Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 812 (2004) 35–51
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Separation strategies of plant constituents–current status

Szabolcs Nyiredy*

Research Institute for Medicinal Plants, P.O. Box 11, H-2011 Budakalász, Hungary

Received 8 August 2004; accepted 31 August 2004

Abstract

The paper summarizes the state of art of different separation methods which are used for the analysis and isolation of plant constituents. An overview about the extraction methods which are frequently used for the non-volatile constituents of plants is given. Special attention is paid to the identification possibilities of non-volatile and volatile compounds, since generally the role of identification of plant's constituents is undervalued. We would emphasize the facts that, for correct identification, the various chromatographic and spectroscopic methods have to be used in conjunction. The application of two different methods from each field is usually sufficient. For quantitative information, two independent methods are necessary and are acceptable if the results are within 3% of each other. If only one method is available for quantitative analysis, the results can only be accepted if, using the global optimum, the ratios of the components determined are identical to one decimal place with the ratios of three measurements (local optima) using different mobile phases with different selectivities. Based on our own 20-year experience and more than a hundred isolated compounds, we give an isolation strategy where the structures and properties of the compounds to be isolated do not have to be known. It is pointed out that without analytical monitoring, the results of preparative separations cannot be guaranteed.

Keywords: Reviews; Extraction; Purification; Phytoanalysis; Medicinal plants; Secondary constituents; Bioactivity

Contents

* Tel.: +36 26 340 203; fax: +36 26 340 426.
E-mail address: rimp@axelero.hu.

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.08.046

1. Introduction

When we look back upon the last 2000 years of the history of medicine we can see that for most of this period, mankind had no other source of medicine than plants, either fresh or dried. Over 248,000 species of higher plants have been identified and from these 12,000 plants are known to have medicinal properties. However, less than 10% of all plants have been investigated from a phyto-chemical and/or pharmacological point of view [1]. Nevertheless, from this small percentage innumerable therapeutically indispensable compounds have been isolated such as alkaloids (the fever-reducing quinine from the Peruvian bark, the antispasmolyticum papaverine and the narcotic paregoric morphine from the poppy capsule); various heart glycosides, produced from *Digitalis* sp., applied widely in cardiac insufficiency syndrome; plant steroids which constitute the base of modern contraceptives; plant vitamins and flavonoids; and a large range of antibiotics [2]. Although synthetic aspirin has been available for more than 100 years, few people realize that the effective agent originated from willow bark.

From the 1994 WHO data, it appears that 90% of the world's population use medicinal plant for curing, and 81% have no access to synthetic drugs! Although this is unfortunate for the less developed countries in Africa, Asia and South-America, these data show that in many parts of the world, drugs from plants are of serious therapeutical importance a fact not sufficiently appreciated in developed countries [3].

The significance of medicinal plant research and research on compounds of natural origin is acknowledged by the pharmaceutical company Bayer AG Pharma Research known mostly for its synthetic medicines. When giving a summary of its 1999 data it stated "A mere 90,000 natural compounds make up about 40% of the total possible new drugs, while several million synthetics comprise the remaining 60%." [4].

The importance of medicinal plants is demonstrated by the fact that even in developed countries, about 35% of prescribed drugs are of natural origin and 50% of OTC drugs are of plant origin. During the last decade, consumption of medicinal plants has doubled in Western Europe [3].

The aims of medicinal plant research can be summarized as follows [5]:

- qualitative and quantitative analysis of the constituents of medicinal plants;
- isolation of plant-originated, biologically active, purified fractions and molecules with new structures;
- optimization of the amount and/or ratio of medicinal plant compounds responsible for therapeutic effects.

Keeping in mind that one single plant can contain up to several thousand secondary metabolites, high-performance and rapid separation methods are absolutely necessary for all three categories.

2. Classifications

2.1. Classification of plant constituents

Any living organism, like plants, may be considered to be a biosynthetic laboratory for chemical constituents such as

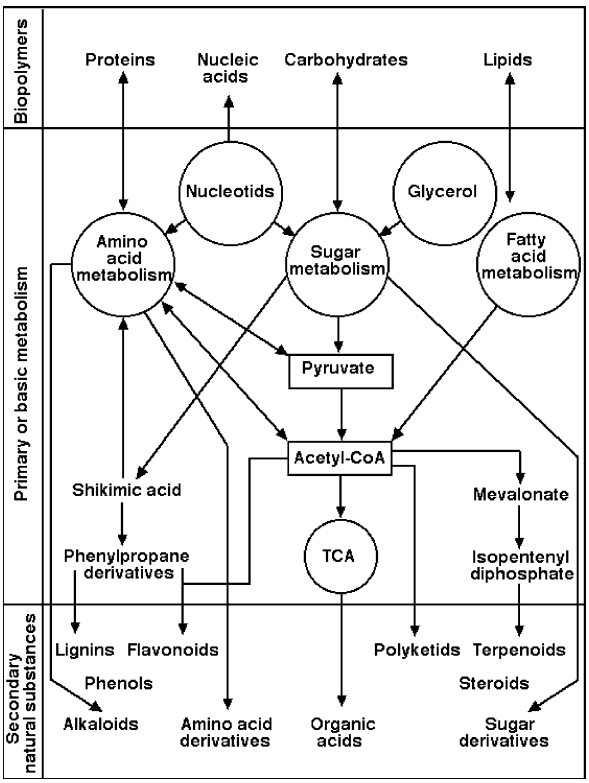

Fig. 1. Relationships of biosynthetic pathways leading to secondary constituents in plants.

biopolymers (proteins, nucleic acids, carbohydrates, lipids), primary or basic metabolites and secondary natural substances (Fig. 1) [2]. These chemical compounds give plants their therapeutic properties. The pharmacologically active constituents which are responsible for the therapeutic effects are differentiated from inert constituents, which also occur in plants. Often, the presence of inert compounds may modify or prevent the absorbability or potency of active constituents [6].

The secondary constituents of plants are influenced by three principal factors: heredity (genetic composition), ontogeny (stage of development) and environment (e.g., climate, associated flora, soil, method of cultivation). Genetic effects include both qualitative and quantitative changes, but those caused by ontogeny and environmental influences are primarily quantitative [6]. The new discipline which concentrates on the study of primary and secondary metabolites such as lignins, flavonoids, phenols, alkaloids, amino acid derivatives, organic acids, polyketids, terpenoids, steroids and sugar derivatives in plants, including ferns, moss, fungi and algae is known as phytomics [7].

2.2. Classification of separation methods

Separation of the constituents of dried, powdered plants can be classified into three main categories: extraction, purification, and chromatography. In general, the first two belong to sample preparation, while the various chromatographic methods ensure qualitative and quantitative analysis as well as isolation.

In order to obtain the plant constituents, the first separation method is the solid–liquid extraction of the dried and powdered plants, which contain many, chemically different classes of compounds. A schematic view of plant tissue structure with damaged and undamaged cells is shown in Fig. 2 indicating which extraction process is decisive for the different types of cells [3]. Using various solvents (A, B, C, D, . . .) with increasing polarity, different extracts (Extracts A, B, C, D, . . .) can be obtained. By applying an appropriate purification method, a certain extract (e.g., Extract C) can be divided into fractions (e.g., Fraction I, Fraction II), as the different shapes indicate it in the figure. Using chromatographic methods, the fractions can be separated into their constituents, in ideal cases baseline separations can be achieved.

For research purposes, extraction methods can be classified according to whether the aim of the extraction is analytical or preparative. In the first case, exhaustive methods have to be used, or such methods through the repetition of which we obtain quantitative results. For the preparative isolation of plant constituents, exhaustive extraction is not essential but is obviously advantageous.

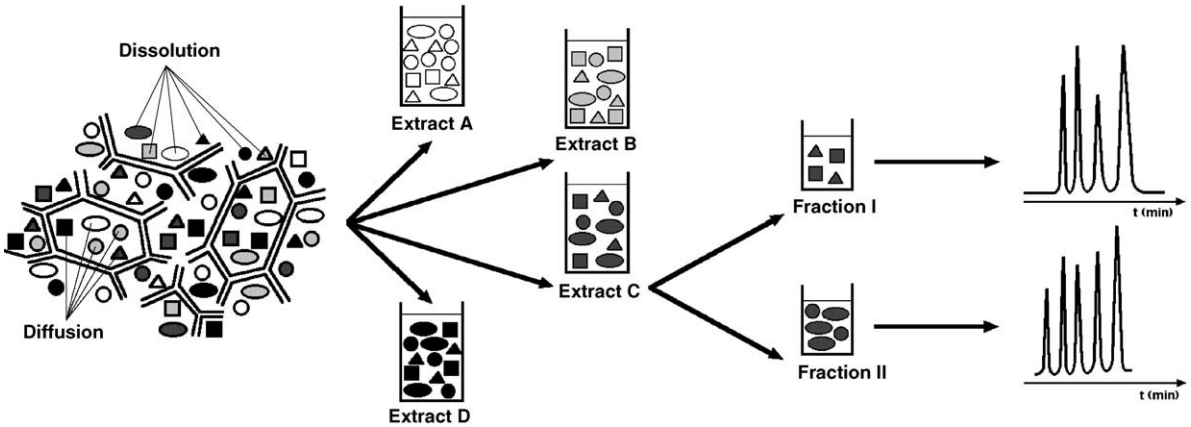

Fig. 2. Separation methods for the analysis and isolation of plant constituents.

The same guidelines are also valid for purification processes, independently of the applied method. Here solvent partition by liquid–liquid extraction is the most frequently applied method, since it is able to remove a large proportion of extraneous constituents and, especially when used in conjunction with a bioassay, fractions enriched in the sought-for constituent can be rapidly obtained [8].

