Determination of Nicotine, Cotinine and Nicotine N-Oxide in Human Blood, Plasma, Urine, Semen and Sperm by LC-Orbitrap MS: Application to Clinical Study

Dissertation

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Summery

A high throughput, robust, simple, and economic bioanalytical method for simultaneous determination of nicotine (Nic), cotinine (Cot) and nicotine N-oxide (Nox) in human urine, plasma, semen, sperm and dried blood spot (DBS) was developed and validated by LC ESI-orbitrap-MS. The current described method was successfully developed using deuterated nicotine (nicotine-d3) as internal standard (IS) following single extraction step by trichloroacetic acid solution (TCA) for protein direct precipitation, providing top extraction recovery and short run time with accurate mass measurements. The method was then validated according to the European and American guideline for bioanalytical method validation in terms of specificity, matrix effect, linearity, sensitivity, precision, accuracy and stability. Chromatographic conditions were optimized using Kinetex-C18 column (150 × 2.1mm, 5µm) and eluted by mobile phase of methanol:water:formic acid (10:90:0.1%, v/v/v).

Accurate mass measurements were obtained by ESI-orbitrap-MS at positive m/z 163.1235, 177.1028, 179.1188 and 166.1423 for Nic, Cot, Nox and IS, respectively. The established calibration range for Nic, Cot and Nox in urine analysis was linear between 50 and 3000 ng/mL, in plasma, semen and DBS was linear between 5-250 ng/mL for Nic and Nox, and between 10-500 ng/mL for Cot. The within and between days measurements precision and accuracy were all within the acceptance criteria according to the European and American guidelines for bioanalytical method validation, and the extraction recovery was higher than 95% for the studied matrixes. The ionic suppression test was achieved and discussed for the all investigated fluidic matrices.

The established method was intended to investigate three objectives, including study the influence of pomegranate and licorice test drinks on Nic metabolism rate. This was done by urinary measurements for metabolic index of Nic/Cot, in addition to new introduced metabolic index Nic/Nox, where urine samples were collected from twenty four smoker volunteers under pomegranate and licorice drink conditions, and compared their measurements to corresponding control condition.

The second study objective was intended to investigate Nic with its major metabolites Cot and Nox availability in human plasma, semen and sperm, where Nic has extensive distribution among human fluids and tissues, but still nobody knows whether Nic is available in sperm or not. Herein, sperm bodies were separated quantitatively from each 1 mL of twelve human's semen samples by centrifugation and extracted. As a result, Nic and Cot were detected in all samples, and Nox was below the LLOQ in plasma but detectable in all semen samples and 10 out of 12 sperm samples. Interestingly, Nic was significantly higher in semen compared to plasma (2.3-fold, $p<0.001$). The measured Nic and Cot concentrations in sperm samples were comparable to the corresponding measurements in plasma, and Nic was significantly lower in sperm compared to semen (2.1-fold, $p<0.001$).

The third study objective was intended to find a suitable bioanalytical method using DBS technique as an alternative to the traditionally used methods. A 6.35 mm disk diameter was cut out of the filter cards and extracted by TCA solution in a single extraction step. Chromatographic effect was investigated in current study and demonstrated that the overall variation accounted due to decentralized punch and hematocrit effects are caused a minimal variation factor in Nic and Cot quantitation by DBS. The optimized method was then applied to collect blood samples from twelve smoker volunteers, and compared their DBS measurements with the corresponding plasma measurements.

Zusammenfassung

In dieser Arbeit wurde eine durchsatzstarke, robuste, einfache und ökonomische bioanalytische Methode für die simultane Analyse von Nikotin (Nic), Cotinin (Cot) und Nikotin N-oxid (Nox) in menschlichem Urin, Plasma, Samenflüssigkeit, Sperma und getrockneten Blutproben (engl. *dried blood spot*, DBS) mit LC-ESI-orbitrap-MS entwickelt und validiert. Zum Ausfällen der Proteine wurde ein einzelner Extraktionsschritt mit Trichloressigsäure (TCA) eingesetzt und als interner Standard (IS) diente deuteriertes Nicotin (nicotine-d3). Die entsprechend der europäischen und amerikanischen Richtlinie für bioanalytische Methodenvalidierung hinsichtlich Spezifität, Matrixeffekt, Linearität, Sensitivität, Präzision, Genauigkeit und Lagerstabilität validierte Methode zeigt hohe Wiederfindungsraten, weist kurze Analysenzeiten auf und liefert akkurate Massen der Analyten. Nach Optimierung der chromatographischen Bedingungen wurde eine Kinetex-C18 Säule (150 x 2.1 mm, 5 µm) mit Methanol:Wasser:Ameisensäure (10:90:0,1 %, v/v/v) als Laufmittel eingesetzt.

Mittels ESI-orbitrap-MS wurden akkurate Massen im Positivmodus aufgenommen. Bei der Auswertung wurden die m/z-Verhältnisse 163,1235, 177,1028, 179,1188 und 166,1423 für die Substanzen Nic, Cot, Nox bzw. IS herangezogen. Der lineare Bereich für Nic, Cot und Nox in Urin lag bei 50-3000 ng/mL. In Plasma, Samen und bei den DBS wurde ein linearer Bereich von 5-250 ng/mL für Nic und Nox sowie von 10-500 ng/mL für Cot festgestellt. Präzision und Genauigkeit der Messungen entsprachen den Kriterien der angewandten Richtlinien und die Wiederfindung nach der Extraktion lag für alle Matrices bei über 95 %. Die Ionensuppression wurde für alle untersuchten Matrices getestet und bewertet.

Mit der entwickelten Methode wurden in dieser Arbeit drei Fragestellungen bearbeitet. Als erstes wurde der Einfluss von Granatapfel- und Lakritzgetränken auf die Metabolisierungsrate von Nikotin anhand von Bestimmungen des metabolischen Index Nic/Cot sowie eines zusätzlichen neuen, in dieser Arbeit erstmalig verwendeten Index Nic/Nox, in Urin untersucht. Im Vergleich mit den entsprechenden Kontrollbedingungen

zeigte sich bei den 24 Raucherprobanden unter dem Einfluss von Granatapfel- und Lakritzgetränken eine beschleunigte Metabolisierungsrate von Nikotin.

Im zweiten Teil dieser Arbeit wurde der Gehalt von Nikotin und seinen Hauptmetaboliten Cotinin und Nikotin-N-Oxid in menschlichem Plasma, Samenflüssigkeit und Sperma untersucht. Nikotin verbreitet sich im menschlichen Körper in Flüssigkeiten und Geweben, aber es ist bisher nicht bekannt, ob Nikotin in Sperma vorhanden ist. Dazu wurden Spermienzellen aus je 1 mL Spermienflüssigkeit von 12 Probanden durch Zentrifugation quantitativ abgetrennt und mittels der optimierten Methode extrahiert. Nikotin und Cotinin wurde in allen Proben detektiert. In Plasma lag der Gehalt von Nikotin-N-Oxid unterhalb der Quantifizierungsgrenze, in allen Samenflüssigkeitsproben und in 10 von 12 Spermaproben konnte Nox aber detektiert werden. Der Nikotin- und Cotiningehalt in Sperma war mit dem Gehalt in Plasma vergleichbar. In Samenflüssigkeit war der Gehalt an Nikotin signifikant höher als in Plasma (2,3-fach, $p<0.001$). Im Sperma lag die Konzentration von Nic und Cot im gleichen Bereich wie für Nic im Plasma. Die Nic-Konzentrateion im Sperma war jedoch signifikant niedriger als in der Samenflüssigkeit (2,1-fach, $p<0,001$).

Im dritte Teil dieser Arbeit wurde eine bioanalytische Methode unter Verwendung von getrockneten Blutproben (DBS) als Alternative für traditionell eingesetzte Methoden entwickelt. Die auf Filterpapieren getrockneten Blutproben wurden als Plättchen von 6,35 mm Durchmesser ausgestanzt und mit Trichloressigsäure in einem einzelnen Extraktionsschritt extrahiert. Die Untersuchung chromatographischer Effekte zeigte, dass Variationen der DBS Methode durch nicht exakt zentriertes Ausstanzen der Bluttropfen und hämatokritische Effekte nur zu minimalen Schwankungen der ermittelten Nikotin- und Cotiningehalte führen. Anschließend wurde die optimierte Methode auf Blutproben von zwölf Rauchern angewandt und die Messungen mittels DBS wurden mit den entsprechenden Plasmaproben verglichen.

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1 Introduction

1.1 Nicotine Overview

Nicotine (Nic), chemically 3-(1-methyl-2-pyrrolidinyl) pyridine, is a tertiary amine structured from a pyridine and a pyrrolidine ring ($C_{10}H_{14}N_2$, Figure 1.1 A) and a colorless to pale yellow oily liquid with boiling point 247 °C and exact mass 162.115698 g/mol [1]. Nic occurring naturally as a major alkaloid compound in tobacco leaves and consists about 95% of the total alkaloid content [2, 3].

Nic has historically known since centuries, it was described and reported in 1571 [4], and first determined from tobacco leaves was in 1828 [5]. In spite of negative effect for Nic on human health, it has a high consumption rate around the world, and it becomes provided commercially in many products including tobacco, pesticide [6–8] and Nic replacement therapy (NRT) [9].

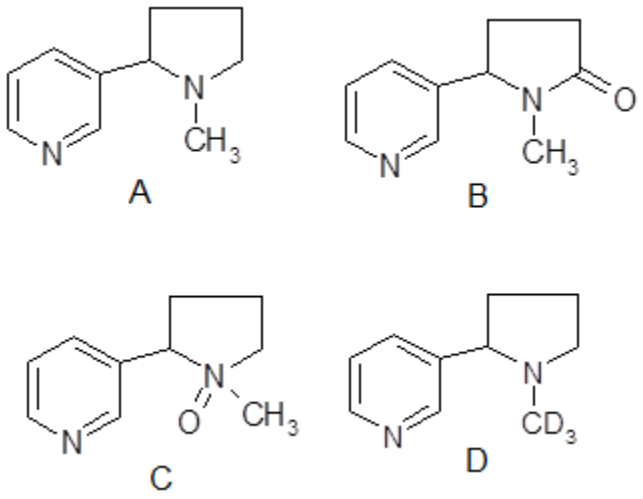

Figure 1.1 Chemical structure of Nicotine (Nic, A), Cotinine (Cot, B), Nicotine-N-oxide (Nox, C) and deuterated Nicotine (Nic-d3, D).

1.1.1 Nicotine Absorption and Distribution

Nic is a weak base with a pK_a of 8.0, it will be absorbed rapidly through biological membranes in basic medium (in non-ionized form), it can be absorbed through oral cavity, skin, lung, urinary bladder, and gastrointestinal tract depending on the absorption environmental pH [10]. In smokers, the suspended Nic in smoking main stream mainly absorbed in lung and mouth cavity [11], and the highest absorption fraction through inhalation occurs in lung (60 % to 80 % of Nic intake [3]), due to the high surface area of lung alveoli, and facilitated absorption across cell membranes through basic medium (pH ≈ 7.4), whereas 30% of Nic being non ionized, and its binding affinity to plasma proteins is around 5% [11].

Following Nic absorption, it enters bloodstream and reaches the systemic circulation, then crosses the placental barrier easily to reach brain within 20 s.

Nic with its metabolites distributed extensively among human fluids and tissues and determined in smoker's autopsy tissues of brain, lung, liver, kidney, muscle [12, 13], spleen, abdominal, thigh, adipose [13]. *Tricker* has described Nic occurrence in human body in details [14], and he also described a variety of analytical techniques used in biomarkers determination derived from Nic and its metabolites, where most of biological fluids and matrices were investigated such as blood, saliva, urine, follicular fluid, fetal fluids, seminal plasma, sweat [14], hair [15], breast milk [16], cervical mucus [17], toenail and the deciduous teeth [18] and bones [19]. But still no described investigation whether Nic or its biomarkers cotinine (Cot) and nicotine N-oxide (Nox) are available in the sperm bodies or not, and only limited studies were reported by *Tricker* for Nic or Cot analysis in seminal plasma [20–22].

1.1.2 Nicotine Metabolism

Nic metabolism occurs in human liver mainly by cytochrome p450 (CYP) 2A6 to its major metabolites Cot and Nox [23–25] and many other metabolites as illustrated in Figure 1.2 [26, 27]. CYP2B6 is another responsible isoform about Nic metabolism, but it accounts around 0.2% of human liver CYPs [29] and plays a minor and conditional role in Nic metabolism [25, 30], especially at high Nic concentration [28]. CYP2A13 is also another minor metabolizer for Nic in a certain conditions [31].

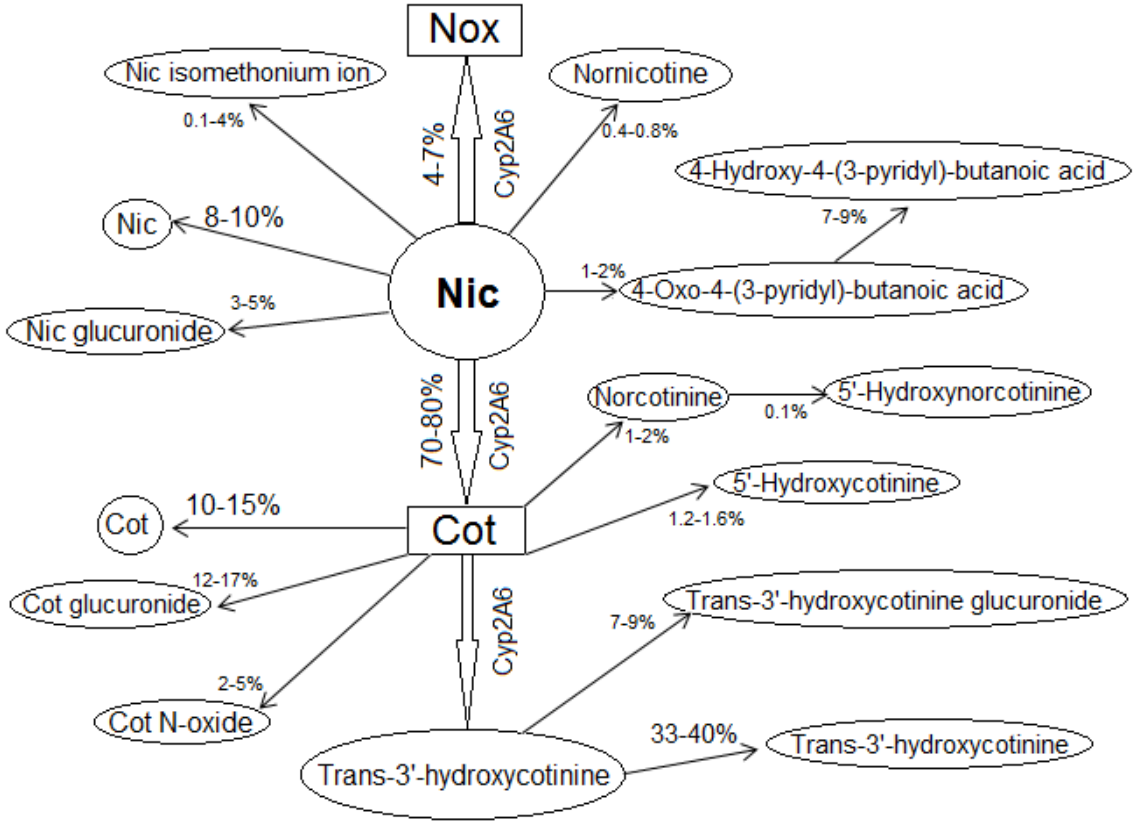

Figure 1.2 Percentage quantitative illustration scheme for Nic metabolism, estimated from total urinary excretion, modified by [26, 27].

The CYP2A6-catalyzed metabolism for Nic involves two steps; first, C-oxidative transformation to produce Nic-iminium ion, in equilibrium with hydroxyl-Nic. The second step is an aldehyde oxidation to produce the principal metabolites Cot in 70–80%, and Nox in 4–7% [32]. Cot undergoes further metabolism steps to produce 3-hydroxy-Cot (3HC) in 33–40%. Nox has no further metabolism evidence, except its back conversion to Nic in the intestines in low recycling level [11].

Nic metabolism is variable among human subjects, in addition to genetic wild-type CYP2A6 [30] and CYP2A6 genetic variability factor [33], there are many other factors affecting Nic metabolism rate, including physiological influences, such as diet and meals [34, 35], age, gender, pregnancy [36], menstrual cycle in women, kidney disease and sleeping [11].

Synchronized ingestion of medications or phytochemicals with Nic intake is also a significant factor that affecting Nic metabolism, where such chemicals are metabolized mainly in liver, and they have interactions with hepatic CYPs [36] which could lead to inhibit Nic metabolism or to stimulate its metabolism rate by induction effect.

There are many examples from the literatures for inhibited Nic metabolism by phytochemicals derived from grapefruit [37], mint drink [38] and many other compounds [28], and the accelerated Nic metabolism by the mean of induction effect has also reported, such as phenobarbital and other anticonvulsant drugs [11]. On the other hand, the accelerated drugs metabolism by induction effect has occurred by Nic [39, 40], and the inhibited metabolism rate for some other drugs were also reported [41].

1.1.3 Nicotine's Adverse Health Consequences

Nic has a wide adverse health consequences [42, 43], especially if taken in high doses [44, 45]. It's an addictive drug [46], which makes difficulties in stop smoking cigarettes [47, 48]. In addition to its health consequences, Nic has been used as insecticide due to its poisoning capabilities [8, 49–51], where insects are not reaching tobacco leaves and farms in nature.

In spite of no evidence for Nic impact on human health - as shown from epidemiological studies relating to the use of Swedish type moist snuff (as Nic supplement) [52] - a lot of studies were correlated using smokeless tobacco to adverse health consequences [53], e.g. the induction of many cancers by Nic [54], disability and early death [55].

1.1.4 Nicotine Impact on Human Reproduction

In addition to Nic's adverse health consequences profile, it has a particular impact on human reproduction as well [56, 57]. Many studies are correlating smoking cigarettes to males infertility, in terms of influenced testicular function and semen quality [58], reduced sperm production, sperm motility, sperm normal forms and sperm fertilizing capacity through increased seminal oxidative stress and DNA damage [59–66]. More accurate in vitro-based studies for Nic effect on human infertility was also correlated with Nic intake on sperm membrane, DNA integrity and viability [67]. In addition to its

genotoxic impact on chromosomes of human fetal cells [68], another animals-based study about Nic oral dose-dependent effect on sperm characteristics and fertility of male rats showed a significant decrease in sperm motility and count for treated rates compared with untreated control rates. More indications were also reported by measuring Nic [69] and Cot [21] levels in seminal plasma, but still nobody knows whether or not Nic or its biomarker Cot or Nox is available in sperm bodies, which could be directly correlated to human infertility.

1.2 Pomegranate Fruit Identification and Enzymatic Interactions

A highly popular functional food of pomegranate fruit, has been considered as super fruit, due to its antioxidant constituents and diseases resistant [70] with many of its therapeutic applications [71].

Chemically, the phytochemicals derived from pomegranate are ellagitannins (punicalagin), pelargonidin, punicalin, anthocyanins, cyanins (cyanins day), ellagic acid as shown their chemical structures in figure 1.3 [72] and certain species of flavonoids (figure 1.4) [73].

The studies on pomegranate inhibition effect for CYP 3A4 [74] found a comparable inhibition potency with grapefruit juice [75, 76], in addition to its minor inhibitory effect for CYP 2C9, CYP 1A2 and CYP 2E1 [77].

Therefore, pomegranate juice has a potential interactions with the drugs [78], such as warfarin [79], tolbutamide [80] and carbamazepine [75], which impairs their metabolic activity pathway.

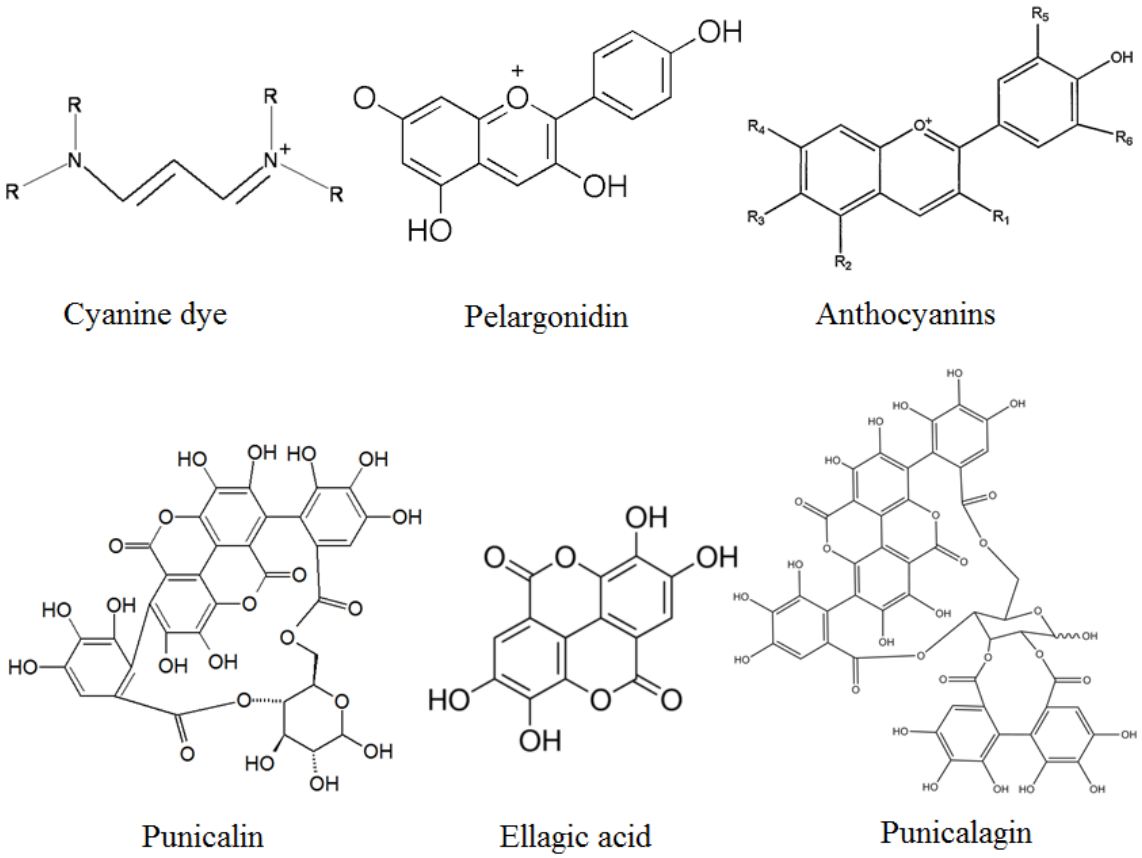

Figure 1.3 Chemical structure for phytochemicals derived from pomegranate fruit.

