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Distinct composition and pharmacokinetic profiles of smoke and smokeless tobacco products:

Development of an analytical method to highlight nicotine consumption as a doping agent

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PREFACE

This report reflects work done at the Swiss Laboratory for Doping Analysis in Lausanne, Switzerland. All analytical work was performed during the period of October 2009- May 2010. The external supervisor of this work was Francois Marclay, PhD student at the Swiss Laboratory for Doping Analysis, and the internal supervisor was Professor Einar Jensen at the University of Tromsø.

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Lausanne, 20.05.2010
Ida Lange

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1 ABSTRACT

Nicotine is a pharmacological active compound found in the tobacco plant, and works as a a stimulant of the central nervous system. Evidence is presented that use of nicotine may have beneficial effects on athletes' performance. Nicotine is known to cause weight loss and to improve mental capacity by enhancing concentration and relieving anxiety. Additionally, improved exercise endurance after nicotine administration has been demonstrated. By using nicotine containing medications or smokeless tobacco products, athletes can benefit from these effects without most of the health risk associated with smoking.

Nicotine is primarily consumed in the form of tobacco, but a large variety of nicotine containing medications exists as well. The most common forms of tobacco consumption patterns are smoking as cigarette and pipes, and smokeless tobacco, in the form of chewing tobacco, snus and snuff. Depending on the type of product, the tobacco compositions of nicotine, and other compounds naturally present in tobacco, differ to a large extent. Levels of each compound measurable in biological fluids depend on the chemical composition and the distinct pharmacokinetic profile of the consumed product. Theoretically, these variations in levels of investigated compounds could be used to differentiate between nicotine consumption patterns.

In this project, compounds of interest for discriminating between the use of smoke and smokeless tobacco were identified and an LC-MS/MS method involving SPE sample preparation was developed and validated in order to determine concentration levels of these compounds in urine specimens. Further, concentration levels of these molecules were measured in urine specimens from smokers and smokeless tobacco users, and interpretation of preliminary results indicated some variability. Still there is a long way to go before the method is suited to highlight nicotine consumption for a doping purpose.

2 ABBREVIATIONS

EPO: erythropoietin

ESI: electron spray ionization

FDA : U.S Food and Drug Administration

HLB : hydrophilic lipophilic balance

ICH: International Comitte of Harmonistion

IOC: The International Olympic Committee

LLE: liquid phase extraction

LOQ: limit of quantification

LTQ: linear ion trap quadrupole

MCX : mixed mode cation exchange)

MRM: multiple reaction monitoring

MS: mass spectrometry

SIM: single ion monitoring

SPE: solid phase extraction

Trans-OH-cot : trans-3-hydroxycotinie

TSNA: tobacco specific nitrosamines

TSQ: triple stage quadrupole

ULOQ: upper limit of quantification

WADA: World Anti-Doping Agency

3 INTRODUCTION

3.1 Doping in sports

The term doping can be defined as a practice that consists in the absorbtion of specific substances or the practice of specific medical acts in order to artificially enhance one's physical or mental capacities[2]. Doping creates different conditions of competition, and might as well represent an actual or potential health risk to the athlete[3].

Doping is not a new phenomenon, either in sport or society in general. Doping has been accepted in many cultures, including the western one. In some sports doping has a long tradition, and the trend has accelerated from 1960 to the present day. The number of different types of substances that are used has increased considerably. In particular, the misuse of blood doping has become more common. In association with Tour de France in 1998, it was revealed extensive abuse of EPO, growth hormones, anabolic steroids and amphetamines. Both public authorities and sports organizations realized then that it was necessary to unite the forces in the fight against doping. This led to the creation of the World Anti-Doping Agency (WADA) 10 November 1999[4]. Its mission is to promote, coordinate and monitor the fight against doping in sport in all forms. WADA's key activities include scientific research, education, development of anti-doping strategies, and monitoring of the World Anti Doping Code- the document harmonizing anti-doping policies in all sports and all countries[5]. This document contains the list of substances and methods that are prohibited in sports[6].

Doping controls are carried out at all international championships and major events to enforce in practice this lists of prohibited drugs, narcotics and methods. In addition unannounced spot checks in the training periods are also conducted. The doping analyses are performed on urine and / or blood samples by one of the 35 WADA approved doping laboratories around the world. If there is found traces of banned drugs (positive samples), the penalty is usually exclusion from all sports for a period of two years[4].

3.2 Aim of the project

The purpose of this study is to develop an analytical method to highlight nicotine consumption as a doping agent.

The first phase of this project is to identify compounds of interest for discriminate between the use of smoke and smokeless tobacco.

In the second phase, an analytical method to determine concentration levels of these compounds of interest in urine specimens should be developed and validated.

Then, the concentration levels of these molecules should be determined in urine specimens from smokers and smokeless tobacco users, in order to highlight variability due to either form of consumption.

3.3 Tobacco, general background

3.3.1 *Different tobacco consumption patterns*

Tobacco is a common term for a variety of products made from dried leaves of tobacco plants [7]. Tobacco has two principal use patterns. Either it is smoked, or used as smokeless tobacco. Smoke tobacco exists in various forms as cigarettes, cigars and pipes. This kind of tobacco is inhaled as smoke. There are three main forms of smokeless tobacco; snus, snuff, and chewing tobacco. Snus is a finely ground tobacco, packaged as dry, moist, or in sachets.

The user places a pinch or dip between the cheek and the gum. Sniffing dry snuff through the nose is common in some European countries.

Chewing tobacco is available in loose leaf, plug, or twist forms, with the user putting a wad of tobacco inside the cheek. Smokeless tobacco is sometimes called "spit" or "spitting" tobacco because people spit out the tobacco juices and saliva that build up in the mouth[8].

3.3.2 *The chemical components of tobacco*

The tobacco plant is a species of the nightshade family, *Solanaceae*, which includes numerous varieties. The tobacco plant is not a typically tropical or subtropical growth, it can be grown in temperate areas with sufficient warm summers[7]. This plant is naturally rich in a variety of chemical components, such as tobacco alkaloids and tobacco specific nitrosamines (TSNA). In addition to the chemical components found in the tobacco leaves themselves, about 600 different additives are being added to the tobacco products during fabrication[9]. These additives and production methods vary from factory to factory[10]. Analyses of cigarette smoke have discovered over 4000 different chemical compounds[11]. About 30 carcinogens

have been identified in chewing tobacco and snuff. The tobacco specific N-nitrosamines (TSNA) and the polycyclic aromatic hydrocarbons (PAH) are among the major contributors to the carcinogenic activity of tobacco products[12].

3.3.2.1 **Tobacco alkaloids**

Tobacco alkaloids are a large group of nitrogen-containing compounds found in plants. Many of them affect the central nervous system and are strong poisons, or used as drugs[13]. The addictiveness of nicotine is the cause of the continuing use of tobacco products.

Nicotine is occurring to the extent of about 1.5% by weight in commercial cigarette tobacco and accounting for about 95% of the total alkaloid content [14, 15].

Snus and pipe tobacco contain concentrations of nicotine similar to cigarette tobacco, whereas cigar and chewing tobacco have only half of the nicotine concentration of the cigarette tobacco[14].

Nornicotine, anabasine, myosmene, nicotyrine and anatabine make up for 8-12 % of the total alkaloid content of tobacco products. In some varieties of tobacco, nornicotine concentrations exceed those of nicotine[16].

In animals, some of these alkaloids other than nicotine are pharmacologically active, but less potent than nicotine. Little is known about their effects in humans [15].

During the production and storage some of the tobacco alkaloids are chemically converted to carcinogenic nitrosamines compounds [15, 17].

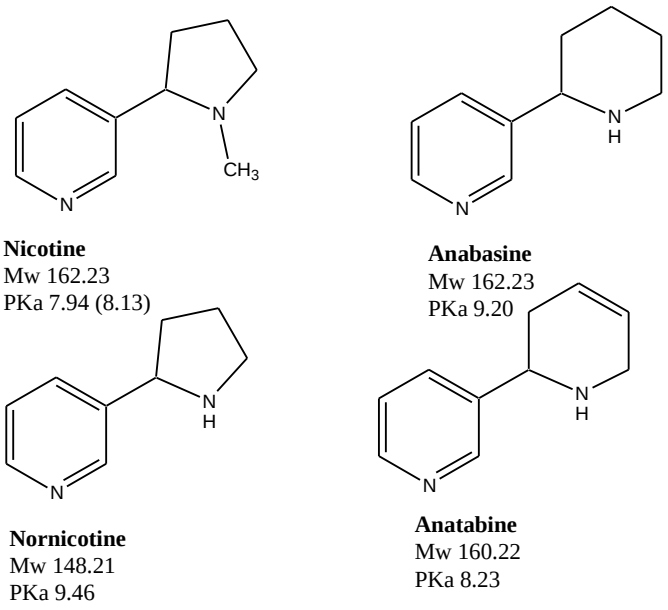


Figure 1 Structure of some tobacco alkaloids [14, 15, 17]

3.3.2.2 **Tobacco specific nitrosamnines**

Tobacco specific nitrosamines (TSNA) are compounds exclusively formed from the previously mentioned tobacco alkaloids. These derivatives of tobacco alkaloids arise by the action of nitrous acid on nicotine, nornicotine, anabasine, and anatabine during the processing, fermentation and aging of tobacco. Small amounts of these compounds, also called N'-methyl derivatives, are found in smoke and smokeless tobacco [12, 14]. The TSNAs are among the major contributors to the carcinogenic activity of tobacco use[12].

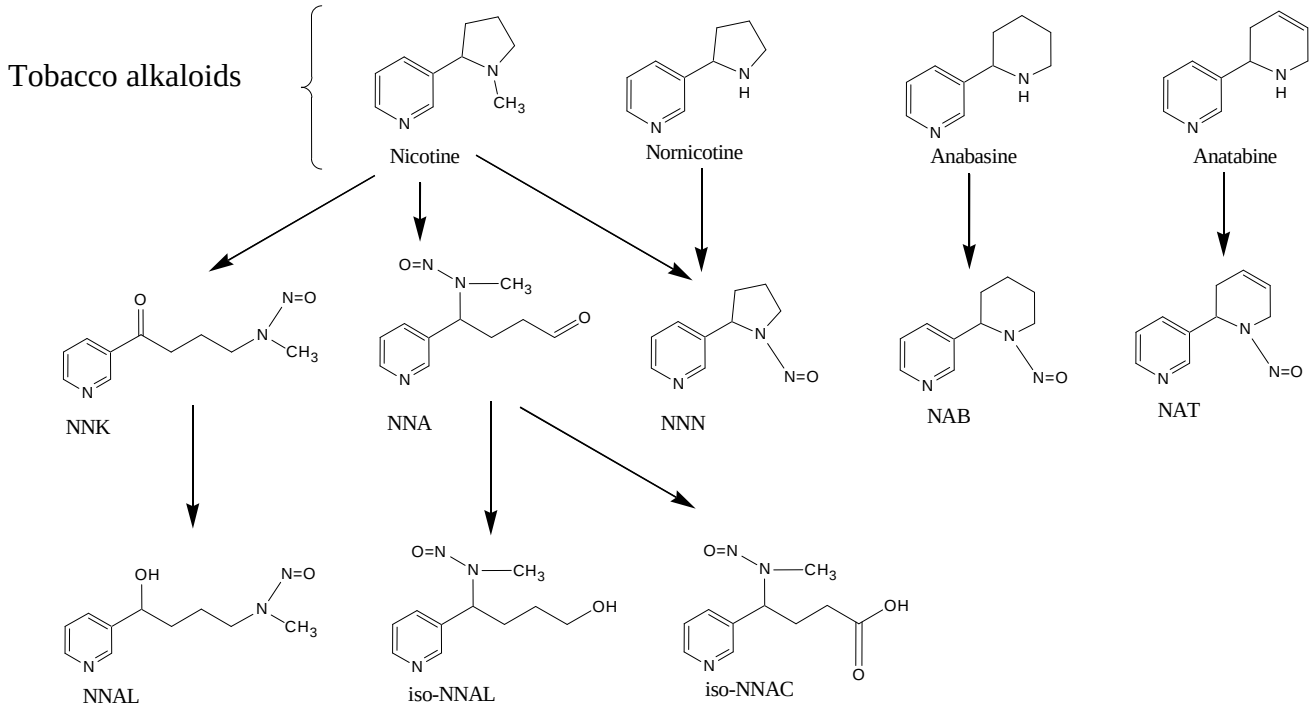


Figure 2 Structures of tobacco-specific nitrosamines and their precursors [18].

3.3.2.3 **Tobacco additives**

Natural herbs, spices, essences and artificial additives have been added to tobacco products for several years. Over 600 different additives are being added to tobacco products during fabrication[9]. These additives vary amongst the different tobacco products and from factory to factory[10].

A great number of tobacco ingredients are added to enhance the natural tobacco taste and to give the distinctive flavour characteristics of specific products and brands[19]. Other substances are added to tobacco in order to prevent rapid loss of moisture. These additives are called moisturizers. These make the tobacco moist and preserve physical and sensory properties for a long time. Frequently used substances in this group are glycerol, propylene glycol and dietylenglycol. In order to improve the physical properties of tobacco, texturizing agents are added. These compounds enable tobacco products to withstand mechanical stress during manufacturing and transportation. Naturally occurring components such as cellulose, dextrin, gum, pectin and starch are commonly used. Combustion modifiers are another group of additives, used in cigarette paper to control the combustion properties of cigarettes. Citric acid and phosphoric acid are examples of such compounds.

In cigarette paper, adhesives are another necessity. Starch, polyvinyl acetate and certain vinyl-based copolymers are commonly used. Last but not least, preservatives are added to protect the tobacco product against microbial decomposition. Relevant substances in this group are potassium salts of propionic acid, sorbic acid, benzoic acid and 4-hydroxsybenzoacid.

The amount of additives does in some cases exceed 10 % of the weight in U.S. cigarettes, of which sugars, flavouring and moisturizing agents make up the majority [20].

3.4 Nicotine

3.4.1 *Pharmacological effects and doping potential*

To ensure that the initiation of this project is justified, the effects of nicotine and its potential as a doping agent must be addressed.

If nicotine or any other substance or method shall be considered for inclusion on the Prohibited List of WADA at least two of the following criteria must be met:

- The substance or method (alone or in combination with other substances or methods) has the potential to enhance sport performance,
- the use of the substance or method represents an actual or potential health risk to the athlete
- the use of the substance or method is against sports ethics[21].

This must be supported by medical or other scientific evidence, pharmacological effect or experience. Whether or not nicotine in the form of smokeless tobacco meets these criteria is further discussed in this section.

Most athletes avoid using cigarettes because of the negative physiological effects of smoking. Smoking impairs performance by causing airway constriction, additionally the carbon monoxide in smoke combines with haemoglobin, making less haemoglobin available for oxygen transport, resulting in a decreased maximal oxygen uptake for heavy smokers[22]. Thus, professional athletes rarely smoke tobacco.

The prevalence of smokeless tobacco use amongst athletes is suspected to be high in certain sports on the other hand. Participants in team sports were remarkable smokeless tobacco users already in the 1980s, especially ice hockey and floor ball players. By the mid 1980s and early 1990s the prevalence of smokeless tobacco use among professional baseball players was about twice high that of the general population of males in the same age range. In the early 90s it was estimated that about 45 % of the premiere professional athletes in USA were using smokeless tobacco[23]. In 2003 the prevalence was still high, with 30–36% of the major league players reporting regular use[24]. Without doubt, smokeless tobacco use is popular amongst athletes, and incidences of smokeless tobacco use in athletics are likely to increase in parallel with the expanded use in society as a whole[25]. This trend could be explained by the athletes' repulsion of cigarettes, but there is some evidence supporting nicotine might be performance enhancing.

Nicotine action is mediated trough the nicotinic acetylcholine receptors. These receptors respond endogenously to acetylcholine in the periphery and central nervous system. Nicotine acts on these receptors and cause neuronal excitation.

The peripheral effects of nicotine are mainly caused by the excretion of catecholamine, such as adrenaline and noradrenaline from the medulla. When reaching receptors in heart and lung tissues, the catecholamine's causes cardiovascular effects. These effects consist of an increase in pulse rate and blood pressure and mobilisation of blood sugar and fatty acids [26]. Repeated exposure leads to development of tolerance to these effects, thus other mechanisms are more likely responsible of the performance enhancing capacities of nicotine.

It is suggested that nicotine might prolong endurance by a central mechanism that involves nicotinic receptor activation and/or altered activity of dopaminergic pathways[27]. This was demonstrated by a study of nicotine administration on exercise endurance. A significant improvement in performance was observed, with ten out of twelve subjects being able to cycle for a longer period with nicotine administration (17% ±7%, $P<0.05$)[27]. Additionally, nicotine is reported to improve coordination and produce faster motor responses[28]. These effects of nicotine could be beneficial in most sports.

Evidence is presented supporting the involvement of nicotinic acetylcholine receptors in relieves of anxiety[29]. As well as reducing anxiety, nicotine is reported to cause increased cognitive performance, arousing and alertness. These beneficial effect on mental capacity, has been demonstrated by a reduced stress-related decline in performance after nicotine consumption[26, 30] [29]. In sports like ski jumping, where control of the nerves is essential; use of nicotine could provide a considerable advantage.

Another effect of nicotine is weight loss associated with serotonin release[28]. In sports competing in weight categories such as different martial arts, a lower bodyweight would be beneficial. As well in many endurance sports lowering the bodyweight could be advantageous.

The suspicion about the doping potential of nicotine is confirmed by another study in which baseball players using smokeless tobacco were asked about the reason for their tobacco use. This study revealed use of smokeless tobacco because it helped them to relax. Players reported smokeless tobacco use as a coping strategy, or as a concentration or weight control aid, and some even believed that it helped to improve their field performance[24].

If nicotine has the potential to enhance performances, use of smokeless tobacco enables the athletes to benefit of them without most of the harmful effects associated with smoking.
If this is the case, nicotine may be used with the intention of artificially enhancing physical or mental capacities. The intentional use of a substance in order to enhance performance is defined as doping, and would violate the spirit of sports.
This evidence suggests that nicotine could meet one of the previously mentioned WADA criteria for inclusion of new substances and methods on the Prohibited List[21].

As previously mentioned, smokeless tobacco is associated with less risk than cigarettes; however the risk is still appreciable. A systematic review of the relation between smokeless tobacco and cancer in Europe and North America addressed the risk of developing pancreatic cancer. In this case, the authors concluded that the overall data suggest a possible indication that "smokeless tobacco" use increases the risk of developing pancreatic cancer[31]. Other published studies also support these findings [32, 33].

Snus has also been blamed for increasing the risk of developing a number of other cancers such as prostate, biliary cancer, bladder cancer, kidney cancer, larynx cancer, nasal cancer, haematopoietic and lymphoid cancers, however, more evidence is needed before one can deny or confirm this hypothesis[31]. There are as well great uncertainties concerning the potential risks for cardiovascular events associated with snus use. A systematic review concluded that it seems possible that there is a moderate risk of cardiovascular disease associated with the use of snus[34].

This opens for the discussion whether or not nicotine, meets the second criteria for inclusion on the Prohibited list; that the use of the substance or method represents an actual or potential health risk to the athlete[21].

If a substance meets two of the previously mentioned criteria, it should be considered for addition to WADA's "Prohibited List of Substances and Methods". However, lack of prevalence data on use and means to distinguish between smoke and smokeless tobacco consumptions is the main reason nicotine has not been considered for addition to this list. Development of such an analytical method is thereby valuable, and the initiation of the project is justified.

3.4.2 *Absorbtion and metabolism*

Nicotine is a weak base with a pKa around 8.0, but the presence of both a pyrrolidine and pyridine nitrogen give nicotine dibasic properties. At pH lower than 2.7, nicotine exists in the diprotonated form (two charges), at pH ranges from 4.5 to 7.0 it is dominated by the monoprotonated form (1 charge), and at pH above 7.0 nicotine is mostly neutral[35].

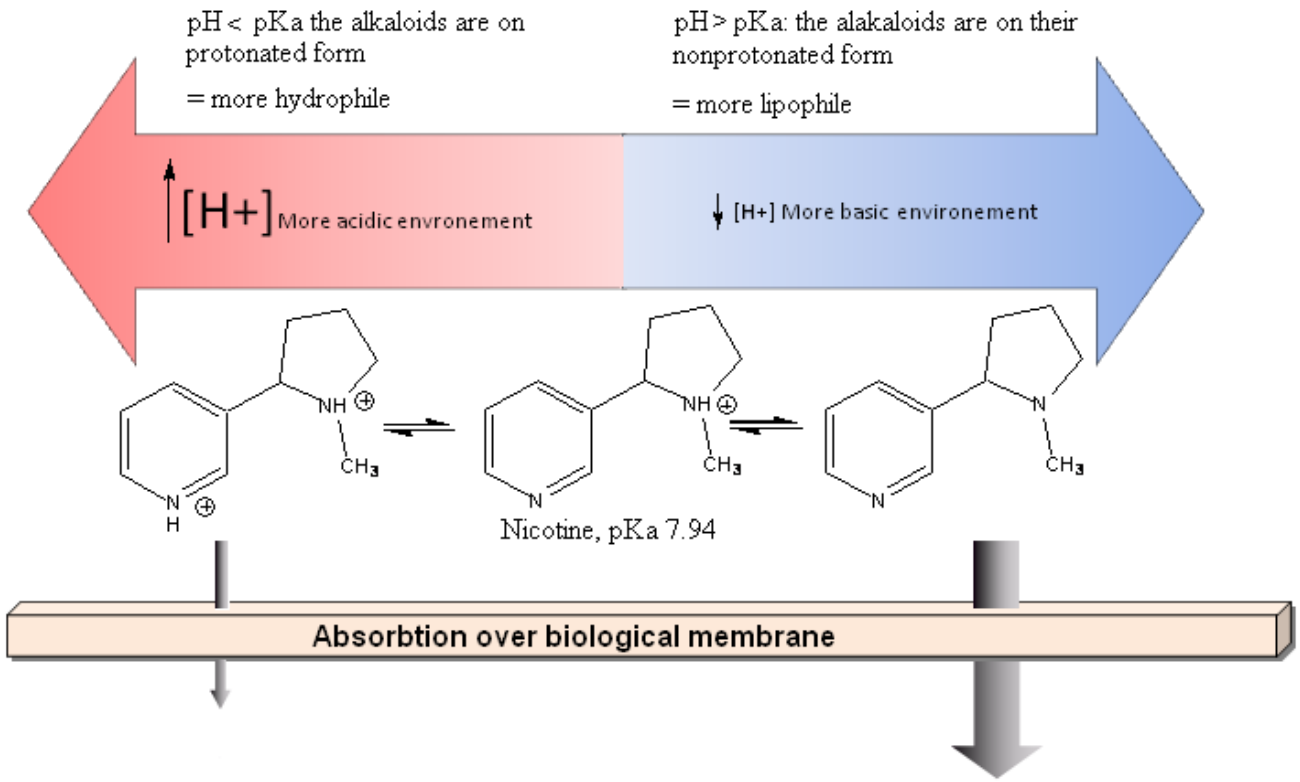


Figure 3 Illustration of the pH dependent absorbtion of nicotine

The rate of nicotine absorbtion through the biological membranes is a pH dependent process because unionised organic bases are lipophilic (fat soluble) while ionized organic bases are hydrophilic (water soluble). By increasing the amount unionised nicotine in relation to ionized nicotine, the physiological effects of nicotine are enhanced[19].

In tobacco smoke, the pH is acidic (about 6). As a consequence, only a negligible amount of nicotine is absorbed from the mucous membranes in the mouth because less than 1% of nicotine is unionised (free). By increasing the pH of smoke tobacco, the amounts of free nicotine are increasing. At pH 8.0, the amount of free nicotine is increased to approximately 50%. The main routes of nicotine absorbtion in smokers who inhale, are trough the alveoli of the lungs. When tobacco smoke reaches the small alveoli of the lung, nicotine is rapidly absorbed independently of smoke pH. This is presumably because of the huge surface area of the alveoli and small airways, which facilitate transfer across cell membranes.

The oral bioavailability of nicotine is about 45 %. Bioavailability is incomplete because of first pass metabolism. The nicotine absorbtion through the gastrointestinal tract is poor because of the acidic nature of the stomach juice, but is well absorbed in the small intestine, which has a more alkaline pH and a larger surface area[16, 26]. Snus and nicotine-containing medications are buffered to alkaline pH, thus absorbtion of nicotine through the oral mucosa is facilitated[19].

