Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 852 (2007) 459–464
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous identification of 2-carboxy-tetrahydrocannabinol, tetrahydrocannabinol, cannabinol and cannabidiol in oral fluid

Christine Moore *, Sumandeep Rana, Cynthia Coulter

Toxicology Research and Development, Immunalysis Corporation, 829 Towne Center Drive, Pomona, CA 91767, USA

Received 27 September 2006; accepted 4 February 2007
Available online 15 February 2007

Abstract

Tetrahydrocannabinol (THC) is an important psychoactive ingredient in marijuana, which is the most widely used illegal recreational drug in the USA. Since it is generally smoked, the constituents of the plant material, as well as THC may be present in oral fluid specimens collected for the purposes of drug testing. We present an analytical procedure for the simultaneous determination of the pyrolytic precursor Δ^9-tetrahydrocannabinolic acid A, tetrahydrocannabinol, cannabinol and cannabidiol in human oral fluid specimens using gas chromatography mass spectrometry (GC/MS). Solid phase extraction and GC/MS/EI with selected ion monitoring were used, and the linearity of the method ranged from 0–16 ng/mL of neat oral fluid. The recovery of the cannabinoids from the collection pad into the transportation buffer was greater than 70% for all cannabinoids tested at 4 ng/mL, and the intra- and inter-day precision was less than 10.3 and 15.2% for all analytes. The stability of the drugs in oral fluid and of the extracted derivatives was investigated. The procedure was applied to oral fluid specimens taken from habitual marijuana smokers. We have previously reported the presence of the metabolite 11-nor-Δ^9-tetra-hydrocannabinol-9-carboxylic acid in oral fluid, but this is the first report of the plant constituent 2-carboxy-THC being detected in saliva.

Keywords: Oral fluid; Tetrahydrocannabinol; Cannabinol; Cannabidiol; 2-Carboxy-THC

1. Introduction

The marijuana plant is a complex material containing over 400 different chemical compounds including more than 50 different cannabinoids. The primary psychoactive constituent of marijuana, the most widely used illegal drug in the USA, is tetrahydrocannabinol (THC). Generally, it is administered via smoking, resulting in increased heart-rate at low doses; euphoria and hallucinations at higher doses. Reports have shown that some marijuana effects may be due to THC (Fig. 1(i)) in combination with other constituents of the plant, such as cannabinol (CBN) (Fig. 1(ii)) and cannabidiol (CBD) (Fig. 1(iii)). Various cannabinoids have been analyzed in plasma, blood and urine [1–3], but their detection in the more esoteric matrices such as sweat, oral fluid and hair has only recently been addressed. Kim et al. [4] report an analytical method for the detection of CBN, CBD and THC in hair, with a detection limit of 6 pg/mg for THC. Other authors have analyzed cannabinoids in hair, using various techniques, such as solid-phase micro-extraction [5] and two-dimensional chromatography [6].

Oral fluid is becoming increasingly popular as a specimen for the detection of drugs at the roadside, and in workplace testing. Several publications have reported the presence of THC in saliva using various collection devices such as the Intercept® [7], Salivette™, stimulated expectoration [8,9], and Quantisal™ [10]. Recently, we reported the presence of the metabolite 11-nor-Δ^9-tetra-hydrocannabinol-9-carboxylic acid (THC-COOH) in oral fluid specimens for the first time [11]. However, the presence of CBN and CBD in the marijuana plant material, and therefore possibly in the oral fluid sample collected, has not been reported previously and may be of importance for screening and confirmatory assays.

