The role of hexamethyldisilazane as silylation and acylation reagent in the analysis of biological compounds and their metabolites by GC-MS

PhD theses

Blanka Fodor

Semmelweis University

Doctoral School of Pharmaceutical Sciences

Supervisor: Ibolya Molnár-Perl, D.Sc., professor emerita

Reviewers: Mária Mörtl, Ph.D, head of department
Ágnes Alberti-Dér, Ph.D, associate professor

The Complex Examination Committee:
Chairwoman: Romána Zelkó, D.Sc., professor
Members: Krisztina Kurin-Csörgei, Ph.D., associate professor
László Őrfi, Ph.D., associate professor

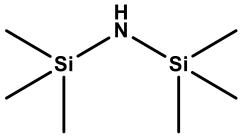
Budapest
2020

1. Introduction

The first gas chromatograph was presented by A.T. James and A.J.P. Martin at the meeting of the Biochemical Society on October 20, 1950. The technique gas chromatography (GC) associated with the instrument, is still regarded as one of the most frequently used methods in modern separation science. First, it was applied to separate volatile fatty acids. The determination of the crude oil composition, which proved to be the biggest challenge in analytical chemistry, was solved in the 1950s. Subsequently, the petroleum industry implemented amazing improvements to the method, which resulted in the increasing utilization of this technique in other disciplines (e.g. biochemistry, bioanalytics and food analytics). The real breakthrough and popularity was obtained after the 1980s due to the appearance of capillary columns.

The comprehensive spread of GC-MS identification and quantifications required a continuous improvement in the sensitivity and selectivity of measurements. Numerous derivatization techniques have been developed to solve this problem. The number of separable components in one run, the efficiency of separation, the binding of the compounds to the column, thermal stability of the molecules, as well as the volatility and apolar nature of the substances are amended by replacing active hydrogen in “offline” methods prior to the separation. In consequence of these modifications, polar and low volatility compounds that had not been measured before by GC-MS became quantifiable. Among the derivatization techniques, trialkylsilylation, acylation with halogenated carboxylic acid derivatives, esterification, alkylation, oximation and the conversion of bifunctional compounds into ring derivatives have become part of the routine labour work.

The colourless, water-miscible liquid, hexamethyldisilazane (HMDS), also known as bis(trimethylsilyl)amine (Fig. 1), was produced by Sauer and Hasek, reacting trimethylchlorosilane with ammonia. The aforementioned reagent is commonly used to produce trimethylsilyl (TMS) derivatives.



Hexamethyldisilazane
(HMDS)

CAS number: 999-97-3
M = 161.2 g/mol
Boiling point: 125 °C
Density: 0.77 g/cm^3

Fig 1. Structure, physical and chemical properties of hexamethyldisilazane

HMDS was applied at first in the early 1960s by Brochmann and colleagues to convert sympathomimetic amines into TMS derivatives prior to their gas-liquid chromatographic separation. Nowadays its utilization is widespread, either alone or in the presence of a trifluoroacetic acid (TFA) catalyst, mainly to produce TMS derivatives. Due to its special symmetrical structure and unique reactivity of the HMDS & perfluorocarboxylic acid couples, it was applied at first in our laboratory as an acylating reagent to derivatize primary phenylalkyl amines.

The aim of my PhD study was to investigate applicability of the HMDS, in which its efficacy as a trimethylsilylating reagent has been compared with other derivatizing agents in a comprehensive study. The first purpose of my work, along with the method development, was to investigate an extraction free, direct sample preparation working strategy to quantify the active ingredients of cannabis. My second objective was a) to analyse the HMDS' acylating property ensured by its symmetric structure, as the novelty of derivatization techniques, and b) to extend acylation with the simultaneous trimethylsilylation of amino acid, amino alcohols, amino sugar, oligopeptide and biogenic monoamine compounds prior to their identification and quantification by GC-MS.

