AppNote 2/2014

Fully Automated SPE-GC/MS Determination of Δ9-Tetrahydrocannabinol (THC) and its Metabolites in Serum Samples

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Keywords
THC, THC-OH, THC-COOH, Serum, Solid Phase Extraction (SPE), Automation, GC/MS

Abstract
This note presents a fully automated analysis system for the determination of Δ9-tetrahydrocannabinol (THC) and its metabolites in blood serum. Automation is based on the GERSTEL MultiPurpose Sampler (MPS) equipped for solid phase extraction (GERSTEL SPE) and a module for automated eluate evaporation (GERSTEL MultiPosition Evaporation Station, mVAP).

A validated, semi-automated analysis method used for routine analysis was transferred and automated using the described system. Improvements were realized such as a reduction of the sample volume used from 1 to 0.5 mL serum and the use of smaller 1 mL format SPE cartridges. The analysis method has been validated according to GTFCh guidelines (Society of Toxicological and Forensic Chemistry). Limits of quantification below 1 ng/mL for THC and THC-OH, extraction efficiencies between 70 and 93 % and relative standard deviations between 3.3 and 10 % were achieved. The SPE system performs sample preparation in

parallel with the chromatographic run, enabling the GC/MS-system to operate at maximum productivity and full capacity.

Introduction

Δ9-tetrahydrocannabinol (THC) is the main psychoactive compound in cannabis leaves. After its consumption it is metabolized in the body to the active metabolite 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH) and further to the inactive metabolite 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH). Since cannabis consumption has a negative influence on a person's driving abilities [1] driving under the influence of THC is forbidden in Germany and many other countries. A driving ban can be imposed, if a concentration level higher than 1 ng/mL in serum is determined. In this context the consumption pattern can play an important role. This can be estimated from the metabolite concentrations since a high level of THC-OH reveals recent consumption of cannabis and a high level of THC-COOH indicates frequent cannabis consumption.

For these reasons the determination of THC and its metabolites in blood serum is a commonly performed task in forensic laboratories. Solid phase extraction (SPE) is often employed for extraction of analytes and for adequate cleanup. Many laboratories perform GC/MS(/MS) analysis which requires a derivatization step in order to make the compounds (especially THC-COOH) GC-compatible [2-6]. The sample preparation normally comprises several manual steps. This represents a significant workload for laboratory staff with exposure to potentially toxic solvents and reagents and it means that errors are more likely to occur. Therefore, complete automation of the analysis is preferable.

Experimental

Instrumentation. Sample preparation was performed on a MultiPurpose Sampler (MPS) in the Dual Head version equipped with a Solid Phase Extraction (SPE) module and a MultiPosition Evaporation Station mVAP (all GERSTEL) mounted on a GC/MS system (figure 1).

Figure 1. Instrument setup for the automated determination of THC and metabolites in blood serum. Dual Head MultiPurpose Sampler (MPS) equipped with agitator, standard wash station, trays for eluate vials, SPE cartridges and samples, SPE module, two solvent filling stations (SFS), Multi-Position evaporation station (mVAP) and solvent bottle station (from left to right). Left head with 10 µL syringe for injection, right head with 2.5 mL syringe for sample preparation steps. GC/MS system: Agilent GC 7890 / MSD 5977 (Agilent Technologies).

The autosampler head on the right was equipped with a 2.5 mL syringe used for all sample preparation steps (except the MSTFA addition for derivatization); the one on the left was equipped with a 10 µL syringe used for injection into the GC. All solvents and samples were delivered by the 2.5 mL syringe providing a controlled and repeatable flow over the SPE cartridge. Standard SPE cartridges cut off at the top and equipped with transport adapters and syringe needles were used for extraction (figure 2). Such cartridges are commercially available from a number of vendors (Macherey & Nagel, Agilent, Sigma-Aldrich, Phenomenex, Bekolut etc.). Previous work has shown that since standard SPE cartridge sorbent bed dimensions are used established manual SPE procedures can be transferred directly and automated. The complete process is conveniently controlled using the GERSTEL MAESTRO software [7,8]. The automated workflow is depicted in figure 3. SPE cartridge drying is possible by using a gas supply line connected to the syringe. Sealed vials and cartridges minimize the risk of sample contamination and loss of solvents. The mVAP module facilitates the evaporation of eluates under controlled vacuum, temperature and agitation conditions.

