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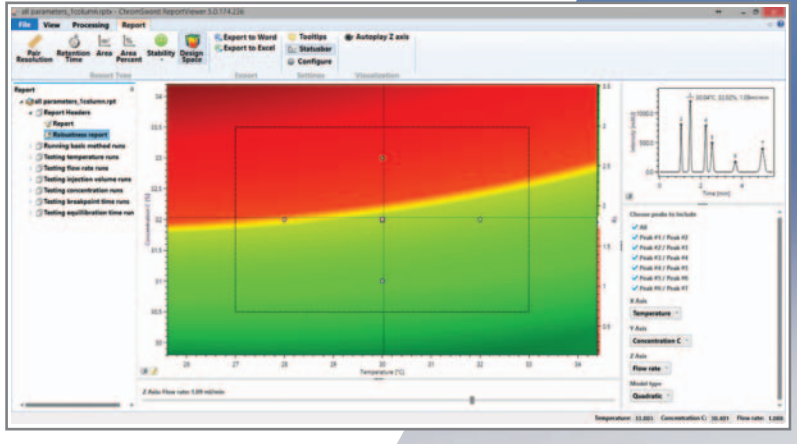

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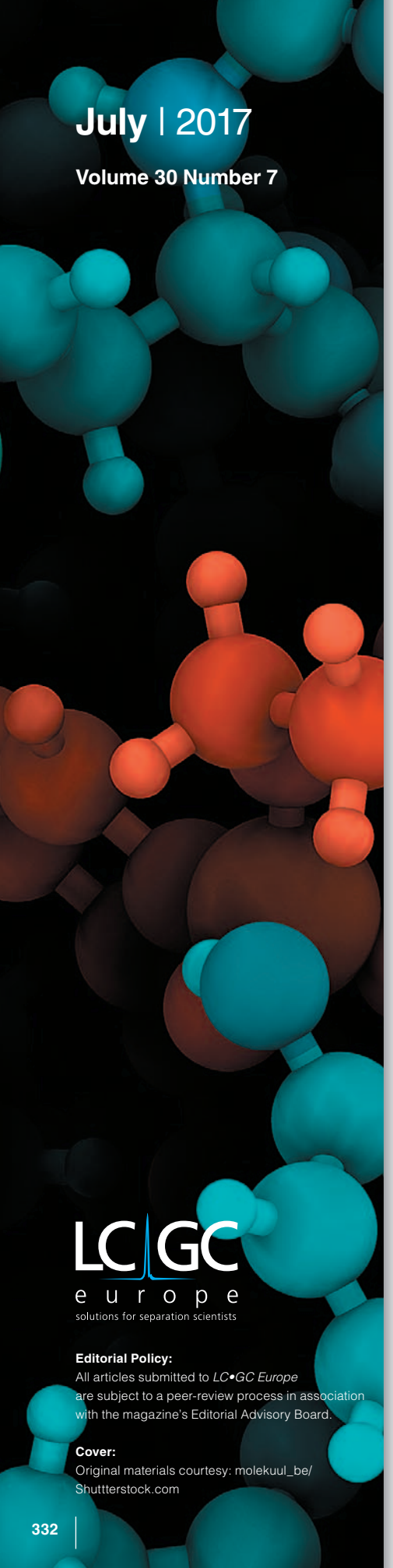

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A Review of MS-Based Approaches to Analyze Erectile Dysfunction Drugs in Botanical Dietary Supplements: **Are Those Products All Natural?**

Lukas Vaclavik[1] and Katerina Mastovska[2], [1]Covance Food Solutions, Harrogate, North Yorkshire, UK, [2]Covance Food Solutions, Madison, Wisconsin, USA

Botanical dietary supplements and herbal medicines advertised to naturally enhance sexual performance have become popular with consumers globally. A relatively high retail price and the availability of these products in the anonymous environment of the internet have made them a target for adulteration. Phosphodiesterase type 5 (PDE-5) inhibitors are widely used prescription drugs used to treat erectile dysfunction. Defrauders often use designer analogues of PDE-5 inhibitors synthesized by minor modifications of the parent molecules to avoid detection of the adulteration. This review highlights current mass spectrometry-based approaches and recent developments in screening and quantification of PDE-5 inhibitors in dietary supplements, and summarizes the role of this technique to detect the novel designer analogues.

The popularity and use of botanical dietary supplements has increased considerably over the past decade worldwide (1). The global dietary supplement industry is a growing business with sales that reached 84.5 billion US dollars and 5.4% annual growth in 2010 (2). Botanical dietary supplements represent a significant part of marketed items with relatively high retail value. These products are widely available and easily accessible to consumers in pharmacies, grocery stores, and on-line. The current legislation implemented in both European Union (EU) countries and the United States (US) does not require dietary supplements, including those containing botanicals, to be subjected to any specific regulatory pre-approval or safety assessment before they are introduced commercially (3).

It is commonly assumed that the consumption of botanical dietary supplements and herbal remedies is a safe and natural way to prevent various diseases and maintain good health, but there are several risks associated with the use of these products (3). As well as the potential presence of pathogens, toxins, contaminants, or chemical residues (4–7), botanical dietary supplements may be adulterated with active pharmaceutical ingredients (APIs), such as approved prescription drugs, their designer analogues, and patented drugs not undergoing clinical trials or pharmaceuticals that have been discontinued or withdrawn because of their adverse side effects (1,3). The aim of this fraudulent practice is to develop immediate pharmacological action that is in agreement with the claimed effect of the supplement. Such outcomes cannot typically be achieved by natural constituents alone, and the consumer may perceive the adulterated item to be more effective compared to natural products free of synthetic drugs. Marketing adulterated supplements can therefore result in increased sales and financial gain for the manufacturer or distributor. Considering the increasingly globalized supply chain of both dietary supplements and ingredients, the presence of adulterated products in the market is a global concern.

The botanical supplements and remedies most frequently targeted for adulteration with pharmaceuticals are for weight loss, body-building and athletic performance enhancement, and supplements intended to boost male sexual performance (3). The last group of products may be doped with phosphodiesterase type-5 (PDE-5) inhibitors, drugs that are widely used to treat erectile dysfunction (ED) (1,8,9). These drugs block the phosphodiesterase enzyme, which normally breaks down cyclic guanosine monophosphate (cGMP). cGMP causes relaxation of smooth muscle cells, allowing the flow of blood into the penis and producing the erection. PDE-5 inhibitors currently approved for treatment of ED in the EU and US are sildenafil citrate (Viagra, Pfizer Inc.), tadalafil (Cialis, Eli Lilly and Company), vardenafil hydrochloride (Levitra, Bayer HealthCare Pharmaceuticals Inc.), and

KEY POINTS

- Dietary supplements advertised for natural sexual performance enhancement may be adulterated with PDE-5 inhibitors and analogues.
- Liquid chromatography–mass spectrometry (LC–MS) is the most frequently employed strategy for the analysis of PDE-5 inhibitors and their analogues in dietary supplements.
- LC–MS instruments with accurate mass measurement capabilities are currently the most suitable tools for detection of new designer analogues.

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Figure 1: Chemical structures of sildenafil, tadalafil, vardenafil, and selected analogues. The grey areas highlight the structural differences between the analogue and respective parent PDE-5 inhibitor.

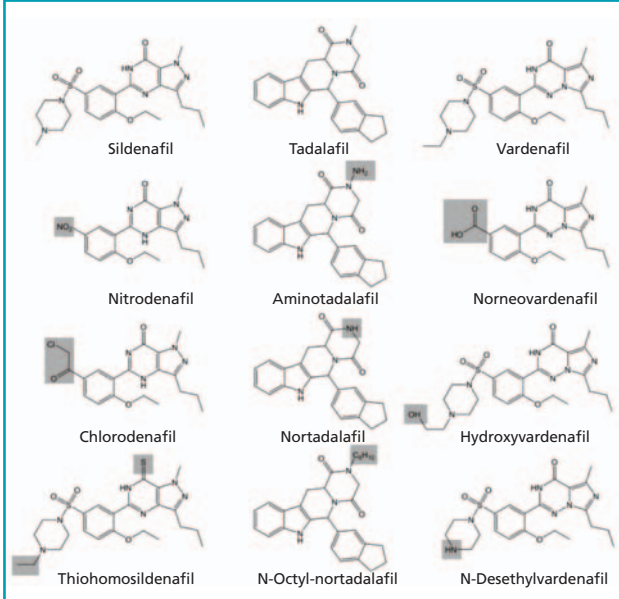
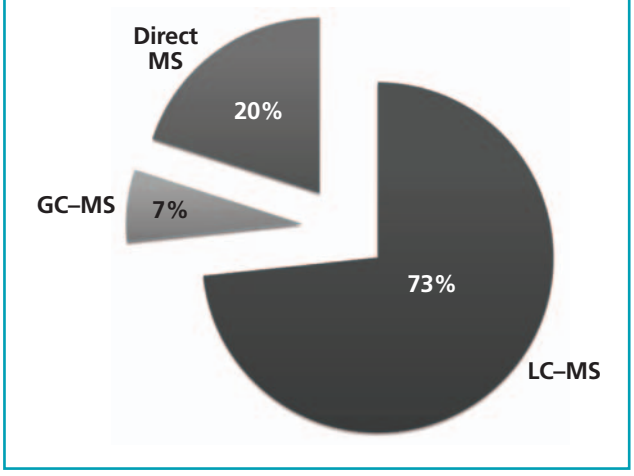


Figure 2: The percentage of MS applications focused on targeted qualitative and quantitative analysis of PDE-5 inhibitors in dietary supplements published between 2001 and 2017.



avanafil (Stendra or Spendra, Metuchen Pharmaceuticals) (10). The use of products with PDE-5 inhibitors obtained outside of the official health system and that promise to deal with symptoms of ED is increasing (8). According to Rocha *et al.* (1), 81 cases of PDE-5 inhibitor drug detection in products labelled dietetic foods, food supplements, or fortified food were reported in the EU Rapid Alert System for Food and Feed (RASFF) between 2010 and 2015. In the same period of time, the US Food and Drug Administration issued 229 notifications associated with the presence of PDE-5 inhibitors in dietary supplements. As a result of failure or lack of good manufacturing practices, adulterated supplements are often of poor quality in terms of purity, homogeneity, and API dosage (3). The recommended therapeutic doses for the approved PDE-5 inhibitors mentioned above range from 10 mg to 200 mg (11,12), but it is not unusual that the tainted supplements contain significantly higher concentrations for each single dosage form (for example, tablet or capsule) and occasionally may be spiked with multiple APIs. Needless to say, the uncontrolled intake of PDE-5 inhibitors can result in undesirable side effects, including potentially fatal hypotension, especially when taken concomitantly with other pharmaceuticals that lower the blood pressure, such as nitrates or α-blockers (8).

As well as the approved PDE-5 inhibitor drugs, their analogues are increasingly being used to adulterate the dietary supplement products, as defrauders attempt to avoid detection of adulteration by routine, targeted control testing (9). Analogues are substances with a chemical structure similar to that of sildenafil, tadalafil, and vardenafil (Figure 1), and may retain the desired pharmacological action. Most of these compounds represent a byproduct of the drug discovery process, in which numerous structurally related compounds containing the active moiety responsible for the pharmacological effect were evaluated. The information on the structure and synthesis of the vast majority of analogues therefore comes from disclosed patent literature describing the development of approved drugs (6,9). New designer analogues can also be developed *de novo* in small clandestine laboratories (6). In 2014, Patel *et al.* (9) reported that more than 50 different analogues have been used as adulterants in dietary supplements. Since PDE-5 inhibitor analogues have not undergone clinical trials, their efficacy or safety profiles are not known for these compounds, and their presence in dietary supplements may cause serious adverse effects to consumers.

To ensure that botanical dietary supplements and remedies are safe and free of PDE-5 inhibitor adulterants, there is an increased need for flexible analytical methods that allow rapid and reliable detection, identification, and quantification of these compounds. Mass spectrometry (MS) and especially hyphenated techniques, such as liquid chromatography–mass spectrometry (LC–MS), have become a primary tool in this endeavour (Figure 2). This review paper highlights current approaches and recent developments in PDE-5 inhibitor MS-based analyses, including those that aim at screening and structural elucidation of novel designer analogues. The potential of workflows using new technologies, such as ambient ionization MS (AIMS), is also discussed.

Mass Spectrometry-Based Approaches to Analysis of PDE-5 Inhibitors and Analogues

Sample Preparation: Determination of representative test portion and appropriate homogenization of the test material are critical factors that can influence the outcome of both qualitative and quantitative analyses. Considering potentially large and unpredictable variations in the adulterant concentration in single dosage forms, selection of too small a sample portion can result in false negative results or underestimation of the health risk associated with that particular adulterated product (13). Ideally, separate analyses of as many single doses (tablets, capsules) as possible should be performed. In practice, however, separate analyses are not always a conceivable approach and a

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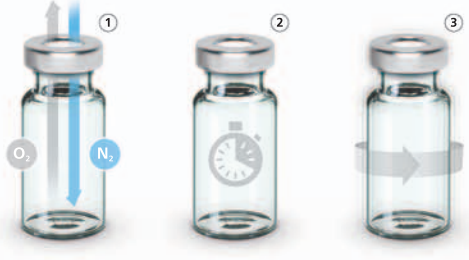

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Table 1: Overview of selected multianalyte LC–MS-based methods for targeted qualitative and quantitative analysis of PDE-5 inhibitors and analogues in dietary supplements and related products

Analytes	Analytical Column	Mobile Phase	Elution Program	Run Time	MS System	Ionization-Detection Mode	Quantitative	Ref.
50 PDE-5 inhibitors and analogues	100 × 2.1 mm, 2.6-µm Accucore aQ C_{18} (Thermo)	Acetonitrile–methanol mixture (1:1, *v/v*) with 10 mM ammonium formate and 0.1% formic acid–water with 10 mM ammonium formate and 0.1% formic acid	Gradient	24 min	Q-orbitrap	ESI+/full MS, all ion fragmentation MS^2 and data-dependent MS^2	Yes	26
Sildenafil, tadalafil, vardenafil, homosildenafil, hydroxyhomosildenafil, pseudovardenafil, yohimbine	150 × 2.1 mm, 5.0-µm PolymerX PSDVB (Phenomenex)	Acetonitrile with 0.1% formic acid–water with 0.1% formic acid	Gradient	30 min	QTOF	ESI+/full MS and data-dependent MS^2	Yes	17
28 PDE-5 inhibitors and other pharmaceutical adulterants	150 × 2.1 mm, 5-µm Zorbax Eclipse C_{18} (Agilent)	Acetonitrile with 0.1% acetic acid–water with 0.1% acetic acid	Gradient	45 min	IT-TOF	ESI+ and ESI-/full MS and data-dependent MS^2 and MS^3	No	25
Sildenafil, tadalafil, vardenafil, and other pharmaceutical adulterants	100 × 2.1 mm, 2.2-µm Acclaim RSLC 120 18 (Thermo)	Methanol–water mixture (9:1, *v/v*) with 0.1% formic acid–methanol with 0.1% formic acid	Gradient	14.5 min	orbitrap	ESI+/ full MS	Yes	22
82 PDE-5 inhibitors and analogues (synthetic and natural)	100 × 2.1 mm, 2.6-µm Accucore aQ C_{18} (Thermo)	Acetonitrile with 0.1% formic acid–water with 0.1% formic acid	Gradient	10 min	LIT-orbitrap	ESI+/ full MS and data-dependent MS^2	Yes	27
Sildenafil, tadalafil, vardenafil, other pharmaceutical adulterants, plant toxins and secondary metabolites	100 × 2.1 mm, 1.8-µm Acquity HSS T3 (Waters)	Methanol with 5 mM ammonium formate and 0.1% formic acid–water with 5 mM ammonium formate and 0.1% formic acid	Gradient	15 min	Q-orbitrap	ESI+/ full MS and data-dependent MS^2	Yes	18
38 PDE-5 inhibitors and analogues	100 × 2.0 mm, 3.0-µm Capcell MG2 C_{18} (Shiseido)	Acetonitrile–water with 2 mM ammonium formate	Gradient	15 min	QqQ-LIT	ESI+/ MRM (2 transitions per analyte)	Yes	23
18 PDE-5 inhibitors and analogues	100 × 2.0 mm, 2.2-µm Shim-pack XR-OSD II C_{18} (Shimadzu)	Acetonitrile–water with 12 mM ammonium formate and 0.01% acetic acid	Gradient	18 min	QqQ	ESI+/ MRM (2 transitions per analyte)	Yes	19
Sildenafil, tadalafil, vardenafil, homosildenafil, hydroxyhomosildenafil, sulfoaildenafil	150 × 2.1 mm, 3.5-µm Zorbax C_{18} (Agilent)	Acetonitrile with 4 mM ammonium formate and 0.05% formic acid–water with 4 mM ammonium formate and 0.05% formic acid	Gradient	16 min	QqQ	ESI+/ MRM (2 transitions per analyte)	No	20

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Table 1: (Continued)

Analytes	Analytical Column	Mobile Phase	Elution Program	Run Time	MS System	Ionization-Detection Mode	Quantitative	Ref.
24 PDE-5 inhibitors and analogues	75 × 2.0 mm, 2-µm Shim-pack XR-OSD II C_{18} (Shimadzu)	Mixture of acetonitrile and water (95:5, *v/v*) with 2 mM ammonium formate and 0.2% formic acid–water with 2 mM ammonium formate and 0.2% formic acid	Gradient	15 min	QqQ-LIT	ESI+/ MRM (1 transition per analyte) data-dependent product ion scan and precursor ion scan	Yes	24
Sildenafil, vardenafil, tadalafil, homosildenafil, acetildenafil, hydroxyhomosildenafil	150 × 2.0 mm, 5-µm Luna C_{18} (Phenomenex)	Mixture of acetonitrile and water (62:38, *v/v*) with 10 mM ammonium formate	Isocratic	16 min	QqQ	ESI+/ MRM (2 transitions per analyte)	Yes	15

compromise has to be found. In this regard, analysis of a composite sample prepared by pooling individual doses may decrease the probability of false negative results (13). Capsules and softgels should always be homogenized and further processed with the shell because the adulterants can be embedded in this part of the dosage form to avoid detection (14). Disintegration in the presence of dry ice or liquid nitrogen may be necessary to allow for sufficient homogenization of whole capsules and softgels.

Simple and straightforward "dilute and shoot" procedures that involve sample extraction with organic solvent, such as methanol, acetonitrile, or their mixtures with water (up to 50% aqueous component), followed by centrifugation and filtration and dilution, are usually performed prior to MS-based analysis of PDE-5 inhibitors in solid botanical supplements and similar products (1,3). In some studies, acetonitrile was preferred as the extraction and diluent solvent over methanol because of solubility issues of tadalafil and analogues in the latter solvent (15,16). Decrease of signal suppression during electrospray ionization (ESI) of herbal supplement extracts was reported for PDE-5 inhibitors and analogues after the addition of formic acid into the extraction mixture (1% in 50:50 [*v/v*] water–acetonitrile), probably because of precipitation of some matrix components (17). Liquid samples are usually diluted with a suitable solvent or analyzed directly (18). Since the adulterants are added externally to the sample, these compounds are not bound to the matrix components. Relatively short extraction times are sufficient to obtain acceptable recovery of the analytes from tested samples. The majority of available procedures do not involve any cleanup step, and direct analysis of crude extracts is performed.

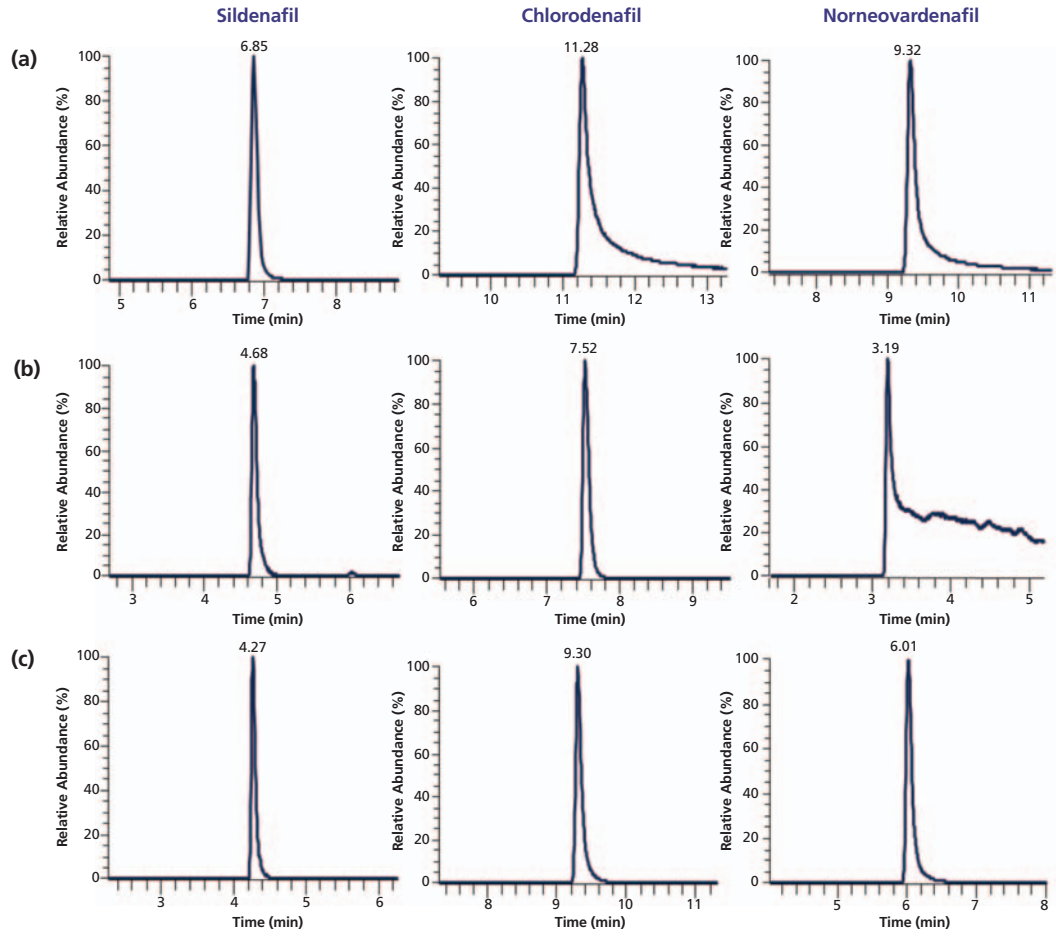
Liquid Chromatography–Mass Spectrometry:

LC–MS is the most frequently used strategy to analyze PDE-5 inhibitors and their analogues in botanical dietary supplements and similar samples (1,3). The popularity of LC–MS relates to its selectivity along with its high sensitivity and the ability to provide information on analyte structures in complex mixtures. Depending on the objective of the analysis, PDE-5 inhibitors and analogues have been tested using different LC–MS systems equipped with ESI and various mass analyzers allowing low or high resolution mass measurements. These included triple quadrupole (QqQ) (15,19,20), time-of-flight (TOF) (21), and orbitrap (22). Hybrid MS systems have also been used, such as triple quadrupole-linear ion trap (QqQ-LIT) (23,24), quadrupole-time-of-flight (QTOF) (17), ion trap-time-of-flight (IT-TOF) (25), quadrupole-orbitrap (18,26), and LIT-orbitrap (27). The LC–MS technique—especially in its high mass resolution mode—also plays a crucial role in screening for and elucidation of the structure of novel analogues (3,9), as discussed later in this article.

LC preceding ionization and MS analysis of the analyte molecules is an important step in a typical PDE-5 inhibitor determination workflow. State-of-the-art mass spectrometers provide a level of selectivity, but chromatography remains important in preventing suppression of analyte signals and interferences caused by bulk sample matrix. LC is also crucial when it comes to the analysis of isobaric compounds with similar structures and similar or identical fragmentation patterns, and is needed for unequivocal identification of novel PDE-5 inhibitor analogues. Chromatographic systems with an octadecylsilyl (C_{18}) stationary phase and mobile phases consisting of mixtures of water with acetonitrile or methanol in combination with gradient elution were the most widely used in studies focused on LC–MS-based analysis of PDE-5 inhibitors.

As can be seen in Table 1, which shows a selection of recently published LC–MS methods, acetonitrile was the preferred organic component of the mobile phase, particularly in methods that aimed at analysis of a higher number of analytes. Employing acetonitrile in gradient elution programmes allows sharper peaks and significantly better chromatographic resolution between structurally similar compounds (sildenafil and vardenafil and their analogues) to be achieved, as compared to methanol, which may result in partial or complete coelution of these analytes (23,26). Multiple basic nitrogen groups in the structures of PDE-5 inhibitors and their analogues (Figure 1) are prone to pH-dependent chromatographic problems, such as tailing or poor peak shape caused by the presence of analytes in both neutral and ionized forms and secondary interactions with the stationary phase (26). Various additives, including ammonium formate or acetate (2–20 mM)—either with or without formic or acetic acid (0.01–0.1%)—were added to the mobile phase to fix the above issues. Diethylamine and trimethylamine represent basic mobile phase additives commonly used in

Figure 3: Impact of the mobile phase composition on peak shape and retention of selected PDE-5 inhibitors. (a) Mobile phase A–B: 0.1% formic acid in water–0.1% formic acid in methanol. (b) Mobile phase A–B: 5 mM ammonium formate in water–5 mM ammonium formate in acetonitrile. (c) Mobile phase A–B: 10 mM ammonium formate and 0.1% formic acid in water–10 mM ammonium formate and 0.1% formic acid in acetonitrile–methanol mixture (1:1, *v/v*). Authors' unpublished results.

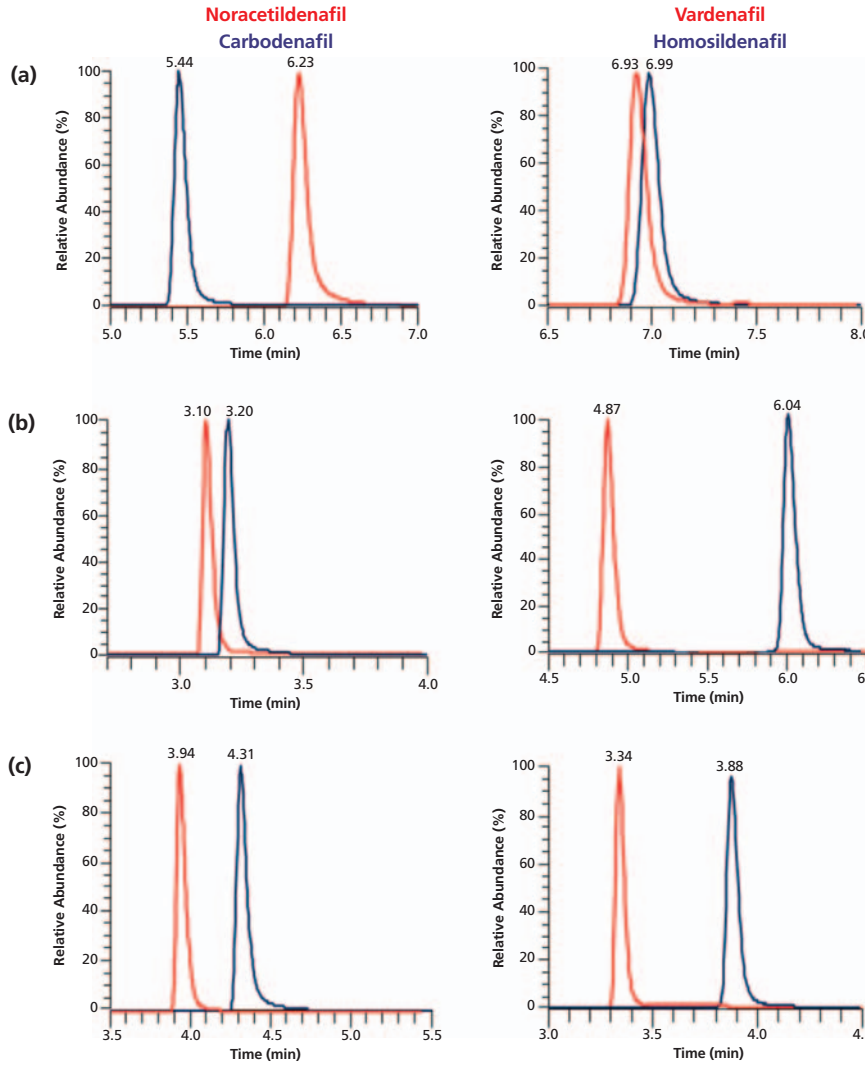


the LC analysis of compounds containing *N* atoms and can also improve peak shapes of PDE-5 inhibitors. However, the use of these additives is detrimental to analyte detectability in the positive mode ESI (15,28). In several studies, formic or acetic acid without any additional modifier was employed, resulting in good analyte peak shapes (17,22,25). This indicates that the peak shape of PDE-5 inhibitors is not dictated entirely by the pH value of the mobile phase, but also largely depends on the properties of the C18 stationary phase (residual silanol groups, endcapping) that govern the extent of secondary interactions.

Figures 3 and 4 illustrate the impact of the mobile phase composition on the peak shape of selected PDE-5 inhibitors (sildenafil, chlorodenafil, and norneovardenafil) and chromatographic resolution between structurally similar isobaric analytes (noracetildenafil–carbodenafil and vardenafil–homosildenafil), respectively (26). When using gradient elution with the mobile phase composed of water and methanol both with 0.1% formic acid, tailing was observed for chlorodenafil and norneovardenafil, while acceptable peak shape was obtained for sildenafil. Sildenafil eluted much earlier than the other two compounds and therefore did not notably suffer from tailing caused by secondary interactions (Figure 3[a]). The use of an identical elution programme with aqueous acetonitrile mobile phase and 5 mM ammonium formate resulted in considerable peak shape improvement for chlorodenafil. However, this was not the case for norneovardenafil as retention time shift and excessive tailing were observed. Norneovardenafil contains a carboxylic functional group that remains charged under the higher pH value conditions (Figure 3[b]). Finally, good peak shape and retention was achieved for all analytes under optimal pH value facilitated by the 10 mM ammonium formate–0.1% formic acid buffer added to both aqueous and organic mobile phase components (Figure 3[c]).

