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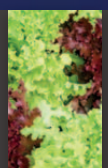

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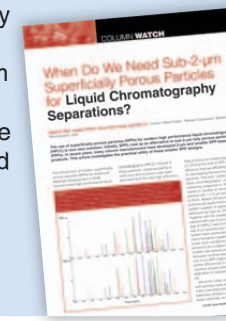

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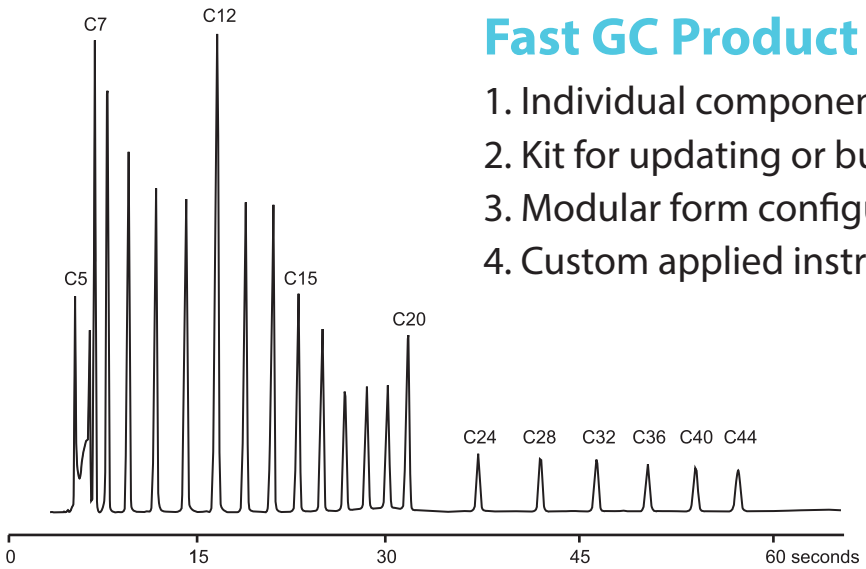

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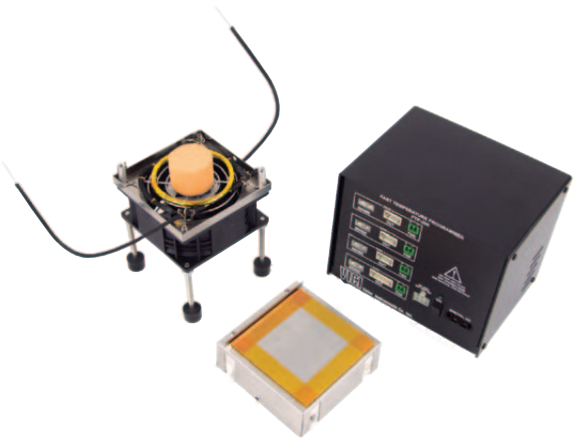

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Fundamental and Practical Aspects of Liquid Chromatography and Capillary Electromigration Techniques for the Analysis of Phenolic Compounds in Plants and Plant-Derived Food (Part 1): Liquid Chromatography

Danilo Corradini[1], Francesca Orsini[1,2], Laura De Gara[2], and Isabella Nicoletti[1], [1]CNR, Institute of Chemical Methodologies, Area della Ricerca di Roma 1, Monterotondo Stazione (Rome), Italy, [2]Unit of Food Science and Nutrition, University Campus Bio-Medico of Rome, Rome, Italy

Column-based liquid phase separation techniques, such as liquid chromatography (LC) in reversed phase separation mode and capillary electromigration techniques, using continuous electrolyte systems, are widely used for the identification and quantification of phenolic compounds in plants and food matrices of plant origin. This paper is the first of a two-part review article discussing fundamental and practical aspects of both LC and capillary electromigration techniques used for the analysis of phenolic compounds occurring in plant-derived food and in edible and medicinal plants. The chemical structure and distribution of the major phenolic compounds occurring in the plant kingdom, as well as the main methods used for their extraction and sample preparation, are also discussed. Part 1 will focus on liquid chromatography.

Plant secondary metabolites, also known as specialized metabolites, are organic compounds produced along with the primary biosynthetic and metabolic routes that are believed to be mainly produced in response to the interactions of the plant with the environment.

These compounds protect the plant against biotic and abiotic stresses, including pathogens, predators, ultraviolet light, and drought (1). In addition, secondary metabolites may confer specific sensory characteristics to food products and play important roles in disease prevention and health-promoting effects of edible plants and plant-derived food products (2–5). Based on their biosynthetic origins, plant secondary metabolites are usually classified into three major classes: terpenoids, alkaloids, and phenolic compounds, the last of which are one of the most important and widespread class of secondary metabolites in the plant kingdom (6).

Phenolic compounds form an integral part of the human diet, contributing to the sensorial properties of food products and to the beneficial effects of the Mediterranean diet on human health, mainly as a result of their antioxidant properties (7). These compounds, as well as many other plant secondary metabolites, are also important as bioactive components in medicinal plants and have numerous biological activities and a variety of health benefits for chronic and degenerative human diseases (8).

KEY POINTS

- This article discusses the main separation modes of LC used to identify and quantify phenolic compounds in plants and food matrices of plant origin.
- Part 1 of the article also briefly describes the chemical structure and distribution of the major phenolic compounds occurring in the plant kingdom and the main methods used for their extraction and sample preparation.
- Reversed phase chromatography, either with HPLC or UHPLC columns, is the technique of choice for the analysis of phenolic compounds.
- HILIC is both a valuable alternative to reversed phase chromatography and the main separation modes used in multidimensional chromatography of phenolic compounds.

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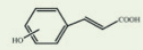
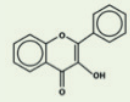
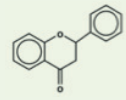
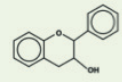
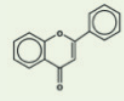
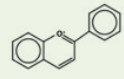
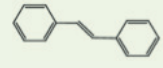
Table 1: Main classes of plant phenolic compounds

Phenolic acids (C_6-C_1) (C_6-C_3)	Hydroxybenzoic acid derivatives	
	Hydroxycinnamic acid derivatives	
Flavonoids (C_6-C_3-C_6)	Flavonols	
	Flavanones	
	Flavanols (or flavan-3-ol)	
	Flavones	
	Anthocyanidins	
Stilbenes (C_6-C_2-C_6)	*trans*-Resveratrol derivatives	

Important steps for the assay of phenolic compounds in plants and plant-derived food are sample preparation and extraction, followed by identification and quantification using various instrumental analytical methods, most of which use column-based high performance liquid phase separation techniques coupled to a suitable detection method. Traditionally, total phenolic compounds are determined using spectrophotometric methods based on the Folin-Ciocalteau reaction (9), which generate a coloured product as the result of the oxidative titration of phenolate anions by phosphotungstate and phosphomolybdate. More sophisticated methods, based on instrumental analytical techniques, are needed to identify and quantify each of the main phenolic compounds present in a given sample. Among them, liquid chromatography (LC), generally in the reversed phase separation mode, is the technique of choice, while capillary electrophoresis (CE) is gaining increasing acceptance because of its high separation efficiency, short analysis time, and extremely small sample and reagent volume requirements.

This is the first of a two-part review article aimed at discussing fundamental and practical aspects of both LC and capillary electromigration techniques used for the analysis of phenolic compounds occurring in edible and medicinal plants and in plant-derived food and dietary supplements. Part 1 will focus on liquid chromatography of these compounds and the chemical structure and distribution of the major phenolic compounds, as well as the main methods used for sample preparation and extraction.

Major Phenolic Compounds

Phenolic compounds, also referred to as phenolics, comprise a large number of heterogeneous structures that range from simple molecules to highly polymerized compounds, which are commonly bound to other molecules, frequently to sugars, although phenolics in free form also occur. Among glycosylated phenolic compounds, both *C*- and *O*-glycosylations are found. A common structural characteristic of phenolics is the presence of at least one aromatic ring hydroxyl-substituted. They are commonly divided into different subclasses according to the number of aromatic rings, the structural elements that bind these rings to each other, and the substituents linked to the rings (see Table 1).

The simplest form of phenolics are the phenolic acids, which can be divided into benzoic acid and cinnamic acid derivatives, with basic carbon skeletons C_6-C_1 and C_6-C_3, respectively. Other main phenolic compounds comprise flavonoids, with carbon skeletons C_6-C_3-C_6, and a variety of nonflavonoid phenolic compounds with basic skeletons C_6-C_2-C_6 (stilbenes, anthraquinones), $(C_6$-C_3-$C_6)_n$ (condensed tannins), $(C_6$-$C_3)_2$ (lignans), $(C_6$-$C_3)_n$ (lignins), C_6-C_4 (naphtochinones), and C_6-C_1-C_6 (xanthones), just to mention the main subclasses.

Flavonoids are the largest group of phenolic compounds and are distributed in several subclasses of structurally diverse composition. Their classification is determined by the arrangements of the three-carbon atoms group occurring in the C_6-C_3-C_6 structure. Several studies have reported that flavonoids possess a variety of beneficial effects on human health, including the properties of acting as chemopreventive agents interfering with several cancer mechanisms (10).

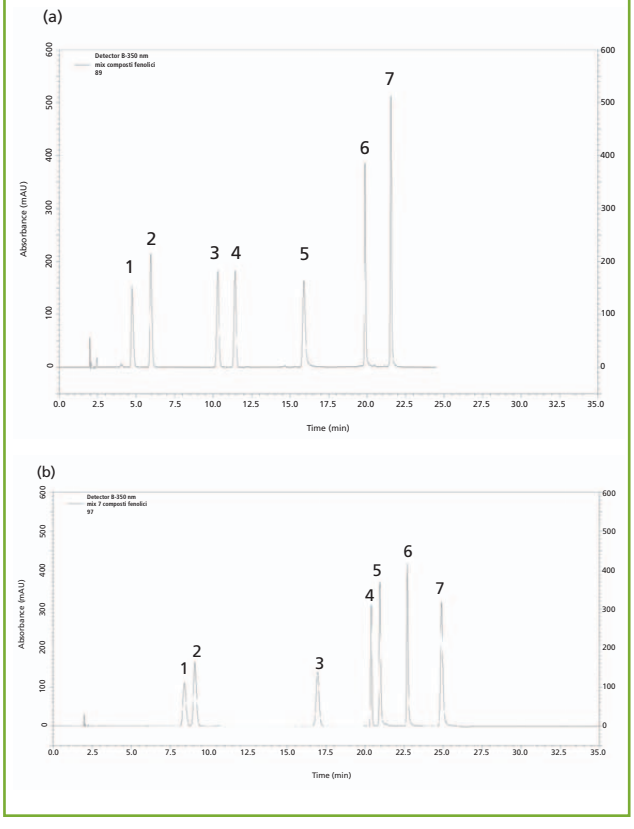
One of the most abundant and widely distributed flavonoid subclasses are flavonols, which comprise quercetin, myricetin, kaempferol, and fisentin, and are found in wine, onion, apples, and a variety of leafy vegetables. Flavanones, commonly found in plants and plant-derived foods and beverages, include naringenin, naringin, narirurin, hesperidin, and hesperitin. A high intake of flavanones in the diet has been associated with a reduced risk of degenerative and cardiovascular diseases (11).

Flavanols (or flavan-3ol) include catechin and epicatechin, which can be hydroxylated to form gallocatechins, and are the building blocks of oligomeric and polymeric proanthocyanidins, which are called procyanidins when they consist exclusively of epicatechin units. Flavanols are found in wine, broccoli, and other food products, such as cocoa, tea, beans, and a variety of fruits, such as apples, pomegranate, blackberries, and red grapes.

Flavones are widely found in many medicinal plants, spices, fruits, and leafy vegetables. Common flavones include apigenin, luteolin, and their glycosylated forms, apigenin-O-glucuronide, apigenin-O-glucoside, and luteolin-C-glucoside, which have proven to have potential antioxidant and anti-inflammatory activity (12).

