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Development of an electrospray LC-MS/MS method for quantification of Δ^9-tetrahydrocannabinol and its main metabolite in oral fluid

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A fast and sensitive reference method for quantification of Δ^9-tetrahydrocannabinol (THC) and its main metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in oral fluid is described in this study. Samples were collected using an oral specimen collection device, followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry analysis. Chromatographic separation of the analytes was achieved by gradient elution on a reversed-phase column with subsequent detection by electrospray triple quadrupole mass spectrometry in positive ionization multiple reaction monitoring mode. Quantification was performed by means of deuterated analogues of the analytes as internal standards. Total run time of the assay was 12 min. The method allowed sensitive quantification of both analytes at a limit of quantification of 0.2 ng/ml. This sensitivity is essential for analysis of samples collected with the Intercept Oral Fluid Collection device (OraSure) and an assay for simultaneous quantification of THC and THCCOOH in saliva has not yet been described. The calibration curves for THC and THCCOOH were linear in the range between 0.25 and 8 ng/ml ($r^2 > 0.99$). Ion suppression effects from endogenous or exogenous interferences were investigated using selected model substances (albumin, ascorbic acid, bilirubin, hemoglobin, breath spray, cigarette, chewing gum, chewing tobacco, candy, tooth whitening, and Tums antacid). These substances were chosen because of the high probability of their presence in the collected samples. None of the 11 endogenous model interferences altered the accuracy of analysis, demonstrating good robustness of the method with respect to interferences in common hygiene products, medicine, tobacco and naturally occurring endogenous substances. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: Δ^9-Tetrahydrocannabinol (THC); 11-nor-9-carboxy-Δ^9-Tetrahydro-cannabinol (THCCOOH); oral fluid; SPE; LC-MS/MS

Introduction

The number of traffic accidents caused by drivers under the influence of drugs of abuse is strongly on the rise.[1] This has prompted the DRUID (Driving Under the Influence of Drugs) programme in the United States and the equivalent ROSITA (RoadSIde Testing Assessment) project in Europe to improve traffic safety regulations.[2] The most common drug of abuse in Europe, the United States, and Australia is cannabis. The active component of cannabis is Δ^9-tetrahydrocannabinol (THC), which is primarily metabolized to 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH).

Oral fluid has become a very popular matrix for roadside testing for THC over the past few years because oral fluid can be collected in a simple, non-invasive manner as opposed to blood or urine. There is no need for medical personnel to be present and the samples can be collected under close supervision, thus reducing the risk of sample adulteration and contamination. The legally permissible concentration of THC in neat oral fluid according to the Substance Abuse and Mental Health Services Administration (SAMSHA) is 4 ng/ml.[3] THC permits only a short detection window of ~12 h [4] in oral fluid, reflecting recent consumption of marijuana. The concentration of THC in oral fluid is closely related to the concentration in blood.[4–6] Disadvantages of this matrix are the small sample volumes and low analyte concentrations. Moreover, possible contamination of samples from food or other exogenous or endogenous substances may affect the results of the analyses. Analytical methods to quantify drugs in oral fluid must therefore be highly sensitive. After collection, samples are usually sent to analytical laboratories for initial screening by immunoassay, followed by confirmation *via* chromatography and mass spectrometry (MS) if needed.[7] A number of liquid chromatography (LC) and gas chromatography (GC) separation methods combined with MS have already described in the literature for oral fluid.[8–22] Several of these methods utilize oral fluid sampling by direct expectoration;[23] other methods collect samples using certified collection devices (e.g. Intercept® OraSure, Salivette® Sarstedt, OmniSal® Cozart Biosciences, Saliva-sampler, StatSure Diagnostics).[11,14–16]

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In this study, oral fluid samples were collected with the Intercept Oral Fluid Collection device. This device has been certified by the United States Food and Drug Administration (FDA). It consists of a cotton pad impregnated with citric acid and other salts to increase oral fluid excretion. During sampling, the pad is placed in the mouth of the human subject for several minutes and then transferred to a tube containing buffer solution and preservatives. The dilution factor by the buffer solution is four; the target concentration for THC is therefore set to 1 ng/ml in the sample solution. A few methods in the literature describe quantification of THC,[9,11] THC in combination with other substances,[5,12] or the main metabolite THCCOOH [8,24] from samples collected with the Intercept collection device. Simultaneous measurement of THC and THCCOOH in a single LC-MS assay has not been reported but is important to differentiate between active and passive marijuana consumption.