After absorbtion, nicotine enters the bloodstream and is distributed to body tissues. The plasma half life of nicotine after cigarette smoking averages about 2 hours because of the extensive metabolism of nicotine[14]. The nicotine metabolic pathway is complex and involves the formation of a number of Phase I metabolites through oxidation, hydroxylation and N-demethylation, and, also Phase II metabolites through conjugation with glucuronic acid[36] There are evidence of interindividual differences in nicotine metabolism. Polymorphism of the CYP 2A6 gene has a major impact on nicotine clearance. Gender-related effects, disease states, and various inhibitors and inducers affect individual rates of nicotine metabolism[14]. Quantitative aspects of the pattern of nicotine metabolism have been elucidated in humans. About 90% of a systemic dose of nicotine can be accounted for as nicotine and nicotine metabolites in urine[14] In average, 75% of the nicotine dose is converted to cotinine, which in turn is also extensively metabolized. About 10-15% of this cotinine dose is excreted unchanged in urine. The remainder is converted to other metabolites, mainly trans-3-hydroxycotinie [14, 16]. Nicotine, cotinine and trans-3-hydroxycotinieare all subjected to Phase II metabolism and are transformed to their respective glucuronide acid conjugates (See Figure 4). In most smokers, nicotine and these five metabolites constitutes > 80 % of the absorbed nicotine dose [37].

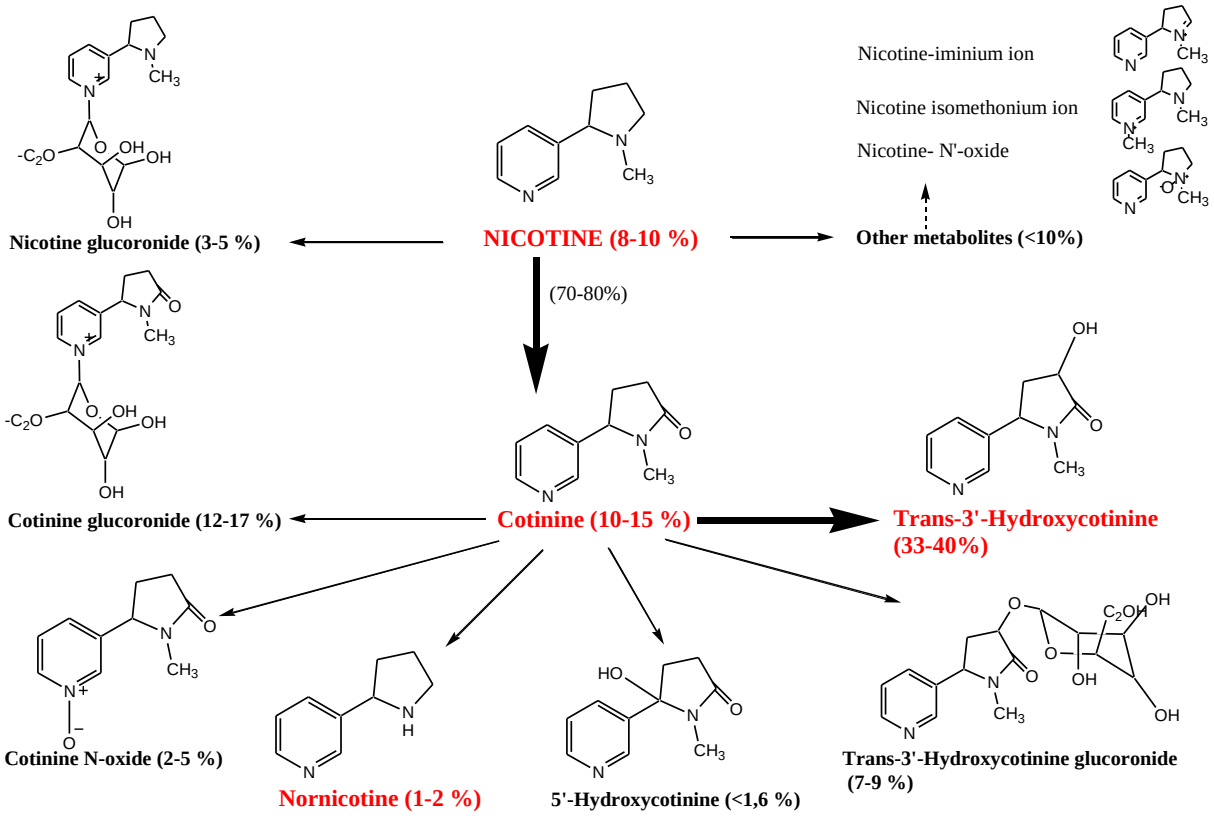


Figure 4 Primary routes of nicotine metabolism [25]

3.5 **Choosing Biomarkers for tobacco exposure**

In order to develop a method with sufficient discriminating power to distinguish between different tobacco consumption patterns, compounds of interest must be identified.

This molecule should meet following requirements on its own or paired with other molecules;

- tobacco specific, not measurable in significant amounts after exposure from other sources
- specific to a particular form of tobacco
- present in sufficient amounts to be detected in body fluids, preferentially urine

In addition, it would be beneficial if the compound was not metabolically derived from nicotine. This would be necessary in order to differentiate between nicotine from tobacco and nicotine containing medicine.

The amount of the different chemical compounds measured in body fluids will vary depending on the formulation of the tobacco product and their pharmacokinetic properties.

In the following sequences, different strategies of choosing these compounds will be considered. From this section on, these compounds are referred to as biomarkers.

3.5.1 *Major tobacco alkaloids*

A better strategy would be to have a closer look at the composition of the tobacco plants. This would greatly enhance the chances of finding a tobacco selective biomarker.

In general the presence of the tobacco alkaloids in human tissues can be attributed to use of tobacco products[11]. Though low levels of nicotine have been found in tea and edible plants, nicotine derived from dietary sources is insignificant compared with the amounts derived from tobacco use [11, 38]. Concentrations of nicotine and its metabolites in biological fluids are frequently used to ascertain whether or not a person is using tobacco and to estimate nicotine intake [15]. Because nicotine is almost extensively metabolized only a small percentage is excreted unchanged in urine (See section 3.4.2). Analysis of nicotine alone in biological samples can provide insufficient information due to its short half-life and the dependency of the analytical result on the time of sampling. Therefore it would be more useful to test for nicotine metabolites which have longer half-lives and detection windows compared to nicotine[36]. Cotinine and trans-3'-hydroxycotinine have longer half-lives and are therefore considered as potentially more useful biomarkers for the assessment of exposure to nicotine[36].

The ratio of nicotine:cotinine in urine of smokeless tobacco users and cigarette smokers showed a significant difference between groups in one study [38].This is a good indication that it might be possible to distinguish between consumption of the different tobacco products when comparing the ratio of these metabolites in urine samples. In most smokers, measurements of urinary nicotine, cotinine, trans-3-hydroxycotinine and their respective glucuronic acid conjugates accounts for more than 80 % of the absorbed nicotine dose[37]. Therefore nicotine and these 5 metabolites are of great interest as biomarkers in this project, (Figure 4). Because the glucuronic acid conjugate requires a more sophisticated and time consuming sample preparation than the other biomarkers of interest, they were not further investigated.

However, nicotine and its metabolites are present in nicotine-containing medications to various degrees. Discrimination between nicotine from smoke, smokeless tobacco and nicotine containing medication using only these biomarkers is probably insufficient.

3.5.2 *Minor tobacco alkaloids*

Minor alkaloids are present in tobacco, but not in nicotine-containing medications.

In most tobacco strains, nornicotine and anatabine are the most abundant of the minor alkaloids, followed by anabasine. This order of abundance is the same in cigarette tobacco and oral snuff, chewing, pipe and cigar tobacco. Nornicotine levels are the highest in cigar tobacco, and anatabine levels are lowest in chewing tobacco and oral snuff [14]. Anabasine as a percentage of the total alkaloids was significantly lower in oral snuff than in cigarette and pipe tobacco [15]. The low levels of anabasine in oral snuff are due to the conversion to N-nitrosanabasine and/or other degradation [15, 17]. Urine levels of the minor alkaloids are shown to correlate well with systemic nicotine intake from various tobacco products and could be useful to determine the origin of nicotine [15]. Urinary anabasine can be used as a biomarker of tobacco use to monitor compliance to nicotine therapy. Patients abstaining from tobacco typically use to have a urinary anabasine concentration < 2 μg/L. The mean anatabine concentrations in urine of cigarette smokers (22ng/ml) were about half those found in urine of smokeless tobacco users(41-45ng/ml), despite similar nicotine and cotinine levels, and although smokeless tobacco products contain considerably lower levels of anatabine than cigarette tobacco. A likely explanation is that anatabine is decomposed to a much greater extent than is nicotine in burning tobacco, resulting in lesser absorbtion by cigarette smokers than by smokeless tobacco users [38]. In subjects abstaining from tobacco but using nicotine gum, anabasine and anatabine levels have been found to be below the cut off point of 2 ng/mL[38]. This makes the minor tobacco alkaloids promising as biomarkers to distinguish between smoke and smokeless tobacco, as well as the use of nicotine containing medications.

3.5.3 *Tobacco specific nitrosamines*

The total amount of the TSNAs; NNN, NAT, and NAB, were significantly higher in urine of smokeless tobacco/snus users compared to smokers[39]. Daily exposure to tobacco-specific nitrosamines is estimated to about 20 μg in smokers and 68 μg in smokeless tobacco users. This is presumably due to the conversion of tobacco alkaloids into nitrosamines during the manufacturing and storage of smokeless tobacco products [15] (See Figure 5). Comparative

studies have generally shown lower levels of tobacco-specific nitrosamines in Swedish snus than in similar products sold on the American continent[32]. Because of the considerable differences in the concentration of the nitrosamines in the different brands of snus the nitrosamines are less promising as biomarkers. Therefore these compounds were not further investigated.

3.5.4 *Tobacco additives as biomarkers*

One possibility to discriminate between nicotine from smoke and smokeless tobacco could theoretically be based on differences in additives used during the manufacturing of the tobacco products. Due to legal requirements, any tobacco company that sells cigarettes in a European country must submit a list of additives to the Department of Health in the current country. A serious attempt to get access to this complete list of the chemical composition of the different tobacco products in Norway was performed. The tobacco industry considers these additives as a trade secret, and prohibits anyone who views the lists from sharing the content with external toxicologists or others, therefore this request was denied.

In 1994, a list of the 599 additives used in the manufacture of cigarettes by the five major American cigarette companies was published[9]. This list does not specify which compounds are used in the different brands and makes it impossible to identify ingredients common to one type of tobacco product.

Many of these substances are expected to be transformed formed during heating and / or combustion as well[19]. This would further complicate the use of additives as biomarkers.

In addition, most of the tobacco additives are used in the manufacture of cigarettes and other tobacco products approved for use by the FDA GRAS list[20]. This would mean that most of the additives are commonly used in food and commercial products; thereby their specificity as biomarkers to tobacco exposure is most likely insufficient.

Therefore neither tobacco additives were selected as biomarkers in this project.

3.5.5 *The selected biomarkers*

In order to develop a relatively time and cost efficient sample preparation step with a high discriminating potential, the following compounds were chosen to investigate their potential as biomarkers to distinguish between smoke and smokeless tobacco use:

- ✓ **nicotine** (major tobacco alkaloid)
- ✓ **cotinine** (nicotine metabolite, major tobacco alkaloid)
- ✓ **trans-3-hydroxycotinine** (nicotine metabolite, major tobacco alkaloid)
- ✓ **anatabine** (minor tobacco alkaloid)
- ✓ **anabasine** (minor tobacco alkaloid)
- ✓ **nornicotine** (minor tobacco alkaloid as well as nicotine metabolite)

Table 1 Concentration of the selected biomarkers expected to be measured in urine [15-17, 38, 40]

	Expected urinary concentration levels (ng/mL):			
	low	medium	high	non tobacco user
nicotine	2	250	1000	< 20
cotinine	2	375	1500	<20
trans-OH-cot	10	625	2500	<50
anatabine	2	50	200	<2
anabasine	2	50	200	<2
nornicotine	2	50	200	<15

3.6 Basic principles of the analytical procedure

3.6.1 *Sample preparation*

Urine is often the body fluid of choice for human exposure assessment due to a broad detection window and relatively non-invasive aspect of sample collection[41]. Urine is an aqueous solution consisting of mostly water, and about five percent metabolic wastes such as urea, dissolved salts, and organic compounds. In humans, all the water soluble wastes are excreted by the kidneys, highly diluted in the form of urine. The sample preparation step allows isolation of the compounds of interest and adequate up-concentration necessary in order to reach the limit of detection looked for.

3.6.1.1 **Solid phase extraction (SPE)**

In solid phase extraction the analytes are being retained on a sorbent that contains different functional groups depending on the type of column. As the solution pours trough the column, the analytes are being retained by strong interactions with these groups on the surface of the sorbent. The general setup for any SPE procedure consists of four steps: (1) conditioning the SPE material by means of methanol or acetonitrile to rise the functional groups, followed by water to remove the organic solvent form the cartridge, (2) application of the aqueous

biological sample to the SPE material, (3) removal of interferences by a washing step, and (4) eluting the analytes. The most widely applied packings are based on silica or chemically-modified silica. On these C18 – or C8-materials, the analyte retention is based on hydrophobic interactions. Another popular packing is copolymers in mixed mode materials, where the retention is based on combined hydrophobic interaction and ion-exchange interactions[42].

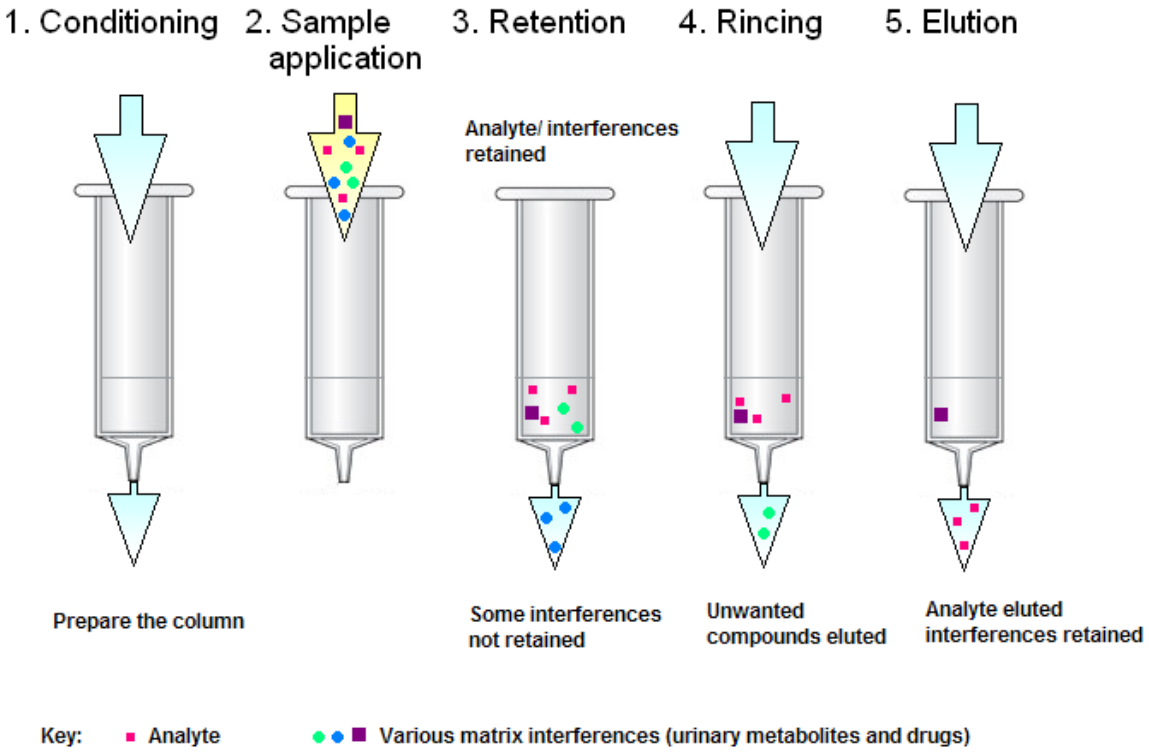


Figure 5 Schematic diagram of a solid-phase extraction procedure[1]

3.6.2 *HPLC (High performance liquid chromatography)*

Chromatography is a physical separation method in which the components to be separated are selectively distributed between two immiscible phases: a mobile phase flowing through a stationary bed. In liquid chromatography, this mobile phase is a liquid[42]. High performance liquid chromatography is basically a highly improved form of column chromatography where the solvent is being forced through the column under high pressures, which allows the use of smaller particle size for the column packing material. This provides faster and better separation of the analytes. The column is a steel tube filled with fine-diameter packing material. Liquid chromatography columns are typically 100-300-mm long and have an

internal diameter of 3-4.6 mm. The mobile phase is pumped from a bottle, trough an injector, into the column, and out to the detector. Before injection to the column, the sample should be dissolved in the mobile phase or a similar solvent. When injected, the sample flows with the mobile phase trough the column[42]. The chromatographic process occurs as a result of repeated sorption/desorption steps between the liquid phase and the stationary phase, during the movement of the analytes along the stationary phase. Figure 6 illustrates the intra- and intermolecular interactions between analyte molecules and mobile and stationary phase important in sorption/desorption.

HPLC can be divided into two categories depending on the relative polarity of the solvent and the stationary phase: Normal phase and Reversed phase.

In Normal phase HPLC, the column is filled with tiny silica particles, and the solvent is non-polar. Polar compounds in the mixture are tighter retained to the polar silica than non-polar compounds when eluting through the column. Therefore non-polar compounds will elute more quickly from the column[43].

Reversed phase HPLC is the most applied form of HPLC. By attaching long hydrocarbon chains to the surface of the silica, the columns are modified to become non-polar. Frequently used are the silica C18 columns, containing hydrocarbon chains with 18 carbon atoms. When a polar solvent is used, strong attractions between the polar solvent and polar molecules in the mixture will occur. Spending most of their time moving with the solvent, the polar molecules are less retained will elute from the column first[43]. Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of Van der Waals interactions (Figure 6).

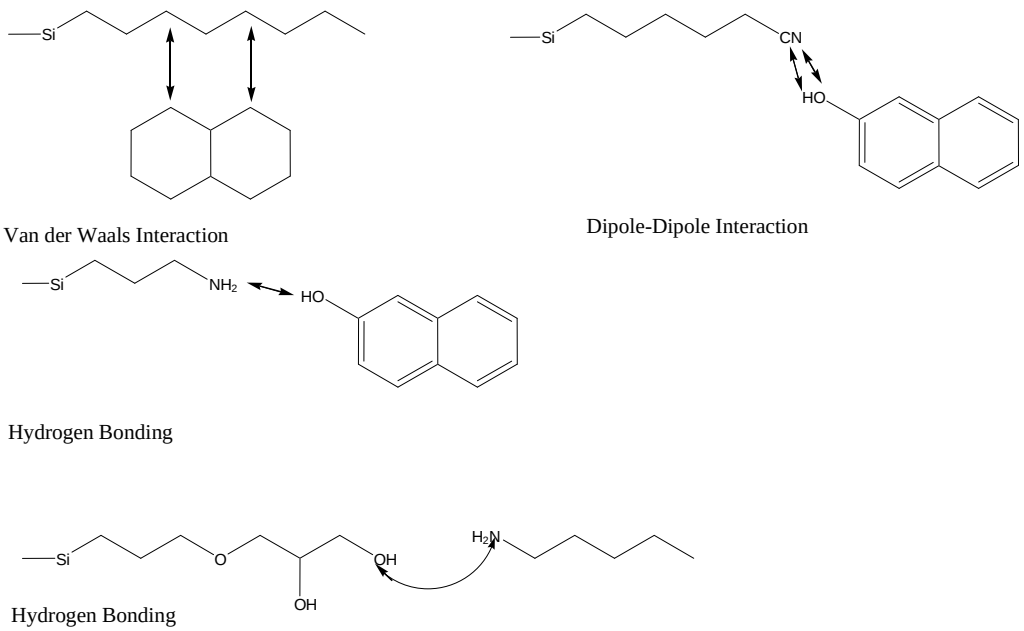


Figure 6 Various functional groups bound to the silica surface and various interaction between theese groups and different analytes[42].

The time taken for a particular compound to travel through the column to the detector is known as its retention time. This time is measured from the time at which the sample is injected to the point at which the analyser measures maximum peak intensity for that compound. Different compounds have different retention times. For a particular compound, the retention is influenced by the nature of the stationary phase, the eluent/solvent composition and pH together with the column temperature[42].

3.6.3 *Mass analyser (ESI-MS/MS)*

The separated analytes enter the mass spectrometer via the electrospray source. The eluent from the HPLC is nebulized into small droplets by a combined action of a strong electric potential between needle and counter electrode, and a high speed concurrent N_2 flow. This results in small droplets with an excess charge (positive or negative depending on the operation mode of the ion-source.) In their flight between the ESI needle and the ESI source block, neutral solvent molecules evaporate from the droplet surface. As a result, the droplet size decreases. This reduces the distance between the excess charges at the droplet surface. After some time, the surface tension of the liquid can no longer accommodate the increasing Coulomb repulsion between the excess charges at the surface. At this point, a Coulomb

explosion (Figure 9) leads to disintegration of the droplets. This process may be repeated a number of times, leading to smaller and smaller offspring droplets. Eventually gas phase ions are generated and can be mass analyzed[42].

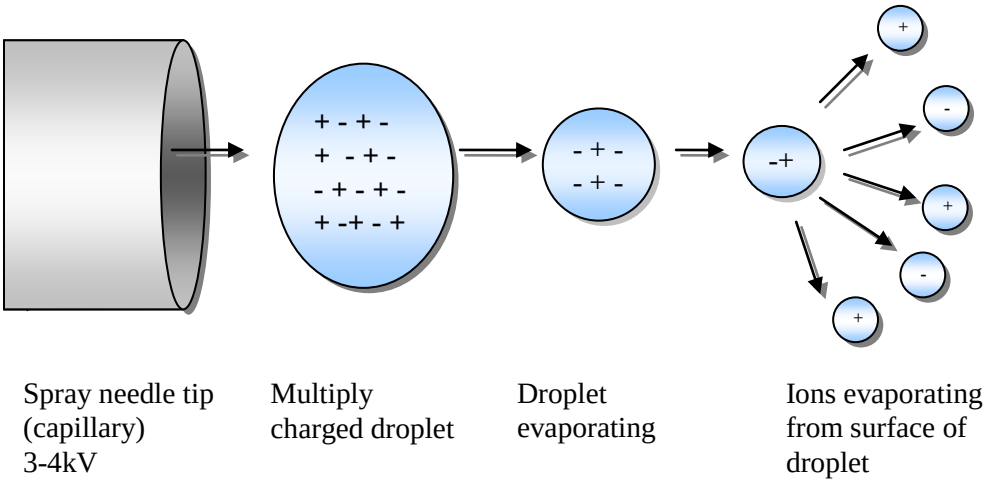


Figure 7 Illustration of the mechanism of Electron spray ionization [1]

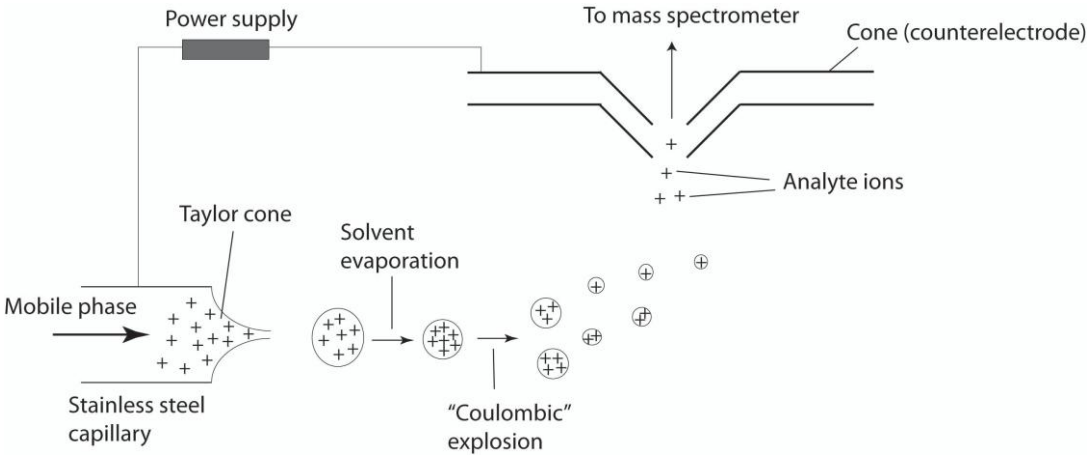


Figure 8 Electron spray ion source, reprinted with permission from Terje Wasskog

The mass spectrometer basically consists of five parts: sample introduction, ionization, mass analysis, ion detection, and data handling[42].

