9.02 High Performance Liquid Chromatographic Separation Methods

Reinhard I. Boysen and Milton T. W. Hearn, Monash University, Melbourne, VIC, Australia

9.02.1 Introduction

The properties of natural products obtained from plants, fungi, animals, and other organisms of terrestrial or marine origin have intrigued mankind for millennia, in particular those products that exert an effect (i.e., have a particular bioactivity) on humans or other organisms. Such compounds have long been used as nutraceuticals, social elixirs, intoxicants, drugs of abuse, or as therapeutics.

At the highest level of classification, natural (= biological) products can be divided into two super classes, namely, primary and secondary metabolites. The primary class of natural products include nucleic acids,

proteins, carbohydrates, lipids, and their precursors, all produced by metabolic pathways essential for life. Primary metabolic pathways therefore encompass all of the modes of synthesis, interconversion, or degradation associated with the production of primary metabolites.

The secondary metabolites are produced from key intermediates of the primary metabolism pathways. They are often found in limited quantity, can occur transiently in the cell cycle and can be unique for a particular group of organisms or even species. Secondary metabolites typically represent a chemically very diverse group of small molecules (molecular mass <2000 amu) and include (1) products from overflow metabolism as a consequence of nutrient limitation, (2) compounds for defense, (3) regulatory molecules, (4) signaling molecules, or (5) molecules that serve the requirements of evolutionary exploration within the physicochemical space available on this planet. Secondary metabolites can be grouped according to the primary metabolic pathway from which they are derived or in terms of their structural similarity.

The isolation of small quantities of unknown secondary metabolites from complex biological matrices and their unambiguous structural elucidation represent formidable analytical and purification challenges. Such tasks become even more demanding if they are used in conjunction with dereplication algorithms (i.e., using natural product libraries) to avoid time and resource allocation based on already known compounds.[1–4] These studies can, in some cases, be bioassay guided, which involves the testing of individual aliquots during a separation process in order to indicate which fractions are associated with a specific bioactivity.[5] Such methods increasingly involve high-throughput, robotic instrumentation and procedures.

The process of natural product isolation and identification can be split into several subprocesses of extraction, fractionation, isolation, and final purification to a level that enables structural elucidation and appropriate functional evaluation and discrimination. After bulk extraction (e.g., maceration, boiling, Soxhlet extraction, supercritical fluid extraction) from the biological material, a crude extract is usually fractionated by a variety of different chromatographic techniques, which take into account the chemical diversity and the low-molecular-mass ranges of the secondary plant metabolites. These fractionations can be performed with a variety of chromatographic techniques, including preparative thin-layer chromatography (PTLC), high-speed countercurrent chromatography (HSCC), open column chromatography (CC), flash chromatography (FC), or solid-phase extraction (SPE).[6] For further isolation, preparative or semipreparative high-performance liquid chromatography (HPLC) is usually applied, in conjunction with online ultraviolet (UV), evaporative light scattering, fluorescence, electrochemical detection (ED), or mass spectrometric detection. Nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, Fourier transform infrared (FTIR) spectroscopy, or multiple stage mass spectrometry (MS^n) may then allow the unambiguous determination of the molecular structure.

The workflow integration of HPLC in the extraction, fractionation, isolation, and structural elucidation of natural products is illustrated in **Figure 1**. After the extraction of a natural product from a biological matrix (level 1), the crude extract is clarified (i.e., by filtration, centrifugation) to be free from particulate matter suitable for chromatography and in an appropriate (noninterfering) buffer compatible with the mobile phase(s) of the particular chromatographic mode used in the next steps. This is followed by an enrichment step (level 2), preferably with SPE or with restricted access materials (RAMs) in a step elution mode to eliminate the majority of macromolecular material and to drastically reduce the volume of the sample. The next purification step (level 3) comprises the intermediate purification of target compound(s) and a final chromatographic purification and uses a variety of high-performance (HP) chromatographic modes (i.e., reversed-phase chromatography (HP-RPC), normal-phase chromatography (HP-NPC), ion-exchange chromatography (HP-IEX), hydrophilic interaction chromatography (HP-HILIC), size exclusion chromatography (HP-SEC), or affinity chromatography (HP-AC)), to yield the desired compound(s) in the required amount and degree of purity. At the detection level (level 4), besides diode array detection (DAD), electrospray ionization (ESI) mass spectrometry (MS) can often be applied for the identification and – depending on the type of mass spectrometer – may allow quantification of the separated compound(s). Further structural elucidation can be performed with MS^n, that is, with an ion trap mass spectrometer, quadruple mass spectrometer, or FT mass spectrometer together with proton, carbon, or heteronuclear NMR spectroscopy.

Over the last two decades, HPLC has to a large extent superseded the classical modes of open column, thin-layer or paper chromatography previously used for natural product separation and has become an integral part of natural product analysis and preparative isolation. This can be attributed to various factors, including (1) availability of numerous chromatographic modes, robust high-resolution chromatographic materials and

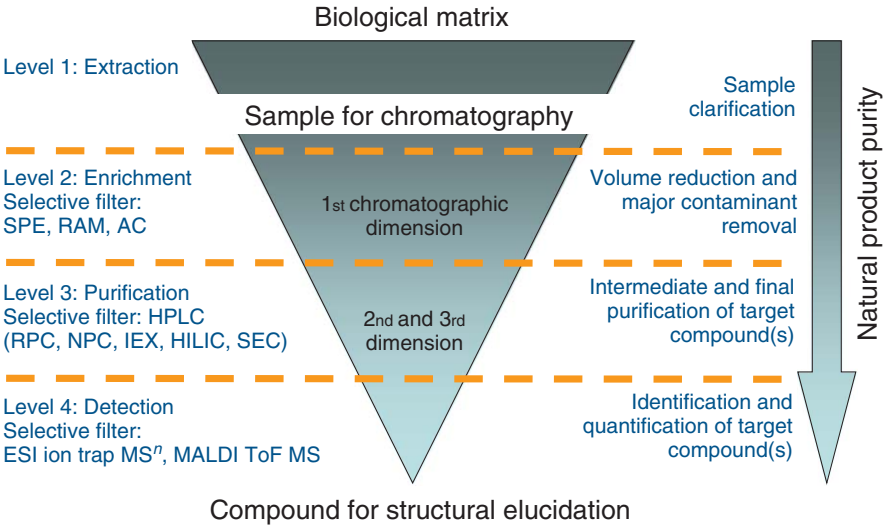


Figure 1 Example of workflow in natural product isolation from a complex biological matrix using high-performance liquid chromatography for the target compound purification and identification. With successive application of several chromatographic modes of different selectivity (i.e., hydrophobicity/hydrophilicity, charge, molecular size) the chromatographic separation can become multidimensional.

reliable instrumentation, (2) availability of well-established optimization procedures, (3) ease of scalability from analytical to preparative separation, (4) ease of integration with spectroscopic methods (e.g., DAD, UV, fluorescence), (5) the ability to use fully automated high-throughput screening (HTS) modes, (6) the ease of in-line integration with MS, and (7) the possibility of hyphenation with NMR spectroscopy.[5] An illustrative example, where these chromatographic capabilities have been used with finesse, has been reported by Dugo *et al.*[7] to resolve a highly complex, crude extract of carotenoids present in red orange juice. In this example (**Figure 2**), the crude extract was partly separated by two-dimensional (2D) normal-phase × reversed-phase

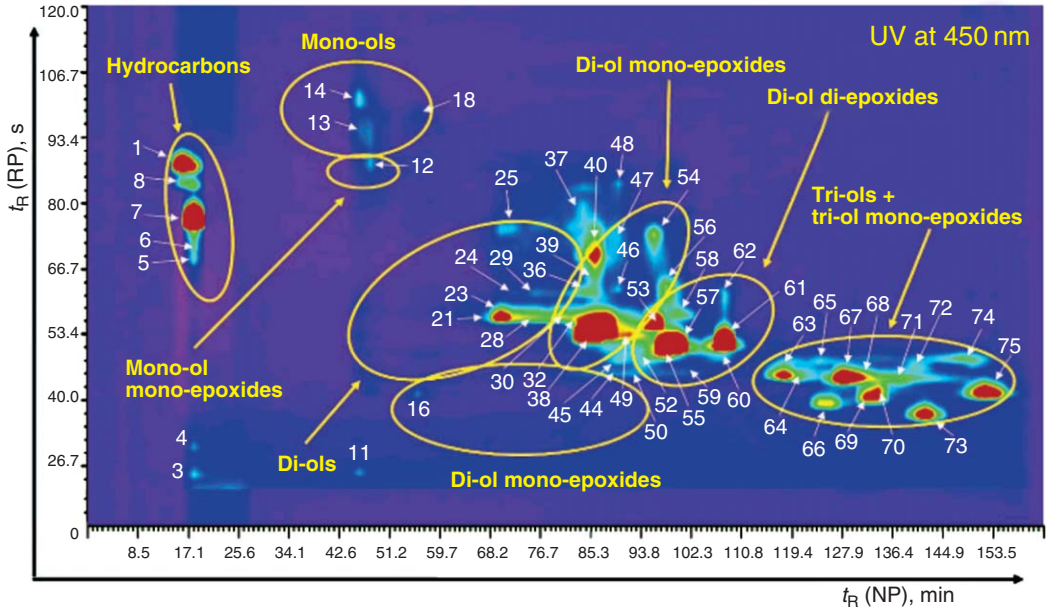


Figure 2 Contour plot of the comprehensive normal-phase × reversed-phase liquid chromatography analyses of carotenoids present in red orange juice with peaks and compound classes indicated. Reproduced from P. Dugo; V. Skerikova; T. Kumm; A. Trozzi; P. Jandera; L. Mondello, *Anal. Chem.* **2006**, *78*, 7743–7750.

liquid chromatography (LC), where the authors used a capillary 300 mm × 1.0 mm 5 μm normal-phase silica column in the first dimension with linear gradient elution (eluent A: *n*-hexane and eluent B: ethyl alcohol) at a flow rate of 10 μl min^{-1}. In the second dimension, they employed a monolithic 100 mm × 4.6 mm C_{18} column, with linear gradient elution (eluent A: 2-propanol and eluent B: 20% (v/v) water in acetonitrile (ACN)) at a flow rate of 4.7 ml min^{-1}. The incompatibility of the solvents that were used in the two dimensions (normal-phase chromatography (NPC) and reversed-phase chromatography (RPC)) and their effects on the separation were overcome by using a combination of a capillary column in the first dimension and a (larger) analytical monolithic column in the second dimension. Using flow splitting, the detection was performed in parallel using photodiode array and mass spectrometric detection. This approach then allowed establishment of a 2D contour plot, permitting group separations as well as identification of individual compounds.

The six chromatographic modes available in HPLC for the isolation and purification of natural products are described in more detail in Section 9.02.2. Since the groups of natural products differ in their molecular properties, certain chromatographic modes have been shown to work better with particular natural product groups. However, in order to take full advantage of a specific HPLC mode for a separation task and to effectively utilize time and resources, comprehensive method development should be performed. An example of such method development from the analytical to the preparative stage is described for reversed-phase chromatography (HP-RPC), the most frequently employed mode in natural product purification, in Section 9.02.3.

Since natural products are generally isolated from complex biological matrices, often more than one chromatographic step is needed for their purification. The successive application of several suitable different chromatographic modes must consider their applicability to the compound (class) of interest and the compatibility of the chromatographic modes to be integrated with the detection procedures. The design and implementation of a two- or higher-dimensional separation scheme for natural product purification is discussed in Section 9.02.4.

A variety of different approaches lead to successful natural product separation schemes. Representative (as well as some unusual) HPLC separation methods for selected natural product groups (isoprenoids, phenolics, and alkaloids) are reviewed in Section 9.02.5.

9.02.2 Modes of Separation by HPLC in Natural Product Isolation

There are six modes of HPLC currently in use for secondary metabolite analysis, namely, HP-RPC, HP-NPC, HP-IEX, HP-HILIC, HP-SEC, and HP-AC. The principles of these different modes are explained below. All of these various chromatographic modes can be operated under isocratic (i.e., fixed eluent composition), step gradient, or gradient elution conditions (variable step or continuous changes in eluent composition), except for SEC, which is usually performed under isocratic conditions. All modes can be used in analytical, semipreparative,[8] or preparative[9–14] situations.

9.02.2.1 High-Performance Reversed-Phase Chromatography

HP-RPC separates compounds according to their relative nonpolarity or hydrophobicity. In RPC, the polarity of the stationary and mobile phase is to the reverse of that used in NPC. HP-RPC is performed on porous or nonporous stationary phases with immobilized nonpolar polymers (i.e., *n*-alkylsilicas) or nonpolymer polymers (i.e., microparticulate polystyrenes). The most commonly accepted retention mechanism in RPC is based on the solvophobic theory, which describes the hydrophobic interaction between the nonpolar surface regions of the analytes and the nonpolar ligands/surfaces of the stationary phase.[15,16]

Typically, the nonpolar ligands are immobilized onto the surface of spherical, porous, or nonporous silica particles, although nonpolar polymeric sorbents (e.g., those derived from cross-linked polystyrene–divinylbenzene) can also be employed. Silica-based packing materials of 3–10 μm average particle diameter and 70–80 Å pore size, with *n*-butyl, *n*-octyl, or *n*-octadecyl ligands are widely used for the separation of natural products. Silica particles of 1 μm to over 65 μm have been developed in various size distributions and configurations, for example, spherical, irregular, with various pore geometries and pore connectivities; and in pellicular, fully porous, or monolithic structures by a variety of routes of manufacture and with different silica

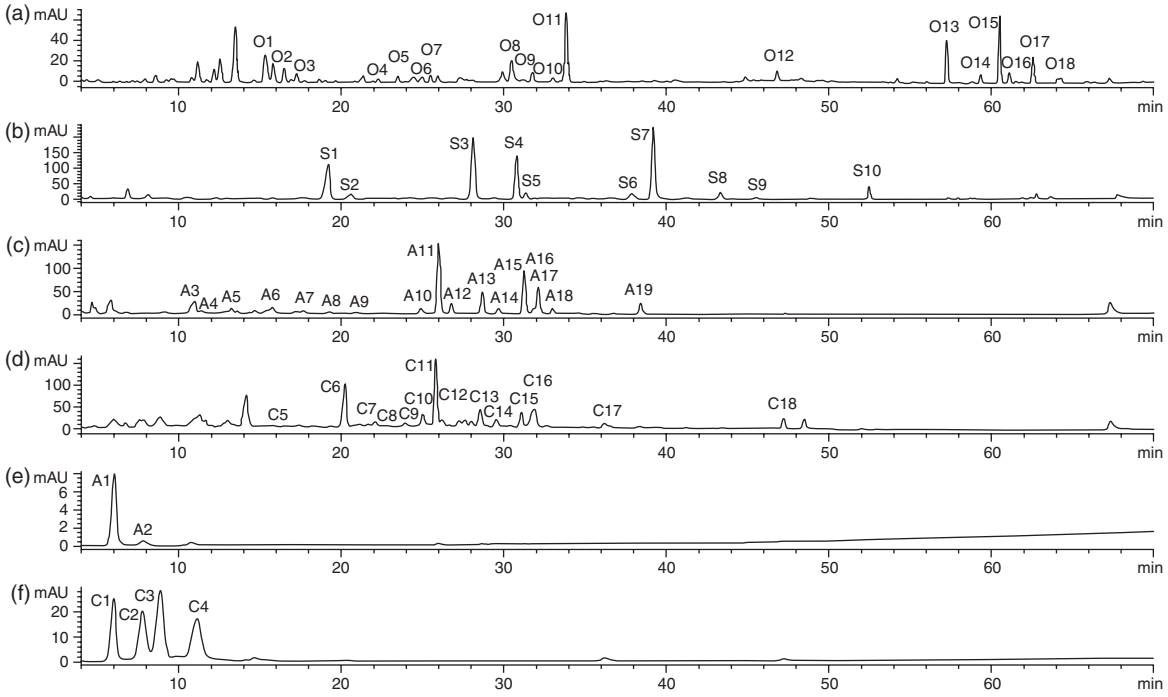


Figure 3 High-performance reversed-phase chromatography of glycosylated flavonoids and other phenolic compounds with UV absorption: (a) navel orange peel (350 nm), (b) soybean seeds (270 nm), (c) Fuji apple peel (270 nm), (d) cranberry (270 nm), (e) Fuji apple peel (520 nm), and (f) cranberry (520 nm). Reproduced from L.-Z. Lin; J. M. Harnly, *J. Agric. Food Chem.* **2007**, *55*, 1084–1096.

types, that is, whether they are based on type I, type II, or type III silica according to the classification of Unger.[17] For low-molecular-mass natural products (molecular mass <4000 Da) silica materials of 70–80 Å pore size and 3–5 μm average particle diameter are often used.

In HP-RPC, an organic solvent (i.e., methanol (MeOH), ethanol, ACN, *n*-propanol, tetrahydrofuran (THF)) is used as a surface tension modifier in the chromatographic eluent, which has a particular elution strength, viscosity, and UV cutoff. Mobile phase additives (i.e., acetic acid (AA), formic acid (FA), trifluoroacetic acid (TFA), and heptafluorobutyric acid (HBFA)) are used to obtain a particular pH value, typically at low pH (e.g., ~pH 2 for silica-based materials) with the exception of polymeric stationary phases, which have an extended pH range from pH 1 to 12. Some mobile phase additives may also function as ion pair reagents, which interact with the ionized analytes to form overall neutral eluting species and also suppress silanophilic interactions between free silanol groups on the silica surface and basic functional groups of the analytes. This property determines the suitability of a particular mobile phase additive for use with ESI MS, since strong ion pair interactions between analytes and mobile phase additives can suppress the ionization of the analytes. As noted above, HP-RPC can be operated in the isocratic, step gradient, or the continuous gradient elution mode, whereby the retaining mobile phase is aqueous and the eluting mobile phase is an organic solvent or an aqueous organic solvent mixture.

