The analytical landscape of cannabis compliance testing

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ABSTRACT
Owing to the lack of federal oversight of recreational and medical cannabis in the United States, a patchwork of regulatory guidelines exists for compliance testing. Adding to this complexity is the fact that Canadian cannabis regulations differ from those in any of the state mandated regulatory jurisdictions and, at the time of writing, cannabis was only recently legalized in Mexico. Therefore, from a North American perspective, cannabis testing represents a significant regulatory landscape to navigate. This not only makes things confusing for those involved in cannabis production and processing, it also creates challenges for those in the analytical testing world when they have to understand and develop methods to be compliant with these various regulatory jurisdictions. In this review article, the current state of analytical chemistry knowledge for cannabis compliance testing is summarized, with an emphasis on suitable techniques and some common problems to avoid. This includes summaries of analytical methods for potency, terpenes, pesticides, mycotoxins, residual solvents, heavy metals and microbiology.

GRAPHICAL ABSTRACT

Introduction
Widely known and studied, Cannabis sativa L. (cannabis) produces a class of secondary metabolites called phytocannabinoids (heretofore referred to simply as cannabinoids); terpenophenolic compounds that are concentrated in a resinous secretion produced in the glandular trichomes of cannabis. While only a handful of cannabinoids have been objectively characterized for their biological activity, more than 100 cannabinoid variants have been identified in cannabis. Though used for its medicinal properties for hundreds of years, the characterization of the cannabinoids found in cannabis (and hemp) has been a focus of law
enforcement for decades, as tetrahydrocannabinol (THC) has been classified as a Schedule 1 substance under the United States Controlled Substances Act since 1970. More recently, the US effectively legalized hemp, defined as having “the plant Cannabis sativa Linnaeus… with a delta-9 tetrahydrocannabinol concentration of not more than 0.3 percent on a dry weight basis.” in the Agriculture Improvement Act of 2018, commonly referred to as the Farm Bill. Similar measures for hemp have been adopted by the European Union. Meanwhile, the move to legalize cannabis consumption, for medical and/or recreational use in many North American regulatory jurisdictions has spurred research and development pertaining to cannabis. It has also lead to a burgeoning analytical testing landscape to support consumer safety and regulatory compliance.

Included in the various regulatory frameworks are requirements to test cannabis and cannabis-derived products for endogenous metabolites and exogenous contaminants. Paramount among the endogenous metabolites included in legislation are cannabinoids, and in some regulations, terpenes. For exogenous contaminants, all jurisdictions mandate testing for residual solvents (in derivative products), pesticides, mycotoxins, heavy metals and microbial contaminants. The particulars of these testing requirements change as a function of regulatory jurisdiction.

Due to its nascent status and heterogeneous regulatory requirements, the analytical testing infrastructure to support cannabis testing is evolving. This makes things confusing for those involved in cannabis production and processing and creates challenges for those in the analytical testing world when they have to understand and develop methods to be compliant with various (and evolving) regulatory requirements. In this review article, the current state of analytical chemistry in support of cannabis compliance testing is discussed. This includes thorough evaluations and commentary on published methods for potency, terpenes, pesticides, mycotoxins, residual solvents, heavy metals and microbiology.

**Potency**

There is a growing interest in cannabinoids beyond THC and (cannabidiol) CBD, led currently by 8-THC. As a result, it has become increasingly important to implement methods for the quantitative measurement of cannabinoids in a variety of matrices. This includes so-called potency testing, defined here as the quantitation of THC, CBD, their respective acidic forms (tetrahydrocannabinolic acid [THCA] and cannabidiolic acid [CBDA]) and cannabinol (CBN), as well as the quantitation of more complex cannabinoid profiles (vide infra). The discussion herein on the quantitation of cannabinoids is a distinct analytical process from determining total THC on a dry weight basis. For viewpoints on dry weight determination, a critical aspect of hemp and cannabis legislation, we refer the reader to the work currently being done by the ASTM International Committee D37 and the Cannabis Analytical Science Program (CASP) coordinated by AOAC International.

Sample preparation for cannabinoid quantitation has recently gained focus as new methods and matrices reach the consumer marketplace. Traditionally, cannabis oil and flower samples used liquid-liquid and liquid-solid extraction procedures, respectively. The solvent systems have evolved from methanol/chloroform extractions, most recently replaced by similar performing methanol/water or acetonitrile/methanol procedures to reduce the health risks for laboratory personnel when using chloroform. Sampling and homogenization of plant materials is also a critical factor, especially for heterogenous materials like cannabis flower and concentrates. While sonication is the most common and supported technique, a thorough understanding of sampling procedures and homogeneity should underpin all cannabis compliance testing programs. As more matrices containing cannabinoids enter the marketplace (e.g., pet food, shampoo, bath bombs, cereal, etc.), there may be an evolving need for modified extraction procedures. For example, recent publications have focused on edible matrices like chocolates, where matrix effects have been shown to have an important impact on method accuracy. Modified extraction procedures will have a measurable impact on method performance for such challenging matrices.

The quantitative analysis of cannabinoids may be performed using high-pressure liquid chromatography (HPLC) and gas chromatography (GC) for analytical separation prior to detection, but their comparative suitability is a widely discussed topic. Early focus of analytical analyses were related to the legality and criminalization of cannabis, led by forensic chemistry. Therefore, the focus was on the total THC content of a sample, defined as the sum of THC and THCA. Since GC relies on the thermal volatilization of samples during injection, it converts THC into THCA. However, determining total THC in this way requires the acidic cannabinoids to undergo quantitative and reproducible thermal decarboxylation in the GC. The degree of decarboxylation during GC analysis depends on many factors including, but not limited to, inlet and oven temperatures, injection volume and diluents. This typically makes it difficult to accurately quantitate THC and CBD using GC, let alone the quantitation of complex cannabinoid profiles.

A systemic analysis of cannabinoid decarboxylation during GC analysis has, to the authors knowledge, not been published. Derivatization, usually in the form of trimethylsilylated compounds, can be used to stabilize acidic cannabinoids for GC analysis, but in a modern fee-for-service cannabis testing environment, the added material and labor costs associated with derivatization limit their adoption when throughput and testing costs are primary market drivers for cannabinoid quantitation.

Recently, HPLC and ultra-high pressure liquid chromatography (UHPLC) have been implemented in laboratories in order to provide faster analysis times and additional information to the market. Complex profiling of the acidic and neutral cannabinoids can provide important information about the status of the plant material, including harvest maturity and storage conditions that could be valuable to manufacturers and cultivators. While potency assays
typically have 5–11 target cannabinoids, more cannabinoids of interest and importance, biologically and economically, are being added to cannabinoid profiling analyses.\[^{5,16}\] The addition of more cannabinoids is currently limited by the availability of analytical standards, which at the time of writing includes 24 analytes. The stability of mixed calibration standards should be monitored, as examples of significant changes in cannabinoid concentration at room temperature have been reported.\[^{6}\] There are many published HPLC potency methods, with the vast majority of them using conventional C18 reversed phase column chemistry.\[^{12}\] Both methanol and acetonitrile can be used as organic modifiers, yielding different retention factors due to elutropic strength and possibly divergent selectivity depending on the column phase.\[^{17}\] It is clear from the literature that in order to achieve robust HPLC analyses with consistent chromatographic retention time, peak shape and resolution, precise pH and temperature control are required.\[^{5,6,13}\] Buffer concentration and pH are especially important, as most methods prefer a pH $\sim 3.3$ and many of the acidic cannabinoids have a $pK_a \sim 3.4$,\[^{13}\] leading to the potential for peak shape and RT shifting since a given acidic cannabinoid will exist in solution in different charge states close to its $pK_a$.

