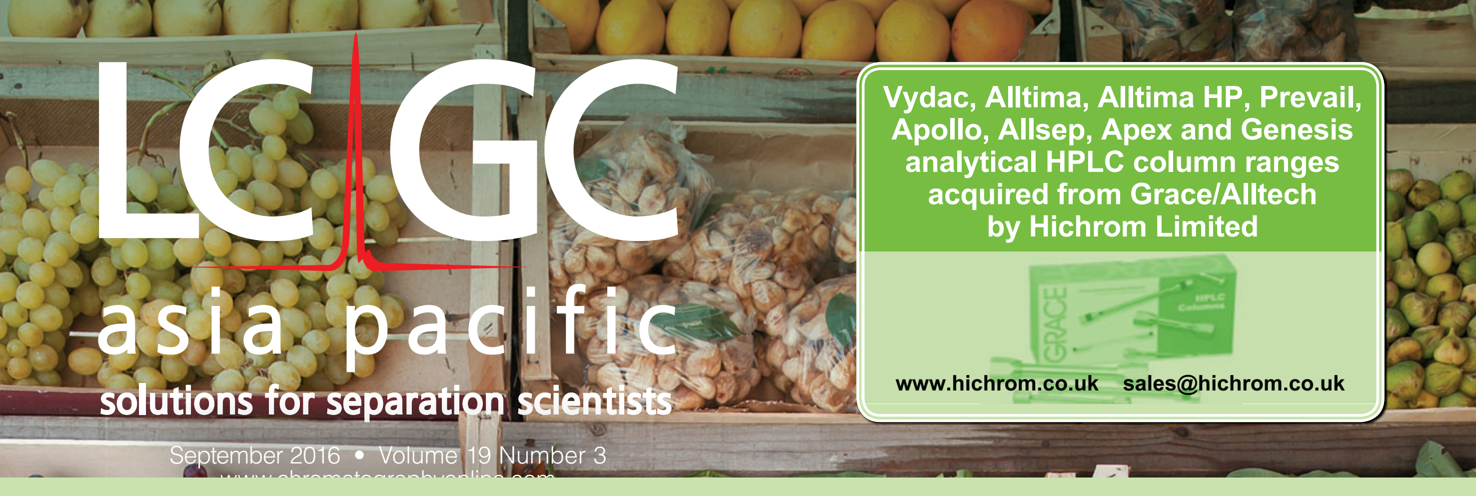

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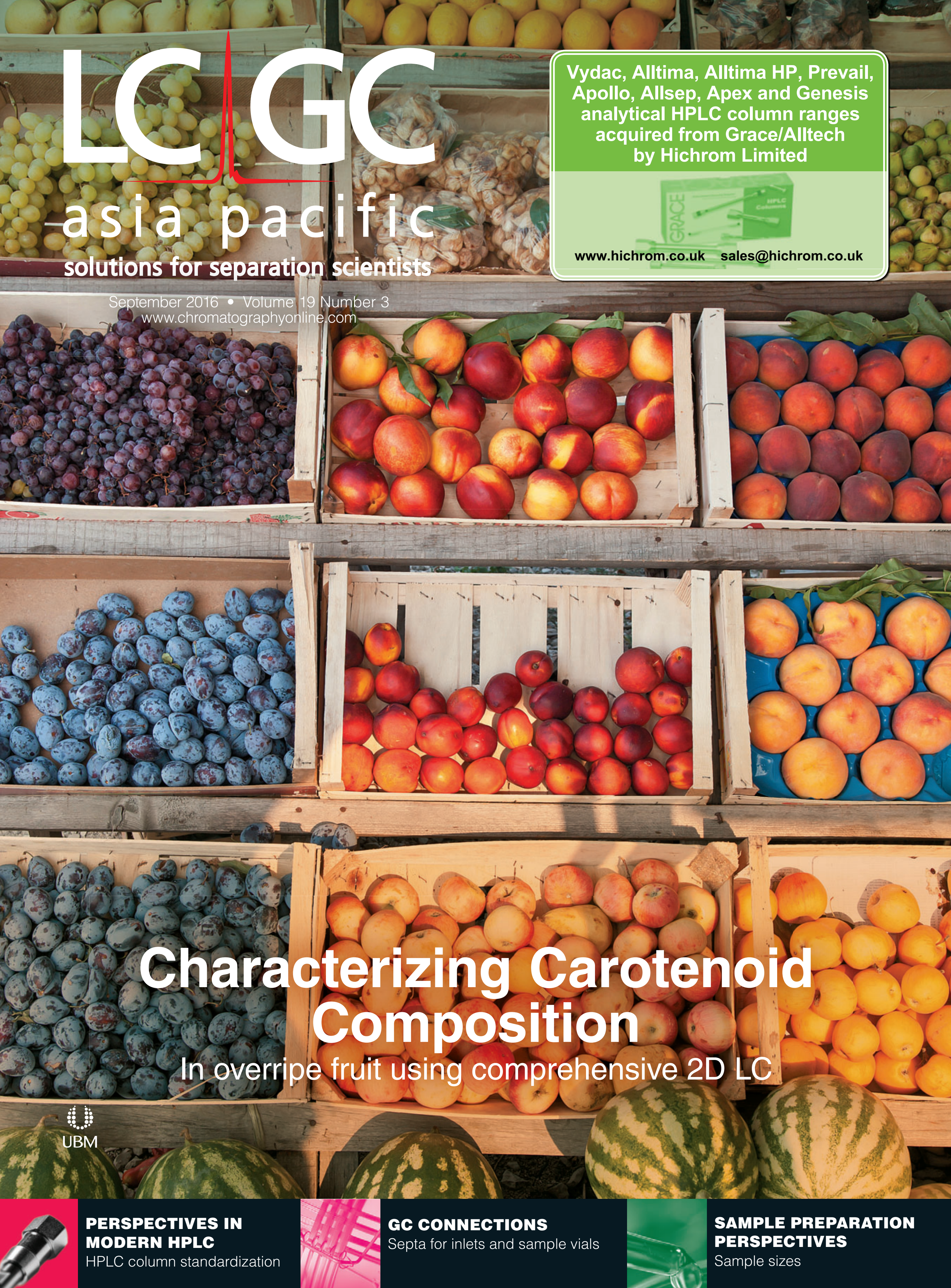
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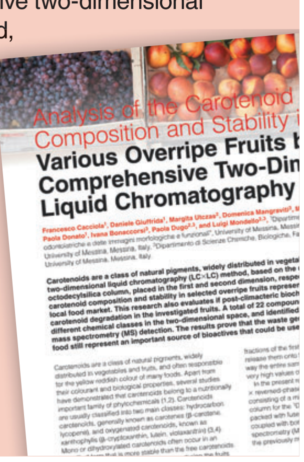
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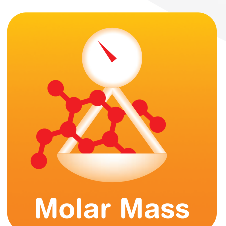

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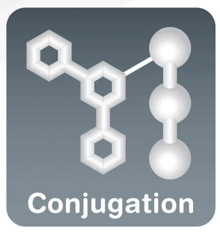

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Analysis of the Carotenoid Composition and Stability in Various Overripe Fruits by Comprehensive Two-Dimensional Liquid Chromatography

Francesco Cacciola[1], Daniele Giuffrida[1], Margita Utczas[2], Domenica Mangraviti[3], Marco Beccaria[2], Paola Donato[1], Ivana Bonaccorsi[3], Paola Dugo[2,3], and Luigi Mondello[2,3], [1]Dipartimento di "Scienze biomediche, odontoiatriche e delle immagini morfologiche e funzionali", University of Messina, Messina, Italy, [2]Chromaleont s.r.l., c/o University of Messina, Messina, Italy, [3]Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali, University of Messina, Messina, Italy.

Carotenoids are a class of natural pigments, widely distributed in vegetables and fruits. A comprehensive two-dimensional liquid chromatography (LC×LC) method, based on the use of a cyano and an octodecylsilica column, placed in the first and second dimension, respectively, was applied to evaluate carotenoid composition and stability in selected overripe fruits representing the waste generated by a local food market. This research also evaluates if post-climacteric biochemical changes are linked to carotenoid degradation in the investigated fruits. A total of 22 compounds was separated into seven different chemical classes in the two-dimensional space, and identified by photodiode array (PDA) and mass spectrometry (MS) detection. The results prove that the waste generated by the large distribution of food still represent an important source of bioactives that could be used for other purposes.

Carotenoids are a class of natural pigments, widely distributed in vegetables and fruits, and often responsible for the yellow reddish colour of many foods. Apart from their colourant and biological properties, several studies have demonstrated that carotenoids belong to a nutritionally important family of phytochemicals (1,2). Carotenoids are usually classified into two main classes: hydrocarbon carotenoids, generally known as carotenes (β-carotene, lycopene), and oxygenated carotenoids, known as xanthophylls (β-cryptoxanthin, lutein, violaxanthin) (3,4). Mono or dihydroxylated carotenoids often occur in an esterified form that is more stable than the free carotenoids. Moreover, esterification greatly increases during the fruits ripening process (5).

The wastes generated by the large distribution of food still represent an important source of bioactives, which could be diverted towards further uses, either in the animal feed production, or to the recovery of purified molecules for nutraceutical purposes. In the current study the carotenoid composition and stability in three overripe fruits, namely hybrid persimmon-apple, banana (pulp and peel), and nectarine was evaluated for the first time, thus also evaluating whether post-climacteric biochemical changes are linked to carotenoid degradation in the investigated fruits.

Comprehensive two-dimensional liquid chromatography (LC×LC) is a technique based on the combination of two independent separation steps with orthogonal selectivities. In LC×LC, a primary column is connected to one or more secondary columns by means of a switching valve as an interface. The function of the latter is to isolate continuous fractions of the first dimension column (^{1}D) effluent and then release them onto the second dimension (^{2}D) column; in this way the entire sample is analyzed in both dimensions, and very high values of peak capacities are obtained (6–10).

In the present research, a normal-phase LC × reversed-phase LC application has been developed, consisting of a micro-bore 250 × 1.0 mm, 5-μm cyano

KEY POINTS

- A comprehensive two-dimensional liquid chromatography method, based on the use of a cyano and an octodecylsilica column, was applied for the evaluation of the carotenoid composition and stability in selected overripe fruits.
- A total of 22 compounds were separated into seven different chemical classes in the two-dimensional space, and identified by photodiode array and mass spectrometry detection.
- The waste generated by the large distribution of food still represents an important source of bioactives.

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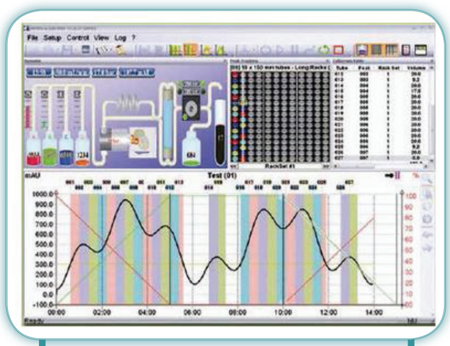

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Figure 1: Normal-phase LC × reversed-phase LC contour plots, indicating the chemical classes of the samples investigated: (a) hybrid persimmon-apple; (b): banana pulp; (c): banana peel; (d): nectarine. For experimental conditions see the text.

column for the [1]D separation, interfaced to a [2]D C18 column packed with fused-core particles (30 × 4.6 mm, 2.7-µm), coupled with both photodiode array (PDA) and mass spectrometry (MS) detectors for the carotenoids evaluation in the previously mentioned fruits matrices.

Experimental

Chemicals: All the reagents and solvents used were of analytical- or HPLC-grade and were purchased from Sigma-Aldrich. Carotenoids standards, namely β-carotene, lycopene, β-cryptoxanthin, lutein, and zeaxanthin, were purchased from Extrasynthese.

Sample and Sample Preparation: Overripe nectarine, hybrid persimmon-apple, and banana samples were purchased in a market place. For the extraction of intact carotenoids, 1 g of each lyophilized sample was treated with 4 mL of methanol, and then shaken with a magnetic stirrer for 15 min. An equal volume of hexane (Hex) (4 mL) was added to the mixture and shaken for another 15 min, as before. Subsequently, 3 mL of water was added to the mixture and shaken again. The mixture was centrifuged at 3000 × g for 15 min, and the organic layer was recovered in a volumetric flask and dried under vacuum. The dry residue was dissolved in methanol/methyl tert-butyl ether (MTBE) (1:1, *v/v*) mixture and then filtered through a 0.45 µm Acrodisc nylon membrane filter (Pall Life Sciences) prior to LC analysis.

