

Valencia, February 2021



Fundación CANNA

presents

***Composition of CBD e-liquids marketed in Europe;
quantification of CBD and Vitamin E Acetate***

Introduction

On 25 February 2020, the Center for Disease Control prevention (CDC), with the U.S. Food and Drug Administration (FDA), state and local health departments, and other clinical and public health partners, published a final update report on the (inter)national outbreak (August 2019) of lung injuries (EVALI) associated with the use of e-cigarette and/or vaping products.

Of the 2807 total hospitalized cases, 68 were fatal, with an average of 50 years of age. The fatal lung damages have been attributed to Vitamin E acetate, found both in the vaporization products and the lung fluids of affected subjects.

Both EVALI subjects (51 s.) and group of volunteers (99 s.) were tested for the accumulation of the compound in pulmonary tissues through vaporization and it was found in almost 100% of the cases. {1}

After the first confirmed case in Brussels (November 2019), the Direction of Fundació CANNA felt the need to know more about conditions in Europe, which inspired a study to screen the materials sold on the European market, searching for Vitamin E acetate or any other compound known, or suspected, to be harmful to human health.

The sample group would be formed with the fifteen best-selling e-liquids of 2019 and would represent the products that a consumer may legally purchase from physical/online shops all over Europe. The samples were all bought anonymously from different shops to avoid any potential cheating.

The main analysis would be performed with MS techniques, by Fundació CANNA's partner laboratory, PhytoPlant Research S.L.

At the same time, the Director shared his intention to find another laboratory capable of performing another set of independent analyses with his close circle of partners and collaborators. The Institut de Recerca i Tecnologia Agroalimentàries, IRTA, answered with enthusiasm, ready to perform an independent GC-MS analysis using a FFAP column (the method used for oils).

The first samples of a 10-vape-group were shipped to PhytoPlant Research S.L. in the first week of January. Upon arrival, the samples were split between two separate GC-MS analysis (one with head-space sampler) and one HPLC-DAD analysis, for a precise quantification of the

cannabinoids in the e-liquids. Another batch, of five vape liquids, was analyzed in early February and the data were merged with the previous ones.

The first GC-MS data were verified, as before, prior to Christmas 2019. The final data were collected, and the sample shipped to IRTA for the second part of this project. By August 2020, we were able to collect all the data and merge them into this report.

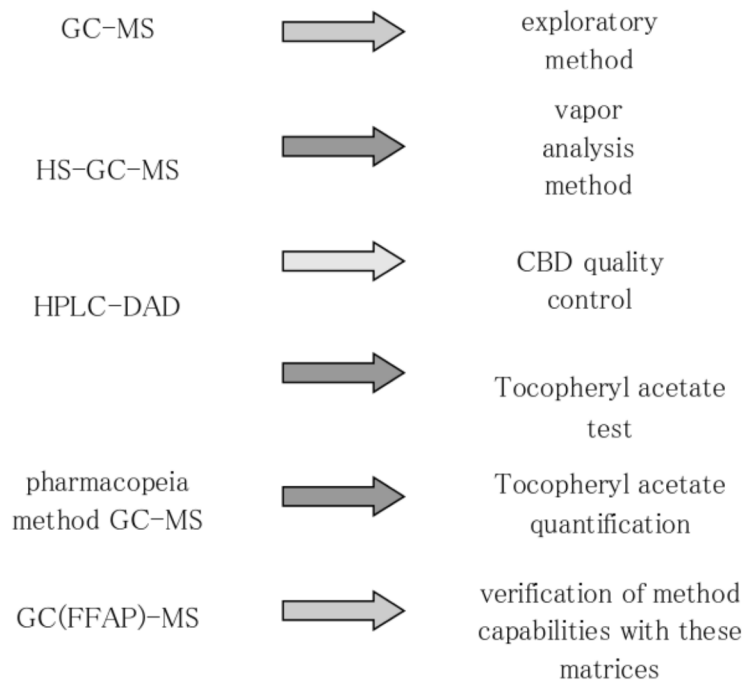


Figure 1: Graphic summary of the objectives of this work

Methods and Sample Preparation:

GC-MS and HS-GC-MS analysis

This first series of tests was performed to scan the major compounds/constituents of the e-liquid samples.