Most published methods\[^{1,4,6,7,13,14}\] use optical detection, typically photodiode arrays (PDAs), due to their ease of use and relatively low cost of operation. Additionally, PDAs can provide UV spectra for identification and peak purity information.\[^{13}\] However, optical detection has some limitations in specificity, as many cannabinoids are structurally similar, and thus have similar UV spectra. While chromatography is able to address many interference challenges with optical detection, there are many examples of cannabinoids and/or other interferences co-eluting, causing problems in identification and quantification.\[^{18}\] Such considerations become more important as the panel of cannabinoids is expanded and the number of matrices are increased, as the potential for chromatographic interferences increases. This is especially true when quantitating lower concentration cannabinoids like cannabigerol (CBG) or tetrahydrocannabivarin (THCV), where the relative impact of chromatographic interferences on quantitative accuracy would be higher. Recent publications are utilizing the advantages of mass spectrometry (MS) in series with liquid chromatography to overcome these challenges.\[^{15}\] LC-MS methods come with selectivity advantages via the added dimension of mass-to-charge analysis and product ion ratios (i.e., multiple reaction monitoring [MRM]). Even in single-ion-monitoring (SIM) experiments, many selectivity challenges posed by interferences can be reduced or eliminated.\[^{18}\]

LC-MS methodologies, however, have their own limitations. The largest of these being the cost increase and added laboratory expertise required for successful implementation of LC-MS methods.\[^{6}\] Since cannabinoids like $\Delta^8$-THC, $\Delta^9$-THC, exo-THC, CBD, CBC, and CBL are structural isomers (i.e., isobaric), MS detection still requires chromatographic resolution, as these compounds all have the same mass-to-charge ratio and their product ion spectra are, for practical purposes, indistinguishable. Although, in general LC-MS methods are faster, despite the required isomer resolution since mass-resolved analytes typically can co-elute without negatively impacting method performance. Another MS-based method that could help overcome isomeric separation limitations is ion mobility. While not extensively explored, there is evidence that ion mobility can successfully differentiate THC and CBD.\[^{19}\]

### Terpenes

Second to cannabinoids as the dominant (by weight percent) secondary metabolite produced by cannabis, terpenes may constitute 0.5–5% w/w of the inflorescence.\[^{9,20}\] Terpenes are classified according to the repeating number of five-carbon isoprene units used for their biosynthesis: mono- (2), sesqui- (3), di- (4) and triterpenoids (5).\[^{21}\] While the term terpene includes compounds that are simple hydrocarbons (e.g., pinene) and terpenoids refers to compounds containing heteroatoms (e.g., linalool), it is common practice to use “terpenes” more generically to refer to this family of secondary metabolites. This nomenclature convention is used herein. To date more than 200 terpenes have been identified in cannabis, but the 58 monoterpenes and 38 sesquiterpenes confirmed represent the compound classes found in the greatest frequency and abundance.\[^{8}\] Parsing down these exhaustive lists further, cannabis chemotypes are generally dominated by a subset of 10–20 highly prevalent and highly abundant terpenes, with the most studied and frequently observed being $\beta$-myrcene, $d$-limonene, $\alpha$-terpinene, $\gamma$-terpinolene, $x$/-$\beta$-pinene, linalool, $\beta$-caryophyllene (the most abundant sesquiterpene), $\alpha$-humulene and Caryophyllene oxide (the chemical that narcotics control dogs smell when searching for cannabis).\[^{8}\],\[^{9,22}\]

Beyond their natural functions in plants (pest defense, etc.), terpenes represent the dominant organoleptic contributors to cannabis—it is these compounds that drive consumer preference for certain types of cannabis due to their distinct aroma and flavor profiles. Savvy cannabis brands have recognized this as an opportunity to curate a diverse product portfolio with consistent sensory attributes, that is highlighted not just by cannabinoid profiles and concentrations, but also terpene profiles and their relative concentrations. Notwithstanding their importance to consumer preference, it is their potential synergy with cannabinoids (and other secondary metabolites in cannabis) to elicit an “entourage” biological effect\[^{21}\] that underscores their importance to the collective scientific understanding of cannabis. For example, while only a few terpenes have been adequately studied for their biological effects, pinene has been reported to aid memory, which may counteract some of the effects of THC, and $\beta$-caryophyllene interacts with the endocannabinoid system and may be responsible for some of the anti-inflammatory effects of cannabis.\[^{22}\] Further, it has been proposed that terpene profiles, while highly influenced by environmental conditions during plant maturation,\[^{23}\] could be leveraged to aid in the classification of cannabis chemotypes beyond the typical high THC and high CBD designations.\[^{9}\] The need for accurate and precise quantitation of terpenes,
therefore, is driven by consumer preference, their potential biological impact and also the potential to aid in the classification of cannabis. As more is discovered about the biological impact of terpenes in cannabis products, it is also possible that policies similar to some states, where terpene quantitation is part of regulatory compliance testing, will be more widely adopted.

Sample preparation for terpene analysis is divided into two categories: alkane-based solid-liquid extraction (SLE) and headspace-based (HS) techniques. To date, the most prevalent approach for the analysis of terpenes in cannabis (and indeed, in other plants and derivative products) has been SLE using a hydrocarbon solvent, typically pentane, hexane or ether. The one exception to this was the recent work of Ibrahim et al., who found that ethyl acetate provided the highest extraction efficiency. SLE is favored due to its scalability for throughput and the fact that it is simple and cost-effective. While it is generally assumed that quantitative extraction of terpenes is achieved with this approach, the dearth of certified reference materials (CRMs) for terpenes in cannabis and derivative products makes this assertion a challenge to validate. The principal drawback of SLE for terpene analysis is the fact that it is nonselective, so co-extracted matrix components that are not compatible with a given analytical technique can limit throughput capabilities due to an increased need for instrument maintenance (e.g., the GC inlet will become dirtier more readily than with more selective sample preparation techniques).

Several recent studies have eschewed SLE in favor of static HS or HS solid-phase microextraction (HS-SPME). These techniques have the advantage of only sampling volatile constituents in the sample matrix, so fouling of analytical instrumentation is minimized. HS-SPME takes this a step further and only samples volatile analytes that adsorb to a chosen stationary phase. Recent reports of bias in terms of the terpenes that adsorb to the SPME fiber suggest that there are still method parameters to be evaluated with this approach before it could see wide adoption in a compliance testing environment. Moreover, SPME is frequently used as a preconcentration step. For terpenes, this is not necessary based on their concentrations in cannabis and could actually lead to instances of column overloading and/or detector saturation. These problems are potentially exacerbated in cannabis, as it can be a challenge to tell, a priori, what an appropriate sample size would be given the variability in terpene profiles and concentrations in cannabis. Static HS, on the other hand, will sample all analytes in an equilibrated gas phase, but may experience a sampling bias for high boiling terpenes, as such compounds may not have sufficient equilibrium partial pressures, especially in matrix. Nguyen et al. overcame this by using high-temperature HS sampling (180 °C) to force higher boiling terpenes (e.g., guaiol and z-bisabolol) into the gas phase. This approach was utilized to successfully pass a terpene proficiency testing sample, indicating that acceptable bias for higher boiling terpenes was attained. It should be noted though, that not all HS autosamplers are capable of such high temperature incubations, nor do all laboratories have SPME hardware, so these techniques would not necessarily be accessible to a wide swath of compliance testing laboratories.

