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Review

Role of advances in chromatographic techniques in phytochemistry

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Abstract

Chromatography is the lynchpin of phytochemistry and is the key to obtaining pure compounds for structure elucidation, for pharmacological testing or for development into therapeuticals. It also plays a fundamental role as an analytical technique for quality control and standardisation of phytotherapeuticals. Although liquid chromatography is barely 100 years old, an extraordinary variety of instrumental and ancillary equipment is available, notably in the domain of high-performance liquid chromatography. It is impossible to touch all areas of chromatography in such a review but certain areas are worthy of mention: HPLC, HPTLC, UPLC and countercurrent chromatography.

Another important addition has been the development of hyphenated techniques involving HPLC: LC/UV, LC/MS, LC/MS^n and LC/NMR. These are indispensable nowadays for the early detection and identification of compounds in crude plant extracts.

Keywords: Chromatography; Liquid chromatography; Countercurrent chromatography; Thin-layer chromatography; High-performance liquid chromatography

Contents

1. Introduction

Over the past 100 years, chromatography has developed from a rudimentary tool for the separation of pigments into an array of techniques capable of dealing with the

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Table 1
The major chromatographic methods used in phytochemistry

Chromatographic method	Separation mechanism
Liquid–solid chromatography	Adsorption
Paper	(Adsorption), partition
Gas liquid chromatography (GLC)	Adsorption, partition
Thin-layer chromatography (TLC)	Adsorption, partition
High-performance liquid chromatography (HPLC)	Adsorption, partition
Ultra-performance liquid chromatography (UPLC)	Adsorption, partition
Supercritical fluid chromatography (SFC)	Adsorption, partition
Liquid–liquid chromatography (LLC)	Partition
Countercurrent chromatography (CCC)	Partition
Ion-exchange chromatography (IEC)	Ion exchange
Capillary electrophoresis	Charge
Ion-pair chromatography	Ion pair formation, ion interaction
Hydrophobic interaction chromatography (HIC)	(Adsorption), partition
Size exclusion chromatography (SEC)	Size of analyte
Affinity chromatography	Biological affinity

most complex analytical and purification problems in phytochemistry. The developments can basically be divided into three important landmarks: the first introduction of chromatography; the contribution played by Martin in the 1950s; and the introduction of commercially-available high-performance liquid chromatography (HPLC) equipment in the 1970s.

A list of the most frequently employed chromatographic methods is given in Table 1.

In a review of this type, it is impossible to include all aspects of chromatography and attention will be concentrated on certain key areas, such as HPLC.

2. Historical aspects

Tswett introduced column adsorption chromatography at the beginning of the twentieth century, initially for the separation of plant pigments. The first printed description of the method came with a lecture in 1903 at the Warsaw Society of Natural Sciences entitled "On a new category of adsorption phenomena and their application to biochemical analysis" (Engelhardt, 2004). This was the starting point of liquid chromatography and in a paper published in 1906, Tswett took the subject further with the notion of the "chromatogram" and its development by using different eluents (Tswett, 1906). By the end of the 1930s, column adsorption chromatography (nowadays known as normal phase chromatography) had become a widely used separation technique for plant extracts and natural products.

Although the method provided access to numerous compounds, resolution was low and difficulties were experienced with water-soluble samples. It was not until the work of Martin and co-workers that further significant advances were made in the chromatography of natural products, with the invention of partition chromatography (liquid–liquid chromatography, LLC) (Martin and Synge, 1941). Martin described partition chromatography, in which a liquid stationary phase is immobilized on a solid support, as a marriage between Tswett's adsorption-based chromatography and countercurrent solvent extraction. In the 1941 paper, gas–liquid chromatography (GLC) was also anticipated: "the mobile phase need not to be a liquid but may be a vapour. Very refined separations of volatile substances should therefore be possible in a column in which a permanent gas is made to flow over a gel impregnated with a non-volatile solvent".

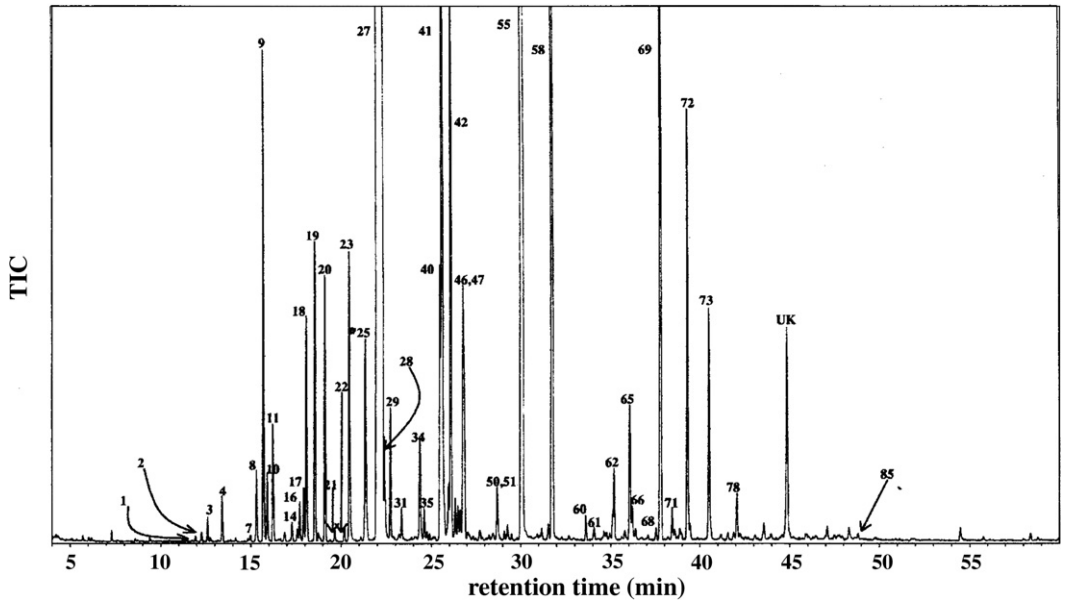


Fig. 1. GC–MS of lavender essential oil (reproduced from Shellie et al., 2002).

The development of partition chromatography earned Martin and Synge the 1952 Nobel Prize in chemistry.

Martin and co-workers also introduced paper chromatography using filter paper sheets impregnated with water or other liquids; this became the first chromatographic microanalytical technique (Consden et al., 1944). However, the slow migration rates of paper chromatography have resulted in this technique being overtaken by thin-layer chromatography (TLC), in which the application of thin layers of adsorbent on glass plates originated in Russia (Izmailov and Shraiber, 1938).

GLC was finally introduced in 1952 (Martin and James, 1952) and became widely available in the 1960s. It is suitable for small, volatile compounds. Sensitive universal detection was provided by flame ionization (FID). GLC (or GC) is ideal for the analysis of complex mixtures such as those found in essential oils. In one run, it is possible to separate hundreds of constituents and identify them by comparison with a data base. Quantitation is performed using the FID or by GC–MS, although many operators prefer FID data to total ion current (TIC) response data, since MS response factors for different analytes often vary. An example is given in Fig. 1, in which a lavender essential oil is analysed by GC–MS and 85 components were identified.

3. High-performance thin-layer chromatography (HPTLC)

Thin layer chromatography (TLC) is certainly not to be forgotten. It is the only chromatographic method offering the option of presenting the result as an image. Furthermore, TLC is the sole technique in which all the components of the sample are included in the chromatogram. In contrast, HPLC and GC are selective and not all of the compounds in the sample are included in the display.

