Webinar Summary

Residual Pesticide Screening in Cannabis Matrices: Tips and Tricks

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This paper covers a number of tips and tricks to optimize the process of residual pesticide testing in the the sample preparation and test methodology stages of cannabis samples. It is a summary of a webinar conducted by Dr Macherone and Dr Stone on the 19th February 2020.

Introduction

Residual pesticide quantitation is challenging, regardless of sample type. But with cannabis, the issue is even more pronounced. Cannabis species biosynthesize thousands of chemicals including cannabinoids, terpenes, flavonoids, and fatty acids. Many of these chemicals are present in extremely high concentrations, making it a very complex matrix to analyze – especially when measuring part per billion levels of pesticides.

This webcast summary presents some key techniques and tips to test cannabis samples for residual pesticides and mycotoxins. It includes techniques for sample preparation and provides an overview of testing using two different methodologies: liquid chromatography/tandem mass spectrometry (LC/MS/MS), and gas chromatography/tandem mass spectrometry (GC/MS/MS).

Given the heterogeneous and fast-evolving regulatory environment, it is essential to set in place a testing protocol that can enable laboratories to rapidly adapt.

Key terminology used

Action Level	The quantitative concentration that a pesticide cannot exceed: defines pass/fail results.
Limit of Quantitation (LOQ)	The empirically determined lowest concentration of a pesticide within the sample that is confidently quantifiable. It is not necessarily the action level.
Limit of Detection (LOD)	The empirically determined lowest concentration of a pesticide that is confidently differentiated from noise. It is always lower than the LOQ.

Sample preparation

The key to success in pesticide screening is careful sample preparation. Cannabis is one of the most difficult plant matrices to extract trace analytes from. To be successful, getting the sample preparation right is the essential first step.

We describe below a single stream sample preparation routine that is suitable for both LC/MS/MS and GC/MS/MS techniques. Single stream sample preparation can also be categorized as superior winterization or "dilute and shoot"; delivering higher quantitative sensitivity, robustness, and throughput. It also decreases the need for instrument maintenance.

LC/MS sample preparation

Step 1: Weigh 1.0 g of chopped cannabis flower material into 50 mL tube. Add two ceramic homogenizer pellets and shake for 5 min at high speed.

Step 2: Add 15 mL acetonitrile and shake for a further 5 min at high speed.

Step 3: Place in centrifuge at 5000 rpm for 5 min.

Step 4: Transfer supernatant solvent into unconditioned Agilent SampliQ C18 EC SPE cartridge (P/N 5982–1365) for gravity elution.

Step 5: Add a 5 mL aliquot of acetonitrile to the original tube, stirring up the pellet of plant material and shake for 3 min at high speed. Transfer all solids and ACN to the SPE cartridge. Finally, wash tube with a further 5 mL ACN and transfer to SPE cartridge.

Step 6: Bring the collected eluent (extract) up to 25 mL with acetonitrile (25-fold dilution.)

Step 7: For LC/MS/MS: Aliquot 50 µl extract. Add 450 µl of 25/75% (v/v) mobile phase A/mobile phase B.

For GC/MS/MS: Aliquot 100 µl extract. Add 400 µl high-purity pesticide-grade acetonitrile.

Tips and tricks for successful sample preparation:

1. Transfer of acetonitrile extracts and washes (described in Steps 4 and 5 above) should be made with glass transfer pipettes. Avoid plastic types completely as they are a possible cause for ion suppression of several specific nonpolar pesticides.

   We investigated the use of plastic pipettes and tips and compared our scan results with those obtained using glass transfer pipettes.

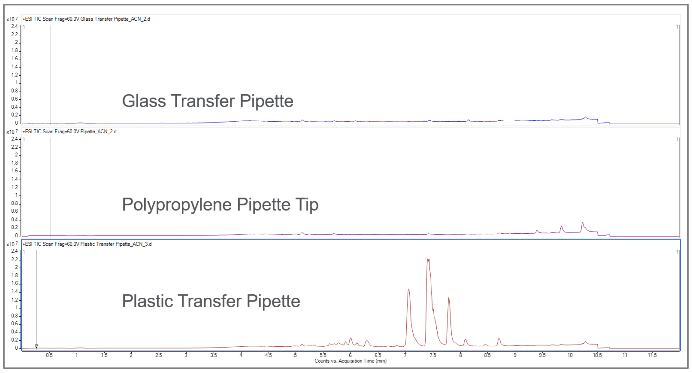

Figure 1. Oleamide plasticizer contamination via plastic pipettes

   Figure 1 shows three different types of transfer pipettes used during sample preparation and the respective total ion chromatogram (TIC) obtained from each material when transferring neat acetonitrile. These TICs are scaled identically. It is clear from Figure 1 that there are significant interference peaks that elute towards the end of the chromatography gradient, the exact region where some nonpolar pesticides were being suppressed. These contaminants were identified as oleamide plasticizers in the plastic transfer pipettes.

