Development of Procedures for Screening for, Identification and/or Validated Quantification of Herbal Drugs in Blood or Urine Using GC-MS, LC-MS or LC-MS/MS

Dissertation
zur Erlangung des Grades
des Doktors der Naturwissenschaften
der Naturwissenschaftlich-Technischen Fakultät III -
Chemie, Pharmazie, Bio- und Werkstoffwissenschaften
der Universität des Saarlandes

von
Jochen Beyer
Saarbrücken
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Die folgende Arbeit entstand unter der Anleitung von Herrn Professor Dr. Hans H. Maurer in der Abteilung Experimentelle und Klinische Toxikologie der Fachrichtung 2.4 Experimentelle und Klinische Pharmakologie und Toxikologie der Universität des Saarlandes in Homburg/Saar von Februar 2003 bis September 2006.

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Meinen Eltern

NICHT WEIL ES SCHWER IST, WAGEN WIR ES NICHT,
SONDERN WEIL WIR ES NICHT WAGEN, IST ES SCHWER.

LUCIUS ANNAEUS SENECA

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1 GENERAL PART

1.1 INTRODUCTION

Poisonings with plants or plant ingredients as well as their abuse are widespread and play an important role in clinical and forensic toxicology. According to the annual report of 2005 of the poison information center of northern Germany, 12 % of the medicinal consultations and advice related to plants or plant ingredients (Fig. 1.1.). Although many plants contain pharmacologically active ingredients, severe poisonings with plants are relatively rare and limited to a few toxic plant species.[1]

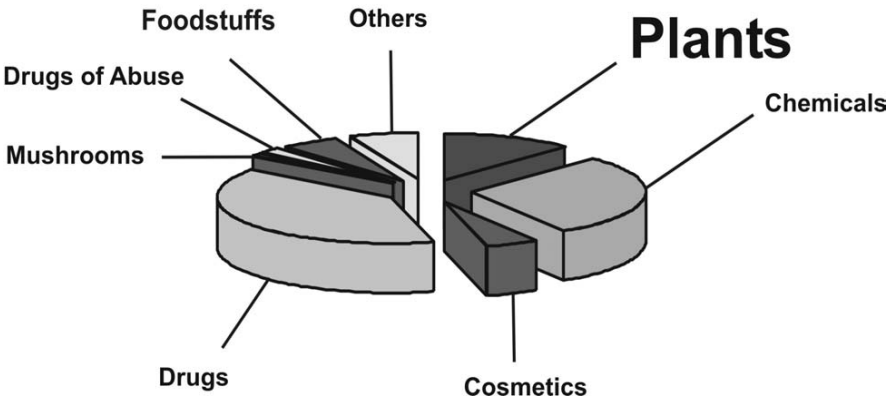


Fig. 1.1. Distribution of medical consultions of the Poison Information Center of Northern Germany

Besides plant poisonings, a lot of these consultations concern the abuse of plants and plant ingredients. The main reasons of such an abuse are the wish to experience stimulative or hallucinogenic effects, to induce or support loosing weight, or simply habitual misuse. In contrast to classical drugs of abuse such as tetrahydrocannabinol (THC), opiates, cocaine, and amphetamines, the so-called “herbal” or “natural” drugs are often thought to be relatively harmless and propagated as "safe". For these reasons and because of a well-organized publicity in the internet, they have become increasingly popular among drug abusers in recent years. However, as in case of classical drugs of abuse, abuse of plants or plant ingredients may cause psychiatric problems like addiction or chronic illnesses. Depending on the pharmacological activity of the

contained active ingredients, they may even cause serious acute intoxications and poisonings, especially in overdose situations. In the following, an overview of herbal drugs leading to an abuse and/or intoxication is given.

1.1.1 Laxatives

The stimulant laxatives of the anthraquinone type (e.g. rhein) contained in plant extracts of cascara, senna, rhubarb, frangula, and aloe as well as of the diphenol-type phenolphthalein, bisacodyl, and sodium picosulfate are widely used and abused drugs. They are freely available over-the-counter drugs which are well tolerated for self medication and generally viewed as harmless, because their occasional use is associated with only few unwanted side effects.[2] The chemical structures of the above-mentioned laxatives are given in Fig. 1.2.

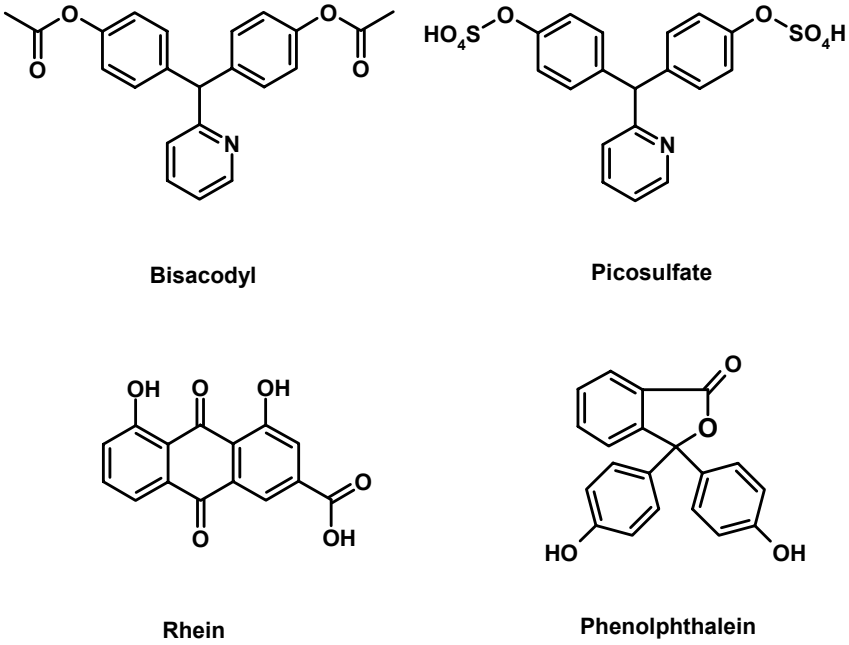


Fig. 1.2. Chemical structures of bisacodyl, picosulfate, rhein, and pheolphthalein.

However, the above-mentioned drugs are also widely abused for various reasons. On the one hand, there are surreptitious laxative abusers, whose abuse is often associated with an eating disorder like bulimia nervosa or anorexia[3-10] or with Munchhausen syndrome.[11] In case of eating disorders, a study of Pryor et al. showed that more than 50 % of patients had abused laxatives at some point.[12] On the other hand, there are habitual abusers, which are usually middle-aged and start using these drugs for treatment of constipation due to poor diet, decreased mobility or concomitant drug therapy. Such a chronic use or abuse can induce hypokalemia, which in turn leads to constipation, thus leading to a vicious circle of laxative abuse and constipation. Laxative-induced hypokalemia may even lead to life threatening disorders like torsade de pointes.[10] Other unwanted side effects of chronic laxative abuse are abdominal pain, chronic diarrhea, dehydration, disturbance of acid-base balance, and reversible or irreversible damages as well as inflammatory changes of the colonic mucosa.[2,7,9,13-15] Because of the heterogeneity of these side effects and their similarity to symptoms of gastrointestinal disorders, a toxicological screening for laxatives should be part of the differential diagnosis of such disorders.[3,4] This may also help to avoid extensive and expensive diagnostic work.

1.1.2 Nutmeg

Nutmegs, the seeds of the evergreen tropical tree *Myristica fragrans* Houtt., are mainly used as a spice. They are described to be psychoactive when administered in high doses.[16] Psychoactive properties were already described by Hildegard von Bingen in the Middle Ages.[17] More recently, different psychotropic effects have been described in case reports after suspected intake of high doses of nutmegs.[17-21] The main ingredients of the volative oil of nutmeg are the alkenebenzene derivatives elemicin (1-(3',4',5'-trimethoxyphenyl)-prop-2-ene), myristicin (1-(3',4'-methylenedioxy-5'-methoxyphenyl)-prop-2-ene), and safrole (1-(3',4'-methylenedioxyphenyl)-prop-2-ene).[22] The structures of these ingredients are given in Fig. 1.3.

Fig. 1.3. Chemical structures of the main ingredients of the volative oil of nutmeg, safrole, myristicin, and elemicin

In 1966, Shulgin hypothesized that the possible psychotropic effects of myristicin may be caused by metabolic addition of ammonia to the allyl side chain leading to the amphetamine derivative 3,4-methylenedioxy-5-methoxyamphetamine (MMDA)[23] and that of elemicin by conversion to 3,4,5-trimethoxyamphetamine (TMA).[24] These proposed metabolic steps are given in Fig. 1.4, but have not been proven so far.

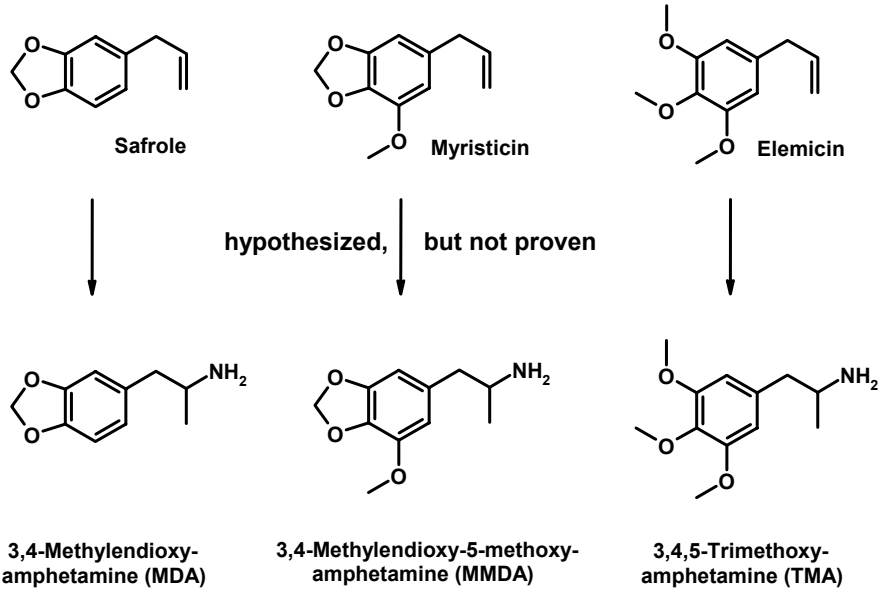


Fig. 1.4. Postulated metabolic pathways of safrole, myristicin, and elemicin to 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxy-5-methoxy-amphetamine (MMDA), and 3,4,5-trimethoxy-amphetamine (TMA).

1.1.3 Herbal Phenalkylamines

Herbal drugs of *Ephedra* species have been abused as stimulants for centuries. *Ephedra* is a shrub-like evergreen plant found in arid regions of Europe, central Asia, and other parts of the world. Major species of *Ephedra* include *Ephedra sinica* Stapf., *E. equisetina* Bunge, *E. intermedia*, and *E. distachya*.[25] The traditional Chinese medicine Ma Huang, derived from aerial parts of *Ephedra*, and has been used for the treatment of asthma, bronchial spasms, and as stimulant or diaphoretic.[26]

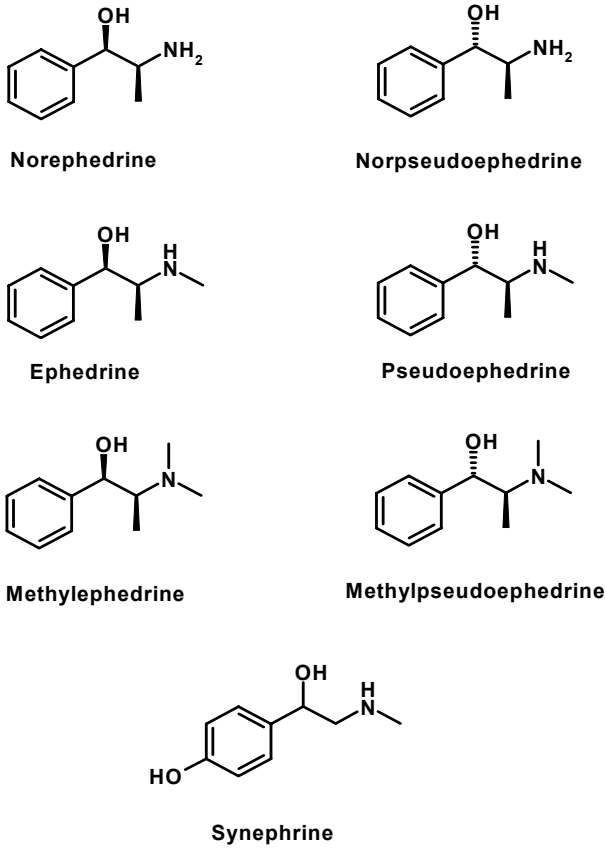
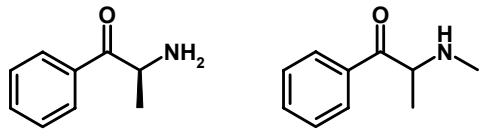


Fig. 1.5. Chemical structures of norephedrine, norpseudoephedrine (cathine), ephedrine, pseudoephedrine, methylephedrine, methylpseudoephedrine, and synephrine.

The main pharmacologically active ingredients of *Ephedra* species are the alkaloids ephedrine and pseudoephedrine as well as norephedrine, norpseudoephedrine (cathine), methylephedrine, and methylpseudoephedrine.[27-30] These compounds are potent central nervous system (CNS) stimulants and also have sympathomimetic effects in the peripheral nervous system. Ephedrine, norpseudoephedrine and methylephedrine were banned as doping agents by the International Olympic Committee. Because of the peripheral effects, ephedrine, pseudoephedrine, or norephedrine are often contained in cold medications. Norephedrine and norpseudoephedrine are often contained in anorectic medications. Such medicaments are often abused as well as supplements containing the anorectic compound synephrine (oxedrine).[31,32] The chemical structures of all mentioned ephedrines and synephrine are depicted in Fig. 1.5.

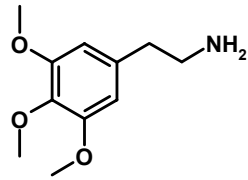
The psychostimulant herbal drug khat (*Catha edulis* Forsk.) is also abused. Khat is cultivated and abused mainly in East Africa and the Arab Peninsula. Emigrants from these countries try to maintain this habit, and large quantities of fresh khat are imported into other areas in the world. For example, >2300 kg of khat were confiscated at the Frankfurt airport (Germany) in 1998.[33] The main alkaloids of khat are the psychostimulants cathinone, norpseudoephedrine and norephedrine.[33-35] Cathinone is also a CNS stimulant and has sympathomimetic effects in the peripheral nervous system. Cathinone and norpseudoephedrine are controlled substances in many countries due to the khat abuse. A structurally related drug of relevance in this context is the synthetic designer drug methcathinone.[36-42] The chemical structures of cathinone and methcathinone are depicted in Fig. 1.6.



Cathinone **Methcathinone**

Fig. 1.6. Chemical structures of cathinone and methcathinone.

Finally, one of the oldest known and best studied herbal drugs of abuse containing phenalkylamines is *Lophophora williamsii* Coult. (peyote). It contains the hallucinogenic alkaloid mescaline.[43,44] Mescaline acts as partial agonist at 5-HT_{2A} and other 5-HT_2 receptors in the central nervous system. The noradrenergic locus coeruleus and the cerebral cortex are among the regions where hallucinogens have prominent effects through their actions upon 5-HT_{2A} receptors.[44-48] The chemical structure of mescaline is depicted in Fig. 1.7.

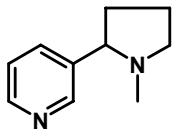


Mescaline

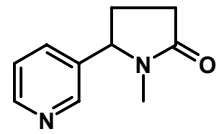
Fig. 1.7. Chemical structure of mescaline.

1.1.4 Toxic Alkaloids

The most common herbal drug of abuse is *Nicotiana tabacum* L. A description of all the severe side effects of smoking is found in many handbooks. Intoxications do usually not occur after smoking of tobacco products, but may occur after oral ingestion of tobacco, e.g. by small children eating cigarettes. Nicotine acts by stimulation of the N-cholinoreceptors and is quickly metabolized to cotinine. The Strucures of nicotine and its metabolite cotinine are depicted in Fig. 1.8.



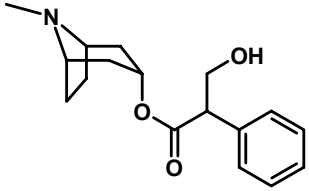
Nicotine



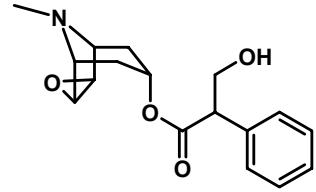
Cotinine

Fig. 1.8. Chemical structures of nicotine and cotinine.

Apart from the above-mentioned drugs of abuse, very popular herbal drugs abused for psychoactive reasons are plants of the nightshade family like *Atropa belladonna* L. (deadly nightshade), *Datura stramonium* L. (Jimson weed), or *Datura arborea* L. (angles trumpet). These plants contain as main active ingredients scopolamine and hyoscyamine which easily racemizes to atropine during storage, isolation and/or gastro-intestinal passage. These substances are potent anticholinergic substances, blocking the M-subtypes of acetylcholine receptors.[49,50] Due to the common abuse, these substances were banned in many countries. The structures of atropine and scopolamine are given in Fig. 1.9.



Atropine

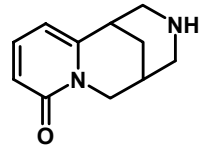


Scopolamine

Fig. 1.9. Chemical structures of atropine and scopolamine.

Such plants may also cause severe or even fatal poisonings for example after unintentional ingestion. An unintentional ingestion of plants is most common among young children who often eat plants which seem attractive to them. *Atropa belladonna* L. is attractive for children due to its colorful and even sweet berries.

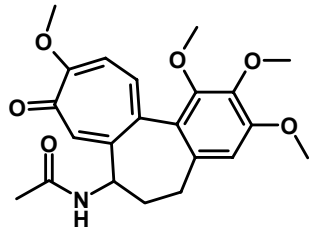
Another plant, attractive for children is *Laburnum anagyroides* L. with fruits similar to beans or peas. The plant contains the alkaloid Cytisine as main active compound. Cytisine acts like nicotine by stimulation of N-cholinoceptors and a resulting overstimulation as a blocking agent on the CNS.[49,51] The chemical structure of cytisine is given in Fig. 1.10.



Cytisine

Fig. 1.10. Chemical structure of cytisine

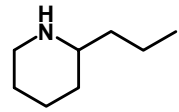
An unintentional or accidental ingestion can also be caused by mix-up of toxic plants with medicinal or nutritional plants. In Germany people often mix-up *Colchicum autumnale* L. containing colchicine with *Allium ursinum* known as Ramsons. Such accidental ingestion due to a mix-up is described in many case reports.[52-59] *Colchicum autumnale* is a very toxic plant due to its main alkaloid colchicine. Colchicine acts via disruption of microtubules.[49,51,60] The chemical structure of colchicine is given in Fig. 1.11.



Colchicine

Fig. 1.11 Chemical structure of colchicine

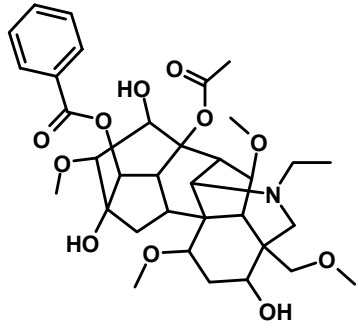
Another plant sometimes mixed-up is *Conium maculatum* L. The seeds of *Conium maculatum* L. resembel those of anis or fennel. This plant is also mixed-up with the root of horseradish. *Conium* contains the alkaloid Coniine. The exact mechanism of action of coniine is not known.[51] The chemical structure of coniine is given in Fig. 1.12.



Coniine

Fig. 1.12. Chemical structure of coniine

Sometimes, plants or their parts are also mixed up in alternative medicines. *Aconitium napellus* may be part of Chinese herbal medicines, and contains the alkaloid Aconitine. Intoxications are frequent after consumption of *Aconitum*, due to mix-up with other plants or wrong preparation. It has been suggested that aconitine acts as a potassium channel blocking agent. The complex chemical structure of aconitine is given in Fig. 1.13.



Aconitine

Fig. 1.13. Chemical structure of aconitine.

1.2 Overview of Published Procedures and Aims for new Studies

In the following, an overview is given on analytical methods published for the different studied herbal drugs.

1.2.1 Laxatives

Previously published procedures for screening for laxatives in urine samples used thin-layer chromatography (TLC),[61-64] high performance liquid chromatography (HPLC) with diode array[65,66] or ultraviolet (UV)[67] detection, or gas chromatography-mass spectrometry (GC-MS) after acetylation[68] or trimethylsilylation.[69] Part of these procedures[61,63-66] allowed simultaneous screening for all the above-mentioned analytes, but required large volumes of urine,[63,64] involved time consuming sample preparation,[61,66] or lacked sensitivity,[63,65] which limits their applicability in routine work. Therefore, there is still need for a comprehensive and sensitive screening procedure for simultaneous detection of an ingestion of diphenol- and anthraquinone-type laxatives in urine samples. The aim of the presented study was to adapt the previously published screening procedure for acidic drugs[70-73] in urine for this purpose. This procedure employed GC-MS, the gold standard for toxicological screening analysis, after extractive methylation of urine samples. The target analytes for screening for laxatives were bisacodyl diphenol, the common metabolite of bisacodyl and sodium picosulfate, and phenolphthalein for the diphenol-type laxatives and rhein for the anthraquinone-type laxatives.

1.2.2 Nutmeg

The main ingredients of the volative oil of nutmeg are the alkenebenzene derivatives elemicin, myristicin, and safrole.[22] As already mentioned, Shulgin had hypothesized that the possible psychotropic effects of myristicin may be caused by metabolic addition of ammonia to the allyl side chain leading to the amphetamine derivative MMDA[23] and that of elemicin by conversion to TMA.[24] Years later, some authors reported the detection of MMDA in liver homogenisate after incubation with myristicin using TLC and fluorescence detection after dansylation.[74,75] In case of safrole and myristicin, detection of different 3-amino-1-(3',4'-methylenedioxyphenyl)-1-propanone derivatives in urine of rat and guinea pig was reported. TLC, mass spectrometry (MS), and nuclear magnetic

resonance spectroscopy (NMR) were used after sample treatment with sodium borohydride.[76,77] However, in metabolism studies of the structurally related estragole (1-(4'-methoxyphenyl)-prop-2-ene), the corresponding 4'-methoxyamphetamine (PMA) could not be detected by GC-MS.[78]

In a case of suspected nutmeg abuse, urine samples submitted to our laboratory for toxicological analysis were analyzed using our systematic toxicological analysis (STA) procedure,[79-84] but neither the above-mentioned amphetamine derivatives (detection limit 5-50 ng/ml[85,86]), nor the main nutmeg ingredients could be detected in urine, but a number of unknown mass spectra suspected to result from metabolites of the nutmeg ingredients. Urine screening for amphetamines by the Abbott TDx fluorescence polarization immunoassay amphetamine/methamphetamine II was negative (below the detection limit of 100 ng/ml).

Therefore, the aim of the study here presented was to identify the metabolites of elemicin, myristicin, and safrole in rat urine and to confirm their presence in human urine of the nutmeg abuser using GC-MS in the electron ionization (EI) mode. In addition, the detectability of nutmeg ingredients and/or their metabolites within our STA procedure[79-84] in urine by full-scan EI GC-MS will be described in order to confirm a nutmeg abuse or intoxication.