For the identification of the compounds via mass analysis (GC-MS and HS-GC-MS), the group merged the data from the NIST library with the information collected in the internal database.

GC-MS

Chromatographic separation is carried out using an Agilent 5-MS Ultra Inert capillary cast column (30m x 0.25mm ID, 0.25 µm film thickness) placed in the Agilent 7890B gas chromatographic oven, using helium (99.9 %) at a flow rate of 1.3 ml/min.

The oven temperature program starts at 50°C and is maintained for 0.5 minutes, then the temperature is increased to 160°C with a first ramp of 5°C/min, maintaining the temperature for two minutes. The temperature then increases to 232°C with the same ramp as the previous one and, without stopping, it increases to 242°C with a ramp of 1.5°C / min and up to 250°C every 2°C/min. Finally, the temperature increases to 300°C with a ramp of 5°C/min and is maintained for five minutes to ensure that all the compounds have been eluted.

The injection is done with an Agilent 7693 autosampler, to improve the precision of the method, working and in splitless mode. The injector temperature is 250°C and the injected volume is 1 µL. The detector used is an Agilent 5977B mass spectrometer. The transfer line is kept at 300 °C. The detector is set to scan mode in the mass range of 40 to 400.

The samples for the GC-MS analysis were diluted, starting from a variable number of drops, to fit the concentration ranges allowed by the column and the detector. Each sample was diluted to reach an approximate concentration of 200 ppm, so the approximate concentration of a drop of each sample has been calculated using the labeled values. For example, the calculated concentration for a sample containing 400mg of CBD in 10ml container is:

$$\text{Concentration (ppm)} = \frac{400000 \mu\text{g}}{10 \text{ mL}} = 40000 \text{ ppm}$$

If a droplet of product has a volume of 0.05 ml, we can approximate the concentration of a drop as:

$$CBD/drop (\mu g/drop) = 40000 \frac{\mu g}{mL} * 0.05 ml = 2000 \mu g /drop$$

if we dilute the single drop of product in 10 ml of methanol:

$$Concentration (ppm) = \frac{2000 \mu g}{10 mL} = 200 ppm$$

With this calculation, we were able to weight different amounts of sample, resulting in dilutions with similar values:

Sample	Nº drops	weight (g)
Sample 01	1	0.02083
Sample 02	2	0.03339
Sample 03	4	0.12146
Sample 04	1	0.02004
Sample 05	10	0.14710
Sample 06	1	0.01798
Sample 07	1	0.01885
Sample 08	1	0.03122
Sample 09	1	0.03228
Sample 10	3	0.04709
Sample 11	1	0,01007
Sample 12	1	0,01905

Sample 13	1	0,01820
Sample 14	8	0,16136
Sample 15	3	0,06250
Table 1: Nr. of drops and weights of the samples used to reach the first level of dilution.		

Starting from these solutions, we prepared a further dilution of around 100ppm

$$M \cdot V = M' \cdot V'$$

$$V = \frac{100 \text{ ppm} \cdot 1000 \mu\text{L}}{200 \text{ ppm}} = 500 \mu\text{L of sample}$$

$$V_{\text{MeOH}} = 1000 \mu\text{L} - 500 \mu\text{L} = 500 \mu\text{L of pure methanol}$$

These diluted solutions were transferred to darkened 1ml vials and agitated @1500rpm for thirty seconds. The resulting vials were kept chilled until the analysis was performed.

HS-GC-MS

Chromatographic separation is carried out using an Agilent 5-MS Ultra Inert capillary cast column (30m x 0.25mm ID, 0.25 μm film thickness) placed in the Agilent 7890B gas chromatographic oven, using helium (99.9 %) at a flow rate of 1.3 ml / min. The oven temperature program is identical to the previous case.

