

Natural product isolation – how to get from biological material to pure compounds

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Covering: 2008 to 2012

Since the last comprehensive review by Otto Sticher on natural product isolation in NPR (O. Sticher, *Nat. Prod. Rep.*, 2008, **25**, 517), a plethora of new reports on isolation of secondary compounds from higher plants, marine organisms and microorganisms has been published. Although methods described earlier like the liquid-solid chromatographic techniques (VLC, FC, MPLC, HPLC) or partition chromatographic methods are still the major tools for isolating pure compounds, some developments like hydrophilic interaction chromatography (HILIC) have not been fully covered in previous reviews. Furthermore, examples of using different preparative solid-phase extraction (SPE) columns including molecular imprinting technology have been included. Special attention is given to chiral stationary phases in isolation of natural products. Methods for proper identification of plant material, problems of post-harvest changes in plant material, extraction methods including application of ionic liquids, de-replication procedures during natural product isolation are further issues to be discussed by the review. Selected work published between 2008 and mid-2012 is covered.

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1 Introduction

Despite of substantial developments of extraction and separation techniques, isolation of natural products (NPs) from plants, marine organisms or microorganisms is still a challenging task. Undoubtedly hybrid methods like LC-NMR or LC-MS made on-line structure elucidation possible and provided impressive examples of NP identification without prior isolation,² however, in many cases the necessity to get the purified compounds in hand is still a fact. Full chemical structures including stereochemistry of new NPs most likely need isolated, highly purified compounds, however the amounts needed have significantly decreased and 2D NMR spectra of small molecules now can be obtained with less than 100 µg within a reasonable

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time. Furthermore, testing for biological activity *in vitro* and *in vivo* has to be done after a purification process in order to exclude interference with accompanying compounds. Last but not least, reference standards for quality control of herbal medicinal plants and herbal medicines largely depend on isolated compounds with documented purity. In recent years, NPs have experienced a renaissance in drug-discovery programmes, mainly due to their superior chemical diversity over synthetic compound libraries³ and their drug-like properties.⁴ The classical way of isolation of NPs starts with identification, collection and preparation of the biological material, usually by drying. Extraction with different solvents from low to higher polarity follows. Prior to isolation of pure compounds, often by (semi-) preparative HPLC or liquid-liquid chromatographic techniques, several purification steps are necessary to remove most of the unwanted matrix. This review will go through these essential

steps except liquid-liquid chromatographic techniques and illustrate developments in these areas during the time since 2008 by selected examples of secondary metabolites, *i.e.* the review is focusing on small molecule NPs ($M_r < 2000$). In recent years, a trend towards isolation strategies driven by biological or pharmacological activity can be recognized. However, by following this approach extensively, our picture of the vast chemical diversity of plants, microbes or marine organisms will be narrowed and explorative work on the chemistry of living organisms and interactive changes of their metabolic profiles should be encouraged.

Bioassay-guided isolation strategies connecting information on the chemical profiles of extracts and fractions with their activity data in *in vitro* bioassays performed at micro-scale significantly reduced the time for hit discovery. In principle it seems to be a straightforward procedure to get from a plant to an active compound, however there are some critical steps which have to be kept in mind, such as correct plant identification, consideration of transformations during preparation and extraction of the material, or de-replication of already known compounds at the earliest stage of the fractionation procedure. These issues will be discussed briefly in this review. The majority of studies aiming at isolation of significant amounts (mg to g quantities) of pure NPs still use the wide range of liquid chromatographic methods like VLC, MPLC and HPLC, taking advantage of improved separation capacities due to smaller particle size and different selectivity (*e.g.* HILIC stationary phases). Solid-phase extraction, originally established as a purification method prior to HPLC or GC analysis, is increasingly recognized as a method for rapid fractionation of crude plant extracts or for trapping pure compounds eluted after HPLC separation transferred to capillary NMR (*cap*NMR) analysis for *de novo* structure elucidation.

Since the comprehensive review by Sticher¹ on NP isolation which still represents a valuable overview of currently available



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methods, the topic has been covered by the recently published 3rd edition of *Natural Products Isolation*⁵ which outlines a selection of methods including protocols for extraction and application of chromatographic techniques for NP isolation. Detection, isolation and bioactivity testing of NPs is also covered by the book edited by Colegate and Molyneux.⁶ A review by Beek *et al.*⁷ covers methods for rapid analysis of plant constituents including miniaturized liquid-liquid extraction techniques. Aside from analytical methods, different modes of sample preparation are covered by a review on Chinese plants used for medicinal and food purposes.⁸

2 Authentication and preparation of plant material/marine organisms

Unequivocal identification of the investigated biological material is without doubt the key to all following steps in NP isolation. In a comprehensive review on marine organisms by Blunt *et al.*,⁹ major concerns regarding the recent trend to publish compounds isolated from unidentified microorganisms from sources that are not clearly defined is expressed, illustrating this important issue. In the medicinal plants area, the awareness of the necessity of authentication of biological material has been given a boost by the significantly increasing emergence of herbal drugs from traditional Chinese medicine and products derived thereof on the European market.¹⁰ As a result, in an international research programme, GP-TCM (www.gp-tcm.org), authentication projects involving the Chinese Medicinal Plant Authentication Centre at RBG Kew have been established for economically important plant species.¹¹ Characteristics of Chinese medicinal plants and their corresponding herbal drugs have been recently illustrated.¹² When collecting plant material, selection criteria might be based on ethnomedicinal data, chemosystematic relationships or ecological observations. Legal and ethical issues like the convention on biodiversity (CBD, <http://www.cbd.int/>) have to be respected.^{4,13,14}

In the following section, major tools which are used in plant authentication will be discussed. A wide range of methods is available for identification of biological materials (plants, marine organisms, microorganisms) which are applicable to different degrees for authentication of unknown material. A combination of several methods might be necessary for unequivocal authentication. In any case, a voucher specimen of authenticated reference material is an indispensable prerequisite. In order to keep track of investigated material of each study a voucher specimen should be kept locally and also be stored in a major herbarium.

2.1 Morphological/anatomical analysis

The primary way of authentication is by morphological as well as anatomical analysis. Both methods need profound expertise and training. One major concern for the authors is the disappearance of classical pharmacognosy, including training in morphological and anatomical analysis of herbal drugs, from curricula, and thus a lack of expertise in this area can be expected in the future. However, if strong anatomical characters

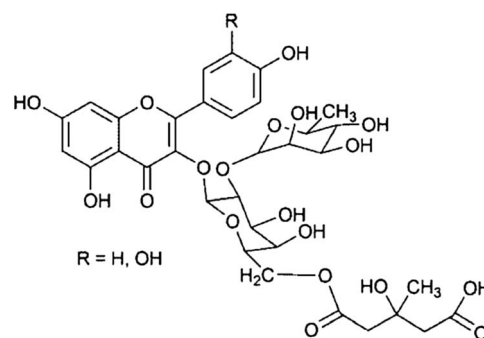
like trichomes or calcium oxalate crystals are present, the classical light microscopic analysis of plant material is still a valuable and inexpensive method. Recently, microscopic characteristics of medicinal plants have been published by Upton *et al.*¹⁵ and Rahfeld.¹⁶ Morphological characterization of microorganisms usually is combined with genetic markers for identification.^{17–19}

2.2 TLC/HPTLC analysis

For rapid comparison of a series of samples with reference material, fingerprint analysis by TLC or in the more sophisticated version by HPTLC is an option. In order to make results comparable between different laboratories and literature references, a number of parameters like saturation of the TLC chamber, mobile-phase composition, water content of the silica stationary phase, *etc.* have to be controlled. Meanwhile HPTLC can be regarded as an established method with application in GMP-compliant quality control of herbal drugs and preparations thereof.^{20–23} As exemplifying applications of HPTLC, the detection of 5% adulterations of black cohosh (*Cimicifuga racemosa*) with other *Cimicifuga* species,²⁴ the identification of *Hoodia gordonii*²⁵ or the differentiation between *Arctostaphylos uva-ursi* and *A. pungens*²⁶ might serve. Coupling TLC/HPTLC with mass spectrometry either by compound extraction with specific interfaces or by ambient mass spectrometry significantly increased the spectral information on selected compounds.^{7,27,28} Recently, using a TLC-MS extraction interface and coupling to NMR, rutin, caffeic acid and chlorogenic acid could be identified and quantitatively determined.²⁹

2.3 HPLC analysis

Hyphenation of HPLC separation with different spectroscopic detection methods like PDA, MS or NMR offers two ways of identification of plant material. On the one hand, specific marker compounds can be used for chemotaxonomic applications, *e.g.* 3-hydroxy-3-methylglutaric acid in flavonol acyl glycosides in the genus *Rosa*;³⁰ on the other hand, HPLC fingerprints in combination with pattern recognition analysis can be applied for identification of the plant of origin in extracts.



