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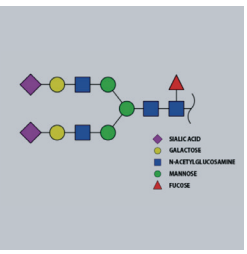

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NOTE FROM THE CEO

WELCOME, CHROMATOGRAPHY ENTHUSIASTS, to the March 2024 issue of LCGC International, brimming with insightful articles and updates from the world of separation science. This edition covers a diverse range of topics, from troubleshooting in liquid chromatography to the latest advancements in sample preparation, peak integration in gas chromatography, food analysis, and the analysis of coumarin in smoking products. Additionally, we provide a sneak peek into an upcoming event that promises to be a highlight for chromatographers worldwide.

Let's delve into the enriching content awaiting you:

In this month's "LC Troubleshooting" column, Dwight Stoll explores "The Gradient Delay Volume, Part III: Practice; Effects on Throughput." This installment explains the crucial yet often overlooked parameter of gradient delay volume in liquid chromatography, offering valuable insights into its impact on throughput and method development.

Douglas E. Raynie continues our journey in "Sample Prep Perspectives," focusing on "Trends in Sample Preparation." In this article, Raynie analyzes the latest trends in sample preparation techniques, with a special emphasis on sample size, automation, and solid-phase extraction devices.

Nicholas H. Snow deciphers peak integration in gas chromatography in his "GC Connections" column titles "From Detector to Decision, Part Four: Demystifying Peak Integration." Snow examines the various parameters affecting automated peak integration and peak area determination, providing essential guidance for quantitative analysis.

In our "Focus on Food Analysis" column, Merlin K. L. Bicking presents "An Efficient Procedure for Determining Simple Sugars in Fruit Juices." This article introduces a fast and simplified procedure for sugar analysis in liquid samples, offering significant improvements over standard methods.

This month's peer-reviewed article by Jingcun Wu tackles the "Analysis of Coumarin in Tobacco, Smokeless Tobacco Products and Electronic Cigarette Liquids by Isotope Dilution LC-MS/MS." Wu's study highlights a robust LC–MS/MS method for coumarin analysis, crucial for ensuring product safety in the tobacco industry.

We hope you find this issue as enlightening and informative as we do. Happy reading and stay tuned for more exciting updates from the world of chromatography! ■

Mike Hennessy, Jr.
President & CEO, MJH Life Sciences®

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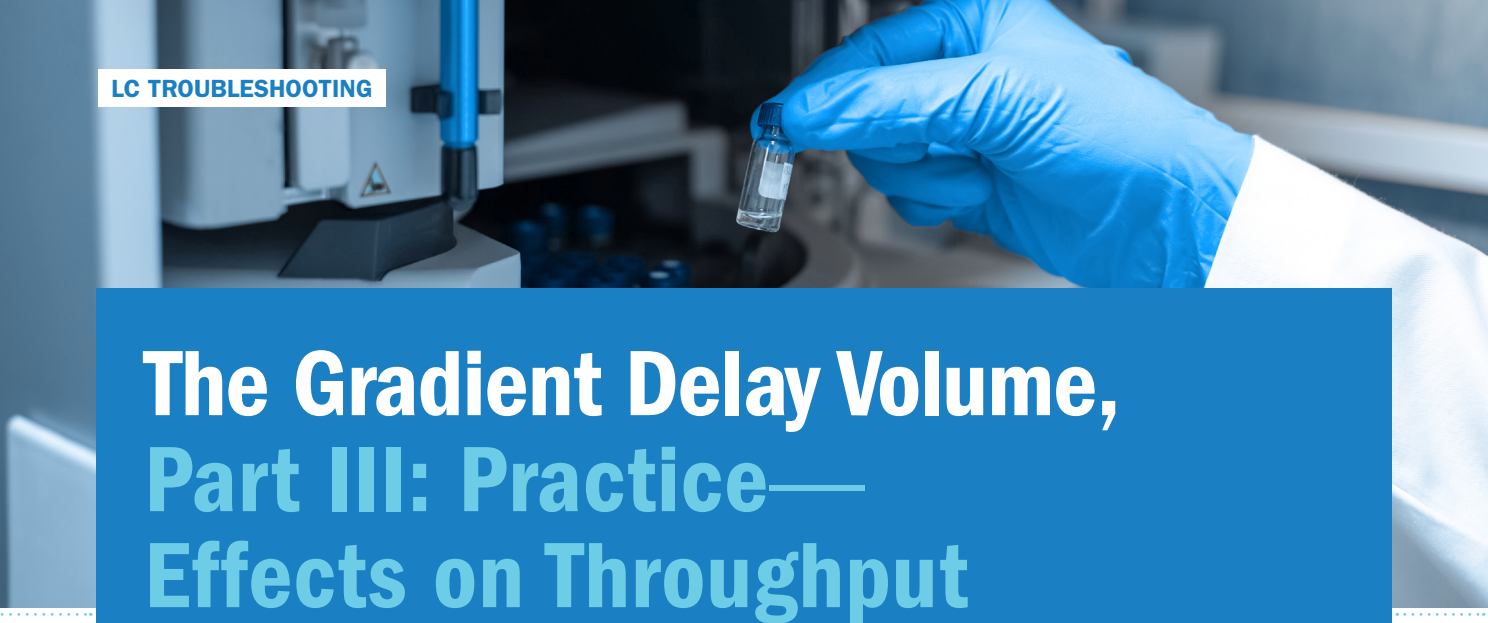

The Gradient Delay Volume, Part III: Practice—Effects on Throughput

Dwight R. Stoll

The concept of gradient delay volume (GDV) in liquid chromatography (LC) poses challenges for both beginners and experienced practitioners. The GDV, which affects the arrival time of mobile phase composition changes at the column inlet, can have a significant impact on method throughput, influencing the time required for mobile phase changes at both the beginning and end of the LC method. Different pump designs and column characteristics affect efficient use of the available analysis time, as well as overall throughput. Notably, achieving repeatable equilibration, rather than full equilibration of LC columns following mobile phase gradients, is often sufficient for many LC applications, which can also be leveraged to increase method throughput.

IN MY INTERACTIONS with people learning about various aspects of liquid chromatography (LC), I find that the concept of *gradient delay volume* (GDV) is one of the most difficult ideas to grasp and apply in practice. I find this to be the case both for true beginners—students who are just learning the basics of LC—and for more experienced scientists who have always dealt with GDV, knowingly or unknowingly, but are perhaps having to think about its impacts on their work in new ways. The GDV concept has been important since the very first time LC separations involving changes in mobile phase composition were made during an analysis. This is a phenomenon now know as a *gradient elution separation*. However, given the various ways that GDV can impact the practice of LC, and that we continue to see changes in commercial instrumentation that affect the way we interact and think about GDV, I think a dive into the details is warranted here.

In the last two installments of "LC Troubleshooting," I reviewed the basic elements of the GDV concept and discussed how we understand that GDV affects characteristics of LC separations from a theoretical point of view. I then discussed the practical implications of these ideas, with an emphasis on how the differences in GDVs between instruments can impact how a particular method will function on those instruments. In this month's installment, I will discuss the impact of GDV on method throughput, since GDV leads to a time where we need to wait for changes in the solvent composition delivered to the LC column at both the beginning and the end of a gradient elution method.

The gradient delay volume is commonly referred to by others as the *gradient dwell volume*, or sometimes just *dwell volume*. I prefer the inclusion of "gradient" to make it clear what we are talking about, and I prefer "delay" over "dwell," because "delay" communicates one of the most important impacts of GDV—that it delays the arrival of a programmed change in mobile phase composition at the column inlet. Nevertheless, from my point of view, "gradient delay volume" and "gradient dwell volume" refer to the same thing.

Finally, readers interested in learning more about GDV will not have a hard time finding good resources, and are encouraged to consult them. A short list includes several articles in *LCGC* Magazine and the book by Snyder and Dolan that is focused entirely on gradient elution LC (1). The relatively recent books, edited by Stavros Kromidas, have rich sections written by major instrument vendors that explain in some detail the software- and hardware-oriented approaches they have taken to effectively achieve variable GDV in their instruments (2,3). Searching the "LC Troubleshooting Bible" website (https://lctsbible.com/) for the keyword "dwell volume" will immediately return about a dozen articles from the last 20 years.

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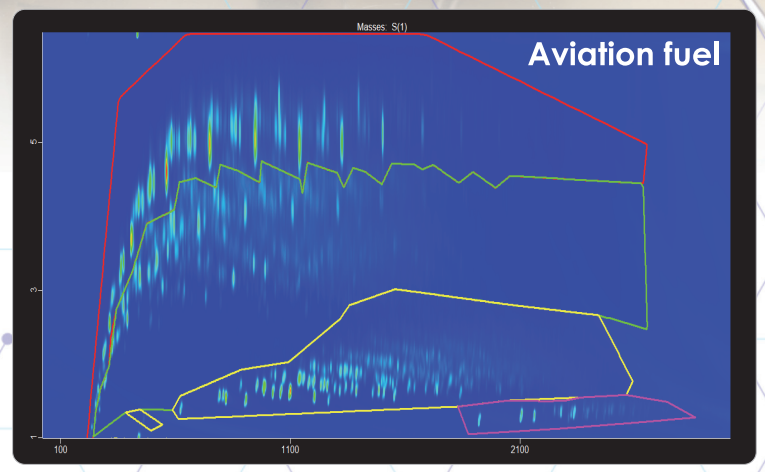

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Changes in Mobile Phase Composition at the Beginning and End of a Gradient Elution Method

In the previous two installments in this series, the focus of the impact of GDV has been on its effect on the arrival of the change in mobile phase composition at the column during the early stages of the gradient. However, we should not overlook the impact of the GDV on what happens at the end of the gradient. Here, when the pump is instructed to change the mobile phase composition back to the initial level used in the gradient (ϕ_i), we have to wait for the "strong solvent" used in the gradient to be washed out of the pump and other components leading to the column. Only when the mobile phase composition used as the initial level in the gradient actually reaches the column can the column actually start equilibrating with this mobile phase in preparation for the next analysis. The relationship between the solvent gradient program—that is, the instructions we give to the pump—and what the column actually experiences at the inlet is illustrated in Figure 1.

The delay in the arrival of a change in composition on the front side of the gradient is $t_d = \frac{V_d}{F}$. On the back side of the gradient, the time required to flush the strong solvent from the pump and connecting components is t_{flush}. Given the exponential profile of this flushout, for practical purposes. we assume that t_{flush} is about 2 × t_d, or $t_{flush} = \frac{2 \cdot V_d}{F}$. Additionally, we see that $t_{re\text{-}eq}$ is a bit longer than t_{flush}. Here, we define the re-equilibration time $t_{re\text{-}eq}$ as the time required to re-equilibrate the column, including t_{flush}. So, we understand that the difference between $t_{re\text{-}eq}$ and t_{flush} is the time we allow the column to equilibrate with the mobile phase composition used as the starting point in the gradient (ϕ_i). Later on in this installment, I'll discuss more of what constitutes "enough" time for re-equilibration of the column itself; for the remainder of this section, we'll assume that two column volumes of equilibration is enough, such that $t_{re-eq} = t_{flush} + \frac{2 \cdot V_m}{F}$, where V_m is the dead volume of the column. Finally, when thinking about the throughput of analyses involving gradient elution, it is useful to define a fraction α that quantifies the portion of the analysis time that is actually used for separating things under solvent gradient conditions (5) (nominally, t_g; peaks can elute during t_d as well, but we assume here that elution during the isocratic pre-gradient phase is generally not as useful as elution during the actual gradient).

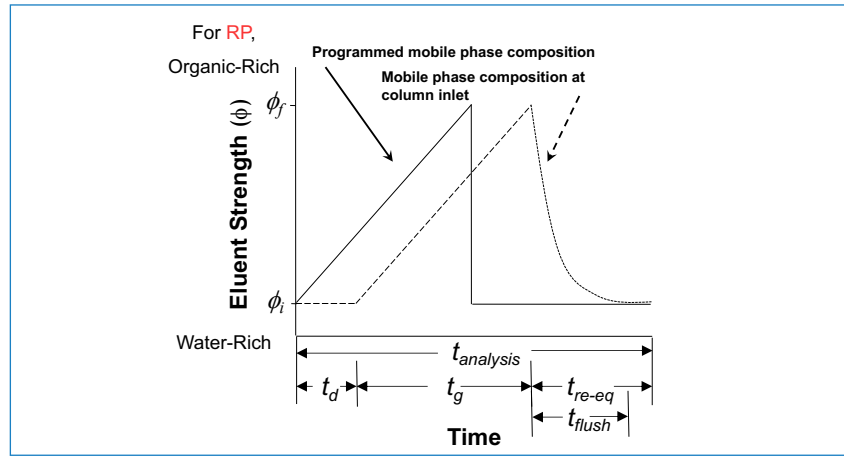

FIGURE 1: Solvent program instruction delivered to the LC pump (solid line), and the mobile phase composition observed at the column inlet (dashed line). Adapted from reference (4).

$$\alpha = \frac{t_g}{t_{analysis}} = \frac{t_g}{t_d + t_g + t_{re-eq}} \quad [1]$$

Having defined α and all the times involved, we can think about the effects of different variables on throughput and the fraction of the analysis time that is actually used for separation, including V_d, F, and V_m. Table I shows some different combinations of these variables, along with a description of where these combinations are found most often in practice. First, in Scenario A, we see that when using a modern binary pump characterized by a small GDV, a relatively short, narrow column, and a reasonably fast gradient time of 2 min, the fraction of analysis time that is the gradient time is about 70%, which is not too bad. Now, if we use the exact same conditions in Scenario B, but change the pump to a quaternary, low-pressure mixing design characterized by a large GDV, we see that α drops to around 40%. If there is no other choice due to resource constraints, then this is how it has to be, but using less than 50% of the analysis time for the gradient separation is far from optimal. In Scenario C, we suppose that the focus is more on performance as measured by peak capacity or resolution, as derived from the use of a longer column. If we stick with the same pump as in Scenario B, the α value increases only marginally, mainly because when we move to the longer column, we have to use a lower flow rate to avoid going over the pressure limit of the system, which, in turn, leads to larger values of t_d and $t_{re\text{-}eq}$. The takeaway from Scenario D is that efficient usage of the analysis time (as measured by a large α) is indeed possible, even with an older pump with a large GDV; however, this requires much higher flow rates, and thus, a larger diameter column. The high flow rate reduces t_d and $t_{re\text{-}eq}$, while maintaining a gradient slope, similar to that in Scenario A. Scenario E illustrates what happens when we try to use a modern, short, narrow column with an old quaternary pump with a large GDV. The α value drops to around 24%, which will be unacceptably low in most cases.

The final two scenarios in Table I (F and G) are relevant to gradient elution conditions used in the second dimension of comprehensive 2D-LC separations. In this case, the performance requirements are relatively unusual—very short analysis times on the order of 30 s are highly valuable. Scenario F shows that if a short,

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CASE STUDY CORNER

This month, I am rolling out a new feature in the "LC Troubleshooting" column, The "Case Study Corner." Here, I will provide a short description of a real problem I've observed, and ask readers to send me their diagnosis of the root cause of the problem and a proposed solution. The first person to propose the correct diagnosis and solution will receive an "Analytically Speaking" podcast coffee mug. In a subsequent installment, I will then discuss the correct diagnosis and solution in some detail. I encourage educators and lab managers to consider assigning these case studies as "homework" to their students and scientists to help them develop their troubleshooting skills. Enjoy!

Case Study #1

The focus of this month's case study is a problem with a LC autosampler, and this time, we have two major troubleshooting clues. The first clue is the pressure trace measured at the LC pump, as shown in Figure A. Here, I've included data from two full analysis cycles in the plot, and we see that the pressure drops dramatically at the end of the first analysis, but then rises quickly to the nominal operating pressure very early in the second analysis. When I was observing this instrument, I saw that this pattern was repeated over tens of injections. The second clue is that I observed a puddle of liquid on the benchtop in front of the LC, as shown in Figure B. This particular sampler involves a flow through needle design (Agilent G4226A). Readers are welcome to email me at **dstoll@gustavus.edu** with clarifying questions, in addition to sending their proposed diagnoses and solutions to the problem.

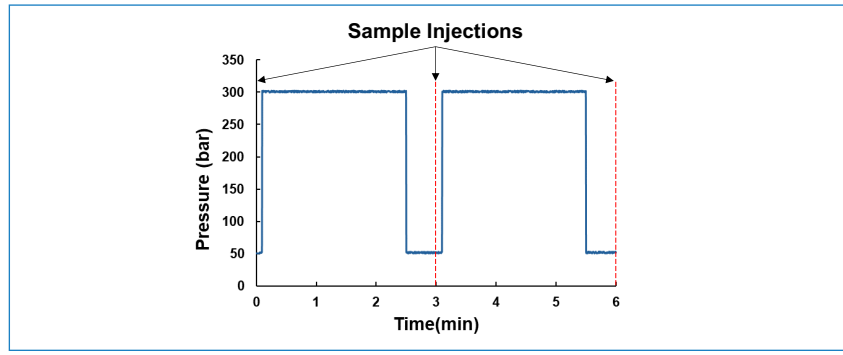

FIGURE A: Pressure profiles measured at the pump over two analysis periods; sample injections were made at 0, 3, and 6 min.

