Graduate engineer Dr. Hans Henke

Preparative Low Pressure Liquid Chromatography

Vogel Buchverlag

Biographical sketch:
Dr. techn. Dipl.-Ing. **Hans Henke**, born in 1934, studied technical chemistry at the Technical University of Graz, Austria, from 1956 to 1964. His Diploma Thesis was entitled Quantitative Paper Chromatographic Determination of Vitamin C in Fruit and Vegetables and his Doctoral Thesis addressed the analysis of components of nucleic acids (first quantitative gel Chromatographic determination on Sephadex G gels). During 1964 to 1970, Dr. Henke was Research Assistant at the Institute of Biochemistry, Food Chemistry, and Microchemistry of the Technical University of Graz. From 1970 to 1994 he was group leader of the analytical department (general analysis, gas and liquid chromatography) of the research facility of AKZO Nobel AG at Obernburg/Main, Germany. During this time he authored numerous publications and internal reports on analytical and preparative LC methods.

A brief selection from these publications:

- New column liquid Chromatographic analysis of spinning preparations and fiber extracts
- New analytical methods for polyurethanes
- New and highly accurate preparative and analytical LC separations of alkylpolyglycosides, sugar derivatives, rubber chemicals, etc.

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ISBN 978-3-8343-3135-9
1st edition 2011

In recognition of the achievements of
the brilliant inventor of
Column Adsorption Chromatography
(1903/1906)
Michail Semenovich TSWETT
(1872–1919)

Preface

Preparative column liquid chromatography was and remains an important subdiscipline of chromatography, whether as an indispensable component of instrumental analysis in the isolation of unknown compounds for identification or as a gentle separation or purification procedure, or also as a method for the isolation of sensitive natural products.

On mention of preparative column liquid chromatography, the mind immediately turns to the numerous laboratories engaged in industrial research and those in university institutes grappling with many different separation problems, in the gram range and not on a production scale because that is a different world. In particular, these laboratories require straightforward, versatile, and above all relatively inexpensive separation systems. The samples or substance mixtures to be separated can be of synthetic or natural origin. The latter are generally plant extracts.

The predominant role of thin-layer chromatography in qualitative testing in preparative organic laboratories, as well as for plant constituents, has remained unchallenged for many decades. After TLC monitoring of a synthesis, it usually becomes necessary to isolate the desired synthesis products or even the by-products in preparative amounts.

The principal component of all separation units remains the separation system, consisting of the column packing materials and the mobile phase. Preparative column liquid chromatographic set-ups must be equipped with an economical separation system.

A separation system is economical if neat solvents are used as mobile phases and very cheap or long-lived compound packing materials are used. The feasibility of fulfilling both criteria while producing highly selective separation systems will be demonstrated in this book with the aid of numerous examples.
Use of neat solvents as mobile phases has yet another great advantage for the practitioner:

- Simple transfer of all analytical TLC separations from a plate to an almost identical preparative system in a column is guaranteed.
- Probably the most important task in preparative column liquid chromatography is to establish the best neat solvent for the sample, i.e. the substance mixture.
- The next very important step is to obtain a qualitative impression of the composition of the sample by TLC and/or HPLC.

These qualitative analyses suffice to provide important information about the substance mixture and permit a straightforward and rapid decision about how to solve the separation task. In preparative low-pressure column liquid chromatography the column packing materials are packed in glass columns of various lengths

and IDs. The phases used may be pressure-stable silica gels or swellable organic polymers with an average particle size of ≥ 20 µm. The range of available column packing materials is large but only few of them are versatile in use.

This book has been written in keeping with the motto:
"By the practitioner for the practitioner"

with the aim of encouraging the user of preparative methods to solve his own separation problems with the aid of simple and readily reproducible examples taken from everyday practice.

A critical remark is in place at this juncture: Preparative separations with unnamed peaks are completely unsuitable as illustrative examples!

This book presents various laboratory-scale preparative separations, which, however, can be readily scaled-up to pilot plant dimensions.

Complex substance mixtures, whether of synthetic or natural origin, can generally be separated according to the following scheme:

- Enrichment of the desired products with one of the systems shown and
- Separation and isolation of the desired components with the aid of a selective separation system.

The most important attributes of this book are the use of neat solvents as mobile phases for all separations and the unconditional guarantee of transfer of analytical TLC separations to a preparative column.

