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Determination of cannabinoids in cannabis products using liquid chromatography–ion trap mass spectrometry

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Abstract

A method was developed and validated for the simultaneous determination of five cannabinoids, viz. cannabidiol (CBD), cannabidiol acid (CBD-COOH), cannabinol (CBN), Δ^9-tetrahydrocannabinol (THC), and 3′-carboxy-Δ^9-all-*trans*-tetrahydrocannabinol (THC-COOH) in cannabis products. The cannabinoids were extracted from the grinded cannabis samples with a mixture of methanol–chloroform and analysed using liquid chromatography with ion-trap-mass-spectrometry (LC–IT-MS^n). For quantification the two most abundant diagnostic MS–MS ions of the analyte in the sample and external standard were monitored. For confirmation purposes the EU criteria as described in Commission Decision 2002/657/EC were followed. Fully satisfactory results were obtained, that is, unequivocal confirmation according to the most stringent EU criteria was possible. The limits of quantification were 0.1 g/kg for CBD, 0.04 g/kg for CBD-COOH, 0.03 g/kg for CBN, 0.28 g/kg for THC and 9.9 g/kg for THC-COOH. The repeatabilities, defined by R.S.D., were 2% for CBN, THC and THC-COOH at the concentration levels of respectively 0.023, 3.3 and 113 g/kg and 5% for CBD-COOH at the level of 0.34 g/kg (n = 6).

Keywords: Multi-analyte; Validation; Confirmation; Cannabinoids

1. Introduction

The use of hashish and marijuana in Europe and the United States, respectively, surpasses that of the other illegal psychoactive substances. Its source is *Cannabis sativa*, variety *Indica*, the hemp plant. Cannabis contains more than 400 compounds including more than 60 cannabinoids. The supposed main psychoactive agent is Δ^9-tetrahydrocannabinol (THC); its concentration varies, depending on the formulation type. Considerable evidence has emerged suggesting that the effects of marijunana are not due to THC alone [1,2]. Al least one other constituent, cannabidiol (CBD) was found to cause pharmacological effects [2,3]. The cannabinoid acids of THC and CBD, THC-COOH and CBD-COOH respectively are quantitatively important cannabinoids present in the plant [4,5]. As THC is thermolabile and photolabile, the storage of cannabis leads to a cumulative decrease in THC content through oxidation of THC to cannabinol (CBN). See Fig. 1 for the structures of some cannabinoids.

In The Netherlands, since September 2003, cannabis for therapeutic use is available on medical prescription. The medicinal cannabis has to be of a specific type and quality. The criteria are described by the Dutch Office of Medicinal Cannabis [6]. For the characterisation of the cannabis and the determination of its quality it is obligatory to determine concentrations of CBD, THC and CBN [6]. For this reason there is a need for an analytical method to detect different cannabinoids in one single run with high selectivity. The expected concentrations for the cannabinoids in dry cannabis material are for THC and CBD at least 100 g/kg and 1 g/kg respectively and for CBN <10 g/kg. During smoking (heating) the carboxylic acids of THC and CBD are decarboxylated and inhaled as THC and CBD. Preferably, both, the concentrations of THC and CBD and of their carboxyl acids have to be determined.

Slijkhuis et al. [6] describe a liquid chromatographic (LC) method in combination with UV or diode-array detection

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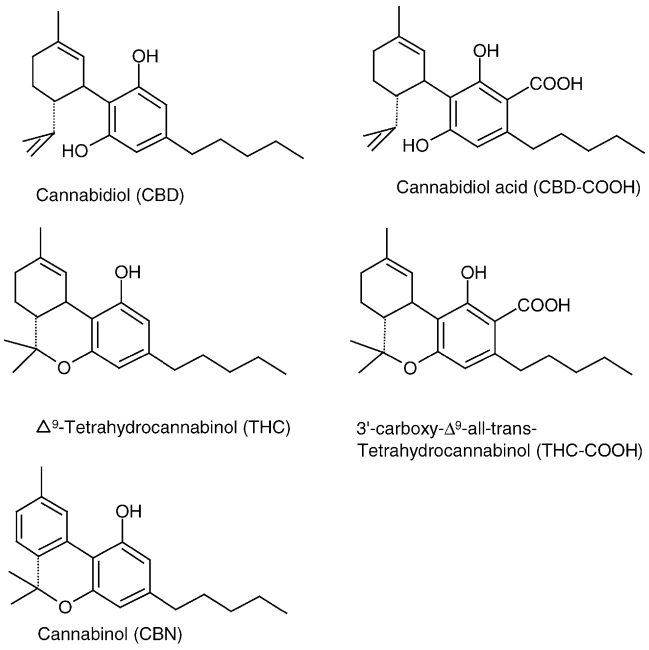


Fig. 1. Structures of some cannabinoids.