Poor separation of vardenafil and homosildenafil was obtained with methanol organic eluent (Figure 4[a]). In line with information provided above, excellent resolution was observed after switching to acetonitrile. However, under these conditions previously well-resolved noracetildenafil and carbodenafil (analogues of sildenafil) coeluted (Figure 4[b]). In this study, organic eluent prepared by mixing equal volumes of acetonitrile and methanol allowed for sufficient resolution between both isobaric pairs (Figure 4[c]).

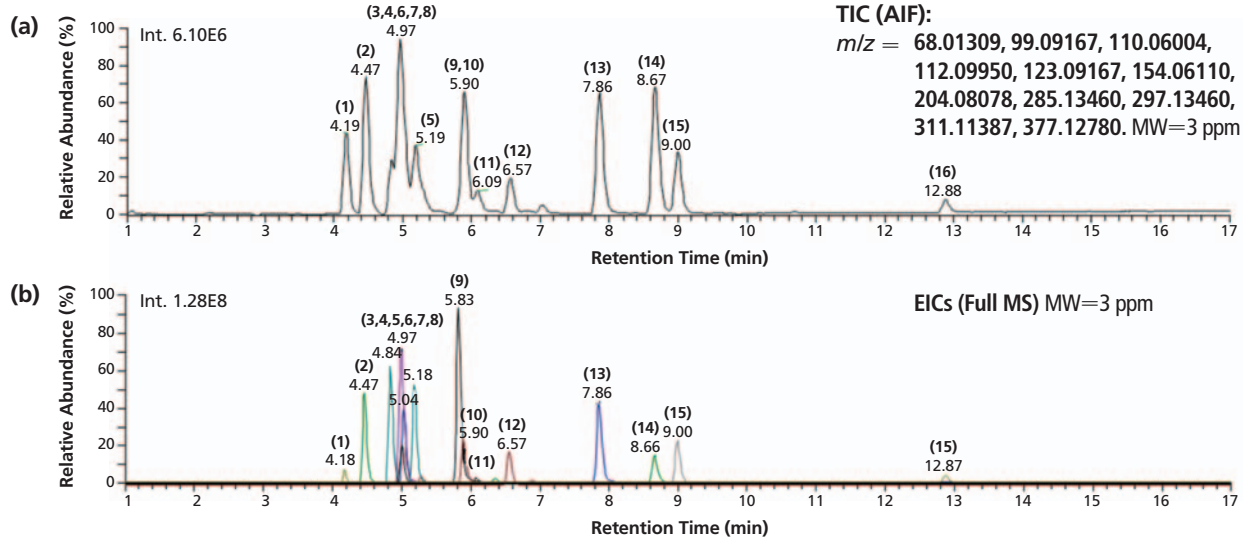
Figure 4: Impact of the mobile phase composition on chromatographic resolution between isobaric analytes. (a) Mobile phase A–B: 0.1% formic acid in water–0.1% formic acid in methanol. (b) Mobile phase A–B: 5 mM ammonium formate in water–5 mM ammonium formate in acetonitrile. (c) Mobile phase A–B: 10 mM ammonium formate and 0.1% formic acid in water–10 mM ammonium formate and 0.1% formic acid in acetonitrile–methanol mixture (1:1, *v/v*). Adapted with permission from reference 26.



Positive mode ESI is the technique of choice for the LC–MS analysis of PDE-5 inhibitors and has been almost exclusively used for ionization of these analytes. Under positive ESI conditions, these basic compounds readily form abundant $[M+H]^+$ ions. In methods targeting PDE-5 inhibitors along with other classes of pharmaceutical adulterants that favour negative ESI, a separate negative mode chromatographic run or simultaneous data acquisition under positive and negative ionization mode in a single LC–MS run (fast polarity switching) have been used (25,29).

There are several MS data acquisition modes that can be used in targeted detection, identification, and quantification of pharmaceutical adulterants in complex biological samples, such as botanical dietary supplements. The selection of the optimal MS experiment is dictated by the type of information required and, of course, the MS system available. Instruments equipped with QqQ or hybrid QqQ-LIT that allow mass measurement at low or medium resolution are usually operated in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode (12,19,20,23,24,29). In SRM and MRM, high selectivity and sensitivity for target analytes are achieved through monitoring of characteristic product ion(s) formed within collision-induced dissociation of a particular precursor ion. It is a good practice to monitor at least two precursor-to-product ion transitions for each analyte and identify positive hits based on the comparison of transition relative intensities with those obtained in reference standards (30). The maximum number of transitions that can be concurrently monitored with sufficient detectability is dictated by the time spent for acquisition of each transition (dwell time). This can become a limiting factor when analyzing a larger number of

Figure 5: LC–HRMS chromatograms of a botanical dietary supplement extract spiked with 16 PDE-5 inhibitors at 100 mg/kg: (a) Total ion current (TIC) representing the sum of all characteristic fragment ions extracted with a 3 ppm mass window from full MS/MS data. (b) Extracted ion chromatograms (EICs) obtained by extracting $[M+H]^+$ ions of individual analytes from full MS data with a 3 ppm mass window. (1) Hydroxyacetildenafil, (2) Acetildenafil, (3) Vardenafil, (4) Hydroxyhomosildenafil, (5) Avanafil, (6) Sildenafil, (7) Homo sildenafil, (8) Acetaminotadalafil, (9) Udenafil, (10) Propoxyphenyl homohydroxysildenafil, (11) Tadalafil, (12) Isotope-labelled internal standard (Pyrazole N-demethyl sildenafil-d_3), (13) Mirodenafil, (14) Hydroxythiohomo sildenafil, (15) Thiohomosildenafil, (16) Lodenafil carbonate. Authors' unpublished results.



analytes, but can be dealt with by using a time-scheduled MRM algorithm that allows monitoring of target analytes only around the expected retention time (24). An alternative approach relying on the acquisition of a single transition for each analyte and a product ion scan triggered for analyte pseudomolecular $[M+H]^+$ ion at the apex of the peak has been used in a study by Lee *et al.* for 24 PDE-5 inhibitors and analogues (23). A hybrid QqQ-LIT instrument enabled data from both MS experiments to be collected in a single LC–MS run. While the SRM data channel was used for quantification of target analytes, the product ion spectrum obtained in the data-dependent product ion scan experiment facilitated identification based on matching with the in-house developed spectral library (23).

The use of LC–HRMS in the PDE-5 inhibitor analysis was initially limited to structure elucidation of new analogues (9); however, in the past few years this technique has been increasingly used in targeted screening and quantitative applications (see Table 1). The indisputable advantages of modern high-resolution mass spectrometers include excellent sensitivity in full MS mode and accurate mass measurement capabilities with typical mass errors <5 ppm. Full MS and tandem MS (MS/MS) data acquisition enables retrospective evaluation of the records and opens the door to post-run targeted and nontargeted screening applications. High-mass resolving power can considerably increase selectivity because it allows analyte ion chromatograms with narrow mass windows to be extracted and enables identification through elemental formulae calculations and isotopic profile matching. This approach was successfully demonstrated for PDE-5 inhibitors and stimulant adulterants by Strano-Rossi *et al.*, who used a single-stage orbitrap LC–MS system (22).

Additional confidence in identification can be obtained through acquisition of high-resolution data-dependent product ion scan (17,18,26,27) or data-dependent multiple-stage fragmentation (MS^n) spectra of analyte ions (25) using hybrid instruments (QTOF, IT-TOF, Q-orbitrap, or LIT-orbitrap). In targeted screening applications, the data-dependent fragmentation experiments are performed with the use of a predefined inclusion list that provides precursor ion masses and retention times of analytes (17,18,26). The data-processing workflows are often facilitated by software tools that automatically search for target analytes in the experimental LC–HRMS data and apply various detection and identification criteria to reduce both false positive and false negative results. These criteria usually define tolerances for peak retention time shift, peak area and intensity threshold, mass error of the pseudomolecular ion, isotope profile match in terms of isotope spacing and relative intensities, and, last but not least, fragmentation pattern match with MS/MS spectra in a spectral library or with experimental spectra obtained by analysis of reference standards or, alternatively, match of measured fragment ion masses with exact masses calculated from their elemental formulae (25,26).

Gas Chromatography–Mass Spectrometry:

Considering the rather poor volatility, thermal instability, and difficult derivatization of PDE-5 inhibitors and analogues with standard reagents, the applications of gas chromatography–mass spectrometry (GC–MS) to the analysis of these compounds is rather limited (9). A derivatization-free GC–MS method for high-throughput screening of sildenafil, tadalafil, and vardenafil was introduced by Man *et al.* (31), who used a short 10-m capillary column and optimized temperature gradient to

obtain a total run time below 8 min. Detection with a mass spectrometer equipped with an electron ionization source and a single quadrupole mass analyzer was performed in selected ion monitoring (SIM) mode by recording characteristic fragment ions; full MS mode was used for identification (31).

Direct Mass Spectrometry Techniques: To increase the throughput of MS-based analyses of PDE-5 inhibitors in botanical dietary supplements, several techniques, which allow (chromatographic) separation of sample components to be omitted and reduce the requirements for sample preparation, have been applied to this analytical problem (3). The drawbacks often encountered with these approaches are extensive matrix effects resulting in analyte signal suppression and spectral interferences, as well as a higher risk of false positive results (retention time information is not available).

In flow injection mass spectrometry (FI-MS), the sample extract is introduced into a suitable carrier and transferred to the mass spectrometer via the capillary tubing connecting the autosampler outlet and the ion source (32). Besides the detection settings of the mass spectrometer, the composition and flow rate of the carrier solution and capillary tubing dimensions represent important parameters that have a major impact on peak shape and detectability of target analytes. FI-MS with a QqQ MS system operated in positive ESI and MRM mode was successfully used for semiquantitative analysis of sildenafil, tadalafil, and vardenafil in adulterated dietary supplement materials with the total run time less than 1 min (20). The authors concluded that the method is suitable for high-throughput screening of PDE-5 inhibitors, but cannot discriminate analytes that share the same MRM transitions.

Ambient ionization–desorption techniques coupled to MS detection facilitate direct sample analysis in an open environment at atmospheric pressure and represent excellent tools for high throughput measurements (33,34). In ambient MS, solid, liquid, or gaseous samples are introduced into the ionization region and exposed to a stream of desorbing or ionizing medium. Analyte ions arising from ionization processes similar to ESI–atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) are subsequently transferred through the open air to the inlet of the mass spectrometer (34). Among more than 30 ambient ionization–desorption techniques described to date, direct analysis in real time (DART) (35), desorption corona beam ionization (DCBI) (36), and atmospheric solids analysis probe (ASAP) (37) were used to determine various pharmaceutical adulterants in botanical dietary supplements (38–40). In ASAP, the liquid or solid sample is loaded on a glass probe and inserted into a conventional APCI source to be exposed to a stream of hot nitrogen gas. Vapourized sample components are ionized through corona discharge-based APCI processes (34). The use of ASAP coupled to TOF and QTOF instruments operated in positive mode for direct analysis of PDE-5 inhibitors in adulterated herbal capsules was demonstrated by Twohig *et al.* (40). The sample preparation was limited to brushing the solid glass probe against the capsule contents and the removal of excess material from its surface prior to ASAP-MS analysis. Mass spectra of counterfeit supplement were dominated by $[M+H]^+$ of tadalafil, sildenafil, and an unknown compound. The unknown compound was identified as thiohomosildenafil based on high-resolution product ion spectra acquired in a separate ASAP-QTOF-MS experiment and information in the literature. The level of sensitivity achieved with this approach is significantly lower (approximately 500-fold) compared to LC–MS/MS.

Screening and Structure Elucidation of New Analogues: Screening for new, previously unreported and characterized PDE-5 inhibitor analogues is a challenging analytical problem. Considering the structural relations between parent pharmaceuticals and designer analogues, both groups of compounds usually have similar physicochemical properties, and may share absorbance maxima in their ultraviolet (UV) spectra and certain features in MS fragmentation patterns (3,8,9). Currently, LC–MS optionally coupled with on-line diode array detection (DAD) represents the most appropriate tool for this purpose (3,8,9). Screening for PDE-5 inhibitor analogues based on monitoring of characteristic MS fragment ions common to analytes with identical substructure was used in several studies using both low- and high-resolution mass spectrometers. MS acquisition combining precursor ion scan of a common fragment ion of 16 sildenafil and vardenafil analogues (*m/z* 283) and data-dependent product ion scan was performed by Lee *et al.* (24), who used a QqQ-LIT system. An LC–HRMS approach that allowed collection of MS and MS/MS data for both targeted and nontargeted PDE-5 inhibitors in a single chromatographic run was introduced by Vaclavik *et al.* (26). In addition to full MS and data-dependent product ion scan, full MS/MS (all ion fragmentation) was included in the data acquisition method. A combined search for marker fragment ions selected based on a review of fragmentation spectra of 50 PDE-5 inhibitors in full MS/MS records was suggested to screen novel analogues based on their structural similarity to known PDE-5 inhibitors. The use of this strategy for detection of 16 adulterants related to sildenafil, tadalafil, and vardenafil in spiked botanical dietary supplement is demonstrated in Figure 5.

Structure elucidation of newly detected unknown analogues cannot usually be accomplished based on MS experiments alone and requires the application of additional techniques, such as nuclear magnetic resonance (NMR) spectroscopy (41,42). Isolation of the unknown compound, collection of MS, UV, and NMR data, and its comparison with records obtained for known PDE-5 inhibitors are typical steps used in the structure elucidation workflow (3,8,9).

Conclusions

Adulteration of botanical dietary supplements with PDE-5 inhibitors has become a major area of concern to regulatory authorities, industry stakeholders, and consumers. MS-based methods have been demonstrated to play key roles in both qualitative and quantitative analysis of these pharmaceutical adulterants. To keep pace with defrauders who continue to develop novel designer analogues, applications that allow broad scope, nontargeted, and high-throughput screening are needed. LC–MS instruments with accurate mass measurement capabilities are currently the most suitable tools to address this analytical challenge. In addition, there is

a need for rapid and simple workflows that can be used in the field. As well as handheld spectroscopic instruments, portable mass spectrometers allowing ambient sampling represent a promising approach.

References

(1) T. Rocha, J.S. Amaral, and M.B.P.P. Oliveira, *Compr. Rev. Food. Sci. Food. Saf.* **15**, 43–62 (2016).
(2) V. Gilard, S. Balayssac, A. Tinaugusus, N. Martins, R. Martino, and M. Malet-Martino, *J. Pharm. Biomed. Anal.* **102**, 476–493 (2015).
(3) L. Vaclavik, A.J. Krynitsky, and J.I. Rader, *Anal. Bioanal. Chem.* **406**, 6767–6790 (2014).
(4) D.H. Phua, A. Zosel, and K. Heard, *Am. J. Emerg. Med.* **2**, 69–76 (2009).
(5) K. Mastovska and P.L. Wylie, *J. Chromatogr. A* **1265**, 155–164 (2012).
(6) L. Vaclavik, M. Vaclavikova, T.H. Begley, A.J. Krynitsky, and J.I. Rader, *J. Agric. Food Chem.* **61**, 4822–4830 (2013).
(7) E.S.J. Harris, S. Cao, B.A. Littlefield, J.A. Craycroft, R. Scholten, Y. Liu, T. Kaptchuk, Y. Fu, W. Wang, Y. Liu, H. Chen, Z. Zhao, J. Clardy, A.D. Woolf, and D.M. Eisenberg, *Sci. Total Environ.* **409**, 4297–4305 (2001).
(8) B.J. Venhuis and D. de Kaste, *J. Pharm. Biomed. Anal.* **69**, 196–208 (2012).
(9) D.N. Patel, L. Li, C.L. Kee, X. Ge, M.Y. Low, and H.L. Koh, *J. Pharm. Biomed. Anal.* **87**, 176–190 (2014).
(10) C.F. Codevilla, T.S Castilhos, and A.M. Bergold, *Braz. J. Pharm. Sci.* **49**, 1–12 (2013).
(11) European Medicines Agency, http://www.ema.europa.eu/ Accessed 8 Aug 2016
(12) https://www.drugs.com/ Accessed 8 Aug 2016
(13) B.J. Venhuis, M.E. Zwaagstra, P.H.J. Keizers, and D. de Kaste, *J. Pharm. Biomed. Anal.* **89**, 158–165 (2014).
(14) A. Lanzarotta, J.B. Crowe, M. Witkovski, and B.M. Gamble, *J. Pharm. Biomed. Anal.* **67–68**, 22–27 (2012).
(15) P. Zou, S.S.Y. Oh, P. Hou, M.Y. Low, and H.L. Koh, *J. Chromatogr. A* **1104**, 113–122 (2006).
(16) S.R. Gratz, C.L. Flurer, and K.A. Wolnik *J. Pharm. Biomed. Anal.* **36**, 525–533 (2004).
(17) C. Bortolini, A. Pivato, S. Bogialli, and P. Pastore, *Anal. Bioanal. Chem.* **407**, 6207–6216 (2015).
(18) L. Vaclavik, A.J. Krynitsky, and J.I. Rader, *Anal. Chim. Acta* **810**, 45–60 (2014).
(19) Y. Ren, C. Wu, and J. Zhang, *J. Sep. Sci.* **35**, 2847–2857 (2012).
(20) F. Song, A. El-Demerdash, and S.J.S.H. Lee, *J. Pharm. Biomed. Anal.* **70**, 40–46 (2012).
(21) A.A. Savaliya, R.P. Shah, B. Prasad, and S. Singh, *J. Pharm. Biomed. Anal.* **52**, 406–409 (2010).
(22) S. Strano-Rossi, S. Odoardi, E. Castrignano, G. Serpelloni, and M. Chiarotti, *J. Pharm. Biomed. Anal.* **106**, 144–152 (2015).
(23) E.S. Lee, J.H. Lee. K.M. Han, J.W. Kin, I.S. Hwang, S. Cho, S.Y. Han, and J. Kim, *J. Pharm. Biomed. Anal.* **83**, 171–178 (2013).
(24) H. Lee and B.J. Lee, *Food Addit. Contam. A* **28**, 396–407 (2011).
(25) B. Guo, M. Wang, Y. Liu, J. Zhou, H. Dai, Z. Huang, L. Shen, Q. Zhang, and B. Chen, *J. Agric. Food Chem.* **63**, 6954–6967 (2015).
(26) L. Vaclavik, J.R. Schmitz, J.-F. Halbardier, and K. Mastovska, *J. AOAC Int.* **99**, 55–72 (2016).
(27) P. Lebel, J. Gagnon, A. Furtos, and K.C. Waldron, *J. Chromatogr. A* **1343**, 143–151 (2014).
(28) X. Zhu, S. Xiao, B. Chen, F. Zhang, and S. Yao, *J. Chromatogr. A* **1066**, 89–95 (2005).
(29) M.J. Bogusz, H. Hassan, E. Al-Enazi, Z. Ibrahim, and M. Al-Tufail, *J. Pharm. Biomed. Anal.* **41**, 554–564 (2006).
(30) Commission Decision 2002/657/EC, Off. J. Eur. Communities. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF
(31) C.N. Man, N.M. Nor, R. Lajis, and G.L. Harn, *J. Chromatogr. A* **1216**, 8426–8430 (2009).
(32) H.G.J. Mol and R.C.J. van Dam, *Anal. Bioanal. Chem.* **406**, 6817–6825 (2014).
(33) J. Hajslova, T. Cajka, and L. Vaclavik, *TRAC-Trends Anal. Chem.* **30**, 204–218 (2011).
(34) L. Vaclavik, T. Cajka, W.L. Zhou, and P.G. Wang, *High-Throughput Analysis for Food Safety, 1st Ed.* (John Wiley & Sons, Hoboken, New Jersey, USA, 2010), pp. 15–72.
(35) R.B. Cody, J.A. Laramee, and H.D. Durst, *Anal. Chem.* **77**, 2297–2302 (2005).
(36) H. Wang, W. Sun, J. Zhang, X. Yang, T. Lin, and L. Ding, *Analyst* **135**, 688–695 (2010).
(37) C.N. McEwen, R.G. McKay, and B.S. Larsenm, *Anal. Chem.* **77**, 7826–7831 (2005).
(38) Z. Zhou, J. Zhang, W. Zhang, Y. Bai, and H. Liu, *Analyst* **136**, 2613–2618 (2011).
(39) H. Wang, Y. Wu, W. Sun, L. Ding, B. Guo, and B. Chen, *Food Addit. Contam. A* **29**, 1194–1201 (2012).
(40) M. Twohlig, J. Skilton, G. Fujimoto, N. Ellor, and R.S. Plumb, *Drug Test. Anal.* **2**, 45–50 (2010).
(41) G. Zhang, Y. Yu, X. Wu, and J. Li, *J. Pharm. Biomed. Anal.* **94**, 210–214 (2014).
(42) S.E. Kern, E.A. Nickum, R.A. Flurer, V.M. Toomey, and J.J Litzau, *J. Pharm. Biomed. Anal.* **103**, 99–103 (2015).

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The Role of Surface Coverage and Orthogonality Metrics in Two-Dimensional Chromatography

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The enhanced separation power of two-dimensional (2D) chromatography has become accessible thanks to the commercialization of dedicated two-dimensional systems. However, with great separation power comes great system complexity. All two-dimensional systems require a means for collecting and transferring fractions of the first dimension to the second dimension typically via a loop-based interface in on-line methods. It is important to collect a sufficient number of fractions to prevent loss of the first dimension resolution; that is, the sampling rate must be sufficient to prevent undersampling. Another key parameter to consider is selectivity. By coupling two selectivities that have unrelated retention mechanisms we are able to exploit the different physiochemical characteristics of the sample we wish to separate. This is the concept behind the term *orthogonality*. By coupling orthogonal selectivities and reducing under-sampling, our system should be able to achieve the theoretical maximum two-dimensional peak capacity. Unfortunately, this is virtually impossible to achieve with current technology. It follows that it is important to be able to calculate the actual (conditional) peak capacity of our two-dimensional chromatographic system. To calculate this, we need to know the first dimension sampling time and the proportion of the separation space occupied by peaks; the latter is referred to as *surface coverage*. This review discusses the role of orthogonality metrics and surface coverage metrics and their relationship to selectivity and peak capacity in two-dimensional chromatography.

Setting up a two-dimensional (2D) chromatographic system involves more than building a system that uses two columns to separate a sample into its components. To achieve this effectively, an understanding of the concept of dimensionality in chromatography is required.

There are three key aspects to this concept: sample dimensionality, apparent sample dimensionality, and the system dimensionality (1). The relationship between these concepts is illustrated in Figure 1. All of these aspects should be considered when setting up a 2D system. The first aspect, sample dimensionality, refers to the number of independent factors that can be used to characterize or separate the sample (1). For example, the number of carbon units in the structure of the various sample components is a factor that can be used to describe or separate the components of a sample of alkyl benzenes.

For a sample containing a mixture of proteins, sample components could be defined by their molecular weight, their isoelectric point, or their affinity for a certain antibody for example. Each factor that can be used to characterize the individual components of the sample is regarded as one dimension. It follows that the protein sample mentioned above can be described as multidimensional whereas the alkyl benzene sample is one-dimensional. The apparent sample dimensionality follows the same logic as described above. However, it refers to the number of factors that the analyst is interested in or are actually used to separate the sample into its components (1). For example, if we again consider a mixture of proteins, and the molecular weight was the only property used to separate the sample components, then the apparent sample dimensionality would be one, even though the sample itself is multidimensional.

When the sample dimensionality is understood, the various factors of this dimensionality should be considered when choosing appropriate separation techniques or retention mechanisms to combine to build the 2D system most appropriate for exploiting the sample dimensionality. In other words, the system dimensionality should be appropriate for the (apparent) sample dimensionality. System dimensionality is defined as the number of different separation stages where different retention mechanisms are employed (1). For example, a chromatographic system employing a C18 column and an ion exchange column would be considered 2D since it incorporates two separation stages with two different retention mechanisms. Conversely, a system comprised of two C18 columns using the same mobile phase for both dimensions could be considered a one-dimensional (1D) system, despite the involvement of two columns since both dimensions would separate the sample based primarily on hydrophobicity. Such a system would

KEY POINTS

- Orthogonality and surface coverage metrics are useful for evaluating 2D-LC separations.
- Orthogonality metrics consider the degree of similarity of the retention mechanisms within a given 2D-LC system.
- Surface coverage metrics consider the distribution of peaks in a geometric manner.
- Surface coverage metrics can be used to gauge orthogonality but the reverse is generally not valid, depending on the metric.

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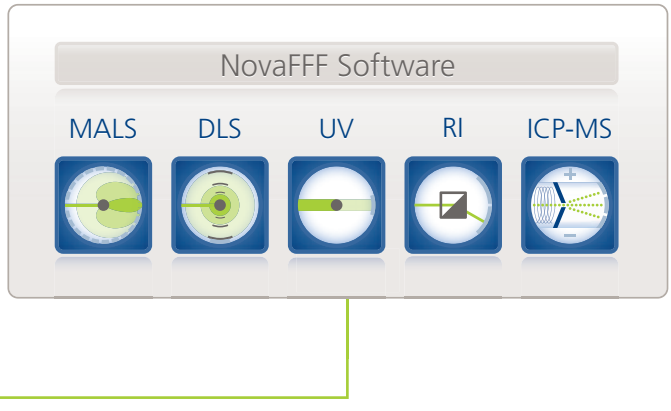

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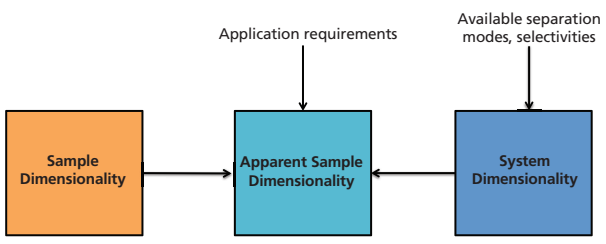
Figure 1: Illustration of the relationship between sample, apparent sample, and system dimensionality. Apparent sample dimensionality is affected by the sample and system dimensionality as well as the requirements of the application. System dimensionality is affected by the components used to construct the system.



essentially be equivalent to a long C18 column. It should be noted that the stationary phase selectivity in each dimension is not the only factor that can determine whether the system is one-dimensional or multidimensional: the mobile phases used in each dimension play an equally important role. For example, a two-dimensional system can consist of two C18 columns when mobile phases are used to generate a selectivity difference in each dimension, if the sample itself is multidimensional. Such is the case for peptides, which can be separated based on hydrophobicity, size, and charge. The latter property was exploited by employing an acidic mobile phase (pH 2.6) in the first dimension and a basic mobile phase (pH 10) in the second dimension with both dimensions using C18 stationary phases (2). In acidic mobile phase conditions, acidic peptides (pK_a 3 or below) would be protonated and therefore retained on the C18 stationary phase, whilst "neutral" and basic peptides (pK_a above 7) would be ionized, consequently having reduced retention. In basic conditions, the reverse is true. This system produced greater separation power, in terms of practical peak capacity, compared to the commonly used reversed phase × strong cation exchange 2D system.

It follows that when setting up an appropriate 2D system for a particular sample, the analyst chooses the appropriate separation mechanisms (stationary phase and mobile phase) to exploit the dimensionality of the sample. Such a system would have one dimension of the system exploiting one aspect of the sample dimensionality and a second dimension that makes use of another aspect of the sample dimensionality. Ideally, there would be no overlap between the sample dimensions that the system dimensions exploit. Such a system would be considered orthogonal. An orthogonal 2D system could approach the theoretical maximum peak capacity, which is the product of the peak capacities in the first and second dimension (1,3).

Orthogonality and Selectivity in Two-Dimensional Chromatography

While it is known that maximum separation power—in terms of peak capacity—can be achieved by selecting orthogonal selectivities for the first and second dimension, it is not always a simple process to choose appropriate selectivities. Many stationary phases share a certain degree of similarity between their retention mechanism and the retention mechanism of other stationary phases. This is particularly the case when combining reversed-phased liquid chromatography phases in a 2D system. For example, it is possible to use a cyano column and a C18 column for the separation of coffee and still achieve a reasonable degree of orthogonality because the cyano column is capable of participating in π-π interactions with the aromatic components of coffee (4,5). However, the cyano stationary phase is also capable of interacting with solutes based on their degree of hydrophobicity, which is a retention mechanism it has in common with the C18 stationary phase. While these two selectivities exploit two different sample dimensions and form a system with a dimensionality equal to 2 according to the theory of Giddings (1), the system is not completely orthogonal and the theoretical maximum peak capacity cannot be equated to the actual peak capacity. It follows from this example that orthogonality is not a binary "yes or no" concept. Rather, orthogonality comes in degrees and cannot be entirely predicted by coupling two systems that in principle separate with different retention mechanisms. This is where orthogonality metrics become useful. These metrics allow chromatographers to assess how effectively their chosen selectivities distribute sample components throughout the 2D separation space. It has been argued that experienced chromatographers can adequately assess orthogonality themselves, without using metrics. While this is true to a degree, it should be appreciated that orthogonality metrics provide an assessment unbiased by user inclinations or day-to-day variability and this makes them particularly valuable for inclusion in industrial quality assurance. In addition, orthogonality metrics can serve as a guide to help the analyst keep track of the success of their method development procedures. For example, an analyst may be testing a number of different selectivities to determine which will give the most optimal 2D system for their particular sample. By calculating the orthogonality for each selectivity couple, they can gain a better understanding of the physiochemical aspects that play a role in separating the sample. This may lead to the selection of columns whose retention mechanisms target these physiochemical properties, eventually leading to the development of the most orthogonal system possible for their sample.

