Fully Automated Determination of Cannabinoids in Hair Samples using Headspace Solid-Phase Microextraction and Gas Chromatography–Mass Spectrometry

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Abstract

This paper describes a fully automated procedure using alkaline hydrolysis and headspace solid-phase microextraction (HS-SPME) followed by on-fiber derivatization and gas chromatographic–mass spectrometric (GC–MS) detection of cannabinoids in human hair samples. Ten milligrams of hair was washed with deionized water, petroleum ether, and dichloromethane. After the addition of deuterated internal standards the sample was hydrolyzed with sodium hydroxide and directly submitted to HS-SPME. After absorption of analytes for an on-fiber derivatization procedure the fiber was directly placed into the headspace of a second vial containing *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) before GC–MS analysis. The limit f detection was 0.05 ng/mg for Δ^9-tetrahydrocannabinol (THC), 0.08 ng/mg for cannabidiol (CBD), and 0.14 ng/mg for cannabinol (CBN). Absolute recoveries were in the range between 0.3 and 7.5%. Linearity was proved over a range from 0.1 to 20 ng/mg with coefficients of correlation from 0.998 to 0.999. Validation of the whole procedure revealed excellent results. In comparison with conventional methods of hair analysis this automated HS-SPME-GC–MS procedure is substantially faster. It is easy to perform without use of solvents and with minimal sample quantities, but with the same degree of sensitivity and reproducibility. The applicability was demonstrated by the analysis of 25 hair samples from several forensic cases. The following concentration ranges were determined: THC 0.29–2.20 (mean 1.7) ng/mg, CBN 0.55–4.54 (mean 1.2) ng/mg, and CBD 0.53–18.36 (mean 1.3) ng/mg. 11-nor-Δ^9-Tetrahydrocannabinol-9-carboxylic acid could not be detected with this method.

Introduction

Hair analysis for drug-of-abuse testing has been established as an important instrument in clinical and forensic toxicology (1,2). Various methods have been described for the determination of cannabinoids in hair samples. Gas chromatography coupled with mass spectrometry (GC–MS) appears to be the method of choice (3–12). It has been shown that electron capture derivatives give an enhanced sensitivity in negative ion chemical ionization (NCI) mode (6,7). The highest sensitivity was reached by use of tandem mass spectrometry (MS–MS) (5,11,12).

Besides the parent drug Δ^9-tetrahydrocannabinol (THC) the determination of the main metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) is recommended (13), because the proof of this metabolite in hair samples is considered as evidence of active cannabis use. Three main factors influence the drug incorporation and retention in hair: melanin affinity, lipophilicity, and basicity of the taken substance (14). Normally lipophilic parent drugs are found at higher concentrations in hair samples than their more hydrophilic metabolites. From 20 tested drug compounds, THC-COOH was demonstrated to have the lowest affinity for hair matrix (15). In authentic hair samples THC was detected in concentrations ranging from 0.009 to 9.9 ng/mg, and THC-COOH was found in the range between 0.05 and 5.0 ng/mg (1,2). Even with MS–MS, it was not possible in a lot of cases to detect THC-COOH in the hair of known cannabis users with positive test results for THC (5). There are great differences in THC-COOH concentrations reported from various working groups, some researchers measured concentrations in the low picogram-per-milligram range, others in the low nanogram-per-milligram range. In our own experience, THC-COOH was seldom identified even in THC-positive cases using our previous routine method for hair analysis. However, in addition THC, the presence of cannabinol (CBN) and cannabidiol (CBD), which are normal constituents of cannabis, was demonstrated in hair samples in concentrations between 0.01–1.07 ng/mg (CBN) and 0.03–14.1 ng/mg (CBD) (8,16). First, Cirimele et al. (8) developed a simple rapid and economic method for the simultaneous identification of THC, CBN, and CBD.

