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Extractable and Leachable Testing for Pharmaceutical Packaging, Finished Pharmaceutical Products, and Medical Devices: An Analytical Perspective

Gyorgy Vas, Louis Fleck, Katie Comstock, and Jason Cole

Extractable and leachable (E&L) testing for finished pharmaceutical products, bioprocess manufacturing systems, and medical devices is currently a subject of intense interest. The majority of the challenges encountered in analytical workflows are related to the highly complex matrices and relatively low analyte evaluation thresholds seen in E&L studies. This paper provides options for executing E&L testing to supporting regulatory submissions. There are multiple compliant routes possible, and the presented workflows and analytical solutions are only one of multiple successful approaches.

Comprehensive Gas Chromatography Coupled to Simultaneous Dual Detection (TOF-MS/FID) as a Confirmatory Method for MOSH and MOAH Determination in Food

Sebastiano Pantò, Maurine Collard, and Giorgia Purcaro

Food contamination from mineral oil saturated hydrocarbons (MOSHs) and mineral oil aromatic hydrocarbons (MOAHs) is problematic and requires a sensitive analytical technique. These contaminants were analyzed using GC×GC with flame ionization detection (FID) and time-of-flight–MS (TOF–MS) parallel dual detection. The method provides enhanced chromatographic separation, along with the full mass spectra information, and overcomes difficult interferences, resulting in reduction of false positives over conventional GC–MS methods.

Novel LC–MS/MS Method with a Dual ESI and APCI Ion Source for Analysis of California-Regulated Pesticides and Mycotoxins in Medium-Chain Triglyceride (MCT) Oil Cannabis Tinctures

Avinash Dalmia, Charles Johnson, Saba Hariri, Jacob Jalali, Erasmus Cudjoe, Joey Kingstad, and Feng Qin

Analysis of 66 pesticides and 5 mycotoxins regulated by the State of California in cannabis tinctures were analyzed using LC–MS/MS with an ESI source, and LC–MS/MS with an APCI source. A simple, fast, and cheap acetonitrile solvent extraction method was used for sample preparation for good recovery and high throughput, and internal standards were used to compensate for ion suppression effects from the hydrophobic matrix.

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Extractable and Leachable Testing for Pharmaceutical Packaging, Finished Pharmaceutical Products, and Medical Devices: An Analytical Perspective

The purpose of extractable and leachable (E&L) impurity testing is to provide identity and quantity of observed species released from the pharmaceutical container-closure, or from manufacturing equipment for toxicological risk assessment. E&L testing for finished pharmaceutical products, bioprocess manufacturing systems, and medical devices is currently a subject of intense interest for the industry. The majority of the challenges encountered in analytical workflows are related to the highly complex matrices and relatively low analyte evaluation thresholds in E&L studies. Component identification requires state-of-the-art analytical instrumentation and highly skilled analytical scientists. Additionally, this step is heavily reliant on high resolution chromatography instrumentation (both gas chromatography [GC] and liquid chromatography [LC]) hyphenated with mass spectrometry systems. Obtaining consistent high-quality data requires multiple types of systems. While the data packages generated by different systems may not be comparable or equivalent, if instrumentation is used according to the recommendation of USP <1663>, the data package will satisfy the regulatory expectations.

Gyorgy Vas, Louis Fleck, Katie Comstock, and Jason Cole

Drug manufacturing or container-closure systems should not release chemicals that can accumulate, or leach, into the drug product in quantities sufficient to present a risk of toxicity, or affect its stability or efficacy. The purpose of extractable and leachable (E&L) impurity testing is to provide answers for identity and quantity of observed species for toxicological risk assessment. E&L testing for finished pharmaceutical products, bioprocess manufacturing systems, and medical devices is currently a subject of intense interest for the industry. This paper intends to provide options for executing E&L testing to provide fully compliant data for regulatory submissions. There are multiple compliant routes possible, and the presented workflows and analytical solutions are only one of multiple successful approaches.

Regulatory Landscape
The U.S. Food and Drug Administration (FDA) Packaging Guidance (1) was published almost 20 years ago in 1999, and leachable testing is part of the 21 CFR 211 (2) regulation. However, recently regulatory guidance has
began to evolve. In the past, E&L analytical testing results were only part of regulatory submission packages for certain high risk dosage forms, such as inhalation and nasal route of administration products. Recently, the FDA has increased its focus, and has issued several deficiency letters for absence, or low quality, of E&L data for parenteral, injectable, transdermal, and even for liquid oral dosage formulations.

In the past few years, a significant level of work has been completed by the United States Pharmacopeia (USP) packaging committee, to provide detailed instruction and guidance for performing extractable and leachable testing (3). The related USP chapters were written with a different philosophy than the earlier monograph chapters, providing useful and comprehensive guidance rather than a full detailed methodological approach. The USP general chapter <1663> allows multiple approaches to complete the testing, with the expectation that certain pieces of analytical test data must be in the data package. A similar approach was taken to guidance for analytical testing of medical devices (4). The analytical workflow consists of multiple steps, resulting in a fairly complex testing regimen where high-end, complex analytical instrumentation is used to solve the problems.

**Analysis of Extractables and Leachables**

The majority of the challenges encountered in analytical workflows are related to the highly complex matrices and relatively low analyte levels in E&L studies. The component identification aspect requires state-of-the-art analytical instrumentation and highly skilled analytical scientists. Additionally, this step is heavily reliant on high resolution chromatography instrumentation (both gas chromatography [GC] and liquid chromatography [LC]) hyphenated with mass spectrometry (MS) systems. Obtaining consistent and high-quality data requires multiple types of systems. Often, the data packages generated by different systems are not directly comparable or equivalent; however, if instrumentation are used according to the recommendation of USP <1663>, the data package will satisfy the regulatory expectations.

**Study Design and Workflow**

A general analytical workflow presented in Figure 1, is one of the possible way to comply with the latest regulatory expectations, and a few key points must be considered:

- The extractable study design must be based on the finished drug product (DP) risk category.
- Container−closure system (CCS) components must be evaluated individually.
- Testing must be based on a final packaging configuration.
- Contact type of the material must be considered (direct or indirect).
- Contact period and temperature must be considered.
- Drug product formulation must be evaluated. (If the formulation is extremely complex or challenging, a well justified simulation media may be used.)

The extraction should be designed to target extractable species with different polarity and at different concentration levels; therefore, extraction solvents with different polarities are recommended. In some cases, mixtures of solvents can be used to fine-tune the polarity of the extraction solvent.

Typical laboratory solvents included to cover a range of polarities:

- **Non-polar**: hexanes, heptane, or cyclohexane

![Composite structure](https://example.com/structure.png)
• **Mid-polarity**: dichloromethane, isopropanol, or ethanol (it should be noted that for some acidic extractables, ethyl-esters could form in the presence of ethanol)
• **Polar**: pH adjusted water with or without buffer is acceptable
• **Solventless**: highly suitable for volatiles and semi-volatiles

The extraction needs to be designed so that appropriate concentrations of the extractables can be achieved. This may require a concentration step prior to the instrumental analysis. For practical considerations, 2–5 ng of an individual analyte is desired on-column, which is translated to 2–5 µg/mL concentration for GC–MS analysis and 0.2–0.5 µg/mL concentration for LC–MS analysis.

Solventless sample preparation techniques such as solid phase microextraction (SPME) (5) and stir bar sorptive extraction (SBSE) (6) can provide high quality complimentary data for the solvent based extractions, especially when a complex drug formulation is being simulated, or the actual finished DP is used for the extractions. Solventless sample preparation allows direct analysis of the broadest range of finished drug products, as they are compatible with highly complex finished drug products formulations, and can extract targets at extremely low concentrations. When state-of-the-art, highly-efficient sample preparation is combined with high resolution accurate mass (HRAM)-based mass spectrometry, a number of sample processing steps can be eliminated from the workflow, and high value information can be generated from a single analytical run.

### Analytical Evaluation Threshold

In order to provide meaningful data, the analytical evaluation threshold (AET) must be calculated based on the dosage and the route of administration of the finished DP (7). For inhalation and nasal products, the AET is calculated based on the Safety Concern Threshold (SCT) of 0.15 µg/day. For other administration routes, a 1.5 µg/day SCT can be used.

For a high number of doses in a single dispensing unit, the AET will increase, and it will be easier to perform analytical testing. Inhalation products typically contain multiple days of supply in a single unit, resulting in a relatively high AET. The chemical species detected above the AET needs to be identified. If the calculated AET in the extract is below 200–500 ng/mL, a concentration step is applied to reach the desired concentration for proper analytical evaluation. The concentration step is critical, and can introduce a significant level of error to the testing, and has a high potential to lose volatile species. For example, 95% of styrene can be lost during a concentration step even when gentle flow of nitrogen is used at room temperature.

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The evaporation step is less critical for inhalation products where the AET is generally relatively high, but more significant for large volume parenteral (LVP) dosage forms, where the formulations are mainly aqueous, and solvent-based back extraction is commonly employed utilizing a large volume of solvent.

### Sample Preparation

The extracts can be generated by using instrument or non-instrument based techniques. USP general chapter <1663> lists 12 different types of extraction methods appropriate for extractable studies (extraction methods not listed in USP <1663> can be used if justified). The non-instrument based techniques are currently the most commonly used in the industry (including, but not limited to, Soxhlet, reflux, sonication augmented, and pressurized vessel), since they require lower capital investment. However, there can be some disadvantages:

- Extraction times can be lengthy (up to 8–48 h).
- Highly manual processes, documentation according to current good manufacturing practices (cGMP) compliance can be burdensome.
- Data integrity can be an issue in a cGMP environment for manual based extraction techniques.