Separation processes in practice
Liquid chromatography is performed by two different yet complementary procedures: thin-layer and column chromatography. The former is carried out mainly in the analytical mode and the latter both in the analytical and in the preparative mode. The complementary nature of the two procedures follows from the way in which fast analytical TLC separations can serve as an optimization method for preparative column liquid chromatography. Optimization amounts to determining the most suitable separation system for preparative separation of a substance mixture.

Thin-layer chromatography

This is not the place for a detailed account of thin-layer chromatography (TLC) because several specialized monographs are available; instead we shall focus our attention on transfers from a TLC plate to a preparative column.

It is common knowledge that thin-layer chromatographic separations are generally performed with solvent mixtures as developers. In the case of development with solvents containing two or more components, gradients are generated during development on the silica gel layer. This means that the developing solvent contains less and less polar components the higher the front rises.

Thin-layer chromatographic analysis and sample origin, whether essential oils and plant extracts or samples of synthetic origin, are of enormous practical importance in connection with preparative separations.

It is not always easy to transfer analytical TLC separations to preparative columns, but transfer can prove to be straightforward if two conditions are met:

- The sample or substance mixture must undergo at least incipient separation on a silica gel plate when developed with a neat solvent.
- As a rule it is advisable to pre-sort the sample components by stepwise elution (see Section V.4.), i.e. to perform sample preparation or enrichment.

A TLC separation with dichloromethane on a silica gel plate is generally very helpful because it provides good qualitative information about the polarity distribution of the sample components. Information is often available at an early stage about the qualitative composition of a sample and that is extremely helpful for separation or enrichment of desired products by stepwise elution.

Problem-free, i.e. guaranteed transfer from an analytical silica gel TLC plate to a preparative column is possible on exclusive use of neat developer. Developers of choice are dichloromethane, ethyl acetate, acetone, and methanol, which are also good solvents for numerous substance mixtures of various compositions – an important prerequisite in preparative column-liquid chromatography.

It is understandable that transfer from silica gel plate to silica gel column is still accepted as a matter of course, or not accepted.

Matters become more problematical on transfer of a separation from a silica gel plate to a column packed with the hydroxypropylated dextran gel Sephadex LH-20, because a transfer from an inorganic to an organic gel can hardly be expected to work. In fact, the opposite could repeatedly be proved true in practice, with the enormous additional benefit that Sephadex LH-20 forms separation systems of exceptional selectivity with neat organic solvents.

Erlenbach — Hans Henke

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I Introduction

When speaking of preparative column liquid chromatography, it is necessary to distinguish between the separation of synthesis products on a laboratory scale as well as the isolation of pharmaceutically relevant plant constituents on the one hand and separations and purifications of various products on a production scale on the other. Use of the heading 'preparative chromatography' in both cases is inappropriate because the subject matter will only become apparent from the content.

The products of organic syntheses (reaction mixtures and extracts) and plant constituents are generally separated or purified with the aid of low-pressure column liquid chromatography for reasons of cost. Preparative column liquid chromatography becomes an expensive process if the separations or purifications incur high costs compared to classical methods such as distillation, crystallization, or extraction. These higher costs arise from expensive column packing materials and the large quantities of solvent mixtures required. The lack of versatility and low loadability of the separation systems are also cost factors.

Whenever the main emphasis is placed on industrial column liquid chromatography in the relevant monographs, a systematic treatment of this method in the low pressure range often becomes impossible or is perhaps even undesired (?).

These separation methods most commonly used in the gram, or higher gram, ranges have been, and still are, wantonly neglected. The firm persuasion prevailed, and still prevails, that everyone masters this separation technique. This important area of LC is also overlooked because there are no offers of seminars or workshops held by independent experts which would provide attendees with separation proposals.

Another reason is that laboratory staff generally receive a basic training in classical, i.e. manual, column chromatography. Consequently, there is a dearth of qualified experts required for systematic education in preparative LC. The excellent and highly promising techniques of flash chromatography and liquid-liquid partition chromatography alone with a limited number of documented separations will not suffice to solve the problem at hand.

Simple and powerful separation systems of versatile selectivity employing neat solvents as eluents are required for the economical separation of numerous substance mixtures of widely differing composition. The feasibility of achieving this aim is apparent from the many examples presented in this book.