Headspace solid-phase microextraction (HS-SPME) is a sampling technique that allows an extraction from small amounts

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of biological material. HS-SPME is based on the partitioning of analytes between the sample, the headspace above the sample, and a coated fused-silica fiber. Analytes are absorbed and concentrated onto the fiber until the three-phase equilibrium is reached. Then the fiber can be directly injected into a GC injection port for thermal desorption (17,18). In contrast to the direct extraction from an aqueous medium (direct immersion, DI-SPME), the headspace technique (HS-SPME) particularly shows a great advantage because of the avoidance of organic solvents, the simple technical performance, and the very low chromatographic background. HS-SPME has been used in hair analysis for the determination of methadone and EDDP (19,20), amphetamines (21,22), lidocaine (23), benzodiazepines and other psychotropic drugs (24). Also cannabinoids have been determined in different matrices by means of SPME. THC, CBD, and CBN have been analyzed so far by direct immersion (DI) in water and human saliva (25) and in hair samples (16). Only Sporkert and Pragst (26) reported on an HS-SPME method for the determination of THC, CBD, and CBN in hair samples, nevertheless with unsatisfactory limits of detection. However, these methods (16,25,26) did not include a derivatization step, which is highly recommended for GC–MS determination of cannabinoids.

Using a multipurpose sampler we have developed a fully automated procedure for the determination of THC, CBN, and CBD in hair samples combining alkaline hydrolysis, HS-SPME with on-fiber derivatization followed by GC–MS. The reliability of the procedure for the analysis of other drugs was also evaluated.

Experimental

Reagents and materials

The following substances were purchased from Promochem (Wesel, Germany): CBD, CBN, THC, and THC-d_3. *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). An SPME device for autosampler with a replaceable 100-µm polydimethylsiloxane (PDMS) fiber was obtained from Supelco (Deisenhofen, Germany). The fiber was conditioned at 250°C for 1 h in the injection port of the GC according to the supplier's instructions. Chemicals were purchased from Merck (Darmstadt, Germany).

Subjects

Hair samples were obtained from deceased subjects with presumed drug abuse during medicolegal autopsy, as well as from persons in cases of driving liability examination directly in our institute. Negative control samples were obtained from staff members. The samples were analyzed within 2–4 weeks.

Hair was collected from the back of the head as close as possible to the skin. The samples were stored under dry conditions at ambient temperature. Before analysis, longer hair samples were cut into 3-cm segments.

GC–MS method

The GC–MS system used for analysis was a model 6890 series Plus GC (Agilent, Waldbronn, Germany) in combination with a CTC-Combi-PAL-Autosampler (Chromtech, Idstein, Germany) and a model 5973 N mass selective detector (MSD). Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation). Substances were separated on a fused-silica capillary column (HP-5MS, 30 m × 0.25-mm i.d., 0.25-µm film thickness). Temperature program: 160°C hold for 1 min, 15°C/min up to 190°C, hold for 10 min, 5°C/min up to 250°C, hold for 3 min, 13°C/min up to 300°C, hold for 3 min. The temperatures for the injection port, ion source, quadrupole, and interface were set at 250°C, 230°C, 150°C, and 280°C, respectively. Splitless injection mode was used and helium with a flow rate of 1.0 mL/min was used as carrier gas.

To determine the retention times and characteristic mass fragments, electron impact (EI) mass spectra of the analytes were recorded by total ion monitoring. For quantitative analysis the chosen diagnostic mass fragments were monitored in the selected ion monitoring (SIM) mode: *m/z* 303, 371, 386 for THC-TMS; *m/z* 301, 337, 390 for CBD-di-TMS; *m/z* 367, 368, 382 for CBN-TMS; and *m/z* 315, 374, 389 for THC-TMS-d_3 as internal standard (target ions are bolded). For quantitation, peak-area ratios of the analytes to the internal standard were calculated as a function of the concentration of the substances.

HS-SPME method

The washing of the hair samples was performed according to a modified procedure of Kauert et al. (9): The samples were subsequently washed for 5 min in 5 mL of deionised water, petroleum ether and finally dichloromethane using a Vortex Genie 2 mixer (Bender & Hobein AG, Zurich, Switzerland). After drying the hair samples were cut into small pieces of about 1 mm. The washing solutions were analyzed by conventional GC–MS procedures to exclude a contamination.