To avoid those drawbacks, instrument-based extraction technologies are available on the market to perform the extractable testing. For example, one possible solution for solvent mitigated extraction is the accelerated solvent extraction (ASE) technique. The criteria for selection of this instrument-based option include the following:

1. **USP** <1663> lists ASE as a possible option to perform extractable studies.
2. ASTM D7210 also lists ASE as one of the options to extract antioxidants from polymeric matrices (8).
3. System allows automated, unattended extraction with full control and recording of all parameters.
4. System has intelligent solvent management.
5. Reduced extraction times.
6. Reduce solvent consumption.
7. Increase extraction efficiency.
8. Nitrogen flush gas minimizes the oxidation of the extractables.
9. It works very well for wide range of polymers, especially for ultra-high molecular weight cross-linked polyethylene (9).
10. System comes with compliant-ready software control.

There is a significant difference in the efficiency of extraction when using ASE versus Soxhlet. Figure 2 shows 48-h Soxhlet extraction compared to an ASE extraction for an Irganox-1010 treated ultra-high molecule weight polyethylene (UHMWPE) sample. The peak at 13.8 min is Irganox-1010, the other peaks are Irganox 1010 degradation products formed during the polymer cross-linking process.

The ASE technique provides comparable results for polyethylene (PE), silicone-tubes, and polypropylene (PP) materials. Its performance is especially advantageous for highly crosslinked polymeric systems used to manufacturing medical devices. Usually, lower levels of oxidation products are observed with ASE compared to reflux or Soxhlet methods, explainable by the inert nitrogen-filled extraction path and the shorter extraction time.

### Static Headspace (SHS) and Solid-Phase Microextraction (SPME) for GC–MS

It is also feasible to perform extraction studies without presence of any kind of solvent (non-solvent mediated extractions). The most common of these is static headspace. However, this method is not the most useful for analyzing polymer samples directly. Modern GC–MS systems can now be equipped with a robotic autosampler, which can perform multiple sample preparations including direct liquid injection (DI), static headspace (SHS), and solid-phase microextraction (SPME). Besides the outstanding performance of SPME, it is used less often in E&L testing than SHS. Figure 3 shows a polymer sample headspace extracted with SHS and with SPME. The headspace of a 1
cm² piece of polymeric manufacturing bag was extracted. Both chromatograms are scaled to the same intensity level, and show greater extraction efficiency using SPME. Both SHS and SPME extractions were performed at 80 °C with 15 min extraction time for SPME.

Component Identification
Since its development, mass spectrometry has been one of the most heavily used techniques for component identification. The relatively high scan speed (multiple scans per s), relatively easy connection to chromatographic techniques, high level of specificity and the extreme low detection limit, make it an ideal tool for identifying and quantifying low level impurities in a complex matrix. The development of benchtop high resolution systems (both orbital trap- and time-of-flight [TOF]-based), gradually replaced the large floor standing magnetic sector type systems. These benchtop instruments offer an affordable high resolution accurate mass solution, even for small size analytical laboratories. Implementing high resolution accurate mass system into the identification workflow improves compliance with regulations such USP <1663> and European Union (EU) guidances (10). USP <1663> provides a detailed flow-path to perform the characterization of extracted components. The recommended process is a four-step procedure as indicated below:

- Scouting: Provide information about bulk properties (ultraviolet [UV], Fourier-transform infrared spectroscopy [FT-IR], total organic carbon [TOC], pH) of the packaging materials.
- Discovery: Employ multiple analytical techniques to analyze the sample extracts. These steps usually involve separation techniques, and individual compound response evaluation.
- Identification: Searching for the answer to “What is the compound?” Identification is a complex process, requiring expertise and advanced instrumentation. This is often the most time-consuming step. At this step, advanced instrumentation can make a significant impact on the toxicological risk assessment and the overall quality of the data package.
- Quantitation: Searching for the answer to “How much of the compound is in the sample?” involves the determination of specific target components, sometimes at very low levels. Here, method validation needs a high level of expertise and the lack of authentic reference materials is also a challenge (11,12). When authentic reference material is not available, semi-quantitation is usually performed against a single or multiple individual surrogate standards, either
as internal standard or external standard calibration.

The identification step is one of the most complex tasks (13), requiring high-end analytical instrumentation and analytical skills; therefore, it will be discussed in detail.

The USP general chapter <1663> recognizes three identification categories for extracted chemical species:

- **Tentative:** A tentative identification means that data have been obtained that are consistent with a class of molecule only, usually achieved only with data available from automated library search or fragmentation behavior by expert interpretation.
- **Confident:** A confident identification means that the tentative identification has been bolstered by additional and sufficient confirmatory information to preclude all but the most closely-related structures, usually achieved only with data available from automated library search or fragmentation behavior by expert interpretation. Confidence can come from confirmation of molecular weight, exact mass elemental composition, or orthogonal spectral confirmation such as nuclear magnetic resonance (NMR).
- **Confirmed:** A confirmed identification means that the preponderance of evidence confirms that the entity in question can only be the identification that is provided. This can usually only be achieved with data available from automated library search or fragmentation behavior by expert interpretation, confirmation of molecular weight elemental composition, and a mass spectrum and chromatographic retention index match with an authentic reference compound. Confident elemental composition determination requires HRAM.

According to the identification categories defined in the USP chapter, a tentative identification does not demand any additional characterization. However, it may not provide sufficient support for the toxicological risk assessment. For hydrocarbons such as alkenes or alkanes, this information may be sufficient, since those components have low toxicity. In general, a tentative identification would not be sufficient for species containing aromatic rings or ring systems, halogen atoms, or even heteroatoms.

The confirmed category, on the other hand, is very challenging, since this category effectively requires authentic reference materials, which in some cases are not available or extremely difficult to obtain. Since, in most cases, confident identification can be performed, it is important to know and understand the instrument requirements to support meaningful toxicological assessment. While the automated library search for a peak above the AET requires minimal effort, to move beyond this point requires sig-

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**Figure 6:** El Spectrum, previously misidentified as 2,6-di-tert-butyl-4-(prop-1-en-1-yl)phenol \((C_{17}H_{28}O)\), confirmed as \(C_{16}H_{22}O_{2}\) using HRAM-MS.

**Figure 7:** HRAM Spectrum of Compound C, showing higher accuracy elemental composition match for \(C_{16}H_{22}O_{2}\) than for \(C_{17}H_{26}O\).
significant nonroutine testing together with expert interpretation.

**Commercial and Proprietary Library Databases**

Automated library searches have limitations, with many extractable compounds not being present in proprietary libraries, such as the National Institute of Standards and Technology (NIST) Mass Spectral Library. Overdependence on limited libraries also has issues where high intensity peaks overload the column and bias the spectral search. This can happen in highly concentrated extracts, or where peaks coelute. It is a preferred approach that laboratories may contribute spectra for a public database (such as NIST), using a common library database format by sharing knowledge across the industry. This approach could reduce the identification burden, and would be beneficial for the entire industry. It is also desirable that, when such records are shared, a common format and an appropri-

![Figure 8: Positive and negative APCI spectra of Irganox-1010 degradant peak, with proposed structure.](image)
Interpretation of spectral fragments from CID experiment of \( m/z = 292 \) ion for \( C_{17}H_{26}O \) rubber oligomer using Mass Frontier software.

Figure 9: Interpretation of spectral fragments from CID experiment of \( m/z = 292 \) ion for \( C_{17}H_{26}O \) rubber oligomer using Mass Frontier software.

A large number of ions are recorded and stored in the database, enhancing opportunity to evaluate and review the identification process.

The first step of the confident identification is to confirm the molecular ion. For GC–MS, electron ionization (EI) is the most common ionization mode. EI is an aggressive ionization mode that results in extensive fragmentation and sometimes provides weak molecular ion signal, with insufficient intensity for unequivocal molecular weight confirmation. Here, chemical ionization (CI) can be used to enhance the intensity of the molecular ion and to confirm the molecular ion using adduct ion species. From a laboratory operation perspective, it is very important to have the CI option available without venting the mass spectrometer in order to perform CI testing as a part of the routine workflow, otherwise a separate dedicated instrument is recommended.

Figure 4 shows EI and CI spectra of propylparaben. CI was performed in positive mode, with methane used as the reagent gas. The molecular ion in the EI spectra has very low intensity. The CI spectrum acquired in positive ion clearly shows the quasi-molecular ion; \([M+H]^+\) proton adduct. Here, we are able to confirm the molecular ion of \( m/z = 180.0781 \) corresponding to \( C_{10}H_{12}O_3 \). The CI spectrum shows less fragmentation compared to EI conditions.

It should be noted that interpretation of CI spectra can be challenging, as additional \([M+29]^+\) and \([M+43]^+\) ions can be observed in the spectra. Moreover, some species have a tendency to provide \([M-H]^+\) ions through hydride abstraction. Nonetheless, with experience, such signature ions can be used for structural elucidation. Exact mass capabilities provided with HRAM-MS systems provide confidence in the elemental composition determination of each measured ion and is a valuable tool in the confirmation process.