After the ions have been produced, separation and filtration according to their mass-to-charge (m/z) is performed on a mass analyser. Most commonly used for mass analysis, is the quadrupole mass filter. This mass analyser consists of four hyperbolic or cylindrical rods that are placed in parallel in a radial array. Opposite rods are charged by a positive or negative

direct current potential at which an oscillating radiofrequency alternating-current voltage is superimposed. Ions are introduced into the quadrupole filter, and begin to oscillate in a plan perpendicular to the rod length as they traverse trough the quadrupole filter. At a given combination of direct-current and alternating current applied to the rods, the trajectories of the ions of one particular m/z are stable, and are transmitted towards the detector. Ions with other m/z do not pass the filter because of their unstable trajectories, and are discharged on the rods and lost in the vacuum system[42]. Triple stage quadrupole (TSQ) system was designed to cleave ions into their daughter ions. Use of this triple quadrupole system allows so-called MS/MS analysis.

The triple-quad system consists of a scanning (Q1) quadrupole analyser for separating the original ion(s), an unscanned quadrupole that serves as a collision cell to fragment the ions sent to it by collision with a heavy gas molecule, and a scanning (q) quadrupole(/hexapole) that can separate the fragments produced in the (Q2) unit[43]. (Figure 9)

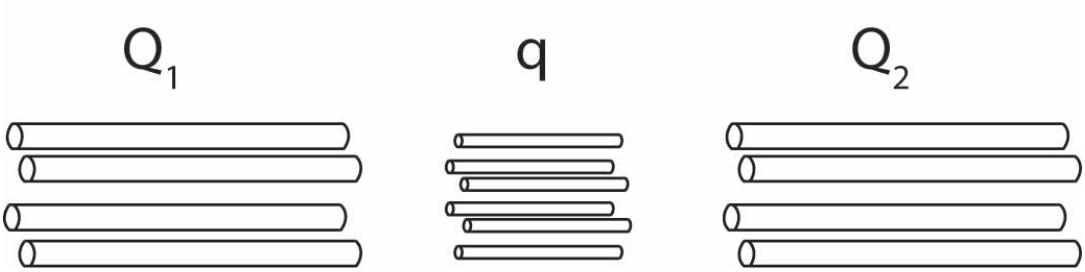


Figure 9 Triple quadrupole system, reprinted with permission from Terje Wasskog

The mass analyser system used in this project, a linear ion trap quadrupole (LTQ), is a square array of precision-machined and precision-aligned hyperbolic rods. Each rod is cut into three sections. Ions are ejected during scan out trough the center section rods. Quartz spacers act as electrical isolators between adjacent rods. The four rods of each section can be considered to be two pairs of two rods each. Application of voltage to the rod pairs produces a two dimensional quadrupole field with the mass analyser cavity which drives ionic motion in the redial direction[44]. Mass separation further based on the same principles as the TSQ.

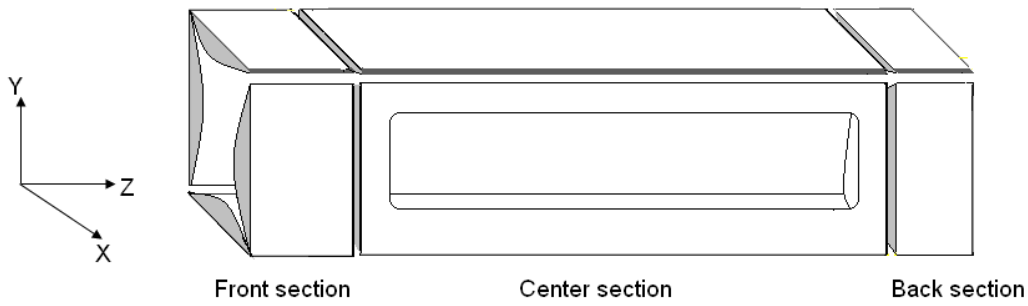


Figure 10 Linear ion trap quadrupole (LTQ) rod assembly [44]

Mass spectrometry can be performed in two general data-acquisition modes: full-spectrum analysis, where a series of mass spectra is acquired, and selected-ion monitoring (SIM), where the ion abundances of preselected ions are acquired. In quadrupole instruments, acquisition SIM mode provides a substantial gain in signal-to-noise ratio(S/N)[42, 43]. The following four different MS/MS operating modes exist:

Table 2 MS/MS operating modes

Q1	Q2	Technique
SIM	Scan	MRM
SIM	SIM	Product ion scan
Scan	SIM	Parent ion scan
Scan	Scan	Neutral loss

3.6.4 *LC-MS/MS*

An LC/MS system is an HPLC pumping system, injector and column coupled to a mass spectrometer through some type of evaporating ionizing interface (ESI). A computer system coordinates the components of the system together by providing control of the HPLC for flow, solvent gradient and remote starting of injection and the gradient run. It also provides control of the ion source parameters, mass spectrometry scan range and lens in addition to access and process data from the ion detector amplifier. The digital data is then processed by the computer software to provide a total ion chromatogram and the molecular weights of the compounds in the peaks detected using mass spectrometer's spectral data[42, 43]. Coupling MS with LC as a separation technique provides a valuable mean for identification and quantitation.

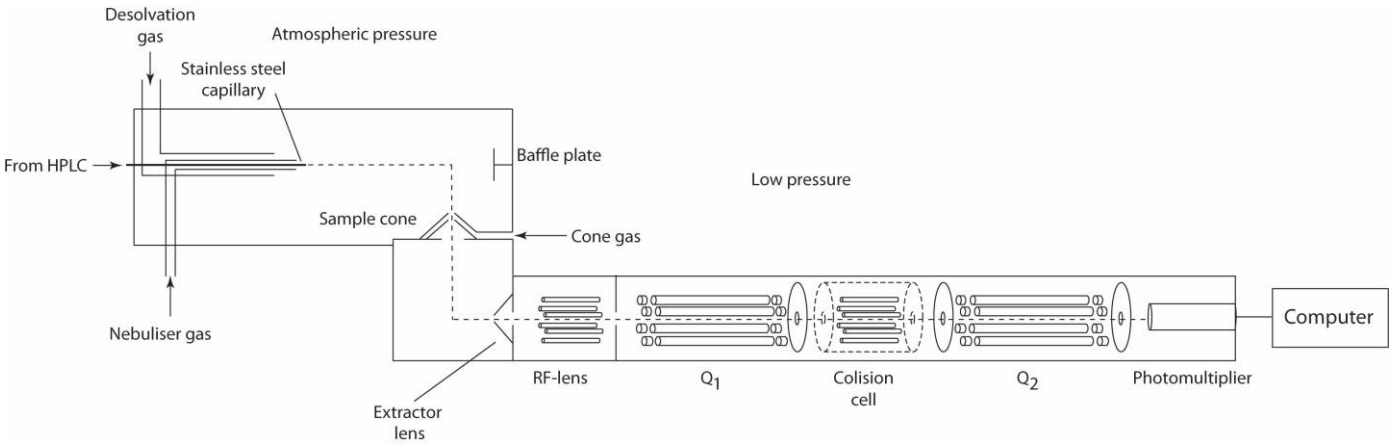


Figure 11 Overview triple quadruple-MS, Reprinted with permission from Terje Wasskog, the Finningan LTQ mass analyser is based on same principle, except separation is 2D instead of 3D as demonstrated in this figure.

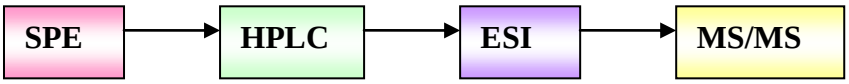


Figure 12 Overview of the analytical procedures used in this thesis

3.7 **Method validation**

Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurements of analytes in a given biological matrix is reliable and reproducible for its intended use. This following section is a brief presentation of important parameters of such a validation process.

The *precision* of an analytical method describes the closeness of individual measures of an anlayte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. In order to meet both FDA [45] and IHC[46] guidelines, precision should be measured using a minimum of 9 determinations over a minimum of 5 concentrations levels covering the specified range. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (or 20% for the LLOQ). Precision is subdivided into estimates of repeatability (CVr), and intermediate imprecision (CVR). Repeatability expresses the precision within the same run, while

intermediate imprecision express precision between measurements performed in different days. Precision is for both categories expressed as RSD, relative standard deviation (%).

Trueness, is described as the closeness of agreement between the average value obtained from a large serious of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias. Trueness is related to the systemic errors of an analytical procedure[46].

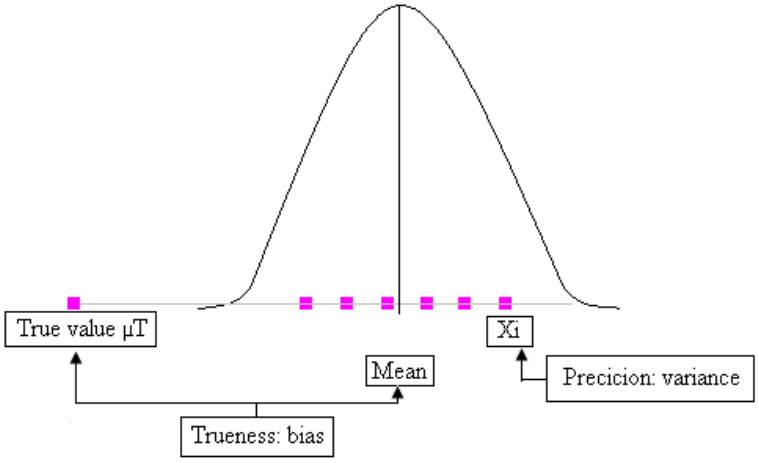


Figure 13 Illustration of validation parameters, trueness

The *accuracy* of an analytical method describes the closeness of mean test results obtained by the method to the true value of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of 5 determinations per concentration. A minimum of 3 concentration in the in the expected concentration range is recommended by the FDA[45] ICH, on the other hand suggests a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range[46]. The mean value should be within 15% of the actual value (20% for LLOQ). The deviation of the mean from the true value serves as the measure of accuracy. Accuracy is defined as the percent relative error (%RE) and was calculated using the following formula $\%RE = (E - T)(100/T)$ where E is the experimentally determined concentration and T is the theoretical concentration[45]

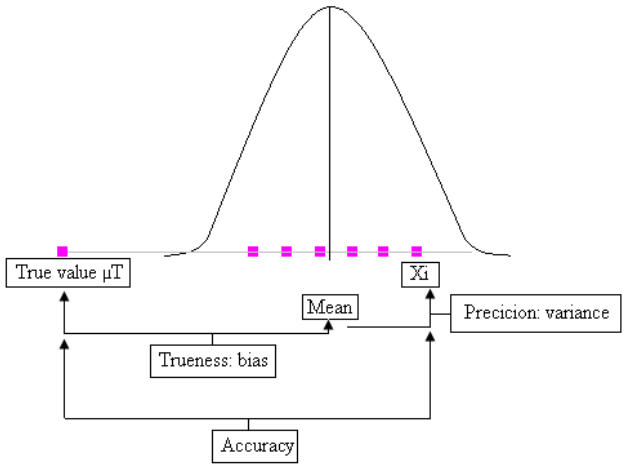


Figure 14 Illustration of validation parameters, accuracy

The *linearity* of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Method linearity should be determined concurrently during the accuracy study. Correlation between recalculated values and theoretical values, expressed as y = ax + b, y is the recalculated values, x is the slope of the curve, x is the theoretical values and b is the value where the curve crosses the y axis. Slope values should be as close to 1 as possible, and R^2 values above 0,995, for the method to be considered linear and valid over the selected range. The measured values should be as close as possible to the theoretical values[45].

The true performance of the method-bias and precision are unknown. The experiments performed during the validation phase will only provide estimates of bias and precision. How reliable those estimates are depends on the experiments. Design and sample size have to be carefully chosen in order to obtain reliable estimates.

Detection limit (LO) is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value[46].

Quantitation limit (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantititative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products [46].

Selectivity is defined as the ability of a method to differentiate an analyte in the presence of other components in the sample. Selectivity should be assessed by analyzing at least six sources of blank samples of appropriate matrix. Each blank should then be tested for interference and selectivity should be established at the LOQ[45].

The r*ecovery* of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector respons obtained for the true concentration of the pure standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but consistent, precise and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentration levels (low, medium and high) with unextracted standards that represents 100% recovery.

Stability of the analyte in biological matrix at intended storage temperatures should be established. The influence of freeze-thaw cycles should be tested by a minimum of 3 cycles at 2 concentrations in triplicate[45].

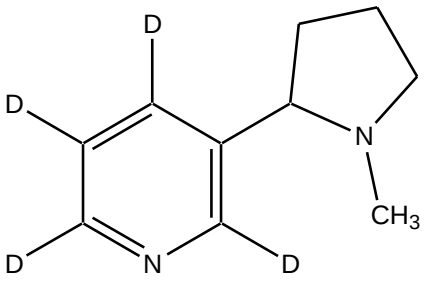
4 EXPERIMENTAL

4.1 Reagents and chemicals

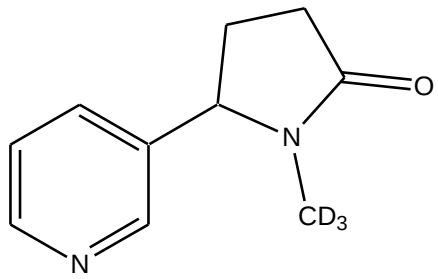
Nicotine, cotinine, anabasine and anatabine and were purchased from Sigma-Aldrich (produced by Fluka). Trans-3'-hydroxycotinine and anatabine were obtained from Toronto Research Chemicals. The internal standards (R,S)-Anatabine-2,4,5,6-d4 and Trans-3'-hydroxycotinine- methyl-d3 were purchased from Toronto Research Chemicals, whereas Nicotine-D4 was supplied by LGC Standards, (produced by Cerillant) and Cotinine-D3 was supplied by Prochem, (produced by Cerillant). Methanol (> 99, 9%) was obtained from Merck Chemicals (Darmstadt, Germany). Acetonitrile HPLC (> 99, 9%) was purchased from Biosolve Chemicals (Valkenswaad, Netherlands). Ammonium hydroxide (>25 %) was supplied by Sigma-Aldrich (produsedby Fluka Analytical)
KH_2PO_4(99.5-100.5 %) , Na_2HPO_4 (>98 %) and HCl(>37 %) fuming was purchased from Sigma-Aldrich (St. Louis, USA). Formic acid was supplied by Sigma-Aldrich, produced by Fluka Analytical. NaOH pellets (>99 %) was purchased from VWR International (Leuven Belgium). Liquid nitrogen TP 100 and Helium (99%) Alphagaz-HE (collision gas) was

supplied by Carbagas(Domididier, Switzerland). The nitrogen gas was produced by the N2-Mistral-Ofrom DBS nitrogen generator. Purchased from Analythical Instrument (Vigonza, Italy). All chemicals and reagents were HPLC grade (≥99% purity)

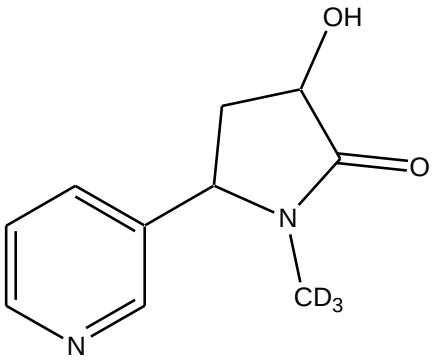
Ultrapure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a Quantum™ EX Ultrapure organex cartridge purchased by Millipore Corp.



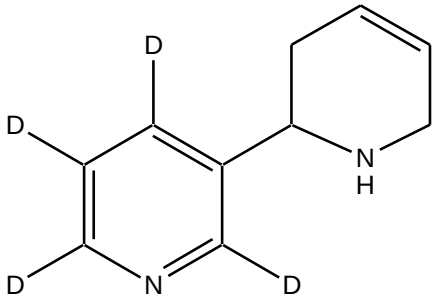
Nicotine-d4
Mw 166.26
pKa 7.94 (8.13)



Cotinitine-d3
Mw 179.11
PKa 4.88



Trans-3'-hydroxycotinine- methyl-d3
Mw 195.23
PKa 4.50



(R,S)-Anatabine-2,4,5,6-d4
Mw 164.24
PKa 8.23

Figure 15 Chemical structures of reference standards

4.2 Material and equipment

The LC-MS/MS system used for the assay comprised a Rheos 2000 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and a HTS Pal autosampler (CTC analytics AG,

Zwingen, Switzerland) coupled to a linear ion trap mass spectrometer LTQ-MS (ThermoFinnigan, San Jose, CA, USA), equipped with an atmospheric pressure ionisation(API) interface, Ion MAX™. Mass spectrometric analysis was conducted using a Quattro Premier XE™ triple quadrupole mass spectrometer (Waters ® Corporation, Milford, MA) with MassLynx™ v 4.1 software. The separation was performed on a Luna® HILIC Phenomenex 150 x 2.0 mm column with 3 µm particles, 200 Å pore diameters.

For evaporation of the samples, a Pierce ReactiVap$_{TM}$ III Evaporator with 24 ports, (Pierce&Rochford, USA) was used. For centrifugation a Multifuge 3S from Thermo electron corporation (Osterode, Germany) or a Heraeus Multifuge 3S+ from Thermo Scientific (Osterode, Germany) was used. Oasis® HLB (1cm3 x 30 mg) SPE cartridges was obtained from Waters Corporation (Milford, MA, US) Visiprep^{TM} 24 was used for extraction of all the samples prepared by SPE, and the vacuum pump used was a KNF neuberger vacuumpump (Balterswil, Switzerland).

4.3 **Sample preparation and extraction**

One ml urine sample and 1 ml phosphate buffer (0. 2 M, pH 7) were transferred into a tube and 10 µL of a 10ng/mL internal standard solution was added. The samples were centrifuged for 10 min at 3500 rpm.

The Oasis HLB (1cm^3 x 30 mg) SPE cartridges were conditioned with 1.0 ml of methanol, 1 ml of H_20 prior to loading with 2 ml of buffered sample solution.

1 ml of 2% NH_4OH/H_2O solution was used to rinse the cartridges prior to eluting with 1 mL MeOH.

Extracts were collected in conical tubes and evaporated carefully to dryness under a stream of air at 50 °C. Extracted urine residues were reconstituted in 500µL of a solution of the HPLC mobile phase constituents' acetonitrile and formate buffer (0.1 M, pH 3) in 90:10 (v/v), and transferred into microvials. This SPE technique was developed on the basis of a previously published method:[41].

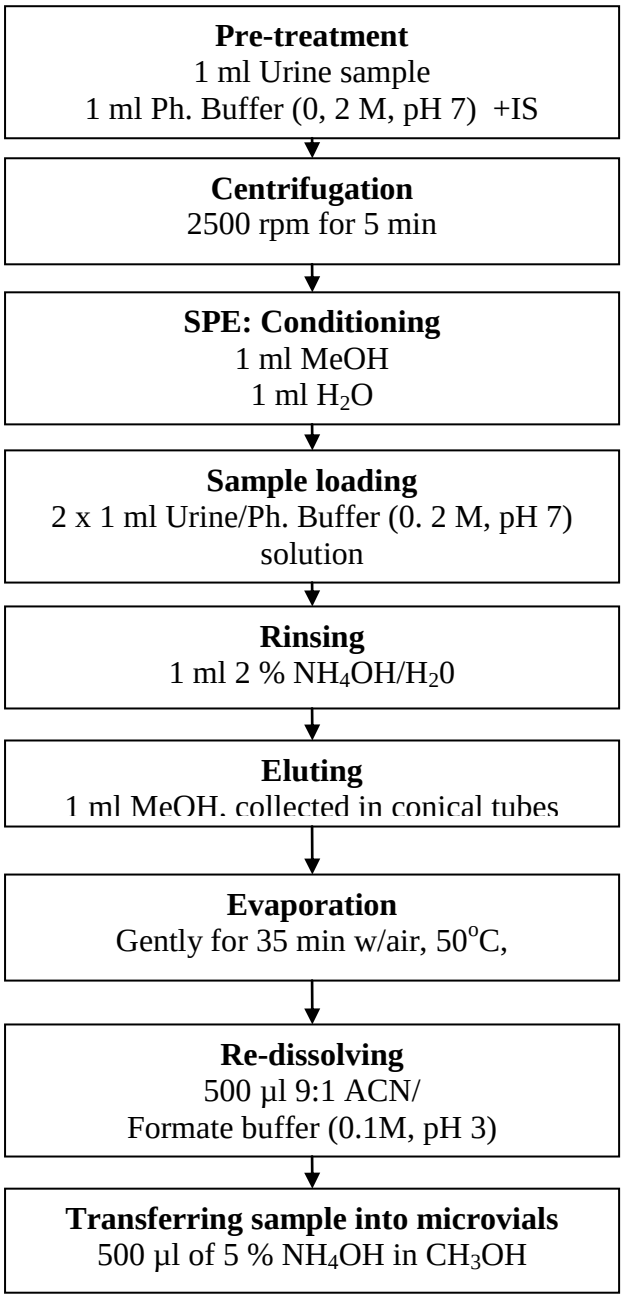


Figure 16 Extraction method used on Oasis HLB cartridges

4.4 **Liquid chromatography- tandem mass spectrometry conditions**

For the chromatographic separations, 0,1M ammonium formate buffer, pH 3 and acetonitrile were used as mobile phase with the following gradients:

Table Table 3 HPLC optimal gradient table

No	Time (min)	% Solvent A	% Solvent B	Flow rate (µl/min)
0	0	2	98	250
1	3	2	98	250
2	7	65	35	250
3	10	65	35	250
4	11	2	98	250
5	13	2	98	250

Solvent A: Formiate buffer pH 3
Solvent B: Acetonitrile

Before every set of analyses a conditioning step was performed at a flow rate of 250 µL min−1 for 10 min, the injected volume was 10 µL. The column and the autosampler tray temperatures were set at 30 °C and 4 °C, respectivley.

The mass spectrometer was operated in positive ESI mode. One MRM transitions was monitored for each analyte. MS operating conditions were set as follow: spray voltage= 5.0 kV; heated capillary voltage and temperature of 10V and 320°C, respectively; ionization width of 1.5 Da; activation time= 30 ms; activation q of 0.250 and scan time was fixed at 30 ms. Sheath gas, auxiliary gas and sweep gas (nitrogen) were set at 20, 5 and 1,5 respectively. Analyte-specific normalized collision energies, MRM transitions and retention times are provided in table 5.

Table 4 Liquid chromatography-electro spray ionization-tandem mass spectrometry parameters

Analyte	**tR (min)**	**tR SD (min)**	**Collision energy (AU)**	**MRM transitions**
Nicotine	3.38	0.19	25	163.10 → 132
Nicotine-d3	3.39	0.04	25	167.10 → 134
Cotinine	2.19	0.01	28	177.10 → 98
Cotinine-d3	2.16	0.18	28	180.10 → 100
Trans-OH-cot	2.36	0.01	26	193.10 → 134
Trans-OH-cot-d3	2.37	0.01	26	196.10 → 134
Anatabine	5.28	0.15	23	161.10 → 144
Anatabine-d4	5.3	0.14	23	165.10 → 148
Anabasine	6.21	0.16	25	163.10 → 146
Nornicotine	7.03	0.07	26	149.10 → 132

4.4.1 *Method Validation*

4.4.1.1 **Collection of anayte free urine**

Analyte-free urine used in method development and validation was collected from nicotine-abstinent members of the Swiss anti doping laboratory, unexposed to environmental smoke the last 48 h.

For the quantitative validation, urine samples were collected from two individuals at 9 am of the initiation day. The urine was merged in one bottle, stored in the fridge and used during the following days of the validation.

4.4.1.2 **Choice of internal standards**

As internal standard the deuterated standards of nicotine, cotinine, trans-3-hydroxycotinie and anatabine, respectively nicotine-d4, cotinine-d3, trans-3-hydroxycotinie -d3 and anatabine-d4 were purchased. These are presented with structural formulas in Table 16.

When calculating the peak area ratios, the peak area of the standard was divided on the peak area of the respective internal standard. The internal standard was not available for the compounds anabasine and nornicotine, thus anatabine-d4 was used as refrence standard for these two analytes as well. Since anatabine, anabasine and nornicotine have quite similar chemical properties, it was defendable to use anatabine-d4 as internal standard for anabasine and nornicotine as well.

4.4.1.3 **Assessment of specificity**

The specificity of the method was assessed by the analysis of analyte-free urine samples from six individuals. All individuals were nicotine abstinent and non-exposed to significant amounts of environmental smoke the last 48 hours. The urines were collected from both males and females, all personnel within the Swiss anti doping laboratory. In addition, urine of one child was analysed as well. Each sample was treated according to the developed method to highlight the absence of interfering peaks observed at the retention times for nicotine, nicotine-d3, cotinine, cotinine d-3, trans-3-hydroxycotinie, trans-3-hydroxycotinie -d3, anatabine, anatabine-d4, anabasine and nornicotine. For each individual urine, following analysis were performed; one analysis of pure (analyte free) urine, a second analysis of pure urine spiked with 100 ng/mL of the respective IS. And a third analysis of pure urine spiked spiked with100ng/mL of anabasine and nornicotine standards, since their respective internal standards were not available. In total18 urine samples were extracted and treated to assess the specificity of the analytes.