The aim of the present study was the development of a fast and sensitive method for the simultaneous quantification of THC and THCCOOH in oral fluid using a combination of solid-phase extraction (SPE) and liquid chromatography- tandem mass spectrometry (LC-MS/MS).

Experimental

Reagents and chemicals

Stock solutions of Δ^9-tetrahydrocannabinol (THC), Δ^9-tetrahydrocannabinol-d_3 (THC-d_3), 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol (THCCOOH) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol-d_3 (THCCOOH-d_3) were purchased from LGC Standards (Wesel, Germany) at 0.1 mg/ml in methanol and were stored at 4 °C. LC-MS grade solvents were from Biosolve (Valkenvaard, the Netherlands). Formic acid (98–100%), acetic acid (100%) and sodium acetate were from Merck (Darmstadt, Germany). Negative calibrator oral fluid was provided by OraSure Technologies (Bethlehem, PA, USA); toluene was from Riedel de Häen (Seeltze, Germany), dichloromethylsilane (>99%) from Fluka Analytical (Sigma-Aldrich, Steinheim, Germany) and *n*-hexane from Sigma-Aldrich (Steinheim, Germany). Water was generated using a Millipore Milli-Q-Plus purification system (Eschborn, Germany).

Sample preparation

Silanization of glassware

Because of the high affinity of THC to glass,[21] all glass surfaces were deactivated through silanization. A solution of 5% dichlorodimethylsilane in toluene was filled into the containers and incubated for 1 h at 60 °C. The silanized glassware was then washed twice with methanol and air-dried.

Sample collection and pretreatment

Oral fluid samples were collected with the OraSure Intercept collection device. After centrifugation (3000 *g*) for 10 min, the content of the device was transferred to 15 ml Falcon polypropylene tubes. Four hundred µl of samples were stored at −20 °C in glass vials prior to analysis. Before SPE, 100 µl of internal standard solution at 10 ng/ml was added to the samples.

Sample extraction

For SPE, 1 ml acetonitrile was added to 400 µl of sample. After vortexing, solutions were diluted with 0.1 M acetate buffer at pH 7 to obtain total sample volumes of 5 ml, followed by vortexing. Extraction of samples was performed using Bond Elut Certify II cartridges (200 mg, 10 ml, Varian, Amstelveen, the Netherlands). The cartridges were first conditioned with 3 ml methanol, followed by 3 mL of 0.1 M sodium acetate buffer at pH 7.0 (+ 5% methanol). The samples were subsequently added to the cartridges and rinsed with 3 ml methanol/water 50:50 (*v/v*). After vacuum drying for 1 min, cartridges were eluted with 3 ml *n*-hexane/ethylacetate 75/25 (*v/v*, + 1% acetic acid). Eluates were collected in silanized glass vials and evaporated to dryness at 40 °C under a gentle stream of nitrogen. Dry residues were dissolved in 150 µl of acetonitrile/ water 30/70 (*v/v*) and transferred to injection vials with silanized inserts.

Extraction recovery

Standard solutions of THC and THCCOOH in a concentration range from 0.1 to 10 ng/ml were used for external calibration. For determining the extraction recoveries, six oral fluid blanks were spiked with 1.0 ng/ml THC and THCCOOH without their internal standards. The extraction recovery was calculated by comparing the absolute response (peak area) of an analyte blank spiked with analyte after extraction with that of a sample spiked prior to extraction.