Chromatography used for the separation of plant constituents can be classified from different points of view [9]:

2.2.1. Physical state of the phases employed for separation

If the mobile phase is a gas and the stationary phase is a solid or liquid, the separation techniques are known as gas–solid chromatography or gas–liquid chromatography (GSC and GLC or often simply GC). In liquid chromatography (LC), the mobile phase is a liquid, ranging from a single pure solvent to a multi-component mixture. Supercritical fluid chromatography (SFC) uses a mobile phase (usually CO_2) in the supercritical state at high temperature and pressure.

2.2.2. Geometry of the stationary phase

In GC, SFC and the majority of LC separations, the stationary phase is contained in a column. If the stationary phase is distributed as a thin layer on a flat support, the method is referred to as planar chromatography (PC).

2.2.3. Method of flow of the mobile phase

The mobile phase can migrate through the stationary phase under the influence of forced flow or by capillary action. Forced flow can be achieved either by the use of pressure as in GC, or in the different types of LC. Centrifugal force is used in rotation planar chromatography (RPC). The forced-flow techniques have the advantage that the mobile-phase velocity can be adjusted to give optimum separation conditions. In thin-layer chromatography (TLC), capillary action is the only driving force so optimum flow rate over the whole solid phase cannot be achieved.

2.2.4. Mechanism of separation

The stationary phase facilitates separation by adsorption, partition, ion-exchange, or size-exclusion processes. Some types of stationary phases cannot be classified into these four basic groups and separation may involve a combination of two or more of these four basic mechanisms.

2.2.5. Polarity relationship between the mobile and stationary phases

In normal-phase (NP) LC, the sorbent is more polar than the mobile phase, whereas in reversed-phase (RP) LC, the stationary phase is less polar than the mobile phase. Since plant extracts can generally contain many compounds, NPLC – which gas a good separation power – plays a very important role, especially in isolation strategies.

2.2.6. Aim of the separation

The aim of a separation may be for qualitative identification or for quantitative determination of the compounds separated (or both). LC may be used for preparative separation to isolate or purify substances. Preparative column LC techniques have seen great advances in recent years and are now extensively used in pharmaceutical research and development [reference to Novasep]. They are often used for the separation of chiral mixtures. Preparative TLC, OPLC and RPC are also used for purification and/or isolation on a laboratory scale.

2.2.7. On-line or off-line operation

All forced-flow techniques (GC, HPLC, MPLC, LPLC, FC, OPLC) are fully on-line methods, where the principal steps are performed as continuous operations and the separated compounds are eluted from the stationary phase. If OPLC is equipped with an injector and a detector, the various off-line and on-line operations can also be combined [10]. Similarly OPLC and also RPC may be used by connection to a flow-through detector, recorder and/or collection of isolated compounds with a fraction collector [11]. TLC and HPTLC for analytical purposes are typically fully off-line processes where the principal steps of sample application, development, evaporation of the solvent system, and densitometric evaluation are performed as separate operations. In preparative TLC the separated substances are not subjected to in situ quantification, instead the zones are scraped off the support and the separated compounds are eluted from the sorbent, using a solvent of high solvent strength [12].

2.3. Classification of solvents

The solvent-selectivity triangle classification method of Snyder [13] is the most enduring approach to solvent characterization used by chromatographers, although several other systems have been described [14–16]. In the Snyder system more than 80 solvents are represented by each of three solvent selectivity coordinates [proton acceptors (x_a) and proton donors (x_d), and their dipole interactions (x_n)]. Plotting the results on the surface of a triangle results in the classification of solvents into eight selectivity groups, as shown in Fig. 3. Solvents of similar solvent strength (s_i) from different groups have different selectivities and are capable of providing changes in the separation order.

Individual solvent strengths (s_i) and calculated individual selectivity values (s_v) for the characterization of different solvents in NP liquid chromatography have been published by Nyiredy et al. [17]. For characterization of a single solvent, s_v is defined as the quotient of the proton-acceptor (x_e) and proton-donor (x_d) values from Snyder's solvent classification data.

For characterization of multi-component mobile phases, the total solvent strength (S_T) can be defined as the sum of the s_i values of the components, weighted by multiplication

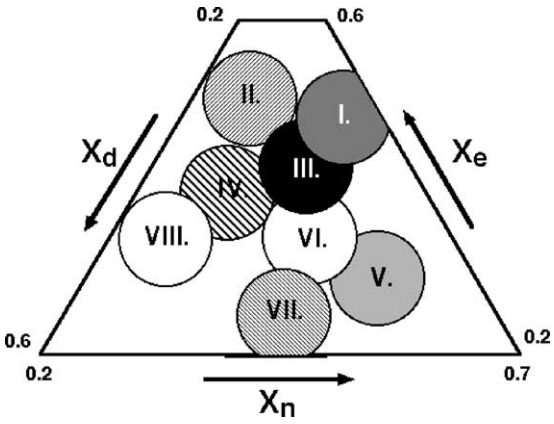

Fig. 3. Snyder's solent classification scheme for normal phase chromatography.

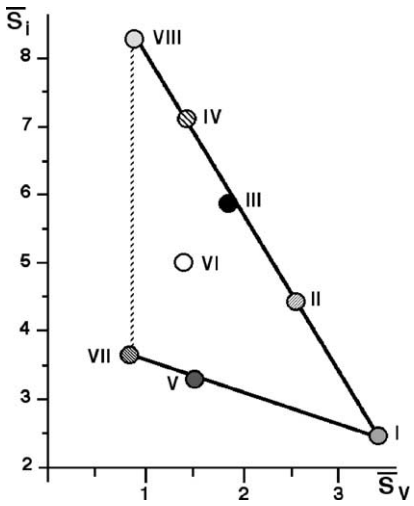

Fig. 4. Correlations between average individual selectivity values and average solvent strength of 80 solvents, used in normal phase chromatography.

with their volume fraction in the solvent mixture [17]:

$$S_T = \phi_1 s_1 + \cdots + \phi_n s_n = \sum_{i=1}^{n} \phi_i s_i$$

where ϕ_1 is the proportion of component 1 in the solvent mixture, ϕ_i the proportion of component i in the solvent mixture, s_1 = individual solvent strength of component 1, s_i the individual solvent strength of component i, i = 1, 2, . . ., n.

The total selectivity value (S_V) can be also calculated in a similar manner [17]:

$$S_V = \phi_1 s_{v1} + \cdots + \phi_n s_{vn} = \sum_{i=1}^{n} \phi_i s_{vi}$$

where ϕ_1 is the proportion of component 1 in the solvent mixture, ϕ_i the proportion of component i in the solvent mixture, s_{v1} the individual solvent strength of component 1, s_{vi} the individual solvent strength of component i, i = 1, 2, . . ., n.

When the average solvent strengths and selectivity values are calculated for each of Snyder's solvent groups, linear correlations are found between solvent groups I, II, III, IV, and VIII and between solvent groups I, V, and VII. For both groups, the r^2 values are greater than 0.998 [18]. Solvents which belong to group VI cannot be classified by these two correlations (Fig. 4). Literature data show that the solvent classes most often used for NP chromatographic separations are those in groups I, VII, and VIII, at the corners of the selectivity value–solvent strength (S_V–S_T) triangle, and solvents from group VI, for which all three properties (proton acceptor, proton donor, and dipole interaction) are approximately equal (Table 1).

This characterization of solvents can be used not only to describe pure solvents and multi-component mobile phase mixtures for normal-phase LC separations, but also to characterize the solvents for solid–liquid extraction procedures (see Section 3.3 below), as well as for liquid–liquid–liquid purification processes (see Section 4.2 below).

Table 1
Classification and characterization of solvents for NP chromatography

Group	Solvent	s_i	x_e	x_d	s_v
–	*n*-Hexane	0.1	–	–	–
I	*n*-Butyl ether	2.1	0.44	0.18	2.44
	i-Propyl ether	2.4	0.48	0.14	3.43
	Diethyl ether	2.8	0.53	0.13	4.08
	Methyl *t*-butyl ether	2.7	0.49	0.14	3.50
II	*n*-Butanol	3.9	0.59	0.19	3.11
	i-Propanol	3.9	0.55	0.19	2.89
	n-Propanol	4.0	0.54	0.19	2.84
	Ethanol	4.3	0.52	0.19	2.74
	Methanol	5.1	0.48	0.22	2.18
III	Tetrahydrofuran	4.0	0.38	0.20	1.90
	Pyridine	5.3	0.41	0.22	1.86
	Methoxyethanol	5.5	0.38	0.24	1.58
	Methylformamide	6.0	0.41	0.23	1.78
	Dimethylformamide	6.4	0.39	0.21	1.86
IV	Acetic acid	6.0	0.39	0.31	1.26
	Formamide	9.6	0.36	0.33	1.09
V	Dichloromethane	3.1	0.29	0.18	1.61
	Ethylene chloride	3.5	0.30	0.21	1.43
VI	Ethyl acetate	4.4	0.34	0.23	1.48
	Methyl ethyl ketone	4.7	0.35	0.22	1.59
	Dioxane	4.8	0.36	0.24	1.50
	Acetone	5.1	0.35	0.23	1.5
	Acetonitrile	5.8	0.31	0.27	1.15
VII	Toluene	2.4	0.25	0.28	0.89
	Benzene	2.7	0.23	0.32	0.72
VIII	Chloroform	4.1	0.25	0.41	0.61
	Water	10.2	0.37	0.37	1.00

3. Extraction of plant constituents

The extraction methods and strategies used depend on whether the aim of the extraction is analytical or preparative and whether the plants to be extracted contain known

compound/s, or unknown structure/s, where thermal stability may be important.

