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THC and THC-COOH hair concentrations: Influence of age, gender, consumption habits, cosmetics treatment, and hair features

Fabio Vaiano [a,b,*], Lapo Scuffi [a], Alessio Lachi [c], Claudia Trignano [d], Antonina Argo [e], Francesco Mari [b], Elisabetta Bertol [b]

[a] Forensic Toxicology Unit, Department of Health Science, University of Florence, Italy
[b] U.R.I.To.N – Unit of Research, University of Florence, Italy
[c] Department of Statistics, Computer Science, Applications "G. Parenti", University of Florence, Florence, Italy
[d] Department of Biomedical Sciences, University of Sassari, Sassari, Italy
[e] PROMISE Department, University of Palermo, Palermo, Italy

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ABSTRACT

Evaluation of Cannabis consumption is required for many purposes (i.e., workplace drug testing and driving license renewal). Hair analysis represents the most adopted and reliable approach for the investigation of repeated or chronic exposure to Cannabis. The main markers are the Δ9-tetrahydrocannabinol (THC) and its main metabolite, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), as stated by the Society of Hair Testing (SoHT) and the European Workplace Drug Testing Society (EWDTS). In this paper we presented an observational study on the hair concentrations of THC and THC-COOH and influences due to age, gender, consumption habits, and hair features. Data were collected from analysis of scalp hair samples (3-cm proximal segment) provided by subjects tested for THC consumption for personal purposes (i.e., workplace drug testing, personal use proving). The subjects provided an informed consent and a short questionnaire. A new analytical method was previously developed and then adopted. It consisted in a hydrolysis (1 mL of 1 M NaOH at 65 °C, 20 min) and a liquid-liquid extraction (with hexane/ethyl acetate,90/10, v/v in presence of 1.5 mL of H_2SO_4 1 M) of 25 mg of hair. A liquid chromatograph – tandem mass spectrometer (LC-MS/MS) equipped with a C18 column was used. The acquisition was in multiple reaction monitoring for the following transitions: 315→259, 193 *m/z*, for THC; 318→196, 123 *m/z*, for THC-d3; 345→299, 193 *m/z* for THC-COOH; 348→196, 302 *m/z* for THC-COOH-d3. Correlation between THC and THC-COOH hair concentrations was analyzed by Spearman's rank correlation coefficient. In order to study the influences of several variables, a new value, *Sqrt(THC*THCCOOH)*, was adopted. Its effectiveness and reliability were proved by the Principal Component Analysis. Relationships between the *Sqrt(THC*THCCOOH)* and the variables were studied through the Stepwise regression ($p = 0.05$). The normality of data distribution was tested by the Shapiro-Wilk test. The Lower limits of quantification were 10.0 (THC) and 0.2 (THC-COOH) pg/mg. Accuracy and precision always met the acceptable criteria. Recoveries were > 78% and ion suppression was observed for both the compounds. Data from 126 hair samples were included in this study: 54 subjects(42.9%) were positive both for THC and THC-COOH; none of the samples was positive for a single substance. Concentrations ranged from 0.18 to 1.75 ng/mg (median: 0.78 ng/mg) for THC and from 0.04 to 0.85 ng/mg (median: 0.31 ng/mg) for THC-COOH. Cannabinoids levels seemed to decrease with the age, with lower amounts in the subjects aged > 40 years ($p < 0.05$). Also years of consumption seemed to have a significant impact on hair concentrations, as higher levels were observed in consumers from > 10 years ($p = 0.013$). Moreover, this study further provided evidences of a significant reduction of THC and THC-COOH in bleached hair ($p = 0.042$).

* Correspondence to: Forensic Toxicology Division, Department of Health Science, University of Florence, Largo Brambilla 3, Italy.
E-mail address: fabio.vaiano@unifi.it (F. Vaiano).

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1. Introduction

Cannabis is the most used drug of abuse worldwide both for recreational and therapeutic scopes [1]. Given its great spread, monitoring its use/abuse represents a key task for forensic and clinical toxicology. In forensic toxicology, evaluation of Cannabis consumption is required for many purposes, such as workplace drug testing and driving license renewal. In these cases, hair analysis represents the most adopted and reliable approach for the investigation of repeated or chronic exposure to Cannabis. The main marker is Δ^9-tetrahydrocannabinol (THC) but, since it is present in Cannabis smoke or on contaminated objects, its identification cannot be unequivocally indicative of active use of cannabinoids. For this reason, detection of THC metabolites is strongly recommended to exclude passive smoking and/or external deposition on hair. In their guidelines, the Society of Hair Testing (SoHT) and the European Workplace Drug Testing Society (EWDTS) suggest the determination of the 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) to confirm the Cannabis intake [2–4]. Unfortunately, THC-COOH identification in hair is hampered by the low incorporation rate due to its acidic nature. In order to overcome this limitation, forensic toxicologists have explored new markers or developed more sensitive analytical methods. Among the most interesting markers, 11-hydroxy-Δ^9-tetrahydrocannabinol (OH-THC) has been recently investigated providing good agreement with THC-COOH results [5–7]. However, evaluation of its sensitivity is still ongoing and needs to be evaluated by further studies. Regarding the analytical procedure, the gold standard technique for THC and THC-COOH determination in hair is the gas chromatography-mass spectrometry (GC-MS) equipped with electronic impact (EI) or negative chemical ionization (NCI) sources [8–12]. Besides these methods, recently liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been increasingly applied to cannabinoid hair quantification for its low time and resource consumption [13–16]. Indeed, LC- MS/MS has proved to be less demanding than GC-MS for sample preparation. However, the moderate ionization efficiency for cannabinoids negatively affects the sensitivity [17,18]. Sensitivity plays a key role in THC and THC-COOH hair quantification as their concentrations can range from 0.1 to 100 pg/mg. SoHT set the THC cut-off value at 50 pg/mg and the minimum required limit of quantification for THC-COOH at 0.2 pg/mg [2]. This observational study aimed to explore the relationship between THC and THC-COOH levels in hair and several variables, such as age, gender, consumption habits, and hair features. To the best of our knowledge, only a few studies are available on this topic and mainly focused on hair treatments [19–23].

