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Cold injections that beat the heat

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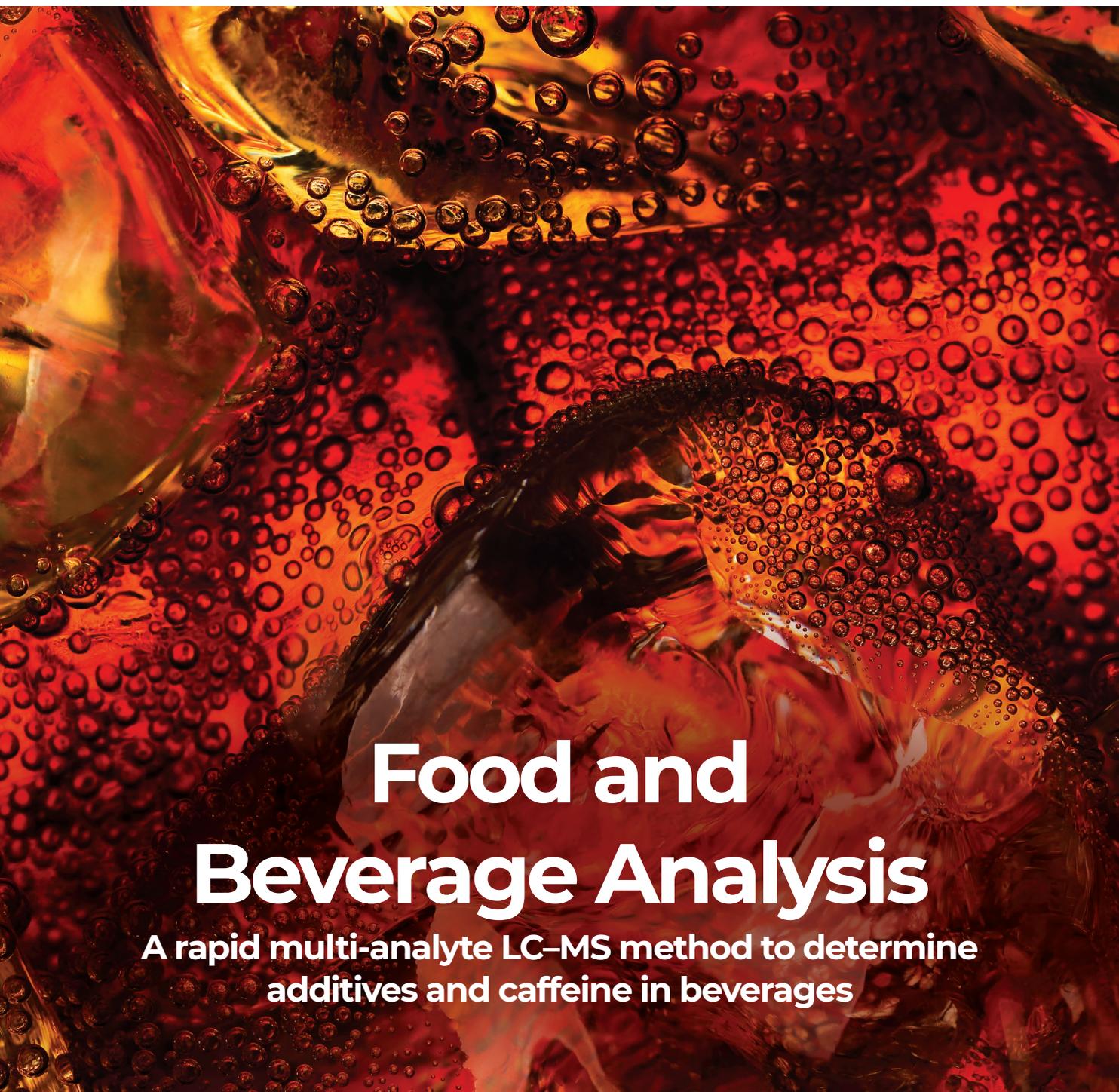
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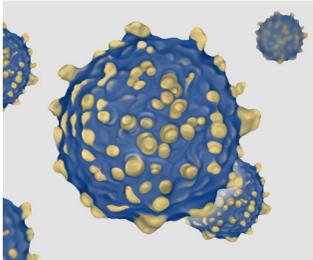
A rapid multi-analyte LC-MS method to determine
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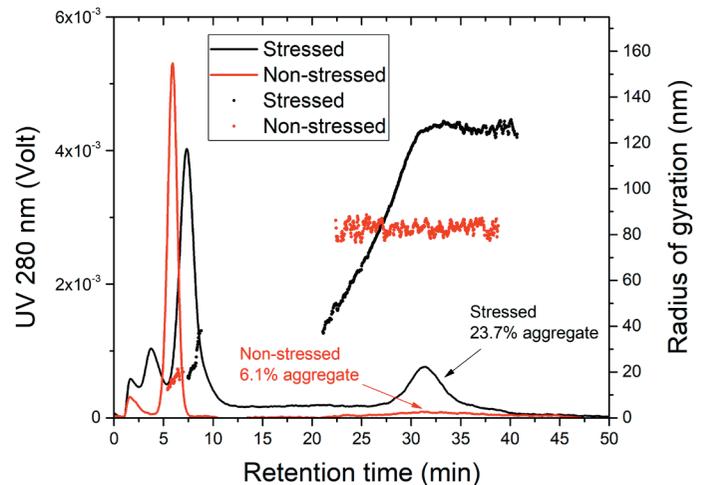


POSTNOVA



Adeno-associated viruses (AAVs) are increasingly used for gene therapy due to their versatility and safety. One of the biggest concerns for manufacturing a uniform AAV suspension is the presence of viral aggregates, which can create problems with transduction efficiency, biodistribution, and immunogenicity. These large AAV aggregates are challenging to separate and characterize by traditional column-based chromatography techniques such as size exclusion chromatography (SEC).

Asymmetrical Flow Field-Flow Fractionation with Multi Angle Light Scattering (AF4-MALS) can separate and size large AAV aggregates, and discern a difference in aggregate concentration due to the stressing protocol. Some or all of the large aggregates would be filtered out by SEC, resulting in incorrect determination of the aggregate content or the false conclusion that no aggregates are present.



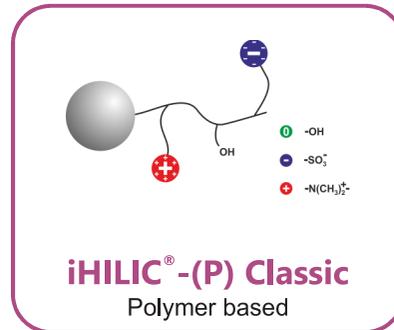
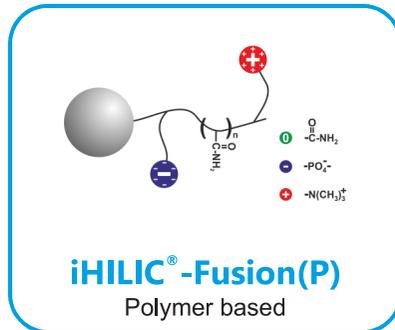
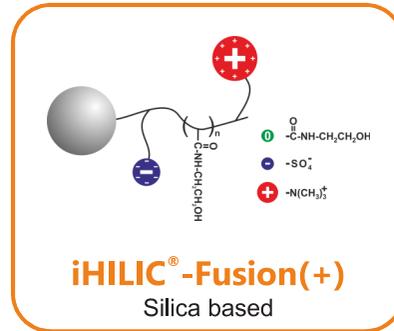
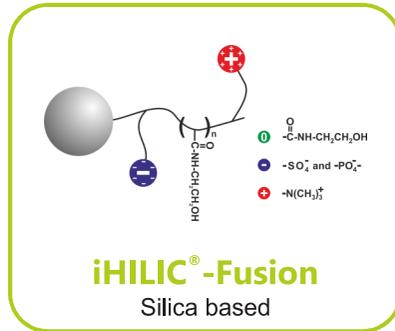
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COVER STORY**PEER REVIEW**

327 **A Simple LC–MS Multi-Analyte Method to Determine Food Additives and Caffeine in Beverages**

Ales Krmela, Aliaksandra Kharoshka, Vera Schulzova, Jana Pulkrabova, and Jana Hajslova

This article describes a simple multi-analyte UHPLC–MS method for the simultaneous determination of 18 synthetic food additives and caffeine in soft and energy drinks, and various alcoholic beverages.

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Alasdair Matheson

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Cory E. Muraco, Hillel K. Brandes, and David S. Bell

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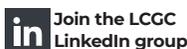
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THE LCGC BLOG

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The nature of surface silanol groups is very important to determine the selectivity of a stationary phase. This article illustrates what analysts should be looking at on the silanol surface to assess the separation mechanism.

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A Simple LC–MS Multi-Analyte Method to Determine Food Additives and Caffeine in Beverages

Ales Krmela, Aliaksandra Kharoshka, Vera Schulzova, Jana Pulkrabova, and Jana Hajslova, Department of Food Analysis and Nutrition, University of Chemistry and Technology, Prague, Czech Republic

A simple LC–MS method has been developed and validated for the simultaneous determination of 18 synthetic food additives and caffeine in soft and energy drinks, and in various alcoholic beverages. Nine food colours (tartrazine, sunset yellow FCF, azorubine, ponceau 4R, allura red AC, patent blue V, brilliant blue FCF, green S, brilliant black BN), two preservatives (sorbic and benzoic acid) and seven sweeteners (acesulfame K, aspartame, cyclamic acid, saccharin, sucralose, neohesperidin DC, neotame) were targeted food additives. The method employs reversed-phase ultra-high performance liquid chromatography (UHPLC) for analyte separation and a single quadrupole mass spectrometer for their detection. The limits of quantification were low enough to enable a reliable control of maximum limits set for some additives (Regulation [EC] No. 1333/2008). The method was applied for analysis of a wide range of samples collected at a typical supermarket: 14 soft drinks, 19 energy drinks, and 43 alcoholic beverages.

KEY POINTS

- Reversed-phase chromatography coupled with simple quadrupole MS can be used to analyse multiple food additives.
- 18 synthetic food additives representing various preservatives, sweeteners and food colours are involved in the method.
- The performance parameters of the method validated for soft and energy drinks/alcoholic beverages allow effective control of legislative requirements.

Food additives involve a wide group of compounds that differ in their physico-chemical properties. According to their function, they are classified as food colours, preservatives, sweeteners, and antioxidants. The use of food additives is regulated by Regulation (EC) No 1333/2008 of European Parliament and of the Council on food additives, which sets maximum limits for various commodities. To control these regulatory requirements, reliable analytical methods are needed (1,2).

High performance liquid chromatography (HPLC) using reversed-phase columns and neutral or acidic mobile phases are the most common systems for food additive separation. Conventional detectors like ultraviolet-visible (UV–vis) spectrophotometers are often used for the detection of various food additive groups such as colours, preservatives, sweeteners and/or caffeine (3–8). Nevertheless, in some recent studies, mass spectrometry (MS) was preferred for its versatility and better selectivity, and both simple and tandem mass spectrometry were employed (9–13). Although studies introducing multi-detection methods enabling simultaneous analysis of sweeteners, preservatives, and caffeine (6,7), or colours, preservatives, and caffeine (8), were published in the past, LC–MS-based methods suitable for simultaneous determination of all the groups mentioned above have not yet been published.

This study presents a multi-detection method employing ultra-high performance liquid chromatography (UHPLC) coupled to a simple quadrupole mass spectrometer for the simultaneous determination of several groups of food additives—nine food colours, seven sweeteners, two preservatives, and caffeine in a wide range of beverages.

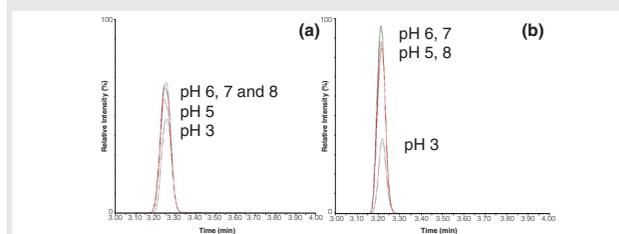
Materials and Methods

Chemicals: Standards of tartrazine, sunset yellow FCF, azorubine, ponceau 4R, allura red AC, patent blue V calcium salt, brilliant blue FCF, green S, brilliant black BN, sorbic acid, benzoic acid, sodium cyclamate, sodium saccharin, sucralose, neohesperidin dihydrochalcone, and neotame were purchased from Sigma Aldrich (Germany). Anhydrous caffeine and acesulfame K were purchased from Fluka (Germany), aspartame from Supelco (USA). HPLC-grade methanol was obtained from Sigma Aldrich (Germany). Analytical grade ammonium acetate was purchased from Sigma Aldrich (Germany). Water was purified using a Milli-Q Ultrapure water purification system from Millipore (USA).

Samples: In total, 76 samples of beverages were sampled, including 14 soft drinks (including 2 cola type sodas), 19 energy drinks (including five so-called "sugar-free" energy drinks), and 43 alcoholic beverages consisting of 23 liquors, 14 spirits and 6 ciders obtained on the Czech market.

Standard solutions preparation: Stock solutions of 2000 mg/L of each analyte were prepared by dissolution of 10 mg of standard in 5 mL of methanol–water (50:50, v/v) mixture. A standard mixture of 100 mg/L in the same solvent composition was prepared. Calibration solutions in the range from 0.1 to 10 mg/L were also prepared. For the analysis of soft and energy drinks, calibration solutions were diluted with

FIGURE 1: Comparison of pH influence on response of (a) brilliant blue FCF and (b) azorubine, 5 mg/L standard aqueous solutions with pH values of 3, 5, 6, 7, and 8.



deionized water. For alcoholic beverage analysis, calibration solutions in methanol–water (25:75, v/v) were prepared.

Samples preparation: In the case of soft and energy drinks, 40 mL of sample were placed in 100 mL beaker and degassed using the ultrasonic bath for 10 mins. The pH value of the degassed sample was then adjusted to 6 using 1% aqueous solution of ammonium hydroxide. The sample was then transferred to a 50 mL volumetric flask and the volume was adjusted by deionized water. Prior to LC–MS analysis, the sample was filtered through a 0.22- μ m syringe filter. If necessary, samples were further diluted with deionized water.

Analyzed alcoholic beverage samples differed in alcohol content, so their composition was adjusted to the level of alcohol–

TABLE 1: Overview of separation and detection parameters for targeted food additives

Compound	Summary formula	Retention time (min)	Ion type	Ionization mode	<i>m/z</i> measured
Tartrazine (E 102)	C ₁₆ H ₉ Na ₃ O ₉ S ₂	0.88	[M - 3Na + 4H] ⁺	ESI ⁺	469.0
Acesulfam K (E 950)	C ₄ H ₄ KNO ₄ S	1.12	[M - K] ⁻	ESI ⁻	162.0
Benzoic acid (E 210)	C ₇ H ₆ O ₂	1.46	[M - H] ⁻	ESI ⁻	121.0
Saccharin (E 954)	C ₇ H ₅ NO ₃ S	1.79	[M - H] ⁻	ESI ⁻	182.0
Sorbic acid (E 200)	C ₆ H ₈ O ₂	1.95	[M - H] ⁻	ESI ⁻	111.1
Ponceau 4R (E 124)	C ₂₀ H ₁₁ N ₂ Na ₃ O ₁₀ S ₃	2.21	[M - 3Na + 2H] ⁻	ESI ⁻	537.0
Brilliant black BN (E 151)	C ₂₈ H ₁₇ N ₅ Na ₄ O ₁₄ S ₄	2.32	[M - 4Na + 3H] ⁻	ESI ⁻	778.0
Cyclamic acid (E 952)	C ₆ H ₁₃ NO ₃ S	2.40	[M - H] ⁻	ESI ⁻	178.1
Sunset yellow FCF (E 110)	C ₁₆ H ₁₀ N ₂ Na ₂ O ₇ S ₂	2.46	[M - 2Na + H] ⁻	ESI ⁻	407.0
Caffeine	C ₈ H ₁₀ N ₄ O ₂	2.68	[M + H] ⁺	ESI ⁺	195.1
Allura red AC (E 129)	C ₁₈ H ₁₄ N ₂ Na ₂ O ₆ S ₂	2.73	[M - 2Na + 3H] ⁺	ESI ⁺	453.0
Sucralose (E 955)	C ₁₂ H ₁₉ Cl ₃ O ₈	2.83	[M - H] ⁻	ESI ⁻	395.0
Green S (E 142)	C ₂₇ H ₂₅ N ₂ NaO ₇ S ₂	2.96	[M - Na + 2H] ⁺	ESI ⁺	555.1
Aspartame (E 951)	C ₁₄ H ₁₈ N ₂ O ₅	3.03	[M + H] ⁺	ESI ⁺	295.1
Azorubine (E 122)	C ₂₀ H ₁₂ N ₂ Na ₂ O ₇ S ₂	3.24	[M - 2Na + H] ⁻	ESI ⁻	457.0
Brilliant blue FCF (E 133)	C ₃₇ H ₃₄ N ₂ Na ₂ O ₉ S ₃	3.24	[M - 2Na + 3H] ⁺	ESI ⁺	749.2
Neohesperidine dihydrochalcone (E 959)	C ₂₈ H ₃₆ O ₁₅	3.49	[M - H] ⁻	ESI ⁻	611.2
Patent blue V calcium salt (E 131)	C ₂₇ H ₃₁ N ₂ O ₇ S ₂ Ca _{1/2}	3.71	[M - 1/2Ca - Na + 2H] ⁺	ESI ⁺	561.2
Neotame (E 961)	C ₂₀ H ₃₀ N ₂ O ₅	4.19	[M + H] ⁺	ESI ⁺	379.2



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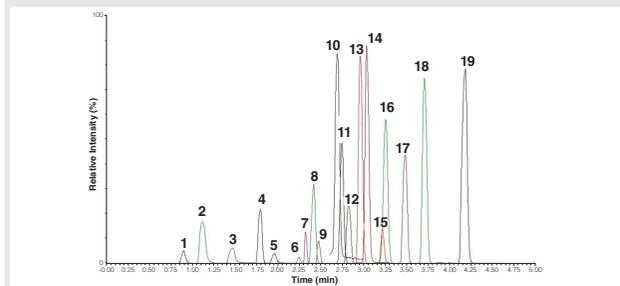
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FIGURE 2: Chromatogram of standard solution in distilled water (pH 6): 1) Tartrazine, 10 mg/L; 2) Acesulfame K, 10 mg/L; 3) Benzoic acid, 10 mg/L; 4) Saccharine, 10 mg/L; 5) Sorbic acid, 10 mg/L; 6) Ponceau 4R, 10 mg/L; 7) Brilliant black BN, 10 mg/L; 8) Cyclamic acid, 10 mg/L; 9) Sunset yellow FCF, 10 mg/L; 10) Caffeine, 0.5 mg/L; 11) Allura red AC, 10 mg/L; 12) Sucralose, 10 mg/L; 13) Green S, 5 mg/L; 14) Aspartame, 1 mg/L; 15) Azorubine, 10 mg/L; 16) Brilliant blue FCF, 10 mg/L; 17) Neohesperidin dihydrochalcone, 10 mg/L; 18) Patent blue V, 1 mg/L; 19) Reotame, 0.3 mg/L).



water (25:75, v/v) by dilution using methanol and deionized water. After dilution, the pH was adjusted to the value of 6.