4.4.1.4 **Quantitative analysis**

Quantitative analysis was performed for all the selected biomarkers within the range of 10 to 10000 ng/mL for nicotine, cotinine and trans-3-hydroxycotinie, and the range of 10 to 500 ng/mL for anatabine, anabasine and nornicotine.

The calibration process was performed over three days. Calibration standards at two concentration levels (k=2) and validation standards at three concentration levels (k=3) were prepared in triplicate (n=3) for each day. Concentrations levels of the calibration and validation standards are given in Table 6.

Table 5 Table of spiked amounts of calibration and validator standards (ng/ml)

Analyte	Calibration standards (ng/mL)	Validator standards (ng/ml)
Nicotine	10, 10000	10, 5000, 8500
Cotinine	10, 10000	10, 5000, 8500
Trans-OH-cot	10, 10000	10, 5000, 8500
Anatabine	10, 500	10, 250, 400
Anabasine	10, 500	10, 250, 400
Nornicotine	10, 500	10, 250, 400

Calibration curves for each analyte were plotted with peak ratio values of analyte peak area over internal standard peak area. Estimates of trueness, repeatability and intermediate precision were calculated at each concentration level. Trueness was expressed in percent as the closeness of agreement between the average concentration obtained from the measurements and the theoretical concentrations. Assessment of precision was performed by calculating repeatability (CVr), expressed the precision within the same run, and intermediate imprecision express as precision between the measurements of different days. Both repeatability and intermediate precision was expressed in percent as relative standard deviation (RSD).

4.4.2 ***Method application to snus and cigarette samples***

The present analytical procedure was applied for the determination of nicotine, cotinine, trans-3-hydroxycotinie, anatabine, anabasine and nornicotine in urine of one smoker (female) and one snuser (male). Both volunteers had been nicotine deprived over night.

The first urine sample was collected one hour after consuming of one unit of the respective tobacco product. The next three urine samples were collected throughout the day. Two calibrators at the LOQ and ULOQ determined during method validation were used for the generation of the calibration curve. The ability of the assay to determine reliable concentrations of the analytes was assessed by using Quality Control (QC) samples. Two quality control (QC) samples were prepared at a one concentration level (100ng/mL, of each analyte and internal standard). The QC samples were used as a reference to determine suitability of the calibration curve to calculate concentration levels in unknown samples. The obtained calibration curve was used to back-calculate the concentrations of samples. Trueness, expressed as bias, was determined at each concentration level.

5 RESULTS AND DISCUSSION

5.1 Method development

5.1.1 *Chromatography and mass spectrometry*

5.1.1.1 Selecting MS parameters

Each compound was infused separately by a Hamilton syringe (250µl) to the MS for detection of the precursor and fragment ions. The daughter ion with highest intensity that was specific for its representive biomarker. See table 3

Collision energies (AU) were selected for the individual analytes through manual tuning. The optimal collision energy that optimized the fragmentation of the precursor ion towards the fragment ion response was chosen in order to obtain the highest abundance response of the fragment ion. See Table 3 and 4. Appendix for the respective ms-spectres for the chosen transitions for each analyte.

5.1.1.2 Selecting chromatographic parameters

When choosing HPLC column, a Waters X Bridge C18 column, (150 x 2.0 mm, with 3 µm particles) was compared to a Luna® HILIC Phenomenex (150 x 2.0 mm, 3 µm particles) column. On the Waters X Bridge column, which is a traditional reversed- phase chromatography column, the retention decreases with increasingly polarity of the analyte. In this project, tobacco alkaloids should be separated. The tobacco alkaloids are basic

compounds, and are on ionic form when the pH is below their pKa value. On ionic form, such analytes show little retention on traditional C-18 sorbents.

A buffer is frequently used to control the pH, and indirectly the degree of ionization of such polar compounds. Phosphate buffers are widely applied in this purpose, since they cover a wide pH range and show good buffer capacity. However, in LC-MS/MS application, phosphate buffers must be replaced by volatile alternatives, such as ammonium formate[42]. These buffer solutions have inferior buffer capacities than the phosphate buffers, thus they are less suited to prevent ionization of the analytes. Because of this problem, the polar alkaloids are expected to have poor retention on this column, which was demonstrated during method development experiments. On this column, it was difficult to obtain good separation of the analytes. Compositions of mobile phase are given in table 4 in the appendix.

The Luna® HILIC Phenomenex columns retain a water-enriched layer on the surface of the silica, which facilitates the transfer of polar compounds into the stationary phase for increased retention. Separation is achieved through the partitioning of polar solutes from a high concentration, water-miscible, organic mobile phase into this hydrophilic environment, resulting in increased retention of polar solutes and elution in the order of increasing hydrophilicity[47]. On this column, ionization of the alkaloids is an advantage, providing optimal condition for separating the selected biomarkers by hydrophilic interactions with the sorbent of the column. This column allows retention for polar compounds such as the alkaloids, and the elution order is typically the opposite of that for reversed phase with the most polar compounds eluting after the non-polar compounds, resulting in an alternative selectivity[2, 47]. As expected, better separation was observed on this column compared to the Waters-X-bridge. Satisfactory separation of all the analytes was obtained; therefore this column was further used in this project. Ammonium phosphate buffer solution (0,1M pH 3) and acetonitrile was chosen as mobile phases. Experiments with different mobile phase compositions were performed, demonstrating better separation obtained by graded mobile phase composition than with isocratic. The best suited gradients for separating the analytes on the Luna® HILIC Phenomenex column is given in Table 2.

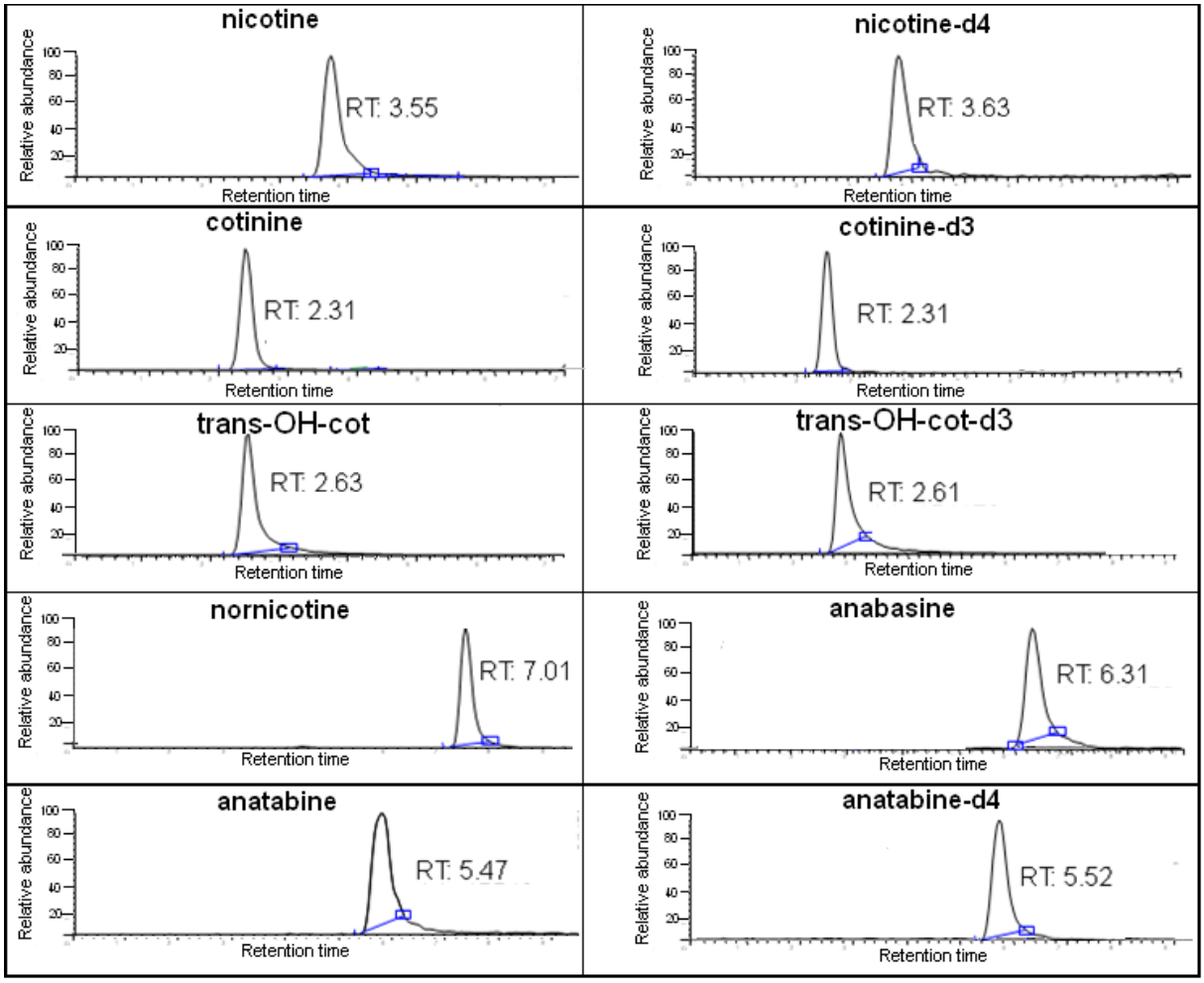


Figure 17 Chromatogram presenting separation of the selected analaytes obtained on the Luna® HILIC Phenomenex column. RT: retention time.

5.1.2 *Sample preparation*

5.1.2.1 **LLE vs. SPE**

Several sample preparation techniques for nicotine and its metabolites in biological specimens have been described in the literature; protein precipitation, centrifugal clarification, solid phase extraction and liquid-liquid extraction [41, 48-50].

Solid phase extraction methods gave the best peak-to-noise ratios in initial experiments, and were therefore chosen for further investigation.

5.1.3 *SPE*

5.1.3.1 **HLB**

The alkaloids to be extracted with the same sample preparation step had pKa values between the range of 4.5 (trans-3 `hydroxycotinine) and 9.5(nornicotine).

The Oasis HLB sorbent is a copolymer designed with both hydrophilic and lipophilc groups. This allows retention of respectively both hydrophilic and lipophilic substances. All the 6 biomarkers selected in this project are basic compounds. Subjected to an acidic environment, the alkaloids are on their ionised form. This allows a stronger interaction to the hydrophilic parts of the HLB sorbent.(Figure 18) In the selected sample preparation method, phosphate buffer (0.2 M, pH 7) was used to stabilise the urine samples. Trans-3 `hydroxycotinine and cotinine have pKa values below this pH. However, the abundance of these metabolites is relatively high compared to the minor tobacco alkaloids like anabasine, anatabine and nornicotine. Therefore a potential lower theoretical recovery of these biomarkers could be acceptable.

Oasis HLB

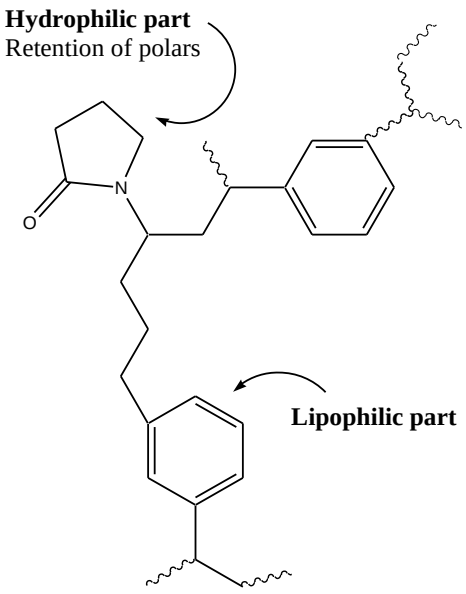


Figure 18 Illustration of the sorbent used in the Oasis HLB cartridges[51]

5.1.3.2 **Selecting buffer solution**

During the method development of the HLB extraction, a triiodophosphate(pH 13) buffer solution was compared with a phosphate(0.2 M , pH 7) buffer solution. The exact same SPE

procedure and sample analysis was performed for the two different buffer solutions (See figure 18).

After diluting the urine sample with the tiiodophosphate buffer, the pH of the sample was about 12. At this pH, all six compounds are almost entirely on their unionised form. Under such conditions, the alkaloids are expected to be better retained by the lipophilc groups of the sorbent. Theoretically this would further enhance the recovery of the six biomarkers. However, experimentally, using the triiodophosphate buffer, demonstrated only a marginal improvement of peak intensity compared to the phosphate buffer.

The phosphate buffer was selected for this SPE procedure for two reasons; 1) the phosphate buffer solution had lower cost than the triiodophosphate buffer, and 2) the triiodophosphate buffer had a greater tendency to clog the SPE cartridges during the extraction than the phosphate buffer, causing greater concerns regarding the reproducibility of the procedure.

5.1.3.3 **HLB vs MCX**

The Oasis MCX (Mixed mode Cation eXchange) polymeric sorbent are designed for solid-phase extraction of basic compounds from biological matrices (Figure 19). The anionic group on the sorbent allows strong ionic interactions with the alkaloids when they are on their protonated from. Theoretically, this sorbent would provide better recovery compared to HLB where the retention of the analytes is only based on the weaker lipophilic/ hydrophilic interactions (Figure 18), thus following MCX extraction method was investigated:

The principal behind this method consisted in acidifying the urine with a HCl solution to ionize the alkaloids, and prevent them from binding with proteins. In the next step, the cartridges were washed with HCl to assure that the ionized alkaloids got attatched on the ion exchanger and proteins removed. Then a second washing step was performed in order to remove neutral and acidic interferences trapped by the reversed-phase mechanism of MCX. In order to neutralize and elute the alkaloids, a methanolic base was used. The elute was collected and further treated as described in the method used on the HLB cartridges. See Figure 35, in appendix for illustration of the method.

Osais MCX

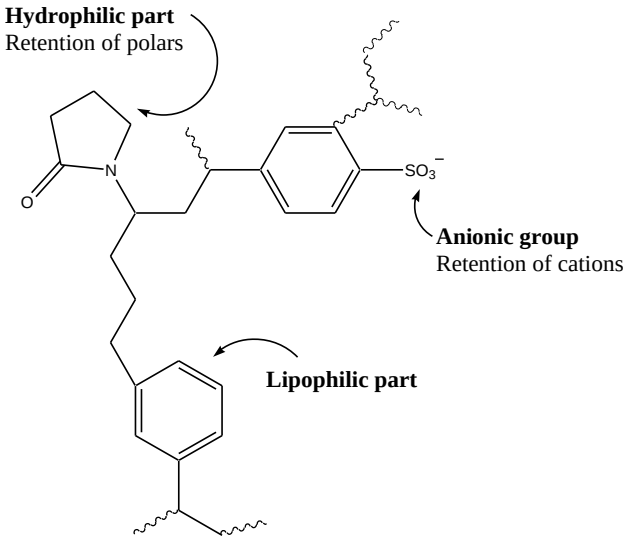


Figure 19 Illustration of the sorbent on Oasis MCX cartridges[51]

The first time the MCX method was tried out, it demonstrated great improvements compared to the HLB method results. Same procedure was conducted several times, but these results were not reproducible in consecutive experiments, which demonstrated a peak-to-noise ratio considerably lower than what was obtained on the previously developed HLB extraction method. Why the results from the first attempts were not reproducible is still uncertain. The most likely hypotheses are that the centrifugation-step prior to the extraction was forgotten, or that some of the solution used in the extraction procedure got mixed up.

Developing and improving a new solid phase extraction method, is very difficult, time consuming and expensive. Due to these issues the MCX method was not further improved. When tried out on real samples from smokers, the pre-existing HLB method provided sufficient up concentration allowing detection of all analytes. Therefore, the project continued with this method. The final HLB method was established after experimentally deciding which solution was best suited for the different steps of the extraction. Because of the differences in chemical properties of the six detected biomarkers, this decision was based on compromises. While deciding the amount of acetonitrile:formiate buffer solution best suited to redissolve the evaporated elutent from the SPE, re-dissolving in respectively 100, 250, 500, and 1000µL was tested. For the minor tobacco alkaloids, a low amount gave higher peak intensity, whereas a high amount was more fitted to nicotine. As a compromise, 500 µL was chosen.

5.2 **Method Validation**

5.2.1 ***Selectivity***

The selectivity of the four the internal standars was visually determined by absence of interfering peaks at the selected MRM transitions in the negative urine samples. In the samples spiked with the IS, one peak appeared in each chromatogram absent in the samples of blank urine. This experiment suggests sufficient selectivity of all internal standards in urine (Figure 20) Selectivity of nicotine, cotinine, trans-3'-hydroxycotinine, anatabine, anabasine and nornicotine was confirmed by the absence of interfering peaks at the selected MRM transitions with good peak shape and a S/N of >3 with a RT within ±2% of the corresponding deuterated analogue.(Figure 21) Whereas for anatabine and nornicotine, selectivity was established by comparing blank urine to urine spiked with the respective standards, since the deuterated analogues were not available. Peaks appeared in the urine spiked with the standards, and absence of interfering peaks at the selected MRM transitions with a RT within ±2% of the corresponding analogue demonstrated selectivity of these compounds (Figure 22). This experiment ensures the selectivity of the method.

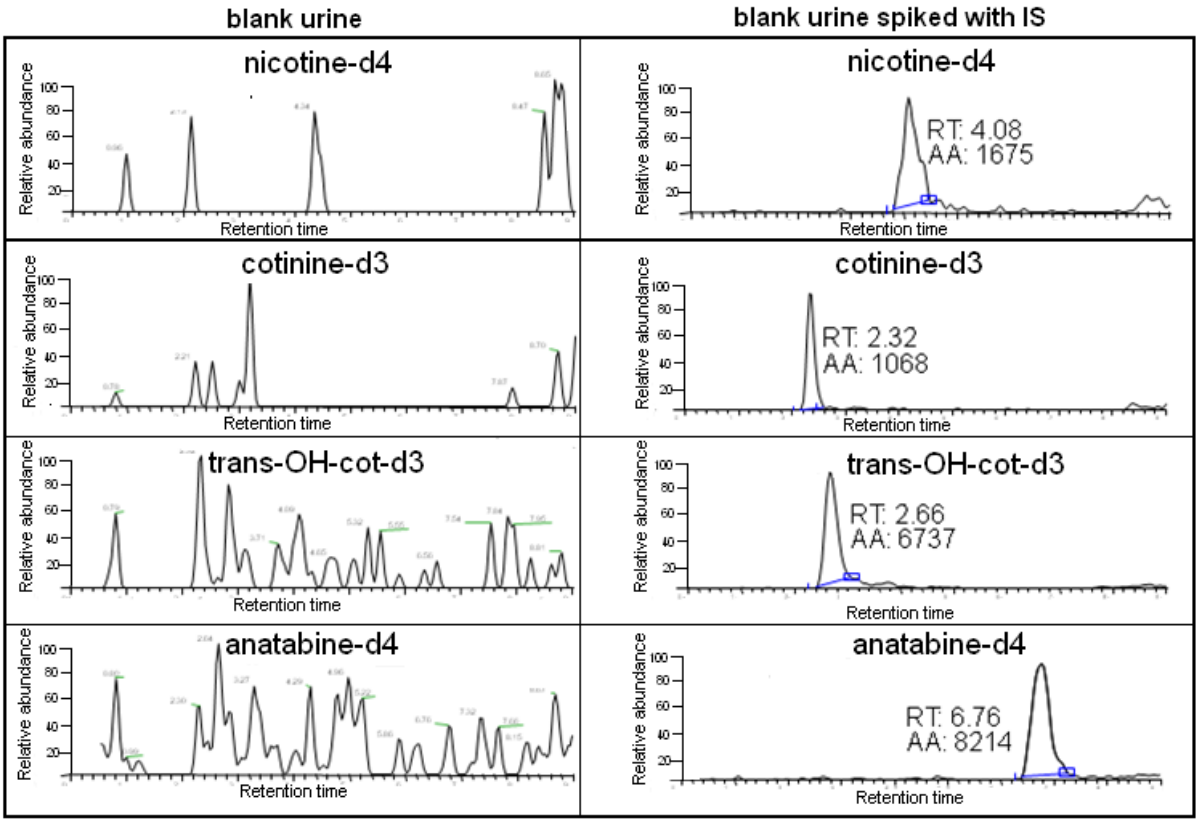


Figure 20 Selectivity test of internal standards, no interferences observed in the blank urine at selected MRM transitions at ±2% of the RT observed in the samples spiked with IS

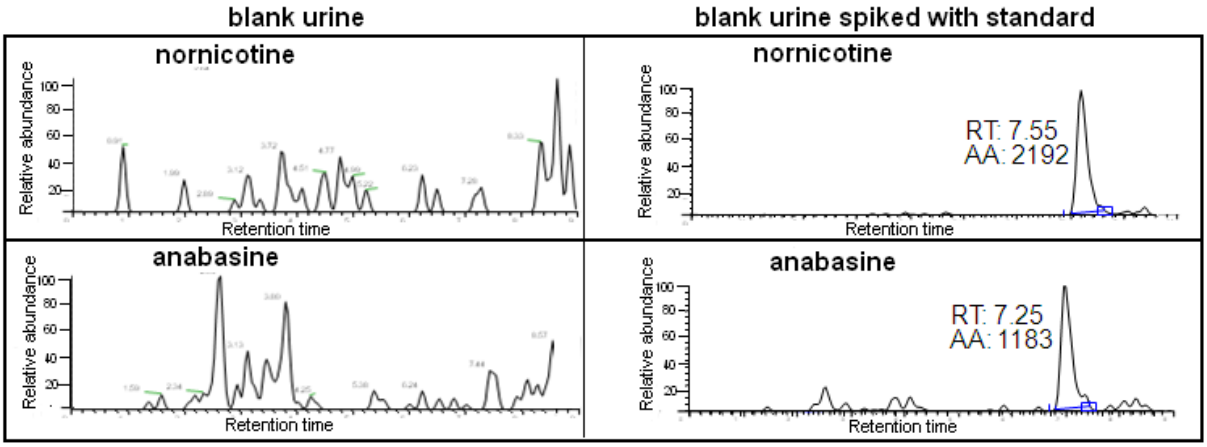


Figure 21 Selectivity test nornicotine and anabasine, blank (negative) urine compared to spiked urine, no interferences observed in the blank urine at selected MRM transitions at ±2% of the RT observed in samples spiked with standards

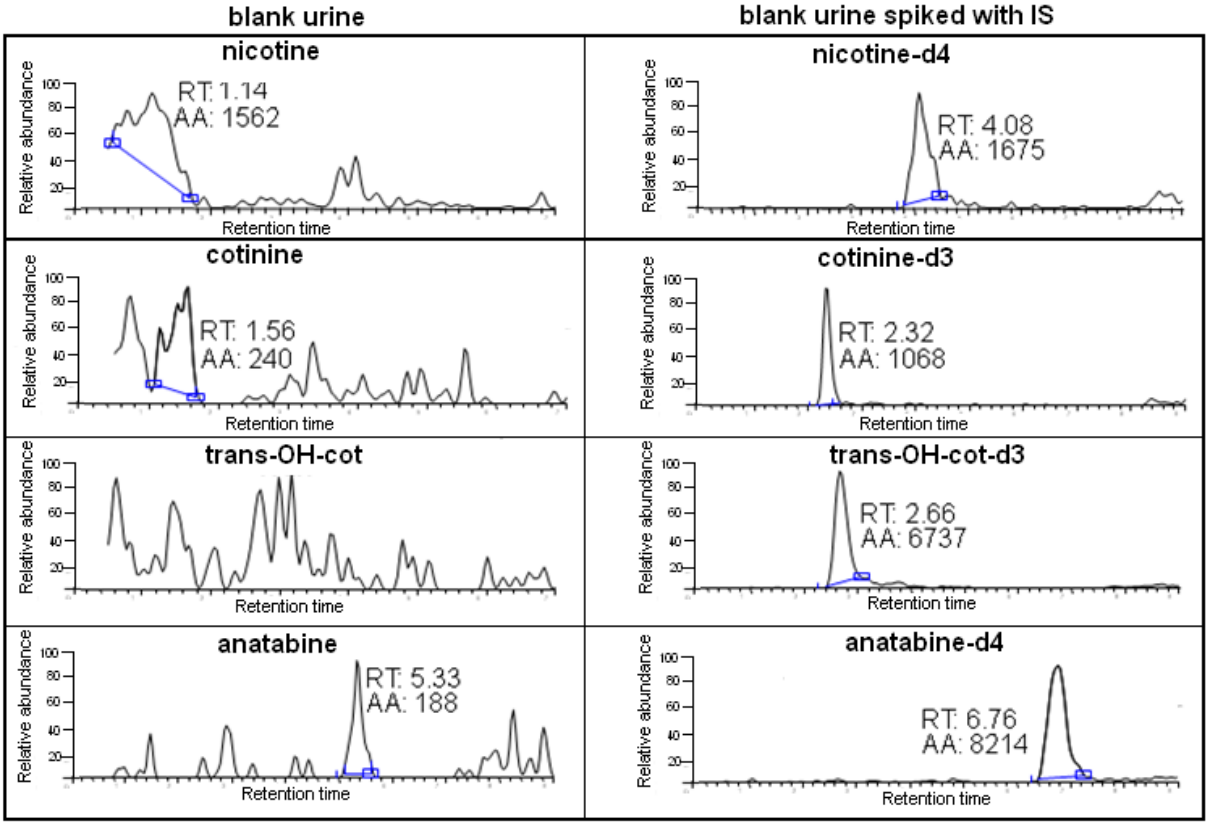


Figure 22 Selectivity test nicotine, cotinine, trans-OH cotinine and anatabine. Negative urine was compared to the negative urine spiked with IS-only, no interferences observed in the negative urine at selected MRM transitions with a RT within ±2% of the corresponding analogue.