Anthocyanins are glycosylated derivatives of a flavylium cation carrying a positive charge on the heterocyclic oxygen, which, owing to its conjugated double bonds, absorbs visible light and is therefore responsible for the intensive red-orange to blue-violet colour of many fruits and flowers. The aglycon of anthocyanins, also known

Figure 1: Reversed-phase HPLC separation of standard phenolic compounds. Column: 2.1 mm × 150 mm, 5-µm Polaris C-18 (Agilent Technologies) eluted by a linear gradient of increasing concentration of either (a) acetonitrile or (b) methanol in water containing 0.1% (*v/v*) formic acid; flow rate: 0.2 mL/min. UV detection at 280 nm. Standard phenolic compounds: 1. chlorogenic acid, 2. caffeic acid, 3. ferulic acid, 4. rutin, 5. myricetin, 6. quercetin, 7. kaempferol (personal data).



as anthocyanidins, occurring more frequently in nature and of dietary importance are cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (13). Other subclasses of flavonoids include chalcones, aurones, dihydrochalcones, isoflavonoids, neoflavanoids, and biflavonoids (7,10).

Sample Preparation and Extraction

Edible plants and a variety of processed food and beverages of plant origin are major sources of bioactive secondary metabolites in the human diet. Very often, such matrices need to be processed by selected sample preparation methods before the extraction of the targeted secondary metabolites and, in some cases, also after their extraction (sample clean up and sample enrichment). The preparation of the samples and the extraction of the compounds of interest are critical steps in obtaining accurate analytical data and reliable interpretation of their values. The proper selection of the above processes depends on the nature of the sample matrix and the chemical properties of the targeted secondary metabolites, including their molecular structure and polarity. Other factors to be considered are the chemical stability of the compounds of interest during sample preparation, storage, and extraction, in addition to their concentration in the matrix, which is frequently washed, milled, dried, and homogenized before sample preparation and extraction.

The extraction of phenolics from the huge number of plant species and food matrices is performed by a variety of techniques, involving the use of solvents, steam, or supercritical fluids (14). These techniques include the conventional solid-liquid extraction (SLE) methods, such as maceration, infusion, percolation, hydrodistillation, decoction, and boiling under reflux (Soxhlet extraction). Most of these techniques, which are based on the application of heat or mixing, are cumbersome, time-consuming, and require the use of relatively large volumes of expensive hazardous organic solvents. In addition, their extraction yield is very often limited.

An alternative extraction technique, particularly suitable for thermolabile compounds, is supercritical fluid extraction (SFE) (15), which can be operated at room temperature and uses as the extracting media a supercritical fluid such as carbon dioxide. This compound, as well as all supercritical fluids, possesses liquid-like density and extraction power, as well as gas-like properties of viscosity, diffusion, and surface tension that facilitate its penetration to the sample matrix, with the result of improved extraction efficiency. However, carbon dioxide is nonpolar and therefore, because most phenolic compounds are polar, is generally used in combination with a polar cosolvent, such as ethanol, ethyl acetate, or acetone. The extraction rate can be further enhanced by using ultrasound during SFE (ultrasound-assisted SFE).

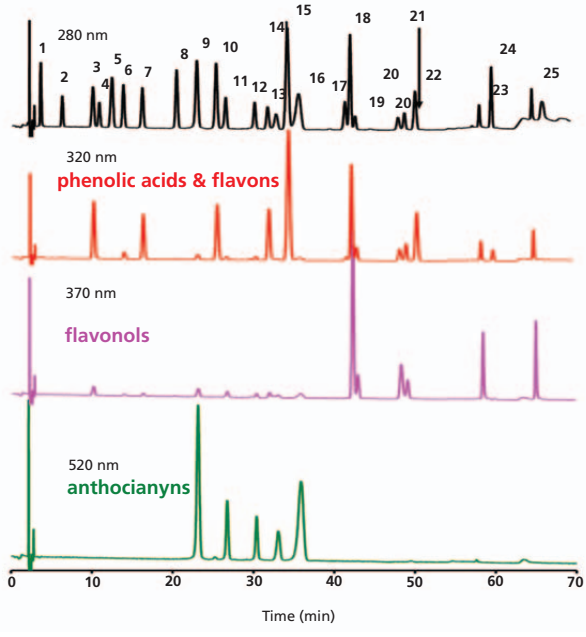
Other recent and efficient extraction techniques include ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), pulsed-electric field extraction (PEF), and pressurized liquid extraction (PLE). Few of these modern extraction techniques have been further advanced, such as MAE, with the recent development of high-pressure MAE (HPMAE), nitrogen-protected MAE (NPMAE), vacuum MAE (VMAE) ultrasonic MAE (UMAE), solvent-free MAE (SFMAE), and dynamic MAE (DMAE) (16).

The removal of potential interferents (sample cleanup), and the enrichment of the target analytes in the extracted samples are traditionally performed by liquid–liquid extraction (LLE) and solid-phase extraction (SPE). With the advent of miniaturization, these methods have been evolved in a variety of microscale extraction techniques, referred to as liquid–liquid microextraction (LLME) and solid-phase microextraction (SPME). High-molecular-weight polymeric phenolic compounds or individual low-molecular-weight phenolics associated to macromolecules, the so-called nonextractable phenolics (NEPs), are usually extracted after acid, alkaline, or enzymatic hydrolysis, which is performed to release NEPs from the matrix. Other advanced cleanup methods use liquid membrane extraction (LME), pipette-tip SPE (PT-SPE), molecular imprinted SPME (MI-SPME), and microfluid extraction systems (17).

HPLC and UHPLC

Conventional high performance liquid chromatography in reversed phase separation mode is the technique of choice

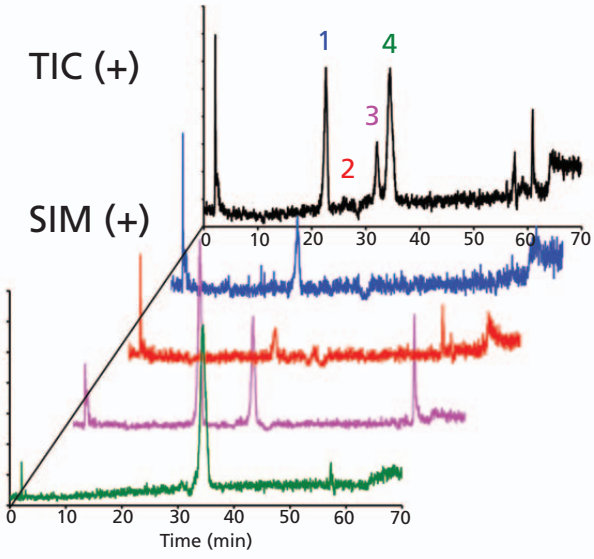
Figure 2: Reversed-phase HPLC separation and identification of phenolic compounds extracted from grape berries, variety "Uva di Troia". Column: 2.1 mm × 150 mm, 5-µm Polaris C-18 (Agilent Technologies) eluted by a multisegment gradient of increasing concentration of acetonitrile in water containing 0.5% (*v/v*) formic acid; flow rate: 0.2 mL/min; photodiode array detection at 280 nm, 320 nm, 370 nm, and 520 nm. Peak identity (confirmed by ESI-MS detection): 1. gallic acid, 2. protocatechuic acid, 3. caftaric acid, 4. p-hydroxybenzoic acid, 5. catechin, 6. m-salicylic acid, 7. caffeic acid, 8. epicatechin, 9. delphinidin 3-O-glucoside, 10. p-coumaric acid, 11. cyaniding 3-O-glucoside, 12. pelargonidin 3-O-glucoside, 13. sinapic acid, 14. peonidin 3-O-glucoside, 15. *trans*-piceid, 16. malvidin 3-O-glucoside, 17. naringenine-7-glucoside, 18. rutin, 19. quercetin 3-O-glucoside, 20. myricetin, 21. kaempferol 3-O-glucoside, 22. *trans*-resveratrol, 23. quercetin, 24. naringenin, 25. kaempferol (personal data).



for the analysis of phenolic compounds, which is generally performed using analytical size columns with an internal diameter (i.d.) in the range of 4.0–4.6 mm. Narrow-bore columns, with an i.d. of 2.0 mm or 2.1 mm, have recently gained increasing acceptance as a result of their positive impact on the environment and the analysis costs, which is a result of the reduced consumption of hazardous and expensive organic solvents. Additional advantages of using narrow-bore columns include the flow rate compatibility with mass spectrometry (MS) detection, in addition to the expected higher sensitivity of UV–vis absorbance detection, owing to the minor dilution of samples during separation, in comparison to using a conventional analytical size column.

Columns packed with microparticulate (2.5–5.0 µm) spherical porous octadecyl (C18) bonded silica are very popular, but other bonded stationary phases are also used including octyl (C8), phenyl-hexyl, pentafluorophenyl, and diphenyl bonded silica. Efficient and rapid separations

Figure 3: Separation and identification of antocyanins extracted from grape berries, variety "Merlot". Column: 2.1 mm × 150 mm, 5-µm Polaris C-18 (Agilent Technologies) eluted by a multisegment gradient of increasing concentration of acetonitrile in water containing 0.5% (v/v) formic acid; flow rate: 0.2 mL/min; ESI-MS detection in positive ionization mode. Peak identity: 1. delphinidin-3-glucoside (*m/z* 466.4), 2. cyanidin-3-glucoside (*m/z* 450.4), 3. peonidin-3-glucoside (*m/z* 464.4), 4. malvidin-3-glucoside (*m/z* 494.4) (personal data).



are also obtained using superficially porous particles, also known as fused-core or core–shell particles, consisting of an impenetrable inner core surrounded by a layer of fully porous silica, which provide higher efficiency and more homogeneous packing density for the same particle diameter than conventional fully porous silica particles.

As well as packed silica-based columns, polymeric microparticulate packing materials and monolithic columns (either polymeric or silica-based) are also used in reversed-phase HPLC of phenolic compounds. Monolithic columns consist of a continuous rod of the chromatographic support with bimodal porosity. They are synthesized using either organic or inorganic precursors and exhibit enhanced mass transfer characteristics in comparison to conventional columns (18). Both core–shell packed and monolithic columns can be operated at higher mobile phase flow rates, with lower back pressures, than conventional columns, while providing high efficiency and resolving power for a variety of analytes, including phenolic compounds of natural origin (19–20).

A more advanced form of HPLC, namely ultrahigh-pressure liquid chromatography (UHPLC), uses narrow-bore columns (1.0–2.1 mm [i.d.]), packed with sub-2-µm particles, which are eluted at high flow rates and require the use of a chromatographic system that withstands pressures up to 600 and even 1000 bar (60–100 MPa). According to theory (van Deemter equation), the use of columns packed with sub-2-µm particles implies a significant gain in efficiency, even at high values of the mobile phase linear velocity, which is proportional to the mobile phase flow rate. Because of this higher efficiency, the chromatographic peaks are narrower and the maximum number of resolvable peaks (peak capacity) is larger and the detection limits are lower, which means that both resolving power and sensitivity are expected to be higher in UHPLC than in conventional HPLC.

A further advantage of performing the chromatographic separation at high flow rates is the significant decrease in the analysis time, while the more evident disadvantage is the high column back pressure that can easily reach the upper pressure limits of conventional HPLC systems. The column back pressure can be lowered by running the chromatographic separation at higher than ambient temperature, with the advantage of the possible use of a conventional HPLC instrument. However, to take full advantage of UHPLC, the separation should be perfomed using dedicated instrumentation with extended pressure capability, a sampling valve with a fast injection cycle and low injection volume, tubing ensuring minimum extra-column volume, and a detector with fast time constant and acquisition rate.

Most of the reversed-phase columns used in either HPLC or UHPLC analysis of phenolic compounds are operated under gradient elution mode with the starting eluent and the gradient former consisting of a water-rich and an organic solvent-rich solution, respectively. A suitable acid is generally incorporated into the starting eluent and, less frequently, into the gradient former solution to control the protonic equilibrium at acidic pH values. Acidic conditions are requested to improve the hydrophobic interactions of the phenolic compounds with the stationary phase by ensuring that both carboxyl and hydroxyl groups of the analytes are in their protonated form. Acetonitrile and methanol are the organic solvents generally used as the gradient former. The two solvents exhibit different elution strength and separation selectivity (Figure 1). However, whenever possible, acetonitrile is preferred to methanol because of its lower UV cutoff and viscosity.

The primary detection method used in LC of phenolic compounds is based on the absorbance of UV or, as in the case of anthocyanins, visible light. These detectors comprise fixed-wavelength, variable-wavelength, scanning, and photodiode array (PDA) detectors. Low-pressure discharge lamps are used as the source of intensive line UV radiations, such as mercury (254 nm) or cadmium (229, 326 nm), whereas deuterium lamps, covering the range 190–700 nm, are used in variable-wavelength and photodiode array detectors, where a tungsten-halogen lamp may also be used to improve the performance in the visible region.