Analytes separation: A Waters Acquity UPLC iClass system (Waters, USA) with a BEH C18 analytical column (2.1 mm × 100 mm, 1.7- μ m, Waters, USA) was used for analytes separation. The

mobile phase system consisted of 5 mM ammonium acetate aqueous solution (A) and methanol (B). The linear gradient elution was programmed as follows: 0–0.5 min, 2% B; 0.5–1.4 min, 2–15% B; 1.4–2 min, 12–30% B; 2–3 min, 30–60% B; 3–4 min, 60–70% B; 4–5 min, 70–98% B; 5–8 min, 98% B; 8–10 min, 2% B. Mobile phase flow rate was set at 0.4 mL/min, column temperature was 60 °C. The injection volume was 3 μ L.

Analytes detection: A simple quadrupole MS system (QDa, Waters, USA) was used for the detection of target analytes. Electrospray ionization (ESI) was used, while this MS system was operated in selected ion recording (SIR) mode with ion polarity switching, allowing the detection in both positive and negative ionization mode within a single run. Nitrogen gas was used as both desolvation and cone gas, and the probe temperature was set at 600 °C. Capillary voltage was set to 0.8 kV for both positive and negative ionization mode. Cone voltage was set at 10 V. Detector gain was set to 1. Acquisition and data processing were carried out using Masslynx v.4.1 software (Waters, USA). Monitored *m/z* values of target analytes together with retention times under experimental conditions are summarized in Table 1.

Validation: The Method was validated by the analysis (six replicates) of soft drink 10 (lemon soda, 9.5 % sugar content) to which targeted analytes were added at levels of 1 or 5 mg/L

TABLE 2: Method validation parameters

Compound	Addition level (mg/L)	Linear range (mg/L)	LOQ (mg/L)	Recovery (%)	RSD (%)
Tartrazine (E 102)	5	0.5–10	0.625	87.0	2.0
Sunset yellow FCF (E 110)	5	1–10	1.250	99.8	4.4
Azorubine (E 122)	5	1–10	3.750	103.2	2.6
Ponceau 4R (E 124)	5	3–10	3.750	96.0	3.1
Allura red AC (E 129)	1	0.1–10	0.125	92.0	2.5
Patent blue V (E 131)	1	0.1–10	0.125	90.7	2.8
Brilliant blue FCF (E 133)	1	0.3–10	0.375	93.8	4.5
Green S (E 142)	1	0.1–10	0.125	87.8	1.7
Brilliant black BN (E 151)	5	3–10	3.750	96.9	3.5
Sorbic acid (E 200)	5	3–100	3.750	101.0	2.8
Benzoic acid (E 210)	5	1–100	1.250	102.6	0.8
Acesulfame K (E 950)	1	0.1–10	0.125	100.7	0.4
Aspartame (E 951)	1	0.1–10	0.125	83.8	3.7
Cyclamic acid (E 952)	1	0.5–10	0.625	101.3	1.1
Saccharin (E 954)	1	0.3–10	0.375	103.0	0.7
Sucralose (E 955)	1	0.5–10	0.625	106.2	2.7
Neohesperidine dihydrochalcone (E 959)	1	0.3–10	0.125	85.7	1.9
Neotame (E 961)	1	0.1–10	0.125	96.8	2.5
Caffeine	1	0.1–10	0.125	96.8	2.5

depending on the limit of quantification (LOQs) which, together with recoveries and repeatabilities are summarized in Table 2.

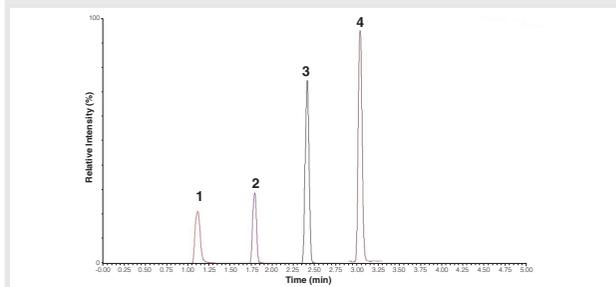
Results and Discussion

As the set of target analytes includes compounds largely differing in their polarity and presence of ionisable groups, universal, trifunctionally bonded (ethylene bridged) BEH C-18 (100 × 2.1 mm; 1.7 μm) ultra-high performance liquid chromatography column was employed for separation. Poor peak shapes and low responses were obtained for preservatives when using ammonium formate as a mobile phase modifier. Also, low retention times for tartrazine and acesulfame K were obtained when solvent of lower polarity than methanol was used as a mobile phase B. A wide range of mobile phases were tested, and the best results regarding peak shapes (low width, minimal tailing) and short separation time (4.5 min), were obtained when using a 5-mM aqueous solution of ammonium acetate and methanol gradient.

In the next step of the method development, detectability of target analytes was tested. Interestingly, lower peak intensities were observed for some food colours such as brilliant blue FCF, and azorubine, when the standard was injected in acidic solution of pH 3 (most of the tested drinks had similar pH). The peak intensity increased with growing pH until the range of 6–8, see

FIGURE 3: Chromatogram of Soft drink 3 sample:

1) Acesulfame K, 100 × diluted; 2) Saccharine, 10 × diluted; 3) Cyclamic acid, 100 × diluted; 4) Aspartame, 10 × diluted).



Figures 1(a) and 1(b), illustrating the influence of this parameter for brilliant blue FCF and azorubine, respectively. It is assumed that more ionic forms of these analytes were present in the samples, as the pH value of 3 is close to their pKa. To optimize analysis conditions, a pH value of 6 was chosen as suitable for the preparation of both calibration solutions and samples.

In the case of alcoholic beverages, the possible impact of alcoholic strength of injected sample on analyte responses was investigated. Compared to an aqueous solution, equal or higher responses were obtained when using a mixture of

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TABLE 3: Mini survey of food additives in soft and energy drinks

Sample	Label-declared food additives	pH	Preservatives (mg/L)		Sweeteners (mg/L)					Caffeine (mg/L)
			Sorbic acid	Benzoic acid	Acesulfame K	Aspartame	Cyclamic acid	Saccharin	Sucralose	
			(E 200)	(E 210)	(E 950)	(E 951)	(E 952)	(E 954)	(E 955)	
Maximum limit set by Regulation (EC) No 1333/2008			300*	150	350	600	250	80**	300**	
Fruit Soda 1	E 950, E 951, E 952, E 954	3.2			33.7	43.5	266.2	49.1		
Fruit Soda 2	E 950, E 951, E 952, E 954	3.2			21.9	44.2	250.8	45.8		
Fruit Soda 3	E 950, E 951, E 952, E 954	3.2			32.3	44.1	254.8	45.9		
Fruit Soda 4	E 202, E 211	3.4	131.5	127.2						
Fruit Soda 5	E 211, E 950, E 951, E 954	3.2		110.7	84.5	72.2		45.3		
Cola Type 1	E 950, E 951, E 952	2.8			88.6	93.6	193.6			27.1
Cola Type 2		2.9								358.2
Energy Drink 1		3.0								328.4
Energy Drink 2		2.9								328.8
Energy Drink 3	E 202, E 211, E 950, E 951	3.1	139.9	84.8	30.9	28.5				328.9
Energy Drink 4	E 202, E 211	3.0	132.2	84.1						309.1
Energy Drink 5	E 202, E 211	3.0	143.1	83.7						332.4
Energy Drink 6	E 202, E 211	2.9	133.3	80.1						321.9
Energy Drink 7	E 202	3.2	261.6							303.5
Energy Drink 8	E 202, E 211	3.0	213.0	126.8						311.0
Energy Drink 9	E 202	2.8	7.6							326.2
Energy Drink 10		3.4								296.8
Energy Drink 11		3.5								314.5
Energy Drink 12		3.2								314.0

Continued on Page 333

Continued from Page 332

TABLE 3: Mini survey of food additives in soft and energy drinks (continued)

Sample	Label-declared food additives	pH	Preservatives (mg/L)		Sweeteners (mg/L)					Caffeine (mg/L)
			Sorbic acid	Benzoic acid	Acesulfame K	Aspartame	Cyclamic acid	Saccharin	Sucralose	
			(E 200)	(E 210)	(E 950)	(E 951)	(E 952)	(E 954)	(E 955)	
Energy Drink 13	E 202	3.2	8.7							340.1
Energy Drink 14		3.0								296.8
Energy Drink: Sugar-free 1	E 202, E 211, E 950, E 951	2.9	138.3	82.3	126.8	79.9				286.9
Energy Drink: Sugar-free 2	E 202, E 211, E 950, E 951, E 955	3.1	223.4	123.6	206.2				155.0	288.6

Continued on Page 334

ethanol and water as a solvent for standard preparation. The most distinct difference was observed in the case of green S, the signal of which, when injected in 25% (v/v) ethanol was higher by 48.4%; after this point, the signal intensity slowly dropped. To standardize sample conditions, dilution of ethanol content 25% (v/v) with distilled water and ethanol, and pH adjustment to the value of 6 was always performed prior to injection.

The matrix effects when analysing real life samples were relatively low, recoveries of targeted analytes ranged from 83.8 to 103.0%. Repeatability of injections, expressed as relative standard deviation, ranged from 0.4 to 4.5%. Limits of quantification (LOQs) ranged from 0.1 to 3.7 mg/L, these values are two to three orders of magnitude lower than limits set by Regulation (EC) No 1333/2008. All validation parameters are summarized in Table 2. The example of chromatographic separation is demonstrated in Figure 2, on standard mixture in distilled water (pH 6).

Beverage samples analysis: The method developed was applied to the analysis of 76 samples of beverages collected at the Czech market. The sample set included 14 soft drinks, 19 energy drinks and 43 alcoholic beverages. In the case of soft drinks, up to four different sweeteners were detected. Samples "Fruit Soda" 1, 2, and 3 (see Table 3) produced by the same company, differed in flavouring component but contained almost the same concentrations of sweeteners; the ratio probably best simulates the taste profile of natural sugar. Sweeteners were also detected in Fruit Soda 5 and Cola Type Soda 1, which also contained saccharides. The presence of preservatives was confirmed in Fruit Soda samples 4 and 5. Caffeine was detected in Cola Type Soda samples, as expected. An example chromatogram is presented in Figure

3 for Fruit Soda 3. Remaining targeted analytes were neither detected in remaining soda samples nor label-declared.

In energy drinks, caffeine was present in large quantities, in line with declarations on the drink labelling. In total, three different sweeteners (always two in combination) were detected in seven samples, of which, five were labelled as "sugar-free";



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TABLE 3: Mini survey of food additives in soft and energy drinks (continued)

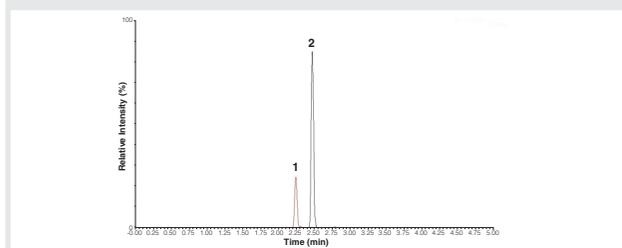
Sample	Label-declared food additives	pH	Preservatives (mg/L)		Sweeteners (mg/L)					Caffeine (mg/L)	
			Sorbic acid	Benzoic acid	Acesulfame K	Aspartame	Cyclamic acid	Saccharin	Sucralose		
			(E 200)	(E 210)	(E 950)	(E 951)	(E 952)	(E 954)	(E 955)		
Energy Drink: Sugar-free 3	E 202, E 950, E 955	3.3	9.8		138.2					46.9	306.7
Energy Drink: Sugar-free 4	E 950, E 951	3.0			119.1	153.9					322.1
Energy Drink: Sugar-free 5	E 955	3.2								66.8	297.3

* Applies only if benzoic acid is not also used, otherwise lower legal limit 250 mg/L is valid
 ** Valid only for energy-reduced or with no added sugar drinks

TABLE 4: Mini survey of food additives in alcoholic beverages (n = 43) – positive findings only

Sample	Type	Colour	Alcohol content (% v/v)	pH	Food colours (mg/L)						Preservatives (mg/L)
					Tartrazine (E 102)	Sunset yellow FCF (E 110)	Azorubine (E 122)	Ponceau 4R (E 124)	Patent blue V (E 131)	Brilliant blue FCF (E 133)	Sorbic acid (E 200)
Maximum limit set by Regulation (EC) No 1333/2008					200*	100	200*	170	200*	200*	200
Liquor 1	Mint liquor	Green	19	4.5	10.3					2.6	
Liquor 2	Herbal liquor	Black	35	5.2							13.1
Liquor 3	Cherry liquor	Red	14.5	3.9							134.5
Liquor 4	Bitter liquor	Red	25	4.7	5.4		24.4			0.3	
Liquor 5	Lemon liquor	Blue	21	3.8					27.8		
Liquor 6	Mint liquor	Green	20	4.8	8.2					4.8	
Liquor 7	Punch	Red	20	4.2			78.5				
Liquor 8	Mint liquor	Green	19	5.2	11.4					2.5	
Liquor 9	Punch	Red	30	4.9			30.5			0.7	
Liquor 10	Bitter liquor	Red	11	5.3		34.3		34.9			
Spirit	Herbal spirit	Blue	50	5.8						2.4	

FIGURE 4: Chromatogram of liquor 10 sample: 1) Ponceau 4R, 10 × diluted; 2) Sunset yellow FCF, 10 × diluted).



in the remaining two, sugar was present. Preservatives were detected in 10 out of 19 energy drink samples. The results of food additive determination in soft and energy drink samples are summarized in Table 3 – only positive findings are presented here.

In addition to alcohol-free products, a set of 43 different alcoholic beverages were analysed, consisting of 23 liquors, 14 spirits, and six ciders (see Table 4). Overall, six different food colours were determined in 10 liquors and one spirit sample. Colours are used in combinations in order to achieve a specific desired shade of the final product (for example, the green colour in Liquor sample 1 was the result of a mixture of brilliant blue FCF and tartrazine). Table 4 summarizes the results for samples containing some of the targeted additives. Additionally, sorbic acid was used for preservation of Liquors 2 and 3. The chromatographic analysis of Liquor 10 is demonstrated in Figure 4.

In analysed samples, the targeted additives did not exceed the maximum level set for some of them by Regulation (EC) No 1333/2008 (see Tables 3 and 4). The limits of quantification (LOQs) achieved in this study were, as mentioned above, lower than these regulatory limits, thus the developed method is suitable for a rapid and reliable control of beverages. It's worth noting that, in all cases, the information on the use of food additives was provided on the label.

Conclusion

A multi-analyte UHPLC–MS method for simultaneous determination of nine food colours, seven sweeteners, two preservatives and caffeine has been implemented and validated. Contrary to studies presented so far, this method allows a single-run determination in only 10 min, and with regards to a simple sample preparation, it represents an effective tool for beverage quality control. An analysis of a large set of 69 beverages was achieved. Although a single quadrupole is less selective than more expensive tandem mass analyzers, it still provides a better selectivity and versatility compared to conventional detectors. The achievable limits of quantification are low enough for a reliable control of regulatory limits of targeted food additives.

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Recovering from a COVID-19 Shutdown: Tips and Tricks for Starting Up, Part 2

Dwight R. Stoll¹ and Tony Taylor², ¹LC Troubleshooting Editor, ²Chief Science Officer of Arch Sciences Group and Technical Director of CHROMacademy

COVID-19-related laboratory shutdowns are sure to cause a myriad of problems with liquid chromatography (LC) instrumentation across the globe. Taking a systematic approach to restarting these systems will save money and time in the long run by preventing problems that may otherwise appear in days or weeks following startup.

In March of this year, many organizations took unprecedented steps to halt the spread of COVID-19, including severely restricting work in laboratories, or even shutting down entire laboratories, buildings, and worksites for weeks at a time. While some of these shutdowns were planned days in advance and executed well, I have heard many stories from scientists indicating that the shutdowns were sudden, and did not allow time to properly prepare their analytical instrumentation to be idle for weeks or months at a time. Unfortunately, this means that these scientists are going to encounter many challenges when they return to the laboratory that will necessarily include a lot of troubleshooting to figure out why their systems are not working properly before they can return to their normal experience of producing high quality data. For last month's instalment of "LC Troubleshooting", I asked Tony Taylor to join me in pulling together advice for starting up liquid chromatography (LC) systems after they have been idled for weeks or months at a time (1). In that instalment, we mainly addressed

challenges with microbial growth in solvent bottles and different parts of the LC system itself, and obstruction of the LC flow path that can occur as a result of precipitation of buffer salts or other debris. For this month's instalment, I've asked Tony to join me again, this time sharing advice related to the health of columns, qualifying system performance, and a little about restarting work with LC-mass spectrometry (MS) systems in particular. I hope that these suggestions are helpful as you return to the laboratory, but I am sure many strange things will be observed after so many LC users have been away from the laboratory for prolonged periods. If you've encountered a problem and gained some troubleshooting experience that you think others might be able to learn from, please don't hesitate to send your story my way.

- Dwight Stoll

The Column: Is It Still Okay?

Last month, we noted that the column is the heart of any chromatography system, and as such we need to ensure that our columns are in a healthy condition prior

to performing analyses. It is possible that the shutdown period may lead to mechanical or chemical problems, and the solutions to these problems will be different. Until you know that the column has been properly flushed, the column outlet should not be connected to the rest of the system. Disconnecting the column outlet from the flow path will avoid any unwanted compounds or debris that may come out of the column from causing problems with any other components in the system. You can either attach a waste line to the column outlet to collect the effluent, or simply let it drip into a beaker or similar container.

Mechanical Problems

If your column was left on the system when the laboratory was shut down, there is a possibility that it will now be full of air, because the mobile phase solvent may have evaporated over time. Similarly, if the column was removed from the system, but not plugged at both ends, it will again be full of air, which can lead to problems for many types of columns. We need to carefully guard against applying high pressures

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TABLE 1: Approximate void volumes of some column sizes in common use (2)

Column Dimensions (mm × mm i.d.)	Column (Void) Volume (mL)
250 × 4.6	2.5
150 × 4.6	1.5
150 × 3.0	0.64
150 × 2.1	0.28
50 × 4.6	0.50
30 × 4.6	0.30
15 × 4.6	0.15

to columns that are dry, as this may cause mechanical reorganization of the packed bed of particles, leading to unwanted voids, channelling, and significantly reduced column performance. In the following discussion, please bear in mind that the pressure applied to the column inlet should be increased gradually when first turning the flow back on (in steps of <10 bar where possible); this can be achieved by starting the pump at a very low flow rate (for example, 10 µL/min. for 2.1 to 4.6 mm i.d. columns), and increasing the flow rate in steps of 10 µL/min (larger steps can be used provided the corresponding pressure increase is not much more than 10 bar per step). Some newer systems also provide the option to specify a flow rate ramp rate that is used when the pump is turned on.