2. Objectives

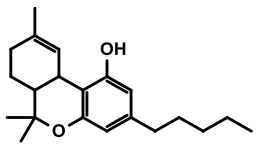
Based on the literature overview, the aims of my doctoral work were:

- the optimization of seven plant cannabinoids trialkylsilylations in a reagent and a reaction conditions related study, strictly under the same conditions, using a fast chromatographic elution program, evaluated by selective fragment ions.
- to select an easily available plant matrix, like *Cannabis ruderalis*, suitable to prove our extraction free, direct sample preparation strategy prior to the GC-MS determination of the plant cannabinoids.
- the investigation of the acylating properties of HMDS and extension with simultaneous trimethylsilylation to derivatize chemically multifunctional molecules, such as amino acids and biogenic monoamines.
- to optimize the new derivatization technique, varying reagent composition including solvent selection, time and temperature conditions to define optimum analytical circumstances, confirming stoichiometric behaviour of the novel method.
- demonstrating the selective fragmentation patterns in various types of the multifunctional group containing organics monitored by GC–MS and confirm their main analytical performance characteristics.
- to define the optimum HMDS & perfluorocarboxylic acid couple, with special emphasis on the retention properties of the biogenic monoamines and their corresponding acidic metabolites.
- highlighting practical utilization of our green working strategy, demonstrating the main reaction performance characteristics by limit of quantification, reproducibility, linearity and recovery data for the target compounds both in standard solution and urine samples.

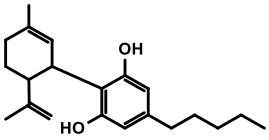
3. Experimental

3.1 Samples

The examined plant samples were female species of *C. ruderalis* (Fig. 2), and were collected in flowering stage from their natural habitats in Somogy county (Hungary) in 2016 and 2017. Their storage, until the quantification, was provided by the Department of Plant Anatomy of Eötvös Lóránd University.



Tetrahydrocannabinol
(THC)



Cannabidiol
(CBD)

Fig 2. The bud of cannabis and the structure of its two main active ingredients

Branched-Chain Amino Acid (BCAA, the nutritional supplement), which was ordered via Internet, served as the matrix for the standard addition processes.

Female urine of a healthy volunteer (blank urine) and those of patients diagnosed with metabolic disorder, serving to prove the advantages of the direct derivatization method without the need of preliminary extraction processes, were ensured by the Department of Forensic Medicine, Semmelweis University (Budapest, Hungary).

Fifteen-membered peptides ATE and ETA (AS composition denoted by one-letter codes: ETA = KDQYASNVVVGETA, ATE = ATEGVVNSAYQDK) were synthesized by the MTA-ELTE Peptide Chemistry Research Group for research purposes.

3.2 Instrumentation

3.2.1 Sample preparation implements

The exact masses were weighed on an analytical balance (Sartorius, Goettingen, Germany) with ± 0.01 mg accuracy. Calibrated volumetric flasks were used for the preparation of standard and sample stock solutions. Hamilton (Bonaduz, Switzerland) and Trajan (Victoria, Australia) microsyringes with a precision of ± 1% were applied to pipet

accurately the volume of reagents. The dissolution of the compounds was assisted by a Sonorex (Bandelin electronic, Berlin, Germany) temperature controlled ultrasonic water bath. The lyophilization was performed with a Modulyo freeze-dryer (Jencons, UK), the centrifugation on a Hettich EBA 21 (Tuttlingen, Germany) centrifuge, the dry evaporation on a Büchi Rotavapor R-200 (Flawil, Switzerland) rotary vacuum evaporator and the thermostable stove applied to derivatization was originated from Kutesz (Hungary). The exact volume of the urine samples was measured with a 100-1000 µl volume Biohit Proline (Helsinki, Finland) automatic pipette set up with a disposable tip.

3.2.2 Gas chromatography and mass spectrometric conditions

The instrument consists of a Varian 450 gas chromatograph equipped with a CP-8400 (Varian, Walnut Creek, USA) autosampler and a programmable injector. As detector a Varian 240 MS / MS mass spectrometer was operated with an ion trap detector. The column used for the separation of the cannabinoids, the biogenic monoamines and the amino acids, was an Agilent (Victoria, Australia) HP-5MS (5% phenylmethyl-polysiloxane stationary phase) capillary column. Helium of 6.0 (99.9999 %) purity was used as a carrier gas, with a flow rate of 1 ml/min.

The temperatures of the transfer line, ion trap and manifold were in order of listing: 300 °C, 210 °C and 80 °C, respectively. The injector and oven temperature operational data are summarized in Table 1. The general MS parameters were: filament current: 25 µA; Target TIC: 20000 counts; Prescan Ion Time: 1500 µs; Scan mode: Fast; Scan Time: 0.59 s/scan; electron energy: 70 eV. FS acquisition were performed in mass range of *m/z* 50 - 1000. Setting the voltage of the electron multiplier, the adjusted value by the auto calibration (auto tune) was increased by 100 V. The parameters of the instrument were controlled by the Varian MS Workstation 6.9 software.

