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Liquid Chromatography-Mass Spectrometry for the Geographical Sourcing of *Cannabis sativa* L.

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Abstract. Marijuana, dried and ground *Cannabis*, is the most consumed illicit drug in the world, despite many undesirable and risky effects to human health that are caused by its use. The medicinal use and/or legal recreational use of *Cannabis* has also been rising worldwide. Therefore, traceability methodologies are increasingly gaining importance whether for forensic use, such as drug trafficking eradication, or for quality control purposes of legal medicinal *Cannabis*. The goal of this study was to analyze *Cannabis* samples seized by the Brazilian Federal Police in eradication operations occurred in 2014 and 2017, by means of liquid chromatography-mass spectrometry (LC-MS) and linear discriminant analysis (LDA), aiming to trace their geographical origin. The LDA showed 39.13% accuracy in sourcing the samples from 2014, and 80.95% for the 2017 samples, which indicates that high temperature in the drying step and plant age negatively affect data accuracy.

Keywords: LC-MS; Hemp; Marijuana; THC; CBN; LDA.

1. Introduction

Cannabis is by far the most widely used drug worldwide: approximately 228 million people used it in 2022[1]; not surprisingly, it is the most used illicit drug worldwide, despite its use being illegal in several countries[2].

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According to the United Nations Office on Drug and Crime, there were 6,168 tons of *Cannabis* herb and 1,194 ton of *Cannabis* resin seized in 2022 around the world[1]. *Cannabis* cultivation did not continue its upward trend in 2022: outdoor *Cannabis* cultivation declined while indoor cultivation seems to have reached a plateau, although at a high level.

Surveys applied in various member countries of the European Union[3] show that 4.3% of adults between 15 and 64 years of age and 12% of 15 and 16-year-old students in Malta have consumed marijuana in their lifetime, whereas 44.8% of the French population between 15 and 64 years of age and 23% of 15 and 16-year-old students had used *Cannabis* during their lifetime. These results for Malta and France are the lowest and highest percentages, respectively, among the surveyed European member countries.

Regarding illicit drug seizures, around one million seizures were reported in the European Union in 2020, with *Cannabis* products being the most often seized[3]. Herbal *Cannabis* had a 278% increase in quantity seized between 2010 and 2020, only after methamphetamine (+477%), amphetamine (+391%)[3]. In addition, there is an increasing tendency of the amounts of marijuana being seized in Brazil and in the United States of America. In Brazil, the amount of marijuana seized by the Brazilian Federal Police shows a tendency of growth since 1995[4], despite the fact that *Cannabis* cultivation and the recreational use of marijuana are prohibited.

Even though the medicinal and recreational use of marijuana are being progressively legalized in many countries, several *Cannabis*-based products have become available for purchase, and concerns regarding their safety are rising[5-13], such as the mislabeling of cannabidiol extracts[5], health problems due to taking *Cannabis* products[6,10,11,12,13], adulteration and/or contamination of *Cannabis* products[7,8,9].

Considering all these issues, it is clear that the development of methodologies able to trace the geographical origin of *Cannabis*, whether for forensic or safety purposes has become crucial, especially considering that over the past decade the number of people who use *Cannabis* increased by approximately 28%[1]. Approaches such as isotopic quantification[14-18], genetic analyses[19-22] and entomological evaluation[23,24] have been tested as to *Cannabis* traceability. Analytical methods[25-30], however, seem to be a more sensitive option, detecting subtle differences between samples and being adequate to analyze samples from close geographical origins, in contrast with the aforementioned approaches.

Liquid chromatography-mass spectrometry (LC-MS) is a well-stablished analytical technique that has already been employed in the authentication of beverages[31], certification of green tea[32], among other uses. Liquid chromatography is a separation technique that isolates the constituents of a given sample according to its differences in affinity with a mobile phase and stationary phase, whereas the mass spectrometry is responsible for three main functions: ionization of molecules, separation of these molecules according to their mass-to-charge ratio, as well as the determination of the abundance of each resulting ions[33].

When the geographical origin of a sample, i.e. its traceability, is a goal of a study, once the samples are analyzed, a statistical analysis is necessary to segregate the data. Linear discriminant analysis (LDA) models differences among samples assigned to groups[34] by maximizing the between-group variance and minimizing the within-group variance. LDA transforms data linearly in order to reduce the number of dimensions required to represent them[35] by operating in three steps: (1) calculating the separability between different classes (the distance between the means of different classes), also known as between-class variance or between-class matrix; (2) calculating the distance between the means and the samples of each class, which is called within-class variance or within-class matrix; and finally (3) constructing the lowest dimensional space possible, which maximizes the between-class variance and minimizes the within-class variance[36].