3.1. Analytical extraction methods

Sample preparation methods representing the first stage in an analytical procedure are of great importance. For volatile compounds generally steam distillation is the standard method [19], however, many new modern methods have been published [e.g., [20]]. Since as plant constituents the non-volatile compounds are mainly interesting, in the following these are shortly summarized.

Recently, Smith [21] published an overview on solvent extraction, including supercritical fluid and superheated water extraction [22]. Immuno-based sample preparation has been discussed by Hennion and Pichon [23], and a review by Saito and Jinno has also appeared [24]. Although these methods have so far only been partly used for the sample preparation of plant constituents, the trends are in this direction.

An overview of sample preparation used for solid samples was published by Majors [25] including the traditional methods, e.g., homogenization, vortex-mixing, and Soxhlet extraction, and some modern methods, e.g., supercritical fluid extraction (SFE). Papers have also been published describing extraction strategies using ultrasonic extraction (USE) [26], microwave-assisted extraction (MAE) [27], accelerated solvent extraction (ASE) [28] and automated Soxhlet extraction [29]. Because the majority of biologically active compounds from medicinal plants are non-volatile, only those methods will be discussed which are regularly used for solid–liquid extraction of medicinal plants.

For classic ultrasonic extraction, acoustic vibrations with frequencies above 20 kHz are applied to the sample when vibrations are transmitted through the liquid and cavitation occurs, that is bubbles with negative pressures are formed. Implosion of cavities creates high pressures and temperatures in the microenvironment [26]. Sonication may cause decomposition or oxidation of compounds, which has to be borne in mind during development of the extraction method [30]. Due to the inefficient recovery, the extraction is generally performed in three steps and the solvent consumption is therefore relatively high.

For microwave-assisted extraction, introduced by Ganzler et al. [31], typically the microwave sources operate at 2.45 GHz. Atmospheric pressure methods employ solvents with low dielectric constants, which are essentially microwave-transparent. The extraction can be carried out in open vessels, because the solvents absorb only a small amount of energy. The temperature of the sample increases during the process, because of the water content (6–10%) of the air-dried plants. This extraction procedure is mild, so this type of MAE may be used for the extraction of thermally labile compounds [32]. For pressurized MAE, a microwave-transparent vessel and solvents with high dielectric constants are used. The solvents absorb the microwave radiation and are therefore heated under pressure to a temperature exceeding their normal boiling points [33,34]. This extraction method is similar to accelerated solvent extraction, because the elevated temperature and pressure facilitate extraction of compounds from the plants.

Using the recently developed ASE, also referred to as pressurized fluid extraction (PFE), the solid sample is enclosed in a sample cell that is filled with the extraction solvent. After the cell is sealed, the sample is permeated by the extracting solvent under elevated pressure and temperature for 5–10 min. Typically, the samples are extracted under static conditions, however dynamic or flow-through techniques have also been described [35,36]. Comparisons between ASE, USE and Soxhlet extraction show that the performance of ASE is equivalent or better than the other methods [37].

SFE is an exhaustive extraction method, since the applied fresh supercritical fluid may be continuously forced to flow through the sample. A remarkably high selectivity can be achieved, because the solvation power of the supercritical fluid can be changed by varying pressure and/or temperature and by the addition of small quantities of more polar compounds [38]. Other additional advantages of SFE over conventional methods are that it can be automated, it has short extraction times and has a very small solvent consumption. Therefore, SFE is suitable for fast, routine analysis, but method development is extremely labor-intensive [39]. A review of the use of SFE in the preparation and analysis of Chinese herbal medicines was presented by Chen and Ling [40], while a critical review of the analytical usefulness of SFE has been published by Zougagh et al. [41].

A comparison of different analytical solid–liquid extraction methods for plant constituents is given in Table 2.

Table 2
Comparison of different analytical solid–liquid extraction methods for plant constituents

Factor	Soxhlet	USE	ASE	MAE	SFE
Type of extraction	Exhaustive	Not exhaustive	Not exhaustive	Not exhaustive	Exhaustive
Investment	Small	Small	Large	Medium	Large
Extraction time	6–48 h	<30 min	<30 min	<30 min	<60 min
Solvent consumption (mL)	200–600	<50	<100	<40	<10
Method development	Simple	Simple	Simple	Simple	Difficult
Sample treatment	Required	Required	Required	Required	Not required

USE: ultrasonic extraction; ASE: accelerated solvent extraction; MAE: microwave-assisted extraction; SFE: supercritical fluid extraction.

An up-to-date summary of sample preparation of plants for the chromatographic analysis has been given by Namiesnik and Górecki [42]. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants was recently published by Huie [43].

3.2. Preparative extraction methods

Preparative extraction methods include all techniques where the aim of the extraction is the isolation of the major and/or a biologically active compound(s) from a particular plant. Typically a few milligrams of the isolated compound are sufficient for research purposes (structure elucidation, in vitro and/or in vivo test for bioactivity): a few grams are required for animal experiments and/or for clinical purposes.

The production of phyto-pharmaceuticals includes standardized extraction procedures, since this is essential for quality assurance [44]. The quantity of major compounds or their relative abundance is assayed by different liquid chromatographic methods. When choosing major substances from particular plants or phyto-pharmaceuticals it is essential that analytically well-characterized standards (certified reference materials) [45] are available for their quantification. Because it is often impossible to separate all the compounds in a plant extract completely, purity of major constituents must be proven with a different and independent method of separation to avoid doubts arising from co-elution.

Generally, for the isolation of plant constituents for research purposes little attention is given to the efficiency of the extraction methods used. The most commonly used methods are the maceration, remaceration (shaking) and Soxhlet extraction for stable substances [8]. Solid–liquid extraction methods for industrial purposes have been summarized by List and Schmidt [46]. The principles of two recently published novel forced-flow solid–liquid extraction methods developed mainly for laboratory purposes are summarized below.

With the rotation planar extraction (RPE) method, the solid–liquid extractions are carried out in a closed circular chamber with a special geometric design of the planar support to ensure linear solvent flow [47]. Although the extraction distance is relatively short (11 cm) compared with other preparative techniques, the separation efficiency is higher due to the very fine particle sizes of the powdered plant material to be extracted and the variable centrifugal force (rotation speed). The RPE method clearly has all of the advantages of both forced-flow and exhaustive separation and should gain rapid acceptance as an efficient, on-line preparative technique for laboratory purposes.

The method can easily be used for the preparation of fractions containing constituents of different polarities. The extraction can be started with *n*-hexane using a low rotation speed (ca. 400 rpm). After the *n*-hexane fraction has been obtained, the rotation speed is increased to ca. 1500 rpm for a few minutes in order to expel the remaining solvent and to dry the plant material. The next extraction solvent with increasing polarity can then be applied at 400 rpm. Between changing the solvents the centrifugal force has always to be increased for the expulsion and drying process. Clearly, many fractions with increasing polarity can be prepared in this way [47].

Recently, a novel variant of continuous relative counter-current extraction, called medium-pressure solid–liquid extraction (MPSLE) was introduced as a new preparative separation method [48]. On using MPSLE the extraction column – a medium pressure liquid chromatographic column – is filled with the finely powdered plant material to be extracted, and extraction solvent/s is/are forced through the stationary bed by means of a pump. This method constitutes relative counter-current extraction, and results in exhaustive and rapid extraction. The efficiency is achieved not just because the extraction solvent moves through the sample, but also due to the overpressure in the extraction column and by changes in the dissolution and diffusion periods of the process. The method can be used for the rapid extraction of various substance classes occurring as complex solid matrices. Experiments carried out with MPLC columns show that 100–3000 g of finely powdered plant material can be extracted and concentrated with an automated equipment within a few hours [48]. Further advantages of this environment-friendly process performed in a closed system are full automation and operation with a small quantity of extracting solvent.

3.3. Proposed extraction strategy

Little attention has, unfortunately, been given to the selection of appropriate extraction solvents or solvent systems for solid–liquid extraction of plant constituents and solid–liquid extraction of plant is generally based on trial and error [46]. Based on the “PRISMA” optimization model [e.g., [49]] a general useful extraction strategy for plant constituents was published recently [50].

In this suggested strategy for handling the solvents in Snyder’s eight selectivity groups [13], 13 are commonly used for the solid–liquid extraction of plant constituents and/or as representatives of their groups on the basis of their individual s_i and s_v values. In the following, the Snyder’s solvent groups are indicated in parenthesis: *n*-hexane (−), *i*-propyl ether (I), ethanol and methanol (II), tetrahydrofuran and methoxyethanol (III), dichloromethane (V), ethyl acetate and acetone (VI.a), acetonitrile (VI.b), toluene (VII), chloroform and water (VIII). The individual s_i and s_v values can be taken from Table 1. Because the s_i value of *n*-hexane is approximately 0.1, to simplify the calculation the solvent strength of this solvent can be regarded as zero. Because *n*-hexane has no s_v value, a selectivity value of 0.01 can be used for the calculation.

