# Natural product isolation†

**Otto Sticher***

*Received 15th October 2007*
*First published as an Advance Article on the web 11th March 2008*
**DOI: 10.1039/b700306b**

Covering: 2000 to mid-2007

Since the 1990s, interest in natural product research has increased considerably. Following several outstanding developments in the areas of separation methods, spectroscopic techniques, and sensitive bioassays, natural product research has gained new attention for providing novel chemical entities. This updated review deals with sample preparation and purification, recent extraction techniques used for natural product separation, liquid–solid and liquid–liquid isolation techniques, as well as multi-step chromatographic operations. It covers examples of papers published since the *NPR* review 'Modern separation methods' by Marston and Hostettmann,[1] with major emphasis on methods developed and the research undertaken since 2000.

1 Introduction
2 Preparation and purification of samples
3 Extraction techniques used for separation and isolation
3.1 Supercritical fluid extraction (SFE)
3.2 Pressurised liquid extraction (PLE)
3.3 Microwave-assisted extraction (MAE)
3.4 Brief comparison of SFE, PLE and MAE with Soxhlet extraction
4 Liquid–solid isolation techniques
4.1 Preparative planar chromatography
4.2 Vacuum liquid chromatography (VLC)
4.3 Preparative pressure liquid chromatography (PPLC)
4.3.1 Flash chromatography (FC)
4.3.2 Low-pressure LC (LPLC)
4.3.3 Medium-pressure LC (MPLC)
4.3.4 High-pressure LC (HPLC)
5 Liquid–liquid isolation techniques
5.1 Terminology
5.2 Instruments and advantages of counter-current chromatography
5.3 High-speed counter-current chromatography (HSCCC)
5.3.1 Examples of natural product isolation by HSCCC
6 Concluding remarks
7 Acknowledgements
8 References

## 1 Introduction

Natural products are expected to play an important role as one of the major sources of new drugs in the years to come because of (i) their incomparable structural diversity, (ii) the relatively small dimensions of many of them (<2000 Da), and (iii) their "drug-like" properties, *i.e.* their ability to be absorbed and metabolised.[2] Isolation of natural products from higher plants, marine organisms and microorganisms is therefore still urgently needed, calling for state-of-the-art methodologies for separation and isolation procedures. Taking into consideration that a plant may contain thousands of constituents, the separation and isolation process can be long and tedious. Isolation of natural products generally combines various separation techniques, which depend on the solubility, volatility and stability of the compounds to be separated. The choice of different separation steps is of great importance and an analytical-scale optimisation of the separation parameters is worthwhile.

The separation methods described by Marston and Hostettmann[1] are centrifugal thin-layer chromatography (CTLC), overpressure layer chromatography (OPLC), flash chromatography (FC), liquid chromatography [low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC), high-pressure liquid chromatography (HPLC)], and counter-current chromatography [droplet counter-current chromatography (DCCC), rotation locular counter-current chromatography (RLCC), centrifugal partition chromatography (CPC)]. Evaluation of recent literature shows that CTLC, OPLC, RLCC, and DCCC have been rarely used since 2000. FC is still used often but mainly as part of a multi-step isolation procedure. The main separation technologies used in recent years are methods of liquid chromatography such as MPLC and semi-preparative HPLC, as well as CPC, mainly as high-speed counter-current chromatography (HSCCC) or high-performance centrifugal partition chromatography (HPCPC). Multi-step chromatographic operations have mostly been used, *e.g.* a combination of FC for pre-purification and semi-preparative HPLC for final purification.

## 2 Preparation and purification of samples

Several sample preparation, pre-purification and clean-up steps are used prior to isolation and/or analysis of natural products. Initial extraction with low-polarity solvents yields the more

*Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology, Zurich, 8093, Switzerland*

† Electronic supplementary information (ESI) available: Table S1 (complete version of Table 11) and Table S2 (complete version of Table 12). See DOI: 10.1039/b700306b

lipophilic components, while ethanolic solvents obtain a larger spectrum of non-polar and polar material. If a more polar solvent is used for the first extraction step subsequent solvent partition allows a finer division into different polarity fractions. Extraction methods (see Section 3) are therefore used as a pre-purification step to selectively remove interfering components and/or to isolate the active compounds. Other pre-purification methods are filtration, precipitation, removal of chlorophyll, waxes and tannins, solid-phase extraction (SPE) using pre-packed cartridges with a variety of packing material, both normal- and reversed-phase silica gel, or short columns with other suitable packing material such as alumina, Celite, Amberlite resins and Sephadex LH-20. Pre-packed cartridges for SPE operate on the principle of liquid–solid extraction and may be used in one of two modes: a) the interfering matrix elements of a sample are retained on the cartridge while the components of interest are eluted; b) the components of interest are retained while the interfering matrix elements are eluted. In the latter case, a concentration effect can be achieved. The required compounds are then eluted from the cartridge by changing the solvent.[3]

## 3 Extraction techniques used for separation and isolation

The first step in the analysis and isolation of natural products is extraction to separate the compounds from the cellular matrix. Extraction and recovery of a solute from a solid matrix may be regarded as a five-stage process: (i) desorption of the compound from the active sites of the matrix; (ii) diffusion into the matrix itself; (iii) solubilisation of the analyte in the extractant; (iv) diffusion of the compound in the extractant; (v) collection of the extracted solutes. Ideally, an extraction process should be exhaustive with respect to the constituents to be analysed or isolated, rapid, simple, inexpensive, and – at least for routine analysis – amenable for automation. The increasing interest in plant and marine secondary metabolites makes it necessary to expand and modify the arsenal of the traditional extraction protocols. Conventional methods for the extraction of natural products include Soxhlet extraction, maceration, percolation, turbo-extraction and sonication. These traditional methods present major drawbacks, including long extraction times, labour-intensive procedures, large amounts of organic solvents, unsatisfactory extraction efficiency, and potential degradation of labile compounds. In recent years new extraction techniques with significant advantages over conventional methods have been developed for extracting analytes from solid matrices, *e.g.* reduction in organic solvent consumption and in sample degradation, reduction of extraction and clean-up times or even elimination of additional sample clean-up and concentration steps, improvement in extraction efficiency, selectivity, and/or kinetics, ease of automation, *etc.*[4–9] These recent extraction techniques include supercritical fluid extraction (SFE), pressurised liquid extraction (PLE), microwave-assisted extraction (MAE), solid-phase microextraction (SPME), ultrasound-assisted extraction (UAE), superheated liquid extraction, and extraction with supercritical or subcritical water. Most of these methods have similar pros and cons with regard to solvent volume, extraction time and extraction efficiency. Numerous review articles have been published on several of these new extraction techniques for natural products, *e.g.* SFE,[6,10–15] PLE and MAE.[6,7] To date these extraction methods (with exception of SFE) have been mainly used in analytical protocols and rarely for the isolation of natural compounds. But it may be assumed that PLE and MAE will be used more frequently in the future and increasingly applied to the isolation of new compounds. For modern hyphenated isolation, screening and structure elucidation techniques, as well as mechanism-based and cellular assays, only milligram quantities are needed. This review describes PSE, PLE and MAE.

### 3.1 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) represents an interesting alternative technique to conventional solid–liquid extraction (*e.g.* Soxhlet extraction) with lower solvent consumption and lower working temperature. It is a form of liquid extraction where the usual liquid solvent phase has been replaced by a supercritical fluid—a substance that is above its critical point. Amongst a wide variety of supercritical fluids, carbon dioxide is essentially the only convenient supercritical extraction solvent used because of its comparatively low critical temperature (31.1 °C) and pressure (73.8 bar/7.38 MPa) (Fig. 1). None of the other

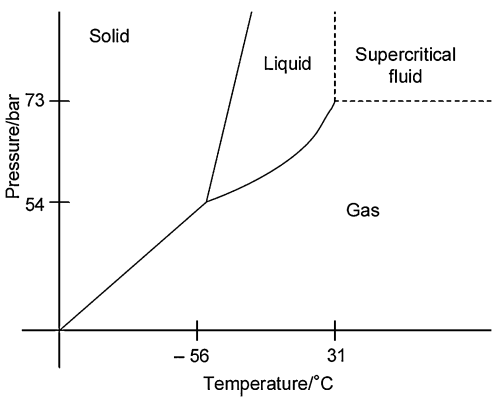
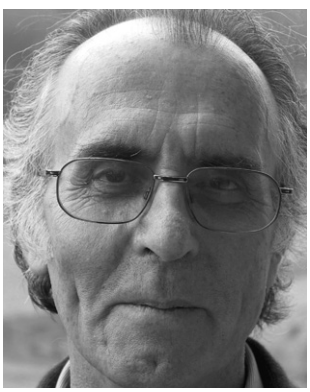



**Fig. 1** A supercritical fluid: the phase diagram of carbon dioxide.



**Otto Sticher**

*Otto Sticher studied pharmacy and received his PhD in natural sciences from the Swiss Federal Institute of Technology (ETH) in Zürich, Switzerland. He was Professor of Pharmacognosy and Phytochemistry at ETH from 1972 to 2002. Since Spring 2002 he has been Professor Emeritus. His research interests were the detection, isolation, structure elucidation and biological screening of natural products, the quality control of crude drugs and phytomedicines, the development of new technologies for isolation and separation of natural products, as well as ethnobotany and ethnopharmacology.*

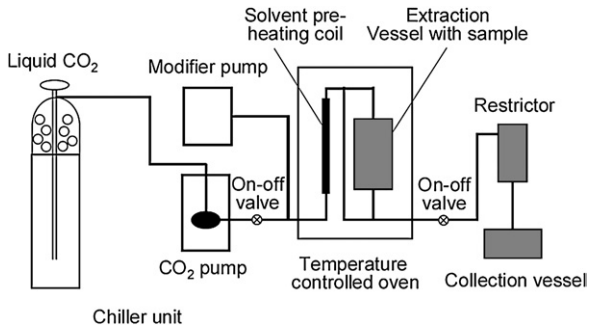



**Fig. 2** Schematic diagram of the basic components of an off-line SFE system.[12,17]

supercritical materials have shown sufficient advantages for general use in comparison with the ready availability, low cost, low toxicity (safety) and readily obtained critical conditions offered by $CO_2$. An organic solvent (also called modifier) may be added to the supercritical fluid to enhance its solvating properties. In case of $CO_2$, volatile polar solvents such as ethanol, methanol or acetonitrile are preferred. By using $CO_2$ as the supercritical fluid, extractions can be performed under mild conditions, thus reducing both the risks of thermal degradation and the poor collection efficiencies of volatile analytes. $CO_2$ is most effective for dissolving organic compounds, particularly molecules displaying some degree of lipophilicity, such as esters, ethers and lactones.[5,10,13,16]

SFE is conceptually simple to perform and does not require sophisticated instrumentation. A schematic diagram of the basic components of an SFE system is shown in Fig. 2. A pump is used to supply a known pressure of extraction fluid (*e.g.* liquid $CO_2$) to the extraction vessel held above the critical temperature of the fluid. The fluid flows through the sample matrix and exits *via* a backpressure regulator or restrictor into a collection device (off-line SFE) or in case of analytical SFE into another instrument such as a chromatograph (on-line SFE) where it depressurises and evaporates, allowing for collection of the extract or subsequent separation. Off-line SFE enables direct collection of extract analytes, while on-line SFE generally refers to the direct coupling of the SFE system to a chromatograph [gas chromatograph (GC), supercritical fluid chromatograph (SFC) or HPLC]. The modifier component may be introduced into the fluid either using a separate pump and suitable mixing device or may be added to the sample matrix in the extraction cell prior to pressuring with $CO_2$. Frequently, an off-line valve is incorporated between the pump and the extraction vessel and between the vessel and the restrictor. In this set-up static or dynamic extraction or a combination of the two may be performed. The restrictor maintains the pressure within the extraction vessel by flow control.[5,12]

Beginning in the 1970s, interest in extracting plant matrices with SFE has been prominent. Initially, supercritical $CO_2$ was used for the decaffeination of coffee and the large-scale isolation of compounds from hops and spices. More recently, there has been a trend towards the application of SFE as an analytical extraction method for sample preparation prior to chromatographic systems, such as SFC and GC. The interest in SFE can be charted in surveys of publications. Since 1982 there has been a rapid increase of SFE, with a peak in 1996/97, but a decline in publications in recent years. The use of SFE both at the analytical and processing scales is quite widespread in the food industry for the extraction of fats and oils from seeds, foodstuffs, and other materials. The technique has also been applied to the extraction of active compounds from medicinal plants, such as steroids, terpenes, alkaloids, various oxygen-containing heterocyclic compounds, as well as aromatic and phenolic compounds (see ref. 10–13,15,18).

**Table 1** Recent preparative-scale applications of SFE to natural product extraction

| Compounds | Matrix[a] | Ref.[b] |
|---|---|---|
| Catechins (EGCG, ECG) | *Cratoxylum prunifolium*, leaf | 19 |
| Methylxanthines | *Ilex paraguariensis*, leaf | 20 |
| Free fatty acids | Grape seed | 21 |
| Triterpenoid esters | *Calendula officinalis*, flower | 22 |
| Psoralen and isopsoralen | *Psoralea corylifolia*, seed | 23 |
| Microcystins | *Microcystis aeruginosa* | 24,25 |
| Aurentiamide acetate | *Patrinia villosa* | 26 |
| Coumarins | *Stellera chamaejasme* | 27 |
| Flavonoids | *Patrinia villosa* | 28 |
| Quinolizidine alkaloids | *Sophora flavescens*, root | 29 |
| Lignans, cinnamic acid | *Schisandra chinensis*, stem, leaf | 30 |

[a] Systematic plant name and/or crude drug or systematic name of cyanobacterium. [b] References: 2000–2007.

Recent preparative-scale applications of SFE to natural product extraction are listed in Table 1. Cao *et al.*[19] reported SFE of tea catechins including epigallocatechin-3-*O*-gallate (EGCG) and epicatechin-3-*O*-gallate (ECG) from *Cratoxylum prunifolium*. An analytical-scale SFE system was used for optimisation of the extraction parameters. Then the extraction was scaled-up 100-fold using a preparative-scale SFE system: 485 g of leaves were extracted statically and dynamically for 1 h each under optimised conditions at 40 °C and 25 MPa, with $CO_2$ containing 80% aqueous ethanol as modifier. A yield of 3.7 g of ethanol-soluble extract with 6.8% EGCG and 6.5% ECG was obtained. Fig. 3 shows the HPLC analysis of the ethanol-soluble SFE extract from preparative SFE extraction without any further treatment (A) and of the crude catechin mixture derived from SFE extract after cleaning-up by distributing it between chloroform and water and extraction of the water phase with ethyl acetate. Ling *et al.*[29] have also used SFE to extract quinolizidine alkaloids from *Sophora flavescens* roots. Under optimised SFE conditions determined by an orthogonal test design, the extraction was scaled-up 30-fold with a preparative system. A 165 g sample was extracted statically for 1 h followed by dynamic extraction for 3 h by flowing liquid $CO_2$ (75% ethanol and 25% water as modifier) at a rate of 2 l $min^{-1}$. The extract flowed directly into a collection vessel and stored in a refrigerator for subsequent HPLC analysis (Fig. 4). 12.9 g of SFE extract were obtained (6.65% matrine, 17.18% oxysophocarpine, 51.95% oxymatrine).

### 3.2 Pressurised liquid extraction (PLE)

A new extraction technique appeared about 10 years ago, variously called pressurised liquid extraction (PLE), accelerated solvent extraction (ASE™, a Dionex trade mark), pressurised

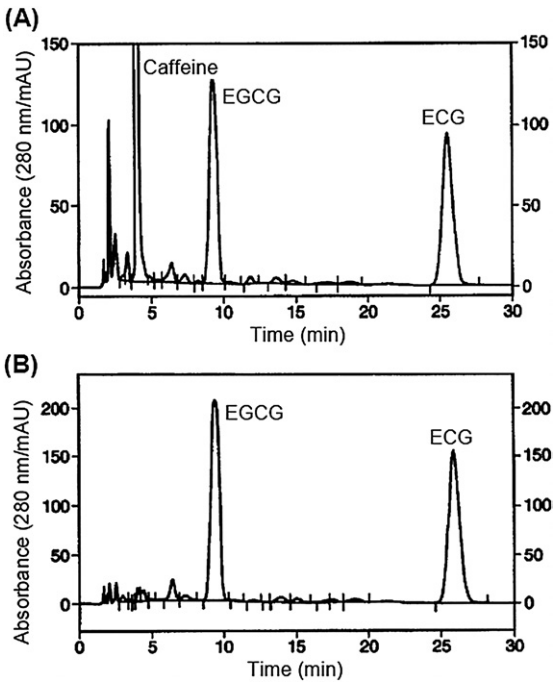



**Fig. 3** HPLC analysis of the SFE extract from *Cratoxylum prunifolium* leaves. (A) Sample of the ethanol-soluble part of the extract from preparative SFE extraction without any further treatment. (B) HPLC analysis of the crude catechin mixture derived from SFE extract after cleaning-up. EGCG = epigallocatechin-3-*O*-gallate, ECG = epicatechin-3-*O*-gallate. Reprinted from X.-L. Cao, Y. Tian, T.-Y. Zhang and Y. Ito, 'Supercritical fluid extraction of catechins from *Cratoxylum prunifolium* Dyer and subsequent purification by high-speed counter-current chromatography', *J. Chromatogr., A*, 2000, **898**, 75–81, Copyright (2000), with permission from Elsevier.

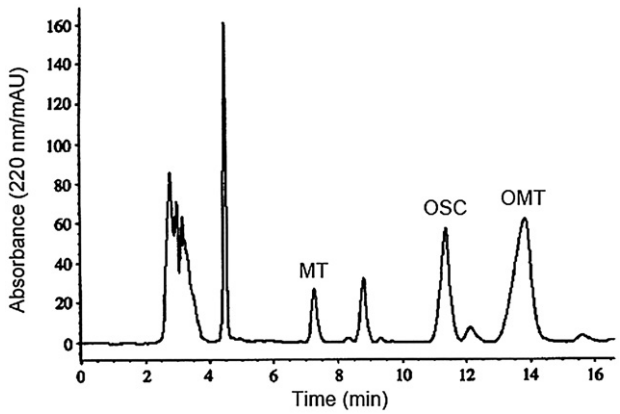



**Fig. 4** HPLC chromatogram of the SFE extract from *Sophora flavescens* roots. Sample: ethanol solution of preparative SFE extraction without any further treatment. MT (matrine), OSC (oxysophocarpine), OMT (oxymatrine). Reprinted from J. Y. Ling, G. Y. Zhang, Z. J. Cui and C. K. Zhang, 'Supercritical fluid extraction of quinolizidine alkaloids from *Sophora flavescens* Ait. and purification by high-speed counter-current chromatography', *J. Chromatogr., A*, 2007, **1145**, 123–127. Copyright (2007), with permission from Elsevier.

fluid extraction (PFE), pressurised solvent extraction (PSE) or enhanced solvent extraction (ESE), and is partly derived from SFE.[5,31] PLE is a solid–liquid extraction process using organic solvents at an elevated temperature (usually between 50 and

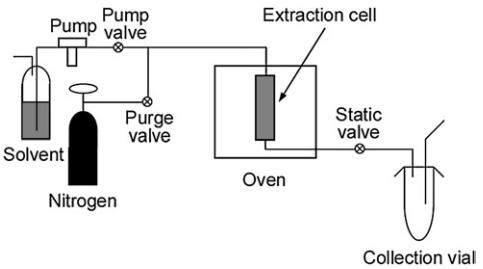



**Fig. 5** Schematic illustration of a PLE system.[31]

200 °C) and applying higher pressure (between 10–15 MPa) to extract samples in an extraction cell. Extractions are carried out under pressure in order to maintain the solvent in its liquid state, even at temperatures above boiling point. Moreover, pressure allows the extraction cell to be filled more quickly, and helps to force the solvent into the matrix pores. Thus, the efficiency of the extraction process is improved. Extraction at elevated temperatures increases solubility, diffusion rate, and mass transfer, coupled with the ability of the solvent to disrupt the analyte–matrix interactions. PLE thus allows fast extraction owing to increased solubility, better desorption and enhanced diffusion, and the extraction is generally completed within a few minutes. PLE was developed especially for laboratories with increased sample throughput. Comparison with conventional extraction methods has demonstrated faster extraction, higher extraction efficiency, and lower solvent consumption, along with comparable recoveries in most cases. No evidence was seen for thermal degradation during the extraction of temperature-sensitive compounds.[5,17,32,33] The scheme of a typical PLE unit is given in Fig. 5. There are two ways to perform PLE. The first is the static mode in which the extraction cell is filled with a solvent, followed by heating to generate pressure in the cell. After a period of time (5–10 min is usually sufficient), the system is rinsed with fresh solvent to ensure that all of the extract reaches the collection vials, and is purged with gas to avoid any losses or 'memory' effects. In the second method, the dynamic mode, fresh solvent is continuously percolated through the cartridge under pressure at a constant flow rate for a fixed period of time. The extraction cell is placed in a thermostatted oven. In both cases, under conditions of elevated pressure and temperature, the mass transfer rates are accelerated according to Fick's law of diffusion. The typical volume collected depends on the cell size. Volumes between 10 and 100 ml may be required, and hence repeated evaporation steps are needed to concentrate the final extracts.[5,34] Both commercially available and laboratory-assembled PLE systems are used.

A large number of applications have been reported for the extraction of environmental and food samples using PLE. However, in recent years reports in phytochemical applications have increased (see Table 2 for examples). The cited papers use PLE for identification and quantification of active constituents in natural products, mainly medicinal plants. In one case a preparative method for the isolation of paclitaxel from *Taxus cuspidata* is described.[49] Preparative PLE (Dionex ASE-200, 10.13 MPa, 140 °C, 15 min) with water was conducted, and the paclitaxel was isolated from the water extract by chromatographic methods. The final recovery of paclitaxel from bark

**Table 2** Recent applications of PLE to natural product extraction

| Compounds | Matrix[a] | Ref.[b] |
|---|---|---|
| Berberine, aristolochic acids I, II | Coptidis rhizoma, Aristolochiae radix | 35 |
| Iridoid glycosides | *Veronica longifolia*, leaf | 36 |
| Hydrastine, berberine | Goldenseal (*Hydrastis canadensis*) | 37 |
| Cocaine, benzoylecgonine | *Erythroxylum coca*, leaf | 32 |
| Withanolides | *Iochroma gesnerioides*, leaf | 34 |
| Artemisinin, artemisinic acid | *Artemisia annua*, leaf | 17 |
| Berberine, strychnine | Coptidis rhizoma, *Strychnos nux-vomica*, seed | 33 |
| Curcuminoids | *Curcuma longa*, root | 38 |
| Ginsenosides | American ginseng roots (*Panax quinquefolium*) | 39 |
| Isoflavones | Soybeans | 40 |
| Furanocoumarins | *Archangelica officinalis*, fruit | 41 |
| Furanocoumarins | *Pastinaca sativa*, fruit | 42 |
| Hypericin, hyperforin | St. John's wort (*Hypericum perforatum*) | 43 |
| Flavonoids | American skullcap (*Scutellaria lateriflora*) | 44 |
| Terpenoids, sterols | Tobacco (*Nicotiana tabaccum*) | 45 |
| Capsaicinoids | Cayenne pepper | 46 |
| Hypericins, flavonoids, hyperforin | St. John's wort (*Hypericum perforatum*) | 47 |
| Flavonoids | *Lysimachia clethroides* | 48 |

[a] Systematic plant name and/or crude drug. [b] References: 2000–2007.

was 0.022%, and the isolated material had NMR spectroscopic data that matched those of an authentic sample. HPLC analysis of the water extract produced by PLE proved the high extractivity of paclitaxel using this procedure (0.082% w/w recovery from the bark). Extraction with water can be useful in the treatment of effluent and for the reduction of solvent costs. These results using PLE expand the potential for industrial application of direct extraction of paclitaxel from *Taxus* species.

### 3.3 Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is a recent technology for extracting soluble products into a fluid from a wide range of materials using microwave energy. It provides a technique which allows one to extract compounds more selectively and more rapidly (usually in less than 30 min) with similar or better recovery than traditional extraction processes. Microwaves directly heat the solvent or solvent mixture, thus accelerating the speed of heating. Besides the advantage of high extraction speed, MAE also enables a significant reduction in the consumption of organic solvent.

The application of microwave energy to the samples may be performed using two technologies: either closed vessels under controlled pressure and temperature, or open vessels at atmospheric pressure. The two technologies are commonly named pressurised MAE (PMAE), with a multi-mode cavity, or focused MAE (FMAE) using the waveguide as a single-mode cavity, respectively. Both systems are shown in Fig. 6. Whereas in open vessels the temperature is limited by the boiling point of the solvent at atmospheric pressure, in closed vessels the solvent can be heated above its boiling point at atmospheric pressure by simply applying suitable pressure, thus enhancing both extraction speed and efficiency. However, after extraction with closed

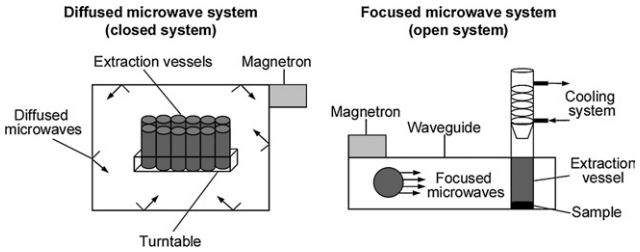



**Fig. 6** Scheme of the two microwave systems, using diffused or focused microwaves.[5]

vessels, one needs to wait for the temperature to decrease before opening the vessel, thereby increasing the overall extraction time (by approximately 20 min). Open systems use focused microwaves, resulting in homogenous and very efficient heating of the sample. In closed systems using diffuse microwaves, the electric field in the cavity is non-homogenous, and therefore the vessels are placed on a turntable. Recently, the respective advantages of high-pressure vessels and focused microwave heating have led to the development of systems that combine both approaches. These so-called "focused high-pressure, high-temperature microwave systems" comprise an integrated closed vessel and a focused microwave-heated system operating at very high pressure and temperature. The closed vessel MAE system is quite similar to the PLE technology, as the solvent is heated and pressurised in both systems. The main difference is in the means of heating, either by microwave energy or by conventional oven heating. Consequently, as for PLE, the number of influential parameters is reduced, thus making the application of this technique quite simple to use.[5,6,50,51]

Microwaves are a non-ionising electromagnetic radiation with a frequency from 300 to 300 000 MHz. In order to avoid interference with radio communications, domestic and commercial systems generally operate at 2450 MHz. Even though the use of microwave energy as a source of heat has been used in analytical laboratories since the late 1970s, their application to enhance extraction is very recent. Extensive use began around 10 years ago, with the commercialisation of several extraction instruments.[5,7] Due to the particular effects of microwaves on matter (namely dipole rotation and ionic conductance), heating with microwaves is instantaneous and occurs in the heart of the sample, leading to very fast extractions. The results obtained so far have concluded that microwave radiation causes no degradation of the extracted compounds, unless the temperature in the vessel rises too high. At the same time, a specific effect of microwaves on plant material has been found. They interact selectively with the free water molecules present in the solid matrix, leading to rapid heating and temperature increase, resulting in rupture of the plant tissue and release of solutes into the solvent.

Among others, MAE has mainly been successfully used for environmental applications, but in recent years also in the phytochemical field (see Table 3 for examples). In the first applications of MAE, domestic microwave ovens were used. However, as the application of microwave energy to flammable organic solvents can present serious hazards, it is strongly recommended to use commercial equipment approved for MAE only.

MAE (especially PMAE) is used in analytical protocols and very often to investigate extraction parameters including

**Table 3** Recent applications of MAE to natural product extraction

| Compounds | Matrix[a] | Ref.[b] |
|---|---|---|
| Glycyrrhizic acid | Licorice (*Glycyrrhiza glabra*), root | 52 |
| Tanshinones | *Salvia miltiorrhiza*, root | 53 |
| Withanolides | *Iochroma gesnerioides*, leaf | 54 |
| Cocaine, benzoylecgonine | *Erythroxylum coca*, leaf | 55 |
| Tanshinones | *Salvia miltiorrhiza*, root | 56 |
| Ginsenosides | Ginseng (*Panax ginseng*), root | 57 |
| Ginsenosides | Ginseng (*Panax ginseng*), root | 58 |
| Saponins, isofraxidin | *Acanthopanax senticosus*, root ('*ciwuja*' in TCM) | 59 |
| Polyphenols, caffeine | *Camellia sinensis*, leaf | 60 |
| Ginsenosides | *Panax quinquefolium*, root | 61 |
| Polyphenolic acids | *Eucommia ulmoides*, bark | 62 |
| Furanocoumarins | *Archangelica officinalis*, fruit | 41 |
| Piperin, hesperidin, trimyristin, betulinic acid | Black and white pepper (*Piper* spp.), orange peel (*Citrus* spp.), nutmeg (*Myristica fragrans*), plane bark (*Platanus acerifolia*) | 9 |
| Saponins | Chickpea seeds (*Cicer arietinum*) | 8 |
| Camptothecin | *Nothapodytes foetida* | 63 |
| Sanguinarine and chelerythrine | *Macleaya cordata*, fruit | 64 |
| Curcumol, curdione, germacrone | Curcumae rhizoma from *Curcuma wenyujin*, *C. phaenocaulis*, *C. kwangsiensis* | 65 |
| Camphor and borneol | *Chrysanthemum indicum*, flower | 66 |
| Paeonol | *Cynanchum paniculatum, Paeonia suffruticosa*, root | 67 |
| Solanesol | Tobacco | 68 |
| Oleuropein and related biophenols | *Olea europaea*, leaf | 69 |
| Ferulic acid | *Angelica sinensis*, root | 70 |
| Volatile constituents | Ginger (*Zingiber officinale*), rhizome | 71 |
| Secoisolariciresinol diglucoside | *Linum usitatissimum*, seed | 72 |
| Phenolic compounds | *Linum usitatissimum*, seed | 73 |
| Emblin | *Emblia ribes*, fruit | 74 |
| Anthraquinones | *Morinda citrifolia*, root | 75 |
| Triterpenoid saponins | *Ganoderma atrum* | 76 |

[a] Systematic plant name and/or crude drug, systematic name of mushroom. [b] References: 2000–2007. For earlier reports see Kaufmann and Christen.[7]

pressure and temperature, extraction time, microwave power, solvent nature and volume, or in comparative studies of this and other recent techniques (such as SFE or PLE) with classical extraction methods for particular applications. The compared performances regarding extraction efficiency and susceptibility to matrix effects, selectivity, time and solvent reduction, level of automation and simplicity of the operating procedures are discussed in the literature.[5,50] MAE may also be used in isolation protocols. Recently, MAE was used for the isolation of various natural products such as saponins from chickpea,[8] piperin from pepper, hesperidin from orange peel, trimyristin from nutmeg, and betulinic acid from plane bark.[9]

Nüchter *et al.*[9] describe a detailed protocol using MAE for the isolation of betulinic acid from plane bark (*Platanus acerifolia*). They used the microwave oven ETHOS 1600 (MLS, Germany) with a multi-mode cavity (42 l) and reactor type HEF 270 (segment rotor, 3 segments) with an automatic steering device (5 min, 500 W, 100 °C then 25 min, 400 W, 100 °C). Additional parameters are pressure: 5 bar/0.5 MPa; power: 197 W; solvent: MtBE (methyl *tert*-butyl ether, 80 ml in each of the rotor segments); sample: 30 g powdered bark (10 g in each rotor segment). The yield of betulinic acid depends on the quality of the bark powder. A yield of 3.4–3.7% raw material was obtained (1.0–1.4% pure betulinic acid after crystallisation). The same authors constructed a pilot reactor filtEX for the MAE of 150 g plant material using the same ETHOS 1600 microwave system. The filtEX reactor allows the use of a maximum of 450 ml solvent for extraction. Using this pilot reactor 60 g orange peel and 90 g plane bark were subjected to MAE. The yields obtained are shown in Table 4. The comparison of MAE with Soxhlet extraction for the isolation of natural products shows clear advantages of MAE, namely reduction of extraction time and solvent consumption as well a higher yield of pure compounds;[9] see also www.oc-praktikum.de.

