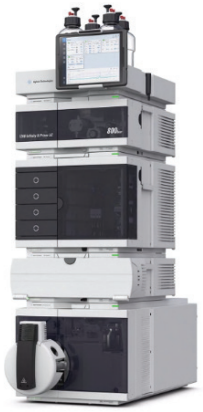
Application Note

Food Testing

Meeting California Cannabis Regulations with the Agilent 1260 Infinity II Prime LC and Agilent Ultivo Triple Quadruple MS

Pesticides, potency, and mycotoxins: one extraction, one analysis



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Abstract

The flowering buds of *cannabis sativa* are extracted and analyzed for the regulated LC/MS amenable pesticides and mycotoxins at sub-ppm levels. The same extract and analysis is used to determine the required cannabinoids for potency at the percent level using the lower response of the carbon-13 isotopes of their pseudomolecular ions and product ions. This Application Note is not intended as a proposed workflow solution from Agilent. Instead, it is an indication from an experienced test laboratory of their strategy to reduce sample turnaround time and lab resourcing, in assessing cannabis flowers for safety and potency. Sample preparation is simply a solvent extraction followed by SPE cleanup and a 250-fold dilution. The required method detection limits for the mycotoxins at 20 ppb and for the pesticides at 100 ppb are met. Recoveries in matrix for pesticides range from 75 to 105%, and for the mycotoxins between 60 and 80%. Accuracy for potency spikes was between 85 and 105% for the six cannabinoids.

Introduction

Presently, 21 states in the USA have legalized the use of medical marijuana. In addition, nine states and the District of Columbia have legalized its use for adult recreation. Although more may follow, the federal government still classifies marijuana as a schedule I substance. Schedule I designates that the substance has considerable potential for abuse, can lead to physical or psychological dependence, and has no medical use. Recently, the US Food and Drug Administration (FDA) has approved a cannabinoid in marijuana for treatment of children with epilepsy.[1] However, until the Federal government changes its marijuana laws, each state must determine its own regulations without guidance from the FDA or the Environmental Protection Agency (EPA). The US EPA is responsible for regulating pesticides in food and setting maximum residue limits for those approved pesticides.

With the legalization of cannabis in California for both medical (Compassionate Use Act of 1996)[2] and recreational use (The adult Use of Marijuana Act),[3] requirements for safe use have been put in place. Stipulations regarding the use of allowed pesticides have been made,[4] and those pesticides that cannot be used have be identified.[5] Because mold has been found on marijuana, specifically *Aspergillus*, the testing for aflatoxins and ochratoxin has been required (see section of 5721 of the final California regulations).[6]

Although required analytes have been specified, validated methods have not, and it is the responsibility of the testing laboratory to provide valid methods of analysis. A method for the analysis of 214 pesticides in the flower of cannabis using both GC/MS and LC/MS has been reported using a relatively simple extraction and cleanup procedure.[7] A similar method has been developed to meet the Canadian regulations,[8] and that method has been applied to the California regulations.[9] We used a modification of that procedure to analyze the 52 pesticides amenable to LC/MS in the California required list.

As outlined elsewhere, it is the opinion of Weck labs that certain pesticides on the California list are not reliable to be analyzed through electrospray ionization (ESI) or atmospheric chemical ionization (APCI), and are best analyzed using GC/MS. For example, chlordane ($C_{10}H_6Cl_8$, monoisotopic mass 405.8) has been shown to respond with APCI. However, the precursor ion of *m/z* 266.8 is the dissociative loss of four chlorine atoms. Thus, the MRM transitions are not specific to the analytes, defeating a primary function of soft ionization techniques. In addition, with the high number of congeners of chlordane, the results would not be accurate. For this compound and others with similar challenges, Weck Labs successfully apply GC/MS/MS. Compounds that could be analyzed by LC/MS but provide better results by GC/MS are also not required for this LC/MS method.

In addition to the pesticides in scope, four aflatoxins (B1, B2, G1, and G2), and ochratoxin are analyzed in the same extract along with the cannabinoids required for potency. Using LC/MS/MS for potency adds specificity not obtained with other methods and makes the analysis much more efficient. The determination of all these analytes in one method of analysis poses challenges in both their widely varied chemistry and concentrations. The sum of the four aflatoxins cannot exceed 20 ng/g while the pesticides require a detection limit of 100 ng/g, and the cannabinoids are found at percent levels.

The standard approach for assessing cannabinoids in cannabis is to make a separate analysis using HPLC with UV detection.[10] This approach is well proven and robust, but Weck wanted to see if this assessment could be condensed within the preceding pesticide and mycotoxin analysis. Using the much less abundant ^{13}C isotopes as precursors and product ions, the ability to analyze high-concentration compounds along with trace compounds is demonstrated.

This Application Note describes the methodology used to determine these varied compounds and concentrations in one analysis, and provides some of the performance characteristics of the method. We plan to test this multiplex approach in the coming years for robustness and cost effectiveness.

Experimental

Reagents and standards

Water was obtained from a Milli-Q polishing system. Acetonitrile and isopropanol were from Thermo Fisher Scientific (Waltham, Massachusetts), and acetic acid was from Millipore Sigma (Burlington, Massachusetts). The SPE cartridges were a 200 mg/6 mL reversed-phased polymeric sorbent. Pesticide standards were from AccuStandard (New Haven, Connecticut), mycotoxins from Millipore Sigma, and cannabinoids from Cerilliant (Round Rock, Texas).

Sample preparation

Flowers of cannabis were ground with a ceramic homogenizer. A 1.0 g homogenized sample was placed in a 50 mL centrifuge tube, and 15 mL of acetonitrile was added; the tube was then shaken for 120 seconds. The liquid was next decanted into the SPE cartridge (not conditioned), and the eluent collected.