2. When transferring the two acetonitrile 5 mL wash aliquots (Step 5), it is important to ensure that the previous extract aliquot meniscus has disappeared below the SPE sorbent surface to avoid a recirculation of pesticides into subsequent aliquots.

3. Since gravity feed SPE filtering is used, not all the 25 mL will flow through into the collection vessel in Step 6. Directly add sufficient acetonitrile via a glass transfer pipette to make up the extract solution in the collection vessel to 25 mL.

4. Use a mixture of 25% aqueous mobile phase A and 75% methanolic mobile phase (v/v) for final sample dilution in Step 7. This is done in order to compensate for the lack of solubility of various pesticides in high aqueous percentiles. Methanol is the preferred organic diluent, and acetonitrile should be avoided for final dilutions.

California Pesticide List	Solvent Composition for Acceptable Recovery (% Aqueous)
Abamectin B1a	50
Abamectin B1b	50
Acephate	95
Acequinocyl	25
Acetamiprid	95
Aldicarb	95
Azoxystrobin	75
Bifenazate	75
Bifenthrin	25
Boscalid	75
Carbaryl	95
Carbofuran	95
Chlorantraniliprole	75
Chlorfenapyr	50
Chlorpyrifos	50
Clofentezine	50
Coumaphos	50
Cyfluthrin	25
Cypermethrin	25
Daminozid	95
DDVP (Dichlorvos)	95
Diazinon	50
Dimethomorph I	50
Dimethomorph II	50
Dimethoate	95
Ethoprophos	75
Etofenprox	25
Etoxazole	25
Fenhexamid	75
Fenoxycarb	50
Fenpyroximate	25
Fipronil	75
Flonicamid	95
Fludioxonil	95
Hexythiazox	95
Imazalil	95
Imidacloprid	95
Kresoxim-methyl	50
Malathion	75
Metalaxyl	95
Methiocarb	75
Methomyl	95
Methyl Parathion	75
Mevinphos	95
MGK-264	50
Myclobutanil	75
Oxamyl	95
Paclobutrazol	50
Permethrins	95
Phosmet	75
Piperonylbutoxide	25
Prallethrin	95
Propiconazole	25
Propoxur	95
Pyrethrins†	50
Pyridaben	25
Spinetoram L	25
Spinetoram J	25
Spinosin A	25
Spinosin D	25
Spiromesifen	50
Spirotetramat	75
Spiroxamine	95
Tebuconazole	50
Thiacloprid	95
Thiamethoxam	95
Trifloxystrobin	50

Figure 2. Maximum pesticide solubility in aqueous diluent content

Figure 2 illustrates the acceptable maximum percentage (v/v) of aqueous diluent required for near 100% recovery. The analytes highlighted represent the least aqueous-soluble ones, which precipitate out of the solution if the aqueous content percentage is increased.

Five separate batches of cannabis flower extract were prepared using this routine and figure 3 below illustrates the recoveries observed. California state legislation, for example, requires sample preparation recoveries of between 70 and 130%, and this was achieved using the sample preparation routine outlined herein.