In terms of usage, due to its versatility and flexibility, HP-RPC techniques dominate the separation of secondary metabolites at the analytical, laboratory-scale, and preparative levels, since the majority of secondary metabolites possess some degree of hydrophobicity.[18,19] **Figure 3** shows the analysis of glycosylated flavonoids and other phenolic compounds using HP-RPC.[20]

9.02.2.2 High-Performance Normal-Phase Chromatography

HP-NPC can be performed on unmodified silica that separates analytes according to their intrinsic polarity. HP-NPC can also be operated in isocratic, step gradient, or gradient elution mode, where the retaining mobile

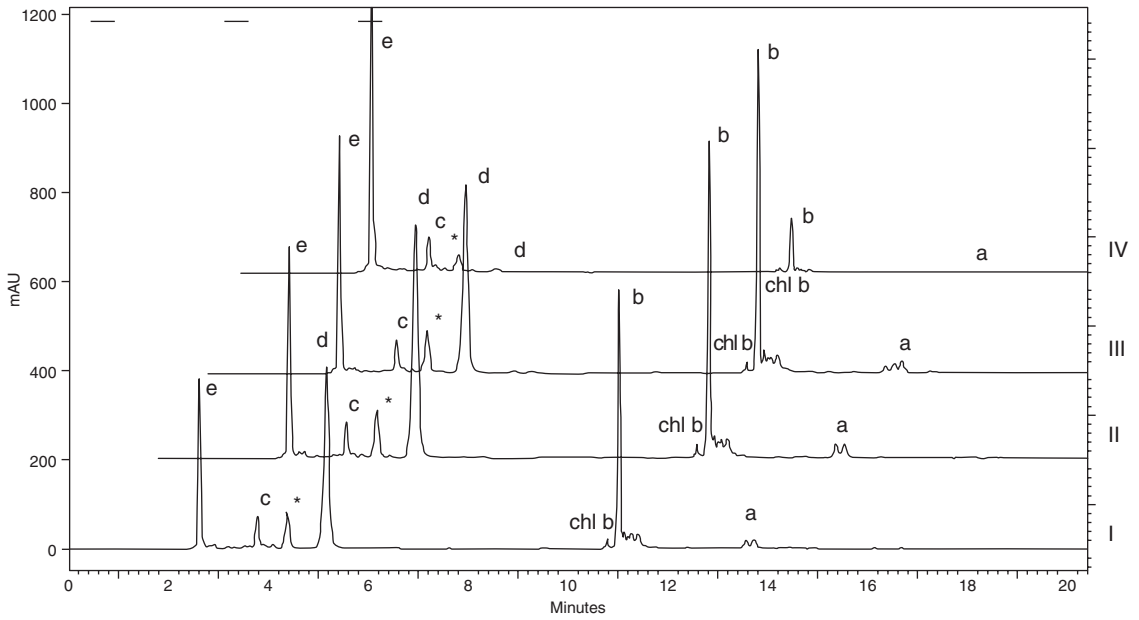


Figure 4 High-performance normal-phase chromatography using the four different solvent mixtures. I, 0h/100de; II, 20h/80de; III, 50h/50de; IV, 100h/0e (h, hexane; de, diethyl ether). a, Neoxanthin polar fraction; b, lutein polar fraction; c, internal standard; d, unknown (415, 435 nm); e, β-carotene; chl b, chlorophyll b (λ_{max} 435; 458 nm; 2nd D 458). Reproduced from M. M. Mendes-Pinto; A. C. S. Ferreira; M. B. P. P. Oliveira; P. Guedes de Pinho, *J. Agric. Food Chem.* **2004**, *52*, 3182–3188.

phase contains less polar organic solvents and the eluting mobile phase consists of more polar organic solvents and on occasions, water. **Figure 4** shows a separation of carotenoids in grapes using HP-NPC.[21]

9.02.2.3 High-Performance Ion-Exchange Chromatography

HP-IEX is performed on stationary phases with immobilized charged ligands and separates according to electrostatic interactions between the charged surface of the analyte(s) and the complementary charged surface of the sorbent. HP-IEX can be operated in isocratic, step gradient, or gradient elution mode. In high-performance anion-exchange chromatography (HP-AEX), analytes are separated according to their net negative charge, where the retaining mobile phase is aqueous, of high pH and low salt concentration and the eluting mobile phase is either aqueous, of high pH and high salt concentration, or aqueous, and low pH. In contrast, high-performance cation-exchange chromatography (HP-CEX) separates according to the net positive charge of the analytes, where the retaining mobile phase is aqueous, of low pH and low salt concentration and the eluting mobile phase is either aqueous, of low pH and high salt concentration, or aqueous, and high pH. With low-molecular-weight natural products, the anisotropy of the charge usually does not affect chromatographic resolution, although with conjugates and other metabolite derivatives charge distribution effects can influence the selectivity. **Figure 5** shows the isolation of ephedrine alkaloids and synephrine in dietary supplements using HP-IEX.[22]

9.02.2.4 High-Performance Hydrophilic Interaction Chromatography

HP-HILIC is performed on porous stationary phases with immobilized hydrophilic ligands and separates analytes according to their hydrophilicity. HP-HILIC can be operated in isocratic, step gradient, or gradient elution mode, where the retaining mobile phase is organic and the eluting mobile phase is aqueous. Being more suited to the isolation of polar substances, HP-HILIC, when linked to electrospray MS, has mainly found application for evaluation of polar compound mixtures for drug discovery. **Figure 6** shows an analysis of the polar components in a fermentation extract using HP-HILIC.

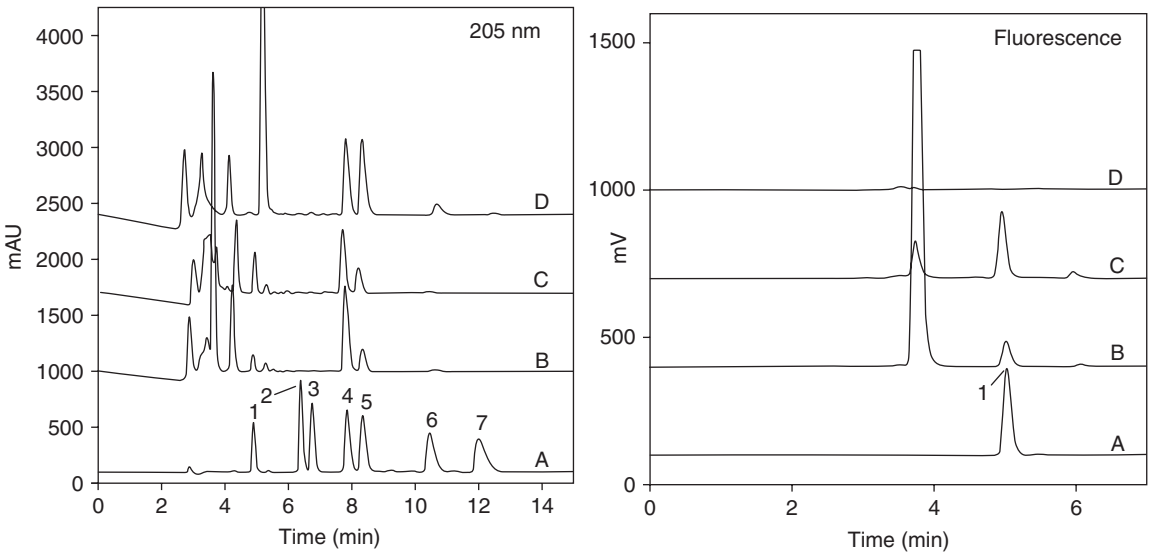


Figure 5 High-performance strong cation-exchange chromatography of ephedrine alkaloids and synephrine in dietary supplements. UV and fluorescence chromatograms of (A) standard solution (~3 μg per component, except 1.6 μg of component 1), (B) Xenadrine RFA-1, (C) Xetalean, and (D) Ultra Diet Pep. Analytes: 1, (±)-synephrine; 2, (−)-norephedrine; 3, (+)-norpseudoephedrine-HCl; 4, (−)-ephedrine-HCl; 5, (+)-pseudoephedrine; 6, (−)-*N*-methylephedrine; 7, (+)-*N*-methylpseudoephedrine. Reproduced from: R. A. Niemann; M. L. Gay, *J. Agric. Food Chem.* **2003**, *51*, 5630–5638.

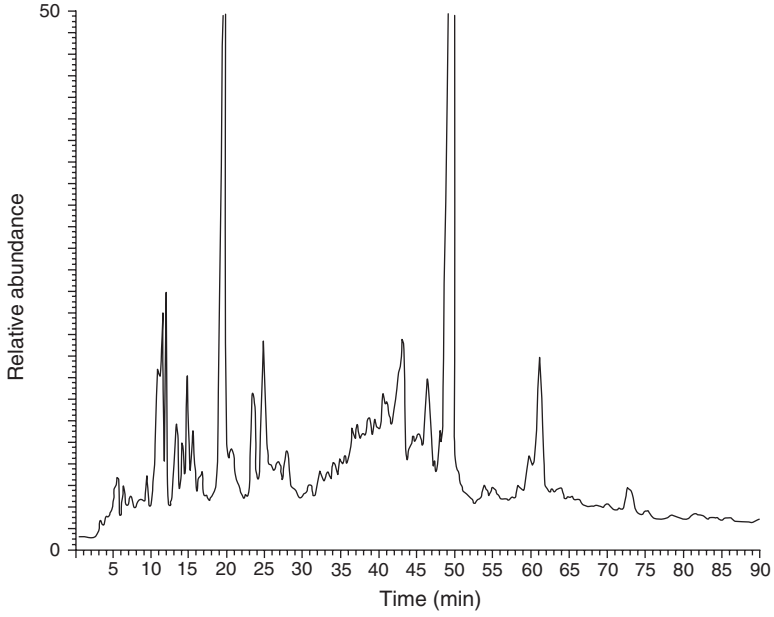


Figure 6 HILIC-ESI-MS separation of the polar components (unretained by reversed-phase solid-phase extraction pretreatment) of a fermentation extract, represented as a total ion chromatogram in positive ion ESI. The chromatogram was obtained using a TSKGel Amide 80 packing, 6.5 mmol l^{-1} ammonium acetate pH 5.5-buffered mobile phases, and a 90 min 10–40% aqueous gradient. Reproduced from M. A. Strege, *Anal. Chem.* **1998**, *70*, 2439–2445.

9.02.2.5 High-Performance Size Exclusion Chromatography

HP-SEC, also called high-performance gel-permeation chromatography (HP-GPC), is performed on porous stationary phases and separates analytes according to their molecular mass or their hydrodynamic volume. As a nonretentive separation mode, HP-SEC is usually operated with isocratic elution using aqueous low salt mobile

Figure 7 One-dimensional chromatogram of a concentrated Qingkailing injection. SEC; column: Toyopearl HW-40S, 300 mm × 8 mm ID; mobile phase: 0.05 mol l^{-1} Tris-HCl (pH 6.90); flow rate: 0.4 ml min^{-1}; injection volume: 20 μl; detection: UV 254 nm. Reproduced from S. Ma; L. Chen; G. Luo; K. Ren; J. Wu; Y. Wang, *J. Chromatogr. A* **2006**, *1127*, 207–213.

phases. The separation of analytes of different sizes is based on the concept that molecules of different hydrodynamic volume (Stokes radius) permeate to different extents into porous HP-SEC separation media and thus exhibit different permeation coefficients according to differences in their molecular masses/hydrodynamic volumes. **Figure 7** shows an analysis of the complex Traditional Chinese Medicine (TCM), Qingkailing using HP-SEC.[23]

9.02.2.6 High-Performance Affinity Chromatography

HP-AC is performed on stationary phases containing immobilized biomimetic or biospecific ligands and separates according to principles of molecular recognition. Into this category can also be included the resolution of enantiomeric compounds by chiral HPLC. In HP-AC, analytes are usually eluted by step gradient or gradient elution, where the capture (loading) mobile phase is aqueous and of low ionic strength and the eluting mobile phase is aqueous and of higher ionic strength or different pH value or alternatively, contains a mobile phase additive that competes with the target compound for binding to the immobilized biospecific ligand. For chiral HPLC, organic solvent mixtures usually form the mobile phase eluents. In both HP-AC and chiral HPLC, separations can be performed with immobilized chemical or biological ligands, as well as with molecularly imprinted polymers (MIPs). In terms of achieving maximal selectivity and highest affinity for the interaction between the target substance(s) and the chromatographic sorbent, HP-AC provides the greatest gain as a trade-off between flexibility and versatility, that is, each affinity sorbent has to be tailored to the specific compound. Nevertheless, HP-AC has found application in natural product isolation. **Figure 8** shows the isolation of scoparone (6,7-dimethoxycoumarin) and capillarisin, an extract from *Artemisia capillaris* using this technique.[24]

9.02.2.7 Summary

In order to achieve optimal selectivity and hence resolution of natural products in HP chromatographic separation, irrespective of whether the task at hand in analytical or preparative, the choice of the chromatographic mode must be guided by the properties of the analytes (i.e., their hydrophobicity/hydrophilicity, charge, molecular size). These attributes can often be rationalized in terms of structural features, solubility profiles, and source. A more detailed overview of existing methods that have been applied for seven classes of natural products is given in Section 9.02.5.

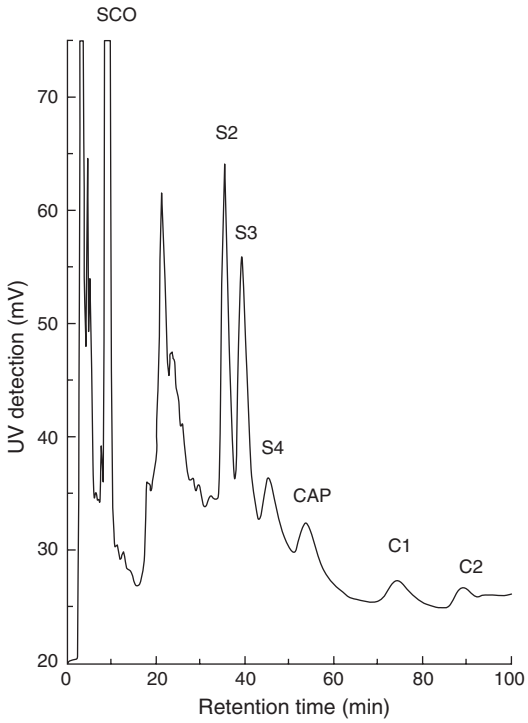


Figure 8 Affinity chromatography for the analysis of the methanol extract of *Artemisia capillaris* under optimized conditions. SCO, scoparone; CAP, capillarisin. Experimental conditions: the column used was of 150 mm × 4.6 mm ID packed with HSA immobilized on silica (7 μm), column temperature was 35 °C, flow rate was 0.8 ml min^{-1}, and UV detection wavelength was set to 238 nm. Initially, 10 min isocratic elution with mobile phase of 1.5% acetonitrile in 10 mmol l^{-1} phosphate buffer (pH 6.0); then, 5 min linear gradient elution from 1.5 to 12% acetonitrile in 10 mmol l^{-1} phosphate buffer (pH 6.0) with the elution of the latter mobile phase kept for an additional 45 min; and finally, another 45 min linear gradient elution from 12% acetonitrile in 10 mmol l^{-1} phosphate buffer (pH 6.0) to 15% acetonitrile in 10 mmol l^{-1} phosphate buffer (pH 7.4). Reproduced from H. L. Wang; H. F. Zou; J. Y. Ni; L. Kong; S. Gao; B. C. Guo, *J. Chromatogr. A* **2000**, *870*, 501–510.

9.02.3 From Analytical to Preparative Scale Illustrated for HP-RPC

As noted above, HP-RPC is currently the most frequently used HP liquid chromatographic mode for the analysis and preparative purification of secondary metabolites, in particular for applications that involve off-line or online ESI MS.

The development of a method for preparative HP-RPC purification for the purpose of isolation of one or more component(s) from a natural product sample (or alternatively the purification of a synthesized product from natural occurring precursors) is usually performed in four steps: (1) development, optimization, and validation of an analytical method, (2) scaling up of this method to a preparative chromatographic system, (3) application of the preparative method to the fractionation of the product, and (4) analysis of the individual fractions.

9.02.3.1 Development of Analytical Method

The development of an analytical method for the separation of a natural product encompasses the selection of the stationary and mobile phase taking into consideration the analyte properties (hydrophobicity/hydrophilicity, acid–base properties, charge, temperature stability, molecular size) and is followed by a systematic optimization of the (isocratic or gradient) separations, using either aliquots of the crude extract or, if available, analytical standards.

In the selection of the stationary and mobile phase, a variety of chemical and physical factors of the chromatographic system that may contribute to the variation in the resolution and recovery of natural products need to be considered. The stationary phase contributions relate to the ligand composition, ligand density, surface heterogeneity, surface area, particle size, particle size distribution, particle compressibility, pore diameter, and pore diameter distribution. The mobile phase contributions relate to the type of organic solvents, eluent composition, ionic strength, pH, temperature, loading concentration, and volume.

Typically, a particular HP-RPC material will be selected empirically as the starting point for the separation, taking into consideration its suitability for the separation task at hand, published procedures for similar types of natural products, availability of the stationary phase material for preparative chromatography, and if information is available, on the analyte properties.

Since the quality of a separation is determined by resolution of individual peak zones, method development always aims at optimization of the resolution. The resolution of adjacent peak zones for a two-analyte system can be defined as follows:

$$R_S = \frac{t_{R2} - t_{R1}}{(1/2)(w_1 + w_2)} \tag{1}$$

where t_{R1} and t_{R2} are the retention times, while w_1 and w_2 are the peak widths of two adjacent peaks corresponding to the analytes. To develop good resolution in the analytical separation of a complex mixture of natural products, method development always focuses on the least well-resolved peak pair(s) of interest.

In isocratic elution, resolution depends on the column efficiency or plate number N, the selectivity α, and the retention factor k, all of which can be experimentally influenced through systematic changes in individual chromatographic parameters. In the isocratic mode of separation, resolution is determined from

$$R_S = \left(\frac{1}{4}\right) N^{1/2} (\alpha - 1) \left(\frac{k}{(1+k)}\right) \tag{2}$$

The plate number, N, is the efficiency of the column and is a measure of the column performance. The selectivity α describes the selectivity of a chromatographic system for a defined peak pair and is the ratio of the k values of the second peak to the first peak. The retention factor k is a dimensionless parameter and is defined as $k = (t_R - t_0)/t_0$, where t_R is the retention time of a particular peak and t_0 is the column void time. In this manner, normalization of the relative retention can be achieved for columns of different dimensions. While N and α change only slightly during the solute migration through the column, the value of k can be readily manipulated through changes in the elutropicity of the mobile phase by a factor of 10 or more. The best chromatographic separations for low- or mid-molecular-weight analytes are generally achieved with mobile phase–stationary phase combinations that result in a k value between 1 and 20.

In gradient elution, in contrast to isocratic elution, $\overline{N}$, $\overline{\alpha}$, and $\overline{k}$ are the median values for N, α, and k, since they change during the separation as the shape and duration of the gradient changes. The 'gradient' plate number $\overline{N}$ has no influence on the selectivity or the retention (except for temperature change). The selectivity $\overline{\alpha}$ and the retention factor $\overline{k}$ usually have only a minor influence on $\overline{N}$. While $\overline{N}$ and $\overline{\alpha}$ change only slightly during the solute migration through the column, the $\overline{k}$ value can change by a factor of 10 or more depending on the gradient steepness. Again, the best chromatographic separation is generally achieved with a $\overline{k}$ value between 1 and 20. Although resolution in isocratic and gradient elution is mainly influenced by the mobile phase variables α (or $\overline{\alpha}$) and k (or $\overline{k}$) and nearly independent of $\overline{N}$, for a given column, an optimization strategy should nevertheless start with appropriate selection of the stationary phase. This is because many initial choices (like column dimensions, choice of ligand) are determined by the overall strategy (i.e., separation optimization for quantification of several analytes or separation optimization for planned scaling up to preparative purification of specific target compounds) and by the purification goals. A number of computer-assisted, expert systems can be used to guide this selection, for example, for further insight into this field and the choice of different algorithmic expert systems approaches, see I *et al.*[25]

Based on these considerations and in view of the implication of Equation (2), the separation optimization for a natural product sample requires three steps to be performed: (1) the optimization of the column efficiency N, (2) the optimization of the selectivity α, and (3) the optimization of the retention factor $\overline{k}$ values.

Step 1: Optimization of the column efficiency N

The optimization of the peak efficiency, expressed as the theoretical plate number, N, requires an independent optimization of each of the contributing factors that influence the band broadening of the peak zones due to column and the extra-column effects. With a particular sorbent (ligand type, particle size, and pore size) and column configuration, this can be achieved through optimization of linear velocity (flow rate), the temperature, detector time constant, column packing characteristics, and by minimizing extra-column effects, by, for example, using zero dead volume tubing and connectors. The temperature of the column and the eluents should be thermostatically controlled in order to facilitate the reproducible determination of the various column parameters and to ensure resolution reproducibility. The flow rate (or alternatively the linear flow velocity) to achieve the minimum plate height, H, for a particular column can be taken from the literature or experimentally determined according to published procedures.

Step 2: Optimization of the selectivity α

Change in selectivity of the separation is the most effective way to influence resolution. This is mainly achieved by changing the chemical nature or concentration of the organic solvent modifier (ACN, MeOH, isopropanol, etc.) in conjunction with the appropriate choice of mobile phase additive(s). As noted above, this can be realized in both isocratic or gradient elution. Moreover, the interconversion of isocratic data to gradient data and vice versa can be achieved through the use of algorithms[25] based on linear and nonlinear solvent strength theory.

A good starting point is the solvent selectivity triangle approach (see **Figure 9**). Here, solvents are classified according to their relative dipole moment, basicity, or acidity. Combinations of three different solvents, plus water to provide an appropriate retention factor range, are selected to differ as much as possible in terms of their polar interactions. This selection permits the solvent combinations to mimic the selectivity that is possible for any given solvent, and defines the boundaries of the triangle.[26,27] At the same time, these solvents must be totally miscible with each other and with water. Three solvents, which best meet these requirements, are MeOH, ACN, and THF. Four solvent mobile phase optimization using water plus three organic solvents provides significant control over α-values in reversed-phase HPLC. However, if different organic solvents are used, different eluotropic strengths must be considered in order to allow elution of the analytes of the sample in the appropriate retention factor range.[28,29]

Once the selectivity parameter is fixed due to the initial choices of the mobile and stationary phase, further optimization should concentrate on resolution optimization via achieving the most appropriate retention factor for the different natural products in the mixture.