1.3 Licorice Root Identification and Enzymatic Interactions

Licorice originated in the Mediterranean, native to southern Europe and parts of Asia and has been used medically to treat many health disorders [81]. It is the most frequently used herbal medicines over worldwide, and known as herbs grandfather, due to its vast biomedical applications [82], it contains to glycyrrhizin, which is 50 times sweeter than sucrose.

Chemically, licorice contains phytochemicals (as shown in figure 1.4) of triterpene glycosides (including glycyrrhizin or glycyrrhitic acid), polysaccharides, flavonoids, coumarin derivatives, formononetin [83] and glabridin [84].

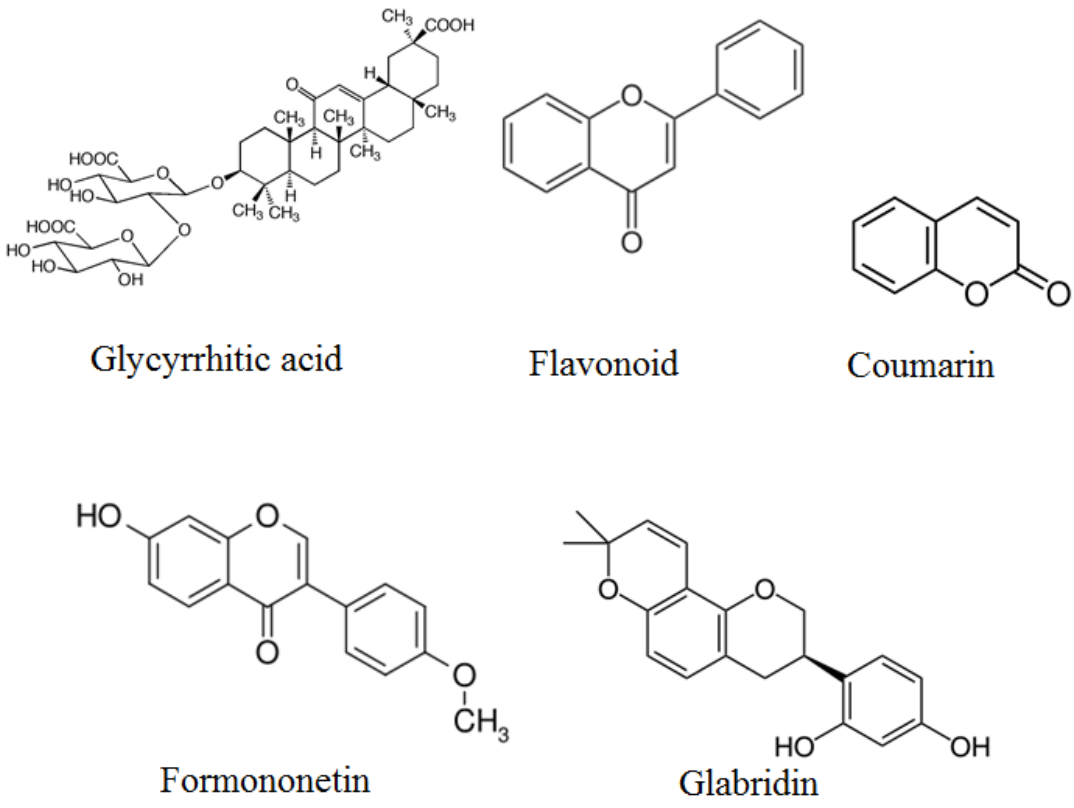

Figure 1.4 Chemical structure for phytochemicals derived from licorice root.

The phytochemicals of isoflavan and glabridin derived from licorice root inhibit the activities of human CYP 3A4, 2B6, 2C9 [29], 1A2, 2C19 and 2D6 [82], which are responsible for 80% of known drugs metabolism [85]. Many studies are demonstrating positive effect for licorice in therapeutic applications [86, 87], due to its strong and variable interactions with hepatic CYPs, that lead to clinical-relevance for the prescribed drugs [88].

Licorice metabolism occurred mainly by CYP 3A4, which metabolizes more than 50% of clinically used drugs [29]. There are many inhibited drugs metabolism in presence of licorice [89], and its induction effect was also reported [90].

1.4 Pomegranate, Licorice and Nicotine Interactions

Nutrition (including food, herbs, fruit juice and drugs)-drug interactions have recently received a wide attention in the clinical investigations research area for in vivo drugs kinetics when administrated concurrently with such phytochemicals.

Pomegranate [74] and licorice [29] are exhibiting a potential inhibition effect for CYP3A4, in addition to a minor inhibitory effect by licorice for CYPs of 2B6, 2C9 [29], 1A2, 2C19 and 2D6 [82], and pomegranate has also a minor inhibitory effect for CYP2C9, CYP1A2 and CYP2E1 [77], while Nic metabolism occurs mainly by CYP 2A6 [23–25], in addition to CYP2B6 [25, 30] and 2A13 [31], which play a minor role under certain conditions, especially at high Nic concentrations [28].

Table 1.1 Summarizes all CYPs which are responsible about metabolism for each of Nic, pomegranate and licorice, where each CYP correlated by tick sign with corresponding inhibitor.

From Table 1.1, there is no common CYP between Nic and pomegranate or licorice in the major metabolism path. CYP2B6 plays a minor metabolism role as a common metabolizer between Nic and licorice, but it accounts 0.2% of human liver CYPs [29], where it metabolizes around 3% of all drugs, and it plays a minor role in Nic metabolism [25, 30], especially at high Nic concentrations [28].

Upon the intensive search from the literature for inhibited CYPs by Nic, pomegranate or licorice, there is no inhibitory metabolic competition between Nic and each of pomegranate and licorice as summarised in Table 1.1, such interactions for CYP3A4 with pomegranate or licorice could induce CYP2A6, that lead to accelerates Nic metabolism, and the interactions mechanism for CYP's with phytochemicals which lead to inhibition or induction effect [91, 92] attributes such outcome.

Table 1.1 Correlation of CYPs with corresponding inhibitor.

CYP	Nic	Pomegranate	Licorice
	Major metabolic path		
3A4		✔	✔
2A6	✔		
	Minor metabolic path		
2A13	✔		
1A2		✔	✔
2B6	✔		✔
2C9		✔	✔
2C19			✔
2D6			✔
2E1		✔	

1.5 Nicotine Analysis in Human

Nic with its metabolites has long been determined analytically in many applications of medicinal, pharmaceutical, environmental and industrial research fields. In vivo investigations for Nic is a challenging analytical mission, due to the vast and variable application demands, such as matrix separation, sensitivity of lower limit of quantification (LLOQ) and validity of the optimized analytical method.

1.5.1 Methods of Extraction

Excellent methods for Nic determination in human fluids like liquid-liquid (LL) extraction [93] and LL-back extraction [38], solid phase extraction (SPE) [94] and micro-SPE [95] in addition to online-SPE for human tissues [12] and dry blood spots (DBS) [96] are reported. Urine direct injection into analytical system was also used for Nic determination [97–99], but not possible for plasma or other thick fluids injection.

Simpler and more economic methods with less extraction steps and higher sample throughput are still required to manage large-scale human bio monitoring studies with low sample volume.

Protein direct precipitation with agents like methanol [100], trichloroacetic acid (TCA) [101], acetonitrile [102], hydrochloric acid as extraction method for the analytes from human fluids has been used for drugs determination in human plasma in many applications. The mechanism of action for protein precipitation still not clearly understood. But many proposed mechanisms were described protein separation from its biological fluid by van-der-Waal's force between protein molecules and precipitation agent or by protein's dehydration in peripheral layer, which leads to cross-linking complexes formation and electrostatic hydrophobic interactions [103]. Another mechanism of action for protein precipitation was described by the salting out procedure [104]. Figure 1.5 shows how protein constituents were precipitated from human plasma by addition 20% w/v of TCA solution to the samples, and figure 1.6 demonstrates how protein constituents were precipitated from human semen by addition 20% w/v of TCA solution to the samples.

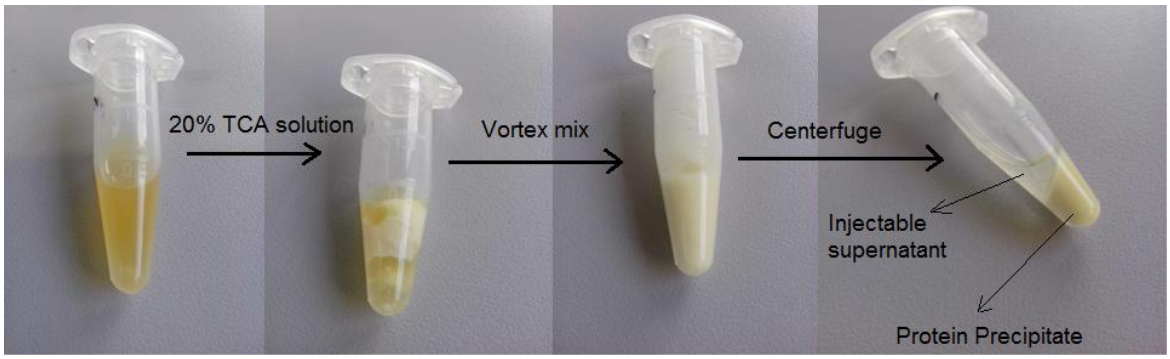

Figure 1.5 Illustration for protein direct precipitation procedure applied on human plasma sample from the current study.

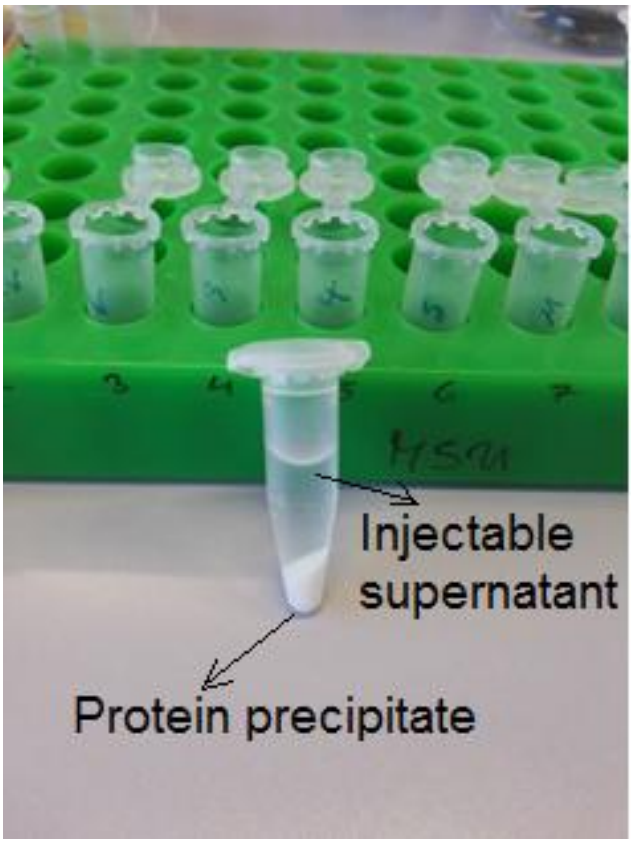

Figure 1.6 Application of protein direct precipitation procedure on human semen sample from the current study.

1.5.2 Analytical Techniques

Many analytical systems have involved in Nic analysis including fluorometric detection and postcolumn UV-photoirradiation for Nic and Cot determination in human serum [105], high performance liquid chromatography with ultraviolet detection (HPLC-UV) for Nic and Cot determination from human plasma [106], colorimetry and immunoassay methods for Nic and Cot determination in human saliva, urine, and blood [107], comparative radioimmunoassay with LC-MS method for Nic and Cot determination from smoker's serum and saliva [108], liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for simultaneous determination of Nic and its nine metabolites in human urine [98], comparative study between reversed phase (RP) – HPLC-photodiode array (PDA) with gas chromatography mass spectrometry (GC-MS)

for determination of Nic and Cot in Jordanian smoker's blood and urine [109], ion trap MS with quadrupole time-of-flight (QTOF) MS for Nic characterization with related compounds through electrospray ionization (ESI) [110] and hybrid tandem quadrupole time of flight (QqTOF) MS was used for simultaneous determination of Nic and its major metabolites Cot and trans-3'-hydroxycotnine in human urine [111].

1.5.3 Orbitrap MS

Orbitrap-MS has first introduced in 2000 [112] by Dr. Alexander Makarov and becomes commercially available in 2004 [113]. Orbitrap device consisted from central inner electrode which aligned horizontally inside of cylindrically shaped outer electrode as shown in Figure 1.7, these electrically charged electrodes are forming a cylindrical hole that confines ions revolving around the inner electrode, which allowing fractionation to be occur for ions with different m/z values into rings surrounding the electrode through their orbital motion [112, 113]. Consequently, the pulsed ions into orbitrap device are oscillating along with axial frequencies of 50–150 kHz [114].

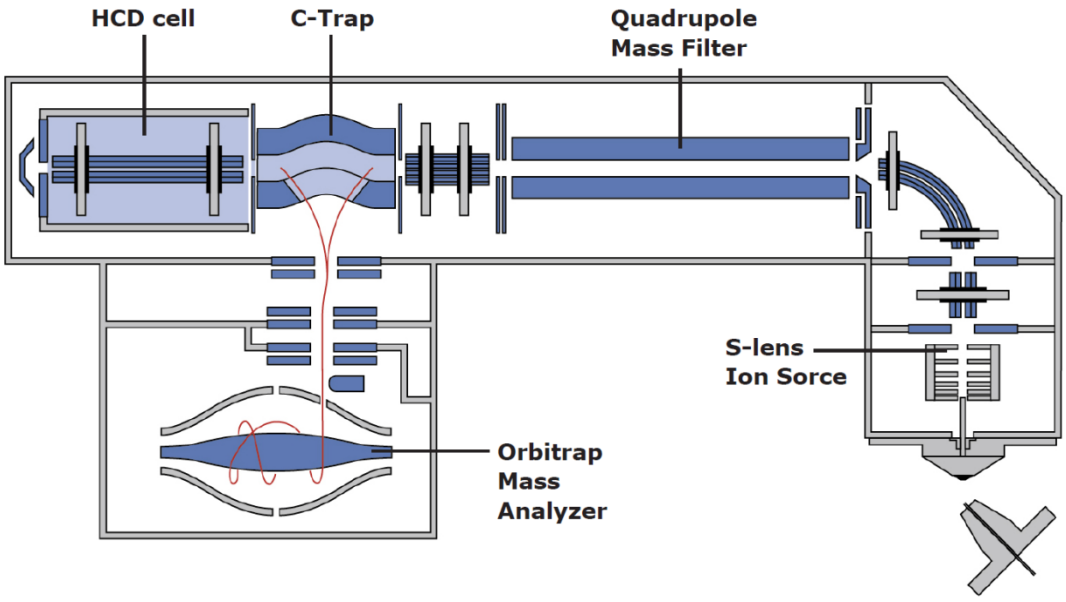

Figure 1.7 Schematic diagram for orbitrap mass analyzer with C-Trap device [113, 115].

1.5.4 Dried Blood Spot

DBS have been used for screening of metabolic disorders in newborns for decades, and have already become a popular sampling method for the quantitation of small molecules in blood [116]. This technique has more recently been applied to drug metabolism (DM) [117], pharmacokinetic (pk) [118], therapeutic drug monitoring (TDM) [119], and toxicokinetic (TK) [120] studies. DBS has applied in tobacco smoke research field as well as an alternative sampling method for Cot determination in human blood as a biomarker for Nic intake in metabolism studies [121], secondhand smokers [122], newborn [123]. Recently DBS has also applied in an automated sample extraction system for purpose of doping relevant compounds determination, using Nic with its major metabolites of nornicotine, Cot and trans-3-HCOT as an applicable example for such drugs testing in sport applications [96].

The use of DBS technique has increased to manage high population-based studies, especially those of large-scale human bio monitoring, as well as studies involving children or small animals, since the skin-stick procedure is technically simple and do not require extensive training or expensive equipment. Only a tiny volume of blood is enough, and handling blood specimens is relatively non-hazardous against pathogens risk, such as HIV.

Many punching-extraction systems are involved in DBS analysis, including the simplest punching equipment like manual puncher in figure 1.8 which has used in current study, automatic mechanical puncher [124] and automatic puncher by laser [124, 125]. Also the extraction methods are vary for the punched DBS such as direct reconstitution in single extraction step, which was used in this study as illustrated in figure 1.8. More extraction steps are also reported for DBS by LLE [126] and SPE [127] including online SPE [96].

The commercially available DBS's cards are manufactured mainly from cellulose and non-cellulose material in a specified thickness and pores. Recently, among these commercially available filter cards, few types are approved by United State (US) food and drug administration (FDA) for quantitative analysis work, which are Ahlstrom 226-K062932, Whatman 903 and PerkinElmer 226 [128]. The most recommended filter cards for drug metabolism and pharmacokinetics (DMPK) in addition to newborns screening and other quantitative routine analysis work are general electronics (GE) Whatman 903

FTA cards with its sub-type A, B, C as a satisfactory filter paper for quantitative analysis requirements [128].

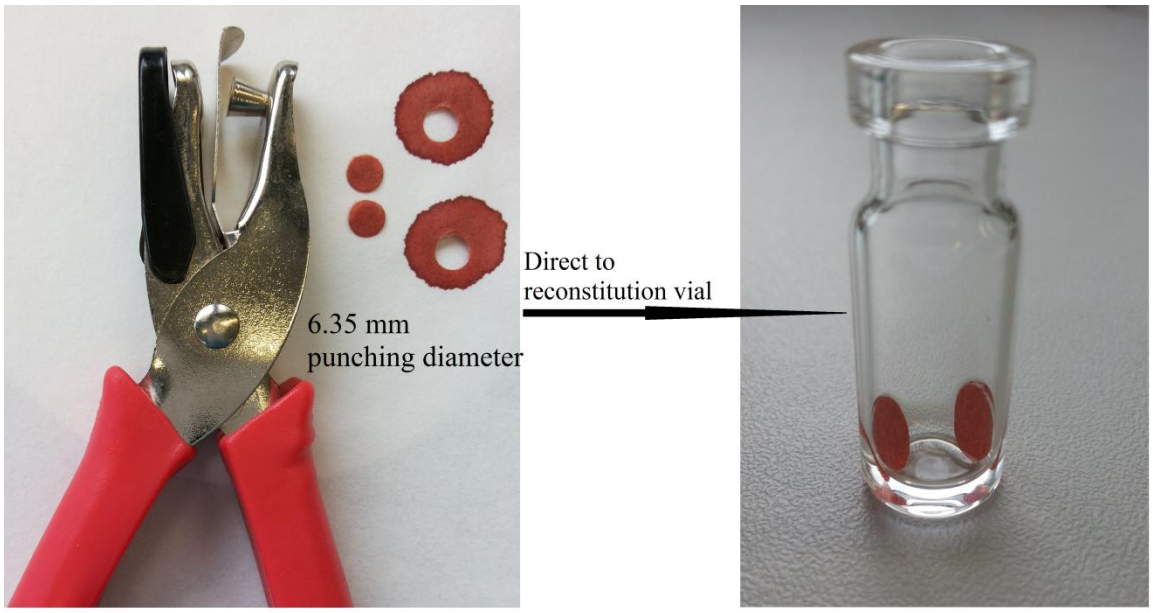

Figure 1.8 Illustration for DBS punching and reconstitution procedure from the current study.

1.5.5 Investigated Nicotine in Human Fluids and Tissues

Nic is distributed extensively in human body, it was determined with its metabolites in smoker's autopsy tissues of brain, lung, liver, spleen, kidney, skeletal muscle, abdominal, thigh, adipose [13], hair [129], nails, teeth [18] and bones [19]. A limited studies were described Nic availability in human solid tissues in spite of its significant levels were found, and not as much as those reports for Nic investigation in human fluids, such as human plasma [130], saliva [131], urine [132], female breast milk [16] and cervical mucus [17]. A significant concentration for Nic and its major metabolites were also found in human's seminal fluid, but limited studies were described its analysis in seminal plasma using non- validated analytical method [20–22], and no further studies were reported for Nic determination in human semen or sperm.

1.6 Bioanalytical Method Validation

The demands for analytical methods validation was reviewed and discussed in 1991, by Karnes et al. [133] and described a guidance for bioanalytical method validation. US FDA -center for drug evaluation and research (CDER) - was released their first guidline on bioanalytical methods validation in 2001 [134], and discussed validation sections including the accebtable criteria, using biological fluids by most analytical techniques (such as HPLC, LC-MS, GC and GC-MS). European medicine agency (EMEA) was also in agreement with the US FDA's guideline on bioanalytical method validation. Their first released guideline on validation of bioanalytical methods was in 2011 [135], then followed by the latest version of EMA in 2013 [136].

Both of US FDA and EMA are discussing the full validation sections in terms of selectivity (specificity), accuracy, precision, linearity and stability. US FDA guideline adds more recovery and system suitability sections which are not discussed in EMA. According to both guidelines, when some of the method parameters are changed, a partial validation could be applied to the methods of which are fully validated. The acceptable criteria for measurements accuracy and precision in all validation sections described in US FDA and EMA guidelines are in the same range, where ± 15% biased measurement's accuracy are accepted for each quality control (QC) and standard calibrator sample, and ± 20% biased measurement's accuracy for LLOQ. The described and discussed validation sections in both guidelines as the following:

1.6.1 Selectivity (Specificity) and Carryover

Specificity of any analytical method is described as the ability of an analytical method to quantify the targeted analyte peaks in presence of other components in the sample matrix. The specificity of the method should be evaluated by screening six individual sources of the appropriate blank matrix, which are individually analyzed as replicates, and evaluated for interference with comparison to LLOQ. By roles, the accepted interfering peaks should have less than 20% of the LLOQ's response for the analyte, and 5% for the IS in the analyte matrix. Less matrix samples and lower replicate analysis in specificity or

selectivity test is allowed in cases of difficultly available matrices, like human semen or tissues.

