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Simultaneous analysis of THC and its metabolites in blood using liquid chromatography–tandem mass spectrometry

Maria del Mar Ramirez Fernandez[a,*], Gert De Boeck[a], Michelle Wood[b], Manuel Lopez-Rivadulla[c], Nele Samyn[a]

[a] Federal Public Service Justice, National Institute of Criminalistics and Criminology, Chaussée de Vilvorde 100, 1120 Brussels, Belgium
[b] Waters Corporation, MS Technologies Centre, Manchester, UK
[c] Forensic Toxicology Service, Institute of Legal Medicine, University of Santiago de Compostela, Spain

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ABSTRACT

Cannabis is considered to be the most widely abused illicit drug in Europe. Consequently, sensitive and specific analytical methods are needed for forensic purposes and for cannabinoid pharmacokinetic and pharmacodynamic studies. A simple, rapid and highly sensitive and specific method for the extraction and quantification of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy- Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol (THC-COOH) in blood is presented. The method was fully validated according to international guidelines and comprises simultaneous liquid–liquid extraction (LLE) of the three analytes with hexane:ethyl acetate (90:10, v/v) into a single eluant followed by separation and quantification using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Chromatographic separation was achieved using a XBridge C_{18} column eluted isocratically with methanol:0.1% formic acid (80:20, v/v). Selectivity of the method was achieved by a combination of retention time, and two precursor–product ion transitions. The use of the LLE was demonstrated to be highly effective and led to significant decreases in the interferences present in the matrix. Validation of the method was performed using 250 μL of blood. The method was linear over the range investigated (0.5–40 μg/L for THC, 1–40 μg/L for 11-OH-THC, and 2–160 μg/L for THC-COOH) with excellent intra-assay and inter-assay precision; relative standard deviations (RSDs) were <12% for THC and 11-OH-THC and <8% for THC-COOH for certified quality control samples. The lower limit of quantification was fixed at the lowest calibrator in the linearity experiments. No instability was observed after repeated freezing and thawing or in processed samples. The method was subsequently applied to 63 authentic blood samples obtained from toxicology cases. The validation and actual sample analysis results show that this method is rugged, precise, accurate, and well suited for routine analysis.

1. Introduction

Cannabis is considered to be the most widely abused illicit drug in Europe. Indeed, statistical information shows that 30% of the under-forties age group have already consumed this drug [1,2].

Δ^9-Tetrahydrocannabinol (THC) is the main psychoactive constituent. During marijuana smoking, THC is rapidly absorbed in larger amounts than when taken orally and, due to its strong lypophilic nature, it spreads rapidly throughout the body. It is mainly metabolized to 11-hydroxy- Δ^9-tetrahydrocannabinol (11-OH-THC) by the human body. This metabolite is still psychoactive and is further oxidized to 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol (THC-COOH). In humans and animals more than 100 metabolites could be identified but 11-OH-THC and THC-COOH are the most predominant. Metabolism mainly occurs in the liver by cytochrome P450 enzymes CYP2C9, CYP2C19 and CYP3A4 [3].

Urine drug concentration data do not provide adequate answers to demanding clinical and forensic questions. These are more readily answered with quantitative blood data which provides more information related to the current state of impairment. However, the analysis of blood can be more challenging due to the presence of lipophilic and proteinaceous compounds not usually found in urine, the need for substantially lower sensitivity limits and the lower sample volume available.

Due to the high specificity and the increased signal-to-noise in combination with short chromatographic run times, liquid

* Corresponding author. Tel.: +32 2 240 05 00; fax: +32 2 241 61 05.
E-mail address: mariadelmar.ramirezfernandez@just.fgov.be (M. del Mar Ramirez Fernandez).

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chromatography–tandem mass spectrometry (LC–MS/MS) allows for specific, selective and sensitive analysis of compounds with a wide polarity range in samples of various nature. It offers the possibility to simplify sample preparation, although this approach should be treated with caution due to the possibility of ion suppression or enhancement as a result of the matrix. Consequently, attention must be paid to the choice of the sampling method and the influence of the collected matrix on the LC–MS/MS analysis.

