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The Heat is On: The Isolation of Capsaicin for Therapeutic Treatments.

The burning associated with ingesting or touching a chili pepper has been well documented as originating from the organic molecule, capsaicin. Capsaicin is widely accepted as the pepper’s natural defense system, but has also been exploited by the medical community. Capsaicin research has indicated that it and its analogs contribute to reduced cholesterol, blood lipids, blood sugar, and has anti-oxidative, anti-inflammatory, anti-obesity, and analgesic properties.

Conversely, capsaicinoids were implicated as carcinogenic in some studies. Yet further investigation has led to findings that show apoptotic actions in breast and prostate cancers indicating complex interactions. To further complicate matters, capsaicin, has been shown to be a chemotherapy sensitizing agent when combined with 5-Fu, cisplatin, and pirarubicin, thereby enhancing the efficacy of these therapeutic techniques.

With these applications in mind, we set out to isolate the major capsaicinoids utilizing the Hamilton PRP-C18 reversed-phase HPLC column (5 μm). Formic acid (0.1%) was used as the aqueous mobile phase due to the compatibility with both mass spectroscopy and UV detection. As a test of the isocratic method, Bishops Crown peppers were dried, macerated to a fine powder and extracted with ethanol, followed by filtration and injection of the sample. The results showed good correlation with literature values at 10190 Scoville Heat Units.

The excellent peak shape can be attributed to beneficial interactions between the capsaicinoids and the PS-DVB core as well as hydrophobic interactions associated with the octadecyl alkyl chains traditionally used for this separation. Unlike traditional ODS columns which cannot usually be reconditioned, the Hamilton PRP-C18 column can be easily regenerated making the stationary phase an indispensable tool in the synthetic chemists’ arsenal.


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Publisher’s Note

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

Chemometrics tools are powerful, but many analytical chemists find the idea of using these approaches daunting. It does not have to be that way. In this month’s installment of “The Next Generation,” our special article series highlighting the work of younger scientists, Leandro Wang Hantao and co-authors from the University of Campinas, in Brazil, make this case quite eloquently. Using examples from food chemistry, Hantao illustrates three core chemometrics approaches—pattern recognition, regression, and classification—showing their value and demonstrating that they are not hard to apply, even for those who are unfamiliar with using them.

Our regular columns this month also elucidate valuable tools for analytical chemists. In “LC Troubleshooting,” Dwight Stoll and Ken Broeckhoven wrap up a three-part series on kinetic plots for liquid chromatography, showing how what they explained in earlier installments all comes together for practical use. They demonstrate how kinetic plots help us understand how different combinations of parameters (particle size and length) will perform in terms of the time needed to get to a particular column efficiency (and thus resolution). This information can facilitate well-informed decisions about column choice.

In “Fundamentals,” Tony Taylor, the Technical Director of CHROMacadey, LCGC’s online learning partner, explains how a little detective work can ease the challenges of adopting or transferring a new method—and even make it fun! “GC Connections” then considers how to choose the best glass inlet liner—one of the most important, yet least understood and most often ignored, components of a gas chromatographic experiment.

Looking at the new techniques, “Focus on Biopharmaceutical Analysis,” discusses charge detection mass spectrometry (CDMS). CDMS is an emerging tool that is gaining traction for a current challenge: characterizing larger, more complex biopharmaceuticals, like bispecific antibodies and antibody-drug conjugates.

Doug Raynie, in “Sample Prep Perspectives,” considers another new technique, the quick polar pesticides (QuPPe) method. QuPPe builds on what has been learned with QuEChERS to address the particular challenges of highly polar compounds. This piece is an excellent follow on to the discussion of the QuEChERSER mega-method presented in our January issue.

Happy reading!

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But Why Doesn’t It Get Better?
Kinetic Plots for Liquid Chromatography, Part III: Pulling It All Together

Choosing a liquid chromatography (LC) column for a particular application can be a surprisingly challenging task. On one hand, column manufacturers give us many options to choose from, including particle types, pore sizes, particle sizes, and different lengths and diameters. On the other hand, we usually don’t have time to experimentally evaluate many combinations of these parameters, and sometimes we end up picking something similar to the columns that are already in the drawer. The “kinetic plot” is a powerful graphical tool that can help leverage the best available theory to help us understand how different combinations of parameters (that is, particle size and length) will perform in terms of the time needed to get to a particular column efficiency (and thus resolution), and therefore make well-informed decisions when choosing columns.

Ken Broeckhoven, Caden Gunnarson, and Dwight R. Stoll

In the last two installments of “LC Troubleshooting,” we reviewed the basic idea of a “kinetic plot” (1) and how to make the plots from experimental data or data from the literature (2). Ultimately, this graphical tool can be used to make informed decisions when choosing columns and to understand why a column might not be delivering expected performance improvements. This month, we conclude this series of articles by discussing the so-called “Knox-Saleem limit” (KSL), application of kinetic plots to gradient elution conditions, and the impact of extracolumn dispersion on kinetic plots. Finally, we introduce a web-based application that pulls together all of the theory discussed in these installments into a convenient and flexible web-based calculator that allows you to explore the impact of many variables on the kinetic plot on your own.

The Knox-Saleem Limit (KSL)
In last month’s installment, we began discussing the effect of particle size on kinetic plots by showing the kinetic performance limit (KPL) curves for different particle sizes (Figure 1a). Interestingly, these curves cross in the kinetic plot, which means that at any given combination of \( t_0 \) and \( N \), there is one particle size that provides superior performance compared to the others. In other words, there is no single particle size that is superior to all others over the entire range of analysis times of practical interest. Whereas the smallest particles (1.7 µm) show the best kinetic performance at short analysis times, the larger particles (5 µm) are the better choice to obtain high efficiencies at long analysis times, which is a direct result of the improved performance at higher flow rates for smaller particles (fast analysis). However, the small particles also lead to large pressure drops that limit their use to relatively short columns (lower efficiency).

In Figure 1a, we see that each of the KPL curves touches an oblique asymptote (dashed lines) below which one cannot work regardless of the choice of column length, particle size, and velocity because the pressure drop will exceed the chosen pressure limit. This oblique asymptote is the KSL and in fact touches the KPLs for different particles sizes at their respective optimal mobile phase velocities (that is, \( u_{0,min} \)) (3). The point where the KPL and KSL curves touch represents the optimal choice of not only mobile phase velocity and column length, but also of the particle size for each combination of \( t_0 \) and \( N \). For the particle sizes represented in Figure 1, we see that the KPL curves come very close to the KSL, which indicates that at least one of these particles is close to optimal for plate numbers in the range of \( 10,000 < N < 200,000 \). When there is a gap in the available particles sizes (for example, jumping from 1.7 to 3.5 µm), we see a gap between the points at which the two KPL curves cross with the KSL. This occurrence indicates a gap between the truly optimal performance that is possible for a given combination of \( t_0 \) and \( N \), and what can actually be realized with the available particle sizes. Fortunately, these differences are rather small, as has been discussed in detail by Matula and Carr in the literature (4).
The KSL can be calculated using equation 1 if the dynamic viscosity of the mobile phase (\(\eta\)), the minimum reduced plate height (\(h_{\text{min}}\)), and the \(u_0\)-velocity-based flow resistance (\(\Phi_0\)) for a certain stationary phase support are known using (2,3,5):

\[
t_0 = \frac{\eta \cdot \Phi_0 \cdot h_{\text{min}}}{\Delta P_{\text{max}}} N^2
\] [1]

This relationship makes clear that the kinetic performance can be improved by increasing the maximum operating pressure (\(\Delta P_{\text{max}}\)); that is, UHPLC vs. HPLC), decreasing the mobile phase viscosity (for example, through the use of high temperatures in LC, or low viscosity eluents in supercritical fluid chromatography [SFC]), reducing the flow resistance (for example, by using monolithic or chip-based columns), or decreasing the minimum reduced plate height (for example, with superficially porous particles, chip-based, or 3D-printed columns) (6). A change in any of these parameters will shift the KSL (and also the KPL curves) to the right, allowing for both faster and more efficient separations. When all other parameters are fixed, doubling \(\Delta P_{\text{max}}\) results in a decrease in \(t_0\) by a factor of two. In other words, doubling the available pressure allows the same efficiency to be realized in half the time, which is illustrated in Figure 1b where the effect of the operating pressure on the KPL curve for the 1.7 µm particles is shown. The curve shifts to the bottom right of the kinetic plot, showing how even faster analyses and higher efficiencies can be obtained when operating at this higher maximum pressure. In fact, when comparing the use of 1.7 µm particles at 1000 bar with 3.5 and 5 µm particles used at 400 bar, the smaller 1.7 µm particles outperform the 3.5 µm particles in the part of the efficiency analysis time range where the latter outperforms the 5 µm particles at 400 bar. The 1.7 µm particles at 1000 bar even outperform the 5 µm particles up to approximately \(N \sim 100,000\). Of course, this comparison changes if the 3.5 and 5 µm particles can also be used at 1000 bar. Similarly to the KPL curve, the KSL also shifts with an increase in maximum pressure, as expected from equation 1.

Application of the Kinetic Plot Concept to Gradient Elution Conditions

For fundamental comparisons of the separation performance of different column types, it is most practical to use isocratic elution conditions, which is
why our discussion of kinetic plots has so far focused on the kinetic plots with $t_0$ and $N$ as the axes. In practice, however, most applications use mobile phase composition gradients to optimize separation time and resolution. Thus, it is desirable to apply the kinetic plot concept to the gradient elution condition as well, which can be done by replacing the plate number with the peak capacity ($n_p$) as the measure of separation performance (5,8). When making experimental measurements of retention time and peak width under gradient elution conditions for the purpose of making kinetic plots from experimental data, several details are important to keep in mind. These are mentioned briefly here. Readers interested in learning more about them are referred to the literature for detailed protocols (9).

- Gradient time should be scaled inversely proportional to the flow rate so that the gradient slope remains constant (10,11).
- If the mobile phase composition is held constant at the beginning of the separation, or at any other point in the elution program, these so-called hold times should also be scaled with the inverse of the flow rate.
- If columns with the same stationary phase chemistry from the same vendor are compared, there is usually little to no difference in selectivity and the same gradient range (initial and final composition) can be used. However, when comparing columns from different vendors, differences in retention may be observed, and it is advisable to tune the initial and final composition of the gradient in such a way that the first and last eluted compounds have similar retention factors (9,11). As previously mentioned, in the case of gradient elution, the peak capacity ($n_p$) is usually the preferred measure of separation performance rather than the column plate count ($N$). Calculation of the column dead time and retention time at the kinetic performance limit (that is, $t_{0,KPL}$, and $t_{R,KPL}$) is similar in isocratic and gradient elution, however calculation of the peak capacity at the KPL is slightly different, as shown in equations 2 and 3 (9,10,12,13):

$$\lambda = \frac{\Delta P_{\text{max}}}{\Delta P_{\text{exp}}} \quad [2]$$

$$n_{p,KPL} = 1 + \lambda \cdot (1 - n_{p,exp}) \quad [3]$$

The square root dependence in equation 3 is the direct result of the square root dependence of the peak capacity on the column plate number (10). As a result, increasing the column length by a factor of four will only increase the peak capacity by a factor of two. Please note that also in this case the value for $\Delta P_{\text{exp}}$ should include the extracolumn pressure drop as discussed in the next section.
FIGURE 4: Illustration of the kinetic plot tool output that illustrates the significant effect of extra-column dispersion (ECD) on kinetic plots, which is mathematically convenient. However, peak dispersion outside of the column is often too large to ignore. We discussed the details associated with dispersion in different parts of the LC system in a prior multipart series of articles in this magazine (14–17) and elsewhere (18), and readers interested in these details are referred there. Here, we focus on adjustments that must be made to the kinetic plot calculations to account for both the dispersion that occurs in the LC system outside of the column, and the pressure drop that occurs in different parts of the system.