Another approach to reduce bias based on boiling point is to use a technique called full evaporative technique (FET) HS sampling. In this approach, a small quantity of sample is used to ensure complete evaporation into the gas phase. This low sample quantity facilitates a full transfer to the gas phase due to an overall reduced partial pressure from other volatile analytes present in the sample (and diluent, etc.). At the same time, it eliminates disparity in gas phase equilibria for matrix samples in relation to solvent calibrators. Shay et al. successfully used this approach to quantitate terpenes in cannabis. However, the impact of small sample sizes relative to sample homogeneity and representative sampling need to be considered when using FET. For example, Shay, et al only used 5 mg of solid sample or 10 µL of standard for their analyses. The potential for sampling bias and variability with such small sample quantities need to be systematically verified before FET methods are taken through method validation.

Chromatographic separation and detection of terpenes has historically been done using GC-FID, a cost-effective detector with excellent dynamic range and a response that is directly proportional to carbon number (amenable to the analysis of hydrocarbon-based analytes like terpenes). However, with fee-for-service terpene panels of 30–40 analytes being the norm, and the fact that 93 have been reported in other methods and more than 200 identified overall, the potential for chromatographic interferences with a nonspecific detector technology like FID is high. For this reason, more current methods have focused on GC-MS and GC-MS/MS, both of which have improved selectivity in comparison to FID. But MS is not always the final solution to selectivity challenges for terpenes. This is due to the number of terpene isomers that can be present in a given sample and the high energy nature of electron impact (EI) ionization used for GC-MS analyses. These high energy reactions break the terpenes down to fragments that are generally shared within a given class and will also be shared for structural isomers. For example, pinene and myrcene are structural isomers (both have a formula of C10H16) and will have the same EI mass spectra, so they need to be chromatographically resolved. To provide an additional level of confirmation in terpene identification, especially in the absence of analytical standards, many methods use retention indexing (e.g., Kovats) in tandem with EI mass spectral matching. An alternate approach, while seemingly more academic at present, is the use of cold EI, which is analogous to chemical ionization for GC in that it provides a more stable molecular ion to aid compound identification.

A final point worth mentioning, is that all terpene analyses discussed thus far will report a sum of enantiomers present in a given sample, as conventional chromatographic columns do not resolve stereoisomers. This is an area of research that is likely to get more attention as the biological impact of new terpenes are investigated, especially since it is
known that enantiomers can elicit disparate biological effects (e.g., α/β-pinene have different perceived odors). Moreover, a proof-of-concept using terpene enantiomer profiles for product authenticity testing has been reported,\[^{[6]}\] which is consistent with other terpene-rich products like tea tree oil where enantiomers have been used for geographic characterization.\[^{[32]}\]

**Pesticides**

Pesticides are compounds manufactured with the intent of destroying insects or microorganisms that are harmful to plants. Pesticides are typically categorized based on their class of use or chemical class. The seven classes of use include insecticides, herbicides, fungicides, rodenticides, acaricides, molluscicides, and nematocides.\[^{[33]}\] Pesticides can also be divided by their chemical structures, with organo-phosphorus compounds, carbamates, chlorinated hydrocarbons, pyrethroids, and heterocyclic compounds being the dominant groups. The Federal Insecticide Fungicide and Rodenticide Act (FIFRA) of 1947 requires pesticide registration for specific uses and establishes labeling requirements for these pesticides.\[^{[34]}\] In the US, prior to its use, a pesticide must first be granted a residue tolerance or receive a tolerance exemption from the US Food and Drug Administration (FDA). Allowed uses for pesticides must be supported by data including toxicological, environmental fate, and lifecycle analyses. Given the federal status of cannabis as a Schedule 1 drug, none of these processes used for other plant commodities have been initiated and very little is known about best practices of pesticide application and use in cannabis cultivation.\[^{[35]}\] While hemp has been effectively legalized in the US (vide supra), it too has the same lack of codified knowledge regarding best practices for pesticide application. As a result, most maximum residue limits (MRLs; often in the low part-per-billion range) are not due to toxicity studies or risk management, but often are based on the ability of laboratories to achieve them analytically.\[^{[36,37]}\]

Pesticides are typically applied as a foliar spray during cannabis cultivation and their residues may remain with harvested flowers after cultivation. Interestingly, pesticide use may not even be beneficial for the cannabis plant, rendering their use a risk introduced during cultivation.\[^{[38]}\] Even if pesticides are not applied directly to the plant though, they could still be introduced through the use of soil or irrigation sources contaminated with pesticides. If the cannabis inflorescence is further processed into cannabis oils, concentrates, or infused products, these pesticide residues could be concentrated, removed altogether, or chemically transformed depending on which manufacturing processes are utilized. Pesticide residues can also be altered after application due to photo oxidation, biological degradation (within the plant, or due to soil microbes, etc.), or volatilization\[^{[39]}\] leading to variable concentrations throughout the cultivation and manufacturing processes.

Many of the pesticides found in cannabis and hemp samples are deemed moderately hazardous by the World Health Organization\[^{[33]}\] and most of the documented toxic effects of pesticides are based on dermal contact. Cannabis, however, is frequently inhaled and there is scarce toxicological research on the fate of pesticides and pesticide metabolites during and after inhalational exposure. Some studies have measured the pesticide levels in mainstream smoke from 10% to 70% of the initial concentration on the flower.\[^{[40]}\] While there is data on pesticide inhalation from cigarette smokers, cannabis users typically inhale up to two-thirds larger puffs by volume and hold the puff for much longer than cigarette smokers.\[^{[33]}\] This indicates that pesticide exposure data from cigarettes may not correlate to cannabis users. As well, during pregnancy, the placenta and fetal brain are potential targets for both cannabinoids and organophosphate pesticides.\[^{[41]}\] This is worrisome given that estimates from the Colorado Department of Public Health and Environment found 8.2% of new mothers had used cannabis shortly before their pregnancy. These data suggest that the risks of pesticides in cannabis and derivative products need to be better understood. One way to support this effort is the development of accurate quantitative methods for pesticides.

