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The Column

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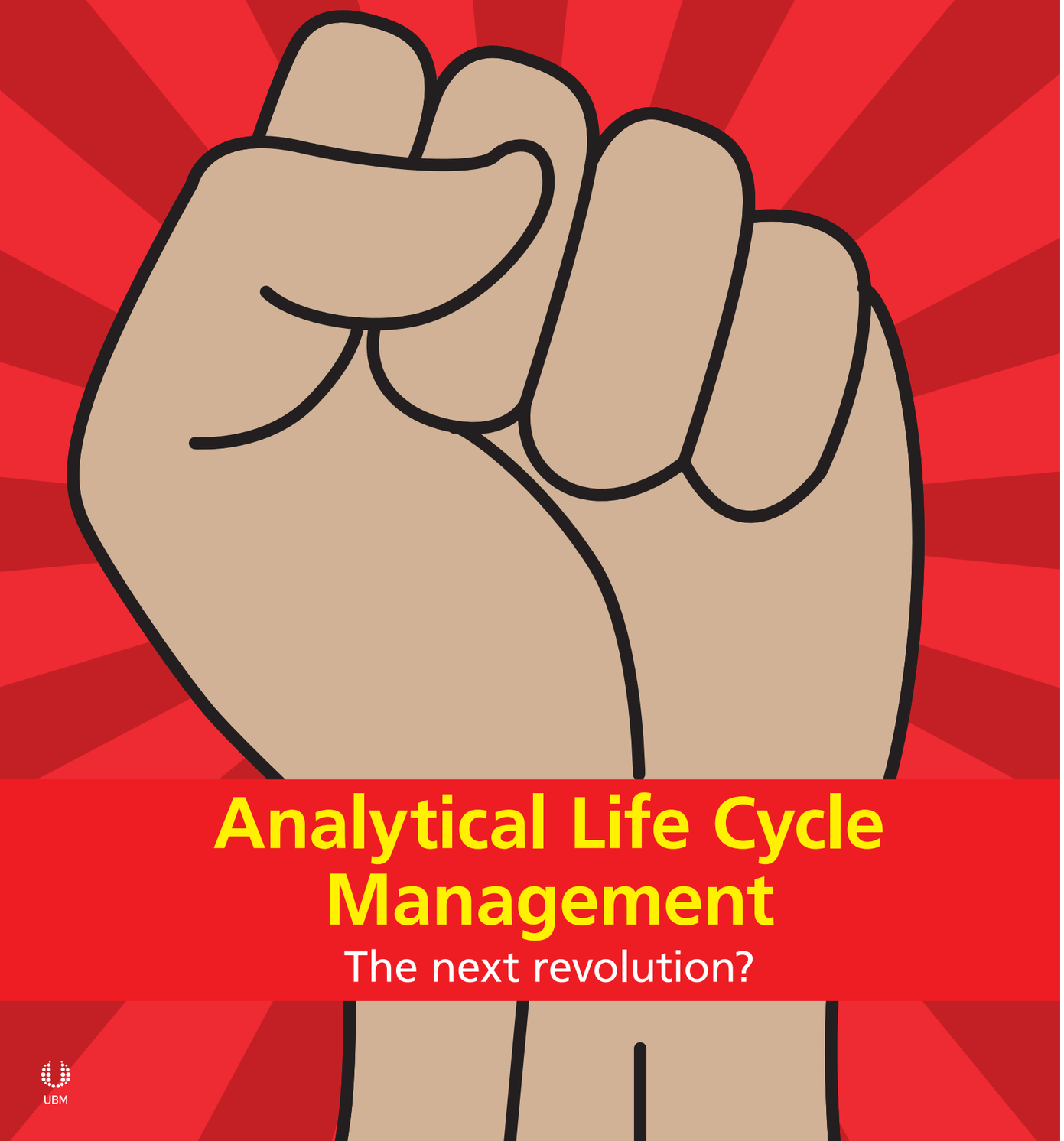

Analytical Life Cycle Management

The next revolution?

UBM

Cover Story

Features

Regulars

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Analytical Life Cycle Management— The Coming Revolution

Incognito looks to a paradigm shift.

A storm of three letter acronyms (TLAs) is on the way, which is set to radically update the way in which analytical methods are developed, validated, and verified, and the pharmacopeial bodies and regulators are at the heart of the storm.

Those of you working in the pharmaceutical industry may already be aware of some of these proposed changes, however those who do not, but whose work may ultimately be influenced by the new ways of working, should take note; the momentum is gathering and the changes herald a new dawn in analytical measurement for product quality control and beyond.

Some of the TLAs that represent the new approach include:

ACS (Analytical Control Strategy): The ACS is a planned set of controls, derived from an understanding of the requirements for fitness for purpose of the reportable value, an understanding of the analytical procedure as a process, and the management of risk, all of which ensure the performance of the procedure and the quality of the reportable value, in alignment with the ATP, on an ongoing basis (1,2).

ATP (Analytical Target Profile): The ATP states the required quality of the results produced by a procedure in terms of the acceptable error in the measurement; in other words, it states the allowable target measurement uncertainty (TMU) associated with the reportable value. Because the ATP describes the quality attributes of the reportable value, it is applied during the procedure life cycle and connects all of its stages (3).

TMU (Target Measurement Uncertainty): *TMU* is a more comprehensive term than the traditional term *precision* to represent random errors, and *bias* is a term traditionally used to represent systematic errors or accuracy. These terms (*uncertainty* and *bias*), when examined holistically, can be considered to represent the TMU associated with the reportable value generated by the procedure (2).

AQbD (OK, there are also some FLAs!) (Analytical Quality by Design): Quality by design (QbD) is a systematic approach to development that begins with predefined

objectives and emphasizes understanding and control, based on sound science and quality risk management (3). QbD principles when applied to the development of analytical methods are known as analytical QbD (AQbD). The outcome of AQbD is a well characterized method that is fit for purpose, robust, and will consistently deliver the intended performance throughout its life cycle.

FEMA (Failure Effect Modes Analysis): A step-by-step approach for identifying all possible failures in a process (here the total analytical process). Failures are prioritized according to how serious their consequences are, how frequently they occur, and how easily they can be detected. The purpose of the FEMA is to take action to eliminate or reduce failures, starting with the highest priority factors, and to document current knowledge and actions regarding mitigating the risk of failure for the purpose of continuous improvement.

DoE (Design of Experiments): Sometimes also called *experimental design*, DoE is the design of any task that aims to describe or explain the variation of information under conditions that are hypothesized to reflect the variation. Using an optimum number of variable combinations (often set as high, medium, low combinations), the primary effects and secondary interactions of variables may be efficiently investigated and described. This approach is more time efficient and more powerful than the one factor at a time (OFAT) approach and typically uses analysis of variance (ANOVA) statistical analysis to interpret the results of the DoE and highlight the variables and combinations of variables that have the largest effect on uncertainty.

MODR (Method Operable Design Region): A multidimensional space derived from AQbD investigations, which defines those combinations of experimental variables that produce a valid measurement as defined by the ATP.

There are also a number of stimuli articles and pharmacopeial documents that you will need to become familiar with:

- *ICH Q9, Quality Risk Management* (November 2005) (4)
- *ICH Q12, Technical and Regulatory Considerations for Pharmaceutical Product Lifecycle Management* (Draft November 2017, currently out to industry for comments) (5)
- *USP General Chapter <1210> "Statistical Tools for Procedure Validation"* (6)

Stimuli articles in pharmacopeial forum:

- *Stimuli Article: Analytical Control Strategy* (1)
- *Stimuli Article: Analytical Target Profile, Structure and Application Throughout the Analytical Lifecycle* (3)
- *Proposed New USP General Chapter <1220> "The Analytical Procedure Lifecycle"* (2)

There are several other relevant documents to be considered, however they are all referenced in the articles or guidance documents cited above.

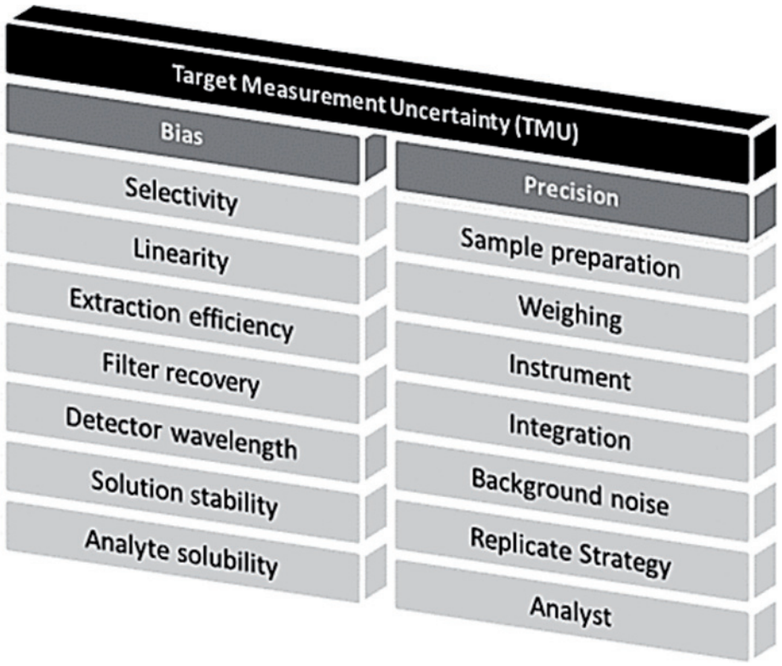
The bottom line here is to adopt a risk management approach to analytical methods so that the fitness for purpose of a reportable value and the performance of the analytical procedure are assured on an ongoing basis. By first defining the required performance of the analytical method in its ultimate intended use (that is, assay of drug products for potency prior to release), QbD principles are applied to analytical method development, validation, and ongoing verification to ensure better method performance and control. The ability of the analytical method to deliver a fit for purpose result according to a predefined specification drives every stage of the analytical method development, validation, and ongoing performance verification.

Is your brain hurting already? Well, as this is an opinions column, let's start with my opinion on the proposed paradigm shift and what impact the changes will have on the quality of the information that we produce. For that, I first refer you back to two of my previous articles (see references 7 and 8).

Well, in a nutshell, someone must have been listening, because essentially the principles of QbD are going to be used to help ensure that analytical measurements are made to within a specified level of measurement of uncertainty throughout the lifetime of the method. In short, I'm very much in favour of this new paradigm. What I'm sure many of us will be more daunted about is the extra work involved in understanding the guidance and regulations, adopting new ways of working, and acquiring the knowledge and skills required to comply.

It's taken me a long time to become familiar with the principles of the ACS and the documents which outline how we should develop and define ATP, as well as reading all of the stimuli articles and ICH regulations that interlock to define the new approaches. Add to this the ability to produce FEMA or Ishikawa analyses, use these to inform the statistical DoE and ANOVA, which will assess the risk in my analysis, and then use QbD principles to define ranges of key analytical variables, which must be controlled in order to produce data to comply with the ATP.

Figure 1: Precision and bias factors which are consolidated within the Target Measurement Uncertainty (TMU). Adapted with permission from reference 3. © 2016 The U.S. Pharmacopeial Convention (USP).

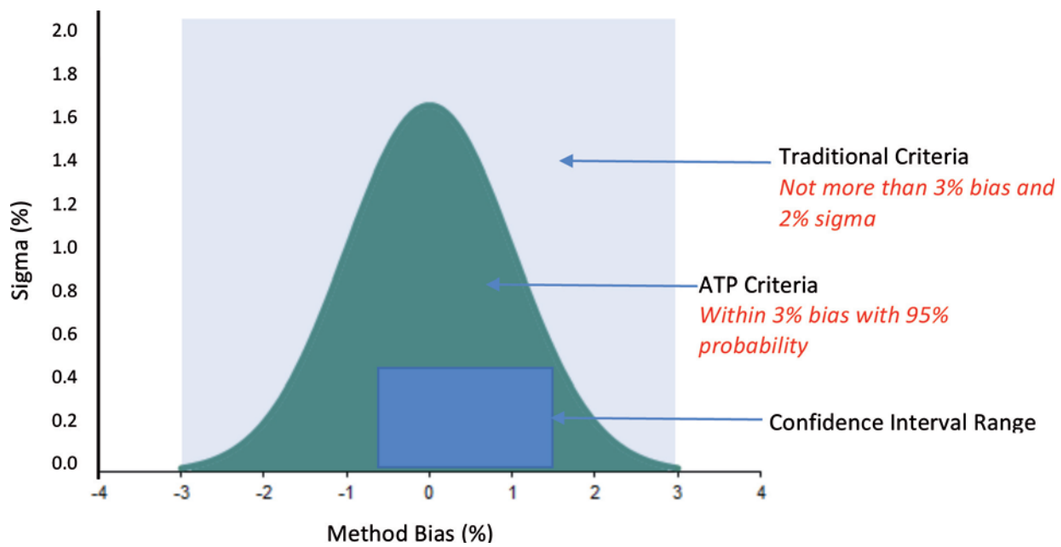


A single Incognito column isn't long enough to discuss everything that we need to consider and implement in order to produce an analytical method which follows an ACS or analytical life cycle management approach. What follows are some brief notes and comments from my own (albeit brief) experience, in the hope that they might help you to focus on the important challenges that may lie ahead.

