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SOLID PHASE EXTRACTION AND FAST GAS CHROMATOGRAPHY FOR A RESIDUE ANALYSIS OF SEDATIVE-HYPNOTIC DRUGS IN BIOLOGICAL SAMPLES

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ABBREVIATIONS

ACN – acetonitrile;

BSTFA – N,O-bis(trimethylsilyl)trifluoroacetamide;

1-BuOH – 1-butanol;

EtOH – ethanol;

GC/EI-MS – gas chromatography/mass spectrometry with electron impact ionization;

GC/NICI-MS – gas chromatography/mass spectrometry with negative-ion chemical ionization;

hc-poly(St-DVB) – hypercrosslinked poly(styrene-divinylbenzene);

HPLC – high performance liquid chromatography;

IS – internal standard;

LOD – the limit of detection;

LOQ – the limit of quantification;

LC-MS/MS – liquid chromatography coupled with tandem mass spectrometry;

LLE – liquid-liquid extraction;

MSTFA – N-methyl-N-(trimethylsilyl)trifluoroacetamide;

MTBSTFA – N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide;

m/z – mass-to-charge ratio;

poly(NVP-DVB) – poly(N-vinylpyrrolidone-divinylbenzene);

poly(St-DVB) – poly(styrene-divinylbenzene);

1-PrOH – 1-propanol;

2-PrOH – 2-propanol;

QC – quality control;

RRF – relative response factor;

SIM – selected ion monitoring;

SPE – solid phase extraction;

TBDMS – tert-butyldimethylsilylation;

TMCS – trimethyl-chlorosilane;

TMS – trimethylsilyl derivative.

INTRODUCTION

Lithuania's integration into West Europe caused a rapid spread of addictions for psychotropic materials. According to the data of epidemiological research, a lot of Lithuanian people often use psychoactive materials such as benzodiazepines and as well as other newest medications [1, 2]. Main causes are difficult social and economical conditions, good accessibility of the drugs and the lack of motivation.

Benzodiazepines are a large class of drugs with some 35 controlled by the United Nations Convention (1971), and many more available throughout the world [3, 4]. They are an important class of drugs with a broad range of therapeutic effects, including sedative-hypnotic, anxiolytic, muscle-relaxant, and anticonvulsant [3 - 6]. Because of their wide use, benzodiazepines have a potential of interaction with other central nervous system depressants, which can result in life-threatening conditions. Benzodiazepines are among the most commonly-prescribed drugs nowadays in Europe including Lithuania [1, 2], and are often found in a combination with other drugs in drug-related fatalities [3, 7 - 9].

Zaleplon and zopiclone are sedative-hypnotic drugs, and are used to induce sleep in short-term treatment of insomnia. These newest medications are less safe than other benzodiazepines, and have tendency to induce physical dependence [3, 10 - 12]. For this reason, the analysis of benzodiazepines and newest medications is of great interests to forensic and clinical toxicologists.

In forensic and clinical toxicology, demands for analytical laboratories are growing along with the rapidly changing methods for the determination of sedative-hypnotic drugs in biological samples. The chemist or toxicologist must be able to detect and identify drugs and their metabolites that could be abused or cause intoxication, and to quantitative them at concentration levels [3, 4]. In this analysis, comprehensiveness, positive identification and quantification are of great importance.

Simple and fast sample preparation is a very important step in the development and investigation of methods for the analysis of sedative-hypnotic drugs in biomedical materials. Direct determination of drugs in biological samples (especially derived from human urine or whole blood) is complicated due to significant matrix interferences and relatively high concentrations of the analytes [13, 14]. However, there is a limited number of sample preparation procedures suitable for effective isolation/preconcentration of the analytes from biological samples. Nowadays, the most widely used sample preparation techniques are liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [10, 15, 16]. LLE offers the advantages of low sample consumption, simple device, easy operation, and low costs [16]. However, insufficient repeatability of the results and co-extracted matrix constituents have been observed [15, 16].

Meanwhile, SPE has the advantages of high selectivity, cleaner extracts, no emulsions, reduced solvent usage and higher throughput by automatization [17, 18]. Existing SPE procedures for drugs cover a broad range of sorbents (apolar, ion-exchange and polymeric sorbents or a combination of different sorbents) and allow the development of extraction procedures for specific needs. SPE is also very important for the clean-up of complex samples (whole or hemolyzed blood) meaning the removal of interfering compounds from the matrix in order to increase the overall selectivity and/or specificity of the analytical method [18].

In recent years, the new generations of hydrophilic-lipophilic balance polymeric [19] and mixed-mode [20] SPE sorbents have also been used for sample preparation of several sedative-hypnotic drugs in biological specimens. These SPE sorbents were designed to simplify and improve sample preparation by combining the correct sorbent chemistry, device format and methodology. When compared to the traditional SPE sorbents, they have the advantages of being more time-efficient, yielding cleaner extracts, showing increased selectivity for the compounds of interest, and smaller volumes of solvent may be used for extraction [19, 20]. However, for the most of these methods the

sensitivities, accuracies and repeatabilities of the analytical results are still in need of improvement in the case of residue analysis.

The main aim of this study was to develop a new SPE procedures/methods for a residue analysis of some sedative-hypnotic drugs in biological samples by fast gas chromatography with negative-ion chemical ionization mass spectrometry (GC/NICI-MS). For this purpose, two analytically important the analyte/sample matrix systems were selected: 15 benzodiazepines in whole blood, and zaleplon and zopiclone in hemolyzed blood.

The main tasks set to achieve the aim were the following:

1. To develop the optimal derivatization procedure for the determination of benzodiazepines.
2. To optimize fast GC/NICI-MS conditions for the efficient separation and detection of benzodiazepines, zaleplon and zopiclone.
3. To develop and to select the best SPE methods for multi-residue extraction of benzodiazepines from whole blood, as well as zaleplon and zopiclone from hemolyzed blood samples.
4. To validate the developed SPE-GC/NICI-MS methods in whole and hemolyzed blood.
5. To apply the developed methods in the analysis of real blood samples.

Statements for defence:

1. Fast GC/NICI-MS technique for the separation of benzodiazepines, zaleplon and zopiclone are more selective and faster in comparison to the conventional techniques.
2. Derivatization of benzodiazepines after the SPE procedure improves the sensitivity of their determination.
3. The investigated SPE methods are selective, accurate and precise with good recoveries and more effective than the conventional currently used SPE and LLE methods.

4. New fully validated SPE-GC/NICI-MS methods can be used for extraction of complex biological samples, such as whole blood or hemolyzed blood.

1. LITERATURE SURVEY

1.1. An SPE method

Sample preparation prior to analysis is very important to the success of an assay as the analytical technique. This is especially true when low levels of detection are required, or for analytes in complex matrices, such as biological fluids, tissue, foodstuffs, agricultural products and environmental samples. Solid-phase extraction (SPE) is a fast, cost-effective sample preparation technique for purification of complex samples before their analysis by gas or liquid chromatography [21]. The technique removes undesirable matrix compounds that can interfere with the analyte. SPE has been used extensively to concentrate the analytes, and this method can efficiently extract different types of the analytes from biological specimens [18, 21, 22]. Therefore, SPE is currently one of the most widespread extraction methods for the pretreatment of liquid biological samples.

The SPE was developed and first introduced in the mid-1970s [23, 24], becoming commercially available in 1980. In the SPE technique, silica gel, modified silica gel, hydrophobic polymer, hydrophilic polymer or mixed-mode polymer is embedded in a column as a sorbent material. In SPE methodology, analytes are partitioned between a solid phase and a liquid phase, and the stationary phase usually is chosen to have a greater affinity for the analytes than for the sample matrix [21, 25]. SPE is based on the principle of the analytes transfer from an aqueous phase to the absorption sites of the adjacent solid phase. The analytes are adsorbed onto the surface of the sorbent when the sample solution flows through the column. Endogenous interferences can be removed by passing an aqueous solution through the column. The analytes are

eluted from the solid medium with appropriate organic solvents and then are determined using different chromatographic techniques [25 - 27].

Compared to traditional LLE techniques, SPE is simpler, more convenient, and easier to automate [26, 27]. In addition, SPE possesses other distinct advantages [18, 22, 27]:

(i) It requires a lower volume of solvent than traditional LLEs;

(ii) It involves simple manipulations, which are not time consuming;

(iii) High, reproducible analyte recovery;

(iv) No foaming or emulsion problems;

(v) Easier operation;

(vi) Compatibility with instrumental analysis.

Sorbent materials are constantly being developed [19, 22], and can be selected according to the behavior of the analytes. Although method development for SPE is not as straightforward as for LLE [21], this technique offers appealing advantages. Because of SPE's high extraction efficiency, even very small sample volumes are sufficient [18, 22, 26], thereby reducing solvent consumption.

SPE is a very effective method, employing disposable extraction columns (Figure 1.1), which are available in a wide range of reservoir volumes, formats and sorbents.

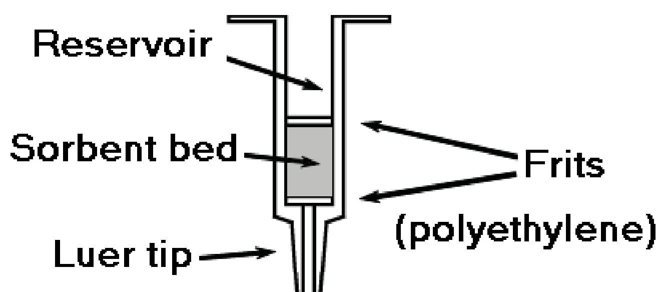


Figure 1.1. Components of an SPE column.

1.2. The classification of sorbents in an SPE method

1.2.1. Classic sorbents in SPE

The most important sorbents previously used in SPE are the existing forms of carbon, such as graphitized carbon blacks and porous graphitic carbon [25, 28]. These materials have a low specific surface area (~100 m^2 g^{-1}) and are characterized by their great adsorption capacity, and their chemical, thermal, and mechanical resistance [28]. However, the disadvantage of these sorbents is that some compounds show excessive, or even irreversible, adsorption [25, 28].

Silica chemically bonded with various groups has been the most common material in SPE. These sorbents can be classified as reversed-phase sorbents with octadecyl (C_{18}), octyl (C_8), ethyl (C_2), phenyl, cyclohexyl or as normal-phase sorbents with cyanopropyl, aminopropyl functional groups [24 – 26, 29]. Their interaction mechanisms are mainly based on reversed-phase interactions, such as Van der Waals forces, between the analytes and the stationary phase. However, silica sorbents have several disadvantages, such as low recovery extracting polar and apolar compounds, unstable at extreme pH values ($2 \leq pH \geq 8$), and the presence of some residual silanol groups [21, 26, 29].

1.2.2. Hydrophobic polymeric sorbents

The traditional hydrophobic macroporous polymers overcome some silica- and carbon-based sorbents problems, such as instability throughout the pH range and difficulties of analyte elution [19, 25, 26]. The most widely used polymeric sorbent is macroporous poly(styrene-divinylbenzene) (poly(St-DVB)), which presents a hydrophobic structure with specific surface area up to 500 m^2 g^{-1} [19, 26]. These sorbents interactions with the analytes are basically through Van der Waals forces, and the π–π sites of the aromatic rings that form the sorbent structure [19].

In the extraction of drugs or other types of the analytes using these poly(St-DVB) hydrophobic sorbents, one of the most important parameter is the specific surface area (m^2 g^{-1}), as the higher specific surface area enables the larger number of π–π sites interact with the analytes [19, 30]. Therefore, in order to improve the extraction efficiency of these hydrophobic sorbents the increase of the specific surface area is required.

In this doctoral dissertation, a wide range of polymeric sorbents, together with their chemical and morphological properties, which have been progressively developed in recent years to be used as SPE materials are described.

1.2.3. Hypercrosslinked hydrophobic polymeric sorbents

The new generations of hypercrosslinked polymeric sorbents are the most suitable because of their chemical stability and broad range of physico-chemical characteristics [19, 31]. The type of sorbent, its structure and its interactions with the solute are clearly related to the efficiency of the extraction process. Thus, when new sorbents are being developed, it is equally important to define both their chemical structure, which determines the type of interactions, and their morphology (specific surface area), which determines the mechanical properties of sorbent [19, 32, 33].

These sorbents are hypercrosslinked poly(styrene-divinylbenzene) (hc-poly(St-DVB)) polymers that are prepared with optimised conventional methods, but with a high loading of crosslinking agent (poly-DVB), which results in increase of specific surface areas of sorbents up to 2000 m^2 g^{-1} [32, 34]. Hypercrosslinked sorbents were obtained using method introduced by Davankov and Tsyurupa [35] in the early 1970s. Despite the fact that these hypercrosslinked sorbents materials, as well as their structure are not fully known, these sorbents are widely used because of their high specific surface area, and excellent sorption properties [19].

To the best of my knowledge, there are several studies have shown that the hypercrosslinked column HySphere-SH (hc-poly(St-DVB) sorbent, ~1000

$m^2\ g^{-1}$) gave better recoveries up to 96 %, than conventional macroporous column PLRP-S-10 (poly(St-DVB) sorbent, 500 $m^2\ g^{-1}$) with a lower crosslinking and twice lower surface area of sorbent, for the determination of phenols [36] and anilines [37].

1.2.4. Hydrophilic polymeric sorbents

The hydrophobic hypercrosslinked sorbents had high specific surface areas up to 2000 $m^2\ g^{-1}$, and finally their interactions with the analytes are only reversed-phase, which leads to poor sorption properties in the extraction of polar and medium-polarity compounds [38 - 40]. One of the solutions to this problem is to introduce polar groups into the sorbents structure. Thus, favoring the polar interaction between the analyte and sorbent, and at the same time improving the recoveries and repeatability of the analytical results [38]. In recent years, most of the research in the field of new SPE materials has focused on the development of new hydrophilic polymeric materials.

The hydrophilic sorbent can be obtained by copolymerizing two monomers, containing appropriate polar and/or medium polarity functional groups. These types of sorbents are macroporous copolymers that are mainly made from a balanced ratio of a hydrophilic monomer, such as N-vinylimidazole, and a crosslinked agent, which is generally divinylbenzene (DVB) [39, 40]. In this doctoral dissertation, I discuss several methods with hydrophilic sorbents and describe how they can be applied in SPE.

The first available hydrophilic polymeric sorbents were created out of methacrylate and DVB monomers, which enhances/promotes hydrophilic and lipophilic interactions between the analytes and sorbents, and finally improves sorption properties of polar and medium-polarity compounds [19, 41]. A polar monomer (methacrylate) and a crosslinking monomer DVB help to increase the specific surface area of sorbent up to 450 $m^2\ g^{-1}$. Furthermore, these hydrophilic polymeric columns were the series of Amberlite XAD-7 and Amberlite XAD-8 [19]. A recent study [41] compared the sorption properties of phenolic compounds with Amberlite XAD-8 (poly(methacrylate-DVB)

sorbent, 310 m^2 g^{-1}) and conventional Amberlite XAD-4 (poly(St-DVB) sorbent, ~750 m^2 g^{-1}) columns. It was demonstrated that the polarity of XAD-8 columns dominated (recovery up to 80 %) in the sorption properties of phenolic compounds as compared with the conventional larger specific surface area XAD-4 column.

More recently, another column Abselut Nexus with a hydrophilic sorbent based on the copolymer of methacrylate and DVB was tested. This hydrophilic sorbent has a higher surface area of 575 m^2 g^{-1} [19]. Abselut Nexus column has been used to clean up complex matrices, such as blood [42], urine [42], blood plasma [43, 44] and animal feed [45]. Thus, an extra advantage of Abselut Nexus column is that conditioning of the column before SPE extraction is not necessary. Hua He and co-workers [42] compared the feasibility of both Abselut Nexus (poly(methacrylate-DVB) sorbent, 575 m^2 g^{-1}) and Oasis HLB (poly(NVP-DVB) sorbent, 830 m^2 g^{-1}) columns without the conditioning step. The authors stated that the application of these columns redused the time of analysis and facilitated the analysis protocol in comparison to previously published methods. Based on these observations, Abselut Nexus column was selected for further studies without the step of sorbent activation with methanol and water. Overall, Abselut Nexus column provided the highest recoveries up to 101 %, and sufficiently good repeatability of results (RSD ≤ 5 %) for the determination of benzodiazepines, methadone and its major metabolites in samples of biological origin [42]. On the other hand, some studies [46, 47 - 50] demonstrated that the extraction efficiency decreased up to 70 - 85 % using an Abselut Nexus column without the conditioning step.

1.2.4.1. The new generation of hydrophilic polymeric sorbent

One of the hydrophilic sorbent recognized world-wide is Oasis HLB (Waters, Milford, MA, USA) [19, 22]. The Waters Corporation has designed Oasis HLB polymeric sorbent for sample extraction, which overcomes limitations of silica-based and hydrophobic polymeric sorbents used in SPE,

and finally streamlines the sample preparation process [22]. The key to this advancement was the development of a novel patented hydrophilic polymeric sorbent. This type of sorbent is macroporous copolymer, which was produced from a balanced ratio of a hydrophilic monomer – N-vinylpyrrolidone (NVP) and a crosslinking agent – divinylbenzene (DVB), and finally it has a specific surface area of 830 m^2 g^{-1} [19, 22]. For this reason the new generation sorbent is characterized by both hydrophilic and reversed-phase (lipophilic) sorption characteristics. A macroporous poly(NVP-DVB) sorbent is shown in Figure 1.2. A hydrophilic-lipophilic Oasis HLB column describes two major features of this sorbent [51]:

1. The unique abilities to remain wetted with water;
2. To retain a wide spectrum of compounds.

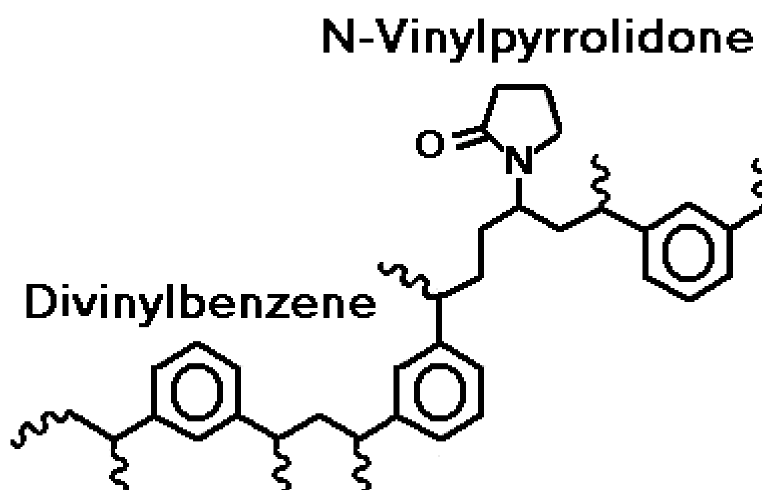


Figure 1.2. The structure of Oasis HLB hydrophilic polymeric sorbent.

The new generation of Oasis HLB column has been widely used in SPE. Some of this column application example is shown in Table 1.1. The most of the mentioned studies showed the potential of Oasis HLB in the extraction of the analytes with medium and high polarity. Basically, it has been used to extract and clean up several types of pharmaceuticals from aqueous samples [52 - 57] and biological matrices (the vitreous humor) [58].

Table 1.1. Sample preparation for the determination of drugs of abuse and related compounds in aqueous and biological samples and analysis using the Oasis HLB column.

Columns and sample extraction conditions	LOQ (ng L^{-1})	Recovery ± SD (%)	Technique, reference
Oasis 200 mg HLB Condition: 2 mL of n-heptane; 2 mL of acetone; 9 mL of MeOH; 8 mL of water; Loading of the sample: 100 mL of wastewater; Wash was not proposed; Elution: 8 mL of acetone.	BE – 10 COD – 20 DICOD – 20 MET – 50 MOR – 10 OXYC – 20 TRAM – 20 DIAZ – 10 OXAZ – 20 TEM – 10	BE 40 ± 5 COD 48 ± 2 DICOD 36 ± 1 MET 44 ± 2 MOR 29 ± 1 OXYC 50 ± 5 TRAM 20 ± 1 DIAZ 18 ± 1 OXAZ 42 ± 5 TEM 19 ± 7	LC-MS/MS, [52]
Oasis 200 mg HLB Condition: 10 mL of MeOH; 10 mL of water; Loading of the sample: 100 mL of wastewater; Wash: 8 mL of 5 % MeOH; Elution: 6 mL of MeOH.	COC – 0.2 BE – 0.2 AMP – 1.0 MAMP – 0.9 MDA – 1.0 MDEA – 2.1 MDMA –1.5 KET – 5.0 FENT – 4.0	COC 86 ± 6 BE 92 ± 6 AMP 70 ± 7 MAMP 80 ± 4 MDA 74 ± 5 MDEA 101 ± 5 MDMA 88 ± 6 KET 85 ± 5 FENT 80 ± 4	LC-MS/MS, [53]
Oasis 200 mg HLB Condition: 5 mL of MeOH; 5 mL of water; Loading of the sample: 200 mL of wastewater; Wash: 3 mL of water; Elution: 8 mL of MeOH.	HER – 20.0 6ACM – 3.1 MOR – 7.1 COD – 2.5 MET – 0.3 FENT – 1.7	HER – 65 6ACM – 90 MOR – 83 COD – 94 MET – 75 FENT – 72	LC-MS/MS, [54]
Oasis 500 mg HLB Condition: 3 mL of MeOH; 3 mL of water; Loading of the sample: 100 mL of wastewater; Wash: 3 mL of 5 % MeOH; Elution: 8 mL of MeOH.	COC – 0.5 BE – 1.0 EME – 20.0	COC 96 ± 6 BE 92 ± 2 EME 73 ± 5	LC-MS/MS, [55]
Oasis 60 mg HLB Condition: 2 mL of MeOH; 2 mL of water; Loading of the sample:	AMP – 4.4 MAM – 2.9 MDMA – 2.4 MDA – 9.7 MDEA – 2.2	AMP – 86 MAM – 87 MDMA – 84 MDA – 76 MDEA – 90	LC-MS/MS, [56]

100 mL of wastewater; Wash was not proposed; Elution: 4 mL of MeOH.	EPH – 3.1 VENL – 5.0	EPH – 81 VENL – 125	
Oasis 200 mg HLB Condition: 5 mL of MeOH; 5 mL of water; Loading of the sample: 250 mL of surface water; Wash: 10 mL of water; Elution: 6 mL of MeOH.	COC – 0.1 BE – 0.2 EM – 1.4 AMP – 0.4 MAM – 0.8 MDMA – 0.4 MDA – 1.4 MOR – 0.1 HER – 0.2 6ACM – 0.3 COD – 0.1 MET – 0.1	COC 121 ± 12 BE 100 ± 9 EME 96 ± 10 AMP 96 ± 10 MAM 113 ± 11 MDMA 125 ± 8 MDA 105 ± 10 MOR 75 ± 10 HER 100 ± 12 6ACM 85 ± 14 COD 113 ± 8 MET 100 ± 9	LC-MS/MS, [57]
Oasis 60 mg HLB Condition: 2 mL of MeOH; 2 mL of water; Loading of the sample: 0.5 mL of the vitreous humor at pH 9.0; Wash: 2 mL of 20 % MeOH; Elution: 2 mL of dichloromethane.	The limit of quantification was set at 100 ng mL^{-1} for all the drugs studied.	COC – 97.0 BE – 91.9 COE – 94.4 MOR – 90.5 COD – 94.6 6ACM – 97.1 MET – 78.6	HPLC-DAD, [58]

Abbreviations of the analytes: amphetamine (AMP), 6-acetylmorphine (6ACM), benzoylecgonine (BE), cocaethylene (COE), cocaine (COC), codeine (COD), diazepam (DIAZ), ecgonine methyl ester (EME), ephedrine (EPH), fentanyl (FENT), heroin (HER), ketamine (KET), methamphetamine, (MDA), 3,4-methylenedioxyamphetamine (MAM), 3,4-methylenedioxy-N-methyl-amphetamine (MDMA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), methadone (MET), morphine (MOR), oxazepam (OXAZ), oxycodone (OXYC), temazepam (TEM), tramadol (TRAM), venlafaxine (VENL).

It can be summarized that usually the analytes were analyzed through the usage of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Since the most drugs of abuse were present in wastewater samples at ng L^{-1} range or higher (at 100 ng mL^{-1}) in the vitreous humor [58], the effective sample pre-concentration procedure is of a crucial importance in order to make samples amenable to LC-MS/MS quantification. This is most commonly carried out through the use of an SPE method with Oasis HLB

column. The procedure subsequently involves: extraction of sample, evaporation of extract, reconstitution and injection into the LC-MS/MS system. However, not all currently used SPE methods for the analytes extractions are characterised by the sufficient extraction efficiencies and the sensitivity of the developed method. The sample preparation method selected by the D. Hummel et al (Table 1.1) gave insufficient recoveries of the analytes, which were in the range of 18 – 50 %, while limits of quantification were higher than 10 ng L^{-1} [52]. Therefore, this method of analyte pre-concentration was ineffective for the determination of drugs of abuse in wastewater samples. On the other hand, the effective extraction of the analytes highly depends on the used eluent. In the most methods the use of pure methanol or dichloromethane as effective eluent is recommended, and sufficiently high recoveries of the analytes (≥ 65 %) were obtained [53 - 58]. Moreover, the cleanest extracts of drugs of abuse were achieved using all methods [52 - 58].

To the best of my knowledge, there are several studies on the comparison of different sorbents. Maria J. López and co-workers [59] compared several columns for the extraction efficiency of estrogens and progestogens from the water samples. The experiments were performed using polymeric columns, such as: HySphere (hc-poly(St-DVB), >1000 m^2 g^{-1}), PLRP-S-10 (poly(St-DVB), 500 m^2 g^{-1}), and Oasis HLB (poly(NVP-DVB), 830 m^2 g^{-1}). Sufficiently higher recoveries in the range of 63 - 100 % for most of the analytes were achieved on HySphere and PLRP-S-10 columns, but Oasis HLB provided the best overall recoveries [59]. The following results clearly show that polarity of the sorbent and its specific surface area contributed to the sorption of the analytes.

Several other research studies also reported that Oasis HLB column is better able to analyse various types of drugs, amines or pesticides, than the conventional columns. Consequently, most of the authors compared several columns, such as: Isolute C_{18} (silica-based phase with bonded octadecyl ligand, sorbent mass of 200 mg) [60], Hypercarb (graphitized carbon black, sorbent mass of 80 mg) [61], Amberlite XAD-4 (poly(St-DVB) sorbent, ~750 m^2 g^{-1})

[62], and Lichrolut EN (hc-poly(St-DVB) sorbent, 1200 m^2 g^{-1}) [60, 61, 63] for the analytes extraction efficiency. The following studies tested how both specific surface area and polarity of the sorbent contributed to the sorption of the analytes. These results again confirm that the most suitable sorbent for extracting polar and medium-polarity compounds should have a proper balance between specific surface area and polar group content. In all cases, the better results were obtained for Oasis HLB column (absolute recoveries ≥ 70 %) [60 - 62], as well as the equivalent results for the Lichrolut EN column [61] was obtained.

1.2.5. Mixed-mode sorbents in SPE

1.2.5.1. Classic mixed-mode sorbents

The analytes that are ions, or which can be converted to ions by adjusting the sample pH, are adsorbed onto the sorbent due to ion-exchange mechanism [20, 29]. Mixed-mode sorption mechanisms have been observed during the SPE method of the basic analytes using conventional reversed-phase silica-based sorbent containing residual silanols groups at the sample pH when both analyte and sorbent are ionised [24, 25, 29].

The analytes usually contain functional groups and many of them can be of cationic or anionic form depending on the sample pH. Around 80 % of sedative-hypnotic drugs contain nitrogen (amino and/or amide groups) that may be readily protonated [3, 6]. This feature of the drugs has been exploited to make sorbents that contain reversed-phase alkyl chains and ion exchanger interacted on the same solid-phase. The traditional commercially available, and still popular sorbents containing octyl (C_8) chains and ion-exchange groups are: Bond-Elut Certify I (silica-based/C_8-benzenesulfonic acid), and Bond-Elut Certify II (silica-based/C_8-quaternary amine), both columns are from Varian Corporation [24, 29].

C. S. Torre and co-workers [64] compared the performance of two different SPE columns, such as Bond-Elut Certify I, and Chem Elut

(diatomaceous earth) for the multi-residue extractions of different pharmaceuticals from whole blood. These two columns presented similar recoveries for the neutral analytes in the range of 21 - 76 %, whereas basic pharmaceuticals provided higher recoveries up to 97 % with Bond-Elut Certify I column. The use of mixed-mode Bond Elut Certify I columns showed advantages comparing with Chem Elut columns: cleaner extracts, sufficient precision (RSD ≤ 15 %) and less solvent consumption [64].