The real breakthrough in TLC came through the work of Stahl, who introduced the use of calcium sulphate as binder, and standardized layer thickness and chromatographic development (Stahl, 1958).

Not only does the technique give visual results but it excels in its simplicity and is low in cost. Parallel analysis of samples is possible, sample capacity is high and results are obtained rapidly. TLC is flexible and multiple detection is possible. It is an ideal screening method in biological and chemical analysis, providing identification and qualitative results, determination of adulteration, together with quantitative and semi-quantitative determination. In conjunction with microorganisms and other biological agents, TLC bioautography can be used to screen for bioactivities (Hostettmann et al., 1997). The disadvantages of TLC are a lack of automation, the problems of reproducibility which sometimes occur and the lack of accuracy in quantitation. Nevertheless, TLC will remain a fast and simple micro technique of chromatography.

In HPTLC, the plates are precoated with stationary phase with a typical mean particle size of 5 μm. The plates give better separations and reproducibility than normal precoated TLC plates (mean particle size 12 μm) and they also allow more sensitive detection. Shorter developing distances are required. The number of theoretical plates is in the 5000 range (Reich and Schibli, 2007), while for HPLC the range is 6–10,000. The separation power of HPTLC is still lower than that of HPLC and the latter is preferred for quantitative determination. Merck also offers HPTLC plates with spherical particles, which gives faster chromatography and better separation power. A water-resistant layer is available from Merck and for RP-18 W supports, 100% water can be used with these plates.

For herbal extracts, regulatory agencies often recommend fingerprint chromatography for proper identification purposes. HPTLC is ideal in this instance and excellent examples can be found in the literature (Wagner and Bladt, 1995; Reich and Schibli, 2007). HPTLC is also ideally suited for the preliminary screening of plant extracts before HPLC analysis.

4. High-performance liquid chromatography (HPLC)

The most remarkable advances in chromatography have occurred in the domain of HPLC, despite the fact that the technique itself has only been in existence for about 40 years. The year 1967 was a landmark in the introduction of HPLC, with papers from Horvath, Huber and Scott, but the first automatic liquid chromatograph with gradient elution was an amino acid analyser described by Moore and co-workers in 1958 (Spackman et al., 1958).

Until the advent of HPLC, most phytochemical separations were performed by open-column, paper or thin-layer chromatography. Open-column chromatography was time-consuming and tedious, often requiring a large amount of sample. With paper chromatography and TLC, very small samples could be analysed and the resolution and reproducibility improved. However, quantitation was still inadequate and resolution of similar compounds difficult. Gas chromatography provided excellent resolution but the restriction to volatile samples (less than 20% of organic compounds can be separated by gas chromatography) meant that derivatization was often necessary. A technique was needed which could separate water-soluble, thermally-labile, non-volatile compounds with speed, precision and high resolution. HPLC fulfilled these criteria and is now one of the most powerful tools in analytical chemistry, with the ability to separate, identify and quantitate the compounds present in any sample that can be dissolved in a liquid. The viscosity of liquids is higher than that of gases by a factor of 100 – hence the need for pressure in the columns and the original name ''high-pressure liquid chromatography''. But ''pressure'' was replaced by ''performance'' as particles got smaller and columns became shorter.

The wide variety of stationary and mobile phases should give a large potential for finding suitable separation conditions. However, at first, only relatively large particles were available. The introduction of small porous silica particles

with a diameter of approximately 10 μm radically changed the situation, as did the production of chemically-bonded phases, notably the reversed-phase (RP) octadecyl (RP-18) and octyl (RP-8) materials. Standardization of silica surfaces by defined hydroxylation, application of very pure silica, improvement in bonding and end-capping procedures now gives very versatile reversed-phase systems, with high selectivity, high separation power, robustness, high stability and efficiency. Reviews on equipment and instrumentation are published periodically in journals such as Analytical Chemistry – for example, by LaCourse (2002).

High-performance liquid chromatography has become by far the most widely used chromatographic technique. In fact, the liquid chromatography, mass spectrometry and thermal analysis segments account for USD 4.4 billion of the global USD 20 billion analytical instrumentation market.

4.1. Analytical HPLC

HPLC has been the biggest revolution in analytical chemistry over the past 40 years (Brown, 1990). The implications are enormous, with applications of HPLC being found in hundreds of areas, not least of which is phytochemistry. The following examples give an idea of its potential.

For chemotaxonomic purposes, the botanical relationships between different species can be shown by chromatographic comparison of their chemical composition. Chromatograms, which are used as fingerprints, are compared with authentic samples and known substances to permit identification of drugs and/or search for adulteration. HPLC is thus the best suited technique for an efficient separation of the crude extracts, as shown by Sakakibara et al. (2003) who claim to have found a method capable of quantifying every polyphenol in vegetables, fruits and teas. For this purpose they used a Capcell pak C18 UG120 (250 × 4.6 mm, S-5, 5 μm) column at 35 °C. Gradient elution at a flow-rate of 1 ml/min was performed over 95 min with solution A (50 mM sodium phosphate (pH 3.3) and 10% methanol) and solution B (70% methanol), as follows: initially 100% of solution A; for the next 15 min, 70% A; for 30 min, 65% A; for 20 min, 60% A; for 5 min, 50% A; and finally 100% B for 25 min. Vegetable material was extracted with 90% methanol containing 0.5% acetic acid. A typical HPLC profile for 28 reference polyphenols is shown in Fig. 2. The method allowed the determination of aglycones separately from glycosides. Information could also be obtained about simple polyphenols in the presence of more complex polycyclic polyphenols. Quantitative determination was achieved for a total of 63 different food samples.

High performance liquid chromatography (HPLC) is used routinely in phytochemistry to "pilot" the preparative isolation of natural products (optimization of the experimental conditions, checking of the different fractions throughout the separation) and to control the final purity of the isolated compounds.

4.2. Hyphenated HPLC techniques

Chemical screening can involve simple TLC analysis but a much more selective and predictive method is HPLC coupled to different detectors (hyphenated techniques). This provides efficient separation of metabolites and valuable

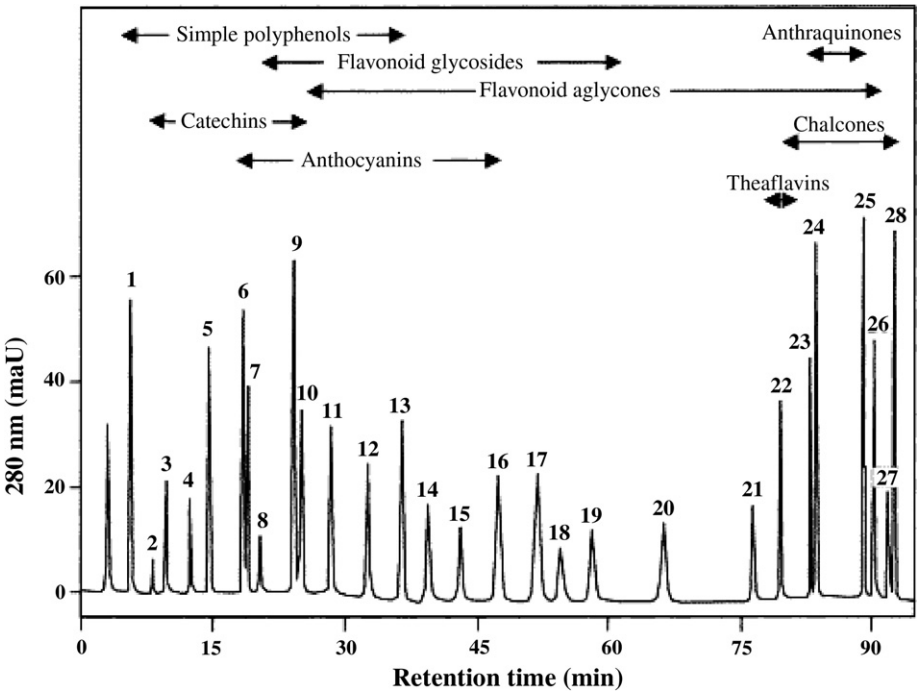


Fig. 2. HPLC profile for 28 different polyphenols on a C_{18} column. Classes of compound are shown in the upper part of the chromatogram. For separation conditions, see text (reproduced from Sakakibara et al., 2003).

structure information at the same time, using only several micrograms of sample. Coupling HPLC with mass spectrometry (LC/MS) and UV diode array (LC/UV) detection has now been complemented by the connection of HPLC to nuclear magnetic resonance (LC/NMR). The latter allows the complete on-line structure determination of numerous plant metabolites (Wolfender et al., 2001).