Each class of phenolic compounds has unique spectral characteristics. Therefore, general information on the different classes of phenolic compounds occurring in a complex sample mixture can be obtained by performing UV–vis detection at the wavelength corresponding to the absorption maxima of the phenolic compounds expected to occur in the sample. An example of this approach is depicted in Figure 2, which displays the reversed-phase HPLC separation of phenolic compounds extracted from grape berries and detected by PDA at wavelength values corresponding to the absorption maxima of flavonols

(370 nm), anthocyanins (520 nm), and phenolic acids and flavons (320 nm), respectively.

Fluorescence detection is used to detect the limited number of phenolic compounds that naturally fluoresce or that are chemically modified to produce molecules containing a fluorescent tag, usually using on-line post-column derivatization methods. On the other hand, indirect detection, performed by incorporating a fluorescent probe into the mobile phase, as well as chemiluminescence detection with post-column addition of suitable reagents has found limited application.

The hyphenation of either HPLC or UHPLC with MS or high resolution mass spectrometry (HRMS), mainly using time-of-flight (TOF) or orbital trap mass analyzers, allows the elucidation of the chemical structure of the investigated phenolic compounds. Prominent among the different ionization sources used in LC–MS is electrospray ionization (ESI) in negative ionization mode, although ESI in positive ionization mode is also used. For example, anthocyanins are glycosylated derivatives of a flavylium cation that are detected by ESI-MS in positive ionization mode (21) (Figure 3). Other, less common, atmospheric pressure ionization (API) interfaces used in LC–MS of phenolics include atmospheric-pressure chemical ionization (APCI) (22) and, to a minor extent, atmospheric-pressure photochemical ionization (APPI) (23).

The hyphenation of liquid chromatography with nuclear magnetic resonance spectroscopy (NMR), which is widely used in analytical chemistry for the unambiguous identification of known and novel organic compounds, is also promising. Direct on-line hyphenation can be realized using flow NMR probes, either of the double saddle Helmholtz coil design or of solenoidal microcoil design, also in combination with methods designed to improve the otherwise low-detection sensitivity of NMR, which is negatively affected by the background absorption of the liquid phase used in the separation step (24). Noticeable among these methods is the hyphenation of LC with NMR using SPE. According to this approach, each phenolic compound eluting the chromatographic column as a separated peak is trapped on an SPE cartridge, which is subsequently dried with nitrogen and then eluted with a deuterated solvent into proper tubes for off-line NMR analysis. An application of this approach has been reported by Goulas *et al.* for the identification of the methoxylated flavones hispidulin, salvigenin, and cirsimaritin in extracts of *Salvia fruticosa*, exhibiting antifungal activity (25).

Hydrophilic interaction chromatography (HILIC) is receiving increasing attention, either as a valuable alternative to reversed phase chromatography or as one of the separation modes used in multidimensional chromatography. The chromatographic retention in HILIC is governed by the hydrophilic partitioning of the analyte between an organic-rich mobile phase and a water layer formed at the surface of a polar stationary phase, with the possible contribution of hydrogen bonding, dipole-dipole interactions, and ion-exchange mechanism. Therefore, HILIC can be considered orthogonal to reversed phase chromatography, whose retention mechanism is based on hydrophobic interactions. On the other hand, similar to reversed phase chromatography, HILIC uses hydro-organic mobile phases that are fully compatible with MS detection. Further advantages of HILIC include its suitability for the analysis of polar phenolic compounds that are not sufficiently retained in reversed phase chromatography, and the availability of an alternative separation mechanism, which implies that compounds not easily separated by reversed phase chromatography may be resolved in HILIC. Examples of the applicability of HILIC to the analysis of phenolics include the resolution of individual oligomeric and polymeric procyanidins in apples and apple extracts (26), and the separation and identification of anthocyanins in blueberries, red grape skins, black beans, red cabbage, and red radish (27).

Comprehensive Two-Dimensional Liquid Chromatography

HILIC is very promising for the development of two-dimensional liquid chromatographic methods for the analysis of phenolic compounds in food and beverages (28). This technique is performed by passing the sample through two columns, each containing a different stationary phase that separate the analytes either according to a diverse separation mechanism or with different selectivity. The technique is performed according to various approaches, which comprise “heart-cutting” and “comprehensive” two-dimensional LC, also referred to as LC-LC and LC×LC, respectively. In LC-LC systems, only the fractions of the effluent from the first column containing the analytes of interest are further separated in the second column, whereas in comprehensive two-dimensional LC the entire effluent from the first column is transferred to the second one.

Comprehensive two-dimensional LC systems, created by coupling HILIC and reversed phase chromatography, combine the different selectivity and resolving power of the two independent separation mechanisms operating in the first (HILIC) and second (reversed phase chromatography) columns, which are based on hydrophilicity and hydrophobicity, respectively. HILIC×reversed phase chromatography techniques are generally operated according to the on-line approach, using a multi-port switching valve with one or two sampling loops, whereas the off-line mode is less popular. On-line, off-line, and stop-flow modalities of HILIC×reversed phase chromatography have been deeply investigated and successfully applied for the analysis of cocoa procyanidins (29) and for the separation and identification of anthocyanins and their derived pigments in aged red wine (30). Recently, 265 compounds, comprising 196 potentially new phenolic acids, were separated and tentatively characterized in *Salvia miltiorrhiza* by an off-line two-dimensional HILIC×reversed phase chromatography system, hyphenated to an ion trap time-of-flight mass spectrometer (31). However, because mobile phases with a high content of acetonitrile, such as those used in HILIC, are strong eluents in reversed phase chromatography, system compatibility problems may arise in comprehensive two-dimensional HILIC×reversed phase chromatography systems.

A less problematic approach of comprehensive two-dimensional liquid chromatography to the analyses of phenolic compounds is based on the use of two reversed-phase columns of different selectivity. According to this method, phenolic acids and flavonoid antioxidants

have been successfully separated in beer and wine using combinations of single or serially coupled polyethylene glycol (PEG)-, phenyl-, and C18- reversed-phase columns in the first dimension and single or two alternating C18 or Zr-carbon columns in the second dimension (32–33). The use of a microbore phenyl column in the first dimension coupled to a monolithic or superficially porous C18 column in the second dimension for comprehensive reversed phase chromatography×reversed phase chromatography analysis of phenolic compounds in red wine has also been reported (34). The same group has described a comprehensive two-dimensional reversed phase chromatography×reversed phase chromatography system for the analysis of phenolics in sugarcane leaf extract, consisting of a micro-cyano column and a partially porous C18 column as the first and the second dimension, respectively (35). A total of 34 phenolic compounds, comprising phenolic acids, ellagitannins, flavan-3-ols, flavonols, and ellagic acid conjugates, have been identified in the shoots of *Rubus idaeus* "Glen Ample" by a two-dimensional reversed phase chromatography×reversed phase chromatography system consisting of a C18 silica column in the first dimension and a pentafluorophenyl column in the second dimension (36).

Besides reversed phase chromatography and HILIC, other chromatographic separation modes, such as size exclusion (SEC) or ion exchange chromatography (IEC), have found limited applications in both LC-LC and LC×LC of phenolic compounds. Also limited is the use of dual-retention mechanism columns (37). The combination of SEC with reversed phase chromatography has been reported for the analysis of green and black teas (*Camellia sinensis*) (38). The developed comprehensive two-dimensional SEC×reversed phase chromatography method has allowed the separation and identification of a variety of phenolic compounds, including catechins, theaflavins and their gallate derivatives, kaempferol, quercetin and myricetin mono-, di-, tri-, and tetraglycosides, esters of quinic acid, and gallic or hydroxycinnamic acids. More recently, a zwitterionic polymethacrylate monolithic column, which provides a dual-retention mechanism, HILIC at high concentrations of acetonitrile, and reversed phase in water-rich mobile phases, has been used in the first dimension of either HILIC×reversed phase chromatography or reversed phase chromatography×reversed phase chromatography comprehensive two-dimensional separations of flavones and related phenolic compounds (39).

Conclusions

To summarize, the expanding interest in phenolic compounds and their positive effects on human health has promoted the development of a variety of techniques for the analysis of these compounds in edible and medicinal plants and in plant-derived food products and dietary supplements. The complexity of such matrices and the heterogeneous chemical structures of the variety of phenolic compounds occurring in the plant kingdom require, besides reliable analytical methods, well-designed sample preparation methods and extraction processes.

Specialized LC separation techniques, combined with state-of-art mass spectrometric detection, are currently used for the separation, identification, and quantification of phenolic compounds in plants and in plant-derived food products. Such techniques comprise HPLC, performed with either analytical size or narrow-bore columns, and UHPLC, with dedicated columns and instrumentation. The majority of HPLC and UHPLC methods currently used for the analysis of phenolic compounds are performed in reversed phase separation mode, although HILIC is also used, either as a valuable alternative to reversed phase chromatography or as one of the chromatographic modes used in in 2D LC, in combination with reversed phase chromatography or with other chromatographic techniques.

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Tips, Tricks, and Troubleshooting for Separations of Biomolecules, Part 1: Contemporary Reversed-Phase Protein Separations

Szabolcs Fekete[1], Davy Guillarme[1], and Dwight R. Stoll[2], [1]University of Geneva, Geneva, Switzerland, [2]LC Troubleshooting Editor

Several new materials and columns have been introduced in recent years for reversed-phase separations of proteins. How do I know which one to choose, and which separation conditions will be best for my protein separation?

Over the past decade, there has been tremendous growth in the development of biologics for the purpose of treating diseases ranging from cancer to inflammatory disorders such as ankylosing spondylitis. Currently, the majority of these biologics are proteins, and many of them are monoclonal antibodies. This increase in development activity has, in turn, resulted in a dramatic increase in the number of researchers engaged in protein separations—perhaps more than any other time in history. For those who learned the principles of chromatography by doing experiments involving small molecules, the increased importance of protein separations in the chromatography community presents both tremendous opportunities and challenges. Whereas particle and column technologies designed for small-molecule applications have steadily improved over the past 20 years (for example, with the refinement of superficially porous particles and development of high-performing stationary-phase chemistries), until recently there has not been as much development of materials for large-biomolecule separations, and many users are still using column technologies developed more than 20 years ago. This lag in development is both an opportunity—advances in materials technologies will inevitably improve separation performance for large biomolecules—and a challenge, because many of the best practices relevant to these separations will have to be rewritten as particle and column technologies evolve.

In my laboratory, we made a major shift about four years ago from focusing mainly on small-molecule separations (for example, environmental contaminants and forensic applications) to focusing more than 75% of our effort today on large-biomolecule separations. As I find myself saying often these days, shifting from small-molecule to large-molecule separations is not as simple as just injecting different samples. Researchers with a lot of experience with large biomolecules are painfully aware of this difficulty, but chromatographers who are transitioning from small- to large-molecule analysis may not appreciate the importance of seemingly minor details for achieving high-quality biomolecule separations.

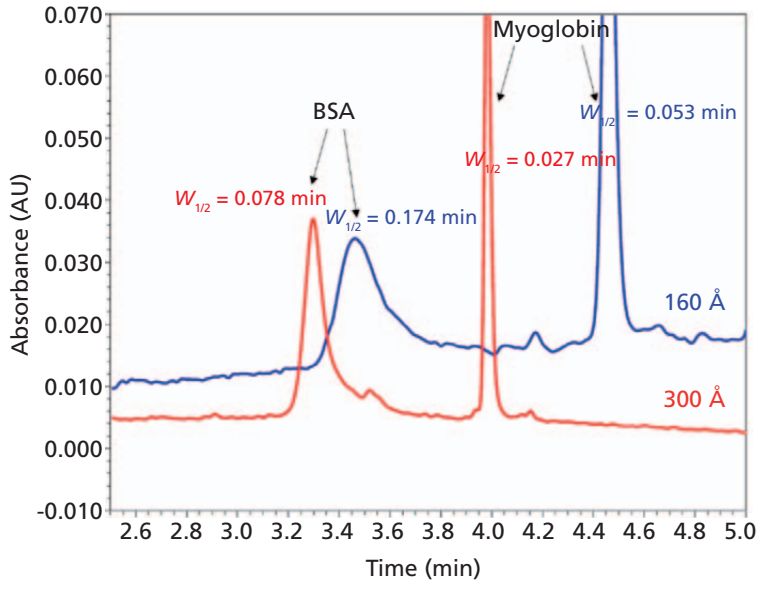
For this instalment of "LC Troubleshooting", I have asked two of my collaborators in the biomolecule application space to join me in writing about some of the details that we have found to be particularly important to reversed-phase separations of proteins.