Corrections to the kinetic plot calculations to account for extra-column effects can be made using values for the extra-column dispersion and pressure drop obtained from experiments, or some means of estimation. When it comes to experimental measurements, the column is replaced by a zero dead volume union in order to obtain the extracolumn time ($t_{ec}$) and peak variance ($\sigma^2_{ec}$) at different flow rates. It is important to understand that extrapolation of the plate number from a FL curve to the KPL using $\lambda = \Delta P_{max} / \Delta P$ (as discussed in Part II of this series) should only be done using plate numbers that have been corrected for ECD. Then, after the extrapolation, the extra-column variance is added back to the peak variance contributed by the column to give an effective plate number ($N_{eff}$) as shown in equation 4. Similarly, the column dead time must be corrected to account for the time the analyte spends traveling from the injector to detector, but outside of the column, as shown in equation 5:

$$N_{eff} = \left( \frac{t_{col} + t_{ec}}{\sigma^2_{col} + \sigma^2_{ec}} \right)^2$$  \hspace{1cm} [4]

$$t_{0,eff} = t_{0,col} + t_{ec}$$  \hspace{1cm} [5]

In addition to the effect of the LC system on dispersion of peaks, some of the available operating pressure is also lost because of pressure drops along the connecting tubes, especially when narrow diameter tubes are used. To account for this, the value of $\Delta P_{max}$ used in calculating the kinetic curves should be reduced by the value of the extra-column pressure drop ($\Delta P_{ec}$) at the corresponding flow rate, as shown in equation 6:

$$\Delta P_{max,eff} = \Delta P_{max} - \Delta P_{ec}$$  \hspace{1cm} [6]

Pulling It All Together—A Web-based Application for You

Although no single mathematical step in calculating the kinetic curves is particularly difficult, there are many details to keep track of, and building a calculator correctly from scratch takes some time. Thus, we have built a freely available web-based calculator (www.multidlc.org/kinetic_plot_tool) that incorporates all of the theory discussed in this series of articles, including consideration of extracolumn effects discussed in the previous section. Here, we briefly demonstrate use of the tool by way of an example that shows how it can be used to explore the effects of different variables on the curves, and perhaps develop hypotheses for troubleshooting situations where column performance does not live up to one’s expectations.

Figures 2 and 3 show screenshots of the inputs to the tool. Up to three different conditions can be compared simultaneously. Pre-set configurations for zero, low (~1–2 µL²), and normal (~10–15 µL²) levels of extracolumn dispersion enable quick configuration of the extracolumn inputs; however, each of the system parameters (that is, injector, tubing, and detector) are fully adjustable as well.

Figure 4 shows screenshots of the kinetic plots produced by the tool for two different cases (A and B). In both cases the comparison is between columns packed with fully porous 1.7 µm particles and columns packed with superficially porous 2.7 µm particles. In case A, the tool is configured using the pre-set parameters for a low dispersion system (~1–2 µL²) for both the FPP and SPP columns. Here, we see that the 1.7 µm FPP columns outperform the 2.7 µm SPP ones at the KPL over the range of 5,000 < $N$ < 30,000, though the difference is small ($t_{0,FPP} = 0.28$ min vs. $t_{0,SPP} = 0.31$ min for 15,000 plates). At approximately $N = 30,000$ plates, the curves cross over and the SPP columns become superior for higher efficiencies as a result of their higher permeability.

However, when the tool is reconfigured using the preset parameters for a normal dispersion system (~10–15 µL²), we get the curves shown in Figure 4b, where the SPP columns are superior to the FPP ones at the KPL over the entire range of efficiencies shown. On one hand, the superiority of SPP columns is not surprising: manufacturers of sub-2-µm columns have been working to educate users for years about the importance of using these columns in low dispersion systems to maximize their performance potential. On the other hand, this comparison shows the utility of the kinetic plot tool both for making informed choices about column selection, and troubleshooting situations where a column in use does not live up to user expectations.
Summary

In this installment of “LC Troubleshooting,” we have continued our discussion of kinetic plots and their utility when selecting column technologies and formats, and troubleshooting columns that appear to not live up to our expectations. The KSL quantifies the best achievable performance (as measured by plate number) in a given analysis time when the particle size is allowed to vary. Kinetic plots can also be used to compare technologies under gradient elution conditions, and when the effects of extracolumn dispersion are accounted for. Finally, we have introduced a freely available web-based kinetic plot tool that leverages all of the theory discussed in this series and enables comparison of up to three different sets of conditions simultaneously. This tool is useful for quickly comparing different column technologies and LC system configurations, and developing troubleshooting hypotheses when things don’t seem to be quite right. It is important to note that all calculations in this series have been done with diffusion coefficients typical of small molecules. When working with large molecules their diffusion coefficients will be very different, and thus the kinetic plots will be very different as well.

References

Part 6: Potency Testing in Baked Goods, Candies, Beverages, and Topicals—Who Said it Had to be Painful?

In the sixth and final part of this series, Cannabis Science & Technology sat down with Anthony Macherone, senior scientist and cannabis technical lead for Agilent Technologies, to speak with Christophe Deckers, a sample prep application scientist, and Jean-Francois Roy (JF), an MS application scientist at Agilent Technologies, about quantifying cannabinoids in edible products.

MACHERONE: We hear many labs complain about potency testing in fatty samples like chocolates, brownies, and cookies. What do you recommend for these types of samples for accurate and repeatable potency testing?

DECKERS: When customers complain about fatty samples, they usually say, “I have high backpressure, or my LC column doesn’t last as long as I want.” They also say they get strange results that are not reproducible, or “We sent our edibles to different testing labs, and they come back with different results.” This is interesting, so in our Montreal Center of Excellence, we investigated this growing consensus in the scientific community that lipids are responsible for those problems. In essence, the more lipids you have, the less accurate your quantification can be, both by liquid chromatography (LC)-UV and LC mass spectrometry (MS/MS).

How can we remove those lipids without losing cannabinoids because they are fat-soluble? We found a way to simplify the grinding and filter those lipids out without losing our cannabinoids using a technology called Captiva enhanced matrix removal (EMR)-Lipid. Our results have been published in an application note.

MACHERONE: For these fatty-type samples, can we still use high-performance liquid chromatography (HPLC) with UV detection for analysis?

ROY: Yes, LC-UV can be used, but we have a few recommendations. The first is to do what’s called matrix-matched calibrators. In other words, use a non-infused matrix and spike it with known concentrations of the cannabinoids you are trying to quantify. Make sure the calibration curves reflect the matrix you are analyzing as well as the sample preparation used to treat that matrix. The fact that edibles are made from matrices that are easily purchased in stores without being infused makes matrix-matching somewhat easy.

The second recommendation is to evaluate your existing LC-UV method: do you clean your column and system enough toward the end of the gradient? We repeatedly hear from customers, “My method used to work, but after a few months, the results were wrong, reproducibility was down, and the accuracy was bad.” We’ve found that, in most cases, the LC-UV method was not adapted to the reality of the edible matrix. By cleaning an LC system and LC column—i.e., increase the amount of time high organic content is pumped through the column toward the end of the gradient—you remove a lot of undesired matrix components that accumulate after each injection. Those are our two main recommendations for potency testing and edibles matrix-matching for calibrants and enhanced cleaning of the LC column and system.

MACHERONE: Let’s move to hard candies and gummies, which don’t have a lot of fat in them. Do you need to perform a similar sample preparation for these samples?
**DECKERS:** Gummies are low in fat, but they’re sticky, and they don’t melt very well in acetonitrile or methanol. Most labs use methanol for edibles, but high sugar edibles don’t melt. We found water melts candies, and if you use warmer water or sonicate, they melt even faster. Although water helps, it’s not that simple.

Some candies have difficult chemical mixtures—some are made from gelatin and pectin, others are made from starch, for example, non-transparent gummies in raspberries or peach—and react differently in solvents. To make things even more challenging, candies or gummies are sand-sanded—they have sugar around them, or they have coconut or palm oil, carbowax, and beeswax—which interferes with the quality of your analysis. So, we recommend taking the inside of the gummy and dissolving it in water. Then add acetonitrile and a QuEChERS extraction salt, shake it, and it creates two layers: the bottom layer has the water and starch (if you used a starch-based candy), and the top layer has the cleaner acetonitrile extract with all the cannabinoids. Next filter it and inject it onto your LC system, and that’s it—a simple procedure specifically for those high-sugar analytes. If you have a hard candy, crush it first with a hammer or pestle and use this procedure.

**ROY:** I would complement your comments by stressing the importance of matrix-matching the calibrants. Here, non-infused gummies or hard candies are readily available. Gummies are often not uniform due to sanding with sugar or oiling, so talk with the manufacturer to adapt your reporting units.

Often, we see milligrams of cannabinoid per candy. The issue is when weighing multiple candies from a lot, there is a lot of variation in the weight, which calls for accurate weighing of the gummy or the gummy portion you’re analyzing. Also, make sure the reporting units of your assay are relevant to the manufacturing process of the product you’re analyzing.

**DECKERS:** One final thing to note is the impact of high pH. Gummies, especially pectin candies, need low pH to solidify. To our surprise, we had a faster and a homogeneous dissolution when we used 2% ammonia to dissolve the gummies. The pH is high during the shaking time, so it melts the gummy, but when you add the extraction salt, there’s a buffer. I don’t know if it’s powerful enough to neutralize it completely, but I’ve seen a change in color, meaning that it does neutralize the pH. We also found stability in our results over time, as pH is the key element to ensuring the stability of the analytes, accuracy, and precision. Over eight days in those pH conditions, we did not see any variation in the concentration of those different cannabinoids.

**MACHERONE:** THC- or CBD-infused beverages can be challenging. Is LC-UV sensitive enough for those samples because they usually have lower concentrations?

**ROY:** Yes, LC-UV is sensitive enough. But again, sample preparation needs to be fully aligned with the nature of the matrix. With beverages, you’re dealing with a water-based matrix in which we solubilize molecules, CBD and THC, that are not soluble in water.

When Christophe and I worked on stability and potency testing for beverages, we looked at the content of those beverages, especially the nano-emulsifier agents that are used to keep CBD, THC, and other cannabinoids in solution. For efficient extraction, separate the cannabinoids from the fatty emulsifying agents, making sure you don’t dilute a lot of the beverage. Your sample preparation should reflect the nature of the beverage and the presence of those nano-emulsifying agents.

**DECKERS:** Our customers tell us they thought beverages would be easy to work with, but they complain about the back pressure increasing in their systems and unrepeatable results because the emulsifiers build up on the instrument and cause inaccuracy in the quantification of THC. Some drinks use ethyl alcohol or Tween 80, modified palm oil, or lecithin. But when we remove those emulsifiers, the UV signal goes up right away, which is a testament to the accuracy of the method we developed.

