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RESEARCH PAPER

Pitfall in cannabinoid analysis—detection of a previously unrecognized interfering compound in human serum

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Abstract In clinical and forensic toxicology, high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS) is increasingly used since it allows the development of sensitive and fast drug analysis procedures. During development of a LC-MS/MS method for determination of the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (THC) and of its two metabolites 11-hydroxy-THC (THCOH) and 11-nor-9-carboxy-THC (THCCOOH) in serum, a previously unrecognized interfering compound was detected. Extending the fast gradient elution program by an isocratic phase leads to sufficient separation of the interfering compound, initially co-eluting with THCCOOH and exhibiting the same fragments. For characterization, product ion scans and precursor ion scans were performed. Samples from cannabis users were analyzed to estimate the abundance of the interfering compound. The mass spectrometric experiments showed that the interfering compound exhibited the same molecular mass as THCCOOH and a similar fragmentation pattern except for relative fragment intensities. This compound was exclusively detectable in authentic samples. Concentrations were in the range of 4.5 to 51 % (median 14.6 %, n=73) of those of THCCOOH. After further optimization of the gradient, the method was sufficiently selective and sensitive and validation parameters were within acceptance limits. A new compound related to cannabis use was detected in human serum, and data suggest an isomeric structure to THCCOOH. Considering the rather high amounts observed, it was surprising that this compound had not been detected previously. Further studies on its structure and origin are necessary.

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Introduction

Cannabis is the most commonly used illicit drug in the European Union [1] and worldwide [2] and is, therefore, frequently detected in forensic samples. For evidence and evaluation of cannabis intake as well as for pharmacokinetic studies, a number of well-established GC/MS methods have been published [3–8]. Over the last years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is increasingly used as a method of choice in forensic and clinical toxicology since high sensitivity in small sample volumes can be obtained with less effort in sample preparation yielding short turnaround times (c.f. recent reviews [9–11]). This technology has also been applied to the analysis of the main psychoactive cannabinoid Δ^9-tetrahydrocannabinol (THC) of its major psychoactive metabolite 11-hydroxy-THC (THCOH) and the non-psychoactive metabolite 11-nor-9-carboxy-THC (THCCOOH) in various biological matrices such as whole blood [12–17], plasma/serum [18, 19], urine [13, 20], and hair [21, 22]. LC-MS/MS is especially used if polar metabolites such as the non-psychoactive glucuronide of THCCOOH or THC glucuronide are to be assayed.

In our laboratory, we also changed from the GC-MS to the LC-MS/MS method for routine quantification of THC, THCOH, and THCCOOH in human serum. In the last stage of method validation, we observed a shoulder on the THCCOOH peak, exclusively in authentic samples from cannabis users but not in spiked samples (Fig. 1). Therefore, we had to optimize the chromatographic separation for revalidation. As major result, an unknown, but apparently abundant interfering compound in serum of cannabis users, was

identified that has not been detected during the validation process of other reported LC-MS/MS methods.

Materials and methods

Chemicals and reagents

Reference substance of 1.0 g/L Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC (THCOH), 11-nor-9-carboxy-THC (THCCOOH), 0.1 g/L of their deuterated analogs THC-d_3, THCOH-d_3, and THCCOOH-d_3 as well as of 1.0 g/L cannabinol (CBN), cannabidiol (CBD), and 0.1 g/L of 11-nor-Δ^9-THC-9-carboxylic acid glucuronide (THCCOOH-glucuronide) were purchased from LGC Standards GmbH (Wesel, Germany) as methanolic solutions. THC glucuronide (0.01 g/L) was from ElSohly Laboratories, Inc. (Oxford MS, USA). LC grade water was from LGC Promochem (Wesel, Germany) and LC grade acetonitrile from Karl Roth GmbH (Karlsruhe, Germany). All other chemicals or solvents were obtained from Sigma-Aldrich (Munich, Germany) and were of analytical or LC grade. Drug-free serum was obtained from blood provided by the clinic's blood bank and was tested for the absence of drugs of abuse and medical drugs.