Quantification

Calibration samples were prepared by spiking drug-free oral fluid samples with standard solutions of analytes, obtained by diluting stock solutions with methanol. Five ml of negative calibrator oral fluid (OFD) were spiked with THC and THCCOOH at concentrations between 0.1 and 10 ng/ml. Deuterated internal standards THC-d_3 and THCCOOH-d_3 were spiked to each sample at 2.5 ng/ml. Five hundred µl of each calibration sample was transferred to 15 ml polypropylene tubes and used for SPE.

The compounds were quantified by multiple reaction monitoring (MRM, see below). The calibration curves were constructed using analyte/internal standard peak area ratios for both analytes. Unknown samples were quantified by comparing the ratio of the integrated area of the analyte and the corresponding internal standard to the calibration curve.

Instrumentation

Chromatography

The chromatographic system was a Dionex (Germering, Germany) U3000 binary pump system, column oven and autosampler. Analytes were separated on Waters (Eschborn, Germany) XTerra C_{18} columns (2.1 x 50 mm, 3.5 µm) using gradient elution with water + 0.1% formic acid (eluent A) and acetonitrile + 0.1% formic acid (eluent B) at 300 µl/min. The column temperature was maintained at 30 °C during analysis. An injection volume of 20 µl was used. The gradient started with 40% B and increased to 100% B in 5 min. The final solvent composition was maintained for 2 min, before reconditioning the column at the initial solvent composition for 5 min. The total run time of an analysis was 12 min.

Mass spectrometry

The mass spectrometer was a Thermo Scientific (Bremen, Germany) TSQ Vantage triple quadrupole MS equipped with a heated electrospray ionization (HESI) source. The sprayer voltage was set to 3 kV; capillary and vaporizer temperature were

maintained at 220 °C and 50 °C, respectively; nitrogen (60 psi) was used as sheath, argon (1 mTorr) as collision gas. Analyses were performed in multiple reaction monitoring (MRM) mode. The collision energy (CE) for each compound's MRM transition was optimized: THC, *m/z* 315➔193 (CE, 22 V); internal standard THC-d_3, *m/z* 318➔196 (CE, 22 V). The transitions for THCCOOH were *m/z* 345➔299 (CE, 19 V) and *m/z* 345➔327 (CE, 15 V); internal standard THCCOOH-d_3 *m/z* 348➔302 (CE, 15 V) and *m/z* 348➔330 (CE, 19 V). Total acquisition time was divided into three segments: for the first 4.5 min, only transitions for THCCOOH and the internal standard THCCOOH-d3 were monitored, followed by 5.5 min acquisition of transitions for THC and THC-d_3 in positive ion mode. For the final 2 min, ionization was switched to negative ion mode to eliminate charging effects in the ion optics and quadrupole rod assembly. This procedure has proven to improve precision on subsequent runs.

Method validation

Linearity and LLOQ

Duplicate measurements were performed for each sample. The linearity of the method was investigated by extending the calibration range to 80% of the lowest and 120% of the highest concentration values. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a signal-to-noise ratio ≥ 1:10.

Precision

Intra-day and the inter-day (1 day) precision was determined from six spiked sample solutions (THC and THCCOOH at 1 ng/ml), which were extracted and analyzed in duplicate.

Matrix similarity and stabilities

Analyte free oral fluid was collected with the Intercept device from seven donors and pooled. The donors were in the range of 21–56 years old, both male and female. Samples from this oral fluid pool (OFD) were spiked with THC and THCCOOH in concentrations of 1, 5, and 7.5 ng/ml. The recovery of both analytes in native oral fluid was determined by comparison to a sample of the same concentration prepared in OFD. Three repetitions were carried out for each experiment.

To determine the stability of unprocessed samples, a pool of analyte free oral fluid samples was spiked with THC and THCCOOH at 1 ng/ml and stored in glass, silanized glass and polypropylene tubes at 4 °C, -20 °C, and −80 °C for five days. An aliquot of the sample pool was analyzed immediately and used as reference to determine the recovery for both analytes. The same oral fluid pool was used to investigate the freeze-thaw stability of the samples. Three aliquots with a concentration of 1 ng/ml THC and THCCOOH were frozen at −20 °C in standard glass high performance liquid chromatography (HPLC) vials, and made to undergo freeze-thaw cycles with a storage period of one day.