There are a wide range of orthogonality metrics. Most of these were recently compared by Schure and Davis (6). Their study compared the assessment of 20 orthogonality metrics applied to 47 experimental chromatograms. The assessments of the orthogonality metrics were compared to those given by expert reviewers who assessed the chromatograms visually based on their experience in 2D chromatography. A couple of important key points from this study include the observation that while the expert reviewers agreed on which were the best and which were the worst chromatograms, their assessment on the "mediocre" chromatograms were variable. This implies that the value of orthogonality metrics is their ability to provide constant, reliable assessments of orthogonality throughout the range of possible degrees of orthogonality. The other important point from their study was that no single metric stood out as the best for assessing orthogonality. Methods reporting metrics that appeared as good indicators of orthogonality included the convex hull, dimensionality, and information theory. Recently developed metrics that were not tested in the study of Schure and Davis were the asterisk equations (7) and the maximal information coefficient (8). In the interests of conciseness, we will briefly discuss the convex hull, dimensionality, asterisk, maximal information coefficient, and the bin counting methods. The latter have proven very popular in chromatography.

The bin counting methods are intuitive, simple to use, and are effective in assessing the orthogonality of 2D separations.

There are two versions of the bin counting methods that are conceptually very similar (2,9). Both methods divide the separation space into boxes or bins, where the number of bins equals the number of components within the sample. The width of the peaks corresponds to the average peak width. In the original method, the number of bins containing peaks is summed up and compared with the total number of bins via equation 1 (2):

$$O = \frac{\Sigma bins - \sqrt{P_{max}}}{0.63\,P_{max}} \quad [1]$$

Where P_{max} is the total number of bins. $O = 1$ for an orthogonal separation based on the observation that systems close to orthogonal have a ratio of bins occupied or total bins = 0.63. Bins are also summed up in the second version of the bin counting methods (9). The difference between this method and the original one is that firstly a "fence" is drawn around the area containing bins with peaks. The bins within this enclosed area are summed whether they have peaks or not. The number of bins within the enclosure is compared to the total number of bins to produce the value of orthogonality. Again, this value will range from 0 for a nonorthogonal system and reach a maximum of 1 for a fully orthogonal system where each bin contains one peak (9). While these methods are intuitive and easy to implement, the key limitation that they face is the necessity to know the number of components within the sample. This is not always possible for complex samples, such as protein digests. The consequence is that an insufficient number of bins may be used causing an inflated value of orthogonality. Alternatively, using too many bins will artificially deflate the value of orthogonality.

Dimensionality as an orthogonality metric also uses bins to divide the separation space into sections. Yet unlike the bin counting method described above, it is not necessary to know the number of sample components. The size of the bins or intervals are scaled relative to the first eluting and last eluting peaks in the dimension being considered, using equation 2 (10):

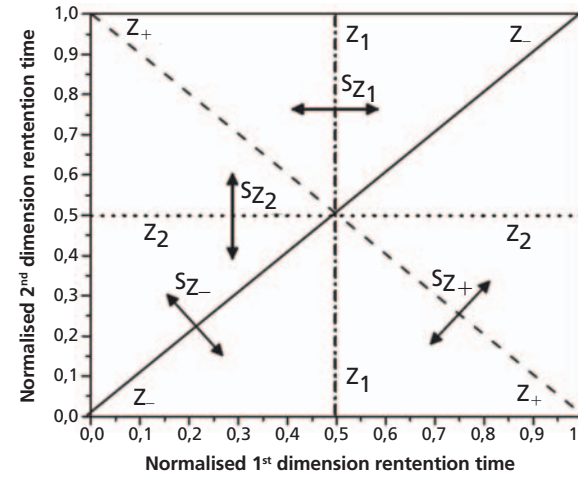
$$\varepsilon_i = \frac{t'_{max} - t'_{min}}{i} = \frac{1}{i} \quad [2]$$

Where ε_i is the interval width, t'_{max} and t'_{min} are the normalized retention times for the last and the first eluting peak, respectively. Retention times are normalized as per equation 3 (10), which is the first step in calculating orthogonality for most—if not all—metrics.

$$t'_i = \frac{t_i - t_{min}}{t_{max} - t_{min}} \quad [3]$$

t'_i is the normalized retention time of peak i, t_i is the retention time of peak i, and t_{min} and t_{max} are the retention times of the first and last eluting peaks, respectively. It follows that the normalized separation space would range from 0 to 1 on each axis or dimension hence the $1/i$ in equation 2. The value i in equation 2 varies in value from 1 up to some maximum value. The number (N) of bins or intervals required to cover the separation space at a given interval width is determined by the user. A plot of $log\ N$ versus $log\ \varepsilon_i$ is constructed and in the least squares regression slope of the plot is multiplied by -1 to give the value of dimensionality (D) (10). For a 2D separation, the width of the intervals varies with respect to both dimensions. A completely orthogonal 2D separation gives a value of 2.00. Conversely, a nonorthogonal separation would give a value of 1.00 for D

Figure 2: Illustration of the concept underlying the asterisk equation. Adapted with permission from reference 7.



indicating that the separation is in fact 1D in agreement with the Giddings concept of dimensionality discussed earlier (10). One limitation of the dimensionality orthogonality metric is apparent when insufficient data is distributed throughout the separation space. To compensate for this the user can include a "step" value within the value of i so that the corresponding $log\ N$ versus $log\ \varepsilon_i$ plot is more smooth, improving the reliability of the calculation of D (10). User defined variables such as i in this method can introduce a source of variability into the reported metric, which is undesirable when different users are comparing orthogonality values. Recently another orthogonality metric that also scales the bin width was developed; in this case by changing the grid resolution. The method is almost identical to the method of Zeng, Hugel, and Marriott (11) with the exception that the maximal informational coefficient (*MIC*) is used in place of the least squares linear regression coefficient (R^2). The metric for orthogonality using the *MIC* is calculated via equation 4 (8).

$$O = \frac{\sum_i bins}{0.63\ P_{max}} \times (1 - MIC) \quad [4]$$

Where Σ_{bins} is the sum of bins containing peaks and P_{max} is the maximum theoretical peak capacity according to Giddings's theory (1). The authors put forward *MIC* as a replacement for R^2 on the basis that it considers nonlinear correlation as well as linear correlation. While it was shown that using equation 4 with *MIC* rather than R^2 improved the method (8), they did not compare this metric with other metrics nor did they investigate the effect of the number of sample components on O, which is known to affect almost all orthogonality metrics.

There are a number of metrics that do not require the use of bins or intervals. The convex hull is one of them (12). There are numerous types of convex hulls but they all share the same concept: a polygon of the smallest possible size is used to fence the area containing peaks. Naturally the fenced area will contain some portion of the separation space that does not contain peaks, which may add some bias to the reported value of orthogonality. Some versions of the convex hull, such as the α-hull and the local convex hull, require some user input in setting certain parameters that govern the size of the hull (12). Another metric that does not require the division of the separation space is the asterisk equations (7). These equations

are based on the distance of peaks from 4 lines that cross over the separation space and act as a reference rather than creating divisions as illustrated in Figure 2.

The standard deviation of the distances of every peak from each Z line is determined using equations 5–8 (7).

$$S_{Z-} = \sigma \left\{ {}^1t_{R,norm(i)} - {}^2t_{R,norm(i)} \right\} \quad [5]$$

$$S_{Z+} = \sigma \left\{ {}^2t_{R,norm(i)} - \left(1 - {}^1t_{R,norm(i)}\right) \right\} \quad [6]$$

$$S_{Z_1} = \sigma \left\{ {}^1t_{R,norm(i)} - 0.5 \right\} \quad [7]$$

$$S_{Z_2} = \sigma \left\{ {}^2t_{R,norm(i)} - 0.5 \right\} \quad [8]$$

In equations 5–8, the expression in the curly brackets calculates the distance of peak, i, from the Z line in question, the standard deviation of these distances is determined as indicated by the σ outside the curly brackets. ${}^1t_{R,norm(i)}$ and ${}^2t_{R,norm(i)}$ are the normalized retention times for peak i in the first and second dimension, respectively. Retention times are normalized using equation 3. To express the resulting standard deviation of distances on the same scale for all Z lines, these S values are transformed to Z values using equations 9–12 (7).

$$Z_- = \left| 1 - 2.5|S_{Z-} - 0.4| \right| \quad [9]$$

$$Z_+ = \left| 1 - 2.5|S_{Z+} - 0.4| \right| \quad [10]$$

$$Z_1 = 1 - \left| 2.5\ S_{Z_1} \sqrt{2} - 1 \right| \quad [11]$$

$$Z_2 = 1 - \left| 2.5\ S_{Z_2} \sqrt{2} - 1 \right| \quad [12]$$

Because the Z values range from 0 to 1, they can readily be reported as a percentage. Each Z value describes the degree of clustering with respect to that line. This can be used to pinpoint regions of the separation space that have a relatively high degree of clustering of peaks. The Z values are combined in equation 13 to give the metric for orthogonality, A_O (7).

$$A_O = \sqrt{Z_- Z_+ Z_1 Z_2} \quad [13]$$

Since the Z values are reported as a percentage, it follows that A_O ranges from 0 to 100% where a value of 100% indicates a completely orthogonal 2D separation. The benefits of the asterisk equations are that they are easy to implement in simple spreadsheet software such as Microsoft Excel, they are intuitive, they are not biased by user defined parameters, and it is not necessary to know the number of sample components. That said, one limitation of this method is that A_O is supressed when there are equal to or less than 25 peaks in the separation (7). In such cases it should only be used to compare chromatograms for the one sample with different conditions in a qualitative manner.

Surface Coverage and its Relationship with Orthogonality

There are numerous metrics for surface coverage (2,9,12) and orthogonality (2,7,8,10,13–23). Conceptually, they are related. If two selectivities are combined that produce a nonorthogonal separation for a given sample then the surface coverage will be reduced compared to that of an orthogonal separation for the same sample. Consequently, surface coverage metrics can be used to report orthogonality. That being said the reverse is generally accepted as not valid. This is because orthogonality metrics generally only consider the degree of similarity of the selectivities of the two dimensions. On the other hand, surface coverage metrics consider the distribution of peaks within the separation space in a geometric manner. This gives us an idea of the proportion of space that is accessible by sample components and therefore peaks. For example, the asterisk equations and dimensionality metric discussed above describe the distribution of peaks throughout the separation space but do not describe the proportion of space accessed by peaks. Conversely, the convex hull and bin counting methods describe the proportion of space accessed by peaks and consequently describe the surface coverage. While the distinction between orthogonality and surface coverage does not make much difference in choosing selectivities, it does make an impact in our ability to calculate the actual peak capacity of a 2D system. In practice the actual peak capacity of a comprehensive 2D system, known as the conditional peak capacity ($n^{o'}_{c,2D}$), is given by equation 14 (24).

$$n^{o'}_{c,2D} = {}^1n_c\ {}^2n_c\ f_{coverage}\left(\frac{1}{\langle\beta\rangle}\right) \quad [14]$$

Where ${}^1n_c$ and ${}^2n_c$ denote the peak capacity of the first and second dimension, respectively. This equation considers the two practical aspects that limit us from achieving the theoretical peak capacity: coverage of the separation space by peaks ($f_{coverage}$) and undersampling of the first dimension (<β>). Since $f_{coverage}$ describes the proportion of the separation space that is accessible to peaks, it follows that surface metrics can act as $f_{coverage}$ in equation 14 so long as they range in value from 0 to 1 as is required in the equation. The undersampling parameter <β> is given by equation 15 (24).

$$\langle\beta\rangle = \sqrt{1 + 0.21\left(\frac{t_S}{{}^1\sigma}\right)^2} \quad [15]$$

Where t_s is the first dimension sampling time and ${}^1\sigma$ is the peak standard deviation in the first dimension prior to sampling. It follows that this value must be an average across all peaks since there is only one value for <β> in equation 14. While orthogonality metrics are useful for assessing the various combinations of selectivities to construct an optimal 2D system, if calculating peak capacity is important for your application then surface coverage metrics are useful.

Conclusion

Ideally the peak capacity of a 2D chromatographic system should equate to the product of the peak capacities of the first and second dimension. In reality this is virtually impossible to achieve. Two key limitations preventing many 2D separations from achieving ideal peak capacity are undersampling of the first dimension and the ability of the system to allow peaks to evenly distribute throughout the separation space. The former is relatively well understood and can be accounted for in the computation of the conditional 2D peak capacity using the sampling time. The ability of the system to distribute peaks evenly throughout the separation space is not so easily accounted for. Surface coverage metrics can be used to determine the proportion of separation space accessible to peaks. These metrics typically consider the distribution of peaks relative to the total separation space without accounting for the effect of peak width on consuming separation space. However, if the goal is to screen a number of selectivities to gauge which

combination will provide the most optimum 2D separation then orthogonality metrics are useful.

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References

(1) J.C. Giddings, *J. Chromatogr. A.* **703**, 3–15 (1995).
(2) M. Gilar, P. Olivova, A.E. Daly, and J.C. Gebler, *Anal. Chem.* **77**, 6426–34 (2005).
(3) J.C. Giddings, *Anal. Chem.* **56**, 1258A–1260A, 1262A, 1264A passim (1984).
(4) M. Mnatsakanyan, P.G. Stevenson, D. Shock, X.A. Conlan, T.A. Goodie, K.N. Spencer, *et al.*, *Talanta* **82**, 1349–1357 (2010).
(5) M. Mnatsakanyan, P.G. Stevenson, X.A. Conlan, P.S. Francis, T.A. Goodie, G.P. McDermott, *et al.*, *Talanta* **82**, 1358–1363 (2010).
(6) M.R. Schure and J.M. Davis, *J. Chromatogr. A.* **1414**, 60–76 (2015).
(7) M. Camenzuli and P.J. Schoenmakers, *Anal. Chim. Acta.* **838**, 93–101 (2014).
(8) A. Mani-Varnosfaderani and M. Ghaemmaghami, *J. Chromatogr. A.* **1415**, 108–114 (2015).
(9) M. Gilar, J. Fridrich, M.R. Schure, and A. Jaworski, *Anal. Chem.* **84**, 8722–8732 (2012).
(10) M.R. Schure, *J. Chromatogr. A.* **1218**, 293–302 (2011).
(11) Z.-D. Zeng, H.M. Hugel, and P.J. Marriott, *Anal. Chem.* **85**, 6356–63 (2013).
(12) S.C. Rutan, J.M. Davis, and P.W. Carr, *J. Chromatogr. A.* **1255**, 267–76 (2012).
(13) Z. Liu and D. Patterson Jr, *Anal. Chem.* **67**, 3840–3845 (1995).
(14) P.J. Slonecker, X. Li, T.H. Ridgway, and J.G. Dorsey, *Anal. Chem.* **68**, 682–689 (1996).
(15) J.W. Dolan, A. Maule, D. Bingley, L. Wrisley, C.C. Chan, M. Angod, *et al.*, *J. Chromatogr. A.* **1057**, 59–74 (2004).
(16) E. Van Gyseghem, I. Crosiers, S. Gourvénec, D.L. Massart, and Y. Van der Heyden, *J. Chromatogr. A.* **1026**, 117–128 (2004).
(17) E. Van Gyseghem, M. Jimidar, R. Sneyers, D. Redlich, E. Verhoeven, D.L. Massart, *et al.*, *J. Chromatogr. A.* **1042**, 69–80 (2004).
(18) P. Forlay-Frick, E. Van Gyseghem, K. Héberger, and Y. Van der Heyden, *Anal. Chim. Acta.* **539**, 1–10 (2005).
(19) U.D. Neue, J.E. O'Gara, and A. Méndez, *J. Chromatogr. A.* **1127**, 161–174 (2006).
(20) C. West and E. Lesellier, *J. Chromatogr. A.* **1203**, 105–113 (2008).
(21) R. Al Bakain, I. Rivals, P. Sassiat, D. Thiébaut, M.C. Hennion, G. Euvrard, *et al.*, *J. Chromatogr. A.* **1218**, 2963–2975 (2011).
(22) W. Nowik, M. Bonose, S. Héron, M. Nowik, and A. Tchapla, *Anal. Chem.* **85**, 9459–9468 (2013).
(23) W. Nowik, S. Héron, M. Bonose, M. Nowik, and A. Tchapla, *Anal. Chem.* **85**, 9449–9458 (2013).
(24) D.R. Stoll, X. Wang, and P.W. Carr, *Anal. Chem.* **80**, 268–278 (2008).

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Column Care for the Long Haul—Considerations for Column Storage

Dwight Stoll, LC Troubleshooting Editor

Several factors influence the useful lifetime of high performance liquid chromatography (HPLC) columns. In this instalment we consider some of the details associated with preparing a column for storage, with an eye towards choices that will pay dividends in future use of the column.

It seems simple. We finish our work with a particular high performance liquid chromatography (HPLC) column, put it in the drawer for safe keeping, and move on to the next column for the next project, or the next step in method development. But, what exactly should we do with that column before it goes in the drawer? Stopping to think about the details for a bit, we recognize that there is actually quite a lot to consider. In this instalment, I summarize some definite do's and don'ts for column storage, and try to make sense of the variety of advice that is available on the topic.

Backing up a bit to think about column care in a broader sense, following best practices for the way we treat HPLC columns can have big effects on the performance of these columns in our work, especially over time. John Dolan has addressed various aspects of column care in his instalments of "LC Troubleshooting" over the years. Given their importance to column lifetime, I've summarized some of the important ones briefly again here. Readers interested in more detailed discussions of these topics can follow the references to previous issues of "LC Troubleshooting" (1).

- Avoid mobile phases that will cause chemical damage to the column. For silica-based columns with bonded stationary phases, this advice means two things: avoid very acidic conditions (<<pH 2) that cause hydrolysis of siloxane bonds between the silica and stationary phase ligand, and avoid alkaline conditions (>>pH 8) that can cause dissolution of the silica particle material itself (2). New silica-based materials introduced in the last decade have made chemical damage less of an issue (3), but users need to be aware of the limits of the particular columns they are working with (4).
- "Don't inject junk" (5). Injecting things into the HPLC column that don't come out of the column during the analysis is generally bad for performance. This can be particulate debris that gets stuck at the column inlet, increasing pressure drop across the column and causing uneven flow distribution, or chemical constituents of the sample that tend to be very strongly retained and cause changes in column chemistry as they accumulate on the stationary phase. The problem with particulates can be minimized by filtering the sample before injecting it into the HPLC system. Particulates can also originate from the column or the mobile phase itself, or by shedding of various parts of the instrument. Using an in-line filter upstream from the HPLC column can significantly reduce the impact of these particulates on the column as well (6). Finally, using guard columns can minimize the impact of sample constituents that tend to adsorb strongly to the column under the conditions of the analysis. The role of the guard column is to "catch" these components, and the guard is simply thrown away and replaced after a specified number of injections, ultimately extending the life of the analytical column.

In the Beginning: Establishing a Baseline

A common problem in troubleshooting the behaviour of HPLC columns is that we don't have a good reference point to help us understand how and when the behaviour of a column has changed. For example, in the course of method development we might observe that the resolution of a critical pair of analytes has decreased. This situation leads to a bunch of questions—When did the change start to happen? What are the likely causes for the change? How did the column behave when it was brand new relative to its behaviour now? Likewise, if we start work with a column that has been in the drawer for a month, how do we know that column will behave like it did when it was new? One easy thing to do in this situation is to try to reproduce the separation indicated on the quality control (QC) sheet that comes with the column inside the box. For reversed-phase columns this QC sample is typically a simple mixture of small neutral molecules separated in a simple organic solvent–water mobile phase. For columns designed for separations of biomolecules, this QC separation might involve a standard mixture of proteins that are readily available (for example, myoglobin). If we can reproduce the separation on the QC sheet with retention factors, selectivities, and plate numbers that are similar to what was obtained by the manufacturer, that most certainly should increase our confidence that the column is working like it

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was when it was new. The problem, however, is that it is very unlikely that the molecules used in the mixtures for these QC separations will interact with the stationary phase in exactly the same ways that the molecules in our analytical samples do. The only way to really address this problem is to establish the baseline performance of the column by injecting a mixture of compounds that is relevant to the separation we are using or trying to develop, and separating the mixture using conditions that are relevant to the conditions we plan to use. In my laboratory this approach is our standard practice—although we may never use that baseline information again in the life of the column, when we do need it, it is invaluable. For example, if we are working with a new column that we hope to use for separations of antibody proteins, the very first thing we do with that column is inject an antibody standard under conditions that are likely to be similar to the final operating conditions we use for that column. Then, if we suspect that something is not right with the HPLC column or instrument, we can always check things out by repeating this separation and comparing results to what we obtained with the column when it was new out of the box.

Column Storage: Avoiding Major Pitfalls

There is a short list of definite must do's when preparing a column for storage, all of which involve flushing the most recently used mobile phase out of the column and replacing it with a solvent suitable for storage. What constitutes a suitable storage solvent is discussed in more detail below, because these solutions are stationary phase specific.

- Do flush strongly acidic or alkaline mobile phases from the column (<<pH 2 or >>pH 7). This step will minimize the possibility of chemical degradation of the stationary phase during storage (see above for the mechanisms of degradation).
- Do flush mobile phases containing high concentrations of salt (for example, >30 mM sodium chloride) or ion-pairing reagents (for example, octanesulfonate) from the column. Chloride salts in particular are very corrosive to stainless steel, and will attack the column wall, and inlet–outlet frits (7). The metal ions that are released when the metal surfaces corrode or erode can lead to numerous problems, including contamination of the stationary-phase material and interference with analyte detection (8). Although high concentrations of salts are not so commonly used in reversed-phase separations, they are very commonly used in ion-exchange separations, and are essential in hydrophobic interaction chromatography (HIC), which is becoming widely used for protein separations. The other major concern with high salt concentrations is that if the column begins to dry out during storage, the column can be turned into a giant salt crystal, which is impossible to recover from.

Storing Reversed-Phase Columns

Now, when we think about how to care for specific types of phases, things become a bit more nuanced. One of the first things to consider in the case of reversed-phase columns is how to flush the mobile phase from the column to prepare for the storage solvent. One problem we want to avoid in this step is precipitation of buffer salts. Although acetonitrile is the most commonly used organic solvent in reversed-phase separations, and phosphate salts are among the most commonly used buffers, they are not compatible at high levels of acetonitrile. A study by Schellinger and Carr (9) mapped out the solubility of different buffer systems (for example, ammonium phosphate, potassium phosphate, and so forth) in different organic solvents, including acetonitrile. For example, they found that a 30 mM potassium phosphate buffer at pH 3 was soluble in mixtures of buffer and acetonitrile only when the acetonitrile level was less than 75%. For most reversed-phase separations this solubility issue is not a problem because compounds of interest are typically eluted at percentages less than this level. However, this means that when flushing the buffer to prepare the column for storage we should not use high acetonitrile levels, and certainly not 100% acetonitrile, as it will cause the buffer to precipitate both in the pump and connecting tubing, as well as the column.

In preparing for this column instalment, I informally surveyed the recommendations of about 30 manufacturers of reversed-phase columns by going through column boxes in my laboratory and reading the column care sheets provided by the manufacturers. This informal survey was interesting because on one hand the advice in those sheets is more varied than I would have expected given that these were all reversed-phase columns. On the other hand, it seems that some of the advice has been handed down through different generations of manufacturers, kind of like an old family recipe. The first thing that surprised me is that several of the care sheets recommend flushing the column first with pure water to remove buffers in preparation for storage. Although this step will undoubtedly be effective in removing the buffering agents, it may also cause the stationary phase to "dewet" (10,11). Here, dewetting means that the water is expelled from the bonded phase, and sometimes entirely from the pores of the particle, leading to a dramatic loss in retention if the column is used in this state, simply because analytes cannot enter the vacated stationary phase. The good news is that retention can usually be fully restored by reconditioning the column with a mobile phase containing more than about 50% organic solvent. But, it is probably best to avoid this situation altogether when possible. Thus, it is generally advisable to first flush buffering agents from the column with about 10 column volumes of mobile phase containing about 10% organic solvent in water. This approach will be effective and avoid both the precipitation and dewetting problems.

After we have flushed the most recently used mobile phase from the column, we must decide what solvent will be used for actual storage of the column. Going again back to the column care sheets, I found that in the 30 sheets I surveyed about 35% of them recommended storing the column in pure acetonitrile or methanol, and the other 65% recommended storing the column in a mixture of organic solvent and water, where the recommended ratio ranged from 50:50 to 80:20 organic–water. A study by Mowery (12) of the rates of erosion and corrosion of stainless steel components for HPLC in reversed-phase mobile phases showed that acetonitrile and

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methanol were far more erosive when used as pure solvents compared to when they were mixed with water. Even adding a few percent of water slowed the erosion by at least a factor of 10. Given the large surface area of the porous stainless steel frits that are typically used to retain the particles in the column bed, even a small amount of erosion or corrosion can lead to contamination of the stationary phase metal ions liberated upon oxidation of the bulk metal surface. Indeed, Euerby, Tennekon, and colleagues (13) showed that contamination of the reversed-phase stationary phases with metal ions seemed to promote epimerization of the molecule tipredane on-column. Furthermore, the amount of metal liberated from the column hardware during storage in pure organic solvents was enough to increase the rate of on-column epimerization, and lead to very bad peak shapes for molecules having chelating moieties.

In the end, the "right" choice of storage conditions is dictated by the application at hand. There undoubtedly are applications where column performance is unaffected by storage conditions, so long as the major pitfalls described above are avoided. However, a little work on the front end of method development to see if column storage conditions affect the selectivity of the column for the analytes at hand may well save a lot of trouble (and troubleshooting) later on in the life of the method.

Storing Other Columns: Ion-Exchange, Mixed-Mode, and HILIC Columns

With other column types the potential major problems discussed above (that is, chemical attack, corrosion, and precipitation of salts) still apply. Here, I briefly discuss some details to be aware of that are specific to ion-exchange, mixed-mode, and hydrophilic-interaction chromatography (HILIC) phases. After dealing with the complications that can arise from the use of very salty mobile phases in ion-exchange columns, the next biggest issue is that ion-exchange mobile phases are very often entirely aqueous, and can be very friendly environments for microbes. Steps should be taken to minimize growth of these bugs during storage. As discussed in my most recent column on filtration (6), adding a small amount of organic solvent (on the order of 10%), or adding sodium azide at a low concentration (for example, 0.05%) in the storage solvent can be sufficient to prevent microbial growth. I've seen both approaches recommended in the manufacturers column care sheets.

With these columns, the rate of reequilibration after storage may depend on the storage conditions. For example, if an ion-exchange column is stored with a solution containing counterions that are strongly retained by the stationary phase, then it will take a long time or a high concentration of the counterion in the mobile phase used for the separation method to reequilibrate the stationary phase. Likewise, storing a HILIC column in an acetonitrile–water mixture may take a long time to reequilibrate if a low ionic strength buffer (for example, 5 mM ammonium acetate) is used for the analytical method. Some manufacturers of HILIC columns recommend storage in solvents containing 80–90% acetonitrile, and buffers containing 5–10 mM ammonium acetate or ammonium formate.

The last consideration I'll mention here is that some stationary-phase chemistries may be susceptible to chemical modification by the storage solution that is different from the types of chemical attacks discussed above for reversed-phase columns. One notable example of this is the potential for esterification of ion-exchange and mixed-mode phases containing carboxylic acid functional groups (for example, weak cation-exchange phases) by alcohols. Although this esterification will be very slow at room temperature, it can lead to significant changes in separation selectivity. For this reason some manufacturers of these phases explicitly advise against storage of these phases in solutions containing alcohols.

How Long Is Too Long?

Most sources of advice on the topic of column storage suggest that the column can be stored in mobile phase for two to four days without any major ill effects. Beyond four days, the column should be flushed and prepared for long-term storage as discussed above. Anecdotally, many users I know report storing their columns for long periods in mobile phase (specifically, the initial mobile phase when gradient elution is used), especially if weakly acidic mobile phases are used (for example, 0.1% phosphoric acid), without any known problems. Thinking towards the other extreme, though, raises the following question: How long is too long? Or perhaps, what happens when the column dries out? As a final step before storage the column should be sealed tightly by screwing in the endplugs supplied by the manufacturer in the column box. Given enough time, or if this is not done, the solvent will eventually evaporate. I am not aware of any published long-term studies of column storage, but I can say that we have inadvertently collected some data on this point in my laboratory as part of our work with the Product Quality Research Institute (PQRI) column selectivity database that is built upon the hydrophobic subtraction model (14). In a few cases, we have reevaluated the selectivity of columns that have been sitting on the shelf for more than five years, and observed no statistically significant changes in selectivity over this time period. We assumed that the columns had dried out, and rewetted them by first flushing with 100% acetonitrile, and then equilibrating in mobile phase for about 1 h before making any selectivity measurements.