Several methods have been described for the quantification of THC and its metabolites in blood. Immunochemical methods, mainly ELISA, are generally used as screening tools for cannabis use [4]. However, for workplace drug testing, driving under the influence of drugs and for forensic cases, the confirmation of positive immunoassay test results is necessary. It is usually performed by gas chromatography–mass spectrometry (GC–MS) methodologies [5–7]. However, GC requires time-consuming sample preparation and the need to use various derivatization techniques. In contrast to GC, no decomposition of the cannabinoids occurs during liquid chromatography and hence the cannabinoid acid forms may be analyzed directly. Several papers report the application of LC–MS(/MS) [8–14]. Most of them require high sample volume (1 mL) to achieve high sensitivity, they are focused on just one compound and/or the method is not fully validated (following all criteria for chromatographic assays). All of these aspects are significant at the moment of development of a method since (a) the amount of sample required is an analytical limitation, (b) forensic toxicologist may be required to analyze THC and other hydroxylated metabolites in blood to evaluate an impairment status and (c) complete validation is required to guarantee the robustness of the method.

Our aim was to develop and fully validate a simple, fast and sensitive LC–MS/MS method for the confirmation of THC, 11-OH-THC and THC-COOH in blood which required only a minimal volume of sample and with an efficient sample clean-up procedure.

2. Experimental

2.1. Chemicals

Individual stock solutions of THC, 11-OH-THC and THC-COOH (all certified at a concentration of 1 mg/mL in methanol), and the internal standards (I.S.) [$^2H_3$]THC (THC-d_3), [$^2H_3$]11-OH-THC (11-OH-THC-d_3) and [$^2H_9$]THC-COOH (THC-COOH-d_9) (certified concentration of 0.1 mg/mL in methanol) were obtained from LGC Promochem (Molsheim, France).

Methanol (LC–MS grade), 0.1% formic acid in water (UPLC grade) and water (HPLC grade) were purchased from Biosolve (Valkenswaard, The Netherlands). N-hexane (for chromatography), ethylacetate (for chromatography) and acetic acid (glacial) were obtained from Merck (Darmstadt, Germany).

External quality control (QC) samples were obtained from Medichem World (Steinenbronn, Germany).

2.2. Specimens

Pooled blank blood samples were used for development and validation of the procedure and were obtained from a local blood bank. Authentic samples were obtained from toxicology cases.

2.3. Preparation of standard solutions and sample extraction

Separate working solutions of the drugs, for tuning and selectivity experiments, were prepared in the laboratory at a concentration of 4 mg/L in methanol. A mixed working solution of non-deuterated compounds at 4 mg/L for THC and 11-OH-THC and of 16 mg/L for THC-COOH, in methanol was used for the preparation of calibrators. A mixed I.S. working solution of 1 mg/L for THC and 11-OH-THC and of 4 mg/L for THC-COOH, was prepared in methanol. Working solutions were stored at 4 °C, and were prepared monthly.

To obtain the lower concentrations needed for internal standardization and validation of each experiment, further dilutions in methanol were prepared the same day.

The extraction procedure was carried out in 10 mL disposable screw top vials of high quality glassware (Chromacol, Herts, UK) using 250 μL of blood. Fifty microliters of the I.S. working solution, 750 μL of deionised water and 200 μL of 10% acetic acid (glacial) were added. After adding 4 mL of hexane:ethyl acetate (90:10, v/v) mechanical shaking was carried out for 30 min. Then, the samples were centrifuged (10 min at 3000 × *g*), the organic phase was transferred to a 5 mL disposable screw top vial (Chromacol) and then evaporated to dryness with a vacuum centrifuge (Jouan, Saint Herblain, France). The extract was reconstituted in 120 μL of mobile phase and 30 μL was injected into the LC–MS/MS system.