It may be possible that, upon starting the column flushing procedure, a high back pressure is encountered due to blockages in either the column inlet frit (pressure increase will be immediately noticeable) or the outlet frit (pressure will build more gradually over time). In the case of the former, it may be possible to reverse the direction of the column prior to turning on the flow, in order to back-flush the debris from the frit. Overall, this may have the longer-term effect of slightly reducing the efficiency of the packed bed, but the column should be usable for your application, unless it was heavily voided prior to the instrument shutdown.

Chemical Problems

In most cases, the following column flushing routine can be applied to reversed-phase, silica-based stationary phases as a kind of generic column cleanup step. Approximate column volumes for several different dimensions of columns in common use are given in Table 1.

- Set the column thermostat compartment to 60 °C.
- Flush with the following solvents in the order shown. For 2.1 mm i.d. columns use a flow rate of about 0.1 mL/min; for 4.6 mm i.d. columns use a flow rate of about 0.5 mL/min.
- 10:90 methanol:water for 20 column volumes (to remove any precipitated buffers)
- Increase organic composition to 100% methanol, and flush for 20 column volumes.
- Flush with 20 column volumes of 75:25 acetonitrile:isopropyl alcohol (IPA). Flush with 20 column volumes of the starting mobile phase of your method.

The rationale behind this series of flushing solvents is to cover a wide range of solvent polarities that give the best chance of dissolving and eluting anything that has adsorbed to the stationary phase. Only after these flushing steps should the column be reconnected to the detector. If the column flow direction has been reversed as discussed above, switch

back to the original orientation prior to reconnecting the column to the system.

In this brief discussion, we have focused on suggestions for restarting work with reversed-phase columns, and in a very generic way. Readers interested in a more detailed discussion of cleaning procedures for reversed-phase columns are referred to the excellent “Column Watch” article on this topic by Ron Majors (2).

Is It Safe to Collect Data Again?

At a minimum, before collecting important data again, one should run the system suitability test that is appropriate to the analysis at hand. The topics of system suitability and operational qualification have been discussed in prior instalments of “LC Troubleshooting” by John Dolan, and readers interested in more detail are referred to these articles (3,4).

In general terms, system suitability tests are designed to provide data that indicate an LC system is functioning in a way that it can be expected to produce reliable data for a particular analysis. In other words, it is application- or method-specific. In many situations, these data are sufficient to give the user confidence that the system is “ready to go”. However, in other situations, under certain regulatory frameworks, it may be necessary to carry out a more extensive operational qualification (OQ) of the instrument, which will verify instrument performance regardless of the methods being run. While doing OQ tests will undoubtedly take more time when restarting an LC system, we feel strongly that this time spent at startup can save a lot of time in the long term by catching problems early and addressing them before data acquisition resumes. In other words, a little extra effort now will decrease the likelihood that we encounter unwelcome surprises down the line!

A typical OQ verification routine will include some or all of the following tests, which we have annotated briefly to give an explanation of what the test does, and the information it provides toward an assessment of operational performance. Wherever a “reference compound” is referred to in these descriptions, caffeine is very commonly used, particularly for systems with ultraviolet (UV) detectors.

Pump Flow Rate Accuracy and Precision

Typically, a number of flow rate measurements are made at different flow rates using a digital flow meter. Flow rate accuracy is important for transfer of methods between systems and retention time agreement with standard methods of test. Flow rate precision has a direct impact on the repeatability of peak height and area.

Column Temperature Accuracy and Stability

Typically, a temperature sensor is used to measure either the column compartment temperature, or the temperature of the column effluent. Temperature is measured over time at two different setpoints, and the absolute difference between them, as well as the variability, are compared to manufacturers or regulatory criteria. Temperature accuracy can be critical for chromatographic selectivity, especially when separating ionizable analytes (5), and temperature stability has a strong influence on the repeatability of retention times.

UV Wavelength Accuracy

Typically, a caffeine or holmium oxide standard is used under conditions of no mobile phase flow, and the absorbance is recorded at several wavelengths across the range of the

detector. The measured positions for the peaks (maxima) and troughs (minima) of the standard are compared to the known (expected) values for the standard. The difference between the measured and expected wavelengths of maximum and minimum absorption is compared to manufacturers specifications. Wavelength accuracy is vital for both qualitative and quantitative work, and transferability of methods between systems.

Detector Noise and Drift

Short- and long-term variation in the detector signal is determined with a fixed eluent composition (typically water). The detector signal is measured over a certain period of time and at a particular frequency to determine the short-term variation in the signal, referred to as the *noise*. The slope of the detector signal over a longer time period (tens of minutes) is also measured, and this is referred to as the *detector drift*. The magnitudes of the noise and drift directly influence the ability to differentiate between real peaks for low concentration analytes and random variation in the detector signal.

Signal-to-Noise Ratio (S/N)

The detector sensitivity is also evaluated using a reference compound under specific conditions and compared to a target specification. There are many different manufacturer and regulatory recommendations on the topic of signal-to-noise ratio (S/N), and you should follow the guidance most appropriate for your situation.

Injection Precision

Using a reference standard, the peak height and area are measured for several replicate injections, sometimes for both small and large injection volumes. The absolute values for the

peak height and area, as well as the relative standard deviation of each value, are compared to manufacturers specifications. Injection precision is particularly important for quantitative analysis; the better the injection precision is (that is, smaller standard deviation), the better is the ability of the method to differentiate between samples having similar analyte concentrations.

Detector Response Linearity

Typically a reference standard is injected multiple times in a range of concentrations that cover the normal operating range of the detector (for example, up to 1.5 AU for a UV detector). Statistical assessment of linearity is performed using a combination of linear regression, residuals analysis, *F*-tests, and relative standard deviation of detector response for each analyte concentration, as well as ratios of signals. Linearity of detector response is critical for quantitative analysis, and has a direct impact on the accuracy of analyte concentrations reported based on use of calibration curves.

Solvent Gradient Composition

Typically, a tracer compound such as acetone is added to one of the mobile phase solvents (usually the “B solvent”), and a method is used that steps through different mixtures of two solvents, one of which contains the tracer compound that can be observed by the detector (for example, acetone absorbs well at 265 nm). For example, a method may start at 0% B, and increase in steps of 5% B until 100% B is reached. The signal due to the presence of the tracer compound is used as an indirect measure of the ratio of the volumes of the A and B solvent that are combined by the pump to make the mobile phase mixture of A and B. The average detector signal at each % B level is

compared to the expected value, and the short-term variation at each step may also be evaluated. Finally, some OQ routines call for the analysis of a linear gradient profile using the same tracer compound. The accuracy and repeatability of the gradient profile are critical for both qualitative and quantitative analysis, transfer of methods between instruments, and repeatability of retention times.

Suggestions for LC Systems with Mass Spectrometric (MS) Detectors

MS detectors can be particularly susceptible to problems on startup following extended periods in standby mode or shutdown, and one needs to pay particular attention to these detectors prior to restarting work with them. As there is a lot of variation in maintenance protocols for different MS manufacturers and instrument types, it is essential that you carefully follow the manufacturer's guidance when considering the following steps.

First, thoroughly clean the ionization source, preferably when the instrument is not under vacuum. However, it is not necessary to vent the instrument if it is under vacuum at the time of cleaning. In any case, follow the manufacturer's recommended procedure for cleaning the source. The emitter (that is, sprayer or nebulizer) should also be checked carefully prior to re-establishing flow from the LC system, as residual eluent solvents evaporate during extended storage periods, often leaving residues or even blocking the flow path entirely, and can be difficult to remove. If significant residue or an obstruction is observed, remove the nebulizer and sonicate for 10 min (first in water, and then in IPA). Be careful to suspend the nebulizer tip in the cleaning solvent in such a way that it does not contact the bottom or walls of

the container, as this could damage the tip itself, and affect spray performance.

If the instrument has been vented, carefully monitor the vacuum levels in the instrument when pumping it back down, and check the vacuum levels against the manufacturer's specifications.

Before using the MS for data acquisition, a verification of its performance will be required. At a minimum this will include a full tune (autotune), and this is typically achieved using the manufacturer's recommended tuning compound or solution and performance criteria. If your instrument includes an on-board tuning solution that can be activated using the control software, make sure there is enough tuning solution in the reservoir prior to starting the autotune routine. The autotune routine will tune the electrostatic lenses within the instrument as well as the voltages applied to the mass filtering device in order to optimize and verify, amongst other things, mass accuracy across a wide range of values, instrument sensitivity, and response profile.

While the on-board autotune is very useful to set the detector parameters and check them against the manufacturer's performance requirements, one may also need to carry out further "whole-system" performance checks with a typical set of performance criteria involving: 1) response linearity (or response profile if it is expected to be non-linear based on previous experience); 2) injection precision; 3) carryover; 4) signal-to-noise ratio; and 5) minimum detection limits.

Summary

Given the variety of ways different laboratories were shutdown early on in the global COVID-19 outbreak, it

is likely that LC users will encounter a wide variety of problems with their instruments when they return to their laboratories and resume work with their instruments. In this instalment of "LC Troubleshooting", we have provided suggestions specific to handling LC columns and LC systems with MS detectors when restarting work after a long time away. We have also discussed the value of running system suitability and operational qualification tests before starting to collect important data again. These tests will be helpful for identifying problems that may have been caused by the shutdown, so that they can be resolved before causing trouble later on.

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High-Throughput and High-Efficiency Separations of Antibodies by Reversed-Phase Chromatography Using Organic Alcohols

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Biomacromolecules, especially monoclonal antibodies, are complex molecular species that have a myriad of functional groups. This complexity has led to much discussion on strategies for improving efficiency and analyte recovery of chromatographic methods used to characterize these molecules. This edition of “Column Watch” will focus on the roles of temperature and organic modifier for yielding improved efficiency in analyzing biomacromolecules. It is shown that these variables can be used together to achieve higher throughput, resolution, and recovery when analyzing monoclonal antibodies and antibody-drug conjugates.

Monoclonal antibodies (mAbs) are a promising class of biologics for the treatment of several autoimmune diseases and cancers. An additional application of mAbs, however, is when a cytotoxic payload (such as a drug) is attached to the mAb, allowing for the mAb to target a certain cell type or tissue and deliver the payload to a specific target. This combination of mAb plus cytotoxic drug, connected through an organic linker, is known as an *antibody-drug conjugate* (ADC). As of January 2020, there are 89 ADCs in the pharmaceutical pipeline (1).

One downside of mAb-based drugs, however, is that, due to their structural complexity, there is significant heterogeneity. This heterogeneity can arise as a result of the presence of charge variants, glycosylation variants, or phosphorylation variants; these arise by the nature of the biological production process.

Several different chromatographic strategies are applied to investigate and resolve the structural and chemical variants of mAbs. Size-exclusion chromatography is used as one method to assess the aggregation of a mAb sample. Ion-exchange chromatography is a suitable method to conduct charge variant analysis. Both chromatographic modes can present issues of compatibility with electrospray ionization mass spectrometry (ESI-MS), which is routinely used for protein characterization. Hydrophobic interaction chromatography (HIC) is routinely employed for analysis and characterization of protein glycans. Reversed-phase chromatography has long been a method of choice for analyzing proteins because of its high resolution and compatibility with MS. Reversed-phase chromatography of proteins, however, has its own issues. Of primary significance is that

protein structures can be flexible in comparison to structures of small organic molecules. This fact may present a chromatographic challenge, because various structural conformations may differentially interact with the stationary phase. With proteins, peak shape in reversed-phase chromatography is generally enhanced by parameters that stabilize a single denatured state (2–4). Temperature is one parameter that can dramatically affect the tertiary and quaternary structure of proteins, and could lead to a “denatured state”.

Another aspect of protein and peptide reversed-phase chromatography is that, for most applications, elution must be by utilization of a solvent strength gradient. This requirement is due to at least two reasons: 1) polypeptides are generally polyionic, and, therefore, can present problems of secondary interactions with the silica surface, potentially causing

FIGURE 1: Analysis of mAb reference material by reversed-phase chromatography with varying amounts of 1-butanol. Conditions: Column: BIOshell A400 Protein C4, 10 cm × 4.6 mm i.d., 3.4-µm; Mobile Phase: (A) 70:30 0.1% TFA in water: 0.1% TFA in acetonitrile; (B) 60:40 0.1% TFA in water: 0.1% TFA in acetonitrile; (C) 70:25:5 0.1% TFA in water: 0.1% TFA in acetonitrile: 0.1% TFA in alcohol; (D) 60:35:5 0.1% TFA in water: 0.1% TFA in acetonitrile: 0.1% TFA in alcohol; Gradient: For x = 0, 20, 40, 60, 80, or 100; [(100-x)% A, 0% B, x% C, 0% D] to [0% A, (100-x% B), 0% C, x% D] in 15 min; Flow Rate: 1.0 mL/min; Column Temp.: 55 °C; Detector: UV, 215 nm; Injection: 3.0 µL; Sample: mAb reference material, 1 g/L, 0.05% TFA in water.

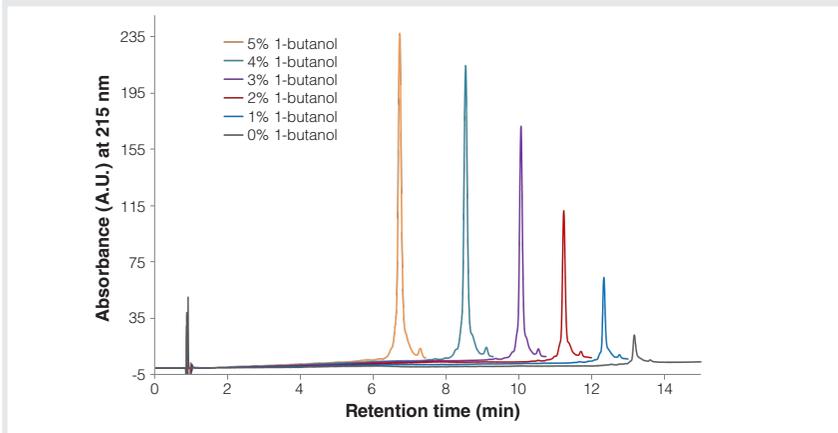
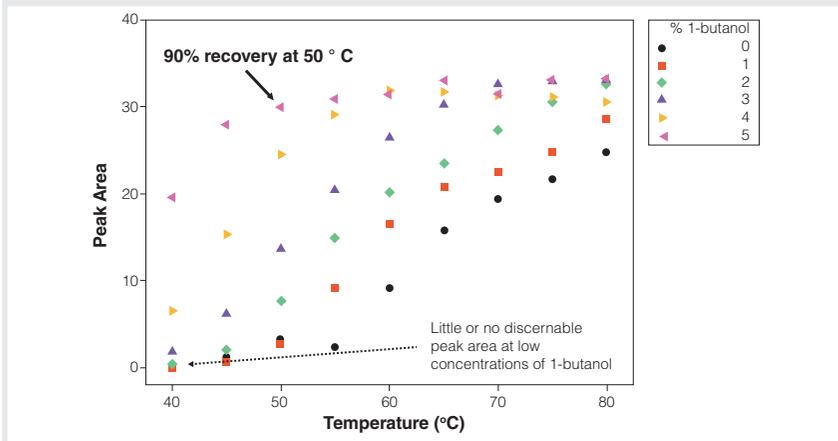


FIGURE 2: Recovery, measured by peak area, as a function of temperature and 1-butanol concentration. Note that the maximum recovery of the analyte occurs at much lower temperatures as the concentration of 1-butanol increases. Chromatographic conditions are the same as those described in Figure 1 except for temperature, which was varied as indicated in the figure.



issues of peak tailing; and 2) partitioning of polypeptide analytes between the mobile and stationary phase occurs over a narrow window of solvent strengths (as compared to most small molecules), therefore exhibiting much more of an on-off adsorption phenomenon. With the

requirement for gradient elution comes the requirement for column re-equilibration prior to injection of a sample. Column re-equilibration can be sped up by reducing changes to the solvation state of the silica surface, as has been shown by inclusion of low levels of small, primary

alcohols in the mobile phase (5,6). How this mechanistically takes place has not been defined, but computer modelling of short, primary alcohols in binary mobile phase systems is consistent with intercalation of the alcohol into the stationary phase, with the hydroxyl hydrogen-bonding to the surface silanols or an adsorbed water layer (7). This phenomenon may have additional benefits in masking silanols, therefore improving peak shape. Scott and Simpson reported that 1-butanol can form a simple monolayer on a C18-bonded silica surface; this also fits with a model of a small, primary alcohol hydrogen bonding to the surface silanols (8).

High temperature has been shown to be necessary in achieving optimal analyte recovery and peak shape of mAbs in reversed-phase chromatography (9). This fact has been confirmed to be the case, irrespective of the column used or the specific mAb sample (10). Additionally, Fekete and associates have shown that inclusion of low levels of 1-butanol reduced the temperature optimum for the reversed-phase chromatography of the mAb (9). Thus, the mechanism of any conferred benefits of inclusion of low levels of primary alcohols in the mobile phase is not entirely clear. These previously published data suggest a primary mechanism of masking of the silica surface; another possibility might be imagined to explain the effects on antibody chromatography in which the intercalated 1-butanol is oriented with the hydroxyl facing the bulk mobile phase, thus lending some polarity to the environment at the antibody-stationary phase interface. Such models might be elucidated by inspecting results with analogues of 1-butanol.

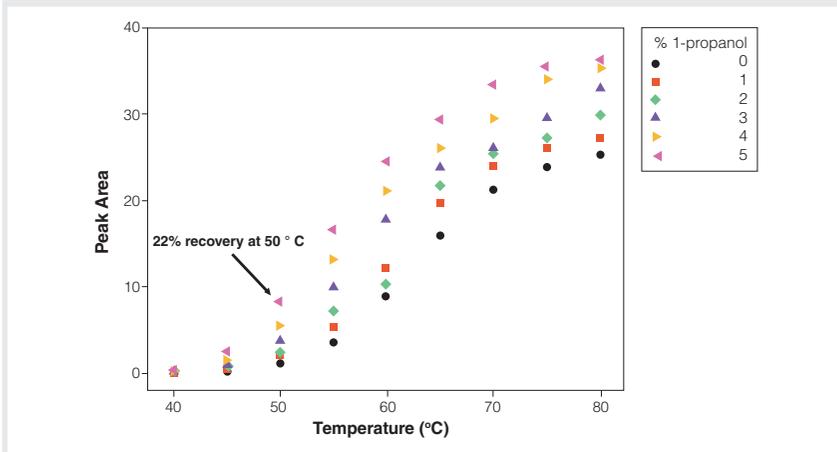
Effect of Organic Alcohol on Monoclonal Antibody Characterization

These ideas were investigated further using a commercially available mAb

reference material. Initially, the goal was to at least confirm previous reports on the chromatographic effects of 1-butanol on the reversed-phase chromatography of mAbs. Two primary alcohols, 1-propanol and 1-butanol, were investigated. Figure 1 shows chromatographic results of the recovery of the mAb at varying percentages of 1-butanol at 55 °C.