For sample carryover, the evaluation test occurs mainly to ensure that the rinsing solution used to clean up the injection port is able to avoid the injected sample residuals efficiently on subsequent injections, especially at high concentration. Carryover test should be checked through method development, and its evaluation in the method validation should be examined under extreme conditions by injecting blank sample following injection of the highest used concentration. Carryover acceptance criteria in the subsequent blank sample injection after the upper limit of quantification (ULOQ) shouldn't exceed 20% of the LLOQ and 5% for the internal standard.

1.6.2 Matrix Effect and Recovery

Sample's extraction recovery and its matrix effect are the factors that affecting the overall analytical signal intensity by sample's total ions count (TIC). The recovery validation section is described by US FDA guideline on bioanalytical method validation, page number 6, lines 233-240 [137], but it doesn't mentioned in guideline for bioanalytical method validation of EMA [136].

To reflect the extraction efficiency for the analytes from their biological matrix, a set of extracted QC samples should be analyzed in replicate, then comparing their peak area to the corresponding non-extracted ones (prepared by spiking of equivalent amount from QC samples in extracted blank).

The endogenous compounds in any biological matrix could affect the aerosol formation efficiency or droplet evaporation in the ion source of mass spectrometry (MS) [138, 139], where less volatile matrix components negatively affect the sample evaporation in terms of aerosol's droplet transformation into gaseous phase and releasing free ions as a result. Matrix effect should be evaluated by using six different non-pooled sources of biological matrix, given freshly from individual donors.

The matrix impact on ion suppression or ion enhancement should be assessed for each targeted analyte including IS at two different QC levels (at low and high levels). In order

to determine the matrix factor magnitude, a set of extracted blank matrix should be spiked at low and high of QC levels, and compered directly to corresponding prepared QC samples in matrix free solvent.

The differences in analytical signal between injected samples in presence of matrix and in absence of matrix are considered as matrix factor (MF), and internal standard-normalized MF (IS-NMF) should also be calculated by dividing analyte MF by the measured IS MF. The acceptance criteria for the coefficient of variation (CV %) of IS-NMF, calculated from the replicate analysis, shouldn't be more than 15 %.

1.6.3 Calibration Curve Linearity

The linear relationship of concentration with corresponding instrumental response should be studied by regression function over specified concentration points. A calibration curve (CC) should be prepared for each analyte studied in the method validation and it should be included with each analytical run.

CC in bioanalytical methods determinations should be established from blank sample (extracted blank sample without analytes or IS), zero concentration (extracted blank sample with IS) and at least six calibrator samples including LLOQ and ULOQ. The used dynamic range for any application should be established upon the expected in vivo concentration for the targeted analyte by literature aid, to quantitate most of the designed study samples, and the selected LLOQ's concentration as the lowest analyte level in the quantitative CC should be decided upon the investigational targeted level as well. The accepted LLOQ's analytical signal must be more than 5 times of any interfering or baseline noise peak, in other words it should has signal to noise ratio more than 5. LLOQ measurements have the most tolerant acceptable range, where the acceptance criteria for individual measurement accuracy must be more than 80 %, and its replicate analysis CV % shouldn't exceed 20 %.

The specified concentrations that built up a CC should be back calculated upon the established regression function, and the parameters of slope and intercept should be reported for at least three CC's replicate analysis during validation. All calibrators

accuracy should be within ±15% biased from there specified concentration, and ± 20% for LLOQ. At least 75% of the CC's points with at least six calibrator points as minimum acceptable number should satisfy this criterion.

1.6.4 Precision and Accuracy

Accuracy and precision measurements should be derived from replicate analysis for each of QC level in addition to LLOQ, which are independently prepared from different working solution of the calibration standards. The biased calculation accuracy for individual QC measurements shouldn't exceed 15% from corresponding specified concentration and 20% for LLOQ. The intra-or within-run measurements precision should be evaluated from at least five replicate analysis per QC level with a minimum of four QC levels including LLOQ, where QC low should be assigned its concentration as three times higher than LLOQ, QC mid on the middle of ULOQ and 80% of ULOQ for QC high, to cover most of studied dynamic range. The same measurement acceptance criteria are applied for QC measurements in all validation sections with respect of the biased measurement accuracy (±15% for QC and ±20% for LLOQ). The measurements precision should also meet same criteria in terms of CV% (15% for QC and 20% for LLOQ). Within single analytical sequence, 67% of QC samples should pass through the acceptance criteria.

The between-run or inter-run accuracy and precision measurements should be evaluated by running three sets of within-run sequences. LLOQ measurements accuracy and precision are considered as indicator for the method sensitivity.

1.6.5 Stability

Stability test in bioanalytical method validation is carried out to assure that all steps in sample preparation are stable, including repeatable samples re-entry to deep freezer (freeze-thaw cycles) and short term storage conditions of pre- and post-samples extraction. Samples stability under long term storage conditions should also be studied to ensure that long term storage conditions are preservative enough for the samples.

Stability evaluations should be carried out in replicate analysis for QC low and QC high which are matching storage conditions, then analyzed immediately after each stability test with freshly prepared CC. More than 66% of QC replicate analysis should fit within measurement accuracy of ±15% after each stability test.

1.7 Work Objectives and Significance

A new bioanalytical method for determination of Nic, Cot and Nox in human urine, plasma, semen and sperm, by using LC-Orbitrap MS should be developed and validated in this study, in order to apply three objectives dealing with:

1- Study the Influence of Licorice and Pomegranate Drinks on Nicotine Metabolism in Human Urine.

2- Simultaneous determination of nicotine, cotinine and nicotine N-oxide in human plasma, semen and sperm by LC-Orbitrap MS.

3- Determination of nicotine and cotinine in human blood by dried blood spot-LC-Orbitrap MS technique.

For all objectives in this study, twenty-four Jordanian volunteers will be participated in a clinical study at Jordan center for pharmaceutical research, Amman, Jordan, (http://jcpr-jo.com/Home.aspx) to provide their urine samples under designed conditions to first objective, synchronized with blood and semen samples from twelve volunteer of them for the second study objective in addition to their blood droplets collection over the filter cards for the third study objective.
The applied bioanalytical method in the current objectives will be modified as each analytical objective demands, then validated according to European [136] and US [137] guideline for bioanalytical method validation.

The significance of first objective entitled “Study the Influence of Licorice and Pomegranate Drinks on Nicotine Metabolism in Human Urine” is to evaluate the safely

use of pomegranate and licorice with Nic intake. For this study purpose, a clinical study will be established including twenty-four healthy smoker volunteers, and early morning urine samples will be collected from each participant volunteer under licorice drink condition, pomegranate drink condition and control condition in separate periods. A new bioanalytical method for simultaneous determination of Nic, Cot and Nox in human urine will be developed and validated.

In the second work objective, entitled with "Simultaneous determination of nicotine, cotinine and nicotine N-oxide in human plasma, semen and sperm by LC-Orbitrap MS", a twelve healthy smoker volunteers will provide their semen samples synchronized with blood samples, and the sperm samples will be separated quantitatively from each 1 ml of semen samples from each participant volunteers. Each separate matrix of plasma, semen and sperm will be extracted by TCA solution in single extraction step, and Nic, Cot and Nox concentrations will be determined in human sperm and correlated corresponding levels in semen and plasma samples.

In the third work objective, entitled with "Determination of nicotine and cotinine in human blood by dried blood spot/ LC-Orbitrap MS technique" a blood samples will be collected and dried onto filter cards as spots, these filter cards are designed especially for quantitative drugs analysis. The proposed bioanalytical method by DBS will be validated for quantitative analysis of Nic and Cot in human blood following single extraction step, and then it will be applied for blood samples collection from twelve healthy and smoker volunteers, synchronized with blood samples collection from each participant volunteer following a conventional method (taking blood into tubes for plasma separation), then the measured Nic and Cot concentrations by DBS techniques will be correlated to corresponding measurements by conventional method from plasma analysis.

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2 Study the Influence of Licorice and Pomegranate Drinks on Nicotine Metabolism in Human Urine by LC-Orbitrap MS

2.1 Introduction

Nicotine (Nic) 3-(1-methyl-2-pyrrolidinyl) pyridine (Figure 2.1A) is the major alkaloid component in tobacco leaves [1]. It absorbed in lung immediately by smoking [2], causing disability and early death [3], due to its addiction and toxicological profile [4,5], even it has been used as insecticide due to its poisoning capabilities [6]. Nic metabolism occurs in human liver mainly by cytochrome P450 (CYP) 2A6 to its major metabolites of cotinine (Cot) and nicotine N-oxide (Nox), in addition to other miner metabolites [7]. Also CYP2A13 is responsible about Nic metabolism in certain conditions [8]. Food is reported to increase Nic metabolim rate by increased hepatic blood flow [9], and some of food items are reported to inhibit CYP2A6 competitively with Nic, like mint drink [10] and grapefruit [11]. on the other hand, some of dietary substances like isoflavan and glabridin derived from licorice products [12,13] and certain species of flavonoids and anthocyanins derived from pomegranate [14,15] could accelerate Nic metabolism by induction effect of CYP 3A4, which is mainly metabolizes these phytochemicals. Herein, it is a particular of importance to evaluate the influence and safely use of pomegranate or licorice products when they consumed concurrently with Nic, where Nicotinell®, moist plug and loose leaf chew [16] are Nic-licorice combination product, in addition to many other commercially available licorice products, like sweets, flavors and herbal medicines over worldwide, and such products are subject to influence CYP2A6 activity. In this study, we intend to evaluate the influence of pomegranate and licorice test drinks on Nic metabolism rate, by measuring urinary Nic/Cot, in addition to measure Nic/Nox as a new introduced metabolic index in this study, following a previously reported study design [10] for evaluation the effect of mint drink on Nic metabolism as measured by Nic to Cot ratio in human urine. Furthermore, a new bioanalytical method was developed and validated in this study for simultaneous determination of Nic with its major metabolites Cot and Nox in human urine by LC-ESI-orbitrap-MS, providing high resolution and

accurate mass measurements, top extraction recovery, following single extraction step with short run time (2.3 min), where direct urine injection into LC–MS system was reported [17], but with not less than 8 min run time, and multiple extraction steps were also reported, like liquid–liquid (LL) extraction [18] and LL-back extraction [10], solid phase extraction and micro-SPE [19,20], but more throughput and economic method with less extraction steps are still required to manage large-scale human bio monitoring studies with low sample volume, short run time and minimum running cost as the current described method.

2.2 Experimental

2.2.1 Chemicals and reagents

Nic (purity 98%), Nox (purity 98%) and deuterated nicotine-d3(purity 98%) were obtained from Toronto Research Chemical (TRC) Inc. (Toronto, Canada), Cot (purity 98%) was obtained from Fischer Scientific (Schwerte, Germany), blank of urine samples were harvested from non-smoker donors and stored immediately in a freezer at −40◦C after centrifugation. Deionized water, methanol and formic acid are in LC/MS-quality and purchased from Sigma Aldrich (Schnelldorf, Germany).

2.2.2 Instrumentation

The LC–MS system was constructed from Exactive bench-top orbitrap mass spectrometer (MS) (Thermo Fisher Scientific, Bremen-Germany), equipped with electrospray ionization ESI source and protected by a waste/detector Rheodyne divert valve, attached to (Accela^{TM}) LC system from Thermo Fisher Scientific,1250 Pump model, and controlled by Xcalibur 3.0.63 software for data management system.

2.2.2.1 HPLC conditions

An isocratic elution system was applied by methanol:water:formic acid (10:90:0.1, v/v/v) at constant flow rate of 0.4 mL/min through an analytical (Phenomenex, Kinetex-C18

column 150 × 2.1 mm, 5 _m). The auto-sampler (AS) tray temperature was set at 5 ◦C, and the injection volume was 1 µL.

2.2.2.2 Mass spectrometric conditions

The electrospray ion source was operated in positive ion mode for targeted analytes. The adjusted ion source nitrogen gas was sat for sheath, auxiliary and sweep gases flow at 45, 20, 5 units, respectively. The ion spray voltage was sat at 4500 V and the ion transfer capillary temperature at 300◦C. The MS resolution option was sat at high-50,000 @ 2 Hz with a mass window of 0.1 D for each analyte (Nic, Cot, Nox and Nic-d3).

2.2.3 Standard solutions, calibration curve and QC Samples

Stock solutions of 1.0 mg/mL for Nic, Cot, Nox and Nic-d3 as an internal standard (IS) were prepared separately in methanol and further diluted in 50% methanol for working serial dilution at 50, 100, 200, 500, 1000, 2000 and 3000 ng/mL for Nic, Cot and Nox. For preparation of spiked calibration curve, 50 µL of these solutions were added to 450 µL of urine. Similarly, another set of serial dilution were prepared for quality control (QC) samples at 50 ng/mL for LLOQ, 150 ng/mL for QC low, 1500 ng/mL for QC mid and 2400 ng/mL for QC high.

2.2.4 Sample preparation

Each 100 µL of urine sample was transferred to an eppendorf tube and 20 µL from 10 µg/mL IS solution was added, and vortex mixed, then 500 µL of 10% w/v of TCA solution was added and vortex mixed for 30 s, then the mixture was centrifuged for 10 min at 14,000 rpm by Eppendorf centrifuge (MiniSpin® Plus, 12 × 1.5/2 mL). The supernatant was then transferred into an AS vial.

2.2.5 Bioanalytical method validations

The developed method was validated following the European and United States food and drug administration (FDA) guidelines [21,22]. Full validation was achieved for urine analysis in terms of specificity, carryover, sensitivity, linearity, accuracy, precision, matrix effect, recovery and stability.

2.2.5.1 Linearity, accuracy and precision

Within-run linearity, accuracy and precision were evaluated by running analytical sequence containing six replicates of LLOQ, QC low, QC mid and QC high spiked in urine, with calibration curve including blank and zero. Between-run linearity, accuracy and precision were evaluated by running three sets of within-run sequences in three separate days. The linearity was assessed by a weighted (1/x) least squares regression function.

2.2.5.2 Ion suppression and matrix effect

The ion suppression was checked for urine matrix by synchronized injection of extracted blank, online through post-column infusion for Nic, Cot and Nox standard solution by T-connection [23]. Moreover, matrix effect test was achieved from six different sources of urine (analytes-free blanks) and expressed in terms of matrix factor (MF) through QC low and QC high samples. This was done by comparing the peak area of spiked blank with analyte (after extraction) to the peak area in absence of matrix (matrix-free analyte). IS normalized MF (IS-N MF) was also calculated by dividing the MF of the analyte by the MF of the IS.

2.2.5.3 Recovery and stability

The recovery of extracted Nic, Cot and Nox was determined in urine by duplicate analysis at QC low, QC medium and QC high, and compared their peak area to corresponding concentration (prepared by spiking of equivalent amount from QC samples in extracted blank). All stability sections in the validation were performed in triplicate analysis at QC low and high samples, and calculated upon freshly spiked calibration curve. The stock solution and spiked urine samples were kept under room temperature for 48 h to check short term stability. Freeze–thaw cycle's stability of the samples was obtained over four freeze–thaw cycles by thawing from frozen state at room temperature for 1 h and refrozen for 24 h. Other processed QC samples were delayed to 48 h and injected to evaluate their stability under AS condition at 5◦C.

2.2.6 Preparation of pomegranate juice and licorice drink

Pomegranate juice was prepared by seeding, the seeds were then filled into fruit squeezer machine which doesn't crash the inner hard seeds, and then the fresh juice was provided daily to the volunteers. Licorice drink was prepared from its dry roots. Each kg of licorice roots powder was soaked for 6 hr at room temperature in 300 mL of water contains 1 g of sodium bicarbonate. The slurry mixture was then diluted by 3 L of drinking water and filtered to be provided daily and freshly prepared to the volunteers.

2.2.7 Study Design

In this study, 24 healthy and smoker Jordanian volunteers were participated and selected upon inclusion/exclusion criteria. The informed consent and consent form were obtained from all participant volunteers in Jordan center for pharmaceutical research, Amman, Jordan. The selected adult males were participant in two groups; twelve volunteers were selected for each group of A and B depend on their demographic data in Table 4 including fagerström score for each volunteer. In an open-label, two-period crossover design for each of pomegranate and licorice drink treatment, group A was assigned to drink 150 mL of licorice beverage three times a day, for seven successive days in the first period, while group B was avoided from licorice products and asked to drink water instead of licorice, then both groups were switched their drink treatment in the second period. Similarly, after 7-days wash up period, pomegranate fresh juice was provided three times a day for each volunteer in group A, and group B were avoided from pomegranate products in first period, then both groups were switched their drink treatment in subsequent period. Early morning urine samples were collected from all volunteers within 30 min and before taking the first cigarette and all samples were then frozen immediately at −40 °C. The collected samples from avoided test drink group were considered as control samples. The frozen samples were then shipped in ice box to Germany (Duisburg-Essen University) and kept under −40 °C deepfreeze until the analysis day. The medicines which have impact on Nic metabolism [1] were avoided, and fruit or food items which reported to influence on Nic metabolism was also avoided through whole study periods, such as grapefruit [11]. All volunteers asked to smoke their

usual preferred tobacco brand excepting those of licorice-tobacco combination products during the study.

2.3 Results and discussion

2.3.1 Orbitrap-MS analysis

Mass spectrum in Figure 2.1 shows m/z molecular ion measurements for Nic, Cot, Nox and Nic-d3 (IS), detected by Orbitrap-MS in ESI positive scan mode with high mass accuracy, sensitivity and selectivity at m/z 163.1237, 177.1031, 179.1187 and 166.1426, respectively, the current mass measurements are in agreement with high resolution detection by Q-TOF MS [24].

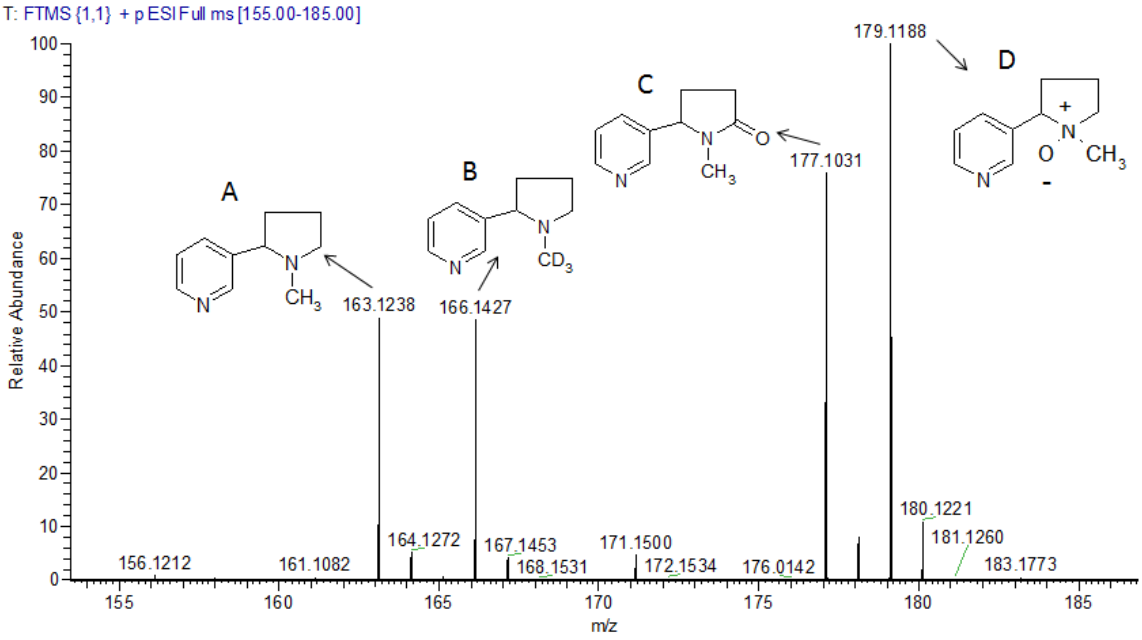

Figure 2.1 Accurate measurements mass spectrum for precursor ions of Nic (A), Nic-d3 (B), Cot (C) and Nox (D), by Orbitrap-MS ESI positive detection mode.

2.3.2 Chromatographic separation specificity

The optimized chromatographic conditions for Nic, Cot, Nox and IS were good enough to obtain sharp peaks with minimum matrix effects and ionic suppression negative peak (Figure 2.3), assuring high throughput by short run time. The observed retention times from LLOQ chromatogram for Nic, IS, Cot and Nox were at 1.8, 1.79, 2.02 and 1.94 min

as shown in Figure 2.2A–D, respectively. The specificity of extracted urine for Nic, Cot and Nox determination was indicated from blank analysis in six deferent lots, and all samples were clean and no endogenous peaks were observed from urine blank chromatogram in Figure 2.2E–H for Nic, IS, Cot and Nox respectively, with comparison to corresponding LLOQ chromatogram.

2.3.3 Ion suppression and Matrix effect

The ionic suppression test indicated a separate analyte peaks as shown in Figure 2.3A for analyte's total ion chromatogram (TIC) from ionic suppressant negative peak that caused by urine matrix in Figure 2.3B–D for Nic, Cot and Nox, respectively. Further influences for urine matrix were investigated and reported in Table 2.1.

2.3.4 Standard calibration curve linearity

The calibration dynamic range was linear for Nic, Cot and Nox between 50 and 6000 ng/mL in urine. Table 2.2 summarizes the linear regression function of $y = ax \pm b$ parameters for Nic, Cot and Nox for 6 replicates analysis in different analytical runs. The linear least squares regression weighted function of 1/x was applied for Nic, Cot and Nox calculations.