1.2.3 Herbal Phenalkyamines

In case of abuse or intoxication of herbal phenalkylamines, their detection and quantification in plasma is necessary in clinical and forensic toxicology.[87] Furthermore, it can be important in the assessment of a probable abuse to differentiate an intake of Ephedra or khat from an intake of corresponding cold or anorectic medications. Cold medications contain either ephedrine or its diastereomer pseudoephedrine and anorectics either norephedrine or its diastereomer norpseudoephedrine, while *Ephedra* species always contain all four diastereomers and additionally small amounts methylephedrine and methylpseudoephedrine. Hence, differentiation should be possible either by detection of methylephedrine or methylpseudoephedrine or by differentiation of the corresponding diastereomers ephedrine/pseudoephedrine or norephedrine/norpseudoephedrine. Differentiation of a khat or methcathinone abuse from intake of the mentioned medications should be possible by detection of cathinone or methcathinone. For the above-mentioned reasons, an analytical procedure to be

used in this context should allow selective detection of all analytes including differentiation of the diastereomers as well as their reliable quantification.

So far, procedures for separation, detection and quantification of all the above-mentioned phenalkylamines in blood plasma have not been published in contrast to methods for quantification of single substances of this group or combinations of a few of them. For analysis of ephedrine and related compounds in biological fluids, various procedures were published using HPLC,[26,88-90] liquid chromatography coupled with a single-stage mass spectrometer (LC-MS)[91,92] or tandem mass spectrometer (LC-MS/MS),[31,93-95] or GC-MS.[28,94] For the detection and quantification of mescaline, several procedures were published[96-98] as well as for monitoring khat abuse.[33,35,99]

The aim of the presented study was to develop a multi-analyte procedure for separation, detection and quantification of all the above-mentioned phenalkylamines in blood plasma using LC-MS/MS in the electrospray ionization (ESI) mode. The quantification procedure had to be validated and tested for applicability in clinical and forensic toxicology.

1.2.4 Toxic Alkaloids

Based on the statistics of the 2005 annual report of poison information center of northern Germany,[1] besides cardiac glycosides, the following alkaloids were most frequently involved in plant poisonings: aconitine, atropine, colchicine, coniine, cytisine, nicotine, physostigmine, scopolamine.

For diagnosis and prognosis of such poisonings, analytical methods for detection and quantification of the respective toxic alkaloids are required in clinical and forensic toxicology.[87] As blood plasma concentrations correlate best with the pharmacologic/toxicologic effects, this sample matrix should be used for determination whenever possible. While many methods have been described for plasma analysis of cardiac glycosides,[100-104] only few are available for the above-mentioned alkaloids. For plasma analysis of aconitine, procedures were published using GC-MS[105,106] or LC-MS/MS,[107] for atropine and/or scopolamine using GC-MS[105,106,108] or LC-MS(/MS),[109-111] for physostigmine (also used as antidote in treatment of atropine and/or scopolamine poisoning) using HPLC with fluorescence[112,113] or electrochemical[114,115] detection, for nicotine and its main metabolite cotinine using GC-MS[116-118] or LC-MS(/MS),[119-125] and finally for colchicine using GC-MS[126] or LC-MS(/MS).[57,127-129] However, none of these methods covered more than two of the above-mentioned alkaloids and methods for

plasma analysis of coniine and cytisine are not available in the literature at all. Therefore, the first aim of the presented study was to develop a multi-analyte procedure for detection and validated quantification of aconitine, atropine, colchicine, coniine, cytisine, nicotine and its metabolite cotinine, physostigmine, and scopolamine in blood plasma. Development of an LC-MS-based assay seemed most promising due to soft ionization, and high selectivity and sensitivity. Tandem MS apparatus are more sensitive and selective, but much more expensive than single stage MS. This raised the question whether tandem MS is actually necessary for detection and quantification of these alkaloids. Therefore, the second aim of the study was to compare a single stage vs. a tandem MS instrument with respect to selectivity, sensitivity, accuracy and precision after identical sample preparation.

1.3 Summary of the Aims of the Presented Studies

In summary, it can be stated that for some of the presented single substances and substance classes a variety of analytical procedures has been developed over the years, but universal procedures covering more than a few toxic herbal drugs remain to be elaborated due to the different physiochemical properties of the active ingredients of herbal drugs. One systematic toxicological procedure will not cover all compounds of interest. Therefore, development or adaption of different procedures is necessary.

The aim of the presented thesis was to develop methods for detection and validated quantification. The following drugs or drug clases were included in this method development:

- Laxatives
- Ingredients of Nutmeg
- Herbal Phenalkylamines
- Toxic alkaloids

2 EXPERIMENTAL PROCEDURES

2.1 CHEMICALS AND REAGENTS

2.1.1 Laxatives

Bisacodyl diphenol was obtained from Sigma, Taufkirchen (Germany). Rhein, phenolphthalein, diethyl ether, ethyl acetate, methanol and methyl iodide were obtained from Merck, Darmstadt (Germany). Tetrahexylammonium hydrogen sulfate and toluene were obtained from Fluka, Neu-Ulm (Germany). All chemicals except methyl iodide (for synthesis) were of analytical grade. The solid-phase extraction (SPE) columns (Isolute Diol, 500 mg, 10 ml XL) were obtained from Separtis, Grenzach-Wylen (Germany).

For the synthesis of dimethylated bisacodyl diphenol (bisacodyl diphenol-2ME) and dimethylated phenolphthalein (phenolphthalein-2ME), 1 ml of a methanolic 1 mg/ml solution of each analyte was transferred to a 10 ml glass tube, each. After addition of 2 ml of freshly prepared diazomethane solution in diethyl ether, the glass tube was closed and the solution was incubated for 48 hours at room temperature in the dark. Diazomethane was synthesized according to the procedure of McKay et al. using 1-methyl-3-nitro-1-nitroso-3-nitroguanidine, KOH and diethyl ether.[130] After incubation, the solution was evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 1 ml of methanol. The purity of the methyl derivatives was checked by GC-MS.

Trimethylated rhein (rhein-3ME) was synthesized by extractive methylation. Two ml of an aqueous solution containing 50 mg/ml of rhein were extracted as described below (see 2.3.1). After evaporation, the residue was redissolved in water and extractive methylation was repeated. After the second evaporation, the residue was weighed and dissolved in methanol. The purity of the derivative was checked by GC-MS.

2.1.2 Ingredients of Nutmeg

Elemicin was provided by cc chemical consulting (Berlin, Germany) for research purposes. Myristicin and safrole were obtained from Sigma (Taufkirchen, Germany) and all other chemicals and biochemicals (analytical grade) were obtained from Merck (Darmstadt, Germany).

2.1.3 Herbal Phenalkylamines

Ammonium formate (analytical grade), free bases of ephedrine, methylephedrine, methylpseudoephedrine, norephedrine, norpseudoephedrine, pseudoephedrine, and synephrine were obtained from Fluka (Neu-Ulm, Germany). Methanolic solutions of cathinone, methcathinone, mescaline and of the internal standards (IS) ephedrine-d_3, norephdrine-d_3 and mescaline-d_9 were obtained from Promochem (Wesel, Germany). Acetonitrile and water (both HPLC grade) and all other chemicals (analytical grade) were obtained from E. Merck (Darmstadt, Germany). Varian Bond Elute Certify cartridges (130 mg; 3 ml) were obtained from Varian (Darmstadt, Germany).

2.1.4 Toxic Alkaloids

Aconitine, atropine, colchicine, and scopolamine were obtained from Fluka (Neu-Ulm, Germany), cytisine from ChromaDex (St. Ana, USA), and physostigmine from Koehler Chemie (Alsbach-Haehnlein, Germany). Coniine was a kind gift of the Institute of Pharmaceutical Biology (Saarbruecken, Germany). Methanolic solutions of cotinine and nicotine, as well as the IS cotinine-d_3, benzoylecgonine-d_3, and trimipramine-d_3 were obtained from Promochem (Wesel, Germany). Acetonitrile and water (both HPLC grade) and all other chemicals (analytical grade) were obtained from E. Merck (Darmstadt, Germany). Varian Bond Elute Certify cartridges (130 mg; 3 ml) were obtained from Varian (Darmstadt, Germany).

2.2 BIOSAMPLES

2.2.1 Urine Samples for the Study on Analysis of Laxatives

Blank urine samples used for method development as well as for stability, recovery, and limit of detection (LOD) experiments were obtained from healthy drug-free volunteers. Duration of detectability experiments were carried out using urine samples from healthy volunteers who received one lowest therapeutic dose of senna extract containing 7 mg sennoside B or of one lowest therapeutic dose of sodium picosulfate (5 mg) after informed consent according the declaration of Helsinki. Urine samples from authentic cases had been submitted to our laboratory for toxicological analysis.

2.2.2 Urine Samples for the Study on Analysis of Ingredients of Nutmeg

The investigations were performed using urine of male rats (Wistar, Ch. River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German Animal Protecting law. The rats were administered a single 100 mg/kg body mass (BM) dose of elemicin, myristicin, and safrole for metabolism studies or a single 500 mg/kg BM dose of grounded nutmeg from two different batches for the STA study in aqueous suspension (final volume 1 ml each) by gastric intubation (n = 2 for each substance and dose). Urine was collected separately from the faeces over a 24 h period. All samples were directly analyzed. Blank rat urine samples were collected before drug administration to check whether they were free of interfering compounds.
Human urine samples were submitted to our laboratory for toxicological analysis. They were collected from an inpatient of a psychiatric hospital who stated after being informed on the result of the urine screening that he had taken powder of about five nutmegs. The only registered symptom was vomiting.

2.2.3 Plasma Samples for the Study on Analysis of Herbal Phenalkylamines

Pooled human blank plasma samples were used for development and validation of the procedure and were obtained from a local blood bank. Blood samples for selectivity experiments were from therapeutic drug monitoring (TDM) cases or intoxication cases and had been submitted to our laboratory by various hospitals.

Applicability experiments were carried out using plasma samples from healthy volunteers taken one hour after application of 30 mg of pseudoephedrine (contained in Aspirin complex®), 6.2 mg of ephedrine (contained in Wick MediNait®), or 10 g of an aqueous extract of *Herba Ephedra* after informed consent according to the declaration of Helsinki. *Herba Ephedra* was obtained from a local pharmacy and decocted for 5 min with boiled water. The dose was selected according to recommendations of using *Herba Ephedra* as stimulant.[25]

2.2.4 Plasma Samples for the Study on Analysis of Toxic Alkaloids

Human blank plasma samples and blood samples from drug free volunteers were used for development of selectivity experiments and validation of the procedure. They were obtained from a local blood bank. Applicability experiments were carried out using plasma samples from poisoning cases sent to our laboratory for toxicological analysis.

2.3 Sample Preparations

2.3.1 Sample Preparation for Analysis of Laxatives

A 2-ml portion of urine was adjusted to pH 5.2 with acetic acid (1 mol/L) and incubated in a water bath at 52°C for 90 min with 100 µl of a mixture (100 000 Fishman units per ml) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1) from *Helix pomatia* then mixed in a centrifugation tube with 2 ml of the phase-transfer reagent consisting of 0.02 M tetrahexylammonium (THA) hydrogensulfate in 1 M sodium phosphate solution (pH 12). After addition of 6 ml of 1 M methyl iodide in toluene, the closed tube was shaken in a heating block at 50°C for 30 min. After phase separation by centrifugation at 1500 × *g* for 3 min, the organic phase containing the analytes and THA salts was transferred to the SPE column conditioned as follows: 5 ml of methanol at a flow rate of 10 ml/min, drying the column under vacuum for 15 seconds, 5 ml of toluene at a flow rate of 10 ml/min. The organic phase was rinsed through the sorbent bed at a flow rate of 3 ml/min to remove the THA salts from the organic phase and the eluate was collected. The part of the analytes also adsorbed on the sorbent was selectively eluted with 5 ml of diethyl ether/ethyl acetate (95: 5, v/v) at a flow rate of 3 ml/min and the eluate was collected. The combined eluates were carefully evaporated to dryness at 60°C (reduced pressure, 30-50 kPa). The residue was dissolved in 50 µl of ethyl acetate and a 1 µl-aliquot of this extract was injected into the GC-MS system.

2.3.2 Sample Preparation for Analysis of Ingredients of Nutmeg

2.3.2.1 Sample Preparation for Identification of Metabolites by GC-MS

A 5-ml portion of rat or human urine was adjusted to pH 5.2 with acetic acid (1 mol/l) and incubated at 37°C for 12 h with 100 µl of a mixture (100 000 Fishman units per ml) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1) from *Helix pomatia*, then adjusted to pH 8-9 and extracted with 5 ml of a dichloromethane-isopropanol-ethyl acetate mixture (1:1:3; v/v/v). After phase separation by centrifugation, the organic layer was transferred into glass flasks and carefully evaporated to dryness at 75°C under vacuum. The residue was derivatized by acetylation or left underivatized and dissolved in 100 µl of methanol. Acetylation was

conducted with 100 µl of an acetic anhydride-pyridine mixture (3:2; v/v) for 5 min under microwave irradiation at about 440 W. After careful evaporation, the residue was dissolved in 100 µl of methanol and 2 µl of this solution were injected into the GC-MS. The same procedure with the exception of enzymatic hydrolysis was used to study whether metabolites of nutmeg ingredients were excreted as glucuronide and/or sulfate conjugates.

A second urine sample was worked up as described above, but pH was adjusted to 4-5 and the corresponding extract was methylated and subsequently acetylated. After reconstitution of the extraction residue in 100 µl of methanol, methylation was conducted with 200 µl of a solution of diazomethane in diethyl ether, synthesized according to the procedure of McKay et al.[130] The reaction vials were sealed and left at room temperature for 15 min. Thereafter, the mixture was once again carefully evaporated to dryness under a stream of nitrogen, the residue acetylated as described above, and finally the evaporated residue redissolved in 100 µl of methanol and 2 µl of this sample was injected into the GC-MS.[84]

2.3.2.2 Sample Preparation for Systematic Toxicological Analysis by GC-MS

The urine samples (5 ml) were divided into two aliquots. One aliquot was refluxed with 1 ml of 37% hydrochloric acid for 15 min. Following hydrolysis, the sample was mixed with 2 ml of 2.3 mol/l aqueous ammonium sulfate and 1.5 ml of 10 mol/l aqueous sodium hydroxide to obtain a pH value of 8-9. Before extraction, the other aliquot of unhydrolyzed urine was added and this solution was extracted with 5 ml of a dichloromethane-isopropanol-ethyl acetate mixture (1:1:3; v/v/v). After phase separation by centrifugation, the organic layer was transferred into glass flasks and carefully evaporated to dryness at 75°C under vacuum. The residue was derivatized by acetylation with 100 µl of an acetic anhydride-pyridine mixture (3:2; v/v) for 5 min under microwave irradiation at about 440 W. After careful evaporation of the derivatization mixture, the residue was dissolved in 100 µl of methanol and 2 µl of this sample were injected into the GC-MS.

2.3.3 Sample Preparartion for Analysis of Herbal Phenalkylamines

Plasma samples (1 ml) were diluted with 2 ml of 5 mM aqueous ammonium formate solution adjusted to pH 3 with formic acid. After addition of 0.1 ml of a methanolic solution of the IS containing 1000 ng/ml of ephedrine-d_3, norephedrine-d_3, and mescaline-d_9 each, the samples were briefly mixed (15 s) on a rotary shaker, centrifuged for 3 min at 1000 g and loaded on mixed-mode SPE cartridges previously conditioned with 1 ml of methanol and 1 ml of purified water. After extraction, the cartridges were washed with 1 ml of purified water, 1 ml of 0.01 M aqueous hydrochloric acid and 2 ml of methanol. Reduced pressure was applied until the cartridges were dry, and the analytes were eluted with 1 ml of methanol–aqueous ammonia (98:2, v/v) into 1.5 ml polypropylene reaction vials. The eluates were evaporated to dryness under a stream of nitrogen at 56 °C. Then, 0.1 ml of 5 mM aqueous ammonium formate solution (pH 3) was added and the vials were shaken on a rotary shaker for 3 min. After centrifugation for 2 min at 10 000 g, the solution was transferred to autosampler vials and 5 µl were injected into the LC-MS/MS system.

2.3.4 Sample Preparartion for Analysis of Toxic Alkaloids

Plasma samples (1 ml) were diluted with 2 ml of 5 mM aqueous ammonium formate solution adjusted to pH 3 with formic acid. After addition of 0.1 ml of a methanolic solution of the IS containing 1000 ng/ml of cotinine-d_3, 100 ng/ml of benzoylecgonine-d_3, and 10000 ng/ml trimipramine-d_3, the samples were mixed for 15 s on a rotary shaker, centrifuged for 3 min at 1000 g and loaded on mixed-mode SPE cartridges previously conditioned with 1 ml of methanol and 1 ml of purified water. After extraction, the cartridges were washed with 1 ml of purified water, 1 ml of 0.01 M aqueous hydrochloric acid and 2 ml of methanol. Reduced pressure was applied until the cartridges were dry, and the analytes were eluted with 1 ml of methanol–aqueous ammonia (98:2, v/v) into 1.5 ml polypropylene reaction vials. The eluates were evaporated to dryness under a stream of nitrogen at 56 °C. Then, 0.1 ml of 5 mM aqueous ammonium formate solution (pH 3) was added and the vials were shaken on a rotary shaker for 3 min. After centrifugation for 2 min at 10.000 g, the solution was transferred to autosampler vials and 10 µl each were injected into the LC-MS and LC-MS/MS systems.

2.4 APPARATUS AND CONDITIONS

2.4.1 Screening and Identification of Laxatives

2.4.1.1 Apparatus

The samples were incubated in a heating block fixed to a multifix shaker S 300 (Schwinherr, Schwäbisch Gmünd, Germany). A Vac-Master V-10 (ICT, Bad Homburg, Germany) was used for manual SPE. The samples were analyzed using a Hewlett Packard (Agilent, Waldbronn, Germany) HP 6890 Series GC system combined with an HP 5972 Series mass selective detector, an HP 6890 Series injector and an HP Chem Station G1701AA version A.03.00.

2.4.1.2 GC Conditions

The GC conditions were as follows: split-less injection mode; column, HP-1 capillary (12 m x 0.2 mm I.D.), cross-linked methyl silicone, 330 nm film thickness; injection port temperature, 280°C; carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100-310°C at 30°/min, initial time 3 min, final time 8 min.

2.4.1.3 MS Conditions for Screening Procedure

The MS conditions for the screening procedure were as follows: full scan mode, mass to charge ratio (*m/z*) 50-550 u; EI mode: ionization energy, 70 eV; ion source temperature, 280°C.

2.4.1.4 MS Conditions for Recovery Experiments

Mass fragmentography in selected-ion monitoring (SIM) mode was used with the following program: solvent delay, 3 min; time window A, 3.00-9.00 min, *m/z* 305 (target ion, t), 290 for bisacodyl diphenol-2ME; time window B, 9.01-18.00 min *m/z* 311 (t), 326 for rhein-3ME and *m/z* 271 (t), 346 for phenolphthalein-2ME.

2.4.1.5 GC-MS Screening Procedure

The laxatives and/or their metabolites were screened for by mass chromatography extracting characteristic fragment ions from the total ion current. The following ions were used for this purpose: *m/z* 305, 290, 335, 320, 365, 350, 311, 326, 271 and 346. For matters of convenience, this operation was carried out by a user-defined macro. Positive peaks were identified by visual or computerized comparison of the peaks underlying mass spectra with reference spectra.

2.4.2 Screening and Identification of Ingredients of Nutmeg

2.4.2.1 Apparatus

The extracts were analyzed using a Hewlett Packard (Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with a HP 5972A MSD mass spectrometer and a HP MS ChemStation (DOS series) with HP G1034C software version C03.00. Analyte separation was achieved on a fused silica capillary column (HP-1MS, 12 m x 0.2 mm i.d., film thickness 0.33 µm). The GC conditions were as follows: split-less injection mode; column, injection port temperature, 280°C; carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100-310°C at 30°/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full-scan mode, *m/z* 50-550 u; EI mode, ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface, heated at 280°C.

2.4.2.2 GC-MS Procedure for Identification of Metabolites

The urinary metabolites of elemicin, myristicin, and safrole were separated by GC and identified by EI MS after enzymatic hydrolysis, extraction, acetylation, trifluoroacetylation, methylation plus acetylation or without derivatization. The postulated structures of the metabolites were deduced from the fragments detected in the EI mode which were interpreted in correlation to those of the parent compound according to the rules described by e.g. McLafferty and Turecek [131] and Smith and Busch [132].

2.4.2.3 GC-MS Procedure for Systematic Toxicological Analysis

The extracted and derivatized metabolites of elemicin, myristicin, and safrole were separated by GC. They were screened for and identified as follows: mass chromatography with the selected ions *m/z* 150, 164, 165, 180, 194, 252, and 266 was used for screening. These ions were selected from the mass spectra recorded during this study. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros.[79,133] The identity of the peaks in the mass chromatograms was confirmed by computerized comparison[134] of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study.

2.4.3 Screening and Identification of Herbal Phenalkylamines

2.4.3.1 Apparatus

The studied analytes were separated using a Shimadzu integrated HPLC system which consisted of a Shimadzu CBM 20 A controller, two Shimadzu LC 20 AD pumps including a degasser, a Shimadzu SIL 20 AC autosampler, and a Shimadzu CTO 20 AC column oven. The analytes were detected using an Applied Biosystems 3200 Q TRAP Linear Ion Trap Quadrupole Mass Spectrometer with Analyst Software (Version 1.4.1) equipped with a Turbo V™ Ion Source operated in the ESI mode.

2.4.3.2 LC Conditions

Gradient elution was performed on an Agilent Zorbax® strong cation exchange (SCX) column (5 µm, 150 x 2.1 mm) as stationary phase and a Wicom SCX guard column (Heppenheim, Germany). The mobile phase consisted of 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid (eluent A) and acetonitrile (eluent B). Before use, the mobile phases were degassed for 30 min in an ultrasonic bath. During use, the mobile phase was degassed by the integrated Shimadzu Prominence degasser. Before starting analysis, the HPLC system was equilibrated for 10 min with a mixture of 95 % of eluent A and 5 % of eluent B. The flow rate and gradient were programmed as follows: flow rate: 1.5 ml/min; 0.00-7.00 min: 5% eluent B, 7.01-10.00 min: gradient increase to 30% eluent B, 10.01-11.00 min: 30% eluent B, 11.01-17.00 min: 5% eluent B for re-equilibration of the HPLC column. The column oven was set at 55°C, the autosampler was cooled at 15°C and operated without rinsing.

2.4.3.3 MS/MS Conditions

For detection and quantification, the following ESI inlet conditions were applied: gas 1, nitrogen (45 psi; 310.3 kPa); gas 2, nitrogen (90 psi; 620.5 kPa); ion spray voltage, 5500 V; ion source temperature, 630°C; curtain gas, nitrogen (30 psi; 206.8 kPa).
The mass spectrometer was operated in multiple-reaction monitoring (MRM) mode with the following settings: collision gas was set at medium, the collision cell exit potential was 4.00 V, the dwell time was set at 50 ms. All other settings were analyte specific and were determined using Analyst® software in Quantitative Optimization mode. The

settings are summarized in Table 2.1. The method was checked for cross-talk by injection of the single analytes. Q1 and Q3 were operated in Unit resolution.

Table 2.1. Analytes, MRM transitions and parameter settings including declustering potential (DP), entrance potential (ENP), collision cell entrance potential (CEP), and collision energy (CE). Target transitions are marked with (t), qualifier transitions marked with (q).