Flavonol 3-hydroxy-3-methylglutaryl glycosides in the genus *Rosa*

In a chemosystematic study of *Taxus* spp., LC-PDA-MS fingerprint chromatograms were analysed by hierarchical

cluster analysis (HCA) and principal component analysis (PCA) leading to differentiation of eight investigated species to six well-supported groups and correct assignment of most species.³¹ Combination of PCA of two fingerprints of LC and ¹H NMR with a pharmacological fingerprint was used for comprehensive characterization of commercial willow (*Salix* spp.) bark extracts.³² Differentiation of six *Ganoderma* species, fungi used in traditional Chinese medicine, was possible by combination of HILIC (see 4.2.5) and reversed-phase columns.³³ HPLC analysis still plays a major role in phytochemical analysis including identification of crude plant extracts.^{8,23,34}

Aside from identification purposes directed to organisms, a major application of HPLC methods is de-replication, *i.e.* the identification of known metabolites in extracts ideally at an early stage of the fractionation process. This is largely done by hyphenated techniques such as LC-NMR, LC-MS, LC-PDA³⁵ and combinations thereof. Using a UHPLC-PDA-TOF-MS setup in *Lippia* spp. 14 compounds could be unambiguously and further 28 compounds tentatively identified.³⁶ For analytical purposes UHPLC (UPLC), *i.e.* the application of stationary phases of sub-2 µm particle size combined with high speed elution and instrumentation capable of coping with high backpressures, has resulted in remarkable improvements of analysis of complex mixtures like plant extracts, as clearly outlined in a review by Eugster *et al.*³⁷

A significant increase in sensitivity of NMR analysis could be gained by using micro-coil NMR which made successful recording of two dimensional NMR spectra (HMBC, HSQC) of 100 µg NP samples (M_r ca. 500) in overnight runs possible, as outlined in a recent review on LC-NMR methods by Sturm and Seger.²

2.4 GC analysis

In case of analysing biological material containing volatile constituents like essential oils, GC-MS analysis still represents the method of choice, taking advantage of the unsurpassed peak capacity of capillary GC columns. Headspace solid-phase micro-extraction or steam distillation extraction can be used to collect the volatile fractions from small amounts of plant material.³⁸ Comprehensive two dimensional GC (GC × GC) and multidimensional GC (MDGC),^{39,40} combining different GC instruments, columns and detectors and selective transfer of individual peaks in combination with multivariate data analysis (MVDA), made fingerprint analysis of volatiles even more informative.^{38,41,42} Identification of bacteria by GC analyses of bacterial fatty acid methyl esters is still a frequently used authentication technique.⁴³

2.5 Spectroscopic methods: NMR, MS, NIR, FT-IR

Advances in data analysis of complex signal patterns enabled application of spectroscopic techniques to crude plant extracts for metabolic fingerprinting without prior HPLC separation.^{35,44,45} By using ¹H-NMR metabolic fingerprinting in combination with PCA five different *Verbascum* species were divided in two groups, group A (*Verbascum phlomoides* and *Verbascum densiflorum*) and group B (*Verbascum xanthophoeniceum*, *Verbascum*

nigrum and *Verbascum phoeniceum*).⁴⁶ A similar approach of ¹H-NMR-based metabolic profiling was used for discrimination of *Ilex* species and varieties.⁴⁷

NIR direct measurements of fresh and dry samples without prior extraction is possible, but samples may also include hydrodistillates and extracts. For quality control of the fruiting bodies of *Ganoderma lucidum*, NIR diffuse reflectance spectroscopy could be used in combination with chemometric techniques to discriminate the samples according to their cultivation area.⁴⁸ Exploration of different IR techniques for identification of *Epilobium* spp. and *Hypericum* spp. from whole leaf samples showed that the morphological properties of the plant material have to be taken into consideration when developing the appropriate IR-based identification method.⁴⁹ A review by Alvarez-Ordóñez *et al.* covers the potential of FT-IR-based methods as rapid and non-invasive techniques for assessment of membrane composition and changes due to environmental and other stress factors in food-borne bacteria.⁵⁰ Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has revolutionized *in situ* identification of microorganisms by analysing them in a short time from colonies grown on culture plates.^{51,52}

2.6 Molecular biological methods

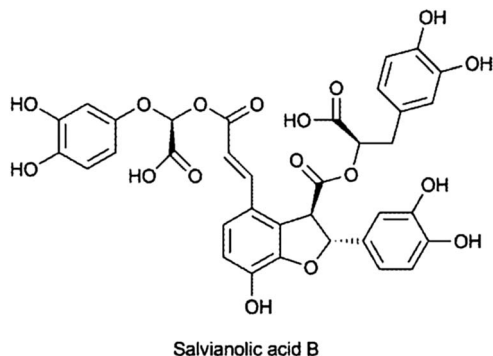
Omics techniques have gained increasing importance in authentication of biological material during the last decades.¹⁰ DNA-based approaches to authenticate plant materials include comparison of internal transcribed spacer (ITS) sequences, random amplified polymorphic DNA (RAPD) markers, the use of sequence characterised amplified region (SCAR) markers or high resolution melting analysis (HRM). In a study by Ruzicka *et al.*⁵³ on the problematic genus *Verbena*, which includes about 40 species with frequently occurring natural hybrids, it was possible to differentiate *Verbena officinalis* by SCAR markers from all species except the closest *V. hastata* while HRM even enabled discrimination from the latter species.

In commercialized plant material, admixtures with different plant species represent a serious problem. By HRM, Mader *et al.*⁵⁴ were able to detect the adulteration in a ratio of 1 : 1000 with unknown plant species and a ratio of 1 : 200 000 of added *Veratrum nigrum*. As a major drawback for the detection of unknown adulterations, the authors suggested that since universal primers might not react with all species it is important to design assays for specific contaminants or at least for higher level taxa (*e.g.* plant families).

SCAR markers were also applied for identification of important Indian medicinal *Phyllanthus* species, namely *P. amarus*, *P. fraternus*, *P. debilis* and *P. urinaria*.⁵⁵ The issue of post-mortem alteration of DNA in herbarium material has been investigated. Although DNA modifications, most likely due to hydrolytic deamination of cytosine during long-term herbarium storage, were observed, herbarium specimens are considered a valuable source of reliable sequence data.⁵⁶ Molecular identification methods of microorganisms include amplified and non-amplified nucleic acid probes and have been reviewed several times.^{57–59}

2.7 Post-harvest changes in plant material

Post-harvest alteration of plant metabolites has to be taken into consideration as it can lead to significant changes due to plant immanent enzymes like hydrolases (glycosidases), peroxidases or polyphenol oxidases (PPO).⁶⁰ Early studies by Janecke and Henning⁶¹ could identify a number of enzymes in dried plant material which can be reactivated after extraction with aqueous solvents even if lower percentages of ethanol or methanol are present.⁶² Especially caffeic acid derivatives seem to be subject to oxidative changes. Cichoric acid (2*R*,3*R*-*O*-dicaffeoyltartaric acid), a marker compound in *Echinacea purpurea* products, was shown to be highly susceptible to degradation by PPO.⁶³ Salvianolic acid B was found only as minor component in fresh roots of *Savia miltiorrhiza* but significantly increased during drying.⁶⁴



Similar processes were observed in rhizomes of *Ligusticum chuanxiong* when studying the influence of post-harvest drying and processing methods on nine major components. By drying at 60° or under the sun, the contents of senkyunolide A, coniferylferulate and *Z*-ligustilide significantly decreased while the content of corresponding compounds increased.⁶⁵ Detailed studies of post-harvest changes of St. John's wort (*Hypericum perforatum*), marjoram (*Majorana hortensis*) and peppermint (*Mentha x piperita*) have been performed by the group of Boettcher *et al.*^{66–68} In addition, perishing of plant material by microbes or fungi has to be scrutinized. Not only can enzymatic degradation be caused by microbial enzymes, secondary metabolites can be induced if plant material was contaminated during life-time, as known for isoflavonoid phytoalexins in legumes.⁶⁹

The problem of artefact formation during the isolation procedure was also discussed by Jones and Kinghorn.¹³

3 Extraction methods

Extracting the compounds of interest from the non-soluble matrix in which they are embedded needs several issues to be taken into account. These include the polarity and stability of the extractives and the solvent, the toxicity, volatility, viscosity and purity of the extraction solvent, the probability of artefact formation during the extraction process, and the amount of bulk material to be extracted. The issue of artefact formation due to solvents has been reviewed recently.⁷⁰ In plant material secondary metabolites

usually are found inside cells, thus grinding of the raw material and breaking tissue and cell integrity before extraction increases extraction yield. In the following section the most important methods for extraction of secondary metabolites from biological material applied in laboratory scale will be discussed.

3.1 Classical solvent extraction procedures

The majority of isolation procedures still utilize simple extraction procedures with organic solvents of different polarity, water and their mixtures.^{1,71,72} The methods include maceration, percolation, Soxhlet extraction, ultrasound-assisted extraction and turbo-extraction. Maceration is carried out at room temperature by soaking the material with the solvent with eventual stirring. It has the advantage of moderate extraction conditions but suffers from high solvent consumption, long extraction times and low extraction yields. Extraction yield is improved by percolation, *i.e.* packing the pre-soaked plant material in a container which allows the constantly controlled removal of the extract *via* a valve at the bottom and adding fresh solvent from the top. Soxhlet extraction is a popular method for extraction due to its reduced solvent consumption; however, thermo-labile compounds might be degraded during the extraction process. For liquid samples extraction by organic solvents or heterogeneous solvent mixtures can be done, either simply in a separating funnel or similar to a Soxhlet apparatus in a perforator. On a smaller scale, extraction of the liquid sample absorbed on a porous matrix (like diatomaceous earth) packed in a column with non-miscible solvents is an option (*e.g.* Extrelut® columns).

3.2 Ultrasound-assisted extraction (UAE)

In UAE the plant material, usually in a glass container, is covered by the extraction solvent and put into an ultrasonic bath. It decreases extraction time and improves extraction yields due to mechanical stress which induces cavitations and cellular breakdown, and has gained increasing popularity. Examples of NPs extracted by UAE include anthocyanidins, flavonols and phenolic acids from *Delonix regia*,⁷³ capsaicinoids from *Capsicum frutescens* in lab and pilot-plant scale,⁷⁴ cyanidin-3-rutinosid from *Litchi chinensis*⁷⁵ or essential oils from laurel, rosemary, thyme, oregano and tuberos.⁷⁶ In the latter study by Roldan-Gutierrez *et al.*,⁷⁶ dynamic UAE, *i.e.* where the solvent (in this case ethanol) is pumped through the plant material which is placed in an extraction tube in a temperature-controlled water bath connected to an ultrasound probe, showed superior extraction efficiency compared to steam distillation or superheated water extraction.