Modern column liquid chromatography is based on the method of adsorption chromatography developed by the Russian botanist Michael Semenovich Tswett and used by him for the separation of vegetable dyes. In 1906 M.S. Tswett reported the separation of chlorophyll with ligroin on a calcium carbonate column. Separation of the components of the green leaf coloring matter with a neat solvent constituted a decisive improvement in the separation procedure. And although Richard Willstätter may have considered the separation of plant dyes by chro-

matographic adsorption analysis according to Tswett to be unsuitable for preparative work, the method is justifiably regarded as a preparative technique by today's chromatographers.

What do we mean by preparative chromatography?
The term preparative chromatography is applied generally to a separation technique, in liquid (LC) or gas chromatography (GC), which can be used to isolate pure substances from a mixture of natural or synthetic origin in comparatively large, i.e. preparative, quantities (mg to kg).

Why are preparative separations performed?
In general: The amount of substance to be isolated depends upon its intended use.

- Structure elucidation requires 30–50 mg of pure substance.
- If the substance is to be used as analytical standard then one hundred or more milligrams have to be isolated.
- If it is intended to undertake further reactions with the pure substance then isolation has to be performed on a gram scale.
- However, if the preparative method is used to obtain pure substances in production quantities, hundreds of grams or kilograms have to be separated or isolated.

Some of the above reasons for performing preparative separations will now be considered in detail.

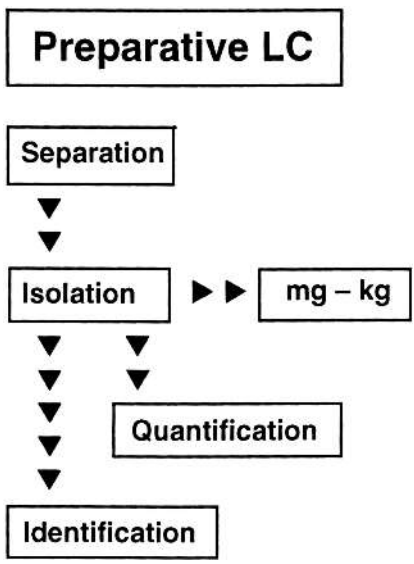
1) Isolation of pure substances for identification purposes, e.g. the main and/or side components of a synthesis or a mixture of natural products. The amount of substance required will also depend upon the equipment available in a laboratory. As a rule, structure elucidation by ^{1}H-, ^{13}C-NMR, FD-MS, IR, and elemental analysis requires 30–50 mg of pure substance.
2) Isolation of standard substances, whether main or side products, for analytical methods such as HPLC or GC.
3) Isolation of one or more compounds of a reaction mixture (several hundred milligram or gram quantities) for further reactions before performing a synthesis on a large scale.
4) Quantification of the individual peaks by gravimetry (a highly accurate method of determination). This provides quantitative information about a mixture of substances even before the qualitative composition of the sample is known. A prerequisite for the success of this method is: one peak = one substance. Precise sample evaluation by mass determination can indicate whether several components are present in roughly equal proportions or whether just one main component (desired or undesired) is present alongside a number of minor components in the reaction mixture. If the compounds are unknown or not available as standards it is not possible to deduce the amount of substance present

from the peak height of peak area in the case of RI or spectrophotometric detection.

5) Gentle purification of a reaction product. In this way, time consuming and sometimes difficult purification operations such as distillation, crystallization, re-precipitation, etc. can be replaced by this more effective method. It provides a means of gently removing, e.g., a high-boiling solvent or excess reaction component.
6) Straightforward isolation of intermediates in multistep preparations.
7) Active principles of plant origin or drugs can readily be quantitatively isolated in large amounts from plant extracts or from pharmaceuticals such as tablets or dragees.
8) Isolation or enrichment of side components of a reaction or constituents of natural products present only in small quantities (e.g. <1%).
9) Preparative isolation of a substance in gram quantities is often less expensive than optimizing the preparation reaction.

Bearing in mind that it permits separation and isolation at room temperature, the above reasons for carrying out a preparative separation and isolation of pure substances also underline the significance and advantages of this method in comparison with other separation techniques: even sophisticated distillation methods (with scope for variation of temperature, vacuum, high performance columns, etc.) are unable to separate many compounds from their mixture. Nor do other methods of isolating pure substances such as extraction, fractional crystallization, or sublimation always lead to the desired result.