LC×LC Instrument: LC×LC analyses were performed on a Prominence LC-20A (Shimadzu), consisting of a CBM-20A controller, four LC-20AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A photo diode array detector (2.5 µL detector flow cell volume), a CTO-20AC column oven, and a SIL-20A autosampler (all Shimadzu). The two dimensions were connected by means of a 10-port two-position switching valve equipped with a micro-electric actuator (Sigma-Aldrich/Supelco) placed inside the column oven and equipped with two 0.254 mm i.d. stainless steel sample loops of identical volume (10 µL). Both dimensions and the switching valve were controlled by LCMSsolution software (Version 3.60.361, Shimadzu). The LC×LC data were visualized and elaborated into two and three dimensions using Chromsquare ver. 2.0 RC2 software (Chromaleont). The LC×LC system was coupled to an LCMS-2010 mass spectrometer through an atmospheric pressure chemical ionization (APCI) source (Shimadzu).

LC×LC–PDA-MS Conditions:

[1]D: [1]D analyses were performed on an Ascentis ES Cyano column (250 × 1.0 mm, 5-µm Sigma-Aldrich/Supelco).

[1]D Mobile Phase: (A) Hex; (B) Hex/butyl-acetate/acetone

Table 1: List of carotenoids identified by LC×LC analysis in the overripe fruits.

Peak Numbering	UV–vis, λ max (nm)	$[M+H]^+$	Identification
1	453, 476	791	β-Cryptoxanthin-C16:0
2	452, 477	763	β-Cryptoxanthin-C14:0
3	452, 476	789	β-Cryptoxanthin-C16:1
4	452, 476	537	β-Carotene
5	447, 473, 504	537	Lycopene
6	448, 473	n.d.	n.d.
7	453, 476	553	β-Cryptoxanthin
8	424, 446, 474	795	Anteraxanthin-C14:0
9	424, 446, 474	1005	Anteraxanthin-C14:0-C14:0
10	420, 444, 470	989	Lutein-C14:0-C14:0
11	427, 451, 477	989	Zeaxanthin-C14:0-C14:0
12	421, 444, 473	537	α-Carotene
13	421, 446, 472	751	Iso-lutein-C12:0
14	422, 445, 473	723	Lutein-C10:0
15	421, 445, 472	751	Lutein-C12:0
16	422, 446, 472	779	Lutein-C14:0
17	422, 445, 473	807	Lutein-C16:0
18	420, 440, 468	601	Violaxanthin
19	414, 438, 467	601	Neoxanthin
20	422, 446, 472	569	Lutein
21	339, 445, 473	537	Z-β-Carotene
22	453, 476	817	β-Cryptoxanthin-C18:1

(80:15:5, *v/v/v*). Gradient: 0.01 min, 0% B; 34 min, 0% B; 50 min, 50% B; 60 min, 100% B; 180 min, 100%B. Flow rate: 10 µL/min adjusted by means of a flow splitter, (split ratio 1:20). Column oven: 40 °C. Injection volume: 5 µL.
²D: ²D analyses were performed on an Ascentis Express C18 column (50 × 4.6 mm, 2.7-µm Sigma-Aldrich/Supelco).
²D Mobile Phase: (A) H_2O/acetonitrile (10:90, *v/v*); (B) (isopropanol) IPA. Gradient: 0.01 min, 20% B; 0.05 min, to 70% B; 0.63 min, 80% B; 0.75 min, to 90% B; 0.76 min, 20% B. Flow rate: 3 mL/min (splitted to 0.75 ml/min).
PDA Detection: 250–550 nm (sampling rate, 12.5 Hz; time constant, 0.08 s; cell temperature 40 °C).
MS Detection (APCI-Positive and Negative Ionization Modes): m/z range: 450–1100 amu; nebulizing gas (N_2) flow: 2.0 mL/min; detector voltage: 2.0 kV; interface temperature: 450 °C; CDL temperature: 300 °C; heat block temperature: 300 °C; event time: 0.15 s; scan speed: 6000 amu/s; CDL temperature: 300 °C; heat block temperature: 300 °C.

Results and Discussion

The aim of this study was to evaluate the carotenoid composition and stability in three overripe fruits, namely hybrid persimmon-apple, banana (pulp and peel), and nectarine for the first time by the use of an LC×LC method, consisting of normal phase and reversed phase chromatography in the ¹D and in the ²D, respectively. The two dimensions were both optimized independently, then combined and tuned together.

A micro-bore cyano column was chosen for the ¹D separations and it was operated with a step-wise gradient, starting from 100% Hex (A, 0.01 min) reaching 100% (B) Hex/butyl-acetate, acetone (80:15:5, *v/v/v*) in 60 min. The mobile phase flow rate was set to 10 µL/min, to give the best results in terms of peak overlap and sampling, performing a carotenoid separation into classes of increasing polarity. The experiments were performed on an LC×LC instrument configured with an electrically actuated 10-port two-position switching valve, allowing automated fraction collection/reinjection of the two 10 µL loops. Aliquots eluted of the 1D were sampled in one of the sampling loops and transferred into the ²D via the 10-port valve, while the second sampling loop is filled. So, the whole effluent from the ¹D column was transferred on-line to the ²D column, an octadecylsilica column every 60 s, and resolved using a gradient elution program, starting with a low concentration of the stronger solvent (IPA), 20% at mobile phase flow of 3.0 mL/min. Figure 1(a–d) shows the LC×LC chromatograms of the carotenoid extracts in (a) overripe hybrid persimmon-apple, (b) banana pulp and (c) peel, and (d) nectarine at 450 nm, with the location of the different identified carotenoid classes in the ²D space. Figure 2(a–d) shows the normal-phase LC × reversed-phase LC contour plots, indicating the peak numbering of the samples investigated (a: hybrid persimmon-apple; b: banana pulp; c: banana peel; d: nectarine). Table 1 reports the

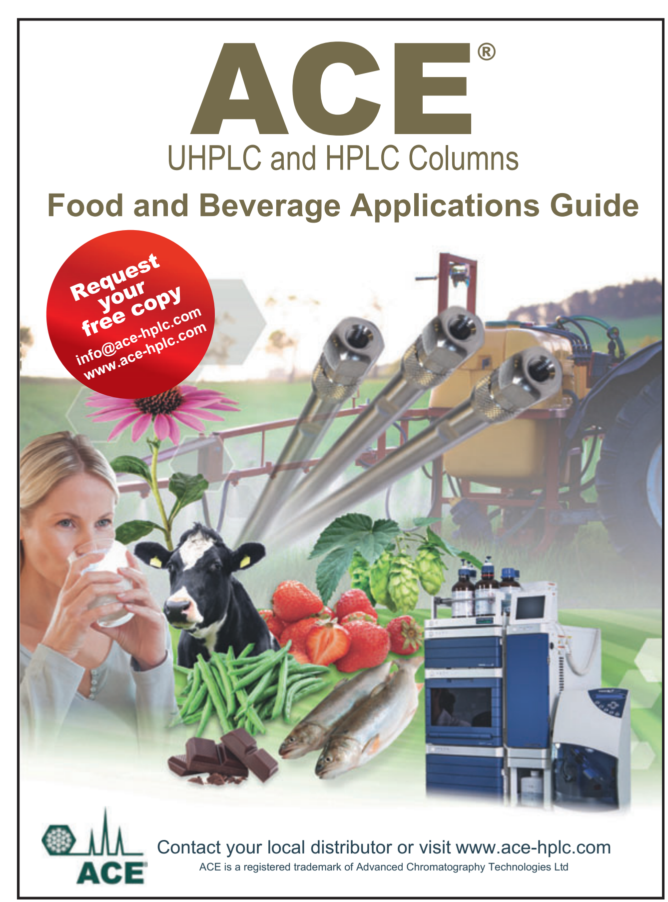
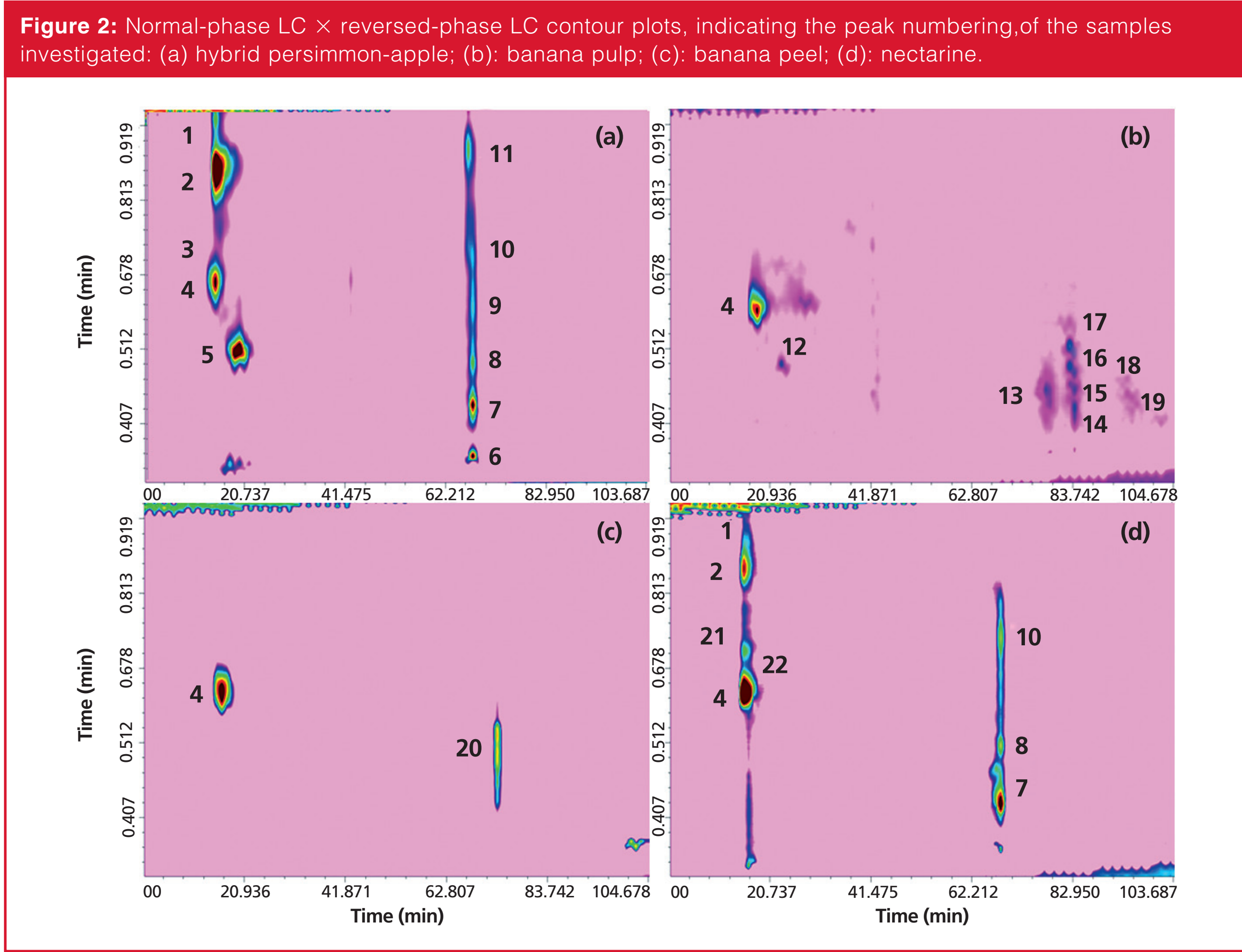


Figure 2: Normal-phase LC × reversed-phase LC contour plots, indicating the peak numbering,of the samples investigated: (a) hybrid persimmon-apple; (b): banana pulp; (c): banana peel; (d): nectarine.



relative peak assignment for the spots numbering shown in Figure 2.

Chromatography on the cyano stationary phase allowed a good separation of the carotenoids in seven groups of different polarity in the first dimension, as can be seen from the circles in Figure 1, namely hydrocarbons, mono-ol-esters, di-ol-di-esters, di-ol-mono-esters, free-mono-ols, free-di-ols, and free-epoxy-di-ols. On the other hand, the secondary C18 column allowed the separation of carotenoids within each class, according to their increasing hydrophobicity and decreasing polarity (for components of the same class, the elution order increases with the number of carbon atoms of the fatty acid chain).