Table 1. Heating rate of the injector and oven, data acquisition delay time during the elution program

Compounds	**Injector**		**Oven**			**Data acquisition delay time, *min***
	Temp., *°C*	**Time, *min***	**Temp., *°C***	**Heating speed, *°C/min***	**Hold, *min***	
Cannabinoids	Elution time: 13 min (in case of TBDMS 20 min.)					
	300	4.00	100	-	-	3.00
			300	20	- (7)	
Amino acids, -alcohols, -sugar, 2,6-Diamino-heptanedioic acid and oligopeptides	Elution time: 20.20 min					
	280	3.00	100	-	1,00	2.00
			145	10	-	
			195	5	1.00	
			280	50	2.00	
Biogenic monoamines and their metabolites	Elution time: 19.35 min					
	280	3.00	100	-	1.00	5.00
			145	10	-	
			195	5	1.00	
			280	100	2.00	

Indications: Temp. = temperature

3.3 Methods

3.3.1 Standard solutions

The concentration of stock solutions, prepared from analytically pure standard compounds, was 0.1-10 mg/ml in 5-10 ml total volume solved in methanol, 2 M or 0.25 mM hydrochloric acid. The solid standards were measured with ± 0.01 mg accuracy. Prior to the experiments, the solutions were diluted to 10-500-fold using the appropriate solvent, then the 3-100 µl volume of diluted stock solution was transferred by a microsyringe to reaction tube fitting to the rotavapor instrument. The evaporation was performed at 30-40 °C.

3.3.2 Derivatization

Residues of standard solution were reconstituted in aprotic organic solvent (pyridine, ethyl-acetate or acetonitrile) and then derivatization reagents were added. The ratio of solvent to reagents was chosen based on the previous experiences of our research group. Trimethylsilylation was performed in a total volume of 375 µl and one-step acylation and silylation in a total volume of 200 µl. The reaction time and temperature used in the optimization were varied between 10-120 min. and 60-100 °C. After derivatization, the solutions were cooled down to room temperature, then 1-1 µl aliquots of the undiluted or five-tenfold diluted solutions were injected into the GC–MS system. Dilution was performed with the derivatizing reagent in each case and all tests were performed in parallel and each injected three times.

3.3.3 Sample preparation

The plant tissues were freeze dried then pulverized in an agate mortar. Aliquots of 0.5-2.0 mg of the sample were measured with ± 0.001 mg precision in a 2 ml volume reaction tube and without preliminary extraction were converted directly to TMS derivatives. Urine samples, stored in a freezer in 10 ml plastic centrifuge tubes, after thawing were centrifuged (6000 rpm, 10 min) and 100-300 µl clear supernatant were pipetted into the reaction tubes, then evaporated to dryness under a vacuum before derivatization and GC-MS analysis (Fig. 3). Further sample preparation steps were performed according to the details described in the previous paragraph.

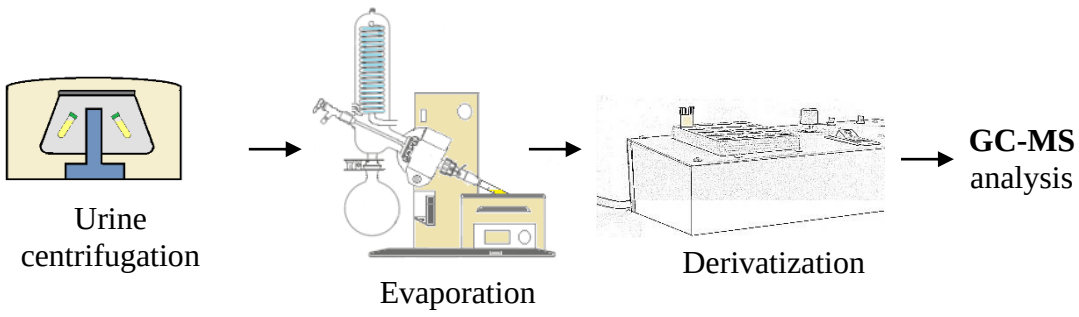


Fig 3. The main stages in the preparation of urine samples

The accurately weighed BCAA nutrition supplement powder was dissolved in 2 M hydrochloric acid. 910 and 900 μg of ATE and ETA synthetic peptides were hydrolysed in 6 M hydrochloric acid in a sealed vial at 105 °C for 24 h. The hydrolysates were washed quantitatively with 2 M hydrochloric acid in a 5 ml volumetric flask and stock solution was prepared. The required volume was taken from the samples with a microsyringe, which was derivatized according to 3.3.2 after evaporation to dryness.