In addition, Δ^9-tetrahydrocannabinolic acid A (2-carboxy-THC), is a pyrolytic precursor to tetrahydrocannabinol. 2-Carboxy-THC is present in marijuana plants, accounting for up to 100% of the THC level [12]. The cannabinoid dibenzofuran numbering system and the chemical structure of 2-carboxy-THC are shown in Fig. 2. 2-Carboxy-THC concentrations determined

* Corresponding author. Tel.: +1 909 482 0840; fax: +1 909 482 0850.
E-mail address: cmoore@immunalysis.com (C. Moore).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.02.016

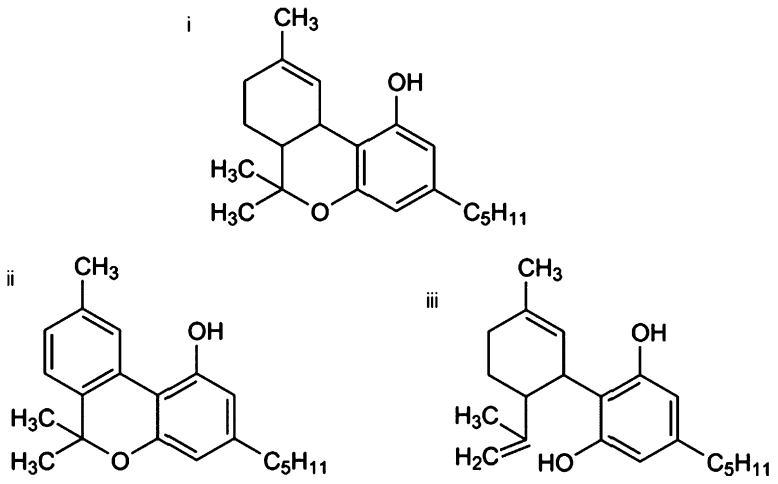

Fig. 1. Cannabinoid chemical structures: (i) tetrahydrocannabinol (THC); (ii) cannabinol (CBN); (iii) cannabidiol (CBD).

by Zoller et al., in various foods containing hemp, showed 0.08 ng/μL in 50 μg of hempseed extract, and 0.09 ng/μL in 2 mg of infused hemp tea [13]. Dussy et al. [14] report that the decarboxylation of 2-carboxy-THC to THC during smoking converts only approximately 70% of the precursor to the active form, so the potential presence of 2-carboxy-THC in oral fluid specimens was considered and included in this project. This report describes for the first time, the simultaneous determination of tetrahydrocannabinol, cannabinol, cannabidiol and 2-carboxy-THC in oral fluid.

2. Experimental

2.1. Collection devices, reagents and standards

Quantisal™ devices for the collection of oral fluid specimens were obtained from Immunalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when 1 mL of oral fluid (±10%) has been collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases where the specimen is positive for more than one drug and the volume of specimen available for analysis may be an issue. The drug concentrations detected were adjusted accordingly.

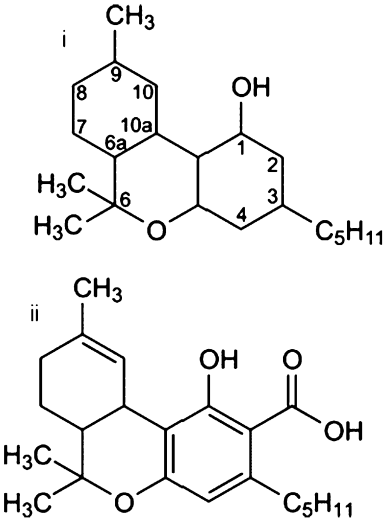

Fig. 2. (i) Cannabinoid numbering system; (ii) structure of Δ^9-tetrahydrocannabinolic acid A (2-carboxy-THC).

Methanol, acetonitrile, toluene, ethyl acetate, hexane, glacial acetic acid and methylene chloride were obtained from Spectrum Chemicals (Gardena, CA). All solvents were HPLC grade or better and all chemicals were ACS grade. The positive pressure extraction manifold and the Trace-N 315 solid phase extraction columns were obtained from SPEWare (San Pedro, CA). The derivatizing agent, N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Pierce (Rockford, IL). Gas chromatographic columns were obtained from J & W Scientific, an Agilent Company (Palo Alto, CA).