Identification of the separated compounds was achieved by means of both PDA and MS detection (through APCI ionization). The latter represents a powerful analysis tool for unknown molecules; particularly in the case of carotenoids, operation of the interface under both positive and negative mode offers the double advantage of improved sensitivity and identification power. MS spectra obtained under negative ionization mode are in fact dominated by the presence of very intense pseudo-molecular ions $[M^{\cdot}]^{-}$, which makes identification of low-abundant components easier; on the other hand, abundant fragmentation is generally observed under positive APCI ionization, especially for carotenoid esters, whose fragment ions can help in structure elucidation. It must also be stressed that a better front-end LC separation is highly beneficial before MS analysis because clearer spectra are obtained.

The combined use of PDA and MS data allowed 22 carotenoids contained in the samples to be distinguished, either in their free or in the esterified form (Table 1); it is noteworthy that the complementary information attained allowed compounds showing similar (or nearly identical) UV-absorption properties, arising from the same chromophore groups, to be discriminated between. An example is represented by the two mono-ol-esters labelled as 2 and 1 in Table 1, namely β-cryptoxanthin-C14:0 and its superior homologue β-cryptoxanthin-C16:0. The absorption spectra of these two molecules in fact overlap, while the *m/z* pseudo-molecular ions are easy to distinguish one from the other. The studied fruits are representative of tropical and temperate zone fruits and are considered as climacteric fruits. In fact these fruits can be ripened after harvest; thus the present study also aims to evaluate whether post-climacteric biochemical changes are linked to carotenoid degradation in the investigated fruits. Although some reports are available in the literature on the carotenoid composition of fresh apples (11) and persimmon (12), the carotenoid profile of their hybrid fruit had never been previously investigated. From the

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evaluation of the carotenoid profile shown in Figure 2(a) for the overripe kakimela, it can be concluded that both the hydrocarbons, β-carotene and lycopene, and the various identified xanthophyll mono- and di-esters were stable in the overripe sample; moreover, the carotenoids pattern in the hybrid persimmon-apple was more similar to the reported carotenoid profile of native kaki fruit then to the reported carotenoid profile of native apple fruits, thus indicating that prevailing metabolic pathways may occur in the carotenoid production of the investigated fruit. In agreement with the report by Lokesh *et al.* (13), β-carotene, lutein, and α-carotene were the main carotenoid detected in banana as shown in Figure 2(b) and (c); moreover, it can be observed here that free lutein was only detected in the pulp and that a series of lutein esters were only detected in the peel. It can also be observed that those carotenoids were stable in the overripe banana fruit. The stability of the main carotenoid in nectarine (14), β-carotene, in overripe nectarine was observed in this study together with other minor — here detected for the first time — carotenoid in this fruit, such as *cis* β-carotene and the xanthophylls esters.

Conclusions

The applied LC×LC methodology has enabled the identification of different carotenoids, including various esters, in selected overripe fruits and its application in the analysis of other complex carotenoid matrices could be a future objective of research. The results showed that no post-climacteric carotenoid losses occurred and that provitamin A carotenoids and lutein were indeed stable in the overripe stage of the studied fruits. Thus, those matrices still represent an important source of bioactives, which could be diverted towards further uses, either in animal feed production, or to the recovery of purified molecules for nutraceutical purposes.

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UHPLC Instrument Variations and Approaches to **Ease the Method Transfer Process**

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Ultrahigh-pressure liquid chromatography (UHPLC) instruments from different manufacturers and instruments with different configurations can produce significant variations in chromatographic separation. The variety in instrument configuration increases the complexity of the method development process, which now requires a more thorough evaluation of the effect of instrument variations on the method. The studies presented here determined the typical interinstrument variations in dwell volume, extracolumn dispersion, and mixing efficiency as measured by mobile-phase compositional accuracy. Additionally, the dwell volume and extracolumn dispersion were independently and systematically varied to evaluate the resulting impact on resolution for a small-molecule test mixture during gradient elution. To account for these interinstrument variations, dwell volume and wash-out volume method translation and adjustment techniques were evaluated.

To support the need to get products to market more quickly and under tighter cost constraints, increased externalization of manufacturing and analytical development has occurred within the pharmaceutical and biotechnology industries (1,2). As activities are shifted to external laboratories, the diversity of instruments and configurations dramatically increases as compared to when all activities occurred within the same internal laboratory that typically had one instrument model and configuration. This instrument diversity has led to the observation of increased chromatographic separation variation.

This trend in conjunction with the shift from high performance liquid chromatography (HPLC) to ultrahigh-pressure liquid chromatography (UHPLC) platforms has driven the need to characterize the expected interinstrument variations (3). Although variations between HPLC instruments from different manufacturers or with different module configurations contribute to chromatographic differences, typically the impact is not significant because of the inherent total volume of the system and the efficiency of the columns (4–6). With the shift to higher efficiency columns, the instrumental variations have a significant impact on the chromatographic performance. For example, the dwell volume to void volume ratio (V_D/V_M) varies between 2.1 to 2.8 for a 100 mm × 3.0 mm, 3.5-μm d_p column on a binary versus quaternary pump HPLC. While using a 50 mm × 2.1 mm, 1.7-μm column on a binary versus quaternary UHPLC, the ratio can vary between 1.8 and 3.8. This difference illustrates the increased relative impact of instrumental variations on UHPLC methods.

Recommendations in the literature suggest adjustment of dwell volume, column temperature, and wash-out volume to produce equivalent chromatographic results on different instruments (5,7–10). While these suggestions are available, the amount of expected interinstrument variation and the tolerability of these variations have had limited discussion. Additionally, the success rate of using these method translation and adjustment techniques has had limited unbiased evaluation, and the regulatory implications and required method validation studies to allow method adjustments require consideration.

The goals of this research are to understand the impact of instrumental parameters on the retention and resolution of analytes and better identify the cause of observed chromatographic differences between instruments for gradient separations. Additionally, method translation and adjustment techniques are evaluated with the goal of developing a framework to build quality into UHPLC methods to ease the method transfer process.

Experimental

All studies were performed using Waters H-Class instruments or Agilent 1290 instruments (binary and quaternary). All mobile phases were prepared using HPLC-grade solvents. The gradient test mixture was purchased and used as is (Waters gradient test mix as part of the Acquity UPLC Absorb Start-up solution). The gradient test mix was selected to represent a simple small-molecule mixture for which retention would span the typical range of a pharmaceutical method. The gradient test mix method used

KEY POINTS

- The study presented here determined the typical interinstrument variations in dwell volume, extracolumn dispersion, and mixing efficiency, and assessed techniques to account for the variation.
- The aim of this research was to understand the impact of instrumental parameters on the retention and resolution of analytes, as well as to identify the cause of chromatographic differences between instruments for gradient separations.

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Table 1: Summary of the available UHPLC instruments and configuration options.

Parameter	Agilent 1290 Infinity (23–25)	Thermo Scientific Dionex UltiMate 3000 RSLC (26–29)	PerkinElmer Flexar UHPLC (30–31)	Shimadzu Nexera X2 (32–33)	Thermo Fisher Scientific Vanquish (34–36)	Waters UPLC (Acquity, H-Class, I-Class) (37–39)
Pump	Binary, quaternary	Binary, quaternary	Isocratic, binary, quaternary	Binary, quaternary	Binary, quaternary	Binary, quaternary
Mixer volume	35 µL, 100 µL, 380 µL	35 µL, 100 µL, 200 µL, 400 µL, 800 µL, 1550 µL	50 µL, 150 µL, 250 µL, 350 µL, 500 µL, 750 µL, 1000 µL	20 µL, 40 µL, 100 µL, 180 µL	25 µL (B), 200 µL (B), 400 µL (Q)	50 µL (A, I), 100 µL (H,I), 380 µL (I)
Detector cell volume	0.6–4 µL	2.5 µL, 13 µL	2.4 µL	2.5 µL, 12 µL	2 µL, 13 µL	0.5 µL
Tubing configurations	Default, ultralow dispersion	Default	Default	Default	Default	Default
Injection type	Flow through needle	In-line split loop (flow through)	Fixed loop	Flow through needle (30, 30ACMP) or fixed loop (30)	Split loop (flow through)	Flow through needle (H, I), fixed loop (A)
Injector volume	20–120 µL	25–500 µL	2–1000 µL	50 µL–2 mL	25–100 µL	10–1000 µL
Column heater	Peltier	Peltier with forced air	Peltier	Peltier with forced air or still air	Peltier with forced air or still air	Peltier

a 50 mm × 2.1 mm, 1.7-µm Waters Acquity BEH C18 column at 40 °C. The mobile phase, multistep gradient, and flow rate were as follows: mobile-phase A: water; mobile-phase B: acetonitrile; 0–0.25 min, 10% B; 0.25–2.5 min, 10–95% B; 2.5–2.6 min, 95% B; 2.6–3.0 min, 95–10% B; 3.0–5.0 min, 10% B; 0.6 mL/min. The pharmaceutical sample used was a small-molecule peak identification solution, which contained molecules varying in acidity or basicity and hydrophobicity. This sample was selected because of its complex nature and sensitivity to variations in method conditions.

For all experiments, a 50 mm × 2.1 mm, 1.7-µm column was used. This column was selected based on the majority of the methods that are used within our laboratory. Compared to other typical column dimensions used for UHPLC methods (excluding 1.0-mm i.d. columns), these column dimensions represent a worst-case scenario in terms of impact from instrumental parameters.

Dwell Volume: The interinstrument variation in dwell volume was assessed by using a gradient of water and 0.1% acetone in water (11). The gradient was a 0–100% B linear ramp over 10 min. The column was replaced with a 1000 cm × 0.018 cm i.d. piece of polyetheretherketone (PEEK) tubing and was accounted for (248 µL) in determining the dwell volume of the system. The intersection of the isocratic (zero slope) and gradient slope of the chromatogram was used as the dwell time. For each UHPLC system, the dwell time was determined in triplicate and the average dwell time was used for the determination of the dwell volume. The dwell volume was calculated as shown in equation 1, based on the measured dwell time.

$$\text{dwell volume} = \left(\text{dwell time (min)} \times \text{flow rate} \left(\frac{\text{mL}}{\text{min}}\right)\right) - \text{PEEK tubing volume (mL)} \qquad [1]$$

To evaluate the impact of dwell volume on the retention time and resolution of the analytes in the gradient test mix, the dwell volume was physically modified by adding different lengths of 0.17-mm i.d stainless steel tubing between the pump and the injector. Under the method conditions stated above, the gradient test mix was injected in triplicate and the average retention time of each analyte and the average resolution for each pair was determined. The average change in resolution was plotted as a function of the total dwell volume of the system (system dwell volume and volume of tubing added) for each analyte pair. The average change in resolution as a function of the change in dwell volume was calculated based on the experimental data.

Extracolumn Dispersion: The column was replaced with a zero-dead-volume union, 50:50 water–acetonitrile was used as the mobile-phase, and 1 mL of 0.1% acetone in water was injected. The extracolumn dispersion was determined by measuring the 4σ peak width of the acetone peak (12). For one UHPLC system, the extracolumn dispersion (ECD) was measured at different flow rates between 0.4–1.0 mL/min. The variation in ECD as a function of flow rate was found to be less than 1 µL. Therefore, only one flow rate was used for the measurement of ECD on the remaining instruments. For each UHPLC system, the measurement was determined in triplicate at 1.0 mL/min and the average ECD was calculated.