3.4 Determination of the performance characteristics of the methods

Specificity and selectivity were confirmed by injecting a "blank" (solution containing the reagents treated according to the sample preparation procedure) solution and a "blank" urine sample as a reference originated from a healthy volunteer was also injected. At the target compounds' retention no other interfering peak appeared neither in the chromatogram and nor in the mass spectra. The linearity of the developed methods was investigated by standard solutions and in the presence of the matrix. The exact concentration of the calibration solutions was calculated with respect to the accurately measured masses and, in the case of HCl salts, with respect to the base.

Limit of quantification (LOQ) values were determined empirically. That point of the calibration was chosen as LOQ, where the signal/noise (S/N) ratio was > 10. The reproducibility of the method was investigated by parallel sample preparations and injections.

Analytical performance characteristics for urine matrices were calculated by using the standard addition method, applying the urine of a healthy volunteer and also analyzing urines of patients diagnosed with neurological diseases. The recovery value was calculated based on the responses of standard solutions prepared with the same concentration. *Cannabis ruderalis* dried and pulverized plant tissues were weighed to the evaporated standard solution and derivatized without preliminary extraction. The model solution and the corresponding amounts of urine or BCAA nutrition supplement samples were rotary evaporated to dryness at 30-40 °C. The residues were treated according to the 3.3.2 paragraph. Thereafter, the derivatives' solutions were diluted with HMDS and transferred into the autosampler vial.

The acquisition was performed in full scan mode, the chromatograms extracted on the basis of SFIs were used for the evaluation. The peak areas were integrated by the software.

4. Results

As a first step of my research, I performed a comprehensive derivatization study, investigating the behaviour of the most well-known silylating reagents, completed with HMDS and examined the role of catalysts on the derivatization of plant cannabinoids in the same chromatographic system with simultaneous quantitative measurements of neutral and acidic compounds within thirteen minutes. HMDS and TFA, which were never used before to derivatize cannabiboids, have been proven to be an ideal choice to determine the cannabinoid content of plant samples with low active ingredient level.

I developed a new selective and quantitative analytical method based on the unique reactivity of HMDS and perfluorocarboxylic acid pairs, which allows for trialkylsilylation of amino acids, amino alcohols, amino sugars, 2,6-diaminoheptanedioic acid, oligopeptides, biogenic monoamines and in turn moderates the basicity of their free amino groups and facilitates subsequent acylation (Fig. 4).

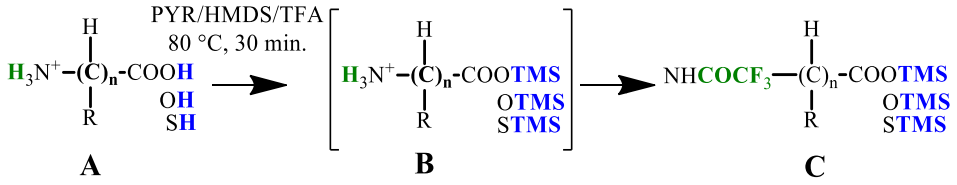


Fig 4. Assumed sequence of simultaneous trimethylsilylation and trifluoroacylation of multifunctional compounds as exemplified by amino acids

Simultaneous trimethylsilylation and trifluoroacylation reaction conditions were optimized and analytical performance characteristics were established. The practical utility of the proposal was evidenced 1) by the quantitation (including recovery studies with standard additions) of the nutrition supplement's BCAA, 2) by the determination of

various amounts of females' urine free amino acid content and 3) measuring the composition of peptide hydrolysates.

By optimizing the perfluorocarboxylic acid member of the reagent pair, I separated the biogenic monoamines from their acidic metabolites. The practical applicability of the method was confirmed by determining the content of the target compounds using the standard addition method in the presence of a matrix. The VMA content of patients' urine diagnosed with a neuronal disorder was quantified from three different starting volume samples, as well as the ST concentration in the presence of a huge excess of 5-HIAA metabolite.

A detailed fragmentation pattern analysis was performed and the main performance characteristics, like linearity, reproducibility, LOQ and recovery with a standard solution and in the presence of a matrix were determined.

I recommended a new sampling protocol and I recognized the importance of solvent in stock solution preparation. Applying the investigated and optimized methods, the loss of target compounds can be avoided (can be performed without time-consuming and labour-intensive extraction steps) and a fast, selective, sensitive, time-, labour-, cost- and solvent-efficient work strategy is resulted in accordance with green chemical requirements.