Dwight Stoll

Why Do Proteins Behave So Differently?

Perhaps the simplest view of why proteins behave so differently from small molecules in chromatographic systems has to do with their (sometimes very) large size. Whereas a small molecule like ibuprofen has a mass of about 300 Da, proteins are on the order of 20- to 1000-fold larger; monoclonal antibodies (mAbs), which currently dominate the list of top-selling biologic therapeutics, have a mass of roughly 150,000 Da. The large size of these proteins leads to much slower diffusion in solution. They are composed of thousands of atoms, tens of different functional groups, and are sometimes quite reactive. They are invariably produced by living organisms (for example, bacteria or mammalian cells), unlike small molecules, which are generally synthesized from simple starting

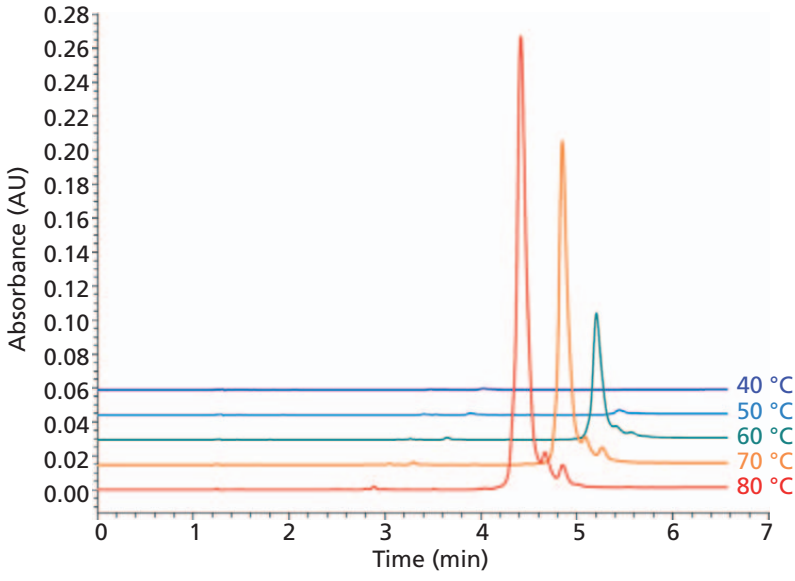
Figure 1: Impact of nominal pore diameter on peak width for BSA and myoglobin. Columns: 150 mm × 2.1 mm C18; mobile-phase A: 0.1% trifluoroacetic acid in water; mobile-phase B: 0.1% trifluoroacetic acid in acetonitrile; gradient: 30–70% B in 10 min; flow rate: 400 µL/min; temperature: 50 °C; injected volume: 1 µL (unpublished results).



materials. Subsequent purification steps, which can be very good, are not perfect, meaning that the analyte we are interested in may be chemically heterogeneous. Finally, these large molecules can adopt secondary, tertiary, and even quaternary structures (that is, shapes) that may give rise to interactions with chromatographic media that are difficult to understand.

In the following sections, we have summarized what we and others have learned about how to work effectively with these proteins under reversed-phase conditions. Some of the issues we discuss are better understood at a fundamental level than others, and many laboratories around the world are actively engaged in trying to understand them better. In most cases, there is both a lot we can learn from older literature (1), and a lot that remains to be discovered as we experiment with new technologies being introduced by manufacturers (2).

Figure 2: Chromatograms obtained from reversed-phase separations of intact rituximab at temperatures ranging from 40 to 80 °C. Column: 150 mm × 2.1 mm BEH300 C4; mobile-phase A: 0.1% trifluoroacetic acid in water; mobile-phase B: 0.1% trifluoroacetic acid in acetonitrile; gradient: 30–37% B in 6 min; flow rate: 0.3 mL/min; injected volume: 0.5 µL; detection: UV absorbance at 280 nm. Adapted with permission from reference 10.



The Pore Size of the Chromatographic Media Is Very Important

One of the fundamental concepts we learn about liquid chromatography (LC) early on is that diffusion of analytes into and out of the pores of porous particles is important, and when it is too slow, it leads to broadening of chromatographic peaks. Whereas the pores of typical porous particles (nominally 10 nm [100 Å] in diameter) are large enough to accommodate a 300 Da molecule without significantly hindering its diffusion into and out of the pores, these pores are simply not big enough to accommodate a protein. In the best case, the diffusion of the protein will be obstructed, leading to peak broadening. In the worst case, the proteins can effectively become "stuck" inside the particle, leading to serious peak tailing and symptoms that could be interpreted as analyte carryover from one analysis to the next.

Column manufacturers are aware of this problem, of course, but from the perspective of the analyst one of the most important practical questions is "How big is big enough?" In the limit of very large pores, the mechanical stability of the porous particle will be compromised, and the surface area of the stationary phase will be decreased to the point where mass overload (that is, increased peak width that results from injecting too much analyte mass [3,4]) becomes a serious problem. In this way, solving one problem can create a different one if not done carefully.

Figure 1 shows a comparison of the peak shapes observed for the two model proteins bovine serum albumin (BSA, 66 kDa) and myoglobin (17 kDa) obtained under reversed-phase conditions using columns prepared with particles having two different nominal pore diameters—one 160 Å, and the other 300 Å.

The pore diameter clearly has a profound impact on the peak width. For myoglobin, the peak width decreases by about 50% when the pore diameter is increased from 160 Å to 300 Å. For BSA, which is more than twice the size of myoglobin, the improvement is even better, with a decrease in peak width of about 56%. We note here that the retention also drops significantly with the wider pore diameter material, presumably because of the lower surface area of the particle. This kind of improvement in peak width, which is even more dramatic for larger proteins such as mAbs, has motivated manufacturers to introduce wider pore materials in the 400–500 Å range, and even up to 1500 Å. The most reliable way to determine which particle will perform best for a particular protein is to experimentally measure peak width and retention time. When making these measurements, it is important to make them over a range of injected masses (for example, from 0.1 to 10 µg protein injected) because these data can be used to assess the compromise between pore size and stationary-phase surface area. Of course, it is not always practical to make these measurements; readers interested in more detail on this topic, or looking for guidance without having to make their own measurements, are referred to a number of recent articles on this topic (3–7).

Column Temperature Is Important, Too

The temperature of the column and mobile phase used during reversed-phase separations of proteins is important as well. There is a compelling case for increasing the temperature well above ambient, because this increased temperature increases the molecular diffusivity of proteins in solution, which can mitigate peak broadening that occurs as a result of slow mass transfer. This approach can improve the efficiency and peak capacity of protein separations in general, and can also be used to improve separation speed through the reduction in mobile-phase viscosity at higher temperatures (4,8). On the other hand, using a temperature that is too high—especially with the acidic mobile phases commonly used for reversed-phase separations of proteins—can cause hydrolysis of the siloxane bond that tethers stationary-phase ligands to the silica surface in the case of silica-based phases. Advances in stationary-phase chemistry over the years have considerably improved the chemical stability of some silica-based phases at low pH (4,9). Finally, prolonged exposure of protein analytes to these conditions can also cause on-column degradation of the protein. So, as was the case with pore size discussed above, the question here, too, is "How hot is hot enough?"

First and foremost, users should abide by the guidance of the column manufacturer with respect to the prescribed temperature limits for the column. Then, we consider the following questions:

- Recovery of protein from the column—in other words, does everything that we inject come out within the analysis time?
- Stability of the protein during the separation—is there any detectable degradation inside the column?

Although we don't believe the cause of incomplete recovery is well understood, what we do know from experiments is that using column temperatures that are too low can lead to incomplete recovery of the protein from the column, and that increasing the column temperature improves the recovery. Figure 2 shows a compelling example of this effect from our own work in the case of reversed-phase separations of an intact mAb protein (10). Whereas at 40 °C the protein peak is barely detectable, it becomes more apparent at 50 °C and keeps increasing in size up to 80 °C, which was the maximum temperature explored in this work. Based on these data and other results from our own work (11), as well as those from other groups (4), we generally use column temperatures of 70–90 °C for reversed-phase separations of proteins in our laboratories. However, when working at these temperatures, we also try to limit on-column times (that is, retention times) to less than about 20 min to minimize the likelihood that proteins will degrade inside of the column. Very recent results suggest a future where this requirement of elevated column temperature may be relaxed to some extent through development of new stationary-phase materials (7).

To some extent these issues of incomplete recovery (adsorption) and on-column degradation of the protein are protein- and column-specific. This situation means that, while we use the guidelines described above as a starting point in method development, it is also a good idea to experimentally determine the conditions needed to maximize recovery and minimize degradation for the protein or sample of interest. Realistically, the inherently complex and dynamic nature of protein structures limits our ability to predict interactions between proteins and their surroundings, and thus their adsorption and retention properties under reversed-phase conditions. For these reasons, screening methods have become quite popular as a means to empirically evaluate some specific protein properties, including chromatographic behaviour and developability as therapeutic molecules.

What About the Stationary-Phase Chemistry?

Many stationary phases marketed for reversed-phase protein separations are commercially available. Some of these are based on particles composed entirely of organic polymers. Others are based on silica and other metal oxides that have stationary phases either covalently bonded to or coated on the particle surface. As a practical matter, it is reasonable to ask how the stationary-phase chemistry affects reversed-phase separations of proteins. In the case of silica-based phases, the type of alkylsilane bonded to the surface can influence the retention of proteins and can therefore be used to manipulate the retention and, to a lesser extent, selectivity. Although the detailed molecular basis of the effect of stationary-phase ligand structure on protein retention is not fully understood, we know from

experiments that a number of factors influence retention. These factors include the relative hydrophobicity of the ligand, surface coverage, ligand density, carbon load, flexibility of the ligand, and degree of exposure of the surface silanols. In addition, the choice of ligand can also influence the recovery and conformational integrity of the protein analytes.

Historically, it was assumed that shorter and less hydrophobic n-butyl ligands provide better recovery than octyl or octadecyl ligands (C8 and C18 phases, respectively). Therefore, in the past mostly butyl (that is, C4) and propyl (C3) phases were used for protein separations. However, with modern commercially available phases, it now seems that there is no significant dependence of protein recovery on the length of the stationary phase alkyl ligand (10). Significant differences in the retention of proteins are sometimes observed when comparing columns with different bonded phases. For example, in several cases, it has been observed that proteins are more retained on C4 stationary phases than they are on C8 or C18 phases. The reason for this behaviour may be that large solutes (proteins) do not penetrate into the bonded-phase layer like small molecules do (12), because the proteins are simply too big compared to the available space between ligands. In this way, proteins probably interact with the stationary phase mainly at the interface between the bonded-phase layer and the bulk mobile phase—in other words, proteins only experience a "bird's-eye view" of the stationary phase (1). In most cases, the ligand density of shorter chains is larger than that for longer chains, thus the accessible hydrophobic surface area is larger for phases modified with short alkyl ligands compared to longer ligands. In addition, if there are residual unbonded silanols present on the silica surface, they will be more accessible in cases where the stationary phase is composed of short-chain ligands (hydrogen-bonding and ion-exchange interactions have longer interaction distances than dispersive interactions).

Our perspective is that most alkyl bonded phases (for example, C3, C4, C8, and C18) are viable options for proteins separations, provided that conditions are optimized for a particular material. This optimization should consider—at a minimum—pore size, mobile-phase temperature, and mobile-phase additives (for example, trifluoroacetic acid). Some recently introduced materials look promising from the point of view of ease of use (7), and it will be interesting to see how these offerings continue to evolve in the near future.

Summary

Over the past decade, many new materials and columns have been introduced for reversed-phase separations of proteins. The good news from this trend is that users now have many more commercially available materials to choose from. The challenge, though, is sorting out which one will be best for a particular application, as well as finding optimal separation conditions. In this instalment, we have briefly discussed the importance of pore size, column temperature, and stationary-phase chemistry for reversed-phase separations of proteins. The ideas discussed here should be helpful to users beginning method development, or troubleshooting the performance of an existing method. Much remains to be discussed, both in terms of additional considerations for reversed-phase separations, and for other separation modes including ion-exchange and size-based separations, and we look forward to discussing these issues in future instalments.

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Split, Splitless, and Beyond—Getting the Most From Your Inlet

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While capillary gas chromatography has been undergoing a renaissance, with new columns, detectors, data systems, and multidimensional separations, the classical inlets have remained the same: We are still injecting liquid samples with syringes into split and splitless inlets, as we have for nearly 50 years. Split and splitless injections present several well-known and some not-so-well known challenges, mostly arising from heating of the inlet, that make sample injection and inlets a major hurdle for gas chromatographers. These challenges and some ideas for mitigating them are discussed and a case is made for renewed exploration of the cool inlets and injection techniques: cool on-column and programmed temperature vaporization.