Once the molecular ion is established, the next step of the identifi-
ever, when representative samples were reanalyzed using a HRAM-MS system, Figures 6 and 7, the proposed elemental composition was inconsistent with the previously assigned structure. The HRAM-MS data supported an elemental composition of C_{12}H_{28}O_{5}, and not the earlier proposed C_{11}H_{26}O. The mass difference between the two compositions is “only” 36 mmu (mDa); however, that is enough to confidently disprove the earlier proposed structure and propose an alternative structure.

**Non-Volatile Organic Components**

An LC–MS E&L data package should contain both positive ion and negative ion data sets to support detection and identification of as wide a range of components as possible. Positive and negative data can easily be generated in parallel in a single run on Orbital trap-based MS instrumentation (also known as **polarity switching**). The positive and negative data package is complementary. Unlike with many TOF-based MS instruments, polarity switching on Orbital trap-based MS is quick, which adds efficiency, maintains accurate mass/charge determination, and does not require lengthy stabilization times. With many TOF systems, a second analytical run must typically be performed to generate data in both positive and negative modes, thus doubling E&L analysis times and using precious sample.

An example is presented in Figure 8, related to an Igmanox-1010 degradation study. Such studies are essential for generating a wide variety of degradation products, and they are an excellent basis for a compound specific database.

For LC–MS, electrospray (ESI) or atmospheric pressure chemical ionization (APCI) is generally used. Resulting spectra typically contain either protonated pseudomolecular ions or other cation adducts (for negative ion detection usually deprotonated species are being observed). The observed mass error is below 1 ppm in the presented example, which provides excellent support to assign elemental composition. The additional capability to perform CID fragmentation with HRAM data acquisition mode provides a valuable tool for structure elucidation. To provide even a tentative chemical structure for toxicological risk assessment when authentic reference standard is not available is the only option for high resolution based analytical packages.

**Data Processing**

LC–MS data sets represent additional challenges over GC–MS data sets when it comes to component identification for multiple reasons:

- The ionization for LC–MS is typically soft (ESI or APCI) resulting in molecular ions or quasi molecular ions with little fragmentation to support confident library searching.
- Databases and spectral libraries are not readily available (13).
- Additional MS/MS modes are required to generate fragmentation either by collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD), which provide information for substructural characterization.
- MS/MS dissociation energy values vary by instruments and vendors. Therefore, it is difficult to have a consistent fragmentation library.

To counter these challenges, some instrument vendors are building LC–MS HRAM mass spectral libraries, such as the free-to-search mzCloud (15) database from HighChem. These are particularly useful. However, they are currently limited to approximately 200 E&L compounds with approximately 40,000 E&L compound HRAM-MS and MS/MS spectra.

Spectral interpretation is an important step in component identification assignment, especially where the library search does not provide any usable hits. The interpretation is often augmented with software packages, such as Thermo Scientific Mass Frontier Software and Thermo Scientific Compound Discoverer Software. These software packages provide tentative interpretation for ions present in the spectra, and assist the analytical expert with fragmentation assignment. These software packages can be applied to both EI fragmentation spectra and CID fragmentation spectra for both GC and LC–MS. An example of GC–MS peak interpretation is presented in Figure 9, where spectral peaks present in the CID spectra of C_{21}H_{40} rubber oligomer for the molecular ion of m/z 292 fragments are assigned, to propose a reasonable and justifiable structure.

Many of the software products available to support compound identification providing the following features to the scientist:

- automated deconvolution
- component retention time alignment
- unknown peak detection
- integrated compound identification
- elemental composition
- automated parallel searching of multiple libraries; including online libraries such as ChemSpider and mzCloud.
- The ideal software product should support data analysis from MS/MS data across multiple instrument platforms such as orbital trap or ToF, with full fragment interpretation toolset.

**Method Validation**

Analytical method validation is a critical part to complete the leachable assessment. It is recommended that this part of the testing be completed under cGMP rules and regulations. Analytical method validation provides objective evidence that the analytical method designed for quantitative assessment of a qualified leachable is specific for the target analytes, has sufficient detection or quantitation limit, accuracy, precision, and linearity. The method validation part only addresses identified target components. If certified reference standard is not available, an appropriate surrogate with strong scientific justification can be used (7). The method validation
can be a straightforward analytical exercise at part-per-million (ppm) level, or extremely challenging at parts-per-billion (ppb) or parts-per-trillion (ppt) levels. The level of validation required depends on the daily administered dose, and the permissible daily exposure of the component; however, in some cases, the regulatory agency can recommend testing at even lower levels to minimize the potential toxicological risk to patients.

MS is often required to achieve sufficient specificity at sub-ppm levels. However, use of mass spectrometry presents certain challenges. U.S. FDA published a guidance several years ago (16), and the EU has a comprehensive guidance as well (9). The messages for the analytical chemists are:

- When a mass spectrometer is being used as a detection method, to achieve specificity for various matrices, multiple ions (ion ratios) need to be monitored (1 quantifier and 2 or 3 qualifiers).
- Multiple stage (MS/MS) or HRAM detection is preferred if possible to achieve 4 points of identification (10, 18).
- Acceptance criteria for method precision or reproducibility depends on the analyte concentration level. Lower levels can use larger acceptance ranges (1 ppm level is about 10%; for 1 ppb level, 25–30% is acceptable) (11,12).
- Accuracy (recovery) is in a range of 80 to 110% at 1 ppm level, and in a range of 40 to 120% at 1 ppb level (11,12).
- S/N based LOD sometimes cannot be calculated as the noise can be zero (for example, HRAM based methods), therefore an attempt to divide a number with zero generates an undefined mathematical operation. To avoid the “zero noise issue,” IDL or MDL (instrument detection limit or method detection limit) can be calculated based on a statistical approach using as one-tail Student-t test (17,18).

It is an important message for trace level validation that the system blank is almost never a flat baseline. If modern systems are performing well, trace levels of common target analytes can typically be detected in the laboratory background.

**Summary**

The analytical solutions presented in this paper provide a highly effective and powerful workflow for testing extractables and leachables for regulatory submissions.

Orbital trap-based HRAM mass spectrometry coupled to gas or liquid chromatography provides a powerful tool supporting rapid and confident identification on unknown leachable components.

**Disclaimer**

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Comprehensive Gas Chromatography Coupled to Simultaneous Dual Detection (TOF-MS/FID) as a Confirmatory Method for MOSH and MOAH Determination in Food

Mineral oil hydrocarbons (MOHs) are very complex mixtures of isomers mainly associated with two classes of compounds: mineral oil saturated hydrocarbons (MOSHs) and mineral oil aromatic hydrocarbons (MOAHs). The analysis of such contaminants in food is a challenging task, especially without a mass-spectrometry-based confirmatory method. Using gas chromatography–mass spectrometry (GC–MS) alone fails, because the MS spectra of the fractions considered and interferences are often difficult, if not impossible, to distinguish. Therefore, the use of comprehensive multidimensional GC (GC×GC) with parallel dual detection, namely a flame ionization detector (FID) and time-of-flight–MS (TOF–MS), has been proposed. The enhanced chromatographic separation, along with the full mass spectra information, allows reducing the detection of false positives. The method was optimized to obtain completely overlapped two-dimensional (2D) plots from the two detectors, simplifying the use of a classification tool, including a spectrum-based filter, to translate the findings in the MS to the FID trace, and thus correct any quantification issue due to coelution with naturally present components.

Sebastiano Pantò, Maurine Collard, and Giorgia Purcaro

Mineral oil hydrocarbon (MOH) contamination has been described as a complex mixture of isomers mainly related to two classes of compounds, namely mineral oil saturated hydrocarbons (MOSHs) and mineral oil aromatic hydrocarbons (MOAHs). The former class includes aliphatic hydrocarbons, both linear and branched (alkanes and isoparaffins), and cyclic compounds (cycloalkanes or naphthenes) with possible lateral alkylation. The MOAH class includes one or more benzene rings, mainly alkylated, which usually represent up to 25% of the MOSH fraction (1). Humans can be exposed to MOH through ingestion, skin contact, or inhalation, but the former is the most common. Many sources of food contamination can be listed, either as intended and unintended. A well-presented compendium can be found in (2).

Although still under investigation, the toxicity of MOSH and MOAH is of different concern. MOAHs, mainly the three-to-seven ring polycyclic aromatic compounds with a low degree of alkylation, are potentially mutagenic and tumor promoters (1,3). More de-
bate is open on the effects of MOSH: those in the C16 to C35 range can be selectively accumulated and cause microgranulomas in several rat tissues (such as lymph nodes, spleen, and liver) (1,4,5). Nevertheless, the correlation between exposition and granuloma formation or inflammatory response is still not clear, and data on humans are scarce (6).

The European Commission has required an effort to re-evaluate the toxicology of MOH based on a detailed characterization rather than on physicochemical properties (1), and to gather more information regarding the occurrence and possible sources of contamination in food (7). In this regard, the focus is on the analytical methods for MOSH and MOAH determination, which is rather demanding, not just in terms of sample preparation (8–10). Both MOSH and MOAH appear on a chromatographic trace as an unresolved complex mixture (UCM) or a “hump.” Several off-line methods have been proposed (11–13), the method of election for MOH determination is hyphenated liquid–gas chromatography (LC–GC) method (14–16). Although the method is very efficient, many interferences from different sources (including, but not limited to, polyolefins from plastic, carotenoids, olefins, and terpenoids from the sample itself), may be present in both of the fractions, which, in some cases, are not easily distinguished by the MOSH and MOAH UCMs, thus leading to possible false positives. Complicating the scenario even more is the lack of a confirmatory method with MS as requested by Decision 2002/657 (17). The lack of standards requires the use of a flame ionization detector (FID) for quantification rather than MS, because MS virtually provides the same response for all hydrocarbons (18). Such an issue is a hot topic of discussion in the field, and, although a GC–MS has been proposed to fill the gap, the lack of specific diagnostic fragments to differentiate MOSH and MOAH from other possible contaminants or analogous (such as MORE, mineral oil refined product, PAO, polyalphaolefins, POSH, and polymer oligomeric hydrocarbons) leaves the issue open (2,19,20). The use of comprehensive multidimensional GC (GC×GC) has been proposed (21,22), and, until now, it seems the most promising solution. However, all the applications using GC×GC had been presented and optimized before the publication of the last Joint Research Center (JRC) Guidelines in 2019 (23), which required extending the data reported to the C10 to C50 range.

