Hellmut Jork, Werner Funk,
Walter Fischer, Hans Wimmer

Thin-Layer Chromatography:

Reagents and Detection Methods

Volume 1
Physical and Chemical Detection Methods
(in several parts, part 1c in preparation)

Volume 2
Biochemical and Biological Detection Methods
(in preparation)

Distribution:
VCH, P.O. Box 101161, D-69451 Weinheim (Federal Republic of Germany)
Switzerland: VCH, P.O. Box, CH-4020 Basel (Switzerland)
United Kingdom and Ireland: VCH (UK) Ltd., 8 Wellington Court, Cambridge CB1 1HZ (England)
USA and Canada: VCH, 220 East 23rd Street, New York, NY 10010-4604 (USA)
Japan: VCH, Eikow Building, 10-9 Hongo 1-chome, Bunkyo-ku, Tokyo 113 (Japan)

ISBN 3-527-28205-X (VCH, Weinheim) ISBN 1-56081-103-X (VCH, New York)

Hellmut Jork, Werner Funk,
Walter Fischer, Hans Wimmer

Thin-Layer Chromatography:

Reagents and Detection Methods

Volume 1b
Physical and Chemical Detection Methods:
Activation Reactions, Reagent Sequences,
Reagents II

Translated by Frank and Jennifer A. Hampson

Weinheim · New York
Basel · Cambridge · Tokyo

Prof. Dr. H. Jork
Universität des Saarlandes
Fachbereich 12
Stadtwald
D-66123 Saarbrücken

Dr. W. Fischer – c/o E. Merck
Abteilnng Lab Chrom 1
D-64271 Darmstadt

Prof. W. Funk
Fachbereich Technisches Gesundheitswesen
der Fachhochschule Gießen-Friedberg
Wiesenstraße 14
D-35390 Gießen

Hans Wimmer
Eckhardt-Straße 23
D-64289 Darmstadt

Editorial Directors: Dr. Christina Dyllick-Brenzinger, Karin Sora
Production Manager: Elke Littmann

Library of Congress Card No. applied for.

A catalogue record for this book is available from the British Library.

Deutsche Bibliothek Cataloguing-in-Publication Data:
Thin layer chromatography : reagents and detection methods /
Hellmut Jork ... – Weinheim ; New York ; Basel ; Cambridge ;
Tokyo : VCH.
ISBN 3-527-28666-7
NE: Jork, Hellmut
Vol. 1. Physical and chemical detection methods.
b. Activation reactions, reagent sequences, reagents II / transl. by
Frank and Jennifer A. Hampson. – 1994
ISBN 3-527-28205-X (Weinheim ...)
ISBN 1-56081-103-X (New York)

Printed on acid-free and chlorine-free paper.

Composition: Filmsatz Unger nnd Sommer GmbH, D-69469 Weinheim. Printing: Colordruck Kurt Weber GmbH, D-69181 Leimen. Bookbinding: IVB Heppenheim GmbH, D-64646 Heppenheim.
Printed in the Federal Republic of Germany

Foreword

Chromatographic methods often develop in a cyclic manner: The discovery of a new separation technique naturally stimulates interest concerning the method; this attention wanes when another technique appears on the horizon and soon interest is directed at the new technique. There is then a confrontation between the methods and a critical comparison of the advantages and disadvantages of the two methods. This sometimes leads to a renaissance of the older method, which has been the subject of further development in the meantime. In this context discoveries made in connection with the modern technique are often used to advantage in the older one.

This is what happened in liquid chromatography, as it was influenced by the instrumentation developed for gas chromatography. A similar process has occurred in thin-layer chromatography. It has experienced a new impetus during the last 10 years as a result of instrumentation and automation together with the availability of improved stationary phases and working techniques. Nevertheless, one of the greatest advantages of thin-layer chromatography is that it provides a wealth of information rapidly and economically without the necessity for expensive equipment. The large numbers of publications are a proof of this popularity: According to Sherma 3800 articles were published during the years 1990–1991 in which thin-layer chromatography was used to separate mixtures of substances, for identification and purity testing or in conjunction with quantitation. Thus TLC/HPTLC is a standard analytical method today. The applications are far more numerous than the publications. This results in the method frequently not being described in detail in the literature. There it often says tersely: "the identification or the determination was carried out by means of planar chromatography".

Thin-layer chromatography is a separation technique: Emphasis is laid on the possibility of separating substances and characterizing them, initially based on their mobility in a system of two phases. The components are then detected. Earlier this was only done by chemical reactions on the layer or by the measurement of absorption or fluorescence in short- or long-wavelength light. Later the palette of possibilities was enlarged so that thin-layer chromatography now possesses a wide variety of detection methods. This is the great advantage of the method over column techniques (HPLC, CZE, GC). The rational choice from numerous general, selective or specific detection methods provides a wealth of information concerning the structure of the substance being analysed, which culminates in the

greatly enhanced probability of the identification of the separated substance. All this is achieved relatively simply and very cheaply with the sensitivity of the method often equalling that of HPLC.

For these reasons great emphasis has been placed, from the very beginning, on detection in planar techniques. First compilations on this subject can be found in our monograph on paper chromatography. Methods for 221 detection reagents and advice on their proper use were described forty years ago. These reagents were then modified for thin-layer chromatography by Waldi in 1962 and by Wimmer, Heusser and Krebs in 1966 and collected in the already classical monograph by Egon Stahl. Zweig and Sherma enlarged the collection ten years later. It also appeared in the Merck company brochure „Anfärbereagenzien für die Dünnschicht- und Papier-Chromatographie". Unfortunately little attention was paid in the later literature to the important combination of physical separation and chemical detection. It is only in recent years that efforts have been made to develop more sensitive detection reagents to improve the selectivity and increase the precision of the quantitation that follows.

It is therefore very much to be welcomed that the four authors – all specialists in the field of thin-layer chromatography – have devoted themselves to the production of a monograph covering this complex of topics. This assignment is no mean task, but it is as current as ever. The planned, detailed description in 5 volumes has no parallel in the world literature. It can only be attempted by colleagues who have many years of personal experience of thin-layer chromatography and have lovingly accompanied the development of the method for over 35 years with their own research. The methods described in this book are so clearly set out that they can be followed without recourse to the original literature. In addition the interested worker will also find a wealth of literature references, to serve as a basis for personal study. The authors are to be congratulated on their achievement. It is to be hoped that this monograph will not only ease routine work in the laboratory but will also act as a stimulus for the further development and growth of thin-layer chromatography.

Prague, September 1993 Karel Macek

Preface to Volume 1b

This volume is the second of a series of practice-orientated TLC/HPTLC books published in excellent quality by VCH Publishers. As in the first volume, a series of reagents and detection methods have been reviewed with the intention of helping the practical analyst increase the detection specificity of routine samples separated by thin-layer chromatography.

This volume is divided into two parts which encompass about the same amount of material as Volume 1a. Thus Part I begins with specific detection methods including the known photochemical, thermochemical and electrochemical activation methods. Here microchemical reactions are described that are carried out without the use of reagents. Detection involves the use of light, heat and electric current.

Then follows a selection of group-specific reagents, in response to requests from practical workers after the publication of Volume 1a. This part should be seen in reference to the monographs that follow or have already been published.

The section on "Reagent Series" has also been included at the request of practical workers. There are many publications describing the sequential application of a series of different reagents to the same chromatogram. Reagents intended for the preparatory reaction of certain substances so that the final reagent applied can yield specific detection results are dealt with in this volume. Independent reagents, each capable of detection, combined on the same chromatogram to increase selectivity, e.g. by specifically altering certain colors (= potentiated multi-detection) will be treated in Volume 1c. Such combinations are frequently used in the fields of clinical and forensic chemistry and in the analysis of natural product extracts.

Part I, which contains tested examples together with more than 220 literature references, is followed by Part II; this consists of 65 reagent monographs in alphabetical order. Once again, each includes an example that has been tested in the laboratory and is supplemented by numerous literature references. In the past it is just these references that have helped provide the practical worker with an entry to the earlier literature.

Great importance has also been attached to the inclusion of photographs of original chromatograms in the examples tested along with absorption photometric or fluorimetric scans. These show at a glance that modern thin-layer chromatography is a microanalytical separation method that should be taken seriously and that its development certainly conforms to the state of the analytical art. Those of us more senior in years will immediately recognize the advances made in the method over the last decade.

Particular attention has been devoted to the compilation of the cumulative index. Every reference work is only as good as its indexing system. For this reason a presentation has been chosen which allows one to recognize immediately in which volume the key word appears. The same also applies to named reagents which can be traced back to the original publication in almost all cases in order to be able to correct any errors that have crept in. This type of presentation will be continued in future volumes.

This volume includes a new feature in the collection of reagents into groups that are discussed comparatively. Such groups include the chloroamine T, the diaminobenzaldehyde or the vanillin reagents. The iodine reagents are also dealt with as a group. We have not yet been able to make a similar comparison of the Dragendorff or the ninhydrin reagents. They will follow in Volume 1c.

The fact that our treatment of group-specific reagents is still incomplete illustrates the enormous amount of work involved in compiling a reagent series of value to the practical worker. Those who have also been confronted with such a task appreciate our difficulties. Thanks and recognition are due to J. Ganz, I. Klein and Bl. Meiers and to S. Netz for their tireless work. Thanks are also expressed to the numerous undergraduate and graduate students who have assisted in checking the derivatization reactions, together with E. Otto, G. Schon and Dipl. Ing. M. Heiligenthal in whose capable hands lay the technical preparation of this book.

Prof. Dr. H.-J. Kallmayer (University of Saarland) and Dr. H.-O. Kalinowski (Giessen University) provided generous assistance in the formulation and interpretation of often difficult reaction paths. We had always wished such a cooperation, and it proved to be of great benefit to the resulting work.

We also thank Baron, J.T. Baker, Camag, Desaga, Macherey-Nagel, Merck and Riedel de Haën for their generous support of the experimental work. The monographs would never have been written without their aid.

Our especial thanks are due to the ladies of VCH Publishers, Mrs. Banerjea-Schulz, Dr. Dyllick and Mrs. Littmann for the way they have converted our ideas for the design and layout of this book into reality and for the pleasant cooperation over the past four years.

In spite of all our efforts and careful work errors are bound to remain. We would appreciate our readers sending us their suggestions for improvements. The positive reaction we received to Volume 1a gave us enormous pleasure and has provided us with the motivation to continue our work on the series.

Saarbrücken, Gießen and Darmstadt, December 1992

Hellmut Jork
Werner Funk
Walter Fischer
Hans Wimmer

Preface to Volume 1a

This book is the result of cooperation between four colleagues, who have been working in the field of thin-layer chromatography for many years and, in particular, took an active part in the development from hand-coated TLC plates to commercially available precoated plates and instrumental thin-layer chromatography. This development was accompanied by improvements in the field of detection of the separated zones. In particular, it became necessary to be able to deal with ever decreasing quantities of substance, so that the compilation "Anfärbereagenzien" by E. Merck, that had been available as a brochure for many, many years, no longer represented the state of the art of thin-layer chromatography.

It was against this background and in view of the fact that there is at present no contemporary monograph on thin-layer chromatography that this book was produced. It is intended as an introduction to the method, a reference book, and a laboratory handbook in one, i.e., far more than just a "Reagent Book".

The first part of the book consists of a detailed treatment of the fundamentals of thin-layer chromatography, and of measurement techniques and apparatus for the qualitative and quantitative evaluation of thin-layer chromatograms. In situ prechromatographic derivatization techniques used to improve the selectivity of the separation, to increase the sensitivity of detection, and to enhance the precision of the subsequent quantitative analysis are summarized in numerous tables.

Particular attention has been devoted to the fluorescence methods, which are now of such topicality, and to methods of increasing and stabilizing the fluorescence emissions. Nowhere else in the literature is there so much detailed information to be found as in the first part of this book, whose more than 600 literature references may serve to stimulate the reader to enlarge his or her own knowledge.

Nor has a general introduction to the microchemical postchromatographic reactions been omitted: it makes up the second part of the book.

This second part with its 80 worked-through and checked detection methods forms the foundation of a collection of reagent reports (monographs), which will be extended to several volumes and which is also sure to be welcomed by workers who carry out derivatizations in the fields of electrophoresis and high-pressure liquid chromatography. Alongside details of the reagents required and their handling and storage, the individual reports also contain details about the reaction concerned.

Wherever possible, dipping reagents have been employed instead of the spray reagents that were formerly commonplace. These make it easier to avoid contaminating the laboratory, because the coating of the chromatogram with the reagent takes place with less environmental pollution and lower health risks; furthermore, it is more homogeneous, which results in higher precision in quantitative analyses.

It is possible that the solvents suggested will not be compatible with all the substances detectable with a particular reagent, for instance, because the chromatographically separated substances or their reaction products are too soluble. Therefore, it should be checked in each case whether it is possible to employ the conditions suggested without modification. We have done this in each report for one chosen class of substance by working through an example for ourselves and have documented the results in the "Procedure Tested"; this includes not only the exact chromatographic conditions but also details concerning quantitation and the detection limits actually found. Other observations are included as "Notes". Various types of adsorbent have been included in these investigations and their applicability is also reported. If an adsorbent is not mentioned it only means that we did not check the application of the reagent to that type of layer and not that the reagent cannot be employed on that layer.

Since, in general, the reagent report includes at least one reference covering each substance or class of substances, it is possible to use Part II of this book with its ca. 750 references as a source for TLC applications. Only rarely are earlier references (prior to 1960), which were of importance for the development of the reagent, cited here.

There is no need to emphasize that many helpful hands are required in the compilation of such a review. Our particular thanks are due to Mrs. E. Kany, Mrs. I. Klein, and Mrs. S. Netz together with Dipl.-Ing. M. Heiligenthal for their conscientious execution of the practical work.

We would also like to thank the graduate and postgraduate students who helped to check the derivatization reactions and Mrs. U. Enderlein, Mrs. E. Otto, and Mrs. H. Roth, whose capable hands took care of the technical preparations for the book and the production of the manuscript. We would particularly like to thank Dr. Kalinowski (Univ. Gießen) for his magnificent help in the formulation of the reaction paths for the reagent reports. Our thanks are also due to Dr. F. Hampson and Mrs. J. A. Hampson for translating the German edition of the book into English.

We thank the Baron, J. T. Baker, Camag, Desaga, Macherey-Nagel and E. Merck companies for their generous support of the experimental work.

Our particular thanks are also due to Dr. H. F. Ebel and his colleagues at VCH Verlagsgesellschaft for the realization of our concepts and for the design and presentation of the book and for the fact that this work has appeared in such a short time.

In spite of all our care and efforts we are bound to have made mistakes. For this reason we would like to ask TLC specialists to communicate to us any errors and any suggestions they may have for improving later volumes.

Saarbrücken, Gießen and Darmstadt, October 1989

Hellmut Jork
Werner Funk
Walter Fischer
Hans Wimmer

Contents

Part II
Reagents in Alphabetical Order

Introduction

Modern thin-layer chromatography is a microanalytical separation method whose importance has been increasing steadily since the 1970s [1]. UNGER has spoken of a renaissance of the 30-year-old liquid chromatographic method [2] and MAJORS has postulated a positive continuation of the development on the basis of a poll of experts [3]; this development has been confirmed in a review of organic analysis in the year 1990 [4]. KELKER writes that the former "poor man's chromatography" remains practically irreplaceable and is used at the bench in almost every single organic chemical synthetic/research laboratory [5].

The currently most important fields of application of thin-layer chromatography can be seen in Fig. 1. The proportion of publications in the fields of pharmacy and environmental analysis has increased over that in previous years. There has also been an appreciable increase in the fields of clinical and forensic chemistry and in biochemistry.

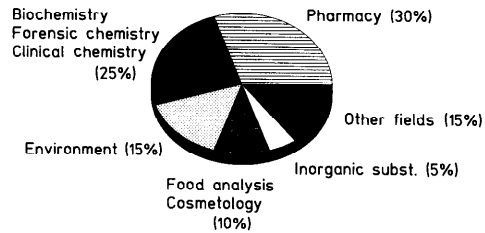


Fig. 1: Fields of application of thin-layer chromatography (TLC/HPTLC) during the period 1988–1991.

The reason for this lies not least in the increasing instrumentalization and deliberate automation of all those processes which were earlier particularly subject to errors (Fig. 2). Modern high performance thin-layer chromatography (HPTLC) is no longer inferior to other liquid chromatographic techniques with respect to precision and sensitivity (Fig. 3) [6].

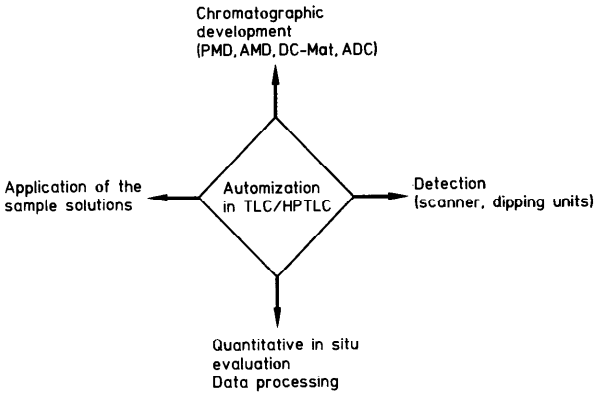


Fig. 2: The steps in the process of thin-layer chromatography that have been instrumentalized and automated to a large degree in the recent past. PMD = Programmed Multiple Development, AMD = Automated Multiple Development, DC-Mat or ADC = Automatic Development Chamber.

The development of methods of coupling TLC with other chromatographic methods and with physical methods of measurement has brought enormous advantages. The first attempts to couple gas chromatography on-line with thin-layer chromatography were made by NIGAM [7], JANAK [8–10] and KAISER [11]. VAN DUK [12] described the on-line coupling of column chromatography with thin-layer chromatography as early as 1969. He divided the eluent stream with a splitting system and demonstrated afterwards that at least three different components could be detected thin-layer chromatographically in an apparently uniform fraction of column eluate.

Today 80–90% of all HPLC separations are carried out on RP phases, while silica gel layers are used for more than 90% of all thin-layer chromatography. This provides the possibility of coupling different separation mechanisms together.

Separation by adsorption chromatography takes place preferentially as a result of hydrogen bonding or dipole-dipole interactions. Hence, separation of mixtures of substances on silica gel layers by lipophilic solvents primarily takes place according to polarity differences. Further separation within a polarity group can then be achieved either two-dimensionally or off-line by partition chromatography on another TLC plate (Fig. 4).

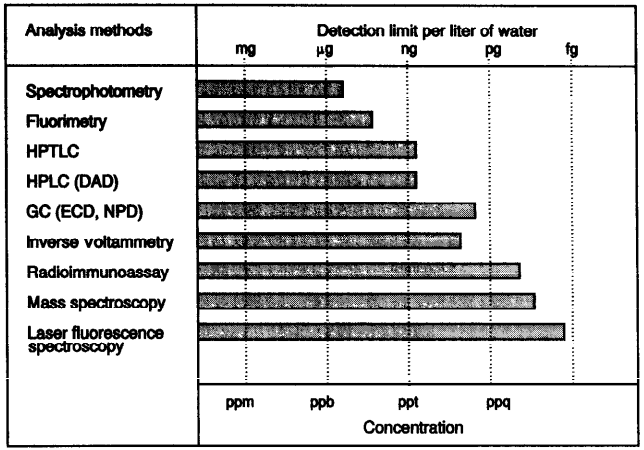


Fig. 3: Sensitivity of various methods of determination.

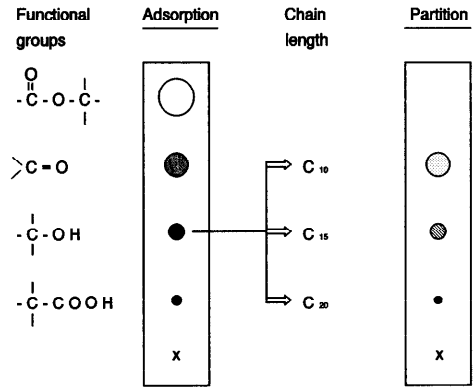


Fig. 4: Coupling the separation principles of adsorption and partition chromatography.

For the same reason it is also possible to use Over Pressure Layer Chromatography (OPLC) on-line for prefractionation or as a clean-up method for HPLC [13, 14]: A group separation according to polarity is followed by a differentiation of the substances according to their differing lipophilicities (Fig. 5).

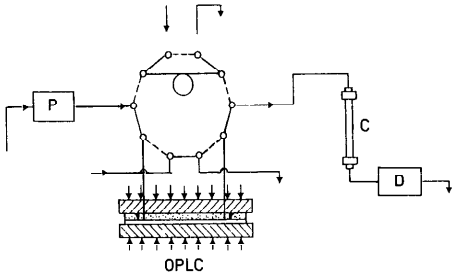


Fig. 5: Schematic representation of OPLC-HPLC coupling; P = pump system, C = column, D = detector.

Conversely successful on-line coupling of HPLC to TLC is also possible. HOFSTRAAT [15–17] and BAEYENS and LING[18] have described suitable apparatus. BURGER, for instance, was able to demonstrate that adsorptive separation of selective cut fractions of an HPLC eluate from RP partition chromatography could be separated into up to 700 individual peaks [19, 20]. Here the thin-layer chromatographic step employed the Automated Multiple Development (AMD) technique. These investigations and the results of KROKER, FUNK and EISENBEISS [21, 22] demonstrate the enormous power of such on-line coupling techniques in a very impressive manner.

In their investigations of caramel MÜLLER et al. [23, 24] demonstrated that such combinations can also be applied to purely aqueous fractions of column eluates: A column-chromatographic separation was made on TSK gels according to hydrophobic interactions, the eluates of individual peaks were then led directly into an on-line sample preparator (OSP 2) equipped with small Polyspher® RP 18 CAT cartridges (OSP 2) [25, 26], in which the organic components were enriched (Fig. 6). A brief rinsing and drying process was followed by elution with a little organic solvent and on-line application of the eluate to silica gel 60 HPTLC plates using the Linomat C (Fig. 7). This was followed by thin-layer chromatographic separation and detection of the individual components.

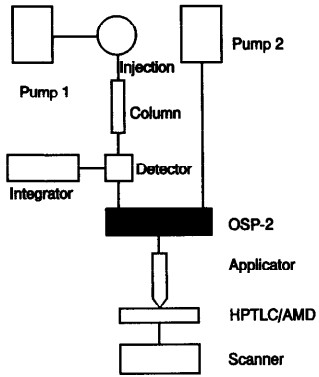


Fig. 6: Schematic representation of HPLC-HPTLC coupling by means of the OSP-2 system (MERCK) for "post-column enrichment" of the column eluate fractions.

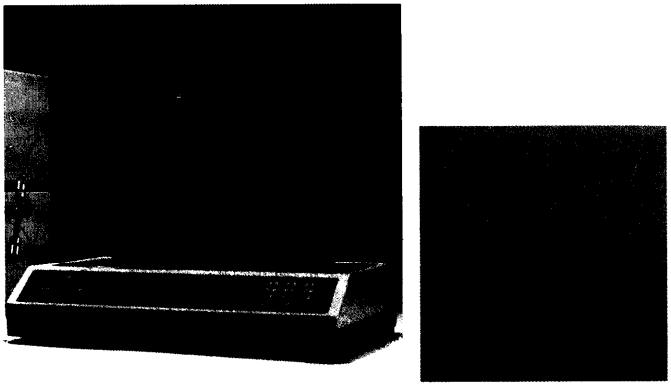


Fig. 7: Linomat C (CAMAG) for on-line transfer of column eluate fractions to TLC/HPTLC plates (A) and application scheme (B).

The on-line principle has also been extended into the field of detection (Fig. 8). Thus, it is now possible to record FTIR [27–31] and Raman spectra in situ [32, 33], and there have been considerable advances in the on-line coupling of thin-layer chromatography with mass spectrometry. Here it has been, above all, the research groups of WILSON [34–36] and BUSCH [37–40] that have made the necessary instrumental and methodological advances, so that TLC must no longer be viewed as merely a clean-up method. Rather it forms the essential central point for all these on-line coupling techniques.

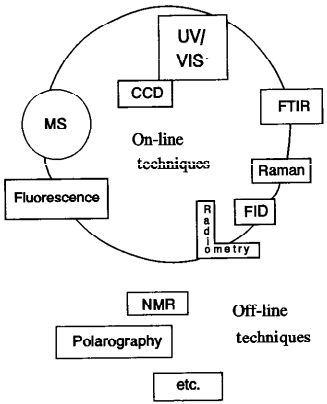


Fig. 8: Possibilities for on-line coupling of thin-layer chromatography with physical measurement and determination methods. CCD = Charge Coupled Device Detection.

The range of microchemical pre- and postchromatographic derivatization methods has also been enlarged, for instance photo- and thermochemical methods have yielded unexpected results. Group-specific reagents have been refined and new ones suggested. Reagent series are receiving greater attention and more sensitive reagents have been developed. These have led logically to the organization of this volume.

References

[1] Jork, H.: *J. Planar Chromatogr.* **1992**, *5*, 4–5.
[2] Unger, K. K.: *GIT Spezial 1 „Chromatographie"* **1991**, 3.
[3] Majors, R. E.: *LC-GC Internat.* **1990**, *3*, 8–16.
[4] Linscheid, M.: *Nachr. Chem. Techn. Lab.* **1991**, *39*, 132–137.
[5] Kelker, H.: *GIT Fachz. Lab.* **1992**, *36*, 2–3.
[6] Fonds der Chemischen Industrie: Brochure „Umweltbereich Wasser", Frankfurt/Main, 1990.
[7] Nigam, I. C., Sahasrabudhe, M., Levi, L.: *Can. J. Chem.* **1963**, *41*, 1535–1539.
[8] Janák, J.: *J. Gas Chromatogr.* **1963**, *1 (10)*, 20–23.
[9] Janák, J.: *J. Chromatogr.* **1964**, *15*, 15–28.
[10] Janák, J., Klimes, I., Hana, K.: *J. Chromatogr.* **1965**, *18*, 270–277.
[11] Kaiser, R.: *Z. Anal. Chem.* **1964**, *205*, 284–298.
[12] Van Dijk, J. H.: *Z. Anal. Chem.* **1969**, *247*, 262–266.
[13] Mincsovics, E., Garami, M., Tyihak, E.: *J. Planar Chromatogr.* **1991**, *4*, 299–303.
[14] Tyihak, E., Mincsovics, E., Kalász, H.: *J. Chromatogr.* **1979**, *174*, 75–81; **1980**, *191*, 293–300; **1981**, *211*, 54.
[15] Hofstraat, J. W., Engelsma, M., Van de Nesse, R. J., Gooijer, C., Velthorst, N. H., Brinkman, U. A. Th.: *Anal. Chim. Acta* **1986**, *186*, 247–259.
[16] Hofstraat, J. W., Engelsma, M., Van de Nesse, R. J., Gooijer, C., Velthorst, N. H., Brinkman, U. A. Th.: *Anal. Chim. Acta* **1987**, *187*, 193–207.
[17] Hofstraat, J. W., Griffion, S., Van de Nesse, R. J., Brinkman, U. A. Th., Gooijer, C., Velthorst, N. H.: *J. Planar Chromatogr.* **1988**, *1*, 220–226.
[18] Bacyens, W. R. G., Ling, B. L.: *J. Planar Chromatogr.* **1988**, *1*, 198–213.
[19] Burger, K.: Lecture given at Merck Forum, Leverkusen, 1990.
[20] Burger, K., Protze, B.: Results from a thesis, Fachhochschule Niederrhein, 1986.
[21] Kroker, B.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1991.
[22] Eisenbeiß, F., Kroker, B., Funk, W.: Lecture InCom '92, Düsseldorf 1992.
[23] Müller, E.: Dissertation, Universität des Saarlandes, Fachrichtung 12.5, Saarbrücken, 1994.
[24] Müller, E., Jork, H.: *J. Planar Chromatogr.* **1993**, *6*, 21–28.
[25] Wotschokowsky, M., Witzenbacher, M., Godau, S.: 5th. Int. Symp. on Sample Handling of Environmental and Biol. Samples in Chromatography. Poster No. 36/91, Baden-Baden 1991.
[26] Wotschokowsky, M., Witzenbacher, M., Godau, S.: *GIT Fachz. Lab.* **1991**, *35*, 404–409.
[27] Chalmers, J. M., Mackenzie, M. W., Sharp, J. L., Ibbett, R. N.: *Anal. Chem.* **1987**, *59*. 415–418.
[28] Glauninger, G.: Dissertation, Eberhard-Karls-Universität, Tübingen, 1989.
[29] Glauninger, G., Kovar, K. A., Hoffmann, V.: *Fresenius Z. Anal. Chem.* **1990**, *338*, 710–716.
[30] Kovar, K. A., Enßlin, H. K., Frey, O. R., Rienas, S., Wolff, S. C.: *J. Planar Chromatogr.* **1991**, *4*, 246–250.
[31] Kovar, K. A., Enßlin, H. K., Frey, O. R., Rienas, S., Wolff, S. C.: *GIT Spezial 2 „Chromatographie"* **1991**, 95–101.
[32] Koglin, E.: *J. Planar Chromatogr.* **1989**, *2*, 194–197.
[33] Koglin, E.: *J. Planar Chromatogr.* **1990**, *3*, 117–120.
[34] Wilson, I. D., Lafont, R., Wall, P.: *J. Planar Chromatogr.* **1988**, *1*, 357–359.
[35] Wilson, I. D., Lafont, R., Kingston, R. G., Porter, C. F.: *J. Planar Chromatogr.* **1990**, *3*, 359–361.

[36] Wilson, I. D., Morden, W.: *J. Planar Chromatogr.* **1991**, *4*, 226–229.
[37] Duffin, K. L., Busch, K. L.: *J. Planar Chromatogr.* **1988**, *1*, 249–251.
[38] Doherty, S. J., Busch, K. L.: *J. Planar Chromatogr.* **1989**, *2*, 149–151.
[39] Busch, K. L.: *J. Planar Chromatogr.* **1989**, *2*, 355–361.
[40] Brown, S. M., Busch, K. L.: *J. Planar Chromatogr.* **1991**, *4*, 189–193.

Part I

Specific Detection Methods

1 Activation Reactions

Every reaction chain is only as strong as its weakest link. It was LIEBIG who illustrated this truism with a barrel (Fig. 9): The shortest stave determines how high the barrel can be filled.

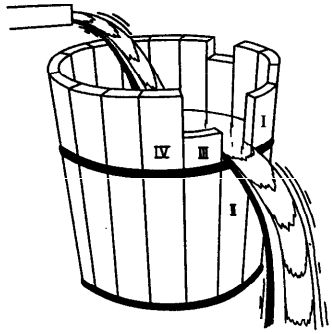


Fig. 9: LIEBIG's barrel.

This principle also applies to chromatography. For instance, the best of separation methods is of no avail if the results of the separation cannot be detected.
It is well known that the sorbents used in thin-layer chromatography possess large specific surface areas [1], that can interact with the substances being separated. This applies to the time taken for development and naturally when the chromatogram has been dried. The additional effect of energy (heat, visible light, UV, X-rays, γ-radiation, high potential) can be used to bring about desired photo- and thermochemical reactions. The following are among the phenomena that have been observed:

- dehydrogenations and dehydrations
- the formation of oxidation products in the presence of oxygen that are readily detected by the SRS technique (separation – reaction – separation)
- rearrangement of *trans* to *cis* compounds
- production of ions and radicals which then react further e.g. in chain reactions, to yield stable higher molecular weight substances that can be colored and/or can emit fluorescent light
- pyrolysis phenomena [2].

The inorganic sorbents act as catalysts in all this [3, 4]. The pH also probably plays a role. Reactions that do not otherwise occur are observed on acid silica gel [5] or basic aluminium oxide layers. Reactions of this type have also been observed for amino [6–8] and RP phases [9]. The products of reaction are usually fluorescent and can normally be used for quantitative analysis since the reactions are reproducible.

Such reactions can be promoted by exposing the chromatogram to the vapors of hydrogen halides, to nitric acid fumes [4], to ammonia or oxides of nitrogen [2] in suitable reaction chambers [10]. Ammonium hydrogen carbonate, first proposed by SEGURA and GOTTO is also suitable [11].

Impregnation with ammonium acetate or ammonium hydrogen sulfate serves the same purpose [11–13]. In conjunction with the TLC separation previously carried out it is even possible to obtain group-specific and sometimes substance-specific information.

The reactions discussed in the next section are those carried out without any application or impregnation with reagent solutions or exposure to reagent vapors.

References

[1] Halpaap, H.: *J. Chromatogr.* **1973**, *78*, 63–75.
[2] Heidbrink, W.: *Fette, Seifen, Anstrichm.* **1964**, *66*, 569–573.
[3] Egg, D., Huck, H.: *J. Chromatogr.* **1971**, *63*, 349–355.
[4] Zhou, L., Shanfield, H., Wang, F.-S., Zlatkis, A.: *J. Chromatogr.* **1981**, *217*, 341–348.
[5] Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin-layer Chromatography, Reagents and Detection Methods*, Vol. 1a, VCH-Verlagsgesellschaft, Weinheim, Cambridge, New York, 1990.
[6] Okamoto, M., Yamada, F.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1982**, *5*, 163–164.
[7] Okamoto, M., Yamada, F., Omori T.: *Chromatographia* **1982**, *16*, 152–154.
[8] Klaus, R., Fischer, W., Hauck, H.E.: *Chromatographia* **1989**, *28*, 364–366; **1990**, *29*, 467–472; **1991**, *32*, 307–316.
[9] Maxwell, R.J., Unruh, J.: *J. Planar Chromatogr.* **1992**, *5*, 35–40.
[10] Heisig, W., Wichtl, M.: *Dtsch. Apoth. Ztg.* **1990**, *130*, 2058–2062.
[11] Segura, R., Gotto, A.M.: *J. Chromatogr.* **1974**, *99*, 643–657.
[12] Maxwell, R.J.: *J. Planar Chromatogr.* **1988**, *1*, 345–346.
[13] Kupke, I.R., Zeugner, S.: *J. Chromatogr.* **1978**, *146*, 261–271.

1.1 Photochemical Activation

It was observed relatively early that chemically labile compounds – such as vitamins, carotenes – decompose, either on application to the TLC layer or during the TLC separation that follows. This phenomenon was primarily ascribed to the presence of oxygen (oxidation) and exposure to light (photochemical reaction) in the presence of the active sorbents, which were assumed to exert a catalytic effect (photocatalytic reaction).

Today all automatic sample applicators blanket the plate with nitrogen; firstly this has the effect that the applied starting zones dry quickly and secondly serves to prevent oxidation of the applied substances.

Some application instruments possess light-absorbent covers to prevent or reduce the action of UV and visible light (Fig. 10).

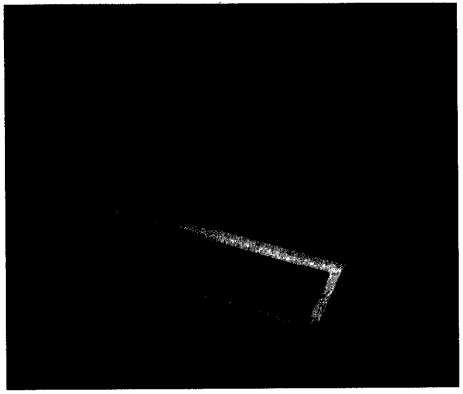


Fig. 10: Linomat IV with protective gas atmosphere (CAMAG).

It has been recommended that the outer walls of the separation chamber be covered with black foil or that the work be carried out in a dark room under green or red light [1].

More recent chromatogram chambers – e. g. the AMD system (Fig. 11) – only possess a small observation window and this can, if necessary, be covered with a black cloth. Development in the DC-Mat (Fig. 12) or the ADC (Fig. 13) automatic development chambers is carried out entirely in the dark.

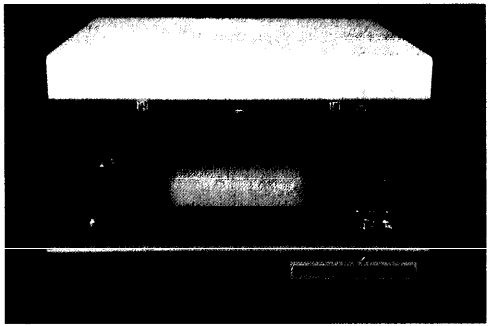


Fig. 11: AMD system (CAMAG).

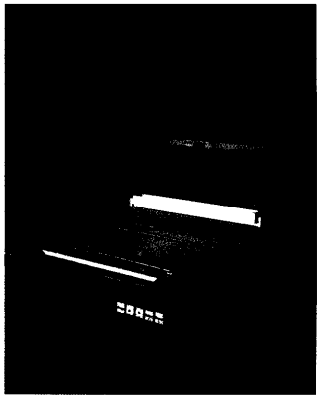


Fig. 12: DC-Mat (BARON, DESAGA), opened. The "flap" bearing the HPTLC plate is folded upwards during chromatography, so that the development can take place in the dark.

Fig. 13: Automatic development chamber (ADC chamber, CAMAG).

In general photochemical reactions only occur when the affected substance absorbs radiation, i. e. when π or n electrons are raised to an excited π^* state. Interaction of the electric light vector with the electronic shell of the molecule brings about a change in the structure of the electronic shell. This change takes place during one period of oscillation of the light (ca. 10^{-15} s). The altered structure of the electronic shell corresponds to a higher energy state of the molecule. Hence, the molecule is in an electronically excited state [2] This excited singlet state S only has a short life. Excess oscillation energy is immediately conducted away (Fig. 14). Activated molecules return to the ground state once again, whereby one of the following processes can occur [3]:

- Energy rich π^* electrons experience a spin reversal so that the molecule involved passes from the singlet to the corresponding triplet state (Fig. 14, 15/I).
- The excited molecule passes instantaneously from the singlet to the gound state S_o with the emission of light (fluorescence) (Fig. 14, 15/II).
- The excess energy of excited molecules is transferred, by collision, to acceptor molecules, which are converted to an excited state while the initially excited molecules return to the ground state (Fig. 15/III).

- The excited singlet or triplet state returns to the ground state by a radiationless deactivation process (Fig. 15/IV).
- A chemical process occurs involving the formation of a new substance with corresponding energetic ground state N (Fig. 14, 15/V). For instance, on aluminium oxide or silica gel layers in the presence of oxygen, anthracene initially yields anthraquinone, that is then oxidized further to yield 1,2-dihydroxyanthraquinone [4, 5]. Alizarin and chrysazin are also formed depending on the properties of the aluminium oxide used [6].
- Starting from the energy level of the triplet state a further spin reversal leads to the ground state S_0 (phosphorescence radiation, Fig. 14, 15/VI).

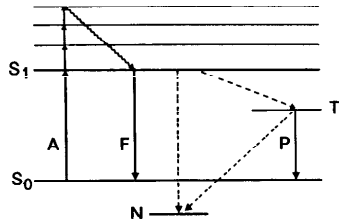


Fig. 14: Schematic representation of the electronic transitions of photochemically excited substances S_0 = ground state, S_1 = first excited singlet state, T = "forbidden" triplet transition, N = ground state of a newly formed compound, A = absorption, F = fluorescence, P = phosphorescence.

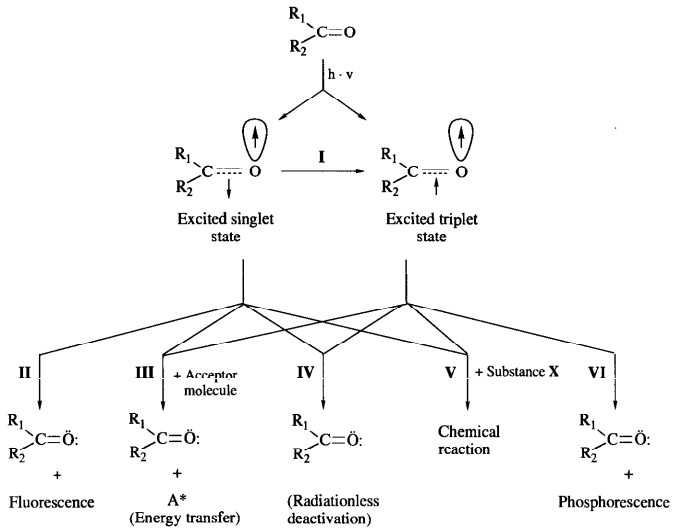


Fig. 15: Possibilities for photochemically induced reactions, using a carbonyl compound as example. ↓↓ and ↓↑ = electron spins parallel and antiparallel respectively.

This short discussion should provide an indication of the versatility of photochemical reactions. For example it is possible to synthesize, in a simple manner, complicated ring systems that are difficult to produce by conventional synthetic methods. For these reasons it is only rarely possible to make unequivocal predictions concerning the chemical structures of the products formed particularly if oxygen is present during the course of the reaction.

It is often possible to detect such photochemical reactions with the aid of the SRS technique (separation – reaction – separation) [1, 7]. An initial thin-layer chromatographic separation is followed by irradiation of the chromatogram. The irradiated chromatogram is then developed perpendicular to the first direction of development using the same mobile phase. In the absence of any reaction all the chromatogram zones lie on a diagonal. However, if reaction has occurred, the hR_f values of the affected substances are displaced into the regions above or below the diagonal during the second development.

STAHL, for instance, was able to demonstrate that on irradiation with long-wavelength UV light the naturally occurring contact insecticides pyrethrin I and II, cinerin I and II and jasmolin I and II present in *Chrysanthemum cinerariifolium* are converted to inactive pyrethrin oxides by the incorporation of oxygen [7].

UV irradiation of piperine, the most important hot substance of pepper, does not lead to the incorporation of atmsopheric oxygen [8]. The all-*trans* compound is converted to the *cis-trans* isomer, this can be seen in the chromatogram above the all-*trans* piperine (Fig. 16).

In this case the excited molecules produced on interaction with radiation undergo spin reversal to yield a triplet state with a much longer lifetime than that of the singlet excited state. One or more π-bonds are broken in the triplet state since one of the π-electrons affected is in an antibonding π* molecular orbital. This means that the σ-bond is free to rotate and *cis* and *trans* isomers can be formed next to each other on recombination of the double bond.

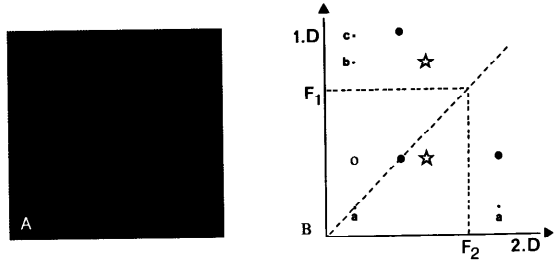


Fig. 16: Detection of *cis/trans* isomerization of piperine by the SRS technique after UV irradiation: (A) original chromatogram, (B) schematic representation.
F_1, F_2 = mobile phase front after development in the first and in the second dimension; a, b, c = positions of application of the *trans/trans*-piperine before the first (1D) and before the second development 2D; ☆ = *cis/trans*-piperine, ● = *trans/trans*-piperine, ○ = position of the *trans/trans*-piperine after the first development. Irradiation of the chromatogram with long-wavelength UV light after application of *trans/trans*-piperine to position b after the first development (position c was not irradiated!).

SCHUNACK and ROCHELMEYER have described such a *cis/trans* isomerization of annuloline, a weakly basic alkaloid from *Lolium multiflorum* LAM [9]. Irradiation with UV light after the first TLC development simultaneously causes a *cis→trans* and a *trans→cis* isomerization, so that the SRS technique yields four blue fluorescent

chromatogram zones (λ_{exc} = 365 nm, λ_{fl} = 422 nm) at the corners of a rectangle. Detailed investigations carried out in the complete absence of light revealed that the plant produces exclusively *trans*-annuloline and that only this is fluorescent. Hence, there are evidently four blue fluorescent spots on the SRS chromatogram because *trans→cis* isomerization occurs during work-up of the plant extract and application of the sample solution and *cis→trans* and *trans→cis* isomerizations occur simultaneously during the UV irradiation after the first TLC development.

Similar processes occur with azo compounds [10]. *trans*-Dimethylaminoazobenzene (butter yellow) yields some of the *cis* isomer on irradiation with long-wavelength UV light and this possesses a lower hR_f than the *trans* compound on rechromatographing with the same mobile phase using the two-dimensional SRS technique (Fig. 17). IR and MS measurements were used to confirm that no oxygen had been incorporated into the molecule.

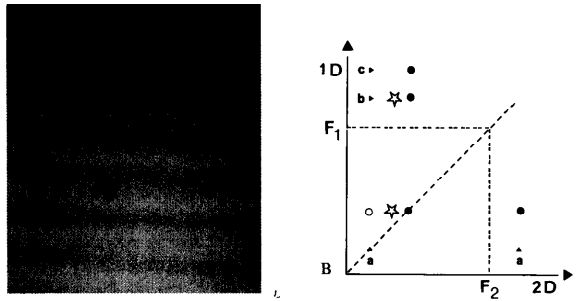


Fig. 17: Detection of the photochemical *cis/trans* isomerization of butter yellow after UV irradiation by using the SRS technique. (A) original chromatogram – treated with hydrochloric acid vapor for better recognition (yellow then turns red) – and (B) schematic representation.
F_1, F_2 – mobile phase front after development in the first and in the second dimension; a, b, c = positions of application of the *trans*-butter yellow before the first (1D) and before the second development (2D); ☆ = *cis*-butter yellow, ● = *trans*-butter yellow, ○ = position of the *trans*-butter yellow after the first development. Irradiation of the chromatogram with long-wavelength UV light after application of *trans*-butter yellow to position b after the first development (position c was not irradiated!). In contrast to Figure 16 the photochemically produced reaction product lies below the starting compound.

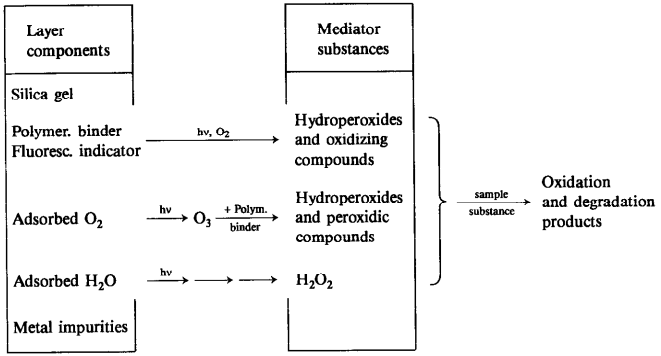
These few examples illustrate impressively how a range of substances can undergo chemical reaction when they are exposed to light while on the TLC plate:

- Wavelengths of about $\lambda = 200$ nm excite olefinic structures in particular, causing $\pi \rightarrow \pi^*$ transitions.
- Wavelengths between $\lambda = 280$ nm and 290 nm or longer are absorbed by carbonyl compounds. Here the free n-electrons of the oxygen enter the antibonding π^* molecular orbital.
- It is possible for homolysis to occur when the light energy absorbed by a molecule reaches or exceeds the bonding energy. Radicals are formed or, in the case of halogens, atomic halogen:

$$Cl_2 \rightarrow 2\ Cl^{\bullet}$$

Further characteristic assignments of substance structures to wavelength ranges that are absorbable are to be found in the specialist literature [2, 11–14]. The publications of the research groups of FASSLER [15, 16] and OELKRUG [17–19] reveal that the sorbent can exert a considerable additional effect.

TAKÁCS et al. [27] have also studied the effects of sorbents. They demonstrated that the irradiation of the sorbent layers before use ("activation") causes changes to occur in the stationary phase chemically altering the chromatographic behavior of 3,5-pyrazolidindione derivatives. The authors attributed these "memory" effects to photochemical oxidation of the binders and other materials. According to the following scheme (p. 21) the water film of the layer yields hydroperoxide and the oxygen ozone, these two then react – possibly under the influence of metallic impurities or fluorescence indicators – with the acrylate and methacrylate polymers. In this manner transmitter substances are produced that greatly increase the reactivity of the layer and which remain active for days. This "post-photo effect" generally leads to the same reaction product as that produced by direct irradiation of the plate after application or after chromatography of the sample under investigation.



FRIJNS has demonstrated this possibility with reserpine and rescinnamine by irradiating at the start zone for two hours and obtaining a characteristic zone pattern (fingerprint) after TLC separation of the photochemically produced derivatives [20]. HUCK and DWORCAK exposed developed chromatograms with vanilmandelic acid and homovanillic acid zones to diffuse daylight and observed the formation of fluorescent metabolites that were suitable for direct quantitative analysis [21].

FUNK et al. have used a low-pressure mercury lamp without filter to liberate inorganic tin ions from thin-layer chromatographically separated organotin compounds; these were then reacted with 3-hydroxyflavone to yield blue fluorescent chromatogram zones on a yellow fluorescent background [22]. Quantitative analysis was also possible here ($\lambda_{exc} = 405$ nm, $\lambda_{fl} = 436$ nm, monochromatic filter). After treatment of the chromatogram with Triton X-100 (fluorescence amplification by a factor of 5) the detection limits for various organotin compounds were between 200 and 500 pg (calculated as tin).

Fifteen β-blockers have also been activated photochemically with the same radiation unit (HERAEUS, Hanau; OSRAM STE 501; UV lamp TNN 15-3200/721)[23]. Their detection limits, the working range and associated standard deviation of the method are listed in Table 1 below. The blue fluorescence of the chromatogram zones ($\lambda_{exc} = 313$ nm, $\lambda_{fl} > 390$ nm) was measured after dipping the chromatogram in liquid paraffin - *n*-hexane (1+2). Figure 18 illustrates the separation of seven β-blockers.

The reactions described above also explain reactions that occasionally occur during TLC and are frequently regarded as interferences. Of course, they can also be deliberately employed for photochemical activation of applied or thin-layer chromatographically separated samples.

Table 1: Detection limits, working ranges and method standard deviation V_{xo} for quantitative analysis of β-blockers.

Substance	Detection limit [ng/chromatogram zone]	Working range [ng/chromatogram zone]	V_{xo} [%]
Acebutolol	5	8–70	±3.7
Atenolol	100	n.a.*)	n.a.*)
Bupranolol	50	60–220	±2.3
Carazolol	5	10–80	±4.5
Nadolol	50	n.a.*)	n.a.*)
Pindolol	5	10–90	±1.7

*) not available

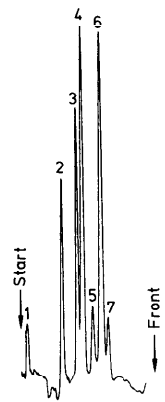


Fig. 18: Fluorescence scan of a chromatogram track with 250 ng each of atenolol (1), acebutolol (2), cartelol [3], pindolol (4), bunitrolol (5), alprenolol (6) and penbutolol (7) per chromatogram zone.

While FUNK et al. did not use temperatures above 30 °C during the irradiation times discussed above, SISTOVARIS combined UV irradiation with simultaneous heating (70 °C, 2 h) of the TLC layers [24]. After this treatment nomifensine and its metabolites appeared as intense yellow fluorescent chromatogram zones on a dark background.

This chapter ends with a tested procedure to represent the many photochemical reactions on silica gel.

Procedure Tested

Chelidonine in the Greater Celandine [25]

Layer: Silica gel **Irradiation: λ = 254 nm**

Reaction

The reaction mechanism has not been elucidated. The processes occurring are presumably those already discussed in Section 1.1.

Sample preparation: Dried greater celandine was pulverized and briefly boiled in 0.05 mol sulfuric acid. After cooling to room temperature the mixture was placed in a separating funnel and adjusted to pH 10 with ammonia solution and extracted once with chloroform. The organic phase was dried with sodium sulfate and evaporated to dryness under reduced pressure. The residue was taken up in methanol and used as the sample solution for TLC.

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation and the exclusion of light.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK), before application of the samples the layer was developed to its upper edge with chloroform – methanol (50 + 50) to precleanse it and then dried at 110 °C for 30 min.
Mobile phase	Toluene – methanol (90+10)

Migration distance 7 cm

Running time 15–20 min

Detection and result: The developed chromatogram was dried for 15 min in a stream of warm air and then examined under long-wavelength UV light ($\lambda = 365$ nm):

A whole range of separated celandine extract components are visible as intensely fluorescent chromatogram zones; however, chelidonine does not emit fluorescent light at this stage, but fluorescence quenching is likely to occur under short-wavelength UV light ($\lambda = 254$ nm) (Fig. IA and IB).

The chromatogram was then irradiated with short-wavelength UV light ($\lambda = 254$ nm) for 3–5 min and examined again under long-wavelength UV light ($\lambda = 365$ nm).

Now chelidonine produced an intense green fluorescent chromatogram zone; in addition there were other intensely fluorescent zones in the track of the celandine extracts – some of which were not previously visible or had another color shade (Fig. IC). In addition the general fluorescence was increased as a result of the UV irradiation. Figure II illustrates the corresponding fluorescence scans.

In situ quantitation: The fluorimetric scan was carried out at $\lambda_{exc} = 313$ nm and the fluorescence emission was measured at $\lambda_{fl} > 400$ nm (cut off filter) (Fig. II).

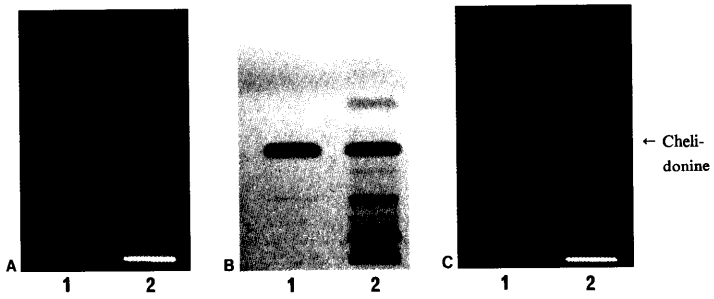


Fig. I: Chromatogram of celandine extract (Track 2) and a chelidonine standard (Track 1): (A) detection of fluorescent zones in long-wavelength UV light, (B) detection of UV absorbing zones in short-wavelength UV light by fluorescence quenching and (C) detection of photochemically activated chromatogram zones after irradiation of the chromatogram with short-wavelength UV light.

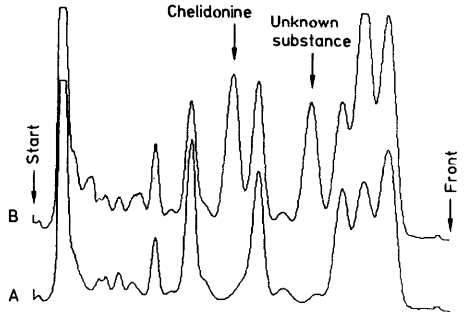


Fig. II: Fluorescence scan of a *Chelidonium* extract chromatogram track with ca 5 µg chelidonine (A) before and (B) after 1 h irradiation with short-wavelength UV light; two new zones are apparent that were not previously detected [25, 26].

References

[1] Stahl, E.: *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch*. 2. Ed., Springer, Berlin 1967.

[2] Henning, H., Rehorek, D.: *Photochemische und photokatalytische Reaktionen von Koordinationsverbindungen*, Akademie-Verlag, Wissenschaftliche Taschenbücher Bd. 300, Berlin (DDR) 1987.

[3] Christen, H. R.: *Grundlagen der organischen Chemie*, Sauerländer-Diesterweg-Salle, Aarau-Frankfurt 1970.

[4] Kortüm, G., Braun, W.: *Liebigs Ann. Chem.* **1960**, *632*, 104–115.

[5] Kortüm, G. in: Symposiumsband *„Optische Anregung organischer Systeme"*, Proc. 2. Int. Farbsymp. 1964, Verlag Chemie, Weinheim 1966.

[6] Voyatzakis, E., Jannakoudakis, D., Dorfmüller, T., Sipitanos, C., Stalidis, G.: *Compt. Rend.* **1960**, *251*, 2696–2697.

[7] Stahl, E.: *Arch. Pharm.* **1960**, *293*, 531–537.

[8] Jork, H., Kany, E.: GDCh-Training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1992.

[9] Schunack, W., Rochelmeyer, H.: *Arch. Pharm.* **1965**, *298*, 572–579.

[10] Jork, H., Ganz, J.: GDCh-Training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1991.

[11] Kortüm, G.: *Kolorimetrie – Photometrie und Spektrometrie*, Springer, Berlin-Göttingen-Heidelberg 1962.

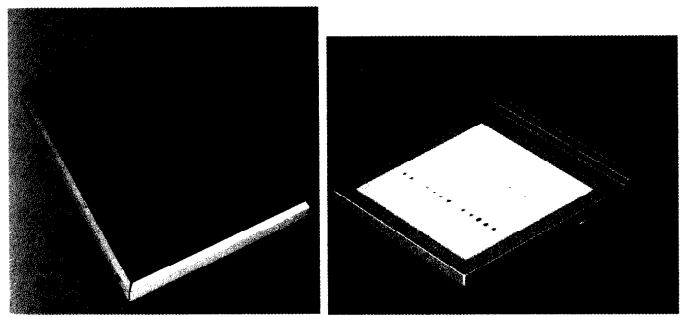
[12] Gauglitz, G.: *Praktische Spektroskopie*, Attempto Verlag, Tübingen 1983.

[13] Rücker, G.: *Spektroskopische Methoden in der Pharmazie*, Bd. 1, Wissenschaftliche Verlagsgesellschaft, Stuttgart 1976.
[14] Staab, H. A.: *Einführung in die theoretische organische Chemie*, Verlag Chemie, Weinheim 1959.
[15] Faßler, D., Günther, W.: *Z. Chem.* **1977**, *17*, 429-430.
[16] Faßler, D., Günther, W.: *Z. Chem.* **1978**, *18*, 69-70.
[17] Oelkrug, D., Erbse, A., Plauschinat, M.: *Z. Phys. Chem. N.F.* **1975**, *96*, 283-296.
[18] Kessler, R. W., Oelkrug, D., Uhl, S.: *Le Vide, les Couches Minces* **1981**, *290*, 1338-1341.
[19] Krablichler, G., Schlüter, I., Oelkrug, D., *Proc. IX. IUPAC Symp. Photochem.*, Pau 1982, 188.
[20] Frijns, J. M. G. J.: *Pharm. Weekblad* **1971**, *106*, 605-623.
[21] Huck, H., Dworzak, E.: *J. Chromatogr.* **1972**, *74*, 303-310.
[22] Funk, W., Kornapp, M., Donnevert, G., Netz, S.: *J. Planar Chromatogr.* **1989**, *2*, 276-281.
[23] Funk, W., Azarderakhsh, M.: *GIT Fachz. Lab. Supplement „Chromatographie"* **1990**, 31-39.
[24] Sistovaris, N.: *J. Chromatogr.* **1983**, *276*, 139-149.
[25] Hahn-Deinstrop, E.: Private communication, Fa. Heumann, Abt. Entwicklungsanalytik, D-8500 Nürnberg 1.
[26] Handloser, D., Jänchen, D.: Private communication, Muttenz 1992.
[27] Takács, M., Kertész, P., Wiener, E., Reisch, J.: *Arch. Pharm.* **1985**, *318*, 824-832.

1.2 Thermochemical Activation

As is well known chemical reactions are accelerated by increasing the temperature. This also applies to heterogeneously catalyzed reactions taking place on the surface of polar sorbents such as aluminium oxide or silica gel (Tables 2.1 and 2.2). Such reactions have also been reported on the moderately polar NH_2 layers. ALPERIN et al. have described the activation of cellulose to yield specific information concerning the substances chromatographed [1].

In the simplest case the developed chromatograms are heated to the required temperature on a hot plate (Fig. 19) or in a drying cupboard. More rarely infrared heaters are used to heat the system [2]. Gas chromatograph ovens can be used if exact adjustment of the temperature is required [3].



A B

Fig. 19: TLC plate heater III (CAMAG) (A), (DESAGA) (B).

When the compounds are heated close to their decomposition temperatures, in contact with the surface of the active sorbents, while fluorescent substances are produced. Further heating can, however, lead to complete carbonization. The details of the reactions taking place are not currently known [4].

SEGURA and GOTTO have postulated that nitrogen-containing compounds form derivatives of the type R-N=CH-CH=CH-NH-R, in a similar manner to the reaction

of malonaldehyde with amino acids to yield SCHIFF's bases – a hypothesis that is supported by the occurrence of appropriate IR bands [5].

In general compounds with heteroatoms (N, O, S and P) are more amenable to "fluorescence reactions" than pure hydrocarbons. Under the influence of the catalytic sorbents substances rich in π-electrons are formed, that conjugate to "rigid" reaction products that are fluorescent when appropriately excited. The formation of fluorescent derivatives is frequently encouraged by gassing with nitrogen or carbon dioxide.

Changes of pH can also yield specific evidence. Thus, it is frequently possible to alter the excitation and fluorescent wavelengths of many fluorescing compounds in this manner. In addition there is a range of nonfluorescent substances that can be derivatized by exposure to ammonia gas, ammonium hydrogen carbonate or acids (e.g. HCl, HBr) to yield products that are able to fluoresce. The impregnation of the layer with ammonium acetate or hydrogen sulfate, that is frequently recommended, serves the same purpose. Examples of this behavior are to be found in the reagent monographs.

The following Tables 2.1 to 2.3 summarize some examples based exclusively on thermochemical reactions on the sorbent surface which lead to the formation of fluorescent reaction products. The derivatives formed frequently remain stable for weeks [6] and the fluorescence can frequently be intensified and/or be stabilized by treatment with viscous liquids (liquid paraffin, Triton X-100, polyethylene glycol etc.).

Quantitation is possible in many cases [6–15]. However, the activation reaction does not always yield a single reaction product (check by SRS method!), so the dependence of the linear response interval on temperature and duration of heating must be checked for each product. It can be taken as a rule of thumb that there will be a linear response between measurement signal and amount applied over the range 10 to 100 ng substance per chromatogram zone [5].

Since the literature cited did not reveal a significant effect of the differing pore systems of the various types of layer the aluminium oxide and silica gel types (60, 80, 100, etc.) are not specified. The same applies to binders, fluorescence indicators and trace impurities in the sorbents.

Table 2.1: Summary of some examples of detection after merely heating aluminium oxide layers (Types 150/T or 60/E) after chromatography.

Substances	Temperature/time	Remarks	Ref.
Pesticides, e.g. aminocarb, captan, difolatan, landrin, rotenone	200 °C, 45 min	Induction of fluorescence in weakly fluorescent or nonfluorescent pesticides and amplification of natural fluorescence. There are some differences between basic and acidic aluminium oxide layers.	[16]
Δ^4-3-Ketosteroids, e.g. testosterone and *epi*-testosterone in urine	180 °C, 20 min	Pale blue induced fluorescence (λ_{fl} = 440 nm) for Δ^4-3-ketosteroids, detection limit: 5 ng.	[8]
Δ^4-3-Ketosteroids, e.g. trimethylsilyl-testosterone	180 °C, 20 min or 150 °C, 20 min	Conversion of Δ^4-3-ketosteroids or their trimethylsilyl or acetyl derivatives in fluorescent components, whereby the detection limits were improved by 65% for the acetates. Δ^5-3-keto- and Δ^5-3-OH-steroids also react with the same sensitivity.	[17]
Testosterone	180 °C, 20 min	Induced fluorescence ($\lambda_{fl} > 430$ nm, cut off filter) by thermal treatment of the chromatogram, the fluorescence increased by a factor of 2.5 by dipping in a solution of Triton X-100 – chloroform (1+4). Working range: 2–50 ng substance per chromatogram zone. Prewashing the layers with methanol-ammonia solution (25%) (50+50) increased the precision.	[15]
Testosterone	180 °C, 20 min	Induced fluorescence and fluorescence amplification by a factor of 25 by dipping the chromatogram in a solution of Triton X-100 – chloroform (1+4).	[9]
Δ^4-3-Ketosteroids, e.g. progesterone in plasma	150 °C, 20 min	Conversion of Δ^4-3-ketosteroids into fluorescent derivatives (λ_{fl} = 440 nm). Relatively selective for progesterone at 150 °C, detection limit: 2–5 ng.	[4]

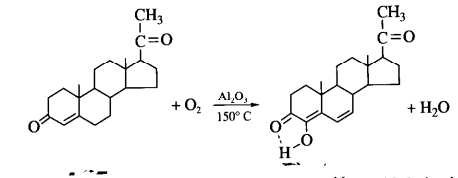
Procedure Tested

Testosterone [9, 15]

Layer: Aluminium oxide **Temperature: 180 °C**

Reaction

At elevated temperatures in the presence of oxygen the aluminium oxide layer catalyzes the formation of blue fluorescent "aluminium oxide surface compounds" with 4-hydroxy-3-oxo-$\Delta^{4,6}$-steroid structures [4]. Aluminium oxide acts as an oxidation catalyst for an activated methylene group.



Progesterone 4-Hydroxy-3-oxo-$\Delta^{4,6}$-steroid derivative

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	TLC plates Aluminium oxide 150 F_{254} (MERCK); before application of the samples the layer was developed twice to its upper edge with methanol – ammonia solution (25%) (50 + 50) to precleanse it and then dried after each development at 120 °C for 30 min.
Mobile phase	Toluene – 2-propanol (10+1)
Migration distance	8 cm
Running time	25 min

Detection and result: The dried chromatogram was heated in the drying oven at 180 °C for 20 min. After cooling to room temperature it was dipped twice for 1 s into a solution of Triton X-100 – chloroform (1+4) which stabilized the fluorescence and increased its intensity by a factor of 2.5. Between the two dipping steps the chromatogram was air-dried in the dark for 30 min until the chloroform had completely evaporated.

Testosterone (hR_f: 65 –70) appeared under long-wavelength UV light (λ= 365 nm) as a pale blue fluorescent zone on a dark background.

The detection limit was less than 2 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric scan was carried out at λ_{exc} = 365 nm and the fluorescence emission was measured at λ_{fl} > 430 nm (cut off filter Fl 43, Fig. I).

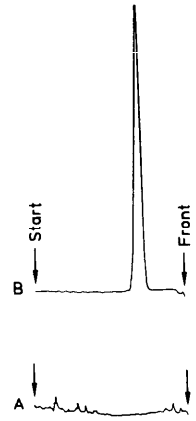


Fig. I: Fluorescence scan of a blank track (A) and of a chromatogram track with 4 ng testosterone.

Analogous examples have been described for "silica gel chromatograms". Table 2.2 gives an overview.

Table 2.2: Summary of some examples of fluorimetric detection after merely heating silica gel layers after chromatography.

Substances	Temperature/time	Remarks	Ref.
Essential oil components	800–900 °C	Induction of fluorescence in a special apparatus.	[18]
Steroids, e.g. cholesterol, triolein, androsterone; sugars, e.g. fructose, glucose, ribose; amino acids, pyrimidines, purines, alkaloids	110–150 °C, 2–12 h	Conversion to fluorescent derivatives by heating.	[5]
Alkaloids, e.g. raubasine and its metabolites in plasma, urine and bile	120 °C, 1 h	Amplification of the natural fluorescence of raubasine ($\lambda_{fl} = 482$ nm), detection limit 20 ng.	[10]
Alkaloids, e.g. reserpine, rescinnamine	105 °C, 2 h	Induced fluorescence ($\lambda_{fl} > 500$ nm, cut off filter). Possibly formation of 3-dehydro derivatives.	[19]
Alkaloids, e.g. reserpine, ajmaline, rescinnamine	105 °C, 2 h or 105 °C, 15 h	Induction of stable fluorescence ($\lambda_{fl} > 480$ nm, cut off filter), detection limits 5–20 ng.	[12]
Alkaloids, e.g. cocaine, ecgonine, benzoylecgonine, ecgonine methyl ester	280 °C, 8 min or 260 °C, 10–30 min	Pale blue induced fluorescence ($\lambda_{fl} > 390$ nm, cut off filter), fluorescence amplification by a factor of 2 on dipping in liquid paraffin solution; detection limits: <10 ng.	[13]
Alkaloids, e.g. lupanine, angustifoline, sparteine, lupinine, hydroxylupanine	130 °C, 17–35 h	Induced blue fluorescence ($\lambda_{fl} = 400$ nm), detection limits: 10 ng.	[6]
Pesticides, e.g. dursban, azinphos-methyl, menazon, imidan, phosalone, zinophos	200–225 °C, 20–120 min	Induced fluorescence or amplification of natural fluorescence; detection limits: 10–300 ng.	[20]
Organophosphorus pesticides, e.g. coumaphos, menazon, maretin, dursban	200 °C, 45 min	Induced fluorescence or amplification of natural fluorescence, detection limits: 1–80 ng.	[21]

Table 2.2: (continued)

Substances	Temperature/time	Remarks	Ref.
Pesticides, e.g. fuberidazol	200 °C, 45 min	Amplification of the natural fluorescence of some pesticides and bathochromic shift of the excitation and emission maxima; detection limits: 5–100 ng.	[22]
Pesticides, e.g. coumatetralyl, methabenzthiazuron, propylisom, naptalam, thioquinox, warfarin etc.	200 °C, 45 min	Induced fluorescence ($\lambda_{fl} > 430$ nm, cut off filter); detection limits: 6–600 ng.	[23]
Coumaphos	200 °C, 20 min	Residue analysis; induced fluorescence on heating ($\lambda_{fl} > 400$ nm); detection limit: 1 ng.	[11]
Potasan, coumaphos, coroxon	200 °C, 20 min	Induced blue fluorescence ($\lambda_{fl} = 430$ nm or 450 nm), identification of the fluorescent derivatives as chlorferon or 4-methylumbelliferone.	[24]
Coumaphos	200 °C, 20 min	Residue determination in honey, induced fluorescence ($\lambda_{fl} > 400$ nm, cut off filter); detection limit: 0.5 ng.	[14]
Rubratoxin B	200 °C, 10 min	Induced fluorescence that can be intensified by gassing the previously heated chromatogram plates with ammonia vapors (10 min). This also alters the color of the emitted light to pale blue.	[25]
Glucose or methylglucosides	135 °C, 3 min or 140 °C, 10 min	Induced yellow fluorescence.	[26]
Sugar derivatives	"Mild heating over a Bunsen burner"	No details of whether fluorescence was produced or if a carbonization reaction occurred.	[27]
Sugars, e.g. glucose, fructose, galactose, mannose etc.	160 °C, 10 min	Production of fluorescence by heating the chromatogram after covering it with a glass plate. Sugar alcohols and C_1-C_1 bonded oligosaccharides do not react; detection limit: 10 ng.	[28]

Table 2.2: (continued)

Substances	Temperature/time	Remarks	Ref.
Sugars, e.g. glucose, glucosamine, fucose, raffinose, cellobiose, methylated sugars	80 → 260 °C, gradient or 200 °C, 5 min	Production of fluorescence by temperature gradients (10 °C/30 s) to determine the optimum heating temperature for the individual substances. Oligosaccharides require higher temperatures than monosaccharides. Detection limit: 1 nMol. The fluorescence colors are characteristic particularly for the methylated sugars.	[3]
Lipids, e.g. β-sitosterol, geraniol, dolichol, squalene, cholesterol	200 °C, 15 min	Induced fluorescence; detection limits: <1 µg cholesterol.	[3]
C-Nucleosides	Moderate heating on a hot plate	No details of whether fluorescence or carbonization was produced.	[29]
Nomifensine and metabolites	70 °C, 2 h + UV_{254}	Heating and simultaneous UV irradiation produced intense yellow fluorescence ($\lambda_{fl} > 460$ nm, cut off filter).	[30]

Procedure Tested

Tropane Alkaloids [35, 36]

Cocaine, ecgonine, ecgonine methyl ester, benzoylecgonine

Layer: Silica gel **Temperature: 260 °C**

Reaction

At elevated temperatures and possibly under the catalytic influence of the sorbent surface there is probably elimination of functional groups to yield aromatic ring systems that are excited to fluorescence under long-wavelength UV light ($\lambda = 365$ nm).

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK) .
Mobile phase	Methanol – water – dioxane – sodium acetate solution (aqueous, 0.2 mol/L, pH 8.0) (60+28+12+10)
Migration distance	5 cm
Running time	30 min

Detection and result: The chromatogram was briefly dried in a stream of cold air then heated for 10 to 30 min at 260 °C in a drying oven. After cooling to room temperature (ca. 15 min) it was dipped in a solution of liquid paraffin – *n*-hexane (1 + 2) for 3 s. This stabilized the fluorescence and intensified it by a factor of about 2.

On examination under long-wavelength UV light ($\lambda = 365$ nm) ecgonine methyl ester (hR_f 30–35), cocaine (hR_f 45–50), ecgonine (hR_f 55–60) and benzoylecgonine (hR_f 70–75) appeared as pale blue fluorescent chromatogram zones on a dark background.

The detection limits were less than 10 ng substance per chromatogram zone.

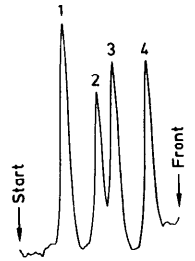


Fig. 1: Fluorescence scan of a chromatogram zone with 300 ng each of ecgonine methyl ester (1), cocaine (2), ecgonine (3) and benzoylecgonine (4) per chromatogram zone.

Note: The sodium acetate was added to the mobile phase solely to improve the separation. It had no detectable effect on the production of fluorescence during thermal activation, since the fluorescence reaction also occurred in the absence of sodium acetate.

In situ quantitation: The fluorimetric scan was carried out at $\lambda_{exc} = 313$ nm and the fluorescence emission was measured at $\lambda_{fl} > 390$ nm (cut off filter Fl 39) (Fig. I).

The last example for thermal activation to be discussed involves amino phases. Table 2.3 lists the publications concerning the specific detection of sugars and creatine derivatives by means of the fluorescence obtained on heating mobile phase-free "amino layer chromatograms".

Table 2.3: Summary of some examples of fluorimetric detection after thermal treatment of amino layers after chromatography.

Substances	Temperature/time	Remarks	Ref.
Sugars, e.g. lactose, glucose, fructose	120 °C, 15 min	Violet fluorescence on a dark blue background.	[31]
Sugars, e.g. lactose, glucose, fructose	120 °C, 15 min	Induced fluorescence; detection limits in nanogram range.	[32]
Glucose, fructose	Infrared lamp or 170 °C each for 3 min	Heating produced stable bluish-white fluorescence ($\lambda_{exc} = 365$ nm and $\lambda_{fl} > 400$ nm, cut off filter K 400), detection limits; 5–10 ng.	[33]
Sugars, e.g. glucose, rhamnose, xylose etc.	160 °C, 3–4 min or infrared lamp	Induction of brilliant stable fluorescence $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 400$ nm, (cut off filter K 400), sugar alcohols do not fluoresce; detection limits: 5–10 ng.	[2]
Creatine, creatinine, uric acid in urine and serum	150 °C, 3–4 min	Stable fluorescence $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 400$ nm, (cut off filter K 400).	[7]
Sugars, e.g. sucrose, ribose, xylose	150 °C, 3–4 min	Induced fluorescence $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 400$ nm, (cut off filter K 400).	[34]

Procedure Tested

Catecholamines, Serotonin and Metabolites [37]

Layer: NH_2 modified silica gel **Temperature: 150 °C**

Reaction

Heteroaromatic ring systems are formed presumably with loss of functional groups, at elevated temperatures and probably under the catalytic influence of the aminopropyl groups on the sorbent surface. The compounds so formed are excited to fluorescence by long-wavelength UV light ($\lambda = 365$ nm).

Method	Ascending, one-dimensional two-fold development (10 min intermediate drying in stream of cold air) in a trough chamber without chamber saturation.
Layer	HPTLC plates NH_2 F_{254s} (MERCK).
Mobile phase	Chloroform – 1-propanol – formic acid (50+10+5)
Migration distance	2 × 7 cm
Running time	2 × 30 min

Detection and result: The chromatogram was dried for 10 min in a stream of warm air and heated to ca. 150 °C under an infrared lamp, on a hot plate or in a drying cupboard for 3 to 4 min.

Noradrenaline ("hR_f" ~15)*), adrenaline ("hR_f" ~20), serotonin ("hR_f" ~35), vanilmandelic acid ("hR_f" ~45), creatinine ("hR_f" ~50), hydroxyindoleacetic acid ("hR_f" ~55) and homovanillic acid ("hR_f" ~85) appear on examination in long-wave-

*) The figures given here are calculated in the same manner as hR_f even though two developments were carried out.

length UV light ($\lambda = 365$ nm) as brilliant pale blue fluorescent chromatogram zones on a dark background (Fig. IA). Vanillic acid ("hR_f" ~90), on the other hand, only fluoresces weakly, but produces appreciable fluorescence quenching on NH_2 layers containing a fluorescence indicator (Fig. IB).

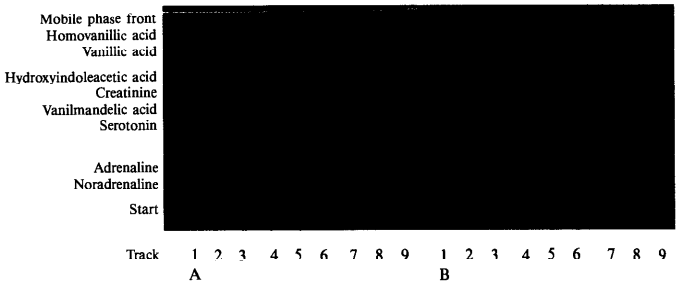


Fig. I: Chromatogram of catecholamines, serotonin and some metabolites together with creatinine: A) examination at $\lambda = 365$ nm, B) examination at $\lambda = 254$ nm.

The visual detection limits for fluorimetric detection are substance-dependent and lie between 5 ng (adrenaline, noradrenaline) and 30 ng (homovanillic acid) substance per chromatogram zone.

In situ quantitation: Fluorimetric evaluation was carried out by excitation at several wavelengths and by measuring the fluorescence emission. (Fig. II).

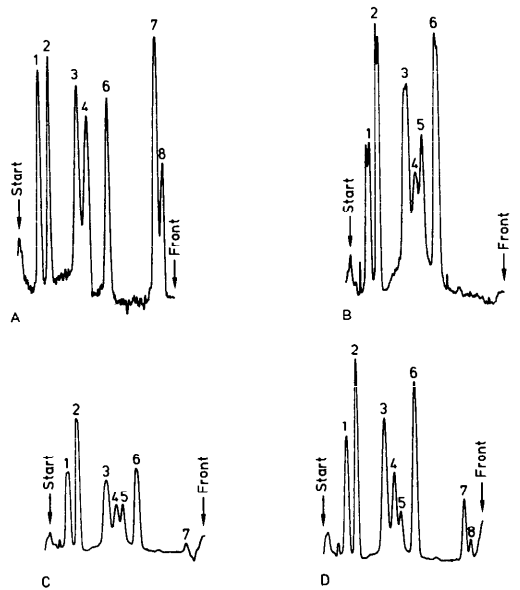


Fig. II: Fluorescence scan of a chromatogram track with 500 ng each of noradrenaline (1), adrenaline (2), serotonin (3), vanilmandelic acid (4), 5-hydroxyindoleacetic acid (6), homovanillic acid (7) and vanillic acid (8) together with 230 ng creatinine, all per chromatogram zone: measurement at $\lambda_{exc} = 313$ nm and $\lambda_{fl} > 390$ nm (cut off filter Fl 39 (A)), $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 430$ nm (cut off filter Fl 43 (B)), $\lambda_{exc} = 405$ nm and $\lambda_{fl} > 460$ nm (cut off filter Fl 46 (C)) and at $\lambda_{exc} = 436$ nm and $\lambda_{fl} > 560$ nm (cut off filter Fl 56 (D)). Under the conditions used for scans B and C some substances appear as double peaks on account of fluorescence quenching at the center of the chromatogram zones as a consequence of the substance concentration being too high.

References

[1] Alperin, D. M., Idoyaga-Vargas, V. P., Carminatti, H.: *J. Chromatogr.* **1982**, *242*, 299–304.
[2] Klaus, R., Fischer, W., Hauck, H. E.: *Chromatographia* **1990**, *29*, 467–472.
[3] Alperin, D. M., Carminatti, H., Idoyaga-Vargas, V. P., Couso, R. O.: *J. Chromatogr.* **1983**, *265*, 193–200.

[4] Egg, D.: *J. Chromatogr.* **1973**, *86*, 151–157.
[5] Segura, R., Gotto, A. M.: *J. Chromatogr.* **1974**, *99*, 643–657.
[6] Karlsson, E.-M., Peter, H. W.: *J. Chromatogr.* **1978**, *155*, 218–222.
[7] Klaus, R., Fischer, W., Hauck, H. E.: *Chromatographia* **1991**, *32*, 307–316.
[8] Egg, D., Huck, H.: *J. Chromatogr.* **1971**, *63*, 349–355.
[9] Funk, W.: *Fresenius Z. Anal. Chem.* **1984**, *318*, 206–219.
[10] Löhr, J. P., Bartsch, G. G.: *Arzneim. Forsch.* **1975**, *25*, 870–873.
[11] Mallet, V., Brun, G. L.: *Bull. Environm. Contam. Toxicol.* **1974**, *12*, 739–744.
[12] Duez, P., Chamart, S., Vanhaelen, M., Vanhaelen-Fastré, R., Hanocq, M., Molle, L.: *J. Chromatogr.* **1986**, *356*, 334–340.
[13] Funk, W., Dröschel, S.: *J. Planar Chromatogr.* **1991**, *4*, 123–126.
[14] Taccheo, M. B., De Paoli, M., Spessotto, C.: *Pestic. Sci.* **1989**, *25*, 11–15.
[15] Funk, W., Schanze, M., Wenske, U.: *GIT Fachz. Lab. Supplement 3 „Chromatographie"* **1983**, 8–16.
[16] Caissie, G. E., Mallet, V. N.: *J. Chromatogr.* **1976**, *117*, 129–136.
[17] Huck, H.: *J. Chromatogr.* **1975**, *110*, 125–131.
[18] Heidbrink, W.: *Fette, Seifen, Anstrichm.* **1964**, *66*, 569–573.
[19] Duez, P., Chamart, S., Hanocq, M.: *J. Planar Chromatogr.* **1991**, *4*, 69–76.
[20] Brun, G. L., Surette, D., Mallet, V.: *Intern. J. Environ. Anal. Chem.* **1973**, *3*, 61–71.
[21] Brun, G. L., Mallet, V.: *J. Chromatogr.* **1973**, *80*, 117–123.
[22] Mallet, V., Surette, D., Brun, G. L.: *J. Chromatogr.* **1973**, *79*, 217–222.
[23] Mallet, V., Surette, D.: *J. Chromatogr.* **1974**, *95*, 243–246.
[24] Volpe, Y., Mallet, V.: *Anal. Chim. Acta* **1976**, *81*, 111–116.
[25] Whidden, M. P., Davis, N.D., Diener, U. L.: *J. Agric. Food Chem.* **1980**, *28*, 784–786.
[26] Dubra, M. S., Alperin, D. M., Sagedahl, A., Idoyaga-Vargas, V. P., Carminatti, H.: *J. Chromatogr.* **1982**, *250*, 124–128.
[27] Azhayev, A. V., Smrt, J.: *Collect. Czech. Chem. Commun.* **1978**, *43*, 1520–1530.
[28] Büchele, B., Lang, J.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1979**, *2*, 585.
[29] Szabó, I. F., Somsák, L., Farkas, I.: *Acta Chim. Hung.* **1984**, *115*, 319–325.
[30] Sistovaris, N.: *J. Chromatogr.* **1983**, *276*, 139–149.
[31] Okamoto, M., Yamada, F.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1982**, *5*, 163–164.
[32] Okamoto, M., Yamada, F., Omori, T.: *Chromatographia* **1982**, *16*, 152–154.
[33] Klaus, R., Fischer, W., Hauck, H. E.: *Chromatographia* **1989**, *28*, 364–366.
[34] CAMAG: *Application note* A-58, 1992.
[35] Dröschel, S.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[36] Dröschel, S.: German patent No. 4037686.
[37] Klaus, R., Fischer, W., Hauck, H. E.: Private communication, Darmstadt, 1992.