Cannabis products are also much more diverse than those offered by the tobacco or alcohol industries. Flowers are inhaled; oils and concentrates are dabbed at high temperatures or used in vaporizer cartridges and made into numerous infused products, beverages, topicals, and edibles. Most coil-based vaporizer cartridges can be operated at variable battery settings leading to vaporization temperatures ranging from 212 degrees F to 630 degrees F, depending on the degree of wick wetting.\[^{[42]}\] This large temperature range covers pyrolysis temperatures and can lead to thermo-oxidative degradation of many pesticides and can yield toxic by-products. One of the most famous by-products is hydrogen cyanide, formed from the pyrolysis of the pesticide myclobutanil.\[^{[43]}\]

The United States Pharmacopeia (USP) lists 561 compounds (including 108 pesticides) that consumable crops should be tested for, the National Organic Program (NOP) 2611-1 lists 188 residues, and the United States FDA lists over 750 pesticide residues in their compliance tests.\[^{[44]}\] Given the high number of analytes, the lack of federal guidance on which residues to look for and the lack of any toxicological and environmental data, states are left with little guidance on how to figure out how to regulate pesticide use on cannabis on their own. Providing some guidance is a 2013 release by the American Herbal Pharmacopeia that included pesticides that were most likely to be used on cannabis and highlighted acaricides, insecticides, fungicides and plant growth regulators.\[^{[45]}\] This document has helped some states begin to regulate pesticides, but variation between regulatory jurisdictions is the norm, rather than the exception. For example, California have mandated pesticide testing for 66 pesticides,\[^{[46]}\] Oregon has 58 on their list,\[^{[47]}\] Colorado has only 15 on their list, while Canada mandates testing for 96 pesticides.\[^{[37]}\]

Beyond the challenges of inconsistent regulations, sample preparation is another area of concern for pesticide analysis.
The cannabis flower matrix is heterogeneous and includes over 550 compounds, many of which are capable of interacti-
ing[46]—flavonoids, fatty acids, phenols, phytosterols, alco-
hols, ketones, and lactones can all interfere with various extraction strategies and introduce ion suppression and ion enhancements when using MS for analyte detection. Common techniques include liquid-liquid extraction (LLE), solid-liquid extraction (SLE), solid-phase extraction (SPE), solid-phase microextraction (SPME), and quick, easy, cheap, effective, rugged, and safe (QuEChERS) extractions. LLE and/or SLE are quick and easy, but they are nonspecific, include high levels of solvent consumption and can require significant dilution schemes prior to injection to mitigate matrix effects. SPE uses less solvent, but is more laborious and can be costly depending on the SPE phase selected. SPME is a solvent free technique that is typically used only in GC analyses, limiting its usefulness in analyzing broad pesticide panels. Although, when its use is appropriate, SPME reduces background interferences that may have been present in LLE or SLE approaches. QuEChERS is a popular technique, appearing in a significant amount of the literature on pesticide quantitation in cannabis flower and derivative products. Given the many variables in using QuEChERS, this technique needs to be validated based on the type of salt, buffer, and organic/water phases used, as the chemis-
tries of the pesticide panel under investigation will impact method recoveries (e.g., PSA will reduce the recovery of acidic pesticides and GCB will do the same for planar pesti-
cides). A study investigating the effects of buffers on the QuEChERS preparation proved no clear trend in extraction efficiencies and recoveries across the three methods.[49] Another issue with QuEChERS is the fact that the organic solvents will also extract many hydrophobic compounds, like cannabinoids. Since the concentration difference between cannabinoids and pesticides in a given sample will likely be large, even with heavy dilution prior to analysis, the cannabinoids can present chromatographic interferences that are challenging to overcome. Indeed, while such inter-
fences can frequently be addressed by the selectivity afforded by tandem mass-spectrometry analyses, this approach is not always successful. In some instances, it may require altered sample preparation and/or chromatography to remove such interferences. Therefore QuEChERS may not be the method of choice for some laboratories.

Given the variability in the physical and chemical properties of pesticides (polarity, solubility, thermal lability, pK_a, etc.), as well as the variability in maximum residue limits (MRLs) by regulatory jurisdiction, different technologies are utilized to quantitate pesticide residues. Since many pesticide MRLs are in the low part-per-billion range, most peer-
reviewed articles have focused on LC-MS/MS and GC-MS/ MS to quantitate these residues in cannabis and derivative products. GC is sensitive and selective for hydrophobic and volatile pesticides, but struggles with polar or thermally labile compounds. LC-MS/MS, excels at the analysis of polar and ionic compounds. [50] Such capital expenditures must be carefully considered, with financial capacity and testing needs influencing which instrumentation to purchase. One study from 2021 used LC-MS/MS to measure 82 of the 96 pesticide residues required by Health Canada.[56] This method shows how difficult pesticide testing can be when regulatory requirements need to be met. Fourteen pesticides were not able to meet Canadian requirements (seven were detectable but not quantifiable and the other seven were not detectable using ESI). A 2018 paper from Health Canada used three methods to validate and quantitate pesticides from various cannabis matrices and then inspect licensed cultivation sites to reveal unauthorized pesticide control products in use.[51] The first method detected 39 pesticides in cannabis leaves, the second 32 pesticides in cannabis flowers, and the third 40 pesticides in cannabis oils. All of these methods used a combination of LC/GC-MS/MS. Another interesting paper outlines possible pesticide remedi-
ation techniques for the cannabis industry.[52] The German research group used computer modeling to look at simultane-
ous CBD isolation and pesticide removal using liquid-liquid chromatography. The group outlines three solvent systems capable of achieving both goals and even produces a flow-sheet on which solvent system to use based on the pesticide responsible for the contamination. Perhaps the most com-
prehensive study of pesticides in cannabis though, looked at 367 pesticides using QuEChERS cleanup and both LC-MS/ MS (290 pesticides) and GC-MS/MS (78 pesticides) analyses. Most analytes had recoveries in the 70–120% range.[47]

Generally, the peer-reviewed literature sheds light on a couple analytical trends in cannabis testing laboratories regarding pesticides: (1) pesticide testing is difficult and requires advanced analytical instrumentation and highly skilled staff to meet regulatory demands with a robust, accurate and precise test method, (2) LC-MS/MS and GC-MS/MS are preferred by most groups, and (3) QuEChERS is the preferred sample clean up step.[53,54]

Mycotoxins

Cannabis and hemp, as with other commodity crops, is sus-
tceptible to contamination from fungi during growth, pro-
cessing and storage.[55,56] Fresh cannabis has a water activity (A_w) of 0.95 A_w, but to prevent most fungal growth a water activity below 0.65 A_w is required.[57] For example, the Botrytis genus of fungi is most commonly found postharvest and has a water activity threshold of 0.85 A_w.[57] There are currently at least 88 known fungal species affecting cannabis during the growth stage.[58–60] Commonly found fungal gen-
ergens include Alternaria, Aspergillus, Cladosporium, Fusarium, Penicillium and Trichothecium.[61] These pathogens can be found in the foliar and stem regions of the plant, but can also be soilborne.[61] It is for this reason microbial testing (vide infra) of cannabis is required in all regulated regions in Canada and the US. Certain genera of these fungi have the ability to produce secondary metabolites known as mycotoxins, which can elicit various forms of acute toxicity if consumed. For this reason, analysis of mycotoxins is also required in many regulatory jurisdictions.

The simplest and most common approach for mycotoxin sample preparation employs a broad spectrum solvent
extraction, typically with acetonitrile, using anywhere between 5 and 10 mL of solvent per gram of cannabis. Other solvents including toluene, ethyl acetate and methanol have shown reduced extraction efficiencies. It has been found that wetting the sample prior to acetonitrile addition with a small volume (roughly 20% of the acetonitrile volume) of water or isopropyl alcohol can improve extraction yields and precision. The addition of this wetting step can increase the polar matrix co-extractables, which could result in matrix effects during analysis (i.e., by LC-MS/MS). The addition of QuEChERS salts to separate the acetonitrile and water layers can mitigate this, while still showing high extraction efficiencies for mycotoxins.

As mycotoxins are surface contaminants typically found at the foliar and stem junctions, it has been reported that measuring the whole flower bud without grinding was key for high extraction recoveries. Of course, measuring exposure from a single flower presents questions about homogeneity across a given plant, row, crop, etc that would need to be weighed by regulatory agencies if/when they define sampling procedures for mycotoxin testing. Moreover, differences have been reported for measured extraction efficiencies between spiked samples (i.e., those used for method validation) and samples with incurred mycotoxins, especially without the inclusion of a wetting step. For this reason, matrix-matched calibrations can be important for method accuracy and to compensate for matrix effects during analysis. This also highlights the importance for the development of certified reference materials to support cannabis testing programs.