Sample preparation and extraction procedure

The extraction of serum samples was performed as described previously [7]. Briefly, aliquots of 0.2 ml serum were diluted with 4.8 ml of deionized water and 50 μl of internal standard solution (a mixture containing 50 μg/L THC-d_3 and THCOH-d_3 and 200 μg/L THCCOOH-d_3 in methanol) was added. After brief mixing, automated solid-phase extraction was performed using the robot GX-274 ASPEC from Gilson Inc. (Middleton, WI, USA) with 3 ml Bakerbond extraction columns (500 mg) from Baker (Griesheim, Germany). The diluted serum samples were slowly loaded onto the extraction cartridges preconditioned with methanol (3 ml) and water (3 ml). After washing with water, 0.25 M acetic acid, and 70 % acetonitrile (1 ml each), the extraction cartridges were dried with air for 15 min. Cannabinoids were eluted with 2 ml of acetone which were evaporated with air to dryness at 25 °C. The dry residue was reconstituted in 100 μl of acetonitrile/methanol/water (6:6:4, *v*/*v*/*v*) of which 5 μl was injected into the LC-MS/MS system.

Calibration standards and quality controls

Calibration standards were prepared from 0.2 ml of drug-free serum by spiking with diluted methanolic working solutions (10 μl) of a mixture of THC, THCOH, and THCCOOH to obtain final concentrations of 1.0, 3.0, 6.0, 12.5, 25.0, and 50.0 μg/L (THC, THCOH) and 3.0, 9.0, 18.0, 37.5, 75.0, and 150.0 μg/L (THCCOOH). Three levels of quality control samples (QC) were prepared from drug-free serum and stored in aliquots of 0.2 ml at −18 °C until use. The concentrations of low, medium, and high QC samples were 2.0, 7.5, and 40.0 μg/L (THC, THCOH) and 6.0, 22.5, and 120.0 μg/L (THCCOOH), respectively. Accuracy was assessed for all analytical series using external serum reference material (Medidrug DOA-I S low, MEDICHEM Diagnostica GmbH & Co. KG, Steinenbronn, Germany). Calibration standards and quality control samples were processed as described above.

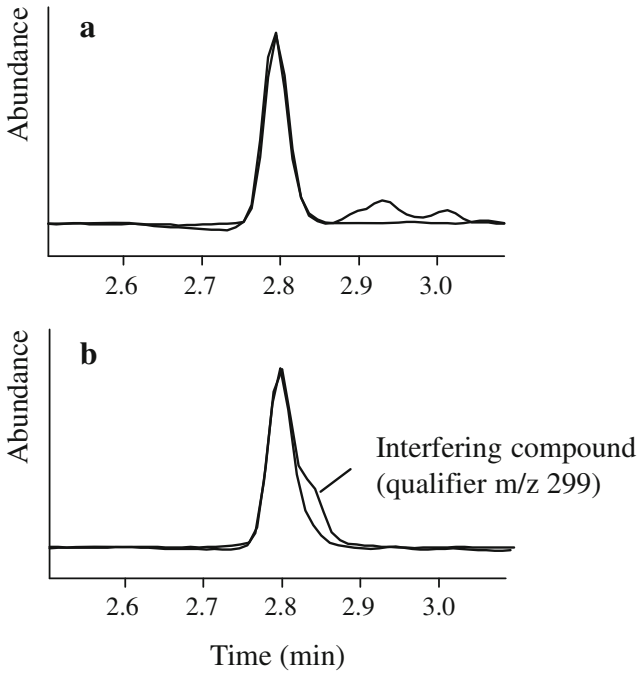

Fig. 1 Overlaid chromatograms of quantifier (*m*/*z* 327) and qualifier (*m*/*z* 299, abundance normalized to quantifier) of THCCOOH in a calibrator (3 μg/L, **a**) and in an authentic sample (**b**) using a short gradient method (starting from 20 % and increasing to 100 % acetonitrile in 3 min) without an initial isocratic phase