Understanding the Concept and Defining the ATP

The concept of the ATP lies at the heart of the ACS. QbD principles are used to define the performance of the analytical determination in terms of the acceptable error in the measurement and consider all aspects of the TMU. The TMU encompasses the precision and accuracy (bias) factors that are considered

Figure 2: Graphical representation of method performance as defined by traditional and ATP approaches.



during analytical method validation (Figure 1).

An example of an ATP may look something like this: An analytical procedure was developed to determine drug substance (Y) in film coated tablets containing [major excipients or other significant ingredients] in the range from 80% to 120% of the specification value. The reported results should fall within ± 3% of the true value level at the 95% level of confidence.

One should note here that the required performance contains limits for both accuracy and precision of the analytical measurement and the required performance under these new constraints will avoid the "acceptance" of results that show both high bias and low precision (which is possible using traditional approaches to the assessment of analytical performance).

In Figure 2 the light blue box area represents any combination of bias and precision estimates allowed under typical method validation criteria. The shaded area beneath the normal distribution curve shows the combinations of bias and precision allowed under the ATP described above.

Whilst it is possible to estimate method accuracy and precision using a few limited measurements as is typical under current method validation guidelines, it is also possible to estimate the confidence interval of the measurement of both accuracy and precision such that the estimate of uncertainty is known. Statistical distributions (such as the t-distribution or chi squared [χ^2] distribution) can be used to estimate the confidence interval (I have used the 95% level of confidence above) from experimental data to define a range of either accuracy or precision that will be 95% certain to contain the true accuracy or precision of the procedure. The area defined by the combined intervals for both accuracy and precision will contain a defined percentage of "true values". If the 95% level of confidence is used to generate both intervals, around 90% ($0.95^2 = 0.9025$) of all measurements in this area will contain the true value of the accuracy and precision of the determination. Hence this type of exercise can be used to derive the ATP statement according to the acceptable limits for the type of measurement being made. Figure 2 also shows a confidence interval range for a procedure with accuracy confidence interval -0.5 to +1.5% and precision 0.0–0.5%.

One would need a reasonable grasp of basic statistics in order to determine the confidence intervals using the appropriate distribution; the statistical power of the model will increase with the number of degrees of freedom, that is, the number of results generated using the procedure that are used to determine the confidence intervals. Often the ATP will be generated at the end of the development process and is defined further below.

As with method validation, it is often the case that the ATP criteria may be further verified or amended after the following steps have been implemented and evaluated.

Initial Screening Studies

For the sake of brevity, I've assumed that we have decided to use a chromatographic technique for the determination; however, the initial stage in any QbD-based strategy would need to include an assessment of the desired method performance alongside the analyte and matrix properties to evaluate the most appropriate analytical technique. The need to consider the desired analytical performance as the fundamental driver for analytical development is critical to the QbD approach.

Initial screening, in which several combinations of columns, organic solvents, additives, eluent pH, and perhaps column temperature are screened against criteria such as number of peaks within the chromatogram and minimum resolution, may be familiar to many. The outputs may be visually assessed for suitability, chromatography optimization software may be used, or a statistical approach using a full factorial DoE with a restricted number of levels (variables) may be employed. If the separation requires a gradient, some initial experimentation to find the optimum gradient conditions may also be undertaken to arrive at a separation believed to have the basic characteristics that can be further developed into a fit for purpose analytical method.

The findings obtained during initial screening studies, especially when derived from DoE approaches, can be very useful in the primary risk assessment. Once again, a good working knowledge of applied statistics is necessary to conduct a DoE approach with ANOVA to interpret the results and highlight the important variables or combinations of variables from the initial experimentation.

Conducting a Primary Risk Assessment Using Factorial Analysis

The next stage in the process is to perform a factorial analysis of the method using cause and effect diagrams (Ishikawa or Fishbone diagrams) and to classify the relevant factors.

All factors within the high performance liquid chromatography (HPLC) analysis should be taken into account; this will cover sample preparation as well as the instrumental analysis. Each factor is considered (effect of pH, column packing variability, accuracy of volumetric eluent preparation), alongside the mode of failure that may result (irreproducible retention times, changes in selectivity) and the set point of the variable where know (eluent pH 2.8, 10 mM ammonium formate, 55% organic). An NCX code (Noise/Controllable/Experimental) is then assigned to each factor depending upon which of the factors may be mitigated through proper control (C = type of buffer salt used, for example), factors that are difficult to control and need measures to reduce their impact on measurement quality (N = pH adjustment accuracy, for example), and those that need to be investigated experimentally to assess their impact on the quality of the separation and therefore the quality of the data (X = column temperature, for example).

Secondary Risk Assessment, FEMA Analysis, and MODR Definition

All factors assigned as category X are then evaluated in a further screening experiment (DoE 2), perhaps based on success criteria such as a minimum resolution for any peak pair. This DoE can then be used to establish the method operable design region (MODR). This is a well-documented approach to the application of AQbD and essentially investigates the range in which the combination of values of each critical variable will result in a fit for purpose measurement according to the ATP. This produces a "control space" (the MODR) in which the ranges of each critical variable may be defined.

All of the factors from risk assessment 1, which are assigned as category N, are then considered using FEMA or a similar approach to identify all of the possible causes of failure of the analytical procedure. This process is informed by the DoE results from the secondary risk assessment and evaluates each factor in terms of severity (magnitude of the effect on the quality of the analysis), occurrence (how the failure might occur), and detectability (how easy it is to spot the potential problem should it occur). The "score" values of S, O, and D are well documented and are designed to produce clearly defined risk factors.

An example here may be the effect of the inter-batch variability of the column packing material:

- The S factor effect may be a change in peak efficiency or the selectivity of the separation. A score of 5 may be assigned.
- The O factor effect may be assigned as a failure of the manufacturer to implement proper batch control measures and may attract a score of 3.
- The D factor effect may be assigned a score of 4 (which is high) as without proper control the issue may remain undetected.

The risk priority number (RPN) of 60 is a result of all three scores being multiplied together. Once preventative or control measures are put in place within the analysis, the RPN score is then re-evaluated. Here, there is little the end user can do other than choose columns produced by reputable manufacturers, however, the detectability may be improved by implementing a system suitability test, which may reduce the detectability score to 1, resulting in an overall RPN number of 15. This new number is then evaluated against set criteria to define what controls should be in place to ensure fit for purpose results on an ongoing basis. Typical criteria may be:

- Low (RPN 1–35): sufficiently acceptable risk level; generally, a further reduction of the risk is not required.

- Medium (RPN 36–59): acceptable risk level; however, some measures to further reduce the risk are desirable.
- High (RPN 60 or more): unacceptable risk level; some measures to reduce the risk are required.

ATP Verification and Ongoing Control Strategies

The DoE 2 experiments may highlight critical variables or interactions between variables (such as an interdependence on method performance between mobile phase pH and gradient time [slope]), which may need to be further investigated using a final DoE experiment to modify the MODR. Furthermore, at this point several analyses might be undertaken to investigate the method bias and precision to ensure that the method performance can meet the criteria outlined in the ATP. If method performance cannot meet the required performance, the ATP criteria may need to be modified or further method improvements implemented. This may also involve altering the method control strategy, such as optimization of the bracketing interval for standards within the sequence to reduce the potential for bias, or setting tighter specification limits for the system suitability test specification.

At this point, a method validation according to ICH Q2 guidelines may also be undertaken to verify the method is fit for purpose in the more traditional sense, although the data generated in the previous experiment is likely to provide much of the necessary information.

The method performance will now be evaluated during its lifetime, and the use of control charts and other measures are required to indicate that the method performance is satisfactory over time and that trends in method performance are identified, understood, and controlled. This may be achieved by longer term monitoring of control sample results, resolution, relative standard deviation (RSD) of system precision data, routine sample results, quality control sample data, and performance data measured against the ATP specification (both within and out of specification data should be included in this analysis). If the method is found to contain critical variables not identified during development and validation, or is found to be inconsistent, the method or control strategy may need to be updated to meet the ATP specification.

These changes should be carried out under the auspices of a change control process, which evaluates the results of the change against the TMU defined in the ATP and determines that proper re-qualification of the impact of the change is undertaken. Risk assessment tools should be used in order to define the level of method-requalification necessary to establish that the changes will result in improved method performance.

Summary

It is clear that QbD principles used for the design and implementation of an ACS change the way in which we will approach method development, validation, and in-use performance monitoring in the future. Whilst current practice tends to focus on the verification of the analytical method "at that time", the new approach is more concerned with the quality of the data produced (as opposed to the performance of the analytical system) over time, and wherever the method is being used.

Whilst I see this as a very positive step forward, I'm also concerned for those of us who work in laboratories without access to statisticians, who are not used to "six sigma" type risk assessment and risk control paradigms, and who don't have highly automated HPLC systems capable of switching columns or eluents to automated, often complex series of experiments defined by DoE for ANOVA data analysis. These are big changes, a paradigm shift in fact, and anyone who is not aware of the requirements should use this introduction to start their journey.

The revolution is coming, it's a change for good, but there will be pain before we can all see the brand-new dawn.

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News

Sciex Donates to World Cancer Research Fund

Sciex (Framingham, Massachusetts, USA) has donated $17,500 to the World Cancer Research Fund (WCRF) to help fund urgently-needed research investigating the relationships between food, nutrition, and physical activity with cancer prognosis and outcome in cancer survivors.

A donation of $11,000 was made as the result of 98% participation in the annual Sciex employee engagement survey, where Sciex committed to donating to this cause based on the number of survey responses collected. An additional $6500 was donated by the Danaher Foundation, the parent company of Sciex.

"Sciex is very proud to support organizations that promote healthcare research and discovery, community engagement, and efforts towards innovative approaches to improve quality of life around the world," said Inese Lowenstein, President of Sciex

Gerard Cousins, Director of Fundraising for WCRF UK, commented: "WCRF is proud to have been working with Sciex, an organization that shares our aim of empowering people to make healthier lifestyle choices, since 2015. Sciex fundraising has helped to fund much-needed research into cancer prevention, which has been reflected in our latest report, Diet, Nutrition, Physical Activity and Cancer: a Global Perspective, which was launched in May."

For more information, please visit **www.sciex.com**

Forensic Profiling of Human Odour Using GC×GC–MS

Researchers from ESPCI Paris and the Institut de Recherche Criminelle de la Gendarmerie Nationale have developed and optimized a comprehensive two-dimensional gas chromatography–mass spectrometry (GC–MS) method for the forensic profiling of human hand odour (1).

Like fingerprints, human odour is said to be specific to each individual, consisting of hundreds of molecules, and has long played a role in crime investigation through the highly trained and unscrupulous nose of police dogs. However, during court proceedings more conclusive evidence is required and thus the development of an objective analytical strategy to characterize human odour is needed to complement existing canine capabilities.

The complexity of human odour is one of the major challenges to the development of such a technique, with comprehensive reviews of human-originating volatiles finding a wide variety of chemical compounds, including: acids, alcohols, aldehydes, esters, hydrocarbons, ketones, heterocyclic compounds, and sulphur-containing compounds (2,3).

Researchers reasoned that GC–MS would be the most appropriate method because of the nature of the molecules involved, but based upon the sample complexity and the peak capacity limitations of classic one-dimensional GC–MS it was determined that GC×GC–MS would be better suited. In order to optimize the method, 27 different analytical setups were evaluated based upon the criteria of column set, gradient, and a modulation time.