5.2.2 *Quantitative validation*

The calibration curve was plotted with nominal concentration to peak area ratios (peak area of standard/peak area of internal standard). The peaks were automatically integrated on the Xcalibur program, and calibration graphs were generated using Microsoft® Office Excel 2007. Un-weighted and weighted (1/x, $1/x^2$ and log x) standard curve fittings were evaluated, and un-weighted linear regression was established as the best suited for the detected analytes. With un-weighted linear regression, linearity determination coefficients (R^2) were obtained of > 0.985 for nicotine, >0.985 for cotinine, >0.982 for trans-3-hydroxycotinie, >0.980 for anatabine and >0.976 for anabasine, and > 0.961 for nornicotine, respectively. Repeatability values were estimated to be within ranges of 7.1-13.6 % at each concentration level for all the analytes, and intermediate imprecision was estimated at 9.0-15.0%, respectively. Accuracy profile for each analyte is given in the Appendix. At all concentration ranges for each analyte, the accuracy was estimated to be within acceptance criteria of ± 30 %. In this simplified validation method, calibration standards were prepared at only two concentration levels (k=2). Additionally, the mean recalculated value should be within 15% of the theoretical value at all concentration ranges except for the LLOQ (within 20%). The selected acceptance criteria of ± 30% is outside these recommendations. For the aim of this project a wider accuracy acceptance range was tolerated.

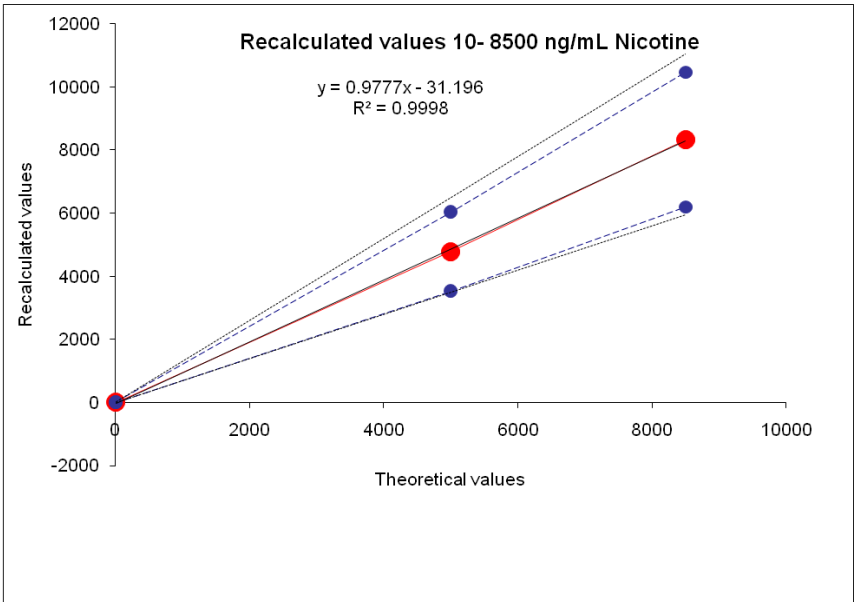


Figure 23 Correlation between recalculated values and theoretical values of nicotine. The red points represent the mean measured values; the blue lines represent the estimated deviation from the mean. The theoretical values should be as close as possible to the measured values. Correlation curves of cotinine, trans-3-hydroxycotinie, anatabine, anabasine and nornicotine are given in the appendix.

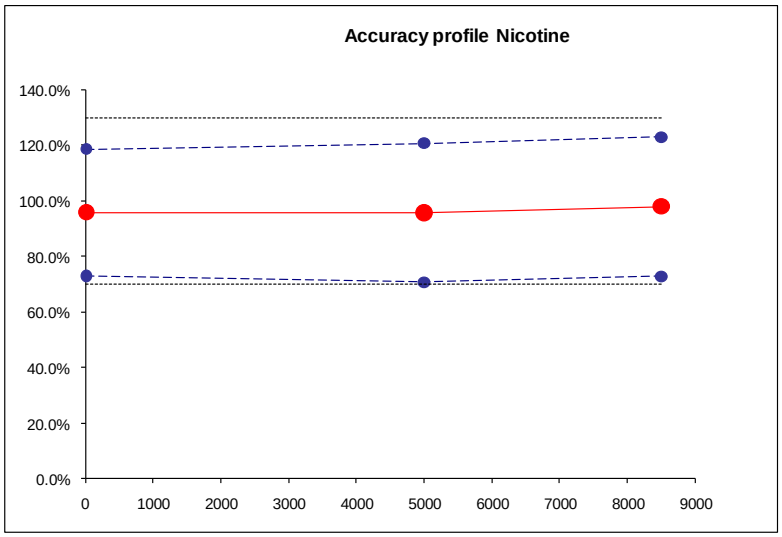


Figure 24 Accuracy profile of nicotine, See appendix for accuracy profile of the other analytes.

According to the FDA and ICH guidelines, the standard curve should consist of a minimum of six standards points over the entire range of expected concentrations. In order to meet this criteria with the selected validation design, the calibration standards should have been prepared at three concentration levels (k=3), instead of two.

In the first attempt of validation, the calibration was performed with three successive series, at six calibration standards levels (k=6) and five validation standards concentration levels (k=5), prepared in triplicate (n=3). However, sufficient linearity was not obtained. In the first attempts, this was due to wrong composition of solvent used in re-dissolution of the extracted samples. The second attempt was unsuccessful presumably due to inaccurate measurements. This suspicion was confirmed by six consecutive reinjection of same sample that revealed >20% variation in the peak area of standard/peak area of internal standard ratios. The equipment got fine tuned, and quantitative validation was reattempted. Satisfying accuracy profiles were obtained for all three series. Unfortunately, the working solutions had been incorrectly prepared. This was discovered first after fully validation had been completely conducted and all data processed. These problems encountered during quantitative validation required introduction of a simplified validation. If the method should be applied to routine analysis, a more robust quantitative validation must be preformed, including determinations of matrix effect and total extraction recovery for each analyte as well.

5.2.3 *Method sensitivity*

Method sensitivity was experimentally determined during the quantitative validation. The LOQ was defined as the lowest standard in the calibration graph that produced a S/N ratio of ≥10 for the selected MRM transitions with acceptable precision and accuracy. The LOQ was determined as 10ng/mL for nicotine, cotinine and trans-3-hydroxycotinie, anatabine, anabasine and nornicotine. Whereas the ULOQ was experimentally demonstrated at 10000 ng/mL for nicotine, cotinine and trans-OH-cotinine and 500 ng/mL for anatabine, anabasine and nornicotine.

5.3 **Application of the method**

At least one of the QC samples at each concentration levels (>3) should be within 15% of the nominal value. In this experiment, only one concentration level of QC samples was prepared, of which neither of the duplicates passed the acceptance criteria set by FDA guidelines. According to FDA requirements, the run should have been rejected if it was applied in routine analysis.

This experiment was conducted with objective to evaluate whether or not the developed method could work as intended, thus the result could still provide useful information. Regardless of these concerns in relation to the reliability of the method, the results were interpreted.

In urine of the cigarette smoker, the analytes were detected within the following concentration ranges; 10.5-676.6 ng/mL for nicotine, 202.9-339,0 ng/mL for cotinine, 550-1047 ng/mL for trans-3-hydroxycotinie, 9.9-19.8 ng/mL for anatabine, 6.6-15.3 ng/mL for anabasine and 13.3-63.9 ng/mL for nornicotine. Whereas for the snuser, the analytes were detected within the ranges of 6.1-63.7 ng/mL for nicotine, 133.4-201.8 ng/mL for cotinine, 345.4-416.4 ng/mL for trans-3-hydroxycotinie, 345.4-450.1 ng/mL for anatabine, 5.4-7.0 ng/mL for anabasine and 7.5-31.9 ng/mL for nornicotine. (See table 5 for further information)

Table 6 Measured analyte concentration (ng/mL), (* = < LOQ, - = < LOD)

		measured analyte concentration (ng/ml)					
		nicotine	**cotinine**	**trans-OH-cot**	**anatabine**	**anabasine**	**nornicotine**
cigarette samples	1. sample	676.6	339	1047.5	19.8	15.3	63.9
	2. sample	109.5	202.9	550	9.9*	6.6*	25.8
	3. sample	14.7	269.5	725	-	-	13.3
	4. sample	10.5	299.4	937.7	-	-	18.3
snus samples	1. sample	39.4	201.8	416.4	-	-	7.5*
	2. sample	63.7	180.9	345.4	5.4*	6.4*	19.6
	3. sample	6.8*	163.1	356.7	7*	6.3*	31.9
	4. sample	6.1*	133.4	450.1	-	-	12.7

These measurements suggest that smokers accumulate higher levels of nicotine, cotinine and trans-3-hydroxycotinie than snusers. The measured urinary concentration of tobacco alkaloids is influenced by variations in tobacco product composition, as well as inter-individual differences in the pharmacokinetic of the respective alkaloids measured. When urine is collected from smokers and snusers after free tobacco use, they tend to have about the same nicotine and cotinine levels despite these differences in tobacco composition.This may be attributed to individual adjustments of tobacco consumption frequency in order to obtain most favourable nicotine plasma concentrations. The participants of this experiment consumed only one tobacco-unit each, thus they were not able to compensate for any such influencing factors as they presumably would have done in "real" life. Due to these issues, comparing ratio of the measured analytes would provide more useful information.

According to the literature, best chances of finding ratio differences are by comparing nicotine:cotinine, nicotine:anatabine, and nicotine:anabasine ratios[38]. When interpreting the measured results, no such difference was observed in the nicotine:cotinine ratio between the snus and cigarette samples(See table 8). However, differences in nicotine:anatabine, cotinine:anatabine and trans-3-hydroxycotinie:anabasine were observed between the two groups. The nicotine:anatabine and trans-3-hydroxycotinie:anabasine ratio values were remarkable higher in the cigarette samples than in the snus samples, indicating a higher consentation of the minor tobacco alkaloids compared to the major tobacco alkaloids in the urine of snus users. The same trend was seen in the nicotine:anatabine ratios as well, even though there was a slightly overlap between the groups. These findings correlates well with

results presented in a published study, where higher nicotine:anatabine ratio in urine of smokers than smokeless tobacco users was observed [38].

Table 7 Ratio range intervals. √: overlap of concentration ranges between the two compared groups, ×: no overlap in ratio ranges between the groups (This table is a simplified presentation of information from table 10, see appendix.)

investegated ratios	ratio ranges		
	cigarette samples	snus samples	overlap
nicotine: cotinie	0.0-2.0	0.0-0.4	√
nicotine: trans-OH-cot	0.0-0.7	0.0-0.2	√
nicotine: anatabine	11.1-34.2	1,0-11.8	(√)
nicotine: anabasine	16.6-44.2	1.1-10.0	×
nicotine: nornicotine	0.6-10.6	0.2-5.3	√
cotinine: anatabine	17.1-20.5	23.3-33.5	×
cotinine: anabasine	22.2-30.7	25.9-28.3	√
cotinine: nornicotine	5.3-20.3	5.1-29.9	√
trans-OH-cot: anatabine	52.3-55.6	51.0-64.0	√
trans-OH-cot: anabasine	68.5-83.3	54.0-56.6	×
trans-OH-cot: nornicotine	16.4-54.5	11.2-55.5	√

However, these are only preliminary results, and no conclusions can be drawn due to inadequate reliability of the method and limited number of samples measured. Still, these findings suggest that some of the selected biomarker ratios, especially nicotine:anabasine, nicotine:anatabine, cotinine:anatabine, cotinine:anabasine and trans-3-hydroxycotinie:anabasine could be used to distinguish between the different tobacco consumption patterns.

6 CONCLUSION

A LC-MS/MS method for the simultaneous extraction and quantification of nicotine, cotinine, trans-3-hydroxycotinie, anatabine, anabasine and nornicotine from human urine has been developed and validated. The applied SPE technique, allowed a simple and cost-reduced sample preparation. Such considerations are important to make when developing a novel methods intended for use in routine screening of prohibited substances.

Some preliminary results were obtained, especially in concentration ranges of the major alkaloids; nicotine, cotinine and trans-3-hydroxycotinie in relationship to the minor tobacco

alkaloids; anatabine and anabasine, indicating a higher concentration of minor tobacco alkaloids compared to major tobacco alkaloids in the urine of snusers than smokers. These findings correlate well with results presented in a published study [38]. These elevated amounts are presumably caused by heat induced decomposition of minor tobacco alkaloids when the tobacco is burned, such as the case for the smoked tobacco products. This would correspondingly lead to lower absorbtion of respective alkaloids by cigarette smokers than by smokeless tobacco users, as indicated by these preliminary results.

Despite promising potential, the developed method was demonstrated as unreliable during the method validation for its application in routine work. However, analysis were performed on a Luna® HILIC Phenomenex column, thus this project provides interesting information concerning its capacity to separate ionic compounds. This column is relatively new on the market; hence there is a lack of published studies in which it has been used.

7 FURTHER PERSPECTIVES

If a better sample extraction procedure was obtained, the presented method could have great potential to differentiate between the nicotine consumption patterns. The main issues concerning this method are inadequate sensitivity and high degree of bias, both parameters most plausible linked to the sample preparation step. If there was more time available, the MCX sample preparation method described in the method development discussion, would be reattempted and improved. If not satisfying result were obtained, there could still be ways to achieve better and more accurate sample preparation on the HLB columns. Finding a more appropriate buffer solution not clogging the HLB cartridges could be a good strategy to improve this method. If it was possible to develop a well functioning SPE method, it should further be validated according to FDA and IHC guidelines. Finally, the method must be applied in a clinical study of optimal size and designed in order establish whether or not these reported differences in ratios between the groups really are significant or attributed by confounders.