As seen in Figure 1, the peak area and height of the analyte increased as the concentration of 1-butanol increased. In addition, as the concentration of 1-butanol increased, one can begin to resolve impurities from the main analyte peak. Finally, the retention time of the mAb decreased with increasing 1-butanol concentration. This phenomenon was further investigated by looking at how temperature played a role in the recovery of the mAb. Figure 2 displays the results of this analysis.

FIGURE 3: Recovery, measured by peak area, as a function of temperature and 1-propanol concentration. Note the much-reduced recovery at 50 °C with 1-propanol versus 1-butanol. Chromatographic conditions were the same as those described in Figure 1 except for temperature which was varied as indicated in the figure.



As can be seen in Figure 2, it should become obvious that one of the main advantages of including 1-butanol in the mobile phase is the much lower

temperature required to achieve maximum recovery of the analyte. The data, however, cannot differentiate effects due to possible mitigation against

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FIGURE 4: Recovery, measured by peak area, as a function of temperature and 2-butanol concentration. Chromatographic conditions are the same as those described in Figure 1 except for temperature which was varied, as indicated in the figure.

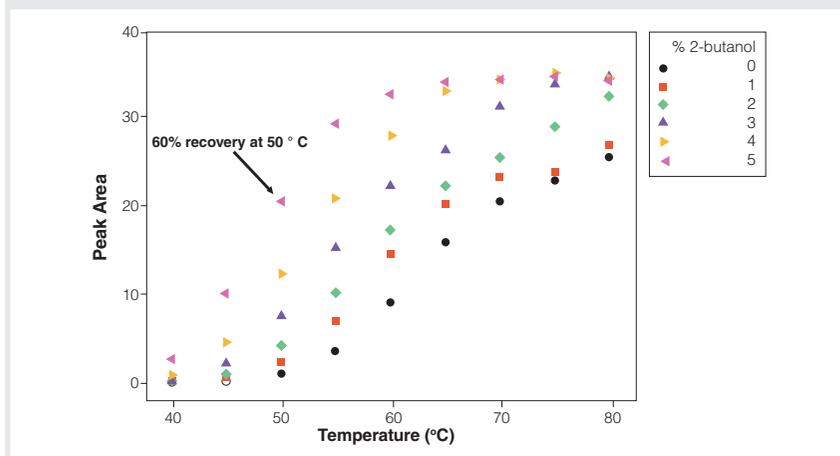
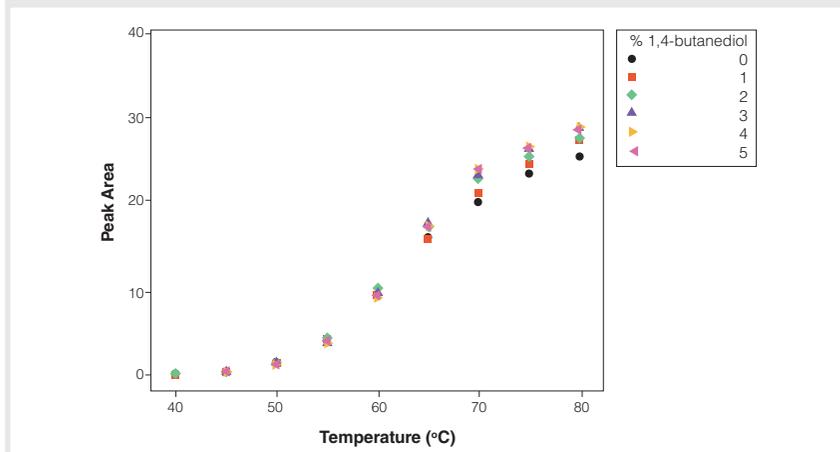


FIGURE 5: Recovery, measured by peak area, as a function of temperature and 1,4-butanediol concentration. Note that the concentration of 1,4-butanediol does not appear to influence recovery. Chromatographic conditions are the same as those described in Figure 1, except for temperature which was varied, as indicated in the figure.



thermal degradation or impacts on the actual chromatography of the mAb. The experiment was repeated, this time with 1-propanol as the mobile phase modifier. Figure 3 shows the results of this analysis.

As shown in Figure 3, the temperature required to achieve the maximum recovery of the analyte was much higher (around 80 °C) in comparison to 1-butanol. While 1-propanol has been shown to have similar benefits as 1-butanol for keeping the silica surface solvated, and shielding

surface silanols, it clearly did not provide the same chromatographic benefits in this case, as compared to 1-butanol (5,6).

Continuing the investigation, the effect of type of alcohol (primary, secondary, and so forth) on analyte recovery was examined. As noted in Figure 4, the secondary alcohol elicits good recovery of the antibody, albeit not as good as 1-butanol. It could be inferred that, due to steric effects, 2-butanol should be not as effective as primary alcohols in

hydrogen-bonding with surface silanols or an adsorbed water layer. However, 2-butanol achieved higher recovery of the analyte at low to moderate temperatures than with 1-propanol, suggesting that the mechanistic explanation is not as simple as hydrogen-bonding to the silica surface.

A final test was to examine the use of 1,4-butanediol. The idea is that this alcohol could possibly play a dual role of hydrogen-bonding to the silica surface, as well contributing additional polarity to the interface where protein adsorption takes place on the stationary phase. The results are shown in Figure 5.

As can be deduced from Figure 5, there appears to be no advantage in adding 1,4-butanediol to the mobile phase.

Perhaps the two terminal hydroxyls render it sufficiently polar such that it no longer readily distributes within the stationary phase to provide any performance benefit to the reversed-phase chromatography. In fact, the collective results beg the question, could the difference in the effects simply be due to the solubility of the alcohol within the stationary phase?

It also suggested to the authors that it would be most interesting to do a comparative study with an identical column or particle, but with a bonded butanol phase (the terminal hydroxyl exposed to the bulk solvent). Such an experiment could shed light on this mechanistic question regarding the conferred impacts of added butanol in the mobile phase. As mentioned earlier, perhaps the hydroxyl faces the bulk mobile phase to lend polarity to the environment at the antibody-stationary phase interface.

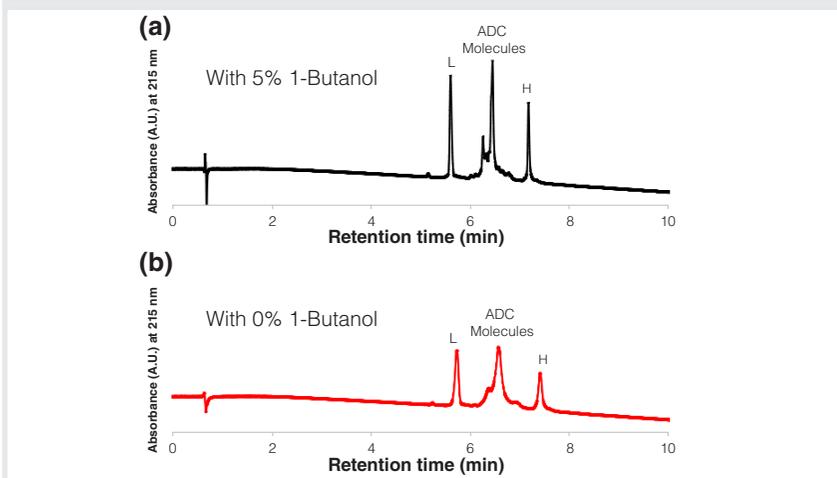
Effects of 1-Butanol on Analysis of an ADC

In addition to all the inherent complexity associated with a mAb, characterization of an ADC introduces further complexity to the separation challenge due to the

addition of the cytotoxic drug (payload) and organic linker. Creation of a cysteine-linked ADC, where the payload is attached to cysteine amino acid residues, leads to a mixture of ADC molecules with different amounts of payload attached to the mAb vehicle. Commonly seen permutations of a cysteine-linked ADC molecule include the native mAb molecule (no payload attached to the mAb), followed by ADCs with two, four, six, or eight payloads attached. In addition, impurities from the mAb production process and unbound cytotoxic drug are also present in ADC samples.

As was discussed in the previous section, incorporating 1-butanol into the mobile phase yielded improved recoveries, higher throughput, and higher efficiency than with mobile phases without 1-butanol. To see if this observation can

FIGURE 6: Analysis of an ADC by reversed-phase chromatography (a) with or (b) without 1-butanol. Conditions: Column: BIOshell A400 Protein C4, 10 cm × 4.6 mm i.d., 3.4- μ m; Mobile Phase: (A) 0.1% DFA in water; (B) 0.1% DFA in acetonitrile; (C) 95:5 0.1% DFA in acetonitrile; 0.1% DFA in 1-butanol; Gradient: 25% B to 45% B in 10 min (no butanol method) OR 25% C to 45% C in 10 min (with butanol); Flow rate: 0.5 mL/min; Column temp.: 55 °C; Detector: UV, 215 nm; Injection: 2.5 μ L; Sample: ADC SR-388, 1 mg/mL, 0.1% DFA in water.



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be translated to a more complex protein molecule, 1-butanol was incorporated into the mobile phase used for the analysis of an ADC by reversed-phase chromatography. Figure 6 displays the chromatographic results of this analysis. Note the improved resolution and recovery of some of the peaks corresponding to labelled heavy and light chains of the ADC. Interestingly, there does not appear to be a reduction in retention time as was seen with the mAb analyte.

However, there is an effect on the separation as the efficiency of the method with 1-butanol was higher (as indicated by more narrow peak widths) than without the 1-butanol. An explanation for this result may possibly be tied with the tertiary structure of the protein. The structural integrity of cysteine-linked ADCs is less than in native mAbs, due to some of the cysteines used in disulfide bonds between the light and heavy chains of the mAb being reduced to add the payload molecules. Since the method conditions would permit denaturing of the protein, less energy would be required to cause denaturation. Denatured proteins expose more hydrophobic amino acids to the stationary phase, and thus could lead to peak broadening due to a stronger interaction between the stationary phase and the protein. Perhaps the 1-butanol is solvating the protein molecule and minimizing protein denaturation (or minimizing the number of denatured forms of the protein) leading to sharper peaks. Additional investigations are needed to fully test these possible explanations.

Conclusions

Despite these seemingly conflicting results, what emerges is a picture in which the role of the alcohol in conferring a chromatographic benefit to the reversed-phase chromatography of mAbs and ADCs (or maybe most any other immunoglobulin G molecule) is not as simple as has

been suggested from other chemical and computer modelling studies—that something other than masking the silica surface is at play here. Perhaps there is a separate effect on the thermal stability of the mAb in this common mobile phase. Nevertheless, as shown in Figures 1 and 2, the addition of 1-butanol to the mobile phase can elicit excellent recovery of an antibody standard at far lower temperatures than in its absence, and can serve as a general method for reversed-phase chromatography of antibodies, or perhaps any other proteins that exhibit poor peak shape at moderate temperatures.

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Beat the Heat: Cold Injections in Gas Chromatography

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In capillary gas chromatography (GC), we heat the most common inlets (split and splitless) to vaporize the samples and transfer them to the column. While heating and vaporizing the sample in the inlet presents the most convenient means for transferring it to the capillary column head, several problems that result in sample losses and quantitative reproducibility problems occur. We will briefly discuss the problems with sample heating in classical split and splitless inlets, followed by an introduction to cold inlets, which have been available since the 1970s, but are underutilized. Next, we will discuss modes of cold sample introduction, including cold on-column, cold split and splitless, cold splitless with solvent venting, and large volume injection. We will close with key points and considerations, and argue that users should consider cold injection as a purchase option or upgrade for any gas chromatograph.

This instalment of "GC Connections" follows up and builds on the basic discussion presented in LCGC Europe in May, 2018 (1).

Many gas chromatographers would agree that inlets and sample introduction are the most challenging aspects of capillary gas chromatography, and we see that most gas chromatographers are still using the same inlets (split and splitless) and techniques, especially injecting with a 10 μL syringe, developed in the 1950s and 1960s (2). We make a fundamental, yet incorrect, assumption that the inlet must be heated in order to rapidly vaporize the sample, mix it with the carrier gas, and transfer it to the column. This assumption led to the development of both the split and splitless techniques in common use today. It also leads to several of the main causes of reproducibility problems and the need for troubleshooting in gas chromatography.

Figure 1, a photograph first published in the May, 2018 instalment of "GC Connections," illustrates some of the key

problems. It shows the result when a few millilitres of water (like a sample solvent) is transferred using a spoon to a hot cast iron skillet on a stove (like a heated inlet liner). The picture was taken a few seconds after the water was introduced to the skillet. There are several take-home points about hot sample injections from this photo.

- No matter how hot and massive the inlet, the solvent does not evaporate instantly. This is a major cause of discrimination, or the preferential loss of some analytes.
- The layer of gas between the solvent and the surface also causes some of the analytes to be exposed directly to the hot surface, causing analyte decomposition.
- In splitless inlets, the sample requires up to a minute or more to transfer to the column, leading to band broadening.
- Note the discolouration of the water on the surface. The solvent can actually re-introduce contaminants from a dirty inlet. This causes contamination or *ghost peaks*.
- While not shown here, some of the water remained in the spoon, analogous

to a syringe. The syringe needle is also a leading cause of discrimination and apparent sample losses.

Although they are convenient to use, heated split and splitless inlets can clearly cause many problems and challenges for method development and analysis by GC. This is enough of a challenge that the book by Grob, the classic text on split and splitless inlets, is nearly 500 pages long (3)!

Figure 2 shows diagrams of the pressure, temperature and flows in classical split (Figure 2[a]) and splitless (Figure 2[b]) injections. These show the relative simplicity and convenience of the two techniques, and provide the main reason why there has been resistance in the community to others. In split injections (Figure 2[a]), once the split ratio (total flow) and column flow rate are set, none of the parameters change during the run. The inlet temperature is constant and hot throughout the injection and the analytical run (often 250 $^{\circ}\text{C}$), and the purge flow, through the inlet liner, is constant and high (often on the order of

FIGURE 1: A few mL of water on a hot cast-iron skillet shows how injected samples evaporate in a heated inlet.



100 mL/min). To save carrier gas, the gas saver feature on modern inlets can be used to reduce the purge flow once the injection process is complete. In splitless injections (Figure 2[b]), the purge flow through the inlet liner is low at the beginning of the run and at the injection. It stays low for a specified time, and then is rapidly raised by switching a solenoid valve. During the “purge-off” time, vapours in the inlet liner are transferred slowly to the column, with no other outlet. The oven temperature starts low (this is a requirement in splitless injections) and a temperature programme is then performed. As in split injections, the purge flow can be reduced later in the run to save carrier gas. The added parameter of the purge valve starting “off” and coming “on” after a specified time is still relatively straightforward to optimize.

A solution to the problems with split and splitless injections has existed since the late 1970s with the invention of programmed temperature vaporization inlets, termed PTV, by Vogt (4,5). Major instrument vendors have offered PTV and cool-on column inlets as an add-on to new gas chromatographs, but they have never become popular. In the rest of this instalment, we will discuss the fundamentals of programmed temperature injections using PTV and cool on-column inlets, and follow up with key points and discussion that should lead you to consider cold inlets with your next GC.

Most of the instrument vendors and a few specialty vendors offer solutions for

cold inlets in GC. Some vendors have their own terminology for the classical PTV inlet, some sticking with PTV, while others use “multi-mode inlet” or “cooled injection system.” Nearly all vendors use cool or cold on-column to describe their on-column inlet offerings. With varying capabilities, they all allow a user to inject a sample into a cooled inlet, and then rapidly heat the inlet to transfer the sample to the column. Following the injection and heated transfer, and before the next injection, the inlet is cooled by a jet of air, carbon dioxide or liquid nitrogen.

Cool On-Column

The simplest and least expensive means for cold injections is to add a cool on-column inlet to the GC. This inlet, as the name implies, is cooled during injection, and allows sample to be injected directly onto the column. Following injection, the inlet (which includes the column head) is heated along with the column via temperature programming. The cool on-column inlet is the best of all inlets for ensuring that the entire sample that leaves the syringe needle reaches the column. However, it is the worst of all inlets for keeping the column clean; the entire sample, including matrix components that cause glass sleeves in other inlets to need replacement, reaches the column. Cool on-column is especially useful for “clean” samples in non-polar solvents, or solvents whose polarity closely matches that of the column. A retention gap, a short (typically

5 m or so) length of uncoated fused-silica tubing is often used between the inlet and analytical column, to provide a polar surface to allow the use of polar solvents, and an easily replaced guard column to protect the analytical column. If used with columns smaller than 0.53 mm inside diameter, cool on-column requires a special tapered syringe needle, which is both more costly and more fragile than traditional syringes. Large volume injections can be performed with cool on-column using a solvent vapour exit (6). Several vendors commercialized this process in the 1990s, so solvent vapour exit upgrade solutions may be available for cool on-column equipped systems. Check with the vendor.

Programmed Temperature Vaporization (PTV)

The most important difference between a PTV inlet and a typical split or splitless inlet is a much lower thermal mass to allow rapid heating and cooling. A glass liner, much like the glass liners used in split and splitless inlets, is used, as is a septum or septum-less head. Some PTV inlets have both a split vent, like the split or splitless inlet, and a solvent vent, used for purging larger volumes of injected solvent commonly used in large volume injections. They also have a septum purge if a septum is used. A PTV inlet can be installed at instrument purchase, or it can be retrofitted. The inlets offered by some specialty vendors are designed to be easily retrofitted onto existing systems, as are inlets offered by the major vendors.

The PTV inlet is often termed a “multi-mode” inlet because it can perform several injection and sampling techniques on a single platform, using a single inlet. In addition to the traditional hot split and splitless techniques familiar to all of us, it can perform cold split and splitless, and cold splitless with solvent venting (large volume injection). Detailed summaries of inlet capabilities and a thorough

description of large volume injection can be found on-line (7,8). As the heart of a comprehensive on-line sampling system and GC, a PTV inlet can serve as an interface for thermal desorption, pyrolysis, headspace, SPME and sorptive extractions, and even on-line HPLC-GC (9).