(DAD) for the determination of THC, THC-COOH, CBD, CBD-COOH and CBN. Although this method can be used for the quality control of medicinal cannabis the authors concluded that due to the limited selectivity of UV and DAD sometimes the concentration of CBD is overestimated. Ross et al. [7] described the determination of THC using gas chromatography in combination with mass spectrometry (GC–MS). GC–MS has the advantage of selective MS detection but due to the injector temperature (>100 °C) of the GC the acids of THC and CBD are decarboxylated, consequently only the THC and CBD are detected and not their corresponding carboxylic acids. Segura et al. [4] published a review describing several procedures for the analysis of THC and its metabolites in blood, hair and urine. All procedures are based on a derivatisation with, e.g. BSTFA, HFBA in combination with GC–MS analysis. Bacigalupo et al. [8] used a time-resolved fluoroimmunoassay for the detection of the same cannabinols. Szabady et al. [9] published a separation method for neutral cannabinoids in hemp samples using overpressured-layer chromatography. This procedure is of special interest for rapid quantitative screening of different types of hemp sample.

Several studies demonstrate the feasibility of LC–MS and LC–MS–MS for the determination of cannabinoids in biological fluids [10,11]. To our knowledge no LC–MS methods are published for the determination of cannabinoids in cannabis products. The use of LC–MS for the determination of cannabinoids in cannabis products combines the advantages of LC–UV(DAD) and GC–MS. The use of LC makes the derivatisation step, necessary for GC analysis, superfluous, makes it possible to detect the carboxyl acids and the selective MS technique enables the combination of quantification and confirmation of cannabinoids in one single method.

This study reports on the development and validation of a method for the quantification and confirmation of CBD, CBD-COOH, THC, THC-COOH and CBN in cannabis products by LC–IT-MS^n. At this moment there are no specific confirmation criteria for the cannabinoids in cannabis products for that reason the confirmation criteria were used as described by the EU Commission Decision 2002/657/EC [12] for the LC–MS–MS confirmation analysis of veterinary drugs and growth promoting agents.

2. Materials

2.1. Chemicals and reagents

Standards of CBD, CBD-COOH, CBN, THC and THC-COOH were obtained from Leiden-Amsterdam Center for Drug Research (LACDR) (Leiden, The Netherlands). Ammonium acetate was obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands), chloroform, methanol and formic acid were obtained from Merck (Amsterdam, The Netherlands).

2.2. Solutions

HPLC-standard solution containing 1 μg/ml CBN and CBD, 150 μg/ml THC and 990 μg/ml THC-COOH in methanol was stored at −18 °C for a maximum of 1 year.

2.3. Samples

Hop pellets and cannabis were used as sample material and obtained from the Institute of Medical Marijuana (Rotterdam, The Netherlands). Hop is a biologically related species to cannabis and was used as a blank matrix in order to dilute the cannabis samples to obtain an appropriate concentration of cannabinoids for the quality control (QC) samples used for method validation.

2.4. Quality control samples

QCs – containing a subselection of cannabinoids – were used to determine repeatability, reproducibility, and accuracy of the method. The QCs were prepared using cannabis legally available in The Netherlands.

QC1: grinded hop pellets were spiked with CBD at 0.1 g/kg, CBN at 0.08 g/kg, THC at 0.81 g/kg and THC-COOH at 0.56 g/kg.
QC2: grinded hop pellets were mixed with cannabis (9:1, m/m) containing CBD-COOH at 0.04 g/kg, THC at 0.28 g/kg and THC-COOH at 9.2 g/kg.
QC3: cannabis with CBD-COOH at 0.3 g/kg, CBN at 0.03 g/kg, THC at 3.3 g/kg and THC-COOH at 108 g/kg.

The concentrations of QC2 and QC3 were determined by the developed method.

2.5. Sample storage

QCs were stored at less than −18 °C for a maximum of 5 years. Normally, samples can be stored for short period of time (<4 weeks) at room temperature; nevertheless quality control samples were stored at lower temperature because at longer time-intervals the concentration CBN will increase as a result of sample deterioration.