The aim of this work is to investigate the potential of a GC×GC system coupled in parallel to an FID and time-of-flight (TOF) MS as a confirmation tool for MOSH and MOAH analysis within the JRC guidelines requirements.

**Materials and Methods**

**Reagents, Standards, and Samples**

All the solvents were purchased from MilliporeSigma. The n-hexane, acetone, and dichloromethane were distilled before use.

The MOSH and MOAH internal standards, containing 5α-cholestane (Cho, 0.6 mg/mL), n-C11 (0.3 mg/mL), n-C13 (0.15 mg/mL), cyclohexyl cyclohexane (CyCy, 0.3 mg/mL), n-pentyl benzene (5B, 0.30 mg/mL), 1-methyl napthalene (1-MN, 0.30 mg/mL), 2-methyl napthalene (2-MN, 0.30 mg/mL), tri-tert-butyl benzene (TBB, 0.3 mg/mL) and perylene (Per, 0.6 mg/mL) in toluene, were provided by Restek. The MOSH/MAOH retention time standard, containing a standard mixture of n-alkane C10-C40 (50 mg/L each) plus C50 was also from Restek.

All the glassware was carefully washed and rinsed with distilled solvents (acetone and n-hexane) before use.

Palm oil and a spice extract were provided by a private laboratory (which asks to not be disclosed).

**GC×GC-FID/TOF-MS Analysis**

The primary column was a Rxi-17Sil MS 12 m × 0.25 mm i.d. × 0.25-μm df connected through a tee-union (Agilent) to two Rxi-1 MS 1.4 m × 0.1-mm i.d. × 0.1-μm df linked to the FID and the TOF-MS.

GC oven temperature program: 50 °C (hold 1 min) to 350 °C (hold 1 min) at 5 °C/min. A 10 °C offset was applied between the first and secondary ovens. The carrier gas, helium, was supplied at an initial pressure of 275 kPa (constant flow).

Injection temperature: 310 °C. Injection mode and volume: splitless injection (1 min); 3μL. Modulation was performed every 10 s, applying variable hot and cold pulse durations based on the wide range of volatilities in the sample. The FID was operated as follows: H2 flow: 40 mL/min; airflow: 400.0 mL/min; make up (He): 30.0 mL/min; Temperature: 350 °C. MS parameters: 40–750 m/z; spectra generation frequency (TOF-MS and FID): 100 Hz; interface and ion source temperatures were 250 °C and 340 °C, respectively. MS ionization mode: electron ionization (EI) at 70 eV.

Data were acquired and elaborated using the ChromTOF brand software (v5.50).

**Results and Discussion**

**GC×GC-FID/TOF-MS: Chromatographic Optimization**

The optimization of the GC×GC system with dual detection started from a series of considerations, namely the separation efficiency, the sensitivity, and the goal to obtain a perfect match between FID and MS retention times. Based on the outcomes of previous work by Cordero and coworkers (24), the use of two secondary columns was selected to maximize separation efficiency and two-dimensional (2D) space exploitation. Differently from the calculations done elsewhere with quadrupole MS systems (22,24), the use of the TOF-MS mentioned above, which consists of a slightly longer transfer line (31 cm) required extra care regarding the flow and MS/FID split calculations. In fact, our initial calculation suggested the use of a 1.6 m × 0.1 mm segment con-
nected to the MS and 1.4 m × 0.1 mm to the FID, as also reported in (24) for a split between the two detectors of ~50% and a match of the retention times. However, the experimental results did not confirm the calculation, because an extra resistance has to be accounted for, due to the length of the transfer line constantly maintained at high temperature (340 °C). The final optimal column lengths were 1.4 m × 0.1 mm for both of the secondary columns. Although the use of pressure control mode rather than flow control mode for the carrier gas would have ensured a constant split all over the chromatographic area, the retention time match would have been negatively affected. Therefore, the flow control mode was used and the split ratio variation over the entire oven ramp (50–350 °C) was experimentally evaluated analyzing a mixture of n-alkanes in the GC×GC-TOF-MS/FID system and comparing the signal with the data obtained in a GC×GC-FID system, without any splitting device. An average splitting of 56/44 at FID-MS was measured, with a variation between C10 and C50 within the experimental variability of the three replicates (Figure 1). The chemical-class structure in the 2D chromatogram was maintained up to the elution of the C50.

The overall separation performance of the two parallel separations was compared measuring the peak capacity (n), the net separation used (S₁, S₂, and S₉₉×GC), and the percentage of usage (25–28). The results are reported in Table I, showing that the performance of the two parallel separations is highly comparable.

Finally, the alignment of the two plots obtained from the FID and the TOF-MS acquisition was positively confirmed on the base of 1D and 2D retention time of the MOSH and MOAH internal standards (Figure 2).

### Classification and Template Match

The good retention time matches between the MS and the FID trace made it possible to create a template model based on both the enhanced separation power of the GC×GC and the reliable information obtained from the TOF-MS trace. The classification of the different chemical families (n- and i-paraffins, cycloalkanes, 1-ring, 2-ring MOAH) was created simply by drawing the edges of each class within the GC×GC-TOF-MS contour plot. The latter was then applied to the GC×GC-FID trace for quantification purposes (Figure 3).

The idea behind the development of this strategy is to confirm the identity of the compounds of interest at the TOF-MS, but even more to exclude the presence of substances other than MOSH and MOAH that may lead to false positives or higher contamination values when examining the corresponding FID trace. In fact, when compounds other than the targeted ones are identified in the TOF-MS trace, the perfect retention time match with the FID trace allows to easily pick and remove them from the sum. This procedure can be simplified even more by applying an additional MS spectral filter based on custom-built spectral

#### Table I: 1D and 2D separation measure (S₁ and S₂, respectively), comprehensive separation measure (S₉₉×GC), peak capacity (n), and percentage of 2D space used.

<table>
<thead>
<tr>
<th>Performance Parameters</th>
<th>S₁</th>
<th>S₂</th>
<th>S₉₉×GC</th>
<th>n</th>
<th>% of Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>1423</td>
<td>100</td>
<td>142414</td>
<td>8901</td>
<td>87</td>
</tr>
<tr>
<td>FID</td>
<td>1495</td>
<td>94</td>
<td>140930</td>
<td>8808</td>
<td>89</td>
</tr>
</tbody>
</table>

![Figure 1](image1.png)

**Figure 1:** FID response (n = 3) obtained for a mixture of n-alkanes analyzed in the GC×GC-TOF-MS/FID system, normalized versus the response of the same mixture analyzed by GC×GC-FID.

![Figure 2](image2.png)

**Figure 2:** Reconstructed excel 2D plot on the basis of the 1D and 2D retention times of the MOSH and MOAH internal standard mixture in the FID and ToF-MS traces.
rules, such as the identification of a particular class of substances based on the presence of one or multiple diagnostic $m/z$ or ion ratios. As an example, all the compounds belonging to the family of $n$-$i$-paraffins can be easily identified by their characteristic main fragments 43 $m/z$ and 57 $m/z$. The same concept can be applied to as many as needed classes of components such as olefins, hopanes, steranes, dibenzothiophenes, DIPN, and terpenes. The extra filter was very useful in the case of classes of compounds eluted in the same 2D space, helping to quickly pinpoint the compounds belonging to one or the other class (Figure 4).

Application to Real-World Samples

The accurate determination of MOSH and MOAH in food is of high importance, since this contamination represents a concern from a toxicological viewpoint and an economic impact for food companies as well. Therefore, it is highly essential to avoid false positives to limit unnecessary alarmism. At the same time, as required by the EU, it would be beneficial to collect data on the occurrence of the different sub-classes within the MOSH and MOAH fractions, and to highlight the presence of possible markers of specific contamination sources to allow the stakeholders to take actions to prevent the contamination in the food chain. This last goal is challenging, because contamination may occur at different levels, from the raw materials to the final product and from primary or secondary packaging (2). The profile of the contamination may change during the processing of the food, making the identification of the source of contamination for corrective action very challenging. Moreover, a limited number of markers is known at present. Nevertheless, some consideration can be taken in this direction by carefully examining the results. For instance, the presence of POSH (deriving from plastic food contact material), as occurred in the spice sample, can be easily chromatographically separated in the GC×GC contour plot, without requiring expert evaluation of the LC–GC–FID trace and not causing possible overestimation of the amount of MOSH (Figure 5).

The coupling of the chromatographic separation with a sensitive MS instrument allowed us to simultaneously detect the presence
of hopanes (a well-known marker of petrogenic origin [29]) in the MOSH fraction of the palm oil sample.