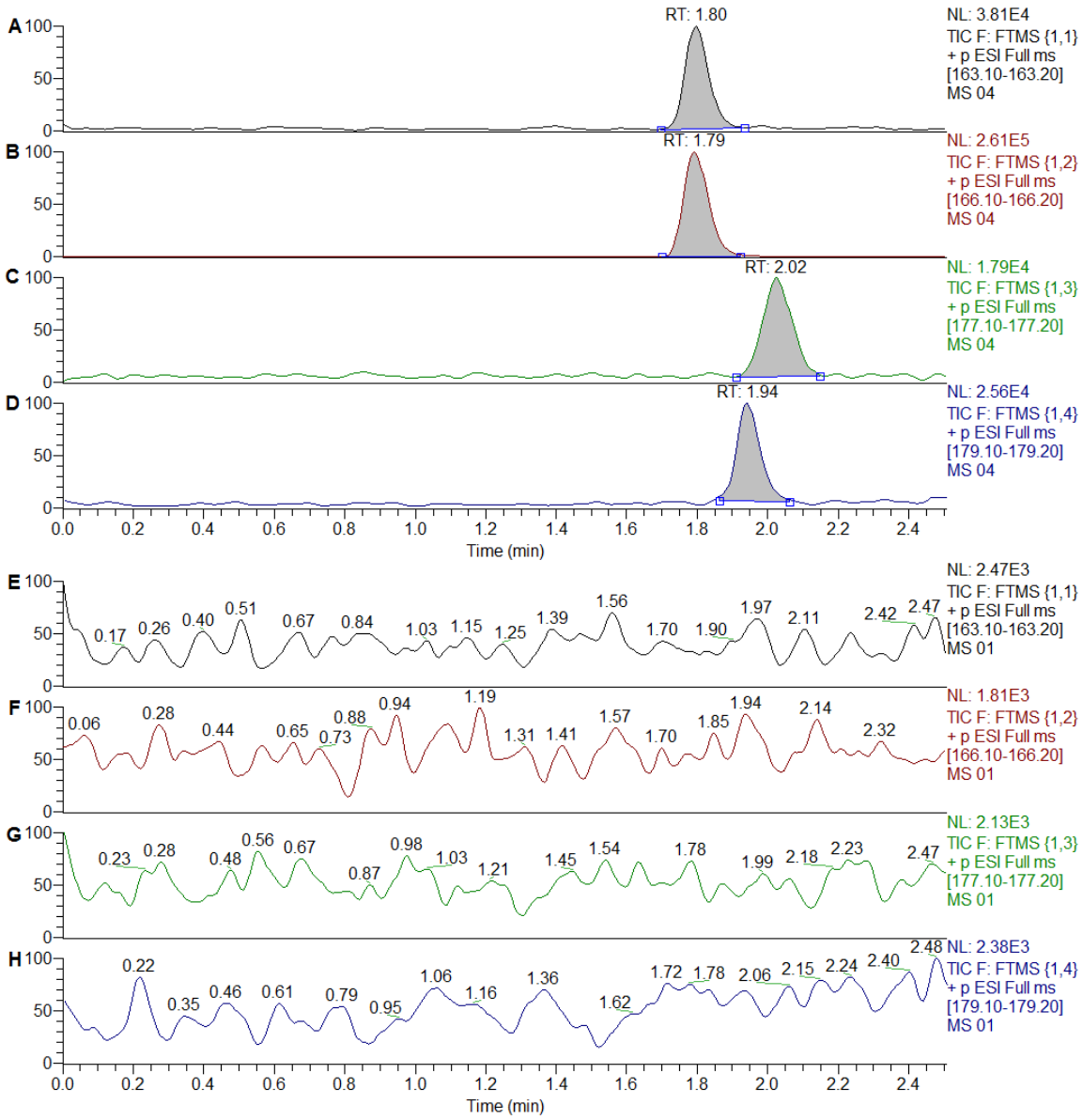

Figure 2.2 Chromatogram for LLOQ (50 ng/ml) of Nic (A), IS (B), Cot (C) and Nox (D), with corresponding blank in (E), (F), (G) and (H), respectively, in human urine.

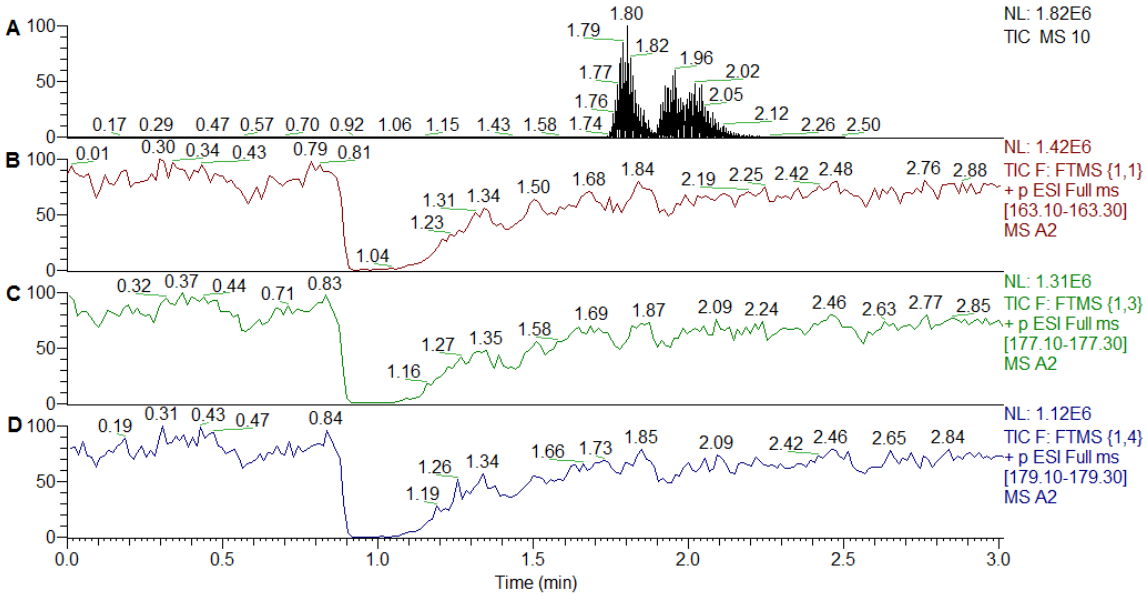

Figure 2.3 Synchronized urine blank injection with Post-column infusion for standard mixture of Nic (B), Cot (C) and Nox (D), in comparison to TIC for further standard mixture injection in (A).

Table 2.1 MF and IS-N MF measurements from different sources of urine (analyte-free blank), at QC low and QC high.

	MF (n=6)				IS-N MF (n=6)			
	QC low	**CV%**	**QC high**	**CV%**	**QC low**	**CV%**	**QC high**	**CV%**
Nic	0.96	5.76	0.92	4.2	1.04	8.61	1.00	7.72
Cot	0.94	7.50	0.97	4.25	1.02	9.77	1.06	7.81
Nox	0.96	5.66	0.95	0.26	1.05	8.56	1.04	6.48
IS	0.92	6.52	0.93	5.42				

Table 2.2 The linear regression function parameters for the analytes by mean the of between run analysis during validation in urine.

Analyte	Slope (n=6)	Intercept (n=6)	R-square (n=6)
Nic	0.000845 ± 0.000020	-0.000805 ± 0.002419	0.999017 ± 0.000763
Cot	0.000876 ± 0.000022	-0.007575 ± 0.001225	0.998950 ± 0.000853
Nox	0.000751 ± 0.000021	-0.000889 ± 0.002332	0.999467 ± 0.000225

2.3.5 Within-and between-run sensitivity, accuracy and precision

Within- and between-run accuracy and precision values for Nic,Cot and Nox measurements are given in Table 2.3.

Table 2.3 Within- and between-run sensitivity, accuracy and precision for Nic, Cot and Nox measurements in LLOQ and QC samples.

Nominal concentration	Nic		Cot		Nox	
	Within-run measurements (n=6)					
	Accuracy %	Precision %	Accuracy %	Precision %	Accuracy %	Precision %
LLOQ	99.07 [92-109]	6.67	101.32 [84-119]	12.01	99.95 [89-110]	6.55
QC Low	103.50 [94-111]	6.44	98.21 [90-110]	7.57	102.50 [91-110]	6.54
QC mid	100.39 [95-105]	3.35	101.58 [94-107]	6.13	101.88 [90-111]	6.29
QC high	98.60 [93-106]	4.28	98.16 [91-108]	5.44	100.19 [96-106]	3.79
	Between-run measurements (n=18)					
LLOQ	98.29 [83-109]	5.47	100.59 [83-119]	7.81	100.81 [88-112]	6.21
QC Low	104.06 [94-111]	4.96	101.24 [90-110]	6.59	101.38 [88-111]	5.90
QC mid	99.48 [93-108]	3.52	100.23 [87-110]	7.11	101.19 [90-114]	6.26
QC high	100.57 [89-111]	5.68	98.61 [91-112]	5.04	99.80 [94-106]	3.55

2.3.6 Recovery and stability

The described extraction procedure was extracted the analytes in recovery mean of 100 ± 4% from urine, and it was higher than direct injection of unprocessed urine. Stability tests of short term under room temperature, freeze-thawing cycles and post preparation under AS conditions for analytes were stable within the specified test time, and the calculated accuracies for Nic, Cot and Nox after corresponding stability test were higher than 85%.

2.3.7 Study applications

All participant volunteers had a prior history of smoking, and their demographic data of age and body mass index (BMI) including fagerström test [25] have reported in Table 2.4. All urine samples under licorice and pomegranate drink conditions with corresponding control samples were measured and reported their summary in Table 2.5. The measurements of control samples (avoided from test drink) in urine were in agreement with previous studies [10,26]. An increased metabolism rate for Nic was observed from Table 2.5, indicated from decreased Nic/Cot ratio under pomegranate or licorice drink with comparison to their corresponding control conditions, whereas Nic/Cot ratio has used as metabolic marker and reflected CYP2A6 activity [10]. Moreover, the increased metabolism rate was also observed from decreased Nic/Nox ratio under test drinks, and more information about Nic metabolism were obtained from Nic/Nox and Cot/Nox, where urinary measurements of Cot/Nox ratio remains almost stable under different drink conditions, this approach confirms that both of Cot and Nox have similar metabolic pathway. P-values from paired t-test were all less than 0.05 as calculated to correlate Nic/metabolite ratio derived from each test drink measurements with its corresponding control measurements.

Table 2.4 Demographic data for all volunteers as group A and B, including Fagerstrom score for Nic dependence on cigarettes.

Group A						Group B					
Volunteer no	Age (Year)	mass (kg)	Tall (m)	BMI (kg/m2)	fagerström score	**Volunteer no**	Age (Year)	Mass (kg)	Tall (m)	BMI (kg/m2)	fagerström score
1	25	110	1.93	29.53	8.00	**3**	21	65	1.66	23.59	4.00
2	19	78	1.73	26.06	9.00	**4**	44	72	1.70	24.91	6.00
5	38	68	1.73	22.72	9.00	**7**	27	93	1.78	29.35	7.00
6	33	88	1.72	29.75	10.00	**8**	28	62	1.74	20.48	10.00
9	45	74	1.85	21.62	8.00	**11**	32	102	1.83	30.46	9.00
10	29	78	1.69	27.31	9.00	**12**	39	113	1.93	30.34	9.00
13	38	83	1.72	28.06	8.00	**15**	22	68	1.70	23.53	7.00
14	48	92	1.74	30.39	7.00	**16**	43	79	1.72	26.70	7.00
17	41	58	1.77	18.51	2.00	**19**	46	76	1.92	20.62	4.00
18	35	103	1.81	31.44	8.00	**20**	38	78	1.88	22.07	10.00
21	28	81	1.79	25.28	4.00	**23**	34	93	1.74	30.72	8.00
22	44	62	1.88	17.54	7.00	**24**	47	74	1.81	22.59	9.00
Mean	35.25	81.25	1.78	25.68	7.42	**Mean**	35.08	81.25	1.78	25.45	7.50
STD	8.78	15.45	0.07	4.65	2.27	**STD**	9.16	15.69	0.09	3.92	2.07
***p* (t test)**	0.480	0.500	0.455	0.449	0.451						

Table 2.5 Statistical analysis summery for all volunteer's urine measurements under pomegranate and licorice drink with their corresponding control condition.

	Pomegranate Control measurements						Measurements under pomegranate drink condition					
	Nic	Cot	Nox	Nic/Cot	Nic/Nox	Cot/Nox	Nic	Cot	Nox	Nic/Cot	Nic/Nox	Cot/Nox
Mean (n=24, ng/mL)	1298	1460	958	0.99	1.60	1.81	862	1614	1169	0.62	1.01	1.79
STD	774	1032	616	0.39	0.76	1.13	577	941	877	0.50	0.88	0.98
Min	237	339	126	0.29	0.66	0.76	166	686	267	0.10	0.13	0.38
Max	2797	3920	2217	1.87	3.32	5.72	1996	3940	3288	2.33	3.88	5.12
P-(t test)										0.0041	0.0021	0.4659
	Licorice control measurements						Measurements under licorice drink condition					
Mean (n=24, ng/mL)	1326	1382	969	0.92	1.47	1.83	804	1710	1107	0.50	0.76	1.78
STD	1014	521	539	0.55	0.94	0.97	509	941	627	0.32	0.36	0.82
Min	197	385	137	0.23	0.45	0.79	196	722	347	0.11	0.11	0.66
Max	4174	2520	2139	1.91	4.43	4.19	1794	3807	2350	1.37	1.69	3.58
P-(t test)										0.0006	0.0008	0.3974

2.4 Conclusion

The current described method for simultaneous determination of Nic, Cot and Nox in human urine by LC-Orbitrap-MS was successfully validated, following single extraction step, providing accurate mass measurements, top extraction recovery with short run time. Nic metabolism rate was accelerated in presence of licorice or pomegranate test drink, indicated from urinary measurements of Nic/Cot ratio, in addition to measure a new introduced metabolic marker of Nic/Nox in this study. The graphical illustration (Figure 2.4) shows the metabolic indexes change against different drink treatment. This is the first report demonstrating induced Nic metabolism by the mean of induced CYP2A6 activity.

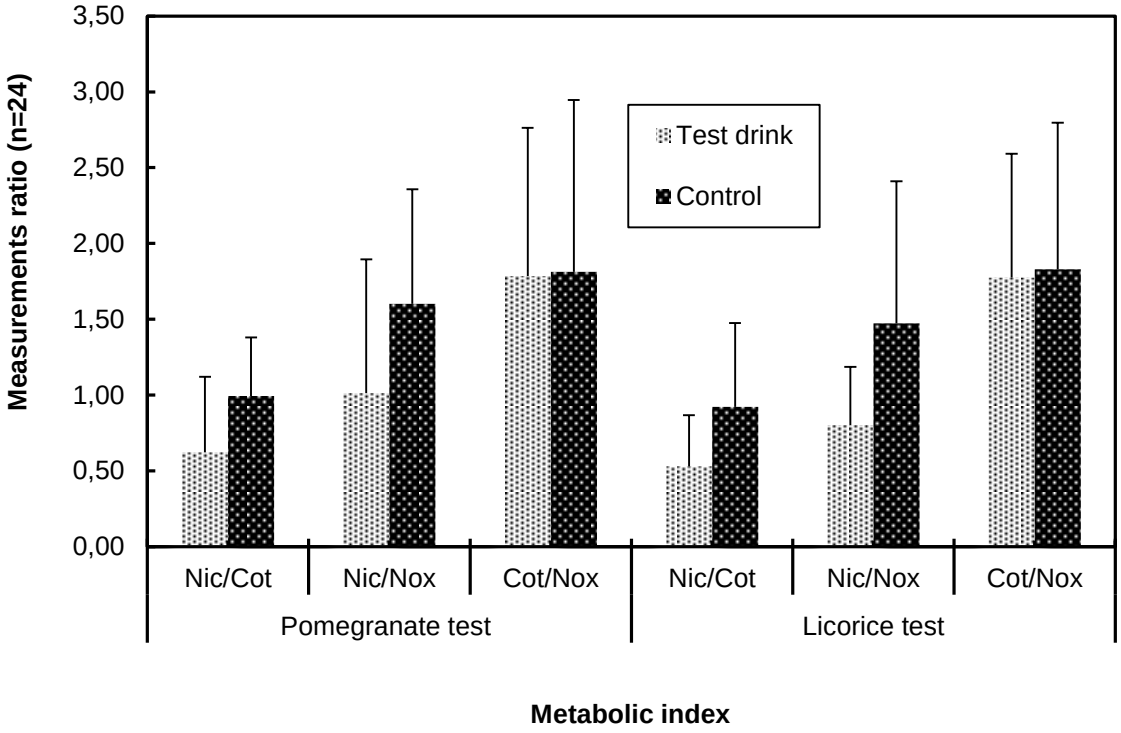

Figure 2.4 Metabolic index under pomegranate and licorice drink with their corresponding control condition in human urine for 24 volunteers.

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3 Simultaneous determination of nicotine, cotinine, and nicotine N-oxide in human plasma, semen, and sperm by LC-Orbitrap MS

3.1 Introduction

Nicotine (Nic) has long been investigated in human body due to its addiction profile [1], and smoking cigarettes, which leads to many kind of cancers [2, 3], is an important way of intake. Nic shows a toxic effect if taken in high doses [2, 3], even it has been used as insecticide due to its poisoning capabilities [4]. In an epidemiological study about the use of Swedish type moist snuff [5], no evidence for Nic impact on human health was found, but in limited studies smokeless tobacco was correlated to adverse health consequences [6]. For example, in vitro-based studies for Nic effect on human health indicated a deleterious influence on sperm membrane, DNA integrity, and viability [7], or its genotoxic impact on chromosomes of human fetal cells [8]. In addition, an animalbased study for Nic oral dose-dependent effect on sperm characteristics and fertility of male rats showed a significant decrease in sperm motility and count for treated rates compared to untreated control rates [9]. Nic enters the bloodstream immediately by smoking through lung absorption, and metabolized in liver mainly by CYP 2A6 to cotinine (Cot), nicotine N-oxide (Nox), and other metabolites [10]. Nic was distributed extensively among human fluids and tissues and was determined in smoker’ s autopsy tissues of the brain, lung, liver, kidney, muscle [11, 12], spleen, abdominal, thigh, and adipose [12]. Tricker has described the Nic occurrence in human body in details [13], and he also described a variety of analytical techniques used in biomarkers determination derived from Nic and its metabolites, where most of biological fluids and matrixes were investigated such as blood, saliva, urine, hair, breast milk, cervical mucus, follicular fluid, fetal fluids, seminal plasma, sweat, toenail, and the deciduous teeth. Limited studies were also reported by Tricker’ s review [13] for Nic or Cot analysis in seminal plasma [14‑16] but without validated methods and the determination of Nox. To the best of our knowledge, there is no investigation whether Nic or its biomarkers Cot and Nox are available in the sperm bodies or not, which could be directly correlated to the human infertility. Recently published excellent methods for Nic determination in human fluids like liquid-liquid (LL)

extraction [17] and LL-back extraction [18], solid-phase extraction (SPE) [19], and micro-SPE [20] in addition to online-SPE for human tissues [11] and dry blood spots [21] are reported, but simple and economic methods with less extraction steps and higher sample throughput are still required to manage large-scale human biomonitoring studies with low sample volume. Therefore, we developed and validated a novel bioanalytical method for simultaneous determination of Nic, Cot, and Nox in human plasma, semen, and sperm bodies by LC-ESI-orbitrap-MS, following single extraction step from 100 μL of sample volume by protein direct precipitation.

3.2 Experimental

3.2.1 Chemicals and reagents

Nic (purity 98 %), Nox (purity 98 %), and deuterated nicotined3 (purity 98 %) were obtained from Toronto Research Chemical (TRC) Inc. (Canada). Cot (purity 98 %) was obtained from Fischer Inc.; blank of plasma and semen samples were harvested from non-smoker donors and stored immediately in a freezer at −40 °C after centrifugation. Plasma was obtained by centrifugation of blood, treated with sodium heparin.

Deionized water, methanol, and formic acid are in LC/MSquality and purchased from Sigma-Aldrich (Germany).

3.2.2 Instrumentation

The LC‑MS system was constructed from benchtop orbitrap mass spectrometer (Exactive, Thermo Fisher Scientific, Bremen, Germany), equipped with electrospray ionization (ESI) source and protected by a waste/detector Rheodyne divert valve, attached to (Accela™) LC system from Thermo Fisher Scientific, 1250 Pump model, and controlled by Xcalibur 3.0.63 software for data management system.

3.2.2.1 HPLC conditions

Chromatographic conditions were identical for analysis of plasma, semen, and sperm samples. An isocratic elution was applied by methanol:water:formic acid (10:90:0.001,

v/v/v) at constant flow rate of 0.4 mL/min through an analytical column (Phenomenex, Kinetex-C18 column 150 × 2.1 mm, 5 μm). The auto sampler tray temperature was set at 5 °C, and the injection volume was 2 μL.

3.2.2.2 Mass spectrometric conditions

The ESI source was operated in positive ion mode for targeted analytes. The adjusted ion source nitrogen gas was set for sheath, auxiliary, and sweep gases flow to 45, 20, 5 units, respectively. The ion spray voltage was set to 4500 V and the ion transfer capillary temperature to 300 °C. The MS resolution option was high at 50,000 at 2 Hz with a mass window of 0.1 Da for each analyte (Nic, Cot, Nox, and Nic-d3).

3.2.3 Standard solutions, calibration curve, and QC samples

Stock solutions of 1.0 mg/mL for Nic, Cot, Nox, and Nic-d3 as an internal standard (IS) were prepared separately in methanol and further diluted in 50 % methanol for working serial dilution at 5, 10, 20, 40, 80, 150, and 250 ng/mL for Nic and Nox and at 10, 20, 40, 80, 160, 300, and 500 ng/mL for Cot. For preparation of spiked calibration curves, 50 μL of these solutions were added to 450 μL of plasma and semen. Similarly, another set of serial dilution were prepared for quality control (QC) samples at 5 ng/mL for LLOQ, 15 ng/mL for QC low, 125 ng/mL for QC mid, and 200 ng/mL for QC high for Nic and Nox and at 10 ng/mL for LLOQ, 30 ng/mL for QC low, 250 ng/mL for QC mid, and 400 ng/mL for QC high for Cot. For quantitative data calculations, a built up processing method was used by processing setup-Xcalibur software, where all targeted analytes were identified with respect to corresponding mass, calibration levels, and related IS, then the peak area of the analytes was divided over the peak area of IS and plotted against specified concentrations to obtain linear regression function with 1/× weighting factor, which was applied for unknown samples calculation.