Figure 2. Top: Solid phase extraction cartridge configured for automated GERSTEL SPE.
Bottom: Standard solid phase extraction cartridge.

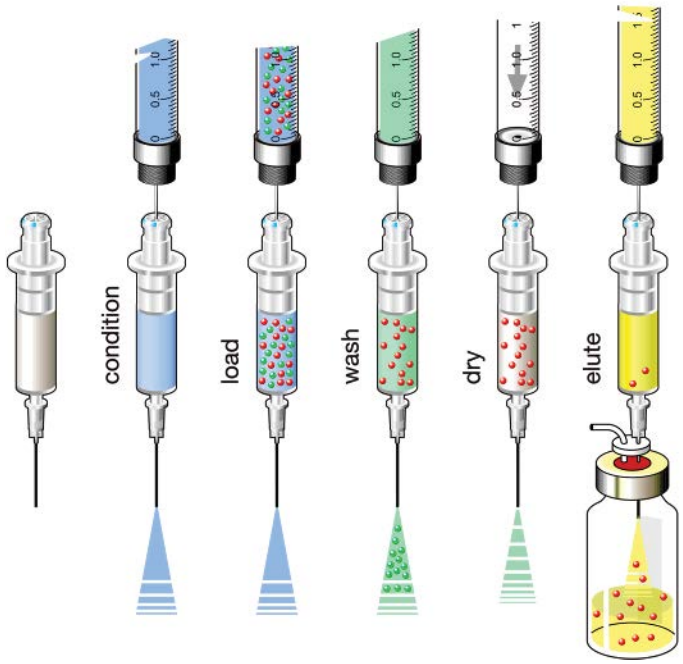

Figure 3. Workflow of automated SPE with the GERSTEL SPE module.

The MPS was mounted on a 6890 GC coupled to a 5973 MSD for GC/MS analysis. Sample introduction was done via a hot split/splitless injector onto the analytical column, VF-1ms 25 m, d_i = 0.2 mm, d_f = 0.33 µm (all Agilent Technologies).

Materials, Solvents and Chemicals. Samples were extracted on 1 mL C18ec 100 mg solid phase extraction cartridges (Macherey & Nagel, 730011MPS, Germany). These standard SPE cartridges can be directly purchased equipped with transport adapter and syringe needle facilitating the SPE automation.

All solvents and salts were of analytical grade. N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Macherey & Nagel. A silicone solution in isopropanol (Serva Electrophoresis, 35130, Germany) was used to rinse eluate vials and calibrator vials, which were subsequently dried at room temperature or in an oven before being used for the analysis.

Preparation of Standards and Solutions. Standards of THC, THC-OH and THC-COOH each 1 mg/mL in methanol were purchased from Sigma-Aldrich. Three working solutions of 500, 50 and 5 ng/mL per analyte in methanol were prepared from the stock solutions (Please note: THC-COOH was present in all solutions at 10 times higher concentration!). These working solutions were used for calibration and for spiking control samples.

Standards of 0.1 mg/mL for THC-D3 and THC-OH-D3 and 1 mg/mL for THC-COOH-D3 were purchased from Sigma-Aldrich. Via an intermediate dilution of 1:100 an internal standard working solution of 60 ng/mL for THC-D3 and THC-OH-D3 and 600 ng/mL for THC-COOH-D3 respectively was prepared. A volume of 50 µL of this solution was added to every sample, calibrator and control sanple.