In the same way, analyzing the results obtained from the MOAH fractions, several factors can be considered. The presence of diisopropylnaphthalenes (DIPN, markers of contamination from recycled paperboard fiber) was easily highlighted in the LC–GC-FID trace, although no confirmation was possible. The GC×GC-TOF-MS information made it possible to confirm the identity of such markers and, moreover, the presence of sulfur compounds was detected, pointing toward jute bags as an additional source of contamination, both for the spice and the palm oil (Figure 6).

Regarding the palm oil sample, it underwent an additional purification step, namely epoxidation, to remove the presence of interferences such as carotenoids, terpenoids, and olefin, which generally sit on top of the MOAH hump. In the LC–GC-FID chromatogram, the MOAH UCM appeared smooth with a limited number of well-shaped peaks on top of the hump, leading to the conclusion that the interferences were removed and that only the remaining few peaks needed to be removed for accurate quantification of the MOAH. However, based on the information obtained from the classification and the MS spectral filter feature explained in section 3.2, few compounds were classified as aromatic within the classification area (Figure 7). From a careful evaluation of the TOF-MS spectra of the peaks populating the 2D space in the aromatic classification area (the most abundant class of MOAH present), although not a univocal and precise identification was possible, it was clear that the peaks were not only belonging to the MOAH fraction, but specifically to terpenoids.

This is a particular critical finding, since it supports an already open discussion on the efficiency of the epoxidation process and doubts on many possible false positives found after carrying out such an extra purification step, which is known to cause the loss of MOAH themselves at a certain extent but that seems also to not be efficient in the removal of the interferents in some specific circumstances that have not been determined yet.

**Conclusion**

This work is a proof-of-concept study to prove the capabilities of the use of a GC×GC-TOF-MS/FID system as a confirmatory method of the occurrence of MOSH and MOAH contamination in food. For the first time, a method able to maintain the chemical-structure of the 2D plot up to C50 was presented. Moreover, the ultimate goal will be to provide a method that can be used both for routine and confirmatory purposes. Research is ongoing to validate the quantification based on the GC×GC-FID trace, possibly corrected based...
Comparison of the LC–GC-FID chromatogram and the GC×GC-ToF-MS/FID of the MOAH fraction of the palm oil sample. The MS spectrum of a peak eluted in the diaromatic classification area of the chromatogram but not belonging to this class is highlighted in the box.

on the information deriving from use of the TOF-MS instrument.

Acknowledgment
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References
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Oil-based tinctures are an increasingly popular means of consuming cannabis, because they are relatively simple to both dose and intake. These tinctures are generally formulated by dissolving cannabis concentrates in medium chain triglyceride (MCT) oil (1). The cannabis concentrates are prepared by the extraction of cannabinoids and other compounds from cannabis plant material using solvents such as butane, ethanol, and supercritical carbon dioxide (2). This preparation concentrates not only cannabinoids, but also any pesticides and mycotoxins found on the plant (3). Both pesticides and mycotoxins present a significant public health risk, and therefore robust analysis of cannabis products must be implemented across the supply chain to protect consumers. With the changing legal nature of the cannabis drug, stricter regulations for cannabis products are coming into force. California has now enforced some of the most stringent regulations, including maximum residue limits for a total of 66 pesticides and 5 mycotoxins in cannabis products in the United States (4). While sample preparation methods have been reported for extracting pesticides from cannabis (such as “quick, easy, cheap, effective, rugged, and safe” [QuEChERS] methods with solid-phase extraction [SPE] and dispersive SPE [dSPE]), these methods are time consuming, expensive, slow, and show poor

A novel liquid chromatography–tandem mass spectrometry (LC–MS/MS) method with a dual electro-spray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) source was developed for analysis of 66 pesticides and 5 mycotoxins regulated by the State of California in medium-chain triglyceride (MCT) oil cannabis tinctures. The limits of quantitation (LOQs) of all of the pesticides and mycotoxins were well below California state action limits of these analytes in cannabis-based non-inhalable or edible products. A total of 62 (out of 66) pesticides and 5 mycotoxins were analyzed using LC–MS/MS with an ESI source, and the remaining 4 pesticides were determined using LC–MS/MS with an APCI source. A simple, fast, and cheap acetonitrile solvent extraction method was used for sample preparation to get good recovery and achieve high throughput for this analysis. For late-eluted analytes, a number of internal standards were used to compensate for ion suppression effects from the hydrophobic matrix.

Avinash Dalmia, Charles Johnson, Saba Hariri, Jacob Jalali, Erasmus Cudjoe, Joey Kingstad, and Feng Qin
recoveries for a few analytes, such as daminozide and others (5–8). Furthermore, many of the existing pesticide analysis methods rely on the use of both liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography–tandem mass spectrometry (GC–MS/MS), substantially increasing the cost, complexity, and turnaround time of analysis (8,9).

This article presents a novel, cost-effective, and fast LC–MS/MS method to analyze pesticides and mycotoxins in edible cannabis products such as MCT oil tinctures, with low detection limits and good recoveries. The method uses a simple solvent extraction and LC–MS/MS method with a dual electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) source, making it possible to detect the very hydrophobic and chlorinated pesticides that are typically analyzed using GC–MS/MS. With this new method, it was possible to measure all of the 66 pesticides and 5 mycotoxins spiked into MCT oil tinctures well below the maximum residue limits specified by the State of California in cannabis non-inhalable or edible products (4).

**Experimental Hardware/Software**
Chromatographic separation was conducted on a PerkinElmer LX50 ultra-high-performance liquid chromatography (UHPLC) system, and detection was achieved using a PerkinElmer Q-Sight 420 MS/MS detector with a dual ionization ESI and APCI source, which operates independently with two separate inlets. All instrument control, data acquisition, and data processing was performed using the Simplicity 3Q software platform.

**Sample Preparation Method**
Below is the step-by-step sample preparation procedure with a 50-fold dilution for the ESI method and a 30-fold dilution for the APCI method:
1. Approximately 5 g of MCT oil cannabis tincture was weighed out as a representative for each sample batch.
2. One gram of the representative sample was weighed out and placed into a 50 mL centrifuge tube.
3. Next, 29 mL of LC/MS grade acetonitrile with 0.1% formic acid was added to the tube, which was then capped. The formic acid was added to the acetonitrile to minimize degradation of a number of analytes including capton, ochratoxin A, and several others.
4. The tube was placed in a multitube vortex mixer and allowed to vortex for 10 min.

### Table I: LC Method and MS source conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Parameter Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LC Conditions</strong></td>
<td></td>
</tr>
<tr>
<td>LC Column</td>
<td>PerkinElmer Quasar SPP Pesticides (4.6 × 100 mm, 2.7 µm)</td>
</tr>
<tr>
<td>Mobile Phase A (ESI method)</td>
<td>2 mM ammonium formate + 0.1% formic acid (in water)</td>
</tr>
<tr>
<td>Mobile Phase B (ESI method)</td>
<td>2 mM ammonium formate + 0.1% formic acid (in methanol)</td>
</tr>
<tr>
<td>Mobile Phase A (APCI method)</td>
<td>LC–MS grade water</td>
</tr>
<tr>
<td>Mobile Phase B (APCI method)</td>
<td>LC–MS grade methanol</td>
</tr>
<tr>
<td>Mobile Phase Gradient</td>
<td>The run time for the optimized gradient elution method including analytical column reconditioning was 18 mins for the ESI method, and 6 mins for the APCI method. The final method ensured separation of the MCT oil cannabis tincture matrix from the analytes for improved quantitation.</td>
</tr>
<tr>
<td>Column Oven Temperature</td>
<td>45 ºC</td>
</tr>
<tr>
<td>Auto sampler Temperature</td>
<td>20 ºC</td>
</tr>
<tr>
<td>Injection Volume</td>
<td></td>
</tr>
<tr>
<td>Injection Volume (ESI method)</td>
<td>3.0 µL for the LC–MS/MS method with the ESI source</td>
</tr>
<tr>
<td>Injection Volume (APCI method)</td>
<td>10 µL for the LC–MS/MS method with the APCI source</td>
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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Parameter Setting</th>
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</thead>
<tbody>
<tr>
<td><strong>MS Source Conditions for the ESI and APCI sources</strong></td>
<td></td>
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<tr>
<td>ESI Voltage (Positive)</td>
<td>+5100 V</td>
</tr>
<tr>
<td>ESI Voltage (Negative)</td>
<td>-4200 V</td>
</tr>
<tr>
<td>APCI Corona Discharge</td>
<td>-3 µA</td>
</tr>
<tr>
<td>Drying Gas</td>
<td>150 arbitrary units</td>
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<td>Nebulizer Gas</td>
<td>350 arbitrary units</td>
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<tr>
<td>Source Temperature (ESI method)</td>
<td>315 ºC</td>
</tr>
<tr>
<td>Source Temperature (APCI method)</td>
<td>250 ºC</td>
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<td>HSID Temperature (ESI method)</td>
<td>200 ºC</td>
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<td>HSID Temperature (APCI method)</td>
<td>180 ºC</td>
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<tr>
<td>Detection mode</td>
<td>Time-managed MRM</td>
</tr>
</tbody>
</table>
Table II: LOQs and action levels for California Category II Pesticides in MCT oil cannabis tinctures analyzed by an LC–MS/MS method. **Red/Green**: Pesticides typically analyzed by GC–MS/MS, **Red/Black**: Pesticides analyzed on LC–MS/MS with ESI source, **Green**: Pesticides analyzed on LC–MS/MS with APCI source