2.6. Equipment

For analysis a LC–IT-MS^n system, LCQ-Classic from ThermoFinnigan (Breda, The Netherlands) equipped with the ThermoFinnigan APCI interface in the (+)-mode and an Alliance (Waters, Chromatography, Etten-Leur, The Netherlands) pump and autosampler were used. Separations were obtained at 30 °C using a C18 LC-column, Hypersil BDS, 150 mm × 2.1 mm, 3 μm particles. The step gradient used (solvent A, 10 mM ammonium acetate and 0.2% formic acid (v/v) in methanol; solvent B, 10 mM ammonium acetate and 0.2% formic acid (v/v) in water; flow 0.2 ml/min) was: 0–0.5 min: 60% A; 0.5–1.0 min linear increase to 80% A; 21–21.5 min linear increase to 95% A, with final hold of 95% A for 7 min; 28.529.0 min linear decrease to 60% A, with a 6 min stabilisation time. Injection volume was 10 μl and the temperature of the autosampler was set at 10 °C. MS acquisition parameters were: APCI(+) ionisation mode; corona discharge 5 μA; capillary temperature 225 °C; vaporiser temperature 225 °C; sheath gas nitrogen flow 20 l/h; divert valve 0–10 min to waste, 10–34.5 min to source.

Acquisition parameters were optimised by 0.2 ml/min infusion of 200 μg/ml CBD in methanol.

3. Methods

3.1. Standard materials

Standard materials as well as cannabis samples are regulated according to the Dutch Opium Law. This means that the use of these materials needs to be registered and a mass-balance should be available at all times. Purity of the standards was checked (qualitative and quantitative) by NMR.

3.2. Extraction procedure

To 0.5 g of grinded sample 100 ml of a methanol–chloroform (9:1, v/v) mixture was added. To extract the analytes the sample was shaken for 30 min and placed in an ultrasonic water bath at ambient temperature for 3 min. After 30 min an aliquot of the clarified extract was injected into the LC–APCI(+)IT-MS^n.

3.3. Quantification

The final extract was analysed using LC–APCI(+)IT-MS^n. Calibration curves for the cannabinoids were constructed by the injection of six standard solutions from two stock solutions. To check the linearity, calibration curves were measured on six different days. Calibration curves were constructed by plotting the ratios of heights of the specific MS–MS ions of analyte/external standard analogous against the concentration. For the samples of cannabis the same ratio was calculated and by using linear regression method the concentrations of cannabinoids were estimated. Calibration samples, blank samples of hop fortified with standards or hop/cannabis samples and external standard solutions at concentrations corresponding to levels between 0.04 and 108 g/kg were analysed. See Table 1 for the specific ions monitored.

3.4. Confirmation

The identities of the cannabinoids detected in the cannabis products were confirmed by applying the EU criteria for the LC–MS–MS analysis. The ion ratio between the abundances of the two diagnostic fragment ions was calculated and compared with the ratio obtained for the reference standard or QC. The ratio in the sample has to be within a specific tolerance interval defined by the EU [12]. Table 1 presents the analytes in combination with the parent ion and the corresponding fragment ions. Furthermore the LC (relative) retention time of the cannabinoid has to correspond to that of the reference with a tolerance of ±2.5%.

3.5. Method validation

For method validation the repeatability, reproducibility, accuracy and LOQs were established and the qualitative parameters specificity and robustness were checked.

Table 1
LC–APCI(+)IT-MS^n acquisition parameters

Analyte	t_R (min)	Parent ion [M(—COOH) + H]$^+$ (m/z)	MS–MS ions for quantification[a] (m/z)	Coll. energy (%)
CBD	13.5	315	193, 259	38
CBD-COOH	14.9	315	193, 259	38
CBN	19.5	311	223, 293	54
THC	22.6	315	193, 259	28
THC-COOH	29.7	315	193, 259	38

[a] MS–MS ions were also used for confirmation of the identity.

Table 2
Partial factorial design to investigate the robustness of the LC–IT-MS^n method for cannabinoids

Factor/experiment	1	2	3	4	5	6	7	8
(A) Sample weight (+ = 0.5 g; − = 1.0 g)	+	+	+	+	−	−	−	−
(B) Extraction volume (+ = 100 ml; − = 75 ml)	+	+	−	−	+	+	−	−
(C) Extraction time (+ = 30 min; − = 15 min)	+	−	+	−	+	−	+	−
(D) Extraction fluid (+ = 10%; − = 5% dichloromethane)	+	+	−	−	−	−	+	+
(E) Ultrasonication (+ = 30 min; − = 0 min)	+	−	+	−	−	+	−	+
(F) Injection volume (+ = 10 μl; − = 20 μl)	+	−	−	+	+	−	−	+
(G) Flow (+ = 0.20 ml/min; − = 0.18 ml/min)	+	−	−	+	−	+	+	−
Result	s	t	u	v	w	x	y	z

Repeatability, quantified by the intra-day variation, was determined by analyzing QC1 and QC3 in six-fold.