1.3 Electrochemical Activation

Electromagnetic radiation energy can be used to stimulate substances to fluorescence after separation by thin-layer chromatography. Its action makes it possible to convert some nonfluorescent substances into fluorescent derivatives. The "active" sorbents often act as catalysts in such processes (cf. Chapter 1.1).

The supply of thermal energy by a heater or IR lamp is a second method of converting the separated substances into fluorescent derivatives. Here too, at about their decomposition temperatures, many substances react to form fluorescent derivatives, generally with the catalytic participation of the "active sorbent". These fluorescent derivative often provide specific evidence concerning the nature of the substances being detected (cf. Chapter 1.2).

A third possibility of converting nonfluorescent substances to fluorescent derivatives, without the use of additional reagents apart from the stationary phase on which the separation has been carried out, is to place the plate in a "plasma chamber" for some time.

The application of high tension (e.g. 20 kV, 0.5 MHz) in an evacuated system (0.2 ... 8 torr) causes the residual gas to form a highly ionized mixture of positive and negative ions, electrons, photons and neutral gas molecules. In the presence of "active" sorbents this plasma reacts with the chromatographically separated substances to yield reactive ions and radicals.

Depending on the structures of the substances being investigated the chromatograms are exposed to the effects of the plasma chamber for 5 to 300 s and then heated to ca. 130 °C for 1 to 2 min [1] or irradiated with long-wavelength UV light for 3 min [2]. This causes the formation of fluorescent derivatives with the sorbent again acting as a welcome catalyst.

The residual gas in the plasma chamber can either be a noble gas, nitrogen, methane or hydrogen. Argon yields less intensely fluorescent substances and oxygen permits oxidation reactions that usually lead to nonfluorescent final products. The results obtained by exposing to activated ammonia vapor [1] or acid vapors [3] reveal that pH displacements can also have positive effects here. Ammonium hydrogen carbonate vapors also behave favorably. SEGURA and GOTTO [4] used them for the induction of fluorescence using thermal activation many years ago.

Some examples of substances present in chromatogram zones being made highly fluorescent by exposure to electrochemical stimulation in an atmosphere of nitrogen are listed in Table 3 [1]. The plates used were "Permakote" silica gel layers containing no organic binder. However, silica gel 60 HPTLC plates (MERCK) were also employed; these yield a considerable background fluorescence on account of the organic binder they contain.

It is also possible to ionize the gases at normal atmospheric pressure instead of using a plasma chamber [2]. In this case the gas is passed through a high tension field at a flow rate of 5 to 30 L/min (spark discharge: 20 kV, 0.5 MHz) and blown onto the chromatogram (Fig. 20). As in the case of the electric vacuum discharge-chamber described above the chromatographed substances are activated to intense fluorescence emission. Under favorable conditions (low background layer fluorescence) it is still possible to detect 1 ng chlorpromazine or n-$C_{22}H_{46}$ visually. Hence this mode of activation is just as sensitive as the thermochemically generated fluorescence described in Chapter 1.2 [3].

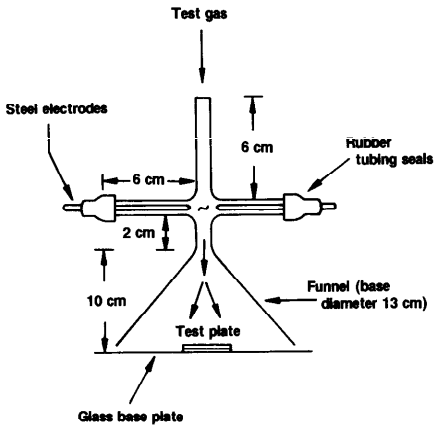


Fig. 20: Schematic representation of an electric spark discharge chamber for the activation of gases at normal atmospheric pressure for the production of fluorescence in substances separated by thin-layer chromatography [2].

Table 3: Some substances that produce intense fluorescence when treated with ionized nitrogen after they have been chromatographed [1].

Substance	Exposure time [s]	Substance	Exposure time [s]
Cholesterol	60	Oleic acid	180
Cholesteryl pelargonate	60	Morphine	180
Progesterone	60	Codeine	180
Testosterone	60	Cocaine	180
Dieldrin	60	Dimerol	180
Tetrahydrocannabinol	60	Phenobarbital	180
Inositol	60	Chlorpromazine	180
Laurylalcohol	180	d-Amphetamine sulfate	180
n-$C_{22}H_{46}$	180	Methadone	180
Phenol	180		

References

[1] Shanfield, H., Hsu, F., Martin, A. J. P.: *J. Chromatogr.* **1976**, *126*, 457–462.
[2] Shanfield, H., Lee, K. Y., Martin, A. J. P.: *J. Chromatogr.* **1977**, *142*, 387–397.
[3] Zhou, L., Shanfield, H., Wang, F.-S., Zlatkis, A.: *J. Chromatogr.* **1981**, *217*, 341–348.
[4] Segura, R., Gotto, A. M.: *J. Chromatogr.* **1974**, *99*, 643–657.

2 Reagents for the Recognition of Functional Groups

The aim of most screening methods is to produce a yes/no decision, concerning whether the concentration of a certain substance in a sample exceeds a given limiting concentration or not. For instance, if the concentration of a substance lies below a permitted maximum concentration then there is probably no need to analyse the sample. However, if the content is in the region of or above the permitted limit, then the result must be confirmed by means of an exact quantitative determination.

Such yes/no decisions are of great importance in foodstuffs control and environmental analysis. They also play an important role in pharmacy in the form of content uniformity tests. Without suitable screening methods for rapid detection of positive samples it would scarcely be possible to carry out economic doping controls and toxicological investigations or to recognize medicament abuse.

Thin-layer chromatography is an excellent screening method because:

- Many samples can be chromatographed alongside each other.
- It is possible to chromatograph reference substances on the same TLC/HPTLC plate and thus compare unknown samples in the same defined system.
- Only a few milliliters of mobile phase are required for the separations so that there are scarcely any disposal problems.
- The whole chromatogram can be taken in at a glance and an immediate comparative evaluation can be made.
- It is often possible to carry out any clean-up step that may be necessary in the concentrating zone of a suitable chromatographic plate, in any event clean-up is less complex than for other forms of chromatography.
- There is no necessity to regenerate the sorbent since TLC/HPTLC plates are not generally used twice.

The unequivocal recognition or exclusion of particular substances in question is of especial importance for such screening methods. As far as the chemist is concerned this can involve a deliberate search for substances with particular functional groups; particular questions that might require answering might include the following:

- Does the sample contain a substance with a carbonyl group or one that has been produced by oxidation of an alcoholic OH group?
- Does the reduction of the sample molecule lead to the formation of substances containing amino groups?

- Are there substances in the sample capable of coupling reactions and where are they to be found in the chromatogram?

These few questions serve to demonstrate that there must be great interest in characterizing chemical compounds by means of their reactive functional groups. The most important group-specific reagents for postchromatographic derivatization are listed alphabetically in Table 4 below:

Table 4: Reagents suitable for the recognition of functional groups.

Functional group	Reagent	Remarks	Reference
Acetylene compounds	Dicobaltoctacarbonyl	Formation of colored complexes. After the reagent excess has been washed out, reaction with bromine vapor yields cobalt bromide, which reacts with α-nitroso-β-naphthol to yield red chromatogram zones on an almost colorless background.	[11]
Aldehydes	4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald reagent)	Aldehydes yield violet chromatogram zones on a whitish-yellow background. Some alcohols form yellow to orange-colored chromatogram zones.	[2]
Aldehydes	2,4-Dinitrophenyl-hydrazine	Formation of colored hydrazones or osazones. It is possible to distinguish between saturated and unsaturated hydrazones using potassium hexacyano-ferrate(III) [5].	[3, 4]
Aldehydes	Hydrazine sulfate + hydrochloric acid	Aromatic aldehydes yield colored hydrazones.	[6]
Alcohols	4-(4-Nitrobenzyl)-pyridine	Amino compounds, esters and ethers do not interfere, but phenols and acids as well as epoxides, olefins and substances containing labile halogen probably do.	[7]
Alcohols (diols, polyols, sugars)	Lead(IV) acetate – dichlorofluorescein	Diol cleavage of vicinal diols, e.g. sugars, sugar alcohols. The lead tetraacetate consumed is no longer available to decompose the fluorescent dichlorofluorescein.	[3, 8]

Table 4: (continued)

Functional group	Reagent	Remarks	Reference
Amines (primary)	Ninhydrin	Reddish or bluish chromatogram zones are produced, amino sugars and amino acids also react. Unexpectedly ascorbic acid also reacts.	[3, 9]
Amines (primary aliphatic and aromatic)	Diphenylboric anhydride + salicylaldehyde (DOOB)	Fluorescent reaction products are produced.	[3, 10, 11]
Amines (primary)	*o*-Phthalaldehyde (OPA)	In the presence of mercaptoethanol *o*-phthalaldehyde reacts with primary amines and amino acids to yield fluorescent isoindole derivatives.	[3, 12]
Amines (primary)	Trinitrobenzenesulfonic acid (TNBS)	On heating primary amines react with TNBA to yield intensely colored MEISENHEIMER complexes. Amino acids also react.	[3, 13]
Amines (primary)	Fluorescamine	Primary aliphatic and aromatic amines yield fluorescent derivatives. Primary aromatic amines yield stable yellow-colored derivatives that can be eluted from the TLC layer [16].	[3, 14], [15]
Amines (primary aromatic)	Sodium nitrite + α-naphthol or BRATTON-MARSHALL reagent	Diazotization of the primary amine followed by coupling with α-naphthol or N-(1-naphthyl)-ethylenediamine. Sulfonamides also react [18].	[3, 17]
Amines (primary aromatic)	4-(Dimethylamino)-benzaldehyde + acid	Alkaloids and indole derivatives also react [19].	[17]
Amines (capable of coupling)	Fast blue salt B, fast blue salt BB, fast black salt K, diazotized sulfanilic acid (PAULY's reagent), diazotized sulfanilamide or 4-nitroaniline	Intensely colored azo dyes are produced. Catecholamines [20], imidazoles [21] and phenols also react.	[3, 17]

Table 4: (continued)

Functional group	Reagent	Remarks	Reference
Amines (primary and secondary)	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	Fluorescent 4-nitrobenzofurazan derivatives are produced. Phenols and thiols also react.	[3]
Amines (primary and secondary aromatic)	*p*-Chloranil	The reaction depends on the catalytic effect of silica gel. Monochlorobenzene, as solvent for the reagent, also contributes. There is no reaction on cellulose layers.	[17, 22]
Amines (secondary aliphatic and alicyclic)	Sodium nitroprusside + acetaldehyde	Secondary aliphatic and alicyclic amines yield blue-colored chromatogram zones (e.g. morpholine, diethanolamine).	[23, 24]
Amines (long-chain primary, secondary and tertiary plus quaternary ammonium salts	Cobalt(II) thiocyanate	Long-chain primary, secondary and tertiary amines and long-chain quaternary ammonium salts yield blue chromatogram zones on a pink background.	[25]
Carboxyl groups (carboxylic acids)	Indicators, e.g. bromocresol green, bromocresol green + bromophenol blue + potassium permanganate, bromocresol purple, methyl red + bromothymol blue	Detection depends on the color change of the indicator in acid medium. Quaternary ammonium salts give a color change in some cases [2].	[2, 26] [3, 27]
Carboxyl groups (carboxylic acids)	2,6-Dichlorophenolindophenol (TILLMANN's reagent)	Organic acids release the red undissociated acid from the blue mesomerically stabilized phenolate anion. Reductones reduce the reagent to a colorless compound.	[3] [28, 29]
Carboxyl groups (carboxylic acids)	Aniline + aldose (e.g. glucose)	The action of acid causes glucose to be converted to furfural which reacts with aniline to yield a colored product.	[3]

Table 4: (continued)

Functional group	Reagent	Remarks	Reference
Halogen derivatives	Silver nitrate, ammoniacal (DEDONDER's, TOLLENS' or ZAFFARONI's reagent)	Halogen compounds yield black chromatogram zones on a pale gray background.	[2]
Ketones	2,4-Dinitrophenylhydrazine	Formation of colored hydrazones or osazones. It is possible to distinguish between saturated and unsaturated hydrazones using potassium hexacyanoferrate(III) [5].	[3, 4]
Nitro derivatives	Benzylcyanide + benzyltrimethylammonium hydroxide	Nitro compounds, e.g. explosives, or pesticides containing nitro groups yield gray to bluish-green chromatogram zones on a brownish background.	[30]
Peroxides	1-Naphthol + N^4-ethyl-N^4-(2-methanesulfonamidoethyl)-2-methyl-1,4-phenylenediamine (peroxide reagent)	A quinonimine dyestuff is produced on reaction with peroxides.	[3]
Peroxides	Iron(II) sulfate + ammonium thiocyanate	Peroxides rapidly oxidize iron(II) to iron(III) ions which react to yield brown-red iron(III) thiocyanate complexes.	[31, 32]
Peroxides	Potassium iodide + starch	Peroxides release free iodine which forms a blue complex with the starch.	[17, 33]
Peroxides	N,N-Dimethyl-1,4-phenylenediamine (N,N-DPDD), N,N,N′,N′-tetramethyl-1,4-phenylenediamine (TPDD)	Peroxides, e.g. alkyl hydroperoxides, oxidize N,N-DPDD to WURSTER's red and TPDD to WURSTER's blue.	[17] [34]
Phenols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	Fluorescent 4-nitrobenzofuran derivatives are produced. Primary and secondary aromatic amines and thiols also react.	[3]

Table 4: (continued)

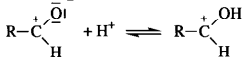
Functional group	Reagent	Remarks	Reference
Phenols (capable of coupling)	Fast blue salt B, fast blue salt BB, fast black salt K, diazotized sulfanilic acid (PAULY's reagent) diazotized sulfanilamide or 4-nitroaniline	Intensely colored azo dyes are formed. Catecholamines [20, 35], imidazoles [21] and amines capable of coupling also react.	[3, 17]
Thiols, thioethers, disulfides	Sodium metaperiodate + benzidine	Substances with divalent sulfur yield white chromatogram zones on a blue background.	[36]
Thiols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	Fluorescent 4-nitrobenzofuran derivatives are formed. Primary and secondary aromatic amines and phenols also react.	[3]

It should be remembered that the group-specific reagents listed in Table 4 are rarely completely selective, for there are almost always a few substances that do not contain the particular group and yet give a comparable reaction! The detection of ascorbic acid with ninhydrin is a spectacular example of this. Ninhydrin usually reacts with primary amino groups. The other side of the coin is that there is scarcely a more sensitive reagent for vitamin C even though this substance does not contain an amino group.

In addition the role played by the sorbent on which the chromatography is carried out must not be neglected. For instance, it is only on aluminium oxide layers and not on silica gel that it is possible to detect caffeine and codeine by exposure to chlorine gas and treatment with potassium iodide – benzidine [37]. The detection limits can also depend on the sorbent used. The detection limit is also a function of the hR_f value. The concentration of substance per chromatogram zone is greater when the migration distance is short than it is for components with high hR_f values. Hence, compounds with low hR_f values are more sensitively detected.

These reactions at particular functional groups of the sample molecule are closely related in an inverse sense with those reagents which bring their own functional group into the molecule. The numerous "aldehyde – acid" reactions are an example. Numerous monographs of such reactions are already included in Volume Ia. Their reactivity depends on the ready polarizability of the carbonyl group as a result of the inductive effect of the carbonyl oxygen.

The carbonyl group also possesses electrophilic properties at the carbon atom and nucleophilic properties at the oxygen atom. Nucleophilic attack of the carbonyl group is favored if this is attached to an aromatic ring (inductive effect) and there is also a methoxy or phenolic OH group present in the 4-position. Changing a neutral reaction medium by proton addition has the same effect.



Three large groups of substances are suitable nucleophilic reaction partners, namely:

- bases
- C-H acidic compounds
- crypto bases.

The boundaries between these groups are not always easily delineated. Nevertheless, the classification is useful in practice [38]. A selection of *"base" reactions* is listed in Table 5.

Table 5: Reactions of bases with carbonyl compounds, a selection.

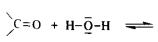
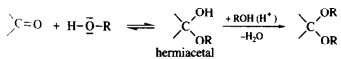
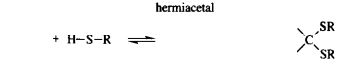
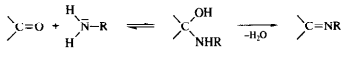
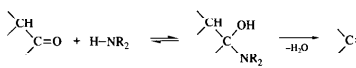
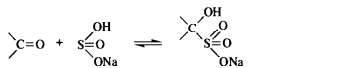
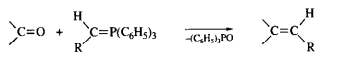
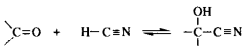
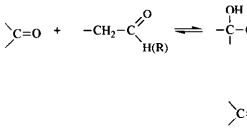
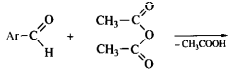
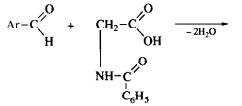
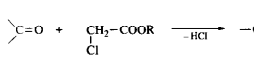
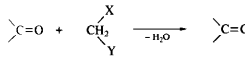
Reactants	Reaction/reaction product
	Hydrates
	Acetals and ketals
	Mercaptals

Table 5: (continued)

Reactants	Reaction/reaktion product
	SCHIFF's bases
	Oximes
	(Substituted) hydrazones
	Semicarbazones
	Enamines
	Bisulfite addition compounds
	WITTIG reaction
	Hydrogenation to alcohols

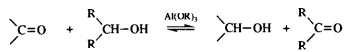
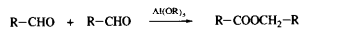
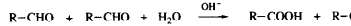
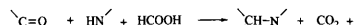
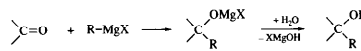
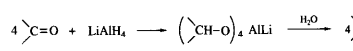
C-H acidic compounds do not possess any basic properties. But they can form anions in the presence of strong bases, and these possess sufficiently strong nucleophilic properties to be able to add to a polarized carbonyl group. Examples are listed in Table 6.

Table 6: Reaction of carbonyl compounds (aldehydes, ketones) with C-H acidic compounds, a selection.

Reactant	Reaction/reaction product
	Cyanhydrins
	Ethinylization
	Aldol addition Aldol condensation
	PERKIN reaction
	ERLENMEYER reaction
	DARZEN's glycide ester synthesis
	KNOEVENAGEL condensation

It is not possible to draw a sharp boundary between the reaction of C-H acidic substances (e. g. aldol reactions) and the reactions of *cryptobases*. The cryptobases include organometallic compounds and metal hydrides, whose alkyl residues or hydrogen atoms are rendered negative by the +I effect of the metal and, hence, are readily transferred to a carbonyl group together with their bonding electrons. Hydrogen atoms attached to carbon atoms can also react when they are subject to great electron pressure. Here the presence of LEWIS acids induces reactions which usually take place via a cyclic transition state where all electron transfers are simultaneous. The named reactions listed in Table 7 are examples.

Table 7: Reactions of carbonyl groups with cryptobases.

Reactant	Reaction/reaction product
	MEERWEIN-PONNDORF reduction OPPENAUER oxidation
	CLAISEN-TIŠCENKO reaction
	CANNIZZARO reaction
	LEUCKART-WALLACH reaction
	GRIGNARD reaction
	Reduction with complex hydrides

These basic reaction mechanisms occur with many of the reagents treated in Volumes 1a and 1b. The following examples can be listed:

- 4-Aminobenzoic acid + sugars
- 2-Aminodiphenyl – sulfuric acid + carbonyl compounds
- 4-Aminohippuric acid + monosaccharides
- 4-Aminohippuric acid – phthalic acid + sugars
- Aniline – diphenylamine – phosphoric acid + sugars
- Aniline – phosphoric acid + carbohydrates
  Aniline – phthalic acid + sugars
- Anisaldehyde – phthalic acid + sugars
- *p*-Anisidine – phthalic acid + oligosaccharides
- 4-(Dimethylamino)-cinnamaldehyde – hydrochloric acid + indole derivatives
- 2,4-Dinitrophenylhydrazine + carbonyl compounds
- EHRLICH'S REAGENT + indole derivatives
- EP reagent + terpenes
- MARQUIS' reagent + alkaloids
- 1,2-Phenylenediamine – trichloroacetic acid + ascorbic acid
- PROCHAZKA's reagent + indole derivatives
- VAN URK's reagent + indole derivatives

References

[1] Schulte, K. E., Ahrens, F., Sprenger, E.: *Pharm. Ztg.* **1963**, *108*, 1165–1169.
[2] De Kruif, N., Schouten, A.: *Parfümerie und Kosmetik* **1991**, *72*, 386–398.
[3] Reagent monograph in: Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin-Layer Chromatography Reagents and Detection Methods*, Vol. 1a, VCH-Verlagsgesellschaft, Weinheim, Cambridge, New York, 1990.
[4] Holloway, P. J., Challen, S. B.: *J. Chromatogr.* **1966**, *25*, 336–346.
[5] Mehlitz, A., Gierschner, K., Minas, T.: *Chemiker Ztg.* **1963**, *87*, 573–576.
[6] Sundt, E., Saccardi, A.: *Food Techn.* **1962**, *16 (4)*, 89–91.
[7] Pomonis, J. G., Seversom, R. F., Freeman, P. J.: *J. Chromatogr.* **1969**, *40*, 78–84.
[8] Wassermann, L., Hanus, H.: *Naturwissenschaften* **1963**, *50*, 351.
[9] Fahmi, R. A., Niederwieser, A., Pataki, G., Brenner, M.: *Helv. Chim. Acta* **1961**, *44*, 2022–2026.
[10] Hohaus, E.: *Fresenius Z. Anal. Chem.* **1982**, *310*, 70–76.
[11] Hohaus, E.: *Fresenius Z. Anal. Chem.* **1984**, *319*, 533–539.
[12] Lindeberg, E.G.G.: *J. Chromatogr.* **1976**, *117*, 439–441.
[13] Munier, R. L., Peigner, A., Thommegay, C.: *Chromatographia* **1970**, *3*, 205–210.
[14] Singh, A. K., Granley, K., Ashraf, M., Mishra, U.: *J. Planar Chromatogr.* **1989**, *2*, 410–419.
[15] Nowicki, H. G.: *J. Forensic Sci.* **1976**, *21*, 154–162.
[16] Rinde, E., Troll, W.: *Anal. Chem.* **1976**, *48*, 542–544.
[17] Compare the corresponding reagent monograph in this volume.
[18] Bicán-Fišter, T., Kajganovic, V.: *J. Chromatogr.* **1963**, *11*, 492–495.
[19] Heacock, R. A., Mahon, M. E.: *J. Chromatogr.* **1965**, *17*, 338–348.
[20] Segura-Cardona, R., Soehring, K.: *Med. exp.* **1964**, *10*, 251–257.
[21] Grimmett, M. R., Richards, E. L.: *J. Chromatogr.* **1965**, *20*, 171–173.
[22] Pires, L. M., Roseira, A. N.: *J. Chromatogr.* **1971**, *56*, 59–67.
[23] Macek, K., Hacaperková, J., Kakác, B.: *Pharmazie* **1956**, *11*, 533–538.
[24] Kloubek, J., Marhoul, A.: *Collect. Czech. Chem. Commun.* **1963**, *28*, 1016–1021.
[25] Lane, E. S.: *J. Chromatogr.* **1965**, *18*, 426–430.
[26] Kirchner, J. G., Miller, J. M., Keller, G. J.: *Anal. Chem.* **1951**, *23*, 420–425.
[27] Touchstone, J. C., Dobbins, M. F.: *Practice of Thin Layer Chromatography*, J. Wiley & Sons, New York, Chichester, Brisbane, Toronto 1978.
[28] Franc, J., Hájková, M., Gehlicka, M.: *Chem. Zvesti* **1963**, *17*, 542.
[29] Passera, C., Pedrotti, A., Ferrari, G.: *J. Chromatogr.* **1964**, *14*, 289–291.
[30] Ebing, W.: *Chimia* **1967**, *21*, 132–133.
[31] Stahl, E.: *Chemiker Ztg.* **1958**, *82*, 323–329.
[32] Maruyama, K., Onoe, K., Goto, R.: *Nippon Kagaku Zasshi* **1956**, *77*, 1496–1498; *Chem. Abstr.* **1958**, *52*, 2665b.
[33] Stahl, E.: *Arch. Pharm.* **1960**, *293*, 531–537.
[34] Knappe, E., Peteri, D.: *Fresenius Z. Anal. Chem.* **1962**, *190*, 386–389.
[35] Wagner, G.: *Pharmazie* **1955**, *10*, 302–304.
[36] Stephan, R., Erdman, J. G.: *Nature* **1964**, *203*, 749–749.
[37] Gänshirt, H.: *Arch. Pharm.* **1963**, *296*, 73–79.
[38] *Organikum*, VEB Deutscher Verlag der Wissenschaften, Berlin (DDR) 1977.

3 Reagent Sequences

Thin-layer chromatography has the great advantage that the result of the separation is stored – usually invisibly – on the TLC/HPTLC plate as on a diskette. In such cases it needs developing or detecting, rather like an exposed film. This can now be done on-line or off-line so that the analyst can decide which method to use to detect the separated substances.

Physical methods, some of which can be applied in the on-line mode (Fig. 8) and *physiological methods* providing information concerning the effectivity of the separated substance can be used. A later volume will treat these bioautographic methods which can be subdivided into bioassays (off-line) and bioautogrammes (on-line).

The *microchemical detection* methods are the most economical methods and the simplest for the chemist to carry out. No expensive apparatus is required and in certain circumstances they can be combined with the two detection methods mentioned above.

The aim of microchemical investigations on TLC/HPTLC plates is to provide information concerning identity and/or purity. Quantitation is not usually carried out but is possible at any time. The simple absence of a reaction is frequently taken as evidence of the absence of a particular substance.

If color reactions occur these serve to help characterize the substance. They can only ever act as a pointer to the presence of a substance, but never as proof even when accompanied by a separation process. Unequivocal identification requires a mosaic of many pieces of information (hR_f values, color reactions UV/VIS, IR, Raman, mass spectra etc).

As is well known the difficulty of analysis of a sample increases as its complexity increases. Analysis usually commences with a rather nonspecific clean-up step and requires that the separation step that follows be highly selective and depends on a detection step that is as specific as possible. As the selectivity of detection increases there is also an increase in the reliability of the identification and it is possible to reduce the demands made on the selectivity of the preceding separation method. This is the case for radiometric and enzymatic methods and also explains the popularity of fluorescence measurements. The latter obtain their selectivity from the freedom to choose excitation and measurement wavelengths.

Color reactions are more or less clearly defined reactions of the substances with suitable reagents. Substance-specific reactions are not usually available, e. g. many compounds with aromatic skeletons give both a positive VITALI-MORIN reaction [1–4] and a positive MARQUIS reaction [4]. Again, numerous aldehydes react with electron-rich compounds in acidic medium to yield colored substances (cf. Chapters 2 and 3.1).

Reactions can be exploited more specifically if it is known that particular functional groups are present [cf. Chapter 2]. They still do not allow direct identification, but they increase the specificity of the evidence. The chromatographic separation carried out before detection also contributes to this. This reduces the number of potential components. However, this does not exclude the possibility that there might be several substances in the particular part of the chromatogram involved. This not only applies to thin-layer chromatography but also applies with equal force to other microanalytical separation methods (GC, HPLC).

It is often possible to increase the selectivity of detection by carrying out a sequence of reactions on one and the same chromatographic plate – a technique that is only possible in thin-layer chromatography. In principle it is possible to distinguish between two sorts of reagent sequence ("TYPE A" and "TYPE B"), which are discussed in this and the next volume.

The examples that are treated below are those sequences where all steps – except the last – are preparations for a color or fluorescence derivatization reaction which is carried out in the last step, i.e. they can be regarded as a sort of selective in situ pretreatment for a final detection reaction. Such reaction sequences are frequently necessary because all the reagents cannot be mixed together in a single solvent, or because it is necessary to dry, heat or irradiate with UV light between the individual reaction steps. The detection of aromatics by the reaction sequence "nitration – reduction – diazotization – couple to form an azo dye" is an example of this type (Fig 21).

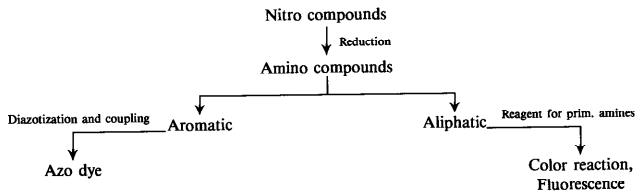


Fig. 21: Reaction scheme for the detection of aromatics, by means of the reaction sequence, nitration, reduction, diazotization and coupling to an azo dye, and of aliphatic nitro compounds by detection of the primary amino group produced on reduction.

The "true" reaction sequences that will be described in Volume 1c are frequently used in toxicological analysis, since the unequivocal identification of medicaments, intoxicants and addictive drugs in body fluids almost always requires the simultaneous detection of many possible substances with completely different chemical characteristics. For

this purpose various staining reagents, each of which can be used individually for a particular substance class, are applied consecutively to the same chromatogram. After each application the result is evaluated under visible or UV light and the result recorded photographically, if necessary, for it is possible that the result obtained might be destroyed or made unrecognizable by the next reaction step. (Fig. 22).

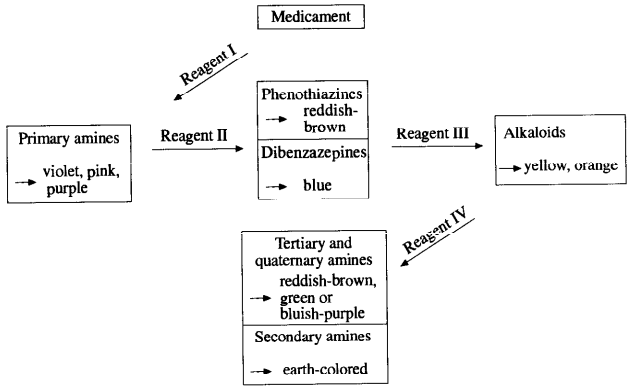
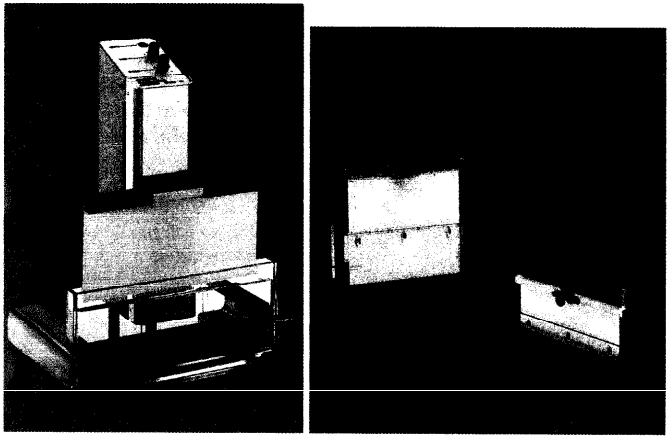


Fig. 22: Scheme for type B reagent sequence (cf. the reagent sequence "ninhydrin – iron(III) chloride – DRAGENDORFF – potassium iodoplatinate" [6]).

Therefore, such sequential in situ reactions are always carried out either in order to prepare a substance for a color reaction that is to follow or to increase the amount of information that is obtained by exploiting a combination of different independent reactions. This provides information that could not be obtained using one single reagent.

Both types of reagent sequence are frequently used when the samples are complex in nature (plant extracts, urine, environmental samples etc.). It can happen that an excess of reagent A could interfere with the reaction of the following reagent B. With the dipping technique it is also possible that reagent transfer might occur making it only possible to use reagent B once.

In such cases it is possible to use intermediate "rinsing troughs" in the form of appropriately prepared dipping chambers (Fig. 23) or diffusion destaining apparatus, such as is used in electrophoresis (Fig. 24). These can also be used sometimes to destain the layer background when single reagents are used (cf. potassium hexaiodoplatinate reagent).



A B

Fig. 23: Chromatogram dipping device III (CAMAG) (A), (DESAGA) (B).

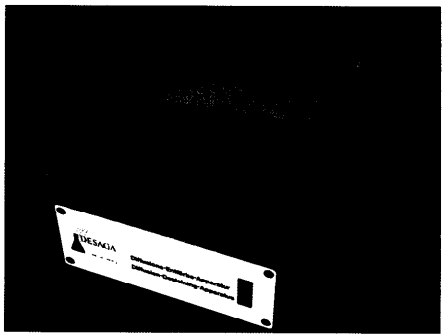


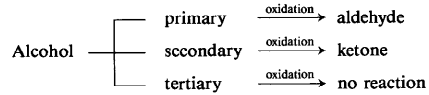
Fig. 24: Diffusion destaining apparatus (DESAGA). The dish contains the wash liquid and is periodically tilted so that the reagent excess is removed from the chromatogram plate.

Thin-layer chromatography usually involves the adsorption chromatographic separation of substance mixtures into polarity groups. It is well known that clean looking chromatographic peaks can "hide" several substances. For instance, primary, secondary and tertiary alcohols are to be found at very nearly the same hR_f.

It is only possible to distinguish them on the basis of prechromatographic reactions. Here it is possible to acetylate primary alcohols without difficulty while leaving tertiary alcohols unreacted under the conditions chosen.

Secondly it is possible to carry out "functional chromatography" within the framework of a two-dimensional development [6–8]. The first separation is followed by an in situ reaction of the sample substance on the layer; the chromatogram is then developed perpendicular to the direction of the first chromatogram (SRS technique). The decision concerning the type of alcohol, is then made on the basis of the positions of the chromatogram zones: esters migrate appreciable further than their parent alcohols and acids.

Thirdly, if it is not possible to apply the SRS technique, it can be established whether a primary, secondary or tertiary alcohol is present by oxidizing the alcohol on the chromatographic zone and then subjecting the oxidation product to a detection reaction. On oxidation primary alcohols form aldehydes, secondary alcohols ketones and tertiary alcohols are not oxidized.



If a group-specific reagent is now used, e.g. one that is chosen to react specifically with the reducing properties of aldehydes (ammoniacal silver nitrate solution) or to react with ketones (2,4-dinitrophenylhydrazine [9]) it is very simple to determine which form of alcohol is present in the sample.

This example demonstrates the following:

- The derivatization is always carried out with an aim in mind,
- Group-specific reagents can provide evidence to characterize the substance,
- The desired results can be obtained by multiple chromatography or multiple in situ reaction at the same chromatogram zone.

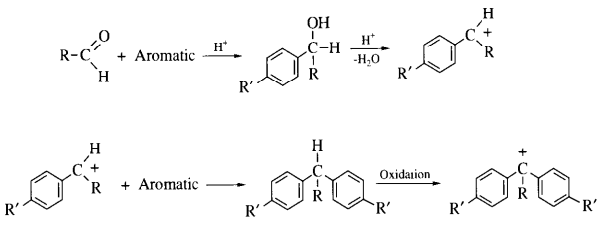
This leads to savings of time and materials.

The specific detection of aromatic nitro compounds is a second example. These can be converted by reduction to primary amines, which are then diazotized and coupled to yield azo dyes (cf. reagent sequence "Titanium(III) chloride – BRATTON-MARSHALL reagent"). Sodium nitrite–naphthol reagent, diazotized sulfanilic acid and other reagents specific for amino groups (e.g. ninhydrin, fluorescamine, DOOB, NBD chloride [9]) can also be used in the second stage of the reaction (Fig. 21).

Finally some reagent sequences are included that lead to a selectivity increase on detection. These frequently yield cationic, anionic or neutral polymethynes, azo dyes, quinonoid or indigoid dyes [4]. Polymethyne radicals, polyenes and metal and charge transfer complexes are also represented [4]. With such a large number of possibilities it is self-evident that any list will be incomplete and that some reaction sequences can be assigned to various reaction categories. The examples included are taken from the literature and have been included without their having been checked by the authors. A gray bar at the side of the text makes this evident.

3.1 Electrophilic Substitutions

Many known color reactions involve electrophilic substitution at an electron-rich aromatic or heteroaromatic (cf. "4-(dimethylamino)-benzaldehyde–acid reagents" and "vanillin reagents"). Here aliphatic or aromatic aldehydes react in acid medium to yield polymethyne cations which are intensely colored di- or triarylcarbenium ions [4, 10].



Fomaldehyde (MARQUIS [11] and PROCHAZKA reagent), furfural, 4-methoxybenzaldehyde, 4-(dimethylamino)-cinnamaldehyde and 4-(dimethylamino)-benzaldehyde (EHRLICH'S, VAN URK'S, MORGAN-ELSON or EP reagents) react according to this scheme with

phenols, pyrrole or indole derivatives as reaction partners [4]. Examples are to be found in the reagent monographs.

Primary alcohols can be selectively detected using reagent sequences involving an initial oxidation to yield aldehydes that are then reacted in acid medium with electron-rich aromatics or heteroaromatics, according to the above scheme, to yield intensely colored triphenylmethane dyes.

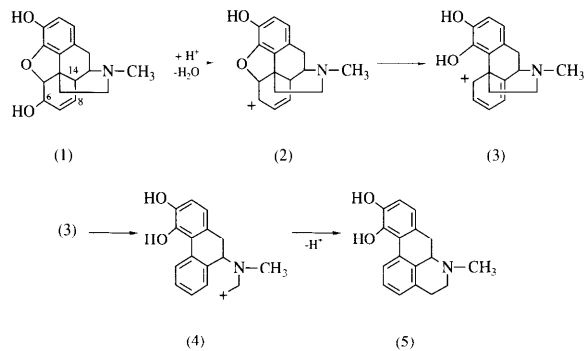
Secondary alcohols yield ketones on oxidation and these can be reacted with 2,4-dinitrophenylhydrazine to yield the corresponding colored hydrazones.

These examples form a link with derivatizations depending on redox reactions.

3.2 Oxidations and Reductions

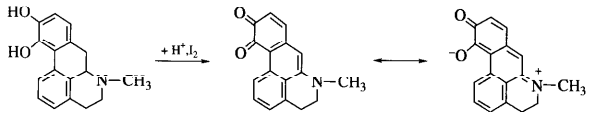
Substances that can oxidize to form a system of conjugated double bonds are frequently oxidized by atmospheric oxygen, iodine or iron(III) salts. The products are chromophoric systems frequently containing *ortho*- or *para*-quinoid structure.

For instance morphine (**1**) can be detected by the formation of various quinones via apomorphine as intermediate [4, 12, 13]. All morphines with an OH group in the 6-position and a $\Delta^{7,8}$ double bond (codeine, ethylmorphine etc.) first undergo an acid-catalyzed rearrangement according to the following scheme [12]:



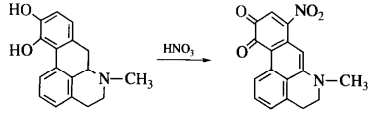
Here the alcoholic hydroxyl is first protonated and then eliminated as water. The allylcarbenium ion (**2**) is initially stabilized by elimination of the proton at C-14. Then the ether link is opened after protonation of the ring oxygen with the formation of carbenium ion (**3**), whereby the neighboring C-C bond of the piperidine ring is cleaved with aromatization of the C ring. The carbenium ion (**4**) formed is stabilized by elimination of a proton and ring closure to apomorphine (**5**).

The *ortho* diphenolic structure of apomorphine makes it a strongly reducing substance; hence, in acid medium it forms the blue colored *ortho*-quinone (**6**) with iodine or other oxidizing agent which is in equilibrium with its zwitterionic limiting structure (**7**) (PELLARGI's reaction [14]).



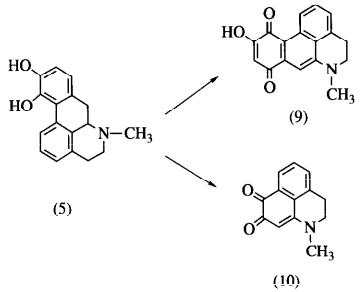
(5) (6) (7)

The HUSEMANN and ERDMANN identification reactions for morphine and codeine in the DAB 9 (German Pharmacopoeia) involve the formation of the red-colored *ortho*-quinone (**8**) via apomorphine (**5**) under the influence of nitric acid with the simultaneous nitration of the benzene ring [15].



(5) (8)

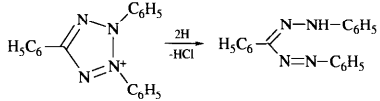
In alkaline medium, in contrast, apomorphine (**5**) yields the two quinones (**9**) and (**10**) [16]:



(5) (9) (10)

Redox reactions can naturally lead to the formation of numerous other colored substances in addition to quinonoid and radical chromophores. These include:

- The thalleiochin reaction for the specific detection of quinine alkaloids carrying an oxygen group at C-6 of the quinoline nucleus (e. g. quinine and quinidine) [17], or the
- KOBER reaction of phenolic steroids with strong acids leading to polymethyne dyes [18, 19].
- The well-known triphenyltetrazolium chloride (TTC) reaction for the detection of α-ketolsteroids, pyridinium carbinols and pyridinium glycols can also be included here [20–23]. The chromophore system of the red-colored formazan dye produced by reduction of the TTC is composed of highly conjugated double bonds resulting from the combination of a phenylhydrazone group with an azo group:



TTC Formazan

The following examples of reagent sequences, which include the reagent "Ammonium monovanadate – *p*-anisidine" described in the second part of the book can also be classified as redox reactions.

4-Aminobenzenesulfonic Acid/ 8-Hydroxyquinoline–Thionyl Chloride–Ammonia Vapors

Vries, G., Brinkman, U.A.T.: "A Sensitive and Selective Reaction for Nitrate-Application in Thin-Layer Chromatography", *Mikrochim. Acta (Vienna)* 1984, 47–52.

Reagent Sequence for:

- Nitrate and nitrite ions

Preparation of the Reagents

Reagent 1 **Coupling reagent**
Dipping solution 1: Dissolve 500 mg 4-aminobenzenesulfonic acid and 150 mg 8-hydroxyquinoline in 100 ml of a mixture of acetone – diethyl ether – water (12 + 12 + 1). This solution is stable for several months. It should be topped up with diethyl ether from time to time to make up for evaporation losses.

Reagent 2 **Reduction**
Dipping solution 2: Dissolve 3 g thionyl chloride in 100 ml decane.

Reagent 3 **Adjustment of pH**
Ammonia vapor: Place 5 to 10 ml conc. ammonia solution in the free trough of a twin-trough chamber.

Reaction

Nitrates and nitrites are first reduced to nitrosyl chloride with thionyl chloride. The volatile nitrosyl chloride then reacts with 4-aminobenzenesulfonic acid to yield a diazonium salt that then couples with 8-hydroxyquinoline to form a colored azo compound. Hence, the coupling reagent is applied to the chromatogram first.

Method

The dried chromatogram is first dipped in reagent solution 1 for 1 s, dried briefly in a stream of cold air and then dipped in reagent solution 2 for 1 s. The TLC/HPTLC plate is then held upright on tissue paper to allow excess reagent to drain away; when the layer appears matt it is covered with a glass plate and kept at room temperature for 5 min. Afterwards it is dried in a stream of hot air and exposed to ammonia vapor.

Nitrate and nitrite ions yield orange-brown to magenta-red chromatogram zones on a pale yellow background immediately on treatment with ammonia; these zones are stable for days in an atmosphere of ammonia.

Note: When combined with thin-layer chromatographic separation the reagent provides a specific detection method for nitrate and nitrite. The color development is often completed within a few minutes on silica gel plates. In the absence of ammonia vapor traces of oxides of nitrogen in the laboratory atmosphere can slowly cause the background to become reddish-brown. The simultaneous presence of the following ions in the chromatogram zones interferes with the detection of nitrate/nitrite: I^-, IO_3^-, IO_4^-, MoO_4^{2-} and $H_2PO_2^-$.

The detection limits are slightly dependent on the layer involved, lying between 5 and 10 ng (RP 8, RP 18, silica gel 60) and 50 ng (cellulose, NH_2) per chromatogram zone. The detection of 20 ng nitrate is not prevented by a one hundred-fold excess of the following ions: NH_4^+, Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, Hg^{2+}, Al^{3+}, Sb^{3+}, Bi^{3+}, Zr^{3+}, Ag^+, Ni^{2+}, Mn^{2+}, UO_2^{2+}, F^-, CLO_4^-, Br^-, CN^-, NCS^-, BO_2^-, SiO_3^{2-}, SiF_6^{2-}, AsO_4^{2-}, AsO_4^{3-}, SO_3^{2-}, $S_2O_3^{2-}$, SO_4^{2-}, OH^- and CH_3COO^-. Colored ions such as Fe^{3+}, Cu^{2+}, Cr^{3+} etc. can, however, interfere.

The reagent can be employed on silica gel, kieselguhr, Si 50000, RP, NH_2 and cellulose layers.

tert-Butyl Hypochlorite–Potassium Iodide/Starch

Purkayastha, R.: "Simultaneous Detection of the Residues of Atrazine and Linuron in Water, Soil, Plant and Animal Samples by Thin-Layer Chromatography", *Internat. J. Environ. Anal. Chem.*: **1971**, *1*, 147–158.

Reagent Sequence for:

- *s*-Triazine and urea herbicides
  e. g. atrazine, linuron

Preparation of the Reagents

Reagent 1 **Chlorination**
Spray solution 1: Mix 1 ml *tert*-butyl hypochlorite with 100 ml cyclohexane.

Reagent 2 **Oxidation**
Spray solution 2: Dissolve 1 g potassium iodide and 1 g soluble starch in 100 ml water.

Reaction

The *s*-triazines undergo chlorination at nitrogen to yield reactive N-chloro derivatives which oxidize iodide to iodine in the second step. This then forms an intense blue iodine-starch inclusion complex with starch.

Method

First spray the dried chromatogram homogeneously with reagent 1. Then remove excess reagent in a stream of cold air in the fume cupboard (ca. 30 min for silica gel and 3 h for aluminium oxide layers). Then spray the chromatogram lightly with reagent 2.

Urea herbicides give pale yellow and *s*-triazines blue chromatogram zones on a colorless background.

Note: The reagent sequence is a modification of the "chlorine–potassium iodide-starch" reagent.

The detection limits – dependent on the TLC layer – lie between 0.2 and 0.5 µg substance per chromatogram zone for atrazine and linuron.

The reagent can be employed on aluminium oxide, silica gel, kieselguhr and Si 50000 layers.

tert-Butyl Hypochlorite–Potassium Iodide/*p*-Tolidine

[1] Halstrom, J., Brunfeldt, K., Thomsen, J., Kovács, K.: "Synthesis of the Protected C-Terminal Lys^5-Heptapeptide of Eledoisin by the Merrifield Method", *Acta Chem. Scand.* **1969**, *23*, 2335–2341.
[2] Halstrom, J., Kovács, K., Brunfeldt, K.: "Synthesis of the N-Trityl Hexapeptide Hydrazide Corresponding to the Sequence 152–157 of the Coat Protein of Tobacco Mosaic Virus. Comparison of the Homogeneous and the Solid Phase Syntheses", *Acta Chem. Scand.* **1973**, *27*, 3085–3090.

Reagent Sequence for:

- Protected amino acids and peptides [1, 2]

Preparation of the Reagents

Reagent 1 **Chlorination**
Spray solution 1: tert-Butyl hypochlorite solution.

Reagent 2 **Oxidation**
Spray solution 2: Solution of potassium iodide and *p*-tolidine in glacial acetic acid/water.

Reaction

Reactive chloramine derivatives are produced in the first reaction step as a result of chlorination of the nitrogen by the *tert*-butyl hypochlorite; in the presence of potassium iodide these derivatives oxidize *p*-tolidine to a deep blue semiquinonoid dye (cf. reagent monograph "chlorine – *o*-tolidine – potassium iodide").

Method

The dried chromatogram is evenly sprayed with the first and then with the second reagent.

Intensely colored chromatogram zones are produced on a colorless background.

Note: The reagent sequence can be employed on silica gel, kieselguhr and Si 50000 layers.

tert-Butyl Hypochlorite–Potassium Iodide/*o*-Toluidine

Heitz, W., Höcker, H., Kern, W., Ullner, H.: „Darstellung und Eigenschaften von linearen Oligourethanen aus Diethylenglykol und Hexamethylendiisocyanat", *Makromol. Chem.* 1971, *150*, 73–94.

Reagent Sequence for:

- Oligourethanes

Preparation of the Reagents

Reagent 1 **Chlorination**
tert-Butyl hypochlorite vapor: Place 10 ml *tert*-butyl hypochlorite in one half of a twin-trough chamber.

Reagent 2 **Oxidation**
Spray solution: Slowly mix a solution of 1.6 g *o*-toluidine in 30 ml glacial acetic acid with a solution of 2 g potassium iodide in 500 ml water.

Reaction

First there is chlorination of nitrogen to yield reactive N-chloro derivatives, which oxidize iodide to iodine in the next step. Finally oxidation of the *o*-toluidine probably yields colored quinonoid toluidine derivatives.

Method

The chromatogram is freed from mobile phase in the drying cupboard (10 min 160 °C) and placed while still hot in the chamber with *tert*-butyl hypochlorite vapor for 5 min. After removal of excess reagent (15 min stream of warm air) the chromatogram is sprayed with reagent 2.

This yields deeply colored chromatogram zones on a pale background.

Note: The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Danger warning: *o*-Toluidine is suspected of possessing carcinogenic properties. Therefore, the dipping method should be used if possible, if it is decided to use the method in spite of this (cf. reagent monograph "Chlorine–*o*-toluidine reagent").

Calcium Hypochlorite–Formaldehyde–Potassium Iodide/Starch/Triton X-100

[1] Schwarz, D.P., Sherman, I.T.: "Improved N-Chlorination Procedure for Detecting Amides, Amines and Related Compounds on Thin-Layer Chromatograms", *J. Chromatogr.* **1982**, *240*, 206–208.
[2] Schwartz, D.P., McDonough, F.E.: "Practical Screening Procedure for Chloramphenicol in Milk at Low Parts per Billion Level", *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 563–565.

Reagent Sequence for:

- Amines, amino acids, amides [1, 2]
  e.g. chloramphenicol, creatine, adenine, guanine
  histidine, phenylalanine, sphingosine

Preparation of the Reagents

Reagent 1 **Chlorination**
Chlorine gas: Cover the base of a tightly sealable chromatographic trough with solid calcium hypochlorite to a depth of ca. 0.5 cm. This salt must be renewed weekly [1, 2].

Reagent 2 **Reduction of excess chlorine**
Formaldehyde gas: Fill one trough of a twin-trough chamber with 20 ml formalin solution (37%) [1, 2].

Reagent 3 **Oxidation and complex formation**
Spray solution: Dissolve 1 g potato starch, 1 g potassium iodide and 50 mg Triton X-100 in 100 ml water with warming [1].

Reaction

Primary and secondary amines and amides are first chlorinated at nitrogen by the chlorine released by the gradually decomposing calcium hypochlorite. Excess chlorine gas is then selectively reduced in the TLC layer by gaseous formaldehyde. The reactive chloramines produced in the chromatogram zones then oxidize iodide to iodine, which reacts with the starch to yield an intense blue iodine-starch inclusion complex.

Method

The chromatogram is dried in a stream of warm air and placed in the trough chamber with the calcium hypochlorite (reagent 1) for 2 min followed by 30–45 s in the free trough of a twin-trough chamber with the formalin solution (reagent 2). The chromatogram is then sprayed with reagent 3.

Intense blue-black chromatogram zones are produced on a colorless background.

Note: This reagent sequence is a modification of the reagent "chlorine – potassium iodide – starch". Mobile phases containing ammonia must be removed completely before treatment with the reagent sequence, since otherwise the background will be colored too. Some secondary amines (e.g. diphenylamine) and some amides (e.g. 2,4-dinitrobenzamide) and methionine sulfoxide do not give reactions even in quantities of up to 1 to 2 μg.

The detection limits for primary and secondary amines and for the amides are 10 to 60 ng substance per chromatogram zone [1]. The detection limits on layers with fluorescence indicators are about double the amount of substance [1].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Cerium(IV) Sulfate/Sodium Arsenite/ Sulfuric Acid–Methylene Blue– Ammonia Vapor

Naidoo, S.: "Separation of Acetic and Propionic Acid Analogs of L-Thyroxine and L-Triiodothyronine by Thin-Layer Chromatography", *Anal. Biochem.* **1978**, *91*, 543–547.

Reagent Sequence for:

- Iodide ions and organic iodine compounds
  e.g. thyroxine, triiodo- and tetraiodothyronine

Preparation of the Reagents

Reagent 1 **Redox reaction**

Spray solution 1: Mix solutions 1 and 2 in a volume ratio of 2 + 3 immediately before use.

Solution 1: Dissolve 10 g cerium(IV) sulfate in 100 ml sulfuric acid (10%).

Solution 2: Dissolve 5 g sodium metaarsenite in 100 ml water.

Reagent 2 **Redox reaction**

Spray solution 2: Dissolve 50 mg methylene blue in 100 ml water.

Reagent 3 **Neutralization of excess acid**

Ammonia vapor: Concentrated ammonia solution in one half of a twin-trough chamber.

Reaction

The course of the reaction has not been elucidated. Probably redox reactions involving cerium(IV) and arsenic(III) are catalyzed by iodide ions and organic iodine compounds with methylene blue acting as a redox indicator.

Method

The dried chromatogram is sprayed homogeneously first with spray solution 1 and then with reagent 2. Finally the chromatogram is exposed to an atmosphere of ammonia.

Iodides and organic iodine compounds produce brilliant blue chromatogram zones on a yellow background.

Note: The detection limits for iodides and organic iodine compounds are reported to be 50 to 100 ng substance per chromatogram zone.

The reagent can be employed on silica gel II layers.

Phosphoric Acid–Phosphomolybdic Acid

[1] Mikac-Devic, D., Mišic, M., Stankovic, H.: "Quantitative Method for the Determination of 17-Oxosteroid Fractions by Thin-Layer Chromatography", *Z. Klin. Chem. Klin. Biochem.* **1970**, *8*, 361–363.
[2] Detter, F., Kollmeier, W., Klingmüller, V.: „Das Muster der neutralen 17-Ketosteroide des Harns aufgrund der Dünnschicht-Chromatographie", *Z. Klin. Chem. Klin. Biochem.* **1967**, *5*, 153–155.

Reagent Sequence for:

- 17-Oxosteroids (17-ketosteroids) [1, 2]
  e. g. dihydroxyandrosterone, androsterone, aetiocholanolone, 11-oxoandrosterone

Preparation of the Reagents

Reagent 1 **pH adjustment**
Spray solution 1: Phosphoric acid (30%).

Reagent 2 **Redox reaction**
Spray solution 2: Alcoholic phosphomolybdic acid (10% in ethanol).

Reaction

In spite of the numerous publications the reaction mechanism is still not finally clarified. A large number of organic compounds can be oxidized by phosphomolybdic acid, with the reduction of some of the Mo^{VI} to Mo^{IV}, which then reacts with the remaining Mo^{VI} to yield a blue-gray mixed oxide (= molybdenum blue). The reduction of phosphomolybdic acid is pH-dependent.

Method

The dried chromatograms are first homogeneously sprayed with reagent 1 and then heated to 110 °C for 10 min. After cooling to room temperature they are sprayed with reagent 2 and then heated again to 110 °C for 10 min.

17-Oxosteroids produce blue-black chromatogram zones on colorless to pale yellow backgrounds.

Note: The derivatized steroids can be extracted from the blue chromatogram zones with alcohol and quantitatively determined by means of the ZIMMERMANN reaction, which is not interfered with by the presence of phosphoric acid and phosphomolybdic acid. A yellow background can be bleached by exposure to ammonia vapor [2].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide–4-Aminoantipyrine–Potassium Hexacyanoferrate(III)

Gosselé, J.A.W.: "Modified Thin-Layer Chromatographic Separation of Preservatives", *J. Chromatogr.* **1971**, *63*, 433–437.

Reagent Sequence for:

- Antioxidants
  e.g. 4-hydroxybenzoic acid and its esters

Preparation of the Reagents

Reagent 1 **Hydrolysis**
Spray solution 1: Dissolve 10 g sodium hydroxide pellets in 100 ml water.

Reagent 2 **Condensation**
Spray solution 2: Dissolve 2 g 4-aminoantipyrine in 100 ml ethanol.

Reagent 3 **Oxidation**
Spray solution 3: Dissolve 8 g potassium hexacyanoferrate(III) in 100 ml water.

Reaction

When oxidized by iron(III) ions 4-aminoantipyrine reacts with phenols to yield colored quinonoid derivatives (cf. 4-aminoantipyrine – potassium hexacyanoferrate(III) reagent in Volume 1a). It is an oxidative coupling based on the EMERSON reaction.

Method

The dried chromatogram is first sprayed homogeneously with spray solution 1 and then heated to 80 °C for 5 min. After cooling to room temperature the TLC plate is sprayed with water and heated to 80 °C for another 5 min, after which it is homogeneously lightly sprayed with reagent 2 and then with reagent 3.

Red to reddish-brown chromatogram zones are produced on a pale background.

Note: It is recommended that only small quantities of reagents be sprayed.

The detection limits lie between 0.25 and 1 µg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide-Cobalt(II) Acetate–*o*-Tolidine

Patil, V.B., Sevalkar, M.T., Padalikar, S.V.: "Thin-layer Chromatographic Detection of Endosulfan and Phosphamidon by Use of Cobalt Acetate and *o*-Tolidine", *J. Chromatogr.* **1990**, *519*, 268–270.

Reagent Sequence for:

- Insecticides
  e.g. endosulfan, phosphamidon

Preparation of the Reagents

Reagent 1 **Hydrolysis**
Spray solution 1: Dissolve 5 g sodium hydroxide pellets in 100 ml water.

Reagent 2 **Oxidation**
Spray solution 2: Dissolve 5 g cobalt(II) acetate in 100 ml water.

Reagent 3 **Oxidation**
Spray solution 3: Dissolve 1 g *o*-tolidine in 100 ml acetic acid (10%).

Reaction

The sulfite group present in the heterocyclic ring of endosulfan is rapidly hydrolyzed by alkali. In the presence of atmospheric oxygen it then oxidizes divalent cobalt to cobalt(III). Phosphamidon, which contains a 2-chloro-2-diethylcarbamoyl group, oxidizes divalent cobalt to trivalent, which then oxidizes *o*-tolidine to a blue-colored quinonoid derivative.

$$2\ Co^{2+} + 4\ OH^- + SO_3^{2-} + O_2 \longrightarrow 2\ CoO(OH) + SO_4^{2-} + H_2O$$

Method

Firstly, the dried chromatograms are homogeneously sprayed sequentially with spray solutions 1 and 2 and then, after being allowed to stand for 5 min, they are sprayed with reagent 3.

Intense blue chromatogram zones are produced on a colorless background; in acid medium they remain stable for ca. 30 min and then slowly fade.

Note: When the concentration of substance is sufficiently high endosulfan and phosphamidon sometimes appear as yellowish-brown zones after application of the first two reagents of the sequence.

The reagent sequence is specific for endosulfan and phosphamidon. Other insecticides, e.g. organochlorine insecticides, such as endrin, aldrin, dieldrin, DDT and BHC, organophosphorus insecticides, such as malathion, parathion, dimethoate, quinalphos, phorate and fenitrothion, or carbamate insecticides, such as baygon, carbaryl and carbofuran do not react. Neither is there interference from amino acids, peptides or proteins which might be extracted from the biological material together with the pesticides.

Warning: The substances benzidine and *o*-dianisidine, which are classified as carcinogenic, react in a similar manner to *o*-tolidine, which is also suspected of causing cancer.

The detection limits are 1 µg substance per chromatogram zone (corresponding to 10 µg/g biological material).

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide–Iodine/ Potassium Iodide/ Sodium Azide–Starch

Vandamme, E.J., Voets, J.P.: "Separation and Detection of Degradation Products of Penicillin and Cephalosporins by Means of Thin-Layer Chromatography", *J. Chromatogr.* 1972, *71*, 141–148.

Reagent Sequence for:

- Antibiotics with a thiazolidine ring
  e.g. penicillins, cephalosporins and their degradation products
  such as penicillin V, penicillin G, oxacillin, cloxacillin, ampicillin, methicillin, cephalosporin C
- Sulfur-containing amino acids
  e.g. cysteine

Preparation of the Reagents

Reagent 1 **Hydrolytic ring opening**
Spray solution 1: Sodium hydroxide (c = 2 mol/L).

Reagent 2 **Redox reaction (iodazide reaction)**
Spray solution 2: Dissolve 1 g sodium azide in a solution of 127 mg iodine and 200 mg potassium iodide in 100 ml water.

Reagent 3 **Iodine starch complex formation**
Spray solution 3: Dissolve 1 g soluble starch in 100 ml water.

Reaction

Detection depends on the "iodine – azide reaction" which is normally extremely slow but is accelerated in the presence of divalent sulfur (cf. iodine-potassium chloride solution – sodium azide – starch reagent, Awe's reagent). The reaction involves the conversion of iodine to iodide, so that the iodine is no longer available for formation of the intense blue-colored iodine starch inclusion complex. The sodium hydroxide used as reagent 1 presumably acts to open the thioether linkage or the thiazolidine ring. The sulfhydryl groups accelerate the iodine-azide reaction appreciably more than the sulfur bound to the thiazolidine ring.

Method

The dried chromatogram is homogeneously sprayed successively with reagents 1, 2 and 3.

Pale chromatogram zones are produced on an intense blue-colored background.

Note: Sulfides, thiols and thioethers also react. The blue background of the chromatogram treated with the reagent sequence fades with time.

The detection limits for penicillin derivatives and cephalosporins are 0.5 to 1 μg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sodium Hydroxide–4-Nitrobenzaldehyde–1,2-Dinitrobenzene

Khazanchi, R., Handa, S.K.: "Detection and Separation of Fenpropathrin, Flucythrinate, Fluvalinate and PP 321 by Thin-Layer Chromatography", *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 512–514.

Reagent Sequence for:

- Pyrethroids with α-cyano ester groups
  e.g. fenpropathrin, flucythrinate, fluvalinate, PP 321

Preparation of the Reagents

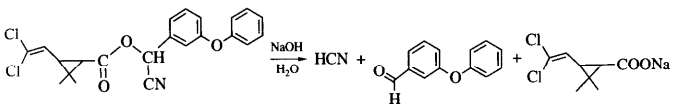
Reagent 1 **Hydrolysis**
Spray solution 1: Dissolve 1 g sodium hydroxide in 5 ml water and make up to 50 ml with methanol.

Reagent 2 **Cyanhydrin formation**
Spray solution 2: Dissolve 0.3 g 4-nitrobenzaldehyde in 10 ml 2-methoxyethanol (methylcellosolve).

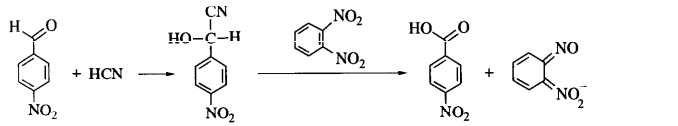
Reagent 3 **Formation of an *o*-quinoid di-anion**
Spray solution 3: Dissolve 0.25 g 1,2-dinitrobenzene in 10 ml 2-methoxyethanol.

Reaction

Synthetic pyrethroids with α-cyano ester group react with sodium hydroxide to yield hydrogen cyanide, which reacts with 4-nitrobenzaldehyde and 1,2-dinitrobenzene to yield a pink-colored derivative (*o*-quinonoid di-anion) according to the following scheme:



Cypermethrin
(NRDC 149)



4-Nitrobenzaldehyde cyanhydrin

4-Nitrobenzoic acid

o-Quinonoid dianion

Method

The chromatograms are dried in a stream of cold air and first sprayed homogeneously with reagent 1 and allowed to stand for 3 min. Then they are sprayed with reagent 2 followed immediately by reagent 3.

Pink-colored chromatogram zones are produced on a colorless background.

Note: Spray solutions 2 and 3 can be applied in any order or as a mixture.

The detection limits are 100 ng substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Tin(II) Chloride–Ammonium Thiocyanate

Gaibakian, D.S., Rozylo, J.K., Janicka, M.: "Thin-Layer Chromatography Method for the Investigations of Re(VII), Mo(VI) and V(V) ions mixtures", *J. Liq. Chromatogr.* **1985**, *8*, 2969–2989.

Reagent Sequence for:

- Transition metal anions
  e.g. $Re^{(VII)}$, $Mo^{(VI)}$, $V^{(V)}$

Preparation of the Reagents

Reagent 1 **Reduction**
Spray solution 1: Dissolve 10 g tin(II) chloride in 100 ml 6 mol hydrochloric acid.

Reagent 2 **Complex formation**
Spray solution 2: Dissolve 5 g ammonium thiocyanate in 10 ml water.

Reaction

The initial step of the reaction with tin(II) chloride reduces the highly oxidized metal in the transition metal anions to low valency cations; these are capable of forming stable colored complexes with thiocyanate.

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then, after a short interval, with reagent 2.

Rhenium, molybdenum and vanadium ions yield orange, pink and yellow-colored chromatogram zones respectively on a colorless background.

Note: Iron(III) cations also react and give the well known deep red iron(III) isothiocyanate.

No details of the detection limits were provided.

The reagent can be employed on aluminium oxide, silica gel, kieselguhr, Si 50000, RP and cellulose layers.

Tin(II) Chloride–Borate Buffer–Fluorescamine

Haefelfinger, P.: "Determination of Nanogram Amounts of Primary Aromatic Amines and Nitro Compounds in Blood and Plasma", *J. Chromatogr.* **1975**, *111*, 323–329.

Reagent Sequence for:

- Nitroaromatics
  e.g. funitrazepam

Preparation of the Reagents

Reagent 1 **Reduction**
Spray solution 1: Dissolve 4 g tin(II) chloride dihydrate in 100 ml acetic acid (5%) and add 1 ml of a solution of 0.5 g phenolphthalcin in 100 ml dioxane.

Reagent 2 **pH adjustment**
Spray solution 2A: Sodium hydroxide solution (2%).
Spray solution 2B: Dissolve 31 g boric acid in 100 ml 1 mol sodium hydroxide solution and dilute with 800 ml water. Adjust the pH of this solution to 8.4 and make up to 1000 ml with water.

Reagent 3 **Condensation**
Spray solution 3: Dissolve 100 mg fluorescamine in 100 ml acetone.

Reaction

In the first step tin(II) chloride in acetic acid solution reduces the aromatic nitro groups to amino groups. The aromatic amines produced then react with fluorescamine in weakly basic medium to yield fluorescent derivatives (cf. reagent monograph "Fluorescamine Reagent", Volume 1a).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until the layer begins to become transparent and then heated to 105 to 110 °C for 10 min. After cooling to room temperature they are then sprayed with reagent 2A until the background is pale purple (color change of the pH indicator phenolphthalein) and then sprayed with reagent 2B until the layer begins to appear transparent. When the layer is evenly wetted it is then sprayed with reagent 3 and dried in a stream of warm air (ca. 70 °C).

On examination under long-wavelength UV light ($\lambda = 365$ nm) there are yellow fluorescent chromatogram zones on a dark background.

Note: The detection limits for flunitrazepam and its 7-nitrodesmethyl metabolites are 1 to 2 ng/ml plasma or 0.5 ng substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Titanium(III) Chloride–4-(Dimethylamino)-benzaldehyde

Suzuki, T., Uchiyama, M.: "Pathway of Nitro Reduction of Parathion by Spinach Homogenate", *J. Agric. Food Chem.* **1975**, *23*, 281–286.

Reagent Sequence for:

- Oxidized aromatic amines
  e.g. metabolites of parathion

Preparation of the Reagents

Reagent 1 **Reduction**
Spray solution 1: Dissolve 0.5 g titanium(III) chloride in 100 ml N hydrochloric acid.

Reagent 2 **Condensation**
Spray solution 2: Dissolve 0.5 g 4-(dimethylamino)-benzaldehyde in a mixture of 50 ml ethanol and 50 ml glacial acetic acid.

Reaction

In a first step oxidized aromatic amines are reduced with titanium(III) chloride in glacial acetic acid solution and then condensed to a colored SCHIFF's base with 4-(dimethylamino)-benzaldehyde (cf. Chapter 2).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then, after an interval of a few minutes, with reagent 2.

The oxidized aromatic amines yield yellow-colored chromatogram zones on a colorless background.

Note: The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

3.3 Azo Coupling

Coupling reactions with diazonium salts to yield intensely colored azo derivatives have often been used for the detection of phenols, primary aromatic amines and electron-rich heterocyclics.

These reactions can be opened up to all substances that can yield aniline derivatives in acid or basic medium. Carbamic acid derivatives, numerous variations of which are used as plant treatment agents, provide a striking application. As do urea herbicides and a variety of drug substances e. g. benzodiazepines or phenylbutazone derivatives.

The aromatic nitro compounds make up another group of substances. These can also be formed directly on the TLC layer as a result of the frequently used VITALI reaction [24]. They are detected – as shown in Fig. 21 – by reduction and coupling to azo dyes.

The examples reproduced below have been taken from the literature.

Hydrochloric Acid Vapor–Sodium Nitrite/Hydrochloric Acid–Amidosulfonic Acid–N-(1-Naphthyl)-ethylenediamine

Bernhard, W., Jeger, A.N., De La Vigne, U.: "Identification and determination of hydrochlorothiazide [Esidrix®]", *J. Planar Chromatogr.* **1989**, *2*, 77–79.

Reagent Sequence for:

- Diuretics
  e. g. hydrochlorothiazide (Esidrix®)

Preparation of the Reagents

Reagent 1 **Hydrolysis**
Hydrochloric acid vapor: This can be generated by placing 10 ml fuming hydrochloric acid (37%) in one trough of a twin-trough chamber.

Reagent 2 **Diazotization**
Spray solution 1: Dissolve 4 g sodium nitrite in 40 ml water. The solution is stable over a long period.
Spray solution 2: Hydrochloric acid (10%).

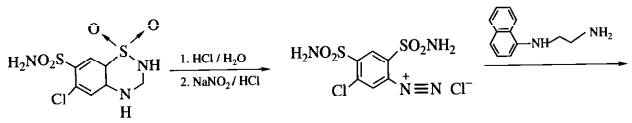
Reagent 3 **Destruction of excess nitrite**
Spray solution 3: Dissolve 500 mg ammonium amidosulfonate (amidosulfonic acid ammonium salt) in 100 ml water.

Reagent 4 **Coupling**

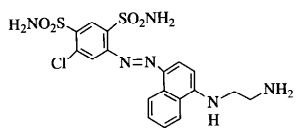
Spray solution 4: Dissolve 100 mg N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml ethanol. The solution should always be made up fresh even though it remains stable for several days.

Reaction

In the first reaction step the heterocyclic ring is opened by hydrolysis to yield a primary amino group, that is then diazotized and finally coupled with N-(1-naphthyl)-ethylenediamine to yield an azo dye.



Hydrochlorothiazide Diazonium salt



Red azo dye

Method

The dried chromatograms are first exposed to hydrochloric acid vapor (reagent 1) for a few minutes and then heated to 120 °C for 5 min to remove excess hydrochloric acid. After cooling to room temperature the TLC/HPTLC plates are successively sprayed homogeneously with spray solutions 1 and 2. They are then dried briefly in a stream of cold air, sprayed with reagent 3, dried once again in a stream of cold air and finally sprayed with reagent 4 and dried in a stream of warm air.

Red chromatogram zones are produced on a light background.

Note: When this reagent sequence is combined with the in situ measurement of the UV spectra of the chromatogram zones before derivatization this reagent sequence becomes virtually specific for hydrochlorothiazide and its related derivatives in the analysis of urine. Hence, it is possible to use it for drug monitoring and doping control.

The detection and determination limits are less than 0.1 and 0.2 mg hydrochlorothiazide per liter body fluid and thus appreciably lower than the therapeutic levels which are reported to be between 0.2 and 1.6 mg/l.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Iodine–Sodium Carbonate–Sulfanilic Acid, Diazotized

[1] Ritter, W., Plempel, M., Pütter, J.: „Drei Methoden zur Konzentrationsbestimmung von Clotrimazol in biologischem Material", *Arzneim. Forsch.* **1974**, *24*, 521–525.
[2] Ritter, W.: "Post-Chromatographic Derivatizations in Quantitative (HP)TLC", *Proc. 2nd Int. Symp. Instrum. High Perform. Thin-Layer Chromatogr. (HPTLC)* p. 100–113, Interlaken, 1982.

Reagent Sequence for:

- Imidazole antimycotics
  e.g. clotrimazole [1, 2]

Preparation of the Reagents

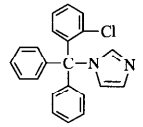
Reagent 1 **Cleavage by iodine**
Spray solution 1: Dissolve 1 g iodine in 100 ml ethanol.

Reagent 2 **pH adjustment**
Spray solution 2: Dissolve 20 g sodium carbonate in water and make up to 100 ml.

Reagent 3 **Coupling with PAULY's reagent**
Solution A: Dissolve 0.3 g sulfanilic acid in 100 ml 10% hydrochloric acid.
Solution B: Dissolve 10 g sodium nitrite in water and make up to 100 ml.
Spray solution 3: Mix 8 parts by volume solution A with 2 parts by volume solution B immediately before use; this mixture can be used for about 1 h.

Reaction

The mechanism of the reaction has not been elucidated. Presumably iodine eliminates the imidazole ring from N-substituted imidazole derivatives such as clotrimazole, and this then couples with diazotized sulfanilic acid to yield an azo dye.



Clotrimazole (Bay b 5097)

Method

The dried chromatograms are sprayed homogeneously with reagent 1, then heated up to 100 °C in a vacuum drying cupboard, first at atmospheric pressure for 15–20 min and then under vacuum for a further 5–10 min (removal of excess iodine). After cooling to room temperature the chromatograms are sprayed with reagent solution 2 and then dried at 100 °C (atmospheric pressure). The chromatograms are finally sprayed with reagent solution 3 and dried at 100 °C [1, 2].

Red to violet chromatogram zones, that can be recorded photometrically at $\lambda = 530$ nm, are produced on a colorless background.

Note: The reaction is very specific for N-substituted imidazole derivatives. In serum investigations the detection limit was 50 ng clotrimazole per milliliter serum. The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Nitric Acid–Sodium Dithionite–Sodium Nitrite–N-(1-Naphthyl)-ethylenediamine

Haefelfinger, P.: "Determination of Amitriptyline and Nortriptyline in Human Plasma by Quantitative Thin-Layer Chromatography", *J. Chromatogr.* **1978**, *145*, 445–451.

Reagent Sequence for:

- Aromatic substances
  e.g. antidepressives
  such as amitriptyline, nortriptyline

Preparation of the Reagents

Reagent 1 **Nitration**
Spray solution 1: Mix equal volumes of nitric acid (65%) and methanol while cooling with ice. The reagent solution may be kept for several weeks.

Reagent 2 **Reduction**
Spray solution 2: Dissolve 4 g sodium dithionite ($Na_2S_2O_4$) in 100 ml 0.5 mol phosphate buffer solution (pH 6.5). This solution is only stable for about 1 h, so it should always be made up fresh.

Reagent 3 **Diazotization**
Spray solution 3: Dissolve 400 mg sodium nitrite in 20 ml 1 mol hydrochloric acid solution. The solution should always be made up fresh.

Reagent 4 **Conpling**
Spray solution 4: Dissolve 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml methanol. This solution should be made up fresh, even though it is reported to be stable for several days.

Reaction

The first step of the reaction involves nitration of the aromatic skeleton of the substance to be detected. Then the aromatic nitro derivatives so produced are reduced with sodium dithionite, in acid medium, to the corresponding amines; these are then diazotized and coupled with N-(1-naphthyl)-ethylenediamine to yield an azo dye (cf. Fig. 21).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until the layer begins to become transparent and are then heated to 125–130 °C for 15 min. After cooling to room temperature the TLC/HPTLC plates are homogeneously sprayed with reagent solution 2 and heated to 80–85 °C for 8 min. After cooling to room temperature the plates are sprayed homogeneously with reagent 3 and then thoroughly dried (ca. 10 min) in a stream of cold air. Finally the chromatograms are sprayed with reagent 4 and then dried in a stream of warm air for 1 min.

Reddish chromatogram zones are formed on a light background.

Note: Derivatization with this reagent sequence in combination with extraction and TLC separation is specific for amitriptyline and nortriptyline in the analysis of plasma; furthermore its high sensitivity allows its employment in pharmacokinetic studies, e.g. after the oral administration of a single dose of 25 mg amitriptyline.

The plate should be heated as rapidly and as evenly as possible after the first spraying step; this is best done on a thick preheated aluminium plate in a drying cupboard equipped with ventilation allowing the nitric acid vapors to be removed using a water pump vacuum. Sodium dithionite is better than titanium(III) chloride or tin(II) chloride for the reduction of the nitro derivatives of amitrityline and nortriptyline.

The amino derivatives of amitriptyline and nortriptyline produced at the second heating stage exhibit intense pale yellow fluorescence on examination in long-wavelength UV light (λ – 365 nm), but this is not sufficiently reproducible for quantitative in situ work.

The detection limits for amitriptyline and nortriptyline are ca. 500 pg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Nitric Acid/Sulfuric Acid–Titanium(III) Chloride–Sodium Nitrite–N-(1-Naphthyl)-ethylenediamine

[1] Haefelfinger, P.: "Determination of Nanogram Amounts of Aromatic Compounds by Spectrophotometry on Thin-Layer Chromatograms", *J. Chromatogr.* **1976**, *124*, 351–358.

[2] Albet, C., Sánchez, M.G., Colomé, J.: "Determination of a new mucolytic drug Adamexina in biological liquids by photodensitometry", *J. Chromatogr.* **1980**, *181*, 504–511.

Reagent Sequence for:

- Aromatic substances
  e.g. antitussives [1]
  such as chlorpheniramine, codeine
  e.g. mucolytics [2]
  such as adamexine, bromexine

Preparation of the Reagents

Reagent 1 **Nitration**

Spray solution 1: Cautiously and with cooling mix 30 ml nitric acid (65%) with 10 ml sulfuric acid and add the mixture with cooling and mixing to 40 ml methanol. The reagent solution may be kept for several weeks.

Reagent 2 **Reduction**
Spray solution 2: Dilute 4 ml of a solution of 15 g titanium(III) chloride in 100 ml hydrochloric acid (4%) to 20 ml with methanol. This solution is only stable for about 1 h and should, therefore, always be made up fresh.

Reagent 3 **Diazotization**
Spray solution 3: Dissolve 400 mg sodium nitrite in 20 ml 1 mol hydrochloric acid. This solution should always be made up fresh.

Reagent 4 **Coupling**
Spray solution 4: Dissolve 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml methanol. The solution should always be made up fresh even though it remains stable for several days.

Reaction

The aromatic skeleton is nitrated in the first reaction step. Then titanium(III) chloride in acid medium is used to reduce the aromatic nitro compounds so produced to the corresponding amines, which in turn are diazotized and coupled to N-(1-naphthyl)-ethylenediamine to yield an azo dye (cf. Fig. 21).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until they start to become transparent and then heated to 110–115 °C for 10–15 min. After cooling to room temperature the TLC/HPTLC plates are sprayed with reagent 2 and heated to 110–115 °C for 5 to 30 min. The layers are cooled to room temperature, sprayed with reagent 3 and then dried in a stream of cold air for 10 min. Finally the chromatograms are treated with reagent 4 and dried in a stream of warm air (ca. 50–60 °C) for 1 min.

Chlorpheniramine produces purple zones and codeine bluish-gray zones on a light background [1]. Adamexine and bromexine produce pinkish-violet zones, that are stable for ca 24 h in the dark, on a yellowish-white background [2].

Note: Derivatization with this sequence of reagents in combination with extraction and TLC separation is virtually specific for chlorpheniramine in the analysis of plasma [1]; its high sensitivity also makes it suitable for pharmacokinetic studies after the oral administration of a single therapeutic dose. If excited with long-wavelength UV light ($\lambda_{exc} = 365$ nm) after the first reaction step codeine produces a fluorescent emission ($\lambda_{fl} > 578$ nm) which can be used for quantitative measurement.

The plate should be heated as rapidly and homogeneously as possible after the first spraying step; this is best done on a thick preheated aluminium plate in a drying cupboard equipped with ventilation allowing the nitric acid vapors to be removed using a water pump vacuum [1].

The detection limits are 1 to 2 ng/ml plasma for chlorpheniramine [1] and 50 and 250 ng per chromatogram zone for adamexine and bromexine respectively [2].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Nitrous Fumes–N-(1-Naphthyl)-ethylenediamine

[1] Singh, A.K., Ashraf, M., Granley, K., Mishra, U., Rao, M.M., Gordon, B.: "Screening and Confirmation of Drugs in Horse Urine by Using a Simple Column Extraction Procedure", *J. Chromatogr.* **1989**, *473*, 215–226.
[2] Heuser, E., Reusche, W., Wrabetz, K., Fauss, R.: „Zur Bestimmung von freien, monomeren Toluylendiisocyanaten und Hexamethylendiisocyanat in Polyurethan (=PUR)-Anstrichstoffen", *Fresenius Z. Anal. Chem.* **1971**, *257*, 119–125.