Results indicated that based upon the analytical criteria, including six complementary orthogonality criteria, the developed method was appropriate for the analysis of human hand odour.

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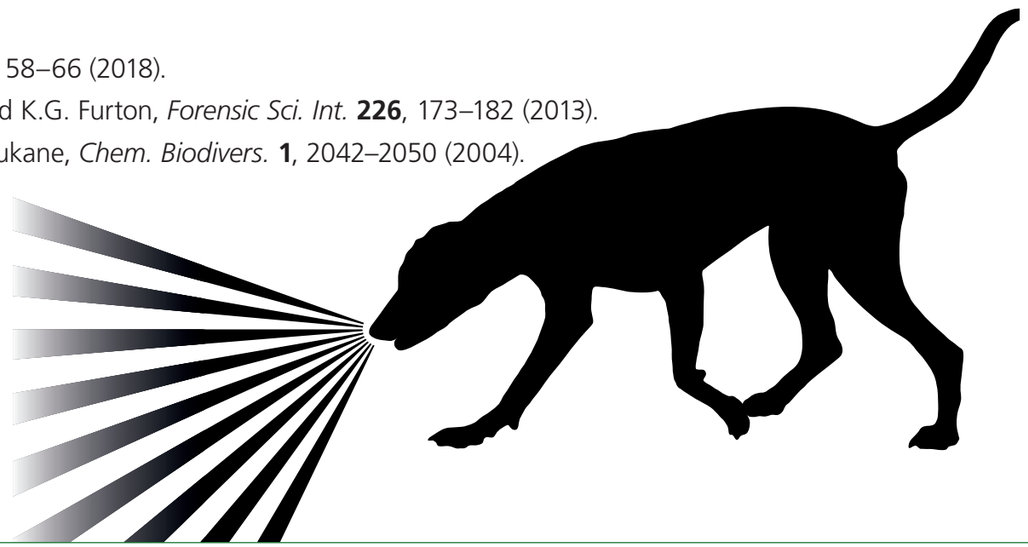

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New Teaching and Research Centre for Separation Science is Launched

Agilent Technologies (Santa Clara, California, USA) and the University of Duisburg-Essen (Germany) will collaborate to combine the company's analytical technologies with the university's researchers. As part of the collaboration, the company will support the university with a broad range of instruments to equip the new Teaching and Research Center for Separation (TRC).

The focus of the TRC will be teaching students, industry employees, technicians, managers, graduates, and postdocs about separation science, and training them in the use of modern analytical equipment. The TRC will support an extensive cross section of research activities in biomedicine, nanotechnology, and other life science specialties.

The company has developed a global network of world-class Centers of Excellence; the University of Duisburg-Essen is the fifth university to join this network.

For more information about the TRC, please visit: **www.trc-separation.com/home-en**

Biodiesel-Diesel Blend Analysis Using UFGC

Researchers from the College of the Holy Cross (Worcester, Massachusetts, USA) have developed an ultrafast gas chromatography (UFGC) and chemometric method for the analysis of biodiesel blends (1).

The reduction of greenhouse gas emissions is an internationally important topic with countries looking for methods to reduce emissions without significantly affecting the daily life of millions. One method to emerge has been the addition of biodiesel to petroleum diesel fuel. Added to diesel fuel to intentionally decrease greenhouse gas emissions, studies have indicated that the use of 100% biodiesel (B100) results in 74% fewer emissions when compared to petroleum diesel (2). Similar reductions are also seen in blended diesels where even a small addition of 20% biodiesel (B20) can decrease hydrocarbon emissions by around 20% and carbon monoxide emissions by around 13% (2). With countries looking to incentivize biodiesel addition, the accurate blending of diesel and biodiesel becomes vital as regulatory requirements must be met. Furthermore, retail outlets may want to change the blending percentage during different periods to maximize profits or reductions. Thus, the analysis of these fuels has become and will continue to become increasingly important.

Fuel adulteration is another major concern surrounding biodiesel-diesel blends because the addition of motor oil or vegetable oil to diesel can allow unscrupulous sellers to increase profit margins while impacting on consumers, with potential negative impacts on engine performance (3).

Currently, diesel fuel is analyzed using the UFGC method D7798, while biodiesel analysis is conventionally performed using spectroscopy or GC (4,5). Containing both the saturated hydrocarbons, aromatic hydrocarbons, and unsaturated hydrocarbons of diesel and the variety of fatty acid methyl esters (FAMEs) of biodiesel, biodiesel-diesel blends present a unique analysis challenge. This is amplified by the variety of biodiesel composition, which can vary based upon the feedstock used in production.

In this study researchers used the D7798 UFGC method, traditionally used for diesel, along with chemometric methods for the analysis of biodiesel-diesel blends.

Researchers reported that the method was successful for the analysis of biodiesel-diesel blends. If the focus was on common plant and tallow sources of biodiesel, the UFGC method required only a run time of 2.5–3 min with a cycle time of under 5 min. The study showed that a method currently used by many sectors for the analysis of diesel can be utilized for the analysis of biodiesel-diesel blends. With concerns about adulteration, the method also provides authorities with a quick and trusted technique to apply to get accurate results quickly.

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Peaks of the Week

- **The *LCGC* Blog: Optimizing Sensitivity in Splitless Capillary GC with FID Detection**—It is often possible to achieve better sensitivity and lower limits of detection and quantitation using standard gas chromatography (GC) equipment—here I'm referring to a standard split/splitless injection port and a flame ionization detector (FID). Paying attention to some of the fundamental variables as well as some of the more esoteric considerations can lead to much improved method performance. **Read Here>>**

- **Antibody–Drug Conjugates: Perspectives and Characterization**—This instalment of "Perspectives in Modern HPLC" provides an overview of antibody–drug conjugates (ADCs) as a new class of biotherapeutics and describes their analytical characterization for quality assessment with examples from extensive applications libraries. **Read Here>>**

- **Split, Splitless, and Beyond—Getting the Most From Your Inlet**—While capillary gas chromatography has been undergoing a renaissance, with new columns, detectors, data systems, and multidimensional separations, the classical inlets have remained the same: We are still injecting liquid samples with syringes into split and splitless inlets, as we have for nearly 50 years. **Read Here>>**

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- **Injecting Water onto a GC Column: Solving the Mystery of Poor Chromatography**—This article describes a robust approach to analyze glycols in aqueous samples, which reduces downtime and maintains sensitivity. **Read Here>>**

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- **Generic GC–FID for Volatile Amine Quantitation in Pharma**—Researchers have developed a simple and fast generic gas chromatography–flame ionization detection (GC–FID) method for the quantitation of volatile amines in pharmaceutical drugs and synthetic intermediates. **Read Here>>**

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News In Brief

Researchers from Guangzhou Center for Disease Control, Guangzhou, China, have developed an ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method for the simultaneous determination of five glycopeptide antibiotics in food and biological samples. The clinical abuse and passive exposure to antibiotics has led to the rise of antibiotic resistance with governments and health organizations looking for ways to combat it. The reduction of passive exposure to antibiotics and their residues from drinking water and food is one such method. This method uses SPE-based UHPLC–MS/MS to monitor given glycopeptide antibiotics in food and biological samples with an analysis time of 6 min.
DOI: 10.1016/j.chroma.2018.01.036

A paper published in the *Journal of Chromatography A* has detailed the effect of salts on retention in hydrophilic interaction chromatography (HILIC). The research studied the effect of the anion with four triethylammonium salts across varying pH values and concentrations. The effect of the cation was examined by comparing retention of a tryptic peptide containing either phosphoserine or aspartic acid at the same position. Results demonstrated that counterions that are well hydrated serve to promote partitioning of charged solutes into the immobilized aqueous layer in HILIC, while poorly hydrated counterions have the opposite effect.
DOI: 10.1016/j.chroma.2018.01.038

Optimizing Splitless GC Injections

Linx Waclaski, Restek Corporation, Bellefonte, Pennsylvania, USA

Splitless injections are sometimes necessary for trace analyses, where the analyst hopes to recover 100% of the analytes that are injected. Unfortunately, splitless injections can be challenging and using an imperfect method can lead to loss of analytes and poor peak shapes. The choice of inlet liner can have an impact on the data and one must consider the effects of geometry, packing, deactivation, and volume on introduction of analytes into the system. Other important inlet parameters to consider include inlet temperature, splitless hold time, and initial oven temperature.

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Split and splitless injections are two very common techniques used for the introduction of samples into a gas chromatograph. Split injections generally provide superior chromatography because of the rapid transfer of the sample through the inlet onto the column, leading to sharp peaks with little time for adverse interactions to occur within the inlet. The drawback of split injections is that the majority of the sample is vented and lost, making it sometimes necessary to use a splitless injection when performing trace analyses.

During a splitless injection, the split vent is closed, with the total inlet flow essentially being equal to the column flow. Because capillary column flows are typically slow in relation to the total inlet volume, it can be difficult to transfer the sample to the column in a tight band, leading to broadened or poor peak shapes. The longer residence time of the sample in the hot inlet can also lead to adverse interactions, such as adsorption and chemical reactivity. Optimizing splitless injections requires careful selection of an inlet liner, as well as using an appropriate inlet temperature, splitless hold time, initial oven temperature, and solvent.

Liner Selection

Geometry and Packing: Glass inlet liners serve as a chamber in which a liquid sample is vapourized and transferred to the column. The shape of a liner, as well as the presence or absence of packing material, can affect its heat capacity and flow dynamics, ultimately affecting both the responses of compounds, as well as the injection to injection repeatability (1).

Figure 1 shows a comparison of hydrocarbon peak area responses for liners commonly used with splitless injections. The single taper liner containing glass wool, as well as the double taper cyclo liner, which features

Figure 1: Splitless liner configuration comparison for hydrocarbons ranging from C_8 to C_{40} at 5 ng. n = 5 liners per configuration with five replicate injections per liner. Inlet temperature: 280 °C; splitless hold time: 1 min; column flow: 1.5 mL/min; instrument: Agilent 7890 with split–splitless inlet and FID detector.