Analyte	Q 1 Mass, u	Q 3 Mass, u	DP, V	ENP, V	CEP, V	CE, eV
Cathinone	150.06	107.10 (t)	36.00	10.00	14.43	35.00
		91.20 (q)	36.00	10.00	14.43	25.00
		77.00 (q)	36.00	10.00	14.43	49.00
Ephedrine	166.07	117.10 (t)	10.00	5.00	14.88	27.00
		91.00 (q)	10.00	5.00	14.88	43.00
		133.10 (q)	10.00	5.00	14.88	27.00
Methcathinone	164.08	130.90 (t)	10.00	4.00	14.82	27.00
		130.10 (q)	10.00	4.00	14.82	43.00
		77.00 (q)	10.00	4.00	14.82	59.00
Methylephedrine	180.12	117.10 (t)	10.00	4.50	15.27	27.00
		147.10 (q)	10.00	4.50	15.27	27.00
		91.10 (q)	10.00	4.50	15.27	41.00
Mescaline	212.09	195.10 (t)	10.00	6.00	16.17	23.00
		180.20 (q)	10.00	6.00	16.17	23.00
		77.10 (q)	10.00	6.00	16.17	59.00
Methylpseudoephedrine	180.10	147.20 (t)	10.00	3.00	15.27	27.00
		91.10 (q)	10.00	3.00	15.27	47.00
		146.00 (q)	10.00	3.00	15.27	33.00
Norephedrine	152.08	117.00 (t)	10.00	4.00	14.49	23.00
		115.00 (q)	10.00	4.00	14.49	29.00
		91.00 (q)	10.00	4.00	14.49	39.00
Norpseudoephedrine	152.08	117.10 (t)	10.00	4.50	14.49	23.00
		115.00 (q)	10.00	4.50	14.49	23.00
Norpseudoephedrine	152.08	91.00 (q)	10.00	4.50	14.49	37.00

Pseudoephedrine	166.10	91.00 (t)	10.00	4.00	14.88	45.00
		133.00 (q)	10.00	4.00	14.88	27.00
		115.00 (q)	10.00	4.00	14.88	35.00
Synephrine	150.08	117.00 (t)	10.00	3.00	14.43	29.00
		105.10 (q)	10.00	3.00	14.43	25.00
		77.10 (q)	10.00	3.00	14.43	47.00
Ephedrine-d_3	169.18	117.10 (t)	21.00	10.50	14.96	27.00
		115.10 (q)	21.00	10.50	14.96	37.00
		91.10 (q)	21.00	10.50	14.96	45.00
Mescaline-d_9	221.26	204.20 (t)	21.00	9.00	16.42	15.00
		186.20 (q)	21.00	9.00	16.42	25.00
		170.20 (q)	21.00	9.00	16.42	31.00
Norephedrine-d_3	155.19	119.10 (t)	16.00	9.00	14.57	25.00
		120.10 (q)	16.00	9.00	14.57	25.00
		117.10 (q)	16.00	9.00	14.57	33.00

2.4.4 Screening and Identification of Toxic Alkaloids

2.4.4.1 Apparatus

The LC-MS system was as follows: Agilent Technologies (AT, Waldbronn, Germany) AT 1100 Series HPLC system which consisted of a degasser, a binary pump and an autosampler. As detector an AT 1100 MSD Mass Spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source was used. In the following, for this system the term single stage MS is used.

The LC-MS/MS system was as follows: Shimadzu integrated HPLC system which consisted of a Shimadzu CBM 20 A controller, two Shimadzu LC 20 AD pumps including a degasser, a Shimadzu SIL 20 AC autosampler, and a Shimadzu CTO 20 AC column oven. As detector an Applied Biosystems 3200 Q TRAP Linear Ion Trap Quadrupole Mass Spectrometer with Analyst Software (Version 1.4.1) equipped with a Turbo V™ Ion Source operated in the ESI mode was used. In the following, for this system the term tandem MS is used.

2.4.4.2 LC Conditions

The following LC conditions were identical in both systems. Gradient elution was performed on a Merck LiChroCART column (125 x 2 mm internal diameter) with Superspher®60 RP Select B as stationary phase and a LiChroCART 10-2 Superspher 60 RP Select B guard column. The mobile phase consisted of 50 mM aqueous ammonium formate adjusted to pH 3.5 with formic acid (eluent A) and acetonitrile (eluent B). Before use, the mobile phases were degassed for 30 min in an ultrasonic bath. During use, the mobile phase was degassed by the corresponding integrated degasser. Before starting analysis, the HPLC systems were equilibrated for 10 min with a mixture of 90 % of eluent A and 10 % of eluent B. The gradient and the flow rate were programmed as follows: 0.00-2.00 min 10% B (flow: 0.4 ml/min), 2.01-5.00 min gradient increase to 80% B (flow: 0.6 ml/min), 5.01-7.00 min 80% B (flow: 0.60 ml/min) 7.01-10.00 min 10% B (flow: 0.4 ml/min) for re-equilibration of the HPLC column. The column oven was set at 25°C.

2.4.4.3 Single stage and Tandem MS Conditions

For single stage MS, the following APCI inlet conditions were selected: drying gas, nitrogen (12 l/min, 350°C) and nebulizer gas, nitrogen (25 psi; 172.5 kPa); capillary voltage, 4000 V; vaporizer temperature, 400°C; corona current, 5.0 µA. The MS was operated in positive scan mode with a scan range from *m/z* 50 to 800 on MSD 1 for screening and identification, and in SIM mode on MSD 2 for quantification. For quantification, the SIM mode at 100 and 200 V fragmentor voltage with different gain values was used. The settings are given in Table 2.2. Tuning of the MS was performed with the help of the autotune feature of the LC-MS ChemStation software (rev. A.08.03) using the APCI acetonitrile solution tuning mix supplied by the manufacturer.

Table 2.2. Analytes, monitored ions and parameter settings used in LC-APCI-MS

MSD 1 (Full Scan)		Mass Range, *m/z*	Fragmentor Voltage, V	Gain	
		50-800	100	50	
MSD 2 (SIM)	Analyte	Ion, *m/z*	Fragmentor Voltage, V	Gain	Dwell time, ms
	Aconitine	646	100	5.0	28
	Atropine	290	100	5.0	28
	Colchicine	400	100	5.0	28
	Coniine	128	100	1.0	39
	Cotinine	177	100	1.0	39
	Cytisine	191	100	1.0	39
	Nicotine	163	100	1.0	39
	Physostigmine	276	100	5.0	28
	Scopolamine	304	100	5.0	28
	Cotine-d_3	180	100	1.0	39
	Benzoylecgonine-d_3	293	200	5.0	28
	Trimipramine-d_3	298	200	5.0	28

For tandem MS, the following ESI inlet conditions were selected: gas 1, nitrogen (45 psi; 310.3 kPa); gas 2, nitrogen (90 psi; 620.5 kPa); ion spray voltage, 5500 V; ion source temperature, 630°C; curtain gas, nitrogen (30 psi; 206.8 kPa). The MS was operated in MRM mode with the following settings: collision gas was set at medium, the dwell time was set at 50 ms. All other settings were analyte specific and were determined using Analyst® software in Quantitative Optimization mode. The transitions used are given in Table 2.3. The method was checked for cross-talk by injection of the single analytes. Q1 and Q3 were operated in Unit resolution.

Table 2.3. Analytes, MRM transitions and parameter settings including declustering potential (DP), entrance potential (ENP), collision cell entrance potential (CEP), collision energy (CE), and collision cell exit potential (CXP) used in LC-ESI-MS/MS. Target transitions are marked with (t), qualifier transitions marked with (q).

Analyte	Q 1 Mass, u	Q 3 Mass, u	DP, V	ENP, V	CEP, V	CE, eV	CXP, V
Aconitine	646.52	105.1 (t)	66	9.5	26	93	4
		586.4 (q)	66	9.5	26	45	4
		368.2 (q)	66	9.5	26	55	6
Atropine	290.31	124.4 (t)	51	5.5	14	33	4
		93.1 (q)	51	5.5	14	43	4
		91.1 (q)	51	5.5	14	55	4
Colchicine	400.31	152.2 (t)	51	8.5	24	129	4
		358.2 (q)	51	8.5	24	29	4
		165.2 (q)	51	8.5	24	103	4
Coniine	128.19	69.1 (t)	31	9.0	10	23	2
		55.1 (q)	31	9.0	10	31	4
		83.1 (q)	31	9.0	10	21	4
Cotinine	177.19	80.2 (t)	41	5.0	12	35	4
		98.1 (q)	41	5.0	12	33	4
		53.0 (q)	41	5.0	12	67	4
Cytisine	191.21	148.2 (t)	51	5.0	14	27	4
		80.1 (q)	51	5.0	14	51	2
		91.3 (q)	51	5.0	14	55	2
Nicotine	163.19	132.1 (t)	31	3.5	12	21	4
		117.2 (q)	31	3.5	12	35	4
		130.2 (q)	31	3.5	12	25	4
Physostigmine	276.27	162.3 (t)	26	4.0	28	29	4
		219.3 (q)	26	4.0	28	17	4
		147.2 (q)	26	4.0	28	47	4
Scopolamine	304.27	138.3 (t)	46	5.0	16	27	4
		103.2 (q)	46	5.0	16	53	4

Scopolamine	304.27	156.3 (q)	46	5.0	16	23	4
Benzoylecgonine-d_3	293.29	171.2 (t)	46	4.5	18	29	4
		105.0 (q)	46	4.5	18	47	4
		76.8 (q)	46	4.5	18	65	4
Cotinine-d_3	180.19	80.0 (t)	51	6.5	12	39	4
		101.0 (q)	51	6.5	12	35	4
		53.0 (q)	51	6.5	12	31	4
Trimipramine-d_3	298.28	61.1 (t)	41	5.5	19	53	6
		103.1 (q)	41	5.5	19	27	4
		193.1 (q)	41	5.5	19	59	4

2.5 VALIDATION OF THE DESCRIBED METHODS

2.5.1 Laxatives

2.5.1.1 Limit of Detection Experiments

Urine samples (2 ml) were spiked with decreasing concentrations of analytes and analyzed as described above. The lowest concentrations at which the analytes could still be successfully identified by library search and for which *S/N* ≥ 3 were obtained for at least three characteristic fragment ions per analyte were the estimated LODs of the procedure.

2.5.1.2 Recovery Experiments

Aliquots (2 ml) of urine (n = 6) were spiked to 50 ng/ml with bisacodyl diphenol, phenolphthalein and to 125 ng/ml with rhein. These samples were analyzed as described above (see 2.3.1). The peak areas of the derivatized analytes obtained from these samples were compared with the corresponding peak areas obtained from solutions of bisacodyl diphenol-2ME, phenolphthalein-2ME (2 000 ng/ml, each) and rhein-3ME (5 000 ng/ml) in ethyl acetate (concentrations correspond to 100% recovery).

2.5.1.3 Stability Experiments

For estimation of the stability of the analytes in buffer solution, 10 ml of urine spiked with 100 µg/ml of each analyte (bisacodyl diphenol, phenolphthalein, rhein) was incubated with 10 ml extraction buffer at room temperature. At time points 0, 30, 120, 240, and 480 min 4 ml-aliquots of the solution were taken and extractive methylation was performed as described above (2.3.1 Sample Preparation of Laxatives).

2.5.2 Herbal Phenalkylamines

2.5.2.1 Preparation of Stock Solutions, Calibration Standards, and Control Samples

Stock solutions of each analyte were prepared at a concentration of 1 mg/ml by separate weighings using eluent A as solvent. Working solutions of each analyte were prepared by independent dilution from each stock solution at the following concentrations: 0.001, 0.01, and 0.1 mg/ml. The calibration standards were prepared using pooled blank plasma and spiking solutions prepared from the working solutions as mixtures of the ten analytes in eluent A at concentrations ten times higher than the corresponding calibration standards. The quality control samples (concentrations as given in chapter 2.5.2.4) were prepared using pooled blank plasma and independently prepared mixtures of the ten analytes at concentrations hundred times higher than the concentrations of the corresponding quality control samples. All solutions were stored at 4°C.

2.5.2.2 Selectivity

Ten blank plasma samples from different sources were analyzed and checked for peaks interfering with the detection of the analytes or the IS. In addition, to check for possible interferences from other common drugs and/or their metabolites, plasma samples routinely submitted to our laboratory for TDM or toxicological analysis were analyzed by the described procedure. The plasma samples did not contain any of the analytes as checked by comprehensive screening of the corresponding urine samples.

2.5.2.3 Linearity

Aliquots of blank plasma (1 ml) were spiked with 0.1 ml of the corresponding spiking solutions and 0.1 ml of IS solution to obtain calibration standards with concentrations of 10, 20, 100, 250, 500, 750, 1000 ng/ml. Replicates (n = 6) at each concentration level were analyzed as described above. The regression line was calculated using a weighted $[1/(\text{concentration})^2]$ least-squares regression model. A weighted second-order model with the same weighting factors was also calculated. Daily calibration curves

using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

2.5.2.4 Accuracy and Precision

Qualitiy control (QC) samples were prepared at five concentration levels, namely near the lower limit of quantification (LLOQ) at 10 ng/ml (LQC), 20 ng/ml (LOW), 500 ng/ml (MED), 800 ng/ml (HIGH), and above the calibration range at 4000 ng/ml (ACR). They were analyzed according to the procedure described above in duplicate on each of eight days. The concentrations of the analytes in the quality control samples were calculated via the daily calibration curves. Accuracy was calculated for each analyte in terms of bias as the percent deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Precision data (given as relative standard deviations) for within-day (repeatability), and time-different intermediate precision (combination of within and between day effects) of the method were calculated according to references[135,136] using one-way analysis of variance (ANOVA) with the grouping-variable "day". The acceptance intervals of within-day (repeatability) and intermediate precision were ≤ 15% RSD (≤ 20% RSD at LLOQ) and for bias ± 15% (± 20% at the LLOQ) of the nominal values.[137]

2.5.2.5 Processed Sample Stability

For estimation of stability of processed samples under the conditions of LC-MS/MS analysis, LOW and HIGH quality control samples (n = 10 each) were extracted as described above. The resulting extracts at each concentration level were pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected under the conditions of a regular analytical run at time intervals of 2 h. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration versus injection time. Instability of processed samples would be indicated by a negative slope significantly different from zero ($p \leq 0.05$).[138]

2.5.2.6 Freeze/Thaw Stability and Bench Top Stability

For evaluation of freeze/thaw stability, quality control samples (LOW and HIGH) were analyzed prior to (control samples, n = 6 each) and after three freeze/thaw cycles (stability samples, n = 6 each). For each freeze/thaw cycle, the samples were frozen at -20°C for 21 h, thawed and kept at ambient temperature for 3 h. The experiments were carried out together with the accuracy and precision experiments and the concentrations of the control and stability samples were calculated via daily calibration curves. Stability was tested against an acceptance interval of 90-110% for the ratio of the means (stability samples vs. control samples) and an acceptance interval of 80-120% from the control samples' mean for the 90% confidence interval (CI) of stability samples.[138]

2.5.2.7 Long-term Stability

The experimental design and procedure for evaluation of long term stability were similar to those used for freeze/thaw stability. Analyte stability for long-term storage was tested by analyzing spiked samples at two concentrations of the analytes (LOW/HIGH) before (control samples, n = 6 each) and after storage for one month at -20 °C (stability samples, n = 6 each).

2.5.2.8 Lower Limits of Quantification

The LLOQ in the MRM mode was defined as the lowest point of the calibration curve (concentration 10 ng/ml) and fulfilled the requirement of LLOQ, signal to noise ratio of 10:1. The noise data from the assay of blank matrices was taken from the selectivity experiments. Furthermore, the accuracy and precision data of the LQC sample were compared with the criteria for the parameters at the LLOQ established by Shah et al.[137] (accuracy within ≤ 20% of the nominal value and a RSD < 20%). The LOD was not systematically evaluated.

2.5.2.9 Extraction Efficiencies, Matrix Effects, and Process Efficiencies

Extraction efficiencies, matrix effect, and process efficiencies were estimated in a post-extraction addition approach as proposed in references.[139,140] Three sets of samples were prepared at low and high concentrations. Samples in set 1 consisted of neat

standards containing the analytes at concentrations of 200 ng/ml and 8000 ng/ml in eluent A, respectively. For preparation of the samples in set 2, blank plasma samples from five different sources (1 ml, each) were first extracted as described above. Then, the dry residues were reconstituted in 100 µl of eluent A containing the analytes at concentrations of 200 ng/ml and 8000 ng/ml, respectively. For preparation of the samples in set 3, blank plasma samples (1 ml) from the same sources as those in set 2 were spiked with 100 µl of eluent A containing the analytes at concentrations of 200 ng/ml and 8000 ng/ml, respectively. Thereafter, they were extracted as described above and the dry residues were reconstituted in 100 µl of eluent A.

Extraction efficiencies were estimated by comparison of the peak areas from the samples from set 3 to those from the corresponding samples of set 2 and reported in %. Matrix effects were estimated by comparison of the peak areas from the samples from set 2 to those from the corresponding samples of set 1 and reported in %. Hence, values below 100% indicate ion suppression while values above 100% indicate ion enhancement. Finally, process efficiencies (combination of extraction efficiencies and matrix effects) were estimated by comparison of the peak areas from the samples from set 3 to those from the corresponding samples of set 1 and also reported in %.

2.5.2.10 Proof of Applicability

Applicability experiments were carried out using plasma samples from healthy volunteers taken one hour after application of 10 g of an aqueous extract of *Herba Ephedra* (common dosage), 30 mg of pseudoephedrine (contained in Aspirin complex®), or 6.2 mg of ephedrine (contained in Wick MediNait®) after informed consent according to the declaration of Helsinki.

2.5.3 Toxic Alkaloids

For comparison of the two apparatus, the following validation experiments were performed using identical plasma extracts. After extraction of the plasma samples for validation, the extracts were first injected into the single stage MS, and directly after into the tandem MS.

2.5.3.1 Preparation of Stock Solutions, Calibration Standards, and Control Samples

Stock solutions of each analyte were prepared at a concentration of 1 mg/ml by separate weighings using eluent A as solvent. Working solutions of each analyte were prepared by independent dilution from each stock solution at the following concentrations: 0.001, 0.01, and 0.1 mg/ml. The calibration standards were prepared using pooled blank plasma and spiking solutions prepared from the working solutions as mixtures of the nine analytes in eluent A at concentrations ten times higher than the corresponding calibration standards. The quality control samples (concentrations as given in chapter 2.5.3.4) were prepared using pooled blank plasma and independently prepared mixtures of the nine analytes at concentrations hundred times higher than the concentrations of the corresponding quality control samples. All solutions were stored at 4°C.

2.5.3.2 Selectivity

Ten blank plasma samples from different sources were analyzed using both apparatus and checked for peaks interfering with the detection of the analytes or the IS. A zero sample (blank sample + IS) was analyzed to check for absence of analyte ions in the respective peaks of the IS.

2.5.3.3 Linearity

Aliquots of blank plasma (1 ml) were spiked with 0.1 ml of the corresponding spiking solutions and 0.1 ml of IS solution to obtain calibration standards with concentrations of 1, 50, 100, 250, 500, 750, 1000 ng/ml of cotinine, coniine, cytisine and nicotine, and concentrations of 0.1, 5, 10, 25, 50, 75, 100 ng/ml of aconitine, atropine, colchicine, scopolamine, and physostigmine. Replicates (n = 6) at each concentration level were analyzed as described above. Using single stage MS, the lowest concentration level could not be determined due to lower sensitivity of the apparatus. The regression line was calculated using a weighted $[1/(\text{concentration})^2]$ least-squares regression model. A weighted second-order model with the same weighting factors was also calculated to check for possible non-linearity. Daily linear calibration curves using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

2.5.3.4 Accuracy and Precision

QC samples were prepared at three concentration levels. The concentrations were as follows: 100 ng/ml (LOW), 500 ng/ml (MED), and 800 ng/ml (HIGH) for coniine, cotinine, cytisine, and nicotine and 10 ng/ml (LOW), 50 ng/ml (MED), and 80 ng/ml (HIGH) for aconitine, atropine, colchicine, physostigmine, and scopolamine. They were analyzed using both apparatus according to the procedure described above in duplicate on each of eight days. The concentrations of the analytes in the quality control samples were calculated via the daily calibration curves. Accuracy was calculated for each analyte in terms of bias as the percent deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Precision data (given as relative standard deviations) for within-day (repeatability), and time-different intermediate precision (combination of within and between day effects) of the method were calculated according to references[135,136] using one-way ANOVA with the grouping-variable "day". The acceptance intervals of within-day (repeatability) and intermediate precision were ≤ 15% RSD and for bias ± 15% of the nominal values.[137]

2.5.3.5 Processed Sample Stability

For estimation of stability of processed samples under the conditions of single stage MS or tandem MS analysis, LOW and HIGH quality control samples (n = 10 each) were extracted as described above. The resulting extracts at each concentration level were pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected under the conditions of a regular analytical run of the corresponding apparatus at time intervals of 2 h. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration versus injection time. Instability of processed samples would be indicated by a negative slope significantly different from zero ($p \leq 0.05$).[138]

2.5.3.6 Freeze/Thaw and Bench Top Stability

For evaluation of freeze/thaw stability, quality control samples (LOW and HIGH) were analyzed using both apparatus prior to (control samples, n = 6 each) and after three freeze/thaw cycles (stability samples, n = 6 each). For each freeze/thaw cycle, the samples were frozen at -20°C for 21 h, thawed and kept at ambient temperature for 3 h. The experiments were carried out together with the accuracy and precision experiments

and the concentrations of the control and stability samples were calculated via daily calibration curves. Stability was tested against an acceptance interval of 90-110% for the ratio of the means (stability samples vs. control samples) and an acceptance interval of 80-120% from the control samples' mean for the 90% CI of stability samples.[138]

2.5.3.7 Long-term Stability

The experimental design and procedure for evaluation of long term stability were similar to those used for freeze/thaw stability. Analyte stability for long-term storage was tested by analyzing spiked samples at two concentrations of the analytes (LOW/HIGH) before (control samples, n = 6 each) and after storage for one month at -20 °C (stability samples, n = 6 each).

2.5.3.8 Lower Limits of Quanification and Limit of Detection

The LLOQ in the SIM mode for single stage MS or the MRM mode for tandem MS was defined as the lowest point of the calibration curve (concentrations of 50 ng/ml, and 5 ng/ml respectively for single stage MS and concentrations of 1 ng/ml, and 0.1 ng/ml respectively for tandem MS) and fulfilled the requirement of LLOQ, signal to noise ratio of 10:1. The noise data from the assays of blank matrices were taken from the selectivity experiments. Plasma samples (1 ml) were spiked with decreasing concentrations of analytes and analyzed as described above. The LODs of the corresponding procedures were defined as the lowest concentrations at which identification was still possible by library search for single stage MS or $S/N \geq 3$ were obtained for all monitored ions in the MRM mode.

2.5.3.9 Extraction Efficiencies, Matrix Effects, and Process Effieciencies

Extraction efficiencies, matrix effects, and process efficiencies were estimated in a post-extraction addition approach as proposed in references.[139,140] Three sets of samples were prepared at LOW and HIGH concentrations. Samples in set 1 consisted of neat standards containing the analytes at concentrations of 1000 ng/ml and 8000 ng/ml for coniine, cotinine, cytisine, and nicotine, as well as 100 ng/ml and 800 ng/ml for aconitine, atropine, colchicine, physostigmine, and scopolamine in eluent A, respectively. For preparation of the samples in set 2, blank plasma samples from five

different sources (1 ml, each) were first extracted as described above. Then, the dry residues were reconstituted in 100 µl of eluent A containing the analytes at concentrations of 1000 ng/ml and 8000 ng/ml for coniine, cotinine, cytisine, and nicotine, as well as 100 ng/ml and 800 ng/ml for aconitine, atropine, colchicine, physostigmine, and scopolamine in eluent A, respectively. For preparation of the samples in set 3, blank plasma samples (1 ml) from the same sources as those in set 2 were spiked with 100 µl of eluent A containing the analytes at concentrations of 200 ng/ml and 8000 ng/ml, respectively. Thereafter, they were extracted as described above and the dry residues were reconstituted in 100 µl of eluent A.