Reagent Sequence for:

- Aromatic amines
  e.g. clenbuterol [1]
  N-methyl-N-(4-aminobenzyl)-amino derivatives of isocyanates [2]

Preparation of the Reagents

Reagent 1 **Diazotization**

Nitrous fumes: Nitrous fumes can be generated in one trough of a twin-trough chamber by mixing a solution of 1 g sodium nitrite in 5 ml water with 5 ml fuming hydrochloric acid.

Reagent 2 **Coupling**

Spray solution: Dissolve 10 mg N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml ethanol [1]. Alternatively it is possible to use 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml dimethylformamide – hydrochloric acid (c = 1mol/L) (1+1) [2].

Reaction

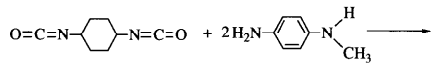
The aromatic amino group is diazotized in the first reaction step. The diazonium compound so formed is then coupled with N-(1-naphthyl)-ethylenediamine to yield an azo dye.

Method

The dried chromatograms are first treated with nitrous fumes for about 5 min. The excess nitrous fumes are then removed in a stream of cold air (ca. 5 min) and the TLC/HPTLC plates are sprayed homogeneously with reagent 2.

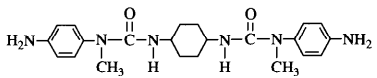
Clenbuterol, for example, yields pink-colored chromatogram zones on a light background.

Note: This reagent is a modification of the BRATTON-MARSHALL reagent, Isocyanates can be made accessible to this modification of the BRATTON-MARSHALL reagent by reacting prechromatographically with N-methyl-N-(4-aminophenyl)-amine to give the corresponding urea derivatives with primary aromatic amino groups:



1,4-Diisocyanato-cyclohexane

N-Methyl-N-(4-aminophenyl)-amine



Urea derivative with primary amine groups

This combination of the modified BRATTON-MARSHALL reaction with prechromatographic derivatization with N-methyl-N-(4-aminophenyl)-amine allows specific detection of isocyanates, that is especially applicable to aliphatic isocyanates.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Tin(II) Chloride–Sodium Nitrite–1-Naphthol

[1] Pugge, H.: Dissertation, Universität des Saarlandes, Saarbrücken, in preparation.
[2] Pugge, H., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1992.

Reagent Sequence for:

- Aromatic nitro compounds [1]
  e.g. herbicides
  such as trifluralin, pendimethalin
  e.g. fungicides
  such as dinocap

Preparation of the Reagents

Reagent 1 **Reduction**
Dipping solution 1: Dissolve 2 g tin(II) chloride dihydrate in 20 ml hydrochloric acid (25%) and dilute with 30 ml methanol. The reagent should always be made up fresh.

Reagent 2 **Diazotization**
Nitrous fumes: Mix a solution of 1 g sodium nitrite in 5 ml water with 5 ml fuming hydrochloric acid in one trough of a twin-trough chamber to generate nitrous fumes. The reagent should always be made up fresh.

Reagent 3 **Coupling**
Dipping solution 2: Dissolve 1 g 1-naphthol in 100 ml ethanol. The solution may be stored in the refrigerator for several weeks.

Reaction

Aromatic nitro compounds are reduced to the corresponding amines by tin(II) chloride in acidic medium. These are then diazotized via the gas phase with nitrous fumes and finally coupled with 1-naphthol to yield an azo dye.

Method

The chromatograms are dried in a stream of cold air and then dipped in reagent 1 for 2 s, dried in a stream of cold air for 10 min and then cooled to −20 °C for 15 min. The cold chromatogram is then placed in the free trough of a twin-trough chamber containing reagent 2 for 10 min for the purpose of diazotization via the gas phase. After removal of excess nitrous fumes by exposure to a stream of cold air for 5 min, the chromatograms are dipped in reagent 3 for 2 s and dried in a stream of cold air for 5 min.

Chromatogram zones of various colors are produced on a colorless background.

Note: The treated chromatogram should not be exposed to UV light or heat, neither should it be covered with a glass plate, since this causes it to turn dark brown in a short period of time. Nitrous fumes are corrosive so the diazotization and azo coupling should be carried out in the fume cupboard (rubber gloves).

The selectivity of the detection reaction can be increased by exposing the chromatogram to ammonia vapor after it has been treated with reagents 1 to 3; this can be done by placing 10 ml ammonia solution (25%) in the free trough of a twin-trough chamber. The result in general is that all chromatogram zones acquire a red to yellow-brown color, with the detection sensitivity of some substances being increased, while it can be reduced for others.

The color shades of the chromatogram zones and above all the pale background produced by this technique are stabilized by dipping the chromatogram in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s. The color shades produced on silica gel and RP layers are not identical.

The detection limits for 2,6-dinitroaniline herbicides are between 20 and 200 ng substance per chromatogram zone (Table 1). Similar results are also obtained with methyl and ethyl parathion (pink-colored zones).

The reagent can be employed on silica gel, kieselguhr, Si 50000 and RP layers.

Table 1: Absorption maxima and detection limits for some 2,6-dinitroaniline herbicides on silica gel layers after treatment with the reagent sequence and after additional exposure to ammonia vapor.

Substance	Without ammonia vapor		With ammonia vapor	
	λ_{max} [nm]	Detection limits [ng]	λ_{max} [nm]	Detection limits [ng]
Oryzalin	455	20	485	20
Nitralin	444	20	481	20
Dinitramin	440	200	452	100
Pendimethalin	583	5	496	20
Butralin	572	5	504	20
Fluchloralin	498	20	498	20
Isopropalin	437	20	441	20
Trifluralin	448	40	436	50

Procedure Tested

2,6-Dinitroaniline Herbicides [2]

Method Ascending, one-dimensional double development in a twin-trough chamber without chamber saturation and with 10 min drying (cold air stream) after the first run.

Layer HPTLC plates Silica gel 60 (MERCK), that were prewashed by immersing them in 2-propanol overnight and then drying at 110 °C for 60 min. Immediately before the first development the HPTLC plates were conditioned for 20 min at 30% relative humidity, e. g. over sulfuric acid (50%, g/g).

Mobile phase 1. *n*-Hexane – toluene (17+10)
2. Toluene – methanol (85+15)

Migration distance 1. 8.5 cm
2. 1.5 cm

Running time 1. 35 min
2. 3 min

Detection and result: The chromatogram was dried for 10 min in a stream of cold air and dipped in reagent 1 for 2 s and dried for 10 min in a stream of cold air. The yellow

2,6-dinitroaniline compounds were converted to colorless derivatives by this process. The chromatogram was then cooled to −20 °C for 15 min and exposed – still cold – to nitrous fumes for 10 min for diazotization; these fumes were generated in the empty trough of a twin-trough chamber by mixing 5 ml aqueous sodium nitrite solution (20%) with 3 to 5 ml fuming hydrochloric acid. Excess nitrous fumes were removed by exposing the layer for 5 min to a stream of cold air and the chromatogram was dipped in reagent 3 for 2 s and dried in a stream of cold air for 5 min.

The chromatogram zones produced were brown for oryzalin (migration distance 4–6 mm) and nitralin (10–15 mm), yellowish-brown for dinitramin (18–22 mm) and isopropalin (58–62 mm), blue for pendimethalin (38–42 mm), violet for butralin (43–48 mm), red for fluchloralin (50–55 mm) and orange for trifluralin (65–70 mm); the background was colorless (Fig 1A).

These chromatograms acquired a dark brown coloration within seconds if they were heated or exposed to UV light; the same effect was observed on covering the chromatogram with a glass plate!

The chromatogram was then placed in a twin-trough chamber with ca. 10 ml ammonia solution (25%) to increase the sensitivity. Afterwards the chromatogram zones were red in the case of oryzalin, nitralin, dinitramin, pendimethalin, butralin and fluchloralin and yellowish-brown in the case of isopropalin and trifluralin (Fig. IB). The detection sensitivity was sometimes increased and sometimes decreased (Table 1).

The colors obtained in this manner, and above all the pale background, were stabilized by dipping the chromatogram finally in a solution of liquid paraffin – *n*-hexane (1+2).

The detection limits are 20–200 ng substance per chromatogram zone (cf. Table 1).

In situ quantitation: The adsorption photometric evaluation in reflectance, before exposure of the chromatograms to ammonia, was carried out at $\lambda = 460$ nm (Fig. IIA) and $\lambda = 580$ nm (Fig. IIB), that after ammonia treatment at $\lambda = 490$ nm (Fig. IIC).

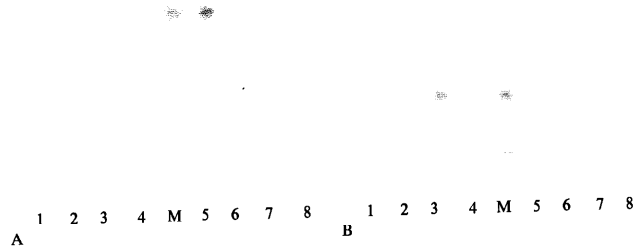


Fig I: Chromatogram of 2,6-dinitroaniline herbicides after treatment with the reagent sequence (A) and after additional treatment with ammonia vapor (B): 1 = oryzalin, 2 = nitralin, 3 = dinitramin, 4 = pendimethalin, 5 = butralin, 6 = fluchloralin, 7 = isopropalin, 8 = trifluralin, M = mixture.

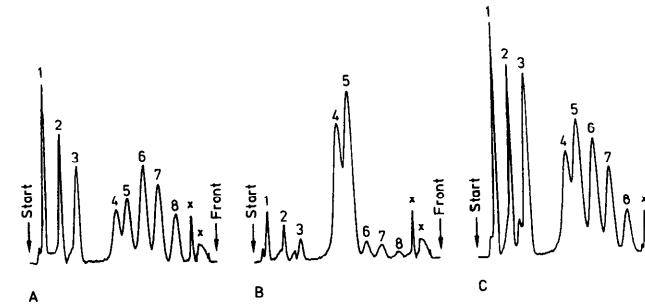


Fig. II: Reflectance scans of a chromatogram track with 200 ng g each of oryzalin (1), nitralin (2), pendimethalin (4), butralin (5), fluchloralin (6), isopropalin (7), trifluralin (8) and of 1000 ng dinitramin (3), per chromatogram zone; measurement at λ = 460 nm (A), 580 nm (B) and 490 nm (C); x = dipping fronts.

Titanium(III) Chloride–Bratton Marshall

[1] Pitzer, H.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[2] Schütz, H.: „Möglichkeiten der Reaktionschromatographie, dargestellt am Beispiel des reaktionschromatographischen Nachweises von Nitrazepam (Mogadan®) und seiner Hauptmetaboliten", *J. Chromatogr.* **1974**, *94*, 159–167.
[3] Sperlich, H.: „Nachweis und Unterscheidung von E 605; Methyl-E 605 und Chlorthion", *Dtsch. Apoth. Ztg.* **1968**, *100*, 774–777.

Reagent Sequence for:

- 4-Nitrophenyl esters
  e.g. thiophosphoric acid insecticides [1]
- Benzodiazepines [2]

Preparation of the Reagents

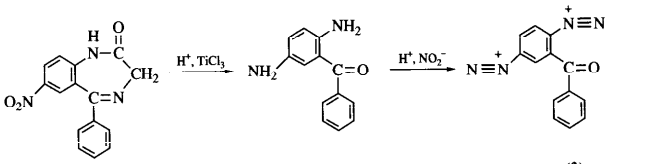
Reagent 1 **Reduction**
Dipping solution 1: Make 10 ml titanium(III) chloride solution (c = 15% in 10% hydrochloric acid) up to 50 ml with acetone [1].

Reagent 2 **Diazotization**
Dipping solution 2: Dissolve 0.5 g sodium nitrite in 10 ml water and make up to 50 ml with a mixture of 8.5 ml hydrochloric acid (32%) and 41.5 ml ethanol [1].

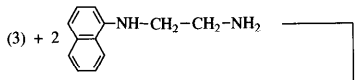
Reagent 3 **Coupling**
Dipping solution 3: Dissolve 0.5 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 5 ml water and make up to 50 ml with ethanol [1].

Reaction

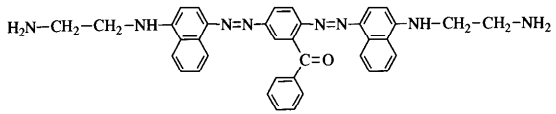
Titanium(III) chloride (particularly in slightly alkaline medium) reduces the *p*-nitro groups of the thiophosphate insecticides to amino groups, which are then reacted with nitrite in acid medium in a second step to yield a diazonium compound as intermediate. This is then coupled to N-(1-naphthyl)-ethylenediamine dihydrochloride to yield an azo dye [3]. In the case of benzodiazepines the first reaction step includes an additional acid hydrolysis to the corresponding benzophenone derivative [2].



(1) Nitrazepam — (2) Aromat. amine — (3) Diazonium compound



(4)
N-(1-Naphthyl-)-ethylenediamine



(5)
Azo dye

Method

After the chromatograms have been freed from mobile phase in a stream of warm air for 3 min they are immersed in dipping solution 1 for 3 s or homogeneously sprayed with it and then dried in a stream of warm air. Then they are dipped in reagent solution 2 for 3 s or homogeneously sprayed with it and finally, after drying in a stream of warm air, they are dipped in reagent solution 3 for 3 s or homogeneously sprayed with it.

Thiophosphoric acid insecticides and benzodiazepines yield reddish to bluish-violet-colored chromatogram zones on a colorless background.

Note: The reagent sequence can also be deployed in two stages with an intermediate chromatographic development using the SRS technique (separation – reaction – separation) [2]. When carrying out the acidic, reducing hydrolysis of benzodiazepines it is recommended that, after treatment with titanium(III) chloride in hydrochloric acid, the TLC plate be covered with a glass plate and heated to 100 °C for ca. 10 min; afterwards the amines that have been formed can be released for subsequent separation by exposing the plate to ammonia vapor [2].

The diazotization reaction can also be initiated via the vapor phase, e. g. with ethyl nitrite that can be generated in one trough of a twin-trough chamber by adding a few drops of conc. hydrochloric acid to a mixture of ethanol and saturated aqueous sodium nitrite solution (1+1) [3]; the less volatile amyl nitrite can be used as an alternative [3].

The detection limits for thiophosphoric acid insecticides are 100 ng and for benzodiazepines 20 ng substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr, Si 50000, RP and cellolose layers.

Procedure Tested

Organophosphorus Insecticides [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} extra thin layer (Merck).
Mobile phase	*n*-Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (909+40+26+25+1)
Migration distance	8 cm
Running time	28 min

Detection and result: The developed chromatogram was dried for 3 min in a stream of warm air and first immersed for 3 s in dipping solution 1 and dried in a stream of warm

air. It was then immersed in dipping solution 2 for 3 s and finally treated with dipping solution 3.

Azinphos methyl (hR_f: 15–20), azinphos ethyl (hR_f 20–25, parathion methyl (hR_f: 40–45), fenitrothion (hR_f: 45–50), parathion ethyl (hR_f: 60–65) and phoxim (hR_f: 60–65) appear as red-colored chromatogram zones on a colorless background.

The detection limits are 80–100 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 550$ nm (Fig. I).

Fig. I: Reflectance scan of a chromatogram track with 300 ng substance per chromatogram zone: 1 = azinphos methyl, 2 = azinphos ethyl, 3 = parathion methyl, 4 = fenitrothion, 5 = parathion ethyl + phoxim.

Titanium(III) Chloride–Nitrous Fumes–N-(1-Naphthyl)-ethylenediamine

VDI Richtlinie 2467: *VDI-Handbuch „Reinhaltung der Luft"*, Vol. 3, Beuth Verlag, Berlin.

Reagent Sequence for:

- Aromatic nitro compounds
  e. g. dinitrophenyl derivatives of primary and secondary amines

Preparation of the Reagents

Reagent 1 **Reduction**

Spray solution: Dilute 1 ml titanium(III) chloride solution (c = ca. 15% in hydrochloric acid (10%)) to 10 ml with hydrochloric acid (20%) or sulfuric acid (20%) and mix with 10 ml pyridine and 5 ml glacial acetic acid.

Reagent 2 **Diazotization**

Nitrous fumes: These can be generated in one trough of a twin-trough chamber by mixing a solution of 1 g sodium nitrite in 5 ml water with 5 ml fuming hydrochloric acid.

Reagent 3 **Coupling**

Spray solution: Dissolve 0.5 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml water, containing 2 drops conc. hydrochloric acid.

Reaction

The dinitrophenyl derivatives are reduced by titanium(III) chloride in acidic medium in the first step of the reaction to the corresponding aromatic amines; these are then diazotized and coupled with N-(1-naphthyl)-ethylenediamine to yield an azo dye (cf. Fig. 21).

Method

The dried chromatograms are first sprayed homogeneously with reagent 1, then dried for 2 to 3 min in the air and for ca. 10 min at 60 °C in a drying cupboard until the pyridine has completely evaporated. Afterwards they are briefly sprayed with a little hydrochloric acid (25%) and exposed to nitrous fumes (reagent 2) for several minutes. Finally after removal of the excess nitrous fumes in a stream of cold air the chromatograms are sprayed with reagent 3.

Blue-violet chromatogram zones are produced immediately on a pale background.

Note: This reagent sequence can be employed to great advantage after the prechromatographic derivatization of primary and secondary amines with 2,4-dinitrofluorobenzene; this makes for virtually specific detection of aliphatic amines.

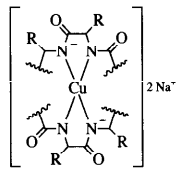
The detection limits for the aliphatic amines methyl, ethyl and isopropylamine are a few nanograms substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

3.4 Metal Complexes

The cations of some transition metals are electron acceptors that are capable of complexing with colorless organic compounds having electron donor properties. Colored complexes are formed as a result of changes occurring in the electron orbitals of the central metal atom [4]. The most important of these chelate formers are Cu^{2+}, Fe^{3+} and Co^{2+} ions, which have a great affinity for compounds containing oxygen or nitrogen.

The frequently used biuret reaction of proteins results in the formation of the following reddish-violet complex in alkaline medium [4, 25, 26]:



Aromatic ethanolamine derivatives, e.g. ephedrine, also yield blue-colored chelates with Cu^{2+} ions (CHEN-KAO reaction [4, 27]).

Mono- and polyhydric phenols and enols frequently form characteristically colored complexes with Fe^{3+} ions [4, 28, 29]. Here monohydric phenols usually produce reddish-violet colors, while pyrocatechol derivatives yield green chelates [4]. Detection of acetone using LEGAL's test is based on the formation of an iron complex [4]. The same applies to the thioglycolic acid reaction of the German Pharmacopoeia (DAB 9) [4, 30].

The ZWIKKER reaction involving Co^{2+} salts is frequently used for the detection of barbituric acid derivatives [31–35], but some purine, pyridine and piperidine derivatives and heterocyclic sulfonamides also yield colored derivatives. The ZWIKKER reaction is particularly sensitive when it is possible to form a tetrahedral complex $[Co(Barb)_2 \cdot X_2]$ (X = donor ligand, e.g. amine) [4].

Hydroxylamine–Iron(III) Chloride

Schwarzmaier, U.: „Hydroxylamin/Eisen(III)-chlorid, ein stereospezifisches Farbreagenz für Mandelsäurenitrilglycoside", *J. Chromatogr.* **1975**, *111*, 430–433.

Reagent Sequence for:

- Mandelonitrile glycosides (cyanoglycosides)
  e.g. amygdalin, prunasin, taxiphyllin, vicianin

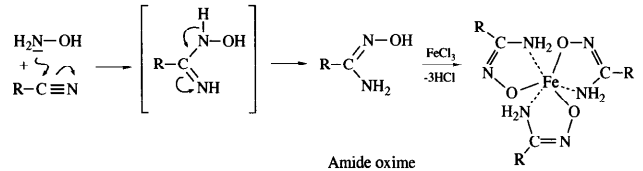
Preparation of the Reagents

Reagent 1 **Nucleophilic substitution**
Spray solution 1: Add 9.5 g finely ground anhydrous potassium carbonate portionwise to an ice-cooled solution of 14 g hydroxylamine hydrochloride in 20 ml water. When the evolution of CO_2 gas has stopped add 80 ml ethanol. Stir for a further 30 min at 0 °C and filter. The spray solution 1 that is produced has a pH of 5.0 to 5.5 and may be stored in the refrigerator for ca. 10 days.

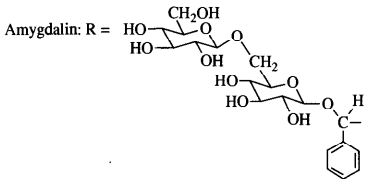
Reagent 2 **Complex formation**
Spray solution 2: Dissolve 1.5 g iron(III) chloride in 100 ml methanol.

Reaction

Firstly there is nucleophilic attack of the nitrile carbon atom by hydroxylamine. An amide oxime is produced; this then forms an intensely colored complex with the iron(III) chloride.



Amide oxime



Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then allowed to stand at room temperature for 15 min. They are then sprayed homogeneously with reagent 2.

Mandelonitrile glycosides with D-configuration (e.g. prunasin, taxiphyllin, *p*-O-methyltaxiphyllin, amygdalin vicianin) immediately yield reddish-brown chromatogram zones on a colorless background. L-isomers (e.g. sambunigrin, dhurrin, *p*-O-methyldhurrin, neoamygdalin) gradually yield chromatogram zones that are pale brown initially.

Note: The colors of the chromatogram zones produced by the mandelonitrile glycosides change with time: Those of the D-isomers turn steel blue within an hour, while the L-isomers turn reddish-brown during this time. It is possible to distinguish between D- and L-forms on the basis of these typical color differences and color changes. Both isomeric forms have a dark brown color after about 24 hours.

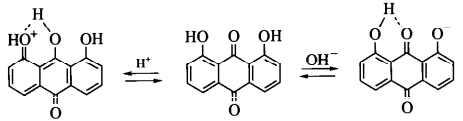
The detection limits for mandelonitrile glycosides are 3 to 5 µg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

3.5 Halochromism and Charge-Transfer Complexes

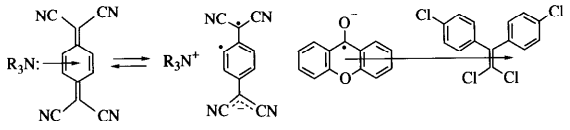
Numerous colorless organic compounds with extended π-electron systems can be converted to colored cations or anions with polymethyne chromophors by protonation or deprotonation. The intense coloration of the corresponding "salts" is usually attributable to the fact that the lone pairs of electrons of the heteroatoms participate in the mesomerism of the conjugated π-electron systems [4].

The well-known BORNTRÄGER reaction for the detection of 1,8-dihydroxyanthraquinones is a characteristic example of such halochromism [36–38]:



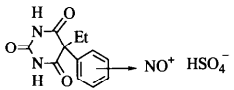
The reaction between 4-nitrophenacyl esters and DMSO/diethylamine, described by KALLMAYER et al., is also a halochromic reaction [39].

Charge transfer complexes (CT complexes) primarily occur in planar organic molecules with conjugated π-electron systems [4]. Examples include:

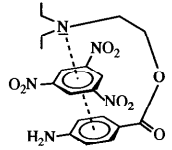


CT complex:
tert.-Amine + Tetracyanoquinodimethane

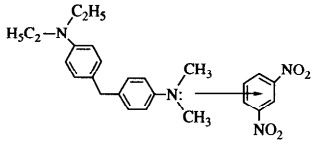
CT complex:
Xanthydrol + DDT



CT complex:
Phenobarbital + Sodium nitrite/H_2SO_4



CT complex
Procaine + 1,3,5-Trinitrobenzene



CT complex
4-(Dimethylamino)-4′-(diethylamino)-diphenylmethane
(ARNOLD's reagent) + 1,3-Dinitrobenzene

3.6 Reagent Sequences with Complex Reaction Patterns

In addition to the reagent sequences with clearly detectable reaction mechanism, which have already been described, many sequences of reagents not covered by any of the reaction types described have also found application in thin-layer chromatography. The reaction sequences that remain to be described were all designed to provide as specific a detection of the separated substances as possible.

Borate Buffer–Fluorescamine–Taurine

Nakamura, H., Tsuzuki, S., Tamura, Z., Yoda, R., Yamamoto, Y.: "Fluorescence Detection of Secondary Amines on Thin-Layer Plates Using a Fluorogenic Reaction with Fluorescamine" *J. Chromatogr.* 1980, 200, 324–329.

Reagent Sequence for:

- Secondary amines
  e. g. benzimidazole, sarcosine, morpholine

Preparation of the Reagents

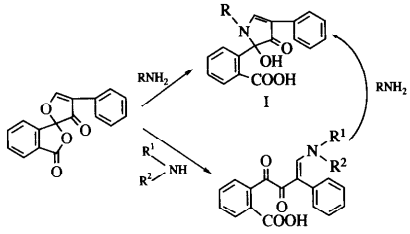
Reagent 1 *Spray solution 1*: 0.05 mol sodium borate buffer (pH 10.5).

Reagent 2 *Spray solution 2*: Dissolve 20 mg fluorescamine in 100 ml acetone.

Reagent 3 *Spray solution 3*: 0.2 mol taurine in 0.2 mol sodium phosphate buffer (pH 7.5).

Reaction

Fluorescamine reacts directly with primary amines to yield fluorescent derivatives of the general formula I. On the other hand, secondary amines react in weakly basic medium to yield nonfluorescent derivatives of type II; after the hydrolysis of excess fluorescamine, these are converted to fluorescent products of type I by reaction with a primary amine, e. g. taurine.



Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then heated to 110 °C for 15 min. After cooling to room temperature the layer is sprayed with reagent 2 and then left in the dark at room temperature for 10 min. Finally the chromatogram is sprayed with reagent 3 and heated to 60 °C for 5 min.

Observation under UV light ($\lambda = 254$ or 365 nm) reveals intense fluorescent chromatogram zones on a dark background.

Note: Primary amines yield fluorescent chromatogram zones even before the application of reagent 3. Secondary amines do not yield fluorescent derivatives until they have been treated with reagent 3. Hence, the reagent sequence allows the stepwise detection of primary and secondary amines. Taurine is preferred as the essential component of reagent 3 over the multiplicity of other possibilities because it produces intense fluorescence; it is also not very volatile and is readily available. Amides and substances with peptide linkages, e. g. hippuric acid, are not detected, neither are secondary amines that are volatile at high temperatures.

All traces of mobile phase (and of ammonia in particular) must be removed from the layer, e. g. by heating the chromatogram (10 min 110 °C). The first reagent treatment, including the heat treatment, should be carried out twice if a mobile phase containing acetic acid is used for development.

The detection limits for secondary amines lie between 2 ng (morpholine) and 500 ng (benzimidazole) substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Preparation of the Reagents

Reagent 1 *Spray solution 1*: Dissolve 2 g 1-chloro-2,4-dinitrobenzene in 100 ml ethanol.

Reagent 2 *Spray solution 2*: Dissolve 12 g sodium hydroxide pellets in 100 ml methanol.

Reagent 3 *Ammonia vapor*: Concentrated ammonia solution in the free trough of a twin-trough chamber.

Reaction

Nicotinic acid and related compounds react with 1-chloro-2,4-dinitrobenzene in the manner of the cyanogen bromide reaction to yield derivative I, which possibly also decarboxylates at elevated temperature. In alkaline medium this derivative first adds an hydroxyl ion and then undergoes ring opening to yield the colored derivative II.

1-Chloro-2,4-dinitrobenzene–Sodium Hydroxide–Ammonia Vapor

Wachtel, I.: Eine neue dünnschicht-chromatographische Bestimmungsmethode für Nicotinsäure und Nicotinsäureamid auf Grund der Fleckenfarbe", *Mikrochim. Acta (Vienna)* 1970, 621–627.

Reagent Sequence for:

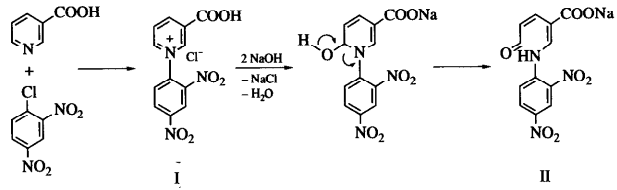
- Vitamins
  e.g. nicotinic acid, nicotinamide

on a colorless to slightly yellow background.

Note: The colors of the chromatogram zones fade relatively quickly. A temperature of 180 °C should not be exceeded in the first heating step, otherwise the sensitivity of detection will be reduced.

The detection limits for nicotinic acid and nicotinamide are 200 ng substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.



I II

Method

The chromatograms are dried in a stream of warm air, then sprayed homogeneously with reagent 1 and heated to 180 °C for 30 to 45 min. After cooling to room temperature they are homogeneously sprayed with reagent 2 and then exposed to ammonia vapor (reagent 3).

Nicotinic acid produces violet-red and nicotinamide orange-red chromatogram zones

Diphenylamine/Iron(III) Chloride/ Sulfuric Acid–Silver Nitrate/ Ammonia

Chawla, H.M., Raihan, N.N., Garg, N.K., Chibber, S.S.: "TLC Separation and Identification of Some Monovalent Anions" J. High Resolut. Chromatogr. Chromatogr. Commun. 1980, *3*, 651–652.

Reagent Sequence for:

- Univalent inorganic anions
  e.g. chloride, bromide, iodide, nitrate, thiocyanate

Preparation of the Reagents

Reagent 1 *Spray solution 1*: Dissolve 1 g iron(III) chloride and 1 g diphenylamine in 100 ml conc. sulfuric acid.

Reagent 2 *Spray solution 2*: Ammoniacal silver nitrate solution (precise composition not specified).

Reaction

The mechanism of the reaction has not been elucidated. Presumably several reactions occur simultaneously. Thiocyanates react with iron(III) salts with the formation of red-colored complexes. In sulfuric acid medium nitrate or nitrite oxidize diphenylamine to tetraphenylhydrazine, which then undergoes a benzidine rearrangement and the product is oxidized to a blue-colored N,N′-diphenyldiphenoquinonediimine sulfate. In sulfuric acid medium iodide and bromide are oxidized to the elementary halogens, which are also probably capable of oxidizing diphenylamine to colored derivatives. On the other hand halides are also capable of decomposing the silver tetramine complex (reagent 2) to yield elementary silver, which produces brownish-black chromatogram zones.

Method

The dried chromatograms are sprayed homogeneously with reagent 1 and then with reagent 2 and finally dried in a stream of warm air for 5 to 10 min.

Chromatogram zones of various colors are produced (bromide: yellow, iodide: pale red, thiocyanate: red, nitrate: blue and chloride: black) on a colorless background.

Note: The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Fluorescein Isothiocyanate–Ninhydrin

Laskar, S., Basak, B.: "Detection of Amino Acids on Thin-Layer Plates", *J. Chromatogr.* **1988**, *436*, 341–343.

Reagent Sequence for:

- Amino acids

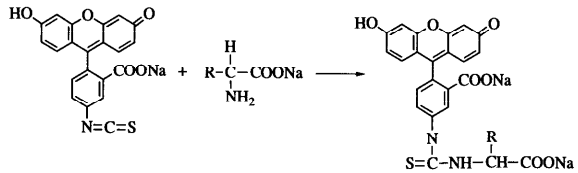
Preparation of the Reagents

Reagent 1 **Formation of thiourea derivatives**
Spray solution 1: Dissolve 10 mg fluorescein isothiocyanate in 100 ml 0.1 mol sodium hydroxide solution.

Reagent 2 **Ninhydrin solution**
Spray solution 2: Dissolve 250 mg ninhydrin in 100 ml acetone.

Reaction

The amino acids probably react with the fluorescein isothiocyanate to yield fluorescein thiourea derivatives. These are hydrolyzed at elevated temperature in alkaline medium so that the amino groups that are produced can then react with ninhydrin.



Fluorescein isothiocyanate Amino acid Fluorescein thiourea derivative

Method

The chromatograms dried in a stream of warm air are first sprayed homogeneously with reagent 1 and then heated to 90 °C for 10 min. After cooling to room temperature they are sprayed with reagent 2 and dried in a stream of cold air. Finally they are heated again to 90 °C for 10 min.

The reagent sequence produces colored chromatogram zones of substance-dependent color, some of which appear before the final heating step. The background remains colorless. Some of the zones fluoresce with various colors, when examined under UV light (λ = 280 nm).

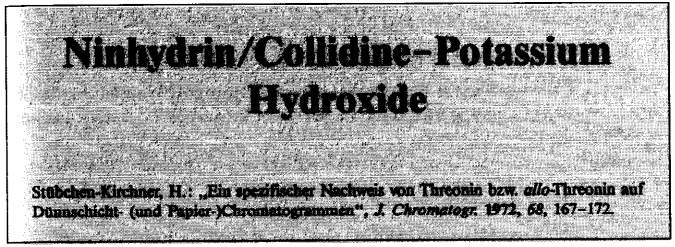
Note: This reagent sequence is less sensitive than ninhydrin alone. However, it possesses the advantage that the colors produced by the individual amino acids vary (Table 1), whereas ninhydrin alone only produces reddish-violet colored zones.

The visual detection limits are 0.3 to 1 µg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Table 1: Color reactions of amino acids with fluorescein isothiocyanate – ninhydrin (extract from original table).

Amino acid	Color of the chromatogram zones in visible light	
	Before heating	After heating
Alanine	grayish-pink	gray
Arginine	mauve	grayish-pink
Asparagine	yellow-orange	orange-yellow
Aspartic acid	pale mauve	dark violet
Cysteine	mauve	brown
Cystine	light beige	yellow
Hydroxyproline	orange-yellow	yellow
Lysine	mauve	grayish-pink
Proline	straw-colored	deep yellow
Serine	pink-violet	grayish-pink



Reagent Sequence for:

- Hydroxyamino acids
  e.g. threonine and *allo*-threonine

Preparation of the Reagents

Reagent 1 **Condensation and decarboxylation**
Spray solution 1: Dissolve 0.4 g ninhydrin and 5 ml 2,4,6-collidine in 2-propanol and make up to 100 ml.

Reagent 2 **Deprotonation**
Spray solution 2: 1 percent ethanolic potassium hydroxide solution.

Reaction

The mechanism of the fluorescence reaction has not been elucidated. See Volume 1a "Ninhydrin – Collidine Reagent" for the ninhydrin reaction.

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 until they begin to be transparent and then heated to 80 °C for 20 min. After cooling to room temperature the layers are sprayed several times with reagent 2. After this treatment the chromatograms are stored for an extended period (24 h or longer), under the influence of daylight, in a twin-trough chamber, whose spare trough is filled with 72 percent glycerol (by volume).

Examination under long-wavelength UV light ($\lambda = 365$ nm) reveals pink to brilliant red fluorescent chromatogram zones on a dark background for threonine and *allo*-threonine.

Note: Detection is reported to be specific for threonine and *allo*-threonine. The presence of collidine in reagent 1 is reported to have a favorable effect on the production of the fluorescence. When the substance concentration is high the red fluorescence only occurs at the outer edges of the chromatogram zones (quenching as a result of high concentration). Immediately after treatment with reagent 2 threonine and *allo*-threonine produce characteristic pale green or sometimes yellow green to dirty blue-green fluorescence emissions in long-wavelength UV light ($\lambda = 365$ nm), but these are only visible for 10 to 20 min.

The detection limits for threonine and *allo*-threonine are less than 100 ng substance per chromatogram zone.

The reagent can be employed on cellulose layers.

Potassium Hexaiodoplatinate–Sodium Hydroxide–1,2-Naphthoquinone-4-Sulfonic Acid

Wallace, J.E., Hamilton, H.E., Skrdlant, H., Burkett, L. Schwertner, H.: "Identification of Selected Antihypertensive Drugs by Thin-Layer Chromatography", *J. Chromatogr.* 1977, *138*, 111–118.

Reagent Sequence for:

- Pharmaceuticals and metabolites
  e.g. diuretics, antihypertensives
  such as chlorothiazide, hydrochlorothiazide, methyldopa

Preparation of the Reagents

Reagent 1 *Spray solution 1*: Treat 1 ml of a platinum chloride solution (5%) with 1.5 g potassium iodide and mix with 3 ml conc. hydrochloric acid and 11 ml water.

Reagent 2 *Spray solution 2*: Sodium hydroxide (c = 1 mol/L).

Reagent 3 *Spray solution 3*: A saturated solution of 1,2-naphthoquinone-4-sulfonic acid sodium salt in ethanol – water (1+1).

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are dried in a stream of warm air, then lightly sprayed successively with reagent 1 and reagent 2, followed by reagent 3. Finally they are heated to 110 °C for 5 to 10 min.

Chlorothiazide, hydrochlorothiazide and methyldopa produce pink-colored chromatogram zones on a pale background.

Note: The three reagents should be applied as quickly as possible after each other. In combination with the R_f value, and with UV detection before application of the reagent sequence this procedure allows the identification of therapeutic quantities of thiazide diuretics and methyldopa in urine together with a series of other therapeutic agents. Mobile phase residues e. g. acetic acid, should be completely removed from the chromatograms before application of the reagent sequence.

The detection limits for chlorothiazide, hydrochlorothiazide and methyldopa are 5 µg substance per milliliter urine (working with 5 ml samples).

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Sulfuric Acid–Potassium Hexaiodoplatinate

Jukofsky, D., Verebey, K., Mulé, S.J.: "Qualitative Differentiation Between Cocaine, Lidocaine and Cocaine-Lidocaine Mixtures ("Rock Cocaine") Using Thin-Layer Chromatography", *J. Chromatogr.* 1980, *198*, 534–535.

Reagent Sequence for:

- Nitrogen-containing compounds
  e. g. alkaloids
  such as cocaine, lidocaine

Preparation of the Reagents

Reagent 1 *Spray solution 1*: Sulfuric acid (5%).

Reagent 2 *Spray solution 2*: Treat 3 ml hexachloroplatinic(IV) acid solution (10%) with 100 ml aqueous potassium iodide solution (6%) and dilute with 97 ml water.

Reaction

The reaction is a redox reaction whose individual steps have not been elucidated.

Method

The dried chromatograms are first sprayed homogeneously with reagent 1 and then oversprayed with reagent 2.

Cocaine and lidocaine instantly produce purple-blue chromatogram zones on a colorless background.

Note: The colors of the chromatogram zones change gradually and peripheral rings of color form. The lidocaine zone, for example, becomes brown with a blue-gray ring and the cocaine zone becomes brown with a very weak purple ring, that becomes paler with time. When the cocaine and lidocaine zones are not adequately separated a characteristic blue-gray band-shaped zone appears above the lidocaine and becomes more emphatic as the cocaine zone disappears with time.

The color differences between lidocaine and cocaine zones become clearly apparent after 4 h and are stable for about 24 h. They make it possible to identify cocaine and lidocaine in mixtures, even when the two substances are scarcely separated from each other.

The reagent can be employed on silica gel, kieselguhr, Si 50000, RP and cellulose layers.

References

[1] Schwenker, G.: *Arch. Pharm.* **1965**, *298*, 826–838.
[2] Kovar, K.-A., Pichler, H., Pieper, R., Walter, D.: *Identifizierung von Arzneistoffen*, Wissenschaftl. Verlagsgesellschaft, 5. Aufl., S. 65. Stuttgart 1985.
[3] Kovar, K.-A., Schlecht, I., Weber, R., Guarnieri, A., Varoli, L.: *Arch. Pharm.* **1985**, *318*, 149–157.
[4] Pindur, U., Witzel, H.: *Dtsch. Apoth. Ztg.* **1988**, *128*, 2127–2136.
[5] Stead, A. H., Gill, R., Wright, T., Gibbs, J.P., Moffat, A.C.: *Analyst* **1982**, *107*, 1106–1168.
[6] Kaess, A., Mathis, C.: *Ann. pharmac. franç.* **1966**, *24*, 753–762.
[7] Mathis, C., Ourisson, G.: *J. Chromatogr.* **1963**, *12*, 94–96.
[8] Kaess, A., Mathis, C.: *Ann. pharmac. franç.* **1965**, *23*, 267–274; 739–747.
[9] Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin-layer Chromatography Reagents and Detection Methods*, Vol. 1a, VCH-Verlagsgesellschaft, Weinheim, Cambridge, New York, 1990.
[10] Pindur, U.: *Pharm. Unserer Zeit* **1982**, *11*, 74–82.
[11] Rehse, K., Kawerau, H. G.: *Arch. Pharm.* **1974**, *307*, 934–942.
[12] Müller, A. in Hartke, K., Mutschler, E.: *DAB 9 Kommentar*, Wissenschaftliche Verlagsgesellschaft, Stuttgart 1988.
[13] Erhardt, P. W., Smith, R. V., Sayther, T. T., Keiser, J. E. *J. Chromatogr.* **1976**, *116*, 218–224.
[14] Rehse, K.: *Naturwissenschaften* **1968**, *55*, 390.
[15] Rehse, K.: *Arch. Pharm.* **1972**, *305*, 625–629.
[16] Rehse, K.: *Arch. Pharm.* **1969**, *302*, 487–494.
[17] Auterhoff, H., Pankow, H.-J.: *Arch. Pharm.* **1967**, *300*, 103–110.
[18] Huck, H., *Naturwissenschaften* **1976**, *63*, 90.
[19] Kimura, M., Kawata, M., Akiyama, K., Harita, K., Miura, T.: *Chem. Pharm. Bull.* **1973**, *21*, 1720–1726; 1741–1746.
[20] Auterhoff, H.: *Dtsch. Apoth. Ztg.* **1962**, *102*, 765–767.
[21] Möhrle, H., Schittenhelm, D.: *Pharm. Ztg.* **1967**, *112*, 1400–1405.
[22] Roth, H. J., Möhrle, H.: *Arch. Pharm.* **1966**, *299*, 315–320.
[23] Heintz, B., Kalusa, R.:*Dtsch. Apoth. Ztg.* **1978**, *118*, 1000–1006.
[24] Schwenker, G.: *Arch. Pharm.* **1965**, *298*, 826–828.
[25] Kurzer, F.: *Chem. Rev.* **1956**, *56*, 95–197.
[26] Freeman, H. C., Smith, W. L., Taylor, J. C.: *Nature* **1959**, *184*, 707–710.
[27] Winde, E.: *Dtsch. Apoth. Ztg.* **1970**, *110*, 123–124.
[28] Vejdelek, Z. J., Kakáč, B.: *Farbreaktionen in der spektrophotometrischen Analyse*, Erg. Bd. II, G. Fischer, Jena 1982.
[29] Heesterman, J. E.: *Pharm. Weekblad* **1935**, *32*, 463.
[30] Geffken, D., Surborg, K.-H.: *Dtsch. Apoth. Ztg.* **1988**, *128*, 1235–1236.
[31] Büchi, J., Perlia, X.: *Pharm. Acta Helv.* **1954**, *29*, 183–199, 290–310.
[32] Bult, A., Klasen, H. B.: *Spectrosc. Lett.* **1976**, *9*, 81.
[33] Bult, A., Klasen, H. B.: *Pharm. Weekblad* **1974**, *109*, 109–123, 513–522; **1975**, *110*, 533–538, 1161–1163.
[34] Schwenker, G.: *Dtsch. Apoth. Ztg.* **1957**, *97*, 238–240.
[35] Schmidt, F.: *Dtsch. Apoth. Ztg.* **1978**, *118*, 443–444.

[36] Bornträger, H.: *Z. Anal. Chem.* **1880**, *19*, 165–167.
[37] Auterhoff, H., Boehme, K.: *Arch. Pharm.* **1968**, *301*, 793–799.
[38] Boehme, K., Auterhoff, H.: *Arch. Pharm.* **1969**, *302*, 801–802.
[39] Kallmayer, H.-J., Plener, H.-U., Binger, M.: *Pharm. Ztg.* **1987**, *132*, 3265–3269.

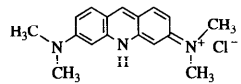
Part II

Reagents in Alphabetical Order

Acridine Orange Reagent

Reagent for:

- Dicarboxylic acids
  e. g. succinic acid, glutaric acid [1, 2]
- Aryl- and heteroarylpropionic acids
  e. g. carprofen, naproxen [3]



$C_{17}H_{20}ClN_3$

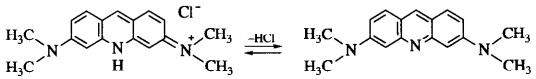
$M_r = 301.82$

Preparation of the Reagent

Dipping solution	Dissolve 0.02 g acridine orange (Basic Orange 14, C.I. 46005) in 100 ml ethanol [3].
Spray solution	Dissolve 0.05 g acridine orange in 100 ml ethanol [1, 2].
Storage	The reagent solutions may be stored for a longer period.
Substances	Acridine orange Ethanol Hydrochloric acid (32%)

Reaction

Acridine orange changes its fluorescence color from pale yellow-green to yellow in a specific pH range (pH 8–10) [1, 4].



Method

The chromatograms are freed from mobile phase (30 min, 120 °C), cooled to room temperature, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution, dried in a stream of cold air for 10 min and then heated to 120 °C for 30 min [1].

Observation under short- and long-wavelength UV light (λ = 254 nm or 365 nm) reveals yellow to orange fluorescent chromatogram zones on a yellow-green fluorescent background.

Note: Other acridine derivatives can be used as reagents instead of acridine orange (Table 1) [1, 5]. The detection limits lie between a few ng and 100 ng substance per chromatogram zone depending on the substance [1, 3]. If 2-methoxy-9-isothiocyanatoacridine is used as reagent fatty acids can also be detected, whereby the visual detection limits are in the nanogram range at 0.1 µg substance per chromatogram zone [5].

The reagent can, for instance, be employed on silica gel, paraffin-impregnated silica gel, kieselguhr and Si 50000 layers.

Table 1: Fluorescence colors of the chromatogram zones and of the layer background as a function of the reagent employed and of the excitation wavelength [1].

Fluorescence excitation at:	254 nm		365 nm	
Color of:	Background	Zone	Background	Zone
Acridine	pale blue	yellow-green	pale blue	pale yellow
9-Phenylacridine	blue-green	yellow	blue-green	yellow
Acridine orange	yellow-green	red-violet	yellow-green	red
Dibenz[*aj*]acridine	deep blue	pale blue	deep blue	pale blue
9-Phenoxyacridine	deep blue	pale blue	deep blue	pale blue
9-(*p*-Methylphenoxy)acridine	deep blue	pale blue	deep blue	pale blue
9-(*m*-Methylphenoxy)acridine	deep blue	pale blue	deep blue	pale blue
9-(*p*-Aminophenoxy)acridine	deep blue	pale blue	deep blue	pale blue

Procedure Tested

Carprofen and Naproxen [3]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation. After sample application the HPTLC plate was preconditioned for 30 min in the mobile phase-free trough of a twin-trough chamber (without filter paper lining).

Layer HPTLC plates Silica gel 60 (MERCK).

Mobile phase Hexane – diethyl ether – 1-butanol – ethyl acetate (65+15+11+9).

Migration distance 6 cm

Running time 20 min

Detection and result: The developed chromatogram was dried for 30 min at 120 °C, cooled to room temperature and immersed in the dipping solution for 2 s. Then it was dried in a stream of cold air for 5–10 min.

On visual inspection in daylight carprofen (hR_f 40–45) was recognizable as a pink-colored chromatogram zone on a yellow background. In long-wavelength UV light (λ = 365 nm) carprofen appeared as an orange and naproxen (hR_f 55–60) as a yellow fluorescent chromatogram zone on a yellow-greenish fluorescent background.

In situ quantitation: For fluorimetric evaluation there was excitation at $\lambda_{exc} = 313$ nm and the fluorescence emission was measured at $\lambda_{fl} = 365$ nm (monochromatic filter M 365). This arrangement yielded the most intense signals. (The emission beam at $\lambda = 365$ nm is appreciably more intense than the visible yellow fluorescence.) Further treatment of the chromatogram with liquid paraffin – *n*-hexane (1+2) is not to be recommended.

The photometric detection limit of carprofen was at 4 ng substance per chromatogram zone; 100 ng substance per chromatogram zone could be detected visually.

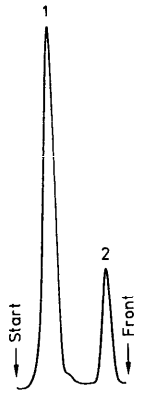


Fig. 1: Fluorescence scan of a chromatogram track with 200 ng each of carprofen (1) and naproxen (2) per chromatogram zone.

References

[1] Sârbu, C., Horn, M., Marutoiu, C.: *J. Chromatogr.* **1983,** *281,* 345-347.
[2] Kreuzig, F. in: L. R. Treiber, *Quantitative TLC and its Industrial Applications,* Chrom. Science Series (J. Cazes, Ed.), Vol. 36 S. 262-263, Marcel Dekker, New York, Basel, Hong Kong, 1986.
3] Honermann, U.: Thesis, Fachhochschule Gießen-Friedberg, Fachbereich Technisches Gesundheitsesen, 1991.
[4] Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin-layer Chromatography – Reagents and Detection Methods,* Vol. 1a, p. 91, VCH Verlagsgesellschaft, Weinheim, 1990.
[5] Sârbu, C., Coman, V., Marutoiu, C.: *J. Planar Chromatogr.* **1991,** *4,* 325-326.

Ammonium Monovanadate–*p*-Anisidine Reagent

Reagent for:

- Phenols [1-3]

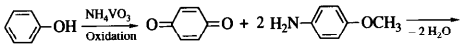
NH_4VO_3	C_7H_9NO
$M_r = 116.98$	$M_r = 123.16$
Ammonium monovanadate	*p*-Anisidine

Preparation of the Reagent

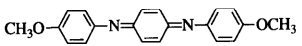
Solution I	Dissolve 2.5 g ammonium monovanadate (ammonium metavanadate) in 20 ml water and add 20 ml ethanol. Filter off the precipitate that is formed.
Solution II	Dissolve 0.5 g *p*-anisidine in 50 ml ethanol, add 2 ml phosphoric acid (85%) and make up to 100 ml with ethanol. Filter off the precipitate that is formed.
Dipping solution	Mix equal volumes of solutions I and II immediately before use.
Storage	Reagent solutions I and II may be stored for a longer period.
Substances	Ammonium monovanadate Ethanol absolute *p*-Anisidine Phosphoric acid (85%)

Reaction

The reaction mechanism has not been elucidated; the ammonium monovanadate presumably oxidizes the phenols to quinones, that then react with *p*-anisidine to form quinonimines.



Phenol Quinone *p*-Anisidine



Quinonimine

Method

The chromatogram is dried for 10 min in a stream of warm air and then immersed in the dipping solution for 2 s or sprayed homogeneously with it, dried for 2 min in a stream of warm air and then heated to 110 °C for 1 min.

Dimethylphenols yield turquoise-colored chromatogram zones on a violet background, that are converted to blue-violet zones on a flesh-colored background on brief exposure to ammonia vapor.

Note: The detection limits are 20 ng substance per chromatogram zone.

The reagent can, for instance, be employed on silica gel, kieselguhr and on Si 50000 layers.

Procedure Tested

Dimethylphenols [1-3]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer	HPTLC plates Silica gel 60 (MERCK); before application of the samples the layer was developed once to its upper edge with chloroform – methanol (50 + 50) to precleanse it and then dried at 110 °C for 30 min.
Mobile phase	Toluene
Migration distance	6 cm
Running time	10 min

Detection and result: The chromatogram was dried for 10 min in a stream of warm air and immersed in the dipping solution for 2 s, dried for 2 min in a stream of warm air and heated for 1 min to 110 °C (Thermoplate DESAGA). 2,5-Dimethylphenol (hR_f 25-30) and 2,6-dimethylphenol (hR_f 40-45) appeared as turquoise-colored chromatogram zones on a violet background and were converted to blue-violet zones on a flesh-colored background on exposure to ammonia vapor for 5 min.

In situ quantitation: The absorption photometric scan in reflectance was carried out at λ = 590 nm after exposure to ammonia vapor. The detection limits were 20 ng substance per chromatogram zone (Fig. 1).

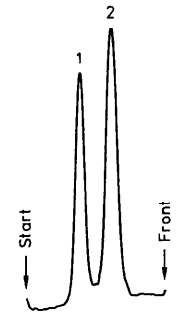


Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone: 1 = 2,5-dimethylphenol, 2 = 2,6-dimethylphenol.

References

[1] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1989.
[2] Hoffmann, A., Funk, W.: *GIT Fachz. Lab. Supplement 3 "Chromatographie"* **1988**, 12–19.
[3] Hoffmann, A.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1987.

Ammonium Thiocyanate Reagent
(Ammonium Rhodanide Reagent)

Reagent for:

- Cations
  e.g. cobalt(II), iron(III) [1]
  rhenium(VII), molybdenum(VI), vanadium(V) [2]

NH_4SCN
CH_4N_2S
$M_r = 76.12$

Preparation of the Reagent

Dipping solution	Dissolve 500 mg ammonium thiocyanate in 45 ml acetone and add 5 ml glacial acetic acid [1].
Storage	The dipping solution may be stored for at least 1 week.
Substances	Ammonium thiocyanate Acetone Acetic acid (100%)

Reaction

Thiocyanate ions form stable complex salts, e.g. intense red with iron(III) and pale blue with cobalt(II) cations.

Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 2 s or homogenocously sprayed with it and then dried for 3 min in a stream of cold air.

Cobalt produces pale blue and iron red-brown chromatogram zones on a colorless background.

Note: Substances that form more stable colorless complexes with the metal ions (e.g. EDTA, phosphates, phosphonic acids etc.) than thiocyanate interfere with the reaction.

Re(VII), Mo(VI) and V(V) cations are detected by first spraying the chromatogram with tin(II) chloride solution (10% in 6 N hydrochloric acid) and then with ammonium thiocyanate solution (50% in water). This leads to the formation of orange, pink or yellow-colored complexes [2].

The detection limits for iron and cobalt cations on cellulose layers are 2 and 20 ng substance per chromatogram zone [1].

The reagent can be used, for example, on aluminium oxide and on cellulose layers.

Procedure Tested

Iron(III) and Cobalt(II) Ions [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Cellulose F_{254s} (MERCK); before application of the samples the layer was developed once to its upper edge with chloroform – methanol (50 + 50) to precleanse it and then dried at 110 °C for 15 min.
Mobile phase	Methanol – hydrochloric acid (32%) – water (80+16+4).
Migration distance	5 cm
Running time	30 min

Detection and result: The developed chromatogram was dried for ca. 5 min in a stream of cold air, immersed in the dipping solution for 2 s and then dried for 3 min in a stream of cold air.

Cobalt(II) ions (hR_f 65–70) yielded pale blue and iron(III) ions (hR_f 85–90) red-brown chromatogram zones on a colorless background. The detection limits per chromatogram zone were 2 ng for iron and 20 ng for cobalt.

In situ quantitation: The absorption photometric scan in reflectance was carried out at λ_{max} = 610 nm for cobalt and at λ_{max} = 480 nm for iron (Figure 1).

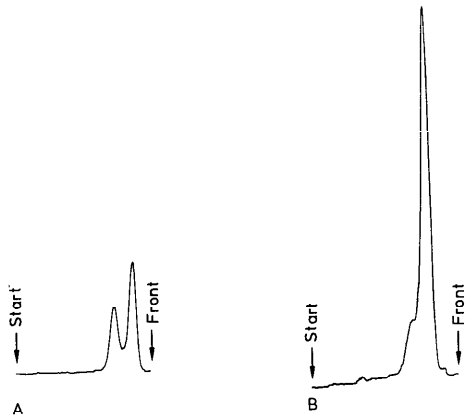


Fig. 1: Reflectance scans of a chromatogram track with 200 ng cobalt(II) chloride and 50 ng iron(III) chloride per chromatogram zone. A) scannned at λ = 610 nm and B) at λ = 480 nm (different reproduction scales!): 1 = cobalt(II) ions, 2 = iron(III) ions

References

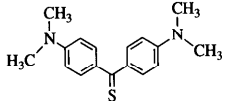
[1] Netz, S., Funk, W., Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, Private communication 1990.

[2] Gaibakian, D. S., Rozylo, J., Janicka, M.: *J. Liq. Chromatogr.* **1985**, *8*, 2969–2989.

4,4′-Bis(dimethylamino)-thiobenzophenone Reagent (Michler's Thioketone)

Reagent for:

- Organomercury compounds [1-5]



$C_{17}H_{20}N_2S$

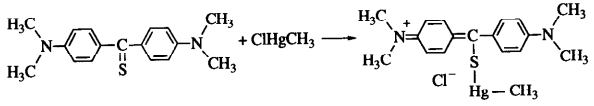
$M_r = 284.43$

Preparation of the Reagent

Stock solution	Dissolve 1 g 4,4′-bis(dimethylamino)thiobenzophenone (MICHLER's thioketone) in 100 ml chloroform.
Dipping solution	Dilute 1 ml stock solution to 100 ml with chloroform.
Storage	The stock solution is stable for several weeks in the refrigerator. The dipping solution is light-sensitive and should, therefore, always be made up fresh.
Substances	4,4′-Bis(dimethylamino)thiobenzophenone Chloroform

Reaction

The reaction has not been elucidated. Presumably MICHLER's thioketone reacts with organomercury compounds to yield intensely colored, mesomer-stabilized diphenylmethane derivatives.



Method

The developed chromatogram is dried in a stream of cold air, immersed in the dipping solution for 2 s, then dried in a stream of warm air for 5 min and heated to 110 °C for 1-2 min until the coloration reaches its maximum.

Violet chromatogram zones are formed on a pale yellow to pale green background.

Note: The detection limits are 1-2 ng substance per chromatogram zone.

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Organomercury Compounds [1-3]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK); before application of the samples the layer was developed once to its upper edge with chloroform – methanol (50 + 50) to precleanse it and then dried at 110 °C for 30 min.

Mobile phase *n*-Hexane – diisopropyl ether – tetrahydrofuran (85+10+5).

Migration distance 5 cm

Running time 16 min

Detection and result: The chromatogram was freed from mobile phase for 15 min in a stream of cold air and immersed in the dipping solution for 2 s, dried for 5 min in a stream of warm air and then heated to 110–130 °C for 1–2 min, until the color of the chromatogram zones reached maximum intensity.

Methylmercuric chloride (hR_f 20–25), ethylmercuric chloride (hR_f 25–30), phenylmercuric chloride (hR_f 35–40), dimethylmercury (hR_f 65–70) and diphenylmercury (hR_f 75–80) appeared as violet zones on a pale yellow to pale green background.

Note: With the mobile phase described a pale colored ß-front appeared at hR_f 5–10, but it did not affect the interpretation of the chromatogram.

In situ quantitation: The absorption photometric scan in reflectance was carried out at $\lambda = 560$ nm. The detection limits lie between 1 and 2 ng substance per chromatogram zone.

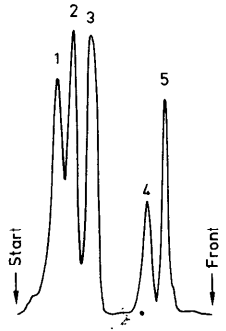


Fig. 1: Reflectance scan of a chromatogram track with 30 ng substance (calculated as Hg) per chromatogram zone: 1 = methylmercuric chloride, 2 = ethylmercuric chloride, 3 = phenylmercuric chloride, 4 = dimethylmercury, 5 = diphenylmercury.

References

[1] Enders, A.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1989.
[2] Funk, W., Cleres, L., Enders, A., Pitzer, H., Kornapp, M. in Henschel, P., Laubereau, P. G. (Hrsg.): Water Pollution Research Report 17, Commission of the European Communities 1989.
[3] Funk, W., Enders, A., Donnevert, G.: *J. Planar Chromatogr.* **1989**, *2*, 282–284.
[4] Westöö, G.: *Acta Chem. Scand.* **1966**, *20*, 2131–2137.
[5] Fishbein, L.: *Chromatogr. Rev.* **1970**, *13*, 83–162.

N-Bromosuccinimide Reagent

Reagent for:

- α-Hydroxyquinones, 5-hydroxyflavones [1]
- Amino acids, Z-protected amino acids
  e.g. histidine and histidine derivatives [2, 3]
  carnosine [2]

$C_4H_4BrNO_2$

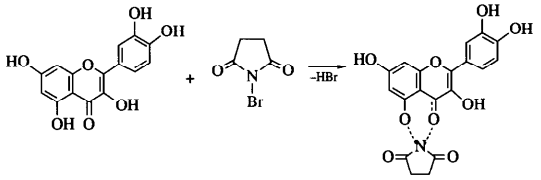
$M_r = 177.99$

Preparation of the Reagent

Dipping solution	Dissolve 0.5 g N-bromosuccinimide in 100 ml acetone [3].
Spray solution	Dissolve 0.5 g N-bromosuccinimide in 25 ml 1-butanol [1].
Storage	The reagent solutions may be stored for ca. 1 week in the refrigerator [3].
Substances	N-Bromosuccinimide Acetone 1-Butanol

Reaction

The mechanism of the reaction has not yet been elucidated [4]. In the case of 5-hydroxyflavonoids it is assumed that colored adducts are formed [1].



Quercetin N-Bromosuccinimide Colored adduct

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and then heated to 120 °C for 20 min.

This yields yellow to brownish colored chromatogram zones, which emit pale blue fluorescence on a dark background when excited with long-wavelength UV light ($\lambda = 365$ nm).

Note: The detection limits for α-hydroxyquinones and 5-hydroxyflavones are 5 μg [1] and for histidine 20 ng substance per chromatogram zone [3].
The reagent can be used on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Histidine and N-α-Z-L-Histidine [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Chloroform – methanol – ammonia solution (32%) (20+16+10).
Migration distance	7 cm
Running time	30 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air for 15 min, immersed in the dipping solution for 3 s, dried in a stream of cold air for 5 min and then heated to 120 °C for 20 min. Finally, the chromatogram was immersed for 2 s in a solution of liquid paraffin – *n*-hexane (1+2) in order to stabilize and enhance the fluorescence (factor ca. 4).

Histidine (hR_f 35–40) and N-α-Z-L-histidine (hR_f 45–50) yielded brown chromatogram zones, with a pale blue fluorescence on a dark background under long-wavelength UV light ($\lambda = 365$ nm). The detection limits lay at 20 ng substance per chromatogram zone.

In situ quantitation: Fluorimetric evaluation was carried out with excitation at $\lambda_{exc} = 365$ nm and the fluorescence emission was measured at $\lambda_{fl} > 400$ nm (cut off filter K 400).

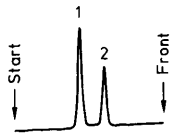


Fig. 1: Fluorescence scan of a chromatogram track with 200 ng each of histidine (1) and N-α-Z-L-histidine (2) per chromatogram zone.

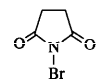
References

[1] Tiwari, K. P., Masood, M.: *Indian J. Chem. Sect. B*, **1979**, *18B*, 97–98; *Chem. Abstr.* **1979**, *91*, 116888d.
[2] Carlo Erba, Company literature *Derivatizing Agents*, p. 79.
[3] Zeller, M.: Private communication, Ciba-Geigy AG, Zentrale Analytik/FD 2.3, K-127.270, Basel, 1991.
[4] Brand, L., Shaltiel, S.: *Biochem. Biophys. Acta* **1964**, *88*, 338–351.

N-Bromosuccinimide-Robinetin Reagent

Reagent for:

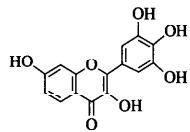
- Pesticides
  e.g. thiophosphoric acid insecticides [1, 2]



$C_4H_4BrNO_2$

$M_r = 177.99$

N-Bromosuccinimide



$C_{15}H_{10}O_7$

$M_r = 302.23$

Robinetin

Preparation of the Reagent

Solution I Make 3.2 ml bromine up to 100 ml with carbon tetrachloride in a measuring cylinder [1].

Dipping solution I Dissolve 50 mg N-bromosuccinimide in 50 ml acetone [3].

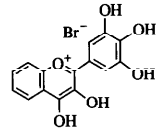
Dipping solution II Dissolve 50 mg robinetin in 70 ml ethanol [3].

Storage The dipping solutions may be stored in the refrigerator at 4 °C for ca. 2 weeks [3].

Substances
N-Bromosuccinimide
Robinetin
Ethanol
Acetone
Bromine
Carbon tetrachloride

Reaction

It is assumed that the hydrogen bromide released on the oxidation of thiophosphoric acid insecticides with N-bromosuccinimide or bromine vapors forms intensely fluorescent salt-like derivatives with 3-hydroxyflavones – such as robinetin [1, 2, 4].



Method

The chromatograms are freed from mobile phase in a stream of warm air and then at 105 °C for 5 min, immersed in the dipping solution I for 3 s [3] or placed still warm for 10 s in a twin-trough chamber, whose vacant trough has been filled with 10 ml of solution I [5, 6]. Then after drying in a stream of cold air (after evaporation of the excessive bromine vapor) the chromatograms are immersed in dipping solution II for 3 s or evenly sprayed with it until the layers begin to be transparent and finally heated to 105 °C for 5 min.

In long-wavelength UV light ($\lambda = 365$ nm) yellow-green fluorescent chromatogram zones are visible on a weakly fluorescent background.

Note: A range of pesticides can be detected on cellulose layers using 3-hydroxyflavones without prior bromination. Thus, the natural fluorescence of robinetin or fisetin, which is weak in a non-polar environment, is significantly enhanced by the presence of polar pesticides [2, 5, 7, 8].

The detection limits for thiophosphate insecticides are 40–100 ng substance per chromatogram zone [1].

The reagent can be used on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Thiophosphoric Acid Insecticides [3]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254}, extra thin layer (MERCK), that had been prewashed by immersing overnight in 2-propanol and then dried at 110 °C for 30 min.
Mobile phase	*n*-Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (543+200+130+127+1).
Migration distance	8 cm
Running time	35 min

Detection and result: The chromatogram was dried in a stream of warm air for 3 min, immersed in dipping solution I for 5 s, dried in a stream of cold air for 3 min and then immersed in dipping solution II for 3 s. It was then heated to 110 °C for 5 min, cooled to room temperature and dipped in a solution of liquid paraffin – *n*-hexane (1+2) for 1 s in order to stabilize and enhance the fluorescence. The chromatogram was then dried in a stream of cold air and evaluated after allowing to stand for ca. 30 min.

In long-wavelength UV light ($\lambda = 365$ nm) oxydemeton-methyl (hR_f 5–10), omethoate (hR_f 15–20), demeton-S-methylsulfon (hR_f 30–35), methamidophos (hR_f 40–45), dimethoate (hR_f 55–60), *trans*-mevinphos (hR_f 60–65), *cis*-mevinphos (hR_f 70–75), dichlorophos (hR_f 75–80) and trichlorfon (hR_f 85–90) appeared as yellow fluorescent chromatogram zones on a weakly fluorescent background.

The detection limits lay at 100 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric evaluation was carried out at $\lambda_{exc} = 365$ nm and the fluorescence emission $\lambda_{fl} > 430$ nm was measured (cut off filter Fl 43).

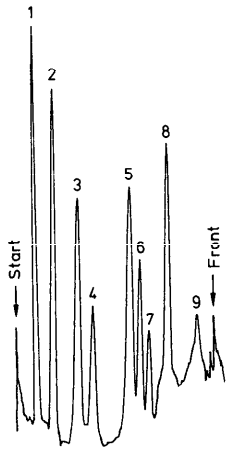


Fig. 1: Fluorescence scan of a chromatogram track with 300 ng of each substance per chromatogram zone:
1 = oxydemeton-methyl, 2 = omethoate, 3 = demeton-S-methylsulfon, 4 = methamidophos, 5 = dimethoate, 6 = *trans*-mevinphos, 7 = *cis*-mevinphos, 8 = dichlorophos, 9 = trichlorfon.

References

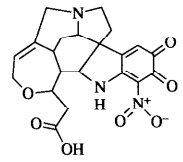
[1] Frei, R. W., Mallet, V., Pothier, C.: *J. Chromatogr.* **1971**, *59*, 135–140.
[2] Mallet, V. N., in J. Harvey, G. Zweig (Ed.): *Pesticide Analytical Methodology*, ACS Symposium Series **1980**, *136*, 127–157.
[3] Pitzer, H.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[4] Lawrence, J. F., Frei, R. W.: *J. Chromatogr.* **1974**, *98*, 253–270.

[5] Mallet, V., Frei, R. W.: *J. Chromatogr.* **1971**, *56*, 69–77.
[6] Sherma, J. in: J. Sherma, G. Zweig (Ed.): *Analytical Methods for Pesticides and Plant Growth Regulators*, Vol. 7, S. 38, Academic Press, 1973.
[7] Mallet, V., Frei, R. W.: *J. Chromatogr.* **1971**, *54*, 251–257.
[8] Mallet, V., Frei, R. W.: *J. Chromatogr.* **1971**, *60*, 213–217.

Cacotheline Reagent

Reagent for:

- Ascorbic acid [1, 2]



$C_{21}H_{21}N_3O_7$

M_r – 427.42

Preparation of the Reagent

Dipping solution	Dissolve 50 mg cacotheline in 50 ml water and make up to 100 ml with ethanol [2].
Spray solution	Dissolve 1 g cacotheline in 50 ml water [1].
Storage	The reagent solutions should always be made up fresh.
Substances	Cacotheline Ethanol

Reaction

Cacotheline is a redox indicator which is yellow in the oxidized form and reddish-violet in the reduced form.

Method

The chromatograms are dried in a stream of warm air, immersed in the dipping solution for 10 s or sprayed homogeneously with the spray reagent and then dried in a stream of cold air [2] or heated briefly to 110 °C [1].

Ascorbic acid produces reddish-brown to violet chromatogram zones on a yellow background [2].

Note: Dehydroascorbic acid does not react [2, 3].

The detection limit for ascorbic acid is less than 100 ng substance per chromatogram zone [2].

The reagent can be employed on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Ascorbic Acid [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Ethanol (96%) – acetic acid (10%) (95+5).
Migration distance	5 cm
Running time	30 min

Detection and result: The dried chromatogram was immersed in the dipping solution for 3 s and then heated briefly to 110 °C.

Ascorbic acid (hR_f 50–55) appeared as a brown-red chromatogram zone that was only stable for ca. 20 min; the background was yellow. The detection limit was less than 100 ng substance per chromatogram zone.

Note: Dehydroascorbic acid, the decomposition product of ascorbic acid, does not react. But it can be detected as a yellow-orange chromatogram zone (hR_f 65–70) by further treatment of the chromatogram with 2,4-dinitrophenylhydrazine. This sequence of reagents, which can also be applied in the reverse order, leads to the disappearance of the red-brown coloration of the ascorbic acid zone within 15 to 20 min!

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 500$ nm (Fig. 1).

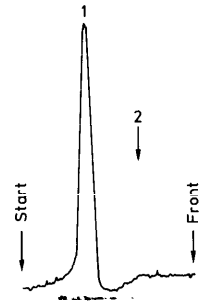


Fig 1: Reflectance scan of a chromatogram track with 500 ng each of ascorbic acid (1) and dehydroascorbic acid (2) per chromatogram zone.

References

[1] E. MERCK, Company brochure *Staining Reagents for Thin-Layer and Paper Chromatography*, Darmstadt 1980.

[2] Schnekenburger, G.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1987.

[3] Ganz, J., Jork, H.: InCom, short course, Düsseldorf 1993.

Chloramine T Reagents

A variety of chloramine T reagents, involving the use of chloramine T for chlorination or oxidation reactions, have been described in the literature. These are described in detail individually in the following monographs.

In these reagents chloramine T – a white powder containing approximatcly 25% active chlorine – is used as a substitute for hypochlorite or chlorine gas. The reactions take place in either acid (hydrochloric acid, sulfuric acid, trichloroacetic acid) or alkaline medium (sodium hydroxide).

Comparative investigations have revealed that varying the acid used or replacing it by a base is generally accompanied by changes – sometimes drastic – in the sensitivity of detection. The information that follows is intended to help the TLC user to choose and optimize the most suitable chloramine T reagent for a particular application:

1. When using chloramine T-mineral acid reagents care should be taken to treat the layer with chloramine T first and then with mineral acid. The sensitivity is considerably reduced if the plate is, for example, merely dipped once in a combined reagent consisting of chloramine T in 5 percent sulfuric acid.
2. Care must be exercised in the choice of acid employed in chloramine T – mineral acid reagent since the detection sensitivity and also the color of the fluorescences produced depend to a significant extent on the choice of acid. This is illustrated for the purine derivatives caffeine, theobromine and theophylline in Figure 1 and Table 1.
3. Only theophylline yields an intensely fluorescent derivative under long-wavelength UV light when treated with chloramine T – sodium hydroxide reagent. The purine derivatives caffeine and theobromine investigated at the same time fluoresce very weakly or not at all.
4. *Digitalis* glycosides that react well with various chloramine T – trichloroacetic acid or mineral acid reagents are not excited to fluorescence after treatment with chloramine T – sodium hydroxide.
5. The phenols pyrocatechol, resorcinol and hydroquinone can be detected with all chloramine T reagents. The detection sensitivity is about the same with chloramine T – sodium hydroxide and chloramine T – trichloroacetic acid. In all cases the detection limits are ca. 75 ng substance per chromatogram zone after the plate has been subsequently dipped in a paraffin oil solution. Somewhat less favorable detection limits of 150 to 200 ng substance per chromatogram zone are obtained after treatment with chloramine T – hydrochloric acid and chloramine T – sulfuric acid.
6. Exposure to hydrochloric acid vapor instead of application of 5% methanolic hydrochloric acid leads to approximately comparable results.

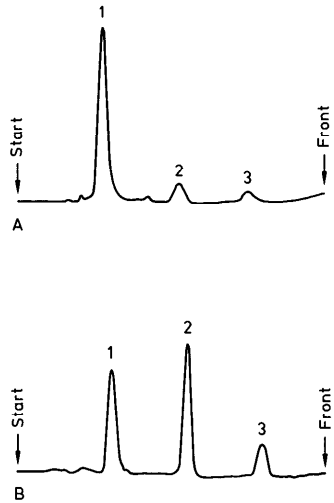


Fig. 1: Comparison of the detection sensitivity after derivatization of three purine derivatives with chloramine T – sulfuric acid (A) and chloramine T – hydrochloric acid (B). Measurement: λ_{exc} = 365 nm, λ_{fl} = 440 nm (monochromatic filter M 440): 1 = theophylline, 2 = theobromine, 3 = caffeine.

Table 1: Fluorescence color and detection limits of the derivatives formed by reaction with various reagents incorporating chloramine T.

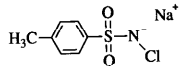
Substance*	Chloramine T reagent with HCl	H_2SO_4	CCl_3COOH	NaOH
Theophylline	yellow-green	blue	blue	blue
	100	100	100	50
Theobromine	blue	yellow	yellow	no fl.
	100	50	300	–
Caffeine	blue	yellow	blue	no fl.
	200	300	300	–
Digoxin	blue	blue	blue	no fl.
	50	50	50	–
Digitoxin	yellow	yellow	yellow	no fl.
	50	50	50	–
Pyrocatechol	pale yellow	pale yellow	yellow-orange	yellow
	100	75	75	75
Resorcinol	pale yellow	pale yellow	yellow	yellow-orange
	100	100	75	75
Hydroquinone	yellow	yellow	red-brown	red-brown
	100	75	75	75

* Fluorescence colors produced on excitation at $\lambda = 365$ nm; detection limits in ng per chromatogram zone, after dipping in liquid paraffin – *n*-hexane (1+2).

Chloramine T–Mineral Acid Reagent

Reagent for:

- Purine derivatives [1–3]
  e.g. caffeine, theophylline, theobromine
- Steroids, sterols [4, 5]
  e.g. dehydroepiandrosterone, cholesterol, solasodine, estriol, testosterone



HCl	H_2SO_4	$C_7H_7ClNNaO_2S \cdot 3H_2O$
$M_r = 36.46$	$M_r = 98.08$	$M_r = 281.69$
Hydrochloric acid	Sulfuric acid	Chloramine T

Preparation of the Reagent

Dipping solution I Dissolve 2.5 g chloramine T in 20 ml water and dilute with 30 ml methanol [6, 7].

Dipping solution II Cautiously mix 47.5 ml methanol with 2.5 ml conc. sulfuric or hydrochloric acid with cooling [6, 7].

Spray solution I Dissolve 10 g chloramine T in 100 ml water [1–3].

Spray solution II Hydrochloric acid (c = 1 mol/L) [1–3].

Spray solution III Dissolve 2 g chloramine T in 100 ml conc. sulfuric acid [4, 5].

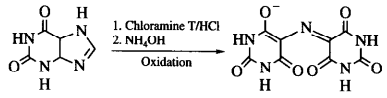
Storage Contrary to what literature reports spray solutions I and III should always be made up fresh; as should dipping solution I. Dipping solution II and spray solution II may be stored for an extended period.

Substances

Chloramine T trihydrate
Hydrochloric acid, 1 mol/L
Hydrochloric acid (32%)
Sulfuric acid (95–97%)
Methanol

Reaction

Purine derivatives (e.g. xanthine) are oxidized by chloramine T in the presence of hydrochloric acid and form purple-red ammonium salts of purpuric acid (murexide) with ammonia. Whether the murexide reaction is also the cause of the fluorescence is open to question.



Xanthine Murexide

Method

Purine derivatives: The chromatograms are freed from mobile phase in a stream of warm air, immersed in dipping solution I for 2 s and either placed while still damp in an atmosphere of hydrochloric acid for 10 min [6] or immersed in dipping solution II for 1 s after brief intermediate drying. Alternatively, they can be sprayed homogeneously with spray solution I and then, after brief intermediate drying with spray solution II [1–3]. In both cases the chromatograms are then heated to ca. 100 °C – until the chlorine odor disappears – and then placed for ca. 5 min in the empty trough of a twin-trough chamber which has been filled with ammonia solution (25%).

Purine derivative yield pinkish-red chromatogram zones on an almost colorless background [1–3]. The chromatogram is then heated to ca. 100 °C again until the color development reaches maximum intensity (yellow to orange). On excitation with long-wavelength UV light ($\lambda = 365$ nm), blue or yellow fluorescent zones become visible now.

Steroids: The developed chromatograms are freed from mobile phase in a stream of cold air, then either immersed for 2 s in dipping solution I and, after brief intermediate drying in a stream of warm air, immersed in dipping solution II for 2 s or homogeneously sprayed with spray solution III and, after being left for 5 min at room temperature, heated at 110 °C for 5 min and evaluated.

Even before heating steroids frequently yield characteristic, pale yellow to dark purple colored zones, whose colors generally change on heating [4] and which are usually excited to fluorescence in long-wavelength UV light ($\lambda = 365$ nm) [6].

Note: The detection of steroids is more sensitive than with the LIEBERMANN-BURCHARD reaction. Visual detection limits of 0.2 to 5 µg substance per chromatogram zone have been reported [4]; photometric detection limits are appreciably lower being a few nanograms substance per chromatogram zone [6]. The reagent with sulfuric acid is more sensitive than that containing hydrochloric acid for the detection of estrogens [6]. However, the hydrochloric acid-containing chloramine T reagent yields the best coloration results for purine detection; here it is more sensitive than the reagents containing trichloroacetic acid or sulfuric acid [6]. The fluorescence occurring with purines can be increased (factor 2) and stabilized by finally dipping the chromatogram in liquid paraffin – *n*-hexane (1+2) [6].

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers as well as on RP, CN, NH_2 and DIOL phases. Cellulose and polyamide 11 layers char on heating.

Procedure Tested 1

Xanthine Derivatives [6, 7]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Ethyl acetate – methanol – ammonia solution (25%) (16+4+3).
Migration distance	6 cm
Running time	15 min

Detection and result: When viewed under short-wavelength UV light ($\lambda = 254$ nm) dark zones were visible due to fluorescence quenching.

With larger amounts of substance red zones appeared on a white background. When viewed under long-wavelength UV light ($\lambda = 365$ nm) clear fluorescence could be seen with lower concentrations if the chromatogram was freed from mobile phase for 5 min in a stream of cold air, immersed in dipping solution I for 2 s and immediately exposed, while still damp, to hydrochloric acid vapors for 10 min (twin-trough chamber, whose second trough had been filled with 10 ml hydrochloric acid (32%)). The chromatogram was heated to 110 °C for 10 min and then, after cooling to room temperature, placed in the vacant trough of a twin-trough chamber filled with 10 ml ammonia solution (25%) for 10 min. Then it was heated once again to 110 °C for 10 min and, after cooling, dipped in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s in order to increase (factor 2) and stabilize the fluorescence.

In the concentration range above 1 µg substance per spot, red-colored chromatogram zones (murexide reaction) could be seen on a pale background; these could be excited to blue (caffeine, hR_f 75- 80: theobromine, hR_f 55-60) or yellow (theophylline hR_f 35-40) fluorescence on a dark background in long-wavelength UV light ($\lambda = 365$ nm).

If the fluorescence was excited at a wavelength of $\lambda_{exc} = 313$ nm, then the detection limits were 200 ng (caffeine, theophylline) to 400 ng (theobromine) substance per chromatogram zone. At an excitation wavelength $\lambda_{exc} = 365$ nm the detection limits were appreciably lower at 100 ng substance per chromatogram zone for theobromine and theophylline. The sensitivity remained unchanged in the case of caffeine.

In situ quantitation: The fluorimetric evaluation was made either at $\lambda_{exc} = 313$ nm and $\lambda_{fl} > 390$ nm or at $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 390$ nm (Fig. 1).

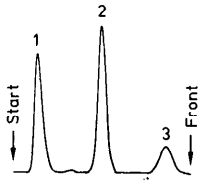


Fig. 1: Fluorescence scan of a chromatogram track with 500 ng each of theophylline (1), theobromine (2) and caffeine (3) per chromatogram zone.

Procedure Tested 2

Estrogens [6]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Toluene – ethanol (90+10).
Migration distance	7 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed in dipping solution I for 2 s and dried in a stream of warm air for 1 min. It was then immersed in dipping solution II for 2 s and heated to 110 °C for 10 min. The chromatogram was then placed for 5 min in a twin-trough chamber, whose second chamber had been filled with 10 ml ammonia solution (25%), and then heated to 110 °C for 5 min. After cooling the chromatogram was immersed for 2 s in liquid paraffin – *n*-hexane in order to increase (factor 2) and stabilize the fluorescence.

After treatment with dipping solution II and subsequent heating the estrogens already appeared as earth brown (estriol, estradiol) and yellow-brown (estrone) chromatogram zones, which could be excited to pale yellow fluorescence on a colorless background in long-wavelength UV light ($\lambda = 365$ nm).