The original PTV inlets were little more than split or splitless inlets with the large thermal block removed and heating tape installed. This simple fundamental lies at the heart of PTV inlets today. When selecting a PTV inlet, there are a few instrumental options to consider. First, a PTV inlet can be ordered with or mounted on a new GC; it can also be retrofitted onto an existing system. The most important factors are how the inlet is heated and cooled. Is the heating rate and maximum temperature sufficient for the needed application? A typical PTV inlet can be heated up to temperatures in excess of 500 °C. The final temperature should be high enough to desorb all analytes of interest into the column. The high temperatures, often well above the stated column maximum temperature, do not damage the column, as the highest temperatures are not maintained for more than a few seconds before the inlet is cooled. The second choice is about cooling. Air cooling is simpler and less expensive, but requires more time than cryogenic cooling. Cryogenic cooling also allows rapid cooling to below room temperature for more effective use of highly volatile solvents or analytes.

PTV inlets can perform the same roles as traditional split and splitless inlets, hence the common term multi-mode now used to describe them. The lower thermal mass of the inlet causes a compromise in heating for the traditional hot split and splitless techniques. It is well-known that when a liquid evaporates, heat from the surroundings is needed to force the evaporation, causing the surroundings to cool. Limiting cooling

during evaporation is the reason for the high thermal mass (heavy metal block) that is used for the housing of traditional inlets. Even with this high thermal mass, there is some cooling as the solvent evaporates, especially for polar solvents such as methanol, with high enthalpies of vaporization. The cooling is uncontrolled and usually non-reproducible, and is

one of the causes of inlet discrimination and mysterious reproducibility problems in traditional split and splitless injections. The PTV inlet does not have the large thermal mass, so in some cases it may be subject to additional cooling as the solvent evaporates, when traditional hot split and splitless injections are performed.

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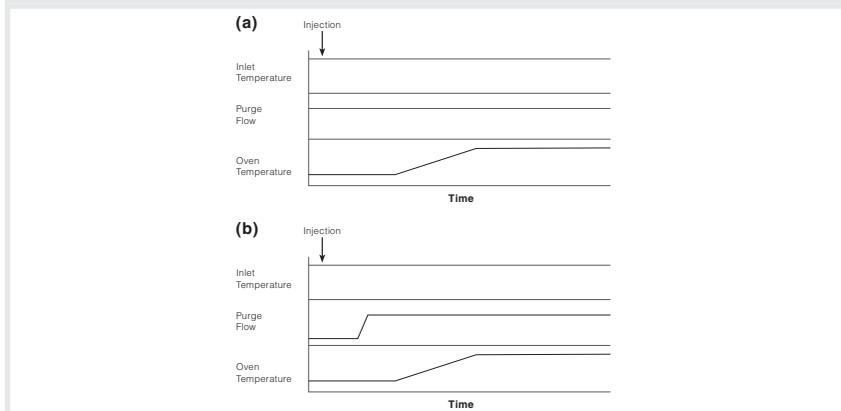


- study degradation processes



- characterize the structure of polymers

FIGURE 2: Diagrams of temperatures and flows in (a) classical split, and (b) splitless injections.



To limit this problem, and control solvent evaporation, split and splitless injections can be performed cold. The sample is injected with the inlet cooled, and the inlet is then heated rapidly to control the evaporation process. Cold injections also reduce or usually eliminate syringe needle discrimination, often seen in hot split and splitless injections. Since a traditional split or splitless inlet comes as standard on new GCs, and since you are already using traditional split and splitless injections, a new GC with a PTV can easily be mounted with the traditional split or splitless inlet as well, and parallel studies can be performed to validate the multi-mode inlet.

Have you ever wanted to simply inject a larger volume of sample? Perhaps the most important limitation of classical splitless injection is the limitation of injection volume to 1 (or a few) μL . The ability to inject larger sample volumes, up to hundreds of microlitres or even millilitres, is the most important aspect of using a PTV inlet. Injecting the sample cold, into a packed inlet liner at a temperature below the boiling point of the solvent, venting most of the solvent to waste and then rapidly heating the inlet to transfer the remaining sample and solvent to the column allows the inlet to accept much larger sample volumes.

Large volume injection is a lot like splitless on steroids. Figure 3 shows a

simplified schematic of the process. Prior to the injection, the inlet is cooled and the purge flow through the inlet liner is high. The oven is also cooled in preparation for temperature programming. A large volume, say 100 μL , is injected using a standard syringe and an autoinjector into an inlet liner packed with an adsorbent to provide a large surface area, and possibly some selectivity. The open purge valve removes about 95–99% of the solvent. Analytes remain sorbed on the packing, and are concentrated in the solvent that remains in the inlet. Timing this purging period to remove most, but not all, of the solvent, is one of the most crucial steps in large volume injection. Inlet vendors provide calculators to assist with determining the correct timing. Once the solvent is purged, the purge valve is closed and the inlet is rapidly heated, to transfer the remaining solvent and now-concentrated sample into the column. Like splitless, the purge vent is then opened again to clean the inlet and the inlet is cooled following the run. In short, the inlet acts as a sample concentrator and splitless inlet all in one.

If a PTV inlet can handle a single injection of 100 μL , then why not inject more? It is actually possible to inject samples up to millilitres (yes, mL) using a PTV inlet by taking advantage of

the packed liner, the cooled inlet and the vapour pressure of the solvent. A diagram of the process is shown in Figure 4. Consider each injected sample as an aliquot. Prior to the run, the inlet and column oven are cooled, and the purge flow through the inlet liner is high. With the inlet cooled and in its solvent vent mode, the solvent evaporates out the vent once the sample is injected. Once most of the solvent has evaporated, another aliquot can be injected. The solvent evaporates, leaving the analytes adsorbed in the packed liner. If this process is repeated 10 times using a 100 μL syringe, a 1000 μL sample has been injected. After the final injection and solvent venting, the solvent vent valve is closed, flow goes through the column, the inlet is heated, and the analytes are transferred to the column as in a splitless injection. In method development, the timing of the injections with the solvent vent flow rate must be carefully optimized. If all of the solvent is evaporated at any point, the vapour pressure of the analytes and the high solvent vent flow rate may cause some of them to desorb out through the solvent vent and be lost. This capability allows a PTV inlet to be the transfer device in on-line LC–GC (9).

A cooled inlet with large volume injection capability, combined with a robotic auto-sampler (a “rail”), offers many intriguing possibilities for automated on-line sample preparation and injection. Since the inlet is cooled, exchanging liners and performing maintenance is much faster than with heated inlets. Simply remove the top nut, and replace the septum and liner. No extra cooling and subsequent heating and equilibration, which are time consuming, are required. This is especially useful for laboratories that run “dirty” samples requiring inlet maintenance often.

Further, the top nut and seal can be configured to allow the inlet to be opened, and the inlet liner exchanged

automatically. This is termed automatic liner exchange, and it opens up several opportunities. A PTV inlet with automatic liner exchange can double as a pyrolysis or thermal desorption unit. Samples can be loaded directly into the inlet liners either on a rail-type auto-sampler, or on a separate instrument. Automated liner exchange is then used to load each inlet liner into the inlet, one at a time, for analysis. The PTV inlet is then heated to desorb analytes into the column. Most PTV inlets can rapidly heat to 500 °C or more, providing some capability for pyrolysis as well as thermal desorption.

We close with some questions and discussion points that you should explore with your vendor when considering a cold inlet.

- How are they equipped to support and provide method development assistance? Especially with PTV inlets, there are method development considerations that most gas chromatographers have not learned. Who will be providing technical support and how available will they be?
- Is this their own inlet, or is it provided by a third party? If a third party vendor provides the inlet, you may wish to work with the third party directly for purchasing the inlet and associated sampling devices, such as a rail auto-sampler.
- It is common to pair a PTV inlet with a rail-type autosampler. Be sure to discuss technical support and maintenance for the rail and the inlet in addition to the GC. Most likely, these will be additional operating costs. In my opinion, if a cold inlet is not a “must have”, it is a “must consider” for any GC purchase today, and is worth considering for a retrofit. By reducing the possibilities for discrimination and other problems associated with traditional heated inlets, the extra upfront cost and learning time is more than offset by the greater capabilities and reduced troubleshooting.

FIGURE 3: Diagram of temperatures and flows in a PTV large volume injection.

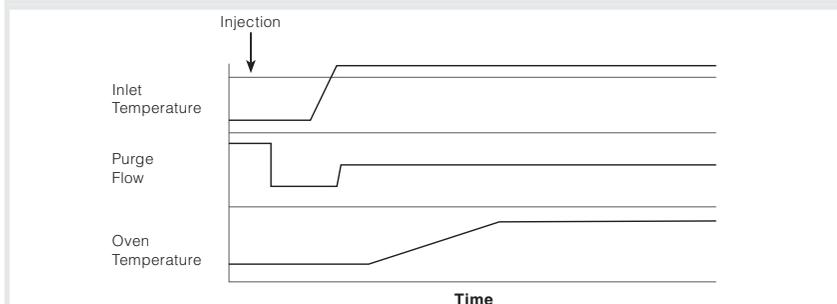
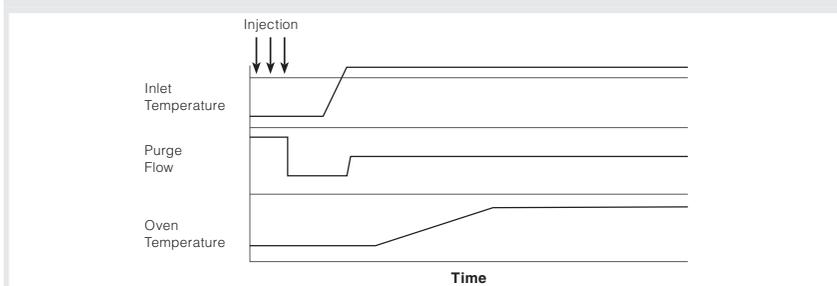


FIGURE 4: Diagram of temperatures and flows in a PTV large volume injection process with multiple injections to generate a very large volume injection.



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Nicholas H. Snow is the Founding Endowed Professor in the Department of Chemistry and Biochemistry at Seton Hall University, and an Adjunct Professor of Medical Science. During his 30 years as a chromatographer, he has published more than 70 refereed articles and book chapters and has given more than 200 presentations and short courses. He is interested in the fundamentals and applications of separation science, especially gas chromatography, sampling, and sample preparation for chemical analysis. His research group is very active, with ongoing projects using GC, GC–MS, two-dimensional GC, and extraction methods including headspace, liquid–liquid extraction, and solid-phase microextraction. Direct correspondence to: amatheson@mjhlifesciences.com

Practical Two-Dimensional Liquid Chromatography in Drug Metabolism Studies and Bioanalysis

Filip Cuyckens from Janssen R&D in Belgium spoke to *LCGC Europe* about recent innovative approaches he and his team developed to support drug metabolism and pharmacokinetic studies, and the inventive role that two-dimensional liquid chromatography (2D-LC) plays in his laboratory to boost sensitivity, solve recovery issues, and increase overall efficiency.

Interview by Alasdair Matheson, Editor-in-Chief, *LCGC Europe*



Filip Cuyckens is Scientific Director and Fellow at Janssen R&D, Drug Metabolism and

Pharmacokinetics, Beerse, Belgium.

He earned a pharmacist degree in 1998, a degree in industrial pharmacy in 2002, and a Ph.D. in pharmaceutical sciences in 2003 at the University of Antwerp, Belgium. He has more than 20 years of experience in analytical chemistry, particularly in liquid chromatography, and mass spectrometry. At Janssen R&D he is currently responsible for an Analytical Sciences team in the Drug Metabolism and Pharmacokinetics department. His team focuses on metabolite profiling and identification from discovery to late development, providing support for studies such as metabolic soft spot analysis, reactive metabolite trapping, and animal and human mass balance studies. Another part of the team performs imaging mass spectrometry and sensitive LC–MS analysis for the quantification of drug candidates, metabolites, and biomarkers. He has authored or co-authored 80 scientific publications.

Q. When did your laboratory start working with 2D-LC and when did you start using this technique routinely in your laboratory? What applications is it being used for?

A: We started with the first implementation around 2012, primarily focusing on improving sensitivity in radioactive drug metabolism studies by concentrating the samples online (1). Often we only consume a small portion of the samples we have at our disposal or that can be easily harvested. Injection of a much larger volume of sample can be a way to improve the sensitivity of your analysis.

We gradually expanded the approach with additional pumps, valves, and columns allowing online preconcentration in combination with heart-cut two-dimensional liquid chromatography (2D-LC) (2), providing additional selectivity in cases where we encounter co-eluting radioactive metabolite peaks or to speed up the tedious purification process when structure elucidation with nuclear magnetic resonance (NMR) is required.

A downscaled micro-ultrahigh-pressure liquid chromatography–mass spectrometry (micro-UHPLC–MS) version was built based on the experience we obtained with the systems used for

radioactive and ultraviolet (UV) analyses. This system is primarily applied for very sensitive LC–MS bioanalysis (3).

Q. You developed an online preconcentration method using high-volume injections of biological samples for sensitive metabolite profiling and quantification (2). What benefits does this approach offer and could it be useful in other applications?

A: Radiolabelled mass balance studies are a critical component of drug development to provide insight in the absorption, distribution, metabolism, and excretion (ADME) of a new drug. As radioactivity can be counted in exhaled air and excreta, and “what goes in, should come out”, it allows us to make up a mass balance and know when the majority of drug-related material has left the body, and from which route. In most studies, especially for the in vivo human study, the amount of radioactivity dosed is kept to a minimum, while sample volumes are rather large. Therefore, it is common practice to concentrate the samples prior to injection. We used to perform this step in an offline fashion, but more frequently encountered recovery issues potentially related with advances in formulation science,

that allow to also advance drug molecules that are “sticky” and/or have low solubility. Using an online preconcentration approach we could easily avoid losses of radioactivity, that is, compound-related material, and save a lot on otherwise time-consuming method development. Since the purpose of the drug metabolism studies is the identification and quantification of drug metabolites, the compounds we are analyzing are still unknown prior to analysis. Therefore, we cannot check their stability and recovery, and should avoid any related risk, such as drying steps, during offline sample concentration. Another approach we routinely apply is partial drying of a sample, for example, after protein precipitation with acetonitrile or methanol extraction of faeces. A fixed volume of dimethyl sulfoxide (DMSO) is added to the sample and dried to the DMSO content. This way, complete drying of the sample is avoided at which point the majority of degradation usually occurs and also makes reconstitution of the compounds in solution much more difficult. The DMSO solution can be injected afterwards without affecting chromatographic performance thanks to an online dilution during sample loading with a high content of water.

The approach we use for online preconcentration was, of course, already widely used in many applications. The main

difference with “trap-elute” approaches routinely applied, is that we use a much larger 4.6×50 mm high-performance liquid chromatography (HPLC) column for sample loading. This makes it possible to inject up to 100 mL of urine and tens of mL protein-precipitated plasma or blood, faeces extracts, and so on, without deterioration of the chromatographic separation. Moreover, LC resolution often improves as a result of the back-flushing mode we use to elute the trapped sample compounds to the analytical column. We also notice a longer life-time of our analytical columns when this approach is used. Often a replacement of the trapping HPLC column is sufficient to restore deteriorating LC performance after multiple large volume injections.

Q. You combined the method using very high-volume injections with heart-cut 2D-UHPLC for selective drug metabolite trace analysis

(2). What is novel about this approach?

A: This is an extension of the online preconcentration approach discussed previously, which allows large volume injections (1–100 mL) in combination with heart-cut 2D-LC. This setup combines ultimate sensitivity with improved selectivity. The setup



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uses four columns: two 3-mm-id. analytical columns for the first and second dimension separation, each preceded by a 4.6-mm-id. column to concentrate the sample or trap the compounds of interest. This way we were able to reach a quantification limit of 770 attogram/mL of a tritiated imipramine metabolite by injection of 100 mL of urine (2). For context, 770 attogram/mL is the same concentration as one 4 g sugar cube dissolved in more than 2 million Olympic swimming pools of 2.5 million litres each.

Q. What were the main challenges you encountered when developing this method?

A: These setups are not readily-available, “off-the-shelf” systems. Most UHPLC users and vendors consider a few tens of μL already a large injection volume. Therefore, ten years ago there were not many UHPLC systems available providing the flexibility to go beyond 50–200 μL injection volume. Since the ability to inject larger volumes is the only way to make a combination of UHPLC with online radioactive detection work for metabolism studies (4), we had already tackled this problem in previous years. Using a modular approach with separate autosamplers, pumps, valves, and so on, a lot of powerful combinations can be built. The most challenging part, however, is not the hardware configuration but finding the right software solution to control all the different modules in an easy and robust way. For this we heavily rely on the vendors, and especially their expertise in supporting less routine applications.

Q. What applications is this technique being used for in your laboratory and what benefits does it offer the analyst? Could this technique be useful in other applications?

A: Since the constituents of a metabolism sample are still unknown prior to analysis and often differ from sample to sample, we regularly encounter situations where new metabolites pop up that were not found earlier in other in vitro or in vivo samples. If these metabolites co-elute with other metabolites, we can use the heart-cut 2D-LC approach to separate these, which is the only way for accurate quantification in radiochemical detection for which baseline separation is required.

Another application is selectivity enhancement, separating analytes of interest from interfering matrix components. Despite the fact that we are dealing with relatively dirty in vitro and in vivo samples (such as urine, blood, faeces, bile, and tissue), we usually keep sample preparation to a minimum since we don't know the metabolites we are analyzing yet and any sample preparation might introduce loss or degradation of some or all of the metabolites. The online preconcentration approach previously discussed is very good if a selective

detection method such as radioactive detection or qualitative MS is used. If, however, the detection suffers from selectivity (such as UV) or matrix effects (for example, quantitative MS), heart-cut 2D-LC can be a solution. We use this, for example, to get baseline separated UV peaks of metabolites for which we don't have authentic standards. UV detection is more reliable to estimate the relative abundance of metabolites (if the chromophore is not dramatically changing relative to the parent drug) compared to MS, for which the response is more structure dependent. The combination with an HPLC trapping column prior to the first dimension, allows us to further boost the sensitivity of our analyses. Another application is the purification of metabolites for NMR structure identification, requiring relatively large amounts of compound in high purity. The combination of online preconcentration with heart-cut 2D-LC offers a way to purify large volumes of sample with high chromatographic performance on 3-mm-id. columns packed with sub-2.5- μm particles. The purification could also be done with offline 2D-LC. The main benefits of the online approach, however, is that it reduces manipulation of the sample and, thus, reduces the time and potential errors and artefacts during sample preparation. A larger volume injection and trapping between the two dimensions is preferred over drying and reconstitution in a small volume, with the risk of degradation or incomplete recovery of the analyte of interest.

Q. The 4-column multidimensional micro-UHPLC–MS method you developed offers high sensitivity and selectivity for the quantitative analysis of drugs in biological samples (3). What is novel about this method and what benefits does it offer the analyst?