When compared to the classical use of UV, MS and NMR spectroscopy applied to pure natural products, ideally the integration of all these techniques in their hyphenated forms (LC/UV LC/MS and LC/NMR) in a single setup, with centralised acquisition of the spectroscopic data should permit the complete spectroscopic characterisation of different metabolites in a mixture during a single analysis. Furthermore, other existing hyphenated techniques such as LC/IR (Visser et al., 1997) or LC/CD (Bringmann et al., 1999) may also bring valuable complementary information. In practice, however, many factors may hinder on-line detection and structure determination of an unknown plant metabolite and often only partial structure information will be obtained. These on-line data however already provide very precious information for targeting the isolation of new compounds or for the dereplication of known constituents.

HPLC coupled with UV photodiode array detection (LC/UV), introduced by George and Maute (1982), allows the running of a chromatographic separation with simultaneous detection at different wavelengths. It has been used for more than two decades by phytochemists in the screening of the plant extracts and is now widely employed in many laboratories. The UV spectra of natural products give useful information on the type of constituents and also, as is the case for polyphenols, information on the oxidation pattern. New instruments allow the recording of UV spectra of matching compounds, which can be performed automatically when screening for known constituents.

At present, LC/MS is one of the most sensitive analytical methods and with the high power of mass separation of a MS detector, very good selectivities can be obtained. Moreover, this technique has the potential to yield information about the molecular weight as well as the structure of the analytes, using the MS/MS or MS^n capabilities of the analyser available. Because of the basic incompatibilities between HPLC and mass spectrometry, online linking of these instrumental techniques has been difficult to achieve; to cope with these problems, different LC/MS interfaces have been conceived. Each of these interfaces has its own characteristics and range of applications and several of them are suitable in phytochemistry. For the analysis of natural products, the most commonly used LC/MS interfaces are atmospheric pressure chemical ionization (APCI) and electrospray (ESI). Each of these interfaces has its own characteristics and range of application but their combined use permits the analysis of small non-polar natural products to very large polar molecules.

Direct on-line coupling of a NMR spectrometer as a detector for chromatographic separation has required the development of special interfaces called flow probes, as well

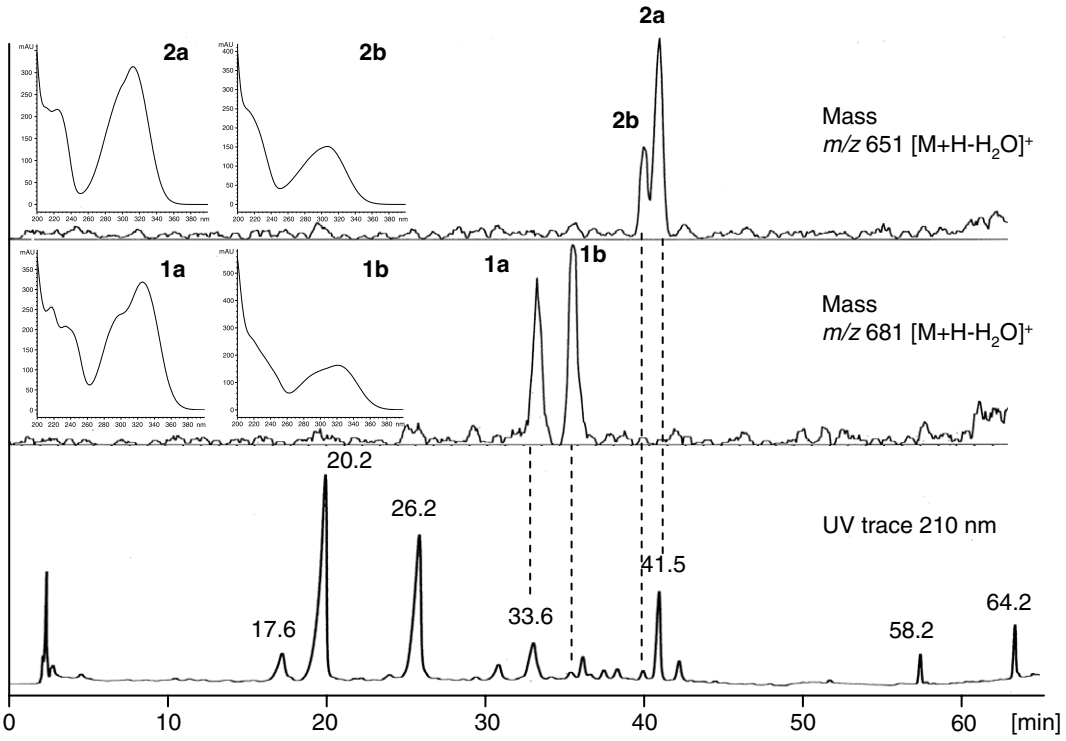


Fig. 3. LC/UV/ESI-MS of the methanol extract of *Jamesbrittenia fodina* (Scrophulariaceae). HPLC conditions: Inj. 50 µg; Col. Symmetry C-18 (250 × 3.9 mm i.d., 5 µm); MeOH–H_2O gradient (28:72–46:54 in 30 min, 46:54–62:38 in 16 min, 62:38–100:0 in 14 min); 1 ml/min. UV spectra were recorded between 200 and 400 nm.

as dedicated pieces of NMR hardware and software. Modern LC/NMR systems consist of a high resolution NMR instrument (400–800 MHz) with a dedicated HPLC system equipped with a valve or a loop collector for stop-flow experiments and a LC/NMR flow probe. One of the drawbacks of LC/NMR is its relatively low sensitivity but the technique has many valuable applications. One of these is the on-line characterisation of unstable cinnamic ester derivatives in the African plant *Jamesbrittenia fodina* (Scrophulariaceae). A preliminary metabolite profiling study performed with LC/UV-DAD and LC/ESI-MS on the methanolic extract revealed the presence of the common cinnamic ester derivative verbascoside as well as other unknown compounds of the same type (Cogne et al., 2003). Based on these first chemical screening results, attention was focused on two pairs of isomeric compounds **1a/1b** and **2a/2b** displaying characteristic UV spectra for cinnamic esters and having protonated molecular ions at *m/z* 699 $(M+H)^+$ and *m/z* 669 $(M+H)^+$, respectively together with intense dehydrated ions at *m/z* 681 and *m/z* 651 (Fig. 3).