LC-MS/MS instrumentation and analytical conditions

For analysis, a LC-MS/MS system from Agilent (Waldbronn, Germany) was used consisting of a 1290 Infinity LC coupled via JetStream electrospray interface (ESI) to a 6460 Triple Quadrupole Mass Spectrometer. Analytes were separated at 50 °C on a Kinetex™ XB-C18, 100 Å, 100×2.1 mm ID column equipped with a guard column (Kinetex™ XB-C18) from Phenomenex (Aschaffenburg, Germany). The mobile phase consisted of 0.01 % formic acid containing 5 mM ammonium formate (A) and acetonitrile containing 0.1 % formic acid (B). The final elution program started isocratic with 50 % B for 1.5 min and increased to 100 % B within 2.5 min, was held for 0.4 min, followed by re-equilibration for 1 min at a flow rate of 0.5 ml/min. Electrospray parameters

were gas flow 13 l/min (250 °C), nebulizer 20 psi, sheath gas flow 12 l/min (400 °C), and capillary voltage 4000 V. The MS/MS was operated in multiple reaction monitoring mode (MRM) with one transition for internal standards and two transitions recorded for each analyte (*m/z*, collision energy in parentheses, quantifier underlined): THC-d_3, 318.2→196.1 (18); THC 315.2→193.1 (28), 315.2→123.0 (40); THCOH-d_3 334.1→316.3 (6); THCOH 331.2→313.2 (16), 331.2→193.1 (32); THCCOOH-d_3 348.1→330.2 (10); THCCOOH 345.1→327.2 (16), 345.1→299.2 (24).

Data evaluation was performed using the Agilent Mass Hunter Software (B 06.01.). For identification, a deviation of ± 0.1 min of the expected retention time compared to calibrators and a quantifier/qualifier ratio within 20 % of the ratio measured in calibrators were required.

Method validation

Validation was performed according to current guidelines [23, 24]. For statistical evaluation, Valistat 2.0 software (Arvecon GmbH, Walldorf, Germany) was used. Selectivity was assessed with six drug-free human serum samples from different sources with and without addition of IS (blank and zero samples). Interference by exogenous substances was assessed by analysis of serum samples spiked with 48 illicit and common therapeutic drugs, their metabolites, and related compounds. Linearity was assessed by analysis of six calibration levels (each $n=6$) ranging from 1.0 to 50.0 μg/L (THC, THCOH) and 3.0 to 150.0 μg/L (THCCOOH). Sensitivity was evaluated in terms of limit of detection (LOD) and lower limit of quantification (LLOQ). Eight evenly spaced calibration levels in a range from 0.2 to 1.6 μg/L (THC, THCOH) and 0.6 to 4.8 μg/L (THCCOOH) were analyzed. LOD and LLOQ were calculated as described in the guideline of the German Institute for Standardization (DIN 32645, c.f. Kolb et al. [25]). For evaluation of precision and accuracy, low, medium, and high QC samples were used. QC samples were analyzed daily in duplicate for 10 days. Mean concentrations and coefficients of variation (CV) were used for the determination of intra-day und inter-day precision and accuracy (bias, deviation of measured from spiked concentration). CVs and a bias below 15 % were regarded as acceptable. Matrix effects and recovery were determined according to the approach described by Matuszewski al. [26] at two levels (2.0 and 8.0 μg/L (THC, THCOH) and 6.0 and 24.0 μg/L (THCCOOH)). The stability of analytes in extracts kept on the autosampler at 5 °C was evaluated by five repeated analyses of QC samples over 5 days. Data on stability during freeze-thaw cycles and storage stability can be found elsewhere [12, 27]. Cross-contamination during automated solid-phase extraction and LC-MS/MS analysis was excluded by analysis of a negative specimen containing the internal standard only in succession to a specimen containing two times the highest calibrator concentration.