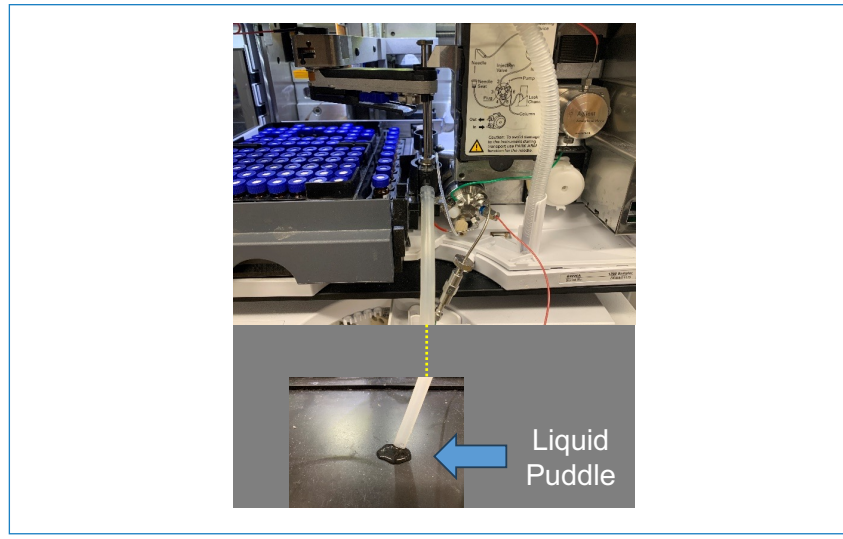

FIGURE B: Picture of the sampler in question and the puddle of liquid observed on the benchtop in front of the instrument.

narrow column is used, along with a modern binary pump and a relatively high flow rate, the α fraction can actually be quite high at around 70%, which is similar to what we see with conventional 1D-LC separations. However, if we imagine trying to do the same 30-s separations using an old quaternary pump, the α value drops again to an unacceptably low 28%. This application effectively requires the use of modern binary pumps.

How Long Do We Actually Need to Re-Equilibrate the Column?

In the mid-2000s, I began looking into this question deeply, along with Adam Schellinger and Peter Carr, motivated by our interest in superfast gradient elution conditions along the lines of Scenario F in Table I (6–8). One of the most important things we learned from our work at that time is that, when talking about re-equilibration of LC columns following a mobile phase composition gradient, we really have to make a distinction between two different types of equilibration:

1. *A state of repeatable equilibration*. In this case, the column is not actually fully equilibrated with the initial mobile phase used in the gradient before starting the next analysis, but the condition of the column is consistently achieved between analyses, such that highly repeatable retention times are observed.
2. *A state of full equilibration*. In this case, the column is fully equilibrat-

TABLE I: Comparison of the fraction of analysis time used for actual separation during the solvent gradient in different scenarios.

Scenario	Description	Pump	Column Dimensions (mm x mm i.d.)	V_d (µL)	V_m (µL)	F (µL/min)	t_m (min)	t_d (min)	t_{flush} (min)	$t_{re\text{-}eq}$ (min)	t_g (min)	α
A	Contemporary high-throughput analysis	Modern, Binary	50 x 2.1	100	100	500	0.20	0.20	0.40	0.8	2.0	0.67
B	Contemporary high-throughput analysis	Modern, Quaternary	50 x 2.1	400	100	500	0.20	0.80	1.60	2.0	2.0	0.42
C	Contemporary high performance analysis	Modern, Quaternary	150 x 2.1	400	300	200	1.50	2.00	4.00	7.0	9.0	0.50
D	High-throughput analysis with old pump and large column	Old, Quaternary	50 x 4.6	1000	500	3000	0.17	0.33	0.67	1.0	2.0	0.60
E	Attempted high-throughput with old pump and small column	Old, Quaternary	50 x 2.1	1000	100	500	0.20	2.00	4.00	4.4	2.0	0.24
F	Second dimension of comprehensive 2D-LC	Modern, Binary	30 x 2.1	100	60	3000	0.02	0.03	0.07	0.1	0.4	0.74
G	Attempted second dimension of comprehensive 2D-LC	Old, Quaternary	30 x 2.1	1000	60	3000	0.02	0.33	0.67	0.7	0.4	0.28

ed, as indicated by the observation that retention time observed in the gradient elution method is independent of the re-equilibration time between analyses.

Our realization at the time was that for many applications of gradient elution methods in LC, we care far more about having highly repeatable (that is, precise) retention times than we care about starting with a column that is in a fully equilibrated state.

Once we realized the importance of this distinction between states, we found that for reversed-phase separations of small molecules, it usually does not take more than two column volumes (or two dead times of flushing with ϕ_i) to get to a state of repeatable equilibration. For large columns operated at conventional flow rates (for example, a 150 mm x 4.6 mm i.d. column operated at 2 mL/min), this is about 1.5 min. But for short columns at high flow rates, this can be remarkably short at just a few seconds (for example, as in Scenario F in Table I).

This finding has since been confirmed by other groups for reversed-phase separations (9,10), and also for other modes of separation, such as hydrophilic interaction liquid chromatography (HILIC) (4,11). Frankly, modern comprehensive 2D-LC separations would not exist as we know them today if it were not possible to repeatedly equilibrate LC columns in a matter of seconds (12)

Summary

In this installment of "LC Troubleshooting," I have discussed the effects of gradient delay volume (GDV) on the throughput of LC methods that involve mobile phase composition gradients. While we very often focus on the impact of the gradient delay time and its effects on selectivity and resolution, as discussed in last month's installment, the more important impact of GDV on throughput occurs at the end of the method, where we instruct the LC to return the mobile phase to the initial condition used in the gradient. Understanding the interactions between pump characteristics, column characteristics, and other method parameters and performance goals is useful when developing a new method with throughput in mind, troubleshooting variations in retention time, and optimizing existing methods to improve throughput. ■

This article has additional supplemental information only available online.
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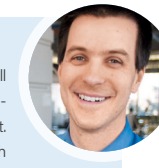

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is the editor of "LC Troubleshooting." Stoll is a professor and the co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 75 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of *LCGC*'s editorial advisory board.

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Trends in Sample Preparation, Part II: Sample Considerations and Techniques

Douglas E. Raynie

In the October 2023 installment of "Sample Prep Perspectives," we reported results obtained from a survey on sample preparation techniques, and compared those to results from previous surveys. The results uncovered trends in the field over the last generation and their impact in areas such as which technologies are currently being used, sample types, and sample load. This installment will continue our look at the sample preparation survey, focusing on sample sizes, laboratory techniques used, automation, the use of solid-phase extraction (SPE) devices (cartridges, disks, plates, tips), SPE chemistries and selection criteria, and problems encountered in SPE.

PERIODICALLY, *LCGC International* surveys readership concerning trends in sample preparation. Over the 30 years of this survey, several developments in the field have been uncovered. In the October 2023 issue, we discussed several of these developments (1). Here we discuss considerations with sample type and size, laboratory techniques, including solid-phase extraction (SPE).

Sample Considerations

Previously, we noted several trends regarding samples in the current survey (1). The survey showed an increase in the number of liquid samples analyzed, with a decrease in solid samples, and fewer sample types per laboratory. However, more samples per instrument per week are characterized, though whether this trend will continue is uncertain. We postulated that, combined with post-pandemic considerations, laboratories are becoming more selective in the types of analyses they perform. The top sample matrices reported included pharmaceutical and over-the-counter drugs, foods, water, soils, polymers, biomass, inorganics, metals, and fruits, vegetables, and grains.

Sample Sizes

Continuing on the sample theme, it appears that sample sizes are getting smaller and analytes are less concentrated. The size distributions (masses and volumes) of different sample types are shown in Figure 1, and seems to reiterate the trend of fewer environmental samples, whose heterogeneity demands larger sample sizes. For liquid samples, just over three quarters (75.4%) of samples are between 0.5 and 20 mL. While only 3.9% of liquid samples are less than 0.5 mL (down from 14.4% a decade earlier [2]), 57.7% (normalized to exclude those who do not analyze liquids) are less than 5 mL, compared with 39.5% in 2013. Turning to gaseous samples, the initial sample size distribution is somewhat similar to that reported in 2016 (3), while most samples are less than 5 mL (58.6%, normalized), fewer samples less than 1 mL (28.6%) are noted compared to 2016 (38%). On the other hand, there are also fewer large samples. In 2023, only 6.5% of samples are greater than 500 mL, compared with over twice that many (14%) in 2016. Finally, with solid samples, about 10% more samples less than 1 g (65.1% in 2023 vs. 59% in 2016) are observed over the past seven years. However, for samples greater than 10 grams, we saw a significant decrease to 10.4% in 2023, compared with 17% in 2016.

Sample Concentrations

The trend in sample concentrations, the distribution from the 2023 survey is shown in Figure 2, skews toward trace levels of analyte. In 2023, nearly three-fourths of samples (71.8%) are less than one part per million, significantly greater than over the previous two decades (47% in both the 2013 and 2002 [4] surveys). Just over a third of samples, 35.6%, are in the 1–100 parts per billion range. One would think that sample concentration would help dictate the final sample volume prior to injection into a chromatograph, but this assumption is only partially correct. Over the past decade,

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the amount of samples at 2 mL or less (compatible with most standard autosampler vials) is fairly constant (71.3% in 2023, 68.7% in 2013). But the number of samples whose concentration is adjusted to less than 1 mL has dropped significantly, to 14.8% in 2023, compared with 38% in 2013 and 25% in 2002. The distribution of final sample dilutions from the 2023 survey is presented in Figure 3.

Techniques Used

Laboratory Techniques

Before delving into sample preparation procedures used by survey respondents, we queried the techniques used in the laboratory. Results are shown in Figure 4. This question was also asked 10 years ago (2). In both surveys, respondents could select more than one answer (in 2023, respondents were limited to three selections, while 2013 had no restrictions). When normalized to the total number of responses, it appears that the number of techniques used in a given laboratory has decreased significantly, from 3.9 in 2013 to 2.7 in 2023. This is in line with our hypothesis that laboratories are becoming more specialized. The two most widely used techniques, each used in about a third of the respondent laboratories, are liquid chromatography-mass spectrometry (LC–MS) and gas chromatography (GC). Looking at the normalized data, some type of LC and some form of GC are of similar popularity and used

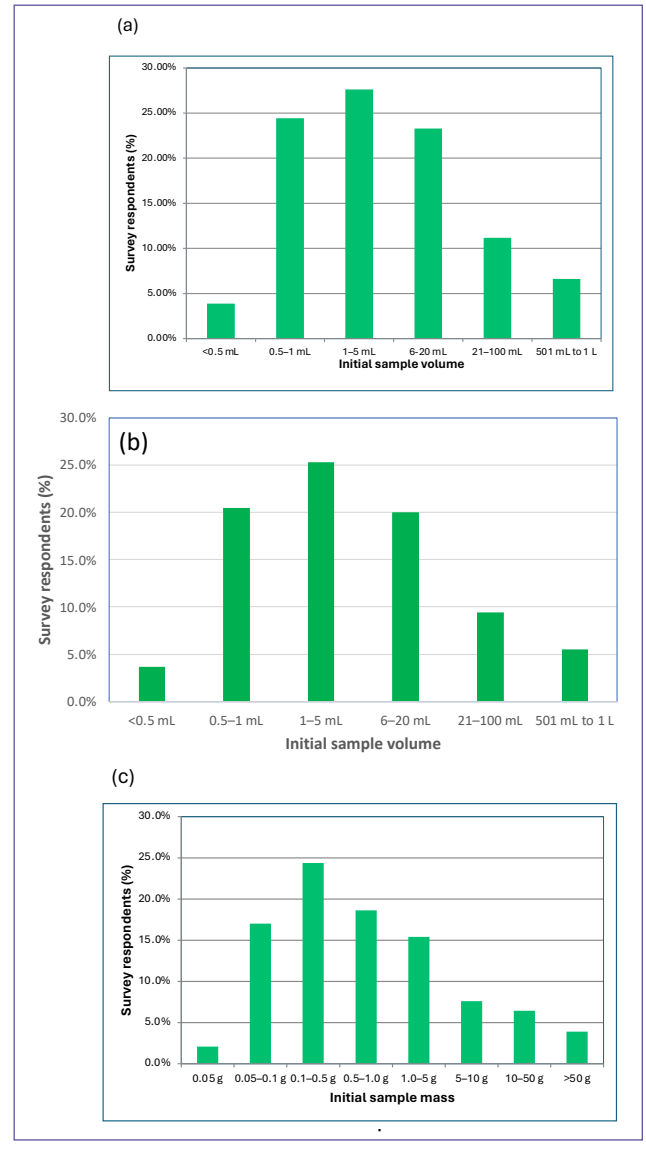

FIGURE 1: Initial amounts (volume or mass) of (a) liquid, (b) gaseous, and (c) solid samples typically analyzed by survey respondents.

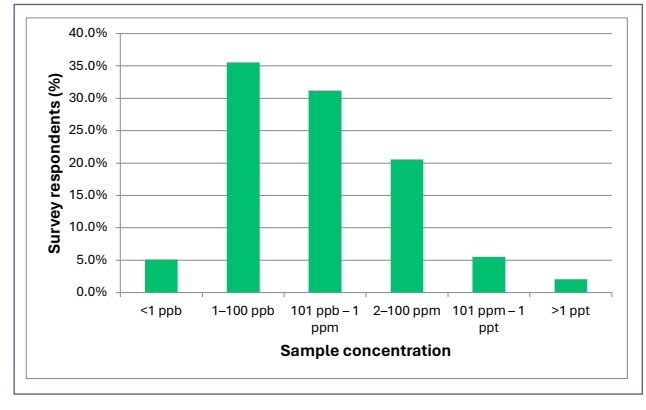

FIGURE 2: Initial sample concentrations typically analyzed by survey respondents.

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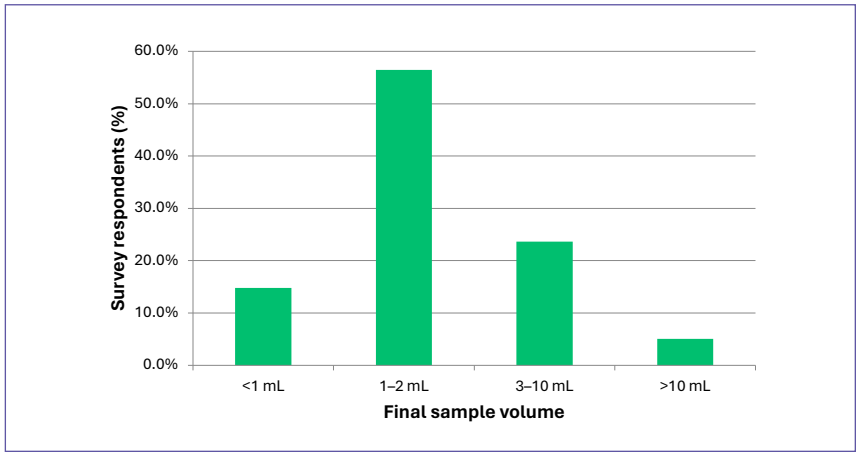

FIGURE 3: Distribution of final sample volumes prior to chromatographic injection reported by survey respondents.

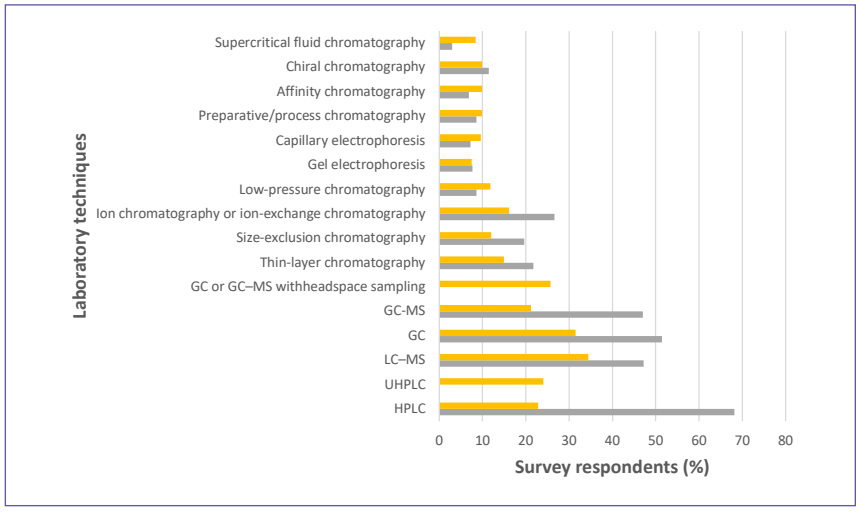

FIGURE 4: Laboratory techniques currently in use (yellow) and those reported in 2013 (grey) as a percentage of survey respondents.