Gas chromatography (GC) has seen some tremendous advances over the past two decades. New instruments have come to take much fuller advantage of the capabilities of capillary columns. New technologies include sorptive microextraction techniques such as solid-phase microextraction (SPME), fast GC, comprehensive multidimensional GC (GC×GC), capillary columns that are highly inert (mass spectrometry [MS] designated) and selective (specialty and ionic liquid), and new detectors, including benchtop MS-MS and vacuum ultraviolet (VUV) detectors. They demonstrate that new research on GC is still coming. It is poised to remain a staple analytical technique well into the future.

With all of that development, it is ironic that the one area that perhaps baffles chromatographers the most, the inlet, has received little attention. Four inlets are used on main-line instruments: split, splitless, on-column, and programmed temperature vaporization (PTV). By far the most commonly used inlets are split and splitless, which were developed in the 1950s and 1960s; the most recently developed inlet, PTV, was invented in 1979. Although computer control of pneumatics and highly inert glass sleeve materials have marginally improved split and splitless as inlets, the fundamental problems that have baffled chromatographers for 50 or more years remain.

Split and Splitless—The Old Reliable Standbys

The vast majority of gas chromatographs manufactured today include a single inlet, often called a split–splitless inlet, that performs two types of liquid sample introduction: split and splitless. To varying degrees, split and splitless injections address five fundamental problems with injecting liquid samples into a capillary GC system:

- The needle problem: Most syringe needles do not fit into a capillary column.
- The mass problem: You can only inject small amounts of sample onto a capillary column (1 µL of a liquid sample weighs about 1 mg) and you never know how much sample you actually injected.
- The time problem: You never know exactly how long the injection process takes or how wide the peaks will be at the head of the column.
- The contamination problem: Dirty samples are bad for the column, and contaminants can collect in the inlet or at the column head and interact or react with analytes. Analytes can also interact with the surfaces inside the inlet.
- The discrimination problem: Heating of the inlet or syringe needle can cause some analytes to remain in the needle or inlet while others transfer to the column.

A schematic diagram of a split–splitless inlet is shown in Figure 1. Note that the inlet has a high thermal mass and is typically heated to a temperature well above the normal boiling point of most samples, often 250 °C. As seen in the figure, the five fundamental problems are partially addressed by the inlet design and how it is operated. A simple review of some basic principles can be accessed online at *LCGC Asia Pacific*'s ChromAcademy (1). For a discussion of the basics of split and splitless inlet maintenance, see the May 2018 issue of *LCGC Europe* (2). Grob's classic book on split and splitless injection provides over 800 pages of fundamentals, theory, and practice related to split and splitless injections (3).

The needle problem is addressed by a glass sleeve, which serves as a venue for the transfer of the sample from the syringe needle to the column.

Figure 1: Schematic diagram of a split–splitless inlet.

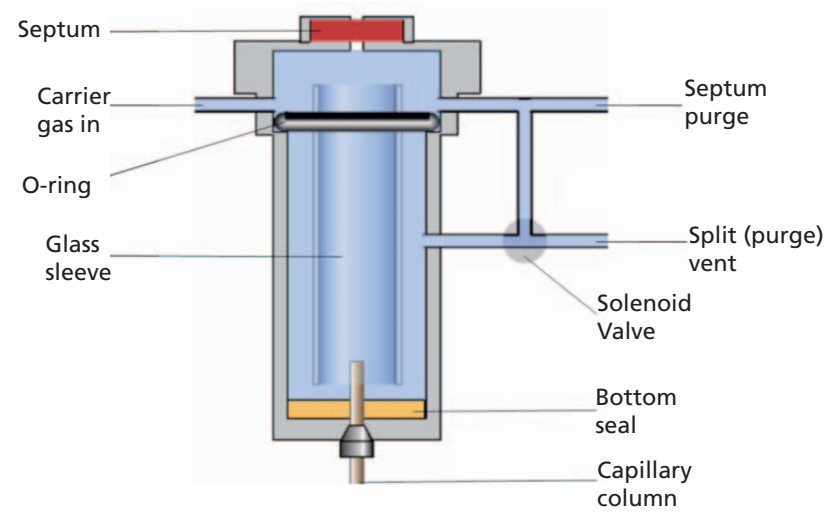
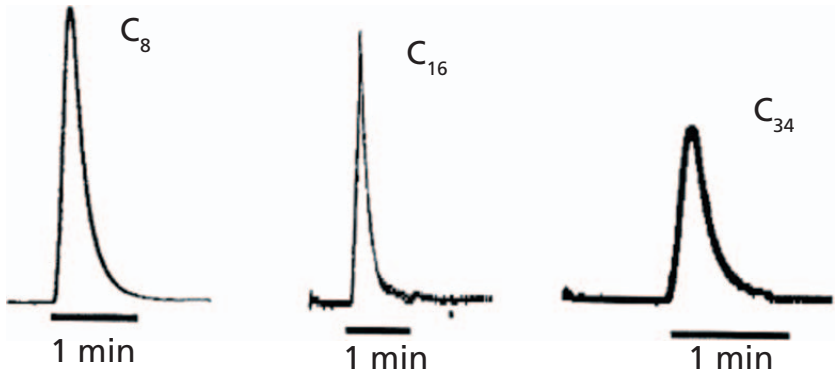


Figure 2: Injection profiles for three alkanes under splitless conditions from SPME using a 2-mm i.d. glass sleeve.



All common syringe needles can easily fit into the glass sleeves used in split and splitless inlets. The mass problem is clearly evident in a split injection, as can be illustrated with a simple example. A 1-mg (1-µL) amount of a liquid sample with a 100:1 split ratio allows about 10 µg of sample to reach the column. If the analyte concentration is 1 ppm, then the mass reaching the detector, assuming none is lost in the column or inlet, can be estimated as 10 pg, which is below the detection limits of many detectors. Split injection, although considered simple, is very limited for trace analysis.

Splitless injection partially mitigates this problem because it allows introduction of nearly all of the injected sample into the column. In the above example, the split ratio would not be included, so the mass going onto the column is estimated as 1 ng, which is much more suitable for most detectors. A second mass problem arises with the term "estimated" to describe the mass of analyte reaching the column in both of the above examples. Although calibration techniques can mostly mitigate this problem for most quantitative analysis, the actual mass of analyte reaching the column is not accurately known in either split or splitless injection.

Recently, Bai and colleagues used vacuum ultraviolet detection, which allows for pseudo-absolute quantitation without using standards, to examine the efficiency of split and splitless injections (4). They analyzed several variables, including split ratio, splitless "purge off" time, and injection volume. In an investigation of split ratio, in all cases with split ratios ranging from 5:1 to 200:1, the actual mass measured by GC–VUV was significantly lower than the expected mass calculated using the sample concentration, injection volume, and split ratio. Performance was seen to get worse at lower split ratios.

In both split and splitless injection, it is important to avoid overloading the glass sleeve with too much vapour when the sample evaporates. Many typical glass sleeves have a volume of 1 mL or less, but depending on the solvent, a 1-µL liquid volume can have a volume of 200 µL to 1.2 mL or more when it is fully evaporated. To assist in addressing this problem, there are several solvent vapour volume calculators available online (1,5–6). These calculators are useful for estimating the solvent vapour produced during the injection process and comparing it to the volume of typical glass sleeves. Be sure to keep the solvent-vapour volume lower than the glass-sleeve volume. This effect can also contribute to mass problems if solvent vapour backflushes into the gas lines that feed the inlet, taking some of the vaporized analytes with it.

In splitless injections and to a small extent in split, especially at low split ratios, the time problem presents a significant challenge, termed by Grob (3) as "band broadening in time". During the "purge off" time period in a splitless injection, gas flow through the glass sleeve is reduced to match flow in the column. For many typical capillary columns, this flow rate is about 1 mL/min. If the glass sleeve has a volume of about 1 mL then it can take about 1 min for the carrier gas to sweep the injected sample into the column, so the initial peak reaching the column is about 1-min wide!

Figure 2 shows some profiles from actual peaks as they leave the inlet under splitless conditions (7,8). These profiles were obtained by placing a very short empty fused-silica transfer line between the inlet and a flame ionization detector, and using SPME to perform the injection (so there is no solvent present). Note that the peaks are about 1-min wide and are not fully symmetrical. The bulk of the peak broadening is due to the time required for the sample to traverse from the SPME fibre through the glass sleeve and into the column. Note also that traces of the sample may remain in the inlet for a long period of time, often

much longer than the "purge off" time. Minimizing the time required for the sample to traverse the glass sleeve should be a method development goal.

Clearly, the peaks resulting from most splitless injections do not end up 1 min wide at the detector and on chromatograms. There are several peak-focusing mechanisms at play, all described in great detail in Grob's book (3). These mechanisms lead to some important optimization points specific to splitless injections:

- Use cold trapping to refocus high-boiling solutes. If possible, start your temperature program at least 100 °C below the normal boiling point of the analytes.
- Use solvent effects to refocus low-boiling solutes. Start your temperature program 30–40 °C below the normal boiling point of the solvent. For most solvents and for convenience I start it at 40 °C. The impact of solvent effects decreases as the analyte normal boiling point increases.
- Use a narrow-bore straight-tube glass sleeve, but beware of possible liner overload as the solvent evaporates. Obstructions and materials such as glass wool collect dirt and can cause as many problems as they solve.
- Match the polarity of the solvent to the polarity of the column. Use nonpolar solvents with nonpolar columns.

To take advantage of these optimizations, all methods using splitless injection should be temperature programmed.

Contaminant problems relate to the composition of the sample, and the chemistry of the surfaces inside the inlet and column can be especially challenging. Obviously, dirty samples can shorten column life by fouling the column or the inlet. The design of split–splitless inlets that use a glass sleeve does prevent some column fouling by providing a landing place for nonvolatile sample components. However, it is possible for nonvolatile sample components or residual components left behind after splitless injections to remain on the surfaces and contaminate them.

Thinking about samples interacting with the surfaces within the inlet leads to a simple question with a surprisingly complicated answer.

What Really Happens When a Liquid Sample Ejects From a Syringe Into a Split–Splitless Inlet?

Most short courses and books introducing GC tell us that the injected sample is flash vaporized and then mixed with the carrier gas and transferred as a homogenous mixture in the vapour phase into the column. Numerous glass sleeves have been designed to facilitate this concept. However, two common sense experiments demonstrate that this description of the process is not accurate and that care is required to avoid reactions and contamination in the inlet.

Figure 3 shows the result when a small volume of about 5 mL of water—analogous to the sample—is poured onto a hot cast iron skillet, analogous to the hot inlet surfaces. This experiment was done at home, but it also works in the laboratory with a hot plate. Not only does the water not evaporate immediately, but, because of the formation of a vapour layer between the water and the surface, it is seen to dance. This is what really happens when a liquid sample strikes the surfaces within a heated inlet. It is clear that the evaporation processes in heated inlets are not easily controlled and certainly are not reproducible. This effect is also the primary cause of syringe needle discrimination, which is common in manual injections and is caused by the heating of the syringe needle in the inlet.

The second simple experiment is to simply see how far you can shoot some water with a typical 10-µL syringe, used for GC. It is easy to shoot the liquid much farther than the length of the inlet. This experiment demonstrates that the liquid leaving the syringe is not likely to spread out and flash vaporize upon exiting the syringe. It is likely to shoot straight to the first surface it encounters, then evaporate in a manner analogous to the skillet example. Grob's book (3) includes a DVD with several videos demonstrating this process. A few of these videos can be seen online (9).

These heating effects are the primary cause of the fifth challenge: discrimination. They can cause some analytes to preferentially evaporate and be carried to the column, whereas other components do not evaporate as efficiently and are only partially transferred or do not enter the column at all. Because the heating is not controlled well, neither is the discrimination. Discrimination and contamination in the inlet are leading causes of precision and accuracy problems in gas chromatography.