After initial experiments with these pure solvents, the amount/s of compound/s of interest, the extraction yield and/or their ratio must be measured by means of a suitable

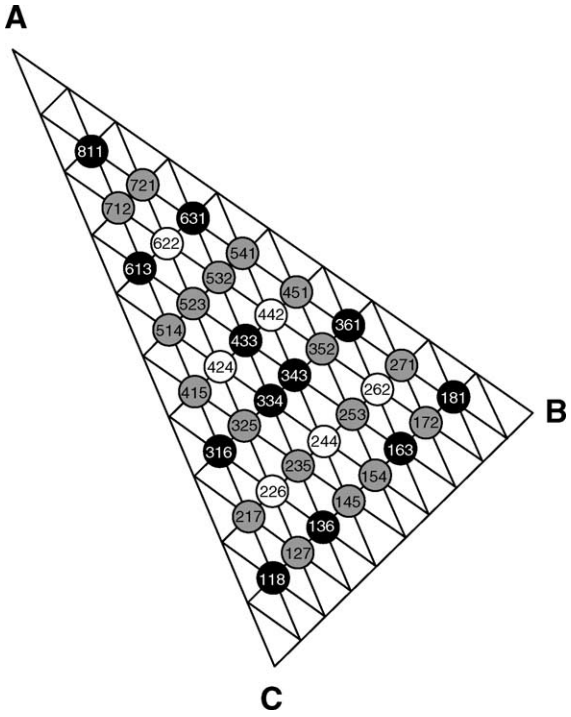

Fig. 5. Proposed optimization strategy for the solid–liquid extraction procedure ($s_A > s_B > s_C$) (black rings indicate measured data, grey rings indicate calculated data, and white rings indicate predicted extraction data).

optimized analytical method (e.g., HPLC, TLC). Occasionally satisfactory results can be achieved by extraction with the pure solvents. If solvents give good results, their homologs or other solvents from the same Snyder's group [13] might also be tested.

Generally, between two and four solvents are mixed to give the best results. When three solvents are selected, the optimization triangle can be visualized as the top (irregular) triangle of the "PRISMA" model. The extraction solvent combinations [representing three solvents along the edges of the triangle between the basic selectivity points (811–181–118) and the selectivity points around the center of the triangle (433–343–334)] are measured.

In Fig. 5, the 12 black rings (e.g., 811, 631, 361, 181) indicate the selectivity points to be tested and the 18 grey rings (e.g., 721, 541, 451, 271) show those selectivity points at which the extraction values can be calculated with the quadratic functions obtained from the measured values. For the remaining six selectivity points, indicated by the white rings (e.g., 622, 442), the extraction values can be predicted by functions obtained from the measured and calculated data. Thus, 12 measurements are necessary to obtain the global optimum. Data from non-polar (*Heracleum sphondylium* L.) and polar (*Betulae pubescens* L.) constituents have been used to demonstrate the validity of the proposed strategy [50].

The major advantages of the proposed method are that the structures and properties of the compounds to be extracted do not have to be known and the method may be used for analytical as well as for preparative extractions.

4. Purification of plant constituents

After solid–liquid extraction, the next step in sample preparation is the purification of the raw extract, which is a crucial step for medicinal plants. Thus, the development of modern purification techniques with significant advantages over conventional methods for the extraction and analysis of medicinal plants is likely to play an important role in the overall effort to provide high quality herbal products to consumers worldwide.

Recent developments and applications of modern sample preparation techniques for the clean-up, and concentration of analytes from medicinal plants or herbal materials are reviewed in reference [42].

4.1. Analytical purification methods

There is no doubt that for analytical purposes the most widely used purification method is solid-phase extraction (SPE), which appeared as an alternative to liquid–liquid extraction [7]. SPE is widely used for pre-concentration and clean-up of analytical samples and the purification of medicinal plant extracts. The method offers a variety of sorbents based mainly on silica. Pre-packed cartridges may be used in one of two modes, such that the interfering plant constituents of a sample are retained on the cartridge while the components of interest are eluted or vice versa. The method is widely used for sample preparation prior to GC and HPLC analysis. The new directions in the application of SPE, like immunoassay, molecularly imprinted polymers and stir bar sorptive extraction have been summarized in reference [30].

4.2. Preparative purification methods

Liquid–liquid extraction, based on partition between solvents is the most commonly applied preparative purification method where a large proportion of extraneous constituents need to be removed. The two (basically) immiscible phases which are frequently used are various ratios of diethyl ether/water, ethyl acetate/water, dichloromethane/water and chloroform/water. Every research group has experience of two-phase systems for different classes of substances.

The multiple partition steps (Craig counter-current distribution, various counter-current chromatographic methods), filtration and precipitation for the preliminary purification of raw extracts are discussed in detail by Hostettmann et al. [8].

It would appear that the most effective and simple purification method for isolation purposes is forced-flow multiphase liquid extraction (FFMLE) [51], which is an absolute counter-current distribution method, based on the use of three immiscible liquid phases. The operating principle and set-up of FFMLE are shown schematically in Fig. 6. The extraction column (e.g., an MPLC column) is filled with an appropriate volume of all three phases. The lowest of the three immiscible liquid phases (shown in grey in Fig. 6) is then pumped

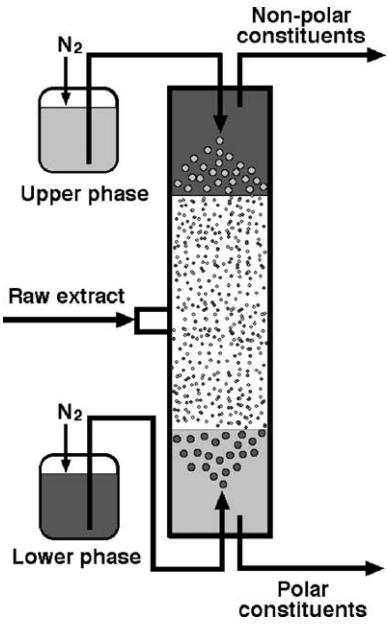

Fig. 6. Operating principle and set-up of FFMLE (upper mobile phase is black, middle stationary phase is white, lower mobile phase is grey).

from the top of the column through the stationary (middle) phase (shown in white in Fig. 6).

It moves as droplets from the top of the column to the bottom, where it is collected and removed from the system. The uppermost of the three phases (shown in black in Fig. 6) is pumped from the bottom to the top of the extractor, where it is collected and removed. The adjustable injector systems are located such that the upper phase is injected into the lower phase and the lower phase into the upper phase. At the phase boundary, the droplets can disintegrate into numerous much smaller droplets, significantly increasing their surface area. In the middle, stationary (white) phase millions of tiny droplets are present, these can only be seen schematically in Fig. 6. The sample has to be injected into the middle phase.

Beside the applied forced-flow, the driving force for the droplets is the difference between the specific gravities of the phases traveling in opposite directions. The separation power is excellent, because the distance traveled by a tiny droplet is significantly longer than the length of the column, since the droplets experience many collisions.

4.3. Proposed purification strategies

For both, analytical and preparative purposes, multi-phase liquid extraction can be applied with high efficiency. For analytical sample preparation, the shaking method is acceptable, while for large amounts of extract to be purified, the forced-flow multi-phase liquid extraction method is suggested.

Typical three-phase systems can be obtained on mixing water (for RP chromatography $s_i = 0$), acetonitrile (group VI) and *n*-hexane (for NP chromatography $s_i = 0$) as the basic standards for a ternary system, with a modifier solvent (selector), which mainly influences the selectivity of the systems. It

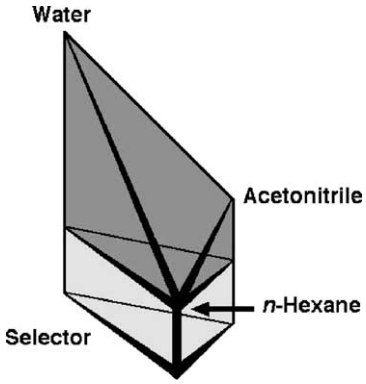

Fig. 7. The applied parts of the "PRISMA" model for demonstrating the creation of three immiscible phases, using quaternary solvent systems (upper part of the model where the top irregular triangle represents the solvent combinations for the basic of ternary systems, lower part of the model demonstrate the selector for modifying the selectivity of the systems).

can be symbolized by the upper part of the "PRISMA" model [49], where the top irregular triangle represents the solvent combination possibilities of the three basic solvents, while the selector is the socle (substructure) illustrated in Fig. 7.

The selector which influences the selectivity can be diethyl ether (I), dichloromethane (V), ethyl acetate (VI), toluene (VII) and chloroform (VIII). Typical quaternary systems can be produced if the ratio of water:acetonitrile:*n*-hexane:any of the suggested modifier = 2:4:4:2.

For both, analytical and isolation purposes all five quaternary three-phase systems have to be tested by a pre-assay in stirring cones. Dry samples have to be introduced in the system, otherwise the immiscibility of the solvents could be jeopardized. The suggested ratio for analytical purification is 1:50 (all three phases together). Since the shaking method is a non-exhaustive method, it has to be repeated three times.

For special problems, or for industrial production purposes the selectivity of the three-phase system can be tuned, according to the possibilities offered by the "PRISMA" model [49].

5. Analytical chromatographic strategies

For a phytoanalyst working with plants with possibly many unknown constituents, two questions about these constituents require answering: What is it and how much? Generally, the role of identification is undervalued. Even in the Encyclopedia of Separation Sciences [52] and in the newest textbooks on chromatography [53], the word "identification" does not appear in the subject index.

5.1. Identification strategy for volatile compounds

The identification strategy depends on whether only an FID is available or if MS and FTIR spectra are also available (Fig. 8).