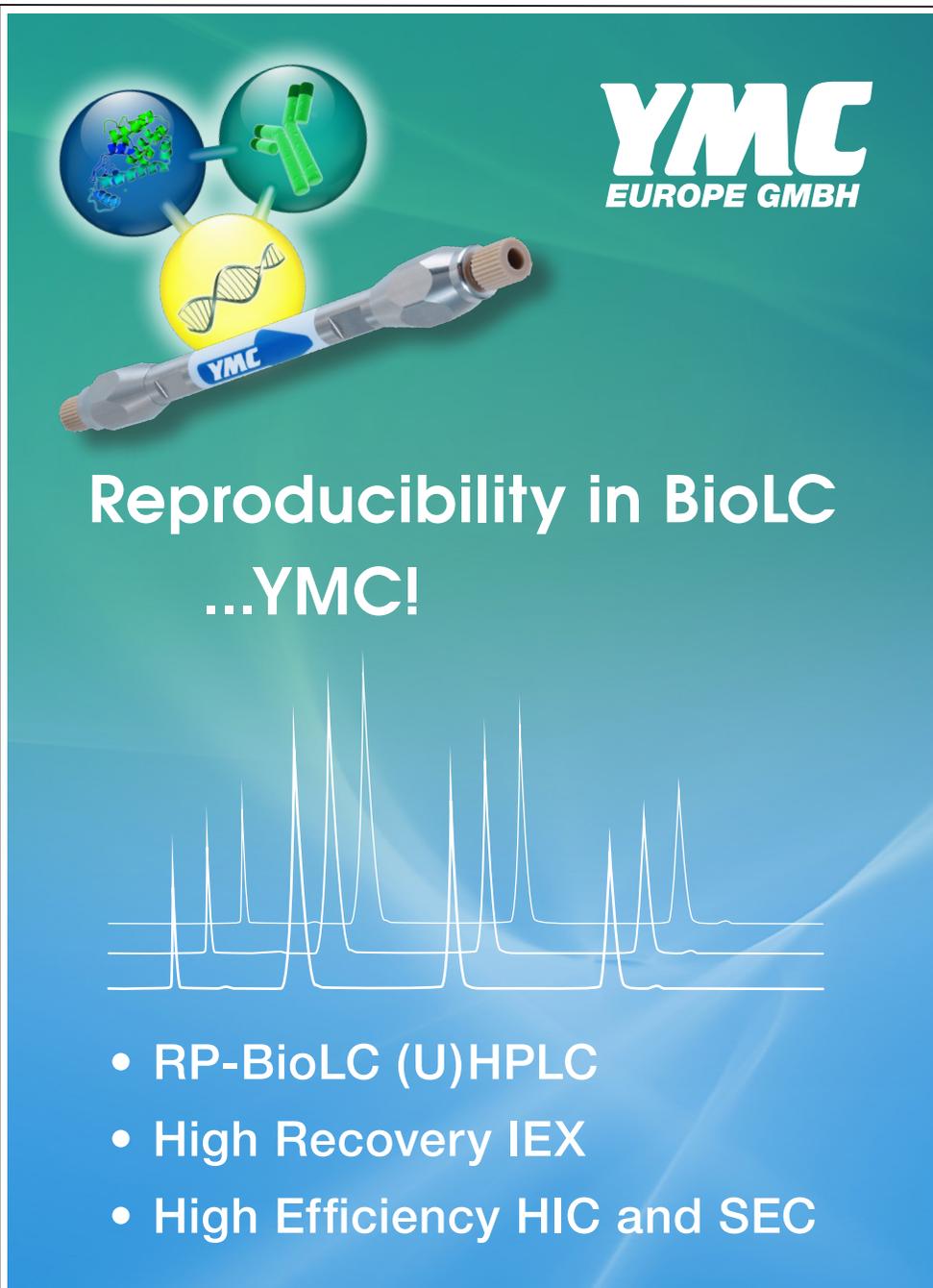
A: The micro-UHPLC-MS setup was built based on the setup and experience obtained with the system using two 4.6-mm-id. trapping columns and two 3-mm-id. analytical columns. While the previous setup was aiming for sensitive and selective LC–radioactive and LC–UV detection, this setup was targeting ultrasensitive LC–MS quantification. Since sampling efficiency in electrospray ionization (ESI) largely improves with decreasing flow rates to the ESI emitter (5,6,7), a gain in MS sensitivity can be obtained in going to columns with a smaller internal diameter. Although in this perspective, nano-LC should provide the biggest gain in sensitivity, we are making use of micro-LC in this setup because it is considered the best compromise between sensitivity and throughput, which is also very important in the bioanalytical environment in which we are working. The sensitivity gain, often advocated for nano- and micro-LC–MS, should also be put in perspective of the sample volume

available. If the sample volume is not the limiting factor, the sensitivity gain in going to nano- or micro-LC will usually be negligible compared to an injection of a larger volume of the same sample on a larger bore column that has a higher sample loadability. Trap-elute is a potential solution to increase loading on smaller bore columns, but we often observe a less than proportional gain in sensitivity, for example, a 10-fold higher sample loading often results in only three-fold higher sensitivity. This can be explained by the equally concentrated sample matrix constituents negatively affecting sensitivity by matrix effects (ion suppression). Using heart-cut 2D-LC, the analyte of interest can not only be concentrated but also separated from the sample matrix. Therefore, we usually see a proportional gain in sensitivity when using the 4-column micro-UHPLC-MS setup. By gradually decreasing column internal diameters from 2.1 mm (first trapping column) to 1 mm (first dimension analytical column) to 0.5 mm (second trapping column) and finally to 0.15 mm (second dimension analytical column) we are able to inject much larger sample volumes onto micro-LC with a reasonable throughput (± 15 min total run times). As illustrated in reference 3 the lower limit of quantification (LLOQ) we can obtain with this setup are about 250- to 500-fold better than with direct injection on micro-LC and 10–50 times better compared to trap-elute. For midazolam and its 1'-OH-metabolite we were able to reach an LLOQ of 100 fg/mL. This is comparable to a concentration of one 4 g sugar cube in 16 000 Olympic swimming pools of 2.5 million litres. In the midazolam example, we were limiting ourselves to the maximum injection volume feasible on the nano-LC system due to its maximum loop size and syringe volume.

We are confident that, based on the experience we have with the larger-bore 4-column system, we can inject even higher volumes using multiple injection cycles without affecting the chromatographic performance.

Q. How is this approach used in your laboratory?

A: This 4-column micro-UHPLC-MS setup requires more expertise, is more cumbersome to use, and has a lower throughput than our standard UHPLC-MS methods applying 2.1 mm \times 50 mm columns and 1–3 min run times. Therefore, we only use it for samples where the absolute sensitivity is required to get results. Typical



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examples where this setup can be of value in our field are quantification of drug candidates in microdose studies, extremely potent compounds, and (protein and other) biomarkers. Offline 2D-LC and different sample preparation protocols are also considered and can offer some benefits, for example, when multiple analytes are targeted. The main benefits of the online 2D-LC approach in this micro-LC setup are identical to that discussed for the system using larger bore columns, that is, increased automation and reduced sample manipulation, resulting in less chances for errors and degradation or loss of the analyte of interest.

Q. 2D-LC is often regarded mainly as a research tool and not suitable for routine analysis. Is this view changing in practice? Is there more scope to use 2D-LC routinely in pharmaceutical and biopharmaceutical analysis?

A: There is clearly a trend towards increased use of 2D-LC in pharmaceutical analyses, especially heart-cut 2D-LC (rather than comprehensive 2D-LC). While currently 2D-LC is primarily exerted in less routine, non-GXP environments. I am confident that the use in more routine analyses will grow further, especially in areas such as large molecule analyses (such as proteins and oligonucleotides) where sample preparation can be challenging and one dimensional LC does not offer the required separation power.

Recently, more and more LC vendors are offering 2D-LC instrumentation as a full package or as an optional expansion of regular LC systems including the right software to warrant better robustness (for example, communication between the different modules) as well as improved user friendliness. This proves the increasing interest of the industry in 2D-LC and significantly lowers the threshold for new users and application in more regulated and routine environments.

In order to be successful in the implementation of technology such as 2D-LC in the (bio)pharmaceutical industry, it is important to make its use as simple and straightforward as possible. There is no time to tweak the system for every new study, nor is there time to allow elaborate method development for every sample. This is also clear in the choices we made for the setups and methods we are using. The valve setup is chosen in such a way that it allows switching between the larger volume injections (large loop size) using online preconcentration and smaller direct injections (small loop size) without any replumbing. We use trap (5–10 μm particles) and analytical columns (2.2–2.5 μm particles) packed with the same stationary phase avoiding that any analyte of interest would be more retained on the trap than on the analytical column. We use stationary phases in the

first and second dimension that might not be extremely orthogonal but work for most drug-like molecules, such as, C18 and phenyl-hexyl, and always combine this with an as orthogonal as possible pH (for example acidic and basic) between both dimensions. The use of a trapping column in combination with solvent dilution between both dimensions might look more complex than a simple loop approach but allows the use of orthogonal conditions in the second dimension without having to worry about elution strength (8,9,10). The only thing that needs some, but little optimization is the solvent strength for loading the sample and the dilution ratio with water to avoid breakthrough of analytes. A more apolar analyte will generally require a higher solvent strength to avoid loss by adsorption to the loop wall. Since metabolites are usually more polar than the candidate drug, we use the candidate drug for optimization and to test potential losses or carry over. Next, we adjust the loading pump/dilution pump flow rate ratio to get the right dilution factor depending on the expected polarity of the metabolites.

Q. Do you have any advice for analysts who are using 2D-LC for the first time (or thinking about it)?

A: Every implementation of new technology should, of course, bring value. Therefore, it is important to define your current challenges and weigh the pros and cons of an offline sample preparation and/or offline 2D-LC approach versus an online approach. If, for example, instrument availability is the major bottleneck in your laboratory, an online 2D-LC approach that generally consumes more instrument time might not be the best way forward.

When building your own instrument, it helps if you can start from existing, ideally commercially-available, 2D-LC setups. It is also good to go step-by-step and get fully acquainted with every step before you go to the next step. The systems we are currently using were not configured at once, but built step-by-step over many years. Every additional step was only taken when we got fully acquainted with the previous installation of an additional valve, etc., to allow additional functionality. Only when we got full control and enough experience, were we thinking about the next potential improvement. We first optimized the UHPLC-radioactive detection coupling. Then we added two pumps, two valves and a column for the online preconcentration and expanded this approach to three other systems. Then the initial setup was complemented with some additional pumps, columns and valves to allow heart-cutting 2D-LC. The knowledge obtained with this was used to get the 4-column micro-UHPLC-MS system up and running. We added another pump providing

a make-up flow to the MS, to get a larger split ratio going into the radioactive detector. And now we are thinking about the next step where we want to add an extra UV detector to follow both the first and second dimension in UV simultaneously to improve the efficiency in metabolite purification.

Q. Are there any areas of chromatography that you think are particularly exciting or innovative at the moment?

A: The increasing number of commercially available “plug and play” nano- and micro-LC–MS systems is an interesting trend. The new low flow MS sources with integrated column technology or “dummy proof” LC connections allow (almost) trouble-free application of micro- and nano-LC–MS by non-expert users to gain sensitivity or reduce sample consumption. Hopefully, we can also welcome some smaller footprint systems in the future, integrating nano-LC and MS in one optimized system, thereby further reducing dead volumes to allow higher throughput. This might convince more analysts to use micro- or nano-LC–MS to also reduce solvent consumption for more “environment-friendly” analyses. If, however, peak capacity is what you are looking for this can now be better achieved in combination with 2 m columns

filled with a stationary phase based on micropillar array technology (11) for high-resolution separation power, which is definitely another promising and exiting innovation in the field.

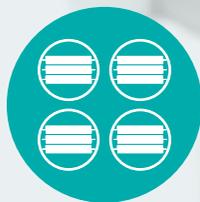
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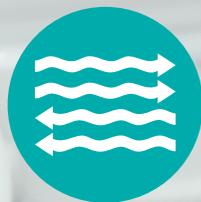
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The Applications Book

The Applications Book

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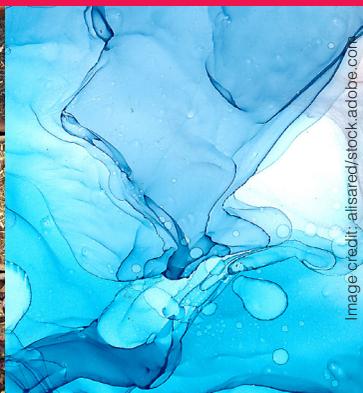
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Wyatt Technology Corp.



Cylinder Free Auto-GC-MS for Continuous SVOCs, VOCs, and PAHs Monitoring in Air

Franck Amiet, Chromatotec Group, France

Ambient air is polluted by many semi-volatile and volatile organic compounds (SVOCs and VOCs) originating from anthropogenic and natural sources. These compounds contribute significantly to the formation of tropospheric (ground-level) ozone (O_3), resulting primarily from the reaction of VOCs with NO_x (NO and NO_2) in the presence of sunlight, and secondary organic aerosol (SOA) by oxidation.

Automatic, continuous identification and quantification of VOCs in ambient air is possible through artificial intelligence: software compares results from FID and MS systems for validation of results and identifies molecules by their ion's spectrum. The system does not need an expert to interpret and analyze data.

Due to the large number of molecules, the complete separation of all compounds is difficult to perform using gas chromatography (GC) columns. Mass spectrometry (MS) detection must be added to identify a list of VOCs with expertise, also allowing identification of unknown compounds with FID or MS.

To respond to those needs, a new Field Dual Thermal Desorption (TD)-GC-MS/Flame Ionization Detector (FID) solution has been developed. This standalone system provides automatically- and continuously-validated results for the identification and quantification of more than 120 different VOCs as standard, directly on the field without the need of any daily operator.

Introduction

The airmOzone expert has been selected by the United States Environmental Protection Agency (USEPA). The USEPA requires Photochemical Assessment Monitoring Stations (PAMS) to collect ambient air pollutant and meteorological data to attain the National Ambient Air Quality Standard (NAAQS) for ozone nationwide.

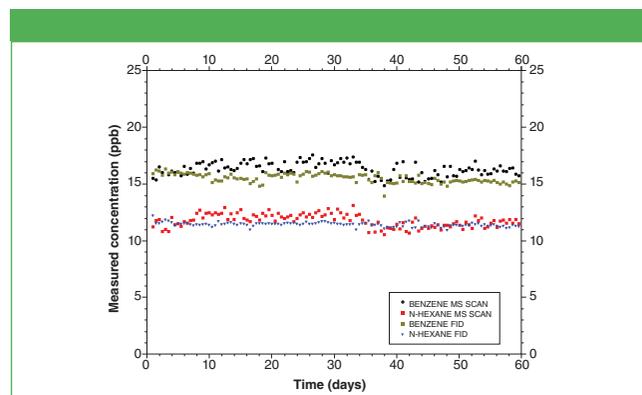


Figure 1: Repeatability on internal calibration with airmOzone + DET QMS (120 days).

The VOCs measured are gaseous aliphatic and aromatic hydrocarbons in ambient air up to C_{20} , including the components of interest at the PAMS programme as well as the extra chlorinated compounds covered by Compendium Methods TO (Toxic Organic)-14 (EPA 625/R-96/010b) and TO-15. The data collected at the PAMS sites include measurements of a target list of VOCs such as paraffins, olefins, aromatics, and alkynes (1).

Additionally, the system is used by various reference institutes and universities to monitor Polycyclic Aromatic Hydrocarbons (PAHs) in industrial sites, tropical forests, and on-site under decontamination. Volatile PAHs measured in standard are: naphthalene, 2-methyl-naphthalene, 1-methyl-naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, and anthracene. Benzo(a)pyrene can now also be analyzed.

Instrumentation

The system consists of two automatic gas chromatographs (airmVOC C2-C6 and airmVOC C6-C20) both equipped with a FID detector, one for light compounds (C_2 – C_6) and the other for heavy compounds (C_6 – C_{20}). Both TD-GCs are coupled to a single Quadrupole MS.

Air generators (airmPure, Chromatotec®) were used for the flames of FIDs, valve actuations of the auto-TD-GC-FIDs, and drying of samples for C_2 to C_6 analysis, and hydrogen generators 99.9999% with dew point below $-15\text{ }^\circ\text{C}$ (Hydroxychrom, Chromatotec®) were used as carrier gas and to fuel the FID flames.

It can automatically identify and quantify VOCs down to ppt levels. Results are obtained every 30 min for molecules from 2 to 20 carbon atoms simultaneously. LOQ for Benzene is as low as 1 ppt in automatic.

The equipment is specially designed for field applications with the best performance and flexibility, optimizing the space. It can be placed in one cabinet or in two smaller ones, each one occupying less than one square metre of floor space. This miniaturized system is transportable, thanks to its wheels, and the inside is easily accessible thanks to the rack sliders, perfect for installation in mobile laboratories for on-site analysis.

Results

Characteristics of the analytical method

The stability and accuracy of the system is automatically checked every day by the internal calibration system, with benzene permeation tube for validation of the complete system and to provide automatic data validation. Both detectors gave an excellent correlation between their response and the concentration ($>R^2=0,999$).

The robustness and long-term stability of the complete system allow validating data without the need for frequent calibration. The results are extremely stable at 10 ppb level with, for example, on

benzene peak area RSD at 3.77% for MS detector and 2.51% for FID detector over more than 120 days without tuning or calibration during this period (Figure 1). The concentrations are automatically computed using VistaMS software which calculate the compounds concentration analyzed using high intensity on selected ion for each species.

The performance of the system was studied using permeation tubes, certified cylinders containing VOC mixtures (PAMS, TO14 and TO15), and a dilution system equipped with two mass flow controllers and a dilution chamber. With this system, concentrations ranging from 0 ppb up to 40 ppb were generated.

Solving coelutions in ambient air samples

Compounds taking part in a coelution can be identified by FID coupled to MS detection. When a single FID detector is used, interferences may occur but the additional MS can identify individual components of a peak by choosing the compound with the best matching mass spectra pattern available in the NIST library. Thus, the peak at 680 s, identified as only N-Octane in Figure 2(a), could be deconvoluted into N-Octane and tetrachloroethene in Figure 2(b).

Validation of the results

The determination of validated results by the automatic intercomparison of FID and MS results was performed automatically by VistaMS software following an algorithm that privileges the result obtained by FID because it is a very accurate and sensitive detector. In case of coelution, validated results are considered those obtained using the MS detector, due to its ability to identify individual compounds. Thus, dual-detection improved the accuracy of the identification and quantification of the compounds in ambient air by 14%, with precision values expressed as RSD better than 5%.

Identification of unknown compounds

Two unidentified peaks of less than 1 ppb were found in the GC-MS data. Compound identification was performed automatically by comparison of the mass spectrum (*m/z* values and the relative intensities) obtained with those from the NIST Library by VistaMS software. Figure 3 shows the excellent spectral match obtained for Hexanal and Furfural by C6-C20 analysis.