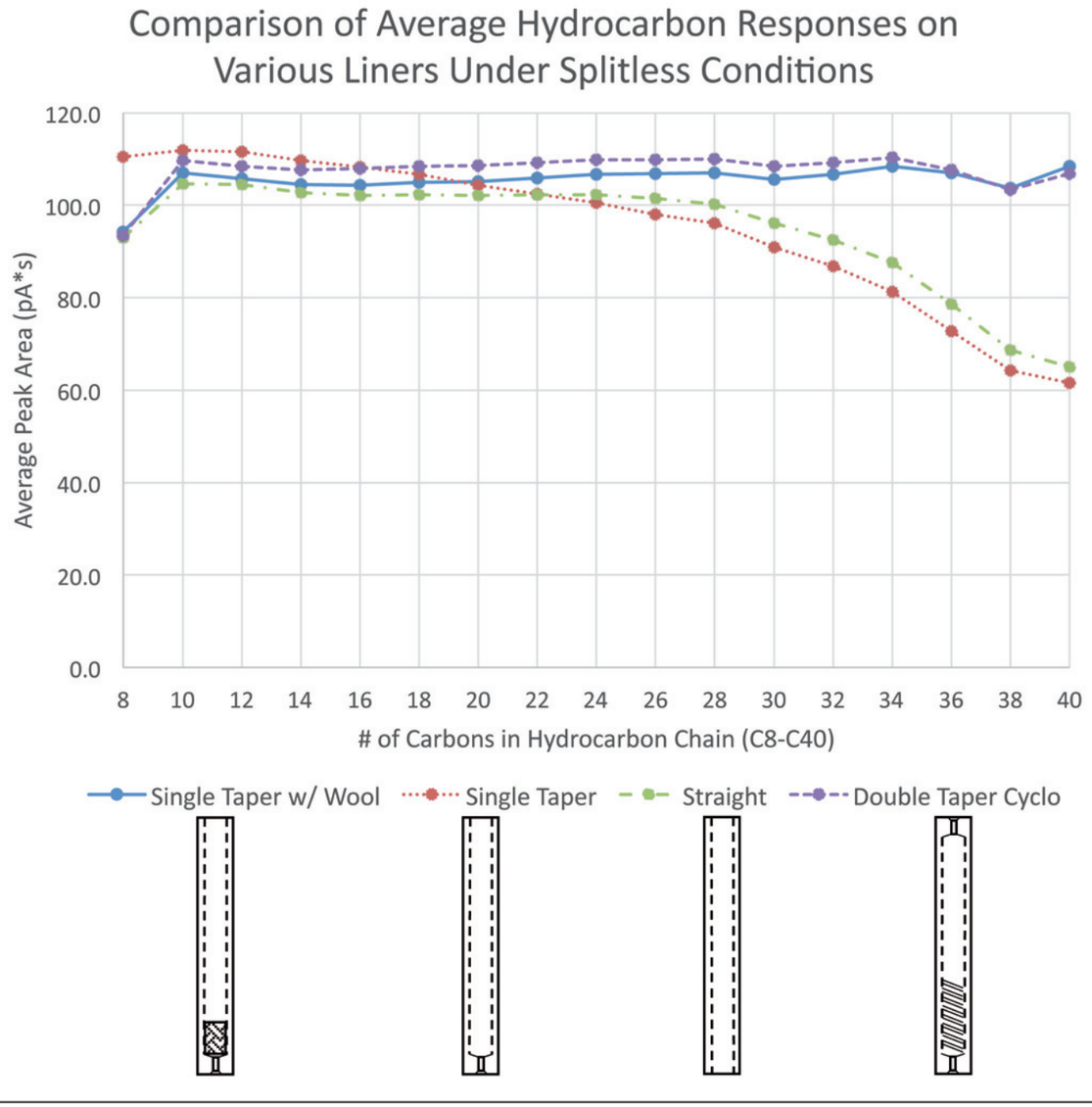
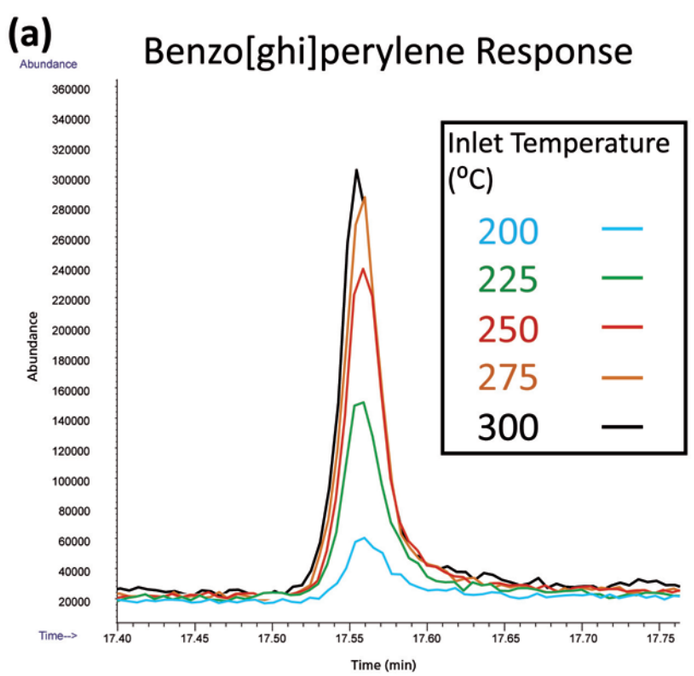


Figure 2: Effect of inlet temperature on analyte performance using a single taper liner with wool. (a) Benzo[ghi]perylene response increases with inlet temperature. (b) Endrin, an "active" pesticide, shows increased degradation as inlet temperature increases.



(b) Endrin % Degradation

Inlet Temperature (°C)	Endrin % Degradation
200	3.6%
225	4.3%
250	5.8%
275	8.8%
300	10.3%

a corkscrew obstruction, demonstrated the best overall responses across all hydrocarbons ranging from C_8 up to C_{40}. Both the wool and the corkscrew provide additional surface area, which increases the heat capacity of the liner and allows for better vapourization of analytes with high boiling points. Wool and the corkscrew can also help to increase reproducibility by enhancing mixing with the carrier gas. In addition to these benefits, wool can protect the column by trapping relatively nonvolatile matrix material as well as particles and pieces of septa.

In contrast, the empty straight liner and single taper liner show increasingly poor vapourization potential as the analyte boiling points increase. This phenomenon is known as *molecular weight discrimination*. If analyzing analytes with relatively low boiling points, using wool or some type of obstruction may not be necessary for complete vapourization; however, column maintenance intervals may be more frequent, with shorter overall lifetime, because of the lack of protection from nonvolatile matrix components.

A bottom taper is recommended when performing splitless injections because it helps to direct the sample to the column, while also minimizing contact with the seal. The inlet seal is often cooler and contact with it can condense high molecular weight analytes, leading to losses.

Inertness: Liners are usually constructed from borosilicate glass, which contains active sites such as silanols, as well as metallic impurities. These active sites may interact with sensitive analytes, leading to adsorptive loss, as well as chemical reactivity. Adverse interactions are far more likely to occur with splitless injections because inlet flows are slow and analyte residence times are relatively long. A surface treatment, or deactivation, is needed to cover active sites and produce an inert surface with which analyte integrity will remain.

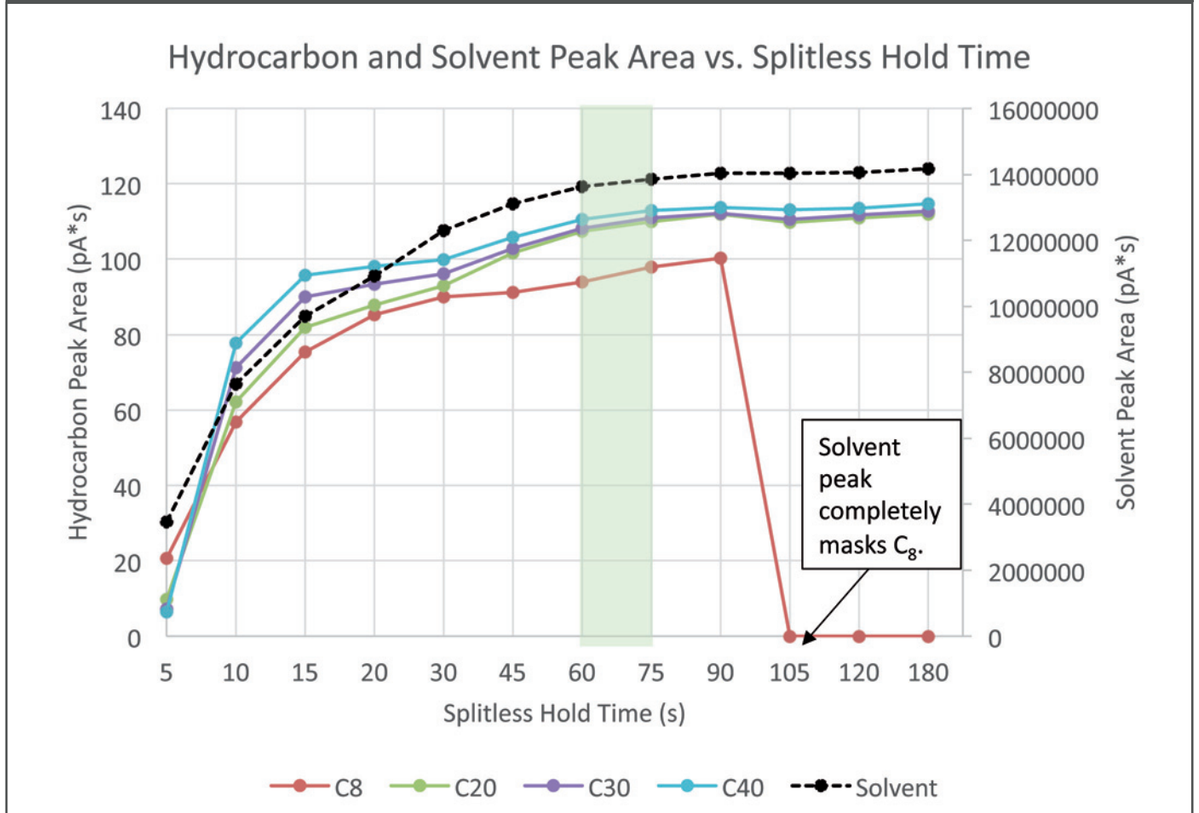
Deactivation typically involves the silanization of the liner surface and can be performed with either liquid or gas phase reagents. Dimethyldichlorosilane (DMDCS), a commonly used glassware deactivation reagent, is used as a liquid deactivant and the procedure is simple enough that most laboratories can perform it in-house. Many instrument and consumables manufacturers use a chemical vapour deposition of proprietary deactivation reagents instead because superior inertness is often achieved. Deactivation quality can vary depending on the process used, and will affect responses of active analytes, so it's important to seek out a deactivation that works for your particular analytes.

The benefits of using wool were discussed earlier; however, the high surface area of wool can also make it difficult to thoroughly deactivate, potentially exposing analytes to active sites. Both borosilicate and quartz wool are used in liners, with quartz offering superior inertness because it contains less impurities. There are liners pre-packed with quartz wool that has been deactivated, allowing for the use of wool, even with active analytes. Nonetheless, in some cases it may be necessary to select a liner without wool.

Using a liner with a bottom taper is beneficial for splitless injections because it helps to lessen or prevent contact with the bottom metal seal of the inlet, where analytes can also react.

Volume: Upon injection of a liquid sample into a hot inlet liner, solvents will greatly expand as they are transferred to a gas. If the solvent expands beyond the confines of the liner, gas lines can potentially become contaminated and reproducibility

Figure 3: Peak area response for select hydrocarbons and solvent vs. splitless hold time. After approximately 60–75 s, there is negligible growth in analyte area by continuing to extend hold time. Eventually C_8 is no longer separated from the solvent. Inlet temperature: 280 °C; flow: 1.5 mL/min; liner volume: ~1 mL.



may suffer. This phenomenon is referred to as *backflash*. The expansion volume will depend on the solvent, inlet temperature, and inlet pressure. Using a liner with a larger inner diameter (3–5 mm) is usually preferred for splitless injections because it allows the most expansion volume for the solvent.

If using a solvent with a low expansion coefficient, it may be possible to use a liner with a narrower inner diameter. The advantage of this is faster transfer of analytes to the column, which helps to prevent band broadening and allows less time for adverse interactions to occur within the liner.

Inlet and Oven Parameters

Inlet Temperature: Inlet temperature can have a profound impact on analyte response because higher temperatures provide increased thermal energy for transferring semivolatile compounds to a gaseous state. Unfortunately, high inlet temperatures can also cause increased degradation of thermolabile compounds. It is often necessary to find an inlet temperature that provides a good balance of response for high boilers as well as minimal degradation of sensitive compounds.