The injection is performed by an Agilent Headspace, in which the headspace-sample (20ml vial), the loop, and the transfer-line were equilibrated for 7min. at 200°C. The injection time was 0.5 min in split mode.

The injector temperature is 250°C and the injected volume, defined by the loop volume, is 1 ml. The detector used is an Agilent 5977B mass spectrometer. The transfer line is kept at 300°C., working in scan mode in the mass range of 40 to 400 m/z.

Sample preparation was straightforward: a single drop of product was transferred in a 20 ml HS-vial and rapidly encapsulated. Approximate sample weight may be calculated with the data in Table 1. The resulting vials were kept chilled until the analysis was performed.

CBD Test

A proprietary method (Phytoplant *Research S.L.*) was used to determine the concentrations of CBD. Samples were prepared with 100 µL of sample diluted in MeOH. After homogenization, the dilutions were analyzed, without further treatment, via HPLC-DAD. The HPLC detector was operated at 210 nm.

Tocopheryl acetate test

The samples for the **tocopheryl acetate test** were prepared according to the procedure described in the European *Pharmacopoeia*.^{2} The solvent used for the dilution was modified: our samples were not soluble in cyclohexane, so it was substituted with acetone.

The six solutions described in the method were prepared as follows:

- Test A: this solution contains the sample at a concentration of 10,000 ppm and the internal standard (squalane) at a concentration of 10,000 ppm. The solution is prepared weighting 0.1 g of sample that we will dissolve in 10 ml of a solution previously prepared with the internal standard (1 g of squalane in 100 ml of acetone). With this solution we will confirm whether or not the sample is positive for tocopheryl acetate.
- Test B: this solution contains the sample at a concentration of 10,000 ppm absence of internal standard. The solution is prepared weighting 0.1 g of sample that we dissolve in 10 ml of acetone.

- Reference solution A: this is the standard tocopheryl acetate solution at a concentration of 10,000 ppm and the internal standard (squalane) at a concentration of 10,000 ppm. We dissolve 0.1 g of α -tocopheryl acetate CRS (Purity 98.4%, Sigma Aldrich) in 10 ml of the internal standard solution (the same as in Test A, 1 g of squalane in 100 ml of acetone).
- Reference solution B: solution containing the sample at a concentration of 100 ppm and α -tocopherol at a concentration of 100 ppm. We dissolve 10 mg of sample and 10 mg of α -tocopherol CRS (Purity 98.7%, Sigma Aldrich) in 100 ml of acetone.
- Reference solution C: standard solution of tocopheryl acetate at a concentration of 10,000 ppm. This solution is used for the identification of the analytical signal produced by the analyte, as well as possible interference due to isomer impurities (peak identification). We dissolve 10 mg of all-rac- α -tocopheryl acetate CRS (containing impurities A-B-E, Sigma Aldrich) in 1 ml of acetone.
- Reference solution D: dilution of Test B solution to 1:1000. We dilute 1 ml of Test B solution in 100 ml of acetone. Subsequently, we take 1 ml of this intermediate solution and we dilute it again in 10 ml of acetone.
- All these solutions were prepared for each sample of e-liquids.

Tocopheryl acetate GC-MS quantification

Chromatographic separation is carried out using an Agilent 5-MS Ultra Inert capillary cast column (30m x 0.25mm ID, 0.25 μ m film thickness) placed in the Agilent 7890B gas chromatographic oven, using helium (99.9 %) at a flow rate of 1.0 ml/min. The oven temperature was kept constant at 280 $^{\circ}$ during the analysis (20 minutes). The injector temperature was 290 $^{\circ}$ C in split mode (1: 100), injecting 1 μ L of sample. The injection was

performed using an Agilent 7693 autosampler, to make the method more precise. The detector used was an Agilent 5977B mass spectrometer: the transfer line temperature was kept at 290°C. The mass spectrometer working mode was in SIM mode.