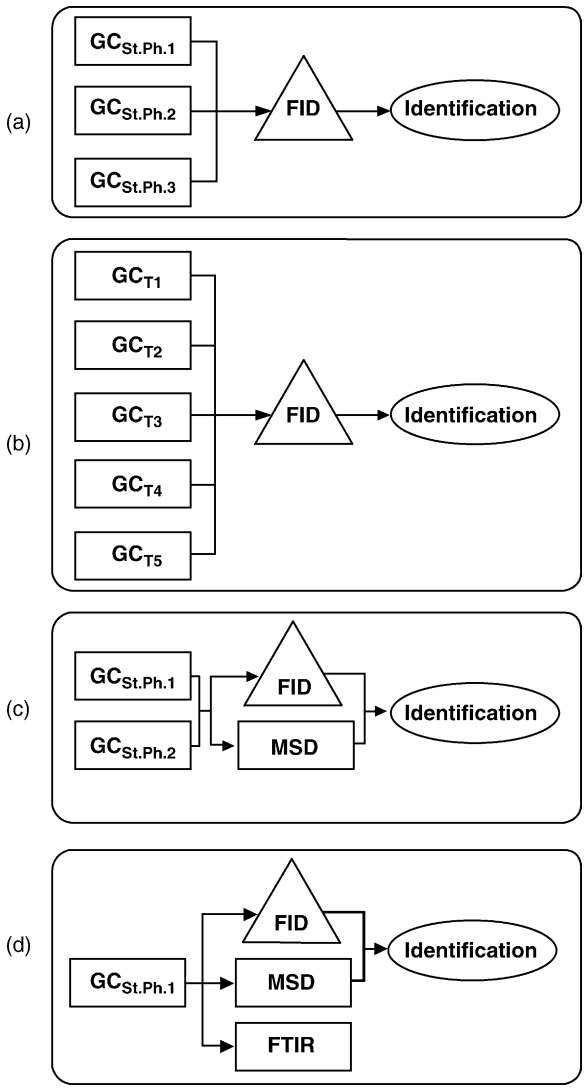

Fig. 8. Identification strategies for volatile constituents.

5.1.1. Identification using retention data

Reliance on a single retention value, even when using the same capillary column is totally unsatisfactory and, in our experience, in many publications the identification given is incorrect.

The Kováts [54] retention index system offers an excellent means of GC identification provided that two ways of using retention indices are followed. Either the retention indices have to be identical (within ±0.2 index units) using three stationary phases with different selectivities (Fig. 8a), or if using only a single column, the dependence of retention index with temperature has to be identical (Fig. 8b) at not less than five different temperatures [55]. In both cases, identification can be carried out without reference compounds if reliable data can be found in the literature.

5.1.2. Identification using hyphenated techniques

Generally, separated components are identified on the basis of their mass spectra. Nowadays high resolution MS and MS–MS are pretty well nigh infallible [56,57]. Recently, Tarján et al. [58] showed that reliable qualitative identification is doubtful without the use of retention data in conjunction with low resolution MS and standards wherever possible. By identification of the essential oil component of marjoram, *cis*-sabinene hydrate acetate, they demonstrated that the mass spectrum alone could lead to incorrect identification if retention indices are not taken into consideration. Without reference compound, the certainty of identification can be increased if, beside the MS spectra, retention indices on two different stationary phases are used (Fig. 8c) or, if only a single column is applied, FTIR spectra are available in addition to mass spectra (Fig. 8d).

5.2. Identification strategy of non-volatile compounds

Generally, the identification procedures of non-volatile plant constituents are carried out with three different methods:

- For known compounds using references three different separation methods plus one spectroscopic method.
- For known compounds without references two different separation methods plus two spectroscopic methods.
- For unknown compounds one high-performance separation method plus three different spectroscopic methods.

5.2.1. Identification of known compounds using reference standards

The identification of plant compounds by TLC is the basic technique given in pharmacopoeias. The TLC is performed under standard conditions and the spot of the substance being examined, with or without derivatization, is compared with that of similarly developed reference material applied at approximately the same concentration [e.g. [56]].

Using TLC for identification, the plant extract and the reference substances have to be always applied on the same chromatoplate and the hR_F values have to be calculated from the densitograms. Using a single solvent system, two compounds can be considered as identical if the variance of the ΔhR_F values of the compounds to be identified and the reference substances is less than ±3. If the hR_F value of the unknown compound has practically the same value as the reference substance in three mobile phases with different selectivities, these hR_F values can be depicted as a triangle in a coordinate system. The area of the triangle (the $I_{P(Chr)}$ value), which can be calculated according to the rules of coordinate geometry, characterizes the goodness of the chromatographic identification (Fig. 9) [17].

The higher the value of $I_{P(Chr)}$, the better is the probability that two compounds are identical. If the $I_{P(Chr)}$) value is less than 0.1, the identification is inadequate; for routine laboratory work it has to be between 0.1 and 0.5. If the value of $I_{P(Chr)}$ is higher than 0.5 then the substances are chromatographically identical with a high degree of probability. The

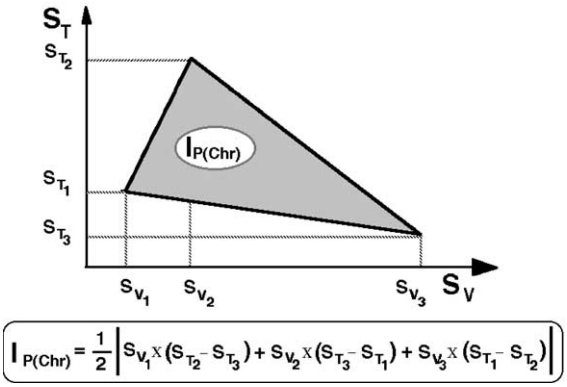

Fig. 9. Chromatographic identification probability [$I_{P(Chr)}$] for non-volatile constituents.

identification scheme using three different mobile phases and in situ UV–vis spectra is depicted in Fig. 10a.

For satisfactory identification of known components of plant extracts not only the chromatographic but also the spectroscopic data must be identical. For in situ spectroscopic identification from TLC plates, two criteria must be fulfilled. Firstly, every minimum and maximum of the UV and/or vis spectra must be practically identical and secondly the ratio of the local absorbance minima and maxima must be identical. All values of local minima and maxima (λmin, λmax), and the relative absorbance ratios must, therefore, be given. The illustrated correlations between the reference substance and the substance to be identified must be linear in the coordinate systems applied. Regression coefficients ($r2$) are used to characterize the probability of spectroscopic identification [59].

Using RP-HPLC for known compounds and if reference compounds are present, generally three different mobile phase compositions (different solvent strength and selectivity) and DAD detection have to be used for the identification [60]. To create significantly different mobile phase compositions is a real challenge, because only a few solvents (methanol, ethanol, propanol, acetonitrile, tetrahydrofuran and water) can be used for reversed-phase separations (Fig. 10b).

The advantage of NP-TLC is the large number of solvents which can be combined to vary the selectivity of the separations, while the disadvantage of the method is the low separation power. Using RP-HPLC, fewer solvents can be used, however, the separation power is much greater. Therefore, in practice, a good combination is the use of two different NP-TLC mobile phases and one RP-HPLC mobile phase (Fig. 10c). For dubious identifications, three different separation methods (e.g., NP-TLC, RP-HPLC and CE) have to be used (Fig. 10d).

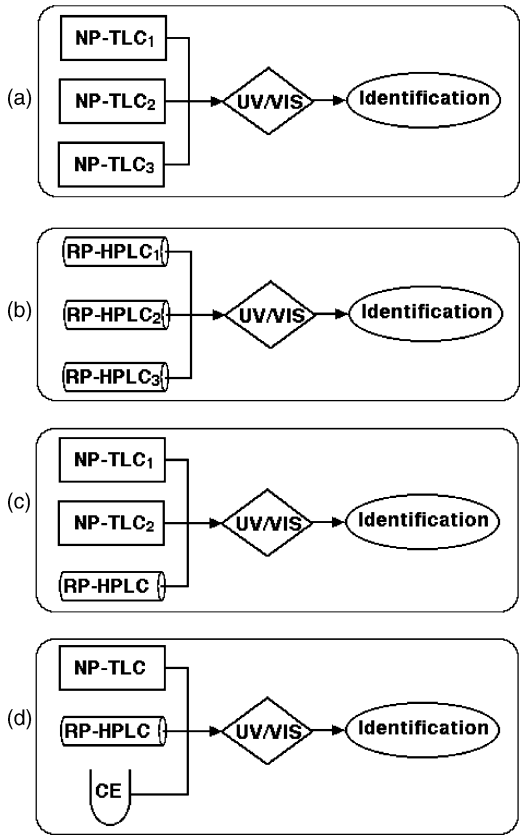

Fig. 10. Identification strategies for known, non-volatile constituents using references.

5.2.2. *Identification of known compounds without reference standards*

Use of retention data alone is insufficient for unambiguous identification because of the high risk of co-elution of the compound in question with many other compounds in any separation system. Combined or coupled techniques, for example HPLC–DAD–MS [61], or HPLC–DAD–MS–MS [62] are frequently used for structural elucidation and for identification of a known compound from a plant. The recent introduction of HPLC coupled to nuclear magnetic resonance (HPLC–NMR) [63,64] represents a powerful complement to HPLC–DAD–MS screening. However, in many cases, two different separation methods, like RP-HPLC and NP-TLC (Fig. 11a), or HPLC and CE (Fig. 11b), or TLC

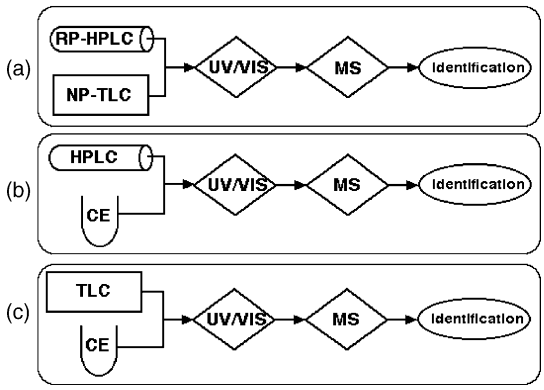

Fig. 11. Identification strategies for known, non-volatile constituents without references.

and CE (Fig. 11c) and the use of DAD and MS techniques are adequate for identification. Using TLC as one of the separation methods, UV/VIS can be carried out in situ on the chromatoplate, but MS can only be carried out off-line.

In practice, three types of identification arise in the search for major compounds in medicinal plants:

- identification of a known compound,
- identification of an unknown compound,
- verification of the presence of particular medicinal plants in an extract.