**Table 4** Yields of hesperidin and betulinic acid using HEF 270 and filtEx reactor systems[9]

| | HEF 270 | filtEx |
|---|---|---|
| Hesperidin from orange peel | 10 g peel = 3.8% after second extraction | 60 g peel = 2.4% after second extraction |
| Betulinic acid from plane bark | 30 g bark = 3.6% raw material (1.2–1.4% pure betulinic acid) | 90 g bark = 2.5% raw material (1.0% pure betulinic acid) |

### 3.4 Brief comparison of SFE, PLE and MAE with Soxhlet extraction

A brief summary of the experimental conditions for SFE, PLE and MAE methods compared with Soxhlet extraction can be found in Table 5. The application of supercritical fluids such as carbon dioxide, or organic solvents at elevated temperature and pressure, improves the speed of the extraction process drastically from 6–24 h (Soxhlet) to 10–45 min (SFE), and to less than 30 min for PLE and MAE. The extraction process is up to 60 times more efficient in terms of operation time and

**Table 5** A brief summary of the experimental conditions for recent extraction techniques (SFE, PLE, MAE) for natural products compared with Soxhlet extraction[5,77]

| Technique | Common solvents used | Solvent volume/ml | Sample size/g | Cells[a] | Temperature/°C | Pressure applied | Time required | Investment |
|---|---|---|---|---|---|---|---|---|
| Soxhlet | MeOH, EtOH, or mixture of alcohol and water | 100–500 | 1–50 | 1 | Depends on solvent used | Atmospheric | 6–24 h | Very low |
| SFE | $CO_2$ or $CO_2$ with modifiers such as EtOH, MeOH | 2–5 (solid trap); 15–60 (liquid trap) | 1–5 | 1–2 | Depends on supercritical fluid used | 25–45 MPa | 10–45 min | High |
| PLE | MeOH | 10–100 | 1–30 | 1 | 80–200 | 1–10 MPa | 10–30 min | High |
| MAE | MeOH, EtOH, or mixture of EtOH and water | 10–50 (PMAE); 30–70 (FMAE) | 1–20 (PMAE); 1–10 (FMAE) | 6–50 (PMAE); 1–3 (FMAE) | 80–150 | Variable (PMAE); Atmospheric (FMAE) | 10–30 min | Moderate (PMAE); Low (FMAE) |

[a] Number of cells that can be simultaneously extracted using the same apparatus.

generally completed within a few minutes. These recent methods reduce the large amount of organic solvents or even make them unnecessary. The recovery of analytes is comparable to conventional extraction methods. In contrast to SFE, PLE and MAE seem particularly promising for the extraction of components with medium to high polarity. Non-polar to moderately polar compounds can be extracted using SFE with supercritical $CO_2$, while modified $CO_2$ is necessary for extraction of more polar compounds. SFE using supercritical $CO_2$ allows the extraction of thermolabile compounds at low temperature in an oxygen-free environment while PLE and MAE are less (or not) suitable for heat-sensitive compounds. However, SFE or PLE requires greater financial investment compared to the moderate or even low cost of Soxhlet extraction or MAE.

## 4 Liquid–solid isolation techniques

Most separations in liquid chromatography are performed with liquid–solid isolation techniques such as various forms of planar chromatography and column chromatography. The diversity of these methods is tremendous, and many newer books are available describing the basics of the techniques as well as their application in natural product isolation (see, for example, ref. 3,78–83). The present review only discusses in detail examples of classical preparative thin-layer (planar) chromatography and of preparative liquid chromatography under pressure such as flash chromatography, low-pressure LC (LPLC), medium pressure LC (MPLC) and high-pressure LC (HPLC).

### 4.1 Preparative planar chromatography

Several planar chromatographic (PC) techniques are applicable to natural product isolation. Some of these involve mobile phase migration through a stationary phase by capillary forces [preparative thin-layer chromatography (PTLC)], and some are forced-flow methods (FFPC), such as rotation planar chromatography (RPC)/centrifugally accelerated thin-layer chromatography (CTLC) and overpressure layer chromatography (OPLC). In order to overcome some of the drawbacks of classical PTLC, such as the removal of purified substances from the plate and their subsequent extraction from the sorbent, the length of time required for separation, and the presence of impurities and residues deriving from the plate itself, RPC (in its various forms) and OPLC were developed in the last decades of the 20th century and frequently used. For reviews of these methods and examples of separation see ref. 1, 3 and 84.

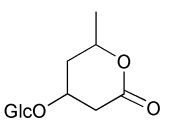
The application of preparative FFPC methods has declined in recent years, while classical PTLC is still used frequently. The equipment for classical PTLC is most basic, requiring minimal outlay. Although gram quantities of material can be separated by PTLC, most applications involve milligram quantities. PTLC, in conjunction with open-column chromatography, is still found in many publications on the isolation of natural products. Various studies have investigated the most important parameters regarding method development in thin-layer (planar) chromatography such as selection of stationary phase, vapour phase, suitable solvents, mode of development as well as mobile phase optimisation and mobile phase transfer (see, for example, ref. 84). Silica gel is the most common adsorbent and is employed for the separation of both lipophilic and hydrophilic substance mixtures. The most frequently employed thicknesses of the chromatography plates are 0.5–2 mm; the format is generally 20 × 20 or 20 × 40 cm. Limitations to the thickness of the layer and the size of the plates naturally reduce the amount of plant material that can be separated by PTLC. A maximum sample load for a 1.0 mm silica layer is about 5 mg $cm^{-2}$. PTLC plates may either be self-made or purchased with the adsorbents already applied. Preparing plates oneself makes the accommodation of any thickness (up to 5 mm) or any composition of plates possible. Thus, silver nitrate, buffers *etc.* can be incorporated into the adsorbent. The band in which the sample is applied must be as narrow as possible, as the resolution depends on the width of the band. As a general guideline, 10–100 mg of sample can be separated on a 1 mm thick 20 × 20 cm silica gel or aluminium oxide layer.[3] Choice of eluent is determined by preliminary analytical TLC investigation. Mobile-phase optimisation can be done using the "PRISMA" model based on Snyder's solvent selectivity triangle, which was developed by Nyiredy in the 1980s.[84–86] Frequently applied binary mobile phases in varying proportions are: *n*-hexane–ethyl acetate, *n*-hexane–acetone, and chloroform–methanol. Addition of an acid or diethylamine in small amounts is useful for the separation of acidic and basic compounds, respectively. The bands, having been localised after elution in glass tanks, are scraped off the plate with a spatula. To remove

binders and fluorescent indicators, which may be extracted together with the scraped off compounds, a final purification step by gel filtration on Sephadex LH-20 is highly recommended.[3]

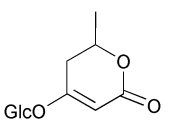
Recent applications of PTLC to natural product isolation are shown in Table 6.

PTLC is used mainly in conjunction with other preparative-scale separation methods (see Table 6, column 4). Frequently, PTLC is merely part of a multi-step procedure. Repetitive PTLC separations and the use of RP- and NP-adsorbents are state-of-the-art for the isolation of pure compounds. Open columns filled with aluminium oxide, Amberlite XAD resins and other adsorbents are used for pre-purification. Further purification is mainly carried out by chromatography on a Sephadex LH-20 column.

Steam distillation of *Nepeta cataria* (catnip) afforded an essential oil containing *Z*,*E*- and *E*,*Z*-nepetalactone in a 6 : 1 ratio. Together these isomers comprised 98% of the steam distillate. PTLC plates with a solvent system of hexane–ethyl acetate (19 : 1, v/v) were used for separation. The plates were run several times and allowed to dry completely between runs. The products were visualized under UV (254 nm), and the silica gel was scraped off the plates and washed three times with diethyl ether. The ether was removed by rotary evaporation and the purity of the isomers was assessed by HPLC.[91] Polar and non-polar TLC adsorbents, binary mobile phases, and a combination of RP- and NP-plates were used for the separation and isolation of the coumarins from *Peucedanum tauricum* leaves. The first step of the separation of coumarins from the methanolic extract was performed on silanised silica gel 60 RP-2. This procedure allowed the separation of coumarin-containing fractions from ballast compounds (especially chlorophylls). Separated bands were observed under UV light at 366 nm and monitored by densitometric scanning. Bands were then scraped from the plates and extracted with methanol–acetone (1 : 1, v/v) in an ultrasonic bath. Repeated chromatography of coumarin-containing fractions by NP-TLC with more selective mixtures of dichloromethane–acetonitrile (99 : 1; 97.5 : 2.5, v/v) enabled the isolation of crystalline bergapten and scopoletin.[109] Application of RPC and MPLC methods for the direct isolation of parasorboside (**1**) and gerberin (**2**), two structurally closely related 2-pyrone glucosides, did not lead to a satisfactory resolution, thus necessitating the use of PTLC as a purification method. RPC and MPLC enabled complete elimination of chlorophylls and other non-polar compounds from the extract from the floral stems and leaves of *Gerbera hybrida*. However, the two glucosides were not completely separated. Therefore, fractions containing either **1** or **2**, or a mixture of the two, were combined and the resulting mixture subjected to further purification and subsequent isolation of the pure compounds by silica gel PTLC using *n*-hexane–ethyl acetate–methanol–tetrahydrofuran–formic acid (3 : 9 : 8 : 80 : 1, v/v) as eluent.[95]



Parasorboside (**1**)



Gerberin (**2**)

### 4.2 Vacuum liquid chromatography (VLC)

VLC may be considered as a preparative TLC run as a column, with a vacuum applied to speed up eluent flow-rates. As opposed to flash chromatography, the column is allowed to run dry after each fraction is collected. This is similar to preparative TLC where plates can be dried after a run and then re-eluted. In the last decade VLC has been increasingly used in the field of natural products because of its simplicity of operation. Separations of up to 30 g of extract are possible. Different chromatographic supports have been employed in VLC: silica gel (both normal- and reversed-phase), $Al_2O_3$, CN, diol and polyamide.[3] The most popular eluent is hexane with increasing proportions of ethyl acetate. VLC is mainly used for the fractionation of natural products prior to other separation steps such as RPC, MPLC, and HPLC (for recent applications see Table 7).

In some cases VLC is used as the only separation step. An example is a study by Villaseñor and Domingo.[124] Repeated VLC was used for the isolation of spinasterol, an antimutagen from squash flowers (*Cucurbita maxima*). The chloroform extract obtained after partition of the methanol extract between hexane and water and extraction of the aqueous layer with chloroform was subjected to VLC using silica gel 60 G from Merck. A 60 mm column was dry-packed by using suction to make a 4–5 cm bed onto which the sample powder was added. The column was eluted with hexane, 2–6% ethyl acetate in hexane in 2% gradient ratios, 10–50% ethyl acetate in hexane in 5% gradient ratios, 60% ethyl acetate in hexane–ethyl acetate in 10% gradient ratios, 50% ethanol in ethyl acetate, and ethanol in numbered 250 ml Erlenmeyer flasks. Crystalline spinasterol (94% purity) was obtained after additional VLC of fraction FwB2 and repeated recrystallisation from dichloromethane and methanol. Similarly, García-Argáez *et al.*[132] isolated zapotin (5,6,2′,6′-tetramethoxyflavone), 5,6,2′,3′,4′-pentamethoxyflavone, casimiroin (alkaloid) and zapoterin (limonoid) by means of VLC using silica gel and a step gradient of hexane in ethyl acetate. Landreau *et al.*[127] isolated four marine mycotoxines (peptaibols; peptides) produced by the fungus *Trichoderma koningii* in two steps (VLC and analytical HPLC), whereas peptaibols are usually obtained after three purification steps. Kalász *et al.*[137] isolated ecdysteroids from the roots of *Serratula wolffii* with a combination of VLC and RPC. Earlier isolation methods consisted of several chromatographic steps, with both column and planar techniques and DCCC, while preparative TLC and/or HPLC were used for final purification. The effective clean-up and optimised combination of RP-VLC and repeated preparative RPC on silica gel resulted in pure, biologically active ecdysteroids.

### 4.3 Preparative pressure liquid chromatography (PPLC)

The PPLC methods discussed in this section are: flash chromatography (*ca.* 2 bar/30 psi/0.2 MPa); low-pressure LC (*ca.* 5 bar/75 psi/0.5 MPa); medium-pressure LC (*ca.* 5–20 bar/75–300 psi/0.2–2.0 MPa); and high-pressure LC (>20 bar/300 psi/2.0 MPa).

There is a considerable overlap between low-pressure, medium-pressure and high-pressure LC, and they are treated as three classes for convenience only. For the basic principles of PPLC as well as for details of the columns, stationary phases,

**Table 6** Recent applications of PTLC to natural product isolation

| Compounds | Matrix[a] | Sorbent [b] (thickness/mm) | Eluent (volume ratio)[c] | Other chromatographic methods used[d] | Ref.[e] |
|---|---|---|---|---|---|
| Methoxylated flavones | *Primula veris*, flower | Si gel (1) | Hex–EtOAc (70 : 30) | CC ($Al_2O_3$), MPLC, prep. RP-HPLC | 87 |
| Polypeptide antibiotics | *Stilbella flavipes* CBS 146.81 | Si gel (2) | $CHCl_3$–MeOH (75 : 25) | CC (Amberlite XAD-2, Sephadex LH-20) | 88 |
| Antibacterial compounds | *Carpobrotus edulis*, leaf | Si gel (0.5) | EtOAc–MeOH–water (100 : 13.5 : 10) | CC (Sephadex LH-20) | 89 |
| Bisphenol derivatives | *Aspergillus niger* | Si gel | $C_6H_6$–EtOAc (9 : 1) | CC (Si gel) | 90 |
| Nepetalactone isomers | *Nepeta cataria*, aerial part | Si gel (1) | Hex–$Et_2O$ (19 : 1) | — | 91 |
| Quassinoids | *Quassia africana*, root bark | Si gel (1) | $Et_2O$–MeOH (9 : 1) | CC (Si gel) | 92 |
| Palmitic acid | *Pentanisia prunelloides*, leaf, root | Si gel (0.25) | Hex–EtOAc (3 : 1) | VLC | 93 |
| Taxoids | *Taxus baccata*, twigs | Si gel (0.5) | $CH_2Cl_2$–dioxane–$Me_2O$–MeOH (84 : 10 : 5 : 1) | SPE (Si gel), CC (Si gel) | 94 |
| 2-Pyrone derivatives | *Gerbera hybrida*, floral stem, leaf | Si gel | Hex–EtOAc–MeOH–THF–HCOOH (3 : 9 : 8 : 80 : 1) | RPC, MPLC | 95 |
| Kava lactones | *Piper methysticum*, root | Si gel + Si gel $C_{18}$ (0.25, 0.5) | Various solvent mixtures | MPLC, prep. RP-HPLC | 96 |
| Epoxybergamottin | Grapefruit (*Citrus paradisi*), peel | Si gel (2) | Hex–$Et_2O$–$CHCl_3$ (2 : 1 : 1) | FC, RPC | 97 |
| Lipopeptide antibiotics | *Bacillus subtilis* strain KS03 | Si gel | $CHCl_3$–MeOH–water (65 : 25 : 4) | CC (DEAE Sepharose CL-6B) | 98 |
| Harmalin | *Peganum harmala*, seed | Si gel | $CHCl_3$–MeOH–$NH_3$ (50 : 50 : 3) | CC (Si gel) | 99 |
| Coumarins | *Angelica sylvestris*, seed | Si gel | 20% EtOAc in Hex | VLC | 100 |
| Ecdysteroids | *Silene italica* ssp. *nemoralis*, aerial part | Si gel | EtOAc–EtOH (96%)–water (16 : 2 : 1) | SPE ($Al_2O_3$), DCCC, LPLC, prep. NP- + RP-HPLC | 101 |
| Umbelliprenin | *Ferula persica* var. *persica*, root | Si gel | PE–EtOAc (2 : 1) | — | 102 |
| Lolitrem B | Endophyte-infected ryegrass (*Lolium perenne*) | Si gel | $CH_2Cl_2$–$CH_3CN$ (90 : 10) | CPC, LPLC | 103 |
| Persicasulfides A, B | *Ferula persica* var. *persica*, root | Si gel | PE–EtOAc (2 : 1) | — | 104 |
| Polysaccharides | *Citrus grandis*, fruit flavedo | Si gel | $CH_2Cl_2$–$Me_2CO$–MeOH–water (5 : 3 : 3 : 0.5), BuOH–AcOH–MeOH–water (2 : 1 : 0.5 : 1) | CC (Si gel, Sephadex LH-20) | 105 |
| Various compounds | *Polyalthia longifolia* var. *pendula*, root bark | Si gel | $CHCl_3$–MeOH (9.5 : 0.5) | FC, VLC | 106 |
| Alkaloids | *Boophane disticha*, leaf | Si gel | EtOAc–MeOH–water (90 : 20 : 10) | VLC | 107 |
| Coumarins | *Peucedanum verticillare*, fruit, root | Si gel (0.5) | Hept–$CH_2Cl_2$–EtOAc (40 : 50 : 10), Hept–$CH_2Cl_2$–EtOAc (30 : 40 : 30), Hept–diisopropyl ether–iPrOH (80 : 20 : 12.5) | CC (Si gel + $Mg^{2+}$-Si gel), RP-HPLC | 108 |
| Coumarins | *Peucedanum tauricum*, leaf | Si gel (0.5) | MeOH–water (40 : 60), $CH_2Cl_2$–$CH_3CN$ (99 : 1), (97.5 : 2.5) | SPE (Si gel $C_{18}$) | 109 |
| Phenolic compounds | *Quercus aucheri*, leaf | Si gel | $C_6H_6$–$Me_2CO$ (8 : 2) | CC (Sephadex LH-20) | 110 |
| Lignans | *Styrax camporum*, stem | Si gel | $CHCl_3$–MeOH (92 : 8) | CC (Si gel, Sephadex LH-20), FC | 111 |
| Verrucarin A | *Myrothecium verrucaria* | Si gel | Hex–$CH_2Cl_2$–propan-2-ol (8 : 4 : 1) | FC, RP-HPLC | 112 |
| Various compounds | *Rubia cordifolia*, root | Si gel (0.25) | $CH_2Cl_2$–MeOH (10 : 0.1) | CC (Si gel), RP-HPLC | 113 |
| Various compounds | *Citrus grandis*, fruit albedo | Si gel | Appropriate solvents for each sample (see ref.) | CC (Si gel, Sephadex LH-20) | 114 |
| Phenanthrenes | *Tamus communis* | Si gel | $C_6H_6$–$Et_2O$–PE (2 : 1 : 1), $CHCl_3$–$Me_2CO$ (19 : 1) | VLC, CC (Sephadex LH-20), RP-HPLC | 115 |
| Aristolactams | *Piper marginatum*, leaf | Si gel (1) | $CHCl_3$–MeOH (99 : 1), (98 : 2) | CC (Si gel) | 116 |
| Flavonoids | *Cistus laurifolius*, leaf | Si gel | $CHCl_3$–MeOH (9 : 1) | CC (Si gel, Sephadex LH-20) | 117 |
| Diterpenes | *Hyptis suaveolens*, leaf | Si gel | Hex–$Et_2O$–MeOH (2 : 7 : 1), $Et_2O$–$CH_2Cl_2$–toluene (3 : 6 : 1) | CC (Si gel) | 118 |
| Pachypodol (flavonoid) | *Croton ciliatoglanduliferus*, leaf | Si gel | Hex–EtOAc (80 : 20) | CC (Si gel) | 119 |

**Table 6** *(Contd.)*

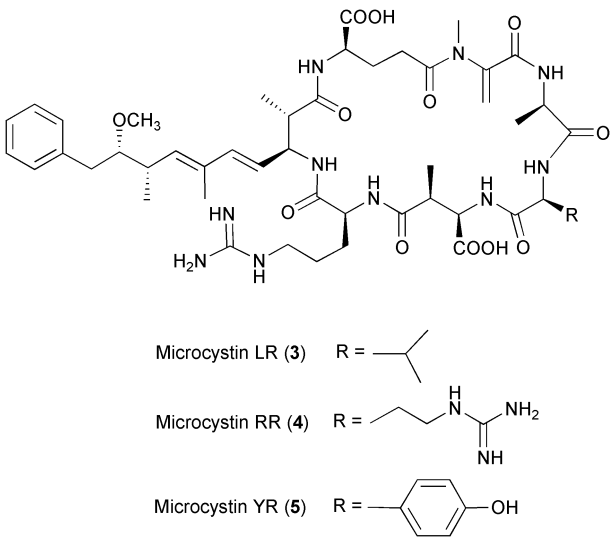
| Compounds | Matrix[a] | Sorbent [b] (thickness/mm) | Eluent (volume ratio)[c] | Other chromatographic methods used[d] | Ref.[e] |
|---|---|---|---|---|---|
| Phenolic compounds | *Prunus mume*, fruit | Si gel | Hex–$CHCl_3$–MeOH (6 : 2 : 1), Hex–$CHCl_3$–EtOAc–MeOH (6 : 2 : 1 : 1) | CC (Si gel $C_{18}$), RP-HPLC | 120 |
| Phenylpropanoids | *Pimpinella aurea*, aerial part | Si gel | Hex–EtOAc–AcOH (40 : 60 : 0.5) | VLC | 121 |
| Chemopreventive agents | Green onion (*Allium cepa*) | Si gel (1) | 30% EtOAc in toluene | Prep. RP-HPLC | 122 |
| Ecdysteroids | *Serratula wolffii*, aerial part | Si gel | Various solvents | CC (Si gel, polyamide, Sephadex LH-20, $Al_2O_3$), VLC, NP-HPLC | 123 |

[a] Systematic plant name and crude drug or systematic name of bacterium, mould or fungus. [b] Si gel: silica gel. [c] AcOH: acetic acid; $Me_2CO$: acetone; $CH_3CN$: acetonitrile; $NH_3$: ammonia; $C_6H_6$: benzene; BuOH: 1-butanol; $CHCl_3$: chloroform; $Et_2O$: diethyl ether; EtOAc: ethyl acetate; HCOOH: formic acid; Hept: *n*-heptane; Hex: *n*-hexane; iPrOH: isopropanol; MeOH: methanol; $CH_2Cl_2$: methylene chloride; PE: petroleum ether; THF: tetrahydrofuran. [d] $Al_2O_3$: aluminium oxide; CPC: centrifugal partition chromatography; CC: column chromatography; DEAE: diethylaminoethyl; DCCC: droplet counter-current chromatography; FC: flash chromatography; LPLC: low-pressure liquid chromatography; MPLC: medium-pressure liquid chromatography; RPC: rotation planar chromatography; VLC: vacuum liquid chromatography; SPE: solid-phase extraction. [e] References: 2000–2007.

column packing methods, mobile phases, sample introduction, collection of separated materials and other technical features, see ref. 3.

**4.3.1 Flash chromatography (FC).** The concept of FC is exceptionally simple. This modification of conventional column chromatography (CC) is very easy to employ for preparative separations, using readily available and cheap laboratory glassware. Therefore, FC is very popular among researchers who are confronted with straightforward separation problems. The performance of FC is lower than that of MPLC systems (which have a similar loading capacity). But considerations of simplicity and costs often dominate and make it a method of choice in many cases. The principle of FC is that the eluent is rapidly pushed through a short glass column with large inner diameter under gas pressure (usually nitrogen or compressed air). The glass column is packed with an adsorbent of defined particle size. The most widely used stationary phases are silica gel 35–70 μm or 40–63 μm, but obviously other particle sizes can be used as well. Particles smaller than 25 μm should only be used with very low viscosity mobile phases, as otherwise the flow-rate would be very low. FC is occasionally used for final purification of natural products on silica gel. More frequently, however, crude extracts of mixtures are pre-purified by FC before applying other techniques with greater resolution. In other words, FC provides a rapid preliminary fractionation of complex mixtures. FC has become a frequent, routine technique and thus, except for the eluent, details such as column dimensions, granulometry of the sorbent and flow-rates are rarely mentioned in the experimental part of published papers.[3] Recent applications of FC are shown in Table 8.

Pyo and Lee[24] reported a rapid and efficient method for extraction and isolation of microcystin LR (**3**) from the cyanobacterium *Microcystis aeruginosa*. The method involves supercritical fluid extraction (SFE; see Section 3.1) and silica gel FC for the purification of the compound. The unique feature of this method is that it uses only one-step SFE and one-step FC instead of multiple extractions with organic solvents and multistep column chromatography. The crude extract obtained by SFE was applied to a $C_{18}$ cartridge. The cartridge, which contained microcystins, was rinsed with 14 ml of a mixture of methanol and 0.005 M phosphate buffer solution (pH 2.4), followed by 20 ml of water. Microcystins were finally eluted from the $C_{18}$ cartridge with 30 ml of methanol. The eluate was evaporated and the residue was dissolved in 2 ml of methanol. The solution was then applied to FC. A silica gel column was used with a mobile phase of EtOAc–iPrOH–water (30 : 45 : 25, v/v) and a flow-rate of 2 ml $min^{-1}$. Two fractions contained **3** purified by semipreparative HPLC (Fig. 7). The same procedure but without the need for a further HPLC step was applied for the isolation of microcystins RR (**4**) and YR (**5**).[25]




**4.3.2 Low-pressure LC (LPLC).** In LPLC, a mobile phase is allowed to flow through a densely packed sorbent. The separation mechanism is adsorption or size exclusion depending on the choice of packing material for the stationary phase (adsorption: silica gel, bonded-phase silica gel, alumina, polystyrene; size-exclusion: polyacrylamide, carbohydrates). Silica gel is the most commonly used stationary phase in LPLC for the separation of natural products. Silica gel may be regarded as a typical

**Table 7** Recent applications of VLC to natural product isolation

| Compounds | Matrix[a] | Sorbent[b] | Eluent (volume ratio)[c] | Other chromatographic methods used[d] | Ref.[e] |
|---|---|---|---|---|---|
| Spinasterol | *Cucurbita maxima*, flower | Si gel | Hex, 2–6% EtOAc in Hex in 2% gradient ratios, 10–50% EtOAc in Hex in 5% gradient ratios, 60% EtOAc in Hex–EtOAc in 10% gradient ratios, 50% EtOH in EtOAc, and EtOH | — | 124 |
| Methoxyflavones | *Psiadia dentata*, leaf | Si gel | Step gradient of EtOAc in $CH_2Cl_2$ | LC (Si gel) | 125 |
| Antimutagen | *Mentha cordifolia*, leaf | Si gel | Hex, 5% gradient ratios of EtOAc in Hex, EtOAc, and 5% gradient ratios of EtOH in EtOAc; 30% EtOAc–Hex | CC (Si gel) | 126 |
| Marine mycotoxins | *Trichoderma koningii* | Nucleoprep 100–30 OH | Gradient of $CH_2Cl_2$–EtOH (100 : 0 to 50 : 50) | Anal. RP-HPLC | 127 |
| Palmitic acid | *Pentanisia prunelloides*, leaf, root | Si gel | Hex–EtOAc gradient (starting at 100% Hex, decreasing to 85% and then further decreasing in steps of 5% per fraction to 60%. Thereafter, the % Hex was decreased by 10% in each fraction | PTLC | 93 |
| Iridoids, phenylethanoids | *Verbascum macrurum*, leaf | Si gel | $CH_2Cl_2$–MeOH gradient | RP-MPLC | 128 |
| Norditerpenoid alkaloid | *Aconitum balfourii*, root | Si gel, $Al_2O_3$ | Gradient elution in increasing polarity with Hex, $Et_2O$ and MeOH. $Et_2O$–MeOH (95 : 5), (90 : 10), 85 : 15) fractions were pooled | RPC | 129 |
| Corymbiferan lactones | *Penicillium hordei* | Si gel | MeOH–water (10 : 90), (25 : 75), (50 : 50), (75 : 25) and 100 : 0 (+0.05% TFA) | Semiprep. RP-HPLC | 130 |
| Isoflavones | Soybean paste | Si gel | Hex, EtOAc, MeOH and step gradient of Hex–$CH_2Cl_2$–EtOAc–MeOH | CC (Sephadex LH-20), prep. RP-HPLC | 131 |
| Coumarins | *Angelica sylvestris*, seed | Si gel | Solvent mixtures of increasing polarity: Hex, Hex–EtOAc, EtOAc, EtOAc–MeOH, MeOH | PTLC | 100 |
| Various compounds | *Casimiroa pubescens*, seed | Si gel | Step gradient of Hex in EtOAc | — | 132 |
| Flavonoids | *Opuntia dillenii*, flower | Si gel | $CHCl_3$, $CHCl_3$–EtOAc, EtOAc, EtOAc–MeOH | CC (Sephadex LH-20, Si gel), PPC | 133 |
| Various compounds | *Polyalthia longifolia* var. *pendula* | Si gel | PE, EtOAc with 5–10% rise in polarity | FC, PTLC | 106 |
| Alkaloids | *Boophane disticha*, leaf | Si gel | Hex, Hex–EtOAc (50 : 50), (25 : 75), EtOAc, EtOAc–MeOH (90 : 10) (80 : 20), (70 : 30), (60 : 40), (50 : 50), (40 : 60), (30 : 70), (20 : 80), (10 : 90), MeOH | PTLC | 107 |
| Phenolic compounds | *Quercus aucheri*, leaf | Si gel $C_{18}$ | 17.5% MeOH | CC (Sephadex LH-20), RP-MPLC, PTLC | 110 |
| Triterpene saponins | *Polygala ruwenzoriensis*, root | Si gel $C_{18}$ | Water containing increasing amounts of MeOH | MPLC | 134 |
| Prenylated phenolics | *Humulus lupulus*, strobile | Si gel | PE–EtOAc–MeOH and $CHCl_3$–MeOH gradient | CCC | 135 |
| Various compounds | *Lomatium californicum*, root | Si gel | EtOAc extract: Water–MeOH (100% to 100%), EtOH, EtOAc; hexane extract: Hex–EtOAc (1.0 to 0.1) | CC (Sephadex LH-20), semiprep. HPLC | 136 |
| Phenylpropanoids | *Pimpinella aurea*, aerial part | Si gel | Step gradient of Hex–EtOAc (100 : 0, 90 : 10, 80 : 20, 60 : 40, 40 : 60, 20 : 80, 100 : 0) | PTLC | 121 |
| Phenanthrenes | *Tamus communis*, rhizome | Si gel | Gradient system of Cyclohex–EtOAc–EtOH (9 : 1 : 0, 8 : 2 : 0, 7 : 3 : 0, 70 : 30 : 1, 70 : 30 : 2, 70 : 30 : 5, 50 : 50 : 10) | CC (Sephadex LH-20), RP-HPLC, PTLC | 115 |
| Ecdysteroids | *Serratula wolffii*, root | Si gel $C_{18}$ | Step gradient of MeOH–water | SPE (Polyamide 6), RPC | 137 |
| Ecdysteroids | *Serratula wolffii*, aerial part | Si gel, Si gel $C_{18}$ | EtOAc–MeOH–water (85 : 10 : 5); step gradient 30% to 60% aqueous MeOH and $CH_2Cl_2$, $CH_2Cl_2$–EtOH | CC (Si gel, polyamide, Sephadex LH-20, $Al_2O_3$), PTLC, NP-HPLC | 123 |

[a] Systematic plant name and crude drug, commercial formulation, systematic name of mould or fungi. [b] Si gel: silica gel; $Al_2O_3$: aluminium oxide. [c] $CHCl_3$: chloroform; $Et_2O$: diethyl ether; EtOAc: ethyl acetate; EtOH: ethanol; Hex: *n*-hexane; Cyclohex: cyclohexane; MeOH: methanol; $CH_2Cl_2$: methylene chloride; PE: petroleum ether; TFA: trifluoroacetic acid. [d] FC: flash chromatography; HPLC: high-pressure liquid chromatography; LC: Liquid chromatography, MPLC: medium-pressure liquid chromatography; PPC: preparative paper chromatography; PTLC: preparative thin-layer chromatography; RPC: rotation planar chromatography; SPE: solid-phase extraction. [e] References: 2000–2007.