The stability of processed oral fluid samples was investigated over 48 h. The autosampler of the LC system was maintained at a temperature of 4 °C during this period.

Ruggedness, exogenous and endogenous interferences

Eleven possible exogenous and endogenous interferences were added to oral fluid samples from five different donor subjects (healthy volunteers), which had been spiked with THC and THCCOOH at a concentration of 1 ng/ml, and compared to a reference oral fluid sample, which was collected from a donor who knowingly had not consumed any food during the previous 2 h. As for exogenous interferences, oral fluid samples were collected 15 min after subjects had been administered the substance. The inferences and their concentration levels are summarized in Table 1.

Results and discussion

The aim of the study was to develop a rapid method for quantification of a frequently used drug of abuse, Δ^9-tetrahydrocannabinol (THC) and its major metabolite, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) (Figure 1). Both target molecules are acidic, non-polar compounds, which initially had to be extracted from the complex sample matrix. This sample matrix consisted of a mixture of oral fluid and buffer solution from the collection device. Finally, extracts were separated and analyzed by LC-MS/MS.

Solid-phase extraction

The analytes were extracted from oral fluid samples using a mixed-mode SPE material, composed of a non-polar C_8 phase and a strong anion exchange sorbent (see Experimental section), similar to established procedures in the literature, which usually utilize mixed-mode cartridges containing cation exchange[14,18,23,25] and/or reversed-phase sorbents.[9,13] In our experiments, the chosen combination showed excellent efficiency for simultaneous extraction of THC and its metabolite THCCOOH. The washing step was optimized using mixtures of methanol and H_2O at different concentrations. The optimum methanol content was determined to be 50%, considering both washing efficiency and recovery of the analytes. Recoveries were 56% for THC, and 85% for THCCOOH ($n = 6$). As SPE was performed using cartridges made of polypropylene, this could potentially be the reason for lower recoveries seen for THC as compared to THCCOOH, since THC exhibits high affinity for plastic surfaces.[19]

Mass spectrometry

Multiple reaction monitoring optimization

THC and THCCOOH were ionized by electrospray ionization (ESI) and structure-specific product ions for MRM generated by collision-induced dissociation (CID) in a triple quadrupole

Table 1. Investigated endogenous and exogenous interferences during the analysis of THC and THCCOOH and their concentration levels in saliva.

Endogenous substances	
Albumin	4939 µg/ml
Ascorbic acid	9831 µg/ml
Bilirubin	460 µg/ml
Hemoglobin	1702 µg/ml
Exogenous substances	
Breath spray	
Cigarette	Smoking of one cigarette
Chewing gum	Chewing for 5 min
Chewing tobacco	Chewing for 10 min
Candy	1 piece
Tooth whitening	1 strip
Tums antacid	1 tablet

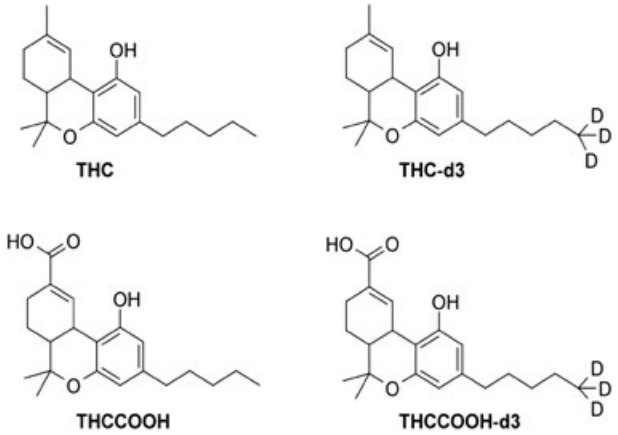

Figure 1. Structure of the analytes THC and THCCOOH and their deuterated internal standards.