The internal standard, tri-deuterated THC (d3) (100 μg/mL in methanol) as well as unlabelled drugs (1 mg/mL in methanol) were obtained from Cerilliant, (Round Rock, TX). 2-Carboxy-THC was purchased from Lipomed (Cambridge, MA).

2.2. Calibrators and controls

The deuterated internal standard (THC-d3) stock solution and the unlabelled drug standards for THC, CBN, CBD and 2-carboxy-THC were prepared in methanol at a concentration of 100 μg/mL. The working solutions were diluted from the stock to a concentration of 10 μg/mL in methanol. The solutions were stored at −20 °C when not in use. Controls were prepared by fortifying drug free synthetic oral fluid with various concentrations of cannabinoids. Drug free negative specimens, positive controls at 1 and 4 ng/mL were included in every batch.

2.3. Sample preparation

Calibrators were prepared in oral fluid at concentrations of 1, 2, 4, 8 and 16 ng/mL for all analytes. Trace-N 315 solid phase

extraction columns were conditioned with methanol (0.5 mL) and 0.1 M glacial acetic acid (0.1 mL). 0.1 M sodium acetate buffer (pH 4.5, 1 mL) was added and the samples were loaded onto the columns and allowed to dry. The columns were washed with deionized water (2 × 3 mL) and allowed to dry for 5 min. The columns were washed with deionized water:glacial acetic acid (80:20 v/v; 1 mL) and deionized water:methanol (40:60 v/v; 1 mL). The columns were allowed to dry at 30 psi for 5 min. The columns were finally rinsed with hexane: glacial acetic acid (98:2 v/v; 0.8 mL) in order to elute the cannabinoids into silanized glass tubes. The entire extraction procedure was carried out using a positive pressure manifold, which allows the flowrate through the columns to be highly uniform. The eluent was evaporated to dryness under nitrogen at 40 °C, and reconstituted in ethyl acetate (30 μL). The derivatizing agent BSTFA + TMCS was added (20 μL) and the vials were heated at 60 °C for 15 min. The samples were injected into the GC/MS system.

2.4. Gas chromatography–mass spectrometry (GC/MS)

An Agilent 6890 gas chromatograph coupled to a 5975 mass selective detector was used for the analysis, operating in electron impact mode. The column was a DB5-MS (15 m × 0.25 mm × 0.25 μm) and the injector was operated in splitless mode at a temperature of 250 °C. The oven ran from 125 °C for 0.5 min to 250 °C at a rate of 40 °C/min where it was held for 1.3 min, then ramped at 70 °C/min to 300 °C. The selected ions monitored were **374** and 389 for the tri-deuterated internal standard (D3-THC); **371**, 386 and 303 for unlabelled THC; **390** and 301 for CBD; **367**, 382 and 310 for CBN; **487**, 488 and 489 for 2-carboxy-THC. The quantitation ions are shown in bold type. The retention time for deuterated THC was 4.27 min; THC 4.28 min; CBD 3.88 min; CBN 4.61 min; and 2-carboxy-THC 5.66 min.

2.5. Data analysis

Calibration using deuterated internal standardization was carried out using linear regression analysis over a concentration range of 0–16 ng/mL. Peak area ratios of target analytes and the internal standard were calculated for each concentration using Agilent MSD software. The data were fit to a linear least squares regression curve, not forced through the origin, and with equal weighting. For confirmation, three ions were monitored for THC, CBN and 2-carboxy-THC and two ion ratios determined, which were required to be within 20% of those of the known calibration standards (calculated at 4 ng/mL) in order to be acceptable. For CBD only two ions were monitored since the third ion showed severe interference from co-eluting substances associated with the oral fluid extract. The ion ratio had to be within ±20% of that established at the calibration point of 4 ng/mL.

2.6. Selectivity

Five drug free oral fluid specimens were collected using the Quantisal™ device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer.