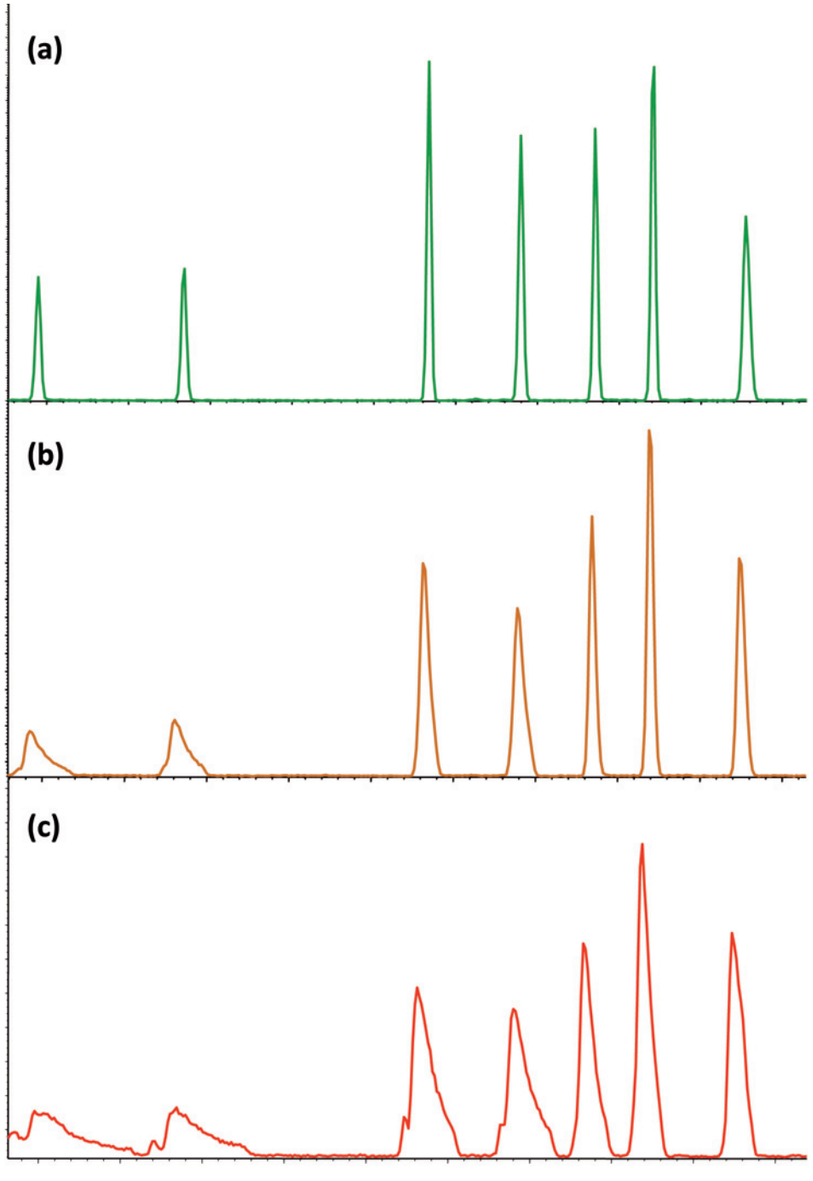
Figure 2(a) shows the response of benzo[ghi] perylene, a polycyclic aromatic hydrocarbon (PAH) with a boiling point of 550 °C, at various inlet temperatures ranging from 200 °C to 300 °C. Note that the gains in peak area become increasingly less significant as the temperature continues to increase beyond 250 °C. On the other hand, endrin, a thermolabile chlorinated pesticide, shows greater chemical reactivity at higher inlet temperatures, leading to increased formation of its degradation products, endrin aldehyde and endrin ketone (Figure 2[b]).

Splitless Hold Time: During the splitless hold time, all inlet flow is directed to the column, as analytes evaporate within the inlet and transfer to the column. This will result in a large amount of solvent being transferred to the column relative to the analytes. If too much solvent is loaded onto the column, it can potentially interfere with early-eluting analytes or lead to an elevated baseline; therefore, at a predetermined time, the split vent must be opened, allowing excess solvent to exit the inlet. On the other hand, if the splitless hold time is too short, there may be insufficient vapourization and transfer of analytes.

Figure 3 demonstrates the effect of splitless hold time on analyte and solvent response. Allowing a 1.5 to 2 times sweep of the total inlet liner volume with carrier gas before opening the split vent should provide time for most analytes to achieve sufficient evaporation, assuming all other parameters are also optimized. A shorter hold time may be needed if the solvent peak interferes with the most volatile compounds.

Initial Oven Temperature: The low flow rates inherent to splitless injections mean that complete transfer of the analytes to the column can be slow, leading to diffusion, or band broadening, of analytes. This ultimately leads to wider peaks, affecting quantitation and detection limits. To counteract this unwanted effect, it is necessary to condense the analytes at the head of the analytical column. This is achieved by using a low initial oven temperature.

Figure 4: Effect of initial oven temperature on peak shape for splitless injections. (a) Ideal starting oven temperature; peaks sharp and narrow. (b) Initial oven temperature slightly too high; early eluting peaks show poor focusing. (c) Initial oven temperature too high; all peaks show signs of poor focusing.



Starting at an oven temperature below the boiling point of the solvent will condense the solvent at the head of the column, which will then trap analytes ("solvent effect"). This will yield a narrow starting band from which the chromatographic separation can begin. If the analytes of interest have a substantially higher boiling point than the solvent (>200 °C), an alternative method is to set the starting oven temperature below the boiling point of the earliest eluting analyte. This will ensure analytes condense at the head of the column in a tight band, even if the solvent does not. Figure 4 demonstrates the effect of initial oven temperature on peak shape.

Solvent Polarity: Matching the solvent polarity to the column polarity is important for good peak shape. The general chemistry rule of "like dissolves like" applies here, as injecting a polar solvent like methanol onto a nonpolar column such as a 1-type (dimethyl polysiloxane) will result in the solvent beading up rather than dissolving into the column's phase. This can lead to split and deformed peak shapes with low reproducibility.

Final Considerations

Successful splitless analyses require careful optimization of a number of parameters. Keep in mind that many of these parameters are interrelated and the choice of one can affect others. For instance, using a liner with a higher heat capacity may lead to the ability to use a lower inlet temperature set point. Using a lower inlet temperature may require a longer splitless hold time and so on. Understanding the effect of adjusting each parameter is the ultimate goal for effective method development; there isn't a "one-size-fits-all" method that will work for every analysis. The chemical properties of the analytes, as well as the solvent used, will impact the optimal conditions for a particular splitless method.

Reference

1. L. Waclaski, *American Laboratory* **48**(6), 24–26 (2016).

Linx Waclaski is a chemist in Restek's GC Applications group, with his primary focus being on the GC accessories product line. Prior to joining Restek in 2013, he worked in an environmental testing laboratory performing various semivolatile organics analyses using GC. Linx received a master's degree in forensic science and a bachelor's degree in biochemistry from Duquesne University (Pennsylvania, USA).

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The 32nd International Symposium on Chromatography—ISC 2018

The 32nd International Symposium on Chromatography (ISC 2018) will be held on 23–27 September 2018 in Cannes-Mandelieu, France. This preview will shed a little light on what to expect.

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ISC 2018 is one of the premier meetings to discuss all modes of chromatography and separation sciences with a broad coverage of techniques and applications.

Through a harmonious combination of oral and poster presentations, tutorials, short courses, vendor lectures and seminars, and an international exhibition on instruments and services, **ISC 2018** will provide visitors with the advances, fundamentals, challenges, trends, and applications of separation techniques, chromatography, and mass spectrometry in a wide range of topics.

So far the conference has five confirmed Plenary Lectures to be presented by Alain Beck (Center of Immunology, France), Attila Felinger (University of Pécs, Hungary), Fabrice Gritti (Waters Corporation, USA), Robert Kennedy (University of Michigan, USA), and Peter Schoenmakers (University of Amsterdam, The Netherlands), alongside 33 keynote speakers, six short courses, and six tutorials. The short courses will begin on Sunday 23 September 2018 and will include:

- *Analytical Characterization of Protein Biopharmaceuticals* with Davy Guillarme (Université de Genève, Switzerland) and Koen Sandra (Research Institute for Chromatography, Belgium)
- *Flavours and Fragrances and Analytical Chemistry: An Endless Story* with Frédéric Begnaud (Firmenich S.A, Switzerland), and Philippe Darriet (ISVV—Université de Bordeaux, France)
- *Development and Control of Robust HPLC Methods by Modeling* with Szabolcs Fekete (Université de Genève, Switzerland) and Imre Molnár (Molnár-Institute for applied chromatography, Germany)
- *GC×GC: Fundamental Principles, Processes, and Applications* with Philip Marriott (ACROSS, Australia)
- *Microextraction—The "Green" Sample Preparation Choice of Next Generation Analytical Chemists* with

Stig Pedersen-Bjergaard (UiO: School of Pharmacy, Norway) and Janusz Pawliszyn (University of Waterloo, Canada)
- *Introduction to Metabolomics Workflow* with Serge Rudaz (Université de Genève, Switzerland) and Coral Barbas (Universidad CEU-San Pablo, Spain)

As the Symposium will take place in Cannes-Mandelieu, Côte d'Azur, a fabulous setting in the French Riviera, the organizers hope that the charm and sweetness of the region in September will give **ISC 2018** a special flavour.

The Congress and Exhibition Centre (Mandelieu Centre Expo Congrès) is easily accessible via International Airport Nice Côte d'Azur by shuttle, taxi, and train. The area also features more than 1000 hotel rooms that are within walking distance from the Congress Centre.

Mandelieu, the Mimosa Capital, is situated on the Esterel Massif. It offers a fantastic setting for a unique destination in the heart of the Côte d'Azur midway between Saint-Tropez and the Italian border.

In addition to the very exciting five-day conference programme, the organizers hope attendees will find time to sample the attractions of the region. The warm temperature in September in Esterel Massif is an ideal location for trekking. For a scientific adventure visit the Sophia Antipolis Science and Technology Park, where many fragrances and perfumes are created. The organizers recommend tasting the southern version of "French cuisine" and experiencing the hyphenation of the local food with the local wines (in moderation).

The symposium chairpersons are Didier Thiébaut (CNRS/Université PSL, France), Valérie Pichon (Sorbonne Université-PSL, France), and Jean-Luc Veuthey (Université de Genève, Switzerland). For more information, please visit: **www.isc2018.fr**

E-mail: **info@isc2018.frv**
Website: **www.isc2018.fr**

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Advancing Chromatography Methods for Cannabis Analysis

The cannabis industry has been taking a hard look at the science behind their products and the various contaminants that can inadvertently be added to consumer goods. As testing laboratories begin to implement new state-mandated regulations and other consensus methods for best practices, there is a need to review the analytical instruments and methods that can deliver the most accurate results in a timely fashion. Anthony Macherone, senior scientist at Agilent Technologies and visiting scientist at the Johns Hopkins University School of Medicine, recently spoke to us about his research in this area and the chromatographic techniques he has found to be most effective for profiling cannabinoids and terpenes, detecting pesticides, and residual solvents testing.

—Interview by Megan L'Heureux, Editor-in-Chief of Cannabis, Science, and Technology

Q. You recently published an article about the use of liquid chromatography–time-of-flight mass spectrometry (LC–TOF-MS) for cannabinoid profiling and quantitation in hemp oil extracts (1). Can you tell us about the method development involved in this project? How does the LC–TOF-MS method improve upon previous methods used to analyze hemp oil extracts?

A: The purpose of the project was to demonstrate a semi-targeted method to profile cannabinoids in hemp oil, quantitate a targeted list of cannabinoids in the samples, and to begin the development

of a spectral library that can be used for annotation of cannabinoids found in unknown samples.

An Agilent-developed high performance liquid chromatography–ultraviolet (HPLC–UV) method used for potency testing provided the foundational LC parameters for method development. For example, a smaller injection volume, a slightly different mobile phase gradient, and a narrower column internal diameter with a 1.8-µm particle size were used in the LC–TOF-MS method. We also took advantage of a quaternary pump to maintain a constant percentage of aqueous modifiers (ammonium formate and formic acid) over the analytical run time. The quaternary pump helped to maintain a flat baseline across the chromatogram and reduce methanol degradation, which is known to occur when buffers are added directly to the organic mobile phase.

In most laboratories providing safety and compliance testing for cannabis and cannabinoid products, HPLC with UV detection (230 nm) provides a stable, robust, and relatively fast platform for day-to-day productivity work. In the United States and Canada, 4–6 cannabinoids are required to be quantitated by regulation. These include Δ9-tetrahydrocannabinol (THC), its carboxylated analogue THCA, cannabidiol (CBD), its carboxylated analogue CBDA, cannabinol (CBN), and cannabigerol (CBG). Many laboratories will also quantitate 5–8 more cannabinoids in their assay, but they are not required to do so by the regulatory authorities. However, when using HPLC with UV detection, a laboratory runs the risk of misidentifying an interfering compound, such as a terpene for a cannabinoid. There are two factors that mitigate this risk: (a) sample preparation for cannabinoid analyses includes very high dilution factors (1000-fold to as much as 2000-fold), and (b) many terpenes, which can be present in the 1–2% range by weight, poorly absorb at a wavelength of 230 nm. Even so, small terpene peaks are often seen in HPLC–UV assays.

To differentiate cannabinoids from other endogenous compounds, and identify as many as 120–140 different cannabinoids in a given genetic strain, LC–TOF-MS leverages high-resolution accurate mass (HRAM) spectrometry. HRAM mitigates matrix and endogenous chemical interferences, and facilitates identification of chemical components through high-precision mass assignment and formula generation. Of course, HRAM is not perfect. If a chemical component with the same accurate mass (empirical

formula) is eluted at the same time as a cannabinoid, it cannot differentiate between the two, but this type of coelution is generally a rare occurrence. Nonetheless, to ensure lot-by-lot quality control, LC–TOF-MS can provide a wealth of information about a given strain. Moreover, drug discovery and development laboratories researching cannabinoids or modified cannabinoid scaffolds use LC–TOF-MS for metabolic identification and drug metabolism and pharmacokinetics (DMPK) studies.