They could not be identified directly based on the LC/UV/MS analysis and their isolation was undertaken. The purification steps led to two enriched fractions, each containing one pair of isomeric peaks. These isomers were well separated by semi-preparative HPLC, but it was noticed that the resulting fractions still contained the two isomeric peaks in the same proportions. These results suggested that the constituents were probably unstable upon drying of the fraction.

In order to obtain more information about these unstable compounds, stop-flow LC/NMR analyses of fractions **1a/1b** and **2a/2b** were performed. The LC/^{1}H NMR spectra of compounds **2a** and **2b** showed many similarities (Fig. 4).

Both isomers displayed two multiplets between δ 2.20 and δ 2.60, and a number of signals from δ 3.20 to δ 5.20, suggesting the presence of two sugars. Moreover, the presence of a methyl group at about δ 1.1 indicated that one of the sugars might be rhamnose. Integration of the signal at δ 3.82 for **2a** showed the presence of three protons and thus suggested the presence of a methoxyl group. Seven aromatic protons were observed between δ 5.8 and δ 7.8. Six of these were attributed to a cinnamic acid moiety, the presence of which had already been suggested by the DAD-UV spectra: two signals corresponding to four *ortho*-coupled protons at δ 7.01 (2H, *d*, $J = 8.8$ Hz) and δ 7.61 (2H, *d*, $J = 8.2$ Hz) in **2a** and δ 6.89 (2H, δ, $J = 8.8$ Hz) and δ 7.50 (2H, *d*, $J = 8.8$ Hz) in **2b** could be attributed to a 1,4-disubstituted aromatic ring. The most notable difference between the spectra of **2a** and **2b** concerned the signals attributed to the cinnamoyl double bond: δ 7.72 (1H, *d*, $J = 15.9$ Hz) and δ 6.42 (1H, *d*, $J = 15.9$ Hz) were characteristic of a *trans* double bond in the case of **2a**, whereas in **2b**, the two corresponding doublets at δ 7.04 (1H, *d*, $J = 12.6$ Hz) and δ 5.84 (1H, *d*, $J = 12.6$ Hz) were typical of a *cis* double bond (Fig. 4).

The fraction containing **1a** and **1b** was submitted to the same analysis and similar deductions could be made.

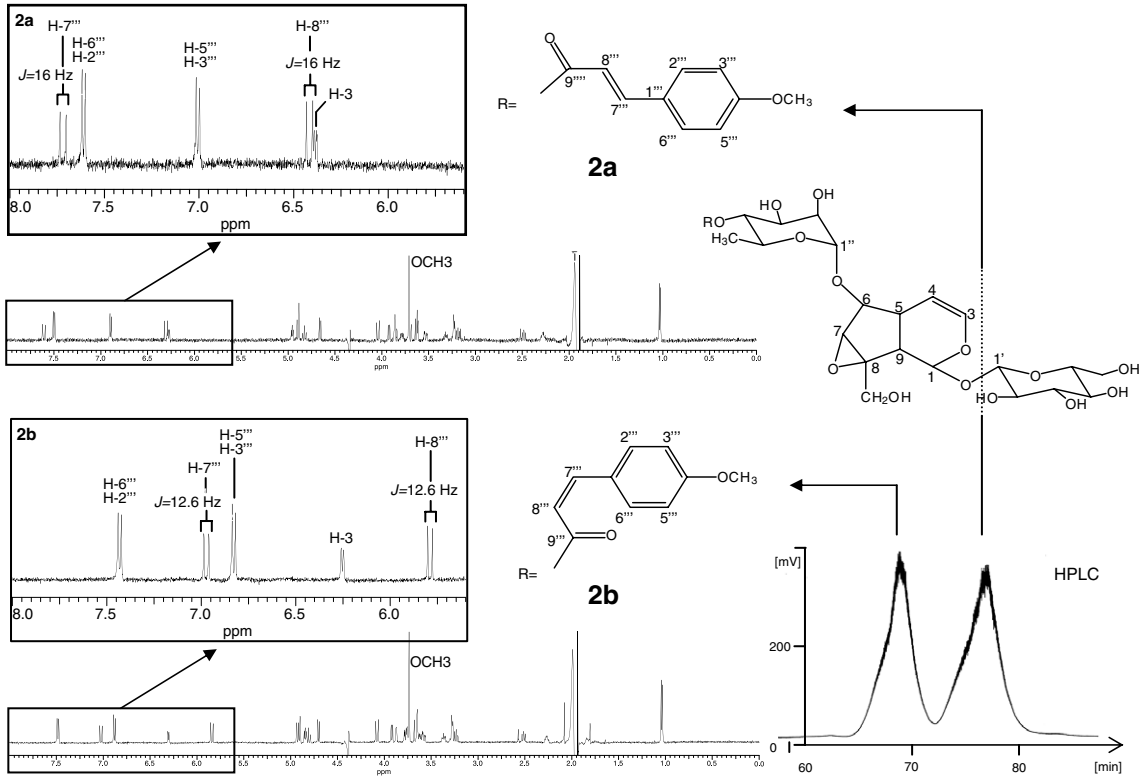


Fig. 4. Stop-flow LC/NMR spectra of the unstable catalpol derivatives **2a** and **2b** (in D_2O:MeCN). LC/NMR conditions: 256 scans, flow cell (60 μl, 3 mm i.d.), 500 MHz.

The difference between **1a/1b** and **2a/2b** was related to the presence of one additional methoxyl in the case of **1a/1b.**

The LC/NMR spectra thus suggested that the two fractions consisted of mixtures of *cis*- and *trans*-cinnamoyl glycoside derivatives. Based on the LC/^{1}H NMR spectra it was not possible, however, to deduce the complete structure of both pairs of constituents because information on the core nucleus of these molecules was lacking. The mixtures of compounds **1a/1b** and **2a/2b** were thus submitted to classical 1D and 2D NMR analysis recorded in CD_3OD. Interpretation of the 2D NMR in-mixture experiments (gCOSY, gHMBC and gHSQC) allowed assignment of the core nucleus of these molecules to catalpol and the HMBC correlation proved the attachment positions of the sugars and cinnamoyl moieties (Cogne et al., 2003).

Based on the on-line and in-mixture experiments, the structures of **2a** and **2b** were established to be *trans* and *cis* forms of 6-*O*-(4-*O*-(4-methoxycinnamoyl)-α-L-rhamnopyranoside)catalpol, and **1a** and **1b** were identified as *trans* and *cis* forms of 6-*O*-(4-*O*-(3,4-dimethoxycinnamoyl)-α-L-rhamnopyranoside)catalpol, respectively. The *trans* forms of these compounds have been described in other members of the Scrophulariaceae and Buddlejaceae families but there has been no mention of the *cis* forms.

4.3. Preparative HPLC

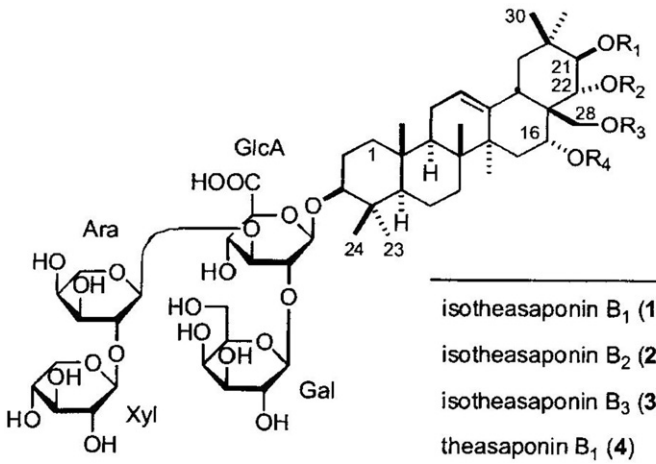
In preparative HPLC (pressure >20 bar), larger columns and packing materials are needed. The aim is to isolate or purify compounds, whereas in analytical work the goal is to get information about the sample. In analytical HPLC, the important parameters are resolution, sensitivity and fast analysis time; in preparative HPLC, the degree of solute purity is important, as is the amount of compound that can be produced per unit time (throughput), although this latter concerns mainly production-scale chromatography. The load is another important parameter.