Reproducibility, quantified by the inter-day variation, was determined by analyzing QC2 and QC3 in duplicate at six different days by two different technicians.

The accuracy was determined in two ways: (a) blank hop samples were spiked and the average recoveries of the cannabinoids were determined in six-fold on the same day; (b) cannabis was mixed with hop (1:9, m/m); analysed in six-fold on different days and the average recoveries were compared with the concentrations in cannabis (100%).

The extraction efficiency was checked by consecutive extraction of the cannabis product and the second extract was analyzed for residual cannabinoids.

LOQs were determined as the lowest concentration at which repeatable analysis was possible with sufficient recovery.

Robustness was determined by investigating seven critical steps in the analysis procedure according to the Plackett-Burmann schedule given in Table 2.

Effect of factor A;

$$D_A = \tfrac{1}{4}(s + t + u + v - w - x - y - z) \quad (1)$$

and analogous for the other factors mentioned in Table 2.

Estimation of variance:

$$\sigma_{n-1} = \sqrt{\left(\frac{2}{7}\sum (D_i)^2\right)} \quad (2)$$

with $\sum D_i$ the sum of effects of all factors.

Factors are considered significant ($p < 0.05$) if:

$$|D_i| > \sqrt{2\sigma_{n-1}} \quad (3)$$

4. Results and discussion

4.1. Standards

Quality control of the standards by NMR revealed that CBD-COOH was not available at high purity. Therefore the CBD-COOH standard was primarily used qualitatively to determine the retention time and mass spectrum. Quantitative analysis for CBD-COOH was performed by using CBD as standard material with the additional assumption that the response factor CBD/CBD-COOH equals the response factor THC/THC-COOH.

Table 3
LC–APCI(+)IT-MS^n method characteristics

Sample	Analyte	Concentration (g/kg)	Repeatability (n = 6) R.S.D. (%)	Reproducibility (n = 6) R.S.D. (%)	Recovery (%)	LOQ (g/kg)
QC1	CBD	0.1	4	–	93	0.1
	CBD-COOH	<0.04	–[a]	–	–	
	CBN	0.08	1	–	84	0.03
	THC	0.81	2	–	83	0.28
	THC-COOH	0.56	4	–	63	
QC2	CBD	<0.1	–	–	–	
	CBD-COOH	0.04	–	9	81	0.04
	CBN	<0.02	–	–	–	
	THC	0.28	–	7	86	
	THC-COOH	9.2	–	4	85	9.9
QC3	CBD	<0.1	–	–	–	
	CBD-COOH	0.3	5	5	2[b]	
	CBN	0.03	2	13	4[b]	
	THC	3.3	2	5	3[b]	
	THC-COOH	108	2	3	3[b]	

[a] Not determined.
[b] Results of the second extraction.