3.3 Microwave-assisted extraction (MAE)

Nowadays extraction employing either diffused microwaves in closed systems or focused microwaves in open systems are established methods. Principles of these technologies, their pros and cons as well as extraction protocols have been outlined

in detail by Sticher¹ and by Delazar *et al.*⁸⁸ MAE has been modified in different ways leading to vacuum microwave-assisted extraction (VMAE), nitrogen-protected microwave-assisted extraction (NPMAE), ultrasonic microwave-assisted extraction (UMAE) or dynamic microwave-assisted extraction (DMAE), which are discussed in a review by Chan *et al.*⁸⁹ Some recent examples of application of MAE to NP isolation employing ionic liquids are mentioned below (section 3.4).

3.4 Extraction with ionic liquids

In recent years, application of ionic liquids (ILs) for UAE, MAE or simple batch extraction of plant metabolites at room temperature or elevated temperature has gained increasing attention and has been recently reviewed extensively.⁹⁰ These ILs, also designated as “designer solvents”, are organic salts in the liquid state consisting of an organic cation and an organic or inorganic anion. ILs are able to dissolve a wide range of polar to non-polar compounds, have a low vapour pressure, show a high thermal stability and low combustibility, and some of them are biodegradable. Table 1 presents applications of ionic liquids with different extraction technologies like liquid-liquid extraction (LLE), UAE, MAE or liquid-phase micro-extraction (LPME). An exemplifying study was performed for extraction of artemisin by IL, *N,N*-dimethylethanolammonium octanoate (DMEA oct) and bis(2-methoxyethyl)ammonium bis(trifluoromethylsulfonyl)imide (BMOEA bst) showing the best performance.⁷⁹ Artemisin was recovered from the extract after addition of water and crystallisation in 82% yield compared to the total extracted amount. The purity of artemisin crystals was 95% as determined by NMR. Meanwhile a number of studies have been performed mainly with the aim of enriching extracts

for analysis by HPLC. Immobilized ILs for solid-phase extraction is discussed in section 3.7. Application of ILs as new solid-phase micro-extraction (SPME) stationary phases caused problems due to contamination of the GC injector when directly inserted into the system.⁹⁰ *N,N*-dimethylammonium *N,N'*-dimethylcarbamate (DIMCARB) proved to be a distillable IL, and could be more easily removed from the extract compared to the majority of ILs which are minimally volatile.⁷⁷ Another feature of ILs which is still insufficiently investigated is their biodegradability and impact on the environment if used at industrial scale,¹⁰⁰ and this needs future attention. In eco-toxicological studies using a *Vibrio fischeri* bioluminescence quenching assay, longer side-chains, non-aromatic head groups and the anion BF₄ showed the highest toxicological risk,¹⁰¹ but the potential to design more hydrophobic ILs with lower toxicity by avoiding aromatic substructures was indicated.¹⁰²

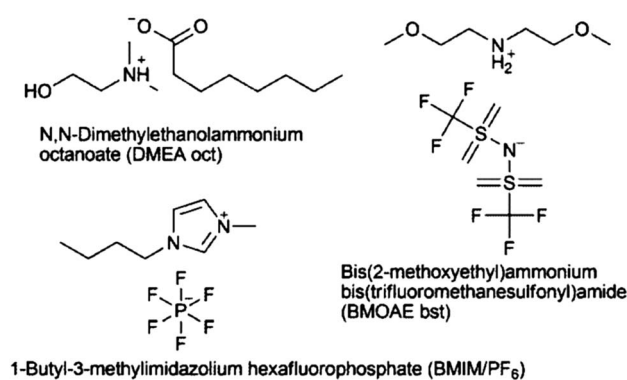


Table 1 Recent applications of ionic liquids in extraction of plant constituents

Plant	Compound	Extraction method ^a	IL ^b	Reference
<i>Acacia catechu</i>	Hydrolysable tannins	LSE	DIMCARB, removable from extract by distillation	77
<i>Apocynum venetum</i>	Hyperoside, isoquercitrin	MAE	BMIM/BF ₄	78
<i>Artemisia annua</i>	Artemisinin	LSE	DMEA oct; BMOEA bst	79
<i>Cynanachum bungei</i>	Acetophenones	UAE	BMIM/BF ₄	80
<i>Glaucium flavum</i>	Alkaloids	LSE	C _n MIM/Cl; Br; Sac; Ace	81
<i>Nelumbo nucifera</i>	Phenolic alkaloids	MAE	C _n MIM/Cl; Br; BF ₄	82
<i>Polygonum cuspidatum</i>	<i>trans</i> -Resveratrol	MAE	BMIM/Br	83
<i>Psidium guajava</i>	Gallic acid, ellagic acid, quercetin	MAE	C _n MIM/Cl; Br; a.o.	84
<i>Rheum</i> spp. (rhubarb)	Anthraquinones	UMAE	C _n MIM/Cl; Br; BF ₄	85
<i>Salvia miltiorrhiza</i>	Cryptotanshinone, tanshinone I, tanshinone II A	UAE	Aqueous OMIM/Cl; analytes concentrated by anion metathesis to OMIM/PF ₆	86
<i>Smilax china</i>	<i>trans</i> -Resveratrol, quercetin	MAE	C _n MIM/Cl; Br; a.o.	84
<i>Sophora flavescens</i>	Oxymatrine	1. LSE; 2. SPE	1. Silica-confined IL; 2. MeOH	87
<i>Terminalia chebujia</i>	Hydrolysable tannins	LSE	DIMCARB	77

^a LSE: liquid-solid extraction; MAE: microwave-assisted extraction; SPE: solid-phase extraction; UAE: ultrasound-assisted extraction; UMAE: ultrasound/microwave-assisted extraction. ^b a.o.: and other anions; BMIM/BF₄: 1-butyl-3-methylimidazolium tetrakisfluoroborate; BMOEA bst: bis(2-methoxyethyl)ammonium bis(trifluoromethylsulfonyl)imide; C_nMIM/Cl; Br; Sac; Ace: 1-alkyl-3-methylimidazolium chloride, bromide, saccharinate, acesulfamate; DIMCARB: *N,N*-dimethylammonium *N,N'*-dimethylcarbamate; DMEA oct: *N,N*-dimethylethanolammonium octanoate; OMIM/Cl 1-octyl-3-methylimidazolium chloride; OMIM/PF₆ 1-octyl-3-methylimidazolium hexafluorophosphate.

3.5 Accelerated (pressurized) solvent extraction (ASE)

In comparison to most other extraction systems which need an additional step for separation of the remaining non-soluble matter from the liquid extract, on-line filtration within the automatized extraction process of accelerated (or pressurized) solvent extraction (ASE, a patented system by Thermo/Dionex®) is included. The methodology is applied to solid and semisolid samples in 1–100 g scale using common solvents at elevated temperature and pressure.¹⁰³ Up to 24 samples can be extracted automatically. In a study on altitudinal variation of phenolic compounds in *Calluna vulgaris*, *Vaccinium myrtillus* and *Sambucus nigra*, 205 samples of dried and ground material mixed 1 : 1 with diatomaceous earth (DE) or sea sand were extracted with 80% MeOH for their flavonoids and phenolic acids, illustrating the necessity of serial extraction under controlled conditions when doing comparative studies.¹⁰⁴ In ASE, sequential extraction with solvents of different polarity and mixing of solvents is possible, as illustrated by Cicek *et al.*¹⁰⁵ Consecutive extraction of subaerial parts of *Actea racemosa* with petroleum ether for defatting followed by dichloromethane led to isolation of 2.2 g enriched triterpene saponin fraction from 50 g of plant material. Although ASE usually is mainly used as a sample preparation method for analytical purposes,^{106–110} preparative scale application of ASE was performed with *Hypericum perforatum* to obtain the phloroglucinols adhyperforin and hyperforin as well as three caffeoyl quinic acid derivatives.¹¹¹ Due to increased capacities of extraction cells in the latest version of ASE instrumentation, this type of application is likely to increase in the future. ASE or similar instrumentation can also be used for subcritical water extraction (SWE) employing temperatures of 100–280 °C. Subcritical water (superheated water, pressurized hot water) is heated to a temperature between the boiling point at atmospheric pressure (100 °C) and the critical temperature (374 °C) under pressure, thereby increasing its solution properties for organic lipophilic compounds. In the NPs field SWE has been employed to extract phenolic compounds from pomegranate (*Punica granatum*) seed residues,¹¹² gallic acid and ellagitannins from *Terminalia chebula*,¹¹³ the flavonol quercetin from onion (*Allium cepa*) skin,¹¹⁴ phenolic compounds from potato (*Solanum tuberosum*) peels¹¹⁵ or essential oil from *Cinnamomum ceylanicum*.¹¹⁶ For phenolic type of compounds, SWE seems to be an attractive alternative to organic solvent extraction, however, artefact formation and degradation has to be scrutinized, as shown by Plaza *et al.* who observed formation of degradation products due to Maillard reaction, caramelization and thermo-oxidation when SWE was applied to extraction of different organic matter including microalgae, algae and plants.¹¹⁷

3.6 Supercritical fluid extraction (SFE)

Replacing extraction with organic solvents by extraction technologies which are less detrimental to environment and meet the increasing regulatory requirements certainly can be considered as a driving force for the increasing application of supercritical fluid extraction, above all using supercritical CO₂. An overview of methodology including extraction protocols and applications in NP isolation and extraction is given by Nahar and Sarker¹¹⁸ as well

as Sticher.¹ Mathematical models which represent the mass transfer mechanisms and the extraction process in order to design the SFE application properly have been reviewed by Huang *et al.*¹¹⁹ Recent reports on SFE for extraction of NPs and modelling include phloroglucinol and benzophenone derivatives from *Hypericum carinatum*,¹²⁰ essential oils,¹²¹ gallic acid, quercetin and essential oil from the flowers of *Achyrocline satureioides*¹²² or phenolics including anthocyanidins from grape peels (*Vitis labrusca*).¹²³

The utilization of organic solvents as modifiers for supercritical CO₂ to increase its solvating capabilities to medium-polar and polar compounds has broadened the spectrum of NP compound classes accessible to SFE, accepting the ecological problems related to organic solvent extractions which increase to a small extent.