2.4. LC–MS/MS

2.4.1. Chromatography

LC was performed using a Waters Alliance 2695 separation module (Waters, Milford, MA, US). Analytes were separated on a XBridge C_{18} column (150 mm × 2.1 mm, 3.5 μm) (Waters), eluted isocratically with methanol:0.1% formic acid (80:20, v/v), delivered at a flow rate of 0.3 mL/min. The total run time of the method was 13 min.

2.4.2. Mass spectrometry

A Quattro Ultima tandem MS (Waters) fitted with a Z-Spray ion interface was used for all analyses. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis: capillary voltage, 1.0 kV; source block temperature, 120 °C, desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 800 L/h. The appropriate multiple reaction monitoring (MRM) conditions for the individual analytes and their respective deuterated analogues were determined by direct infusion into the MS/MS. The cone voltage (CV) was adjusted to maximize the intensity of the protonated molecular species $[M+H]^+$ and collision-induced dissociation of each protonated molecule was performed. Collision gas (argon) pressure was maintained at 2.7×10^{-3} mbar and the collision energy (eV) adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis.

All aspects of system operation and data acquisition were controlled using MassLynx NT 4.2 software with automated data processing using the TargetLynx™ software (Waters). The statistical treatment of data was carried out using Excel 2000 (Microsoft).

2.5. LC–MS/MS assay validation

The analytical validation was performed according to the recommendations of Peters and Maurer [15,16], Shah et al. [17] and the SOFT/AAFFS Laboratory Guidelines [18].

2.5.1. Linearity, limit of quantification (LOQ), limit of detection (LOD), precision and accuracy

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogues. Freshly prepared working solutions of 200, 50, 12.5 and 2.5 μg/L for THC and 11-OH-THC, and of 800, 200, 50 and 10 μg/L for THC-COOH in methanol were used to prepare blood calibrators at a concentration of 40, 20, 15, 10, 5, 2, 1

and 0.5 μg/L for THC and 11-OH-THC, and of 160, 80, 60, 40, 20, 8, 4, and 2 μg/L for THC-COOH. Standard curves, freshly prepared with each batch of QC samples and authentic samples, were generated using a least-squares linear regression, with a $1/x$-weighing factor for all compounds.

The limit of quantification (LOQ) was estimated by replicate analysis ($n = 2$) over eight different days and it was defined as the concentration of the lowest calibrator that was calculated within ±20% of the nominal value and with a relative standard deviation (RSD) less than 20%.

The limit of detection (LOD) was estimated from blank blood samples, spiked with decreasing concentrations of the analytes. It was defined as the concentration where the response of the qualitative ion could reliably be differentiated from background noise, i.e. signal-to-noise ratio (S/N) equal to or greater than 3:1, and with acceptance criteria for ion ratios equal to or lower than 30% and retention time deviations lower than 3.5% relative to that of the corresponding control or calibrator [18].

Three blood QCs for THC and 11-OH-THC and four blood QCs for THC-COOH at three and four different concentrations, respectively, were included and analyzed in every batch: (a) external QC1 (Medichem): concentration THC (2.0 μg/L), 11-OH-THC (2.7 μg/L), THC-COOH (26.1 μg/L), (b) external QC2 (Medichem): concentration THC (2.9 μg/L), OH-THC (2.2 μg/L), THC-COOH (53.1 μg/L), (c) "in house" QC 'low' at 3 μg/L for each compound and (d) "in house" QC 'high' at 120 μg/L for THC-COOH. The "in house" QCs were prepared by different operators from different working solutions of the calibrators and it was stored at −20 °C until use.

Intra-assay and inter-assay precision was evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days. Precision (expressed as $\%RSD_r$ for intra-assay precision and $\%RSD_t$ for inter-assay precision) was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level—α of 0.05). Comparing the mean of calculated concentrations of QC samples to their respective nominal values, provided data on the accuracy of the method.