Based on the five challenges, the following quick-hitting keys can help analysts reduce discrimination and contamination and get the most from classical split and splitless inlets:

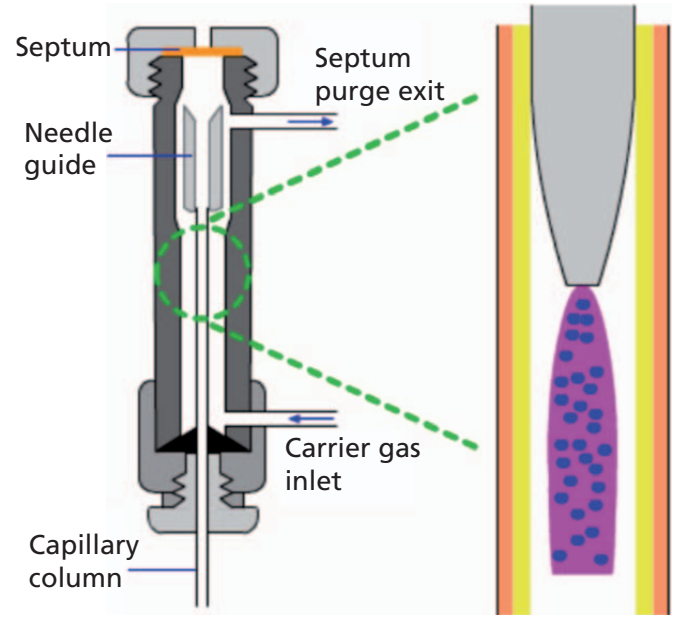
1. Keep the inlet heated and with carrier gas flow at all times to reduce contamination. Gas saver mode between runs will reduce carrier gas consumption.
2. Use a fast autosampler for liquid sample injections—faster is better.
3. Use the best glass sleeve for your sample. The sleeves for split and splitless are different. There is no magic bullet sleeve for all samples. You should test several different sleeves when optimizing your method.
4. Check cleanliness and clean the inlet on a regular basis. Use a flashlight to look for debris at the bottom of the inlet, and clean it out if you see any.
5. Change the septum often. The wide, blunt needles used with many autosamplers can core them quickly and cause leaks.

Why Are We Still Doing Hot Injections?

Most of the challenges involved with using split and splitless inlets occur because the inlet is heated. The reasons for heating these inlets arose from packed-column GC, in which strong heating is the only way to make analytes pass through the column. In classical packed columns, the large mass of stationary phase present in the column in gas–liquid chromatography, or a large surface area in the case of gas–solid chromatography, generally means that analyses are carried out at temperatures above the normal boiling points of the analytes. Strong heating throughout the separation, including injection, separation, and detection, is therefore required. Packed-column inlets are also generally very simple: The syringe needle fits directly into the column, so many of the problems with capillary inlets described above do not occur.

Figure 3: Result of about 5 mL of water added to a hot cast iron skillet. Full evaporation of the water required about 10 s. Note that some droplets are coloured, which is the result of contamination on the skillet surface.

Figure 4: Schematic of a cool on-column inlet showing major components and the syringe needle entering the column.



In capillary GC, the low mass of stationary phase present in the column changes the equilibrium conditions on which GC separations are based. With most thin and moderate film thicknesses, separations are conducted at temperatures below the normal boiling points of the analytes. Classical split and splitless injections therefore involve ejection of the sample from the syringe, evaporation in the glass sleeve, transfer of the vapour through the glass sleeve into the column, and condensation into the stationary phase in the column. Each of these steps may involve complex chemical interactions and, as shown in Figure 3, they may occur in an uncontrolled fashion. This possible problem leads

to the question, "Why are we still doing hot injections?"

A false answer to that question is that there are no alternatives. There are two alternatives available for nearly every capillary gas chromatograph: on-column inlets and PTV. Both of these inlets eliminate most of the contamination and discrimination problems related to the hot injection and surface of the glass sleeve by injecting the sample into a cool inlet instead of a hot inlet. This approach keeps both the syringe needle and inlet cool throughout the injection process. The inlet is then heated to move analytes into the column after the syringe is removed. On-column and PTV inlets have been available for decades, yet they remain niche techniques even though they can reduce or eliminate the major complaints about split and splitless inlets.

An on-column inlet is exactly that: The syringe needle is inserted directly into the end of the column and the liquid sample is deposited onto the column head. The inlet is then temperature programmed along with the column to generate the separation. Using this inlet completely eliminates heating during the injection and the glass sleeve, removing all of the problems described above that these steps can generate. Cool on-column inlets are especially useful for trace analysis of thermally labile analytes, especially where an inert inlet is required. The greatest strength of an on-column inlet, that the entire sample that exits the syringe enters the column, is also its greatest weakness. If the entire sample enters the column, so do any nonvolatile matrix components that can easily foul the column after only a few injections.

Besides the possibility of column fouling, the other major drawback of on-column inlets is the syringe. Traditional syringes may be used with 530-µm i.d. megabore columns; however, columns with smaller inside diameters require special syringes with tapered needles or fused-silica needle extensions that can be very fragile and difficult to handle.

Figure 4 illustrates the components of a typical on-column inlet. Similar to other inlets, during an injection the syringe passes through a septum. A needle guide ensures that the syringe needle is properly lined up with the column so that the needle can pass into the column without catching or bending. In a manner similar to a packed column inlet, carrier gas passes around the outside of the column, then through the needle guide and into the column. A septum purge is used to keep the septum clean, which is especially important because the inlet is cooled most of the time. A liquid sample leaving the syringe is depicted on the right side of Figure 4. As the liquid exits the syringe, it will coat the first several centimetres of the inside of the column. As the column temperature is raised, the liquid will be removed in a manner similar to the solvent effects in a splitless injection.

Addition of a precolumn followed by a tee connector with outlets to the analytical column and an external vapour exit adds solvent vapour exit capability that can allow an on-column inlet to be used with large-volume injections up to several hundred microlitres, with the accompanying improvements in detection limits (10).

The advantages of a cool on-column inlet are clear, because the sample is injected without heating. This approach allows samples to exit the syringe without undergoing losses resulting from needle heating, and it deposits the entire injected sample into the column with no losses caused by the heated inlet and glass sleeve. Simplicity and low carrier-gas usage are additional advantages. An on-column inlet is the inlet of choice if inertness and trace analysis are analytical requirements. The major disadvantage is that the column is directly exposed to the entire sample, so "dirty" samples may quickly foul the column, resulting in much greater need to trim or replace columns.

In 1979, Vogt and colleagues modified a split–splitless inlet, similar to the one shown in Figure 1, to allow for rapid heating and cooling of the glass sleeve (11,12). In short, they removed the high-thermal-mass block containing the glass sleeve and replaced it with a low-thermal-mass tube wrapped in heating tape. This modification allows the inlet to be operated in four modes: traditional hot split and splitless plus cold split and splitless. In the two cold modes, injection is followed by rapid heating of the inlet following injection, which transfers the sample to the column under much more controlled conditions than in traditional hot split and splitless modes. This inlet and process, PTV, is highly versatile but requires additional training and method development to use effectively. The PTV inlet became widely available in the 1990s, when it generated considerable attention in the literature. It is still available today, but it has not yet become popular, perhaps because of its additional cost and complexity.

Schematically, a PTV inlet is similar to the split–splitless inlet shown in Figure 1. The low thermal mass surrounding the glass sleeve allows rapid heating and cooling, and allows multiple modes of operation:

- Hot split is the same as traditional split.
- Hot splitless is the same as traditional splitless.
- Cold split and splitless involve injecting the sample under split or splitless conditions into a cooled inlet, typically into a packed or baffled glass sleeve. This technique allows the sample to eject from the syringe and land on the surfaces inside the inlet while cool, avoiding the evaporative effects similar to those shown in Figure 3. The inlet is then rapidly heated to drive the sample into the column. These techniques can be especially useful for thermally labile or sensitive analytes because the sample heating rate can be controlled. Cold split and splitless also reduce or eliminate sample losses resulting from vapour overload and syringe needle discrimination.
- Cold splitless solvent vent or large-volume injection is a variant of the cold splitless technique that allows injection of much larger sample volumes. A volume of up to hundreds of microlitres is injected into a cool packed glass sleeve. The packing serves to hold the liquid in place while the solvent is evaporated by carrier gas flow to a vent. There is no flow from the inlet to the column during this evaporation process. After about 95% of the solvent has evaporated, the remaining sample, including the now-concentrated analytes, is transferred to the column under splitless conditions.

On most gas chromatographs, a PTV inlet is not standard equipment. It offers the advantages of versatility, reduction or elimination of the contamination and discrimination problems that

challenge split and splitless, and large volume injection capability. The main disadvantage is that it is not as simple to operate. Method development for large-volume injections involves several steps. There are several introductory guides and application examples freely available online (13).

Classical heated split and splitless inlets and injection techniques have served gas chromatographers well for nearly 50 years. However, these inlets are heated continuously and strongly, and their effectiveness for sample introduction into capillary columns is limited. Heating of the inlet throughout the injection process causes several problems, including unnecessary sample losses, band broadening, and possible contamination. In hot split and splitless injections, the amount of sample actually reaching the column can only be estimated. These challenges make a case for gas chromatographers to explore cool on-column and PTV injections. Cool on-column and PTV have been available for almost as long as split and splitless, but have not been nearly as widely used. The several possibilities for cool on-column and PTV described here are only beginnings.

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When Do We Need Sub-2-µm Superficially Porous Particles for **Liquid Chromatography Separations?**

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The use of superficially porous particles (SPPs) for modern high performance liquid chromatography (HPLC) is now very common. Initially, SPPs rose as an alternative to sub-2-µm fully porous particles (FPPs). In recent years, many column manufacturers have developed 2-µm and smaller SPP-based products. This article investigates the practical utility of these smaller SPP designs.

The introduction of modern superficially porous particles (SPPs) by Advanced Materials Development in 2006 revolutionized high performance liquid chromatography (HPLC). Interest in these particles, characterized by a solid core and a porous outer layer, stemmed from the very high efficiency they produce at modest back pressures (1). During the time of SPP emergence, efforts to improve efficiency focused on decreasing the size of packed particles. The term "ultrahigh-pressure liquid chromatography", or UHPLC, was coined by Jorgenson in 1997, and was based on studies of nanobore columns packed with 1–1.5 µm particles (2). In 2004, Waters introduced the first commercial liquid chromatography system capable of operating at pressures as high as 1000 bar. Together with the availability of columns packed with sub-2-µm particles, the age of UHPLC was born. The small sub-2-µm particle size resulted in high system back pressures that required instrumentation capable of operating under such conditions. The SPP design proved to be a viable alternative to smaller fully porous particles (FPPs), because columns packed with sub-3-µm SPP materials provided similar efficiencies to sub-2-µm FPP columns at back pressures that were attainable on standard HPLC systems (3).

Since the initial rise of SPP columns with particle sizes around 2.7 µm, many manufacturers have developed stationary phases using smaller and larger particles, as well as particles

Figure 1: Comparison of 1.8- and 2.7-µm superficially porous particle columns for the separation of 34 perfluorinated compounds. Columns: (a) Raptor C18, 1.8 µm, (b) Raptor C18, 2.7 µm; column dimensions: 50 mm × 2.1 mm; mobile-phase A: 5 mM ammonium acetate in water; mobile-phase B: methanol; gradient: 20–95% B in 6 min; flow rate: 0.4 mL/min; column temperature: 40 °C; detection: mass spectrometry.