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Category II</th>
<th>LOQ</th>
<th>Action Level (µg/g)</th>
<th>Action Level/LOQ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>LC–MS/MS (µg/g)</td>
<td>%CV (n = 7)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Abamectin</td>
<td>0.2</td>
<td>15.5</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>Acephate</td>
<td>0.025</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Acequinocyl</td>
<td>0.05</td>
<td>9.2</td>
<td>4</td>
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<tr>
<td>4</td>
<td>Acetamiprid</td>
<td>0.005</td>
<td>4.5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Azoxystrobin</td>
<td>0.005</td>
<td>6.9</td>
<td>40</td>
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<tr>
<td>6</td>
<td>Bifenazate</td>
<td>0.010</td>
<td>5.7</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Bifenthrin</td>
<td>0.025</td>
<td>10.7</td>
<td>0.5</td>
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<td>8</td>
<td>Boscalid</td>
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<td>9</td>
<td>10</td>
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<td>9</td>
<td>Captan</td>
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<td>5</td>
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<tr>
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<td>Carbaryl</td>
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<td>8.4</td>
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<td>0.01</td>
<td>10.2</td>
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<td>Clofentezine</td>
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<td>8.5</td>
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<td>Diazinon</td>
<td>0.01</td>
<td>7.9</td>
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<td>16</td>
<td>Dimethomorph</td>
<td>0.01</td>
<td>8.4</td>
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<td>Etoxazole</td>
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<td>Fenhexamid</td>
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<td>Fenpyroximate</td>
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<td>Fonicamid</td>
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<td>2</td>
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<tr>
<td>21</td>
<td>Fludioxonil</td>
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<tr>
<td>22</td>
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<td>Imidacloprid</td>
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<tr>
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<tr>
<td>25</td>
<td>Malathion</td>
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<td>0.5</td>
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<td>31</td>
<td>Pentachloronitrobenzene</td>
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<td>Permethrin</td>
<td>0.025</td>
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<td>33</td>
<td>Phosmet</td>
<td>0.01</td>
<td>7.1</td>
<td>0.2</td>
</tr>
<tr>
<td>34</td>
<td>Piperonylbutoxide</td>
<td>0.01</td>
<td>4.0</td>
<td>6.6</td>
</tr>
<tr>
<td>35</td>
<td>Prallethrin</td>
<td>0.01</td>
<td>7.9</td>
<td>0.4</td>
</tr>
<tr>
<td>36</td>
<td>Propiconazole</td>
<td>0.01</td>
<td>9.3</td>
<td>20</td>
</tr>
<tr>
<td>37</td>
<td>Pyrethrins</td>
<td>0.01</td>
<td>11.1</td>
<td>1</td>
</tr>
<tr>
<td>38</td>
<td>Pyridaben</td>
<td>0.01</td>
<td>8.4</td>
<td>3</td>
</tr>
<tr>
<td>39</td>
<td>Spinetoram</td>
<td>0.01</td>
<td>9.5</td>
<td>3</td>
</tr>
<tr>
<td>40</td>
<td>Spinosad</td>
<td>0.01</td>
<td>13.6</td>
<td>3</td>
</tr>
<tr>
<td>41</td>
<td>Spiromesifen</td>
<td>0.025</td>
<td>6.6</td>
<td>12</td>
</tr>
<tr>
<td>42</td>
<td>Spirotetramat</td>
<td>0.01</td>
<td>5.7</td>
<td>13</td>
</tr>
<tr>
<td>43</td>
<td>Tebuconazole</td>
<td>0.01</td>
<td>5.7</td>
<td>2</td>
</tr>
<tr>
<td>44</td>
<td>Thiamethoxam</td>
<td>0.010</td>
<td>4.3</td>
<td>4.5</td>
</tr>
<tr>
<td>45</td>
<td>Trifloxystrobin</td>
<td>0.005</td>
<td>7.5</td>
<td>30</td>
</tr>
</tbody>
</table>
Table III: LOQs and action levels for California Category I pesticides in MCT oil cannabis tinctures analyzed by LC–MS/MS. 

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Category I Residual Pesticide</th>
<th>LOQ (µg/g)</th>
<th>%CV (n = 7)</th>
<th>Action Level (µg/g)</th>
<th>Action Level/LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aldicarb</td>
<td>0.050</td>
<td>13.9</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Carbofuran</td>
<td>0.005</td>
<td>7.8</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Chlorfenvyr</td>
<td>0.015</td>
<td>10.2</td>
<td>0.1</td>
<td>6.6</td>
</tr>
<tr>
<td>4</td>
<td>Chlorpyrifos</td>
<td>0.01</td>
<td>14.9</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Coumaphos</td>
<td>0.01</td>
<td>10.1</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Daminozide</td>
<td>0.01</td>
<td>11.9</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>DDVP (Dichlorvos)</td>
<td>0.025</td>
<td>10.3</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>Dimethoate</td>
<td>0.01</td>
<td>9.3</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>Ethoprop(hos)</td>
<td>0.01</td>
<td>12.2</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>Etofenprox</td>
<td>0.01</td>
<td>11.5</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Fenoxy carb</td>
<td>0.005</td>
<td>5.5</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>Fipronil</td>
<td>0.025</td>
<td>19.2</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>13</td>
<td>Imazalil</td>
<td>0.010</td>
<td>16.3</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>14</td>
<td>Methiocarb</td>
<td>0.010</td>
<td>9.2</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>Methyl parathion</td>
<td>0.015</td>
<td>7.5</td>
<td>0.1</td>
<td>6.6</td>
</tr>
<tr>
<td>16</td>
<td>Mevinphos</td>
<td>0.01</td>
<td>10.1</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>17</td>
<td>Paclorbutrazol</td>
<td>0.01</td>
<td>4.3</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>18</td>
<td>Propoxur</td>
<td>0.01</td>
<td>8.6</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>19</td>
<td>Spiroxamine</td>
<td>0.005</td>
<td>9.2</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>Thiacloprid</td>
<td>0.005</td>
<td>9.5</td>
<td>0.1</td>
<td>20</td>
</tr>
</tbody>
</table>

5. The extract was then centrifuged in the tube for 10 min at 3000 rpm.
6. Following centrifugation, the supernatant was filtered into another 50 mL centrifuge tube using a PTFE syringe filter. The 50 mL centrifuge was then capped.
7. The tube was labeled with the sample ID.
8. For the APCI method, 990 µL of extracted sample (from step 7) was transferred into a 2 mL HPLC vial, which was then spiked with 10 µL of internal standard.
9. For the ESI method, 600 µL of extracted sample (from step 7) was transferred into a 2 mL HPLC vial, which was then spiked with 10 µL of internal standard and diluted with 390 µL of LC–MS grade acetonitrile with 0.1% formic acid. The HPLC vial was then mixed on a vortex mixer.
10. The sample was injected for LC–MS/MS analysis.

**LC Method and MS Source Conditions**

The LC method and MS source parameters are shown in Table I.

**Results and Discussion**

**Detectability and Reproducibility**

With this LC–MS/MS method, 62 out of 66 pesticides and 5 mycotoxins were analyzed using the ESI source, and the remaining 4 pesticides regulated by the State of California were measured with the APCI source. According to the California Bureau of Cannabis Control’s text of regulations, the 66 pesticides are divided into categories I and II. Category I pesticides are banned for use on cannabis, due to the risks they present to human health and the environment, whereas category II pesticides are eligible for use on cannabis plants. This categorization reflects the varying action limits and corresponding limit of quantitation (LOQ) requirements for these pesticides and five mycotoxins in different cannabis-related products (4). The LOQs and response reproducibility at LOQ level for each of the pesticides categorized and mycotoxins in MCT oil cannabis tinctures are summarized in Table II, III and IV. The LOQs were determined by ensuring that the signal-to-noise ratio for each analyte was greater than 10. The response relative standard deviation (RSD) for each pesticide and mycotoxin at its LOQ level in the cannabis matrix was less than 20% for seven replicates. As demonstrated in Tables II–IV, the LOQs determined in this study are well below the California action limits (by a factor of 1.5 to 8000) for all of the analyzed pesticides and mycotoxins. This shows that the method is sufficiently sensitive and reproducible for pesticide and mycotoxin analysis in MCT oil cannabis tinctures at the regulatory limits specified by the State of California. In addition, the matrix-matched calibration curves showed excellent linearity for all of the analytes in the tinctures with coefficients of determination ($R^2$) greater than 0.99.
Recovery Studies with Solvent Extraction

Sample preparation is the main bottleneck for the high throughput analysis of pesticides and mycotoxins in cannabis-related matrices. Traditional sample preparation techniques like SPE and QuEChERS are time-consuming and laborious, requiring multiple steps and expensive sorbents, with low recovery for some pesticides (5–8). Solvent extraction was therefore used in this method as a cheap, fast, and simple alternative that could achieve high extraction recovery. The cannabis tincture samples were tested to confirm the absence of pesticides and mycotoxins before they were spiked at two levels (low and high) for all pesticides (0.1 and 1 µg/g) and mycotoxins (0.02 and 0.2 µg/g). These spiked samples were used to determine pesticide and mycotoxin recovery. The absolute recoveries of all 66 pesticides and 5 mycotoxins at two different levels were within an acceptable range of 80 to 120% with an RSD less than 20%. No recovery data was obtained for the pesticides captan, abamectin, and cyfluthrin at the lower level of 0.1 µg/g, since the LOQ of these pesticides is higher than 0.1 µg/g.