After exposure to ammonia vapor and treatment with paraffin oil estriol (hR_f = 15-20), estradiol (hR_f 30-35) and estrone (hR_f 35-40) appeared as white-yellow fluorescent chromatogram zones on a colorless background, when excited in long-wavelength UV light ($\lambda = 365$ nm). The detection limits for all three estrogens were in the 50 ng range on absorption photometric measurement and 10 ng substance per chromatogram zone on fluorimetric evaluation.

In situ quantitation: The absorption photometric measurement was made at $\lambda = 380$ nm (Fig. 2A) and the fluorimetric evaluation was carried out with excitation at $\lambda_{exc} = 365$ nm and measurement at $\lambda_{fl} > 560$ nm (Fig. 2B).

Fig. 2: Reflectance scan (A) and fluorescence scan (B) of a chromatogram track with 1 µg (A) or 200 ng (B) each substance per chromatogram zone: 1 = estriol, 2 = estradiol, 3 = estrone.

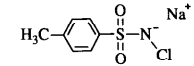
References

[1] Gänshirt, H., Malzacher, A.: *Arch. Pharm.* **1960,** *293,* 925–932.
[2] Baerheim Svendsen, A.: *J. Planar Chromatogr.* **1989,** *2,* 8–18.
[3] E. Merck, Company brochure *Dyeing Reagents for Thin-layer and Paper Chromatography,* Darmstadt 1980.
[4] Bajaj, K. L., Ahuja, K. L.: *J. Chromatogr.* **1979,** *172,* 417–419.
[5] Ahuja, K. L., Bajaj, K. L.: *J. Am. Oil Chem. Soc.* **1980,** *57,* 250–251.
[6] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1990.
[7] Ganz, J., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1991.

Chloramine T–Sodium Hydroxide Reagent

Reagent for:

- Polybasic phenols
  e. g. 1,2- and 1,3-dihydroxybenzene derivatives,
  vicinal trihydroxybenzene derivatives [1]
- Flavonoids [1]
- α-Nitroso-β-naphthol [1]



NaOH	$C_7H_7ClNNaO_2S \cdot 3H_2O$
$M_r = 40.00$	$M_r = 281.69$
Sodium hydroxide	Chloramine T

Preparation of the Reagent

Dipping solution Dissolve 5 g chloramine T in 50 ml water with shaking. Add 500 mg sodium hydroxide to this solution and dissolve also with shaking. Then dilute with 50 ml methanol.

Spray solution Dissolve 5 g chloramine T in 100 ml 0.5 percent sodium hydroxide solution [1].

Storage The dipping reagent may be stored in the refrigerator for several weeks.

Substances Chloramine T trihydrate
Sodium hydroxide pellets
Methanol

Reaction

The mechanism of the reaction has not yet been elucidated; it is assumed that 1,2-dihydroxybenzene is oxidized to quinone and vicinal trihydroxybenzene derivatives to hydroxyquinones [1].

Method

The chromatograms are freed from mobile phase in a stream of cold air, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution, allowed to stand at room temperature for 5 min and then heated to 120 °C for 10 min before examination. 1,2-Dihydroxybenzene derivatives yield yellow zones, 1,3-dihydroxybenzene derivatives purple zones and vicinal trihydroxybenzene derivatives brown chromatogram zones on an almost colorless background [1]. The zones fluoresce under long-wavelength UV light ($\lambda = 365$ nm) [2]; α-resorcinic acid with a peach color, flavonoids and phloroglucinol with a yellowish-red color [1].

Note: Monohydroxybenzene derivatives and vicinal hydroxymethoxybenzene derivatives do not react under these conditions but can be made visible by spraying afterwards with FOLIN-CIOCALTEU's reagent [1]. Flavonoids, anthraquinone derivatives and α-nitroso-ß-naphthol yield a red color with alkalis alone [1]. Steroids and aromatic amines do not react [1].

The fluorescence of the chromatogram zones of some phenols can be increased by a factor of 2 and stabilized by dipping the chromatogram in liquid paraffin – *n*-hexane (1+2) [2].

The visual detection limits for polyphenols and flavonoids are 0.2 to 1 µg substance per chromatogram zone [1]. The photometric detection limits are appreciably lower (see "Procedure Tested").

The reagent can be used on silica gel, kieselguhr, Si 50000, NH_2 and cellulose layers.

Procedure Tested

Dihydroxybenzenes [2, 3]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer HPTLC plates NH_2 F_{254s} (MERCK).

Mobile phase Toluene – ethyl acetate – ethanol (20+10+10).

Migration distance 6 cm

Running time 15 min

Detection and result: The chromatogram was dried in a stream of cold air for 5 min. Observation under short-wavelength UV light ($\lambda = 254$ nm) revealed dark zones on a pale blue, fluorescent background. Immersion in the dipping solution for 2 s, brief drying in a stream of warm air and then heating to 110–120 °C for 10 min yielded, on examination under long-wavelength UV light ($\lambda = 365$ nm) pale yellow (pyrocatechol, hR_f 35–40), yellow (resorcinol, hR_f 45–50) and red-brown (hydroquinone, hR_f 50–55) fluorescent chromatogram zones on a pale blue, fluorescent backgound. After dipping in liquid paraffin – *n*-hexane (1+2) the detection limits were between 50 and 100 ng substance per chromatogram zone.

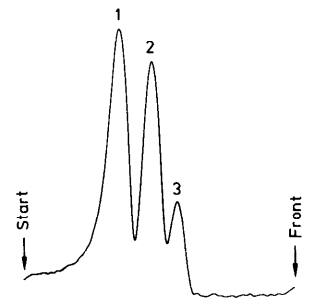


Fig. 1: Fluorescence scan of a chromatogram track with 400 ng each of pyrocatechol (1), resorcinol (2) and hydroquinone (3) per chromatogram zone.

In situ quantitation: Fluorimetric evaluation was carried out under long-wavelength UV light with excitation at $\lambda_{exc} = 436$ nm and detection at $\lambda_{fl} > 560$ nm (Fig. 1).

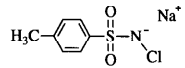
References

[1] Bajaj, K. L., Arora, Y. K.: *J. Chromatogr.* **1980**, *196*, 309–313.
[2] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1990.
[3] Ganz, J., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1991.

Chloramine T–Trichloroacetic Acid Reagent (Jensen's Reagent)

Reagent for:

- *Digitalis* glycosides [1–11]
  e.g. digoxin, digitoxin, β-methyldigoxin, α- and β-acetyldigoxin, gitoxin, acetyldigitoxin, acetylgitoxin



CCl_3COOH	$C_7H_7ClNNaO_2S \cdot 3H_2O$
$M_r = 163.39$	$M_r = 281.69$
Trichloroacetic acid	Chloramine T

Preparation of the Reagent

Dipping solution	Dissolve 10 g trichloroacetic acid and 0.4 g chloramine T (N-chloro-4-methylbenzenesulfonamide sodium salt) in a mixture of 80 ml chloroform, 18 ml methanol and 2 ml water [11].
Solution I	Dissolve 3 to 5 g chloramine T in 100 ml water.
Solution II	Dissolve 25 g trichloroacetic acid in 100 ml ethanol.

Spray solution Mix 10 ml solution I with 40 ml solution II immediately before use [1–5].
Other proportions, e.g. 1+15 [6, 10] and 2+8 [7], have also been recommended.

Storage Solution I should always be made up fresh. The dipping solution may be stored in the refrigerator for several days [11]. Solution II may be stored for longer periods.

Substances
Chloramine T trihydrate
Ethanol absolute
Trichloroacetic acid
Chloroform
Methanol

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 to 2 s or homogeneously sprayed with the spray solution and then heated to 100–150 °C for 5–30 min [2–7, 10, 11].

Digitalis glycosides yield yellow or blue fluorescent chromatogram zones on a dark background when examined under long-wavelength UV light ($\lambda = 365$ nm) [1, 4, 5–7].

Note: If the mobile phases contains formamide the chromatograms should be freed from it by heating to 130–140 °C in the drying cupboard for 1 h before applying the reagent [6].

The detection limits for *digitalis* glycosides are 12–50 ng substance per chromatogram zone [2, 8].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, cellulose and RP layers.

Procedure Tested 1

Cardenolides in Lily of the Valley Extract [12]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer TLC plates Silica gel 60 F_{254} (MERCK). The layer was prewashed before use by developing with chloroform – methanol (50+50) and then drying at 110 °C for 30 min.

Mobile phase Ethyl acetate – methanol – water (81+11+8).

Migration distance 10 cm

Running time 25 min

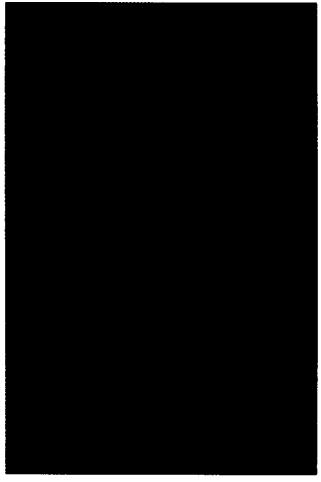


Fig. 1: Chromatogram of a lily of the valley extract – left before and right after treatment with JENSEN's reagent, photographed in long-wavelength UV light ($\lambda = 365$ nm) [13].

Detection and result: The dried chromatogram was half covered with a glass plate in the direction of development and the other half was homogeneously sprayed with the spray solution. The glass plate was then removed and the whole chromatogram heated to 120 °C for 5–10 min, allowed to cool to room temperature and examined under long-wavelength UV light ($\lambda = 365$ nm).

In Fig. 1 it can be seen that the reagent applied to the right-hand side of the chromatogram has, on the one hand, intensified the fluorescent zones of the cardenolides, but that there are, on the other hand, other substance zones whose fluorescence, compared with the natural fluorescence on the left-hand side of the chromatogram, has been weakened appreciably on treatment with the reagent. The reagent is not suitable for in situ quantitation.

Procedure Tested 2

Digitalis Glycosides [14]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} with concentration zone (RIEDEL DE HAEN, MERCK).
Mobile phase	Acetone – dichloromethane (60+40).
Migration distance	5 cm
Running time	8 min

Detection and result: The dried chromatogram was immersed in the dipping solution for 2 s and heated to 110 °C for 10 min. Observation in long-wavelength UV light revealed fluorescent zones for digoxin (hR_f 30–35) blue and digitoxin (hR_f 40–45) yellow.

The fluorescence can be stabilized and enhanced by ca. 20% by dipping in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s. The detection limits after dipping in the paraffin solution are 50 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric evaluation was carried out under long-wavelength UV light at $\lambda_{exc} = 365$ nm and the fluorescence emission was measured at $\lambda_{fl} > 400$ nm (cut off filter K 400) (Fig. 2).

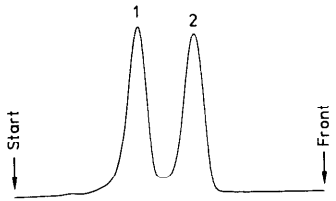


Fig. 2: Fluorescence scan of a chromatogram track with 500 ng each digoxin (1) and digitoxin (2) after treatment of the chromatogram with reagent and paraffin oil solution.

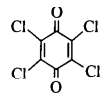
References

[1] Winsauer, K., Buchberger, W.: *Chromatographia* **1981**, *14*, 623–625.
[2] Bloch, D. E.: *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 707–708.
[3] Sabatka, J. J., Brent, D. A., Murphy, J., Charles, J., Vance, J., Gault, M. H.: *J. Chromatogr.* **1976**, *125*, 523–525.
[4] Kibbe, A. H., Araujo, O. E.: *J. Pharmac. Sci.* **1973**, *62*, 1703–1704.
[5] E. MERCK, Company brochure *Dyeing Reagents for Thin-layer and Paper Chromatography*, Darmstadt 1980.
[6] Czerwek, H., Hardebeck, K., Kaiser, F., Schaumann, W., Wolfahrth-Ribbentrop, A.: *Arzneim. Forsch.* **1971**, *21*, 231–234.
[7] Pachaly, P.: Dünnschicht-Chromatographie in der Apotheke, 2. Aufl., Wissenschaftliche Verlagsgesellschaft, Stuttgart 1983, 188.
[8] Lutz, U.: *Österr. Apoth. Ztg.* **1971**, *25*, 250.
[9] Balbaa, S. I., Hilal, S. H., Haggag, M. Y.: *Planta Med.* **1971**, *20*, 54–59.
[10] Krüger, D., Wichtl, M.: *Dtsch. Apoth. Ztg.* **1985**, *125*, 55–57.
[11] Horvath, P., Szepesi, G., Hoznek, M., Vegh, V., Mincsovics, E.: *Proc. Int. Symp. Instrum. High Perform. Thin-Layer Chromatogr. (HPTLC), 2nd, Interlaken* 1982, 295–304.
[12] Hahn-Deinstrop, E.: Privatmitteilung, Heumann-Pharma, Abt. Entwicklungsanalytik, Heideloffstraße 18–28, D-90478 Nürnberg 1, 1990.
[13] Hahn-Deinstrop, E.: *GIT Fachz. Lab. Supplement 3 „Chromatographie"* **1989**, 29–31.
[14] Ganz, J., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1991.

p-Chloranil Reagent

Reagent for:

- Aromatic amines [1]
  e.g. aniline, diphenylamine, anisidine
- Alkaloids [2, 6]
  e.g. ephedrine, emetine, cephaëline, cytisine, coniine, γ-coniceine, anabasine, brucine, symphytine, harmaline, berberine, reserpine, papaverine, morphine
- *tert*-N-Ethyl derivatives
  e.g. local anesthetics such as procaine, lidocaine, tolycaine [7]
- Phenothiazines
  e.g. phenothiazine, promazine, promethazine [8]
- Benzodiazepines
  e.g. chlordiazepoxide, diazepam, prazepam [2]
- Steroids
  e.g. 17β-estradiol, dienestrol, estrone, stilbestrol, 2α-xanthatocholestan-3-one [9]
- Antibiotics
  e.g. penicillins [10] such as ampicillin, amoxycillin, cloxacillin, pivampicillin, oxacillin, nafcillin
- Diuretics
  e.g. acetazolamide, ethoxolamide, quinethazone, furosemide, benzthiazide, methazolamide [11]
- Antidiabetics [11]
  e.g. gliclazide [11]



$C_6Cl_4O_2$

$M_r = 245.88$

Preparation of the Reagent

Dipping solution Dissolve 0.5 g *p*-chloranil in 90 ml ethyl acetate, cautiously make up to 100 ml with 10 ml sulfuric acid (ca. 96%) and homogenize for 5 min in the ultrasonic bath or by vigorous shaking.

Spray solution I *For alkaloids, phenothiazines, penicillins, diuretics, tertiary aliphatic amines and steroids*: Dissolve 200 mg to 1 g *p*-chloranil in 100 ml dioxan, acetonitrile or toluene [2–11].

Spray solution II *For aromatic amines*: Dissolve 200 mg *p*-chloranil in 100 ml chlorobenzene [1].

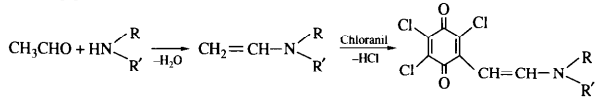
Spray solution III *For sec. amines*: Dissolve 100 mg *p*-chloranil in 100 ml dioxan or acetonitrile [2].

Storage The reagent solutions may be stored in the refrigerator for at least one week [2].

Substances *p*-Chloranil
Ethyl acetate
Sulfuric acid (95–97%)
Acetonitrile
Acetaldehyde
Dioxan
Toluene
Chlorobenzene

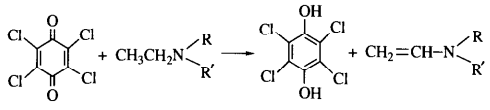
Reaction

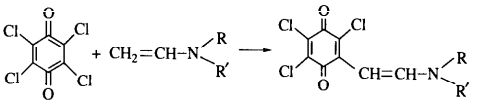
Secondary amines react at room temperature with acetaldehyde and *p*-chloranil according to the following scheme to yield intensely blue-colored dialkylaminovinylquinone derivatives [2]:



Dialkylaminovinylquinone derivative

In the case of tertiary N-ethylamine derivatives the N-ethyl group is first selectively oxidized by *p*-chloranil to an enamino group which then condenses with excess *p*-chloranil to a blue aminovinylquinone derivative [7]. Secondary N-ethyl derivatives do not yield blue aminovinylquinone derivatives; they probably react directly with chloranil by nucleophilic attack at one of the four chlorine atoms to yield aminoquinones of other colors [7]. It has also been suggested that some classes of substances react to yield charge transfer complexes [1, 5, 8, 12].





In the case of aromatic amines there is an initial nucleophilic substitution catalyzed by the silanol groups of the silica gel layer to yield arylaminobenzoquinone derivatives, that undergoe oxidative cyclization to the corresponding dioxazines [1].

Method

The chromatograms are freed from mobile phase in a stream of warm air, then immersed in the dipping solution for 2 s or homogeneously sprayed with the appropriate spray solution. Then, in the case of N-ethyl derivatives, the plate is heated to 105–110 °C for 2 min to accelerate the reaction [7]. Heating (e.g. to 80–105 °C for 15 min) can also lead to color intensification and color change in the case of other alkaloids [5, 6].

Sec. "amine alkaloids" yield blue-colored chromatogram zones on a colorless to pale yellow backgound at room temperature [2]. Aromatic amines, phenothiazines, steroids, diuretics and penicillins yield yellow, orange or reddish-brown to purple chromatogram zones at room temperature [1, 8–11]. On heating tertiary N-ethyl derivatives also yield blue-colored chromatogram zones, this time on a pale yellow background [7]. Other N-containing pharmaceuticals, e.g. some benzodiazepines, yield grey, brown or violet-brown zones on heating [7].

Note: It is reported that the use of chlorobenzene as solvent is essential when the reagent is to be used to detect aromatic amines [1]. In the case of steroids, penicillins, diuretics and alkaloids the reaction should be accelerated and intensified by spraying afterwards with dimethylsulfoxide (DMSO) or dimethylformamide (DMF), indeed this step makes it possible to detect some substances when this would not otherwise be possible [5, 9–11]; this latter treatment can, like heating, cause color changes [5, 9]. Penicillins and diuretics only exhibit weak reactions if not treated afterwards with DMF [10, 11]. Steroids alone also yield colored derivatives with DMSO [9]. Treatment afterwards with diluted sulfuric acid (c = 2 mol/L) also leads to an improvement in detection sensitivity in the case of a range of alkaloids. In the case of pyrrolizidine alkaloids it is possible to use *o*-chloranil as an alternative detection reagent; however, in this case it is recommended that the plate be treated afterwards with a solution of 2 g 4-(dimethylamino)-benzaldehyde and 2 ml boron trifluoride etherate in 100 ml anhydrous ethanol because otherwise the colors initially produced with *o*-chloranil rapidly fade [12].

The blue derivatives formed with the reagent by alkaloids remain stable for at least one day and usually much longer (cover the chromatogram with a glass plate) [2, 6, 7]. The shade of color produced can be affected by fluorescence indicators incorporated in the silica gel layer [7]. Tertiary amine alkaloids do not react at room temperature with the acetaldehyde-containing reagent [2].

The detection limits (substance per chromatogram zone) are 10 to 20 ng for aromatic amines [1], 100 ng for phenothiazines [8], 0.5 to 2 µg for secondary amine alkaloids [2], 5 to 50 µg for N-ethyl derivatives [7], 1 to 3 µg for penicillins [10], 1 to 4 µg for diuretics and 1 to 2 µg for a range of steroids [9]. There have been some reports of appreciably lower detection limits of 40–400 ng substance per chromatogram zone and even less for alkaloids [6].

The dipping reagent can be used, for example, on silica gel, kieselguhr, Si 50000, RP 18, NH_2, Diol and CN layers. It is not possible to detect aromatic amines on cellulose layers [1].

Procedure Tested

Opium Alkaloids [13]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Acetone – toluene – ethanol – ammonia solution (25%) (40+40+6+2).
Migration distance	7 cm
Running time	7 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s and then heated to 140 °C for 5 min. (Caution! The back of the HPTLC plate is contaminated with sulfuric acid.)

Narceine (hR_f 1–5) yielded red-brown, morphine (hR_f 5–10), codeine (hR_f 15–20) and thebaine (hR_f 35–40) brown-violet, papaverine (hR_f 60–65) light brown and narcotine red chromatogram zones on a colorless background. Since the colors fade in the air it is recommended that the chromatogram be covered with a glass plate.

After treatment with the reagent the detection limits for opium alkaloids are 50–500 ng per chromatogram zone, this is sometimes a somewhat lower sensitivity than that obtained by direct measurement of the UV absorption (cf. Table 1). However, the color reactions provided additional specificity.

Table 1: Comparison of the detection sensitivities

Substance	Detection limits (ng)	
	UV_{280}	*p*-Chloranil
Narceine	50	100
Morphine	50	50
Codeine	50	50
Thebaine	20	20
Papaverine	5	500
Narcotine	50	50

In situ quantitation: Direct measurement of the UV absorption at wavelength $\lambda = 280$ nm was preferred for quantitative in situ evaluation since the reagent treatment did not yield more exact results.

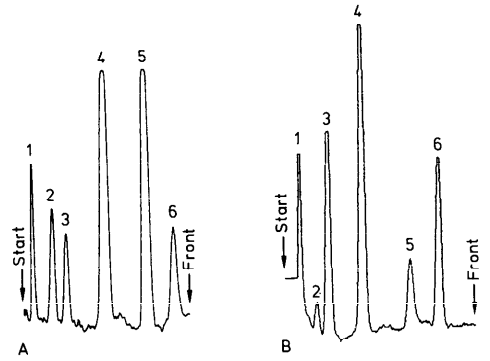


Fig. 1: Reflectance scan of a chromatogram track with 1 µg of each substance per chromatogram zone (exception: papaverine: 5 µg). Chromatogram (A) before and (B) after reagent treatment (registration of B with double sensitivity): 1 = narceine, 2 = morphine, 3 = codeine, 4 = thebaine, 5 = papaverine, 6 = narcotine.

References

[1] Pires, L. M., Roseira, A. N.: *J. Chromatogr.* **1971**, *56*, 59–67.
[2] Taha, A. M., Rücker, G., Gomaa, C. S.: *Planta Med.* **1979**, *36*, 277–278.
[3] Stahl, E., Schmitt, W.: *Arch. Pharm.* **1975**, *308*, 570–578.
[4] Unger, W.: *Planta Med.* **1977**, *31*, 262–265.
[5] Agarwal, S. P., Abdel-Hady Elsayed, M.: *Planta Med.* **1982**, *45*, 240–242.
[6] Huizing, H. J., De Boer, F., Malingré, Th. M.: *J. Chromatogr.* **1980**, *195*, 407–411.
[7] Taha, A. M., Abd El-Kader, M. A.: *J. Chromatogr.* **1979**, *177*, 405–408.
[8] Forrest, J. E., Heacock, R. A.: *J. Chromatogr.* **1973**, *75*, 156–160.
[9] Agarwal, S. P., Nwaiwu, J.: *J. Chromatogr.* **1984**, *295*, 537–542.
[10] Agarwal, S. P., Nwaiwu, J.: *J. Chromatogr.* **1985**, *323*, 424–428.
[11] Agarwal, S. P., Nwaiwu, J.: *J. Chromatogr.* **1986**, *351*, 383–387.
[12] Molyneux, R. J., Roitman, J. N.: *J. Chromatogr.* **1980**, *195*, 412–415.
[13] Klein, I., Jork, H.: GDCh-training course Nr. 300 „Einführung in die Dünnschicht-Chromatographie", Universität des Saarlandes, Saarbrücken, 1990.

Chlorine–Potassium Iodide–Starch Reagent

Reagent for:

- Amino, imino, amido groups
  - e.g. peptides [1–4]
    such as vasotocin analogues [5]
  - e.g. phenylalkylamines
    such as chlorphentermine, cloforex [6]
  - e.g. parbendazole and its metabolites [7]
  - e.g. phenylbutazone, prenazone [8]
- Phthalimide and derivatives [9]
- Pesticides
  - e.g. fungicides [10]
    such as benomyl, 2-aminobenzimidazol (2-AB), methyl-2-benzimidazolyl carbamate
  - e.g. triazine herbicides
    such as simazine, atrazine, propazine prometryn, prometon, ametryn [11]

KI	$(C_6H_{10}O_5)_n$
$M_r = 166.01$	$M_r \approx 7000$
Potassium iodide	Starch

Preparation of the Reagent

Solution I Dissolve 3 g potassium permanganate in 100 ml water.

Solution II Dilute 25 ml hydrochloric acid (32%) with 50 ml water.

Dipping solution Dissolve 250 mg potassium iodide in 25 ml water, mix with a solution of 750 mg starch (soluble starch according to ZULKOWSKY) in 25 ml water and dilute with 30 ml ethanol (99.5%) [12].

Spray solution Mix a solution of 0.5–4 g potassium iodide in 50 ml water with a solution of 1.5–2 g starch in 50 ml water [8] and add 20 ml ethanol [9, 10].

Storage The spray solution should always be made up fresh [10].

Substances
Potassium iodide
Starch, soluble according to ZULKOWSKY
Potassium permanganate
Hydrochloric acid (32%)
Ethanol

Reaction

Treatment with chlorine gas converts amines to chloramines, whose active chlorine oxidizes iodide to iodine. This then forms the well-known, deep blue iodine-starch complex [13].

Method

The chromatograms are freed from mobile phase in a stream of warm air and treated with chlorine gas for 1–5 min, for example, by placing in the vacant trough of a twin-trough chamber filled with 10 ml each of solution I and solution II [10]. After the excess chlorine has been removed (ca. 5–10 min stream of cold air) the chromatograms are immersed in the dipping solution for 1 s [12] or homogeneously sprayed with the spray solution [10].

In general, deep blue chromatogram zones are produced immediately on a colorless background.

Note: Instead of chlorine gas treatment the chromatograms can be exposed to bromine vapor [8] or sprayed with bleach solution. In the case of phthalimide derivatives the chromatograms are heated for 1 h at 180 °C before chlorine treatment [9]. The colored

chromatograms remain unchanged for ca. 2 to 3 hours, then they begin to darken [10, 12]. The color of the chromatogram zones turns brown on drying; the original blue color can be regenerated by moistening the chromatogram with water vapor [12].

The starch according to ZULKOWSKY yields a clear solution in cold water without heating; this solution can be diluted with ethanol without precipitation of insolubles. For instance 10 ml of a 3% aqueous starch solution can be mixed with 9 ml ethanol (99.5%) without the precipitation of starch [12].

The detection limits for triazine herbicides are 10–20 ng [11, 12], for phthalimide derivatives 50–100 ng [9] and for fungicides 50–500 ng substance per chromatogram zone [10].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, cellulose, Diol and RP layers [12]. NH_2 phases are not suitable, since the dipping solution elutes a brown-violet solution from the NH_2 layer, which itself remains white [12].

Polyamide and CN phases are not suitable either because the layer background is colored yellow [12].

Procedure Tested

Triazines [12]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Toluene – acetone (85+15).
Migration distance	5 cm
Running time	8 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed for 1 min in a twin-trough chamber in which a chlorine gas atmosphere had previously been generated by pouring ca. 3 ml hydrochloric acid (25%) over ca. 0.2 g potassium permanganate in the vacant trough. The chromatogram was immersed for 1 s in the dipping solution after removal of the excess chlorine (5 min stream of cold air).

The triazines atraton (hR_f 15–20), cyanazin (hR_f 30–35), terbutylazin (hR_f 45–50) and anilazin (hR_f 70–75) immediately appear as violet-blue chromatogram zones on a colorless background (Fig. 1A). In order to prevent spot diffusion when the chromatogram is dried the TLC plate is placed on a hot plate at 50 °C and a stream of warm air was also directed at the layer from a fan (2 min). On drying the color of the chromatogram zones changes from blue-violet to brown (Fig. 1B) and the whole layer becomes dark after a prolonged period. The original blue coloration of the chromatogram zones can be regenerated by moistening the layer with water vapor.

In situ quantitation: The absorption photometric evaluation was carried out in reflectance at wavelength $\lambda = 550$ nm (Fig. 1C).

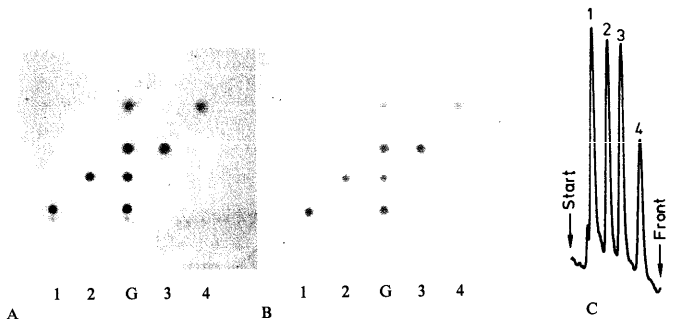


Fig. 1: Chromatogram of triazines (A) after immersion in the dipping solution and (B) after additional heating and (C) reflectance scan of a chromatogram track with 170 ng each of atraton (1), cyanazin (2), terbutylazin (3) and anilazin (4) per chromatogram zone.

References

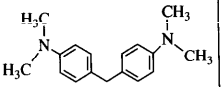
[1] Bryan, W. M., Hempel, J. C., Huffman, W. F., Marshall, G. R., Moore, M. L., Silvestri, J., Stassen, F. L., Stefankiewicz, J. S., Sulat, L., Webb, R. L.: *J. Med. Chem.* **1988**, *31*, 742–744.
[2] Hocart, S. J., Nekola, M. V., Coy, D. H.: *J. Med. Chem.* **1988**, *31*, 1820 1824.
[3] Allen, M. C., Fuhrer, W., Tuck, B., Wade, R., Wood, J. M.: *J. Med. Chem.* **1989**, *32*, 1652–1661.

[4] Callahan, J. F., Ashton-Shue, D., Bryan, H. G., Bryan, W. M., Heckman, G. D., Kinter, L. B., McDonald, J. E., Moore, M. L., Schmidt, D. B., Silvestri, J. S., Stassen, F. L., Sulat, L., Yim, N. C. F., Huffman, W. F.: *J. Med. Chem.* **1989,** *32,* 391–396.
[5] Buku, A., Gazis, D., Eggena, P.: *J. Med. Chem.* **1989,** *32,* 2432–2435.
[6] Bülow, M., Dell, H.-D., Fiedler, J., Kamp, R., Lorenz, D.: *Arzneim. Forsch.* **1971,** *21,* 86–93.
[7] DiCuollo, C. J., Miller, J. A., Mendelson, W. L., Pagano, J. F.: *J. Agric. Food Chem.* **1974,** *22,* 948–953.
[8] Schütz, C., Schütz, H.: *Arzneim. Forsch.* **1973,** *23,* 428–431.
[9] Ackermann, H., Faust, H., Kagan, Y. S., Voronina, V. H.: *Arch. Toxikol.* **1978,** *40,* 255–261.
[10] Balinova, A.: *J. Chromatogr.* **1975,** *111,* 197–199.
[11] Delley, R., Friedrich, K., Karlhuber, B., Székely, G., Stammbach, K.: *Fresenius Z. Anal. Chem.* **1967,** *228,* 23–38.
[12] Meiers, B., Jork, H.: GDCh-training course No. 302 „Möglichkeiten der quantitativen Auswertung von Dünnschicht-Chromatogrammen", Universität des Saarlandes, Saarbrücken, 1992.
[13] Rydon, N. H., Smith, P. W. G.: *Nature* **1952,** *169,* 922–923.

Chlorine–4,4′-Tetramethyldiaminodiphenylmethane Reagent (TDM Reagent)

Reagent for:

- Amino acids, amides, peptides [1]
- Secondary amines [1, 2]
- Phenols
  e.g. phenol, *p*-nitrophenol, chromotropic acid
  N-hydroxysuccinimide, pentachlorophenol [1]
- Triazines
  e.g. atrazine, trietazine, prometryn, simazine [2]
- Chloranils [3]



$C_{17}H_{22}N_2$

$M_r = 254.38$

Preparation of the Reagent

Solution I Dilute 20 ml sodium hypochlorite solution (13–14% active chlorine) with 100 ml water [1].

Solution II Dissolve 2.5 g 4.4′-tetramethyldiaminodiphenylmethane (TDM, MICHLER's base, N,N,N′,N′-tetramethyl-4,4′-diaminodiphenylmethane) in 10 ml glacial acetic acid (yields a greenish solution) and make up to 100 ml with 80% 2-propanol.

Solution III Dissolve 5 g potassium iodide in 100 ml 80% 2-propanol.

Solution IV Dissolve 300 mg ninhydrin in 10 ml glacial acetic acid and dilute to 100 ml with 80% 2-propanol.

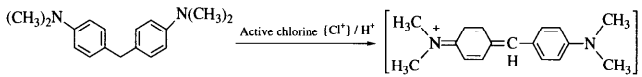
Dipping solntion Mix solutions II and III together and add 1.5 ml solution IV.

Storage Solutions I to IV may be stored in the refrigerator for several weeks. The dipping solution may be kept in the dark for 1 month at room temperature [1].

Substances
Sodium hypochlorite solution
4,4'-Tetramethyldiaminodiphenylmethane
Potassium iodide
Ninhydrin
Acetic acid (100%)
2-Propanol

Reaction

Presumably the active chlorine of the chloramines formed by reaction with chlorine gas or hypochlorite reacts with TDM in the presence of acetic acid to yield dark blue, mesomerically stabilized quinoid reaction products that possibly rearrange to yield triphenylmethane dyestuffs.



Phenols are probably initially oxidized to quinones, which then presumably react further to yield triphenylmethane dyestuffs.

Method

The chromatograms are freed from mobile phase (15 min 100 °C), placed in the empty chamber of a twin-trough chamber containing 20 ml solution I (chlorine chamber) for 1 min or homogeneously sprayed with solution I until the layer begins to be transparent. They are then freed from excess chlorine in a stream of warm air for 30 min and immersed in the dipping solution for 3 s or sprayed homogeneously with it.

Yellow chromatogram zones are first formed at room temperature; the color of these zones generally changes to green and then to blue-black. This color change can be accelerated by heating or irradiating with long-wavelength UV light (λ = 365 nm) [1]; at the same time the background also takes on a greenish color.

Note: The TDM reagent can be used everywhere, where *o*-tolidine is employed. It can also be used on chromatograms, that have already been treated with ninhydrin, PAULY or ammonia perchlorate reagent or with iodine vapor [1]. Water may be used in place of 80% 2-propanol when making up solutions II, III and IV. The chlorine gas atmosphere in the chromatography chamber can also be created by pouring 5 ml hydrochloric acid (ca. 20%) onto 0.5 g potassium permanganate in a beaker; such a chlorine chamber is ready for use after 2 min.

Chloranils, which are formed from polychlorine phenols by heating briefly with conc. nitric acid, can be detected, without chlorine treatment, with TDM reagent, followed by heating (10 min 110 °C) [3]. Phenols yield variously colored chromatogram zones (e.g. phenol: mauve, chromotropic acid: grey, 8-hydroxyquinoline: light brown, 4-*tert*-butyl-pyrocatechol: red [1]).

Before treatment with chlorine gas or hypochlorite solution, pyridine-containing mobile phases must be removed completely from the chromatograms, if necessary, by prolonged drying in a stream of warm air [1]. The colored chromatograms may be stored for several days in the dark under cool conditions [1].

In the case of protected peptides, it is necessary to remove the protecting groups by spraying the chromatograms with conc. hydrochloric acid and then heating (20 min, 110 °C), before applying the reagent [1].

The detection limits for triazines are 20 ng [2] and for amino acids – with a few exceptions – 0.1 to 1 µg substance per chromatogram zone [1]. In the case of peptides 50 ng can be detected visually.

The reagent can be used on silica gel, kieselguhr, Si 50000, aluminium oxide and cellulose layers.

Warning: 4,4'-Tetramethyldiaminodiphenylmethane is thought to be carcinogenic [4]. Therefore, the dipping solution should be used if possible (gloves!). It is only in this way that it is possible to guarantee that the spray vapors do not come into contact with the skin or respiratory tract.

Procedure Tested

Triazines [5]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (RIEDEL DE HAEN)
Mobile phase	Cyclohexane – dichloromethane – dioxan – tetrahydrofuran (80+10+5+5).
Migration distance	7 cm
Running time	20–25 min

Detection and result: The chromatogram was freed from mobile phase and placed in an atmosphere of chlorine gas (twin-trough chamber, containing 20 ml solution I in the second chamber) for 1 min. Then the excess chlorine was removed (30 min stream of warm air), the treated chromatogram immersed in the dipping solution for 3 s and dried on a hotplate (60–70 °C).

Depending on the duration of heating yellow-green to dark green chromatogram zones were produced on a weakly colored background. The detection limits of the triazine herbicides cyanazine (hR_f 25–30), simazine (hR_f 30–35), atrazine (hR_f 40–45), terbutylazine (hR_f 45–50) and anilazine (hR_f 60–65) were 20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at $\lambda = 353$ nm (Fig. 1).

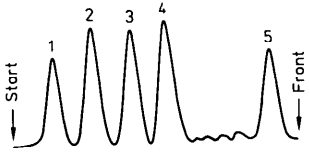


Fig. 1: Reflectance scan of a chromatogram track with 100 ng each of 1 = cyanazine, 2 = simazine, 3 = atrazine, 4 = terbutylazine, 5 = anilazine per chromatogram zone.

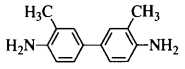
References

[1] Von Arx, E., Faupel, M., Brugger, M.: *J. Chromatogr.* **1976,** *120,* 224–228.
[2] Székely, G., Weick, P., Abt, B.: *J. Planar Chromatogr.* **1989,** *2,* 321–322.
[3] Ting, H.-H., Quick, M. P.: *J. Chromatogr.* **1980,** *195,* 441–444.
[4] Fourth Annual Report on Carcinogens (NTP 85-002, 1985) S. 130.
[5] Ehlert, W., Jork, H.: GDCh-training course Nr. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken 1990.

Chlorine-*o*-Tolidine-Potassium Iodide Reagent (Reindel-Hoppe Reagent)

Reagent for:

- Compounds that can be converted to chloramines [1-4]
  e.g. amino acids, peptides, proteins and derivatives [5-13]
  such as cyclochlorotin, simatoxin [14]
  aspartic acid derivatives [15]
  carbobenzoxyamino acids [16]
  caprolactam oligomers [17]
  phenyl carbamate herbicides
  such as chloropropham [18, 19]
  urea and urea derivatives [20, 21]
  carbamide derivatives [10]
- Pesticides
- Triazines and their hydroxy derivatives [22, 23]
  e.g. atrazine, simazine [18, 19, 24]
  metribuzine [25]
  prometryn [18, 24, 26, 27]
- Vitamins [28-30]
  e.g. vitamins B_1, B_2, B_6, nicotinamide, panthenol
- Pharmaceuticals
  e.g. sulfonylurea derivatives [31, 32]
  meprobamate, barbiturates [33]
  sulfonamides [34]
  central stimulants [35]



$C_{14}H_{16}N_2$

$M_r = 212.30$

o-Tolidine

Preparation of the Reagent

Solution I Dissolve 0.5 g *o*-tolidine (3,3'- dimethylbenzidine) in 5 ml acetic acid (100%) and make up to 250 ml with water or 80% 2-propanol [26, 27].

Solution II Dissolve 2 g potassium iodide in 10 ml water.

Dipping solution Combine solutions I and II and make up to 500 ml with water or 80% 2-propanol [26, 27].

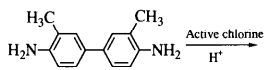
Spray solution Dissolve 160 mg *o*-tolidine in 30 ml glacial acetic acid and make up to 500 ml with water; dissolve 1 g potassium iodide in this solution [4, 14, 16, 17, 28-30].

Storage Dipping and spray solutions may be stored in the refrigerator for 1 week [24, 27].

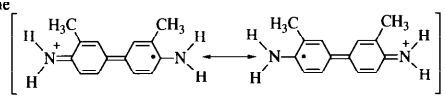
Substances
o-Tolidine
Potassium iodide
Potassium permanganate
Hydrochloric acid (32%)
Acetic acid (100%)
2-Propanol

Reaction

The action of chlorine gas produces, for example, chloramine derivatives from herbicides, amino acids, peptides and proteins; the active chlorine of these derivatives then



o-Tolidine



Diphenoquinondimine radical

oxidizes *o*-tolidine in the presence of acetic acid and potassium iodide to produce a deep blue dyestuff (diphenoquinonediimine radical) [1], that exhibits semiquinonoid character [2]. The active chlorine possibly also reacts with the potassium iodide to liberate iodine which is capable of complex formation with the semiquinonoid reaction product, thus deepening its color.

Method

The chromatogram is freed from mobile phase in a stream of cold air and then exposed to an atmosphere of chlorine gas for 30 s – 1 h. This chlorine gas can be generated in a trough chamber by pouring 5 ml ca. 20% hydrochloric acid onto 0.5 g potassium permanganate placed in a beaker; the chlorine gas chamber is ready for use after ca. 2 minutes.

The chromatogram is then completely freed from excess chlorine in a stream of warm air for 30 min, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and dried for 5 min in a stream of warm air [26].

Usually blue-grey colored chromatogram zones are produced on a colorless [19, 24, 25] to pale grey background [26]; cyclochlorotin and simatoxin yield brilliant yellow zones [14] and urea derivatives yellow, green or blue colors [21].

Note: The dipping solution can also be used as a spray reagent. The quantitative scan should not be delayed for more than 1 h since the background begins to darken after this [26]. The chromatogram should not be completely freed from water before exposure to chlorine gas [2], moistening in water vapor has even been suggested [1, 16]. The chromatogram should first only be sprayed or dipped in the reagent at one corner. If the background becomes blue this is a sign that traces of chlorine gas are still present; in such cases the chromatogram should be exposed to air for longer after treatment with chlorine gas [16, 24, 28–30].

Instead of exposing the chromatogram to chlorine gas it can be dipped in a solution of chlorine in carbon tetrachloride [24] or, in some instances, pretreated with sodium hypochlorite [4] or *tert*-butyl hypochlorite solution [8]. Treatment with chlorine gas is not necessary for chloramines; here the chromatogram can be treated with the spray solution immediately after development and drying [36]. A modified reagent includes sodium tungstate solution in making up the spray solution [20]. In some cases the colored zones fade within a few minutes [24]. An additional treatment with 1% ammonium molybdate in acetic acid (c = 1 mol/L) stabilizes the colored zones [1]. A series of chlorinated pesticides and biphenylenes can be detected with *o*-tolidine followed by exposure to light (see *o*-tolidine – UV light reagent).

The detection limit is 1 ppm for cyclochlorotin and 12–100 ng substance per chromatogram zone for triazines [18, 19, 25–27].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000 and cellulose layers.

Caution: *o*-Tolidine is very toxic and possibly carcinogenic! For this reason the dipping method should be preferred. This is the only way to ensure that the spray mist does not reach the respiratory tract or skin.

Procedure Tested

Triazines [26, 27]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK, RIEDEL-DE-HAEN); before application of the samples the layer is immersed in 2-propanol for 12 hours (preferably overnight) for purification purposes and then activated on a hot plate for 60 min at 110 °C.
Mobile phase	1. n-Pentane – chloroform – acetonitrile (50+40+10) (Fig. 1). 2. Cyclohexane – dichloromethane – dioxan – tetrahydrofuran (80+10+5+5) (Fig. 2).
Migration distance	5–7 cm
Running time	20 –25 min

Detection and result: The chromatogram was freed from mobile phase for 5 min in a stream of cold air and then for 15 min on a hot plate (Thermoplate DESAGA) at 60 °C. It was then exposed for 30 s to an atmosphere of chlorine gas, that had been generated in a twin trough chamber by pouring 5 ml hydrochloric acid (ca. 20%) onto 0.5 g potassium permanganate placed in one of the troughs (waiting time 2–5 min after the acid had been poured onto the potassium permanganate). The chromatogram was then freed from excess chlorine for 30 min in a stream of warm air, immersed in the dipping solution for 3 s and dried for 5 min in a stream of warm air.

The substances aziprotryn (hR_f 80–85), dipropretryn (hR_f 70–75), prometryn (hR_f 65–70), ametryn (hR_f 55–60), desmetryn (hR_f 40–45) and methoprotryn (hR_f 30–35) separated using mobile phase 1 and the components terbutylazine (hR_f 45–50), atrazine (hR_f 35–40), simazine (hR_f 30–35) and cyanazine (hR_f 20–25) chromato-

graphed with mobile phase 2 all yielded blue-grey chromatogram zones on a pale grey background. Anilazine (hR_f 60–65, mob. ph. 1) did not produce a color, but it could be quantified well without reagent treatment at $\lambda = 223$ nm.

Note: Since the plate background begins to darken after 1 h it is necessary to carry out quantitation within this time.

The detection limits for triazines are in the range 10–20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric measurement in reflectance was carried out at a mean wavelength of $\lambda = 650$ nm (Fig. 1) and 495 nm (Fig. 2).

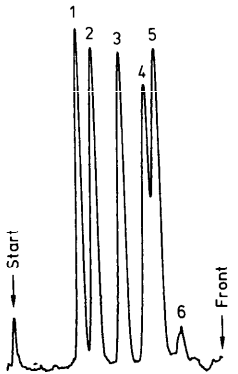


Fig. 1

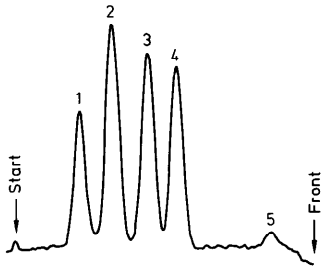


Fig. 2

Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone: 1 = methoprotryn, 2 = desmetryn, 3 = ametryn, 4 = prometryn, 5 = dipropretryn, 6 = aziprotryn

Fig. 2: Reflectance scan of a chromatogram track with 100 ng substance per chromatogram zone: 1 = cyanazine, 2 = simazine, 3 = atrazine, 4 = terbutylazine, 5 = anilazine

References

[1] Barrollier, J.: *Naturwiss.* **1961,** *16,* 554.
[2] Reindel, F., Hoppe, W.: *Chem. Ber.* **1954,** *87,* 1103–1107.

[3] Krebs, K. G., Heusser, D., Wimmer, H. in Stahl, E. (Ed.) *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch,* 2. Ed., Springer, Berlin, Heidelberg, New York 1967, S. 822.
[4] E. Merck, Company brochure "Dyeing reagents for Thin-layer and Paper Chromatography", Darmstadt 1980.
[5] Humphries, J., Wan, Y.-P., Fisher, G., Folkers, K., Bowers, C. Y.: *Biochem. Biophys. Res. Commun* **1974,** *57,* 675–682.
[6] Stverteczky, J., Hollósi, I., Bajusz, S.: *Acta Chim.* **1975,** *87,* 269–283.
[7] Stverteczky, J., Bajusz, S.: *Acta Chim. Acad. Sci. Hung.* **1976,** *88,* 67–74.
[8] Halstrom, J., Kovács, K., Brunfeld, K.: *Acta Chem. Scand.* **1973,** *27,* 3085–3090.
[9] Kajtár, M., Hollósi, Riedl, Z.: *Acta Chim. Acad. Sci. Hung.* **1976,** *88,* 301–308.
[10] Szabó, A.: *Analyst* **1981,** *106,* 602–604.
[11] Arnold, W.-H., White, W., Flouret, G.: *J. Med. Chem.* **1973,** *16,* 1054–1055.
[12] Flouret, G. R., Arnold, W.-H., Cole, J. W., Morgan, R. L., White, W. F., Hedlund, M. T., Rippel, R. H.: *J. Med. Chem.* **1973,** *16,* 369–373.
[13] Flouret, G. R., Morgan, R. L., Gendrich, R., Wilber, J., Seibel, M.: *J. Med. Chem.* **1973,** *16,* 1137–1140.
[14] Ghosh, A. C., Manmade, A., Bousquet, A., Townsend, J. M., Demain, A. L.: *Experientia* **1978,** *34,* 819–820.
[15] Schön, I.: *Acta Chim. Acad. Sci. Hung.* **1982,** *109,* 219–222.
[16] Pataki, G.: *J. Chromatogr.* **1963,** *12,* 541.
[17] Reinisch, G., Gohlke, U.: *Faserforsch. u. Textiltechn.* **1972,** *23,* 415–420.
[18] Engst, R., Noske, R.: *Nahrung* **1970,** *14,* 623–629.
[19] Ambrus, A., Hargitai, É., Károly, G., Fülöp, A., Lantos, J.: *J. Assoc. Off. Anal. Chem.* **1981,** *64,* 743–748.
[20] Kynast, G.: *Z. Anal. Chem.* **1970,** *251,* 161–166.
[21] Ludlam, P. R.: *Analyst* **1973,** *98,* 116–121.
[22] Valk, G., Kamel, M., Abou-Zeid, N. Y.: *Acta Chim. Acad. Sci. Hung.* **1976,** *90,* 69–73.
[23] Sherma, J., Zweig, G.: Analytical Methods for Pestizides and Plant Growth Regulators, Vol. VII, Academic Press, New York, London 1973.
[24] Koudela, S.: *J. Chromatogr.* **1970,** *53,* 589–591.
[25] Wirthgen, B., Raffke, W.: *Nahrung* **1977,** *21,* K27–K29.
[26] Battenfeld, R.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[27] Ehlert, W., Jork, H.: GDCh-training course Nr. 301, Universität des Saarlandes, Saarbrücken 1990.
[28] Thielemann, H.: *Pharmazie* **1977,** *32,* 51.
[29] Thielemann, H.: *Z. Anal. Chem.* **1974,** *271,* 365–366.
[30] Thielemann, H.: *Pharmazie* **1981,** *36,* 574.
[31] Speck, U., Mützel, W., Kolb, K. H., Acksteiner, B., Schulze, P. E.: *Arzneim. Forsch.* **1974,** *24,* 404–409.
[32] Kopitar, Z.: *Arzneim. Forsch.* **1975,** *25,* 1455–1460.
[33] Thielemann, H.: *Fresenius Z. Anal. Chem.* **1989,** *334,* 64.
[34] Pauncz, J. K.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1981,** *4,* 287–291.
[35] Ryrfeldt, A.: *Acta pharmacol. et toxicol.* **1970,** *28,* 391–405.
[36] Thielemann, H.: *Pharmazie* **1977,** *32,* 182–183.

Chlorine-*o*-Toluidine Reagent

Reagent for:

- Amines, amides
  e.g. hexamine, urea derivatives, melamine resins [1, 2]
  peptides [3–6]
- Triazines [7]
- Vitamin B_1, B_2, B_6

C_7H_9N

$M_r = 107.16$

Preparation of the Reagent

Dipping solution	Dissolve 2.5 ml *o*-toluidine in a mixture of 45 ml diethyl ether and 5 ml glacial acetic acid [7].
Spray solution	Dissolve 5 g *o*-toluidine in 100 ml glacial acetic acid [1, 2].
Storage	The reagent solutions may be stored for several weeks [1].
Substances	*o*-Toluidine Acetic acid (100%) Potassium permanganate Hydrochloric acid (25%) Diethyl ether

Reaction

Peptides, for example, yield chloramine derivatives on exposure to chlorine gas; these derivatives oxidize *o*-toluidine to a blue semiquinonoid dyestuff in the presence of acetic acid.

Method

The chromatograms are freed from mobile phase and then treated for 5–10 min with chlorine gas. This can be produced in the vacant trough of a twin-trough chamber by pouring 5 ml hydrochloric acid (ca. 20%) over 0.5 g potassium permanganate. After the excess chlorine has been removed the TLC plates are immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution and laid out in the air.

Yellow, green, gray or blue chromatogram zones are produced on a colorless background [1, 2, 7].

Note: Chloramines do not require exposure to chlorine gas before application of *o*-toluidine. A range of halogen-containing substances (e.g. bromazine, hexachlorocyclohexane isomers) can be detected with *o*-toluidine (1% in ethanol) after subsequent irradiation with UV light ($\lambda = 254$ or 366 nm; 10–15 min) [1, 8].

The detection limits for triazines are 300 ng [7] and for urea formaldehyde reaction products they are 1 to 5 µg substance per chromatogram zone [1].

The reagent can be used on silica gel, kieselguhr and Si 50000 layers. RP, CN, Diol, NH_2 and cellulose layers are not suitable. Amino layers, for example, turn yellow under influence of the reagent [7].

Warning: *o*-Toluidine is highly poisonous and possibly carcinogenic! Therefore, the dipping solution should be employed if possible. This is the only way to ensure that spray vapor is kept away from the respiratory tract or skin.

(ca. 3 min in a stream of warm air) the chromatogram was immersed for 1 s in the dipping solution and left in the air for a few minutes.

Cyanazin (hR_f 5–10) appeared as gray, terbutylazin (hR_f 20–25) as violet and anilazin (hR_f 35–40) as pale blue chromatogram zones (Fig. 1). The intensity of the spots increased during one hour but did not change thereafter. The detection limits for all three substances were 300 ng per chromatogram zone. These amounts could also readily be detected visually.

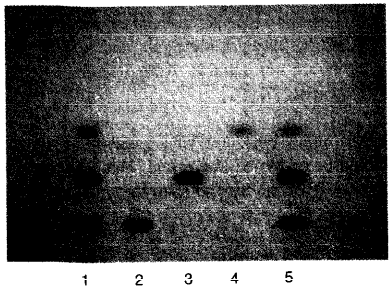


Fig. 1: Thin-layer chromatogram of triazines (amount applied: 4 µg each substance per chromatogram zone):Tracks 1 and 5 = mixture, Track 2 = cyanazin, Track 3 = terbutylazin, Track 4 – anilazin.

Procedure Tested

Triazines [7]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Cyclohexane – dichloromethane – tetrahydrofuran – dioxane (80+10+5+5).
Migration distance	7 cm
Running time	14 min

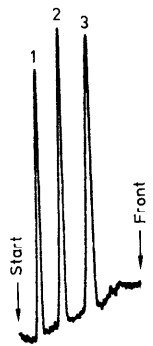
Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed for 5 min in a twin-trough chamber in which a chlorine gas atmosphere had been produced (by pouring ca. 6 ml hydrochloric acid (20%) over 0.4 g potassium permanganate in the vacant trough). After removal of the excess chlorine

Fig. 2: Reflectance scan of a chromatogram track with 2 µg each of cyanazin (1), terbutylazin (2) and anilazin (3) per chromatogram zone.

References

[1] Ludlam, P. R.: *Analyst* **1973,** *98,* 107–115.
[2] Schindlbauer, H., Dabernig, G.: *Fresenius Z. Anal. Chem.* **1984,** *319,* 399–402.
[3] Davídek, J., Velísek, J.: *Proc. Int. Congress of Food Science and Technology, 6th, Dublin* 1983, 219–220.
[4] Gáll-Istók, K., Zára-Kaczián, E., Kisfaludy, L., Deák, G.: *Acta Chim. Acad. Sci. Hung.* **1982,** *110,* 441–446.
[5] Baláspiri, L., Papp, G., Tóth, M., Sirokmán, F., Kovács, K.: *Acta Phys. et Chem.* **1979,** *25,* 179–185.
[6] Mezö, I., Seprödi, J., Erchegyi, J., Horváth, A., Nikolics, K., Teplán, I., Vigh, S., Kovács, M., Flerkó, B., Coy, D. H., Pedroza, E., Nekola, M. V., Schally, A. V.: *Acta Chim. Hung.* **1984,** *116,* 173–187.
[7] Jork, H., Meiers, B.: GDCh-training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1992.
[8] Hauck, H. E., Amadori, E. in J. Harvey, G. Zweig (eds.): *Pesticide Analytical Methodology,* ACS Symposium Series Nr. 136, 1980.

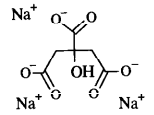
In situ quantitation: Absorption photometric evaluation in reflectance was carried out at wavelength λ= 650 nm (Fig. 2).



Copper(II) Sulfate–Sodium Citrate Reagent (Benedict's Reagent)

Reagent for:

- Reducing sugars [1]
- Flavonoids [2–5]
- Cumarins [4]



$CuSO_4 \cdot 5H_2O$
$M_r = 249.68$
Copper sulfate

$C_6H_5Na_3O_7 \cdot 2H_2O$
$M_r = 294.10$
Sodium citrate

Preparation of the Reagent

Solution I Dissolve 17.3 g copper(II) sulfate pentahydrate in 100 ml water.

Solution II Dissolve 173 g *tri*-sodium citrate dihydrate and 270 g sodium carbonate decahydrate in 600 ml water.

Dipping solution Slowly add solution I to solution II with stirring and make up to 1 L with water [1].

Storage The dipping solution can be stored for several weeks at room temperature.

Substances Copper(II) sulfate pentahydrate
tri-Sodium citrate dihydrate
Sodium carbonate decahydrate

Reaction

Reducing sugars convert copper(II) salts to red copper(I) oxide. Evidently the phenolic OH groups of many flavonoids and cumarins are also capable of reducing copper(II), probably leading to strongly fluorescent quinoid systems. Substances with *ortho*-phenolic OH-groups have their natural fluorescence reduced, those with isolated OH-groups have this enhanced [6].

Method

After development the chromatogram is freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 s or homogeneously sprayed with it, dried for 5 min in a stream of cold air and, in the case of flavonoids and cumarins, is immediately examined under long-wavelength UV light ($\lambda = 365$ nm) [7]. Reducing sugars are detected by heating to 105 °C for 30 min after dipping or spraying [1].

When examined under long-wavelength UV light ($\lambda = 365$ nm) cumarins yield light blue [7] and flavonoids yellow-green [2, 3] fluorescent chromatogram zones on a dark background. Reducing sugars yield brilliant orange-colored zones on a colorless to pale beige background.

Note: The dipping solution can also be used as a spray solution [7]. Chromatograms of natural product extracts should always be examined under UV light before using BENEDICT's reagent, since some natural fluorescences are reduced to a greater or lesser degree by the reagent.

The detection limits for cumarins are 5 ng substance per chromatogram zone [7]. They can be appreciably lowered by dipping the mobile phase-free chromatogram in a solution of liquid paraffin – *n*-hexane (1+9) [8].

The reagent can be used on silica gel, kieselguhr, Si 50000, cellulose and polyamide layers.

Procedure Tested

Cumarins in Plant Extracts [7, 8]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK); before application of the samples the layer was developed with methanol to precleanse it and then dried at 110 °C for 30 min.
Mobile phase	Acetone – water – ammonia solution (25%) (90+7+3).
Migration distance	7.5 cm
Running time	17 min

Detection and result: The chromatogram was dried in a stream of warm air. Blood-red fluorescent chlorophyll zones were visible in the region of the solvent front. In the case of *Orthosiphon* leaf extract there was an intense pale blue fluorescent sinensetin zone (hR_f 90–95) immediately below this, followed by a series of usually weaker blue fluorescent zones extending right down to the start zone (Fig. 1A).

After application of BENEDICT's reagent (dipping time: 3.5 s; 5 min drying in a stream of warm air) the fluorescence intensity of many of the chromatogram zones is appreciably reduced. At the same time the fluorescence of other zones is increased (Fig. 2), so that in stinging nettle extract, for instance, the scopoletin zone (hR_f 48–53) fluoresced most strongly (Figure 1B). Dipping for 3 s in liquid paraffin – *n*-hexane (1+9) followed by drying in a stream of cold air caused the fluorescence intensity to increase by a factor of 2.

Note: Allowing the sprayed chromatograms to stand for a longer time and, in particular, exposing them to heat, reduces the intensity of the fluorescence of the chromatogram zones.

In situ quantitation: The fading of the fluorescence on exposure to heat and on allowing the chromatograms to stand makes this reagent unsuitable for in situ quantitation. Dipping the chromatograms in paraffin solution does not improve this (Fig. 2).

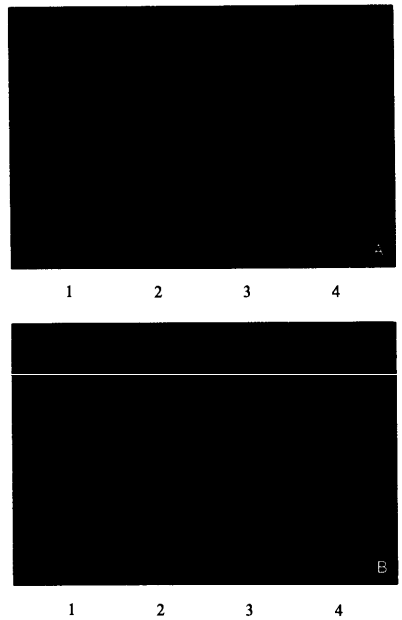


Fig. 1: Chromatograms of two natural product extracts and associated reference substances A. before and B. after application of BENEDICT's reagent.
Track 1: sinensetin (hR_f 90–95), scopoletin (hR_f 50–55); track 2: Extr. Urticae (extract of stinging nettle leaves); track 3: *Orthosiphon* extract; track 4: mixture of stinging nettle and *Orthosiphon* extracts.

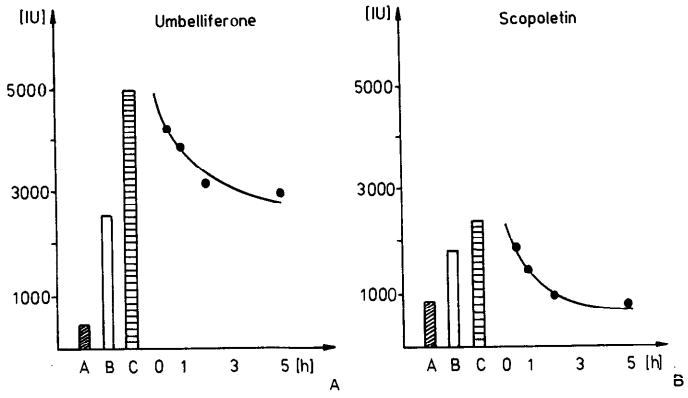


Fig. 2: Increase in fluorescence of the genuine fluorescence (A) by treatment with BENEDICT's reagent (B) and immersion in a paraffin solution (C) and reduction of emission intensities with time for the two cumarins umbelliferone and scopoletin (curves).

References

[1] Ohlenschläger, I., Berger, I., Depner, W.: *Synopsis der Elektrophorese-Techniken*, GIT-Verlag, Darmstadt 1980, S. 183.

[2] Mues, R., Zinsmeister, H. D.: *Phytochemistry* **1975**, *14*, 577; **1976**, *15*, 1757–1760.

[3] Theodor, R., Zinsmeister, H. D., Mues, R., Markham, K.: *Phytochemistry* **1980**, *19*, 1695–1700.

[4] Krause, J., Reznik, H.: *Z. Pflanzenphysiol.* **1972**, *68*, 115–120.

[5] Oettmeier, W., Heupel, A.: *Z. Naturforsch.* **1972**, *27b*, 177–183.

[6] Reznik, H., Egger, K.: *Z. Anal. Chem.* **1961**, *183*, 196–199.

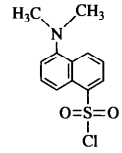
[7] Hahn-Deinstrop, E.: Private communication, Fa. Heumann Pharma GmbH & Co., Abt. Entwicklungsanalytik, D-90478 Nürnberg 1.

[8] Ganz, J., Jork, H.: Private communication, Universität des Saarlandes, Fachbereich 12, Saarbrücken 1990.

Dansyl Chloride Reagent

Reagent for:

- Primary and secondary amines [1–4]
  e.g. cactus alkaloids [1–5]
  such as hordenine, tyramine, synephrine
- Amino acids
- Phenols



$C_{12}H_{12}ClNO_2S$

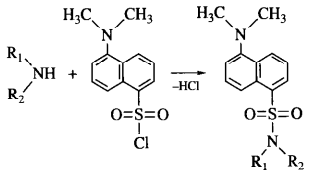
$M_r = 269.75$

Preparation of the Reagent

Dipping solution I	Dissolve 2 to 5 g sodium carbonate in 50 ml water and make up to 100 ml with methanol [6].
Dipping solution II	Dissolve 100 mg dansyl chloride (5-(dimethylamino)-naphthalene-1-sulfonyl chloride) in 100 ml ethanol [6].
Spray solution	Dissolve 50 mg dansyl chloride in 100 ml acetone [3–5].
Storage	Dipping solution I can be stored for a longer period. Dipping solution II and the spray solution should be made up fresh daily and protected from light.
Substances	5-(Dimethylamino)-naphthalene-1-sulfonyl chloride Sodium carbonate, anhydrous Methanol Ethanol (96%)

Reaction

Dansyl chloride that exhibits a blue intrinsic fluorescence, reacts with many amines and phenols to yield derivatives with fluorescence of another color.



Method

The chromatogram is freed from mobile phase, immersed in dipping solution I for 1 s or sprayed homogeneously with it, dried in a stream of warm air and immersed immediately after cooling for 1 s in dipping solution II or sprayed homogeneously with the spray solution and then heated to 110 °C for 2 min.

Under long-wavelength UV light ($\lambda = 365$ nm) yellow-orange fluorescent chromatogram zones are observed on a pale light-blue fluorescent background.

Note: Tertiary amines do not react with dansyl chloride and can be detected by spraying afterwards with WAGNER's reagent [1]. The detection limits for amines are in the lower nanogram range.

The reagent can be used on silica gel, kieselguhr, Si 50000, aluminium oxide and RP layers; amino phases are unsuitable.

Procedure Tested

Biogenic Amines [6]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer HPTLC plates Silica gel 60 (MERCK).

Mobile phase Ethyl acetate – 1-propanol – ammonia solution (25%) (12+9+3).

Migration distance 6 cm

Running time 20 min

Detection and result: The chromatogram was freed from mobile phase (the ammonia must be removed completely) and immersed in dipping solution I for 1 s, dried in a stream of warm air for 2 min and immersed immediately after cooling in dipping solution II for 1 s and then heated to 110 °C for 2 min.

Phenylethylamine (hR_f 60–65), tyramine (hR_f 45–50), serotonin (hR_f 35–40) and histamine (hR_f 20–25) yielded yellow-orange fluorescent zones on a pale light-blue fluorescent background under long-wavelength UV light ($\lambda = 365$ nm).

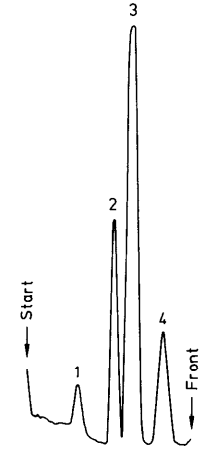


Fig. 1: Fluorescence scan of a chromatogram track with a mixture of biogenic amines with 1 ng substance per chromatogram zone: 1 = histamine, 2 = serotonin, 3 = tyramine, 4 = phenylethylamine.

In situ quantitation: The fluorimetric quantitation was carried out in long-wavelength UV light at $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 560$ nm (Fig. 1).

It is not recommended that the chromatogram then be treated with liquid paraffin – n-hexane (1+4) since the intensity of the pale light blue fluorescent background is also increased, so that the difference in emission of the chromatogram zones is reduced.

References

[1] Kelley Hornemann, K. M., Neal, J. M., McLaughlin, J. L.: *J. Pharmac. Sci.* **1972,** *61,* 41–45.
[2] Dingerdissen, J. J., McLaughlin, J. L.: *J. Pharmac. Sci.* **1973,** *62,* 1663–1665.
[3] Sato, P. T., Neal, J. M., McLaughlin, J. L.: *J. Pharmac. Sci.* **1973,** *62,* 411–414.
[4] Keller, W. J., McLaughlin, J. L., Brady, L. R.: *J. Pharmac. Sci.* **1973,** *62,* 408–410.
[5] McLaughlin, J. L., Paul, A. G.: *Lloydia* **1966,** *29,* 315–327.
[6] Jungblut, E., Kany, E., Jork, H.: GDCh-training course Nr. 302 „Möglichkeiten der quantitativen Auswertung von Dünnschicht-Chromatogrammen", Universität des Saarlandes, Saarbrücken 1988.

Dimedone–Phosphoric Acid Reagent

Reagent for:

- Ketosugars [1–4]
  e.g. fructose, sucrose, raffinose, lactose
- Aryl- and heteroarylpropionic acids
  e.g. flurbiprofen, ketoprofen [5]

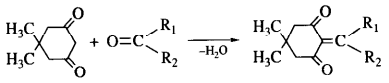
$C_8H_{12}O_2$	H_3PO_4
$M_r = 140.18$	$M_r = 98.00$
Dimedone	Phosphoric acid

Preparation of the Reagent

Dipping solution	Dissolve 0.3 g dimedone (5,5-dimethylcyclohexane-1.3-dione) in 90 ml ethanol and mix with 10 ml *ortho*-phosphoric acid (85%) [1, 5].
Storage	The dipping solution may be stored for an extended period.
Substances	Dimedone Ethanol *ortho*-Phosphoric acid (85%)

Reaction

Dimedone reacts with carbonyl compounds with the elimination of water yielding the condensation product [1]. The reaction is specific for ketoses; aldoses do not react or only weakly [6].



Dimedone | Carbonyl compound | Condensation product

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 4 s or homogeneously sprayed with it until the layer begins to be transparent and then heated to 110 °C for 15 to 20 min, after briefly drying in a stream of cold air.

Yellow chromatogram zones are formed on a colorless background; these zones mostly fluoresce blue when excited with long-wavelength UV light ($\lambda = 365$ nm).

Note: In the case of aryl- and heteroarylpropionic acids the chromatograms are irradiated with unfiltered UV light for 30 min before application of the reagent [5]. The chromatograms can then be immersed in a solution of liquid paraffin – *n*-hexane (1+2) in order to stabilize and enhance the fluorescence [5].

The detection limits for aryl- and heteroarylpropionic acids are in the lower nanogram range [5, 7]. In the case of ketosugars 10–40 ng substance can be detected per chromatogram zone [1].

The reagent can, for instance, be used on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Flurbiprofen and Ketoprofen [5]

Method	Ascending, one-dimensional development in a trough chamber without filter paper lining. The development was commenced 30 min after charging the chamber with 5 ml mobile phase.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	*n*-Hexane – diethyl ether – 1-butanol – ethyl acetate (65+15+11+9).
Migration distance	6 cm
Running time	20 min

Detection and result: The chromatogram was freed from mobile phase for 10 min in a stream of cold air, irradiated for 30 min with unfiltered UV light, then immersed in the dipping solution for 4 s and finally heated to 110 °C for 15 min. The chromatogram

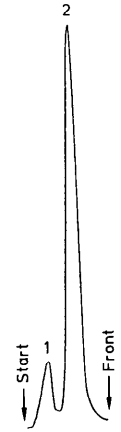


Fig. 1: Fluorescence scan of a chromatogram track with 500 ng each substance per chromatogram zone: 1 = ketoprofen, 2 = flurbiprofen.

was then immersed in a solution of liquid paraffin – *n*-hexane (1+2) for 2 s in order to stabilize and enhance the fluorescence by a factor of about 2.

On excitation with long-wavelength UV light ($\lambda = 365$ nm) ketoprofen (hr_f 35–40) and flurbiprofen (hR_f 50–55) appeared as yellow or blue fluorescent chromatogram zones on a pale blue fluorescent background. The detection limits of, for instance, flurbiprofen were 10 ng subtance per chromatogram zone.

In situ quantitation: The fluorimetric evaluation was carried out under long-wavelength UV light at $\lambda_{exc} = 313$ nm and the fluorescence emission was measured at $\lambda_{fl} > 390$ nm (cut off filter FL 39).

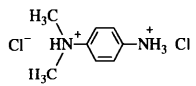
References

[1] Patzsch, K., Netz, S., Funk, W.: *J. Planar Chromatogr.* **1988**, *1*, 39–45.
[2] Washüttl, J., Riederer, P., Bancher, E., Wurst, F., Steiner, K.: *Z. Lebensm. Unters. Forsch.* **1974**, *155*, 77–80.
[3] Kröplin, U.: *J. Agric. Food Chem.* **1974**, *22*, 110–116.
[4] E. Merck, Company brochure *Dyeing Reagents for Thin-layer and Paper Chromatography*, Darmstadt 1980.
[5] Honermann, U.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1991.
[6] Adachi, S.: *Anal. Biochem.* **1964**, *9*, 224–227.
[7] Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin Layer Chromatography – Reagents and Detection Methods*, Vol. 1a, p. 92–93, VCH-Verlagsgesellschaft, Weinheim, 1990.

N,N-Dimethyl-1,4-phenylenediamine Reagent (Wurster's Red Reagent)

Reagent for:

- Peroxides
  - e.g. alkyl hydroperoxides and their esters,
    dialkyl and diacyl peroxides, ketone peroxides [2]
    cumol hydroperoxide [2, 3]
    nonanoyl peroxide, *tert*-butyl perbenzoate [3]
    sterol hydroperoxides [4]
    linoleic acid hydroperoxides [5]
    pregnen-17α-hydroperoxides [6]
- Halogen-containing substances
  - e.g. chlorine-containing insecticides [1, 7]
    such as aldrin, dieldrin, DDT, perthane,
    hexachlorocyclohexane, methoxychlor [7]
  - e.g. bromine-containing hypnotics [1]
  - e.g. antimicrobials
    such as triclosan (Irgasan) [8]
- Steroids [4]
  - e.g. Δ^4- and Δ^5-3-ketosteroids, Δ^4-ketosteroid-α-ketols
    cholest-5-en-3β,7α(or 7β)-diol
- Triazines [9, 10]



$C_8H_{14}Cl_2N_2$

$M_r = 209.12$

Preparation of the Reagent

Solution I Dissolve 1 g N,N-dimethyl-1,4-phenylenediammonium dichloride (N,N-DPDD) in 100 ml ethanol at 40 °C.

Solution II Dilute 10 ml ethanolic sodium ethylate solution (20%) to 100 ml with ethanol.

Dipping solution Mix equal volumes of solutions I and II; filter off the precipitate that forms.

Spray solution *For peroxides:* Dissolve 1 g N,N-DPDD in a mixture of 50 ml methanol, 50 ml water and 1 ml glacial acetic acid [4, 6].

Reagents with other compositions are also in use, e.g. 1.5 g N,N-DPDD in methanol – water – glacial acetic acid (128 + 25 + 1) [1, 2] or 0.1% N,N-DPDD in chloroform – glacial acetic acid – water (50+50+10) [5].

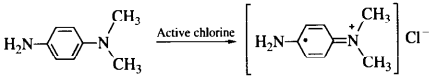
For halogen-containing compounds: Dissolve 0.5 g N,N-DPDD in a mixture of 50 ml solution II and 50 ml ethanol [1, 8].

Storage Solution I and the dipping solution should always be freshly made up. The spray solution and solution II can be stored for a longer period in the refrigerator.

Substances N,N-Dimethyl-1,4-phenylenediammonium dichloride
Sodium ethylate (20% in ethanol)
Ethanol

Reaction

Peroxides oxidize N,N-DPDD to WURSTER's red, a semiquinone diimine derivative [4]. Similarly WURSTER's red is also produced from N,N-DPDD by reaction with halogen-containing substances in the presence of sodium ethylate and UV light and by reaction with the chlorinated triazines produced by reaction with chlorine [7].



WURSTER's red

Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and then dried in a stream of cold air. Triazines must be converted to chlorinated derivatives by exposing the chromatogram to chlorine gas (see "Procedure Tested") before application of the reagent.

In the case of halogen-containing substances (e.g. insecticides) the chromatograms are moistened by spraying with water after treatment with the reagent and then exposed to unfiltered UV light for ca. 1 min [1, 7] or to sunlight for 30 min [8].

Peroxides yield reddish-pink to purple-red chromatogram zones on a pale pink-colored background [2, 4, 5] and halogen-containing substances dirty violet, ultramarine-grey to greenish zones [7, 8]. Triazines yield intense grey to brown zones on a light brown background, observed from the back of the plate they are intense purple-red. A series of steroids also react; e.g. Δ^4- and Δ^5-3-ketosteroids produce a yellow to brown color and Δ^4-3-ketosteroid-α-ketols (e.g. cortisone) orange to pink-orange zones while the two cholest-5-en-3ß,7α(and 7ß)-diols only react slowly to yield a blue color [4].

Note: The contrast between the colored zones and the layer background can be improved by warming the chromatogram gently [4]. Di-*tert*-butyl peroxide does not react [3]. N,N,N′,N′-tetramethyl-p-phenylenediamine (*q.v.*) can also be used instead of N,N-DPDD for the detection of peroxides [3]. The spray solution for peroxides gradually turns dark red in color but it still retains its ability to react for several weeks [4].

The detection limits for peroxides are about 500 ng or with N,N,N′,N′-tetramethyl-p-phenylenediamine reagent 50 ng substance per chromatogram zones [4]. The detection limits for insecticides are 5 µg per chromatogram zone in the most unfavorable cases [7].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Triazines [10, 11]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254s} (MERCK, RIEDEL-DE-HAEN); before application of the samples the layer is immersed in 2-propanol for 12 hours (preferably overnight) and then activated on a hot plate for 15 min at 110 °C.
Mobile phase	1. Pentane – chloroform – acetonitrile (50+40+10) (Fig. 1). 2. Cyclohexane – dichloromethane – dioxan – tetrahydrofuran (80+10+5+5) (Fig. 2).
Migration distance	7 cm
Running time	25 min

Detection and result: The chromatogram was first dried in a stream of cold air for 5 min and then for 15 min on a hot plate at 60 °C. It was then exposed for 1 min to an atmosphere of chlorine gas, that had been generated in a trough chamber by pouring 5 ml hydrochloric acid (ca. 20%) onto 0.5 g potassium permanganate placed in a small beaker (waiting time ca. 2 min before insertion of the plate). The chromatogram was then freed from excess chlorine for exactly 5 min in a stream of cold air (prolonged ventilation makes the result worse), immersed in the dipping solution for 3 s and dried for 5 min in a stream of cold air.

The substances methoprotryn (hR_f 30–35), desmetryn (hR_f 40–45), ametryn (hR_f 55–60), prometryn (hR_f 65–70) and dipropetryn (hR_f 70–75) separated using mobile phase 1 and the components cyanazine (hR_f 20–25), simazine (hR_f 30–35), atrazine (hR_f 35–40), terbutylazine (hR_f 45–50) and anilazine (hR_f 60–65) chromatographed with mobile phase 2 all yielded intense grey to brown-colored zones on a light brown background, that appear intense purple-red when viewed from the back of the plate (WURSTER's red).

In situ quantitation: The photometric evaluation was carried out in reflectance at a wavelength of $\lambda = 460$ nm (Fig. 1) and 545 nm (Fig. 2). The detection limits lay at 15 ng substance per chromatogram zone.

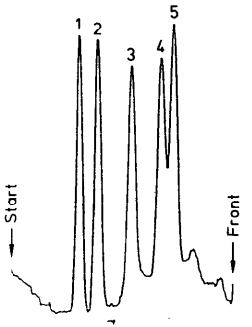


Fig. 1

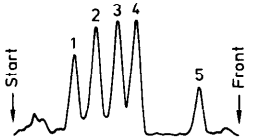


Fig. 2

Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone: 1 = methoprotryn, 2 = desmetryn, 3 = ametryn, 4 = prometryn, 5 = dipropretryn.

Fig. 2: Reflectance scan of a chromatogram track with 100 ng substance per chromatogram zone: 1 = cyanazine, 2 = simazine, 3 = atrazine, 4 = terbutylazine, 5 = anilazine.

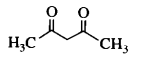
References

[1] E. MERCK, Company brochure "Dyeing reagents for Thin-layer and Paper chromatography", Darmstadt 1980.
[2] Knappe, E., Peteri, D.: *Z. Anal. Chem.* **1962**, *190*, 386–389.
[3] Cornish, L. A., Ferrie, R., Paterson, J. E.: *J. Chromatogr. Sci.* **1981**, *19*, 85–87.
[4] Smith, L. L., Hill, F. L.: *J. Chromatogr.* **1972**, *66*, 101–109.
[5] Heimann, W., Schreier, P.: *Helv. Chim. Acta* **1971**, *54*, 2794–2803.
[6] Hrcycay, E. G., O'Brien, P. J., van Lier, J. E., Kan, G.: *Arch. Biochem. Biophys.* **1972**, *153*, 495–501.
[7] Bäumler, J., Rippstein, S.: *Helv. Chim. Acta* **1961**, *44*, 1162–1164.
[8] Matissek, R.: *Dtsch. Lebensm. Rundsch.* **1981**, *77*, 282–285.
[9] Mobini, K.: Dissertation (in preparation), Universität Gießen, Institut für Rechtsmedizin.
[10] Battenfeld, R.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[11] Ehlert, W., Jork, H.: GDCh-training course Nr. 301, Universität des Saarlandes, Saarbrücken 1990.

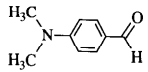
4-(Dimethylamino)-benzaldehyde–Acetylacetone–Reagent (Morgan-Elson Reagent)

Reagent for:

- Amino sugars
  e. g. glucosamine, galactosamine



$C_5H_8O_2$
$M_r = 100.12$
Acetylacetone



$C_9H_{11}NO$
$M_r = 149.19$
4-(Dimethylamino)-benzaldehyde

Preparation of the Reagent

Solution I Mix 5 ml potassium hydroxide solution (50%) wth 20 ml ethanol [1].

Solution II Mix 0.5 ml acetylacetone and 50 ml 1-butanol [1].

Reagent solution Mix 0.5 ml solution I with 10 ml solution II immediately before use [1, 2].

Dipping solution Dissolve 1 g 4-(dimethylamino)-benzaldehyde in 30 ml ethanol, add 30 ml hydrochloric acid (37%) and dilute with 180 ml 1-butanol [2].

Spray solution Dissolve 1 g 4-(dimethylamino)-benzaldehyde in 30 ml ethanol and add 30 ml hydrochloric acid (37%) [1].

Storage The reagent solution should always be made up fresh [1], since it can be stored for only a few hours.

Substances
4-(Dimethylamino)-benzaldehyde
Acetylacetone
Potassium hydroxide
Ethanol
1-Butanol
Hydrochloric acid, fuming (37%)

Reaction

The mechanism of the reaction has not been elucidated.

Method

The dried chromatograms are dipped in the reagent solution for 3 s or sprayed homogeneously with it and then heated to 105 °C for 5 min. After cooling to room temperature the chromatograms are then immersed in the dipping solution or homogeneously sprayed with the spray solution. They are finally dried at 90 °C for 5 min [1, 2].

Red to brown chromatogram zones are produced on a colorless to yellow background [1].

Note: The reagent is not very sensitive. Detection is also evidently affected by external influences (pH, temperature, heating time etc.), which have an effect on the detection sensitivity and on the colors of the chromatogram zones [3].

If the order of application of the reagents is reversed and all other conditions kept the same, lemon-yellow chromatogram zones are produced on a pale yellow background.