The effect of both precolumn and postcolumn ECD on the resolution of each analyte pair in the gradient test mix was evaluated. A known length of 0.12-mm i.d. PEEK tubing was placed between the injector and the column inlet to assess precolumn ECD and between the column outlet and the detector for postcolumn ECD. The same gradient test mix method conditions listed above were used

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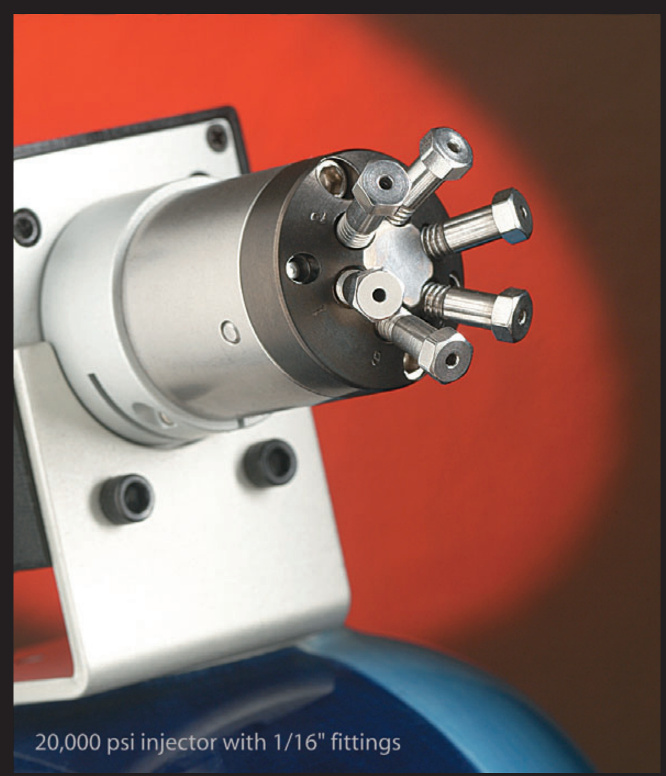
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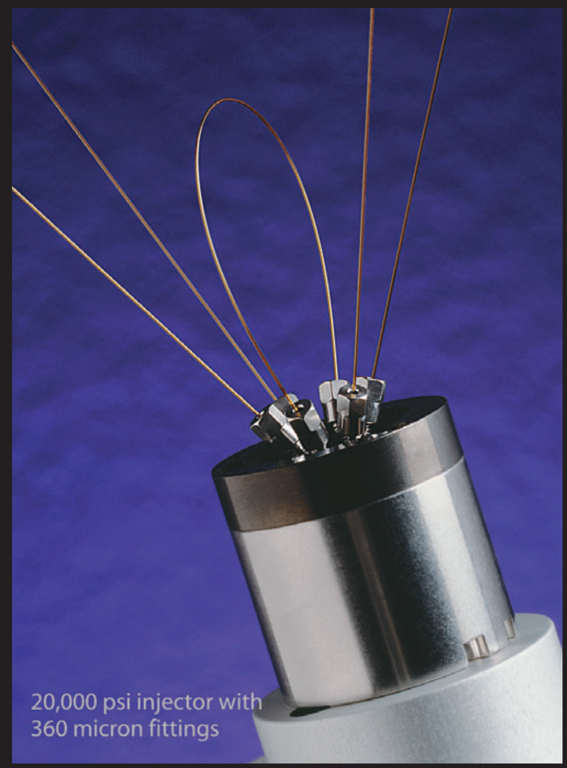
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Figure 1: Effect of dwell volume on resolution.

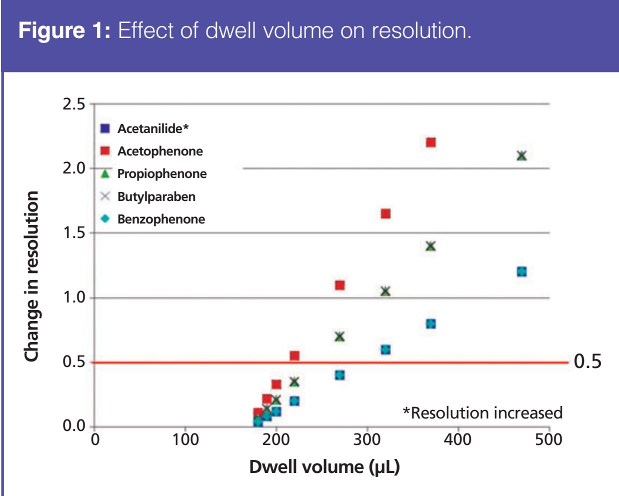
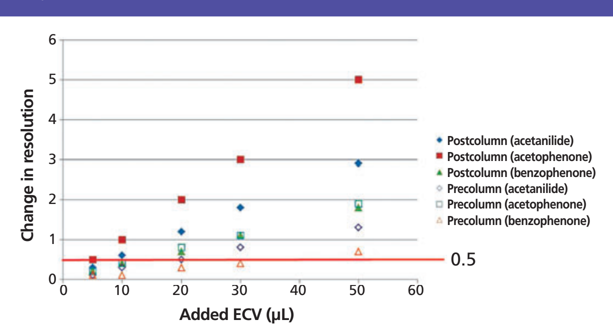


Figure 2: Effect of precolumn and postcolumn extracolumn dispersion on resolution.



for these experiments. The change in resolution for each analyte pair was plotted as a function of ECD added to the system. Based on this, the average change in resolution as a function of change in ECD was calculated for precolumn and postcolumn ECD.

Mixing Efficiency: For several instruments with different mixing volume and mixer type, the column was replaced with a zero-dead-volume union and a step gradient of 0.1% acetone in mobile-phase B was generated as described by Medvedovici and David (13). The following mobile-phase combinations were assessed: water–methanol, water–acetonitrile, and water–isopropanol. In all cases, %B was increased in increments of 10% and held at each level for 10 min. After the pump was delivering 100% mobile-phase B, the step gradient decreased in increments of 10% B until the pump was generating 0% mobile-phase B. The deviation from the theoretical gradient set point was determined by taking the difference between the instrumental set point and the $\%B_{plateau}$:

$$\%B_{plateau} = (\bar{A}_{plateau} - \bar{A}_{\%B=0}) \times \frac{100}{(\bar{A}_{\%B=100} - \bar{A}_{\%B=0})} \quad [2]$$

The average absorbance at the chromatographic plateau, $\bar{A}_{plateau}$, was corrected for the absorbance at 0% B, $\bar{A}_{\%B=0}$ and normalized against the difference between the absorbance at 100% B, $\bar{A}_{\%B=100}$ and the absorbance at 0% B. For each UHPLC system–mixer configuration and mobile-phase combination, the average percent deviation was calculated. The measurement of the mobile-phase compositional accuracy as determined above was deemed suitable to assess the mixing efficiency since larger variations were observed for different mixer types than when measuring baseline noise.

Method Translation: For method translation and adjustment by dwell volume, the initial isocratic hold time was increased when moving from a system with larger dwell volume to a system with smaller dwell volume. The required isocratic hold time was determined by calculating the interinstrument difference in dwell volume and dividing by the flow rate to produce the time required to generate the required dwell volume. When moving from a system with a smaller dwell volume to a system with a larger dwell volume, an injection delay was added to the method. Within the software, the dwell volume difference was entered and the required injection delay was automatically calculated based on the method flow rate. The gradient test mix and the pharmaceutical sample were assessed for retention time and resolution consistency between the two instruments under evaluation. Moving from a system with a smaller dwell volume to a system with a larger dwell volume and vice versa was evaluated for each sample.

In addition to adjusting for dwell volume, the wash-out volume was also used as a method adjustment technique. A gradient of water and 0.1% acetone in acetonitrile from 0% to 100% B was generated with an additional isocratic hold at 100% B. The time required to transition from the gradient-slope region to the zero-slope region was used as the wash-out time for the system (7). The wash-out time and the flow rate were used to calculate the wash-out volume for each UHPLC system. The ratio of the wash-out volume for the two instruments of interest was used to scale every step in the gradient programme. The gradient test mix was used to evaluate the consistency of retention time and resolution when adjusting for dwell volume and wash-out ratio simultaneously.

Results and Discussion

Instrument configuration variations can arise from differing pump type, mixer volume, detector cell volume, and tubing configuration — to name a few. When taken in combination, the potential for interinstrumental differences becomes quite high. Table 1 lists the common configurations available for several UHPLC systems currently on the market.

Dwell Volume: Dwell volume differences arise from instrumental parameters such as the pump configuration (binary or quaternary), the presence or absence of an in-line filter, mixer volume, and tubing configuration. The resulting dwell volume difference can affect the retention, selectivity, and resolution (14). The dwell volume and flow rate dictate the length of time required for the gradient to reach the column inlet, which affects the initial isocratic hold time experienced by the column and the speed of gradient change. Although dwell volume affects analytes throughout the entire separation space, variation in the dwell volume typically has a greater impact on weakly retained analytes; the elution of weakly retained analytes can vary from isocratic elution to gradient elution depending on the dwell volume of the system and the retention time of the analyte (9). Dwell volume can impact analytes throughout the

Figure 3: Comparison of mixing efficiency (compositional accuracy) for typical reversed-phase mobile-phase combinations.

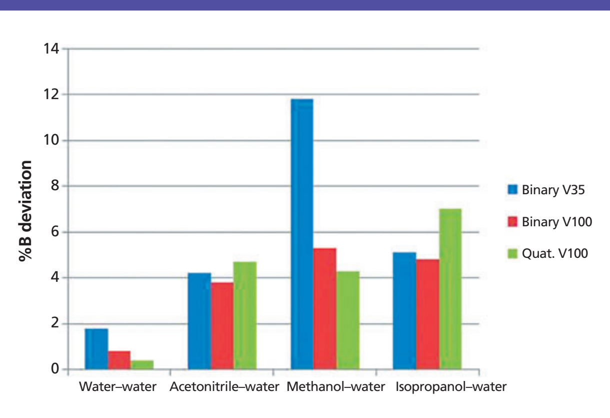
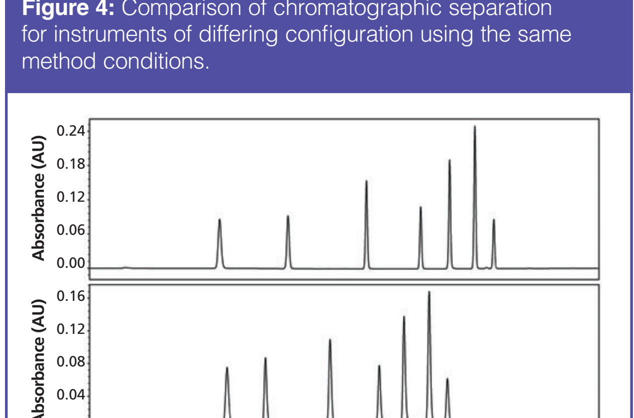


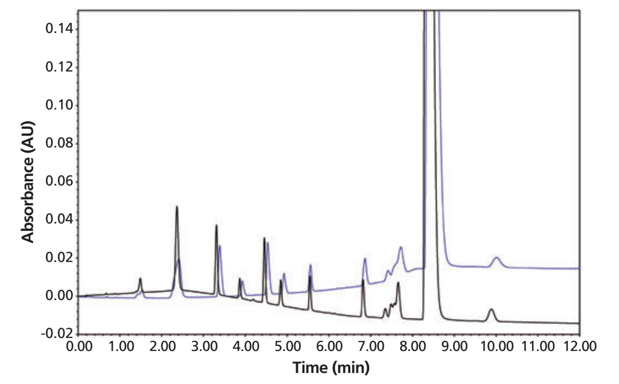
Figure 4: Comparison of chromatographic separation for instruments of differing configuration using the same method conditions.



retention window because of the resulting gradient shape dictated by the dwell volume to void volume ratio (10).

The dwell volume was measured for the UHPLC instruments within our laboratories to determine the amount of interinstrument variation that can be expected. The dwell volume for the instruments within our laboratories varied between 140 µL and 560 µL. For binary instruments, the dwell volume ranged from 140 µL to 220 µL and between 380 µL to 560 µL for quaternary instruments, depending on the configuration. To put this in perspective, for a method flow rate of 0.6 mL/min, the initial isocratic hold time would vary between 0.2 min and 0.9 min depending on the dwell volume of the system. Therefore, an analyte eluted between 0.2 and 0.9 min could be eluted under either isocratic or gradient conditions depending on the system used. Based on these potential variations, differences would be expected in the chromatographic separation between UHPLC instruments.

Figure 5: Comparison of chromatographic separation of a pharmaceutical sample on instruments of differing configuration with dwell volume adjustment. Blue trace: manufacturer 2, binary, original method; black trace: manufacturer 1, quaternary, injection delay equivalent to a 100-µL dwell volume.



To evaluate the extent to which dwell volume affects analytes throughout the retention window, the dwell volume was physically modified by adding tubing between the pump and the injector. The resolution of the analyte pairs in the gradient test mix was measured as a function of system dwell volume (Figure 1). A change in resolution of 0.5 was chosen as an acceptable amount of variation. At this level of resolution variation, a dwell volume change up to 30 µL should not require method translation and adjustment. However, if the separation of interest cannot tolerate a resolution change of 0.5 between the critical pair, the amount of dwell volume variation acceptable would decrease as well.

As expected, instruments with the same configuration and from the same manufacturer typically will not require method adjustment because of dwell volume. However, variations in tubing internal diameter between the pump and injector can produce a dwell volume difference that would require method translation and adjustment to maintain the chromatographic separation. Based on these findings, it is recommended to measure the dwell volume of the UHPLC system in which the method will be run and define the dwell volume used during method development within the analytical method.