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9 APPENDIX

9.1 Method validation

9.1.1 *Fragmentation patterns*

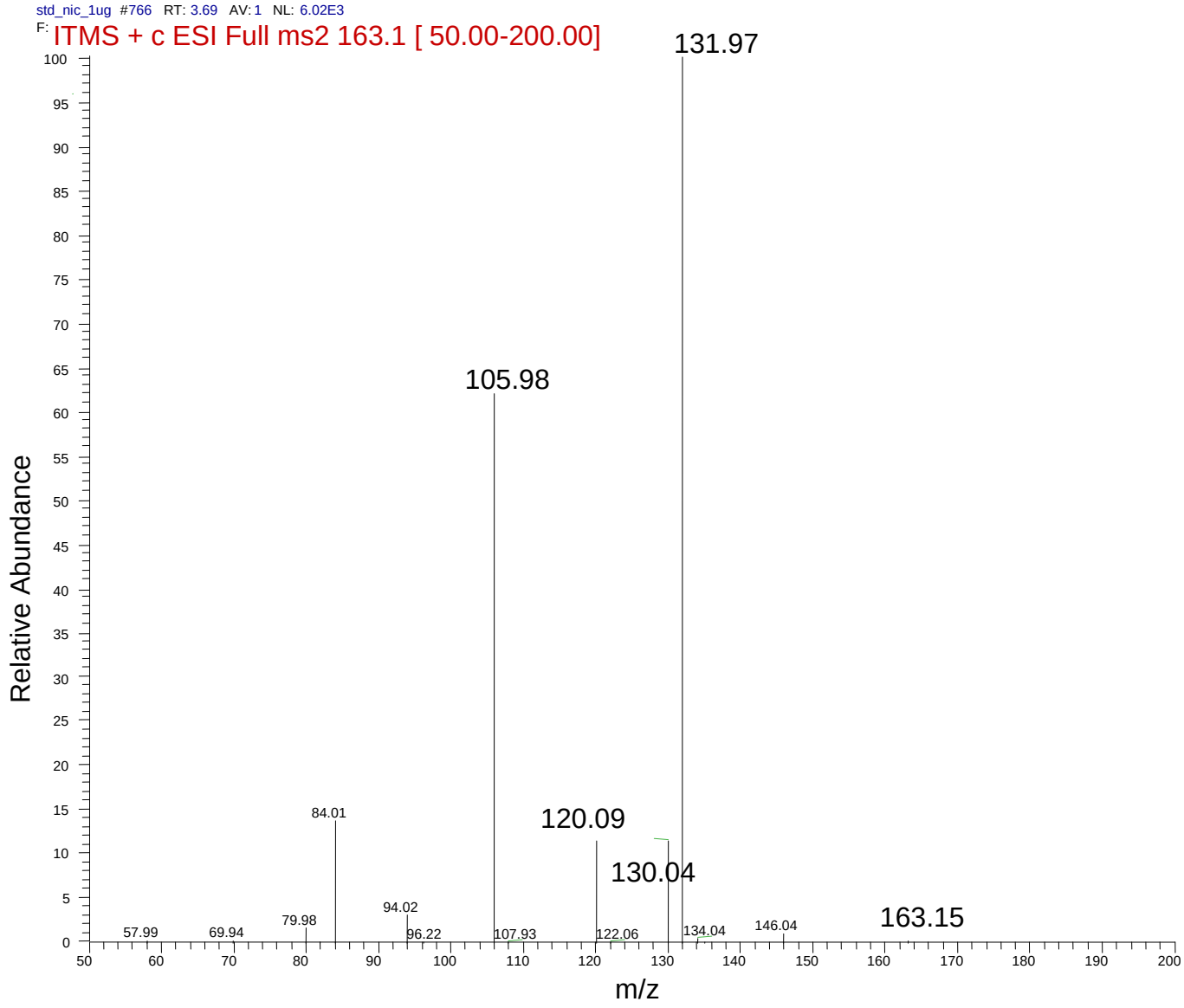

Figure 25 Fragmentation pattern of nicotine

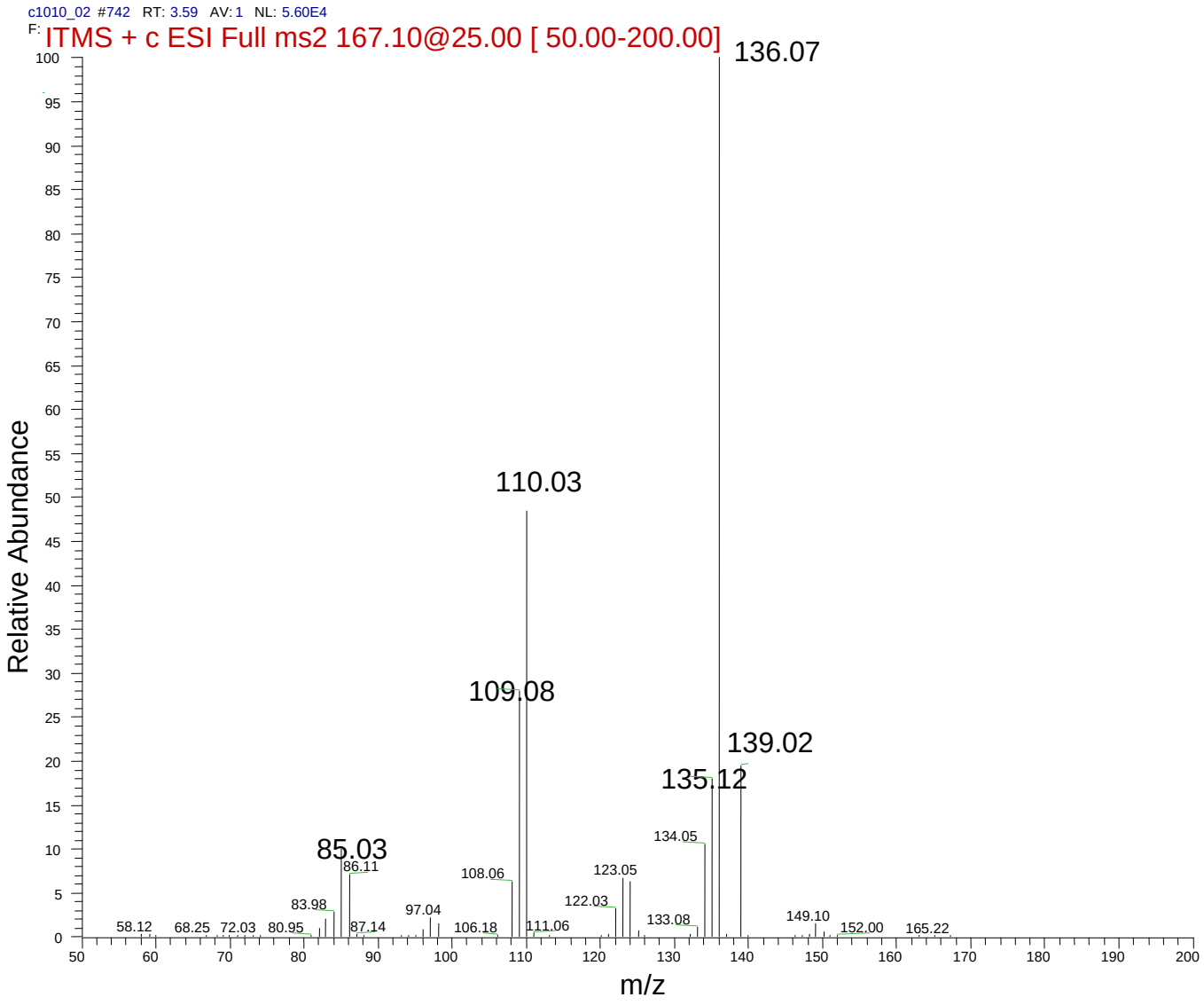

Figure 26 Fragmentation pattern of Nicotine-d4

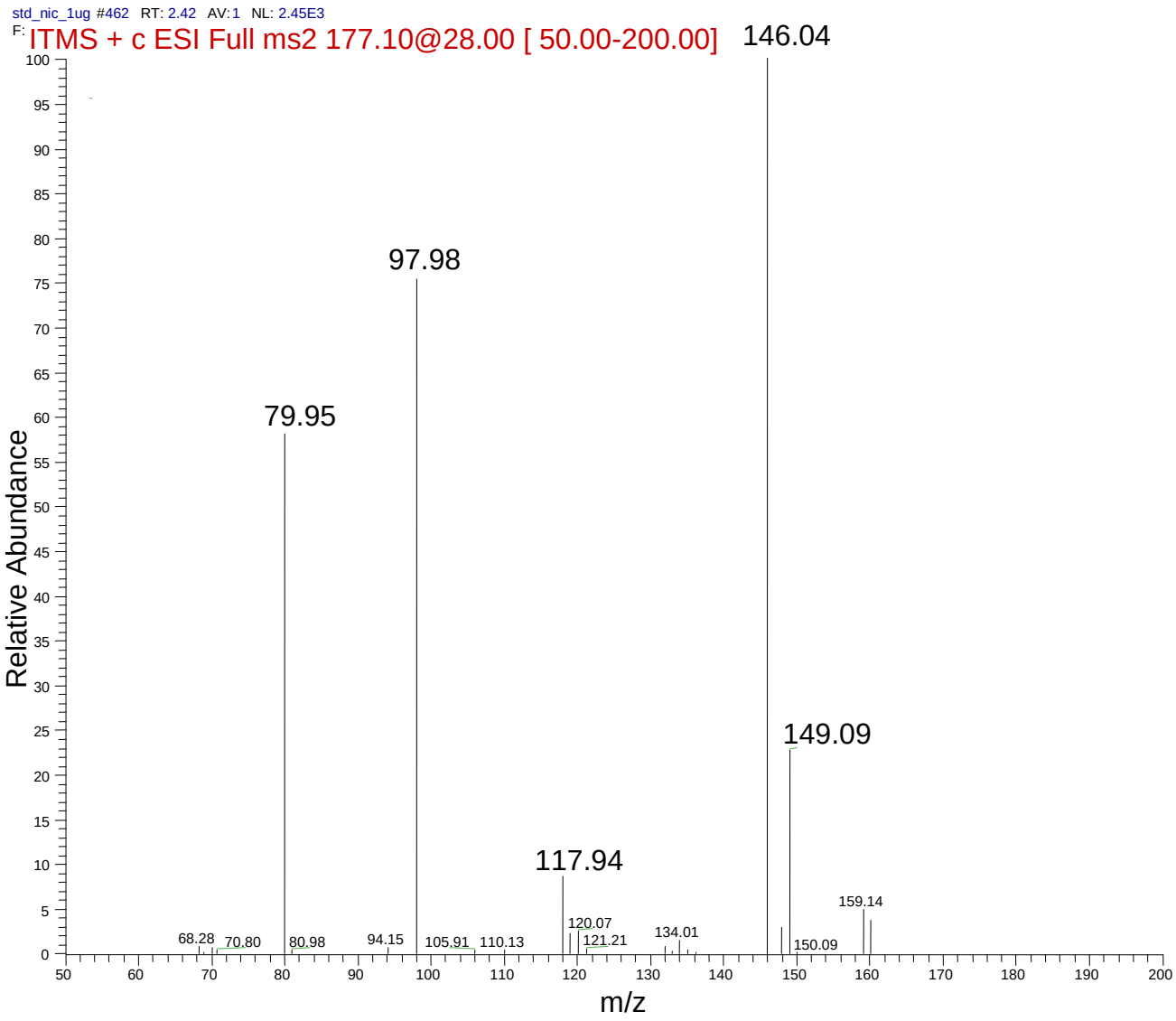

Figure 27 Fragmentation pattern of cotinine

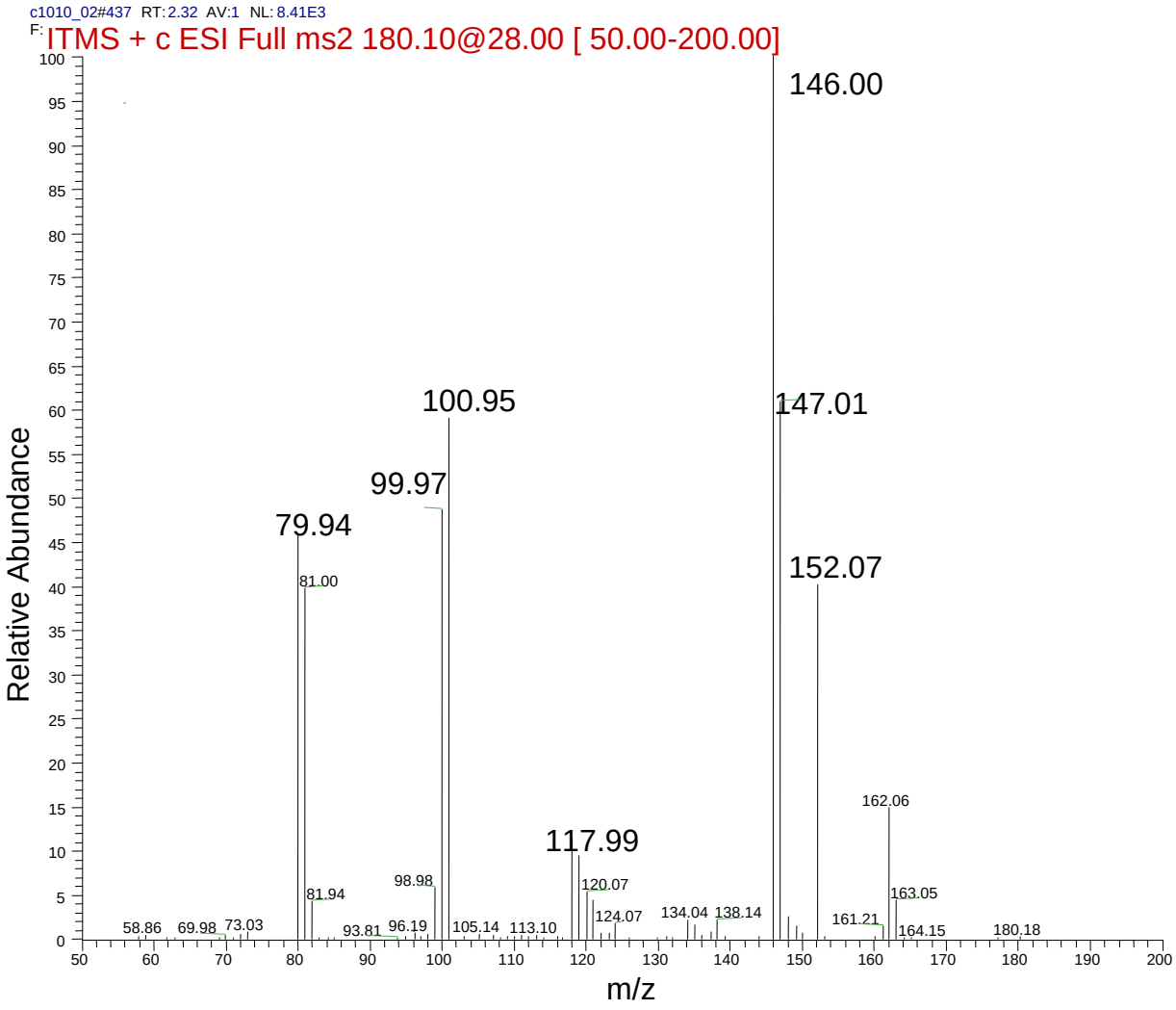

Figure 28 Fragmentation pattern of cotinine d-3

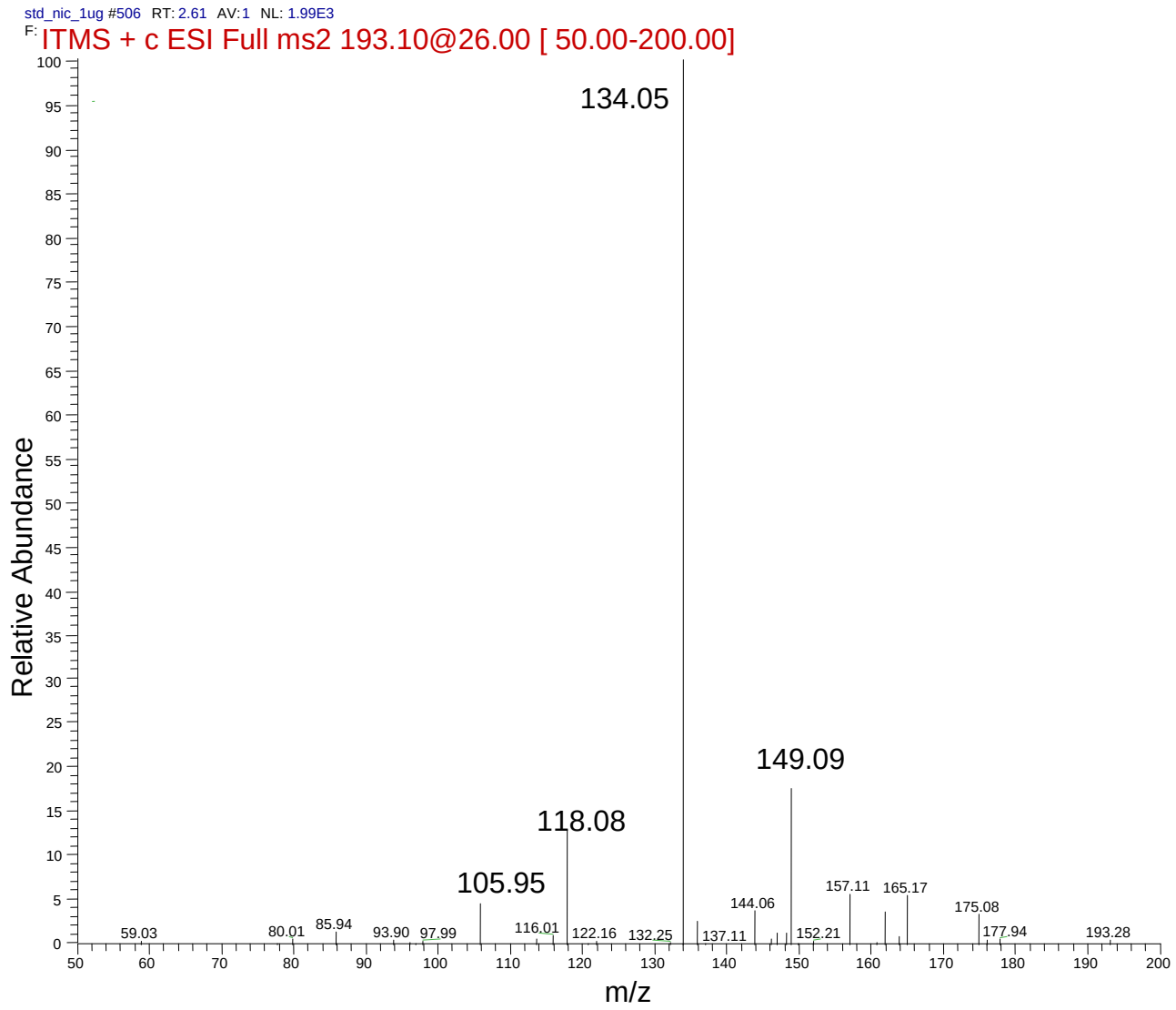

Figure 29 Fragmentation pattern of tans-oh-cotinine

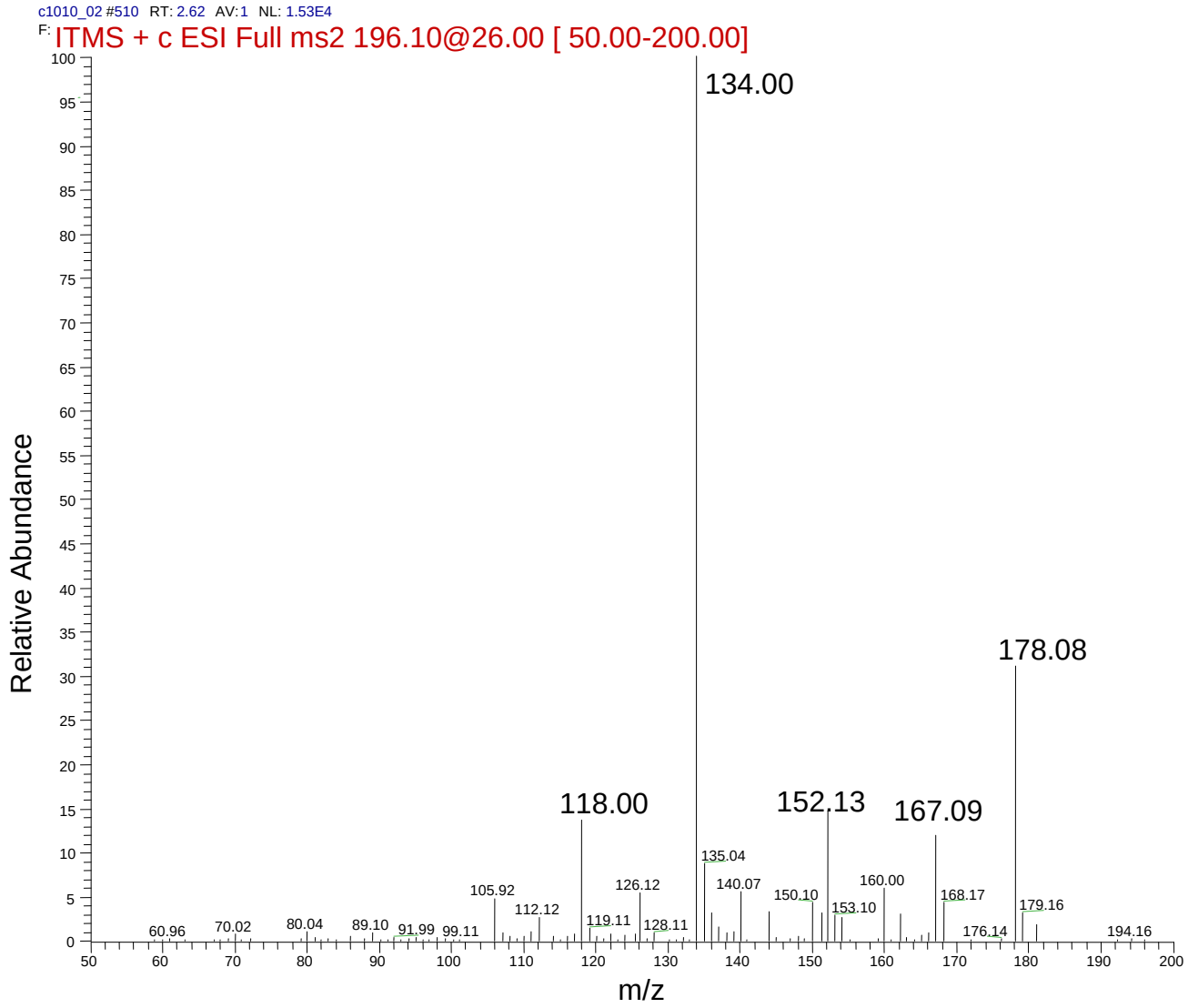

Figure 30 Fragmentation pattern of trans-oh-cotinine-d3

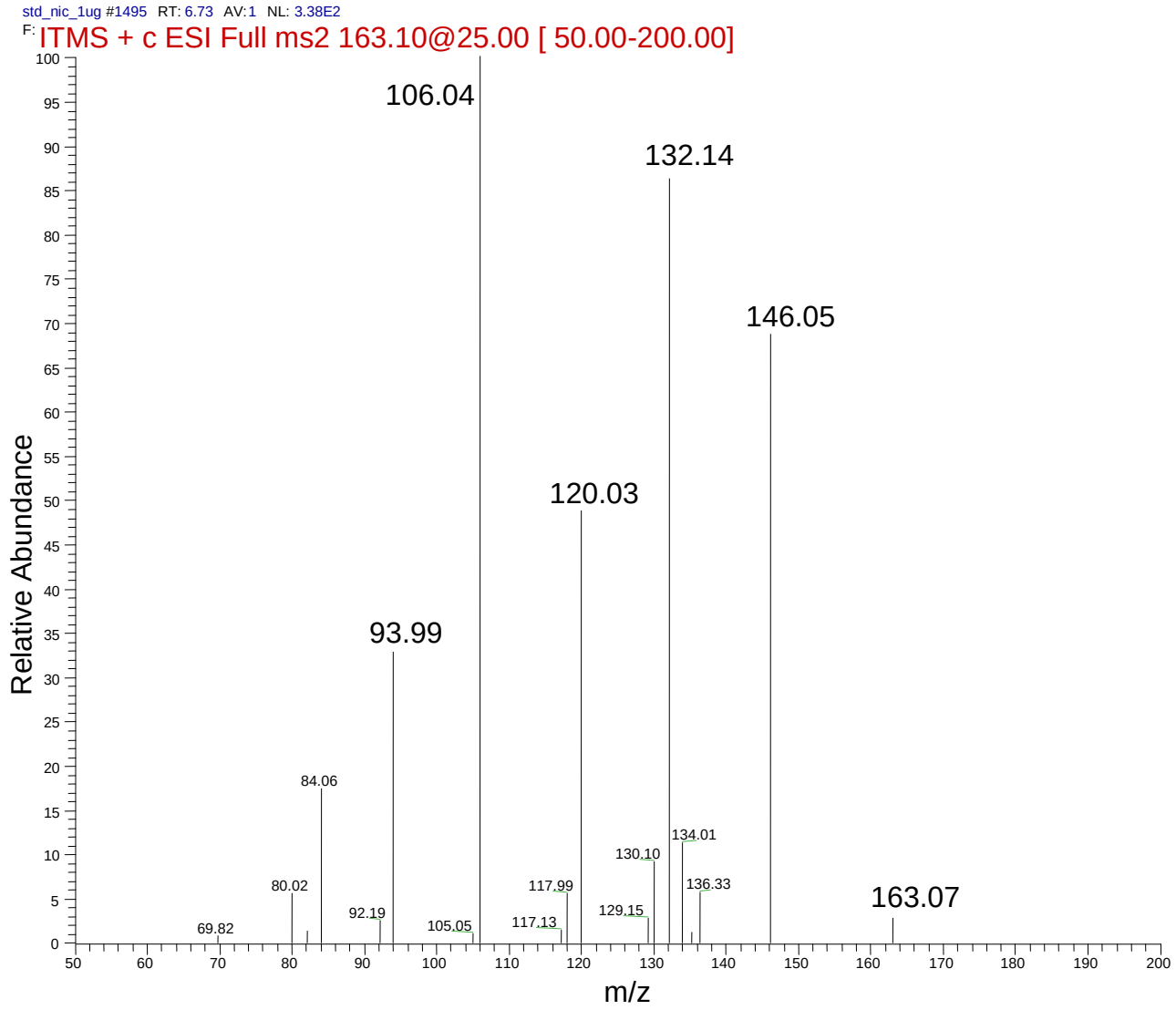

Figure 31 Fragmentation pattern of anatabine

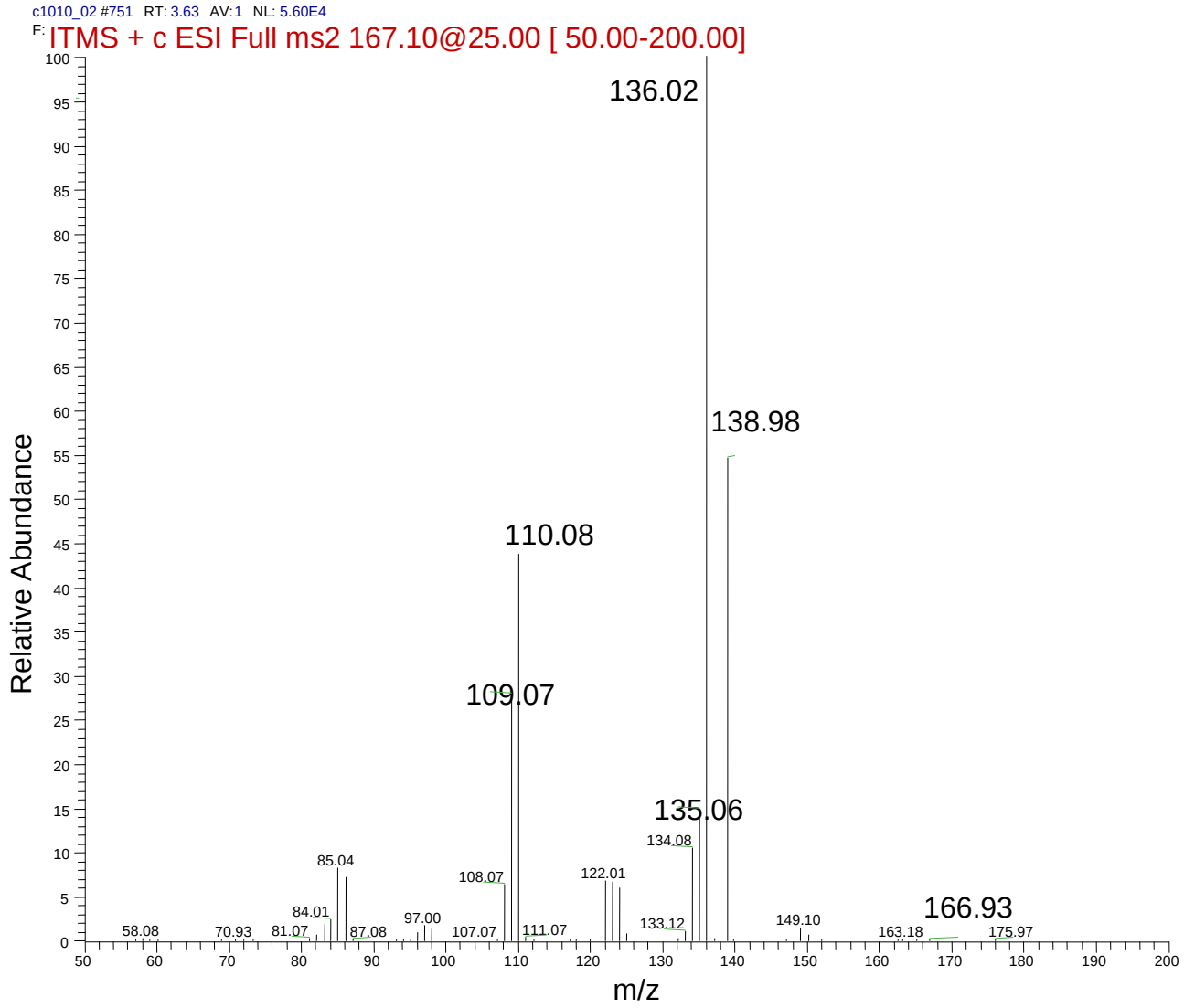

Figure 32 Fragmentation pattern of anatabine-d4

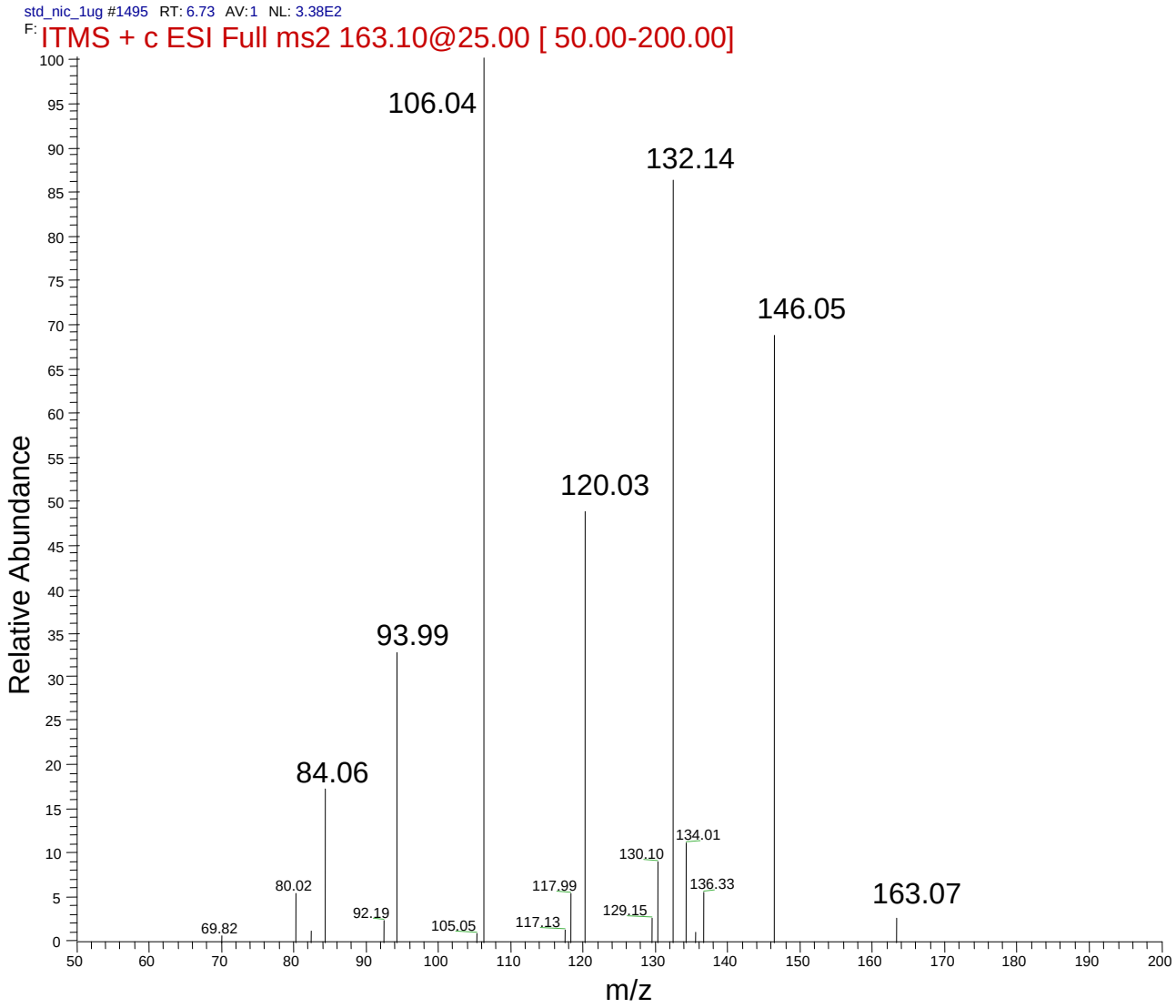

Figure 33 Fragmentation pattern of anabasine

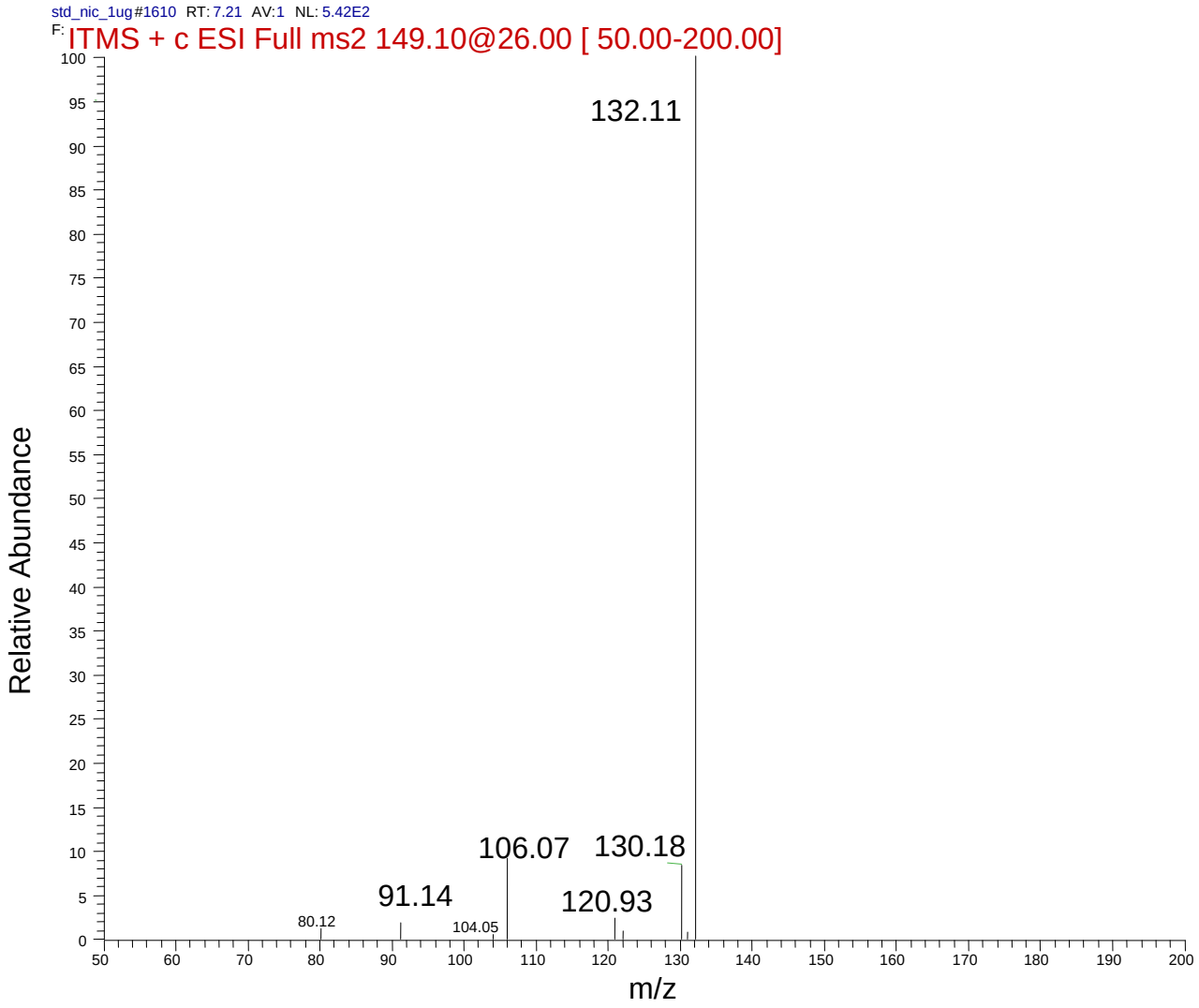

Figure 34 Fragmentation pattern of nornicotine

9.1.2 *Mobile phase gradients*

Table 8 HPLC gradient table used on the Waters X Bridge C18 column (3 µm particles, 200 Å pore diameters) Solvent A: 0,1 M Formiate buffer pH 3, Solvent B: Methanol

No	Time (min)	% Solvent A	% Solvent B	Pressure (µl/min)
0	0	90	10	400
1	3	90	10	400
2	7	30	70	400
3	10	30	70	10
4	11	90	10	10
5	13	90	10	10

9.1.3 *MCX solide phase extraction method*

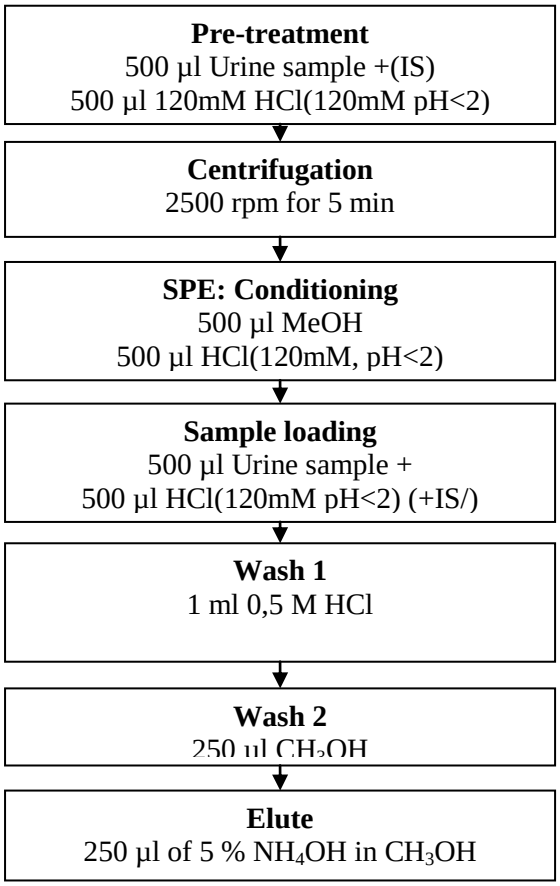

Figure 35 MCX extraction procedure This solid phase extraction method was performed using a Oasis® MCX extraction plate 30 mg, 96-well.

9.2 **Method validation**