3.7 Extraction on solid phases

Extraction processes which take advantage of adsorption of the analytes or unwanted impurities on a solid phase have gained a dominant role in purification of NP extracts, not least due to its integration into automated sample preparation and isolation systems. Most applications utilize solid-phase extraction (SPE) which employs a wide range of stationary phases with diverse chemistry like silica gel, reversed-phase material, ion-exchange resins or mixed-mode material and HILIC stationary phases in pre-packed glass or plastic columns. For HILIC, hydrophilic interaction chromatography, see section 4.2.5. Usually a forced flow technique using a vacuum manifold is applied. Several strategies can be used in SPE. Either unwanted impurities (like chlorophylls) are removed by adsorption on the stationary phase or the analytes of interest are adsorbed on the stationary phase, whereas impurities are eluted. In the latter version, a second step of elution will remove the concentrated analytes from the column. Elution of the compounds of interest might be done stepwise by applying a gradient with increasing eluting power, *i.e.* the procedure is then related to VLC (vacuum liquid chromatography). An exciting development of recent years was the design of molecularly imprinted polymers (MIP) to be used in SPE applications for selective enrichment of various compounds. Either ionic liquid-imprinted silica particles or copolymers of acrylamide and ethylene glycol dimethacrylate with the respective template compounds are used to create material which will have a high affinity to the template structures. In a first elution step the unwanted material is removed from the SPE column whereas target compounds bound to the solid phase are obtained in a concentrated solution usually upon elution with organic solvents like methanol, though additional purification steps might be necessary. Recent reports on isolation of NPs with MIP-SPE are summarized in Table 2.

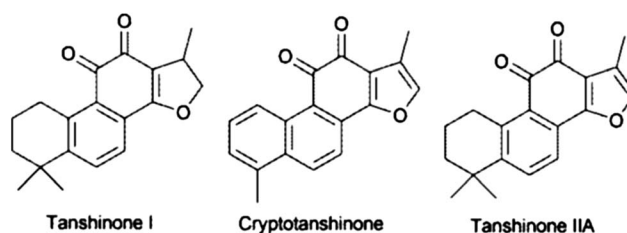
Aside from SPE as sample purification before LC or GC analysis, trapping compounds on SPE columns for off-line LC-NMR coupling has gained increasing importance for structure elucidation, metabolic profiling and de-replication strategies.^{2,124–126} As part of automated isolation systems SPE is combined with preparative HPLC like in the Sepbox® instrument¹²⁷ or as proposed by Tu *et al.*¹²⁸ A sophisticated combination of SPE columns representing strong anion and cation

Table 2 Recent applications of solid-phase extraction with molecularly imprinted polymers (MIP-SPE)

Compound (template)	Plant	MIP ^a	Polymerization	SPE eluent	Ref.
Podophyllotoxin	<i>Dysosma versipellis</i> ; <i>Sinopodophyllum hexandrum</i> ; <i>Diphylleia sinensis</i>	Fm: AA Cl: EDMA + divinylbenzene Pg: AcCN In: AIBN	Microwave heating initiated precipitation polymerization, 60 °C	MeOH; MeOH/acetic acid (9 : 1, v/v)	91
Andrographolide	<i>Andrographis paniculata</i>	Fm: AA Cl: EDMA Pg: ACN-toluene (3 : 1, v/v) In: AIBN	Precipitation polymerization, 60 °C	MeOH/water (3 : 2, v/v); MeOH	92
Quercetin	<i>Cacumen platycladi</i> (<i>Platycladus orientalis</i>)	Fm: AA Cl: EDMA Pg: 1,4-dioxane, THF, acetone, ACN In: AIBN	Batch polymerization; 60 °C	MeOH; MeOH/acetic acid (9 : 1, v/v)	93
Kireinol	<i>Siegesbeckia pubescens</i>	Fm: AA Cl: EDMA Pg: THF In: AIBN	Batch polymerization; 60 °C	MeOH/acetic acid (9 : 1, v/v)	94
Berberine	<i>Phellodendron wilsonii</i>	Fm: AA Cl: EDMA Pg: CHCl ₃ , DMSO, MeOH In: AIBN	Batch polymerization, 60 °C	MeOH-CHCl ₃ (1 : 60, v/v)	95
Protocatechuic acid	<i>Homalomena occulta</i>	Fm: AA Cl: EDMA Pg: ACN In: AIBN	Precipitation polymerization, 60 °C	MeOH/acetic acid (9 : 1, v/v)	96
18β-glycyrrhetic acid	<i>Glycyrrhiza glabra</i>	Fm: MAA Cl: EDMA Pg: CHCl ₃ In: AIBN	Batch polymerization, 60 °C	MeOH	97
Protocatechuic acid; caffeic acid; ferulic acid	<i>Salicornia herbacea</i>	Fm: IL monomer (AEIB) Cl: EDMA Pg: n-BuOH/H ₂ O (9 : 1, v/v) In: AIBN	Batch polymerization, 60 °C	Aqueous HCl (0.5 mol L ⁻¹)	98
Cryptotanshinone, tanshinone I, tanshinone IIA, template: 9,10-phenanthrenequinone	<i>Salvia miltiorrhiza</i>	IL: 3-aminopropyl-trimethoxysilane + 3-chloropropionylchloride + imidazole; immobilized on silica	—	n-hexane (washing step) MeOH (elution)	99

^a AA: acrylamide; ACN: acetonitrile; AEIB: 1-allyl-3-ethylimidazolium bromide; AIBN: 2,2'-azo-bis-isobutyronitrile; CHCl₃: chloroform; Cl: cross linker; DMSO: dimethylsulfoxide; EDMA: ethylene glycol dimethacrylate; Fm: functional monomer; IL: ionic liquid; In: initiator; MAA: methacrylic acid; MeOH: methanol; n-BuOH: n-butanol; Pg: porogene; THF: tetrahydrofuran.

exchangers, a mixed-mode polymeric RP-anion exchanger with a poly(divinylbenzen-co-vinylpyrrolidone) backbone and a size exclusion column of a hydroxypropylated dextran gel (Sephadex LH-20®) were used for explorative fractionation of extracts from microorganisms.¹²⁹ SPE might also be carried out by adding spatially separated anion and cation exchange resins in sachets to organic extract solutions for separating acidic, basic and neutral compounds.¹³⁰ For micro-scale isolation, variants of SPE like SPME or stir-bar sorptive extraction (SBSE) can be used. For isolation of the volatile fraction of herbal teas SPE was used in comparison to hydro distillation,^{131,132} but headspace-SPME and SBSE are attractive alternatives for this type of application, as reviewed recently.¹³³



3.8 Distillation methods

Volatiles such as essential oils are still obtained mainly by distillation techniques, although working at elevated temperatures can

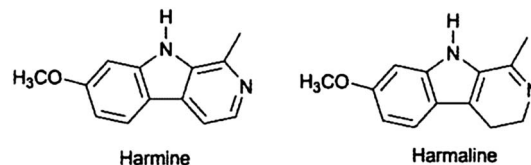
lead to chemical changes, most obvious in essential oils of chamomile (blue chamazulene originating from colourless matricin) or other proazulene-containing plants (e.g. yarrow, *Achillea* spp.). Recent developments in distillation methodology include microwave steam distillation (synonym: microwave steam diffusion) which applies microwaves to increase disruption of glands and cells while steam is passing through the plant material and carrying the essential oil.^{134,135} In a comparative study of the essential oil isolated from *Salvia rosifolia*¹³⁶ by microwave-assisted hydrodistillation in 45 min an essential oil of similar yield and composition as the one obtained after 180 min of conventional hydrodistillation (HD) was obtained. Microdistillation was preferable for isolation of the most volatile fraction of monoterpene hydrocarbons.¹³⁶ For characterisation of representative chamomile volatiles in the vapour upon inhalation, a combination of HD and RP18-SPE in a circulating apparatus (SD-SPE) was applied and compared to simultaneous distillation extraction (collecting the volatiles in a water non-soluble solvent) and HD. It could be shown that actually a much higher percentage of the more polar en-in-dicycloethers and bisabolol, important ingredients for the anti-inflammatory activity of chamomile oil, could be obtained by SD-SPE.¹³⁷

4 Isolation by liquid-solid chromatography techniques

A wide range of liquid chromatographic methods with solid stationary phases either as planar or column chromatography is available for further fractionation and final purification of NPs. The choice largely depends on the stage of purity of the extract or fraction, and the final purpose of the isolated NP. High sample capacity combined with relatively low costs made low pressure liquid chromatography (LPLC), vacuum liquid chromatography (VLC) or flash chromatography (FC) popular for fractionation of crude extracts, and in rare cases even pure compounds could be obtained by these single fractionation steps. However, in many cases medium-pressure liquid chromatography (MPLC) or semi-preparative and preparative HPLC with higher peak resolution power had to be applied for final purification.

4.1 Preparative planar chromatography (PPC)

Due to its simplicity in use and relatively low costs for isolation of small molecule NPs, PPC is still a frequently used technique although the number of applications is lower than those of column chromatography. An attractive feature of PPC is the wide range of chemical detection methods characteristic for compound classes which can be carried out on a narrow section of the plate leaving most of the compound unchanged and available for isolation. In bioassay-guided isolation strategies, planar chromatography has the advantage of direct application of bioassays on TLC plates, making the rapid localisation of bioactive compound zones possible. So far, bioautographic methods include antifungal and antibacterial activity, acetyl cholinesterase (AChE) inhibition, α - and β -glucosidase inhibition and radical scavenging or antioxidant activity, as reviewed recently by Marston.¹³⁸ The search for AChE inhibitors by TLC bioautography can be illustrated by studies of the genus *Peganum* identifying harmine and harmaline as potent compounds.¹³⁹



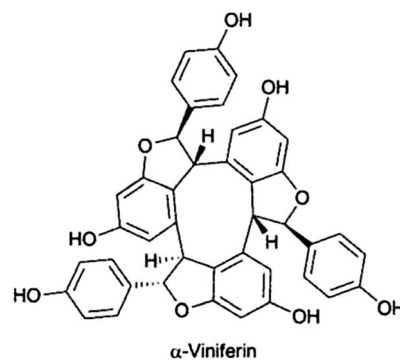
In addition to the optimization of growth media for bioautographic detection of antimicrobial activity of *Cordia giletti*, the ability to quench the bioluminescence of *Vibrio fischeri*, indicating toxicity, was checked in another TLC bioautographic approach.¹⁴⁰ A review by Sherma¹⁴¹ on developments in planar chromatography between 2009 and 2011 presents some illustrative examples too.