**Table 8** Recent applications of FC to natural product isolation

| Compounds | Matrix[a] | Sorbent[b] (granulometry/ μm) | Column dimensions/ mm | Eluent (volume ratio)[c] | Other chromatographic methods used[d] | Ref.[e] |
|---|---|---|---|---|---|---|
| Various compounds | *Anethum graveolens*, herb | Si gel | 35 × 550 | Solvents of increasing polarity: *i.e.* Pent–$Et_2O$, EtOAc, MeOH | CC (Amberlite XAD-2), MLCCC, prep. + anal. NP- and RP- HPLC | 138 |
| Acid diterpenes | *Copaifera cearensis*, oil | Si gel KOH | — | Hex, $CH_2Cl_2$, MeOH | Semiprep RP-HPLC | 139 |
| Various glycosides | *Xanthoxylum piperitum*, leaf | Si gel $C_{18}$ | 30 × 200 | Stepwise elution: 5, 20, 30, 40, 50, and 100% MeOH–water | CC (Amberlite XAD-2), RP-HPLC | 140 |
| Guaianolides | *Achillea asiatica*, herb | Si gel | 50 × 200 | $CH_2Cl_2$, $CH_2Cl_2$–$Me_2CO$ (9 : 1, 8 : 2, 7 : 3, 6 : 4, 1 : 1); $CH_2Cl_2$–MeOH (9 : 1, 8 : 2, 7 : 3, 6 : 4) | CC (Si gel), semiprep. RP-HPLC | 141 |
| Triterpene saponins | *Quercus petraea, Q. robur*, chips of the heartwood | Si gel | 23 × 550 | $CHCl_3$–MeOH–water (80 : 25 : 1, 50 : 50 : 4) | Sempiprep. + anal. RP-HPLC, CC (Sephadex LH-20) | 142 |
| Taxane derivatives | *Taxus brevifolia*, bark | Si gel | — | Step gradient of (1) 75% Hex, 25% EtOAc, (2) 50% Hex, 50% EtOAc, (3) 100% EtOAc, (4) 75% EtOAc, 25% MeOH, (5) 50% EtOAc, 50% MeOH | Prep. RP-HPLC | 143 |
| Microcystin LR | *Microcystis aeruginosa* | Si gel | 10 × 330 | EtOAc–iPrOH–water (30 : 45 : 25) | SPE ($C_{18}$ cartridge), semiprep. RP-HPLC | 24 |
| Microcystin RR, YR | *Microcystis aeruginosa* | Si gel | 10 × 330 | EtOAc–iPrOH–water (30 : 45 : 25) | SPE ($C_{18}$ cartridge) | 25 |
| Glucocerebrosides | *Euphorbia nicaeensis*, aerial part | Si gel $C_{18}$ | 10 × 330 | MeOH | CC (Si gel) | 144 |
| Epoxybergamottin | Grapefruit peel (*Citrus paradisi*) | Si gel (40–63) | 25 × 500 | $CHCl_3$, EtOAc, $Me_2CO$, $Me_2CO$–MeOH (1 : 1) | RPC, PTLC | 97 |
| Azadirachtin-A | Neem AzalT/S | Si gel | — | $Et_2O$–MeOH (49 : 1) | Semiprep. RP-HPLC | 145 |
| Celastrol | *Celastrus orbiculatus*, root | Si gel | 50 × 150 | LtPet–EtOAc (1 : 0, 1 : 0.25, 1 : 0.5, 1 : 1, 0 : 1) | HSCCC | 146 |
| Flavonoids | Grapefruit molasse | Si gel $C_{18}$ (35–70) | 75 × 300 | Step gradient of 19% $CH_3CN$ to 22% $CH_3CN$ | CC (Dowex-50, SP-70 resin) | 147 |
| Lignans | *Styrax camporum*, stem | Si gel | — | $CHCl_3$–MeOH (98 : 2, 96 : 4) | CC (Si gel, Sephadex LH-20), PTLC | 111 |
| Limonoid glucosides | Grapefruit seed | Si gel $C_{18}$ (35–70) | 75 × 300 | MeOH–$CH_3CN$–water (10 : 15 : 75) | — | 148 |
| Various compounds | *Polyalthia longifolia* var. *pendula* | Si gel | — | Mixture of $PetEtO_2$–EtOAc–MeOH with increasing polarity | VLC, PTLC | 106 |
| Verrucarin A | *Myrothecium verrucaria* | Si gel | — | Hex–$CH_2Cl_2$–propan-2-ol (8 : 4 : 1) | PTLC, RP-HPLC | 112 |
| Cyclic peptide | *Streptomyces nobilis* | Si gel + Si gel $C_{18}$ | — | Hex–EtOAc (1 : 1), $CHCl_3$–MeOH (50 : 1), $CHCl_3$–MeOH (20 : 1); step gradient of aqueous MeOH | Prep HPLC | 149 |
| Phase II enzyme-inducing agents | Freeze-dried onion (*Allium cepa*) | Si gel + Si gel $C_{18}$ | — | Various eluents | PTLC, RP-HPLC | 150 |
| Streptokordin | *Streptomyces* sp. KORDI-3238 | Si gel | — | Gradient mixture of Hex and EtOAc (40%, 60% EtOAc) | RP-HPLC | 151 |
| Chemopreventive agents | Green onion (*Allium cepa*) | Si gel + Si gel $C_{18}$ | 48 × 300, 25 × 600 | NP: step gradient of 2,5%, 5%, 10%, 30%, and 100% MeOH in $CH_2Cl_2$; RP: linear gradient from 2% to 30% of $CH_3CN$ in 1% AcOH | Prep. RP-HPLC, PTLC | 122 |
| Monogalactosyl diacylglycerols | *Sargassum thunbergii* | Si gel $C_{18}$ | — | MeOH–water (70 : 30, 80 : 20, 90 : 10); 100% MeOH, $Me_2CO$, EtOAc | RP-HPLC | 152 |
| Flavonol derivatives | *Euphorbia stenoclada*, aerial part | Si gel $C_{18}$ | 40 × 150 | Stepwise elution with MeOH–water from 10% to 100% MeOH | Semiprep. RP-HPLC | 153 |
| Flavonol tetraglycosides | *Lens culinaris*, seed | Si gel (32–63) | 40 × 150 | EtOAc–PrOH–water (2 : 7 : 1) | CC (Diaion HP-20 beads), semiprep. RP-HPLC | 154 |

**Table 8** *(Contd.)*

| Compounds | Matrix[a] | Sorbent[b] (granulometry/ μm) | Column dimensions/ mm | Eluent (volume ratio)[c] | Other chromatographic methods used[d] | Ref.[e] |
|---|---|---|---|---|---|---|
| Urukthapelstatin A | *Mechercharimyces asporophorigenens* YM11-542 | Si gel $C_{18}$ | — | MeOH–water | CC (Si gel), prep. RP-HPLC | 155 |
| Hydroxyanthra-quinones | *Rheum tanguticum*, root | Si gel | — | PE–EtOAc (95 : 5, 8 : 1, 3 : 1, 1 : 1); EtOAc | — | 156 |

[a] Systematic plant name and crude drug, commercial formulation (Neem AzalT/S), systematic name of alga, bacterium, cyanobacterium or fungus. [b] Si gel: silica gel. NP: normal phase; RP: reversed-phase. [c] AcOH: acetic acid; $Me_2CO$: acetone; $CH_3CN$: acetonitrile; $CHCl_3$: chloroform; $Et_2O$: diethyl ether; EtOAc: ethyl acetate; Hex: *n*-hexane; MeOH: methanol; $CH_2Cl_2$: methylene chloride; Pent: pentane; PE: petroleum ether; LtPet: light petroleum; PrOH: *n*-propanol; iPrOH: isopropanol; TFA: trifluoroacetic acid. [d] CC: column chromatography; FC: flash chromatography; HSCCC: high-speed counter-current chromatography; RP-HPLC: reversed-phase high-pressure liquid chromatography; MLCCC: multilayer counter-current chromatography; MPLC: medium-pressure liquid chromatography; PTLC: preparative thin-layer chromatography; SPE: solid-phase extraction. [e] References: 2000–2007.

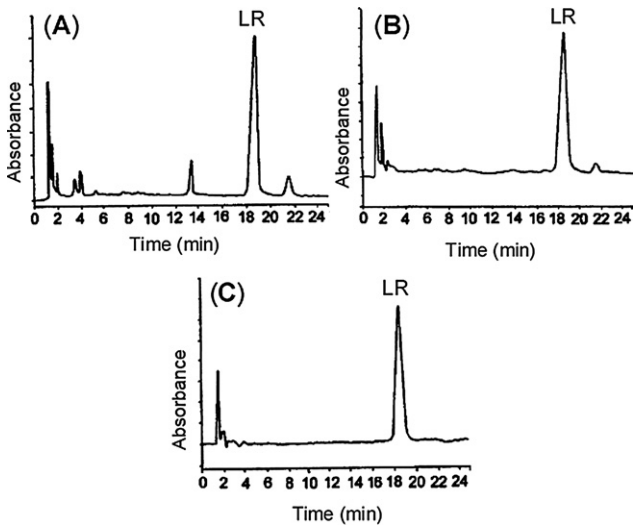
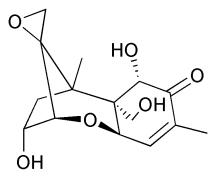



**Fig. 7** HPLC chromatograms of microcystin LR (**3**) fractions: after silica gel FC (A), after the first (B) and the second (C) semipreparative HPLC purification step. HPLC conditions: MeOH–0.05 M phosphate buffer (pH 3) (55 : 45), 1 ml $min^{-1}$, λ 235 nm. Purity of **3**: 95%. LR represents microcystin LR (**3**). Reprinted from *Anal. Lett.* (http://www.informaworld.com), with permission from Taylor & Francis.[24]

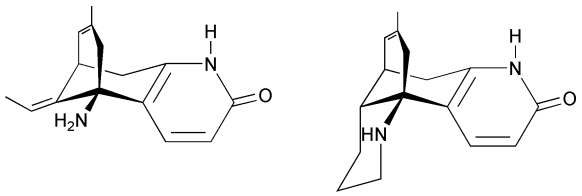
polar sorbent. For LPLC, the particle size of the silica gel is normally in the range of 40–60 μm, which allows one to achieve high flow-rates with low pressures. Silica gel can be chemically modified in a variety of ways to alter both its physical properties and chromatographic behaviour. The silica gel surface consists of exposed silanol groups and these hydroxyl groups form the active centres. The silanol groups can be blocked with a variety of silyl chlorides to produce either a non-polar (reversed-phase) or an intermediate polarity (bonded normal phase) chromatography support. The reversed-phase stationary phase is prepared by treating silica gel with chlorodimethylalkylsilanes or chloroalkoxysilanes of different chain lengths. Most chromatographers prefer $C_8$ or $C_{18}$ materials.[3,157] For recent applications see Table 9.

LPLC is generally used in combination with other separation methods and may form the intermediate or final steps of purification. In some cases, LPLC is applied as the only separation step. Clifford *et al.*[158] used it for the isolation of the mycotoxin deoxynivalenol (**6**) from the fungus *Fusarium graminearum*. Silica gel LPLC readily facilitated the purification of large quantities of **6**. The use of a hexane–acetone gradient (4 : 1, 7 : 3, 3 : 2, 1 : 1, 2 : 3, and 1 : 3, v/v) eliminated the need for repeated partitioning (water–ethyl acetate or water–methylene chloride), charcoal–alumina columns, Florisil columns, or Sephadex LH-20 columns, as in previous cases. Additional clean-up prior to crystallisation was also not necessary. Repeated crystallisation yielded >99% pure **6**, determined by HPLC analysis. Li *et al.*[162] developed a rapid, facile, and environmentally friendly process for the purification of huperzine A (**7**) and B (**8**) from the herb *Huperzia serrata*. The process consisted of two successive steps of LPLC on two polystyrene-based resins. The first step removed a large amount of impurities and captured **7** and **8** using Amberlite XAD-4 from the herbal extraction prepared by 1% aqueous sulfuric acid. This was more efficient than multi-cycle liquid–liquid extraction as an initial separation step. In the second step it was possible to separate **7** and **8**, employing a polystyrene-based porous microsphere (PST, average particle size 30 μm), as packing material. The PST column demonstrated a better separation and shorter run time than a $C_{18}$ column. The mobile phases used in both LPLC separations consisted of ethanol and water. Combination of XAD-4 and PST chromatography and one crystallisation step enabled purification of **7** and **8** from 0.18% and 0.08% to 98.2% and 98.8%, respectively, with recoveries of 82.8% and 84.3%.




Deoxynivalenol (**6**)

Similar isolation protocols were used for the purification of icariin from a crude extract of *Epimedium* species and of paclitaxel from a crude extract of *Taxus* species. In the case of icariin purification, comparison between the PST medium and a commercially available $C_{18}$ material showed that the PST medium demonstrated a higher resolution and better selectivity than the $C_{18}$ column. Fig. 8 shows the profiles of the




Huperzine A (**7**) Huperzine B (**8**)

chromatographic separation of the two columns. The PST column was run at low pressure of 0.005 MPa while the $C_{18}$ column was run at 0.5 MPa. The PST column produced a better separation within a shorter time. A crude extract of 20% icariin can be purified to 90% with a recovery of 99.9% under optimised conditions. After crystallisation, the purity of icariin can reach more than 98% with a total recovery of 93%.[160]

Traditional methods of isolation and purification of paclitaxel (**9**) involve multiple steps of liquid–liquid partitioning, LPLC and preparative HPLC. Sun *et al.*[161] developed a two-column LPLC process using $Al_2O_3$ and PST as stationary phases. The first column ($Al_2O_3$) separated **9** from a majority of unwanted compounds and removed 10-deacetyl-7-*epi*-paclitaxel, which is difficult to separate from **9**. Other more polar taxane analogues with structures similar to **9** could be removed by subsequent LPLC with PST medium resulting in a paclitaxel content of 90.6%. The final purity of **9** after a single crystallisation step was more than 98%, with a recovery of 86%.

Recently, Pyo *et al.*[163] reported an efficient and low-cost large-scale purification procedure of three taxane derivatives from

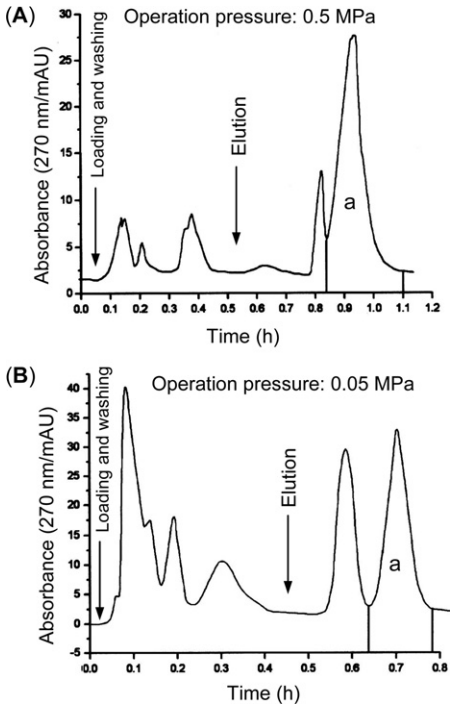


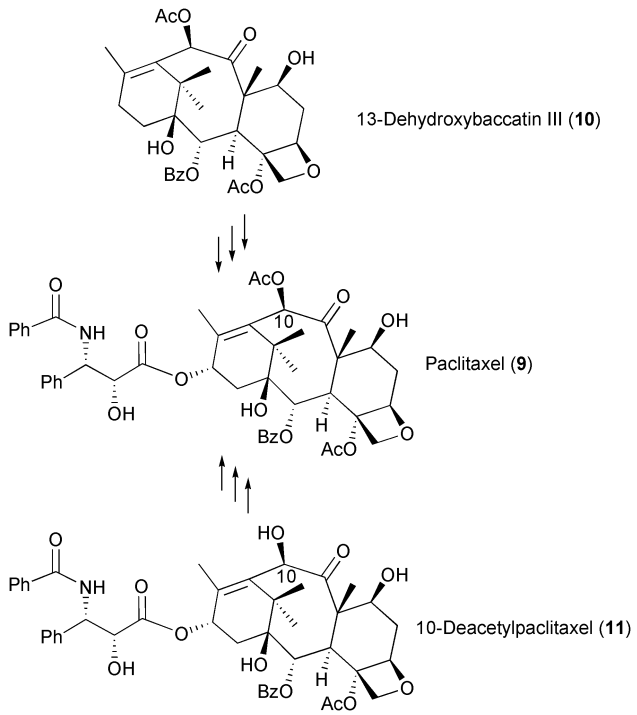

**Fig. 8** The profiles of the chromatographic purification of icariin from a crude extract of an *Epimedium* species: (A) by a $C_{18}$ column, (B) by a PST column. a = icariin. Reprinted from H. Sun, X. Li, G. Ma and Z. Su, *Chromatographia*, 2005, **61**, 9–15, with permission.[160]

**Table 9** Recent applications of LPLC to natural product isolation

| Compounds | Matrix[a] | Sorbent[b] (granulometry/μm) | Column dimensions/mm | Eluent (volume ratio)[c] | Other chromatographic methods used[d] | Ref.[e] |
|---|---|---|---|---|---|---|
| Mycotoxin deoxynivalenol | *Fusarium graminearum* | Si gel | 37 i.d. | Hex–$Me_2CO$ gradient (7 : 3, 3 : 2, 1 : 1, 2 : 3, 1 : 3) | — | 158 |
| Triterpenoid esters | *Calendula officinalis*, flower heads | Si gel $C_{18}$ (40–63) | 37 × 440 | MeOH | CC (Si gel), RP-HPLC | 22 |
| Lolitrem B | Endophyte-infected ryegrass (*Lolium perenne*) | Si gel (50) | 20 × 240 | $CH_2Cl_2$–$Me_2CO$ (97 : 3, 94 : 6) and $CH_2Cl_2$–EtOAc (95 : 5) | CPC, PTLC | 103 |
| Ecdysteroids | *Silene italica* ssp. *nemoralis*, aerial part | Si gel $C_{18}$ (60–200) | 16 × 900 | Stepwise gradient: 30–60% aqueous MeOH | SPE ($Al_2O_3$), DCCC, NP- and RP-HPLC | 101 |
| Soyasaponins | Soybean flour (*Glycine max*) | Si gel $C_{18}$ | 25 × 150, 25 × 250 | EtOH, EtOH–water and MeOH–water mixtures | SPE (Sep-Pak $C_{18}$ cartridge) | 159 |
| Icariin (flavonol) | *Epimedium* sp. | Si gel $C_{18}$ (10–40) + PST (30) | 16 × 100 | Isocratically with 70% MeOH (Si gel), 60% MeOH (PST) | — | 160 |
| Paclitaxel | *Taxus* sp. | $Al_2O_3$, Si gel + PST (30) | 25 × 250, 16 × 90 | $CHCl_3$–MeOH (97 : 3; $Al_2O_3$), $CHCl_3$–MeOH (96 : 4; Si gel), isocratically with 80% MeOH (PST) | — | 161 |
| Huperzine A, B | *Huperzia serrata*, herb | Amberlite XAD-4 + PST (30) | 25 × 250, 16 × 100 | Isocratically with 40% EtOH | — | 162 |
| Taxane derivatives | *Taxus chinensis*, cell cultures | Si gel + Si gel $C_{18}$ (100) | 16 × 900 | 1.5 and 5% MeOH in $CH_2Cl_2$; 62% MeOH in water | NP- + RP-HPLC | 163 |

[a] Systematic plant name and crude drug or systematic name of fungus. [b] Si gel: silica gel. $Al_2O_3$: aluminium oxide. PST: spherical styrene–divinylbenzene polymeric resin (laboratory-made). [c] $Me_2CO$: acetone; $CHCl_3$: chloroform; EtOAc: ethyl acetate; EtOH: ethanol; Hex: *n*-hexane; MeOH: methanol; $CH_2Cl_2$: methylene chloride. [d] CPC: centrifugal partition chromatography; CC: column chromatography; DCCC: droplet counter-current chromatography; PTLC: preparative thin-layer chromatography; SPE: solid-phase extraction. [e] References: 2000–2007.

a plant cell culture of *Taxus chinensis*. Paclitaxel (**9**), 13-dehydrobaccatin III (**10**) and 10-deacetylpaclitaxel (**11**) were readily isolated using mainly LPLC. A schematic diagram of the purification process is shown in Fig. 9. Crude compounds with purities of 21.5% (**10**), 28.7% (**9**) and 25.3% (**11**) were isolated by solvent extraction and silica gel LPLC using isocratic elution with 1.5 and 5% methanol in dichloromethane in one chromatographic step. During further purification of **10** and **11** by RP-LPLC, methanol and water were used as solvents; these solvents are the same as those used in purification of **9** and **11** by HPLC. Thus, **9** and the paclitaxel precursors **10** and **11** can be simply and economically produced on an industrial scale with purities of >99% and overall recoveries between 87 and 98%.




For the chromatography of labile natural products as well as for purification steps, one of the most commonly used materials is an inert polymer of carbohydrates (Sephadex). In natural product separation, the most extensively used gel is Sephadex LH-20, a hydroxypropylated form of Sephadex G-25 (for examples see Tables 6–11, "other chromatographic methods used").

**4.3.3 Medium-pressure LC (MPLC).** MPLC involves longer columns with large internal diameters and requires higher pressures than LPLC to enable sufficiently high flow-rates. MPLC fulfils the requirement for a simple complementary or supplementary method to open-column chromatography (CC) and flash chromatography (FC) with both higher resolution and shorter separation times.[3] Nyiredy *et al.*[164] tried to find optimal MPLC conditions on silica gel columns. The PRISMA model was applied to determine optimal solvent systems.[86] These conditions can be transposed directly to MPLC. Recent applications of MPLC separations are depicted in Table 10.

A medium-pressure liquid chromatographic method has been effectively employed to obtain three of the major azadirachtin

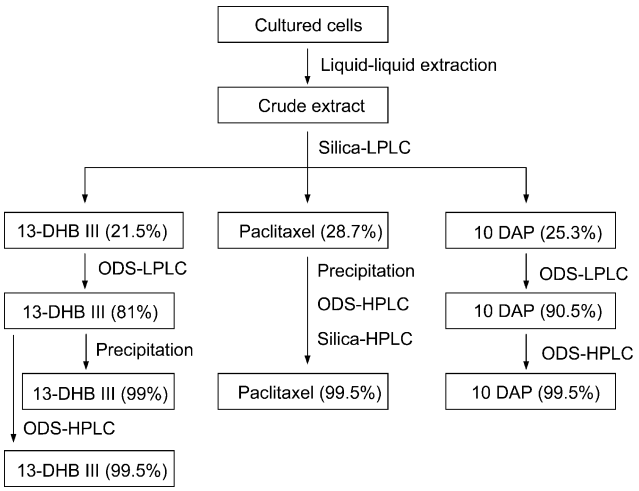
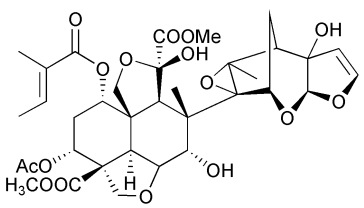
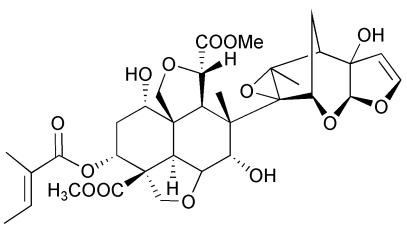



**Fig. 9** Schematic diagram of the purification process for 13-dehydroxybaccatin III (13-DHB III; **10**), paclitaxel (**9**) and 10-deacetylpaclitaxel (10-DAP; **11**) from plant cell cultures of *Taxus chinensis*.[163]

congeners (A, B, H) by Sharma *et al.*[168] 750 mg of azadirachtin A concentrate with a purity of 60%, obtained from *Azadirachta indica* seed kernels through repeated precipitation with hexane from a methanolic solution, was purified by MPLC using a 15 × 25 mm guard column and a 40 × 600 mm glass column packed with $C_{18}$ material (40–63 μm) and eluted with methanol–water (50 : 50, v/v) at a flow-rate of 2 ml $min^{-1}$. The fractions containing azadirachtins A **12**), B (**13**), and H (**14**) were pooled and evaporated. Pure **14** (10 mg), **12** (256 mg), and **13** (15 mg) were isolated as white powders from the pooled fractions. Most of the previously reported preparative HPLC procedures for the


Azadirachtin A (**12**)


Azadirachtin B (**13**)

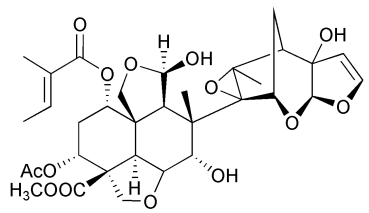

Azadirachtin H (**14**)

**Table 10** Recent applications of MPLC to natural product isolation

| Compounds | Matrix[a] | Sorbent[b] (granulometry/μm) | Column dimensions/mm | Eluent (volume ratio)[c] | Other chromatographic methods used[d] | Ref.[e] |
|---|---|---|---|---|---|---|
| Methoxylated flavones | *Primula veris*, flower | Si gel (25–40) | 25 × 500 | Hex–EtOAc (70 : 30) | CC ($Al_2O_3$), PTLC, RP-HPLC | 87 |
| Various compounds | *Avena fatua*, root | Si gel | — | Gradient of Hex–$CHCl_3$ (4 : 1 to 1 : 4), $CHCl_3$, $CHCl_3$–MeOH (49 : 1 to 1 : 4) | — | 165 |
| Phenolic compounds | Onion (*Allium cepa*), bulb | Si gel $C_{18}$ (15–25) | 26 × 460 | Gradient: $CH_3CN$ + 1% aqueous HCOOH | — | 166 |
| Kava lactones | *Piper methysticum*, root | Si gel + Si gel $C_{18}$ (25–40) | — | Hex–$Me_2CO$ (10 : 1, 6 : 1, 3 : 1, 1 : 1), 100% $Me_2CO$, MeOH; $CH_3CN$–water gradient, 100% $CH_3CN$ | PTLC, RP-HPLC | 96 |
| Diterpene | *Croton zambesicus*, leaf | Si gel | 15 × 750 | Toluene–EtOAc (90 : 10) | HSCCC | 167 |
| 2-Pyrone derivatives | *Gerbera hybrida*, stem, leaf | Si gel (15) | 26 × 230 | Step gradient of increasing solvent strength: MeOH–EtOAc–THF at selectivity point $P_s$ 111 with 1% HCOOH | CPC, PTLC | 95 |
| Iridoids, phenylethanoids | *Verbascum macrurum*, leaf | Si gel $C_{18}$(20–40) | — | MeOH–water (20 : 80, 70 : 30), $CH_2Cl_2$–MeOH (75 : 25), water, water–MeOH (70 : 30) | VLC | 128 |
| Azadirachtin A, B, H | *Azadirachta indica*, seed kernels | Si gel $C_{18}$ (40–63) | 40 × 600, 15 × 25 (guard column) | MeOH–water (50 : 50) | — | 168 |
| Acylated saponins | *Polygala myrtifolia*, bark, root | Si gel (15–40) | — | $CHCl_3$–MeOH–water (8 : 5 : 1, 13 : 7 : 2), lower phase | CC (Sephadex LH-20) | 169 |
| Carotenoids | *Carica papaya*, fruit | Si gel (40–63) | 15–100 | Hex–$CH_2Cl_2$ (100 : 0. 96.875 : 3.125, 93.75 : 6.25, 87.5 : 12.5, 75 : 25, 50 : 50) | — | 170 |
| Acylated triterpene saponins | *Polygala arenaria*, root | Si gel (15–40) | 25 × 460, 15 × 460, 15 × 110 (pre-column) | $CHCl_3$–MeOH–water (65 : 40 : 8) | CC (Sephadex LH-20) | 171 |
| Quinic acid derivatives | *Baccharis* sp., aerial part | Si gel $C_{18}$ (40–63) | 25 × 270 | Stepwise gradient of MeOH–water (95 : 5 to 100 : 0) | Semiprep. RP-HPLC | 172 |
| Phenolic compounds | *Quercus aucheri*, leaf | Si gel $C_{18}$ | — | 45% MeOH, 20% MeOH | CC (Sephadex LH-20), PTLC, VLC | 110 |
| Triterpene saponins | *Polygala ruwenzoriensis*, root | Si gel | — | $CHCl_3$–MeOH–water (13 : 7 : 2), lower phase | VLC | 134 |
| Carvacrol | *Eupatorium glutinosum*, leaf, twig | Si gel (40–63) | — | Continuous gradients running from Hex, through $CH_2Cl_2$ to MeOH | — | 173 |
| Azadirachtin A | *Azadirachta indica*, seed kernels | Si gel $C_{18}$ | 400 × 600, 15 × 25 (guard column) | MeOH–water (55 : 45) | — | 174 |
| Capsaicin glucosides | *Capsicum* sp., fruit | Si gel $C_{18}$ (38–63) | 10 × 200 | $CH_3CN$–water (1 : 1) | RP-HPLC | 175 |
| Arbutin derivative | *Myrothamnus flabellifolia*, herb | Si gel $C_{18}$ (18–32–100) | 36 × 500 | MeOH 25% | CC (Sephadex LH-20, MCI gel CHP 20 P) | 176 |

[a] Systematic plant name and crude drug. [b] Si gel: silica gel. [c] $Me_2CO$: acetone; $CH_3CN$: acetonitrile; $CHCl_3$: chloroform; EtOAc: ethyl acetate; EtOH: ethanol; Hex: *n*-hexane; MeOH: methanol; $CH_2Cl_2$: methylene chloride; THF: tetrahydrofuran. [d] $Al_2O_3$: aluminium oxide; CPC: centrifugal partition chromatography; CC: column chromatography; HSCCC: high-speed counter-current chromatography; RP-HPLC: reversed-phase high-pressure liquid chromatography; PTLC: preparative thin-layer chromatography; VLC: vacuum liquid chromatography. [e] References: 2000–2007.

separation of azadirachtin congeners were complicated, time-consuming, and involved the use of numerous preparative HPLC columns. Unlike these earlier methods, the MPLC isolation procedure is simpler, more convenient, more cost-effective, and less time-consuming.

**4.3.4 High-pressure LC (HPLC).** In the literature the terms "analytical", "semi-preparative (semi-prep)" and "preparative (prep)" HPLC can be found. For isolation of natural products, semi-prep (for the separation of about 1 mg to 100 mg mixtures) and prep HPLC are commonly used. If only microgram quantities of compound are needed, *e.g.* for initial bioassay screening, purifications can sometimes be carried out using analytical-scale HPLC systems.