TABLE I: The 10 most prevalent sample preparation techniques reported in 2023, and their ratings in previous surveys

Preparation Technique	1991	2002	2013	2023
Centrifugation	7	9	3	1
Pressurized Fluid Extraction	---	34	37	2
Cooling	30	28	27	3
Filtration	2	3	2	4
Dilution	4	4	4	5
Concentration	5	10	9	6
Internal Standard Addition	3	5	5	7
Evaporation	4	7	5	8
Weighing	1	1	1	9
Digestion	20	19	16	10

in proportions similar to a decade ago; in 2023, 30.0% of respondents used some form of LC and 28.9% use some form of GC, compared with 33.3% (LC) and 28.4% (GC) in 2013. Ion chromatography (5.9% in 2023, vs. 7.7% in 2013) and size exclusion chromatography (4.4% vs. 5.6%) slightly dipped in popularity, while various forms of electrophoresis saw a small uptick (6.3% vs. 4.3%). This was the first year in which ultra-high pressure liquid chromatography (UHPLC) and headspace sampling (combined with GC) were queried in our survey and each of these techniques tallied use by about a quarter of respondents.

Sample Preparation Procedures

Figure 5 turns to procedures specific to sample preparation. The survey results are interesting, and somewhat surprising, though they seem to support the observation of bioanalysis taking a higher priority than environmental analysis among amount survey respondents. First, we can see the widespread adoption of each of the surveyed techniques. In prior surveys, the lesser utilized techniques found use among about 10–15% of the most widely employed techniques. This year, the least frequently used technique was employed at a rate at least 50% of the most widely used technique. Next, for every technique queried, if not currently being used in a given laboratory, it is under consideration. Often those considering using these techniques exceeds those using them. Several techniques were queried for the first time in this year's survey, namely cloud-point and coacervative extraction, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), β-glucuronidase removal, phospholipid removal, stir-bar sorptive extraction (SBSE), and protein precipitation. Each of these techniques are currently used by about a third of survey respondents, with another 40–50% contemplating their use. Three of these approaches (β-glucuronidase and phospholipid removal and protein precipitation) are used to remove biological macromolecules which interfere with LC–MS separations, perhaps leading to

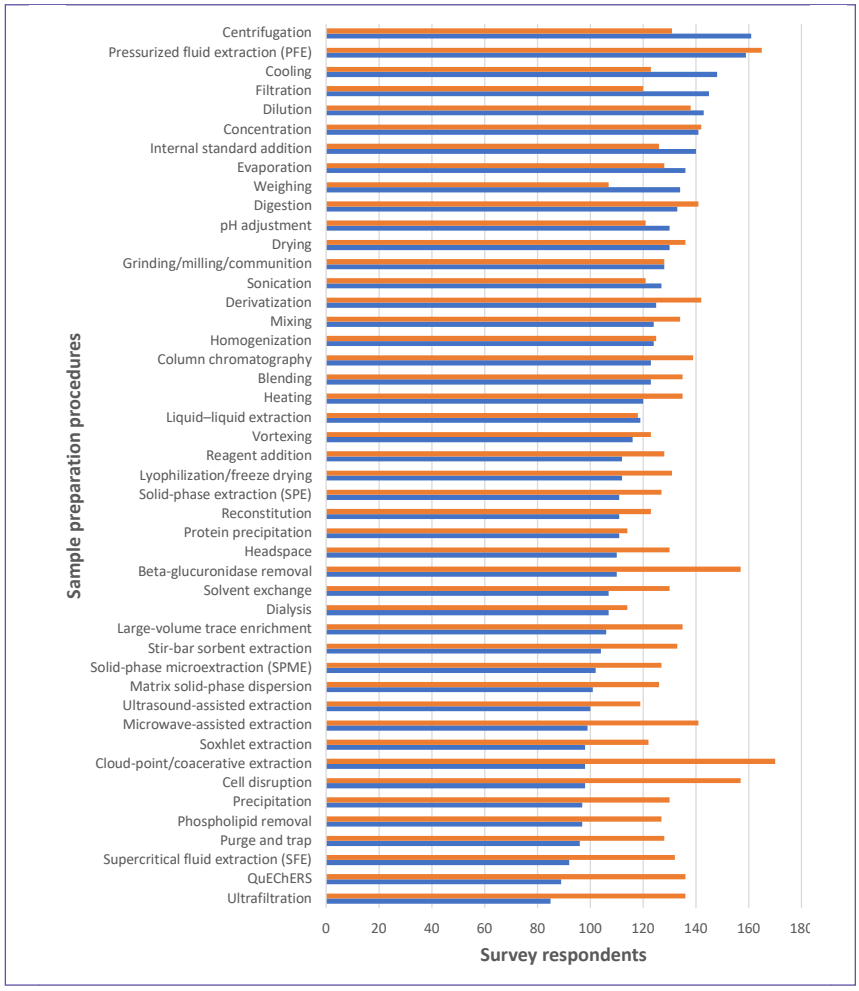

FIGURE 5: Sample preparation procedures currently in use (blue) or planned to use (red) by survey respondents.

other problems like ion suppression. This is consistent with the support of bioanalysis and increased use of LC–MS over the past 10–20 years. It is also encouraging that newly developed techniques such as SBSE and QuEChERS are being readily adopted by the analytical community, as both of these techniques were introduced after the advent of this survey series, SBSE in 1999 (5) and QuEChERS in 2003 (6).

The biggest surprises in the trends in sample preparation procedures can be gleaned from Table I, which compares the ten most widely used procedures every ten years of the survey history. Historically, weighing, filtration, centrifugation, and internal standard addition are the most common sample preparation procedures. While this trend continues, notably, weighing dropped from its historic perch as the top s ample preparation procedure to ninth on the list. Meanwhile, centrifugation, which was in the ninth spot 20 years ago, is now the most common procedure. Cooling jumped from a less common procedure to the third most common, and digestion continues a steady climb up the list. Each of these techniques are more common to bioanalysis, reiterating our observation on the overall trend in chemical analysis practice.

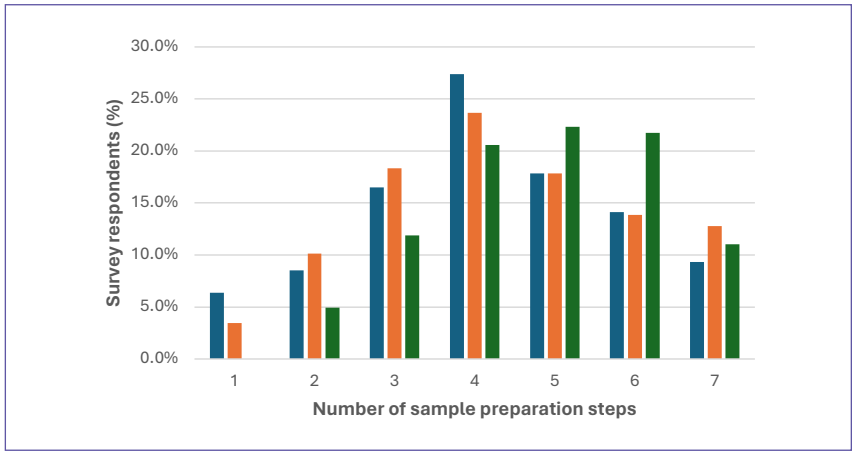

FIGURE 6: Average number of sample preparation steps from raw sample to chromatographic injection reposted by survey respondents for solid (blue), liquid (red), and gaseous (grey) samples.

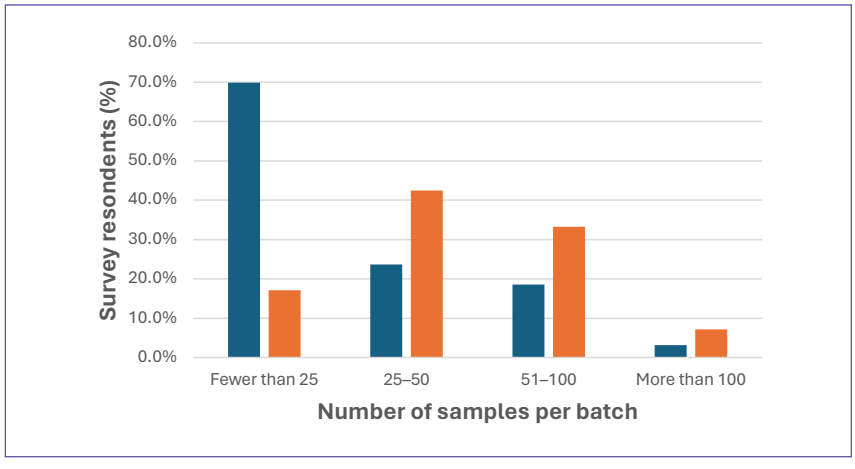

FIGURE 7: Average number of samples per batch reported by survey respondents for sample prepared batchwise in 2013 (blue) and 2023 (red).

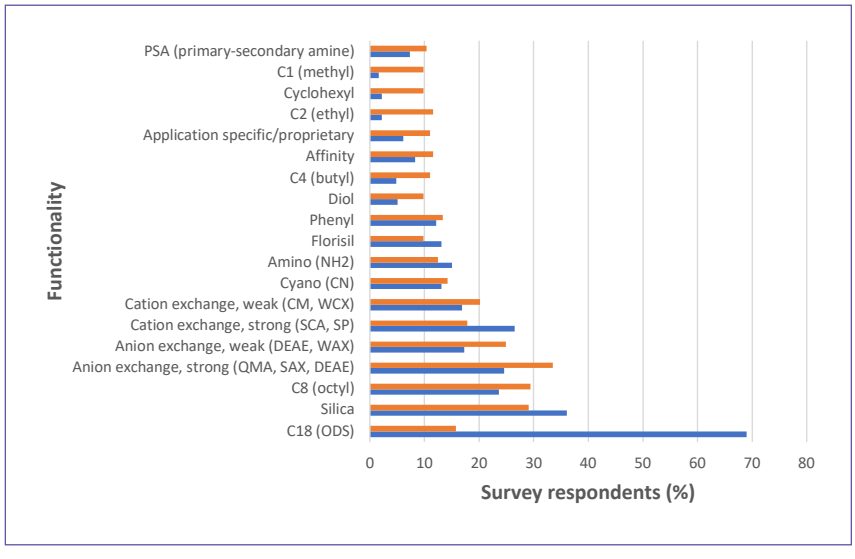

FIGURE 8: Silica-based SPE chemistries used, as reported in 2013 (blue) and 2023 (red).

A final surprise is concerns pressurized fluid extraction (PFE). Introduced by Dionex (now part of ThermoFisher) as accelerated solvent extraction in 1996 (7), subsequent to the beginning of this survey series, it has jumped from very low in usage to the second most common procedure. While there may be anomalies in this, or any, survey, PFE has demonstrated utility in a number of application areas and been accepted by regulatory agencies worldwide. In recent years updates and competitors to the original systems have emerged which may lead to more its widespread use.

Sample Loads

Apparently, laboratories are performing more sample preparation steps for each sample. This trend is shown in Figure 6. An average of 4.35 steps per sample was reported in this survey. This is an increase from previous years, as the 2002 survey reported 3.43 steps (4) and 2013 reported 3.10 steps per sample (2). This perhaps can be explained by looking at the steps involved in each sample type. Our survey found that solids averaged 4.2 sample preparation techniques per sample, up from 4.0 in 2016 (3), liquid samples increased from 3.4 techniques in 2016 to 4.3 in 2023, and gases jumped from 1.8 techniques in 2016 to 4.5 in 2023. The jump for gases was remarkable as, in 2016, no more than four steps were used in this analysis. Perhaps this may be attributed to determination of trace levels of environmental pollutants, off-flavors, and related samples.

In Part I of our 2023 survey results (1), we reported an increased number of samples processed in analytical laboratories. This impacts how the samples are processed. Generally, samples are processed individually as needed (40.7% of samples) or batchwise (54.8% of samples); only 4.6% of samples are processed serially. This is unchanged since 2013 (2). However, what has changed is the size of batches processed in this manner. Figure 7 demonstrated the tremendous shift to larger size batches. A decade ago, essentially all samples

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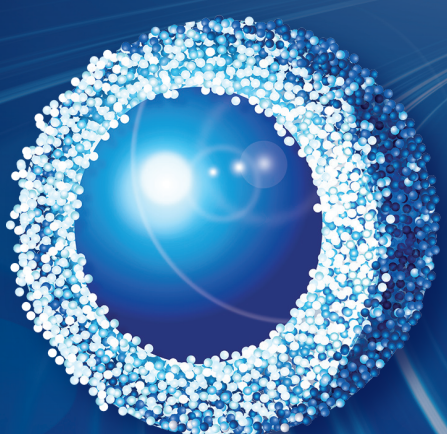

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TABLE II: Areas for further improvement in the field of sample preparation

Improvement Area	Percent of Survey Respondents
Minimize the need for sample preparation	23.6
Miniaturized sample preparation techniques	39.2
Green techniques	42.4
Ease of use	28.1
Time and labor intensity	30.8
Automation	22.9
Performance	12.1
Lower cost	7.4

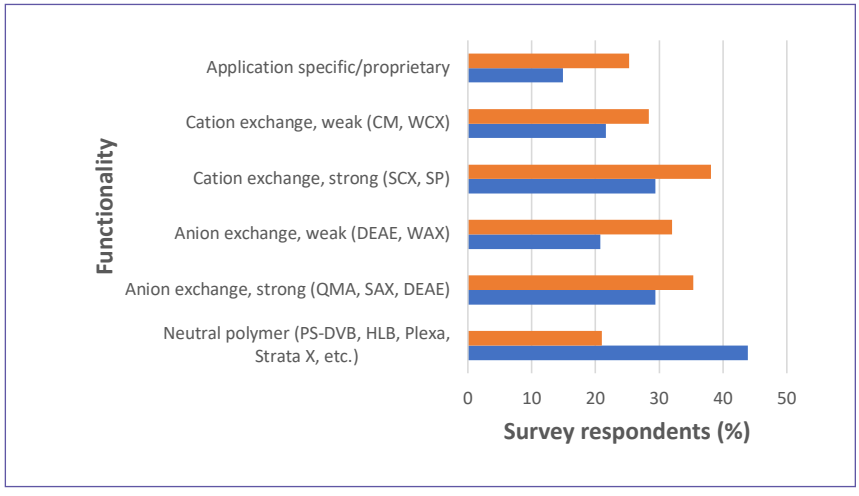

FIGURE 9: Polymeric-based SPE chemistries used, as reported in 2013 (blue) and 2023 (red).

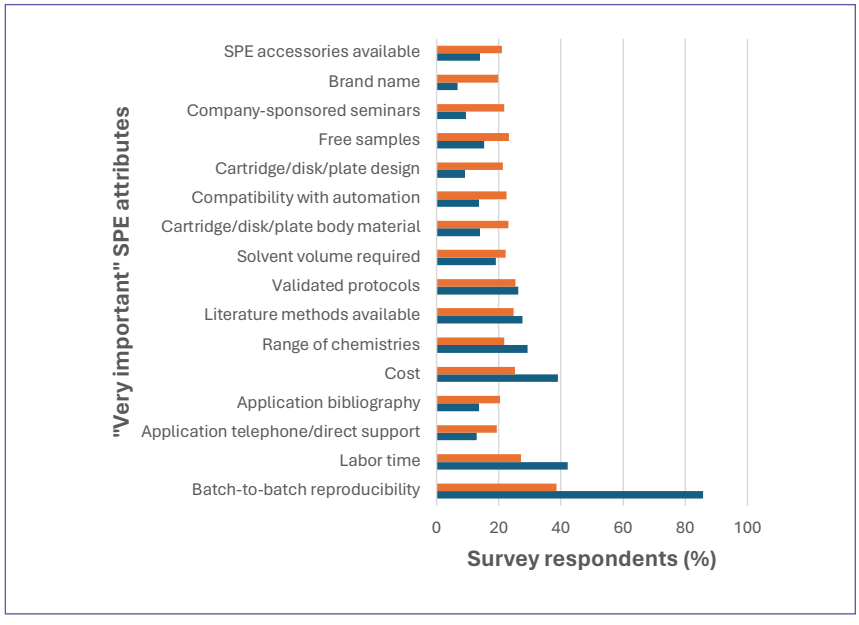

FIGURE 10: SPE characteristics considered "very important" by survey respondents in 2013 (blue) and 2023 (red).

(93.3%) processed as batches were in batches of 50 or less. Currently, 59.6% of samples are in batches of 50 or less, with more samples being processed in larger batch sizes.

Automation

No more than about a quarter of the sample preparation procedures previously discussed are performed in an automated manner, according to survey respondents. But with the larger reported number of samples, the use of automation has increased dramatically, from 29% of respondents in 2013 to 39% in 2016 to 84% of current survey respondents. Additionally, 42% of respondents claim to be planning or considering use of automation in the coming year, up from just 15% a decade ago. This follows the survey finding that only 25% of respondents do not have the sample throughput to justify automation, down from 55% a decade earlier. Of those using automated sample preparation systems, nearly a third (32.4%) use an autosampler with sample preparation capabilities and a quarter (24.5%) use an automated solid-phase extraction (SPE) system. Lesser numbers of laboratories use a full laboratory robot (14.6%), an automated liquid handling system (12.3%), or an autosampler (9.1%).