Preparation of analytical samples:

We choose a four-point calibration: the sample itself and three standard-added solutions.

Sample 11 and the tocopheryl acetate standard were diluted as follows:

- Sample dilution (M): 250 mg of the sample are weighed and diluted in 5 ml of acetone.
- Standard dilution (S): 50.8 mg of tocopheryl acetate standard (98.4% pure) are diluted in 1 ml of acetone.

In this way, the final concentration of acetate tocopheryl is 50000 mg / L.

With these solutions we built the calibration curve as follows:

	M(μL)	S(μL)	Acetone(μL)	Vfin(μL)
M	50	0	950	1000
M+S	50	10	940	1000
M+2S	50	20	930	1000
M+5S	50	50	900	1000

Table 2: Concentration used for the calibration curve

Where M is the sample diluted solution, and S is the standard diluted solution, both expressed in micro-liters. The solutions, prepared in duplicate, yield the standard at concentrations of, respectively, 0, 500, 1000 and 2500 ppm.

GC(FFAP)-MS analysis

Chromatographic separation was carried out using a FFAP column measuring 50 m x 0.2 mm x 0,33 μm. The temperature of the oven was set to 80°C for 1 min, then raised by 30°C/min for nineteen min until it reached 230 °C. The injector temperature was 240°C, injecting 1 μL of sample. The transfer line temperature was kept at 250°C. The mass spectrometer recording between 40<m/z<500.

The results show fragments compatible with Vitamin E-like molecules in two more samples, but the exact nature of the molecules could not be determined. The common and specific FFAP column does not look like a possible ready-to-use method to separate/identify Vitamin E-derivatives in these matrices.

Results

GC-MS and HS-GC-MS analysis

The table that follows summarizes the compounds separated and identified with correlations over 80%. It should be obvious that the compounds identified represent just a small fraction of the chemicals present in the liquids.

Decomposition compounds were found in each and every sample analyzed; their presence was widely anticipated and is attributed both to natural aging of the mixtures and the effect of the heated elements on the GCs.

	Compounds\Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Frequency %
Matrix	Propilenglycol	x	x	x	x	x	x	x	x	x	x		x	x	x	x	93.3
	Glycerine	x	x	x	x	x	x	x	x	x	x		x	x	x	x	93.3
	Squalane										x	x		x			20.0
	Glycerol tricaprilate											x		x			13.3
Terpenes	α -pinene		x		x								x		x		26.7
	Limonene	x		x	x			x			x	x		x	x		53.3
	β -myrcene	x			x			x			x	x		x	x		46.7
	Caryophyllene	x	x		x		x	x	x		x	x	x	x			66.7
	Caryophyllene oxide	x	x		x						x	x	x	x	x		53.3
	Humulene	x	x					x									20.0
	α -Bisabolol		x						x					x			20.0
	Terpinene										x						6.7
	α -Terpineol				x			x									13.3
	p-Cymene			x				x	x		x	x	x				40.0
	Farnesene/Farnesol	x	x				x	x			x	x		x			46.7
	trans-Nerolidol														x		6.7
Cann. Compounds	CBD	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	100.0
	Δ^9 -THC	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	100.0
	CBDV	x	x	x	x	x	x		x	x		x	x	x		x	80.0
	THCV													x			6.7
	CBG		x											x		x	20.0
	CBC														x	x	13.3
	CBV													x			6.7
	CBE											x	x	x	x	x	33.3
Aromas	CBN	x														x	13.3
	Ethyl 3-methylbutanoate			x													6.7
	Ethyl 2-methylbutanoate					x											6.7
	Methyl N-hydroxybenzenecarboximidoate			x		x	x	x	x	x							40.0
	γ -Decalactone			x		x											13.3
	Hedione					x											6.7
	Metil-cynamate					x											6.7
Others	Benzyl alcohol			x	x												13.3
	1-Butanol									x							6.7
	1-Hexanol									x							6.7
	dl- α -Tocopherol											x					6.7

Table 3: Major compounds identified via MS analysis

CBD Test

The concentrations quantified via HPLC-DAD are summarized in the table that follows.