Setting Up for Success

With most things in analytical laboratories, simplicity leads to consistency of execution. It will be more likely that columns are stored properly if we have a plan for doing so that is straightforward and easy to implement. In my laboratory we have an old pumping system from a retired HPLC instrument that we've dedicated to the purpose of flushing columns and preparing them for storage. Several laboratory managers I know have related that they do the same thing. Taking this approach one step further, one can set up a series of methods on a pumping system that is dedicated for the purpose of flushing, or on each instrument so that these methods can simply be run at the end of a series of analyses if it is expected that the column will be taken

out of use after the run. For example, a method could involve an initial flush with something like 10:90 acetonitrile–water for 10 column volumes to remove buffer salts, a flush at high organic solvent to remove strongly adsorbed compounds and ion-pairing reagents that had accumulated during the run, and finally a switch-over to the actual storage solution (for example, 50:50 acetonitrile–water for reversed-phases). This strategy will increase the likelihood that columns are properly prepared for storage, extend the lives of columns, and reduce the amount of troubleshooting needed later on in the life of the column.

Acknowledgements

I want to thank Tony Taylor of Crawford Scientific for some insightful discussion around the topic of column storage.

References

(1) J.W. Dolan, *LCGC North America* **23**(11), 1174–1181 (2005).
(2) H.A. Claessens, M.A. van Straten, and J.J. Kirkland, *J. Chromatogr. A* **728**, 259–270 (1996). DOI: 10.1016/0021-9673(95)00904-3.
(3) E.M. Borges and M.R. Euerby, *J. Pharm. Biomed. Anal.* **77**, 100–115 (2013). DOI:10.1016/j.jpba.2013.01.013.
(4) J.W. Dolan, *LCGC Europe* **29**(2), 82–84 (2016).
(5) J.W. Dolan, *LCGC Europe* **27**(12), 640–644 (2014).
(6) D.R. Stoll, *LCGC Europe* **30**(2), 80–83 (2017).
(7) K. Collins, C.H. Collins, and C. Bertran, *LCGC North Am.* **18**(6), 600–608 (2000).
(8) S. Liu, C. Zhang, J.L. Campbell, H. Zhang, K.K.-C. Yeung, V.K.M. Han, and G.A. Lajoie, *Rapid Commun. Mass Spectrom.* **19**, 2747–2756 (2005). DOI: 10.1002/rcm.2105.
(9) A.P. Schellinger and P.W. Carr, *LCGC North Am.* **22**(6), 544–548 (2004).
(10) T.H. Walter, P. Iraneta, and M. Capparella, *J. Chromatogr. A* **1075**, 177–183 (2005). DOI:10.1016/j.chroma.2005.04.039.
(11) M.R. Schure, J.L. Rafferty, J.I. Siepmann, and L. Zhang, *LCGC Europe* **27**(1), 18–27 (2014).
(12) R.A. Mowery, *J. Chromatogr. Sci.* **23**, 22–29 (1985). DOI:10.1093/chromsci/23.1.22.
(13) M.R. Euerby, C.M. Johnson, I.D. Rushin, and D.A.S.S. Tennekoon, *J. Chromatogr. A* **705**, 229–245 (1995). DOI:10.1016/0021-9673(95)00273-P.
(14) L.R. Snyder, J.W. Dolan, and P.W. Carr, *Anal. Chem.* **79**, 3254–3262 (2007). DOI:10.1021/ac071905z.

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Effects of External Influences on GC Results

John V. Hinshaw, GC Connections Editor

Small differences in process gas chromatography (GC) results from the same sample stream over time can indicate corresponding changes in target analyte concentrations, or the fluctuations might be due to external influences on the instrument. This instalment of "GC Connections" explores ways to examine such results and better understand their significance.

I sometimes become involved in conversations that start out with casual observations about data variability and the closeness or lack thereof between two or more sets of analytical results originating from the same material source. Sometimes differences may be expected, especially when, for example, two very different methodologies are compared. In other cases, a lack of closeness between sets of results could indicate a problem that needs attention. This instalment of "GC Connections" explores some of the basics and then examines some real-world data to see what can be learned or at least inferred.

The Problem of External Influences

A collection of experimental data with multiple external influences comes with a problem: Is the apparent meaning of the observations influenced by unaccounted experimental factors? In chromatography, as in other experimental methods, we try to control as many external factors as possible. For example, a tank pressure regulator may be susceptible to the gas flow rate through it, causing its outlet pressure to change significantly as flow changes. The inlet pressure and flow controllers in a gas chromatography (GC) instrument are designed to compensate for this variability. However, if the tank regulator is not configured correctly with an outlet pressure at least 10% higher than the highest column inlet pressure, the ability of the GC system pneumatics to perform accurately may be compromised. This inaccuracy in turn can lead to irreproducible retention times and thus result in poor performance.

A list of some possible external influences includes:

- environmental temperature and pressure,
- main power line voltage and frequency,
- carrier and detector gas purity,
- condition of gas generators,
- service state of in-line gas filters,
- condition of pressure regulators, and
- leaks in connecting tubing.

A collection of experimental data with multiple external influences comes with a problem: Is the apparent meaning of the observations influenced by unaccounted experimental factors?

Factors internal to a GC system that can influence chromatographic results also include:

- inlet liner type and condition,
- state of the detector, such as flame jet cleanliness,
- internal gas leaks or blockages,
- column inlet and outlet connections,
- column contamination and age, and
- inlet, oven, and detector temperature control.

Chromatographic and other experimental results benefit tremendously by users understanding and controlling as many of these factors as possible. The influences listed above are not intended to be comprehensive lists, but rather points of discussion. Considerations for the influence of sampling and sample preparation as sources of error are beyond this discussion, and I am sure readers can name even more factors to worry about.

A real problem arises when such influences are either not identified or cannot be compensated for. Let's review some data with an external influence that can be readily identified and understood.

Table 1 gives measured concentrations and simple statistics for a single component measured by a process GC system during two contiguous intervals of two days each. Visual inspection appears to confirm that the two data sets measure different concentrations. The arithmetic means differ by about the same amount as the standard deviation of the second set of data, and by about twice the standard deviation of the first set of data. But how significant are the differences? Can the conclusion be drawn that the concentration being measured has changed from one set of data to the next?

Most readers will be familiar with Student's *t*-test. An interesting point of fact: the attribution to *Student* refers to the pseudonym used by Willam S. Gosset who in 1908 published the test as a way to monitor the quality of stout beer at Guinness in Dublin, Ireland.

The *t*-test infers information about a larger population from relatively few samples. It is based on the assumption that the population being sampled falls close to a normal or Gaussian distribution of values. The *t*-distribution is a probability density function of the number of degrees of freedom (*df*)

in a sample set. For a single set of n measurements, $df = n - 1$. As degrees of freedom increase beyond about 60, the *t*-distribution approaches a normal distribution. At lower levels, it predicts the entire population's characteristics on the basis of the fewer available samples. As we shall see, and much like chromatographic peaks, this assumption can be incorrect for real-world data.

The *t*-test is most often applied to a single set of data in comparison to a single known value, to determine the significance of the hypothesis that the data represents the same value as the known amount. The *t*-test can also be applied to two data sets in comparison to each other, but it assumes that the variances of the sampled populations are the same, and it works best if the number of samples or degrees of freedom of each sample set are the same as well. This last assumption is true for the data in Table 1, but the *variances*, which are the squares of the standard deviations, are obviously not the same. This difference is an indication that some unaccounted influence may be at work inside the data.

The *t*-test is most often applied to a single set of data in comparison to a single known value, to determine the significance of the hypothesis that the data represents the same value as the known amount.

There are several alternatives to the basic *t*-test. In the present case, Welch's unequal variances *t*-test seems the most appropriate. This modification accommodates unequal population variances, although it still assumes that the population variances are normal. Performing Welch's *t*-test gives a null-hypothesis probability (*p*-value) of ~2×10^{-6} that the mean values are not different or, to put it another way, the probability that the sample means are different seems to be greater than 99.999%.

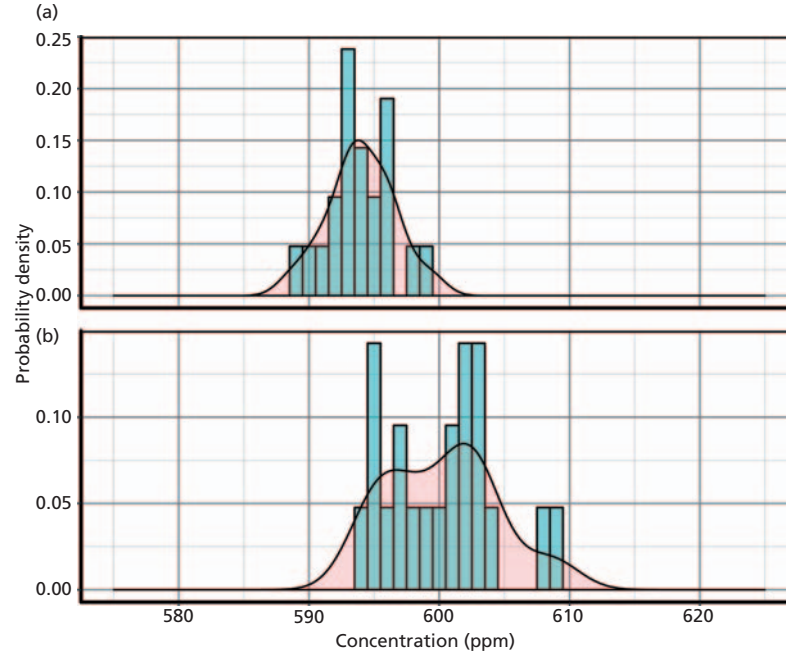
The data analysis might stop at this point, and we might conclude that the quantity being measured has changed from the first sampled interval to the second. However, the significantly different variances or standard deviations of the two sample sets should lead to further investigation.

The two sample data sets are plotted in Figure 1 as histograms, where the height of each bar represents the number of samples with values between regular intervals along the *x*-axis. In this case, the intervals are spaced at 1-ppm increments. For the first set of data, there are two values at 595 ± 0.5 ppm at the points 595.3 and 595.4, while for the second set there are three values in the same interval, at 594.9, 595.0, and 595.4. The smooth filled curve in each plot shows a calculated probability density that a sample falls at a particular concentration, and helps visualize the distribution of the measured values. The values have a normal-looking

Table 1: On-line GC results for one component measured during two different intervals

Sample Number	Concentration (ppm)	
	March 28–29	March 30–31
1	588.6	594.1
2	595.1	600.7
3	597.9	603.5
4	590.7	595.7
5	596.1	602.3
6	593.4	599.9
7	593.9	598.1
8	596.5	596.7
9	592.1	594.9
10	591.7	595.4
11	594.3	597.4
12	595.4	602.2
13	599.5	603.9
14	590.2	609.1
15	596.4	602.1
16	595.8	608.0
17	593.5	603.3
18	594.3	601.4
19	592.7	602.6
20	593.0	595.0
Average	594.06	600.32
Standard deviation	2.67	4.31

Figure 1: Histogram plots of GC measurements over contiguous two-day intervals: (a) First two days of data, and (b) second two days of data. The vertical bars show the total number of results falling within ±0.5 ppm of each concentration level. The filled curve shows a smoothed cumulative probability density across all of the values.



distribution for the first sample set but definitely not for the second one.

Another useful visualization is a time-series plot of the data. This plot can help you see if there is some systematic factor that varies over time and has an influence on the measurements. Figure 2 is a time-series plot, with the measurement data in the upper panel and the bulk sample-stream temperature in the lower panel. There is a clear correlation between sample temperature and measured

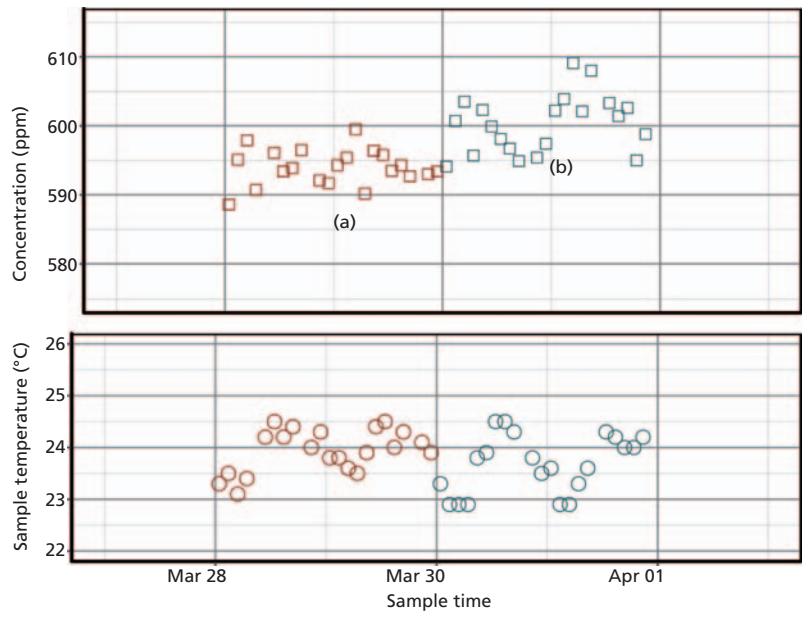
A High-Pressure Tank Fitting Hazard

Recently, I was asked to help troubleshoot a multigas-dilution system that had eight individual gas tanks and regulators. It's the kind of equipment that produces precise gas dilution of a standard into a bulk gas, such as nitrogen or air, used for calibration or other precision studies. Most of the regulators had been purchased within the past year, and I didn't pay much attention to them other than to check the outlet pressure setting and see that the tank and regulator valves were actually turned on.

It became necessary to change one of the gas standard tanks after working with the system for a bit, and I cheerfully volunteered to do so. I brought over the new tank on a tank cart, closed the tank valve on the empty tank, and proceeded to unscrew the high-pressure fitting. I was very surprised and then alarmed to find that only one and a half turns were needed to remove the threaded regulator-side collar from the tank fitting. Thinking that this couldn't be right—perhaps the empty tank's fitting was defective or cross-threaded—I carefully attached the regulator onto the new tank's fitting. However, I found the same situation: fewer than two turns were needed until the regulator fitting would have to be tightened with a tank wrench. This is a serious problem because this fitting is tasked with sealing pressures up to 3000 psig (20.7 MPa) across the cross-sectional area of the nipple of about 0.5 in.^{2} (3.2 cm^2). I'm not a mechanical engineer, but assuming this setup amounts to a linear force along the axis of the fitting, it means that the threads would have to restrain around 1500 lb (680 kg). That's not something I am confident that two threads of the regulator nut could withstand. The regulator was red-tagged and quarantined. Some analysis revealed that the fitting was not cross-threaded, but in fact the inner metal nipple seemed to be too large to engage properly with the tank fitting.

I hadn't observed this problem before, but now I always check that regulator nuts thread onto tank fittings properly, with at least 4–5 turns required before final tightening with a wrench. It is very important that those who work with high-pressure gas tanks receive adequate training and update their procedures accordingly.

Figure 2: Time-series plot of the experimental data: (a) First two days of data, and (b) second two days of data. The upper panel shows the measured data values and the lower panel shows the corresponding observed sample-stream temperatures.



concentrations. The peak-to-peak sample temperature fluctuates a bit more in the second sample set than in the first, which could explain the larger observed standard deviation in the second set. The peaks and valleys of the concentration measurements tend to lag behind the sample temperatures by some hours. This time lag is an expected behaviour in the process system under test because of the flows and volumes involved, although there is no room here to provide more detail. A clear upward trend in sample concentration is also apparent in the second set of observations, while the sample temperature moves about a relatively constant value.

The upward trend makes simple *t*-test results less meaningful. We no longer have an unchanging population to sample; it has changed while we observe it. This fluidity strongly contributes to the apparent variance of the test data. How to proceed with data analysis depends on the measurement goal. Do we want to know whether the concentration changes over a shorter or longer time span? Smoothing or removing the thermal influence from the data could remove much of the periodic nature of the results and reveal a more clear picture of how the results increase over longer time spans, while making measurements more frequently would improve the short-term characterization. There may be, and probably are, other external influences on the results. As a whole, the external factors tend to couple together, as well, which correlation techniques such as principal component analysis can help unravel.

Conclusion

This brief data analysis shows the influence of temperature on measured results. Although the system under test was not a typical laboratory setup, it demonstrates how a simple statistical analysis of measured results can provide misleading information about the variability of the results and the influence of external sources. It also shows that analysts can better understand how their systems are affected by outside influences, and then proceed to take control of the variables they can while accommodating those they cannot change.

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New Advice on an Old Topic: **Buffers in Reversed-Phase HPLC**

Sharon Lupo and Ty Kahler, Restek, Bellefonte, Pennsylvania, USA

Buffers are commonly used in reversed-phase liquid chromatography (LC) to control the ionization state of analytes. However, the addition of buffers is much more complex than simple pH control. Complex equilibria exist between these mobile-phase additives, the analytes, the silica surface, and even the stationary phase in certain circumstances. The addition of mass spectrometry (MS) as a primary detection technique makes decisions about mobile-phase additives even more crucial. In this column instalment, we use a model set of analytes and selected applications to demonstrate the effects that buffers can have not only on the selectivity of a separation, but also on the sensitivity of a reversed-phase analysis when using MS detection.

The successful use of buffers can be critical for the retention and separation of ionic sample mixtures containing any combination of acidic, basic, or neutral compounds, by reversed-phase liquid chromatography (LC). According to the Brønsted-Lowry definition, an *acid* is a substance that donates a proton (hydrogen ion, H^+) and a *base* is a substance that accepts a proton. The product that results when the acid loses the proton is called the *conjugate base* of the acid. The product that results when the base gains a proton is called the *conjugate acid* of the base.

$$\underset{\text{Acid}}{HA} + \underset{\text{Base}}{B} \leftrightarrow \underset{\text{Conjugate base}}{A^-} + \underset{\text{Conjugate acid}}{HB^+} \quad [1]$$

Acids differ in their proton-donating ability. Most acids are weak and only partially ionize in solution. The strength of an acid in solution can be expressed by the *acid dissociation constant*, K_a, as shown in equation 2. Acid strengths are typically expressed as pK_a, or the negative logarithm of the acid dissociation constant. A stronger acid has a lower pK_a (higher K_a) whereas a weaker acid has a higher pK_a (lower K_a) (1).

$$K_a = \frac{[H^+]\ [A^-]}{[HA]} \quad [2]$$

Buffer systems typically consist of a weak conjugate acid–base pair. For example, formic acid is a weak organic acid and ammonium formate is a salt containing its conjugate base. When in solution, this pair of compounds resists changes in pH because they contain both an acidic species to neutralize OH^- ions and a basic one to neutralize H^+ ions (2). In reversed-phase LC, additives such as formic acid, acetic acid, and ammonium hydroxide are commonly used to prepared mobile-phase solutions. Although they are not true buffers (at all operational pH values), these additives will maintain a relatively constant pH upon dilution.

Buffer Considerations

The Analyte: One of the primary functions of a buffer solution in reversed-phase LC is to maintain a constant mobile-phase pH so that acidic or basic analytes can be kept in a single ionization state. Using the Henderson-Hasselbalch equation, the relationship between pH and K_a is demonstrated:

$$pH = pK_a + \log \frac{[\text{base}]}{[\text{acid}]} \quad [3]$$

where [acid] and [base] refer to the equilibrium concentrations of the conjugate acid–base pair. As shown in Figure 1, the further the mobile-phase pH is from the pK_a of a compound, the less impact small changes in mobile-phase pH will have on the compound's ionization state. When the pH and pK_a are equal, the compound is considered to be 50% ionized and small changes in mobile-phase pH will have more drastic effects on the compound's degree of ionization. As a molecule increases in polarity, its retention will decrease. Therefore, when an acid or base becomes ionized the molecule becomes more polar, or hydrophilic, and retention is reduced (3). Differences in retention are demonstrated in Figure 2 where the retention factor (k') of three representative compounds (acid, base, and neutral) are plotted against mobile-phase pH. In this example, acetylsalicylic acid (weak acid) loses a proton, or dissociates, and becomes ionized when the mobile-phase pH is increased. Alternatively, nortriptyline (weak base) gains a proton and becomes ionized as the mobile-phase pH decreases. The retention of acetophenone (neutral) remains largely unaffected by the changing mobile-phase pH. Through this example it becomes apparent that mobile-phase pH can be a powerful strategy for controlling selectivity in ionic sample mixtures. A common approach in reversed-phase LC is to adjust the mobile-phase pH so that ionizable compounds are

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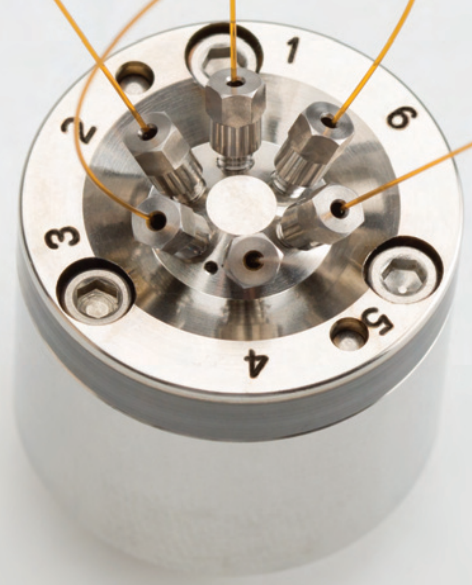

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Figure 1: The relationship between percent ionization for acids and bases as a function of pH in reversed-phase LC.

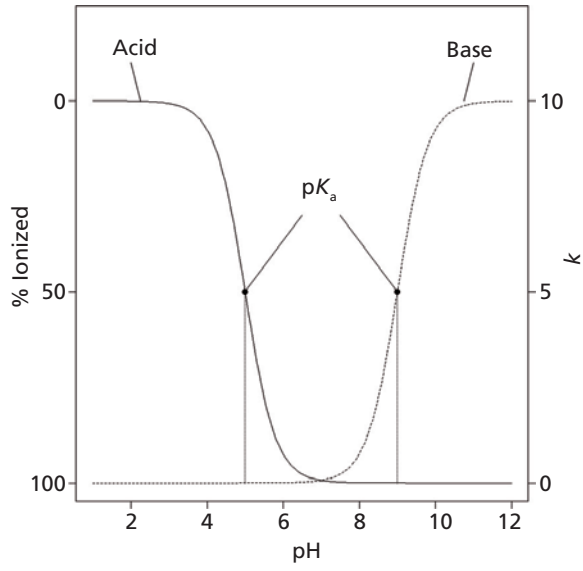
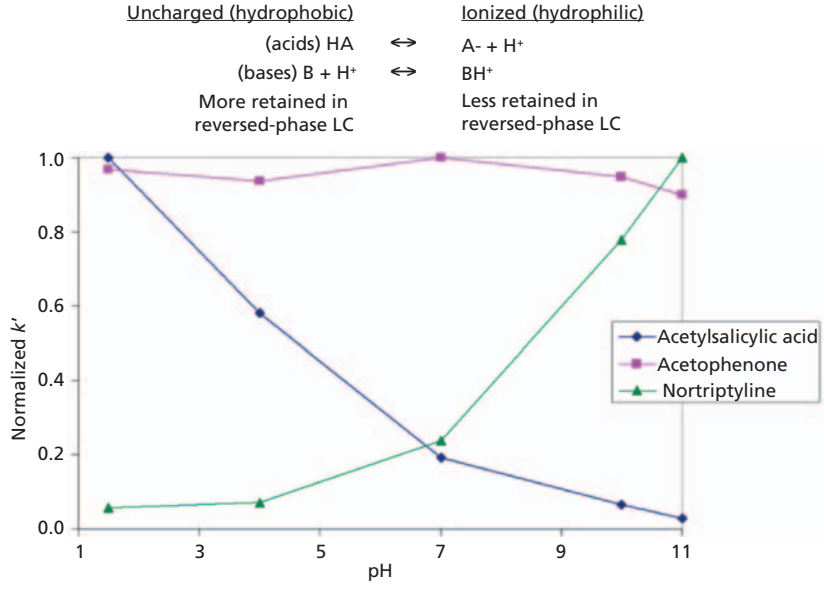


Figure 2: The acid dissociation equilibrium for acidic and basic molecules and how it correlates with retention as a function of mobile-phase pH in reversed-phase LC.

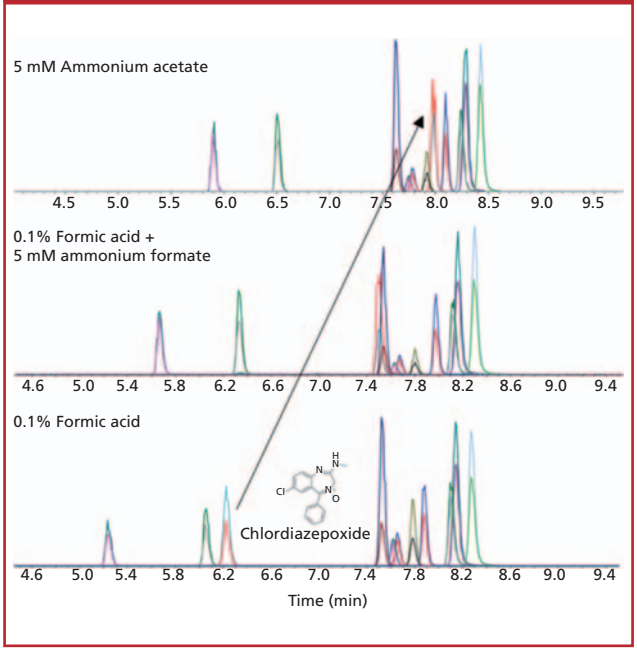


in their neutral state for maximum retention. This means for acidic compounds, the mobile-phase pH should be below its pK_a and vice versa for basic compounds. As a rule, the mobile-phase pH should be at least ±1.5 pH units above or below the pK_a values of the analytes to avoid pH-related retention issues. Although using a mobile phase within 1.0 pH units of the compound pK_a will provide chromatographers with the most control over selectivity, working in this region can result in an irreproducible separation since a minimal change in pH will result in a maximum change in retention.

When selecting a buffer for pH control, one must consider its buffer capacity and solubility. The ability of a buffer to maintain a constant pH is its *buffer capacity*, which is dependent on the buffer pK_a value, buffer concentration, and the desired pH of the mobile phase. An appropriate buffer should have a pK_a value within 1.0 unit of the desired mobile-phase pH. Outside of this range, buffering becomes less effective and an increased buffer concentration will be required to achieve the same buffer capacity (3). Likewise, buffer solubility must also be taken into consideration, particularly when gradient elution is performed; buffers can precipitate from solution when they are mixed with organic solvents. Buffer solubility is dependent on the buffer concentration, counter-ion, and organic solvent. Obviously, lower buffer concentrations will be more soluble. In general, ammonium salts are the most soluble and sodium salts are the least, while methanol tends to exhibit greater solubility than acetonitrile does.

The impact of buffer type and the associated change in mobile-phase pH on relative retention is demonstrated in Figure 3. Here, a mixture of benzodiazepines and their metabolites are separated with gradient elution using three different mobile-phase modifiers of varying pH: 0.1% formic acid (pH ~2.7), 0.1% formic acid and 5 mM ammonium formate (pH ~3.0), and 5 mM ammonium acetate (pH ~6.8). As the pH of the mobile phase increases, several of the early eluting compounds (7-aminoclonazepam, 7-aminoflunitrazepam, and chlordiazepoxide) become more retained while the retention of the remaining benzodiazepines remains constant. In particular, chlordiazepoxide, with a pK_a of ~4.6 (4), displays a drastic increase in retention that results in an elution order switch. The majority of the benzodiazepines are weak bases with pK_a values ranging from 8 to 10. By raising the mobile-phase pH above its pK_a, chlordiazepoxide becomes less ionized and more retained; orthogonal selectivity is observed as the mobile-phase pH crosses the pK_a of chlordiazepoxide. The other benzodiazepines are unaffected because the mobile-phase pH does not approach their pK_a and their

Figure 3: The effect of buffer type on the gradient elution of several benzodiazepines and their metabolites. Column: 100 mm × 2.1 mm, 2.7-µm superficially porous biphenyl; mobile-phase A: water + additive; mobile-phase B: methanol + additive; gradient: 10–100% B over 9 min with a 1-min hold at 100% B; flow rate: 0.4 mL/min.



ionization state remains unchanged. In this example, the use of different buffers allows the mobile-phase pH to be adjusted while maintaining sufficient buffer capacity by working within its buffer range.

The Column: In addition to analyte interactions, the effects of buffer on the silica support of the column must be considered. Most reversed-phase LC columns are stable for a mobile-phase pH between 2 and 8. Using mobile phases outside of this range can result in shortened column lifetime. Low-pH mobile phases (pH ≤2) can cause hydrolysis of the stationary phase from the silica support while high-pH mobile phases (pH ≥8) can dissolve the silica packing. Silica is less susceptible to dissolution when low concentration organic buffers are used in conjunction with low oven temperatures (5). Specialty columns exist that enable analysis outside this range by protecting the silica from chemical attack through steric hindrance or the use of hybrid silica supports.