1.2.5.2. The new generation of a mixed-mode polymeric sorbent

The new generation of Oasis MCX column is designed to overcome the limitations of traditional silica-based mixed-mode SPE columns [20, 65]. The Oasis MCX extraction column contains a mixed-mode, water-wettable, polymeric sorbent produced by a Waters Corporation, and patented the novel process for the enhancement of the reproducibility of the analytical results. This commercially available sorbent is produced of 30 and 60 µm particles with reversed-phase (hydrophilic-lipophilic) and strong ion-exchange functionalities [20, 22]. The Oasis MCX is composed of the strong anion-exchange sulfonic acid groups, which are on the surface of the Oasis HLB (poly(NVP-DVB)) sorbent (Figure 1.3.) [22, 66]. The anionic groups from the sorbent improve ion-exchange interactions with cationic analytes in the sample. The major difference between the Oasis HLB and Oasis MCX columns is the high selectivity of the Oasis MCX sorbent for basic compounds [67].

In addition to the pH stability common to polymers, Oasis MCX has far greater binding capacity than other hydrophilic polymers or silica-based mixed-mode SPE sorbents. The ability to fully manipulate pH (pH 0 – 14) during the development processes and the use of SPE method on mixed-mode sorbent enable not only fast straightforward method development, but also help to ensure very rugged and robust procedures [20, 22, 68].

The new generation of a mixed-mode SPE sorbent is characterized by sufficient sorption of polar and aromatic compounds due to hydrophilic-

lipophilic interactions, and strong ion-exchange groups for basic compounds due to ion-exchange interactions [20, 22]. Consequently, matrix interferences and the analytes were eluted separately during the washing and the elution steps, respectively, by carefully choosing the sample pH, and the solvent in each SPE step [67 - 71]. The major interactions of the analyte with the investigated sorbent are shown in Figure 1.3.

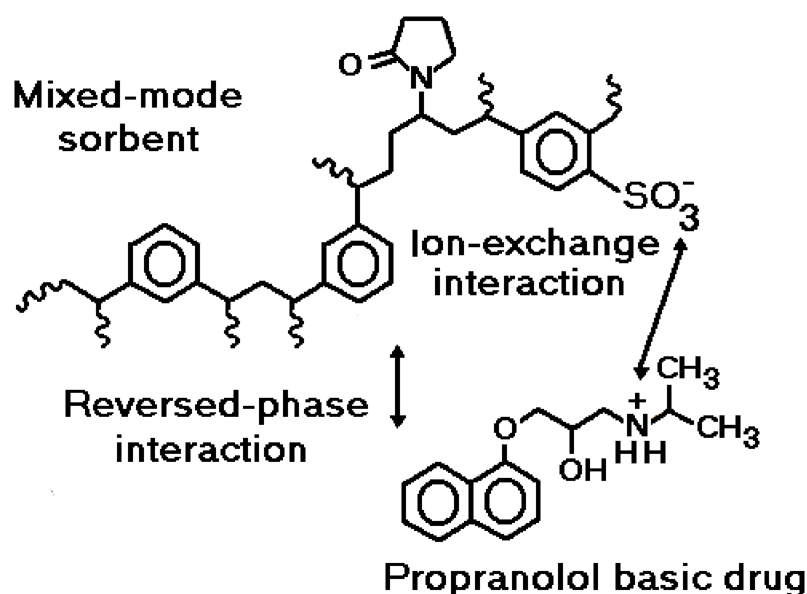


Figure 1.3. The structure of Oasis MCX sorbent and two major interactions of basic drug on Oasis MCX sorbent.

On the basis of the interactions presented in Figure 1.3, it can be stated that evaluating ion-exchange phases, the sample pH during the loading and the eluting step is very important. The sample pH during the loading step has to be two pH units lower than the pK_a of the analyte, and one or two pH units higher than the sorbent. At this pH, approximately 99 % of the groups are charged [22]. Furthermore, a very selective analyte and sorbent interactions can occur when ion-exchange is used as the predominant sorption mechanism. Actually, a mixed-mode mechanism is the result of the strong ionic interaction between the negatively charged groups on the sorbent and the positively charged groups on the analyte (e.g. propranolol) along with the reversed-phase (hydrophilic-lipophilic) interactions between the organic portion of the analyte of interest

and the organic basis of the polymer itself. Of course, ionic interactions are much stronger than reversed-phase interactions (Table 1.2.) [26], but both types of interactions occur to some degree.

Table 1.2. Energetic of interactions in SPE.

Interaction type	Energy (kcal moL^{-1})
Dispersion	1 – 5
Dipole-induced dipole	2 – 7
Dipole-dipole	5 – 10
Hydrogen bonding	5 – 10
Ionic	50 – 200
Covalent	100 – 1000

The new generation of Oasis MCX column has been widely used in SPE, and some of applications are shown in Table 1.3. The ion-exchange groups provide high selectivity for basic drugs allowing to obtain clean extracts from water [72 - 75], urine [76] and blood plasma [77] for analysis by LC-MS/MS. Mixed-mode Oasis MCX columns gives high and reproducible recoveries for the basic and most of the neutral compounds (≥ 81 %). Using acidic sample solution (pH ~ 2) the analytes in the cationic form were adsorbed onto the sorbent due to ion-exchange interactions. In addition, in the washing step, which optionally includes acidic aqueous solution or neutral water (pH ~ 7), the most water-soluble interferences poorly attached onto the sorbent by hydrophilic-lipophilic interactions were removed.

According to the previously published results, the most of the target analytes were extracted in their cationic form [72 - 77], so they interacted with Oasis MCX specifically through ionic interactions. Effective use of a mixed-mode SPE sorbent for extraction a wide range of the analytes from the complex matrices requires high capacity of both reversed-phase for the sorption of interferences, and ion-exchange phase for the selective sorption of the analytes. Additionally, the major advantage of a mixed-mode polymeric material is ease of use, since there is no need to keep the phase moistened to

maintain interactions [72 - 77]. The Oasis MCX column was used for all stages of drugs monitoring: screening, identification/confirmation, and quantification.

Table 1.3. Sample preparation for the determination of drugs of abuse and related compounds in aqueous and biological samples and analysis using the Oasis MCX column.

Columns and sample extraction conditions	LOQ (ng L^{-1})	Recovery ± SD (%)	Technique, reference
Oasis 60 mg MCX Condition: 6 mL of MeOH; 3 mL of water; 3 mL of water (pH 2); Loading of the sample: 50 mL of water (pH 2); Wash was not proposed; Elution: 3 mL of MeOH; 3 mL of MeOH:2 % NH_3.	COC – 1.4 BE – 1.9 COE – 0.9 AMP – 5.4 MAM – 3.7 MDA – 8.7 MDMA – 6.3 MDEA – 4.2 MOR – 3.9 6ACM – 5.3 MET – 1.1	COC 96 ± 5 BE 107 ± 9 COE 109 ± 4 AMP 110 ± 5 MAM 112 ± 7 MDA 102 ± 3 MDMA 104 ± 2 MDEA 107 ± 4 MOR 88 ± 7 6ACM 106 ± 5 MET 112 ± 7	LC-MS/MS, [72]
Oasis 150 mg MCX Condition: 6 mL of MeOH; 3 mL of water; 3 mL of water (pH 2); Loading of the sample: 50 mL of water (pH 2); Wash: 5 mL of water; Elution: 8 mL of MeOH:2 % NH_3.	COC – 3.0 BE – 0.3 COE – 2.0 AMP – 5.4 MAM – 7.0 MDMA – 8.0 MDA – 9.1 MDEA – 4.0	COC – 92 BE – 90 COE – 88 AMP – 85 MAM – 85 MDMA – 95 MDA – 90 MDEA – 88	LC-MS/MS, [73]
Oasis 60 mg MCX Condition: 6 mL of MeOH; 4 mL of water; 4 mL of water (pH 2); Loading of the sample: 50 mL of water (pH 2); Wash: 3 mL of water; Elution: 4 mL of MeOH; 4 mL of MeOH:5 % NH_3.	COC – 1.0 BE – 1.0 AMP – 2.0 MAM – 1.0 MDMA – 1.0 MET – 1.0 6ACM – 2.0	COC 102 ± 6 BE 87 ± 3 AMP 102 ± 6 MAM 99 ± 4 MDMA 100 ± 4 MET 103 ± 3 6ACM 92 ± 4	LC-MS/MS, [74]
Oasis 60 mg MCX Condition: 2 mL of MeOH; 2 mL of 2 % formic acid; Loading of the sample:	COC – 0.7 BE – 0.7 COE – 0.9 AMP – 5.1 MAM – 0.6	COC 91 ± 2 BE 103 ± 19 COE 88 ± 2 AMP 81 ± 17 MAM 86 ± 2	LC-MS/MS, [75]

100 mL of water (pH 1.8); Wash: 2 mL of 2 % formic acid; 2 mL of 0.6 % formic acid in MeOH; Elute: 3 mL of MeOH:7 % NH_3.	MDA – 4.2 MDMA – 0.7 MDEA – 1.1 6ACM – 2.6 COD – 3.7 MOR – 4.8 MET – 0.8 FENT – 0.8 DIAZ – 6.0 VENL – 3.5	MDA 90 ± 3 MDMA 86 ± 3 MDEA 86 ± 6 6ACM 139 ± 9 COD 103 ± 4 MOR 85 ± 6 MET 97 ± 6 FENT 96 ± 7 DIAZ 105 ± 13 VENL 91 ± 15	
Oasis 30 mg MCX Condition: 2 mL of MeOH; 2 mL of water; Loading of the sample: 0.5 mL of urine (pH 2.4); Wash: 2 mL of water; 1 mL of 0.1 M HCl; 2 mL of MeOH; Elution: 2 mL of dichloromethane:2-propanol:NH_3 (80/20/2).	LOQ (ng mL^{-1}) COC – 3.5 BE – 2.9 6ACM – 3.2 COD – 7.0 OXYC – 5.0 MOR – 7.9	Recoveries of 90 – 95 %	LC-MS/MS, [76]
Oasis 30 mg MCX Condition: 2 mL of MeOH; 1 mL of water; 2 mL of 10 mM citrate buffer (pH 3.0); Loading of the sample: 0.25 mL of blood plasma (pH ~ 3.0); Wash: 0.5 mL of water (pH 3.0); Elution: 0.5 mL of 0.5 % ammonium acetate in MeOH solution (1:20 v/v)	The lower limit of quantification was set at 5.0 ng mL^{-1} for all the drugs studied.	6ACM 89 ± 4 HER 91 ± 5 MET 81 ± 5 MOR 90 ± 10	LC-MS/MS, [77]

Abbreviations of the analytes: amphetamine (AMP), 6-acetylmorphine (6ACM), benzoylecgonine (BE), cocaethylene (COE), cocaine (COC), codeine (COD), diazepam (DIAZ), fentanyl (FENT), heroin (HER), methamphetamine, (MDA), 3,4-methylenedioxyamphetamine (MAM), 3,4-methylenedioxy-N-methyl-amphetamine (MDMA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), methadone (MET), morphine (MOR), oxycodone (OXYC), venlafaxine (VENL).

To the best of my knowledge, there are several studies on the comparison of different sorbents: Oasis HLB, Oasis MCX, XAD-16 and XAD-16/7. For example, Y. Yu and co-workers [78] compared the performance of four different hydrophilic polymeric SPE sorbents (Oasis HLB, Oasis MCX, XAD-16 and XAD-16/7) in the multi-residue extraction of 10 different pharmaceuticals in wastewater samples. The authors proposed two active methods by Oasis HLB and Oasis MCX, when the samples pH was adjusted to pH 2, and two passive methods by XAD-16 and XAD-16/7. These four sorbents are characterized by the similar recoveries for the neutral analytes, whereas for basic pharmaceuticals higher sorption properties with Oasis MCX were obsereved. The mean recoveries of the target analytes obtained by Oasis MCX ranged from 37.6 to 87.6 % [78]. Furthermore, no negative effect on the recoveries of the target analytes without the washing step was observed. On the other hand the authors were unable to remove neutral and/or acidic compounds, which were attached by the reversed-phase interactions to the Oasis MCX sorbent.

1.3. The factors influencing the efficiency of an SPE method

1.3.1. Selectivity of the sorbent

The extensive range of sorbent chemistry provides one of the most important parameter for the SPE – high selectivity. Selectivity is the level to which an extraction technique can separate the analyte of interest from the interferences in the original sample [19 - 21, 23, 29]. The highly selective nature of SPE is due to two factors [19, 26, 29]: extraction sorbent chemistry, which offers unique and distinctive sorption properties to determine a wide range of the analyte characteristics, and extraction mechanism.

In LLE, the two liquids phases must be immiscible with each other. Thus, an aqueous sample cannot be extracted with pure methanol. However, in SPE one phase is a solid sorbent, and is therefore by definition immiscible with any extraction solvent used. This results in a high variety of possible sorbent

and solvent combinations, with potential to achieve highly selective extractions [19 - 21, 29, 79].

1.3.2. Capacity of the sorbent

The capacity of the sorbent is defined as the total mass of strongly adsorbed analyte that can be adsorbed by a given sorbent mass under the optimum conditions [29]. When determining the amount of the sorbent required for an extraction, it is essential to consider not only the capacity requirement for the analyte, but also for any undesired sample components (interferences), which may be co-extracted with the analyte using the same sorption mechanism [29, 80]. Thus, the proper choice of a more selective sorption mechanism increased the relative capacity of the sorbent, as well as reduced the amount of the sorbent required for a given extraction. All of this has a benefit in reducing the amount of solvent used, and increasing the final concentration of the analyte in the extract [29, 80, 81]. Maximum selectivity and capacity are achieved through the proper sorbent interaction with functional groups present on the analyte, but not on the other sample components (interferences).

Typically, non-polar and polar traditional SPE sorbents have a capacity of 1 – 5 % of the sorbent mass (e.g. sorbent mass of 100 mg can strongly adsorb up to 5 mg of the analyte under the optimal conditions) [29]. In addition, a mixed-mode sorbent capacity measured in milli-equivalents per gram of sorbent (meq g^{-1}) is based on the amount of available ionic groups on the sorbent. For example, the Oasis MCX has an ion-exchange capacity of 1.0 meq g^{-1}. This means that 1 g of Oasis MCX column can retain up to 1.0 mmol of basic (cationic form) compound [20, 66].

1.4. Practical aspects of an SPE method development

Once an appropriate sorbent type and sorption mechanism has been selected, the main steps of the method have to be considered. An SPE procedure typically consists of six steps (Figure 1.4.) [20, 22 - 25, 29, 81]:

1. The sample pre-treatment;
2. SPE sorbent solvation (condition);
3. SPE sorbent equilibration;
4. The sample application;
5. SPE sorbent washing (interferences elution step);
6. The analytes elution from the SPE sorbent.

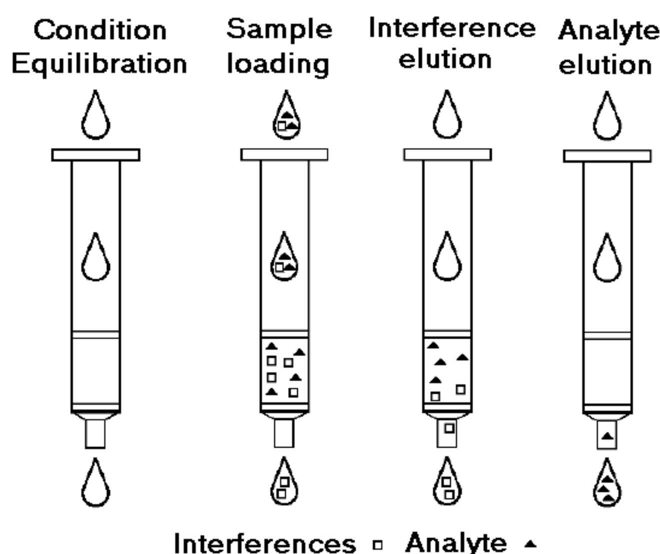


Figure 1.4. Typical SPE procedures.

The sample pre-treatment

The sample pre-treatment may simply be a dilution of the sample with an appropriate solvent in order to reduce viscosity [29], or it could involve addition of a buffer to control the pH of the sample prior to sorption by non-polar or ion-exchange sorbents [20, 24, 29, 81]. Thus, the analytes are in solution and are available for interactions with the sorbent. If the sample contains particulate matter to which the analytes absorbed, it is essential to first desorbt the analytes from the particulates before applying the sample to the SPE column [22, 29]. Similarly, if the analytes are bound to large molecules in the sample (e.g. sedative-hypnotic drugs bound to proteins present in biological fluids [10, 15]), these bindings must be disrupted to achieve high extraction efficiencies of the analytes. Usually it can be achieved by the addition of a

small amount (in the range of 50 – 200 μL) of the organic solvent to the sample. The most commonly used organic solvents are methanol [82, 83], acetonitrile [84, 85], and isopropanol [86]. In addition, pH adjustment of the sample was proposed [87] in order to improve the extraction efficiencies of the analytes.

It is important to ensure that the ionic strength of the sample would not be so high as to weaken the ionic interactions on a mixed-mode sorbent. To overcome this problem, the authors recommended dilution of the sample with low ionic strength buffer (e.g. 0.05 or 0.1 M buffer) [18, 29]. Failure to deal with high and variable ionic strength samples can result in the low recoveries up to 60 %, when mixed-mode sorbents were used [20, 22] for extraction of ionizable drugs from the biological fluids.

SPE sorbent condition and equilibration

Prior to the sample loading to the column, the SPE sorbent should be so prepared that matched the conditions of the pre-treated sample. For this purpose, two steps of preparation are used: solvation (conditioning) and equilibration. This will ensure the sufficiently high recoveries of the analytes [20, 29, 81]. For example, if the sample pH is adjusted up to the ionization state of the analytes, the buffer of the same pH and the ionic strength has to be used to equilibrate the sorbent [20, 29].

The sample loading to the column

The sample is applied to the SPE column, where the analytes of interest bind to the sorbent and under the ideal conditions the most of the sample matrix (interferences) is discarded to the waste [24, 29]. An alternative method [81], which is less frequently used, is the sorption of interferences materials on the sorbent whereas the analytes of interest are collected. In both cases, the liquid sample is passed through the SPE sorbent. The extraction efficiency is highly dependent on the flow rate of the liquid sample [29, 81], which should be reasonably constant. The flow rate is very important part of the method

development process. According to the literature, most authors proposed to use the flow rate of 1 mL min^{-1}, when a small amount of sorbent in the range of 30 – 60 mg [22, 29, 81] was analyzed.

Elution of the interfering compounds

The sorbent is washed with a suitable solvent and/or mixture of solvents in order to remove interfering matrix compounds that might interfere with the analytes during the following chromatographic step. On the other hand, the analytes of interest must be unchanged and they have to interact with the investigated sorbent. In addition, sufficiently high extraction efficiencies of the analytes, and their sorption properties (e.g. liquid sample pH and ionic strength) should be maintained during this step [24, 29, 81].

The analytes elution

The final step of the process is the quantitative elution of the analytes from the SPE sorbent. A good eluent should be strong enough to elute the analytes of interest in a limited volume, but on the other hand the stronger the eluent, the more interfering (matrix) compounds are also eluted [24, 29, 81]. Therefore, an optimized wash and elution steps are necessary.

The authors also recommend [20, 24, 29, 81] to use a solvent or mixture of solvents in which the analytes are highly soluble. Furthermore, the elution solvents should discontinue both primary and any secondary interactions by which the analytes are adsorbed on the sorbent. If an evaporation step is used after the elution [29, 81], it's possible to elute analytes with a stronger solvents of the larger volume and thus to improve extraction efficiencies of the analytes.

All of the SPE steps mentioned above are subjected to the optimization during the method development with regards to the type of the sorbent, sample pH value, solvents types, solvents ionic strength. All steps are dependent on the matrix used as well as the physical and chemical properties of the analytes [20, 22 - 25, 29, 81].

1.5. Sedative-hypnotic drugs – benzodiazepines

Depression is a common mental disorder that affects about 120 million people worldwide [3, 88, 89]. According to the World Health Organisation this mood disorder will be the second leading contributor to the global burden of disease, calculated for all ages and both sexes by the year 2020. Depression is a chronic or recurrent illness that affects both economic and social functions of the patient and can eventually lead to suicidal behaviour [3, 6, 89].

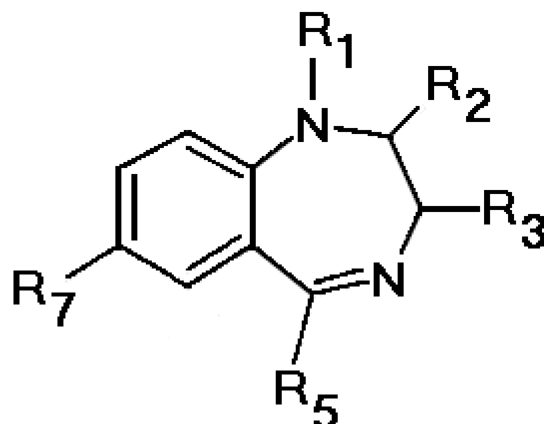
Between 1960 and 1980, depression was treated with barbiturates and tricyclic antidepressants. The side effects, high toxicity and strong drug – drug interactions of these compounds in combination with the remarkable advances in the understanding of the central nervous system lead to the imposition of several benzodiazepines [3, 6].

Benzodiazepines are a large class of drugs with some 35 controlled by the United Nations Convention (1971) [4], and many more are available throughout the world. Over 50 years of these drugs have been investigated worldwide for biological activity since the introduction of chlordiazepoxide in 1960 year [3, 16]. Benzodiazepines are an important class of drugs with a broad range of therapeutic effects, including sedative-hypnotic, anxiolytic, muscle-relaxant, and anticonvulsant [3, 6, 90]. The major advantage of benzodiazepines, except the barbiturates, was the lower risk of toxicity in overdose, although prolonged use of benzodiazepines led to abuse and dependence in some pre-disposed individuals. Due to their positive features these medications are among the most commonly prescribed drugs today in Europe and in Lithuania [1, 2]. Because of their wide use, benzodiazepines have a potential of interaction with other central nervous system depressants drugs, which can result in life-threatening conditions, and are often found in a combination with other drugs/narcotics in drug-related fatalities [91 - 94]. For this reason, the analysis of benzodiazepines is of great interest to forensic and clinical toxicologists.

These medications are clinically effective and represent a large range of potencies at low doses ranging from less than 1 – 30 mg to over 100 mg,

resulting in whole blood concentrations ranging from sub-nanogram per milliliter (2 – 100 ng mL^{-1}) to near-microgram per milliliter levels [3, 95 - 97]. Therefore, it is essential for a laboratory to develop a rapid and sensitive method for the determination of this class compounds. The structures of selected classical benzodiazepines are shown in Table 1.4.

Table 1.4. The structures of selected classical benzodiazepines.



Benzodiazepines	R_1	R_2	R_3	R_5	R_7
Bromazepam	-H	=O	-H	-2'-pyridyl	-Br
Clonazepam	-H	=O	-H	-2-Cl-phenyl	$-NO_2$
Diazepam	$-CH_3$	=O	-H	-Phenyl	-Cl
Fludiazepam	$-CH_3$	=O	-H	-2-F-phenyl	-Cl
Flunitrazepam	$-CH_3$	=O	-H	-2-F-phenyl	$-NO_2$
Lorazepam	-H	=O	-OH	-2-Cl-phenyl	-Cl
Nordiazepam	-H	=O	-H	-Phenyl	-Cl
Nitrazepam	-H	=O	-H	-Phenyl	$-NO_2$
Oxazepam	-H	=O	-OH	-Phenyl	-Cl
Temazepam	$-CH_3$	=O	-OH	-Phenyl	-Cl

1.5.1. Chromatographic methods for the determination of benzodiazepines

In recent years, several chromatographic methods for the determination of benzodiazepines in biological matrices, such as, human plasma [15, 16, 98 - 105], whole blood [14 - 16, 106 - 119], urine [13, 15, 16, 42, 118, 120 - 122], oral fluid [123 - 125] and human hair [15, 126] have been developed. These

analytical methods have been employed to determine the concentrations of drugs in biological specimen including high-performance liquid chromatography (HPLC) with ultraviolet or diode-array detections [15, 16, 42, 100 - 102, 104, 106], and finally with tandem mass spectrometry (LC-MS/MS) [13 - 16, 98, 99, 110, 111, 115, 117 - 121, 124 - 126].

Among the widely used detectors, ultraviolet is still the most popular for HPLC benzodiazepines assays due to high absorption in the range of 200 – 240 nm. However, the use of ultraviolet detection for the determination of several benzodiazepines in biological specimens provided a high limit of detection values within the range 1.0 – 200.0 ng mL^{-1} [15, 100 - 102, 104]. On the other hand, the combination of MS/MS provides a good example of the analytes separation mode of HPLC with sufficiently high sensitivity and specificity of MS detection. The limit of detection was found sufficiently low within the range 0.1 – 1.0 ng mL^{-1} for the determination of most benzodiazepines along with other drugs of abuse in whole blood interfacing MS/MS with electrospray positive ionization mode [14, 110, 115, 117, 119]. These analytical methods enable to reach the highest specificity for the major analytes and meet the requirements of good laboratory practice, especially when applied to pharmacodynamic investigations.

As recently reviewed by R. Verplaetse and co-workers [118], LC-MS/MS is the technique of choice for the determination of twenty-six benzodiazepines, because of its sufficiently good sensitivity (LOQ – 2.0 ng mL^{-1}) without need of the analytes' derivatisation. However, LC-MS/MS methods have to face the well-known problem of matrix effects when analyzing complex samples, such as biological specimens. Particularly, strong signal suppression effects (11 – 110 %) have been reported during the analysis of benzodiazepines and benzodiazepine-like hypnotics in urine and whole blood [118], and finally in human hair [126] samples. Though deuterated internal standards are available for most of these drugs/metabolites, they cannot always completely compensate this problem [15, 118, 126], and even so, they do not avoid the inherent loss of sensitivity.

In both clinical and forensic toxicology, gas chromatography/mass spectrometry using electron impact (GC/EI-MS) technique is still the method of choice as it is sensitive and selective, providing the best separation of benzodiazepines and their metabolites, which are volatile under GC conditions [16, 107, 108, 112, 127]. In addition, the GC with electron capture detection has previously been used to measure several analytes in human plasma [103] and whole blood [109] samples due to its excellent sensitivity (LOQ $\leq$ 10.0 ng mL^{-1}) to compounds with high electron affinities. However, co-elution interferences from biological matrix species reduce the usefulness of this method.

Gas chromatography with negative ion chemical ionization (GC/NICI-MS) is a sensitive and selective MS ionization technique for the analysis of benzodiazepines. The sensitivity of GC/NICI-MS is better than the conventional GC/EI-MS technique and it has been successfully applied for the detection of different benzodiazepines in human plasma [105] and whole blood samples [113]. The mechanism of NICI-MS is characterized as a "soft" ionization technique, whereby NICI-MS spectra exhibit information about prominent molecular anions and therefore about their molecular weight. The use of NICI-MS can improve sensitivity by a factor of several thousands [128] when compared with positive ion chemical ionization MS and EI-MS detection, especially for the determination of compounds with electronegative moieties, such as halogen and/or nitro atoms in the xenobiotic itself, or after appropriate derivatization [129, 130]. The high sensitivity and selectivity of GC/NICI-MS confer a significant benefits in terms of increased signal to noise rations and this technique also allow a reliable analysis from microsample (e.g. 0.1 mL of whole blood) [113].

Derivatization procedures

Derivatization steps are necessary to improve the gas chromatographic characteristics of polar and non-polar compounds as well as drugs [16, 131]. Furthermore, mass spectra of several compounds are altered during

derivatization step, so they contain more typical ions, e.g. the molecular ion. The sensitivity of the method after derivatization can be improved by introduction of halogen and/or nitro atoms into the molecule if NICI-MS mode is used [113, 132, 133].

For the determination of benzodiazepines by GC/NICI-MS analysis, a derivatization step prior the measurement is necessary for improving their chromatographic performance and resolution [105, 113]. Hence, the analytes after derivatization allow a significant reduction in peak tailing, providing much sharper chromatographic peaks than corresponding underivatized benzodiazepines. It significantly increases the signal-to-noise ration of the peaks, and reduces the detection limits of the related analytes [16, 127]. Either N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) or N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) can be used as a silylation reagent leading to the formation of trimethylsilyl (TMS) derivatives. A catalyst, such as trimethyl-chlorosilane (TMCS), is usually added to enhance the derivatization process performance [108, 134]. The silylation reagent N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) has also been widely used as a derivatization reagent for the analysis of benzodiazepines [16, 107 - 109, 113]. In general, compared with other silylated derivatives, t-butyldimethylsilylation (TBDMS) derivatives formed by MTBSTFA have superior properties, such as more specific mass fragmentation, greater hydrolytic stability and higher mass-to-charge ratio (m/z) values in both EI-MS and NICI-MS ionization modes [108, 127, 134].