3.2.4 Sample preparation

3.2.4.1 Subjects

Plasma and semen samples were obtained from the clinical research center of the Jordan center for pharmaceutical research (JCPR), Amman, from 12 healthy smokers following a period of sexual abstinence of at least 3 days. Demographic data including Fagerström test are shown in the appendix of online supplementary material. From each volunteer, an early morning plasma sample was taken before smoking the first cigarette. Subsequently, semen samples were obtained by masturbation. But of course, sperm count among volunteers is different, depending on many factors of health, diet, environmental considerations, and many other factors [22]. Each 1 mL of semen sample was centrifuged at 1500 rpm for 15 min by Eppendorf centrifuge (MiniSpin® Plus, 12 × 1.5/2 mL) to avoid damage of sperm bodies. Then, the separated sperm samples were weighted and washed twice by gentle shaking with 0.5 mL of water to remove seminal carryover by decantation of washing water. Each sperm sample was then mixed quantitatively in 1:2 (w/v) ratio with 20 % (w/v) of trichloroacetic acid (TCA) solution, containing 50 ng/mL of IS. The mixture was vortexed for 2 min in a 2-mL Eppendorf tube, then kept in an ultrasonic for 10 min and vortexed again for 2 min before centrifugation at 14,400 rpm. The supernatant was then transferred into an autosampler (AS) vial.

3.2.4.2 Semen and plasma extraction

Each 100 μL of sperm-free semen and plasma sample was mixed quantitatively with 200 μL of 50 ng/mL nicotine-d3 internal standard (IS) in 1:2 (v/v), where IS solution contains 20 % (w/v) of TCA solution for protein direct precipitation. The mixture was vortexed for 1 min and centrifuged for 10 min at 14,400 rpm. The supernatant was then transferred into an AS vial.

3.2.5 Bioanalytical method validations

The method was validated following the European and US FDA guideline [23, 24]. Full validation was achieved for plasma analysis in terms of specificity, carryover, sensitivity, linearity, accuracy, precision, matrix effect, recovery, and stability. Partial validation was

applied on semen analysis in terms of specificity, matrix effect, recovery, and linearity. The outcome acceptance criteria for all validation sections were based upon European guideline for bioanalytical method validation [23].

3.2.5.1 Linearity, accuracy, and precision

Within-run linearity, accuracy, and precision were evaluated by running analytical sequence containing six replicates of LLOQ, QC low, QC mid, and QC high, separately for plasma and semen, with calibration curve including blank and zero. Between-run linearity, accuracy, and precision were evaluated by running three sets of within-run sequences on three separate days for plasma and semen. The linearity was assessed by a weighted (1/×) least-squares regression analysis.

3.2.5.2 Matrix effect, recovery and stability

The ionic suppression was checked for plasma and semen, by parallel injection of extracted blank and a constant flow of standard solution, containing 1 μg/mL Nic, Cot, and Nox through a T-connection [25]. Matrix effect test was achieved from six different sources of plasma and semen (analytes-free blanks). For each analyte and the IS, the matrix factor (MF) was expressed through QC low and QC high samples, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked after extraction with analyte) to the peak area in absence of matrix (pure solution of the analyte). The IS normalized MF (IS-N MF) was also calculated by dividing the MF of the analyte by the MF of the IS.

Extraction recovery for Nic, Cot, and Nox was determined (duplicate analysis) for plasma and semen samples by comparison of the peak areas from extracted QC at low, medium, and high, with corresponding unprocessed samples (prepared by spiking of equivalent amount from QC samples in extracted blank).

All stability sections in the validation were performed in triplicate analysis for QC samples at low and high levels, and then the measurements were back calculated upon freshly spiked calibration curve. The stock solution and spiked plasma samples were kept under room temperature for 48 h to check short-term stability. Freeze–thaw cycle's stability of the samples were obtained over four freeze–thaw cycles by thawing from

frozen state at room temperature for 1 h and refrozen for 24 h. For another stability test, the processed samples were stored for 48 h under AS cooling conditions (5 °C) before injection.

3.3 Results and discussion

3.3.1 Orbitrap–MS analysis

Figure 3.1 shows the analysis of the protonated molecular ions for Nic, Cot, Nox, and Nic-d3 (IS) by Orbitrap-MS in ESI positive mode with high mass accuracy, sensitivity, and selectivity at m/z 163.1237, 177.1031, 179.1187, and 166.1426, respectively. The current mass measurements were in agreement with high-resolution detection by Q-TOF MS [26].

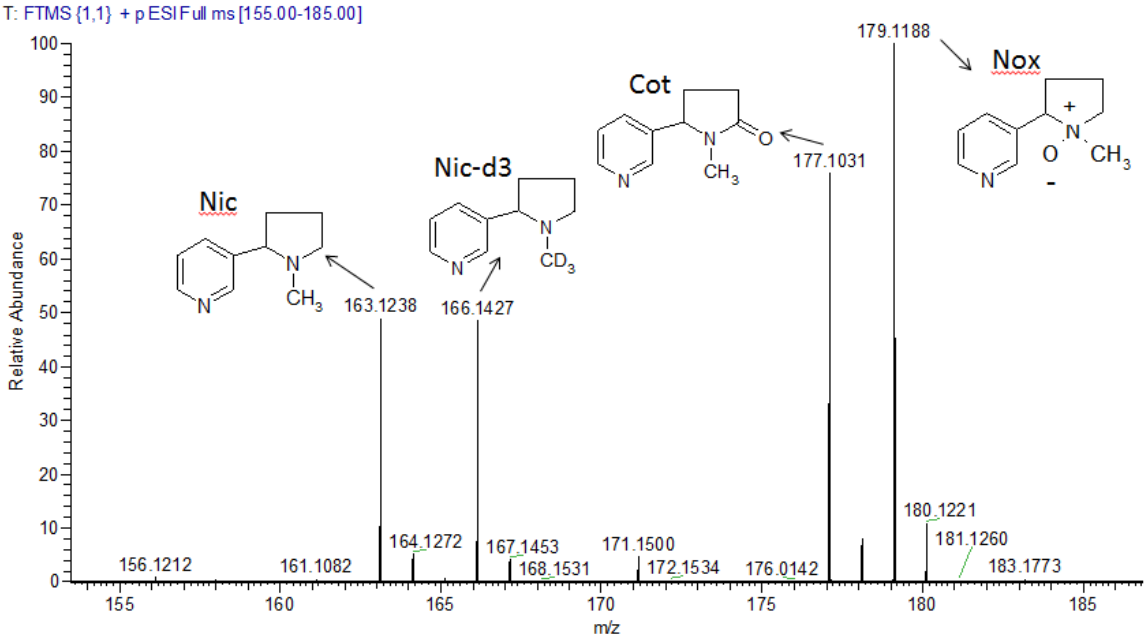

Figure 3.1 Orbitrap mass spectrum with ESI positive detection mode for m/z protonated molecular ions of Nic, Cot, Nox and Nic-d3.

3.3.2 Chromatographic separation and matrix effect

The optimized chromatographic conditions for Nic, Cot, Nox, and IS were good enough to obtain sharp peaks with minimum matrix effects and ionic suppression by a short analysis time. The retention times for Nic, Cot, and Nox were at 1.8, 2.02, and 1.9 min, respectively, as shown in Figure 3.2A, b for LLOQ in plasma and semen, respectively.

The specificity of extracted plasma and semen for Nic, Cot, and Nox determination was indicated from blank analysis in six different lots. All blank samples were clean, and no endogenous interfering peaks were observed from blank screening (Figure 3.2C) compared to LLOQ (Figure 3.2B).

Due the fact of thermal decomposition of N-oxide to its parent amine [27], we checked Nox thermal back conversion to Nic which could be induced by heated ion transfer capillary by injection of Nox alone, and we did not find any noticeable peak at Nic's mass filter.

Ionic suppression test in Figure 3.3A, b, for plasma and semen, respectively, demonstrates that Nic, Cot, and Nox were eluted from the column later than ionic suppression negative peak.

Further matrix was investigated, where both MF and IS-N MF measurements were all below 10 % for each analyte in plasma and semen.

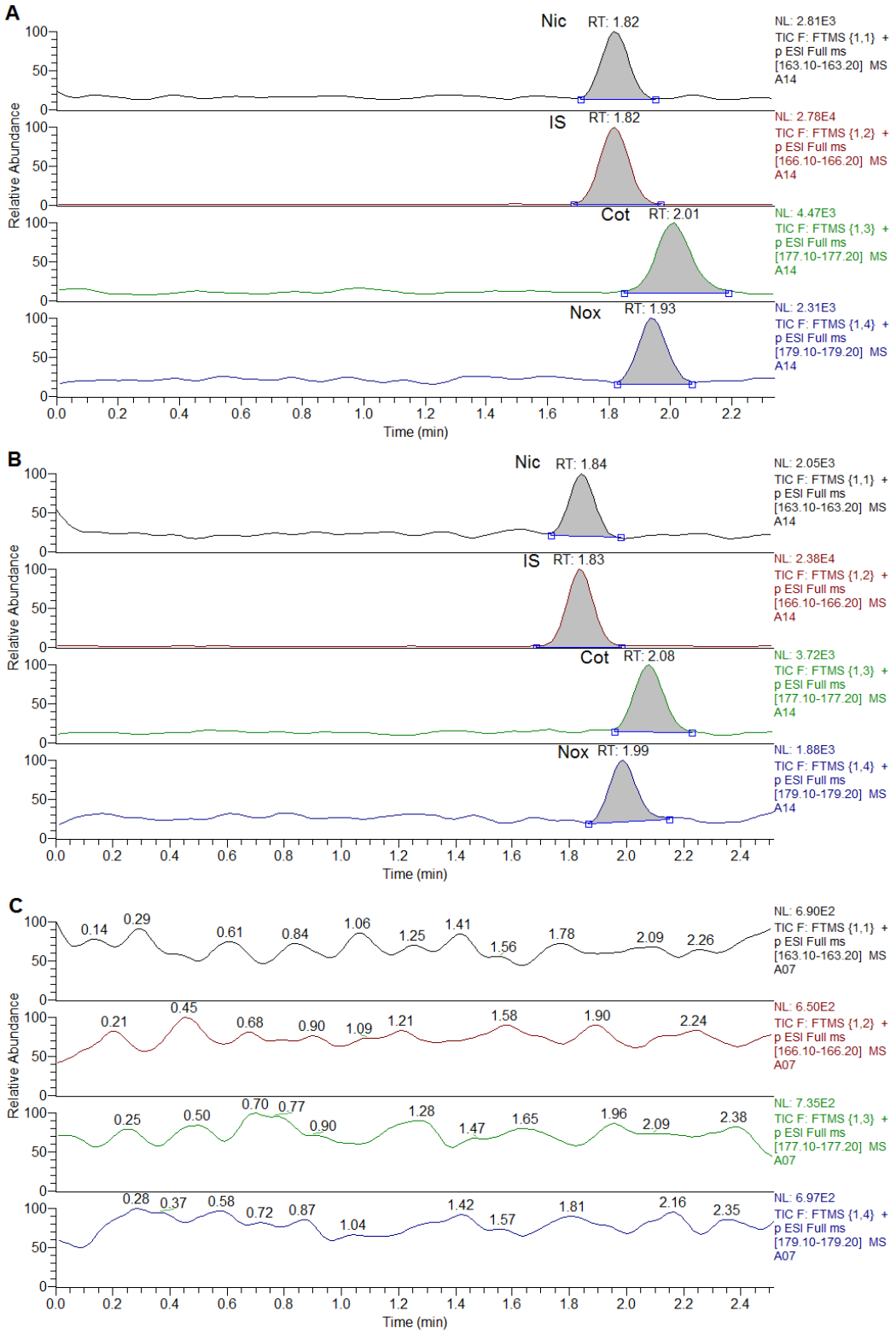

Figure 3.2 Raw ion chromatograms analyzed in localized accurate mass at high resolution (50,000 at 2 Hz) for LLOQ from plasma analysis **A**, LLOQ from semen analysis **B**, and extracted semen blank chromatogram **C.**

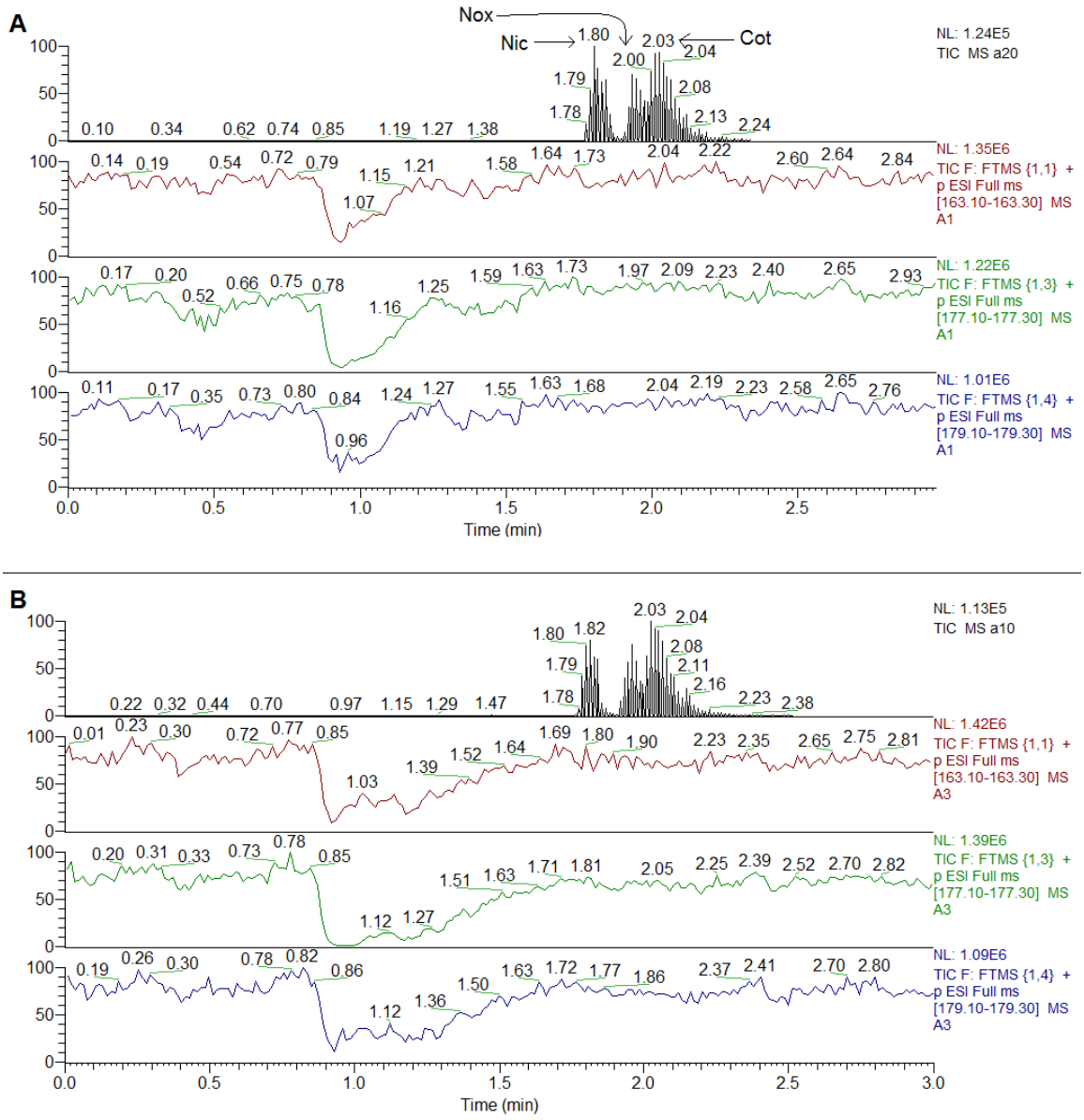

Figure 3.3 Separation of Nic, Cot and Nox in TIC from ionic suppression negative peak for plasma (**A),** and for semen (**B)**.

3.3.3 Standard calibration curve linearity

The calibration dynamic range for Nic and Nox was linear between 5 and 250 ng/mL in plasma and semen, and for Cot between 10 and 500 ng/mL. Table 3.1 summarizes the linear regression function parameters for Nic, Nox, and Cot in plasma and semen analysis. The linear least-squares regression weighted 1/× function was applied for Nic, Cot, and Nox calculations.

Table 3.1 The linear regression function parameters for Nic, Cot and Nox, in plasma and semen analysis during validation.

	Plasma (n=6)			Semen (n=3)		
Analyte	Slope (CV %)	Intercept	R (CV %)	Slope (CV %)	Intercept	R (CV %)
Nic	0.0131 (3.89)	0.0147	0.9993 (0.07)	0.0131 (6.13)	0.0179	0.9985 (0.08)
Cot	0.0141 (3.90)	0.0511	0.9981 (0.11)	0.0142 (9.04)	0.0926	0.9968 (0.17)
Nox	0.0121 (4.96)	0.0157	0.9986 (0.08)	0.0123 (7.78)	0.0139	0.9986 (0.09)

3.3.4 Sensitivity, accuracy, and precision

Accuracy and precision values for Nic, Nox, and Cot measurements in plasma and semen during validation are given in Table 3.2. Precision run includes six replicates for LLOQ, QC low, QC mid, and QC high.

Table 3.2 Within-day measurements accuracy and precision in plasma and semen.

QC level	Measurements in plasma (ng/mL, n=6)											
	Nic				Cot				Nox			
	Spiked	Measured	Accuracy %	CV%	Spiked	Measured	Accuracy %	CV%	Spiked	Measured	Accuracy %	CV%
LLOQ	5	4.87	97.35 (85-105)	7.80	10	9.91	99.09 (82-113)	11.17	5	5.13	102.50 (87-118)	10.4
Low	15	15.65	104.32 (96-112)	5.60	30	30.41	101.38 (90-114)	9.81	15	15.22	100.13 (87-114)	9.17
Mid	125	121.77	97.41 (93-100)	2.63	250	246.89	98.76 (88-106)	6.42	125	123.42	98.74 (92-105)	4.33
High	200	197.38	100.82 (94-106)	5.23	400	394.63	98.66 (89-104)	6.44	200	202.07	101.04 (91-106)	5.81
	Measurements in semen (ng/mL, n=6)											
LLOQ	5	5.21	101.84 (92-108)	8.96	10	9.79	98.39 (88-110)	8.81	5	5.07	99.21 (90-107)	8.62
Low	15	15.66	103.49 (97-110)	5.35	30	31.57	104.38 (98-110)	4.61	15	15.12	102.49 (92-110)	6.55
Mid	125	124.58	100.63 (93-106)	5.19	250	249.66	98.54 (94-107)	5.64	125	126.60	100.81 (93-106)	4.39
High	200	198.01	98.81 (91-110)	6.86	400	391.40	99.22 (88-106)	7.66	200	198.08	98.39 (95-103)	4.29

3.3.5 Recovery and stability

Protein direct precipitation by TCA solution in plasma and semen leads to high extraction efficiency with recovery values higher than 97 % for each analyte. The described recovery test was not applied for sperm samples because solid bodies cannot be homogeneously spiked, but to assure that the applied extraction procedure was efficient enough for analytes extraction, the extracted sperm samples were extracted again, and no detectable peaks were observed in the second extract.

For short-term stability, the test under room temperature, freeze-thawing cycles, and post preparation at 5 °C show that Nic, Nox, and Cot were stable within the specified test time, and the accuracies for Nic, Cot, and Nox after corresponding stability test were higher than 85 %.

3.3.6 Application

The method was successfully applied for analyses of plasma semen and sperm of smokers. Nic and Cot were detected in all samples, whereas Nox was below the LLOQ in plasma but detectable in all semen samples and 10 of 12 sperm samples (Table 3.3). The concentration of Nic and Cot in semen and plasma were largely in agreement with two previous studies [15, 28]. Interestingly, Nic was significantly higher in semen compared to plasma (2.3-fold, $p < 0.001$). In sperm, the concentrations of Nic and Cot were in the same range as for Nic in plasma, and Nic was significantly lower in sperm compared to semen (2.1-fold, $p < 0.001$). To our knowledge, the presence of Nox in semen as well as sperm has not been reported up to now. Nox is routinely detected in urine of smokers, snuffers, and even nonsmokers [29] and makes up about 4 % of total nicotine equivalents in urine [13]. Figure 3.4C, D, respectively, shows no correlation for Nic and Cot concentration between sperm and semen, because many factors are affecting sperm count in semen [41]. And there is also only a weak correlation for Nic concentration between plasma and semen in Figure 3.4A, but a much better correlation for Cot concentration between plasma and semen could be observed in Figure 3.4B, which is in agreement with similar work for Nic and Cot correlations between semen and plasma [28].

Table 3.3 Measurements of Nic, Cot and Nox in human plasma, semen and sperm.