Additionally, to other aliquots of the drug free fluid, common drugs of abuse were added at concentrations of 500 ng/mL. Codeine, morphine, 6-acetylmorphine, amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), pseudoephedrine, phentermine, phencyclidine, cocaine, benzoylecgonine, hydrocodone, propoxyphene, meperidine, tramadol and methadone were all added to drug free oral fluid, extracted and analyzed as described.

2.7. Linearity and sensitivity

The limit of quantitation (LOQ) of the method was defined as the lowest point at which the signal-to-noise ratio (peak height) was at least 5, and the chromatography in terms of peak shape and resolution, retention time (within 2% of calibration standard) and qualifier ion ratio (±20%) compared to the 4 ng/mL calibration standard were acceptable. Since all specimens were to be quantitated, the limit of detection (LOD) was not determined.

2.8. Precision

The precision of the assay at four levels was evaluated. Specimens fortified with all the cannabinoids simultaneously at concentrations of 1, 2, 4 and 8 ng/mL were prepared and six aliquots of each concentration were analyzed according to the described procedure each day (inter-day precision) for four consecutive days (intra-day precision).

2.9. Recovery from the collection pad

Six synthetic oral fluid specimens fortified with all the cannabinoids at a concentration of 4 ng/mL were prepared. The collection pad was placed into the samples until 1 mL (±10%) had been collected, as evidenced by the blue volume adequacy indicator incorporated into the stem of the collector, then the pad was transferred to the Quantisal™ buffer, capped and stored overnight to simulate transportation to the laboratory. The following day, the pads were removed with a serum separator, and an aliquot of the specimen was analyzed as described. The amount recovered from the pad was compared to an absolute concentration (100%) where drug was added to the buffer and left overnight at room temperature without the pad, then subjected to extraction and analysis.

2.10. Stability of the cannabinoids in oral fluid and as derivatized extracts

The stability of THC in oral fluid has been previously reported [10]. The stability of other cannabinoids in oral fluid was determined over 10 days at both room temperature and at 4 °C. Oral fluid samples were fortified with 4 ng/mL of each cannabinoid. The samples were allowed to remain at room temperature and

Table 1
Limits of quantitation and calibration curves for cannabinoids in oral fluid

Analyte	LOQ (ng/mL)	Linear equation*	*Correlation, r^2	Ion ratio* (acceptable range)
THC	0.5	$y = 0.0266x + 0.00273$	0.998	386/371: 69.7–104.5% 303/371: 44.0–66.0%
Cannabinol	0.5	$y = 0.138x + 0.0022$	0.999	382/367: 7.4–11.2% 310/367: 5.7–8.5%
Cannabidiol	1	$y = 0.0271x + 0.00178$	0.998	301/390: 17.1–25.7%
2-Carboxy-THC	1	$y = 0.0571x + 0.0195$	0.998	488/487: 31.7–47.5% 489/487: 11.0–16.6%

* Reported values are the mean of five determinations over 5 days.

Table 2
Inter-day ($n = 4$) and intra-day ($n = 6$) precision for the determination of cannabinoids in oral fluid

Concentration (ng/mL)	THC CV (%)		CBN		CBD		2-Carboxy-THC	
	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
1	0	4.8	5.26	15.3	7.07	6.08	5.73	15.2
2	0	2.53	2.21	2.41	2.82	3.12	10.3	8.3
4	1.39	1.46	5.96	4.20	4.08	4.52	7.03	8.5
8	0.68	1.77	4.66	5.58	1.66	6.84	2.99	2.25

at 4 °C for 10 days. On days 1, 4, 7 and 10 an aliquot was removed and analyzed as described. The stability of the derivatized extracts was also investigated. Autosampler vials, after analysis, were re-capped and stored at 4 °C overnight, before being re-analyzed. The concentration change between the days was noted.

2.11. Application to authentic samples

Specimens were collected from volunteers, who were habitual marijuana smokers using a Quantisal™ oral fluid collection device, and analyzed using the described procedure.