Because mycotoxins can be analyzed alongside pesticides using LC-MS/MS, employing selective clean-up steps increases the risk of analyte loss, especially in a multiresidue method. Although, depending on instrument sensitivity, it may be necessary to use SPE sorbents that are selective for mycotoxins, they do not retain pesticides, and as such, are typically not used when LC-MS/MS is used for detection. They are, however, common when HPLC with fluorescence detection is used for mycotoxin quantification.

Residual solvents

As of the writing of this review, 34 states have legalized medical and/or recreational use of cannabis and cannabis products in the United States. Among these states, 22 of them developed laboratory or state mandatory residual
solvents testing for product safety.\[^{82,83}\] Canada and some European countries also started to regulate residual solvent testing for cannabis oil, concentrate or inhalation products.\[^{84,85}\] Because of its Schedule I status in the United States, the unified safety measures at the federal level are not applied to cannabis, leading to a lack of standardization in residual solvent testing. As a result, each state has selected its own residual solvents and associated MRLs. For example, in Colorado, eleven residual solvents are listed in the test regulations for cannabis concentrates with the action limits ranging from 2 ppm to 1000 ppm.\[^{86}\] In California, twenty residual solvents are required to be tested in cannabis pre-rolls and products. The 20 residual solvents are divided into two categories based on their health concerns. Category 1 compounds have an MRL of 1 ppm and Category 2 compounds have MRLs ranging from 290 to 5000 ppm.\[^{87}\] MRLs for residual solvents are generally established by referencing state standards (e.g., the Department of Cannabis Control in California) and federal organizations such as the Office of Environmental Health Hazard Assessment (OEHHA), the United States Environmental Protection Agency (USEPA), the Agency for Toxic Substances and Disease Registry (ATSDR), National Institute for Occupational Safety and Health (NIOSH) and the United States Pharmacopeial Convention (USP). Research from most common solvents and processing chemicals used in the cannabis industry and the results of exploratory laboratory testing are also used to establish MRLs. In Canada, twenty-eight residual solvents are required to be tested in cannabis oil with a blanket MRL for each compound of 5000 ppm.\[^{85}\]

Certain chemicals and solvents, such as propane, butane, hydrocarbons, water, ethanol, isopropanol, acetone, and hexane are frequently employed during cannabis extraction processes.\[^{40,88}\] Such solvents are used to reduce production costs and preserve incurred terpenes, in addition to their use by illicit extraction facilities.\[^{88–91}\] Chemicals used during the processing of cannabis from machine operations and product packaging may also contaminate the end products,\[^{92}\] with a recent study of 57 cannabis samples conducted by Raber et al. showing that over 80% of the concentrates evaluated contained residual solvents.\[^{40}\] Producers of cannabis concentrates and derivative products are adding terpenes to tinctures, vaping oils, lotions, foods, and beverages to enhance the flavor, make claims of health benefits, or replicate the original terpene profile lost during the cannabis extraction process. Also, thinning/cutting agents such as propylene glycol, polyethylene glycol, or medium-chain triglycerides are added to vape oil to change product viscosity, and/or reduce the cost of production.\[^{93}\] These added terpenes, be they cannabis, botanical, or synthetic in origin, create another potential source of residual solvents in cannabis products. Furthermore, added terpenes and thinning/cutting agents may interact with each other or undergo thermoxidative degradation during the vaping process to form, among other things, analytes included in residual solvent compliance testing.\[^{94}\] Residual solvents formed in this manner are beyond the scope of current compliance regulations but nevertheless represent an important public health consideration.

Most of the published residual solvents test regulations reference USP <467>, which is a gold standard for pharmaceutical products.\[^{95}\] The common solvents present in drug substances, excipients, and finished products were well defined and the testing methods described in USP <467> have been implemented for decades. However, manufacturing of cannabis products differs greatly from pharmaceuticals, especially in their use of compounds such as propane, butane and isobutane during the extraction process. Moreover, cannabis is a complex matrix compared to typical pharmaceuticals, and derivative products further complicate things with matrices including joints, concentrates/distillate, e-juice, topicals, oral supplements, tinctures, capsules, and infused edibles such as candies, chocolates, mint/chews, dried meat, crackers, dairy products and baked goods. Given these differences, USP <467> is a good starting point, but does not have the necessary scope to deal with cannabis products.

There are limited methodologies for residual solvent testing in cannabis products. Gravimetric analysis (e.g., for class 3 volatile solvents specified in USP <467>) is simple, but needs larger amounts of samples and lacks specificity to identify the volatile analytes.\[^{96}\] Spectroscopic and spectrometric tests have low sensitivity in general and are therefore not applicable for regulatory requirements that specify any Category 1 analytes. GC as a chief and preferred method of solvent analysis has been used by pharmaceutical manufacturers for decades and is the most appropriate, specific analysis for residual solvent testing.\[^{97}\] Similarly, GC is the most dominant and successful tool in cannabis research and testing for residual solvents.\[^{91,96}\] Many methods for residual solvent testing using headspace (HS) GC-MS or GC-FID to utilize sample introduction, GC separation, and analyte detection.\[^{88,98–101}\]

Sample introduction has three main procedures and they are direct injection, HS analysis, and solid-phase microextraction (SPME). Direct injection is simple, reliable and a standard autosampler can be used. The samples are usually dissolved in an appropriate extraction solvent (i.e., not an analyte) prior to analysis.\[^{97,102}\] Direct injection introduces liquid samples into the GC system and so care must be taken to mitigate matrix interferences and the potential for more frequent system maintenance due to contamination of the inlet and/or head of the analytical column. Because of these drawbacks, the direct injection method has become less popular and replaced by the HS sampling in residual solvent testing in recent years.

HS sampling is an indirect testing procedure. The analysis is conducted when a volume of gas above the sample matrix is collected and analyzed by a GC. The major types of headspace-sampling techniques are: dynamic HS sampling (also referred to as purge-and-trap) and static HS sampling. In dynamic HS sampling, a continuous gas flow is swept over the surface of a sample matrix. Volatiles from the sample matrix are conveyed into a trap where the volatile residual solvents are accumulated prior to analysis. Whereas
dynamic HS sampling is particularly suited for the determination of analytes at very low concentrations. Static HS sampling is the most widely used technique for residual solvent analysis in the cannabis industry. In static HS, a liquid or sometimes a solid sample is placed into a sealed vial where it is heated until a thermodynamic equilibrium between the sample and the gas phase is reached. A volume of the headspace gas is then sampled for analysis. The advantage of the HS sampling is simplified sample preparation, as homogenized or diluted samples can be directly aliquoted for analysis. Since only the gas phase of the samples are injected into GC system, matrix interferences are significantly reduced. The nonvolatile matrix components remain in the sample vial, which limits GC maintenance relative to other. However, one disadvantage of the HS sampling is the possibility of over saturation of the detectors for analytes with high sensitives or concentrations depending on the liner dynamic range of the detector used. Another potential bias possibly introduced by HS techniques is that some residual solvents have higher boiling point, and this may contribute to partial pressure change in headspace gas phase due to incurred volatiles, especially in complex cannabis matrices. To overcome these potential matrix effects, full evaporation technique (FET) can be implemented into HS sampling. FET sample preparation involves the use of a very small sample amount (e.g., 5–20 mg), which effectively creates a single-phase gas system in the headspace vial at equilibrium, effectively mitigating the matrix issues discussed above.