To the best of my knowledge, there are several studies on the silylation of benzodiazepines by MTBSTFA with different derivatization conditions and solvents: n-butyl acetate at 90 °C for 30 min [113], ethyl acetate at 80 °C for 30 min [114], ACN at 80 °C for 30 min [107 - 109]. However, in some studies [105, 122] the application of BSTFA in pyridine (at 1:1 v/v) at 50 °C for 30 min [105] or BSTFA with 1 % TMCS in ethyl acetate (at 1:1 v/v) at 70 °C for 30 min [122] for the derivatization of several benzodiazepines also has been demonstrated.

1.5.2. Extraction methods for the analysis of benzodiazepines

Extraction methods for the separation of benzodiazepines and their metabolite from biological matrices can be devided into three distinct types:

1. Direct introduction into a chromatographic system;
2. Liquid-liquid extraction;
3. Solid-phase extraction.

Direct injection

Direct determination techniques used for benzodiazepines involve dialysis [135], and clean chromatograms can be obtained without sample pretreatment. Direct injection of sample after protein precipitation (blood) [14, 136] and filtration (urine) [13, 121] also has been applied for LC-MS/MS. While these techniques avoid an extraction step the application of more instrumentation is required. However, direct injection of complex biological samples leads to contamination of chromatographic columns, and impairing their performance. Direct injection methods are complicated due to significant matrix interferences and relatively high concentrations of several benzodiazepines (LOQ of 0.1 – 50 ng mL^{-1}) and insufficient repeatability of the results (RSD up to 15 %) in biological specimens [13, 14, 121]. In order to avoid problems identified, sample clean-up procedure is required.

Liquid-liquid extraction

A simple and fast sample preparation is a very important step in the development and application of analytical methods for the analysis of benzodiazepines and their metabolites in biomedical materials. Conventional LLE [15, 16, 98, 99, 106 - 110, 112, 113, 127] is still used for the extraction of benzodiazepines from biological specimens. When the analytes are extracted from the biological sample by LLE, the physicochemical characteristics of the analytes, such as: pK_a values and hydrophobicity or hydrophilicity, are very important. Under slightly alkaline conditions achieved by the use of 0.5 M phosphate buffer [107 - 109, 112, 113], 0.63 M borate buffer [98, 110] or

0.1 M aqueous sodium hydroxide solution [106] at pH value of 9 – 10, most neutral benzodiazepines are transformed to the organic phase – n-butyl acetate [107 - 109, 113], ethyl acetate [106, 110] or chloroform [112]. Organic solvent polarity and pH of the aqueous phases are the major factors to be considered. However, some benzodiazepines were subjected to a double extraction (sample extracted two times with a mixture of diethyl ether and ethyl acetate (1:1, v/v)) owing to their lower hydrophobic solubility, and evaporation of obtained extracts to dryness before LC-MS/MS analysis [99]. This method is characterized by sufficiently high recoveries of 39.4 – 104.7 % for the determination of 23 benzodiazepines.

The selectivity of analytical methods allows to achieve high sensitivities for the specific target analytes. Furthermore, more selective sample preparation methodologies that result in a lower amount of co-extracted matrix constituents are highly desirable. However, for the most benzodiazepines the improvement of methods are still required, especially in the cases of residue analysis, because accuracies and recoveries were over 90 % exclusively for the several analytes, and LOQ $\geq$ 10 ng mL^{-1} [15, 16, 98, 107, 109, 112, 113, 127] in biological specimens using LLE methodologies.

Solid-phase extraction

SPE has several advantages: high selectivity, cleaner extracts, no emulsions, reduced solvent usage and higher throughput by automatization. Existing SPE procedures for the determination of benzodiazepines cover a broad range of sorbents (apolar, ion-exchange and polymeric sorbents or a combination of different sorbents) and allow the development of extraction procedures for specific needs [15, 16, 127].

Classic sorbents in SPE

The traditional commercially available, and popular to date sorbents based on silica-based phases with bonded C_1 (sorbent mass of 100 mg) [100], C_2 (sorbent mass of 50 mg) [137, 138], C_{18} (sorbent mass of 500 mg) [101 -

104, 139] or phenyl (sorbent mass of 200 mg) [114, 140] ligands were applied for the determination of benzodiazepines in human biological fluids. Furthermore, a mixed-mode sorbent containing non-polar C_8 and strong ion exchanger functionalities – Bond-Elut Certify I column (sorbent mass of 130 mg) has also been used for sample preparation before LC-MS/MS analysis of benzodiazepines and other illicit drugs in oral fluid [124]. These traditional silica-based sorbents provided quite high recoveries in the range of 75 – 105 % and they selectively adsorbed benzodiazepines and their metabolites at sample pH of 6.0 – 9.5, except for a mixed-mode sorbent (pH 4.1). However, insufficient repeatability of the results (RSD of 1.0 – 13.7 %) for most of the analytes was obtained [102, 103, 124, 137 - 139]. In addition, matrix interferences were not removed from the columns sufficiently [101 - 103, 139, 140].

M. Casas and co-workers [138] studied the extractability and cleanliness of a number of SPE columns. The authors concluded that the silica-based C_2 sorbent provided the best combination of high recovery (95 ± 5 %) and clean extracts from human urine and blood samples, compared to C_8, C_{18}, phenyl and cyclohexyl phases. However, insufficient repeatability of the results (RSD up to 12 %) was obtained. A similar system was used satisfactorily for the determination of four benzodiazepines in plasma using C_2 columns [137].

Recently, N. Badawi and co-workers [124] proposed simultaneous 29 drugs screening method using the fully automated Gilson SPE method for oral fluid. This method used Bond-Elut Certify I columns. Recoveries of most benzodiazepines were higher than 85 % using with 1.5 mL of a mixture of acetonitrile and NH_4OH (98:2 v/v) as eluent. In the present study the authors emphasized two main advantages over other reported methods: time savings and effective removal of matrix from the samples. However, insufficient repeatability of the results (RSD up to 10 %) was obtained [124].

The new generation of sorbent in SPE

The new generation of mixed-mode SPE columns, such as, Bond-Elut Plexa PCX (poly(St-DVB)-SO_3H sorbent, 550 $m^2 g^{-1}$, 60 mg) [118], Strata X-C (poly(St-DVB)-SO_3H sorbent, 800 $m^2 g^{-1}$, 30 mg) [105, 119], and Oasis MCX (poly(NVP-DVB)-SO_3H sorbent, 810 $m^2 g^{-1}$ (30 or 60 mg)) [122, 141, 142] columns have been used for biological sample preparation. A hydrophilic-lipophilic balance Oasis HLB (poly(NVP-DVB) sorbent, 830 $m^2 g^{-1}$ (30 or 60 mg)) column was successfully applied for sample preparation before HPLC or LC-MS/MS analysis of benzodiazepines in human blood [143, 144], urine [42, 120], and oral fluid [125].

Mixed-mode polymeric sorbents offered some advantages for the determination of benzodiazepines. They can be used at pH of 2 – 7 and with many different polar and/or non-polar organic solvents (e.g., methanol, ethyl acetate, diethyl-ether, dichloromethane or chloroform) [15, 127]. Contrary to classical reversed phase silica-based extraction sorbents, it is easier to find an appropriate extraction condition for a specific compound, for example midazolam [105, 141] or flunitrazepam [142], and especially for a mixture of more than twenty benzodiazepines [118, 119, 122] with different chemical properties, such as polarity, pH and affinity. All this allows to determine benzodiazepines at higher recoveries up to 95 %. The following SPE procedures not caused any problems in the determination of benzodiazepines and their metabolites, and no matrix interferences were observed. However, a few methods were insufficient for determination of several benzodiazepines. When Bond-Elut Plexa PCX [118], Strata X-C [119] or Oasis HLB [125] columns were used the extracts were less clean, and extraction recoveries in the range of 45 – 83 % were constantly lower compared to Oasis MCX [122, 141, 142]. The new generation column (Oasis MCX) showed many advantages over the other tested SPE sorbents as it ensured rapid, reproducible (RSD $\leq$ 5 %) [122], simple, clean extraction and gave sufficient sample purification as well as extraction yields of the most benzodiazepines (up to 99 %) in biological specimens [122, 141, 142].

Furthermore, the low (LOQ of 1 – 5 ng mL^{-1}) therapeutic concentrations of benzodiazepines require a highly sensitive method for their determination. However, the sensitivities of the most currently used methods do not meet this requirement [42, 101 - 104, 107 - 109, 112 - 114, 121, 139, 144].

1.6. Sedative-hypnotic drugs – zaleplon and zopiclone

Sedative-hypnotics drugs represent a pharmacologically diverse group of compounds, such as benzodiazepines, barbiturates, and other newest agents (e.g. zaleplon and zopiclone), that are used clinically [3, 145]. Currently there is no scientifically precise or universally accepted classification scheme for these drugs. Sedative-hypnotics drugs are often prescribed in primary care for insomnia [3, 145, 146].

Zaleplon and zopiclone are sedative-hypnotic drugs, which are used to induce sleep in short-term treatment of insomnia. These newest medications are less safe than other benzodiazepines, and have tendency to induce physical dependence [3, 10 - 12]. Currently these drugs are the most commonly prescribed sedative-hypnotics agents in the United States of America and the European Union [145]. Chemical structures of both drugs are reported in Figure 1.5.

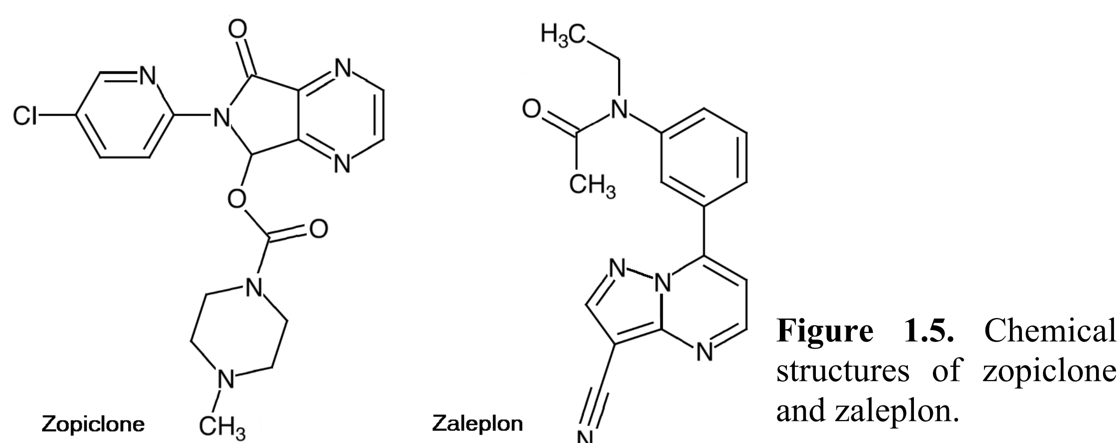


Figure 1.5. Chemical structures of zopiclone and zaleplon.

Although these drugs are widely prescribed for patiens, the certain side-effects were identified. It should be noted an adverse cognitive (such as,

memory loss) and psychomotor (such as, road traffic crashes) effects, daytime fatigue, addiction, and excess of mortalities with no significant difference from the side-effects of typical benzodiazepines [11, 147 - 149]. However, with increased use of zaleplon and zopiclone drugs, reports of misuse and possible dependence began to appear in the literature, particularly considering people with a history of drugs and/or narcotics misuse and comorbid psychiatric illness [145, 147]. For this reason, it is essential to develop a fast and sensitive method for the determination of both analytes in the biological matrix.

1.6.1. Chromatographic methods for the determination of both analytes

In recent years, several chromatographic methods for the determination of zaleplon and/or zopiclone in biological matrices, such as, human plasma [98, 99, 150 - 153], whole blood [108 - 110, 113, 115, 117 - 119], urine [13, 118, 120, 154 - 156], oral fluid [123 - 125], human hair [126], and postmortem specimens (central blood, vitreous, liver, bile and urine) [157] have been developed. Many methods dealing with the determination of both drugs by HPLC with different detectors have been reported [10]. Determination of the analytes in human plasma by reversed-phase HPLC with ultraviolet [10, 150] or fluorescence detections [153] has been demonstrated. Finally, LC-MS/MS [10, 13, 98, 110, 115, 117 - 120, 124 - 126, 151, 155, 156] or atmospheric pressure chemical ionization tandem mass spectrometry [10, 99, 152] determination of both drugs has been reported. Several methods have been described for detection of the analytes by GC/EI-MS [108, 109, 123, 154] or electron capture ionization techniques [109, 157], and finally, only one study by GC/NICI-MS [113]. As in the case of benzodiazepines, the GC/NICI-MS is a sensitive and selective technique for the analysis of zaleplon and zopiclone with electronegative moieties, such as, halogen atom in the xenobiotic itself or original structure (Figure 1.5.) [113].

The highest sensitivities of methods for analytes of interest detection are of major interest to meet the requirements of good laboratory practice. However, some previously published methods do not detect low concentrations

of both analytes in low-volumes biological specimens. Furthermore, some methods published previously have shown quite high LODs in the range of 2.5 to 10.0 ng mL^{-1} in biological samples [99, 108, 109, 113, 123, 150, 153, 156].

1.6.2. Extraction methods for the analysis of both analytes

Various extraction methods for the determination of zaleplon and/or zopiclone in biological specimens have been successfully applied. These techniques can be separated into three distinct types:

1. Direct introduction into a chromatographic system;
2. Liquid-liquid extraction;
3. Solid-phase extraction.

Direct injection

Direct determination of zaleplon and zopiclone in biological samples is complicated due to significant matrix interferences and relatively high concentrations of the analytes [10]. The use of diluted urine sample was suitable for direct analysis of both analytes, and benzodiazepines by LC-MS/MS, with significant matrix effect [13]. Although this method avoids an extraction step it requires more instrumentation. The main advantage over other reported techniques is potential time saving. Moreover, sufficient accuracies (in the range of 96.3 - 105.4 %), and precision (RSD $\leq$ 8.5 %) were obtained for quantitative determination in diluted urine samples [13].

Currently direct injection methods are not popular. They have negative effects for the determination of the analytes, such as, significant increase of matrix interferences, relatively high concentrations of the analytes determined, and limitations of biological samples [10, 16].

Liquid-liquid extraction

An important step of an analytical method is the extraction of the analytes of interest from the biological matrix. The standard procedure for extracting both analytes is based on a LLE before alkalinization (pH $\geq$ 9) with

0.63 M borate buffer [98, 110], 2.0 M sodium hydroxide solution [99, 151, 152], 0.2 M ammonium carbonate buffer [115] or 0.5 M phosphate buffer [108, 109, 113]. A variety of organic solvents, such as, ethyl acetate [110, 151, 152], a mixture of diethyl ether and ethyl acetate (1:1 v/v) [99], a mixture of ethyl acetate and heptane (4:1 v/v) [115] or n-butyl acetate [108, 109, 113] were used for LLE with sufficient extraction efficiency (recovery $\geq$ 80 %). When insufficient extraction [153, 158] was applied, the extraction efficiency for zopiclone was found to be low (recovery $\leq$ 65 %). Under these conditions (sample pH ~ 7) zopiclone was partially ionized (pK_a – 6.79), and finally was determined by HPLC with fluorescence [153] or GC with nitrogen phosphorus [158] detections.

According to previously published methods insufficient repeatability of the results for zopiclone and zaleplon detection was obtained. Between-day precision for the analytes ranged from 5.7 to 19.8 %, whereas accuracy values were in the range of 59.0 – 123.8 % [99, 108 - 110, 113, 115, 151]. Thus, there are a limited number of sample preparation procedures, which are suitable for effective isolation/preconcentration of both analytes from biological samples. The SPE is most effective sample preparation method for the determination of both analytes [10].

Solid-phase extraction

Recently, several SPE methods for either one or a mixture of several analytes extraction have been developed. The SPE has several advantages: high selectivity, cleaner extracts, no emulsions, reduced solvent usage, and higher throughput by automatisation [10, 116]. In addition, a large variety of sorbents for determination of both analytes (polar, non-polar, mixed-mode and polymeric sorbents) allows the development of extraction procedures for specific needs. The sorbents used include: i) polar silica-based phases $(Si\text{-}OH)_n$ [157]; ii) apolar silica-based phases with bonded C_8 [155] and C_{18} [156] ligands. The traditional, but popular to date, mixed-mode sorbents containing non-polar C_8 and strong ion exchanger functionalities – Bond-Elut Certify I

column (sorbent mass of 130 mg) [124] and Isolute HCX column (sorbent mass of 130 mg) [117] have also been used for oral fluid [124] and whole blood [117] samples preparation before LC-MS/MS analysis of zaleplon and zopiclone and other illicit drugs.

The new generation of mixed-mode SPE columns, such as, Bond Elut Plexa PCX (poly(St-DVB)-SO_3H sorbent, 550 m^2 g^{-1}, 60 mg) [118], Strata X-C (poly(St-DVB)-SO_3H sorbent, 800 m^2 g^{-1}, 30 mg) [119] have also been used for whole blood [118, 119] and urine [118] samples preparation. A hydrophilic–lipophilic balanced column Oasis HLB (poly(NVP-DVB) sorbent, 830 m^2 g^{-1}, 60 mg) was successfully applied for sample preparation before LC-MS/MS analysis of zopiclone and other illicit drugs in urine [120] and oral fluid [125].

During SPE method development much attention should be paid to the optimization of biological sample preparation step that diminish matrix effects as much as possible, and to increase the absolute recovery. A mixed-mode Bond Elut Plexa PCX column [118] showed lower absolute recovery result up to 45.6 % for the determination of zopiclone, however, sufficient extraction efficiency of zaleplon in the range of 80.8 – 91.7 % was obtained. On the other hand, Isolute HCX column [117] led also to the lowest absolute recovery result up to 6 % for the determination of zaleplon, however, in the analysis of zopiclone sufficient extraction efficiency of 97 % was obtained. In this method [117], zaleplon was only one drug from a mixture, which was mainly eluted in the washing step with a mixture of toluene and ethyl acetate (80:20 v/v). Under these conditions (the sample pH of 4.1) zaleplon was non-ionized, and ion-exchange mechanism has not been dominated. Since the absolute recovery was reproducible, it was concluded that only the alkaline extract 5 % of NH_4OH in ACN should be collected to minimize the total amount of samples for LC-MS/MS analysis [117].

Higher absolute recoveries results more than 83.5 % for the determination of zopiclone and without any interference from endogenous compounds were achieved using Strata X-C [119], Bond-Elut Certify I [124],

and Oasis HLB [120] columns. The extraction efficiencies of zopiclone were reproducible (RSD ≤ 7.5 %), when these three SPE columns were used, and finally obtained extracts were suitable for LC-MS/MS analysis.

When Bond Elut Plexa PCX [118] and Isolute HCX [117] columns were tested, the obtained the extracts were less clean. Some endogenous interference compounds was seen for the determination of zopiclone (signal enhancement of 64 %) [118]. However, the use of deuterated internal standards diminished the effect of the matrix, and the results for linearity, precision, and accuracy were acceptable for the two analytes [117, 118]. In addition, both Oasis HLB [120, 125] and Strata X-C [119] extraction methods showed clear advantages over the other previously tested SPE methods. For these both methods an effective/appropriate sorbents washing and elution steps were used. Summarizing the previously published methods, it can be concluded that the SPE procedure is also very important for the clean-up of biological samples that means the removal of interfering compounds from matrix to increase the selectivity in comparison with the conventional LLE methods.

The selectivity of analytical methods allows to achieve high sensitivities for the specific targeted analytes. Furthermore, the low (LOQ of 1 – 2 ng mL^{-1}) therapeutic concentrations of zaleplon and zopiclone required a highly sensitive method for their determination. However, the sensitivities of the most currently used methods do not meet this requirement [117, 155 - 157].

1.7. The biological specimens

The choice of biological specimen is often predetermined by the forensic medicine (toxicology) and/or clinical situation. The most common specimens used for the determination of benzodiazepines as well as zaleplon and zopiclone are blood, serum, plasma and urine [10, 15]. Blood is a typical sampling matrix and suitable for detecting, and quantifying sedative-hypnotic drugs [3]. Serum and plasma are over 90 % water and also contain ions, dissolved gases, proteins and tissue products like creatinine, urea and lactate, in addition to drugs and their metabolites [3]. Numerous methods have been

reported in the literature and consolidated in several review articles that describe the quantitation of sedative-hypnotic drugs in whole blood and plasma. Both biological specimens are irreplaceable in the most analytical methods [159, 160]. Conversely, many drugs and their metabolites are present in urine as conjugates covalently bound to glucuronic acid, sulphate or glycine, and must be released prior to extraction, for example by enzymatic hydrolysis [10, 15].

Drugs present in whole blood and inside the blood cells should be released before extraction. Hydrophilic drugs are usually free in solution, whereas lipophilic drugs (e.g. benzodiazepines) are noncovalently bound to proteins or particles [3]. Noncovalent bonds can be broken by dilution with appropriate buffer, changing sample pH or selecting the organic solvents. Whole blood analysis is very important in forensic toxicology and clinical medicine cases, because drug concentration in blood represent an acute drug effect and can be used to estimate the probability of intoxication [3, 160].

2. EXPERIMENTAL

2.1. Experimental materials for the determination of benzodiazepines

2.1.1. Reagents

Diazepam, nordiazepam, midazolam, flunitrazepam, bromazepam, oxazepam, nitrazepam, temazepam, lorazepam, clonazepam, alprazolam, α-OH-midazolam, triazolam, α-OH-alprazolam, 7-aminoclonazepam and fludiazepam (internal standard - IS) powders were obtained from Lipomed - Services to Medicine (Arlesheim, Switzerland). Internal standards in ampoules of clonazepam-d4, oxazepam-d5 and 7-aminoclonazepam-d4 (100.0 $\mu g\ mL^{-1}$ in MeOH) were obtained from Cerilliant Corporation (Round Rock, USA). Silylation reagents: a commercial mixture of 99 % BSTFA with 1 % trimethyl-chlorosilane (TMCS), MSTFA and MTBSTFA of analytical grade was purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Methanol (MeOH), ethanol (EtOH), 1-propanol (1-PrOH), 1-butanol (1-BuOH), acetonitrile (ACN), ethyl acetate, hydrochloric acid (HCl), potassium hydrogen phosphate, sodium tetraborate, ammonium hydroxide (NH_4OH) were obtained from Merck (Darmstadt, Germany). Other chemicals and solvents were of analytical grade. SPE was carried out with Oasis MCX (30 mg, 1 ml), a mixed-mode sorbent. The column purchased from Waters Corp. (Waters, UK).

Fresh human whole blood (the National Blood Center of Lithuania) and stored (drug-free) human whole blood from authentic samples submitted to the authors' laboratory were used in the study. The real blood samples were collected from ten volunteers, after long periods of time of a single oral administration of some benzodiazepines (diazepam 5 mg, clonazepam 2 mg and lorazepam 1 mg). The blood samples were collected and kept until their analysis in test tubes containing anti-coagulant (sodium fluoride/potassium oxalate). The volunteers gave written informed consent to participate in the investigation.

2.1.2. Calibration and quality control samples

Stock solutions of each benzodiazepine were prepared at a concentration of 1.0 mg mL^{-1} in MeOH, and stored at -70.0 ± 1.0 °C. Standard solutions with different concentrations of each analyte were prepared by dilution with MeOH from corresponding stock solutions. Appropriate amounts of standard solutions were added to a volumetric flask and evaporated to dryness under a gentle stream of nitrogen. Then the residues were reconstituted with drug-free human whole blood, which was thawed to room temperature (22.5 ± 0.5 °C) in advance, to yield final benzodiazepine concentrations. These spiked human whole blood calibration standards were considered to be matrix-matched and three concentration levels in whole blood were considered as quality control (QC) samples. Concentrations of the analytes were used in calibration and the four internal standards (ISs) chosen for each analyte are shown in Table 2.1.

2.1.3. GC/NICI-MS analysis and determination of benzodiazepines

Analysis of benzodiazepines was performed using an Agilent Technologies-7890A (Folsom, CA, USA) gas chromatograph with a mass selective detector (5975C NICI-MS mode). The ion source was operated in the chemical ionization mode and methane (purity 99.9995 %) was used as reagent gas in all MS measurements. The flow controller of methane was set to 2.50 mL min^{-1}. The NICI-MS multiplier voltage was 1625 ± 50 V, emission – 49.0 ± 1.0 μA, electron energy – 149.0 ± 1.0 eV, repeller – 2.8 ± 0.2 V and ion focus – 130 ± 2 V. The mass spectrometer detector transfer line temperature was 300 °C; MS quadrupole and MS source temperatures were 150 °C.

Fast chromatographic separations were performed using a DB-5HT capillary column (30 m × 0.320 mm I.D., 0.10 μm film thickness) from Agilent (Folsom, CA, USA). The injection was performed automatically at an injector temperature of 250 °C. The initial temperature of the analytical column was 180 °C, and was gradually increased by 50 °C per min up to 325 °C and stable heating was maintained for one min. The carrier helium gas (purity 99.9996 %)

had a constant flow rate of 3.5 mL min^{-1} after a pulsed flow injection in a split-less mode (1.0 μL).

Table 2.1. Selection of quantitative parameters for the determination of the analytes.

Compound	Calibration levels (ng mL^{-1})	Internal standard
Fludiazepam (IS)	20	-
Diazepam	2, 5, 10, 20, 30, 50, 100, 200	Fludiazepam
Nordiazepam	2, 5, 10, 20, 30, 50, 100, 200	Clonazepam-d4
Midazolam	1, 5, 10, 20, 30, 50, 70, 100	Fludiazepam
Flunitrazepam	1, 2, 5, 10, 20, 30, 40, 50	Fludiazepam
Bromazepam	2, 5, 10, 20, 30, 50, 70, 100	Clonazepam-d4
Oxazepam-d5 (IS)	20	-
Oxazepam	2, 5, 10, 20, 30, 50, 100, 200	Oxazepam-d5
Nitrazepam	2, 5, 10, 20, 30, 50, 70, 100	Clonazepam-d4
Temazepam	2, 5, 10, 20, 30, 50, 100, 200	Oxazepam-d5
7-aminoclonazepam-d4 (IS)	20	-
7-aminoclonazepam	1, 5, 10, 20, 30, 50, 70, 100	7-aminoclonazepam-d4
Lorazepam	1, 5, 10, 20, 30, 50, 70, 100	Oxazepam-d5
Clonazepam-d4 (IS)	20	-
Clonazepam	1, 5, 10, 20, 30, 50, 70, 100	Clonazepam-d4
Alprazolam	1, 5, 10, 20, 30, 50, 70, 100	Fludiazepam
α-OH-midazolam	1, 2, 5, 10, 20, 30, 40, 50	Oxazepam-d5
Triazolam	1, 5, 10, 20, 30, 50, 70, 100	Fludiazepam
α-OH-alprazolam	1, 5, 10, 20, 30, 50, 70, 100	Oxazepam-d5

2.1.4. Sample preparation for the determination of benzodiazepines

Before SPE whole blood samples (calibrators, QC and real samples) should be prepared. HPLC grade water (2.0 mL at pH 7.0) and 20 μL of the four ISs – fludiazepam, oxazepam-d5, 7-aminoclonazepam-d4 and clonazepam-d4 (a mixture of 200.0 ng mL^{-1} in MeOH) – were added to 0.2 mL of blood sample. After acidification with 0.15 mL of 1.5 M HCl, the mixture was vortex-stirred and left to incubate for 2 min at the temperature of 22.5 ± 0.5 °C. The sample, with resulting pH 1.0, was then centrifuged at 1233 g (3500 rpm) for 5 min and the supernatant was subjected to SPE.

SPE was carried out using the Oasis MCX column. Conditioning was performed with 1.0 mL of MeOH and equilibration with 1.0 mL of 0.1 M HCl

at pH 1.0, both at a flow rate of 1 mL min^{-1}. The acidified blood sample having pH 1.0 was slowly passed through the column at a flow rate of 1 mL min^{-1}, without vacuum. After that, the column was immediately washed three times with: i) 1.5 mL of 0.1 M HCl at pH 1.0; ii) 1.5 mL of 1-PrOH and acidified water at 0.15 M HCl mixture (60:40 (v/v)) and finally iii) 1.0 mL of ACN at a flow rate of 1 mL min^{-1}. The column was dried for 2 min and followed by elution with 2.0 mL of 5 % NH_4OH in MeOH at a 1 mL min^{-1} flow rate. The extract was evaporated to dryness (35 ± 2 °C, N_2). The residue was silylated with 100 µL of the MTBSTFA : ACN : ethyl acetate mixture (20:40:40 (v/v/v)) at 85 °C for 30 min. This was done in borosilicate glass tubes covered by special caps (having a Teflon layer) to avoid of evaporation of the aliquot. After the derivatization and cooling at room temperature, a 1.0 µL aliquot was injected into the GC/NICI-MS system.