All subjects were male, habitual marijuana users and smoked at least every other day. Samples were collected almost immediately after the subjects smoked, then at various time points including 30 min, 1, 2, 8, 12, 16, 24, 36 and 48 h after smoking. Not all time points were collected for all subjects.

3. Results and discussion

3.1. Method validation

The method was adapted for oral fluid from a published procedure for plasma to include marijuana plant constituents and the pyrolytic precursor to THC, 2-carboxy-THC [1]. The cannabinoids were derivatized before injection into the instrument, to improve chromatographic response, and also to prevent degradation of the 2-carboxy-THC to THC as is the case when marijuana is smoked or heated. The method was validated using drug free oral fluid specimens diluted in 0.1% bovine serum albumin (BSA) buffer, fortified with various concentrations of the analytes, bracketing the concentration range of the proposed Federal cut-off concentration (2 ng/mL) [15]. Specimens from real users with concentrations higher than the range analyzed, were diluted into the linear range of the assay.

No endogenous interference was noted from drug free extracts; or for exogenous interference from any of the commonly encountered drugs, which were analyzed at high concentration, however, there was interference present from the oral fluid buffer extract on the third ion for CBD. Linearity was obtained for all the analytes over the range 1–16 ng/mL and the equations of the calibration curves, inter-day and intra-day precision of the assay, acceptable ion ratio ranges and the limits of quantitation are shown in Tables 1 and 2.

3.2. Extraction from the pad and stability of the analytes

The recovery of the cannabinoids from the collection pad was determined to be 89.2% (CV 9.0%) for THC, 71.9% (CV 19.1%) for CBD, 79.7% (CV 7.8%) for CBN and 78.2% (CV 11.8%) for 2-carboxy-THC, at a concentration of 4 ng/mL ($n = 6$). The stability of the cannabinoids at room temperature over a period of 10 days is presented in Fig. 3. Overall, THC, CBD and 2-carboxy-THC were significantly degraded over 10 days, losing almost 50% of the drug; CBN appeared to be stable. At 4 °C, none of the cannabinoids showed any degradation, indicating

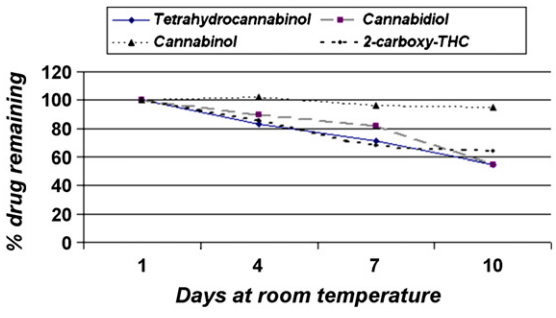

Fig. 3. Degradation of cannabinoids stored in oral fluid collection buffer at room temperature for 10 days.

Table 3a
Stability of cannabinoids in oral fluid as derivatized extracts ($n = 3$) after 24 h

Concentration (ng/mL)	THC		CBN		CBD		2-Carboxy-THC	
	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
1	1.03	5.58	1.03	5.41	1.06	5.41	0.8	12.5
2	2.03	2.83	2.03	2.83	1.93	2.98	1.86	3.09
4	3.96	1.45	3.86	1.49	4.03	1.43	4.06	2.83
8	8.06	1.43	8.16	3.53	8.06	2.58	8.3	8.09

Table 3b
Stability of cannabinoids in oral fluid as derivatized extracts ($n = 3$) after 48 h

Concentration (ng/mL)	THC		CBN		CBD		2-Carboxy-THC	
	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
1	0.96	5.97	1.06	5.41	1.1	9.08	1.06	5.41
2	2.03	2.83	2.16	2.66	1.96	5.87	2.13	5.41
4	3.83	3.01	4.0	2.5	4.06	1.41	4.83	10.2
8	8.06	2.58	8.56	4.5	8.2	2.11	9.6	14.4

that collected specimens should be stored at refrigerated temperatures prior to extraction. The derivatized extracts were stable over 48 h, with a maximum variation of 5.9, 9.08, 5.41 and 14.4% for THC, CBD, CBN and 2-carboxy-THC, respectively (Table 3).