Analysis Conditions

MPS:	3 µL injection volume
Inlet Temperature:	280°C
Inlet Liner:	Deactivated, single taper (Agilent)
Injection Mode:	Splitless, 2 min
Pneumatics:	134.5 kPa He, constant pressure
Oven:	160°C (1 min); 10°C/min; 180°C (8 min); 5°C/min; 220°C (4 min); 15°C/min; 270°C (5 min); 10°C/min; 300°C (5 min)
Post Run:	325°C (2 min), 350 kPa
Column:	VF-1ms (Agilent) 25 m, d_i = 0.2 mm, d_f = 0.33 µm
Transfer Line:	300°C
MSD Mode:	Selected ion monitoring (SIM)
Source Temp.:	230°C
SIM Masses:	m/z (THC): 386, 371, 303 m/z (THC-D3): 389, 374, 306 m/z (THC-OH): 371, 474, 459 m/z (THC-OH-D3): 374, 477, 462 m/z (THC-COOH): 371, 473, 488 m/z (THC-COOH-D3): 374, 476, 491

Sample Preparation

Manual Sample Preparation Steps

- Dilute a 500 µL sample of serum with 500 µL of 10 % acetic acid and 50 µL internal standard working solution.
- Centrifuge the mixture if precipitate is visible, transfer the supernatant to a clean vial and place it on the autosampler tray.

These steps could also have been automated since a suitable centrifuge is available for the MPS.

Automated Sample Preparation Steps

- Condition the SPE cartridge with 2 mL each of methanol, deionized water and 0.1 M acetic acid.
- Load the entire sample onto the SPE cartridge.
- Wash the SPE cartridge with 2 mL each of 0.1 M acetic acid and a mixture of water and acetonitrile (40/60 v/v).
- Dry the SPE cartridge by purging it with a flow of nitrogen for 1 min.
- Elute the analytes with 2 x 200 µL acetonitrile.
- Evaporate the eluate to dryness at 60°C, 100 mbar under shaking with 250 rpm for 5 min in the mVAP module.
- Reconstitute in 25 µL MSTFA under shaking (agitator at 750 rpm) for 5 min at room temperature.
- Inject 3 µL of the solution into the hot inlet resulting in simultaneous silylation of the analytes and transfer to the GC column.

Results and Discussion

The original validated routine method employed 3 mL C18ec 200 mg SPE cartridges from UCT (USA) and a sample volume of 1 mL. Since these cartridges are not readily available in the GERSTEL format cartridges from Macherey & Nagel with nominally the same sorbent material and sorbent weight were tested. Analyte and background traces were equivalent for all compounds and both cartridges (THC shown in figure 4). Also analytical results were essentially equivalent (table 1). Therefore, Macherey & Nagel cartridges were employed for all subsequent measurements.

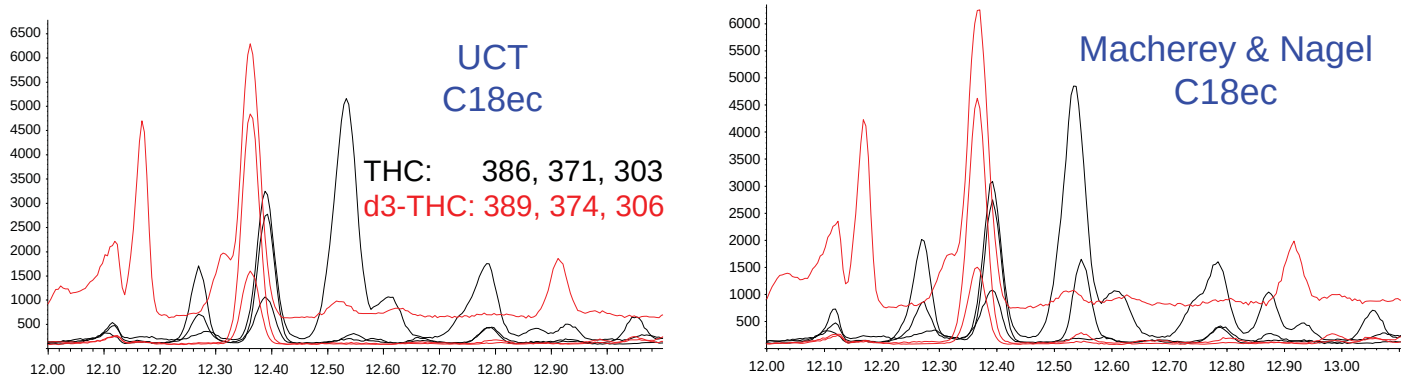

Figure 4. Comparison of chromatograms using UCT (left) and Macherey & Nagel C18ec sorbents for the extraction of THC (shown), THC-OH and THC-COOH. The sorbent materials are equivalent.