Extracolumn Dispersion: In addition to dwell volume, the extracolumn dispersion can be found to vary between instruments producing differences in peak variance. The length and internal diameter of the connecting tubing and detector cell volume contribute to the ECD of the system (15). Although ECD contributes to band broadening in HPLC separations, as the variance related to the column decreases — as is the case with most UHPLC methods — the variance contributions from the system have an increased impact on the total peak broadening (14):

$$\sigma^2_{total} = \sigma^2_{ec,before} + \sigma^2_{column} + \sigma^2_{ec,after} \quad [3]$$

Although a large amount of research has addressed the effect of ECD on isocratic separations, few studies have addressed the impact on gradient separations. It is expected that the impact of the ECD will be less for gradient separations than for isocratic separations because of the gradient focusing that occurs, but this focusing cannot completely negate the contributions from the extracolumn dispersion (4,14).

The extracolumn dispersion for the UHPLC instruments within our laboratories varied between 12 µL and 50 µL. The majority of the instruments were found to have an ECD of 12–19 µL. A couple of instruments were found to have an ECD of approximately 50 µL, which resulted from tubing modifications that were made after installation. This excessive ECD can be easily remedied by changing the tubing, but the variability in ECD should be expected when transferring methods to external laboratories that may have many different system configurations.

Because the ECD was found to vary between the UHPLC instruments measured, the effect of ECD changes on resolution was evaluated. For both precolumn and postcolumn ECD, the change in resolution for the analyte pairs in the gradient test mix was measured as a function of added ECD (Figure 2). As expected, the change in resolution caused by additional precolumn ECD was less than the resolution change with added postcolumn ECD. Although the resolution change was more significant for postcolumn volume, the gradient focusing effect did not completely negate the effects of the precolumn ECD. Again, assuming that a resolution change of 0.5 is acceptable, changes of 10 µL and 4 µL are acceptable for the precolumn and postcolumn ECD, respectively. Therefore, based on the typical variation in ECD (7 µL) for the instruments measured, method translation and adjustment would not be required.

Comparing the effect of dwell volume and ECD on changes in resolution, the resolution change per volume change in ECD is more significant than that for dwell volume changes. However, the magnitude of interinstrument dwell volume variation is much greater than the ECD. Therefore, the dwell volume will have a more significant effect than the ECD on the separation.

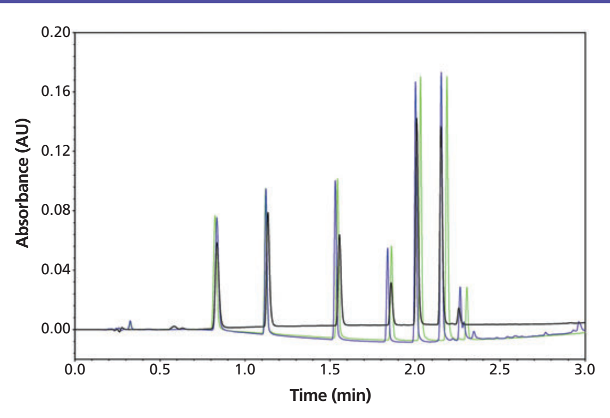
Mobile-Phase Mixing: Historically, the mixing efficiency of high-pressure mixing (binary) instruments and low-pressure mixing (quaternary) instruments has been debated. Typically, greater mixing volumes or complex mixers are put in place to compensate for the mixing inefficiencies of high-pressure mixing instruments for solvents of differing viscosity (16). However, high-pressure mixing is more suitable if outgassing occurs during mixing (17). As technology has improved, mixing inefficiencies have decreased. In practice, concerns are still present, particularly in cases when mixing volume is decreased to accommodate fast separations.

The mobile-phase mixing efficiency was assessed by measuring the %B deviation from the theoretical set point (Figure 3). For binary and quaternary systems with the same mixing volume (100 µL), the %B deviation was similar and without a trend. Furthermore, in most cases similar mixing efficiency was achieved for a high-pressure mixing system with a 35-µL mixer and a 100-µL mixer on a low- or high-pressure mixing system. When mixing water and methanol, the 35-µL mixer on the high-pressure mixing system was not sufficient to give adequate mixing as measured from compositional accuracy. In cases when

water and methanol will be used, the mixing volume for a binary system should be at least 100 μL. For separations on the 5-min time scale, the additional dwell volume due to the 100-μL mixer as compared to the 35-μL mixer does not hinder separation speed, but can ensure sufficient mixing. Very fast separations (1–2 min) can use the smaller mixing volumes, but they may lead to mixing inconsistencies in the case of water and methanol mixing.

Method Translation Techniques: Running the gradient test mix method on instruments with differing configurations was found to produce significantly different chromatography (Figure 4). Because of this variation, method translation and adjustment is required. As previously discussed, several method translation techniques have been suggested in the literature and by instrument manufacturers (5,7–10). One approach is to adjust the isocratic hold time or injection delay to account for the interinstrument differences in dwell volume. Using the gradient test mix and pharmaceutical samples, this method was found to produce equivalent chromatographic retention. Although the retention was comparable, in some cases the critical pair resolution was inconsistent (Figure 5). This resolution variation may be due to other system differences such as actual column temperature, column axial temperature gradient, and extracolumn dispersion, which are not accounted for by adjusting the dwell volume. For highly complex methods and methods that are highly sensitive to method conditions, the ability to translate or adjust the method by simple techniques becomes more difficult.

Figure 6: Comparison of chromatographic separation of gradient test mix on instruments with differing configuration. Black trace: system manufacturer 2, binary, original method; blue trace: manufacturer 1, quaternary, injection delay for dwell volume; green trace: manufacturer 1, quaternary, injection delay for dwell volume and gradient adjustment for wash-out volume.



In addition to the dwell volume, analysts may need to account for the wash-out volume of the system. This volume is related to the mixer and manifests as a difference in the volume (time) over which the gradient changes (7). The difference results in an S-shaped transition or a

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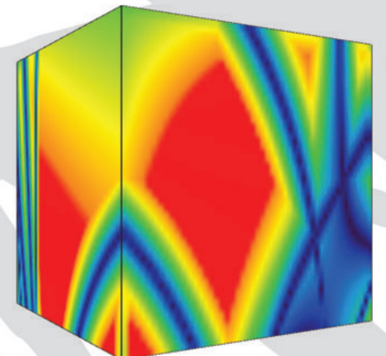

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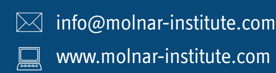

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Z-shaped transition depending on the wash-out volume. Upon determination of the wash-out volume ratio between the instruments of interests, the method was modified to account for the dwell volume differences and the wash-out volume ratio. Using the gradient test mix to assess this method translation technique, it was found that the interinstrument retention time deviation was greater than when using dwell volume alone (Figure 6). The wash-out volume ratio could be modified to align the chromatographic retention times on the two instruments, but this approach deviates from first principles of method translation and should be considered method development.

Based on the comparison of these method translation and adjustment techniques, the modification of the method based on dwell-volume differences has proven to be useful for the separations evaluated. To allow for dwell-volume adjustments in the method, an initial isocratic hold should be included in the method that can be adjusted to accommodate instruments with differing dwell volumes. Additionally, the dwell volume of the method development system should be included in the method to allow for accurate adjustments. This method does not account for thermal differences, however, and they must be considered during the method validation studies if they are expected to affect the chromatographic separation.

Regulatory Considerations

Although method translation and adjustment can help ensure consistency of the chromatographic separation across differing UHPLC configurations, the method and modifications still need to comply with regulatory guidelines. According to *United States Pharmacopeia* (*USP*) <621>, the method can be adjusted for dwell volume and column temperature (±10 °C) (18). Although these modifications are acceptable, the adjustments should still be covered by the method validation robustness studies to allow for translation of the method during method implementation. Additionally, a method equivalency focus group as part of the IQ Consortium suggested that method modifications, including dwell volume, should be included in method validation robustness studies to allow for adjustments between instruments (19).

Additional method translation techniques have been developed by instrument manufacturers to compensate for instrumental differences and are promoted as not requiring additional robustness studies, but to date the acceptability by regulatory agencies is unknown (20–22). Although these translation techniques are based on first principles, their use without method validation robustness study coverage has yet to be accepted by the regulatory agencies. This lack of acceptance is not specifically because of the agencies disagreeing about the principles, but because of the hesitation of companies to submit methods that use this technology.

To stay within the regulatory guidelines and also allow method flexibility, the method should be evaluated over the entire range of expected dwell volumes. Within the method robustness studies, physically alter the dwell volume of the system and adjust the initial method isocratic hold time to maintain a constant isocratic step. Also, if a method is sensitive to the column temperature, assess the method at varying temperatures and include a temperature range within the method to allow for modifications to maintain the chromatographic performance specified within your system suitability criteria. Although most often the extracolumn dispersion should not vary by more than is tolerated by a typical UHPLC gradient separation, knowing the different system configurations that the method will be run on will allow the method developer to assess the potential impact.

Conclusions

The variation in dwell volume, extracolumn dispersion, and mixing efficiency were evaluated for the Waters H-Class and Agilent 1290 instruments present in our laboratories. For these method conditions, the greatest interinstrument variation was found to be caused by the dwell volume. Variations in ECD and mixing efficiency were observed, but at the expected level of variation neither is expected to significantly affect gradient separations. A dwell-volume variation of less than 30 µL, a postcolumn ECD variation less than 4 µL, and a precolumn ECD variation less than 10 µL are not expected to require method translation or adjustment for the majority of gradient separations. Because the impact of these instrumental variations is dependent on the specific separation, these generalizations should be confirmed for the specific method.

In this study, the dwell volume and wash-out volume ratio method translation and adjustment methods were evaluated. The method found to be most useful for the methods evaluated was adjustment of dwell volume. To accommodate the need to adjust dwell volume, adding an initial isocratic hold in the method can ease the method translation–adjustment process. A further alternative is to develop methods on a column with a more modest column volume (that is, increased column internal diameter) to reduce the impact from instrumental parameters. Because of varying method complexity and analyte sensitivity to instrumental parameters, the specific method required for adjustment is expected to vary. To allow these types of method adjustments, the expected variations should be evaluated during the method validation robustness studies. The advantages in increased efficiency and shorter analysis time for UHPLC methods with columns of small volume and particle size are evident, but the time benefits of this technology have not been fully realized in a regulated environment because of the additional time required to assess method robustness. This missed opportunity points to the balance required between efficiency gains and increased impact from instrumental variations when using smaller column volumes.

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Sealing It With **Septa**

John V. Hinshaw, GC Connections Editor

While gas chromatographers may take their septa for granted, in fact these small and seemingly unremarkable polymer disks keep air out of the carrier-gas stream when used in an inlet and keep samples intact and uncontaminated when used in sample vials. Choosing the wrong septa can compromise method accuracy and repeatability as well as reduce column life in extreme cases. This instalment addresses septa for inlets and sample vials.

In gas chromatography (GC), septa form part of the critical juncture between internal passages and the external ambient atmosphere with its oxygen and water. Unlike other sealing components such as ferrules, o-rings, or inlet ring seals, septa are mechanically challenged by a syringe needle once — or in the case of autosampling more than once — with every injection. To add insult to these injuries, inlet septa are subjected to continuous high temperatures and vial septa are exposed to solvent vapours, either of which can destroy the integrity of an inappropriate septum. Septum technology and chemistry have undergone extensive development and improvement during the more than half-century since septa first met gas chromatography. A number of septum-less solutions are also available.

Septa for Inlets

Arguably the most stressed-out parts of a GC system, inlet septa are tasked with maintaining a leak-tight seal between the atmosphere and carrier gas while not introducing significant contaminants into the carrier-gas stream — all of this while heated to upwards of 250 °C and being repeatedly punctured by sharp needles. I've only otherwise encountered this situation at the acupuncturist's office, and it is an experience I'm not likely to repeat. While concentrating life-force energies may be good for us humans, in the case of septa it's all about the chemistry.

Excluding gas-injection valving, which is not addressed here, a number of GC inlet systems do not have septa. Some on-column inlets have used various nonseptum sealing arrangements. Capsule-based inlets do not have septa, either. They work by sealing a liquid or solid sample into a metallic or glass capsule, placing it inside the inlet, purging with carrier gas while the capsule heats up, and then puncturing the capsule to release the vaporized sample. I had thought of capsule inlets as out-of-date, but a recent patent (1) describes a new type of capsule inlet that uses a heat-sealed polymeric capsule. One type of septumless inlet accessory works with heated inlets and has two seals that are activated by passage of the syringe needle.