The use of prep HPLC has become a mainstay in the isolation of most classes of natural products. Prep HPLC is a robust,

**Table 11** Selected recent applications of semipreparative and preparative HPLC to natural product isolation. For the complete version of this table, containing further references appearing in Section 11 (References),[178–230] see ESI (Table S1)†

| Compounds | Matrix[a] | HPLC[b] | Sorbent[c] (granulometry/μm) | Column dimensions/mm | Eluent (volume ratio)[d] | Flow-rate/ ml min$^{-1}$ | Other chromatographic methods used[e] | Ref.[f] |
|---|---|---|---|---|---|---|---|---|
| ***Flavonoids*** | | | | | | | | |
| Anthocyanins | *Eugenia umbelliflora*, fruit | SP | Si gel $C_{18}$ (12) | 21.2 × 250 | Linear gradient: (A) $CH_3CN$, (B) 10% $H_3PO_4$, 5% AcOH, 10% $CH_3CN$, 5% MeOH, water | 10 | CC (Amberlite XAD-7), SPE Sep-Pak $C_{18}$ cartidge) | 178 |
| Apigenin acyl glucosides | *Chamomilla recutita*, flower | P | Si gel $C_{18}$ (5) | 21.2 × 250, 21.2 × 60 (guard column) | Gradient: water containing 0.1% TFA (A), $CH_3CN$ (B) | 5 | CC (Polyamide) | 181 |
| Flavonoid glycosides | *Trollius ledebouri*, flower | SP | Si gel $C_{18}$ (10) | 10 × 200 | $CH_3CN$–0.1% aqueous AcOH (15 : 85) | 1.2 | HSCCC | 183 |
| Flavones | *Scutellaria baicalensis*, root | P | Si gel $C_{18}$ (10) | 10 × 250 | Gradient: $CH_3CN$ + 0.1% TFA (10 : 90 → 90 : 10) | 6 | — | 184 |
| Flavonoids | *Crataegus* sp., leaf and flower | P | Si gel $C_{18}$ (5) | 22 × 250 | Gradient: water–HCOOH (9 : 0.5) and MeOH–water–HCOOH (5 : 4 : 0.5) | 14 | CC (Amberlite XAD-7, Sephadex LH-20) | 190 |
| Procyanidins | *Theobroma cacao*, seed | P | Si gel (100) | 50 × 300 | Gradient: $CH_3CN$–AcOH (99 : 1) (A), MeOH–water–AcOH (95 : 4 : 1) (B) | 55 | — | 192 |
| Catechins, procyanidins | *Anisophyllea dichostyla*, root | SP | Si gel $C_{18}$ | 10 i.d. | Gradient: 2% AcOH in water (A), $CH_3CN$ (B) | 3 | CC (Si gel) | 193 |
| ***Coumarins*** | | | | | | | | |
| Coumarins | *Peucedanum verticillare*, fruit, root | SP | Si gel $C_{18}$ (5) | 4.6 × 250 | $CH_3CN$ gradient | 1 | CC (Si gel + $Mg^{2+}$ Si gel), PTLC | 108 |
| ***Phenolic compounds*** | | | | | | | | |
| Punicalagin | *Punica granatum*, fruit | P | Si gel $C_{18}$ (5) | 20 × 250 | EtOAc–MeOH–water (5 : 25 : 70) | 5 | CC (Si gel) | 197 |
| Quinic acid derivatives | *Baccharis* sp., aerial part | SP | Si gel $C_{18}$ (10) | 25 × 100 | MeOH–water (20 : 80) | 10 | RP-MPLC | 172 |
| Phenolic compounds | *Magnolia officinalis*, bark | SP | Si gel $C_{18}$ (5) | 9.4 × 250 | MeOH–water (70 : 30) | 2.5 | — | 201 |
| ***Alkaloids*** | | | | | | | | |
| Bisbenzylisoquinoline alkaloids | *Cissampelos mucronata*, root | P | Si gel $C_{18}$ (5), Si gel CN (7) | 8 × 250 | Various eluents | 0.6–2.5 | CC (Si gel) | 203 |
| Alkaloids | *Nicotiana tabaccum*, cell cultures | SP | Si gel $C_{18}$ (10) | 7.8 × 150 | 30% MeOH (A), 70% $(NH_4)HCO_3$ buffer (10 mM, pH 9, adjusted with $NH_4OH$) | 2.8 | — | 204 |
| Ungeremine | *Nerine bowdenii*, bulb | P | Si gel $C_{18}$ (10) | 7.8 × 300 | MeOH–water–THF (30 : 68 : 2) | 2.5 | CPC, CC (Sephadex LH-20), CPC | 205 |
| Alkaloids | *Penicillium rivulum* | P | Si gel $C_{18}$ (5) | 10 × 250 | $CH_3CN$–water (1 : 1) | 5 | HSCCC | 206 |
| ***Lignans*** | | | | | | | | |
| Lignans | *Arctium lappa*, leaf | SP | Si gel $C_{18}$ (5) | 4 × 250 | Linear gradient: $CH_3CN$–1% aqueous AcOH | — | CC (Polyamide 6) | 207 |
| Lignans | *Centaurea americana*, seed | P | Si gel $C_{18}$ (10) | 21.2 × 250 | Linear gradient: MeOH–water (60 : 40) to (80 : 20) followed by 80% MeOH | 2, 1 | SPE (Sep-Pak $C_{18}$ cartridge) | 208 |
| ***Saponins*** | | | | | | | | |
| Saponins | *Aesculus chinensis*, seed | P | Si gel $C_{18}$ (5) | 50 × 300 | Gradient: MeOH–0.1% AcOH (20 : 80) | 20 | CC (D-101 resin) | 210 |
| Ginsenosides $Rb_3$, Rc | *Panax notoginseng*, leaf, caudex | SP | Si gel $C_{18}$ (25–45) | 50 × 300 | Gradient: MeOH–water (70 : 30) → (65 : 35) | 30 | — | 211 |

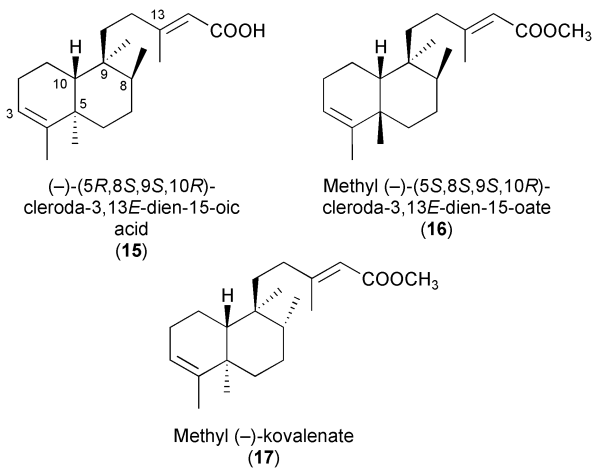
**Table 11** *(Contd.)*

| Compounds | Matrix[a] | HPLC[b] | Sorbent[c] (granulometry/μm) | Column dimensions/mm | Eluent (volume ratio)[d] | Flow-rate/ ml min$^{-1}$ | Other chromatographic methods used[e] | Ref.[f] |
|---|---|---|---|---|---|---|---|---|
| ***Terpenoids*** | | | | | | | | |
| Clerodane diterpenes | *Hymenaea courbaril* var. *stilbocarpa* | SP | Si gel $C_{18}$ (5) | 10 × 250 | MeOH–water–HCOOH (85 : 15 : 1) | 2 | CC (Si gel), PTLC | 212 |
| Secoiridoid glycosides | *Centaurium erythraea*, aerial part | P | Si gel $C_{18}$ (10) | 21.2 × 250 | Linear gradient: $CH_3CN$–water, $CH_3CN$ | 20 | SPE (Sep-Pak $C_{18}$ cartridge) | 213 |
| Triterpenoid esters | *Calendula officinalis*, flower head | P | Si gel $C_{18}$ (5 and 7) | 4.6 × 250<br>25 × 250 | MeOH; MeOH–iPrOH (85 : 15) | 1, 8, 10 | CC (Si gel), RP-LPLC | 22 |
| Iridoid glycosides | *Eremostachys glabra*, rhizome | P | Si gel $C_{18}$ (10) | 21.2 × 250 | $CH_3CN$–water gradient | 20 | SPE (Sep-Pak $C_{18}$ cartidge) | 214 |
| Sesquiterpene lactones | *Laurus nobilis*, leaf | SP | Si gel (5) | 9.4 × 250 | Hex–EtOAc (92 : 8) | 3 | CC (Si gel) | 215 |
| Diterpenoids | *Coprinus heptemerus* | P | Si gel $C_{18}$ (7) | 25 × 250 | Gradient: water to MeOH | 7.5 | CC (Diaion HP21 resin, Si gel) | 216 |
| Ganodermic acids | *Ganoderma lucidum* | SP | Si gel $C_{18}$ (5) | 10 × 250 | 99.5% MeOH–0.5% AcOH, mixed with water at a ratio of 9 : 1 | 4 | — | 218 |
| Triterpenoids | *Decalepis hamiltonii*, root | SP | Si gel $C_{18}$ (5) | 4.6 × 250 | MeOH–water (94 : 6) | 1 | CC (Si gel) | 220 |
| Tetranortriterpenoids | *Azadirachta indica*, seed | P | Si gel $C_8$ (5) | 21 × 250,<br>4.6 × 50 (pre-column) | MeOH–water (50 : 50) and MeOH–water (60 : 40) | 6, 8 | CCC | 221 |
| Oleuropein | *Syringa oblata*, leaf | P | Si gel $C_{18}$ (15) | 25 × 200 | Linear gradient: 10–30% $CH_3CN$ in 0.1% HCOOH | 8.5 | — | 222 |
| ***Miscellaneous*** | | | | | | | | |
| Microcystin LR | *Microcystis aeruginosa* | SP | Si gel $C_{18}$ (5) | 10 × 250 | MeOH–0.05 M sodium sulfate (55 : 45) | — | SPE ($C_{18}$ cartridge), FC | 24,25 |
| Ecdysteroids | *Silene italica* ssp. *nemoralis*, aerial part | SP | Si gel + Si gel $C_{18}$ (5) | 9.4 × 250 | $CH_3CN$–water (23 : 77) | 1 | SPE ($Al_2O_3$), DCCC, LPLC, PTLC | 101 |
| Cyclic peptide antibiotics | *Bacillus subtilis* | P | Si gel $C_{18}$ | 25 × 250 | Gradient: $CH_3CN$ and water, both containing 0.1% TFA | 10 | — | 224 |
| Cyclic depsipeptides | *Fusarium avenaceum* (contamination of rice cultures) | SP | Si gel $C_8$ (5) | 19 × 150 | Gradient: starting with $CH_3CN$–water (30 : 70) rising to 100% $CH_3CN$ | — | — | 228 |
| Capsaicin glucosides | *Capsicum* sp., fruit | SP | Si gel $C_{18}$ | 7.5 × 300,<br>4.6 × 250 | $CH_3CN$–water (30 : 70), $CH_3CN$–water (40 : 60) | 2, 1.5 | RP-MPLC | 175 |
| Gambogic and epigambogic acid | *Garcinia hanburyi*, resin | P | Si gel $C_{18}$ (5, 10), Si gel $C_8$ (5) | 22 × 250,<br>4.6 × 250 | $C_{18}$: MeOH–0.1% $H_3PO_4$ (90 : 10) and $CH_3CN$–0.1% AcOH (90 : 10); $C_8$: $CH_3CN$–0.1% AcOH (75 : 25) | 1 | CC (Sephadex LH-20) | 229 |
| Ecdysteroids | *Serratula wolffii*, aerial part | SP | Si gel (5) | 9.4 × 250 | $CH_2Cl_2$–iPrOH–water (125 : 50 : 5), (125 : 40 : 3), 125 : 30 : 2), (125 : 25 : 2); Cyclohex–iPrOH–water (100 : 40 : 3) | 2, 4 | CC (Si gel, polyamide, Sephadex LH-20, $Al_2O_3$), PTLC, VLC | 123 |

[a] Systematic plant name and crude drug, systematic name of bacterium, cyanobacterium, fungus or mushroom. [b] P: Preparative high-pressure liquid chromatography; SP: semipreparative high-pressure liquid chromatography. [c] Si gel: silica gel. [d] AcOH: acetic acid; $CH_3CN$: acetonitrile; $(NH_4)HCO_3$: ammonium hydrogencarbonate; Cyclohex: cyclohexane; EtOAc: ethyl acetate; HCOOH: formic acid; Hex: *n*-hexane; iPrOH: isopropanol; MeOH: methanol; $H_3PO_4$: phosphoric acid; THF: tetrahydrofuran; TFA: trifluoroacetic acid. [e] $Al_2O_3$: aluminium oxide; CPC: centrifugal partition chromatography; CC: column chromatography; FC: flash chromatography; HSCCC: high-speed counter-current chromatography; PTLC: preparative thin-layer chromatography; SPE: solid-phase extraction. [f] References: 2004–2007 (with 6 exceptions).

versatile, and usually rapid technique by which compounds from complex mixtures can be purified. The main differences between prep HPLC and other "lower pressure" column chromatographic systems are the consistency and size of the particles in the stationary phase. Particle size distribution is critical when trying to separate a mixture of two compounds: the separation between the two compounds improves with smaller particle size. The average particle size of prep HPLC stationary phases, typically between 3 and 10 μm, is substantially smaller than other stationary phases. Because of the small particle size, high pressures are necessary to push the mobile phase through the system. However, the high surface area available for the solutes to interact with the stationary phase results in a chromatography with high powers of resolution that are necessary for purifying complex natural product mixtures. Column diameters usually range from 10 to 100 mm. If gram quantities are called for, then typically pilot-plant-scale HPLC systems with internal column diameters >100 mm are needed.[177] Some selected recent prep HPLC separations are listed in Table 11. Generally, prep HPLC is the final purification step in these examples. Very often particle sizes and column dimensions are identical or very similar in prep and semi-prep HPLC applications. In Table 11 only the term prep HPLC is used, with the abbreviations P (prep) and SP (semi-prep) in an additional column for the interested reader.

Nogueira *et al.*[212] isolated clerodane diterpenes from the seed pods of *Hymenaea courbaril* var. *stilbocarpa* by a combination of column chromatography (silica gel) followed by preparative TLC on $SiO_2/AgNO_3$ (5%). One of the resulting fractions, containing a mixture of compounds **15–17**, was submitted to further purification by prep HPLC using octadecyl-bonded silica gel with methanol–water–formic acid (85 : 15 : 1, v/v) as mobile phase (Fig. 10). The separation of these types of compounds is not easy, due to their closely related structures.




(–)-(5*R*,8*S*,9*S*,10*R*)-cleroda-3,13*E*-dien-15-oic acid (**15**)

Methyl (–)-(5*S*,8*S*,9*S*,10*R*)-cleroda-3,13*E*-dien-15-oate (**16**)

Methyl (–)-kovalenate (**17**)

Anthocyanin pigments in the berries of *Eugenia umbelliflora* were extracted with 0.1% HCl in ethanol, and the crude anthocyanin extract was purified by Amberlite XAD-7 CC. After elution of the pigments by using a gradient from MeOH–water (8 : 92, v/v) to MeOH–water (65 : 35, v/v), the eluate was concentrated and passed through a Sep-Pak $C_{18}$ cartridge. Anthocyanins and other phenolics were adsorbed on the surface of the Sep-Pak, whereas sugars, acids, and other water-soluble compounds were eluted with 2 × 5 ml of 1% aqueous acetic acid. The pigments were finally

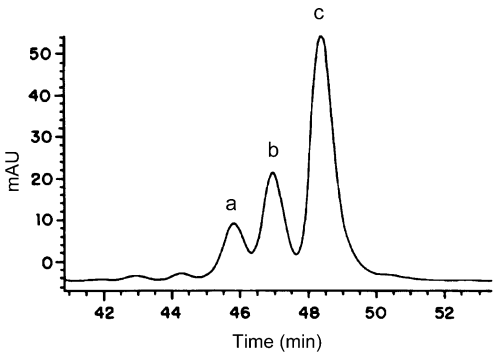



**Fig. 10** Chromatogram obtained for the diterpenes **15–17**, isolated from the seed pods of *Hymenaea courbaril* var. *stilbocarpa*. Chromatographic conditions: column Spherisorb ODS (end-capped, 5 μm, 10 × 250 mm); mobile phase MeOH–water–HCOOH (85 : 15 : 1); UV detection at 240 nm; flow-rate at 2 ml $min^{-1}$. a = (–)-(5*R*,8*S*,9*S*,10*R*)-cleroda-3,13*E*-dien-15-oic acid (**15**); b = methyl (–)-(5*S*,8*S*,9*S*,10*R*)-cleroda-3,13*E*-dien-15-oate (**16**); c = methyl (–)-kovalenate (**17**). Reprinted from *J. Liquid Chromatogr. Relat. Technol.* (http://www.informaworld.com), with permission from Taylor & Francis.[212]

eluted with methanol–water–acetic acid (89 : 10 : 1, v/v) resulting in a methanol extract from which six major anthocyanins were isolated by prep HPLC using a Supelcosil $C_{18}$ column (21.2 × 250 mm, 12 μm). The solvents used were (A) 100% acetonitrile and (B) 1% phosphoric acid, 5% acetic acid, 10% acetonitrile, 5% methanol, and water. The program followed a linear gradient from 0 to 22% A in 35 min. The flow-rate was 10 ml $min^{-1}$.[178]

For the separation of the complex mixtures of structurally related bisbenzylquinoline alkaloids from the roots of *Cissampelos mucronata*, a combination of several types of column chromatography proved to be suitable. In a first step, open CC with normal-phase silica gel and gradient elution with dichloromethane and methanol yielded 17 fractions from the alkaloid-containing root extract. Selected fractions from these were then separated using HPLC on $C_{18}$ material (Spherisorb ODS, 5 μm) with mixtures of methanol, water, and trifluoroacetic acid as eluent. Monomeric isococlaurine was the only compound isolated in pure form at the end of this phase of separation; all other 15 alkaloids were isolated only after at least one further HPLC separation was completed using a CN phase (Eurospher-100 CN, 7 μm). The great advantage of the CN phase over normal- and reversed-phase material is that it can be used in either mode depending on the eluents employed. In this case it was used with lipophilic and hydrophilic eluent mixtures, enabling diastereomeric and enantiomeric compounds to be separated.[203]

Two secoiridoid glycosides, swertiamarin and sweroside, were isolated from the aerial parts of *Centaurea erythraea*. The methanol extract was run through a Sep-Pak $C_{18}$ cartridge with 100% methanol to remove any non-polar material. Prep HPLC (Luna $C_{18}$, 10 μm) was performed using a linear gradient of acetonitrile–water (20 : 80) to (0 : 100) over 30 min, followed by 100% acetonitrile for 10 min with a flow-rate of 20 ml $min^{-1}$.[213]

Four isomeric saponins (escins and isoescins) were purified and isolated from a crude extract of the seeds of *Aesculus chinensis* by prep HPLC. The water-soluble fraction of an extract, obtained by solvent extraction and partition between ethyl acetate and water, was subjected to a D-101 macroreticular resin

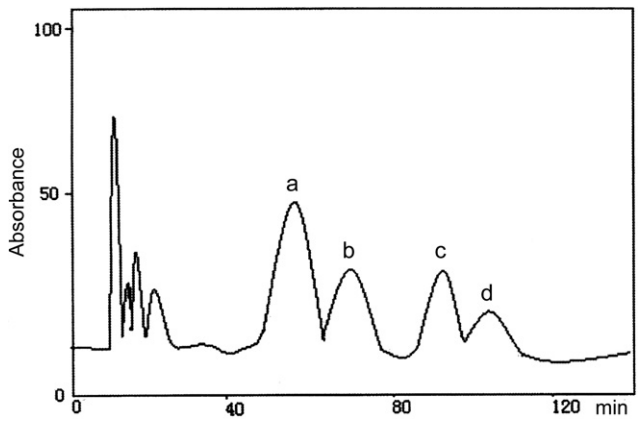
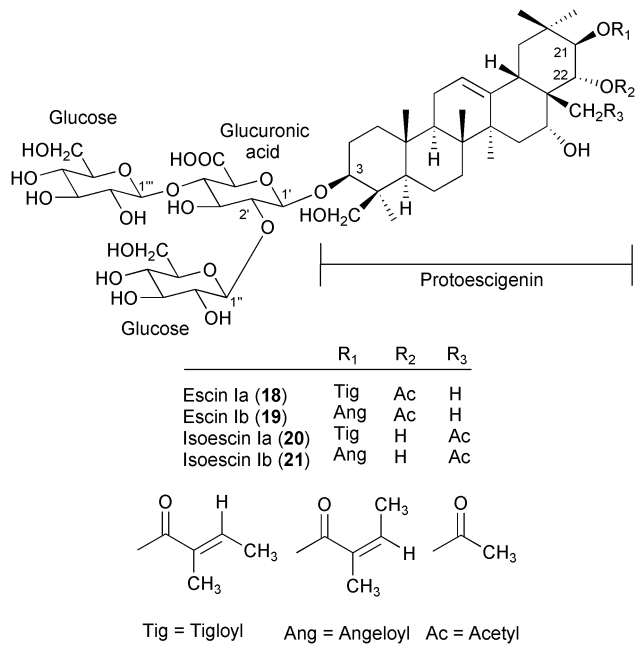
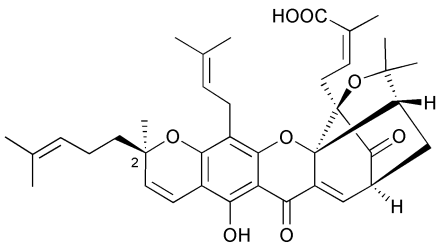



**Fig. 11** Chromatogram obtained of four isomeric escins isolated from the seeds of *Aesculus chinensis* by preparative HPLC. Chromatographic conditions: column $C_{18}$ (5 μm); mobile phase methanol–0.1% aqueous acetic acid (20 : 80) gradient; flow-rate = 20 ml $min^{-1}$. a = escin Ia (**18**); b = escin Ib (**19**); c = isoescin Ia (**20**); d = isoescin Ib (**21**). Reprinted from *J. Liquid Chromatogr. Relat. Technol.* (http://www.informaworld.com), with permission from Taylor & Francis.[210]

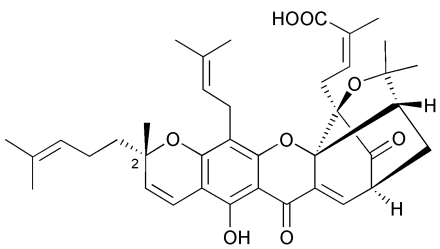
column and eluted successively with water, 30%, 70% and 95% ethanol, giving four fractions. The fraction obtained with 70% ethanol was evaporated to dryness. A 50 g quantity of the crude extract (containing 80% saponins) was dissolved in methanol–water (1 : 5, v/v) to get a sample solution, which contained about 100 mg $ml^{-1}$ saponins. Then, every 20 ml sample solution was injected and purified by prep HPLC on $C_{18}$ material (5 μm) using methanol–0.1% aqueous acetic acid (20 : 80, v/v) gradient (flow-rate: 20 ml $min^{-1}$). Four isomeric saponins **18–21** were separated (Fig. 11). The eluates were separated repeatedly by prep HPLC to yield 5.2 g **18** (99.7% purity), 3.8 g **20** (99.5% purity), 2.8 g **19** (99.3% purity, and 1.69 g **21** (99.1% purity).[210]




Gambogic acid, obtained from the resin of various *Garcinia* species, was until recently believed to be an inseparable C-2 epimeric mixture, repeated efforts having been made to separate and determine the two epimers. 90 mg of *Garcinia hanburyi* resin was dissolved in 2 ml acetone and loaded on a prep HPLC column (Altima $C_{18}$, 10 μm) using methanol–0.1% phosphoric acid (90 : 10, v/v) as mobile phase (flow-rate: 1 ml $min^{-1}$) to yield crude gambogic acid. Additional Sephadex LH-20 CC to remove the acid by eluting with water resulted in 35 mg of gambogic acid (mixture of two epimers), which appeared as one peak on a $C_{18}$ column (Altima $C_{18}$, 5 μm) eluting with acetonitrile–acetic acid (90 : 10, v/v). However, it appeared as two completely separated peaks on a $C_8$ column (Altima $C_8$, 5 μm) eluting with acetonitrile–0.1% acetic acid (75 : 25, v/v). The two peaks were separated under the same analytical conditions to yield pure gambogic acid (**22**; *R*-epimer; 12 mg) and pure epigambogic acid (**23**; *S*-epimer; 10 mg).[229]




Gambogic acid (**22**)




Epigambogic acid (**23**)

## 5 Liquid–liquid isolation techniques

Liquid–liquid isolation techniques such as counter-current chromatography (CCC) are all-liquid methods, without solid phases, which rely on the partition of a sample between two immiscible solvents to achieve separation. The relative proportion of solute passing into each of the two phases is determined by the respective partition coefficients. CCC originates from pioneering work by Ito *et al.*[231]

### 5.1 Terminology

The terminology for liquid–liquid isolation techniques is rather confusing. The main terms found in the literature are counter-current chromatography (CCC) and centrifugal partition chromatography (CPC). The first instrument (Sanki, Kyoto, 1982), which consisted of twelve cartridges arranged around the rotor of a centrifuge, was called the centrifugal counter-current chromatograph (CCCC). This resulted in confusion with the patent series of two-axis gyration apparatus, called CCC. Although neither instrument involves true counter-current motion, since one phase is kept stationary by centrifugal force, the more appropriate name CPC was adopted in 1986 as a generic name

for one-axis centrifugal systems. The term "counter-current" remained for the numerous designs of two axis-instruments invented by Ito.[232] In this review, the term CCC is mainly used, which is accepted worldwide for all separation techniques using a support-free liquid stationary phase,[233] for both technologies.

### 5.2 Instruments and advantages of counter-current chromatography

All modern CCC apparatuses use a centrifugal field to maintain one of the liquid phases in the "column", acting as the stationary phase. The other liquid phase is pumped through it and thus acts as the mobile phase. Two types of CCC apparatuses, hydrodynamic and hydrostatic machines, are commercially available. The hydrodynamic CCC machines use a variable-gravity field produced by a two-axis gyration mechanism and a rotary seal-free arrangement for the column (spools containing coiled PTFE tubes). Due to the planetary motion of the apparatus spools, the centrifugal field changes in intensity and direction. When the centrifugal field is high, phase decantation occurs, and when the centrifugal field direction reverses, the separated liquid phases mingle in an emulsion-like state, so alternating decantation and mixing zones appear in the spool. These apparatuses, mainly developed by Ito and co-workers, are referred to as CCC instruments (see Section 5.1). The hydrostatic CCC machines use a constant-gravity field produced by a single-axis rotation mechanism and two rotary seal joints as the inlet and outlet for the mobile phase. The column itself consists of a series of discrete partition cells engraved in the rotor and connected by ducts in a cascade. The mobile phase is pumped from cell to cell and flows through the stationary phase in the centrifugal direction if it is the denser phase (this operating mode is called the descending mode) or in the centripetal direction if it is the less dense phase (the ascending mode). Hydrostatic CCC apparatuses, mainly developed by Nunogaki (Sanki Engineering, Japan) are usually named CPC instruments.[234] For details, see the books by Ito and Conway[235] and by Berthod.[236]

CCC has several advantages over the more traditional liquid–solid separation methods: (i) no irreversible adsorption of the sample; (ii) quantitative recovery of the injected sample; (iii) tailing is minimised; (iv) low risk of sample denaturation; (v) low solvent consumption; (vi) the technique is very economical (after the initial investment in an instrument, no expensive columns are required and only common solvents are used). Although the efficiency cannot match that of HPLC, it is more than compensated by the high selectivity and the high ratio of stationary to mobile phase. In HPLC, around 20% of the volume of the column is the stationary (bonded) phase around the silica support, available for interaction with the solute. In CCC the ratio of stationary phase content can be as high as 80%. An additional advantage of CCC is the ability to reverse the flow direction and interchange the mobile and stationary phases (reversed-phase or dual-mode operation).[237]

CCC has evolved rapidly in the last decade from the initial, time-consuming applications with droplet counter-current chromatography (DCCC) and rotation locular counter-current chromatography (RLCC) to the new generations of instruments, referred to as high-speed counter-current chromatography (HSCCC) and high-performance (or fast centrifugal) partition chromatography (HPCPC or FCPC). Since the 1980s CCC has gained more and more popularity as an isolation tool for natural products, with a peak in 2005. Both crude extracts and semi-pure fractions can be chromatographed with sample loads ranging from milligrams to grams. For reports on the CCC techniques used in the isolation of natural products, see various reviews (*e.g.* ref. 1,238–245) and books (*e.g.* ref. 3,235,236). HSCCC is discussed in detail in Section 5.3, and recent examples of isolation of mainly plant-derived natural products are presented.

### 5.3 High-speed counter-current chromatography (HSCCC)

HSCCC is a CCC method radically improved in terms of resolution, separation time and sample loading capacity. HSCCC yields a highly efficient separation of multi-gram quantities of samples in several hours. It is an efficient preparative technique, and widely used for separation and purification of natural products. However, it requires some simple but specific technical knowledge, since the selection of experimental conditions and the practical separation procedure are quite unique.[244]

A practical and effective strategy for a step by step selection of HSCCC conditions including the selection of two-phase solvent systems, determination of partition coefficient ($K$) of analytes, preparation of two-phase solvent system and sample solution, selection of elution mode, flow-rate, rotation speed, and on-line monitoring of the eluate, is presented by Ito.[244] The selection of a suitable solvent system is the most important step in CCC method development and may be estimated as 90% of the entire work. In contrast to conventional liquid chromatography, the CCC technique uses a two-phase solvent system made of a pair of mutually immiscible solvents, one used as the stationary phase and the other as the mobile phase. The use of two-phase solvent systems results in an enormous number of possible combinations of solvents to choose from, enabling separation of compounds with a wide range of polarities. The selected solvents should satisfy the following requirements: (i) the analyte(s) should be stable and soluble in the system; (ii) the solvent system should form two phases with an acceptable volume ratio to avoid wastage; (iii) the solvent system should provide a suitable $K$ value to the analytes (suitable $K$ values for HSCCC are $0.5 \leq K \leq 1.0$); (iv) the solvent system should yield satisfactory retention of the stationary phase in the column.

Additionally, various scales for selection of appropriate biphasic solvent systems have been reported in the literature, such as the Arizona liquid system[246] or the GUESS approach.[247,248]

**5.3.1 Examples of natural product isolation by HSCCC.** A *selection* of recent research on natural products is summarised in Table 12 (the literature between 2000 and 2007 includes several hundred papers), followed by some key examples regarding the various elution modes or methods. Although CCC has been shown to be a powerful tool in the preliminary stages of crude extract fractionation, examples of this kind of work are not listed in Table 12.

Extracts from natural products usually contain a high number of different compounds with a broad range of hydrophobicity. Most often, only one or two compounds can be separated from the others using a single solvent system by one-step elution.

**Table 12** Selected recent applications of HSCCC/HPCPC to natural product isolation from medicinal plants and algae. For the complete version of this table, containing further references appearing in Section 8 (References),[249–380] see ESI (Table S2)†

| Compounds | Matrix[a] | Solvent systems (volume ratio)[b] | Mode[c] | MP[d] | Ref.[e] |
|---|---|---|---|---|---|
| ***Flavonoids*** | | | | | |
| Flavonoids | *Ampelopsis grossedentata*, leaf | Hex–EtOAc–MeOH–water (1 : 6 : 1.5 : 7.5) | — | LP | 249 |
| Anthocyanins (sambubiosides) | *Vaccinium myrtillus*, fruit | MtBE–BuOH–$CH_3CN$–water–TFA (1 : 4 : 1 : 5 : 0.01) | — | LP | 251 |
| Catechin, stilbene derivative | *Rheum tanguticum*, root and rhizome | EtOAc–EtOH–water (25 : 1 : 25) → (5 : 1 : 5) | Stepwise | LP | 253 |
| Baicalein, wogonin, oroxylin A | *Scutellaria baicalensis*, root | Hex–EtOAc–BuOH–water (1 : 1 : 8 : 10) | Stepwise | LP | 256 |
| Baicalin, wogonoside | *Scutellaria baicalensis*, root | EtOAc–MeOH–1% AcOH (5 : 0.5 : 5) | — | LP | 257 |
| Flavonoid glycosides | *Trollius ledebouri*, herb | EtOAc–BuOH–water (2 : 1 : 3) | — | LP | 183 |
| Flavonoids | *Oroxylum indicum*, seed | Hex–EtOAc–MeOH–water (1 : 1.2 : 1 : 1), (1 : 2 : 1 : 1), (1 : 8 : 1 : 8) | — | LP | 262 |
| Liquiritigenin, isoliquiritigenin | *Glycyrrhiza uralensis*, root | Hex–EtOAc–MeOH–$CH_3CN$–water (2 : 2 : 1 : 0.6 : 2) | — | LP | 267 |
| Flavonoid glycosides, phloroglucinol derivatives | *Hypericum japonicum*, herb | EtOAc–EtOH–water (5 : 1 : 5); Hex–EtOAc–EtOH–water (1 : 1.2 : 1.2 : 1) | Stepwise; 2-step | LP | 269 |
| Hyperoside | *Hypericum perforatum*, herb | EtOAc–EtOH–water (5 : 1 : 5) | — | LP | 270 |
| Flavonoids | *Patrinia villosa*, herb | Hex–EtOAc–MeOH–water (5 : 6 : 6 : 6) | Stepwise | LP | 28 |
| Xanthohumol | *Humulus lupulus*, hop cones | Hept–toluene–acetone–water (24.8 : 2.8 : 50 : 22.4) | Dual mode | UP, LP | 275 |
| Casticin | *Artemisia annua*, leaf | Hex–EtOAc–MeOH–water (7 : 10 : 7 : 10) | — | LP | 277 |
| Hyperosid, luteolin glucoside | *Agrimonia pilosa* | EtOAc–MeOH–water (50 : 1 : 50) → (5 : 1 : 5) | Stepwise | LP | 278 |
| Biflavonoids | *Garcinia kola*, seed | Hex–EtOAc–MeOH–water (3 : 5 : 3.5) | Stepwise | LP | 281 |
| Prenylflavonoids | *Artocarpus altilis* | Hex–EtOAc–MeOH–water (5 : 5 : 7 : 3), (5 : 5 : 6.5 : 3.5) | MDCCC | LP | 283 |
| ***Coumarins*** | | | | | |
| Osthol, xanthotoxol | *Cnidium monnieri*, fruit | Hex–EtOAc–MeOH–water (1 : 1 : 1 : 1) → (5 : 5 : 6 : 4) | Stepwise | LP | 285 |
| Psoralen, isopsoralen | *Psoralea corylifolia*, fruit | Hex–EtOAc–MeOH–water (1 : 0.7 : 1 : 0.8) | — | LP | 23 |
| Coumarins | *Peucedanum praeruptorum*, root | LtPet–EtOAc–MeOH–water (5 : 5 : 5 : 5) → (5 : 5 : 6.5 : 3.5) | Grad | LP | 287 |
| Coumarins | *Cnidium monnieri* | LtPet–EtOAc–MeOH–water (5 : 5 : 5 : 5) → (5 : 5 : 6 : 4) → (5 : 5 : 6.5 : 3.5) | Stepwise | LP | 288 |
| Coumarins | *Angelica dahuria*, root | Hex–MeOH–water (5 : 5 : 5) → (5 : 7 : 3) | Grad | LP | 289 |
| Bergapten, imperatorin | *Cnidium monneri*, fruit | Hex–EtOAc–EtOH-water (5 : 5 : 5 : 5) | Stepwise | LP | 290 |
| Coumarins | *Cnidium monnieri*, fruit | Hex–EtOAc–EtOH–water (5 : 5 : 4 : 6) → (5 : 5 : 6 : 4) | Stepwise | LP | 291 |
| Coumarins | *Angelica dahurica*, herb | Hex–EtOAc–MeOH–water (1 : 1 : 1 : 1), (5 : 5 : 4.5 : 5.5) | MDCCC | LP | 295 |
| Coumarins | *Stellera chamaejasme*, root | Hex–EtOAc–MeOH–water (10 : 13 : 13 : 10) | Stepwise | LP | 27 |
| ***Phenolic compounds*** | | | | | |
| Honokiol, magnolol | *Magnolia officinalis*, bark | Hex–EtOAc–MeOH–water (1 : 0.4 : 1 : 0.4) | — | LP | 299 |
| Cannabinoids | *Cannabis sativa*, leaf | Hex–MeOH–water (5 : 3 : 2) acidified with 25 mM formic acid; linear grad MeOH–water from (3 : 2) to (4.5 : 0.5) | Grad | LP | 303 |
| Kava lactones | *Piper methysticum*, root | Hex–EtOAc–MeOH–water (6 : 5 : 6 : 5) | — | LP | 304 |
| Phenylethanoid- and iridoid glycosides | *Stachytarpheta cayennensis*, root | EtOAc–BuOH–water (1:X : 1); X = 0.05 → 0.2 → 0.5 → 1.0 | Step-grad | LP | 308 |
| Prenylated phenolics | *Humulus lupulus*, hop cones | Hex–EtOAc–MeOH–water (8 : 2 : 8 : 2), (6 : 4 : 6 : 4), (5 : 5 : 5 : 5) | Sample cutting | LP | 135 |
| Salvianolic acids | *Salvia miltiorrhiza*, root | Hex–EtOAc–MeOH–water (1.5 : 5 : 1.5 : 5) | — | LP | 310 |
| Salvianolic acid B | *Salvia miltiorrhiza* | 36% PrOH–8% phosphate system with the ratio between $NaH_2PO_4$:$K_2HPO_4$ = 6 : 94 | — | UP | 311 |
| Ferulic acid | *Angelica sinensis*, root | Hex–EtOAc–MeOH–water (3 : 7 : 5 : 5) | — | LP | 70 |
| Honokiol, magnolol | *Magnolia officinalis*, bark | LtPet–EtOAc–$CCl_4$–MeOH–water (1 : 1 : 8 : 6 : 1) | — | LP | 201 |
| Honokiol, magnolol | *Magnolia officinalis*, bark | Hex–EtOAc–MeOH–water (1 : 0.4 : 1 : 0.4) | — | UP | 315 |
| Isomeric polyphenols | *Parthenocissus laetevirens*, root | Hex–EtOAc–MeOH–water (1 : 2 : 1 : 2) | MDCCC | LP | 318 |
| ***Alkaloids*** | | | | | |
| Protoberberine alkaloids | *Enantia chlorantha*, bark | $CH_2Cl_2$–MeOH-water (48 : 16 : 36) containing $KClO_4$ (1st run) or NaOH (2nd run) | 2 step, dual mode | LP, UP | 234 |
| Ungeremine | *Nerine bowdenii*, bulb | EtOAc–MeOH–water (45 : 20 : 35) | — | UP | 205 |
| Diterpene alkaloids | *Aconitum coreanum*, root | Hex–EtOAc–MeOH–0.2 M HCl (1 : 3.5 : 2 : 4.5) | — | LP | 324 |
| Quinolizidine alkaloids | *Sophora flavescens*, root | $CHCl_3$–MeOH–$2.3 \times 10^{-2}$ M $NaH_2PO_4$ (27.5 : 20 : 12.5) | — | LP | 29 |