instrument. Based on the structure of the analytes, THC was expected to predominately generate positive ions during ESI and THCCOOH was predicted to form negative ions due to the presence of the carboxylic acid group. Surprisingly, signals for THC and THCCOOH were larger in the positive than in the negative ionization mode and both analytes were therefore monitored in the positive ionization mode. Figure 2 shows product ion mass spectra for THC and THCCOOH after CID of the ($[M+H]^+$ precursor ions. The most intense product ion at *m/z* 193 for THC and the two most abundant product ions at *m/z* 327 and 299 for THCCOOH were used for quantification.[26] Two ions were chosen for THCCOOH to increase selectivity as these products ions resulted from less specific small molecule neutral losses as compared to the more specific ion at *m/z* 193 for THC. For the internal standards of THC and THCCOOH, equivalent MRM transitions were chosen (see Experimental section). Typical MRM chromatograms are shown in Figure 3. The polarity of the ESI source was switched to negative ionization mode for 2 min at the end of each analysis to avoid charging effects in the ion optics and rod assembly. This step has been shown to be effective for improving the method's precision. Duplicate measurements of the same sample exhibited much improved accuracy after incorporating this polarity reversal into the instrumental method. This was verified by analyzing a standard solution of THC at a concentration of 1 ng/ml. The coefficient of variation was calculated over ten injections for the same sample with and without polarity-switching. The CV obtained for the method without the switching step was 4.1%, but was reduced to only 2.1% when the 2-min polarity switching was included.

Ion suppression

The specificity of the method and matrix-dependent ion suppression phenomena were assessed by continuous post-column infusion of a standard solution of analyte after injection of a processed blank oral fluid sample. Any increase or decrease of signal intensity during analysis at or near the retention times of the analytes and the internal standards are direct indicators for substances present in the sample matrix directly interfering with the analytes.[27] Importantly, no major ion suppression phenomena were evident for this method indicating that the samples did not contain a significant amount of substances interfering with the quantification of THC and THCCOOH. Importantly, small losses of analyte from suppression by endogenous interferences were compensated by means of isotope standards for the analytes.

Deuterated isotope standards

The d_3-labelled internal standards were chosen because of their commercial availability and affordable prices compared to d_4 or