The centrifuge tube was washed with 5 mL of acetonitrile two times, and each was passed through the SPE cartridge. The fractions were then combined and brought to a total of 25 mL with acetonitrile for a final dilution of 1 g to 25 mL, then 100 µL of this extract was diluted to 1.0 mL, resulting in an overall dilution of 250×.

Instrument and operational parameters

This method used an Agilent 1260 Infinity II Prime LC with:

- Agilent 1260 Infinity II flexible pump with pressure to 800 bar
- Agilent 1260 Infinity II multisampler
- Agilent 1260 Infinity II multicolumn thermostat

The LC system was coupled to an Agilent Ultivo triple quadrupole LC/MS (LC/TQ). Injections were made with a sandwich of solvent as follows: 10 µL water, 2 µL sample (acetonitrile), and 10 µL water. After each run, the injection loop was rinsed with 30 µL of isopropanol. Table 1 shows the conditions for the analysis of pesticides, mycotoxins, and the six cannabinoids required for potency.

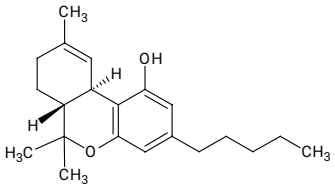
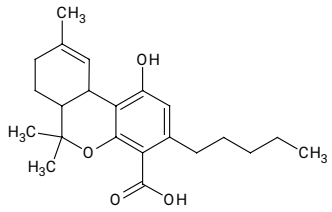
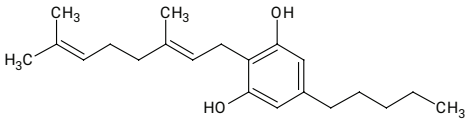
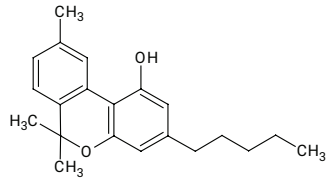
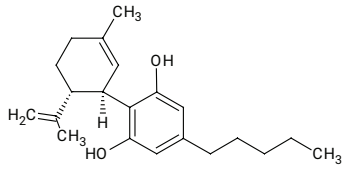
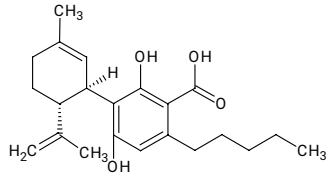
Table 1. LC/MS/MS conditions.

LC Conditions Agilent 1260 Infinity II Prime LC	
Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 mm × 50 mm, 1.9 µm (p/n 699675-902)
Column Temperature	25 °C
Injection Volume	2 µL
Mobile Phase	A) H_2O with 5 mM ammonium acetate and 0.1% acetic acid B)100% acetonitrile
Run Time	8 minutes, data collection to 6.5 minutes
Flow Rate	0.650 mL/min
Gradient	Time (min) %B 0–0.5 5 4 76 5.5 83 7.5 100 Hold to 8 minutes
MS Conditions With Positive/Negative Switching	
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Gas Temperature	250 °C
Gas Flow	9 L/min
Nebulizer Pressure	35 psi
Capillary Voltage	4,000 V in positive and 3,500 V in negative
Nozzle Voltage	0 V in positive and negative mode

Results and discussion

Table 2 lists the cannabinoids specified by California regulations for potency. The analysis of these compounds was performed by calibrating the instrument with ^{12}C monoisotopic mass using its protonated or deprotonated ion. This allows the use of small quantities of these chemicals for calibration as the concentrations needed to calibrate are ng/mL. A conversion calibration was then used in the analysis of samples using the ^{13}C or $^{13}C_2$ isotopic mass of the protonated or deprotonated ion to reduce the response so that the detector was not saturated. This is shown in Figure 1, where the response is dramatically reduced. Table 3 gives the conditions for the isotope measurement. Figure 2 shows the calibration of ^{12}C and ^{13}C isotopes. Because ionization is the same, the relative responses are accurate. The conversion between the two calibrations is shown in Figure 3, and is generated by correlating the response of highly dilute standards (10K × dilution) using the ^{12}C transitions with the concentrations found from the sample preparation described (a 250-fold dilution) using the ^{13}C transitions.

Table 2. Names, CAS numbers, and chemical structures of regulated cannabinoids for potency.

Analyte Name Abbreviation Monoisotopic Mass	CAS Number	Chemical Structure
Tetrahydrocannabinol THC 314.224579	1972-08-3	
Tetrahydrocannabinolic acid THCA 358.214417	23978-85-0	
Cannabigerol CBG 316.240234	25654-31-3	
Cannabinol CBN 310.193268	521-35-7	
Cannabidiol CBD 314.224579	13956-29-1	
Cannabidiolic acid CBDA 358.214417	1244-58-2	

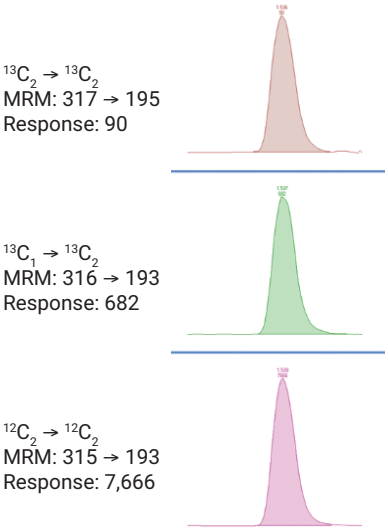


Figure 1. The relative response of THC using ^{12}C and ^{13}C transitions.