**Table 12** *(Contd.)*

| Compounds | Matrix[a] | Solvent systems (volume ratio)[b] | Mode[c] | MP[d] | Ref.[e] |
|---|---|---|---|---|---|
| Verticine, verticinone | *Fritillaria thunbergii*, bulb | $CHCl_3$–EtOH–0.2 mol $L^{-1}$ HCl (3 : 2 : 2) | — | LP | 325 |
| Benzylisoquinoline alkaloids | *Coptis chinensis* | Hex–EtOAc–MeOH–1% AcOH (1 : 1 : 1 : 1) | — | LP | 326 |
| Sesquiterpene alkaloids | *Tripterygium wilfordii*, root | PE–EtOAc–EtOH–water (6 : 4 : 5 : 8) | — | LP | 327 |
| ***Anthraquinones*** | | | | | |
| Anthraquinones and phenolic compounds | *Polygonum cuspidatum*, root | LtPet–EtOAc–MeOH–water (2 : 5 : 4 : 6)→ I + II<br>I: LtPet–EtOAc–water (1 : 5 : 5)<br>II: LtPet–EtOAc–MeOH–water (3 : 5 : 4 : 6) → (3 : 5 : 7 : 3) | Grad | LP | 329 |
| Anthraquinones | *Polygonum multiflorum*, root | Hex–EtOAc–MeOH–water (3 : 7 : 5 : 5 → (9 : 1 : 5 : 5)<br>EtOAc–MeOH–water (50 : 1 : 50)<br>EtOAc–BuOH–water (20 : 1 : 20) | Stepwise | LP | 330 |
| Aloin A and B | *Aloe vera*, Aloe powder | $CHCl_3$–MeOH–water (4 : 2 : 3), EtOAc–MeOH–water (5 : 1 : 5), BuOH–EtOAc–water (1 : 3 : 4) | — | LP | 332 |
| ***Lignans*** | | | | | |
| Schizandrin, gomisin A | *Schisandra chinensis*, fruit | Hex–EtOAc–MeOH–water (1 : 0.9 : 0.9 : 1) | — | LP | 335 |
| Deoxyschisandrin, γ-schisandrin | *Schisandra chinensis*, fruit | Hex–MeOH–water (35 : 30 : 3) | — | LP | 336 |
| ***Saponins*** | | | | | |
| Saponin and flavonoid glycosides | *Clinopodium chinensis*, herb | EtOAc–BuOH–water (5 : 0.8 : 5)<br>EtOAc–MeOH–water (5 : 1 : 5) | 2-step | LP | 344 |
| Ginsenosides | *Panax ginseng*, root | $CH_2Cl_2$–MeOH–iPrOH–water (6 : 6 : 1 : 4) | — | LP | 345 |
| Triterpene saponins | *Clematis mandshurica*, root and rhizome | EtOAc–BuOH–EtOH–0.5% TFA (5 : 10 : 2 : 20) | — | LP | 346 |
| ***Terpenoids*** | | | | | |
| Celastrol | *Celastrus orbiculatus*, root | LtPet–EtOAc–$CCl_4$–methanol–water (1 : 1 : 8 : 6 : 1) | — | LP | 146 |
| Rupestonic acid | *Artemisia rupestris*, root | Hex–EtOAc–MeOH–water (6 : 4 : 3.5 : 6.5) with 0.5% AcOH in starionary phase | 2-step | LP | 350 |
| Costunolide, dehydrocostuslactone | *Aucklandia lappa*, root | LtPet–MeOH–water (5 : 6.5 : 3.5) | — | LP | 351 |
| Triterpenoids | *Adenophora tetraphylla*, root | Hex–EtOAc–$CH_3CN$ (5 : 1 : 5), (5 : 1 : 4), (5 : 2 : 5)<br>LtPet–EtOAc–EtOH–water (6 : 4 : 5 : 5) | — | LP | 360 |
| Diterpenoids (oridonin, ponicidin) | *Rabdosia rubescens* | Hex–EtOAc–MeOH–water (1 : 5 : 1 : 5), (3 : 5 : 3 : 5) | MDCCC | LP | 362 |
| Sesquiterpene lactones | *Xanthium macrocarpum*, leaf | Hept–EtOAc–MeOH–water (1 : 1 : 1 : 1) | — | UP | 364 |
| ***Miscellaneous*** | | | | | |
| Shikonin | *Lithospermum erythrorhizon*, root | Hex–EtOAc–EtOH–water (16 : 14 : 14 : 5) | — | LP | 365 |
| Tripdiolide | *Tripterygium wilfordii*, herb | Hex–$CH_2Cl_2$–MeOH–water (3 : 22 : 17 : 8), $CHCl_3$–MeOH–water (4 : 3 : 2) | MDCCC | LP | 368 |
| Aurentiamide acetate | *Patrinia villosa* | Hex–EtOAc–MeOH–water (1 : 1.2 : 1.2 : 1) | — | LP | 26 |
| Atractylon, atractylenolide III | *Atractylodes macrocephala*, root | LtPet–EtOAc–EtOH–water (4 : 1 : 4 : 1) | Dual mode | LP, UP | 371 |
| Mangiferin | *Anemarrhena asphodeloides*, rhizome | EtOAc–iPrOH–water (3 : 2 : 5) | — | LP | 374 |
| Gambogic acid, epigambogic acid | *Garcinia hanburyi* | Hex–MeOH–water (5 : 4 : 1) | MDCCC | LP | 377 |
| Mangiferin, neomangiferin, 5-HMF | *Anemarrhena asphodeloides* | BuOH–water (1 : 1) | Stepwise | LP | 378 |

[a] Systematic plant name and crude drug. [b] AcOH: acetic acid; $CH_3CN$: acetonitrile; BuOH: 1-butanol; $CHCl_3$: chloroform; $CH_2Cl_2$: methylene chloride; $CCl_4$: carbon tetrachloride; EtOAc: ethyl acetate; EtOH: ethanol; Hept: heptane; Hex: n-hexane; iPrOH: isopropanol; LtPet: light petroleum; MtBE: methyl *tert*-butyl ether; MeOH: methanol; PE: petroleum ether; PrOH: *n*-propanol; TFA: trifluoroacetic acid. [c] 2-step: two-step elution using either twice the same, or different solvent systems. Between 2 steps, the sample is dried and re-dissolved. Stepwise: either by changing the solvent (→) or by increasing the flow-rate of the solvent. Grad: linear gradient elution. Step-grad: step gradient elution (→). Dual mode: dual-mode elution (LP → UP, or reversed). MDCCC: multidimensional CCC. [d] LP: lower phase; MP: mobile phase; UP: upper phase. [e] References: 2004–2007.

Very often, a silica gel clean-up chromatography before separation by HSCCC or a final purification by preparative HPLC is necessary. On the other hand, HSCCC is also applied for final purification of semi-crude samples. When two peaks overlap in CCC separation, it is common practice that each peak is pooled, dried and rechromatographed with the same or a slightly modified solvent system to improve the yield and purity of a target compound (two-step elution). In order to separate compounds with a larger difference in hydrophobicity and shorten the separation time, stepwise elution and gradient elution are applied. Additionally, dual-mode elution, multidimensional HSCCC (MDHSCCC), high-capacity HSCCC

(HCHSCCC), three-phase solvent systems, pH-zone refining and ion-exchange displacement CCC are common. Examples for each of these possibilities are presented below. Further recently developed methods, such as elution-extrusion CCC[381–383] and the cocurrent CCC,[384] are not discussed in this review, as to date they have been applied for the validation of methods using various model compounds only.

#### *5.3.1.1 One-step, two-step, stepwise and gradient elution modes.*

• *One-step elution*: Preparative isolation of monomeric anthocyanin glycosides by HSCCC requires solvent systems of high polarity such as methyl *tert*-butyl ether–1-butanol–acetonitrile–water–trifluoroacetic acid (1 : 4 : 1 : 5 : 0.01, v/v), as was used for the isolation of two sambubiosides from a crude extract of bilberry (*Vaccinium myrtillus*) (Fig. 12). The principal advantage of HSCCC for anthocyanin separation is the elution of other much more polar matrix constituents, *i.e.* oligomeric and polymeric proanthocyanidins as well as polysaccharides, immediately from the HSCCC coil system due to a lower stationary phase affinity. The study reported by Du *et al.*[251] demonstrates that a single chromatographic separation by HSCCC is able to yield pure anthocyanin-3-*O*-disaccharides from a complex matrix of natural products on a preparative scale. In this case, for the recovery of anthocyanins, time-consuming clean-up procedures before HSCCC separation (*i.e.* size-exclusion chromatography on Sephadex LH-20, or adsorbance to Amberlite XAD-7 resin material) was not necessary. For the separation of the two compounds from a 500 mg sample, only 500 ml of the lower mobile phase was consumed, whereas 18 l of 30% methanol are necessary for the separation of the two compounds by preparative HPLC.

HSCCC is frequently used for final purification of semi-crude extracts. Examples are betulinic acid and epigallocatechin (EGC). The purification of betulinic acid normally requires multiple-stage cleaning by complex procedures, involving column or thin-layer chromatography. A high-yield of betulinic acid (up to 17% from the ethanolic extract) was found in the leaves of *Eugenia florida*. Semi-crude leaf extracts were subjected to HSCCC using *n*-hexane–ethyl acetate–methanol–water (10 : 5 : 2.5 : 1, v/v) to yield betulinic acid with up to 98% purity.[385] Degallation of epigallocatechin gallate (EGCG) by tannase at 35 °C yielded a mixture of EGC and gallic acid.

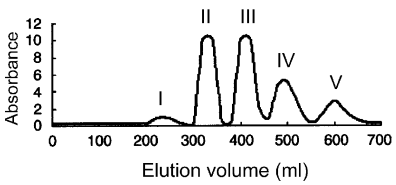



**Fig. 12** HSCCC chromatogram of 500 mg crude extract from bilberry fruit. Two-phase solvent system: MtBE–BuOH–$CH_3CN$–water–TFA (1 : 4 : 1 : 5 : 0.01, v/v); SP: UP; MP: LP; flow-rate: 1.5 ml $min^{-1}$; fraction II = 130 mg of delphinidin-3-*O*-sambubioside, fraction III = 77 mg of cyanidin-3-*O*-sambubioside. Reprinted from Q. Du, G. Jerz and P. Winterhalter, 'Isolation of two anthocyanin sambubiosides from bilberry (*Vaccinium myrtillus*) by high-speed counter-current chromatography', *J. Chromatogr., A*, 2004, **1045**, 59–63. Copyright (2004), with permission from Elsevier.[251]

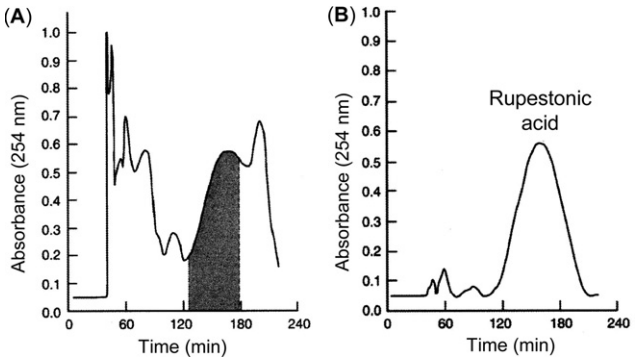


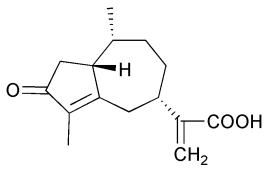

**Fig. 13** HSCCC separations of a crude extract from *Artemisia rupestris*. Solvent system: Hex–EtOAc–MeOH–water (6 : 4 : 3.5 : 6.5, v/v) with 0.5% AcOH in the SP (UP); MP: LP; flow-rate: 2 ml $min^{-1}$; (A) 200 mg of crude extract, (B) HSCCC fraction corresponding to the rupestonic acid peak (shaded) of (A), dried and redissolved. Reprinted from Y. Ma, H. A. Aisha, L. Liao, S. Aibai, T. Zhang and Y. Ito, 'Preparative isolation and purification of rupestonic acid from the Chinese medicinal plant *Artemisia rupestris* L. by high-speed counter-current chromatography', *J. Chromatogr., A*, 2005, **1076**, 198–201. Copyright (2005), with permission from Elsevier.[350]

The separation of these two compounds was performed by HSCCC using *n*-hexane–ethyl acetate–water (1 : 9 : 10, v/v) as a two-phase solvent system. After degallation and HSCCC separation, 290 mg of EGC with a purity of 97% was obtained from 500 mg EGCG. These results demonstrate that EGC can be successfully prepared by degallation of EGCG with tannase, and completely recovered by preparative HSCCC separation with high purity.[386]

• *Two-step elution*: A two-step HSCCC procedure using *n*-hexane–ethyl acetate–methanol–water (6 : 4 : 3.5 : 6.5, v/v) as a two-phase solvent system with 0.5% acetic acid in the stationary phase was applied for the separation of the sesquiterpene rupestonic acid (**24**) from 200 mg of a crude extract from the roots of *Artemisia rupestris* (Fig. 13). After the first separation step (Fig. 13A), the fractions containing **24** (shaded peak) were collected, dried, redissolved and purified by a second HSCCC step with the same solvent system (Fig. 13B). This second separation step yielded 27.9 mg of **24** at more than 98% purity.[350] A similar two-step HSCCC procedure but with different solvents was applied for the separation of two flavone glycosides and a saponin from *Clinopodium chinensis*. In this case, ethyl acetate–1-butanol–water (5 : 0.8 : 5, v/v) was used as the two-phase solvent system in the first step; nairutin was purified, didymin and clinopodiside A were eluted together. In the second step, after collection of the fractions and drying, ethyl acetate–methanol–water (5 : 1 : 5, v/v) was used as the solvent system; didymin and clinopodiside A were separated and purified. The two-step separation yielded 15 mg of nairutin, 39.1 mg of clinopodiside A and 20.6 mg of didymin from 100 mg of crude extract with purities of 96.5%, 98.4% and 99.1%, respectively.[344]

• *Stepwise elution*: In order to separate several different compounds, stepwise elution or increasing the flow-rate of the mobile phase might be chosen. A preparative HSCCC method was applied to isolate the two coumarins osthol and xanthotoxol from a crude fruit extract from *Cnidium monneri* by stepwise




Rupestonic acid (**24**)

elution using a pair of two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water (1 : 1 : 1 : 1 and 5 : 5 : 6 : 4, v/v) (Fig. 14). 308 mg of the crude extract yielded 88.3 mg of osthol and 19.4 mg of xanthotoxol at a high purity of over 98%.[285] From the fruit extract of the same plant the two coumarins bergapten and imperatorin were isolated using the two-phase solvent system *n*-hexane–ethyl acetate–ethanol–water (5 : 5 : 5 : 5, v/v) by stepwise increasing the flow-rate of the mobile phase. This one-step separation of 500 mg crude extract yielded 45.8 mg of bergapten at 96.5% purity and 118 mg imperatorin at 98.5% purity.[290] Five coumarins in total could be isolated from the crude extract of *C. monneri* by stepwise elution using three different ratios of the two-solvent system light petroleum–ethyl acetate–methanol–water: 5 : 5 : 5 : 5 (v/v) in the first 150 min, 5 : 5 : 6 : 4 (v/v) in the second 100 min, and finally 5 : 6 : 6.5 : 3.5 (v/v). HSCCC of 150 mg crude sample thus yielded 7.6 mg of xanthotoxol, 7.6 mg of isopimpinellin, 9.7 mg of bergapten, 60.5 mg of imperatorin, and 50.6 mg of osthol with purities of 95.0%, 99.6%, 99.7%, 100.0% and 100.0%, respectively.[288]

- *Gradient elution*: HSCCC isolation and purification of coumarins from a crude extract of *Peucedanum praeruptorum* by using light petroleum–ethyl acetate–methanol–water at volume ratios of 5 : 5 : 5 : 5 and 5 : 5 : 6.5 : 3.5 were used in gradient elution mode (Fig. 15). Four kinds of coumarin and an unknown compound were obtained from a 110 mg sample and yielded 5.3 mg of qianhucoumarin D (**25**), 7.7 mg of Pd-Ib (**26**), 35.8 mg of (+)-praeruptorin A (**27**), 31.9 mg of (+)-praeruptorin B (**28**) and 6.4 mg of the unknown compound with purities of 98.6%, 92.8%, 99.5%, 99.4% and 99.8% in a one-step separation.[287]

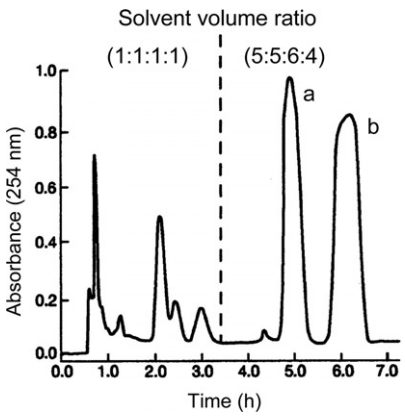



**Fig. 14** Preparative HSCCC separation of a crude fruit extract from *Cnidium monneri*. Solvent system: Hex–EtOAc–MeOH–water (1 : 1 : 1 : 1, v/v) and (5 : 5 : 6 : 4, v/v); SP: UP; MP: LP; flow-rate: 1 ml $min^{-1}$. The separation was started with the 1 : 1 : 1 : 1 solvent system and, after most of the polar impurities had been eluted (3 h and 20 min at the dotted line), the MP was switched to the 5 : 5 : 6 : 4 solvent. a = xanthotoxol, b = osthol. Reprinted from Y. Wei, T. Zhang and Y. Ito, 'Preparative isolation of osthol and xanthotoxol from Common Cnidium fruit (Chinese traditional herb) using stepwise elution by high-speed counter-current chromatography', *J. Chromatogr., A*, 2004, **1033**, 373–377. Copyright (2004), with permission from Elsevier.[285]

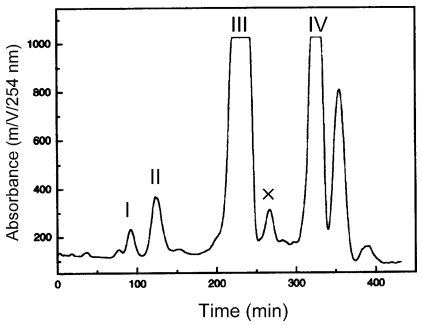
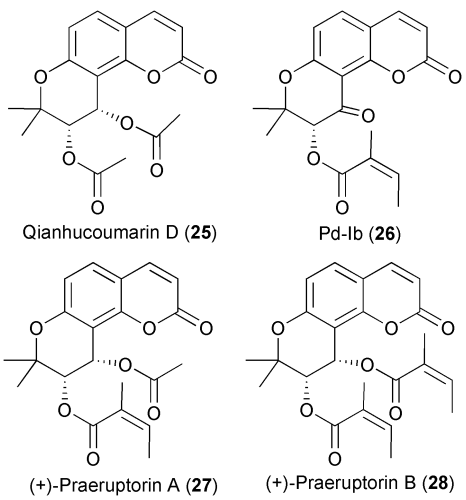



**Fig. 15** HSCCC chromatogram of a crude extract from *Peucedanum praeruptorum*. Solvent system: SP = UP of LtPet–EtOAc–MeOH–water (5 : 5 : 5 : 5, v/v); MP = LP of the same solvent system, volume ratios 5 : 5 : 5 : 5 and 5 : 5 : 6.5 : 3.5 in gradient elution mode; flow-rate: 2 ml $min^{-1}$. The volume ratios were changed as follows: 0–150 min, 100 : 0; 150–300 min: 100 : 0 to 0 : 100; after 300 min, 0 : 100. Flow-rate: 2 ml $min^{-1}$. I = qianhucoumarin D (**25**), II = Pd-Ib (**26**), III = (+)-praeruptorin A (**27**), IV = (+)-praeruptorin B (**28**), × = unknown compound. Reprinted from R. Liu, L. Feng, A. Sun and L. Kong, 'Preparative isolation and purification of coumarins from *Peucedanum praeruptorum* Dunn by high-speed counter-current chromatography', *J. Chromatogr., A*, 2004, **1057**, 89–94. Copyright (2004), with permission from Elsevier.[287]




***5.3.1.2 Dual-mode elution.*** The CCC technique allows fractionation to be carried out in a normal-phase mode, followed by a reversed-phase mode or *vice versa* during the same run. This is possible because both phases are liquids. In practice, switching the CCC-valve between descending and ascending modes reverses pumping of the stationary/mobile phase. Dual-mode elution allows the fractionation of molecules with very different polarities from complex initial materials such as a crude plant extract with short run-times and without sample loss.[387]

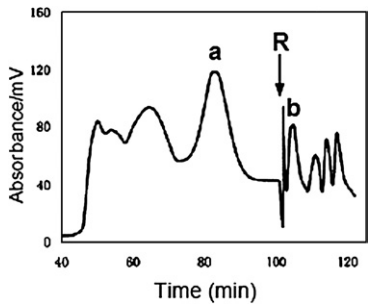
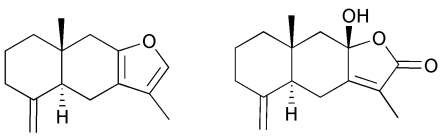



**Fig. 16** Chromatogram of the crude root extract from *Atractylodes macrocephala* by dual-mode HSCCC. Solvent system: LtPet–EtOAc–EtOH–water (4 : 1 : 4 : 1, v/v); flow-rate: 5 ml $min^{-1}$; (a) atractylenolide III (**30**), (b) atractylon (**29**). Phases are reversed at 102 min (R). MP: 0–102 min, LP; 102–125 min, UP. Reprinted from C. Zhao and C. He, 'Preparative isolation and purification of atractylon and atractylenolide III from the Chinese medicinal plant *Atractylodes macrocephala* by high-speed counter-current chromatography', *J. Sep. Sci.*, 2006, **29**, 1630–1636. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.[371]

Atractylon (**29**) and atractylenolide III (**30**) were isolated from a crude root extract of *Actractylodes macrocephala* using the two-phase solvent system light petroleum–ethyl acetate–ethanol–water (4 : 1 : 4 : 1, v/v) in dual-mode elution. Compared with the separation using normal-mode elution, the dual-mode HSCCC elution can be achieved with shorter elution time (Fig. 16). Applying dual-mode elution, the separation started with the organic phase as the stationary phase, and the aqueous mobile phase allowed elution of **30** (peak a in Fig. 16). After about 100 min of separation in the head–tail mode, the elution was reversed to tail–head, and the upper phase was pumped into the column. The phase reversal permitted elution of **29** (peak b in Fig. 16). The separation in the normal-mode HSCCC would take about seven hours, but only about two hours in the dual-mode HSCCC.[371] Recently, a new semi-continuous development mode CCC, named multiple dual-mode (MDM) has been developed. MDM separation consists of a succession of dual-mode runs (*i.e.* multiple inversion of stationary and mobile phase), with or without sample re-injection between each of the runs.[387] The diagrams in Fig. 17 readily explain the principles of MDM separation as applied to two poorly resolved analytes. The main point is the inversion of the elution mode before any collected product becomes impure.




Atractylon (**29**) Atractylenolide III (**30**)

***5.3.1.3 Multi-dimensional counter-current chromatography.*** In 1998, Yang *et al.*[388] developed multidimensional counter-current chromatography (MDCCC) for the separation of isorhamnetin, kaempferol and quercetin from a crude mixture of flavone aglycones of *Ginkgo biloba* and *Hippophae rhamnoides*. The first preparative separations using MDCCC were

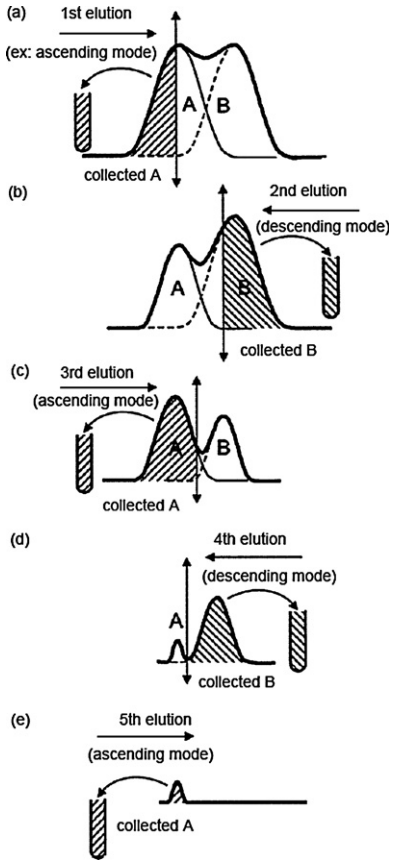



**Fig. 17** Schematic principles of MDM separation of two poorly resolved analytes. Reprinted from E. Delannay, A. Toribio, L. Bourdesocque, J.-M. Nuzillard, M. Zèches-Hanrot, E. Dardennes, G. Le Dour, J. Sapi and J.-H. Renault, 'Multiple dual-mode centrifugal partition chromatography, a semi-continuous development mode for routine laboratory-scale purifications', *J. Chromatogr., A*, 2006, **1127**, 44–51. Copyright (2006), with permission from Elsevier.[387]

reported by Tian *et al.*[389] and Wei *et al.*[390] Recently, MDCCC was successfully used for the isolation and purification of tripdiolide from *Tripterygium wilfordii*,[368] of three coumarins from *Angelica dahurica*[295] as well as of two diterpenoids from *Rabdosia rubescens*.[362]

Wei and Ito[295] applied MDCCC for the isolation and purification of coumarins from *Angelica dahurica* using two preparative identical multilayer coil planet centrifuge units and a pair of two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water at volume ratios of 1 : 1 : 1 : 1 and 5 : 5 : 4.5 : 5.5. A schematic diagram of this MDCCC system is shown in Fig. 18. The chromatograms of this separation are presented in Fig. 19. The crude extract was eluted with the solvent system at a volume ratio of 1 : 1 : 1 : 1 (Fig. 19A). After three and half hours, when peak 1 (shaded) started to elute, the effluent from CCC 1 was cut and introduced into the CCC 2 column. After peak 1 was completely introduced from CCC 1 into CCC 2 (about 30 min), the elution of the cut peak 1 was resumed by pump 2 with the second solvent at a volume ratio 5 : 5 : 4.5 : 5.5. Meanwhile, the rest of the components (peaks 2 and 3), still remaining in the CCC 1 column, were continuously eluted with the solvent system at a volume ratio of 1 : 1 : 1 : 1

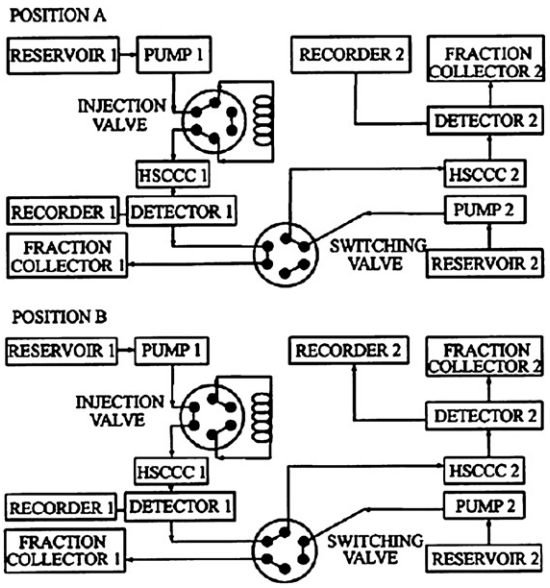
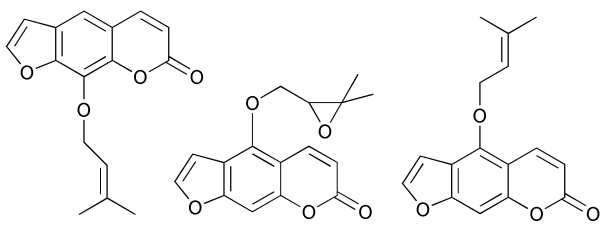



**Fig. 18** Schematic diagram of the repeated HSCCC system with two sets of HSCCC chromatographs. Two constant-flow pumps were used to elute the MP while continuous monitoring of the effluent was achieved with two UV monitors at 254 nm. Two manual six-port valves, one with a 20 ml loop used as the injection valve and the other without loop used as the switching valve, were used to introduce the sample into the column. Two portable recorders were used to draw the chromatogram. Reprinted from Y. Wei and Y. Ito, 'Preparative isolation of imperatorin, oxypeucedanin and isoimperatorin from traditional Chinese herb "bai zhi" *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook using multidimensional high-speed counter-current chromatography', *J. Chromatogr. A*, 2006, **1115**, 112–117. Copyright (2006), with permission from Elsevier.[295]

using pump 1. Fig. 19B shows the chromatogram obtained from CCC 1 yielding 8.6 mg of oxypeucedanin (**32**) and 10.4 mg of isoimperatorin (**33**). The chromatogram in Fig. 19C was obtained by the cut fraction of CCC 1 (the shaded part of the peak 1 in Fig. 19A) introduced into and eluted from the CCC 2 column. This separation yielded 19.9 mg of imperatorin (**31**) at over 98% purity. MDCCC improves both yield and separation time by directly introducing the desired effluent from the first column into the head of the second column, *i.e.* separating it in tandem.