To overcome the disadvantage of classical TLC of uncontrolled flow rates of the mobile phase, forced-flow techniques such as centrifugal planar chromatography or over-pressured layer chromatography have been developed enabling elution and on-line detection of compounds.^{142,143} A comprehensive outline of the application of PPC to isolation of NPs has been provided by Gibbons recently.¹⁴⁴

4.2 Column chromatographic methods

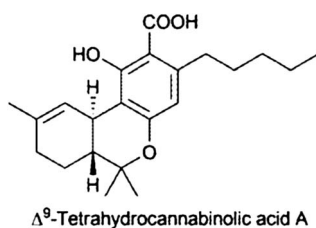
4.2.1 Vacuum liquid chromatography (VLC). In contrast to other forced-flow column chromatographic techniques, not pressure but vacuum is applied in VLC to increase flow rate and hence speed up the fractionation procedure. Column beds in VLC usually consist of silica of 40–60 μm particle size or reversed-phase silica. The open end of the column is easily accessible for the sample (as liquid or adsorbed to inactivated silica or diatomaceous earth) and the mobile phase, which is frequently a stepwise gradient with increasing elution power (e.g. hexane to methanol for silica columns). VLC is a popular method for fractionation of crude extracts due to its ease of use and high sample capacity. Eluted fractions are usually analysed by TLC for their composition. The review by Sticher¹ illustrated the application of VLC to different compound classes such as sterols, flavonoids, alkaloids, triterpene saponins or coumarins; the methodology was also discussed by Reid and Sarker.¹⁴⁵

Recently, VLC was part of the isolation procedure of α -viniferin and hopeaphenol, trimeric and tetrameric stilbenes from *Shorea ovalis*,^{146,147} anthraquinones, naphthalenes and naphthoquinones from *Asphodeline lutea*,¹⁴⁸ alantolactone and isoalantolactone from *Inula helenium*,¹⁴⁹ the antifungal sakurasosaponin from *Jacquinia flammea*,¹⁵⁰ and antimalarial diterpene formamides from the marine sponge *Cymbastela hooperi*.¹⁵¹



4.2.2 Flash chromatography (FC). Similarly to VLC, FC is mainly used for rapid fractionation of crude extracts or coarsely purified fractions. By applying nitrogen or compressed air, the mobile phase is flushed through the stationary phase in a tightly closed glass column or prepacked cartridges. In comparison to open-column chromatography, smaller particle size (*ca.* 40 μm in case of silica) can be used, hence increasing peak resolution. On-line peak detection is possible, usually by coupling to a UV detector. Supercritical fluid chromatography is a promising new option not only for HPLC but also for FC, however it will need significantly higher expenditure of equipment.¹⁵² Examples for successful application of FC have been shown.^{1,145} For FC method development, TLC separations on corresponding stationary phases were suggested.^{153,154} Excellent separations of compounds from *Curcuma zanthorrhiza* (curcumin, xanthorrhizol), *Piper nigrum* (amides) and *Salvia miltiorrhiza* (tanshinones) could be obtained by FC on prepacked RP-18 cartridges (Sepacore®) based on empirical rules involving HPLC separations.¹⁵⁵ By stepwise up-scaling, a method for separation of tasteless limonin glucoside from bitter-tasting limonin on a gram scale on a Biotage® C-18 cartridge with ethanol and water mixtures as eluents could be developed, impressively showing the sample capacities of FC.¹⁵⁶ Some recent examples of FC as part of the isolation strategy include acylphloroglucinols from *Hypericum empetrifolium*, which were isolated by FC on silica, RP-18 and a final purification on RP-HPLC,¹⁵⁷ antiplasmodial aporphine alkaloids and sesquiterpene lactones from *Liriodendron tulipifera*,¹⁵⁸ and microbial stress-induced resveratrol oligomers from *Vitis vinifera* leaves using ENV+® and Toyopearl HW 40S® resins.¹⁵⁹ In the case of the macrolide antibiotics oligomycins A and C isolated from *Streptomyces diastaticus*, FC on RP-18 material was used as a final purification step.¹⁶⁰

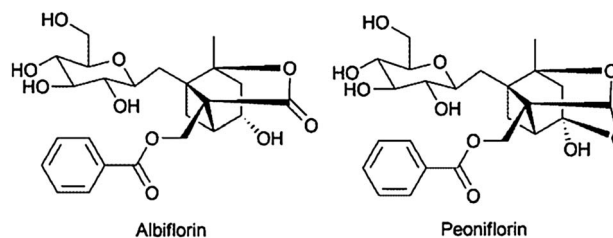
Two independent flash chromatography systems on normal phase and reversed phase were developed for the rapid isolation of Δ^9 -tetrahydrocannabinolic acid A (THCA) from *Cannabis sativa*.¹⁶¹ By normal-phase FC and gradient elution with cyclohexane and acetone 1.8 g, crude cannabis extract yielded 0.6 g THCA, whereas using an RP-18 phase with an isocratic elution with MeOH–formic acid (0.554%, pH 2.3), 85 : 15 v/v, 0.3 g extract resulted in 51 mg THCA; purity of THCA with both methods was >98.8%.¹⁶¹



Another example of the separation power of FC was provided by Uckoo *et al.*¹⁶² isolating four structurally similar polymethoxyflavones, *i.e.* tangeretin, nobiletin, tetramethoxyflavone and sinensitin, from peels of *Citrus reshni* and *C. sinensis* by FC on silica with a hexane–acetone gradient. A mixture of diterpenes from the mollusc *Thuridilla splendens*, thuridillins D–F, was obtained by silica FC but could be finally separated by preparative TLC on AgNO₃-impregnated silica gel plates.¹⁶³

4.2.3 Low-pressure liquid chromatography (LPLC). Column chromatographic methods which allow flow of the mobile phase at atmospheric pressure without additional forces either by vacuum or pressure are still a major tool in the fractionation protocols for NP isolation. There are a plethora of stationary phases with different separation mechanisms, such as adsorption, liquid–liquid partition (cellulose), ion exchange, bioaffinity or molecular sieving, available, which will not be discussed in this review but have been recently summarized by Reid and Sarker¹⁴⁵ and Ghisalberti.⁷² When using the frequently applied hydroxy-propylated dextran gel Sephadex LH-20® it has to be considered that not only molecular sieves but also adsorption effects contribute to the separation mechanism.¹⁶⁴

4.2.4 Medium-pressure liquid chromatography (MPLC). MPLC is commonly used to enrich biologically active secondary metabolites before further purification by HPLC due to its lower cost, higher sample loading and higher throughput. Cheng *et al.*¹⁶⁵ used normal-phase (NP)-MPLC as a pre-treatment method to enrich ginsenoside-Ro from the crude extract of *Panax ginseng* and purified it by high-performance counter-current chromatography. Interestingly, this two-step purification process resulted in a 79.2% total recovery of ginsenoside-Ro. Successful fractionation of the acetone extract of the aquatic macrophyte *Stratiotes aloides* with MPLC using RP-18 and polyamide CC 6 stationary materials afforded highly pure flavonoid glycosides after final semi-preparative HPLC on RP-18 columns including those with polar endcapping.¹⁶⁶ Some studies have revealed the potential and suitability of MPLC for direct isolation of pure natural compounds which failed to be achieved by other chromatographic methods. Yang *et al.*¹⁶⁷ managed to separate the anthraquinones, 2-hydroxy-emodin-1-methylether and 1-desmethylchrysoobtusin from the seeds of the Chinese medicinal plant *Cassia obtusifolia* using RP-18 MPLC after various unsuccessful attempts to purify them by recycling counter-current chromatography. Similarly, an octadecyl-phase MPLC was employed to get the cyanopyridone glycoside acalyphin from the inflorescences and leaves of the Indian copperleaf *Acalypha indica*.¹⁶⁸ Peoniflorin and albiflorin, the main constituents of *Paeonia lactiflora*, are well known for their immunoregulating and blood circulation improving functions. Wang *et al.*¹⁶⁹ have developed an efficient and economical MPLC method for large scale purification of these monoterpene glycosides. Isocratic elution of the enriched extract with H₂O/0.1%HOAc–MeOH (77 : 23) using an RP-18 column at a flow rate of 100 ml min⁻¹ afforded pure compounds of peoniflorin and albiflorin.