Table 3. ^{13}C transitions for cannabinoids and their dynamic MRM settings.

Compound	Precursor Ion	Product Ions	Fragmentor Voltage (V)	Collision Energy (V)	Retention Time (min)	Polarity
TCH	317	193 123	100	20 36	5.88	Positive
THCA	359	245 313	128	36 24	5.51	Negative
CBD	316	193 123	128	24 32	5.31	Positive
CBDA	359	245 339	134	32 20	4.97	Negative
CBN	311	279 222	162	32 50	5.63	Negative
CBG	318	123 193	118	32 12	5.28	Positive

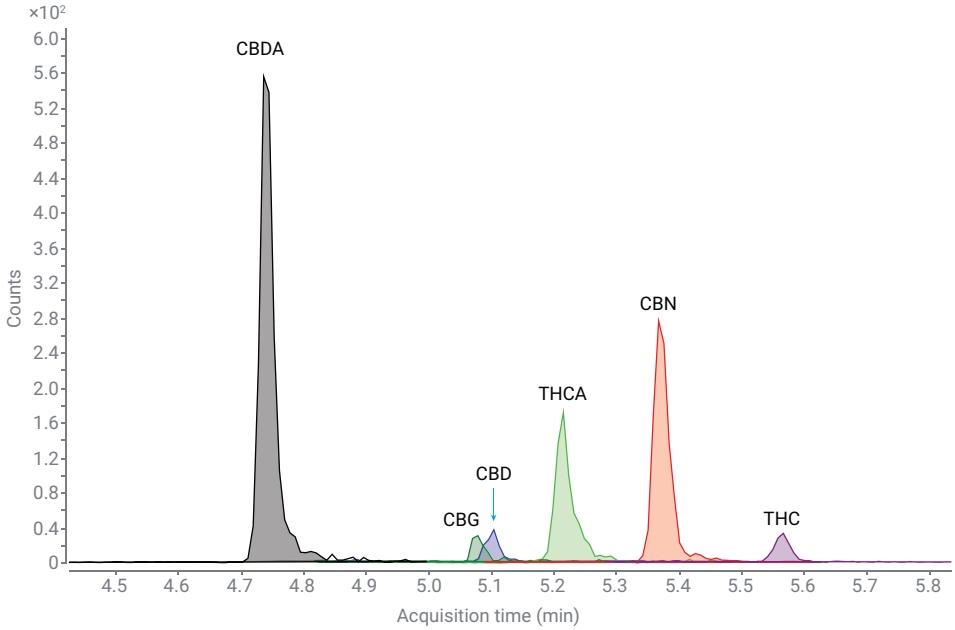


Figure 2. Separation of six cannabinoids using the method for potency, mycotoxins, and pesticides. The responses are from the ^{13}C transitions in the method for a hops spike at 0.125% concentration, the California regulatory limit. Note that the peaks are between three and five seconds wide.

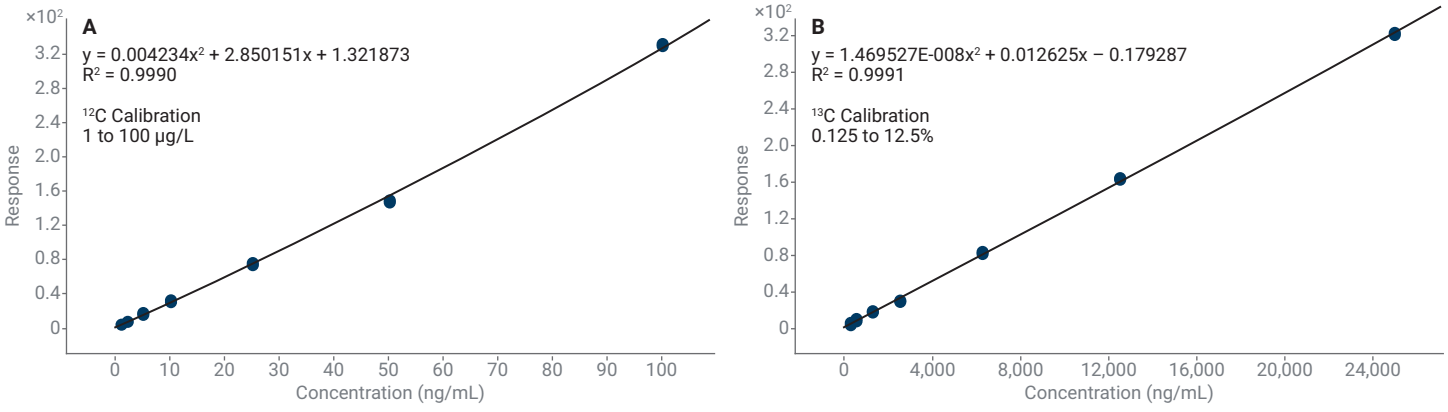


Figure 3. Calibration example of cannabinoids; THC is shown.

The mycotoxins and pesticides were determined directly from their respective calibrations. The mycotoxin structures, transitions, MRM conditions, and method detection limits are given in Tables 4 to 6, respectively. These analytes have the lowest required detection limits, and this method meets those requirements.

The California regulated pesticides amenable to ESI LC/MS are the largest list of analytes in this method. Table 7 gives their dynamic MRM transitions and conditions. The California regulated limits for these compounds in inhalable products are given in Table 8, as are the signal-to-noise ratios (S/Ns) at the regulated limits. The method can easily meet the required limits for cannabis flower, and often, are much lower.

Table 4. Regulated mycotoxins, their CAS number, and structure.