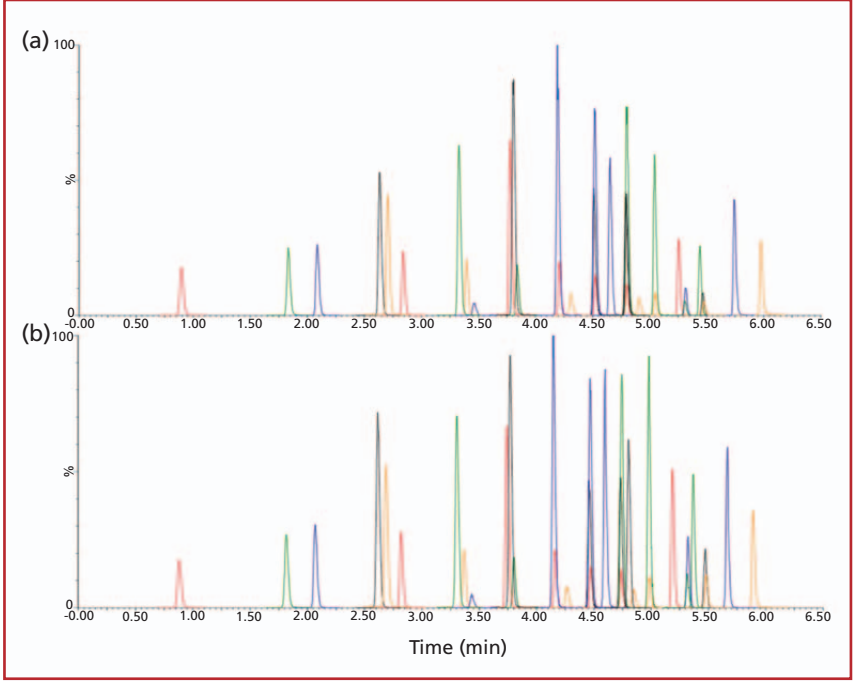
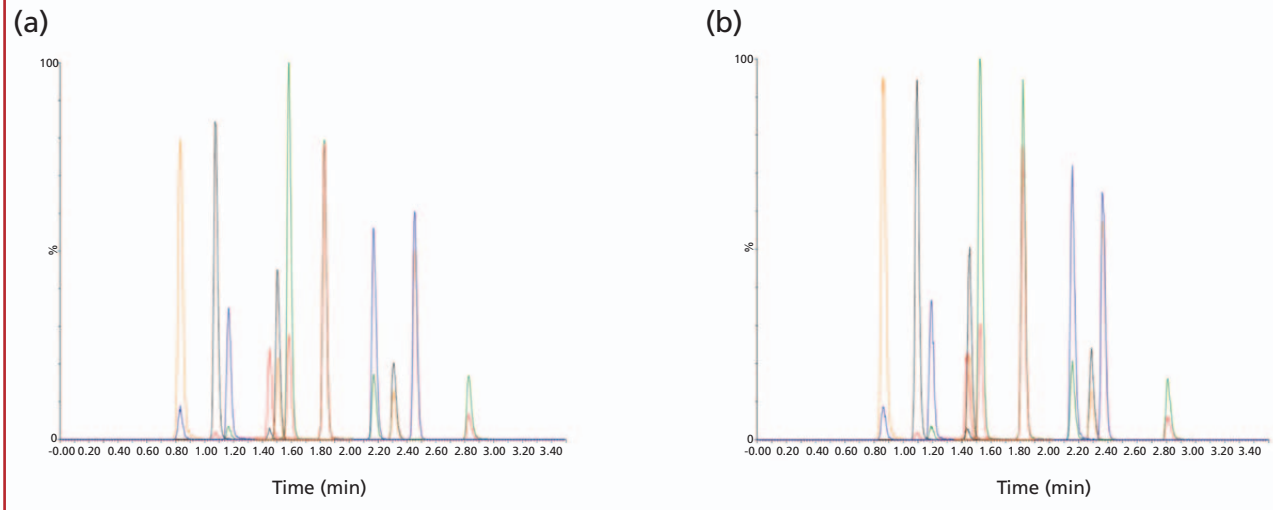


Figure 2: Comparison of 1.8- and 2.7-µm superficially porous particle columns for the separation of 11 artificial sweeteners. Columns: (a) Raptor Biphenyl, 1.8 µm, (b) Raptor Biphenyl, 2.7 µm; column dimensions: 50 mm × 2.1 mm; mobile-phase A: 0.1% formic acid in water; mobile-phase B: 0.1% formic acid in acetonitrile; gradient: 5–55% B in 3 min; flow rate: 0.3 mL/min; column temperature: 40 °C; detection: mass spectrometry.



with various pore structures. In 2014, Fekete and coauthors predicted that one future trend for UHPLC would be the incorporation of sub-2-µm SPP columns (4). The authors note that, because of significant improvements in eddy diffusion, moderate decrease in longitudinal diffusion, and some improvement in mass transfer resistance, SPPs offer a 30–50% increase in efficiency over same particle size FPPs, regardless of the particle size. Efficiency gains for smaller SPPs should thus trend similarly with what has been observed for FPPs. It was also noted that only two sub-2-µm columns were commercially available at that time.

In another paper, Fekete and Guillarme evaluated a 1.3-µm SPP phase, noting kinetic advantages over fully porous packed columns. Efficiencies of greater than 500,000 plates/m were observed as compared to 300,000 typically generated by a well-packed sub-2-µm FPP column. The authors noted that the performance of the column was limited by the current instrumentation. Lower extracolumn variance and higher pressure limits for both the column and the instrument were noted as potential improvements (5).

DeStefano and coauthors noted that the advantages of smaller SPPs include higher efficiency, improved detection sensitivity, less mobile-phase consumption per analysis, higher peak capacity, and the perception of state-of-the-art technology. The authors also noted the following disadvantages:

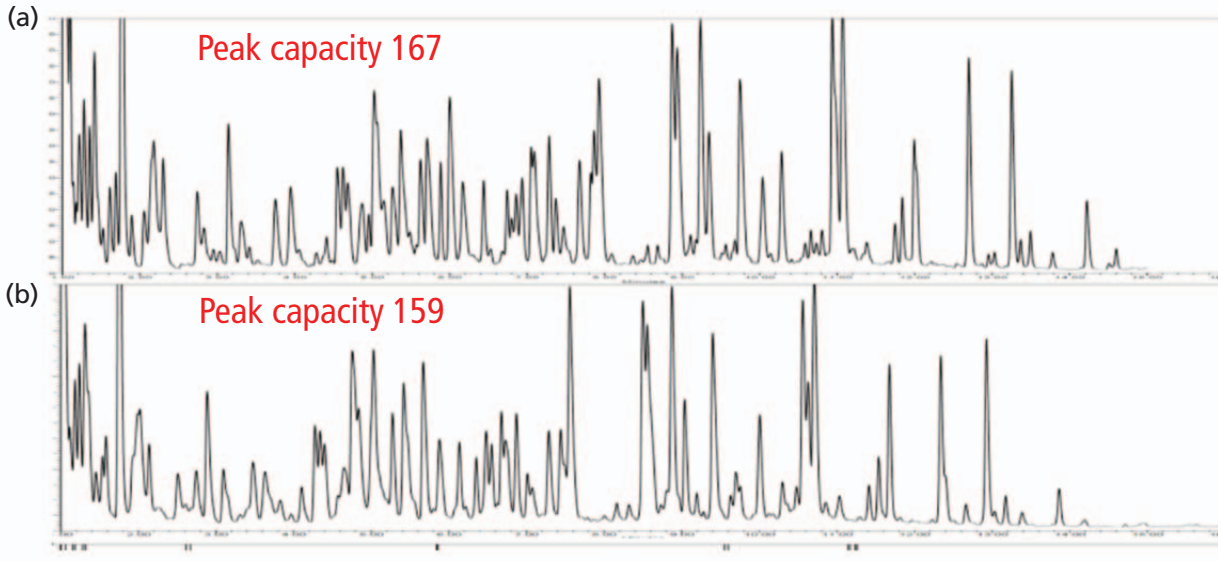
- Smaller particles require higher-pressure instruments, with minimal extracolumn volume, and small internal diameter connection tubing (plugs, increased back pressure);
- High-pressure operation may equal more frequent instrument repair;
- Smaller column frits may clog more easily than larger frits used for larger particle size columns;
- Columns may not exhibit expected efficiencies because smaller particles are more difficult to pack.

The authors noted that smaller particles do provide utility for some applications, but the efficiency advantage often does not overcome the disadvantages (6).

The availability of commercial 2-µm and sub-2-µm columns mid-year 2015 was still limited. Advanced Material Technologies and Supelco had introduced 2-µm column lines with several surface modifications. Phenomenex offered both a 1.3-µm (C18) and a 1.7-µm version of the Kinetex brand. Thermo Fisher Scientific commercialized a 1.4-µm C18 phase, and Waters launched a 1.6-µm platform with bare silica and C18 surface chemistries (7). In a 2016 review, no new providers entered into the 2-µm and sub-2-µm SPP market; however, two additional stationary phases, Kinetex EVO-C18 and HALO Peptide ES C18 (Mac-Mod), were made available (8). In 2017, Agilent introduced a number of chemistries on 1.9-µm SPPs, including bare silica for HILIC. ChromaNik introduced SunShell in a 2-µm format, Waters introduced 1.6-µm Cortecs with additional chemistries, and Phenomenex introduced a 1.7-µm SPP column intended for characterization of RNA and DNA (9). It is clear from this list that the trend towards smaller SPPs has indeed continued.

There have been many publications in recent years about studies employing SPP phases of all sizes for applications in a variety of disciplines. Within the literature scanned for this instalment of "Column Watch", no "applied" publications could be found that compared sub-3-µm SPP phases to sub-2-µm SPP phases explicitly. In a systematic evaluation of commercially available UHPLC columns for drug metabolite profiling, Dubbelman and coauthors found 1.3-µm and 1.7-µm SPPs to provide highly efficient separations. However, the system pressure experienced using the 1.3-µm particles was noted as an issue when higher flow rates were employed in an attempt to shorten run times (10).

Figure 3: Comparison of 1.8- and 2.7-µm superficially porous particle columns for the separation of trypsin-digested BSA. Columns: (a) Raptor ARC18, 1.8 µm, (b) Raptor ARC18, 2.7 µm; column dimensions 100 mm × 2.1 mm; mobile-phase A: 0.1% trifluoroacetic acid in water; mobile-phase B: 0.1% trifluoroacetic acid in acetonitrile; gradient: 10–20% B in 6 min, 20–40% B at 15 min; flow rate: 0.3 mL/min; column temperature: 60 °C; detection: mass spectrometry.



So the question still remains, "Does one need to use 2-µm or sub-2-µm SPP phases, or do sub-3-µm SPP phases suffice?" The answer depends on the analysis at hand. For example, Figure 1 shows a reasonably complex separation of perfluorinated compounds (PFCs) on both a 1.8-µm SPP and a 2.7-µm SPP. With very little difference observed in terms of sensitivity, resolution, or peak shape, it would be difficult to justify running this method at the higher back pressure using the 1.8-µm phase. Figure 2 shows a comparison of 1.8-µm and a 2.7-µm SPP-based biphenyl columns performing a separation of artificial sweeteners. In this case, the 1.8-µm phase shows additional resolution over the 2.7-µm phase. The more complex the sample, the higher the probability that higher efficiency will be needed. Figure 3 provides a comparison of a bovine serum albumin (BSA) tryptic digest run on both a 1.8-µm and a 2.7-µm SPP-based C18 column. As indicated by the higher peak capacity, using the smaller SPP column, the increased efficiency may become more important for such complex samples.

As with many so many questions when it comes to chromatography, the answer to whether one needs to enter into the 2-µm and sub-2-µm SPP realm is "It depends". It appears that, as the complexity of the separation increases, the probability of needing the extra efficiency of a smaller SPP increases. In many cases, however, closely eluted compounds, even in a two- or three-analyte separation, may require added efficiency and thus smaller SPP-based phases. In practice, the use of sub-3-µm SPP columns is typically a suitable compromise for method development. Adjustment to smaller (or larger) particle sizes during method optimization may provide a more suitable final method.

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David S. Bell is a director of Research and Development at Restek. He also serves on the Editorial Advisory Board for *LCGC* and is the Editor for "Column Watch". Direct correspondence to: LCGCedit@ubm.com

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Determination of Pharmaceuticals from Serum

Hans Rainer Wollseifen, Johannes Brand, and Detlef Lambrecht, MACHEREY-NAGEL GmbH & Co. KG

This application note describes the determination of pharmaceuticals from serum using solid-phase extraction (SPE) with the hydrophilic-lipophilic balanced SPE phase CHROMABOND® HLB for analyte enrichment and for sample cleanup. The eluates from SPE are finally analyzed by HPLC–MS/MS on a NUCLEOSHELL® PFP core–shell phase.

Nowadays, people suffer from various diseases, and are prescribed many types of pharmaceuticals as part of their treatment, for example, anesthetics, antibiotics, anticholinergics, anticonvulsants. In order for the treatment to be successful, it is necessary to keep controlling the levels of the pharmaceuticals to provide an accurate dosage. This has led to an increasing demand for the development of accurate and sensitive analytical methods to analyze the pharmaceuticals from serum to protect human health.