2.5.2. Selectivity and specificity

The selectivity of the method against endogenous interferences was verified by examination of the chromatograms obtained after the extraction of eight different blank blood samples from different origin. Moreover, specificity was also assessed by the analysis of blood samples spiked at 200 μg/L with other drugs of abuse and their metabolites (amphetamine, methamphetamine, MDA, MDMA, MDEA, ephedrine, PMA, benzoylecgonine, cocaine, morphine, codeine, 6-MAM, hydromorphone, hydrocodone, dihydrocodeine, oxycodone, oxymorphone, ethylmorphine, norcodeine, buprenorphine, methadone, EDDP, meperidine, fentanyl, pholcodine and tramadol) usually found in Belgium.

2.5.3. Stability

The stability of the drugs in blood samples that had been processed and then stored in the autosampler awaiting LC–MS/MS analysis was monitored; 250 μL blank blood spiked at the initial concentration of 34 μg/L ($n = 12$) and extracted. The I.S. was added to control samples ($n = 6$) and the concentrations were determined immediately. Another pool of samples was kept in the autosampler at 6 ± 2 °C and analyzed, previously spiked with the I.S., after 48 h ($n = 6$).

For an evaluation of freeze/thaw stability, the control samples at 34 and 2.5 μg/L ($n = 6$, at each concentration) were spiked with the I.S. and analyzed immediately. The stability samples, spiked at 34 and 2.5 μg/L ($n = 6$, at each concentration), were subjected to three freeze/thaw cycles. For each freeze/thaw cycle, the samples were frozen at −20 °C for at least 24 h, thawed, and then maintained at ambient temperature for 4 h. After the three cycles, the samples were spiked with the I.S. and analyzed. Stability was tested against a lower percentage limit corresponding to 90–110% of the ratio (mean value of stability samples/mean value control samples) with a 90% of the confidence interval of the stability samples between 80% and 120% of the mean of the control samples.

2.5.4. Assessment of matrix effects

To assess any potential suppression or enhancement of ionization due to the sample matrix, two different analyses were carried out. The first one involved a post-column infusion experiment. The study was based on a continuous post-column infusion of a mixture of the analytes and their internal standards (10 μg/L at a flow rate of 10 μL/min) to produce a constant elevated response in the MRM channels. The interference of this constant response was monitored in the whole run following the injection of extracted blood samples from different origin ($n = 6$) and compared to the response following the injection of mobile phase only. A second type experiment consisted of a comparison of the peak responses of the analysis of a blank sample spiked at 37.5 and 2.5 μg/L ($n = 6$, for each concentration) with those obtained from mobile phase spiked at the same concentration levels.

2.5.5. Recovery

Extraction recoveries were estimated by comparing the ratio of the peak areas of the non-deuterated compounds to the peak areas of the I.S. (i.e. responses) of blood samples spiked at 37.5 and 2.5 μg/L ($n = 6$, for each concentration) when the non-deuterated compounds were added before the extraction step with those obtained when the non-deuterated analytes were added after sample extraction. In both cases, the deuterated analogues were added after the extraction.

3. Results and discussion

The method was validated for specificity, linearity, LOQ, LOD, precision, accuracy, recovery and matrix effect by the analysis of spiked blood samples. In each case, a weighted ($1/x$) linear regression line was applied. Other weighting factors were tested before validation of the method but they did not give significantly better results. Correlation coefficients higher than 0.998 were achieved in the range investigated, i.e. from 0.5 to 40 μg/L for THC, from 1 to 40 μg/L for 11-OH-THC and from 2 to 160 μg/L for THC-COOH. Fig. 1 shows the MRM chromatograms obtained following the analysis of the lowest calibrator (0.5 μg/L for THC, 1 μg/L for 11-OH-THC and 2 μg/L for THC-COOH). At this concentration a S/N ratio >10:1 was observed for the qualifier and the criteria for LOQ were satisfied.

The applied isocratic mobile phase (methanol:0.1% formic acid, 80:20, v/v) ensured the elution of all the drugs examined within 11 min and produced chromatographic peaks of acceptable symmetry. In the early stages of method development an isocratic gradient of 90:10 methanol: 0.1% formic acid (v/v) was tested: although the compounds eluted earlier, giving a shorter run time, many interferences were observed at the retention time (2.5 min) of 11-OH-THC and THC-COOH when analysing blank blood samples.