5. Conclusions

GC proposals published between 2000 and 2020 were reviewed. Procedures, without derivatization and applying various alkylsilyl, acylation and/or esterification techniques were listed, compared and criticized **(I)**. New sample preparation techniques were developed to derivatize biologically active compounds prior to their GC-MS determination.

- A novel, direct sample preparation approach was developed, HMDS was applied for the first time to convert cannabinoids to their TMS derivatives, including simultaneous quantification of seven plant cannabinoids by GC-MS, within a 13 min run time. **(I, II)**
- Direct, full derivatization was achieved utilizing the unique reactivity of the HMDS and perfluorocarboxylic acid couples leading to the simultaneous stoichiometric acylation, etherification and esterification. A selective and quantitative novel analytical process was presented, providing unified, full derivatization of amino acids, amino alcohols, amino sugar, amino carboxylic acid, oligopeptides and biogenic monoamines, without extraction, in a single step. The derivatives structures were confirmed by mass spectra. **(III, IV)**
- By optimizing the HMDS and perfluorocarboxylic acid type, one-step acylation and silylation was first applied for separation of the biogenic monoamines from their acidic metabolites present in huge excess. Based on detailed studies, the HMDS and pentafluoropropionic acid couple proved to be the reagent of choice. **(IV)**
- The mass fragmentation patterns were described in detail for all derivatization principles related to silylation and acylation properties of HMDS. Analytical performance characteristics both in model solutions and in urine matrices such as repeatability, linearity and limit of quantitation were confirmed.
- The main analytical performance characteristics of the developed sample preparation methods were determined both in the model solutions and in the urine matrices. **(II, III, IV)**

- The practical applicability of derivatization without extraction was confirmed. The cannabinoid-content of the dried plant tissues, the amino acids and biogenic monoamines concentrations of the dietary supplement and the urine were measured in the presence of the matrix. The time-consuming procedure for collecting acidic and neutral fractions separately, presented in the literature, was simplified. Cannabinoids, amino acids and biogenic monoamines were directly derivatized without preliminary extraction in the presence of their matrices before the GC-MS analysis: minimizing the use of organic reagents according to the requirements of green chemistry. **(II, III, IV)**

6. Publications

Publications related to the PhD thesis:

Fodor, B., Molnar-Perl, I. (2017) The role of derivatization techniques in the analysis of plant cannabinoids by gas chromatography mass spectrometry. Trends Anal. Chem., 95: 149-158. **(I)**

Fodor, B., Boldizsár, I., Molnár-Perl, I. (2018) Alkylsilyl speciation and direct sample preparation of plant cannabinoids prior to their analysis by GC-MS. Anal. Chim Acta, 1021: 51-59. **(II)**

Fodor, B., A. Csampai, Molnar-Perl, I. (2020) Hexamethyldisilazane and perfluorocarboxylic acid couples achieve trialkylsilylation and acylation of active proton containing organics in a single step. Microchem J., 154: 104554 **(III)**

Fodor, B., Üveges, E., Molnár-Perl, I. (2020) Direct sample preparation and simultaneous perfuoroacylation - Trimethylsilylation of biogenic monoamines along with their acidic metabolites for a single step analysis by GC-MS. Anal. Chim. Acta, 1127: 9-19. **(IV)**

Additional publications:

Zürn, M., Tóth, G., Kraszni, M., Sólyomváry, A., Mucsi, Z., Deme, R., Rózsa, B., **Fodor, B**., Molnár-Perl, I., Horváti, K., Bősze, Sz., Pályi, B., Kis, Z., Béni, Sz., Noszál, B., Boldizsár, I., (2019) Galls of European Fraxinus trees as new and abundant sources of valuable phenylethanoid and coumarin glycosides. Ind. Crops Prod., 139: 111517

Molnár, B., **Fodor, B.**, Boldizsár, I.; Molnár-Perl, I., (2016) Trimethylsilyl speciations of cathine, cathinone and norephedrine followed by gas chromatography mass spectrometry: Direct sample preparation and analysis of khatamines. J. Chromatogr. A, 1440: 172-178.

Molnár, B., **Fodor, B.**, Csámpai, A., Hidvégi, E., Molnár-Perl, I., (2016) Structure-related new approach in the gas chromatography/mass spectrometry analysis of cathinone type synthetic drugs. J. Chromatogr. A, 1477: 70-75.

Molnár, B., **Fodor, B.**, Boldizsár, I., Molnár-Perl, I., (2015) Quantitative silylation speciations of primary phenylalkyl amines, including amphetamine and 3,4-methylenedioxyamphetamine prior to their analysis by GC/MS. Anal. Chem., 87: 10188-10192.