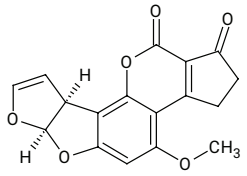
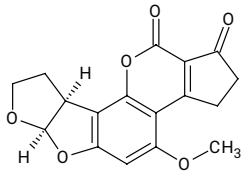
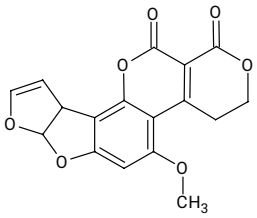
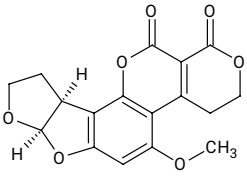
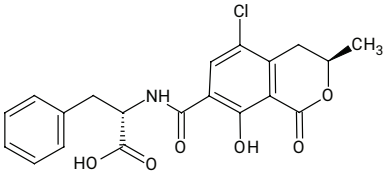
Analyte Name Monoisotopic Mass	CAS Number	Chemical Structure
Aflatoxin B1 312.063385	1162-65-8	
Aflatoxin B2 314.079041	7220-81-7	
Aflatoxin G1 328.058289	1402-68-2	
Aflatoxin G2 330.073944	1262849-73-9	
Ochratoxin A 403.082275	303-47-9	

Table 5. Mycotoxin transitions and MRM conditions.

Compound	Precursor Ion	Product Ions	Fragmentor Voltage (V)	Collision Energy (V)	Retention Time (min)	Polarity
Aflatoxin B1	313	285.1 241.1	159	16 36	3.13	Positive
Aflatoxin B2	315	287.1 259	167	20 24	3.01	Positive
Aflatoxin G1	329	243 200	153	24 40	3.00	Positive
Aflatoxin G2	331	313.1 189.1	153	20 40	2.88	Positive
Ochratoxin A	404	239 221	115	16 32	3.62	Positive

Table 6. California required limits of quantitation for the mycotoxins studied, accounting for both MRM transitions. The aflatoxins cannot exceed a total of 20 ng/g.

Compound	LOQ (ng/g)	S/N at LOQ
Aflatoxin B1	5	210
Aflatoxin B2	5	2,300
Aflatoxin G1	5	3,900
Aflatoxin G2	5	1,000
Ochratoxin	20	1,100

Table 7. Pesticide transitions and their MRM conditions.

Compound	Precursor Ion	Product Ions	Retention Time	Fragmentor	Collision Energy	Polarity
Abamectin B1a	890.5	567 305	5.63	130	20 0	Positive
Abamectin B1b	876.5	553 291	5.49	100	12 0	Positive
Acephate	184	143 49.2	0.39	75	0 12	Positive
Aldicarb	116	89 70	2.81	75	5 5	Positive
Azoxystrobin	404	372 344	4.02	110	4 20	Positive
Bifenazate	301.2	198 170	4.17	95	0 12	Positive
Boscalid	343	307 271	4.07	145	12 28	Positive
Carbaryl	202.1	145.1 127	3.33	106	0 24	Positive
Chlorantraniliprole	484	453 286	4.39	105	8 4	Positive
Carbofuran	222.1	165.1 123	3.23	83	4 16	Positive
Clofentezine	303	138 102	4.61	97	4 36	Positive
Coumaphos	363	307 227	4.6	111	28 40	Positive
Daminozide	161.1	143.1 43.9	0.21	83	0 20	Positive
Diazinon	305.1	169 153	4.92	115	16 16	Positive
Dimethoate	230	199 124.9	2.41	74	0 16	Positive
Dimethomorph	388.1	301 165	3.85	120	12 28	Positive
Ethoprop (Ethoprophos)	243.1	130.9 96.9	4.05	100	15 35	Positive
Etofenprox	394.5	177 107	5.88	111	4 44	Positive
Etoxazole	360.2	141 113	5.22	109	28 55	Positive
Fenhexamid	302.1	97.2 55.1	4.07	100	35 60	Positive
Fenoxycarb	302.1	116 88	4.26	115	0 12	Positive
Fenpyroximate	422.2	366 138	5.17	118	8 28	Positive
Fipronil	435	330 250	4.44	120	22 40	Negative
Flonicamid	230.1	203 174	1.54	112	12 12	Positive
Fludioxonil	247	126	3.89	141	28	Negative
Fludioxonil	229	185	3.89	120	8	Positive
Hexythiazox	353.1	228 168	5.09	112	4 20	Positive

Compound	Precursor Ion	Product Ions	Retention Time	Fragmentor	Collision Energy	Polarity
Imazalil	297.1	200.9 159	3.41	124	20 16	Positive
Imidacloprid	256.1	209 175	2.4	89	8 12	Positive
Jasmolin I	331.2	126.9 98.9	4.42	97	4 20	Positive
Malathion	331.1	126.9 98.9	3.55	103	4 20	Positive
Metalaxyl	280	220 192	3.49	120	4 8	Positive
Methiocarb	226.1	169 121	3.82	86	0 12	Positive
Methomyl	163.1	106 88	1.15	75	0 0	Positive
Mevinphos	225	193.1 127.1	2.68	74	0 8	Positive
Myclobutanil	289.1	125 70	4	112	16 16	Positive
Oxamyl	237	90 72	1.1	75	0 10	Positive
Paclobutrazol	294.1	125 70	3.81	112	36 12	Positive
Phosmet	318	160 133	4.09	75	8 36	Positive
Piperonyl Butoxide	356	177 119	5.39	90	0 32	Positive
Propiconazole	342	159 69	4.3	115	32 12	Positive
Propoxur	210.1	110.9 92.9	3.19	83	4 20	Positive
Pyridaben	365.2	309 147	5.43	80	0 20	Positive
Spinetoram	760.5	142 98	4.55	150	20 48	Positive
Spinosyn	732.5	142 98	4.96	80	24 50	Positive
Spiromesifen	371.2	355.1 255.1	6	97	0 16	Positive
Spirotetramat	374.2	330 302	3.95	120	4 8	Positive
Spiroxamine	298.3	143.9 100	3.66	115	12 28	Positive
Tebuconazole	308.2	125 70	4.14	100	40 12	Positive
Thiacloprid	253	125.9 98.9	2.81	100	16 40	Positive
Thiamethoxam	292	211 181	1.91	77	4 16	Positive
Trifloxystrobin	409.1	206 186	4.82	120	4 8	Positive

Table 8. Pesticide minimum required levels in µg/g for inhalable products and the corresponding S/N of each pesticide at the minimum level (ML).