Extraction efficiencies were estimated by comparison of the peak areas from the samples from set 3 to those from the corresponding samples of set 2 and reported in %. Matrix effects were estimated by comparison of the peak areas from the samples from set 2 to those from the corresponding samples of set 1 and reported in %. Hence, values below 100% indicate ion suppression while values above 100% indicate ion enhancement. Finally, process efficiencies (combination of extraction efficiencies and matrix effects) were estimated by comparison of the peak areas from the samples from set 3 to those from the corresponding samples of set 1 and also reported in %.

2.5.3.10 Proof of Applicability

Applicability experiments were carried out using plasma samples from poisoning cases sent to our laboratory for toxicological analysis. Patient one accidentally ingested leaves of *Colchicum autumnale* (Meadow saffron) containing colchicine due to a mix-up with vegetable *Allium ursinum*, known as Ramsons. Patient two abused *Datura stramonium* containing atropine and scopolamine.

3 RESULTS AND DISCUSSION

3.1 LAXATIVES

3.1.1 Sample Preparation

During preliminary studies, bisacodyl diphenol, phenolphthalein and rhein and/or their metabolites were found to be excreted in urine partly in conjugated form. Comparison of the peak areas of these analytes in authentic urine samples prepared with and without enzymatic conjugate cleavage showed that the extent of conjugation was about 50% for rhein and about 90% for bisacodyl diphenol and phenolphthalein. A further differentiation in glucuronides and sulfates was not possible from these data because a mixture of glucuronidase and arylsulfatase had been used for enzymatic hydrolysis. These results showed that cleavage of conjugates was necessary before extraction to increase sensitivity. In previously published procedures, acid hydrolysis had been used for this purpose.[62,68] The cleavage rate of both hydrolysis procedures was similar. However, it is known that certain compounds may be altered or completely destroyed during acid hydrolysis. Among these are some which can also be analyzed using extractive methylation, e.g. diclofenac,[73] buprenorphine,[141] and some diuretics.[142] Because such compounds may also be present in authentic samples and because the described procedure should also be useful for STA, more time consuming but gentle enzymatic hydrolysis was preferred.

Extractive alkylation (methylation) has proved to be suitable for analysis of different classes of more or less acidic compounds.[70-73,141,143,144] It is based on the principle of phase transfer catalysis. The analytes were extracted as ion pairs, with the phase-transfer catalyst at pH 12, into the organic phase. Reaching the organic phase the phase-transfer catalyst could easily be solvated because of its lipophilic hexyl groups. The poor solvatization of the anionic analytes leads to high reactivity against the methylation reagent methyl iodide. Part of the phase-transfer catalyst could also reach the organic phase as an ion pair with the iodide anion formed during the methylation reaction or with anions of the urine matrix. Part of these THA salts remained in the organic phase, so they had to be removed for maintaining the GC column´s separation power and to exclude interactions with analytes in the GC injection port. SPE with diol sorbent had successfully been used to remove THA salts from the organic phase,[70-73]

whereas the major part of the analytes passed through the column without being adsorbed. The part of the analytes which had also been adsorbed on the diol sorbent were selectively eluted with diethyl ether/ethyl acetate (95:5, v/v) to increase recovery, while THA salts remained adsorbed on the diol sorbent under these conditions.[73] Major advantages of the diol columns over those described by Lisi et al.[143] are their commercial availability and easy handling.[70-73]

3.1.2 GC-MS Analysis

The laxatives and/or their metabolites were separated by GC and identified by EI MS after enzymatic hydrolysis and extractive methylation. Fig. 3.1 shows the EI mass spectra, the proposed structures and the retention indices (RIs) of the methylated compounds or their metabolites. The RIs provide preliminary indications and may be useful to gas chromatographers without a GC-MS facility. They were recorded during the GC-MS procedure and calculated in correlation with the Kovats' indices[145] of the components of a standard solution of typical drugs which is measured daily for testing the GC-MS performance.[146] The reproducibility of retention indices measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats.[145]

The presence of the laxatives and/or their metabolites was screened for via characteristic fragment ions. User defined macros extracting these fragment ions from the total ion current were used to facilitate this procedure. For all analytes, the base peak and an additional qualifier ion were used for screening: *m/z* 305, 290 for bisacodyl diphenol-2ME (mass spectrum no. 1 in Fig. 3.1), *m/z* 335 and 320 for bismethylated methoxy-bisacodyl diphenol (mass spectrum no. 2 in Fig. 3.1), *m/z* 365 and 350 for bismethylated bis-methoxy-bisacodyl diphenol (mass spectrum no. 3 in Fig. 3.1), and *m/z* 271 and 346 for phenolphthalein-2ME (mass spectrum no. 4 in Fig. 3.1), *m/z* 311 and 326 for rhein-3ME (mass spectrum no. 5 in Fig. 3.1).

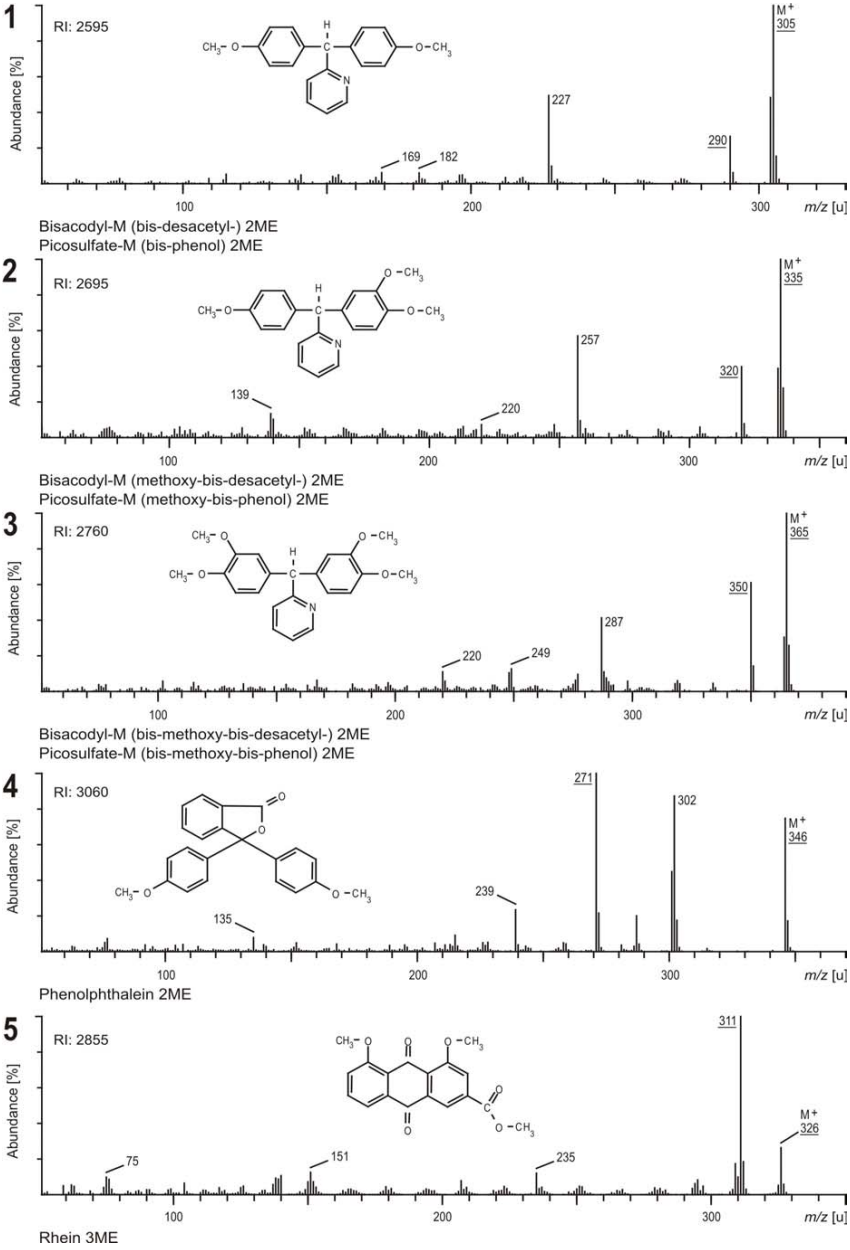


Fig. 3.1. EI mass spectra, gas chromatographic retention indices (RI), structures and predominant fragmentation patterns of stimulant laxatives and/or their metabolites after methylation. The ions used for mass chromatography are underlined.

Fig. 3.2 shows typical reconstructed mass chromatograms of an authentic urine sample after extractive methylation, which had been sent to our laboratory for toxicological analysis. Peak 1 indicates the presence of bisacodyl diphenol, peaks 2 and 3 indicate the presence of methoxy-bisacodyl diphenol and bis-methoxy-bisacodyl diphenol in the sample. For the latter two, differentiation of metabolic and extractive methylation was not possible with the described procedure.

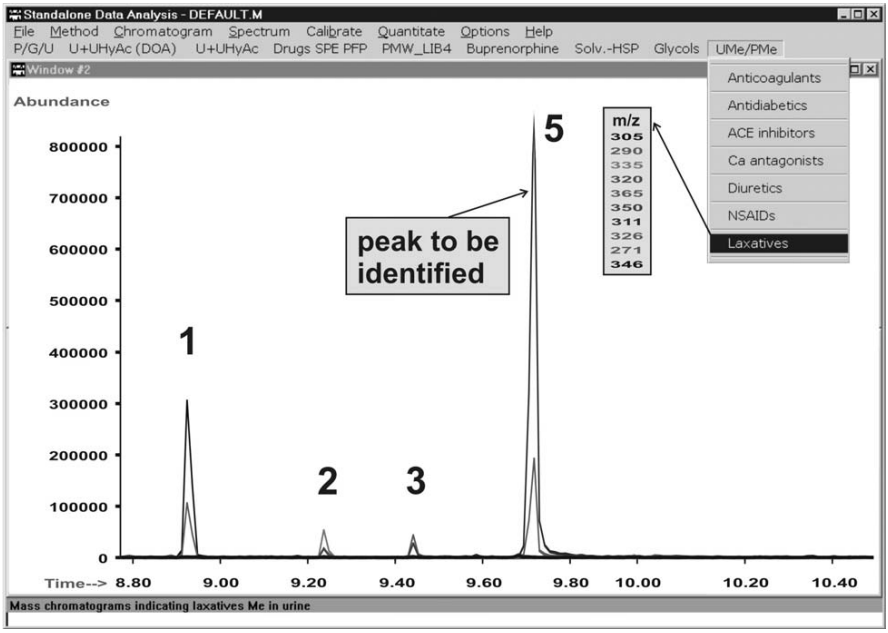


Fig. 3.2. Typical reconstructed mass chromatograms with the ions *m/z* 305, 290, 335, 320, 365, 350, 311, 326, 271, and 346 of a urine extract after extractive methylation of a patient who has taken an unknown amount of bisacodyl or picosulfate and an anthraquinone containing plant extract.

However, analysis of the same urine sample according to ref.[68] showed that these two metabolites were indeed excreted in urine as conjugates of methoxy-bisacodyl diphenol and bis-methoxy-bisacodyl diphenol. Peak 5 indicates the presence of rhein.

As illustrated in Fig. 3.3, the identity of positive peaks in corresponding mass chromatograms was confirmed by visual and computerized comparison of the underlying spectrum (here of peak 5 in Fig. 3.2) with reference spectra, which were already contained in the used reference library or had been recorded during the presented study. In order to avoid misinterpretation of the mass spectral analysis, proper use of the Pfleger/Maurer/Weber library[147] is indispensable. The "@" sign

indicates that the compound can also be found after intake of other compounds given in the corresponding handbooks.[148,149] This is of special importance for the presented study, because bisacodyl diphenol, methoxy-bisacodyl diphenol, and bismethoxy-bisacodyl diphenol are common metabolites of bisacodyl and picosulfate, whereas rhein is a common metabolite of a series of active compounds in anthraquinone containing plant extracts. Therefore, detection of one or more of the common metabolites of bisacodyl and picosulfate does not allow to differentiate which of these two drugs have been ingested by the patient. The same is true for rhein detection and ingestion of anthraquinone-type laxatives. However, such a differentiation is of little relevance from a clinical point of view.

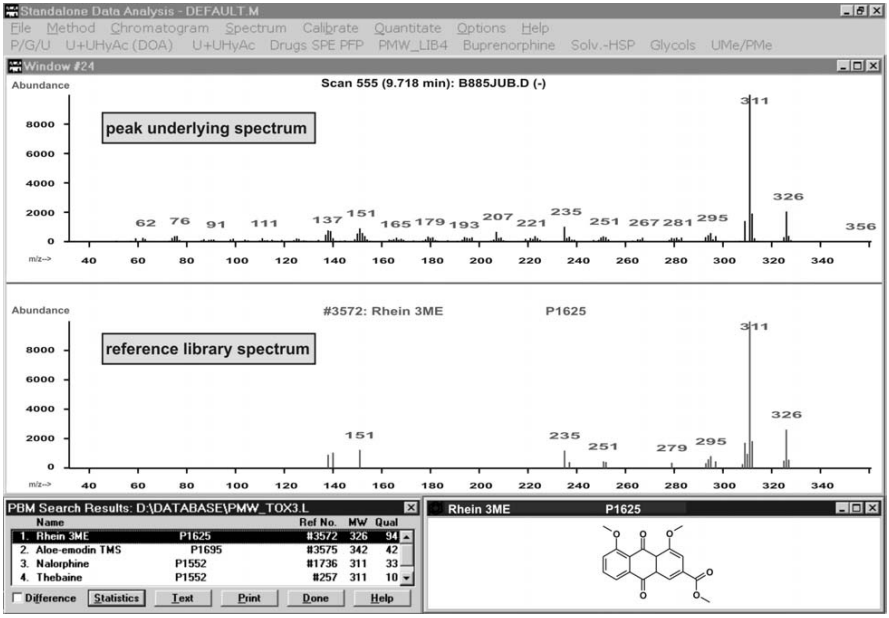


Fig. 3.3. Mass spectrum underlying peak 5 in Figure 3.2, the reference spectrum, the structure, and the hit list found by computer library search.

Interference by biomolecules or further drugs indicated in the reconstructed mass chromatograms could be excluded, because these compounds have different gas chromatographic and/or mass spectrometric properties. The corresponding RIs and reference spectra are included in the used reference library.

3.1.3 Assay Validation

Recoveries, analyte stability, and LODs were only determined for the analytes with the longest duration of detectability after ingestion of the above-mentioned laxatives. These are bisacodyl diphenol (main metabolite of bisacodyl and picosulfate), rhein (main metabolite of various active compounds in anthraquinone containing plant extracts) and phenolphthalein.[62,68] Recovery experiments were carried out using SIM mode to allow more precise determination of peak areas. LOD experiments were carried out using full-scan mode to allow estimation of LODs under routine conditions. The results for both parameters are given in Table 3.1. All three analytes showed reproducible recoveries as indicated by the low standard deviations. The lower recovery of rhein in comparison to the two other analytes could in part be explained by the instability of this analyte in buffer solution, even at room temperature.

Table 3.1. Overall Recoveries with Coefficient of Variation and LOD (*S/N*= 3), determined in the SIM Mode

Target Analyte	Recovery	LOD
Bisacodyl diphenol	62.2 ± 7.35 %	10 ng/ml
Phenolphthalein	89.5 ± 14.64 %	10 ng/ml
Rhein	33.0 ± 4.22 %	25 ng/ml

Fig. 3.4. shows the extent of degradation of the analytes in a urine sample incubated with buffer solution at room temperature. For all three analytes a significant decrease of peak areas could be observed for aliquots taken and worked up after increasing intervals of time. Degradation of rhein was especially pronounced and followed a mathematical model of monoexponential decay. These results show that samples have to be worked up immediately after addition of buffer solution to prevent considerable losses of analytes. At the elevated temperature of extractive methylation (50 °C) degradation can be expected to be even faster. Therefore, one might consider a reduction of shaking time during sample preparation. However, since recoveries were reproducible and LODs were satisfactory even for rhein, there was no reason to change the described procedure which is also routinely used for screening analysis of acidic

compounds in our laboratory.[70-73] Finally, with the described procedure, rhein was still detectable in a urine sample of a healthy young volunteer taken 24 h after ingestion of the lowest therapeutic dose of senna extract. bisacodyl diphenol was still detectable in a urine sample of a volunteer collected 52 h after ingestion of the lowest therapeutic dose of sodium picosulfate.

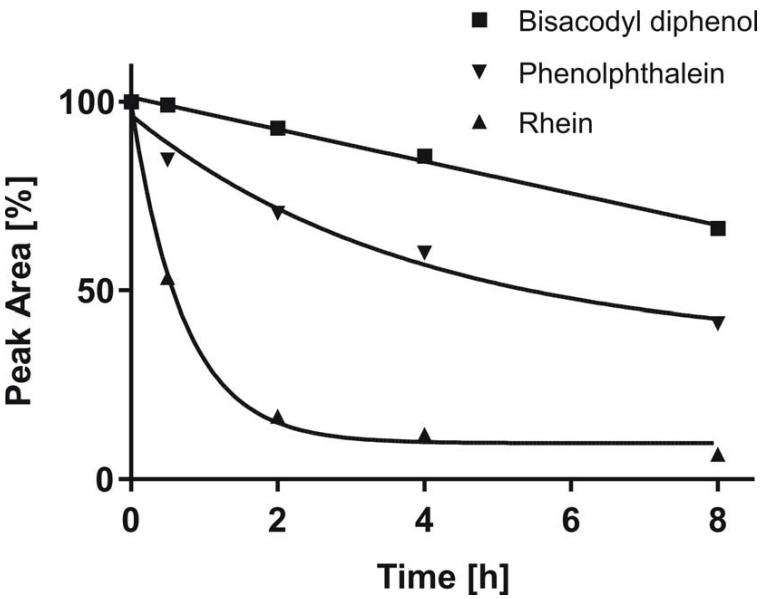


Fig. 3.4. Degradation of the analytes, expressed as peak areas in relation to the initial values, over time in a urine sample incubated with buffer solution at room temperature.

3.2 INGREDIENTS OF NUTMEG

3.2.1 Identification of Metabolites of Elemicin, Myristicin and Safrole in Rat Urine

The urinary metabolites of the nutmeg ingredients were separated by GC and identified by EI MS after enzymatic hydrolysis, extraction and acetylation. Fig. 3.5 A-C shows reconstructed mass chromatograms of acetylated rat urine extracts after administration of 100 mg/kg BM each of safrole (A), myristicin (B), or elemicin (C). The peak numbers correspond to the numbering in Fig. 3.6. The mass spectra underlying the numbered peaks were interpreted in correlation to those of the parent compound according to the rules described by e.g. McLafferty and Turecek[131] and Smith and Busch.[132] The corresponding EI mass spectra, the gas chromatographic RI, the concluded structures and postulated predominant fragmentation patterns of safrole, myristicin, elemicin, and their acetylated metabolites are shown in Fig. 3.6. After administration of safrole (mass spectrum no. 1), the following acetylated metabolites could be identified (the mass spectra numbers in Fig. 3.6 are given in brackets): 1-(3'-methoxy-4'-hydroxyphenyl)-prop-2-ene (2), 1-(3',4'-methylenedioxy-5'-hydroxyphenyl)-prop-2-ene (3), 1-(3',4'-dihydroxyphenyl)-prop-2-ene (4), 1-hydroxy-1-(3',4'-methylenedioxyphenyl)-prop-2-ene (5), 1-(3',4'-dihydroxy-5'-methoxyphenyl)-prop-2-ene (6), and 2,3-dihydroxy-1-(3',4'-methylenedioxyphenyl)-propane (7). After administration of myristicin (8), the following metabolites could be identified: 1-(3',4'-methylenedioxy-5'-hydroxyphenyl)-prop-2-ene (3), 1-(3',4'-dihydroxy-5'-methoxyphenyl)-prop-2-ene (6), 1-(3',5'-dimethoxy-4'-hydroxyphenyl)-prop-2-ene (9), 1-hydroxy-1-(3',4'-methylenedioxy-5'-methoxy-phenyl)-prop-2-ene (10), and 2,3-dihydroxy-1-(3',4'-methylenedioxy-5'-methoxyphenyl)-propane (11). After administration of elemicin (12), the following metabolites could be identified: 1-(3',4'-dihydroxy-5'-methoxyphenyl)-prop-2-ene (6), 1-(3',5'-dimethoxy-4'-hydroxyphenyl)-prop-2-ene (9), 1-(3',4'-dimethoxy-5'-hydroxyphenyl)-prop-2-ene (13), 1-hydroxy-1-(3',4',5'-trimethoxyphenyl)-prop-2-ene (14), 2,3-dihydroxy-1-(3',4',5'-trimethoxyphenyl)-propane (15), and two isomers of 2,3-dihydroxy-1-(dimethoxy-hydroxyphenyl)-propane (16 and 17). In none of the urine extracts, the corresponding amphetamine derivatives MMDA, TMA, or 3,4-methylenedioxyamphetamine (MDA), could be detected. No further metabolites of safrole, myristicin or elemicin could be detected in the acidic extracts after methylation and acetylation.