3.3. Authentic specimens

The procedure was applied to specimens collected from habitual marijuana smokers and the results are shown in Table 4 and Fig. 4. In one subject, THC was detectable in oral fluid for 16 h after intake, even in the initial specimen taken directly before smoking; the precursor 2-carboxy-THC was identified up to 8 h after intake. The presence of THC in specimens collected before smoking demonstrates the limitation of the study in terms of self-reported marijuana use. The subjects were habitual smokers, so THC was likely residual from a previous intake. No CBD was detected in any of the samples; CBN cannabinol was measurable for only 2 h. The subject admitted to using marijuana the day prior to the sample collection. An extracted ion chromatogram of the sample collected 2 h after smoking from Subject 1 is presented in Fig. 5. The extracted ions for CBD were not included since there was none present in any of the specimens.

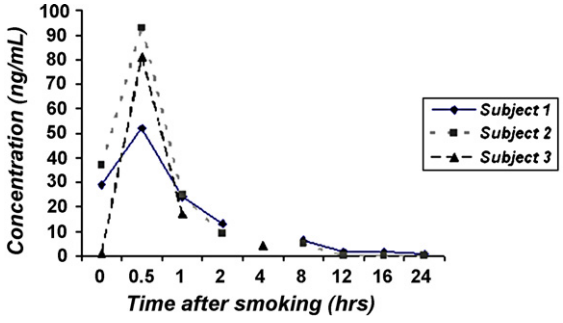

Fig. 4. Concentration of THC, CBN and 2-carboxy-THC detected in oral fluid collected from a marijuana user.

In the other two subjects, THC was detectable for 4 h in one case, but samples after the 4-h time point were not collected in this patient. In the other set of specimens, THC was detectable for 8 h; 2-carboxy-THC diminished rapidly and CBD was not detected in any of the samples.

3.4. Limitations of the study

The application of the Quantisal™ device to the collection of oral fluid for the analysis of cannabinoids has

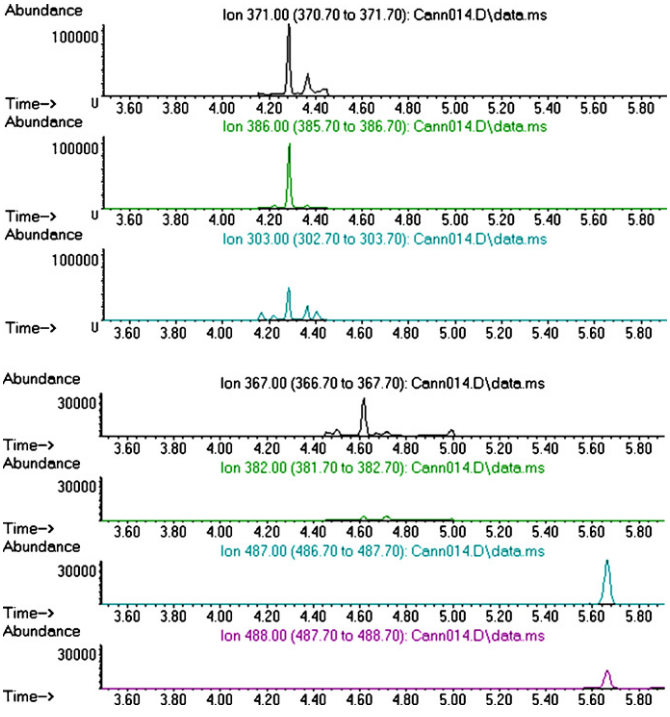

Fig. 5. Extracted ion chromatogram (EIC) of an oral fluid specimen collected from a marijuana user 2 h after smoking. Extracted ions shown: THC 371, 386, 303; CBN 367, 382; 2-carboxy-THC 487, 488.