Buffer–silica interactions have been shown to reduce retention and improve peak shape of positively charged analytes by suppressing the ion-exchange retention mechanism that can occur between positively charged bases and the anionic silanols (pK_a ~3.5–7) of the stationary phase support. This retention mechanism was pronounced in older type-A silica columns because of the presence of heavy metals that increased the acidity of the silanol groups. As a result, a higher concentration of ionized silanols occurred regardless of the mobile-phase pH causing severe peak tailing. Silanol ionization can be minimized by utilizing low-pH mobile phases; however, this can, in turn, protonate basic analytes. An increase in mobile-phase ionic strength has been shown to reduce silanol activity through masking of the silanol active sites. Ionic strength can be elevated through increased buffer concentration or by using a mobile-phase pH, which will optimize buffer capacity while maintaining the same buffer concentration. Most columns today consist of purer type-B silica, which is more reproducible with less tailing; however, they can still suffer from poor peak shapes. When protonated bases are analyzed in conjunction with low-pH mobile phases, charge repulsion can occur between the retained ionized molecules because of column overloading (6). This type of column overload can be overcome by reducing the injection volume or by increasing the mobile-phase ionic strength (7). In Figure 4, a sample mixture containing 4-hexylaniline (primary amine) is analyzed with two different phosphate buffer concentrations, 1 mM and 10 mM, both adjusted to pH 2.5. The mixture of 4-hexylaniline displays a peak shape indicative of column overload when 5 µL are injected with the 1 mM buffer concentration. When the same volume is injected with the 10 mM buffer concentration, the peak shape for 4-hexylaniline improves combined with a loss in retention. Here, peak shape is improved by increasing the ionic strength of the mobile phase. The increased buffer concentration results in a concurrent change in selectivity caused by a reduction in the available ionized silanols capable of ion exchange. Lacking a charge, the neutral compounds are unaffected by ionic forces of repulsion and attraction, and therefore their retention and peak shape remain consistent.

Figure 4: The effect of buffer concentration on reversed-phase LC retention for a sample mixture containing representative basic and acidic solutes. Column: 150 mm × 4.6 mm, 5-µm fully porous C18; mobile-phase A: potassium phosphate buffer, pH 2.5; mobile-phase B: acetonitrile; gradient: 50–100% B over 15 min; flow rate: 1.5 mL/min; injection volume: 5 µL; oven temperature: 30 °C; detection: absorbance at 210 nm. Peaks: 1 = isopropylbenzyl alcohol, 2 = benzene, 3 = 4-hexylaniline (basic), 4 = toluene, 5 = ethylbenzene, 6 = 4-hexylbenzoic acid (acid), 7 = 4-pentylbenzaldehyde, 8 = 4-pentylbenzonitrile.

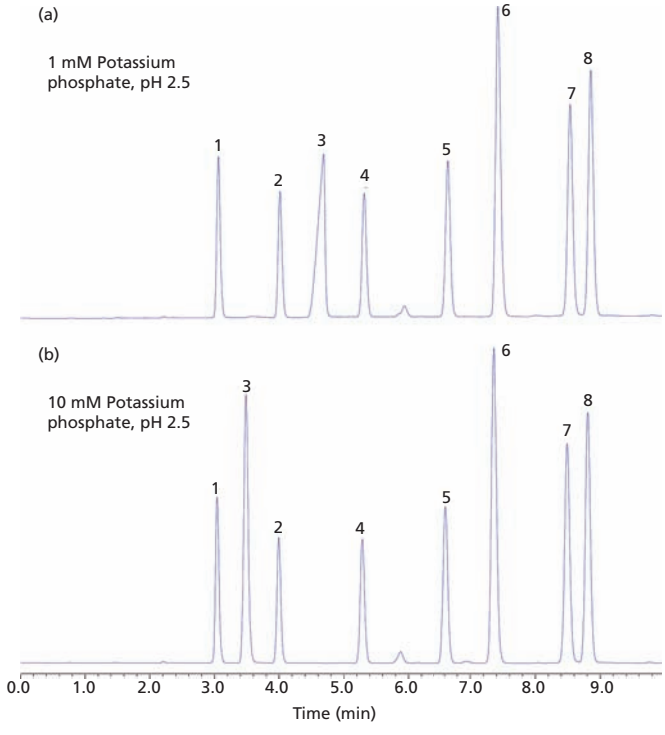
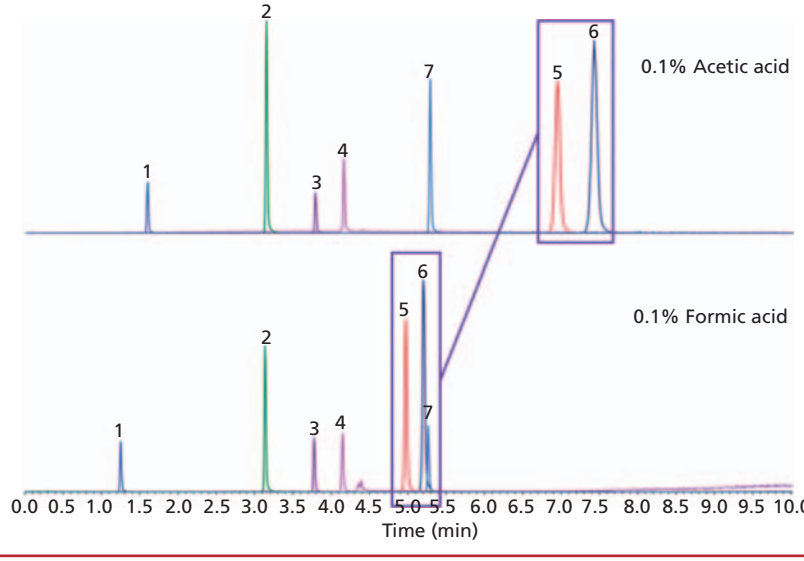


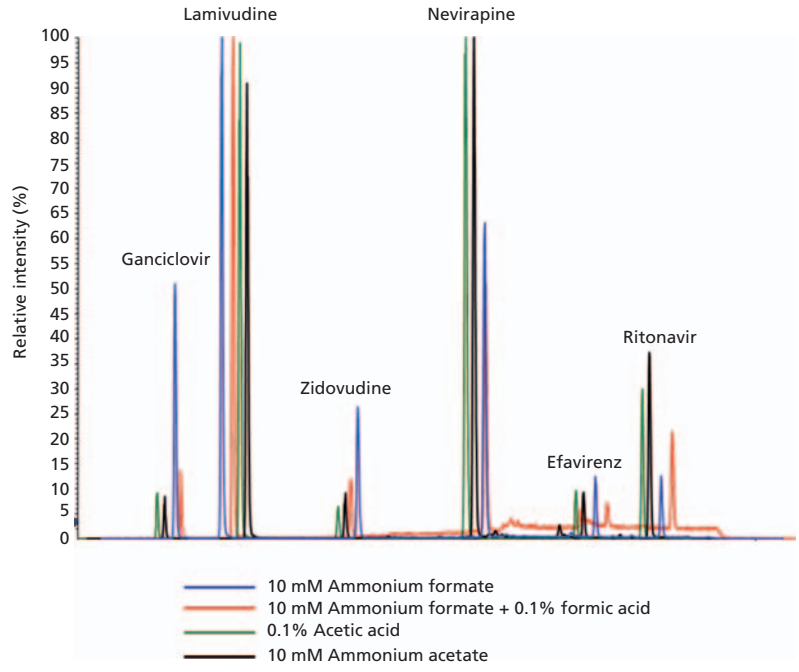
Figure 5: Comparison of selectivity as a function of acidic modifier on a superficially porous PFPP column. Column: 100 mm × 2.1 mm, 2.7-µm superficially porous PFPP; mobile-phase A: 0.1% acid in water; mobile-phase B: 0.1% acid in acetonitrile; gradient: 5–100% B over 10 min; flow rate: 0.8 mL/min. Peaks: 1 = atenolol, 2 = phenytoin, 3 = trenbolone, 4 = testosterone, 5 = nortriptyline, 6 = amitriptyline, 7 = celecoxib.



In some instances, buffers can enhance stationary-phase selectivity. This phenomenon is particularly true for perfluorinated phenyl phases where the electronegative fluorine ring can intensify the ion-exchange capability of the base silica over that of an alkyl column (8). Contrary to the previous example where increased mobile-phase ionic strength was used to suppress the ion-exchange retention of ionized bases, preferential selectivity of bases can be achieved by enhancing this mechanism. In a sample composed of acidic, basic, and neutral probes, reversed-phase selectivity is compared for water and acetonitrile mobile phases modified with 0.1% formic acid and 0.1% acetic acid on a superficially porous pentafluorophenylpropyl (PFPP) column (Figure 5). When switching from formic acid to acetic acid there is a dramatic increase in retention for nortriptyline (pK_a ~10.5) and amitriptyline (pK_a ~9.7), two tricyclic antidepressants both of which contain an amine functionality. The use of acetic acid as mobile-phase modifier results in a decrease in ionic strength and an increase in mobile-phase pH from ~2.7 (0.1% formic acid) to ~3.5. At this pH, it is likely that the number of active silanols will increase while nortriptyline and amitriptyline remain charged, resulting in an increase in retention. The neutral and acid probes are not capable of ion exchange with the silanols so their retention remains constant.

Mass Spectrometry Detection: Another consideration when choosing an appropriate buffer is the intended means of detection. Characteristics such as absorbance and volatility make some buffers incompatible with certain detectors. For example, if ultraviolet (UV) detection is performed, it is important to choose a buffer with a low UV cutoff to avoid a significant increase in the UV absorbance of the mobile phase (typically a buffer with a UV cutoff <210 nm is preferred). For liquid chromatography–mass spectrometry (LC–MS) methods, a volatile buffer is required to prevent excessive background noise, contamination, and fouling of the MS detector.

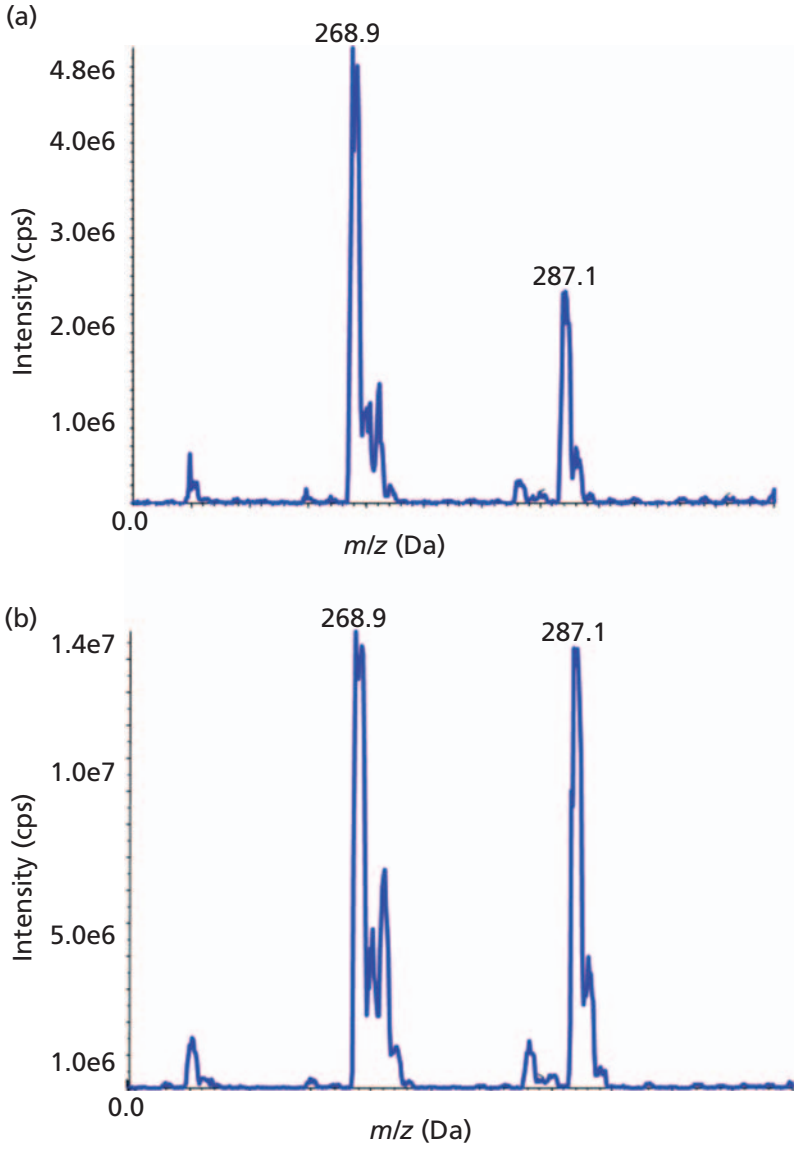
Figure 6: Antiretroviral drug response as a function of mobile phase additive when analyzed by LC–ESI-MS in positive-ion mode. Column: 100 mm × 2.1 mm, 2.7-µm superficially porous biphenyl; mobile-phase A: water + additive; mobile-phase B: methanol; gradient: 5–95% B over 9 min; flow rate: 0.5 mL/min. Peaks: 1 = ganciclovir (weak base), 2 = lamivudine (weak base), 3 = zidovudine (weak acid), 4 = nevirapine (amphoteric), 5 = efavirenz (weak acid), 6 = ritonavir (weak base).



Sensitivity in LC–MS directly relates to *ionization efficiency*, or the effectiveness of producing gas-phase ions from analyte molecules in solution (10). There are several ionization techniques available; however, the focus of this discussion is on electrospray ionization (ESI). In ESI, positive and negative ions are separated by the presence of an electrostatic field at the tip of the sample capillary in the ionization source. In positive ESI, the negative ions are neutralized on the capillary wall, and positive ions drift downfield to the surface of the liquid front at the capillary tip where a Taylor cone is formed (11). At the surface of the Taylor cone, electrostatic repulsion overcomes the surface tension of the liquid and causes the cone to break up into small electrically charged droplets. These droplets travel towards the atmospheric pressure interface, and as they do, their surface area decreases because of evaporation of the solvent from the droplet. As a result, the surface charge density of the droplet increases. Once the droplet reaches a certain radius, called the *Rayleigh limit* (12), repulsion forces once again exceed the surface tension and cause an explosion of even smaller droplets to form (13). The process repeats itself until the droplet is so small that gas-phase ions are emitted (14).

ESI has many advantages, including its applicability to analytes over a large polarity range and its compatibility with large molecules or proteins and thermally labile compounds. The primary disadvantage of ESI is that it is susceptible to the reduction of detector response due to competition for ionization efficiency in the ionization source, also known as *ion suppression* (or enhancement in the case of elevated ion efficiency). There are multiple

Figure 7: Comparison of MS response for the infusion of two estrogens (50 ng/mL) when prepared in (a) 15:85 water–methanol, 0.2% acetic acid and (b) 15:85 water–methanol, 0.2% ammonium hydroxide. Sample: estrone (286.9 *m/z*) and estriol (287.1 *m/z*). Infusion rate = 7 µL/min.



causes for this phenomenon, including competition for available charge or surface area of the droplet, increased surface tension, which can reduce the rate of solvent evaporation (15), coprecipitation of the analyte with nonvolatile solutes (16), and neutralization of the analyte by ion pairing with mobile-phase additives or by gas-phase reactions (17). Ionization by ESI is complex and can be influenced by the characteristics of the mobile-phase buffers selected. In many cases, the mobile-phase system that is optimal for analyte retention and resolution is not optimal for analyte ionization because neutral analytes tend to display the best retention in reversed-phase LC. Therefore careful selection of mobile-phase buffer type and concentration is paramount for a sensitive and selective assay.

Common choices for LC–MS mobile-phase additives are acetic acid, formic acid, and their ammonium salts. These buffers are volatile and allow for adequate coverage of the working pH range of 2–8 for reversed-phase LC. Alternatively, ammonium hydroxide or ammonium bicarbonate can be used for high pH applications (pH >8). If pH <2 is required, trifluoroacetic acid is a viable option; however, strong acids such as this are capable of ion-pairing, which can have deleterious effects on method sensitivity and can be difficult to flush from the instrument if used for prolonged periods of time.

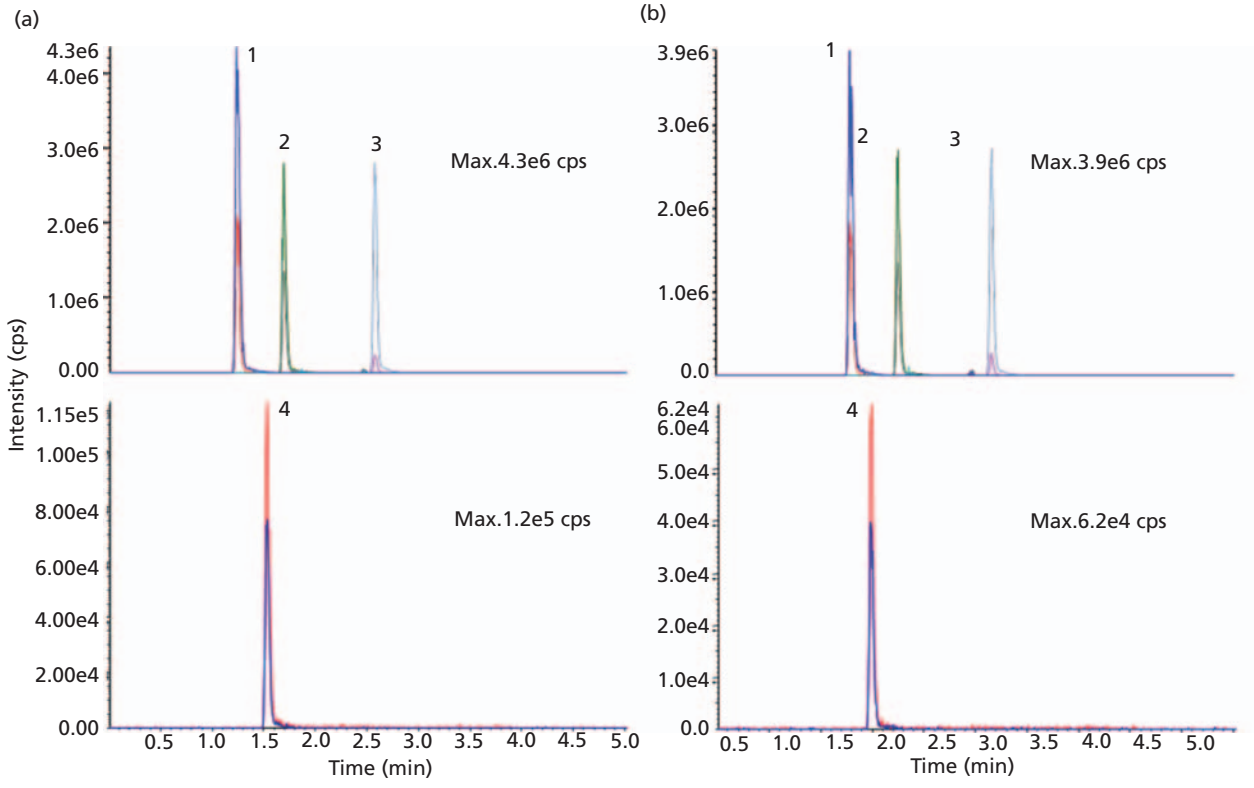
In addition to increasing the reproducibility of the chromatographic method, these additives can serve to enhance ionization. The effects of different buffers on ionization is not always well understood and can be compound dependent, as shown in Figure 6. The response for several antiretroviral drugs analyzed by LC–ESI-MS in positive-ion mode are compared as a function of additive composition in the mobile phase. Ganciclovir, a weak base, displays optimal response with a 10 mM ammonium formate buffer. Surprisingly, so does zidovudine, a weak acid. Lamivudine appears to ionize well regardless of the buffer used, whereas efavirenz shows poor ionization under all circumstances. The overall best responses are achieved with a 0.1% acetic acid modification. It would be reasonable to believe that an acidic modifier would provide the best analyte response in ESI positive-ion mode because of its ability to donate protons, while a basic modifier would be preferred in negative-ion mode as a proton acceptor. This theory holds true for the negative ionization of two neutral estrogens, estrone and estriol (Figure 7). The response for the estrogens triples when they are prepared in diluent containing 0.2% ammonium hydroxide compared to one that contains 0.2% acetic acid. However, this is not always the case. It has been shown that abundant protonated molecules can be produced in basic conditions and abundant deprotonated molecules in acidic conditions. This phenomenon is referred to as *wrong-way-round ionization* (18).

Buffer salts containing ammonia (for example, ammonium formate or ammonium acetate) can increase the ionization efficiency of polar neutral compounds that cannot be

ionized on their own. Ammonium adducts can boost the ionization efficiency of these compounds and can be formed by including a constant supply in the mobile phase (1–2 mM). In a recent example from our laboratory, we analyzed digoxin and digitoxin at low levels. These two cardiac glycosides exhibit very poor sensitivity in the absence of ammonium buffers; however, they show significantly enhanced sensitivity through adduct formation. Often the formation of adducts is unwanted since they can diminish the signal of the protonated molecule $[M+H]^+$. Sodium and potassium adducts are ubiquitous in LC–MS since contamination of metal ions can occur from glassware, solvents, and analysts themselves. One way to reduce the level of metal adducts in ESI positive-ion mode is to lower the pH of the mobile phase with formic acid. The excess in protons provided by the acid will drive the majority of ion formation to the protonated molecule $[M+H]^+$ (19).

The concentration of mobile-phase modifiers used in LC typically range from 10–100 mM; however, loss in signal has been observed in ESI at this level. To avoid ion suppression, buffer concentrations not exceeding 10 mM are recommended for LC–MS applications. The inverse relationship between response and buffer concentration in ESI may be attributed to an increase in repulsive forces because of increased charge density. These forces cause spreading of the spray plume in the source and a reduction of ions in the centre of the spray with ultimately less ions collected by the atmospheric pressure ionization (API) source for detection (20). Decreased sensitivity could also be caused by competition at the surface of the droplet due to an increase in bulk ions from the mobile phase (21). The effects of buffer concentration are demonstrated in Figure 8 where cardiac drugs flecainide, verapamil, and amiodarone were analyzed by LC–ESI-MS in positive-ion mode and furosemide was analyzed in negative-ion mode. As the concentration of ammonium formate is increased in the mobile phase from 2 mM to 10 mM, the overall intensities in positive and negative-ion mode decrease approximately 9.3% and 48.3%, respectively. Mallet and colleagues (22) devised experiments to benchmark common volatile mobile-phase additives and their effect on ESI response. Although there was a strong compound dependency, for the mobile-phase acids, bases, and buffer salts tested, Mallet found that acidic additives and buffer salts showed an inverse relationship between concentration and ESI signal for acidic and basic compounds analyzed in positive and negative-ion mode; as the concentration is increased a decrease in response is observed. On the other hand, ammonium hydroxide displayed a positive correlation with an increase in sensitivity for basic compounds in positive-ion mode with increased

Figure 8: The effect of buffer concentration on LC–ESI-MS signal intensity for representative cardiac drugs in positive and negative-ion modes. Mobile phases consisted of water and methanol each modified with 0.1% formic acid and ammonium formate at a concentration of (a) 2 mM or (b) 10 mM. Peaks: 1 = flecainide, 2 = verapamil, 3 = amiodarone, 4 = furosemide.



concentration (22). In this scenario, ionization is believed to take place through proton transfer reactions in the gas-phase, since an increase in electrolytes has been shown to cause suppressed ionization of analytes while in the liquid-phase (20). The relationship reverses itself once again for the analysis of acids in negative-ion mode using ammonium hydroxide with a decrease in response observed with an increase in concentration of the additive.

Conclusion

It is clear that the relationship between analyte retention, ionization, and the mobile-phase buffer system is complex. For improved retention, the application of acid–base theory as it relates to the analyte structure, pK_a, and analytical column chemistry is required. In reversed-phase LC, buffers can be used to neutralize charged acids and bases for improved retention. In turn, an understanding of LC–MS ionization theory needs to be used to avoid unnecessary ion suppression. Although ionization efficiency is largely analyte specific, the use of mobile-phase additives in low concentrations can improve ESI response. Predicting the effects a particular additive will have on a reversed-phase LC method is not absolute; however, the correctly chosen buffer can help create a sensitive, selective, and reproducible method.

References

(1) J. McMurry, *Organic Chemistry*, 3rd Edition (Brooks/Cole Publishing Company, Pacific Grove, California, USA, 1992), pp. 50–53.
(2) T.L. Brown, H.E. LeMay, Jr., and B.E. Bursten, *Chemistry The Central Science*, 6th Edition (Prentice Hall, Englewood Cliffs, New Jersey, USA, 1994), pp. 629–630.
(3) L.R. Snyder, J.J. Kirkland, and J.W. Dolan, *Introduction to Modern Liquid Chromatography*, 3rd Edition (John Wiley & Sons, Hoboken, New Jersey, USA, 2010), pp. 304–331.
(4) T.H Shayesteh, M. Radmehr, F. Khajavi, and R. Mahjub, *Eur. J. Pharm. Sci.* **69**, 44–50 (2015).
(5) J. Nawrocki, *J. Chromatogr. A* **779**, 29–71 (1997).
(6) S.M.C Buckenmaier, D.V. McCalley, and M.R. Euerby, *Anal. Chem.* **74**, 46–72 (2002).
(7) D.V. McCalley, *J. Chromatogr. A* **1075**, 57 (2005).
(8) D.S. Bell and A.D. Jones, *J. Chromatogr. A* **1073**, 99–109 (2005).
(9) C.R. Mallet, A. Lu, and J.R. Mazzeo, *Rapid Commun. Mass Spectrom.* **18**, 49–58 (2004).
(10) J.S. Page, R.T. Kelly, K. Tang, and R.D. Smith, *J. Am. Soc. Mass Spectrom.* **18**, 1582–1590 (2007).
(11) G.I. Taylor, *Proc. R. Soc. Lond. A.* **280**, 383 (1964).
(12) L. Rayleigh, *Philos. Mag.* **14**(1), 184 (1882).
(13) A. Gomez and K. Tang, *Phys. Fluid.* **6**, 404 (1994).
(14) P. Kebarle, *J. Mass Spectrom.* **35**, 804 (2000).
(15) I. Fu, E.J. Wolf, and B.K. Matuszewski, *J. Pharm. Biomed. Anal.* **18**, 347 (1998).
(16) R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, and T. Olah, *J. Am. Soc. Mass Spectrom.* **11**, 942 (2000).
(17) M.H. Amad, N.B. Cech, G.S. Jackson, and C.G. Enke, *J. Mass Spectrom.* **35**, 784 (2000).

(18) S. Zhou and K.D. Cook, *J. Am. Soc. Mass Spectrom.* **11,** 961 (2000).
(19) F. Klink, MS Solutions #3, Sepscience.com/Information/Archive/MS-Solutions.
(20) R. Kostiainen and T.J. Kauppila, *J. Chromatogr. A* **1216**, 685–699 (2009).
(21) R. Kostiainen and A.P. Bruins, *Rapid Commun. Mass Spectrom.* **8**, 549–558 (1994).
(22) C.R. Mallet, Z. Lu, and J.R. Mazzeo, *Rapid Commun. Mass Spectrom.* **18**, 49–58 (2004).

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Ty Kahler joined Restek as a Senior Innovations Chemist in 2008 and has since become the Manager of the HPLC R&D and Applications group. In addition to overseeing the HPLC lab, his responsibilities include LC methods development, quality investigation and implementation, and phase design and research. Before joining Restek, Ty worked as a manager, study director, and principal investigator for a contract research laboratory conducting methods development, validation, and routine analysis of pharmaceuticals and agrochemicals. He has been in the field of liquid chromatography for more than 17 years, including time as a quality chemist and instrumentation metrologist.

David S. Bell is a manager in pharmaceutical and bioanalytical research at MilliporeSigma (formerly Sigma-Aldrich/Supelco). With a B.S. degree from SUNY Plattsburgh and a PhD in analytical chemistry from The Pennsylvania State University, Dave spent the first decade of his career within the pharmaceutical industry performing analytical method development using various forms of chromatography and electrophoresis. During the past 20 years, working directly in the chromatography industry, Dave has focused his efforts on the design, development, and application of stationary phases for use in HPLC and hyphenated techniques. In his current role at MilliporeSigma, Dr. Bell's main focus has been to research, publish, and present on the topic of molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. Direct correspondence to: dave.bell@sial.com

Measuring **Mycotoxins**

***LCGC* spoke to Rudolf Krska from the University of Natural Resources and Life Sciences in Vienna, Austria, about the latest analytical techniques and challenges facing analysts involved in the evolving field of mycotoxin analysis.**

Interview by Alasdair Matheson

Q. Mycotoxin analysis is a major field in food analysis at the moment. Why has mycotoxin analysis become important?
A: The occurrence of mycotoxins (secondary fungal metabolites) in various crops is a global concern because it has significant implications for food and feed safety, food security, and international trade. Despite huge research investments, prevention and control of these toxic secondary metabolites remains difficult and the agriculture and food industries continue to be vulnerable to problems of contamination. In addition, extreme weather conditions because of climate change is increasingly affecting the mycotoxin map in Europe and worldwide. The EU's Rapid Alert System for Food and Feed (RASFF) (EC, 2016) showed that of the total border rejections in 2015, 18.3% were due to mycotoxin contamination exceeding the EU legislative limits, accounting for the most frequently reported chemical hazard (1).

In recent years, research on cumulative risks, exposure, and long-term effects has raised awareness for the control of these health risks. As a result of the potential danger of mycotoxins to humans and livestock, strict regulatory controls determine the sale and use of contaminated food and feeds. Thus, grain and other foodstuff buyers increasingly demand more rigorous and timely food safety testing. Failure to achieve a satisfactory performance may lead to unacceptable consignments being accepted or satisfactory batches being unnecessarily rejected. Recent reports have also demonstrated that the range of mycotoxins present in the food and feed chains goes beyond the list of regulated toxins. In fact, some 140 different fungal metabolites have been found in feed and feed ingredients by the use of a multi-toxin method based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). As a result of such findings, the demand for quantitative and rapid screening tools for the determination of (multiple) mycotoxins has increased tremendously.

Q. What are the biggest challenges in mycotoxin analysis?
A: The biggest challenge in mycotoxin analysis is still the sampling issue. Despite recent available guidance (2), it is still a difficult and tedious task to obtain a representative sample. Appropriate extraction solvents matching the range of (multiple) mycotoxins to be determined is another crucial step followed by proper cleanup. The latter is dependent on the final determination step. The use of LC–MS/MS instruments combined with optimized chromatographic separation reduces the need for sample cleanup. This is especially true for regulated toxins for which fully C13 labelled internal standards can be used to compensate for matrix effects. Ensuring comparability of measurement results is another challenge, particularly for mycotoxin-commodity combinations for which no certified reference materials exist.