Fig. 12 summarizes the identification strategies used for all three types. In the second type of identification, when the compounds have been isolated and the structure elucidated, the task becomes the same as for the first type [65].

5.2.3. *Identification of compounds with unknown structures*

The identification of compounds with unknown structures is a very difficult analytical task. Plant constituents of interest are usually isolated following a fractionation (mainly bioactivity-guided) procedure. In order to render this approach more efficient, the monitoring of plant extracts (crude or purified) with HPLC-hyphenated techniques avoids finding known compounds and targets the isolation of new biologically active compounds [66,67].

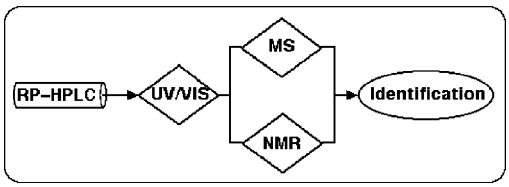

Fig. 13. Identification strategy for unknown, non-volatile constituents.

To provide a precise fingerprinting of the secondary metabolites in a given plant extract directly, on-line hyphenated techniques such as HPLC–DAD–MS–NMR are necessary, as illustrated in Fig. 13. This combination represents a valuable tool for further detailed metabolomic studies on either genetically modified plants or stressed plants [68].

HPLC–DAD–MS–NMR allows a rapid structural determination of known compounds with only a minute amount of plant material. Simple bio-autographic assays for screening biologically active compounds can also be performed directly on-line by collecting HPLC peaks and measuring the activity. These bioassays permit a rapid location of the biologically active compounds. With such a combined approach, the time-consuming isolation of common natural substances

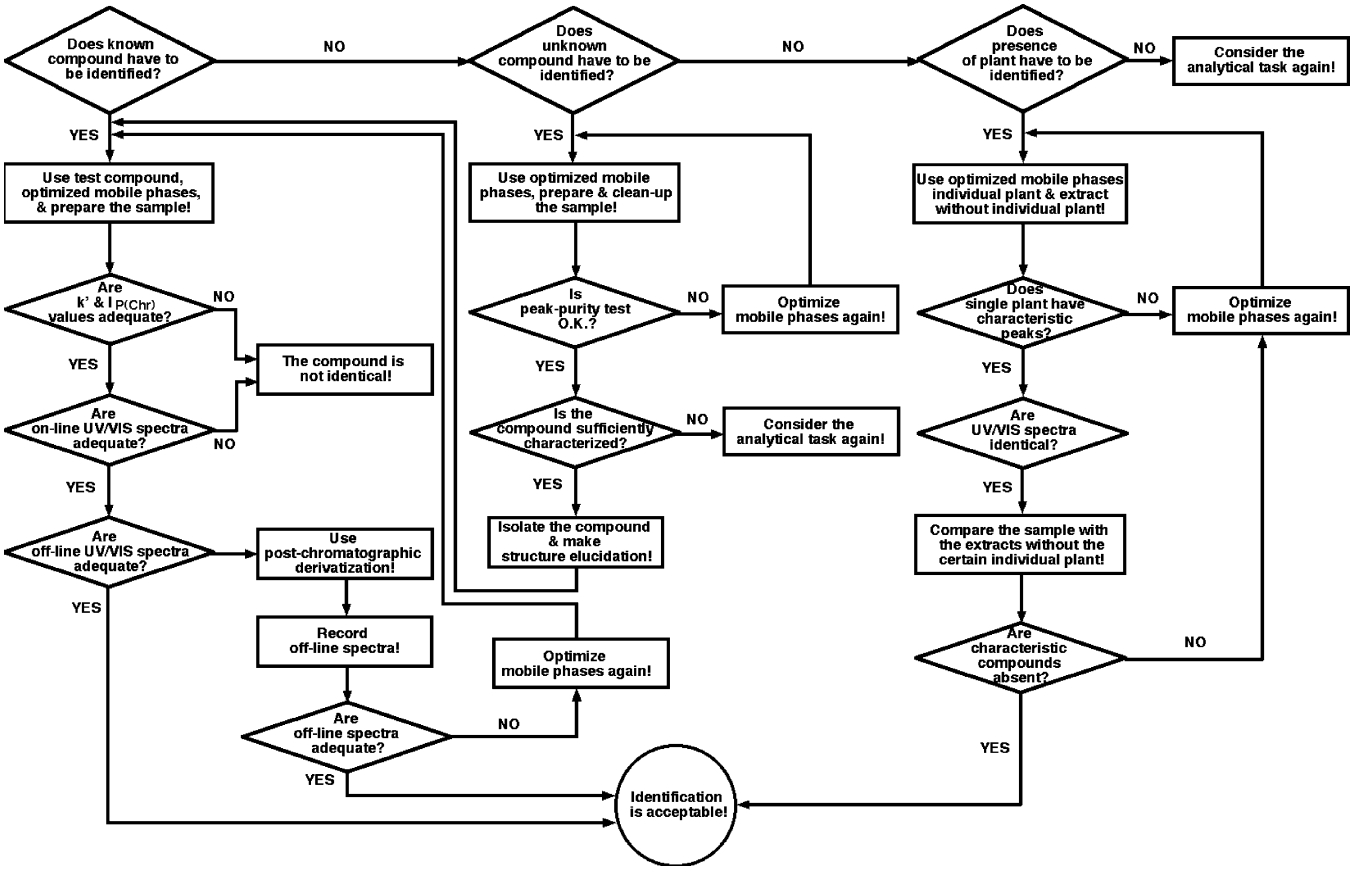

Fig. 12. Flow chart of various identification procedures for non-volatile major compounds.

is avoided and an efficient targeted isolation of compounds with interesting biological and/or spectroscopic features is performed [69].

6. Quantitation

Keeping in mind that a single medicinal plant can contain up to several thousand secondary metabolites, the co-elution of compounds with similar or identical UV spectra cannot be excluded. Therefore, there exists two ways to produce correct quantitative data.

6.1. Quantitation with different methods

An analysis has to be not only correct, but also rapid and economical, however the most important is the correct result. Generally, for routine phytoanalytical determinations, by the use of two independent methods with different separation mechanisms (e.g., RP-HPLC and NP-TLC, CE and GC), the results are acceptable if for major compounds of the plant they agreed within 3%. As was demonstrated for the determination of Betulae flavonoids [5] the difference between planar (off-line OPLC) and column (HPLC) LC determinations are not generally significant, with standard deviations of 0.062%, 0.075% and coefficients of variation of 3.6% and 4%, respectively.

In certain cases, the use of a third, confirmatory method may be necessary. Szűcs et al. [70] published a high throughput strategy, for the quantification of ca. 10,000 samples per year, per genotype. The procedure described comprised consecutive LC methods, where the quantification parameters are different:

- planar (OPLC, HPTLC) and column (HPLC) methods,
- open (HPTLC) and closed (OPLC, HPLC) systems,
- different equilibration of the stationary phases (non-equilibrated for OPLC, saturated vapour phase for HPTLC, and a mobile phase equilibrated system for HPLC),
- different mobile and stationary phases (amino phase for OPLC, silica for HPTLC, and a C18 phase for HPLC),
- different detection modes (visual off-line for OPLC, off-line densitometric for HPTLC, and on-line for HPLC).

The strategy is demonstrated in Fig. 14. The first step is a multi-layer OPLC separation [71] using a circular separation mode, in which the mobile phase migrates radially from the centre to the periphery. Using the multi-layer technique on two amino TLC plates 144 samples were applied and simultaneously developed, from which two samples were identified containing standard mixtures of alkaloids at the limiting concentration (1.6% morphine) for visual semi-quantitative evaluation. The reduced number of samples (ca. 80%), where the alkaloid content seems to be more than or equal to 1.6%, were analysed on silica HPTLC plate with densitometric evaluation at 280 nm.

All samples where the first quantitative method showed higher alkaloid content than 2.0% (ca. 6000 samples) were verified with a rapid RP-HPLC method, using a short column (33 mm × 4.6 mm i.d.) filled with 1.5 μm Kovasil MS-C_{18} material. If the discrepancy between the two quantitative results was too large, a third, confirmatory RP-HPLC method was used, applying a longer column (120 mm × 4.6 mm i.d.) with larger particle size (5 μm) C_{18} material. Only ca. 6% of all samples required the third analysis. The authors [70], who gave the strategy in the form of a flow-chart, assumed this was necessary because of minor compounds which appeared during the elimination procedures.

6.2. Quantitation with a single method and different parameters

Generally, only a single method, mainly HPLC is used for the quantitative determination of medicinal plant extracts. First the separation of reference compounds is optimised, then the global optimum conditions are applied to the analysis of the plant samples. Before the quantitative determination, the peak purities of all compounds of interest are

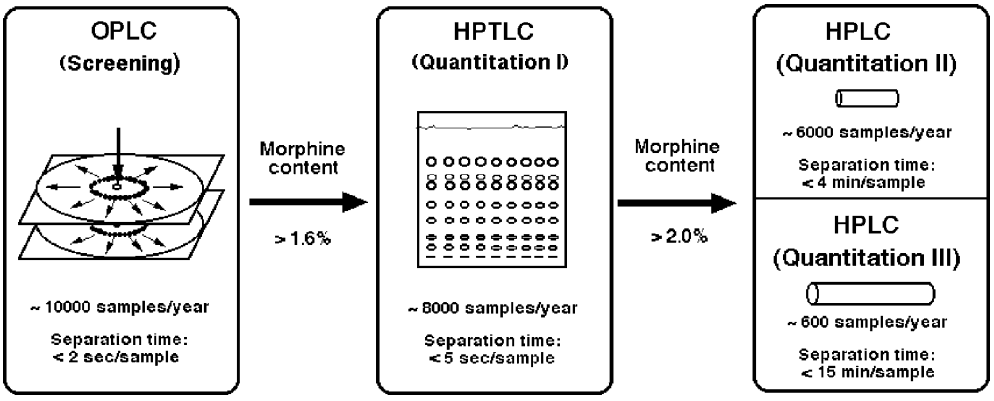

Fig. 14. Combined LC strategy for the determination of morphine content of poppy straw.

tested using diode-array detection. If all three spectra (up, apex, down) are identical then the quantities of the individual components can be determined from calibration curves. This procedure generally works satisfactorily but our experience is that, in certain cases, there can be an error of over 50%! [72]. The reason for this is in the difficult identification of the UV spectra of certain compound classes, like flavanolignans. Therefore, the suggested strategy is as follows:

(1) determination of the global optimum separation using reference compounds,
(2) confirmation of the purity of all compounds of interest,
(3) quantitative determination of all compounds of interest,
(4) calculation of the ratios of the determined compounds,
(5) repetition of steps (2)–(4) using three local optima, with different selectivities of the mobile phases.