It is simple: start with 10 mL of your drink. Sonicate at 50 - 60° degrees C – this helps to start to break down those emulsifiers. Then add 10 mL of acetonitrile, shake for a few minutes, and the original Agilent QuEChERS salt that is unbuffered. Shake that for a minute, spin it, and you have two layers: The bottom layer has the water, sugars, and some emulsifiers; the top layer has acetonitrile with cannabinoids, but also, unfortunately, some fatty emulsifiers.

That top layer is cleaned up on an EMR cartridge. It works as a filter—pour it in with a bit of water and all the emulsifiers are gone. You’re ready to inject and use right away. You have that robustness back. The strange results are gone, and you have more confidence in the results you generate.

**MACHERONE:** One other question about beverages. What about carbonated beverages? Do you have to remove the CO₂, sonicate or something similar, before you start to break down the emulsion?

**DECKERS:** We found that decarbonation is regularly done in labs that test carbonated beverages. That’s why we also included a 20-minute sonication ahead of time in our procedure because it decarbonates. It’s good practice to sonicate first and to degas, so you don’t have that extra variability in your analysis.

**MACHERONE:** Have you worked with lotions and balms—these types of topicals?

**DECKERS:** I have personally not worked with them, but we heard quite a deal about them. It’s one of the next things we’ll be working on. We hear even the physical consistency is challenging: The balms are harder at room temperature, and the lotions are thicker. You wonder how you can work with that on a very sensitive instrument such as a UV-VIS or an LC-MS/MS. The answer is that we do have an existing procedure for sunscreen/sunblock. It’s designed to work for small molecules in sunblock. Some cosmetic labs need to track some of those compounds and balms, lotions, etc. We used that existing application note with some of our customers, and they came back with successful results for potency testing. We’re looking forward to those collaborations and maybe one day making an application note on that.

You need to warm your sample with water and then extract and pass it onto EMR technology that selectively removes lipids but not cannabinoids. The key is to get that robustness and accuracy back.
Quick Polar Pesticides (QuPPe): Learning from and Expanding on the Work of Others

In January, Steve Lehotay from the U.S. Department of Agriculture provided an update on the quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method, originally developed for multiresidue pesticide analysis. Like any method, QuEChERS had some deficiencies, which were addressed with the new QuEChERSER (adding “efficient” and “robust” to the acronym) mega-method. Meanwhile, the European Union (EU) Reference Laboratories developed a new method for the multiresidue analysis of highly polar pesticides, called the quick polar pesticides (QuPPe) method. This month, we take a look at QuPPe, comparing and contrasting it with QuEChERS and noting how we can learn from previously developed methods as we strive for improvements.

Douglas E. Raynie

Steve Lehotay (1) engaged us with a summary of the current state of the quick, easy, cheap, effective, rugged, and safe (QuEChERS) multiresidue extraction method in our last “Sample Preparation Perspectives” column. Lehotay and Anastassiades developed QuEChERS approximately 20 years ago as a multi-class, multiresidue method, primarily for determining pesticides in produce. Because the method developed employed a wider range of flexibility (that is, it was simple and developed for the analytical instrumentation widely used at the time), modifications to the approach led it to become a model for the extraction and chromatographic sample preparation of more diverse analysis needs, such as for veterinary drugs, environmental contaminants, and mycotoxins. The still growing popularity of QuEChERS is evidenced by close to 4500 peer-reviewed publications since its inception. The simplicity and flexibility inherent in the QuEChERS method also results in its limitations. For example, the minimal or “just enough” clean-up steps avoiding indirect matrix effects make the approach both applicable as a template for an array of analyses and provide some limits on the range of residue polarities amenable to the technique. Additionally, analytical instrumentation, such as liquid chromatography–tandem mass spectrometry (LC–MS/MS), has improved dramatically since the turn of the century. With this in mind, Lehotay updated the technique to include “efficient and robust,” and update the method name to QuEChERSER, and this updated method was the topic of the January column (1). The QuChERSER upgrade accommodates a broader scope of polar and non-polar analytes, creating what Lehotay describes as a mega-method. A comparison of the workflows for QuEChERS and its offspring QuEChERSER is shown in Figure 1. From these schematics, the major differences between the methods can be gleaned.

Quick Polar Pesticide Extraction (QuPPe)

Despite the improvements afforded by QuEChERSER, there is still a need for the simple and effective extraction of residues of polar pesticides incurred in produce and other foodstuffs. Working with the European Union (EU) Reference Laboratories for Residues of Pesticides, Michelangelo Anastassiades, co-inventor of the original QuEChERS method, created a new method that was aimed specifically at the highly polar pesticides not amenable to extraction via QuEChERS (or QuEChERSER) (2). He called the new method quick polar pesticide (QuPPe) extraction. The workflows for the QuPPe family of methods are shown in Figure 2. Although starting with goals similar to those used with the original QuEChERS extraction, what is immediately evident in these workflows is that Anastassiades learned and reapplied concepts from the previous development of QuEChERS and added his knowledge of chemistry in the development of QuPPe.

Four QuPPe methods are described, one each for most plant-based commodities and honey; cereals, pulses, nuts, and oily seeds; liver, kidney, muscle, and milk; and animal fat. In these methods, the sample is weighed and water may also be added. The acidified method is used for the extraction, and heating (fats) or cooling (lipids and proteins) is used to remove biological

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Icon image: Joe Zugcic, Zugcic Photographers, Inc.

Sample Prep Perspectives

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Icon image: Joe Zugcic, Zugcic Photographers, Inc.
SCOPE
LC and GC-amenable pesticide, environmental contaminants, mycotoxins, and more relatively polar analytes.

BULK SAMPLE COMMUNITION
1-Step room temperature (or dry ice)

TEST PORTION
10-15 g
Add water to dried or oily samples

EXTRACTION
1 mL/g: acetonitrile (optional buffer)

SHAKING TIME
5-10 min (up to 60 min)

CENTRIFUGATION
3 min at >3000 rcf

SALTING OUT
4 g 1:1 (w/v) MgSO4:NaCl per 10 g sample
Shake 1 min and Centrifuge 3 min

CLEAN-UP
dSPE with 0.25 g 3:1:1 (w/w/w) anh. MgSO4:PSA:C18 per g equivalent sample

FINAL EXTRACT
1 g/mL, adjustable

(Many options available from vendors.)

SCOPE
LC and GC-amenable pesticide, environmental contaminants, mycotoxins, vet. drugs, and more relatively polar analytes.

BULK SAMPLE COMMUNITION
1-Step liquid nitrogen or dry ice, 2-step room temperature

TEST PORTION
<0.25 g (2-5 g)
May add extra to long-term dried samples

EXTRACTION
5 mL/g 4:1 (v/v) acetonitrile:water (buffer probably not needed)

SHAKING TIME
5-10 min (up to 60 min)

CENTRIFUGATION
3 min at >3000 rcf

SALTING OUT
For GC, decant 10 mL into 2 g 4:1 (w/w) MgSO4:NaCl per g sample
None for LC (take 200 µL)

CLEAN-UP
For LC, evaporate MeCN, add initial mobile phase, ultracentrifuge for 5 min
For GC, dSPE with 45 mg 20:12:1:1 anh. MgSO4:PSA:C18 per 0.3 mL extract

FINAL EXTRACT
LC, adjustable
GC, 0.25 g/mL

FIGURE 1: Workflow diagrams for the (a) QuEChERS, and (b) QuEChERSER methods. Notable differences between the methods are found in the sample comminution, extraction solvent, salting out, and clean-up steps are observed.

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macromolecules. The clean-up process is performed prior to LC–MS/MS or ion chromatography (IC)–MS/MS. Ethylene-diaminetetraacetic acid (EDTA) is added, as necessary, to the complex metal ions. Isotopically labeled internal standards are added at the beginning of the procedure to compensate for volume deviations, analyte loss, or matrix effects on recovery rates. Where internal standards are not possible, quantification by standard addition is used. Because of the highly polar nature of the target analytes, plastic vials are used to minimize loss of analyte because of surface adsorption onto glass vials, though some carry-over is still possible depending on the analyte. Because of the simplicity of the method, strong matrix effects are frequently observed. Prior to QuPpe, samples are ground to less than 500 µm and homogenized to a free-flowing mixture.

During the development of QuPpe, the analytical team spiked a host of highly polar pesticides (Table I) at levels of 0.005–20 mg/kg per sample and conducted matrix-matched and matrix isotope-labeled internal standards (ILIS) calibrations with interlaboratory calibrations. The mean recoveries reported were between 70–120%, with a 0–20% relative standard deviation. Analysis of spiked samples of a number of foods of plant- and animal-origin were reported, including citrus fruits, pome fruits, stone fruits, and herbs, stem vegetables, legumes and animal-origin were reported, including citrus fruits, pome fruits, stone fruits, soft and small fruits, dried fruit, root and tuber vegetables, leek plants, fruiting vegetables, cabbages, leafy vegetables and herbs, stem vegetables, legumes or pulses, cereals, oily seeds, nuts, animal tissues, whole fat and skimmed milk, honey, eggs, and animal fats.
Split/Splitless Inlets in Gas Chromatography: What’s Up with All Those Different Glass Inlet Liners?

Over the winter break at the university where I work, I was ordering consumables for the upcoming semester. As I was browsing the web pages at a major vendor for glass inlet liners, I was again impressed by the many glass inlet liner options and geometries available for split and splitless inlets, still by far the most used today. In this installment, we review the processes that happen in the inlet when a sample is injected, and use this to make some sense of the many available inlet liner configurations. We generate the web pages at a major vendor for glass inlet liners, I was again impressed by the many glass inlet liner options and geometries available for split and splitless injections, still by far the most used today. In this installment, we review the processes that happen in the inlet when a sample is injected, and use this to make some sense of the many available inlet liner configurations. We generate some best practices and guidelines in selecting an inlet liner, but we see that there is no one single liner that fits all situations. Selecting the best inlet liner should be a step in all method development involving split or splitless injections.

Nicholas H. Snow

Capillary gas chromatography (GC) has always suffered from some fundamental challenges regarding sample introduction into the chromatograph. First, the liquid sample must be transferred from a syringe or other sampling device to an open tubular column that most often has an inside diameter smaller than the syringe. Second, the injected sample must be vaporized and homogeneously mixed with the carrier gas stream. The sample/carrier gas mixture must be quantitatively and reproducibly transferred into the open tube of the capillary column. Finally, the inlet must be large enough to accommodate the full vapor volume generated when the liquid sample is evaporated. As described in the classical book by Grob, Split and Splitless Injection in Capillary Gas Chromatography, the many processes involved with solving these challenges can be very complex, to the tune of this book being nearly 500 pages long (1).

A final challenge involved in transferring a liquid sample into a capillary column is that the entire flow path must be inert. In his book, Grob describes that much research has been done over the years to ensure that the flow path is inert and does not react with or adsorb compounds from the samples. The flow path must also be easy to clean because it will eventually collect nonvolatile contaminants resulting from sample degradation. All of these challenges led to the development of glass inlet liners to provide an inert flow path that can accommodate the other challenges with vaporization of the sample, mixing with the carrier gas, and transferring that mixture to the column. Figure 1 shows a split/splitless inlet from above, with the top flanges, including the septum and septum nut, removed. Note that the inlet block and gas flow lines are all metal, which can be highly reactive with organic compounds when heated, and would be very difficult to clean. The inlet liner is glass, and usually fits loosely into the inlet body to allow for carrier gas to flow both through the inside of the liner and around the outside. An O-ring provides a gas-tight seal when the flanges and nuts are replaced. Details of the flow paths for split (and splitless) inlets and on general troubleshooting and maintenance of inlet systems are discussed in most texts including gas chromatography and on ChromAcademy, LCGC North America’s online learning platform (2). As a general maintenance and troubleshooting rule, the glass inlet liner and O-ring should be replaced together on a regular schedule, depending on the cleanliness of the samples being analyzed.