Thanks to NIST library, all molecules SVOCs, VOCs, and PAHs from 2 to 20 carbon molecules can be identified by MS and quantified by FID using theoretical response factors. Thus, the standard parameter list can be adapted according to the needs of the measurement campaign (for example, depending on the geographical area) or if the requirements of the PAMS, TO14, and TO15 methods change.

Conclusion

In this study, 120 different VOCs from C2 to C20 range were quantified at ppb and ppt level. Potentially coeluted compounds were identified and validated by VistaMS software thanks to the combination of FID and MS detectors. Any unexpected or unknown compounds are automatically identified thanks to the internal NIST library linked to VistaMS in the internal computer of the GC-MS system.

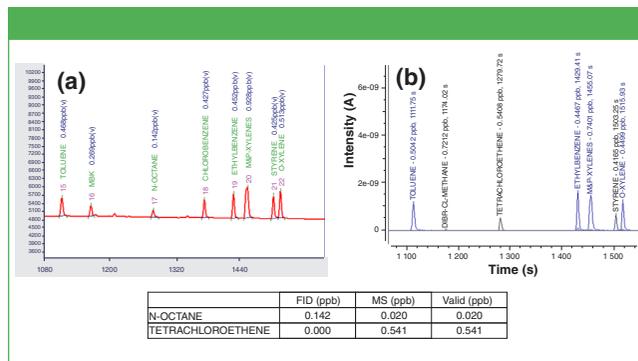


Figure 2: Chromatograms, intensity of the signal [A] vs. retention time (s), of the N-Octane and tetrachloroethene coelution observed by FID detection (a) solved with the MS (b) for C6-C20 analysis.

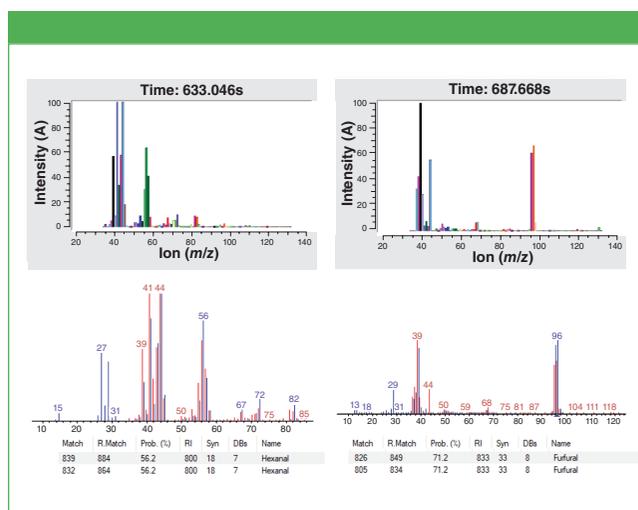


Figure 3: Automatic comparison between mass spectra obtained (top) and reference (bottom).

This fully automatic, intrinsically linear, precise, and very stable system with data validation allows non-specialist operators to access expertise-level results. No cylinders are required: the FID can be operated continuously using hydrogen and zero air generators. All data collected are stored to assure their full traceability and availability.

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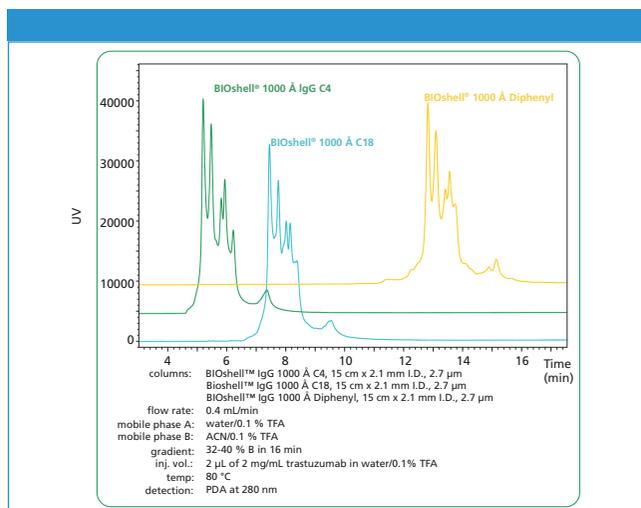


Figure 1: IgG separation on 1000 Å SPP particles.

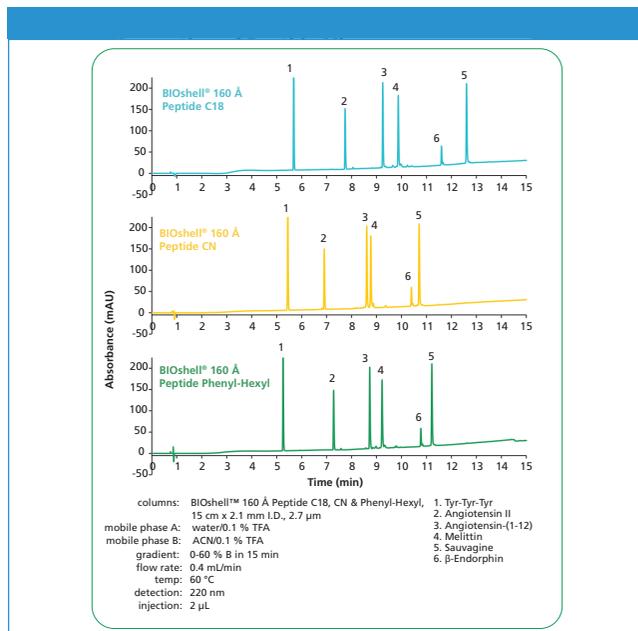


Figure 2: Separation of peptide mix.



Figure 3: BIOshell™ UHPLC and HPLC columns.

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High Throughput, Routine and Comprehensive Proteome Analysis using a Micro Pillar Array Column (μ PACTM)-based Capillary-flow LC–MS Workflow

Jeff Op de Beeck and Geert Van Raemdonck, PharmaFluidics, Gent, Belgium

In this application note, optimal LC methods for a variety of LC-MS based proteome analysis needs are described. PharmaFluidics' μ PACTM capLC column is evaluated for different user needs ranging from 12 samples per day in a comprehensive mode and maximizing the data throughput to as high as 65 samples per day for high sample throughput analyses. 2 μ g of tryptic digest sample (HeLa cell lysate) was injected and separated using reversed phase liquid chromatography over a range of flow rates (2–10 μ L/min) and solvent gradients (15–120 min).

High throughput proteome analysis: For short LC–MS methods where high sample turnover is required, the relative time that is spent on sample injection and column equilibration can become a crucial factor affecting data productivity. Working at higher flow rates is considered a valid strategy to reduce the impact of these processes. A high throughput capillary flow LC method at 10 μ L/min has been defined that provides optimal MS time use within a time frame of nearly 21 min. Figure 1 and Table 2 summarize the metrics that could be obtained for a tryptic HeLa cell digest sample. Base peak chromatograms obtained for triplicate injections of 2 μ g HeLa cell digest clearly indicate the high level of chromatographic repeatability that can be achieved. Up to 83% of the total time was effectively used to identify peptides at a sample turnover rate of 65 samples per day, making this among the most effective ever reported within capillary/microflow LC–MS based proteomics. Recent literature reports on percentages between 65 and 80% for similar sample turnover workflows (1–3). Combining the output of all 3 runs resulted in a total number of peptide and protein groups of respectively 3397 and 804. With values down to 0.12% CV or 0.75s average variation in retention time, an exceptionally reproducible and robust high throughput LC–MS based proteomics workflow is demonstrated (Table 2).

Routine proteome analysis: An optimal μ PACTM capLC–MS method was developed that can be used to maximize the output of routine proteome analyses with run times in the order of 60 min. Operating the column at

Table 1: Experimental conditions	
LC	
LC system	Thermo Scientific TM Ultimate 3000 LC system
Analytical column	PharmaFluidics μ PAC TM capLC C18
Mobile phase	A: Water (100%) with 0.1% (v/v) FA B: Water/acetonitrile (20/80) with 0,1% (v/v) FA
Loading buffer	Water/acetonitrile (99/1) with 0,1% (v/v) TFA
Flow rate	2–10 μ L/min
Gradient profiles	non-linear 1–35% B in 17.5 - 54 - 108 min gradient
Temperature	50 °C
Sample	500 ng/ μ L HeLa cell digest
Injection	4 μ L user defined prog injection (direct injection)
Source	
ESI source	Thermo Scientific TM nanoflex ion source
ESI emitter	Thermo Scientific TM electropolished stainless steel emitter 30 μ m tip (ES542)
Polarity	Positive
Capillary temperature	275 °C
Spray voltage positive ion	2100 V
MS settings	
MS instrument	Thermo Scientific TM Orbitrap Elite TM
Acquisition mode	DDA
MS1	
Resolution	240 000
Maximum IT	30 ms
Scan range	300–1600 m/z
MS2	
AGC target	100000
Maximum IT	250 ms
TopN	20
Isolation window	2.0 m/z
NCE	35
Charge states rejected	unassigned and 1
Charge states not rejected	2+,3+,4+
Dynamic exclusion	20 sec
Lock mass	445.120020 Da (polysiloxane peak) (internal calibration)

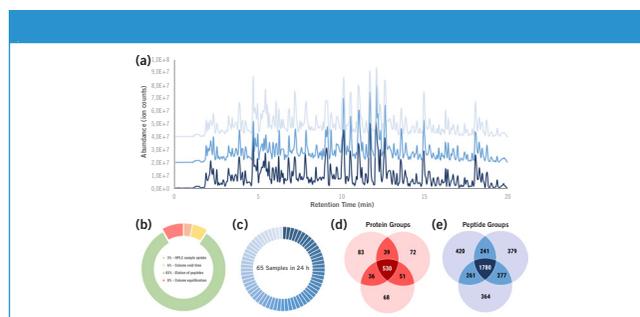


Figure 1: High throughput proteome analysis with a μ PACTM capLC column.

Table 2: Overview of chromatographic metrics and proteome coverage results

		Total analysis time	Runs/day	Flow rate	Elution window	Effective time use	Peak width FWHM / 4 σ	Peak capacity FWHM / 4 σ	Δ Retention time	Δ Retention time	Peptide ID's	Protein ID's
		(min)	(/)	(μ L/min)	(min)	(%)	(s)	(/)	(s)	(%CV)	(/)	(/)
Method	Through-put	20.7	65	10	17.1	83	4.0/6.7	259/153	0.75	0.12	3397	804
	Routine	60.7	24	5	54.3	89	8.3/13.9	395/234	1.13	0.08	10003	1972
	Compre-hensive	120.7	12	2	101.5	84	11.6/19.6	526/311	1.81	0.07	15954	3105

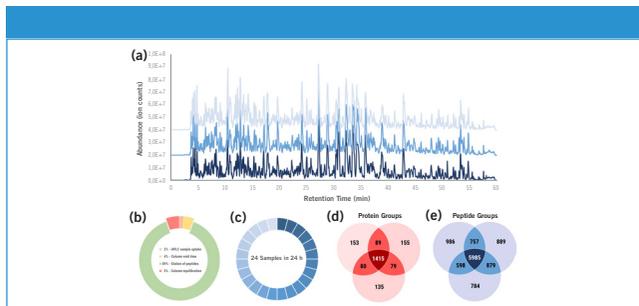


Figure 2: Routine proteome analysis with a μ PAC™ capLC column.

a flow rate of 5 μ L/min will produce the highest peak capacity (and thus the sharpest peptide peaks) for this gradient time. Using this method, the first and last peptide peak elute respectively around 3.4 and 57.7 min, generating an elution window of nearly 55 min and nearly yielding an effective MS time use of 90% (Figure 2). At a sample turnover rate of 24 samples per day, a little more than 10 000 peptide groups and close to 2000 protein groups could be identified by combining three replicate injections. An average FWHM of 8.25s was calculated from the apQuant node, equivalent to a peptide peak width at the base of 14s or 0.24 min and delivering a peak capacity value close to 250. As can be expected for methods that give an increased elution window, the relative variation in retention time drops even further (0.08% CV) even though a slight increase in absolute value is observed (going from 0.75s for the high throughput method, to 1.13s for the routine method – Table 2).

Comprehensive proteome analysis: When aiming for highest peak capacity that can be achieved with this column, it is advised to operate the column in the lower range of the capillary flow rate regime (1–3 μ L/min). Even though the contribution of overhead time to the total analysis time becomes more significant, the combination of a low flow rate and long gradient time will be most beneficial when comprehensive proteome analysis is required. Using this method, the first and last peptide peak elute respectively around 8.3 and 110 min, generating an elution window of over 100 min and nearly yielding an effective MS time use of 84% (Figure 3). At a sample turnover rate of 12 samples per day, close to 16 000 peptide groups and 3105 protein groups could be identified by combining three replicate injections. An average FWHM of 11.58s was calculated from the apQuant node, equivalent to a peptide peak width at the base of 19.7s or 0.33 min and delivering a peak capacity value of 311 (measured at the peak base – Table 2). In accordance with the observations when comparing the high throughput and routine method, a decrease in relative retention time

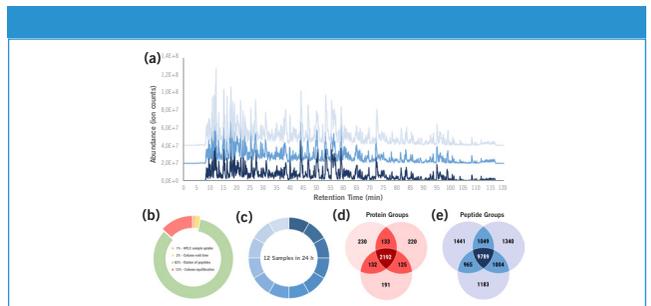


Figure 3: Comprehensive proteome analysis with a μ PAC™ capLC column.

variation is observed (0.07% CV) even though the absolute retention time variation increases to 1.81s (based on all shared PSM's).

Conclusions

Using optimal LC methods for a variety of LC–MS based proteome analysis needs, the recently launched PharmaFluidics' μ PAC™ capLC column provides versatile output in terms of separation performance and throughput. As a consequence of the low column back pressure, the column can be operated over a range of flow rates, enabling analytical scientists to tune LC methods according to the sample complexity or to the size of the sample set. Three common separation needs in LC–MS proteome research have been identified, and tailored LC methods with optimal output in terms of data productivity and sensitivity have been developed. In addition to the flexible operation benefits, the perfect order and unique control of stationary phase design result in excellent chromatography at the highest level of LC reproducibility.

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Size-Exclusion Chromatography for the Impurity Analysis of Adeno-Associated Virus Serotypes

Stephan M. Koza and Weibin Chen, Waters Corporation

Monitoring the size heterogeneity of AAV-based gene therapy therapeutics is potentially important to ensure consistent product quality and efficacy. We demonstrate that the levels of both high-molecular weight (HMW) and low-molecular weight (LMW) impurities in AAV capsid preparations can be separated on a Waters™ 450 Å Protein BEH SEC column for a series of AAV serotypes.

As the development of gene therapy products accelerates, the need to develop sound and efficient analytical strategies to help guide the development of manufacturing processes and evaluate the quality of clinical adeno-associated virus-(AAV)-based gene therapy materials has become more important. Among other critical quality attributes, the levels of potential AAV high molecular weight (HMW) species and AAV fragments or low molecular weight (LMW) species may also require monitoring (1). Here we present optimized non-denaturing size-exclusion chromatography (SEC) methods that can separate soluble AAV for several AAV-CMV-GFP serotypes including AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9.

Experimental Conditions

AAV sample: AAV serotypes ranging from 1.6×10^{12} to 6.7×10^{13} GC/mL

LC system: ACQUITY™ UPLC™ H-Class Bio
 Sample temp.: 6 °C
 Column temp.: 25 °C
 Injection volume: 3 µL
 Column: XBridge™ Protein BEH SEC, 450 Å, 3.5 µm, 7.8 × 300 mm
 Fluorescence detector: Excitation: 280 nm; Emission: 350 nm
 Mobile phase: 10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 200–400 m KCl, pH 6.6 (HCl)
 Flow rate: 0.6 mL/min

Results and Discussion

The upper analyte size limitation for SEC separations of proteins is approximately 100–200 nm (subvisible) depending on SEC particle size. Above these limits, the analyte may be altered due to shear forces or trapped by the frits or packed bed of the column. Therefore, these subvisible entities are analyzed using dynamic light scattering and nanoparticle tracking analysis (NTA) methods, among others (2). With these considerations, the SEC separation of soluble AAV monomer, and HMW, and LMW was evaluated on a 450 Å pore-size BEH diol-bonded SEC column. This column was previously demonstrated to be effective in the separation of IgM pentamer

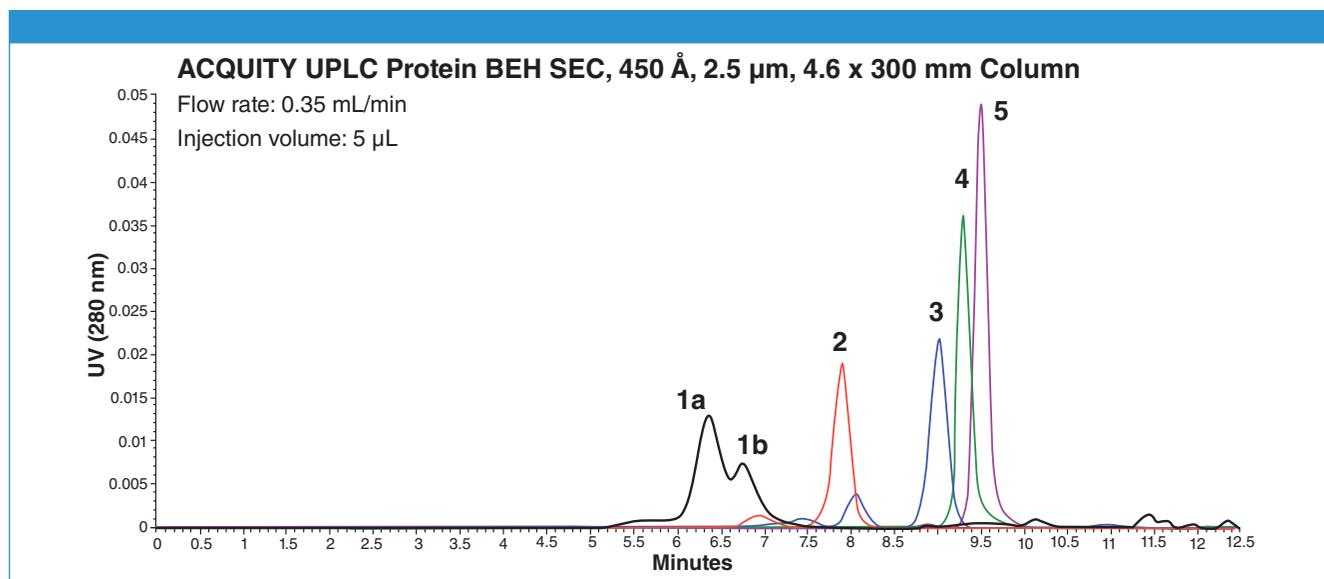


Figure 1: Shown is the SEC separation of several protein structures. Compounds are: 1a. IgM dipentamer (1.8 kDa), 1b. IgM pentamer (900 kDa), 2. thyroglobulin (667 kDa), 3. apoferritin (443 kDa), 4. β-amylase (200 kDa), 5. IgG (150 kDa). UV absorbance (280 nm) and the identities of the peaks observed for the IgM sample were confirmed by SEC–MALS analysis.

and dipentamer in Waters laboratories (Figure 1) and has a reported diameter of 35 nm (3). AAV has a total protein and ssDNA molecular weight of 5500 kDa; however, the compact structure has a diameter of only 25 nm (4). Therefore, it was predicted that these high-efficiency SEC particles could provide the accessible pore volume needed. A 3.5 μm particle size was selected over 2.5 μm particles to reduce potential sample sieving and shearing effects. Intrinsic protein fluorescence detection was also employed to provide maximum optical sensitivity.