	Measurements in plasma (ng/mL)			**Measurements in semen (ng/mL)**			**Measurements in sperm (ng/mL)**		
Subject	Nic	Cot	Nox	Nic	Cot	Nox	Nic	Cot	Nox
1	10.90	106.85	< LLOQ	19.56	102.95	5.89	15.00	110.00	6.23
2	23.16	220.18	< LLOQ	48.98	234.53	12.54	11.79	89.82	< LLOQ
3	18.28	188.11	< LLOQ	23.00	203.82	6.38	13.12	113.84	< LLOQ
4	17.57	195.05	< LLOQ	53.87	232.47	14.31	21.00	213.40	5.43
5	34.30	314.75	< LLOQ	76.14	350.98	21.85	26.34	190.19	10.22
6	25.08	213.52	< LLOQ	56.61	248.38	12.24	14.32	157.78	5.96
7	13.95	117.03	< LLOQ	44.57	146.74	12.05	17.73	142.90	6.91
8	29.16	220.87	< LLOQ	77.06	278.29	13.47	28.48	238.77	8.91
9	16.65	183.24	< LLOQ	57.16	203.53	17.23	21.83	203.58	7.79
10	23.24	182.29	< LLOQ	43.50	221.00	13.57	48.59	307.26	18.98
11	23.89	136.30	< LLOQ	38.00	175.12	11.65	33.52	147.79	10.35
12	33.51	276.49	< LLOQ	81.26	331.68	10.74	43.29	331.19	11.69
Mean	22.47	196.22	--------	51.64	227.46	12.66	24.58	187.21	9.25
STD	7.38	60.49	--------	19.87	70.80	4.27	12.00	76.26	4.01
CV%	32.82	30.83	--------	38.47	31.13	33.69	48.82	40.74	43.41

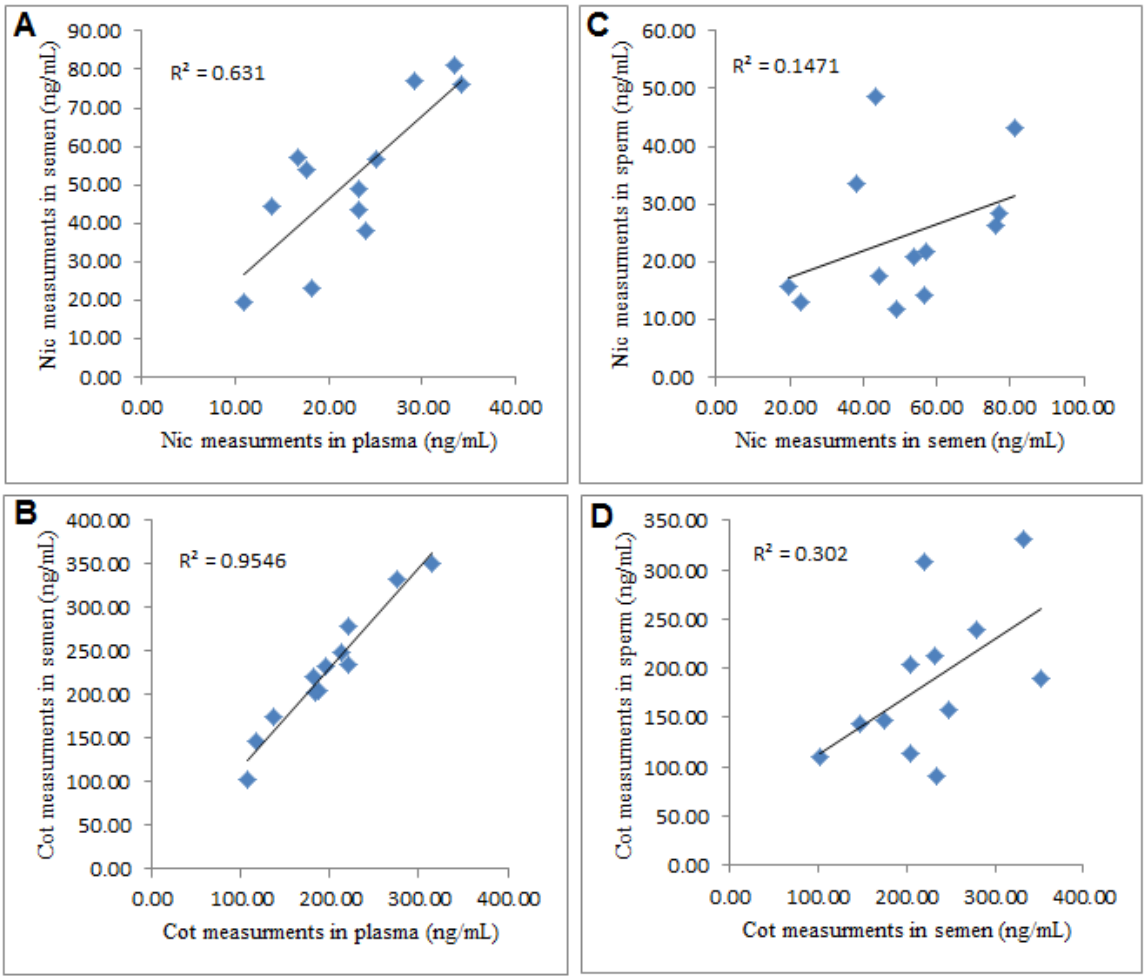

Figure 3.4 Nic correlation between plasma and semen (**A**), Nic correlation between semen and sperm (**C**), Cot correlation between plasma and semen (**B**), Cot correlation between semen and sperm (**D**).

3.4 Conclusion

Avery fast LC‑MS method was developed and validated for the quantitative analysis of Nic and Cot in human plasma, Nic, Cot, and Nox in human sperm and semen. Protein direct precipitation in a single extraction step was successfully applied for 100 µL of plasma and semen by TCA solution. This is the first time that Nic, Cot, and Nox were determined in human sperm and Nox in semen. Nic and Cot were found in sperm samples at comparable concentrations to plasma samples. Nox was not detected in plasma at the established calibration range, but it was detected in semen and sperm.

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4 Determination of nicotine and cotinine in human blood by dried blood spot- LC-Orbitrap MS technique

4.1 Introduction

Nicotine (Nic, Figure 4.1) has long been investigated in human body due to its adverse health consequence and addiction profile [1]. It has also been used as insecticide due to its poisoning capabilities [2]. Nic enters bloodstream immediately through smoking by lung absorption [3], and metabolized in liver mainly by CYP 2A6 to cotinine (Cot, Figure 4.1), and other metabolites. For Nic determination in human blood, a traditional sampling procedure of drawing blood by venous cannula or syringe followed by multiple processing steps is usually used, but many restrictions of hospitalization demands, ethical agreements, and high costs are making challanges in Nic research area from human blood.

Dried blood spot (DBS) have been used technique for screening of metabolic disorders in newborns for decades, and have already became a popular sampling method for the quantitation of small molecules in blood [4]. Using DBS technique has increased to manage high population-based studies, especially those for large-scale human bio monitoring, as well as studies involving children or small animals, since the skin-stick procedure is technically simple and doesn't need extensive training or expensive equipment, only a tiny volume of blood is enough, and handling blood specimens is relatively non-hazardous against pathogens risk, such as HIV. This technique has more recently been applied to drug metabolism (DM) [5], pharmacokinetic (pk) [6], therapeutic drug monitoring (TDM) [7] and toxicokinetic (TK) [8] studies. DBS has applied in tobacco smoke research field as well, to provide an alternative sampling method for Cot determination in human blood as a biomarker for Nic intake in metabolism studies [9], secondhand smokers [10] and newborn screening [11]. Recently, Tretzel et al. has described an excellent application for DBS by automated samples extraction system, for determination of doping relevant compounds, using Nic and its major metabolites as an example for drugs testing in sport applications [12], which has achieved by multiple extraction steps (based on solid phase extraction), relatively low extraction recovery (25-

40%) and 10 min run time. The current study demonstrates a validated bioanalytical method for simultaneous determination of Nic with Cot in human blood based on DBS technique, following a single extraction-reconstitution step. Subsequently, the validated method was then successfully applied to measure Nic and Cot levels in 12 healthy and smoker volunteers, and compared their DBS measurements with corresponding measurements from whole plasma (traditional blood sampling procedure), using LC-Orbitrap MS technique as a highly accurate and sensitive detection system, to satisfy the sensitivity demand of small sample volume, where the current described method has the advantages of low running costs, single extraction step through direct reconstitution by trichloroacetic acid solution (TCA), short run time (2.5 min) and good extraction recovery from DBS. Furthermore, we investigated the influence of chromatographic effect for first time in Nic analysis by DBS technique.

4.2 Experimental

4.2.1 Chemicals, reagents and materials

Nic (purity 98%) and deuterated Nic-d3 (purity 98%) were obtained from Toronto research chemical (TRC Inc., Canada). Cot (purity 98%) was obtained from Fischer Inc., Germany. Blood blank samples were harvested in heparinized tubes from healthy and non-smoker donors. Deionized water, methanol and formic acid are in LC/MS-quality and purchased from Sigma Aldrich (Germany). FTA DMPK- filter cards, (Whatman™ 903, US-FDA approved type for quantitative analysis [13]), were obtained from GE Healthcare.

4.2.2 Instrumentation

The LC–MS system was constructed from benchtop Orbitrap-MS (Exactive, Thermo Fisher Scientific, Bremen-Germany), equipped with a the heated-electrospray ionization source (H-ESI) and protected by a waste/detector Rheodyne divert valve, attached to (Accela™) LC system from Thermo Fisher Scientific (1250 Pump model) and controlled by Xcalibur 3.0.63 software for data management system.

4.2.2.1 HPLC conditions

An isocratic elution with methanol:water:formic acid (10:90:0.001, v/v/v), delivered at constant flow rate of 0.4 mL/min through an analytical Kinetex-C18 column (150 × 2.1 mm, 5 µm) from of Phenomenex™ (Germany) was used. The auto sampler tray temperature was set at 5 °C and the injection volume was 20 µL.

4.2.2.2 Mass spectrometric conditions

The heated-electrospray ionization (H-ESI) source was operated in positive scan mode was used. The adjusted ion source parameters of nitrogen gas for sheath, auxiliary and sweep flow were at 45, 20, 5 unit, respectively. Ion transfer capillary temperature and auxiliary gas heating temperature was at 300 °C and 400 °C, respectively. The ion spray voltage was set at 4500 V. MS resolution option was sat at high-50000@ 2Hz with a mass window of 0.1 D for each analyte (Nic, Cot and Nic-d3).

4.2.3 Standard solutions, calibration curve and QC Samples

Stock solutions of 1.0 mg/mL for Nic, Cot and Nic-d3 were prepared separately in methanol, and further diluted in 50% methanol in order to prepare working serial dilution at 5, 10, 20, 40, 80, 150 and 250 ng/mL for Nic and at 10, 20, 40, 80, 160, 300 and 500 ng/mL for Cot. For preparation of spiked calibration curves 50 µL of these solutions were added to 450 µL of blood. Similarly, another set of serial dilution were prepared for quality control (QC) samples at 5 ng/mL for LLOQ, 15 ng/mL for QC low, 125 ng/mL for QC mid and 200 ng/mL for QC high for Nic and at 10 ng/mL for LLOQ, 30 ng/mL for QC low, 250 ng/mL for QC mid and 400 ng/mL for QC high for Cot.

The DBS for standard and QC samples were prepared from freshly spiked whole blood and 60 µL were spotted immediately on filter cards. After that, the spots were dried under room temperature for 3 h.

4.2.4 Sample preparation

A single extraction step was followed for DBS analysis from a standard 6.35 mm punch. Two punches from each level were placed in a polypropylene micro-centrifuge tube and

100 µL of 10 % (w/v) TCA, containing 20 ng/mL IS, were added for extraction. The mixture were then vortexed for 1 min and centrifuged for 10 min at 16,400 rpm by Eppendorf centrifuge (MiniSpin® Plus, 12 × 1.5/2 mL). The supernatant was then transferred into autosampler (AS) micro vial.

4.2.5 Method validations

The developed DBS-based method was validated following the European Medicines Agency (EMA) and United States FDA guideline [14, 15], in terms of specificity, carryover, sensitivity, linearity, accuracy, precision, matrix effect, recovery and stability. DBS handling process was following a reported procedure [16, 17]. Hematocrit effects for Cot were studied by *Sosnoff* and reported previously [10], and blood spot volume effect was also studied previously [18, 19]. A chromatographic effect (or distribution effect) for the analytes due to a decentralized DBS punching area was studied in the current validation to determine the influence of used filter paper on spread of analytes across the spot [17].

4.2.5.1 Linearity, accuracy and precision

Within-run linearity, accuracy and precision were evaluated by running analytical sequence containing six replicates from each of LLOQ, QC low, QC mid and QC high with calibration curve including blank and zero. Between-run linearity, accuracy and precision were evaluated by running three sets of within-run sequences in three separate analytical batches within two days. For quantitative data calculations, a built up processing method was used by processing setup-

Xcalibur software, where targeted analytes were identified with respect to corresponding accurate mass, calibration levels and related IS. The peaks area of the analytes were then divided over corresponding IS peak area and plotted against specified concentrations to obtain linear regression function using 1/x weighting factor.

4.2.5.2 Ion suppression, matrix effect

The ion suppression was checked for DBS by synchronized injection of extracted blank, online through post-column infusion for Nic and Cot standard solution. The ion

suppressed negative peak was then compared to the analytes total ion chromatogram (TIC) [20]. Matrix effect test was achieved from six different sources of blood (analytes-free blanks) and expressed in terms of matrix factor (MF) through QC low and QC high samples by calculating the ratio of the peak area in the presence of matrix. This was done by comparing the peak area of blank matrix, spiked after extraction with analyte, to the peak area in absence of matrix (pure solution of the analyte). The IS normalized MF (IS-N MF) was also calculated by dividing the MF of the analyte by the MF of the IS.

4.2.5.3 Recovery

Nic and Cot extraction recovery from DBS was investigated by applying a second extraction for the reconstituted punches at QC high level, to assure that first reconstitution step has released Nic and Cot in maximum extraction efficiency.

4.2.5.4 Stability

All stability sections in the validation were performed in triplicate analysis for QC samples at low and high levels, the measurements were then back calculated upon freshly prepared calibration curve. The stock solution and DBS samples were kept under room temperature for 48 h to check their short term stability and the supernatant stability for the extracted DBS samples at 5 °C was checked for 48 h. For long term stability another set of QC samples were kept in deep freezer at – 20 °C for 3 months before analysis.

4.2.5.5 Chromatographic effect and decentralized DBS punching area

DBS decentralized punching area could lead to a variable measurement factor, due to the interaction possibility of analyte in blood with the used filter paper materials and through stretching of liquid blood spot over cards. Such interactions could lead to differences in analytes concentration between the center and peripheral areas within a spot. Here, we evaluated the chromatographic effect by punching DBS at peripheral areas (but not touching the spot edge) in six replicate analysis at QC low and QC high, and then compared their measurements to corresponding centralized punched samples.

4.3 Results and discussion

4.3.1 Orbitrap–MS analysis

Figure 4.1 shows the detection of protonated molecular ions for Nic, Cot, and Nic-d3 by Orbitrap-MS in H-ESI positive mode, with high mass accuracy, sensitivity and selectivity at m/z 163.12354, 177.10287 and 166.14235, respectively. The current mass measurements were in agreement with high resolution detection by Q-TOF MS [110].

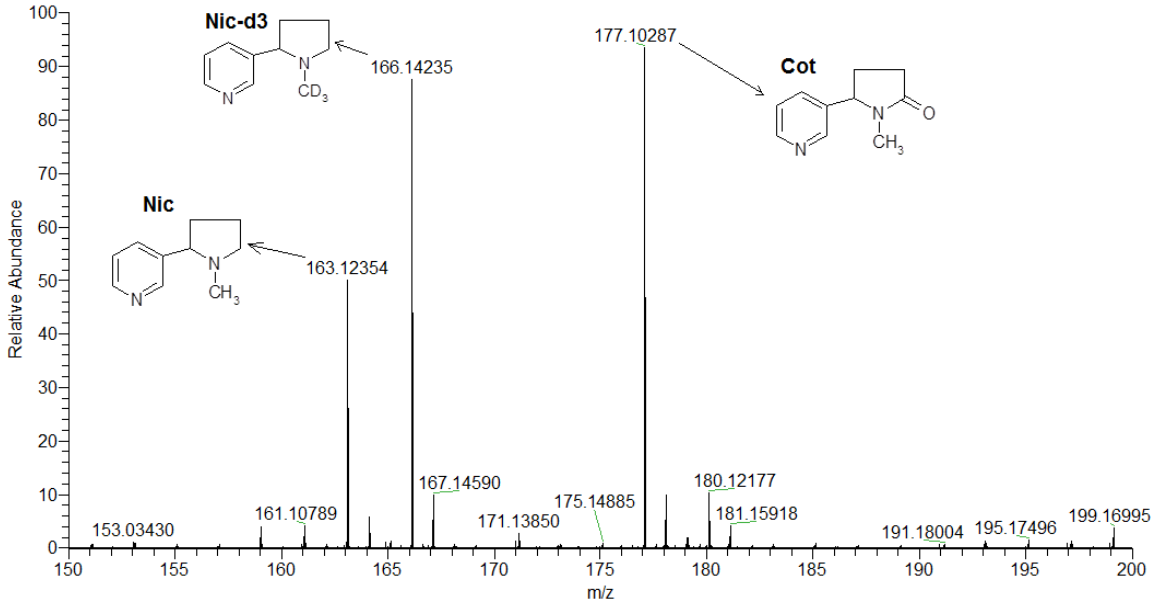

Figure 4.1 Orbitrap mass spectrums by ESI positive detection mode for protonated molecular ions m/z of Nic, Cot and Nic-d3.

4.3.2 Chromatographic separation, ion suppression and matrix effect

The chromatographic conditions for Nic, Cot and IS were optimized for sharp peaks with minimum matrix effects and ion suppression, assuring high throughput by short run time. The retention times for Nic and Cot were 1.8 and 2.0 min, respectively, as shown in Figure 4.2A for LLOQ by DBS. The specificity of extracted DBS for Nic and Cot determination in blood was indicated by analysis the DBS blank from six different blood sources. All blank samples were clean and no endogenous interfering peak were observed from blank screening (Figure 4.2B) compared to LLOQ (Figure 4.2A).

Figure 4.3 demonstrates that Nic and Cot peaks in total ion chromatogram (TIC, Figure 4.3A) are eluted from the column later than ion suppression negative peaks, where the synchronized injection for DBS blank caused a negative peaks through post-column infusion for Nic and Cot solution as shown in Figures 4.3 B and C, respectively. More information about matrix effect was obtained from Table 4.1 for matrix effect test and reported in terms of MF and IS-N MF for each analyte in blood.

Table 4.1 MF and IS-N MF for DBS measurements from different sources of human blood (analyte-free blank) at QC low and QC high.

	MF (n=6)				**IS-N MF in plasma (n=6)**			
	QC low	CV %	QC high	CV %	QC low	CV %	QC high	CV%
Nic	1.01	1.74	0.99	2.15	1.02	2.18	0.98	1.16
Cot	1.00	1.98	0.99	2.33	1.01	3.27	0.98	1.60
IS	0.99	1.59	1.00	2.28				

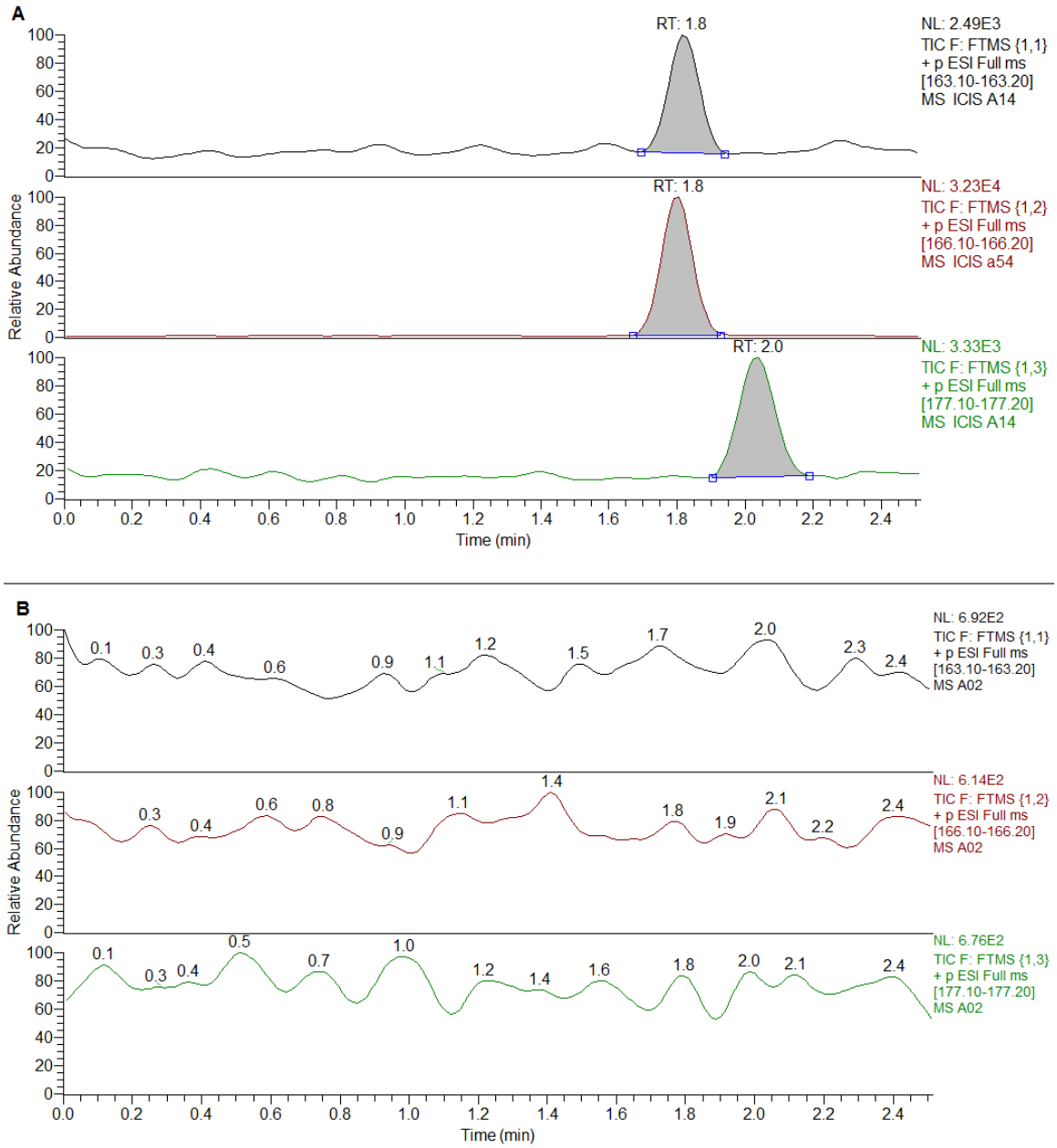

Figure 4.2 Chromatograms for LLOQ from DBS analysis (**A)**, and extracted blank from DBS (**B)**.