Step 3: Optimization of the retention factor $\overline{k}$ values

In the isocratic elution mode of HP-RPC, resolution optimization can take advantage of the relationship between the retention time of an analyte (expressed as the retention factor k) and the volume fraction of the

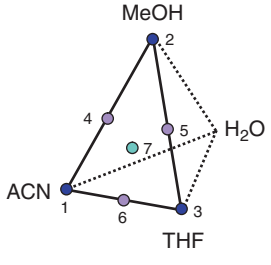


Figure 9 Solvent selectivity triangle approach for the selectivity optimization in HP-RPC. First, three initial experiments (1–3) with three mobile phases (binary mixtures of ACN/water, MeOH/water, and THF/water, respectively) are performed. If necessary, a further three experiments (4–6) with three mobile phase blends (ACN/MeOH/water, MeOH/THF/water, and THF/ACN/water) are performed. If necessary, a further experiment (7) with a mobile phase blend ACN/MeOH/THF/water is performed.

organic solvent modifier, φ. Although typically these dependencies are curvilinear, that is, not first order, for practical convenience they are often treated as linear relationships. Thus, the change in retention factor as a function of φ can be represented by

$$\ln k = \ln k_0 - S\varphi \tag{3}$$

where k_0 is the retention factor of the solute in the absence of the organic solvent modifier and S is the slope of the plot of $\ln k$ versus φ. The values of $\ln k_0$ and S can be calculated by linear regression analysis. Greater precision in the quality of fit of the experimental data, and thus improved reliability in the prediction of the retention behavior of analytes in HP-RPC systems for mobile phases of different solvent composition, can be achieved[30] through the use of an expanded form of Equation (3), that is,

$$\ln k = \ln k_0 - S\varphi + S'\varphi^2 - S''\varphi^3 + \cdots \tag{4}$$

Similarly, in gradient elution HP-RPC, resolution optimization can take advantage of the relationship between the gradient retention time of an analyte (expressed as the median retention factor $\overline{k}$) and the median volume fraction of the organic solvent modifier, $\overline{\varphi}$, in regular HP-RPC systems based on the concepts of the linear solvent strength theory,[15,31] such that

$$\ln \overline{k} = \ln k_0 - S\overline{\varphi} \tag{5}$$

A mapping of the dependence of analyte retention (expressed as the natural logarithm of the retention factor, k) on the mobile phase composition (expressed as the volume fraction of solvent in the mobile phase, φ) in isocratic elution (or as $\overline{k}$ versus $\overline{\varphi}$ in gradient elution) with a minimum of two initial experiments can be used to define the useful range of mobile phase conditions, and can indicate the mobile phase composition at which the band spacing is optimal (see **Figure 10**).

Irrespective of whether the data are obtained through isocratic or gradient elution, techniques employing two initial experiments (differing only by their mobile phase composition or gradient run times, respectively) with tracking and assignment of the peaks, a relative resolution map (RRM) can be established, which plots resolution R_S against the separation time (or gradient run time t_G). In the case of gradient elution, the RRM then allows determination of the optimal gradient run time (and gradient range). Such a procedure can be conveniently performed in any laboratory using Excel spreadsheets containing the relevant equations (see below) as macros or through software packages (e.g., DryLab, LabExpert). Such strategies greatly reduce the time to achieve an optimal separation, as well as saving on solvent, reagent, and analyte consumption. Moreover, as shown in various studies, the more sophisticated of these methods permit[24] instrumentation to be operated in a nearly fully automated, unattended fashion 24 h/7 days per week.

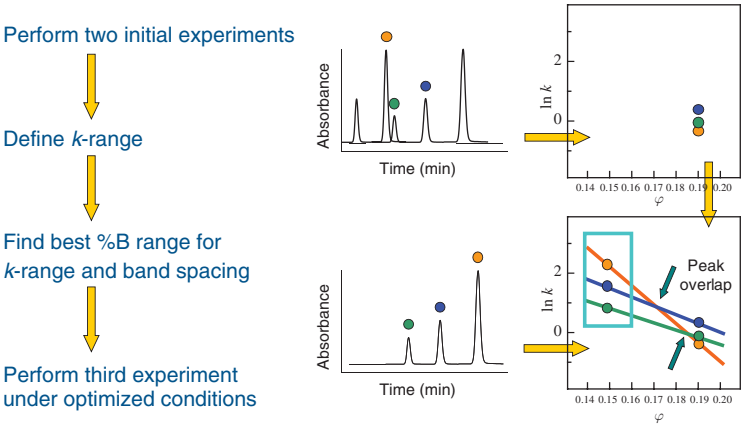


Figure 10 Optimization of isocratic elution. Two chromatograms obtained for 19 and 14% (v/v) of organic solvent modifier in the mobile phase (corresponding to $\varphi = 0.19$ and 0.14, respectively) can be used to plot the resulting retention factors versus the volume fraction of the organic solvent modifier in order to identify the mobile phase composition with optimal peak spacing.

In more advanced applications, optimization can be performed via computer simulation software (e.g., Simplex methods, multivariate factor analysis programs, DryLab G/plus, LabExpert). In such procedures, resolution of peak zones is optimized through systematic adjustment of mobile phase composition by successive change in the φ value (or equivalent parameters, such as the concentration of the ion pairing reagent employed). In gradient elution, advantage is taken of a strategy with the following eight steps: (1) performing of initial experiments, (2) peak tracking and assignment of the peaks, (3) calculation of $\ln k_0$ and S values from initial chromatograms, (4) optimization of gradient run time t_G over the whole gradient range, (5) determination of new gradient range, (6) calculation of new gradient retention times t_g, (7) change of gradient shape (optional), and (8) verification of results.

(1) Initial experiments: In initial experiments, the natural product sample is separated using two linear gradients differing by a factor of three in their gradient run times (all other chromatographic parameters being held unchanged) to obtain the HP-RPC retention times for each of the natural product compounds.[32] Irrespective of what optimization strategy will be used, it is advisable to separate any sample with at least two different gradient run times, in order to identify overlapping peaks. For optimization of gradient shape and to achieve maximum resolution between adjacent peak zones, the ability to determine retention times of the natural compounds and to classify the parameters that reflect the contributions from the mobile phase composition and column dimensions is essential.[33–35] The determination of the volume, V_{mix}, is useful,[36] determination of dead volume and gradient delay are crucial.[31] With various inputs regarding stationary and mobile phase parameters, algorithms, for example, that of DryLab G/plus, can generate the RRM, based on calculation of corresponding S and k_0 values for each component. If no computer program is available the resolution information can be plotted directly from the distances of the individual peak zones of adjacent peak pairs ($R_S = t_{R2} - t_{R1}$) against the gradient run time t_G.

(2) Peak tracking and assignment of the peaks: Complex chromatograms from reversed-phase gradient elution can often exhibit changes in peak order when the gradient steepness is changed. Before $\ln k_0$ and S values are calculated, or computer simulation is used, the peaks from the two initial runs need to be correctly assigned. Several approaches to peak tracking have been described, using algorithms based on relative retention and peak areas,[37] or alternatively, based on DAD.[38,39]

(3) Calculation of $\ln k_0$ and S values: The retention times t_{g1} and t_{g2} for a solute separated under conditions of two different gradient run times (t_{G1} and t_{G2}, where $t_{G1} < t_{G2}$) can be given by the following equations:[33,40]

$$t_{g1} = \left(\frac{t_0}{b_1}\right) \log\left(2.3 k_0 b_1\right) + t_0 + t_D \tag{6a}$$

$$t_{g2} = \left(\frac{t_0}{b_2}\right) \log\left(2.3 k_0 b_2\right) + t_0 + t_D \tag{6b}$$

with

$$\frac{b_1}{b_2} = \frac{t_{G2}}{t_{G1}} = \beta \tag{7}$$

where t_{G1},t_{G2} are the gradient run time values of t_G for two different gradient runs, resulting in different values of $b(b_1,b_2)$, and $t_g\left(t_{g1},t_{g2}\right)$ are the gradient retention times for a single solute in two different gradient runs; b_1,b_2 are the gradient steepness parameters for a single solute over the two differing gradient run times; k_0 is the solute retention factor at the initial mobile phase composition; β is the ratio of t_{G2} and t_{G1}, which is equivalent to the ratio of b_1 and b_2; t_0 is the column dead time; and t_D is the gradient delay time. Steep gradients correspond to large b values and small $\overline{k}$ values.

For small molecules there is an explicit solution[40] for b and k_0, namely,

$$b_1 = \frac{t_0 \log \beta}{\left[t_{g1} - \left(\frac{t_{g2}}{\beta}\right) + (t_0 + t_D)\left(\frac{(1-\beta)}{\beta}\right)\right]} \tag{8}$$

$$\log k_0 = \left(\frac{b_1}{b_2}\right)\left(t_{g1} - t_0 + t_D\right) - \log(2.3 b_1) \tag{9}$$

From the knowledge of b and k_0 the values of $\overline{k}$ and $\overline{\varphi}$ can be calculated.[31]

$$\bar{k} = \left(\frac{1}{1.15\, b_1}\right) \tag{10}$$

$$\overline{\varphi} = \frac{\left[t_{g1} - t_0 - t_D - \left(\frac{t_0}{b_1}\right)\log 2\right]}{t^0_{G1}} \tag{11}$$

where $\overline{k}$ is the value of k (retention factor) for a solute when it reaches the column midpoint during elution, φ the volume fraction of solvent in the mobile phase, $\Delta\varphi$ the change in φ for the mobile phase during gradient elution ($\Delta\varphi = 1$ for a 0–100% gradient), $\overline{\varphi}$ the effective value of φ during gradient elution and the value of φ at band center when the band is at the midpoint of column, and t^0_{G1} the normalized gradient time with $t^0_{G1} = t_{G1}/\Delta\varphi$.

By linear regression analysis, using $\overline{k}$ and $\overline{\varphi}$, the S value (empirically related to the hydrophobic contact area between solute and ligand) can be derived from the slope of the $\log \overline{k}$ versus $\overline{\varphi}$ plots, and $\ln k_0$ (empirically related to the affinity of the solute toward the ligand) as the y-intercept.[15]

$$S = \frac{\left(\ln k_0 - \ln \overline{k}\right)}{\overline{\varphi}} \tag{12}$$

(4) Optimization of the gradient time, t_G, over the entire gradient range: The retention factor $\overline{k}$ is a linear function of the gradient run time t_G if $\Delta\varphi$ is kept constant. Hence,

$$\frac{\overline{k}}{t_G} = \frac{0.87F}{V_m \times \Delta\varphi \times S} = \text{const.} = C \tag{13}$$

The optimized gradient run time t_{GRRM} can be obtained from the RRM or alternatively, from the plot of R_S versus t_G, and yields for each analyte the new values of $\overline{k}_{new}$ by t_{GRRM} being multiplied with C:

$$Ct_{GRRM} = \overline{k}_{new} \tag{14}$$

(5) Determination of the new gradient range: If the gradient run time t_{GRRM} is changed in relation to $\Delta\varphi$ with $t^0_{G1} = \text{const.}$, the $\overline{k}$ values do not change, as can be seen from the following equation:

$$t^0_{G1} = \frac{t_{GRRM}}{\Delta\varphi} = \frac{V_m S\overline{k}}{0.87 \times F} \tag{15}$$

where

$$\Delta\varphi_{opt} = \frac{t_{Gopt}}{t^0_{G1}} \tag{16}$$

and where the retention time t_g of the first peak is $> (t_0 + t_D)$ and the retention time t_g of the last peak is $< \Delta t_{Gopt}$.

(6) Calculation of the new gradient retention times t_g: Based on the knowledge of the S and the $\ln k_0$ values, new gradient retention times can then be calculated.

(7) Change of gradient shape (optional): Multisegmented gradients should only be used once the gradient delay has been measured. With multisegmented gradients, an error in the gradient delay will reoccur at the beginning and end of each gradient step. In addition, the effect of V_{mix} (which can be determined according to the procedures described in Ghrist *et al.*,[33] which modifies the composition of the gradient at the start and end (rounding of the gradient shape)) can lead to deviation of the experimentally determined retention times from the predicted 'ideal' values as derived, for example, with DryLab G/plus simulations.

(8) Verification of the results: After completion of the optimization process, the simulated chromatographic separation can now be verified experimentally using the predicted chromatographic conditions.

Examples where such systematic method development has been used for the analytical separation of natural products can be found in studies performed on flavones,[41] flavonoids,[42] and secoiridoids.[43] Computer-assisted method development was applied to anthranoids,[44] carotenoids,[45] coumarins,[46,47] flavonoids,[48,49] and other natural products.[50]

9.02.3.2 Scaling Up to Preparative Chromatography

While analytical HPLC aims at the quantification and/or identification of compounds (with the sample going from the detector to waste), preparative chromatography aims at the isolation of compounds (with the sample going to the fraction collector). For preparative separations, method development always focuses on the peaks of interest, and the two adjacent eluting peaks. In many cases, all other peaks can be viewed as superfluous, and directed to the waste. Optimization of the resolution of the peak of interest from the adjacent peaks has to take into account the sample size and the relative abundances of the three components that form the basis of the separation task. Once an analytical method is established, it can be scaled up[8] to a preparative separation by taking into consideration the operating ranges of the column (see **Table 1**) or used for scaling up by deliberate column overloading.

The concept of parity in scaling up or down implies that the performance features, selectivity behavior, and recyclability of the stationary phase material used for the analytical and the preparative separation are identical, with the exception of particle size. Both robust experimental methods as well as rules of thumb, acquired by experienced investigators, have been developed that enable such comparisons to be made. An extensive scientific literature is now available to indicate sound foundations for such scaling up strategies, coupled with suitable experimental methods for their validation. **Table 1** summarizes some of this information.

In order to obtain an equivalent elution profile, the flow rate needs to be adjusted for columns with different internal diameter, according to the following formula:

$$F_{\text{preparative}} = \left[\frac{r_{\text{preparative}}}{r_{\text{analytical}}}\right]^2 \times F_{\text{analytical}} \tag{17}$$

where F is the flow rate and r the column radius of the preparative or analytical column.

Estimates of the loading capacity of a particular column material can be usually obtained from the manufacturer. The mass loadability for a scaled up separation can be calculated with the following formula:

$$M_{\text{preparative}} = \left[\frac{r_{\text{preparative}}}{r_{\text{analytical}}}\right]^2 \times M_{\text{analytical}} \times C_{\text{L}} \tag{18}$$

where M is the mass, r the column radius of the preparative or analytical column, and C_{L} the column length ratio.

In many cases, despite some loss of resolution, column overloading is an economic and viable method for compound purification. In analytical LC, the ideal peak shape is a Gaussian curve. If under analytical conditions a higher amount of sample is injected, peak height and area change, but not peak shape or the retention factor. However, if more than the recommended amount of sample is injected onto the column, the adsorption isotherm becomes nonlinear. As a direct consequence, resolution decreases, and peak retention

Table 1 Operating ranges of column types in HPLC with sample amount, inner column diameter, column length, and flow rate range

Column type	*Sample amount*	*Column ID (mm)*	*Column length (mm)*	*Flow rate range (ml min^{-1})*
Preparative	mg–g	>4	15–250	5–20
Analytical	μg–mg	2–4	15–250	0.2–1
Capillary	μg	1	35–250	0.05–0.1
Nano	ng–μg	<1	50–150	<0.05
Chip	ng	<0.1	<50	<0.01

times and peak shapes may change. There are two methods of column overloading: concentration overloading and volume overloading. In concentration overloading, the volume of the injected sample is maintained, while the sample concentration is increased. The retention factors of the compound(s) decrease, and the peak shape may become triangular and fronting. The applicability of this method is limited by the solubility of the target compound(s) in the mobile phases employed. In volume overloading, the concentration of the sample is maintained, but the sample volume is increased. The retention factor of the compound(s) decrease(s), with broadened peak shape. Once a suitable method is established, it can be applied to the preparative purification of the target compound(s).

9.02.3.3 Fractionation

There are four types of fraction collection:[51] (1) manual, with a manually pressed button to start and stop collection, (2) time based, with a fraction collecting during fixed preprogrammed time intervals, (3) peak based, based on a chosen threshold of the up- and down-slope of a detector signal, and (4) mass based, with fraction collection occurring only if the specific mass of a trigger ion is detected by MS. In addition, a recovery collection can be performed, by which everything that is not collected as a fraction goes into a dedicated container where it can be easily recovered. Whatever the type of fraction collection, careful attention has to be given to the fraction collection delay times and a delay time measurement performed. For a peak with start time t_0 and end time t_E, fraction collection needs to be started when the start of the peak arrives at the diverter valve ($t_0 + t_{D1}$) and ended when the end of the peak arrives at the needle tip ($t_E + t_{D1} + t_{D2}$), where t_{D1} is the delay time between detector and valve and t_{D2} the delay time between valve and needle tip.

9.02.3.4 Analysis of the Quality of the Fractionation

In the absence of online MS, fractionation is usually accompanied by an off-line mode of quality analysis. The typical workflow comprises of (1) a prepreparative analysis of the unpurified material, (2) the purification/fractionation of the compound, and (3) a postpreparative analysis of the individual fractions. The pre- and postpreparative analysis can be performed with analytical HPLC, MS, and activity testing of the fractions, if an assay is available.

After the fractions have been collected, the solvent needs to be removed by using a freeze-dryer, rotary evaporator, or high-throughput parallel evaporator. Nonvolatile components can be removed with reversed-phase SPE procedures prior to solvent removal if the aqueous portion of the buffer is sufficiently large.

9.02.4 Multidimensional High-Performance Liquid Chromatography

Although HPLC is a powerful separation technique for the fractionation of natural products from complex biological mixtures, very often more then one chromatographic step is necessary to achieve a required degree of purity of the target compounds. In practice, this is achieved through a series of purification steps. As there are material losses associated with each purification step in these procedures (see **Figure 11**), the overall recovery of the product has to be optimized. This can be achieved if the number of employed purification steps is minimized. Thus, strategies and techniques that reduce the number of unit operations are to be preferred since they will lead to minimization of product loss(es), and save on capital costs for equipment or operational costs for staff, reagents, and other consumables.

After initial extraction, the enrichment and purification of the target compound can be typically achieved in two or three chromatographic dimensions using a combination of different chromatographic modes. For natural products, ion-exchange chromatography-reversed-phase chromatography (IEX-RPC), size exclusion chromatography-reversed-phase chromatography (SEC-RPC), normal-phase chromatography-reversed-phase chromatography (NPC-RPC), affinity chromatography-reversed-phase chromatography (AC-RPC) combinations are described in the literature.

Multidimensional (multistage, multicolumn) high-performance liquid chromatography (MD-HPLC) offers the possibility of cutting the elution profiles into consecutive fractions, where these fractions can be treated

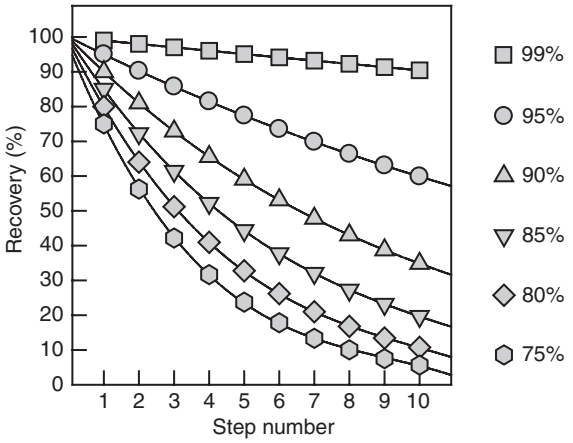


Figure 11 Effect on overall recovery at a fixed recovery per step value due to the additional steps in multidimensional chromatography.

independently from each other. One important consequence of this strategy is the gain in peak capacity (PC), defined as the number of peaks that can be accommodated between the first and the last peak in a separation of defined resolution.[52] Multidimensional LC has developed from column switching and related techniques[53] for a specific target or class of targets.[54] MD-HPLC has the potential of independent optimization of the separation conditions for each fraction and allows a relative enrichment/depletion/peak compression of components. An advanced conceptual framework of multidimensional LC has been developed for small molecule separations.[55–59]

MD-HPLC can be applied to the purification of a particular natural product or comprehensive fractionation of complex natural product mixtures.[60] **Figure 2** illustrated an example where such approaches have been employed. The two applications have some similarities in terms of basic separation science principles; however, they have fundamental differences in system design, optimization, and operation.