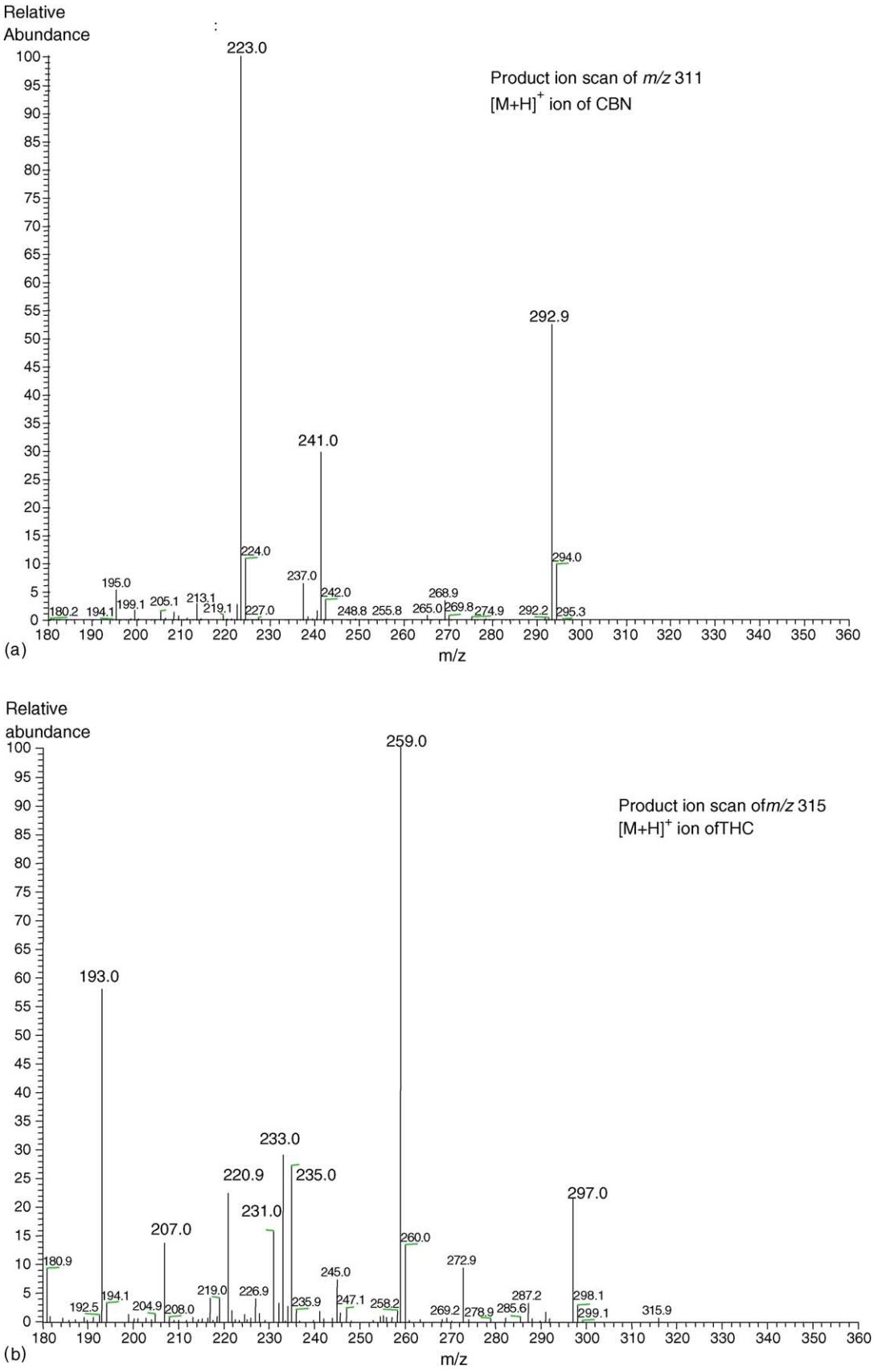


Fig. 2. APCI(+)–MS product ion scans of (a) $[M + H]^+$ ion of CBN; *m/z* 311, t_R = 19.5 min and (b) $[M + H]^+$ of THC; *m/z* 315, t_R = 22.7 min.