Solid-Phase Extraction

A large percentage (82.8%) of survey respondents use SPE and historically this survey has looked into trends in SPE. We continue exploring SPE trends with the current survey. While, based on previous surveys, it is highly likely that practitioners use more than one SPE format, this year's survey showed a fairly even distribution of those using the traditional cartridge form of SPE (26.3%), the disk format (28.6%), and well plates (28.1%), with fewer using the pipette tip approach (15.5%). Of these formats, cartridge users prefer sorbent beds of 500 mg or less (71%), with 100-mg (31.2%) and 500-mg (29.1%) beds the most commonly used. Nearly 60% of those using SPE disks use 47-mm disks, with approximately equal number of users with either larger or smaller disk sizes.

The SPE phases used are summarized in Figures 8 and 9, for silica-based and polymer-based sorbents, respectively. With silica-based SPE, a large dropoff in the use of octadecyl (ODS) is observed, perhaps offset by increasing use of octyl (C8) and sorbent functionalities which traditionally have seen little use, such as diol, amino, and butyl, among others. With polymer-based systems, use of neutral polymers has dropped off while ion exchange and application-specific columns have seen increased use, perhaps reflecting their utility across a broader pH range. When looking at users' preferences in selecting SPE products, the trend in the past decade shows less discernment in which factors are considered "very important" in this choice (Figure 10), though batch-to-batch reproducibility remains the top factor, reflecting the major concern during the developmental stages of SPE and a lingering concern.

Future Directions and Conclusions

This year's survey asked readers, "As the field of analysis and sample preparation continues to evolve, which of the following would you like to see further developed?" The results are shown in Table II. The top two desired features are the development of green or miniaturized techniques, trends which have taken increasing prominence in recent years. Other concerns (including ease of use, time and labor intensity, performance, and cost) follow from the most frequently identified problems with sample preparation reported previously (1). Meanwhile, respondents were asked to identify up to three emerging technologies that may become commonplace in the next five years. Not surprisingly, primary among these are green approaches to sample preparation, namely greener solvents and solventless methods and recycling solvents, reagents, and materials. Other emerging technologies identified are use of three-dimensional printing, flow injection, nanoparticle technology, and vacuum heating.

Over the past thirty years, *LCGC* has polled readership concerning trends and practices in the area of sample preparation. In this two-part summary of the 2023 survey, we see the shift to bioanalysis as the driving force of new practices. Concern over green chemistry and sustainability is rising to the forefront and will force new developments in the near-term future of the field. ■

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Transferring Methods to Compact and Portable HPLC

The Axcend Focus LC® enables laboratories to optimize space without compromising quality.

Greg Ward
CEO
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Space is an invaluable resource in the laboratory, so recent trends have led to the miniaturization of instruments to optimize that space. Smaller high-performance liquid chromatography (HPLC) instrumentation, for example, has the same benefits as analytical-scale equipment, but additionally offers a smaller footprint, more affordability, and portability. To learn more, *LCGC* sat down with Greg Ward (CEO of Axcend) to discuss the advantages of the Focus LC.

LCGC: Liquid chromatography has been around for decades. Can you explain Axcend's mission and how it differs from the "big box" chromatography vendors?

WARD: Today's chromatography systems are here to stay, but Axcend's mission and vision is to provide advantages to our customers who are trying to dramatically reduce the time, cost, and complexity of their analytical research. The Focus LC from Axcend was the brainchild of Dr. Milton Lee from Brigham Young University, who is well known in the field of analytical chemistry and separation science. The Focus LC is the smallest and lightest system on the market. As a result, it can fit in a fume hood, can be used in a mobile lab, and can go in a BSL space. The same qualities that make the Focus LC applicable for niche areas also make it work well for pharma and industry. It uses very small amounts of solvent, takes up minimal space, and runs on the well-known software platforms or CDS systems that pharma labs are required to use.

LCGC: What are some of the specific benefits of the Focus LC that enable users?

WARD: Let's talk about five benefits that are essentially our value propositions. First, the Focus LC is compact. That's the ability to put the tool where you need it because it doesn't take up much space. Second, it's portable, which means it can be moved around within a lab or from lab to lab. For one customer, that lab-to-lab movement was from North Carolina to Utah, and they could easily bring the instrument with them and test a contract manufacturer's product that they were making. It also could be an oil platform in the North Sea or in the field. There are several applications for portability beyond a small footprint. The third benefit is ease of use. I mentioned we work with existing CDS systems that are required in areas like pharma, where there are high regulations, but there are also instances in pharma where labs just want fast data. We have developed our own software that is simple to use and allows people to get the data they want quickly. It can be used in academic settings with brand new users who have never seen CDS before and don't want a high learning curve. We also have made a version of this that we call the Axcend Analyzer LC, where we put it in the hands of non-chromatographers. For example, we delivered some instruments to the US Air Force and, in that case, an 18-year-old airman was running it. These were certainly not chromatographers; they just needed a go-no-go tester, and we were able to do that. Next, the total cost of ownership is a benefit. This doesn't just include acquisition costs, but also what it costs to run the instrument. I mentioned that the maintenance contracts are less; the consumables are divided by a thousand in acquisition and disposable. We simply stretch a customer's budget by making the cost of ownership much lower. The last benefit is that the instrument is eco-friendly. Environmental, social, and governance (ESG) goals have been talked about in corporations for at least a decade, but it's getting to the point where they're becoming actionable. I read an article from Ernst and Young recently that said these goals are here to stay, not a fad.[1] Companies and employees are beginning to be measured on what they're doing to reach their ESG goals, and that could be achieved with green chemistry. The Focus LC helps customers and corporations reach those ESG goals.

"[ESG is] not a fad, but an imperative."

– Ernst and Young

EY

LCGC: (Bio)pharma is definitely a significant portion of the LC market and highly invested in those large LC companies that we discussed earlier. Where does the Focus LC fit in with Pharma today, and where do you see it going tomorrow?

WARD: Pharma companies are trying to figure out how to bring their products to market faster. They're moving from what were large-batch reactions to micro-reactors where the reactor is being put inside a fume hood. The LC or other instrumentation is put directly in the fume hood next to that reactor as opposed to using tubes that go to the ceiling. The emerging technology consortium from the pharma community sent out a request for proposal for compact HPLC. We were able to win that bid and work directly with them, and they're using this in drug discovery and drug development. It doesn't have to just be used in pharma, but even small or large molecule discovery and development. We even had one customer who had a robotic platform where the chemistry was on the platform and the robot could draw sample, dilute, heat, stir, and then push the sample directly into the LC. In none of these cases could you put a legacy HPLC inside of a fume hood, but with the Focus LC you can. We're also finding that the Focus LC expands available bench space. Bench space is becoming increasingly expensive. Instead of building a new building, you can maximize the efficiency of your bench space, which is very attractive. The compact footprint allows labs to put more instruments per linear foot of bench space. Some of these pharma labs have multi-floor buildings, but they are restricted in the number of LCs they can put on floors two and three because of regulations on flammable materials. It would take more than 70 of our instruments to equal one legacy instrument in terms of the amount of solvent. With our 15-mL vials, you can now use floors two and three and expand the available bench space to pharmas without having to build new facilities.

LCGC: Looking outside of pharma, what markets and applications do you see the Focus LC addressing?

WARD: There are a few additional markets, and they keep expanding. Some that come to mind are industrial cleaning validation for kettle cleaning and industry, fuels, and different areas in oil and gas. We also have really good traction in academia because it's an easy-to-use tool. It's flexible, it's portable, and users like the minimal maintenance and service contracts we have. There are applications in government or military, like for explosives or biomarkers, which could be done with or without a small-footprint mass spectrometer. The instrument could be applied to point of care, drugs of abuse, or remote clinics. Basically, it can be applied to anything that is at line, online, or truly portable.

The Focus LC is a robust instrument that delivers the same chromatographic results as legacy HPLC, but with the benefits of being compact, inexpensive to operate, and green.

LCGC: You mentioned mass spectrometry. Can the Focus be used with mass spectrometers or other technologies? What else is important for our listeners to know about the Focus LC?

WARD: LCMS is state-of-the art in the industry. Even if it's not all the time, many customers need it, especially when they have an unknown or when they have very low concentrations. We have been able to pair our instrument with a broad range of mass spectrometers from single quads, triple quads, ion traps, time of flight, and high-resolution mass spectrometers. Any mass spectrometer that has an appropriate source and works with the capillary flow rates will match well with the Focus LC. It comes down to the fluidic connection and the input-output (IO), and we have flexibility for that communication. On the electrical side, the IO and the plumbing are straightforward. We get data quickly with a broad range of LCMS or LCMS-MS. Recently, one of our customers had a peer-reviewed paper published in the *Journal of Separation Science*, and they demonstrated that they could get the same data from the Axcend Focus LC coupled with a range of mass spectrometers as they could with traditional legacy HPLC and mass spectrometers. This proved the tool works and that users can get what they need when they want a mass spectrometer, and they can get all the benefits of low solvents. Additionally, it is relatively well known in industry that capillary is an ideal flow rate in these single-digit µL/minute. With mass spec, you get higher efficiency out of that instrument in and of itself. Overall, the Focus LC is a robust instrument that delivers the same chromatographic results as legacy HPLC, but with the benefits of being compact, inexpensive to operate, and green.

Reference:

1. How C-suites and boards can seize the opportunity in their ESG risk strategy. EY. Accessed February 6, 2024. https://www.ey.com/en_us/consulting/esg-risk-strategy-for-c-suites-and-boards

From Detector to Decision Part IV: Demystifying Peak Integration

Nicholas H. Snow

Most quantitative analysis in chromatography is performed based on the peak area, the integrated area underneath the curve defining a peak. There are many variables and settings in a data system that can impact this determination. In this installment, we will examine several of the common parameters that can affect automated peak integration and the resulting peak areas. We will consider how the data system detects the beginning and end of the peak, how it determines the peak maximum, how real peaks are differentiated from noise, and how signals at individual time intervals are summed to generate the peak area. We will also briefly look at techniques for determining the areas of unusually shaped peaks.

IN PREVIOUS INSTALLMENTS, we have discussed what happens between the generation of signal by the detector and the appearance of a chromatogram on the screen or in a report. We discussed the basics of how the detector signal is converted into data and reports by the data system, how modern data systems automate operation of the instrument, and calibration techniques (1–3). We now take a step back from calibration and look at peak integration, which is one of the most important functions that happens behind the scenes.

Calibration and quantitative analysis based on peak areas are not possible without integration, and we will see that, while integration is now performed automatically by the data system, there are several parameters that should be understood by the user to ensure reliable and reproducible results.

What is Integration?

On a two-dimensional plot, such as a line or a traditional chromatogram, integration involves calculating the sum of the *y*-axis data points over a chosen range of the curve. A typical equation describing an integral is shown in equation 1:

$$\int_a^b f(x)dx \qquad [1]$$

The integral sign and the variables *a* and *b* represent that this is a definite integral with lower limit *a*, and the upper limit *b*, and the summation occurring between *a* and *b* on the *x*-axis. The function being integrated, called the integrand is *f*(*x*). The term *dx* represents the variable being integrated and can be thought of one slice or data point of that variable. We can also think of the integral as the sum of the *y*-axis data points, expressed in equation 2 as a summation.

$$\sum_{i=1}^{n} f(x) \qquad [2]$$

For a chromatographic peak, the limits of the summation or integration are given by the beginning and end of the peak, and the integer increments for the summation are given by each individual data point recorded by the detector.

Why Integrate? Origins of Chromatographic Peaks

Most modern quantitative chromatographic methods use the area underneath a peak, known as the *peak area*, to ultimately calculate the mass or concentration of each analyte of interest. Calibration techniques, including area percent normalization, response factors, internal standard, external standard, and standard additions were reviewed in a recent column (3). All these techniques rely on the peak area to provide the *y*-axis data used in plotting the calibration curve or calculating the estimated analyte mass or concentration.

Figure 1 shows a chromatographic peak, divided into slices, to show a simplified graphical example of peak area determination. The peak area is simply the sum, or integral of the signals. The signal is generated, usually as a voltage, by the detector, which is then converted to a series of data points versus time through sampling by an analog to digital conversion, and then transmitted as a digital signal to the data system, where the series of data points is stored in a file for analysis and reporting, including integration.

Main Factors Affecting Integration

There are several detector and data system settings that can impact integration, peak detection and peak areas. A full description of each of these could more than fill a "GC Connections" column, so we will summarize some key points with each and briefly discuss the origins of each of their effects on integration.

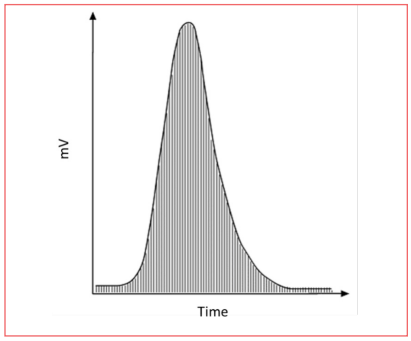

FIGURE 1: Integration of a peak. Each slice is a data point. Adapted from CHROMacademy, *LCGC International*'s online learning platform (5).

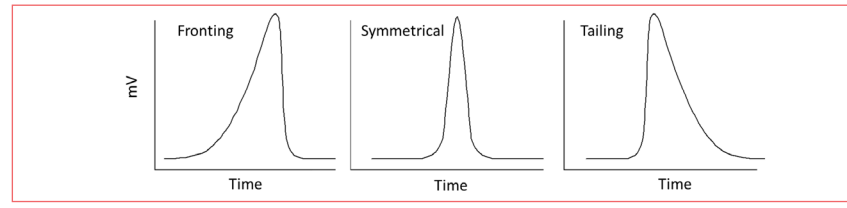

FIGURE 2: Symmetrical, fronting and tailing peaks.

The detector data acquisition rate will usually not change the peak area, but it does change the width of each of the slices seen in Figure 1. *Threshold* is a measure of the change in the slope of the curve seen in Figure 1 (the second derivative of the curve), and is used to determine when the peak starts and stops. A peak width setting can prevent integration of peaks, such as short-term noise spikes, that are too sharp to be analyte peaks. The last two factors, peak shape and peak overlap, are not detector or integrator settings, but they can have a major impact on integration. There still is no universally effective means for precisely and accurately integrating overlapped peaks and separating the overlapped signals.

The units of an integrated peak, as seen in Figure 1 where the *y*-axis is in mV and the *x*-axis is sec are: mV × sec. Each slice is a rectangle with the long side (*y*-axis) in mV and the short side (*x*-axis) in sec.

Data Acquisition Rate

The data acquisition rate determines the number of *x*-axis data points for any given period of time on a chromatogram. For a flame ionization detector (FID), a typical data acquisition rate is 20–50 data points/s. If a peak is two seconds wide, then there are 40 data points at 20 points/s. In performing the integration, the signals measured for these 40 data points are summed by the data system, with each signal being multiplied by 1/20 s to give the area of the slice in mV s. If the acquisition rate is 50 data points/s, then there are 100 data points, but each is multiplied by 1/50 s to give the area of each slice. The two peak areas then add the same.

Integration and peak area can be affected by acquisition rate in two situations. First, higher acquisition rate usually leads to increased noise, which may not affect the average peak area determined for multiple samples, but can impact the precision of that average. Second, if the peak is narrow or the acquisition rate is too slow, there may not be enough data points to precisely define the peak. This is most often seen in full-scan gas chromatography–mass spectrometry (GC–MS), where the acquisition rate may only allow a few scans to determine a peak.

Threshold

Examining the peak in Figure 1, in order to determine the beginning and end of the peak and the maximum, for reporting the retention time, the integrator must examine the slope of the curve and determine changes. Before the peak is eluted (to its left on the plot in Figure 1), all we see is baseline, the slope of the curve (first derivative) is zero, and the slope is not changing (second derivative is zero). As the peak starts to rise, the slope becomes positive and is increasing; both the first and second derivatives are positive.

Threshold is a measure of these two derivatives that tells the integrator when the peak starts, and therefore when to start summing the peak area. This is an adjustable parameter that can clearly affect the summed peak area. If the threshold is too sensitive, then noise and baseline may be included in peak areas, and small, spurious peaks due to noise and baseline drift may be integrated. If the threshold is not sensitive enough, then small peaks of interest may not be counted, and peak areas may be slightly low.

As we follow the peak rise, there is an inflection point where the slope is still positive but the change in slope goes to zero and then the slope, while still positive, is decreasing. When the slope and the change in the slope then reach zero, this is the peak maximum and the retention time is recorded. Following the peak maximum, the slope starts to increase in the negative direction, again passing through an inflection point, and eventually both the slope and the change in slope return to zero. This marks the end of the peak, and integration is stopped. Threshold impacts the location where the peak starts and stops, the number of data points used to calculate the peak area, and the peak area itself.