Q. What novel approaches has your group contributed to the field of mycotoxin analysis, and what advantages do they offer?
A: Within the frame of (inter-) national efforts to obtain new and comprehensive data on the occurrence of (multiple) mycotoxins in food and feed chains, we have developed a unique multi-analyte approach based on LC–MS/MS. The developed analytical method, which does not require any cleanup, has continuously been extended and fully validated for the quantification of more than 300 fungal and bacterial metabolites, including all regulated mycotoxins in various cereals, food, and feed matrices. This highly cited mass spectrometric method is crucial both to elucidate the occurrence of various mycotoxins potentially being present in agricultural and food commodities and to study their metabolization by plants, animals, and humans. Our mass spectrometric work has also included the development of a fast, easy-to-handle, and highly accurate isotope dilution mass spectrometric assay.

The biggest challenge in mycotoxin analysis is still the sampling issue. despite recent available guidance, it is still a difficult and tedious task to obtain a representative sample.

The latter has become feasible through the production of fully isotopically labelled mycotoxins by means of a patented technology developed in our laboratory in cooperation with Romer Labs. We have also provided the first scientific proof of the occurrence of glucose-bound (masked) deoxynivalenol in naturally

contaminated wheat and maize, as part of the plant's defense strategy to detoxify this most prevalent *Fusarium* mycotoxin. The potential threat to consumer safety from masked mycotoxins has, as a result, been addressed by institutions such as ILSI Europe (Washington, D.C., USA) and the European Food Safety Authority (EFSA) (Parma, Italy). Utilizing the power of the advanced multi-biomarker LC–MS/MS method, we have also been able to provide new insights into the human metabolism of *Fusarium* mycotoxins by identifying and quantifying appropriate biomarkers in human urine.

Within the frame of the EU-funded project MYCOSPEC, we have developed a mid-infrared spectroscopic sensing method using tunable quantum cascade lasers and thin-film waveguides obtained from the University of Ulm (Ulm, Germany). This novel method showed its great potential for the rapid on-site classification of contaminated wheat, maize, and peanuts at the concentration levels established in the EU for deoxynivalenol and aflatoxin B_1, respectively.

Q. You are coordinator of an EU project www.mytoolbox.eu dealing with integrated management strategies to tackle mycotoxins. Can you tell us more about your role in this project and what this project involves.

A: In fact, there is still a pressing need to mobilize the wealth of knowledge from the international mycotoxin research conducted over the past 25–30 years, and to perform cutting-edge research where knowledge gaps still exist. We believe that this knowledge needs to be integrated into affordable and practical tools for farmers and food processors along the chain to reduce the risk of mycotoxin contamination of crops, feed, and food. This is the mission of MyToolBox—a four-year project that has received funding from the European Commission and which I have the pleasure to coordinate. It mobilizes a multi-actor partnership (academia, farmers, technology SMEs, food industry, and policy stakeholders) to develop novel interventions aimed at achieving a significant reduction in crop losses caused by mycotoxin contamination. Besides a field-to-fork approach, MyToolBox also considers safe use options of contaminated batches, such as the efficient production of biofuels. Within a range of novel preharvest interventions we will investigate the genetic resistance to fungal infection, cultural control, the use of novel biopesticides suitable for organic farming, competitive biocontrol treatment, and the development of novel modelling approaches to predict mycotoxin contamination. Research into postharvest measures includes real-time monitoring during storage, innovative sorting of crops using hyperspectral vision-technology, novel milling technology, and the study of the effects of baking on mycotoxins at an industrial scale. Again, our multi-toxin screening method will be crucial to verify the success of all these intervention strategies.

Q. What areas of mycotoxin analysis will your group focus on next?

A: In the past few years, our team has continuously moved from the target analysis of individual mycotoxins to untargeted metabolite profiling and metabolomics of (ideally) all secondary metabolites that are involved in plant–fungi interactions. This methodology is based on *in vivo* stable isotopic ^{13}C-labelling and subsequent measurement of biological samples by full scan high-resolution LC–MS.

We plan to continue and expand our interdisciplinary and comprehensive strategy to study plant–fungi interactions and the metabolism of mycotoxins by moving our research to the next level, a fully integrated "omics-based" approach. In this context, we intend to pursue our efforts to characterize the analytically ascertainable metabolome of wheat and maize genotypes differing in their *Fusarium* resistance level and ideally to link their metabolite profile to resistance criteria or markers. A major goal within this endeavour is the development of a standardized metabolomics platform to study primary and secondary metabolites produced by microorganisms and plants and to understand the interactions between plants, fungi, mycotoxins, and other secondary metabolites at a molecular level.

Q. Does your group focus on other areas of food analysis using chromatography?

A: In the area of food and feed safety my colleague Professor Rainer Schuhmacher and I aim for the fingerprinting of food and feed samples. The development of standardized conditions is a prerequisite for the

development and establishment of robust fingerprinting methods. For the description of defined conditions, again *in vivo* ^{13}C-labelling of the matrix (for example, grains) is a promising approach, which can be achieved through growing plants (matrix) under $^{13}CO_2$ atmosphere. We also intend to combine this approach with the labelling of tracers (for example, different food contaminants). This would facilitate the recognition of changes of the labelled contaminant itself, but also of the tracers or contaminants on the matrix as a result of, for example, food or feed processing. Moreover, the *in vivo* ^{13}C-labelling of the food or feed matrix would enable endogenous and exogenous compounds to be differentiated between. Stable isotopic labelling can be used to detect deviations of secondary metabolites of fungi, plants, and bacteria from normal patterns, flagging suspicious samples for further analysis and confirmation, and for a more accurate quantification and identification of compounds.

Q. Do you think the time will come when there will be no need for the chromatography component in food analysis?

A: In view of the amazing sensitivity and high resolution achievable with novel mass spectrometry, this is certainly a valid question. Nonetheless, chromatography will probably stay forever until (or unless) someone develops a radically different approach to separate complex mixtures. With the advent of small particles and ultrahigh-pressure LC (UHPLC), we can now process smaller amounts of samples faster than ever. And with the wealth of potential compounds, which we aim to quantify in our food and feed chain in highly complex matrices, separation remains as important as ever.

References

(1) European Commission (EC), 2016. The Rapid Alert System for Food and Feed 2015 Annual Report. Available at: http://tinyurl.com/h7l6v9x

(2) FAO's Mycotoxin Sampling Tool: http://www.fstools.org/mycotoxins/ (FAO 2013≠2017)

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www.shimadzu.eu
Shimadzu Europa GmbH, Duisburg, Germany.

Thermal desorption platform

The new TDU 2 from Gerstel is a flexible GC–MS sample introduction platform optimized for ruggedness and ultra-low LODs. According to the company, the MPS automation platform processes 240+ samples with barcode reading capability. The system performs thermal desorption, spiking of adsorbent tubes, liquid sample introduction, headspace (HS), dynamic headspace (DHS), DHS large (1 L), SPME, desorption of Gerstel Twisters (SBSE), and pyrolysis.
www.gerstel.com
Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany.

Noise reduction

Laboratories are often noisy—from vacuum pumps to ultrasonic cleaners, all laboratory equipment contributes. That noise makes working conditions around equipment uncomfortable and tiring. The noise enclosures from MS Noise are equipped with acoustic foam for a -15dB(A) noise reduction, fans for excellent heat exchange, and offer easy access to the device, according to the company.
www.msnoise.com
MS Noise, 89100 Sens, France.

MALS detector

The new Postnova PN3621 Maximum Angle MALS detector sets a new standard for most precise multi-angle light scattering detection for size-exclusion chromatography (SEC) and field-flow fractionation (FFF), according to the company. The detector simultaneously measures the scattering intensity at a maximum of 21 angles, which enables excellent determination of absolute molecular weight and size of proteins, polymers, and nanoparticles.
www.postnova.com
Postnova Analytics GmbH, Landsberg, Germany.

Differential refractometer

The AZURA RID 2.1L is a sensitive and competitively priced differential refractometer, according to the company. It is suitable for detecting compounds with little or no UV activity, such as alcohols, sugars, lipids, or polymers. This instrument is designed for use in analytical HPLC, GPC and FPLC applications. The unit has advanced temperature control and ensures high sensitivity, fast baseline stabilization, and excellent reproducibility. Furthermore, the long-life LED, highly pressure-resistant flow cell, improved safety features, and enhanced diagnostics functions reportedly offer easy handling and maintenance.
www.knauer.net
Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany.

Thermal desorption systems

The latest thermal desorption instruments from Markes International enhance the capability of GC for the analysis of VOCs and SVOCs. According to the company, each instrument, including the flagship TD100-xr fully-automated 100-tube system (pictured), offers all of Markes's innovations from the last 20 years, in addition to a suite of new productivity and performance benefits, including extended re-collection, analyte range, and reliability.

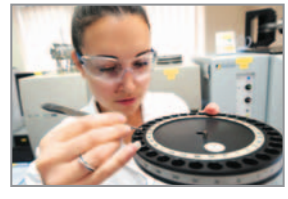
http://chem.markes.com/XR
Markes International Ltd., Llantrisant, UK.

On-line viscometry

On-line intrinsic viscometry is a valuable technique for the characterization of polymers, to assess conformation, branching, and size. Wyatt Technology's ViscoStar III incorporates multiple innovations, according to the company, including the proprietary impedance matching of the capillary bridge (patent pending), which reduces pressure fluctuations from pump pulses without the common artificially induced electronic signal smoothing that adversely affects chromatographic resolution. Sensitivity to 100 ng of 100 kDa polystyrene in THF under typical GPC conditions is achieved, with a dynamic range of 135,000:1, temperature regulation from 4–70 °C, and drift of under 2.5 Pa/h.

www.wyatt.com/ViscoStar
Wyatt Technology, Santa Barbara, California, USA.

Analytical testing



Providing high-tech independent consultancy and analytical testing services, Minerva Scientific aims to offer clients more than just analytical results. The company holds both MHRA GMP approval for the chemical testing of pharmaceutical and veterinary products with particular expertise in the release testing of generics and metered dose inhalers, and ISO17025 accreditation for food testing specializing in honey and mycotoxin analysis. Recognizing that quality, cost and speed of delivery of results are the major client drivers, the company strives to offer high-quality data on time.

www.minervascientific.com
Minerva Scientific Ltd, Derby, UK.

ELSD

The VWR ELSD 100 detector combines the high sensitivity, reliability, and accuracy for the required analyses as a result of its low-temperature technology. The detector can be connected to any HPLC or SFC system, and it can be controlled locally or via a PC for a fully integrated system using a broad range of drivers. According to the company, it is an ideal addition to any chromatography instrument.

vwr.com/chrom-applications
VWR International GmbH, Darmstadt, Germany.

Microchip-based column

The µPAC from Pharmafluidics is a microchip-based chromatography column with an ordered pillar array as separation bed. µPAC columns facilitate a significant increase in peak capacity and sensitivity at moderate column pressure. According to the company, these columns enhance the detection of molecules in tiny, complex biological samples in the field of biomarker discovery and development of biopharmaceuticals. Furthermore, µPAC columns are compatible with any third-party nano LC–MS system.

www.pharmafluidics.com
Pharmafluidics, Ghent, Belgium.

Hydrogen generator

The VICI DBS FID Station combines the reliability of the hydrogen and zero air generators into one compact and convenient package, according to the company. Available in high and ultrahigh purity for all GC detector and carrier gas applications, the generator is offered in two styles: flat for placement under a GC, or the Tower, and operates at flow rates up to 1000 mL/min.

http://www.dbsinstruments.com/en/prodotti/fid_tower_plus/
VICI AG International, Schenkon, Switzerland.

The 23rd International Symposium on Separation Science (ISSS 2017)

The **23rd International Symposium on Separation Science (ISSS 2017)** will be held in **Vienna, Austria,** from **19–22 September 2017.** The bellwether event of the Central European Group for Separation Sciences (CEGSS), **ISSS 2017** will again offer a stage for the presentation and discussion of exciting new research developments in the field of separation science. Complementing the scientific programme, the associated company exhibition will also showcase the most up-to-date examples of analytical applications, instrumentation, supplies, and analytical services.

Leading scientists from across the field will be giving presentations at the conference. The list of plenary and keynote speakers and a preliminary conference schedule are available on the conference website. The conference will particularly focus on:

- Advances in chromatographic and nonchromatographic techniques
- New stationary phases for GC and HPLC
- Sample preparation
- Multidimensional separation techniques
- Novel instrumentation in separation sciences
- Emerging detection schemes
- Green chromatography
- Environmental and industrial applications
- Food and consumer product safety
- Natural products, flavour, and fragrance analysis
- Separation sciences in cultural heritage and art analysis
- "Omics" and nontargeted analysis
- Fundamental aspects of separation sciences

An exciting social programme and the unique atmosphere of the historic centre of Vienna promise a memorable conference. The members of the Scientific Committee and the organizers look forward to seeing you in Vienna!

E-mail: info@isss2017.at
Website: www.isss2017.at

ChromSoc: Advances in Gas Chromatography VI

ChromSoc's Advances in Gas Chromatography meeting will take place at **The Heath Business and Technical Park, Runcorn, Cheshire, UK,** on the **18 October 2017.** Gas chromatography (GC) is still the technique of choice for analyzing both volatile and semivolatile compounds, but as we move to more difficult matrices, more is being asked of the technique in terms of separation and limits of detection. The meeting sets out to give an overview of current GC technologies and methods, as well as information on newer GC solutions. The meeting has been put together to showcase work from industry, academia, and the instrument companies and is designed to accommodate the needs of both experienced chromatographers and those new to the technology and its applications. The expansive one-day event has been structured to bring attendees up to speed with the "state of play" in gas chromatography, its associated instrumentation, and support products. In previous years it has been a "full house" and booking early is recommended. For further information on the meeting please visit: **www.chromsoc.com/chromsocevents.aspx**

Telephone: +44 (0)141 945 6880
E-Mail: chromsoc@meetingmakers.co.uk

10–13 September 2017
24th International Symposium on Electro- and Liquid Phase-Separation Techniques (ITP 2017)
Sopot, Poland
E-mail: symposium@itp2017.com
Website: www.itp2017.com

18–19 September 2017
4th Stir-Bar Sorptive Extraction Technical Meeting
Novotel Paris Sud, France
E-mail: david.benanou@veolia.com
Website: www.sbsetechnicalmeeting.com

9–12 October 2017
17th International Nutrition & Diagnostics Conference
Hotel Duo, Prague, Czech Republic
E-mail: info@indc.cz
Website: www.indc.cz

5–9 November 2017
International Symposium for High-Performance Liquid Phase Separations and Related Techniques (HPLC 2017 Jeju)
Jeju, Korea
E-mail: hplc2017@gmail.com
Website: www.hplc2017-jeju.org

7–10 November 2017
8th International Symposium on Recent Advances in Food Analysis
Prague, Czech Republic
E-mail: rafa2017@vscht.cz
Website: www.rafa2017.eu

24–26 January 2018
15th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-15)
Cardiff, UK
E-mail: info@ilmexhibitions.com
Website: www.ilmexhibitions.com/htc

17–18 April 2018
International Scientific Conference Ion Chromatography and Related Techniques
Zabrze, Poland
E-mail: rajmund.michalski@ipis.zabrze.pl
Website: http://ipis.pan.pl/en/

Please send any upcoming event information to Lewis Botcherby: lewis.botcherby@ubm.com

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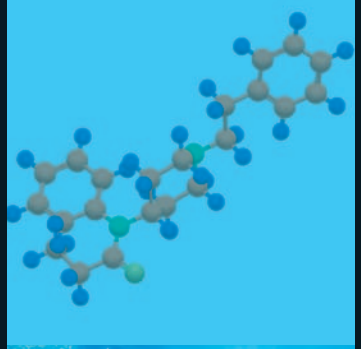
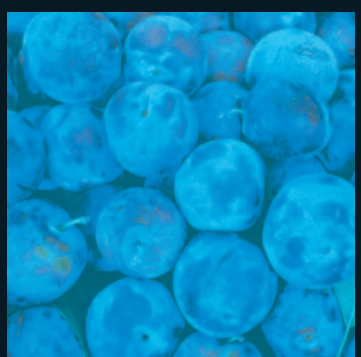
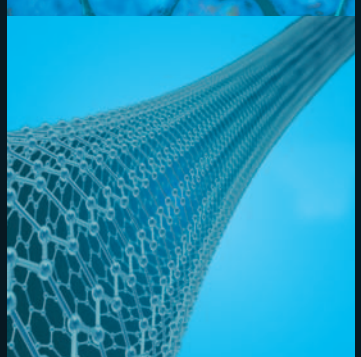
LC GC
europe
solutions for separation scientists



THE APPLICATIONS BOOK





July 2017
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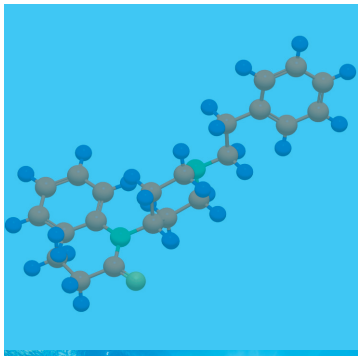

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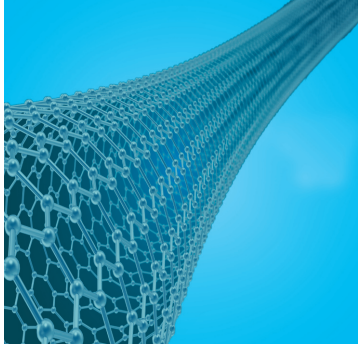

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THE APPLICATIONS BOOK

Environmental

Food and Beverage

Medical/Biological

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Automated Solid-Phase Extraction (SPE) and GC–MS Analysis of Pond Water Samples According to EN16691 for PAHs

Alicia Cannon and Michael Ebitson, Horizon Technology, Inc.

Polycyclic aromatic hydrocarbons (PAHs) are found worldwide and are emitted from a number of sources including fossil fuel, coal and shale oil derivatives, coke production, and burning wood for home heating, and generally arise from incomplete combustion. Surface water supplies, such as water in ponds, may be used for recreational purposes or become a drinking water source. Characterization of PAHs and their concentration is of interest in maintaining public health.

PAH measurement in water should be accurate, precise, and sensitive enough to measure low concentrations. Method EN 16691 is a recently developed method that uses solid-phase extraction to isolate organic compounds from 1 L of water using a divinylbenzene (DVB) solid-phase extraction disk. PAHs are eluted from the disk with dichloromethane and dried to remove water before evaporation, solvent exchange into toluene, and introduction into GC–MS. The method specifies the use of the whole water sample, ensuring that any analyte adsorbed on the particulate matter will be extracted along with the water sample. Disks are a particularly well suited SPE format for samples containing particulates because the increased surface area does not become clogged with particulate as easily as a cartridge format might, even for larger water samples, such as 1 L. In addition, the particulates are rinsed with solvent at the same time as the bottle is rinsed in an automated system (SPE-DEX® 4790 or new SPE-DEX 5000), including compounds adsorbed on the particulate surfaces in the extraction process (1).

The recoveries of a suite of PAH compounds spiked into a pond water sample using this methodology are shown in Table 1 (2). A slow flow rate through the disk (25 mL/min) is specified in the method and excellent recoveries are shown in the full application note. However, the flow rate through a disk does not need to be limited in the same way as a cartridge for good equilibrium. The recoveries shown in Table 1 result from extraction at full speed (approximately 100 mL/min). Recoveries of the surrogate compounds are very good ensuring the method is operating properly. The recoveries of spiked compounds from the matrix is excellent.

The performance of the SPE-DEX 4790, using Atlantic® DVB disks for the extraction of PAHs, was shown to comply with method requirements and provided excellent recoveries of the full suite of PAH analytes.

Table 1: Spike recoveries for pond water samples using the SPE-DEX 4790 with fast sample application

IS (surrogates) Spiked at 5 µg/mL	Blank Concentration (µg/mL)	% Recovery	Sample Spike Concentration (µg/mL)	% Recovery
Anthracene-d10	4.21	84.2	4.08	81.6
Fluoranthene-d10	4.27	85.4	4.14	82.8
Benzo(b)fluoranthene-d12	4.19	83.8	4.07	81.4
Benzo(k)fluoranthene-d12	4.57	91.4	4.56	91.2
Benzo(a)pyrene-d12	4.48	89.6	4.41	88.2
Indeno(1,2,3-c,d) pyrene-d12	4.38	87.6	4.25	85.0
Benzo(g,h,i)perylene-d12	4.12	82.4	3.98	79.6
Targets Spiked at 0.5 µg/mL				
Anthracene	0.14		0.69	110
Fluoranthene	2.23		2.86	126
Benzo(b)fluoranthene	1.43		1.91	96.0
Benzo(k)fluoranthene	1.59		1.08	102
Benzo(a)pyrene	0.94		1.50	112
Indeno(1,2,3-c,d) pyrene	0.70		1.13	86.0
Benzo(g,h,i)perylene	0.76		1.21	90.0

References

(1) Application Note AN1091606_01, Automated Solid Phase Extraction (SPE) and GC/MS Analysis of Whole Water Samples According to EN16691 for PAHs, available from www.horizontechinc.com.

(2) Application Note AN1101606_01, Automated Solid Phase Extraction (SPE) and GC/MS Analysis of Pond Water Samples According to EN16691 for PAHs.

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Rapid Perfluorinated Alkyl Acid Analysis by LC–MS/MS Increases Sample Throughput

Restek Corporation

- ***Raptor C18 SPP 5 µm core–shell silica particle columns offer excellent resolution for fluorochemicals with short total cycle times. For even faster analysis, 2.7 µm core–shell particles are available.***
- ***Meets EPA Method 537 requirements.***
- ***Unique, robust Raptor C18 column design increases instrument uptime.***

Perfluorinated alkyl acids are man-made fluorochemicals used as surface-active agents in the manufacture of a variety of products, such as firefighting foams, coating additives, textiles, and cleaning products. They have been detected in the environment globally and are used in very large quantities around the world. These fluorochemicals are extremely persistent and resistant to typical environmental degradation processes. As a result, they are widely distributed across the higher trophic levels and are found in soil, air, groundwater, municipal refuse, and landfill leachates. The toxicity, mobility, and bioaccumulation potential of perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), in particular, pose potential adverse effects for the environment and human health.

Perfluorinated alkyl acid analysis can be challenging because these compounds are chemically different from most other environmental contaminants. They are difficult to quantify because some are more volatile than others, and they also tend to be more hydrophilic and somewhat reactive. In addition, fluorochemicals are present in polytetrafluoroethylene (PTFE) materials, so excluding the use of any PTFE labware throughout the sampling and analytical processes (including HPLC solvent inlet tubing) is essential for accurate analysis. Typically, perfluorinated alkyl acids are analyzed by LC–MS/MS methods, such as EPA Method 537, but long analysis times can significantly limit sample throughput.

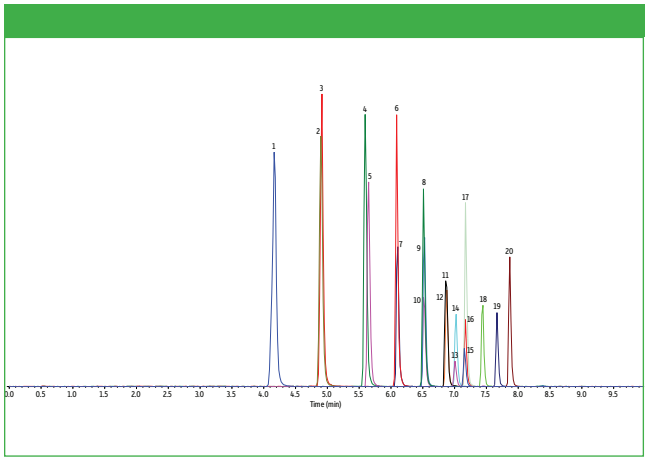

Figure 1: Column: Raptor C18 (cat.# 9304512); Dimensions: 100 mm × 2.1 mm i.d.; Particle size: 5 µm; Pore size: 90 Å; Temp.: 40 °C; Sample: Diluent: Methanol–water (96:4); Conc.: 5–10 ng/mL; Inj. vol.: 5 µL; Mobile phase: A: 5 mM ammonium acetate in water; B: Methanol; Gradient (%B): 0.00 min (10%), 8.00 min (95%), 8.01 min (10%), 10.0 min (10%); Flow: 0.4 mL/min; Detector: MS/MS; Ion source: Electrospray; Ion mode: ESI-; Mode: MRM.

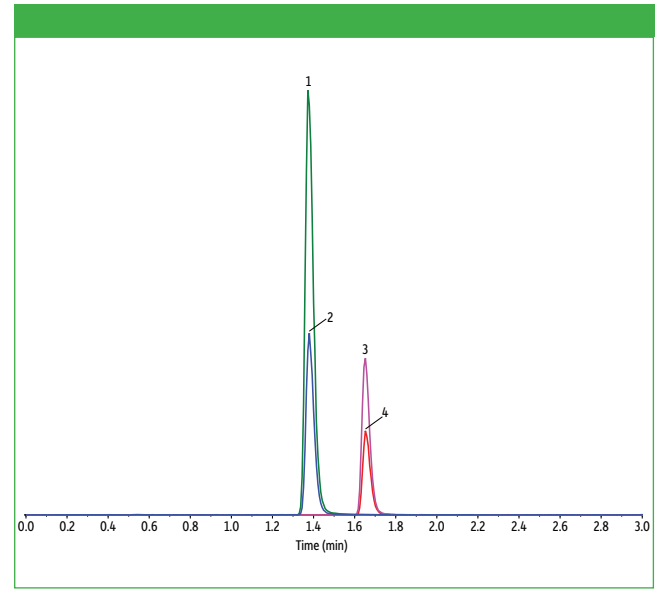

Figure 2: Column: Raptor C18 (cat.# 9304512); Dimensions: 100 mm × 2.1 mm i.d.; Particle size: 5 µm; Pore size: 90 Å; Temp.: 40 °C; Sample: Diluent: Water–methanol (50:50); Conc.: 5–10 ng/mL; Inj. vol.: 5 µL; Mobile phase: A: 5 mM ammonium acetate in water; B: Methanol; Gradient (%B): 0.00 min (60%), 2.50 min (95%), 2.51 min (60%), 4.50 min (60%); Flow: 0.4 mL/min; Detector: MS/MS; Ion mode: ESI-; Mode: MRM; Instrument: UHPLC.

As written, the EPA 537 requires a 27-min cycle per sample, but the method does allow flexibility in the column used as long as there is sufficient resolution for the MS dwell time for all compounds in a specific retention time window. In Figure 1, all target perfluorinated alkyl acids were analyzed on a Raptor C18 column in under 8 min with a total cycle time of 10 min—resulting in an approximately threefold faster analysis than the EPA method. While this analysis is significantly faster, there is no sacrifice in peak resolution or selectivity, meaning all fluorochemicals are easily identified and they elute as highly symmetrical peaks that can be accurately integrated and quantified by MS/MS. If PFOA and PFOS are the only target fluorochemicals, the analysis can be further optimized, which

Table 1: Peak identifications for Figure 1

Peaks	t_R (min)	Conc. (ng/mL)	Precursor Ion	Product Ion
1. Perfluorobutanesulfonic acid (PFBS)	4.17	5	298.9	79.9
2. Perfluoro-*n*-[1,2-$^{13}C_2$]hexanoic acid (^{13}C-PFHxA)	4.90	5	314.8	269.8
3. Perfluorohexanoic acid (PFHxA)	4.91	5	312.7	268.9
4. Perfluoroheptanoic acid (PFHpA)	5.59	5	362.8	318.8
5. Perfluorohexanesulfonic acid (PFHxS)	5.65	5	398.8	79.8
6. Perfluoro-[1,2-$^{13}C_2$]octanoic acid (^{13}C-PFOA)	6.09	5	414.8	369.8
7. Perfluorooctanoic acid (PFOA)	6.10	5	412.7	368.8
8. Perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonic acid (^{13}C-PFOS)	6.51	10	502.7	79.9
9. Perfluorooctanesulfonic acid (PFOS)	6.52	5	498.7	79.9
10. Perfluorononanoic acid (PFNA)	6.52	5	462.6	418.9
11. Perfluoro-*n*-[1,2-$^{13}C_2$]decanoic acid (^{13}C-PFDA)	6.87	5	514.8	469.9
12. Perfluorodecanoic acid (PFDA)	6.88	5	512.7	468.8
13. N-deuteriomethylperfluoro-1-octanesulfonamidoacetic acid (d3-NMeFOSAA)	7.01	10	572.7	418.9
14. N-methyl perfluorooctanesulfonamidoacetic acid (NMeFOSAA)	7.02	10	569.8	418.9
15. N-deuterioethylperfluoro-1-octanesulfonamidoacetic acid (d5-NEtFOSAA)	7.16	10	588.8	418.9
16. N-ethyl perfluorooctanesulfonamidoacetic acid (NEtFOSAA)	7.17	10	583.8	418.9
17. Perfluoroundecanoic acid (PFUnA)	7.17	10	562.8	518.8
18. Perfluorododecanoic acid (PFDoA)	7.44	10	612.7	568.8
19. Perfluorotridecanoic acid (PFTrDA)	7.67	10	662.7	618.8
20. Perfluorotetradecanoic acid (PFTA)	7.87	10	712.8	668.9

Column description

Raptor C18 LC Columns (USP L1)

Description	cat.#
5 µm Columns 100 mm, 2.1 mm i.d.	9304512

results in a fast, <2-min separation with a total cycle time of just 4.5 min, as shown in Figure 2.