9.02.4.1 Purification of Natural Products by MD-HPLC Methods

To purify a particular natural product, it is often possible to select complementary chromatographic modes that allow the natural product to be obtained in high purity with only a few separation steps. In such noncomprehensive MD-HPLC, only a part of the analytes (as a single fraction) eluting from the first column is transferred to a second column for further purification (conventionally expressed by a hyphen, i.e., IEX-RPC). For such a 'heart-cutting' technique, knowledge of the retention properties of the analyte mixture in the first column is needed in order to choose the segment(s). Heart-cutting techniques are fast but not comprehensive, since the majority of analytes are not subjected to a separation in a second dimension. The main advantage of the technique is the improved resolution of compounds that coelute in the first dimension. A key requirement for such a purification scheme is that subsequent stages of the separation are orthogonal, with the two separation modes not correlated to each other in relation to their retention characteristics (i.e., selectivity).

For a single chromatographic dimension, the partly contradictory objectives of speed, resolution, capacity, and recovery usually cannot be maximized simultaneously (i.e., a high resolution can be achieved but at the expense of speed; a high-speed separation can reduce resolution) (see **Figure 12**).

MD-HPLC allows meeting the overall purification objectives, by placing the emphasis for each purification stage on a different pair of objectives and choosing a chromatographic mode that is particularly well suited for the task (see **Figure 13**). At the enrichment stage, the emphasis is on speed and capacity, employing, that is, strong cation exchange (SCX) or AC in the SPE format as a low-resolution step. At the intermediate purification stage, emphasis is placed on capacity and resolution, employing chromatographic modes with intermediate resolution, that is, ion-exchange chromatography (IEX) or SEC. At the final chromatographic polishing step, the emphasis is placed on resolution and recovery, employing high-resolution modes, that is, RPC.

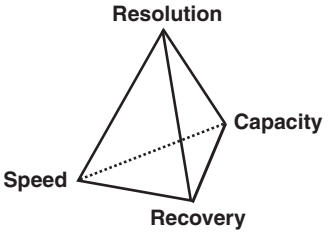


Figure 12 Optimization goals for a chromatographic purification and their interrelationship.

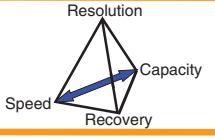
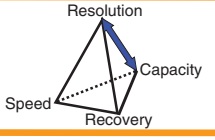
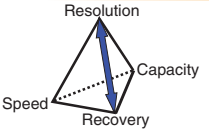
Purification stage	Separation mode	Priority
Enrichment (capture) stage: Rapid, high capacity, low resolution	IEX, SEC, AC	
Intermediate purification stage: High capacity, high resolution, intermediate speed and recovery	IEX, NPC, SEC	
Final purification stage: High resolution and recovery, low capacity and speed	RPC	

Figure 13 Optimization priorities at individual purification stages for a multidimensional fractionation exemplified for natural products and suitable chromatographic modes.

9.02.4.2 Fractionation of Complex Natural Product Mixtures by MD-HPLC Methods

If the objective of a purification scheme is the comprehensive fractionation of a complex, multicomponent natural product mixture, it is of advantage to use orthogonal chromatographic modes, but such extensive fraction collection requires additional, sometimes substantial, infrastructure. In comprehensive MD-HPLC, the entire analyte pool of the first column is transferred to the second column (expressed by a cross, i.e., IEX × RPC) as sequential aliquots, either successively onto one column or alternating onto two parallel columns. The resulting data can be represented as three-dimensional (3D) contour plots, with retention times of the second dimension plotted against retention times of the first dimension. The information content of such comprehensive 2D chromatograms is higher than the information content of individual one-dimensional chromatograms.

The first comprehensive 2D system was developed in the late 1970s by Erni and Frei, who applied IEX × RPC to the analysis of senna glycosides from plant extracts.[61] In the subsequent decades, comprehensive MD-HPLC methods have been further developed, mainly for peptides and proteins,[62,63] but also for separation of various natural products such as phenolic and flavone antioxidants[64] and carotenoids.[65] The theoretical aspects of MD-HPLC techniques have also been further developed.[66–68]

9.02.4.3 Operational Strategies for MD-HPLC Methods

From an operational perspective, multidimensional LC can be carried out off-line or online.[69] Regardless of which operational mode, off-line or online, is used, the compatibility of the mobile phases between successively employed chromatographic modes in a separation scheme needs to be considered (see Section 9.02.4.4.2). As a consequence, it may be necessary to process the fractions between two separation stages (e.g., through buffer exchange, concentration, or dilution) to enhance compatibility of eluent composition of fractions from the first

chromatographic dimension with the retaining mobile phase of the second chromatographic dimension. If a nonretentive chromatographic mode such as SEC is employed in conjunction with a retentive chromatographic mode, such as RPC or IEX, it is usually performed first. This allows (1) relatively large eluent volumes stemming from isocratic elution in the nonretentive mode to be reduced through the capture of analytes under the retaining mobile phase conditions of the subsequent retentive chromatographic mode and (2) reduction of extra-column band broadening with resulting loss of resolution.

9.02.4.3.1 Off-line coupling mode for MD-HPLC methods

The off-line coupling mode in HPLC is comparable with that employed in conventional CC in natural product isolation. In the off-line mode, the eluent of the first column is collected as fractions, either manually or with an automated fraction collector, and reinjected onto the second column. Typical processing steps may include volume reduction by freeze-drying or automated high-throughput parallel evaporation systems taking into account the boiling point(s) or volatility of the target analyte(s) and organic solvent if these are contained within the eluates. The use of volatile mobile phase additives then allows a buffer exchange.

9.02.4.3.2 Online coupling mode for MD-HPLC methods

The online mode uses high-pressure, multiposition, multiport switching valves, which allow selection of pathways for single fractions from the first chromatographic dimension to subsequent column(s) of the second chromatographic dimension. The fractions from the first dimension are either transferred directly, or through one (or more) intermediate trapping columns for the purpose of concentration and automated buffer exchange. This approach requires complex instrumentation, results in increased optimization time and reduced system flexibility. It however has numerous advantages in terms of reproducibility, recovery, speed, and automation.

9.02.4.4 Design of an Effective MD-HPLC Scheme

MD-HPLC for natural products requires thoughtful selection of orthogonal and complementary separation modes, of the order of their utilization and independent optimization with respect to the chromatographic goals (speed, resolution, capacity, and recovery). Furthermore, besides the mobile phase composition of the employed chromatographic modes, the elution mode (isocratic, step, or gradient elution), flow rates, and mobile phase temperatures need to be considered.

9.02.4.4.1 Orthogonality of chromatographic modes

In order to exploit the full PC of a 2D system,[59] it is advantageous if the applied chromatographic modes are orthogonal. It is generally accepted that the dimensions in a 2D separation system are orthogonal, if the separation mechanism of the two dimensions are independent from each other causing the distribution of analytes in the first dimension to be uncorrelated to the distribution in the second dimension. An example of such orthogonality is liquid chromatography–capillary electrophoresis (LC–CE), where totally different separation mechanisms are used, that is, pressure-driven compared to electrically-driven separation.[70] In a similar manner, different separation modes in HPLC can be viewed as being orthogonal, for example, IEX (CEX or AEX) and RPC are orthogonal as they separate according to net charge or hydrophobicity, respectively. A very coarse classification of chromatographic modes commonly applied in the MD-HPLC of natural products according to their separation principles is depicted in **Figure 14**.

In MD-HPLC systems, combinations of chromatographic modes are usually designed to achieve analyte separation according to different characteristic analyte properties.[71]

For an ideal orthogonal, 2D separation, the overall PC is defined as the product of the PCs in each dimension:

$$PC_{2D\ system} = PC_{first\ dimension} \times PC_{second\ dimension} \quad (19)$$

However, if two nonidentical chromatographic modes with some degree of similarity are used in a 2D system, the increase in the PC and the total number of analytes that can be separated is much lower than the product of peak capacities of individual dimensions. The PC also depends on the elution mode. Gradient elution provides a higher PC than isocratic elution and is of advantage in 2D LC.

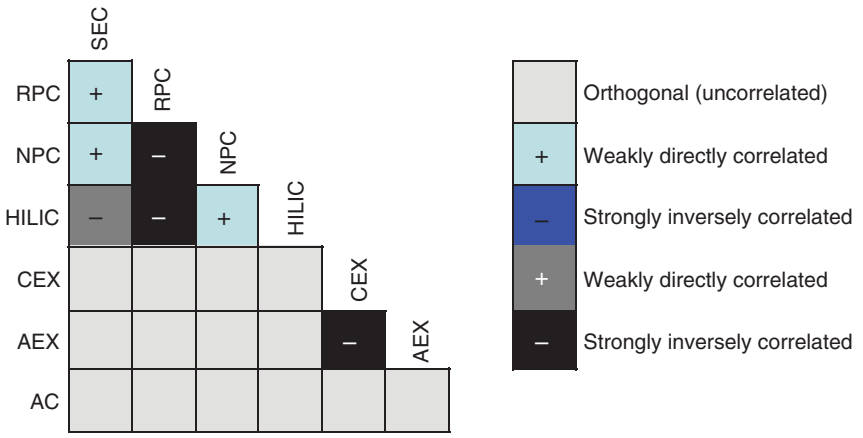


Figure 14 Degree of orthogonality of chromatographic modes employed in the separation of natural products. Shading indicates the degree of correlation of the separation principles of paired modes.

It should be noted that since selectivity in chromatography depends not only on the stationary phase but on the mobile phase as well, orthogonal separations can be achieved through fine-tuning of the separation conditions, even if the principal separation mechanisms of both dimensions are similar. Such tuning removes the inaccessible area from the 2D retention plane and ensures that the remaining retention space is used efficiently.[66]

In addition, the structure of analytes has an effect on the PC. In many separation systems, the contribution of structural units, especially the repeating units, to the Gibbs free energy of association of the analytes with the immobilized chromatographic ligands are additive.[72] Such structural repeating units can be hydrophobic or polar. If one chromatographic system in a 2D LC has no selectivity for a structural element, then the first and the second dimension are noncorrelated (orthogonal) with respect to the repeating structural unit (see **Figure 15**(**a**)). In a completely correlated separation system, with correlated retention factors in two dimensions, the separation space is not utilized (see **Figure 15**(**b**)). Such 2D systems do not provide sufficient selectivity for the separation with respect to the structural property distribution of interest in either dimension and are generally not very useful in practice. In inversely correlated 2D LC × LC separation systems, the retention time increases in the first dimension, but decreases in the second dimension (see **Figure 15**(**c**)). Neither correlated nor inversely correlated 2D LC × LC increase the PC significantly. The selectivity of a 2D LC × LC with respect to hydrophobic or polar repeating units can determine the suitability of chromatographic modes employed in 2D separations and depends on the employed stationary as well as mobile phases. Orthogonal systems with noncorrelated selectivities provide the highest PC and therefore the highest number of resolved peaks. The PC in 2D LC × LC decreases with increasing correlation of the selectivity between the first and the second chromatographic dimension. In practice, however, 2D LC × LC systems are rarely fully orthogonal with respect to each structure distribution type (i.e., hydrophobic, polar).[67] Many partially orthogonal systems are using only part of the theoretically available 2D separation space but can be evaluated using analytes differing in the numbers of hydrophobic or polar structural units or by quantitative structure retention relationships (QSRR).

9.02.4.4.2 Compatibility matrix of chromatographic modes

In designing of 2D LC × LC systems, the selection of the mobile phase for each chromatographic dimension is of fundamental importance, in order to achieve maximal utilization of the 2D separation space. In contrast to off-line 2D LC procedures, where the collected fraction can be subjected to evaporation, dilution, or extraction, before injection onto the column of the second dimension, the compatibility of the mobile phases in online 2D LC × LC in terms of miscibility, solubility, viscosity, and eluotropic strength is much more important. The mobile phases used in SEC × RPC, SEC × NPC, RP × CEX, RP × AEX RP × CEX, NPC × HILIC, NPC × CEX, and NPC × AEX are compatible (see **Figure 16**).

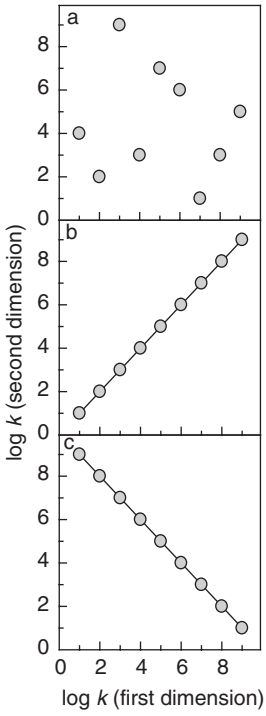


Figure 15 Two-dimensional separation space for a set of natural products utilizing separation systems that are (a) uncorrelated, (b) completely correlated, and (c) inversely correlated, where the retention factors obtained in the second dimension are plotted versus the retention factors obtained in the first dimension.

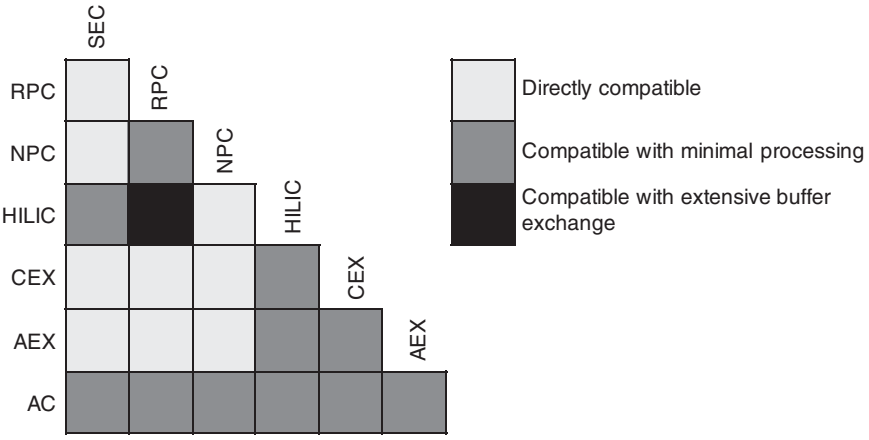


Figure 16 Pair-wise comparison of compatibility between common chromatographic modes. Compatibility is based on miscibility, solubility, and eluotropic strength for a particular class of natural products.

RPC and NPC are fully orthogonal separation modes, which can be useful for complex samples containing a mixture of analytes that are uncharged and possess different polarities or hydrophobicity. However, the use of RPC and NPC in 2D systems needs some consideration especially when NPC is performed with polar chemically bonded columns instead of an unmodified silica column in the first dimension and RPC is performed in the second dimension. When RPC is performed in the first dimension and NPC in the second dimension, the water in the aqueous–organic mobile phase of the first dimension may impact on the resolution of the following NPC separation on unmodified silica gel with nonaqueous mobiles phases.[67,73] When coupling

NPC with RPC, the immiscibility of the employed mobile phase can cause peak broadening in the second dimension. As recently demonstrated for the analysis of carotenoids, NPC × RPC can be performed with immiscible mobile phases, by adjusting the column dimensions and flow rates, for example, using a microbore column in the first dimension and a standard (4.6 mm ID (inner diameter)) analytical column in the second dimension in order to minimize the effects of band broadening.[65,73,74]

Mobile phases in RPC × RPC are usually compatible in terms of miscibility; however, big differences in the viscosities of the employed mobile phases may lead to flow instability at the mixing interface and should be therefore avoided.[75] Nevertheless, RPC × RPC can be performed when the mobile phase/stationary phases employed in the first and second dimension have considerable selectivity differences, as shown for phenolic antioxidants.[76]

A comprehensive AC × RPC approach has been employed for a natural compound extract used in TCM, based on an affinity column with human serum albumin (HSA) covalently linked to a silica column in the first dimension and a monolithic reversed-phase column in the second dimension.[77]

9.02.5 HPLC Separation of Natural Products

As documented in the scientific literature,[78,79] different approaches have attracted interest in the practical separation of natural products. Tabulation of the over 2500 citations for natural product chromatography and >4800 for HPLC purification of such compounds that are accessible through SciFinder Version 2007 using the keywords 'natural product and high performance liquid chromatography' is beyond the scope of this chapter. Rather, the following sections are intended to give an overview of the most frequently and significantly used procedures for the analysis and purification of several major classes of compounds that have long attracted the interest of natural products chemists. For these reasons, the following sections describe the application of HPLC to isoprenoids (with examples of mono-, sesqui-, di-, and triterpenes; iridoids and secoiridoids; carotenoids; saponins; and ecdysteroids), phenolics (with examples of coumarins as well as flavonoids and isoflavonoids), and alkaloids.

9.02.5.1 Mono-, Sesqui-, Di-, and Triterpenes

Terpenoids are the largest class of natural products. These compounds have a variety of roles in mediating beneficial (attracting) or antagonistic (deterring or defending) interactions between organisms.[80,81] Of the ~25 000 terpene structures reported so far, only a small proportion have been fully investigated, mainly those with high commercial (perfumes and flavors) or pharmaceutical value. Terpenoids are distinguished by their number of isoprene (C5) units. Several changes of the basic structures lead to methylations, ring closures, and ring openings, creating a plethora of diversity. Glycosylation is common and presents the molecule in a nonvolatile form. Under the influence of heat or enzymes (plant-derived glycosidases or, e.g., enzymes from yeast in winemaking), the bioactive or fragrant substances are frequently released.

Monoterpenes (C10) are typical ingredients of volatile plant oils and are widely used in the fragrance and flavor industry. However, they also have wide pharmaceutical application, for example, many are excellent bactericides. These plant oils also contain various other substances such as phenolics, lactones, esters, and alcohols, which may present problems during separation of the monoterpenes. Although gas chromatography (GC)–MS was the initial method of choice to separate volatile monoterpenes, the trend toward the use of LC–GC–MS has resulted in the reduction of artifacts and an easier interpretation of the resulting data.[82,83]

Furthermore, the chiral discrimination of monoterpenes has been recognized as one of the most important analytical techniques in flavor chemistry and pharmacology because the optically active stereoisomers have different sensory qualities and biological activities. HPLC offers powerful techniques for separation and quantification of enantiomers because of the progressive improvement of chiral chromatographic materials and chiral detectors such as optical rotatory dispersion (ORD) and circular dichroism (CD) detectors. In contrast, determination of chiral compounds by GC typically requires coinjection of the reference compound with known stereochemistry. An HPLC system equipped with a chiral detector, on the other hand, allows direct determination of the configuration of chiral compounds.[84]

Sesquiterpenes (15C) are a group of pentaprenyl terpenoid substances. Although they are a relatively small group of terpenoids compared to monoterpenes, their sources are widespread, having been isolated from terrestrial fungi, lichens, higher plants, insects, and various marine organisms, especially, sponges.[85] Some have pungent flavors as found in ginger (zingiberene) and pepper (rotundone)[86] and have been highly prized historically. Because of their commercial importance, fast analysis methods are required. In the group of 1000 different sesquiterpene lactones most are bitter (e.g., in absinth from *Artemisia*) and have anti-inflammatory, antibiotic, and in some cases cytotoxic qualities. When they possess exocyclic methyl groups they can react with sulfhydryl groups of, for example, skin or saliva proteins and this may lead to allergic reactions. Thus, sesquiterpenes often protect organisms. For example, in marine green algae (caulerpenyne) they are stored as polyacetates with the deterring sesquiterpene released only upon wounding. The plant hormone abscisic acid is a sesquiterpene and induces fruit ripening and leaf fall.

Diterpenes (C20) can be linear as in the phytol part of chlorophyll or mono- to tetracyclic.[87] Very well researched are the tricyclic taxanes of which taxol A from the *Taxus baccata* tree inhibits cell division and has found use in anticancer treatment. Certain labdanes inhibit blood platelet aggregation and kaurane norditerpene glycosyl ester atractylosides from the Mediterranean thistle block the ATP/ADP translocation – such diterpenes are very toxic when ingested. Ubiquitous plant-derived kaurane-type diterpenes are the plant hormones (gibberellins) or defense substances (phytoalexins).