Q. What other cannabis applications have you used LC methods for?

A: LC–triple quadrupole mass spectrometry (LC–TQMS) is primarily used for targeted residual pesticides quantitation, as well as for mycotoxin and ochratoxin quantification in cannabis and cannabinoid products.

Q. A big issue in cannabis is how each U.S. state where it is legalized has their own testing requirements. You recently addressed this topic in an application note (2), where you used LC–MS/MS and gas chromatography (GC)–MS/MS techniques. Can you describe that research? What sample preparation challenges were faced in this study?

A: Agilent collaborated with Pacific Agricultural Lab in Oregon, USA, to develop a simplified sample preparation workflow that could be shunted to both LC–TQMS and GC–TQMS platforms for comprehensive pesticide residue analysis of more than 210 common pesticides—far more than any U.S. state or country requires. In this way, unsanctioned pesticides can be identified, as well as pesticides that are approved for use through regulation. Again, this approach is not perfect because it is a targeted MS/MS method, and only defined compounds will be identified and quantitated. If a grower applies an unsanctioned pesticide, not on the target list, it will not be found. Here again, is an opportunity for LC–TOF-MS and GC–TOF-MS to screen for large numbers of pesticides in an untargeted mode, then samples determined to be positive for a pesticide or pesticides, can be analyzed by LC–TQMS or GC–TQMS for confirmation and quantitation.

The issue with this TOF screening approach is sensitivity—many pesticides will be present in parts-per-billion (ng/mL) concentrations. TOF-MS sacrifices sensitivity for HRAM, and is generally not as sensitive as triple-quadrupole mass spectrometry. However, in the described sample preparation method, the final dilution factor for both LC–TQMS and GC–TQMS is 500-fold. In this way, the very high sensitivity of these instruments is leveraged, and common problems such as constant GC inlet and column maintenance, carryover, and clogging of HPLC columns are mitigated. This sample preparation approach may open the door for TOF screening applications. For example, a 100-fold dilution factor may provide the sensitivity enhancement needed to detect low levels of pesticides in cannabis and cannabinoid samples.

Q. When developing screening methods for pesticides, do you keep in mind the various states' lists of pesticides?

A: Absolutely. We have U.S. state-specific collaborations, and often tailor our methodology for a given U.S. state's or Canada's list of pesticides. We also keep in mind that there are many compounds on both the California and the Canadian lists that are not well-suited for electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). In fact, 25 pesticides found on at least nine U.S. state lists are recommended for analysis by GC–TQMS by AOAC International. Therefore, forward thinking laboratories and those laboratories in U.S. states, such as California, are aware that to provide accurate and robust testing data to their client, they will need both LC–TQMS and GC–TQMS platforms.

Q. What other cannabis applications have you used GC methods for? Do you see potential for more GC applications in the future?

A: GC-specific methods for cannabis quality and compliance testing include terpene profiling and quantitation for cannabis flower and cannabinoid products. Residual solvents testing is also often required especially for manufactured cannabinoid isolates such as oils, butters, and edible products. Although chemical detection can be performed with flame ionization detection (FID), we advocate simultaneous FID and mass spectrometry detection with a single-quadrupole mass spectrometer. In that way, the large dynamic concentration range of these compounds can be quantified via FID and speciated via single-quadrupole MS. Moreover, to ease sample preparation and create a clean matrix for analyses, headspace auto-sampling should be included in these methods.

Q. What is the biggest analytical science issue to tackle in cannabis?

A: A valid concern is adulteration of dispensed marijuana, or hemp oil products

with illicit chemicals such as synthetic cannabinoids, bath salts (cathinones), or other illegal designer drugs. U.S. state and Canadian regulations only include targeted lists of regulated compounds, and like the example for pesticides given above, if some growers adulterate their crop with a dangerous chemical that is not on the targeted list, it will not be detected, and that product may be released for distribution. Unsanctioned pesticides are often found in marijuana crops, but only by those that look for them. In a similar fashion, laboratories need to develop screening methods to identify adulterants in cannabis products before they reach the retail dispensaries.

Q. What are the next steps in your cannabis research?

A: We continue to identify gaps in the industry's analytical needs. One area of interest to me is the development of TOF-based applications for true comprehensive screening, and to support targeted confirmation and quantification on other analytical platforms.

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Anthony Macherone is a senior scientist at Agilent Technologies and visiting scientist at the Johns Hopkins University School of Medicine (Baltimore, Maryland, USA).

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Tips & Tricks GPC/SEC: UV–vis Detection

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The most commonly applied detector in gel permeation chromatography/ size-exclusion chromatography (GPC/SEC) is the differential refractive index detector, RI. How UV–vis detection, if applicable, adds true value to GPC/SEC applications is discussed in this instalment of Tips & Tricks.

Gel permeation chromatography/ size-exclusion chromatography (GPC/ SEC) is usually used to determine the molar mass distribution (MMD) and molar mass averages of a polymer sample of known chemistry. Peak identification and concentration quantification for each separated peak, the most common tasks in high performance liquid chromatography (HPLC), are often of less interest. To determine the MMD, GPC/SEC requires detectors that measure the relative or—when using light scattering or viscometry detection—absolute concentration in every chromatographic slice.

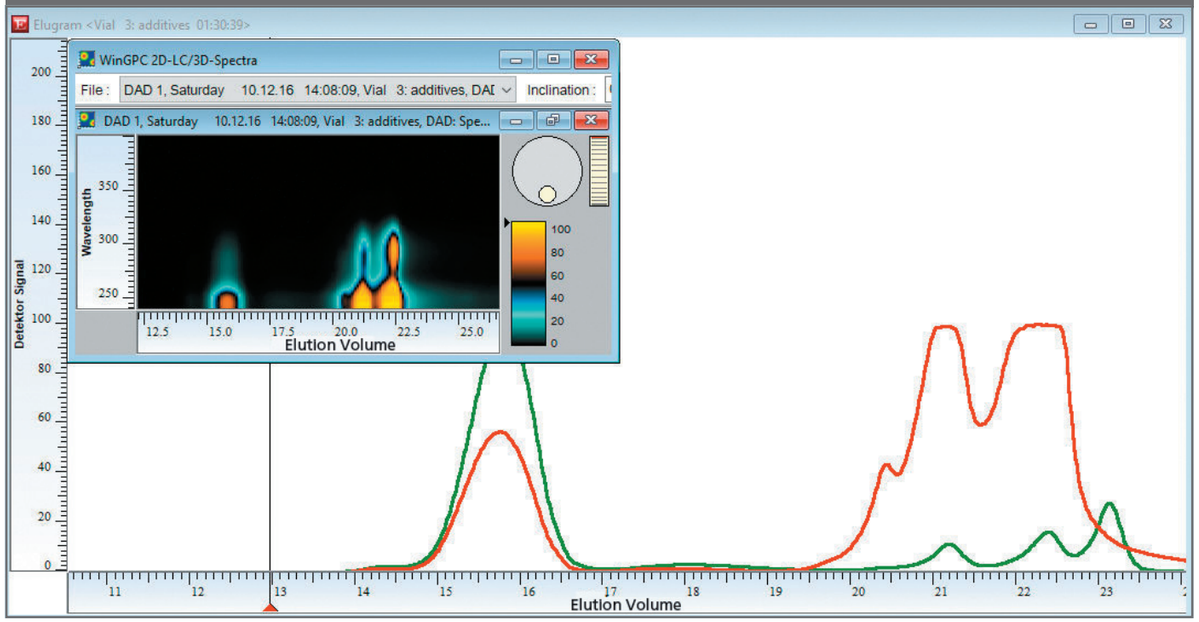
Refractive index detectors, RIs, are the most common GPC/SEC detectors because of their universal applicability; they do not require chromophores to be present in the analyte (1). However, for UV–vis active samples, in general, for analytes containing unsaturated bonds, aromatic groups, or functional groups with heteroatoms, the use of a UV–vis detector offers several advantages.

UV–vis detectors are thus used for both natural (peptides, proteins) and synthetic macromolecules (for comparison see table 1 in reference 2). In addition, the combination of UV–vis and RI detection is widely applied because it offers additional information, for example, about the variation of composition with elution volume.

Types of UV–vis Detectors, Applications, and Advantages

Typically, UV–vis detectors work with light from a deuterium (UV) or tungsten (visible) lamp. The Beer—Lambert law describes the

Figure 1: PDA/DAD spectra (inset) and UV@254 nm (red trace) signals for a sample separated in THF. The RI (green trace) shows an additional peak that cannot be detected by the PDA/DAD as a result of missing chromophores.



relation between the absorbance (*A*) and the analyte concentration (*c*):

$$A = \varepsilon \cdot c \cdot l \qquad [1]$$

ε is the molar absorption coefficient (dm^3 mol^{-1} cm^{-1}) and *l* is the path length of the light through the flow cell (cm). If ε is not known, solutions of known concentration can be used for its determination and for calibrating the detector response (which is required when working with light scattering detectors).

As mentioned above, the most important task in GPC/SEC is determination of the slice concentration. Detection at one specific wavelength is often fully sufficient and the majority of applications can therefore be run with single wavelength UV–vis detectors. Example applications are gelatin–hydrolyzed collagen, which can be easily characterized using a single wavelength detector operated at 214 nm, or synthetic polymers bearing aromatic moieties, which can be detected at 254 nm.

Dual wavelength detection can be applied in protein analysis to additionally quantitate the amount of protein in the solution. The peptide bonds absorb at around 205 nm. Detection at 214 nm and 280 nm allows more detail to be obtained as UV–vis absorbance between different proteins varies strongly at 280 nm and is related to the actual content of tyrosine, tryptophan, and cysteine.

Photodiode array detectors (PDAs or DADs), which can scan a selectable range of wavelengths, are quite common in HPLC. However, there is only a limited number of applications in GPC/SEC where this functionality is required. In those applications spectra are used to identify oligomeric species, for example, in wood resins or polymer additives. Additional concentration detectors (RIs) are often used to obtain the molar mass distribution results simultaneously to the spectral information. Sometimes the PDA/DAD scan functionality is used to determine a wavelength that can later be used for the specific detection.

However, in all cases of UV–vis detection, users should verify upfront that all analyte components can be detected. UV–vis detection alone has a high risk of undetected components because many macromolecules do not exhibit any chromophores. The verification can be done with the additional use of an RI detector.

The inset in Figure 1 shows 3D spectra taken from 240 nm to 390 nm for a GPC separation in tetrahydrofuran (THF). The UV trace (red) at 254 nm detects those components bearing suitable chromophores. The component eluting at 22.5 mL shows an additional absorbance around 300 nm, indicating a chemically different entity. The last component at 23.2 mL is not seen by DAD/PDA because of the absence of suitable chromophores; however, the component is detected by the RI (green trace).

One advantage of UV–vis detectors when compared to other GPC/SEC detectors is that they have a small cell volume. The lower band broadening of smaller cells can be beneficial if the resolution of close peak pairs is of concern. The UV–vis detector can (and should) be placed as a first detector in a detector train because of its lower contribution to band broadening. Its cell is also very pressure stable and the signal does not depend on back pressure (3).

UV–vis detectors have a high linearity, selectivity, and sensitivity and are suitable if scientists have verified upfront that all analyte components can be detected.