Selectivity of the method was achieved by a combination of retention time, precursor and two product ions. The most prominent precursor–product transitions were used for quantification of THC and THC-d_3 and the next most abundant, used as qualifier. For the other compounds, an elevated background was noted when using the MRM transition based on the most prominent product. Improved sensitivity (based on S/N) was achieved when the MRM transition was based on an alternative product ion (Table 1). The

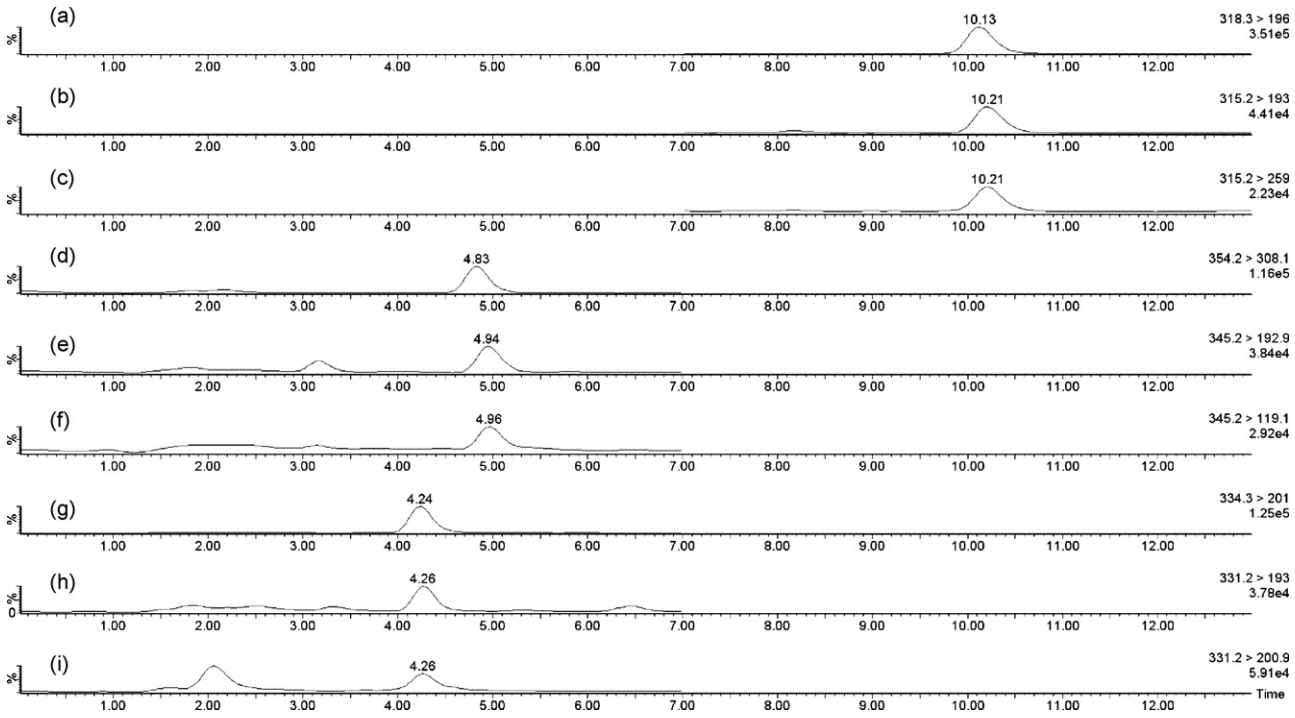

Fig. 1. MRM chromatograms obtained following the analysis of a spiked blood sample at the LOQ for (a) THC-d_3, (b) THC (quantifier), (c) THC (qualifier), (d) THC-COOH-d_9, (e) THC-COOH (quantifier), (f) THC-COOH (qualifier), (g) 11-OH-THC-d_3, (h) 11-OH-THC (quantifier), and (i) 11-OH-THC (qualifier). Peak intensity is shown in the top right-hand corner of each trace.