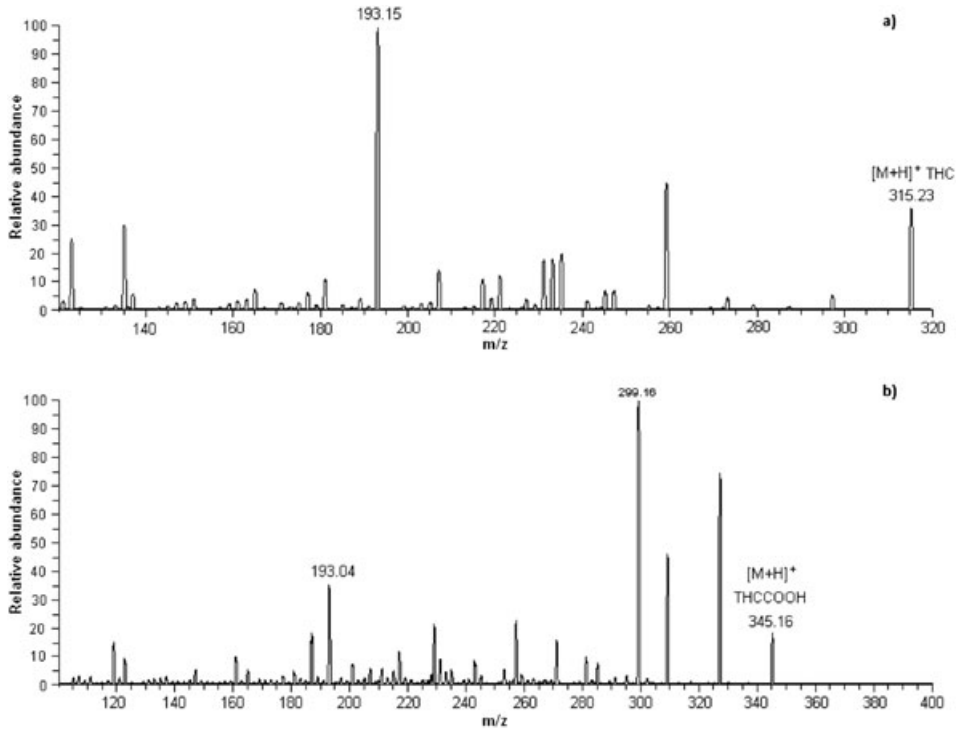

Figure 2. MS/MS spectra of (a) THC ($[M+H]^+$ at *m/z* 315) and (b) THCCOOH (*m/z* 345) standards.

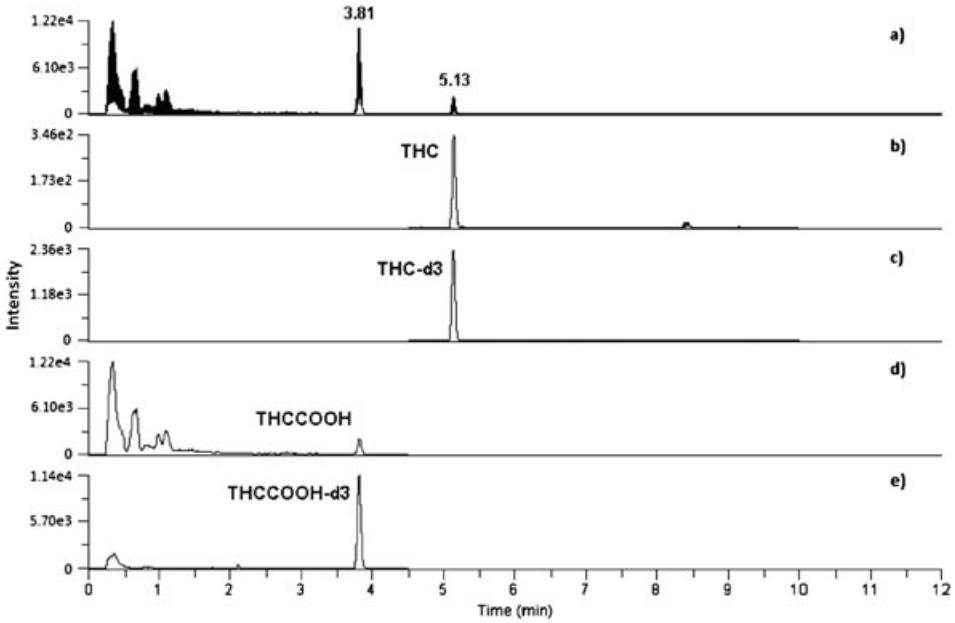

Figure 3. Typical MRM chromatograms of oral fluid spiked with THC and THCCOOH (0.5 ng/ml): (a) TIC; (b) MRM, *m/z* 315➔193; (c) MRM, 318➔196; (d) MRM, 345➔299 + 345➔327; (e) MRM, 348➔302 + 348➔330.

higher deuterated or ^{13}C species. Moreover, the contribution of the natural isotopic abundance of THC and THCCOOH was taken into account and did not significantly influence the response of the internal standards, even for the higher concentrations of the calibration range. The theoretical ^{13}C contribution of THC to the d_3-THC species at *m/z* 318 is 0.27% of the ion current generated via *m/z* 315. That is, even at the upper end of the calibration curve, the isotopic 'cross-talk' is only ≈ 1% (at 2.5 ng/ml for the internal standard; see Experimental). At the lower end of the studied range, representing typical THC or THCCOOH concentration levels in oral fluid, the isotopic contribution from 'cross-talk' is much smaller, on the order of 0.1% or less.