Peak Width

Generally, in chromatography, the peak width increases predictably as retention time increases. In isothermal separations, the peaks get wider and shorter; in temperature programming they are all about the same width, and may get slightly wider with longer retention times (4). The peak width setting on an integrator allows for peaks of unusually wide or narrow peak width to not be integrated, as these are often spurious results and not analyte peaks, or if improperly set can result in too many peaks (such as noise being integrated), or too few (such as some analyte peaks of interest not being integrated). Most data systems today will default this to an appropriate setting based on chromatographic conditions; it may need adjustment if peaks of interest are not integrated, or if too many peaks, including apparent noise spikes, are integrated.

Peak Shape

As seen in Figure 1, most chromatographic peaks are symmetrical, in statistics, representing a Gaussian distribution of mass

around the center of mass, which is also seen at the peak maximum. The peak maximum is also the reported retention time. For a symmetrical peak, the retention time also corresponds to the center of mass of the peak and the peak width, discussed above, represents a function of the width at baseline or at half-height, corresponding to the width at the inflection points described above.

Commonly, chromatographic peaks are not symmetrical; they either front, with more of the mass eluting before the peak maximum, or they tail, with more of the mass eluting after the peak maximum. Examples of symmetrical, fronting, and tailing peaks are shown in Figure 2.

Looking at the fronting and tailing peaks in Figure 2, we immediately see that the front of the fronting peak and the back of the tailing peak have unusual shapes, representing greater mass toward the beginning of the fronting peak and greater mass toward the end of he tailing peak. While, for the same analyte mass, the total peak area is nominally the same as a symmetrical peak, these peaks present several problems for the integrator.

As discussed above, the starting and ending points of the peak for integration are determined by changes in the slope of the curve. As seen in Figure 2, the curve at the start of the fronting peak has a different shape than the symmetrical peak; if the integrator is optimized for detecting the expected slope change for the symmetrical peak, it may not accurately detect the start of the fronting peak. Likewise, this situation would arise for detecting the end of the tailing peak. This can reduce both precision and accuracy of integration.

Peak shape also impacts the accuracy of retention time determination. Note in Figure 2 that the maximum of the fronting peak is shifted to a later retention time for the fronting peak, and to an earlier time for the tailing peak. By inspection, we also see that since the peak is not symmetrical on either side of the peak maximum, that the maximum no longer represents the center of mass. Further, this shift in the peak maximum becomes more pronounced as the tailing or fronting increases. In the rare case where the amount of tailing or fronting is reproducible, the retention time shift may not be a problem, as the retention time, although not representative of the center of mass of the peak, will still be reproducible. In gas chromatography, the situation, especially with tailing, where the amount of tailing varies, usually increasing with time and more column or inlet use, the retention time will vary, becoming shorter as the amount of tailing increases.

Conclusions

Peak integration is one of the many operations that occurs in the background on today's modern gas chromatographs and in their data systems. The peak area values shown in an area percent report are a function of the mass or concentration of analyte that has passed through the detector over a given time, as determined by an integration function in the data system. This integrator and the user must perform several functions to determine the peak area: detecting the start and end of the peak, the limits of integration; determining whether the peak is an analyte peak, baseline drift or noise; and accounting for variations in integration and retention time determination for asymmetrical peaks. These operations, while in the background, are often not simple, and failing to pay attention to them may cause loss of both accuracy and reproducibility in quantitative analysis. ■

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An Efficient Procedure for Determining Simple Sugars in Fruit Juices

Merlin K. L. Bicking

A fast, simplified procedure for determining simple sugars in fruit juices is described. Sample preparation has been generalized to allow for differences in the sample matrices. The use of an evaporative light scattering detector and a different separation column produces separations of fructose, glucose, sucrose, and maltose in less than three minutes, offering a significant improvement over standard methods. Part of the sample preparation can be automated further by using the programming features of many modern autosamplers.

THE DETERMINATION of simple sugars in food and food products is an important step in the production and marketing of these products to consumers. In the United States, current regulations require that manufacturers provide data on "total sugars" as part of the mandatory nutritional labelling (1). Analytical methods for these sugars (fructose, glucose, galactose, sucrose, maltose, and lactose) are not new, with methods appearing in the first books about high performance liquid chromatography (HPLC) (2). Early methods employed a variety of stationary phases, including silica, amino, ion exchange, hydroxylic, and other specialty phases. In combination with the appropriate mobile phase, many of these methods operated in hydrophilic interaction liquid chromatography (HILIC) mode. Detection required either refractive index (RID) or evaporative light scattering (ELSD) for use with the native molecules. Chemical derivatization allowed the use of more common detectors, such as absorbance or fluorescence, and it also added time and complexity to the procedure. Much of the early work on the separation of simple sugars and other carbohydrates has been reviewed and summarized by S. C. Churms (3). More recent method development efforts have focused on other detection options such as pulsed amperometric detection (PAD) (4) and mass spectrometry (MS) (5). Other recent reports have explored operating conditions, sample preparation, and sample types, with most studies using an amino or ligand-exchange column with either refractive index or light scattering detection (6–15).

Typical analysis times are 10 min or more. If information on disaccharides (such as, for example, maltose and lactose) and higher sugars is desired, the retention times on amino phases are even longer, as the retention is directly proportional to the number of sugar units. Current standardized methods used by many laboratories are similar to these procedures (16). Despite their popularity in published studies, most amino stationary phases suffer from reactivity and stability problems (17), resulting in constantly changing retention times and short column lifetimes. When coupled with refractive index detection, the user is further restricted to isocratic separations, resulting in lengthy equilibration and analysis times.

The hydroxylated phases, such as diol, offer better stability profiles but different selectivity toward carbohydrates. Reducing sugars exist in two anomeric forms, where the stereochemistry changes only at the anomeric carbon. These forms are slightly separated on the hydroxylated phases, causing peak splitting (17). Recent reports used a pentahydroxy phase and light scattering detection to produce much faster separations for a wider range of simple sugars and oligosaccharides (18–20). The present report describes using that same column under further optimized conditions to produce separations of four simple plant sugars (fructose, glucose, sucrose, and maltose) in fruit juices in only 3 min with a simpler mobile phase system. Sample processing has been evaluated and adjusted to improve applicability for matrix-specific challenges.

Materials and Methods

Reagents, Standards, and Supplies

Reagent grade (purity >99%) sugar standards (fructose, glucose, sucrose, and maltose) were obtained from Sigma-Aldrich Corporation. HPLC grade solvents (water and acetonitrile) were provided by Tedia, Inc. and Spectrum Chemicals, Inc. Ultra purity nitrogen

was supplied by Airgas. The sample vials, syringe filters (0.2 µm nylon), and plastic filter vials (Thomson, 0.45 µm nylon) were ordered from Chrom Tech, Inc. The filter vial consisted of a bottom section and a top insert which contained the filter element. The total volumetric capacity of the unit was approximately 450 µL.

Equipment

All separation experiments were performed using an Agilent Technologies 1290 HPLC, which included a model G4220A binary pump, a G4226A high performance autosampler, a G1316C heated column compartment, a G4212A diode array, and a G4218A ELSD. Data acquisition, processing, and reporting were completed using the OpenLab ChemStation (version C.01.09). An Agilent 1100 system containing a G1312A binary pump and G1313A standard autosampler was programmed to perform sample dilutions (see "Processing Option 3").

TABLE I: Sample characteristics and processing information

Brand	Serving Size (mL)	Sugars per Serving (g)	Dilution Volume (mL)
Minute Maid orange juice	295	30	25
Lakewood pineapple juice	240	31	50
Lakewood concord grape juice	240	38	50
Mott's apple juice	240	28	50
Simply light lemonade	240	7	25
Simply cranberry cocktail	240	33	50

Samples

Juice samples were obtained from a local retail store. Brands and sugar content are summarized in Table I. These juice types were selected to represent a range of color, complexity, and sugar content.

Sample Preparation

Upon receipt, a 100 mL aliquot of each sample was centrifuged for 10 m at 5000 rpm (ThermoFisher ST8 Centrifuge). The supernatant was carefully transferred to a sealed plastic container and stored at approximately 4 °C when not in use. A measured volume (nominally one mL) of supernatant was measured into a Class A volumetric flask. Flask volumes of 25 mL and 50 mL were used, with the final choice depending on the expected sugar concentration (see Table I). Additional sample processing used one of three general options.

Processing Option 1

An additional volume of water, approximately equal to the volume of sample,

TABLE III: Chromatography settings

Parameter	Setting		
Column	HALO 90 Å Penta-HILIC, 2.7 µm, 3.0 x 50 mm (Advanced Materials Technology)		
Pump flow	0.75 mL/min.		
Pump composition	Time	% A: Water	% B: Acetonitrile
	0.00	8	92
	3.00	23	77
	3.10	50	50
	3.60	50	50
	3.61	8	92
Pump stop time	6.0 min.		
Injection volume	2.0 µL		
Column temperature	65 °C		
ELSD	Evaporator temperature		60 °C
	Nebulizer pressure		45 psi
	Data acquisition rate		10 Hz
	Noise filter		2 sec.
	Gain		7

TABLE II: Autosampler programming for sample dilution

Function	Parameter	Comments
Pump	Flow	0.8 mL/min.
Pump	Composition	A (water): 30%, B (acetonitrile): 70%. Adjust as desired.
Eject	Eject 16 µL to Seat	Reset metering plunger to start position. Not required for all models.
Draw	Draw 16 µL from sample	Sample volume for 25x dilution. Use 8 µL for 50x dilution.
Wash	Use method settings.	Standard method for cleaning outside of needle.
Valve	Switch valve to main flow path into vial location "Sample + 10" for 30 sec	+10 is the relative position of receiving vial to sample. Adjust as needed.
Wash	Use method settings	Standard method for cleaning outside of needle.
Inject	Inject	Finishes the cycle.

was added to the flask with swirling. The desired volume of acetonitrile was then added directly to the flask. For example, if a level of 70% acetonitrile (v/v) was desired, approximately 17 mL was added to the 25 mL flask and approximately 35 mL to the 50 mL flask. Strict control of this volume was not necessary (see "Results" for more details). Following swirling to mix the solution, each sample was allowed to warm to room temperature because of the normal endothermic cooling that occurred when water and acetonitrile were mixed. Each solution was adjusted to its final volume using water. If significant foaming was observed, the final few drops used acetonitrile rather than water. A small aliquot was filtered directly into an HPLC vial and sealed immediately.

Processing Option 2

This option followed the same procedure as Option 1, except that the diluent was a premixed solution of acetonitrile and water. For example, a 70% (v/v) solution of acetonitrile:water would be added to the flask containing the sample and dilution water rather than adding the components separately. After partial dilution and equilibration to room temperature, the solution was adjusted to the final volume using the premix solution and filtered into a vial as in Option 1.

Processing Option 3

The sample supernatant solutions were loaded into the autosampler tray of the Agilent 1100 system. The base of the filter vial was placed at a specific location relative to the corresponding sample. For example, if the sample vials were loaded into locations 11–15, then the base units would be in positions 21–25, ten vial positions after each sample. The system was programmed to dilute all samples, as summarized in Table II. This general approach used the measuring capability of the autosampler to draw the sample and the flow accuracy of the pump to dilute the sample. The total volume of delivery was controlled by adjusting the flow rate and length of time that the autosampler directed the flow to the vial. Each cycle of this program produced one diluted sample and required approximately 2–3 m to complete. Following completion of this sample processing step, the top filter insert was pushed into the bottom section of the vial, causing the diluted sample to be filtered into the upper section of the vial. All vials were then transferred to the analytical autosampler for analysis. Additional information on this general dilution method is available from the author. This procedure is feasible on any systems that have the appropriate programming capabilities, but it is better suited to a standard HPLC system rather than a high pressure ultrahigh-pressure liquid chromatography (UHPLC) system because of the low operating pressures that result.

Preparation of Calibration Standards

Stock solutions of the four sugars were accurately prepared in water:acetonitrile (80:20 v/v) at 5 and 2 mg/mL levels of

each compound. Subsequent dilution of these solutions in water:acetonitrile (50:50 v/v) produced a total of five calibration standards at nominal levels of 0.10, 0.20, 0.50, 1.0, and 2.0 mg/mL levels. A separate check standard was also prepared at approximately 0.50 mg/mL levels of each sugar. The smaller acetonitrile amounts in the initial stock solutions were necessary to ensure solubility of all components.

HPLC Conditions

Table III summarizes the instrumental parameters for all separations. This separation is an example of a HILIC operating mode. Water is a strong solvent in this system, and it is programmed to larger concentrations during the main elution phase, followed by a cleaning step at 50% levels. Programmed cleaning is optional for samples, which are not expected to contain highly retained components such as proteins. The equi-

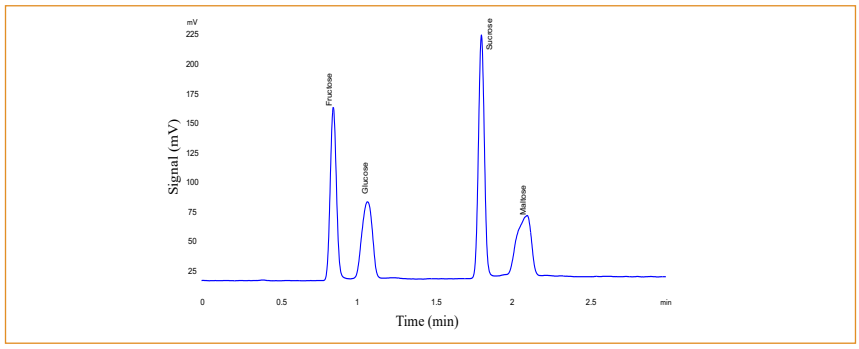

FIGURE 1: Typical chromatogram of 0.50 mg/mL calibration standard.

TABLE IV: Summary of total sugars in orange juice for differing amounts of acetonitrile added during processing

Sugars/Serving (g)[a]					
% ACN	Fructose	Glucose	Sucrose	Total	Label
0	7.0 (3.8)	6.9 (0.84)	13.2 (2.6)	27.1	30.0
30	7.3 (1.2)	6.8 (0.83)	13.9 (0.15)	28.0	30.0
50	7.3 (0.75)	6.4 (1.8)	13.3 (1.1)	27.0	30.0
70	8.0 (1.3)	7.0 (0.54)	13.8 (0.21)	28.8	30.0

[a]Numbers in parentheses are the % Relative Standard Deviations (%RSD) for duplicate injections on each of three preparations.

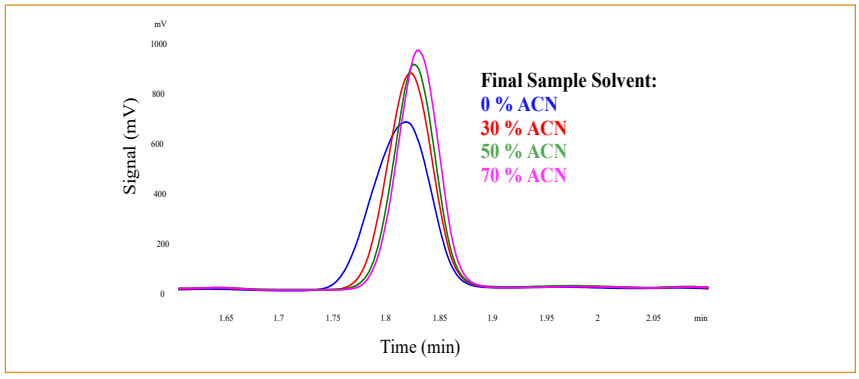

FIGURE 2: Typical chromatography for sucrose in orange juice when the amount of acetonitrile in the final sample solvent is varied. In this region of the separation, the amount of acetonitrile in the mobile phase varies from 92% to 77%.

TABLE V: Results of fruit juice analysis

	Sugars/Serving (g)[a]				
Sample	Fructose	Glucose	Sucrose	Total	Label
Orange juice	8.1	7.3	14.1	29.5	30
Pineapple	7.3	7.9	16.5	31.7	31
Grape	23.7	19.9	ND	43.6	38
Apple	15.9	9.0	4.2	29.1	28
Lemonade	0.6	0.6	5.1	6.3	7
Cranberry	1.3	2.4	25.9	29.6	33

[a] Average of duplicate injections.
ND = not detected at level above lowest standard.

libration time here (from 3.6 to 6.0 min) is optimized for a typical UHPLC system, and other HPLC systems with larger system volumes may require longer equilibration times. The column temperature was chosen to minimize the separation of anomers. At lower temperatures, peak splitting was observed.

System Calibration

Calibration of the instrument was accomplished using duplicate injections of standards from 0.10 to 2.0 mg/mL. Evaporative light scattering detectors (ELSD) are known to produce nonlinear calibration curves. The data from this system required the use of a second order polynomial for the average peak areas, but the correlation coefficients (r^2) for all compounds were greater than 0.999. Given the performance variability of modern ELSD instruments, other systems may require use of a different calibration range. The sample amount and dilution volume can then be adjusted as needed to produce final solutions within the calibrated concentration range.