Hair samples were collected from subjects tested for Cannabis use and analysed by an LC-MS/MS analytical method. This procedure was previously developed to make less resource- and time-consuming the analysis of THC and THC-COOH, since the former routinary GC-MS method required longer and more expensive steps (such as a solid-phase extraction – SPE – and derivatization) [24].

2. Materials and methods

2.1. Chemicals and reagents

Dichloromethane (DCM), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), acetic acid, hexane, and ethyl acetate were purchased from Panreac Quimica S.L.U. (Castellar del Vallès, Spain). Water (H_2O) and acetonitrile (ACN) for LC-MS/MS were acquired from Biosolve Chimie SARL (Dieuze, France). Formic acid was obtained by Merck KGaA (Darmstadt, Germany). THC, THC-d3 (internal standard, IS), THC-COOH, and THC-COOH-d3 (IS) were supplied by Chemical Research 2000 sr.l. (Rome, Italy).

2.2. Sample treatment

Hair sample was washed twice with 2 mL of DCM, dried (in a chemical hood at room temperature), and then cut into short pieces (< 1 mm length) with scissors. A 25-mg aliquot of hair was extracted following a previously published procedure with slight modifications [13]. Briefly, the hair sample was added with 10 μL of IS solutions (0.5 ng/μL) and then hydrolyzed with 1 mL of 1 M NaOH at 65 °C for 20 min. After cooling at room temperature, the mixture was acidified with 1.5 mL of 1 M H_2SO_4 and vortexed. Then, a liquid-liquid extraction (LLE) was performed twice with 5 mL of a hexane/ethyl acetate (90/10, v/v) solution. Once the organic mixture was added, the sample was immediately vortexed and centrifugated at 4000 G for 5 min. The organic phases were mixed and dried under a gentle stream of nitrogen at 30 °C. The residue was reconstituted with 100 μL of mobile phase and 3 μL were injected into the LC-MS/MS system.

2.3. LC-MS/MS

Analysis was conducted using an HPLC Agilent 1290 Infinity system (Agilent Technologies, Palo Alto, CA, USA) interfaced with an Agilent 6460 Triple Quad LC/MS (Agilent Technologies), equipped with an electrospray ion source (ESI) operating in positive mode. The ESI configuration was: gas temperature 325 °C; gas flow rate 10 L/min; nebulizer 20 psi; capillary 4000 V. Acquisition was in multiple reaction monitoring for the following transitions: 315→259, 193 *m/z*, for THC; 318→196, 123 *m/z*, for THC-d3; 345→299, 193 *m/z* for THC-COOH; 348→196, 302 *m/z* for THC-COOH-d3 (Table 1, Fig. 1). Chromatographic separation was performed through a Zorbax Eclipse Plus C18 (2.1 × 50 mm, 1.8 μm, Agilent Technologies). The mobile phase initially consisted of 5 mM aqueous formic acid (A) and ACN (B) 50:50. Gradient of elution was carried out by increasing the %B to 75% within 4 min; to 90% within 1 min (isocratic for 1 min). Post-time was set at 2 min. The flow rate was 0.4 mL/min.

2.4. Validation parameters

Validation was performed following the American Academy of Forensic Sciences' (AAFS) standard practices for method validation in forensic toxicology [25].

2.4.1. Interferences studies

Blank hair samples from 10 different subjects non-consumer of any drug, were analysed in order to evaluate endogenous interfering signals. Interferences due to the deuterated ISs were estimated analysing 10 different blank hair specimens spiked with 5 ng of THC-d3 and THC-COOH-d3. Ten blank samples were also added with 5 ng of several drugs of abuse (cocaine and its metabolites, amphetamines, morphine, 6-monoacethylmorphine, benzodiazepines, methadone and its main metabolite, buprenorphine, ketamine).

2.4.2. Limit of detection (LOD) and lower limit of quantification (LLOQ)

LOD consists in the lowest concentration producing a signal-to-noise

Table 1
LC-MS/MS parameters for THC and THC-COOH. In bold the quantitative transition.