Results and discussion

Modern liquid chromatography coupled to triple quadrupole mass spectrometry enables the separation of analytes in short run times with highly sensitive detection by means of monitoring selective fragments. In an early stage of developing an analytical procedure for the assay of the most relevant cannabinoids in serum for forensic purposes, we observed a distinct shoulder on the THCCOOH peak in samples of cannabis users (Fig. 1, no initial isocratic phase in the gradient), but not in either blank or spike samples or in samples of cannabis abstinent persons. Other cannabinoids that may occur in serum (CBD, CBN, THC glucuronide, THCCOOH glucuronide [12, 18]) were tested for interference, but were chromatographically separated from the three target compounds: THC, THCOH, and THCCOOH. Therefore, a rather short gradient elution program was applied to yield short turnaround times where the interfering compound eluted as a shoulder of the THCCOOH peak. The signal exhibited the same MRMs as THCCOOH with a small deviation in relative abundance. As described below, it was identified as an interfering compound in cannabinoid analysis that has not been described previously [14, 16, 28–30] and which we consider a pitfall in validation of cannabinoid assays. This interfering compound was only detected in authentic samples which have not been used for validation; therefore, this highlights the advice to use authentic samples during validation of selectivity. In most cases, this may not be necessary because metabolites are usually separated chromatographically, are of low intensity and can therefore be neglected, or the metabolites present in relevant concentrations are targets of the assay as assumed in the present case.

Characterization of the interfering compound

A complete chromatographic separation of the compound from THCCOOH by 0.5 min was achieved (Fig. 2) by modifying the mobile phase (0.1 % formic acid as solvent A) and by prolonging the gradient program (initial 4.5 min isocratic phase with 55 % B, followed by increase to 100 % B within 1.5 min, held for 0.5 min, with a flow rate of 0.3 ml/min). This enabled the mass spectrometric characterization of the interfering compound.

The substance initially co-eluting at the same retention time of THCCOOH (Fig. 1) exhibited both MRM transitions of THCCOOH (i.e., *m/z* 345.1→327.2 at 16 V CE as quantifier and 345.1→299.2 at 24 V CE as qualifier, c.f. product ion spectra in Fig. 2), but with a different fragment ion ratio (c.f.