Classical cold on-column inlets may have a septum but generally it is not heated, although in such cases it is still possible for pieces of septum to be displaced into heated areas and act as a contaminant source. The predominant inlet type today remains the heated inlet, and it is usually a split–splitless type. Packed column inlets remain in use as well and experience many of the same problems with septa.

The dichotomy of a heated-inlet septum is that it must simultaneously withstand high temperatures while providing a gas-tight seal over hundreds or more injections. Failure to do so can cause septum bleed, may create active adsorptive sites inside the inlet, and, in severe cases, can compromise the split ratio because of carrier-gas leakage.

Septum Bleed

Most gas chromatographers have heard of or experienced *septum bleed* — the appearance of extra peaks or an offset baseline because of septum materials entering the carrier gas stream and column. These unwanted peaks can then lead to quantitative errors as well as the misidentification of target analytes. Volatilization of the lighter fractions of a septum's polymeric matrix and the deposition of septum particles in the inlet are the predominant sources of septum bleed.

Septa are composed of one or more layers of polymeric materials. Today, essentially all septa for heated inlets are made of various polysiloxane materials. The process of polymerization and cross-linking may leave behind some lower-molecular-weight prepolymeric molecules with fewer than 10 siloxane units in linear, cyclic, or branched configurations. Upon heating, the relatively volatile materials that reside on the surface of a septum will evaporate into the nearby space.

The septum does not get as hot as the bulk of an inlet. The septum nut acts as a heat sink that maintains the septum temperature between 75–100 °C below the inner inlet temperature. Septum temperatures vary a lot between different inlet systems, but typically for a 300 °C split–splitless inlet the septum will run at around 250 °C. Even so, this may be hot enough to cause detectable quantities of septum bleed to evaporate from the septum.

One of the primary functions of an inlet septum purge — the one for which it is named — is the removal of volatized contaminants from the septum area away from the active carrier-gas feed that leads to the column. The septum purge also removes any sample and solvent from injection that may enter the septum area because of inlet overloading. This secondary function prevents flash-back of sample during injection, especially in splitless mode.

Repeated inlet overloading with condensable materials may eventually deposit enough material in the cooler septum-purge exit tube, outside the inlet, to partially or completely block septum-purge flow. A primary symptom

of this situation is the gradual appearance of septum bleed peaks. In extreme cases, sample and solvent may be entrained in the carrier-gas supply line to the inlet and then reappear in subsequent injections as baseline bleed or as discrete peaks carried over from previous injections. The continued presence of sufficient septum-purge flow can be assured by monitoring the flow as part of routine maintenance. And of course, avoid injecting excessively large sample volumes that could overload the inlet.

Septum bleed may also stem from the mechanical breakdown of a septum, caused by abrasion of small septum pieces as the syringe needle passes through. Septum coring by the syringe needle is another less common source of septum pieces. Septum particles that migrate into the inlet liner will immediately experience a much greater degree of volatilization at the higher temperatures in the inlet's active vaporization area. The particles may also selectively absorb higher-boiling sample components and cause sample discrimination effects, where the fraction of sample entering the column is a function of the individual components' molecular weights. In some cases, the presence of septum pieces in the active sampling area may cause the partial decomposition of sensitive components and quantitative errors.

Septa with a fluoropolymer coating on the carrier-gas side may help alleviate septum bleed to some degree, but after a few injections the polymer coating will be compromised and the bulk septum material will be exposed to the carrier-gas flow.

Septum Leakage

A classic test for septum health and lifetime consists of sniffing the needle entry point of an inlet with a helium leak detector. This is a very sensitive check that is probably better performed at the low sensitivity setting of the leak detector. I once tried repeatedly puncturing a new septum with a syringe needle and then checking for the presence of helium. I was surprised to find that some helium leaked away for as much as 30 s after each injection, presumably because of a relatively slow-healing hole in the septum after each injection. Perhaps this effect was also related to the type of septum I used. I do recall that it was thought to be a high-quality silicone septum. After 10 or 20 tests with no differing results I gave up manually injecting and then later repeated the tests with an autosampler. The leakage was about the same, and the syringe needle itself in its standby position near the inlet nut seemed to be another momentary source of helium.

Leak-checking of a septum should be performed regularly, perhaps daily or weekly depending on the sample load. Wait a few minutes after injection to check for leaks. A small leak may not constitute an immediate problem, and it may not be practical to change a septum at the first appearance of any detectable leak. Some inlets' septa are easier and quicker to change than others, and consider the possibility of air incursion when swapping in a new septum. Always ensure the column oven is close to room temperature and the inlet is sufficiently cool. For gas chromatography–mass spectrometry (GC–MS) systems, it may be necessary to vent the vacuum system as well, to avoid drawing air in through the column.

The type of syringe tip and size of the syringe needle are also crucial for the best septum performance. Much has been written on this topic about needle tip bevel angles, blunt needles, conical needles, side-hole needles, and so on. The best choice is to go with the syringe and inlet manufacturer's recommendations for manual or autosampling injections. The best two styles seem to be the classical beveled or the conical blunt-tip styles in a 26-gauge size. However, these are guidelines with many exceptions, so follow the best available recommendations.

Prepunctured septa can yield increased life in some situations; again, follow the manufacturer's recommendations. Multilayer septa are available as well. These have a softer layer of material on the outside for a good external seal to the inlet surfaces, and in the middle have a more robust material that is less prone to leakage after multiple syringe injections.

Generally speaking, an autosampler will inflict less damage on septa than will manual injection. The autosampler's mechanism should hit the septum in a more consistent location and so can avoid multiple puncture points that could start to leak or to break down and pass particles into the inlet.

Septa for Sample Vials

Sample-vial septa have a different set of requirements than do inlet septa. Vial septa are not subject to high temperatures and are liable to be exposed to harsh solvents. Sample-vial septa must withstand multiple punctures during repeated syringe rinsing prior to injection, but there is no long-term requirement for a lasting seal. There are two remaining measures of vial septum performance: leakage and sample contamination.

Vial septa can be made of softer and better sealing materials than inlet septa. One of the crucial tricks for vial sealing is the quality of the mechanical aspects of the seal. A high performance crimper is highly recommended for consistent and reliable sealing. In a laboratory with any kind of normal to high sample load, the cost of a good crimper will be paid back in short order. Some laboratories prefer screw-top vials for ease of use and the possibility of cleaning and reusing the vial. This may be appropriate in some situations, but the crimped cap approach is ultimately more efficient because it avoids the necessity of ensuring that the vials and caps have been cleaned to the required degree.

At the same time, a softer vial septum may come with a different challenge: withstanding the solvent. Some solvents can leach septum polymers into the sample, with undesirable results similar to the effects of septum bleed. The addition of a fluoropolymer layer to the sample side of the septum largely eliminates this problem, but at the expense of making it more difficult to achieve a good seal. Here again, good quality vials, caps, and crimpers go a long way towards improved performance.

Finally, headspace vials present another challenge, that of slightly elevated temperatures and pressures. Sealing is also made more difficult in these vials by their larger diameters.

Overall, the best route for sample vial sealing is to follow the manufacturer's recommendations and to use the vials that are provided by the manufacturer of the autosampling system. This approach may be a bit more expensive, but in the long run it is well worth it.

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Know Your Sample: Size Matters

Douglas E. Raynie, Sample Preparation Perspectives Editor

The past couple instalments of "Sample Preparation Perspectives" have looked at current trends in the field. Another recent trend is dried blood spot analysis and other analysis methods using minute sample amounts. This month we take a quick look at the role of sample homogeneity and the determination of sample size. Microsampling approaches, including dried blood spots, are discussed.

Over the past several years, there has been a trend towards preparing increasingly smaller samples. In many cases, this approach was taken to demonstrate that extractions and sample handling procedures at the microscale and smaller is possible. With the current emphasis on bioanalytical and related technologies, even greater legitimacy is given to these approaches. Hence, the advent of dried-blood spot (DBS) analyses and other approaches. One approach to microsampling for bioanalyis is solid-phase microextraction (SPME), also referred to as bio-SPME, which is discussed here.

Sampling and Sample Heterogeneity

My thoughts as I heard of recent, somewhat controversial, developments in finger-prick sampling for blood tests were concern over the statistics of sample size and homogeneity. Most analysts are widely aware that the standard deviation of sampling and analysis increases with decreasing analyte concentration. Horwitz (1) evaluated interlaboratory validation studies and developed the "trumpet" shown in Figure 1. Although some bias is evident in every sampling protocol, when Meyer (2) presented the relationship between sampling and measurement uncertainty in 2002, she claimed that deviations from the Horwitz curve were caused by the sample matrix and the sample preparation procedure. Meyer provided the following advice: avoid all possible sources of contamination with trace analysis; use large volumes when possible since smaller volumes are difficult to handle and loss of sample material is less severe; mass-based measurements are often more reproducible than volumetric measurements; and use minimal sample handling steps with small-volume samples. Others have also demonstrated the relationship between sampling precision and sampling size. For example, Thiex and colleagues (3) reported the expected relative standard deviation from laboratory subsampling as a function of maximum particle size within the sample. As presented in Table 1 and substantiated by the Horwitz relationship, one cannot simultaneously have good sampling precision and small samples.

Moving beyond sample homogeneity concerns, the Royal Society of Chemistry's Analytical Methods Committee explored representative sampling from an analytical and statistical viewpoint (4). They prefer the term "appropriate sampling" to "representative sampling". They made this distinction because of the survey statistics definition of *representative sample* as "a sample for which the observed values have the same distribution as that in the population", while the analytical definition states "a sample resulting from a sampling plan that can be expected to reflect adequately the properties of interest in the parent population". This concept of adequacy in the analytical definition implies an inherent sampling bias and recognizes that in many, especially regulatory, cases analytical results are compared with a limit value. This limit value is often a "fitness for purpose", which allows the use of analytical results to be used in decision making. Appropriateness of sampling can be improved by increasing the sample size or the number of samples.

Note that these relationships between sample size and heterogeneity are primarily derived from investigations of solid samples, including food and feeds. However, most microsampling applications are used in bioanalysis, especially those involving blood samples. For reasons of diffusion and turbulent flow, liquid samples can be assumed to be considerably more homogeneous than solid samples.

Note that these relationships between sample size and heterogeneity are primarily derived from investigations of solid samples, including food and feeds.

Overview of Microsampling in Bioanalysis, Including BioSPME

Along with analysis of DBS, paper-based and more-conventional microfluidic approaches are gaining popularity. Such procedures are simple, inexpensive, and easy to use. A balance of hydrophobic and hydrophilic treatments controls fluid movement in these devices, resulting in their claimed reliability. One significant advantage of these approaches is their applicability

Figure 1: The Horwitz "trumpet" displaying the inverse relationship between analyte concentration and relative standard deviation of sampling. (Adapted from reference 2.)

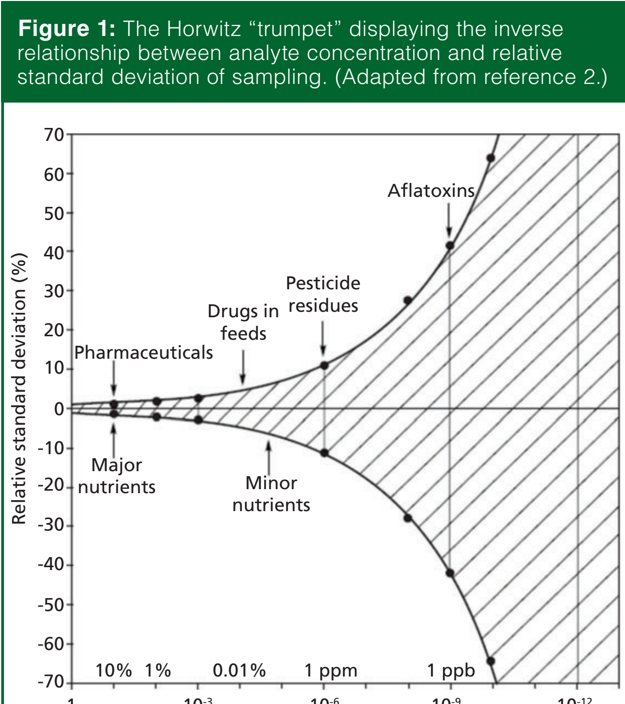

Table 1: Sample size required to obtain a desired sampling uncertainty (relative standard deviation) as a function of particle size (used with permission from reference 3).