The aims and objectives of preparative LC are summarized in Scheme I.1.



Scheme I.1

The various preparative separation methods in LC

In the course of recent years, various LC methods have been developed for the separation and isolation of pure substances from mixtures of varying degrees of complexity, with the scale-up of analytical HPLC separations to columns of correspondingly greater dimensions (ID, length, coarser-grained stationary phases; 30–60 µm average particle size) probably representing the most common approach. In case of very good resolution, analytical columns can also be overloaded for semi-preparative separations. The main drawback of this method is often the insufficient sample capacity (amount of substance/separation = loadability) of the columns in the sense of a truly preparative separation. In addition, insufficient solubility of the sample in the mobile phase can also be an obstacle. It is common knowledge that such adsorption, partitioning, or reversed phase chromatographic separations on silica gel, RP, diol, or CN phases can only be accomplished with organic or organic-aqueous solvent mixtures. Consumption of large amounts of solvents, which is unavoidable in preparative column liquid chromatography, means that recovery and regeneration of the binary or even ternary solvent mixtures is far from simple because distillation rarely leads to the desired effect, except in the case of azeotropic mixtures. The "loss" of mobile phase can be limited on use of a peak-controlled fraction collector by collecting that part of the eluate which is free of peaks in a receiver, i.e. that part where the detector signal corresponds to the baseline. In addition to these numerous variants of column chromatographic methods, use is also made of extraction processes (liquid/liquid partitioning) in the form of a chromatography with complex solvent mixtures as stationary and mobile phases. The sample capacity of the last-mentioned methods is generally even lower. The solvent mixtures used are anything but inexpensive in the quantities used and their recovery is far from straightforward. The fraction collectors often have to be cooled to prevent excessive losses of the frequently highly volatile mobile phase. Regardless of whether preparative column liquid chromatography is performed with a solid or liquid stationary phase, each sample mixture will require a different separation system (stationary and/or mobile phase), i.e. within a given separation operation the chromatographic conditions have to be adjusted to the separation task in question, i.e. optimized, because differently composed samples cannot be separated with a single solvent mixture as eluent on one of the above-mentioned stationary phases.

The total absence of doubt concerning the above separation methods is very evident. There are only few users of preparative LC who frequently have to solve very different separation problems and also have access to different separation procedures so that they are able to use that method which holds the greatest promise for a specific problem. There is more demand for straightforward, universally applicable yet powerful separation methods, i.e. there is a wish to be able to solve a variety of separation problems with just one separation system. It is the author's intention to demonstrate, with the aid of numerous examples taken largely from practice, that Sephadex LH-20 with a single or neat volatile solvent as mobile phase largely fulfills these requirements of the practical separation scientist.

The following preparative separation methods are currently the ones most commonly used:

- ❑ Flash chromatography
- ❑ Low pressure chromatography
- ❑ Medium pressure chromatography
- ❑ High pressure chromatography
- ❑ Droplet countercurrent chromatography (DCCC)
- ❑ Rotation locular countercurrent chromatography (RLCC)
- ❑ Centrifugal countercurrent chromatography (CCC)

Preparative gel chromatographic separations on Sephadex LH-20 with various neat solvents as mobile phases was practiced with great success even several years before introduction of the above preparative methods. The continuing great demand for this separation method is evidenced by the increase in the number of separation units: we operated thirteen complete separation assemblies together with additional sets of columns with various mobile phases and in various dimensions.

Preparative gel chromatography on Sephadex LH-20

Sephadex LH-20 has dual lipophilic (L) and hydrophilic properties (H). This crosslinked dextran gel derives its lipophilic character from the isopropyl groups introduced by hydroxypropylation of Sephadex G-25, which possesses only hydrophilic properties. The hydrophilicity is due to the numerous hydroxyl functions present. As a result of this dual nature, Sephadex LH-20 swells not only in solvents of weak and medium polarity but also in strongly polar ones. The degree of swelling increases with increasing polarity of the solvent (= mobile phase). The principal properties and physical data of the solvents used by the author as mobile phases are listed in Table II.2.