This maintenance rule highlights the need for a removable and replaceable liner for the flow path that also solves the several challenges. Even more challenging, a liner that works for one sample may not work for another. For the best performance, options must be tested and evaluated. In the remainder of this column, we examine principles of split and splitless injections that inform the choice of liner, beginning with some overriding concerns that govern all liners.

The Two Overriding Concerns: Inertness and Volume

Regardless of the injection technique being used, the inlet liner must be inert to the samples, and must have large enough volume to accommodate the vapor generated when the sample is injected into the heated inlet and vaporized. Depending on the solvent, inlet temperature, and inlet pressure, typical sample solvents used in gas chromatography may generate anywhere from 200 µL to over 1 mL of vapor when evaporated in the inlet. If the volume inside the inlet liner is not large enough, vapor can backflow into the carrier gas lines. Because that vapor will also contain vaporized analytes, those analytes can condense in the gas lines and eventually become eluted as ghost peaks or baseline drift. Solvent vapor volume calculators for common solvents and inlet conditions are available online (3,4). These calculators
can be used as guides for choosing the needed volume for the inlet liner. Generally, the chosen liner should have a higher internal volume then provided by the calculator. Table I shows vapor volumes generated using the two calculators for some common conditions. Excessive vapor volume occurs in relatively few situations, usually with polar solvents, high injection volumes, or low head pressure. Note that increased head pressure solved the problem with excessive evaporation of water.

The inlet liner must also be inert to any reactive compounds, both analytes and interferences. Nearly all inlet liners for split and splitless inlets are made from glass, which by itself is not deactivated and can have free silanol groups and other surface contaminants that can react with or adsorb analytes. One of the impressive options seen at the vendors’ websites is the choice of deactivation methods, which also should be matched to the expected reactivity of the analytes. Not surprisingly, deactivated inlet liners come at a higher individual price. Being an academic researcher, when developing a method, I used to start with the most inexpensive option and move to the more expensive deactivated liners as needed. Today, I simply purchase deactivated liners up front, so I do not have to find out the hard way, usually through poor peak shapes or poor quantitative reproducibility that I need to change to a more inert inlet liner. I also purchase deactivated liners from the vendors. I no longer attempt to deactivate them myself.

Next, we discuss glass liner choices for some specific situations, the most common split and splitless injections and some special cases, including solid-phase microextraction (SPME) and programmed temperature vaporization (PTV) systems.

**Liners for Split Injections**

An ideal split injection requires that the sample be injected as quickly as possible, vaporized as quickly as possible, and mixed homogeneously with the carrier gas, with the mixture transferred rapidly to the column head. Rapid injection minimizes band broadening and is the primary cause of the sharp peaks characteristic of split injection combined with temperature programming. To assist in vaporization, split liners have large volume and often have obstructions, baffles or packing (glass wool or adsorbent) to facilitate vaporization. Figure 2 shows a typical cup-design glass inlet liner and two glass wool packed liners.

**TABLE I:** Example solvent vapor expansion calculations using an online calculator and the expected results. Assumes inlet temperature 250 °C, 1 μL injected and typical split injection liner 78.5 mm long with 4 mm inside diameter, with total volume about 1 mL and effective volume 500 μL (half of volume is occupied by carrier gas).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Head Pressure (psig)</th>
<th>Vapor Volume (μL)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>10</td>
<td>630</td>
<td>Excessive backflash</td>
</tr>
<tr>
<td>Methanol</td>
<td>20</td>
<td>450</td>
<td>Good</td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
<td>1400</td>
<td>Excessive backflash</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10</td>
<td>450</td>
<td>Good</td>
</tr>
<tr>
<td>Acetone</td>
<td>10</td>
<td>350</td>
<td>Good</td>
</tr>
<tr>
<td>Hexane</td>
<td>10</td>
<td>200</td>
<td>Good</td>
</tr>
<tr>
<td>Methylene Chloride</td>
<td>10</td>
<td>400</td>
<td>Good</td>
</tr>
<tr>
<td>Iso-octane</td>
<td>10</td>
<td>150</td>
<td>Good</td>
</tr>
<tr>
<td>Toluene</td>
<td>10</td>
<td>250</td>
<td>Good</td>
</tr>
</tbody>
</table>

**TABLE II:** Summary of injection techniques and recommended starting points for choosing a glass inlet liner.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Goals</th>
<th>Liner Characteristics</th>
<th>Start With</th>
</tr>
</thead>
<tbody>
<tr>
<td>Split</td>
<td>Fast vaporization and transfer to column</td>
<td>Large internal volume; high surface area</td>
<td>Inert Analytes: 4 mm inside diameter cup or glass wool; Active analytes: cup glass wool; Dirty samples: glass wool</td>
</tr>
<tr>
<td>Splitless</td>
<td>Slow vaporization and transfer to column</td>
<td>Smaller internal diameter</td>
<td>2 mm inside diameter straight tube</td>
</tr>
<tr>
<td>SPME</td>
<td>Slow vaporization and transfer to column (no solvent)</td>
<td>Smallest internal diameter</td>
<td>0.75 mm or 2 mm inside diameter straight tube</td>
</tr>
<tr>
<td>PTV large volume</td>
<td>Slow vaporization and transfer to column with large solvent volume venting</td>
<td>Large internal diameter; low thermal mass; high surface area</td>
<td>4 mm inside diameter with adsorbent packing</td>
</tr>
</tbody>
</table>
for split injections. I usually start with the cup design, developed by Jennings and often referred to as the “Jennings cup” (5). The cup and associated tubing provide a flow path that assists with vaporization by the large surface area and mixing by the twisted flow path. In Figure 2, the cup is highlighted by the yellow oval. Glass wool and baffles provide a less expensive alternative (especially glass wool for samples with a “dirty” matrix), but both can be challenging with reactive analytes; if active analytes are involved, I recommend the additional expense of deactivated liners. If you look closely at Figure 2c, you will see some contamination in the glass wool, providing evidence of the main challenge of using glass wool.

Liners For Splitless Injections
Splitless injection is a much slower process than split. A rapid injection with the syringe is followed by a slow evaporation and transfer of the vaporized sample to the column head. To reduce the band broadening caused by the slow sample transfer to the column, temperature programming is almost always used with splitless injections. The inlet liner for splitless injections must be a trade-off between adequate internal volume to accommodate solvent vapor and reasonable transfer time for the vapor phase solvent/solute/carrier gas mixture to the column. The classical liner is a straight glass tube with about 2 mm inside diameter, as seen in Figure 3a. A splitless liner usually has a smaller internal volume than for split. This is partially compensated by slower vaporization, but the liner volume should still be confirmed using a vapor volume calculator. The tube ends may be tapered or goosenecked to assist with column installation or syringe insertion. The liner may also be packed with glass wool or an adsorbent material to facilitate solvent and sample vaporization.

Special Liners: SPME and PTV
Most injections using solid-phase microextraction (SPME) are a special application of a split/splitless inlet in which no solvent is used. Because no solvent is used, there is no need for a large volume to accommodate solvent vapor. Since there is no need for a large volume, a small, 0.75 mm, inside diameter straight tube inlet liner is most commonly used. One advantage of SPME injections is that there is no solvent, so there is no need for the inlet liner to have a large internal volume; in fact, the smallest possible volume is best to reduce band broadening during the injection process. Figure 3b shows a typical glass liner for SPME. Note the very narrow inside diameter, which is possible since there is no solvent vapor and the taper at one end, which provides a guide for the needle to fit more easily into the small diameter. In the early days of SPME, my group wrote on the details of optimizing SPME injections using the split/splitless inlet (6). The narrow diameter liner becomes more critical as the analytes become more volatile. For semi-volatile analytes, a traditional straight splitless liner will often work.

You may have a programmed temperature vaporization (PTV) or multi-mode inlet that is...
Choosing a Liner

Basic characteristics of inlet liners for specific injections methods are summarized in Table II along with my recommendations for the left end of the SPME liner which acts as a needle guide for the syringe. When I am choosing inlet liners, I start simple. For split injections, I start with a classical cup design, which, although a little more expensive than some of the other options, provides a large space for vapor expansion and a high heated surface area to aid in sample vaporization. Also, the cup prevents an accidental “lucky shot” in which the liquid stream from the syringe shoots straight into the column. For splitless, I prefer to start with a 2 mm inside diameter straight tube, with no glass wool or other obstructions. I then consider options for a modified flow path, such as glass wool, should my evaluation of the data dictate, usually by seeing unsatisfactory quantitative reproducibility for repeated injections of standards. The challenge with choosing a glass inlet liner is that there is no solution that fits all analyses and situations. Once a starting point is chosen, if the results are not satisfactory, alternatives must be selected and tried by experimentation. Finally, when choosing a glass liner for a specific application, the classic first steps in method development especially apply: check the applications literature, and contact the vendor for advice as well as ask someone who knows.

Conclusions

The glass inlet liner is one of the most important, yet least understood and most often ignored, components of a gas chromatographic experiment. It is the critical link between sample preparation and separation by the column. The best sample preparation and the best column can be ruined by a poor choice of glass liner. Unfortunately, there is no “killer app” for choosing an appropriate glass inlet liner. Users are best served by making an intelligent first choice, usually simple, and of course appropriate to the injection technique (split or splitless) being used, thinking about the expected chemistry of their samples and standards and making changes such as from cup to baffles to inserting glass wool as analytical and chromatographic performance dictate. Not all glass inlet liners are alike, and not all will solve every problem. For best performance, they must be studied by experiment.

References


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FOCUS ON BIOPHARMACEUTICAL ANALYSIS

Charge Detection Mass Spectrometry: What’s the “Big” Deal?

Over the last several years, the landscape of the biopharmaceutical industry has begun to change. Monoclonal antibodies (mAbs) still dominate the pipelines in the biopharmaceutical industry; however, more complex molecules, such as antibody–drug conjugates (ADC), are becoming more predominant. In addition to the complexity of ADCs, one of the fastest growing classes of biopharmaceuticals is cell and gene therapies. Gene therapies, for example, use large viral vectors, such as adeno-associated viruses (AAV), as the preferred vector for performing gene therapy. With the complexity of biopharmaceuticals increasing, especially in their size, new and innovative approaches are needed to address and characterize these molecules. This month’s column reviews how charge detection mass spectrometry (CDMS) is being used to characterize some of these larger more complex molecules in the biopharmaceutical industry.

Jared R. Auclair, Liang Xue, and Anurag S. Rathore

Biopharmaceuticals are products that are specifically made by living cells or biotechnological processes. By the nature of these products being manufactured in living cells can lead to variability in the product produced, that is the “process is the product” (3). In addition, compared to synthetic drugs, biopharmaceuticals are orders of magnitude larger and more complex because they often contain post-translation modifications such as glycosylation and can form higher-order structures (oligomers). These post-translation modifications are largely why biopharmaceutical products are so complex and a diverse set of analytical tools is needed for their characterization (3).