Table 4
Concentration of cannabinoids detected in oral fluid collected from habitual marijuana smokers after a single smoking session

Time after smoking (h)	THC (ng/mL)			CBN (ng/mL)			2-Carboxy-THC (ng/mL)		
	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
Prior	29	37	1.0	1.3	1.1	ND	ND	ND	ND
0.5	52	93	81	3.6	4.1	ND	18.6	3.1	20
1	24	25	17	1.8	1.2	ND	13.2	3.0	6.0
2	13	9	NC	0.9	ND	NC	4.2	2.1	NC
4	NC	NC	4.2	NC	NC	ND	NC	NC	3.1
8	6.4	5.0	NC	ND	ND	NC	1.0	1.0	NC
12	1.6	ND	NC	ND	ND	NC	ND	ND	NC
16	1.6	ND	NC	ND	ND	NC	ND	ND	NC
24	0.7	ND	NC	ND	ND	NC	ND	ND	NC

ND: not detected; NC: not collected.

been demonstrated, however, the study is limited due to self-reported marijuana smoking. THC was detected in the specimens taken prior to the inhalation experiment, in all three subjects, although the concentration increased markedly after smoking. The method is useful and can be applied in future studies of controlled marijuana ingestion.

4. Conclusions

We report a sensitive, specific method for the simultaneous detection of THC, CBN CBD and 2-carboxy-THC in oral fluid for the first time. The procedure is applicable to the analysis of specimens collected using the Quantisal™ device for the presence of cannabinoids in oral fluid, and will be useful for future research studies on marijuana detection in saliva.

References

[1] T. Nadulski, F. Sporkert, M. Schnelle, A.M. Stadelmann, P. Roser, T. Schefter, F. Pragst, J. Anal. Toxicol. 29 (8) (2005) 782.
[2] B. Maralikova, W. Weinmann, J. Mass Spectrom. 39 (5) (2004) 526.
[3] R.D. Scurlock, G.B. Ohlson, D.K. Worthen, J. Anal. Toxicol. 30 (4) (2006) 262.
[4] J.Y. Kim, S.I. Suh, M.K. In, K.J. Paeng, B.C. Chung, Arch. Pharm. Res. 28 (9) (2005) 1086.
[5] F. Musshoff, H.P. Junker, D.W. Lachenmeier, L. Kroener, B. Madea, J. Anal. Toxicol. 26 (8) (2002) 554.
[6] C. Moore, S. Rana, C. Coulter, F. Feyerherm, H. Prest, J. Anal. Toxicol. 30 (3) (2006) 171.
[7] G.F. Kauert, S. Iwersen-Bergmann, S.W. Toennes, J. Anal. Toxicol. 30 (4) (2006) 274.
[8] H. Teixeira, P. Proenca, A. Verstraete, F. Corte-Real, D.N. Vieira, Forensic Sci. Int. 150 (2–3) (2005) 205.
[9] M.A. Huestis, E.J. Cone, J. Anal. Toxicol. 28 (6) (2004) 394.
[10] C. Moore, M. Vincent, S. Rana, C. Coulter, A. Agrawal, J. Soares, Forensic Sci. Int. 164 (2–3) (2006) 126.
[11] C. Moore, W. Ross, C. Coulter, L. Adams, S. Rana, M. Vincent, J. Soares, J. Anal. Toxicol. 30 (7) (2006) 413.
[12] M.A. ElSohly, A.B. Jones, J. Anal. Toxicol. 19 (1995) 450.
[13] O. Zoller, P. Rhyn, B. Zimmerli, J. Chromatogr. A 872 (2001) 101.
[14] F.E. Dussy, C. Hamberg, M. Luginbuhl, T. Schwerzmann, T.A. Briellmann, Forensic Sci. Int. 149 (2005) 3.
[15] Department of Health and Human Services, Federal Register 69 (71) (2004) 19673.