The exclusion limit of Sephadex LH-20 at maximum swelling, such as occurs in water, dimethyl sulfoxide (DMSO), *N*-methyl-2-pyrrolidone, and methanol, is 4000. In other words, all compounds with a molecular mass of ≥ 4000 can only pass through the interparticle spaces and thus elute without separation.

It is not so well known that in addition to molecular sieve separations in order of decreasing molecular mass it is also possible to perform highly selective separations with neat solvents as mobile phases. The most effective of these are selective adsorption chromatographic separations from neat solvents of compounds having molecular masses below 1000. Comparable swelling capacities of Sephadex LH-20 in methanol, dichloromethane, and water make the dextran gel a universal column packing material. The range of applications is further extended by the possibility of using other mobile phases differing in their solvent properties and polarities. Depending upon the polarity of the mobile phase and the compounds to be separated it is possible to perform normal phase, reversed phase, and selective

adsorption chromatography on hydroxypropylated dextran gel. The most commonly used mobile phases are methanol, acetone, dichloromethane, and water. Less common use is made of chloroform, *N*-methyl-2-pyrrolidone, and ethyl acetate, although the last-mentioned mobile phase forms a highly selective separation system with the dextran gel and is thus ideally suited for exceptional separations.

The most straightforward separation systems are well suited for separating structural and positional isomers, as well as diastereomers and *cis/trans* isomers. The elution or retention times are determined primarily by the heteroelements in the form of functional groups. It would appear very important to emphasize that separations are also possible within the pore volume, i.e. in the "classical" molecular sieve range.

Many years of experience with Sephadex LH-20 have adequately demonstrated that no other separation system displays such a pronounced correlation between chemical and molecular structure and elution or retention behavior as does this straightforward system. This will be considered in greater detail in the individual chapters of this book.

We use the dextran gel in columns of various dimensions. The standard columns or column assemblies have an ID of 25/26 mm and the gel bed height or length ranges from 1 cm to 500 cm. The same gel bed lengths are also used for larger ID columns (52, 63, 70, 75, and 100 mm ID).

The samples to be separated are of various origins, and represent a very wide range of chemical classes. Most common are various reaction products followed by various extracts which are sometimes of highly complex composition.

Successful separations are readily predictable because solubility of the sample in one or more of the above solvents essentially guarantees separation. Another invaluable aid is thin-layer chromatography. Preliminary separations on silica gel with dichloromethane, acetone, or ethyl acetate indicate that excellent separations on a truly preparative scale are possible with the same solvents, even on short gel beds. Neither solvent mixtures nor gradients are required to effect highly selective separations. We shall now consider the advantages of Sephadex LH-20 as column packing material after first having examined the principal arguments against using this dextran gel (see below), which, however, in most cases prove to be without substance.

Why is Sephadex LH-20 so "rarely" used as column packing material in preparative LC?

Although Sephadex LH-20 is used more frequently than is generally recognized as column packing material, its applications are limited mainly to biochemistry and natural products chemistry. The gel is used for special separations or for preliminary or intermediate clean-up steps and with the mobile phases used it does not constitute a universal separation system. However, on use of neat solvents as mobile phases Sephadex LH-20 affords extremely selective separation systems with which we can solve a very wide range of preparative separation problems on a day-to-day basis and which we have used at a constant success level for many years.

The main arguments cited against use of Sephadex LH-20 as column packing material will now be briefly presented.

- Substances can only be separated in order of decreasing molecular size on Sephadex LH-20.
  False: With the aid of neat solvents, extremely selective separations of low-molecular substances from all compound classes can be accomplished which have nothing to do with molecular sieve chromatography (MSC).
- Sephadex gels are only suitable as column packing materials for biochemists and natural products chemists.
- Handling of soft gels in large diameter and long columns is beset with problems.
  False: We have demonstrated the very opposite to be true day by day over several decades.
- Chromatography in glass columns, especially in large diameter and long columns, is an obsolete method.
  False, if the real advantages have been recognized.
- Selective separations cannot be performed on short gel bed heights.
  False: We can demonstrate that the opposite is true with the aid of practical examples.