Ten milligrams of hair was submitted to alkaline hydrolysis into a 10-mL headspace vial in the presence of 1 mL of NaOH (1M), 0.5 g of sodium carbonate and 80 µL aqueous internal standard solution (250 ng THC-d_3/mL). The vial was sealed using a silicone/PTFA septum and a magnetic cap and was shaken for 5 min at 90°C in the agitator of the autosampler (650 rpm, agitator on time: 0:05 min, agitator off time: 0:02 min). For absorption the needle of the SPME device containing the extraction fiber was inserted through the septum of the vial, and the fiber was exposed into the headspace of the vial for 25 min. Then for derivatization the fiber was exposed to a second vial containing 25 µL of MSTFA for 8 min at 90°C. The compounds absorbed on the fiber were desorbed by exposing the fiber in the injection port for 5 min and then analyzed.

In order to gain optimal conditions in the sample preparation step, the conditions of hydrolysis, addition of various salts, incubation time and temperature, agitator speed, extraction time, derivatization time and amount of derivatization reagent, desorption time and temperature, depth of fiber insertion into the injection port were determined by testing 3 vials at each temperature and each point. Samples with buffer solutions (phosphate buffer pH 2–10), acids (1M sulfuric acid,

1M hydrochloric acid) or bases (1–10M sodium hydroxide), various additions of salt (0.5 g of ammonium sulfate, sodium carbonate, sodium chloride, or sodium sulfate) containing 2 ng/mg of each analyte were prepared and analyzed as described. Furthermore, spiked hair samples were incubated at different temperatures (60–130°C) for 5 min to determine the optimal incubation temperature. The incubation time was evaluated between 1 and 10 min. The speed of the agitator was varied between 250 and 750 rpm. The absorption times were evaluated between 5 and 45 min, the derivatization times between 1 and 20 min. For the determination of the most efficient desorption time the fiber was exposed to the injection port of the GC for 0.5–10 min, the temperature was varied between 200 and 270°C. Finally the optimal depth of fiber insertion into the injection port was determined by insertion with different depths (46–56 mm).

Table I. Proportional Extraction Yield with Different Salt Additions*

(n = 3)	Without salt†	$(NH_4)_2SO_4$	Na_2CO_3	NaCl	Na_2SO_4
CBD	100	1140	497	144	121
THC	100	117	431	211	98
CBN	100	482	737	502	153

* Sample preparation as indicated above (2 ng/mg of the analytes), in each case 0.5 g salt addition.
† Without salt = 100%.

Spiked samples containing 2 ng of each analyte per milligram of hair, respectively, were prepared and analyzed using the described procedures. For the validation of the method peak purity and selectivity, intra- and interday precision at two different concentrations (0.5 and 20 ng/mg), absolute extraction recovery and sample stability were determined. The linearity of the calibration curve was evaluated between 0.05 and 20 ng/mg. For the determination of the limit of detection (LOD) and the limit of quantitation (LOQ) a separate calibration curve in the range of LOD (0.01–1 ng/mg) was established (27,28). Hair samples (10 mg) spiked with 20 ng of each cannabinoid were analyzed with the HS-SPME procedure and results were compared with a liquid injection of a methanolic solution (20 ng/2 µL) to calculate the absolute recoveries.

Results and Discussion

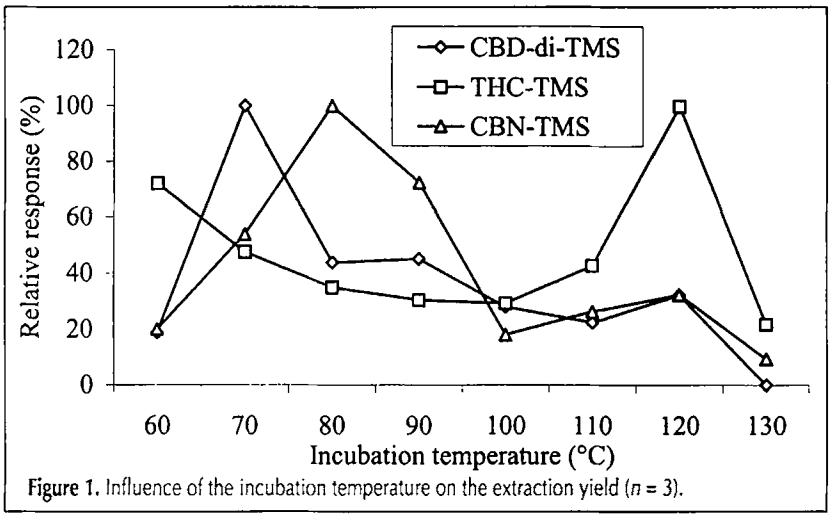

Figure 1. Influence of the incubation temperature on the extraction yield (n = 3).