California Pesticide List	Percent Recovery @ 60ppb
Abamectin B1a	92.5
Abamectin B1b	113.4
Acephate	91.9
Acequinocyl	94.2
Acetamiprid	94.9
Aldicarb	93.7
Azoxystrobin	95.2
Bifenazate	98.8
Bifenthrin	98.0
Boscalid	104.2
Carbaryl	95.7
Carbofuran	94.9
Chlorantraniliprole	98.2
Chlorfenapyr	102.4
Chlorpyrifos	96.4
Clofentezine	100.8
Coumaphos	106.8
Cyfluthrin	97.7
Cypermethrin	96.3
Daminozid	88.4
DDVP (Dichlorvos)	97.3
Diazinon	97.1
Dimethomorph I	107.6
Dimethomorph II	108.2
Dimethoate	97.9
Ethoprophos	103
Etofenprox	101.1
Etoxazole	98.6
Fenhexamid	129.5
Fenoxycarb	102.4
Fenpyroximate	103.2
Fipronil	90.6
Flonicamid	97.9
Fludioxonil	107.5
Hexythiazox	106.3
Imazalil	99.1
Imidacloprid	97.8
Kresoxim-methyl	103.7
Malathion	100.5
Metalaxyl	98.2
Methiocarb	102.7
Methomyl	96.5
Methyl Parathion	110.4
Mevinphos	103.9
MGK-264	109.4
Myclobutanil	104.9
Oxamyl	97
Paclobutrazol	106.9
Permethrins	96.8
Phosmet	101.9
Piperonylbutoxide	100.5
Prallethrin	98.1
Propiconazole	104.7
Propoxur	99.2
Pyrethrins†	70.8
Pyridaben	101
Spinetoram L	108.6
Spinetoram J	102.4
Spinosin A	101.2
Spinosin D	96.5
Spiromesifen	99
Spirotetramat	99.3
Spiroxamine	97.4
Tebuconazole	105.5
Thiacloprid	100.4
Thiamethoxam	97.2
Trifloxystrobin	100.8

$$\% \text{ Recovery} = \frac{\text{Pre — SPE Spiked Sample}}{\text{Post — SPE Spiked Sample}} \text{ x } 100$$

CA Mycotoxin List	% Recovery @ 4ppb
Aflatoxin G1	102.8
Aflatoxin G2	102.7
Aflatoxin B1	104.8
Aflatoxin B2	102.3
Ochratoxin A	100.5

$$\% \text{ Recovery} = \frac{\text{Pre — SPE Spiked Sample}}{\text{Post — SPE Spiked Sample}} \text{ x } 100$$

Figure 3. Sample preparation – recovery studies – CA pesticides and mycotoxins (average of five batches each for three strains of flower)

Tips and tricks for the LC/MS/MS methodology

This section discusses tips to improve results when using the LC/MS/MS methodology for successful detection of pesticides in cannabis inflorescence.

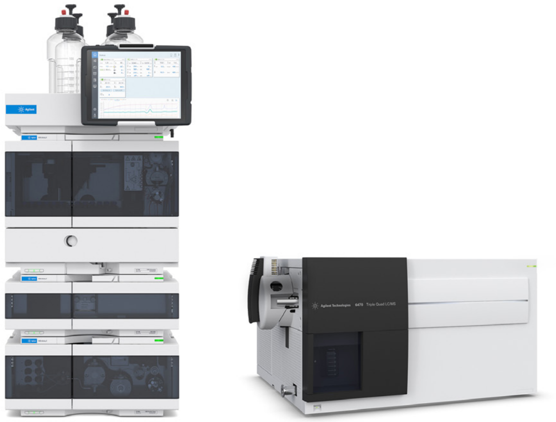

Figure 4. Agilent 1290 Infinity II / Agilent 6470

We recommend using the Agilent 1260 or 1290 Infinity II / Agilent 6470 or Agilent Ultivo LC/MS/MS with the following specifications:

Chromatographic column: Agilent InfinityLab Poroshell 120 column Phenyl-Hexyl 2.7 µm, 3.0 mm x 100 mm

Guard column: Agilent InfinityLab Poroshell 120 column Phenyl-Hexyl 2.7 µm, 3.0 mm x 5 mm

Mobile phase A: 5 mM ammonium formate with 0.1% formic acid in water

Mobile phase B: 0.1 % Formic acid in methanol

The total cycle time (sample injection to sample injection) is approximately 11 minutes.

To calibrate these results, we would recommend matrix-matched calibration (Linear: min. 5 point or quadratic: min. 6 point). This technique minimizes the deviation between the CCD and QC samples. Setting the calibration range to between 50% of each respective analyte action level and approximately five or more times its action level, using 1/X weighting for the calibration curve excluding origin is advised, and a correlation coefficient of >0.99 is required for most US States.

Method performance:

N = 5 replicates were used to determine the limit of quantitation, LOQ defined as having a signal-to-noise ratio >= 10:1

Recoveries were between 70 and 130%, %RSD < 15% for all pesticides. All pesticides detected by this methodology exhibited an LOQ of 50% or less than action limits.[1]

Figure 5 below shows an overlaid chromatogram of pesticides (California list) and mycotoxins in an extracted flower matrix, actual concentration 500 ppt (pre-extraction concentration = 125 ppb).