2.2. Experimental materials for the determination of zaleplon and zopiclone

2.2.1. Reagents

Zaleplon and zaleplon-d5 (IS) were obtained from Toronto Research Chemicals Inc. (Toronto, Canada), while zopiclone was obtained from Lipomed-Services to Medicine (Arlesheim, Switzerland). ACN, MeOH, 1-PrOH, 2-propanol (2-PrOH), ethyl acetate, n-butyl acetate, dichloromethane, toluene, HCl, sodium acetate, acetic acid, potassium hydrogen phosphate, sodium tetraborate, potassium hydroxide and NH_4OH of analytical grade were obtained from Merck (Darmstadt, Germany).

Fresh human whole blood (drug-free) samples from the National Blood Center of Lithuania were used in the study. All whole blood samples were stored at -20.0 ± 1.0 °C, and defrosted at ambient temperature (22.5 ± 0.5 °C) for 30 min before use. As freezing and thawing cause hemolysis of human erythrocytes [143], the samples are referred to as hemolyzed blood samples.

SPE was carried out with Oasis HLB (30 mg, 1 ml) column. For the development of an SPE method Oasis MCX (30 mg, 1 ml) extraction column was used. Both columns were purchased from Waters Corp. (Waters, UK).

2.2.2. Calibration and quality control samples

Stock solutions of zaleplon (1.0 mg mL^{-1}) and zopiclone (2.0 mg mL^{-1}) were prepared in ACN, and stored at -70.0 ± 1.0 °C, until use. During the experiment, stock solutions were diluted and then mixed with ACN. Appropriate amount of new prepared standard solution was added to a volumetric flask and evaporated to dryness. Then the residues were reconstituted in drug-free hemolyzed blood to final concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 40.0, 80.0 ng mL^{-1} of zaleplon, and 2.0, 4.0, 10.0, 20.0, 40.0, 80.0, 160.0 ng mL^{-1} of zopiclone. QC samples were prepared by evaporating standard solution followed by reconstitution in drug-free hemolyzed blood at the final four concentrations of 1.0, 15.0, 30.0, 60.0 ng mL^{-1} of zaleplon, and 2.0, 30.0, 60.0, 120.0 ng mL^{-1} of zopiclone. The calibration and QC samples were considered to be matrix-matched.

2.2.3. GC/NICI-MS analysis and determination of zaleplon and zopiclone

All experiments were performed using Agilent Technologies-7890A (Folsom, CA, USA) gas chromatograph with a mass selective detector (5975C NICI-MS mode). In NICI-MS, methane was used as a reagent gas at flow rate of 2.50 mL min^{-1}. The MS detector transfer line temperature was 300 °C; MS quadrupole and MS source temperatures were set at 110 and 160 °C. The injector temperature was 250 °C. The NICI-MS multiplier voltage was 1600 ± 50 V, emission: 49.5 ± 2.0 µA, electron energy: 148.0 ± 1.5 eV, repeller: 2.8 ± 0.3 V, and ion focus: 129 ± 2 V.

Fast chromatographic separation was achieved using a DB-5HT capillary column (30 m × 0.320 mm id., 0.10 µm film thickness). Helium carrier gas was flowed at a constant flow rate of 3.0 mL min^{-1} after a pulsed flow injection in a split-less mode (1.0 µL). The initial temperature of the

analytical column was set at 200 °C, and was gradually increased by 45 °C per min up to 330 °C, and stable heating was maintained for one min.

2.2.4. Sample preparation for the determination of zaleplon and zopiclone

Frozen sample was thawed at room temperature (22.5 ± 0.5 °C) and vortex-mixed for 30 s. A volume of 2.0 mL of 0.1 M borate buffer at pH 9.0, and 20 μL of IS – zaleplon-d5 (100.0 ng mL^{-1} in ACN) – were added to 0.2 mL of hemolyzed blood, vortex-mixed and sonicated at ambient temperature for 5 min. The sample, with resulting pH 9.0 was then centrifuged at 1610 g (4000 rpm) for 10 min, and the supernatant was subjected to SPE.

SPE was carried out using Oasis HLB column. Conditioning was performed with 1.0 mL of MeOH and equilibrated with 1.0 mL of 0.1 M borate buffer at pH 9.0. The supernatant at pH 9.0 was transferred to the extraction column. After that, the sorbent was immediately washed in two steps: i) 1.0 mL of 0.1 M borate buffer at pH 9.0; ii) 1.0 mL of 1-PrOH and water containing 0.1 % of NH_4OH mixture (30:70 v/v). The column was dried under full vacuum for 10 min. The retained compounds were eluted into a borosilicate glass tube with 1.0 mL of a mixture of n-butyl acetate and 2-PrOH (80:20 v/v). All SPE steps were performed at a flow rate of 1 mL min^{-1}. The eluent was evaporated to dryness at 30 ± 2 °C under the stream of nitrogen, the obtained residue was reconstituted in 120 μL of n-butyl acetate, and 1.0 μL of solution was injected into the GC/NICI-MS system.

2.3. Determination of the effectiveness of sample preparation

The effectiveness of sample preparation, expressed as the relative response factor (RRF), was calculated using the following equation:

$$\mathrm{RRF} = \frac{A_S\,C_{IS}}{A_{IS}\,C_S} \qquad (2.1)$$

where A_S = the target analyte peak area, C_{IS} = IS concentration (ng mL^{-1}), A_{IS} = IS peak area, and C_S = the target analyte concentration (ng mL^{-1}). The effectiveness of sample preparation on the yield of the target analytes

(benzodiazepines, zaleplon and zopiclone) after SPE in relation to solvent type, sample pH value and silylation for benzodiazepines was calculated. The IS was added to all sets of samples after SPE and clean-up procedures. An increase in RRF indicated an increase in the effectiveness of sample preparation.

2.4. Validation of the methods

The developed methods were fully validated. Validation of new methods in human whole blood and/or hemolyzed blood was done following recommendations [161 - 163]. These methods were validated in terms of selectivity, sensitivity, linearity, accuracy, precision, recovery, robustness and stability.

The sensitivity of methods was determined by calculating the limit of detection (LOD), and the limit of quantification (LOQ). The LODs and LOQs for the analytes were determined from drug-free human blood samples (n=5) as the lowest concentrations yielding signal-to-noise ratios of at least 3:1 and 10:1, respectively. They were calculated for average baseline noise using the maximum sensitivity allowed by the system. The LOQ was subsequently determined by analysis of spiked blood samples prepared at their respective concentrations.

3. RESULTS AND DISCUSSION

3.1. Investigation of a mixed-mode SPE method for a multi-residue analysis of 15 benzodiazepines in whole blood by fast GC/NICI-MS

When developing a fast gas chromatography with negative-ion chemical ionization mass spectrometry (GC/NICI-MS) method matrix effects are a major issue. The effect of co-eluting compounds arising from the matrix (whole blood) can result in signal enhancement or suppression [13 - 15, 117 - 120, 124, 125, 143, 144]. During the method development much attention should be paid to diminish matrix effects as much as possible. The present work evaluates matrix effects from whole blood samples in the simultaneous analysis of 15 benzodiazepines.

Furthermore, many widely used screening and confirmation methods do not detect low up to 2.0 ng mL^{-1} concentrations of benzodiazepines in low-volume whole blood samples [15, 16, 42, 99, 101 - 104, 106 - 109, 111 - 113, 116, 127, 139, 144]. Therefore, the main aim of study was development of a new sensitive and specific method based on GC/NICI-MS using a mixed-mode SPE for the identification and quantification of these drugs in whole blood. Moreover, the speed of the analytical separation was emphasized by modifying various GC/NICI-MS parameters. The fully validated method was applied for the quantification of several benzodiazepines in real blood samples.

3.1.1. Derivatization and development of an analytical method

Although benzodiazepines and their metabolites can be determined by GC/EI-MS without derivatization, however several problems are usually encountered for the trace level analysis. Losses of the analytes and peak tailing due to an adsorption in the GC inlet device and/or an interaction of the analytes with active sites on the walls of the capillary column can be observed [16, 103, 109, 127].

The GC/NICI-MS analysis is not sensitive enough for the determination of benzodiazepines without derivatization [113]. Because the analytes are

lipophilic compounds, so they should be converted into volatile derivatives prior to GC analysis. Consequently, for the most GC-MS methods derivatization of the benzodiazepine improves spectral determination/definition and reduces thermal degradation on-column [16, 127]. Thus, derivatization by different reagents: mixtures of MSTFA, BSTFA with 1 % TMCS or MTBSTFA in ACN and ethyl acetate (at 20:40:40 v/v/v) were investigated. The derivatization procedure was optimized in order to obtain the highest sensitivity, repeatability of the derivatives and to avoid interferences from the sample matrix. The greatest peak areas of derivatized analytes were achieved by silylation using 100 μL of a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v). A slightly lower sensitivity was observed after silylation by MSTFA if compared with BSTFA with 1 % TMCS. The peak areas of derivatized analytes using these reagents were smaller in comparison with MTBSTFA, except for temazepam-TBDMS, α-OH-midazolam-TBDMS and α-OH-alprazolam-TBDMS. Chromatograms of 15 benzodiazepines obtained using three different silylation mixtures are summarized in Fig. 3.1.

During silylation by MSTFA or BSTFA with 1 % TMCS many problems arose at the derivatization step because both reagents are very sensitive to humidity. Subsequently, insufficient repeatability of TMS derivatives was observed (Fig. 3.2). Furthermore, the baseline with both silylation reagents showed one peak at the retention time of midazolam and temazepam-TMS derivatives (Fig. 3.1 A and B).

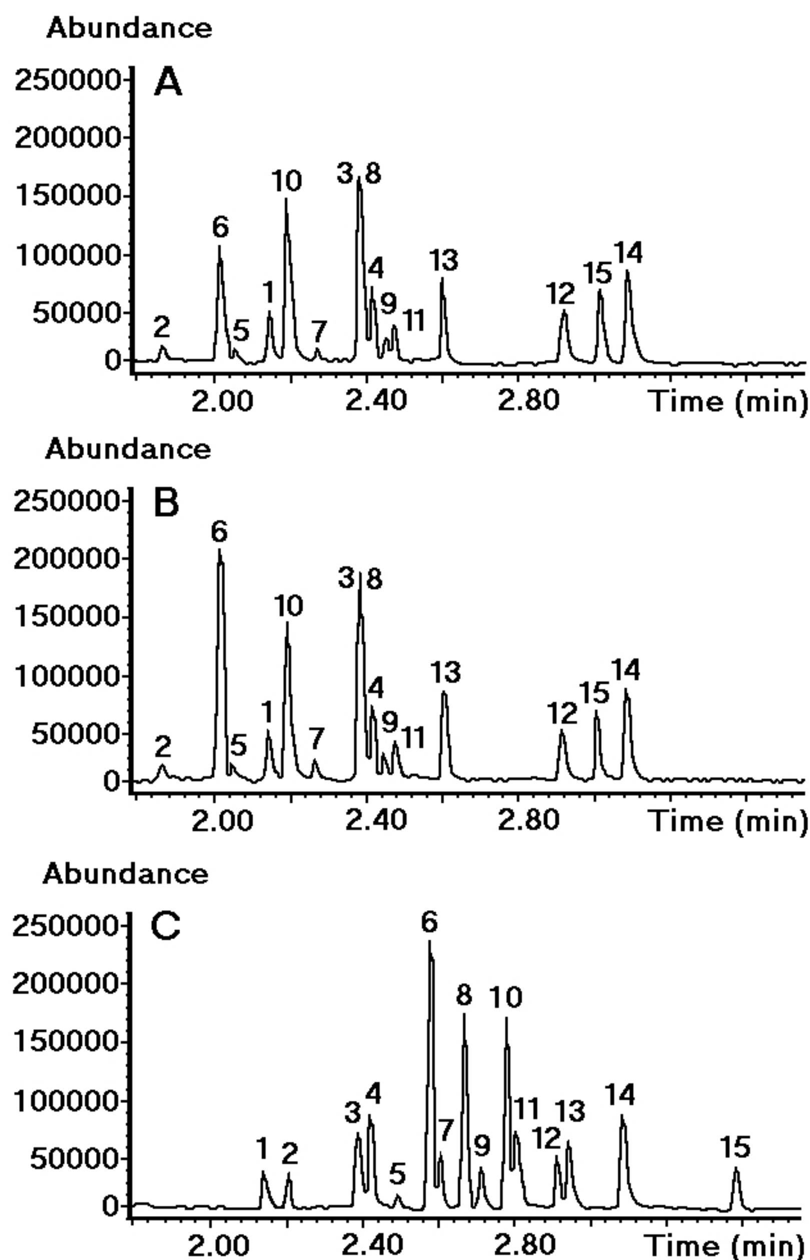


Figure 3.1. Selected ions monitoring chromatograms obtained after derivatization of 15 benzodiazepines at 200.0 ng mL^{-1} of each analyte by fast GC/NICI-MS. Chromatograms of three silylating mixtures obtained after derivatization step: (A) a mixture of MSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v), (B) a mixture of BSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v), (C) a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v). Derivatization was performed at 80 °C for 30 min. The peak numbering refers to: (1) diazepam; (2) nordiazepam; (3) midazolam; (4) flunitrazepam; (5) bromazepam; (6) oxazepam; (7) nitrazepam; (8) temazepam; (9) 7-aminoclonazepam; (10) lorazepam; (11) clonazepam; (12) alprazolam; (13) α-OH-midazolam; (14) triazolam; (15) α-OH-alprazolam.

After silylation by MTBSTFA, benzodiazepines and their metabolites were readily converted to their corresponding t-butyldimethylsilylation-derivatives (TBDMS), via an S_N2 substitution reaction, which yielding a single derivative for each benzodiazepine compound. In addition, TBDMS derivatives of benzodiazepines are hydrolytically stable and less sensitive to humidity. The sensitivity and repeatability of benzodiazepines derivatives using three different silylation mixtures are illustrated in Fig. 3.2.

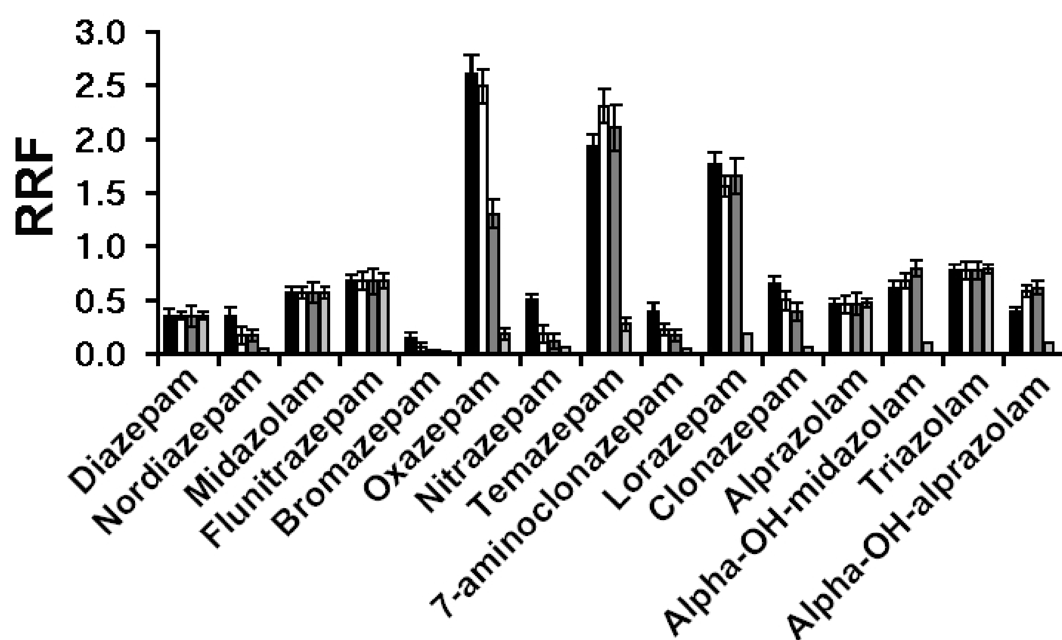


Figure 3.2. Derivatization efficiency of 15 benzodiazepines at three different silylation mixtures, and comparison of derivatized analytes to the non-derivatized analytes (n=5). Derivatization was performed at 80 °C for 30 min, at concentration of 200.0 ng mL^{-1} of each analyte, IS – fludiazepam. Notations: black column – a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v); white column – a mixture of BSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v); grey column – a mixture of MSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v); light grey column – a standard solution of the analytes in MeOH.

As already mentioned above, a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v) was found to be the most suitable silylating reagent for the derivatization of the analytes. In addition to the reduced peak tailing and the analyte adsorption effects, derivatization by MTBSTFA also increases the detection sensitivity and finally improves the chromatographic separation

of the analytes, compared to the other silylating TMS derivatives (Fig. 3.1). For instance, if compared with non-derivatized analytes, the peak areas of derivatized ones increased from approximatelly 5 to 10 times (Fig. 3.2), except for diazepam, midazolam, flunitrazepam, alprazolam and triazolam. These analytes have no active groups to bind to the silylating reagents and thus does not form derivatives (Fig. 3.1 and Fig. 3.2).

Summarizing the obtained results, it can be concluded that derivatization by MTBSTFA decreases the vaporization temperature [108, 134] by lowering the compound's capability to form hydrogen bonds and introducing electronegative moieties to increase the peak intensity in NICI-MS mode. For all of the positive features, a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v) has been used for further investigation.

Derivatization time and temperature

In the next step of research the derivatization time and temperature were optimized. The derivatization conditions including temperature, which was fixed at 60, 70, 80, **85**, 90, and 100 °C, and the duration of the reaction, which was defined at 20, 25, **30**, 35, and 40 min, were tested using standard solutions of the compounds. The optimal conditions are presented in bold. During the optimization of the derivatization temperature, the peak areas of silylated analytes-TBDMS kept increasing with the increase of temperature until the maximum peak area was reached at 85 °C. After this point, an increase in temperature resulted in the decrease of the peak areas of the some analytes: oxazepam-2TBDMS; temazepam-TBDMS; 7-aminoclonazepam-TBDMS; lorazepam-2TBDMS; α-OH-midazolam-TBDMS, and α-OH-alprazolam-TBDMS. The peak areas of the analytes were expressed to the RRF. The obtained results are shown in Figure 3.3.

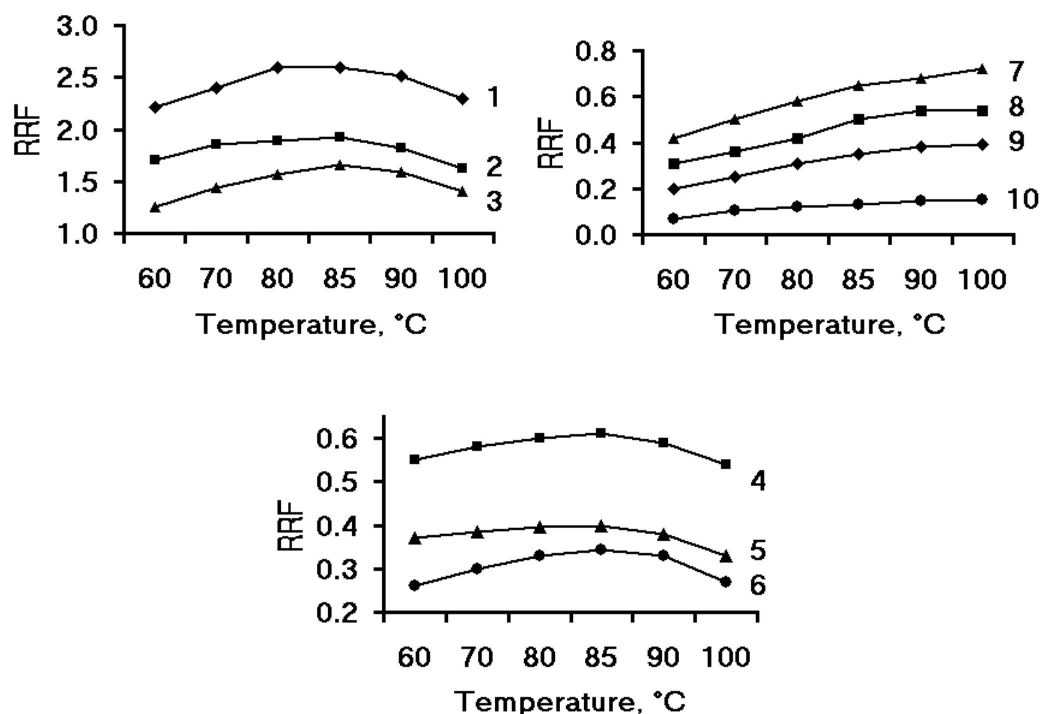


Figure 3.3. Silylation efficiency of benzodiazepines obtained at concentration of 200.0 ng mL^{-1} of each analyte with different reaction temperatures (n=5). Derivatization was performed using a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v) for 30 min, IS – fludiazepam. The analytes numbering refers to: (1) oxazepam; (2) temazepam; (3) lorazepam; (4) α-OH-midazolam; (5) α-OH-alprazolam; (6) 7-aminoclonazepam; (7) clonazepam; (8) nitrazepam; (9) nordiazepam; (10) bromazepam.

The silylation efficiency of benzodiazepines was tested as a function of time. The highest peak areas was achieved when the reaction was carried out for 30 min (Fig. 3.4), and after that increasing reaction time resulting in decrease the peak areas of some analytes. Thus, the silylation time of 30 min was chosen considering the optimal derivatization time. An increase in reaction time from 25 to 30 min had no effect on the peak areas of the most silylated benzodiazepines. Therefore, the derivatization time and temperature for a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v) at 85 °C for 30 min were chosen for further optimization studies.

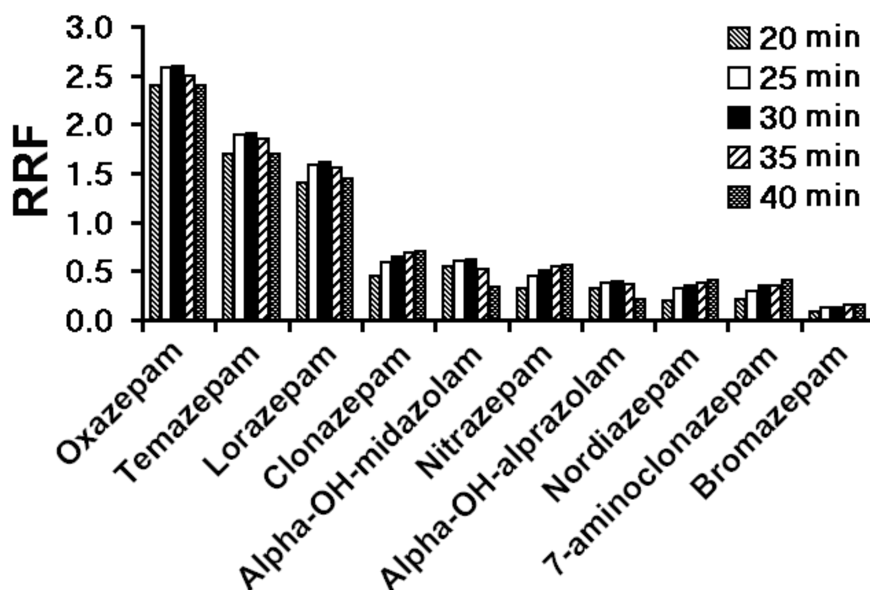


Figure 3.4. Silylation efficiency of benzodiazepines obtained at concentration of 200.0 ng mL^{-1} of each analyte with different reaction times (n=5). Derivatization was performed using a mixture of MTBSTFA, ACN and ethyl acetate (at 20:40:40 v/v/v) at 85 °C, IS – fludiazepam.

3.1.2. Fast gas chromatographic separation of benzodiazepines

High-speed GC provides faster separations of the analytes than conventional GC, leading to an increased sample throughput and cost reductions for the analysis [113, 123]. Fast temperature ramping, high final temperature, above-optimum carrier gas velocity and short column length with small film thickness and diameter are the main parameters commonly applied for faster GC methods [165]. Nevertheless, speeding up the GC separation of the analytes always involves a trade-off between the analysis time and the analytical resolution obtained. Therefore, selective detection techniques, such as MS with NICI or EI detections, are highly suitable for fast GC applications. In fast GC/NICI-MS method the peak widths of the analytes should be several seconds and total analysis time in the range of minutes.

Short and small-diameter analytical columns are characterized by the low sample capacity, which influenced a small sensitivity of the analytical method. Thus, the chromatographic properties of benzodiazepines and their metabolites detection could be significantly improved with analytical GC column having a larger-diameter (0.32 mm) in comparison to smaller-diameter

(0.25 mm), which results in higher reproducibility and sensitivity of the analysis. The same tendency of experiments was observed by other authors [113]. The sensitivity is one of the most crucial and limiting factor during the development of the trace level benzodiazepines concentrations in whole blood analysis method. Other parameters, such as fast temperature programming, allowed sharper peak shapes and reduction in the analyte peak width compared with the conventional GC programmes. The peaks shape of the analytes remained symmetrical and sharp.

The fast chromatographic conditions were tested at: 220, 230, 240, **250,** and 300 °C for injector temperature; 280, 290, **300** and 310 °C for detector temperature; 150, 170, **180** and 250 °C for initial column temperature; 290, 310, **325** and 340 °C for final column temperature; as well as 30, 40, **50** and 60 °C min^{-1} for the column temperature elevation rate. The optimal conditions are presented in bold and have been chosen based on the peak areas of the analytes derivatized with MTBSTFA and not derivatized, as well as on their resolution. The obtained data are summarized in Table 3.1.

Nevertheless, NICI-MS provides a highly specific detection technique for the analytes of interest having electronegative moieties, and therefore the chromatographic separation can be speeded up dramatically compared with conventional GC separations [16, 113, 123]. The fast GC/NICI-MS separation of benzodiazepines and their metabolites was achieved within 3.90 min as shown in figure 3.5. The peak widths of benzodiazepines after derivatization of MTBSTFA varied from 0.020 to 0.042 min leading to approximately 20 – 35 data points per peak, which can be considered a typical number of data points used in fast GC analysis [164, 165].

Table 3.1. Optimization GC column temperatures, in order to obtain fast and efficient GC separation of benzodiazepines and their metabolites.

Temperatures in the column			Separation time, min			
Initial column tempera-ture, °C	The column tem-perature elevation rate, °C min^{-1}	Final column tempera-ture, °C				
150	30/40/50/60	290	5.67	4.50	3.80	3.33
150	30/40/50/60	310	6.33	5.00	4.20	3.67
150	30/40/50/60	325	6.83	5.38	4.50	3.92
150	30/40/50/60	340	7.33	5.75	4.80	4.17
170	30/40/50/60	290	5.00	4.01	3.40	3.00
170	30/40/50/60	310	5.67	4.50	3.80	3.33
170	30/40/50/60	325	6.17	4.88	4.10	3.58
170	30/40/50/60	340	6.67	5.25	4.40	3.83
180	30/40/50/60	290	4.67	3.75	3.20	2.83
180	30/40/50/60	310	5.33	4.25	3.60	3.17
180	30/40/**50**/60	**325**	5.83	4.63	**3.90**	3.42
180	30/40/50/60	340	6.33	5.00	4.20	3.67
250	30/40/50/60	290	2.33	2.00	1.80	1.67
250	30/40/50/60	310	3.00	2.50	2.20	2.00
250	30/40/50/60	325	3.50	2.88	2.50	2.25
250	30/40/50/60	340	4.00	3.25	2.80	2.50

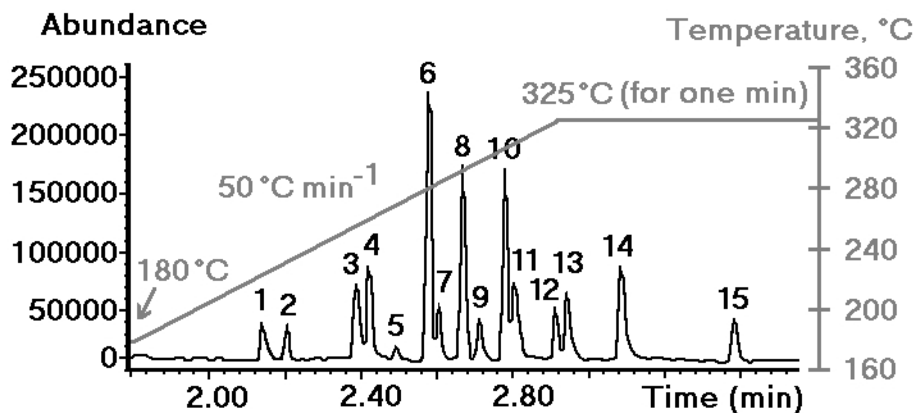


Figure 3.5. Fast and efficient gas chromatographic separation of 15 benzodiazepines. Selected ions monitoring chromatogram of standard solution after derivatization by MTBSTFA at 200.0 ng mL^{-1} of each analyte. The peak numbers in the chromatogram refer to the numbers of analytes provided in Figure 3.1 C.