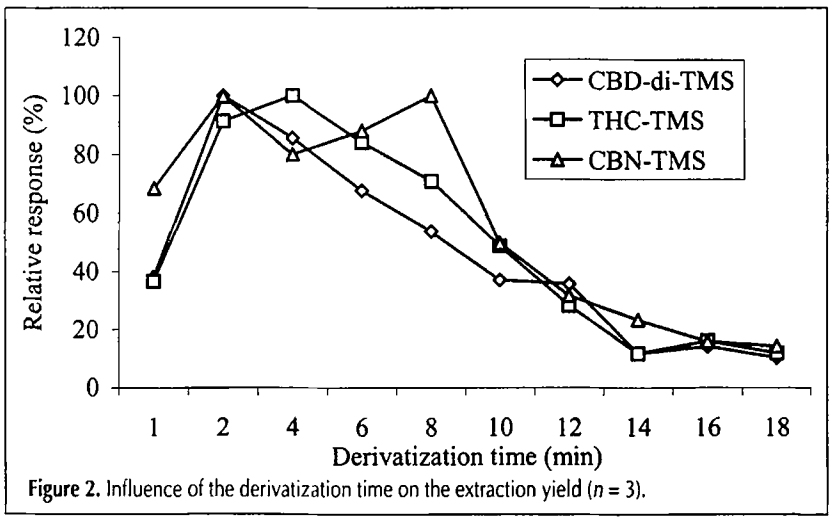

Figure 2. Influence of the derivatization time on the extraction yield (n = 3).

Additions

The fully automated extraction of hair samples either in buffer solutions (phosphate buffer pH 2–10) or after acidic hydrolysis gave none or only very low chromatographic responses, whereas alkaline hydrolysis in the presence of sodium hydroxide gave the highest recovery. The probably occurring deprotonation of phenolic cannabinoids under alkaline conditions had no observable effect on the extraction yields. The influence of different additions of salt on the amounts extracted from the hair samples is shown in Table I. Salting out effects using sodium carbonate optimally increased the sensitivity.

Heating temperature

The incubation of the samples at increased temperatures before the absorption process led to an improvement of sensitivity, because the crossing of the analytes was thereby facilitated from the aqueous into the gaseous phase. With the exception of THC, whose temperature optimum was 120°C, the other analytes showed optimally between 70°C and 80°C. As a compromise, for the procedure a temperature of 90°C was selected. This temperature is also situated below the boiling point of the salt solution because the capillary is contaminated in the piercing area of the vial septum by condensing water simmering at higher temperatures (Figure 1).

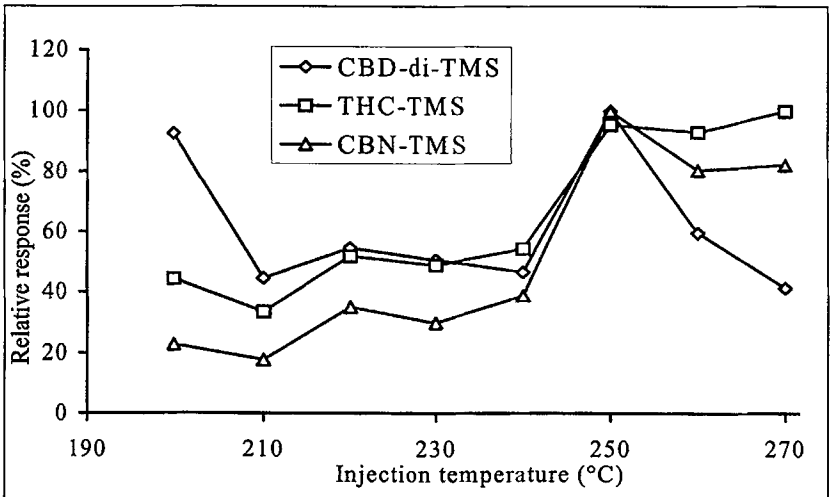

Figure 3. Influence of the injection temperature on the extraction yield (n = 3).