Finally, solid-phase microextraction (SPME) has made much progress in recent years for cannabis related research and testing. In SPME, a silica fiber coated with a sorbent is used to collect and concentrate the volatile components of a sample prior to analysis. The selectivity of the fiber can be modified by changing the phase chemistry and/or thickness according to the characteristics of the analytes. In general, there are two types of SPME - headspace and immersion. Direct HS SPME has been demonstrated to be more sensitive than immersion SPME for polar residual solvents. In recent years, direct HS SPME has gained a sturdy reputation as a valid alternative to HS GC because of the simplicity of the procedure and the low cost of hardware. However, its detection limits may be lower than headspace GC. In addition, the SPME fiber extraction efficiency changes with the wear (number of injections), which can cause poor inter-batch reproducibility. These facts may reduce the applicability of HS SPME in routine determinations of residual solvents in cannabis products.

MS and flame ionization detectors (FID) are the most common detectors for residual solvent testing. GC-FID is adequately used for known specific residual solvents and chemicals in cannabis testing. However, the lack of specificity of the FID and its dependance on hydrocarbons in the analyte can create analytical challenges for certain regulatory jurisdictions. For example, DMSO has low sensitivity using FID and can challenge to chromatographically separate some residual solvents sufficiently (e.g., without the added specificity of an MS detector). In some instances, it may not be possible to overcome these limitations through a single GC column phase. For complicated separations like this, two capillary columns with different stationary phases can be combined in series (tandem) to obtain tunable selectivity. This two-dimensional GC chromatography improves chromatographic separation, which may be necessary in complex cannabis matrices, especially when dealing with large lists of residual solvents. The most utilized capillary columns for GC separation are composed of 6% cyanopropylphenyl, 94% dimethylpolysiloxane stationary phases (624-type columns). This type of MS column is designed specifically for volatiles analysis and is widely used for the analysis of residual solvents. As specified in USP < 467>, USP phase G43 (polyethylene glycol stationary phase; WAX-type columns) are the usual choice, as they represent an orthogonal column chemistry to the 624-type columns when trying to resolve co-eluting residual solvents.

GC-MS can be used for the determination of known residual solvents or processing chemicals in cannabis products and it also has the capability to identify unknowns using mass spectral libraries. Based on the unique mass spectrum of each analyte, GC-MS data processing software can deconvolute coeluting compounds and permit accurate integration of each component. MRLs for residual solvents generally range from 1 ppm (detection limit) to 5000 ppm. To cover such a wide concentration range, both full scan and single ion monitoring (SIM) modes may be used simultaneously in one MS method or a SIM only method can be used. For example, compounds such as propane and ethylene oxide, as well as chloroform, benzene, and 1,2-dichloroethane are notoriously difficult to quantitate accurately due to their high volatility and poor response - SIM can help reduce chemical background to achieve sufficient sensitivity for such compounds. However, GC-MS may suffer from detector saturation for analytes with high sensitivities and/or high MRLs, especially when trying to implement multi-residue methods with Class 1 solvents. In addition, SIM may not have suitable specificity for low molecular weight residual solvents, those that share hydrocarbon backbones (e.g., pentane and hexane) and those with common functional groups. In such cases, orthogonal column chemistries (vide supra) or alternate MS techniques (e.g., GC-MS/MS) may be necessary. The need for these alternate techniques may be exacerbated in the presence of high concentrations of terpenes.

Standard preparation and storage represents a unique challenge for residual solvent quantitation, especially for highly volatile compounds such as propane, butane, ethylene oxide, etc. Gas tight vials and gas tight syringes need to be used during their preparation, and it is advisable to store and open standards cold (e.g., −20 °C) to limit losses due to volatility. Even with these precautions, standards (supplied in sealed ampules) are only good for a single use. Therefore, efficiency from sample preparation to data reporting in residual solvent testing is key. The authors have developed and validated a screening method to detect and semi-quantitate the residual solvents listed in the California test regulations (data not yet published). In this method, a single-point
calibration and average response factor are used to identify and estimate residual solvent concentrations. Owing to this semiquantitative screening approach, a full sequence with multiple calibration points and quality control samples are only carried out for samples above compliance MRLs.

**Heavy metals**

Toxic elements with densities greater than 5 g/cm³ and atomic numbers higher than 11 have traditionally been called heavy metals. Of the 90 naturally occurring elements, 53 can be classified as metals (and one metalloid, As). These elements can include lead, cadmium, arsenic, mercury, cobalt, vanadium, nickel, lithium, antimony, bismuth, molybdenum, copper, tin, and chromium among others. Low concentrations of these metals (lower even than 0.01 mg/L) have been shown to be toxic to humans. Some of these elements are essential plant nutrients (Cr, Mn, Mo, Zn, Fe, Co, Cu, Al, Ni) and some are non-essential elements that can lead to toxicity even at trace levels (Cd, Pb, As, Hg). Many species exist in different oxidation states and will have different biological impacts depending on their route of exposure; inhalation versus ingestion of cannabis products is an excellent example of this, as is the relative toxicity of Cr⁶⁺ versus Cr⁴⁺.

Metals are found in the Earth’s crust in varying concentrations. They are introduced through uncontrolled anthropic activities such as mining, transportation, smelting, and agricultural applications. Typically, they appear in food and cannabis products through plant bioaccumulation, cross contamination during processing, or post-process adulteration. There are ways to use cannabis and cannabis products to remediate soils and water contaminated with toxic elements but those have not been adopted at industrial scales yet. Metals can contaminate soils through runoff from industrial manufacturing plants, direct applications of fertilizers and pesticides, the application of animals wastes or sludges, and the atmospheric deposition of metal-containing particulate matter. It is also known that wildfires and volcanic eruptions in one location can deposit heavy metal containing ash on crops hundreds of miles away.

In soil, where healthy pH is typically 5.5–7, metal cations are bound to negatively charged particles such as clay, humus, and other organic matter. Only when unbound, are metals bioavailable. Several studies have discussed the mobility and availability of metals under specific soil conditions including the effects of rhizosphere specific bacteria on plant uptake. At low pH, metals like lead, copper, mercury, and cadmium are more available for plant uptake. Some specific transport systems can also transport metals, such as phosphate systems for arsenic and cadmium and lead through divalent metal transporters (used for iron, calcium, and zinc typically).

Cadmium has been shown to be enriched in some phosphate-based fertilizers and thus available for plant uptake. High concentrations of cadmium in soil are associated with iron deficiency in some plants leading to higher application of fertilizers and a feedback loop of increasing contaminant concentrations. Similarly, metal uptake can cause signs of disease in plants, such as spots on the leaf surface, which can again cause cultivators to apply more fertilizers, thinking the cause of the disease was a nutrient deficiency. Mercury can enter plant stomatal openings through gas exchange (mercury is volatile at around 30 Celsius) and can also enter plant roots in certain oxidation states. Metals can hyperaccumulate in plants with levels of cadmium and selenium seen over 100 ppm in dry leaf tissue, chromium, and copper over 300 ppm, while lead and arsenic and nickel can reach 1000 ppm. It is not uncommon for metal concentrations in Cannabis to bioaccumulate by tissue location, with higher levels in roots and leaves than in the stems themselves.