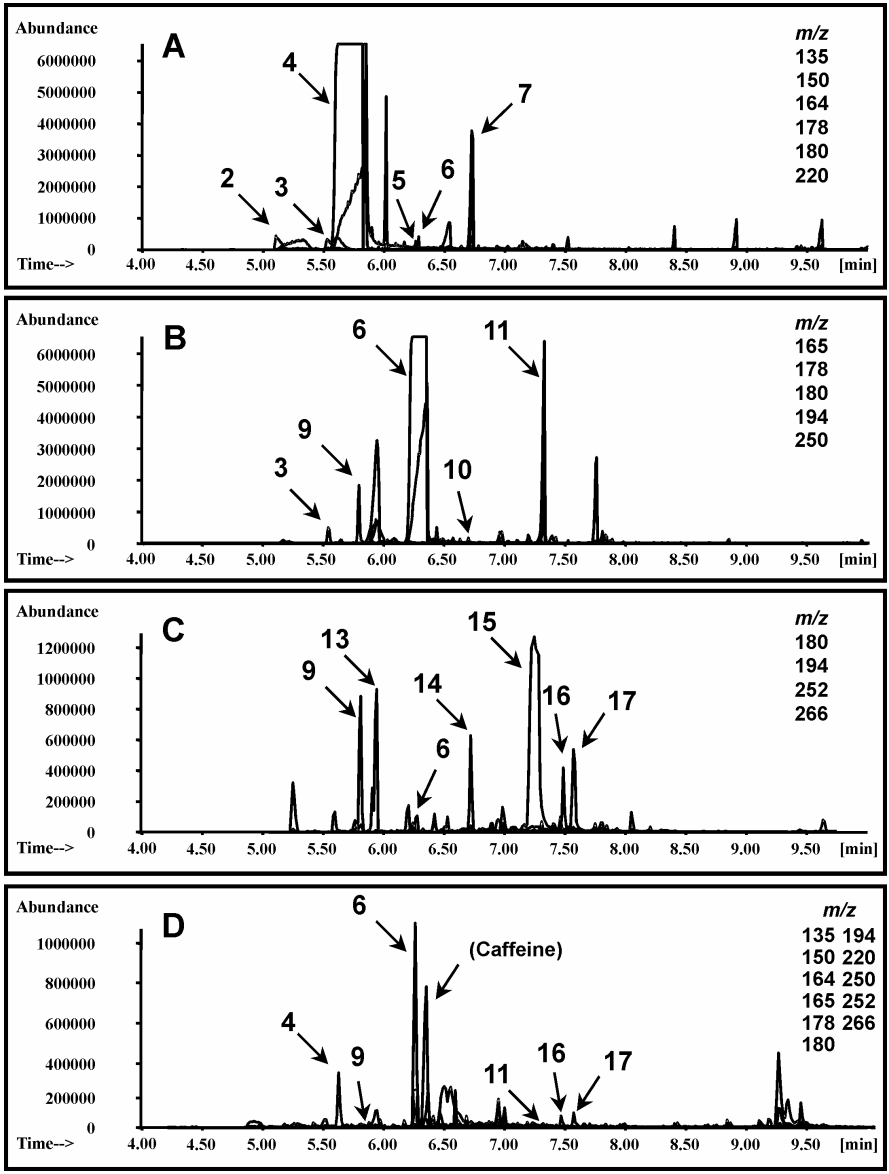
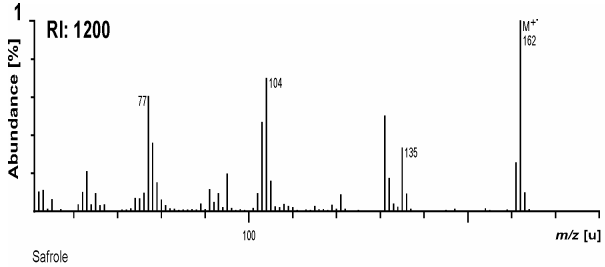
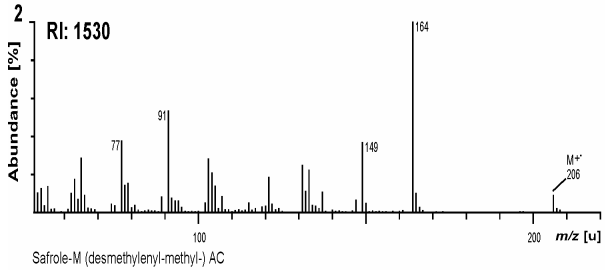


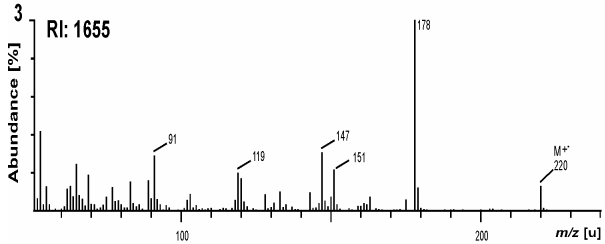
Fig. 3.5. Typical reconstructed mass chromatograms with the given ions of acetylated extracts of enzymatically hydrolyzed rat urine samples after administration of 100 mg/kg BM of safrole (A), myristicin (B), and elemicin (C). Part D: reconstructed mass chromatograms of an acetylated human urine extract after administration of an unknown dose of nutmeg. The peak numbers correspond to those used in Fig. 3.6, Fig. 3.7, and Fig. 3.8. The merged chromatograms can be differentiated by their colors on a color screen.



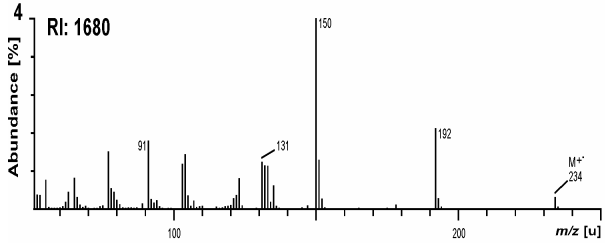
Safrole



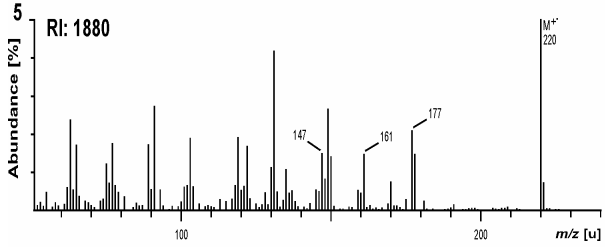
Safrole-M (desmethylenyl-methyl-) AC



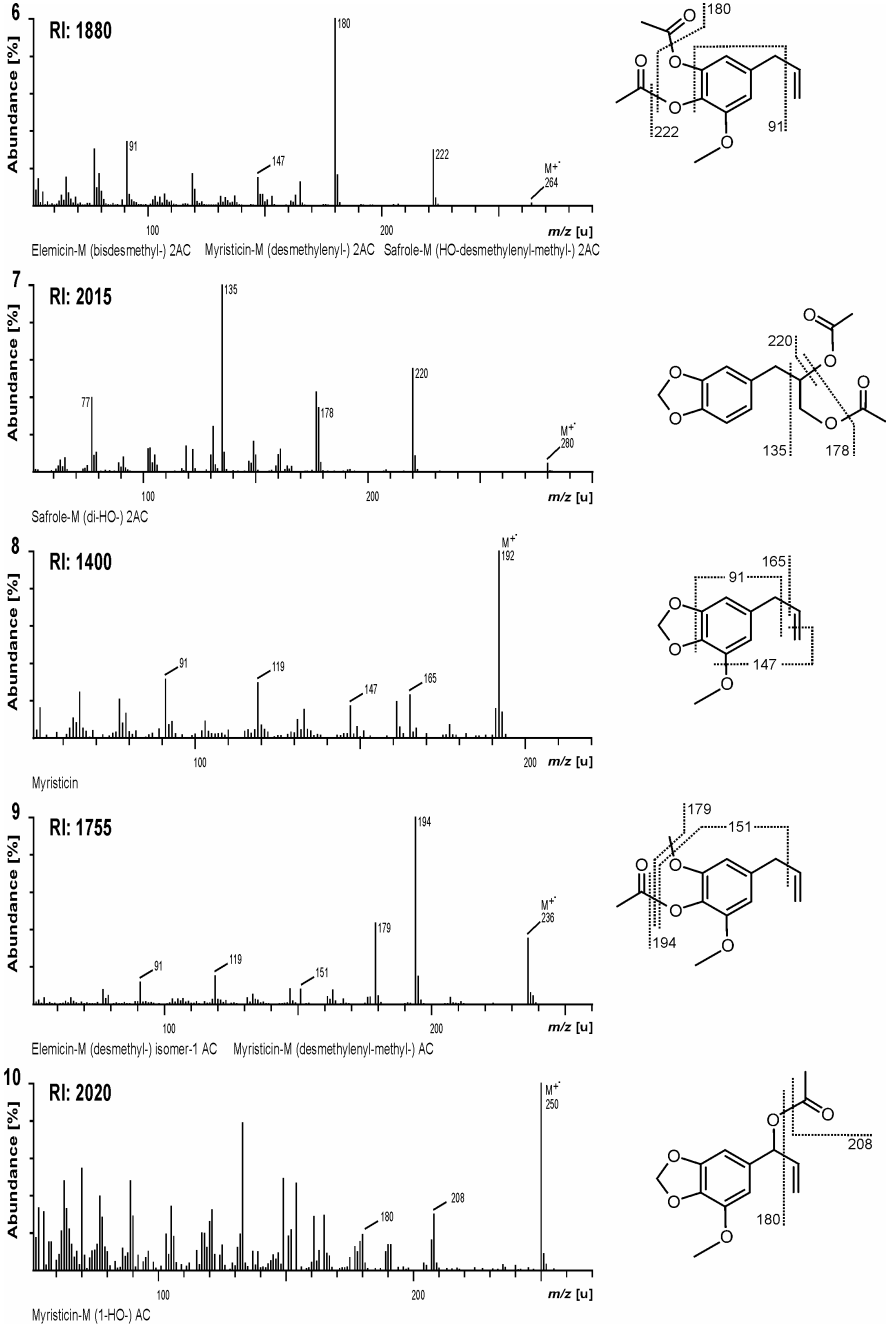
Myristicin-M (desmethyl-) AC Safrole-M (HO-) AC

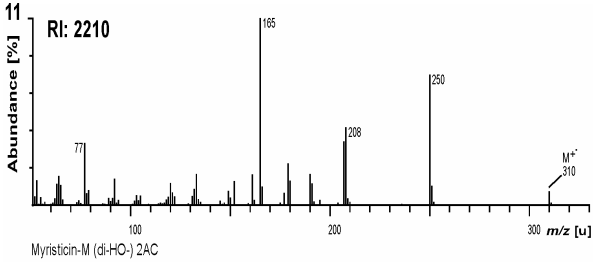


Safrole-M (desmethylenyl-) 2AC

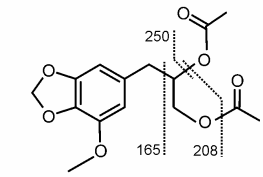


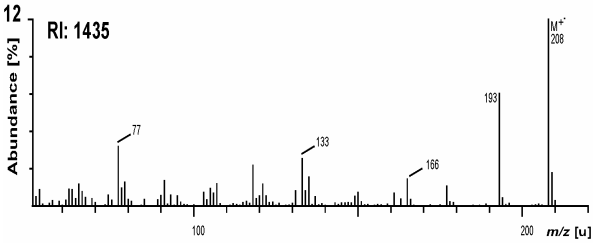
Safrole-M (1-HO-) AC


6
RI: 1880
Abundance [%]
180
91
147
222
M+·
264
100
200
m/z [u]
Elemicin-M (bisdesmethyl-) 2AC
Myristicin-M (desmethylenyl-) 2AC
Safrole-M (HO-desmethylenyl-methyl-) 2AC
180
222
91
7
RI: 2015
Abundance [%]
135
77
178
220
M+·
280
100
200
m/z [u]
Safrole-M (di-HO-) 2AC
220
135
178
8
RI: 1400
Abundance [%]
M+·
192
91
119
147
165
100
200
m/z [u]
Myristicin
165
91
147
9
RI: 1755
Abundance [%]
194
179
M+·
236
91
119
151
100
200
m/z [u]
Elemicin-M (desmethyl-) isomer-1 AC
Myristicin-M (desmethylenyl-methyl-) AC
179
151
194
10
RI: 2020
Abundance [%]
M+·
250
208
180
100
200
m/z [u]
Myristicin-M (1-HO-) AC
208
180

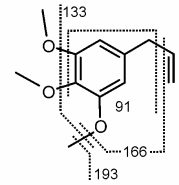


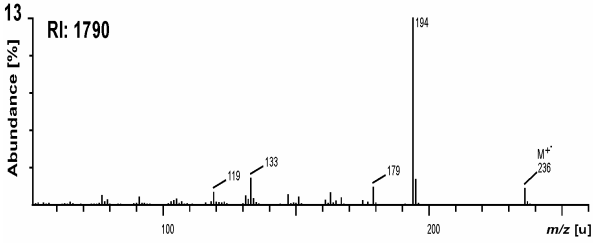
Myristicin-M (di-HO-) 2AC



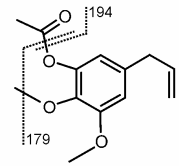


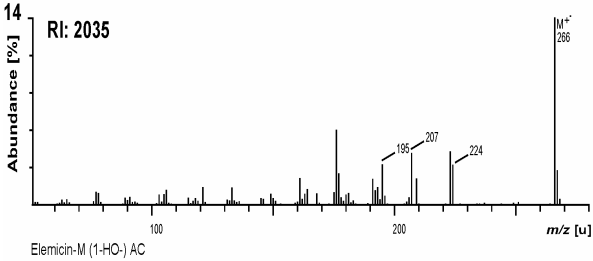
Elemicin



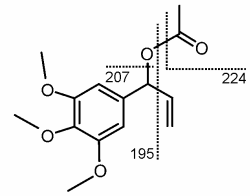


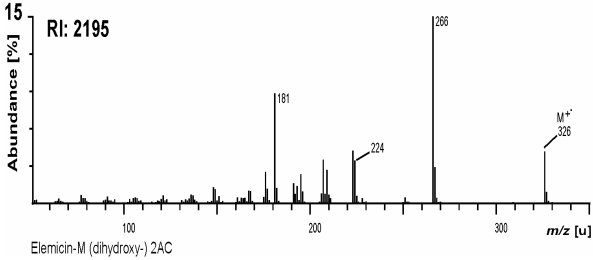
Elemicin-M (desmethyl-) isomer-2 AC



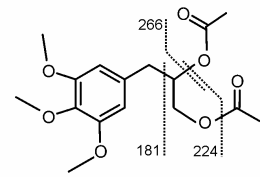


Elemicin-M (1-HO-) AC





Elemicin-M (dihydroxy-) 2AC



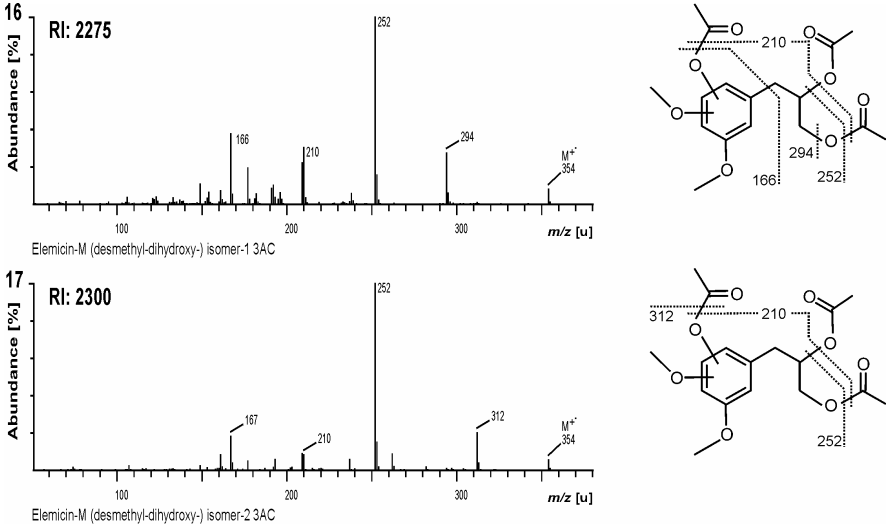


Fig. 3.6. EI mass spectra, the gas chromatographic retention indices (RI), structures and predominant fragmentation patterns of safrole, myristicin, elemicin, and their metabolites after acetylation. The numbers of the spectra correspond to those in Fig. 3.5, Fig. 3.7, and Fig. 3.8.

The position of the hydroxy or methoxy group in metabolites 2 and 9 could not be determined by means of GC-MS, but the given positions were assumed from the metabolic point of view since the methylation step is most probably catalyzed by the enzyme catechol-*O*-methyl transferase (COMT) which predominantly methylates in 3' position. The position of the free hydroxy group in metabolite 13 was assumed because this metabolite had a retention time different form the isomeric metabolite 9, and because metabolite 13 was only found in the urine after administration of elemicin. In the two isomeric metabolites 16 and 17, the position of the ring hydroxy group could not further be elucidated because they were detected only after administration of elemicin.

3.2.2 Identification of Metabolites of Elemicin, Myristicin and Safrole in Rat Urine

Fig. 3.5 (D) shows reconstructed mass chromatograms of an acetylated human urine extract after administration of an unknown dose of nutmeg. The peak numbers correspond to the numbering in Fig. 3.6. Unnumbered peaks represent endogenous biomolecules. As can be seen, metabolites 4, 6, 9, 11, 16, and 17 could be identified also in human urine. While metabolite 4 could only be formed from safrole, metabolite 11 only by myristicin, and metabolites 16 and 17 only by elemicin, metabolite 9 could be

formed from myristicin and elemicin and metabolite 6 from all studied nutmeg ingredients.

3.2.3 Postulated Metabolic Pathways of Elemicin, Myristicin and Safrole in Rats and Humans

Based on the metabolites identified as described above, the following metabolic pathways of safrole, myristicin and elemicin could be postulated as shown in Fig. 3.7 (metabolites found in human urine are marked by "^{h}"): for safrole, myristicin and elemicin, hydroxylation of the side chain to the corresponding 1-hydroxy metabolites 5, 10, and 14, bis-hydroxylation of the side chain to the corresponding 2,3-dihydroxy-metabolites 7, 11, and 15; for safrole and myristicin, demethylenation to the metabolites 4 and 6 followed by methylation to the metabolites 2 and 9; for elemicin, *O*-demethylation at position 3' and 4' to metabolites 9 and 13 followed by side chain hydroxylation to the two isomers 16 and 17. Comparing the peak areas in Fig. 3.5 (A-C), demethylenation seems to be the main metabolic step for safrole and myristicin and side chain hydroxylation for elemicin. All metabolites were partly excreted as glucuronides and/or sulfates, since the peak areas were greater after enzymatic hydrolysis.

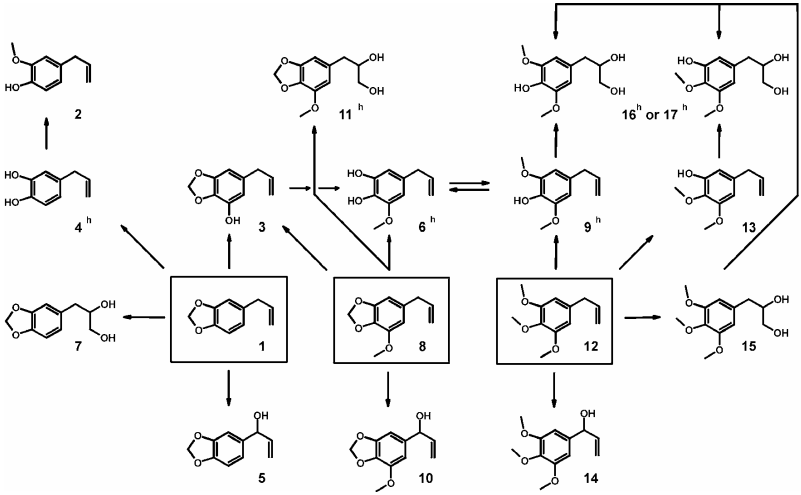


Fig. 3.7. Proposed scheme for the metabolism of safrole (1), myristicin (8), and elemicin (12) in rat and humans. The metabolites marked with ^{h} were also detected in a human urine sample after nutmeg abuse. The numbers of compounds correspond to those in Fig. 3.5, Fig. 3.6, and Fig 3.8.

3.2.4 Monitoring of a Nutmeg Abuse or Intoxication using Systematic Toxicological Analysis

The STA procedure is based on acid hydrolysis for very efficient and fast cleavage of conjugates.[150] However, some compounds were altered or destroyed during hydrolysis.[151,152] Therefore, one part of unhydrolyzed urine was added to the hydrolyzed aliquot before extraction. This modified sample preparation was a compromise between the necessity of a quick cleavage of conjugates and the detectability of compounds destroyed during acid hydrolysis. Although the modification of the STA procedure led to lower extract concentrations of compounds excreted in conjugated form, this modified procedure was sufficient, because of the high sensitivity of modern GC-MS apparatus.[151,152]

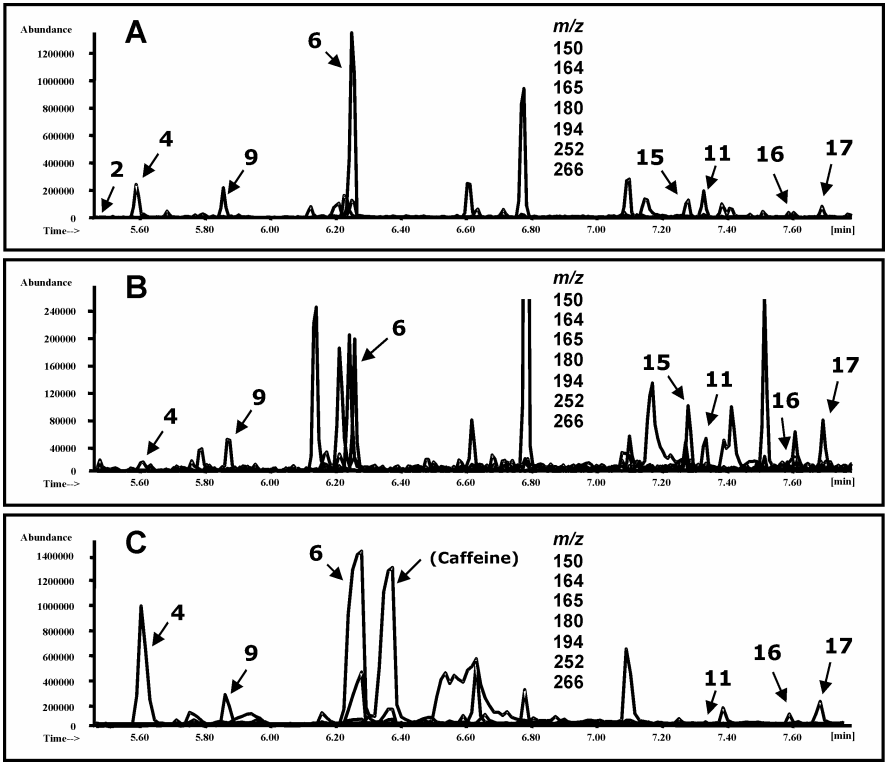


Fig. 3.8. Typical reconstructed mass chromatograms with the given ions of acidic hydrolyzed and acetylated extracts of rat urine sample after administration of 500 mg/kg BM of nutmeg (A and B), and a human urine sample after nutmeg abuse (C). The peak numbers correspond to those used in Fig. 3.5-3.7.

The metabolites of safrole, myristicin, and elemicin were separated by GC and identified by EI MS after acid hydrolysis, extraction and acetylation within the STA.[79-84] Mass chromatography with the following ions *m/z* 150, 165, 180, 194, 252, and 266 was used to indicate the presence of the metabolites of the main ingredients of the volative oil of nutmeg. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros. Fig. 3.8 (A and B) shows typical reconstructed mass chromatograms with the above-mentioned ions of an acetylated rat urine extract after application of nutmeg (500 mg/kg each) from different geographical sources. In part A, the peak area of metabolite 6, the main metabolite of myristicin, but also formed by safrole and elemicin, was about ten times higher than that of the others, while in part B, it was only about twice as high. This can be explained by the fact, that the content of myristicin may vary from source to source.[22] Part C shows typical reconstructed mass chromatograms with the above-mentioned ions of an acetylated human urine extract after suspected nutmeg abuse. The peak numbers correspond to the numbering in Fig. 3.5, Fig. 3.6, and Fig. 3.7. Unnumbered peaks represent endogenous biomolecules. The identity of peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra recorded during this study.[134] The ions *m/z* 150 and 164 were used for indication of the presence of the safrole metabolites 2 and 4, the ions *m/z* 165, 180 and 194 for the metabolites of myristicin 6, 9 and 11, and the ions *m/z* 252 and 266 for the elemicin metabolites 15-17. In our experience, the RIs provide preliminary indications and may be useful to gas chromatographers without a GC-MS facility. In addition, they allow distinguishing between the different isomers.

Therefore, they are also given in Fig. 3.6. The RIs were recorded during the GC-MS procedure and calculated in correlation with the Kovats' indices[145] of the components of a standard solution of typical drugs which is measured daily for testing the GC-MS performance.[146] The reproducibility of RIs measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats. Although interferences by biomolecules or further drugs cannot be entirely excluded, they are rather unlikely, because their mass spectra and/or their RIs should be different and included in the reference library[134] used.