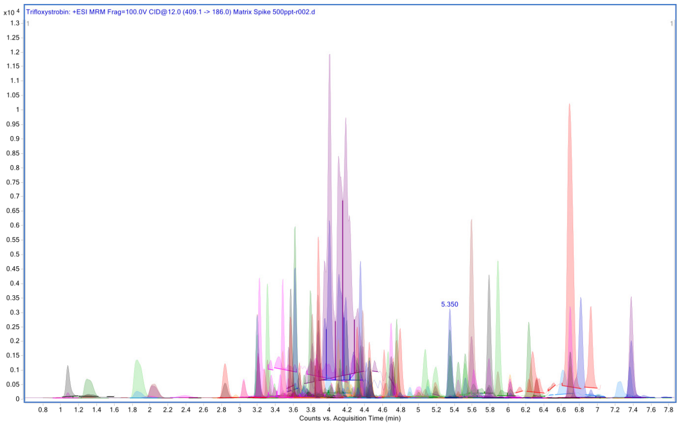

Figure 5. Chromatogram of pesticides and mycotoxins in extracted flower matrix

Tips and tricks for successful analysis:

1. Use the injector pretreatment portion of the acquisition methodology, which dilutes the sample further by one third. This makes it consistent with the starting composition of the reverse phase gradient, ensuring that the early-eluting polar analytes have acceptable and symmetric peak shapes. Any pesticide precipitation due to high aqueous content at this point is negligible, since the sample is already in the system and any precipitate dissolves as the organic gradient content rises during the chromatography process.
2. This final pretreatment stage involves a relatively large volume injection of 30 ul, and therefore to avoid 'volume overloading' of the guard column, it helps to use a column dimension of 3 mm or larger.

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[1] *California Cannabis testing, Agilent Application Note: 5994-0648*

3. Use high-quality LC/MS grade solvents from reputable manufacturers and preferably on-demand de-ionized water of 18.2 Mohm quality rather than bottled equivalents.
4. Source cleaning is another essential practice to achieve consistent and accurate results. We recommend a wipe down of the source chamber every day with a lint-free cloth using a solvent such as Methanol or IPA. This only takes 2–3 minutes. In addition, we advise cleaning the capillary approximately every three to four months. The process is relatively straightforward, by suspending the capillary in a measuring cylinder containing methanol or IPA sonicating for around 20 min.

Tips and tricks for the GC/MS/MS methodology

This section discusses tips to improve results when using the GC/MS/MS methodology for successful analysis of pesticides in cannabis inflorescence.

Figure 6. Agilent Intuvo 9000 GC

We recommend using the Agilent Intuvo 9000 GC with the following specifications:

MultiMode inlet (MMI), Guard Chip, mid-column backflush flow chip, and two Agilent 15 m × 0.25 mm, 0.25 µm HP-5MS UI columns. The 7010B tandem mass spectrometer was configured with the High Efficiency Source (HES) operated in electron ionization (EI) mode.

Tips and tricks for successful analysis:

1. In order to prolong the lifetime of the GC column, eliminate the need for column trimming, save time and improve sample throughput, the Agilent Intuvo 9000 GC includes a disposable consumable called the Agilent Guard Chip. This chip helps keep the system clean and extend column lifetime by ensuring that sample contamination never reaches the head of the Intuvo GC column.
2. Another essential component of this method is the use of analyte protectants (APs) introduced with the sample through a sandwiched injection. The AP solution is a dilute solution of L-gulonic acid γ-lactone (L-gulonolactone, CAS # 1128-23-0), and D-Sorbitol (CAS # 50-70-4). For use with the GC/MS/MS system, this mix must be diluted 1:10 in acetonitrile, and put in position 2 on the autosampler turret.
3. For thermally labile compounds like captan, cold inlet conditions are required. Due to this, we use a cold, solvent vent mode with fast injection parameters. Figure 7 below shows details of this sample setting.