For occasional separations, the target is to isolate a particular compound in a suitable amount, with a certain degree of purity and a certain recovery rate. In production-scale chromatography, throughput depends on parameters such as column dimensions and eluent flow-rate, and is often increased at the expense of purity. Medium-pressure liquid chromatography (MPLC) (5–20 bar), with wider diameter columns, can fulfil the demands of load and throughput. Their packing materials are less expensive than preparative HPLC columns, although the consumption of solvent is higher.

Preparative chromatography for the isolation of pure compounds can, of course, involve many different techniques (HPLC, MPLC, TLC, open-column chromatography, countercurrent chromatography and others). The art of obtaining pure compounds in as short time as possible lies in the design of a suitable separation strategy. Different approaches can be found in Hostettmann et al. (1998).

Preparative separations of natural products that were previously not even conceivable are now possible by HPLC. The resolution of complex mixtures can be attained, as in the case of saponins. Tea saponins, for example, are notoriously difficult to separate but Kobayashi et al. (2006) have purified 3 acyl triterpene tetrasaccharides from *Camellia sinensis* var. *sinensis* (Theaceae) leaves by a procedure involving initial passage of an ethanol–water extract over Sepabeads SP-70 and then over a RP-18 column with a methanol–water gradient. Final purification was by chromatography on different HPLC columns: Develosil ODS-HG-5 (20 × 250 mm, acetonitrile-0.05% aqueous TFA gradient); Develosil Ph-UG-5 (20 × 250 mm, methanol-0.05% TFA gradient). The structures of the resulting saponins are shown in Fig. 5. They include very closely-related isomers (e.g. **1** and **2**) which are normally very difficult to separate.

Another example of a difficult separation is given by a series of peptides from the fruiting bodies of a Malaysian fungus, *Pterula* sp. (Clavariaceae). After solvent partition of a methanol extract of the fungus, semi-preparative HPLC on a RP-18 column (300 × 19 mm, acetonitrile-0.05% TFA gradient) was performed, followed by



	R_1	R_2	R_3	R_4
isotheasaponin B_1 (**1**)	(*E*)-Cin	H	Ac	H
isotheasaponin B_2 (**2**)	Ac	(*E*)-Cin	H	H
isotheasaponin B_3 (**3**)	Ang	(*E*)-Cin	H	H
theasaponin B_1 (**4**)	(*E*)-Cin	Ac	H	Ac

Fig. 5. Structures of saponins isolated from leaves of the tea plant (*Camellia sinensis* var. *sinensis*) by semi-preparative HPLC (reproduced from Kobayashi et al., 2006).

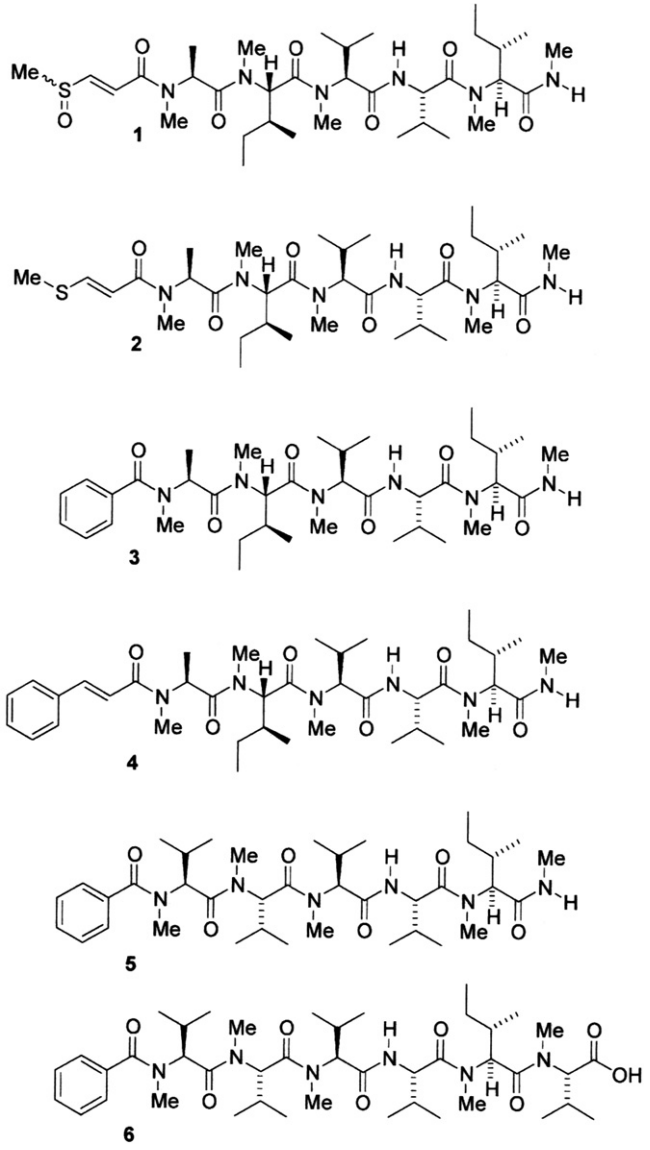


Fig. 6. Structures of peptides isolated by semi-preparative HPLC from fruiting bodies of a species of *Pterula* fungi (reproduced from Lang et al., 2006).

separation on a Phenomenex Luna C18 column (250 × 10 mm, 5 μm, acetonitrile gradient). Six peptides were obtained in all, some of them showing cytotoxic activity (Lang et al., 2006) (Fig. 6).

4.4. HPLC column size, column packings and UPLC

The introduction of spherical particles has led to a reduction in the size of the phase particles to 3 μm in 1978 and 1.5 μm in 1990. This means that column efficiency is improved and shorter columns are possible, leading to shorter separation times (Nguyen et al., 2006). Column lengths of 25 cm (8000–10,000 plates/m) have now dropped to around 6 cm for 3 μm particles in order to attain the same plates/m. The analysis time correspondingly drops by a factor of around 4. The trend is towards columns with 1 μm particles, which would give pressures of around 7000 bar.

In ultra performance liquid chromatography (UPLC), the trade mark of Waters Acquity systems, introduced in 2004, particle sizes of around 1.7 μm are used, at a pressure of 15,000 psi (1000 bar). Very few applications to plants have as yet been published, but as an example, the constituents of raw and steamed ginseng root, *Panax notoginseng*, Araliaceae, have been investigated by this method. UPLC was performed on a Waters Acquity system with a 100 × 2.1 mm C_{18} 1.7 μm column and a mobile phase gradient consisting of (A) 0.1% formic acid in water and (B) acetonitrile containing 0.1% formic acid. The UPLC system was connected to a orthogonal acceleration TOF mass spectrometer and was compared to a classical LC/UV analysis. Identification of peaks was achieved by comparison with an in-house database containing 96 protopanaxadiol-type ginsenosides. The faster chromatography displayed higher reproducibility when compared with traditional HPLC (Fig. 7) (Chan et al., 2007).