In our opinion, quantitative results should only be accepted if, using the global optimum, the ratios of the determined compounds are identical to one decimal point with the ratios of three measurements (local optima) using different mobile phase with different selectivities.

7. Isolation strategies for plant constituents

In the literature, several isolation strategies are reported [e.g., [73–75]], where, depending on the compounds isolated, different separation methods are used. Needless to say, a universal strategy does not exist for the isolation of different secondary metabolites with totally different structures. All laboratories and researchers have their own strategies based on their own experience.

7.1. Structure elucidation before isolation

The hyphenated technique of HPLC–DAD–MS–NMR [68] allows structural determination of plant compounds from the raw extract, without isolation. Simple bioautographic assays for screening bioactive constituents can also be performed on-line by collecting the HPLC peaks and measuring the activity of interest. The advantages of this strategy [69] are that compound/s will be isolated only if the structure elucidation establishes a novel constituent and/or the compound shows high biological activity. The disadvantage of this strategy is that, for lack of isolated compounds, other bioactivity examinations cannot be carried out.

Since the cost of such a combination of techniques is extremely high, only a few laboratories have these facilities at present. If the structure and/or bioactivity data generate interest the guidelines for isolation are as in Section 7.2 below.

7.2. Structure elucidation after isolation

Generally, either the biologically active constituents, or the major compounds are the aim of the isolation procedures [76]. The two strategies from the separation point of view are the same. Using the bioactivity-guided isolation process, the activities have also to be measured at certain points in the procedures, as shown by "BAE" (bioactivity examination) in Fig. 15.

In the following, based on our 20-year experience and more than a hundred isolated compounds, we give an isolation strategy where the structures and properties of the compounds to be isolated do not have to be known.

Before the isolation procedure is started, the collected plant material, identified by at least two independent botanists, has to be carefully dried. Afterwards the chopped

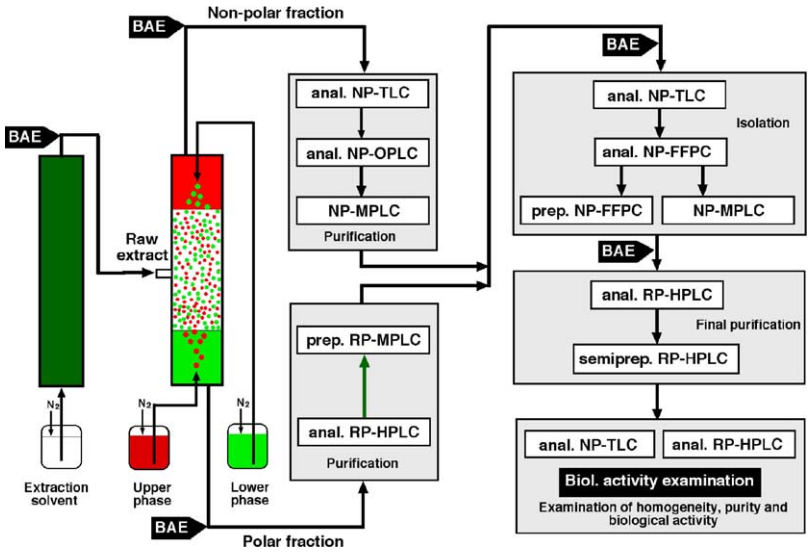

Fig. 15. Proposed strategy for the isolation of secondary constituents of medicinal plants (BAE = bioactivity examination, for all other abbreviations see the text).

and powdered parts of the plant can be used for the solid–liquid extraction procedure. If an unknown plant is involved, the extraction can be carried out using methanol or any exhaustive procedure where only ambient temperature is applied, in order to prevent possible thermal degradation. As a technique we prefer medium-pressure solid–liquid extraction (MPSLE, see Section 3.2), because the efficiency of the extractor is equivalent to other commonly used methods with the advantage that the often difficult filtration of the extract can be omitted. Additionally, depending on the amount of the plant material to be extracted, the choice of an appropriate size of column presents no difficulty.

After the solid–liquid extraction, the fractionation process is started [74]. The concentrated and dried raw extract can be introduced to the forced-flow multi-phase liquid extractor after selection of the appropriate three-phase system. Owing to the absolute counter-current distribution, two fractions can be achieved and the separated compounds continuously leave the extractor. The non-polar compounds emerge from the top of the extractor and the polar ones from the bottom (Fig. 15). Finally, both fractions have to be concentrated at a temperature not exceeding 36 °C. The purification steps of the non-polar fraction can be summarized as follows:

- Optimisation of the mobile phase on silica TLC in an unsaturated chromatographic chamber. The compounds to be isolated have to be between $2 < hR_F < 8$.
- Transfer of the optimised mobile phase to normal phase overpressured layer chromatography (NP-OPLC). A pre-run has to be carried out using *n*-hexane, to eliminate the disturbing zone and equilibrate the "planar column" [75]. The OPLC separation has to be carried out using the optimised NP-TLC mobile phase.
- Scaling-up to normal-phase medium-pressure liquid chromatography (NP-MPLC). The silica (average particle size 15 μm) containing slurry has to be prepared and equilibrated with *n*-hexane. For the preparative MPLC separation, the optimised NP-TLC mobile phase has to be used [76].
- Control of each fractions using NP-TLC.

The purification steps for polar fractions can be summarized as follows:

- Optimisation of mobile phase on analytical reversed-phase HPLC.
- Scaling-up to RP-MPLC. For the preparative separation, the optimised analytical HPLC mobile phase has to be used.
- Control of each fractions using analytical RP-HPLC.

The next steps, which are the same for both polar and non-polar constituents, are as follows:

- Optimisation of mobile phase for each fraction on analytical NP-TLC, in an unsaturated chromatographic chamber. During the optimisation process one solvent has to be incorporated into the mobile phase in which the compound of interest does not migrate. The compound/s to be isolated has/have to be between $2 < hR_F < 8$.
- Transfer of the optimised mobile phase to a suitable NP forced-flow planar chromatographic (FFPC) method. For OPLC, a pre-run has to be carried out using a solvent, in which the compound/s of interest does not migrate. For rotation planar chromatography (RPC), no pre-run is required because the RPC chambers are not completely closed. For both types of analytical FFPC, the NP-TLC mobile phase is used. A flow chart for the selection of an appropriate FFPC method with detailed information is given in [77].
- Depending on the separation problem scaling-up to NP-FFPC or NP-MPLC.
- Control of each fractions using NP-TLC.
- The final purification steps have to be carried out using a reversed phase. Here generally two steps are required:
- Optimisation of the mobile phase by analytical RP-HPLC.
- Scaling up to semipreparative RP-HPLC. For the final isolation, the optimised analytical HPLC mobile phase has to be used.

After the isolation the homogeneity and validity of the compound have to be characterised before starting the structure elucidation. Therefore, the purity of the compound has to be examined for chromatographic uniformity probability [76] using NP-TLC and three mobile phases, characterised with different total solvent strength and total selectivity values. The calculation of Chromatographic Uniformity Probability is similar to the $I_{P(Chr)}$ value described in Section 5.2. If the purity using an RP-HPLC and three NP-TLC mobile phases is at least 95%, the compound is pure enough for structure elucidation.

Needless to say, the suggested strategy is a general guideline that will require modification for specific tasks.

8. Conclusion

Different separation methods such as extraction, purification and chromatography are essential for plant research. The methods presented are not ready-for-use, turnkey operations but serve as guidelines, bearing in mind that without some background knowledge, experience and know-how, the identification and quantitation of individual plant constituents and the isolation of biologically active compounds are not realistic aims.

We would emphasize the facts that, for correct identification, the various chromatographic and spectroscopic methods have to be used in conjunction. The application of two different methods from each field is usually sufficient. For quantitative information, two independent methods are necessary and are acceptable if the results are within 3% of each other. If only one method (e.g., HPLC) is available for quantitative analysis, the results can only be accepted

if, using the global optimum, the ratios of the components determined are identical to one decimal place with the ratios of three measurements (local optima) using different mobile phases with different selectivities. Without analytical monitoring, the results of preparative separations cannot be guaranteed.

Isolation is always a time-consuming, tedious process where the possibilities of thermal instability, photodegradation and oxidation have always to be considered, as must the possibility of the introduction of artifacts somewhere in the process.

This overview on the present status of separation strategies for plant research demonstrates that the various types of separation techniques available cover a range of analytical, small scale and large-scale preparative methods with the newly introduced forced flow methods and hyphenated techniques playing increasingly important roles. All the techniques available are complementary and together enable successful and rapid separations to be obtained. It is our belief that separation methods will always play a vital role in medicinal plant research.