The aim of the present study is to trace the geographical origin of *Cannabis* samples collected from seizure operations in Brazil, by means of liquid chromatography-mass spectrometry and linear discriminant analysis of the data.

2. Material and methods

2.1 Samples

The *Cannabis* samples comprise leaves, flowers and stems, and are originated from two seizure operations performed by the Brazilian Federal Police in illegal outdoor cultivation sites in the states of Pernambuco and Bahia, Brazil. The first seizure operation took place in November 2014, and from it, 26 plant samples were collected. The second operation, in December 2017, seized 21 plant samples.

Figure 1 and Table 1 show the sampling points locations. The approximate distances between sampling points were calculated using the Google Earth Pro software. Points 2 and 9 are 447.7 km (~278.2 miles) apart and are the most distant

ones, in contrast with the 5 and 11 are 0.8 km (~0.5 miles) apart and are the closest points.

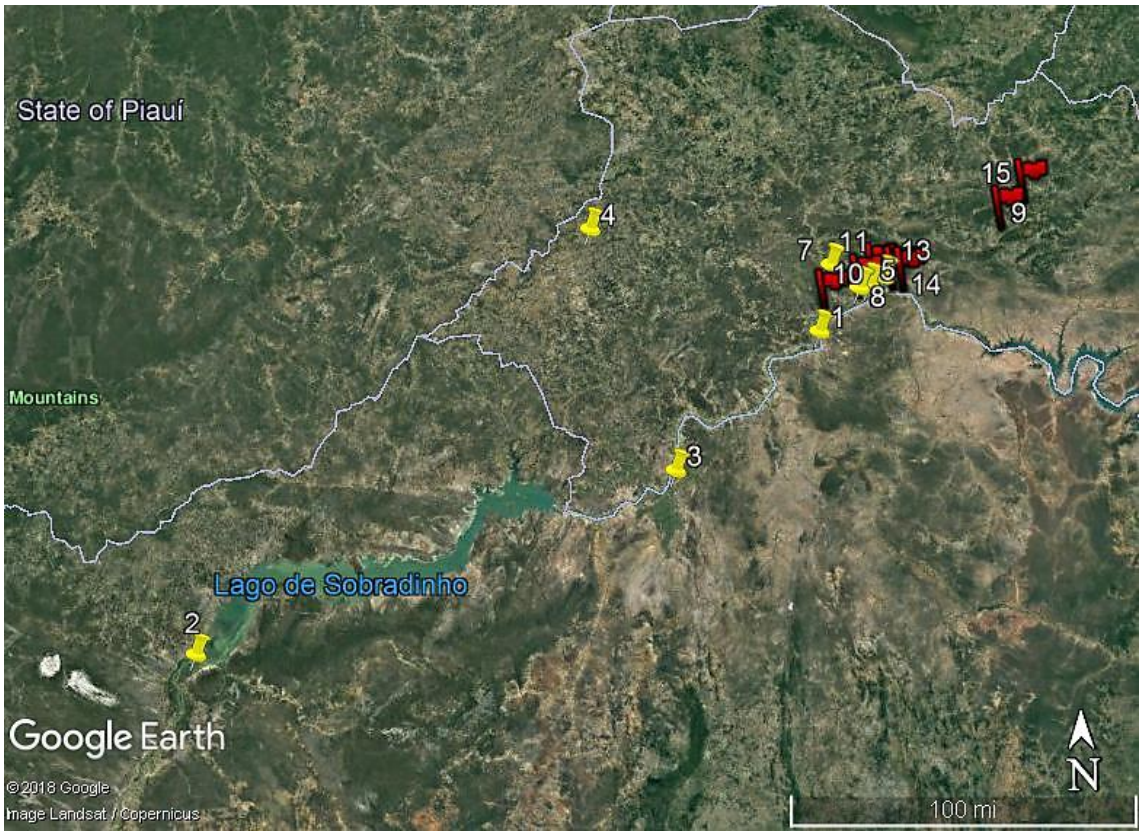

Figure 1. Sampling points from the 2014 and 2017 seizure operations, in yellow and red, respectively.

Table 1. Geographic coordinates of the sampling points.