Triterpenes (C30) are mainly polycyclic[88] and their basic structure is squalene, which was by itself only found in shark liver. Squalene is the building block for the sterane in steroid-like hormones, D vitamins, cholesterol, heart glycosides, and saponins. Modified triterpenes in plants, like the bitter hydroxylated sterol glycosides curcubitacins, are potent phytoalexins and very toxic to insects. They have been removed through breeding from edible parts of the cucumber family. However, investigation of new food sources from the widespread family of Curcubitaceae or Brassicaceae requires precise determination of undesirable triterpenes. As found for monoterpenes, triterpene enantiomers have different activities, for example, in cotton tissues the ratio of (−) to (+) gossypol determines its toxicity to ruminants or human cancer cells.[89] Cardenolides are triterpenes with unusual sugar conjugates and occur in 15 plant families, with heart-stimulating activity; some of the best known examples are the digitoxigenins from *Digitalis*. Saponins are glycosides of polycyclic triterpenes and amphiphilic, as their sterol part is lipophilic and the glycosylated end is hydrophilic. Thus, they can penetrate and interrupt biomembranes, which enables them to be good detergents (soaps) but also to be highly toxic to fish or mammals as they lead to membrane defects and the lysis of erythrocytes. A triterpene saponin of very low hemolytic activity is the very sweet glycyrrhetinic acid from the root of *Glycyrrhiza glabra* (licorice).

As described in more than 310 publications, mono-, sesqui-, di-, and triterpenes have been separated with HPLC, particularly by HP-RPC[89–97] and HP-NPC.[83,86,98]

A representative example of the separation of terpenes with HP-RPC has been described by Song *et al.*,[91] who applied a capillary HPLC–ESI-MS method for the determination of pinane monoterpene glycosides in TCM herbal extracts derived from *Paeonia suffruticosa*. In this study, the authors used a 3 mm × 150 mm 5 µm C_{18} silica column with linear gradient elution (eluent A: 0.1% aqueous FA and eluent B: ACN) at 5 µl min^{-1}. The monoterpenes paeoniflorin, albiflorin, oxypaeoniflorin, benzoyloxypaeoniflorin, galloylpaeoniflorin, and mudanpioside B and C were then detected with a single quadrupole mass spectrometer in negative ESI mode and single ion monitoring and quantified. Similar approaches, employing C_{18} phases with aqueous–organic mobile phases and volatile buffer additives are quite useful for analysis of terpenes by HP-RPC, since they allow rapid online MS.

A selection of examples from the scientific literature on the use of high-resolution chromatographic methods for the isolation and analysis of mono-, sesqui-, di-, and triterpenes (**Table 2**) have been included to demonstrate the commonality of the methodologies, despite the diversity of the structural types involved.

9.02.5.2 Iridoids and Secoiridoids

Iridoids are derivatives of monoterpenes and occur usually, but not invariably, as glycosides.[100,101] Structurally, they are cyclopentano [*c*] pyran monoterpenoids and they provide a biogenetical and chemotaxonomical link between terpenes and alkaloids. The cleavage of the cyclopentane ring of iridoids produces secoiridoids.[102]

Table 2 Mono-, sesqui-, di-, and triterpenes

Compound	*Natural product group*	*Organism/source*	*HPLC method*	*Detection*	*Reference*
α-Pinene, β-pinene, 3-carene, sabinene, limonene	Monoterpenes	Atmospheric aerosol samples	Micro RPC	ESI-MS	90
Paeoniflorin, oxypaeoniflorin, benzoylpaeoniflorin, benzoyloxypaeoniflorin, mudanpiosides B and C	Monoterpene Glycosides	*Paeonia suffruticosa*	Micro RPC	DAD UV, ESI-MS	91
α-Thujene, α-pinene, camphene, sabinene, β-pinene, myrcene, α-phellandrene , 6-3-carene, α-terpinene, β-cymene, limonene, (*Z*)-β-ocimene, (*E*)-β-ocimene, γ-terpinene and terpinolene	Monoterpenes	*Citrus*	Micro NPC	LC-GC-MS	83
Artemisinin	Sesquiterpene	*Artemisia annua*	Analytical RPC	ESI-MS	92
Caulerpenyne	Sesquiterpenes	Seaweed *Caulerpa taxifolia*	Analytical RPC	UV, ESI-MS	93
Euplotin C	Sesquiterpene	Marine ciliate *Euplotes crassus*	Analytical NPC	UV 215 nm, ESI-MS	98
Rotundone	Sesquiterpene	*Vitis vinifera*	Micro NPC	GC–MS–O	86
Various sesquiterpene lactones	Sesquiterpene lactones	*Lychnophora ericoides*	Analytical RPC	DAD UV, ESI-MS	94
Fifteen taxanes	Diterpenes	*Taxus baccata*	Analytical RPC with fluorinated and hydrocarbon phases	DAD UV	95
Bilobalide	Diterpene	*Ginkgo biloba*	Micro RPC	ELSD	99
Cucurbitacins, digitoxigenins	Triterpene sterols	Cucurbitaceae	Analytical RPC	DAD UV, ESI-MS	96
Gossypol	Terpenoid aldehyde	*Gossypium*	Analytical RPC	UV 272 nm	89
Glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester	Triterpene saponins	*Glycyrrhiza glabra*	Analytical RPC	UV 254 nm	97

Iridoids and secoiridoids are secondary metabolites found in terrestrial and marine flora and fauna. They are plant protectants and useful as markers of several gena in various plant families such as *Plantago* (Plantaginaceae), *Galium* (Rubiaceae), and *Scrophularia* (Scrophulariaceae). Many iridoids, for example, those from the genus *Gentiana*, taste bitter. They exhibit a wide range of bioactivities including cardiovascular, anti-inflammatory, antispasmodic, antitumor, antiviral, and immunomodulatory activities.

As shown from the more than 220 relevant references found with SciFinder Scholar or ISI Web of Knowledge, iridoids have been predominantly separated by HP-RPC[103–109] and to a lesser extent by HP-NPC.[110] Secoiridoids have been separated by HP-RPC in both analytical[43,107,109,111–116] and preparative scale.[113,117] Iridoids and secoiridoids have been almost exclusively separated with HP-RPC and detected with UV and MS. An example is the quantitative analysis of iridoids, secoiridoids, xanthones, and xanthone glycosides derived from roots of *Gentiana lutea* by Aberham *et al.*[107] These authors used an analytical 4.6 mm × 150 mm 5 µm C_{18} silica column thermostated to 30 °C with linear gradient elution (eluent A: 0.025% of TFA in water and eluent B: ACN and *n*-propanol, 50:50 (v/v)) at 1.0 ml min^{-1}. The LC system was directly coupled to an ESI mass spectrometer with flow splitting (split ratio 1:3) and the mass spectra were acquired in both positive and negative ionization mode. For the LC–MS method, eluent A was changed to a mixture of water, FA, and AA in a ratio of 99:0.9:0.1, other LC conditions remained unchanged. The described LC–MS method focused on the quantitative analysis of all major, currently known bioactive compounds in gentian roots, rather than on the iridoids (loganic acid), secoiridoids (swertiamarin, gentiopicroside, amarogentin, sweroside), xanthones (gentisin, isogentisin), and xanthone glycosides (gentiosides).

An example of the separation of iridoid glycosides with RPC-RPC chromatography systems has been published by Zhou *et al.*[109] for iridoid glycosides from *Gardenia jasminoides*, a plant used in TCM. For the separation in the first dimension, the authors have used a preparative 10 mm × 100 mm 5 µm C_{18} silica column and isocratic elution (eluent: 0.1 AA water/ACN (93:7) at 9.8 ml min^{-1}. In the second dimension they employed a 10 mm × 250 mm C_{18} silica column, with linear gradient elution (eluent: 0.1 AA water/ACN (93:7)) at 4 ml min^{-1}. Such a 2D column-switching system without sample loop trapping, enabled the isolation and purification of six iridoid glycosides including geniposide, gardenoside, shanzhiside, scandoside methyl ester, deacetylasperulosidic acid methyl ester, and genipin-1.

Additional examples as representative of approaches used for the HPLC separation are shown for iridoids in **Table 3** and for secoiridoids in **Table 4**.

9.02.5.3 Carotenoids

Carotenoids, which are tetraterpenes, are 40-carbon-atoms, aliphatic, conjugated double bond compounds with recurring isoprene units. They can be divided into carotenes (e.g., β-carotene, lycopene), which are formed when the end of the chain is transformed to a cyclic ionone ring, and into xanthophylls (e.g., lutein, cryptoxanthin), which contain hydroxyl-, oxo-, epoxy-, methoxy-, or carboxyl groups produced by oxidation. All carotenoids are very lipophilic and exhibit very low solubility in water. They are mainly formed in the chloro- and chromoplasts of plants and generate a diverse group of yellow-orange pigments, but also exist in other biological systems. They act as antioxidants, attractants, and UV attenuators. Due to their essential function in photosynthesis, they could also be regarded as part of the primary metabolism. They have received attention due to their health benefits and therapeutic value and in the food and perfume industries as they can be transformed into aroma molecules.

As described in more than 930 relevant publications identified by SciFinder, HPLC has been extensively used for the separation of carotenoids,[119–122] particularly in HP-RPC[123–126] and HP-NPC[21,45,127–131] modes. The primary tool of carotenoid separation is HP-RPC. For the separation of naturally occurring carotenoids as well as for carotenoid isomers, C_{30} bonded silica[132–134] exhibits a higher selectivity than the conventionally used C_8 and C_{18} materials.[135,136] The separation behavior of carotenoids on C_{30} silica phases is strongly temperature dependent, with best separations being obtained at lower temperatures.[137–139] The addition of antioxidants such as butylated hydroxytoluene (BHT) to the mobile phase (and to the extraction solvent) has been reported to avoid oxidization.[140] Carotenoids are colored compounds due to their conjugated double bonds, where the maxima in their absorption spectra, spanning from 380 to 550 nm, shifts to longer wavelengths with increasing number of conjugated double bonds. Besides UV and MS detection,[124,135,141–144] ED

Table 3 Iridoids

Compound	*Natural product group*	*Organism*	*HPLC method*	*Detection*[a]	*Other methods*	*Reference*
Ten iridoids	Iridoid glucosides	Genus *Galium* (Rubiaceae)	Analytical RPC	233 nm		105
Five iridoids	Iridoid glycosides	*Gardenia jasminoides*	Analytical RPC	UV at 240 nm, MS		106
Two iridoids	Iridoid glucosides	Noni (*Morinda citrifolia*)	Analytical RPC	UV at 240 nm, MS	HPTLC	108
Ten iridoids	Iridoid glucosides	Genus *Galium*	Analytical NPC	UV at 233 nm		110
Six iridoids	Iridoid glycosides	*Gardenia jasminoides*	RPC-RPC	DAD UV	MS, ^{1}H-NMR and ^{13}C-NMR analysis	109
Various iridoids	Iridoids	Various plants	SEC-RPC	DAD UV, MS		23

[a] If a particular technique, that is, mass spectrometry is listed in this column, it implies online coupling. Off-line application of mass spectrometry is listed in the column as other methods.

Table 4 Secoiridoids

Compound	*Natural product group*	*Organism*	*HPLC method*	*Detection*[a]	*Other methods*	*Reference*
Six secoiridoids	Acylated secoiridoid glycosides	*Gentiana* species (Gentianaceae)	Analytical and semipreparative RPC	DAD UV at 200–400 nm	MS, TLC, NMR spectroscopy	113
Oleuropein, ligustaloside A, ligustaloside B, ligstroside	Secoiridoids	*Ligustrum vulgare* (Oleaceae)	Analytical RPC	DAD UV at 190–450 nm, MS		114
Mangiferin, amarogentin, amaroswerin, sweroside, swertiamarin	Secoiridoid glycosides	*Swertia chirata* (Gentianaceae)	Analytical RPC	MS		115
Oleuropein, ligustroside, angustifolioside B	Secoiridoids	*Chionanthus virginicus* (Oleaceae)	Analytical RPC	MS	NMR spectroscopy	116
Several secoiridoids	Secoiridoids	*Fraxinus* species	Analytical RPC	DAD UV at 254 nm, MS		118

[a] If a particular technique, that is, mass spectrometry is listed in this column, it implies online coupling. Off-line application of mass spectrometry is listed in the column as other methods.

(array)[125,145–147] has been successfully applied for carotenoids. Recently, several comprehensive NPC × RPC methods for the analysis of carotenoids have been described.[7,65]

An example of the separation of free (i.e., not esterified) carotenoids as well as carotenoid esters present in mandarin essential oil with two separate comprehensive normal-phase × reversed-phase LC systems has been published by Dugo *et al.*[65] For the separation of the free carotenoids, the authors used in the first dimension a capillary normal-phase 1.0 mm × 300 mm 5 μm silica column with linear gradient elution (eluent A: *n*-hexane and eluent B: ethyl alcohol) at 10 μl min^{-1}. In the second dimension they employed a monolithic 4.6 mm × 100 mm C_{18} silica column with linear gradient elution (eluent A: 2-propanol and eluent B: 20% (v/v) water in ACN) at 4.7 ml min^{-1}. For the separation of the carotenoid esters, these authors used in the first dimension a capillary normal-phase 1.0 mm × 250 mm 5 μm cyanopropyl silica column with linear gradient elution (eluent A: *n*-hexane and eluent B: *n*-hexane/butyl acetate/acetone 80:15:5 (v/v/v) at 10 μl min^{-1}. The second dimension columns as well as the mobile phases were the same as those used for the free carotenoids analysis, with minor changes to the flow rate and gradient conditions. The incompatibility of the solvents that were used in the two dimensions (NPC and RPC) and their effects on the separation were overcome by using a combination of a capillary column in the first dimension and an analytical monolithic column in the second dimension. Using flow splitting, the detection was performed in parallel with photodiode array and mass spectrometric detection. This approach then allowed the establishment of two independent 2D contour plots, one for the carotenoids and one for the carotenoid esters, allowing group separations as well as identification of the individual compounds. With the first NPC × RPC system, predominantly targeting carotenoids, 19 compounds were identified and grouped separately into mono-ols, mono-epoxides, apo-carotenoids, di-ols, di-ol–mono-epoxides, di-ol–di-epoxides, and tri-ol–mono-epoxides. With the second NPC × RPC system, predominantly targeting carotenoid esters, 23 compounds were identified and grouped separately into hydrocarbons, *β*-cryptoxanthin esters, lutein esters, mutatoxoxanthin esters, isomeric esters, luteoxanthin esters, apocarotenoids, and free xanthophylls. In this study, the information was obtained from (1) analysis of carotenoids in both their free form as well as in their fatty acid esterified form, (2) group separation of the compounds in the respective 2D contour plots, (3) resolution enhancement brought about by the comprehensive NPC × RPC system, and (4) online analysis with MS. Such a comprehensive LC × LC approach demonstrates the paradigm shift from a combined application of open column/HPLC to HP multidimensional chromatographic separation methods in natural product purification.

Table 5 lists additional investigations that are representative of the approaches that have been used for the high-resolution chromatographic separation of carotenoids from diverse sources.

9.02.5.4 Saponins

Saponins consist of an aglycone with carbohydrate moieties. The aglycone can be a triterpene or a steroid and can have a number of different substituents (–H, –COOH, $–CH_3$). The number and type of carbohydrate moieties result in a considerable structural diversity of the saponins. Most carbohydrates in saponins are hexoses (i.e., glucose, galactose), 6-deoxyhexoses (rhamnose), pentoses (arabinose, xylose), uronic acid (glucoronic acid), or carbohydrates with amino functionality (glucosamine). Through the glycosylation of the hydrophobic aglycones, they can act as biological detergents and, when agitated in water, form foams, which gave rise to the name, saponin for this group of compounds. Saponins are widely spread throughout the plant kingdom,[148–150] and have been found in marine animals.[151] The amphiphilic nature of the saponins enables them to act as soaps and detergents as they can dissolve membranes; however, since the saponins can hemolyze erythrocytes they are highly toxic if they reach mammalian blood. Exceptions are the sweet triterpenesaponin glycirrhicin from *Glycirrhiza glabra*, and diosgenin from *Dioscorea* (yam), a steroidal saponin precursor for the synthesis of cortisone and progesterone.

As with many other natural product groups, HPLC has been increasingly applied to saponins, as shown from the 460 reference citations identified by SciFinder using the keywords 'saponins high performance liquid chromatography'. Saponins have predominantly been separated by HP-RPC[152–154] but separation by HP-NPC,[155,156] HP-IEX,[157,158] and HP-HILIC[159] has also been described. Saponins do not have a chromophore, which restricts their UV detection to 200–210 nm. This makes their detection in complex samples containing other analytes, which absorb in this wavelength range, challenging. Notable exceptions are

Table 5 Carotenoids

Compound	*Natural product group*	*Organism*	*HPLC method*	*Detection*	*Reference*
Various compounds	Carotenoids and carotenoid esters	*Capsicum annuum*	Analytical RPC with C_{30} ligand	MS	124
α- and β-Carotene, lycopene	Carotenoids	Vegetable oils	Analytical RPC with C_{30} ligand	ED	125
α- and β-Carotene, β-cryptoxanthin, lutein, zeaxanthin	Carotenoids	Grain: maize, oat, wheat, barley (Poaceae)	Analytical NPC	DAD UV at 350–500 nm	131
Various compounds	Carotenoids	*Citrus* products	NPC $\times$ RPC, first dimension microscale and second dimension analytical scale (monolithic C_{18})	DAD UV	7

DMMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone)-conjugated soya saponins that have UV absorption at 295 nm.[160] Besides UV detection, evaporative light scattering detection (ELSD) is applied as a validated analytical technique. As a consequence of the difficult UV detection, more recently, HPLC has been combined with online MS. Substantial work with LC–MS was performed on saponins from commercially significant plants, that is, black bean (*Vigna mungo*),[161] soybean (*Glycine max*),[162–164] and ginseng (*Panax notoginseng*).[165–167] In some investigations, a combination of ELSD and MS detection methods have been applied.[168–171]

An example of the separation of saponins with HP-RPC has been described by Sun *et al.*,[172] who identified triterpenoid saponins in crude extracts from nine species of *Clematis* with electrospray ionization multistage tandem mass spectrometry (HPLC/ESI-MS^n). These authors used an analytical 4.6 mm × 250 5 μm C_{18} silica column at 25 °C with linear gradient elution (eluent A: water with 0.05% FA (v/v) and eluent B: ACN) at 0.6 ml min^{-1}. Eight triterpenoid saponins, namely, hederacholichiside F, prosapogenin CP11, clematichinenoside B, huzhangoside D, clematiganoside A, clematichinenoside C, huzhangoside B, and HN saponin H, were isolated from the whole plant of *Clematis ganpiniana*, independently characterized and employed as reference compounds. These eight reference compounds were analyzed by HPLC/ESI-MS and HPLC/ESI-MS^n in negative ion mode to establish their MS^n fragmentation pathways ($n = 2$–4). This information provided structural insight into the carbohydrate sequence of the oligosaccharide chains and the mode of attachment of the aglycone of the saponins. This approach demonstrates the power of HP-RPC MS^n methods to allow the rapid discrimination of nonisomeric saponins. As a consequence, these authors have tentatively identified two new compounds hitherto unknown for the genus *Clematis*. This approach, employing C_{18} silica phases with aqueous–organic mobile phases and volatile buffer additives is typical of the procedures followed for the analysis of saponins by HP-RPC and provides, in conjunction with online MS, the methodological framework for the discovery of new compounds.

Table 6 lists examples that are representative of the approaches that have been used for the high-resolution chromatographic separation of saponins.

9.02.5.5 Ecdysteroids

The ecdysteroids are polar, polyhydroxylated steroids that function as the moulting hormones of insects and crustaceans. Ecdysteroids are also found in plants, often in high concentration, where it is thought they contribute to insect deterrence by acting as antifeedants, interfering in ecdysteroid metabolism or mode of action on ingestion by phytophagous insects. The chromatographic behavior of ecdysteroid glycosides is characteristic, as they appear much more polar than their corresponding free aglycones when analyzed by normal-phase HPLC, whereas the presence of glycosidic moieties has a very limited (if any) impact on polarity when using reversed-phase HPLC.[175]

As demonstrated from the 312 relevant SciFinder references, ecdysteroids have been isolated by RP-HPLC,[176–179] HP-NPC,[180–182] HP-IEX,[183,184] or HP-AC.[185] The dominant mode for the separation of ecdysteroids is HP-RPC, with numerous published applications including the use of superheated water as a mobile phase.[186]

An example for the separation of ecdysteroids with HP-RPC systems was published by Louden *et al.*[179] and related to the separation of an ecdysteroids extract from the plant *Lychnis flos-cuculi* (Caryopyllaceae). These authors used an analytical 4.6 mm × 100 mm 5 μm C_{18} silica column with isocratic elution (eluent ACN and D_2O 99.8% isotopic purity 20:80 v/v) at 1.0 ml min^{-1} and DAD between 190 and 360 nm. This study also explored the application of HPLC–NMR spectroscopy and HPLC–NMR spectroscopy–MS to these ecdysteroid-containing plant extracts, showing the advantages and limitations of the use of complex multiply hyphenated detection systems, which incorporate detectors of differing sensitivities.