Silver nitrate-impregnated silica gel was employed for successful separation of the sesquiterpenes (*Z*)- α - and

(*Z*)- β -santalol with >96% purities from the white sandalwood, *Santalum album*.¹⁷⁰ These two sesquiterpene alcohols, which together constitute over 80% of the heartwood oil of matured trees, are responsible for the antifungal, anti-inflammatory, antidepressant and organoleptic properties of white sandalwood essential oil.¹⁷⁰

4.2.5 High-performance (high-pressure) liquid chromatography (HPLC). As shown in Table 3, octadecyl silica (RP-18) columns are still widely used for NP isolation and purification, however various laboratories have benefited from the availability of high-quality modern-generation HPLC columns with diverse modified phases such as cyano, phenyl, trimethylsilane, triazole, secondary and tertiary amines, β -cyclodextrin and dihydroxypropane for successful isolation and purification of NPs. Many of these can be used in HILIC mode. The term “hydrophilic interaction chromatography” (HILIC)¹⁷¹ was introduced about 20 years ago. Among separation principles based on chromatography, HILIC can be regarded as a new type of partition chromatography besides normal-phase and reversed-phase chromatography. The stationary phase of a HILIC column is polar and consists of silanol, amino, or charged groups. The mobile phase must be rich in organic solvent (usually acetonitrile) and should contain low amounts of water. Selectivity can be tuned by pH. Excellent reviews on this chromatographic technique have been published recently.^{172–175} Although its domain is still in the proteomics and glycomics area, HILIC chromatography was applied to small molecule NPs like saponins and flavonoids¹⁷⁶ as well as pro-cyanidins¹⁷⁷ and other polyphenols.¹⁷⁸ Liu *et al.*¹⁷⁹ prepared a click β -cyclodextrin (click-CD) column which enabled them to isolate and purify the anticancer steroids, bufadienolides, from the skin of the toad *Bufo bufo gargarizans*. Since the RP-HPLC method used for the direct isolation of bufadienolides from toad skin did not lead to a satisfactory resolution of arenobufagin and its stereoisomer, the use of RP-HPLC/click-CD orthogonal isolation method was necessitated. The two-dimensional RP/HILIC system with click-CD stationary phase demonstrated a great power to isolate the bioactive bufadienolides. Arenobufagin and its stereoisomer were successfully isolated using the click-CD column with a gradient MeCN/0.1% HCO₂H–H₂O (95 : 5 to 60 : 40). The triazole-bonded silica HILIC column employed by Morikawa *et al.*¹⁸⁰ provided better separation for sesquiterpene glycosides from the Thai medicinal plant *Sapindus rarak* compared to a RP-30 column due to the positively charged triazole stationary phase. A polyamine-II column that possesses secondary and tertiary amine groups bonded to porous silica particles was used for the separation of triterpene glycosides from *Physena sessiliflora* in HILIC mode.¹⁸¹ Van Wagoner *et al.*¹⁸² isolated sulphonated karlotoxins from the microalgae *Karlodinium veneficum* using the reverse-phase Develosil TM-UG-5® C1 phase with a basic eluent. Cyano packing allowed efficient purification of the phytotoxic oleane saponins of the leaves of *Bellis sylvestris* that differ greatly in hydrophobicity, without the need to use gradient elution.¹⁸³

A semi-preparative CN-phase HPLC column was employed to isolate six free amino acids from the aquatic macrophyte *Stratiotes aloides*, the European water soldier.¹⁶⁶ In addition, a

luteolin glycoside was purified from *S. aloides* using a phenyl-bonded silica column. As compared to the aliphatic straight-chain reversed phases such as C18 and C8, the π -electrons of the phenyl group can interact with aromatic residues of an analyte molecule in addition to hydrophobic interaction to increase retention relative to non-aromatic compounds. Thus, phenyl-modified silica gel columns were also employed to isolate lignans from the aerial parts of the Thai medicinal plant *Capparis flavicans*¹⁸⁴ and antiproliferative eupolauridine alkaloids from the roots of *Ambavia gerrardii*.¹⁸⁵

In recent years, a clear trend towards miniaturization of bioassay-guided setups like HPLC-based activity profiling in order to quickly identify metabolites of significant biological activity in crude plant extracts could be recognized.^{186,187} In this respect, a microfractionation strategy combined with activity testing in a zebrafish bioassay in combination with UHPLC-TOF-MS and microfluidic NMR was proposed for rapid detection of pharmacologically active natural products.¹⁸⁸

5 Chiral chromatographic methods in natural products isolation

After isolation of chiral compounds of NPs, often a method to determine absolute configuration is needed. Different models for the requirements of chiral recognition have been discussed. The best known model is the three-point interaction model by Dalglish,¹⁸⁹ which postulates that three interactions have to take effect and at least one of them has to be stereoselective. For enantioseparation at an analytical scale, high-performance separation techniques such as HPLC, GC, CE or SFC have widely been used, however HPLC is applied in most cases. This separation technique allows separating enantiomers either indirectly with chiral derivatization reagents or directly with chiral stationary phases or chiral mobile-phase additives. There are advantages and disadvantages for each of these techniques. Indirect separation is based on derivatization by chiral derivatization reagents to form diastereomeric derivatives. They differ in their chemical and physical behavior and therefore are resolved on achiral stationary phases, such as a reversed-phase column. This approach avoids the need for expensive columns with chiral stationary phases, however derivatization has to be regarded as an additional step which can have side reactions, formation of decomposition products and racemization as undesirable side effects. Furthermore, the chiral derivatization reagent has to be of high enantiomeric purity; also, derivatizable groups in the analyte have to be available. Direct enantioseparation using columns with chiral stationary phases is more convenient and also applicable for separations on preparative scale. On the other hand, a collection of expensive columns is required. Finally, the approach to add a chiral selector to the mobile phase can be regarded as a simple and flexible alternative, however applicability is limited. Since mobile phases containing a chiral selector cannot be reused, this technique should not be applied with expensive chiral additives.²¹⁹ For detection, mostly UV-VIS is used, although polarimetric detectors are advantageous, since they produce a negative peak for (–)-enantiomers. For direct chiral separations, a variety of

Table 3 Isolation and purification of natural secondary metabolites by HPLC

Compounds	Source	Column			Mobile phase	Ref.
		Stationary phase ^a	Dimension (mm)	PD ^b (μm)		
Terpenoids						
Sesquiterpenes	<i>Acorus calamus</i>	Silica gel-Diol	10 × 250	10	Isocratic hexane-2-propanol (97 : 39)	190
		Silica gel C18	30 × 150	5	Gradient H ₂ O-MeOH (50 : 50 to 0 : 100)	
Sesquiterpenes	<i>Rolandra fruticosa</i>	Silica gel-C18	10 × 150, 19 × 150	5	Isocratic H ₂ O-MeOH (50 : 50, 55 : 45)	191
Sesquiterpenes	<i>Artemisia persica</i>	Silica gel-C18	10 × 150	5	Gradient H ₂ O-MeCN (80 : 20 to 0 : 100); H ₂ O-MeOH (70 : 30 to 0 : 100)	192
Diterpenoids	<i>Leonotis leonurus</i>	Silica gel-C18	21.2 × 150	7	100% MeOH	193
		Partisil 10	4.6 × 250	10	Isocratic MeOH-CH ₂ Cl ₂ (1 : 99)	
Diterpenoids	<i>Ajuga bracteose</i>	Silica gel-C18	2.1 × 100	1.7	Gradient H ₂ O/0.1% HCO ₂ H-MeCN (70 : 30 to 5 : 95)	194
Triterpenoids	<i>Lycopodium phlegmaria</i>	Silica gel-C18	20 × 250	5	Isocratic H ₂ O-MeOH (15 : 85)	195
		Silica gel-C18	19 × 250	5	Isocratic H ₂ O-MeOH (15 : 85)	
Triterpenoids	<i>Cogniauxia podolaena</i>	Silica gel-C18	19 × 150	5	Gradient H ₂ O-MeCN (90 : 10 to 0 : 100)	196
Triterpenoid saponins	<i>Aesculus glabra</i>	Silica gel-C18	4.6 × 250	3.5	Isocratic H ₂ O/0.5% HOAc-MeCN (63 : 37, 60 : 40)	197
		Silica gel-C18	22 × 250	10	Isocratic H ₂ O/0.5% AcOH-MeCN (60 : 40, 52 : 48, 45 : 55, 35 : 65)	
Triterpene glycosides	<i>Physena sessiliflora</i>	Silica gel-C18	20 × 100	5	Isocratic H ₂ O-MeCN (70 : 30, 63 : 37)	181
		Silical gel-Polyamine-II	20 × 150	5	Isocratic H ₂ O-MeCN (17.5 : 82.5, 22.5 : 77.5)	
Triterpenoid oligoglycosides	<i>Sapindus rarak</i>	Silica gel-C30	4.6 × 250	5	Isocratic H ₂ O-MeCN/1% AcOH (50 : 50)	180
		Silica gel-Triazole (HILIC)	20 × 250	5	Isocratic H ₂ O-MeCN (5 : 95)	
Terpenoids, phenethyl glucosides	<i>Hyssopus cuspidatus</i>	Silica gel-Phenyl	20 × 250	5	Isocratic H ₂ O-MeOH (25 : 75, 20 : 80, 60 : 40)	198
		Silica gel-C18-Phenyl	10 × 250	5	Isocratic H ₂ O-MeOH (10 : 90, 15 : 85, 25 : 75)	
Sesquiterpenoids, macrolide and diterpenoid	<i>Cyphostemma greveana</i>	Silica gel-C18	10 × 250	5	Isocratic H ₂ O-MeOH (35 : 65)	199
		Silica gel-Phenyl	10 × 250	5	Isocratic H ₂ O-MeCN (55 : 45)	
Oleanane saponins	<i>Bellis sylvestris</i>	Silica gel-C18	10 × 250	10	Isocratic H ₂ O-MeCN-MeOH (50 : 20 : 30)	183
		Silica gel-CN	10 × 250	5		
Alkaloids						
Cyclic diterpene alkaloids	<i>Agelas mauritiana</i>	Silica gel-C18	10 × 250	5	Isocratic H ₂ O-MeCN (46 : 54, 70 : 30, 75 : 25)	200
Quinoline alkaloids	<i>Drummondita calida</i>	Silica gel-C18	21.2 × 150	5	Gradient H ₂ O/0.1%TFA-MeOH/0.1% (90 : 10 to 0 : 100)	201
		Silica gel-Diol	20 × 150	5	Gradient CH ₂ Cl ₂ -MeOH (90 : 10 to 0 : 100)	
Stemona alkaloids	<i>Stemona sp.</i>	Silica gel-C18	4.6 × 250	5	Gradient H ₂ O in 10mM NH ₄ OAc-MeOH (45 : 55 to 10 : 90, 19 min; 10 : 90 to 0 : 100, 1 min; 0 : 100, 10 min)	202
Eupolauridine alkaloids	<i>Ambavia gerrardii</i>	Silica gel-Phenyl	10 × 250	5	Isocratic H ₂ O-MeOH (40 : 60)	185
Flavonoids						
Anthocyanins	<i>Asparagus officinalis</i>	Silica gel-C18	20 × 250	5	Gradient H ₂ O/10%HCO ₂ H 40%MeCN/ 50%H ₂ O/10%HCO ₂ H(75 : 25 to 50 : 50, 23 min)	203
Anthocyanins	<i>Arabidopsis thaliana</i>	Silica gel-C18	20 × 250	5	Isocratic H ₂ O/0.5% AcOH-MeOH (60 : 40)	204
Flavonoid glucuronides, chromone	<i>Stratiotes aloides</i>	Silica gel-phenyl	10 × 250	7	Gradient H ₂ O/0.01%TFA-MeCN 84%/ H ₂ O 16% (100 : 0 to 80 : 20, 10 min; 80 : 20 to 60 : 40, 30 min; 60 : 40 to 50 : 50, 10 min); Gradient H ₂ O/0.01% TFA-MeOH/H ₂ O (84 : 16) (100 : 0, 60 min; 100 : 0 to 0 : 100, 20min)	166
Flavonoid glycosides	<i>Citrus bergamia</i>	Silica gel-C18	21.2 × 100	10	Isocratic H ₂ O/0.1%HCO ₂ H-MeCN (55 : 45, 12 min; 77 : 23, 15 min)	205
Flavones	<i>Mimosa diplotricha</i>	Silica gel-C18	20 × 250	5	Isocratic H ₂ O-MeOH (40 : 60)	206

Table 3 (Contd.)