Method validation

Linearity and LLOQ

The internal calibration curves were shown to be linear over the range of 0.25 to 8 ng/ml (correlation coefficient, $r^2 > 0.99$). The lower limit of quantification (LLOQ) for both analytes (defined as the lowest concentration with a signal-to-noise ratio ≥ 1:10) was determined to be 0.2 ng/ml, similar to other LC-MS methods measuring only THC.[11,14] Importantly, the LLOQ values for both THC and its metabolite THCCOOH were much lower than compared to other methods analyzing more than one analyte.[16,23]

Precision

The intra-day relative standard deviations (RSD) calculated from 12 analyses were 3.5% for THC and 2.8% for THCCOOH. The inter-day precisions were 6% RSD for THC and 3% RSD for THCCOOH.

Matrix similarity and stabilities

To verify that the negative calibrator oral fluid represents a good mimic of real oral fluid samples, analyte free oral fluid was collected with the Intercept device from donors and pooled (see Experimental section). The recoveries for THC and THCCOOH at the three investigated concentration levels were in the range of 95–100%, which demonstrated that the OFD afforded a good substitution for oral fluid.

The stability of the samples was assessed in different containers and at different temperatures over five days. Moreover, freeze-thaw stability was investigated as well as the stability of processed samples in the autosampler over a period of 48 h.

The results for the stability of unprocessed samples are summarized in Figure 4. The measured recovery of THC was lower in plastic tubes than in glass vials, but there were no major differences seen for the three tubes in case of THCCOOH. Storage of samples in plastic containers should therefore generally be avoided. The samples stored at −80 °C showed best recovery. Storage at −20 °C seemed inappropriate, especially for THC. Only a small loss of analyte was observed after storage at 4 °C.

The same oral fluid pool was used to investigate the freeze-thaw stability of the samples. The recovery of THC was 90% after one cycle, 88% after two cycles, and only 84% after three cycles. The recovery of THCCOOH was 90% after one cycle and 89% after the second and third cycle. These results indicate that successive freezing and thawing of oral fluid samples at −20 °C should be avoided.

The stability of processed oral fluid samples was investigated over 48 h at 4 °C and resulted in recoveries for THC and THCCOOH between 90 and 100%.

Ruggedness, exogenous, and endogenous interferences

The possible influence of interferences was studied using oral fluid samples from five different donor subjects. The samples were prepared as described in the Experimental section and compared to a reference oral fluid sample, which was collected from a donor who knowingly had not consumed any food during the previous 2 h. The results of this experiment are illustrated in Figure 5. The measured concentrations of THC were all within 91–100% of the reference value, with the exception of a sample spiked with hemoglobin, for which the value was determined to be only 86% of the reference value. Hemoglobin was added to simulate a serious open wound in the oral cavity. THC most

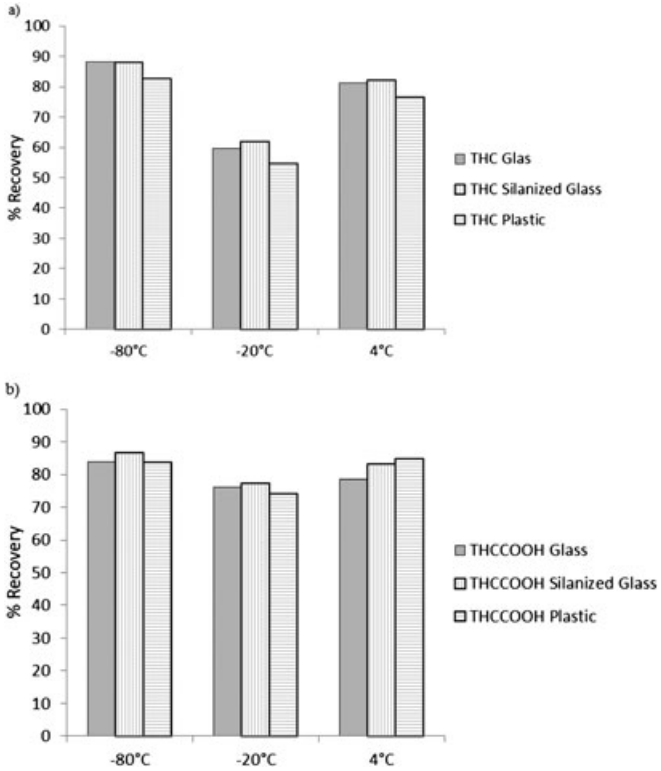

Figure 4. Recoveries for (a) THC and (b) THCCOOH after five days of storage in different containers at different temperatures.