Table 1. Comparison of analytical results using UCT (left) and Macherey & Nagel C18ec sorbents for the extraction of THC (shown), THC-OH and THC-COOH. The sorbent materials are equivalent.

Analyte	THC [ng/mL]	THC-OH [ng/mL]	THC-COOH [ng/mL]
Sample 1	1.7	1.8	17.5
Sample 2	1.7	1.6	18.9
Sample 3	1.9	2	20.5
Sample 4	1.8	1.7	19.4
Sample 5	1.8	1.7	17.5
Mean	1.8	1.8	18.8
RSD [%]	4.4	8.6	6.8

Analyte	THC [ng/mL]	THC-OH [ng/mL]	THC-COOH [ng/mL]
Sample 1	1.8	1.6	18.2
Sample 2	1.8	1.7	19
Sample 3	1.7	1.7	19.5
Sample 4	1.8	1.9	20.5
Sample 5	1.7	1.7	18.6
Mean	1.8	1.7	19.2
RSD [%]	3.3	6.5	4.7

Another important question to be clarified was whether analytical results for the completely automated method and the validated routine method are comparable. To prove this, spiked serum samples at low concentrations were analyzed using both methods. In figure 5 it can be seen that the results obtained using the two methods at THC concentrations near the limit of quantification are in good agreement. Analysis results for the two metabolites also showed good agreement.

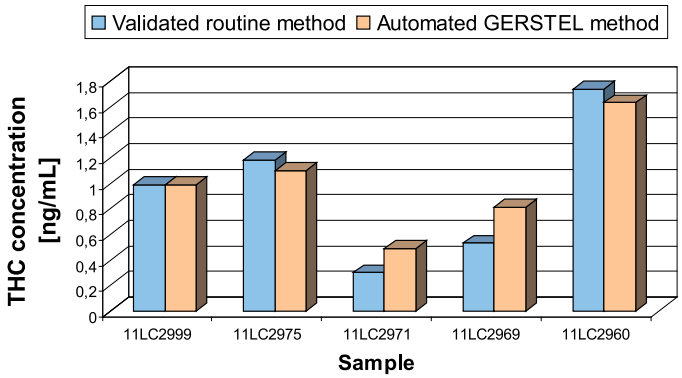

Figure 5. Comparison of analysis results obtained using the validated routine analysis method and the automated GERSTEL method for THC near the limit of quantification.

An important issue for the validation of an automated analysis system is to test for sample to sample carry-over. The system performance with regard to carry-over was tested by analyzing six serum samples spiked to high concentrations of the analytes (60 ng/mL THC and THC-OH, 600 ng/mL THC-COOH). After each sample a blank serum sample was analyzed. No relevant carry-over was detected in the blank sample chromatograms as can be seen in figures 6a-c.

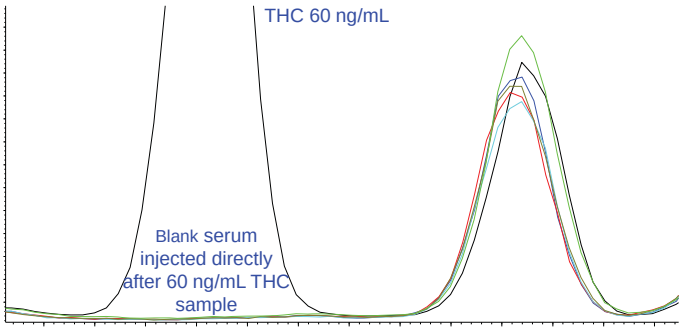

Figure 6a. The fully automated analysis method shows no carry-over for THC.