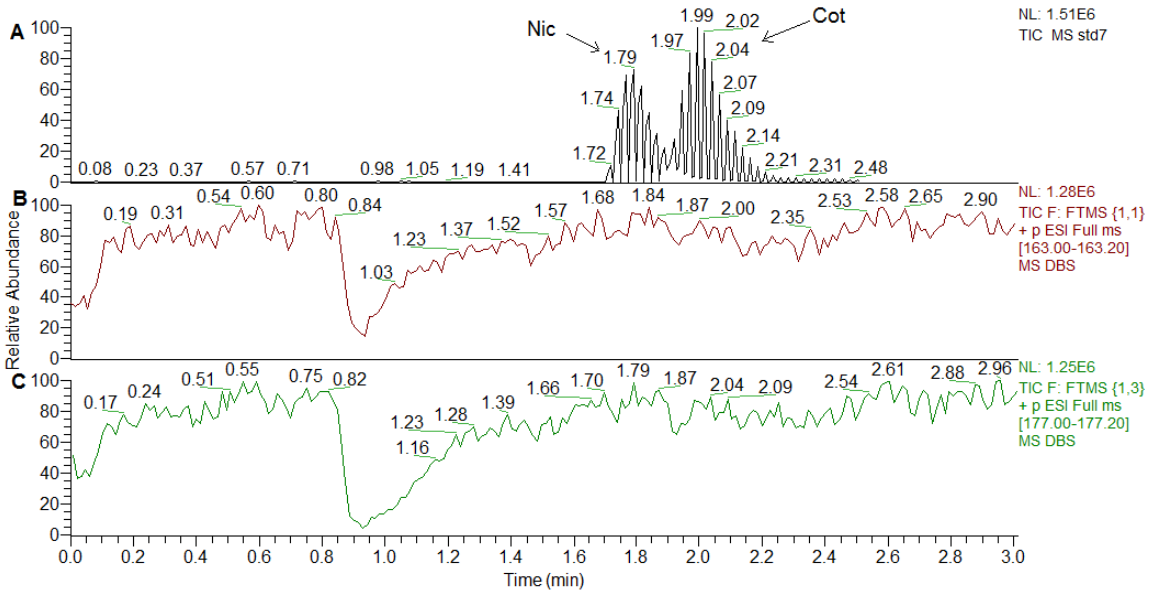

Figure 4.3 A comparison TIC chromatogram in **A** to ionic suppression negative peaks in **B** and **C** for Nic and Cot, respectively.

4.3.3 Standard calibration curve linearity

The calibration dynamic range for Nic was linear between 5-250 ng/mL. The regression function ($Y = a + bX$) parameters of (a = intercept, b= slope and correlation coefficient (R^2)) for Nic (n = 6) were b= 0.0058 ± 0.0001 (relative standard deviation (RSD %) = 2.4), a = -0.0008 ± 0.0016 and R^2 0.9987 ± 0.0012 (RSD % = 0.12). The calibration range for Cot was linear between 10-500 ng/mL, and its regression parameters summary (n = 6) were b= 0.0041 ± 0.0002 (RSD % = 4.14), a = -0.0002 ± 0.0029 and R^2= 0.9987 ± 0.0009 (RSD % = 0.09).

4.3.4 Within-and between-run sensitivity, accuracy and precision

Within- and between-run measurements accuracy for Nic and Cot were all higher than 80 % for LLOQ and 85 % for QC levels. Within- and between-run measurements precision for Nic and Cot were all within 15 % as given in Table 4.2 for precision and accuracy measurements for Nic and Cot in three separate analytical batches from DBS analysis during validation. Each precision batch includes six replicates for LLOQ, QC low, QC mid and QC high.

Table 4.2 DBS measurements accuracy and precision for within-batch and between-batches analytes during validation.

QC Level	Nic				Cot			
	Spiked	Measured	Accuracy %	CV %	Spiked	Measured	Accuracy %	CV %
Within-day measurements (ng/mL, n = 6)								
LLOQ	5	5.27	105.39 (88-114)	10.36	10	9.51	95.08 (81-115)	12.73
Low	15	15.14	100.94 (91-108)	5.35	30	30.28	100.93 (94-106)	3.99
Mid	125	121.95	97.56 (93-103)	3.65	250	246.15	98.46 (93-103)	3.12
High	200	199.16	101.72 (97-107)	12.34	400	388.43	97.11 (95-100)	2.22
Between-days measurements (ng/mL, n = 18)								
LLOQ	5	5.14	102.85 (88-117)	8.42	10	9.74	97.36 (81-115)	8.54
Low	15	14.82	98.77 (91-108)	4.35	30	30.95	103.16 (88-115)	6.46
Mid	125	124.61	99.69 (93-105)	3.53	250	247.15	98.86 (92-114)	4.53
High	200	201.33	101.37 (97-107)	2.87	400	399.23	97.43 (93-102)	2.41

4.3.5 Recovery and stability

The extraction-reconstitution step by TCA solution for the punched DBS disks was efficient enough to recover the analytes from their solid matrix, where the analysis of secondly extracted QC high samples showed that no detectable signals for Nic or Cot were observed, namely, the highest extraction recovery was reached. For short term stability, the test under room temperature and post preparation at 5 °C show that Nic and Cot were stable within the specified test time, and the calculated accuracies for Nic and Cot were higher than 85 %.

4.3.6 Chromatographic effects

The data obtained from decentralized disk punching (from peripheral areas) within the DBS circle was compared to the data obtained from the centralized punching analysis as given in Table 4.3. The data indicated that centralized punches had slightly higher analytes intensities than the decentralized punches. The accuracy and precision for decentralized punching measurements were comparable to corresponding centralized punching measurements. Thus, demonstrate that overall chromatographic variation compares a minimal variation factor in Nic and Cot quantitation by DBS, as long as the punched disks stayed within the confines of the DBS (non-touching the edge).

Table 4.3 Chromatographic effect test by decentralized disk punching for Nic and Cot analysis, in terms of measurements accuracy %.

	Centralized punching measurements accuracy %				Decentralized punching measurements accuracy %			
	Nic		Cot		Nic		Cot	
	QC low	QC high	QC low	QC high	QC low	QC high	QC low	QC high
Test 1	100.98	97.44	98.27	95.56	88.971	91.70	101.06	93.69
Test 2	107.65	106.32	94.54	97.67	99.319	94.54	96.83	95.43
Test 3	101.43	104.16	101.43	99.74	102.168	94.35	91.16	99.52
Test 4	102.75	97.93	101.81	94.51	100.392	95.33	90.04	95.05
Test 5	101.71	103.75	105.94	99.37	94.849	97.06	105.24	95.57
Test 6	91.09	100.71	103.56	95.79	106.165	101.70	92.03	97.76
Mean	100.94	101.72	100.93	97.11	98.64	95.78	96.06	96.17
CV %	5.35	3.54	3.99	2.22	6.09	3.53	6.35	2.18

4.3.7 Application

Early morning blood spots samples were collected from twelve healthy smoking volunteers over filter cards in duplicate from their hand's fingers, following a DBS processing protocol [140, 141], synchronized with drawing blood samples into heparinized tubes for plasma assay [144]. The collected blood spots were left for 3 h under room temperature for drying, and then kept in deep freezer at -20 °C for long term storage till analysis. DBS sampling were synchronized with whole blood collection from each volunteer in a heparinized tube. These blood samples were separated into plasma and kept in deep freezer at -20 °C till analysis.

The measurements of Nic and Cot in DBS with corresponding measurements from whole plasma measurements are listed in Table 4.4.

Long term storage for such volatile compounds (Nic and Cot) could attributes the noticed few scattered measurements from Figure 4.4 for DBS compared to corresponding whole plasma measurements, but in spite of such long term instability, a good correlation in Nic and Cot measurements between both sampling methods was indication from t-test ($p < 0.05$) in Table 5, where p -value for Nic and Cot were 0.0051 and 0.0098, respectively.

Table 4.4 Measurements of Nic and Cot in human DBS with corresponding measurements from whole plasma.

	Measurements by DBS (ng/mL)		Measurements from whole plasma (ng/mL) [21]	
Subject	Nic	Cot	Nic	Cot
1	10.82	103.30	10.90	106.85
2	9.28	123.13	23.16	220.18
3	8.49	105.76	18.28	188.11
4	12.94	182.04	17.57	195.05
5	31.29	271.68	34.30	314.75
6	16.05	157.93	25.08	213.52
7	11.65	114.19	13.95	117.03
8	9.42	100.77	29.16	220.87
9	15.16	175.83	16.65	183.24
10	23.51	183.21	23.24	182.29
11	18.87	128.31	23.89	136.3
12	29.54	256.74	33.51	276.49
Mean	16.42	158.57	22.47	196.22
CV%	47.89	36.68	32.82	30.83
P (t-test)	0.0051	0.0098	------	------

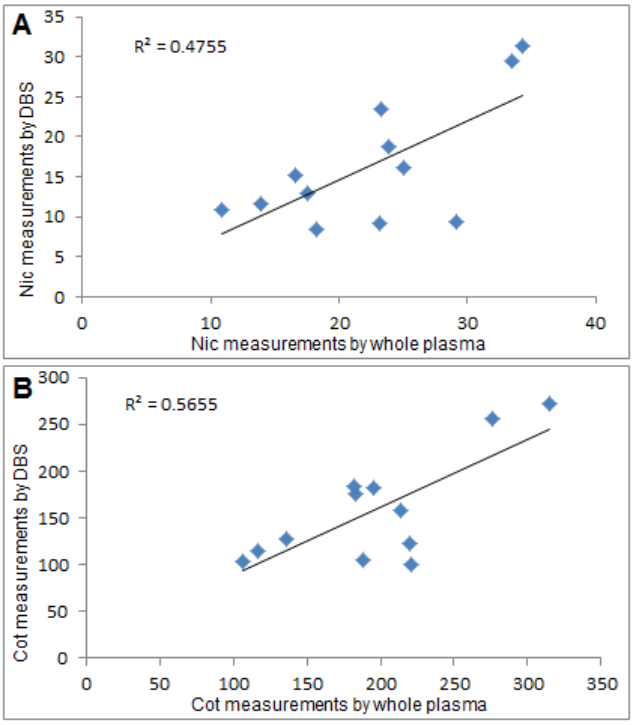

Figure 4.4 Correlation between DBS measurements with their corresponding measurements from whole plasma for Nic (**A**) and Cot (**B**).

4.4 Conclusion

A high throughput, economic and simple bioanalytical method was developed and validated for the quantitative analysis of Nic and Cot in DBS using LC-Orbitrap MS. Single extraction-reconstitution step by TCA solution was successfully applied for DBS extraction.

Chromatographic effect was investigated for first time in this study and demonstrated that overall variation account due to the decentralized punching and hematocrit effect comprised a minimal variation factor in Nic and Cot quantitation by DBS as long as the punched disks stayed within the confines of the DBS (non-touching the edge).

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5 Overall conclusion

The developed method for simultaneous determination of Nic, Cot and Nox in human blood, plasma, urine, semen and sperm using LC ESI-orbitrap-MS was successfully validated according to the European and American guidelines for bioanalytical method validation, following single extraction step by TCA solution for protein direct precipitation, providing top extraction recovery and perfect matrix separation with short run time. Accurate mass measurements were obtained by ESI-orbitrap-MS and the studied validation sections were all satisfying the acceptance criteria following used guidelines. Ion suppression test was achieved and discussed for investigated matrixes and showed a perfect separation for matrix's suppressant compounds from targeted analyte peaks. The established method was successfully applied to investigate three objectives in current study.

In the first study objective for evaluation of pomegranate and licorice drinks effect on Nic metabolism rate as indicated from urinary measurements of Nic/Cot, we found a consistent trend of increased metabolism rate for Nic under pomegranate or licorice test drink comparing to the corresponding control condition in all participant volunteers urine samples ($Nic/Cot_{test\ drink} < Nic/Cot_{control}$). More information about Nic metabolism was obtained from a new measured metabolic index Nic/Nox, which was introduced for first time in this study. Maybe the reason for the accelerated metabolism rate for Nic is that induced CYP 2A6 was occurred by CYP 3A4 induction effect, as discussed induction effect mechanism in the literatures [91, 92].

The second study objective was intended to investigate Nic and its major metabolites Cot and Nox in human plasma, semen and sperm, where Nic has extensive distribution among human fluids and tissues, and we found Nic, Cot and Nox for first time in human sperm, after quantitative separation of sperm bodies from each 1 mL of semen samples by centrifugation as shown in Figure 5.1, then extracted following the optimized method. As a result, Nic and Cot were detected in all samples, and Nox was below the LLOQ in plasma but detectable in semen samples from 10 of 12 sperm samples. Interestingly, Nic

was significantly higher in semen compared to plasma (2.3-fold, $p<0.001$). In sperm the concentrations of Nic and Cot were in the same range as for Nic in plasma and Nic was significantly lower in sperm compared to semen (2.1-fold, $p<0.001$).

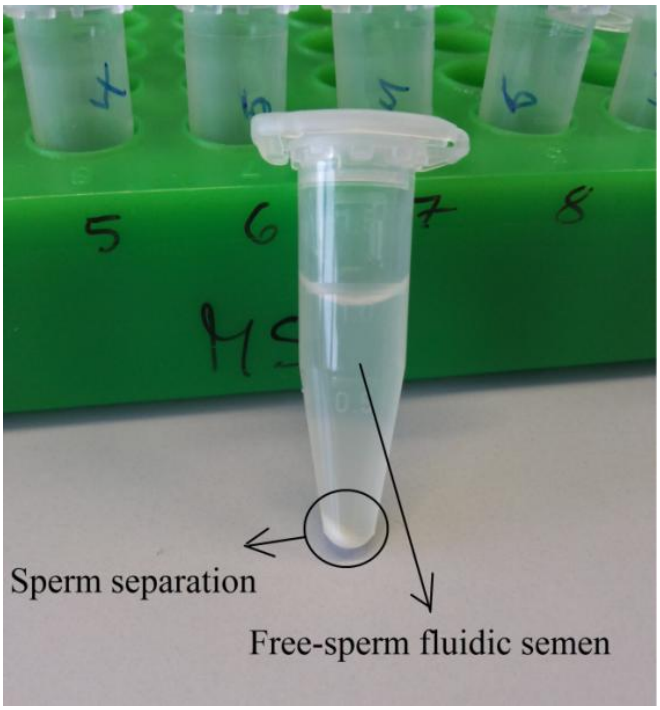


Figure 5.1 Representative separations of sperm bodies as seen in the tube's bottom from whole fluidic semen as achieved in the current study.

The third study objective was intended to find a suitable bioanalytical method for Nic and Cot quantification in human blood using DBS technique alternatively to the traditionally used methods (using separate plasma from blood), and it was successfully validated for simultaneous determination of Nic and Cot following a single extraction-reconstitution step by TCA solution for 6.35 mm disk diameter cut out of DBS. Chromatographic effect was investigated for first time in this study and demonstrated that overall variation account due to the decentralized punching and hematocrit effect comprised a minimal variation factor in Nic and Cot quantitation by DBS as long as the punched disks stayed within the confines of the DBS (non-touching the edge). The validated method was then successfully applied to collect blood samples from twelve smoker volunteers, and compared their DBS measurements with the corresponding plasma measurements.

6 Appendix

6.1 Supplementary material for chapter 3.2.4.1

Abstract

Here detailed information about the demographic data including Fagerström test of all participant volunteers are given.

Demographic data

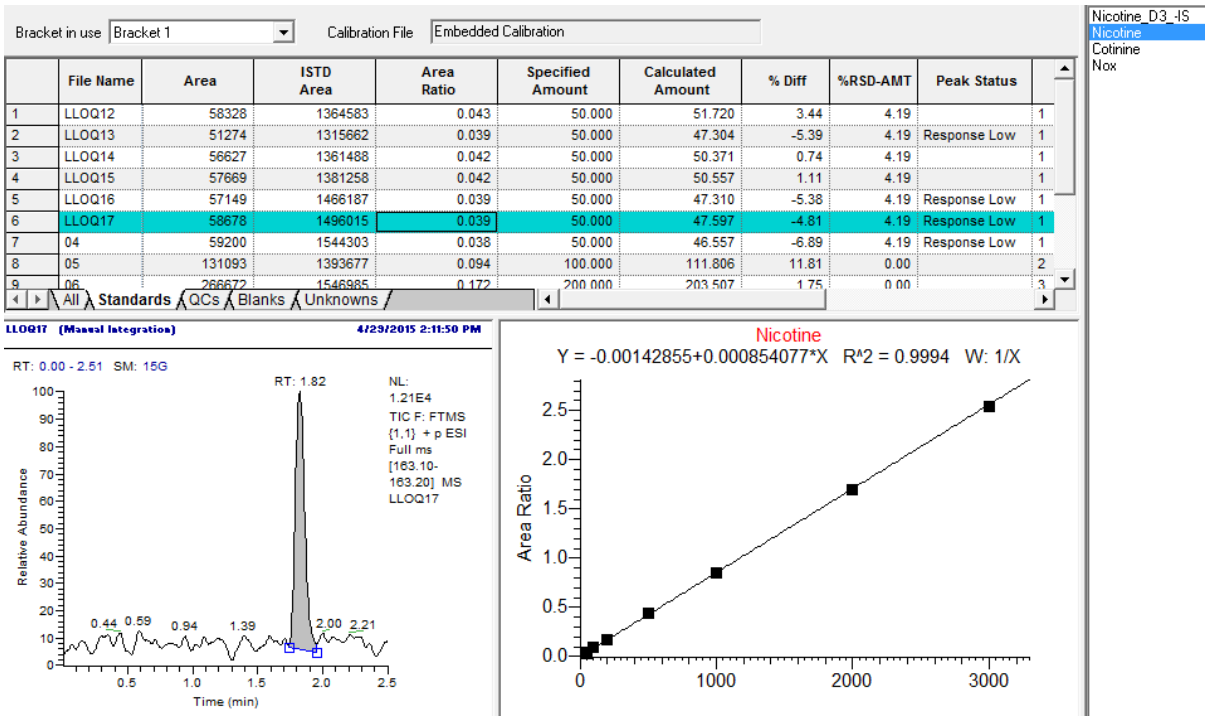
Demographic data including Fagerström test [145] were obtained from all participant volunteers (Supplementary material Table 1).

Demographic data including Fagerström test for all participant volunteers.

Volunteer no	age	mass	tall	BMI	Fagerström score
1	25	110	1.93	29.53	5.00
2	19	78	1.73	26.06	9.00
3	21	65	1.66	23.59	4.00
4	44	72	1.70	24.91	7.00
5	38	68	1.73	22.72	10.00
6	33	88	1.72	29.75	10.00
7	27	93	1.78	29.35	6.00
8	28	62	1.74	20.48	9.00
9	45	74	1.85	21.62	8.00
10	29	78	1.69	27.31	9.00
11	32	102	1.83	30.46	5.00
12	39	113	1.93	30.34	9.00
Mean	31.67	83.58	1.77	26.34	8.17
STD	8.46	17.46	0.09	3.62	1.75

6.2 Preventative calibration curves and function including chromatograms

Nic CC and function including chromatogram from urine analysis



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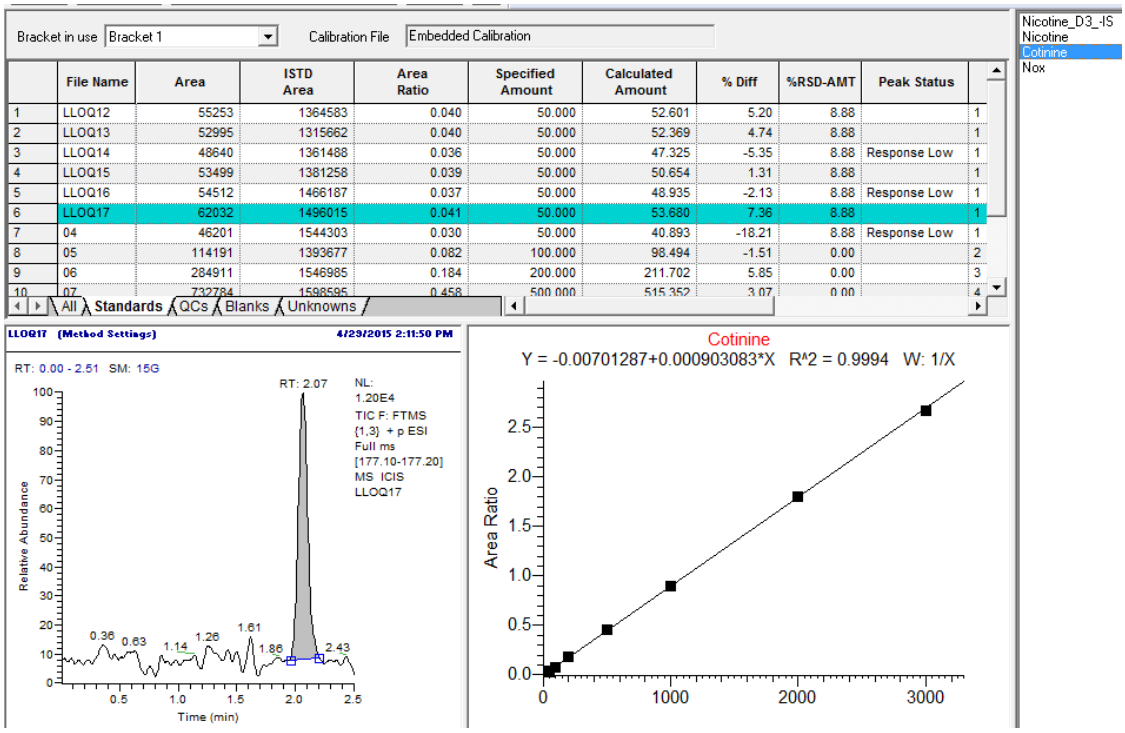
2	LLOQ13	51274	1315662	0.039	50.000	47.304	-5.39	4.19	Response Low	1
3	LLOQ14	56627	1361488	0.042	50.000	50.371	0.74	4.19		1
4	LLOQ15	57669	1381258	0.042	50.000	50.557	1.11	4.19		1
5	LLOQ16	57149	1466187	0.039	50.000	47.310	-5.38	4.19	Response Low	1
6	LLOQ17	58678	1496015	0.039	50.000	47.597	-4.81	4.19	Response Low	1
7	04	59200	1544303	0.038	50.000	46.557	-6.89	4.19	Response Low	1
8	05	131093	1393677	0.094	100.000	111.806	11.81	0.00		2
9	06	266672	1546985	0.172	200.000	203.507	1.75	0.00		3