Table 1
MRM transitions and conditions for all compounds and their deuterated analogues. Underlined transitions were used for quantification.

Compound	Precursor ion (m/z)	Product ions (m/z)	Cone voltage (V)	Collision energy (eV)
THC	315.2	259.0	30	20
		193.0		20
11-OH-THC	331.2	200.9	30	20
		193.0		20
THC-COOH	345.2	192.9	30	30
		119.1		30
THC-d_3	318.3	196.0	30	20
11-OH-THC-d_3	334.3	201.0	30	20
THC-COOH-d_9	354.2	308.1	30	20

ion ratios (quantifier/qualifier) were 2.1, 0.8 and 1.5 for THC, 11-OH-THC and THC-COOH, respectively. For the corresponding deuterated analogues, only one transition was monitored. Injection of single analyte solutions did not produce interference in the other MRM channels. Fig. 2 shows the fragmentation structures of THC (a), 11-OH-THC (b), THC-COOH (c) to the quantifier ion.

Table 2
Dynamic range, LOD, LOQ, relative standard deviation (RSD, %) at the LOQ and equation of a typical calibration curve with the corresponding coefficient of determination (r^2). The limit of quantification (LOQ) was estimated by replicate analysis ($n = 2$) over eight different days.

	THC	11-OH-THC	THC-COOH
Dynamic range (μg/L)	0.5–40	1–40	2–160
LOD (μg/L)	0.5	1	2
LOQ (μg/L)	0.5	1	2
RSD (%) at LOQ	6.8	9.7	6.4
r^2	0.999465	0.999695	0.998958

Linearity data, LOQ and LOD are shown in Table 2. No interference peaks were observed in the cannabinoids MRM channels when blank blood samples spiked with other drugs were analysed. The LOD and the LOQ were identical for all compounds, since lower concentrations did not meet the acceptance criteria for ion ratios. In Belgium, the concentration of THC in a blood sample from a driver

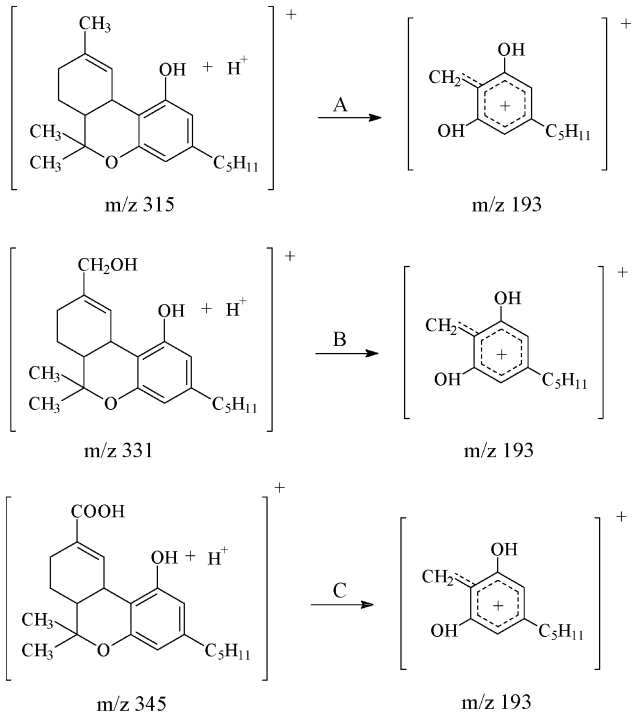

Fig. 2. Fragmentation of THC (a), 11-OH-THC (b), THC-COOH (c) to the quantifier ion.

Table 3
Intra-assay (expressed as RSD_r, %) and inter-assay precision (expressed as RSD_t, %) and accuracy of the QC samples. Intra-assay and inter-assay precision was evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days and it was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level—α of 0.05).