Compound	Fragmentor (V)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Retention time (min)
THC	145	315	**193**	23	4.658
			259	23	
THC-d3	145	318	**196**	20	4.598
			123	21	
THC-COOH	132	345	**193**	17	3.812
			299	21	
THC-COOH-d3	130	348	**196**	20	3.794
			302	14	

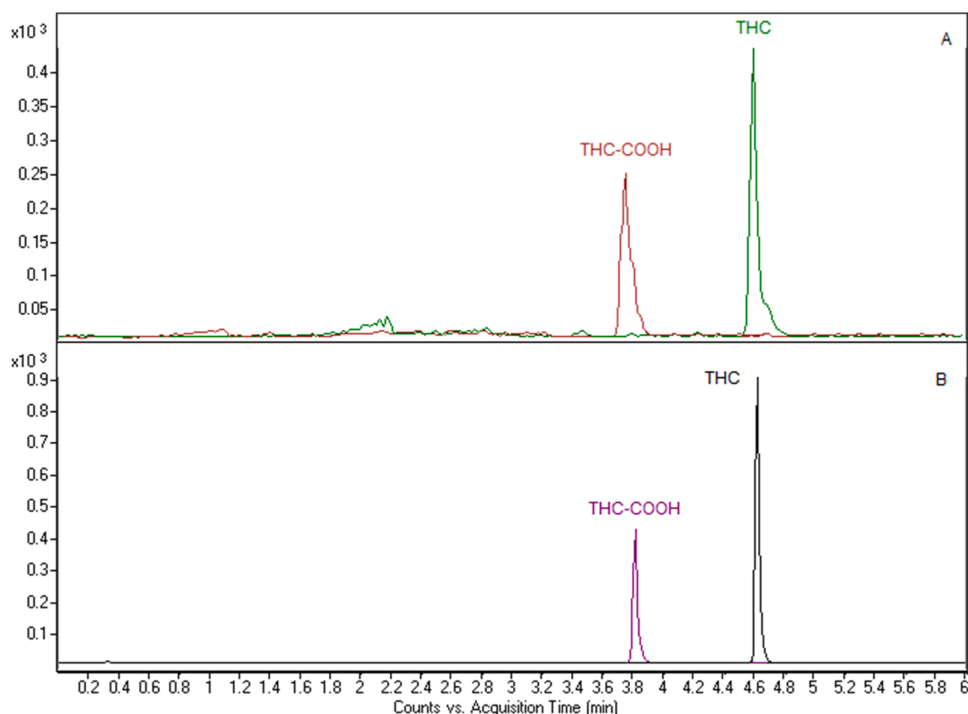

Fig. 1. Chromatograms at LLOQ levels (A) and of a real sample (B, THC: 180 pg/mg; THC-COOH: 50 pg/mg).

ratio (SNR) ≥ 3.3 and meets the identification criteria. Its determination was achieved analysing two replicates of blank matrices (from three sources) spiked with decreasing THC and THC-COOH amounts. The same methodological approach was applied to LLOQ estimation. In addition, the LLOQ had to meet.

all detection, identification, bias, and precision criteria. For this purpose, the decreasing concentrations were added to the calibration range in order to obtain the minimum LLOQ which can be reached by our method. Anyway, the working LLOQ for this study was represented by the lowest calibrator.

2.4.3. Calibration model

Calibration model was investigated for the working ranges 0.050 – 5.0 ng/mg (0.05, 0.20, 0.50, 1.5, 3.0 and 5.0 ng/mg) for THC and LLOQ – 2000 pg/mg (0.2, 1.0, 5.0, 20.0, 100.0, 500.0 and 2000.0 pg/mg) for THC-COOH. Five replicates of blank hair spiked at the proper concentrations were analysed and the least-squares regression procedure applied to the data. Linearity was evaluated by means of the coefficient of determination (R^2, acceptance criterium: ≥ 0.9900).

2.4.4. Bias and precision

Bias was calculated using the following formula:

$$Bias(\%) at concentration_x = \left[\frac{Mean measured concentration_x - nominal concentration_x}{nominal concentration_x}\right] \times 100$$

Evaluation was achieved by the analysis of five replicates of three separated blank hair samples spiked at three different concentrations (quality control, QC): 0.1 (≤ 3 times the first calibration level), 1.0 and 4.0 (~ 80% of the highest calibrator) ng/mg for THC; 0.5 (≤ 3 times the LLOQ), 250.0 and 1600.0 (~ 80% of the highest calibrator) pg/mg for THC-COOH. Bias was acceptable if within ± 20% at each concentration.

Precision was expressed ad coefficient of variation (%CV):

$$\%CV = \frac{standard deviation}{mean response} \times 100$$

Three replicates at QC levels were analysed five times. Within-run precisions were calculated for each QC separately for each of the five runs. For Between-run precision, evaluation for each concentration was performed over the five runs. %CV was accepted if < 20%.

2.4.5. Recovery rate (RR), matrix effect (ME) and carry-over

The estimation of RRs was achieved by the comparison of analytes' slopes from QC1 and QC3 spiked before and after the extraction over 6 replicates.

The post-extraction addition approach was adopted for ME estimation. Ionization suppression (IoS) or enhancement (IoE) were calculated as follows:

$$\%IoS or \%IoE = \left(\frac{mean area of set 2}{mean area of set 1} - 1\right) \times 100$$

Set 1 consisted in two neat standards at QC1 and QC3 concentrations. Each neat standards was injected six times to establish the mean area of set 1.

Set 2 consisted in ten different hair samples extracted in duplicate and then spiked at QC1 and QC3 levels. IoS or IoE should not exceed ± 25%.

Carry-over estimation was achieved by injecting the extracted blank samples into the LC-MS/MS system immediately after the highest calibrator over five runs.

2.5. Enrolled population and sample collection

Data from subjects tested for Cannabis consumption for personal purposes (i.e., child custody, workplace drug testing, driving license issues, personal use proving, etc…) throughout the 2021 were analysed in this study. The informed consent to the use of these data were provided by the individuals on voluntary base. Indeed, at collection time, the subjects were comprehensively informed about all the features of this study (including aims, objectives and methodology) and were asked to provide the written consent. This consent was mandatory and if it was denied, data were not included in our statistics. A short questionnaire was administrated to enrolled subjects in order to collect information about age, gender, consumption habits (i.e., frequency and years of use, Cannabis product), hair treatment (i.e., bleaching, perming, permanent coloring), and hair morphology. Questionnaires were anonymous and were related to hair samples by a code to ensure anonymity.