Sample	Labelled			Analyzed	
	Cbd/flask (mg/flask)	Flask volume (ml)	Conc. (mg/ml)	Conc. (mg/ml)	Label error (%)
1	400	10	40	32,6	-18.5
2	200	10	20	15,6	-22
3	100	10	10	9,4	-6
4	300	10	30	6,5	-78.3
5	30	10	3	3,2	6.7
6	500	10	50	48,3	-3.4
7	600	10	60	58,5	-2.5
8	500	10	50	23,5	-53
9	500	10	50	40,5	-19
10	100	10	10	9,2	-8
11	300	1	300	146	-51.3
12	300	10	30	25,1	-16.3
13	500	10	50	43,0	-14
14	50	10	5	2.1	-58
15	120	10	12	4.1	-65.8

Table 4: CBD quantification summary

The label error represents the discrepancy between the concentrations quantified and the concentrations labeled. It indicates the % (w/V) of CBD missing (negative number) or exceeding (positive number) the concentrations declared.

Each sample was quantified as double; the resulting relative standard deviations for each sample are summarized in the table below.

Sample	RSD(%)
1	0.09
2	0.90
3	1.80
4	2.00
5	5.58
6	4.30
7	11.72
8	6.07
9	11.13
10	0.97
11	40.03
12	5.85
13	1.09
14	1.41
15	0.24
AVG	6.21

Table 5: RSD and its average

Tocopheryl acetate test

The results have been expressed as the ratio of the Test A solution response over response of Ref.Sol.A solution.

Sample	Test A/Ref.Sol.A	Result
1	0	Negative
2	0	Negative
3	0	Negative
4	0	Negative
5	0	Negative
6	0	Negative
7	0	Negative
8	0	Negative
9	0	Negative
10	0	Negative
11	56.7	Positive
12	0	Negative
13	0	Negative
14	0	Negative
15	0	Negative

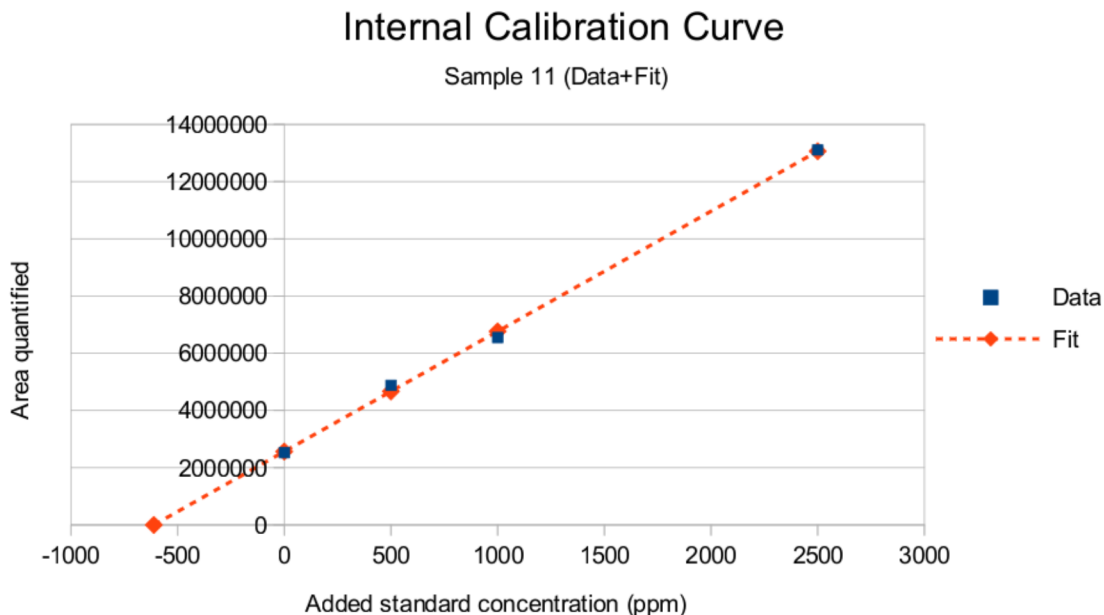
Table 6: Vit-E acetate test results

This test allowed us to clearly see the positive result for Sample 11.