Most metals do not degrade quickly or easily leading to their ability to bioaccumulate up the food chain. Cadmium is a bluish white metal usually from zinc ore processing. Chronic cadmium exposure can cause Itai-Itai disease, characterized by intense bone pain and numbness in extremities. Lead is a silver to gray soft metal. It is widely used and can exist over 30 years in bones. Lead is a calcium analog that interferes with ion channels. In Leipzig Germany, illicit lead was added to Cannabis to increase its mass, and therefore its value. Hundreds of patients exhibited nausea, acute colic, loss of appetite and weight, and chronic fatigue. Mercury is a silver metal that is liquid at standard temperature (0°C). Mercury vapors can cause forgetfulness, irritability, tremors, and paranoia. Arsenic is colorless or gray and can lead to skin cancer and lesions, diabetes, cardiovascular disease and infant mortality.

The International Agency for Research on Cancer (IARC) has classified metals into three categories based on carcinogenicity in humans. As, Cd, Cr, and Ni are all group 1 (proven carcinogens). Inorganic lead and methyl mercury are in group 2 (probable carcinogens), while Organic lead and metallic mercury are in group 3 (possible carcinogens).

Similarly, the international Agency for Toxic Substances and Disease Registry (ATSDR) has ranked As, Pb, Hg, and Cd as the 1st, 2nd, 3rd, and 7th most hazardous substances for toxicological profiles.

Given the high toxicity of metals at low concentrations, it is important to quantitate them at low levels (part per billion or lower). Common methods to prepare samples to run historically included the addition of concentrated acid followed by heating such as EPA methods 200.2, 200.7 or 200.8. More common in high throughput laboratories is to digest material in microwaves (microwave digestion). Many traditional methods to detect metals have included atomic absorption spectrometry, x-ray fluorescence and proton induced x-ray emission but struggle to meet these detection limits. More common in today’s laboratories are inductively coupled plasma (ICP) with either optical emission spectrometry (ICP-OES) or mass spectrometry (ICP-MS). Due to many stringent testing requirements for heavy metals, ICP-MS has become the preferred technique, as it enables higher throughput, can reach lower detection limits, can detect multiple elements concurrently and has a larger linear range than ICP-OES. Some groups...
have turned to electrochemical microfluidics techniques and observed part-per-billion detection levels. These techniques have not seen mass adoption, however, due to high costs and increasing complexity.

Multiple studies have used ICP-MS to detect metal concentrations in cannabis plants and products. Studies in Greece have found low part-per-million concentrations of arsenic, chromium, lead, and cadmium in cannabis flower samples. A screen of CBD products in the UK found lead and arsenic in the low part-per-million range while studies in Poland have detected lead, cadmium, arsenic, aluminum, and nickel in a screen of dietary supplements and dried medicinal plants. Further screens in the United States have also confirmed arsenic, cadmium, and lead in CBD pet products and the authors own network of laboratories regularly finds metal contamination in cannabis and hemp products in multiple states (data not published).

Given the propensity for cannabis plants to accumulate metals from contaminated soils and the fact that the cheapest method of cultivation is to grow outdoors, it is likely metal contamination is a problem that is not going away soon. Without federal oversight, states are left to themselves to regulate which metals to test for and at what levels. Most states are testing for As, Cd, Hg, and Pb, which are categorized as Class 1 elements by the US FDA guidance. Maryland has added chromium, selenium, barium and silver to their list while New York has added nickel, chromium, copper, antimony, and zinc. NIST has recently created hemp reference material which includes lead, cadmium, arsenic, mercury, beryllium, cobalt, vanadium, chromium, manganese, molybdenum, nickel, selenium, and uranium. In 2022, Colorado will mandate the emissions of vaping products be tested for arsenic, lead, cadmium, and mercury. This is increasingly interesting, as the e-juice is in contact with various metal parts in the vaping device, for example, the heating coil. The fate of metals in different oxidation states that may be produced under pyrolysis and vaping conditions (high temperatures but not pyrolysis) is unknown. Due to vaping metal component interactions Cr, and Ni are under study by various groups in cannabis-containing e-juice and may be under consideration for cannabis regulation in states beyond Colorado.

**Microbiological testing**

Identifying and monitoring the diversity of microbiota in cannabis inflorescence and cannabis products can shed light on not only the quality attributes of cannabis, but also the prevalence of pathogens that have the potential to pose public health and safety concerns, especially in immunocompromised populations. Studies on the microbiome of cannabis inflorescence reveal a natural abundance of a wide variety of fungal and bacterial microorganisms, some of which can be pathogenic to humans. Additionally, cultivation practices, product manufacturing processes, and ingredients involved in manufacturing cannabis infused products introduce potential external sources of microbial contamination for consideration. Noting the natural and external sources of microbes to cannabis, it is imperative that adequate methodologies are employed to test cannabis and cannabis products for the presence, and when applicable, concentration of microbes to ensure quality and health standards are met.

In the current regulatory landscape within markets that have legal cannabis industries, Salmonella spp. and Escherichia coli, particularly Shiga toxin-producing Escherichia coli (STEC), are considered to be important enteric pathogens due to their potential to cause human disease and outbreak and their prevalence in a variety of agricultural, cultural, food, and general environmental settings. Salmonella spp. can serve as the causative agents of salmonellosis, typhoid fever, and paratyphoid fever, among other potentially fatal diseases. STEC are also facultative anaerobes that express virulence factors Shiga toxin type 1 and type 2 and when hosted in humans can cause hemolytic uremic syndrome (HUS), often indicated by bloody or non-bloody diarrhea, that can progress to a life threatening state. Additionally, Pseudomonas aeruginosa, Staphylococcus aureus, and bile tolerant gram negative (BTGN) bacteria have been implicated as pathogens of interest when considering testing requirements. Because all of these pathogens have prevalence in the environment and animals and can be transmitted through the production, processing, manufacturing, and dispensing of cannabis and cannabis products, it is critical that these types of products and processes are adequately and efficiently monitored to protect human and environmental health.

Studies regarding the mycoflora present in cannabis inflorescence demonstrate the prevalence of Aspergillus spp., Penicillium spp., Botrytis cinerea, and Fusarium spp., among others. Of these fungal organisms, Aspergillus spp., in particular the human pathogenic species Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, and Aspergillus terreus, have been a focal point of public health concern and subsequent testing regulations for cannabis. This is due to their known ubiquity in the environment, soil, and natural plant products, and incidence in clinical cases as the causative agent of aspergillosis in immunocompromised patients who had reported cannabis use. Aspergillosis is a disease that ranges in medical effects from invasive lung infection to the formation of aspergillomas (fungal balls) to allergic reactions, and cannabis use via inhaled modes of consumption indicates a higher risk for infection, especially in users with already compromised immune function. For this reason, Aspergillus spp. is more commonly required for testing in cannabis and cannabis products, although other fungal pathogens may also be of concern.

As testing for microbial contaminants in cannabis is a relatively nascent development, cannabis testing facilities and method developers have both looked to analogous industries for testing standards and guidance, as well as emerging technologies to meet the evolving needs of the cannabis space. Most commonly, either qualitative presence/absence tests or quantitative general microbial count tests.
are applied to cannabis products, although the need for additional confirmatory tests and metagenomics analyses have proven important in both confirming presumptive results and surveying cannabis materials for the prevalence of certain microorganisms.