Sample were always collected from the posterior vertex region of the head, as close as possible to the scalp, accordingly to the recommendations for hair testing in forensic cases of the SoHT [4]. It must be specified that data included in this study were exclusively related to 3-cm proximal segments for scalp hair samples. Data from body hair or hair segments shorter/longer than 3 cm were excluded.

2.6. Statistical analysis

The correlation between THC and THC-COOH hair concentrations was analysed by Spearman's rank correlation coefficient. In order to study the influences of the several variables, a new value was introduced, *Sqrt(THC*THCCOOH)*, keeping the same unit of measurement of hair concentration (ng/mg):

$$Sqrt(THC*THCCOOH) = \sqrt{THC(ng/mg)*THCCOOH(\frac{ng}{mg})} = \sqrt{THC*THCCOOH(ng/mg)^2} = \sqrt{THC*THCCOOH}(ng/mg)$$

Its effectiveness and reliability were proved by the Principal Component Analysis (PCA). PCA shows that the two variables, THC and THC-COOH, can be reduced into a single variable, also called the "component of PCA". Relationships between the *Sqrt(THC*THCCOOH)* and the variables were studied through the Stepwise regression ($p = 0.05$). All the analyses presented in this paper are performed with STATA17.

3. Results and discussion

3.1. Method optimization

The above-described method was based on the Dulaurent et al.'s procedure [26]. Optimization was performed testing several hydrolysis and acidification conditions in order to obtain the best RRs and MEs (also in terms of interferences). The hydrolysation step was tested at a lower temperature (65 vs 100 °C) and for a longer time (20 vs 10 min).

Table 2
Sensitivity, calibration model and recovery rate (RR) for THC and THC-COOH.

Compound	LOD (pg/mg)	LLOQ (pg/mg)	Calibration ranges (pg/mg)	R^2	RR (%)	
					QC1*	QC3
THC	8	50‡	50–5000	0.9945	78	85
THC-COOH	0.1	0.2	0.2–2000	0.9971	79	88

‡ Working LLOQ
* QC1: 100 pg/mg for THC and 0.5 pg/mg for THC-COOH; QC3: 4000 pg/mg for THC and 1600 pg/mg for THC-COOH.

In this way, a full hydrolysation of conjugated species was obtained at a lower temperature, reducing the risk of matrix components' degradation. Addition of 1.5 mL of 1 M H_2SO_4 provided best RRs (>78%) than other tested acid (20% Acetic acid, RRs from 51% to 77%; 1 M HCl, RRs from 67% to 81%).

3.2. Method validation

The method proved to be linear for both the calibration ranges. The R^2 for THC and THC-COOH were 0.9945 and 0.9971 (> 0.9900), respectively (Table 2). Sensitivity for THC and THC-COOH was in line with previously published methods [13–15]. The LOD and LLOQ values were: 8.0 and 10 pg/mg for THC; 0.1 and 0.2 pg/mg for THC-COOH. However, since the working calibration range for THC started from 50 pg/mg, this concentration should be considered as the LLOQ value for this study. Accuracy, intra-run and between-run precisions always met the acceptable criteria as reported in Table 3. RR was > 78% and ion suppression was observed for both the compounds (from −8.4% to −6.7% for THC and from −11.7% to −9.9% for THC-COOH, Table 3). Carryover was not observed.

The new procedure was also compared to the previous GC-MS method routinely used in our lab [24]. Briefly, it consisted in an overnight acidic digestion (0.1 M HCl, 45 °C, 18 h) of 50 mg of hair, followed by a SPE and a derivatization step. The GC-MS method was more sensitive (LLOQ: 1 pg/mg for THC and 0.1 pg/mg for THC-COOH), even if it required a higher amount of matrix (50 mg vs 25 mg), but it was more time and resource consuming. Indeed, the digestion phase and the LLE for the new method are achieved in a significantly shorter time (~ 30 min vs > 1 day); moreover, LLE is also easier and cheaper than the SPE and do not negatively affect the ME and RR.

3.3. Overall statistics

In this study, 126 hair samples were analysed and 54 (42.9%) were positive both for THC and THC-COOH; none of the samples was positive for a single substance as observed in many studies [6,7,27,28]. Concentrations were from 0.18 to 1.75 ng/mg (median: 0.78 ng/mg) for THC and from 0.04 to 0.85 ng/mg (median: 0.31 ng/mg) for THC-COOH (Table 4); our ranges were comparable to the ones described in literature, even if the metabolite's hair amounts were very high [8,14,15,18,19,22,23,29]. THC levels were always above the threshold suggested by SoHT at 50 pg/mg [2]. The concomitant presence of the two cannabinoids, and the high concentration of THC-COOH, means that an environmental exposure to Cannabis smoke or an external contamination can be excluded for all the individuals [4]. Correlation between THC and THC-COOH hair levels demonstrated a linear increase ($R^2 = 0.8064$) as also observed by Minoli et al. [30]. As mentioned above, a new variable was introduced, *Sqrt(THC*THCCOOH)*, in order to study the effects of several factors on the two substances' concentrations. The new variable proved to be effective and reliable through the PCA analysis and thus, it was applied to this study. PCA shows that the first component explained above 91% of the total variability induced by the two variables THC and THCCOOH. This means that hair concentrations of THC and THCCOOH can explain the same phenomenon in the same way. Moreover, the score produced by PCA has a correlation equal to 1 with the created index *Sqrt (THC*THCCOOH)*. This new variable could be practically considered as the geometric mean for the two variables (hair THC and THC-COOH concentrations) keeping the same unit of measurement (ng/mg). Thus, two almost collinear values were substituted by a single and reliable parameter. A minor limitation could be represented by the 9% of cases not explained by the new parameter; anyway, this low percentage can not negatively affect the statistical analysis.