The N-acetyl derivatives of 2-aminohexoses give a reaction even in the absence of acetylacetone [4].

The detection limits for amino sugars are ca. 0.5 µg substance per chromatogram zone.

The reagent can be employed on silica gel, kieselguhr, Si 50000 and cellulose layers as well as on RP, CN, Diol and NH_2 layers.

Procedure Tested

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	2-Propanol – ethyl acetate – ammonia solution (32%) (10+10+10) [5].
Migration distance	8 cm
Running time	90 min

Detection and result: The chromatogram was dried in a stream of warm air and immersed for 3 s in the reagent solution and then heated to 105 °C for 5 min. After cooling to room temperature it was immersed in the dipping solution for 3 s and then dried at 90 °C for 5 min.

Galactosamine (hR_f 30–35) and glucosamine (hR_f 35–40) produce brownish-red chromatogram zones on a yellow background.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 400$ nm (Fig. 1).

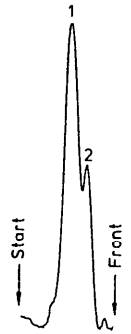


Fig 1: Reflectance scan of a chromatogram track with 5 µg galactosamine (1) and 1 µg glucosamine per chromatogram zone.

References

[1] Krebs, K. G., Heusser, D., Wimmer, H. in E. Stahl (Ed.): *Thin-layer Chromatography – A Laboratory Handbook*, Springer, Berlin, Heidelberg, New York, 1969.
[2] Klein, I., Jork, H.: GDCh-training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1992.
[3] Belcher, R., Nutten, A. J., Sambrook, C. M.: *Analyst* **1954**, *79*, 201–208.
[4] Partridge, S. M.: *Biochem. J.* **1948**, *42*, 238–248.
[5] Kunz, F.-R.: Thesis, Universität des Saarlandes, Saarbrücken, 1988.

4-(Dimethylamino)-benzaldehyde–Acid Reagents

A whole series of derivatization reagents contain 4-(dimethylamino)-benzaldehyde as a fundamental component. They differ in the type and concentration of the mineral acid components used in their preparation. Other components of the reagent generally play a minor role.

The two most commonly used dimethylaminobenzaldehyde reagents bear the names of their "inventors" who first described the acid component used. They are known as

EHRLICH's reagent or VAN URK's reagent

depending on whether hydrochloric acid or sulfuric acid is used in the reagent. Many publications do not follow this naming system – this is particularly evident where the authors give the composition of the dimethylaminobenzaldehyde reagent employed – so that the reaction names for EHRLICH's and VAN URK's reagent have not always been cited correctly in the past.

The fact that many publications just refer to EHRLICH's or VAN URK's reagent without any other reference to the actual composition, necessarily means that the methods in such publications present a probable source of lack of reproducibility when an attempt is made to reproduce the results.

A publication by EHMANN reveals how confused the situation is; here the introduction correctly describes

EHRLICH's reagent as 4-(dimethylamino)-benzaldehyde – hydrochloric acid and

VAN URK's reagent as 4-(dimethylamino)-benzaldehyde – sulfuric acid,

but the experimental section incorrectly refers to a solution of 4-(dimethylamino)-benzaldehyde in hydrochloric acid/ethanol as VAN URK's reagent [1].

It can be concluded from the publications of ROHDE [2] and FREUND and LEBACH [3, 4] that it was EHRLICH who first suggested the use of 4-(dimethylamino)-benzaldehyde in the presence of hydrochloric acid for color reactions with "methylketols" that was found also to apply to indole derivatives [5]. AUTERHOFF [6] designated a reagent for urobilinogen, consisting of a solution of 2 g 4-(dimethylamino)-benzaldehyde in 20 percent hydrochloric acid, correctly as EHRLICH's solution. Hence, it is essentially correct to refer to all reagents which contain these components as EHRLICH's reagent.

The designation VAN URK's reagent can be traced back to a publication in 1929 [7] which describes the detection of ergot alkaloids with 4-(dimethylamino)-benzadehyde in aqueous solution by cautiously underlayering with concentrated sulfuric acid.

AUTERHOFF described a reagent made up of 4-(dimethylamino)-benzaldehyde, sulfuric acid with additional iron(III) ions as "VAN URK's reagent solution". A more correct name would have been VAN URK-SALKOWSKI reagent, since the SALKOWSKI reagent (iron(III) chloride/sulfuric acid) has been used alongside the VAN URK's reagent [8].

Similar errors have been perpetuated in the literature until the present day (cf. [1, 9–11]).

Although more than 150 publications have been reviewed the "dimethylaminobenzaldehyde monographs" that follow only take account of and cite those where there is an unequivocal citation of precise formulations – with respect to the acid used in the reagent – so that they can be assigned to the appropriate named reaction. This procedure was justified in view of the results obtained for the examples tested for the reagent monographs, for there are differences between the two reagents. EHRLICH's reagent is usually more sensitive and yields better method standard deviations on in situ evaluation [12]. However, the plate background is yellow in color while it remains white after the use of VAN URK's reagent [13], or acquires a slight gray color [12]. Furthermore, the layer background does not discolor with standing after the use of the VAN URK reagent as is the case with EHRLICH's reagent [14]. Nevertheless the VAN URK reagent has been used less frequently for TLC in the past but was primarily employed in solution photometry [13], e.g. for the characterization of substances on the basis of aldol condensations. This also applies to determinations made after elution of scraped-off chromatogram zones [11, 15–18].

In rare cases – such as the detection of nafazatrom [19] – 4-(dimethylamino)-benzaldehyde is used without the addition of any acid.

References

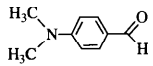
[1] Ehmann, A.: *J. Chromatogr.* **1977**, *132*, 267–276.
[2] Rohde, E.: *Hoppe-Seyler's Z. Physiol. Chem.* **1905**, *44*, 161–170.
[3] Freund, M., Lebach, G.: *Ber. Dtsch. Chem. Ges.* **1903**, *36*, 308.
[4] Freund, M., Lebach, G.: *Ber. Dtsch. Chem. Ges.* **1905**, *38*, 2640–2652.
[5] Ehrlich, P.: *Medizinische Woche*, **1901**, 151 ff.
[6] Auterhoff, H.: *Lehrbuch der Pharmazeutischen Chemie*, Wissenschaftliche Verlagsgesellschaft, Stuttgart 1976.
[7] Van Urk, H. W.: *Pharm. Weekblad* **1929**, *66*, 473–481.
[8] Pilet, P.-E.: *Rev. Gen. Bot.* **1957**, *64*, 106–122.
[9] Stahl, E.: *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch.* 2. Ed., Springer, Berlin 1967.
[10] Klavehn, M., Rochelmeyer, H.: *Dtsch. Apoth. Ztg.* **1961**, *101*, 477–481.
[11] Röder, K., Mutschler, E., Rochelmeyer, H.: *Pharm. Acta Helv.* **1967**, *42*, 407–414.

[12] Schmidt, S.: Thesis, Fachhochschule Gießen-Friedberg, Fachbereich Technisches Gesundheitswesen, 1990.
[13] Dorosiev, I., Simova, M., Kolarova, R., Pangarova, T.: *Pharmazie* **1983**, *38*, 419.
[14] Heacock, R. A., Mahon, M. E.: *J. Chromatogr.* **1965**, *17*, 338–348.
[15] Döhbelin, W., Hartmann, V.: *Dtsch. Apoth. Ztg.* **1980**, *120*, 1821–1823.
[16] Zinser, M., Baumgärtel, C.: *Arch. Pharm.* **1964**, *297*, 158–164.
[17] Maier, W., Erge, D., Gröger, D.: *Planta Med.* **1980**, *40*, 104–108.
[18] Keipert, S., Voigt, R.: *J. Chromatogr.* **1972**, *64*, 327–340.
[19] Ritter, W.: *Proc. Int. Symp. Instrum. High Perform. Thin-Layer Chromatogr. (HPTLC)*, 2nd, p. 100–113, Interlaken 1982.

4-(Dimethylamino)-benzaldehyde–Acetic Acid–Phosphoric Acid Reagent (EP Reagent)

Reagent for:

- Terpene and sesquiterpene derivatives
  e.g. azulenes, proazulenes [1–9]
  such as chamazulene, or matricin
  e.g. bisabolol [2, 3]
- Sesquiterpene esters
  e.g. cinnamoylechinadiol, -echinaxanthol, dihydroxynardol



$C_9H_{11}NO$	CH_3COOH	H_3PO_4
$M_r = 149.19$	$M_r = 60.05$	$M_r = 98.00$
4-(Dimethylamino)-benzaldehyde	Acetic acid	Phosphoric acid

Preparation of the Reagents

Dipping solution Dissolve 0.25 g 4-(dimethylamino)-benzaldehyde in 50 ml glacial acetic acid and add 3 ml *ortho*-phosphoric acid (85%) [10].

Spray solution Dissolve 0.25 g 4-(dimethylamino)-benzaldehyde in a mixture of 50 g glacial acetic acid, 5 g *ortho*-phosphoric acid (85%) and 20–45 ml water [1–3, 5, 8].

Storage The reagent solutions can be stored for months in well-sealed, brown glass bottles [1, 2].

Substances 4-(Dimethylamino)-benzaldehyde
Acetic acid
ortho-Phosphoric acid

Reaction

The mechanism of the reaction has not yet been elucidated.

Method

The chromatograms are dried in a stream of warm air and then immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution and then heated to 80–110 °C for 10–20 min [1, 2, 10].

Generally blue to violet chromatogram zones are formed on a colorless background. Azulene appears pale green and proazulenes produce gray, violet, brown, orange or green chromatogram zones [2].

Note: The EP reagent can be used for the specific detection of matricin in chamomile extract [1].

The detection limits per chromatogram zone are 250 ng for bisabolol and 40 ng for bisabolol dioxide [10].

The reagent can be employed, for example, on silica gel, kieselguhr, Si 50000, CN, diol, RP and cellulose layers.; NH_2 and polyamide phases are not suitable since the whole background acquires a yellow color, and the substances do not react [10].

Procedure Tested

Chamomile oil [10]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer TLC plates Silica gel 60 (MERCK).

Mobile phase Toluene – ethyl acetate (7+3).

Migration distance 6 cm

Running time 7 min

Detection and result: The chromatogram was dried in a stream of warm air, immersed in the dipping solution for 2 s and then heated to 110 °C for 20 min.

Bisabolol oxide (hR_f 40–45) appeared as pink and bisabolol (hR_f 65–70) as mauve-colored chromatogram zones on a pale yellow background. The detection limits per chromatogram zone were 40 ng for bisabolol oxide and 250 ng for bisabolol.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 515$ nm (Fig. 1).

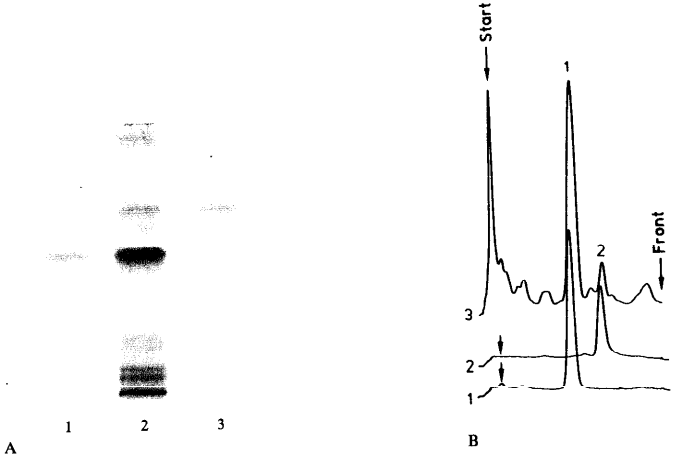


Fig 1: Chromatogram of a chamomile flower extract and of chamomile oil components (A) and reflectance scans (B) of reference tracks with 3.75 µg bisabolol oxide (1) and 9.5 µg bisabolol (2) and a chromatogram track with chamomile flower extract (3).

References

[1] Krebs, K. G., Heusser, D., Wimmer, H. in E. Stahl (Ed.): *Thin-Layer Chromatography – A Laboratory Handbook*, Springer, Berlin, Heidelberg, New York, 1969.
[2] König, H., Walldorf, E.: *Fresenius Z. Anal. Chem.* **1979**, *299*, 1–18.
[3] Messerschmidt, W.: *Dtsch. Apoth. Ztg.* **1973**, *113*, 745–748.
[4] Belliardo, F., Appendino, G.: *J. Liq. Chromatogr.* **1981**, *4*, 1601–1607.
[5] Bauer, R., Khan, I., Wagner, H.: *Dtsch. Apoth. Ztg.* **1986**, *126*, 1065–1070.
[6] Schilcher, H.: *Planta Med.* **1973**, *23*, 132–144.
[7] Stahl, E., Schütz, E.: *Arch. Pharm.* **1978**, *211*, 992–1001.
[8] Stahl, E.: *Dtsch. Apoth. Ztg.* **1953**, *93*, 197–200.
[9] Stahl, E., Schilz, W.: *Chem.-Ing.-Techn.* **1976**, *48*, 773–778.
[10] Meiers, Bl., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken 1992.
[11] Wagner, H., Bladt, S., Zgainski, E. M.: *Drogenanalyse*, Springer Verlag, Berlin, Heidelberg, New York 1983.

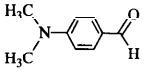
4-(Dimethylamino)-benzaldehyde–Hydrochloric Acid Reagent (Ehrlich's Reagent)

Reagent for:

- Indole derivatives [1, 3]
  - e.g. egot alkaloids [2, 4–12]
    such as ergotamine, ergocristine, ergometrine, ergocornine dihydroergosine,, lysergamide, isolysergamide
  - e.g. ergolinecarboxylic acids [13–16]
  - e.g. *clavine* alkaloids [4, 6, 17, 18]
    such as agroclavine, chanoclavine, penniclavine
  - e.g. harpagophytum alkaloids [19]
    such as harpagoside
  - e.g. hallucinatory drugs
    such as LSD [7, 9, 12], psilocybin, psilocin [12, 20]
  - e.g. auxins [21–23]
    such as 5-hydroxyindole-3-acetic acid, indole-3-acetic acid and their esters
  - e.g. tryptophan derivatives [2, 22–29]
    such as tryptophan, tryptamine, serotonin, tryptophol, N-carbamyltryptophan
  - e.g. yohimbine alkaloids [30]
    such as ajmalicine, rauniticine
  - e.g. pyrrolizidine alkaloids [31]
    such as symphytine N-oxide and echimidine N-oxide
  - e.g. peramine [32]
- Amines [1]
  - e.g. primary aromatic amines [33–36]
- Urea and thiourea derivatives [28, 29, 37]
  - e.g. urea, thiourea
- Drug substances (38, 39)
  - e.g. sulfonamides [40–42]
    such as sulfanilamide, sulfanilthiocarbamide, sulfathiazole

Reagent for:

- Mycotoxins
  e.g. cyclopiazonic acid [43], PR toxin or PR imine [44]
  diacetoxyscirpenol [45]
- Pesticides
  e.g. phenylcarbamate and phenylurea herbicides [34, 36]
  such as asulam, chlorbromuron, chlortoluron, diuron, linuron
- Explosives [35]
  e.g. tetryl, TATB(1,3,5-triamino-2,4,6-tetranitrobenzene)
- Carbapenem antibiotics [46]
- Gangliosides [47, 48]
  e.g. GM1, GM2, Fuc-GM1, GT1b, GD1a, GD1b
- Bitter substances
  e.g. limonin [49], nomilin [50]
- Pyridine derivatives
  e.g. citrazinic acid, citrazinamide [51]



HCl	$C_9H_{11}NO$
$M_r = 36.46$	$M_r = 149.19$
Hydrochloric acid	4-(Dimethylamino)-benzaldehyde

Preparation of the Reagent

Dipping solution Dissolve 300–500 mg 4-(dimethylamino)-benzaldehyde in 25–40 ml methanol and treat with cooling with 10 ml hydrochloric acid (32%); the temperature should not fall below 20 °C or rise above 40 °C [52, 53].
Alternatively combine a solution of 300 mg 4-(dimethylamino)-benzaldehyde with a mixture of 54 ml 1-butanol, 9 ml ethanol and 9 ml conc. hydrochloric acid [46].

Spray solution Dissolve 1–5 g 4-(dimethylamino)-benzaldehyde in a mixture of 50 ml hydrochloric acid (25%) and 50 ml ethanol (96% or 100%) [2, 9, 11, 12, 21, 22,, 27, 32, 42], methanol [3, 10, 54] or 2-propanol [24].

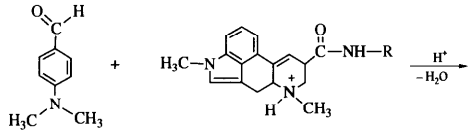
Alternatively make up a stock solution of 10 g 4-(dimethylamino)-benzaldehyde in concentrated hydrochloric acid and dilute one part by volume with 4 to 10 parts by volume acetone immediately before spraying [20, 25, 26, 28, 29, 44].

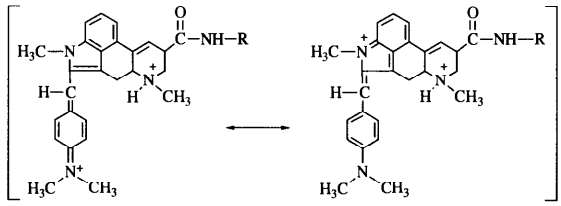
Storage The reagent solutions may be stored for several weeks [4, 53]. The spray solution that has been diluted with acetone is not stable and should therefore always be made up fresh [28].

Substances
4-(Dimethylamino)-benzaldehyde
Hydrochloric acid (32%)
Hydrochloric acid (25%)
Methanol
Ethanol
Ethanol (96%)
1-Butanol

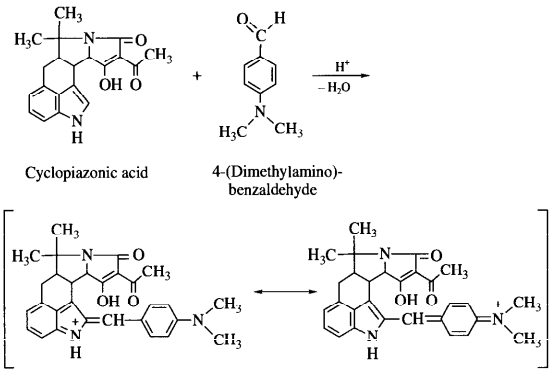
Reaction

Electrophilic substitution, e.g. of the 2-position of the indole ring, followed by the elimination of water leads to the formation of cyanin dyes from ergot alkaloids [53].





The same applies to cyclopiazonic acid [43].



Cyclopiazonic acid 4-(Dimethylamino)-benzaldehyde

Method

The chromatograms are dried in a stream of warm air, then immersed in the dipping solution for 2 s to 20 s or homogeneously sprayed with the spray solution until the layer begins to appear transparent [21, 52, 53]. After allowing the chromatogram to stand for a few minutes it is then heated to 50–120 °C for 2–20 min [12, 24, 47, 52–54]. In the case of gangliosides the chromatograms are covered with a glass plate during heating [47, 48].

Chromatogram zones of different colors (yellow, orange, red, brown, green, blue) are formed – mainly within a few minutes even before heating – on an almost colorless to slightly yellow background [2, 4, 21, 22, 52–54].

For example, ergot alkaloids produce without exception blue chromatogram zones, while clavine alkaloids primarily produce green colors [4]. Urea derivatives and primary aromatic amines yield yellow chromatogram zones [28, 33, 34, 36, 37] and PR toxin and PR imine emit intense blue fluorescence on excitation with long-wavelength UV light ($\lambda = 365$ nm) [44].

Note: Several variants of the reagent have been described in the literature. Thus chromatograms can be sprayed with a solution of 4-(dimethylamino)-benzaldehyde in cyclohexane, ethanol or 1-butanol and then exposed to hydrochloric acid vapor [5, 8, 13–16, 43, 44, 49, 50). Other variants of the reagent involve the addition of a drop of iron(III) chloride solution (10%) [4] or recommend treatment of the chromatogram afterwards with sodium nitrite (1% aqueous) to stabilize the colors [6]. In exceptional cases 4-(dimethylamino)-benzaldehyde reacts alone without the addition of other components to the reagent [55]. The 4-(dimethylamino)-benzaldehyde in the reagent can be replaced by 4-(diethylamino)-benzaldehyde [35]. In the case of pyrrolizidine alkaloids the chromatogram is sprayed with acetic anhydride – petroleum ether – benzene (1+4+5) and heated to 95 °C for 10 min before being treated with EHRLICH's reagent [31]. If zinc powder is incorporated into the layers it is also possible to detect nitroaromatics [35].

Some substances only react slowly at room temperature [20, 22]. The colors that appear initially generally alter over a period of a few hours and then remain stable for a virtually unlimited period [21]. The addition to the reagent of small quantities of oxidizing agents (iron(III) salts, hydrogen peroxide) has been reported to intensify the color tone [2, 4]; the same is also reported to occur if the treated chromatograms are afterwards exposed to UV light or to the vapors of aqua regia or nitric acid [2, 12].

4-(Dimethylamino)-benzaldehyde – hydrochloric acid reacts less sensitively than 4-(dimethylamino)-cinnamaldehyde – hydrochloric acid in the detection of indole derivatives, but the former is better for differentiation of substances on account of the multiplicity of different color shades produced.

The detection limits per chromatogram zone are 4–20 ng substance for aniline derivatives [52] and 3–100 ng substance for indole derivatives [2, 4, 5, 32, 53].But some substances, e. g. dihydroergosine [11] and PR toxin and PR imine [44] can be detected in quantities smaller than 1 ng.

The reagent can be employed, for example, on aluminium oxide, silica gel, silver nitrate impregnated silica gel, kieselguhr, Si 50000 and cellulose layers; RP and CHIR phases are also suitable.

Procedure Tested 1

Aniline Derivatives [52]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).

Mobile phase Toluene

Migration distance 8 cm

Running time 25 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air, immersed in the dipping solution for 2 s, dried briefly in a stream of warm air and then heated on a hot plate to 110 °C for 2 min.
2,4-Dimethylaniline (hR_f 5–10), 4-chloroaniline (hR_f 10–15), 3-chloroaniline (hR_f 20–25), 4-chloro-2-nitroaniline (hR_f 30-35), 2-chloroaniline (hR_f 35–40) and diphenylamine (hR_f 70–75) appeared as yellow chromatogram zones on a pale yellow background. The detection limits were between 4 ng (4-chloroaniline) and 20 ng (diphenylamine) substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength λ = 435 nm (Fig. 1).

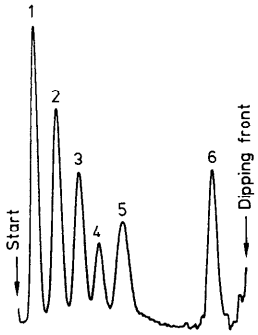


Fig 1: Reflectance scan of a chromatogram track with 100 ng each of 4-chloroaniline (2) and 3-chloroaniline (3) and 200 ng 2,4-dimethylaniline (1), 4-chloro-2-nitroaniline (4), 2-chloroaniline (5) and diphenylamine (6) per chromatogram zone.

Procedure Tested 2

Ergot Alkaloids [53]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer HPTLC plates Silica gel 60 F_{254} (Merck).

Mobile phase Acetonitrile – 1-propanol – water (125 + 28 + 15).

Migration distance 6 cm

Running time 17 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air and immersed twice for 10 s – with brief intermediate drying in a stream of cold air – in the dipping solution and then immediately heated to 115 °C for 15 min in the drying cupboard; the TLC plate was only to be supported on its side on two metal tracks. After cooling to room temperature the chromatogram was immersed for 1 s in a solu-

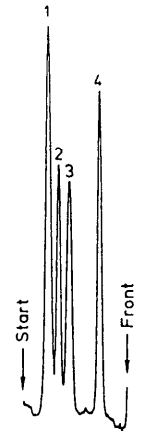


Fig 2: Reflectance scan of a chromatogram track with 90 ng of each substance per chromatogram zone; 1 = lisuride dihydrogen maleate, 2 = methysergide maleate, 3 = dihydroergotamine mesylate, 4 = ergotamine tartrate.

tion of liquid paraffin – *n*-hexane (1+2) and dried for 5 min in a stream of cold air; the purpose of this last immersion was to stabilize the reflectance signal of methysergide in particular.

Lisuride dihydrogen maleate (hR_f 30–35), methysergide maleate (hR_f 40–45), dihydroergotamine mesylate (hR_f 45–50) and ergotamine tartrate (hR_f 70–75) appeared as blue violet chromatogram zones on a yellow background. The detection limits – calculated for free base – were 3–4 ng substance per chromatogram zone.

In situ quantitation: After 1 h the absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 590$ nm (Fig. 2).

References

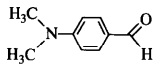
[1] Krebs, K. G., Heusser, D., Wimmer, H. in E. Stahl (Ed.): *Thin-Layer Chromatography – A Laboratory Handbook*, Springer, Berlin, Heidelberg, New York, 1969.
[2] Stahl, E., Kaldewey, H.: *Hoppe-Seyler's Z. Physiol. Chem.* **1961**, *323*, 182–191.
[3] Preobrazhenskaya, M. N., Mikhailova, L. N., Chemerisskaya, A. A.: *J. Chromatogr.* **1971**, *61*, 269–278.
[4] Klavehn, M., Rochelmeyer, H.: *Dtsch. Apoth. Ztg.* **1961**, *101*, 477–481.
[5] Zinser, M., Baumgärtel, C.: *Arch. Pharm.* **1964**, *297*, 158–164.
[6] Genest, K.: *J. Chromatogr.* **1965**, *19*, 531–539.
[7] Fowler, R., Gomm, P. J., Patterson, D. A.: *J. Chromatogr.* **1972**, *72*, 351–357.
[8] Vanhaelen, M., Vanhaelen-Fastré, R.: *J. Chromatogr.* **1972**, *72*, 139–144.
[9] Sperling, A. R.: *J. Chromatogr. Sci.* **1974**, *12*, 265–266.
[10] Van Mansvelt, F. J. W., Greving, J. E., De Zeeuw, R. A.: *J. Chromatogr.* **1978**, *151*, 113–120.
[11] Prošek, M., Katic, M., Koric, J., Kucan, E. in A. Frigerio (Ed.): *Chromatography in Biochemistry, Medicine and Environmental Research*, Elsevier, Amsterdam **1983**, *1*, 27–36.
[12] Stahl, E., Brombeer, J.: *Dtsch. Apoth. Ztg.* **1978**, *118*, 1527–1534.
[13] Cerny, A., Zikán, V., Vlcková, D., Bene, J., Holubek, J., Režábek, K., Aušková, M., Krepelka, J.: *Collect. Czech. Chem. Commun.* **1983**, *48*, 1483–1488.
[14] Bene, J., Cerny, A., Miller, V., Kudrnác, S.: *Collect. Czech. Chem. Commun.* **1983**, *48*, 1333–1340.
[15] Beran, M., Bene, J., Krepelka, J.: *Collect. Czech. Chem. Commun.* **1982**, *47*, 3432–3436.
[16] Krepelka, J., Vlcková, D., Holubek, J., Roubík, J.: *Collect. Czech. Chem. Commun.* **1982**, *48*, 312–317.
[17] Voigt, R., Zier, P., Rabitzsch, G.: *Pharmazie* **1972**, *27*, 175–178.
[18] Voigt, R., Zier, P.: *Pharmazie* **1972**, *27*, 773–776.
[19] Jaspersen-Schib, R.: *Dtsch. Apoth. Ztg.* **1990**, *130*, 71–73.
[20] Beug, M. W., Bigwood, J.: *J. Chromatogr.* **1981**, *207*, 379–385.
[21] Ehmann, A., Bandurski, K.: *J. Chromatogr.* **1972**, *72*, 61–70.
[22] Byrd, D. J., Kochen, W., Idzko, D., Knorr, E.: *J. Chromatogr.* **1974**, *94*, 85–106.
[23] Marchelli, R., Hutzinger, O., Heacock, R. A.: *Pharm. Acta Helv.* **1971**, *46*, 150–155.

[24] Zahn, H.: *Ärztl. Lab.* **1984**, *30*, 279–283.
[25] Gartz, J.: *Pharmazie* **1985**, *40*, 431–432.
[26] Gartz, J.: *Pharmazie* **1985**, *40*, 432.
[27] Bürstell, H., Hilgenberg, W.: *Biol. Zbl.* **1975**, *94*, 389–400.
[28] Heathcote, J. G., Davies, D. M., Haworth, C.: *J. Chromatogr.* **1971**, *60*, 103–109; **1972**, *67*, 325–328.
[29] Haworth, C., Walmsley, T. A.: *J. Chromatogr.* **1972**, *66*, 311–319.
[30] Phillipson, J. D., Hemingway, S. R.: *J. Chromatogr.* **1975**, *105*, 163–178.
[31] Wagner, H., Neidhardt, U., Tittel, G.: *Planta Med.* **1981**, *41*, 232–239.
[32] Fannin, F. F., Bush, L. P., Siegel, M. R., Rowan, D. D.: *J. Chromatogr.* **1990**, *503*, 288–292.
[33] Kovács, G. H.: *J. Chromatogr.* **1984**, *303*, 309–311.
[34] Ambrus, Á., Hargitay, É., Károly, G., Fülöp, A., Lantos, J.: *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 743–748.
[35] Yasuda, S. K.: *J. Chromatogr.* **1970**, *50*, 453–457; **1972**, *71*, 481–486.
[36] Spengler, D., Jumar, A.: *J. Chromatogr.* **1970**, *49*, 329–333.
[37] Mahapatra, G. N., Nath, J. P., Pattnaik, B. K., Rout, D. N.: *J. Chromatogr.* **1980**, *193*, 338–339.
[38] Phillips, G. F., Gardiner, J.: *J. Pharmacy Pharmacol.* **1969**, *21*, 793–807.
[39] Schulte, K. E., Henke, G.: *Arch. Pharm.* **1973**, *306*, 182–197.
[40] Schittenhelm, D., Herrmann, K.: *Dtsch. Apoth. Ztg.* **1970**, *110*, 1441–1446.
[41] Pauncz, J. K.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1981**, *4*, 287–291.
[42] Meakin, B. J., Tansey, I. P., Davies, D. J. G.: *J. Pharmacy Pharmacol.* **1971**, *23*, 252–261.
[43] Rathinavelu, A., Rajabhavani, E., Shanmugasundaram, B.: *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 38–40.
[44] Lafont, P., Debeaupuis, J. P.: *J. Chromatogr.* **1980**, *198*, 481–488.
[45] Eppley, R. M.: *J. Assoc. Off. Anal. Chem.* **1975**, *58*, 906–908.
[46] Okuyama, D., Okabe, M., Fukagawa, Y., Ishikura, T.: *J. Chromatogr.* **1984**, *291*, 464–470.
[47] Gazzotti, G., Sonnino, S., Ghidoni, R.: *J. Chromatogr.* **1984**, *315*, 395–400.
[48] Chigorno, V., Sonnino, S., Ghidoni, R., Tettamanti, G.: *Neurochem. Internat.* **1982**, *4*, 397–404.
[49] Maier, V. P., Grant, E. R.: *J. Agric. Food Chem.* **1970**, *18*, 250–252.
[50] Rouseff, R. L.: *J. Agric. Food Chem.* **1982**, *30*, 504–507.
[51] Cee, A., Horáková, B.: *J. Chromatogr.* **1985**, *331*, 202–203.
[52] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[53] Schmidt, S.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[54] Gont, L. K., Neuendorf, S. K.: *J. Chromatogr.* **1987**, *391*, 343–345.
[55] Ritter, W.: *Proc. Int. Symp. Instrum. High Perform. Thin-Layer Chromatogr. (HPTLC)*, 2nd, p. 100–113, Interlaken 1982.

4-(Dimethylamino)-benzaldehyde–Sulfuric Acid Reagent (Van Urk's Reagent)

Reagent for:

- Primary aromatic amines [1]
- Alkaloids
  - e.g. tropane alkaloids
    such as atropine, scopolamine [2]
  - e.g. ergot alkaloids
    such as ergotamine, ergocryptine, ergometrine [3, 4, 17]
  - e.g. clavine alkaloids
    such as chanoclavine, agroclavine [3, 5, 6]
- Pharmaceutical active ingredients
  - e.g. sulfonamides
    such as sulfasomidine, sulfadiazine, sulfamerazine [1, 7]
- Addictive drugs
  - e.g. lysergic acid diethylamide (LSD) [4, 8–10]
    psilocybin, monomethyltryptamine [9]
- Indole and hydroxyindole derivatives
  - e.g. tryptophan metabolites [11]
    cyclopiazonic acid (mycotoxin) [12]
    4-, 5-, 6- and 7-hydroxyskatoles [13]
- Monoterpene ketones
  - e.g. menthone, pulegone, carvone, piperitenone [14]
- Carbamate pesticides
  - e.g. fenuron, monuron, linuron, carbaryl [15]



H_2SO_4 $M_r = 98.08$

$C_9H_{11}NO$ $M_r = 149.19$

Preparation of the Reagent

Dipping solution

Dissolve 1 g 4-(dimethylamino)-benzaldehyde in a mixture of 45 ml water and 5 ml sulfuric acid (95–97%) and make up to 100 ml with water [2].

Variant 1: Dissolve 2 g 4-(dimethylamino)-benzaldehyde in 45 ml methanol, add 5 ml sulfuric acid (95–97%) cautiously with cooling and make up to 100 ml with methanol [16].

Variant 2: Dissolve 0.3 g 4-(dimethylamino)-benzaldehyde in 90 ml methanol and add 10 ml sulfuric acid (95–97%) cautiously with cooling (ice water). When preparing this dipping solution the temperature should be kept within the 20–40 °C range [17].

Spray solution

For indole derivatives: Dissolve 50 mg 4-(dimethylamino)-benzaldehyde in 1 ml conc. sulfuric acid and make up to 100 ml with 95% ethanol [7].

Variant 1: Dissolve 0.8 g 4-(dimethylamino)-benzaldehyde in a mixture of 90 ml 95% ethanol and 10 ml 98% sulfuric acid [9].

For monoterpene ketones: Dissolve 200 mg 4-(dimethylamino)-benzaldehyde in 20 ml conc. sulfuric acid [14].

Storage

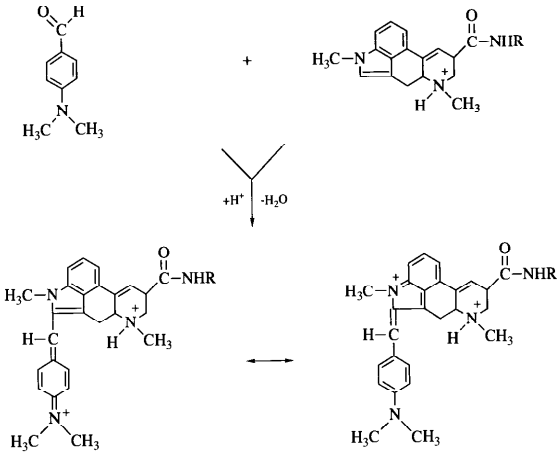
The reagent solutions may be stored over a prolonged period [17].

Substances

4-(Dimethylamino)-benzaldehyde
Sulfuric acid (95–97%)
Methanol
Ethanol

Reaction

4-(Dimethylamino)-benzaldehyde reacts in acidic medium, e.g. with the indole ring of cyclopiazione or ergot alkaloids and forms a cyanin dyestuff by electrophilic substitution in the 2-position followed by the elimination of water [12, 17].



Method

The chromatograms are freed from mobile phase in a stream of warm air, then either immersed briefly in one of the dipping solutions or homogeneously sprayed with one of the spray solutions, until the layer begins to be transparent. Then they are heated to 105–120 °C for 10–30 min [2, 9, 14, 16, 17].

Indole derivatives yield red to blue-violet chromatogram zones on an almost colorless background [2, 3, 12]; these zones gradually fade [12]. Monoterpene ketones yield yellow-gray to red-brown chromatogram zones [14]. Hydroxyskatoles initially yield yellow chromatogram zones but these change color to gray-brown or green-brown on heating or if allowed to lie [13].

Note: The individual components of the reagent can also be applied separately one after the other [12, 15], e.g. the chromatogram is first immersed in an 8% methanolic 4-(dimethylamino)-benzaldehyde solution and then, after intermediate drying, sprayed with 25% sulfuric acid [12]. 4-(Dimethylamino)-benzaldehyde can be replaced in the reagent with 4-(dimethylamino)-cinnamaldehyde [1].

In the case of carbamate pesticides the chromatogram is heated to 150 °C for 20 min after the application of the reagent. Spraying later with a solution of 2 N sodium hydroxide solution to improve the color contrast is recommended [15]. Occasionally a small amount of iron(III) chloride is added to the reagent [13].

Quantitative evaluations are best carried out ca. 15–20 min after heating the chromatogram [2].

The detection limits for sulfonamides lie at 10–50 ng [7], for lysergic acid diethylamide at 50 ng [4] and for tropane alkaloids at 50–500 ng substance per chromatogram zone [2, 16].

In the case of lysergic acid derivatives spraying with sodium nitrite solution afterwards stabilizes the colored chromatogram zones [18].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr and Si 50000 layers; cellulose layers are not suitable.

Procedure Tested 1

Tropane Alkaloids [16]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK) that are prewashed, before application of the samples, by developing in chloroform – methanol (50+50) to the upper edge of the plates and then dried for 30 min at 110 °C.
Mobile phase	Acetone – toluene – ammonia solution (25%) (40+15+5).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of warm air until the ammonia was completely removed (ca. 45 min), cooled in a stream of cold air for 5 min, immersed twice in the dipping solution (variant 1) for 10 s, with brief intermediate drying in a stream of cold air, and then heated to 120 °C for 30 min.

Atropine (hR_f 30–35) and scopolamine (hR_f 60–65) appeared as red chromatogram zones on a reddish-gray background. The detection limits lay at 50 ng substance per spot.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at wavelength $\lambda = 500$ nm (Fig. 1).

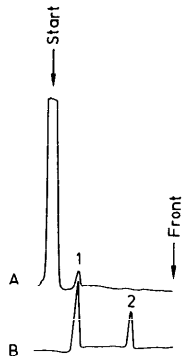


Fig. 1: Reflectance scan of a chromatogram track of an *Atropa belladonna* extract (A) and of a reference track (B) with 200 ng of both atropine (1) and scopolamine (2).

Procedure Tested 2

Ergot Alkaloids [17]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Acetonitrile – 1-propanol – water (125+28+15).
Migration distance	6 cm
Running time	17 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed twice in the dipping solution (variant 2) for 10 s with brief

intermediate drying in a stream of cold air and then heated, while still damp, to 95 °C for 1 min on a hot plate. Then, after cooling to room temperature, it was immersed for 1 s in a solution of liquid paraffin – *n*-hexane (1+2) to stabilize the reflectance signal of dihydroergotamine and dried for 5 min in a stream of cold air.

Lysuride hydrogen maleate (hR_f 30–35), methysergide maleate (hR_f 40–45), dihydroergotamine mesylate (hR_f 45–50) and ergotamine tartrate (hR_f 70–75) appeared as gray-violet chromatogram zones on a colorless background. The detection limits – calculated for the free base – were 15–30 ng substance per chromatogram zone.

In situ quantitation: After waiting for 50 min the absorption photometric evaluation in reflectance was carried out at wavelength $\lambda = 590$ nm (Fig. 2).

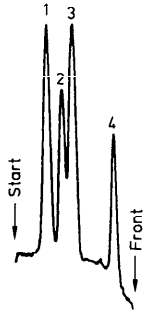


Fig. 2: Reflectance scan of a chromatogram track of 90 ng each lysuride hydrogen maleate (1), methysergide maleate (2), dihydroergotamine mesylate (3) and ergotamine tartrate (4) per chromatogram zone.

References

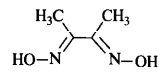
[1] Lee, S.-C.: *J. Chromatogr.* **1974**, *93*, 480–484.
[2] Dorosiev, I., Simova, M., Kolarova, R., Pangarova, T.: *Pharmazie* **1983**, *38*, 419.
[3] Sallam, L. A. R., Naim, N., El-Refai, A. H.: *Z. Anal. Chem.* **1977**, *284*, 47–48.

[4] Cortivo, L. A. D., Broich, J. R., Dihrberg, A., Newman, B.: *Anal. Chem.* **1966**, *38*, 1959–1960.
[5] Hsu, J. C., Anderson, J. A.: *Biochem. Biophys. Acta* **1971**, *230*, 518–525.
[6] Cavender, F. A., Anderson, J. A.: *Biochem. Biophys. Acta* **1970**, *208*, 345–348.
[7] Srivastava, S. P., Dua, V. K., Mehrotra, R. N., Saxena, R. C.: *J. Chromatogr.* **1979**, *176*, 145–147.
[8] Röder, E., Surborg, K. H.: *Z. Anal. Chem.* **1971**, *256*, 362–363.
[9] Brown, J. K., Shapazian, L., Griffin, G. D.: *J. Chromatogr.* **1972**, *64*, 129–133.
[10] Bailey, K., Verner, D., Legault, D.: *J. Assoc. Off. Anal. Chem.* **1973**, *56*, 88–99.
[11] Byrd, D. J., Kochen, W., Bühner, R., Brauer, I., Trefz, F.: *Z. Klin. Chem. Klin. Biochem.* **1972**, *10*, 175–176.
[12] Popken, A., Dose, K.: *Fresenius Z. Anal. Chem.* **1983**, *316*, 47–50.
[13] Heacock, R. A., Mahon, M. E.: *J. Chromatogr.* **1965**, *17*, 338–348.
[14] Rothbächer, H., Suteu, F.: *J. Chromatogr.* **1974**, *100*, 236–239.
[15] Abbott, D. C., Blake, K. W., Tarrant, K. R., Thomson, J.: *J. Chromatogr.* **1967**, *30*, 136–142.
[16] Müller, J.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1987.
[17] Schmidt, S.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[18] Genest, K.: *J. Chromatogr.* **1965**, *19*, 531–539.

Dimethylglyoxime Reagent
(Diacetyldioxime Reagent)

Reagent for:

- Cations
  e.g. nickel [1–7]
  cobalt [4–7]
  copper, iron, manganese, silver [6]
  DMSO complexes of cobalt, nickel [8]



$C_4H_8N_2O_2$

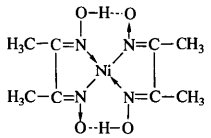
$M_r = 116.12$

Preparation of the Reagent

Dipping solution	Dissolve 1 g dimethylglyoxime (diacetyldioxime) in 100 ml ethanol.
Spray solution	Dissolve 0.1 to 1 g dimethylglyoxime in 100 ml 96% ethanol [1, 5] or in ammonia solution [4, 8].
Storage	The reagent solutions may be stored for longer periods.
Substances	Dimethylglyoxime Ethanol Ammonia solution (25%)

Reaction

A series of metal cations (Ni, Fe, Co, Cu, Pt) form colored complexes with dimethylglyoxime in ammonia solution or weakly acidic medium.



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s or sprayed homogeneously with the spray solution, then dried in a stream of cold air and exposed to ammonia vapor in a twin-trough chamber.

Red-violet (Ni), red-brown (Co, Fe, Cu), flesh-colored (Mn) or pale gray-violet (Mn) chromatogram zones are produced on a colorless background [6, 7].

Note: The detection limits for nickel and cobalt cations are 20 ng substance per chromatogram zone [9].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr, Si 50000, RP and cellulose layers. Sodium molybdate-impregnated phases and zirconium oxide layers are also suitable [1].

Procedure Tested

Nickel and Cobalt Cations [9]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer HPTLC plates Cellulose F_{254s} (MERCK) that have been precleaned by developing once to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.

Mobile phase Ethanol – water – nitric acid (65%) (70+18+12).

Migration distance 6 cm

Running time 45 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air, immersed in the dipping solution for 2 s, dried for 2 min in a stream of cold air and exposed to ammonia vapor (25% ammonia solution in the vacant trough of a twin trough chamber) for 3 min.

Nickel cations (hR_f 35–40) appeared as red and cobalt cations (hR_f 40–45) as yellow chromatogram zones on a colorless background.

The detection limits lay at 20 ng substance per chromatogram zone.

In situ quantitation: The absorption spectrophotometric measurements in reflectance were made at a mean wavelength $\lambda = 480$ nm (Fig. 1A) or at wavelength $\lambda = 450$ nm for cobalt (Fig. 1B) and $\lambda = 510$ nm for nickel (Fig. 1C).

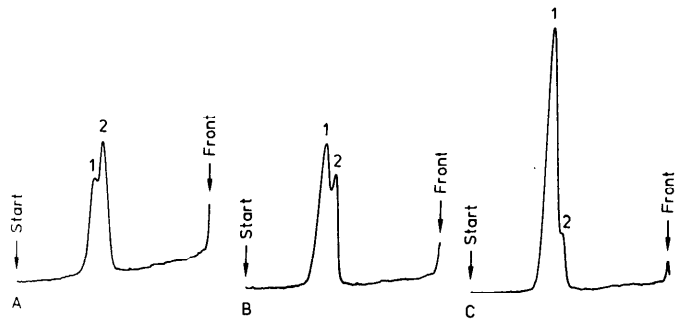


Fig. 1: Reflectance scans of a chromatogram track with 200 ng each of nickel (1) and cobalt (2) cations per chromatogram zone: Scans at $\lambda = 480$ nm (A), 450 nm (B) and 510 nm (C).

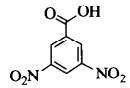
References

[1] Sen, A. K., Das, S. B., Ghosh, U. C.: *J. Liq. Chromatogr.* **1985**, *6*, 2999–3008.
[2] Zetlmeisl, M. J., Haworth, D. T.: *J. Chromatogr.* **1967**, *30*, 637–639.
[3] Deshmukh, L., Kharat, R. B.: *J. Chromatogr. Sci.* **1990**, *28*, 400–402.
[4] Singh, R. P., Saxena, S. K., Kumari, K.: *J. Liq. Chromatogr.* **1985**, *8*, 1319–1326.
[5] Ajmal, M., Mohammad, A., Fatima, N., Ahmad, J.: *J. Planar Chromatogr.* **1988**, *1*, 239–245, 329–335; *J. Liq. Chromatogr.* **1989**, *12*, 3163–3191.
[6] Buchbauer, G., Knie, J.: *Sci. Pharm.* **1983**, *51*, 41–47.
[7] Merkus, F. W. H. M.: *Pharm. Weekblad* **1963**, *98*, 947–957.
[8] Sharma, S. D., Misra, S.: *J. Liq. Chromatogr.* **1985**, *8*, 2991–2998.
[9] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1990.

3,5-Dinitrobenzoic Acid–Potassium Hydroxide Reagent (Kedde's Reagent)

Reagent for:

- Steroid glycosides and aglycones
  e.g. cardenolide glycosides [1–7]
  from *Anodendron* [8], *Digitalis* [9–12],
  Strophantus [13, 14] and
  Convallaria species



$C_7H_4N_2O_6$ KOH

$M_r = 212.12$ $M_r = 56.11$

Preparation of the Reagent

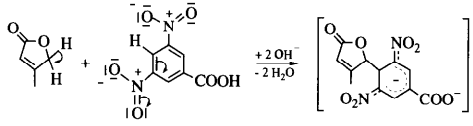
Solution I	Dissolve 2 g 3,5-dinitrobenzoic acid in 100 ml methanol.
Solution II	Dissolve 5.7 g potassium hydroxide in 100 ml methanol.
Dipping solution	Dissolve 0.5 g 3,5-dinitrobenzoic acid in 50 ml ethanol (96%) with gentle warming and mix with 50 ml sodium hydroxide solution (c = 2 mol/L) before use [17].
Spray solution	Mix equal volumes of solution I and solution II before use [1, 8, 10, 14].

Storage Solutions I and II may be stored for extended periods.

Substances 3,5-Dinitrobenzoic acid
Potassium hydroxide pellets
Methanol
Ethanol
Sodium hydroxide solution

Reaction

The γ-lactone ring of the steroid skeleton forms an intermediate cardenolide anion in alkaline medium that nucleophilically adds to the 3,5-dinitrobenzoic acid in the position *ortho* to the two nitro groups. A mesomerically stabilized red-violet anion is produced (MEISENHEIMER complex).



MEISENHEIMER complex

Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution. Alternatively the chromatogram can first be sprayed lightly with solution I and then with an excess of solution II.

Blue to blue-violet chromatogram zones are formed on a colorless background; these gradually fade [11, 16].

Note: In the second spray potassium hydroxide solution can be replaced by sodium hydroxide solution or by a solution of 17 g benzyltrimethylammonium hydroxide in 100 ml 33 percent methanol [4]. The KEDDE reagent [15] can also be applied very successfully to layers that have previously been treated with *p*-toluenesulfonic acid or vanillin – perchloric acid reagent [1]. The instability of the colored derivatives produced makes the reagent unsuitable for quantitative analysis [16].

The detection limits for *Convallaria* glycosides are 20 ng substance per chromatogram zone [16].

The reagent can, for instance, be used on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested I

Cardenolides [16]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer TLC plates Silica gel 60 F_{254} (MERCK) that had been precleaned before applying the samples by developing once with methanol – chloroform (50+50) and then dried at 110 °C for 30 min.

Mobile phase Ethyl acetate – methanol – water (81+11+8).

Migration distance 10 cm

Running time 25 min

Detection and result: The chromatogram was freed from mobile phase and homogeneously sprayed with the spray solution.

The cardenolides g-strophanthin (hR_f 5–10), convallatoxin (hR_f 30–35) and k-strophanthin (hR_f 50–55) immediately formed red-violet chromatogram zones that gradually faded. Hence, the reagent was not always suitable for quantitative work.

The visual detection limits were 20 ng substance per chromatogram zone.

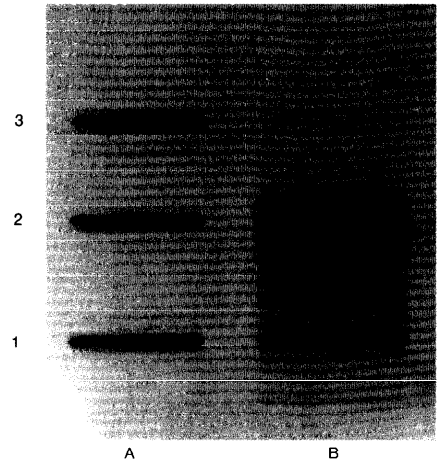


Fig. 1: Chromatograms of reference substances (A) and of a lily of the valley extract (B): 1 = g-strophanthin, 2 = convallatoxin, 3 = k-strophanthin

Procedure Tested II

Digitalis Glycosides [17]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} with concentrating zone (RIEDEL DE HAEN, MERCK).
Mobile phase	Actone – dichloromethane (60+40).
Migration distance	5 cm
Running time	8 min

Detection and result: The chromatogram was freed from mobile phase, immersed in the dipping solution for 2 s and then examined immediately after brief drying in a stream

of warm air. Digoxin (hR_f 30–35) and digitoxin (hR_f 40–45) yielded violet chromatogram zones on a colorless background. The detection limits were 50 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric scan at λ_{max} = 550 nm had to be carried out immediately, since the colors of the derivatives only remained stable for ca. 10–15 min; exact quantitative analysis was not always possible.

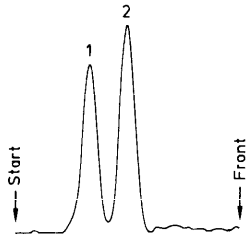


Fig. 2: Absorbance scan of a chromatogram track with 500 ng each of digoxin (1) and digitoxin (2) per chromatogram zone.

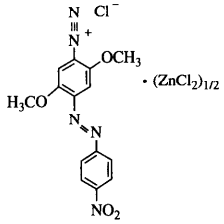
References

[1] Lewbart, M. L., Wehrli, W., Reichstein, T.: *Helv. Chim. Acta* **1963,** *46,* 505–517.
[2] Polonia, J., Jäger, H., Euw, J.v., Reichstein, T.: *Helv. Chim. Acta* **1970,** *53,* 1253–1271.
[3] Junior, P., Krüger, D., Winkler, C.: *Dtsch. Apoth. Ztg.* **1985,** *125,* 1945–1949.
[4] Fung, S. Y.: *Biochem. Systematics and Ecology* **1986,** *14,* 371–373.
[5] Tarng, C. S., Stohs, S. J.: *Planta Med.* **1975,** *27,* 77–82.
[6] Karawya, M. S., Abdel-Wahab, S. M., Niazi, H. M.: *Planta Med.* **1973,** *24,* 234–242.
[7] Bulger, W. H., Stohs, S. J., Wheeler, D. M. S.: *Biochem. Pharmacol.* **1974,** *23,* 921–929.
[8] Abe, F., Yamauchi, T.: *Chem. Pharm. Bull.* **1983,** *30,* 1183–1193; **1983,** *31,* 1199–1206.
[9] Sawlewicz, L., Linde, H. H. A., Meyer, K.: *Helv. Chim. Acta* **1970,** *53,* 1382–1385.
[10] Löffelhardt, W., Kopp, B., Kubelka, W.: *Phytochemistry* **1979,** *18,* 1289–1291.
[11] Krüger, D., Wichtl, M.: *Dtsch. Apoth. Ztg.* **1985,** *125,* 55–57.
[12] Wagner, H., Habermeier, H., Schulten, H.-R.: *Helv. Chim. Acta* **1984,** *67,* 54–64.
[13] Kartnig, Th., Danhofer, R.: *J. Chromatogr.* **1970,** *52,* 313–320.
[14] Kubelka, W., Eichhorn-Kaiser, S.: *Pharm. Acta Helv.* **1970,** *45,* 513–519.
[15] Kedde, D. L.: *Pharm. Weekbl.* **1947,** *82,* 741–757.
[16] Hahn-Deinstrop, E.: Private communication, Heumann-Pharma, Abt. Entwicklungsanalytik, Heideloffstraße 18–28, D-90478 Nürnberg, 1990.
[17] Ganz, J., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1991.

Fast Black Salt K–Sodium Hydroxide Reagent

Reagent for:

- Amines
  - e.g. aliphatic *prim.* and *sec.* amines [1, 2]
    such as α-/β-alanine, ethylamine, diethylamine, piperidine, mescaline desipramine, ephedrine, maprotiline, nortriptyline
  - e.g. aromatic *prim.* and *sec.* amines [1]
    such as aniline, 2,4,6-trimethylaniline, bromhexine, diphenylamine, amethocaine, procaine
  - e.g. aromatic *tert.* amines [1]
    such as N,N-dimethylaniline, N,N-dimethyl-*p*-toluidine
- Phenols
  - e.g. catechin, resorcinol, hydroquinone, morphine [1]
- Salbutanol, isoprenaline, terbutaline [1]
- Tetracyclines [1]
- Aromatic N-heterocyclics
  - e.g. pyrrole, imidazole, indole [1]
- Quaternary ammonium compounds [2]
- Psychopharmaceuticals
  - e.g. chlorprothixene, haloperidol, sulpyride, perphenazine [2]
    oxaflozane and its metabolites [4]
- Analeptics, stimulants [3]
  - e.g. amphetamine, preludin, captagon [6]
    methamphetamine [1, 6]
- Diethyl malonate [1]
- β-Blockers [2, 3, 5]
  - e.g. alprenolol, atenolol, oxprenolol, propranolol, timolol, acebutolol metoprolol, sotalol



NaOH
$M_r = 40.00$
Sodium hydroxide

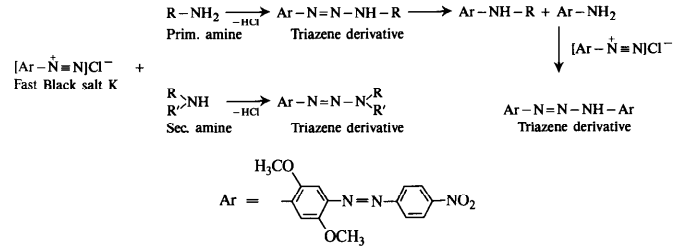
$(C_{14}H_{12}ClN_5O_4)_2 \cdot ZnCl_2$
$M_r = 546.36$
Fast Black Salt K

Preparation of the Reagent

Dipping solution I	Dissolve 500 mg fast black salt K (Echtschwarzsalz K, diazotized 4-amino-2,5-dimethoxy-4'-nitroazobenzene zinc double salt) in 100 ml water with heating. Filter off any undissolved components. Dilute the filtrate with methanol (1+1).
Dipping solution II	Methanolic sodium hydroxide solution (c = 0.5 mol/L).
Spray solution I	Dissolve 500 mg fast black salt K in 100 ml water; filter off any insoluble components [1–3, 5].
Spray solution II	Sodium hydroxide solution (c = 0.0.5 mol/L) [1–3, 5].
Storage	The dipping and spray solutions are stable for up to one day.
Substances	Fast black salt K Sodium hydroxide 0.5 mol/L Methanol

Reaction

Aliphatic primary and secondary amines primarily react with the diazonium compound fast black salt K to yield colored triazene derivatives [1] according to the following scheme:



It is also probable that there is coupling to colored derivatives in the case of aromatic amines and phenols (cf. Fast blue salt B reagent).

Method

The chromatograms are dried in a stream of warm air, sprayed homogeneously with spray solution 1, dried briefly in a stream of hot air, then sprayed lightly with spray solution 2 and finally dried in a stream of warm air. In the case of β-blockers this is followed with a further light spray with spray solution 1 [5].

Aliphatic and aromatic primary amines yield violet to violet-red chromatogram zones and aliphatic and aromatic secondary amines orange-red to brownish-red chromatogram zones on a colorless background; phenols are colored red-violet, light brown or green; pyrrole, imidazole, indole yield violet and diethyl malonate yields orange zones [1]. β-Blockers are colored orange to reddish-violet [5].

Note: Color reactions occur even before application of spray solution II [6]. Tertiary aliphatic amines and phenols with blocked *ortho* and *para* positions and aromatic N-acylated amines, e.g. acetanilide, do not react [1].

The color hues produced in the reaction do not appear to be affected by differing substituents at the amine nitrogen; however electron-attracting substituents at the α-C atom appear to reduce the detection sensitivity of the reaction [1]. The colors produced remain stable for months in the dark. In the light the zones produced by primary amines fade more rapidly than those from secondary amines [1].

The detection limits for analeptics and stimulants are 2 to 5 µg substance per chromatogram zone [6]. β-Blockers can be detected at 50–100 ng per chromatogram zone [5].

The reagent can be employed, for instance, on silica gel, kieselguhr, Si 50000 and RP layers.

Procedure Tested

Tetracyclines [7]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates RP 18 WF_{254s} (MERCK).
Mobile phase	Oxalic acid (c = 0.5 mol/L, aqueous) – acetone – methanol (27+10+6).

Migration distance	8 cm
Running time	60 min

Detection and result: The chromatogram was dried for 15 min in a stream of warm air and first examined under UV light. Tetracycline, chlorotetracycline, doxycycline and oxytetracycline fluoresced red under long-wavelength UV light (λ = 365 nm). These four substances appear as dark zones on a pale blue fluorescent background (fluorescence quenching) under short-wavelength UV light (λ = 254 nm).

The chromatogram was then immersed in dipping solution I for 1 s, dried briefly in a stream of warm air and then immersed in dipping solution II for 1 s. It was then dried in a stream of warm air for 10 min.

Tetracycline (hR_f 35–40) produced blue and doxycycline (hR_f 15–20), chlorotetracycline (hR_f 25–30) and oxytetracycline (hR_f 40–45) produced violet chromatogram zones on a yellow background. The detection limits for all 4 compounds were 2 ng substance per chromatogram zone (λ = 550 nm).

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength λ = 550 nm (Fig. 1A) or = 580 nm (Fig. 1B).

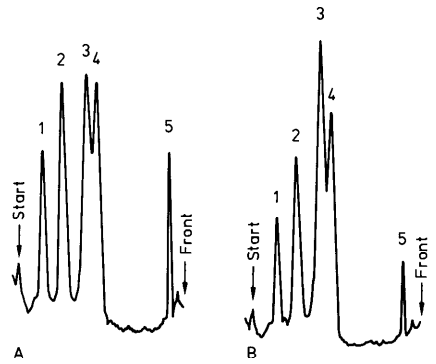


Fig 1: Reflectance scan of a chromatogram track with 16 ng of each substance per chromatogram zone: measurement at λ = 550 nm (A) and at λ = 580 nm (B). Note: The ordinate for (B) has been compressed by ca. 50% in comparison to (A): 1 = doxycycline, 2 = chlorotetracycline, 3 = tetracycline, 4 = oxytetracycline, 5 = unknown.

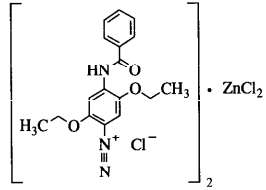
References

[1] Ojanperä, I., Wähälä, K., Hase, T.A.: *Analyst* **1990**, *115*, 263–267.
[2] Ojanperä, I., Vuori, E.: *J. Liq. Chromatogr.* **1987**, *10*, 3595–3604.
[3] Ojanperä, I., Lillsunde, P., Vartiovaara, J., Vuori, E.: *J. Planar Chromatogr.* **1991**, *4*, 373–378.
[4] Constantin, M., Pognat, J. F.: *Arzneim. Forsch.* **1979**, *29*, 109–114.
[5] Ojanperä, I., Ruohonen, A.: *J. Anal. Toxikol.* **1988**, *12*, 108–110.
[6] Eberhardt, H., Debackere, M.: *Arzneim. Forsch.* **1965**, *15*, 969–970.
[7] Klein, I., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken 1990.

Fast Blue Salt BB Reagent

Reagent for:

- Phenols [1]
  e.g. tetracycline antibiotics [2, 3]



$(C_{17}H_{18}ClN_3O_3)_2 \cdot ZnCl_2$

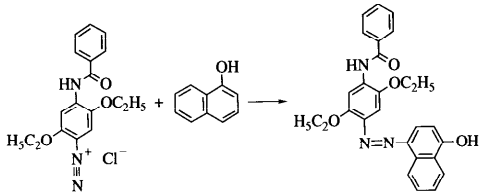
$M_r = 831.88$

Preparation of the Reagent

Dipping solution	Dissolve 0.5 g fast blue salt BB (C.I. 37175) in 25 ml water and make up to 100 ml with methanol [1].
Spray solution	Dissolve 0.5 g fast blue salt BB in 100 ml water [2].
Storage	The reagent solutions should always be prepared fresh and fast blue salt BB should be stored in the refrigerator.
Substances	Fast blue salt BB Methanol

Reaction

Fast blue salt BB couples with phenols, preferably in alkaline medium, to yield intensely colored azo dyes.



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 4 s or homogeneously sprayed with the spray solution and then heated to 110–120 °C for 5–10 min [1, 2].

Chromatogram zones of various colors are produced on a pale yellow-orange colored background [1, 2].

Note: The dipping solution can also be used as a spray solution. RP layers should be treated with the methanol-containing reagent on account of its better wetting properties. In addition it is necessary, particularly after the use of acidic mobile phases, to spray with alkalis, e.g. pyridine, after the heat treatment step [2, 3].

The detection limits per chromatogram zone are 30–50 ng for tetracyclines [2, 3] and 2 ng for 1- and 2-naphthol [1].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000, and RP layers.

Procedure Tested

1-Naphthol and 2-Naphthol [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK) that have been precleaned by developing once to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.
Mobile phase	Toluene – triethylamine (30+10).
Migration distance	6 cm
Running time	12 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, cooled to room temperature, immersed in the dipping solution for 4 s and then heated to 110 °C for 5 min.

2-Naphthol (hR_f 50–55) appeared as a pink and 1-naphthol (hR_f 60–65) as a violet-green chromatogram zone on a pale yellow background. The detection limits were 2 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the absorption maximum of 1-naphthol (λ_{max} = 460 nm, Fig. 1A) or at the absorption maximum of 2-naphthol (λ_{max} = 520 nm, Fig. 1B), as required.

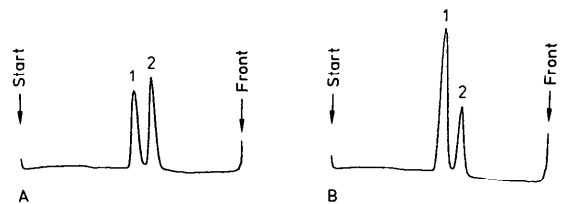


Fig. 1: Absorbance scan of a chromatogram track with 100 ng each of 2-naphthol (1) and 1-naphthol (2) per chromatogram zone: (A) scanned at λ = 460 nm, (B) scanned at λ = 520 nm.

References

[1] Hoffmann, A.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1987.
[2] Oka, H., Ikai, Y., Kawamura, N., Uno, K., Yamada, M., Harada, K.-I., Uchiyama, M., Asukabe, H., Suzuki, M.: *J. Chromatogr.* **1987**, *393*, 285–296.
[3] Oka, H., Uno, K., Harada, K.-I., Hayashi, M., Suzuki, M.: *J. Chromatogr.* **1984**, *295*, 129–139.

Iodine Reagents

It is very often advantageous in thin-layer chromatography to be able to obtain a preliminary impression of a substance separation by first exposing the plate to a rapidly carried out, economically priced universal reaction before passing on to final characterization using group-specific or even better substance-specific reactions.

Iodine is such a universal reagent. It was introduced by MANGOLD [1] as early as 1961 for the analysis of lipids and used again within a year by BARRETT [2] as a "nondestructive reagent".

Detection by iodine is usually based on physical concentration of iodine molecules in the lipophilic chromatogram zones without any reaction occurring. Iodine is more strongly enriched in the substance zones than in the neighboring polar, substance-free silica gel or alumina layer. The result is brown chromatogram zones on a yellow background [3].

Iodine is a less suitable reagent for use on moderately polar phases and RP materials. The chemical modification of the silica gel that such layers have undergone makes them considerably more lipophilic, so that the contrast between substance-coated chromatogram zone and substance-free background is not very strong. The same applies to polyamide layers.

Documentation is carried out as soon as the iodine-colored chromatogram zones can be readily recognized. Then the adsorbed iodine can be allowed to evaporate in the fume cupboard or vacuum desiccator, so that the same chromatograms can be subjected to further reactions and separation steps (e. g. SRS techniques, 2-D separations, coupling techniques such as TLC/GC etc.). The chromatogram zones can also be stabilized by spraying with 0.5 to 1 percent starch solution [4, 5]; the well-known blue clathrates that are formed (starch-iodine inclusion compounds) remain stable for months.

Some substances, e. g. penicillin and pyrazolinone derivatives, are poorly detected by "iodine staining" with detection limits of 2–4 μg substance per chromatogram zone [6, 7]. The limits for lipids and for opium alkaloids lie with 50–500 ng [8] in the middle nanogram range [9].

Exposure of rhodamine 6G-impregnated silica gel layers to iodine vapor for two to five minutes followed by irradiation with UV light leads to the sensitive blue coloration of the chromatogram zones on a greenish fluorescent background [8, 10].

In addition to the "iodine staining" resulting from adsorption or purely physical "solution" of the iodine molecules in the lipophilic chromatogram zones, many substances can also be made visible by chemical reaction with the iodine [9]. In such

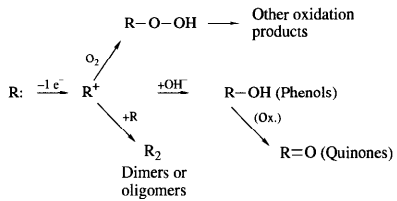
cases the interaction is not reversible since the iodine is covalently bound. It can readily be established whether there has been a chemical reaction between iodine and the test substances by application of the SRS technique [11].

Thus, in spite of its lack of reactivity, iodine reacts chemically with unsaturated compounds, whereby the silica gel of the TLC layer can sometimes be assigned a catalytic role [11, 12]. Irreversible oxidations and electrophilic substitution and addition reactions have been observed on the interaction of iodine with tertiary nitrogen compounds; such reactions possibly depend on particular steric relationships or are favored by particular functional groups [13, 14].

The "iodine reaction" is possibly a one-electron oxidation with the initial formation of a radical cation:

$$\underset{\text{Reactants}}{R: + 1/2\ I_2} \longrightarrow \underset{\text{Radical-cation}}{R^{\ddagger} + I^-}$$

This can then react in various ways. The following products can be derived schematically:



Charge transfer complexes can also be formed, as shown using a tertiary nitrogen compound as an example. An iodine molecule first adds to the nitrogen compound:

$$R_3N + I_2 \longrightarrow R_3N \cdot I_2$$

The complex that is formed can dissociate to form a cation (*n*-σ-complex) and an iodide anion, with the iodide ion reacting with the excess iodine molecules that are present. In addition the decomposition of the *n*-σ-complex can lead to the formation of highly reactive iodine cations, which can initiate further reactions – e.g. oxidations or electrophilic substitutions of aromatic systems [11, 13].

$$R_3N \cdot I_2 \longrightarrow \begin{array}{l} R_3\overset{+}{N}-I + I^- \\ \uparrow\downarrow \\ R_3N + I^+ \end{array}$$

In acidic media the *n*-σ-complex can also produce periodide anions or periodide complexes; these – like the iodide anion – are appreciably less reactive than the iodine cation [13].

$$R_3\overset{+}{N}-I + 2I^- + H^+ \rightleftharpoons \begin{array}{l} R_3\overset{+}{N}-H + I_3^- \\ R_3N \cdot HI_3 \end{array}$$

Table 1 lists examples of the observation and demonstration of such reactions.

Table 1: Examples of treatment with iodine leading to oxidation, addition or substitution products.

Substances	Sorbent	Remarks	References
Estrone derivatives	Silica gel GF_{254}	Iodination to 2-iodoestrone and 2,4-diiodoestrone	[12]
Polycyclic aromatic hydrocarbons, indole and quinoline derivatives, naphthylamines, azulenes	Silica gel G	Formation of oxidation products via the initially formed iodine complexes	[15]
Polycyclic aromatic hydrocarbons	Silica gel G	Monovalent oxidation of the iodine complexes via radical cations yields dimeric or tetrameric aromatics	[16]
Pyridine, pyrrole, indole, quinoline and isoquinoline alkaloids	Silica gel G	Detection by two-dimensional TLC	[11]
Emetine, cephaeline	Silica gel 60	After iodine treatment emetine fluoresces yellow and cephaeline blue	[17]

Table 1: (continued)

Substances	Sorbent	Remarks	References
Pharmaceuticals	Silica gel	Irreversible reaction of iodine with acetylsalicylic acid, aethaverine, amidopyrine, ascorbic acid, benzocaine, quinine, dihydrocodeine, fluorescein, glycine, hydrocortisone acetate, isoniazid, metamizole, papaverine, paracetamol, phenacetin, phenol-phthalein, piperazine, resorcinol, salicylic acid, salicylamide, sulfaguanidine, thymol, triethanolamine, tris buffer; detection by reaction chromatography	[18]
Quinine alkaloids (quinine, cinchonine), barbiturate derivatives, retinol, calciferol and cholecalciferol	Aluminium oxide pH 8.6	Elimination of vinyl groups, addition of iodine to double bonds, ester cleavage; detection by IR	[14]
Morphine, oxymorphone	Silica gel	Iodination	[19]
Opium alkaloids (morphine, codeine), acetylmorphine, oxycodeinone, brucine, phenylbutazone, ketazone, trimethazone	Aluminium oxide pH 8.6	Iodine addition to the tertiary nitrogen of the opium alkaloids and to the OCH_3 group of the brucine with formation of an *o*-quinone derivative, probably ring opening in the case of phenylbutazone, ketazone and trimethazone; detection by IR	[13]
Thiols and thioethers (dithiaden, prothiaden, thiamine etc.)	Aluminium oxide pH 8.3	Oxidation of sulfur and attack of the double bond in the thiazole ring	[20]
Alkaloids (codeine, brucine), phenothiazines (promethazine), sulfonamides (sulfathiazole), vitamins (axerophthol, cholecalciferol)	Aluminium oxide pH 8.6	Irreversible complex formation or saturation of double bonds; detection by IR	[21]

So a check must always be made of whether the universal iodine reagent can be used for nondestructive testing or whether the substances undergo irreversible changes. The iodine reagents are preferentially used for the detection of lipophilic substances (fats, waxes, PAH's etc.) and since this chapter would be inordinately long if all the substances were listed and the references cited the iodine monographs that follow only include those classes of compounds where the use of iodine as a detection reagent seems unusual.

References

[1] Mangold, H. K.: *J. Am. Oil Chem. Soc.* **1961**, *38*, 708–727.
[2] Barrett, G. C.: *Nature* **1962**, *194*, 1171–1172.
[3] Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin-layer Chromatography – Reagents and Detection Methods*, Vol. 1a, VCH-Verlagsgesellschaft, Weinheim, Cambridge, New York, 1990.
[4] Dutta, J., Das, A. K., Ghosh, R.: *J. Chromatogr.* **1981**, *210*, 544–549.
[5] Sliwiok, J., Kowalski, W. J.: *Microchem. J.* **1972**, *17*, 576–587.
[6] Pan, S. C.: *J. Chromatogr.* **1973**, *79.*, 251–255.
[7] Jain, R., Agarwal, D. D.: *J. Liq. Chromatogr.* **1982**, *5*, 1177–1179.
[8] Vroman, H. E., Baker, G.L.: *J. Chromatogr.* **1965**, *18*, 190–191.
[9] Copenhaver, J. H., Cronk, D. R., Carver, M. J.: *Microchem. J.* **1971**, *16*, 472–479.
[10] Milborrow, B. V.: *J. Chromatogr.* **1965**, *19*, 194–197.
[11] Wilk, M., Brill, U.: *Arch. Pharm.* **1968**, *301*, 282–287.
[12] Brown, W., Turner, A. B.: *J. Chromatogr.* **1967**, *26*, 518–519.
[13] Šaršúnová, M., Kakác, B., Krasnec, L.: *J. Chromatogr.* **1970**, *48*, 353–361.
[14] Šaršúnová, M., Kakác, B., Krasnec, L.: *Fresenius Z. Anal. Chem.* **1972**, *260*, 291–292.
[15] Wilk, M., Hoppe, U., Taupp, W., Rochlitz, J.: *J. Chromatogr.* **1967**, *27*, 311–316.
[16] Wilk, M., Bez, W., Rochlitz, J.: *Tetrahedron* **1966**, *22*, 2599–2608.
[17] Stahl, E.: *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch*, 2. Aufl., Springer, Berlin, Heidelberg, New Jork, 1967.
[18] Schmidt, F.: *Krankenhaus-Apoth.*: **1973**, *23*, 10–11.
[19] Barrett, G. C. in: Giddings, J. C., Keller, R. A. (Eds.) *Advances in Chromatography*, Vol. 11, p. 151–152, Marcel Dekker, New York, 1974.
[20] Šaršúnová, M., Kakác, B., Krasnec, L.: *Fresenius Z. Anal. Chem.* **1972**, *262*, 287–288.
[21] Šaršúnová, M., Kakác, B., Maly, V.: *Fresenius Z. Anal. Chem.* **1969**, *245*, 154.

Iodine Vapor Reagent

Reagent for:

- Lipids
  e.g. fats, waxes, hydrocarbons [1–4]
  free fatty acids, diglycerides [5], fatty acid esters [6],
  prostaglandins [7], arene-cyclopentadienyl iron complexes [8]
- Phospholipids
  e.g. lecithin [5, 9–11], sphingomyelin [10]
  phosphatidylcholine [12], dioleylphosphatidylcholine [13]
  ceramides [14], gangliosides [15]
- Steroids [16, 17]
  e.g. cortisone, testosterone, corticosterone [18]
  estrone [19], 19-norsteroids [20]
- Carotenoids [21]
- Antioxidants
  e.g. cyclopentylphenols [22]
- Hydroxyacetophenone [23] and benzophenone derivatives [24]
- N-containing glycolate esters [25]
- Diethyl phenyl phosphate derivatives [26]
- Detergents and emulsifiers
  e.g. dodecyl benzenesulfonate, Triton X-100 [27]
  alcohol ethoxilates [27, 28],
  arlacel A [29]
- Polymers
  e.g. polyethylene glycol derivatives [30, 31]
  polystyrene, polytetrahydrofuran [31]
- Pesticides
  e.g. carbamates [32]
  such as propamocarb [33]
  e.g. aziridine derivatives [34]
- Purine derivatives
  e.g. theophylline, caffeine [35, 36]

Reagent for:

- Pharmaceuticals
  e.g. antibiotics
  such as penicillins [37], maridomycines [38], ferrithiocin [39]
  e.g. amphetamines
  such as phentermine, methamphetamine, ephedrine [40]
  N-cyanobenzylamphetamine [41]
  e.g. benzodiazepines
  such as chlordiazepoxide [35], diazepam [35, 36]
  e.g. miscellaneous pharmaceuticals [42]
  such as propoxyphene [43], barbital [36], phenacetin, antipyrin [35],
  triamcinolone acetonide ester [44]
  germine, germine acetate and diacetate [45],
  xanthone [46], opium alkaloids [47], captopril [45]
- Mycotoxins
  e.g. slaframine [48]
- Carbohydrates [49]

I_2
$M_r = 253.81$

Preparation of the Reagent

Iodine reagent	Place a few iodine cystals on the base of a chamber that can be tightly sealed (e.g. twin-trough chamber). Violet iodine vaporizes and distributes itself homogeneously throughout the interior of the chamber after a few hours. Gentle warming of the iodine chamber accelerates the vaporization of the iodine.
Starch dipping solution	Dissolve 0.5 g soluble starch in 100 ml water with heating.
Storage	The iodine chamber should be stored in the fume cupboard.
Substances	Iodine Starch, soluble

Reaction

Iodine is enriched to a greater extent in chromatogram zones coated with lipophilic substances than it is in a hydrophilic environment. Hence, iodine is only physically "dissolved" or adsorbed. Occasionally a chemical reaction also takes place, such as, for example, with estrone [19] (cf. "Iodine Reagents"). In general it may be said that the longer the iodine effect lasts the more oxidations, additions or electrophilic substitutions are to be expected.

Method

The chromatograms are freed from mobile phase in a stream of warm air, cautiously placed in the iodine chamber and left there for varying periods of time, depending on the substance class (e.g. 5–10 s [1], 15–30 s [17, 50], 1 min [38], 5–15 min [11, 25] or even up to 30 min [22, 24] or for several hours [42].

Brown-violet chromatogram zones are generally formed on a yellow background and, in some cases, these fluoresce when viewed under UV light (λ = 254 or 365 nm) after the evaporation of the excess iodine. Sometimes colorless chromatogram zones are formed on a brown background, for example, if the iodine reacts chemically with the substances that are chromatographed.

Carotenoids immediately form an olive-green complex, which fades irreversibly if the exposure to iodine is prolonged [21].

Note: The iodine evaporates relatively quickly from the layer after a chromatogram has been removed from the iodine chamber. This evaporation of the iodine can be considerably delayed by covering the chromatogram with a glass plate; the edge can also be sealed with adhesive tape, if necessary. Iodine solution can also be used for detection instead of iodine vapor.

Since iodine possesses fluorescence-quenching properties (true fluorscence quencher) iodine-containing chromatogram zones on layers containing fluorecence indicator F_{254} appear as dark zones on a yellow-green fluorescent background when viewed under UV light (λ = 254 nm) – even if there are only traces of iodine in the chromatogram zones.

The chromatogram zones colored by iodine can be fixed later by treatment with a 0.5–1% aqueous starch (amylose) solution. This yields the well known, deep blue iodine-starch inclusion complex which is stable over a prolonged period. This reaction is very sensitive and, hence, should only be carried out if there are only traces of iodine in the chromatogram zones, otherwise the whole background will be colored blue.

Occasionally the starch treatment yields white chromatogram zones on a blue background (cf. procedure tested 2). This is probably a result of iodine being consumed by a chemical reaction with the zone leaving a large quantity of iodine in the background for the formation of a starch-iodine complex. Such effects are not observed on cellulose, CN and water-wetted RP 18 layers, possibly because insufficient adsorbed iodine is available in the neighborhood of the chromatogram zones for the formation of the blue complex or because, for example, the CN phase is not homogeneously wetted by the starch solution.

The detection limits are generally a few µg substance per chromatogram zone. However, the iodine detection is appreciably more sensitive for some substances: it is possible to detect 200 ng glucose [49] and 10 ng propamocarb per chromatogram zone [33].

The reagent can be used most advantageously on aluminium oxide, silica gel, kieselguhr, Si 50000, cellulose, diol and water-wettable RP 18 layers; there is less contrast in color on strongly hydrophobic RP 18 phases. NH_2 and polyamide layers are not suitable because the iodine is too strongly bound and the whole layer is colored green-yellow.

Procedure Tested 1

Fatty Oils [51]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates cellulose (MERCK) that have been impregnated by dipping in a solution of liquid paraffin – petroleum ether (5+95) (40–60 °C) for 4 s and then dried in the air for 5 min, before application of the samples.
Mobile phase	Acetic acid (99%)
Migration distance	12 cm
Running time	1.5 h

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed in an iodine chamber for 5 min. Brown chromatogram zones were

formed on a light brown background (Fig. 1A). After waiting for a few minutes while the excess iodine evaporated from the layer the chromatogram was immersed in the starch solution for 1 s and dried in a stream of warm air. It was then possible to recognize the oil components as blue chromatogram zones on a light background (Fig. 1B).

In situ quantitation: The reagent was not suitable for quantitative in situ evaluations.

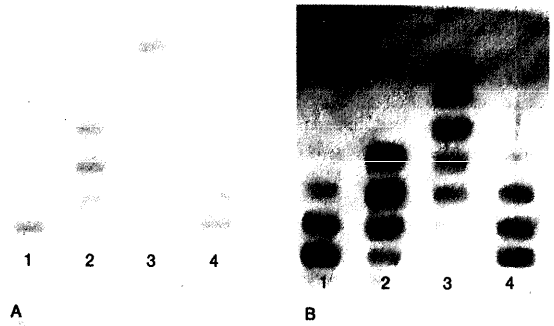


Fig. 1: Chromatogram of fatty oils (9 µg each per 10 mm band) after iodine vapor treatment (A) and after additional immersion in a starch solution (B): Track 1: avocado oil, Track 2: sunflower oil, Track 3: linseed oil, Track 4: almond oil.

Procedure Tested 2

Detergent Dehydol LS 3 [51]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 with concentrating zone (MERCK).
Mobile phase	1-Propanol – glacial acetic acid (90 + 10).