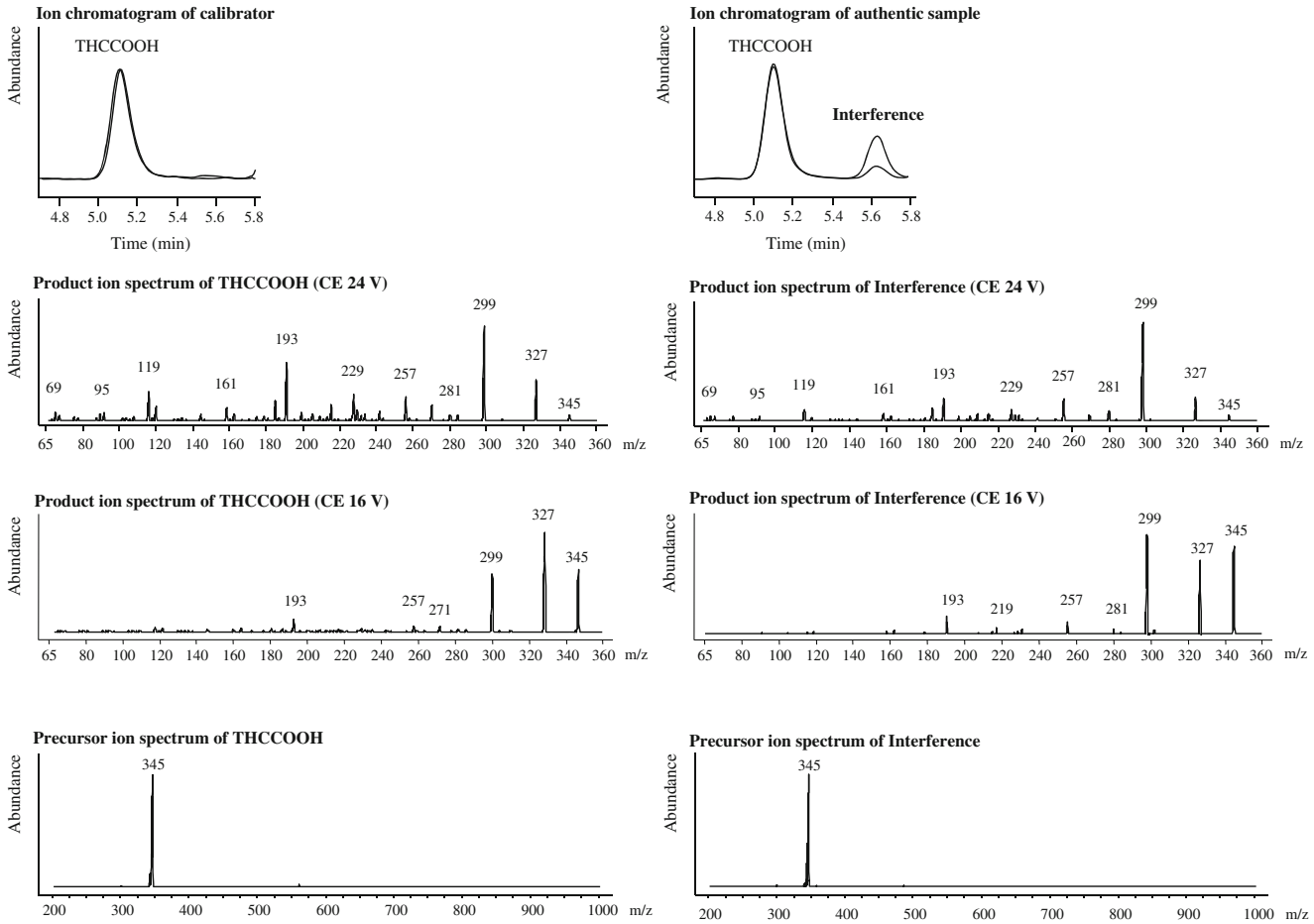

Fig. 2 Overlaid chromatograms of quantifier and qualifier (abundance normalized to quantifier) of THCCOOH in a calibrator (*left*) and an authentic sample (*right*) using an increased initial isocratic phase. The product ion spectra of THCCOOH and of the interfering compound's peaks in a product ion scan experiment and the precursor ion spectra of the two peaks of an precursor ion scan experiment (from fragment *m/z* 327, CE 16 V) are shown below, each from the authentic sample

Figs. 1 and 2). In the prolonged gradient, THCCOOH eluted in extracts of calibrators at 5.11 min and exhibited a qualifier to quantifier ion ratio of 44.1 %, which was also detected in extracted authentic samples. The interfering compound eluted markedly later at 5.63 min exhibiting both MRM transitions, but with a markedly different ion ratio of 184.8 % (c.f. MRM chromatogram in Fig. 2). In order to characterize the nature of this compound, mass spectrometric analyses were performed on spiked and authentic samples to measure the product ion spectra. Complementary precursor ion scans of the MRM fragments *m/z* 327.2 and 299.2 were performed.

In a first attempt, the product ion spectrum of THCCOOH was measured using a product ion scan mode with *m/z* 345.1 as parent, a mass range of *m/z* 65 to 360, and collision energies (CE) of 16 and 24 V. At the lower collision energy, the fragment *m/z* 327 was the base peak, but the higher collision energy yielded a fragment-rich spectrum (Fig. 2), which was used for comparison. The fragments obtained are identical with those described by Bijlsma et al. [31]. The loss of the carboxy moiety is the predominant fragmentation reaction, breaking up ring B or loss of the pentyl side chain are minor reactions. The fragmentation behavior and relative fragment ion intensities of THCCOOH in authentic samples were in agreement with those observed for the THCCOOH reference substance in spiked samples.