Self-reported questionnaires were useful to collect information about the frequency of use over the last three months in order to study the influence of each variable among the single categories of consumption, no use (n = 23, 18.3%), 1/week (n = 38, 30.2%), 2–3/week (n = 23,

Table 3
Accuracy, precision and matrix effect (ME) for THC and THC-COOH.

Compound	Accuracy (%)			Intra-run Precision (%)			Intra-run Precision (%)			ME (%)	
	QC1*	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC3
THC	18.4	15.2	9.4	12.4	14.8	7.4	15.7	13.3	9.7	-8.4	-6.7
THC-COOH	17.6	12.7	10.9	15.4	15.6	8.7	14.1	11.9	12.3	-11.7	-9.9

* QC1: 100 pg/mg for THC and 0.5 pg/mg for THC-COOH; QC2: 1000 pg/mg for THC and 250.0 pg/mg for THC-COOH; QC3: 4000 pg/mg for THC and 1600 pg/mg for THC-COOH.

Table 4
Statistics for THC and THC-COOH hair levels according the evaluated variables.

Variable	Sample population	Positive subjects*	THC, ng/mg (median)	THC-COOH, ng/mg (median)	*Sqrt (THC*THCCOOH)* ng/mg (median)
Whole population	126	54 (42.8%)	0.18–1.75 (0.78)	0.04–0.85 (0.31)	0.09–0.99 (0.51)
Gender					
Male	84 (66.7%)	38 (45.2%)	0.18–1.75 (0.99)	0.04–0.85 (0.43)	0.09–0.99 (0.64)
Female	42 (33.3%)	16 (38.1%)	0.25–1.24 (0.63)	0.06–0.54 (0.28)	0.19–0.82 (0.42)
Age					
< 30 yo	40 (31.7%)	21 (52.5%)	0.18–1.19 (0.95)	0.05–0.85 (0.63)	0.09–0.99 (0.71)
30–40 yo	55 (43.7%)	20 (36.4%)	0.35–1.64 (0.67)	0.06–0.68 (0.31)	0.20–0.96 (0.43)
> 40 yo	31 (24.6%)	13 (41.9%)	0.25–1.75 (0.85)	0.04–0.54 (0.17)	0.19–0.78 (0.33)
Cannabis product					
Marijuana	38 (30.2%)	13 (34.2%)	0.18–1.08 (0.65)	0.05–0.65 (0.26)	0.09–0.84 (0.35)
Hashish	12 (9.5%)	7 (58.3%)	0.63–1.62 (0.68)	0.04–0.45 (0.29)	0.20–0.75 (0.43)
Both	53 (42.1%)	29 (54.7%)	0.25–1.75 (1.05)	0.1–0.85 (0.54)	0.19–0.99 (0.78)
None	20 (15.9%)	5 (21.7%)	0.63–1.02 (0.75)	0.04–0.45 (0.30)	0.20–0.56 (0.47)
Frequency of use					
None	23 (18.3%)	5 (21.7%)	0.63–1.02 (0.75)	0.04–0.45 (0.30)	0.20–0.56 (0.47)
1/week	38 (30.2%)	2 (5.3%)	0.25 and 1.08‡	0.23 and 0.65‡	0.24 and 0.84‡
2–3/week	23 (18.3%)	12 (52.2%)	0.25–1.19 (0.67)	0.06–0.74 (0.28)	0.19–0.90 (0.38)
4–6/week	14 (11.1%)	10 (71.4%)	0.63–1.75 (0.90)	0.17–0.68 (0.29)	0.33–0.96 (0.56)
> 7/week	28 (22.2%)	25 (89.3%)	0.18–1.64 (0.95)	0.04–0.85 (0.39)	0.09–0.99 (0.59)
Timeframe of use					
Never	20 (15.9%)	2 (10.0%)	0.63 and 0.65‡	0.26 and 0.45‡	0.40 and 0.54‡
< 5 y	22 (17.5%)	11 (50.0%)	0.18–1.08 (0.78)	0.05–0.65 (0.32)	0.09–0.84 (0.43)
5–10 y	44 (34.9%)	19 (43.2%)	0.25–1.19 (0.75)	0.06–0.85 (0.30)	0.20–0.99 (0.47)
> 10 y	40 (31.7%)	22 (55.0%)	0.25–1.75 (1.36)	0.04–0.68 (0.31)	0.19–0.96 (0.54)
Hair treatment					
None	78 (61.9%)	34 (43.6%)	0.18–1.75 (1.05)	0.04–0.85 (0.50)	0.09–0.99 (0.71)
Coloring	11 (8.7%)	3 (27.3%)	0.74–1.24 (1.12)	0.39–0.54 (0.54)	0.54–0.82 (0.78)
Bleaching	17 (13.5%)	8 (47.1%)	0.25–0.85 (0.50)	0.14–0.34 (0.21)	0.19–0.54 (0.31)
Permanent	20 (15.9%)	9 (45.0%)	0.43–0.70 (0.63)	0.06–0.29 (0.26)	0.20–0.43 (0.40)
Hair color					
Blonde	72 (57.1%)	34 (47.2%)	0.18–1.64 (0.77)	0.05–0.85 (0.35)	0.09–0.99 (0.51)
Brown	12 (9.5%)	–	–	–	–
Dark	33 (26.2%)	16 (48.5%)	0.25–1.75 (0.80)	0.10–0.68 (0.31)	0.19–0.96 (0.54)
Gray	9 (7.1%)	4 (44.4%)	1.02–1.62 (1.17)	0.04–0.54 (0.09)	0.20–0.78 (0.33)
Hair morphology					
Straight	79 (62.7%)	34 (43.0%)	0.18–1.35 (0.85)	0.05–0.85 (0.50)	0.09–0.99 (0.65)
Frizzy	34 (27.0%)	15 (44.1%)	0.25–1.75 (0.63)	0.06–0.39 (0.26)	0.19–0.79 (0.40)
Curly	7 (5.6%)	1 (14.3%)	1.02	0.04	0.20
Wavy	6 (4.8%)	4 (66.7%)	1.12–1.62 (1.23)	0.04–0.54 (0.34)	0.25–0.82 (0.60)