Table 7 lists studies that are representative of the approaches that have been used for the high-resolution chromatographic separation of ecdysteroids.

9.02.5.6 Coumarins

Coumarins are 1,2-benzopyrones that are derived from the phenylpropanoid pathway; however, major details of their biosynthesis are still largely unknown.[187] Coumarins can also be produced, through the cleavage of meliotoside (the β-glucopyranoside of *o*-hydroxycinnamic acid) in dead plant material, or hay, where in the

Table 6 Saponins

Compound	*Saponin group*	*Organism*	*HPLC method*	*Detection*[a]	*Other methods*	*Reference*
Sixty saponins, differing in carbohydrate substructures	Glycosylated triterpenes	Tree *Quillaja saponaria* var. Molina	Preparative RPC	UV at 206 nm	ESI ion trap multiple stage tandem mass spectrometry	152
Twenty-eight saponins, differing in carbohydrate substructures	Glycosylated triterpenes	Tree *Quillaja saponaria* var. Molina	Preparative RPC	UV at 210 nm	SPE, NMR spectroscopy	154
Protodioscin, tribulosin, terrestrosin D	Steroidal saponins	*Tribulus terrestris* (Zygophyllaceae)	Analytical RPC	UV at 200 nm		173
Periandradulcin A, B and C	Glycosylated triterpenes	*Periandra dulcis* (Leguminosae)	Semipreparative NPC	UV at 207 nm, MS	RPC, MS, NMR spectroscopy	156
Various saponins	Glycosylated	Ginseng roots, *Sapindus mukurossi* and *Anemone rivularis*	Semipreparative IEX	UV		157
Elliptoside A–J	Glycosylated	*Archidendron ellipticum* (Leguminosae)	Preparative HILIC with aminopropyl ligand	DAD UV	SEC, RPC	159
Ginsenosides, notoginsenosides	Dammarane saponins	*Panax notoginseng*	Analytical RPC	ELSD, MS		168
Various saponins		Flos Lonicera, several genera (Caprifoliaceae)	Micro RPC	DAD UV, MS	SPE, IR, ^{1}H-, and ^{13}C-NMR	174

[a] If a particular technique, that is, mass spectrometry is listed in this column, it implies online coupling. Off-line application of mass spectrometry is listed in the column as other methods.

Table 7 Ecdysteroids

Compound	*Natural product group*	*Organism*	*HPLC method*	*Detection*[a]	*Other methods*	*Reference*
Various compounds	Ecdysteroids	*Lychnis flos-coculi* (Caryophyllaceae)	RPC	DAD UV, FT-infrared and ^{1}H-NMR spectroscopy, MS		179
Phyto-ecdysteroids	Ecdysteroids	*Silene* species (Caryophyllaceae)	Semipreparative NPC	UV	MS, bioassay	180
Various compounds	Ecdysteroids		Analytical IEX	UV		183
Various compounds	Ecdysteroids		AC	UV		185

[a] If a particular technique, that is, mass spectrometry is listed in this column, it implies online coupling. Off-line application of mass spectrometry is listed in the column as other methods.

presence of fungi, like *Penicillium* and *Aspergillus* sp., dicumarol, a vitamin K antagonist, can be formed leading to hemophilia in cattle. Coumarins may occur in the oils of some Apiaceae (Umbelliferae), Rubiaceae, and Poaceae and are best known as the bergamot oil from *Citrus bergamia*. In plants, coumarins contribute to the defense against phytopathogens, response to abiotic stresses, regulation of oxidative stress, and probably as signaling molecules.[187] Coumarins can be subclassified into simple coumarins (benzo-α-pyrones), 7-oxygenated coumarins (furanocoumarins), pyranocoumarins (benzodipyran-2-ones), and phenylcoumarins (benzo-benzopyrones). Some furano-coumarins are known to enhance the photosensitivity of human skin. In the presence of UV light, they produce radicals that block enzymes (and thus have found limited use therapeutically) but also lead to inflammation. Unfortunately, they are also incorporated into the cellular DNA via UV-mediated cycloaddition leading to mutations.

As evidenced by the over 360 references retrieved from SciFinder with the keywords 'coumarins high performance liquid chromatography', a variety of approaches have been used for the separation of coumarins. Coumarins have been separated by RP-HPLC,[46,47,188–192] HP-NPC,[193–198] HP-IEX,[199] and/or HP-AC.[24] The dominant mode for the separation of coumarins is HP-RPC and to a lesser extent HP-NPC, used in conjunction with UV and MS detection.

An example of the separation of coumarins with HP-RPC systems was published by Eeva *et al.*[47] for the 21 different coumarins and furanocoumarins isolated from the plants *Peucedanum palustre* and *Angelica archangelica*. In these studies the authors used an analytical 4.6 mm × 100 mm 3 µm C_{18} silica column with linear gradient elution at 1.0 ml min^{-1} and UV detection at 320 nm. A Turbo Method Development program was applied to optimize the mobile phase with two organic solvents (ACN and MeOH) and two aqueous solutions (1.0% FA and 10 mmol l^{-1} ammonium acetate). Optimization of the solvent gradients for the method was performed with the program DryLab, with the aid of coumarin standards. Once the LC methods were established, the techniques were transferred to an LC–MS system, employing a triple quadrupole mass spectrometer taking into consideration the gradient delay volume, and applied to the respective plant extracts containg >45 compounds of interest. Such an approach, employing computer-assisted method development considerably streamlined the method development phase of the separation when speed, high throughput, and method robustness are required.

An example of the separation of coumarins with comprehensive normal-phase × reversed-phase LC systems has been published by Dugo *et al.*[73] for coumarins and psoralens in cold-pressed lemon oil. For the separation in the first dimension, the authors used a capillary normal-phase 1.0 mm × 300 mm 5 µm silica column with isocratic elution (eluent: *n*-hexane:ACN (75:25)) at 20 µl min^{-1}. In the second dimension, a monolithic 4.6 mm × 25 mm C_{18} silica column (including a 4.6 mm × 5 mm guard column) was employed with linear gradient elution (eluent A: water and eluent B: ACN) at 4 ml min^{-1}. The interface between the first and the second dimension was a 10-port, 2-position valve equipped with two storage loops. The incompatibility of the solvents that were used in the two dimensions (NPC and RPC) and its effects in the separation were overcome by using a combination of a capillary column in the first dimension and an analytical monolithic column in the second dimension. With this NPC × RPC system, 11 heterocyclic compounds were analyzed and depicted in 2D contour plots.

Table 8 lists additional investigations that are representative of the approaches that have been used for the high-resolution chromatographic separation of coumarins.

9.02.5.7 Flavonoids and Isoflavonoids

Flavonoids are benzopyrane derivatives with a phenyl group in the second position. Flavonoids are polyphenols, which are biosynthetically derived from phenylalanine and can be O-glycosides, usually in position 3 or 7. They can be grouped into several subclasses, including the anthocyanins, catechins, flavones, isoflavones, flavonols, and chalcones. These subclasses, combined with glycosylation at multiple sites with a variety of different saccharides and further acylation of the saccharides, produce more than 5000 chemically distinguishable compounds.[200] Animals and humans need phenolics to build aromatic amino acids but cannot synthesize them, thus, they or their precursors must be ingested as food from plants. Flavonoids are the largest group of phenolics and contribute to color and oxidative stability of plant parts. They find medicinal uses as antioxidants and are known to increase blood flow and stabilize capillaries.

Table 8 Coumarins

Compound	*Natural product group*	*Organism*	*HPLC method*	*Detection*	*Reference*
Twenty-one different coumarin-type compounds	Coumarins and furanocoumarins	*Peucedanum palustre* and *Angelica archangelica*	Analytical RPC based on computer-assisted optimization	UV, MS	47
Nine coumarin compounds	Coumarins	*Angelica gigas*	Analytical RPC	DAD UV, MS	193
Osthol, corymbo-coumarin	Coumarins	*Seseli* species (Umbelliferae)	Analytical NPC	UV	198
6,7-Dimethoxycoumarin and capillarisin	Coumarins	*Artemisia capillaris*	AC	UV	24
Various compounds	Coumarins	*Citrus* products	NPC $\times$ RPC, first dimension microscale and second dimension analytical scale (monolithic C_{18})	DAD UV	73

As documented from the more than 1820 relevant SciFinder references, flavonoids have been separated by a variety of HPLC modes,[201–205] predominantly by RP-HPLC,[3,20,206–216] but also by HP-NPC,[217–221] HP-SEC,[222–225] HP-AC,[226] or AC with MIPs.[227–229] Separations are almost exclusively performed by HP-RPC using binary elution systems with an aqueous, acidified eluent A (AA, perchloric acid, phosphoric acid, or FA) and a less polar organic solvent eluent B such as MeOH or ACN, possibly acidified. Phenols absorb in the UV region. Two absorption bands are characteristic of flavonoids.[204] Band I, with a maximum in the 300–550 nm range, presumably arises from the B-ring. Band II, with a maximum in the 240–285 nm range, is believed to arise from the A-ring. Anthocyanins show band I and band II absorption maxima in the 465–560 and 265–275 nm regions, respectively.[205] Because there is little or no conjugation between the A- and B-rings, UV spectra of flavanones and isoflavones usually have an intense band II peak but a small band I peak.[230] This lack of conjugation also results in small band I peaks for the catechins. UV spectra of flavones and flavonols have a band I peak around 300–380 nm and a band II peak at around 240–280 nm.[230] Flavonoids have been identified using photo DAD,[231] fluorescence detection,[208] or ED.[216]

An example for the separation for flavonoids with HP-RPC is the screening method employed for the systematic identification of glycosylated flavonoids and other phenolic compounds in plant food materials by Lin *et al.*[20] These authors used an analytical 4.6 mm × 250 mm 5 µm C_{18} silica column at 25 °C with linear gradient elution (eluent A: (0.1% FA in water and eluent B: 0.1% FA in ACN) at 1.0 ml min^{-1}. DAD was performed at 270, 310, 350, and 520 nm to monitor the UV/VIS absorption. The LC system was directly coupled to an ESI mass spectrometer without flow splitting and the mass spectra acquired in the positive and negative ionization mode. The same analytical scheme (aqueous MeOH extraction, reversed-phase liquid chromatographic separation, and diode array and mass spectrometric detection) can be applied to a wide variety of samples and standards and therefore allows the cross-comparison of newly detected compounds in samples with standards and plant materials previously identified in the published literature.

As a further example, Prior *et al.*[221] have demonstrated how procyanidin oligomers can be separated using HP-NPC with UV detection (at 280 nm), fluorescence detection (the excitation and emission wavelengths were 276 and 316 nm), and MS detection. These authors used an analytical 4.6 mm × 250 mm ID 5 µm silica column at 37 °C with linear gradient elution using a ternary mobile phase (eluent A: dichloromethane, eluent B: MeOH, and eluent C: AA and water (1:1 v/v)) at 1.0 ml min^{-1}. For HPLC–MS analysis, 10 mmol l^{-1} ammonium acetate in MeOH was used as an ionization reagent and was added via a tee-junction at 0.1 ml min^{-1} into the eluent stream of the HPLC prior to the mass spectrometer by an auxiliary HPLC pump.

Table 9 lists additional examples that are representative of the approaches that have been used for the high-resolution chromatographic separation of flavonoids and isoflavonoids.

9.02.5.8 Alkaloids

Alkaloids are a very diverse class of secondary metabolites with more than 20 000 known compounds, encompassing a broad variety of chemical structures. Alkaloids are heterocyclic compounds, often possessing tertiary nitrogens, which gives them their basic properties. They are most commonly lipophilic; however, they can form water-soluble salts with acids. Many alkaloids are biosynthetically derived from the amino acids lysine, ornithine, phenylalanine, tryptophan, and tyrosine. Although in the past alkaloids were mostly isolated from plants, where they are sequestered as salts in special tissues to kill predators, they are also found in microorganisms, marine organisms, as well as insects and reptiles. Alkaloids have been classified according to their molecular structure (e.g., indole, isoquinoline, pyridine, pyrrolizidine, steroidal, tropane) or according to their botanical origin, for example, *Nicotina* alkaloids, *Papaver* alkaloids, and *Solanum* alkaloids. Most alkaloids are highly bioactive, and their effects in humans are a direct consequence of their structural similarities to neurotransmitters such as acetylcholine, dopamine, noradrenalin, or serotonin. Alkaloids are of considerable pharmaceutical interest (e.g. codeine, scopolamine, morphine, D-tubocurarine), others are drugs of abuse (mescaline, cocaine, and nicotine), stimulants (caffeine), or poisons employed for pest control (strychnine).

As evident in the literature of the last decade, with more than 1380 references relevant to the theme of this chapter are found within SciFinder Scholar or ISI Web of Knowledge, HPLC has developed into an important tool for the isolation and purification of alkaloids. Alkaloids have been isolated with a variety of HPLC modes,[232–234] including HP-RPC,[235–240] HP-NPC,[241–244] HP-IEX,[22,245–247] HP-HILIC,[248] or HP-AC.[249]

Table 9 Flavonoids and isoflavonoids

Compound	*Natural product group*	*Organism*	*HPLC method*	*Detection*[a]	*Other methods*	*Reference*
Various isoflavones	Isoflavones	*Glycine max* (Fabaceae)	Analytical RPC	MS	SPE	215
Anthocyanins compounds	Flavonoids	*Vitis vinifera*	Analytical RPC	ED		216
Glycosylated flavonoids	Various groups	Various plants	Analytical RPC	DAD UV, MS		20
Various compounds	Isoflavonoids	*Smirnowia iranica*	Analytical RPC	DAD UV	SPE, NMR spectroscopy	3
Various compounds	Procyanidins and anthocyanins	*Vaccinium* spp.	Analytical NPC	DAD UV, MS		221
Various compounds	Pyrano-anthocyanin–flavanols	*Vitis vinifera*	SEC, RPC	UV, MS		225
Quercetin	Flavanols	*Vitis vinifera*	AC with MIP	UV	SPE	228

[a] If a particular technique, that is, mass spectrometry is listed in this column, it implies online coupling. Off-line application of mass spectrometry is listed in the column as other methods.

Several LC–LC methods have been described including AC-SCX[250] where the HP-AC was performed with a MIP column. HP-RPC dominates the analytical and preparative HPLC application with alkaloids, notwithstanding the potential for secondary interaction of their basic primary, secondary, or tertiary amine moiety with residual silanol groups of the *n*-alkylsilica stationary phases, resulting in peak tailing. To overcome this problem, a variety of approaches have been developed, including the suppression of these interactions with mobile phase ion-pairing additives or the employment of end-capped stationary phases with low numbers of free silanol groups. Typically, mobile phases with a pH of 2–4 have been used to shift the equilibrium from the free base into the protonated form of the alkaloid. The detection of alkaloids has been mostly performed with UV detectors; however, fluorescence, electrochemical, or mass spectrometric detection has also been employed. Recently, MS has emerged as the primary tool for alkaloid identification, quantification, and structural elucidation, where structural analysis is usually performed by mass spectrometric fragmentation. Mobile phases suited for LC–MS applications contain preferably only volatile additives, for example, ammonium acetate, ammonium formate, or TFA.

A good example of the separation of alkaloids with HP-RPC is the separation of the protoberberine alkaloids (including berberine, palmatine, and jatrorrhizine) reported by Wu *et al.*[240] These authors used an analytical 4.6 mm × 250 mm 5 μm C_{18} silica column with linear gradient elution (eluent A: water with 0.0 034 mol l^{-1} ammonium acetate and 0.2% AA (v/v) and eluent B: ACN) at 0.5 ml min^{-1} at a temperature of 23 °C. A set of alkaloid standards, permitting external calibration, was employed permitting the quantitative analyses of the protoberberine alkaloids in herbs used in TCM, coupled with the multiple stage mass spectrometric fragmentation information obtained with ESI-FT-ICR-MS^n (Fourier transform ion cyclotron resonance multiple stage mass spectrometry) and ESI-MS^n in the positive ion mode. Such an approach, employing C_{18} silica phases with aqueous–organic mobile phases and volatile buffer additives can be considered typical of techniques now employed for the analysis of alkaloids by HP-RPC and provides, in conjunction with online MS, the methodological framework for fast herbal medicine authentication and the quantification of individual compounds.

The additional examples summarized in **Table 10** are representative of the approaches that have been used for the high-resolution chromatographic separation of alkaloids.

9.02.6 Conclusions

Due to the enormous growth in capability and separation power that has occurred over the past two decades, the benefits of HPLC in natural product chemistry may now seem obvious. However, as there is an immense choice of modes and procedures, further scope exists to improve the quality of such separations and achieve even higher resolutions based on even more efficient optimization procedures. For these reasons, a comprehensive overview of the principles and limitations of contemporary separation methods in various steps of purification and analysis of natural products has been presented at the beginning of this chapter.

In order to solve analytical problems for a particular compound or class of compounds, as well as to save time and resources, it is essential that systematic method development concepts are applied. Such methods then enable a successful scaling up to preparative purifications as well as the design and application of MD-HPLC purification schemes. Moreover, if used in conjunction with dereplication procedures, these advances in high-resolution chromatographic methods may lead to new discoveries that can be used to advance science or medicine and at the same time respect the environment through reduced solvent and reagent usage.

Finally, since many natural product compounds have been investigated with various chromatographic modes and detection techniques, a selection of examples has been summarized in this chapter. This information has been compiled in the form of tables for well-researched classes of secondary metabolites selected from the major subgroups of isoprenoids (mono-, sesqui-, di-, and triterpenes; iridoids and secoiridoids; carotenoids; saponins; and ecdysteroids), of phenolics (coumarins, flavonoids, and isoflavonoids), and of alkaloids.

Despite decades of research, there still remains a vast scope for new natural products to be discovered and isolated from microbial, marine, arthropod, or plant organisms and used as nutraceuticals or pharmaceuticals. Such tasks will require access to more sophisticated and better optimized separation and identification methodologies. To this end, it will also be the responsibility of future generations of natural product scientists

Table 10 Alkaloids

Compound	*Natural product group*	*Organism*	*HPLC method*	*Detection*[a]	*Other methods*	*Reference*
Twenty-two standards	Monoterpenoid indole alkaloids	*Rauvolfia serpentina* or *Rhazya stricta*	Analytical RPC			239
Berberine, palmatine, jatrorrhizine, coptisine	Protoberberine alkaloids	Ranunculaceae	Analytical RPC	DAD UV	FT-ICR-MS	240
Various alkaloids	Alkaloids		NPC	UV		244
Ephedrine alkaloids	Alkaloids	Ephedraceae	IEX	DAD UV and fluorescence		22
Ephedrine, atropine, theophylline, and nicotine	Alkaloids		HILIC	UV at 214 nm		248
Various alkaloids	Vinca alkaloids		AC	UV at 263 nm		249
Atropine and scopolamine	Tropane alkaloids		AC-SCX	UV at 210 nm		250

to ensure that such bioprospecting occurs responsibly and sustainably. It is therefore expected that increasingly the analysis of natural products will use the principles of green analytical chemistry, considering the issues of waste minimization and hazard reduction. Similar criteria will also apply to preparative and process developments. Thus, there is tremendous potential for investigators to pursue new aspects of method development, which hopefully has been encouraged by this chapter.