9.2.1 *Trueness and precision*

Table 9 Trueness and precision calculated at each concentration level over the 3 days of validation

Compound	Target concentration (ng/ml)	Precision: Repeatability/ Intermediate precision (RSD, %)	Trueness (%)
nicotine	10	8.8/11.8	95.80%
	5000	9.1/12.9	95.60%
	8500	13.0/12.9	97.90%
cotinine	10	10.9/12.2	99.10%
	5000	9.9/11.6	94.20%
	8500	10.1/12.0	100.10%
trans-oh-cot	10	13.4/15.0	100.80%
	5000	10.6/10.9	93.20%
	8500	8.8/9.4	100.50%
anatabine	10	12.1/12.6	103.50%
	250	12.6/12.6	96.60%
	400	7.8/10.4	109.50%
anabasine	10	7.1/10.9	96.10%
	250	13.6/14.3	98.10%
	400	10.2/10.0	103.40%
nornicotine	10	8.9/11.8	99.50%
	250	11.4/13.5	103.50%
	400	8.4/9.3	90.00%

9.2.2 *Accuracy profiles and correlation curves*

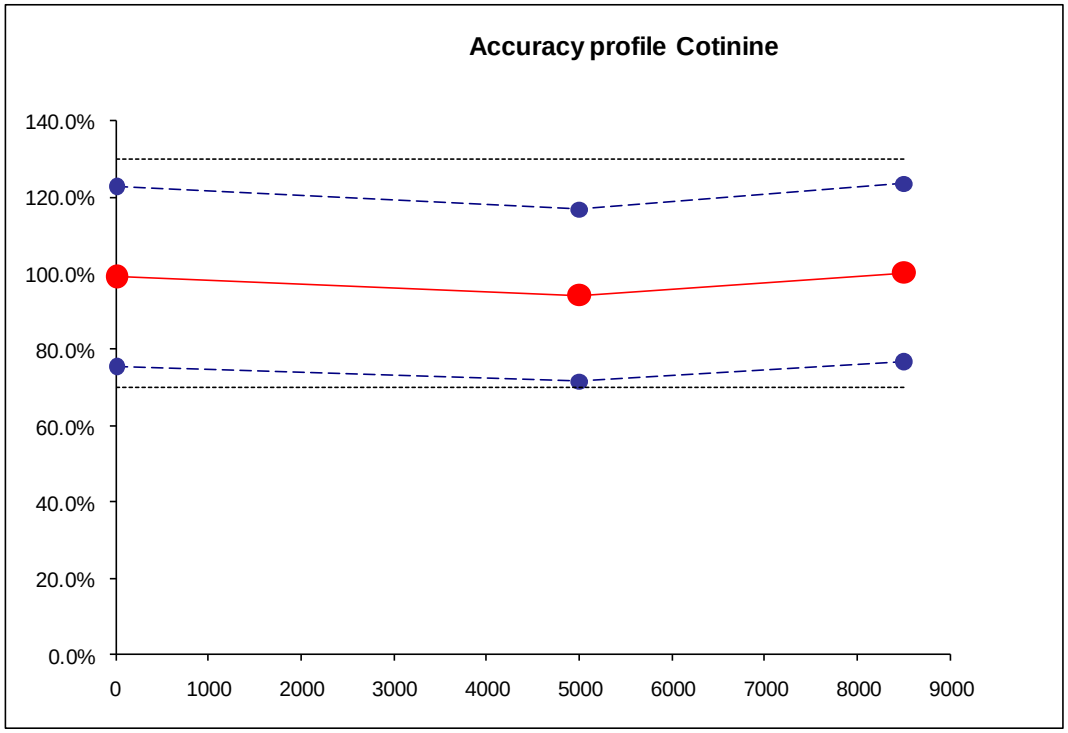

Figure 36 Accuracy profile of cotinine

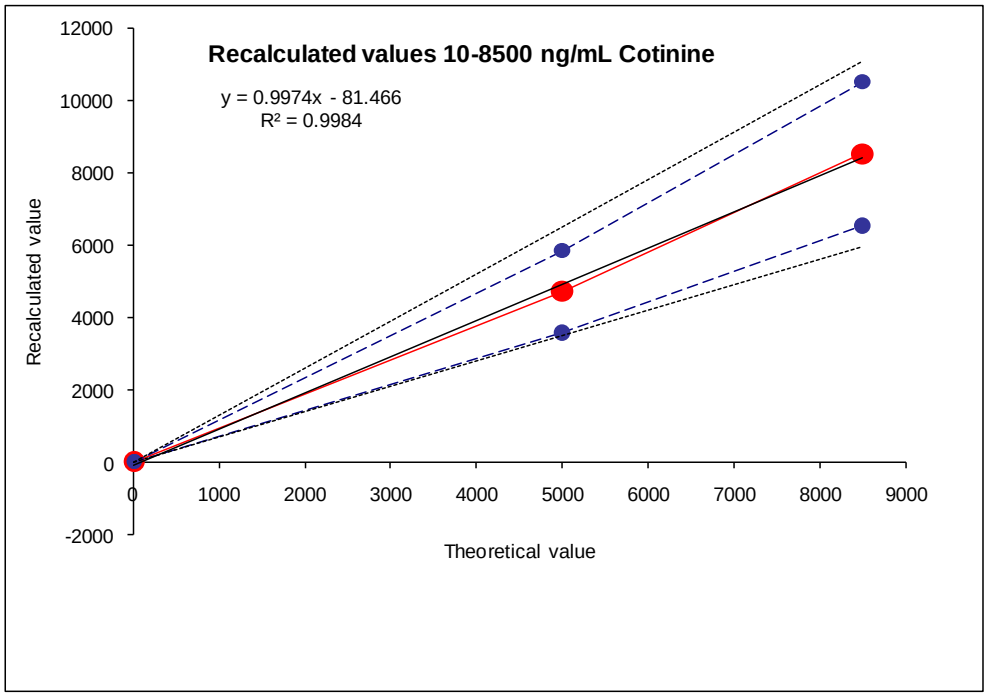

Figure 37 Correlation between recalculated values and theoretical values of cotinine

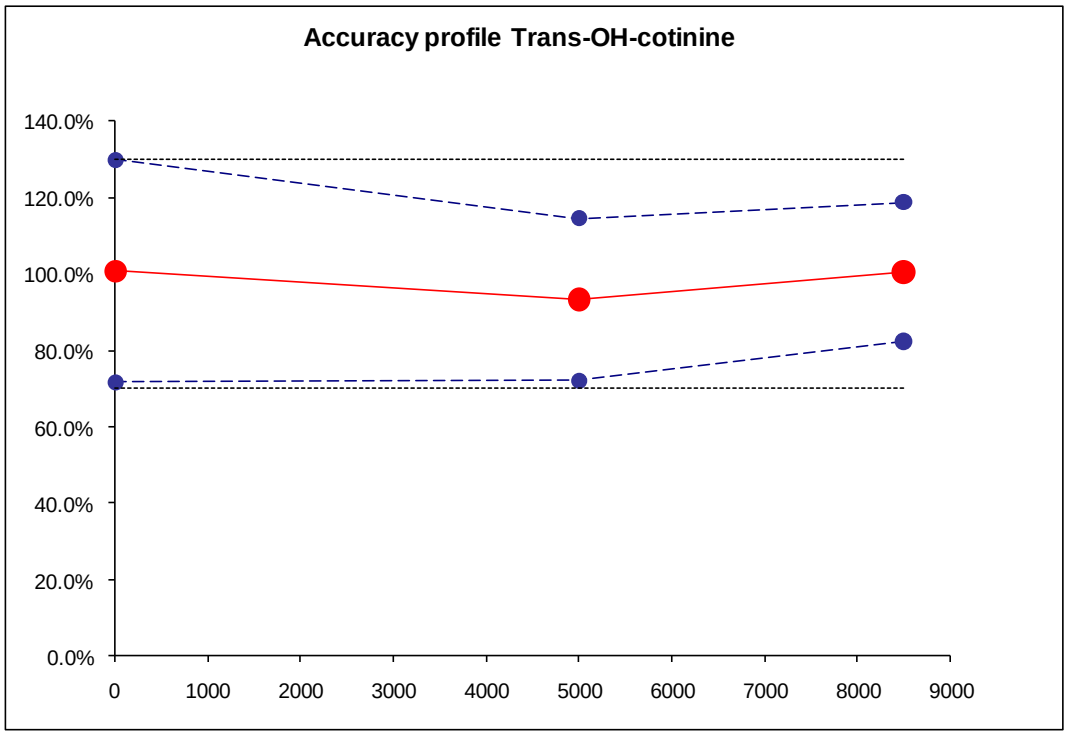

Figure 38 Accuracy profile of trans-OH-cotinine

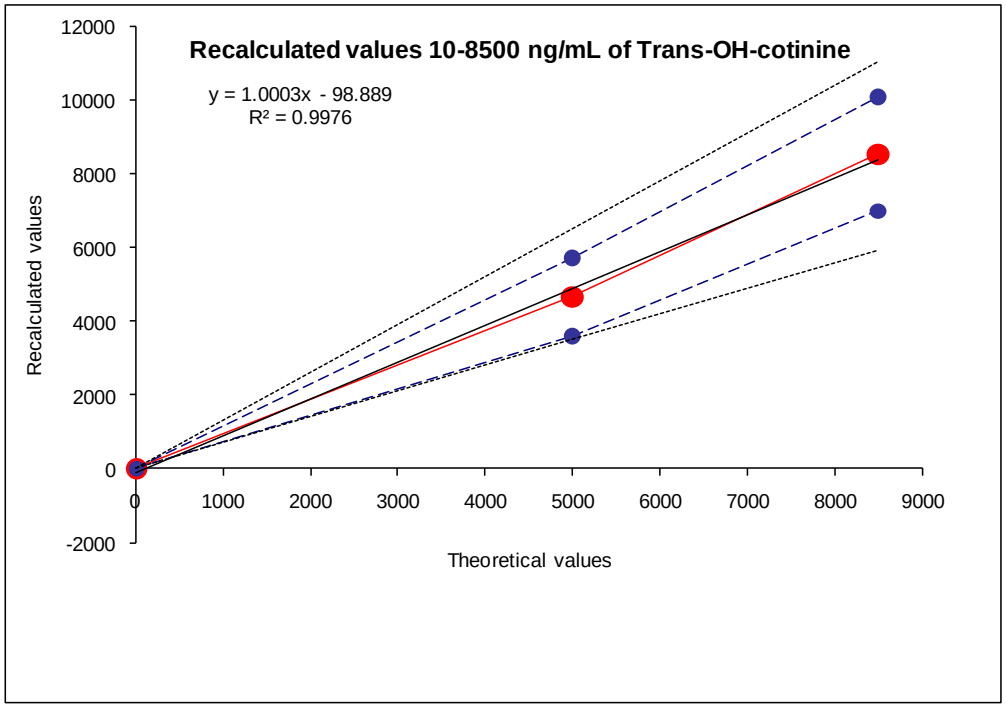

Figure 39 Correlation between recalculated values and theoretical values of trans-OH-cotinine

Appendix

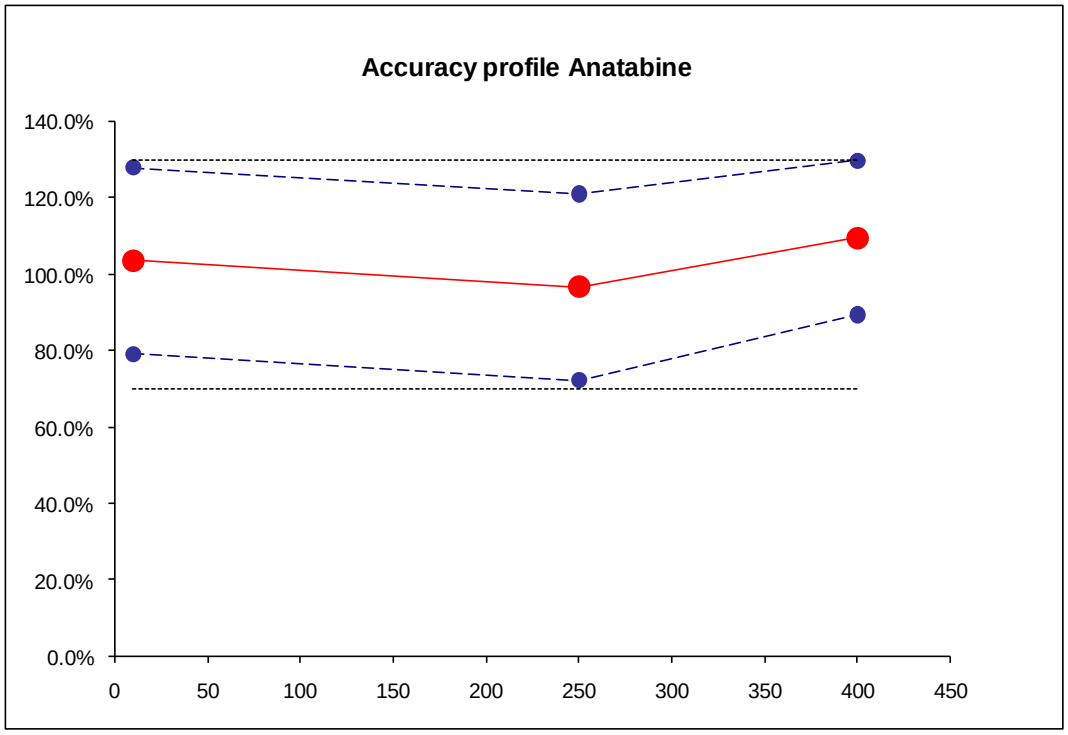

Figure 40 Accuracy profile of anatabine

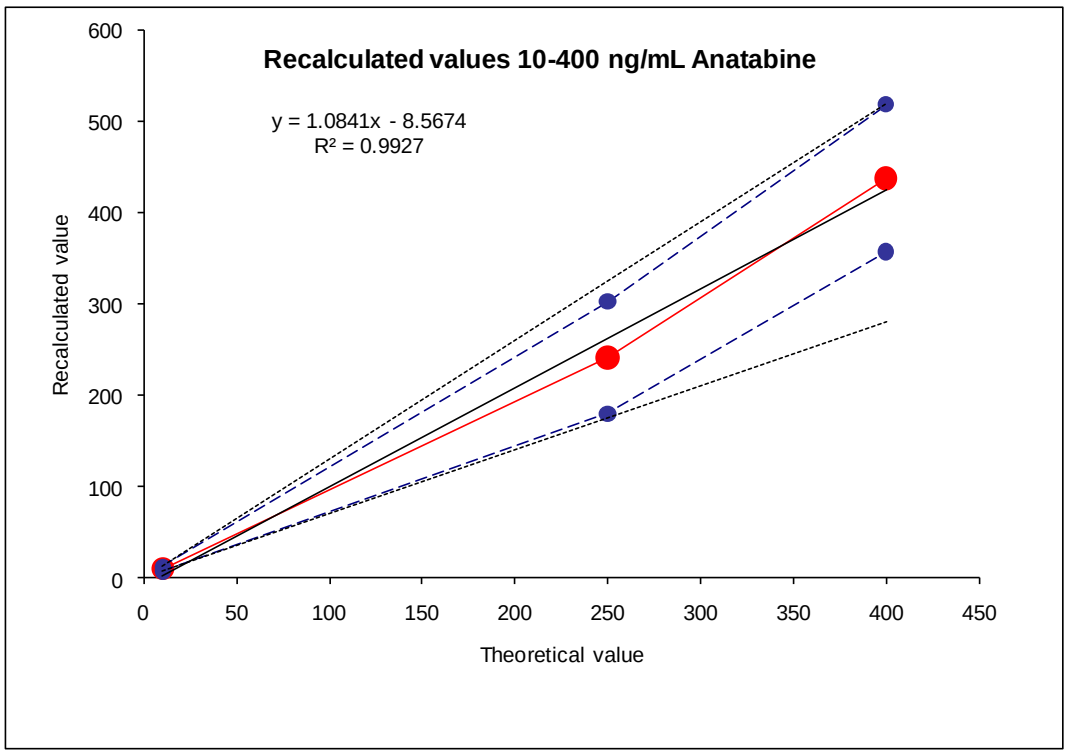

Figure 41 Correlation between recalculated values and theoretical values of anatabine

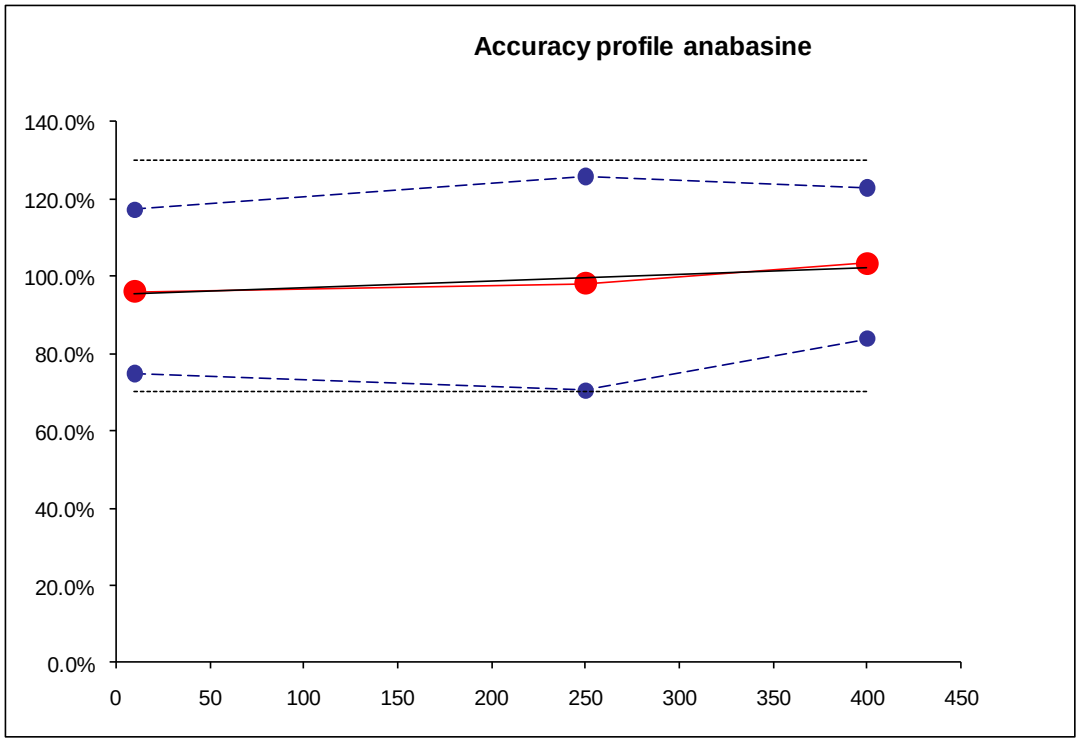

Figure 42 Accuracy profile of anabasine

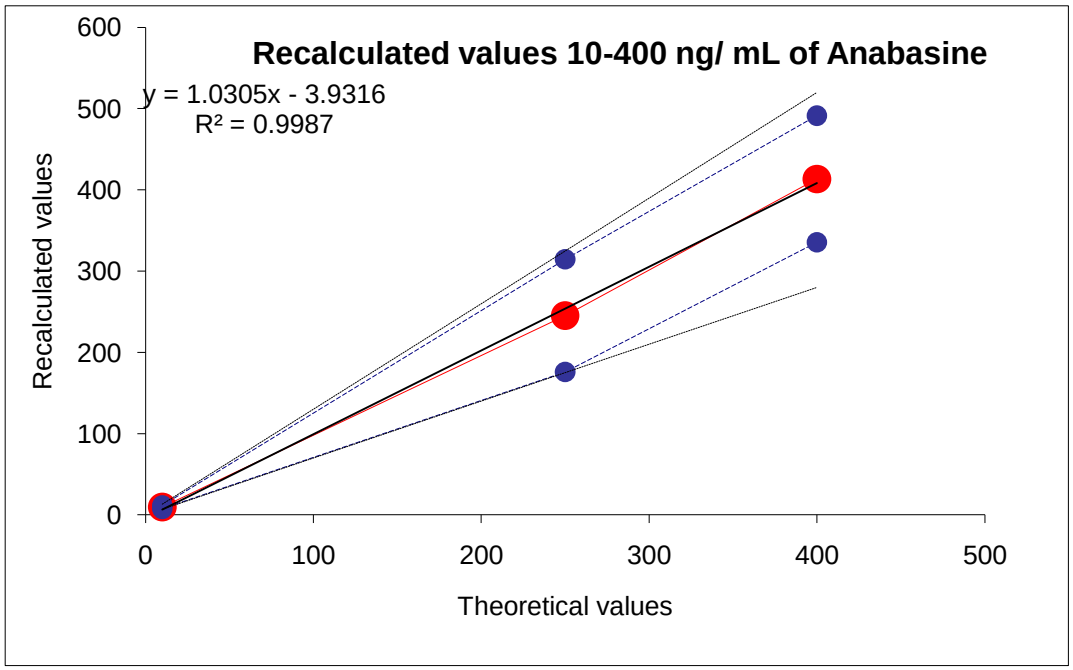

Figure 43 Correlation between recalculated values and theoretical values of anabasine