Detector Combinations with UV–vis Detectors

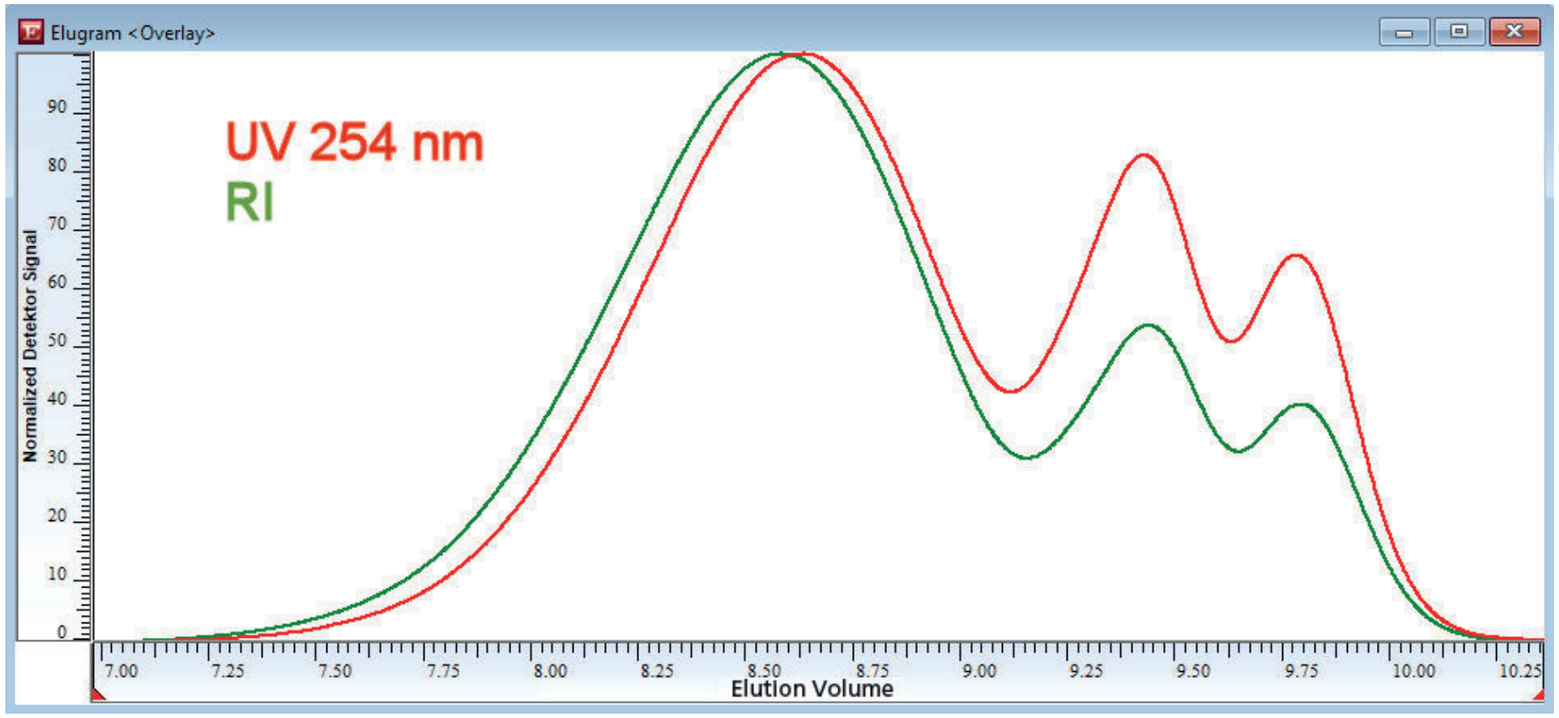
UV–vis detectors have a high selectivity. Appropriate setting of the wavelength may allow selective detection, for example, of only one type of comonomer in a copolymer or of a specific end group. This makes UV–vis detectors a very valuable addition in detector combinations. The protein application mentioned above is an example of UV–vis–UV–vis dual detection; more generally applicable is the combination of a UV–vis detector with an RI detector

UV–vis–RI combinations are often used in copolymer analysis to determine the chemical composition distribution in a given copolymer (4). Figure 2 shows an example of a chromatogram of a graft copolymer consisting of polymethyl methacrylate (PMMA) and polystyrene (PS) measured in THF. The universal RI detector responds to both monomer units, MMA and styrene. However, the UV–vis detector set to a wavelength of 254 nm selectively detects the styrene repetition units, which bear the chromophore. The detector traces appear to be shifted relative to each other although the inter-detector delay between UV–vis and RI has been properly accounted for. This shift of detector traces is a consequence of the variation of the chemical composition with elution volume and the different responses of both detectors.

This difference in response to the same analyte allows more to be learned about copolymers. The simple fact that the (normalized) detector traces of both concentration detectors do not superimpose is an indication of chains of different chemical composition. At low elution volumes, the (normalized) RI signal is higher than the UV signal, while the opposite is true at elution volumes exceeding 8.5 mL. To a first approximation the RI signal is proportional to the total mass concentration of molecules eluting from the column. Thus, at a given concentration the species eluting at low elution volumes bear less absorbing groups than at high elution volumes. In other words: the larger molecules (low elution volumes) are richer in MMA, while the shorter chains (high elution volumes) are rich in polystyrene, as indicated by the higher UV absorbance.

While the above explanation is qualitative only, quantitative information can be obtained as well. This opens up new calibration possibilities for block copolymers. Based on the two detector signals, the chemical composition can be calculated for each elution volume,

Figure 2: UV@254 nm and RI trace for a MMA–styrene graft copolymer with a compositional distribution—chains vary in composition, molar mass, and number of grafts. The UV–vis detector is set to selectively detect the styrene repetition units and thus looks shifted when compared to the RI signal, which detects MMA and styrene alike. The difference between the signals allows more to be learnt about the composition distribution.



allowing a copolymer calibration for the specific composition from the calibration curves of the two homopolymers to be established, which finally permits molar mass determination for copolymers (5).

Another option in UV–RI detection is the selective detection of end groups by choosing a suitable UV–vis wavelength. This approach is applied in the *European Pharmacopoeia* for the molar mass determination of low-molecular-weight heparins. Figure 3 shows an example chromatogram of an end group modified heparin reference material. The end group of this calibration standard can be detected by 234 nm. A UV–vis detector set to this wavelength shows a signal that is related to the number of chains eluting from the column. The RI detector—used in addition—detects every repetition unit of the heparin chains, so that the signal is related to the mass concentration of the chains passing the detector. The fact that the UV signal is proportional to the number concentration, while the RI signal is proportional to the mass concentration of eluting chains is the reason for the strongly differing detector traces. As the number average molar mass (M_n) for this standard is known, it is now possible to create a heparin calibration curve based on the UV–RI ratio. The obtained calibration curve can subsequently be applied for molar mass determination of heparins using the RI signal only.

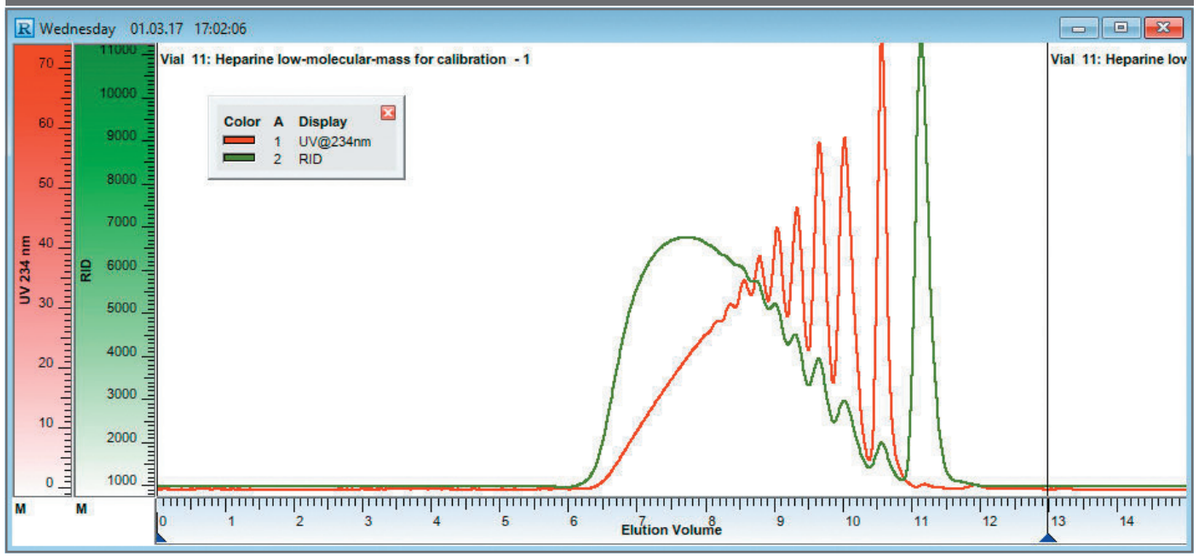
This end group calibration method can be applied to all samples where it is possible to specifically detect end groups and where every chain definitely bears one end group. The only additional information required then is the M_n.

On the other hand, it is also possible to measure the end group distribution within a sample with UV–vis-detectable end groups when using UV–vis–RI dual detection.

Summary

- UV–vis detectors are linear, sensitive, and selective detectors. They are ideal GPC/SEC detectors if all analyte components have chromophores.
- Single wavelength UV–vis detectors are often sufficient. PDA/DAD are only used for a limited number of applications but provide UV–vis spectra for all single components in a run.
- Selecting a specific wavelength to detect

Figure 3: Low-molecular-weight heparin calibration standard with modified end group to allow UV–vis detection. The UV–vis detects every single chain, the RI detects all units.



one comonomer in a copolymer or an end group adds additional value when using dual detection with RI.

- UV–vis detectors can be set to detect the repetition unit (weight average signal) or to detect end groups (number average signal). The latter offers opportunities for calibration to obtain true masses.

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Fast and Simple High Performance Liquid Chromatography–Ultraviolet Assay for the Determination of Cannabinoid Content in Hemp Oil

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The medicinal qualities of cannabinoids contained in hemp have been described in detail. Pain mitigation and reduced severity of nausea and seizures are just a few of the therapeutic benefits reported by medical cannabis patients. There is evidence that a combination of cannabidiol (CBD), a host of other minor cannabinoids, and a complex array of terpenoids as contained in hemp oil may be the most beneficial, which is why CBD-rich oil for oral intake has become increasingly popular. The FDA has issued warning letters to firms that market unapproved new drugs allegedly containing CBD. As part of these actions, the cannabinoid content of some hemp products was determined and many were found to contain levels of CBD largely deviating from the label claim. Like cannabis, hemp oil can be analyzed easily and effectively for its cannabinoid content. This article highlights the use of a fast and simple high performance liquid chromatography–ultraviolet (HPLC–UV) assay for separation and quantification of 11 important cannabinoids, including CBD in hemp oil.

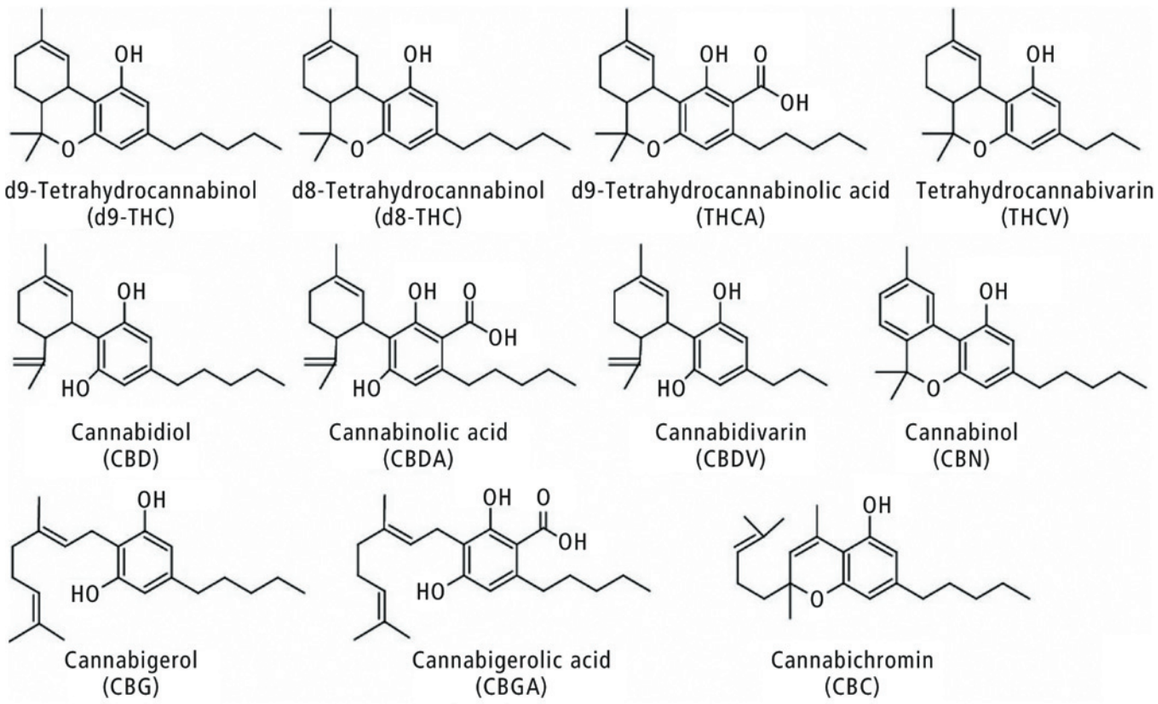
Photo Credit: n_defender/Shutterstock.com

The discussion about legalization of marijuana is a widespread and lively one with a wide range of contrary positions (1–3). While the controversy stems from the psychoactive effect of one of the contained cannabinoids, namely Δ9-tetrahydrocannabinol (d9-THC), the use of other phytocannabinoids in combination has also demonstrated promising results in chronic pain therapy. Efficacy in the treatment of multiple sclerosis, arthritis, and other inflammatory diseases has been indicated (4,5).