Sample 11 was subsequently quantified via a standard addition method (internal calibration).

Tocopheryl acetate GC-MS quantification

After analysis, the areas (response) relative to the Tocopheryl acetate were plotted against the concentrations of standard added to the solutions. The calibration curve relative to Sample 11 quantification is shown below:



Data points	
ppm	Area
0	2524855.5
500	4879803.5
1000	6546718.0
2500	13106006.5

Regression	
Regression Model	Linear
R^2	0.998429010769591
Standard Error	220306.081764232
Slope	4198.35785714286
Intercept	2565988.01785714

Fit curve points	
ppm	Area
0	2565988.0
500	4665166.9
1000	6764345.9
2500	13061882.7

Figure 2: Graphic summary of the elaboration of the data relative to the quantification of Tocopheryl acetate

Extrapolating the values for $y = 0$ leads to:

$$y = ax + b$$

$$y = 0 \quad ; \quad x = C_m = \left| \frac{-b}{a} \right| = \frac{2565988.0}{4198.4} = 611.2 \text{ ppm}$$

Where C_m is

the

concentration of Tocopheryl acetate in the diluted sample analyzed.

Considering the dilution:

$$C_m \times V = C' \times V'$$

Where C' is the concentration in the diluted Solution M and V' is the volume of Solution M used.

Then:

$$C' = \frac{C_m \times V}{V'} = \frac{611.2 \text{ ppm} \times 1000 \mu\text{L}}{50 \mu\text{L}} = 12223.8 \text{ ppm}$$

Solution M was prepared with 250 mg of Sample 11 diluted in 5ml of acetone.

$$12223.8 \text{ ppm} = 12223.8 \text{ mg/L}$$

$$12223.8 \frac{\text{mg}}{\text{L}} * \frac{1 \text{ L}}{1000 \text{ mL}} * 5 \text{ mL} = 61.19 \text{ mg} \quad \text{of analyte in 250 mg of sample.}$$

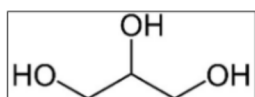
Equal to 244.7 mg of Tocopheryl acetate per gram of Sample 11.

Discussion

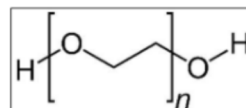
About Matrices

Most of the samples (fourteen of fifteen) showed the presence of propylene glycol (PG) and glycerin (VG), which are the most common matrix of e-liquids. The determination of the exact ratios between PG/VG was beyond the objectives of this study and was not further investigated.

Sample 11 was the only exception: this sample was a solution of glycerol tricaprylate, squalane and a Vitamin E compound that, this same study has identified (and quantified) as Vitamin E acetate.



Glycerine (VG if extracted from vegetal triglycerids)



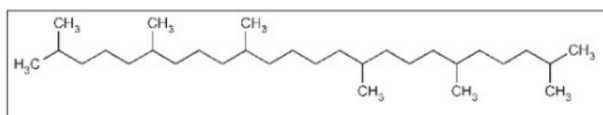
Polyethylene glycol (general formula)

This difference in the matrix offers us the first glance of two distinct product formulation tendencies: PG/VG mixtures and light oils solutions.

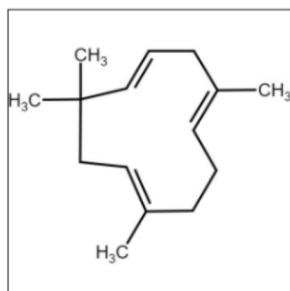
The presence of glycerol tricaprylate, together with compounds like methyl caprylate and methyl palmitate, suggests the use of a refined MCT solution mixed with squalane and other long chain alkanes (the scarcity of alkenes is a data itself).