Particle Size (mm)	15%	10%	5%	2%	1%
0.50	0.06 g	0.13 g	0.50 g	3.0 g	12.5 g
0.75	0.2 g	0.4 g	2.0 g	10.5 g	42 g
1.0	0.4 g	1.0 g	4.0 g	25 g	100 g
2.0	4.0 g	8.0 g	32 g	200 g	400 g
5.0	56 g	125 g	500 g	3130 g	12,500 g

outside of the laboratory, including nonclinical settings, though sample drying of blood spots can present a concern. Capillary microsampling allows collection of microlitre sample volumes along with subsequent steps such as separating plasma and serum. These approaches will be the subject of a future "Sample Preparation Perspectives" column.

Another sample preparation trend we've noticed is interest in SPME, especially since the lapse of patent protection of the initial products. In the case of conventional SPME, a stationary phase, usually a gas chromatography (GC)-type phase, is coated onto a fused-silica fibre encased in a syringe-needle device. The coated fibre is exposed to the sample by either immersion in a liquid sample or exposure to a vapour sample. The adsorbed sample is then desorbed either thermally in a GC inlet or via solvent rinsing into a liquid chromatograph.

Biocompatible SPME (bio-SPME) is a microsampling approach for bioanalysis based on SPME, but featuring some key differences. With bio-SPME, functionalized

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Figure 2: The kinetics of the bio-SPME process, demonstrating an asymptotic approach to quantitative equilibrium. (Courtesy of Supelco.)

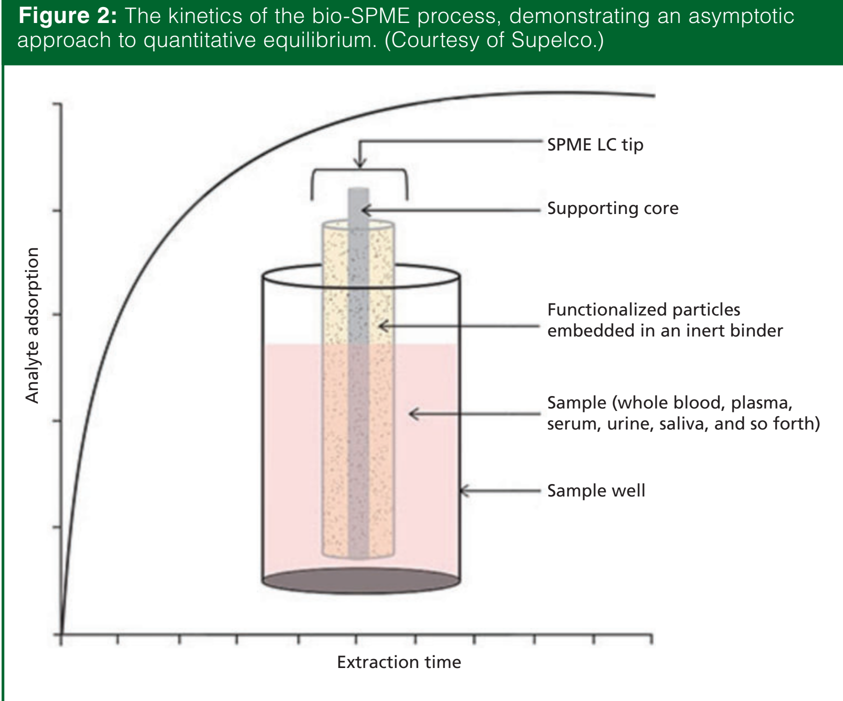
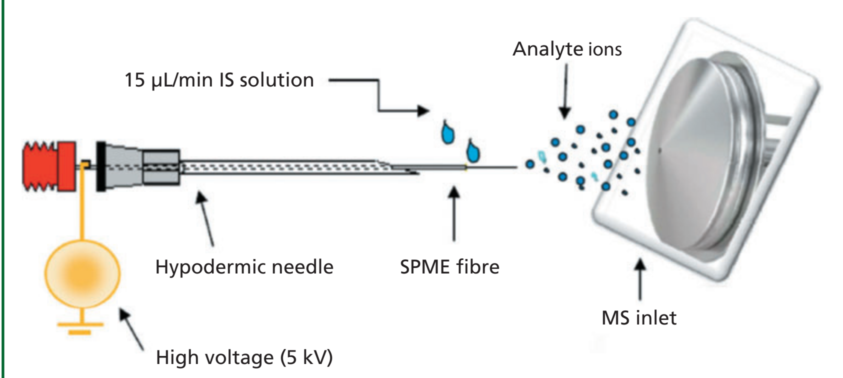


Figure 3: A schematic approach to coupling bio-SPME with mass spectrometry using a spray solvent and high voltage. (Adapted with permission from reference 5.)



silica particles are embedded in an inert binder that is coated or bonded onto metal fibres. The use of the binder minimizes interferences from biomacromolecules. Bio-SPME is available in hypodermic needle and pipette tip formats. Like conventional SPME, the approach is not exhaustive and relies on an equilibrium between the analyte in the biofluid and the fibre materials. Figure 2 displays the kinetics of bio-SPME sampling, which are similar to conventional SPME. Initially, a rapid adsorption of the analyte onto the functionalized silica is observed, followed by an asymptotic approach to the equilibrium amount of analyte isolated.

Two particular advantages of bio-SPME are of special interest. First, the device can be directly inserted into small animals for sampling at or near the point of interaction during physiological studies. This allows multiple analyses per animal, since the animal is not sacrificed, leading to more cost-effective studies and more reliable results since there are multiple analyses per animal. Relative standard deviations around 30% demonstrate the need to strongly consider the uncertainty considerations presented by Horwitz and discussed earlier. The second major advantage is the direct ionization of analytes on the bio-SPME fibres for mass spectrometry, as demonstrated in Figure 3 (5). This schematic shows the ionization occurring when the fibre and spray tip are sharp and a spray solvent carries the analyte into a high-voltage region to create an electric field between the bio-SPME device and the inlet to the mass spectrometer. Quantitative results are similar to other reports of bio-SPME and are 5–10× better than with DBS analysis. Spray solvent flow rates, positioning of the fibre, and other parameters are being optimized.

Biocompatible SPME (bio-SPME) is a microsampling approach for bioanalysis based on SPME, but featuring some key differences.

Conclusions

Microsampling for bioanalysis and other applications is gaining in popularity. One new technique in this area is the reapplication of the SPME approach, designed for biological applications. However, in all microsampling approaches, measurement uncertainty and sample homogeneity concerns must always be considered.

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HPLC Column Standardization in Pharmaceutical Development: A Case Study

Michael W. Dong, Perspectives in Modern HPLC Editor

Significant benefits can be obtained by standardizing high performance liquid chromatography (HPLC) columns in a pharmaceutical development laboratory. Here is a story of how one organization attempted to encourage its staff to develop HPLC methods using fewer column brands and dimensions to reduce waste and efforts in method transfers downstream.

A typical pharmaceutical company may use hundreds or even thousands of high performance liquid chromatography (HPLC) columns each year. This diversity in column dimensions, manufacturers, particle sizes, and bonded phases reflects the preferences of method developers in the organization. In turn, these preferences continue their downstream propagation as methods are transferred to quality control and global manufacturing laboratories (1,2). This freedom of choice may not be the best thing for a global organization because this proliferation of brands, phases, and column geometries adds a considerable amount of cost and wasted efforts for the organization.

With advances in new HPLC and ultrahigh-pressure liquid chromatography (UHPLC) column technologies such as high-purity silica, silica-hybrid particles, smaller and monodispersed particles, superficially porous particles (SPPs), and novel bonded-phase chemistries, there are numerous column choices for method developers (3–6). Nonetheless, selection of the best or most appropriate columns for an intended use does not always happen, because each method developer has his or her unique experience and preferences. Many researchers have their own preferred vendors and column phases from their previous experience. In addition, the column selection process for a method development project is often hurried, allowing no more than a few weeks for an analytical chemist to develop a reasonable stability-indicating method for a drug candidate, conduct forced degradation studies, and begin initial stability studies for that new candidate (1,2). Moreover, after the method has been validated and then submitted in a regulatory filing, the chosen column is subsequently used for product release testing and stability studies, and the corresponding method is eventually transferred to other facilities such as company manufacturing plants or to contract manufacturing organizations (CMOs).

A similar sequence of method development, validation, and transfer processes occurs for analytical methods applied for the quality assessments of starting materials and critical raw materials used in the synthetic process for the drug candidate, although generic broad-gradient purity methods are often sufficient for purity evaluation of these precursor materials (7,8). For these less-demanding assessment methods, there may not be a need for a specific column from a manufacturer, which can allow an organization to achieve a tremendous cost saving by using the same common generic method or set of methods between all projects within the organization (1).

When these scenarios are multiplied by the number of drug development projects, a pharmaceutical laboratory can easily have several hundred LC columns of different brands, bonded phases, particle sizes (d_p), lengths (L), inner diameters (d_c), and usage histories. This "column proliferation" often causes extra technical issues in method validation or transfer and results in an inventory of hundreds of "unique" or "orphan" columns in the facility. The following is a story of how one organization attempted to ameliorate column proliferation by advocating the use of fewer "standardized columns" in the department.

A Column Standardization Technical Focus Group

The organization in this case study was an analytical chemistry and quality control department of a medium-to-large pharmaceutical company that supported chemistry, manufacturing, and control (CMC) in its small-molecule drug discovery programme. It had a staff of more than 50 people, including ~30 laboratory personnel consisting of scientists and research associates working in small teams to support multiple early-phase projects. All laboratory personnel were required to work in the laboratory to develop new methods for raw materials, starting materials, intermediates, drug substances, and drug products to assess purity (both chemical and chiral), stability, and other critical quality attributes.

HPLC was the primary analytical technique, and the cost of HPLC

Table 1: Columns for stability-indicating assays and screening methods.

Bonded Phases	Dimension (mm × mm)	Particle Size (µm)	Support Type
C18 Phases			
C18	150 × 3.0	2.5, 3.0, 3.5	Silica, hybrid, TPP
C18	150 × 3.0	2.6, 2.7	SPP
Orthogonal Phases			
Polar-embedded, PFP, phenyl	150 × 3.0	3.0, 2.5	Silica, hybrid, TPP
Phenyl, phenyl-hexyl	150 × 3.0	2.7	SPP
Columns for IPC, Potency, and Screening Methods			
C18, polar-embedded	50 × 3.0	2.5	Silica, hybrid, TPP
C18, phenyl	50 × 3.0	2.7	SPP

columns was a major fraction of the consumables budget. The laboratory did not use a centralized HPLC column stocking programme, and each individual scientist procured his or her own columns.

Eventually, these columns (used and unused) would wind up in laboratory bench drawers, cabinets, or individuals' offices (see examples in Figure 1). While each individual had a secret stash of his favourite columns for active projects, hundreds of columns from completed and obsolete projects were scattered throughout the laboratory.

Nevertheless, when a new project was started, one never seemed to be able to find the "right" column amidst the myriad columns available. Such a scenario would often result in last-minute group emails to the entire department asking for a specific column from a particular manufacturer. This situation reminds me of the famous quote, "Water, water, every where, nor any drop to drink" (9).

This diversity of column usage, reflecting personal preferences within the group, was not necessarily a bad thing. However, it did get worse when these methods were validated and eventually transferred to CMOs to support production of the clinical trial materials. Since column availability could be a problem for foreign CMOs, it was often necessary to stockpile more columns for those CMOs. Column proliferation probably occurs in most pharmaceutical companies, except in this case a few individuals decided to form a technical focus group to evaluate the issue and propose changes.

Scope and Goals of the Column Standardization Technical Focus Group

The first thing our technical focus group did was to discuss the scope and goals. A consensus was reached to identify a set of primary columns for use in the development of stability-indicating methods and another set of secondary, shorter columns for potency determination, in-process control (IPC), and other screening methods. Initially, our focus for both column sets was on achiral reversed-phase HPLC columns. Columns with limited applications using other chromatographic modes such as supercritical fluid, normal phase, ion-exchange, hydrophilic interaction, and mixed-mode would not be in the standardization programme. Chiral columns, which did constitute a major fraction of the consumables column budget, would be considered later.

The group agreed that the main objective was not to stifle creativity, but rather to find sets of "best" columns for stability-indicating methods and generic IPC–potency methods via a consensus-building process. The recommendations would include column dimensions, particle size, support type (SPPs, totally porous particles [TPPs], hybrids), as well as specific manufacturers or brands. The benefits were expected to be increased productivity (less time and effort in method development and transfer) and cost savings by purchasing columns that could be used across different projects. Another benefit was increased awareness by those in our department of the latest trends and best practices in HPLC column technology.