Pain mitigation and reduced severity of nausea and seizures are just a few of the therapeutic benefits reported. In addition, a large number of studies showed high safety with regard to a wide array of side effects, and no tolerance to cannabidiol (CBD) has so far been demonstrated (6). CBD-rich oil, extracted from the flowers, seeds, and roots of the plant, has therefore become increasingly popular and is administered via sublingual drops, gel capsules, or as a topical ointment.

The Farm Bill of 2014, an agricultural and food policy tool of the United States

Figure 1: Cannabinoids found in hemp and marijuana.



federal government, distinguishes hemp from marijuana, but interpreting the law is difficult since "CBD oil" may be classified as marijuana. However, there are clear definitions depicting the differences in marijuana, cannabis, and hemp (7).

While "cannabis" is the name of the plant itself, marijuana, in its correct use, refers only to the leaves and flowering portions of the plant that contain a wide array of cannabinoids, including more than 0.3% of the psychoactive compound THC. Hemp, on the other hand, describes the sterilized seeds, stems, stalks, and roots of cannabis plants with more than 0.3% THC. Therefore, the CBD oil produced from these seeds and roots exhibits therapeutic benefits without the mind-altering "high" produced by THC (8).

This article describes the application of a fast and simple high performance

Table 1: Results summary of quantitation of total cannabinoid content

		1 Black Label		1 Blue Label		3 Green Label		4 Red Label		5 Yellow Label	
ID#	Name	µg/mL	%	µg/mL	%	µg/mL	%	µg/mL	%	µg/mL	%
1	CBDV	83	0.008	47	0.005	842	0.084	216	0.022	285	0.028
2	CBDA	55	0.006	47	0.005	11,470	1.147	312	0.031	156	0.016
3	CBGA	24	0.002	0	0.000	100	0.010	0	0.000	0	0.000
4	CBG	166	0.017	33	0.003	244	0.024	188	0.019	289	0.029
5	CBD	21,856	2.186	14,956	1.496	22,873	2.287	9722	0.972	16,695	1.670
6	THCV	0	0.000	0	0.000	0	0.000	0	0.000	0	0.000
7	CBN	37	0.004	0	0.000	335	0.034	17	0.002	41	0.004
8	d9-THC	555	0.055	0	0.000	1621	0.162	387	0.039	625	0.063
9	d8-THC	0	0.000	0	0.000	0	0.000	30	0.003	52	0.005
10	CBC	1006	0.101	1194	0.119	1104	0.110	457	0.046	809	0.081
11	THCA	0	0.000	0	0.000	380	0.038	38	0.004	113	0.011
Total cannabinoids %			**2.38**		**1.63**		**3.90**		**1.14**		**1.91**

Table 2: Results summary of quantitation of CBD content

		1 Label Claim CBD: 23,000 µg/mL		2 Label Claim CBD: 16,666 µg/mL		3 Label Claim CBD: 30,000 µg/mL		4 Label Claim CBD: 8000 µg/mL		5 Label Claim CBD: 8333 µg/mL	
		Black Label		Blue Label		Green Label		Red Label		Yellow Label	
ID#	Name	µg/mL	%	µg/mL	%	µg/mL	%	µg/mL	%	µg/mL	%
5	CBD	21,880	2.19	15,243	1.52	24,211	2.42	9721	0.97	16,695	1.67
CBD % of label claim			**95**		**92**		**81**		**122**		**200**
CBD % of Total			**92**		**92**		**59**		**86**		**88**

liquid chromatography–ultraviolet (HPLC–UV) screening test for the separation and quantification of 11 important cannabinoids (Figure 1) in hemp oil, for quality control with regard to the label claim of CBD as well as THC content

Experimental

HPLC–UV Analysis: A high sensitivity HPLC method was used to create standard curves for each of the 11 cannabinoids under investigation, with a minimum acceptable correlation coefficient (R2) of 0.999 over six standard levels. A Prominence-i HPLC system from Shimadzu equipped with a UV detector was used for analysis. A linear dynamic range was established at 0.5 to 100 mg/L (ppm) for each analyte. Gradient elution conditions with acid modified water and acetonitrile were employed with a C18 column chemistry to achieve separation in under 10 min.

Sample Preparation: Hemp oils are typically rich in CBD, with relatively minor concentrations of other cannabinoids. Two dilution factors were used to ensure quantitation within the calibration range. One dilution factor yielded appropriate detector sensitivity to the array of minor cannabinoids. A second, higher dilution factor was applied for the most accurate quantitation of the major component CBD, so that its response was within the linear range determined for that analyte. In practice, it was found that the two approaches yielded quantitative values for CBD that agreed within 0.2%.

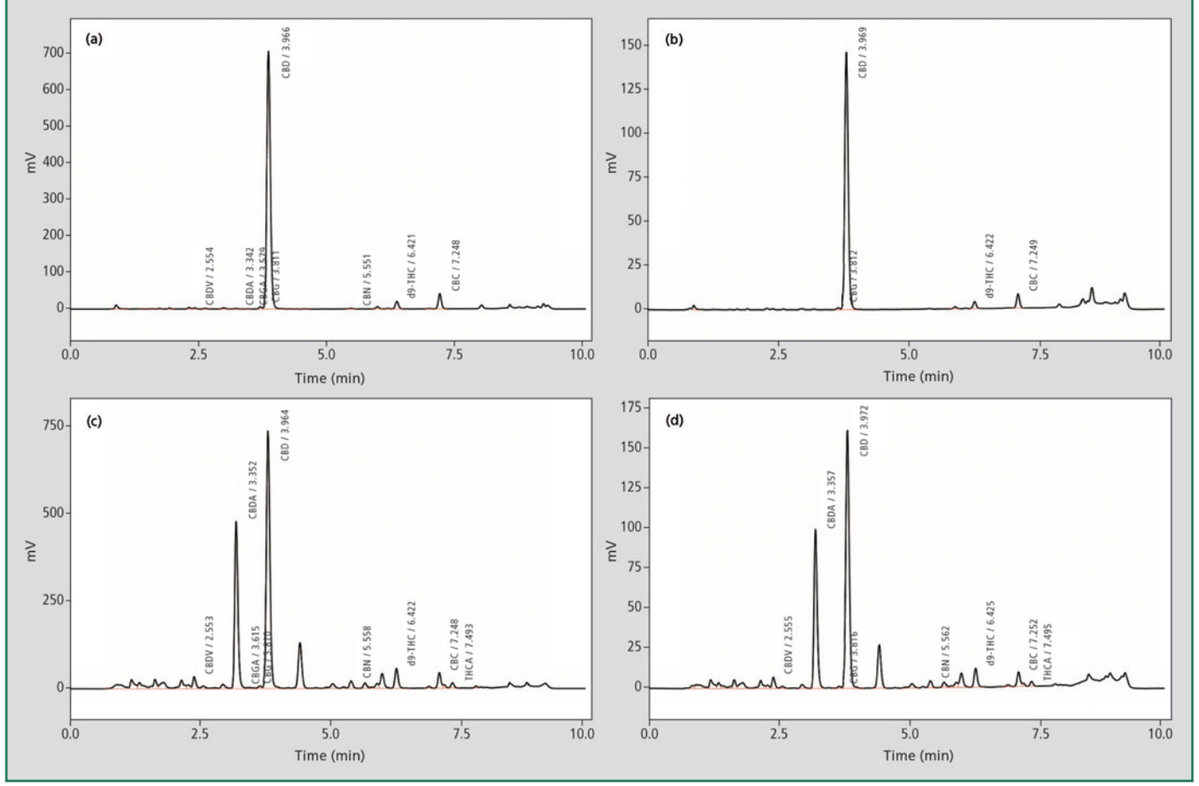
For determination of total cannabinoid content (sample A), 400 µL of isopropanol

Figure 2: Appearance and label information of hemp oils samples.
Hemp Oil #1: Black Label; Label Claim: 23 mg per serving; 100 servings per 100 mL;
Calculation of Label Claim: 23,000 µg/mL or 2.3%
Hemp Oil #2: Blue Label; Label Claim: 500 mg per 30 mL
Calculation of Label Claim: 16,666 µg/mL or 1.7%
Hemp Oil #3: Green Label; Label Claim: 15 mg per 1 serving per 0.5 mL = 15 mg/0.5 mL
Calculation of Label Claim: 30,000 µg/mL or 3.0%

was pipetted into a 2-mL glass vial, followed by 10 µL hemp oil sample. The mixture was agitated for 30 s until the oil sample was completely dissolved. A 400-µL measure of methanol was then added to the mixture and shaken for another 30 s. The mixture was then filtered through a 0.2-µm PTFE syringe filter into an HPLC vial. This resulted in a total dilution factor of 81.

Samples for the quantification of CBD with only a 405-fold dilution were prepared by adding 200 µL of sample A to 800 µL of methanol and thoroughly mixing for 30 s.

Figure 3: Chromatograms for total cannabinoid content and CBD content in hemp oils #1 (a + b) and sample #3 (c + d).



The five hemp oils tested in this study were purchased from various mail order vendors. The appearance and label information for three of the five are shown in Figure 2, referenced as black, blue, and green. Two samples tested but not pictured are referred to as red and yellow.

Results

Figures 3(a), 3(b), 3(c), and 3(d) show the chromatograms obtained from the analysis of hemp oils #1 and #3 for determination of total cannabinoid content (81× diluted) as well as CBD content only (405× dilution).

A summary of results of quantitative determination of total cannabinoid as well as CBD content in five commercially available hemp oils is shown in Tables 1 and 2.

As a general sample observation, hemp oils #1 (black) and #2 (blue) exhibited a transparent, weak yellow–green colouration. This leads to the assumption that each of these oils was a product of multistep purification after extraction; for example, CO_2 or butane extraction followed by steam distillation. Notably, hemp oil #3 (green) was opaque brown–green and gritty in appearance. It also had the most intense smell, a distinctly "earthy" odour. It was concluded that the sample was the result of crude extraction only, with no further refinement.

It is important to note that it has been reported in the literature that the whole plant can be more beneficial to the consumer because it contains not only cannabinoids, but also an array of terpenes providing a synergistic "entourage effect" (4,9). The whole plant can also provide essential fatty acids, plant sterols for lowering cholesterol, and antioxidants, chlorophyll and vitamin E.

Hemp oils #1 (black) and #2 (blue) showed high ratios of CBD to total cannabinoids, both at 92%, and the lowest quantity of other cannabinoids. This finding supported the assumption, along with transparency and colour, that these oils were more highly purified samples. Both samples also tested close to label claim at 95% and 92%, respectively.

Hemp oil #3 (green) revealed the highest content of CBD and total cannabinoids, yet exhibited the lowest ratio of CBD to total cannabinoids (59%). This observation is consistent with the assumption that its crude appearance reflected the least amount of post-extraction purification. Although its CBD percentage of label claim tested the lowest (81%), this sample did contain the highest level of CDB compared to all other oils tested.

Hemp oils #4 (red) and #5 (yellow) tested higher than label claim at 122% and 200%, respectively. The observation is consistent with FDA findings for CBD products, perhaps calling into question the type and accuracy of testing used to justify label claims.

Conclusion

In summary, all samples contained less than 0.3% d9-THC, as expected from hemp products. All samples showed an array of other cannabinoids, but the minor component, THC-V, was not detected in any of the hemp oil samples. From a quality control point of view two samples were within a reasonable range of the label claim at +/- 10%. One sample was well below and two other oils contained CBD levels well above the label claims, one by as much as 200%. This study showed that in three out of five randomly selected samples the actual concentration of CBD did not comply with the stated content. This HPLC method provides

a simple and fast assay for CBD and total cannabinoid content for improved quality control of hemp products.

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