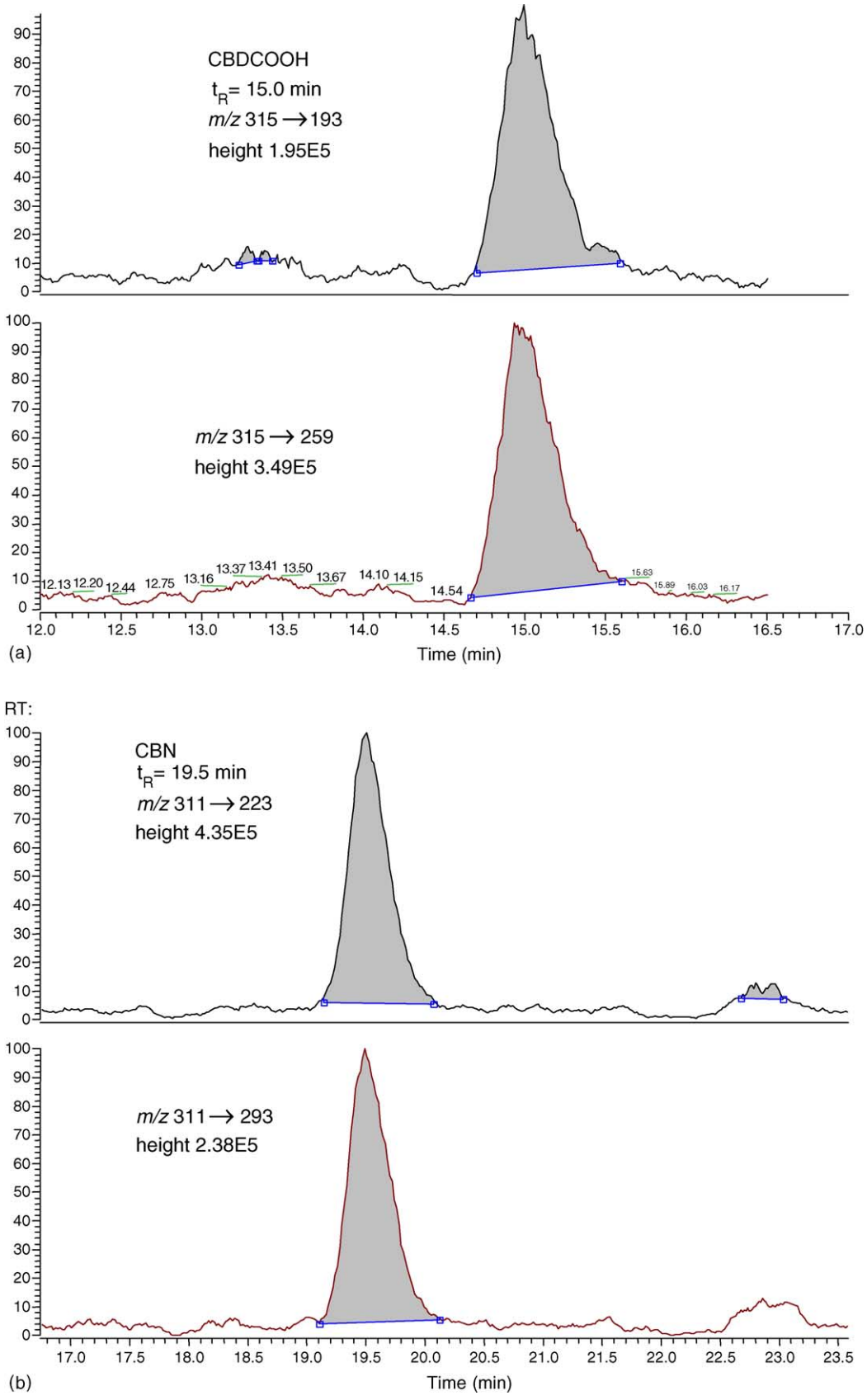
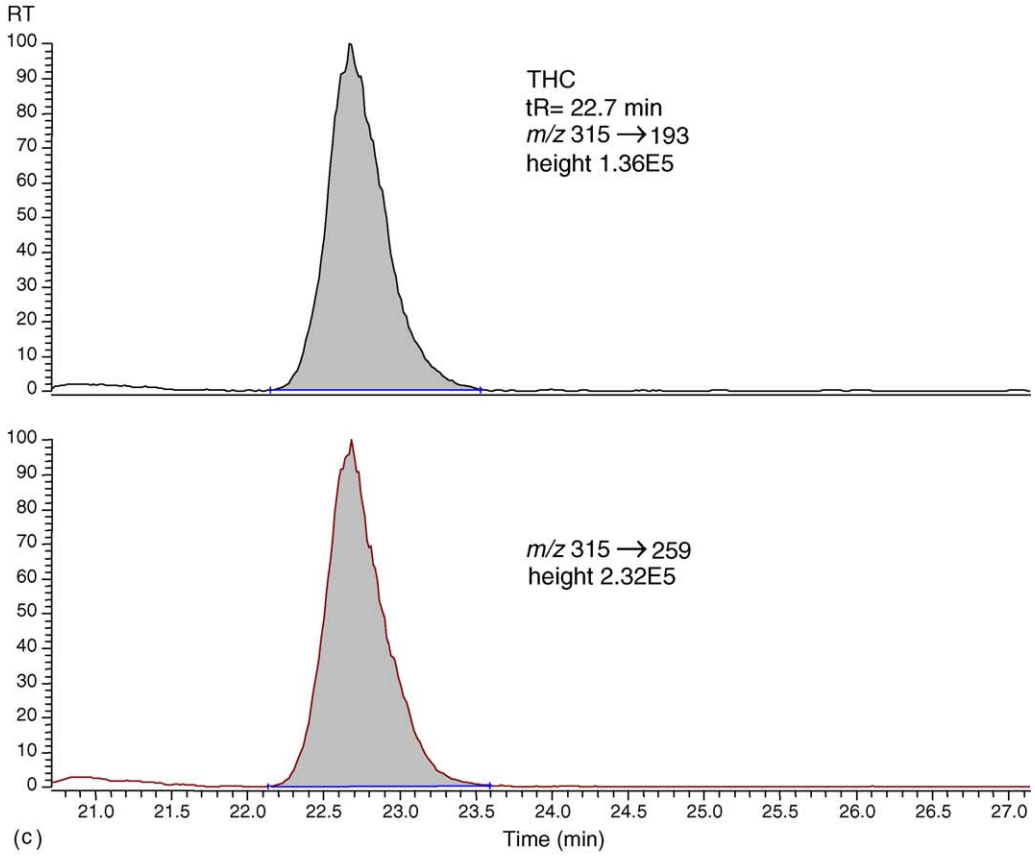


Fig. 3. Illustration of the LC–APCI(+)-MS^2 confirmation analysis of a cannabis product containing (a) CBD-COOH; (b) CBN; (c); THC and (d) THC-COOH. For details, see text.