Pesticide	Minimum Level (µg/g)	S/N at ML
Abamectin	0.1	2600
Acephate	0.1	1400
Acetamiprid	0.1	2200
Aldicarb	0.1	2100
Azoxystrobin	0.1	15000
Bifenazate	0.1	3200
Boscalid	0.1	200
Carbaryl	0.5	4500
Carbofuran	0.1	5900
Chlorantraniliprole	10	1100
Clofentezine	0.1	540
Coumaphos	0.1	200
Daminozide	0.1	500
Diazinon	0.1	4500
Dimethoate	0.1	2000
Dimethomorph	2	960
Ethoprop	0.1	1600
Etofenprox	0.1	190
Etoxazole	0.1	5500
Fenhexamid	0.1	130
Fenoxycarb	0.1	225
Fenpyroximate	0.1	2900
Fipronil	0.1	12000
Flonicamid	0.1	210
Fludioxonil	0.1	7900
Hexythiazox	0.1	220

Pesticide	Minimum Level (µg/g)	S/N at ML
Imazalil	0.1	550
Imidacloprid	5	1300
Jasmolin I (Pyrethrins)	0.5	400
Malathion	0.5	200
Metalaxyl	2	1700
Methiocarb	0.1	3100
Methomyl	1	3400
Mevinphos	0.1	3200
Myclobutanil	0.1	630
Oxamyl	0.5	7400
Paclobutrazol	0.1	1000
Phosmet	0.1	240
Piperonyl Butoxide	3	4500
Propiconazole	0.1	1300
Propoxur	0.1	3600
Pyridaben	0.1	2300
Spinetoram	0.1	1200
Spinosyn	0.1	20000
Spiromesifen	0.1	1300
Spirotetramat	0.1	900
Spiroxamine	0.1	2100
Tebuconazole	0.1	2300
Thiacloprid	0.1	1100
Thiamethoxam	5	2000
Trifloxystrobin	0.1	4300

Method performance

Figure 2 shows the separation achieved for the regulated cannabinoids. Although a Phenyl-Hexyl column was shown to give a better separation for pesticides in cannabis,[7] the column we used provided a better separation for these cannabinoids. Also, the regulation does not specify the need to separate Δ^8-THC from Δ^9-THC. Because cannabinoids are present in any *cannabis* spp. matrix including hemp, the only way to access the accuracy of the measurement is to spike the compounds at twice the level found in a sample, and calculate the results: essentially standard addition. Another way of assessing accuracy is to spike real samples with stable isotope standards (isotope dilution), and measure the accuracy of the stable isotope compared to the same in solvent. Because of the cost of the stable isotopes, however, this would not be practical. To demonstrate the efficacy of using the ^{13}C isotopic transitions for determining the concentrations in the flower of the plant, spikes of each were made into hops. Although the hops matrix is not as complex as cannabis and is not recommended as a surrogate matrix, the 250-fold dilution factor mediates the differences. The hops were then extracted using the all-in-one methodology and the ^{13}C isotopic transitions. The same spiked sample was then extracted with acetonitrile and diluted to the range of the ^{12}C isotopic transitions calibration curve.

The results of those spikes showed good accuracy for the methodology, and are given in Table 9. Three real cannabis flower samples were analyzed with the sample preparation procedure described previously, and with a simple acetonitrile extraction and dilution into the response range of the ^{12}C calibration. Table 10 gives those results, and are in good agreement, showing that this methodology does work for cannabis flower. Interestingly, three unknown cannabis samples showed high concentrations for THCA, and sample 1 was high in THC with lower amounts in the other two samples. For the other four cannabinoids, either none or little was detected. Spike recoveries are shown in Figure 4, and are well within acceptable values.

Table 9. Comparison of spiked sample of hops diluted to the range of the ^{12}C calibration curve and the determined concentration using the all-in-one sample preparation and the ^{13}C isotope transitions. Values are given as a percent of the total sample.

		Sample 1		Sample 2	
	Spiked	Diluted	^{13}C	Diluted	^{13}C
THC	2.5	1.85	2.39	2.31	3.1
THCA	2.5	2.65	1.97	1.7	1.98
CBD	2.5	3.03	3.3	1.85	2.06
CBDA	2.5	2.12	2	2.1	2.41
CBG	2.5	2.71	2.34	3.33	2.91
CBN	2.5	2.78	2.56	2.53	2.18

Table 10. Comparison of real cannabis flower samples both diluted to the range of the ^{12}C isotope calibration versus the all-in-one sample preparation method using the ^{13}C isotope transitions. Values are given as a percent of the total sample.