Incubation time

The duration of the incubation of the samples in the agitator before absorption also has a substantial influence on the extraction yield. A duration of 5 min was found to be optimal.

Agitator speed

The optimum was achieved at 600 rounds per minute.

Extraction

For the HS-SPME it is necessary that a three-phase equilibrium adjusts between the liquid phase of the sample, the gaseous phase, and the solid phase of the fiber. The equilibrium was reached after 25 min.

Table II. Validation Results–Cannabinoids*

				Intraday (n = 6)				Interday (n = 18)					
				Precision§ (%)		Bias# (%)		Precision§ (%)		Bias# (%)		Regression line	
	Extrn. yield† (%)	LOD‡ (ng/mg)	LOQ‡ (ng/mg)	0.5 ng/mg (%)	20 ng/mg (%)	0.5 ng/mg (%)	20 ng/mg (%)	0.5 ng/mg (%)	20 ng/mg (%)	0.5 ng/mg (%)	20 ng/mg (%)	linear range (ng/mg)	corr. coeff.
CBD	1.9	0.08	0.27	7.2	6.1	9.9	4.7	12.6	9.9	15.9	8.6	0.1–20	0.999
THC	7.5	0.05	0.27	5.1	1.9	9.6	2.0	5.5	3.3	15.5	6.7	0.2–20	0.999
CBN	0.3	0.14	0.51	6.8	3.3	9.1	3.6	7.2	6.7	14.6	7.9	0.1–20	0.998

* SPME parameters: 10 mg hair, 1 mL 1M NaOH, 0.5 g Na_2CO_3, incubation (5 min), extraction (25 min) and derivatization (8 min) at 90°C, desorption (5 min) at 250°C, depth of fiber insertion 52 mm.
† Extraction yield: the absolute amount of analytes extracted by SPME was calculated by comparison with the corresponding direct injection of a methanolic sample solution onto the GC column (initial amount: 20 ng, n = 3): yield = peak area SPME / peak area liquid injection x 100.
‡ Limit of detection and LOQ were determined by establishing a specific calibration curve from samples containing the analyte in the range of LOQ. The limits were calculated from the residual standard deviation of the regression line (27,28).
§ Precisions are expressed as RSD (%): precision = standard deviation / mean value x 100 (%).
# Bias = (measured concentration – spiked concentration) / spiked concentration x 100 (%).

Table III. Validation Results–EDDP, Methadone, DHC, and Codeine*

				Intraday (n = 6)				Interday (n = 18)					
				Precision§ (%)		Bias# (%)		Precision§ (%)		Bias# (%)		Regression line	
	Extrn yield† (%)	LOD‡ (ng/mg)	LOQ‡ (ng/mg)	0.5 ng/mg (%)	20 ng/mg (%)	0.5 ng/mg (%)	20 ng/mg (%)	0.5 ng/mg (%)	20 ng/mg (%)	0.5 ng/mg (%)	20 ng/mg (%)	linear range (ng/mg)	corr. coeff.
EDDP	6.4	0.01	0.06	2.4	0.5	6.0	2.2	4.6	1.8	10.3	6.0	0.05–20	0.994
Methadone	9.5	0.59	2.34	4.3	0.9	9.0	6.7	5.5	4.7	9.9	4.0	0.3–20	0.998
DHC	1.3	0.39	1.38	2.6	1.0	9.8	2.7	14.5	6.2	15.2	6.0	1–20	0.998
Codeine	5.7	1.15	2.30	6.4	4.8	9.7	3.4	16.6	6.2	14.6	13.2	0.4–20	0.999

* SPME parameters: 10 mg hair, 1 mL 1M NaOH, 0.5 g Na2CO3, incubation (5 min), extraction (25 min), and derivatization (8 min) at 90°C, desorption (5 min) at 250°C, depth of fiber insertion 52 mm.
† Extraction yield: The absolute amount of analytes extracted by SPME was calculated by comparison with the corresponding direct injection of a methanolic sample solution onto the GC column (initial amount: 20 ng, n = 3): yield = peak area SPME / peak area liquid injection x 100.
‡ Limit of detection and LOQ were determined by establishing a specific calibration curve from samples containing the analyte in the range of LOQ. The limits were calculated from the residual standard deviation of the regression line (27,28).
§ Precisions are expressed as RSD (%): precision = standard deviation / mean value x 100 (%).
# Bias = (measured concentration – spiked concentration) / spiked concentration x 100 (%).