likely binds to hemoglobin as is known for other low molecular weight compounds.[28] A possible explanation of the slightly lower recovery of 86% may involve the different interaction times of native THC with hemoglobin in the sample in comparison to the added internal standard and thus different removal rates from the free solution. Because the exact mechanism and kinetics of this process is not known, it is difficult to give a quantitative interpretation of the loss. For THCCOOH, all measured values were between 97 and 102% of the reference value, justifying the assumption that none of the investigated interferences had a significant influence on quantification of THCCOOH. For the intended use, we considered the recovery rates between 85–100% acceptable.

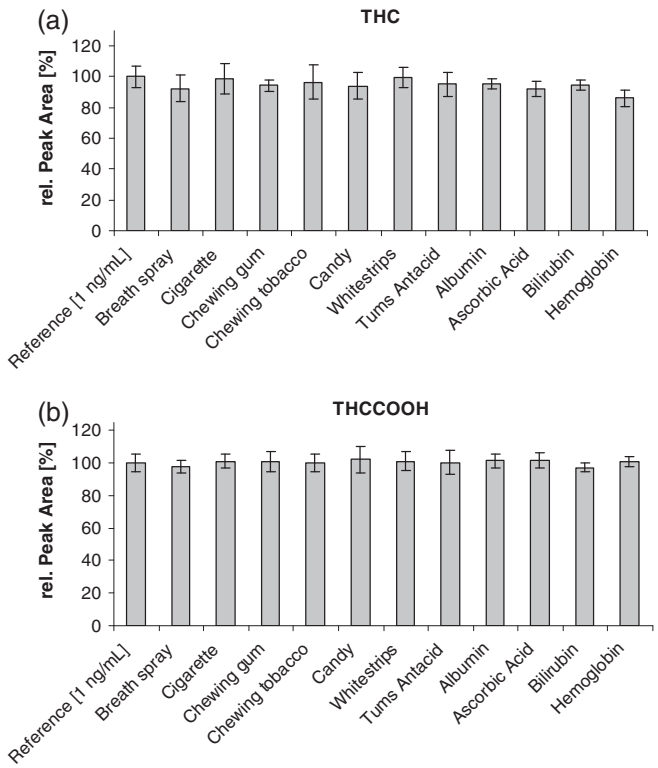

Figure 5. Quantification of THC (a) and THCCOOH (b) in oral fluid samples from six different donors spiked with endogenous and exogenous interferences. Values are shown relative to the reference value (1 ng/ml).

Conclusions

A method for quantifying THC and its main metabolite THCCOOH in oral fluid was developed using the commercial Intercept collection device. Analytes were extracted by SPE, separated by reversed-phase chromatography and quantified by LC-MS/MS using deuterated internal standards. The method was fast and sensitive with intra- and inter-day precisions between 2.5% and 6%; linear over the range 0.25–8.0 ng/ml with a total assay run time of 12 min. The LLOQ values of 0.2 ng/ml for THC and THCCOOH were excellent and lower than values from previous methods, in particular after considering the complex matrix, composed not only of oral fluid but also additional additives from the collection device, such as detergents, preservatives, and colouring agent.

Importantly, the simultaneous quantification of THC and its metabolite provides a useful tool for differentiation of active from passive marijuana consumption. THCCOOH is transferred from blood into oral fluid after metabolic conversion of THC. Thus, it can only be detected if the test person inhaled or ingested cannabis.[13,29]

The method described in this study showed good robustness against possible exogenous and endogenous interferences. To our knowledge, the influence of these interfering substances on quantification of THC and THCCOOH in samples collected with the Intercept device from OraSure has not been investigated before.

Finally, the assay will be used as a reference method for an immunoassay-based drug-screening test in the future.

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