Sampling points' code	Sample code	Geographic coordinates of the sampling points	
		South (S)	West (W)
1	1	8°46'59.50"	39°42'26.60"
	2		
	4		
2	6	10° 5'2.10"	42°22'1.00"
	7		
	8		
	9		
3	10	9°21'11.14"	40°19'27.12"
	11		
	12		
4	13	8°20'32.40"	40°40'2.40"
	14		
	15		
	16		
5	17	8°35'20.82"	39°29'30.66"
	18		
	19		
	20		

	21		
	22		
6	23	8°33'28.02"	39°25'53.58"
	24		
	25		
7	26	8°30'21.00"	39°39'12.78"
	27		
8	29	8°36'7.86"	39°32'5.10"
	30		
	31		
1	32	8°46'59.50"	39°42'26.60"
	35		
2	37	10° 5'2.10"	42°22'1.00"
	38		
	39		
	40		
3	41	9°21'11.14"	40°19'27.12"
	42		
	43		
4	44	8°20'32.40"	40°40'2.40"
	45		
	46		
	47		
5	48	8°35'20.82"	39°29'30.66"
	49		
	50		
	51		
	52		
	53		
6	54	8°33'28.02"	39°25'53.58"
	55		
	56		
7	57	8°30'21.00"	39°39'12.78"
	58		
	59		
8	60	8°36'7.86"	39°32'5.10"
	61		
	62		
1	94	8°46'59.50"	39°42'26.60"
	95		
	96		
-	98	Not identified	Not identified
2	99	10° 5'2.10"	42°22'1.00"
	100		
	101		
3	104	9°21'11.14"	40°19'27.12"
	105		
4	106	8°20'32.40"	40°40'2.40"
	107		
5	110	8°35'20.82"	39°29'30.66"
	111		
	112		
	113		
	114		
	115		
6	116	8°33'28.02"	39°25'53.58"
	117		

	118		
7	119	8°30'21.00"	39°39'12.78"
	121		
8	122	8°36'7.86"	39°32'5.10"
	123		
	124		
9	125	8°11' 23.74080"	38°46'10.92720"
	127		
	128		
15	129	8°18'05.40000"	38°52'19.80120"
	130		
	131		
9	132	8°11' 23.74080"	38°46'10.92720"
	133		
	134		
	135		
10	136	8°38'55.83480"	39°37'47.41680"
	137		
	138		
11	139	8°35'11.73480"	39°29'01.26240"
	140		
	141		
13	142	8°33'16.25760"	39°20'21.91200"
	143		
	144		
14	145	8°33'28.54440"	39°17'44.45160"
	146		
	147		
15	148	8°18'05.40000"	38°52'19.80120"
	149		
	150		
9	151	8°11' 23.74080"	38°46'10.92720"
	152		
	153		
10	154	8°38'55.83480"	39°37'47.41680"
	155		
	156		
11	157	8°35'11.73480"	39°29'01.26240"
	158		
	159		
12	160	8°32'14.60040"	39°25'16.74840"
	161		
	162		
13	163	8°33'16.25760"	39°20'21.91200"
	164		
	165		
14	166	8°33'28.54440"	39°17'44.45160"
	167		
	168		
15	169	8°18'05.40000"	38°52'19.80120"
	170		
	171		
-	Extra 3	Not identified	Not identified

Several samples were soil samples, or their quantities were not enough for chromatographic analysis, therefore, these samples were not used in the present

study. The samples from 2014 had already been oven-dried at 105 ºC for 24 hours and ground with pestle and mortar for a different study (data not shown), whereas the samples from 2017 were dried in an oven at 40 ºC for seven days, ground in a ball mill and then with mortar and pestle. For the LC-MS analysis, 100 mg of each *Cannabis* sample were weighted and sifted.

2.2 Data analysis

LDA was carried out using the MASS Package[37] in RStudio software[38]. Due to the small number of samples, all the samples were considered 'training' samples, there were no 'test' ones. T-test for independent samples by group was performed at 5% significance using the Statistica software, aiming to verify if there were statistical differences between the cannabinoids' concentrations of the samples from 2014 and 2017, considering they had different drying treatments.