Screening for safrole, myristicin, and elemicin themselves was not successful, because the substances could neither be detected in urine after administration of nutmeg nor after high doses of the three substances themselves. The STA procedure allowed

identification of the main metabolites of the nutmeg ingredients safrole, myristicin, and elemicin in rat and human urine after administration of a commonly abused dose thus allowing to monitor a nutmeg abuse and/or intoxication. These findings have been confirmed by Staack and Paul from the Institute of Forensic Toxicology, LMU Munich, Germany (personal communication, to be published elsewhere), who could also detect the described metabolites in urine of a nutmeg abuser using our STA procedure and the reference spectra shown in Fig3.6. The limit of detection could not be determined for these metabolites, because reference substances were not available. As already mentioned above, the postulated amphetamine derivatives MMDA, TMA, and MDA, could not be detected neither in rat, nor in human urine, although, as already mentioned above, amphetamine-derived designer drugs could be detected by STA with a detection limit of 5-50 ng/ml.[85,86]

3.3 Herbal Phenalkylamines

An LC-ESI-MS/MS assay was developed for selective detection as well as for accurate and precise quantification of nine herbal phenalkylamines and methcathinone in human plasma. It allows monitoring of an abuse or intoxication as well as differentiation of an intake of cold medications from herbal drug or methcathinone abuse in contrast to all procedures cited in the Introduction. In addition, the LLOQs were ten times lower than those of the other multi-analyte plasma procedures.[26,94] The assay was validated according international guidelines.[137,140,153,154]

3.3.1 Extraction Procedure

In early development stages of the presented assay, it was intended to extract the ten analytes by our standard plasma mixed-mode SPE procedure,[96,136,155,156] in which the analytes were isolated from 1 ml of plasma after dilution with 2 ml of water. This procedure had been reported to be versatile for the extraction of designer drugs, neuroleptics, and beta-blockers from plasma.[96,136,155,156] However, during method development, it was recognized that the pH of spiked control samples increased by up to 0.75 after freezing and thawing. This caused a decrease in the extraction efficiency of all analytes but mescaline. At higher pH values, the relatively polar ephedrines are not retained by the reversed-phase part of the SPE sorbent and lacking protonation neither

by the ion exchange part. While mescaline also lacks protonation, it was retained well by the reversed-phase sorbent most probably due to its higher lipophilicity. Therefore, the plasma samples were diluted with acidic buffer instead of water. As shown in Table 3.2, the extraction efficiency values ranged from 45 to 93 %. In comparison of previously studied compounds, the extraction efficiencies are relatively low, but due to their reproducibility and the sufficient sensitivity of the LC-MS/MS assay, they were considered acceptable.

Table 3.2. Extraction efficiency, matrix effect, and process efficiency of the LC-MS/MS assay for the studied analytes determined at concentrations of 20 ng/ml (LOW) and 800 ng/ml (HIGH)

Analyte	Extraction efficiency (mean ± SD, %)		Matrix effect (mean ± SD, %)		Process efficiency (mean ± SD, %)	
	LOW (n = 5)	HIGH (n = 5)	LOW (n = 5)	HIGH (n = 5)	LOW (n = 5)	HIGH (n = 5)
Cathinone	92 ± 6.4	85 ± 7.9	93 ± 6.7	96 ± 4.5	85 ± 5.9	82 ± 7.6
Ephedrine	85 ± 4.6	82 ± 5.4	87 ± 4.4	91 ± 3.5	74 ± 4.0	75 ± 5.0
Methcathinone	84 ± 7.2	75 ± 8.5	102 ± 4.3	96 ± 4.4	85 ± 7.4	72 ± 8.2
Methylephedrine	93 ± 5.9	80 ± 8.7	95 ± 4.9	96 ± 2.0	88 ± 5.6	76 ± 8.3
Mescaline	84 ± 5.7	72 ± 9.6	103 ± 3.7	96 ± 4.0	86 ± 5.9	69 ± 9.2
Methylpseudo-ephedrine	92 ± 5.8	72 ± 4.3	93 ± 7.0	100 ± 4.0	86 ± 5.4	72 ± 4.1
Norephedrine	81 ± 9.1	81 ± 6.6	73 ± 3.4	88 ± 5.4	59 ± 6.7	72 ± 5.8
Norpseudo-ephedrine	64 ± 10.3	78 ± 6.8	82 ± 13.3	73 ± 4.8	53 ± 8.5	57 ± 5.0
Pseudoephedrine	80 ± 6.5	71 ± 6.7	93 ± 5.9	96 ± 5.7	75 ± 6.0	68 ± 6.5
Synephrine	45 ± 5.0	55 ± 4.0	92 ± 9.5	94 ± 9.0	41 ± 4.6	52 ± 3.8

3.3.2 Detection and Quantification

The ESI mode was preferred over the APCI mode, because it was found to be more sensitive in preliminary experiments and because no relevant matrix effects were observed as shown in Table 3.2. The highest matrix effect was observed for norephedrine, but this was sufficiently compensated by its deuterated IS, because accuracy and precision were acceptable.

The presence of the drugs was successfully detected in the MRM mode by mass fragmentography using three MRM transitions for each substance. Transitions were selected and their settings were determined using a 1000 ng/ml solution of each analyte in eluent A injected by the integrated syringe pump and using Analyst® Software in Quantitative Optimization mode. The three resulting transitions per analyte and respective settings are given in Table 2.1. Cross-talk was not observed. As shown in Fig. 3.9, not only the parent and product ion masses are important for selective and sensitive detection and quantification, but also the analytes-specific instrument settings. Fig. 3.9 shows chromatograms of MRM transition 150 -> 77 with parameter settings optimized for synephrine (upper part) and for cathinone (lower part). Both 150 -> 77 transitions have nearly the same collision cell energy, but differ in their declustering and entrance potentials. Being located before the collision cell, both potentials are analyte-specific and not fragment-specific. Thus, choosing analyte-specific settings for the MRM transitions was critical for the sensitivity of the assay. Having quite similar mass spectrometric properties, sufficient chromatographic separation of the diastereomer pairs ephedrine/pseudoephedrine, methylephedrine/methylpseudoephedrine, and norephedrine/norpseudoephedrine was necessary for their differentiation. This could be achieved in less than 10 min using an SCX separation column and the described chromatographic conditions.

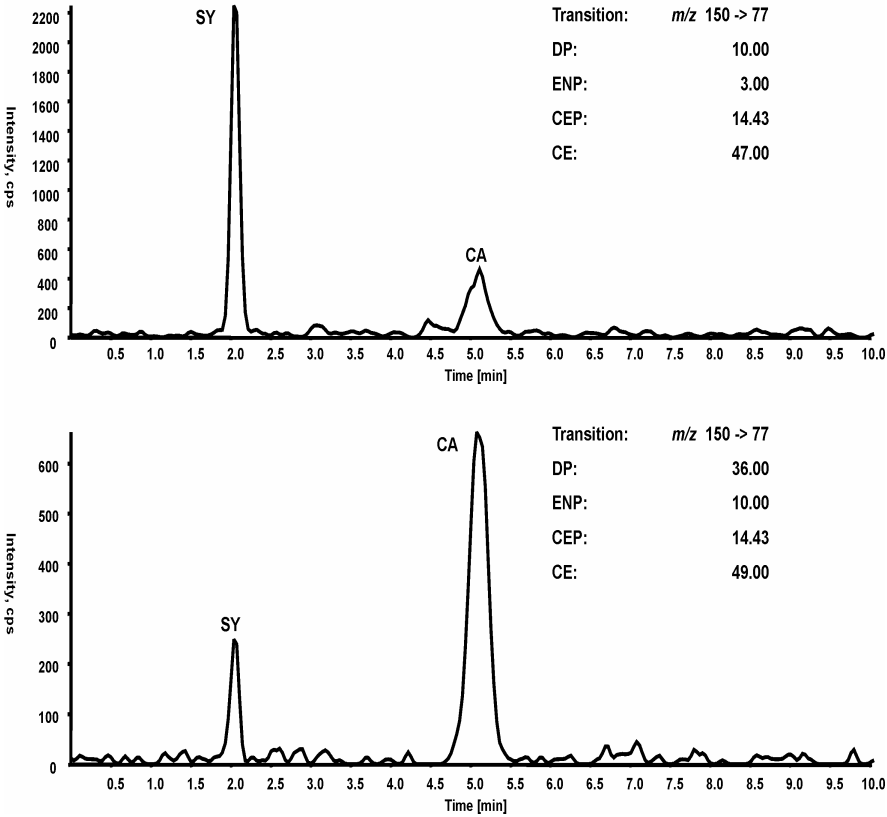


Fig. 3.9. Chromatograms of MRM transition 150 -> 77 with parameter settings optimized for synephrine (SY, upper part) and for cathinone (CA, lower part) using the given declustering potential (DP), entrance potential (ENP), collision cell entrance potential (CEP), and collision energy (CE).

For illustration of the detection and identification procedure, smoothed, normalized, and merged MRM chromatograms of all target transitions of a LQC sample after SPE are shown Fig. 3.10. The peaks were sufficiently separated even in the highest calibrator.

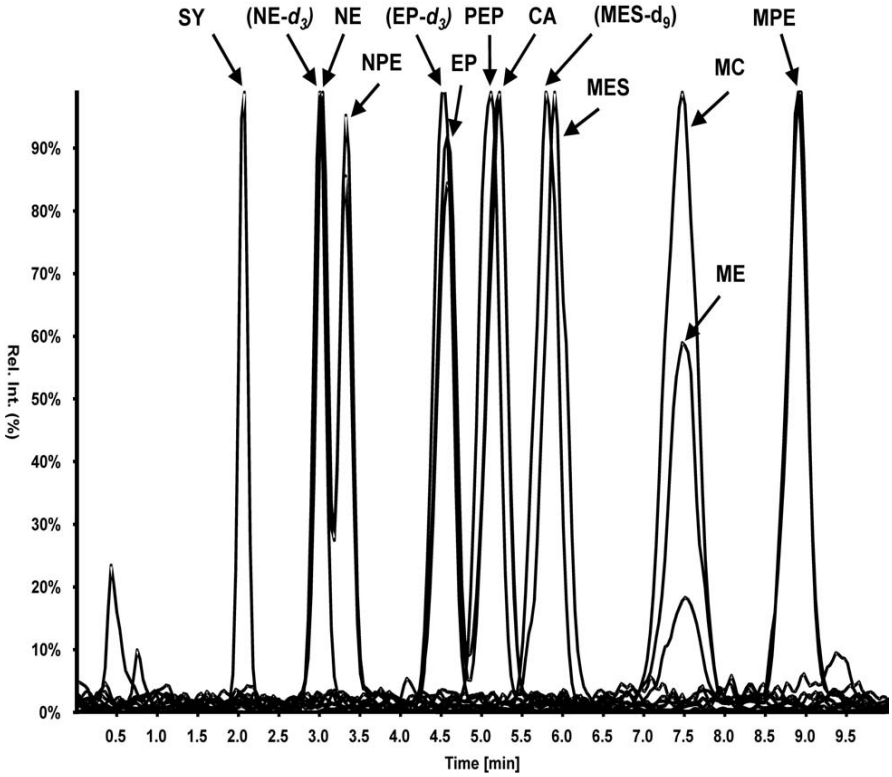


Fig. 3.10. Smoothed, normalized, and merged MRM chromatograms of all target transitions of an extract of a LQC sample containing 10 ng/ml synephrine (SY, oxedrine), norephedrine (NE), norpseudoephedrine (NPE, cathine), ephedrine (EP), pseudoephedrine (PEP), cathinone (CA), mescaline (MES), methcathinone (MC), methylephedrine (ME), methylpseudoephedrine (MPE), norephedrine-d_3 (NE-d_3), ephedrine-d_3 (EP-d_3), and mescaline-d_9 (MES-d_9).

3.3.3 Assay Validation

The described procedure was validated according to internationally accepted recommendations.[137,140,153,154] The validation data are summarized in Tables 3.2 and 3.3. As exemplified in Fig. 3.11 (left), no interfering peaks were observed in the extracts of the different blank plasma samples. Interferences with common drugs of abuse which might be expected to be taken in combination with the analytes were tested and could be excluded.

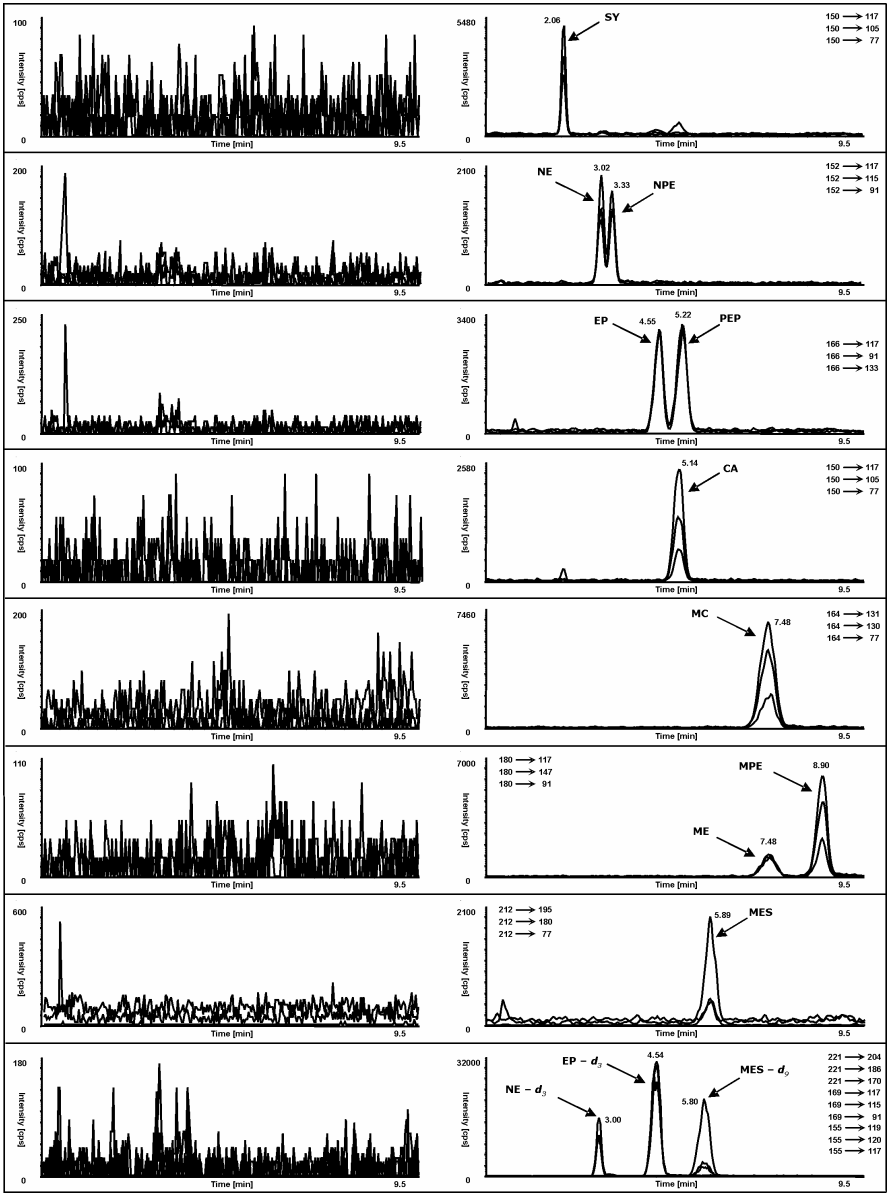


Fig. 3.11. Smoothed, normalized, and merged MRM chromatograms of the given transitions of extracts of a blank plasma (left) and a LQC sample containing 10 ng/ml synephrine (SY, oxedrine), norephedrine (NE), norpseudoephedrine (NPE, cathine), ephedrine (EP), pseudoephedrine (PEP), cathinone (CA), mescaline (MES), methcathinone (MC), methylephedrine (ME), methylpseudoephedrine (MPE), norephedrine-d_3 (NE-d_3), ephedrine-d_3 (EP-d_3), and mescaline-d_9 (MES-d_9). (right).

The assay was found to be selective for all tested compounds. A weighted second-order model was also evaluated to check for a curvature in the data. For all analytes, a linear weighted ($1/c^2$) least squares model was found to be the best and therefore used for calculation of calibration curves. It was linear from 10 to 1000 ng/ml of each compound. The coefficients of determination (R^2) ranged from 0.9948 to 0.9996.

The LLOQs corresponded to the lowest concentrations used for the calibration curves with a signal-to-noise ratio of at least 10. The validation data concerning extraction efficiency, matrix effects, and process efficiency are shown in Table 3.2 and those concerning accuracy and precision in Table 3.3. Accuracy data were determined and all lay within the acceptance interval of ±15% (±20% at the LLOQ) of the nominal values at all concentrations. Within-day (repeatability) and intermediate precision were also determined and lay within the required limits of 15% RSD (20% RSD at LLOQ), at all studied concentration levels.

In extracts, the analytes were stable at low and high concentrations for a period of more than 24 h at 15° C. In the freeze/thaw and long-term stability experiments, the ratio of means (stability vs. control samples) were within 90-110%, whereas the 90% CIs for stability samples were within 80-120% of the respective control means, such fulfilling the acceptance criteria for all analytes at both concentrations.

Table 3.3. Accuracy, intermediate precision and repeatability data of the LC-MS/MS assay for the studied analytes. IS used for quantification are given in brackets. Accuracy: ((mean calculated concentration - nominal concentration)/nominal concentration) x 100. Quality control samples prepared at 10 ng/ml (LQC), 20 ng/ml (LOW), 500 ng/ml (MED), 800 ng/ml (HIGH), and 4000 ng/ml (ACR).

Analyte (IS)	Accuracy					Intermediate Precision					Repeatability				
	LQC	LOW	MED	HIGH	ACR	LQC	LOW	MED	HIGH	ACR	LQC	LOW	MED	HIGH	ACR
Cathinone (Ephedrine-d_3)	5.5	14.8	9.8	4.8	3.3	12.3	10.7	11.1	10.1	13.7	2.9	10.7	6.4	4.8	4.6
Ephedrine (Ephedrine-d_3)	17.0	13.3	6.0	4.6	10.6	11.6	7.3	6.2	7.0	8.1	7.3	7.3	5.0	2.7	3.1
Methcathinone (Ephedrine-d_3)	11.8	15.0	13.6	11.4	11.4	18.0	11.0	8.4	9.3	9.3	8.7	10.6	7.2	3.6	3.6
Methylephedrine (Ephedrine-d_3)	17.2	6.3	10.0	12.6	4.0	15.9	11.4	11.6	7.8	14.4	11.9	6.3	5.8	2.5	5.3
Mescaline (Mescaline- d_9)	11.1	8.2	6.1	9.1	-0.2	12.4	9.2	8.5	9.1	10.7	9.7	5.4	6.0	3.7	6.3
Methylpseudoephedrine (Ephedrine-d_3)	8.3	2.7	8.8	11.1	8.4	17.6	11.9	8.4	11.1	9.9	7.6	9.1	6.2	4.0	5.0
Norephedrine (Norephedrine-d_3)	20.0	12.1	6.8	4.6	2.2	10.9	12.3	9.9	9.9	9.1	6.6	9.7	9.9	5.3	7.2
Norpseudoephedrine (Norephedrine-d_3)	9.9	5.9	10.3	2.8	1.8	15.2	8.8	8.9	6.0	11.5	12.3	8.6	8.4	3.8	11.1
Pseudoephedrine (Ephedrine-d_3)	1.0	2.7	6.2	1.4	5.5	20.0	14.1	5.1	4.6	8.1	9.9	8.6	4.9	2.9	5.5
Synephrine (Norephedrine-d_3)	10.0	1.8	13.3	4.3	-0.8	11.6	10.1	12.9	10.0	9.2	6.8	8.6	8.4	3.8	5.5

3.3.4 Proof of Applicability

Applicability experiments were carried out using plasma samples taken from healthy volunteers one hour after application of common doses of *Herba Ephedra*, pseudoephedrine, or ephedrine. Fig. 3.12 shows smoothed, normalized, and merged MRM chromatograms of extracts of authentic plasma samples after ingestion of *Herba Ephedra* (part 1), of pseudoephedrine (part 2), and of ephedrine (part 3). After ingestion of *Herba Ephedra* the ephedrines norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine, methylephedrine, and methylpseudoephedrine were detected, whereas after ingestion of pseudoephedrine or ephedrine, contained in cold medications, only the applied substances were detected. The quantitative results are summarized in Table 3.4.

Table 3.4. Plasma concentrations (ng/ml) of norephedrine (NE), norpseudoephedrine (NPE), ephedrine (EP), pseudoephedrine (PEP), methylephedrine (ME), and methylpseudoephedrine (MPE) after administration of the given drugs (LLOQ: lower limit of quantification).

Administered drug (dose)	NE	NPE	EP	PEP	ME	MPE
Herba Ephedra	< LLOQ	14.9	20.0	16.0	< LLOQ	-
Herba Ephedra	< LLOQ	20.3	25.5	15.8	< LLOQ	< LLOQ
Pseudoephedrine (30 mg)	-	-	-	107	-	-
Pseudoephedrine (30 mg)	-	-	-	114	-	-
Ephedrine (6.2 mg)	-	-	21.5	-	-	-
Ephedrine (6.2 mg)	-	-	21.6	-	-	-

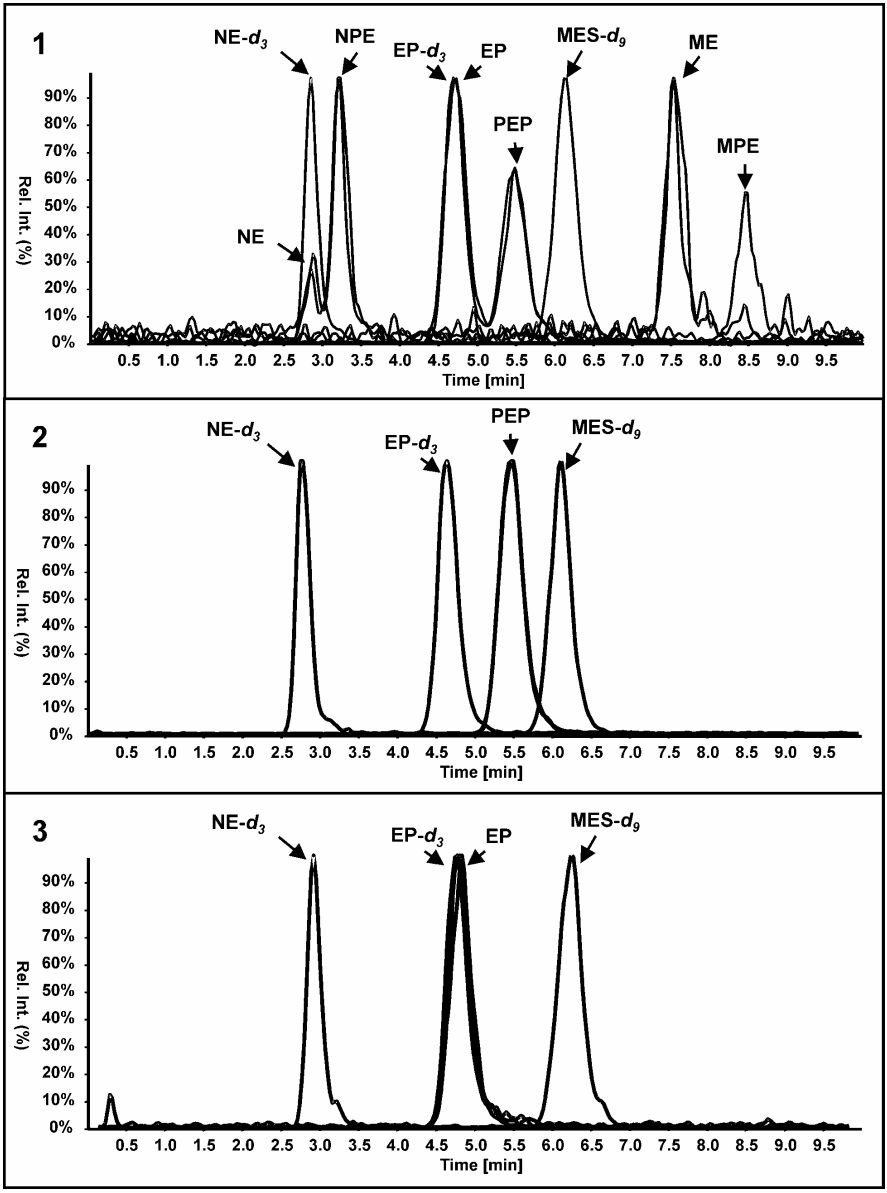


Fig. 3.12. Smoothed, normalized, and merged MRM chromatograms of all target transitions of extracts of authentic plasma samples after ingestion of *Herba Ephedra* (part 1), of pseudoephedrine (part 2), and of ephedrine (part 3) indicating norephedrine (NE), norpseudoephedrine (NPE, cathine), ephedrine (EP), pseudoephedrine (PEP), methylephedrine (ME), and/or methylpseudoephedrine (MPE), and the internal standards NE-d_3, EP-d_3, and MES-d_9.