Matrix Effects

The hydrophobic nature of the MCT oil matrix can lead to significant matrix effects. The ion suppression or enhancement was therefore calculated on the signal of the 62 pesticides and 5 mycotoxins analyzed with the ESI source. The calculation was performed by taking the difference between the signal of the analyte in the matrix and clean solvent, and dividing it by the signal in clean solvent. Figure 1 shows these calculated matrix effects as a function of the analyte retention time on the column. Among the 67 analytes measured with the ESI source, only 3 showed slight signal enhancement, while the rest showed signal suppression effects. Since the MCT oil cannabis tincture matrix is very hydrophobic, significant matrix ion suppression effects of <-20% were only observed for five late-eluting analytes. For those analytes measured with the APCI source, the matrix effects were insignificant (between -20 to 20%) for four analytes. It is generally understood in the literature that fewer matrix effects are observed with the APCI source in comparison to the ESI source (10–12).

For the five analytes measured with the ESI source that showed significant ion suppression effects of less than -20% (cypermethrin, fenpyroximate, etofenprox, bifenthrin, and acequincycl), deuterated internal standards were used. The internal standards were selected to improve the quantitative analysis as well as the overall recovery by compensating for the matrix ion suppression effects and correcting for any analyte loss during sample preparation. Figure 2a shows the overlay of the bifenthrin signal in a clean standard solvent and an extract of MCT oil cannabis tinctures spiked with bifenthrin before and after extraction. The recovery from the sample preparation is calculated by taking the percentage ratio of the signal of analyte in prespiked extract to the signal in solvent standard. Figure 2a clearly demonstrates that the overall recovery of bifenthrin without internal standard is about 29% due to significant ion suppression effects.

Table IV: LOQs and action levels for mycotoxins in MCT oil cannabis tinctures analyzed by LC–MS/MS.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Category II Mycotoxin</th>
<th>LOQ LC–MS/MS (µg/g)</th>
<th>%CV (n = 7)</th>
<th>Action Level (µg/g)</th>
<th>Action Level/LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ochratoxin A</td>
<td>0.005</td>
<td>15.9</td>
<td>0.020</td>
<td>4</td>
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<tr>
<td>2</td>
<td>Aflatoxin B1</td>
<td>0.002</td>
<td>13.7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>Aflatoxin B2</td>
<td>0.002</td>
<td>17.1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>Aflatoxin G1</td>
<td>0.002</td>
<td>18.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>Aflatoxin G2</td>
<td>0.002</td>
<td>17.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>Aflatoxin (B1+B2+G1+G2)</td>
<td>0.008</td>
<td>NA</td>
<td>0.020</td>
<td>2.5</td>
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</tbody>
</table>
caused by the matrix. Bifenthrin-D5, a deuterated analog of bifenthrin, was added in fixed amounts to both the solvent standard and the cannabis tincture matrix to compensate for these effects. The internal standard has a similar chemical structure to the analyte and its elution and ionization efficiency (or ion suppression effect) is therefore very similar to the analyte in both the solvent standard and the sample matrix, meaning the response ratios of the analyte to the internal standard is also similar. According to experimental results shown in Figures 2b and 2c, use of internals standard significantly increased the overall recovery of bifenthrin (calculated based on the extracted concentration of pre-spiked analyte versus the neat solution [unextracted]concentration) from 29% to 101% due to the correction of matrix effects. Finally, the overall recoveries of 70 to 120% with RSD values of less than 20% were achieved for all of the 66 pesticides and 5 mycotoxins with addition of internal standards to MCT oil cannabis tincture matrix.

**Sensitivity and Selectivity of Analysis for Non-Polar Analytes Using an APCI Source**

Several of the hydrophobic and non-polar pesticides (pentachloronitrobenzene [PCNB], chlorfenapyr, methyl parathion, and chlordane) found on the California action list are traditionally analyzed by GC–MS/MS because they do not ionize effectively when analyzed using LC–MS/MS with an ESI source. These analytes either have low proton affinity or lack hydrogen atoms, and are difficult to ionize by an ESI source in either positive or negative ion mode. An APCI ion source is much better suited for ionization of very hydrophobic and nonpolar analytes, and this source was therefore used to determine the detection limits of these hydrophobic analytes in cannabis tinctures. Using a fast 6-min LC–MS/MS method with an APCI source developed earlier by this author’s group (13), the LOQs of PCNB, methyl parathion, chlorfenapyr, and chlordane in cannabis tincture were in the range of 0.015 to 0.045 µg/g, making the method suitable for pesticide analysis in cannabis edible or non-inhalable products. Figure 3a shows an excellent signal-to-noise ratio (S/N = 38) for PCNB spiked at 0.045 µg/g in the cannabis matrix using an LC–MS/MS system with an APCI source. This demonstrates the extreme sensitivity of the APCI method.

Based on the Food and Drug Administration (FDA) method validation guidelines to determine selectivity of analyses, the acceptance criteria for selectivity is that matrix blanks should be free of any matrix interference peaks at the retention time of an analyte (14). In Figure 3b, the blank cannabis tincture matrix response for PCNB shows low background signal with random electrical noise and no matrix interference peak at the retention time of PCNB. This demonstrates that the measurement of PCNB in cannabis tincture matrix is quite selective. Similarly, the matrix blank signal for the other three pesticides measured with

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**Figure 2**: (a) Overlay of response of bifenthrin in solvent (red) and MCT oil cannabis tincture matrix spiked with bifenthrin before (green) and after (blue) extraction without any internal standard. The response ratio (RR) of bifenthrin spiked in cannabis tincture matrix before extraction to solvent standard was 0.29. The response ratio of bifenthrin spiked in cannabis tincture matrix before and after extraction was 0.96. (b) Overlay of response of bifenthrin (green) and bifenthrin-D5 internal standard (red) in pre-spiked cannabis tincture matrix with a RR of 0.168 for analyte to internal standard. (c) Overlay of response of bifenthrin (green) and bifenthrin-D5 internal standard (red) in solvent with a RR of 0.167 for analyte to internal standard.
an APCI source showed no matrix interference peaks at the retention times of these analytes. Figure 4 shows excellent linearity of PCNB response over a concentration range of 0.5 to 100 ppb (corresponding to 15 to 3000 ppb in cannabis tincture) in 30x diluted cannabis tincture extract with an $R^2 = 0.9992$. Since the regression fit value for PCNB is greater than 0.99, it easily meets the requirement of the California Bureau of Cannabis Control for regression fits to be higher than 0.99 (4). The accuracy of the calibration curve was checked by comparing back-calculated concentrations from a calibration curve with known concentrations of PCNB, and the strict criterion of maximum deviation of 10% was met for all concentration levels. Previously, it was claimed without any experimental data that analysis of PCNB with an APCI source may not be selective and may require a quadratic calibration curve with a poor correlation coefficient (15,16). However, this experimental work demonstrates a robust APCI method that exhibits excellent sensitivity, selectivity, and linearity of PCNB analysis in a cannabis sample.

**Ionization Mechanism of PCNB with an APCI Source**

For the ionization of compounds using an APCI source in negative ion mode, different ionization mechanisms such as proton abstraction, anion adduction, electron capture, and dissociative electron capture have been proposed in the past (17). It has been demonstrated that chlorinated nitrobenzene compounds can form phenoxide ions under negative APCI conditions (18). Similarly, we have previously proposed the following mechanism for ionization of PCNB with an APCI source in negative ion mode (19).

$$O_2^- + e^- \rightarrow O_2^-$$

$$M + O_2^- \rightarrow [M - Cl + O]^+ + ClO$$

Where M is PCNB.

Herein, the formation of $[M-CI+O]^+$ can be attributed to the formation of a superoxide ion ($O_2^-$) by electron capture followed by its chemical reaction with PCNB. This mechanism can be explained further by analyzing mass spectra for PCNB analyzed with an APCI source, which revealed a monoisotopic peak at a nominal mass of 274 Da. The nominal monoisotopic mass of a PCNB molecule is 293 Da and the observed mass loss of 19 Da can be explained by the loss of chlorine (nominal monoisotopic mass of 35 Da) and the addition of oxygen (nominal monoisotopic mass of 16 Da) to form a negatively charged ion of PCNB. Also, experimentally observed isotope patterns of the PCNB ion matched very closely to theoretical isotope patterns of the PCNB ion with four chlorine atoms, which further hinted that the PCNB loses one chlorine atom in an APCI ion source. Low mass spectra of an APCI ion source were checked to confirm the formation of superoxide reagent ion species that could react with PCNB to ionize it. We observed that the signal for both superoxide ion ($O_2^-$) and PCNB increased by a factor of roughly 300 and 30, respectively, when the mobile phase was changed from a mixture of methanol and water with 0.1% formic acid and 2 mM ammonium formate to a mixture of methanol and water. This further demonstrated that the superoxide ion plays an important role in the ionization of PCNB in an APCI source.
Conclusions
This study demonstrates a unique, quantitative, rapid, and reliable LC–MS/MS method for the analysis of 66 pesticides and 5 mycotoxins residues in cannabis tinctures. The proposed solvent extraction method is suitable for laboratories to achieve high throughput analysis, with good overall recovery (70–120%; RSD <20%) of all pesticides and mycotoxins. This method enabled identification and quantification of all analytes at low levels (0.002 to 0.5 µg/g), which is well below the action limits set by the State of California in cannabis edible products. The method also demonstrated good precision and reproducibility. In addition, it was possible to demonstrate that analysis of four non-polar pesticides, which are normally analyzed by GC–MS/MS, could be analyzed effectively in a cannabis tincture matrix using an APCI ion source. This analysis was shown to be selective, sensitive and linear. Using this method, the ionization mechanism of PCNB was elucidated. The ability to screen and quantitate all 66 pesticides and 5 mycotoxins, including the very hydrophobic and chlorinated compounds normally analyzed on a GC–MS/MS system, makes this LC-MS/MS method a novel way to screen and quantitate pesticides and mycotoxins in cannabis tinctures with a single instrument.