Optimization of carrier gas flow rate

The effect of carrier gas (helium) flow rate was investigated in the range from 0.5 to 7.0 mL min^{-1}. The way in which flow rate of carrier gas affects column efficiency is the best demonstrated by reference to the Van Deemter curves of benzodiazepines (Fig. 3.6).

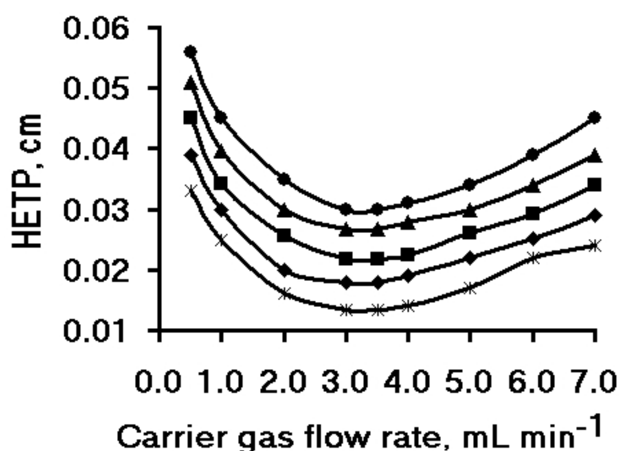


Figure 3.6. The Van Deemter curves of the analytes at 200.0 ng mL^{-1} for a DB-5HT capillary column (30 m × 0.320 mm id., 0.10-µm film thickness) (n=5). The analysis was performed after derivatization by MTBSTFA. The analytes numbering refers to: (1) diazepam; (2) flunitrazepam; (3) oxazepam; (4) lorazepam; (5) α-OH-alprazolam.

According to the obtained results, the column efficiency (minimum height equivalent of a theoretical plate – HETP) was optimal at intermediate flow rate, and that column efficiency was compromised at both low and very high flow rates. Therefore, the instrument operating at 3.5 mL min^{-1} flow rate of helium gas exhibited the best column efficiency and peaks resolution for all benzodiazepines. A small loss in resolution of the analytes for a shorter analysis time (3.90 min) is usually tolerated.

3.1.3. Benzodiazepines mass spectra in the NICI-MS mode

Benzodiazepines-TBDMS derivatives were formed specific mass spectra in NICI-MS mode. In this study, only the two analytes mass spectra – clonazepam-TBDMS and 7-aminoclonazepam-TBDMS were interpreted. The mass spectra of the MTBSTFA derivatives of clonazepam and 7-aminoclonazepam are shown in Figure 3.7.

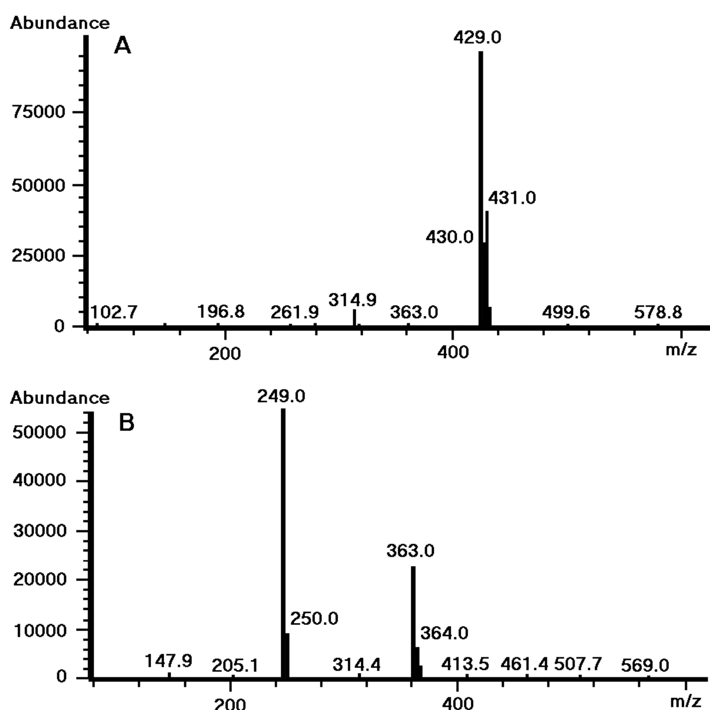


Figure 3.7. Mass spectra of the MTBSTFA derivatives of clonazepam-TBDMS (A) and 7-aminoclonazepam-TBDMS (B). In all scanning mode experiments a mass range of 100 – 600 amu was applied.

Clonazepam-TBDMS derivative in the NICI-MS mode gave the three main m/z 429, m/z 430 and m/z 431 ions. The full fragmentation is illustrated in Fig. 3.7 A. The methane gas induced NICI mass spectrum shows the two isotopic m/z 430 and m/z 431 ions with the peak intensity of 29.7 ± 1.4 % and 41.3 ± 1.0 % (SD_n=10), respectively. The molecular $[C_{15}H_9ClN_3O_3TBDMS]^-$ (m/z 429) ion with 100.0 % intensity was also observed. Virtually, it is the only confirmation factor for clonazepam-TBDMS, resulting from reaction in NICI-MS mode. The fragmental patterns of the most benzodiazepines, such as: diazepam, midazolam, flunitrazepam, bromazepam-TBDMS, nitrazepam-TBDMS, alprazolam, α-OH-midazolam-TBDMS, α-OH-alprazolam-TBDMS were similar to that of clonazepam-TBDMS derivative.

The ions with m/z 249, 363, 364 and intensities of 100.0 %, 39.2 ± 3.6 %, and 12.3 ± 1.1 % (SD_n=10), respectively, are the typical result of 7-aminoclonazepam-TBDMS fragmentation, without the molecular ion. Full fragmentation is illustrated in Figure 3.7 B. The target peak of m/z 249 is assumed to be an ion of $[C_{15}H_{11}N_3O]^-$ derived from the fragmented ion at m/z 363, formed by loss of one TBDMS (tert-butyl-dimethylsilyl) group. This is a typical fragmentation of 7-aminoclonazepam-TBDMS. However, other

investigated analytes, such as: nordiazepam-TBDMS, oxazepam-2TBDMS, temazepam-TBDMS, lorazepam-2TBDMS, and triazolam had unique fragmentation patterns in the NICI-MS mode.

Qualitative and quantitative analysis of benzodiazepines

All quantitative analyses were performed in the selected ion monitoring (SIM) mode. Three characteristic SIM ions, except for ISs (target ion and one or two qualifier(s)), the relative responses of the qualifier ions in relation to the target ion, and the retention time of the analytes were used for the identification of benzodiazepines. In all scanning mode experiments a mass range of 100 – 600 amu, and cycle time 2.60 cps (cycles per sec) was covered for qualitative analysis. Qualitative parameters are shown in Table 3.2.

Table 3.2. Selection of qualitative parameters for the determination of benzodiazepines.

Compound	Retention time, min	SIM ions, m/z[a]
Fludiazepam (IS)	2.094	**302**; 304(32.9±0.4)
Diazepam	2.164	**284**; 286(33.8±1.0), 285(19.0±1.2)
Nordiazepam	2.230	**234**; 384(33.6±4.7), 386(13.6±2.7)
Midazolam	2.401	**325**; 327(34.0±1.1), 326(20.8±0.9)
Flunitrazepam	2.427	**313**; 314(18.4±0.6), 315(2.6±0.4)
Bromazepam	2.487	**431**; 429(89.9±4.3), 432(29.5±2.6)
Oxazepam-d5 (IS)	2.587	**273**; 275(34.1±0.9)
Oxazepam	2.587	**268**; 270(34.0±0.7), 269(17.1±0.4)
Nitrazepam	2.624	**395**; 396(29.3±1.3), 397(8.6±0.5)
Temazepam	2.690	**414**; 282(21.7±2.3), 416(39.7±0.6)
7-aminoclonazepam-d4 (IS)	2.720	**253**; 367(40.2±3.2)
7-aminoclonazepam	2.720	**249**; 363(39.2±3.6), 364(12.3±1.1)
Lorazepam	2.799	**302**; 304(65.7±0.8), 303(17.3±0.7)
Clonazepam-d4 (IS)	2.824	**433**; 435(40.6±0.8)
Clonazepam	2.824	**429**; 431(41.3±1.0), 430(29.7±1.4)
Alprazolam	2.929	**308**; 310(34.6±2.2), 309(21.2±1.9)
α-OH-midazolam	2.965	**455**; 457(40.2±2.1), 456(32.7±2.3)
Triazolam	3.099	**306**; 308(40.4±3.3), 342(2.9±0.5)
α-OH-alprazolam	3.406	**438**; 440(40.4±1.5), 439(30.7±0.9)

[a] The m/z target ion presented in bold and values presented in parenthesis are the relative abundances ± SD (%) of qualifiers ions (n=10).

3.1.4. The effect of reagent gas flow rate

The effect of methane reagent gas flow (which is directly related to the partial pressure of reagent gas in the ion source) on the normalized response of benzodiazepines was used in this study. The methane flow rate was varied from 0.50 to 3.00 mL min^{-1}. The observed detector response increases with increasing methane flow rate for all analytes and implies that increased partial pressure of reagent gas in the source enhances anion production efficiency. Higher flow rates of methane were not evaluated due to the limitation (maximum flow rate of 3.00 mL min^{-1}) of the turbo molecular pump and insufficient repeatability of results. Thus, an instrument operating with a 2.50 mL min^{-1} flow rate of methane gas showed maximal sensitivity and repeatability for all benzodiazepines. The detailed studies are presented in Figure 3.8. In most methods published previously, a 2.00 mL min^{-1} flow rate of methane gas was used [105, 113]. The proposed method gives up to 19.5 % higher responses at the same analyte concentrations and improves sensitivity for all analytes using a 2.50 mL min^{-1} flow rate of reagent gas in comparison with the conventional 2.00 mL min^{-1} flow rate.

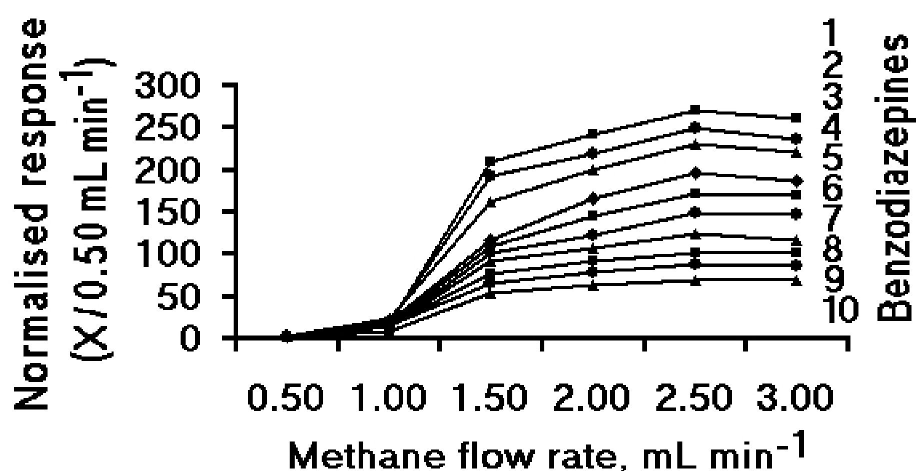


Figure 3.8. The normalised responses to ten analytes obtained by GC/NICI-MS upon gradually increasing the methane reagent gas flow rate (n=5). The carrier gas flow rate was 3.5 mL min^{-1}. The ion source temperature was 150 °C. The analytes numbering refers to: (1) diazepam; (2) midazolam; (3) bromazepam; (4) α-OH-alprazolam; (5) clonazepam; (6) lorazepam; (7) 7-aminoclonazepam; (8) oxazepam; (9) nitrazepam; (10) nordiazepam.

3.1.5. Development of an SPE method for the determination of benzodiazepines

One of the greatest challenges with a multi-residue analysis of benzodiazepines is the selection of a sorbent suitable to achieve an acceptable sensitivity to all compounds characterized by different physicochemical properties. In this work, a mixed-mode polymeric sorbent (Oasis MCX) with ion-exchange properties was applied. This sorbent is capable of hydrophilic-lipophilic and ion-exchange interactions [20, 166] and therefore it is suitable for achieving an efficient extraction of all herein-investigated benzodiazepines by a single extraction step.

3.1.5.1. Optimization of the sample pH

The retention and elution of benzodiazepines could be affected by the pH of the sample solution. For the selection of the optimal pH value of the sample solution, 0.1 M HCl of pH 1.0, 0.1 M phosphate buffer of pH 7.0 and 0.1 M borate buffer of pH 9.0 were used. A ten-times larger volume of HCl solution or buffer was added to the 0.2 mL of aqueous sample solution of each analyte before extraction. The analytes were eluted first with 1.0 mL MeOH and then with 1.0 mL MeOH containing 5 % of NH_4OH.

The adsorption-related behaviour of benzodiazepines was similar. When the acidic solution of pH 1.0 was used, all analytes were adsorbed on the sorbent due to ion-exchange interactions. Benzodiazepines have the basic pK_a values in the range from 1.2 to 6.5 (Table 3.3.) [3, 4, 143]. Analytes were eluted with MeOH containing 5 % of NH_4OH.

Using a neutral (pH 7.0) or basic (pH 9.0) buffer analytes were adsorbed onto the polymeric sorbent in the column due to hydrophilic-lipophilic interactions and were eluted with pure MeOH. The extraction efficiency at pH 7.0 was insufficient for midazolam and triazolam. These analytes with pK_a values of 6.2 and 6.5 were not completely retained/adsorbed under neutral conditions due to lower hydrophobicity than at basic pH (pH 9.0). Typical

extraction efficiencies of 15 benzodiazepines at pH 1.0 and pH 9.0 are shown in Figure 3.9.

Table 3.3. pK_a values of investigated benzodiazepines

Compound	pK_a values	
	Acidic	Basic
Alprazolam	–	2.3
α-OH-alprazolam	13.1	1.2
Bromazepam	11.0	2.9
Clonazepam	11.2	1.5
7-aminoclonazepam	–	3.9
Diazepam	–	3.3
Flunitrazepam	–	1.7
Lorazepam	10.8	1.3
Midazolam	–	6.2
α-OH-midazolam	–	4.4
Nitrazepam	11.4	3.2
Nordiazepam	12.0	3.5
Oxazepam	10.9	1.7
Temazepam	–	1.6
Triazolam	–	6.5

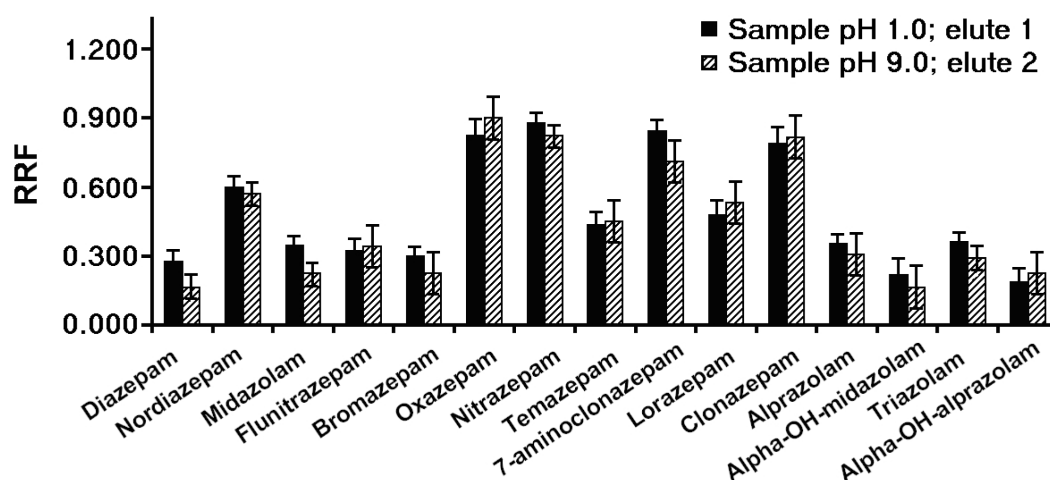


Figure 3.9. Extraction efficiency of 15 benzodiazepines at two pH values. Elute 1 – MeOH containing 5 % (v/v) NH_4OH (n=5). Elute 2 – MeOH (n=5). A volume of eluent was 1.0 mL, the four ISs – fludiazepam, oxazepam-d5, clonazepam-d4, and 7-aminoclonazepam-d4 were used. Aqueous samples were spiked at 20.0 ng mL^{-1} of each analyte.

The adsorption behaviour of benzodiazepines on the SPE sorbent can be explained by the protonation/deprotonation of the analyte in the sample

solution. All analytes, which are in the non-ionic form in a basic buffer at pH 9.0, were adsorbed by reversed-phase interactions. In contrast, analytes that are in the cationic form in an acidic solution, interacted with Oasis MCX specifically through ionic interactions. Additionally, benzodiazepines, which were retained/adsorbed by an ion-exchange phase, could be washed with organic solvents in order to remove acidic and neutral interfering compounds through mixed-mode interactions. After this step, visibly cleaner extracts were obtained in comparison with those obtained using reversed-phase interactions. Therefore, the pH value of 1.0 was chosen for further optimization studies.

3.1.5.2. Acid hydrolysis of benzodiazepines

Efficiency of benzodiazepines extraction from acidic aqueous solutions might be affected by possible hydrolysis of the analytes. The hydrolytic degradation of benzodiazepines was observed at 25.0 ± 0.1 °C in very acidic solution (1.0 M HCl at pH~0) where after 2 h the ring of diazepine is practically opened [167]. In less acidic solutions (0.001, 0.01 and 0.1 M HCl) the ring-opening reaction lasted for up to 4 h until hydrolysis equilibrium was reached [167, 168]. The mechanism of acid hydrolysis of alprazolam is shown in Figure 3.10.

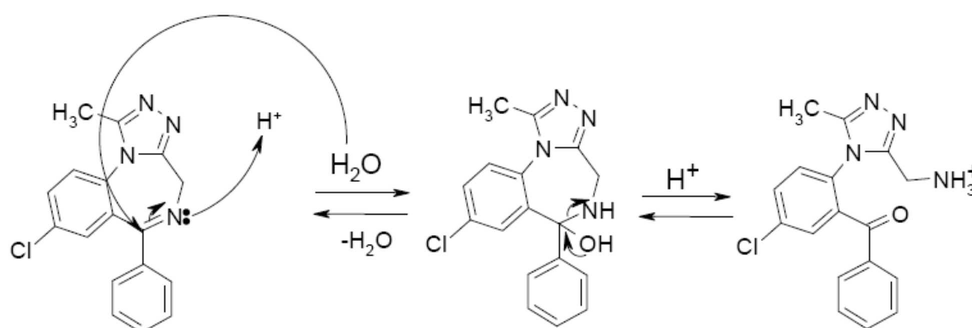


Figure 3.10. The ring-opening reaction of alprazolam [167].

In our study at pH 1.0 the analytes were well retained/adsorbed in the column. However, at such low pH, acid hydrolysis of benzodiazepines can occur. In order to avoid possible degradation of the analytes, an extraction

temperature of 22.5 ± 0.5 °C, and a time length not over 15 min for sample preparation (acidification) before SPE are recommended. Under these conditions, benzodiazepines have proven stable with only a small degradation (Fig. 3.11).

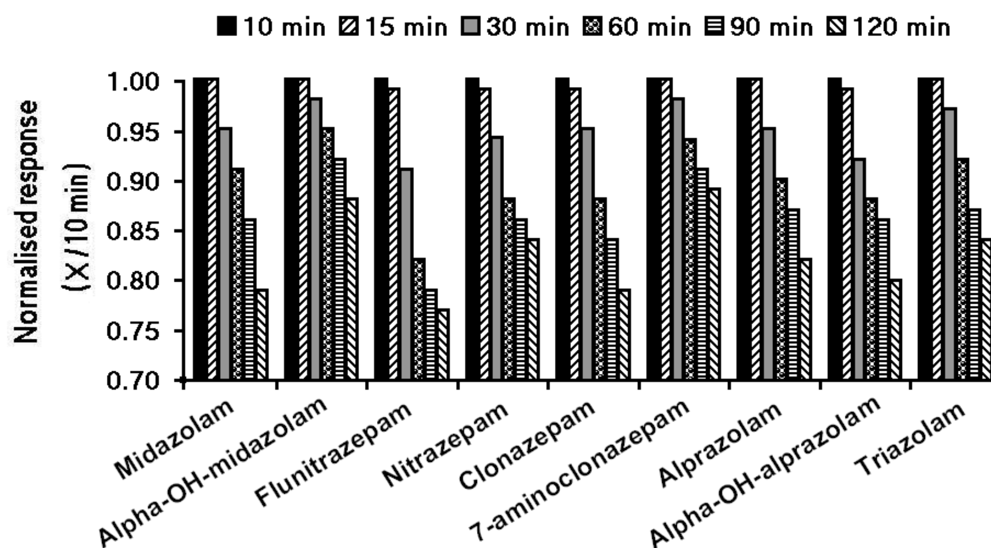


Figure 3.11. Time-dependent changes of normalised responses of benzodiazepines during the ring-opening reaction at pH 1.0 (n=5). Benzodiazepines (at 20.0 ng mL^{-1}) were hydrolyzed in 0.1 M HCl solution at room temperature of 22.5 ± 0.5 °C.

The experimental results showed that an increase in incubation time lead to the decreased extraction efficiency of the analytes. This could be affected by a partial ring-opening of benzodiazepines. For mixed-mode SPE method, similar sample preparation methodologies have been reported previously by other authors [105, 141, 142]: samples were acidified with 2 M HCl (a volume of 50 µL acid was added to 1.5 mL of sample) [105] or concentrated orthophosphoric acid (a volume of 20 µL acid was added to 1.0 mL of sample) [141, 142] for the determination of midazolam, flunitrazepam and their major metabolites.

3.1.5.3. The protein binding to the benzodiazepines

Most benzodiazepines and their metabolites are strongly bound to proteins (e.g. from whole blood sample), mainly to α-1-glycoprotein and to a lesser extent to albumin and lipoproteins [3, 6, 169]. When using SPE as sample preparation method, proteins binding can lower the analyte recovery, as the active sites of the compounds that would normally interact with the sorbent are not available for this interaction. Another problem is the fact that most proteins are large molecules prohibiting penetration of the analytes to the sorbent pores.

Sonication, centrifugation and dilution in combination with a slow sorbent pass-through of the sample seem to be appropriate to demolish the protein binding of drugs [117 - 119]. α-1-glycoprotein binding capacity depends on temperature, pH, protein content and molecules that compete for the same sites on the protein. Thus, dilution, change of pH and centrifugation can also modify the protein binding to the analytes. In this study, whole blood was firstly diluted with 2.0 mL of HPLC grade water at pH 7.0, acidified with 0.15 mL of 1.5 M HCl (pH 1.0 instead of pH 7.0), and finally centrifuged at 1233 g (3500 rpm) for 5 min as a sample preparation procedure before a mixed-mode SPE. All these steps were used in order to decrease protein binding to the analytes.

3.1.5.4. Optimization of the washing step/procedure

Three wash steps were employed to remove the matrix and to get very clean extracts. Firstly, acidified water (0.1 M HCl of pH 1.0) was used, followed by organic solvents. Different volumes (1.5 and 1.0 mL) of both 0.1 M HCl of pH 1.0 and ACN were tested. These reagents did not cause the loss of any benzodiazepines, but the matrix was not sufficiently removed from the sorbent.

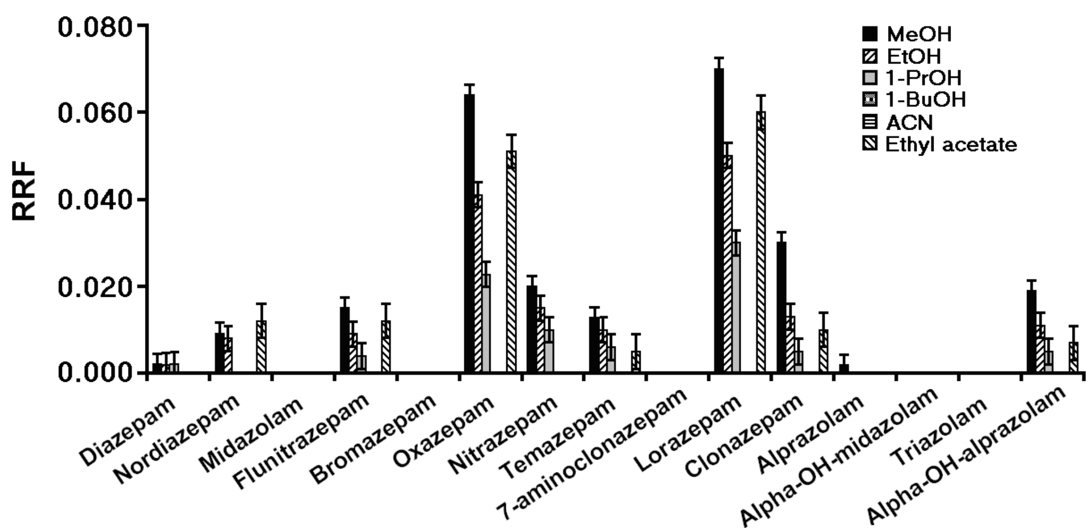


Figure 3.12. Extraction efficiency of 15 benzodiazepines at pH 1.0, eluted with pure organic solvents (n=5). A volume of eluent was 1.5 mL, the four ISs were used. Blood samples spiked at 20.0 ng mL^{-1} of each analyte.

However, 1.5 mL of pure MeOH, EtOH, 1-PrOH, 1-BuOH, or ethyl acetate resulted in removal of some analytes and in effective removal of neutral and weak acidic matrix compounds from the sorbent. The results obtained using these solvents are presented in Figure 3.12. It was observed experimentally that 1-PrOH was the best solvent to remove all matrix compounds from the sorbent. Therefore, in this washing step, mixtures of acidified water (at 0.15 M HCl) and 1-PrOH with a 1-PrOH concentration ranging from 0 to 100 % in intervals of 10 % were tested. The experimental results are shown in Fig. 3.13. The optimal concentration of 1-PrOH to minimize loss of the analytes at this stage was 60 %. It could be concluded that this washing step yielded significantly cleaner extracts and it is the best out of all other procedures tested for this purpose, and subsequently it was used in further studies. According to the obtained experiment results, three wash steps have been used to remove the matrix effects and to get very clean extracts and chromatograms (Fig. 3.14.).

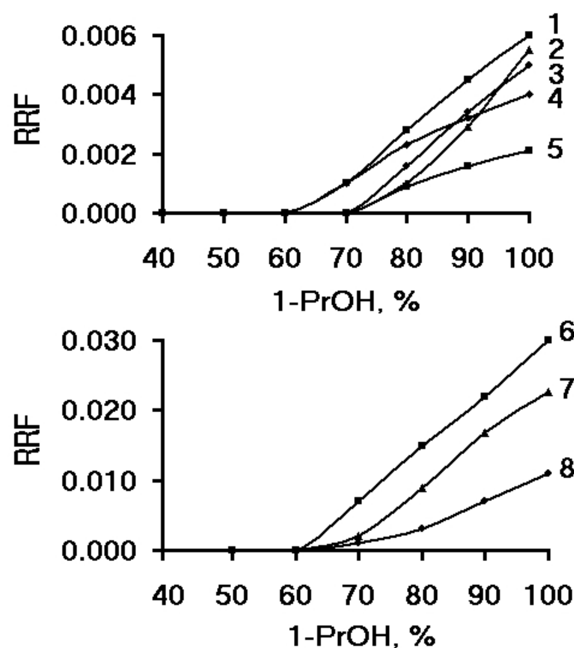


Figure 3.13. The effect of a mixed-mode Oasis MCX sorbent washing on the extraction efficiency (n=5) using 1-PrOH and acidified water at 0.15 M HCl mixtures. Total volume of mixture was 1.5 mL. Blood samples (at pH 1.0) spiked at 20.0 ng mL^{-1} of each analyte. The analytes numbering refers to: (1) temazepam; (2) clonazepam; (3) α-OH-alprazolam; (4) flunitrazepam; (5) diazepam; (6) lorazepam; (7) oxazepam; (8) nitrazepam.