Analysis of Samples

Samples were analyzed by duplicate injections of a single preparation. Multiple preparations were not needed because of the inherent homogeneity of liquids and the observation that the primary source of variability was related to the detector's response rather than the sample preparation procedure. Each sample set included injection of a process blank and a check standard (0.50 mg/mL calibration standard or separate check sample) before and after the sample injections. Analysis results were only acceptable if check standard concentrations were within ten percent of the expected value.

Results

The Halo Penta-HILIC column was chosen for this work because it produced high efficiency separations in a short time, typically generating 10,000 theoretical plates with a 50-mm length. The bonded phase consisted of a penta-hydroxy five carbon phase attached to a superficially porous particle (SPP) with a diameter of 2.7 µm. These particle types are similar in performance to sub-2-µm fully porous particles (FPPs), but generate significantly lower pressures and can be used on typical HPLC instrument with a 600-bar pressure limit. Additionally, this phase did not suffer from the stability problems associated with bonded amino phases. Previous work with the Penta-HILIC phase in this laboratory established gradient elution conditions for separating oligo- and polysaccharides. Those conditions were adjusted to stop the gradient after elution of the disaccharides, resulting in a 3-min gradient program as described above. Retention times for fructose, glucose, sucrose, and maltose were approximately 0.85, 1.05, 1.8, and 2.1 min, respectively. Figure 1 is an example of a typical chromatogram. Note that fructose, glucose, and maltose are reducing sugars and exist in two anomeric forms. In these anomers, the stereochemistry changes at the anomeric carbon, which is the carbon containing the carbonyl functional group (aldehyde or ketone) in the open form of the sugar. These anomers are slightly separated on this column phase, but the peaks coalesce at higher operating temperature. Still, the presence of the anomers is evident from the increase in peak width and small peak shape distortions for these sugars, when comparing to sucrose, which does not have anomeric forms.

The total gradient cycle time was approximately 6 min for a standard UHPLC system. Transferring to a traditional HPLC with a larger system volume would probably require more time for column equilibration, but the additional time should keep the total analysis time under 10 min. Compare this situation to the many literature references above, where the analytes were eluted at 10 min or later.

Optimization of Sample Diluent

In traditional reversed-phase (RP) methods, water is a weak solvent, and direct injection of a filtered aqueous solution is highly advantageous. In HILIC mode, however, water is a strong solvent and direct injection of aqueous solutions can produce peak shape problems (17).

The influence of different amounts of acetonitrile on both peak shape and analysis results for orange juice was evaluated over a range from 0 to 70% (v/v) acetonitrile, using Processing Option 1. Results are summarized in Table IV, including data for individual sugars and total sugars, on a per serving basis. All values for total sugars were within 10% of the nutrition label claim, indicating that the overall procedure produced appropriate results. The lack of a clear trend across these experiments also suggests that the amount of acetonitrile does not appear to be a critical experimental variable. Such results allow the method to be adjusted as needed for a particular sample to improve processing or remove interferences. The general experience in this laboratory for a wide range of samples indicates that the filtration resistance and solution clarity can vary with differing amounts of acetonitrile, and the ability to adjust that processing variable without altering the analytical results is a significant advantage for this procedure.

The impact on peak shape is another consideration when evaluating this experiment. Figure 2 provides an excellent illustration of the impact that sample solvent composition has on peak shape in a HILIC system. Although the general separation is unchanged, there are subtle differences that become apparent when looking closely at any individual peak. The all-aqueous injection has a reduced retention time, and the peak width is noticeably wider. In addition, peak fronting is observed. Such observations are expected when the sample solvent is significantly stronger than the mobile phase. As noted in the previous paragraph, the analytical results are not affected by this change in sample solvent, so the user may decide that the reductions in peak shape and resolution are not more important than the increased cost and time associated with the addition of acetonitrile during processing. On the other hand, if chromatographic interferences are present or other problems are observed during processing, the use of acetonitrile in the sample solvent may be warranted. A secondary benefit of adding acetonitrile is the opportunity to precipitate other sample components, such as proteins, that might otherwise remain on the column, resulting in earlier column failure.

All other results presented here used 70% acetonitrile in sample processing, as it produced the best combination of peak shape, visual sample clarity, and filtration resistance for this type of samples. Clearly, other sample types may require a different optimum concentration, and the user should strongly consider at least a preliminary evaluation during method development.

TABLE VI: Comparison of manual and automated sample processing

Sample	Total Sugars per Serving (g)		
	Manual[a]	Automated[b]	Label
Orange juice	27.5 (2.0)	27.2 (2.0)	30
Apple juice	27.5 (0.63)	27.4 (0.056)	28

[a] Processing option 2.
[b] Processing option 3.
Numbers in parentheses are the % relative standard deviations (%RSD) for triplicate analyses.

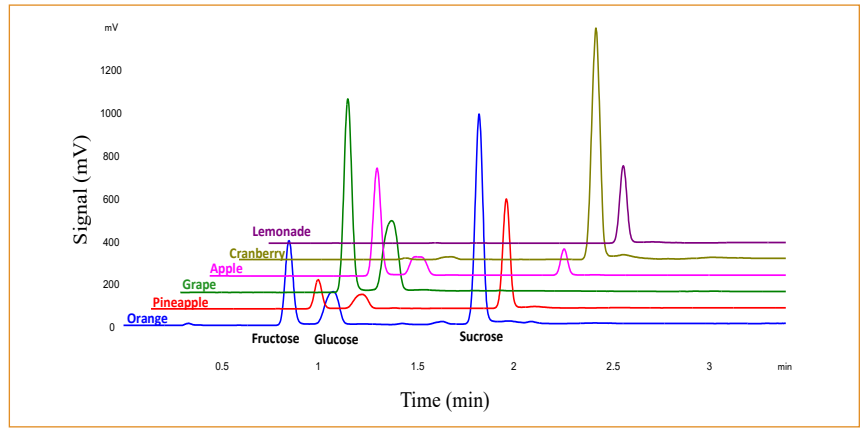

FIGURE 3: Chromatographic examples for fruit juices.

Analysis of Juice Samples

Six juice samples obtained from a local retailer were analyzed using Processing Option 1 with 70% acetonitrile. No unusual problems were observed, and all dilutions were easily filtered prior to analysis. The results are summarized in Table V. This group of samples represented a range of total sugar content as well as the relative amounts of individual sugars. At least two sugars were present in all samples, and the amounts of each varied as expected. Maltose was not found in any of these samples. In each case the calculated value for total sugars was close the label claim for all samples. Given the inherent variability in food products and regulatory labelling requirements, it was expected that the measured values would likely be somewhat lower than the label claim. The analysis results were generally in agreement with the label, except for grape juice, which was somewhat higher.

Figure 3 displays typical chromatographic results for fruit juices. In these samples, no unknown peaks interfered with fructose and glucose. Some small peaks were observed just after the sucrose peaks and careful review of retention times and peak shape was necessary to ensure proper peak identification and construction of the integration baselines. Experience in this laboratory for a wide variety of sugar-containing drinks suggested that

interferences were the exception, not the rule. Of course, more complex samples and actual food samples would likely require additional sample preparation; however, the current procedure provides a good starting point for method development with such sample types.

Automation of Sample Processing

With sample processing and analysis optimized, one final experiment explored the possibility of automating the sample processing step using a programming feature of many modern autosamplers. This procedure, outlined above as Processing Option 3, uses the pump and autosampler of an HPLC system to dilute a sample directly into an HPLC vial.

Modern HPLC autosamplers are capable of accurately drawing known volumes of samples into the needle and loop at volumes between approximately 2 and 100 µL. Meanwhile, the solvent delivery systems can deliver an accurate composition at a carefully controlled flow rate. Since the general sample processing steps in this method involved the simple dilution of a known volume of sample to another known total volume, this procedure could easily be programmed using the Injector Program feature of the software. The total solution was directed into the bottom section of the two-piece filter vial. After completion of the injector cycle, the top section of the filter vial was pushed into the bottom section, forcing the diluted sample into the top section while also filtering out particulates and precipitates. This vial was then immediately transferred to the analytical instrument and analyzed using the established method. A second set of samples was prepared manually for comparison, using Processing Option 2 (dilution with a pre-mixed water-acetonitrile solution). The results are summarized in Table VI.

An analysis of variance for the two data sets indicated no differences at the 95% level. Clearly, the automated procedure generated total sugar values that were not different from the manual method. However, the automated method required no additional volumetric glassware or pipettes and used much less total solvent than the manual method.

Limitations

Although the many advantages of this procedure have been outlined here, it is important to understand the limitations that also exist.

- This method was developed for fast analysis of four components in relatively simple matrices with no significant interference. As the sample becomes more complex, separation problems may arise which might require the use of alternative sample preparation, a longer gradient, or a longer column.
- Most ELSD instruments produce nonlinear calibration curves and require polynomial fits for proper calibration. Although there are no technical issues with using a nonlinear calibration equation if the model properly fits the data, some users are uncomfortable with this approach.
- Glucose and galactose are not well separated, and lactose and maltose are also not completely resolved. Thus, the analysis of dairy sugars is not going to provide a separate value for these components.
- Some sugar alcohols interfere with some of the sugars studied here. Ribitol, xylitol, and arabitol are only partially separated from fructose. Sorbitol, mannitol, and dulcitol co-elute with glucose and maltitol is only partially separated from maltose. Fortunately, the levels of these compounds are expected to be low in most fruit juices. If they are present at interfering levels, then changes to the gradient or column length will probably be necessary.

Conclusions

An HPLC-based method has been described that separates four common sugars in fruit juices in less than 3 min using a simple acetonitrile–water mobile phase, with a total run time of 6 min. The sample preparation procedures were generalized to allow variable amounts of organic solvent depending on the individual needs for the analysis. Furthermore, the processing and dilution scheme could be adjusted as needed to produce analyte concentrations within the range of the calibration system, and automation of this step was feasible. No significant interferences were observed in the six fruit juices.

Future Extensions of this Work

Previous experiments established a general procedure for analyzing some oligo- and polysaccharides, and that work will continue as an alternative to more laborious testing regimens. The general methodology approach that is outlined here has been successfully applied to other juices and drinks containing plant sugars (unpublished results), and extension to raw fruits and vegetables, and even some processed foods, should be successful. Of particular interest is the measurement of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP). This group of compounds includes short-chain carbohydrates that are poorly absorbed in people with irritable bowel syndrome (IBS) and other digestive disorders (21). Foods that have higher levels of fructose compared to glucose can lead to gastric problems in susceptible individuals. The method described here can make that determination with a 6-min analysis.

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Analysis of Coumarin in Tobacco, Smokeless Tobacco Products, and Electronic Cigarette Liquids by Isotope Dilution LC–MS/MS

Jingcun Wu

This article describes a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the analysis of coumarin in various tobacco matrices and electronic cigarette (E-cig) liquids, and highlights the importance of evaluating different MS/MS transitions of an analyte in complex sample matrices to overcome matrix effects. Matrix interfering components were separated from analyte using a C18 ultrahigh-pressure liquid chromatography (UHPLC) column with a larger inner diameter (3.0 mm, or 4.6 mm). Matrix suppressions on analyte responses were corrected by isotope dilution. Four different MS/MS transitions of coumarin were studied in each sample matrix to select a suitable MS/MS transition for analyte quantification based on matrix effects on each MS/MS transition. The method was validated using different tobacco matrices and E-cig liquids.

COUMARIN (1,2-BENZOPYRONE) occurs as a natural component in some plants, and is found as a flavoring ingredient in some foods, tobaccos, and cosmetic products. Coumarin has also been identified and determined as a natural constituent in different types of tobaccos (1). The high content of coumarin in some foods, tobacco products, and other consumer products has received considerable attention due to its hepatotoxic effects found in animal experiments (2). The European food safety authorities have set a maximum limit of 2 mg/kg for foods and beverages in general, and a maximum level of 10 mg/L for alcoholic beverages (3). Coumarin has been banned as a flavor additive in food and other products in the United States (4), and has been included by the U.S. Food and Drug Administration (FDA) on the established list as a harmful and potentially harmful constituent in tobacco and smokeless tobacco products (5).

Electronic cigarettes (E-cigs), also known as vaporizers or vaping products, are battery-powered devices to heat liquid-based nicotine into an inhalable vapor. E-cigs have been widely marketed as a safer alternative to traditional cigarettes, because they can quell smokers' urges for nicotine without using cancer-causing tobacco. However, whether E-cig has less risk or more risk is still debatable due to the devices' high nicotine content and other chemicals and additives in the liquids. E-cigs are by far the most popular tobacco product among teens, according to the 2017 national youth tobacco survey (6). The teens are attracted to vaping by the various flavors in the E-cig liquids. The U.S. FDA is weighing a ban on most flavored E-cigs following a recent national outbreak of E-cig or vaping product use-associated lung injury (EVALI), which is linked to vitamin E acetate used as an additive in some vaping products (7,8).

Although different analytical methods have been used for the determination of coumarin in plant extracts, foods, and fragrance products, the most widely used method in the past was based on high pressure liquid chromatography-ultraviolet spectroscopy (HPLC-UV) with sample clean up and concentrations (9). However, HPLC-UV method suffered from its drawbacks of low selectivity and sensitivity. Because of the low selectivity, the method could easily give false positive results for coumarin due to matrix interfering components, especially for complex sample matrices such as food and tobacco samples, and, therefore, it is required to use a longer analytical column and take longer runtime to separate coumarin from sample matrix components. Due to the low sensitivity of the method, extensive sample clean up and analyte concentration steps are often needed to achieve good separation and sensitive response for coumarin analysis. The gas chromatography-mass spectrometry (GC–MS) method is more selective than the HPLC-UV method and has been

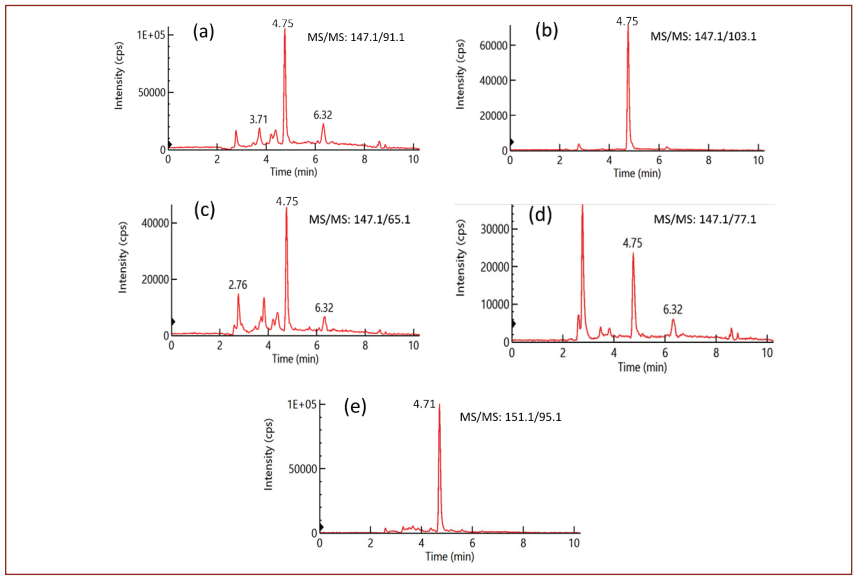

FIGURE 1: (a-e) LC–MS/MS chromatograms for the four MS/MS transitions of coumarin in a smokeless reference tobacco sample CRP3.1.: (a) MS/MS 147.1/91.1; (b) MS/MS 147.1/103.1; (c) MS/MS 147.1/65.1 and (d) MS/MS 147.1/77.1, and for its internal standard (e) MS/MS 151.1/95.1.