Among the many different HPLC column packings available, monolithic columns have been recently introduced. Traditional HPLC columns are packed with tiny silica particles. The difference with monolithic columns is that they contain a single, solid compound as the stationary phase – usually consisting of a network of polymethacrylate or polystyrene copolymers or bonded silica. In particulate columns, the mobile phase can diffuse between the particles, whereas in monolithic columns it flows through the solid stationary phase, which is usually porous. Compounds in the mobile phase are retained to a greater or lesser extent within the pores of the stationary phase. The advantage of monolithic columns is their faster flow rates and quicker separations.

5. Capillary electrophoresis (CE)

Capillary electrophoresis is an analytical technique which provides high separation efficiency and short run times. Several modes of CE are available: (1) capillary zone electrophoresis (CZE), (2) micellar electrokinetic chromatography (MEKC), (3) capillary gel electrophoresis (CGE), (4) capillary isoelectric focusing, (5) capillary isotachophoresis, (6) capillary electrochromatography (CEC) and (7) non-aqueous CE. The simplest and most versatile CE mode is CZE, in which the separation is based on differences in the charge-to-mass ratio and analytes migrate into discrete zones at different velocities. Anions and cations are separated in CZE by electrophoretic migration and electro-osmotic flow (EOF), while neutral species co-elute with the EOF. Applications of CE for the analysis of phytochemicals have been well documented (Tomas-Barberan, 1995; Issaq, 1997, 1999). CE is especially suitable for the separation of flavonoids as they are negatively charged at higher pH values (Tomas-Barberan, 1995).

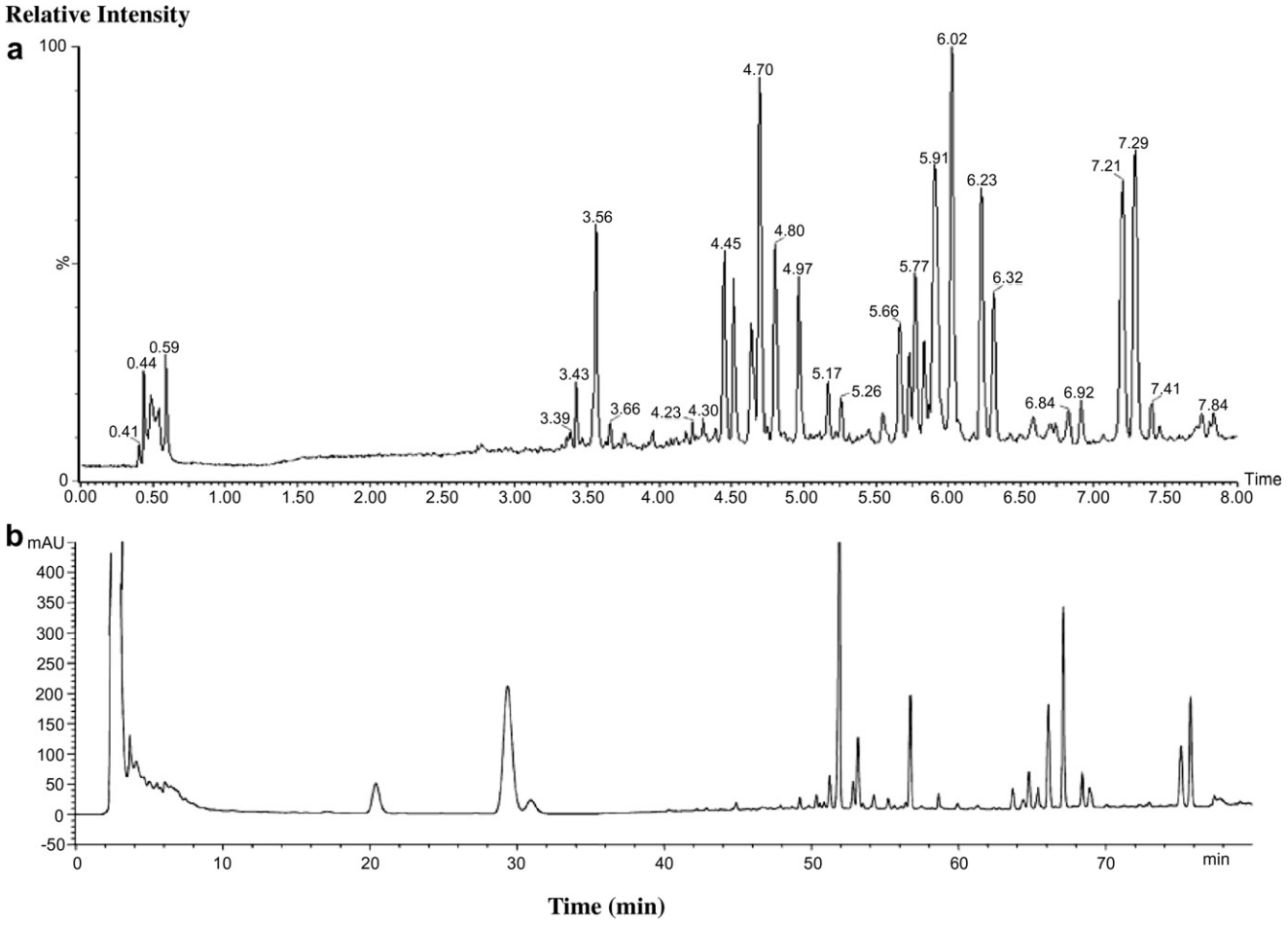


Fig. 7. (a) UPLC/TOFMS electrospray total ion current chromatogram (TIC) and (b) HPLC/UV chromatogram (203 nm) of steamed *Panax notoginseng* (Araliaceae) (reproduced from Chan et al., 2007).

Thin-layer chromatography, HPLC, GC and capillary electrophoresis (CE) are all useful methods for fingerprinting and analysing plant drugs. While HPLC has high precision, sensitivity and reproducibility, it requires lengthy pre-treatment of samples to remove residual solid material and eliminate irreversible adsorptive losses on the solid support matrix. Capillary electrophoresis is becoming increasingly recognised as a fast and efficient analytical separation technique but the reproducibility and selectivity are inferior to HPLC. This is shown by fingerprint studies on an aqueous extract of *Radix Scutellariae*. The electropherogram shown in Fig. 8a gives 11 peaks, while the HPLC chromatogram in Fig. 8b shows 14 major peaks. However, the sample size in CE is smaller (ng) and the run time shorter (12 min), while in HPLC the sample size is larger (μg) and the separation time longer (45 min) (Wang et al., 2005).

6. Countercurrent chromatography (CCC)

Countercurrent chromatography is another advance of the twentieth century with tremendous implications for phytochemistry. Unlike HPLC, there is no solid column packing involved. It is basically a development of countercurrent distribution (CCD), a method developed in the 1940s and 1950s for the batchwise (Craig Distribution) or continuous (O'Keefe Distribution) fractionation of mixtures. Countercurrent chromatography (or centrifugal partition chromatography, as it is sometimes known) is an all-liquid separation technique which relies on the partition of a sample between two immiscible solvents, the relative proportions of solute passing into each of the two phases being determined by the respective partition coefficients. Since it is characterized by the absence of a solid support CCC has the following advantages over other chromatographic techniques:

- no irreversible adsorption,
- total recovery of injected sample,
- tailing minimized,
- low risk of sample decomposition,
- low solvent consumption,
- favourable economics (once the initial investment in an instrument has been made, no expensive columns are required and only common solvents are consumed).