9. Future prospects

Medicinal plant research is aimed at the isolation and identification of naturally occurring substances. Compounds for the treatment of cardiac failure, various cancers and HIV infection have already been isolated from natural products and we must consider all plants – even those currently regarded as weeds – as possible sources of compounds useful to mankind.

Ninety-two percent of all known plant species still await investigation and in the case of the other 8% that have been investigated, researchers have mainly investigated only the major compounds present. To quote Pavlov “We only need to reach out our hands for them” but this does not mean that we must eschew preparedness, specialized knowledge, a vocation and faith. We must develop existing methods of separation and introduce new techniques of higher resolution and effectiveness. In all probability, such developments will give rise to the discovery of new effective compounds from phyto-pharmaceutical sources.

References

[1] J.B. Harborne, H. Baxter, G.P. Moss, Phytochemical Dictionary—A Handbook of Bioactive Compounds from Plants, Taylor & Francis, London, 1999.
[2] R. Haensel, O. Sticher, E. Steinegger, Pharmakognosie–Phytopharmazie, Springer, Berlin, 1999.
[3] Sz. Nyiredy, Magyar Tudomány 12 (2002) 1600.
[4] H. Mueller, in: T. Luijendijk, P. de Graf, A. Remmelzwaal, R. Verpoorte (Eds.), 2000 Years of Natural Products Research: Past, Present and Future, University of Leiden, Leiden, 1999, p. 5.
[5] Sz. Nyiredy, J. AOAC Int. 84 (2001) 1219.
[6] V.E. Tyler, J. Herbal Pharmacother. 1 (2001) 5.
[7] G. Stecher, C.W. Huck, W.M. Stöggl, G.K. Bonn, Trends Anal. Chem. 22 (2003) 1.
[8] K. Hostettmann, M. Hostettmann, A. Marston, Preparative Chromatography Techniques, Springer, Berlin, 1998.
[9] Sz. Nyiredy, in: E. Heftmann (Ed.), Chromatography, Elsevier, Amsterdam, 2004, p. 253.
[10] Sz. Nyiredy, Trends Anal. Chem. 20 (2001) 91.
[11] Sz. Nyiredy, in: Sz. Nyiredy (Ed.), Planar Chromatography—A Retrospective View for the Third Millennium, Springer, Budapest, 2001, p. 177.
[12] Sz. Nyiredy, in: I.D. Wilson, E.R. Adlard, M. Cooke, C.F. Poole (Eds.), Encyclopedia of Separation Science, vol. 2, Academic Press, London, 2000, p. 888.
[13] L.R. Snyder, J. Chromatogr. Sci. 16 (1978) 223.
[14] M. Chastrette, M. Rajzmann, M. Chanon, K.F. Purcell, J. Am. Chem. Soc. 107 (1985) 1.
[15] P.J. Schoenmakers, Optimization of Chromatographic Selectivity, Elsevier, Amsterdam, 1986.
[16] L.R. Snyder, P.W. Carr, S.C. Rutan, J. Chromatogr. A 656 (1993) 537.
[17] Sz. Nyiredy, Zs. Fatér, B. Szabady, J. Planar Chromatogr. 7 (1994) 406.
[18] Sz. Nyiredy, Chromatographia 51 (2000) 288.
[19] European Pharmacopoeia (Ph. Eur.) 4, Suppl. 4.7, EDQM, Strasbourg, 2003, 28 pp.
[20] K.E. Rasmussen, S. Pedersen–Bjergaard, Trends Anal. Chem. 23 (2004) 1.
[21] R.M. Smith, J. Chromatogr. A 1000 (2003) 3.
[22] M.Z. Özel, A.A. Clifford, Flavour Fragrance J. 19 (2004) 354.
[23] M.-C. Hennion, V. Pichon, J. Chromatogr. A 1000 (2003) 29.
[24] Y. Saito, K. Jinno, J. Chromatogr. A 1000 (2003) 53.
[25] R.E. Majors, LC–GC 17 (1999) 8.
[26] J. Carpinteiro, I. Rodriguez, R. Cela, Fresenius J. Anal. Chem. 370 (2001) 872.
[27] B. LeBlane, LC–GC 17 (1999) 30.
[28] B.E. Richter, LC–GC 17 (1999) 22.
[29] S. Arment, LC–GC 17 (1999) 38.
[30] M. Rawa-Adkonis, L. Wolska, J. Namiesnik, Crit. Rev. Anal. Chem. 33 (2003) 199.
[31] K. Ganzler, A. Salgó, K. Valkó, J. Chromatogr. A 371 (1986)299.
[32] L. Yang, J.W. Lam, Anal. At. Spectrom. 16 (2001) 724.
[33] J.S. Yang, D.W. Lee, S. Lee, L. Liq. Chromatogr. Relat. Technol. 25 (2002) 899.
[34] J. Ueberfeld, N. Parthasarathy, H. Zbinden, N. Gisin, J. Buffle, Anal. Chem. 74 (2002) 664.
[35] W.C. Brumley, J. Chromatogr. Sci. 33 (1995) 670.
[36] A. Dbrowski, H. Giergielewicz-Molojska, M. Biziuk, J. Gaca, J. Namiesnik, J. Chromatogr. A 957 (2002) 59.
[37] C. Bandh, E. Bjorklund, L. Mathiasson, C. Naf, Y. Zebutir, Environ. Sci. Technol. 34 (2000) 4995.
[38] S.B. Hawthorne, C.B. Grabanski, E. Martin, D.J. Miller, J. Chromatogr. A 892 (2000) 421.
[39] Ch. Turner, C.S. Eskilsson, E. Björklund, J. Chromatogr. A 947 (2002) 1.
[40] Y.T. Chen, Y.C. Ling, J. Food Drug Anal. 18 (2000) 235.
[41] M. Zougagh, M. Valcárcel, A. Rios, Trends Anal. Chem. 23 (2004) 399.
[42] J. Namiesnik, T. Górecki, J. Planar Chromatogr. 13 (2000) 404.
[43] C.W. Huie, Anal. Bioanal. Chem. 373 (2002) 23.
[44] F. Gaedcke, B. Steinhoff, Phytopharmaka, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 2000.
[45] Ph. Quevauviller, Trends Anal. Chem. 20 (2001) 446.
[46] W. List, P.C. Schmidt, Technologie pflanzlicher Arzneizubereitungen, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1989.
[47] Sz. Nyiredy, J. Planar Chromatogr. 14 (2001) 393.
[48] Sz. Nyiredy, L. Botz, Chromatographia 57 (2003) 291.

[49] Sz. Nyiredy, J. Chromatogr. Sci. 40 (2002) 553.
[50] Sz. Nyiredy, Chromatographia 51 (2000) 288.
[51] Sz. Nyiredy, L. Botz, O. Sticher, J. Chromatogr. 523 (1990) 43.
[52] J. Cases (Ed.), Encyclopedia of Chromatography, Marcel Dekker, New York, 2001.
[53] C.F. Poole, The Essence of Chromatography, Elsevier, Amsterdam, 2003.
[54] E.sz. Kováts, Helv. Chim. Acta 41 (1958) 1915.
[55] J.M. Takács, M. Rockenbauer, I. Olácsi, J. Chromatogr. 42 (1969) 19.
[56] W.M.A. Niessen, J. Chromatogr. A 856 (1999) 179.
[57] J.L. Wolfender, S. Rodriguez, K. Hostettmann, J. Chromatogr. 794 (1998) 299.
[58] G. Tarján, I. Bitter, B. Strasser, M. Szatmáry, Chromatographia 56 (2002) S155.
[59] Sz. Nyiredy, in: B. Fried, J. Sherma (Eds.), Handbook of Thin Layer Chromatography, Marcel Dekker, New York, 2003, p. 307.
[60] H. Vuorela, Ph.D thesis, University of Helsinki, 1988.
[61] E. Gelpi, J. Chromatogr. A 1000 (2003) 567.
[62] C.W. Huck, M. Popp, H. Scherz, G.K. Bonn, J. Chromatogr. Sci. 38 (2000) 441.
[63] K. Albert, J. Chromatogr. A 703 (1995) 123.
[64] K. Albert, J. Chromatogr. A 856 (1999) 199.
[65] Sz. Nyiredy, in: Sz. Nyiredy (Ed.), Planar Chromatography—A Retrospective View for the Third Millennium, Springer, Budapest, 2001, p. 550.
[66] J.-L. Wolfender, K. Ndjoko, K. Hostettmann, Phytochem. Anal. 12 (2001) 2.
[67] J.-L. Wolfender, K. Ndjoko, K. Hostettmann, J. Chromatogr. A 1000 (2003) 437.
[68] J.L. Wolfender, L. Verotta, L. Belvisi, N. Fuzzati, K. Hostettmann, Phytochem. Anal. 14 (2003) 290.
[69] J.-L. Wolfender, Ch. Terreaux, K. Hostettmann, Pharm. Biol. 38 (2000) 41.
[70] Z. Szűcs, B. Szabady, M. Szatmáry, G. Cimpan, Sz. Nyiredy, Chromatographia 56 (2002) S49.
[71] E. Tyihák, E. Mincsovics, T.J. Székely, J. Chromatogr. 471 (1989) 375.
[72] Unpublished results, in preparation.
[73] P. Härmälä, PhD Thesis, Helsinki, 1991.
[74] J.-H. Renault, J.-M. Nuzzilard, G. Le Crouérour, P. Thépenier, M. Zéches-Hanrot, L. Le Men-Oliver, J. Chromatogr. A 849 (1999) 421.
[75] D.H. Lunt, W.F. Hutchinson, G.R. Carvalho, Mol. Ecol. 8 (1999) 891.
[76] Sz. Nyiredy, in: S. Antus, M. Gábor, K. Vetschera (Eds.), Flavonoids and Bioflavonoids, Akadémiai Kiadó, 1995, p. 157.