2.3 Chromatography method

The LC-MS method was developed and validated according to Anvisa's (*Agência Nacional de Vigilância Sanitária* - National Agency of Sanitary Monitoring) resolution nº 166/2017[39]. Specificity, linearity, precision, accuracy, reproducibility and recovery were evaluated[39]. The analyses were carried out in an Agilent 1260 Infinity Series liquid chromatographer equipped with a G1311B quaternary pump, G1329B auto sampler, G1314F UV/VIS detector, G1316A oven for column coupled to an Agilent 6120B mass-quadrupole detector. Chem Station (v.1.4.1.) was used for data acquisition and data analyses. All instrumentation was from Agilent Technologies (Palo Alto, CA, USA) except for the 5430R Eppendorf centrifuge (Hamburg, Germany), that was employed for sample preparation. A Kinetex C18 column (150 x 4.6 mm, 2.6 µm), protected by a C18 pre-column (4 x 2.0 mm, Phenomenex, Torrance, CA, USA) was used, the mobile phase was constituted by methanol and ammonium acetate (buffer solution) 10mM pH 5.0 (85:15 v/v) on isocratic mode, 0.5 mL/min flux, analysis temperature of 30 ºC and 10 µL injection volume. Total run time was 20 minutes.

Stock solutions of ∆9-tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD), cannabinol (CBN), tetrahydrocannabinolic-acid (THCA) and cannabidiolic-acid (CBDA) were prepared to a concentration of 20 µg/mL. The working solution for the internal standard, ∆8-tetrahydrocannabinol (Δ8-THC), was 50 µg/mL and those for the cannabinoids analyzed were 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL. There were no

dilutions for samples that presented concentrations above 10.0 µg/mL. The stock solutions and working solutions were all dissolved in methanol and were kept refrigerated (2 to 8ºC).

The extraction method employed is an adaptation of the United Nations Office on Drugs and Crime's recommended methods for the identification and analysis of *Cannabis* and *Cannabis* products[40]: 100 mg of the sample were weighted, put into glass tubes, added 1.0 mL of methanol while in ultrasonic bath, alternating 5 minutes of the ultrasound with vortex agitation for 10 seconds. This process was repeated three times. Then, the samples were centrifuged for 10 minutes at 3,000 rpm, the supernatant was diluted in methanol (100 µL of extract in 900 µL of methanol and 200 µL of extract in 800 µL of methanol), filtered by a PVDF syringe filter (0.45 µm) and put in LC-MS vials.

The selectivity of the method was tested using a blank sample, a sample containing reference chemical substances (RCS) and a sample of a *Cannabis* extract in order to verify if there were interferers present. The method did not show interference in the peaks of interest; therefore, it was considered selective.

For linearity verification standard curves with 6 different concentrations from the RCS were prepared in triplicates. The concentrations tested were: 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/mL for all analytes. The method was linear, with correlation coefficients of 0.99 for all substances. Variance analysis and statistical regression indicated that the curves for all substances showed significate linear regression and no linearity deviation.

Precision and accuracy were verified by means of nine analyses, employing three concentrations: low (0.5 µg/mL), medium (2 µg/mL) and high (10 µg/mL), with three replicates each. Intraday and between-day precision was tested by calculating the relative standard deviation (RSD), and accuracy was calculated by analyte recovery of known concentration that had been added to the sample. The method was considered precise and accurate once the RSD was below 10% and recoveries (both intraday and between-day) were 85% to 95%. The results proved to be satisfactory, and the method was considered validated.

The quantitative analyses were performed on Single Ion Monitoring (SIM) mode. The mass detector operated with positive electrospray ionization (ESI +) for Δ9-THC, CBD and CBN; and it operated with negative ESI for THCA and CBDA. The following parameters were also used: nebulizer pressure of 55 psi, drying gas flow of

12 L/min, temperature of the drying gas of 350 ºC, capillary voltage at 3,500 V. Gain was kept at 1. For the internal standard, the monitored ions were 318, 262 and 196, with fragmentation voltage of 150, 100 and 100 V, respectively. Peak resolution was considered acceptable (Rs>1.5).

Analytical information such as retention time, quantification ion, confirmation ion and fragmentation voltage are shown below in Table 2.

Table 2. Analytical information. * Retention time (min) ** Quantification ion *** Confirmation ion **** Fragmentation voltage set at LC-MS in volts (V).

Cannabinoid	RT (min)*	Q1 (m/z)**	Frag (V)****	Q2 (m/z)***	Frag (V)****	Q3 (m/z)***	Frag (V)****
CBDA	3.8	357	175	339	150	-	-
THCA	6.2	357	150	312	175	-	-
CBD	7.5	315	100	259	100	193	100
CBN	11.9	311	200	293	100	223	200
∆9-THC	14.6	315	100	293	150	193	200

The samples were classified alphabetically according to their geographical origins in order to perform the LDA, that is, samples from the same geographical locations formed one group. In total, eight groups were formed (from A to H) with the samples from the 2014 seizure operation, whereas seven groups (from A to G) were formed with the samples from 2017 (Table 3).