3.4 TOXIC ALKALOIDS

A single stage MS and a tandem MS assay were developed for selective detection as well as for accurate and precise quantification of toxic alkaloids in human plasma and compared with respect to selectivity, linearity, accuracy, precision, and matrix effects. Both assays were validated according to international guidelines.[137,140,153,154]

3.4.1 Extraction Procedure

In early development stages of the presented assay, it was intended to extract the analytes either by the plasma mixed-mode SPE procedure described for herbal phenalkylamines,[157] or by their standard liquid-liquid extraction (LLE) procedure.[158-160] While colchicine showed better extraction efficiency using LLE, all other studied analytes showed better results using SPE. Therefore, the SPE procedure was preferred over LLE for extraction of these analytes from plasma. As shown in Tables 3.5 and 3.6, the extraction efficiency values ranged from 10 to 90 %. For colchicine the extraction efficiency was relatively low, which can be explained by its chemical properties. Whereas the used mixed-mode SPE procedure is optimized for basic compounds, colchicine shows no basic properties. Nevertheless, the described extraction procedure was considered acceptable due reproducible extraction and the sufficient sensitivity even for this analyte.

3.4.2 Detection and Quantification

For single stage MS, the APCI source was found to be more sensitive than the corresponding ESI source in preliminary experiments. In case of tandem MS coupled with the Turbo V® Source, the ESI mode was preferred over the APCI mode, because it was found to be more sensitive. Consequently, the different apparatus were compared using the respective most sensitive ionization mode.

Table 3.5. Extraction efficiency, matrix effect, and process efficiency of the APCI-LC-MS assay for the studied analytes.

Analyte	Extraction efficiency (mean ± SD, %)		Matrix effect (mean ± SD, %)		Process efficiency (mean ± SD, %)	
	LOW (n = 5)	HIGH (n = 5)	LOW (n = 5)	HIGH (n = 5)	LOW (n = 5)	HIGH (n = 5)
Aconitine	64 ± 6.7	59 ± 9.0	107 ± 9.2	126 ± 10.0	69 ± 9.2	74 ± 5.1
Atropine	61 ± 10.2	78 ± 6.7	99 ± 13.7	91 ± 4.2	60 ± 3.0	71 ± 5.7
Colchicine	10 ± 2.5	16 ± 3.1	101 ± 8.1	105 ± 5.6	11 ± 2.4	17 ± 2.6
Coniine	34 ± 4.5	36 ± 3.9	103 ± 12.5	90 ± 6.4	36 ± 0.9	33 ± 3.5
Cotinine	43 ± 9.1	64 ± 12.5	96 ± 4.8	92 ± 5.3	41 ± 9.8	59 ± 10.1
Cytisine	66 ± 6.5	80 ± 4.5	95 ± 17.0	111 ± 5.0	62 ± 7.6	89 ± 5.8
Nicotine	21 ± 5.1	32 ± 8.4	96 ± 18.7	100 ± 8.9	20 ± 6.0	31 ± 6.4
Physostigmine	85 ± 18.3	85 ± 11.0	70 ± 6.2	88 ± 5.0	58 ± 7.3	75 ± 9.5
Scopolamine	74 ± 6.9	87 ± 4.5	94 ± 5.3	96 ± 2.7	69 ± 5.8	83 ± 3.2

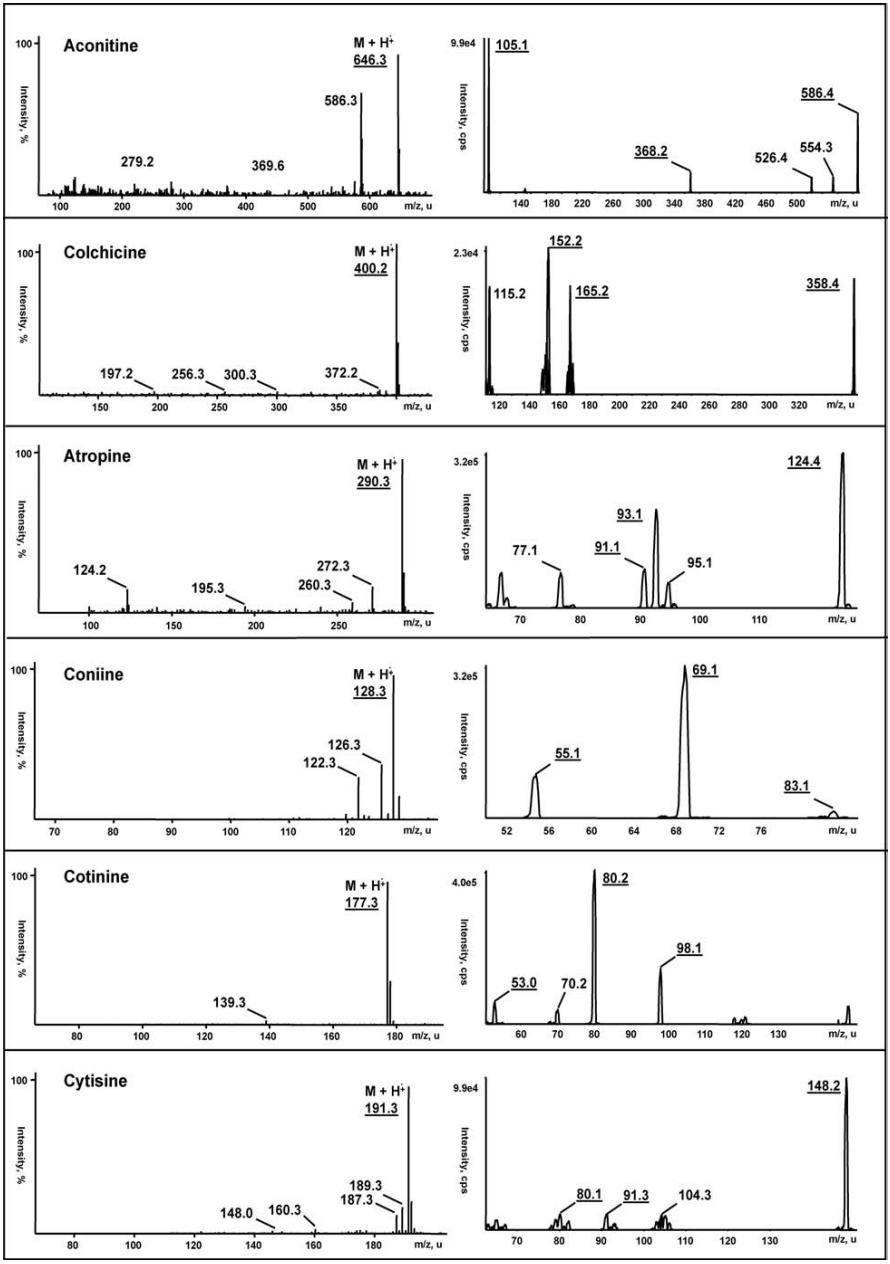
Table 3.6. Extraction efficiency, matrix effect, and process efficiency of the ESI-LC-MS/MS assay for the studied analytes.

Analyte	Extraction efficiency (mean ± SD, %)		Matrix effect (mean ± SD, %)		Process efficiency (mean ± SD, %)	
	LOW (n = 5)	HIGH (n = 5)	LOW (n = 5)	HIGH (n = 5)	LOW (n = 5)	HIGH (n = 5)
Aconitine	66 ± 6.4	55 ± 6.1	86 ± 1.9	89 ± 5.1	57 ± 5.5	49 ± 6.1
Atropine	78 ± 5.8	84 ± 5.2	82 ± 5.9	80 ± 5.0	64 ± 2.5	67 ± 3.3
Colchicine	10 ± 2.4	14 ± 3.5	79 ± 5.6	84 ± 8.7	8 ± 1.8	12 ± 2.5
Coniine	39 ± 2.9	40 ± 5.5	95 ± 2.5	92 ± 2.9	38 ± 5.3	37 ± 1.8
Cotinine	45 ± 7.1	70 ± 2.3	91 ± 6.3	95 ± 2.4	40 ± 5.1	66 ± 3.1
Cytisine	79 ± 5.4	90 ± 5.5	76 ± 5.9	86 ± 3.0	60 ± 4.4	77 ± 4.6
Nicotine	23 ± 2.9	27 ± 6.1	100 ± 3.1	92 ± 9.3	23 ± 3.4	24 ± 5.1
Physostigmine	69 ± 14.3	72 ± 6.8	83 ± 6.7	86 ± 4.0	57 ± 9.4	62 ± 3.5
Scopolamine	64 ± 8.7	72 ± 7.7	72 ± 6.1	78 ± 3.2	41 ± 8.6	56 ± 4.1

In single stage MS, the presence of the analytes was screened for in the full scan mode by mass chromatography in the MSD 1 trace of the same run with the above given parameters. The following ions were used for screening (*m/z*, in order of appearance in the chromatogram): 191, 177, 180 (IS), 163, 128, 304, 293 (IS), 276, 290, 400, 298 (IS), and 646. Positive peaks in the recorded traces were identified by library search comparing the underlying APCI mass spectra with the reference spectra of our LC-MS library of drugs, poisons, pesticides and their metabolites created for the NIST98 search algorithm. The corresponding reference spectra recorded during this study are shown on the left side of Fig 3.13. In this Figure, the ions used for screening are underlined.

In tandem MS, the presence of the drugs was successfully detected in the MRM mode using three MRM transitions for each substance. Transitions were selected and their settings were determined using a 1000 ng/ml solution of each analyte in eluent A injected by the integrated syringe pump and using Analyst® Software in Quantitative Optimization mode. The three resulting transitions per analyte and respective settings are given in Table 2.2. In the right part of Fig. 3.13, the product ion scan spectra, recorded during this study, are shown. The ions chosen as product ions are underlined. Cross-talk was not observed.

For illustration of the detection and identification using the different apparatus, Fig. 3.14 shows the respective chromatograms of a MED sample after SPE. Part A of Fig. 3.14 shows smoothed and merged mass chromatograms of the ions 191, 177, 180 (IS), 163, 128, 304, 293 (IS), 276, 290, 400, 298 (IS), and 646 using single stage MS, part B of Fig. 3.15 shows smoothed, and merged MRM chromatograms of all recorded transitions using tandem MS.


Aconitine
M + H+
646.3
586.3
279.2
369.6
105.1
586.4
368.2
526.4
554.3
Colchicine
M + H+
400.2
197.2
256.3
300.3
372.2
152.2
115.2
165.2
358.4
Atropine
M + H+
290.3
124.2
195.3
272.3
260.3
124.4
93.1
77.1
91.1
95.1
Coniine
M + H+
128.3
126.3
122.3
69.1
55.1
83.1
Cotinine
M + H+
177.3
139.3
80.2
98.1
53.0
70.2
Cytisine
M + H+
191.3
189.3
187.3
148.0
160.3
148.2
80.1
91.3
104.3
Intensity, %
Intensity, cps
m/z, u

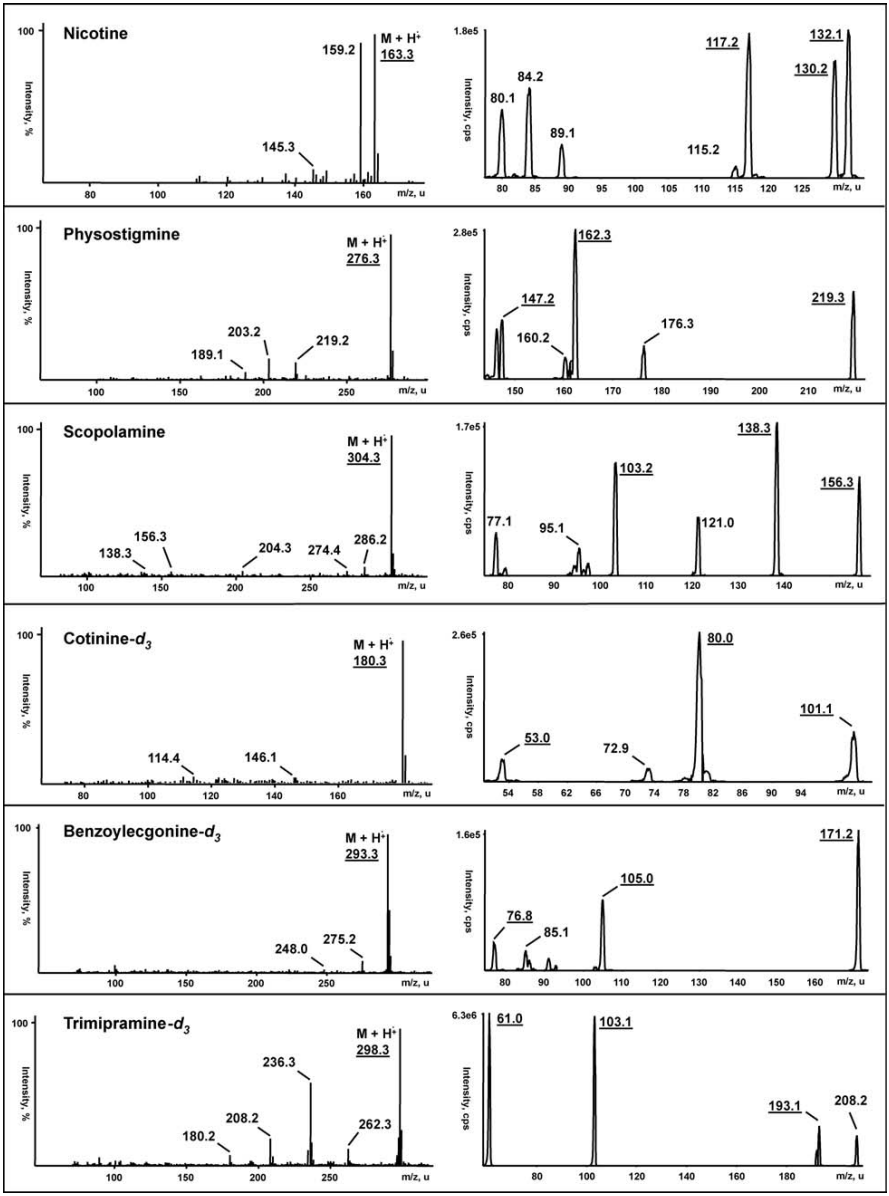
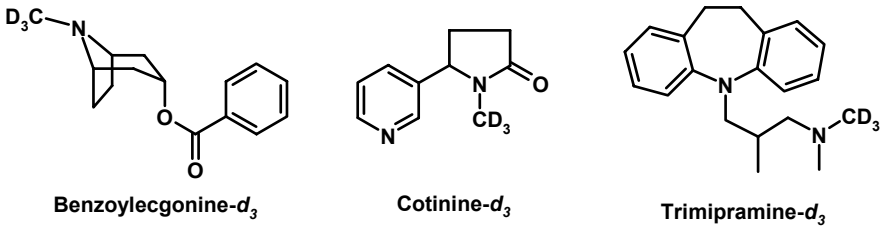


Fig. 3.13. Mass spectra of all studied analytes recorded in the full scan mode using single stage APCI MS (left), and corresponding product ion spectra recorded in the product ion scan mode using tandem ESI MS (right).

For quantification using single stage MS, SIM mode was used at 100 and 200 V fragmentor voltage with different gain values. For the quantification process, the analytes were divided into two different groups according to their expected concentration ranges and each group was assigned to one of three separately recorded traces with specific gain values as given in Table 2.2.

For quantification using tandem MS, MRM mode was used. One of the three transitions of each substance was used for quantification. This target transition is marked with an (t) in Table 2.3.

The peak area ratios of the target ions or the target transition of the drugs vs. those of the corresponding IS were compared with weighted least squares ($1/c^2$) calibration curves in which the peak area ratios (analytes vs. IS) of the calibration standards were plotted versus their concentrations. The different IS were assigned to the different analytes as shown in tables 3.7 and 3.8. The Structures of the IS used for quantification are depicted in Fig. 3.14.



Benzoylecgonine-d_3 **Cotinine-d_3** **Trimipramine-d_3**

Fig. 3.14. Chemical structures of the IS used for quantification.

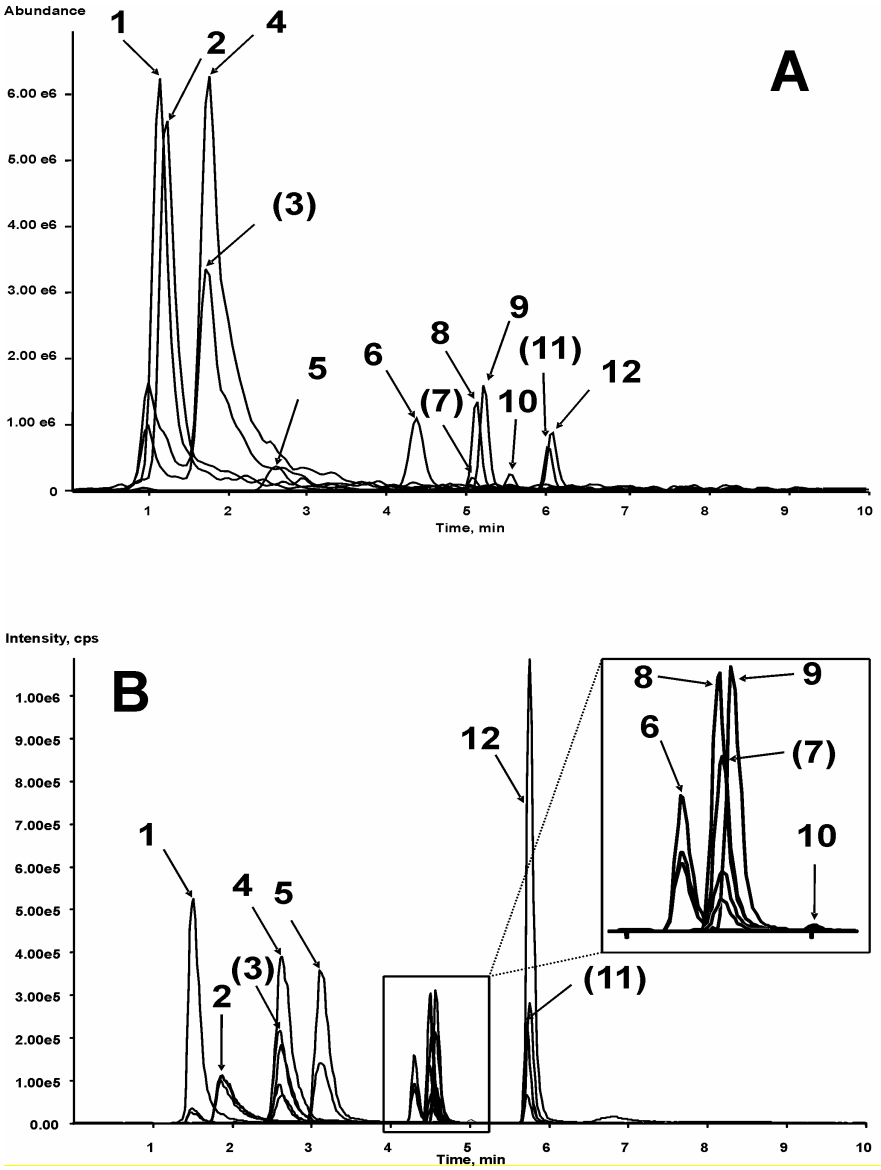


Fig. 3.15. Smoothed and merged chromatograms of the ions 191, 177, 180 (IS), 163, 128, 304, 293 (IS), 276, 290, 400, 298 (IS), and 646, recorded in the full scan mode, of a MED sample containing cytisine (peak 1), nicotine (2), cotinine-d_3 (3), cotinine (4), coniine (5), scopolamine (6), benzoylecgonine-d_3 (7), physostigmine (8), atropine (9), colchicine (10), trimipramine-d_3 (11), and aconitine (12) after SPE using the single stage MS (A). Smoothed and merged MRM chromatograms of all recorded transitions of same MED sample after SPE using the tandem MS (B).

3.4.3 Assay Validation

The described procedures were validated according to internationally accepted recommendations.[137,140,153,154] The validation data are summarized in Tables 3.5-3.8. The assay was found to be selective for all tested compounds using either single stage MS or tandem MS. No interfering peaks were observed in the extracts of the different blank plasma samples using both detectors. When using the APCI mode, the tested IS nicotine-d_4 showed a loss of 4 atomic mass units most probably due to aromatization of the pyrrolidine ring. The resulting fragment ion *m/z* 163 was isobaric to the protonated molecular ion of nicotine and hence interfered with the quantification of the latter. Therefore, nicotine-d_4 could not be used as IS.

As shown in Tables 3.5 and 3.6, no relevant matrix effects were observed for both types of ionization. The highest matrix effect was observed for physostigmine in the APCI mode and for scopolamine in the ESI mode, but considered acceptable due to good reproducibility.

In linearity experiments, a weighted second-order model was also evaluated to check for a curvature in the data. For all analytes, a linear weighted ($1/c^2$) least squares model was found to be the best and therefore used for calculation of calibration curves. Using single stage MS, the assay was linear from 50 to 1000 ng/ml for coniine, cotinine, cytisine, and nicotine, as well as from 5 ng/ml to 100 ng/ml for aconitine, atropine, colchicine, physostigmine, and scopolamine, respectively. The coefficients of determination (R^2) ranged from 0.9894 to 0.9997. Using tandem MS, the assay was linear from 1 to 1000 ng/ml for coniine, cotinine, cytisine, and nicotine, as well as from 0.1 ng/ml to 100 ng/ml for aconitine, atropine, colchicine, physostigmine, and scopolamine, respectively. The coefficients of determination (R^2) ranged from 0.9912 to 0.9994. The linearity was comparable either using single stage MS or tandem MS. In both assays, the worst coefficient of determination was found for nicotine, most probably due to its volatility, whereas the best results were found for cotinine, probably due to the use of its deuterated analogue as IS.

The LLOQs were fixed to the lowest concentrations used for the calibration curves with a signal-to-noise ratio of at least 10. All LOD values were lower or at least equal to half of those of the corresponding LLOQ, either using single stage MS or tandem MS.

The validation data for both apparatus concerning extraction efficiency, matrix effects, and process efficiency are shown in Table 3.5 (single stage MS), and 3.6 (tandem MS) as well as those concerning accuracy and precision in Table 3.7 (single stage MS) and 3.8 (tandem MS). Accuracy data lay within the acceptance interval of ±15% of the nominal values at all concentrations with the exception of those of nicotine most probably due to its volatility. Using nicotine-d_4 as internal standard would probably solve these problems, but as mentioned above, this was not possible. Within-day (repeatability) and intermediate precision lay within the required limits of 15% RSD for both apparatus at all studied concentration levels.