Compounds	Source	Column			Mobile phase	Ref.
		Stationary phase ^a	Dimension (mm)	PD ^b (μm)		
Flavonoids, triterpene saponins	<i>Glycyrrhiza</i> sp.	Silica gel-C18	19 × 100	5	Gradient H ₂ O/0.1% HCO ₂ H-MeCN (85 : 15, 5 min : 85 : 15 to 65 : 35, 55 min; 65 : 35 to 5 : 95, 60 min)	176
		β-CD (HILIC) ^d	30 × 150	5	Gradient H ₂ O-MeCN/0.1% HCO ₂ H (5 : 95 to 10 : 90, 30 min; 10 : 90, 30 min)	
Flavonolignans	<i>Calamus quiquesetinerivius</i>	Silica gel C18	10 × 250	5	Isocratic H ₂ O-MeOH (51 : 49, 65 : 35)	207
Neoflavonoids and Benzofurans	<i>Pterocarpus santalinus</i>	Silica gel-C18	10 × 250	5	Isocratic H ₂ O-MeOH (43 : 57)	208
Steroids Bufadienolides	<i>Bufo bufo gargarizans</i>	Click-CD (HILIC)	4.6 × 150	5	Gradient H ₂ O-MeCN/0.1% HCO ₂ H (5 : 95 to 40 : 60)	179
		Silica gel-C18	4.6 × 150	3	Gradient H ₂ O-MeCN (95 : 5 to 35 : 65, 0–60 min; 35 : 65 to 5 : 95, 60–70 min)	
Lignans Polyhenolic lignans	<i>Capparis flavicana</i> , <i>Vitax glabrata</i>	Silica gel-Phenyl	22 × 250	5	Isocratic H ₂ O-MeCN (85 : 15, 87.5 : 12.5, 90 : 10, 95 : 5)	184
		Silica gel-C18	20 × 250	5	Isocratic H ₂ O-MeCN (95 : 5), H ₂ O-MeOH (90 : 10)	
Lignan glucosides, flavanones	<i>Macaranga tanarius</i>	Silica gel-C18	6 × 250	3	Isocratic H ₂ O-MeCN (90 : 10, 19 : 1, 40 : 10, 41 : : 9, 83 : 17, 40 : 10)	209
Tannins Gallotannins	<i>Eugenia jambolana</i>	Silica gel-C18	10 × 250	5	Isocratic H ₂ O-MeOH (76 : 24, 70 : 30, 67 : 33, 65 : 35)	210
Peptides Cyclopeptides	<i>Annona montana</i>	Silica gel-C18	4.6 × 250	5	Isocratic H ₂ O-MeCN (25 : 75)	211
		Silica gel-C30	20 × 250	5	Isocratic H ₂ O-MeCN/0.5% TFA (25 : 75)	
Cyclodepsipeptides	<i>Lyngbya confervoides</i>	Silica gel-C18	21.2 × 100	10	Gradient H ₂ O-MeOH (70 : 30 to 0 : 100, 40 min, 0 : 100, 10 min)	212
		Silica gel C18	10 × 250	5	H ₂ O-MeOH/0.05% TFA (40 : 60 to 10 : 90, 25 min; 10 : 90 to 0 : 100, 10 min)	
Lipopeptides	<i>Nocardia</i> sp.	Silica gel-C18	10 × 250	5	Gradient H ₂ O-MeCN/CH ₂ Cl ₂ (98 : 2 to 50 : 50)	213
Others Polyketides	<i>Botryosphaeria rhodina</i>	Silica gel-C18	16 × 250	5	Gradient H ₂ O-MeCN (75 : 25 to 0 : 100)	214
		Silica gel-C8	21.2 × 250	5	Gradient H ₂ O-MeOH (100 : 0, 20 min; 80 : 20, 30 min; 0 : 100, 40 min)	168
Cyanopyridone glucosides	<i>Acalypha indica</i>	Silica gel-C8	10 × 250	5	Gradient H ₂ O-MeOH (30 : 70 to 0 : 100)	215
Acetophenone	<i>Acronychia pedunculata</i>	Silica gel-C18	4.6 × 150	3.5	Isocratic H ₂ O-MeCN (62 : 38)	182
Karlotoxins	<i>Karlodinium veneficum</i>	Silica gel-C1	4.6 × 250	5	Isocratic 2 mM NH ₄ Ac-MeCN (64 : 36)	
Picolinic acid derivative	<i>Fusarium fujikuroi</i> sp. Tlau3	Silica gel-C8	19 × 250	5	Isocratic H ₂ O/TFA-MeOH/TFA (45/0.1 : 55/0.1)	216
Stilbenoids, phenanthraquinone	<i>Oncidium microchilum</i> , <i>O. isthmi</i> , <i>Myrmecophila humboldtii</i>	Silica gel-C18	21.2 × 100	5	Gradient H ₂ O/0.05% TFA-MeCN (40 : 60 to 15 : 85)	217
		Silica gel-C18	10 × 250	5	Gradient H ₂ O/0.1% TFA-MeCN (various proportions)	
Polycyclic fatty acids	<i>Beilschmiedia</i> sp.	Silica gel-C18	10 × 250	5	Isocratic H ₂ O/0.05% TFA-MeCN (42 : 58, 45 : 55)	218

^a C1: trimethylsilan chemically bonded to porous silica particle; β-CD: β-cyclodextrin bonded to porous silica particle; Click-CD: β-cyclodextrin bonded to porous silica particle by click chemistry; Diol: dihydroxypropane groups chemically bonded to porous silica particles; HILIC: hydrophilic interaction chromatography; Partisil 10: amino and cyano groups chemically bonded to porous silica particle; Polyamine II: secondary and tertiary amine groups bonded to porous silica particle. ^b PD: particle diameter.

chiral separation principles is available: the most often used principle is based on enantioselective complexation in cavities of a chiral selector.²²⁰ As secondary interactions, hydrogen-bonding, dipole interactions and hydrophobic interactions can be taken into account. For example, cyclodextrin (CD) derivatives of α -CD, β -CD or γ -CD or synthesized chiral crown ethers are suitable. Also, macrocyclic antibiotics such as the glycopeptides vancomycin, ristocetin or teicoplanin are available. The latter compound contains 18 chiral centers and three chiral cavities bridged by 5 aromatic ring structures. As interactions, hydrogen donor and acceptor sites are readily available close to the ring structures. All these selectors can be either fixed on the silica support of a column or can be used as chiral additives to the mobile phase along with an achiral column. Gutierrez *et al.*²²¹ isolated tanikolide seco-acid and tanikolide dimer from the Madagascar marine cyanobacterium *Lynghya majuscula*. They used a chiral HPLC column based on the macrocyclic antibiotic teicoplanin along with mixtures of ethanol/water as mobile phase. Moreover, chiral stationary phases based on polysaccharides are commercially available. They showed a very broad applicability to different compound classes. Since the chiral cavities of native amylose and cellulose are too small, they are not available for interaction and have to be altered by derivatization. These columns have found a wide range of applicability. Besides columns bearing the polysaccharide covalently attached to the silica support, there are also coated polysaccharide CSPs available, however, the latter ones are limited with respect to the solvents that can be used in the mobile phase. Antonov *et al.*²²² report on a new procedure for separation of highly polar glycoside fractions by a Chiralpak IC HPLC column consisting of cellulose tris(3,5-dichlorophenylcarbamate). Batista *et al.*²²³ elucidated the structure and absolute stereochemistry of isomeric monoterpene chromane esters by means of a Chiralcel OD-H HPLC column. In this case cellulose is derivatized by tris(3,5-methylphenylcarbamate). The same selector is also provided by other vendors: a new tyrosine-derived metabolite, namely aspergillusol A, was isolated as well as a methyl ester of 4-hydroxyphenylpyruvic acid oxime and secalonic acid A from the marine-derived fungus *Aspergillus aculeatus* CRI323-04. For chiral HPLC, a Phenomenex Lux Cellulose-1® was used.²²⁴

A further chiral separation principle represents ligand-exchange chromatography, which was one of the first successful separation principles in chiral chromatography. In this case, chiral recognition is based on the formation of ternary mixed metal complexes between the selector and analyte ligand. As can be seen from Table 4, this separation principle was used most frequently. Immobilized amino acids, such as D-penicillamine or amino acid derivatives are complexed by the mobile phase containing Cu(II) for enantio-resolution.^{225,227–230,232–234,236–239}

Adams *et al.*²²⁵ isolated malevamide E, a dolastatin 14 analogue, from the marine cyanobacterium *Symploca laete-viridis*. They used aqueous Cu(II) solutions with acetonitrile as mobile phase. In another approach, Clark *et al.*²²⁸ discovered 6 new acyl proline derivatives and tumonoic acids D–I. Stereostructures were elucidated by chiral HPLC using a Phenomenex

Chirex 3126® column consisting of D-penicillamine bonded on silica backbone. An aqueous solution of 2 mM copper(II) sulfate served as mobile phase. This column showed wide applicability for determination of absolute configuration.^{225,228–230,232,233,236,239} Teruya and coworkers applied another ligand-exchange column, namely a Daicel Chiralpak MA (+), for the determination of a hexapeptide, hexamollamide after bioassay-guided fractionation of the Okinawan ascidian *Didemnum molle*.²³⁷

Another approach for enantioseparation by HPLC represents the use of a so called Pirkle-column or brush-type phase. These columns provide various selectors for ionic or covalent bonding. The chiral selector consists of an optically pure amino acid bonded to γ -aminopropylsilylanized silica. A linking of a π -electron group to the stereogenic center of the selector provides π -electron interactions and one point of chiral recognition. Koyama reports the elucidation of relative and absolute stereochemistry of quinadoline B, an inhibitor of lipid droplet synthesis in macrophages.²³¹ For chiral HPLC, a Sumichiral OA-3100 column with covalently bonded (S)-valine as chiral selector and a mixture of methanol/acetonitrile (95 : 5) containing 1 mM citric acid was used. Further examples for the successful use of chiral HPLC columns can be found in Table 4.