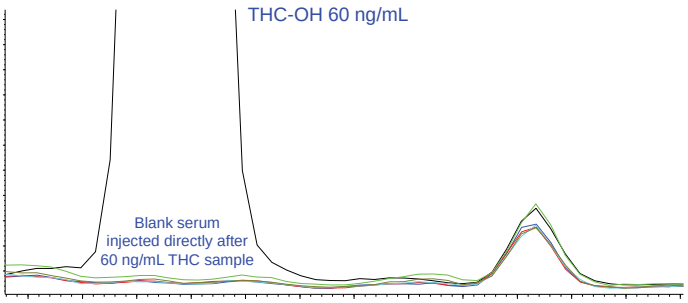

Figure 6b. The fully automated analysis method shows no carry-over for THC-OH.

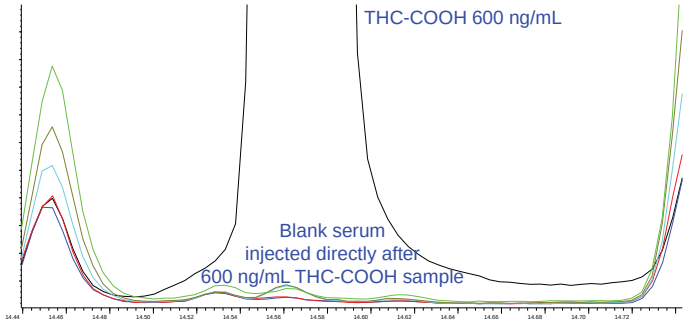

Figure 6c. The fully automated analysis method shows no measurable carry-over for THC-COOH.

After having successfully proven these points further optimization of the automated method was pursued:The method was scaled down by employing a 1 mL 100 mg C18ec cartridge instead of the 3 mL 200 mg C18ec and by reducing all solvent volumes used accordingly. The serum sample volume used was reduced from 1 mL to 0,5 mL. After extraction and cleanup the sample was reconstituted in 25 µL MSTFA instead of 40 µL. As can be seen in figure 7, the results obtained using the two methods were in good agreement. Analyte peak heights were similar the background signal levels appeared slightly reduced when using the smaller volumes.