In the LDAs where samples from both operations were assessed as one dataset, the alphabetical order was followed from the letter H (last group of samples from 2014), i.e., the first samples from 2017 that belonged to the same geographical location formed the group I, the next group was identified as J, and so on.

3. Results and discussion

Cannabinol (CBN) was detected in all samples between 0.14 and 46.28 µg/mL (Table 3). Samples 98, 105, 106, 107, 110, 111, 112, 113, 117, 118, 119, 122, 123, 124 and Extra 3 showed high CBN concentrations and low ∆9-THC concentrations. As ∆9-THC degrades into CBN when the *Cannabis* plant is no longer biologically active[41], this tendency was expected. The average CBN content in the samples from 2014 is of 12.27 µg/mL, and in the samples from 2017 is of 5.01 µg/mL, what suggests that the high temperature employed to dry the samples from 2014 and their age enhanced CBN production, although since none of the samples were refrigerated, this likely

contributed to the increased CBN concentrations, considering that ∆9-THC degrades and forms CBN if the sample is kept under inadequate conditions or during long periods of time[42].

Table 3. *Cannabis* samples codes and groups for LDA. SO=seizure operation.

2014 SO sample code	Group	2017 SO sample code	Group
94	A	151	A
95	A	152	A
96	A	153	A
-	-	-	-
98	-	154	B
99	B	155	B
100	B	156	B
101	B	157	C
-	-	158	C
-	-	159	C
104	C	160	D
105	C	161	D
106	D	162	D
107	D	163	E
-	-	164	E
-	-	165	E
110	E	166	F
111	E	167	F
112	E	168	F
113	E	169	G
114	E	170	G
115	E	171	G
116	F		
117	F		
118	F		
119	G		
-	-		
121	G		
122	H		
123	H		
124	H		
Extra 3	-		

Regarding ∆9-THC content, this cannabinoid was not detected in the samples 94, 96, 114,116 and Extra 3, and in the samples 99, 100, 101, 111, 118 and 153 its concentrations were below the quantification limit. The highest ∆9-THC contents were found in the samples 154, 155, 156, 159, 160 and 169 (Table 3).

The highest THCA concentrations were found in samples 159 and 162. This cannabinoid was detected in samples 94, 96, 98, 99, 100, 101, 107, 110, 111, 112, 113, 117, 118, 119, 121, 124 and extra 3, and was not detected in samples 104, 106 and 116 (Table 3). In the quantifiable samples, the THCA contents were usually lower or similar to those of ∆9-THC.

CBD was detected in samples 157, 158, 169 and 170, and CBDA was detected in samples 123, 152, 154, 155, 156, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169 and 170. Since CBD and CBDA were present in low amounts, not being able to be quantified, these cannabinoids were excluded from the statistical analyses. In general, samples from the same geographical origin seem to have similar cannabinoids concentrations.

Table 4. Cannabinoids concentrations detected in the *Cannabis* samples. BQL: below the quantification limit; - : not detected.

Sample code	∆9-THC	THCA	CBN
	µg/mL		
94	-	BQL	1.41
95	0.18	0.18	6.20
96	-	BQL	6.90
98	0.82	BQL	16.22
99	BQL	BQL	2.81
100	BQL	BQL	3.19
101	BQL	BQL	7.22
104	0.36	-	6.97
105	0.55	-	24.31
106	1.29	-	13.98
107	1.69	BQL	15.14
110	0.66	BQL	12.27
111	BQL	BQL	11.36
112	0.51	BQL	9.86
113	0.19	BQL	13.01
114	-	-	0.99
116	-	-	2.51
117	0.16	BQL	8.18
118	BQL	BQL	29.62
119	0.15	BQL	9.87
121	0.37	BQL	6.39
122	5.45	0.18	21.18
123	2.64	0.40	46.28
124	0.18	BQL	16.05
Extra 3	0.10	BQL	14.96