What are the advantages of Sephadex LH-20 over other column packings?
With neat solvents such as methanol, acetone, dichloromethane, chloroform, ethyl acetate, *N*-methyl-2-pyrrolidone as mobile phases the hydroxypropylated dextran gel forms a straightforward, universal, and powerful separating system. If, on the other hand, silica gel or surface modified silica gel is used then selective separation systems will rarely be attained with neat solvents. The three principal characteristic features mentioned so far are recapitulated below:

Straightforward separation system. Only neat, generally volatile solvents are used as mobile phases. Work-up of the eluate fractions in a rotary evaporator (water bath temperature 50–60 °C/ca. 50 mbar) is easy and fast, and above all gentle on the compounds to be isolated.
Universal separation system. It combines several separation mechanisms such as separation according to decreasing molecular size, separation according to differing, sometimes highly selective adsorption effects, while also offering the possibility of partition chromatographic separations. Universal separation systems are particularly important for the practical scientist who very frequently faces different separation problems.
Powerful separation system. These straightforward and universal separation systems are generally highly selective, i.e. they have high loading capacities. Loadabilities of 300 mg or more of sample per gram of dry gel often encountered in practice are indeed very high.

This book has the aim of contributing to our understanding of the separation mechanisms of low-molecular weight organic compounds, and also of inorganic salts, on the hydroxypropylated dextran gel Sephadex LH- 20 with neat solvents as eluents. The numerous examples of separations taken from everyday practice and selected according to various criteria represent just a fraction of the thousands of separations performed. For reasons of patent protection, many extremely interesting separations of reaction mixtures could not be divulged. Instead, separations of comparable or very similar reaction mixtures are shown. As a rule, the test mixtures were selected in such a way that they showed a pronounced similarity to such reaction mixtures. The nature, number, and position of heteroatoms or functional groups responsible for the differences in elution behavior were identical or essentially comparable.

The following colored test chromatogram (see Fig. I.1) gives a foretaste of the separation performance of Sephadex LH-20.

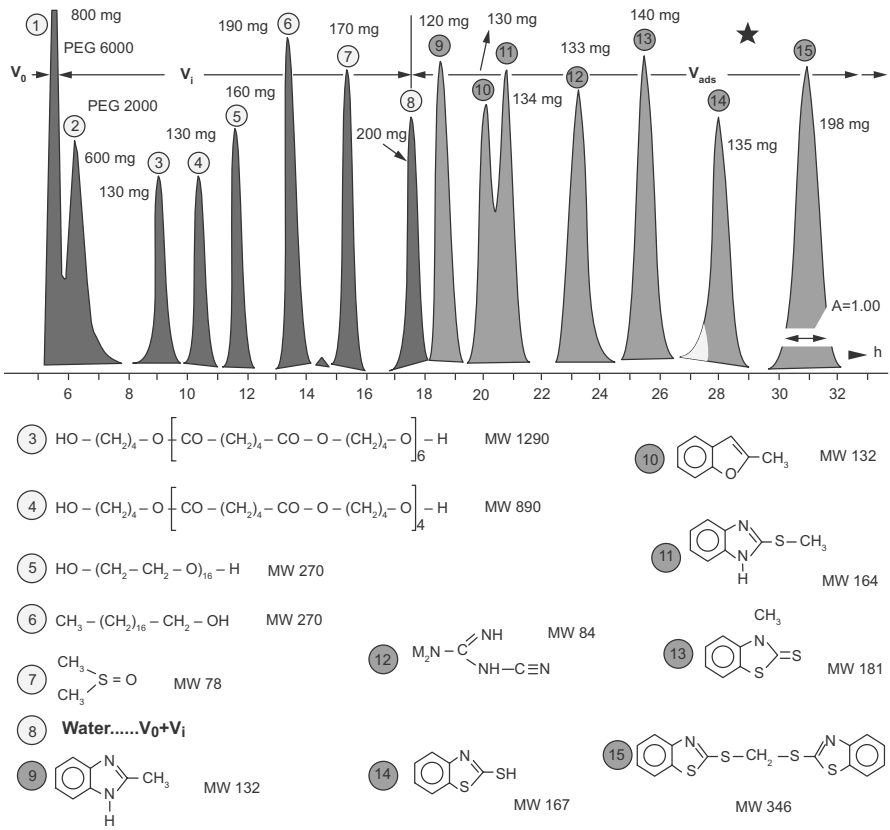


Fig. I.1 Test chromatogram.
A coloured version of this picture you can find in InfoClick on www.vogel-buchverlag.de.