Sample 11 was then analyzed as positive both for Tocopheryl acetate, 244,7 mg/g, and CBD, 146 mg/ml: both over 10% w/w.

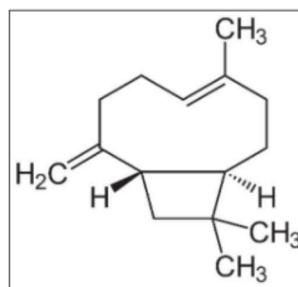
It is not clear why Tocopheryl acetate was added, but this could be related to the stability of the final solution: Tocopheryl acetate may help stabilize CBD in the solution (which is already extremely lipophilic) via aromatic interactions, which are almost absent in MCT derivatives.



Squalane



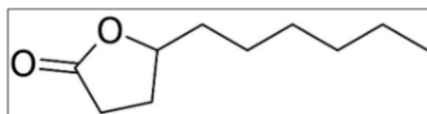
α -Humulene



β -Caryophyllene

Limonene (eight samples), beta-myrcene (seven samples), p-cymene (six samples), and α -humulene (three samples) are also cannabis-related aromas and, modulated in different ratios, are among the compounds responsible for the diverse bouquets produced by different cannabis strains.

Sample 5, in opposition to the other samples, was formulated with a much more complex aroma set: compounds like ethyl 3-methylbutanoate and γ -decalactone are not found in cannabis strains but are well known alimentary degree flavors, used to reproduce fruity aromas.



γ -Decalactone

About Cannabinoids

Fourteen of the fifteen samples presented CBD values lower than those declared in the labeling. If we consider an average 10% uncertainty on the concentrations labeled on analogue products, only four samples were quantified within this range.

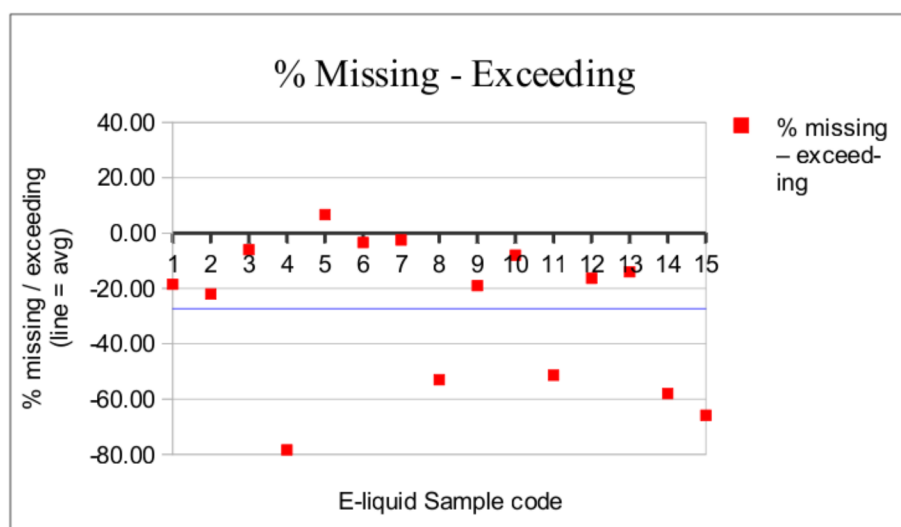


Figure 3: Graphic summary of the CBD discrepancies with the labelled values

On average, CBD was found to be 7% lower than the concentrations stated on labels, with extremes ranging from + 6.67% to -78.33% CBD. Only Sample 5 had higher values than the declared (+6.67%), suggesting that a higher degree of complexity in the formulation (see flavor section) is accompanied by a higher overall quality standard.

In general, the complexity of these mixtures is evident if we analyze the standard deviations of the whole group.

Twelve of fifteen samples were quantified with RSD greater than 1% and three out of fifteen samples over 10%. This statistical pattern is typical of heterogeneous samples, where the sub-sample, needed for the analysis, represents just a particular portion of the entire sample and the accumulation of data (repeated analysis) is the only way to calculate a stable average.