Imperatorin (**31**) Oxypeucedanin (**32**) Isoimperatorin (**33**)

The MDCCC system used by Lu *et al.*[362] differs from the one described earlier in Fig. 18. The authors developed a preparative 2D-CCC system for simultaneous separation and purification of oridonin (**34**) and ponicidin (**35**) from the crude extract of *Rabdosia rubescens* using a high-speed CCC (HSCCC) instrument in the first dimension (1st-D) and a preparative upright

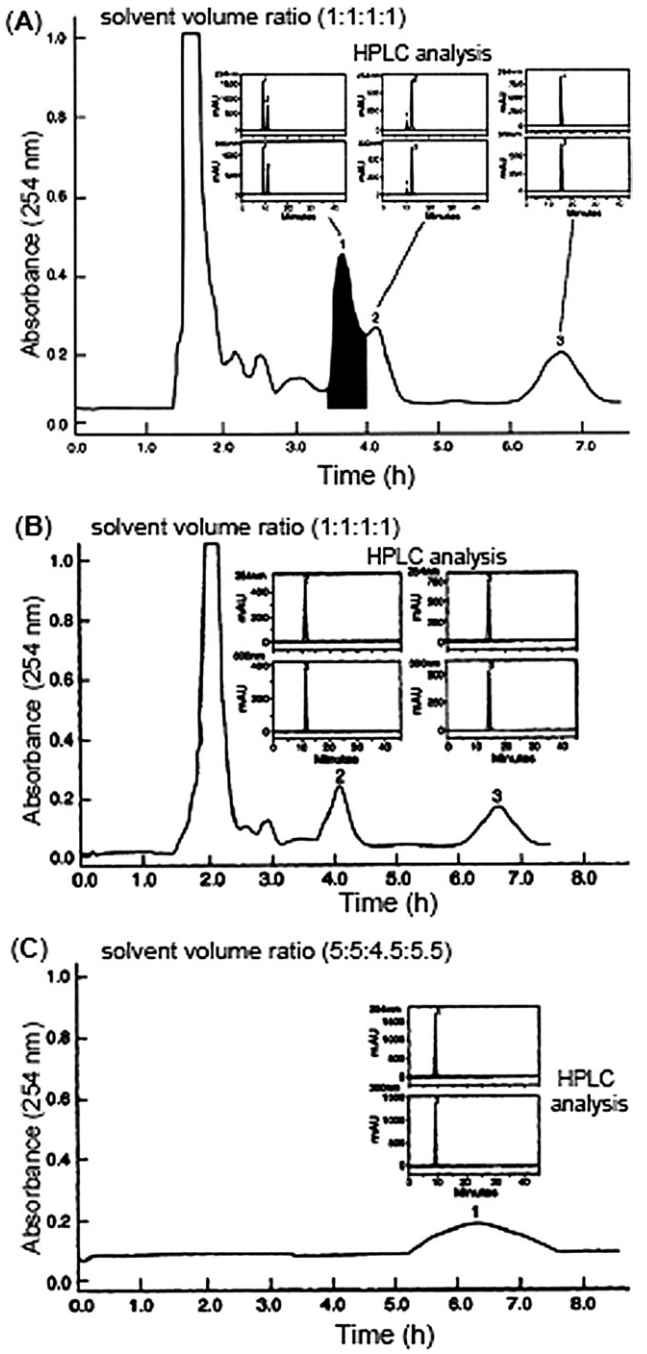



**Fig. 19** Chromatograms of an extract from *Angelica dahurica* by MDCCC. Solvent systems: Hex-EtOAc–MeOH–water in the volume ratios 1 : 1 : 1 : 1 and 5 : 5 : 4.5 : 5.5. SP: UP; MP: LP; flow-rate: 2 ml $min^{-1}$. Separation procedure: see text. Peak 1 = imperatorin (**31**), peak 2 = oxypeucedanin (**32**), peak 3 = isoimperatorin (**33**). Reprinted from Y. Wei and Y. Ito, 'Preparative isolation of imperatorin, oxypeucedanin and isoimperatorin from traditional Chinese herb "bai zhi" *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook using multidimensional high-speed counter-current chromatography', *J. Chromatogr. A*, 2006, **1115**, 112–117. Copyright (2006), with permission from Elsevier.[295]

CCC (UCCC) column in the second dimension (2nd-D). The use of a pair of two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water with volume ratios 1 : 5 : 1 : 5 and 3 : 5 : 3 : 5 in the two dimensions permitted the

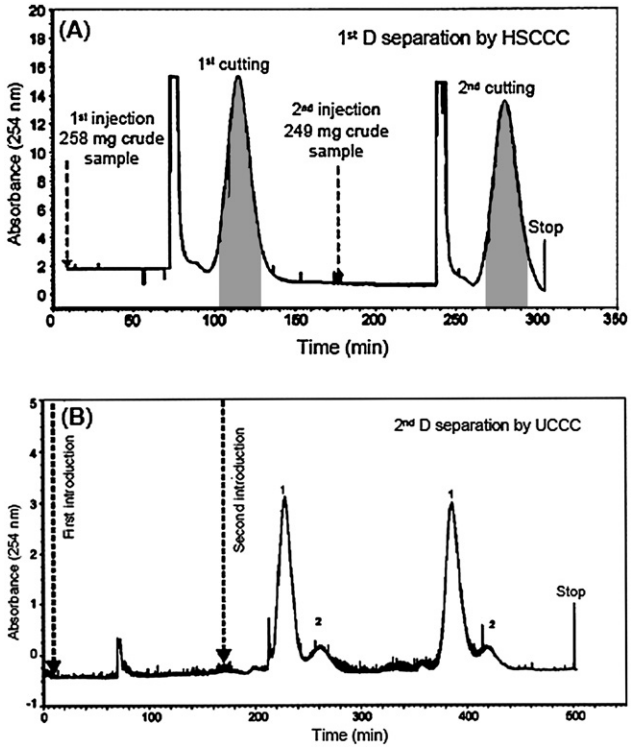
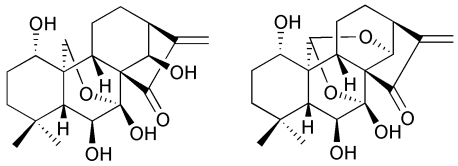



**Fig. 20** 2D-CCC separation of the crude extract from *Rabdosia rubescens*; solvent systems: Hex–EtOAc–MeOH–water with volume ratios 1 : 5 : 1 : 5 and 3 : 5 : 3 : 5. (A) Chromatogram of 1st-D HSCCC separation, volume ratio 1 : 5 : 1 : 5; flow rate: 2.0 ml $min^{-1}$; (B) Chromatogram of 2nd-D UCCC separation by introducing the shaded part from HSCCC volume ratio 3 : 5 : 3 : 5; flow rate: 4 ml $min^{-1}$. Peak 1 = oridonin (**34**), peak 2 = ponicidin (**35**). Reprinted from Y. Lu, C. Sun, R. Lui and Y. Pan, 'Effective two-dimensional counter-current chromatographic method for simultaneous isolation and purification of oridonin and ponicidin from the crude extract of *Rabdosia rubescens*', *J. Chromatogr., A*, 2007, **1146**, 125–130. Copyright (2007), with permission from Elsevier.[362]

simultaneous separation of **34** and **35**. Fig. 20A shows the chromatogram obtained from HSCCC (1st-D). The chromatogram in Fig. 20B was obtained by the cutting fraction of HSCCC (the shaded part in Fig. 20A) introduced and eluted from the UCCC (2nd-D) column. Separation of about 9 h of two injections with a 250 mg amount of the crude extract each yielded 60 mg of **34** and 10 mg of **35** (purity of 97.2 and 96.0%, respectively). The advantages of 2D-CCC as applied in this study are obvious: (i) it is difficult to resolve these two diterpenoids simultaneously using only one two-phase system; this 2D-CCC method greatly improved both resolution and peak capacity; (ii) due to the sufficient column capacity of the UCCC (1500 ml), almost the whole region of HSCCC of interest (about 50 ml) could be introduced to the UCCC without a pre-concentration step, thus obtaining satisfactory yield and peak resolution by the 2D-CCC method. Lu *et al.*[283] applied 2D-CCC also for the preparative separation of prenylflavonoids from *Artocarpus altilis*.

***5.3.1.4 High-capacity high-speed counter-current chromatography.*** HSCCC is very intensively used for preparative separation of natural products in laboratories. Therefore, it is not surprising that scaling-up for industrial use is very attractive.




One way to scale-up CCC is to utilize the slow rotary mode of coiled columns, which was first described in the 1980s. Such apparatus equipped with 10 l or 40 l capacity columns were used for semi-industrial separation of epigallocatechin gallate from crude tea extract, salicin from the extract of white willow bark, and of amygdalin from the extract of bitter almond, all within 20 h.[391,392] Much more promising is a recent development in HSCCC, named dynamic extraction (DE), which was introduced by the Brunel Institute for Bioengineering (Uxbridge, UK), and achieves separations in minutes rather than hours. The DE equipment is more robust than previous HSCCC machines, and scaling-up to pilot scale has been shown to be both quick and easy. The scale of the technology varies between 5 ml (analytical) and 18 l (pilot). Chen *et al.*[315] reported the isolation of honokiol and magnolol from *Magnolia officinalis* bark, which is one of the most popular traditional Chinese medicines. They used an analytical MINI-DE centrifuge to establish the critical parameters required for rapid solvent selection, sample resolution and sample load optimisation. The optimised parameters from the MINI-DE CCC were then used to separate and purify honokiol and magnolol using the 1000 × larger pilot scale MAXI-DE high-capacity HSCCC centrifuge (both are units now available commercially from Dynamic Extractions, Slough, UK). A crude sample of 43 g was successfully separated in one run using the two-phase solvent system hexane–ethyl acetate–methanol–water (1 : 0.4 : 1 : 0.4, v/v). This one-step separation produced 16.9 and 19.4 g honokiol and magnolol with purities of 98.4 and 99.8%, respectively, in only 20 min. This is the first time that high-capacity HSCCC has been used to purify multigram quantities of trial-grade bioactive compounds in less than 1 h with final purified products at such high concentrations (10.8 g $l^{-1}$ for magnolol and 7.0 g $l^{-1}$ for honokiol). The sample concentration of the target compounds was significantly higher than can be achieved with other high-resolution chromatography systems. According to Chen *et al.*[315] the term "high-speed" for HSCCC is a misnomer, as typical separations described as high-speed may take many hours. At the time, HSCCC was first compared to droplet counter-current chromatography (DCCC), the latter would last up to several days and therefore HSCCC was actually the "high-speed" method. High-capacity HSCCC instruments are robust enough to run reliably in high "g" ranges and achieve separation times of minutes as opposed to hours.

***5.3.1.5 Three-phase solvent system in analytical HSCCC.*** Organic solvent mixtures, such as *n*-hexane–methyl acetate–acetonitrile–water at a specific volume ratio (*e.g.* 1 : 1 : 1 : 1, v/v), form three mutually immiscible phases composed of a hydrophobic upper layer (UP), a moderately polar intermediate phase (IP) and a polar aqueous lower phase (LP). A novel HSCCC method using all three phases (UP/IP/LP) of the solvent system *n*-hexane–methyl acetate–acetonitrile–water

(4 : 4 : 3 : 4, v/v) was recently used for the separation of a mixture of fifteen standard compounds with a wide range of hydrophobicity from β-carotene to tryptophan.[393,394] The system successfully resolved all fifteen compounds in a one-step operation within 70 min. Yanagida *et al.*[394] used the above-mentioned three-phase solvent system (volume ratio 4 : 4 : 3 : 4) as an extracting solvent for several crude drugs and teas. Then, using the same three-phase solvent system, HSCCC was applied to the comprehensive separation of a wide variety of secondary metabolites in each extract. The future will show if the use of a three-phase solvent system is also suitable for the preparative HSCCC separation and purification of complex mixtures of natural products.

***5.3.1.6 Counter-current chromatography of polar extracts.*** CCC can be used for all ranges of polarities but has special advantages for the handling of polar extracts, which are often difficult to process with conventional techniques. Zhi *et al.*[311] recently established a hydrophilic organic/salt-containing aqueous two-phase system for the isolation of salvianolic acid B from *Salvia miltiorrhiza*. Following the detailed study of characteristics of organic/salt-containing two-phase systems, *n*-propanol was used to form a biphasic system with sodium dihydrogen phosphate and dipotassium dihydrogen phosphate. Salvianolic acid B was purified to 95.5% purity in a 34% (w/w) *n*-propanol–8% (w/w) phosphate system, the ratio $NaH_2PO_4$:$K_2HPO_4$ being 6 : 94. 108 mg salvianolic acid B was obtained from 285 mg crude extract with a revovery of 89%. Protoberberine quaternary alkaloids such as palmatine, jatrorrhizine, columbamine and pseudocolumbamine, which are very polar compounds and have similar chemical structures, have been isolated in two steps by HPCPC from a crude bark extract of *Enantia chlorantha*.[234] The separations of these alkaloids involved either ion-pairing between the quaternary ammoniums and perchlorate anions, or the ionisation of the phenolic compounds by addition of sodium hydroxide. Two successive biphasic solvent systems composed of dichloromethane–methanol–water (48 : 16 : 36, v/v) were used. The aqueous-rich phase was used as the stationary phase and the organic-rich phase as the mobile phase. The first system containing potassium perchlorate, allowed the isolation of 600 mg of palmatine from 1.47 g of a crude extract with 146 mg of a remaining mixture ($M_2$) containing only jatrorrhizine, columbamine and pseudocolumbamine. The second biphasic system, prepared with water made alkaline with sodium hydroxide, was employed to isolate the $M_2$ components. This system applied to the isolation of 70 mg of $M_2$ allowed a yield of 16 mg of jatrorrhizine and 13 mg of columbamine. To obtain pseudocolumbamine (16 mg), the elution was reversed (dual-mode), the aqueous-rich phase becoming the mobile phase (Fig. 21). The purity of the alkaloids was high (above 95%).

***5.3.1.7 pH-zone-refining counter-current chromatography.*** In the 1990s, Ito and co-workers introduced the pH-zone refining mode in CCC as a variant of displacement chromatography. pH-zone-refining CCC is generally employed as a preparative technique for separating ionisable analytes, whose electric charge is pH-dependent. The method elutes highly concentrated rectangular peaks with minimum overlapping while impurities and

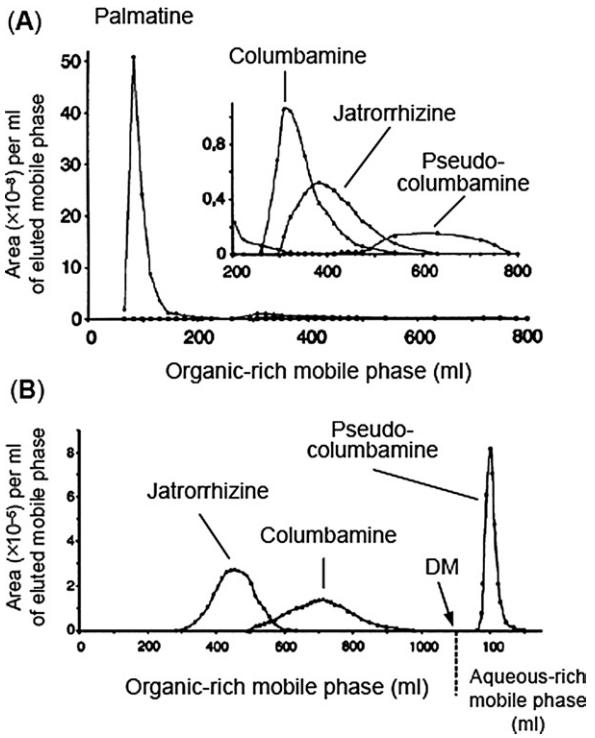



**Fig. 21** (A) HPCPC elution profile of four protoberberine alkaloids from a crude bark extract of *Enantia chlorantha*. Solvent system: $CH_2Cl_2$–MeOH–water (48 : 16 : 36, v/v); flow-rate: 9 ml $min^{-1}$. The injected sample contained $KClO_4$ (molar ratio between perchlorate anions and protoberberine alkaloids equal to 0.5). (B) Elution profile of jatrorrhizine, columbamine and pseudocolumbamine from mixture obtained by the first HPCPC run (A) using the same solvent system containing NaOH (pH 11.8); flow rate: 3 ml $min^{-1}$. Reprinted from M. Bourdat-Deschamps, C. Herrenknecht, B. Akendengue, A. Laurens, R. Hocquemiller, 'Separation of protoberberine quaternary alkaloids from a crude extract of *Enantia chlorantha* by centrifugal partition chromatography', *J. Chromatogr., A*, 2004, **1041**, 143–152. Copyright (2004), with permission from Elsevier.[234]

minor components are concentrated and eluted at the front and rear boundaries. The method uses two components: a retainer such as trifluoroacetic acid (for acidic analytes) or triethylamine (for basic analytes) in the organic stationary phase retains the analytes in the column, whereas an eluter (displacer) such as ammonia (for acidic analytes) or hydrochloric acid (for basic analytes) in the aqueous mobile phase elutes the analytes according to their p$K_a$ values and hydrophobicities. The greatest advantage of this method is its large sample loading capacity in the same separation column, which exceeds that of the standard HSCCC 10-fold. In addition, the method provides various special features such as yielding highly concentrated fractions, concentrating minor impurities for detection, and allowing the separation to be monitored by the pH of the effluent in absence of chromophores. Since the analytes are ionisable compounds, most separations can be performed using a relatively polar solvent system. Furthermore, selection of solvent systems and preparations of the sample are quite different from those used in the standard HSCCC technique.[244] Table 13 shows examples of two-phase solvent systems for pH-zone refining CCC.

**Table 13** Examples of two-phase solvent systems for pH-zone refining CCC/CPC

| Compounds | Matrix, sample[a] | Solvent systems (volume ratio)[b] | Key reagent[b] | | Ref.[c] |
|---|---|---|---|---|---|
| | | | Retainer in SP | Eluter in MP | |
| Curcuminoids | *Curcuma longa*, rhizome extract and crude curcumin | MtBE–$CH_3CN$–water (4 : 1 : 5) | TFA (20 mM) | NaOH (30 mM) | 395 |
| Lappaconitine | *Aconitum sinomontanum*, prepurified alkaloid sample (*ca.* 90% lappaconitine) | MtBE–THF–water (2 : 2 : 3) | TEA (10 mM) | HCl (10 mM) | 396 |
| Benzylisoquinoline alkaloids | *Hydrastis canadensis*, fractions of rhizome extract | $CHCl_3$ saturated with water, reverse displacement mode | HCl (6–25 mM) | TEA (0.05–03%) | 397 |
| Cyclopeptide alkaloid | *Zizyphus lotus*, crude alkaloid bark extract | MtBE–$CH_3CN$–water (4 : 1 : 5) | MSA (10 mM) | TEA (5 mM) | 398 |
| Purine alkaloids (caffeine, theophylline) | *Camellia sinensis*, crude alkaloid extract | MtBE–water (1 : 1) | TEA (10 mM) | HCl (10 mM) | 399 |
| Indole alkaloids (alstonine) | *Picralima nitida*, fruit rind, crude alkaloid extract | MtBE–$CH_3CN$–water (2 : 2 : 3) | TEA (pH 10.7) | HCl (pH 1.7) | 400 |
| Cichoric acid | *Echinacea purpurea*, crude extract | MtBE–$CH_3CN$–water (4 : 1 : 5) | TFA (10 mM) | $NH_3$ (10 mM) | 401 |
| Benzylisoquinoline alkaloids | *Corydalis decumbens*, root, crude alkaloid extract | MtBE–$CH_3CN$–water (2 : 2 : 3) | TEA (5–10 mM) | HCl (5–10 mM) | 402 |
| Tropane aromatic ester alkaloids | *Erythroxylum pervillei*, stem bark, alkaloid fraction | MtBE–water (1 : 1) | TEA (pH 10) | HCl 37% (pH 2) | 403 |
| Seco-dibenzopyrrocoline alkaloids | *Cryptocarya oubatchensis*, alkaloid bark extract | MtBE–$CH_3CN$–water (4 : 1 : 5) | MSA (2 mM) | TEA (1.5 mM) | 404 |
| Sesquiterpene alkaloids (huperzine A, B) | *Huperzia serrata*, whole plant, crude alkaloid extract | Hept–EtOAc–PrOH–water (10 : 30 : 15 : 45) | MSA (6 mM) | TEA (8 mM) | 405 |

[a] Systematic plant name, crude drug or extract. [b] Abbreviations: $CH_3CN$: acetonitrile; EtOAc: ethyl acetate; Hept: *n*-heptane; MP: mobile phase; MSA: methanesulfonic acid; MtBE: methyl *tert*-butyl ether; PrOH: *n*-propanol; SP: stationary phase; TEA: triethylamine; TFA: trifluoroacetic acid; THF: tetrahydrofuran. [c] References: 2000–2007.

Alkaloids are good candidates for pH-zone refining CCC separation. A pre-purified alkaloid sample of *Aconitum sinomontanum* was purified using the following two-phase solvent system: Methyl *tert*-butyl ether–tetrahydrofuran–water (2 : 2 : 3, v/v) with 10 mM triethylamine acid as retainer in the organic stationary phase and 10 mM hydrochloric acid as eluter in the aqueous mobile phase. Fig. 22 shows three typical pH-zone refining counter-current chromatograms of alkaloids from *A. sinomontanum* obtained from the separations of 2.0, 6.5 and 10.5 g of pre-purified sample (with approximately 90% lappaconitine). The target compound, lappaconitine, formed a rectangular peak, whereas impurities or minor alkaloid components were highly concentrated at its front and rear boundaries. Increasing the sample size from 2.0 up to 10.5 g resulted in broadening of the rectangular peak without loss of peak resolution. The pH-zone refining CCC separations yielded 1.75 g (A), 5.6 g (B) and 9.0 g (C) of pure lappaconitine with over 99% purity as determined by HPLC. The purity of lappaconitine obtained by conventional separation and purification methods using several steps such as silica gel column chromatography and recrystallisation, is no more than 95%.[396] Similarly, indole alkaloids from *Picralima nitida*,[400] benzylisochinolin alkaloids from *Corydalis decumbens*[402] and sesquiterpene alkaloids from *Huperzia serrata*[405] were isolated and purified. Fig. 23 shows the pH-zone refining UV chromatogram, pH profile and HPLC control for the separation of 1.4 g of alkaloid extract from *H. serrata* using *n*-heptane–ethyl acetate–*n*-propanol–water (10 : 30 : 15 : 45, v/v) with 6 mM methanesulfonic acid as retainer and 8 mM triethylamine as eluter. This run yielded

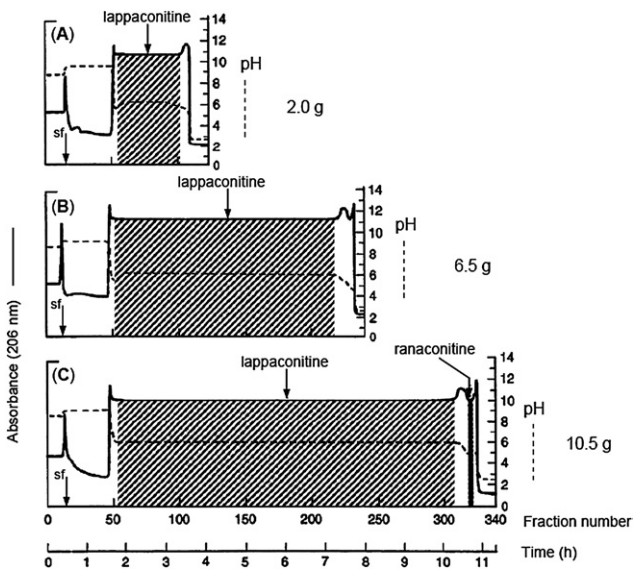



**Fig. 22** Separation of lappaconitine from a pre-purified extract of *Aconitum sinomontanum* by pH-zone refining HSCCC. Solvent system: MtBE–THF–water (2 : 2 : 3, v/v), 10 mM TEA in the ST (UP) and 10 mM HCl in the LP; flow-rate: 3 ml $min^{-1}$. Reprinted from F. Yang and Y. Ito, 'pH-Zone-refining counter-current chromatography of lappaconitine from *Aconitum sinomontanum* Nakai: I. Separation of prepurified extract', *J. Chromatogr., A*, 2001, **923**, 281–285. Copyright (2001), with permission from Elsevier.[396]

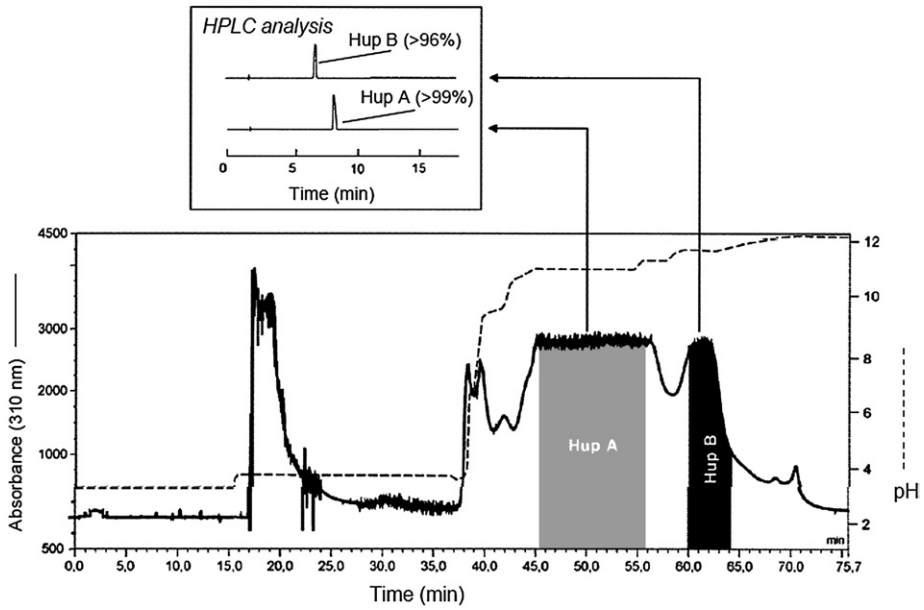



**Fig. 23** pH-zone refining UV chromatogram, pH profile and HPLC of an alkaloid extract from *Huperzia serrata*. Solvent system: Hept–EtOAc–PrOH–water (10 : 30 : 15 : 45, v/v). ST: LP with 6 mM MSA; MP: UP (ascending mode) with 8 mM TEA, flow-rate: 6 ml $min^{-1}$. Hup A = huperzine A, Hup B = huperzine B. Reprinted from A. Toribio, E. Delannay, B. Richard, K. Plé, M. Zèches-Hanrot, J.-M. Nuzillard and J.-H. Renault, 'Preparative isolation of huperzines A and B from *Huperzia serrata* by displacement centrifugal partition chromatography', *J. Chromatogr., A*, 2007, **1140**, 101–106. Copyright (2007), with permission from Elsevier.[405]

105 mg (7.5% of the alkaloid extract) of huperzine A (HPLC purity >99%) and 90 mg (6.5% of the alkaloid extract) of huperzine B (HPLC purity >96%) in one step.[405]

Recently, pH-zone refining CCC was also successfully applied to the separation of an acidic plant constituent, cichoric acid, from a crude extract of *Echinacea purpurea*. A sample of 300 g was separated using methyl *tert*-butyl ether–acetonitrile–water (4 : 1 : 5, v/v) as two-phase solvent system with 10 mM trifluoroacetic acid as retainer and 10 mM ammonia as eluter. Double separations were performed with the same solvent system, yielding 563 mg cichoric acid at 95.6% purity.[401]

***5.3.1.8 Ion-exchange displacement CCC.*** Ion-exchange centrifugal partition chromatography (IXCPC) was recently introduced as a new type of displacement mode. The principle of this method consists of generating lipophilic ion-pairs in the organic stationary phase. Amberlite LA2 was applied as a weak anionic exchanger to the separation of polysulfated polysaccharides (fucans and heparins). Maciuk *et al.*[406] reported the purification of organic acids such as isomers of hydroxycinnamic acid by using benzalkonium chloride as a strong anion-exchanger and sodium iodide as the displacer. The displacement process was characterised by a trapezoidal profile of analyte concentration in the eluate with narrow transition zones. The same methodology was applied to the one-step purification of rosmarinic acid[407] from the crude extract of *Lavandula vera* cell suspension using the ternary biphasic solvent system chloroform–1-butanol–water (4.5 : 1 : 4.5, v/v) with benzalkonium chloride in the organic stationary phase (233 mM) and sodium iodide in the aqueous mobile phase (25 mM). The resulting technique was referred to as SIXCPC (S as in *strong*, IX as in *ion-exchange*). A large yield (3.4% of the extract) of highly pure rosmarinic acid (~90%) was obtained.

***5.3.1.9 On-line monitoring methods in preparative countercurrent chromatography.*** Generally, a UV-VIS detector has become the major detection instrument of CCC to monitor the column effluent as in conventional liquid chromatography. But its application to CCC is limited by its inherent shortcomings. It cannot be used as the detector for separation of non-chromophoric components and makes the application of CCC restricted to some degree. During the past decade, considerable effort has been made to develop first analytical, and later also preparative HSCCC for coupling with mass spectrometry (ESI, APCI), HPLC–DAD as well as ELSD. The introduction of hyphenated online detection and purity systems in HSCCC improved the efficiency of this technique dramatically by overcoming drawbacks of post-analysis in HSCCC isolation. HSCCC instruments were directly interfaced with ESI and APCI mass spectrometry. HSCCC coupled with ESI–MS and ESI–MS/MS was applied to the separation and analysis of (−)-epigallocatechin gallate (EGCG) from crude tea polyphenols[408] and of tanshinone II A from a crude extract of *Salvia miltiorrhiza*, respectively.[409] Chen *et al.*[410,411] used ESI–MS and APCI–MS coupling for the separation and analysis of flavonoids from *Oroxylum indicum*. With ESI a split in the flow of effluent was necessary, but with APCI no splitting was required. In addition, a HSCCC–HPLC–DAD system for online purity monitoring was recently reported. In this system, the effluent from the outlet of HSCCC was split into two parts: one was collected, while the other was introduced directly into an HPLC–DAD system for purity analysis through a switch valve. Thus, the purities of the obtained fractions from HSCCC were monitored, and fractions with high purities were collected. This strategy was successfully demonstrated, *e.g.* with the preparative isolation and purification of hyperoside from *Hypericum perforatum*.[270] The same online HSCCC–HPLC–DAD system was applied to the

isolation and purification of mangiferin and neomangiferin from *Anemarrhena asphodeloides*.[378] HSCCC coupled with ELSD was recently applied, *e.g.* to the isolation and purification of dammarane saponins (ginsenosides) from the roots of *Panax notoginseng* and *P. ginseng*,[412,345] protoberberine alkaloids from *Enantia chlorantha*,[234] peimine and peiminine from the bulbs of *Fritillaria thunbergii*,[413] various triterpenic constituents from the roots of *Adenophora tetraphylla*,[360] diterpene alkaloids from *Aconitum coreanum*,[324] the steroid alkaloids verticine and verticinone from the bulbs of *Fritillaria thunbergii*,[325] and triterpene saponins from *Clematis mandshurica*.[346]

## 6 Concluding remarks

Natural product isolation has undergone many transitions over the years. In the last decades there was a strong shift from the isolation of all compounds present in any extract to the search for bioactive natural compounds. Most of today's isolation protocols comprise *in vitro* assays, frequently coupled on-line to HPLC or MS systems, besides sample preparation and purification steps. An example is the application of a fluorometric flow assay system to an on-line coupled prep HPLC apparatus for the isolation of the acetylcholinesterase inhibitor ungeremine from the bulbs of *Nerine boudenii*.[205] The methanol extract showed a strong inhibitory peak in the on-line assay, and the active compound could be isolated by CPC and prep HPLC. First, the activity was detected in the on-line system with an analytical HPLC column. To obtain a larger amount of the active compound, 1 g of the methanol extract was loaded on a CPC and separated using ethyl acetate–methanol–water (45 : 20 : 35, v/v), with the lower phase as the stationary phase and the upper phase as the mobile phase. The active fraction, identified by TLC in this case, was further separated by a prep HPLC column at a flow-rate of 2.5 ml $min^{-1}$, an analytical HPLC column at a flow-rate of 1.2 ml $min^{-1}$ repeatedly using methanol–water–tetrahydrofuran (30 : 68 : 2, v/v), and purified with a Sephadex LH-20 column. Isolation procedures coupled on-line to a flow assay system are in fashion, as the goal is not only to isolate active compounds but also to obtain research grants.

Comparative studies of preparative isolation and purification using different separation methods are reported in the literature frequently. Lu *et al.*[201] found that CCC is a valid alternative to semi-prep HPLC for the isolation of the two phenolic compounds magnolol and honokiol from the bark of *Magnolia officinalis*. The level of purity of the target compounds separated by CCC is comparable to that obtained by HPLC (Table 14). It is evident that both the chromatographic techniques are highly efficient. However, the selection of a suitable two-phase solvent system is the key element in CCC method development, making such a development more difficult than in the case of HPLC. The choice from an enormous number of possible solvent systems is the main difficulty faced by the analyst. With respect to solvent consumption, the CCC method needs only the half the amount of solvent of semi-prep HPLC, indicating that CCC is much more economical than HPLC. However, the use of tetrachloromethane is the drawback of the presented CCC method.

Isolation of natural products is still mainly carried out using multi-step isolation procedures. Hamburger *et al.*,[22] for example, presented a combination of SFE, LPLC and HPLC for the isolation of faradiol esters from the flower heads of *Calendula officinalis*. Starting with an optimised SFE extract, followed by filtration over silica gel, the LPLC separation afforded highly enriched triterpene ester fractions in multi-gram quantities. Isocratic elution with a single and inexpensive solvent (methanol) was suitable for repeated separations. Also, the last purification step by prep HPLC was carried out under isocratic conditions with methanol or methanol–isopropanol as eluent (Fig. 24). Purities of >96–98% were achieved for the isolated faradiol esters.

**Table 14** Comparison of CCC and semi-preparative HPLC[201]

| | CCC[a] | HPLC |
|---|---|---|
| Stationary phase | Upper phase: LtPet–EtOAc–$CCl_4$–MeOH–water (1 : 1 : 8 : 6 : 1, v/v) | Zorbax Eclipse XDB-$C_{18}$ column, 250 × 9.4 mm i.d., 5 μm |
| Mobile phase | Lower phase | MeOH–water (70 : 30, v/v) |
| Sample capacity per run/g | 2.0 | $1.96 \times 10^{-2}$ |
| Run time/min | 450 | 40 |
| Productivity/mg $min^{-1}$ | 4.44 | 0.49 |
| Purity of isolated compounds | >98.5% | >99.0% |
| Solvent consumption/l $g^{-1}$ | 1.93 | 5.10 |

[a] Abbreviations: LtPet: light petroleum; EtOAc: ethyl acetate; $CCl_4$: tetrachloromethane; MeOH: methanol.

Báthori *et al.*[101] published a complex isolation procedure using a suitable combination of preparative-scale separation methods for the effective clean-up of the ecdysteroids from the aerial part of *Silene italica* ssp. *nemoralis*. The isolation of the minor ecdysteroids from the partially purified extract by solid-phase extraction on alumina is based on the use of both DCCC and RP-LPLC. The purification is completed by PTLC and prep HPLC (Fig. 25). Hunydai *et al.*[123] used a very tedious multi-step procedure for the isolation of 22 ecdysteroids from the herb of *Serratula wolffii*. The isolation process included a great variety of methods, *e.g.* CC columns on NP- and RP-silica gel, polyamide, Sephadex LH-20 and alumina as well as PTLC and NP-HPLC. The isolation of pure compounds required 2–8 steps.