Table 1: SRM transitions for the investigated pharmaceuticals

Analyte	Retention Time (min)	$[M-H]^-$	Q_1 (Quantifier)	Q_2 (Qualifier)
Atenolol	1.12	267.2	145.2	74.1
Sulfapyridine	1.72	242.9	130.9	96.9
Atropine	1.81	290.2	124.2	93.0
Sulfamerazine	1.82	265.1	156.0	91.9
Ketamine	1.87	238.2	125.1	179.1
Chlorpheniramine	2.27	275.1	230.0	167.0
Sulfachloropyridazine	2.47	285.1	156.0	91.9
Sulfadoxine	2.63	311.1	156.0	92.1
Sulfamethoxazole	2.70	254.1	155.8	91.8
Propanolol	2.74	260.2	116.2	182.9
Diphenhydramine	2.94	256.1	166.9	152.1
Amitriptyline	3.04	278.2	223.0	91.0
Sulfaquinozaline	3.14	301.1	156.1	92.1
Nortriptyline	3.32	264.2	232.9	91.1
Verapamil	3.36	455.2	165.0	150.1
Trimipramine	3.41	295.2	100.1	58.0
Carbamazepine	3.50	237.1	194.1	193.0
Clomipramine	3.67	315.1	86.1	58.0
Indapamide	3.77	366.1	132.1	91.1
Ketoprofen	4.28	255.1	77.0	105.0

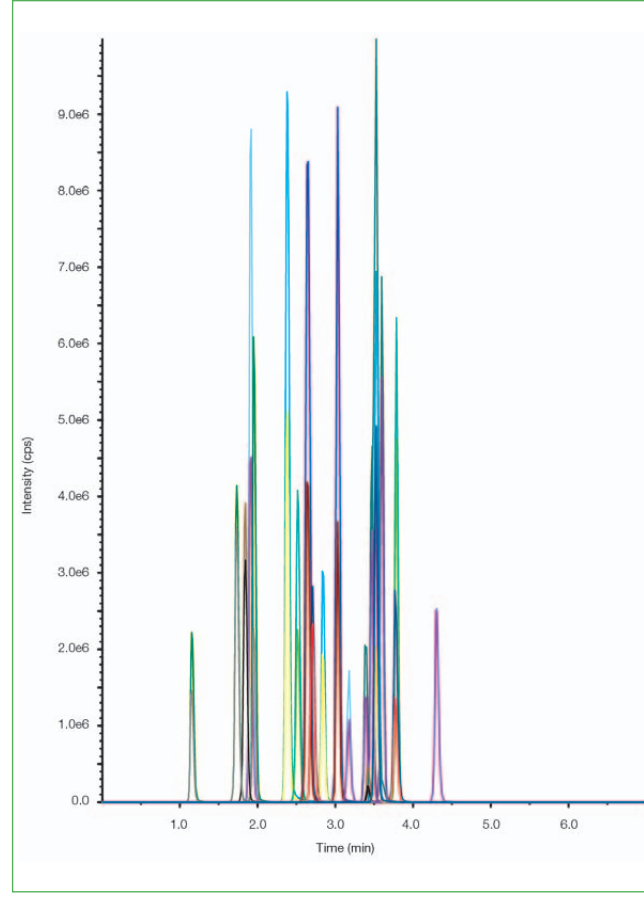

Figure 1: Chromatogram of serum sample spiked with 10 ng/mL for each pharmaceutical.

Solid-Phase Extraction (1)

SPE column: CHROMABOND® HLB, 1 mL, 30 mg, MACHEREY-NAGEL REF 730921
Column conditioning: 1 mL methanol, then 1 mL water
Sample application: 1 mL spiked serum sample is passed through the column by vacuum.
Washing: 1 mL water
Drying: 10 min with vacuum
Elution: 2 mL methanol
Eluent exchange: Eluate is evaporated to dryness at 40 °C under a stream of nitrogen and reconstituted in 1 mL 95:5 (*v/v*) water–acetonitrile.

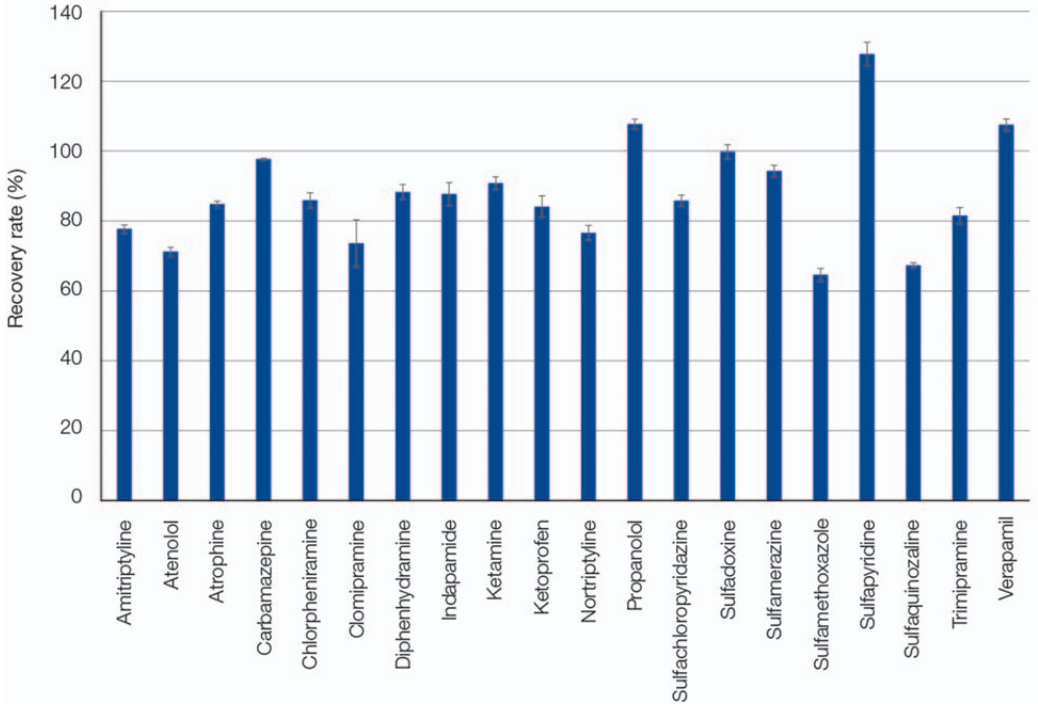

Figure 2: Recovery rates for solid-phase extraction method of pharmaceuticals from serum.

Subsequent Analysis: HPLC–MS/MS (2)

HPLC column: EC 50/2 NUCLEOSHELL® PFP, 2.7 µm, MACHEREY-NAGEL REF 763532.20
Eluent A: 0.1% formic acid in water
Eluent B: 0.1% formic acid in acetonitrile
Gradient: 5–95% B in 7.5 min, 95% B for 1 min, 95–5% B in 0.5 min, 5% B for 5 min
Flow rate: 0.3 mL/min
Temperature: 30 °C
Injection volume: 5 µL
MS/MS detection: API 5500 (AB Sciex GmbH); ion source: ESI; positive ionization mode; scan type: selected reaction monitoring (SRM, for transitions see Table 1); detection window: 90 s; curtain gas: 40 psig; ion spray voltage: 5500 V; temperature: 500 °C; nebulizer gas: 45 psig; turbo gas: 45 psig; CAD: medium.

Results

The recovery rates show that the determination of pharmaceuticals from serum could be carried out successfully (Figure 2). By using SPE with CHROMABOND® HLB, it was possible to recover nearly all pharmaceuticals from serum, with good reproducibility on average. Regarding the different types of pharmaceuticals, the average recovery rates were: for anesthetics 90.8%, antibiotics 94.4%, anticholinergics 84.8%, anticonvulsants 97.7%, antidepressants 77.4%, antihistamines 87.1%, anti-inflammatory drugs 84.1%, beta blockers 89.5%, calcium channel blockers 107.5%, and for diuretics 87.7%.

The identification and quantification of pharmaceuticals in the solid-phase extracts were performed using ESI-MS on an EC 50/2 NUCLEOSHELL® PFP column. The chromatogram in Figure 1 shows the results of solid-phase eluate spiked with 10 ng/mL serum for each pharmaceutical.

Conclusion

The presented application describes a quick and convenient method for the determination of pharmaceuticals from serum by SPE with a hydrophilic-lipophilic balanced phase, followed by HPLC–MS/MS analysis.

References

1. Application No. 306510, MACHEREY-NAGEL, available from www.mn-net.com/apps
2. Application No. 128200, MACHEREY-NAGEL, available from www.mn-net.com/apps

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Investigation of Iron Polysaccharide Complexes by GPC/SEC Using RI- and UV-Detection

PSS Polymer Standards Service GmbH

Gel permeation chromatography (GPC), also known as size-exclusion chromatography (SEC), provides an easy and effective way to measure the molar mass distribution and the amount of free, unbound polysaccharide in iron polysaccharide complexes.

Iron is an essential nutrient in the human body. In case of iron deficiency, complexes of a polysaccharide and iron are applied as drugs to enhance low iron levels. Suitable characterization of these complexes and their formulations are mandatory for regulatory reasons, quality control, and research. In the present investigation, iron polysaccharide complexes from different sources were analyzed on a GPC/SEC system with simultaneous ultraviolet/refractive index (UV/RI) detection.

Experimental Conditions

GPC/SEC was performed using a PSS BioSECcurity SEC system

Columns:	PSS SUPREMA, 5 µm, 30 Å + 2 ×1000 Å (8 × 300 mm, each) PSS SUPREMA precolumn
Eluent:	0.1 n $NaNO_3$, in 0.01 m phosphate buffer at pH = 7
Temperature:	Ambient
Detection:	UV @ 254 nm, refractive index (RI)
Calibration:	PSS Pullulan ReadyCal standards
Concentration:	2 g/L for dry material, approx. 50 g/L for formulations
Injection volume:	25 µL
Software:	PSS WinGPC UniChrom 8.2

Results and Discussion

Figure 1 shows the overlay of the UV-chromatograms of the four different samples A, B, C, and D, while the inset of the figure shows the overlay of the simultaneously measured RI-traces for two of the samples (A and B), which show nearly identical UV-traces.

An advantage of this application is that the iron polysaccharide complex is selectively detected by the UV-detector operated at 254 nm (20–26 mL). All complexes reveal well shaped nearly Gaussian peak shapes, indicating that the PSS SUPREMA column combination is ideal for this molar mass separation range. By applying a calibration curve, established using PSS pullulan standards, the relative molar mass distributions as well as the molar mass averages and the polydispersities are derived.

While UV-detection is sufficient to differentiate between three of the four samples, samples A and B render identical elution profiles. However, when comparing the RI-traces of both samples, it becomes clear that sample A contains a significantly higher amount of the unbound polysaccharide.

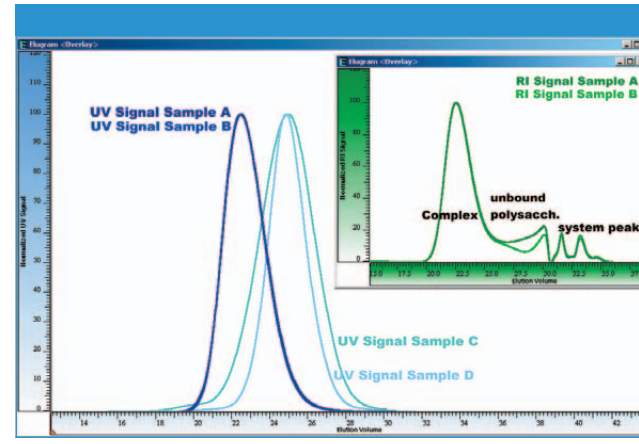

Figure 1: Comparison of the UV-traces of four different iron dextran samples used to determine the molar mass distribution of the iron complexes. While the UV-signals for samples A and B are nearly identical, the inset displaying the RI-traces shows that these samples differ in the amount of unbound polysaccharide.

We can therefore conclude that GPC/SEC with UV- and RI-detection does not only allow the molar mass distribution of iron polysaccharide complexes to be determined, but also provides information on the amount of free, unbound polysaccharide ensuring a more comprehensive characterization of the samples.

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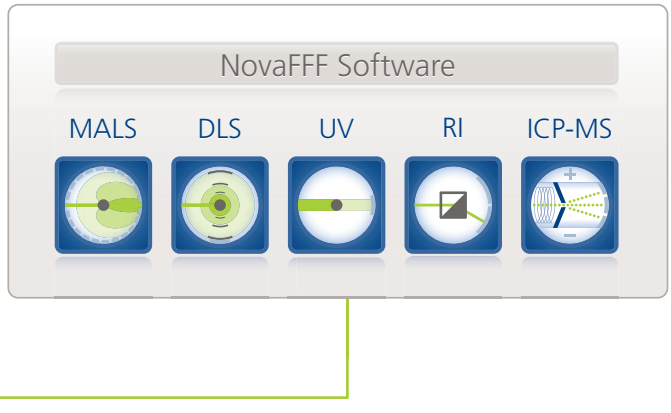

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