In extracts, the analytes were stable at low and high concentrations for a period of more than 24 h. In the freeze/thaw and long-term stability experiments, the ratio of means (stability vs. control samples) were within 90-110%, whereas the 90% CIs for stability samples were within 80-120% of the respective control means, thus fulfilling the acceptance criteria for all analytes at both concentrations.

Table 3.7. Accuracy, intermediate precision and repeatability data of the LC-MS assay for the studied analytes. IS used for quantification are given in brackets. Accuracy: ((mean calculated concentration - nominal concentration)/nominal concentration) x 100

Analyte (IS)	Accuracy			Intermediate Precision			Repeatability		
	LOW	MED	HIGH	LOW	MED	HIGH	LOW	MED	HIGH
Aconitine (Benzoylecgonine-d_3)	7.2	8.5	4.9	12.7	13.5	7.9	8.2	9.7	7.2
Atropine (Benzoylecgonine-d_3)	2.1	5.8	-4.6	8.3	6.9	9.9	6.7	6.9	4.3
Colchicine (Cotinine-d_3)	-6.3	11.0	-12.6	10.4	8.6	7.8	9.3	2.8	2.5
Coniine (Cotinine-d_3)	6.9	5.8	12.5	8.5	7.5	12.5	5.8	6.5	9.8
Cotinine (Cotinine-d_3)	1.9	3.3	-0.8	4.8	5.9	3.0	4.6	5.4	2.8
Cytisine (Cotinine-d_3)	-8.2	-6.1	-9.1	9.2	6.5	9.1	4.4	6.0	8.7
Nicotine (Cotinine-d_3)	-24.0	-35.8	-38.6	11.9	8.4	11.1	9.1	6.2	4.0
Physostigmine (Trimipramine-d_3)	3.7	7.2	1.4	9.1	8.1	7.6	8.6	4.9	2.9
Scopolamine (Trimipramine-d_3)	14.0	-11.6	13.4	10.0	12.4	8.3	9.6	7.2	3.6

Table 3.8. Accuracy, intermediate precision and repeatability data of the LC-MS/MS assay for the studied analytes. IS used for quantification are given in brackets. Accuracy: ((mean calculated concentration - nominal concentration)/nominal concentration) x 100

Analyte (IS)	Accuracy			Intermediate Precision			Repeatability		
	low	med	high	low	med	high	low	med	high
Aconitine (Benzoylecgonine-d_3)	6.0	2.9	1.6	14.7	12.5	9.4	13.8	12.5	3.5
Atropine (Benzoylecgonine-d_3)	2.2	2.9	1.6	9.9	12.5	9.4	9.9	12.5	3.5
Colchicine (Cotinine-d_3)	3.9	-10.4	-1.2	14.1	12.3	12.6	11.3	9.9	9.8
Coniine (Cotinine-d_3)	2.3	-4.7	-4.6	10.2	12.7	8.6	9.7	11.5	8.0
Cotinine (Cotinine-d_3)	7.0	3.4	3.5	5.3	6.6	4.3	3.8	5.8	4.3
Cytisine (Cotinine-d_3)	-1.5	4.3	-4.7	10.9	9.8	9.2	6.7	9.7	9.2
Nicotine (Cotinine-d_3)	-20.5	-38.3	-34.8	11.6	13.0	12.2	8.9	13.0	12.2
Physostigmine (Trimipramine-d_3)	8.3	0.8	-4.5	8.7	13.1	12.3	8.7	13.1	5.8
Scopolamine (Trimipramine-d_3)	4.2	4.1	-4.2	10.3	10.5	13.4	5.5	6.8	6.6

3.4.4 Proof of Applicability

Applicability experiments were carried out using plasma samples from poisoning cases sent to our laboratory for toxicological analysis. Patient one accidentally ingested leaves of *Colchicum autumnale* (Meadow saffron) containing colchicine due to a mix-up with the vegetable *Allium ursinum*, known as Ramsons. In the upper part of Fig 3.16, the corresponding ion fragmentogram with the given ions of the plasma sample extract after SPE using LC-MS (A), as well as the MRM chromatograms with the given transitions using LC-MS/MS (B) are shown. Using single stage MS, a toxic concentration of 24 ng/ml of colchicine was determined and using tandem MS 25 ng/ml.
Patient two abused *Datura stramonium* containing atropine and scopolamine. In the lower part of Fig 3.16 the corresponding ion fragmentogram with the given ions of the plasma sample extract after SPE using LC-MS (C), as well as the MRM chromatograms with the given transitions using LC-MS/MS (D) are shown. Using single stage MS, a therapeutic concentration of 6.4 ng/ml of atropine, a toxic concentration of 5.6 ng/ml of scopolamine, and a common smoker's concentration of 321 ng/ml of cotinine were determined and using tandem MS, 6.1 ng/ml of atropine, 5.9 ng/ml of scopolamine and 319 ng/ml of cotinine.

3.4.5 Comparison of Methods

In all validation experiments of the two procedures, the identical plasma extracts were used. Therefore a comparison was possible of both apparatus operated in the respective most sensitive ionization mode. Both assays (single stage as well as tandem MS) were selective for the tested compounds. Comparing the spectra shown in Fig. 3.13, it can be seen that in tandem MS the identification power was higher due to the higher fragmentation of the precursor ions. Monitoring the transitions allowed limits of detection of about ten times lower in tandem MS than in single stage MS. As shown in Figs. 3.15 and 3.16, tandem MS yielded better signal-to-noise ratios in the identical extracts. In contrast to that advantage of tandem MS, the accuracy and precision data for both apparatus were comparable. No relevant matrix effects were observed in both assays.

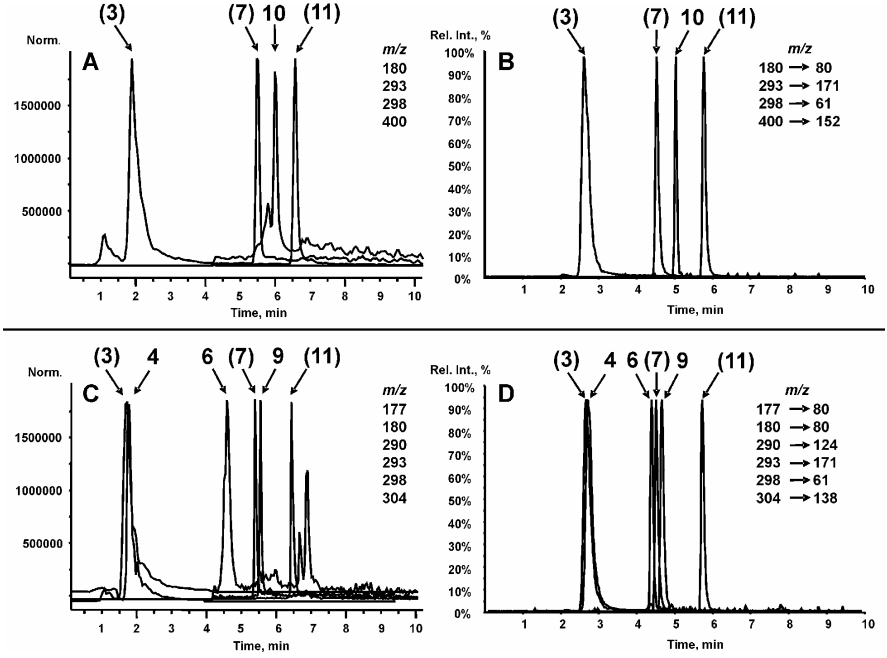


Fig. 3.16. Smoothed, normalized and merged chromatograms with the given ions of a plasma extract after SPE indicating a toxic concentration of 24 ng/ml of colchicine (peak 10) determined using single stage MS (A). Smoothed, normalized and merged chromatograms of the given transitions of the same extract indicating a toxic concentration of 25 ng/ml of colchicine (peak 10) determined using tandem MS (B). Smoothed, normalized and merged chromatograms with the given ions of a plasma extract after SPE indicating a therapeutic concentration of 6.4 ng/ml of atropine (peak 9), a toxic concentration of 5.6 ng/ml of scopolamine (peak 6), and a common smoker's concentration of 321 ng/ml of cotinine (peak 4) determined using single stage MS (C). Smoothed, normalized and merged chromatograms of the given transitions of the same extract indicating a therapeutic concentration of 6.1 ng/ml of atropine (peak 9), a toxic concentration of 5.9 ng/ml of scopolamine (peak 6), and a common smoker's concentration of 319 ng/ml of cotinine (peak 4) determined using tandem MS (D). The peak numbering of the IS is according to that of Fig. 3.15.

4 CONCLUSIONS

The presented GC-MS procedures for the detection of laxatives allowed the identification and differentiation of stimulant laxatives and/or their metabolites in urine after ingestion of therapeutic doses. Therefore, this screening can be used for confirmation of an intake of these drugs during diagnosis or differential diagnosis of various intestinal disorders. Furthermore, introducing enzymatic cleavage of conjugates allowed to integrate successfully the described method into the previously described screening procedures for simultaneous detection of other drugs like first generation anticoagulants,[71] ACE inhibitors and angiotensin II receptor antagonists,[70] dihydropyridine calcium channel blockers,[72] diuretics,[142,143] and NSAIDs.[73]

The study covering the nutmeg ingredients showed that alkenebenzene derivatives elemicin, myristicin and safrole contained in nutmeg were extensively metabolized by rats and humans. However, these metabolites are not amphetamine derivatives as described in the 1970ies. For monitoring a nutmeg abuse or intoxication, the toxicologist should screen for the above-mentioned target analytes, particularly by the described STA.[79-84]

The LC-MS and LC-MS/MS assays presented here for separation, detection and quantification of psychoactive herbal phenalkylamines of interest and methcathinone as well as toxic alkaloids of interest in plasma are the first validated multi-analyte procedures for such substances. All procedures have proven to be selective, linear, accurate and precise for all studied drugs with the exception of nicotine. As expected, the tandem MS is more selective and sensitive than the single stage MS. The accuracy and precision data for both apparatus were comparable. In case of poisoning, both apparatus can be used for detection and quantification. Only if low concentration must be monitored tandem MS is needed due to its higher sensitivity.

All presented assays have also proven to be applicable for clinical and forensic toxicological tasks.

5 SUMMARY

In the presented thesis, procedures are described for screening for, identification and/or validated quantification of herbal drugs in blood or urine using GC-MS, LC-MS or LC-MS/MS. They are needed in in clinical and forensic toxicology, because poisonings with plants or plant ingredients as well as their abuse are widespread. The aims of such an abuse are stimulation, hallucinations, or even for weight loss or habitual use. In both cases, toxicological analysis is the prerequisite for reliable diagnosis, prognosis and monitoring. The following drugs or drugs classes were included in this method development: laxatives, ingredients of nutmeg, herbal phenalkylamines, and toxic alkaloids.

For detection of the acidic laxatives bisacodyldiphenol, phenolphthalein, and rhein in urine, extractive methylation was used. The analytes were derivatized and simultaneously extracted using methyl iodide in toluene and the phase transfer catalyst tetrahexylammonium hydrogen sulfate. The phase transfer catalyst was removed from the organic phase by solid phase extraction. The extracts were analyzed by GC-MS. The developed method allowed the identification and differentiation of stimulant laxatives and/or their metabolites in urine after ingestion of therapeutic doses.[161]

In the study covering the nutmeg ingredients, metabolism and toxicological analysis in urine of elemicin, myristicin, and safrole were investigated. The qualitative metabolism was studied in Wistar rats, that were administered a high dose of the corresponding nutmeg ingredient. The metabolites were identified by GC-MS. The study showed that the nutmeg ingredients were extensively metabolized, and that the parent compounds were not detectable in urine. For toxicological analysis, an established method including acid hydrolysis, liquid-liquid extraction and microwave-assisted acetylation was used. The derivatized metabolites were separated and detected using GC-MS in the full scan mode. Using this procedure, a nutmeg abuse or intoxication can be monitored via detection of the metabolites.[162]

Detection and validated quantification of herbal phenalkylamines ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, methylephedrine, methylpseudoephedrine, cathinone, mescaline, synephrine (oxedrine), and methcathinone in human plasma were based on a standard solid-phase extraction procedure using mixed-mode SPE columns. The analytes were seperated using a liquid

chromatography with a strong cation exchange seperation column as stationary phase, and a gradient elution with 5 mM ammonium formate buffer/acetonitril as mobile phase. The analytes were detected with an LC-ESI-MS/MS operated in the MRM mode. The method allowed the detection of ephedrines after ingestion of therapeutic doses, selective, linear, accurate and precise quantification, as well as the differentiation of an herbal drugs abuse from ingestion of cold remedies.[157]

Detection and validated quantification of the toxic alkaloids aconitine, atropine, colchicine, coniine, cotinine, cytisine, nicotine, physostigmine, scopolamine in human plasma were based on the identical extraction procedure used for the extraction of the herbal phenalkylamines. The analytes were seperated using a liquid chromatography with a C8 base select seperation column as stationary phase, and a gradient elution with 50 mM ammonium formate buffer/acetonitril as mobile phase. The analytes were detected either with an LC-APCI-MS operated in full scan and SIM mode or an LC-ESI-MS/MS operated in the MRM mode. In this study, the use of LC-APCI-MS vs LC-ESI-MS/MS was directly compared. As expected, the tandem MS was more selective and sensitive than the single stage MS. The accuracy and precision data for both apparatus were comparable. In case of poisoning, both apparatus can be used for detection and quantification. Only if low concentration must be monitored tandem MS is needed due to its higher sensitivity.[163]

In summary, all presented methods have proven to be reliable and accurate and are now a valuable tool in clinical and forensic toxicology.

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7 ABBREVIATIONS

ACR	Above calibraton range
ANOVA	Analysis of Variance
APCI	Atmospheric pressure chemical ionization
CE	Collision energy
CEP	Collision cell entrance potential
CNS	Central nervous system
CXP	Collision cell exit potential
DP	Declustering potential
EI	electron ionization
ENP	Entrance potential
ESI	Electrospray ionization
FT	Forensic toxicology
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
IS	Internal standard
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection
LQC	Low quality control
m/z	Mass to charge ratio
MDA	3,4-Methylendioxyamphetamine
MMDA	3,4-Methylendioxy-5-methoxyamphetamine
MRM	Multiple reaction monitoring
MS	Mass spectrometry

NMR	nuclear megnetic resonance
PMA	4´-Methoxyamphetamine
QC	Quality control
RSD	Relative standard deviation
S/N	Signal to noise ratio
SCX	Strong Cation exchange
SIM	Selected-ion monitoring
SPE	Solid-phase extraction
STA	Systematic toxicological analysis
TDM	Therapeutic drug monitoring
THA	Tetrahexylammonium
THC	Tetrahydrocannabinol
TLC	Thin layer chromatography
TMA	3,4,5-Trimethoxyamphetamine
UV	Ultraviolett

8 ZUSAMMENFASSUNG

Die vorliegende Dissertation beschreibt Methoden zur Suchanalyse, Identifizierung und/oder validierten Quantifizierung von pflanzlichen Arzneistoffen in Blut oder Urin unter der Verwendung GC-MS, LC-MS oder LC-MS/MS. Diese Methoden werden sowohl in der klinischen wie auch der forensischen Toxikologie aufgrund der Verbreitung von Pflanzenvergiftungen bzw. Vergiftungen mit Pflanzenwirkstoffen sowie deren Missbrauch benötigt. Die Beweggründe eines solchen Missbrauchs sind Stimulation, Halluzinationen, aber auch Gewichtsverlust oder Gewöhnung. Sowohl zur Diagnose eines Missbrauchs als auch einer Vergiftung sind toxikologische Analysen eine Grundvorrausetzung zur zuverlässigen Diagnose, Prognose und Überwachung. Die folgenden Stoffe oder Stoffklassen wurden bei dieser Methodenentwicklung berücksichtigt: Laxanzien, Inhaltsstoffe der Muskatnuss, pflanzliche Phenalkylamine und giftige Alkaloide.

Zum Nachweis der sauren Laxanzien Bisacodyldiphenol, Phenolphthalein und Rhein in Urin wurde die Extraktive Methylierung eingesetzt. Hierbei werden die Analyten durch Methyliodid in Toluol und den Phasentransferkatalysator Tetrahexylammonium-hydrogensulfat derivatisiert und gleichzeitig extrahiert. Der Phasentransferkatalysator wurde aus der organischen Phase mittels Festphasenextraktion entfernt. Die resultierenden Extrakte wurden unter Zuhilfenahme von GC-MS analysiert. Die im Rahmen dieser Dissertation entwickelte Methode erlaubt die Identifizierung und Differenzierung der stimmulierenden Laxanzien und/oder deren Metaboliten nach Einnahme einer therapeutischen Dosis.[161]

Des Weiteren wurde der Metabolismus sowie der toxikologische Nachweis von Elemicin, Myristicin und Safrol, welche Inhaltsstoffe der Muskatnuss sind, untersucht. Die qualitativen Metabolismusuntersuchungen erfolgten an Wistar Ratten, denen eine hohe Dosis der jeweiligen Substanz verabreicht wurde. Die Metaboliten wurden mittels GC-MS identifiziert. Die Untersuchungen zeigten, dass die Inhaltsstoffe der Muskatnuss aufgrund der ausgeprägten Metabolisierung im Urin nicht nachweisbar waren. Als toxikologisches Nachweisverfahren diente in diesem Fall eine etablierte Methode basierend auf einer sauren Hydrolyse zur Konjugatspaltung, Flüssig-Flüssig-Extraktion und Mikrowellenunterstützter Acetylierung. Die derivatisierten Metaboliten wurden mittels GC-MS im full-scan Modus aufgetrennt und detektiert. Diese Methode erlaubt

den Nachweis einer Vergiftung sowie Nachweis eines Missbrauchs von Muskatnuss durch Detektion der Metaboliten der Muskatnussinhaltsstoffe.[162]

Die Detektion sowie die validierte Quantifizierung der pflanzlichen Phenalkylamine Ephedrin, Pseudoephedrin, Norephedrin, Norpseudoephedrin, Methylephedrin, Methylpseudoephedrin, Cathinon, Mescalin, Synephrin (Oxedrin) und Methcathinon im Blut basierte auf einer standardmäßig verwendeten Festphasenextraktion unter Zuhilfenahme von Mixed-mode Festphasensäulen. Die Analyten wurden mittels Flüssigchromatographie mit einer starken Kationentauscher-Säule als stationäre Phase und einem Gradientengemisch aus 5 mM Ammoniumformiatpuffer/Acetonitril getrennt. Zur Detektion diente ein LC-ESI-MS/MS, welches im MRM Modus betrieben wurde. Die Methode erlaubt die Detektion der Ephedrine nach Einnahme therapeutischer Dosierungen, die selektive, lineare, richtige und präzise Quantifizierung der genannten Substanzen sowie die Unterscheidung eines Missbrauchs von Pflanzen von der Einahme eines Erkältungsmittels.[157]

Die Detektion sowie die validierte Quantifizierung der giftigen Alkaloide Aconitin, Atropin, Colchicin, Coniin, Cotinin, Cytisin, Nicotin, Physostigmin und Scopolamine im Blut basierten auf der identischen Extraktion wie für die pflanzlichen Phenalkylamine. Die Analyten wurden mittels Flüssigchromatographie mit einer C8 base select-Säule als stationäre Phase und einem Gradientengemisch aus 50 mM Ammoniumformiat-puffer/Acetonitril getrennt. Zur Detektion diente entweder ein LC-APCI-MS, welches im full-scan bzw. im SIM Modus betrieben wurde, oder ein LC-ESI-MS/MS, welches im MRM Modus betrieben wurde. Im Rahmen dieser Untersuchung wurden der verwendete LC-APCI-MS sowie der LC-ESI-MS/MS direkt miteinander verglichen. Wie zu erwarten war der Tandem MS selektiver und empfindlicher als der Single Stage MS. Die Richtigkeits und Präzisionswerte hingegen waren vergleichbar. Zum Nachweis und zur Quantifizierung einer Vergiftung sind beide Geräte geeignet. Wenn allerdings niedrigste Konzentrationen der Stoffe überwacht werden müssen, bedarf es der Benutzung eines Tandem MS aufgrund dessen höherer Sensitivität.[163]

Zusammenfassend lässt sich sagen, daß alle hier präsentierten Methoden zuverlässig und genau sind. Diese Methoden sind ein wertvolles Werkzeug sowohl in der klinischen als auch der forensichen Toxikologie.

9 PUBLIKATIONSVERZEICHNIS

Aus der vorliegenden Arbeit sind folgende Publikationen hervorgegangen:

Originalpublikationen (peer reviewed)

1. **Beyer J**, Peters FT, Maurer HH. Screening procedure for detection of stimulant laxatives and/or their metabolites in human urine using gas chromatography-mass spectrometry after enzymatic cleavage of conjugates and extractive methylation. *Ther. Drug Monit.* 2005; **27**: 151.

2. **Beyer J**, Ehlers D, Maurer HH. Abuse of Nutmeg (*Myristica fragrans* Houtt.): Studies on the Metabolism and the Toxicological Detection of its Ingredients Elemicin, Myristicin and Safrole in Rat and Human Urine Using Gas Chromatography/Mass Spectrometry. *Ther. Drug Monit.* 2006; **28**: 568.

3. **Beyer J**, Peters FT, Kraemer T, Maurer HH. Detection and validated quantification of herbal phenalkylamines and methcathinone in human blood plasma by LC/MS/MS. *J. Mass Spectrom.* 2006; in press.

4. **Beyer J**, Peters FT, Kraemer T, Maurer HH. Detection and validated quantification of toxic alkaloids in human plasma - Comprison of LC-APCI-MS with LC-ESI-MS/MS. *J. Mass Spectrom.* 2006; submitted.

Proceedings oder Veröffentlichungen in Bulletins:

1. **Beyer J**, Bierl A, Peters FT, Maurer HH. Simultaneous detection of stimulant laxatives and diuretics in human urine using gc-ms after Enzymatic Cleavage of conjugates and extractive methylation. In: *Proceedings of the 43rd International SOFT/TIAFT Meeting in Washington*, Spiehler V (ed). TIAFT2004: Washington (DC) 2005; in press.

2. **Beyer J**, Peters FT, Maurer HH. Six Poisoning Cases after Intravenous Abuse of Cocaine Adulterated With Atropine. In: *Proceedings of the XIVth GTFCh Symposium in Mosbach 2005*, Pragst F, Aderjan R (eds). Helm-Verlag: Heppenheim (Germany) 2006; 455.

Publizierte Vorträge:

1. **Beyer J**, Bierl A, Peters FT, Maurer HH. Simultaneous Detection of Stimulant Laxatives and Diuretics in Human Urine Using GC-MS after Enzymatic Cleavage of Conjugates and Extractive Methylation. In: *Abstract Book to the 43rd International SOFT/TIAFT Meeting in Washington,* TIAFT2004. Washington, DC (USA).

2. **Beyer J**, Peters FT, Maurer HH. Six Poisoning Cases after Intravenous Abuse of Cocaine Adulterated with Atropine. *Oral Presentation at the 14th Meeting of the Society of Toxicological and Forensic Chemistry in Mosbach (Germany)* Toxichem Krimtech. 2005;**72** (1);14.

3. **Beyer J**, Maurer HH. Abuse of Nutmeg (*Myristica fragrans* Houtt.): Identification of the Metabolites of its Ingredients Elemicin, Myristicin and Safrole in Rat and Human Urine by GC-MS. *Oral Presentation and Poster at the 9th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology, Louiseville, KY (USA) Ther Drug Monit.* 2005;**27**: 212-213.

4. **Beyer J**, Peters FT, Kraemer T, Maurer HH. Detection and validated quantification of herbal phenalkylamines and methcathinone in human blood plasma by LC/MS/MS. In: *Abstract Book to the 45rd International TIAFT Meeting in Ljubljana,* TIAFT2006. Ljubljana (Slowenia).

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