	Sample 1		Sample 2		Sample 3	
	Diluted	^{13}C	Diluted	^{13}C	Diluted	^{13}C
THC	7.03	6.88	0.5	0.86	0.95	0.66
THCA	9.29	9.8	7.17	7.92	4.63	4.42
CBD	ND	ND	ND	ND	ND	ND
CBDA	ND	ND	ND	ND	ND	ND
CBG	0.11	0.15	ND	ND	ND	ND
CBN	0.09	0.12	ND	ND	ND	ND

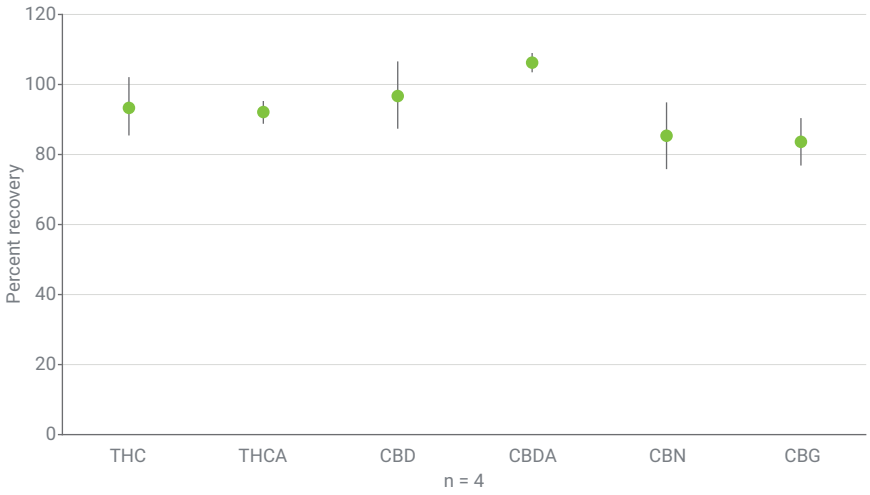


Figure 4. Recovery of the cannabinoids in hops spiked at 5% with four separate extractions.

Figure 5 shows the separation of the five mycotoxins, and demonstrates three-second-wide peaks. The mycotoxins were spiked into a hops extract, and with the 250 fold dilution at a spike level of 1 ppb in the original hops, the LOQ is below the regulated limits. Spikes at a concentration of 15 ppb for the individual aflatoxins and 60 ppb for ochratoxin give recoveries between 60 and 80%, as shown in Figure 6. None of the mycotoxins were detected in the cannabis flower samples.

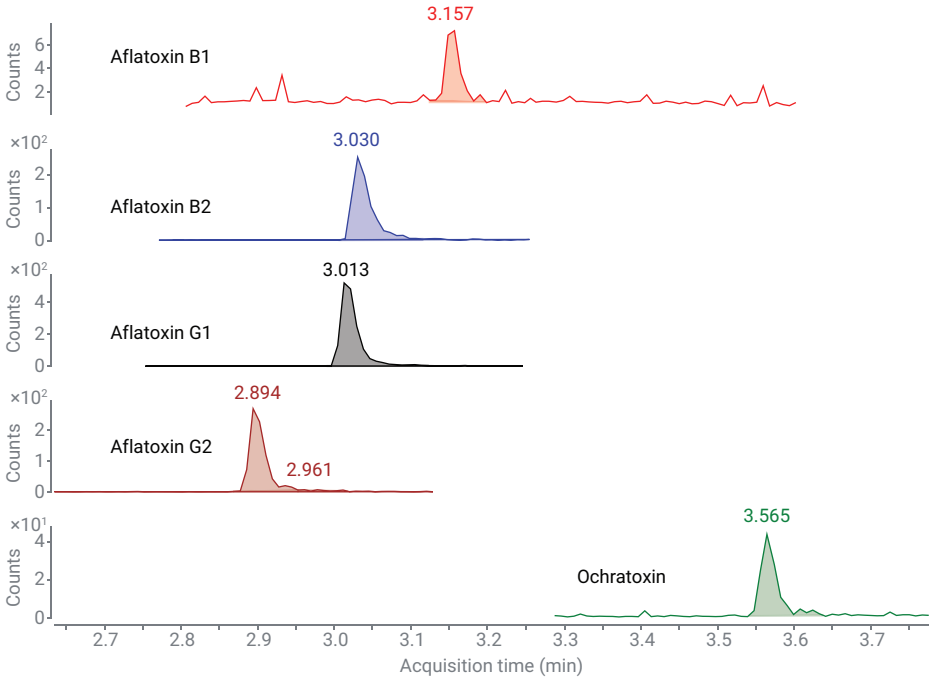


Figure 5. Five mycotoxins spiked into hops at the minimum level (5 ng/g for the aflatoxins and 20 ng/g for ochratoxin A), demonstrating the ability to detect below the required limits.

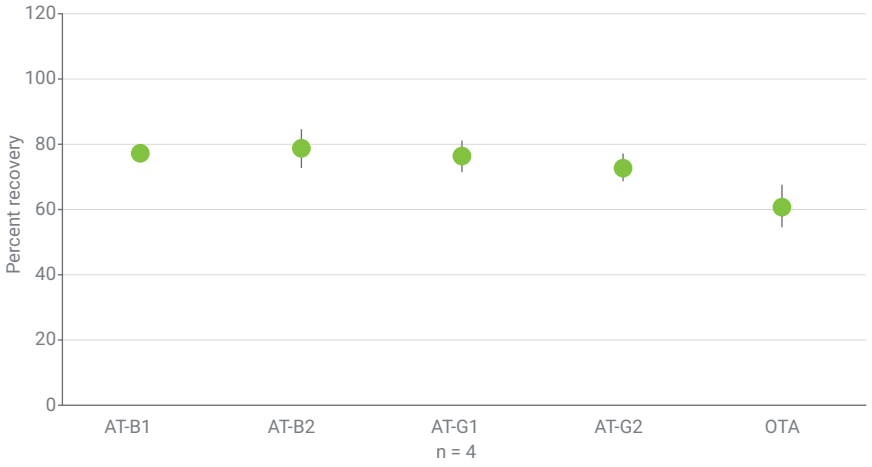


Figure 6. Recovery of mycotoxins in an unknown cannabis sample spiked at 3x the minimum level (not detected in sample).