The biopharmaceutical industry is a billion-dollar industry that is largely dominated by monoclonal antibodies (mAbs). The first mAb was registered in 1986, and the 1990s saw the rapid evolution of biopharmaceutical development and products entering the market. In 2002, the first fully humanized mAb was approved by the U.S. Food and Drug Administration (FDA) (3). Thus, the biopharmaceutical industry is still in its infancy and new, more complex products are in development and will likely dominant the market in the future. For example, several bispecific antibodies are in clinical trials. A bispecific antibody is an artificial protein composed of fragments from two different mAbs, which allows them to bind to different antigens. The bispecific nature of these antibody products makes them promising therapies for certain types of cancer. Another promising new modality is antibody–drug conjugates (ADCs), which are bioconjugated products. In an ADC, a mAb is conjugated with a potent drug, and the mAb targets a specific antigen and delivers the drug to the target. ADCs have proven to be effective at specifically targeting and delivering anti-cancer drugs. Although these are only a few examples of the more complex biopharmaceuticals in development, there are many others such as glycoengineered mAbs, recombinant protein therapeutics, vaccines, and gene therapies (3).

The development and manufacture of these new, more complex biopharmaceuticals drive the need for new analytical technologies to characterize them. Arguably, the most common analytical tool for characterizing biologics is liquid chromatography–mass spectrometry (LC–MS). However, the most common forms of LC–MS have limitations when characterizing large macromolecules (4). Thus, in this column, we discuss the potential for charge detection MS (CDMS) as an analytical tool for characterizing large, complex, and heterogeneous biopharmaceuticals.

Native Mass Spectrometry (Native MS)

Before discussing CDMS, we should briefly discuss native MS, which is perhaps the most commonly used technique to characterize the intact mass of large, complex biomolecules in the megadalton range. As with all MS experiments, sample preparation is of utmost importance, which is also true for native MS. Proteins or protein complexes are isolated, purified, and then often diluted in a solvent such as ammonium acetate for the efficient spraying through the electrospray ionization (ESI) emitter into a MS instrument (5).
When a quadrupole time-of-flight instrument (QTOF) is used in native MS, the ions of the intact molecules pass through the quadrupole and through the collision cell, which is turned off. The parameters of the quadrupole are adjusted to promote the transmission of high mass-to-charge \((m/z)\) ratio ions. For example, in the quadrupole, the pressure of the pumping stages is increased and the frequency is decreased, allowing the transmission of ions in the thousands of \(m/z\) range (5).

These modifications allow for the characterization of protein–protein and protein–ligand complexes. However, there are limitations to this approach. Namely, the molecular mass of the macromolecule can only be determined if the mass and charge state can be resolved for multiple charge states of the molecule. In addition, there is often poor desolvation and broadened mass distributions, which result in overlapping signals between consecutive charge states. These limitations can lead to inaccurate mass assignments preventing the characterization of large heterogeneous proteins or complexes, such as highly glycosylated proteins, aggregates, and genome packed viruses like aden-associated virus (AAVs) (4).

**Charge Detection Mass Spectrometry (CDMS)**

One approach to overcome the convolution of ions, as mentioned above, is to employ single particle, or ion, detection.

When single particle detection is paired with the identification of the charge of the ions, we are left with single-particle MS (sp-MS) measurements. There are several techniques for sp-MS detection, one being CDMS, which has gained momentum over the last few years in analyzing complex biopharmaceuticals (4). As a matter of fact, in October 2021 at the American Association for Mass Spectrometry (ASMS) annual meeting in Philadelphia, TrueMass presented the first commercial CDMS (6,7). Other companies, such as Megadalton Solutions (8), offer fee-for-service analysis of protein assemblies, aggregates, AAV, and vaccines.

In CDMS, the masses of individual ions are determined by the simultaneous measurement of the \(m/z\) ratio of each ion and the charge itself (2). In a CDMS experiment, the \(m/z\) of the individual ions and charge are measured, whereas in traditional MS experiments only the \(m/z\) ratio is measured, and the charge is inferred from the charge state envelope or distribution. The charge in a CDMS experiment is measured by passing the ions through a conducting cylinder where the charge of the ion is induced on the cylinder and detected. The cylinder is often inside an electrostatic linear ion trap (ELIT) instrument, where ions oscillate back and forth. Therefore, the oscillation frequency gives the \(m/z\) and the charge is determined by the magnitude. Historically, the resolving power has been modest, but in recent years and likely in the future, the resolving power will continue to improve (2).

For example, in 2020, Todd and others showed an order of magnitude increase in the resolving power of CDMS by combining dynamic calibration with improvements in an ELIT instrument (2). Briefly, when considering using an ELIT instrument in CDMS, the conducting cylinder is placed in an ELIT instrument, allowing the ions to oscillate in the cylinder many times as opposed to the one time in CDMS experiments where the conducting cylinder is not placed in an ELIT instrument (Figure 1). In dynamic calibration, the charge-sensitive amplifier is calibrated during the measurement by
placing a small antenna in the outer casing of the ELIT instrument (9). This approach of combining dynamic calibration and ELIT improvements, leading to increased resolving power, was benchmarked by the analysis of trapped intermediates in the assembly of the Hepatitis B virus (2), thus showing its applicability to not only large molecule detection but in analysis of heterogeneous samples (Figure 2).

In another study in 2020 by Worner and others, they showed the use of an orbital trap MS in CDMS analysis. In this study, the authors were able to characterize a complex mixture of IgM oligomers and co-occurring empty and genome loaded AAV8 particles. Of particular note in characterizing these molecules, the authors showed the application of CDMS to analyzing heterogenous complexes where conventional native MS cannot be used. Traditional native MS cannot be used because it is unable to resolve the charge states in these heterogenous mixtures, thus preventing molecular mass determination (4).

**What’s the Big Deal?**

So, that leads us to answer the question posed in the title: what’s the big deal? CDMS, a type of sp-MS, allows for the characterization of both larger molecules but also overcomes some of the challenges with heterogeneity in these larger molecules, which is enabled by the ability to detect the m/z of a single ion as well as its charge.

A study by Miller and others was published in JACS in 2021 titled, “Heterogeneity of Glycan Processing on Trimeric SARS-CoV-2 Spike Protein Revealed by Charge Detection Mass Spectrometry” (10). At first glance, the contribution to our understanding of the SARS-CoV-2 protein might seem like the most important finding of the study. However, the ability to analyze the glycoprofile of the Spike protein using CDMS will likely have larger, and longer, implications in the biopharmaceutical industry. The study showed the ability of CDMS to characterize not only large molecules but heterogenous mixtures as well, in this case related to glycosylated proteins, which many biopharmaceuticals are (10).

The Spike protein in SARS-CoV-2 is a trimeric glycosylated protein. The glycosylation of the trimer is determined by the number of different glycans available to occupy the sites of glycosylation. The authors predict based on the number of glycans available that 8.2 × 10^{75} glycosylation forms are possible, which suggests that it is near impossible for any two Spike trimers to have the same glycan distribution (10), thus making it impossible for traditional glycan analysis to give a clear picture of the glycoprofile.

Traditional glycan analysis of glycoproteins cannot give information about where glycans at a particular site are correlated with glycans at other sites on an intact individual glycan molecule. Generally, speaking traditional methods allow for sites of glycosylation to be identified as well as percentages of glycans present in the mixture. On the contrary, Miller and others showed that by using the SARS-CoV-2 Spike protein CDMS can provide information about glycans at a particular site in the molecule (10). Thus, CDMS has the potential to provide a more robust and comprehensive characterization of biopharmaceutical glycoproteins.

**Conclusion**

As biopharmaceuticals become more complex new tools for their characterization are necessary. Over the next several years, more complex molecules such as bi-specific mAbs, vaccines, and gene therapies will be developed, enter the market, and perhaps even begin to dominate the market. CDMS is being used to characterize these molecules more often because of its ability to characterize both large molecules and heterogenous mixtures, such as complex glycoproteins. CDMS is not the only tool, or perhaps, the best tool at this stage, but it is one to watch as the hardware is further refined, new methodologies developed, and applied to other molecules. It will be interesting to see, over the coming years, how CDMS techniques evolve and are applied to biopharmaceuticals in areas mentioned here and others not. At the end of the day, new technologies, such as CDMS, are needed to ensure more complex products are safe, effective, and of good quality for patients.

**References**


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In the fifth episode of this six-part series, Bogdan Budnik, principal scientist at Harvard Center for Mass Spectrometry, discusses common challenges related to nano LC-MS proteomics analysis of very diverse sample types, improvements in proteomics analysis, and how single-cell proteomics will impact the medical field.

**LCGC:** What are the reasons engaging you to continue working in nano LC-MS proteomics?

**BUDNIK:** The field of proteomics is a broad definition of our ability to look at nature and its biology, which is what we’ve been doing for the last 20 years. We try to improve our tools to examine the complexity of biology—how we can learn what it is, what we can do if it’s broken, etc.

**LCGC:** What are the most common challenges related to nano LC-MS proteomics analysis of diverse sample types?

**BUDNIK:** We have a very diverse community of biologists around us who brought their samples into our lab over the years. For the LC-MS unit, it’s a challenge to ensure that each group or individual scientist gets their answer, no matter how complex or not complex their samples are and how much material they have. That's why when scientists come to us with both large and/or small amounts of samples, they need to be properly assigned to an LC-MS system that can deal with such complexity or with a little amount of sample.

**LCGC:** While working with multiple scientific teams, you also improve existing LC-MS methods and setups. What are the drivers for these activities?

**BUDNIK:** Within recent years, proteomics shifted toward a quantitative approach. Instead of asking what is there, the main biological question now is how much is there? Quantitative approaches are mainstream right now, and the improvements over the years have focused on analyzing smaller and smaller amounts of samples. That's the number one priority for our community.

Number two, since there is a large number of samples being analyzed by proteomics, we want to make sure our instruments are working more efficiently, so we can increase the number of samples we can analyze per day.

**LCGC:** How would you describe the role of LC technologies in the overall improvements of LC-MS workflows?

**BUDNIK:** There are different types of approaches. Many use micro-flow rates, but now, everything is done using nano-flow rates. There are two streams of people going in two different directions, answering two different questions: with microflow LCs and clinical proteomics, there will be a lot of improvements with the data-independent acquisition (DIA) approaches via faster turnaround times of large sample amounts, while low nanoflow systems are moving toward single-cell analysis.
LCGC: Can you describe the benefits that bring a tandem nano-LC setup?
BUDNIK: In my lab at Harvard, we've had Tandem Mass Tag (TMT)-labeled breakthroughs for the single-cell analysis. Since then, there are a lot of projects done in this area. We usually analyze eight cells per sample, and the biological questions are in hundreds of cells. This is why you need a lot of LC-MS runs. This is where productivity and sensitivity meet the demand of reproducible runs on an LC-MS system. This is also where a tandem system comes into play. With the help of Thermo Fisher, we've set up a system that is much faster than one-by-one simple analysis with a single pump. Since the implementation of the double system, our instruments work in tandem goes much faster than the conventional approach of the single pump.

LCGC: LC-MS proteomics is moving from identification and semi-quantitative measurements of protein abundance changes to quantitative analysis. What is required from LC technology to support this movement?
BUDNIK: Yes, as I mentioned before, the majority of biologists come to our labs asking how much is there. The quantitative analysis of current samples is an important piece, and not only just quantitative, but the quality of the quantitation is playing a very key role in many biological questions that scientists are trying to answer, which is what we're doing with LC-MS.