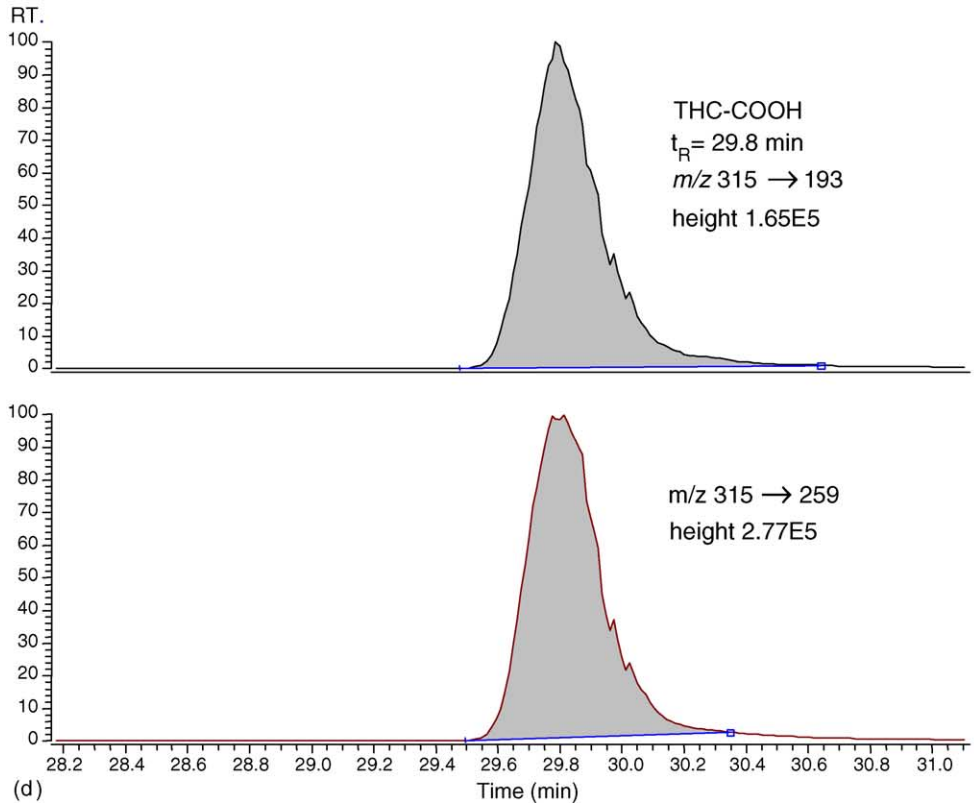


Fig. 3. (*Continued*).

Table 4
Confirmation of the identity of cannabinoids by LC–APCI(+)IT-MS^n

	Sample no.	Concentration (g/kg)	Reference ratio[a]	Tolerances[b] (interval)	Ratio (in sample[c])
CBD-COOH	1	0.3	0.57	20%	**0.52**
	2	0.4		(0.69–0.46)	**0.51**
CBN	1	0.02	0.58	20%	**0.58**
	2	0.01		(0.70–0.47)	**0.64**
THC	1	3.1	0.57	20%	**0.59**
	2	1.4		(0.69–0.46)	**0.58**
THC-COOH	1	118	0.55	20%	**0.58**
	2	130		(0.66–0.44)	**0.61**

[a] Abundance (MS–MS ion/most intense MS–MS ion) of standards, except for CBD-COOH: QC3 is the reference.
[b] According to EU criteria [12].
[c] Bold: result confirmed.

4.2. Sample preparation

The sample extraction was a very simple liquid phase extraction (LPE) with a methanol–chloroform mixture [7]. This approach has the advantage that extension of the method with new cannabinoids is relatively simple. If the cannabinoid is soluble it can be included in the quantitative LC–MS method and the identity is determined by the specific ions of the product ion spectra.

4.3. Quantification

A Hypersil BDS C18 RPLC column and methanol–water containing ammonium acetate and formic acid as the eluent provided adequate retention. The retentions obtained for CBD, CBD-COOH, CBN, THC and THC-COOH were 13.5, 14.9, 19.5, 22.6 and 29.7 min, respectively.