Derivatization

The derivatization was finished after 8 min. A longer derivatization time led to a decrease of the extraction yield (Figure 2). For each sample a separate vial with derivatization reagent has to be used, otherwise carryover was observed. The use of 25 µL MSTFA was sufficient.

Desorption

The thermal desorption of the analytes takes place in the injector of the GC. A desorption time of 5 min appeared to be optimal.

Table IV. THC, CBN, and CBD Concentrations Determined in Hair Samples of Drug Abusers*

	ng/mg	Mean (ng/mg)
THC	0.29–2.20	1.7
CBN	0.55–4.54	1.2
CBD	0.53–18.36	1.3

* n = 25, 18 male, 7 female, age: 29 ± 11.

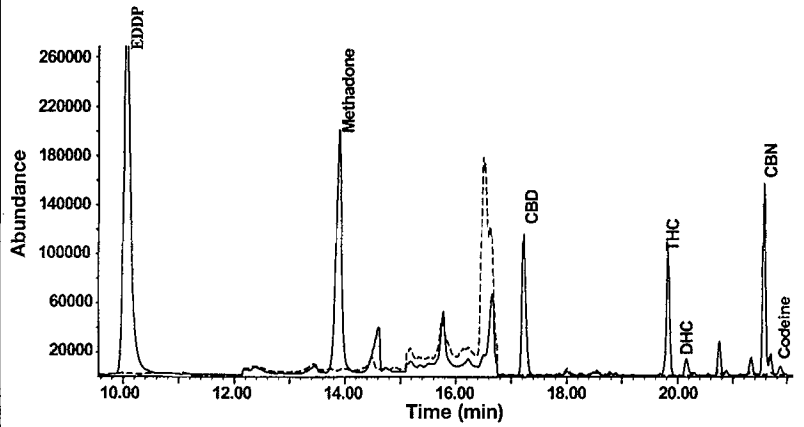

Figure 4. Total ion chromatogram of a spiked hair sample (5 ng/mg) in comparison with a blank hair sample (dotted line).

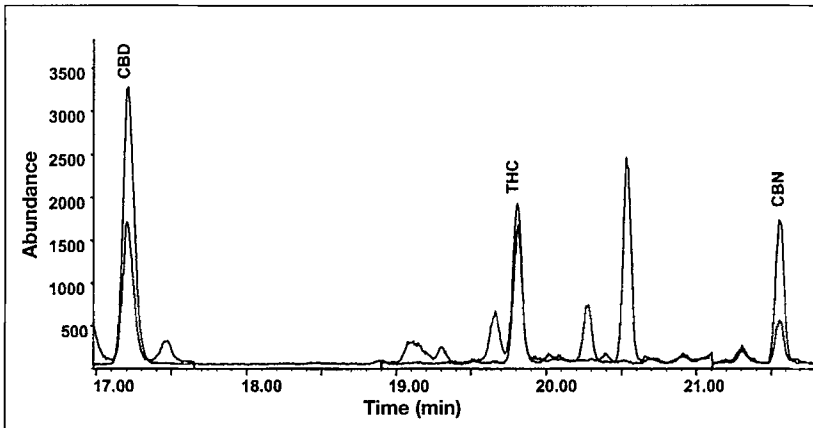

Figure 5. Reconstructed HS-SPME SIM chromatogram of an authentic hair sample containing 1.33 ng/mg CBD, 0.42 ng/mg THC, and 0.91 ng/mg CBN.

Injection port temperature

The extraction yield increased with rising injector temperatures (Figure 3). At 250°C the optimum was reached. Higher temperatures did not increase the chromatographic response, but were disadvantageously regarding the life span of the PDMS fiber.