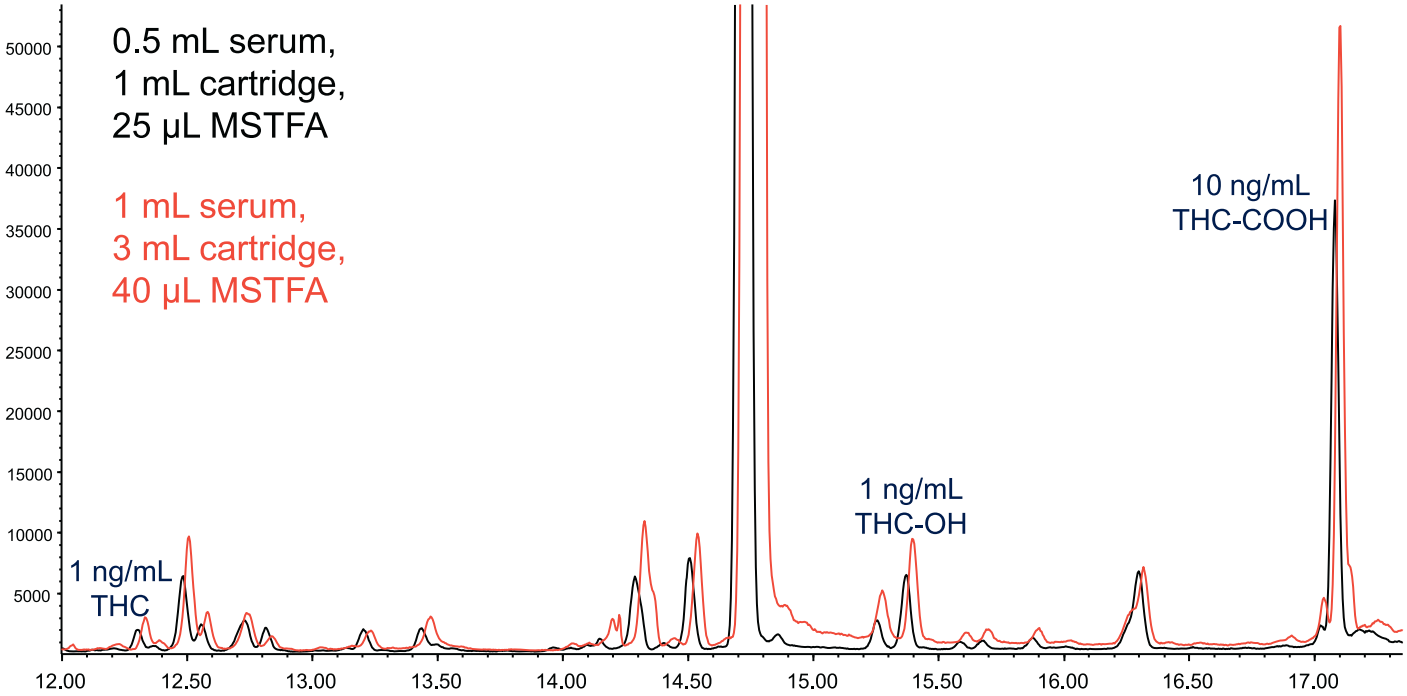

Figure 7. Optimized fully automated method (black chromatogram) produces analyte peak areas similar to the original routine analysis method with slightly less background.

The final method was validated (see table 2). Validation criteria were met, some of the data is shown in table 2. Limits of quantification below 1 ng/mL for THC and THC-OH, extraction efficiencies between 70 and 93 % and repeatabilities between 3.3 and 10 % (inter-day repeatabilities between 6.7 and 16.3 %, respectively) were achieved. Calibration was performed using solvent-based standards, which was possible since a deuterated internal standard was used for every analyte. Furthermore it was proven beforehand that calibration lines from spiked serum and solvent standards are equivalent. Calibration lines and analysis results were calculated based on peak heights. Again, this is possible because internal standards are used. By employing peak heights for calculation, coelution shoulders and the absence of base-line separation - quite likely when using single quadrupole MS detection for complex matrices - do not negatively influence analytical results.

Table 2. Validation data for the fully automated analysis method according to GTFCh guidelines [9].

Analyte	Limit of detection [ng/mL]	Limit of quantification [ng/mL]	Repeatability [%]			Inter-day repeatability [%]			Extraction efficiency [%]		
			1.2 ng/mL	5.5 ng/mL	25 ng/mL	1.2 ng/mL	5.5 ng/mL	25 ng/mL	1.2 ng/mL	5.5 ng/mL	25 ng/mL
THC	0.3	0.7	5.2	7.2	3.8	7.8	7.2	6.7	75	74	70
THC-OH	0.3	0.9	3.5	10	3.5	16.3	10	6.9	93	82	86
THC-COOH	<1	5	3.7	6.6	3.3	8.6	7.1	7.1	83	79	87

The GERSTEL MAESTRO software integrated in the Agilent ChemStation or MassHunter controls the complete sample preparation process. Automated overlapping of sample preparation and chromatographic analysis enables the GC/MS-system to operate at full capacity since the next sample is always prepared and ready to be introduced whenever the GC/MS run has been completed.

Conclusion

- A validated, semi-automated routine analysis method for THC and its main metabolites in serum has been successfully transferred to a completely automated analysis system.
- Analysis results of the fully automated method are precise, accurate and comparable to the semi-automated routine analysis method.
- Validation data in table 2 were collected according to GTFCh guidelines, validation criteria were met.
- The system is highly flexible and opens the possibility of automating further established sample preparation workflows in different application fields.

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