	THC			11-OH-THC			THC-COOH		
	RSD_r (%)	RSD_t (%)	Accuracy (%)	RSD_r (%)	RSD_t (%)	Accuracy (%)	RSD_r (%)	RSD_t (%)	Accuracy (%)
External control 1	3.7	11.5	91.3	4.0	5.2	94.7	3.8	7.8	95.4
External control 2	2.3	7.2	99.4	2.8	5.7	0.0	2.3	4.4	100.8
'In house' QC 'low'	1.4	7.1	96.0	3.9	4.9	105.8	6.4	6.2	99.8
'In house' QC 'high'	–	–	–	–	–	–	3.3	4.6	97.9

Table 4
Matrix effect, matrix effect RSD (%) and extraction recovery (%) evaluated at two different concentrations ($n = 6$, for each concentration).

	THC		11-OH-THC		THC-COOH	
	37.5 μg/L	2.5 μg/L	37.5 μg/L	2.5 μg/L	37.5 μg/L	2.5 μg/L
Mean matrix effect (%)	−8.5	0.4	23.6	26.5	−6.6	0.8
Matrix effect RSD (%)	8.1	8.5	2.6	8.4	6.7	5.4
Recovery (%)	109.4	108.1	110.8	111.5	105.0	104.8

that constitutes an offence is 2 μg/L. For other European counties like France and Germany (for plasma samples) it is fixed at 1 μg/L [19].

The intra- and inter-assay precisions were satisfactory, with all RSDs lower than 12% (Table 3). Results indicated that the accuracy of the assay was >90%.

The stability of processed samples (spiked at 34 μg/L and then stored in the autosampler at 6 ± 2 °C) was monitored after 48 h. No statistical significant difference could be observed for any of the compounds. Furthermore, all compounds, when spiked in blood at two concentrations, i.e. 34 and 2.5 μg/L, were demonstrated to be stable after three freeze/thaw cycles.

The matrix effect, defined as the effect of co-eluting residual matrix components on the ionization of the target analyte, typically results in either signal suppression or enhancement. Moreover, interfering matrix components can affect the reproducibility and accuracy of the developed procedure, leading to compromising or erroneous results. Consequently, in the development of any LC–MS(/MS) method, an efficient sample clean-up and use of appropriate internal standardization is necessary and the potential for any such ion suppression and enhancement should be assessed. Post-column infusion experiments (based on the method described by Bonfiglio et al. [20]) were performed to provide information of the effect of the matrix throughout the course of the elution time for the analytes. Fig. 3(a)–(c) shows the responses obtained following an injection of an extracted blank blood sample and a mobile phase only control (top and bottom chromatograms for each compound, respectively). A second experiment was carried out and we compared peak responses obtained when the cannabinoids were spiked to a blank blood sample with the response obtained when the