151	0.47	0.72	0.55
152	4.31	5.57	5.04
153	BQL	0.29	0.14
154	16.16	4.57	9.03
155	17.93	6.28	12.95
156	17.13	5.22	11.39
157	7.31	7.38	5.88
158	5.96	5.03	3.95
159	12.82	10.96	5.18
160	10.36	5.48	3.50
161	7.12	7.13	2.91
162	9.37	10.23	3.42
163	2.96	1.05	4.38
164	6.90	2.62	4.84
165	3.10	2.21	5.56
166	7.51	2.89	3.38
167	6.83	1.84	2.13
168	7.17	3.07	3.72
169	12.70	6.28	8.47
170	3.56	3.08	4.10
171	9.80	7.87	4.71

The t-test (Table 5) revealed statistical difference between the samples from the 2014 and 2017 seizure operations due to the ∆9-THC, THCA and CBN concentrations, which was probably caused by the high temperature employed to dry the samples from 2014, as well as the age and natural tissue degradation of the plants.

Table 5. T-test of ∆9-THC, THCA and CBN data.

T-tests; Grouping: Secagem Group 1: 2014 Group 2: 2017											
Variable	Mean 2014	Mean 2017	t-value	df	p	Valid N 2014	Valid N 2017	Std.Dev. 2014	Std.Dev. 2017	F-ratio Variances	p Variances
THC	0.612	8.07	-7.12157	44	0.000000	25	21	1.18892	5.083383	18.281	0.000000
THCA	0.030	4.75	-7.98101	44	0.000000	25	21	0.09167	2.962085	1044.021	0.000000
CBN	12.275	5.01	3.17598	44	0.002728	25	21	10.05451	3.169217	10.065	0.000002

The LDA was performed with data from the 2014 and 2017 seizure operations separately and then combined (Table 6). The samples 98 and extra 3 were excluded from the sample dataset because they do not have a known geographical origin. The LDA with the 2014 samples showed 39.13% accuracy, whereas with the 2017 samples it had an 80.95% accuracy; when combined, the accuracy is of 38.63%. The sum of the first two linear discriminants (LD1 and LD2) accounts for 96.34% of data variance

of the 2014 samples dataset, 93.18% of the 2017 samples dataset, and 93.55% of both datasets combined.

Almost all the samples (43.48%) from 2014 that were misclassified were allocated in group E (Table 6). Despite the 2017 samples being from closer geographical origins when compared to the samples from 2014, which would tend to make the classification more difficult, 80.95% all of the 2017 samples were correctly classified, except one sample from group C that was classified as a group E sample, and one sample from the group D was classified as part of the group F, and two samples from the group G that were classified as belonging to the groups C and E. This result indicates that the drying methodology of *Cannabis* influences not only the chemical composition of the sample, but also the accuracy of an LDA, and therefore, the possibility to trace the samples by means of this statistical analysis. When the 2014 and the 2017 samples were assessed together as one dataset, the same tendency of samples from different groups being attributed to the group E was observed, moreover, only the groups E, H, J, K, M, N and O had some samples correctly classified.

Table 6. Distribution of the samples according to their geographical location as designated by the LDA.

Samples from 2014															
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
A	1	0	0	0	0	0	0	0							
B	1	0	0	0	1	1	0	0							
C	0	0	1	0	0	1	0	0							
D	0	0	0	1	0	0	0	0							
E	1	3	1	1	4	1	2	1							
F	0	0	0	0	0	0	0	0							
G	0	0	0	0	0	0	0	0							
H	0	0	0	0	0	0	0	2							
Samples from 2017															
	A	**B**	**C**	**D**	**E**	**F**	**G**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
A	3	0	0	0	0	0	0								
B	0	3	0	0	0	0	0								
C	0	0	2	0	0	0	1								
D	0	0	1	2	0	0	0								
E	0	0	0	0	3	0	1								
F	0	0	0	1	0	3	0								
G	0	0	0	0	0	0	1								
Samples from 2014 and 2017 combined															
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	1	0	0	0	1	1	0	0	2	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	2	3	1	2	4	1	2	1	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	1	0	0	1	0	2	0	0	0	0	0	0	0
I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
J	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
K	0	0	0	0	0	0	0	0	1	0	2	2	0	0	0
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
M	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
N	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0
O	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1

4. Conclusion

The temperature of the drying method and plant age seem to have negatively influenced the accuracy of the LDA, since the 2014 samples showed an accuracy approximately 40 percentage points lower than that of the 2017 samples. Considering the 2017 samples dataset and outcome alone, the LC-MS results and the LDA were able to satisfactorily source *Cannabis*, although it would be necessary to evaluate more samples to further validate or deny this statement.

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