Migration distance	10 cm
Running time	1.75 h

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air and placed in an iodine chamber for 20 min. Brown chromatogram zones were formed on a pale yellow background (Fig. 2A); these faded very rapidly. Therefore, the chromatogram was immediately immersed for 1 s in the starch dipping solution and dried in a stream of cold air.

Pink-colored chromatogram zones appeared on a blue background; these rapidly changed color to white zones (Fig. 2B).

In situ quantitation: The reagent was not suitable for in situ evaluations.

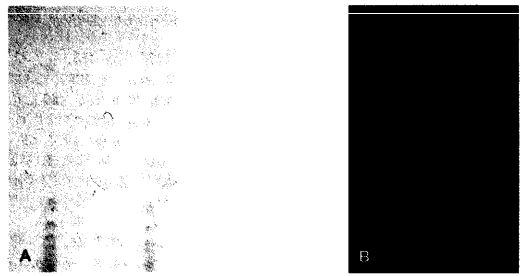


Fig. 2: Chromatogram of the detergent dehydrol LS 3 after iodine treatment (A) and after additional treatment with starch solution (B); amount applied each time 10 µg as spots.

References

[1] Eng, L. F., Lee, Y. L., Hayman, R. B., Gerstl, B.: *J. Lipid. Res.* **1964**, *5*, 128–130.
[2] Stahl, E.: *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch*. 2. Aufl., Springer, Berlin 1967.
[3] Getz, G. S., Jakovcic, S., Heywood, J., Frank, J., Rabinowitz, M.: *Biochem. Biophys. Acta* **1970**, *218*, 441–452.

[4] Andreev, L. V., Belyakovich, T. G.: *J. High Resolut. Chromatogr. Chromatogr. Commun* **1982**, *5*, 100–101.
[5] Nierle, W., El Bayá, A. W.: *Fette, Seifen, Anstrichm.* **1981**, *83*, 391–395.
[6] Vioque, E., Holman, R. T.: *J. Am. Oil Chem. Soc.* **1962**, *39*, 63–66.
[7] Korte, K., Casey, L.: *J. Liq. Chromatogr.* **1983**, *6*, 55–62.
[8] Azogu, C. I.: *J. Chromatogr.* **1981**, *219*, 349–351.
[9] Shiloah, J., Klibansky, C., de Vries, A., Berger, A.: *J. Lipid Res.* **1973**, *14*, 267–278.
[10] De Bohner, L. S., Soto, E. F., De Cohan, T.: *J. Chromatogr.* **1965**, *17*, 513–519.
[11] Kynast, G., Saling, E. Z.: *J. Perinat. Med.* **1973**, *1*, 213–218.
[12] Rivnay, B.: *J. Chromatogr.* **1984**, *294*, 303–315.
[13] Cserháti, T., Szögyi, M., Györfi, L.: *J. Chromatogr.* **1985**, *349*, 295–300.
[14] Sugita, M., Connolly, P., Dulaney, J. T., Moser, H. W.: *Lipids* **1973**, *8*, 401–406.
[15] Kerènyi, L., Kannan, R., Gielen, W., Debuch, H.: *Z. Klin. Chem. Klin. Biochem.* **1974**, *12*, 487–493.
[16] Markert, C., Betz, B., Träger, L.: *Z. Naturforsch.* **1975**, *30c*, *266–270.*
[17] Gänshirt, H. G., Polderman, J.: *J. Chromatogr.* **1964**, *16*, 510–518.
[18] Matthews, J. S., Pereda V., A.L., Aguilera P., A.: *J. Chromatogr.* **1962**, *9*, 331–338.
[19] Brown, W., Turner, A. B.: *J. Chromatogr.* **1967**, *26*, 518–519.
[20] Matlin. S. A.. Abderabbani, N.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1987**, *10*, 401–403.
[21] Wittgenstein, E., Sawicki, E.: *Mikrochim. Acta (Wien)* **1970**, 765–783.
[22] Atkins, T. M., Towles, D. G., Bishara, R. H.: *J. Liq. Chromatogr.* **1983**, *6*, 1171–1174.
[23] Chawla, H. M., Gambhir, I., Kathuria, L.: *J. Chromatogr.* **1980**, *188*, 289–291.
[24] Smith, S. L., Bishara, R. H., Drummond, G. E.: *J. Liq. Chromatogr.* **1981**, *4*, 2205–2212.
[25] Verweij, A., de Jong-de Vos, R., Teisman, H. G. J.: *J. Chromatogr.* **1972**, *69*, 407–410.
[26] Gandhe, B. R., Danikhel, R. K., Purnanand: *J. Chromatogr.* **1984**, *288*, 233–235.
[27] Armstrong, D. W., Stine, G. Y.: *J. Liq. Chromatogr.* **1983**, *6*, 23–33.
[28] Hellmann, H.: *Fresenius Z. Anal. Chem.* **1983**, *315*, 612–617.
[29] Berlin, B. S., Wyman, R.: *Proc. Soc. exp. Biol. Med.* **1971**, *136*, 1363–1368.
[30] Favretto, L., Gabrielli, L. F., Marletta, G. P.: *J. Chromatogr.* **1972**, *66*, 167–171.
[31] Armstrong, D. W., Bui, K. H., Boehm, R. E.: *J. Liq. Chromatogr.* **1983**, *6*, 1–22.
[32] Srivastava, S. P., Reena: *J. Liq. Chromatogr.* **1983**, *6*, 139–143.
[33] Gentile, I. A., Passera, E.: *J. Chromatogr.* **1982**, *236*, 254–257.
[34] Holloway, S. J., Scott, J. G., Casida, J. E., Ruzo, L. O.: *J. Agric. Food Chem.* **1986**, *34*, 1057–1060.
[35] Yuen, S., Lau-Cam, C. A.: *J. Chromatogr.* **1985**, *329*, 107–112.
[36] Gonnet, C., Marichy, M.: *J. Liq. Chromatogr.* **1980**, *3*, 1901–1912.
[37] Hendrickx, S., Roets, E., Hoogmartens, J., Vanderhaeghe, H.: *J. Chromatogr.* **1984**, *291*, 211–218.
[38] Kondo, K.: *J. Chromatogr.* **1980**, *190*, 493–497.
[39] Naegeli, H.-U., Zähner, H.: *Helv. Chim. Acta* **1980**, *63*, 1400–1406.
[40] Cartoni, G. P., Lederer, M., Polidori, F.: *J. Chromatogr.* **1972**, *71*, 370–375.
[41] Salvesen, B., Testa, B., Beckett, A. H.: *Arzneim. Forsch.* **1974**, *24*, 137–140.
[42] Schmidt, N.: *Die Krankenhausapotheke* **1973**, *23*, 10–11.
[43] Souter, R. W., Jensen, E. C.: *J. Chromatogr.* **1983**, *281*, 386–388.
[44] Diamanti, E., Bianchi, G. E.: *Arzneim. Forsch.* **1971**, *21*, 251–252.
[45] Migdalof, B. H., Singhvi, S. M., Kripalani, K. J.: *J. Liq. Chromatogr.* **1980**, *3*, 857–865.

[46] Ghosal, S., Sharma, P. V., Chaudhuri, R. K., Bhattacharya, S. K.: *J. Pharmac. Sci.* **1973**, *62*, 926–930.
[47] Szigeti, J., Mezey, G., Bulyáki, M.: *Acta Pharm. Hung.* **1984**, *54*, 58–63.
[48] Stahr, H. M.: *J. Liq. Chromatogr.* **1983**, *6*, 123–126.
[49] Wahl, R., Maasch, H. J., Geissler, W.: *J. Chromatogr.* **1985**, *329*, 153–159.
[50] Yoshizawa, I., Fukushima, K., Foldes, F. F.: *Arzneim. Forsch.* **1980**, *30*, 928–932.
[51] Meiers, Bl., Jork, H.: GDCh-training course No. 302 „Möglichkeiten der quantitativen Auswertung von Dünnschicht-Chromatogrammen", Universität des Saarlandes, Saarbrücken, 1992.

Iodine Solution, Neutral Reagent

Reagent for:

- Lipids (fats, waxes, hydrocarbons)
  e.g. nitrobenzylarenes [1], cholesteryl esters [2, 3], lipopurothionine [4]
  mono- and diglycerides [5]
  unsaturated lipids [6]
- Phospholipids
  e.g. phosphatidylcholine [7], phosphatidic acid [5]
  phosphonolipids [8, 9]
- Polymers
  e.g. polyisoprene, polybutadiene [10]
  poly(methylmethacrylate) [10, 11]
  polyethylene glycols [12]
- Pyrimidine nucleoside derivatives
  e.g. uridine derivatives [13]
- Alkaloids
  e.g. codeine [14], emetine, cephaeline [15, 16]
  psychotrine [16], hippadine [17]
- Pharmaceuticals [18]
  e.g. benzodiazepines [19], thalidomide [20]
  dithiocarbamoylhydrazine [21], neostigmine [14]
  4-amidinophenylpyruvic acid [22]
  6-mercaptopurine derivatives [23]
  cnicine, artemisiifolin, salonitenolide [24]
- Tertiary amines and quaternary ammonium compounds [25-27]
- Pyrrolidine derivatives [28]
- Imidazole derivatives [29]
- Pentoxifylline [30]

I_2
$M_r = 253.81$

Preparation of the Reagent

Dipping solution Dissolve 250 mg iodine in 100 ml petroleum ether [31].

Spray solution Dissolve 1 to 5 g iodine in 100 ml methanol [2, 3, 5-7, 11, 12, 19], chloroform [1, 4, 16, 18, 20, 24], carbon tetrachloride [15, 28, 29], petroleum ether [17], diethyl ether [26] or acetone [30].

Storage The reagent solutions may be stored in the refrigerator for an extended period.

Substances
Iodine
Methanol
Chloroform
Carbon tetrachloride
Petroleum ether
Diethyl ether
Acetone

Reaction

Iodine is enriched to a greater extent in chromatogram zones with a coating of lipophilic substances than it is in a hydrophilic environment. Hence iodine is only physically "dissolved" or adsorbed. A chemical reaction occasionally takes place too (cf. "Iodine Reagents").

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution. Only in exceptional cases, such as, for example, in the detection of emetine and cephaeline, are the chromatograms then heated to 60–80 °C for 10–20 min [15, 16, 31].

Brown chromatogram zones are generally produced on an almost colorless to pale beige background; the zones occasionally fluoresce when examined under UV light (λ = 254 or 365 nm) [15, 16].

Note: The last traces of mobile phase must be removed completely – if necessary by heating the chromatogram – before the reagent is applied [10]. For documentation it is recommended that the chromatogram treated with iodine be covered with a glass plate in order to prevent evaporation of the iodine.

An aqucous 1% starch (amylose) spray can be sprayed on later to intensify the color contrast between the chromatogram zones and the layer background; the well known blue-colored iodine starch inclusion complexes are formed. This later treatment with starch solution should only be carried out when the iodine excess has evaporated from the layer background so that only traces of iodine remain in the chromatogram zones. Otherwise the whole chromatogram will be colored dark blue (test at a corner of the chromatogram!).

Iodine vapour can be employed as reagent in place of the iodine solution. Water-resistant layers can be treated with 0.5 to 1% aqueous iodine solutions, e.g. 1 g iodine and 2 g potassium iodide dissolved in 30 ml water and made up to 100 ml with ethanol or LUGOL's solution or dilute iodine tincture. These solutions all contain in addition potassium iodide to form the stable complex $KI \cdot I_2$ with the iodine. This greatly reduces the evaporation of the iodine from the chromatogram so that later treatment of the chromatogram with the starch solution can lead to a complete blue coloration of the whole chromatogram, even after an extended period of waiting. Such a potassium iodide-containing iodine solution has been employed for example for the detection of choline [32].

In the case of quaternary ammonium compounds the chromatograms can be treated afterwards with sodium nitrite solution in order to intensify the color [27].

The detection limits are mostly in the microgram range, for cephaeline and emetine they are 200 pg substance per chromatogram zone.

The reagent can be used, for instance, on aluminium oxide, silica gel, kieselguhr and Si 50000 as well as on cellulose, polyamide, RP, CN, Diol and NH_2 phases.

Procedure Tested

Alkaloids in *Ipecacuanha* Tincture [31]

Method	Ascending, one-dimensional, two-fold development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).

Mobile phase	Dichloromethane – methanol (85+15).
Migration distance	2 x 7 cm
Running time	2 x 15 min

Detection and result: The chromatogram was dried in a stream of warm air for 5 min, immersed in the dipping solution for 3 s and then, after brief evaporation of the excess iodine, heated to 60 °C on a hot plate for ca. 10 min. After cooling to room temperature it was placed for a further 5 min in the vacant trough of a twin-trough chamber, whose second chamber had been filled with 25% ammonia solution.

Cephaeline (hR_f 6–11) appeared as blue and emetine (hR_f 10–15) as yellow fluorescent chromatogram zones on a dark background when examined under long-wavelength UV light (λ = 365 nm) (Fig. 1). Emetine – like cephaeline – also yielded a blue fluorescence on RP-2 and RP-18 layers.

The detection limit was ca. 200 pg for emetine.

Note: Under the conditions employed emetine and cephaeline were not well separated but there was good resolution of the subsidiary alkaloids of the *ipecacuanha* tincture (Fig. 1). The separation and quantitative determination of the main alkaloids (Fig. 2) can be carried out under the following conditions: Ascending, one-dimensional development in a trough chamber with chamber saturation; layer: HPTLC plates Silica gel 60 (MERCK); mobile phase: dichloromethane – methanol – ammonia solution (25%) (34+6+1); migration distance: 6 cm; running time: 13 min; hR_f: cephaeline 65–70; emetine 75–80.

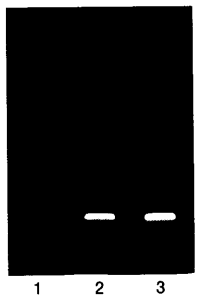


Fig. 1: Chromatogram of *ipecacuahna* tincture and of the standard substances: 1 = emetine, 2 = tincture, 3 = cephaeline.

In situ quantitation: For fluorimetric evaluation excitation was carried out at λ_{exc} = 313 nm and the fluorescence emission of emetine was measured at $\lambda_{fl} > 460$ nm (cut off filter Fl 46).

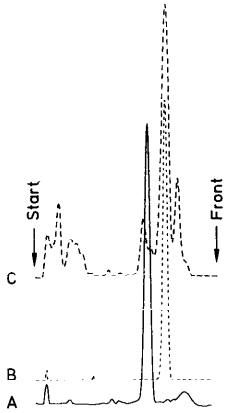


Fig. 2: Fluorescence scan of the chromatogram tracks of the standard substances cephaeline (A) and emetine (B) and of the *ipecacuanha* extract (C). Amounts applied: cephaeline 0.5 µg, emetine 0.7 µg per 10 mm track length.

References

[1] Klemm, D., Klemm, E.: *J. Chromatogr.* **1980**, *188*, 448–451.
[2] Fex, G., Wallinder, L.:*Biochem. Biophys. Acta* **1970**, *210*, 341–343.
[3] Owens, K., Hughes, B. P.: *J. Lipid Res.* **1970**, *11*, 486–495.
[4] Hernandez-Lucas, C., Fernandez de Caleya, R., Carbonero, P., Garcia-Olmedo, F.: *J. Agric. Food Chem.* **1977**, *25*, 1287–1289.
[5] Possmayer, F., Mudd, J. B.: *Biochem. Biophys. Acta* **1971**, *239*, 217–233.
[6] Takayama, K., Schnoes, H. K., Semmler, E. J.: *Biochem. Biophys. Acta* **1973**, *316*, 212–221.
[7] Devor, K. A., Mudd, J. B.: *J. Lipid Res.* **1971**, *12*, 403–411, 412–419.
[8] Moschidis, M. C., Demopoulos, C. A., Kritikou, L. G.: *J. Chromatogr.* **1984**, *292*, 473–478.
[9] Moschidis, M. C.: *J. Chromatogr.* **1983**, *268*, 485–492.
[10] Armstrong, D. W., Bui, K. H., Boehm, R. E.: *J. Liq. Chromatogr.* **1983**, *6*, 1–22.
[11] Bui, K. H., Armstrong, D. W.: *J. Liq. Chromatogr.* **1984**, *7*, 45–58.

[12] Bui, K. H., Armstrong, D. W.: *J. Liq. Chromatogr.* **1984**, *7*, 29–43.
[13] Pischel, H., Holy, A., Wagner, G.: *Collect. Czech. Chem. Commun.* **1981**, *46*, 933–940.
[14] Giebelmann, R.: *Zbl. Pharm.* **1976**, *115*, 485–490.
[15] Habib, M. S., Harkiss, K. J.: *Planta Med.* **1970**, *18*, 270–274.
[16] Stahl, E., Willing, E.: *Planta Med.* **1978**, *34*, 192–202.
[17] El Moghazi, A. M., Ali, A. A.: *Planta Med.* **1976**, *29*, 156–159.
[18] Grady, L. T., Hays, S. E., King, R. H., Klein, H. R., Mader, W. J., Wyatt, D. K., Zimmerer, R. O.: *J. Pharmac. Sci.* **1973**, *62*, 456–464.
[19] Šoljić, Z., Grba, V., Bešić, J.: *Chromatographia* **1977**, *10*, 751–752.
[20] Pischek, G., Kaiser, E., Koch, H.: *Mikrochim. Acta (Wien)* **1970**, 530–535.
[21] Olthoff, U., Matthey, K.: *Pharmazie* **1974**, *29*, 20–25.
[22] Richter, P., Wagner, G., Kazmirowski, H.-G., Usbeck, H.: *Pharmazie* **1976**, *31*, 279–282.
[23] Wagner, G., Oehlke, J., Pischel, H.: *Pharmazie* **1974**, *29*, 160–164.
[24] Vanhaelen-Fastré, R., Vanhaelen, M.: *Planta Med.* **1974**, *26*, 375–379.
[25] Giebelmann, R.: *Zbl. Pharm.* **1976**, *115*, 491–497.
[26] Snyder, L. R., Palamareva, M. D., Kurtev, B. J., Viteva, L. Z., Stefanovsky, J. N.: *J. Chromatogr.* **1986**, *354*, 107–118.
[27] McLean, W. F. H., Jewers, K.: *J. Chromatogr.* **1972**, *74*, 297–302.
[28] Abdulla, R. F., Lahiri, S. K., Crabb, T. A., Cahill, R.: *Z. Naturforsch.* **1971**, *26b*, 95–98.
[29] Van Balgooy, J. N. A., Marshall, F. D., Roberts, E.: *J. Neurochem.* **1972**, *19*, 2341–2353.
[30] Smith, R., Yang, S.-K., Davis, P., Bauza, M.: *J. Chromatogr.* **1983**, *281*, 281–287.
[31] Meiers, B., Jork, H.: GDCh-training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1992.
[32] Shiloah, J., Klibansky, C., de Vries, A., Berger, A.: *J. Lipid Res.* **1973**, *14*, 267–278.

Iodine–Potassium Iodide Solution, Acidic Reagent

Reagent for:

- Alkaloids [1]
- Polyethylene glycols [1]
- Pharmaceuticals
  - e.g. tertiary nitrogen compounds
    - such as phenothiazines [2], phenylalkylamines, chlorphentermine, cloforex [3]
  - e.g. antibiotics
    - such as ampicillin and oligomers [4]
    - penicillin [5]
  - e.g. purine derivatives
    - such as caffeine, theobromine, theophylline [6–8]
- Pesticides
  - e.g. fungicides
    - such as ridomil [9]

I_2	KI
$M_r = 253.81$	$M_r = 166.01$

Preparation of the Reagent

Dipping solution Dissolve 0.4 g potassium iodide and 0.8 g iodine in 40 ml 96% ethanol and add 10 ml 25% hydrochloric acid [8].

Spray solution I *For alkaloids:* Dissolve 1 g iodine and 10 g potassium iodide in 50 ml water with warming, add 2 ml glacial acetic acid and make up to 100 ml with water [1].

Spray solution II *For purine derivatives:*
Solution A: Dissolve 0.1 g potassium iodide and 0.2 g iodine in 10 ml 96% ethanol [6, 7].
Solution B: Mix 5 ml 25% hydrochloric acid with 5 ml 96% ethanol [6, 7].

Spray solution III *For antibiotics:*
Mix 100 ml 1% starch solution with 8 ml acetic acid and 1 ml 0.1 N iodine solution [4].

Storage The reagent solutions may be stored for an extended period.

Substances
Iodine
Potassium iodide
Hydrochloric acid (25%)
Ethanol (96%)
Acetic acid (100%)
Starch, soluble

Reaction

Tertiary nitrogen and iodine initially form a *n*-σ-complex, from which a strongly reactive iodine cation is produced; this cation can bring about electrophilic substitutions on aromatic systems or cause oxidations [2].

$$R_3N + I_2 \longrightarrow R_3\overset{+}{N}-I + I^- \quad \uparrow\downarrow \quad R_3N: + I^+$$

In acidic medium the *n*-σ-complex can also form periodide anions or periodide complexes and these – like the iodide anion – are appreciably less reactive than an iodine cation [2].

$$R_3\overset{+}{N}-I + 2I^- + H^+ \rightleftharpoons \begin{matrix} R_3\overset{+}{N}-H + I_3^- \\ R_3N + HI_3^- \end{matrix}$$

The detection of antibiotics depends on the fact that the iodine contained in the reagent reacts chemically with these and, hence, is no longer available in the chroma-

togram zones for the formation of the deep blue-colored iodine-starch inclusion compound. In the case of penicillin derivatives the ß-lactam ring is initially opened by alkali treatment or with suitable enzymes [10, 11]. The penicilloic acid thus formed reacts rapidly consuming 9 equivalents of iodine [10].

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s or homogeneously sprayed with the suitable spray solution and then dried in air or in a stream of cold air. Alternatively purine derivatives can be treated successively with the two spray solutions IIA and IIB (with 2 min air drying in between) [6, 7].

Spray solution III, which is suitable for the detection of antibiotics, also contains potassium iodide! To detect penicillin derivatives the chromatograms must first be placed – while still damp with mobile phase – for ca. 15 min in an ammonia chamber before treatment with spray solution III [5].

This generally produces red-brown to blue-violet chromatogram zones on a sand-colored background. The detection of antibiotics with spray solution III yields almost colorless chromatogram zones on a blue background [4, 5].

Note: The dipping solution can also be used as spray solution. Since the chromatogram zones slowly fade in the air it is recommended that the chromatograms be covered with a glass plate for long-term storage. Color differentiation is possible with purine derivatives [6, 8]. Diprophylline is not colored [6].

The subsequent treatment with starch solution frequently employed after the iodine treatment for the stabilization and enhancement of the „iodine" chromatogram zones cannot be employed here since the layers – even after lying in the air for several hours (evaporation of the excess iodine) – still contain so much iodine that the whole background is colored blue [8].

The detection limits for purine derivatives are 120–400 ng and for antibiotics 50 ng substance per chromatogram zone [4].

The reagent can, for instance, be employed on silica gel, kieselguhr, Si 50000, cellulose and NH_2 layers; the reaction is appreciably less sensitive on RP 18, CN and Diol phases, neither is there any color differentiation of the purine derivatives [8]. Cellulose and polyamide layers are not suitable, since the whole layer background is colored dark brown [8].

Procedure Tested

Purine Derivatives [6, 8]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Acetone – ethyl acetate – ammonia solution (25%) (75+23+2).
Migration distance	6 cm
Running time	15 min

Detection and result: The chromatogram was dried in a stream of warm air, immersed in the dipping solution for 2 s, dried in the air for 2 min and then dried for a further 2 min in a stream of cold air. Theophylline (hR_f 15–20) and caffeine (hR_f 65–70) were immediately visible as blue-violet chromatogram zones, while etophylline (hR_f 40–45) and proxiphylline (hR_f 60–65) appeared a little later as brown zones on a sand-colored background (Fig. 1A). The detection limits for theophylline and caffeine lay at 120 ng substance and for etophylline and proxiphylline at 400 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric scans were carried out in reflectance at a wavelength of $\lambda = 500$ nm (Fig. 1B).

Fig. 1: A) Chromatogram of xanthine derivatives: Track 1: theophylline, Track 2: proxiphylline, Track 3: mixture, Track 4: etophylline, Track 5: caffeine.
B) Reflectance scan of a chromatogram track with 1.7 µg theophylline (1), 3 µg each etophylline (2) and proxiphylline (3) and 1.6 µg caffeine (4) per chromatogram zone.

References

[1] E. Merck, Company brochure *Dyeing reagents for thin-layer and paper chromatography*, Darmstadt 1980.
[2] Šaršúnová, M., Kakác, B., Krasnec, L.: *J. Chromatogr.* **1970**, *48*, 353–361.
[3] Bülow, M., Dell, H.-D., Fiedler, J., Kamp, R., Lorenz, D.: *Arzneim. Forsch.* **1971**, *21*, 86–93.
[4] Larsen, C., Johansen, M.: *J. Chromatogr.* **1982**, *246*, 360–362.
[5] Bird, A. E., Marshall, A. C.: *J. Chromatogr.* **1971**, *63*, 313–319
[6] Pachaly, P.: *DC-Atlas – Dünnschicht-Chromatographie in der Apotheke*, p. 16, Wissenschaftliche Verlagsanstalt, Stuttgart, 1991.
[7] Kraus, L.: *Kleines Praktikumsbuch der Dünnschicht-Chromatographie*, p. 74, Desaga, Heidelberg, 1985.
[8] Meiers, Bl., Wagner, J., Jork, H.: GDCh training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1992.
[9] Singh, U. S., Tripathi, R. K.: *J. Chromatogr.* **1980**, *200*, 317–323.
[10] Thomas, R.: *Nature* **1961**, *191*, 1161–1163.
[11] Pan, S. C.: *J. Chromatogr.* **1973**, *79*, 251–255.

Iodine–Potassium Iodide Solution–Sodium Azide–Starch Reagent (Awe's Reagent, Iodine Azide Reaction)

Reagent for:

- Sulfur-containing compounds [1–3]
  e.g. thiols [4, 5] and thioethers [5]
  sulfide ions [4]
  thiourea derivatives
  such as phenylethyl- and 4-pentenylthiourea [4]
  N-ethyl-N′-benzylthiourea [6]
  oxazolidinethione derivatives [4]
  thiazolidine derivatives [7]
  S-containing amino acids
  such as cystine, methionine [3], cysteine [8]
  thiohydantoin derivatives [17], PTH amino acids [9–12]
- Penicillin derivatives and cephalosporins
  e.g. penicillic acid, oxacillin, clonacillin,
  methicillin, ampicillin, ceporin, cephalosporin C [8]
- Thiophosphorus compounds
  e.g. phosphordithioates, disulfides, alkylphosphinsulfides [5]
- Pesticides
  e.g. thiophosphoric acid insecticides [5]
  1,4-oxathiine derivatives [13]
  ridomil (acylon) [14]

I_2	NaN_3	$(C_6H_{10}O_5)_n$
$M_r = 253.81$	M_r 65.01	$M_r = 6500–8000$
Iodine	Sodium azide	Starch

Preparation of the Reagent

Dipping solution I Dissolve 0.5 g soluble starch in 100 ml water by heating to boiling [15].

Dipping solution II Dissolve 0.25 g iodine and 0.4 g potassium iodide in 100 ml water. Dissolve 0.4 g sodium azide in this solution [15].

Spray solution II Dissolve 1–3 g sodium azide in 100 ml 0.005 N [8] to 0.1 N iodine solution [1, 13] and dilute with 100 ml ethanol, if necessary [6]. Alternatively, a solution of 1.27 g iodine in 100 ml 95% ethanol can be mixed with a solution of 3.25 g sodium azide in 75% ethanol in the volume ratio 1+1, immediately before spraying [14].

Storage Dipping solution I may be stored for an extended period; dipping solution and spray solution II should be stored in the refrigerator and made up fresh every other day [11].

Substances
Iodine
Sodium azide
Potassium iodide
Iodine solution 0.05 mol I_2/L (= 0.1 N sol'n)
Starch, soluble
Ethanol

Reaction

The detection depends on the "iodine azide reaction" that normally takes place very slowly and during the course of which sodium azide reacts with iodine to form sodium iodide with the production of nitrogen:

$$I_2 + 2NaN_3 \longrightarrow 2NaI + 3N_2$$

This reaction is catalyzed by the presence of divalent sulfur – e.g. sulfur ions, substances with S-S links, thioethers, thiazoles. If such substances are present in a chromatogram zone then the iodine applied with the reagent is consumed by reaction with sodium azide according to the above reaction. It is no longer available for the formation of the deep blue-colored iodine starch inclusion compound (clathrate complex) with the starch solution that is also applied [3, 13, 16].

Method

The chromatograms are freed from mobile phase in a stream of warm air, initially immersed in dipping solution I for 1 s or sprayed homogeneously with it and then, after brief intermediate drying in a stream of cold air, either immersed in dipping solution II for 1 s or homogeneously sprayed with spray solution II.

In the case of penicillins and cephalosporins the chromatograms are first sprayed with 2 N sodium hydroxide solution [8].

This yields colorless to slightly yellow chromatogram zones on a deep blue-colored background.

Note: Alternatively 1% solutions of starch, iodine and sodium azide may be sprayed successively onto the chromatogram in that order [4, 9]. Other orders of application are also referred to in the literature [1, 2, 17] and sometimes the starch is also worked into the layer so that it is not necessary to spray with it [11, 12]. Sometimes the treatment of the chromatograms with starch solution is omitted [5, 6, 14]; in such cases colorless chromatogram zones appear on a brown layer background.

However, the starch solution should not be omitted completely since the color difference between the chromatogram zones, in which the iodine is reduced to colorless iodide according to the "iodine azide reaction" mentioned above, and the background colored brown by unreacted iodine is considerably less than the difference in color between the deep blue background provided by the starch-iodine clathrate complex and the pale chromatogram zones.

The blue coloration of the background rapidly changes to brown [11] and then gradually fades [8]. The color change of the background from blue to brown on complete drying of the layer is reversible: The blue color can be regenerated by treatment with water vapor [15].

Ridomil, with which the treatment with starch solution was not carried out, is said to yield brown chromatogram zones on a pale yellow background [14]. Hence, it may be assumed that this detection is based not on the "iodine azide reaction" but on the physical adsorption and enrichment of iodine in the lipophilic chromatogram zones (cf. "Iodine Reagents").

The detection limits for ridomil are 2.5 µg, for penicillin derivatives and cephalosporins 0.5–1 µg [8], for thiophosphate compounds 30 ng – 10 µg and for PTH amino acids and 1,4-oxathiine derivatives 200 ng substance per chromatogram zone [11, 13].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, NH_2 and Diol layers; RP-18W, CN, polyamide and cellulose phases are not suitable [15].

Procedure Tested

Thiophosphate Insecticides [15]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	*n*-Hexane – tetrahydrofuran (5+1).
Migration distance	7 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air, immersed in dipping solution I for 1 s and then dried for 5–10 min in a stream of warm air. Then the cooled TLC plate was immersed for 1 s in dipping solution II.

Azinphos ethyl (hR_f 20–25), malathion (hR_f 40–45) and diazinone (hR_f 47–52) yielded white chromatogram zones on a blue background immediately. Before in situ quantitation the chromatogram was dried in the air until no film of moisture could be seen on the layer surface. It was then dried completely in a stream of warm air whereby the blue coloration of the background changed to brown (Fig. 1). The visual detection limits were 200 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric measurements in reflectance were made at a wavelength $\lambda = 590$ nm (Fig. 2).

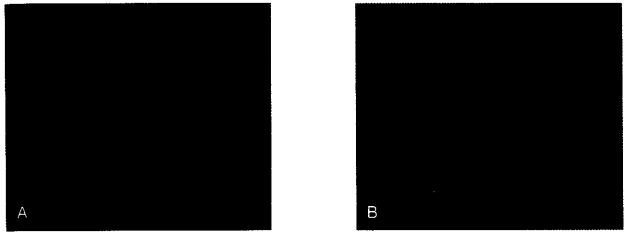


Fig. 1: Chromatogram of the thiophosphate insecticides (each ca. 500 ng) after treatment with dipping solutions I and II (A) before and (B) after complete drying of the TLC plate. Tracks 1 and 5: mixture; Track 2: azinphos ethyl; Track 3: malathion; Track 4: diazinone.

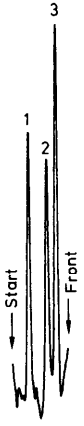


Fig. 2: Reflectance scan of a chromatogram track with 500 ng azinphos ethyl (1), 580 ng malathion (2) and 590 ng diazinone (3) per chromatogram zone.

References

[1] Stahl, E: *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch.* 2. Aufl., Springer, Berlin, Heidelberg, New York 1967.
[2] E. Merck, Company brochure *Dyeing reagents for thin-layer and paper chromatography,* Darmstadt 1980.
[3] Awe, W., Reinecke, I., Thum, J.: *Die Naturwissenschaften* **1954**, *41*, 528.
[4] Daun, J. K., Hougen, F. W.: *J. Am. Oil Chem. Soc.* **1977**, *54*, 351–354.
[5] Kudzin, Z. H., Kotynski, A., Kielbasinski, P.: *J. Chromatogr.* **1991**, *588*, 307–313.
[6] Martin, D., Würbach, G., Hilgetag, G.: *Pharmazie* **1971**, *26*, 15–17.
[7] Hüttenrauch, R., Olthoff, U.: *Pharmazie* **1973**, *28*, 523–526.
[8] Vandamme, E. J., Voets, J. P.: *J. Chromatogr.* **1972**, *71*, 141–148.
[9] King, T. P., Spencer, M.: *J. Biol. Chem.* **1970**, *245*, 6134–6148.
[10] Eyl, A., Inagami, T.: *Biochem. Biophys. Res. Commun.* **1970**, *38*, 149–155.
[11] Solal, M. C., Bernard, J. L.: *J. Chromatogr.* **1973**, *80*, 140–143.
[12] Boigne, J. M., Boigne, N., Rosa, J.: *J. Chromatogr.* **1970**, *47*, 238–246.
[13] Onuska, F. I., Comba, M. E.: *J. Chromatogr.* **1974**, *100*, 247–248.
[14] Singh, U. S., Tripathi, R. K.: *J. Chromatogr.* **1980**, *200*, 317–323.
[15] Meiers, Bl., Jork, H.: GDCh-training course No. 302 „Möglichkeiten der quantitativen Auswertung von Dünnschicht-Chromatogrammen", Universität des Saarlandes, Saarbrücken, 1992.
[16] Awe, W., Neuwald, F., Ulex, G. A.: *Die Naturwissenschaften* **1954**, *41*, 528.
[17] Mahapatra, G. N., Das, H. P.: *J. Chromatogr.* **1981**, *210*, 188–190.

Iron(III) Chloride Reagent

Reagent for:

- Phenols [1–7]
  e.g. flavonoid glycosides and aglycones [8–14]
  catechols [15, 16]
  tannins [17–19]
  such as gallic acid and its esters [16]
  phenolic pharmaceuticals [20]
  such as phenothiazines [20]
  acetylsalicylic acid [22]
  hydroxycinnamic acid derivatives
  such as caffeic acid, chlorogenic acid [23]
  1-hydroxyacridone alkaloids [24]
  gentisyl alcohol [25], gentisyl acid [20]
  fungal toxins
  such as orellanin, orellinin, orellin [26]
  mycophenolic acid [27]
- Enols
  e.g. α-ketolactones [28]
- Hydroxamic acids [7, 29]
- Mycotoxins
  e.g. penitrem A [30]
- Cholesterol and its esters [34]
- Ergot alkaloids [35]
- Inorganic anions
  e.g. nitrite, iodate, chromate, vanadate, selenite, selenate, hexacyanoferrate(II) and (III) ions [31]
  thiocyanate (rhodanide) ions [32]

$FeCl_3 \cdot 6H_2O$

$M_r = 270.30$

Preparation of the Reagent

Dipping solution Dissolve 1 g iron(III) chloride hexahydrate in 5 ml water and make up to 100 ml with ethanol [7].

Spray solution Dissolve 0.1 to 10 g iron(III) chloride hexahydrate in 100 ml ethanol [9, 17, 19, 21, 24], methanol [3, 4, 8, 15, 23, 27], 1-butanol [30], water [5, 20, 31, 32] or dilute hydrochloric acid [7, 29].

Storage The reagent solutions may be stored in the refrigerator (4 °C) for several days.

Substances Iron(III) chloride, hexahydrate
Methanol
Ethanol (95%)

Reaction

Iron(III) chloride forms colored complexes with phenols.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution and then heated to 100–110 °C for 5–10 min.

Variously colored chromatogram zones are produced, usually before heating, on a colorless to pale beige-yellow background.

Flavanones appear red to blue-violet [8], other flavonoid glycosides green, red-brown, wine red and red to blue-violet [11, 13], catechols green to blue [15], tannins blue [17], phenothiazines pink [21], 1-hydroxyacridone alkaloids green [24], penitrem A green to blue-green [30], inorganic anions pale yellow to blue-green [31].

Note: Dilute acetic acid [22], conc. sulfuric acid [25, 34, 35] have also been recommended for making up the reagent, as have anhydrous iron(III) chloride in dioxane or chloroform [5]. A post-reaction treatment with saturated sodium carbonate solution was described in some cases [23].

The detection limits for phenols are 20–100 ng substance per chromatogram zone [33].

The reagent can, for instance, be employed on silica gel, kieselguhr, Si 50000, cellulose and polyamide layers.

Procedure Tested

Aminophenols [33]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer HPTLC plates Silica gel 60 F_{254} (MERCK) that have been precleaned by developing once to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.

Mobile phase Toluene – methanol (18+9).

Migration distance 5 cm

Running time 10 min

Detection and result: The chromatogram was dried in a stream of warm air for 10 min, immersed in the dipping solution for 1 s and then heated to 110 °C for 5 min on a hotplate.

On a beige-yellow background 2-aminophenol (hR_f 55–60) appeared as ochre brown, 4-aminophenol (hR_f 40–45) as violet-brown and 4-aminosalicylic acid (hR_f 20–25) as pale brown-violet chromatogram zones.

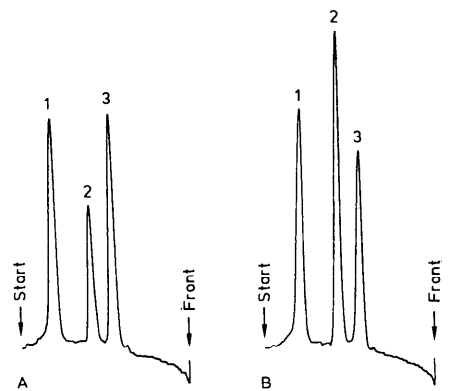
The detection limits lay at 20 ng substance for 2-aminophenol, 50 ng substance for 4-aminosalicylic acid and 100 ng substance for 4-aminophenol per chromatogram zone.

In situ quantitation: The absorption photometric scans were carried out in reflectance at a wavelength of either $\lambda = 460$ nm ($\lambda_{max\ (2\text{-aminophenol})}$) or $\lambda = 520$ nm ($\lambda_{max\ (4\text{-aminophenol})}$) (Fig. 1).

Fig. 1: Reflectance scans of a chromatogram track with 500 ng 4-aminosalicylic acid (1), 1 µg 4-aminophenol (2) and 200 ng 2-aminophenol (3) per chromatogram zone: (A) scanned at $\lambda = 460$ nm; (B) scanned at $\lambda = 520$ nm.

References

[1] Glombitza, K.-W., Stoffelen, H.: *Planta Med.* **1972,** *22,* 391–395.
[2] Stoffelen, H., Glombitza, K.-W., Murawski, U., Bialacek, J.: *Planta Med.* **1972,** *22,* 396–401.
[3] Hadzija, O., Tonkovic, M., Isrkic, S.: *J. Liq. Chromatogr.* **1986,** *9,* 3473–3478.
[4] Kumar, H., Sharma, A., Chibber, S. S.: *J. Chromatogr.* **1982,** *245,* 126–128.
[5] Reio, L.: *J. Chromatogr.* **1958,** *1,* 338–373; **1960,** *4,* 458–476.
[6] Wagner, G., Leistner, S.: *Pharmazie* **1973,** *28,* 25–29.
[7] E. Merck, Company brochure "Dyeing Reagents for Thin-layer and Paper Chromatography", Darmstadt 1980.
[8] Schmidtlein, H., Herrmann, K.: *J. Chromatogr.* **1976,** *123,* 385–390.
[9] Gage, T. B., Douglas, C. D., Wender, S. H.: *Anal. Chem.* **1951,** *23,* 1582–1585.
[10] Poethke, W., Schwarz, C., Gerlach, H.: *Planta Med.* **1970,** *19,* 177–188.
[11] Chawla, H. M., Chibber, S. S.: *Chromatographia* **1976,** *9,* 408–409.
[12] Benjamin, B. D., Mulchandani, N. B.: *Planta Med.* **1976,** *29,* 37–40.
[13] Nagarajan, G. R., Parmar, V. S.: *Planta Med.* **1977,** *31,* 146–150.
[14] Chexal, K. K., Handa, B. K., Rahman, W.: *J. Chromatogr.* **1970,** *48,* 484–492.
[15] Schulz, J. M., Herrmann, K.: *Z. Lebensm. Unters. Forsch.* **1980,** *171,* 278–280.
[16] Friedrich, H., Krüger, N.: *Planta Med.* **1974,** *26,* 327–332, 333–337.



[17] Rossi, F., Barolo, P.: *Rass. Chim.* **1970,** *22,* 73–77.
[18] Gstirner, F., Flach, G.: *Arch. Pharm.* **1970,** *303,* 339–345.
[19] Lund, K., Rimpler, H.: *Dtsch. Apoth. Ztg.* **1985,** *125,* 105–108.
[20] Owen, P., Pendlebury, A., Moffat, A. C.: *J. Chromatogr.* **1978,** *161,* 195–203.
[21] Singh, A. K., Granley, K., Ashraf, M., Mishra, U.: *J. Planar Chromatogr.* **1989,** *2,* 410–419.
[22] Daldrup, T., Rickert, A.: *Fresenius Z. Anal. Chem.* **1989,** *334,* 349–353.
[23] Friedrich, H., Schönert, J.: *Planta Med.* **1973,** *24,* 90–100.
[24] Rózsa, Z., Szendrei, K., Novák, I., Reisch, J.: *J. Chromatogr.* **1972,** *72,* 421–425.
[25] Séquin-Frey, M., Tamm, C.: *Helv. Chim. Acta* **1971,** *54,* 851–861.
[26] Antkowiak, W. Z., Gessner, W. P.: *Experientia* **1985,** *41,* 769–771.
[27] Gainer, F. E., Hussey, R. L.: *J. Chromatogr.* **1971,** *54,* 446–448.
[28] Richter, P., Wagner, G., Kazmirowski, H.-G., Usbeck, H.: *Pharmazie* **1976,** *31,* 279–282.
[29] Rüdiger, W., Nikisch, M., Goedde, H. W.: *J. Chromatogr.* **1971,** *61,* 373–375.
[30] Roberts, B. A., Patterson, D. S. P.: *J. Assoc. Off. Anal. Chem.* **1975,** *58,* 1178–1181.
[31] Franc, J., Kosiková, E.: *J. Chromatogr.* **1980,** *187,* 462–465.
[32] Buchbauer, G., Vasold, I.: *Sci. Pharm.* **1983,** *51,* 54–58.
[33] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1989.
[34] Lowry, R. R.: *J. Lipid. Res.* **1968,** *9,* 397.
[35] Voigt, R., Zier, P.: *Pharmazie* **1971,** *26,* 313.

Iron(III) Chloride–Potassium Hexacyanoferrate(III) Reagent (Barton's Reagent)

Reagent for:

- Aromatic amines [1, 3]
  e.g. anilines
- Phenols [3]
  e.g. salsonilol, dopamine [4],
  lignans, pyrogallol derivatives [5],
  zingerone [7],
  gallic acid, gallotannins [8],
  curare alkaloids (tubocurarine [9])
- Phenolic steroids [10–13],
  e.g. estrone, estradiol, estriol
- Analgesics
  e.g. aminophenazone [14]
- Anti-inflammatories
  e.g. carprofen, zomepirac, diclofenac [15]
- Enamino ketones [16]
- Enol ketones [17]
- Thiosulfates [3]
- Isothiocyanates (mustard oils) [3, 19, 20]
- Thiourea derivatives [19, 20]
- Degradation products of carbamate insecticides [18]
  e.g. mexacarbates, matacil, landrin

$FeCl_3 \cdot 6H_2O$	$K_3[Fe(CN)_6]$
$M_r = 270.30$	$M_r = 329.26$
Iron(III) chloride	Potassium hexacyanoferrate(III)

Preparation of the Reagent

Solution I	Dissolve 1 g potassium hexacyanoferrate(III) in 100 ml water.
Solution II	Dissolve 2 g iron(III) chloride hexahydrate in 100 ml water.
Solution III	Dissolve 0.3 to 2 g potassium hexacyanoferrate(III) in 100 ml water.
Solution IV	Dissolve 0.3 to 10 g iron(III) chloride in 100 ml water.
Dipping solution	Immediately before use mix 10 ml water, 8 ml solution I, 2 ml solution II and 1 ml 32% hydrochloric acid in a measuring cylinder and make up to 100 ml with methanol [2].
Spray solution	Immediately before use solutions III and IV are mixed, generally in equal proportions [1, 3, 6–8].
Storage	Solutions I – IV may be stored in the refrigerator with the exclusion of light for ca. 2 weeks [6].
Substances	Iron(III) chloride hexahydrate Potassium hexacyanoferrate(III) Hydrochloric acid (32%) Methanol

Reaction

Some of the iron(III) ions in the reagent are converted to iron(II) ions by reducing substances and then react to produce Prussian blue or TURNBULL's blue.

$$4Fe^{3+} + 3Fe^{II}(CN)_6^{4-} \rightarrow Fe^{III}[Fe^{III}Fe^{II}(CN)_6]_3$$

Method

The chromatograms are freed from mobile phase in a stream of warm air, then immersed in the dipping solution for 2 s or uniformly sprayed with the spray solution. In the case of anti-inflammatories, the plates are then heated to 110 °C for 5–10 min [15].

Blue chromatogram zones are produced on an almost colorless to pale yellow background.

Note: The dipping solution can also be used as a spray solution. When using the spray solution it is possible to increase the color intensity by spraying afterwards with hydrochloric acid (c = 2 mol/L) [16]. The blue chromatogram zones remain readily visible for ca. 15 to 30 min; then they begin to fade, while the background gradually acquires a blue coloration [14].

The detection limits per chromatogram zone are 100–200 ng for gallic acid and aminophenazone [8, 14] and 10–15 ng for uric acid [6].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Substituted Anilines [2]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	Toluene
Migration distance	8 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for ca. 10 min, immersed in the dipping solution for 2 s, dried for 5 min in a stream of cold air and then heated to 110 °C for 1 min.

2,3-Dimethylaniline (hR_f 5–10), 4-chloro-2-methylaniline (hR_f 15–20), 3,4-dichloroaniline (hR_f 25–30), 3,5-dichloroaniline (hR_f 40–45), 2,3-dichloroaniline (hR_f 45–50) and 2,5-dichloroaniline (hR_f 60–65) appeared as pale blue-turquoise-colored chromatogram zones on a beige-colored background. The detection limits per chromatogram zone ranged from 2 ng (2,3-dimethylaniline) to 10 ng (4-chloro-2-methylaniline).

In situ quantitation: The absorption photometric scan in reflectance was carried out at a mean wavelength of λ = 670 nm (Fig. 1).

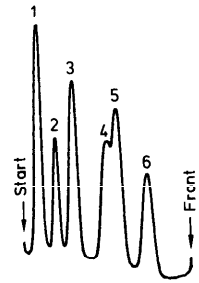


Fig. 1: Reflectance scan of a chromatogram track with 200 ng 2,3-dimethylaniline (1), 100 ng each of 4-chloro-2-methylaniline (2), 3,4-dichloroaniline (3), 3,5-dichloroaniline (4) and 200 ng each of 2,3-dichloroaniline (5) and 2,5-dichloroaniline (6) per chromatogram zone.

References

[1] Gillio-Tos, M., Previtera, S. A., Vimercati, A.: *J. Chromatogr.* **1964,** *13,* 571–572.
[2] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1989.
[3] E. MERCK, Company brochure "Dyeing Reagents for Thin-layer and Paper Chromatography", Darmstadt 1980.
[4] Yamanaka, Y., Walsh, M. J., Davis, V. E.: *Nature* **1970,** *227,* 1143–1144.
[5] MacDonald, B. F., Swan, E. P.: *J. Chromatogr.* **1970,** *51,* 553–555.
[6] Ferrera, R. S., Boese, J. L., Thrasher, J. L.: *J. Assoc. Off. Anal. Chem.* **1986,** *69,* 499–503.
[7] Kucera, M., Kucerová, H.: *J. Chromatogr.* **1974,** *93,* 421–428.
[8] Dadic, M., Van Gheluwe, J. E. A., Weaver, R. L.: *J. Assoc. Off. Anal. Chem.* **1980,** *63,* 1–4.
[9] Clarke, C. J., Raja, R. B.: *J. Chromatogr.* **1982,** *244,* 174–176.
[10] Dannenberg, H., Wolff, T.: *Z. Naturforsch.* **1970,** *25b,* 823–828.
[11] Mirhom, Y. W.: *Clin. Chim. Acta* **1970,** *30,* 347–349.

[12] Breckwoldt, M., Murawec, T., Touchstone, J. C.: *Steroids* **1971**, *17*, 305–316.
[13] Touchstone, J. C., Murawec, T., Brual, O., Breckwoldt, M.: *Steroids* **1971**, *17*, 285–304.
[14] Hüller, G., Haustein, K.-O.: *Pharmazie* **1981**, *36*, 546–548.
[15] Dettwiler, M., Rippstein, S., Jeger, A.: *J. Chromatogr.* **1982**, *244*, 153–158.
[16] Potesil, T., Potesilová, H.: *J. Chromatogr.* **1982**, *249*, 131–137.
[17] Mattsson, O. H., Nyberg, I.: *Acta Chem. Scand.* **1969**, *23*, 2989–2993.
[18] Roberts, R. B., Look, M., Haddon, W. F., Dickerson, Th. C.: *J. Agric. Food Chem.* **1978**, *26*, 55–59.
[19] Wagner, H., Hörhammer, L., Nufer, H.: *Arzneim. Forsch.* **1965**, *15*, 453–457.
[20] Daun, J. K., Hougen, F. W.: *J. Am. Oil Chem. Soc.* **1976**, *54*, 351–354.

8-Mercaptoquinoline Reagent

Reagent for:

- Cations
  e.g. tin, lead, copper [1]

· HCl

$C_9H_7NS \cdot HCl$

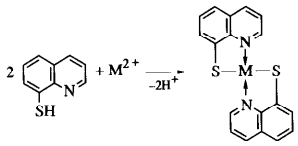
$M_r = 197.69$

Preparation of the Reagent

Dipping solution	Dissolve 100 mg 8-mercaptoquinoline hydrochloride (thiooxine hydrochloride) in 100 ml ethanol [1].
Storage	The dipping solution may be kept in the refrigerator for two weeks [1].
Substances	Thiooxine hydrochloride Ethanol

Reaction

Like 8-hydroxyquinoline [2] 8-mercaptoquinoline forms colored and fluorescent complexes with many metal cations.



Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 3 s or sprayed homogeneously with it and then dried in a stream of cold air for 5 min.

Yellow-colored chromatogram zones are produced that fluoresce yellow when exposed to long-wavelength UV light ($\lambda = 365$ nm).

Note: The detection limits are in the lower nanogram range.

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, cellulose and RP layers.

Procedure Tested

Organotin Compounds [1]

Method	Ascending, one-dimensional stepwise development (10 min intermediate drying in a stream of cold air) in a trough chamber with chamber saturation during the second development.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	1. Diethyl ether – glacial acetic acid (20+1). 2. Isobutyl methyl ketone – glacial acetic acid – *n*-hexane – tetrahydrofuran – dioxane (8+2+1+1+1).
Migration distance	1. 1.5 cm 2. 6 cm
Running time	1. 2 min 2. 15 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air, the organotin compounds were then converted to tin(IV) species by irradiating intensely for 20 min with UV light. Then the layer was immersed in the reagent solution for 3 s and dried for 5 min in a stream of cold air.

Dimethyltin dichloride (hR_f 15–20), trimethyltin chloride (hR_f 55–60), dibutyltin dichloride (hR_f 70–75) and tributyltin methoxide (hR_f 80–85) appeared in long-wavelength UV light ($\lambda = 365$ nm) as yellow fluorescent chromatogram zones on a dark background. The detection limits (calculated as Sn) lay between 5 and 10 ng (dimethyltin dichloride, dibutyltin dichloride, tributyltin methoxide) and 50 ng (trimethyltin chloride) substance per chromatogram zone.

In situ quantitation: Fluorimetric measurements were made by exciting at $\lambda_{exc} = 365$ nm and measuring the fluorescence emission at $\lambda_{fl} > 560$ nm (cut off filter Fl 56).

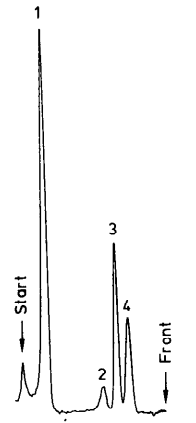


Fig. 1: Fluorescence scan of a chromatogram track with 100 ng substance (calculated as Sn) each per chromatogram zone: 1 = dimethyltin dichloride, 2 = trimethyltin chloride, 3 = dibutyltin dichloride, 4 = tributyltin methoxide.

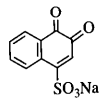
References

[1] Kornapp, M.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1992.
[2] Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin Layer Chromatography – Reagents and Detection Methods*, Vol. 1a, p. 310, VCH-Verlagsgesellschaft, Weinheim, 1990.

1,2-Naphthoquinone-4-sulfonic Acid Reagent (Folin's Reagent)

Reagent for:

- Amino acids, peptides [1]
- Aromatic amines [1, 4, 5]
  e.g. parsalmide [6]
- Piperidine derivatives [7]
  e.g. taurinopiperidine
- Aliphatic amines [8]
- Ergot alkaloids, LSD [9]
- Phenolic aromatic sulfides, sulfoxides, sulfones [10]
- Diuretics [11]
  e.g. chlorthalidon, clorexolone, cyclopenthiazide



$C_{10}H_5NaO_5S$

$M_r = 260.20$

Preparation of the Reagent

Dipping solution *For aromatic amines*: Dissolve 0.5 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 30 ml water and add 65 ml ethanol and 5 ml acetic acid [5].

Spray solution

For amino acids: Dissolve 0.2–0.3 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 100 ml aqueous sodium carbonate solution (5–10%) [1].

For aromatic amines: Dissolve 0.5 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 95 ml water and treat with 5 ml glacial acetic acid [1, 6]; if necessary, filter off the insoluble part [1].

For aliphatic amines: Dissolve 0.6 g 1,2-naphthoquinone-4-sulfonic acid sodium salt in 12 ml water, make up to 200 ml with ethanol (90%) and add 10 ml pyridine [8].

Storage

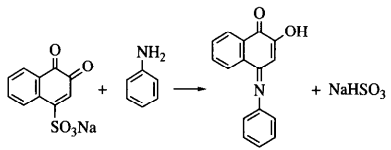
The dipping solution may be stored in the refrigerator for several days [5], the spray solution for amino acids should always be made up fresh [1].

Substances

1,2-Naphthoquinone-4-sulfonic acid sodium salt
Ethanol
Acetic acid (100%)
Sodium carbonate decahydrate

Reaction

Primary amines and substances with reactive methylene groups react with 1,2-naphthoquinone-4-sulfonate to yield intensely colored *p*-quinoid derivatives, which, in the case of aryl amines, are indophenol dyes [12, 13].



1,2-Naphthoquinone-4-sulfonic acid Na Salt — Aniline — *p*-Quinonoid derivative

Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution until the layer begins to be transparent and then dried in a stream of cold air [1].

After a few minutes variously colored chromatogram zones appear on a yellowish background.

Note: It is possible to differentiate amino acids by color on the basis of the markedly different shades produced [2, 3]. Proline and hydroxyproline, that only react weakly with ninhydrin, also yield pink-red colored derivatives [2]. Ergot alkaloids and LSD are detected by spraying with 10% hydrochloric acid and then heating to 110 °C for 20 min after they have been treated with the reagent [9]. Ergot alkaloids and LSD yield red to purple zones when treated in this manner; other alkaloids, e. g. reserpine, emetine, quinine, strychnine, pilocarpine, atropine, scopolamine, cocaine and opium alkaloids, do not give a reaction [9].

In the case of diuretics the chromatogram is first sprayed with sodium hydroxide solution (c = 1 mol/L) and then with a saturated solution of 1,2-naphthoquinone-4-sulfonic acid sodium salt in ethanol – water (50+50) [11]. Stable orange-colored chromatogram zones appear over a period of ca. 15 min, their intensity increases on storage in the dark (1–2 days) [11].

Amino acids yield various colors [1].

Heating the chromatograms after treatment is not generally to be recommended, but it leads to characteristic color changes in some cases [4].

The detection limits per chromatogram zone are 1–3 µg substance for ergot alkaloids [9], 5 µg for diuretics [11] and 5–30 ng for aromatic amines [5].

The reagent can, for instance, be used on silica gel, kieselguhr, Si 50000, aluminium oxide, polyamide and cellulose layers.

Procedure Tested

Aromatic Amines [5]

Method	Ascending, one-dimensional, stepwise development (5 min intermediate drying in a stream of cold air) in a trough chamber at 20 °C without chamber saturation.
Layer	HPTLC plates Silica gel F_{254} (MERCK), which had been prewashed before use by complete immersion overnight in 2-propanol and then dried at 110 °C for 30 min.
Mobile phase	1. Methanol – glacial acetic acid (100+1). 2. Chloroform – *n*-hexane – diisopropyl ether – dichloromethane – formic acid (50+35+10+5+0.45).
Migration distance	1. 0.8 cm 2. 6.5 cm
Running time	1. 1 min 2. 25 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed in the dipping solution for 2 s and then dried in stream of cold air.

After a few minutes 2,4-diamino-6-methylphenol (hR_f 5–10), 3-chloro-4-methoxyaniline (hR_f 25–30), aniline (hR_f 35–40), 4-bromoaniline (hR_f 40–45), 3-chloroaniline (hR_f 50–55), 2,6-dimethylaniline (hR_f 60–65), 2-methyl-6-ethylaniline (hR_f 65–70) and 2-chloroaniline (hR_f 70–75) yielded orange-colored chromatogram zones on a yellow background. The detection limits were between 5 ng (2,4-diamino-6-methylphenol) and 30 ng (2,6-dimethylaniline) substance per chromatogram zone.

In situ quantitation: The absorption photometric analysis in reflectance was carried out at the wavelength $\lambda = 510$ nm (Fig. 1).

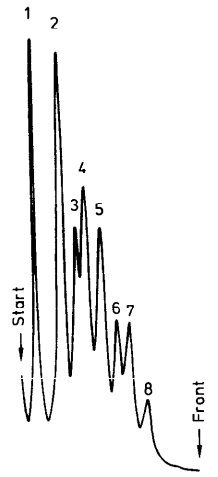


Fig. 1: Reflectance scans of a chromatogram track with 100 ng each of: 1 = 2,4-diamino-6-methylphenol, 2 = 3-chloro-4-methoxyaniline, 3 = aniline, 4 = 4-bromoaniline, 5 = 3-chloroaniline, 6 = 2,6-dimethylaniline, 7 = 2-methyl-6-ethylaniline and 8 = 2-chloroaniline.

References

[1] E. MERCK, Company brochure "Dyeing Reagents for Thin-layer and Paper Chromatography", Darmstadt 1980.
[2] Müting, D.: *Naturwissenschaften* **1952**, *39*, 303.
[3] Giri, K. V., Nagabhushanam, A.: *Naturwissenschaften* **1952**, *39*, 548–549.
[4] Jones, G. R. N.: *J. Chromatogr.* **1973**, *77*, 357–367.
[5] Ditthard, A.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[6] Fleischmann, L.: *Arzneim. Forsch.* **1980**, *30*, 294–298.
[7] Naito, S.-I., Sekishiro, K., Awataguchi, M., Izushi, F.: *J. Pharmac. Sci.* **1972**, *61*, 198–202.
[8] Baudot, P.: *J. Chromatogr.* **1971**, *59*, 203–208.
[9] Güven, K. C., Güneri, T.: *Fresenius Z. Anal. Chem.* **1977**, *283*, 32.

[10] Denisova, L. I., Orlova, V. A.: *Pharm. Chem. J.* **1973**, *7*, 233–235.
[11] Maes, R., Gijbels, M., Laruelle, L.: *J. Chromatogr.* **1970**, *53*, 408–412.
[12] Feigl, F., Anger, V.: *Spot Tests in Organic Analysis*, 7th Ed., p. 153–154, Elsevier, Amsterdam, London, New York, 1966.
[13] Clotten, R., Clotten, A.: *Hochspannungs-Elektrophorese*, p. 146, Thieme Verlag, Stuttgart 1962.

arylazopyrimidinylpyrazoles [7]
benzothiazoles [8]
arylamines [9]
imipramine, desipramine [10, 11]
xanthene derivatives [12]
diazepam [2]

- Testosterone [2]
- Acetylacetonates
  e.g. of Be, Al, V, Fe, Ce, Mn [2]
- Sugars
  e.g. fructose, glucose [2]
- 1,1'-Carbonyldiimidazole [2]
- Phospholipids
  e.g. lecithin, sphingomyelin [14]

HNO_3

$M_r = 63.01$

Nitric Acid Vapor Reagent
(Nitrous Fumes/Nitric Oxide Reagent)

Reagent for:

- Aromatics and compounds with aromatic substituents
  e.g. ephedrine [1, 2]
  methylephedrine, oxeladin citrate [1]
  catecholamine and serotonin metabolites [3]
  insecticides: rotenone, elliptone, deguelin, tephrosin [4]
  arylazothiazoles [5]
  pyrazolin-5-one derivatives [6]

Preparation of the Reagent

Reagent solution	Fuming nitric acid.
Storage	Fuming nitric acid may be stored for several months in a tightly sealed brown glass bottle.
Substances	Nitric acid, fuming (100%)

Reaction

Under the chosen conditions aromatic compounds are nitrated to nitroaromatics [1]. The detection of rotenone [1] (see below) depends on the reduction of silver ions, incorporated into the layer, to metallic silver in the presence of ammonia [4]. The mechanism of the reaction of many substances leading to fluorescent derivatives has not yet been elucidated [2].

Method

The chromatogram is freed from mobile phase in a stream of warm air and placed layer down for 40–45 s [6, 7], 1 min [5, 8], 3 min [1, 4], 10 min [1, 14] or 30–60 min [11] in a conditioning chamber containing 40 ml fuming nitric acid. The chromatogram is then freed from excess nitrous fumes for 5 min in a stream of cold air and occasionally heated to 160 °C [1, 2] or 180 °C [14] for 15 min. In the case of the rotenone insecticides (silver nitrate-impregnated layer!) the chromatogram is exposed to an atmosphere of ammonia for 10 min after it has been reacted with nitrous fumes [4], while for xanthene derivatives reaction is followed by irradiation with short-wavelength UV light (λ = 254 nm) for 5 min [12].

Aromatic compounds generally yield yellow to brown chromatogram zones that usually absorb UV light at λ = 270 nm [1]. These compounds can frequently be excited to fluoresce by long-wavelength UV light (λ = 365 nm) [2, 12]. Rotenoides yield dark [4], arylamines, e.g. imipramine and desipramine, pale yellow to brown-red [9, 11] and catecholamines yellow chromatogram zones on a pale background [3]. A whole range of substances, e.g. xanthene derivatives, diazepam, testosterone, glucose, fructose, ephedrine etc., fluoresce yellow or blue when excited by long-wavelength UV light (λ = 365 nm) [2, 12].

Note: Nitrous fumes can also be generated in a twin trough chamber e.g. by pouring 50% nitric acid [12], hydrochloric acid (c = 2 mol/L) [11] or sulfuric acid onto sodium nitrite.

It can be advantageous to heat the chromatogram to 160 °C for 15 min before treating with nitrous fumes and to place it in the reagent chamber while still hot [1]. Heating to 260 °C has even been recommended for the purpose of reducing the fluorescent background [14], whereby the layer is previously immersed in 1 percent Ludox solution (silicic acid sol) to increase its stability [2]. The fluorescence of the substances detected usually remains stable for at least 2 weeks [2].

Brief exposure to nitrous fumes (up to 3 min) leaves the fluorescent power of the acid-instable fluorescence indicator F_{254}, incorporated into most TLC layers, largely unaffected, so that the nitroaromatics so formed can be detected as dark zones on a green fluorescent background [1]. For purposes of in situ quantitation it is recommended that the fluorescence indicator be destroyed by 10 min exposure to nitrous fumes in order to avoid difficulties in the subsequent evaluation [1].

The visual detection limits per chromatogram zone are 2.5 to 3.5 µg for pyrazolin-5-one compounds [6], 1–2.5 µg for arylazothiazoles and arylazopyrimidinylpyrazoles [5, 7] and 2.5–4 µg for benzothiazoles [8]. Nanogram quantities can be detected by photometric methods [10, 14].

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers as well as on aluminium oxide, cellulose or chiral layers. Neither do difficulties occur on RP 18, Diol, NH_2 and CN phases. Silver nitrate- [4] and calcium oxalate-impregnated layers [9] are also suitable. However, polyamide phases are colored yellow.

Danger: Fuming nitric acid is very aggressive; eye protection and rubber gloves should always be worn!

Procedure Tested

Fungicides [13]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Toluene – *n*-hexane – diisopropyl ether – ethyl acetate (67+20+8+5).
Migration distance	7 cm
Running time	15 min

Detection and result: The dried chromatogram (5 min in a stream of cold air) was heated to 160 °C for 15 min and placed, while still hot, for 10 min in the empty half of a twin trough chamber whose other trough contained 10 to 15 ml fuming nitric acid. After exposure the chromatogram was heated to 160 °C for 15 min to remove excess nitric oxide. Scanning was carried out after cooling. It was not possible to make a visual check of the fungicides (≈ 1 µg each) iprodione (hR_f 10–15), procymidon (hR_f 35–40) and vinclozolin (hR_f 55–60) investigated, due to the yellow coloration of the chromatogram zones.

In situ quantitation: The absorption photometric scan in reflectance was carried out at λ = 270 nm (Fig. 1).

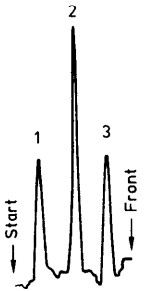


Fig. 1: Reflectance scan of a chromatogram track with 1 µg each per chromatogram zone: 1 = iprodione, 2 = procymidon, 3 = vinclozolin.

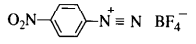
References

[1] Bottler, R., Knuhr, T.: *Fresenius Z. Anal. Chem.* **1980,** *302,* 286–289.
[2] Zhou, L., Shanfield, H., Wang, F.-S., Zlatkis, A.: *J. Chromatogr.* **1981,** *217,* 341–348.
[3] Huck, H., Dworzak, E.: *J. Chromatogr.* **1972,** *74,* 303–310.
[4] Delfel, N. E., Tallent, W. H.: *J. Assoc. Off. Anal. Chem.* **1969,** *52,* 182–187.
[5] Jain, R., Agarwal, D., Goyal, R. N.: *J. Liq. Chromatogr.* **1980,** *3,* 557–560.
[6] Jain, R., Agarwal, D.: *J. Liq. Chromatogr.* **1981,** *4,* 2229–2232.
[7] Jain, R., Agarwal, D., Goyal, R. N.: *Fresenius Z. Anal. Chem.* **1981,** *307,* 207–208.
[8] Jain, R., Agarwal, D.: *J. Liq. Chromatogr.* **1982,** *5,* 1171–1175.
[9] Srivastava, S. P., Dua, V. K.: *Z. Anal. Chem.* **1975,** *276,* 382.
[10] Sistovaris, N., Dagrosa, E. E., Keller, A.: *J. Chromatogr.* **1983,** *277,* 273–281.
[11] Nagy, A., Treiber, L.: *J. Pharm. Pharmac.* **1973,** *25,* 599–603.
[12] Takács, M., Kertész, P.: *Z. Anal. Chem.* **1971,** *254,* 367–368.
[13] Jork, H., Ganz, J.: Private communication, Universität des Saarlandes, Fachbereich 12, Saarbrücken 1990.
[14] Zhou, L., Shanfield, H., Zlatkis, A.: *J. Chromatogr.* **1982,** *239,* 259–264.

4-Nitrobenzenediazonium Tetrafluoroborate Reagent

Reagent for:

- Phenols [1–7]
  e.g. plant phenols [8]
  estrogens [9]
  procyanidins [10]
  catecholamines [11]
- Zearalenone [12]
- Aromatic amines [13]
  e.g. diphenylamine (anti-ageing additive) [14]
- Pesticides
  e.g. carbamate insecticides [1, 4, 7, 8, 15–19]
  such as desmedipham, dioxacarb, phenmedipham, propoxur, pyrazon, aminocarb, aldicarb, carbofuran, carbaryl, oxamyl
  e.g. carbamate herbicides [21]
  such as propanil
  e.g. organophosphorus insecticides [4, 20]
  such as parathion, parathion methyl, coumaphos, dichlorvos, fenitrothion



NaOH	$C_6H_4BF_4N_3O_2$
$M_r = 40.00$	$M_r = 236.92$
Sodium hydroxide	4-Nitrobenzenediazonium tetrafluoroborate

Preparation of the Reagent

Dipping solution I Dissolve 2 g sodium hydroxide [20] or 2.8 g potassium hydroxide [15] in 5 ml water and make up to 50 ml with ethanol or methanol.

Dipping solution II Dissolve 25–50 mg 4-nitrobenzenediazonium tetrafluoroborate in 10 ml diethylene glycol and make up to 100 ml with water [20] or acetone [15].

Spray solution I Dissolve 4–15 g potassium hydroxide [1, 4, 7, 16–18] or sodium hydroxide [8] in 100 ml methanol, ethanol or water.

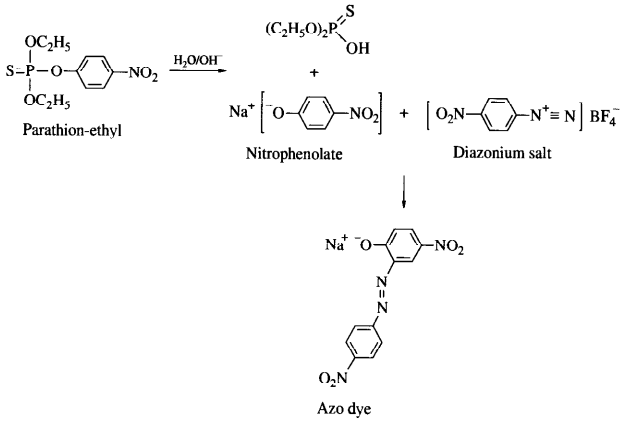
Spray solution II Dissolve 10 to 100 mg 4-nitrobenzenediazonium tetrafluoroborate in 100 ml methanol [7, 17, 18].
Alternatively it is possible to use 0.5 to 2% solutions in acetone [16], acetone – methanol (50+50) [1] or 50% acetic acid [9]. A saturated solution in ethanol – diethylene glycol (90+10) can also be used [8].

Storage The dipping and spray solutions I can be stored over longer periods. The dipping and spray solutions II may only be stored for a few hours with cooling and should, therefore, always be made up freshly before use [7].

Substances
Potassium hydroxide, pellets
Sodium hydroxide, pellets
4-Nitrobenzenediazonium tetrafluoroborate
Diethylene glycol
Ethanol
Methanol
Acetone

Reaction

The hydrolytic action of alkalis on the thiophosphate insecticides, such as parathion, yields free phenols that then couple with the diazonium salt to yield azo dyes.



Method

The chromatograms are freed from mobile phase in a stream of warm air for 3 min, then immersed in dipping solution I for 3–5 s or uniformly sprayed with spray solution I. Then, except in the case of thiophosphate insecticides, the plate is immediately immersed in dipping solution II for 3–5 s or homogeneously sprayed with spray solution II. In the case of thiophosphate insecticides there is a delay of 2 min [4] or the plates are heated to 70–110 °C for 10–15 minutes before the plates are exposed to the second reagent solution.

This yields variously colored chromatogram zones on a colorless background. The zones of phenols are reddish to blue-violet [4, 7, 8].

Note: The first reagent treatment with alkali can be omitted in the case of phenols (e. g. estrogens) [2, 3, 5, 8, 9]. Dipping is preferable to spraying since it yields darker chromatogram zones and, hence, improves the detection sensitivity [15]. The presence of diethylene glycol in the reagent has a favorable effect on the color intensity and stability of the derivatives [15]. Most thiophosphate insecticides do not give any reaction [4].

The detection limits for catecholamines are 10 to 50 ng substance per chromatogram zone [11] and 50 ng substance per chromatogram zone for carbaryl and a series of other

carbamate insecticides [8, 15]. The detection sensitivity is generally better on silica gel than on aluminium oxide layers [4]. It is possible to detect parathion with a sensitivity of 5 to 50 pg after it has been oxidized with bromine to paraoxon, by combination with an enzyme inhibition reaction (choline esterase, substrate: 1-naphthyl acetate). White zones of inhibition are produced on an blue-red background [2, 3].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Thiophosphate Insecticides [20]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254}, extra thin layer (MERCK); before the samples were applied these were immersed overnight in 2-propanol and then dried for 30 min at 110 °C.
Mobile phase	*n*-Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (908 + 40 + 26 + 25 + 1).
Migration distance	8 cm
Running time	30 min

Detection and result: The chromatogram was freed from mobile phase in a stream of warm air for 3 min, immersed in dipping solution I for 3 s, heated to 110 °C for 15 min and then immersed in dipping solution II for 3 s.

After drying in a stream of cold air coumaphos (hR_f 30–35) appeared as an intense red chromatogram zone on a colorless background, while parathion methyl (hR_f 40–45), fenitrothion (hR_f 45–50) and parathion ethyl (hR_f 60–65) yielded yellow zones as they did with sodium hydroxide alone (*q.v.*). The detection limit for coumaphos was 10 ng per chromatogram zone.

In situ qnantitation: The absorption photometric scan in reflectance of parathion methyl, fenitrothion and parathion ethyl was carried out at a mean wavelength of λ_{max} = 406 nm (Fig. 1A). Coumaphos was determined at λ = 540 nm (Fig. 1B).

References

[1] Fuhremann, T. W., Lichtenstein, E. P.: *J. Agric. Food Chem.* **1980**, *28*, 446–452.
[2] Bhaskar, S. U., Nanda Kumar, N. V.: *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 1312–1314.
[3] Nanda Kumar, N. V., Visweswariah, K., Majumder, S. K.: *J. Assoc. Off. Anal. Chem.* **19**
59, 641–643.
[4] Ramasamy, M.: *Analyst* **1969**, *94*, 1075–1080.
[5] Seeboth, H., Görsch, H.: *Chem. Techn. (DDR)* **1963**, *15*, 294–296.
[6] Bollag, J.-M., Czaplicki, E. J., Minard, R. D.: *J. Agric. Food Chem.* **1975**, *23*, 85–90.
[7] Ashworth, R. J., Sheets, T. J.: *J. Agric. Food Chem.* **1972**, *20*, 407–412.
[8] Ambrus, A., Hargitai, É., Károly, G., Fülöp, A., Lantos, J.: *J. Assoc. Off. Anal. Chem.* **19**
64, 743–748.
[9] Krol, G. J., Boyden, G. R., Moody, R. H., Comeau, J. C., Kho, B. T.: *J. Chromatogr.* **19**
61, 187–192.
[10] Lea, A. G. H., Timberlake, C. F.: *J. Sci. Food Agric.* **1974**, *25*, 1537–1545.
[11] Roser, R., Tocci, P. M.: *J. Chromatogr.* **1972**, *72*, 207–211.
[12] Harrach, B., Palyusik, M.: *Acta Vet. Acad. Sci. Hung.* **1979**, *27*, 77–82.
[13] E. Merck, Company brochure "Dyeing Reagents for Thin-layer and Paper Chromatography", Darmstadt 1980.

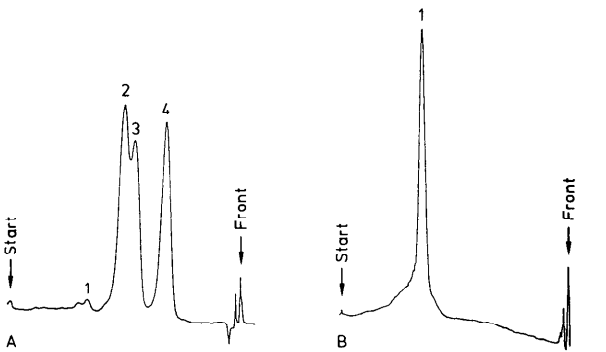


Fig. 1: Reflectance scan of a chromatogram track with 300 ng each substance per chromatogram zone; (A) scan at $\lambda = 406$ nm, (B) scan at $\lambda = 540$ nm: 1 = coumaphos, 2 = parathion methyl, 3 = fenitrothion, 4 = parathion ethyl.

[14] Hofmann, W., Ostromow, H.: *Kautschuk, Gummi, Kunststoffe* **1972**, *25*, 204–206.
[15] Sherma, J., Kovalchick, A. J., Mack, R.: *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 616–620.
[16] Sharma, S. R., Singh, R. P., Saxena, S. K., Ahmed, S. R.: *J. Liq. Chromatogr.* **1985**, *8*, 1327–1346.
[17] Liu, S.-Y., Bollag, J.-M.: *J. Agric. Food Chem.* **1971**, *19*, 487–490.
[18] Yu, C.-C., Booth, G. M., Hansen, D. J., Larsen, J. R.: *J. Agric. Food Chem.* **1974**, *22*, 431–434.
[19] Pree, D. J., Saunders, J. L.: *J. Agric. Food Chem.* **1974**, *22*, 620–625.
[20] Pitzer, H.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1990.
[21] Moilanen, K. W., Crosby, D. G.: *J. Agric. Food Chem.* **1972**, *20*, 950–953.

Palladium(II) Chloride Reagent

Reagent for:

- Thiophosphate esters [1–4]
  e. g. insecticides [5–23]
  such as dimethoate, bromophos-ethyl, Fac, Rogor, parathion, chlorthion, Meta-Systox, diazinon, propetamphos, malathion, terbufos, azinphos-methyl, demeton, phorate, disulfoton, vamidothion
- Sulfur compounds
  e. g. mercaptans, sulfides (thioethers), disulfides [24]
  polysulfides [25]
  phenothiazines [4]
- Antioxidants
  e. g. esters of gallic acid, dialkyl- and diaryldithiophosphates [26, 27]

$PdCl_2$

$M_r = 177.31$

Preparation of the Reagent

Dipping solution Dissolve 500 mg palladium(II) chloride in 2.5 ml hydrochloric acid (32%) and make up to 100 ml with ethanol [10].

Spray solution Dissolve 250 mg to 5 g palladium(II) chloride in 100 ml ethanol [9], water [1], hydrochloric acid (c = 0.2 ... 1 mol/L) [3, 6, 8, 13, 15, 19–21, 23, 27], ethanolic hydrochloric acid [5] or hydrochloric acid – acetone (50+50) [25, 26].

Storage The dipping solution can be stored for ca. 1 month.

Substances Palladium(II) chloride
Hydrochloric acid (32%)
Ethanol
Acetone

Reaction

Palladium(II) chloride forms colored complexes with many aromatic and sulfur-containing compounds [27].

Method

The chromatograms are freed from mobile phase for 3 min in a stream of warm air, immersed for 2 s in the dipping solution [10] or homogeneously sprayed with the spray solution [9], dried in a stream of warm air and then heated to 110–120 °C for 10 to 20 min [9, 10].

Colored zones are formed sometimes without heating [1, 3, 6, 7, 13–15, 18, 24], organophosphorus insecticides forming yellow-brown [2, 6, 9, 10, 14, 18, 28] to black chromatogram zones on a colorless [14, 23] to pale yellow [2] or light brown [6, 10] to pinkish grey [9] background. Mercaptans, sulfides, disulfides and polysulfides appear white, yellow, orange and brown [24, 25] and antioxidants yellow, grey, brown, pink or violet [27].

Note: It is occasionally recommended that sodium acetate be added to the reagent [2]. Thiophosphate insecticides with a simple P–S bond yield yellow chromatogram zones and those with a P=S double bond yield brown ones on a light brown background [10]. Further treatment of the stained chromatogram with iodine vapors increases the detection sensitivity [7] more than does spraying afterwards with caustic soda solution, which is also occasionally recommended [16, 17, 20, 21].

The detection limits for compounds with P=S double bonds are lower than those for substances with single P–S bonds [7]. They are lower on silica gel than on polyamide layers [15] and are, for instance, 10–20 ng substance per chromatogram zone for

organophosphorus pesticides [10]. Higher levels of up to 5 µg are regularly reported the literature [6, 7, 15, 17].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000, polyamid and RP layers.

Procedure Tested

Organophosphorus Insecticides [10, 28, 29]

Method	Ascending, one-dimensional, two-fold development in a troug chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	1. Chloroform – diethyl ether – *n*-hexane – toluen (29.3+25.7+25+20). 2. Ethyl acetate
Migration distance	1. 6 cm 2. 1 cm
Running time	1. 20 min 2. 5 min

Detection and result: The chromatogram was dried for ca. 3 min in a stream of warm air, immersed in the dipping solution for 2 s, dried in a stream of warm air for 3 mi and then heated to 110 °C for 10 min in the drying cupboard.

Demeton-S-methyl sulfone (hR_f 0–5), dimethoate (hR_f 5–10), demeton-S-methy (hR_f 20–25), triazophos (hR_f 40–45), azinphos-methyl (hR_f 40–45), azinphos-ethy (hR_f 50–55), malathion (hR_f 60–65), parathion-methyl (hR_f 75–80) and parathion ethyl (hR_f 80–85) yielded yellow to brown chromatogram zones on a light brown back ground, with thiophosphate insecticides with P=S double bonds appearing as brow zones and those with single P–S bonds as yellow zones.

In situ quantitation: The absorption photometric quantitation was carried out in re flectance at a mean wavelength of $\lambda = 370$ nm. The detection limits per chromatogra zone lay between 10 ng for dimethoate and 20 ng for parathion-ethyl.