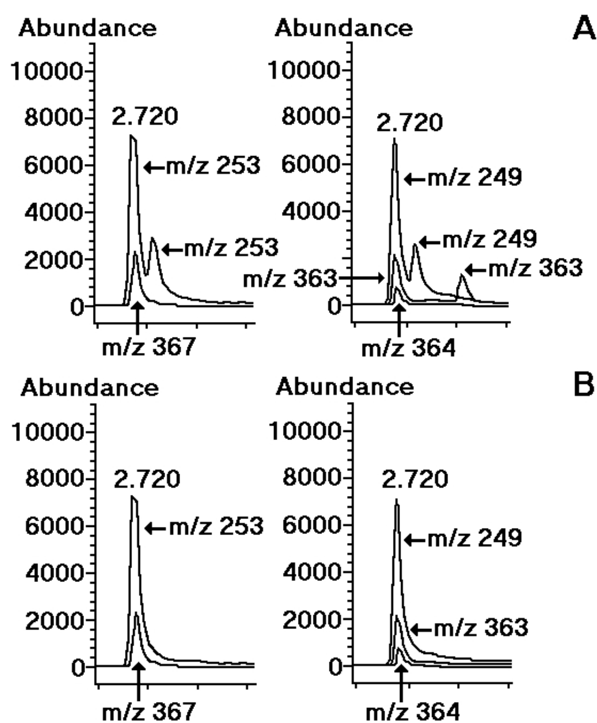


Figure 3.14. Selected ions chromatograms of whole blood samples obtained at 20.0 ng mL^{-1} of both 7-aminoclonazepam-d4 (m/z 253, 367) and 7-aminoclonazepam (m/z 249, 363, 364) after washing steps of SPE: (A) 1.5 mL of 0.1 M HCl at pH 1.0 (acidified water) and 1.0 mL of ACN; (B) 1.5 mL of 0.1 M HCl at pH 1.0 (acidified water), 1.5 mL of 1-PrOH and acidified water at 0.15 M HCl mixture (60:40 v/v), and finally 1.0 mL of ACN. All SPE steps were performed at a flow rate of 1 mL min^{-1}.

3.1.5.5. The benzodiazepines elution from a mixed-mode sorbent

The elution step was the following parameter to be optimised in order to break the ionic interactions between the analytes and a mixed-mode sorbent using basic and organic media. Basic elution mixtures, which contain NH_4OH, were tested to release the basic compounds (benzodiazepines) from a mixed-mode sorbent. Therefore, increasing volumes of 5 % NH_4OH (optimal concentration of NH_4OH was tested) in MeOH, EtOH, 1-PrOH, 1-BuOH, ACN or ethyl acetate over the range of mixture volume from 0.25 to 3.0 mL were investigated for elution of the analytes (Fig. 3.15).

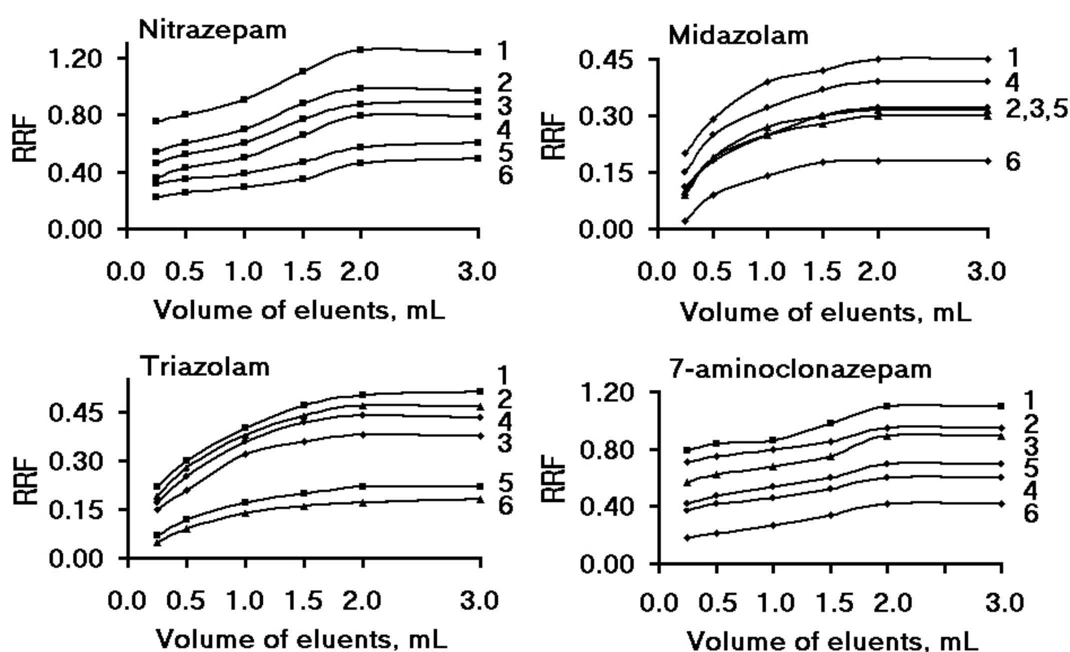


Figure 3.15. The dependence of extraction efficiencies obtained in the elution step for selected analytes on the applied different volumes of eluent (n=5). A mixed-mode Oasis MCX sorbent and whole blood samples spiked at 20.0 ng mL^{-1} of each analyte were used. Eluents numbering refers to: (1) MeOH containing 5 % (v/v) NH_4OH; (2) EtOH containing 5 % (v/v) NH_4OH; (3) 1-PrOH containing 5 % (v/v) NH_4OH; (4) ethyl acetate containing 5 % (v/v) NH_4OH; (5) 1-BuOH containing 5 % (v/v) NH_4OH; (6) ACN containing 5 % (v/v) NH_4OH.

By increasing basic mixtures volumes from 0.25 to 3.0 mL, the analytes extraction efficiencies were increased significantly up to 2.0 mL. However, no significant differences were obtained between 2.0 and 3.0 mL. For the elution

of all compounds, a volume of 2.0 mL of MeOH containing 5 % NH_4OH was determined to be the most effective. The results obtained using different basic mixtures (solvents containing 5 % NH_4OH) are summarised in Fig. 3.16.

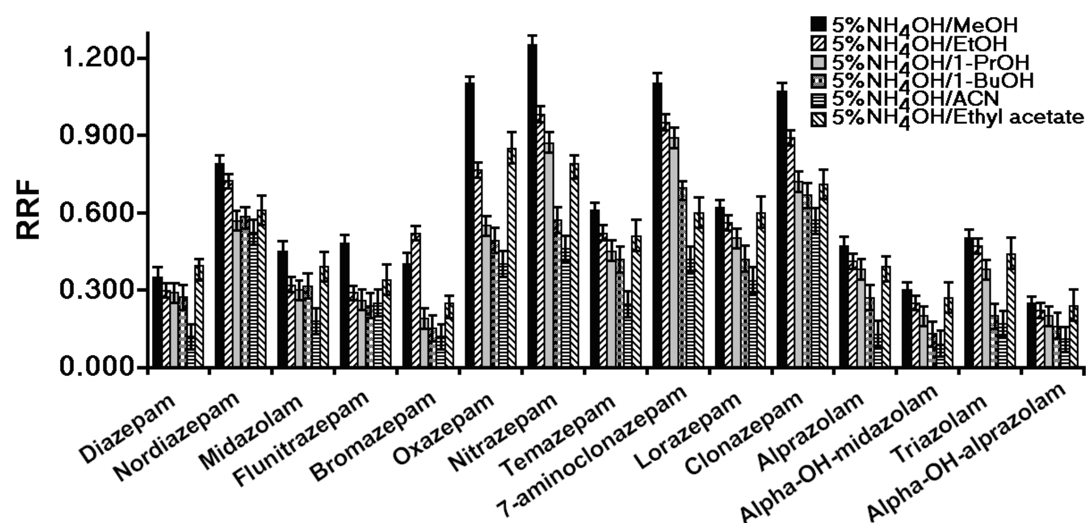


Figure 3.16. Extraction efficiency of 15 benzodiazepines at pH 1.0, eluted with solvents containing 5 % (v/v) NH_4OH (n=5). A volume of eluents was 2.0 mL, the four ISs were used. Blood samples spiked at 20.0 ng mL^{-1} of each analyte.

A volume of 2.0 mL of MeOH containing 5 % NH_4OH was found to be the optimal basic solvent combination suitable for complete elution of analytes from a mixed-mode column (Oasis MCX). This basic mixture ensures the deprotonation of benzodiazepines (the analytes become neutral), while a mixed-mode sorbent is still in the ionic, and the analytes are eluted due to the elution strength of the organic mixture (MeOH containing 5 % NH_4OH). Therefore, it was possible to completely separate all analytes and interference materials from whole blood using the described method and to receive very clean extracts, as shown in the chromatogram (Fig. 3.17). The use MeOH containing 5 % NH_4OH for elution step allowed obtaining sufficient precision for the tested analytes.

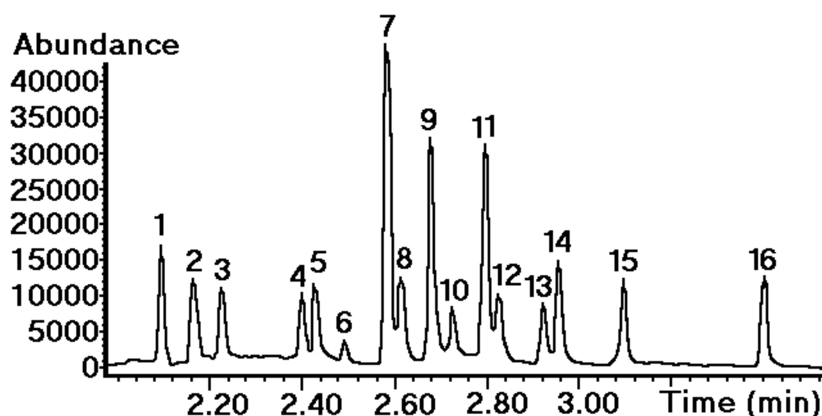


Figure 3.17. SIM chromatogram obtained from the analysis of benzodiazepines by fast GC/NICI-MS. Chromatogram of a whole blood sample (at pH 1.0) spiked at 20.0 ng mL^{-1} of each analyte. The peak numbering refers to: (1) fludiazepam (IS); (2) diazepam; (3) nordiazepam-TBDMS; (4) midazolam; (5) flunitrazepam; (6) bromazepam-TBDMS; (7) oxazepam-2TBDMS; (8) nitrazepam-TBDMS; (9) temazepam-TBDMS; (10) 7-aminoclonazepam-TBDMS; (11) lorazepam-2TBDMS; (12) clonazepam-TBDMS; (13) alprazolam; (14) α-OH-midazolam-TBDMS; (15) triazolam; (16) α-OH-alprazolam-TBDMS.

3.1.6. Validation of an SPE-GC/NICI-MS method for the determination of benzodiazepines in whole blood

The complete method for the determination of benzodiazepines and their metabolites in human whole blood was validated following the recommendation for new methods [161 - 163]. The selectivity, sensitivity, linearity, accuracy, precision, recovery, robustness and stability were validated. The reliability of this method was certified by means of an exhaustive validation study.

The selectivity of the method

For the selectivity study, the interference from endogenous blood compounds were checked by analyzing five different drug-free (blank) human whole blood extracts with and without ISs. The selectivity of the method was adequate, with a minimal matrix effect for all blank samples. Furthermore, no interferences were observed from the tested substances at the retention times of 15 benzodiazepines. The chromatogram of the drug-free sample is shown in Fig. 3.18.

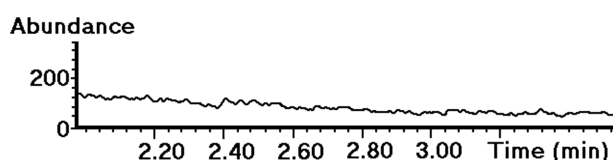


Figure 3.18. Representative SIM chromatogram of the drug-free whole blood sample.

The linearity and sensitivity of the method

The linearity of a typical calibration curve for the each analyte in whole blood was determined by computing a regression line of peak area ratio of a calibrator to the IS versus their concentration ratio using a least-squares fit (Table 3.4). The high number of deuterated analogues and careful selection of suitable ISs helped to obtain large linearity ranges for all analytes tested. Only high concentrations of lorazepam and nitrazepam (at 200.0 ng mL^{-1}) failed to meet the criterion that the back-calculated concentrations should be within ± 20 % of their nominal values. However, the upper limit of 100.0 ng mL^{-1} was therefore used for daily calibration straight lines, which should be high enough for typical blood concentrations of these analytes.

Table 3.4. Calibration data and sensitivity of the method in fresh whole blood (n=5).

Compound	Calibration (Y = aX + b)[a]			LOD, ng mL^{-1}	LOQ, ng mL^{-1}
	a	b	r^2		
Diazepam	0.0091	-0.0181	0.9987	0.55	1.68
Nordiazepam	0.1483	0.2270	0.9990	0.46	1.40
Midazolam	0.0114	-0.0069	0.9994	0.35	0.99
Flunitrazepam	0.0122	0.0021	0.9961	0.30	0.91
Bromazepam	0.0081	0.0003	0.9985	0.62	1.89
Oxazepam	0.0276	0.0057	0.9991	0.24	0.72
Nitrazepam	0.1021	-0.3144	0.9976	0.56	1.69
Temazepam	0.0183	0.0135	0.9997	0.36	1.11
7-aminoclonazepam	0.0226	-0.0133	0.9996	0.33	0.98
Lorazepam	0.0178	-0.0071	0.9994	0.24	0.72
Clonazepam	0.0279	0.0141	0.9994	0.32	0.97
Alprazolam	0.0096	0.0009	0.9990	0.34	0.98
α-OH-midazolam	0.0114	0.0048	0.9987	0.31	0.93
Triazolam	0.0134	-0.0149	0.9981	0.31	0.95
α-OH-alprazolam	0.0090	-0.0096	0.9989	0.33	0.98

[a]Y = area of each compound/area of IS; X = concentration of compound/concentration of IS.

As it is shown in Table 3.4, the linear relationships with the determination coefficients (r^2) are in the range from 0.9961 to 0.9997. LODs and LOQs for the developed method were in the range of 0.24 – 0.62 ng mL^{-1} and 0.72 – 1.89 ng mL^{-1}, respectively. The lowest concentration levels for each compound were considered as the LOQ. Thus, the accuracy of spiked whole blood samples at the same concentration (n=5) ranged from 90.3 to 107.8 %, and the precision (RSD %) respectively was ≤ 15.0 %, in accordance with the most recent regulatory recommendations [161, 162]. LOQs values should be used as the lowest point of calibration curve updated in each analysis and as the cut-off value for reliable quantitation. Thus, obtained results showed that the developed method is very sensitive, selective and specific enough to detect the analytes after a long time of a single oral administration of some drugs.

The accuracy and precision

The accuracy and precision have been evaluated and the obtained data are summarized in Table 3.5 and Table 3.6. The accuracy was determined by comparing the measured concentration in blood obtained by calibration curves with spiked concentrations of QC samples in blood. The results of intra-day (n=5) and inter-day (n=10) accuracy for three different QC concentration levels were in the range of 89.5 – 110.5 % and all RSDs for replicate determinations were ≤ 7.0 %. The intra-day precision is considered to be the repeatability of this method while the inter-day precision is considered to be as the reproducibility. The accuracy and precision in the determination of 15 benzodiazepines were satisfactory. It was determined that at 5.0, 50.0 and 150.0 ng mL^{-1} of diazepam, the intra-day accuracies were 101.8, 97.4 and 91.0 %, respectively, which might be due to calibration slope inaccuracy and/or statistical artefacts. The IS can be extracted using the developed a mixed-mode SPE procedure with a very good extraction efficiency at 20.0 ng mL^{-1} (n=5) for fludiazepam (94.1 ± 3.3 %), clonazepam-d4 (96.3 ± 4.5 %), oxazepam-d5 (97.2 ± 3.5 %) and 7-aminoclonazepam-d4 (97.6 ± 3.3 %). The obtained results suggest that this method is reliable for a multi-residue analysis of benzodiazepines and their metabolites in whole blood samples.

Table 3.5. Intra-day (n=5) precision and accuracy for 15 benzodiazepines and their nominal values at three QC levels.

Compound	Nominal conc., ng mL^{-1}	Intra-day precision and accuracy		
		Measured, ng mL^{-1} Mean ± SD	RSD, %	Accuracy, %
Diazepam	5.0	5.09 ± 0.12	2.36	101.8
	50.0	48.69 ± 1.93	3.96	97.4
	150.0	136.50 ± 6.78	4.97	91.0
Nordiazepam	5.0	5.27 ± 0.21	3.98	105.4
	50.0	49.31 ± 1.44	2.92	98.6
	150.0	156.90 ± 6.13	3.91	104.6
Midazolam	5.0	4.79 ± 0.19	3.97	95.8
	50.0	48.79 ± 2.18	4.47	97.6
	80.0	76.83 ± 2.86	3.72	96.0
Flunitrazepam	2.0	1.81 ± 0.10	5.52	90.5
	10.0	9.95 ± 0.54	5.43	99.5
	30.0	28.51 ± 1.11	3.89	95.0
Bromazepam	5.0	4.89 ± 0.26	5.32	97.8
	50.0	49.01 ± 2.53	5.16	98.0
	80.0	81.60 ± 3.70	4.53	102.0
Oxazepam	5.0	4.75 ± 0.16	3.37	95.0
	50.0	48.47 ± 1.46	3.01	96.9
	150.0	151.50 ± 6.44	4.25	101.0
Nitrazepam	5.0	4.92 ± 0.11	2.24	98.4
	50.0	49.09 ± 1.87	3.81	98.2
	80.0	82.44 ± 2.21	2.68	103.1
Temazepam	5.0	4.84 ± 0.25	5.17	96.8
	50.0	49.87 ± 1.93	3.87	99.7
	150.0	144.00 ± 4.61	3.20	96.0
7-aminoclonazepam	5.0	5.11 ± 0.12	2.35	102.2
	50.0	50.05 ± 2.16	4.32	100.1
	80.0	82.62 ± 2.88	2.76	103.3
Lorazepam	5.0	4.79 ± 0.32	6.68	95.8
	50.0	50.47 ± 2.27	4.50	100.9
	80.0	83.40 ± 3.24	3.88	104.3
Clonazepam	5.0	4.83 ± 0.21	4.35	96.6
	50.0	49.45 ± 2.51	5.08	98.9
	80.0	74.42 ± 2.97	3.99	93.0
Alprazolam	5.0	4.77 ± 0.25	5.24	95.4
	50.0	50.62 ± 1.89	3.73	101.2
	80.0	81.21 ± 2.72	3.35	101.5
α-OH-midazolam	2.0	2.21 ± 0.10	4.52	110.5
	10.0	10.55 ± 0.45	4.27	105.5
	30.0	30.46 ± 1.19	3.91	101.5
Triazolam	5.0	4.88 ± 0.25	5.12	97.6
	50.0	50.09 ± 1.66	3.31	100.2
	80.0	78.81 ± 2.80	3.55	98.5
α-OH-alprazolam	5.0	4.57 ± 0.30	6.56	91.4
	50.0	48.35 ± 2.07	4.28	96.7
	80.0	78.42 ± 3.32	4.23	98.0

Table 3.6. Inter-day (n=10) precision and accuracy for 15 benzodiazepines and their nominal values at three QC levels.

Compound	Nominal conc., ng mL^{-1}	Inter-day precision and accuracy		
		Measured, ng mL^{-1} Mean ± SD	RSD, %	Accuracy, %
Diazepam	5.0	4.99 ± 0.17	3.41	99.8
	50.0	49.19 ± 2.60	5.29	98.4
	150.0	149.85 ± 7.69	5.13	99.9
Nordiazepam	5.0	5.08 ± 0.32	6.30	101.6
	50.0	49.55 ± 2.01	4.06	99.1
	150.0	146.85 ± 7.12	4.85	97.9
Midazolam	5.0	4.79 ± 0.25	5.22	95.8
	50.0	47.78 ± 2.34	4.90	95.6
	80.0	79.08 ± 3.64	4.60	98.9
Flunitrazepam	2.0	1.79 ± 0.12	6.70	89.5
	10.0	10.03 ± 0.64	6.38	100.3
	30.0	27.37 ± 1.86	6.80	91.2
Bromazepam	5.0	4.83 ± 0.32	6.63	96.6
	50.0	48.47 ± 2.86	5.90	96.9
	80.0	80.14 ± 5.08	6.34	100.2
Oxazepam	5.0	4.55 ± 0.20	4.40	91.0
	50.0	48.25 ± 1.91	3.96	96.5
	150.0	149.70 ± 7.57	5.06	99.8
Nitrazepam	5.0	4.82 ± 0.19	3.94	96.4
	50.0	49.78 ± 2.04	4.10	99.6
	80.0	81.08 ± 3.32	4.09	101.4
Temazepam	5.0	4.86 ± 0.31	6.38	97.2
	50.0	50.29 ± 2.78	5.53	100.6
	150.0	147.30 ± 7.81	5.30	98.2
7-aminoclonazepam	5.0	5.03 ± 0.14	2.78	100.6
	50.0	50.47 ± 2.69	5.33	100.9
	80.0	80.28 ± 2.84	3.54	100.4
Lorazepam	5.0	4.72 ± 0.30	6.36	94.4
	50.0	49.28 ± 2.50	5.07	98.6
	80.0	76.68 ± 4.10	5.35	95.9
Clonazepam	5.0	4.75 ± 0.22	4.63	95.0
	50.0	49.72 ± 2.81	5.65	99.4
	80.0	78.16 ± 3.44	4.40	97.7
Alprazolam	5.0	4.85 ± 0.24	4.95	97.0
	50.0	49.98 ± 2.61	5.22	99.9
	80.0	76.96 ± 3.36	4.37	96.2
α-OH-midazolam	2.0	2.17 ± 0.15	6.91	108.5
	10.0	9.74 ± 0.58	5.95	97.4
	30.0	30.05 ± 1.83	6.09	100.2
Triazolam	5.0	4.95 ± 0.27	5.45	99.0
	50.0	49.68 ± 2.17	4.37	99.4
	80.0	77.72 ± 3.66	4.71	97.2
α-OH-alprazolam	5.0	4.53 ± 0.29	6.40	90.6
	50.0	47.45 ± 2.52	5.31	94.9
	80.0	78.92 ± 4.04	5.12	98.7

The recovery

The recovery was determined as the amount of extracted standard solution compared with the amount of standard solution after derivatization. Low (2.0 or 5.0 ng mL^{-1}) and high (30.0, 80.0 or 150.0 ng mL^{-1}) concentrations of standard solutions were tested (n=6). Results are shown in Table 3.7.

Table 3.7. Extraction recovery of 15 benzodiazepines (n=6).

Compound	Nominal conc., ng mL^{-1}	Absolute recovery, %
Diazepam	5.0/150.0	85.5
Nordiazepam	5.0/150.0	86.8
Midazolam	5.0/ 80.0	80.6
Flunitrazepam	2.0/ 30.0	80.7
Bromazepam	5.0/ 80.0	88.2
Oxazepam	5.0/150.0	70.8
Nitrazepam	5.0/ 80.0	88.9
Temazepam	5.0/150.0	80.9
7-aminoclonazepam	5.0/ 80.0	91.4
Lorazepam	5.0/ 80.0	62.2
Clonazepam	5.0/ 80.0	61.8
Alprazolam	5.0/ 80.0	81.8
α-OH-midazolam	2.0/ 30.0	90.6
Triazolam	5.0/ 80.0	81.3
α-OH-alprazolam	5.0/ 80.0	61.1

The average of the absolute recovery was calculated as the percent recovery for each extracted benzodiazepine from standard solutions. The results of absolute recoveries for most of the analytes ranged from 80.6 to 91.4 %, except for oxazepam (70.8 %), lorazepam (62.2 %), clonazepam (61.8 %) and α-OH-alprazolam (61.1 %). Lower recovery results could be due to possible degradation of the analytes at pH 1.0, when reversible ring-opening of benzodiazepines occur (Fig. 3.10). The obtained results have been satisfactory and completely satisfied requirements of the newly method developed [161, 162].

Robustness of the method

Robustness of the method was studied by changing several parameters of the procedure such as: injection temperature (255 instead of 250 °C), oven temperature heating rate (45 instead of 50 °C min^{-1}), extraction pH of the

samples (pH 1.05 instead of pH 1.00), derivatization temperature (80 instead of 85 °C), as well as ratio of the solvents during silylation by MTBSTFA/ACN/ethyl acetate mixtures (20:45:35 and 20:35:45 v/v/v instead of 20:40:40, v/v/v). The robustness was determined from five replicates of spiked samples at the medium (10.0 or 50.0 ng mL^{-1}) QC level. Mean effects of each parameter and standard deviations of all differences were calculated. Neither a single parameter, nor a combination of them showed a significant influence on the results of the method, which was proven to be sufficiently robust, as the mean effect and standard deviation values were found to be adequately low.

The stability of benzodiazepines in autosampler

The stability in autosampler was checked by reinjection of the low (2.0 or 5.0 ng mL^{-1}) and medium (10.0 or 50.0 ng mL^{-1}) QC extracts after 50 h (n=5). This was accomplished by extraction of samples, followed by storing and running them in the chromatographic system. A new standard curve prepared for each experiment was used for the evaluation of stability of test derivatives. Immediate injection of derivatives is always preferable, but conservation at ambient temperature (22.5 ± 0.5 °C) up to 50 h did not significantly alter analytical results, in which the accuracy ranged from 84.6 to 115.5 %, and therefore the precision (RSD %) was less than 12.4 %. In this study, the evaluation of test derivatives stability was performed within 50 h and even after this period a variation of analytical results was within a sufficient level. This period was longer than periods usually tested in stability studies by other authors [105, 112, 113].

3.1.7. Application of an SPE method in real blood samples

The developed method was applied for qualitative and quantitative a multi-residue analysis of benzodiazepines. Real blood samples were collected from ten volunteers: men and women, 23 – 38 years old, after a single oral administration of some benzodiazepines. Determination of drugs level was carried out on fresh or hemolyzed blood of patients in cases of clinical interest.

The obtained results proved it was possible to determine diazepam, clonazepam, lorazepam and their metabolites in the blood of patients within a 1 – 3 day period after the last drug intake. This is in agreement with literature, which reports that lorazepam has an elimination half-life in plasma of 10 – 22 h, clonazepam 20 – 40 h, while the diazepam has the longest half-life 20 – 70 h [95, 170]. In this study, none of these samples caused any problems in the determination of the analytes, and no matrix interferences were observed in any of them. Typical chromatograms of real blood samples are shown in Fig. 3.19.

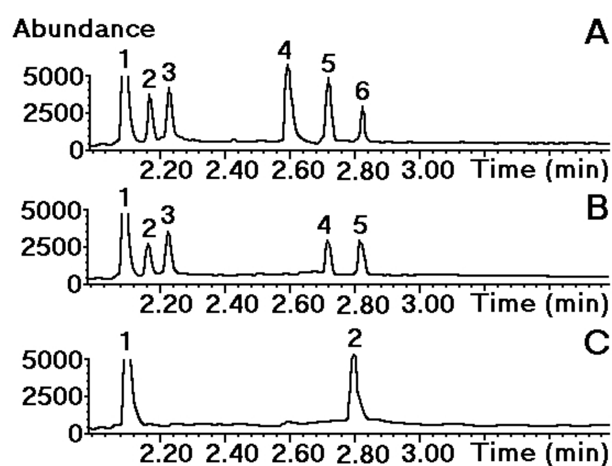


Figure 3.19. Representative SIM chromatograms obtained from real samples. Concentrations and peak numbering refer to: (A) 1) fludiazepam-IS; 2) diazepam 8.48 ng mL^{-1}; 3) nordiazepam 14.23 ng mL^{-1}; 4) oxazepam 2.64 ng mL^{-1}; 5) 7-aminoclonazepam 18.63 ng mL^{-1}; 6) clonazepam 5.50 ng mL^{-1}, (B) 1) fludiazepam-IS; 2) diazepam 5.37 ng mL^{-1}; 3) nordiazepam 10.71 ng mL^{-1}; 4) 7-aminoclonazepam 10.20 ng mL^{-1}; 5) clonazepam 5.47 ng mL^{-1}, (C) 1) fludiazepam-IS; 2) lorazepam 2.62 ng mL^{-1}.

The concentrations of drugs and their metabolites in blood samples were calculated according to the relative calibration curves. Representative results in blood are presented in Table 3.8. Usually diazepam, clonazepam and their degradation products (nordiazepam, oxazepam and 7-aminoclonazepam) and, in some cases, lorazepam were detected in real blood samples. According to the results, the developed a mixed-mode SPE method is accurate, sensitive and specific enough to detect analytes after a long time use of a single oral

administration of some drugs. Furthermore, this method enables to reach the highest specificity for major analytes and meets the requirements of good laboratory practice, especially when applied to pharmacodynamic investigations. Ultimately, the developed an SPE method has been applied in routine toxicological analysis during the investigation of both clinical and forensic cases.

Table 3.8. Results obtained for real samples (n=3).