*The percentage is relative to positive subjects among that specific population.
‡Cannabinoids were found in only two cases.

18.3%), 4–6/week (n = 14, 11.1%), > 7/week (n = 28, 22.2%). Comparing what was declared with the scientific evidence, Cannabis use was observed in 5 non-consumers and in only two cases among the 1/week consumers; moreover, positivity was registered in 52.2%, 71.4%, and 89.3% of 2–3/week, 4–6/week and > 7/week consumers. THC and THC-COOH ranges were reported in Table 4. Regarding the variable *Sqrt (THC*THCCOOH)*, no significant differences ($p > 0.05$) were observed among the groups (Fig. 2). These outcomes may be due to false or erratic reports. For these reasons, statistical analyses were performed on the whole dataset and not for the single consumption habits.

3.4. Gender influence

Positive male subjects (M) were 38 (70.4% of positive and 45.2% of M population) with THC and THC-COOH concentrations of 0.18–1.75 ng/mg (median: 0.99 ng/mg) and of 0.04–0.85 ng/mg (median: 0.42 ng/mg), respectively. In the female (F) positive population (n. = 16, 29.6% of positive and 38.1% of F population), overall lower levels were observed (Fig. 3), but no significant differences ($p = 0.312$) were registered by the variable *Sqrt(THC*THCCOOH)*. In F, THC concentrations were in the range from 0.25 to 1.24 ng/mg (median: 0.63 ng/mg) and THC-COOH from 0.06 to 0.54 ng/mg (median: 0.27 ng/mg). Quite similar results were also observed by Cho et al. for THC-COOH [31]. In this study, hair levels in M (n. 539) were 0.0001 – 0.027 ng/mg (median 0.00091 ng/mg) and in F (n. 47) 0.0001 – 0.014 ng/mg (median 0.00056 ng/mg). Statistical analysis did not highlight significant differences between the genders. Further research is required to study in-depth the reason why these slight differences can be observed; however, hair treatments are supposed to play a key role.

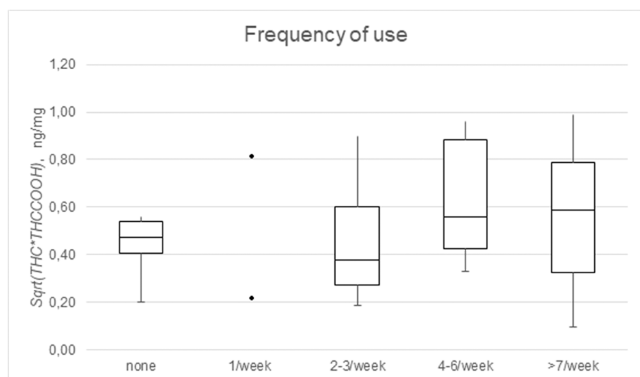

Fig. 2. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the frequencies of use.

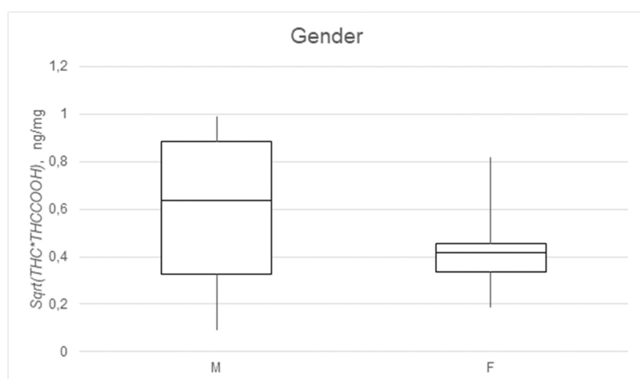

Fig. 3. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the genders.