The product ion spectrum of the interfering compound (precursor ion *m/z* 345.1, CE 16 and 24 V) was measured in an authentic sample. In the fragment-rich spectrum at higher collision energy (Fig. 2), the predominant ions were *m/z* 299, 327, and 193. Less intense fragments were observed as described for THCCOOH. However, the relative intensity of some fragments was markedly different indicating a structural difference. At lower collision energy, the product ion spectrum of the interfering compound revealed *m/z* 299 as the most intense ion in contrast to *m/z* 327 in the spectrum of THCCOOH. The spectrum of this compound also exhibited a higher intensity of the parent ion *m/z* 345, indicating less fragmentation when compared to THCCOOH. Apart from the different relative fragment ion intensity, the mass spectra of THCCOOH and of the interfering compound were

qualitatively identical. It was therefore not possible to establish selective MRMs for the two substances.

The similar fragment spectra suggest that the interfering compound is isomeric to THCCOOH, which was further investigated using the precursor ion scan mode of the MS/MS. By selecting product ions *m/z* 327 and 299 (CE 16 and 24 V), the mass range from *m/z* 200 to 1000 was examined. At the retention time of THCCOOH and of the interfering compound, only the ion *m/z* 345 could be detected as assumed (data for *m/z* 327 at 16 V in Fig. 2, the other results were identical and are not shown).

The interfering compound was reproducibly but, exclusively, detected in serum extracts of cannabis users, even in cases where only very low concentrations of THCCOOH were present. This compound must therefore be related to cannabinoid ingestion with structural similarity to THCCOOH as concluded from the same molecular mass as THCCOOH and the similar fragmentation patterns. One hypothesis was that the compound is formed by hydrolysis of ring B as, e.g., reported for THC to CBD [32]. However, since the compound is chromatographically separated, it obviously is no artifact of THCCOOH; furthermore, it does not occur in calibration samples spiked with THCCOOH (Fig. 2). At present, we suppose that the interfering compound is an isomer of THCCOOH differing in the position of the double bond in ring A.

Relevance of the interfering compound

The signal intensity of the compound varied among the samples and was found in samples containing high concentrations as well as very low concentrations of THCCOOH. Examples in Fig. 3 (5 and 71 μg/L of THCCOOH) were selected to demonstrate this and also to show the range of the relative intensity of the interfering compound's peak in relation to that of THCCOOH. Assuming that this compound was present, but undetected by LC-MS/MS analysis of other work groups influencing the data reported, assaying its amount was attempted to estimate the potential error in the THCCOOH concentrations.

Forensic blood samples (98) from cannabis users were analyzed applying the extended gradient method. The calibration curve for THCCOOH was prepared using the sum of the two most intense fragment ions (*m/z* 327.2 and 299.2), which was also used for quantification of the interfering compound. It was expected that the sum of the fragment areas would compensate the different fragment intensities. In 4 of the 98 samples, no cannabinoids or the interfering compound was detected; in one sample, THCCOOH was present in a concentration below the LLOQ. Therefore, 93 samples of the study were evaluated. The THCCOOH concentrations ranged from 1.2 up to approximately 474 μg/L with a median of 33 μg/L (13–67 μg/L interquartile range, 9 samples exceeded the upper quantification limit of 150 μg/L). In all these samples, the interfering compound was detected; however, 20 samples were excluded from the evaluation due to low integration quality of levels below 1 μg/L. The concentration ratios of the compound vs. THCCOOH were in the range of 4.5 to 51.0 % (median 14.6 %, interquartile range 11.0–20.0 %, $n=73$). A Spearman's rank correlation coefficient of 0.883 indicates a marked and highly significant ($p<0.001$) correlation of the concentration of this compound with that of THCCOOH.