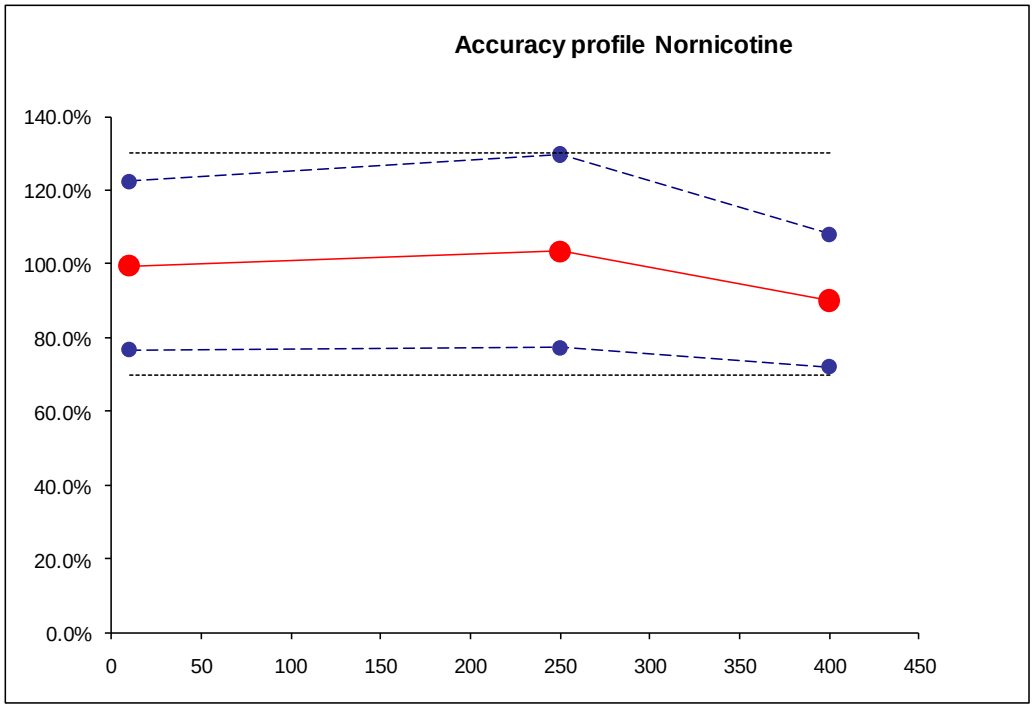

Figure 44 Accuracy profile of nornicotine

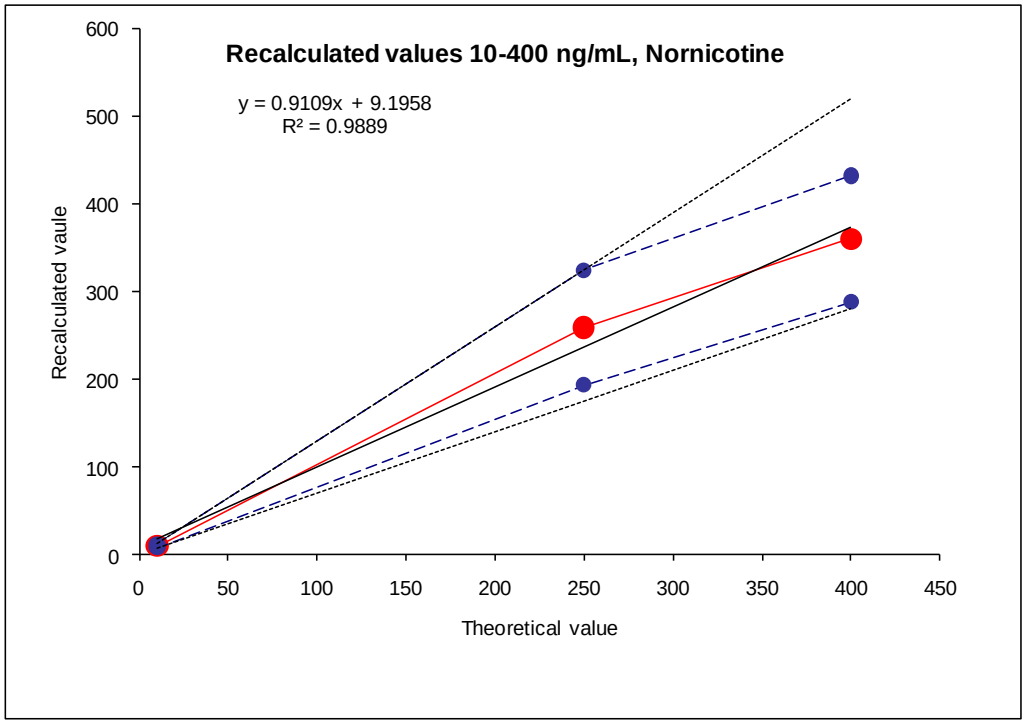

Figure 45 Correlation between recalculated values and theoretical values of nornicotine

9.2.3 *Chromatograms*

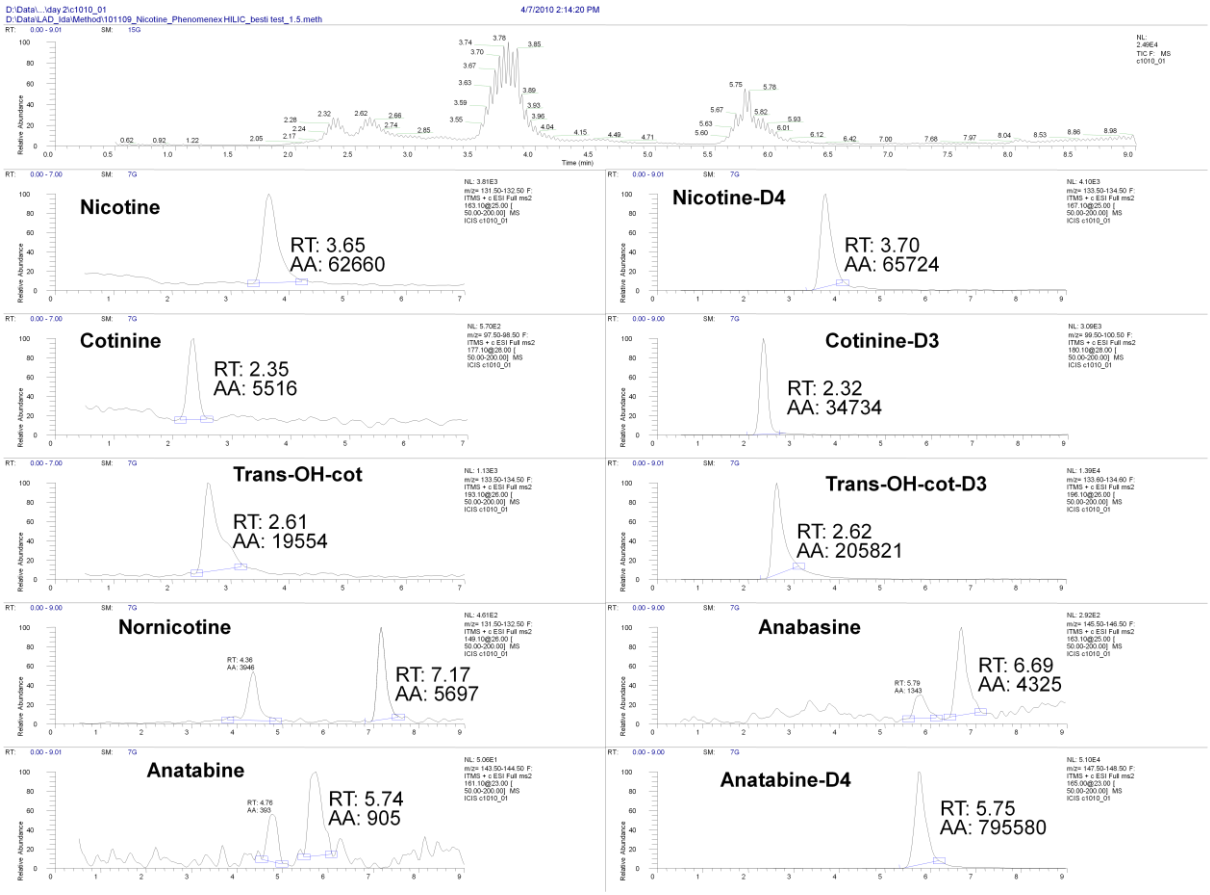

Figure 46 Validation chromatogram, LOQ 10/10 ng /ml

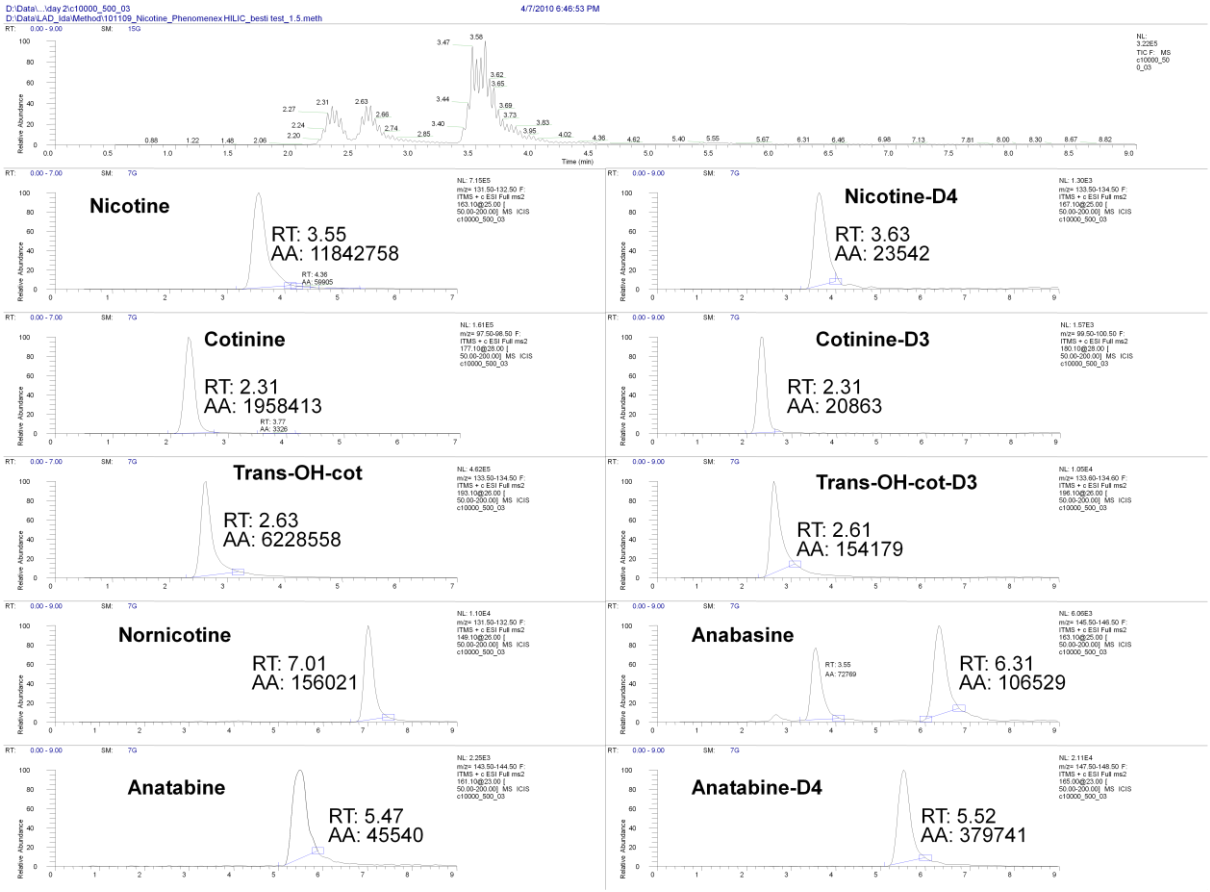

Figure 47 Validation chromatogram, ULOQ 10000/500 ng/ml

9.3 **Method application**

9.3.1 *Concentration ratios*

Table 10 Ratio of the measured analytes in relationship to each other, (calculated from amounts given in table 5)

investegated ratios	measured concentrations (ng/mL)							
	cigarette samples				snus samples			
	1.	2.	3.	4.	1.	2.	3.	4.
nicotine: cotinie	2	0.54	0.05	0.04	0.2	0.35	0.04	0.05
nicotine: trans-oh-cot	0.65	0.2	0.02	0.01	0.09	0.18	0.02	0.01
nicotine: anatabine	34.17	11.06	-	-	-	11.8	0.97	-
nicotine: anabasine	44.22	16.59	-	-	-	9.95	1.08	-
nicotine: nornicotine	10.59	4.24	1.11	0.57	5.25	3.25	0.21	0.48
cotinine: anatabine	17.12	20.49	-	-	-	33.5	23.3	-
cotinine: anabasine	22.16	30.74	-	-	-	28.27	25.89	-
cotinine: nornicotine	5.31	7.86	20.26	16.36	29.91	9.23	5.11	10.5
trans-oh-cot: anatabine	52.9	55.56	-	-	-	63.96	50.96	-
trans-oh-cot: anabasine	68.46	83.33	-	-	-	53.97	56.62	-
trans-oh-cot: nornicotine	16.39	21.32	54.51	51.24	55.52	17.62	11.18	34.44

9.3.2 *Trueness and linearity parameters*

Table 11 Table presenting linearity and estimated amount of bias obtained during method application.

	Calibrators			QC				
	Target concentration (ng/ml)	**Recalculated values (ng/mL)**	**Trueness, Bias(%)**	**Target concentration (ng/ml)**	**Recalculated values (ng/mL)**	**Trueness, Bias(%)**	**Linearity parameters**	
nicotine	10	10.9	8.7%	100	117.2	17.2%	slope	0.1193
	10	9.1	-8.7%	100	114.1	14.1%	intercept	0.0619
	10000	10636.6	6.4%				R^2values	0.9919
	10000	9363.4	-6.4%					
cotinine	10	11.4	14.1%	100	86.0	-14.0%	slope	0.0182
	10000	8.6	-14.1%	100	102.0	2.0%	intercept	0.0385
	100	11036.0	10.4%				R^2values	0.9789
	100	8964.0	-10.4%					
trans-oh-cotinine	10	9.3	-7.3%	100	115.4	15.4%	slope	0.0074
	10	10.7	7.3%	100	90.8	-9.2%	intercept	0.0315
	10000	8598.6	-14.0%				R^2values	0.9621
	10000	11401.4	14.0%					
anatabine	10	11.5	15.4%	100	115.4	15.4%	slope	0.0010
	10	8.5	-15.4%	100	90.8	-9.2%	intercept	0.0024
	500	488.7	-2.3%				R^2values	0.9989
	500	511.3	2.3%					
anabasine	10	11.9	18.8%	100	130.8	30.8%	slope	0.0016
	10	8.1	-18.8%	100	83.0	-17.0%	intercept	0.0011
	500	467.5	-6.5%				R^2values	0.9912
	500	532.5	6.5%					
nornicotine	10	10.2	2.3%	100	119.0	19.0%	slope	0.0024
	10	9.8	-2.3%	100	73.1	-26.9%	intercept	0.0057
	500	504.8	1.0%				R^2values	0.9998
	500	495.2	-1.0%					

9.3.3 *Chromatograms*

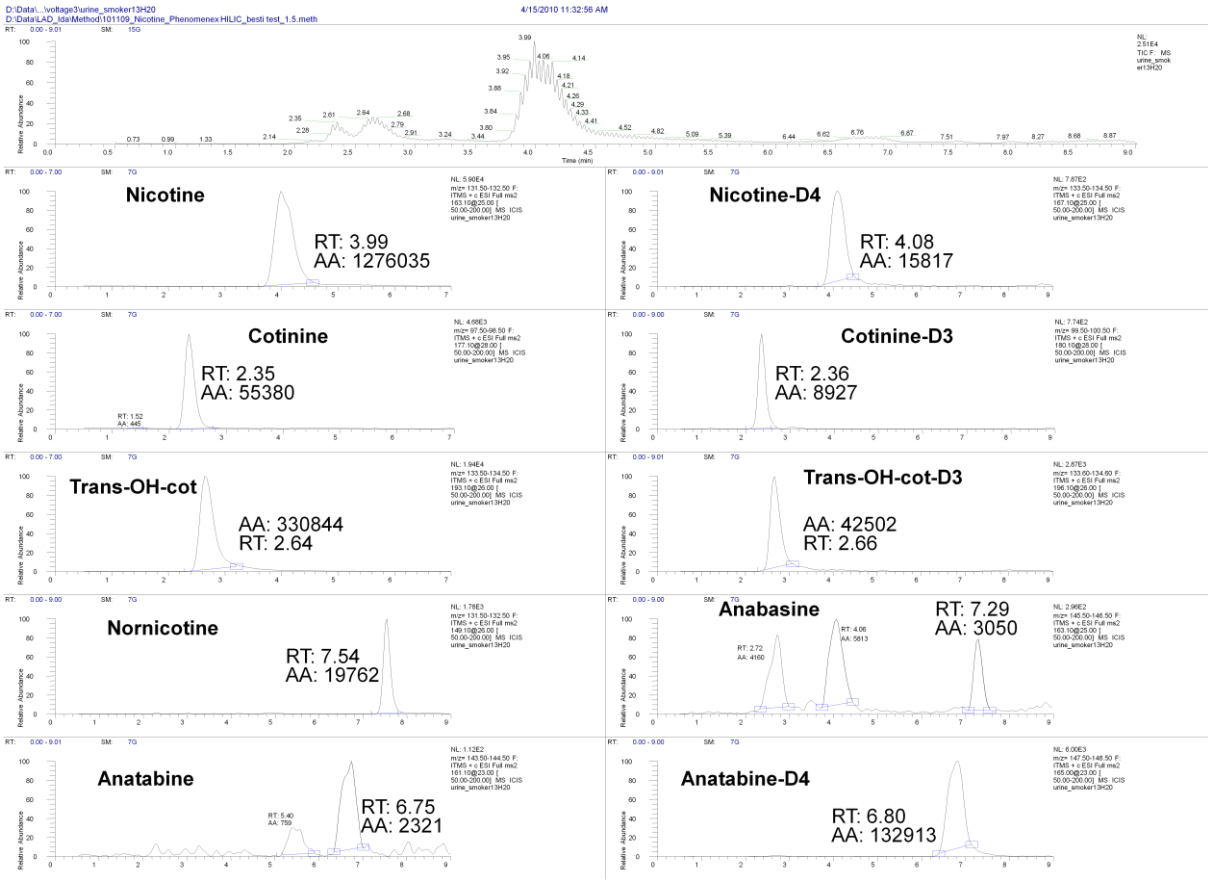

Figure 48 Chromatogram of urine sample of smoker, 1 h after consummation of 1 cigarette

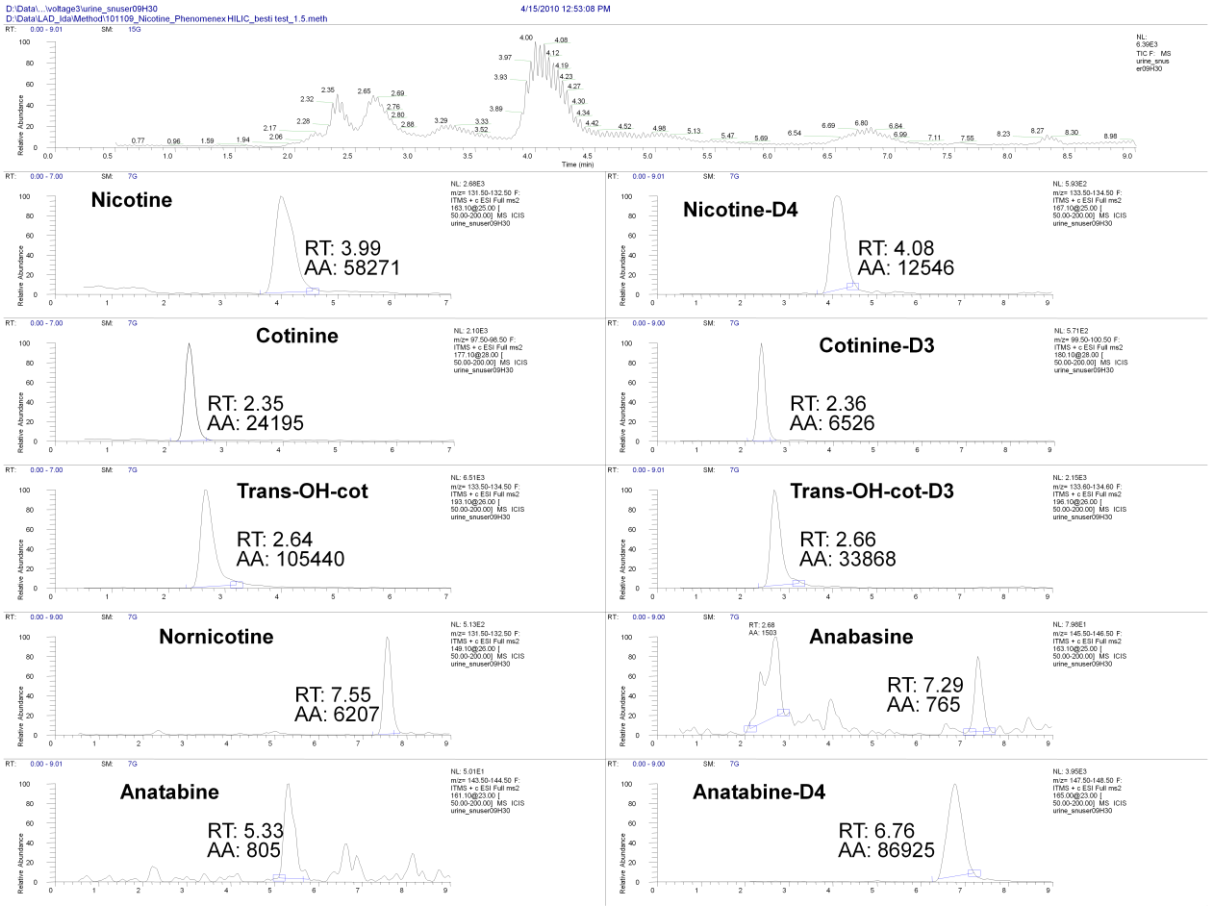

Figure 49 Chromatogram of urine sample from snuser, 1 h after consummation of one snus