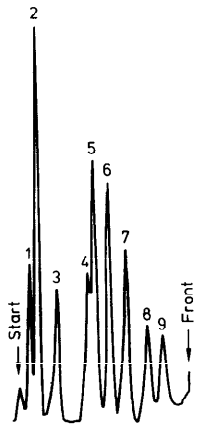


Fig. 1: Reflectance scan of a chromatogram track with 100 ng each substance per chromatogram zone: 1 = demeton-S-methyl sulfone, 2 = dimethoate, 3 = demeton-S-methyl, 4 = triazophos, 5 = azinphos-methyl, 6 = azinphos-ethyl, 7 = malathion, 8 = parathion-methyl and 9 = parathion-ethyl.

References

[1] Eder, F., Schoch, H., Müller, R.: *Mitt. Lebensm.-Unters. Hygiene* **1964**, *55*, 98–131.
[2] Thier, H. P., Bergner, K. G.: *Dtsch. Lebensm. Rundsch.* **1966**, *62*, 399–402.
[3] Bazzi, B., Fabbrini, R., Radice, M.: *J. Assoc. Off. Anal. Chem.* **1973**, *56*, 184–187.
[4] E. MERCK, Company brochure *"Dyeing Reagents for Thin-layer and Paper Chromatography"*, Darmstadt 1980.
[5] Blinn, R. C.: *J. Assoc. Offic. Agr. Chem.* **1964**, *47*, 641–645.
[6] Bäumler, J., Rippstein, S.: *Helv. Chim. Acta* **1961**, *44*, 1162–1164.
[7] Simonovska, B.: *Fresenius Z. Anal. Chem.* **1990**, *336*, 515.
[8] Suzuki, T., Uchiyama, M.: *J. Agric. Food Chem.* **1975**, *23*, 281–286.
[9] Hauck, H. E., Amadori, E. in Harvey, J., Jr., Zweig, G. (Eds.) Pesticide Analytical Methodology, ACS Symposium Series **1980**, *136*, 162–176.
[10] Funk, W., Cleres, L., Pitzer, H., Donnevert, G.: *J. Planar Chromatogr.* **1989**, *2*, 285–289.
[11] Frei, R. W., Mallet, V., Thiébaut, M.: *Internat. J. Environ. Anal. Chem.* **1971**, *1*, 141–146.

[12] Mitchell, T. H., Ruzicka, J. H., Thomson, J., Wheals, B. B.: *J. Chromatogr.* **1968**, *32*, 17–23.
[13] Wells, D. S., Afifi, L. M., Motoyama, N., Dauterman, W. C.: *J. Agric. Food Chem.* **1986**, *34*, 79–86.
[14] Tewari, S. N., Harpalani, S. P.: *J. Chromatogr.* **1977**, *130*, 229–236.
[15] Nagasawa, K., Yoshidome, H.: *J. Chromatogr.* **1969**, *39*, 282–290.
[16] Lichtenstein, E. P., Fuhremann, T. W., Hochberg, A. A., Zahlten, R. N., Stratman, F. W.: *J. Agric. Food Chem.* **1973**, *21*, 416–424.
[17] Grant, D. L., Sherwood, C. R., McCully, K. A.: *J. Chromatogr.* **1969**, *44*, 67–74.
[18] Antoine, O., Mees, G.: *J. Chromatogr.* **1971**, *58*, 247–256.
[19] El-Oshar, M. A., Motoyama, N., Dauterman, W. C.: *J. Agric. Food Chem.* **1987**, *35*, 138–144.
[20] Fuhremann, T. W., Lichtenstein, E. P.: *J. Agric. Food Chem.* **1980**, *28*, 446–452.
[21] Fuhremann, T. W., Lichtenstein, E. P., Stratman, F. W.: *J. Agric Food Chem.* **1978**, *26*, 1068–1075.
[22] Zulalian, J., Blinn, R. C.: *J. Agric. Food Chem.* **1977**, *25*, 1033–1039.
[23] Kovács, G. H.: *J. Chromatogr.* **1984**, *303*, 309–311.
[24] Kaimai, T., Matsunaga, A.: *Anal. Chem.* **1978**, *50*, 268–270.
[25] Hiley, R. W., Cameron, A.: *J. Chromatogr.* **1975**, *107*, 393–395.
[26] Cox, R.: *J. Chromatogr.* **1975**, *105*, 57–64.
[27] Van der Heide, R. F.: *J. Chromatogr.* **1966**, *24*, 239–243.
[28] Pitzer, H.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[29] Cleres, L.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1989.

Phosphoric Acid Reagent

Reagent for:

- Sterols, steroids [1–7]
  e. g. cholesterol [8]
  aldosterone, hydrocortisone, androsterone, estradiol [8]
  contraceptives [9, 10]
  17-spirosteroids [11]
  trenbolone [12]
  liquid crystals [13]
- *Digitalis* glycosides [14, 15]
- Indole derivatives
  e. g. tryptophan, indole-3-acetic acid [16]
  Amanita toxins [17]
- Quinoxalone derivatives of α-ketoacids
  e. g. pyruvic acid [18]
- Components of edible oils (lipids)
  e. g. in groundnut oil, castor oil [19]

H_3PO_4

$M_r = 98.00$

Preparation of the Reagent

Dipping solution Mix 50 ml *ortho*-phosphoric acid (85%) carefully with 50 ml methanol under cooling.

Spray solution A solution of 10 to 50% *ortho*-phosphoric acid in water [1–6, 10, 11, 13, 17, 18], methanol [9, 17, 19], ethanol [12, 16, 17, 20], acetone [17] or carbon tetrachloride – *n*-propanol (3+2) [14].

Storage	Both reagent solutions may be stored, cool and in the dark, several days.
Substances	*ortho*-Phosphoric acid (85%) Methanol Ethanol

Reaction

The mechanism of the reaction has not been elucidated [16].

Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed in the reagent solution for 1 to 2 s or homogeneously sprayed with it until the layer begins to be transparent, then after drying in a stream of warm air it is heated to 105–120 °C for 5–30 min and occasionally (tryptophan derivatives) [16] for 40 min. In exceptional cases evaluation is made without heating (trenbolone [12]).

Variously colored chromatogram zones (grey, blue, brown, orange, violet) are produced on a pale background; the zones frequently fluoresce intensely on exposure to long-wavelength UV light ($\lambda = 365$ nm).

For instance, trenbolone fluoresces yellow [12] and the qinoxalone derivative of pyruvic acid yellow-green [18]. In the case of steroids and *Digitalis* glycosides it is possible to differentiate on the basis of various fluorescence colors [8, 9, 15].

Note: Like sulfuric acid (*q.v.*) *ortho*-phosphoric acid is a universal reagent, with which almost all classes of substance can be detected at high temperatures (150–180 °C) by charring: e. g. high molecular weight hydrocarbons (mineral oils) [20]. The colors and fluorescences produced at lower temperatures (< 120 °C) and their intensities are very dependent on the temperature and period of heating. It is not possible to use meta- or pyrophosphoric acid in place of *ortho*-phosphoric acid, since, for instance, *amanita* toxins react well with alcoholic phosphoric acid only weakly with aqueous phosphoric acid and not at all with meta- or pyrophosphoric acid [17].

The fluorescence can be stabilized by dipping the chromatograms in liquid paraffin – *n*-hexane (1 + 2) [21] or paraffin – carbon tetrachloride (1 + 9) [14]. Quantitative evaluation must generally be carried out rapidly since neither the colors nor the fluorescences are fast [16, 17]. Colored chromatogram zones can be preserved over a longer period by covering the chromatogram with a glass plate [9].

Detection with phosphoric acid at room temperature (with no heating afterwards) is specific for trenbolone, since related steroids such as progesterone and testosterone do not interfere under these conditions [12].

The detection limits per chromatogram zone are below 1 µg for steroids [9] (c. g. 250 pg for trenbolone [12]) and 500 ng for indole derivatives [16].

The reagent can be used on silica gel, silver nitrate-impregnated silica gel [19], kieselguhr, Si 50000, RP, Diol and NH_2 layers. Cellulose layers are less suitable because after application of the reagent the background itself fluoresces strongly, so that fluorescence emission can only be detected after the application of large quantities of substance per chromatogram zone.

Procedure Tested

Steroids [21, 22]

Method	Ascending, one-dimensional development in a trough chamber without (A) or with chamber saturation (B).
Layer	HPTLC plates Silica gel 60 F_{254} (Merck) that were prewashed before application of the sample, by developing once to the upper edge of the plate with chloroform – methanol (50 + 50), and then dried at 110 °C for 30 min. In the case of example A. the layer was conditioned to 0% rel. humidity in a conditioning chamber (over conc. sulfuric acid) after sample application.
Mobile phase	A. Cyclohexane – diethyl ether (50 + 50). B. Chloroform – methanol (98 + 2).
Migration distance	A. 6 cm B. 8 cm
Running time	15 min

Detection and result: The chromatogram was first dried in a stream of cold air, immersed in the reagent solution for 1 to 2 s and then heated to 120–125 °C for 15–20 min. Blue-grey to violet chromatogram zones were produced on a colorless background that could be excited to fluoresce various colors with long-wavelength UV light ($\lambda = 365$ nm).

On a dark background cholesterol (Eluent A, hR_f 20–25) emitted blue, coprostanol (Eluent A, hR_f 25–30) blue, 4-cholesten-3-one (Eluent A, hR_f 40–45) blue, 5α-cholestan-3-one (Eluent A, hR_f 60) blue, coprostanone (Eluent A, hR_f 70) blue, estriol 3-sulfate (Eluent B, hR_f 5–10) yellow, 11-ketoetiocholanolone (Eluent B, hR_f 15–20) blue, estrone (Eluent B, hR_f 20–25) ochre, 11-desoxycorticosterone (Eluent B, hR_f 30–35) yellow, 17α-ethinyl-5-androstene-3β,17β-diol (Eluent B, hR_f 45–50) ochre, 4-cholesten-3-one (Eluent B, hR_f 55–60) faint blue and coprostanone (Eluent B, hR_f 65–70) violet fluorescences.

The detection limits lay between 5 ng and 50 ng substance per chromatogram zone.

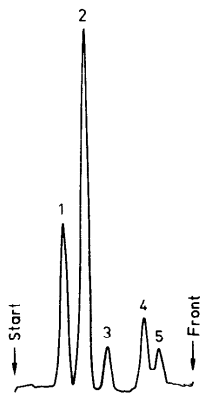


Fig. 1

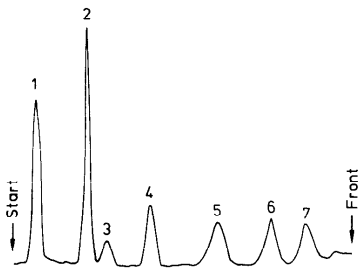


Fig. 2

Fig. 1: Fluorescence scan of a chromatogram track with 255 ng cholesterol (1), 535 ng coprostanol (2), 310 ng 4-cholesten-3-one (3), 320 ng 5α-cholestan-3-one (4) and 220 ng coprostanone (5) per chromatogram zone.

Fig. 2: Fluorescence scan of a chromatogram track with 100 ng each of estriol-3-sulfate (1), 11-ketoetiocholanone (2), estrone (3) 11-desoxycorticosterone (4) and 17α-ethinyl-5-androsten-3β,17β-diol (5), together with 1 µg each of 4-cholesten-3-one (6) and coprostanone (7) per chromatogram zone.

The reagent is not suitable for quantitative determinations because the fluorescences are not stable: In Fig. 1 liquid paraffin – *n*-hexane (1+2) was used to stabilize the fluorescence.

In situ quantitation: The fluorimetric evaluation was carried out with excitation at $\lambda_{exc} = 365$ nm and fluorescense emission was measured at $\lambda_{fl} > 430$ nm (cut off filter Fl 43).

References

[1] Schneider, G., Vincze, I., Hackler, L., Szabo, J., Dombi, G.: *Acta Chim. Acad. Sci. Hung.* **1982,** *110,* 429–440.
[2] Schneider, G., Vincze, I., Vass, A., Hackler, L., Dombi, G.: *Acta Chim. Acad. Sci. Hung.* **1982,** *109,* 71–82.
[3] Schneider, G., Vincze, I., Vass, A.: *Acta Chim. Acad. Sci. Hung.* **1979,** *99,* 51–67.
[4] Agócs, P. M., Czászár, J.: *Acta Chim. Hung.* **1987,** *124,* 541–545.
[5] Bednarski, P. J., Nelson, S. D.: *J. Med. Chem.* **1989,** *32,* 203–213.
[6] Schubert, G., Schneider, G., Schade, W., Dombi, G.: *Acta Chim. Acad. Sci. Hung.* **1982,** *111,* 173–187.
[7] Jansen, G. R., Zanetti, M. E., Hutchinson, C. F.: *Arch. Biochem. Biophys.* **1970,** *138,* 433–442.
[8] Szüts, V. H., Souk, B., Polyák, B., Boross, L.: *Proc. Int. Symp. TLC & OPLC,* Szeged **1984,** 359–363.
[9] Székács, I., Klembala, M.: *Z. Klin. Chem. Klin. Biochem.* **1970,** *8,* 131–133.
[10] Schürenkämper, P., Lisse, K., Lunow, E.: *Zbl. Gynäkol.* **1971,** *93,* 1253–1260.
[11] Szilágyi, I., Sólyom, S., Toldy, L.: *Acta Chim. Hung.* **1984,** *116,* 111–123.
[12] Laitem, L., Gaspar, P., Bello, I.: *J. Chromatogr.* **1978,** *147,* 538–539.
[13] Agócs, P. M., Motika, G., Zsédenyi, P.: *Acta Chim. Acad. Sci. Hung.* **1982,** *110,* 357–362.
[14] Mincsovics, E., Székely, T. J., Hoznek, M., Végh, Z., Zámbó, I., Szepesi, G., Tyihák, E.: *Proc. Symp. Anal. Steroids,* p. 427–431, Eger 1981.
[15] Clarke, C. J., Cobb, P. H.: *J. Chromatogr.* **1979,** *168,* 541–549.
[16] Boctor, F. N.: *J. Chromatogr.* **1972,** *67,* 371–372.
[17] Palyza, V.: *J. Chromatogr.* **1973,** *76,* 499–501.
[18] Andreev, L. V.: *J. Liq. Chromatogr.* **1980,** *5,* 1572–1582.
[19] Srinivasulu, C., Mahapatra, S. N.: *J. Chromatogr.* **1973,** *86,* 261–262.
[20] Chand, S., Srinivasulu, C., Mahapatra, S. N.: *J. Chromatogr.* **1975,** *106,* 475–476.
[21] Schade, M.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1986.
[22] Klein, I., Jork, H.: GDCh-training course No. 300 „Einführung in die Dünnschicht-Chromatographie", Universität des Saarlandes, Saarbrücken 1988.

o-Phthalaldehyde–Sulfuric Acid Reagent

Reagent for:

- Ergot alkaloids and derivatives [1, 2]
  e.g. lysergic acid, LSD, ergotamine, ergocristine
  e.g. hydrogenated ergot alkaloids
  such as dihydrolysergic acid, dihydroergotamine
  dihydroergocristine, dihydroergotoxin
- ß-Blockers [3]
  e.g. propranolol, alprenolol, nadolol
- Histidylpeptides [3]
- Indole derivatives
  e.g. tryptophan, tryptamine,
  serotonin, bufotenin

H_2SO_4	$C_8H_6O_2$
$M_r = 98.08$	$M_r = 134.14$
Sulfuric acid	*o*-Phthalaldehyde

Preparation of the Reagent

Dipping solution Dissolve 0.5–1 g *o*-phthalaldehyde (phthaldialdehyde, OPA) in 45 ml methanol and cautiously add 5 ml sulfuric acid (95–97%) [3].

Spray solution Dissolve 0.2 g *o*-phthalaldehyde cautiously in 100 ml sulfuric acid (95–97%) [1].

Storage The dipping solution is stable for several weeks at room temperature [3], the spray solution may be stored in the refrigerator for 2–3 days [1].

Substances Phthaldialdehyde
Sulfuric acid (95–97%)
Methanol

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 3 s or sprayed homogeneously with the spray solution and – in the case of the ß-blockers – heated to 80 °C for 3 min.

β-Blockers yield yellow to pink-colored chromatogram zones on a colorless background, which, like the zones of the ergot alkaloids and hydrogenated ergot alkaloids, can usually be excited to blue fluorescence on irradiation with long-wavelength UV light ($\lambda = 365$ nm) [1, 3].

Note: *o*-Phthaldehyde in the presence of mercaptoethanol or cysteine has already been discussed as a reagent [4]. The present monograph describes the use of *o*-phthalaldehyde in the presence of sulfuric acid. There are, in addition, a number of applications, which have been described, employing *o*-phthalaldehyde without any additives e.g. for the detection of primary arylamines, histamine, histidine and histidylpeptides [5–7].

The natural fluorescence of ergot alkaloids is considerably augmented by the reagent [1]. Heating for longer than 3 min or to more than 80 °C leads to a reduction in fluorescence intensity and, hence, should be avoided [3].

The fluorescence intensity can be stabilized and enhanced by dipping the chromatograms in a solution of liquid paraffin – *n*-hexane (1+2) [3]. The detection

limits for ß-blockers, ergot and dihydroergot alkaloids and other indole derivatives lie in the lower nanogram range [1, 3, 8].

The reagent can be used, for example, on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

β-Blockers [3]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK), that were prewashed before application of the samples by developing to the upper edge of the plate with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.
Mobile phase	Methanol – ammonia solution (25%) (99+1).
Migration distance	8 cm
Running time	20 min

Detection and result: The chromatogram was dried for 15 min in a stream of warm air, immersed in the dipping solution for 3 s and then dried at 80 °C for 3 min in the drying cupboard. After cooling to room temperature it was immersed for 1 s in a solution of liquid paraffin – *n*-hexane (1+2) to enhance (by a factor of ca. 2) and stabilize the fluorescence intensity and then dried for 3 min in a stream of cold air.

4-Hydroxypropranolol (hR_f 40–45) and propranolol (hR_f 55–60) yielded yellow chromatogram zones on a colorless background that could be excited in long-wavelength UV light (λ = 365 nm) to orange fluorescence on a faint blue fluorescent background. The detection limits were 5 ng substance per chromatogram zone.

In situ quantitation: Fluorimetric measurements were made by exciting at λ_{exc} = 436 nm and measuring the fluorescence emission at λ_{fl} > 560 nm (cut off filter Fl 56).

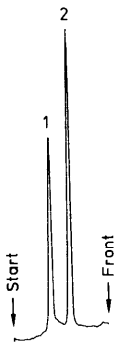


Fig. 1: Fluorescence scan of a chromatogram track with 50 ng each of 4-hydroxypropranolol (1) and propranolol (2) per chromatogram zone.

References

[1] Szabó, A., Karácsony, E. M.: *J. Chromatogr.* **1980**, *193*, 500–503.
[2] Prosek, M., Katic, M., Korsic, J., Kucan, E. in: A. Frigerio (Ed.), *Chromatography in Biochemistry, Medicine and Environmental Research*, Vol. 1, p. 27–36, Elsevier, Amsterdam, 1983.
[3] Azarderakhsh, M.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1988.
[4] Jork, H., Funk, W., Fischer, W., Wimmer, H.: *Thin-Layer Chromatography – Reagents and Detection Methods*, Vol. 1a, VCH-Verlagsgesellschaft, Weinheim, 1990.
[5] Jones, G. R. N.: *J. Chromatogr.* **1973**, *77*, 357–367.
[6] Turner, T. D., Wightman, S. L.: *J. Chromatogr.* **1968**, *32*, 315–322.
[7] Edvinsson, L., Hákanson, R., Rönneberg, A. L., Sundler, F.: *J. Chromatogr.* **1972**, *67*, 81–85.
[8] De Lima, C. G., Pastore, T. C. M., Schwartz, C. A., Cruz, J. S., Sebben, A.: *Talanta* **1991**, *38*, 1303–1307.

Potassium Dichromate–Perchloric Acid–Nitric Acid–Sulfuric Acid Reagent
(Forrest Reagent)

Reagent for:

- Antidepressives
  e.g. imipramine derivatives
  such as imipramine, clomipramine, desipramine, trimipramine [1]
- Neuroleptics
  e.g. phenothiazine derivatives
  such as chlorphenethazine, perazine, promazine [1]
- Antihistamines
  e.g. phenothiazine derivatives
  such as alimemazine [1]

$K_2Cr_2O_7$	$HClO_4$	HNO_3	H_2SO_4
$M_r = 294.19$	$M_r = 100.46$	$M_r = 63.01$	$M_r = 98.08$
Potassium dichromate	Perchloric acid	Nitric acid	Sulfuric acid

Preparation of the Reagent

Solution I	Dissolve 0.2 g potassium dichromate in 100 ml water.
Solution II	Perchloric acid (20%).
Solution III	Nitric acid (50%).
Solution IV	Sulfuric acid (30%).

Spray solution	Carefully mix equal volumes of solutions I to IV.
Dipping solution	Dilute 10 ml of the spray solution with 90 ml water.
Storage	The reagent solutions may be stored over long periods.
Substances	Potassium dichromate Perchloric acid (60%) Nitric acid (65%) Sulfuric acid (95–97%)

Reaction

The mechanism of the reaction is not known. Detection probably depends on the reversible formation of colored radicals [2, 3].

Method

The chromatograms are dried in a stream of cold air, immersed in the dipping solution for 1 s or sprayed homogeneously with the spray solution until the layer becomes transparent and then dried in a stream of cold air for 5 min.

Imipramine and its derivatives produce blue, phenothiazines blue, violet, red or orange to skin-colored chromatogram zones, that fade relatively quickly, on a colorless background (Fig. 1).

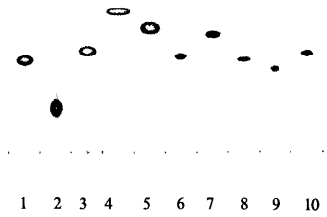


Fig 1: Chromatogram of imipramine and phenothiazine derivatives after staining with FORREST reagent [4]: 1 = imipramine, 2 = desipramine, 3 = clomipramine, 4 = lofepramine, 5 = trimipramine, 6 = thioridazine, 7 = chlorphenethazine, 8 = periciazine, 9 = promazine, 10 = promethazine.

Table 11 Colors of chromatogram zones after treatment with FORREST reagent [1].

Color	Substance			
Edge blue, center pale	clomipramine desipramine	imipramine	lofepramine	trimipramine
Red, orange, pink	alimemazine	dibenzepine	oxypertine	promethazine
	antazoline	dixyrazine	perazine	prothipendyl
	bromhexine	fluphenazine	periciazine	trifluoperazine
	chlorphenethazine	fluspirilene	perphenazine	triflupromazine
	clozapine	homofenazine	promazine	viloxazine
Blue, violet	benperidole	imiclopazine	thiethylperazine	thioridazine
	dihydroergotamine	levomepromazine		
No coloration	acebutolol	butalamine	cytarabine	fenetyline
	amfetaminile	butamirate	dextromethorphane	fenproporex
	aminophenazone	butanilicaine	dextropropoxyphene	fenyramidol
	amitriptyline	chlorazanile	diltiazem	flecainide
	apomorphine	chloroquine	dimetindene	flupentixol
	articaine	chlorphenoxamine	dioxopromethazine	glymidine
	atropine	chlorprothixene	diphenhydramine	haloperidol
	atenolol	chlorotheophylline	diphenylpyraline	lidoflazine
	bamipine	cimetidine	disopyramide	melperone
	benproperine	clemastine	dosulepine	nortriptyline
	benzatropine	clenbuterol	doxapram	oxomemazine
	biperidene	clobutinol	doxepine	pheniramine
	bisacodyl	clomethiazole	doxylamine	pimozide
	bromocriptine	clonidine	drofenine	pipamperone
	brompheniramine	clopamide	eprazinone	tiotixene
	buphenine	codeine	ethenzamide	trazodone
	bupivacaine	caffeine	etozoline	trifluperidol
	bupranolol	cyproheptadine	fendiline	

Note The colors obtained are characteristic for the various substance classes (Table 1). Thus color tones obtained for phenothiazine derivatives are mainly reddish [1]. If the chromatogram zone contains more than 10 µg substance per spot there is only a colored outer ring surrounding an uncolored center (Fig. 1) [1, 4]. The FORREST reagent does not interfere with subsequent detection with the DRAGENDORFF reagent [1, 4].

The detection limits are in the range 100 to 600 ng substance per chromatogram zone [4, 5].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, RP, CN, NH_2, Diol, polyamide and cellulose layers.

Procedure Tested

Dibenzoazepine and Phenothiazine Derivatives [4, 5]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Ethyl acetate – methanol – ammonia solution (25%) (85+10+5).
Migration distance	7 cm
Running time	10–15 min

Detection and result: The chromatogram was dried for 15 min in a stream of cold air, immersed in the dipping solution for 1 s and then dried in a stream of cold air for 5 min.

Immediately after dipping perphenazine (hR_f 28–33) appeared as pink-colored, fluphenazine (hR_f 30–35), dixyrazine (hR_f 33–38) and verophene (= promazine, hR_f 38–43) as skin-colored and periciazine (hR_f 35–40) as orange, promethazine (hR_f 43–48) as bright pink, alimemazine (hR_f 60–65) as skin-colored and dibutil (ethopropazine, hR_f 75–80) as pink-colored chromatogram zones on a colorless background (Fig. 2). The colors faded after some time.

The detection limits of imipramine and its derivatives were 100 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 530$ nm (Fig. 3).

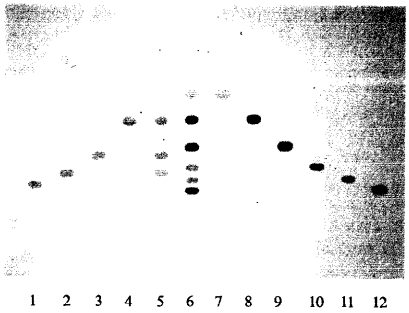


Fig 2: Chromatogram of dibenzoazepine and phenothiazine derivatives after staining with FORREST reagent: 1 = fluphenazine, 2 = periciazine, 3 = promethazine, 4 = alimemazine, 5 = mixture of substances 1 to 4, 6 = mixture of substances 7 to 12, 7 = dibutil, 8 = levomepromazine, 9 = chlorpromazine, 10 = verophene, 11 = dixyrazine, 12 = perphenazine.

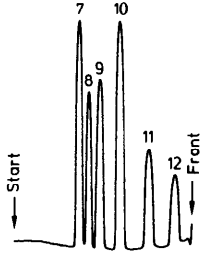


Fig. 3: Reflectance scan of chromatogram track 6 of Figure 2 with 4 µg substance per chromatogram zone each of 7 = dibutil, 8 = levomepromazine, 9 = chlorpromazine, 10 = verophene, 11 = dixyrazine, 12 = perphenazine.

References

[1] Riemer, F., Daldrup, T.: *Pharmazie* **1992**, *47*, 559.
[2] Auterhoff, H., Kühn, J.: *Arch. Pharm.* **1973**, *306*, 241–248.
[3] Forrest, I. S., Forrest, F. M., Berger, M.: *Biochim. Biophys. Acta* **1958**, *29*, 441–443.
[4] Riemer, F.: Private communication, Universität Greifswald, 1992.
[5] Meiers, Bl., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1992.

Potassium Hexaiodoplatinate Reagent

Reagent for:

- Organic nitrogen compounds [1–3]
  - e.g. drug substances and metabolites [4–9]
    - such as benzodiazepines [10, 11], polamidon, dolantin, dilaudid [12]
      carbamazepine, maprotiline, methadone, methaqualone [13]
      phencyclidine [14], imipramine, desimipramine [15]
      morphine [16], naloxone [17], naltrexone [18]
      codeine, 6-acetylmorphine [19], oxaflozane [20]
      pentazocine, tripelennamine [21], chloroquine [22]
  - e.g. alkaloids [23–25]
    - in *Antirrhinum* (26, 27], *Corydalis lutea* [28]
      Hydastis canadensis [29], *Thalictrum polygamum* [30]
      Cinchona ledgeriana [31]
    - such as ajmaline, atropine, ergotamine, raubasine, tropine [23]
      isocorydine, stylopine, bicuculline [28], thebaine [32]
      hydrastine, berberine [29], strychnine [33], cocaine [34]
      methylpalaudinium chloride [30]
  - e.g. quaternary ammonium compounds (surfactants)
    - such as bencetonium chloride [35]
  - e.g. urethanes
    - from the reaction of isocyanates with 1-(2-pyridyl)piperazine [36]
  - e.g. heroin [19, 34]
- Thiols, thioethers and sulfoxides [37–39]
  - e.g. sulfur-containing amino acids
    - such as cysteine, cystine, methionine [37]
  - e.g. antibiotics and derivatives
    - such as penicillin benzathine and -embonate salts [38]
      amoxicillin, penicilloic and penicillic acids, ampicillin,
      tritylpenicillin sulfoxide, benzylpenicillin sulfoxide [39]
- Ketosteroids [3]

Reagent for:

- Vitamins
  - e.g. vitamin D_3 (cholecalciferol), vitamin K_1 [40]
    vitamin B_1 (thiamine) [41]
- Indandione derivatives
  - e.g. pindone, valone [40]
- Thiophosphate pesticides [42]

KI	$H_2(PtCl_6) \cdot 6H_2O$
$M_r = 166.01$	$M_r = 517.92$
Potassium iodide	Hexachloroplatinic acid hexahydrate

Preparation of the Reagent

Dipping solution

Mix 3 ml 10% hexachloroplatinic(IV) acid solution with 97 ml 10% methanol and 100 ml 6% aqueous potassium iodide solution [43].
Variant: Mix 45 ml 10% aqueous potassium iodide solution with 2.5 ml 10% hexachloroplatinic(IV) acid solution and make up to 200 ml with water (pH 2.52) [44].

Spray solution

Add 3 ml 10% hexachloroplatinic(IV) acid solution to 100 ml 6% aqueous potassium iodide solution and dilute with 97 ml water [3, 8, 35, 36, 45].

Storage

The reagent solutions should be stored in brown bottles. They may be kept in the refrigerator for ca. 1 week [8].

Substances

Potassium iodide
Hexachloroplatinic(IV) acid solution (10%)
Methanol

Reaction

The mechanism of the reaction has not been elucidated.

Method

The chromatograms are dried in a stream of warm air, immersed in the dipping solution for 1 to 4 s or sprayed homogeneously with the spray solution and, if necessary, dried in a stream of warm air.

Chromatogram zones of various colors are produced [35], usually immediately, on a pale pink background [36, 39, 44]; they can sometimes also be detected under short-wavelength UV light [λ = 254 nm) [40]. The red coloration of the plate background can be very largely removed by washing out excess reagent with water or with 1% acetic acid [3, 44]. In some cases the colors of the chromatogram zones become deeper if they are heated to 115 °C for 5 min [40].

Alkaloids produce pale yellow, pink, green, brown, blue or violet zones [23]. Urethanes blue-green to dark violet zones [36]. Thiols and penicillin derivatives appear immediately as white zones and sulfoxides only after a few minutes as yellow to yellowish-blue zones on a reddish background [37, 39], which becomes deep purple on spraying with water [37].

Note: Tertiary amines and quaternary ammonium compounds yield stronger colors than primary amines [25]. The dipping solution can also be used as spray solution [44]. Other reagent compositions have also been reported in the literature (1, 3, 6, 12, 13, 15, 18, 21, 23, 41] In some cases the reagents have been made up in acetone [38, 39], methanol [14] or ethanol [37] and/or acidified with hydrochloric acid [3, 33, 37-40]. The concentrations of hexachloroplatinic(IV) acid have been in the range of 0.05-0.4%, those of potassium iodide between 0.5 and 24% [46]. A spray solution containing 2% potassium iodide and 0.23% hexachloroplatinic(IV) acid hexahydrate in N-hydrochloric acid is reported to yield the best coloration results with respect to detection sensitivity and color differentiation in the detection of morphine, codeine, quinine, methadone and cocaine [46]. Acidic reagent solutions have been recommended for benzodiazepines [10, 11]. Sulfones do not react [39].

The detection limits in substance per chromatogram zone are 10 ng for urethanes [36], 10 ng – 1 µg for alkaloids and 50 ng – 1 µg for penicillin derivatives [39].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, RP 18. CN, Diol, polyamide and cellulose layers; NH_2 phases are not suitable – because they decolorize the reagent [44].

Procedure Tested 1

Brucine, Strychnine in Plant Extracts [43]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK), that had been prewashed before application of the samples by developing to the upper edge of the plate with chloroform – methanol [50+50) and then drying at 110 °C for 10 min.
Mobile phase	Acetone – toluene – ammonia solution (25%) (40+15+5).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried for 45 min in a stream of warm air (removal of ammonia!), immersed for 4 s in the dipping solution and dried in a stream of cold air.

Brucine (hR_f 30-35) appeared as a blue chromatogram zone and strychnine (hR_f 50-55) as a brown one on a reddish-brown background. The detection limits were lower than 5 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength of the absorption maximum for brucine λ = 700 nm (Fig. 1).

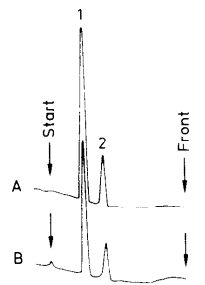


Fig 1: Reflectance scan of a chromatogram of *Nux vomica* extract (A) and of a reference track with 100 ng each of brucine (1) and strychnine (2) per chromatogram zone (B).

Procedure Tested 2

Opium Alkaloids [44]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 WF_{254} (MERCK).
Mobile phase	Acetone – toluene – ethanol – ammonia solution (25%) (40 + 40 + 6 + 2).
Migration distance	7 cm
Running time	10 min

Detection and result: The chromatogram was dried for 30 min at 100 °C and first immersed for 1 s in the dipping solution „variant". Morphine (hR_f 10–15) and codeine (hR_f 15–20) yielded blue-violet chromatogram zones, narceine (hR_f 0), thebaine (hR_f 35–40), papaverine (hR_f 50–55) and narcotine (hR_f 65–70) yielded brown-violet chromatogram zones on a pale red background (Fig. 2A). There was a striking formation of pale half moon-shaped bands over the chromatogram zones in the direction of dipping when the chromatograms were dipped – but not when they were sprayed.

It is advisable to remove excess reagent from the chromatogram before recording the chromatogram zones. This is best done by dipping the treated chromatogram into several fresh 0.5% acetic acid solutions or by „destaining" for two to two and a half minutes (diffusion destaining apparatus, cf. Fig. 24). The chromatogram is then dried in a stream of cold air for 30 min. (Warm air causes fading of the chromatogram zones!)

This procedure yielded a colorless background, on which the colors of the alkaloid zones became pale brown (narceine), blue (morphine) or violet (codeine, papaverine, narcotine) (Fig. 2B).

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 540$ nm (Fig. 3). The detection limits in substance per chromatogram zone were 20 ng for thebaine and papaverine, 200 ng for codeine, 300 ng for morphine and 500 ng for narceine.

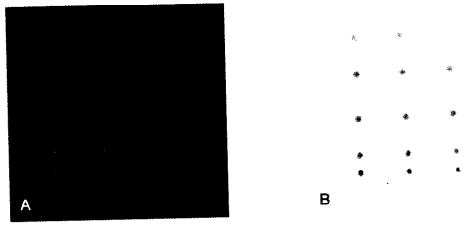


Fig 2: Chromatograms of opium alkaloids (A) after immersion in the reagent solution and (B) after additional washing away of any excess reagent.

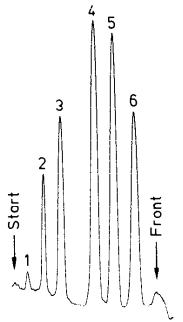


Fig 3: Reflectance scan of a chromatogram track with 1 µg each per chromatogram zone of narceine (1), morphine (2), codeine (3), thebaine (4), papaverine (5) and narcotine (6).

References

[1] Nürnberg, E.: *Arch. Pharm.* **1959**, *292/64*, 610–620.
[2] Verwej, A., De Jong-De Vos, R., Teisman, H. G. J.: *J. Chromatogr.* **1972**, *69*, 407–410.
[3] Krebs, K. G., Heusser, D., Wimmer, H. in E. Stahl (Ed.): *Thin-Layer Chromatography – A Laboratory Handbook*, Springer, Berlin, Heidelberg, New York, 1969.
[4] Viala, A., Estadieu, M.: *J. Chromatogr.* **1972**, *72*, 127–138.
[5] Ho, I. K., Loh, H. H., Way, E. L.: *J. Chromatogr.* **1972**, *65*, 577–579.
[6] Brown, J. K., Shapazian, L., Griffin, G. D.: *J. Chromatogr.* **1972**, *64*, 129–133.

[7] Wallace, J. E., Biggs, J. D., Merritt, J. H., Hamilton, H. E., Blum, K.: *J. Chromatogr.* **1972**, *71*, 135–140.
[8] Berry, D. J., Grove, J.: *J. Chromatogr.* **1971**, *61*, 111–123.
[9] Baselt, R. C., Casarett, L. J.: *J. Chromatogr.* **1971**, *57*, 139–141.
[10] Japp, M., Garthwaite, K., Geeson, A. V., Osselton, M. D.: *J. Chromatogr.* **1988**, *439*, 317–339.
[11] Stead, A. H., Gill, R., Wright, T., Gibbs, J. P., Moffat, A. C.: *Analyst* **1982**, *107*, 1106–1168.
[12] Eberhardt, H., Norden, O.: *Arzneim. Forsch.* **1964**, *14*, 1354–1355.
[13] Daldrup, T. D., Rickert, A.: *Fresenius Z. Anal. Chem.* **1989**, *334*, 349–353.
[14] Rao, K. G., Soni, S. K.: *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 1186–1195.
[15] Ho, B. T., Estevez, V., McIsaac, W. M.: *J. Pharmac. Sci.* **1970**, *59*, 1780–1782.
[16] Hakim, R., Fujimoto, J. M.: *J. Pharmac. Sci.* **1970**, *59*, 1783–1786.
[17] Weinstein, S. H., Pfeffer, M., Schor, J. M., Indindoli, L., Mintz, M.: *J. Pharmac. Sci.* **1971**, *60*, 1567–1568.
[18] Verebey, K., Alarazi, J., Lehrer, M., Mulé, S.J.: *J. Chromatogr.* **1986**, *378*, 261–266.
[19] Yeh, S. Y., McQuinn, R. L., Gorodetzky, C. W.: *J. Pharmac. Sci.* **1977**, *66*, 201–204.
[20] Constantin, M., Pognat, J. F.: *Arzneim. Forsch.* **1979**, *29*, 109–114.
[21] Reid, R. W., Gerbeck, C. M.: *Clin. Chem.* **1981**, *27*, 10–13.
[22] Pachaly, P., Büse, E., Treitner, A., Schick, W.: *Pharm. Ind.* **1992**, *54*, 278–286.
[23] Pothier, J., Galand, N., Viel, N.: *J. Planar Chromatogr.* **1991**, *4*, 392–396.
[24] Baerheim-Svendsen, A.: *J. Planar Chromatogr.* **1989**, *2*, 8–18.
[25] Singh, A. K., Granley, K., Ashraf, M., Mishra, U.: *J. Planar Chromatogr.* **1989**, *2*, 410–419.
[26] Harkiss, K. J.: *Planta Med.* **1972**, *21*, 84–88.
[27] Harkiss, K. J.: *Planta Med.* **1971**, *20*, 108–113.
[28] Preininger, V., Novák, J., Ímánek, V., Ántavy, F.: *Planta Med.* **1978**, *33*, 396–402.
[29] Datta, D. D., Bose, P. C., Ghosh, D.: *Planta Med.* **1971**, *19*, 258–263.
[30] Shamma, M., Moniot, J. L.: *J. Pharmac. Sci.* **1972**, *61*, 295–296.
[31] Mulder-Krieger, T., Verpoorte, R., de Water, A., van Gessel, M., van Oeveren, B. C. J. A.: *Planta Med.* **1982**, *46*, 19–24.
[32] Misra, A. L., Pontani, R. B., Mulé, S. J.: *J. Chromatogr.* **1972**, *71*, 554–556.
[33] Hunter, R. T., Creekmur, R. E.: *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 542–545.
[34] Curry, A. S., Patterson, D. A.: *J. Pharmacy Pharmacol.* **1970**, *22*, 198–201.
[35] Reuvers, T. B. A., Ortiz, G., Ramos, M., De Pozuelo, M. M.: *J. Chromatogr.* **1989**, *467*, 321–326.
[36] Ellwood, P. A., Hardy, H. I., Walker, R. F.: *Analyst* **1981**, *106*, 85–93.
[37] Wong, F. F.: *J. Chromatogr.* **1971**, *59*, 448–451.
[38] Saesmaa, T.: *J. Chromatogr.* **1989**, *463*, 469–473.
[39] Pokorny, M., Vitezic, N., Japelj, M.: *J. Chromatogr.* **1973**, *77*, 458–460.
[40] Opong-Mensah, K., Porter, W. R.: *J. Chromatogr.* **1988**, *455*, 439–443.
[41] Dittmann, J.: *Dtsch. Gesundheitswesen* **1967**, *22*, 1217–1218.
[42] Huang, J. T., Hsiu, H. C., Shih, T. B., Chou, U. T., Wang, K. T., Cheng, C. T.: *J. Pharmac. Sci.* **1968**, *57*, 1620–1621.
[43] Müller, J.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1987.
[44] Jork, H., Klein, I.: GDCh-training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1992.
[45] Hartke, K., Mutschler, E. (Eds.): *DAB 9-Kommentar*, p. 475, Wissenschaftliche Verlagsgesellschaft, Stuttgart 1986.
[46] Frings, C.S., Queen, C.A.: *Clin. Chem.* **1973**, *19*, 664.

Potassium Hydroxide Reagent

Reagent for:

- Cumarin glycosides and their aglycones [1, 2]
  e.g. umckalin, scopoletin [3]
- Anthraquinone glycosides and their aglycones [2, 4–8]
  e.g. chrysophanol, emodin, aloe emodin, rhein [9–12]
  physcione [9, 11, 12], alizarin [10]
- Xanthone glycosides and their aglycones [13]
  e.g. gentisin, isogentisin [14]
- Dalbergion-glycosides and their aglycones [15]
- Pestidices
  e.g. thiophosphate pesticides [16]
  such as bayrusil, fospirate, noltran, menazon
  maretin, dursban, cythioate
- Nitroaryl esters
  e.g. mono and dinitrophenylacetates [17]
- Acetylcholine, adenochrome, nicotinamide [18]
- Cytostatics (antineoplastic agents)
  e.g. 1-ethoxycarbonyl-2-arylazo-2-nitroethane derivatives [19]
- Block copolymers
  e.g. polyacrylonitrile [20]
- Dinitrophenylhydrazones
  e.g. of pyruvic acid, glycolaldehyde [21]

KOH

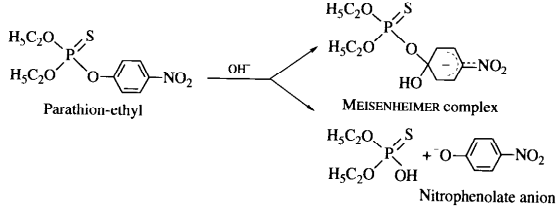
$M_r = 56.11$

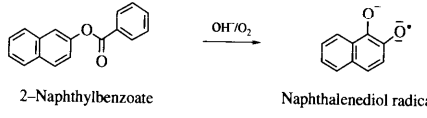
Preparation of the Reagent

Dipping solution	Dissolve 2 g potassium hydroxide in 5 ml water and make up to 50 ml with ethanol [22].
Spray solution	Dissolve 3 to 10 g potassium hydroxide in 100 ml methanol [1, 2, 6, 8–10, 14, 19], ethanol [7, 15, 19] or water [16, 18, 20].
Storage	The reagent solutions can be stored for longer periods.
Substances	Potassium hydroxide pellets Methanol Ethanol

Reaction

The reaction course has not been elucidated (cf. also sodium hydroxide reagent). Hydrolyzation reactions and aromatizations are probably primarily responsible for the formation of colored and fluorescent derivatives. Substituted nitrophenols – e.g. the thiophosphate insecticides – can probably be hydrolyzed to yellow-colored nitrophenolate anions by sodium hydroxide or possibly react to yield yellow MEISENHEIMER complexes. Naphthol derivatives with a tendency to form radicals, e.g. 2-naphthyl benzoate, react with hydrolysis to yield violet-colored mesomerically stabilized 1,2-naphthalenediol radicals.





2-Naphthylbenzoate — Naphthalenediol radical

Method

The chromatograms are dried in a stream of cold air, first sprayed homogeneously with the reagent and, as in the case of anthraquinone derivatives, they are then dried in a steam of warm air for a few minutes [10] or at room temperature for 20 min [11]. In the case of pesticides the chromatogram is covered by a glass plate and heated to 100–200 °C for up to 30 min [16].

Dalbergion glycosides produce green to violet [15], 2-arylazo-2-nitroethane derivatives [19] and polyacrylonitrile (20) yellow to orange-red, dinitrophenylhydrazones yellow to purple-brown and anthraquinone derivatives orange-yellow to purple-colored substance zones [5, 8, 10, 12] that usually emit yellow or pale red to violet fluorescence in UV light ($\lambda = 254$ or 365 nm) [4, 8, 9]. Some thiophosphate pesticides can also be stimulated to fluorescence with long-wavelength UV light ($\lambda = 365$ nm) [16]. Gentisin fluoresces yellow-green [14], cumarins green to dark blue [1].

Note: Methanolic sodium hydroxide solution can replace potassium hydroxide solution [16] (q.v.). The production of color tones and fluorescence is very dependent on the duration and temperature of heating; hence optimal conditions must be determined empirically [16]. Some thiophosphate insecticides do not form fluorescent derivatives [16]. The natural fluorescence of various cumarin derivatives is intensified [1].

It is recommended that the chromatogram treated with reagent be stored for ca. 15 min to allow stabilization of color when undertaking direct quantitation of anthraquinones [10].

The detection limits as substance per chromatogram zone are 1 µg for polyacrylonitrile [20] and 2-arylazo-2-nitroethane derivatives [19] and 6–100 ng for thiophosphate pesticides [16].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, cellulose and polyamide layers as well as on mixed cellulose/polyamide layers [1].

Procedure Tested 1

Thiophosphate Insecticides [22]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer	HPTLC plates Silica gel 60 F_{254} extra thin layer (MERCK), that were prewashed by dipping in 2-propanol overnight and then activated at 110 °C for 30 min.
Mobile phase	*n*-Hexane – diethyl ether – ethanol – ethyl acetate – formic acid (909+40+26+25+1).
Migration distance	8 cm
Running time	30 min

Detection and result: The chromatogram was dried for ca. 3 min in a stream of warm air, immersed in the reagent solution for 3 s and then heated to 110 °C for 15 min.

Parathion-methyl (hR_f 40–45), fenitrothion (hR_f 45–50) and parathion-ethyl (hR_f 60–65) appeared as yellow chromatogram zones on a colorless background. The detection limits lay between 6 ng (parathion, parathion-methyl) and 10 ng (fenitrothion) per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 406$ nm (Fig. 1).

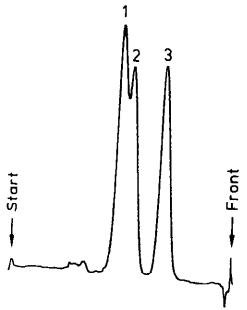


Fig 1: Reflectance scan of a chromatogram track with 300 ng substance per chromatogram zone of parathion-methyl = 1, fenitrothion = 2, parathion-ethyl = 3.

Procedure Tested 2

Cumarin in Asperulae Herba (Woodruff) [23]

Sample solution	A 3 g sample of woodruff was added to 30 ml warm methanol and placed in the ultrasonic bath for 10 min. After filtration the solution was concentrated to ca. 20% of the initial volume under reduced pressure. A portion of the solution was centrifuged at 1200 rpm for 2 min and the clear supernatant was applied to the layer as a band.
Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	Toluene – *tert.*-butyl methyl ether – acetic acid (10%) (40+40+40). The mobile phase is always freshly made up. This is done by mixing the three mobile phase components in a separating funnel and shaking vigorously several times; the top phase is used as mobile phase.
Migration distance	10 cm
Running time	20 min

Detection and result: The chromatogram was dried in a stream of warm air and then inspected under UV light. Cumarin (hR_f 55–60) exhibited fluorescence quenching under short-wavelength UV light ($\lambda = 254$ nm; Fig. 2A); it is not excited to fluorescence emission under long-wavelength UV light ($\lambda = 365$ nm; Fig. 2B). After treatment with the spray solution (1 g KOH pellets in 20 ml ethanol) the cumarin zone produced an intense yellow-green fluorescence when observed under long-wavelength UV light (Fig. 2C), which changed to pale blue after heating the chromatogram (2 min, 100 °C) (Fig. 2D).

It is possible to detect 2 ng cumarin per chromatogram zone visually.

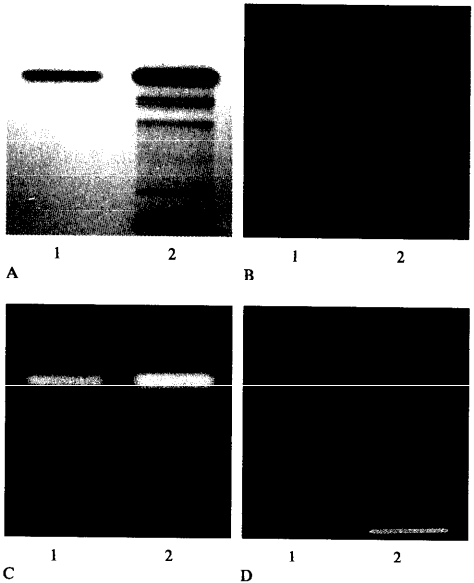


Fig 2: Chromatogram of woodruff extract (track 1: 10 µl 0.1% cumarin solution in methanol, track 2: 100 µl extract; band length 3 cm in each case). Examination in short-wavelength UV light before spraying with the reagent (A) and in long-wavelength UV light before (B) and after spraying (C) and finally after heating (D).

References

[1] Friedrich, H., Zeruhn, E.: *Pharm. Weekblad* **1971**, *106*, 198–206.
[2] E. Merck, Company brochure *Staining reagents for thin-layer and paper chromatography*, Darmstadt 1980.
[3] Bladt, S., Wagner, H.: *Dtsch. Apoth. Ztg.* **1988**, *128*, 292–296.
[4] Haag-Berrurier, M., Garnier, P., Anton, R.: *Planta Med.* **1977**, *31*, 201–211.
[5] Rai, P. P., Turner, T. D., Greensmith, S. L.: *J. Pharmacy Pharmacol.* **1974**, *26*, 722–726.
[6] Tiwari, R. D., Yadava, O. P.: *Planta Med.* **1971**, *19*, 299–305.

[7] Mulder-Krieger, T., Verpoorte, R., De Water, A., Van Gessel, M., Van Oeveren, B. C. J. A., Baerheim Svendsen, A.: *Planta Med.* **1982**, *46*, 19–24.
[8] Kuiper, J., Labadie, R. P.: *Planta Med.* **1981**, *42*, 390–399.
[9] Ma, X., Chen, Y., Hui, R.: *Chromatographia* **1989**, *27*, 465–466.
[10] Rai, P. P., Turner, T. D.: *J. Chromatogr.* **1975**, *104*, 196–199.
[11] Rai, P. P., Shok, M.: *Chromatographia* **1981**, *14*, 599–600.
[12] Friedrich, H., Baier, S.: *Planta Med.* **1973**, *23*, 74–87.
[13] Hostettmann, K., Wagner, H.: *Phytochemistry* **1977**, *16*, 821–829.
[14] Verney, A.-M., Debelmas, A.-M.: *Ann. pharmac. franç.* **1973**, *31*, 415–420.
[15] Dietrichs, H. H., Hausen, B. M.: *Holzforschung* **1971**, *25*, 183–187.
[16] Brun, G. L., Mallet, V.: *J. Chromatogr.* **1973**, *80*, 117–123.
[17] Beyrich, T., Neubauer, E.: *Pharmazie* **1987**, *42*, 824–826.
[18] Galzigna, L., Rizzoli, A. A.: *Clin. Chim. Acta* **1970**, *30*, 5–11.
[19] Upadhyaya, J. S., Upadhyaya, S. K.: *J. Chromatogr.* **1980**, *198*, 224–226.
[20] Gankina, E. S., Efimova, I. I., Kever, J. J., Belenkii, B. G.: *Talanta* **1987**, *34*, 167–174.
[21] Dahms, A. S., Anderson, R. L.: *Biochem. Biophys. Res. Commun.* **1969**, *36*, 809–814.
[22] Pitzer, H.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1990.
[23] Hahn-Deinstrop, E.: Private communication, Fa. Heumann, Abt. Entwicklungsanalytik, D-90478 Nürnberg 1.

$M_r = 166.01$	$M_r \approx 7000$
Potassium iodide	Starch

Preparation of the Reagent

Dipping solution Dissolve 2.8 g potassium iodide and 1.4 g soluble starch according to ZULKOWSKY in 70 ml water and dilute with 30 ml absolute ethanol [10].

Spray solution I *For peroxides*
Solution A: Mix 10 ml 4% aqueous potassium iodide with 40 ml glacial acetic acid and add a spatula-tip of zinc powder. Filter off the zinc powder immediately before using the spray solution [1, 4].
Solution B: Dissolve 1 g soluble starch in 100 ml water with boiling [1, 4].

Spray solution II *For substances containing bromine*: Dissolve 4 g potassium iodide and 2 g soluble starch in 100 ml water with warming [7].

Substances containing active chlorine or bromine oxidize iodide ions – if necessary under the influence of UV light – to iodine, which reacts with starch to yield the well-known intense blue starch-iodine inclusion complex.

Method

For peroxides: The chromatograms are dried in a stream of warm air and immersed in the dipping solution for 2 s [10]. Alternatively they can first be sprayed homogeneously with spray reagent 1A, allowed to stand for 5 min and then sprayed with spray solution 1B until they are transparent [1, 4].

For substances containing bromine: The dried chromatograms are immersed in the dipping solution for 2 s or sprayed homogeneously with spray solution II and then, while still moist, they are irradiated with intense UV light for ca. 1 to 3 min. [7, 10].

For sulfoxides: The dried chromatograms are sprayed homogeneously with spray reagent III. After a few minutes they can then be sprayed with spray solution IB to increase the color contrast [8, 9].

In all cases intense blue or brown-colored chromatogram zones are produced on a colorless to brownish background.

Potassium Iodide–Starch Reagent

Reagent for:

- Peroxides, hydroperoxides [1, 2]
  e.g. (photo)-oxidation products
  of limonene [3]
  linoleic acid [4], methyl linoleate [5]
  methyl oleate and methyl elaidate [6]
- Bromine-containing barbiturates and ureides [7]
- Sulfoxides [8]

Spray solution III *For sulfoxides*: Dissolve 5 g starch and 0.5 g sodium iodide in 100 ml water with warming. Add 1 ml conc. hydrochloric acid to 10 ml of the solution immediately before use [8].

Storage The reagent solutions may be stored for a few days.

Substances
Potassium iodide
Starch, soluble acc. to ZULKOWSKY
Acetic acid (100%)
Zinc powder
Sodium iodide
Hydrochloric acid (32%)
Ethanol

Reaction

KI $(C_6H_{10}O_5)_n$

Note: Separate potassium iodide and starch solutions can also be used successively [2].

The detection limits for bromureides are 40 to 200 ng substance per chromatogram zone [7, 10]. Bromopride, bromazepam, bromhexine and bromocriptine do not react [10].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, cellulose and NH_2 layers. CN, Diol and polyamide phases are unsuitable.

Procedure Tested

Bromureides [10]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 (MERCK).
Mobile phase	Dichloromethane – diethyl ether [17+3].
Migration distance	5 cm
Running time	5 min

Detection and result: The chromatogram was dried in a stream of warm air and immersed in the dipping solution for 2 s. The excess water drops were then removed from the surface of the layer in a stream of cold air. The chromatogram was then intensively irradiated with UV light (mercury lamp St 41, distance from layer 5 cm).

Bromisoval (hR_f 15–20) yielded dark brown chromatogram zones on a light brown background. Bromopride, bromazepam, bromhexine, bromocriptine, caryophyllene epoxide and rose oxide did not react.

The detection limit of bromisoval is 40 ng substance per chromatogram zone.

In situ quantitation: Quantitative evaluation was not possible.

References

[1] Krebs, K. G., Heusser, D., Wimmer, H. in E. Stahl (Ed.): *Thin-Layer Chromatography – A Laboratory Handbook*, Springer, Berlin, Heidelberg, New York, 1969.
[2] Mangold, H. K.: *J. Am. Oil Chem. Soc.* **1961**, *38*, 708–727.

[3] Schieberle, P., Maier, W., Firl, J., Grosch, W.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1987**, *10*, 588–593.
[4] Satoh, T., Matsuda, Y., Takashio, M., Satoh, K., Beppu, T., Arima, K.: *Agric. Biol. Chem.* **1976**, *40*, 953–961.
[5] Morita, M., Fujimaki, M.: *J. Agric. Food Chem.* **1973**, *21*, 860–863.
[6] Sliwiok, J., Kowalski, J., Wasielewska, A.: *Microchem. J.* **1972**, *17*, 576–587.
[7] Vidic, E.: *Arch. Toxikol.* **1970**, *27*, 19–39.
[8] Chatfield, D. H., Hunter, W. H.: *Biochem. J.* **1973**, *134*, 879–884.
[9] Thompson, J. F., Arnold, W. N., Morris, C. J.: *Nature* **1963**, *197*, 380–381.
[10] Meiers, Bl., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1992.

Potassium Nitrate–Sulfuric Acid Reagent

Reagent for:

- Alkaloids
  e.g. colchicine [1]

KNO_3	H_2SO_4
$M_r = 101.11$	$M_r = 98.08$

Preparation of the Reagent

Dipping solution	Dissolve 100 mg potassium nitrate in 5 ml sulfuric acid (95–97%) and make up to 100 ml with ethanol [1].
Storage	The reagent solution can be stored for ca. 1 month.
Substances	Potassium nitrate Sulfuric acid (95–97%) Ethanol

Reaction

The mechanism of the reaction has not been elucidated. Nitration probably takes place.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 4 s or homogeneously sprayed with it, dried in a stream of cold air and then heated to 110 °C for 10 min.

Yellow chromatogram zones are produced on a colorless background.

Note: The detection limit for colchicine is 5 ng substance per chromatogram zone.

The reagent can, for example, be used on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Colchicine [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK) that were prewashed before use by developing once to the upper edge of the plate, before application of the sample, with chloroform – methanol (50+50) and then dried at 110 °C for 10 min.
Mobile phase	Acetone – toluene – ammonia solution (25%) (40+15+5).
Migration distance	5 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of warm air for 45 min in order to remove the ammonia completely, then cooled to room temperature (ca. 5 min), immersed in the dipping solution for 4 s, dried in a stream of cold air and then heated to 110 °C for 10 min.

Colchicine (hR_f 35–40) appeared as a yellow chromatogram zone on a colorless background. The detection limit lay below 5 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation was carried out in reflectance at a wavelength of $\lambda = 380$ nm (Fig. 1).

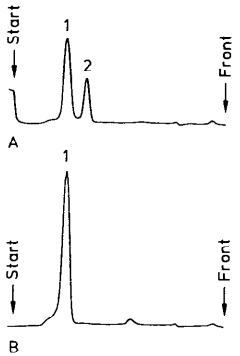


Fig. 1: Reflectance scan of a chromatogram track of a *Colchicum autumnale* extract (A) and of a reference track with 1 µg colchicine (1) per chromatogram zone (B); unknown substance (2).

References

[1] Müller, J.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1987.

Potassium Peroxodisulfate–Silver Nitrate Reagent

Reagent for:

- Aromatic amines and phenols [1–4]
  e.g. resorcinol, catechol, aminonaphthols
- Indole, *m*-dinitrobenzene, pyrene [2]
- Sulfapyridine [2, 3]

$K_2S_2O_8$	$AgNO_3$
$M_r = 270.33$	$M_r = 169.87$

Preparation of the Reagent

Dipping solution	Dissolve 1 g potassium peroxodisulfate and 34 mg silver nitrate in 60 ml water and make up to 100 ml with acetone [5].
Spray solution	Dissolve 1 g potassium peroxodisulfate and 17–34 mg silver nitrate in a mixture of 1 ml acetone and 99 ml water [1, 2] or in pure water [3].
Storage	The reagent solutions may be kept for extended periods.
Substances	Potassium peroxodisulfate Silver nitrate Acetone

Reaction

Peroxodisulfate ions oxidize aromatic amines and phenols to colored derivatives, particularly under the catalytic influence of silver ions [1-4].

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution and then heated to 50-110 °C for 2-5 min.

Yellow, orange to violet or grey-black chromatogram zones are produced, generally before heating. The background is colorless [1-4].

Note: The dipping solution can also be used as a spray solution. Aromatic amines react more sensitively than do phenols [1]. The presence of acetone in the reagent increases the sensitivity for some substances, e.g. for sulfapyridine [2].

The visual detection limits for aromatic amines and phenols are 100-600 ng substance per chromatogram zone [1-3].

The reagent can, for example, be used on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Aminophenols [5]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer HPTLC plates Silica gel 60 F_{254} (MERCK) that were prewashed before use by developing once to the upper edge of the plate, before application of the sample, with chloroform – methanol (50+50) and then dried at 110 °C for 30 min.

Mobile phase Toluene – methanol (10+10).

Migration distance 5 cm

Running time 15 min

Detection and result: The chromatogram was first dried in a stream of cold air for 5 min, then immersed in the dipping solution for 1 s, briefly dried in a stream of cold air and then heated to 110 °C for 2 min.

2-Aminophenol (hR_f 70-75, λ_{max} = 430 nm) appeared as a yellow-green chromatogram zone and 4-aminophenol (hR_f 60-65, λ_{max} = 360-380 nm) as a grey-brown chromatogram zone on a colorless background. The photometric detection limits are 50 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at a mean wavelength of λ = 400 nm (fig. 1).

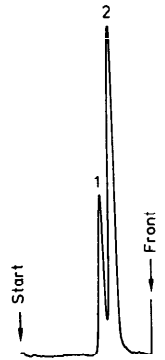


Fig. 1: Absorption scan of a chromatogram track with 200 ng each per chromatogram zone of 4-aminophenol (1) and 2-aminophenol (2).

References

[1] Khulbe, K. C., Mann, R. S.: *Fresenius Z. Anal. Chem.* **1988**, *330*, 642.
[2] Khulbe, K. C., Mann, R. S.: *J. Chromatogr.* **1981**, *208*, 167–169.
[3] Khulbe, K. C., Mann, R. S.: *J. Chromatogr.* **1978**, *150*, 554–556.
[4] Srivastava, S. P., Gupta, R. C., Gupta, A.: *Fresenius Z. Anal. Chem.* **1972**, *262*, 31–32.
[5] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1989.

Selenium Dioxide Reagent

Reagent for:

- Aromatic amines, amino phenols [1]
  e. g. aromatic *o*-diamines [2]
- Polyhydric phenols [1]
  e. g. resorcinol, pyrogallol
- Reducing substances [1]
  e. g. hydrazine, phenylhydrazine, ascorbic acid
- Alkaloids
  e. g. brucine, cinchonine [1]

SeO_2

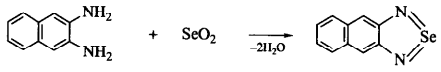
$M_r = 110.96$

Preparation of the Reagent

Dipping solution	Dissolve 0.5 g selenium dioxide in 50 ml methanol and add 1 ml glacial acetic acid [2].
Spray solution	Dissolve 3 g selenium dioxide in 100 ml water [1].
Storage	Both reagents may be stored for longer periods.
Substances	Selenium dioxide Methanol Acetic acid (100%)

Reaction

The reaction mechanism has not been elucidated. Reducing substances presumably release red elementary selenium [1]. Aromatic *o*-diamines yield highly fluorescent selenodiazoles with selenium dioxide.



2,3-Diaminonaphthalene Selenium dioxide Selenodiazole

Method

The chromatograms are freed from mobile phase in a stream of warm air, then immersed twice in the dipping solution for 2 s or sprayed homogeneously with the spray solution and then heated to 120 °C for 15–20 min.

Variously colored chromatogram zones appear, some before heating, on a colorless background [1]; those produced by aromatic *o*-diamines are excited to fluorescence by long-wavelength UV light ($\lambda = 365$ nm) [2].

Note: Reducing sugars do not react [1]. In the course of a few days the chromatogram zones gradually acquire brown-black discoloration, presumably as a result of the production of elementary selenium [1].

The detection limits for aromatic amines are 1–2 µg substance per chromatogram zone [1] and 3 ng substance per chromatogram zone for aromatic *o*-diamines [2].

The reagent can be employed, for example, on silica gel, kieselguhr and on Si 50000 layers.

Procedure Tested

Aromatic *o*-Diamines [2]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 (MERCK).
Mobile phase	2-Propanol – chlorobenzene – *n*-hexane – water – ammonia solution (25 %) (52+34+20+10+5).
Migration distance	6 cm
Running time	35 min

Detection and result: The chromatogram was freed from mobile phase for 5 min in a stream of cold air, immersed twice in the dipping solution for 2 s and then dried for 5 min in a stream of cold air. In order to stabilize and enhance the fluorescence intensity it was then immersed twice for 2 s in a solution of Triton X-100 – chloroform (1+4), with the chromatogram being kept in the dark between and after these dipping processes.

After ca. 30 min, when the chloroform had evaporated, fluorescent chromatogram zones appeared on a dark background on excitation with long-wavelength UV light ($\lambda = 365$ nm): 2,3-diaminonaphthalene (hR_f 70–75), red and 2,3-diaminopyridine (hR_f 55–60), blue. The detection limits were 3 ng substance per chromatogram zone.

In situ quantitation: The fluorimetric scans were carried out at several combinations of excitation and measurement wavelengths (Fig. 1).

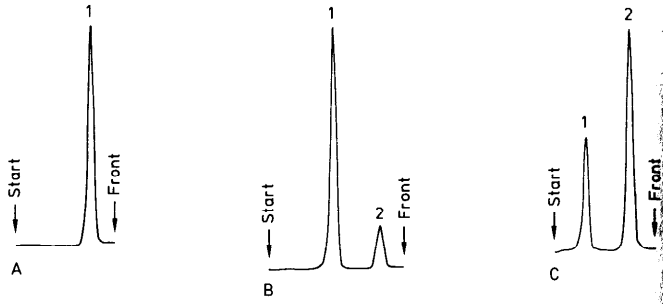


Fig. 1: Fluorescence scans of a chromatogram track with 60 ng each of 2,3-diaminopyridine (1) and 2,3-diaminonaphthalene (2) per chromatogram zone:
A) λ_{exc} = 365 nm and λ_{fl} > 560 nm (cut off filter Fl 56); B) λ_{exc} = 313 nm and λ_{fl} > 390 nm (cut off filter Fl 39); C) λ_{exc} = 365 nm and λ_{fl} > 390 nm (cut off filter Fl 39).

References

[1] Mitchell, S. C., Waring, R. H.: *J. Chromatogr.* **1978**, *151*, 249–251.
[2] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.

Sodium Hydroxide Reagent

Reagent for:

- α-Pyrone derivatives
  e. g. umbelliprenine [1]
  cumarin [2–4]
  dicumarol [5]
- Phenyl-1,3-indandione metabolites [5]
- Mycotoxins
  e. g. patulin as 2,4-DNPH derivative [6]
- Quinones [7]
  e. g. antibiotics
  such as xanthomegnin, viomellein [8]
- Pesticides
  e. g. insecticides
  such as phosalone [9], carbofuran [10], sevin [11]
  organophosphorus pesticides
  such as bayrusil, cythioate, dursban, menazon [12];
  paraoxon [13]
  2-*sec*-butyl-4,6-dinitrophenyl herbicides and acaricides [14]
  such as dinobuton, 2-*sec*-butyl-4,6-dinitrophenol
- Pharmaceuticals
  e. g. in urine [15]
- Steroid and stilbene derivatives
  e. g. *trans*-stilbene metabolites [16]
  Δ^4-3-ketosteroids [17]
- Glucose-8-methionine [18]
- Homogentisic acid [19]
- Arabinosylcytosine [20]
- Sennosides [21]
- α-Naphthol [11]

NaOH

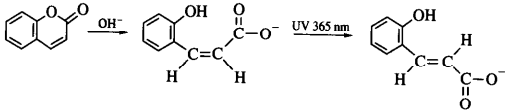
M_r = 40.00

Preparation of the Reagent

Dipping solution	Sodium hydroxide solution (c = 0.1 ... 1 mol/L) in methanol – water (60+40) [22].
Spray solution	Sodium hydroxide solution (c = 1 to 2.5 mol/L) in methanol-water mixtures (e.g. 50+50) [3, 5–7, 9–14, 16, 18, 21].
Storage	The solutions may be stored in the refrigerator for longer periods.
Substances	Sodium hydroxide pellets Methanol

Reaction

The course of the reaction has not been fully clarified. Hydrolytic and aromatization processes are probably responsible for the formation of colored or fluorescent derivatives (cf. Potassium Hydroxide Reagent). For instance, sevin is converted to α-naphthol [11] and paraoxon to the yellow 4-nitrophenolate anion [13]. In the case of α-pyrone derivatives it is assumed that the alkali metal salt of the *o*-hydroxycinnamic acid produced by hydrolytic cleavage of the pyrone ring is converted from the non-fluorescent *cis*- to the fluorescent *trans*-form by the action of long-wavelength UV light (λ = 365 nm) [2].



Cumarin — Anion of *cis-ortho*-hydroxycinnamic acid — Anion of *trans-ortho*-hydroxycinnamic acid

Method

The developed chromatograms are freed from mobile phase in a stream of cold air for 5 min, then immersed in the dipping solution for 2 s or homogeneously sprayed with the spray solution and heated to 80–150 °C for 5–10 min.

Colored or, under long-wavelength UV light (λ = 365 nm), yellow or blue fluorescent chromatograph zones appear, even after drying the chromatogram in a stream of cold air, but sometimes only after heating to 80 °C [17], to 130 °C [9] or 150 °C [22] for 5–10 min.

Note: It is occasionally recommended that after it has been sprayed the plate should be covered with a glass plate for several minutes until optimum reaction has occurred [11], or be irradiated with long-wavelength UV light (λ = 365 nm) [2]. Methanolic potassium hydroxide solution can also be used in place of sodium hydroxide [12] (see Potassium Hydroxide Reagent). The formation of colors and fluorescences depends on the length of heating and on the temperature employed; optimum conditions must be discovered empirically [12].

The 2,4-dinitrophenylhydrazone of patulin and other mono-2,4-dinitrophenylhydrazones form red zones, 2-*sec*-butyl-4-amino-6-nitrophenol appears as a red-orange zone while dinitrophenols and their esters are colored yellow [14]. A whole range of organophosphorus pesticides do not give any reaction [12].

The detection limits per chromatogram zone are 1 ng for sevin and α-naphthol [11], 1 to 100 ng for organophosphorus pesticides, 500 ng for paraoxon [13] and 2 ng for cumarin [3].

The reagent can, for example, be used on silica gel, kieselguhr, Si 50000, NH_2 and diol layers.

Procedure Tested

Δ^4-3-Ketosteroids and Stilbene Derivatives [22]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (Merck).
Mobile phase	Chloroform – methanol (98+2).
Migration distance	8 cm
Running time	15 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air for 5 min, immersed in the dipping solution for 2 s (c = 1 mol/L) and heated to 150 °C for 10 min.

Weakly fluorescent zones were visible under long-wavelength UV light ($\lambda = 365$ n (Fig. 1). Cortisone (hR_f 0–5), dienestrol (hR_f 10–15), 4-androstene-3,17-dione (h 50–55) and 4-cholesten-3-one (hR_f 60–65) had an ochre fluorescence. Diethylsti estrol (hR_f 10–15), 17α-ethinyl-1,3,5-estratriene-3,17β-diol (hR_f 25–30) and estro (hR_f 35–40) had a blue emission.

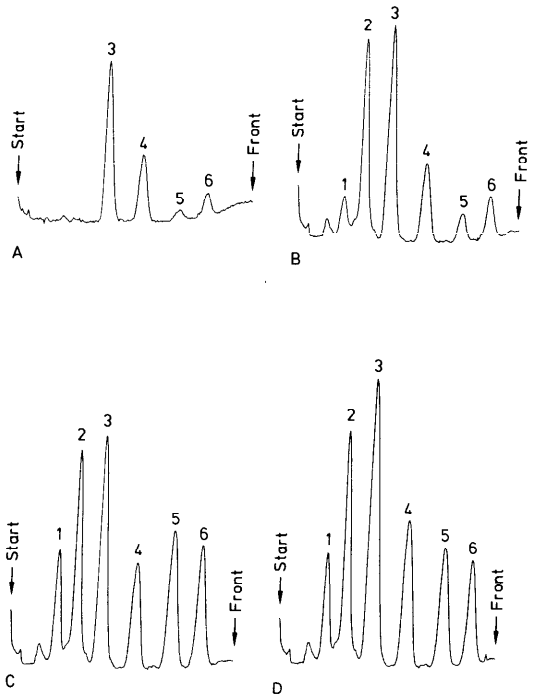


Fig. 1: Fluorescence scan of a chromatogram track with 1 µg cortisone (1), 100 ng dienestrol (2), 300 ng 17α-ethinyl-1,3,5-estratriene-3,17β-diol (3), 100 ng estrone (4) and 1 µg each of 4-androstene-3,17-dione (5) and 4-cholesten-3-one (6): **A.** before immersion in Triton X-100, **B.** after immersion followed by brief drying, **C.** after heating to 120 °C for 10 minutes and **D.** for a further 20 minutes to increase the fluorescence.

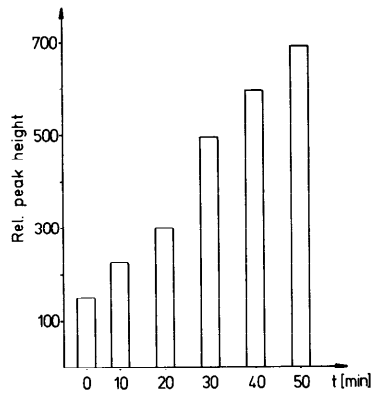


Fig. 2: Increase in the fluorescence intensity of dienestrol as a function of heating time after immersion of the chromatogram in sodium hydroxide solution (c = 1 mol/L) followed by treatment with Triton 100-X and heating to 120 °C.

After heating the chromatogram was immersed in a solution of 2 ml Triton X-100 in 5 ml chloroform plus 35 ml *n*-hexane in order to intensify the fluorescence and then dried in a stream of cold air. This brought about an appreciable increase in the fluorescence intensity of dienestrol and diethylsilbestrol, while the intensities of the other steroids were only marginally increased (Fig. 1).

Heating the chromatogram to 120 °C after treatment with Triton X-100 led to a further increase in sensitivity that was dependent on the length of heating (Fig. 2).

The detection limits lay between 0.5 and 30 ng substance per chromatogram zone.

In situ quantitation: The fluorescence scan was carried out at $\lambda_{exc} = 365$ nm and $\lambda_{fl} > 430$ nm (cut off filter Fl 43).

References

[1] Abu-Mustafa, E. A., El-Bay, F. K., Fayez, M. B.: *J. Pharmac. Sci.* **1971**, *60*, 788–789.
[2] Laub, E., Olszowski, W.: *Z. Lebensm. Unters. Forsch.* **1982**, *175*, 179–181.
[3] Laub, E., Olszowski, W., Woller, R.: *Dtsch. Apoth. Ztg.* **1985**, *125*, 848–850.
[4] Kröller, E.: *Z. Lebensm. Unters. Forsch.* **1973**, *152*, 216–218.
[5] Daenens, P., Boven, M. van: *J. Chromatogr.* **1971**, *57*, 319–321.
[6] Stinson, E. E., Huhtanen, C. N., Zell, T. E., Schwartz, D. P., Osman, S. F.: *J. Agric. Food Chem.* **1977**, *25*, 1220–1222.
[7] Vannini, G. L., Dall'olio, G., Bonava, A.: *Experientia* **1974**, *30*, 203–205.
[8] Sedmera, P., Volc, J., Weijer, J., Vokoun, J., Musilek, V.: *Collect. Czech. Chem. Commun.* **1981**, *46*, 1210–1216.
[9] Guardigli, A., Chow, W., Martwinski, P. M., Lefar, M. S.: *J. Agric. Food Chem.* **1971**, *19*, 742–744.
[10] Stenersen, J., Gilman, A., Vardanis, A.: *J. Agric. Food Chem.* **1973**, *21*, 166–171.
[11] Frei, R. W., Lawrence, J. F., Belliveau, P. E.: *Z. Anal. Chem.* **1971**, *254*, 271–274.
[12] Brun, G. L., Mallet, V.: *J. Chromatogr.* **1973**, *80*, 117–123.
[13] Breuer, H.: *J. Chromatogr.* **1982**, *243*, 183–187.
[14] Bandal, S. K., Casida, J. E.: *J. Agric. Food Chem.* **1972**, *20*, 1235–1245.
[15] Bastos, M. L., Kananen, G. E., Young, R. M., Monforte, J. R., Sunshine, J.: *Clin. Chem.* **1970**, *16*, 931–940.
[16] Sinshcimer, J. E., Smith, R. V.: *Biochem. J.* **1969**, *111*, 35–41.
[17] E. Merck, Company brochure *"Dyeing Reagents for Thin-layer and Paper Chromatography"*, Darmstadt 1980.
[18] Lindsay, R. C., Lau, V. K.: *J. Food Sci.* **1972**, *37*, 787–788.
[19] Humbel, R., Marchal, C., Neu-Fischer, M.: *Z. Klin. Chem. Klin. Biochem.* **1973**, *11*, 447–448.
[20] Notari, R. E., Chin, M. L., Wittebort, R.: *J. Pharmac. Sci.* **1972**, *61*, 1189–1196.
[21] Khafagy, S. M., Girgis, A. N., Khayyal, S. E., Helmi, M. A.: *Planta Med.* **1972**, *21*, 304–309.
[22] Klein, I., Jork, H.: GDCh-training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken 1989.

Sodium Nitrite–Naphthol Reagent

Reagent for:

- Primary aromatic amines [1–3]
  e.g. substituted anilines [4, 5]
- Sulfonamides [3, 6]
  e.g. sulfadiazine, sulfanilamide, sulfathiazole, sulfamerazine, sulfamethazine

$NaNO_2$	$C_{10}H_8O$
$M_r = 69.00$	$M_r = 144.17$
Sodium nitrite	1-Naphthol

Preparation of the Reagent

Dipping solution I Dissolve 5 g sodium nitrite in 20 ml water, add 2 ml hydrochloric acid (32%) and make up to 100 ml with ethanol [7].

Dipping solution II Dissolve 5 g 1-naphthol (α-naphthol) in 100 ml ethanol [7].

Spray solution I Dissolve 1 to 5 g sodium nitrite in 100 ml hydrochloric acid (c = 0.2 to 1 mol/L) [4, 6].

Spray solntion II Dissolve 0.2 to 5 g 1-naphthol [4] or 2-naphthol (ß-naphthol) [5, 6] in 100 ml methanol [4] or sodium hydroxide solution (c = 0.1 mol/L) [6].

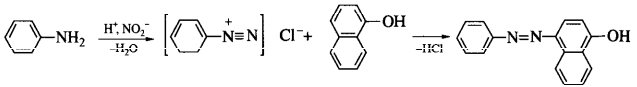
Storage All reagent solutions may be stored in the refrigerator for several days [7].

Substances Sodium nitrite
1-Naphthol
2-Naphthol

Hydrochloric acid (c = 1 mol/L)
Hydrochloric acid (32%)
Ethanol
Methanol

Reaction

Primary aromatic amines are first diazotized by the action of sodium nitrite in acidic solution and then coupled, for instance, with 1-naphthol to form azo dyes (cf. BRATTON-MARSHALL reagent, Vol. 1a).



Prim. aromat. amine — Diazonium salt — 1-Naphthol — Azo dye

Method

The chromatograms are freed from mobile phase in a stream of cold air, immersed in reagent solution I for 1 s or sprayed with it homogeneously until the layer begins to be transparent, dried for 10 min in a stream of cold air (or 5 min at 50–100 °C [4, 6]), immersed in solution II for 1 s or sprayed with it homogeneously and finally dried in a stream of warm air for 5 min.

Pink-red to orange-colored chromatogram zones are formed, usually at once, on a colorless background. Brown to black colors are also sometimes produced [1].

Note: The diazotization of primary aromatic amines can also be carried out by treating the chromatograms for 3–5 min with nitrous fumes in a twin-trough chamber; the fumes are generated by adding 25% hydrochloric acid to 20% sodium nitrite solution in the free trough [5]. Alternatively the diazotization can be carried out by spraying the chromatograms with a solution of 3% pentyl nitrite and 3% formic acid in diethyl ether [1]. The α- or β-naphthol in the reagent can be replaced by N-(1-naphthyl)ethylenediamine (cf. BRATTON-MARSHALL reagent, Vol. 1a).

A few aromatic amines do not react: e.g. *o*-substituted diamines yield benzotriazoles that cannot couple [1].

The detection limits per chromatogram zone are 250 ng for sulfonamides [6] and 80 ng for substituted anilines [7].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Substituted Anilines [7]

Method	Ascending, one-dimensional, two-fold development at 20 °C in a trough chamber without chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK) that are precleaned by immersion overnight in 2-propanol and then dried at 110 °C for 30 min.
Mobile phase	1. Methanol – acetic acid (100+1). 2. Chloroform – *n*-hexane – diisopropyl ether – dichloromethane – formic acid (50+35+10+5+0.45).
Migration distance	1. 0.8 cm 2. 6.5 cm
Running time	1. 1 min 2. 25 min

Detection and result: The chromatogram was dried (5 min in a stream of cold air), immersed in dipping solution I for 2 s and then dried in a stream of cold air. It was then immersed in dipping solution II for 1 s and dried in a stream of warm air for 5 min. Sulfanilic acid (hR_f 10–15), 4-isopropylaniline (hR_f 25–30), 4-chloroaniline (hR_f 40–45) and 3,4-dichloroaniline (hR_f 45–50) yielded pink-colored chromatogram zones on a colorless background.

The detection limits lay between 20 ng (4-chloroaniline) and 80 ng (3,4-dichloroaniline) substance per chromatogram zone.

In situ quantitation: The absorption photometric scans were carried out in reflectance at a wavelength of $\lambda = 520$ nm (Fig. 1).