CBD-COOH and THC-COOH were detected as CBD and THC, respectively. Consequently the diagnostic MS–MS ions of CBD-COOH and THC-COOH were the same as those obtained for CBD and THC. However, based on the specific LC retention times the carboxylic acids were distinguished from CBD and THC and individually quantified.

For all calibrations, the regression resulted in a correlation coefficient above 0.990. The upper limits of quantification were 0.3, 1.3, 0.2, 30 and 200 g/kg for CBD, CBD-COOH, CBN, THC and THC-COOH respectively. In Table 3 the method characteristics are presented. The R.S.D. of the repeatability and reproducibility of the method were <10% for all compounds, the only exception was the reproducibility of CBN at 0.03 g/kg with a reproducibility of 13%. CBN is a marker component for cannabis freshness. Reproducibility is estimated to be sufficiently accurate to make a distinction between fresh cannabis and old cannabis. The repeatability results are the same as those presented by Zoller et al. [13] they use methanol–dichloromethane (9:1, v/v) for the extraction of THC from herbal hemp and use RPLC with UV_{210} nm or fluorescence (FLD) (210/305, emm./ex.) nm for detection. The R.S.D. obtained at 0.4 g/kg were 8.5% for UV and 8.3% for FLD ($n = 4$). The new multi-analyte method shows, at the concentration level of 0.3 g/kg, a R.S.D. of 7% for the same compound.

The accuracy was determined as recovery from spiked samples (repeatability), from hop mixed with cannabis where the recovery is quantified as percentage of the cannabis extract. Results for accuracy are given in Table 3 in the *recovery* column. All recoveries were >80% with the exception of THC-COOH which showed a recovery of 63%.

For the second extraction all recovery results were <5%. These results are very satisfactory for this type of analysis.

4.4. Confirmation

To select the most abundant diagnostic ions the product ion spectra of the cannabinoids were monitored. Fig. 2 shows two representative product ion spectra of the cannabinoids CBN and THC. Although the carboxylic acids, CBD-COOH and THC-COOH were detected as CBD and THC and consequently showed the same MS–MS product ions they were identified by their specific LC retention times. Futhermore CBD and THC have the same parent ion, *m/z* 315 and the same diagnostic MS–MS ions, *m/z* 259 and *m/z* 193 but were also separated by LC demonstrating the importance of chromatography in combination with MS for confirmation of cannabinoids.

The identity of the cannabinoid was confirmed by comparing the ion ratio of the two most abundant MS–MS ions of the cannabinoids in the cannabis products with the ratio calculated for the standards or QCs. Table 4 shows the results obtained for two cannabis products – sample nos. 1 and 2 – and references and also demonstrates the use of the EU criteria. From the results it is concluded that the identity of the cannabinoids, CBD-COOH, CBN, THC and THC-COOH in the samples are confirmed because the ion-ratios are within the tolerance intervals even as the LC retention times. Fig. 3 shows a LC–APCI(+)-MS^2 chromatogram of sample no. 1 used for the confirmation of the identity of the cannabinoids. No signals at the relevant retention times were observed when a blank hop sample was analysed.

4.5. Robustness

The robustness test revealed that two factors were found to influence the final results significantly: (1) for THC-COOH the weight was a significant factor; increase in recovery (20%) was observed if 0.5 g of sample was used instead of 1.0 g; (2) for CBD-COOH and CBN the flow rate of the HPLC was a significant factor, resulting in lower recovery results for a reduced flow rate (0.18 ml/min) of the HPLC eluent. Therefore, in the final operation procedure the sample weight should be 0.5 g and the eluent flow rate should be tuned at 0.2 ml/min.

5. Conclusions

The use of the LC–IT-MS^n makes it possible to combine the quantification and confirmation of the cannabinoids in cannabis products in one single method. With this method simultaneous analysis of CBD, CBD-COOH, CBN, THC and THC-COOH at a broad concentration range from 0.03 to 200 g/kg – depending on the specific cannabinoid – is possible fulfilling the requirements of the Dutch Office of Medicinal Cannabis for quality control of cannabis products. Involving a simple LPE as sample pre-treatment and LC–MS a sample throughput of approximately 30 samples per day is obtained.

The quantitative validation results of this method are very satisfactory and the robustness of the method is also very good. The LC retention time in combination with the specific product ions enable unambiguous confirmation of the identity of the cannabinoid. An additional advantage of the method is that extension of the application field of the method with new cannabinoids or a new type of cannabis is relatively easy, due to the simple extraction and the selective detection and confirmation approach used.

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