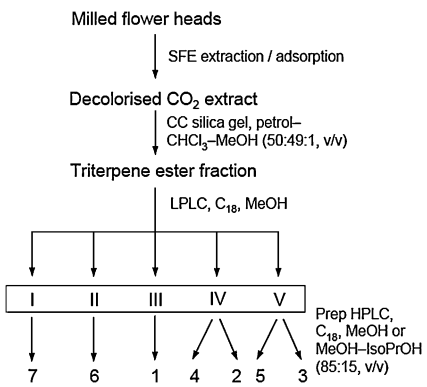



**Fig. 24** Schematic presentation of the purification procedure for faradiol esters. 1 = faradiol-3-*O*-laurate, 2 = faradiol-3-*O*-myristate, 3 = faradiol-3-*O*-palmitate, 4 = maniladiol-3-*O*-laurate, 5 = maniladiol-3-*O*-myristate, 6 = ψ-taraxasterol, 7 = β-amyrin.

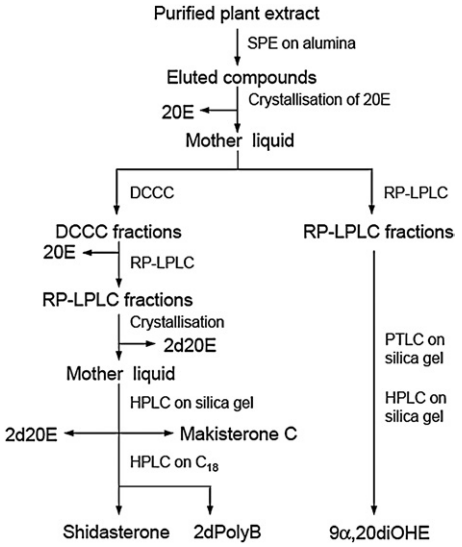



**Fig. 25** Schematic presentation of the isolation of ecdysteroids from *Silene italica* ssp. *nemoralis*. 20E = 20-hydroxyecdysone; 2d20E = 2-deoxy-20-hydroxyecdysone; 2dPolyB = 2-deoxy-polypodine; 9α, 20diOHE = 9α,20-dihydroxyecdysone.

In view of this excessive and complex isolation procedure, the question arises as to which of the applied steps were really necessary due to the different physicochemical properties of the ecdysteroids, and which were chosen by trial and error.

On the other hand, a new trend towards an efficient procedure for extraction, separation, and purification is the application of recently developed extraction techniques such as SFE or MAE in combination with only one separation method. Examples are the isolation of flavonoids from *Patrinia villosa*[28] (SFE→ HSCCC), ferulic acid from *Angelica sinensis*[70] (MAE → HSCCC) as well as coumarins from *Psoralea corylifolia*[23] (SFE → HSCCC) and from *Stellera chamaejasme*[27] (SFE → HSCCC). The results of these four papers demonstrate that SFE/MAE combined with HSCCC are very useful techniques for extraction, isolation and purification with excellent purities of the obtained compounds (~98–99%). Another possibility to optimise and shorten the purification procedure may be to inject the crude drug powder directly into the chromatography system (*e.g.* HSCCC) without prior extraction. The future will tell if this technique, reported by Peng *et al.*[326] for the isolation of benzylisoquinoline alkaloids without describing the necessary experimental details, will become applicable as a general method.

This review clearly shows that prep HPLC and CCC/CPC are the most important and most used chromatographic isolation methods today. Each has advantages and disadvantages, and the analyst must therefore evaluate suitable extraction and isolation procedures on the basis of the physicochemical properties of the expected natural products before starting a new research project.

## 7 Acknowledgements

Special thanks go to Dr D. Kingston, Virginia Tech, Blacksburg, for encouraging and inviting me to write this review. The help of Esther Guggenheim, Jerusalem, with improvements of the English text is gratefully acknowledged.

## 8 References

1 A. Marston and K. Hostettmann, *Nat. Prod. Rep.*, 1991, **8**, 391–413.
2 S. D. Sarker, Z. Latif and A. I. Gray, 'Natural Product Isolation', in *Natural Products Isolation*, ed. S. D. Sarker, Z. Latif and A. I. Gray, 2nd edn, Humana Press, Totowa, New Jersey, 2006, pp. 1–25.
3 K. Hostettmann, A. Marston and M. Hostettmann, *Preparative Chromatography Techniques. Applications in Natural Product Isolation*, 2nd edn, Springer, Berlin/Heidelberg, 1998.
4 B. Benthin, H. Danz and M. Hamburger, *J. Chromatogr., A*, 1999, **837**, 211–219.
5 V. Camel, *Analyst*, 2001, **126**, 1182–1193.
6 C. W. Huie, *Anal. Bioanal. Chem.*, 2002, **373**, 23–30.
7 B. Kaufmann and P. Christen, *Phytochem. Anal.*, 2002, **13**, 105–113.
8 Z. Kerem, H. German-Shashoua and O. Yarden, *J. Sci. Food Agric.*, 2005, **85**, 406–412.
9 M. Nüchter, B. Ondruschka, B. Fischer, A. Tied and W. Lautenschläger, *Chem. Ing. Tech.*, 2005, **77**, 171–175.
10 C. D. Bevan and P. S. Marshall, *Nat. Prod. Rep.*, 1994, **11**, 451–466.
11 P. Castioni, P. Christen and J.-L. Veuthey, *Analusis*, 1995, **23**, 95–106.
12 W. K. Modey, D. A. Mulholland and M. W. Raynor, *Phytochem. Anal.*, 1996, **7**, 1–15.
13 A. P. Jarvis and E. D. Morgan, *Phytochem. Anal.*, 1997, **8**, 217–222.
14 E. Reverchon, *J. Supercrit. Fluids*, 1997, **10**, 1–37.
15 Q. Lang and C. M. Wai, *Talanta*, 2001, **53**, 771–782.
16 R. M. Smith, *J. Chromatogr., A*, 1999, **856**, 83–115.
17 P. Christen and J.-L. Veuthey, *Curr. Med. Chem.*, 2001, **8**, 1827–1839.
18 C. S. Kaiser, H. Römpp and P. C. Schmidt, *Pharmazie*, 2001, **56**, 907–926.
19 X.-L. Cao, Y. Tian, T.-Y. Zhang and Y. Ito, *J. Chromatogr., A*, 2000, **898**, 75–81.
20 M. D. A. Saldaña, R. S. Mohamed and P. Mazzafera, *Braz. J. Chem. Eng.*, 2000, **17**, 251–259.
21 X. Cao and Y. Ito, *J. Chromatogr., A*, 2003, **1021**, 117–124.
22 M. Hamburger, S. Adler, D. Baumann, A. Förg and B. Weinreich, *Fitoterapia*, 2003, **74**, 328–338.
23 X. Wang, Y. Wang, J. Yuan, Q. Sun, J. Liu and C. Zheng, *J. Chromatogr., A*, 2004, **1055**, 135–140.
24 D. Pyo and S. Lee, *Anal. Lett.*, 2002, **35**, 1591–1602.
25 D. Pyo, C. Oh and J. Choi, *Anal. Lett.*, 2004, **37**, 2595–2608.
26 J. Peng, G. Fan and Y. Wu, *J. Chromatogr., A*, 2005, **1083**, 52–57.
27 J. Peng, F. Dong, Q. Xu, Y. Xu, Y. Qi, X. Han, L. Xu, G. Fan and K. Liu, *J. Chromatogr., A*, 2006, **1135**, 151–157.
28 J. Peng, G. Fan, Y. Chai and Y. Wu, *J. Chromatogr., A*, 2006, **1102**, 44–50.
29 J. Y. Ling, G. Y. Zhang, Z. J. Cui and C. K. Zhang, *J. Chromatogr., A*, 2007, **1145**, 123–127.
30 H. Sovová, L. Opletal, M. Bártlová, M. Sajfrtová and M. Křenková, *J. Supercrit. Fluids*, 2007, **42**, 88–95.
31 B. E. Richter, B. A. Jones, J. L. Ezzel and N. L. Porter, *Anal. Chem.*, 1996, **68**, 1033–1039.
32 A. Brachet, S. Rudaz, L. Mateus, P. Christen and J.-L. Veuthey, *J. Sep. Sci.*, 2001, **24**, 865–873.
33 E. S. Ong and S. N. binte Apandi, *Electrophoresis*, 2001, **22**, 2723–2729.
34 B. Kaufmann, P. Christen and J.-L. Veuthey, *Chromatographia*, 2001, **54**, 394–398.
35 E.-S. Ong, S.-O. Woo and Y.-L. Yong, *J. Chromatogr., A*, 2000, **904**, 57–64.
36 J. Suomi, H. Sirén, K. Hartonen and M.-L. Riekkola, *J. Chromatogr., A*, 2000, **868**, 73–83.
37 G. Schieffer and K. Pfeiffer, *J. Liq. Chromatogr. Relat. Technol.*, 2001, **24**, 2415–2427.
38 G. W. Schieffer, *J. Liq. Chromatogr. Relat. Technol.*, 2002, **25**, 3033–3044.
39 M. P. K. Choi, K. K. C. Chan, H. W. Leung and C. W. Huie, *J. Chromatogr., A*, 2003, **983**, 153–162.
40 M. A. Rostagno, M. Palma and C. G. Barroso, *Anal. Chim. Acta*, 2004, **522**, 169–177.
41 M. Waksmundzka-Hajnos, A. Petruczynik, A. Dragan, D. Wianowska and A. L. Dawidowicz, *Phytochem. Anal.*, 2004, **15**, 313–319.
42 M. Waksmundzka-Hajnos, A. Petruczynik, A. Dragan, D. Wianowska, A. L. Dawidowicz and I. Sowa, *J. Chromatogr., B*, 2004, **800**, 181–187.

43 R. Anand, N. Verma, D. K. Gupta, S. C. Puri, G. Handa, V. K. Sharma and G. N. Qazi, *J. Chromatogr. Sci.*, 2005, **43**, 530–531.
44 C. Bergeron, S. Gafner, E. Clausen and D. J. Carrier, *J. Agric. Food Chem.*, 2005, **53**, 3076–3080.
45 J. Shen and X. Shao, *Anal. Bioanal. Chem.*, 2005, **383**, 1003–1008.
46 G. F. Barbero, M. Palma and C. G. Barroso, *J. Agric. Food Chem.*, 2006, **54**, 3231–3236.
47 A. Smelcerovic, M. Spiteller and S. Zuehlke, *J. Agric. Food Chem.*, 2006, **54**, 2750–2753.
48 Y. Jiang, P. Li, S. P. Li, Y. T. Wang and P. F. Tu, *J. Pharm. Biomed. Anal.*, 2007, **43**, 341–345.
49 F. Kawamura, Y. Kikuchi, T. Ohira and M. Yatagai, *J. Nat. Prod.*, 1999, **62**, 244–247.
50 V. Camel, *Trends Anal. Chem.*, 2000, **19**, 229–248.
51 J. L. Luque-García and M. D. Luque de Castro, *Trends Anal. Chem.*, 2003, **22**, 90–98.
52 X. Pan, H. Liu, G. Jia and Y. Shu, *Biochem. Eng. J.*, 2000, **5**, 173–177.
53 X. Pan, G. Niu and H. Liu, *J. Chromatogr., A*, 2001, **922**, 371–375.
54 B. Kaufmann, P. Christen and J.-L. Veuthey, *Phytochem. Anal.*, 2001, **12**, 327–331.
55 A. Brachet, P. Christen and J.-L. Veuthey, *Phytochem. Anal.*, 2002, **13**, 162–169.
56 X. Pan, G. Niu and H. Liu, *Biochem. Eng. J.*, 2002, **12**, 71–77.
57 Y. Y. Shu, M. Y. Ko and Y. S. Chang, *Microchem. J.*, 2003, **74**, 131–139.
58 J.-H. Kwon, J. M. R. Bélanger, J. R. J. Paré and V. A. Yaylayan, *Food Res. Int.*, 2003, **36**, 491–498.
59 J. Wang, P. Shen and Y. Shen, *Chin. J. Chem. Eng.*, 2003, **11**, 231–233.
60 X. Pan, G. Niu and H. Liu, *Chem. Eng. Process.*, 2003, **42**, 129–133.
61 Y. Yang, L. Chen, X.-X. Zhang and Z. Guo, *J. Liq. Chromatogr. Relat. Technol.*, 2004, **27**, 3203–3211.
62 H. Li, B. Chen, L. Nie and S. Yao, *Phytochem. Anal.*, 2004, **15**, 306–312.
63 D. P. Fulzele and R. K. Satdive, *J. Chromatogr., A*, 2005, **1063**, 9–13.
64 F. Zhang, B. Chen, S. Xiao and S.-z. Yao, *Sep. Purif. Technol.*, 2005, **42**, 283–290.
65 C. Deng, J. Ji, N. Li, Y. Yu, G. Duan and X. Zhang, *J. Chromatogr., A*, 2006, **1117**, 115–120.
66 C. Deng, Y. Mao, N. Yao and X. Zhang, *Anal. Chim. Acta*, 2006, **575**, 120–125.
67 C. Deng, N. Yao, B. Wang and X. Zhang, *J. Chromatogr., A*, 2006, **1103**, 15–21.
68 H.-Y. Zhou and C.-Z. Liu, *J. Chromatogr., B*, 2006, **835**, 119–122.
69 R. Japón-Luján, J. M. Luque-Rodríguez and M. D. Luque de Castro, *Anal. Bioanal. Chem.*, 2006, **385**, 753–759.
70 Z. Liu, J. Wang, P. Shen, C. Wang and Y. Shen, *Sep. Purif. Technol.*, 2006, **52**, 18–21.
71 Y. Yu, T. Huang, B. Yang, X. Liu and G. Duan, *J. Pharm. Biomed. Anal.*, 2007, **43**, 24–31.
72 W. Zhang and S. Xu, *J. Sci. Food Agric.*, 2007, **87**, 1455–1462.
73 V. Beejmohun, O. Fliniaux, E. Grand, F. Lamblin, L. Bensaddek, P. Christen, J. Kovenski, M.-A. Fliniaux and F. Mesnard, *Phytochem. Anal.*, 2007, **18**, 275–282.
74 C. Latha, *Biotechnol. Lett.*, 2007, **29**, 319–322.
75 S. Hemwimon, P. Pavasant and A. Shotipruk, *Sep. Purif. Technol.*, 2007, **54**, 44–50.
76 Y. Chen, M.-Y. Xie and X.-F. Gong, *J. Food Eng.*, 2007, **81**, 162–170.
77 E. S. Ong, *J. Chromatogr., B*, 2004, **812**, 23–33.
78 R. J. P. Cannell, *Natural Products Isolation*, Humana Press, Totowa, New Jersey, 1998.
79 C. Tringali, *Bioactive Compounds from Natural Sources. Isolation, characterisation and biological properties*, Taylor & Francis, London/New York, 2001.
80 C. F. Poole, *The essence of chromatography*, Elsevier, Amsterdam, 2003.
81 *Encyclopedia of Chromatography*, 2nd edn, ed. J. Cazes, vol. 1 and 2, Taylor & Francis, Boca Raton, 2005.
82 *Natural Products Isolation*, 2nd edn, ed. S. D. Sarker, Z. Latif and A. I. Gray, Humana Press, Totowa, New Jersey, 2006.
83 *Preparative Layer Chromatography (Chromatographic Science Series: 95)*, ed. T. Kowalska and J. Sherma, CRC Press, Taylor & Francis, Boca Raton, 2006.
84 Sz. Nyiredy, 'Essential guides to method development in thin-layer (planar) chromatography', in *Encyclopedia of Separation Science*, ed. I. D. Wilson, E. R. Adland, M. Corke and C. F. Poole, Academic Press, London, 2000, vol. 10, pp. 4652–4666.
85 Sz. Nyiredy, C. A. J. Erdelmeier, B. Meier and O. Sticher, *Planta Med.*, 1985, **51**, 241–246.
86 Sz. Nyiredy, K. Dallenbach-Tölke and O. Sticher, *J. Planar Chromatogr.*, 1988, **1**, 336–342.
87 C. W. Huck, C. G. Huber, K.-H. Ongania and G. K. Bonn, *J. Chromatogr., A*, 2000, **870**, 453–462.
88 A. Jaworski and H. Brückner, *J. Pept. Sci.*, 2001, **7**, 433–447.
89 E. van der Watt and J. C. Pretorius, *J. Ethnopharmacol.*, 2001, **76**, 87–91.
90 K. C. S. Rao, S. Divakar, A. G. A. Rao, N. G. Karanth and A. P. Sattur, *Appl. Microbiol. Biotechnol.*, 2002, **58**, 539–542.
91 C. J. Peterson, L. T. Nemeth, L. M. Jones and J. R. Coats, *J. Econ. Entomol.*, 2002, **95**, 377–380.
92 S. Apers, K. Cimanga, D. Vanden Berghe, E. Van Meenen, A. O. Longanga, A. Foriers, A. Vlietinck and L. Pieters, *Planta Med.*, 2002, **68**, 20–24.
93 B. T. S. Yff, K. L. Lindsey, M. B. Taylor, D. G. Erasmus and A. K. Jäger, *J. Ethnopharmacol.*, 2002, **79**, 101–107.
94 M. L. Hajnos, K. Glowniak, M. Waksmundzka-Hajnos and S. Piasecka, *Chromatographia*, 2002, **56**, S91–S94.
95 T. Yrjönen, P. Vuorela, K. D. Klika, K. Pihlaja, T. H. Teeri and H. Vuorela, *Phytochem. Anal.*, 2002, **13**, 349–353.
96 D. Wu, L. Yu, M. G. Nair, D. L. DeWitt and R. S. Ramsewak, *Phytomedicine*, 2002, **9**, 41–47.
97 H. Wangensteen, E. Molden, H. Christensen and K. E. Malterud, *Eur. J. Clin. Pharmacol.*, 2003, **58**, 663–668.
98 S.-J. Cho, S. K. Lee, B. J. Cha, Y. H. Kim and K.-S. Shin, *FEMS Microbiol. Lett.*, 2003, **223**, 47–51.
99 H. R. Monsef, A. Ghobadi and M. Iranshahi, *J. Pharm. Pharm. Sci.*, 2004, **7**, 65–69.
100 E. M. Murphy, L. Nahar, M. Byres, M. Shoeb, M. Siakalima, M. M. Rahman, A. I. Gray and S. D. Sarker, *Biochem. Syst. Ecol.*, 2004, **32**, 203–207.
101 M. Báthori, Z. Pongrácz, R. Omacht and I. Máthé, *J. Chromatogr. Sci.*, 2004, **42**, 275–279.
102 M. Iranshahi, A. R. Shahverdi, R. Mirjani, G. Amin and A. Shafiee, *Z. Naturforsch., C*, 2004, **59**, 506–508.
103 D. Grancher, P. Jaussaud, A. Durix, A. Berthod, B. Fenet, Y. Moulard, Y. Bonnaire and S. Bony, *J. Chromatogr., A*, 2004, **1059**, 73–81.
104 R. Mirjani, A.-R. Shahverdi, M. Iranshahi, G. Amin and A. Shafiee, *Pharm. Biol.*, 2005, **43**, 293–295.
105 M. S. Mokbel, *Pakistan J. Biol. Sci.*, 2005, **8**, 1472–1477.
106 R. Saleem, M. Ahmed, S. I. Ahmed, M. Azeem, R. A. Khan, N. Rasool, H. Saleem, F. Noor and S. Faizi, *Phytother. Res.*, 2005, **19**, 881–884.
107 M. Sandager, N. D. Nielsen, G. I. Stafford, J. van Staden and A. K. Jäger, *J. Ethnopharmacol.*, 2005, **98**, 367–370.
108 M. Kozyra, K. Glowniak, A. Zabża, G. Zgórka, T. Mroczek, T. Cierpicki, J. Kulesza and I. Mudło, *J. Planar Chromatogr.*, 2005, **18**, 224–227.
109 M. Bartnik, K. Glowniak, A. Magciąg and M. Ł. Hajnos, *J. Planar Chromatogr.*, 2005, 244–248.
110 M. K. Sakar, D. Şöhretoğlu, M. Özalp, M. Ekizoğlu, S. Piacente and C. Pizza, *Turk. J. Chem.*, 2005, **29**, 555–559.
111 H. L. Teles, J. P. Hemerly, P. M. Pauletti, J. R. C. Pandolfi, A. R. Araújo, S. R. Valentini, M. C. M. Young, V. Da S. Bolzani and D. H. S. Silva, *Nat. Prod. Res.*, 2005, **19**, 319–323.
112 R. El-Kassas, Z. K. El-Din, M. H. Beale, J. L. Ward and R. N. Strange, *Weed Res.*, 2005, **45**, 212–219.
113 J. K. Son, J. H. Jung, C. S. Lee, D. C. Moon, S. W. Choi, B. S. Min and M. H. Woo, *Bull. Korean Chem. Soc.*, 2006, **27**, 1231–1234.
114 M. S. Mokbel and T. Suganuma, *Eur. Food Res. Technol.*, 2006, **224**, 39–47.
115 B. Réthy, A. Kovács, I. Zupkó, P. Forgo, A. Vasas, G. Falkay and J. Hohmann, *Planta Med.*, 2006, **72**, 767–770.
116 M. C. de Oliveira Chaves, A. H. de Oliveira and B. V. de Oliveira Santos, *Biochem. Syst. Ecol.*, 2006, **34**, 75–77.
117 O. Ustün, B. Ozçelik, Y. Akyön, U. Abbasoglu and E. Yesilada, *J. Ethnopharmacol.*, 2006, **108**, 457–461.

118 P. Grassi, T. S. U. Reyes, S. Sosa, A. Tubaro, O. Hofer and K. Zitterl-Eglseer, *Z. Naturforsch., C*, 2006, **61**, 165–170.
119 R. González-Vázquez, B. K. Díaz, M. I. Aguilar, N. Diego and B. Lotina-Hennsen, *J. Agric. Food Chem.*, 2006, **54**, 1217–1221.
120 J. T. Jeong, J.-H. Moon, K.-H. Park and C. S. Shin, *J. Agric. Food Chem.*, 2006, **54**, 2123–2128.
121 A. Delazar, F. Biglari, S. Esnaashari, H. Nazemiyeh, A.-M. Talebpour, L. Nahar and S. D. Sarker, *Phytochemistry*, 2006, **67**, 2176–2181.
122 H. Xiao and K. L. Parkin, *Phytochemistry*, 2007, **68**, 1059–1067.
123 A. Hunyadi, A. Gergely, A. Simon, G. Tóth, G. Veress and M. Báthori, *J. Chromatogr. Sci.*, 2007, **45**, 76–86.
124 I. M. Villaseñor and A. P. Domingo, *Teratogenesis Carcinogen Mutagen*, 2000, **20**, 99–105.
125 T. H. Jakobsen, H. V. Marcussen, A. Adsersen, D. Strasberg, U. W. Smitt and J. W. Jaroszewski, *Biochem. Syst. Ecol.*, 2001, **29**, 963–965.
126 I. M. Villaseñor, D. E. Echegoyen and J. S. Angelada, *Mutat. Res.*, 2002, **515**, 141–146.
127 A. Landreau, Y. F. Pochus, C. Sallenave-Namont, J.-F. Biard, M.-C. Boumard, T. R. du Pont, F. Mondeguer, C. Goulard and J.-F. Verbist, *J. Microbiol. Methods*, 2002, **48**, 181–194.
128 N. Aligiannis, S. Mitaku, E. Tsitsa-Tsardis, C. Harvala, I. Tsaknis, S. Lalas and S. Haroutounian, *J. Agric. Food Chem.*, 2003, **51**, 7308–7312.
129 K. S. Khetwal and S. Pande, *Nat. Prod. Res.*, 2004, **18**, 129–133.
130 D. P. Overy and J. W. Blunt, *J. Nat. Prod.*, 2004, **67**, 1850–1853.
131 J. H. Sung, S. J. Choi, S. W. Lee, K. H. Park and T. W. Moon, *Biosci., Biotechnol., Biochem.*, 2004, **68**, 1051–1058.
132 A. N. García-Argáez, N. M. González-Lugo, H. Parra-Delgado and M. M. Vázquez, *Biochem. Syst. Ecol.*, 2005, **33**, 441–443.
133 M. S. Ahmed, N. D. El Tanbouly, W. T. Islam, A. A. Sleem and A. S. El Senousy, *Phytother. Res.*, 2005, **19**, 807–809.
134 A.-C. Mataine-Offer, T. Miyamoto, C. Jolly, C. Delaude and M.-A. Lacaille-Dubois, *Helv. Chim. Acta*, 2005, **88**, 2986–2995.
135 L. R. Chadwick, H. H. S. Fong, N. R. Farnsworth and G. F. Pauli, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1959–1969.
136 S.-C. Chou, M. C. Everngam, G. Sturtz and J. J. Beck, *Phytother. Res.*, 2006, **20**, 153–156.
137 H. Kalász, E. Liktor-Busa, G. Janicsák and M. Báthori, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 2095–2109.
138 B. Bonnländer and P. Winterhalter, *J. Agric. Food Chem.*, 2000, **48**, 4821–4825.
139 A. C. Pinto, W. F. Braga, C. M. Rezende, F. M. S. Garrido, V. F. Veiga Jr, L. Bergter, M. L. Patitucci and O. A. C. Antunes, *J. Braz. Chem. Soc.*, 2000, **11**, 355–360.
140 L. Jiang, H. Kojima, K. Yamada, A. Kobayashi and K. Kubota, *J. Agric. FoodChem.*, 2001, **49**, 5888–5894.
141 S. Glasl, D. Gunbilig, S. Narantuya, I. Werner and J. Jurenitsch, *J. Chromatogr., A*, 2001, **936**, 193–200.
142 G. Arramon, C. Saucier, D. Colombani and Y. Glories, *Phytochem. Anal.*, 2002, **13**, 305–310.
143 G. R. Eldridge, H. C. Vervoort, C. M. Lee, P. A. Cremin, C. T. Williams, S. M. Hart, M. G. Goering, M. O'Neill-Johnson and L. Zeng, *Anal. Chem.*, 2002, **74**, 3963–3971.
144 F. Cateni, J. Zilic, G. Falsone, F. Hollan, F. Frausin and V. Scarcia, *Farmaco*, 2003, **58**, 809–817.
145 S. Barrek, O. Paisse and M.-F. Grenier-Loustalot, *Anal. Bioanal. Chem.*, 2004, **378**, 753–763.
146 S. Wu, C. Sun, K. Wang and Y. Pan, *J. Chromatogr., A*, 2004, **1028**, 171–174.
147 R. Girija, G. K. Jayarakasha, J. Brodbelt, M. Cho and B. S. Patil, *Anal. Lett.*, 2004, **37**, 3005–3016.
148 R. Girija, M. Cho, J. S. Brodbelt and B. S. Patil, *Phytochem. Anal.*, 2005, **16**, 155–160.
149 K.-y. Sohda, K. Nagai, T. Yamori, K.-i. Suzuki and A. Tanaka, *J. Antibiot.*, 2005, **58**, 27–31.
150 H. Xiao and K. Parkin, *J. Agric. Food Chem.*, 2006, **54**, 8417–8424.
151 S.-Y. Jeong, H. J. Shin, T. S. Kim, H.-S. Lee, S.-k. Park and H. M. Kim, *J. Antibiot.*, 2006, **59**, 234–240.
152 Y. H. Kim, E.-H. Kim, C. Lee, M.-H. Kim and J.-R. Rho, *Lipids*, 2007, **42**, 395–399.
153 M. Chaabi, V. Freund-Michel, N. Frossard, A. Randrinantsoa, R. Andriantsitohaina and A. Lobstein, *J. Ethnopharmacol.*, 2007, **109**, 134–139.
154 W. G. Taylor, P. G. Fields and D. H. Sutherland, *J. Agric. Food Chem.*, 2007, **55**, 5491–5498.
155 Y. Matsuo, K. Kanoh, T. Yamori, H. Kasai, A. Katsuta, K. Adachi, K. Shin-ya and Y. Shizuri, *J. Antibiot.*, 2007, **60**, 251–255.
156 Y.-Z. Zhang, Y.-H. Lu, D.-Z. Wei, G.-X. Chou and E.-Y. Zhu, *Prep. Biochem. Biotechnol.*, 2007, **37**, 185–193.
157 R. G. Reid and S. D. Sarker, 'Isolation of Natural Products by Low-Pressure Column Chromatography', in *Natural Products Isolation*, 2nd edn, ed. S. D. Sarker, Z. Latif and A. I. Gray, Humana Press, Totowa, New Jersey, 2006, pp. 117–157.
158 L. J. Clifford, Q. Jia and J. J. Pestka, *J. Agric. Food Chem.*, 2003, **51**, 521–523.
159 D. M. Gurfinkel, W. F. Reynolds and A. V. Rao, *Int. J. Food Sci. Nutr.*, 2005, **56**, 501–519.
160 H. Sun, X. Li, G. Ma and Z. Su, *Chromatographia*, 2005, **61**, 9–15.
161 H. Sun, X. Li, G. Ma, Z. Su and X. Li, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 605–617.
162 X. Li, R. Ma, H. Su, H. Sun, G. Ma, Z. Su and S. Zha, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 569–582.
163 S.-H. Pyo, H.-J. Choi and B.-H. Han, *J. Chromatogr., A*, 2006, **1123**, 15–21.
164 Sz. Nyiredy, K. Dallenbach-Toelke, G. C. Zogg and O. Sticher, *J. Chromatogr.*, 1990, **499**, 433–462.
165 A. B. Pomilio, S. R. Leicach, M. Y. Grass, C. M. Ghersa, M. Santoro and A. A. Vitale, *Phytochem. Anal.*, 2000, **11**, 304–308.
166 T. Wennberg, J.-P. Rauha and H. Vuorela, *Chromatographia*, 2001, **53**, S240–S245.
167 S. Block, C. Stévigny, M.-C. De Pauw-Gillet, E. de Hoffmann, G. Llabrès, V. Adjakidje and J. Quetin-Leclercq, *Planta Med.*, 2002, **68**, 647–649.
168 V. Sharma, S. Walia, J. Kumar, M. G. Nair and B. S. Parmar, *J. Agric. Food Chem.*, 2003, **51**, 3966–3972.
169 M. Haddad, T. Miyamoto, C. Delaude and M.-A. Lacaille-Dubois, *Helv. Chim. Acta*, 2003, **86**, 3055–3065.
170 U. G. Chandrika, E. R. Jansz, S. M. D. N. Wickramasinghe and N. D. Warnasuriya, *J. Sci. Food Agric.*, 2003, **83**, 1279–1282.
171 A.-C. Mitaine-Offer, T. Miyamoto, V. Laurens, C. Delaude and M.-A. Lacaille-Dubois, *Helv. Chim. Acta*, 2003, **86**, 2404–2413.
172 C. A. Simões-Pires, E. F. Queiroz, A. T. Henriques and K. Hostettmann, *Phytochem. Anal.*, 2005, **16**, 307–314.
173 H. R. El-Seedi, *Nat. Prod. Commun.*, 2006, **1**, 655–659.
174 V. Sharma, S. Walia, S. Dhingra, J. Kumar and B. S. Parmar, *Pest Manage. Sci.*, 2006, **62**, 965–975.
175 F. Higashiguchi, H. Nakamura, H. Hayashi and T. Kometani, *J. Agric. Food Chem.*, 2006, **54**, 5948–5953.
176 G. Engelhardt, F. Petereit, J. Anke and A. Hensel, *Pharmazie*, 2007, **62**, 558–559.
177 Z. Latif, 'Isolation by Preparative High-Performance Liquid Chromatography', in *Natural Products Isolation*, ed. S. D. Sarker, Z. Latif and A. I. Gray, 2nd edn, Humana Press, Totowa, New Jersey, 2006, pp. 213–232.
178 E. M. Kuskoski, J. M. Vega, J. J. Rios, R. Fett, A. M. Troncoso and A. G. Asuero, *J. Agric. Food Chem.*, 2003, **51**, 5450–5454.
179 M. Schwarz, V. Wray and P. Winterhalter, *J. Agric. Food Chem.*, 2004, **52**, 5095–5101.
180 J. Z. Xu, S. Y. V. Yeung, Q. Chang, Y. Huang and Z.-Y. Chen, *Br. J. Nutr.*, 2004, **91**, 873–881.
181 V. Švehlíková, R. N. Bennet, F. A. Mellon, P. W. Needs, S. Paicente, P. A. Kroon and Y. Bao, *Phytochemistry*, 2004, **65**, 2323–2332.
182 Ø. M. Anderson, T. Fossen, K. Torskangerpoll, A. Fossen and U. Hauge, *Phytochemistry*, 2004, **65**, 405–410.
183 X. Zhou, J. Peng, G. Fan and Y. Wu, *J. Chromatogr., A*, 2005, **1092**, 216–221.
184 I. N. Kuzovkina, A. V. Guseva, D. Kovács, É. Szöke and M. Y. Vdovitchenko, *Russ. J. Plant Physiol.*, 2005, **52**, 77–82.
185 R. Byamukama, B. T. Kiremire, Ø. Andersen and A. Steigen, *J. Food Compos. Anal.*, 2005, **18**, 599–605.
186 G. R. Takeoka, L. T. Dao, H. Tamura and L. A. Harden, *J. Agric. Food Chem.*, 2005, **53**, 4932–4937.
187 N. Berardini, R. Fezer, J. Conrad, U. Beifuss, R. Carle and A. Schieber, *J. Agric. Food Chem.*, 2005, **53**, 1563–1570.
188 C. Girotti, M. Ginet, F. D. Demarne, M. Lagarde and A. Gélöen, *Planta Med.*, 2005, **71**, 1170–1172.
189 L. Longo and G. Vasapollo, *J. Agric. Food Chem.*, 2005, **53**, 8063–8067.