Depth of fiber insertion

The depth of the fiber insertion into the injector of the GC also affected the extraction yield. A depth of 52 mm turned out to be optimal.

Absolute recoveries

The extraction yields were between 0.3 and 7.5% which are in the typical range for an SPME procedure (Table II).

Validation

In Figure 4 chromatograms of spiked and blank hair samples are presented. By routine analyses of 30 authentic samples from non-drug-users no interfering peaks from the hair matrix were observed. Additionally 10 hair samples containing drugs as determined by GC–MS or HPLC (opiates, cocaine, methadone, amphetamines, and medicaments) showed no interferences. Peak purity and selectivity are ensured. The stability of the analytes in simultaneously prepared samples after alkaline hydrolysis in 1M NaOH was tested by comparing the results of reference samples at the start and end of a sequence in the autosampler. Additionally the stability under storage conditions was evaluated. No significant loss of analytes was detected. A formation of CBN or CBD from THC in the analytical process was not observed. Further validation data were determined with spiked hair samples and are demonstrated in Table II. The presented method for the determination of THC, CBN, and CBD by means of fully automated HS-SPME and GC–MS after on-fiber derivatization shows LOQs and LODs, which are comparable or situated below to the values indicated in literature, obtained with conventional extraction (8) or SPME (16). The determined absolute recoveries are sufficient because in contrast to a liquid injection, the total amount absorbed from the SPME fiber is transferred onto the GC column. By a liquid injection after a conventional sample preparation (liquid–liquid extraction or SPE) only a fraction of the total extract is injected (e.g., 2 µL of 200 µL, which is 1%). Therefore, even for CBN the extraction yield of 0.3% is sufficient for a valid analysis. Regarding the validating data, the procedure is sensitive, selective, and reproducible.

All in all the new HS-SPME procedure using a multipurpose autosampler seems to be suitable for the determination of THC, CBN, and CBD in hair samples in a convenient one-step

method. All single steps like heating and shaking of the sample, alkaline hydrolysis, absorption, derivatization, and desorption in the injector of the GC are programmable and are automatically executed, whereby the number of sources of error is reduced distinctly concerning the reproducibility. A large advantage of the HS technique in relation to the direct immersion is the protection of the SPME fiber and the exclusion of any matrix effects in chromatography. Approximately 90–100 samplings are possible using the HS technique compared with 20–30 samplings using DI (16). The derivatization of the analytes leads to sharper peaks, an improved resolution and sensitivity. Interfering peaks for THC, often observed in GC–MS analysis after conventional extraction and silylation, are excluded by the lower matrix contamination.

However, THC, CBN, and CBD are present in cannabis smoke, so that a potential contamination of hair by external sources of drugs could probably generate false-positive results. Therefore, in the cases with positive results for one of the cannabis constituents, we analyzed the washing solutions using conventional GC–MS methods. In hair samples from drug abusers only, we found low amounts of THC, CBN, and CBD in the petroleum ether solution, and the third wash with dichloromethane was negative in all cases, which is presumptive evidence of sufficient decontamination. Therefore, a positive result in hair analysis was interpreted as proof of the presence of these substances in the interior of the hair and as indication of active cannabis abuse. If further confirmation is necessary, THC-COOH could be identified using conventional methods (2).

Using the described HS-SPME procedure, in addition to the cannabinoids, codeine, dihydrocodeine (DHC), methadone, and 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine could be determined qualitatively and quantitatively using the same procedure (validation data: Table III, chromatogram: Figure 4).

In order to demonstrate the applicability of the developed method hair samples of 20 individuals with drug abuse in general were analyzed (Table IV). Additional drugs were identified: opiates (two cases) and amphetamine (one case). A HS-SPME chromatogram of an authentic hair sample is shown in Figure 5.

Conclusions

The application of fully automated HS-SPME with following GC–MS for the determination of THC, CBD, and CBN in hair was tested. The method was successfully applied to the analysis of hair samples from drug abusers. The SPME turned out to be a substantially simpler and faster procedure than the conventional sample processing. Regarding sensitivity and selectivity the method meets the requirements of clinical and forensic toxicology.

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