Figure 7 shows the separation of each of the pesticides. The pesticides eluting early in the chromatogram have broad peaks but are still easily detected. Most of the pesticides are well separated. Only one pesticide was detected in one of the flower sample extracts. That pesticide, imidacloprid (a neonicotinoid), is typically used for insect control such as spider mites. The level detected was 1.5 ppm and well below the regulated limit of 5 ppm. The peak is shown in Figure 8 along with a nondetected pesticide imazalil for comparison. Spiked recoveries for the pesticides were typically between 70 to 105%, as shown in Figure 9.

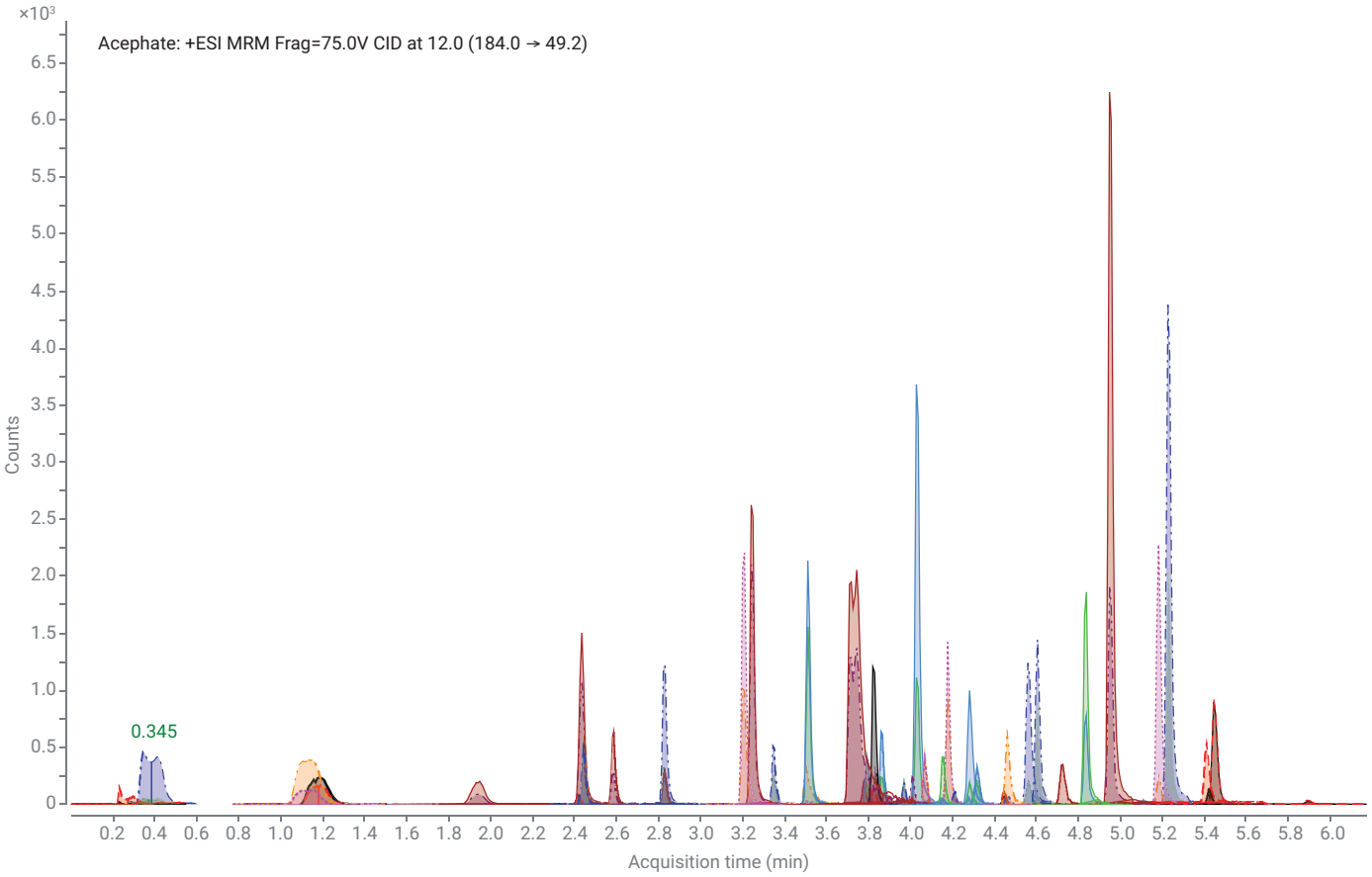


Figure 7. Separation of LC/MS/MS amenable pesticides extracted from hops at a concentration of 0.4 µg/g.

In addition to the previously mentioned performance characteristics of the method, the robustness of the method was examined by making 80 injections of a cannabis extract spiked with 2 ppb of each pesticide (equivalent to 500 ppb in the original sample with a 250-fold dilution). Also, each aflatoxin was spiked at 0.1 ppb (25 ppb in the sample with the 250 × dilution) and 0.4 ppb of ochratoxin. The total average recovery of all spiked analytes including the cannabinoids in the cannabis extract are shown in Figure 10.