It is not clear whether this compound represents a metabolite of THC or another cannabinoid present in cannabis products that may only be detectable using electrospray or APCI MS/MS analysis procedures. It is still unknown to what extent the detection of this substance depends on extraction or analysis techniques. However, because of its similarity with THCCOOH, its presence in any cannabinoid extract can be expected. It may be possible that reported THCCOOH levels may have been biased in excess of 10 %. Therefore, it is recommended to verify whether methods that are currently in use are affected. Further experiments to elucidate the structure of the compound are in progress.

Method validation

For routine analysis, an optimized gradient with a short turnaround time (overall 5.4 min from sample to sample) including an initial isocratic phase of 1 min was finally validated. The validation data were well within acceptance limits. No interfering signals were observed for endogenous compounds, other cannabinoids (cannabinol, cannabidiol, THC glucuronide, and THCCOOH glucuronide), or a variety of other drugs typically found in forensic samples. Calibration curves were linear with $1/x$ weighting up to 50 (THC, THCOH) and 150 μg/L (THCCOOH). LLOQs were satisfactorily low (0.3, 0.2, and 0.6 μg/L for THC, THCOH, and THCCOOH, respectively) to allow the application in forensic analyses or in studies on cannabinoid pharmacokinetics. The analytical recovery was >95 % as expected from the experience with the established extraction procedure [7]. Of the three cannabinoids only for THC and only at low concentration (2 μg/L), a matrix effect was observed (40 %), but this was compensated by using the analyte/internal standard ratio. The intra-day and inter-day precision (10 days) was less than 11 % (mostly less than 6 %) and accuracy was less than 5 %. Reanalyses of three QC samples and of the external reference material yielded CVs of the previously determined concentrations of all three analytes of less than 9 % ($n=5$ days), confirming that reliable results are obtained over at least 5 days.

For quantification of THCCOOH in the presence of the interfering compound, the fragment *m/z* 327.2 was preferred as the quantifier ion since it represents the THCCOOH peak better than the fragment *m/z* 299.2. To demonstrate separation