LCGC: Has LC-MS technology significantly improved over the last several years?
BUDNIK: Yes, there has been a lot of progress in this area. The column is the biggest breakthrough. The new columns use a chip technology instead of packed material, which makes them very reproducible. The packed columns have also improved in quality and reproducibility. Both improvements enable us to analyze more samples per day and week.

LCGC: How would you describe a day in the lab if you had the ideal LC-MS technology for proteomics?
BUDNIK: That would be a very relaxed day. I'd put the samples into the autosampler to start injecting and go home because I could do the analysis online ... the ideal day in the lab would be no day in the lab. All LC and MS systems would be working, running my samples, and we wouldn't have to spend any time on repairs. We'd only need to be in the lab for sample preparation. (How we prepare samples needs improvement.)

LCGC: Where do you see the next big step in the proteomics field? What's the next breakthrough?
BUDNIK: Single-cell proteomics will be the standard type of analysis. Another area we'll see improvement in is a large dataset that will run not only at specialized labs but near hospitals where the analysis for RNA or DNA sequencing is done—this could confirm or defer ideas about the progress of the disease or treatment of each patient. Single cell will be a key area where we can see different types of cells and clinical proteomics. Samples from the patient will be analyzed on the fly in real-time so doctors have answers on how the treatment is or isn't working. These are the two areas I see proteomics playing a key role.
A Suitable Therapeutic Drug Monitoring Method for Amoxicillin in Plasma by High Performance Liquid Chromatography–UV (HPLC–UV) in Neonates

Amoxicillin, a broad-spectrum β-lactam antibiotic, is widely used for treatment of neonatal infections. Despite the unmet need in neonates, an adapted analytical method is still missing in clinical practice. The purpose of this study was to develop and test an easy and credible high performance liquid chromatography–UV (HPLC–UV) method to determine amoxicillin in small volumes of human plasma and use it in routine therapeutic drug monitoring (TDM) of neonates. After a protein precipitation, amoxicillin for standards, quality control samples, and patient samples were separated by LC and measured by UV detection, and tinidazole was used as the internal standard. The calibration range was 0.50–20.0 µg/mL. Intra- and inter-day precisions were less than 4.7%. The acceptance criteria of accuracy (between 85–115%) were met in all cases. A plasma volume of 50 µL was required to achieve the limit of quantification of 0.50 µg/mL. Thus, a simple, rapid, and accurate HPLC–UV method has been developed to detect the concentration of amoxicillin in human plasma. This method was adapted to do TDM of amoxicillin in neonates.

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Amoxicillin (C₁₆H₁₉N₂O₅S, Figure 1) is one of the most commonly prescribed antimicrobial drugs in the world (1,2). Amoxicillin is prescribed for various infections including nose, ear, throat, and skin infections, gonorrhea, genitourinary tract, acute uncomplicated anogenital and urethral infections, and lower respiratory tract infections (3), and it is widely utilized for treating neonatal infections. Amoxicillin is stable in acidic medium and designed for oral use. However, amoxicillin is most commonly administered intravenously (4). It diffuses easily into most of the body fluids and tissues. It also shows low protein binding ability and is principally excreted through glomerular filtration (3).

The pharmacokinetics of amoxicillin showed large variability in neonates (4,5). It is well known that the efficacy of amoxicillin is related to the duration of drug concentrations above the minimum inhibitory concentration (MIC) value (6). Underdosing will speed up bacterial resistance, and overdosing can cause adverse side effects, such as an increase in muscle and liver enzymes, seizures, renal dysfunction, and diarrhea (7). Therefore, therapeutic drug monitoring (TDM) of amoxicillin is required to optimize antimicrobial agents in neonates.

An adapted method is a requisition for neonatal TDM practice. Various bioanalytical methods have been reported to measure amoxicillin concentration, consisting of high performance liquid chromatography (HPLC) paired with UV (8–12), fluorescence (13–15), or mass spectrometry (MS) (16,17). Nevertheless, most of these methods have limitations in neonatal clinical practice. The lower limits of quantification (LOQ) in some of these studies were very low (0.2–1 mg/L), but the volume of the samples were more than 100 µL (9–15). One study on this subject used less volume of samples (40 µL); however, the quantification limit was high (8 mg/L) (8). Other reported studies used either comparatively complicated sample preparation methods (8,9) or utilized expensive methods (16,17). The purpose of the study was to build and test an easy and credible HPLC–UV method to determine amoxicillin in small volumes of human plasma and use it in the routine therapeutic drug monitoring in neonates.

### Methodology

#### Chemicals

Analytical or HPLC grade chemicals and reagents were used. Amoxicillin reference standards and tinidazole (internal standard) were obtained from the National Institute for Drug and Food Control. HPLC-level solvent methanol was procured from Merck Serono. Potassium dihydrogen phosphate (KH₂PO₄) was obtained from a reagent company named Sinopharm. Sodium hydroxide was purchased from the Dalu chemical reagent factory. For HPLC mobile phase filtration, HV membrane filters (0.45 µm in size) were purchased from the Xianan biochemical instruments factory. A Milli-Q water purification system for ultrapure water was purchased from Millipore. Drug-free human plasma was procured from healthy subjects.

#### Quality Control Samples and Standards Preparation

The amoxicillin stock solution (0.86 mg/mL) was prepared in solution A (6.8 g/L concentrated KH₂PO₄ buffer, pH 5.0) and divided into 1 mL aliquots before being stored at a temperature of ~70 °C. After that, calibration standards of amoxicillin (0.5, 0.7, 2, 4, 8, 16, and 20 µg/mL) were prepared in plasma. Quality control (QC) samples (low QC: 1.0 µg/mL; medium QC: 10.0 µg/mL; and high
QC: 17.5 µg/mL) were equipped by adding proper quantity of the main solution to blank plasma. All the QC samples were split into 50 µL aliquots and conserved at −70 °C. The 19.3 µg/mL tinidazole working solution was saved in water, then split into 1.0 mL aliquots and conserved at −70 °C.

Sample Preparation

According to the procedure, 200 µL of methanol and 20 µL of the internal standard working solution were added into a 50 µL sample of plasma to prepare samples. Next, the samples were vortexed for 1 min, trembled and separated at 4000 rpm at 4 °C by using a shaker, and centrifuged for 10 min, respectively. Then, 100 µL of the supernatant was put into an autosampler vial with an insert and diluted with 100 µL of water. Finally, 20 µL of sample volume was analyzed in the LC–UV system via the injector.

![Chemical structure of amoxicillin](image)

**FIGURE 1:** Chemical structure of amoxicillin.

**TABLE I:** Intra-day (n = 5) and inter-day (n = 3) precision and accuracy of amoxicillin plasma concentrations

<table>
<thead>
<tr>
<th>QC Concentrations (µg/mL)</th>
<th>Intra-Day</th>
<th>Inter-Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured Concentrations*</td>
<td>Mean±SD (µg/mL)</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.05 ± 0.05</td>
<td>97.4</td>
</tr>
<tr>
<td>10</td>
<td>9.73 ± 0.46</td>
<td>97.29</td>
</tr>
<tr>
<td>17.5</td>
<td>17.05 ± 0.36</td>
<td>105.22</td>
</tr>
</tbody>
</table>

*Results are expressed as mean±standard deviation. QC: quality control.

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For the HPLC–UV method, a Waters 2695 separations module and a Waters 2487 UV detector were used. For the chromatographic separation, an InertSustain column (RP-18e, 250 mm × 4.6 mm) from Shimadzu was used. The wavelength was set at 230 nm. The gradient of the mobile phase was arranged every day accordingly, degassed by ultrasonication prior to use, and prohibited from recirculating during the procedure.

Method Validation
The method was validated according to the guidelines of the bioanalytical procedure approved by the Food and Drug Administration (FDA) (18) and the European Medicines Agency (EMA) (19). The terms for validation contained selectivity, linearity, LLOQ, matrix effect, accuracy, precision, recovery, and stability.

Selectivity
To assess the selectivity of the method, six different blank plasma samples were analyzed. The method selectivity was defined as the lack of disturbance at the retention times of amoxicillin, the internal standard from the blank plasma components, and the lack of cross-interference among them using the proposed extraction technique and LC–UV conditions.

Linearity
Fresh preparations were used for three days to determine the linearity of the method. Amoxicillin calibration curves were produced by spiking the sample of matrix with a concentration range 0.5–20 µg/mL. The calibration data linear regression analysis was performed by using the equation: 

\[ y = a + b \cdot C \]

where \( y \) is the peak area ratio of amoxicillin to internal standard, \( C \) is the nominal amoxicillin concentration in calibration samples, and \( a \) and \( b \) are the intercept and the slope of the curve, respectively.

For each calibration curve, the slope, intercept, and correlation coefficient \( (r^2) \) were calculated. The calibration curve was tested: a) the mean value should be within ±15% of the theoretical value (LLOQ within ±20%), and b) the precision around the mean value should not exceed a 15% coefficient of variation (CV) (LLOQ within ±20%).
Lower Limit of Quantification (LLOQ)
The LLOQ was ascertained as the signal-to-noise (S/N) ratio being greater than five. The analyte peak should be identifiable, discrete, and reproducible with an accuracy of 80–120%, and a precision of 20%.

Matrix Effect
The potential interference of the matrix compounds in the quantification of amoxicillin was evaluated. A spiked blank plasma sample and a working standard solution were investigated at the LLOQ level.

Accuracy, Precision, and Recovery
Precision, accuracy, and recovery were evaluated at three concentrations (QC1, 1.0 µg/mL; QC2, 10.0 µg/mL; and QC3, 17.5 µg/mL). The intra- and inter-day accuracies were shown using an equation: \[
\left(\frac{\text{measured concentration}}{\text{theoretical concentration}}\right) \times 100
\]\]. Intra-day assay accuracy and precision were computed using five replicates during every analytical procedure. Inter-day assay accuracy and precision were reported by evaluating the three QCs on three different days. The precision had to remain under 15%, and the bias range must be within 85–115%. The recovery of the method was evaluated by the responses of the three extracted concentrations compared with the nonextracted standards (equivalent to 100% recovery).

Stability
To assess the stability of the samples during the sample treatment process, the stability procedures were defined. For QC1 and QC3, short stability was established and assessed by comparing assay values in triplicate at room temperature after 6 hr.

By performing assaying of the QC1 and QC3 freeze-thaw, stability was assessed in triplicate over three freeze–thawing cycles (stored at −70 °C for 24 hr, thawed at room temperature, and refrozen for 24 hr constitutes one cycle). The measured concentrations were then compared to the nominal value.

Post-preparative stability was established by determining the QC1, QC2, and QC3 in an autosampler at 4 °C by injecting extracts immediately after the pretreatment, then re-injecting after 72 hr.

To establish long-term stability, samples stored at −70 °C over 50 days were analyzed.

Application in Therapeutic Drug Monitoring (TDM)
The developed method was routinely implicated in TDM of amoxicillin in neonates. This study was conducted according to the guidelines of the Declaration of Helsinki and with the approval by the local research ethics committee. For this study, 97 neonates receiving amoxicillin as part of their antimicrobial treatment were used. The standard dosing regimen was 30 mg/kg twice daily. The trough concentrations at steady-state (one day after the start of therapy) were drawn according to standard clinical practice. Using EDTA tubes, all the blood samples were collected and centrifuged for 10 min at 4000 rpm at 4 °C to segregate the components of plasma.
the plasma fraction was shifted to a new tube and stored at −70 °C until analysis. All samples were determined in a month.