Case no.	Compound and concentration (Mean ± SD, ng mL^{-1})					
	Diazepam	Nordiazepam	Oxazepam	7-amino-clonazepam	Clonazepam	Lorazepam
1[a]	8.18 ± 0.39	14.63 ± 0.46	2.60 ± 0.17	18.13 ± 0.65	5.33 ± 0.25	–
2[b]	5.49 ± 0.33	10.25 ± 0.48	–	9.92 ± 0.42	5.56 ± 0.29	–
3[b]	–	–	–	–	–	2.70 ± 0.16
4[b]	12.31 ± 0.45	73.88 ± 3.20	2.84 ± 0.18	–	–	–
5[b]	–	–	–	–	–	95.24 ± 2.94
6[a]	16.04 ± 0.44	69.78 ± 1.98	5.66 ± 0.23	50.49 ± 1.92	49.74 ± 2.88	–
7[a]	14.64 ± 0.94	76.94 ± 4.82	11.84 ± 0.36	–	–	–
8[b]	29.97 ± 0.43	2.19 ± 0.11	–	–	–	–
9[a]	–	–	–	55.74 ± 3.28	76.56 ± 3.58	–
10[b]	–	–	–	25.15 ± 0.65	9.82 ± 0.45	–

Information about samples: [a]hemolyzed blood, [b]fresh whole blood.

3.1.8. Review of SPE methods for the determination of benzodiazepines

The developed method has been compared with previously described methods suitable for the determination of benzodiazepines in plasma or whole blood after SPE. The developed method has several advantages, if compared with other previously published data. It shows significant selectivity and robustness, as well as satisfactory recovery, accuracy and precision results. The extraction efficiency of QC is higher in comparison to other published methods [102 - 104, 114]. A review of previously mentioned and already published results with respect to the accuracy of benzodiazepine determination shows accuracies of 81 – 114 % [102], 85 – 115 % [103], 88 – 114 % [104], and 83 – 93 % [114]. Similar results of accuracy towards the determination of midazolam and its main metabolite in the range of 92 – 112 % have been reported with a mixed-mode SPE method [105]. However, some previously

published methods for many benzodiazepines have shown quite high LODs: 0.08 – 0.47 ng μL^{-1} [101], 0.16 – 0.51 ng μL^{-1} [102], 0.2 – 0.5 ng μL^{-1} [139], 1.0 – 2.5 ng mL^{-1} [100], 0.5 – 0.8 ng mL^{-1} [137], or 20 ng mL^{-1} [104] for seven benzodiazepines. The proposed method is highly sensitive and effective for the determination of 15 benzodiazepines in whole blood. Moreover, this method has several advantages: elimination of matrix interferences, low-volume of samples and very fast chromatographic separation of the analytes. Effective sample preparation as well as relatively short time of analyses proved usefulness and practical application in the samples from paediatric patients after a single oral administration, where real concentrations of benzodiazepines are in the range of proposed method.

3.2. Investigation of an SPE method for the determination of zaleplon and zopiclone in hemolyzed blood by fast GC/NICI-MS

Sample preparation is one of the most important steps in the majority of analytical procedures to determine trace compounds in samples with complex matrices. An ideal sample preparation technique should be simple, inexpensive, efficient, selective, and compatible with various analytical techniques. It should give a recovery as high as possible, use the minimum amount of solvent, and be environmentally friendly.

Therefore, simple, fast, and less labour-intensive extraction techniques are needed in both forensic and clinical toxicology. The aim of this study was the development of a new sensitive and specific method based on a fast GC/NICI-MS using SPE for the quantification of zaleplon and zopiclone in low-volume hemolyzed blood samples.

3.2.1. Development of an analytical method

In most previously published GC-MS methods, after LLE or SPE the samples (extracts) were silylated with MTBSTFA, which is the most commonly used derivatization reagent for benzodiazepines and other analytes

[16, 107 - 109, 113, 127]. Thus silylation of samples using 100 μL of a mixture of MTBSTFA in n-butyl acetate (20:80 v/v) at 90 °C for 30 min was investigated in this study. However insufficient recovery of zopiclone at 50.0 ng mL^{-1} concentrations was observed (n=5). The mean recovery of zopiclone was 63.4 (± 4.8) %, and zaleplon – 94.8 (± 1.4) % as shown in Table 3.9.

Table 3.9. Results obtained during the analysis of zaleplon and zopiclone at 50.0 ng mL^{-1} by fast GC/NICI-MS without and with derivatization procedure (n=5). Derivatization was performed using a mixture of MTBSTFA and n-butyl acetate (20:80 v/v) at 90 °C for 30 min. IS – zaleplon-d5.

Compound	Results without derivatization	Results after derivatization
	Mean ± SD, %	Mean ± SD, %
Zaleplon Zopiclone	96.8 ± 1.2 97.4 ± 1.8	94.8 ± 1.4 63.4 ± 4.8

Lower recovery percentage of zopiclone could be influenced/caused by possible degradation of the analyte under a high heating temperature. Both analytes have no active groups enabling them to bind to the silylating reagent, and thus does not form derivatives. A derivatization step is not necessary for the investigated analytes in order to improve the chromatographic characteristics (efficiency). In addition, both analytes are more compatible with the non-polar capillary column stationary phase. The capillary column showed no degradation of the analytes, or other problems during the optimization and validation studies.

3.2.2. Comparison of EI-MS and NICI-MS detections

Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample [3, 16]. Conventional capillary GC/EI-MS is one of the most reliable techniques for the analytes identification [132] due to its excellent chromatographic resolution, high reproducibility of retention times, and the availability of library-searchable spectral information using EI-MS spectra [16, 132]. Figure 3.20 (A

and B) shows mass spectra of the analytes obtained by EI-MS detection. The base peak in EI-MS spectra of zaleplon, and zopiclone was m/z 248 and m/z 143, respectively. The molecular ion of zaleplon was also present at m/z 305, and intensity was of 41.2 ± 2.4 % (SD_n = 10). The ions with m/z 99, 112, 217, 245, and intensities of 33.2 ± 2.0 %, 21.0 ± 1.5 %, 19.8 ± 1.0 %, and 62.9 ± 3.3 % (SD_n = 10), respectively, were the typical results of fragmentation of zopiclone, without the molecular ion.

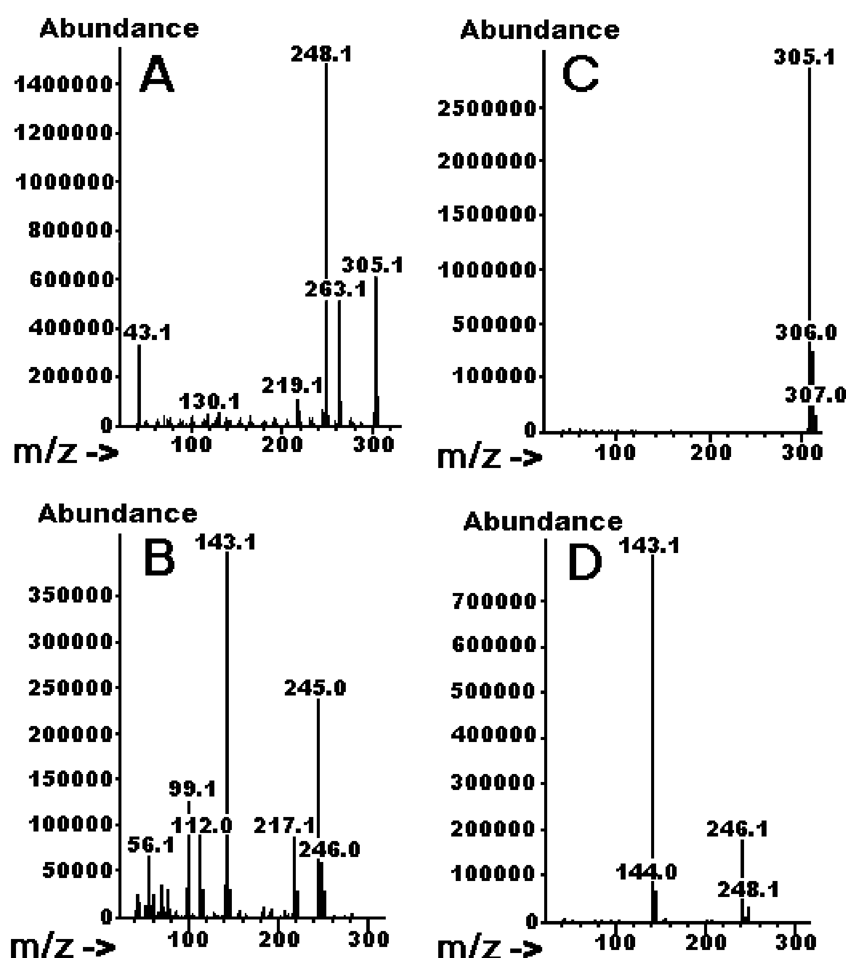


Figure 3.20. Mass spectra of (A) zaleplon, (B) zopiclone obtained by EI-MS mode, and NICI-MS mode mass spectra of (C) zaleplon, (D) zopiclone. In both scanning mode experiments a mass range of 20 – 320 amu was applied. The EI-MS mode was using ionization energy of 70 eV.

The fragmentation of both analytes in the NICI-MS mode is much smaller than using a conventional fragmentation in the EI-MS mode (Fig. 3.20 C and D). In the NICI-MS spectra the base ion of zaleplon, and zopiclone

was m/z 305 $[C_{17}H_{15}N_5O]^-$ the molecular ion, and m/z 143 $[C_6H_{11}N_2O_2]^-$, respectively. Zaleplon in the NICI-MS mode gave two isotopic ions: m/z 306, m/z 307 with the intensities of 20.3 ± 0.3 %, and 4.0 ± 0.2 % (SD_n = 10), respectively. The two main ions of m/z 143 $[C_6H_{11}N_2O_2]^-$ and m/z 246 $[C_{11}H_7N_4OCl]^-$ with the intensities of 100.0 %, 22.7 ± 0.8 % (SD_n = 10), respectively, were the typical results of fragmentation of zopiclone, without the molecular ion. The ions with m/z 144, m/z 248, and intensities of 7.7 ± 0.2 %, 3.4 ± 1.8 % (SD_n = 10), respectively, were the typical isotopic ions of zopiclone (Fig. 3.20 D).

The specific response of NICI-MS mode to the analytes and the virtually nonexistent background interference in MS spectra further assists in the reliable identification of the analytes in the hemolyzed blood samples. The high sensitivity and selectivity encouraged to develop and validate a quantitative GC/NICI-MS method, which confer significant benefits in terms of increased signal-to-noise ratios and decreased background interface, also the technique allow a reliable analysis from micro-volume of samples.

All quantitative analyses were performed in the SIM mode. Three or two characteristic SIM ions were used for the determination of the analytes (Table 3.10).

Table 3.10. SIM parameters for the determination of the analytes (n=20).

Compound	SIM ions, m/z
Zaleplon-d5 (IS)	**310**; 311 (23.1 ± 0.5 %)
Zaleplon	**305**; 306 (20.0 ± 0.4 %), 307 (3.9 ± 0.3 %)
Zopiclone	**143**; 246 (22.9 ± 1.0 %), 144 (7.7 ± 0.2 %)

The target ions are presented in bold and values in parentheses are the relative abundances ± SD (%) of qualifier ions (n=20). One SIM ion time window was used: 1.60 – 3.89 min for all compounds. The dwell time was set at 15 ms per ion and cycle time 6.80 cps (cycles per sec).

3.2.3. Fast gas chromatographic separation of the analytes

The chromatographic conditions for fast GC/NICI-MS were tested at: 150, **200**, and 250 °C for initial column temperature; 300, **330**, and 350 °C for the final column temperature; as well as 30, **45**, and 50 °C min^{-1} for the column temperature elevation rate; and finally 1.0, 2.0, **3.0**, and 7.0 mL min^{-1} for the flow rate of carrier gas. The optimal conditions are presented in bold and have been chosen based on the peak areas of both analytes, and on their resolution. The fast GC/NICI-MS separation of the analytes was achieved within 3.89 min as shown in Fig. 3.21.

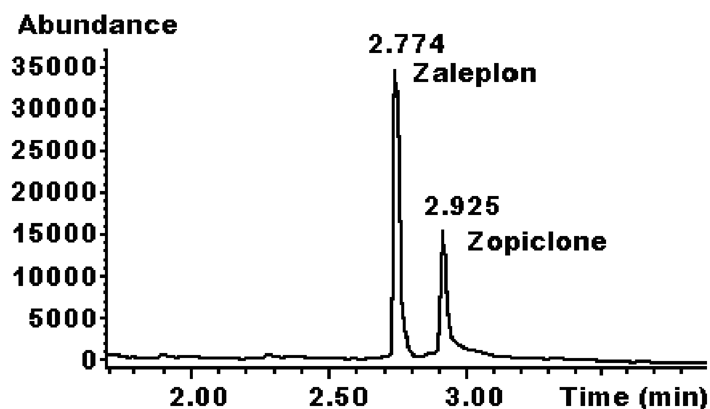


Figure 3.21. Fast and efficient gas chromatographic separation of both analytes. SIM chromatogram of standard solution at high concentration level of zaleplon (100.0 ng mL^{-1}) and zopiclone (200.0 ng mL^{-1}).

3.2.4. Optimization of NICI-MS parameters

Operating with MS detection in the NICI mode is slightly more complicated in comparison to the conventional EI-MS detection. Therefore, the NICI-MS instrument parameters were optimized: reagent gas flow rate (methane partial pressure) and ion source temperature. These data were summarized in order to investigate the instrument sensitivity and repeatability of results. In the case of NICI-MS, a change of septum every one hundred samples is necessary to keep the instrument free from oxygen contamination, which could greatly decrease the intensity of ionization.

Optimization of the methane reagent gas flow rate

The effect of the methane reagent gas flow on the normalized response of both analytes was tested in this experiment. The reagent gas flow rate must be adjusted for maximum stability of the system before tuning the NICI-MS conditions. The analysis of methane flow rate was carried out in an interval from 1.00 to 3.00 mL min^{-1}. Flow rates of methane gas were normalized to the responses observed at a flow rate of 1.00 mL min^{-1}. The observed detector response increased with increasing methane flow rate for both analytes and implies that increased partial pressure of reagent gas in the source enhanced anion production efficiency. A higher flow rate of 3.00 mL min^{-1} of methane gas was not used due to insufficient repeatability of results. The instrument was operated at 2.50 mL min^{-1} flow rate of methane gas to maximize the sensitivity for both analytes. In the previously published method, a 2.00 mL min^{-1} flow rate of methane gas was used [113]. The proposed method enables up to 1.5 times higher responses at 2.50 mL min^{-1} flow rate of methane gas using the same concentration of the analytes in comparison to 2.00 mL min^{-1} flow rate. Results are illustrated in Fig. 3.22.

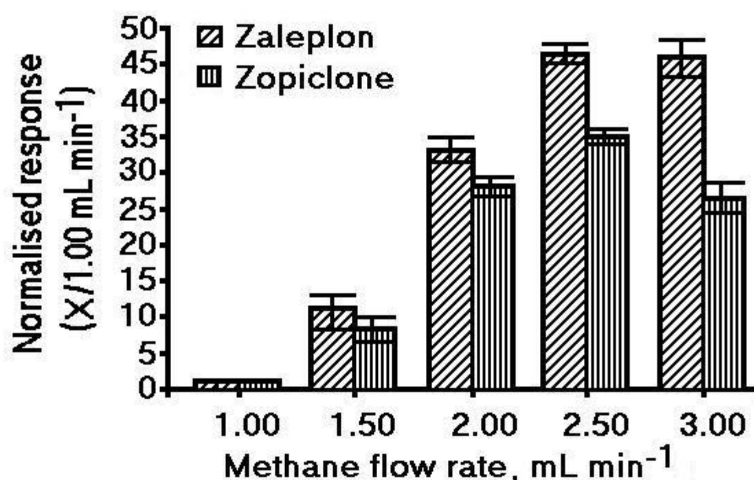


Figure 3.22. The responses to both analytes obtained by fast GC/NICI-MS upon gradually increasing the methane reagent gas flow rate (n=5). The carrier gas flow rate was 3.0 mL min^{-1}. The ion source temperature was 160 °C.

Optimization of the ion source temperature

Figure 3.23 shows the effect of the ion source temperature in the range from 150 to 300 °C on the normalized response for both compounds. Source temperatures below 150 °C were not possible due to the heating of the source from filament operation. The experimental results showed that an increase in ion source temperature lead to the increased noise of the detector for all m/z ions of interest. This noise was the largest for m/z 305 (zaleplon) and m/z 143 (zopiclone) ions, and was not accompanied by a significant increase in responses of both analytes, and in this way resulting in significantly decreased signal-to-noise ratios. When the source temperature was 160 °C, the enhanced signal-to-noise ratios for both analytes were registered. These increased signal-to-noise ratios were reduced by increasing noise at higher ion source temperatures (180 – 300 °C). Furthermore, at a higher ion source temperature (300 °C) zopiclone signal-to-noise ratio was three times lower. Therefore, an ion source temperature of 160 °C was used for both analytes to obtain the best sensitivity and repeatability of the results.

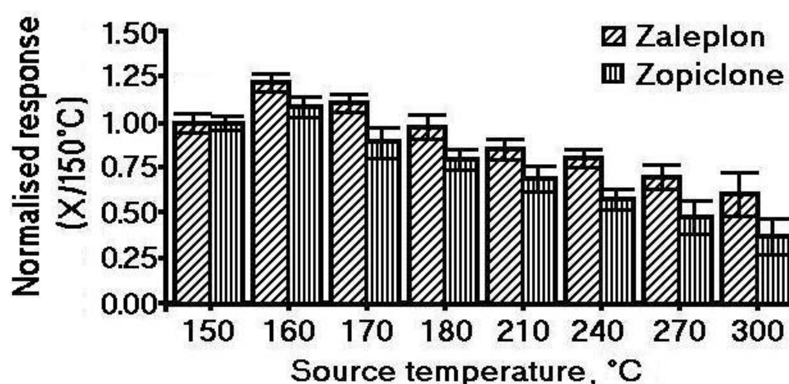


Figure 3.23. The responses to both analytes obtained by fast GC/NICI-MS upon gradually increasing the ion source temperature (n=5). The methane reagent gas flow rate was 2.5 mL min^{-1}. The carrier gas flow rate was 3.0 mL min^{-1}.

3.2.5. Development of an SPE method for the determination of zaleplon and zopiclone

When developing an SPE method, selection of an appropriate SPE sorbent, proper washing and eluting solvents is very important in order to

obtain the final extract that meets the requirements of fast GC/NICI-MS. In comparison to LLE, SPE is more time-efficient, easier to handle, cleaner extracts are obtained, increased selectivity for the compounds of interest is observed, and smaller volumes of solvent may be used for extraction [22]. The SPE column Oasis MCX containing a mixed-mode, water-wettable, polymeric sorbent was used in the experiment. This sorbent is characterized by good adsorption of polar and aromatic compounds due to hydrophilic-lipophilic interactions, and strong ion exchange groups for basic compounds due to ion-exchange interactions [20, 22, 166].

3.2.5.1. Optimization of the sample pH

In the preliminary study, the effect of the sample pH on the extraction effectiveness was determined. It was considered that the adsorption and elution behaviour of both analytes could be affected by the pH of the sample solution. In order to prepare the sample solutions of different pH values, HCl (pH 1.0 – 2.0), acetate (pH 3.0 – 5.0), phosphate (pH 6.0 – 8.0), and borate (pH 9.0 – 10.0) buffers were used. A volume of 2.0 mL of HCl or a buffer solution was added to 0.2 mL of aqueous sample of each analyte (zaleplon 30.0 ng mL^{-1} and zopiclone 60.0 ng mL^{-1}) before analysis. The analytes were eluted first with 1.0 mL of a mixture of n-butyl acetate and 2-PrOH (80:20 v/v), and then with 1.0 mL ACN containing 4 % of NH_4OH. The IS (zaleplon-d5) was added to all sets of samples after SPE and clean-up procedures. Typical extraction efficiency of the analytes is shown in Fig. 3.24.

The adsorption-related behaviour of the analytes was different (Fig. 3.24). Zopiclone is a weak base with pK_a value of 6.79 [3], and data of zaleplon was not readily available. When an acidic (pH 1.0 – 4.0) solution or a buffer was used, zopiclone, which is in the cationic form, was adsorbed on the sorbent due to ion-exchange interactions. However, at such a low pH, acid hydrolysis of the analyte can occur, which, in turn, reduces the recovery. In previously published method acid hydrolysis of zopiclone in a very acidic solution (conc. HCl) at 100 °C for 30 min was determined [154].

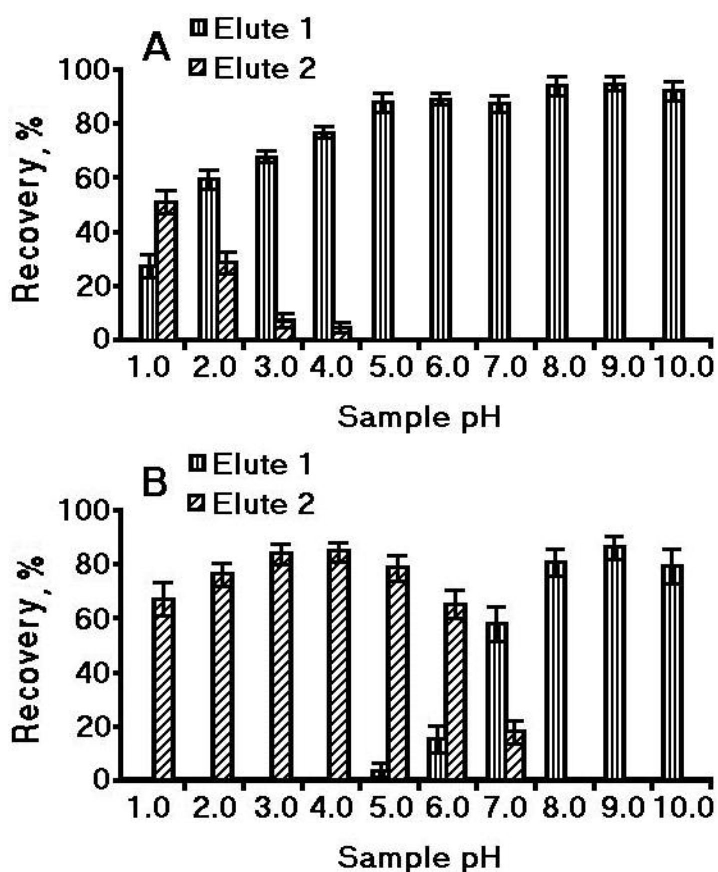


Figure 3.24. Extraction efficiency of zaleplon (A) and zopiclone (B) obtained at different pH values of the sample by Oasis MCX (30 mg) columns. Elute 1 – n-butyl acetate containing 20 % of 2-PrOH (n=5). Elute 2 – acetonitrile containing 4 % of NH_4OH (n=5). A volume of eluent was 1.0 mL.

The adsorption mechanism of zaleplon at the same pH interval is not yet fully understood, and apparently it is different. Zaleplon has not been separated in one fraction, and was eluted in two steps. At neutral or slightly acidic conditions (pH 5.0 – 7.0), zaleplon, which is in the non-ionic form, was adsorbed on the sorbent by an reversed-phase mechanism, and successfully eluted with 1.0 mL mixture of n-butyl acetate and 2-PrOH (80:20 v/v). However, under the same conditions zopiclone was adsorbed on the sorbent due to hydrophilic-lipophilic and/or ion-exchange interactions, and finally was eluted in two steps. When a basic buffer solution (pH 8.0 – 10.0) was used, the analytes in the non-ionic form were adsorbed on the sorbent due to hydrophilic-lipophilic interactions, and were eluted with 1.0 mL mixture of n-butyl acetate and 2-PrOH (80:20 v/v). Thus, the loading of aqueous samples onto the mixed-mode SPE column was the most efficient at pH 9.0 and this value was chosen for further optimization studies. In addition, efficient

extraction of all herein-investigated analytes by a single extraction step was achieved.

3.2.6. Comparison of two SPE columns

In the further experiments Oasis MCX and Oasis HLB columns were compared to assess the extraction efficiency of both zaleplon and zopiclone. It was determined that analytes were well retained on both columns, and also sufficient extraction efficiency was achieved using hemolyzed blood samples spiked with standard solution of zaleplon 30.0 ng mL^{-1} and zopiclone 60.0 ng mL^{-1} at pH 9.0 (n=5). Representative results in hemolyzed blood are presented in Table 3.11.

Table 3.11. The extraction efficiency obtained from two SPE columns (n=5).

Compound	The extraction efficiency (Mean ± SD, %)	
	Oasis MCX	Oasis HLB
Zaleplon	95.5 ± 3.1	94.6 ± 3.4
Zopiclone	82.7 ± 3.7	83.3 ± 3.9

For further study a hydrophilic-lipophilic sorbent Oasis HLB was selected due to the polarity of sorbent surface and its large surface area of 830 m^2 g^{-1}. In addition, the use of Oasis HLB type sorbent reduced the total cost of the experiment. However, when using SPE for sample preparation the binding of different proteins present in hemolyzed blood can lower the analyte recovery, because of the interactions between the active sites of the analytes and proteins occurs. Another problem is the interference from proteins in the penetration of the analytes into the sorbent pores. Therefore, hemolyzed blood was firstly diluted with 2.0 mL of 0.1 M borate buffer at pH 9.0, sonicated for 5 min, and finally centrifuged at 1610 g (4000 rpm) for 10 min as a sample preparation procedure before SPE. All these steps were used in order to decrease protein binding to the analytes. As already has been observed elsewhere, an effective sample in biological matrices preparation [116 - 119],

dilution, sonication and centrifugation is very important and usefull prior to SPE.

3.2.6.1. Optimization of the washing step/procedure

Washing the sorbent before the elution it is necessary to remove interference of endogenous hemolyzed blood compounds and to get very clean extracts. Therefore, this experiment was carried out in two steps: firstly with 0.1 M borate buffer at pH 9.0, and followed by an organic solvent. Different volumes in the range from 0.5 to 2.0 mL of borate buffer at pH 9.0 were investigated. A volume of 1.0 mL was determined to be the most effective for washing the sorbent. This buffer did not cause the loss of the analytes, however, the matrix was not removed from the sorbent sufficiently. As in the case of benzodiazepines, 1-PrOH was selected as the best solvent in the wash step. Therefore, in the washing step, mixtures of 1.0 mL of alkaline water (0.1 % NH_4OH) and 1-PrOH of different concentrations ranging from 0 to 100 % in intervals of 10 % were tested. The optimal amount of 1-PrOH was 30 % resulting in a minimal analyte loss in this stage (Fig. 3.25).

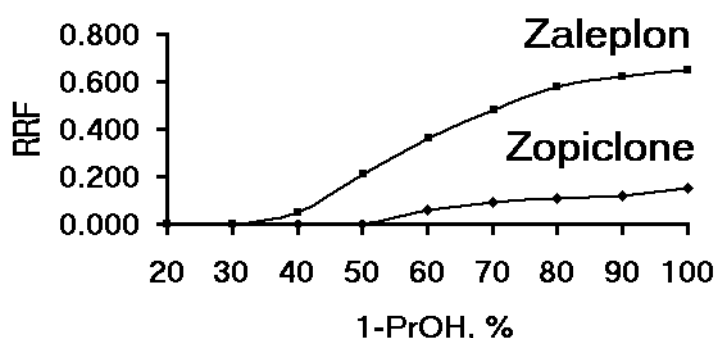


Figure 3.25. The effect of Oasis HLB sorbent washing on the extraction efficiency of zaleplon (30.0 ng mL^{-1}) and zopiclone (60.0 ng mL^{-1}) (n=5) using 1-PrOH and alkaline water (0.1 % NH_4OH) mixtures. Total volume of mixture was 1.0 mL, blood samples pH of 9.0.

In addition, polymeric sorbent (Oasis HLB) also retained a small amount of water, probably due to its hydrophilic character, resulting in a longer drying step (up to 10 min) under vacuum. In this stage, the remaining moisture

of the sorbent was removed. After washing steps neutral interfering compounds were removed, and visibly cleaner extracts were received.

3.2.6.2. The analytes elution from the Oasis HLB sorbent

A good eluent should be strong enough to elute the analytes of interest in a limited volume. On the other hand, stronger eluent can additionally elute more interfering compounds. Therefore, in order to optimize the elution step several solvents were tested to elute both analytes from the Oasis HLB column. Organic solvents of different polarity, such as MeOH, ACN, ethyl acetate, n-butyl acetate, dichloromethane, or toluene, and in a volume range from 0.25 to 2.0 mL were investigated. The results are presented in Figure 3.26. A volume of 1.0 mL of organic solvents was determined to be the most effective for elution of zaleplon from an reversed-phase sorbent. It can be concluded, that MeOH or ACN are not suitable for the analytes elution due to many matrix (blood) interfering compounds co-extraction. Moreover, insufficient recovery of zopiclone up to 36.0 % at 60.0 ng mL^{-1} was obtained (n=5). Thus, these polar solvents were inappropriate for elution of the analytes from an reversed-phase sorbent.