Whether labs conducting perfluorinated alkyl acid analysis by LC use longer target analyte lists or focus just on PFOA and PFOS, the excellent peak shapes and separations achieved here result in consistent, accurate quantification with much shorter analysis times. By switching to a Raptor C18 column, labs can process more samples per hour while still meeting fluorochemical method requirements.

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110 Benner Circle, Bellefonte, Pennsylvania 16823, USA
Tel.: (800) 356 1688 Fax: (814) 353 1309
Website: www.restek.com

The Rapid Determination of Mycotoxins by LC–MS/MS

Advanced Chromatography Technologies

Mycotoxins are secondary metabolites produced by several species of fungi and are considered one of the most significant contaminants of agricultural commodities, both in the field and in storage. Agricultural products that may be affected include cereals, spices, dried fruits, and various nuts. Although hundreds of mycotoxins are known, relatively few are considered to pose a significant health risk. Aflatoxins, in particular aflatoxin B1, are genotoxic and carcinogenic and may cause liver cancer in humans, whilst ochratoxin A and the trichothecenes HT-2 and T-2 can cause various toxic effects. Monitoring and control of certain mycotoxins is important within the food industry because of their potential toxicity at low levels to both humans and animals.

As demonstrated in Figure 1, the separation and identification of seven of the most concerning mycotoxins from a food safety perspective (including the structurally similar aflatoxins) can be achieved using an ACE Excel 2 C18-AR column in less than 5 min. The ACE C18-AR is a novel C18-based phase with enhanced aromatic selectivity. The combination of C18 and aromatic selectivities offers an ideal solution for the separation of mycotoxins. The C18-AR is suitable for a wide range of analytes, including those able to π–π bond, analytes with different dipole moments, those containing electron withdrawing groups (such as halogens, nitro groups, ketones, esters, and acids), analytes with differing hydrophobicity, uncharged acids and bases, and polar to nonpolar analytes. The low bleed characteristic of this phase makes it ideal for use with tandem mass spectrometry (MS) detection, permitting low level detection and identification of these key components.

ACE C18-AR is one of a range of novel selectivities specially engineered by Advanced Chromatography Technologies Limited to provide chromatographers with more choices for alternative selectivity, without compromising stability or robustness. Whilst the ACE C18-AR combines the hydrophobic characteristics of a C18 phase with the aromatic selectivity of a phenyl phase, the ACE C18-PFP combines the hydrophobicity of a C18 phase with the alternative selectivity offered by a pentafluorophenyl phase. This provides π–π, dipole–dipole, and shape selectivity mechanisms in addition to hydrophobic separation mechanisms. Other phases in the ACE range include the ACE C18-Amide and the ACE CN-ES, both offering alternative selectivity and especially recommended for improved retention of polar compounds. In addition, the ACE SuperC18 phase offers extreme inertness and excellent stability across a wide pH range of 1–11, and a new range of novel hydrophilic interaction liquid chromatography (HILIC) selectivities offers a route to systematic and successful HILIC method development. A wide range of application notes are available in a series of LC and LC–MS application guides published by Advanced Chromatography Technologies. Contact us for your free copies.

Advanced Chromatography Technologies
1 Berry Street, Aberdeen, Scotland, UK
E-mail: info@ace-hplc.com Website: www.ace-hplc.com

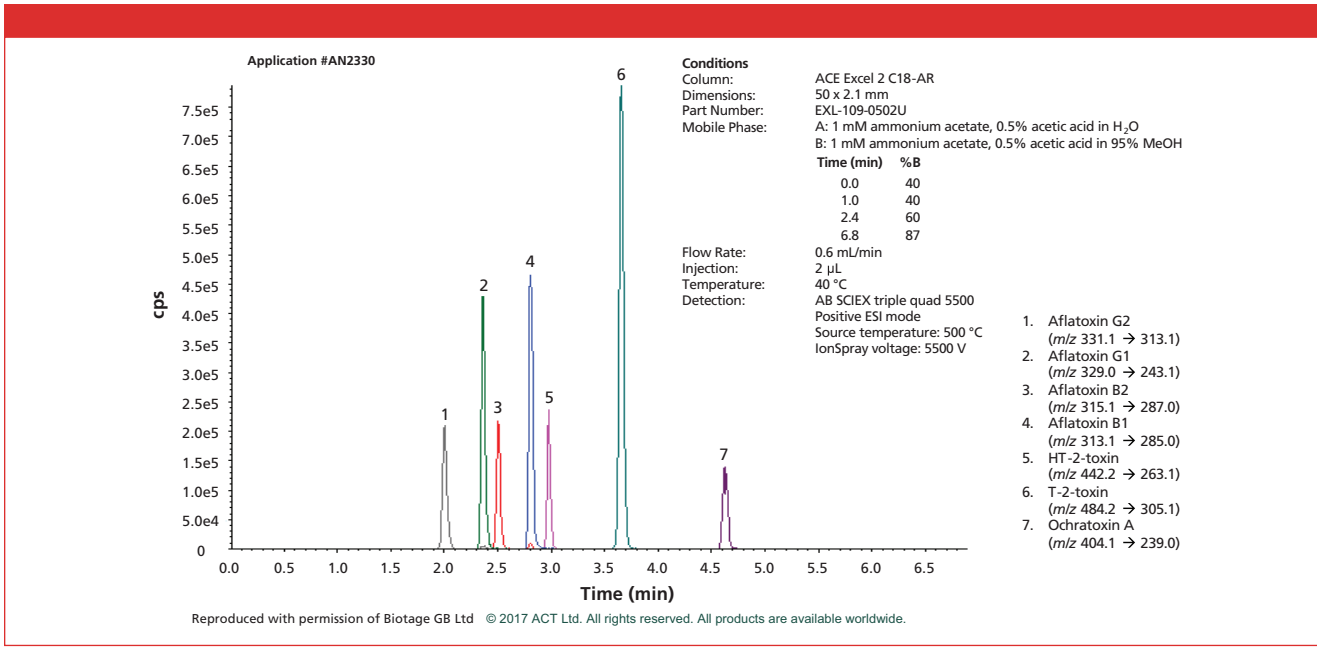

Figure 1: Rapid determination of mycotoxins by LC–MS/MS.

Determination of Sophisticated Honey Adulteration with LC–IRMS

Marian de Reus, Filip Volders, Christian Schmidt, Lutz Lange, and Hans-Peter Sieper,
Elementar Analysensysteme GmbH

Honey is a high-value commodity, whose quality is defined both by its botanical and geographical origin. This generates a strong consumer demand for certain, premium-priced products, which have become the target for adulterations. A useful tool to detect the addition of sugar to honey products is based on the well-documented difference in $\delta^{13}C$ values between C3 (natural honey) and C4 (added sugar) plants. Coupling high performance liquid chromatography (HPLC) with isotope ratio mass spectrometry (LC–IRMS) has the unrivaled advantage of the simultaneous determination of $\delta^{13}C$ values from glucose, fructose, di-, tri-, and oligo-saccharides, allowing the detection of more sophisticated honey adulteration with a simple user-friendly analytical system.

Instrumentation and Experimental Conditions

The system consists of an HPLC (Agilent 1260 Infinity system with 1290 column compartment), an LC–IRMS interface (Elementar iso CHROM LC cube), and an IRMS (Elementar isoprime precisION).

HPLC

Eluent:	Water (LC–MS grade)
Column:	6.5 × 300 mm, 9-µm Dr. Maisch Repromer Ca
Column Temp.:	85 °C
Flow Rate:	0.27 mL/min or 0.60 mL/min
Injection Volume:	5 µL (conc.: 10 mg/mL)

LC-IRMS Interface

Combustion Temp.:	850 °C (at eluent flow rate of 0.27 mL/min)
	1150 °C (at eluent flow rate of 0.60 mL/min)

Results

Unlike the existing LC–IRMS interface solution via chemical oxidation, the iso CHROM LC cube works with high-temperature combustion. Thanks to the low dispersion gas flow path, no significant peak tailing is observed that sacrifices chromatographic resolution. In addition, flow rates higher than 0.3 mL/min are now possible to reduce the run time and increase the number of samples per day.

Table 1: $\delta^{13}C$ isotope ratios for the different sugar fractions in different honey samples. $DIFF_{GLU-FRU}$ is the difference in $\delta^{13}C$ ratio between glucose and fructose, $DIFF_{MAX}$ the maximum difference in $\delta^{13}C$ ratio between all sugar compounds.

	GLUCOSE	FRUCTOSE	$DIFF_{GLU-FRU}$	DISACCHARIDE	$DIFF_{MAX}$
ORIGIN/TYPE	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\Delta\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\Delta\delta^{13}C$ (‰)
Germany	-26.83±0.04	-26.70±0.02	0.13	-28.30±0.02	1.60
Portugal	-27.24±0.06	-28.16±0.01	0.92	-27.12±0.05	1.04
Thailand	-24.28±0.05	-25.17±0.13	0.89	-25.35±0.07	1.07
Japan	-24.26±0.07	-25.02±0.01	0.78	-25.58±0.12	1.33
The Netherlands	-25.93±0.04	-26.69±0.13	0.76	-26.63±0.17	0.76
Adulterated honey #1	-22.99±0.03	-24.67±0.07	1.68	-24.30±0.02	1.68
Adulterated honey #2	-22.68±0.01	-23.56±0.14	0.87	-17.24±0.10	6.31

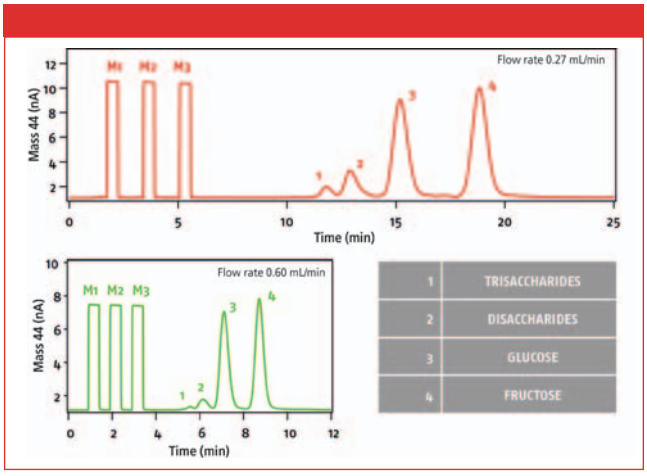

Figure 1: Typical chromatogram obtained at two different flow rates (0.27 mL/min and 0.60 mL/min).

In constant operation, more than 100 different honey samples were analyzed. A typical chromatogram is shown in Figure 1 for two different flow rates. Standards were measured between the honey samples to monitor the system performance. Overall 890 analyses were performed over a period of more than 3 weeks with just a few minor interventions, for example, change of chemical drying agent every 10 days or emptying the waste bottle every 5 days. The standard deviations over the whole period for the standards were as low as 0.07‰ for sucrose, 0.14‰ for glucose, and 0.13‰ for fructose.

Table 1 shows an excerpt of the results including adulterated honey samples. The standard deviation in all cases is excellent for glucose and fructose. For some samples, the disaccharides were also analyzed. A difference in the isotopic ratio between glucose and fructose of larger than 1‰ is evidence for adulteration (adulterated honey #1 in Table 1). A further sign of adulteration is if the isotopic ratio between glucose, fructose, di-, and trisaccharides differs by more than 2.1‰ (adulterated honey #2 in Table 1). The low difference between glucose and fructose for the German honey sample can be explained by the fact that it's a non-commercial, single-source honey from a local beekeeper.

Conclusion

The LC–IRMS results using the iso CHROM LC cube LC–IRMS interface show outstanding performance and precision. The unrivaled robustness and the low intervention and maintenance intervals paired with the ability to significantly reduce the run time make it the perfect choice for any high-throughput laboratory that needs to detect sophisticated honey adulteration.

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Direct Analysis of Amino Acids by HILIC–ESI-MS

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Hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry (HILIC–ESI-MS) has been established as a method to separate and quantify polar and ionic analytes in a direct way for two decades. HILIC separation is based on the polarity of analytes, so the more polar analytes have stronger retention on a HILIC column.

Highly polar amino acids carry great biological weight because they are the basic structural units of proteins or enzymes. Accordingly, they are an essential part of plants, animals, and humans. Quantitative analysis of amino acids is crucial in many fields including clinical diagnostic, biomedical research, and food science (1). Amino acids are classically analyzed by gas chromatography (GC), cation exchange (CEX) or reversed-phase liquid chromatography (LC) with UV absorbance, fluorescence detection (FD), or mass spectrometry (MS) (1). However, pre-column or post-column derivatization is often needed to address either the retention problem or the detection issue. HILIC and ESI-MS is a perfect match for analyzing amino acids in a direct and fast manner (2).

In this application, we separated 14 amino acids with an iHILIC-Fusion(+) column packed with charge modulated hydroxyethyl amide silica. A mixed interaction—for example, hydrophilic partitioning, weak electrostatic interactions, and hydrogen bonding—may be involved in the HILIC separations.

Experimental

LC–MS System: Agilent 1100er LC system and Thermo Fisher LTQ™ equipped with a HESI source, operated in positive ionization mode for analysis of standards. For the dietary supplement, an Orbitrap™ Exactive classic equipped with a HESI source and operated in positive ionization mode.

Column: 150 × 2.1 mm, 3.5-µm iHILIC-Fusion(+) (P/N 100.152.0310, HILICON AB)

Gradient Elution: A) acetonitrile–water–1 M ammonium acetate, pH 5.75 (90:5:5); B) water–acetonitrile–1 M ammonium acetate, pH 5.75 (90:5:5); 0–0.5 min (90:10) A–B; 0.5 to 25 min, gradient elution from (90:10) A–B to (60:40) A–B.

Flow Rate: 0.3 mL/min

Column Temperature: 40 °C

Injection Volume: 5 µL

Amino Acids: Arginine, asparagine, aspartic acid, glutamic acid, glutamine, hydroxyl-proline, isoleucine, leucine, lysine, phenylalanine, proline, tryptophan, tyrosine, and valine. 50 µM of each amino acid was dissolved in water–acetonitrile (25:75) solution.

Dietary Supplement: Whey Prime (Prozis). A 20 mg/mL measure was dissolved in water and then filtered. The sample was further diluted to 2 mg/mL for injection.

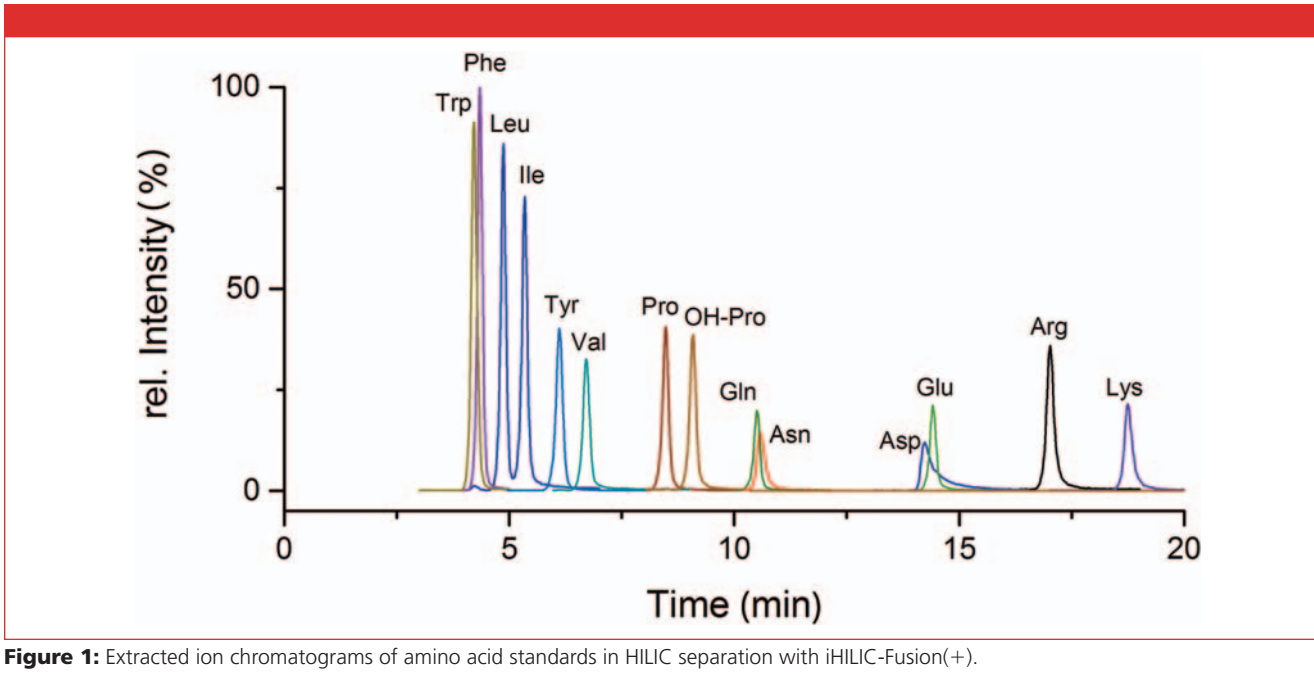

Figure 1: Extracted ion chromatograms of amino acid standards in HILIC separation with iHILIC-Fusion(+).

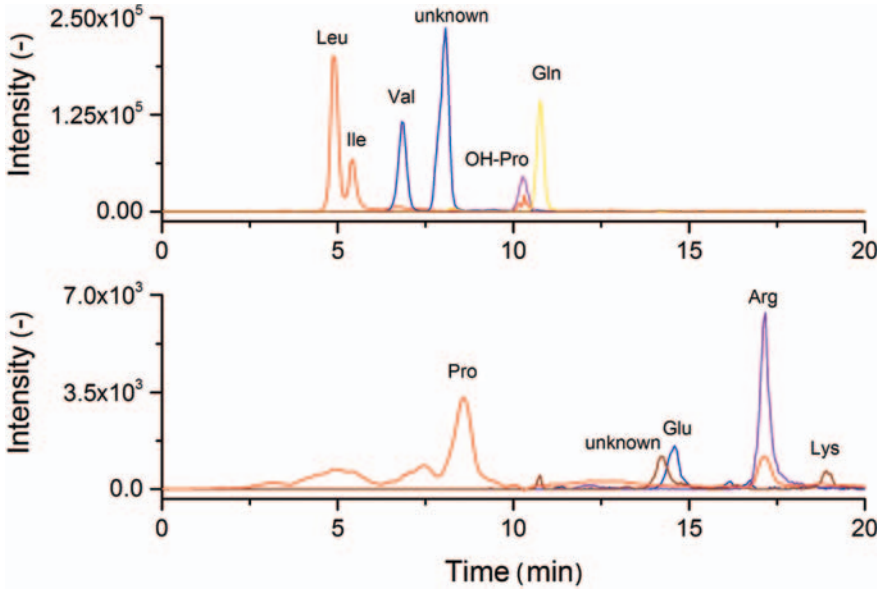

Figure 2: Extracted ion chromatograms of amino acids in dietary supplement.

Table 1: Retention times, classification of amino acids by their side-chain, and pI values

Amino acid	Abbr.	Classification	t_R (min)	pI[a]
Tryptophan	Trp	nonpolar	4.2	6.0
Phenylalanine	Phe	nonpolar	4.3	6.0
Leucine	Leu	nonpolar	4.9	6.2
Isoleucine	Ile	nonpolar	5.3	6.2
Tyrosine	Tyr	polar	6.1	5.5
Valine	Val	nonpolar	6.7	6.2
Proline	Pro	polar	8.5	7.1
Hydroxy-proline	OH-Pro	polar	9.1	6.7
Glutamine	Gln	polar	10.5	5.7
Asparagine	Asn	polar	10.6	5.2
Aspartic acid	Asp	acidic	14.2	3.4
Glutamic acid	Glu	acidic	14.4	2.8
Arginine	Lys	basic	17.0	10.8
Lysine	Arg	basic	18.7	9.8

[a]pI values are based on www.chemicalize.com

Results and Conclusion

As shown in Figure 1, 14 standards of proteinogenic amino acids can be simultaneously determined using a iHILIC-Fusion(+) column coupled with ESI-MS detection. The retention times, classification of amino acids, and pI values are summarized in Table 1. As expected, the amino acids with a nonpolar side chain like tryptophan, phenylalanine, leucine, and isoleucine eluted first with the lowest interactions with the HILIC stationary phase. The polar amino acids like proline, glutamine, or asparagine had more retention and were mainly retained by hydrophilic partitioning. The acidic and basic amino acids are not neutral and had extra electrostatic interactions with the stationary phase. Interestingly, it was found that the constitutional isomers leucine and isoleucine were almost baseline separated in a generic linear gradient elution.

In the second step, we verified the applicability of the newly developed method using a dietary supplement containing various numbers of amino acids. As can be seen in Figure 2, leucine and isoleucine are well separated. Valine, glutamine, and hydroxyproline present intense signals. In addition, proline, glutamic acid, arginine, and lysine were also detectable at lower intensities.

This work demonstrates that amino acids can be easily identified through a HILIC–ESI-MS method. It offers the possibilities to quantify this group of polar analytes in many application areas such as medicine, biology, or nutritional science.

References

(1) H. Kaspar, K. Dettmer, W. Gronwald, and P.J. Oefner, *Anal. Bioanal. Chem.* **393**, 445–452 (2009).

(2) M. Dell'mour, L. Jaitz, E. Oburger, M. Puschenreiter, G. Koellensperger, and S. Hann, *J. Sep. Sci.* **33**, 911–922 (2010).

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Flavour Profiling of Beverages Using Probe-Based Sorptive Extraction and Thermal Desorption–GC–MS

Lara Kelly and David Barden, Markes International

This study shows that the organic compounds giving rise to the flavour of a variety of beverages can be identified by high-capacity sorptive extraction using PDMS probes, with analysis by thermal desorption–GC–MS.

Introduction

Analysis of volatile and semi-volatile organic compounds (VOCs and SVOCs) in foods and beverages is important for quality control and product development and characterization. It is widely studied using GC, but this is hindered by the limitations of existing sample preparation techniques.

Markes International's PDMS HiSorb™ probes allow immersive sorptive extraction from liquids (or headspace sorptive extraction from solids), without the expense of time associated with solvent-extraction methods, and with lower detection limits than SPME.

In these three studies, a HiSorb probe (Figure 1) was immersed into a sample of the beverage in a 20 mL headspace vial. The vial and probe were agitated on a HiSorb Agitator at 40 °C for 1 h, and the probe was then washed, dried, and inserted into a TD tube for desorption. Analysis used a TD100-xr™ thermal desorber (Markes International), with GC–MS. Full details—and further examples showing the analysis of milk and premium teas—are available from http://chem.markes.com/HiSorb

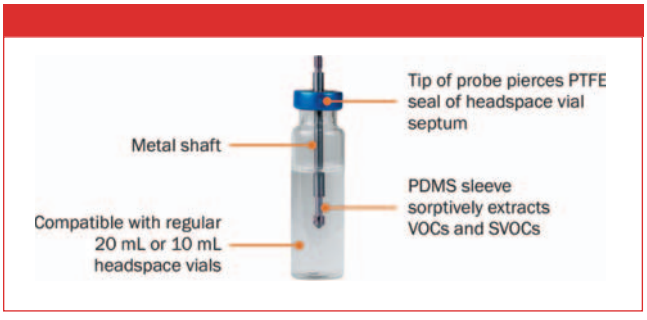

Figure 1: The HiSorb probe.

Sorptive Extraction and TD–GC–MS Analysis of Beverages

Filter Coffee (Figure 2): Furans typically have caramel-like aromas, and a number of these are present in this sample. Nitrogen-containing compounds are also of particular importance to coffee aroma, with pyridine (#4), pyrroles (#11, #13), pyrazoles (#18), and pyrazines (#5, #8) being found in this sample. However, pyrazines can be common in defective coffee beans, and some of them may contribute undesirable flavours.

Herbal Infusions (Figure 3): Monoterpenoids are key components of the aroma and flavour of mint products, and these feature prominently in the peppermint infusion (A). The peppermint and nettle infusion (B) is very similar, except for the loss of two C_8 alcohols (#1, #2) and the addition of the norsesquiterpene cyprotene (#19).

Scotch Whisky (Figure 4): This analysis shows the ability of the HiSorb probe to sample compounds at high and low levels. The

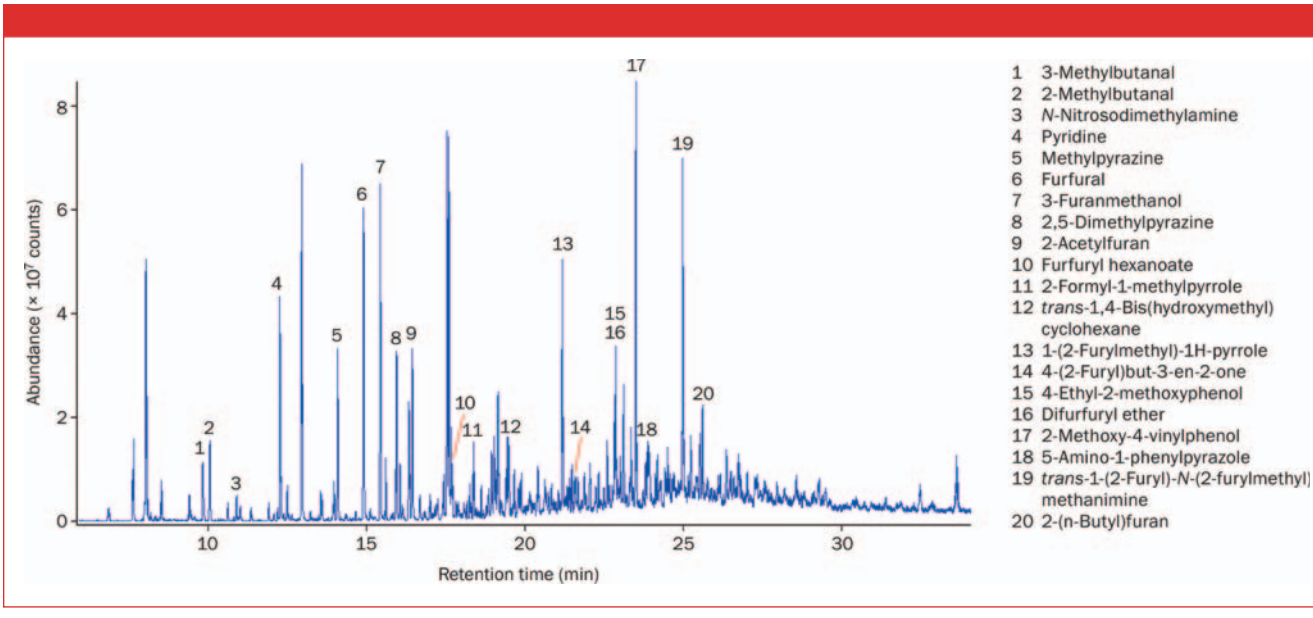

Figure 2: TD–GC–MS flavour profile of filter coffee.

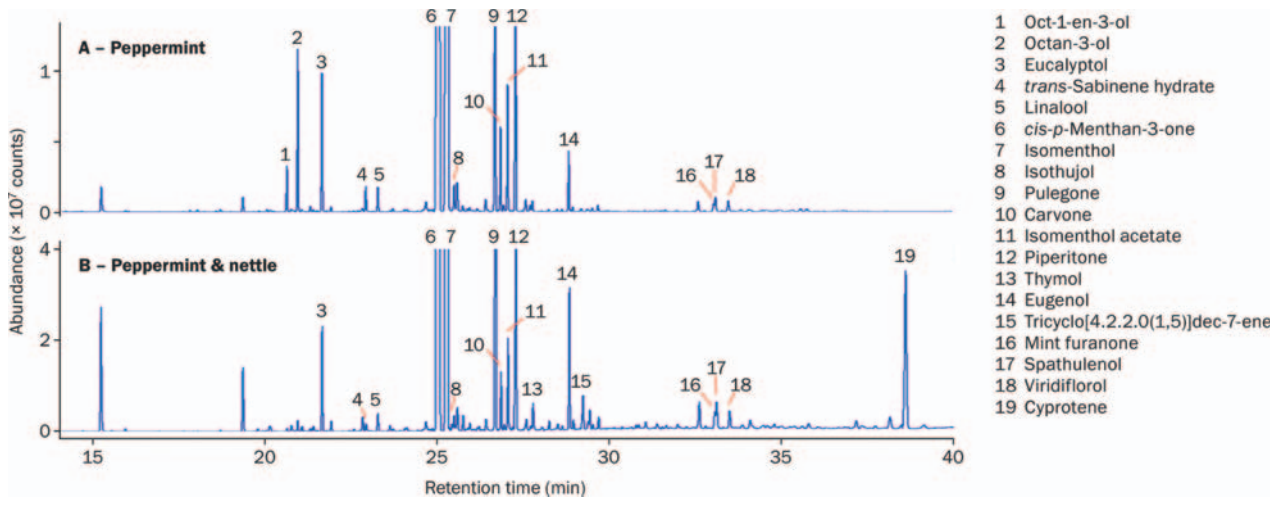

Figure 3: TD–GC–MS flavour profile of two herbal infusions.