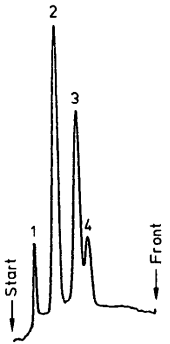


Fig. 1: Reflectance scan of a chromatogram track with 30 ng sulfanilic acid (1) and 100 ng each of 4-isopropylaniline (2), 4-chloroaniline (3) and 3,4-dichloroaniline (4) per chromatogram zone.

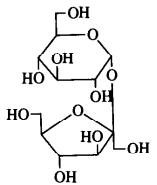
References

[1] Jones, G. R. N.: *J. Chromatogr.* **1973,** *77,* 357–367.
[2] Stahl, E., Zimmer, C., Juell, S.: *Z. Lebensm. Unters. Forsch.* **1982,** *175,* 88–92.
[3] E. Merck, Company brochure *"Dyeing Reagents for Thin-layer and Paper Chromatography"*, Darmstadt 1980.
[4] Gillio-Tos, M., Previtera, S. A., Vimercati, A.: *J. Chromatogr.* **1964,** *13,* 571–572.
[5] Toth, I.: *Helv. Chim. Acta* **1971,** *54,* 1486–1487.
[6] Bican-Fister, T., Kajganovic, V.: *J. Chromatogr.* **1963,** *11,* 492–495.
[7] Ditthard, A.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1990.

Sucrose–Hydrochloric Acid Reagent

Reagent for:

- Phenols [1]



$C_{12}H_{22}O_{11}$ — $M_r = 342.30$ — Sucrose

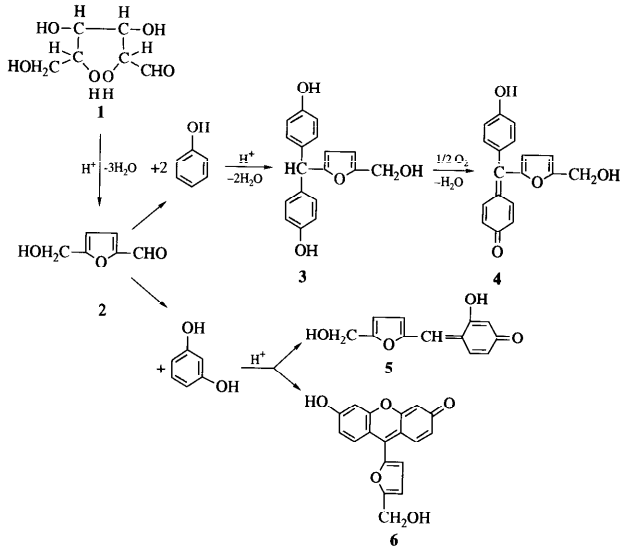
HCl — $M_r = 36.46$ — Hydrochloric acid

Preparation of the Reagent

Dipping solution	Dissolve 2 g sucrose in 5 ml water and 5 ml hydrochloric acid (32%) and make up to 100 ml with ethanol [1].
Storage	The dipping solution can be stored over a longer period.
Substances	Sucrose Hydrochloric acid (32%) Ethanol Ammonia solution (25%)

Reaction

The hexoses that are the initial products of acid hydrolysis of sucrose (1) react at elevated temperature under the influence of acids to yield furfural derivatives (2). These condense, for example, with the phenols to yield triarylmethanes (3), these react further by oxidizing to yield colored quinoid derivatives (2, 4). Polyhydric phenols, e. g. resorcinol, on the other hand, yield condensation products of Types 5 and 6 [2].



Method

The chromatogram is freed from mobile phase in a stream of warm air, immersed in the reagent solution for 2 s or homogeneously sprayed with it and briefly dried in a stream of warm air. It is then heated to 110 °C for 2 min and, after cooling to room temperature, treated with ammonia vapor for 5 min in a twin trough chamber whose empty trough contains 10 ml 25% ammonia solution.

Yellow to brown chromatogram zones are formed on a colorless background.

Note: In long-wavelength UV light ($\lambda = 365$ nm) derivatized phloroglucinol emits a reddish fluorescence that is not suitable for quantitative analysis [1].

The detection limits are in the lower nanogram range.

The reagent can, for example, be used on silica gel, kieselguhr, Si 50000, CN, diol, cellulose and RP layers.

Procedure Tested

"Trihydric" Phenols [1]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	HPTLC plates CN F_{254s} (MERCK).
Mobile phase	Toluene – ethyl acetate (80+20).
Migration distance	6 cm
Running time	10 min

Detection and result: The chromatogram was dried in a stream of warm air for 10 min, immersed in the reagent solution for 2 s, briefly dried in a stream of warm air and heated to 110 °C for 2 min. After cooling to room temperature it was then exposed to ammonia vapors for 5 min (5 ml ammonia solution in the empty trough of a twin trough chamber).

Phloroglucinol (hR_f 15–20) yielded an ochre-colored and pyrogallol (hR_f 35–40) a brown chromatogram zone on a colorless background. The detection limits were 20 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric analysis in reflectance was carried out either at the absorption maximum of the pyrogallol derivative at $\lambda_{max} = 350$ nm (Fig. 1A) or at the absorption maximum of the phloroglucinol derivative at $\lambda_{max} = 420$ nm (Fig. 1B).

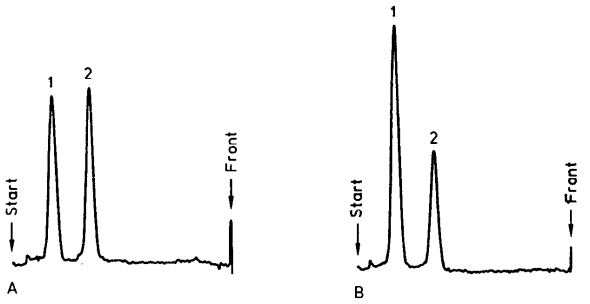


Fig. 1: Reflectance scans of a chromatogram track with 200 ng each of (1) phloroglucinol and (2) pyrogallol per chromatogram zone at wavelengths $\lambda = 350$ nm (A) and $\lambda = 420$ nm (B).

References

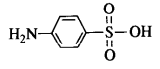
[1] Netz, S., Funk, W.: Private communication, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1989.

[2] Kakáč, B., Vejdelek, Z. J.: *Handbuch der photometrischen Analyse organischer Verbindungen*, Vol. 2, p. 872ff., Verlag Chemie, Weinheim, 1974.

Sulfanilic Acid, Diazotized Reagent
(Pauly's Reagent)

Reagent for:

- Phenols and amines capable of coupling [4, 8]
  - e.g. aniline derivatives, aminophenols [5]
    - such as tyramine [9]
    - phenolcarboxylic acids [1, 3]
    - such as gallic acid, caffeic acid, chlorogenic acid
    - PHB esters [7]
    - phenolic aldehydes
    - such as salicylaldehyde, 4-hydroxybenzaldehyde [3]
    - phenolic alcohols
    - such as coniferyl alcohol [3]
    - chlorophenols [10]
    - naphthylamines [6]
    - flavonoids [3, 27]
    - cumarins [1-3]
- Heterocyclics [4]
  - e.g. imidazole derivatives
    - such as histamine [11], clotrimazole [12]
    - kojic acid [13], imidazole thioethers [14]
    - histidine and metabolites [15]
    - indanedione derivatives [2]
- Proteins
  - e.g. cytochrome C, ovalbumin, aldolase [16]
- Oligo- and polypeptides [18, 21-23, 26]
  - e.g. *Amanita* toxins
    - such as amanitin, phalloidin [17, 20]
    - angiotensin peptides [24, 25]
    - insulin derivatives [19]
- Penicillic acid [13]
- Carboxylic acids [29]
  - e.g. sorbic acid, malic acid, citric acid



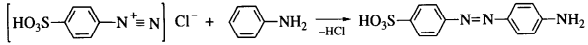
$C_6H_7NO_3S$
$M_r = 173.19$

Preparation of the Reagent

Solution I	Dissolve 4.5 g sulfanilic acid (4-aminobenzenesulfonic acid, aniline-4-sulfonic acid) in 45 ml conc. hydrochloric acid (32%, 10 mol/L) with gentle heating and make up to 500 ml with water.
Solution II	Dissolve 4.5 g sodium nitrite in 100 ml water.
Solution IIa	Dissolve 2.25 g sodium nitrite in 10 ml water and make up to 50 ml with methanol.
Solution III	Combine 10 ml solution I with 10 ml solution II while cooling in ice. This solution is ready for use after 15 min reaction at 0 °C.
Solution IV	Dissolve 10 g sodium carbonate decahydrate in 100 ml water.
Solution IVa	Dissolve 1.5 g anhydrous sodium carbonate in 15 ml water.
Dipping solution	Mix 10 ml portions of solutions I and IIa with cooling to 4 °C, wait 15 min (4 °C) and treat with 15 ml solution IVa [28].
Spray solution	Mix equal volumes of solutions III and IV immediately before use [4, 8, 20].
Storage	The spray and dipping solutions should always be made up fresh. Solution III is stable for up to 3 days at 0 °C. Solutions I, II and IV are stable over longer periods.
Substances	Sulfanilic acid Hydrochloric acid (32%) Sodium nitrite Sodium carbonate decahydrate Sodium carbonate anhydrous Methanol

Reaction

Aromatic amines and phenols couple with diazotized sulfanilic acid to yield azo dyes.



Diazonium salt — Arom. amine — Azo dye

Method

The chromatograms are freed from mobile phase in a stream of warm air and homogeneously sprayed with the freshly prepared spray solution until the layer begins to be transparent.

Chromatogram zones of various colors appear on a colorless background, usually immediately but occasionally after a little time [3, 5, 11]. The colors remain stable over longer periods [20].

Note: The sulfanilic acid can also be diazotized in situ on nitrite-impregnated TLC layers; in this case the chromatograms are merely sprayed with sulfanilic acid solution (2% in 10 mol hydrochloric acid) [5]. Diazotized 4-nitroaniline can also be used in place of diazotized sulfanilic acid [3]. In the case of clotrimazole, the layer is first sprayed with alcoholic iodine solution to cleave the imidazole ring bound to the molecule, then with sodium carbonate solution and finally with diazotized sulfanilic acid [12].

2,4,6-Trichlorophenol and 2,3,4,6-tetrachlorophenol do not form dyestuffs [10].

The detection limits for phenols, phenol carboxylic acids, cumarins and flavonoids are 0.1–1 µg substance per chromatogram zone [1, 3, 7].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000, RP and cellulose layers.

Procedure Tested

Nitrophenols [28]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (MERCK); before the samples were applied these were immersed for 4 h in 2-propanol and then dried for 30 min at 110 °C. After cooling in the desiccator the layers were protected from atmospheric moisture by covering them 1 cm above the start zones with a glass plate.
Mobile phase	Ethyl acetate – *n*-hexane (65+35).
Migration distance	6.5 cm
Running time	13 min

Detection and result: The chromatogram was freed from mobile phase in a stream of cold air (5 min), immersed in the dipping solution for 1 s and then dried for 5 min in a stream of cold air.

2,4-Dinitrophenol (hR_f 0–5, yellow-brown), 2,6-dinitrophenol (hR_f 10–15, yellow-brown), 2,5-dinitrophenol (hR_f 30–35, yellow-brown), 4-nitrophenol (hR_f 40–45, yellow), 3-nitrophenol (hR_f 50–55, light brown) and 2-nitrophenol (hR_f 60–65, pale beige) appeared on a light beige-colored background.

In situ quantitation: The absorption photometric scan in reflectance was carried out at a mean wavelength of $\lambda_{max} = 420$ nm (Fig. 1). The detection limits per chromatogram zone were 5 ng (2,4- and 2,6-dinitrophenol), 10 ng (2,5-dinitrophenol and 3- and 4-nitrophenol) and 80 ng (2-nitrophenol).

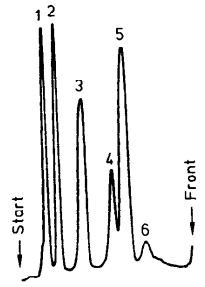


Fig. 1: Reflectance scan of a chromatogram track with 30 ng each of 2,4-dinitrophenol (1) and 2,6-dinitrophenol (2), 60 ng each of 2,5-dinitrophenol (3), 4-nitrophenol (4) and 120 ng each of 3-nitrophenol (5) and 2-nitrophenol (6) per chromatogram zone.

References

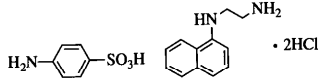
[1] Reichling, J., Beiderbeck, R., Becker, H.: *Planta Med.* **1979,** *36,* 322–332.
[2] Rüssel, H. A.: *Fresenius Z. Anal. Chem.* **1970,** *250,* 125–126.
[3] Jangaard, N. O.: *J. Chromatogr.* **1970,** *50,* 146–148.
[4] E. MERCK, Company brochure *"Dyeing Reagents for Thin-layer und Paper Chromatography"*, Darmstadt 1980.
[5] Dhillon, R. S., Gautam, V. K., Chhabra, B. R.: *J. Chromatogr.* **1988,** *435,* 256–257.
[6] Thielemann, H.: *Mikrochim. Acta (Wien)* **1971,** 748–750.
[7] Thielemann, H.: *Fresenius Z. Anal. Chem.* **1978,** *292,* 237; *Pharmazie* **1980,** *35,* 440.
[8] Thielemann, H.: *Fresenius Z. Anal. Chem.* **1974,** *269,* 125–126; *Pharmazie* **1980,** *35,* 329–330.
[9] Evans, C. S., Gray, S., Kazim, N. O.: *Analyst* **1988,** *113,* 1605–1606.
[10] Thielemann, H.: *Pharmazie* **1970,** *25.* 367.
[11] Grimmet, M. R., Richards, E. L.: *J. Chromatogr.* **1965,** *20,* 171–173.
[12] Ritter, W., Plempel, M., Pütter, J.: *Arzneim. Forsch.* **1974,** *24,* 521–525; Ritter, W.: *Proc. Int. Symp. Instrum. High Perform. Thin-Layer Chromatogr. (HPTLC), 2nd,* Interlaken 1982, 100–112.
[13] Dutton, M. F., Westlake, K.: *J. Assoc. Off. Anal. Chem.* **1985,** *68,* 839–842.
[14] Niebch, G., Schneider, F.: *Z. Naturforsch.* **1972,** *27b,* 675–682.
[15] Humbel, R.: *Clin. Chem.* **1970,** *16,* 58–59.
[16] Jaworek, D.: *Chromatographia* **1970,** *3,* 414–417.
[17] Andary, C., Enjalbert, F., Privat, G., Mandrou, B.: *J. Chromatogr.* **1977,** *132,* 525–532.
[18] Bosshard, H. R.: *Helv. Chim. Acta* **1971,** *54,* 951–958.

[19] Brandenburg, D., Gattner, H.-G., Wollmer, A.: *Hoppe-Seyler's Z. Physiol. Chem.* **1972**, *353*, 599–617.
[20] Palyza, V., Kulhánek, V.: *J. Chromatogr.* **1970**, *53*, 545–558.
[21] Landis, G., Lui, G., Shook, J. E., Yamamura, H. I., Burks, T. F., Hruby, V. J.: *J. Med. Chem.* **1989**, *32*, 638–643.
[22] Neugebauer, W., Elliott, P., Cuello, A. C., Escher, E.: *J. Med. Chem.* **1988**, *31*, 1907–1910.
[23] Samanen, J., Brandeis, E., Narindray, D., Adams, W., Cash, T., Yellin, T., Regoli, D.: *J. Med. Chem.* **1988**, *31*, 737–741.
[24] Bernier, M., Escher, E.: *Helv. Chim. Acta* **1980**, *63*, 1308–1310.
[25] Escher, E., Bernier, M., Parent, P.: *Helv. Chim. Acta* **1983**, *66*, 1355–1365.
[26] Coy, D. H., Coy, E. J., Schally, A. V.: *J. Med. Chem.* **1973**, *16*, 83–84.
[27] Chexal, K. K., Handa, B. K., Rahman, W.: *J. Chromatogr.* **1970**, *48*, 484–492.
[28] Kupper, T.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen 1990.
[29] Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken 1990.

Sulfanilic Acid–N-(1-Naphthyl)-ethylenediamine Reagent

Reagent for:

- Nitrite [1]
- N-Nitrosamines [2, 3]
- Nitro compounds [4]
  e.g. explosives
  such as RDX, HMX, PETN [5]



$C_6H_7NO_3S$
$M_r = 173.19$
Sulfanilic acid

$C_{12}H_{14}N_2 \cdot 2HCl$
$M_r = 259.18$
N-(1-Naphthyl)-ethylenediamine dihydrochloride

Preparation of the Reagent

Dipping solution Dissolve 200 mg sulfanilic acid and 600 mg N-(1-naphthyl)-ethylenediamine dihydrochloride in a mixture of 20 ml hydrochloric acid (32%) and 10 ml water and make up to 200 ml with ethanol (1).

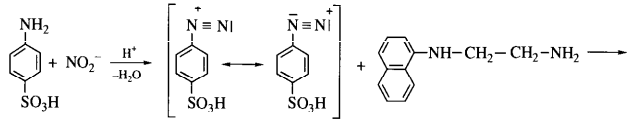
Storage The dipping solution may be stored in the refrigerator at 4 °C for ca. 3 weeks.

Substances

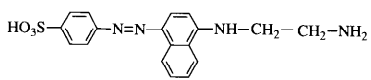
Sulfanilic acid
N-(1-Naphthyl)-ethylenediamine dihydrochloride
Hydrochloric acid (32%)
Ethanol

Reaction

In the presence of acids, sulfanilic acid – like other primary aromatic amines – reacts with nitrite to yield a diazonium compound that can couple with a suitable aromatic amine to yield an azo dye.



Sulfanilic acid | Diazonium compound | N-(1-Naphthyl)-ethylenediamine



Azo dye

Method

The chromatograms are dried in a stream of warm air for 10 min, then heated to 120 °C for 15 min and immersed while still hot (!) in the dipping solution for 1 s or sprayed homogeneously with it and then dried in a stream of cold air for 10 min.

Nitrite, N-nitrosamines and the explosives RDX and HMX yield reddish-violet chromatogram zones on a pale pink-colored background.

Note: Other aromatic amines, e. g. 1- or 2-naphthylamine in acetic acid solution (GRIESS reagent), can be used as coupling agent instead of N-(1-naphthyl)-ethylenediamine dihydrochloride [2, 3, 5]. In the case of N-nitrosamines the chromatograms should be exposed to bright sunlight for 1–2 h before application of the GRIESS reagent [2], or be irradiated with UV light for 10 min while still moist after application of the reagent [3]. Nitro compounds and explosives are detected by first spraying the chromatograms with sodium hydroxide solution and then treating with the reagent [4, 5].

The detection limit for nitrite is 10 ng substance per chromatogram zone.

The reagent can, for example, be employed on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Sodium nitrite [1]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (MERCK).
Mobile phase	2-Butanol – ammonia solution (25%) (8+2).
Migration distance	4 cm
Running time	25 min

Detection and result: The chromatogram was dried in a stream of warm air (10 min) and then heated to 120 °C for 15 min. While still hot (!) it was immersed in the reagent solution (1 s) and then dried in a stream of cold air for 10 min.

Nitrite (hR_f 25–30) yielded a red chromatogram zone on a pale pink background. The detection limit was ca. 10 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 550$ nm (Fig. 1).

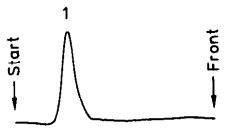


Fig 1: Reflectance scan of a chromatogram track with 50 ng nitrite (1).

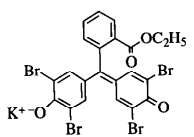
References

[1] Zeller, M.: Private communication, Ciba-Geigy AG, Stofftrennung und Promotion, K-127.270, Basel, 1991.
[2] Vasundhara, T. S., Jayaraman, S., Parihar, D. B.: *J. Chromatogr.* **1975**, *115*, 535–541.
[3] Sen, N. P., Dalpe, C.: *Analyst* **1972**, *97*, 216–220.
[4] Harlow, M. C., Stermitz, F. R., Thomas, R. D.: *Phytochemistry* **1975**, *14*, 1421–1423.
[5] Midkiff, C. R., Washington, W. D.: *J. Assoc. Off. Anal. Chem.* **1976**, *59*, 1357–1374.

Tetrabromophenolphthalein Ethyl Ester–Silver Nitrate–Citric Acid Reagent (Duggan Reagent)

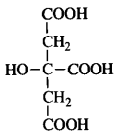
Reagent for:

- Thiophosphate pesticides [1]
- Triazines [2]



$C_{22}H_{13}Br_4O_4K$
$M_r = 700.08$
3,3′,5,5′-Tetrabromophenolphthalein ethyl ester K salt

$AgNO_3$
$M_r = 169.87$
Silver nitrate



$C_6H_8O_7 \cdot H_2O$
$M_r = 210.14$
Citric acid

Preparation of the Reagent

Dipping solution I Dissolve 0.1 g 3,3′,5,5′-tetrabromophenolphthalein ethyl ester potassium salt in 50 ml acetone [2].

Dipping solution II Dissolve 0.5 g silver nitrate in 25 ml water and make up to 100 ml with acetone [2].

Dipping solution III Dissolve 5 g citric acid monohydrate in 50 ml water and make to 100 ml with actone [2].

Storage Dipping solutions I and III can be stored in the refrigerator for at least 1 week. Dipping solution II should be made up fresh daily

Substances
Tetrabromophenolphthalein ethyl ester potassium salt
Silver nitrate
Citric acid monohydrate
Acetone

Reaction

3,3',5,5'-Tetrabromophthalein ethyl ester potassium salt is a pH indicator that changes from blue to yellow in the pH range 4.2–3. It is known that proteins and alkaloids form blue-colored salt-like adsorption compounds with this indicator that are not destroyed by weak acids [3]. Thiophosphate pesticides and triazines possibly form similar compounds.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in dipping solution I for 3 s, dried in a stream of cold air for 15 min and immersed in dipping solution II for 3 s. After drying in a stream of cold air for 2 min they are immersed in dipping solution III for 3 s and finally dried for 5 min in a stream of cold air.

Blue-colored chromatogram zones are formed on a yellow background.

Note: The dipping solutions can also be used as spray solutions [1]. The chromatogram zones are most intensely blue-colored 5 to 10 min after the chromatograms have been treated with citric acid [1]. The whole layer background then gradually acquires a blue-green coloration over a further 10 min period. However, the difference in color between the chromatogram zones and their background can be restored once more by further treatment with citric acid before the blue color of the derivatives completely and irreversibly fades after about 30 to 40 min [1]. The analogous oxygen compounds to parathion, dichlorvos, naled, mevinphos, phosphamidon and trichlorfon do not react [1].

The detection limits for thiophosphate insecticides are 50–100 ng substance per chromatogram zone [1].

The reagent can, for example, be used on silica gel, kieselguhr, aluminium oxide and Si 50000 layers.

Procedure Tested

Triazines [2]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer HPTLC plates Silica gel 60 F_{254} (MERCK), that were precleansed by dipping in 2-propanol for 12 h (overnight) and then dried at 110 °C for 60 min.

Mobile phase *n*-Pentane – chloroform – acetonitrile (50+40+10).

Migration distance 7 cm

Running time 25 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air and for 15 min at 60 °C, then immersed in dipping solution I for 3 s. After drying for 15 min in a stream of cold air it was immersed in dipping solution II for 3 s, dried for exactly 2 min in a stream of cold air, immersed in dipping solution III for 3 s and finally dried in a stream of cold air for 5 min.

The triazines aziprotryn (hR_f 90–95), dipropetryn (hR_f 80–85), prometryn (hR_f 75–80), ametryn (hR_f 65–70), desmetryn (hR_f 50–55) and methoprotryn (hR_f 40–55) yielded blue-colored chromatogram zones on a yellow background, that turned pale yellowish-green after ca. 1 h. However, this did not interfere with the quantitative evaluation. The detection limit was 20 ng substance per chromatogram zone.

In situ quantitation: The absorption spectrophotometric quantitation was carried out in reflectance at the wavelength $\lambda = 565$ nm (Fig. 1).

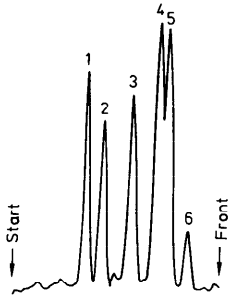


Fig. 1: Reflectance scan of a chromatogram track with 200 ng substance per chromatogram zone 1 = methoprotryn, 2 = desmetryn, 3 = ametryn, 4 = prometryn, 5 = dipropretryn, 6 = aziprotryn

References

[1] Sherma, J. in: J. Sherma, G. Zweig (Eds.), *Analytical Methods for Pesticides and Plant Growth Regulators*, Vol. VII, Academic Press, New York, London, 1973.

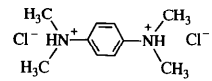
[2] Battenfeld, R.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1989.

[3] Feigl, F., Anger, V.: *Spot Tests in Organic Analysis*, 7th Ed., p. 370ff., Elsevier, Amsterdam, London, New York, 1966.

N,N,N′,N′-Tetramethyl-1,4-phenylenediamine Reagent (Wurster's Blue Reagent)

Reagent for:

- Peroxides
  e.g. sterol hydroperoxides [1],
  such as cholesterol linoleate hydroperoxide [2]
- Steroids [1]
  e.g. Δ^4-3-ketosteroids
- Nitrate esters
  e.g. nitroglycerine, diglycerine tetranitrate
  ethylene glycol dinitrate [3]
- Polynitroaromatics [3]
- Pesticides and pesticide metabolites
  e.g. aniline and urea derivatives [4]
  carbamate and organophosphorus insecticides [4]
  triazines [4, 5]
- Aromatic amines
  e.g. 1,4-phenylenediamine, 2-amino-4-chlorophenol,
  4-nitroaniline, 4-amino-3-nitrotoluene [5]



$C_{10}H_{18}Cl_2N_2$

$M_r = 237.17$

Preparation of the Reagent

Dipping solution Dissolve 500 mg N,N,N',N'-tetramethyl-1,4-phenylenediammonium dichloride (TPDD) in 100 ml methanol [5] or acetone [3].

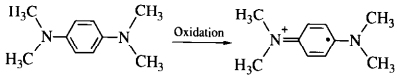
Spray solution Dissolve 1 g TPDD in a mixture of 50 ml methanol, 50 ml water and 1 ml glacial acetic acid [1].

Storage The dipping solution may be stored in the refrigerator for 1 to 2 weeks. It should always be prepared fresh for quantitative work [5].

Substances N,N,N',N'-Tetramethyl-1,4-phenylenediammonium dichloride
Acetone
Methanol
Acetic acid (100%)

Reaction

Peroxides oxidize TPDD to WURSTER's blue, a product with a semiquinone diimine structure [1]. Similarly WURSTER's blue is also produced from TPDD by reaction with halogen-containing substances produced by the reaction of aromatic amines and triazines with chlorine gas.



WURSTER's blue

Method

The chromatogram is freed from mobile phase, lightly but homogeneously sprayed with the spray solution or immersed in the dipping solution for 2 s. Triazines and aromatic amines must first be converted to chlorinated derivatives by exposing the chromatogram to chlorine gas (see "Procedure Tested").

After a few minutes drying in air sterol hydroperoxides, nitrate esters, triazines and aromatic amines yield blue-violet chromatogram zones on a pale blue to violet background [1, 3]. Polynitroaromatics yield yellow to dark beige zones [3].

Note: The substances that will react with TPDD reagent do not all react with the chemically related N,N-dimethyl-1,4-phenylenediamine reagent (N,N-DPDD, q.v.). Hence it is possible to use both reagents at the same time to allow differentiation between substances (cf. Table 1) [4]. The reaction on the reagent-treated layer can be accelerated by brief exposure to UV light [3]. When allowed to stand the spray solution relatively rapidly discolors to a dark violet [1], so it should always be made up freshly. The contrast between the colored zones and the background can be improved by warming the chromatogram. The colors of the chromatogram zones remain stable for several days, while the background gradually darkens [1].

The detection limits per chromatogram zone are 50 ng substance for sterol peroxides [1], 20–100 ng for nitrate esters [3] and 5–25 ng for aromatic amines [5].

The reagent can be used, for example, on aluminium oxide, silica gel, kieselguhr and Si 50000 layers.

Table 1: Comparison of the reaction of pesticides (amounts applied 0.8 µg, without chromatographic development) with N,N-DPDD (WURSTER's Red) and TPDD (WURSTER's Blue) reagents [4]: – = negative, (+) = weakly positive and + + + = positive reaction.

Substance class/Substance	WURSTER's Red Reaction	WURSTER's Blue Reaction
Aniline derivatives		
Alachlor	–	–
Pendimethalin	–	–
Trifluralin	–	–
Metazachlor	–	(+)
Metolachlor	–	(+)
Carbamate pesticides		
Aldicarb	–	+ + +
Carbetamide	–	+ + +
Carbofuran	–	+ + +
Chloropropham (CICP)	–	+ + +
Phenmedipham	–	+ + +
Propham	–	+ + +
Urea pesticides		
Chloroxuron	(+)	+ + +
Chlortoluron	(+)	+ + +
Diuron	(+)	+ + +
Dimefuron	–	+ + +

Table 1 (continued)

Substance class/Substance	Wurster's Red Reaction	Wurster's Blue Reaction
Fenuron	–	+++
Isoproturon	–	+++
Linuron	–	+++
Methabenzthiazuron	–	+++
Metobromuron	–	+++
Metoxuron	–	+++
Monolinuron	(+)	+++
Monuron	(+)	+++
Chlorinated hydrocarbons		
Endosulfan	–	–
Organophosphorus pesticides		
Azinophos-ethyl	–	+++
Chlorphenvinfos	–	–
Parathion-ethyl	–	(+)
Parathion-methyl	–	(+)
Triazines		
Atrazine	+++	+++
Cyanazine	+++	+++
Desisopropylatrazine	+++	+++
Hexazinon	–	(+)
Metamitron	–	–
Metribuzin	(+)	–
Prometryn	+++	+++
Propazine	+++	+++
Sebutylazine	+++	+++
Simazine	+++	+++
Terbutryn	+++	+++
Terbutylazine	+++	+++
Uracil derivatives		
Bromacil	–	+++
Aromatic nitro compounds		
Dinocap	–	–
Dinoseb acetate	–	–
Miscellaneous pesticides		
Amitrole	–	+++
Bentazon	–	+++
Chloridazon	–	+++
Crimidine	–	(+)
Vinclozolin	–	+++

Table 1 (continued)

Substance class/Substance	Wurster's Red Reaction	Wurster's Blue Reaction
Pesticide metabolites		
Aniline	–	+++
4-Bromoaniline	+++	+++
3-Chloroaniline	+++	+++
4-Chloroaniline	+++	+++
4-Chlorophenol	–	+++
Desethylatrazine	–	+++
Dichlobenil	–	–
3,4-Dichloroaniline	+++	+++
2,4-Dichlorophenol	+++	+++
2,6-Dimethylaniline	–	+++

Procedure Tested

Aromatic Amines [5]

Method	Ascending, one-dimensional development in a trough chamber without chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (Merck).
Mobile phase	Toluene – ethanol (19 + 1).
Migration distance	8 cm
Running time	13 min

Detection and result: The chromatogram was first dried in a stream of cold air for 10 min, it was then placed for 2 min in a chamber filled with chlorine gas (cylinder), then freed from excess chlorine in a stream of cold air for **exactly** 5 min (fume cupboard!) and immersed in the dipping solution for 2 s.

After drying for 10 min in a stream of cold air 1,4-phenylenediamine (hR_f 5–10), 2-amino-4-chlorophenol (hR_f 15–20), 4-nitroaniline (hR_f 25–30) and 1,4-amino-3-nitrotoluene (hR_f 50–55) appeared as blue-violet chromatogram zones on a blue background. These could be recognized without difficulty for several days from the back of the chromatogram.

The detection limits per chromatogram zone lay between 5 ng (1,4-phenylenediamine, 4-nitroaniline) and 25 ng (2-amino-4-chlorophenol, 4-amino-3-nitrotoluene).

In situ quantitation: The photometric evaluation was carried out in reflectance at a wavelength of $\lambda = 608$ nm (Fig. 1).

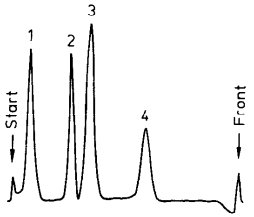


Fig. 1: Reflectance scan of a chromatogram track with 125 ng each substance per chromatogram zone: 1 = 1,4-phenylenediamine, 2 = 2-amino-4-chlorophenol, 3 = 4-nitroaniline, 4 = 4-amino-3-nitrotoluene.

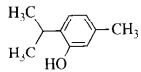
References

[1] Smith, L. L., Hill, F. L.: *J. Chromatogr.* **1972,** *66,* 101–109.
[2] Harland, W. A., Gilbert, J. D., Brooks, C. J. W.: *Biochem. Biophys. Acta* **1973,** *316,* 378–385.
[3] Urbanski, T., Krasiejko, T., Poludnikiewicz, W.: *J. Chromatogr.* **1973,** *84,* 218–219.
[4] Kroker, B.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1991.
[5] Zeller, M.: Private communication, Ciba-Geigy AG, Stofftrennung und Promotion, K-127.2.70, Basel, 1991.

Thymol–Sulfuric Acid Reagent

Reagent for:

- Sugars
  e.g. mono-, di- and trisaccharides [1–10, 13]
  fructo-oligosaccharides [1]
- Polyglycerols [11]
- Sugar alcohols
  e.g. sorbitol [12]
- Uronic acids [2, 4, 6]



$C_{10}H_{14}O$ $M_r = 150.22$ Thymol

H_2SO_4 $M_r = 98.08$ Sulfuric acid

Preparation of the Reagent

Dipping solution	Dissolve 0.5 g thymol in 95 ml ethanol (96%) and cautiously add 5 ml conc. sulfuric acid.
Storage	The reagent solution may be kept for about 2 days [1].
Substances	Thymol Ethanol (96%) Sulfuric acid (95–97%)

Reaction

The mechanism has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of cold air, immersed in the reagent solution for 3 s [1] or sprayed homogeneously with it until the layers begin to become transparent [2, 11, 13]. They are then heated to 110–125 °C for 5 to 20 min [1, 2, 7, 10, 11, 13] or, in the case of sorbitol, to 170 °C for 10–15 min [12].

The sugars appear as chromatogram zones of various colors (yellow, pale pink, red to blue) on an almost colorless background [1–3, 6, 7]. Uronic acids acquire a beige to violet-pink coloration [2, 4, 6].

Note: The various tones of the colors produced by the sugars, that alter during the first hour after reaction [1, 2], make it possible to differentiate according to color. Glucose and galactose are somewhat less strongly pigmented than the other sugars [1]. Prolongation of the heating after dipping or spraying leads to the fading of some zones at least on silica gel layers impregnated with bisulfite [10].

The detection limits for sugars are of the order of 25 ng substance per chromatogram zone [1].

The reagent can, for example, be used on silica gel, sodium acetate, bisulfite- or boric acid-impregnated silica gel layers, kieselguhr and silica gel/kieselguhr layers.

Procedure Tested

Fructo-Oligosaccharides [1]

Method Ascending, one-dimensional development in a trough chamber without chamber saturation.

Layer TLC plates or TLC aluminium sheets silica gel 60 F_{254} (MERCK).

Mobile phase Ethyl acetate – ethanol (96%) – acetic acid (60%) – boric acid solution (cold saturated) (50+20+10+10).

Migration distance 10 cm

Running time 50–55 min

Detection and result: The chromatogram was dried at 55 °C for 30 min, immersed in the reagent solution for 3 s after cooling and then heated to 110 °C for 10 min.

The sugars fructosyl-nystose (hR_f 15–20), maltotetraose (hR_f 15–20), nystose (hR_f 20), 1-kestose (hR_f 20–25), 6-kestose (hR_f 20–25), raffinose (hR_f 20–25), melibiose (hR_f 20–25), maltotriose (hR 20–25), panose (hR_f 20–25), melezitose (hR_f 25), lactose (hR_f 25), sorbose (hR_f 25–30), trehalose (hR_f 25–30), neokestose (hR_f 25–30), turanose (hR_f 25–30), fructose (hR_f 30), maltose (hR_f 30), sucrose (hR_f 30–35), galactose (hR_f 35–40), glucose (hR_f 40), arabinose (hR_f 40–45), mannose (hR_f 40–45), xylose (hR_f 45–50) and ribose (hR_f 50) appeared as reddish-blue chromatogram zones on a pale background (Fig. 1A).

In situ quantitation: The photometric measurement in reflectance was carried out at λ = 525 nm (Fig. 1B). In order to ensure that the zone coloration had stabilized, scanning was not commenced until ca. 30 min after the dipping process. The detection limit for sugars was of the order of 25 ng substance per chromatogram zone.

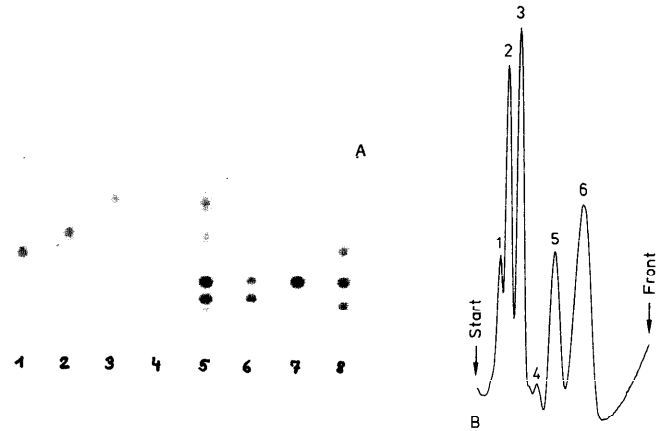


Fig. 1: (A) Chromatographic separation of sugars. Track 1: fructose, 2: sucrose, 3: glucose, 4: mixture of the substances in tracks 1–3, 5: mixture of substances in tracks 1–3 and 6, 6: Fructo-oligosaccharides, 7: 1-kestose, 8: mixture of glucose, maltose, maltotriose and maltotetraose. (B) Absorption scan of track 5 with 200 ng each substance per chromatogram zone: 1 = fructosyl-nystose, 2 = nystose, 3 = 1-kestose, 4 = fructose, 5 = sucrose, 6 = glucose.

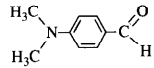
References

[1] Grösz, J., Braunsteiner, W.: *J. Planar Chromatogr.* **1989**, *2*, 420–423.
[2] Kartnig, Th., Wegschaider, O.: *J. Chromatogr.* **1971**, *61*, 375–377.
[3] Lenkey, B., Csányi, J., Nánási, P.: *J. Liq. Chromatogr.* **1986**, *9*, 1869–1875.
[4] Young, D. S., Jackson, A. J.: *Clin. Chem.* **1970**, *16*, 954–959.
[5] Hiller, K., Bader, G., Schulten, H.-R.: *Pharmazie* **1987**, *42*, 541–543.
[6] Kartnig, Th., Wegschaider, O.: *Planta Med.* **1972**, *21*, 144–149.
[7] Krüger, D., Wichtl, M.: *Dtsch. Apoth. Ztg.* **1985**, *125*, 55–57.
[8] Metwally, A. M., Saleh, M. R. I., Amer, M. M. A.: *Planta Med.* **1974**, *23*, 94–98.
[9] Skopp, K., Hörster, H.: *Planta Med.* **1976**, *29*, 208–215.
[10] Adachi, S.: *J. Chromatogr.* **1965**, *17*, 295–299.
[11] Dallas, M. S. J.: *J. Chromatogr.* **1970**, *48*, 225–230.
[12] Grösz, J.: Private communication, Zuckerforschungs-Institut, Zaunergasse 1–3, A-1030 Wien, September 1989.
[13] Kartnig, Th., Gruber, A., Wiedner, I.: *Sci. Pharm.* **1988**, *56*, 161–168.

Tin(II) Chloride–Hydrochloric Acid–4-(Dimethylamino)-benzaldehyde Reagent

Reagent for:

- Aromatic nitrocompounds [1]
  e. g. nitrophenols [2, 3]
  pesticides [2]
  carboxylic acid 2,4-dinitrobenzylesters [4]



$SnCl_2 \cdot 2H_2O$	HCl	$C_9H_{11}NO$
$M_r = 255.63$	$M_r = 36.46$	$M_r = 149.19$
Tin(II) chloride	Hydrochloric acid	4-(Dimethylamino)-benzaldehyde

Preparation of the Reagent

Dipping solution I Dissolve 2 g tin(II) chloride dihydrate in a mixture of 20 ml hydrochloric acid (32%) and 30 ml methanol [3].

Dipping solution II Dissolve 0.25 g 4-(dimethylamino)-benzaldehyde in 50 ml of a mixture of ethanol and 1-butanol (50+50) [3].

Spray solution I Treat 3 ml 15 percent aqueous tin(II) chloride solution with 15 ml hydrochloric acid (32%) and dilute with 180 ml water [1, 2].

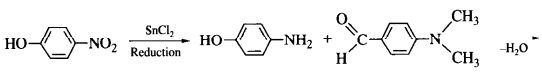
Spray solution II Dissolve 1 g 4-(dimethylamino)-benzaldehyde in a mixture of 30 ml ethanol, 3 ml hydrochloric acid (32%) and 180 ml 1-butanol [1, 2].

Storage The reagent solutions should always be made up fresh.

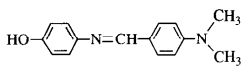
Substances Tin(II) chloride dihydrate
4-(dimethylamino)-benzaldehyde
Hydrochloric acid (32%)
Ethanol
Methanol
1-Butanol

Reaction

Tin(II) chloride reduces aromatic nitro compounds to the corresponding amines, these then react with 4-(dimethylamino)-benzaldehyde to yield colored SCHIFF's bases.



4-Nitrophenol 4-Aminophenol 4-(Dimethylamino)-benzaldehyde



SCHIFF's base

Method

The developed chromatograms are dried in a stream of warm air, immersed in dipping solution I for 1 s or sprayed homogeneously with spray solution I and heated to 105–110 °C for 10 min. After cooling the plates are immersed in dipping solution II for 1 s or sprayed homogeneously with spray solution II and dried for 5 min in a stream of cold air.

Chromatogram zones of various colors are produced on an almost colorless background, most of them can be excited to emit fluorescent light on exposure to long-wavelength UV light ($\lambda = 365$ nm).

Note: The aromatic amines produced by reduction with $SnCl_2$ in acidic medium can be detected with fluorescamine (after neutralization of the layer by spraying with sodium carbonate) instead of 4-(dimethylamino)-benzaldehyde [5].

In the case of dinitrophenols the detection limits are 100 pg to 200 ng substance per chromatogram zone [2, 3].

The reagent can be used, for example, on silica gel, kieselguhr, Si 50000 and cellulose layers.

Procedure Tested

Nitrophenols [3]

Method Ascending, one-dimensional step development in a trough chamber with 5 min drying in cold air betweeen the two development steps (1st development at room temperature without and 2nd development at −20 °C with chamber saturation).

Layer HPTLC plates Silica gel 60 F_{254} (MERCK); before application of the samples the layers were washed by immersing them for 4 h in 2-propanol, then dried at 110 °C for 30 min, before being stored over silica gel in a desiccator. When the samples were being applied the layer above the application zone was covered with a glass plate to avoid adsorption of moisture from the atmosphere.

Mobile phase 1. Methanol
2. Ethyl acetate – *n*-hexane (65+35).

Migration distance 1. 0.7 cm
2. 7 cm

Running time 1. 40 s
2. 20 min

Detection and result: The chromatogram was dried for 5 min in a stream of cold air and immersed in dipping solution I for 1 s and then heated to 110 °C for 10 min. Then after cooling to room temperature, it was immersed in dipping solution II for 1 s and

dried in a stream of cold air for 5 min. Finally the plate was dipped in a solution of liquid paraffin – *n*-hexane (1+2) to stabilize and enhance the fluorescence.

In daylight the nitrophenols appeared as variously colored chromatogram zones on a pale yellow background, under long-wavelength UV light ($\lambda = 365$ nm) they were excited to the emission of fluorescence. The associated hR_f values, colors and fluorescence colors are listed in the table below:

Substance	hR_f	Color	Fluorescence color
2,4-Dinitrophenol	10–15	orange	yellow
2,6-Dinitrophenol	20–25	orange	yellow
2,5-Dinitrophenol	35–40	red	orange
4-Nitrophenol	60–65	yellow	green
3-Nitrophenol	65–70	yellow-green	green
2-Nitrophenol	80–85	yellow	pale green

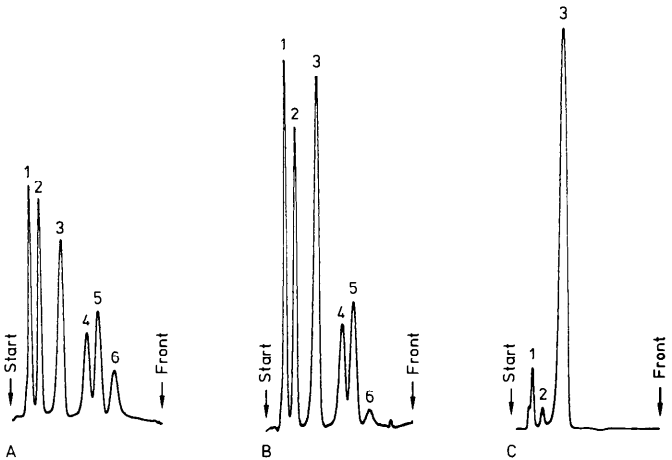


Fig. 1: Reflectance (A) and fluorescence scans (B, C) of a chromatogram track with 30 ng each of 2,4-dinitrophenol (1) and 2,6-dinitrophenol (2), 60 ng each of 2,5-dinitrophenol (3) and 4-nitrophenol (4) and 120 ng each of 3-nitrophenol (5) and 2-nitrophenol (6) per chromatogram zone. Reflectance measurement at $\lambda = 490$ nm (A), fluorescence measurements at $\lambda_{exc} = 408$ nm and $\lambda_{fl} > 460$ nm (B) and at $\lambda_{exc} = 546$ nm and $\lambda_{fl} > 560$ nm (C).

In situ quantitation: The absorption photometric scan in reflectance was carried out, for example, at a mean wavelength of $\lambda = 490$ nm (Fig. 1). Here the detection limits lie between 100 pg (2,4- and 2,6-dinitrophenol) and 2 ng (2- and 3-nitrophenol). The fluorimetric scan, for example, may be carried out at $\lambda_{exc} = 408$ nm and the fluorescence emission be detected at $\lambda_{fl} > 460$ nm (cut off filter Fl 46).

References

[1] E. Merck, Company brochure *"Dyeing reagents for Thin-layer and Paper Chromatography"*, Darmstadt 1980.
[2] Thier, H.-P.: *Dtsch. Lebensm. Rundsch.* **1970**, *66*, 393–398.
[3] Küppet, T.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1990.
[4] Jurecek, M., Churácek, J., Cervinka, V.: *Mikrochim. Acta (Wien)* **1960**, 102–112.
[5] Zakrevsky, J.-G., Mallet, V. N.: *J. Chromatogr.* **1977**, *132*, 315–321.

Tin(IV) Chloride Reagent

Reagent for:

- Sterols, steroids [1, 2]
- Sapogenins [3]
- Triterpenes [2]
- Fatty acids [1]
- Amino acids [1]
- Purines [1]
- Pyrimidines [1]
- Carbohydrates [1]
- Flavonoids [4]
- Phenols, polyphenols [2]

$SnCl_4$

$M_r = 260.50$

Preparation of the Reagent

Dipping solution Add 2.5 ml tin(IV) chloride to 40 ml of a mixture of equal volumes chloroform and glacial acetic acid [4].

Storage The dipping solution may be stored for at least one week.

Substances Tin(IV) chloride
Chloroform
Acetic acid (100%)

Reaction

The reaction mechanism has not been elucidated.

Method

The chromatograms are freed from mobile phase in a stream of cold air and then immersed in the dipping solution for 2 s or sprayed homogeneously with it and then dried for ca. 3 min in a stream of cold air [4].

After reaction the flavonoids, that exhibit weak fluorescence even before derivatization, appear on a colorless background as yellow chromatogram zones; they are excited to yellow to reddish-yellow fluorescence by long-wavelength UV light ($\lambda = 365$ nm) [4].

Note: Many substances do not react until the temperature is raised, e.g. to 160–200 °C [1]. The reagent can also be applied via the vapor phase at 160 °C [1].

The detection limits for flavonoid substances are 5–10 ng substance per chromatogram zone [4].

The reagent can be employed on silica gel, kieselguhr and on Si 50000 layers.

Procedure Tested

Flavonoids [4]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation. After sample application the TLC plates were preconditioned for 30 min at 100% relative humidity and then developed immediately.

Layer HPTLC plates Silica gel 60 F_{254} (MERCK).

Mobile phase Ethyl acetate – water – formic acid (85+15+10).

Migration distance 5 cm

Running time 15 min

Detection and result: The chromatogram was dried for ca. 3 min in a stream of cold air, immersed in the reagent solution for 2 s and then dried for ca. 3 min in a stream of cold air.

The chromatogram zones, that were slightly fluorescent even before derivatization, appeared as yellow zones on a colorless background, under long-wavelength light (λ = 365 nm) they fluoresced, yellow in the case of rutin (hR_f 20–25), red-yellow in the case of quercitrin (hR_f 60–65) and quercetin (hR_f 85–90).

On account of the noisier background the detection limits for fluorimetric determination were twice as high as those for reflectance determination where they were 5 ng substance per chromatogram zone. An additional immersion in liquid paraffin – *n*-hexane (1+2) did not lead to an intensification of the fluorescence.

In situ quantitation: The absorption photometric scan in reflectance was carried out at λ = 420 nm (Fig. 1A), and the fluorescence scan was carried out at λ_{exc} = 436 nm and λ_{fl} > 560 nm (cut off filter Fl 56) (Fig. 1B).

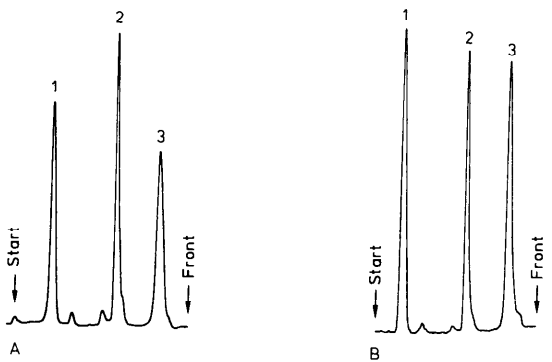


Fig. 1: Reflectance scan (A) and fluorescence scan (B) of a chromatogram track with 200 ng (A) and 100 ng (B) each substance per chromatogram zone: 1 = rutin, 2 = quercitrin, 3 = quercetin.

References

[1] Segura, R., Navarro, X.: *J. Chromatogr.* **1981**, *217*, 329–340.
[2] E. Merck, Company brochure *"Dyeing reagents for Thin-layer and Paper Chromatography"*, Darmstadt 1980.
[3] Glombitza, K.-W., Gielsdorf, W., Eckhardt, G., Koch, M. L.: *Planta Med.* **1975**, *27*, 367–371.
[4] Netz, S., Funk, W.: Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, Private communication, 1989.

Titanium(III) Chloride–Hydrochloric Acid Reagent

Reagent for:

- Ascorbic acid, dehydroascorbic acid [1, 2]

$TiCl_3$
$M_r = 154.27$

Preparation of the Reagent

Dipping solution	Make 15 ml titanium(III) chloride solution (c = 15% in 10 percent hydrochloric acid) up to 100 ml with ethanol (96%) [2].
Storage	The dipping solution can be stored for longer periods.
Substances	Titanium(III) chloride (15% in 10 percent hydrochloric acid) Ethanol (96%)

Reaction

The mechanism of the reaction is unknown.

Method

The dried chromatograms (2 min in a stream of cold air) are immersed in the reagent solution for 10 s or sprayed homogeneously with it and then heated to 110 °C for 10 min.

Yellow chromatogram zones are produced on a colorless background.

Note: The photometric detection limits for ascorbic and dehydroascorbic acids are less than 50 ng substance per chromatogram zone.

The reagent can, for example, be employed on silica gel, kieselguhr and Si 50000 layers.

Procedure Tested

Ascorbic Acid and Dehydroascorbic Acid [3]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	HPTLC plates Silica gel 60 F_{254} (Merck).
Mobile phase	Ethanol (96%) – acetic acid (10%) (95+5).
Migration distance	5 cm
Running time	30 min

Detection and result: The dried chromatogram was immersed in the reagent solution for 10 s and then heated to 110 °C for 10 min.

Ascorbic acid (hR_f 50–55) and dehydroascorbic acid (hR_f 65–70) appeared as yellow chromatogram zones on a colorless background.

The detection limits for both ascorbic acid and dehydroascorbic acid were ca. 50 ng substance per chromatogram zone.

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength $\lambda = 400$ nm (Fig. 1).

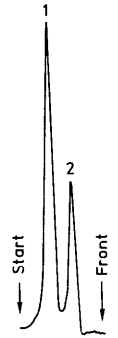


Fig 1: Reflectance scan of a chromatogram track with 2 µg each of ascorbic acid (1) and dehydroascorbic acid (2) per chromatogram zone.

References

[1] Funk, W., Schnekenburger, G.: *GIT Fachz. Lab. Supplement 3 „Chromatographie"* **1989**, 90–96.
[2] Schnekenburger, G.: Thesis, Fachhochschule Gießen, Fachbereich Technisches Gesundheitswesen, 1987.
[3] Ganz, J., Jork, H.: InCom, Training course, Düsseldorf 1993.

Uranyl Acetate Reagent

Reagent for:

- Substances absorbing UV light
  e.g. aromatics [1, 2], purines [1]
- Histidine [2]
- Sterols, fatty acids, triglycerides, essential oil components [3]
- Flavones, quinones

$(CH_3COO)_2UO_2 \cdot 2H_2O$

$M_r = 424.15$

Preparation of the Reagent

Dipping solution	Dissolve 1 g uranyl acetate in 20 ml water with warming and make up to 100 ml with ethanol [3].
Spray solution	Dissolve 1 g uranyl acetate in 100 ml water [1, 2].
Storage	The dipping solution can be stored over a longer period.
Substances	Uranyl acetate dihydrate Ethanol

Reaction

Detection is primarily based on the principle of fluorescence quenching by substances absorbing UV light. It is also possible to detect certain substances whose absorption wavelengths interfere with the uranyl cation [1].

Method

The chromatogram is freed from mobile phase, immersed in the dipping solution for 3 s or homogeneously sprayed with the spray solution and then heated to 100–120 °C for 10–30 min [1].

When examined under UV light (λ = 254 or 365 nm) dark chromatogram zones are observed on a yellow-green fluorescent background.

Note: Uranyl nitrate can be used instead of uranyl acetate [1]. The detection limits for purines are 10 ng substance per chromatogram zone.

The reagent can, for example, be used on silica gel, kieselguhr and Si 50000 layers; cellulose [1] and RP layers are unsuitable.

Procedure Tested

Sterols, Fatty Acids, Triglycerides [3]

Method Ascending, one-dimensional development in a trough chamber with chamber saturation.

Layer HPTLC plates Silica gel 60 F_{254} (MERCK). Before application of the samples the layers were washed by immersing them for 1 h in methanol, then dried at 110 °C for 30 min.

Mobile phase *n*-Hexane – diethyl ether – glacial acetic acid (80 + 20 + 1).

Migration distance 5 cm

Running time 7 min

Detection and result: The chromatogram was heated to 120 °C for 60 min and then cooled to room temperature, dipped in the reagent solution for 3 s and then dried at 120 °C for 30 min. Cholesterol (hR_f 10–15), stearic acid (hR_f 20–25) and tripalmitin (hR_f 45–50) were visible under long-wavelength UV light (λ = 365 nm) as dark chromatographic zones on a yellow fluorescent background. The visual detection limits were 100 ng substance per chromatogram zone for cholesterol and tripalmitin and 500 ng substance per chromatogram zone for stearic acid.

In situ quantitation: The fluorimetric analysis was carried out at λ_{exc} = 313 nm and at λ_{fl} > 560 nm (cut off filter Fl 56). The chromatogram zones gave a negative signal (the fluorescent background was set at 100% emission).

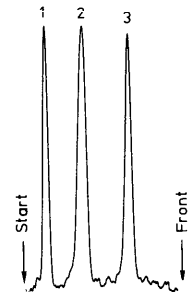


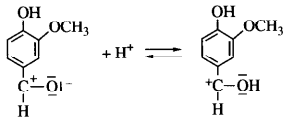
Fig. 1: Fluorescence scan of a chromatogram track with 400 ng cholesterol (1), 200 mg stearic acid (2) and 400 ng tripalmitin (3) per chromatogram zone.

References

[1] Sârbu, C., Măruţoiu, C.: *Chromatographia* **1985,** *20,* 683–684.
[2] Scherz, H., Weisz, H.: *Mikrochim. Acta (Wien)* **1967,** *(2),* 307–309.
[3] Kany, E., Jork, H.: GDCh-training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken 1988.

Vanillin Reagents

The "aldehyde acid" reactions have already been described generally in Chapter 2. There it was pointed out that a nucleophilic attack at a carbonyl group is particularly easy when this is attached to an aromatic ring that bears an "electron withdrawing" group at position 4. The reactivity of the carbonyl group is greatly increased in acid medium:



Classical examples of this type of reaction are the various dimethylaminobenzaldehyde reagents (*q.v.*) and vanillin-acid reagents, of which one, the vanillin-phosphoric acid reagent, is already included in Volume 1a. The aldol condensation of estrogens is an example for the reaction mechanism (cf. Chapter 2, Table 6). According to MALOWAN indole derivatives react in a similar manner [1]. LONGO has postulated that catechins yield intensely colored triphenylmethane dyes [2].

In order to be able to recognize the influences exerted by the various mineral acids used in vanillin reagents the three reagent variants listed below were prepared:

Reagent 1: A solution of 1 g vanillin in 70 ml ethanol (96%) was treated cautiously with 10 ml conc. sulfuric acid.

Reagent 2: A solution of 1 g vanillin in 70 ml ethanol (96%) was treated with 10 ml *ortho*-phosphoric acid (85%).

Reagent 3: A solution of 1 g vanillin in 70 ml ethanol (96%) was treated cautiously with 10 ml fuming hydrochloric acid (37%).

Their reaction was tested on the individual components of the test mixture indole, ergotamine tartrate, ergotaminine and ergobasine [ergometrine) [3]. The results obtained were as follows:

- In the case of reagents 1 and 3 the indole zone could be recognized even before heating, while the ergot alkaloids only became visible on heating to 70°C.
- In the case of reagent 2 the zones for indole and the three ergot alkaloids only appeared as strawberry red, or violet zones on a pale background when the plate was heated to 70°C.
- The coloration of the chromatogram zones was the same for all three variants of the reagents and did not depend on the nature of the mineral acid used.
- In the case of reagent 1 the background turned yellow transiently on heating and then returned to a white color on cooling.
- The detection limits obtained are tabulated below:

Substance	Detection limits in ng per chromatogram zone		
	H_2SO_4	H_3PO_4	HCl
Indole	7	6	8
Ergotamine tartrate	25	25	22
Ergotaminine	8	14	20
Ergobasine	8	6	20

Evidently the reaction of the indoles investigated with fuming hydrochloric acid is less sensitive as is the case for the two other vanillin reagents.

Aromatic aldehydes react in basic as well acidic medium. Thus vanillin and primary amines yield SCHIFF's bases (cf. vanillin-potassium hydroxide reagent in Volume 1a). Colored phenolates are formed at the same time. As would be expected secondary amines, indole derivatives and lysergic acid derivatives do not react.

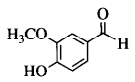
References

[1] Malowan, L. S.: *Biochim. Biophys. Acta* **1948**, *2*, 95-96.
[2] Longo, R.: *J. Chromatogr.* **1970**, *49*, 130-138.
[3] Meiers, Bl., Jork, H.: Private communication, Universität des Saarlandes, Saarbrücken, 1992.

Vanillin-Hydrochloric Acid Reagent

Reagent for:

- Phenols
  e.g. hydroxyindole derivatives [1, 2]
- Catechins, gallocatechins [3-12]
- Alkaloids
  e.g. lysergic acid derivatives [2]



$C_8H_8O_3$
$M_r = 152.15$
Vanillin

HCl
$M_r = 36.46$

Preparation of the Reagent

Dipping solution Dissolve 1 g vanillin in 70 ml ethanol (96%) and cautiously add 10 ml fuming hydrochloric acid (37%).

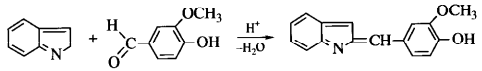
Spray solution Dissolve 1 to 5 g vanillin in 100 ml hydrochloric acid (37%) [1, 7, 12, 13] or 50 percent methanolic hydrochloric acid [2].

Storage The reagents are stable for longer periods.

Substauces
Vanillin
Hydrochloric acid, fuming (37%)
Ethanol (96%)
Methanol

Reaction

In the presence of strong acids catechins react with aromatic aldehydes to yield triphenylmethane dyes [14]; according to Malowan [15] indole derivatives form the following condensation product:



Indole Vanillin Condensation product

Method

The chromatograms are dried in a stream of cold air, immersed in the dipping solution for 1 s or homogeneously sprayed with the spray solution. They are then dried in a stream of cold air.

Alkaloids produce variously colored chromatogram zones (yellow, pink, brown, purple) on a light background [2]. Indole and the catechins appear red [4, 5, 8, 9, 12, 16]. If the catechins are acetylated it is necessary to heat to 105 °C for 5 min after treatment with the reagent [8]. Lysergic acid derivatives should also be heated to 75 °C for 5 min.

Note: The reagent can also be applied by first treating the chromatogram with an unacidified solution of vanillin and then exposing it to hydrochloric acid vapor [3, 9]. Catechin derivatives should be evaluated rapidly (within 10 min), since the red coloration is not stable in daylight and fades relatively quickly [5, 9].

The detection limits in substance per chromatogram zone are 500 ng for alkaloids [2], for indole or ergot alkaloids they are as low as 10 to 25 ng per spot [16].

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000, NH_2, Diol, CN, RP, cellulose and polyamide layers.

Procedure Tested

Indole Derivatives [16]

Method Ascending, one-dimensional, 2-fold development in a trough chamber with 5 min intermediate drying in a stream of cold air. The first development was carried out in an unsaturated normal chamber, the second with chamber saturation. Direct light was excluded.

Layer HPTLC plates Silica gel 60 F_{254} (MERCK), which were prewashed by immersion overnight in 2-propanol and then dried at 110 °C for 20 min before application of the samples.

Mobile phase 1. Dichloromethane – ethanol (99.5%) (50+50).
2. Dichloromethane – *n*-hexane – ethanol (99.5%) (9+2+1).

Migration distance 1. 1 cm
2. 4 cm

Running time 1. 2 min
2. 5 min

Detection and result: After the second development the chromatogram was immersed in the dipping solution for 1 s and then heated to 70 °C for 5 min (hot plate).

Ergobasine (= ergometrine, "hR_f" 20–25)*), ergotamine D-tartrate ("hR_f" 35–40) and ergotaminine ("hR_f" 60–65) produced violet chromatogram zones on a colorless background. Indole ("hR_f" 75–80) acquired a strawberry red color even in the cold. The detection limits in substance per chromatogram zone were 8–10 ng (indole) and 20 to 25 ng (ergot alkaloids).

In situ quantitation: The absorption photometric evaluation in reflectance was carried out at the wavelength λ_{max} = 510 nm for indole and λ_{max} = 580 nm for the ergot alkaloids (Fig. 1).

*) The figures given were calculated as hR_f values even though two developments were involved.

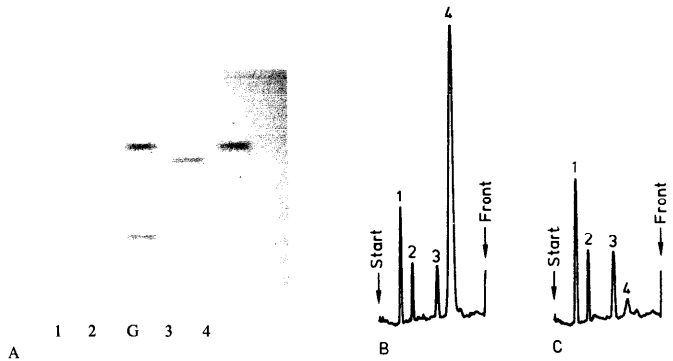


Fig 1: Chromatogram of ergot alkaloids and indole (A) and reflectance scans of chromatogram track G with ca. 80 ng each substance per chromatogram zone measured at $\lambda_{max(indole)}$ = 510 nm (B) and at λ = 580 nm (C): 1 = ergobasine, 2 = ergotamine, 3 = ergotaminine, 4 = indole.

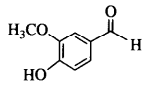
References

[1] Wurziger, J., Harms, U.: *Gordian* **1970**, *70*, 376–378.
[2] Court, W. E., Iwu, M. M.: *J. Chromatogr.* **1980**, *187*, 199–207.
[3] Schulz, J. M., Herrmann, K.: *Z. Lebensm. Unters. Forsch.* **1980**, *171*, 278–280.
[4] Wähner, C., Schönert, J., Friedrich, H.: *Pharmazie* **1974**, *29*, 616–617.
[5] Friedrich, H., Wiedemeyer, H.: *Planta Med.* **1976**, *30*, 223–231.
[6] Friedrich, H., Schönert, J.: *Arch. Pharm.* **1973**, *306*, 611–618.
[7] Mosel, H.-D., Herrmann, K.: *J. Sci. Food Agric.* **1974**, *25*, 251–256.
[8] Friedrich, H., Engelshowe, R.: *Planta Med.* **1978**, *33*, 251–257.
[9] Schulz, J. M., Herrmann, K.: *Z. Lebensm. Unters. Forsch.* **1980**, *171*, 278–280.
[10] Foo, L. Y., Hrstich, H., Vilain, C.: *Phytochemistry* **1985**, *24*, 1495–1498.
[11] Scholtz, K. H.: *Dtsch. Apoth. Ztg.* **1980**, *120*, 175–180.
[12] E. MERCK, Company brochure *Staining reagents for thin-layer and paper chromatography*, Darmstadt 1980.
[13] Zaprometov, M., Grisebach, H.: *Z. Naturforsch.* **1973**, *28c*, 113–115.
[14] Longo, R.: *J. Chromatogr.* **1970**, *49*, 130–138.
[15] Malowan, L. S.: *Biochim. Biophys. Acta* **1948**, *2*, 95–96.
[16] Meiers, Bl., Jork, H.: GDCh-training course No. 301 „Dünnschicht-Chromatographie für Fortgeschrittene", Universität des Saarlandes, Saarbrücken, 1992.

Vanillin–Sulfuric Acid Reagent

Reagent for:

- Steroids [1–4]
  e.g. anabolics [5]
  such as boldenone, testosterone, estradiol
- Bile acid methyl esters [6]
- Essential oil components (4, 7–10)
  e.g. terpene hydrocarbons (3, 11), farnesol (12)
- Sesquiterpene derivatives [3, 13]
  e.g. parthenin, hymenin, xanthinine, xanthumine [14]
- Iridoids [15]
- Carotinoids [3]
- Polyacetylenes (polyolefins) [3]
- Phenols [3, 4]
  e.g. 3-methoxy-4-hydroxyacetophenone [16], holocalin [17]
  e.g. antioxidants [18]
  gallate esters, 2,6-di-*tert*-butylphenol
- Catechins, tannins [1]
- Valepotriates [1]
- Flavonoids [3]
- Cardenolides (cardiac glycosides) [1, 19]
- Ginsenosides [20]
- Higher alcohols [4]
- Fatty acids [3]
- Antibiotics
  e.g. lincomycin [21], primycin [22]
  monensin, narasin, salinomycin, lasalocid [23]
- UV absorbers [24]
  e.g. eusolex, cyasorb, prosolal, parsol, witisol



H_2SO_4
$M_r = 98.08$

$C_8H_8O_3$
$M_r = 152.15$
Vanillin

Preparation of the Reagent

Dipping solution Dissolve 250 mg vanillin in a mixture of 50 ml 1-propanol and 50 ml carbon tetrachloride and cautiously add 2.5 ml conc. sulfuric acid portionwise with stirring and cooling to −15 °C [22].

Spray solution Dissolve 0.5 g vanillin in a mixture of 85 ml methanol, 10 ml acetic acid and 5 ml conc. sulfuric acid that has been prepared under cooling (ice bath) [10].
The literature also contains other compositions, e.g. solutions of 0.5 to 5 g vanillin in 1 to 20% ethanolic [3, 7, 14, 15, 20) or methanolic [18, 21, 23] sulfuric acid, occasionally also in 80 percent ethanolic [4, 6, 25] or undiluted conc. sulfuric acid [4, 5, 11, 12], where the addition of 3 drops acetic acid is recommended in some cases [3, 14].

Storage The spray solutions should always be made up fresh [3]. The colorless dipping solution can be stored in the deep freeze for 2 weeks [22].

Substances
Vanillin
Sulfuric acid (95–97%)
Acetic acid (100%)
Methanol
Carbon tetrachloride
1-Propanol

Reaction

The general aspects of the "aldehyde-acid" reaction were discussed in Chapter 2. Thus it is readily understood that catechins, for example, can react with aromatic aldehydes in the presence of strong acids to yield colored triphenylmethane dyes [26].

Method

The chromatograms are dried in a stream of cold air, immersed twice for 1 s (with intermediate drying in a stream of cold air) in the dipping solution [22] or sprayed once homogeneously with the spray solution and then slowly heated, e. g. on a hot plate, to 70 °C for ca. 10 min [3] or heated to 100–120 °C for 2–15 min [4, 12, 15, 18, 22, 25].

Differently colored chromatogram zones (yellow to dark violet) appear, some before heating, on a light background; the colors alter over a period of 24 h, exceptionally over 48 h (polyolefins) [3]. Monoterpenes steroids and carotinoids yield bright yellow to violet zones [3, 25], fatty acids yield gray colors [3], flavonoids [3], methyl esters of bile acids [6] and lincomycin [21] are colored yellow. The chromatogram zones of farnesol are reddish [12] and primycin gray-violet on a pink-colored background [22]. Cardenolide aglycones, that are not oxidized at the C-14 atom of the steroid skeleton, form blue-colored spots [1].

Note: The chromatogram zones exhibit a broad spectrum of colors [3, 12] that is very dependent on the duration and temperature of heating. Therefore the optimum reaction conditions must be determined empirically. With a few exceptions (ferulic, 4-aminobenzoic and cumarinic acids) aromatic carboxylic acids do not react [3]. The reagent in 80% ethanolic sulfuric acid is reported to be most sensitive for steroids [25].

The detection limits in substance per chromatogram zone are 50–100 ng for sesquiterpene lactones [3] and 100 ng for lincomycin [21] and primycin [22].

It is possible to replace the vanillin in the reagent by 4-dimethylaminobenzaldehyde, 4-hydroxybenzaldehyde, salicylaldehyde, *m*-anisaldehyde, cinnamaldehyde, 4-hydroxybenzoic acid or vanillic acid [3]. However, the range of colors obtained is not so broad.

The reagent can, for example, be employed on silica gel, kieselguhr, Si 50000 and RP layers.

Procedure Tested

Essential Oil Components [10]

Method	Ascending, one-dimensional development in a trough chamber with chamber saturation.
Layer	TLC plates Silica gel 60 F_{254} (MERCK), that had been prewashed before application of the samples by developing once to the upper edge with chloroform – methanol (50+50) and then drying at 110 °C for 30 min.
Mobile phase	Toluene – ethyl acetate (93+7).
Migration distance	13 cm
Running time	30 min

Detection and result: The chromatogram was dried in air (!) and then evaluated under short-wavelength ($\lambda = 254$ nm, Fig. 1A) and under long-wavelength ($\lambda = 365$ nm, Fig. 1B) UV light and documented photographically [27]. It was then sprayed homogeneously with the reagent solution and observed as it was heated to 120 °C on a hot plate. Chromatogram zones of various colors (Fig. 1C, see Table 1 for substance assignment) are produced whose color shades and intensities alter with increasing duration and temperature of heating. Hence the optimum duration and temperature of heating must be determined empirically.

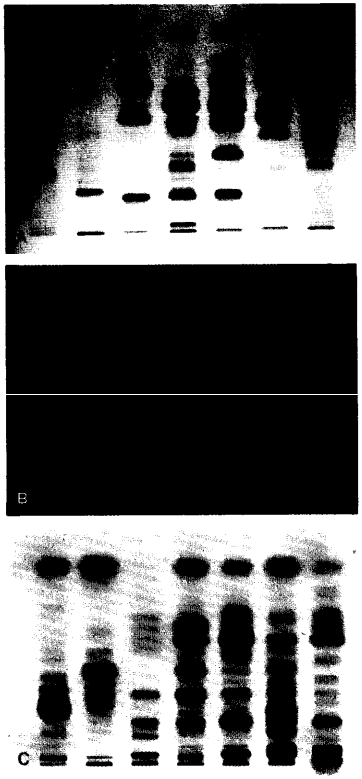


Fig 1: Chromatograms of various essential oils photographed (A) under short-wavelength ($\lambda = 254$ nm) and (B) long-wavelength ($\lambda = 365$ nm) UV light before treatment with the reagent and (C) in daylight after detection with the vanillin – sulfuric acid reagent.

Table 1: Essential oil components with hR_f value, color and detection limits.

Substance	Color on det.	hR_f value	Detection limit
Anethole	brown	55–60	500 ng
Bergamot oil (2 major zones)	reddish brown	20–25	
	reddish brown	45–50	
Bornyl isovaleriate	dark blue	75–80	
Cineole	dark brown	30–35	
Hydroxycitronellal	dark brown	5–10	
Isobornyl acetate	brownish gray	55–60	
Isomenthone	yellowish green	35–40	
Lavender oil (3 major zones)	bluish gray	20–25	
	dark blue	45–50	
	pink	65–70	
Menthol	dark blue	15–20	100 ng
Menthone	yellowish green	45–50	
Menthyl acetate	green	45–50	200 ng
Patchouli oil (2 main zones)	violet	30–35	
	violet	70–75	
Pulegone	dark brown	30–35	500 ng
Rose oil	brownish gray	10–15	
Rose oxide	brownish gray	35–40	
Sandalwood oil (2 major zones)	grayish blue	20–25	
	grayish blue	70–75	
Thymol	strawberry red	40–45	200 ng

Note: In order to document the color changes photometrically during the heating process the hot-plate was placed on the document table of a reproduction camera placed in the fume cupboard (!). The camera was focused when the plate was cold and the heating process was commenced. When the first colored chromatogram zones appeared they were photographed and photographs were then taken at regular intervals, e. g. every 10 s, until the colors on the chromatogram ceased to change.

In situ quantitation The fact that the colors of the chromatogram zones changed means that quantitative in situ evaluation is only meaningful in rare cases.

References

[1] Pauli, G., Junior, P.: *Dtsch. Apoth. Ztg.* **1990**, *130*, 2170–2174.
[2] Schubert, G., Schneider, G., Schade, W., Dombi, G.: *Acta Chim. Acad. Sci Hung.* **1982**, *III*, 173–187.
[3] Picman, A. K., Ranieri, R. L., Towers, G. H. N., Lam, J.: *J. Chromatogr.* **1980**, *189*, 187–198.
[4] E. Merck, Company brochure *Staining reagents for thin-layer and paper chromatography*, Darmstadt 1980.
[5] Weidolf, L. O. G., Chichila, T. M. P., Henion, J. D.: *J. Chromatogr.* **1988**, *433*, 9–21.
[6] Iida, T., Yamauchi, C., Chang, F. C.: *J. High Resolut. Chromatogr. Chromatogr. Commun.* **1983**, *6*, 617–620.
[7] Pobozsny, K., Kernóczi, L., Tétényi, P., Héthelyi, I., Szejtli, J.: *Planta Med.* **1981**, *42*, 255–259.
[8] Hefendehl, F. W.: *Arch. Pharm.* **1970**, *303*, 345–357.
[9] Schilcher, H., Hagels, H.: *Dtsch. Apoth. Ztg.* **1990**, *130*, 2186–2190.
[10] Hahn-Deinstrop, E.: Private communication, Heumann-Pharma, Abt. Entwicklungsanalytik, Heideloffstraße 18–28, D-90478 Nürnberg 1, 1990.
[11] Cartwright, D. W., Langcake, P., Pryce, R. J., Leworthy, D. P., Ride, J. P.: *Phytochemistry* **1981**, *20*, 535–537.
[12] Tyihák, E., Vágujfalvi, D., Hágony, P. L.: *J. Chromatogr.* **1963**, *11*, 45–49.
[13] Bauer, R., Khan, I., Wagner, H.: *Dtsch. Apoth. Ztg.* **1986**, *126*, 1065–1070.
[14] Picman, A. K., Panfil, I., Towers, G. H. N.: *J. Chromatogr.* **1981**, *212*, 379–381.
[15] König, G., Rimpler, H.: *Phytochemistry* **1985**, *24*, 1245–1248.
[16] Háznagy, A., Tóth, L.: *Planta Med.* **1971**, *20*, 136–141.
[17] Möhrle, H., Fangerau, G.: *Pharmazie* **1980**, *35*, 671–677.
[18] Van der Heide, R. F.: *J. Chromatogr.* **1966**, *24*, 239–243.
[19] Junior, P., Krüger, D., Winkler, C.: *Dtsch. Apoth. Ztg.* **1985**, *125*, 1945–1949.
[20] Vanhaelen, M., Vanhaelen-Fastré, R.: *J. Chromatogr.* **1984**, *312*, 497–503.
[21] Stahr, H. M.: *J. Liq. Chromatogr.* **1983**, *6*, 123–126.
[22] Szilágyi, I., Mincsovics, E., Kulcsár, G.: *J. Chromatogr.* **1984**, *295*, 141–151.
[23] Owles, P. J.: *Analyst* **1984**, *109*, 1331–1333.
[24] Eiden, F., Tittel, C.: *Dtsch. Apoth. Ztg.* **1981**, *121*, 910–911.
[25] Matthews, J. S.: *Biochem. Biophys. Acta* **1963**, *69*, 163–165.
[26] Longo, R.: *J. Chromatogr.* **1970**, *49*, 130–138.
[27] Hahn-Deinstrop, E.: *GIT Fachz. Lab. Supplement 3 „Chromatographie"* **1989**, 29–31.

List of Companies

American Instruments Inc., 185 Port Reading Ave., Port Reading, N.J., USA 07064
J. T. Baker Inc., 222 Red School Lane, Phillipsburg, NJ 08865, USA
J. T. Baker Chemikalien, H. S. Richardson Straße, D-64521 Groß-Gerau, Germany
Baron Laborgeräte, Im Weiler 10, D-78479 Insel Reichenau, Germany
Biochem Wissenschaftliche Geräte GmbH, Benzstraße 28a, D-82178 Puchheim, Germany
Camag Scientific Inc., P.O. Box 563, Wrightsville Beach, N.C. 28480, USA
Camag AG, Sonnenmattstraße 11, CH-4132 Muttenz, Schweiz
Camag AG, Bismarckstraße 27–29, D-12169 Berlin, Germany
Desaga GmbH, Maaßstraße 26–28, D-69123 Heidelberg, Germany
Emi Inc., Heritage Park Road, P.O. Box 912, Clinton, CT, USA 06413
Farrand Optical Co., Inc.: Commercial Product Sales, 117 Wall Street, Valhalla, New York 10595, USA
Fluka Feinchemikalien GmbH, Messerschmittstraße 17, D-89231 Neu-Ulm, Germany
Hamamatsu Corp., 420 South Ave., Middlesex, N.J., USA 08846
Hamilton Bonaduz A.G., P.O. Box 26, CH-7402 Bonaduz, Schweiz
Hamilton Deutschland GmbH, Daimlerweg 5A, D-64293 Darmstadt, Germany
Helena Labs, P.O. Box 752, 1530 Lindbergh Dr., Beaumont, Tx, USA 77704
ICN Biomedicals GmbH, Thüringer Straße 15, D-37269 Eschwege, Germany
Joyce-Loebl Ltd., Dukesway, Team Valley, Gateshead, Tyne & Wear, NE 11 OPZ, England
Joyce-Loebl GmbH, Emanuel-Leutze-Straße 1a, D-40547 Düsseldorf, Germany
Kontron Technik GmbH, Industriegebiet 1, D-85386 Eching bei München, Germany
Kratos-Schoeffel Instruments, 24 Booker Street, Westwood, N.J. 07675, USA
Kürner Analysentechnik, P.O. Box 171, D-83004 Rosenheim, Germany
Laboratorium Prof. Dr. Berthold, P.O. Box 160, D-75323 Wildbad, Germany
Fabrik für Laboratorieninstrumente A.G., Thaly Kálmán u. 41, Budapest IX, Ungarn
Macherey-Nagel GmbH & Co. KG, Neumann-Neander-Straße, D-52355 Düren, Germany
Merck, E., Frankfurter Straße 250, D-64271 Darmstadt, Germany
Olympus Optica (Europa) GmbH, Wendenstraße 14–16, D-20097 Hamburg, Germany
Polaroid GmbH, Königsberger Straße 15, D-60487 Frankfurt, Germany
Riedel-de-Haën AG., Wunstorfer Straße 40, D-30926 Seelze, Germany
Schleicher & Schüll Inc., 10 Optical Avenue, Keene N.H. 03431, USA

Schleicher & Schüll GmbH, Postfach 4, D-37586 Dassel, Germany
Schott Glaswerke, Postfach 55014, D-55122 Mainz, Germany
Serva Feinbiochemica, Carl-Benz-Straße 7, D-69115 Heidelberg, Germany
Shandon Labortechnik, Berner Straße 91–95, D-60437 Frankfurt 56, Germany
Shimadzu Europa GmbH, Albert-Hahn-Straße 6–10, D-47269 Duisburg, Germany
Sigma-Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA
Sigma-Chemie GmbH, Grünwalder Weg 30, D-82041 Deisenhofen, Germany
Spectra-Physics GmbH, Siemensstraße 20, D-64289 Darmstadt, Germany
Whatman Ltd., St. Leonard's Road, 20/20 Maidstone, Kent ME16 OLS, U.K.
Zeiss, C., Postfach 1369, D-73447 Oberkochen, Germany

Named Reagents and Reagent Acronyms

Named Reagents

Reagent Acronyms

Index

D