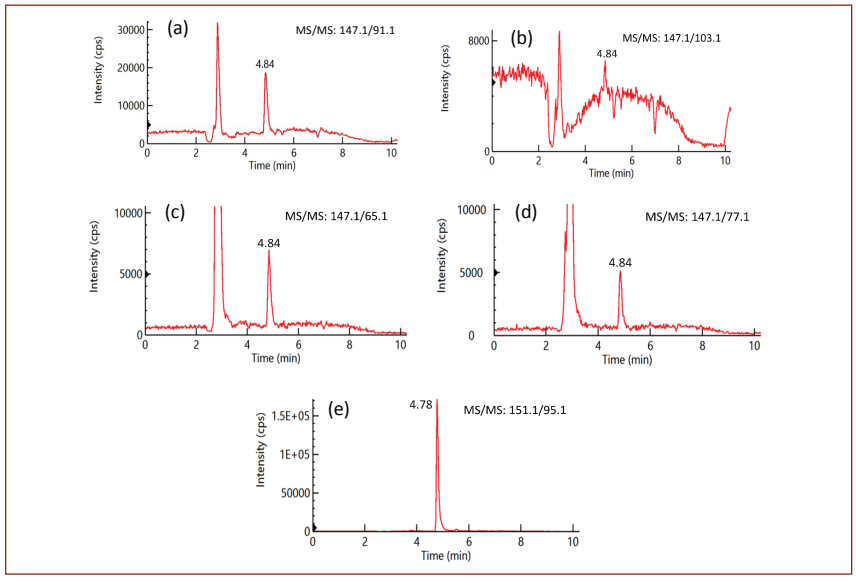

FIGURE 2: (a-e) LC–MS/MS chromatograms for the four MS/MS transitions of coumarin in an E-cig liquid sample: (a) MS/MS 147.1/91.1; (b) MS/MS 147.1/103.1; (c) MS/MS 147.1/65.1 and (d) MS/MS 147.1/77.1, and for its internal standard (e) MS/MS 151.1/95.1.

used for coumarin analysis in tobacco samples (1,10,11). But it still needs sample clean up and analyte concentration to achieve good sensitivity. Recently, the liquid chromatography-tandem mass spectrometry (LC–MS/MS) method has been developed for coumarin analysis in food samples, and demonstrated much higher sensitivity and selectivity with simpler sample preparation (12–16). In this study, a fast, sensitive, selective, and robust LC–MS/MS method has been developed for the analysis of coumarin in various tobacco sample matrices. To obtain accurate results from complex tobacco sample matrices, several reference tobacco samples were used during method validation and multiple MS/MS transitions were evaluated for coumarin identification and quantification. Compared to the GC–MS method, this LC–MS/MS method is much simpler (with no need for sample clean up and analyte concentration), faster, more selective, and sensitive. In addition, using a stable isotope labeled internal standard, this method is more accurate and robust and can be easily applied to coumarin analysis in tobacco, smokeless tobacco products (STP), and E-cig liquid samples in a routine testing laboratory.

Experimental

Chemicals and Materials

Coumarin (≥99% in purity) was obtained from Sigma-Aldrich and deuterium labelled coumarin- 5,6,7,8-d4 (98% in purity) was obtained from Toronto Research Chemicals. LC–MS grade methanol, water, formic acid, and other chemicals were obtained from Sigma-Aldrich. The Kentucky reference cigarette tobacco (KY3R4F) was obtained from the Tobacco and Health Research Institute at University of Kentucky, in Lexington, Kentucky. Smokeless reference tobacco products (1S2 dry snuff and 2S3 moist snuff) and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) recommended reference smokeless tobacco products (CRP) were provided (free of charge) by the Department of Crop Science at North Carolina State University, in Raleigh, North Carolina. Two batches of CRP reference samples were obtained; one batch of samples was produced in 2009 and labeled as: CRP1 (snus), CRP2 (moist snuff), CRP3 (dry snuff) and CRP4 (chewing tobacco), and the other batch was produced in 2016 and labeled as: CRP1.1 (snus), CRP2.1 (moist snuff), CRP3.1 (dry snuff) and CRP4.1 (chewing tobacco). Other testing samples, including five brands of cigarettes and five brands of mini-cigar samples, as well as six brands of E-liquid samples, were obtained from a local store in Toronto, Canada.

Standard Preparation

The primary coumarin standard solution (10 mg/mL) and internal standard (IS) coumarin-*d4* solution (1 mg/mL) were prepared in methanol, separately. The secondary coumarin standard solution (10 µg/mL) and internal standard solution (IS spiking solution, 10 µg/mL) were prepared separately by diluting their primary

standard solutions with 50% methanol solution (in LC–MS grade water, v/v). A tertiary coumarin standard (1.0 µg/mL) was prepared by diluting the secondary solution with 50% methanol solution. Twelve levels of calibration standards containing coumarin at 0.05, 0.1, 0.5, 1, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL were prepared from the secondary and tertiary standard solutions by dilutions with the 50% methanol solution. Each calibration standard contained 100 ng/mL internal standard. Two standard zero solutions were also prepared. Standard 01 was prepared by adding 50% of methanol solution directly into an auto sampler vial to check the background and potential contamination from the vials, and standard 02 contains only the 100 ng/mL of IS, which was prepared to check the isotope purity of the IS.

Tobacco and Smokeless Tobacco Sample Preparation

A 1.0 g sample of the homogenized tobacco product from a freshly opened source was spiked with 200 µL of the IS spiking solution, and then extracted with 20 mL of 50% ethanol solution (in Type I water, 1:1 in v/v) in a 50 mL flask and agitated for 30 min on a shaker. The sample solution was then filtered through a 0.22µm syringe filter into an 8 mL storage vial. A portion of the filtered solution was analyzed directly by the LC–MS/MS method after appropriate dilution with 50% methanol solution.

E-cig Liquid Sample Preparation

A 1.0 g sample of the E-cig liquid sample from a freshly opened source was spiked with 100 µL of the IS spiking solution and then diluted with 10 mL of 50% methanol solution in a 50 mL centrifuge tube and agitated for 10 min on a shaker. The sample solution was analyzed directly by the LC–MS/MS method without further sample treatment.

Quality Control Sample Preparation

To test possible interference or contamination from reagents or materials used and from the sample preparation processes, a laboratory reagent blank (LRB) was prepared per work shift by following the same procedures as for tobacco or E-cig liquid sample preparation described above, without adding tobacco or E-cig liquid sample. To study possible analyte loss or contamination during sample preparations, a Laboratory Fortified Blank (LFB) sample was prepared per work shift by following the same tobacco or E-cig liquid sample preparation procedures as described above, by spiking a known amount of analyte solution. During method validation, LFB samples were prepared by spiking the analyte in three different concentration levels, as shown in Table I,

TABLE I: The coumarin amounts spiked in QC samples and the recovery results

Sample ID	Sample Matrix	Spiked (ng/g)	Recovered (ng/g)*	Recovery (%)
LRB	Lab Reagent Blank	0	0	0
LFB1	Lab Fortified Blank	20	20.2	101
LFB2	Lab Fortified Blank	100	107.8	108
LFB3	Lab Fortified Blank	1000	986.4	98.6
CRP4.1-LFM1	STP (Chewing Tobacco)	20	25.2	116
CRP4.1-LFM2	STP (Chewing Tobacco)	40	33.6	84.0
CRP4.1-LFM3	STP (Chewing Tobacco)	100	110.4	110
CRP4.1-LFM4	STP (Chewing Tobacco)	400	432.0	108
CRP4.1-LFM5	STP (Chewing Tobacco)	1000	991.8	99.2
CRP2.1-LFM1	STP (Moist Snuff)	40	48.1	120
CRP2.1-LFM2	STP (Moist Snuff)	100	108.9	109
CRP2.1-LFM3	STP (Moist Snuff)	400	449.9	112
CRP2.1-LFM4	STP (Moist Snuff)	1000	1141.7	114
CRP3.1-LFM	STP (Dry Snuff)	200	214.7	107
CRP1.1-LFM	STP (Snus)	100	99.7	99.7
1S2-LFM1	STP (Dry Snuff)	100	113.2	113
1S2-LFM2	STP (Dry Snuff)	1000	1060.1	106
2S3-LFM1	STP (Moist Snuff)	100	94.5	94.5
2S3-LFM2	STP (Moist Snuff)	1000	1044.3	104
KY3R4F-LFM	Cigarette Tobacco	100	89.0	89.0
Cigar1-LFM	Cigar Tobacco	200	209.8	105
E-Cig S1-LFM1	E-Cig Liquid	20	18.7	93.5
E-Cig S1-LFM2	E-Cig Liquid	50	47.9	95.8
E-Cig S1-LFM3	E-Cig Liquid	100	106.3	106
E-Cig S6-LFM1	E-Cig Liquid	20	17.9	89.5
E-Cig S6-LFM2	E-Cig Liquid	50	51.3	103
E-Cig S6-LFM3	E-Cig Liquid	100	93.6	93.6

* Average values of three replicate samples.

and three replicates of the LFB samples at each level were prepared. To evaluate sample matrix effects and analyte recovery from sample matrix, a Laboratory Fortified Matrix sample (LFM) was prepared per work shift by following the same tobacco or E-cig liquid sample preparation procedures as described above, using 1.0 gram of a reference tobacco or E-cig liquid sample spiked with a known amount of analyte. The percent recovery is calculated by comparing the difference of the spiked (LFM sample) and non-spiked sample results and the expected (spiked) value. During method validation, the LFM samples were prepared using seven different reference tobacco sample matrices (CRP1.1, CRP2.1, CRP3.1, CRP4.1, 1S2, 2S3 and KY3R4F) and a mini cigar tobacco sample, and different concentrations of analyte were spiked onto each tobacco sample matrix as illustrated in Table I. The LFM samples for E-cig liquids were prepared using two different E-cig liquid sample matrices (with different nicotine contents and flavor components), and three different concentrations of analyte were spiked onto each sample matrix, as shown in Table I.

Analytical Conditions

Chromatographic separation of coumarin from matrix interfering components was conducted using an ultrahigh-pressure liquid chromatography (UHPLC) system (PerkinElmer LX-50) and coumarin

TABLE II: MS/MS transitions, peak area ratio of qualifier to quantifier ions in standard and in sample CRP3.1, and the coumarin results in CRP3.1 obtained from each MS/MS transition

MS/MS Transition	Qualifier Ion/ Quantifier Ion	Area Ratio (Standard)	Area Ratio (CRP3.1)	Coumarin Found (ng/g)
147.1/91.1	-	-	-	1542.5
147.1/103.1	103.1/91.1	0.72	0.71	1572.6
147.1/65.1	65.1/91.1	0.45	0.43	1544.9
147.1/77.1	77.1/91.1	0.21	0.22	1632.1

detection was achieved using a triple quadrupole mass spectrometer (PerkinElmer QSight 220). Two LC–MS/MS systems were used for method robustness validation. Several available UHPLC columns were tested initially; including Phenomenex Kinetex PFP, C8, C18 and XB-C18 columns (2.6 µm, 100 × 4.6 mm), Phenomenex Kinetex C18 column (2.6 µm, 100 × 2.1mm), PerkinElmer Quasa SPP Pesticides column (C18, 2.7µm, 100 × 2.1mm and 100 × 4.6 mm) and PerkinElmer Brownlee SPP C18 (2.7µm, 100 × 2.1mm and 100 × 3.0 mm). The C8 and C18 columns with larger inner diameter (id) such as 3.0 mm or 4.6 mm provided good separation of coumarin from matrix interferences and thus were used during method robustness validation. Mobile phases were water (A) and methanol (B). LC separation was achieved using an isocratic elution of 60% mobile phase B for 6 min at a flow rate of 0.4 mL/min, followed by a gradient to 100% mobile phase B at 6.5 min to wash and clean the column. Finally, the mobile phases were returned to the initial composition at 8 min and then kept under the initial conditions for 4 min for column equilibration. The total run time for each sample including equilibration time was 12 min. Column temperature is 30 °C, and the injection volume is 5 mL. The auto sampler was controlled at 5 °C. Mass detection conditions were as follows: ionization mode: positive ESI/MRM, ion spray voltage: 2000 V; ion source temperature: 400 °C, HSID temperature: 320 °C, drying gas: 120 L/h, nebulizer gas: 400 L/h.

TABLE III: Coumarin results from tobacco samples obtained by each of the four MS/MS transitions

Sample ID	Tobacco Type	Coumarin Amount (ng/g) Measured by Four MS/MS Pairs			
		147.1/91.1	147.1/103.1	147.1/65.1	147.1/77.1
KY-3R4F	Cigarette Tobacco	< LOQ	< LOQ	< LOQ	< LOQ
Cigarettes*	Cigarette Tobacco	< LOQ	< LOQ	< LOQ	< LOQ
Mini - Cigars*	Cigar Tobacco	< LOQ	< LOQ	< LOQ	< LOQ
CRP1 (2009)	STP (Snus)	< LOQ	< LOQ	< LOQ	< LOQ
CRP1.1(2016)	STP (Snus)	< LOQ	< LOQ	< LOQ	< LOQ
CRP2 (2009)	STP (Moist Snuff)	258.3 (5.0) **	245.8 (1.9)	245.9 (13.0)	246.1 (15.5)
CRP2.1 (2016)	STP (Moist Snuff)	662.8 (22.9)	656.8 (7.7)	626.3 (11.6)	702.8 (31.7)
CRP3 (2009)	STP (Dry Snuff)	278.1 (10.3)	275.6 (3.9)	261.7 (20.2)	276.9 (24.8)
CRP3.1 (2016)	STP (Dry Snuff)	1542.5 (22.8)	1572.6 (22.7)	1544.9 (26.7)	1632.1 (46.9)
CRP4 (2009)	STP (Chewing Tobacco)	< LOQ	< LOQ	< LOQ	< LOQ
CRP4.1 (2016)	STP (Chewing Tobacco)	< LOQ	< LOQ	< LOQ	< LOQ
1S2	STP (Dry Snuff)	1094.7 (54.1)	1119.3 (54.1)	1087.4 (48.9)	1166.2 (34.7)
2S3	STP (Moist Snuff)	742.3 (19.9)	790.5 (20.2)	751.7 (33.4)	690.3 (52.5)

* Five brands of cigarettes and five brands of mini cigars obtained from a local store were studied, respectively.

**The values in parentheses are standard deviation, $n = 5$

TABLE IV: MS/MS transitions, peak area ratio of qualifier/quantifier ions in a standard and in a E-Cig liquid sample, and coumarin results in the sample obtained from each MS/MS transition

MS/MS Transition	Qualifier Ion/ Quantifier Ion	Area Ratio (Standard)	Area Ratio (E-cig Liquid)	Coumarin Found (ng/mL)
147.1/91.1	-	-	-	11.07
147.1/103.1	103.1/91.1	0.74	0.095	1.31
147.1/65.1	65.1/91.1	0.45	0.39	9.04
147.1/77.1	77.1/91.1	0.22	0.24	12.25

Compound-dependent parameters, such as collision energies (CE), entrance voltages (EV), and the lens voltages (CCL2), were optimized by infusion of standards and use of the software. Four different fragment transitions (MS/MS) were monitored for coumarin under multiple reaction monitoring (MRM). The following optimized conditions were used for all the four transitions: dwell time, 85 ms; EV, 20 V; and CCL2, -45 V. The following collision energy (CE) was used for each transition, respectively: -33 eV for the first coumarin MS/MS transition (147.1/91.1) and coumarin-d4 (MS/MS: 151.1/95.1); -25 eV for the second coumarin MS/MS (147.1/103.1) and coumarin-d4 (MS/MS: 151.1/107.1); -48 eV for the third coumarin MS/MS (147.1/65.1) and coumarin-d4 (MS/MS: 151.1/68.2); and -36 eV for the fourth coumarin MS/MS (147.1/77.1) and coumarin-d4 (MS/MS: 151.1/80.2). Quantification of coumarin was performed using the ratio of peak area of coumarin to that of internal standard coumarin-*d4*.

Results and Discussion

LC–MS/MS Method Optimization

For mass detection of coumarin, both positive and negative electrospray ionization (ESI) modes were evaluated initially. The results showed that the analyte gave better sensitivity and better signal to noise ratio under positive mode and therefore positive ESI detection was used in this study. For coumarin and its internal standard (IS) coumarin-d4, several product ions were generated at certain collision energies and thus multiple MS/MS transitions could be formed. The optimized MS/MS parameters were listed in the above experimental section in the order of signal intensity.

To separate coumarin from interfering components in different tobacco sample

matrices, several reversed phase UHPLC columns available in our laboratory were evaluated. The Kinetex PFP (pentafluorophenyl propyl) column was confirmed not suitable for this study because very broad and splitting analyte peaks were found with this column. All the tested C8 and C18 columns, including Quasar SPP C18, Brownlee SPP C18, Kinetex C8, C18, and XB-C18 columns, could be applied to the analysis of coumarin. But it was found that the columns with smaller inner diameters (2.1 mm in this study) had difficulty to separate coumarin from interfering components, especially for the third MS/MS transition (147.1/65.1) chromatograms due to relatively stronger interfering component peaks at this transition as shown in Figure 1c. The C8 and C18 columns with larger column inner diameters (4.6 mm or 3.0 mm) provided much better separation efficiency, due to a higher column peak capacity, and therefore were used during method validation and final application. Mobile phase compositions of methanol/water and acetonitrile/water with and without acid were evaluated and it was found that methanol/water provided the highest analyte signal intensity. Figure 1 shows an example of separating coumarin peak from interfering components in a smokeless tobacco sample. Figure 2 gives an example of separating coumarin peak from interfering components in a E-cig liquid sample.

Extraction of Coumarin from Tobacco and Smokeless Tobacco Products (STP)

The method extraction efficiency for the targeted analytes is one of the most important parameters that will influence the method's precision, accuracy, and robustness. The extraction efficiency depends on the solubility of the analytes in a certain solvent or a mixture of solvents, the number of extraction cycles applied, the extraction time in each extraction cycle, extraction temperatures and extraction techniques used. Coumarin has been extracted from tobacco samples by solid-liquid extraction method with different solvents. Various techniques have been used to improve coumarin extraction efficiency, such as stirring the solution using a magnet stir bar or shaking it on a mechanical shaker (1,15), using temperature controlled ultrasonic bath (11,14) and microwaves-assisted solvent extraction (16). However, these studies have shown that there is no significant difference in extraction efficiency among the different extraction techniques used, and higher temperatures can lead to loss of coumarin (1). Therefore, the most convenient technique of shaking sample solutions on a mechanical shaker at room temperature was applied in this study. In addition, we found that more than 90% of coumarin could be extracted from the studied tobacco sample matrices by a single 30 min extraction cycle using a solvent mixture of ethanol/water (1:1 in v/v) (experimental data on extraction study are available upon request).