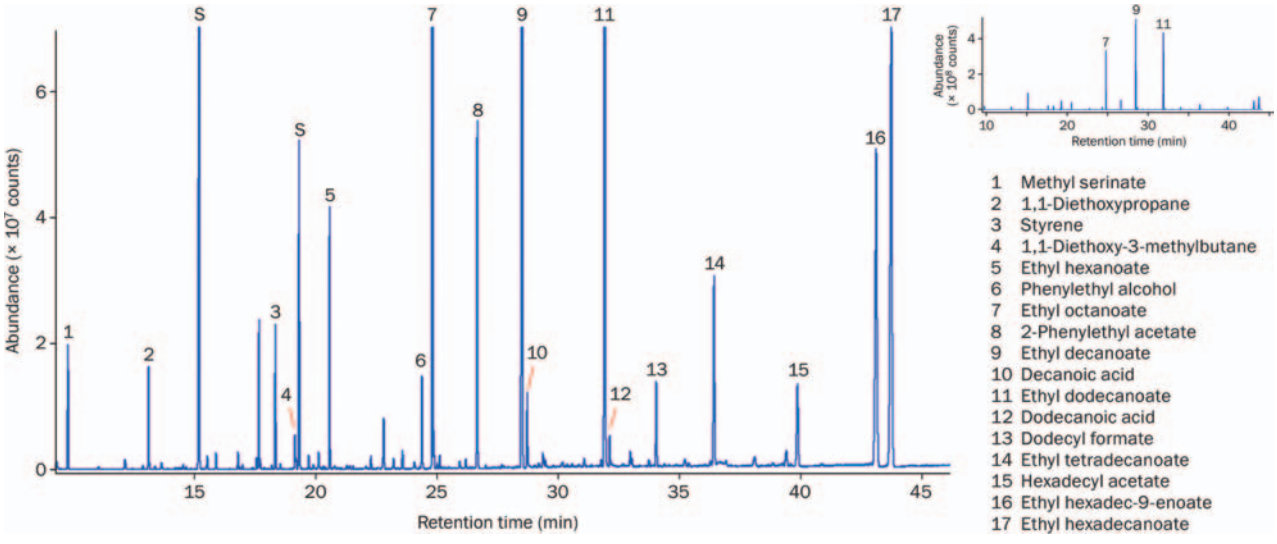

Figure 4: TD–GC–MS flavour profile of Scotch whisky. The inset shows the full-scale profile. S = Siloxane.

profile is dominated by medium-chain esters that likely contribute substantially to the flavour, while phenylethyl alcohol (#6, which is known to impart a floral note) is also of interest.

Conclusions

HiSorb probes are an easy-to-use, highly sensitive approach to the sorptive extraction of VOCs and SVOCs from a range of sample types, including water-based solutions, emulsions, and suspensions, as well as solids. Unattended sample preparation using the HiSorb Agitator, with automated analysis by thermal desorption (TD), ensures maximum productivity, while reusable probes and tubes minimize the cost per sample.

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The Effect of Draw-Out Lens Diameter on Sensitivity of GC–MS Analysis

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Gas chromatography–mass spectrometry (GC–MS) allows isolation and identification of individual analytes within a complex mixture. Helium has traditionally been the first-choice carrier gas, owing to its inertness, performance, and relatively cheap price. Since 2001, however, helium has become increasingly expensive with a reported global increase in price of 500% between 2001 and 2016 (1). In 2012–2013, the global helium shortage increased the number of GC users switching to alternative carrier gases and improved the availability of information on their use.

Hydrogen is half as viscous as helium at the same temperature and pressure, while the diffusion of a sample within the two gases is similar, meaning that hydrogen travels through the GC column more quickly and offers faster analysis than helium. The van Deemter curve (Figure 1) shows the relative efficiencies of hydrogen, helium, and nitrogen at different flow rates and shows how hydrogen has superior column efficiency at higher flow rates. Using method translation software (2,3), it is possible to model the effect of converting a method from helium to hydrogen *in silico* to see what time savings can be made and what changes to the method are required.

To overcome concerns about sensitivity reduction and stabilization times associated with hydrogen carrier gas, hardware changes such as increasing the draw-out lens orifice diameter and baking out the ion source can be conducted. Following analysis of an essential oil mixture run using helium carrier gas with a standard ion volume and hydrogen carrier gas using a larger diameter draw-out lens across a range of flow rates, stabilization time, peak resolution, and signal-to-noise ratio were assessed.

Materials and Methods

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis: All GC–MS analyses were performed using an Agilent Technologies 7890B GC with 5975 mass selective detector. Table 1 shows GC and MSD conditions for essential oils provided by Professor Lauro Barata from UFOPA (Universidade Estadual do Oeste do Pará) VOC mix was purchased from Supelco (EPA VOC Mix 2).

Table 1: GC and MSD conditions for analysis

GC Conditions (all analyses)		MSD Conditions	
Inlet Temperature	270 °C	**Ionization Energy**	70 eV
Split Ratio	100:1	**Full Scan Range**	40–500 amu
Column	HP-5 (30m × 250 µm, 0.25-µm)	**SIM Dwell Time**	15 ms
Oven (Background stabilization, EPA VOC mix 2)	60 °C (10 °C/min) to 138 °C	**Carrier Gas**	
Run Time	50 min	**Helium**	99.9995%, BOC
Oven (Essential oil analysis)	60 °C (3 °C/min) to 210 °C		
Run Time	7.8 min	**Hydrogen**	99.9999%, Peak Scientific Precision H_2 Trace 500cc

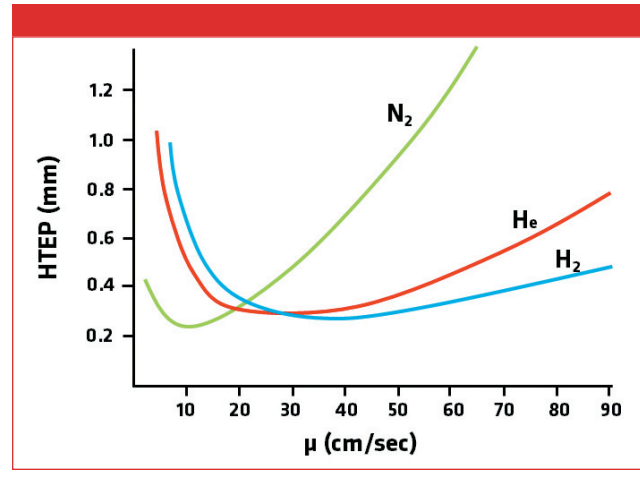

Figure 1: van Deemter curve showing the relative efficiencies of hydrogen, helium, and nitrogen at different flow rates.

Analyses of samples run using helium carrier gas were acquired using an inert 3 mm draw-out plate (G2589-20100). All samples run using hydrogen carrier gas were acquired using an inert 6 mm draw-out plate (G2589-20045).

Background stabilization was assessed by running a volatile organic mixture for 7 days following change of carrier gas. The ion source was baked-out using a slight modification of recommendations (p35–37) by Agilent Technologies (4), with the source temperature set to 300 °C and filament switched on for a period of 3 h.

Results

Effect of Carrier Gas on Signal to Noise: Signal to noise (S/N) and resolution (Rs) were calculated using 1,3,5-trichloro benzene, the last eluting peak of an essential oil mixture (Table 2). Samples run with helium carrier gas in full scan mode showed an inverse relationship between flow rate and S/N, with S/N dropping from 1988.3 at the optimal 1.0 mL/min flow rate to 864.9 at 2.0 mL/min (Table 2). When running samples using hydrogen carrier gas, the opposite relationship between flow rate and S/N was found, with S/N increasing from 106.0 to 209.6 as column flow increased (Table 2).

In SIM mode, there was little variation in S/N regardless of flow rate when using helium carrier gas. However, with hydrogen carrier gas, S/N improved with increasing carrier flow rate, with the S/N

Table 2: Results of resolution and signal-to-noise effects of helium and hydrogen carrier gas. Helium samples were detected with the 3-mm draw-out plate, hydrogen samples were detected using the 6-mm draw-out plate.

		He			H_2		
Flow Rate (mL/min)		Linear Velocity	Full Scan	SIM	Linear Velocity	Full Scan	SIM
Rs	1.0	36.6	4.94	2.84	54.4	2.96	3.20
	1.5	44.8	4.32	2.91	66.6	3.25	4.70
	2.0	51.8	3.20	2.75	76.9	2.60	5.00
S/N	1.0	36.6	1988.3	2690.4	54.4	106.0	798.8
	1.5	44.8	1356.6	2818.6	66.6	180.6	1502.8
	2.0	51.8	864.9	2381.0	76.9	209.6	2359.3

increasing from 798.8 at 1.0 mL/min to 2359.3 at 2.0 mL/min, meaning that S/N with hydrogen at higher flow rates was almost the same with helium (Table 2).

Effect of Carrier Gas on Resolution: In full scan mode, helium carrier gas followed a similar pattern to S/N results, with Rs decreasing as flow rate increased beyond the optimal velocity. When running samples using hydrogen, there was no clear relationship between Rs and flow rate, with the best resolution seen at 1.5 mL/min. When comparing the optimal flow rates of each gas (1.0–He and 2.0–H_2), peak resolution with helium carrier gas was almost double (1.9×) that of hydrogen (Table 2).

In SIM mode, Rs when using helium decreased relative to full scan Rs and was lower than Rs seen with hydrogen carrier gas (Table 2). Hydrogen Rs was vastly improved in SIM mode compared with scan mode (1.9×) and at optimal flow rates, hydrogen gave improved Rs (1.76×) compared to helium.

Background Stabilization: Results showed that background was stable after 3 days, with repeated injections of the EPA VOC mixture being tested for 7 days (Figure 2).

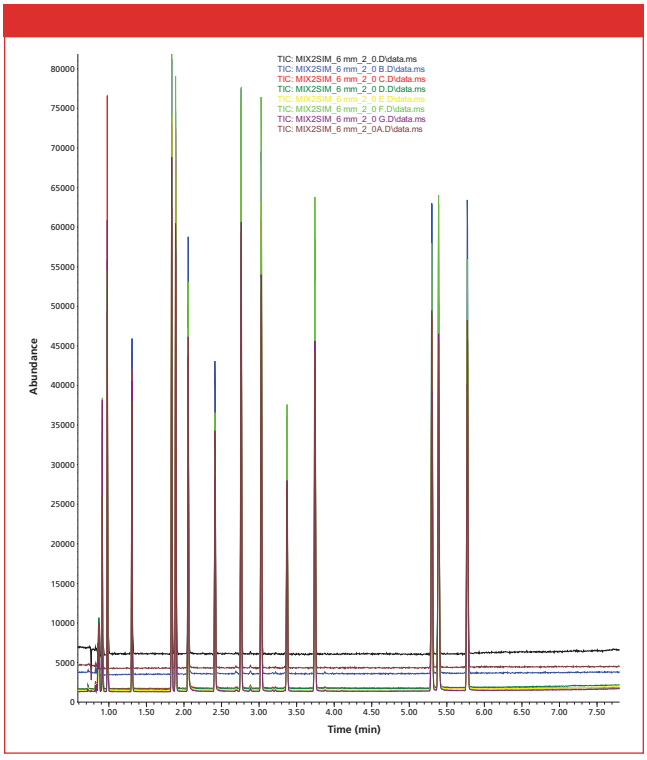

Figure 2: Background stabilization of VOC mixture on consecutive days following carrier gas change from helium to hydrogen.

Discussion

A number of applications use hydrogen carrier gas as a viable alternative to helium. The results of GC–MS when comparing Rs and S/N appear to correspond directly to the carrier gas flow rate relative to the optimal carrier gas velocity of both gases. At an optimal column flow of helium, the best performance for both Rs and S/N were observed in full scan mode. SIM detection appeared to overcome some of the problems of reduced carrier gas efficiency of helium at higher velocities, with little difference found in either Rs or S/N across the range of flow rates tested.

Similarly to helium, running samples with hydrogen carrier gas at a suboptimal flow rate affected Rs and S/N significantly in both scan mode and SIM mode. Interestingly, hydrogen carrier gas showed better peak Rs in SIM mode than helium across all three flow rates. It appears that running in SIM mode largely eliminates background noise that causes interference in full scan mode when using hydrogen. When following the recommendations for preparation of the system when switching to hydrogen, background signal will take at least 3 days to stabilize.

This study clearly demonstrates that hydrogen can be used for routine analysis of known compounds. When using full scan mode, analysts need to be aware that they are likely to see a two- to fivefold reduction in sensitivity (4). When using hydrogen carrier gas for GC–MS, it is essential to initially focus on mitigation of factors causing reduced sensitivity.

References

(1) https://www.theguardian.com/science/2016/jun/28/huge-helium-gas-tanzania-east-africa-averts-medical-shortage
(2) http://www.restek.com/ezgc-mtfc
(3) http://www.agilent.com/en-us/support/gas-chromatography/gcmethodtranslation
(4) http://bit.ly/2rqxQQu

Determination of Pesticides in Coffee with QuEChERS Extraction and Silica Gel SPE Cleanup

Xiaoyan Wang, UCT, LLC

Coffee is one of the most widely consumed beverages in the world, partly because of the stimulating effect of its caffeine content. Like most crops, the application of pesticides in coffee cultivation is a common practice to increase production yields. This application note details an optimized method for the extraction and cleanup of pesticide residues from coffee using a QuEChERS extraction procedure followed by a silica gel solid-phase extraction (SPE) cleanup.

Table 1: Extraction and analytical materials

ECMSSC50CT-MP	50-mL centrifuge tube and mylar pouch containing 4000 mg $MgSO_4$ and 1000 mg NaCl
CUSIL156	Clean-Up® silica gel
GCLGN4MM-5	GC liner - 4 mm splitless gooseneck 4 mm i.d. × 6.5 mm o.d. × 78.5 mm

Procedure

1. Sample Extraction

a) Add 10 mL brewed coffee (pH adjusted to about 8 with 1 N NaOH) and 10 mL acetonitrile (MeCN) to a 50-mL centrifuge tube.

b) Add the QuEChERS extraction salts from the Mylar pouch (ECMSSC50CT-MP) to the 50-mL tube, and shake vigorously for 1 min manually or using a Spex 2010 Geno-Grinder at 1000 strokes/min.

c) Centrifuge at ≥3000 rcf for 5 min.

d) Transfer 5 mL supernatant to a clean test tube, add 1.5 mL toluene, and evaporate to about 1 mL.

2. Sample Cleanup of Extract

a) Add about ½ inch of anhydrous sodium sulfate to a silica gel SPE cartridge (CUSIL156), and attach the SPE cartridge to a glass block or positive pressure manifold.

b) Wash the SPE cartridge with 6 mL dichloromethane, soak for 1 min, drain to waste, and dry the SPE cartridge for 1 min under full vacuum or pressure.

c) Condition the SPE cartridge with 2 × 6 mL hexane by gravity.

d) Insert a glass collection container into the manifold, load the 1 mL concentrated sample onto the SPE cartridge, rinse the test tube with 6 mL of 15% acetone in *n*-hexane, apply the rinsate to the SPE cartridge, and collect.

e) Continue to elute with 3 × 6 mL of 15% acetone in *n*-hexane by gravity.

f) Add 1.5 mL ethyl acetate to the eluate container and evaporate to 1 mL.

g) Add internal standard, vortex for 30 s, and inject 1 µL into the GC–MS system for analysis.

Instrumental

GC–MS/MS: Agilent 6890N GC coupled to a 5975C MSD
Column: 30 m × 0.25 mm, 0.25-µm Restek Rxi®-5Sil MS
Carrier Gas: Helium (1.2 mL/min)
GC Inlet Temperature: 250 °C
Injection Volume: 1 µL (splitless)
Temperature Gradient: 60 °C for 1 min, 10 °C/min to 310 °C, hold for 2 min; 28 min total
Ion Source Temperature: 250 °C
Ionization Mode: EI (70 eV)
Acquisition Mode: SIM

Results

Table 2: Recovery and RSD% from spiked coffee samples

	Spiked at 20 ng/mL		Spiked at 200 ng/mL	
Compound Name	Recovery (%)	RSD (%) (n = 5)	Recovery (%)	RSD (%) (n = 5)
Carbaryl	100.2	5.0	98.7	1.6
Tebuthiuron	95.3	6.3	99.9	2.4
DEET	102.4	5.3	99.1	2.5
Simazine	103.5	5.4	98.6	1.2
Atrazine	103.6	6.5	97.9	2.4
Diazinon	124.4	9.9	99.6	2.2
Pyrimethanil	106.4	6.3	101.6	1.2
Disulfoton	88.1	7.1	92.5	2.2
Acetochlor	103.3	5.6	98.7	1.6
Methyl parathion	91.3	6.3	97.9	1.9
Malathion	103.0	7.7	99.9	3.6
Chlorpyrifos	103.6	6.9	99.4	1.3
Triadimefon	109.3	5.1	101.5	1.6
Cyprodinil	106.4	6.8	102.4	1.0
Endosulfan I	114.0	6.2	98.2	1.7
Flutriafol	74.5	11.6	87.9	4.7
Endosulfan II	103.7	6.1	99.5	1.3
Tebuconazole	92.7	8.5	101.8	1.5
Pyrazophos	98.0	7.5	101.4	1.4
Cypermethrin (sum)	97.0	5.1	101.7	1.0

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Quality and consistency in reagents is critical to successful drug discovery and development. When targeting a particular protein of interest, *in vitro* experiments should be performed with proteins of biological properties similar to those for *in vivo* tests. It is important that molecularity, purity, shape, and degree of heterogeneity remain the same when any alterations are made to the model protein or the formulation buffer. Multi-angle light scattering (MALS) combined with size-exclusion chromatography (SEC-MALS) is a very useful technique to monitor the solution properties of the protein as changes to reagents are made.

As an example, crystallization studies typically have a higher rate of success when the proteins involved are simplified, for example, truncated or expressed in bacteria to minimize post-translational modifications. However, these forms may not be amenable to traditional characterization such as plate-based assays and certain biophysical techniques, where native protein is preferred. Affinity tags or changes to buffer excipients may also be required. All of these differences increase the likelihood of altering solution properties.

The example in this note shows how light scattering data obtained with a DAWN® MALS detector and analyzed by ASTRA® software (both from Wyatt Technology, Santa Barbara, California, USA) were used to elucidate the solution properties of protein expressed from two different constructs. Construct A is the shorter of the two, designed for crystallization efforts.

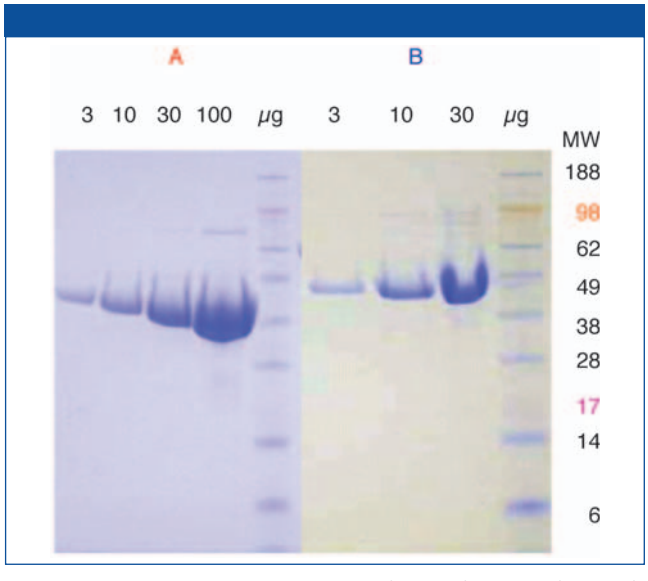

Figure 1: Two protein constructs: A (45 kDa) and B (46 kDa). SDS-PAGE shows similar purity.

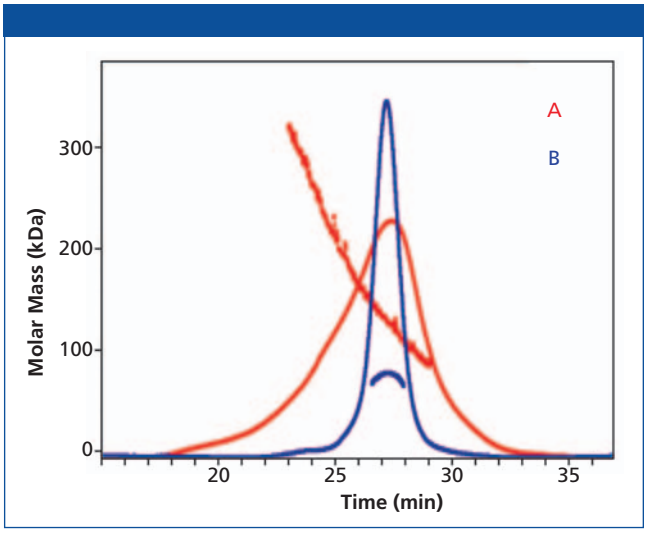

Figure 2: Standard chromatographic trace (UV $_{208nM}$) with MALS data (linear traces within the chromatograms represent the mass distribution across the peak). The enzyme from construct B is much more homogeneous, and is in its biologically active form of a dimer (89 KDa).

Presented with data from Figure 1, combined with knowledge that the enzyme showed activity and was the predominant species in mass spectrometry, one might not question the suitability of this reagent. SEC-MALS data (Figure 2), however, show that this enzyme is in fact quite heterogeneous.

Construct B addressed this; it is the full-length enzyme. MALS data show that the protein is in its biologically active form (dimer) and is highly homogeneous. This information gave the project team confidence to move forward with this construct for crystallography, NMR, and high-throughput screening (HTS) efforts.

This note graciously submitted by Mark Tardie and George Karam; Pfizer Central Research, Groton, Connecticut, USA.

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On-Line MALS-QELS (Quasi-Elastic Light Scattering)

Wyatt Technology

While on-line multi-angle light scattering (MALS) is one of the most important techniques for macromolecular characterization, it can be made even more versatile with the addition of a quasielastic light scattering (QELS, a.k.a. dynamic light scattering) module for determination of hydrodynamic radius. QELS can be added to a Wyatt MALS system as a WyattQELS™ module embedded in the MALS instrument, or by connecting the MALS flow cell to a batch DLS instrument such as a DynaPro® NanoStar® or Mobius® via optical fibre. The QELS instruments can be used to determine the hydrodynamic radius, r_h , for a variety of samples in a continuous-flow mode. The combined MALS-QELS system will measure simultaneously r_g , r_h , and the absolute molar mass.

Since r_g (the root mean square radius) is determined directly from the *angular dependence* of the scattered light intensity, at least three angles are required to make a reliable measurement. On the other hand, r_h is derived from the fluctuations of light scattering intensity because of diffusion of the molecules (aka Brownian motion) and a single measurement angle suffices.

The QELS measurement is performed on-line in the DAWN flow cell. State-of-the-art optical design, high sensitivity, minimal dead volume, and ease-of-use are the hallmarks of the DAWN detectors. These characteristics, among others, enable them to produce superior signal-to-noise, stability, and sensitivity.

An optical fibre receiver is mounted in the read head of the MALS detector at any angular location. The fibre is, in turn, coupled to an avalanche photodiode in an autocorrelator that has been specially modified to accept the signal from the DAWN instrument.

This application note illustrates the results obtained for bovine serum albumin, BSA, and a glycoprotein, which were separated using a size-exclusion chromatograph (SEC). The MALS-QELS detector determined the hydrodynamic radius versus elution time for the two proteins completely independent of the elution time. Figure 1 shows these results with the 90° LS signal superimposed. For these particular samples, r_g was below the MALS r_h measurement limit of 10 nm. However, simultaneous measurements of MALS and QELS in the integrated system provide complete results for r_h and molar mass (even though the small radius cannot be measured by MALS, molar mass measurements are not impacted). Figure 2 shows the molar mass results determined by the DAWN and the RI detector.

The MALS-QELS combination allows the simultaneous determination of absolute molar mass, root-mean-square radius (r_g from about 10–500 nm), and hydrodynamic radius (r_h from about 0.5–300 nm). Conformation results can now be obtained for molecules ranging from 200 g/mol to hundreds of millions of g/mol.

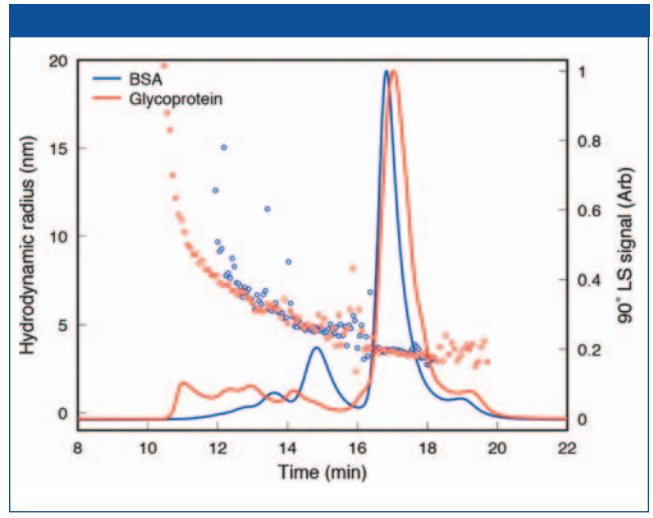

Figure 1: The hydrodynamic radius versus time for BSA and a glycoprotein, obtained from SEC coupled to on-line MALS-QELS detection. The measurements of size (shown here) and molar mass (shown below) can be combined to learn about molecular conformation.

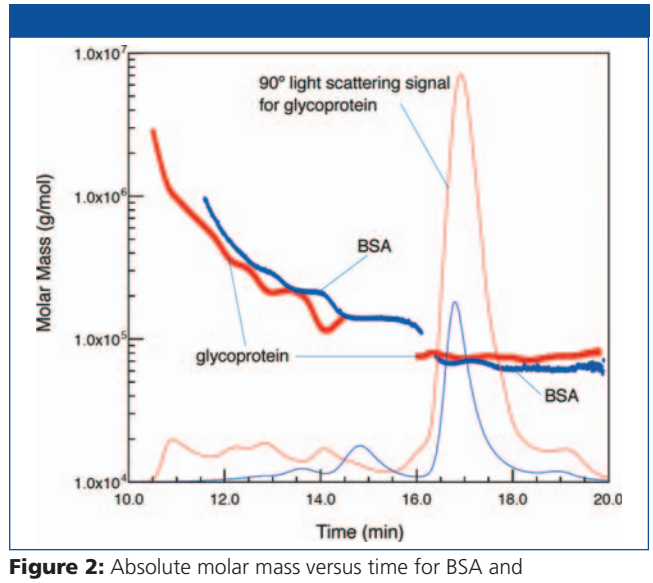

Figure 2: Absolute molar mass versus time for BSA and glycoprotein samples superimposed with the signals from 90° LS detector obtained from size-exclusion chromatography with MALS detection. Glycoprotein aggregates with similar molar mass as BSA aggregates elute earlier due to their extended hydrodynamic size. MALS provides accurate molar mass, regardless of elution time.

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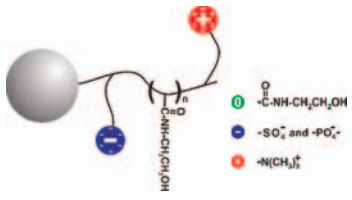

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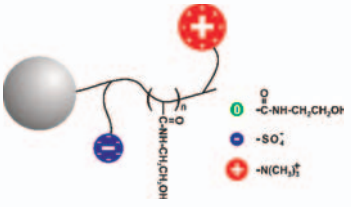

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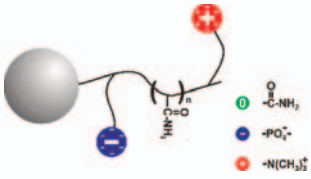

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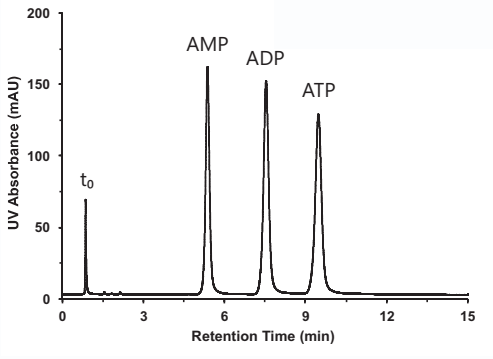

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AppNotes

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GC-MS and LC-MS

Fully Automated Determination of 3-MCPD and Glycidol in Edible Oils by GC–MS Based on the Commonly Used Methods ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10)

Automated determination of 3-MCPD and glycidol in edible oils by GC–MS. An evaporation step helps reach the required LODs using a standard MSD, while removing excess derivatization reagent for improved uptime and stability.

Automated determination of Acrylamide in Brewed Coffee samples by Solid Phase Extraction (SPE)–LC–MS/MS

A manual SPE method used for the determination of acrylamide in brewed coffee was automated. Calibration standards prepared in freshly brewed green (unroasted) coffee produced good linearity and precision.

Characterization of Aroma Compounds in Bread by a 2-Step Multi-Volatile Method (MVM)

A dual step multi-volatiles method (MVM) based on Dynamic Headspace (DHS) analysis provides uniform enrichment of aroma compounds across a wide range of polarities, while eliminating ethanol and water. Bread samples were analyzed.

Analysis of Aroma Compounds in Edible Oils by Direct Thermal Desorption GC–MS Using Slitted Micro-Vials

Hexanal, 2-(E)-nonenal and 2,4-(E,E)-decadienal, edible oil off-flavors derived from unsaturated fatty acid degradation were determined by direct thermal desorption in disposable micro-vials.

Qualitative Analysis of Coconut Water Products Using Stir Bar Sorptive Extraction (SBSE) combined with Thermal Desorption-GC–MS

Flavor compounds, off-flavors, pesticides, antioxidants, and compounds migrating from packaging materials were successfully determined in coconut water products by stir bar sorptive extraction (SBSE)-TD-GC–MS.

For more information about these and other GERSTEL applications, please go to www.gerstel.com/en/apps-food-beverages.htm

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