Besides HPLC, GC and CE can be used for determination of stereostructure as well. Generally, the chiral selectors provided for HPLC are also applicable in GC and CE. For example, malynolide dimer was isolated by Gutierrez *et al.* after the extract of the marine cyanobacterium *Lynghya majuscula* was fractionated.²⁴⁰ The absolute configuration was determined by chiral GC-MS after chemical degradation and results were compared with an authentic sample. Pinto *et al.*²⁴¹ reported the isolation of a new triquinane sesquiterpene, (–)-*epi*-pre-silphiperfolan-1-ol, from the essential oil of *Anemia tomentosa* var. *anthriscifolia*. They elucidated chiral configuration by bi-dimensional GC using 2,3-di-O-ethyl-6-O-tert-butyl-dimethylsilyl- β -cyclodextrin as the chiral stationary phase.²⁴¹ There is a variety of chiral capillaries for GC commercially available. First development of a chiral GC capillary was done by Gil-Av's group.²⁴² An amino acid derivative served as chiral selector for enantioseparation of N-trifluoroacetyl amino acids. Chiral recognition on these phases is based on the formation of multiple hydrogen bonds. Moreover, columns based on the chiral separation principle of metal complexes, cyclodextrins, cyclocholates, calixarenes are used.²¹⁹

6 Isolation by preparative gas chromatography (PGC)

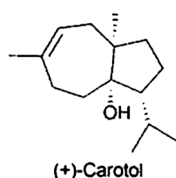
For isolation of volatiles, PGC is an attractive option. Usually packed columns with higher sample capacity but lower peak resolution are employed,^{243,244} however there are an increasing number of successful applications of thick-phase film wide-bore capillaries with capillary GC instrumentation during the last years. PGC was reviewed recently giving also some practical advice to achieve satisfying results.²⁴⁵ Menthol and menthone from peppermint oil (*Mentha x piperita*) have been isolated using a 15 m \times 0.32 mm i.d. DB-5 column (1 μ m film thickness) and an external cryotrap. Flow switching between the cryotrap

Table 4 Chiral HPLC used for isolation and purification of natural secondary metabolites

Compounds	Source	CSP ^a	Chiral stationary phase ^b	Mobile phase	Ref.
Maleamide E	<i>Symploca laete-viridis</i>	LE	Chirex; D-PA on silica	1.7 mM Cu(II) in acetonitrile/water (14 : 86); mobile phase II: 1.9 mM Cu(II) in acetonitrile/water (5 : 95)	225
[8-Ethyl]-chlorophyll c3	<i>Emiliania huxleyi</i>	CIC	Chiralpak IC; cellulose tris (3,5-dichlorophenylcarbamate) on silica	1 : 2 : 2 (v/v/v) methanol-acetonitrile–100 mM aqueous ammonium acetate	226
Monoterpene chromane esters	<i>Peperomia obtusifolia</i>	CIC	Chiralcel OD-H; cellulose tris (3,5-dimethylphenylcarbamate)	n-hexane	223
Cordylheptapeptides C–E	<i>Acremonium persicinum</i>	LE	MCIGEL CRS10W; <i>N,N</i> -diethyl-L(or D)-alanine	2 mM Cu(II)	227
Lyngbyastatins 1 and 3, acyl proline derivatives, tumonoic acids D–I, tumonoic acid A	<i>Blennothrix cantharidosmum</i>	LE	Chirex 3126; D-PA on silica	2 mM Cu(II)	228
Molassamide	<i>Dichothrix utahensis</i>	LE	Chirex 3126; D-PA on silica	2 mM Cu(II) with acetonitrile	229
Carriebowmide	<i>Lyngbya polychroa</i>	LE	Chirex 3126; D-PA on silica	2 mM Cu(II)	230
Tanikolide dimer, tanikolide seco-acid	<i>Lyngbya majuscula</i>	CIC	Chirobiotic T; teicoplanin on silica	40 : 60 water/ethanol	221
Aspergillusol	<i>Aspergillus aculeatus</i>	CIC	Lux Cellulose-1, cellulose tris(3,5-dimethylphenylcarbamate) on silica	2-propanol/hexane (20 : 80)	224
Quinadoline B	<i>Aspergillus</i> sp. FKI-1746	PT	Sumichiral OA-3100; <i>N</i> -(3,5-dinitrophenylaminocarbonyl)-L-valine	methanol/acetonitrile (95 : 5) containing 1 mM citric acid	231
3-Amino-6-hydroxy-2-piperidone	<i>Lyngbya confervoides</i>	LE	Chirex 3126; D-PA on silica	2 mM Cu(II) or 2 mM Cu(II)/acetonitrile (95 : 5)	232
Coibamide A	<i>Leptolyngbya</i> sp.	LE	Chirex 3126; D-PA on silica	2 mM Cu(II) or 2 mM Cu(II)/acetonitrile (95 : 5)	233
Pitipeptolides C–F	<i>Lyngbya majuscula</i>	LE	Chiralpak MA (+); amino acid derivatives on silica	acetonitrile/2 mM Cu(II) (10 : 90)	234
Diarylheptanoids	<i>Alpinia katsumadai</i>	CIC	Daicel Chiralpak IB; cellulose 3,5-dimethylphenylcarbamate on silica	n-Hexane/2-propanol (7 : 3)	235
Kempopeptins A, B	<i>Lyngbya</i> sp.	LE	Chirex 3126; D-PA on silica	2 mM Cu(II) or 2 mM Cu(II)/acetonitrile (95 : 5)	236
Hexamollamide	<i>Didemnum molle</i>	LE	Chiralpak MA (+); amino acid derivatives on silica	2 mM Cu(II)/acetonitrile (80 : 20)	237
Hantupeptin A	<i>Lyngbya majuscula</i>	LE	Chiralpak MA (+); amino acid derivatives on silica	2 mM Cu(II)/acetonitrile (85 : 15);	238
Eudistomides A, B	<i>Eudistoma</i> sp.	LE	Chirex 3126; D-PA on silica	1 mM Cu(II)/acetonitrile (95 : 5)	239

^a CSP: Chiral separation principle; CIC: chiral inclusion complexation; LE: ligand-exchange; PT: Pirklé type. ^b D-PA: D-penicillamine.

and the detector (FID) was gained by an Deans switch device.²⁴⁶ A multidimensional PGC consisting of three GC systems equipped with three Deans switch transfer devices was used for isolation of carotol, an oxygenated sesquiterpene from carrot seed oil (*Daucus carota*).²⁴⁷ By combining 5% diphenyl-polyethylene glycol-ionic liquid stationary phases with diverse selectivity in the preparative MDGC setup, 2.22 mg of carotol were collected in about 230 min.²⁴⁷



Compounds selected in a MDGC setup on the first GC column by microfluidic heart-cut could be enriched from multiple runs by an internal cryogenically cooled trap before transferring to the second column.²⁴⁸ For fractionation of volatiles emitted by *Spodoptera*-infested maize seedlings, which were most attractive to females of the parasitoid *Cotesia marginiventris*, even micro-bore capillary columns were used.²⁴⁹ (*E,E*)-2,4-Undecadienal was identified as the most deodorizing compound in the odor of coriander leaves (*Coriandrum sativum*) with aid of PGC on a 60 m × 0.75 mm column with a polyethylene glycol stationary phase.²⁵⁰

7 Conclusions

In recent years several major developments have been recognized in the field of NP isolation. An increasing number of

methods have been developed by hyphenation of chromatographic and spectroscopic or spectrometric techniques with the aim to elucidate structures of known as well as novel compounds without the need for isolation. In the same direction goes coupling of LC with SPE trapping and transfer to capillary NMR illustrating the trend to downscale isolation procedures. Microwave and ultrasonic-assisted extraction procedures as well as accelerated solvent extraction seem to be established as methods increasing extraction efficacy and shortening extraction time. IL as extraction solvents are also an upcoming field in the natural products area and maybe will result in a more selective enrichment of compounds of interest already in crude extracts. SPE widened its application towards fractionation similar to VLC. However, the most exciting development in SPE seems to be the selective isolation of target compounds by molecularly imprinted stationary phases.

Chiral separations are increasingly also applied at preparative scale, taking the chiral character of many NPs into account. Although the chromatographic principle was known for many years, HILIC is currently experiencing a significant increase of applications in NP isolation and analysis, providing an additional mechanism of separation compared to normal and reversed-phase chromatography. Although isolation of pure compounds from difficult matrices like organic matter is still challenging and we are far from isolation procedures in one step, the application of more selective methods from extraction to fractionation and purification will speed up the time from collection of biological material to final purified compound.

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