3.5. Age influence

Three age ranges were chosen for this study, < 30, 30–40, and > 40 years old. THC and THC-COOH hair levels were respectively: 0.18–1.19 ng/mg (median: 0.95 ng/mg) and 0.05–0.85 ng/mg (median: 0.63 ng/mg) for > 30 years; 0.35–1.64 ng/mg (median: 0.66 ng/mg) and 0.06–0.68 ng/mg (median: 0.30 ng/mg) for 30–40 years; 0.25–1.75 ng/mg (median: 0.85 ng/mg) and 0.04–0.54 ng/mg (median: 0.17 ng/mg) for > 40 years (Table 4). Studying the age influence on hair concentrations by the value *Sqrt(THC*THCCOOH)*, a significant decrease ($p < 0.05$) in the 30–40 and > 40 years-old subjects was observed (Fig. 4). In a previous study, Han et al. also investigated the influence of age on THC-COOH hair levels [19]. The authors stated that the highest average concentrations were found in users aged 20 years, but this difference was not statistically significant. This finding may find explanation in the declared frequency of Cannabis use, since 37.5% of < 30 years-old subjects reported daily consumption of Cannabis. This percentage was lower in the other groups, 14.5% for 30–40% and 16.1% for > 40 years-old individuals.

3.6. Consumption habits

Besides the frequency of use, participants were asked to indicate the Cannabis product consumed and also the timeframe of consumption. Among the whole population, Marijuana use was reported by 38 subjects (30.2%), hashish by 12 (9.5%), and both preparations by 53 (42.1%); 23 (18.3%) subjects did not report the use of Cannabis products (data consistent with the frequency of use declaration, see above). None of the enrolled population declared consumption of edible Cannabis products or use of so-called "Cannabis light" (%THC $< 0.5\%$). THC and THC-COOH concentrations ranged as follows: 0.18–1.08 ng/mg (median: 0.65 ng/mg) and 0.05–0.65 (median: 0.26 ng/mg) for marijuana; 0.63–1.62 ng/mg (median: 0.68 ng/mg) and 0.04–0.45 ng/mg (median: 0.29 ng/mg) for hashish; 0.25–1.75 ng/mg (median: 1.05 ng/mg) and 0.19–0.85 ng/mg (median: 0.78 ng/mg) for both. Regarding people declaring not use of Cannabis products, 5 subjects (21.7%) presented hair THC and THC-COOH levels in the ranges 0.63–1.02 ng/mg (median: 0.75 ng/mg) and 0.04–0.45 ng/mg (median: 0.30 ng/mg), respectively. The variable *Sqrt(THC*THCCOOH)* showed no statistical differences ($p > 0.151$) even if higher levels were found in consumers of both the Cannabis products (Fig. 5).

Most of participants ($n = 84$; 66.7%) declared to be Cannabis users from > 5 years (Table 4). In positive subjects, THC and THC-COOH ranging from 0.25 to 1.75 ng/mg (median: 0.93 ng/mg) and from 0.04 to 0.68 ng/mg (median: 0.30 ng/mg). 22 subjects (17.5%) reported use of this substance from < 5 years and half presented cannabinoids in the ranges of 0.18–1.08 ng/mg (median: 0.78 ng/mg) and 0.05–0.65 ng/mg (median: 0.0.32 ng/mg); non-consumers were 20 (15.9%). Nevertheless, two of the self-reported non-consumers were positive for cannabinoids (Table 4). By the study of *Sqrt(THC*THCCOOH),* a statistically significant influence was observed over 10 years of Cannabis consumption ($p = 0.013$, Fig. 6). To the best of our knowledge, this is the first study on correlation among cannabinoids hair levels and Cannabis products or years of consumption.

3.7. Hair treatment and morphology

As well reported in the literature, bleaching causes a notable decrease in cannabinoids' hair levels [20]. Our study confirms this evidence since it was detected a statistically significant reduction

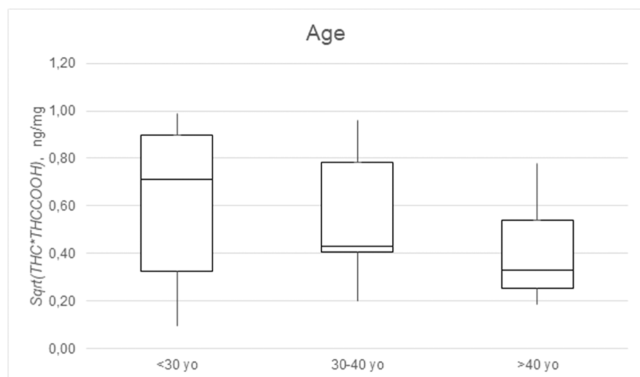

Fig. 4. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the age groups.

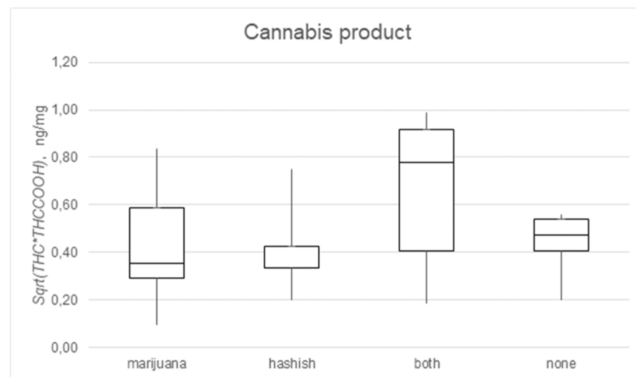

Fig. 5. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the Cannabis products.