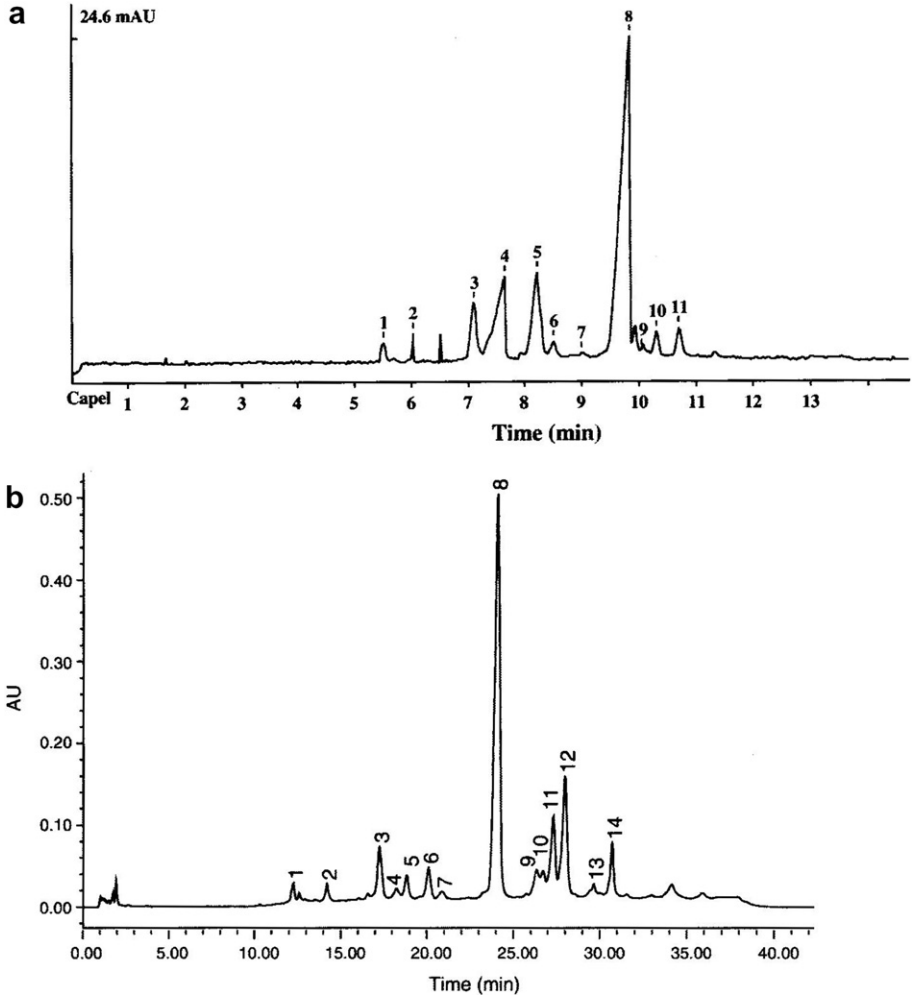


Fig. 8. (a) Electropherogram of a *Radix Scutellariae* water extract. CE conditions: column: uncoated fused-silica capillary (52 cm/62 cm, 50 μm id); running buffer: 50 mmol/L borax solution (pH 8.0); voltage: 20 kV; UV detection 254 nm; pressure injection: 10 s/30 mbar; temperature: 20 °C and (b) chromatogram of a *Radix Scutellariae* water extract. HPLC conditions: column: NovaPak C-18 (150 × 3.9 mm, 5 μm); mobile phase A (MeOH), mobile phase B (H_2O + 0.1% H_3PO_4); gradient: 0–6 min 12% A, 6–9 min 12–34% A, 9–27 min 34–55% A, 27–27.01 min 55–60% A, 27.01–36 min 60% A, 36.01–40 min 12% A; detection 254 nm (reproduced from Wang et al., 2005).

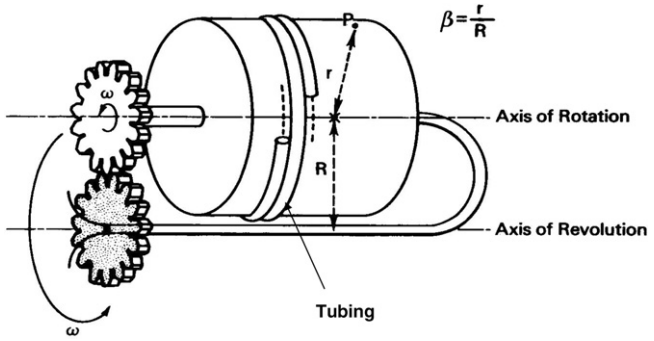


Fig. 9. Schematic drawing of a rotating coil CCC instrument equipped with planetary gear.

Modern countercurrent chromatography finds its origins in pioneering work by Ito at the NIH in the USA (Ito et al., 1966). Subsequent to the initial experiments, CCC has split into two basic directions:

(1) Apparatus with a variable gravity field produced by a double axis gyratory motion and a seal-free arrangement for the column (generally tubing wrapped around bobbins or drums) (Fig. 9).
(2) The second employs a constant gravity field produced by a single axis rotation, together with rotatory seals for supply of solvent. Separation takes place in cartridges or disks.

Although the efficiency (as represented by the number of theoretical plates) cannot match that of HPLC, the high selectivity and the high stationary to mobile phase ratio more than compensate. Another advantage of CCC is the ability to reverse the flow direction and interchange the mobile and stationary phases (“reversed-phase” operation).

Concerning instrumentation, several rotating coil and cartridge-type machines are now on the market. Development is moving fast and the early technical problems

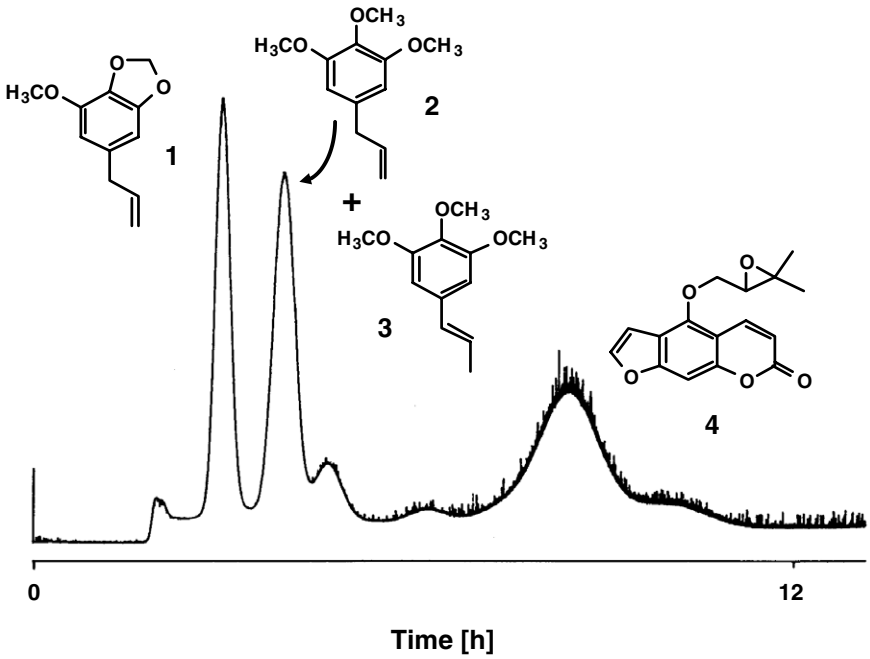


Fig. 10. Initial CCC fractionation of *Diplolophium buchanani* (Apiaceae) leaves (dichloromethane extract). Solvent: hexane-EtOAc–MeOH–H_2O 10:5:5:1 (upper layer as mobile phase); flow-rate 3 ml/min; detection 254 nm; sample 1.7 g (reproduced from Marston et al., 1995).