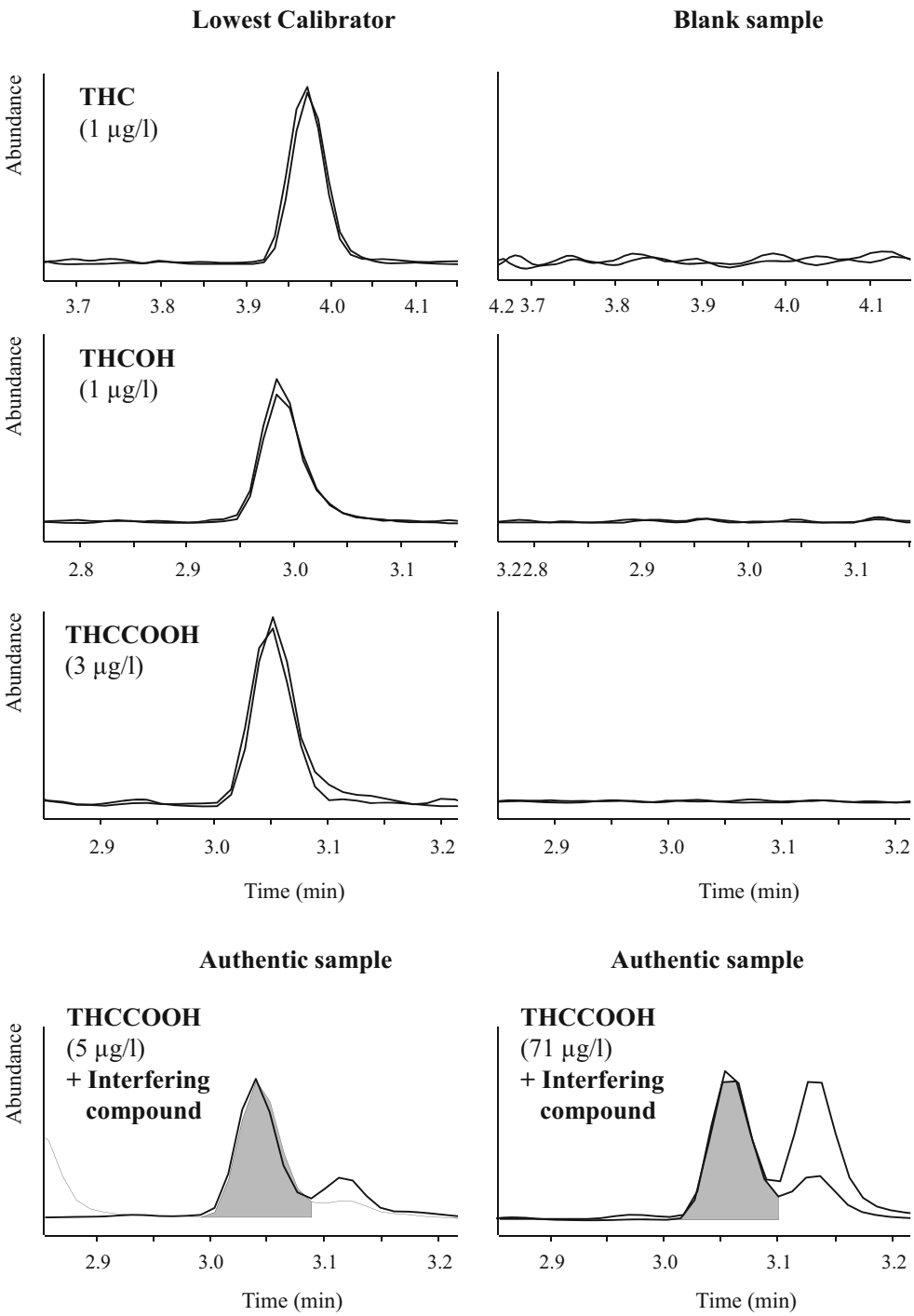

Fig. 3 Overlaid chromatograms of quantifier and qualifier (abundance normalized to quantifier) ions of THC, THCOH, and THCCOOH in the lowest calibrator (*left*) in comparison to those of a blank sample (*right*) using an optimized gradient. The chromatograms of THCCOOH and of the interfering compound are shown below for two typical authentic samples (the integration of the THCCOOH peak is shown in *grey*)

of the compound using an optimized gradient and the reliability of quantification, the 93 samples from routine analysis series containing THCCOOH concentrations above LLOQ were reanalyzed. The results of THCCOOH analysis determined with that gradient method were compared with those obtained with the extended gradient method (see above) and the percentage deviations were calculated. On average, no difference in THCCOOH concentrations between the two chromatographic methods was found (0.01 % mean deviation); the relative standard deviation was only 2.6 %. Therefore, the present analytical procedure is suitable for cannabinoid assays in serum and is not affected by the interfering compound. The reliability of the method has been confirmed by external proficiency tests (www.arvecon.de).

Conclusion

The present study demonstrates that even methods considered to be well established may still cause unexpected problems. During the development of an assay for cannabinoids, one of the most important routine analysis methods in forensic toxicology, we identified a previously unknown substance interfering with the quantification of the major metabolite THCCOOH. Mass spectrometric investigations suggest that this compound is structurally related to THCCOOH where we hypothesize that it may differ in the double bond position in ring A. The interfering compound occurs only, but reproducibly, in samples from cannabis users but has not been detected during validation. This underlines the advice to use authentic samples during validation of the selectivity of new methods.

The estimation of relative concentrations suggests that this compound is present in considerable amounts in a range of 4.5 to 51 % of THCCOOH levels in serum. After optimization of the chromatographic gradient, a selective method was validated. Further studies to elucidate the structure and the origin of this substance are currently in progress.

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