190 S. Rayyan, T. Fossen, H. S. Nateland and Ø. M. Andersen, *Phytochem. Anal.*, 2005, **16**, 334–341.
191 T. K. McGhie, D. R. Rowan and P. J. Edwards, *J. Agric. Food Chem.*, 2006, **54**, 8756–8761.
192 M. A. Kelm, J. C. Johnson, R. J. Robbins, J. F. Hammerstone and H. H. Schmitz, *J. Agric. Food Chem.*, 2006, **54**, 1571–1576.
193 K. Khallouki, R. Haubner, W. E. Hull, G. Erben, B. Spiegelhalder, H. Bartsch and R. W. Owen, *Food Chem. Toxicol.*, 2007, **45**, 472–485.
194 M. M. Tanae, M. T. R. Lima-Landman, T. C. M. De Lima, C. Souccar and A. J. Lapa, *Phytomedicine*, 2007, **14**, 309–313.
195 L.-p. Qu, G.-r. Fan, J.-y. Peng and H.-m. Mi, *Fitoterapia*, 2007, **78**, 200–204.
196 B. Girennavar, S. M. Poulose, G. K. Jayaprakasha, N. G. Bhat and B. S. Patil, *Bioorg. Med. Chem.*, 2006, **14**, 2606–2612.
197 A. P. Kulkarni, S. M. Aradhya and S. Divakar, *Food Chem.*, 2004, **87**, 551–557.
198 G. Li, C.-S. Seo, S.-H. Lee, Y. Jahng, H.-W. Chang, C.-S. Lee, M.-H. Woo and J.-K. Son, *Bull. Korean Chem. Soc.*, 2004, **25**, 397–399.
199 P. Stocker, M. Yousfi, C. Salmi, J. Perrier, J. M. Brunel and A. Moulin, *Biochimie*, 2005, **87**, 507–512.
200 Y. Sudjaroen, R. Haubner, G. Würtele, W. E. Hull, G. Erben, B. Spiegelhalder, S. Changbumrung, H. Bartsch and R. W. Owen, *Food Chem. Toxicol.*, 2005, **43**, 1673–1682.
201 Y. Lu, C. Sun and Y. Pan, *J. Sep. Sci.*, 2006, **29**, 351–357.
202 M. T. S. Trevisan, B. Pfrundstein, R. Haubner, W. Würtele, B. Spiegelhalder, H. Bartsch and R. W. Owen, *Food Chem. Toxicol.*, 2006, **44**, 188–197.
203 J. N. Tshibangu, A. D. Wright and G. M. König, *Phytochem. Anal.*, 2003, **14**, 13–22.
204 S. T. Häkkinen, H. Rischer, I. Laakso, H. Maaheimo, T. Seppänen-Laakso and K.-M. Oksman-Caldentey, *Planta Med.*, 2004, **70**, 936–941.
205 I. K. Rhee, N. Appels, B. Hofte, B. Karabatak, C. Erkelens, L. M. Stark, L. A. Flippin and R. Verpoorte, *Biol. Pharm. Bull.*, 2004, **27**, 1804–1809.
206 P. W. Dalsgaard, J. W. Blunt, M. H. G. Munro, J. C. Frisvad and C. Christophersen, *J. Nat. Prod.*, 2005, **68**, 258–261.
207 S. Liu, K. Chen, W. Schliemann and D. Strack, *Phytochem. Anal.*, 2005, **16**, 86–89.
208 M. Shoeb, S. M. MacManus, Y. Kumarasamy, M. Jaspars, L. Nahar, P. K. Thoo-Lin, H. Nazemiyeh and S. D. Sarker, *Phytochemistry*, 2006, **67**, 2370–2375.
209 V. C. da Silva, G. H. Silvab, V. da S. Bolzani and M. N. Lopes, *Ecletica Quim.*, 2006, **31**, 55–58.
210 F. Wei, L.-Y. Ma, X.-L. Cheng, R.-C. Lin, W.-T. Jin and I. A. Khan, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 763–773.
211 C. Liu, J. Han, Y. Duan, X. Huang and H. Wang, *Sep. Purif. Technol.*, 2007, **54**, 198–203.
212 R. T. Nogueira, S. C. N. Queiroz and P. M. Imamura, *J. Liq. Chromatogr. Relat. Technol.*, 2002, **25**, 59–67.
213 Y. Kumarasamy, L. Nahar, P. J. Cox, M. Jaspars and S. D. Sarker, *Phytomedicine*, 2003, **10**, 344–347.
214 A. Delazar, M. Byres, S. Gibbons, Y. Kumarasamy, M. Modarresi, L. Nahar, M. Shoeb and S. D. Sarker, *J. Nat. Prod.*, 2004, **67**, 1584–1587.
215 F. Fang, S. Sang, K. Y. Chen, A. Gosslau, C.-T. Ho and R. T. Rosen, *Food Chem.*, 2005, **93**, 497–501.
216 M. Kettering, C. Valdivia, O. Sterner, H. Anke and E. Thines, *J. Antibiot.*, 2005, **58**, 390–396.
217 P. R. Sundaresan, S. A. Slavoff, E. Grundel, K. D. White, E. Mazzola, D. Koblenz and J. I. Rader, *Phytochem. Anal.*, 2006, **17**, 243–250.
218 W. Tang, T. Gu and J.-J. Zhong, *Biochem. Eng. J.*, 2006, **32**, 205–210.
219 I. B. Baloch, M. K. Baloch and Q. N. us Saqib, *Planta Med.*, 2006, **72**, 830–834.
220 S. Nagarajan and L. J. M. Rao, *J. Chromatogr. Sci.*, 2007, **45**, 189–194.
221 J. C. T. Silva, G. N. Jham, R. D'arc, L. Oliveira and L. Brown, *J. Chromatogr., A*, 2007, **1151**, 203–210.
222 N. Nenadis, J. Vervoort, S. Boeren and M. Z. Tsimidou, *J. Sci. Food Agric.*, 2007, **87**, 160–166.
223 P. W. Dalsgaard, T. O. Larsen, K. Frydenvang and C. Christophersen, *J. Nat. Prod.*, 2004, **67**, 878–881.
224 G. Hagelin, I. Oulie, Å. Raknes, K. Undheim and O. G. Clausen, *J. Chromatogr., B*, 2004, **811**, 243–251.
225 Y. Zhang, B. Chen, Z. Huang and Z. Shi, *J. Liq. Chromatogr. Relat. Technol.*, 2004, **27**, 875–884.
226 M. P. Mansour, *J. Chromatogr., A*, 2005, **1097**, 54–58.
227 P. W. Dalsgaard, T. O. Larsen and C. Christophersen, *J. Antibiot.*, 2005, **58**, 141–144.
228 L. Ivanova, E. Skjerve, G. S. Eriksen and S. Uhlig, *Toxicon*, 2006, **47**, 868–876.
229 Q. Han, L. Yang, Y. Liu, Y. Wang, C. Qiao, J. Song, L. Du, D. Yang, S. Chen and H. Xu, *Planta Med.*, 2006, **72**, 281–284.
230 S. Rochfort, D. Caridi, M. Stinton, V. C. Trenerry and R. Jones, *J. Chromatogr., A*, 2006, **1120**, 205–210.
231 Y. Ito, M. Weinstein, I. Aoki, R. Harada and E. Kimura, *Nature*, 1966, **212**, 985–987.
232 L. Marchal, J. Legrand and A. Foucault, *Chem. Rec.*, 2003, **3**, 133–143.
233 A. Berthod, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 1447–1463.
234 M. Bourdat-Deschamps, C. Herrenknecht, B. Akendengue, A. Laurens and R. Hocquemiller, *J. Chromatogr., A*, 2004, **1041**, 143–152.
235 *High-Speed Countercurrent Chromatography*, ed. Y. Ito and W. D. Conway, John Wiley & Sons, New York/Chichester/Brisbane/Toronto/Singapore, 1996.
236 A. Berthod, *Countercurrent Chromatography. The Support-Free Stationary Phase*, Elsevier, Amsterdam, 2002.
237 A. Marston and K. Hostettmann, *J. Chromatogr., A*, 2006, **1112**, 181–194.
238 A. Marston, I. Slacanin and K. Hostettmann, *Phytochem. Anal.*, 1990, **1**, 3–17.
239 A. Marston and K. Hostettmann, *J. Chromatogr., A*, 1994, **658**, 315–341.
240 M. Maillard, A. Marston and K. Hostettmann, 'High-Speed Countercurrent Chromatography of Natural Products', in *High-Speed Countercurrent Chromatography*, ed. Y. Ito and W. D. Conway, John Wiley & Sons, New York/Chichester/Brisbane/Toronto/Singapore, 1996, pp. 179–223.
241 T.-Y. Zhang, 'High-Speed Countercurrent Chromatography of Medicinal Herbs', in *High-Speed Countercurrent Chromatography*, ed. Y. Ito and W. D. Conway, John Wiley & Sons, New York/Chichester/Brisbane/Toronto/Singapore, 1996, pp. 225–263.
242 N. L. Fregeau and K. L. Rinehart, 'Isolation of Marine Natural Products by High-Speed Countercurrent Chromatography', in *High-Speed Countercurrent Chromatography*, ed. Y. Ito and W. D. Conway, John Wiley & Sons, New York/Chichester/Brisbane/Toronto/Singapore, 1996, pp. 265–300.
243 T. Zhang, 'Separation and purification of natural products (medicinal herbs) by high speed countercurrent chromatography', in *Countercurrent Chromatography. The Support-Free Stationary Phase*, ed. A. Berthod, Elsevier, Amsterdam, 2002, pp. 201–260.
244 Y. Ito, *J. Chromatogr., A*, 2005, **1065**, 145–168.
245 Y. Pan and Y. Lu, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 649–679.
246 A. Berthod, M. Hassoun and M. J. Ruiz-Angel, *Anal. Bioanal. Chem.*, 2005, **383**, 327–340.
247 J. B. Friesen and G. F. Pauli, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 2777–2806.
248 J. B. Friesen and G. F. Pauli, *J. Chromatogr., A*, 2007, **1151**, 51–59.
249 Q. Du, P. Chen, G. Jerz and P. Winterhalter, *J. Chromatogr., A*, 2004, **1040**, 147–149.
250 M. Sannomiya, C. M. Rodrigues, R. G. Coelho, L. C. dos Santos, C. A. Hiruma-Lima, A. R. M. Souza Brito and W. Vilegas, *J. Chromatogr., A*, 2004, **1035**, 47–51.
251 Q. Du, G. Jerz and P. Winterhalter, *J. Chromatogr., A*, 2004, **1045**, 59–63.
252 J. Peng, G. Yang, G. Fan and Y. Wu, *J. Chromatogr., A*, 2005, **1092**, 235–240.
253 W. Jin and P.-F. Tu, *J. Chromatogr., A*, 2005, **1092**, 241–245.
254 N. S. Kumar and M. Rajapaksha, *J. Chromatogr., A*, 2005, **1083**, 223–228.
255 X. Wang, C. Cheng, Q. Sun, F. Li, J. Liu and C. Zheng, *J. Chromatogr., A*, 2005, **1075**, 127–131.
256 H.-B. Li and F. Chen, *J. Chromatogr., A*, 2005, **1074**, 107–110.
257 S. Wu, A. Sun and R. Liu, *J. Chromatogr., A*, 2005, **1066**, 243–247.
258 R. Liu, A. Li, A. Sun, J. Cui and L. Kong, *J. Chromatogr., A*, 2005, **1064**, 53–57.

259 J. Peng, G. Fan, Z. Hong, Y. Chai and Y. Wu, *J. Chromatogr., A*, 2005, **1074**, 111–115.
260 M. Zhao, Y. Ito and P. Tu, *J. Chromatogr., A*, 2005, **1090**, 193–196.
261 Q. Du, G. Jerz and P. Winterhalter, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **27**, 3257–3264.
262 L. J. Chen, H. Song, X. Q. Lan, D. E. Games and I. A. Sutherland, *J. Chromatogr., A*, 2005, **1063**, 241–245.
263 Q. Du, L. Li and G. Jerz, *J. Chromatogr., A*, 2005, **1077**, 98–101.
264 G. G. Leitão, S. S. El-Adji, W. A. Lopes de Melo, S. G. Leitão and L. Brown, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 2041–2051.
265 Q. Du, Q. Zhang and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 137–144.
266 X. Wang, F. Li, H. Zhang, Y. Geng, J. Yuan and T. Jiang, *J. Chromatogr., A*, 2005, **1090**, 188–192.
267 C.-j. Ma, G.-s. Li, Da-l. Zhang, K. Liu and X. Fan, *J. Chromatogr., A*, 2005, **1078**, 188–192.
268 X. Ma, W. Tian, L. Wu, X. Cao and Y. Ito, *J. Chromatogr., A*, 2005, **1070**, 211–214.
269 J. Peng, G. Fan and Y. Wu, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 1619–1632.
270 T. Zhou, B. Chen, G. Fan, Y. Chai and Y. Wu, *J. Chromatogr., A*, 2006, **1116**, 97–101.
271 L. Qu and J. Peng, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 913–924.
272 J. Peng, G. Fan and Y. Wu, *J. Chromatogr., A*, 2006, **1115**, 103–111.
273 M. Gao, M. Gu and C.-z. Liu, *J. Chromatogr., B*, 2006, **838**, 139–143.
274 D. Rinaldo, M. A. Silva, C. M. Rodrigues, T. R. Calvo, M. Sannomiya, L. C. dos Santos, W. Vilegas, H. Kushima, C. A. Hiruma-Lima and A. R. M. de Souza Brito, *Quim. Nova*, 2006, **29**, 947–949.
275 J.-H. Renault, L. Voutquenne, C. Caron, M. Zeches-Hanrot, S. Berwanger and H. Becker, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 761–771.
276 D. Gutzeit, V. Wray, P. Winterhalter and G. Jerz, *Chromatographia*, 2007, **65**, 1–7.
277 X. Han, X. Ma, T. Zhang, Y. Zhang, Q. Liu and Y. Ito, *J. Chromatogr., A*, 2007, **1151**, 180–182.
278 Y. Wei and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 1465–1473.
279 A. Sun, Q. Sun and R. Liu, *J. Sep. Sci.*, 2007, **30**, 1013–1018.
280 Y. Liang, Z. Huang, H. Chen, T. Zhang and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 419–430.
281 C. Okunji, S. Komarnytsky, G. Fear, A. Poulev, D. M. Ribnicky, P. I. Awachie, Y. Ito and I. Raskin, *J. Chromatogr., A*, 2007, **1151**, 45–50.
282 Q. Zhang, L.-Y. Chen, H.-Y. Ye, L. Gao, W. Hou, M. Tang, G. Yang, Z. Zhong, Y. Yuan and A. Peng, *J. Sep. Sci.*, 2007, **30**, 2153–2159.
283 Y. Lu, C. Sun, Y. Wang and Y. Pan, *J. Chromatogr., A*, 2007, **1151**, 31–36.
284 Q.-E. Wang, F. S.-C. Lee and X. Wang, *J. Chromatogr., A*, 2004, **1048**, 51–57.
285 Y. Wei, T. Zhang and Y. Ito, *J. Chromatogr., A*, 2004, **1033**, 373–377.
286 R. Liu, A. Li, A. Sun and L. Kong, *J. Chromatogr., A*, 2004, **1057**, 225–228.
287 R. Liu, L. Feng, A. Sun and L. Kong, *J. Chromatogr., A*, 2004, **1057**, 89–94.
288 R. Liu, L. Feng, A. Sun and L. Kong, *J. Chromatogr., A*, 2004, **1055**, 71–76.
289 R. Liu, A. Li and A. Sun, *J. Chromatogr., A*, 2004, **1052**, 223–227.
290 H.-B. Li and F. Chen, *J. Chromatogr., A*, 2004, **1061**, 51–54.
291 H.-B. Li and F. Chen, *J. Sep. Sci.*, 2005, **28**, 268–272.
292 R. Liu, Q. Sun, A. Sun and J. Cui, *J. Chromatogr., A*, 2005, **1072**, 195–199.
293 R. Liu, Q. Sun, Y. Shi and L. Kong, *J. Chromatogr., A*, 2005, **1076**, 127–132.
294 J. Yan, S. Tong, L. Sheng and J. Lou, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 1307–1315.
295 Y. Wei and Y. Ito, *J. Chromatogr., A*, 2006, **1115**, 112–117.
296 C. H. Ma, W. Ke, Z. L. Sun, J. Y. Peng, Z. X. Li, X. Zhou, G. R. Fan and C. G. Huang, *Chromatographia*, 2006, **64**, 83–87.
297 A. Sun, L. Feng and R. Liu, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 751–759.
298 Y. Wei and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 1609–1618.
299 X. Wang, Y. Wang, Y. Geng, F. Li and C. Zheng, *J. Chromatogr., A*, 2004, **1036**, 171–175.
300 H.-B. Li and F. Chen, *J. Chromatogr., A*, 2004, **1052**, 229–232.
301 H.-T. Lu, Y. Jiang and F. Chen, *J. Chromatogr., A*, 2004, **1026**, 185–190.
302 J. Yan, S. Tong, J. Chu, L. Sheng and G. Chen, *J. Chromatogr., A*, 2004, **1043**, 329–332.
303 A. Hazekamp, R. Simons, A. Peltenburg-Looman, M. Sengers, R. van Zweden and R. Verpoorte, *J. Liq. Chromatogr. Relat. Technol.*, 2004, **27**, 2421–2439.
304 K. Schäfer and P. Winterhalter, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1703–1716.
305 L. Li, R. Tsao, Z. Liu, S. Liu, R. Yang, J. C. Young, H. Zhu, Z. Deng, M. Xie and Z. Fu, *J. Chromatogr., A*, 2005, **1063**, 161–169.
306 X. Ma, L. Wu, Y. Ito and W. Tian, *J. Chromatogr., A*, 2005, **1076**, 212–215.
307 Y. Lu, C. Sun, Y. Wang and Y. Pan, *J. Chromatogr., A*, 2005, **1089**, 258–262.
308 G. G. Leitao, P. A. de Souza, A. A. Moraes and L. Brown, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 2053–2060.
309 Y. Jiang, P. Tu, X. Chen and T. Zhang, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1583–1592.
310 J. Chen, F. Wang, F. S.-C. Lee, X. Wang and M. Xie, *Talanta*, 2006, **69**, 172–179.
311 W. Zhi and Q. Deng, *J. Chromatogr., A*, 2006, **1116**, 149–152.
312 J. Peng, Y. Jiang, G. Fan, B. Chen, Q. Zhang, Y. Chai and Y. Wu, *Sep. Purif. Technol.*, 2006, **52**, 22–28.
313 Q. Han, J. Song, C. Qiao, L. Wong and H. Xu, *J. Sep. Sci.*, 2006, **29**, 1653–1657.
314 W.-H. Zhao, C.-C. Gao, X.-F. Ma, X.-Y. Bai and Y.-X. Zhang, *J. Chromatogr., B*, 2007, **850**, 523–527.
315 L. Chen, Q. Zhang, G. Yang, L. Fan, J. Tang, I. Garrard, S. Ignatova, D. Fisher and I. A. Sutherland, *J. Chromatogr., A*, 2007, **1142**, 115–122.
316 J. J. Lu, Y. Wei and Q. P. Yuan, *Sep. Purif. Technol.*, 2007, **55**, 40–43.
317 M. Gu, X. Wang, Z. Su and F. Ouyang, *J. Chromatogr., A*, 2007, **1140**, 107–111.
318 S. He, Y. Lu, B. Wu and Y. Pan, *J. Chromatogr., A*, 2007, **1151**, 175–179.
319 Y. Wang and B. Liu, *Phytochem. Anal.*, 2007, **18**, 436–440.
320 J. Lu, Y. Wei and Q. Yuan, *J. Chromatogr., B*, 2007, **857**, 175–179.
321 R. Liu, X. Chu, A. Sun and L. Kong, *J. Chromatogr., A*, 2005, **1074**, 139–144.
322 S. Tong, J. Yan and J. Lou, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 2979–2989.
323 A. C. Leite, E. C. Cabral, D. A. P. dos Santos, J. B. Fernandes, P. C. Vieira and M. F. das G. F. Silva, *Quim. Nova*, 2005, **28**, 983–985.
324 Q. Tang, C. Yang, W. Ye, J. Liu and S. Zhao, *J. Chromatogr., A*, 2007, **1144**, 203–207.
325 Z. Liu, Y. Jin, P. Shen, J. Wang and Y. Shen, *Talanta*, 2007, **71**, 1873–1876.
326 J. Peng, X. Han, Y. Xu, Y. Qi, L. Xu and Q. Xu, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 2929–2940.
327 X.-K. Ou Yang, M.-C. Jin and C.-H. He, *Sep. Purif. Technol.*, 2007, **56**, 319–324.
328 R. Liu, A. Li and A. Sun, *J. Chromatogr., A*, 2004, **1052**, 217–221.
329 X. Chu, A. Sun and R. Liu, *J. Chromatogr., A*, 2005, **1097**, 33–39.
330 S. Yao, Y. Li and L. Kong, *J. Chromatogr., A*, 2006, **1115**, 64–71.
331 Y. Xie, Y. Liang, H.-W. Chen, T.-Y. Zhang and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 1475–1488.
332 X. Cao, D. Huang, Y. Dong, H. Zhao and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 1657–1668.
333 J. Peng, G. Fan and Y. Wu, *J. Chromatogr., A*, 2005, **1091**, 89–93.
334 H.-B. Li and F. Chen, *J. Chromatogr., A*, 2005, **1083**, 102–105.
335 J. Peng, G. Fan, L. Qu, X. Zhou and Y. Wu, *J. Chromatogr., A*, 2005, **1082**, 203–207.
336 T. Huang, P. Shen and Y. Shen, *J. Chromatogr., A*, 2005, **1066**, 239–242.
337 T. Huang, Y. Shen and P. Shen, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 2383–2390.
338 X. Wang, F. Li, Q. Sun, J. Yuan, T. Jiang and C. Zheng, *J. Chromatogr., A*, 2005, **1063**, 247–251.

339 R. Rodrigues de Oliveira, A. P. Heringer, M. R. Figueiredo, D. O. Futuro and M. A. C. Kaplan, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 229–234.
340 C. Y. Kim, M.-J. Ahn and J. Kim, *J. Sep. Sci.*, 2006, **29**, 656–659.
341 S. Feng, S. Ni and W. Sun, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 135–145.
342 Y. Jiang, H.-T. Lu and F. Chen, *J. Chromatogr., A*, 2004, **1033**, 183–186.
343 Q. Du and J. Yuan, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1717–1724.
344 R. Liu, L. Kong, A. Li and A. Sun, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 521–532.
345 Y. W. Ha, S. S. Lim, I. J. Ha, Y.-C. Na, J.-J. Seo, H. Shin, S. H. Son and Y. S. Kim, *J. Chromatogr., A*, 2007, **1151**, 37–44.
346 S. Shi, D. Jiang, M. Zhao and P. Tu, *J. Chromatogr., B*, 2007, **852**, 679–683.
347 Q. B. Han, J. Z. Song, C. F. Qiao, L. Wong and H. X. Xu, *J. Sep. Sci.*, 2007, **30**, 135–140.
348 Q. Du, G. Jerz, P. Chen and P. Winterhalter, *J. Liq. Chromatogr. Relat. Technol.*, 2004, **27**, 2201–2215.
349 T. Zhou, G. Fan, Z. Hong, Y. Chai and Y. Wu, *J. Chromatogr., A*, 2005, **1100**, 76–80.
350 Y. Ma, H. A. Aisha, L. Liao, S. Aibai, T. Zhang and Y. Ito, *J. Chromatogr., A*, 2005, **1076**, 198–201.
351 A. Li, A. Sun and R. Liu, *J. Chromatogr., A*, 2005, **1076**, 193–197.
352 J. Yan, G. Chen, S. Tong, Y. Feng, L. Sheng and J. Lou, *J. Chromatogr., A*, 2005, **1070**, 207–210.
353 F. Chen, H.-B. Li, R. N.-S. Wong, B. Ji and Y. Jiang, *J. Chromatogr., A*, 2005, **1064**, 183–186.
354 R. R. Oliveira, G. G. Leitão, M. C. C. Moraes, M. A. C. Kaplan, D. Lopes and J. P. P. Carauta, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1985–1992.
355 S. Tong, J. Yan and J. Lou, *Phytochem. Anal.*, 2006, **17**, 406–408.
356 Q. Sun, A. Sun and R. Liu, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 113–121.
357 J.-P. Fan and C.-H. He, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 815–826.
358 Y. Lu, C. Sun and Y. Pan, *J. Sep. Sci.*, 2006, **29**, 314–318.
359 H.-B. Li, K.-W. Fan and F. Chen, *J. Sep. Sci.*, 2006, **29**, 699–703.
360 S. Yao, R. Liu, X. Huang and L. Kong, *J. Chromatogr., A*, 2007, **1139**, 254–262.
361 Y. Liang, J. Hu, H. Chen, T. Zhang and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2007, **30**, 509–520.
362 Y. Lu, C. Sun, R. Lui and Y. Pan, *J. Chromatogr., A*, 2007, **1146**, 125–130.
363 C. Y. Kim and J. Kim, *Phytochem. Anal.*, 2007, **18**, 115–117.
364 B. Pinel, G. Audo, S. Mallet, M. Lavault, F. De La Poype, D. Séraphin and P. Richomme, *J. Chromatogr., A*, 2007, **1151**, 14–19.
365 H.-T. Lu, Y. Jiang and F. Chen, *J. Chromatogr., A*, 2004, **1023**, 159–163.
366 H.-B. Li and F. Chen, *J. Chromatogr., A*, 2004, **1047**, 249–253.
367 X. Ma, P. Tu, Y. Chen, T. Zhang, Y. Wei and Y. Ito, *J. Chromatogr., A*, 2004, **1023**, 311–315.
368 J. Wei, T. Zhang and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1903–1911.
369 X. Cao, Y. Dong, H. Zhao, X. Pan and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 2005–2016.
370 S. R. de Paiva, M. R. Figueiredo and M. A. C. Kaplan, *Phytochem. Anal.*, 2005, **16**, 278–281.
371 C. Zhao and C. He, *J. Sep. Sci.*, 2006, **29**, 1630–1636.
372 P. W. Dalsgaard, O. Potterat, F. Dieterle, T. Paulutat, T. Kühn and M. Hamburger, *Planta Med.*, 2006, **72**, 1322–1327.
373 Q. Sun, A. Sun and R. Liu, *J. Chromatogr., A*, 2006, **1104**, 69–74.
374 C. Y. Kim, M.-J. Ahn and J. Kim, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 869–875.
375 D. Zhang, H. Teng, G. Li, K. Liu and Z. Su, *Sep. Sci. Technol.*, 2006, **41**, 3397–3408.
376 J. Yan, S. Tong, J. Li and J. Lou, *J. Liq. Chromatogr. Relat. Technol.*, 2006, **29**, 1271–1279.
377 Q. B. Han, J. Z. Song, C. F. Qiao, L. Wong and H. X. Xu, *J. Chromatogr., A*, 2006, **1127**, 298–301.
378 T. Zhou, Z. Zhu, C. Wang, G. Fan, J. Peng, Y. Chai and Y. Wu, *J. Pharm. Biomed. Anal.*, 2007, **44**, 96–100.
379 C. Han, J. Chen, J. Liu, F. S.-C. Lee and X. Wang, *Talanta*, 2007, **71**, 801–805.
380 Y. Lu, R. Liu, C. Sun and Y. Pan, *J. Sep. Sci.*, 2007, **30**, 1313–1317.
381 A. Berthod, M. J. Ruiz-Angel and S. Carda-Broch, *Anal. Chem.*, 2003, **75**, 5886–5894.
382 A. Berthod, M. Hassoun and G. Harris, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1851–1866.
383 A. Berthod, J. B. Friesen, T. Inui and G. Pauli, *Anal. Chem.*, 2007, **79**, 3371–3382.
384 A. Berthod and M. Hassoun, *J. Chromatogr., A*, 2006, **1116**, 143–148.
385 N. Frighetto, R. M. Welendorf, A. M. Pereira da Silva, M. J. Nakamura and A. C. Siani, *Phytochem. Anal.*, 2005, **16**, 411–414.
386 X. Cao and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2004, **27**, 145–152.
387 E. Delannay, A. Toribio, L. Bourdesocque, J.-M. Nuzillard, M. Zèches-Hanrot, E. Dardennes, G. Le Dour, J. Sapi and J.-H. Renault, *J. Chromatogr., A*, 2006, **1127**, 45–51.
388 F. Yang, J. Quan, T. Y. Zhang and Y. Ito, *J. Chromatogr., A*, 1998, **803**, 298–301.
389 G. Tian, T. Zhang, Y. Zhang and Y. Ito, *J. Chromatogr., A*, 2002, **945**, 281–285.
390 Y. Wei, T. Zhang and Y. Ito, *J. Chromatogr., A*, 2003, **1017**, 125–130.
391 Q. Du, P. Wu and Y. Ito, *Anal. Chem.*, 2000, **72**, 3363–3365.
392 Q. Du, G. Jerz, Y. He, L. Li, Y. Xu, Q. Zhang, Q. Zheng, P. Winterhalter and Y. Ito, *J. Chromatogr., A*, 2005, **1074**, 43–46.
393 Y. Shibusawa, Y. Yamakawa, R. Noji, A. Yanagida, H. Shindo and Y. Ito, *J. Chromatogr., A*, 2006, **1133**, 119–125.
394 Y. Yanagida, Y. Yamakawa, R. Noji, A. Oda, H. Shindo, Y. Ito and Y. Shibusawa, *J. Chromatogr., A*, 2007, **1151**, 74–81.
395 K. Patel, G. Krishna, E. Sokoloski and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2000, **23**, 2209–2218.
396 F. Yang and Y. Ito, *J. Chromatogr., A*, 2001, **923**, 281–285.
397 L. R. Chadwick, C. D. Wu and A. D. Kinghorn, *J. Liq. Chromatogr. Relat. Technol.*, 2001, **24**, 2445–2453.
398 G. Le Crouéour, P. Thépenier, B. Richard, C. Petermann, K. Ghédira and M. Zèches-Hanrot, *Fitoterapia*, 2002, **73**, 63–68.
399 L. M. Yuan, X. X. Chen, P. Ai, S. H. Qi, B. F. Li, D. Wang, L. X. Miao and Z. F. Liu, *J. Liq. Chromatogr. Relat. Technol.*, 2004, **27**, 365–369.
400 C. O. Okunji, M. M. Iwu, Y. Ito and P. L. Smith, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 775–783.
401 X. Wang, Y. Geng, F. Li, Q. Gao and X. Shi, *J. Chromatogr., A*, 2006, **1103**, 166–169.
402 X. Wang, Y. Geng, F. Li, X. Shi and J. Liu, *J. Chromatogr., A*, 2006, **1115**, 267–270.
403 J.-W. Chin, W. P. Jones, T. J. Waybright, T. G. McCloud, P. Rasoanaivo, G. M. Cragg, J. M. Cassady and A. D. Kinghorn, *J. Nat. Prod.*, 2006, **69**, 414–417.
404 A. Toribio, A. Bonfils, E. Delannay, E. Prost, D. Harakat, E. Henon, B. Richard, M. Litaudon, J.-M. Nuzillard and J.-H. Renault, *Org. Lett.*, 2006, **8**, 3825–3828.
405 A. Toribio, E. Delannay, B. Richard, K. Plé, M. Zèches-Hanrot, J.-M. Nuzillard and J.-H. Renault, *J. Chromatogr., A.*, 2007, **1140**, 101–106.
406 A. Maciuk, J.-H. Renault, R. Margraff, P. Trébuchet, M. Zèches-Hanrot and J.-M. Nuzillard, *Anal. Chem.*, 2004, **76**, 6179–6186.
407 A. Maciuk, A. Toribio, M. Zèches-Hanrot, J.-M. Nuzillard, J.-H. Renault, M. I. Georgiev and M. Ilieva, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1947–1957.
408 G. Shi, D. Dai, M. Liu and Z. Wu, Huaxue Tongbao, 2002, **65**, w73/1–w73/6.
409 F.-Y. Wu, D.-S. Dai, Y.-M. Wang and G.-A. Luo, *Gaodeng Xuexiao Huaxue Xuebao*, 2002, **23**, 1698–1700.
410 L. J. Chen, H. Song, Q. Z. Du, J. Li and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1549–1555.
411 L. J. Chen, H. Song, D. E. Games and I. A. Sutherland, *J. Liq. Chromatogr. Relat. Technol.*, 2005, **28**, 1993–2003.
412 X.-L. Cao, Y. Tian, T.-Y. Zhang, Q.-H. Liu, L.-J. Jia and Y. Ito, *J. Liq. Chromatogr. Relat. Technol.*, 2003, **26**, 1579–1591.
413 Y. Jin, P. Shen, J. Zhang, C. Zhuo, C. Xu and Y. Yan, *Zhongyao Yanjiu Yu Xinxi*, 2005, **7**, 13–15.