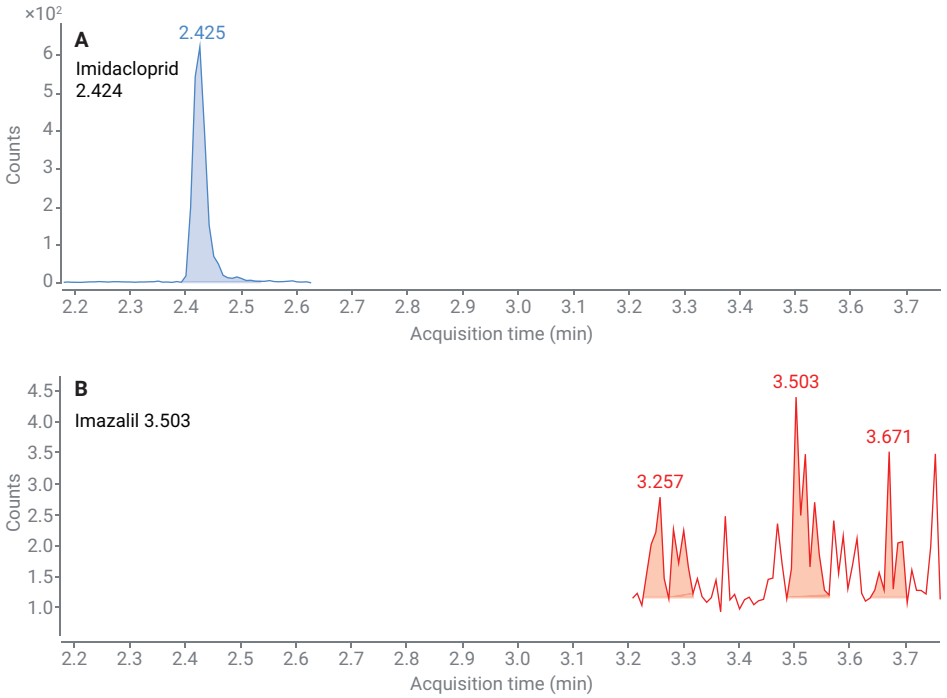


Figure 8. A) Imidacloprid in a marijuana flowering bud sample at 1.5 ppm; the California limit is 5 ppm. B) Imazalil, not detected.

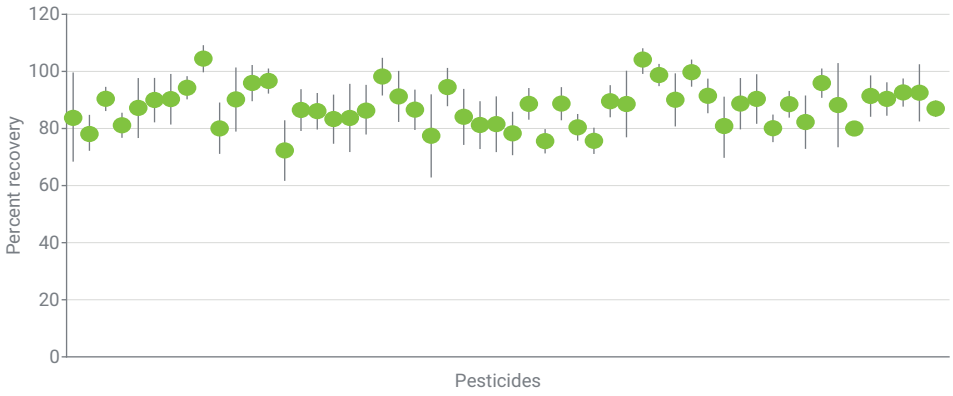


Figure 9. Recovery of pesticides in an unknown cannabis sample spiked at 1 µg/g (not detected in sample).

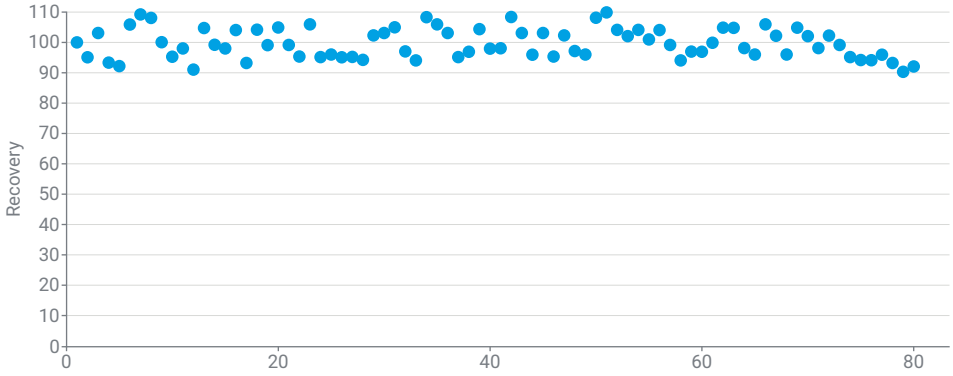


Figure 10. Average recoveries of cannabis extract spiked with 2 ppb pesticides, 0.1 ppb aflatoxins, and 0.4 ochratoxin.

Conclusion

A single method for extraction and analysis is provided for California regulated pesticides amenable to LC/MS, mycotoxins and potency using the Agilent Infinity II Prime LC and Agilent Ultivo LC/triple quadrupole. The method provides the needed accuracy and precision to meet California requirements for marijuana in medical and recreational use.

The method provides one procedure for:

- Potency
- Mycotoxins
- Pesticides

This single method saves time and money, and uses the highly specific MS/MS capability for potency of cannabinoids. The method outlined forms a platform for other experienced labs to explore and develop their own multiplexing approaches. However, developing such methods requires highly skilled analysts moving from familiarity with more standard workflows.

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