Abbreviations

AA	acetic acid
AC	affinity chromatography
ACN	acetonitrile
AEX	anion-exchange chromatography
BHT	butylated hydroxytoluene
CC	(open) column chromatography
CD	circular dichroism
CE	capillary electrophoresis
CEX	cation-exchange chromatography
DAD	diode array detection
DMMP	2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone
ED	electrochemical detection
ELSD	evaporative light scattering detection
ESI	electrospray ionization
FA	formic acid
FC	flash chromatography
FT	Fourier transform
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier transform infrared
GC	gas chromatography
GPC	gel-permeation chromatography
HBFA	heptafluorobutyric acid
HILIC	hydrophilic interaction chromatography
HP	high-performance
HP-AC	high-performance affinity chromatography
HP-AEX	high-performance anion-exchange chromatography
HP-CEX	high-performance cation-exchange chromatography
HP-GPC	high-performance gel-permeation chromatography
HP-HILIC	high-performance hydrophilic interaction chromatography
HP-IEX	high-performance ion-exchange chromatography
HPLC	high-performance liquid chromatography
HP-NPC	high-performance normal-phase chromatography
HP-RPC	high-performance reversed-phase chromatography
HP-SEC	high-performance size exclusion chromatography
HPTLC	high-performance thin-layer chromatography
HSA	human serum albumin
HSCC	high-speed countercurrent chromatography
HTS	high-throughput screening
ID	inner diameter
IEX	ion-exchange chromatography
LC	liquid chromatography
MALDI ToF	matrix-assisted laser desorption/ionization time-of-flight
MD-HPLC	multidimensional high-performance liquid chromatography
MeOH	methanol

MIP	molecularly imprinted polymer
MS	mass spectrometry
MS^n	multiple stage mass spectrometry
NMR	nuclear magnetic resonance
NPC	normal-phase chromatography
O	olfactometry
ORD	optical rotatory dispersion
PC	peak capacity
PTLC	preparative thin-layer chromatography
QSRR	quantitative structure retention relationship
RAM	restricted access material
RPC	reversed-phase chromatography
RRM	relative resolution map
SCX	strong cation exchange
SEC	size exclusion chromatography
SPE	solid-phase extraction
TCM	Traditional Chinese Medicine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
2D	two-dimensional
3D	three-dimensional

References

1. H. L. Constant; K. Slowing; J. G. Graham; J. M. Pezzuto; G. A. Cordell; C. W. W. Beecher, *Phytochem. Anal.* **1997**, *8*, 176–180.
2. M. E. Hansen; J. Smedsgaard; T. O. Larsen, *Anal. Chem.* **2005**, *77*, 6805–6817.
3. M. Lambert; D. Strk; S. H. Hansen; M. Sairafianpour; J. W. Jaroszewski, *J. Nat. Prod.* **2005**, *68*, 1500–1509.
4. Y. Konishi; T. Kiyota; C. Draghici; J.-M. Gao; F. Yeboah; S. Acoca; S. Jarussophon; E. Purisima, *Anal. Chem.* **2007**, *79*, 1187–1197.
5. S. Urban; F. Separovic, *Front. Drug Des. Discov.* **2005**, *1*, 113–166.
6. O. Sticher, *Nat. Prod. Rep.* **2008**, *25*, 517–554.
7. P. Dugo; V. Skerikova; T. Kumm; A. Trozzi; P. Jandera; L. Mondello, *Anal. Chem.* **2006**, *78*, 7743–7750.
8. J. L. Mazzei; L. A. d'Avila, *J. Liq. Chromatogr. Relat. Technol.* **2003**, *26*, 177–193.
9. K. Hostettmann; M. Hostettmann; A. Marston, *Preparative Chromatography Techniques. Applications in Natural Product Isolation*; Springer-Verlag: London, UK, 1986.
10. B. A. Bidlingmeyer, Ed., *Journal of Chromatography Library, Vol. 38: Preparative Liquid Chromatography*; 1987.
11. E. Grushka, Ed., *Chromatographic Science Series, Vol. 46: Preparative-Scale Chromatography*; 1989.
12. K. K. Unger, Ed., *Handbook of HPLC, Part 2: Preparative Liquid Column Chromatography*; 1994.
13. K. Hostettmann; A. Marston; M. Hostettmann, *Preparative Chromatography Techniques: Applications in Natural Product Isolation*, 2nd ed.; 1997.
14. A. S. Rathore; A. Velayudhan, Eds., Scale-Up and Optimization in Preparative Chromatography: Principles and Biopharmaceutical Applications. In *Chromatographic Science Series.* 2003; Vol. 88.
15. C. Horvath; W. Melander; I. Molnar, *J. Chromatogr.* **1976**, *125*, 129–156.
16. C. Horvath; W. Melander; I. Molnar, *Anal. Chem.* **1977**, *49*, 142–154.
17. K. K. Unger, *Journal of Chromatography Library, Vol. 16: Porous Silica, Its Properties and Use as Support in Column Liquid Chromatography*; Elsevier: Amsterdam, The Netherlands, 1979; p 336.
18. P. Stead, *Methods Biotechnol.* **1998**, *4*, 165–208.
19. Z. Latif, *Methods Biotechnol.* **2005**, *20*, 213–232.
20. L.-Z. Lin; J. M. Harnly, *J. Agric. Food Chem.* **2007**, *55*, 1084–1096.
21. M. M. Mendes-Pinto; A. C. S. Ferreira; M. B. P. P. Oliveira; P. Guedes de Pinho, *J. Agric. Food Chem.* **2004**, *52*, 3182–3188.
22. R. A. Niemann; M. L. Gay, *J. Agric. Food Chem.* **2003**, *51*, 5630–5638.
23. S. Ma; L. Chen; G. Luo; K. Ren; J. Wu; Y. Wang, *J. Chromatogr. A* **2006**, *1127*, 207–213.
24. H. L. Wang; H. F. Zou; J. Y. Ni; L. Kong; S. Gao; B. C. Guo, *J. Chromatogr. A* **2000**, *870*, 501–510.
25. T.-P. I; R. Smith; S. Guhan; K. Taksen; M. Vavra; D. Myers; M. T. W. Hearn, *J. Chromatogr. A* **2002**, *972*, 27–43.
26. L. R. Snyder, *J. Chromatogr.* **1974**, *92*, 223–230.
27. L. R. Snyder, *J. Chromatogr. Sci.* **1978**, *16*, 223–234.
28. P. J. Schoenmakers; H. A. H. Biliet; L. D. Galan, *J. Chromatogr.* **1979**, *185*, 179–195.

29. H. B. Patel; T. M. Jefferies, *J. Chromatogr.* **1987**, *389*, 21–32.
30. M. T. W. Hearn, *Handbook of Bioseparations;* 2000; Vol. 2, pp 71–235.
31. L. R. Snyder, Gradient election. In *HPLC – Advances and Perspectives*; C. Horvath, Ed.; Academic Press: New York, 1980; Vol. 1, 208–316.
32. J. W. Dolan; D. C. Lommen; L. R. Snyder, *J. Chromatogr.* **1989**, *485*, 91–112.
33. B. F. D. Ghrist; B. S. Coopermann; L. R. Snyder, *J. Chromatogr.* **1988**, *459*, 1–23.
34. B. F. D. Ghrist; L. R. Snyder, *J. Chromatogr.* **1988**, *459*, 25–41.
35. B. F. D. Ghrist; L. R. Snyder, *J. Chromatogr.* **1988**, *459*, 43–63.
36. M. A. Stadalius; H. S. Gold; L. R. Snyder, *J. Chromatogr.* **1984**, *296*, 31–59.
37. E. P. Lankmayr; W. Wegscheider; K. W. Budna, *J. Liq. Chromatogr.* **1989**, *12*, 35–58.
38. J. C. Berridge, *Techniques for the Automated Optimization of HPLC Separations*; Wiley Interscience: Chichester, 1986.
39. J. K. Strasters; H. A. H. Billiet; L. de Galan; B. G. M. Vandeginste; G. Kateman, *J. Liq. Chromatogr.* **1989**, *12*, 3–22.
40. M. A. Quarry; R. L. Grob; L. R. Snyder, *Anal. Chem.* **1986**, *58*, 907–917.
41. J. Castillo; O. Behavente-Garcia; J. A. Del Rio, *J. Liq. Chromatogr.* **1994**, *17*, 1497–1523.
42. F. Dondi; Y. D. Kahie; G. Lodi; P. Reschiglian; C. Pietrogrande; C. Bighi; G. P. Cartoni, *Chromatographia* **1987**, *23*, 844–849.
43. F. Dondi; T. Gianferrara; P. Reschiglian; M. C. Pietrogrande; C. Ebert; P. Linda, *J. Chromatogr.* **1989**, *485*, 631–645.
44. W. Metzger; K. Reif, *J. Chromatogr. A* **1996**, *740*, 133–138.
45. A. S. Kester; R. E. Thompson, *J. Chromatogr.* **1984**, *310*, 372–378.
46. M. A. Hawryl; E. Soczewinski; T. H. Dzido, *J. Chromatogr. A* **2000**, *886*, 75–81.
47. M. Eeva; J.-P. Rauha; P. Vuorela; H. Vuorela, *Phytochem. Anal.* **2004**, *15*, 167–174.
48. T. H. Dzido; E. Soczewinski; J. Gudej, *J. Chromatogr.* **1991**, *550*, 71–76.
49. M. Krauze-Baranowska; T. Baczek; D. Glod; R. Kaliszan; E. Wollenweber, *Chromatographia* **2004**, *60*, 9–15.
50. T. Wennberg; K. Kreander; M. Laehdevuori; H. Vuorela; P. Vuorela, *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 2573–2592.
51. U. Rosentreter; U. Huber, *J. Comb. Chem.* **2004**, *6*, 159–164.
52. M. Martin, *Fresenius J. Anal. Chem.* **1995**, *352*, 625–632.
53. J. F. K. Huber; R. Van der Linden; E. Ecker; M. Oreans, *J. Chromatogr.* **1973**, *83*, 267–277.
54. L. Mondello; K. Bartle; A. Lewis, *Multidimensional Chromatography;* 2001.
55. J. F. K. Huber; E. Kenndler; G. Reich, *J. Chromatogr.* **1979**, *172*, 15–30.
56. J. C. Giddings, *Anal. Chem.* **1984**, *56*, 1258A–1260A, 1262A, 1264A, 1266A, 1268A, 1270A.
57. J. M. Davis; J. C. Giddings, *Anal. Chem.* **1985**, *57*, 2168–2177.
58. J. M. Davis; J. C. Giddings, *Anal. Chem.* **1985**, *57*, 2178–2182.
59. J. C. Giddings, *J. Chromatogr. A* **1995**, *703*, 3–15.
60. F. Regnier; G. Huang, *J. Chromatogr. A* **1996**, *750*, 3–10.
61. F. Erni; R. W. Frei, *J. Chromatogr.* **1978**, *149*, 561–569.
62. R. J. Simpson, Introduction to Chromatographic Methods for Protein and Peptide Purification. In *Purifying Proteins for Proteomics*; Cold Spring Habor Laboratory Press: Woodbury, N.Y., 2004; p 801.
63. K. K. Unger; K. Racaityte; K. Wagner; T. Miliotis; L. E. Edholm; R. Bischoff; G. Marko-Varga, *J. High Resolut. Chromatogr.* **2000**, *23*, 259–265.
64. F. Cacciola; P. Jandera; Z. Hajdu; P. Cesla; L. Mondello, *J. Chromatogr. A* **2007**, *1149*, 73–87.
65. P. Dugo; M. Herrero; T. Kumm; D. Giuffrida; G. Dugo; L. Mondello, *J. Chromatogr. A* **2008**, *1189*, 196–206.
66. Z. Liu; M. L. Lee, *J. Microcolumn Sep.* **2000**, *12*, 241–254.
67. P. Jandera, *J. Sep. Sci.* **2006**, *29*, 1763–1783.
68. P. Dugo; F. Cacciola; T. Kumm; G. Dugo; L. Mondello, *J. Chromatogr. A* **2008**, *1184*, 353–368.
69. R. E. Majors, *J. Chromatogr. Sci.* **1980**, *18*, 571–579.
70. M. T. W. Hearn, *Biologicals* **2001**, *29*, 159–178.
71. G. Guiochon; L. A. Beaver; M. F. Gonnord; A. M. Siouffi; M. Zakaria, *J. Chromatogr.* **1983**, *255*, 415–437.
72. A. J. P. Martin, *Ann. Rep. Progress Chem.* **1949**, *45*, 267–283.
73. P. Dugo; O. Favoino; R. Luppino; G. Dugo; L. Mondello, *Anal. Chem.* **2004**, *76*, 2525–2530.
74. P. Dugo; M. Herrero; D. Giuffrida; T. Kumm; G. Dugo; L. Mondello, *J. Agric. Food Chem.* **2008**, *56*, 3478–3485.
75. K. J. Mayfield; R. A. Shalliker; H. J. Catchpoole; A. P. Sweeney; V. Wong; G. Guiochon, *J. Chromatogr. A* **2005**, *1080*, 124–131.
76. E. Blahova; P. Jandera; F. Cacciola; L. Mondello, *J. Sep. Sci.* **2006**, *29*, 555–566.
77. L. Hu; X. Li; S. Feng; L. Kong; X. Su; X. Chen; F. Qin; M. Ye; H. Zou, *J. Sep. Sci.* **2006**, *29*, 881–888.
78. G. Cimpan; S. Gocan, *J. Liq. Chromatogr. Relat. Technol.* **2002**, *25*, 2225–2292.
79. M. Lambert; J.-L. Wolfender; D. Strk; S. B. Christensen; K. Hostettmann; J. W. Jaroszewski, *Anal. Chem.* **2007**, *79*, 727–735.
80. K.-H. Wagner; I. Elmadfa, *Ann. Nutr. Metab.* **2003**, *47*, 95–106.
81. A. Aharoni; M. A. Jongsma; H. J. Bouwmeester, *Trends Plant Sci.* **2005**, *10*, 594–602.
82. L. Mondello; K. D. Bartle; G. Dugo; P. Dugo, *J. High Resolut. Chromatogr.* **1994**, *17*, 312–314.
83. L. Mondello; P. Dugo; K. D. Bartle; G. Dugo; A. Cotroneo, *Flavour Fragr. J.* **1995**, *10*, 33–42.
84. J. Mookdasanit; H. Tamura, *Food Sci. Technol. Res.* **2002**, *8*, 367–372.
85. Y. Liu; L. Wang; J. H. Jung; S. Zhang, *Nat. Prod. Rep.* **2007**, *24*, 1401–1429.
86. C. Wood; T. E. Siebert; M. Parker; D. L. Capone; G. M. Elsey; A. P. Pollnitz; M. Eggers; M. Meier; T. Vossing; S. Widder; G. Krammer; M. A. Sefton; M. J. Herderich, *J. Agric. Food Chem.* **2008**, *56*, 3738–3744.
87. J. R. Hanson, *Nat. Prod. Rep.* **2007**, *24*, 1332–1341.
88. J. D. Connolly; R. A. Hill, *Nat. Prod. Rep.* **2007**, *24*, 465–486.
89. C. G. Benson; S. G. Wyllie; D. N. Leach; C. L. Mares; G. P. Fitt, *J. Agric. Food Chem.* **2001**, *49*, 2181–2184.
90. J. Warnke; R. Bandur; T. Hoffmann, *Anal. Bioanal. Chem.* **2006**, *385*, 34–45.
91. Y. Song; Q. He; P. Li; Y.-Y. Cheng, *J. Sep. Sci.* **2008**, *31*, 64–70.
92. P. Sahai; R. A. Vishwakarma; S. Bharel; A. Gulati; M. Z. Abdin; P. S. Srivastava; S. K. Jain, *Anal. Chem.* **1998**, *70*, 3084–3087.
93. A. Raffaelli; S. Pucci; F. Pietra, *Anal. Commun.* **1997**, *34*, 179–182.

94. L. Gobbo-Neto; N. P. Lopes, *J. Agric. Food Chem.* **2008**, *56*, 1193–1204.
95. R. Dolfinger; D. C. Locke, *Anal. Chem.* **2003**, *75*, 1355–1364.
96. S. Sturm; H. Stuppner, *Phytochem. Anal.* **2000**, *11*, 121–127.
97. Y.-C. Wang; Y.-S. Yang, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2007**, *850*, 392–399.
98. D. Savoia; C. Avanzini; T. Allice; E. Callone; G. Guella; F. Dini, *Antimicrob. Agents Chemother.* **2004**, *48*, 3828–3833.
99. M. J. Dubber; I. Kanfer, *J. Pharm. Biomed. Anal.* **2006**, *41*, 135–140.
100. B. Dinda; S. Debnath; Y. Harigaya, *Chem. Pharm. Bull. (Tokyo)* **2007**, *55*, 159–222.
101. B. Dinda; S. Debnath; Y. Harigaya, *Chem. Pharm. Bull. (Tokyo)* **2007**, *55*, 689–728.
102. S. Rodriguez; A. Marston; J. L. Wolfender; K. Hostettmann, *Curr. Org. Chem.* **1998**, *2*, 627–648.
103. B. Meier; O. Sticher, *J. Chromatogr.* **1977**, *138*, 453–457.
104. M. Willems, *Planta Med.* **1988**, *54*, 66–68.
105. K. Chervenkova; B. Nikolova-Damyanova, *J. Liq. Chromatogr. Relat. Technol.* **2000**, *23*, 741–753.
106. L. Ren; X. Xue; F. Zhang; Y. Wang; Y. Liu; C. Li; X. Liang, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3039–3050.
107. A. Aberham; S. Schwaiger; H. Stuppner; M. Ganzera, *J. Pharm. Biomed. Anal.* **2007**, *45*, 437–442.
108. O. Potterat; R. Von Felten; P. W. Dalsgaard; M. Hamburger, *J. Agric. Food Chem.* **2007**, *55*, 7489–7494.
109. T. Zhou; W. Zhao; G. Fan; Y. Chai; Y. Wu, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2007**, *858*, 296–301.
110. N. Dimov; K. Chervenkova; B. Nikolova-Damyanova, *J. Liq. Chromatogr. Relat. Technol.* **2000**, *23*, 935–947.
111. D. Schaufelberger; K. Hostettmann, *J. Chromatogr.* **1987**, *389*, 450–455.
112. A. Romani; P. Pinelli; N. Mulinacci; C. Galardi; F. F. Vincieri; L. Liberatore; A. Cichelli, *Chromatographia* **2001**, *53*, 279–284.
113. R.-W. Jiang; K.-L. Wong; Y.-M. Chan; H.-X. Xu; P. P.-H. But; P.-C. Shaw, *Phytochemistry* **2005**, *66*, 2674–2680.
114. A. Romani; P. Pinelli; N. Mulinacci; F. F. Vincieri; E. Gravano; M. Tattini, *J. Agric. Food Chem.* **2000**, *48*, 4091–4096.
115. S. Suryawanshi; N. Mehrotra; R. K. Asthana; R. C. Gupta, *Rapid Commun. Mass Spectrom.* **2006**, *20*, 3761–3768.
116. L. Boyer; R. Elias; K. Taoubi; L. Debrauwer; R. Faure; B. Baghdikian; G. Balansard, *Phytochem. Anal.* **2005**, *16*, 375–379.
117. D. Schaufelberger; K. Hostettmann, *J. Chromatogr.* **1985**, *346*, 396–400.
118. L. Zhou; J. Kang; L. Fan; X.-C. Ma; H.-Y. Zhao; J. Han; B.-r. Wang; D.-A. Guo, *J. Pharm. Biomed. Anal.* **2008**, *47*, 39–46.
119. M. Ruddat; O. H. Will, III, *Methods Enzymol.* **1985**, *111*, 189–200.
120. F. Khachik; G. R. Beecher; M. B. Goli; W. R. Lusby, *Methods Enzymol.* **1992**, *213*, 347–359.
121. N. E. Craft, *Methods Enzymol.* **1992**, *213*, 185–205.
122. H. Pfander; R. Riesen, *Carotenoids* **1995**, *1A*, 145–190.
123. R. Mateos; J. A. Garcia-Mesa, *Anal. Bioanal. Chem.* **2006**, *385*, 1247–1254.
124. U. Schweiggert; D. R. Kammerer; R. Carle; A. Schieber, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2617–2628.
125. N. L. Puspitasari-Nienaber; M. G. Ferruzzi; S. J. Schwartz, *J. Am. Oil Chem. Soc.* **2002**, *79*, 633–640.
126. A. B. Barua; J. A. Olson, *J. Chromatogr. B: Biomed. Sci. Appl.* **1998**, *707*, 69–79.
127. F. T. Gillan; R. B. Johns, *J. Chromatogr. Sci.* **1983**, *21*, 34–38.
128. S. H. Rhodes; A. G. Netting; B. V. Milborrow, *J. Chromatogr.* **1988**, *442*, 412–419.
129. L. Almela; J. M. Lopez-Roca; M. E. Candela; M. D. Alcazar, *J. Chromatogr.* **1990**, *502*, 95–106.
130. F. Khachik; G. R. Beecher; M. B. Goli; W. R. Lusby; J. C. Smith, Jr., *Anal. Chem.* **1992**, *64*, 2111–2122.
131. G. Panfili; A. Fratianni; M. Irano, *J. Agric. Food Chem.* **2004**, *52*, 6373–6377.
132. L. C. Sander; M. Pursch; B. Maerker; S. A. Wise, *Anal. Chem.* **1999**, *71*, 3477–3483.
133. L. C. Sander; K. E. Sharpless; M. Pursch, *J. Chromatogr. A* **2000**, *880*, 189–202.
134. L. Li; J. Qin; S.-a. Yin; G. Tang, *Chromatographia* **2007**, *65*, 91–94.
135. R. B. van Breemen, *Anal. Chem.* **1995**, *67*, 2004–2009.
136. Q. Su; K. G. Rowley; N. D. H. Balazs, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2002**, *781*, 393–418.
137. K. Albert, *Trends Anal. Chem.* **1998**, *17*, 648–658.
138. K. S. Epler; R. G. Ziegler; N. E. Craft, *J. Chromatogr., Biomed. Appl.* **1993**, *619*, 37–48.
139. M. Dachtler; T. Glaser; K. Kohler; K. Albert, *Anal. Chem.* **2001**, *73*, 667–674.
140. D. J. Hart; K. J. Scott, *Food Chem.* **1995**, *54*, 101–111.
141. R. B. Van Breemen; H. H. Schmitz; S. J. Schwartz, *Anal. Chem.* **1993**, *65*, 965–969.
142. R. B. van Breemen; C.-R. Huang; Y. Tan; L. C. Sander; A. B. Schilling, *J. Mass Spectrom.* **1996**, *31*, 975–981.
143. C. Rentel; S. Strohschein; K. Albert; E. Bayer, *Anal. Chem.* **1998**, *70*, 4394–4400.
144. P. A. Clarke; K. A. Barnes; J. R. Startin; F. I. Ibe; M. J. Shepherd, *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1781–1785.
145. B. Finckh; A. Kontush; J. Commentz; C. Huebner; M. Burdelski; A. Kohlschuetter, *Anal. Biochem.* **1995**, *232*, 210–216.
146. B. Finckh; A. Kontush; J. Commentz; C. Hubner; M. Burdelski; A. Kohlschutter, *Methods Enzymol.* **1999**, *299*, 341–348.
147. F. Ladislav; P. Vera; S. Karel; V. Karel, *Curr. Anal. Chem.* **2005**, *1*, 93–102.
148. W. A. Oleszek, *J. Chromatogr. A* **2002**, *967*, 147–162.
149. W. Oleszek; Z. Bialy, *J. Chromatogr. A* **2006**, *1112*, 78–91.
150. C.-Z. Wang; E. McEntee; S. Wicks; J.-A. Wu; C.-S. Yuan, *J. Nat. Med.* **2006**, *60*, 97–106.
151. J. H. Gil; J. H. Jung; K.-J. Kim; M.-S. Kim; J. Hong, *Anal. Sci.* **2006**, *22*, 641–644.
152. D. C. Van Setten; G. J. Ten Hove; E. J. H. J. Wiertz; J. P. Kamerling; G. Van de Werken, *Anal. Chem.* **1998**, *70*, 4401–4409.
153. T. W. D. Chan; P. P. H. But; S. W. Cheng; I. M. Y. Kwok; F. W. Lau; H. X. Xu, *Anal. Chem.* **2000**, *72*, 1281–1287.
154. N. T. Nyberg; H. Baumann; L. Kenne, *Anal. Chem.* **2003**, *75*, 268–274.
155. R. Kasai; H. Yamaguchi; O. Tanaka, *J. Chromatogr.* **1987**, *407*, 205–210.
156. Y. Ikeda; M. Sugiura; C. Fukaya; K. Yokoyama; Y. Hashimoto; K. Kawanishi; M. Moriyasu, *Chem. Pharm. Bull. (Tokyo)* **1991**, *39*, 566–571.
157. H. Yamaguchi; H. Matsuura; R. Kasai; K. Mizutani; H. Fujino; K. Ohtani; T. Fuwa; O. Tanaka, *Chem. Pharm. Bull. (Tokyo)* **1986**, *34*, 2859–2867.
158. H. Frokiaer; S. A. J. Larsen; A. D. Sorensen; H. Sorensen; J. C. Sorensen; S. Sorensen, *Spec. Publ. – R. Soc. Chem.* **2001**, *269*, 74–76.
159. J. A. Beutler, *J. Liq. Chromatogr. Relat. Technol.* **1997**, *20*, 2415–2426.