We began by conducting a poll of current column preferences, to be followed by a discussion on optimum column dimensions, particle diameter, bonded phase, and brands. Our first deliverables were compiled lists of recommended columns for stability-indicating assays and for potency–IPC assays.

The poll results came back within a few weeks with contributions from at least 20 staff. Trends and observations were tallied. From the poll, our department appeared to have preferences for columns from four manufacturers:

- The first one for its strength in hybrid particles, particularly those with a positively charged surface and its new line of columns packed with sub-3-µm SPPs.
- The second one for its C18 columns known for excellent peak shape for basic pharmaceutical compounds and batch-to-batch reproducibility for quality control applications. This manufacturer also introduced a new set of bonded phases with unique selectivity.
- The third one for its second-generation hybrid and for its substantial product offerings in SPP with particle diameters ranging from sub-2 µm to 5 µm.
- The fourth one for its C18 columns with a long history of robustness and a line of innovative SPPs, including one compatible with high-pH mobile phases.

Not surprisingly, additional details from our poll indicated that 3-µm materials remained the top particle size choice, as well as an increasing preference for columns packed with sub-3-µm SPPs. The most popular inner diameter was the standard 4.6 mm, although many were shifting their preferences towards smaller 3.0-mm i.d. columns.

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Figure 1: Pictures showing column proliferation in an overstocked cabinet, a repository for columns used in automated screening systems, a laboratory bench drawer full of used columns, and a stash of brand new columns allocated to a specific project in a personal office.

Recommendations by the Group

After several meetings and considerable discussions, the technical focus group reached agreement on a list of technical recommendations and two column sets (shown in Table 1). These were presented at a departmental meeting to illicit more open feedback and discussion. Rationales for these recommendations are described in the next section.

Recommended Geometries:

- 150 mm × 3.0 mm columns packed with either 3- or sub-3-µm particles for stability-indicating methods.
- 50 mm × 3.0 mm columns packed with either 3- or sub-3-µm particles for potency, IPC, cleaning verification, and other screening methods.

Recommended Phases:

- A set of C18 and "orthogonal" bonded phases (phenyl, polar-embedded, pentafluorophenyl [PFP], and cyano [CN] phases) (6) for stability-indicating methods. This set would be used with the automated column–mobile phase screening system in the department. A similar set for potency, IPC, and screening methods consisting of mostly 50 mm × 3.0 mm columns packed with C18, phenyl, and pola-embedded phases.

Rationales for the Recommendations

Column Inner Diameter:

The 3.0-mm i.d. column was recommended over the standard 4.6-mm i.d. column for several reasons (10):

- The laboratory was equipped with both HPLC and UHPLC equipment (about 50:50) that worked well with 3.0-mm i.d. columns.
- The optimum flow rate for 3.0-mm i.d. columns packed with 3-µm materials is around 1 mL/min; in contrast, the optimum flow rate for a similar 4.6-mm i.d. column is around 2.0 mL/min with twice the solvent usage (10).
- Although 2.1-mm i.d. columns are commonly used with UHPLC systems, they are much less compatible with conventional HPLC systems from the standpoints of system dispersion and precision for small-volume injections (4).

Particle Size: The 3-µm and sub-3-µm particles were selected because they offered a good balance of efficiency and pressure requirements that were compatible with both HPLC and UHPLC equipment. Sub-2-µm particles would have significant advantages in efficiency and speed, but are less compatible with conventional HPLC equipment, especially for column lengths greater than 50 mm.

Column Length: Our group selected a standard column length of 150 mm for stability-indicating methods. A 150 mm × 3.0 mm column packed with 3- or sub-3-µm materials have column efficiencies of 20,000 to 30,000 plates (or gradient peak capacities of 200–400 within ~30 min) (11), which we considered a good match for ICH-compliant stability-indicating methods. Similarly, 50 mm × 3.0 mm columns would offer a good balance of speed and resolution for less-demanding generic screening methods (1). A 100 mm × 3.0 mm column would also be a viable choice with intermediate speed and efficiency, although the group decided to standardize on the longer 150-mm column instead.

Superficially Porous Particles: The rationales for selecting SPPs over TPPs have been well documented in the literature, because of their superior efficiency performance versus TPPs of the same particle diameter (with reduction of reduced plate heights by ~20–40%) (12,13). With more than 20 manufacturers offering columns packed with SPPs, these materials are quickly becoming

the preferred HPLC–UHPLC support for pharmaceutical analysis.

Bonded Phases: Not surprisingly, C18 remained the dominant bonded phase of choice by most of the staff because of its high hydrophobicity, retention, and batch-to-batch reproducibility (6). There was a strong preference in the department for a C18 bonded phase with a slight positively charged surface and another C18 phase on an SPP designed for high-pH mobile phases. Both bonded phases yielded excellent peak shape for highly basic analytes when used with low-ionic-strength mobile phases (for example, 0.05% formic acid) (1,14,15).

Several "orthogonal" bonded phases with different selectivity than C18 were also selected, notably those with polar-embedded phases (amide or carbamate polar groups) and phenyl phases for their enhanced selectivity for aromatic compounds with π-π interactions (6).

Implementation

It was easy to make recommendations, but it was significantly more challenging to implement these changes to realize any real impact in the department. Since the group had no authority to mandate these proposed changes, we instituted a friendly persuasion approach by emulating casinos that entice customers with free meals and lodging. We encouraged usage of these "preferred" columns by stocking them in the laboratory. Bulk orders were negotiated with manufacturers at deep discounts, and columns were stored in a controlled location so inventory could be maintained and made immediately available when needed.

Summary and Conclusion

Although it would be difficult to quantify the impact of the column standardization programme, our rational column selection strategy had the desired effect of reducing the numerous types of columns used across the projects in the department. The use of polling, open discussions, and consensus building allowed us to compile a list of "best" columns for pharmaceutical analysis in our laboratories. We hope that our story might encourage other laboratories to consider similar programmes to reduce column spending, minimize waste, and improve laboratory productivity.

Acknowledgements

The author would like to thank members of the technical focus group on column standardization for their efforts in developing recommendations based on departmental preferences, and in the implementation of the column stocking programme. Members were Charlotte Tsang, Dawen Kou, Bob Garcia, and Tania Ng of Genentech. The author would like to thank Tom Waeghe of Mac-Mod Analytical, Xiaoli Wang of Agilent, David Van Meter of Rottendorf Pharma, and Davy Guillarme and Szabolcs Fekete of the University of Geneva, for their editorial input and suggestions.

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www.gilson.com
Gilson, Middleton, Wisconsin, USA.

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www.thermoscientific.com/chromatography
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www.vici.com
VICI AG International, Schenkon, Switzerland.

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www.mn-net.com
Macherey-Nagel GmbH & Co. KG, Düren, Germany.

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www.postnova.com
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www.hichrom.co.uk
Hichrom Ltd, Theale, Reading, Berkshire, UK.

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MethodsNow, a CAS solution, is reportedly a single source to search and compare the largest collection of scientific methods. MethodsNow features step-by-step instructions for analytical and synthetic methods in areas like pharmacology, HPLC, food analysis, and more. According to the company, save time with easy access to method details from millions of disclosed procedures and quickly compare analytical methods in easy-to-read table formats.
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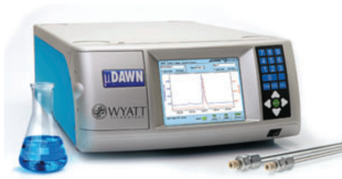
MALS detector



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

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The Precision Hydrogen Trace 250 generator from Peak Scientific is designed primarily for GC carrier gas use and can also be used for detectors requiring hydrogen fuel gas such as FID and FPD. The company reports that one generator is capable of supplying multiple GC instruments. The generator also comes with robust safety features as standard.
www.peakscientific.com
Peak Scientific, Inchinnan, Scotland, UK.

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www.waters.com
Waters Corporation, Milford, Massachusetts, USA.

Antibody Drug Conjugate (ADC) Analysis with SEC–MALS

Wyatt Technology Corporation

There has been a significant resurgence in the development of antibody-drug conjugates (ADC) as target-directed therapeutic agents for cancer treatment. Among the factors critical to effective ADC design is the Drug Antibody Ratio (DAR). The DAR describes the degree of drug addition that directly impacts both potency and potential toxicity of the therapeutic, and can have significant effects on properties such as stability and aggregation. Determination of DAR is, therefore, of critical importance in the development of novel ADC therapeutics.

DAR is typically assessed by mass spectrometry (MALDI–TOF or ESI–MS) or UV spectroscopy. Calculations based on UV absorption are often complicated by similarities in extinction coefficients of the antibody and small molecule. Mass spectrometry, though a powerful tool for M_w determination, depends on uniform ionization and recovery between compounds — which is not always the case for ADCs.

Here we present a method for DAR determination based on size-exclusion chromatography combined with multi-angle light scattering (SEC–MALS) in conjunction with UV absorption and differential refractive index detection. Figure 1 shows UV traces for two model ADCs; molecular weights of the entire ADC complexes are determined directly from light scattering data.

Component analysis is automated within the ASTRA 6 software package by using the differential refractive index increments (dn/dc) and extinction coefficients, which are empirically determined for each species or mined from the literature, to calculate the molar mass of the entire complex as well as for each component of the complex.

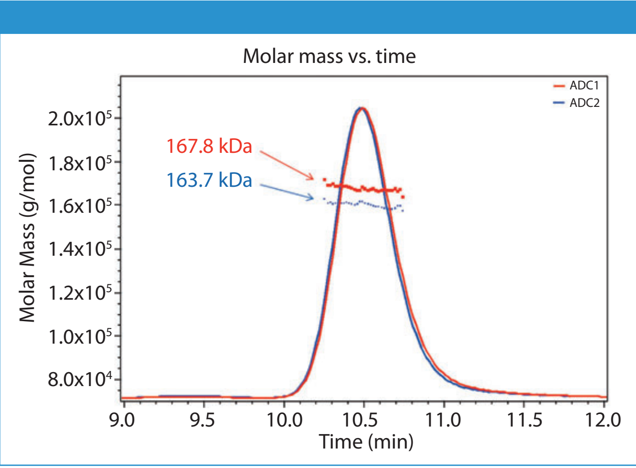
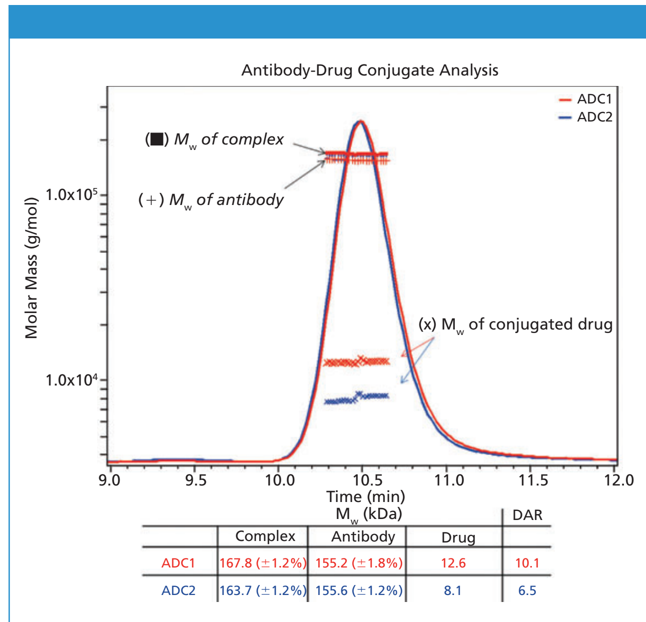


Figure 1: Molar masses for two distinct ADC formulations are determined using SEC–MALS analysis.



	Mw (kDa)			DAR
	Complex	Antibody	Drug	
ADC1	167.8 (±1.2%)	155.2 (±1.8%)	12.6	10.1
ADC2	163.7 (±1.2%)	155.6 (±1.2%)	8.1	6.5

Figure 2: Molar masses for the antibody and total appended drug are calculated in the ASTRA software package based on prior knowledge of each component's extinction coefficent and dn/dc, allowing determination of DAR based on a nominal Mw of 1250 Da for an individual drug.

In this example an antibody has been alkylated with a compound having a nominal molecular weight of 1250 Da (Figure 2). Molar masses of the antibody fractions are similar, which indicates that the overall differences between the two formulations reflect distinct average DARs that are consistent with values obtained by orthogonal techniques. Note that the molar mass traces for the conjugated moiety represent the total amount of attached pendant groups; the horizontal trends indicate that modification is uniform throughout the population eluting in that peak.

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