**Results**

**Selectivity**

The chromatograms of the blank plasma and LLOQ are shown in Figure 2a and 2b, respectively. No interferences were detected at the retention times of amoxicillin and tinidazole.

**Linearity**

The calibration curves were linear over the concentration range of 0.5–20 µg/mL in human plasma. The following equation was obtained: y = 0.0477x + 0.0003 (r² = 0.9948). The average values of calibration concentrations were within 97.7–105.2% of the theoretical values. Other than the LLOQ, bias was within the range of 83.5–102.7% and the precision was 8.8%.

**Lower Limit of Quantification (LLOQ)**

The lower limit of quantification was 0.5 µg/mL.

**Matrix Effect**

The peak areas were evaluated as being similar enough in a way that the matrix effect can be judged insignificant.

**Accuracy, Precision and Recovery**

Intra- and inter-day precision and accuracy are shown in the Table I. Overall, intra-day precision was lower than 4.74%, while inter-day precision was lower than 4.30%. In all cases, the acceptance criteria of accuracy (range, 85 and 115%) were met. Amoxicillin recovery was 107.1 ± 10.7% (range was 94.8–114.7%).

**Stability**

Short-term stability was shown that the QC samples were stable for 6 hr stored at room temperature (biases range, −14.3 and 8.7%). Freeze thawing three consecutive cycles showed no significant degradation for amoxicillin (biases range, 14.8 to −5.0%). In autosampler, the extracted samples were stable for approximately 72 hrs at −4 °C (biases range, −14.9 to 3.4%). From the literature, in case of long-term stability the samples were stable for over 50 days stored at −70 °C, the concentrations of the samples have no significant degradation (deviation <2.03%) (15), which indicated that the samples in this study were stable (measured in a month).

**Therapeutic Drug Monitoring (TDM)**

A total of 97 neonates were included with a median gestational age (GA) of 37.86 weeks (the range was 28.29–41.29 weeks), a median postnatal age (PNA) of six days (the range was 0–18 days), and current weight (CW) of 2.94 kg (the range was 1.06–4.13 kg). Figure 3 showed an original chromatogram from the analyzed patients’ samples. Figure 4 showed the distribution of trough concentrations. Only 51.5% (n = 50) of neonates had trough concentrations higher than 2 µg/mL.

**Discussion**

Because of the unique features in neonates, the analytical method ought to be well adapted and find a balance between small sample volume and high sensitivity. After reviewing previously published methods to determine the amoxicillin concentration, most of these methods have limitations in neonatal TDM, such as cumbersome sample preparation, a large number of plasma samples, a high LLOQ, and an expensive cost. In this study, all of these common problems were resolved. The required plasma volume was approximately 50 µL, which was suitable for neonatal physiological condition, and the LLOQ was only 0.5 µg/mL. At the same time, the sample preparation process was significantly simplified. In addition, the HPLC–UV instrumentation is common in hospitals, so this method is easy to generalize. Obviously, these improvements allow to better interpret TDM results in neonates.

Amoxicillin is not stable in plasma at room temperature as its β-lactam ring is more susceptible to hydrolytic degradation by plasma amidase (20). Considering the water solubility of amoxicillin and its chemical stability, along with the viscosity of the solution (risk of operational error), methanol and water (1:1) were used as the diluent during the solution preparation.

This developed method was further evaluated in neonatal TDM practice. As shown in the results, the trough concentrations were highly variable. As a time-dependent, anti-infective drug, amoxicillin efficacy correlates with the time of drug concentration above the MIC (21). According to the breakpoint tables for interpretation of MICs and zone diameters (22), the MIC breakpoints were 0.5 µg/mL for Viridans streptococci and Gram-negative anaerobes, 1.0 µg/mL for Pasteurella multocida, 2.0 µg/mL for Haemophilus influenzae, 4.0 µg/mL for Enterococcus spp. and Gram-positive anaerobes (except Clostridium difficile), and 8.0 µg/mL for Enterobacteriaceae. Our study showed that 8.4% (n = 47) of patients had a trough concentration of less than 2 µg/mL, indicating a high risk of treatment failure in these patients. In this situation, a higher dose or other antimicrobial treatment ought to be suggested to these patients. Therefore, TDM should be advised to optimize the treatment of amoxicillin in neonates.

**Conclusion**

A simple, rapid, and accurate HPLC–UV method has been developed to determine the amoxicillin concentration in plasma. The small volume of plasma (50 µL) and sensitive LLOQ (0.5 µg/mL) make it well adapted to neonatal TDM practice. Only 51.5% of neonates had trough concentrations higher than 2 µg/mL, so TDM should be advised to optimize the treatment of amoxicillin in neonates.

**Acknowledgments**

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**References**

• Continued from Page 120

Applications of QuPPe

Given the relative newness of the method, reports of QuPPe are just starting to emerge, though the method is accepted by the EU. Perusal of the Scopus database shows 21 literature reports of QuPPe from six papers per year. Although QuPPe is still an emerging technique, the lessons learned from QuEChERS were re-applied in developing this simple method for the extraction of highly polar pesticides from samples of plant and animal origin. This method, and potentially modified versions, bears watching as an important tool for multiresidue monitoring.

Conclusion

Although QuPPe is still an emerging technique, the lessons learned from QuEChERS were re-applied in developing this simple method for the extraction of highly polar pesticides from samples of plant and animal origin. This method, and potentially modified versions, bears watching as an important tool for multiresidue monitoring.

References

(3) Y. Han, L. Song, P. Zhao, Y. Li, N. Zhou, Y. Qin, X. Li, and C. Pan, Food Chem. 197, 730–738 (2016).

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Enter the Matrix: Improving the Interpretation of Separations Data Using Chemometrics in Analytical Investigations

This article describes a compendium of chemometrics applications in separation science to demonstrate the importance and synergy of data handling. We review the main points that must be raised when using multivariate techniques and list references for further reading. With examples in food science, the case studies comprise of applications of pattern recognition (principal component analysis, PCA), regression (partial least squares, PLS), and classification methods (soft independent modelling of class analogies, SIMCA).

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Analysts are often challenged by the multidisciplinary aspects of qualitative investigations, which require a broad set of skills. In this context, it is important to recognize the synergy between sample preparation, separations, and data science (chemometrics) for managing research projects in separation science. Although sample preparation and separation science may share some commonalities like the thermodynamic and kinetic considerations of multi-phase equilibria, chemometrics may frighten some analysts. However, it is not compulsory to be a mathematics expert to become a user of classical multivariate analyses. We start by choosing the right software and toolboxes. Next, one must carefully define the scope and select the most suitable method for the qualitative investigation: pattern recognition, classification, or regression.

Exploratory analysis methods, such as principal component analysis (PCA), are used to extract information and detect patterns in the data matrix based on a multivariate approach (1,2). The dimensionality reduction of the data matrix allows the samples to be described by two or three dimensions (principal components) without losing the native information. A multivariate calibration method must be used when performing a linear regression between the signal (chromatogram and mass spectra) to a property of interest. Partial least squares (PLS) is a multivariate calibration method that uses dimensionality reduction, similar to PCA, for the correlation between the signal and property of interest (3,4). For example, these properties might be concentrations, physicochemical properties, or sensory analysis scores.

Classification methods are used to determine to which class an unknown sample belongs. Classification can vary in format (multiclass or one class) and may include authentication studies (one class), which aim to determine whether an object truly is what it claims to be (5,6). Some algorithms are better suited for authentications, such as soft independent modeling of class analogy (SIMCA). These algorithms distinguish objects of one specific class from all other classes without prior knowledge from the latter. A classic example is distinguishing between original samples and counterfeited samples without knowing exactly how they were counterfeited.

In this article, we share three case studies to provide a concise introduction to the possibilities of these chemometrics approaches using chromatographic and mass spectrometry (MS) data and provide references for the users’ first steps.

Case Study #1: Pattern Recognition Using PCA
PCA is one of the most applied methods in chemometrics (1,7). It aims to project data in a variance space to identify and interpret statistical similarities and differences (1,7). Prior information about samples, such as concentration or class, are not required to train the model (1,8).
In this topic, direct electrospray ionization and high-resolution mass spectrometry (ESI-HRMS) were selected to analyze commercial coffee samples for fingerprinting purposes. Sample preparation consisted of solid-liquid extraction of the six types of coffee capsules (9). To perform PCA, the data were normalized and mean-centered (with the latter being a mandatory preprocessing step that uses the average distance of samples as the origin of the PC-axes). Otherwise, the maximum data variance would be related to the distance between the axis origin and the samples, which is irrelevant information (1).

After data preprocessing, choosing the number of PCs is required. Having too many PCs may introduce noise and errors to the scores and loadings, whereas with fewer PCs, relevant information may be left in the residue matrix (1,7). Figure 1a shows information about the residues, and samples outside the threshold can be defined as outliers. Q residuals are related to non-modeled information and Hotelling’s $T^2$ is related to the distance of the sample from the origin of the data set (1). After outlier removal, the data were remodeled, and the number of PCs was selected again. Two PCs were selected comprising 94.5% of explained variance and low residues.

Scores present the position of samples in the PC space, displaying information related to them and clustering similar samples (Figure 1b). Thus, PCA scores show a clear separation of the six types of coffee capsules. The classes were mainly separated using only PC1, but Italian Ristretto Decaffeinato (RIS-DEC) samples were separated only by PC2. To understand the reason for this separation, scores and loadings must be evaluated together (1,8). Focusing on PC1, the rightmost samples in Figure 1b presents more influence from the most intense positive signals in Figure 1c. In this case, a compound with $m/z$ of 138.0557 is found more in Ethiopian samples than in Palermo Kazaar samples. This compound might be trigonelline, which is an important alkaloid found in coffee (9). Alternatively, PC2 shows that the other samples present more of a compound with $m/z$ 195.0885 than the RIS-DEC samples do. The separation on PC2 is probably promoted by caffeine, which is supported by the fact that RIS-DEC samples are decaffeinated. Therefore, this shows that along with scores, loadings clarify the reason for the observed patterns.

**Case Study #2: Regression Using PLS**

PLS seeks to model an equation to estimate properties using instrumental analysis signals. More details about the theory can be found elsewhere (10). In this study, PLS was used to predict consumer preference based on the volatile organic compound profile of beer samples. The data consisted of a set of total ion chromatograms obtained by comprehensive two-dimensional (2D) gas chromatography coupled to mass spectrometry (GC×GC–MS). The property of interest contained the average score of the analyzed beers, whose values were collected from Untappd (11), a popular beer ranking database. Details about this database can be found in the literature (12).

Prior to PLS model building, preprocessing steps were performed. The first step was orthogonal signal correction, which removes systematic variation unrelated to the properties vector, retaining only the meaningful information (13). Data were then mean-centered. The next step consisted in the selection of an adequate number of latent variables, which are analogous to PCs. This choice should consider the percentage of cumulative variance captured and the residuals. Residuals represent the portion of the validation data that the model could not explain. They may be further analyzed by plotting residuals versus predicted values to observe if there is heteroscedasticity or autocorrelation behavior among them. Residual observation is excellent feedback that indicates if the model needs improvements. These can be done by changing the number of latent variables and preprocessing strategies.