In general, contaminants are known to be pathogenic to humans, such as Salmonella spp., STEC, and Aspergillus spp. require a qualitative presence/absence analysis, whereas tests evaluating the overall quality attributes of cannabis or cannabis products, such as viable yeast and mold count (TYMC) and total aerobic count (TAC) involve a quantitative overview of microbial populations within a given sample. Method references such as the FDA Bacteriological Analytical Manual Chapters 4 and 5 have provided the foundation of Salmonella spp. and STEC analysis for many emerging methods in cannabis.[137,138] Furthermore, entities like AOAC International have developed guidance documents to aid in the establishment of method performance requirements to assist in the development, validation, and approval of microbial contaminant analysis methods specifically in cannabis.[139-141] In addition, USP general chapters <61>, <62>, and <1111> provide direction for the establishment of appropriate limits for general count tests and also methodological guidance.[9,142-144] This has influenced testing methodologies for microbial contaminants in the cannabis space to revolve around culture-based isolation and identification and quantitative polymerase-chain reaction (qPCR) analysis, although other culture-based, mass spectral, and molecular technologies are being developed and implemented.

As cannabis and the process of its cultivation, harvesting, product manufacturing, and distribution are further studied, an increasing understanding of prevalent microbial contaminants and their respective implications on public and environmental health will be better understood. Currently, there is some research into the microbiome populations that occur naturally on the cannabis plant, and as well as other microbial pathogens that have susceptibility to contamination through manufacturing and handling practices in the cannabis and analogous industries that have guided the establishment of testing regulations and corresponding microbial analysis methodologies. As the industry matures, the development of inter-laboratory, reproducible reference methods in conjunction with the evolution of new testing technologies will continue to drive innovation in rapid, accurate, and consistent cannabis microbial analysis to support overall confidence in the quality attributes of cannabis and cannabis products, for both the general consumer and medical patients.

**Certified reference materials and method standardization**

The lack of method standardization in potency testing has led to a variety of test methods being used for cannabinoid quantitation, adding to the potential for variability in results between testing.[5,8,145] Not only is this an issue of analytical accuracy and consumer safety (e.g., does the consumer really know the potency of a product given interlaboratory variability?), it also undermines the confidence that cannabis growers and processors have in the entire testing process. While organizations like AOAC International have codified methods for the quantitation of cannabinoids in flower,[6] concentrates and oils,[146] and more recently in edible chocolates (methods not yet published), regulatory agencies have, as yet, not mandated the use of specific methods. For example, water quality test methods in the US are based on standard Environmental Protection Agency, with all laboratories contracted to do this testing running the same method and adhering to the same method validation and data quality criteria. Moreover, these labs are required to demonstrate ongoing proof of performance for a given test method through participation in interlaboratory studies and proficiency testing programs. This type of standardization would provide a clear quality management framework within which cannabis testing laboratories should operate. Not only would this improve data quality and consistency across the cannabis industry, it would also increase consumer confidence in the analytical results provided by different laboratories. Hindering this type of effort is the secrecy with which laboratories guard their test methods due to the perception that they are private intellectual property. This lack of open scientific communication hinders method standardization (e.g., developing consensus methods). While used as an example, this problem is not unique to potency testing—all of the test methods discussion herein are facing the same challenges when it comes to a lack of method standardization.

Closely tied to the lack of method standardization, is the fact that no consistent guidance on method validation is provided for cannabis compliance testing. To be fair, method validation is inherently ambiguous, with a plethora of ways to approach the evaluation of the core parameters of linearity, accuracy, precision, repeatability and robustness. Not to mention to myriad ways to calculate the limit of detection and/or limit of quantitation for a given test method.[147] This lack of standardization across the industry also adds to the potential for disparate results when comparing data between laboratories (as many cannabis growers and processors rightly do).

Finally, connecting method standardization and method validation is the challenge of developing certified reference materials (CRMs) to support compliance testing labs with materials to document the validity of their method validation results and test methods in general. The importance of CRMs was highlighted in a recent mycotoxin study, where differences were observed for the measured extraction efficiencies between spiked samples and samples with incurred mycotoxins, especially without the inclusion of a wetting step.[62] This study highlights a common limitation of spike-recovery studies that are used for method validation and emphasis the need for CRMs that can be used to assess method performance internally. The same challenge has been raised for pesticides, where the question of sequestration in the plant during maturation and the effect that may have on extraction efficiency is an important consideration. And for volatile analytes (e.g., residual solvent and terpenes),
the development of CRMs may present logistical challenges related to analyte retention during storage, transport and prior to analysis on-site at a cannabis testing laboratory.

It is worth noting that there are several cannabis/hemp proficiency testing programs, and participation in such programs is actually required as part of ISO/IEC 17025 accreditation. Such programs are not a substitute for CRMs though. Rather, they are another arm of a robust quality management system meant to facilitate method validity (versus consensus values) and data transparency. While invaluable, the reliance on a consensus value is not a measure of trueness, per se, as it could be influenced by systematic issues. For example, in 2020 a major supplier of analytical standards provided notification to cannabis testing laboratories that their certificate of authenticity label claim for a particular lot of THC standard was not accurate. This created a systematic bias in their THC values reported to clients, and possibly as part of a given proficiency testing study. A recent initiative by the National Institute for Standards and Technology (NIST) is working to address reference materials through their CannaQAP program,[148] which covers hemp potency and, for those with a DEA exemption, cannabis potency. In the second round of this program, NIST also included toxic elements (i.e., heavy metals) and moisture. Not only is this program free to participate in, but NIST openly publish the aggregate interlaboratory results. This type of transparency will help move the cannabis industry beyond some of the issues observed today, including the much-discussed challenge of “lab shopping.”[149]

Conclusions

Analytical technologies have generally found their respective places as they pertain to potency, terpenes and chemical/biological contaminants in cannabis and derivative products. However, as more testing data are acquired, more proficiency testing programs are evaluated, regulations change, etc., there will be a continuing need to evolve the collective approach to cannabis testing. Doing so will provide continued support to consumer safety and will also guide the evolving regulations and testing requirements for hemp and derivative products. As an example, consider potency, which continues to be a primary driver for broad consumer preference in the cannabis industry. As the cannabis derivatives market matures, increased regulatory control as well as increases in the number of such diverse matrices will continue to challenge testing laboratories and their support services (e.g., proficiency testing providers, certified reference material manufacturers, etc.). Moreover, as more cannabinoids, like δ8-THC, come under regulatory control, new tools in the laboratory may be necessary to meet method specificity requirements. Similar sets of challenges exist for the other test methods discussed herein.

It is also worth noting that secondary metabolites of interest in cannabis are not limited to cannabinoids and terpenes. There are variety of other chemicals of interest, flavonoids,[150] for example, that may enter the cannabis testing space. When these demands arise, the analytical testing community will need to leverage what has been learned about the challenges of cannabis as a matrix to develop suitable testing methods. Luckily, our understanding of the boundaries of analytical testing in cannabis and derivative products has made significant progress in recent years. This is a direct result of changes in the regulatory landscape bringing cannabis science into the commercial marketplace—when there is money to be made, the push toward robust, efficient testing regimes will always be hastened, albeit not without some growing pains due to a lag in the availability of the necessary testing infrastructure.

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Cannabis sativa