The SEC separation of a null control sample (AAV without ssDNA) was evaluated using a Wyatt microDAWN Multi-Angle Light Scattering (MALS) detector and a Waters ACQUITY Refractive Index (RI) detector (Figure 2). The SEC-MALS data confirmed that adequate separation between the dimeric and monomeric AAV forms was observed. Putative multimeric forms preceding dimer were also observed by fluorescence but could not be assigned molecular weights due to their low abundance.

A mobile-phase consisting of 20 mM sodium phosphate, pH 6.6, with varying amounts of KCl (200–400 mM) was found to maximize the recovery of dimer and multimer for serotypes AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9 (Figure 3). We observe that the peak shapes for both the dimer HMW species and monomeric AAV forms are symmetrical and return to baseline appropriately. In addition, detectable levels of multimeric AAV forms were observed for several of the serotypes. Significant amounts of LMW forms were only observed in AAV9 and AAV6.

Conclusions

A BEH SEC column with an average pore size of 450 \AA and a particle diameter of 3.5 μm was demonstrated to be effective in the separation of AAV monomers from their HMW dimers, lower valency multimers, and LMW fragments. The minimal amount of ionic strength (KCl) required for optimal peak shape and recovery varied by serotype, and separations are presented for serotypes AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9.

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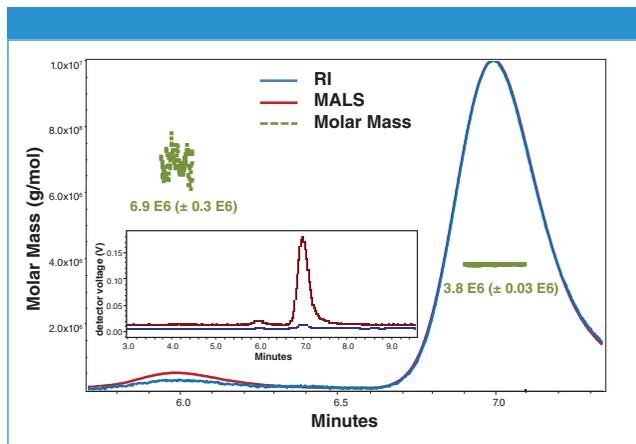


Figure 2: SEC-MALS of AAV8-null sample using refractive index (RI) for concentration measurement are shown. The MALS (red) and RI (blue) signals are normalized and the average and distribution of determined molar masses (green) were determined using Wyatt Astra (v. 7.3.1.9) based on a dn/dc of 0.185 and using a “sphere” model for the icosahedral AAV.

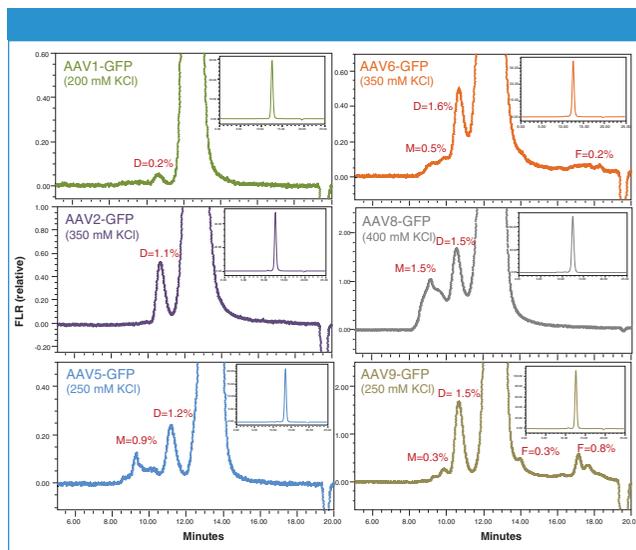


Figure 3: Shown are the SEC separations of a series of AAV serotype control samples containing ssDNA coding for green fluorescent protein (GFP). The peak percentages for dimer (D), multimer (M), and fragments (F) are provided. The chromatogram baselines are zoomed approximately 50x versus the full-scale chromatogram shown in the inset.

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An Anion-Exchange Chromatography Method for Monitoring Empty Capsid Content in Adeno-Associated Virus Serotype AAV8

Hua Yang, Stephan M. Koza, and Weibin Chen, Waters Corporation

The single-stranded DNA (ssDNA) content of AAV capsids in AAV-based gene therapy preparations impacts the efficacy of this treatment modality. We demonstrate that AAV8 capsids without and with full length ssDNA can be separated and their relative abundances determined on a Waters™ Protein-Pak™ Hi Res Q strong anion-exchange column.

During the recombinant adeno-associated virus (AAV) biomanufacturing process, capsids that do not contain ssDNA (empty capsids) are also produced. Empty capsids are unable to deliver ssDNA to the targeted cells and as a result are important to monitor during process development and product manufacturing. An anion-exchange chromatography (AEX) separation driven by differences in surface charge can monitor changes in empty capsid levels (1,2). AEX using fluorescent detection consumes small sample amounts and can provide reliable and reproducible results.

Experimental Conditions

Samples: AAV8-CMV-GFP (full capsid) and AAV8-null (empty capsid) 2×10^{12} capsids/mL; other AAV-CMV-GFP serotypes: ranging from 1.3×10^{13} to 6.7×10^{13} GC/mL = genome copies (physical titer)/mL

LC system: ACQUITY™ UPLC™ H-Class Bio
 Sample temp.: 10 °C
 Column temp.: 30 °C
 Injection volume: 0.2–6 µL
 Column: Waters Protein-Pak Hi Res Q, 4.6 × 100 mm

Fluorescence detector: Excitation: 280 nm; Emission: 350 nm
 Gradient: 70 mM bis-tris propane, pH 9.0, 100–300 mM tetramethylammonium chloride in 20 min at 0.4 mL/min

Results and Discussion

The AEX separation of empty and full AAV8 capsid was optimized for several parameters including pH and salt types (3). In addition, intrinsic protein fluorescence was monitored to provide greater sensitivity and selectivity, and less baseline drift (2). The separation achieved is shown in Figure 1. We observe that the ssDNA “empty” capsids are partially coelute with the ssDNA “full” capsids while showing no significant change in the UV absorbance ratio (280 nm/260 nm, data not shown), indicating the unlikelihood of monitoring AAV8 with partial ssDNA by AEX.

A series of chromatograms for AAV8 samples with differing percentages of empty capsids is presented in Figure 2. The mixtures were generated from >99% pure “empty” and “full” samples. The sample concentrations were normalized based on FLR peak areas from a size-exclusion chromatography (SEC) separation with fluorescence detection and assumed a “full” to “empty” fluorescence response factor ($RF_{F/E}$) of 1.3 (2). For AEX, however, 1.9 was calculated for $RF_{F/E}$ ($Area_{100\% Full} / Area_{100\% Empty}$). The discrepancy in $RF_{F/E}$ values between SEC and AEX may be the result of changes in quantum efficiency under different mobile-phase conditions. $RF_{F/E}$ is then divided into the full capsid peak area to calculate the percentage of

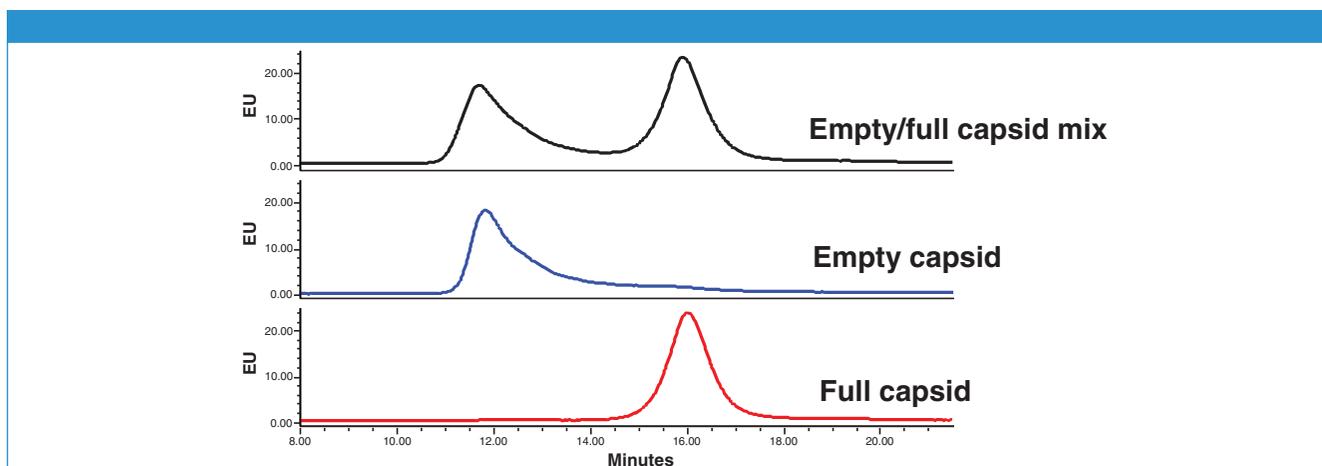


Figure 1: AAV8 ssDNA (CMV-GFP) empty and full capsids are separated on a Protein-Pak Hi Res Q Column using an optimized AEX method (see Experimental Conditions for method details).

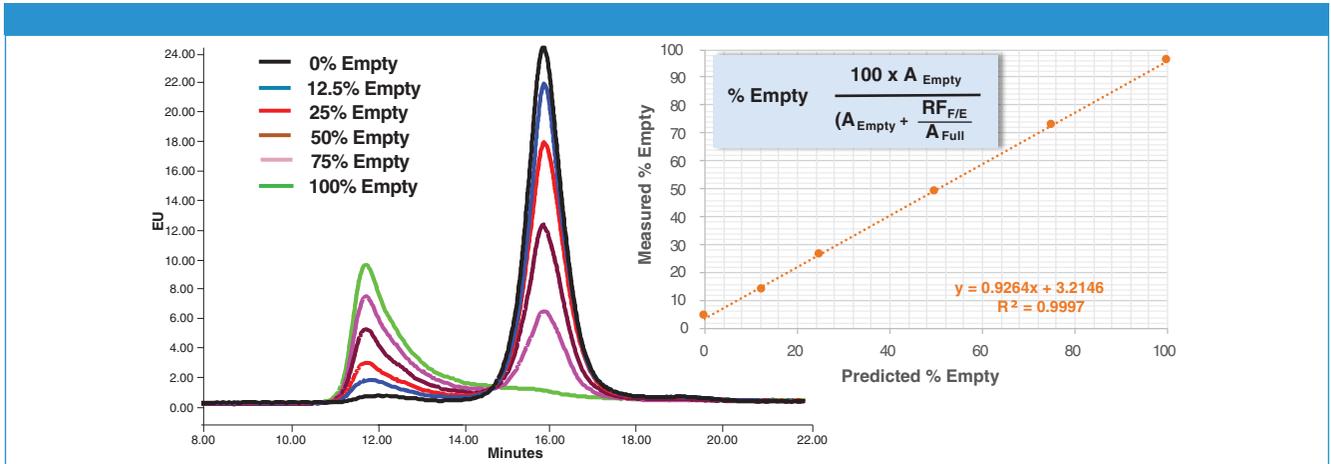


Figure 2: Shown on the left is an overlay of AEX chromatograms for AAV8 samples with differing levels of empty capsid. On the right is the correlation between predicted and measured empty capsid content. Calculation of the percentage of empty capsid was based on the equation shown, which corrects for the difference in fluorescence response between empty and full capsids. A_{Empty} and A_{Full} are the AEX peak areas of the empty and full capsids, and $RF_{F/E}$ is the fluorescence response factor.

empty capsid in a sample (Figure 2). The accuracy of this approach depends on the veracity of $RF_{F/E}$, which requires reasonably pure “full” and “empty” samples, and accurate capsid concentrations. While $RF_{F/E}$ was not rigorously defined in this work, the estimate of $RF_{F/E}$ used here effectively demonstrates the approach to interpretation of the AEX data. Determining $RF_{F/E}$ is not essential, if only relative comparisons of empty capsid levels between samples are required.

Samples of several AAV-CMV-GFP serotypes were also evaluated (Figure 3). All tested serotypes were retained on the column, but their retention varied significantly.

Conclusions

AEX will not likely provide a measurement of AAV8 capsid carrying partial ssDNA, therefore complementary methods such as analytical ultracentrifugation (AUC) would be needed for that analysis. However, due to the technical challenges and costs of these methods, AEX may still provide utility as an initial screening tool in support of manufacturing process development and as a more precise assessment of changes in empty capsid content in product quality testing.

Due to differences in capsid protein surfaces, method optimization may be needed when separating empty and full capsids of other AAV serotypes, and the AEX method described here can serve as a useful starting point.

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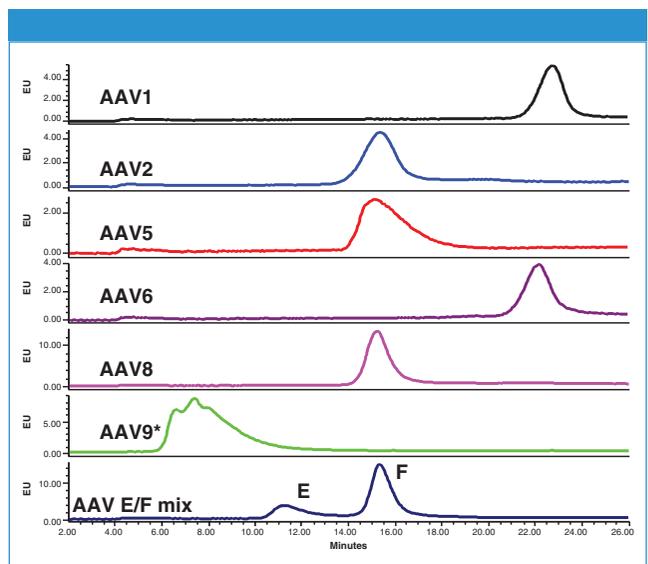


Figure 3: Chromatograms of AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9 serotypes (full capsids, CMV-GFP ssDNA). For comparison, the bottom chromatogram shows the optimized separation of AAV8 empty and full capsid. Separation conditions are as described in the text for all serotypes except for AAV9 (*) where the salt gradient was 0–200 mM KCl.

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LMW Peptide Characterization by SEC-MALS

Wyatt Technology Corp.

Multi-angle light scattering with size-exclusion chromatography (SEC-MALS) is widely used for absolute characterization of proteins and polymers. As shown in this application note, it is also suitable for characterization of low-molecular-weight peptides below 1000 g/mol.

Multi-angle light scattering coupled to size-exclusion chromatography (SEC-MALS) is widely recognized as an essential method for absolute characterization of proteins and polymers, because it is a first-principles method that does not rely on column calibration or assumptions regarding the conformation or column interactions of the analyte. While often used for larger macromolecules, SEC-MALS is perfectly suitable for analysis of the molar mass of small peptides, despite their low molecular weight (LMW). The non-globular conformation of such peptides, which causes them to elute differently than well-folded proteins, is not an obstacle. In fact, a DAWN® or miniDAWN® multi-angle light scattering (MALS) detector is instrumental in obtaining on-line molar mass determinations for peptides of fewer than a thousand g/mol.

Figure 1 shows the chromatograms obtained from a mixture of proteins and peptides separated on a TSK G2000SWXL

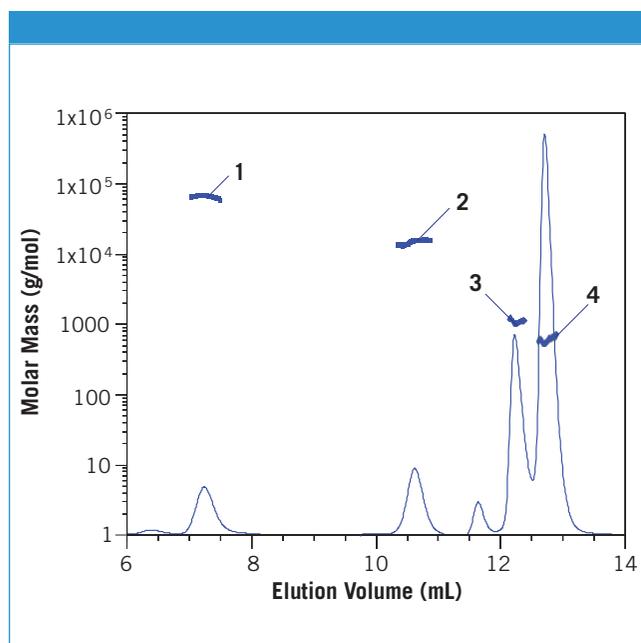


Figure 1: Molar mass determined by SEC-MALS of the four peaks identified, overlaid with the refractive index chromatogram.

size-exclusion chromatography (SEC) column. A miniDAWN MALS detector and Optilab® differential refractive index (dRI) detector are plumbed downstream of the column. The signals are acquired and characterized in terms of molar mass by Wyatt's ASTRA® chromatography software program. The peptide specific refractive index increment (dn/dc) values were measured using the Optilab by injecting a series of known concentrations.

The molar masses for the four major peaks determined by the MALS measurements are listed in Table 1. They compared favourably with those calculated from the amino acid sequences of the peptides and proteins. As seen in the last column, the standard deviation of three consecutive runs was 3% or less. The results indicate that this SEC-MALS method provides a rapid, robust and convenient way to characterize peptides—as well as proteins—absolutely.

Table 1: Peptide and protein molar mass values

Peak No.	Protein Name	Sequence M (kDa)	Measured M (kDa)	M RSD
1	BSA	66,700	64,300	1%
2	Lysozyme	14,400	14,600	1%
3	Bradykinin	1,060	1,090	2%
4	Leucine-Enkephalin	556	592	3%

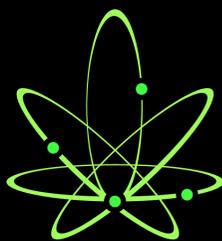


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