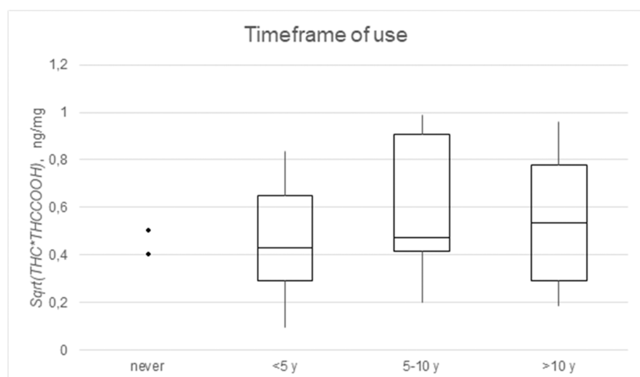

Fig. 6. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the timeframes of use.

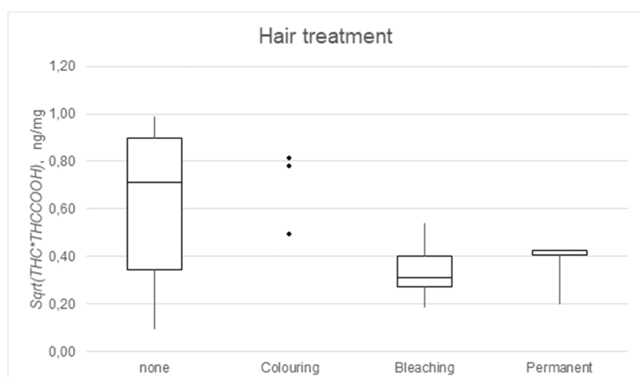

Fig. 7. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the hair treatments.

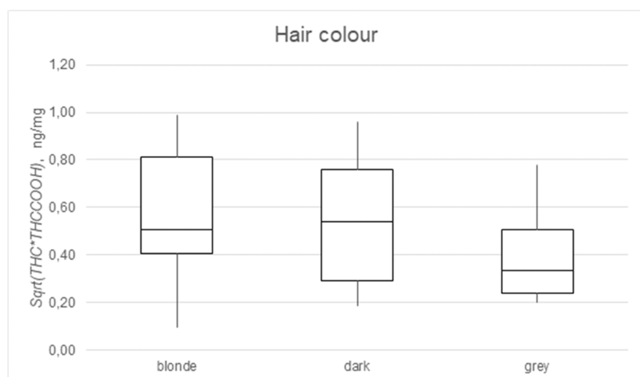

Fig. 8. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the hair colors.

(p = 0.042) of the *Sqrt(THC*THCCOOH)* with respect to the other treatments (Fig. 7). In bleached hair (n. = 8, 47.1%), THC was from 0.25 to 0.85 ng/mg (median: 0.50 ng/mg) and THC-COOH from 0.14 to 0.34 ng/mg (median: 0.21 ng/mg). Levels for the other treatment are reported in Table 4. Regarding hair color and morphology, differences were not statistically significant (p > 0.792) even if in gray and frizzy THC and THC-COOH hair levels were lower (Figs. 8 and 9). Data on color influence are consistent with the Mieczkowski study [22], even if data from brown hair were unavailable in our study. Many studies demonstrated that melanin play a key role in binding of xenobiotics in hair [32–34]. However, influences due to the pigmentation, and morphology as well, need to be investigated more in-depth, in order to explain the mechanism and the specificity of their effects.

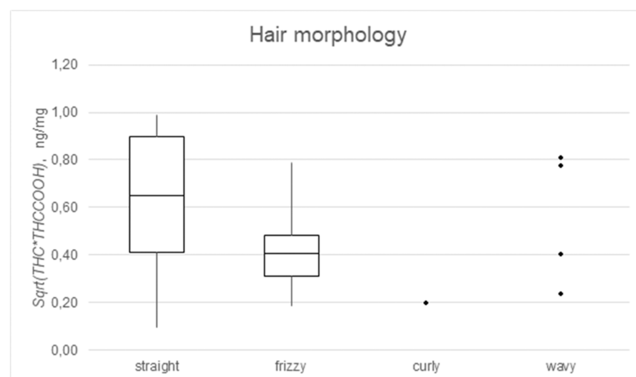

Fig. 9. Box plot of the *Sqrt(THC*THCCOOH)* values' distribution for the hair morphologies.

4. Conclusions

THC and THC-COOH hair levels are still an ongoing issue for forensic toxicologists. Their quantification is determinant in many caseworkers (driving license issue, workplace drug testing, child custody) and interpretation is not always easy. For this reason, the comprehension of the influences of several variables needs to be studied more in-depth. This paper gives new evidence on how age, gender, consumption habit, hair treatment, and morphology may influence the THC and THC-COOH hair amounts. This study demonstrated a statistically significant decrease of them with the age, since lower levels were observed in > 40 years-old subjects. Years of consumption seemed to play a key role, with significant higher amounts were found in consumers from > 10 years. The relevance of this study also lies in the wide number of analysed data and the investigated variables, above all Cannabis product and hair morphology.

These achievements were made possible by the full validation of an LC-MS/MS method which allowed us to reduce time and resource consumption than the previous GC-MS procedure.

CRediT authorship contribution statement

Fabio Vaiano: Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Lapo Scuffi:** Formal analysis, Data curation, Investigation. **Alessio Lachi:** Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Claudia Trignano:** Supervision, Writing – original draft. **Antonina Argo:** Supervision, Writing – original draft. **Francesco Mari:** Conceptualization, Supervision, Writing – original draft. **Elisabetta Bertol:** Conceptualization, Data curation, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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