(leakages, lack of temperature control, etc.) are now more or less solved. Although the solvent capacity of these instruments generally lies in the range 200–1000 ml, large-scale instruments for industrial applications are starting to appear which employ up to 25 l of solvent.

A typical rotating coil instrument consists of a PTFE or Tefzel tube (1.6 or 2.6 mm i.d.) wrapped as a coil around a spool, to form a bobbin. When in motion (generally around 1000 rpm), the coil describes a planetary motion about a central axis (Fig. 9). The planetary motion creates a heterogeneous force field which results in an efficient mixing of the two solvent phases in such a way that about 50,000 partition steps per hour of the analytes in the solvent system can be achieved.

Countercurrent chromatography is an excellent alternative to circumvent the problems associated with solid-phase adsorbents and to preserve the chemical integrity of mixtures subjected to fractionation. With these advantages, CCC is gaining popularity as a purification tool for natural products, and especially in the bioassay-guided fractionation of plant-derived compounds.

CCC has been the subject of numerous research papers, review articles and books (see, for example, Conway, 1990). Practical aspects of the method, such as choice of solvent, have been summarised in an article by Ito (2005), and applications have been reviewed (Ito, 2005; Marston and Hostettmann, 2006).

Countercurrent chromatography is very versatile and can be used for the fractionation of crude plant extracts (in multigram quantities) or for final purification steps.

It is possible to isolate natural products by CCC alone, as shown by the purification of phenylpropanoids and a

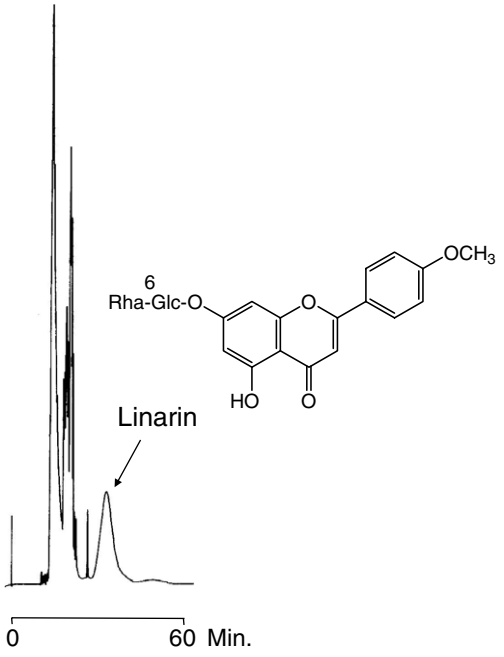


Fig. 11. Separation of a methanol extract of *Buddleia davidii* (Buddlejaceae) leaves by CCC on a DE instrument (17 ml coil); solvent: $CHCl_3$–MeOH–H_2O 45:33:22 (lower layer as mobile phase); flow-rate 1 ml/min; detection 254 nm; sample: 20 mg.

furanocoumarin from a dichloromethane extract of the leaves of *Diplolophium buchanani* (Apiaceae). Initial fractionation (Fig. 10) on a Pharma-Tech CCC-1000 instrument, followed by a second liquid–liquid step, using a different non-aqueous solvent system, gave pure myristicin (**1**) and a mixture of elemicin (**2**) and *trans*-isoelemicin (**3**).

The furanocoumarin oxypeucedanin (**4**) was obtained by simple crystallization of the corresponding CCC fraction. All four isolated compounds had both antifungal and larvicidal activities (Marston et al., 1995).

In addition to different designs and types of CCC apparatus, there are different ways of using the machines. One of these variants is referred to as ph-zone-refining CCC, involving the use of a retainer (such as an acid) in the stationary phase and a displacer (such as a base) in the mobile phase. This method is especially useful for alkaloids and provides a high sample loading capacity, high purity and high concentrations of the collected fractions (Ito and Ma, 1996).

6.1. Analytical CCC instruments

Analytical-scale CCC will never displace the ubiquitous technique of analytical HPLC but it finds certain important uses, notably as a tool for the search for separation conditions in scaled-up HSCCC. In addition to method development, analytical HSCCC is also useful for the screening of new bioactive compounds in crude extracts and for microscale isolations. For the latter, the important fact is that the method involves no sample losses. While separations on 200–1000 ml capacity rotating coil instruments generally take 1–12 h, separations of small quantities (ca. 10–30 mg) of sample can be very efficiently performed on low-volume (10–30 ml), fast rotating (up to 3000 rpm) instruments. For example, the flavone glycoside acetylcholinesterase inhibitor linarin could be isolated in a single step from the leaves of the ornamental bush *Buddleja davidii* (Buddlejaceae) by CCC of the methanol extract. Injection of 20 mg extract gave 3 mg of linarin within 40 min (Fig. 11). This is competitive with semi-preparative HPLC, the only difference being that no clean-up of the methanol extract is required (Fan et al., in press).

Analytical HSCCC is also an attractive method for interfacing with mass spectrometers because of the reduced flow-rates.

7. Perspectives

Without chromatography, it is safe to say that phytochemistry would be a great deal more tedious; analytical procedures would be more complex and certain problems would be impossible to tackle, such as metabolomic analysis. The challenges of proteomics and metabolomics are already omnipresent, with multi-component mixtures of high complexity to analyse. Without doubt, advances in HPLC, via new column technology, such as capillary columns or UPLC, will provide solutions to these problems, in conjunction with mass spectrometry as a detection method. Work is also underway to characterise the complex plant mixtures found in Chinese traditional medicine. This is necessary in order to satisfy requirements from regulatory authorities. There will also be advances in column packings. New chemistries will be developed and greater stability will be sought for.

Another domain that is likely to see rapid development and use in the near future is countercurrent chromatography. When compared to HPLC, the fact that there is no solid phase support in CCC counteracts the lower resolution,. The difference in selectivity is also an important item. There is already true moving bed technology being introduced and continuous injection of sample can be achieved, which of course improves the throughput. The instruments are much more reliable and are moving away from the prototype stage.

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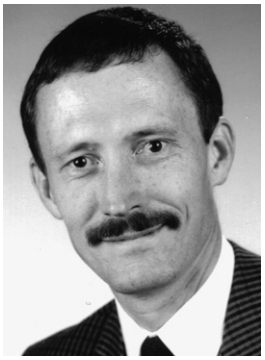

Andrew Marston was born in Zambia (1953). He studied chemistry at University College London and obtained a PhD in the Department of Organic Chemistry at Liverpool University, U.K. He was then awarded a Royal Society / NATO fellowship to perform postdoctoral research on plant-derived tumour promoters at the German Cancer Research Centre in Heidelberg. He is now a senior research scientist in the group of Professor Kurt Hostettmann, working in the fields of pharmacognosy and phytochemistry. This group, originally situated in the University of Lausanne, has recently moved to the Section of Pharmaceutical Sciences of Geneva University, Switzerland.

Andrew Marston is involved in the teaching of pharmacy students and his research includes the study of active principles from plants used in traditional medicine, with special interests in preparative chromatographic separation techniques, saponins and benchtop bioassays. He is author of 170 scientific publications and co-author of several books. His distinctions include the Rhône-Poulenc Rorer Award of the Phytochemical Society of Europe.