160. K. Decroos; J. P. Vincken; L. Heng; R. Bakker; H. Gruppen; W. Verstraete, *J. Chromatogr. A* **2005**, *1072*, 185–193.
161. M.-R. Lee; C.-M. Chen; B.-H. Hwang; L.-M. Hsu, *J. Mass Spectrom.* **1999**, *34*, 804–812.
162. J. Lin; C. Wang, *J. Food Sci.* **2004**, *69*, C456–C462.
163. M. Jin; Y. Yang; B. Su; Q. Ren, *J. Chromatogr. A* **2006**, *1108*, 31–37.
164. M. A. Berhow; S. B. Kong; K. E. Vermillion; S. M. Duval, *J. Agric. Food Chem.* **2006**, *54*, 2035–2044.
165. A.-J. Lau; B.-H. Seo; S.-O. Woo; H.-L. Koh, *J. Chromatogr. A* **2004**, *1057*, 141–149.
166. D. Y. Q. Hong; A. J. Lau; C. L. Yeo; X. K. Liu; C. R. Yang; H. L. Koh; Y. Hong, *J. Agric. Food Chem.* **2005**, *53*, 8460–8467.
167. J.-B. Wan; P. Li; S. Li; Y. Wang; T. T.-X. Dong; K. W.-K. Tsim, *J. Sep. Sci.* **2006**, *29*, 2190–2196.
168. L. Li; T. Rong; J. Dou; F. Song; Z. Liu; S. Liu, *Anal. Chim. Acta* **2005**, *536*, 21–28.
169. Y. W. Ha; Y.-C. Na; J.-J. Seo; S.-N. Kim; R. J. Linhardt; Y. S. Kim, *J. Chromatogr. A* **2006**, *1135*, 27–35.
170. L. Yi; L.-W. Qi; P. Li; Y.-H. Ma; Y.-J. Luo; H.-Y. Li, *Anal. Bioanal. Chem.* **2007**, *389*, 571–580.
171. M. Liang; Z. Zheng; Y. Yuan; L. Kong; Y. Shen; R. Liu; C. Zhang; W. Zhang, *Phytochem. Anal.* **2007**, *18*, 428–435.
172. F. Sun; Q. He; P. Shi; P. Xiao; Y. Cheng, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3743–3750.
173. M. Ganzera; E. Bedir; I. A. Khan, *J. Pharm. Sci.* **2001**, *90*, 1752–1758.
174. J. Chen; Y. Song; P. Li, *J. Chromatogr. A* **2007**, *1157*, 217–226.
175. A. Maria; J.-P. Girault; Z. Saatov; J. Harmatha; L. Dinan; R. Lafont, *J. Chromatogr. Sci.* **2005**, *43*, 149–157.
176. M.-P. Marco; F. J. Sanchez-Baeza; F. Camps; J. Coll, *J. Chromatogr.* **1993**, *641*, 81–87.
177. M. DellaGreca; B. D'Abrosca; A. Fiorentino; L. Previtera; A. Zarrelli, *Chem. Biodiversity* **2005**, *2*, 457–462.
178. V. Rybin; E. Boltenkov; E. Novozhilova, *Nat. Prod. Commun.* **2007**, *2*, 1101–1104.
179. D. Louden; A. Handley; S. Taylor; E. Lenz; S. Miller; I. D. Wilson; A. Sage; R. Lafont, *J. Chromatogr. A* **2001**, *910*, 237–246.
180. Y. Meng; P. Whiting; L. Zibareva; G. Bertho; J.-P. Girault; R. Lafont; L. Dinan, *J. Chromatogr. A* **2001**, *935*, 309–319.
181. N. Kaouadji; R. Lafont, *J. Chromatogr.* **1990**, *505*, 408–412.
182. R. Ho; J.-P. Girault; P.-Y. Cousteau; J.-P. Bianchini; P. Raharivelomanana; R. Lafont, *J. Chromatogr. Sci.* **2008**, *46*, 102–110.
183. R. E. Isaac; N. P. Milner; H. H. Rees, *J. Chromatogr.* **1982**, *246*, 317–322.
184. T. M. Landon; B. A. Sage; B. J. Seeler; J. D. O'Connor, *J. Biol. Chem.* **1988**, *263*, 4693–4697.
185. V. D. Gildengorn, *J. Chromatogr. A* **1996**, *730*, 147–152.
186. D. Louden; A. Handley; R. Lafont; S. Taylor; I. Sinclair; E. Lenz; T. Orton; I. D. Wilson, *Anal. Chem.* **2002**, *74*, 288–294.
187. F. Bourgaud; A. Hehn; R. Larbat; S. Doerper; E. Gontier; S. Kellner; U. Matern, *Phytochem. Rev.* **2006**, *5*, 293–308.
188. K. Glowniak; M. L. Bieganowska, *J. Liq. Chromatogr.* **1985**, *8*, 2927–2947.
189. N. Nykolov; T. Iossifova; E. Vassileva; I. Kostova; G. Stoev, *Phytochem. Anal.* **1993**, *4*, 86–88.
190. T.-T. Wang; H. Jin; Q. Li; W.-M. Cheng; Q.-Q. Hu; X.-H. Chen; K.-S. Bi, *Chromatographia* **2007**, *65*, 477–481.
191. M.-J. Ahn; M. K. Lee; Y. C. Kim; S. H. Sung, *J. Pharm. Biomed. Anal.* **2008**, *46*, 258–266.
192. C. Sproll; W. Ruge; C. Andlauer; R. Godelmann; D. W. Lachenmeier, *Food Chem.* **2008**, *109*, 462–469.
193. H. J. Thompson; S. A. Brown, *J. Chromatogr.* **1984**, *314*, 323–336.
194. M. L. Bieganowska; K. Glowniak, *Chromatographia* **1988**, *25*, 111–116.
195. P. Harmala; H. Vuorela; E. L. Rahko; R. Hiltunen, *J. Chromatogr.* **1992**, *593*, 329–337.
196. P. Vuorela; E.-L. Rahko; R. Hiltunen; H. Vuorela, *J. Chromatogr. A* **1994**, *670*, 191–198.
197. P. Dugo; L. Mondello; E. Sebastiani; R. Ottana; G. Errante; G. Dugo, *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 2991–3005.
198. A. Tosun; M. Baba; T. Okuyama, *J. Nat. Med.* **2007**, *61*, 402–405.
199. K. Hunter; E. A. Sharp; A. Newton, *J. Chromatogr.* **1988**, *435*, 83–95.
200. K. Robards, *J. Chromatogr. A* **2003**, *1000*, 657–691.
201. D. J. Daigle; E. J. Conkerton, *J. Chromatogr.* **1982**, *240*, 202–205.
202. D. J. Daigle; E. J. Conkerton, *J. Liq. Chromatogr.* **1983**, *6*, 105–118.
203. D. J. Daigle; E. J. Conkerton, *J. Liq. Chromatogr.* **1988**, *11*, 309–325.
204. H. M. Merken; G. R. Beecher, *J. Agric. Food Chem.* **2000**, *48*, 577–599.
205. K. Robards; M. Antolovich, *Analyst* **1997**, *122*, 11R–34R.
206. E. Conde; E. Cadahia; M. C. Garcia-Vallejo, *Chromatographia* **1995**, *41*, 657–660.
207. J. L. Wolfender; S. Rodriguez; K. Hostettmann, *J. Chromatogr. A* **1998**, *794*, 299–316.
208. P. C. H. Hollman; J. M. P. Van Trijp; M. N. C. P. Buysman, *Anal. Chem.* **1996**, *68*, 3511–3515.
209. S. H. Hansen; A. G. Jensen; C. Cornett; I. Bjornsdottir; S. Taylor; B. Wright; I. D. Wilson, *Anal. Chem.* **1999**, *71*, 5235–5241.
210. J. Zhang; M. B. Satterfield; J. S. Brodbelt; S. J. Britz; B. Clevidence; J. A. Novotny, *Anal. Chem.* **2003**, *75*, 6401–6407.
211. B. D. Davis; J. S. Brodbelt, *Anal. Chem.* **2005**, *77*, 1883–1890.
212. P. Waridel; J. L. Wolfender; K. Ndjoko; K. R. Hobby; H. J. Major; K. Hostettmann, *J. Chromatogr. A* **2001**, *926*, 29–41.
213. G. Zgorka; A. Hajnos, *Chromatographia* **2003**, *57*, S/77–S/80.
214. M. V. Martinez-Ortega; M. C. Garcia-Parrilla; A. M. Troncoso, *Anal. Chim. Acta* **2004**, *502*, 49–55.
215. C. Cavaliere; F. Cucci; P. Foglia; C. Guarino; R. Samperi; A. Lagana, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2177–2187.
216. P. Kozminski; A. M. O. Brett, *Anal. Lett.* **2006**, *39*, 2687–2697.
217. M. C. Pietrogrande; C. Bighi; G. Blo; Y. D. Kahie; P. Reschiglian; F. Dondi, *Chromatographia* **1989**, *27*, 625–627.
218. M. V. Piretti; P. Doghieri, *J. Chromatogr.* **1990**, *514*, 334–342.
219. F. Buiarelli; G. P. Cartoni; F. Coccioli; E. Ravazzi, *Chromatographia* **1991**, *31*, 489–492.
220. J. C. Herrera; A. J. R. Romero; O. E. Crescente; M. Acosta; S. Pekerar, *J. Chromatogr. A* **1996**, *740*, 201–206.
221. R. L. Prior; S. A. Lazarus; G. Cao; H. Muccitelli; J. F. Hammerstone, *J. Agric. Food Chem.* **2001**, *49*, 1270–1276.
222. M. Lopez-Serrano; Ros A. Barcelo, *J. Chromatogr. A* **2001**, *919*, 267–273.
223. A. Yanagida; T. Shoji; Y. Shibusawa, *J. Biochem. Biophys. Methods* **2003**, *56*, 311–322.
224. K. Hashizume; S. Kida; T. Samuta, *J. Agric. Food Chem.* **1998**, *46*, 4382–4386.
225. J. He; C. Santos-Buelga; N. Mateus; V. de Freitas, *J. Chromatogr. A* **2006**, *1134*, 215–225.
226. D. Bongartz; A. Hesse, *J. Chromatogr. B. Biomed. Appl.* **1995**, *673*, 223–230.
227. J. Xie; L. Zhu; H. Luo; L. Zhou; C. Li; X. Xu, *J. Chromatogr. A* **2001**, *934*, 1–11.
228. R. Weiss; A. Molinelli; M. Jakusch; B. Mizaikoff, *Bioseparation* **2002**, *10*, 379–387.

229. G. Theodoridis; M. Lasakova; V. Skerikova; A. Tegou; N. Giantsiou; P. Jandera, *J. Sep. Sci.* **2006**, *29*, 2310–2321.
230. T. J. Mabry; K. R. Markham; M. B. Thomas, *The Systematic Identification of Flavonoids*; 1970.
231. A. P. Neilson; R. J. Green; K. V. Wood; M. G. Ferruzzi, *J. Chromatogr. A* **2006**, *1132*, 132–140.
232. R. Verpoorte; A. Baerheim Svendsen, *Journal of Chromatography Library, Vol. 23: Chromatography of Alkaloids, Part B: GasLiquid Chromatography and High-Performance Liquid Chromatography*; Elsevier: Amsterdam, The Netherlands, 1984; p 450.
233. J. Stockigt; Y. Sheludko; M. Unger; I. Gerasimenko; H. Warzecha; D. Stockigt, *J. Chromatogr. A* **2002**, *967*, 85–113.
234. P. J. Houghton, *J. Chromatogr. A* **2002**, *967*, 75–84.
235. P. Kulanthaivel; S. W. Pelletier, *J. Chromatogr.* **1987**, *402*, 366–370.
236. G. Bringmann; K. Messer; M. Wohlfarth; J. Kraus; K. Dumbuya; M. Rueckert, *Anal. Chem.* **1999**, *71*, 2678–2686.
237. X. Zhu; B. Chen; M. Ma; X. Luo; F. Zhang; S. Yao; Z. Wan; D. Yang; H. Hang, *J. Pharm. Biomed. Anal.* **2004**, *34*, 695–704.
238. H. Dong; Z. Liu; F. Song; Z. Yu; H. Li; S. Liu, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3193–3199.
239. I. Gerasimenko; Y. Sheludko; M. Unger; J. Stockigt, *Phytochem. Anal.* **2001**, *12*, 96–103.
240. W. Wu; F. Song; C. Yan; Z. Liu; S. Liu, *J. Pharm. Biomed. Anal.* **2005**, *37*, 437–446.
241. M. H. Stutz; S. Sass, *Anal. Chem.* **1973**, *45*, 2134–2136.
242. R. Verpoorte; T. Mulder-Krieger; M. J. Verzijl; J. M. Verzijl; A. Baerheim Svendsen, *J. Chromatogr.* **1983**, *261*, 172–175.
243. G. D. Manners; J. A. Pfister, *Phytochem. Anal.* **1993**, *4*, 14–18.
244. M. Waksmundzka-Hajnos; A. Petruczynik, *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 2247–2267.
245. J. M. Huen; J. P. Thevenin, *HRC & CC J. High Resolut. Chromatogr. Chromatogr. Commun.* **1979**, *2*, 154.
246. L. W. Doner; A. F. Hsu, *J. Chromatogr.* **1982**, *253*, 120–123.
247. R. M. Riggin; P. T. Kissinger, *Anal. Chem.* **1977**, *49*, 530–533.
248. Q.-W. Yu; B. Lin; Y.-Q. Feng; F.-P. Zou, *J. Liq. Chromatogr. Relat. Technol.* **2008**, *31*, 64–78.
249. I. Fitos; J. Visy; M. Simonyi; J. Hermansson, *J. Chromatogr.* **1992**, *609*, 163–171.
250. M. Nakamura; M. Ono; T. Nakajima; Y. Ito; T. Aketo; J. Haginaka, *J. Pharm. Biomed. Anal.* **2005**, *37*, 231–237.

Biographical Sketches

Reinhard I. Boysen holds a Ph.D. in natural sciences (Dr. rer. nat.) from the Faculty of Chemistry at the Freie Universität Berlin, Germany, where he later specialized in analytical biochemistry. Thereafter, he joined the Centre for Bioprocess Technology in the Department of Biochemistry and Molecular Biology of Monash University and worked in the fields of peptide synthesis, separation science, and thermodynamics of peptide/protein-immobilized–ligand interactions. He is currently a research fellow at the ARC Special Research Centre for Green Chemistry at Monash University working in the fields of separation science of chemical and biological molecules (multidimensional capillary-based separation methodologies including separations with molecularly imprinted polymers), mass spectrometry-based medical diagnostics, nanochemistry/nanotechnology (chemical nanoarrays), and in the investigation of interactions between proteins/DNA/cells and nonbiological surfaces.

Milton T. W. Hearn is currently professor of chemistry and director, ARC Special Research Centre for Green Chemistry, Monash University, Australia. He received his B.Sc. (Hons. First Class), Ph.D., and D.Sc. degrees from the University of Adelaide, Australia. Prior to joining Monash University, he held an NRC postdoctoral fellowship at the Department of Chemistry, University of British Columbia; ICI fellow at the Dyson Perrins Laboratory and research fellowships at Christchurch and Wolfson Colleges at Oxford University; MRCNZ senior research fellow at the University of Otago, and NHMRC Principal Research Fellow and McGauran Fellow at the St. Vincent's Institute of Medical Research, University of Melbourne. From 1986 to 2002 he was professor of biochemistry and director, Centre for Bioprocess Technology, Monash University. He has held distinguished professorships at Yale University, University of Paris, Johannes Gutenberg University, and Himeji Institute of Technology. His research interests focus on (1) the analysis, purification, and characterization of chemical and biochemical molecules from the nanoscale through to the process scale, including advanced technologies of importance to the chemical, pharmaceutical, and biotechnological industries, of which many of these developments have been successfully commercialized and (2) the structure/function of bioactive compounds, bioinspired synthesis, molecular imprinting, surface and combinatorial chemistry, and aspects of nanobiotechnology. Professor Hearn has authored 505 scientific publications, several books, and issued patents related to developments in chemistry/biochemistry, (bio)nanotechnology, protein purification, affinity chromatography, other 'downstream' aspects of biotechnology, as well as the development of several lead (bio)pharmaceutical compounds.