E-cig Liquids Sample Preparation

Since both coumarin and the E-cig liquid

solvents (glycerin and propylene glycol) are soluble in water, methanol and the mixture of water and methanol, the solvent mixtures with different ratios of methanol to water were studied for their effect on coumarin determination from E-cig liquid samples. The results showed no significant differences between these solutions and therefore, a 50% methanol aqueous solution was finally used as diluent for coumarin analysis. The sample solutions were mixed on a Vertex Mixer at room temperature before LC–MS/MS analysis.

Sample Matrix Effects and Method's Selectivity

Sample matrix effects have attracted great attention in LC–MS/MS method development and validations since they affect the data quality and method's selectivity, sensitivity, and accuracy, especially for the analyses of analytes in complex sample matrices such as food and tobacco samples (17–21). In this work, three important parameters of sample matrix effects were studied:

Matrix interfering peaks. Several tobacco matrix peaks were observed from chromatograms of tobacco samples, which could interfere with coumarin peak and affect coumarin results if they were not separated from coumarin peak. As illustrated in Figure 1, these matrix peaks could be separated from coumarin using a high efficiency UHPLC column.

Signal suppressions. In previous publications (18–20), the author had studied in detail on tobacco matrix effects and found that tobacco matrix could cause significant analyte signal suppression. These suppression effects were further confirmed in this study by spiking the same amount of IS into pure sample matrices (such as LRB samples without tobacco) and into different tobacco matrices and then comparing the signal intensity of IS in tobacco samples with those in pure samples. The results showed that tobacco matrix effects led to 1.5 to 5 times decrease in IS signal depending on the sample matrices (data not shown, but available upon request). To overcome these suppression effects and achieve accurate results, we used stable isotope labeled IS in all samples to compensate for sample matrix effects.

Matrix effects on different MS/MS transitions of an analyte. Since different samples can have different matrix effects on coumarin, sample matrix effects were studied for each tobacco sample matrix in this work. In addition, four MS/MS transitions of coumarin and its internal standard in each sample were carefully examined and the results showed that matrix interfering peaks and ion suppression effects were different among the four different MS/MS transitions; some transitions showed many matrix peaks and suffered from heavy suppression effects (Figure 1c), while other transitions experienced less matrix effects (Figure 1b and Figure 2a). For example, although the highest intense peak (147.1/91.1 in this study) is usually used for analyte quantification to increase the sensitivity of a method, the second intense peak (147.1/103.1) of coumarin is a better choice for coumarin quantification in tobacco and STP samples due to less matrix interferences for this MS/MS transition as shown in Figure 1b. However, the results are different when analyzing electronic cigarette liquid samples. As illustrated in Figure 2, severe matrix effects and interfering peaks were observed for the second intense peak (147.1/103.1), while the highest intense peak (147.1/91.1) gave the best coumarin results due to less sample matrix effects (Figure 2a). In both cases, the IS peaks (Figure 1e and Figure 2e) had less matrix effects. Therefore, it is important and necessary to examine all MS/MS transitions for each sample matrix before analysis to select the most suitable transition for analyte quantification.

The method's selectivity and analyte confirmation from samples can be evaluated by comparing the analyte retention time and mass spectrum information between reference standard and tobacco samples. According to the regulatory guidance on analytical method validation, at least two structurally specific MS/MS transitions should be used in a method (22,23). In this study, four MS/MS transitions were examined for each sample. As shown in Table II, using a reference smokeless tobacco sample CRP3.1 as an example, the peak area ratios of qualifier to quantifier ions for the tobacco sample are consistent with those of reference standard with variations less than 15%. Similar results were also obtained for other tobacco and STP samples (data not shown, but available upon request), demonstrating good purity of the coumarin peaks for all four MS/MS transitions and great selectivity of the method for coumarin analysis in tobacco samples. Alternatively, the selectivity can also be evaluated by comparing the coumarin results obtained by the calibration methods built from each of the four MS/MS transitions. If the coumarin results obtained from each of the four MS/MS transitions are consistent, it indicates that there are no interfering peaks in any of the four MS/MS transitions used and the method is selective for determining coumarin in the samples. However, if the coumarin result obtained from one MS/MS transition is significantly higher than those obtained from each of the other three MS/MS transitions, it indicates that there are impurity peaks that are coeluted with the coumarin peak in this transition. If the coumarin result obtained from one MS/MS transition is significantly lower than those obtained from each of the other three MS/MS transitions, it indicates that there are severe signal suppression effects in this transition. As shown in Tables II and III, the consistent coumarin results obtained by using any of the four MS/MS transitions as a quantifier for coumarin determination indicate that there are no interfering peaks in any of the four MS/MS transitions and the method is selective for determining coumarin from tobacco and STP samples. However, for E-cig liquid samples, as demonstrated in Table IV and Figure 2b, the second coumarin MS/MS transition (147.1/103.1) suffered from heavy matrix effects in this E-liquid sample, and thus led to much lower coumarin result and lower peak area ratio of qualifier to quantifier ions, while other three MS/MS transitions gave consistent results for both coumarin content and peak area ratio values between standard and sample. Thus, for E-cig liquid samples, the second MS/MS ion pairs should not be used for coumarin quantification. These results demonstrated the advantages of using multiple MS/MS transitions of an analyte in a LC–MS/MS method, which could help compounds confirmation and achieve more accurate results.

Method Validation

Several sets of calibration curves with concentrations ranging from 0.05 ng/mL to 1000 ng/mL were generated on separate days using each of the four MS/MS transitions. All the calibration curves showed good linearity with correlation coefficients (R^2) greater than 0.999. Therefore, all the four MS transitions could be used for coumarin quantification if there are no interfering components in the peaks. The method's limit of detection (LOD) and limit of quantification (LOQ) were determined based on signal to noise ratio (S/N = 3 for LOD and S/N = 10 for LOQ) of the quantifier ion peaks in sample matrices. Although very low LOD (0.02 ng/mL) and LOQ (0.05ng/mL) can be obtained in pure standard samples, the LOD and LOQ for real tobacco matrices are relatively higher due to heavy matrix suppression effects. The LOD and LOQ of the method for tobacco samples are 10 ng/g of coumarin/sample (corresponding to 0.5 ng/mL of coumarin in final solution) and 40 ng/g (which corresponds to 2.0 ng/mL of coumarin in final solution). Similarly, the LOD and LOQ of the method for E-cig liquid samples are 2 ng/g of coumarin/sample (corresponding to 0.2 ng/mL of coumarin in final solution) and 5 ng/g (which corresponds to 0.5 ng/mL of coumarin in final solution). As shown in Table I, no interference or contamination from reagents or glassware was observed in this study as demonstrated by the LRB sample results. Good recoveries were obtained for LFB samples, indicating no analyte loss or contamination during sample preparations. Method precision was assessed based on replicate analyses of a standard and reference tobacco samples (5 replicates) on three days. The precision was then calculated based on the coefficient of variation (RSD %) of the collected data. The RSDs were 2.6 ~ 5.8% for the standard and 3.8 ~ 10.9% for tobacco samples, respectively. Method accuracy assesses how close the experimental value is to the expected value. Method accuracy was evaluated by the recovery of known amount of analyte spiked to samples (LFM samples). As shown in Table I, the recoveries of coumarin from the spiked samples were between 84% and 120%, demonstrating good accuracy of the method. Method robustness was studied by slightly changing the experimental parameters such as the ratio of ethanol and water in extraction solution, mobile phase gradients and LC columns from different suppliers. No significant differences in method's performance and analyte results were observed and thus confirmed the robustness of the method. In addition, the method was validated on two LC–MS/MS systems with equivalent results obtained.

Determination of Coumarin in Tobacco and STP Samples

The method was applied to the determination of coumarin in different tobacco and STP samples including five brands of commercial cigarette tobacco samples, five brands of mini-cigar tobacco samples and various reference tobacco and STP samples. Five replicates for each brand of sample were prepared and analyzed. Although no coumarin was found from the studied cigarette and cigar tobacco samples as well as some STP samples (CRP1, CRP1.1, CRP4, and CRP4.1), significant amount of coumarin was determined from four brands of STP reference products, as listed in Table III. It should be noticed that the coumarin amounts found in CRP2.1 and CRP3.1 (produced in 2016) are much higher compared to coumarin in CRP2 and CRP3 (produced in 2009), indicating the possible loss of coumarin during storage period. Therefore, it is important to measure coumarin from freshly opened samples as quickly as possible and take consideration of their storage conditions (temperature and time) when comparing the results of different or similar tobacco samples.

Determination of Coumarin in E-cig Liquid Samples

Six different brands of E-cig liquid samples were analyzed by the method and significant amount of coumarin (111 ng/g) was found in one of the samples.

Conclusions

The objective of this study is to develop a simple, fast, sensitive, selective, and robust analytical method for the determination of coumarin in various tobacco sample matrices. This goal was achieved by coupling UHPLC with tandem mass spectrometry. The selectivity and accuracy of the method could be improved significantly by using stable isotope internal standard and multiple MS/MS transitions of coumarin. The method was validated with reference tobacco samples and was applied to the analysis of coumarin in cigarette tobacco, cigar tobacco, smokeless tobacco and electronic cigarette liquid samples with good linearity, precision, and accuracy. Compared to the HPLC-UV methods, this LC–MS/MS method is more sensitive, selective, and accurate. Compared with the GC-MS methods, this method is simpler, faster, and without the need for sample cleanup and analyte concentration. ■

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Analysis of Synthetic Cathinones in Biological Samples

Emily Eng, Forensic Technical Specialist at UCT

This application note outlines a solid phase extraction (SPE) procedure and instrument parameters for analyzing new synthetic cathinones and other commonly encountered stimulants from blood and urine.

Introduction

Stimulants boost CNS activity, but misuse can lead to psychosis and addiction. Synthetic cathinones, also known as "bath salts," are designer stimulants related to the naturally occurring alkaloid cathinone, which can be found in Khat. These novel psychoactive substances require forensic labs to continually update their analysis scope. Newly discovered synthetic cathinones include N,N-dimethylpentylone and α-PHP.

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SPHPHO6001-10: Select pH Buffer Pouches 100 mM Phosphate pH 6.0

Instrument Method

LC-MS/MS System	Shimadzu Nexera LC-30AD with MS-8050
Column Temperature	45°C
Flow Rate	0.4 mL/min
Injection Volume	1 µL
Mobile Phase A	5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	5 mM ammonium formate + 0.1% formic acid in methanol
Gradient Program	Conc. B 5% (0 min) - 100% (8-10 min) - 5% (10.20 - 13.20 min)

Target Analytes: Methamphetamine, Amphetamine, Eutylone, Butylone, Pentylone, N,N-dimethylpentylone, MDMA, α-PHP Metabolite, α-PHP

SPE Procedure

Sample Prep: 0.5 mL sample + 1.5 mL 100 mM phosphate buffer pH 6 + ISTDs, vortex and centrifuge
Load: Load sample at 1-2 min/mL
Wash: (a) 1 x 3 mL DI H2O
(b) 1 x 3 mL MeOH
Dry: Dry for at least 5 minutes at full vacuum or pressure
Elute: 1 x 3 mL EtOAc:IPA:NH4OH (78:20:2)

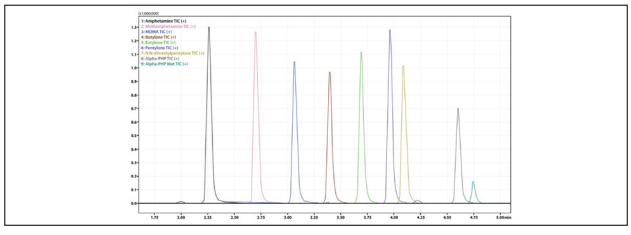

FIGURE 1: Chromatogram of extracted blood sample at 25 ng/mL.

Evaporate: (a) Add 100 µL of 1% HCl in MeOH to help prevent loss of analyte during evaporation
(b) Evaporate samples to dryness at 5 psi and 35°C
Reconstitute: Reconstitute samples in 0.5 mL of 5:95 H2O:MeOH or other appropriate solvent and volume

Results

TABLE I: Analysis of fractions (100 µl injection with 10 mg/ml cannabis extract).

n=5	Blood	Urine
Recoveries	84% - 102%	97% - 106%
Matrix Effects	(-39%) - (-5%)	(-7%) - 1%
RSD	5% - 17%	2% - 7%

*Recoveries were calculated using a pre- and post-spike sample technique. Matrix effects were calculated by comparing post-spike samples and solvent standards.

Conclusion

An optimized SPE method was developed that can be readily implemented by clinical and forensic labs. All analytes were separated using a simple linear gradient with a run time of less than 15 minutes. The biphenyl phase successfully resolved isomers, eutylone and pentylone.

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Pittcon 2024: 75 Years of Analytical Science

Caroline Hroncich, Patrick Lavery, *LCGC International*

This year's conference highlighted how analytical scientists are exchanging research to tackle some of the world's most critical problems.

WHEN OMOWUNMI SADIK, distinguished professor and chair at the New Jersey Institute of Technology, took the stage for her Wallace H. Coulter Lecture at Pittcon 2024 in San Diego, California, she asked the audience how many of them were working on solving complex problems related to per- and polyfluoroalkyl substances (PFAS).

While Sadik didn't get a count, there's a good chance that many of the scientists who traveled to this year's Pittcon are at least thinking about the lasting impacts that forever chemicals will have on the environment and human health over the next decade.

The questions surrounding PFAS for analytical scientists, in particular, are interesting, Sadik said. After all, it was chemists who figured out that the carbon-fluorine bond was one of the strongest found in nature—making it ideal for many uses from nonstick cookware to cleaning products and food packaging. Now scientists are tirelessly working together to find alternatives to, and better tests for, these same chemicals. A quick glance at the technical program indicates that more than 20 of the sessions at the conference this year were about PFAS. One of the final symposiums of the week, "Sustainability and Regulations in the Environmental Lab," brought together experts from Cofience, Indorama Ventures, MilliporeSigma, and PerkinElmer to provide a survey of how the industry side of analytical science is tackling the issue.

IMAGE 1: Martina Catani and Wolfgang Lindner pose with their awards.

IMAGE 2: Wolfgang Lindner and Martina Catani pose for a photo with speakers from their award's symposium at Pittcon 2024.

Pittcon has always been about connecting scientists from all over the world and facilitating an exchange of research—particularly when it comes to the most pressing issues impacting our world, such as PFAS. This year speakers also covered forensic analysis, artificial intelligence, biopharmaceuticals, and much more. The conference celebrated its 75th anniversary with the 2024 edition, and many attendees reminisced about Pittcon's past—fondly remembering nights out celebrating at the Pittcon party or awards lectures recognizing colleagues.

Pittcon is a special time for *LCGC International*. During an award symposium at the conference, the editors of *LCGC* present the Lifetime Achievement in Chromatography Award and the Emerging Leader in Chromatography Award. These annual awards recognize the outstanding achievements of two career chromatographers. This year, Lifetime Achievement Winner Wolfgang Lindner and Emerging Leader Martina Catani organized an awards lecture featuring lectures on chiral stationary phases, untargeted chemical analysis, and more.

Lindner's poignant sit-down with *LCGC* headlines the must-watch interviews that will be featured on the *LCGC International* website in the coming days.

"I am now 80. Now, the question is, how much time do I still have?" Lindner said. "My way is satisfaction, just like the song by the [Rolling] Stones... It's a big opportunity to go to Pittcon and see friends, and also let others see me—what is behind the Lindner column, what person is behind that?"

Other award winners include Susan Olesik, of The Ohio State University, who took home the Dal Nogare Award and Tom Linz, of Wayne State University, who won the Satinder Ahuja Award for Young Investigators in Separation Science. (Interviews with both Olesik and Linz, as well as Sadik and Catani, will be among the videos to watch for in the days ahead.) Philip J. Wyatt, the founder of Wyatt Technology, won the Pittcon Heritage Award.

Scientists working in industry and academia around the world shared their knowledge in posters, lectures, and networking sessions. Alyssa Sanchez, a PhD student at Florida International University, spoke about tracking the decomposition kinetics for the geographic profiling of heroin. Perry Wang from the U.S. FDA chaired a session on high-throughput analysis in the pharmaceutical sector using liquid chromatography–mass spectrometry (LC–MS). For more coverage, visit the *LCGC International* website.■

Check out the *LCGC International* website for more coverage from Pittcon 2024.
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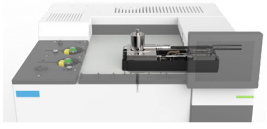

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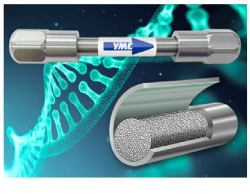

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