Afterwards, the presence of outliers was investigated by plotting Q residuals compared to Hotelling’s $T^2$, as done with PCA. Three outliers were identified (Figure 2a). Outliers are characterized by high values of Hotelling’s $T^2$, that is high leverage. For PLS, that means these samples tend to force the regression line to be closer to them. In this case, outliers belonged to the same brand,
which might exhibit chromatographic features similar to highly rated beers that are not suited to the low average scores it received. Because the predicted property originated from untrained panelists without blind tests, brand recognition may be a factor. To confirm why they were labeled as outliers, further analysis of loadings and comparisons with other beers could be done, but it is not the aim of this article.

After removal of outlier samples, the $R^2$ for the model went from 0.872 to 0.869 when plotting cross-validation and external validation samples (Figure 2b–c), which is a clear sign of the leverage effect of outliers. Finally, it is worth highlighting that predictive modeling based on separation science tend to be hindered because of the presence of a considerable amount of chemical information unrelated to the property of interest (14), requiring careful variable selection.

Case Study #3: Classification Using SIMCA

Classification problems are pattern recognition problems that demand inputs related to the classes of each sample used in the calibration. Class boundaries are then calculated, and unknown samples are classified. If a given sample is within a boundary, it will be classified as belonging to that class. For authentication problems, the idea is to set a boundary for authentic samples and anything outside of them is non-authentic.

One-class classifiers are better suited for authentication and SIMCA is one of the most important of these tools in chemistry (5,6). Its main idea is to use the scores generated by a PCA to set the boundaries of the class. The theory behind it is explained elsewhere (6,15). Anything not similar to the authentic modeled samples is considered as an outlier to the model, thus not belonging to it. The main advantage is that only authentic class samples are needed for modeling.

A SIMCA model was used to classify if beers purchased in local markets could pass as pure malt beers. The SIMCA model was calculated using the toolbox created by Zontov and others (6). Here, 16 different brands were purchased with six of them being pure malted beers, four of which were used for calibration and two to validate the model. Beer samples were analyzed by REIMS in nine replicates for each brand. Only the positive mode was used and $m/z$ ratios whose intensities were lower than 100 units were discarded. As pretreatment, data was normalized, mean-centered, and Pareto-scaled.

To choose the number of PCs for the SIMCA model, a leave-one-out approach to the calibration samples was used and the mean sensitivity was estimated for each number of PCs. The model significance was set to 1%. As can be seen from Figure 3a, the highest leave-one-out sensitivity was obtained when using up to four PCs. Thus, four PCs were chosen as it gives stricter boundaries than with fewer.

FIGURE 1: (a) Identification of outlier samples because of the high values of Q residuals and Hotelling’s $T^2$. After outlier removal, the data was modeled, providing (b) score and (c) loading plots, showing information about samples and features, respectively.

FIGURE 2: (a) Outlier samples identification and their leveraging effect. Characterized by high values of Hotelling’s $T^2$ (blue dashed line) were classified as outliers and were removed. Regression line plots (b) before and (c) after removal of outliers illustrate the leveraging effect caused by the outliers when one observes the relative decrease in the coefficient of determination from (b) to (c).
After calibration, shown in Figure 3b as diamonds, the model was validated with the pure malted beers not used in the calibration procedure. These samples are illustrated in Figure 3b as stars and only one out of 18 replicates was incorrectly classified. Therefore, the model sensitivity was 94.4%. Finally, the model was used in non-pure malted beer samples represented as triangles. From Figure 3b, most of these samples were correctly classified above the boundary. However, 13 out of 90 replicates were incorrectly classified as pure-malted beers, which resulted in a specificity of 85.5%. It is important to highlight that misclassified samples were spread among brands.

Conclusion
We presented three case studies to showcase the chemometric approaches available for pattern recognition (data visualization), regression, and classification. We hope to demonstrate the potential of data handling and raise awareness about the synergy between separation science and chemometrics for the current and upcoming generation of analysts.

Acknowledgments
We dedicate this article to the loving memory of Professor Ronei Jesus Poppi.

References

(10) R.G. Breton, Applied Chemometrics for Scientists (John Wiley & Sons Ltd, Chichester, West Sussex, United Kingdom, 2007).

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The most important part of a chromatography setup is the column, but the final separation also depends strongly on the mobile phase. The choice of mobile phase influences the retention and differential migration (selectivity) of the solutes through the column. Therefore, during method development and optimization, the separation must be fine-tuned by changing mobile-phase parameters such as solvent type, additives (different buffers, ion pair reagents), or operating conditions (gradient time and steepness, temperature, flow rate). Because of all these parameters that require adjustment to achieve a desired separation, there is an inclination to use simpler mobile phases for practical reasons that include increased method robustness, easier method transfer, and ease of use.

Here are key considerations for successful mobile-phase preparation:

The purity of mobile-phase components is crucial
For example, it is critical to use gradient grade solvents and reagents for gradient grade separations to achieve an accurate, reproducible, sensitive, and clean separation. Using the correct grade of solvent based on the application also minimizes the chances of contamination and extends the longevity of a chromatography column. For cases requiring addition of any reagent-like buffer, it is required that the reagent meets the stipulated quality and that it has not expired. Degradants from expired additives may add unknown peaks in the blank chromatogram. Certain additives also expire more quickly, depending on their nature (such as 20 mM pH 7 phosphate buffer) or their improper handling (such as leftover solvent put back into bottle, or the bottle left on the laboratory bench without the cap closed, which spoils chemicals very quickly).

Mix mobile phases correctly
For isocratic separations with premixed mobile phases, solvent volumetric contractions in commonly used mixtures (water/acetonitrile, methanol, or tetrahydrofuran) should be considered during their preparation. The only correct way to prepare such mobile-phase mixtures is to separately measure the volume of each of the components and mix them. For example, to get a 70% organic mobile phase, 300 mL of water and 700 mL of organic solvent should be precisely measured separately and then combined in a flask. But, if only the water is measured precisely and the organic solvent is then added to make up the required final volume, due to the solvent mixture contraction, the resulting solvent strength will be a little higher (or weaker if the organic solvent was added first and water was added later).

In reversed-phase chromatography, HPLC methods with premixed mobile phases, such as 5% acetonitrile in water or 5% water in acetonitrile, are preferred. The rationale behind such preference is to increase degassing effectiveness, avoid mixture heating (for example, methanol in water) or cooling (for example, acetonitrile in water) upon mixing, and improve mixing efficiency by making the two mobile phases more similar in viscosity and surface tension. Typically, it is recommended to use mobile-phase solvents directly out of their delivery containers to prevent additional chances of contamination.

Use the right filter
Filtering removes particles from the prepared mobile phase, preventing clogging of the system and column, and this typically contributes to the degassing of the mobile phase. Recommended are membrane-type filters with pore sizes of at least 0.45 µm for HPLC systems, and 0.22 µm for UHPLC systems. Make sure that the membrane is chemically compatible with the mobile phase, and that it does not have extractables that would show up as unknown peaks in the chromatogram.

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Critical Evaluation of Chromatography Methods: Essential Detective Skills

Tony Taylor

I get excited when there is a new chromatographic method to implement or transfer into the laboratory, and don’t understand why everyone doesn’t feel the same way—perhaps it’s the challenges these processes often entail. Here, I explore how the challenges of new methods can be made easier, and more enjoyable, by taking time for some detective work prior to entering the laboratory.

A starting point of our detective work is to do a thorough reading of the method to gain a sense of whether it stacks up against our previous experience. I’m going to start with a high performance liquid chromatography (HPLC) method I recently came across that hides a surprising number of potential problems:

- Sample diluent: dilute sulfuric acid (approximately pH 2.8)/methanol (50:50)
- Column: endcapped bridged ethylene hybrid ODS (1.7 μm)
- Buffer solution: 1.36 g of potassium dihydrogen phosphate R in 900 mL HPLC-grade water.Add 0.15 g sodium heptanesulfonate R, adjust to pH 7.0 with triethylamine R, and dilute to 1000 mL with HPLC-grade water.
- Mobile phase A: buffer solution/methanol R (90:10 v/v)
- Mobile phase B: buffer solution/methanol R (15:85 v/v)
- Gradient: Illustrated below in Table I

### TABLE I: Gradient

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase A (% v/v)</th>
<th>Mobile Phase B (% v/v)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
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</tr>
<tr>
<td>11</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

- Detection: spectrophotometer at 254 nm
- Analytes: organic compounds with basic functional groups ranging from pKa 7.25 to 10.0

The mobile phase constituents are certainly not what one could call “modern.” Potassium dihydrogen phosphate is a traditional buffer used to stabilize the mobile phase (pH 7.0) when it mixes with the sample diluent (pH 2.8). With a pKa of 7.21, close to the desired pH, it appears to be an excellent choice. However, because at least one of the analytes has a pKa close to 7.0, we should expect changes in retention unless the pH is accurately controlled. It would be better to quote the exact weight of triethylamine required for a more robust pH.

Triethylamine acts as a masking reagent for acidic silanol groups that were not neutralized during column endcapping. Left untreated, these groups cause peak tailing on basic analytes. Unfortunately, triethylamine is volatile and will be lost over time, causing unpredictable drifts in retention. Furthermore, because it inherently alters the stationary phase, extended flushing is needed to re-equilibrate between injections. One must also consider the neutralization of sodium heptanesulfonate, the ion pairing reagent, by triethylamine.

Ion pairing reagents have two modes of action in reversed-phase chromatography. In the mobile phase, these anions form neutral complexes with basic analytes, increasing their retention. Additionally, they bond with the stationary phase, contributing anionic groups (R-SO₃⁻) to the surface. These groups retain basic analytes by electrostatic attraction, but also interact with additives such as triethylamine.

Let’s now look at the column. Bridged ethylene hybrid technology was introduced by Waters around 2004 to improve peak shape, ruggedness under ultra-HPLC (UHPLC) conditions, and resistance to high pH. Acidic silanols were prevalent in early HPLC columns, where “Type A” silicas that were used as stationary phase substrates contained metal impurities known to activate silanol groups. Metals were practically removed with the introduction of “Type B” silica, and novel endcapping treatments were developed to further reduce unwanted silanol groups. Bridged ethylene phases are also known to reduce surface silanols. These innovations have effectively negated the need for triethylamine in modern mobile phases.

But why is there an ion pairing reagent in this separation? Because our column can withstand high pH, we must question whether adjusting the pH to 10.5 or 11 would not neutralize most analytes and enhance their retention. Alternatively, stationary phases designed to retain polar (or even ionized) analytes are available. Routine use of ion pairing reagents has not been necessary for years.

Our column contains 1.7 μm particles, designed for highly efficient UHPLC systems. Phosphate buffers should be avoided in these machines, as crystallization quickly leads to overpressure and blockages. I would recommend prefiltering the eluents, keeping in mind that triethylamine may partially evaporate in the filtration apparatus.

Fixed wavelength detection at 254 nm can be problematic when mobile phases change so dramatically under gradient. Without a reference wavelength to account for changes in refractive index, steep gradients often produce wandering baselines.

Some detective work before donning our lab coats can save us trouble: “exotic” additives should ring alarm bells, as simpler methods are often possible with modern columns. Use a reference wavelength if your system allows, always investigate analyte logD and pKa when selecting buffers, and pre-empt solubility problems under gradient conditions.

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