In the same conditions, homogeneous, monophasic, thin solutions tend to show smaller RSDs with the same number of repetitions, just because of the increase in the reproducibility of the sub-sampling itself.

Δ^9 -THC was found, unsurprisingly, in each sample analyzed, suggesting that the entire sample group was dosed with CBD extracted from biomass and not the synthetic analogue. The abundance of CBDV (found in twelve samples) seems to point in the same direction: CBDV is one of the most common cannabinoids found, as contaminant, in CBD crystals, precipitated out of cannabis extracts/solutions. {3}

CBE was identified in five samples: this particular cannabinoid is the product of light degradation of CBD that may result both from the heating of complex mixtures and/or the aging of the source of CBD used in the product.{4}

About Contaminants

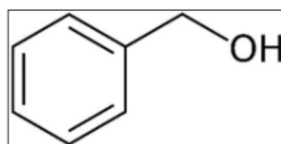
As suspected, both analytical methods were able to identify a large number of compounds that are not directly connected to the practice of vaping and must be considered contaminants. This big group includes compounds that were:

- present in the precursors but not labeled (contaminants)
- generated by aging phenomena (spontaneous decomposition)
- generated by the analytical conditions (induced decomposition)

Keeping in mind that the vaping conditions archived by commercial devices generate a 'chemical stress' much greater than the atomization/ionization conditions in our MS detectors, we may suggest that any (induced) decomposition we introduced should be neglected/ignored in respect to the decompositions that take place while vaping on real devices.

Within the contaminant-group, we have identified a wide variety of thermal decomposition products: mostly alcohols and organic acids. This excess suggests a missive hydrolysis and/or trans-esterification of bio-esters and glyceride-like molecules.

Among the unexpected spices identified by this study is Benzilic Alcohol (BA), found in Sample 4.



Benzilic Alcohol

BA is found in some plants and oils extracted from flowers like jasmine and ylang-ylang, but in minute quantities. Synthetic BA is used as a stabilizer/emulsifier in cosmetics, but its use is restricted within certain concentrations (European Union and International Fragrance Association): at unsafe levels, it can be irritating to the skin. It is not suitable for young children and it can affect the immune system, causing progressive itching and burning. Inhaling effects vary with the vapor pressure of BA (erratic during vaporization practices) and vary from acute irritation to greater degrees of toxicity.{5}

Conclusions

In this study, the presence of Vitamin E acetate was confirmed in one of the fifteen products studied. There is no data on the amount of this compound that can be harmful when inhaled through e-liquids. However, the previous references on the use of these electronic cigarettes, and the presence of Vitamin E acetate in people with serious lung problems, render their consumption inadvisable.

In the same way, the presence of other compounds such as benzyl alcohol, which can cause short- and long-term lung problems, reveal a necessary greater control over the composition of these products, either by limiting their sale or by providing information about them on the label.

The presence of d-9 THC in all samples means that, depending on the amount consumed, this substance can be positive in drug tests.

Regarding the CBD content, the deviation between what is declared and what is quantified in some samples is very high, as is the lack of homogeneity, evidencing the lack of control and good practice when producing the product.

There is also a tendency to add terpene e-liquids found in the cannabis plant, perhaps in an effort to make the aroma of the e-liquid resemble the aroma of the plant.

The overall quality of the products/samples analyzed does not seem to represent an immediate danger for consumers, but the increasing need of a specific regulation should be manifest. D-9 THC levels, matrix details, and flavors data should all be noted on labels and

expressed in terms of the natural uncertainties that are implicit in the production/analysis processes.

Collaborations

The entire team wishes to thank Dr. Carlos Ferreiro, of Phytoplant Research S.L., for the involvement and proficiency demonstrated, and Dr. Joan Noguerol Arias, of Institut de Recerca i Tecnologia Agroalimentàries, IRTA, for his attention and dedication.

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