References
(2) A. Wise, Cann. Sci. & Tech. 2(1), 20–26 (2019).

Avinash Dalmia and Jacob Jalali are with PerkinElmer, in Shelton, Connecticut. Saba Hariri, Erasmus Cudjoe, and Feng Qin are with PerkinElmer, in Woodbridge, Ontario, Canada. Charles Johnson and Joey Kingstad are with Napro Research, in Sacramento, California. Direct correspondence to: avinash.dalmia@perkinelmer.com
### Method-development software

ACD/AutoChrom software is designed to help chromatographers develop methods faster. According to the company, tools (physicochemical-property predictors, pH selectors, and column comparisons) are provided to select initial conditions. Screening and optimization workflows help scientists develop methods using Quality by Design (QbD) principles. The software can implement these workflows automatically by controlling LC instruments, or letting users maintain control. With AutoChrom, chromatographers can develop methods using a rational, consistent, justifiable protocol.

**ACD/Labs, Toronto, Ontario, Canada.**
[www.acdlabs.com](http://www.acdlabs.com)

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### Portable mass spectrometers

BaySpec’s Portability and Continuity portable mass spectrometers are designed to provide high sensitivity, MS/MS capability, and flexibility. According to the company, the spectrometers can perform laboratory quality analysis anytime and anywhere, and can be rapidly deployed and ready for analysis in minutes, requiring minimal sample preparation. Upon sample introduction, results are obtained in real-time. Each mass spectrometry (MS) unit is compatible with ambient ionization sources such as thermal desorption–electrospray ionization (TD–ESI), electrospray ionization (ESI), thermal desorption–atmospheric pressure chemical ionization (TD–APCI), and atmospheric pressure chemical ionization (APCI). Ionization sources can also be customizable according to customer designation.

**BaySpec, Inc., San Jose, CA.**
[www.bayspec.com](http://www.bayspec.com)

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### Solid-phase extraction (SPE) disks

Atlantic ReadyDisks and Biotage VacMaster Disks from Biotage are designed to provide a solid-phase extraction workflow to meet the challenges of any semi-volatile extraction application. According to the company, the VacMaster Disk performs manual disk-based extractions and is ideal for laboratories that require flexibility and efficiency to process a variety of environmental, food and beverage, and agricultural sample matrices. The Atlantic ReadyDisks are single-use solid-phase extraction (SPE) disks, designed for maximum ease-of-use. When paired with the VacMaster Disk, the ReadyDisk allows laboratories to scale their extractions to meet their sample throughput demands without sacrificing data quality.

**Biotage, Salem, NH.**
[www.biotage.com](http://www.biotage.com)

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### GC–MS library

The field-tested Wiley Registry of Mass Spectral Data, 12th Edition reportedly is the largest mass spectral library commercially available, providing more than double the compound coverage of others. According to the company, applications include untargeted gas chromatography–mass spectrometry (GC–MS) screening and accurate mass workflows with mass spectrometry–time-of-flight (MS–TOF) instruments. This latest version of the library was extensively validated to sequester and remove suspect spectra from the main library. The new edition also introduces the inclusion of chemical classifications, calculated Kovats retention indices, and splash IDs. The expansion on this new edition brings an additional 41,450 spectra into the library.

**Wiley, Hoboken, NJ.**
[sciencesolutions.wiley.com](http://sciencesolutions.wiley.com)
There is a high probability that COVID-19 can be detected in the exhaled breath. Moreover, monitoring the impact of treatment or medication is highly relevant. Ionicon PTR-TOF systems have become the de facto standard for real-time breath analysis and a quick, non-invasive SARS-CoV-2 test to identify an infection would be a tremendous step forward.

Advantages of Direct and Real-Time Breath Gas Analysis
Breath gas analysis of volatile organic compounds (VOCs) has progressed in recent years. Researchers are trying to detect volatile biomarkers in exhaled breath that are indicative for a disease or for the response to pharmacological treatments. Several hundred volatile compounds have so far been identified in exhaled breath and their range of concentrations, typically in the parts-per-billion (ppb) range, are a challenge to most modern analyzers.

Ionicon PTR-TOF systems are particularly well suited for breath gas analysis: Their detection limits are in the ppt range (1 ppt = 0.001 ppb) and the high mass resolving power allows separation of isobaric compounds. Moreover, in addition to offline analysis, where breath is collected in a container and then analyzed, the high sensitivity and fast response time of these systems allows for the analysis of breath directly and in real-time. This provides immediate results and avoids complications often arising from sample collection and storage. With Ionicon PTR-TOF analyzers, several hundred compounds can be measured simultaneously from a single exhalation.

A critical add-on is our specialized breath sampling inlet (BET), which employs clinically certified, disposable, non-rebreathing mouthpieces to minimize the risk of contagion between patients.

Applied COVID-19 Breath VOC Pattern Research and Medication Monitoring
Looking at the pathophysiology of COVID-19, there are several indicators that a SARS-CoV-2 infection is detectable in the breath VOC pattern. The disease has been reported to cause a multitude of symptoms and can affect several organs. That supports the assumption that the metabolism is affected in more than one way and that the volatile metabolite distribution is altered.

The rapid detection of COVID-19-specific breath VOCs would be a great step forward for diagnostic purposes and a spectrum of VOCs could be used for monitoring disease progression or response to conventional or investigational drugs. Moreover, since therapies for COVID-19 are experimental at this stage, directly monitoring the effect of a therapeutic drug speeds up the development of an efficient treatment for COVID-19.

Thanks to breath researchers like Dr. Stanislas Grassin Delyle from the Hôpital Foch in Suresnes, France, and University Paris-Saclay (UVSQ), our PTR-TOF solutions are currently deployed directly at the forefront of international scientific efforts:

“We use an Ionicon PTR-TOF system to monitor breath VOCs in ventilated patients with COVID-19. The direct and fast measurement of so many breath VOCs at the same time allows us to study the progression of the disease and the response to therapeutic strategy,” reports Dr. Grassin Delyle. We wish him and all other customers who try gaining insights in SARS-CoV-2 all the best!
Separation of Common Benzodiazepine Metabolites

Benzodiazepines were first discovered in the 1960’s and reached a peak around 1970 with diazepam (Valium). During that time the drug was prescribed for any kind of antihypertensive, analgesic, or psychotropic medication. The overall enthusiasm for the benefits of this class of drug has made a growing trend of addiction more difficult to treat and diagnose. Benzodiazepines became popular due to their safety for the treatment of insomnia and anxiety compared to the preceding sedatives, barbiturates! Benzodiazepines contain a benzene ring fused to a 7-member diazepine ring with variable side chains that can affect potency, duration of action, metabolite activity, and the rate of elimination. Though this class of prescription drugs is considered socially acceptable and generally well tolerated, the addictive effects can be detrimental to life especially when mixed with other drugs. The most commonly mixed drugs with benzodiazepine include over-the-counter cold and flu preparations or ethanol due to the exacerbated effects of mixing.1 Yet, most metabolite toxicology screens of benzodiazepines are only done for oxazepam-glucuronide. The analysis of the metabolites from benzodiazepine use are of importance in the detection of abuse. Though, the majority of benzodiazepines metabolize to a single compound, oxazepam, other metabolites can be detected by liquid chromatography; Temazepam, α-hydroxyalprazolam, 7-aminoindazolazepam, α-hydroxy-midazolam, and the glucuronides of lorazepam, oxazepam, and temazepam (LOT).

In this application note, we examine the chromatography of the aforementioned metabolites from benzodiazepine use. Hamilton’s PRP-1, 5 μm, 50 x 4.1 mm HPLC column with the use of ammonium acetate 20 mM buffer to facilitate a run time of less than 10 minutes with good separation between components was used. If a more sensitive detection limit is required, the use of ammonium acetate enables mass spectrometry detection instead of UV used in this application note.

Column Information

<table>
<thead>
<tr>
<th>Packing Material</th>
<th>P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP-1, 5 μm</td>
<td>79443</td>
</tr>
</tbody>
</table>

Chromatographic Conditions

<table>
<thead>
<tr>
<th>Gradient</th>
<th>0.00–2.00 min, 20% B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.01–10 min, 20–52% B</td>
</tr>
<tr>
<td>Temperature</td>
<td>35°C</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>5 μL</td>
</tr>
<tr>
<td>Detection</td>
<td>UV at 240 nm</td>
</tr>
<tr>
<td>Dimensions</td>
<td>50 x 4.1 mm</td>
</tr>
</tbody>
</table>

Eluent A: 20 mM CH₃COONH₄

Eluent B: CH₃CN:CH₃COONH₄ 20:1

Flow Rate: 3.0 mL/min

Compounds:
1. Oxazepam Glucuronide
2. Lorazepam Glucuronide
3. Temazepam Glucuronide
4. 7-Aminolodazapem
5. Alpha-Hydroxyalprazolam
6. Oxazepam
7. Alpha-Hydroxyalprazolam
8. Temazepam

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