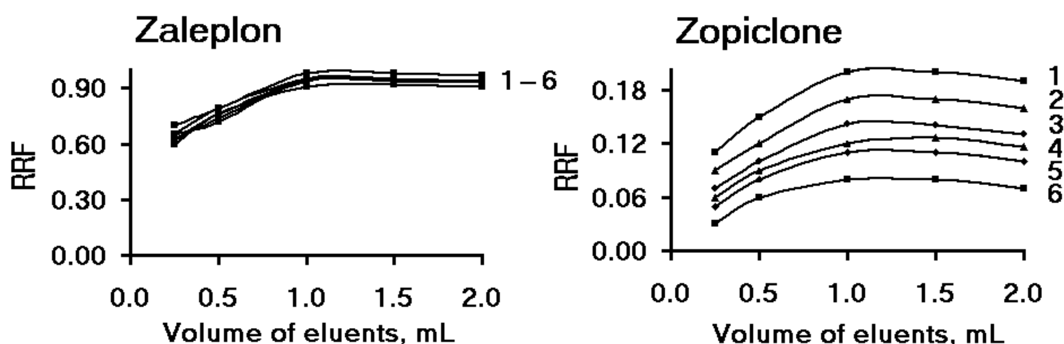


Figure 3.26. The dependence of extraction efficiencies obtained in the elution step for zaleplon (30.0 ng mL^{-1}) and zopiclone (60.0 ng mL^{-1}) on the applied different volumes of eluent (n=5). A polymeric Oasis HLB sorbent and hemolyzed blood samples at pH 9.0 were used. Eluents numbering refers to: (1) n-butyl acetate; (2) toluene; (3) dichloromethane; (4) ethyl acetate; (5) ACN; (6) MeOH.

On the other hand, elution with ethyl acetate, n-butyl acetate, dichloromethane, toluene, or mixtures of these solvents with 2-PrOH (80:20 v/v) was investigated. Experimental results showed that using these eluents slightly lower recoveries of zopiclone (≤ 67.5 %) were obtained in comparison to a mixture of n-butyl acetate and 2-PrOH (80:20 v/v) (Table 3.12). Furthermore, visibly cleaner extracts were obtained using pure non-polar solvents or their mixtures.

Table 3.12. Extraction efficiency of zaleplon 30.0 ng mL^{-1} and zopiclone 60.0 ng mL^{-1} at pH 9.0 by Oasis HLB columns, using organic solvents and their mixtures for the analytes elution (n=5).

Solvents	Compound and recovery (Mean ± SD, %)	
	Zaleplon	Zopiclone
Ethyl acetate	94.50 ± 2.63	36.83 ± 2.61
Ethyl acetate – 2-PrOH (80:20 v/v)	84.67 ± 3.52	47.17 ± 3.15
n-Butyl acetate	93.52 ± 2.42	66.67 ± 2.68
n-Butyl acetate – 2-PrOH (80:20 v/v)	**94.07 ± 3.20**	**83.04 ± 3.55**
Dichloromethane	93.26 ± 5.06	40.74 ± 4.72
Dichloromethane – 2-PrOH (80:20 v/v)	95.70 ± 3.89	67.52 ± 4.57
Toluene	97.24 ± 2.59	43.31 ± 3.78
Toluene – 2-PrOH (80:20 v/v)	85.36 ± 3.31	61.19 ± 3.09

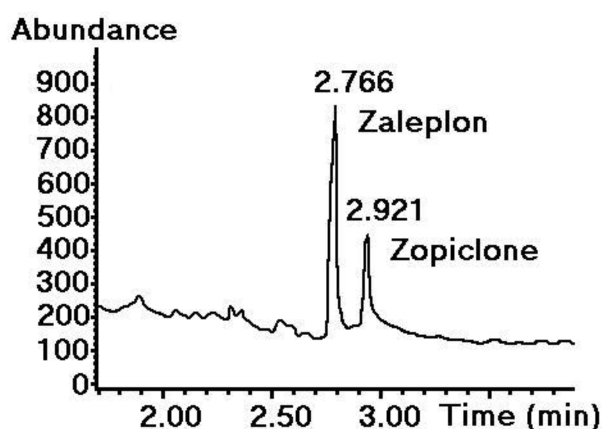


Figure 3.27. Representative SIM chromatogram of a spiked hemolyzed blood sample at LOQ of zaleplon (1.0 ng mL^{-1}) and zopiclone (2.0 ng mL^{-1}).

The use of a mixture of n-butyl acetate and 2-PrOH (80:20 v/v) for the elution step allowed obtaining of the sufficient recovery for both analytes. Typical SIM chromatogram for blood sample containing both analytes is

shown in Fig. 3.27. According to the results, each analyte was completely separated at the baseline and endogenous interfering materials present in the hemolyzed blood did not produce peaks that overlapped with those produced by any of the drugs.

3.2.7. Validation of an SPE-GC/NICI-MS method for the determination of both analytes in hemolyzed blood

The developed method was fully validated according to recommendations [161 - 163]. Various parameters, such as: selectivity, sensitivity, linearity, accuracy, precision, extraction efficiency, robustness, specificity and stability were evaluated. The developed method showed the significant selectivity and specificity, as well as satisfactory accuracy, and precision results for both analytes. Furthermore, peak shapes and resolution were satisfactory, and similar to those obtained by injecting standard solutions.

The selectivity of the method

The selectivity of the method was studied by analyzing five different drug-free hemolyzed blood samples, also the matrix effect was assessed. Because endogenous compounds might still be present in the extracts after SPE and can induce NICI-MS alterations, potential matrix effects were evaluated. All blank blood samples were free of co-eluting peaks at the retention time of the analytes and the IS (zaleplon-d5). The selectivity was further investigated by comparing chromatograms obtained by the injection of drug-free human blood samples spiked at 10.0 ng mL^{-1} of IS (Fig. 3.28 A and E), and drug-free blood samples without IS (Fig. 3.28 B – D). The selectivity of the method was adequate with minimal matrix effect in all drug-free samples (n=5).

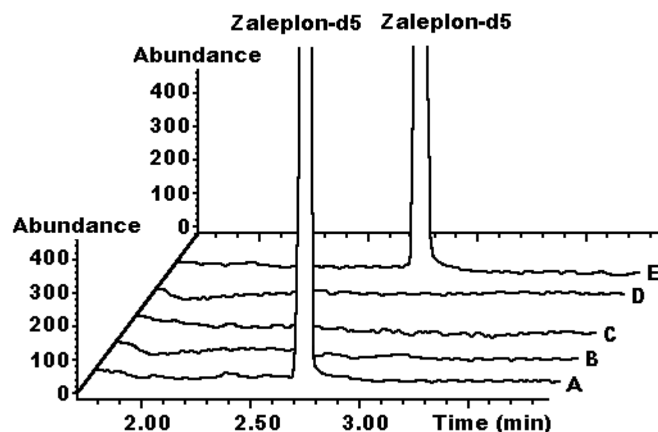


Figure 3.28. Representative SIM chromatograms of extracted five different drug-free human blood. Blood samples spiked at 10.0 ng mL^{-1} of IS (A and E), and without IS (B – D).

The sensitivity of the method

The sensitivity of the method was determined by the calculation of LOD and LOQ. The LOD for the developed method was 0.30 ng mL^{-1} for zaleplon, and 0.60 ng mL^{-1} for zopiclone. The LOQ was determined to be the lowest analyte concentration that could be quantified with acceptable accuracy and precision, which gave rise to a chromatographic peak whose height was equal to ten times the baseline noise. The LOQ was found to be 1.00 ng mL^{-1} for zaleplon, and 2.00 ng mL^{-1} for zopiclone. These limits are sufficient for clinical pharmacokinetic studies following oral administration of therapeutic dose [3, 95, 170]. A chromatogram of spiked hemolyzed blood sample at LOQ concentrations is presented in Fig 3.27.

The linearity of the method

The linearity of the method was evaluated by preparing calibration curves, and calibration points were considered to be matrix-matched. These curves were found to be linear over the concentration range of 1.0 – 80.0 ng mL^{-1} for zaleplon, and 2.0 – 160.0 ng mL^{-1} for zopiclone. The range of calibration curves of the analytes was established from the therapeutic levels of each compound [95, 170]. Linearity was assessed by a weighted ($1/x^2$) least-squares regression analysis. The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor giving a mean

linear regression equation for the calibration curve (Table 3.13). The mean determination coefficient (r^2) of the weighted calibration curve generated during the validation was higher than 0.9985 for both analytes (n=5). Detailed results are listed in Table 3.13.

Table 3.13. Calibration data in hemolyzed blood (n=5).

Compound	Calibration (Y = aX + b)[a]		
	a	b	r^2
Zaleplon	0.106 (±0.004)	-0.031 (±0.002)	0.9994
Zopiclone	0.016 (±0.002)	-0.006 (±0.001)	0.9989

[a]Y = area of each compound/area of IS; X = concentration of compound/concentration of IS.

The accuracy and precision

Intra-day (n=6) and inter-day (n=12) accuracy and precision of the developed method were calculated by analyzing four QC levels, including the LOQ concentration of both analytes, within analyte's linear range. Intra-day and inter-day precision for zaleplon and zopiclone were less than 10.6 % and 9.5 %. Moreover, the intra-day accuracy (bias) ranged from -3.67 to 6.00 % and -7.00 to -0.21 %, whereas the inter-day accuracy ranged from -5.73 to 4.00 % and -3.17 to 6.32 % for zaleplon and zopiclone, respectively (Table 3.14).

Table 3.14. Intra-day (n=6) and inter-day (n=12) coefficients of variation of the proposed method.

Compound	Nominal conc., ng mL^{-1}	Intra-day precision, accuracy			Inter-day precision, accuracy		
		Measured, ng mL^{-1} Mean ± SD	RSD, %	Bias[a], %	Measured, ng mL^{-1} Mean ± SD	RSD, %	Bias[a], %
Zaleplon	1.00	1.06 ± 0.10	9.43	6.00	1.04 ± 0.11	10.58	4.00
	15.00	14.73 ± 0.48	3.26	-1.80	14.14 ± 1.26	8.91	-5.73
	30.00	28.90 ± 0.88	3.04	-3.67	29.11 ± 2.12	7.28	-2.97
	60.00	59.19 ± 2.58	4.36	-1.35	59.62 ± 3.48	5.84	-0.63
Zopiclone	2.00	1.86 ± 0.15	8.06	-7.00	2.10 ± 0.20	9.52	5.00
	30.00	28.53 ± 1.44	5.05	-4.90	30.79 ± 2.21	7.18	2.63
	60.00	58.92 ± 3.46	6.25	-1.80	58.10 ± 4.41	7.20	-3.17
	120.00	119.75 ± 4.88	4.08	-0.21	127.58 ± 7.27	5.70	6.32

[a]Accuracy was calculated as bias values based on the equation: bias (%) = (mean calculated concentration–nominal concentration/nominal concentration) × 100.

The recovery

The extraction efficiency (recovery) of both analytes was estimated by comparing the peak areas obtained after the addition and extraction of a known amount of the analyte in hemolyzed blood, with the peak areas obtained from the corresponding unextracted standards. Then the blood samples were left at room temperature (22.5 ± 0.5 °C) for the adsorption of the analytes onto the blood samples, and later were processed using Oasis HLB column in accordance to the developed method. Low (1.0 or 2.0 ng mL^{-1}) and high (60.0 or 120.0 ng mL^{-1}) concentrations of zaleplon and zopiclone were tested (n=5). The extraction efficiency at low and high QC values is presented in Table 3.15. The obtained results have been satisfactory and completely satisfied requirements of the newly developed method [161, 162].

Table 3.15. Extraction recovery of both analytes (n=5).

Compound	Nominal conc., ng mL^{-1}	Recovery, % Mean ± SD
Zaleplon	1.0	90.1 ± 8.0
	60.0	95.4 ± 3.6
Zopiclone	2.0	82.9 ± 6.2
	120.0	84.6 ± 3.4

Robustness of the method

Robustness of the entire method was studied by changing different parameters of the procedure such as: extraction pH of the samples (pH of samples was adjusted to 8.5 instead of pH 9.0), and evaporation temperature (35 instead of 30 °C), as well as chromatographic parameters: injection temperature (255 instead of 250 °C), oven temperature heating rate (47.0 instead of 45.0 °C min^{-1}), carrier gas flow rate (3.5 instead of 3.0 mL min^{-1}). The robustness was determined from five replicates of spiked hemolyzed blood samples at medium (30.0 or 60.0 ng mL^{-1}), and high (60.0 or 120.0 ng mL^{-1}) QC levels of zaleplon and zopiclone. Mean effects of each parameter and relative standard deviations of all differences were calculated. Neither a single parameter nor combination of them showed a significant influence on the

results of the method. The method was proven to be sufficiently robust, as the mean effect and RSDs values were found to be adequately low.

The specificity of the method

The specificity study performed using hemolyzed blood with the 60.0 ng mL^{-1} concentrations of the following drugs: amitriptyline, nortriptyline, fluoxetine, venlafaxine, clomipramine, mirtazapine, citalopram, clozapine, risperidone, carbamazepine, olanzapine, methadone, fludiazepam, diazepam, nordiazepam, midazolam, flunitrazepam, bromazepam, oxazepam, nitrazepam, temazepam, lorazepam, clonazepam, α-OH-midazolam, triazolam and 7-aminoclonazepam showed no interfere with the both analytes in human hemolyzed blood samples. Furthermore, no interferences materials were observed at the respective retention times of the analytes and IS, thus, the developed method was proven to be specific. However, only the one drug – triazolam with the retention time of 2.872 min, had the same ions as zaleplon. Triazolam ions: m/z **306**; 307 (30.9 ± 0.6 %); 305 (10.6 ± 0.4 %) were identified, however, these ions does not affect the determination of zaleplon, as show in Fig 3.29. The target ion presented in bold and values in parentheses are the relative abundances of qualifier ions (n=10).

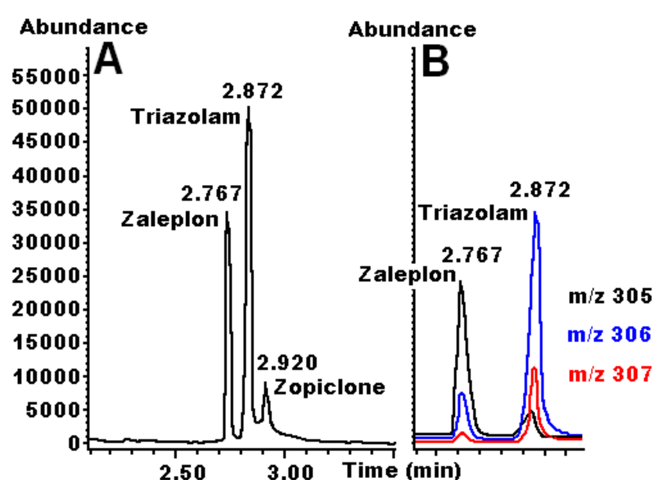


Figure 3.29. Chromatograms obtained during the specificity analysis of zaleplon and zopiclone by fast GC/NICI-MS. (A) SIM chromatogram of a spiked blood sample at 60.0 ng mL^{-1} of each analyte after SPE. (B) Typical reconstructed mass chromatogram of zaleplon and triazolam.

The stability of zaleplon and zopiclone in the blood samples

The stability tests of both zaleplon (30.0 ng mL^{-1}) and zopiclone (60.0 ng mL^{-1}) were performed in hemolyzed blood samples (n=6). The influence of different storage conditions on stability was tested: short-term at ambient temperature up to 24 h, after thirty days at 4.0 ± 0.5 °C, after one and three months at -20.0 ± 1.0 °C, and, finally, long-term at -70.0 ± 1.0 °C for six months. Stability is expressed as a percentage and was calculated by dividing the sample concentration, at each study point, by the sample concentration at the outset of the study and multiplying the resulting value by 100.

In the present study, the short-term stability at room temperature (22.5 ± 0.5 °C) up to 24 h was investigated and the obtained values were 98.5 (± 6.2) % for zaleplon, and 85.8 (± 9.1) % for zopiclone. Blood samples remained stable after storage at 4.0 ± 0.5 °C for thirty days with stability values of 95.7 (± 5.9) % and 75.5 (± 10.5) % for zaleplon and zopiclone. The experiments revealed that zaleplon and zopiclone were also stable at -20.0 ± 1.0 °C in blood for one month, with stability values of 98.4 (± 2.9) % and 85.5 (± 4.9) %. Furthermore, both analytes were stable in blood after three months under the same conditions yielding the stability values of 92.7 (± 3.7) % and 82.2 (± 6.9) % for zaleplon and zopiclone. A similar stability of zopiclone in whole blood samples has been reported previously by other authors [158, 171, 172]. Long-term stability was tested by storing the analytes at -70.0 ± 1.0 °C. Zaleplon and zopiclone were stable in blood for six months, with stability values of 95.7 (± 3.9) % and 80.3 (± 5.2) %. Finally, post-preparative stability was investigated by storing the samples up to 6 h at 24.0 ± 1.0 °C in the autosampler. The stability was found to remain constant.

According to the obtained results, zaleplon was sufficiently stable at different storage conditions, however, degradation of zopiclone can occur. In order to avoid possible degradation of zopiclone, an extraction temperature of 22.5 ± 0.5 °C, and a time of no more than 6 h for sample preparation are recommended. Under these conditions, both analytes have proven stable with only a small degradation.

3.2.8. Application of an SPE method in real hemolyzed blood samples

After developing the SPE procedure the method was applied in real hemolyzed blood samples for a residue analysis. It was verified that the results obtained within this matrix (in terms of baseline shape, chromatogram outline and extraction yields) were almost identical to those obtained in spiked blood samples. In this study, none of these samples caused any problems for the determination of the analytes, and no matrix interferences were observed in any of them. Typical SIM chromatograms of real blood samples are shown in Figure 3.30.

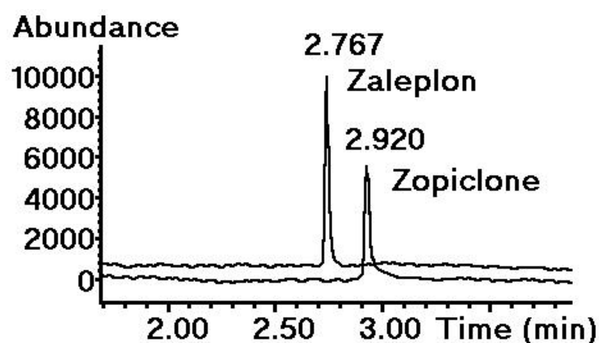


Figure 3.30. Typical SIM chromatograms obtained from real hemolyzed blood samples. Zaleplon (15.25 ng mL^{-1}) was detected in the clinical case 1. Zopiclone (45.39 ng mL^{-1}) was detected in the clinical case 2.

Case report 1: blood sample was obtained from a 25-year-old male within 2 h after the last zaleplon (Sonata® 10 mg) intake. Quantitative analyses of illicit drugs were negative for opiates, amphetamines, cocaine and cannabis detections by GC/EI-MS methods. A therapeutic concentration of zaleplon 15.55 ± 0.72 ng mL^{-1} was determined in the blood (n=4).

Case report 2: blood sample was obtained from a 29-year-old male admitted to the emergency unit of the hospital 4 h after ingestion of zopiclone (Imovane® 7.5 mg). Quantitative analysis of illicit drugs was operated by GC/EI-MS methods. Opiates, amphetamines and cocaine detections were negative (LOQ < 10 ng mL^{-1}). However, low levels of cannabis (11-nor-9-carboxy-delta-9-tetrahydrocannabinol 30.25 ± 1.27 ng mL^{-1}, 11-hydroxy-delta-9-tetrahydrocannabinol 5.01 ± 0.28 ng mL^{-1} and tetrahydrocannabinol 3.16 ± 0.26 ng mL^{-1}) were detected (n=3). A therapeutic level of zopiclone 45.15 ± 2.90 ng mL^{-1} was determined in hemolyzed blood (n=4).

3.2.8.1. Application of the developed method to the clinical whole blood samples

The developed method was applied for quantitative analysis of both analytes in clinical whole blood cases. The determination of the analytes levels was carried out in the blood samples of patients undergoing drug monitoring. Quantitative analyses of illicit drugs were negative for opiates, amphetamines, cocaine and cannabis detections by GC/EI-MS methods.

Case report 1: a 32-year-old woman was taking therapeutically 7.5 mg zopiclone (Imovane®) once a day. A blood sample was taken 2.2 h after the last drug intake and provided for therapeutic drug monitoring. According to quantitative analysis using the developed method, the whole blood concentration of zopiclone was found to be 70.36 ± 4.50 ng mL^{-1} (n=4).

Case report 2: a blood sample was taken 6 h after the last drug intake from a 33-year-old man taking 15 mg zopiclone (Imovane® 2 × 7.5 mg) daily for more than one week. The blood sample was provided for therapeutic drug monitoring as the patient showed symptoms such as: sleepiness, nervousness and dizziness. According to quantitative analysis in the whole blood concentration of zopiclone was 68.90 ± 5.2 ng mL^{-1} (n=4).

Case report 3: a blood sample was taken 5 h after the last drug intake, a single oral administration, from a 36-year-old woman taking one tablet of zaleplon (Sonata® 10 mg). According to quantitative analysis using the developed method, the whole blood concentration of zaleplon was found to be 6.35 ± 0.32 ng mL^{-1} (n=4). The result proved that zaleplon was able to be determined in human blood using the developed method 5 h after the last drug intake. This is in agreement with literature, which mentions that zaleplon has an elimination half-life approximately of 1 h [3, 10, 151, 152].

3.2.9. Review of extraction methods for the determination of both analytes

The developed method has been compared with previously described methods (LLE and SPE) for the detection of both analytes in blood or plasma

samples. The obtained results showed the significant selectivity and robustness, as well as satisfactory recovery, accuracy and precision of the method. The extraction efficiency of the developed method is higher than the other published methods [117, 118, 153, 158]. The mean recoveries of zopiclone were 38.8 % [118], 63.9 % [153] and 50.6 % [158], and the mean recovery of zaleplon was 6.0 % [117]. In some previously published methods slightly wider accuracy limits for determination of zaleplon (-11.7 to 4.0 %) [108], (-14.6 to 23.8 %) [110], and for zopiclone (-5.0 to 14.0 %) [110], (-12.7 to 16.6 %) [117] have been presented. However, quite high LOQs: 5 ng mL^{-1} [99, 113], 10 ng mL^{-1} [109, 117] or 20 ng mL^{-1} [108] for zaleplon, while 10 ng mL^{-1} [109, 113, 117] or 20 ng g^{-1} [158] for zopiclone have been shown.

CONCLUSIONS

1. When optimizing the derivatization conditions it was found that benzodiazepine molecules derivatized by MTBSTFA were characterized by the high sensitivity and repeatability of the results obtained by GC/NICI-MS method. It was determined that the efficiency of benzodiazepines was higher using MTBSTFA in comparison to derivatization with MSTFA and BSTFA. The best results were obtained after derivatization using a mixture of MTBSTFA:acetonitrile:ethyl acetate (20:40:40 (v/v/v)) at 85 °C for 30 min.
2. Sensitive, specific NICI-MS detection combined with optimal fast GC parameters resulted in sharp and symmetric peak shapes of 15 benzodiazepines, as well as zaleplon and zopiclone in chromatographic separation of 3.90 and 3.89 min, respectively. Therefore, the developed fast methods allow high sample throughput and low cost per unit of analysis.
3. The developed SPE method has been used for the first time for the optimization of sample preparation at pH 1.0. For benzodiazepines adsorbed onto the ion-exchange phase and washed by three solutions (0.1 M hydrochloric acid, pH 1.0; 1-propanol and acidified water at 0.15 M hydrochloric acid mixture (60:40 v/v) and acetonitrile) visibly cleaner extracts were obtained in comparison with those obtained using reversed-phase interactions at sample pH 9.0. Benzodiazepines were successfully eluted from a mixed-mode sorbent using 2.0 mL of 5 % (v/v) ammonium hydroxide in methanol.
4. An SPE-GC/NICI-MS method was fully validated for simultaneous determination of 15 benzodiazepines. The limits of detection and quantification of 15 benzodiazepines in whole blood samples ranged from 0.24 to 0.62 ng mL^{-1} and from 0.72 to 1.89 ng mL^{-1}, respectively. The results of accuracy tests for three different concentration levels of quality control were in the range of 89.5 – 110.5 % and all RSDs for replicate determinations were ≤ 7.0 %.
5. About 1.5 times higher sensitivity of GC/NICI-MS method for the determination of zaleplon and zopiclone was achieved using 2.50 mL min^{-1}

methane reagent gas flow rate and 160 °C ion source temperature. The adsorption-related behaviour of zaleplon and zopiclone on the mixed-mode SPE sorbent was dependent on the sample pH in the range of 1.0 – 10.0 due to different pK_a values of both analytes. Investigated analytes were well adsorbed onto Oasis MCX and Oasis HLB sorbents at sample pH 9.0 due to reversed-phase interactions, and sufficient extraction efficiency up to 82.7 % was achieved using 1.0 mL of a mixture of n-butyl acetate and 2-propanol (80:20 v/v).

6. A fully validated SPE-GC/NICI-MS method for the determination of zaleplon and zopiclone shows high sensitivity in hemolyzed blood samples. The limit of quantification for zaleplon was 1.00 ng mL^{-1}, and zopiclone – 2.00 ng mL^{-1}. The mean extraction efficiency was higher than 90.1 % for zaleplon, and 82.9 % for zopiclone. The precision for zaleplon and zopiclone was between 3.04 – 10.58 % and 4.08 – 9.52 % whereas the accuracy was in the range from -5.73 to 6.00 %, and from -7.00 to 6.32 %, respectively.

7. The developed SPE-GC/NICI-MS methods were successfully applied in clinical and forensic cases for the determination of trace concentrations of benzodiazepines, as well as zaleplon and zopiclone after a single oral administration.

THE LIST OF ORIGINAL SCIENTIFIC PUBLICATIONS BY THE AUTHOR

Articles in journals:

1. N. Karlonas, A. Padarauskas, A. Ramanavicius, Z. Minkuviene, A. Ramanaviciene. Rapid and highly sensitive determination of clonazepam and 7-aminoclonazepam in whole blood using gas chromatography with negative-ion chemical ionization mass spectrometry. *Chemija* 23 (2012) 91-99, ISSN 0235-7216.

2. N. Karlonas, A. Padarauskas, A. Ramanavicius, A. Ramanaviciene. Mixed-mode SPE for a multi-residue analysis of benzodiazepines in whole blood using rapid GC with negative-ion chemical ionization MS. *Journal of Separation Science* 36 (2013) 1437-1445, ISSN 1615-9306.

3. N. Karlonas, A. Ramanavicius, A. Ramanaviciene. Development of an SPE method for the determination of zaleplon and zopiclone in hemolyzed blood using fast GC with negative-ion chemical ionization MS. *Journal of Separation Science* 37 (2014) 551-557, ISSN 1615-9306.

Published contributions to academic conferences:

1. N. Karlonas, A. Ramanaviciene, A. Ramanavicius. Gas chromatography-negative-ion chemical ionization mass spectrometry for the determination of diazepam, nordiazepam, oxazepam and temazepam in whole blood. Theses of The conference of Chemistry and Technology of Inorganic materials, Kaunas, Kaunas University of Technology, 27 April 2011, p. 71 - 73, ISBN 978-9955-25-981-7.

2. N. Karlonas, A. Ramanaviciene, A. Ramanavicius. High sensitive analysis method for the determination of alprazolam, midazolam and their α-hydroxy metabolites in blood, using gas chromatography-negative-ion chemical ionization mass spectrometry. 10th International Conference of Lithuanian Chemists, Chemistry 2011, Vilnius, Lithuanian Academy of Sciences, 14 October 2011, p. 63, ISBN 978-9955-634-65-2.

3. **N. Karlonas**, Z. Minkuviene, A. Ramanavicius, A. Ramanaviciene. Separation and sample pretreatment analysis of 15 benzodiazepines in whole blood using GC/NICI-MS. 6th International Scientific Conference, The Vital Nature Sign, Kaunas, Vytautas Magnus University, 1 - 4 June 2012, p. 27.

4. **N. Karlonas**, A. Ramanavicius, A. Ramanaviciene. Development of a mixed-mode solid phase extraction for a multi-residue analysis of 15 benzodiazepines in blood by rapid gas chromatography with negative-ion chemical ionization mass spectrometry. 7th International Scientific Conference, The Vital Nature Sign, Kaunas, Vytautas Magnus University, 16 - 19 May 2013, p. 39, ISSN 2335-8653.

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