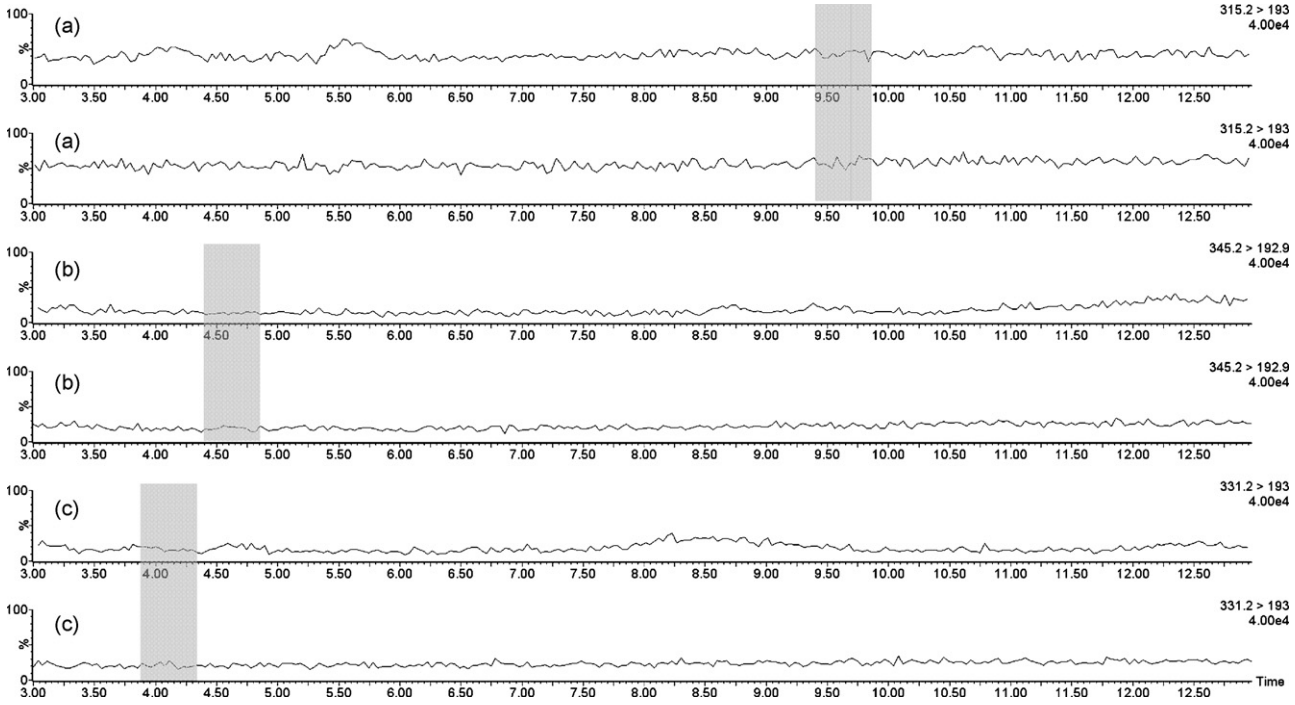

Fig. 3. Evaluation of the effect of the matrix on (a) THC, (b) THC-COOH and (c) 11-OH-THC by post-column infusion following an injection of extracted blank blood (top trace for each compound) and mobile phase only control (bottom trace). The dotted areas indicate the elution-position of each drug. MRM transition and peak intensity are shown on the right-hand corner of each trace.

cannabinoids were added to a mobile phase only at the same concentration. For 11-OH-THC some ion enhancement was observed, nevertheless it has been stated that the use of deuterated I.S. would partially compensante for matrix effects [21,22]. The results of the matrix effects at the two different concentrations are presented in Table 4.

The results of the extraction recovery study are also presented in Table 4. Very high and reproducible recoveries were obtained with this LLE procedure for all analytes. It must be pointed out the importance of adding the solution of 10% acetic acid to achieve a pH lower than 4.5 due to the pK_a of THC-COOH.

The validated LC–MS/MS method was applied to the analysis of 63 authentic samples from toxicology cases. Concentrations of THC-COOH were quite variable and generally high. The corresponding 11-OH-THC concentrations remained quite low. Samples with a concentration above the linear range of the calibration curve were diluted 1:2 with blank blood and re-analyzed. The median and minimum–maximum range (in μg/L), respectively, were as follows: THC (7.45 [1.3–34.1]), 11-OH-THC (2.7 [1.0–13.4]) and THC-COOH (44.8 [7.9–224.3]).

4. Conclusions

Reliable analytical data are a prerequisite for correct interpretation of toxicological findings in the evaluation of scientific studies, as well as in daily routine work. Unreliable analytical data might not only be contested in court, but could also lead to unjustified legal consequences for a defendant. Therefore, new analytical methods to be used in forensic and/or clinical toxicology require careful method development and thorough validation of the final method.

In this report, a fully validated and highly sensitive LC–MS/MS method is described for the simultaneous analysis of THC, 11-OH-THC and THC-COOH in blood. The method combined LLE with LC–MS/MS and provided a thorough clean-up of the matrix to minimize ion suppression and enhancement, in combination with high recovery, excellent precision and accuracy in the linear range investigated, using 250 μL of sample. The method was successfully applied to authentic samples.

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