MMI Program	Solvent Vent
Septum Purge Flow	3 mL/minute in switched mode with a total flow of 54.3 mL/minute
Gas Saver	20 mL/min after 3 minutes
Purge Flow to Split Vent	50 mL/min at 1.5 minutes
Vent Flow	25 mL/min
Vent Pressure	5 psi for 0.3 minutes
Cryo Type	Air
Inlet Temperature Program	60 °C for 0.35 minute, then 600 °C/min to 280 °C (14.8 minute hold time), then 600 °C/min to 300 °C (0.25 minute hold time)

Figure 7. Using cold inlet conditions with fast injection parameters

4. As a best practice, it is recommended to use internal standards and surrogates. The internal standard corrects for extraction and instrument bias while the surrogate helps determine extraction recoveries and provides a measure of method performance. α-BHC-d_6 and parathion-d_{10} were used as internal standards, and triphenylphosphate (TPP) as the surrogate, to monitor method performance over time and for recovery studies.

5. Using Agilent backflush capillary flow technology will remove heavy matrix and high-boiling interferences from the system between injections. Benefits of this include reduced source maintenance, retention time stability, and increased column longevity. Equipped with a high-efficiency source, it also provides the sensitivity and selectivity needed to measure part-per-billion levels of pesticides masked by the dense cannabis matrix.
6. Once an analytical method is developed, its performance must be verified to ensure accuracy, precision, and robustness over time.

Agilent employs a vetting paradigm that collects hundreds of data points over multiple days. Matrix-matched, quintuplet replicates are made at all calibrator and QC levels within a defined batch. Multiple batches are collected, resulting in a data-rich packet of information that is statistically analyzed to determine accuracy, range, linearity, accuracy, precision, LOD, and LLOQ for each pesticide. Data analysis is performed using Agilent MassHunter software and Agilent proprietary Quant-My-Way tools within the software package. Figures 8, 9, 10 and 11 show summaries of the statistical calculations used and sample results.

Statistical Calculations

Five replicate injections at each level permitted statistical calculations of MDL, LOQ, accuracy, precision, and average quantitative accuracy. This study used these primary equations:

- Average = $\sum x_i / n$
- Sample Standard Deviation, (s) = $\left[\frac{\sum (x - x)^2}{n - 1} \right]^{1/2}$
- MDL = (s) x (Student t-value, n — 1, 99% Confidence)
- Logical Test = Calculated MDL < Spike Level < 10 x Calculated MDL
- LOQ = 10 x (s)
- Precision, (%RSD) = [(s)/Average] x 100
- Average Quantitative Accuracy = (calculated average concentration/spiked concentration) x 100

The n — 1 degrees of freedom Student t-statistic was 3.747 at the 99% confidence level. The Calculated MDL < Spike Level < 10 * Calculated MDL equation was used to evaluate the empirically determined MDL and ensure its validity.

Figure 8. Summary

Compound	Target Calibration Level (ppb)	Empirical Average and 99% Confidence Interval	Percent Accuracy	Precision (%RSD)
PCNB	0.25	0.23 +/— 0.019	91	6.96
Methyl Parathion	0.25	0.28 +/— 0.013	111	4.71
Captan	4.00	4.31 +/— 0.50	108	10.00
Chlordane 1	1.00	0.86 +/— 0.071	86	7.61
Chlordane 2	1.00	0.99 +/— 0.081	99	7.02
Total Chlordanes	1.00	0.93 +/— 0.076	93	7.32
Chlorfenapyr	1.00	1.02 +/— 0.059	102	5.08

Figure 9. Accuracy and precision

Compound	In-Vial Range (ppb)	Number of Calibator Levels	Curve	Weighting
PCNB	0.016 - 64.00	8	Linear	1/x
Methyl Parathion	0.016 - 64.00	8	Linear	1/x
Captan	1.00 - 64.00	5	Linear	1/x
Chlordane 1	0.016 - 64.00	8	Linear	1/x
Chlordane 2	0.016 - 64.00	8	Linear	1/x
Chlorfenapyr	0.016 - 64.00	8	Linear	1/x

Figure 10. Range

Compound	CA Category	CA LOQ (ppb)	Empirical LOD In-Vial (ppb)	Empirical LOD In-Matrix (ppb)	Empirical LOQ In-Vial (ppb)	Empirical LOQ In-Matrix (ppb)
PCNB	II	100.00	0.061	7.59	0.16	20.25
Methyl Parathion	I	>LOD	0.031	3.88	0.084	10.50
Captan	II	700.00	1.64	204.75	4.37	546.38
Chlordane 1	I	>LOD	0.23	29.00	0.62	77.38
Chlordane 2	I	>LOD	0.26	32.75	0.70	87.38
Chlorfenapyr	I	>LOD	0.19	23.88	0.51	63.63

Figure 11. MDL

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