All | Standards | QCs | Blanks | Unknowns

Nicotine_D3_-IS
Nicotine
Cotinine
Nox

Cot CC and function including chromatogram from urine analysis



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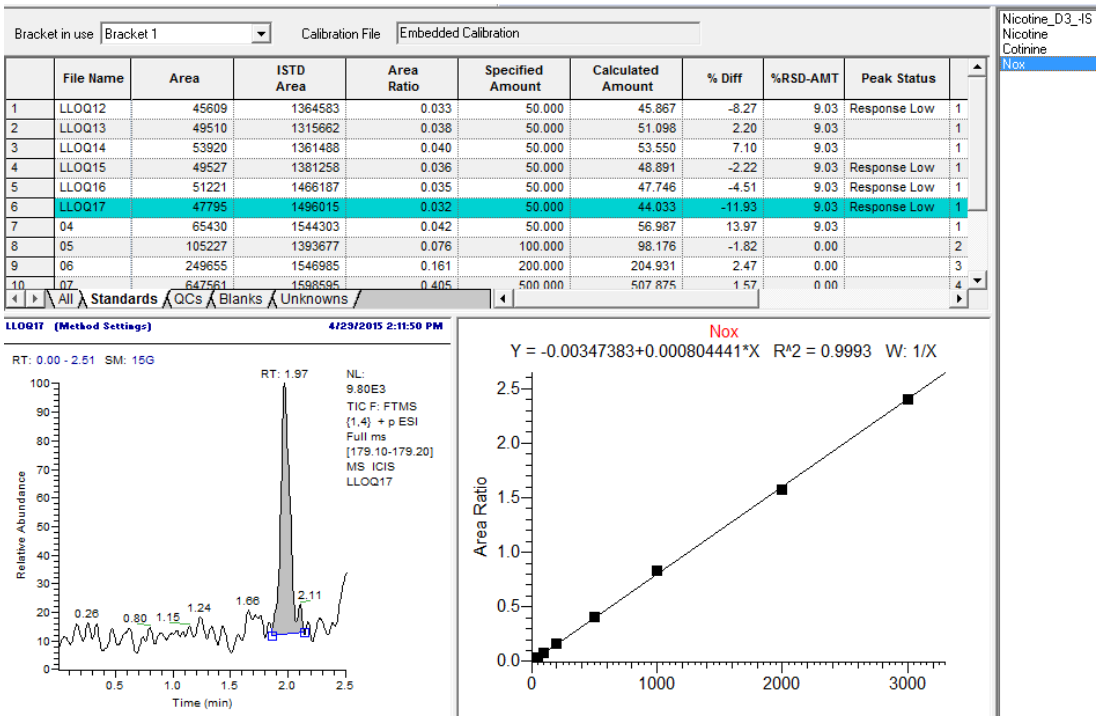
3	LLOQ14	48640	1361488	0.036	50.000	47.325	-5.35	8.88	Response Low	1
4	LLOQ15	53499	1381258	0.039	50.000	50.654	1.31	8.88		1
5	LLOQ16	54512	1466187	0.037	50.000	48.935	-2.13	8.88	Response Low	1
6	LLOQ17	62032	1496015	0.041	50.000	53.680	7.36	8.88		1
7	04	46201	1544303	0.030	50.000	40.893	-18.21	8.88	Response Low	1
8	05	114191	1393677	0.082	100.000	98.494	-1.51	0.00		2
9	06	284911	1546985	0.184	200.000	211.702	5.85	0.00		3
10	07	732784	1598595	0.458	500.000	515.352	3.07	0.00		4

Nox CC and function including chromatogram from urine analysis



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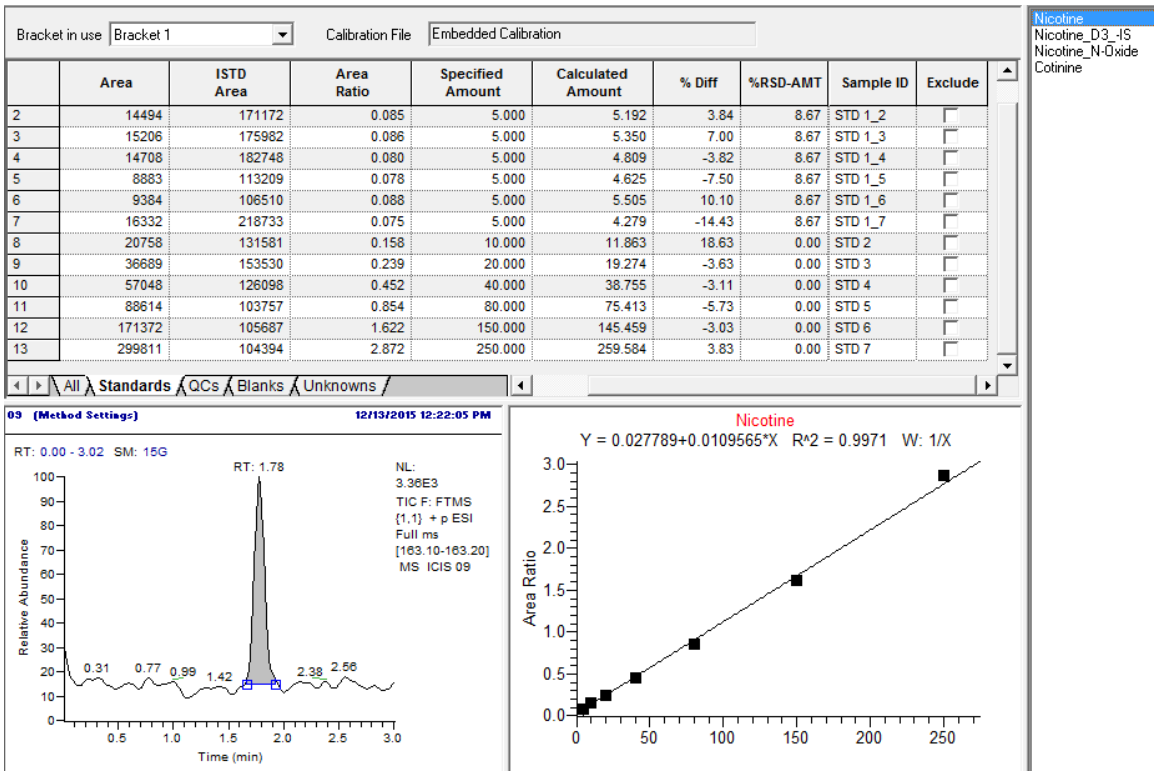
3	LLOQ14	53920	1361488	0.040	50.000	53.550	7.10	9.03		1
4	LLOQ15	49527	1381258	0.036	50.000	48.891	-2.22	9.03	Response Low	1
5	LLOQ16	51221	1466187	0.035	50.000	47.746	-4.51	9.03	Response Low	1
6	LLOQ17	47795	1496015	0.032	50.000	44.033	-11.93	9.03	Response Low	1
7	04	65430	1544303	0.042	50.000	56.987	13.97	9.03		1
8	05	105227	1393677	0.076	100.000	98.176	-1.82	0.00		2
9	06	249655	1546985	0.161	200.000	204.931	2.47	0.00		3
10	07	647561	1598595	0.405	500.000	507.875	1.57	0.00		4

Nic CC and function including chromatogram from plasma analysis



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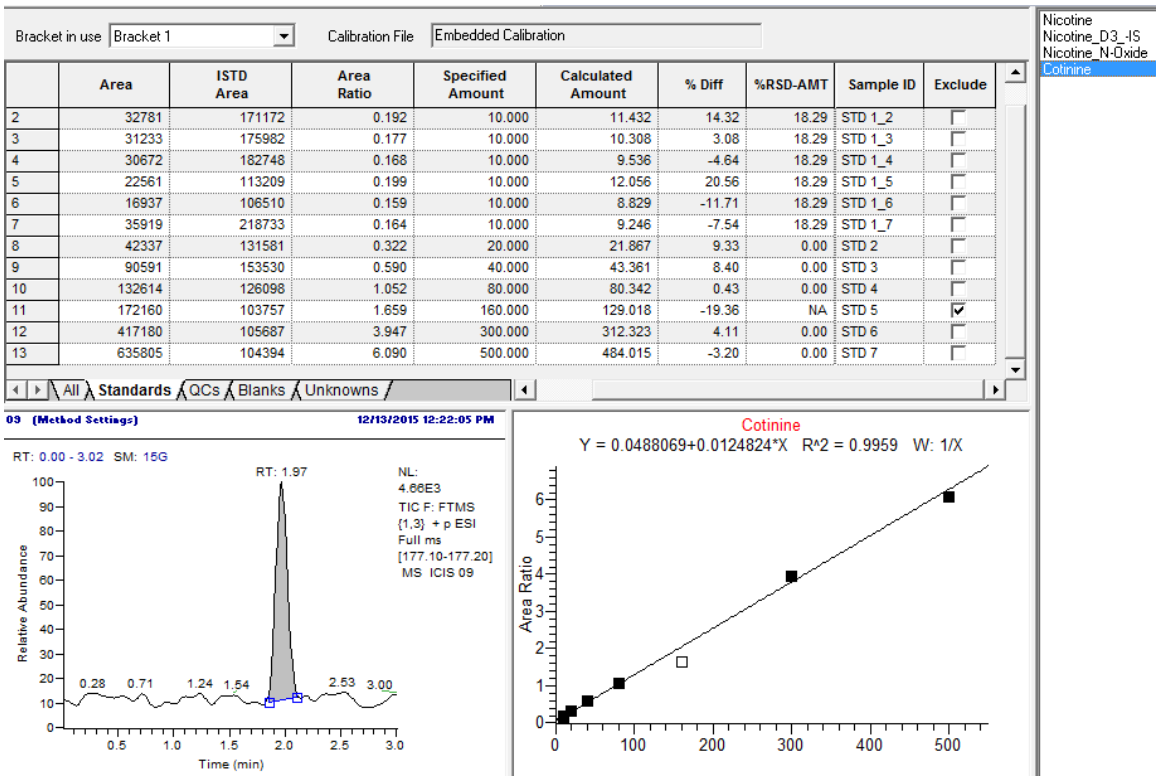
4	14708	182748	0.080	5.000	4.809	-3.82	8.67	STD 1_4	
5	8883	113209	0.078	5.000	4.625	-7.50	8.67	STD 1_5	
6	9384	106510	0.088	5.000	5.505	10.10	8.67	STD 1_6	
7	16332	218733	0.075	5.000	4.279	-14.43	8.67	STD 1_7	
8	20758	131581	0.158	10.000	11.863	18.63	0.00	STD 2	
9	36689	153530	0.239	20.000	19.274	-3.63	0.00	STD 3	
10	57048	126098	0.452	40.000	38.755	-3.11	0.00	STD 4	
11	88614	103757	0.854	80.000	75.413	-5.73	0.00	STD 5	
12	171372	105687	1.622	150.000	145.459	-3.03	0.00	STD 6	
13	299811	104394	2.872	250.000	259.584	3.83	0.00	STD 7	

All | Standards | QCs | Blanks | Unknowns

Nicotine
Nicotine_D3_-IS
Nicotine_N-Oxide
Cotinine

Cot CC and function including chromatogram from plasma analysis



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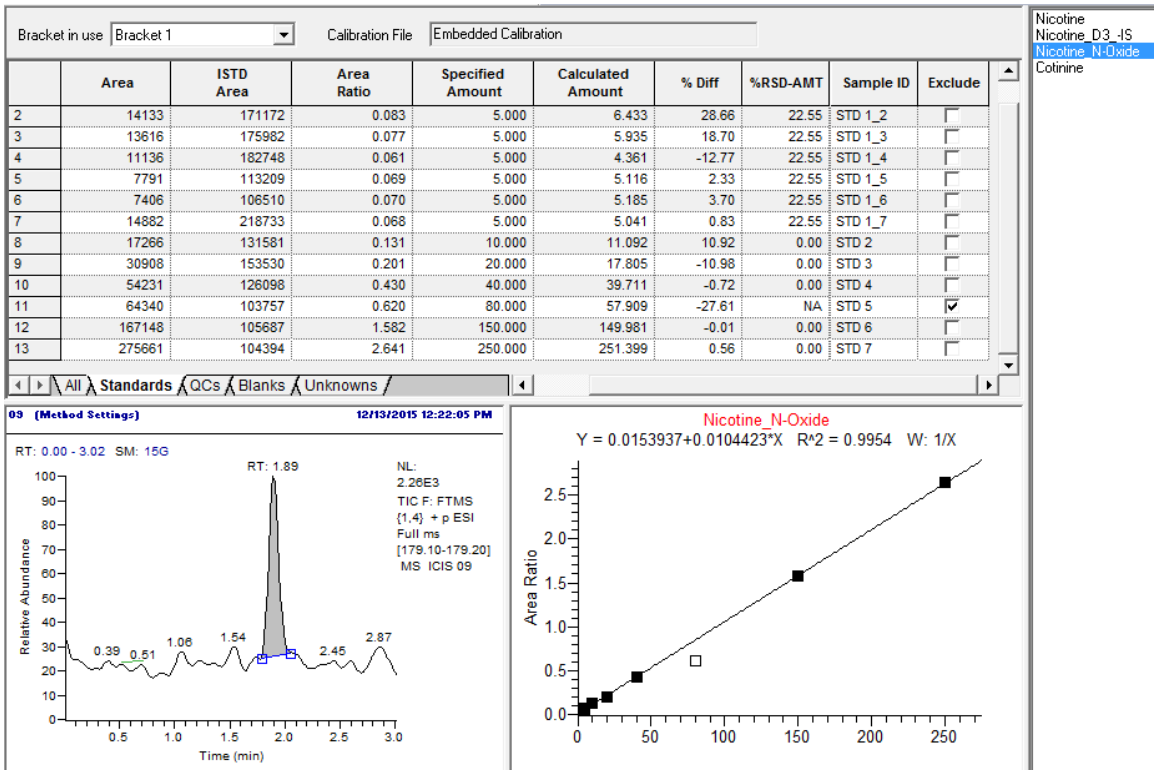
3	31233	175982	0.177	10.000	10.308	3.08	18.29	STD 1_3	
4	30672	182748	0.168	10.000	9.536	-4.64	18.29	STD 1_4	
5	22561	113209	0.199	10.000	12.056	20.56	18.29	STD 1_5	
6	16937	106510	0.159	10.000	8.829	-11.71	18.29	STD 1_6	
7	35919	218733	0.164	10.000	9.246	-7.54	18.29	STD 1_7	
8	42337	131581	0.322	20.000	21.867	9.33	0.00	STD 2	
9	90591	153530	0.590	40.000	43.361	8.40	0.00	STD 3	
10	132614	126098	1.052	80.000	80.342	0.43	0.00	STD 4	
11	172160	103757	1.659	160.000	129.018	-19.36	NA	STD 5	☑
12	417180	105687	3.947	300.000	312.323	4.11	0.00	STD 6	
13	635805	104394	6.090	500.000	484.015	-3.20	0.00	STD 7	

Nox CC and function including chromatogram from plasma analysis



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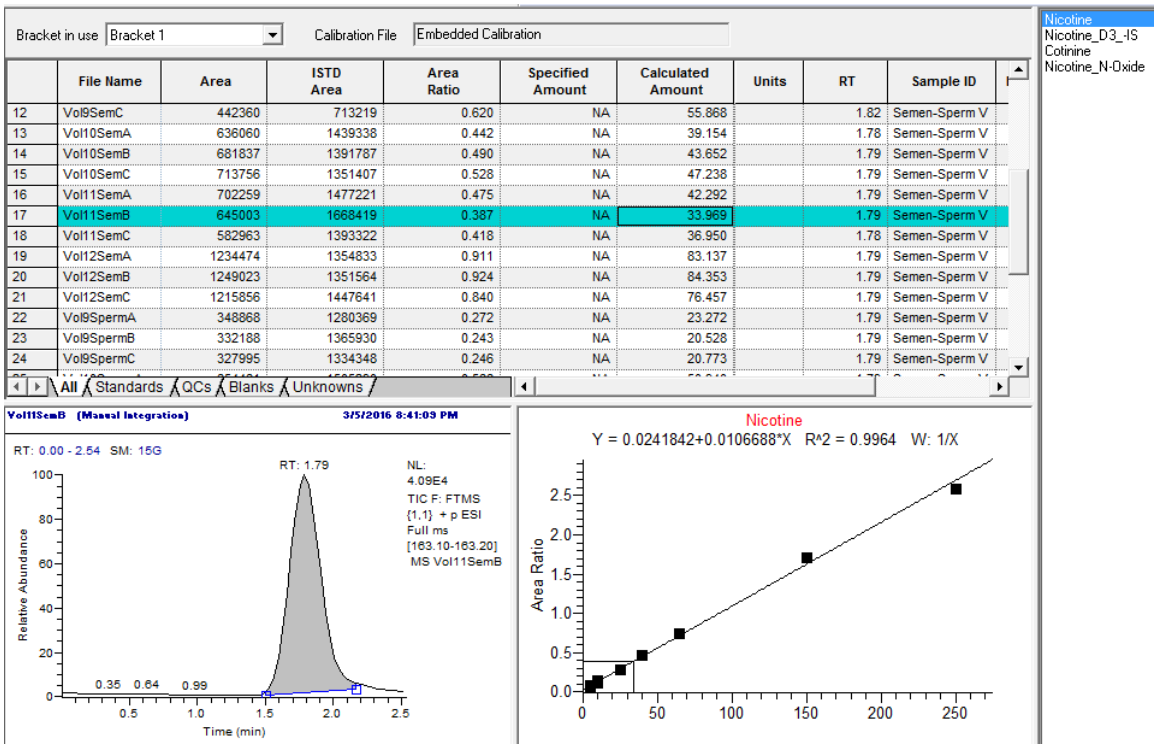
3	13616	175982	0.077	5.000	5.935	18.70	22.55	STD 1_3	
4	11136	182748	0.061	5.000	4.361	-12.77	22.55	STD 1_4	
5	7791	113209	0.069	5.000	5.116	2.33	22.55	STD 1_5	
6	7406	106510	0.070	5.000	5.185	3.70	22.55	STD 1_6	
7	14882	218733	0.068	5.000	5.041	0.83	22.55	STD 1_7	
8	17266	131581	0.131	10.000	11.092	10.92	0.00	STD 2	
9	30908	153530	0.201	20.000	17.805	-10.98	0.00	STD 3	
10	54231	126098	0.430	40.000	39.711	-0.72	0.00	STD 4	
11	64340	103757	0.620	80.000	57.909	-27.61	NA	STD 5	☑
12	167148	105687	1.582	150.000	149.981	-0.01	0.00	STD 6	
13	275661	104394	2.641	250.000	251.399	0.56	0.00	STD 7	

Nic CC and function including chromatogram from semen analysis



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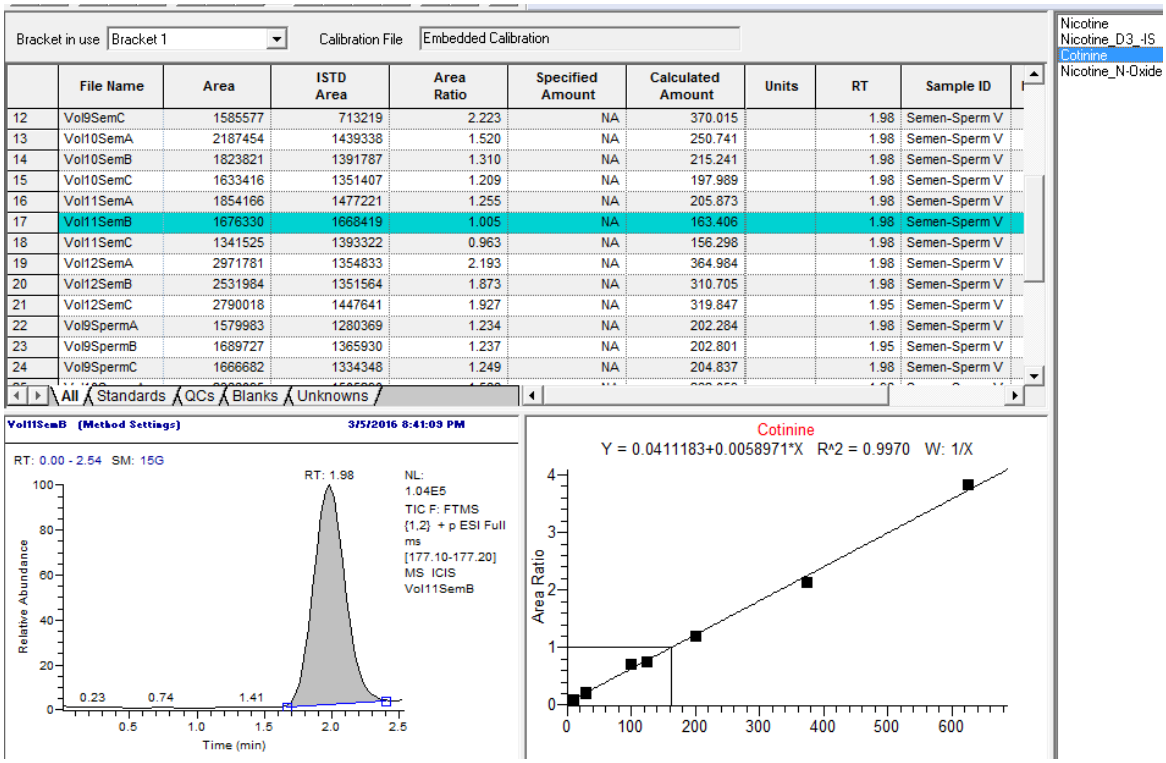
13	Vol10SemA	636060	1439338	0.442	NA	39.154		1.78	Semen-Sperm V
14	Vol10SemB	681837	1391787	0.490	NA	43.652		1.79	Semen-Sperm V
15	Vol10SemC	713756	1351407	0.528	NA	47.238		1.79	Semen-Sperm V
16	Vol11SemA	702259	1477221	0.475	NA	42.292		1.79	Semen-Sperm V
17	Vol11SemB	645003	1668419	0.387	NA	33.969		1.79	Semen-Sperm V
18	Vol11SemC	582963	1393322	0.418	NA	36.950		1.78	Semen-Sperm V
19	Vol12SemA	1234474	1354833	0.911	NA	83.137		1.79	Semen-Sperm V
20	Vol12SemB	1249023	1351564	0.924	NA	84.353		1.79	Semen-Sperm V
21	Vol12SemC	1215856	1447641	0.840	NA	76.457		1.79	Semen-Sperm V
22	Vol9SpermA	348868	1280369	0.272	NA	23.272		1.79	Semen-Sperm V
23	Vol9SpermB	332188	1365930	0.243	NA	20.528		1.79	Semen-Sperm V
24	Vol9SpermC	327995	1334348	0.246	NA	20.773		1.79	Semen-Sperm V

Cot CC and function including chromatogram from semen analysis



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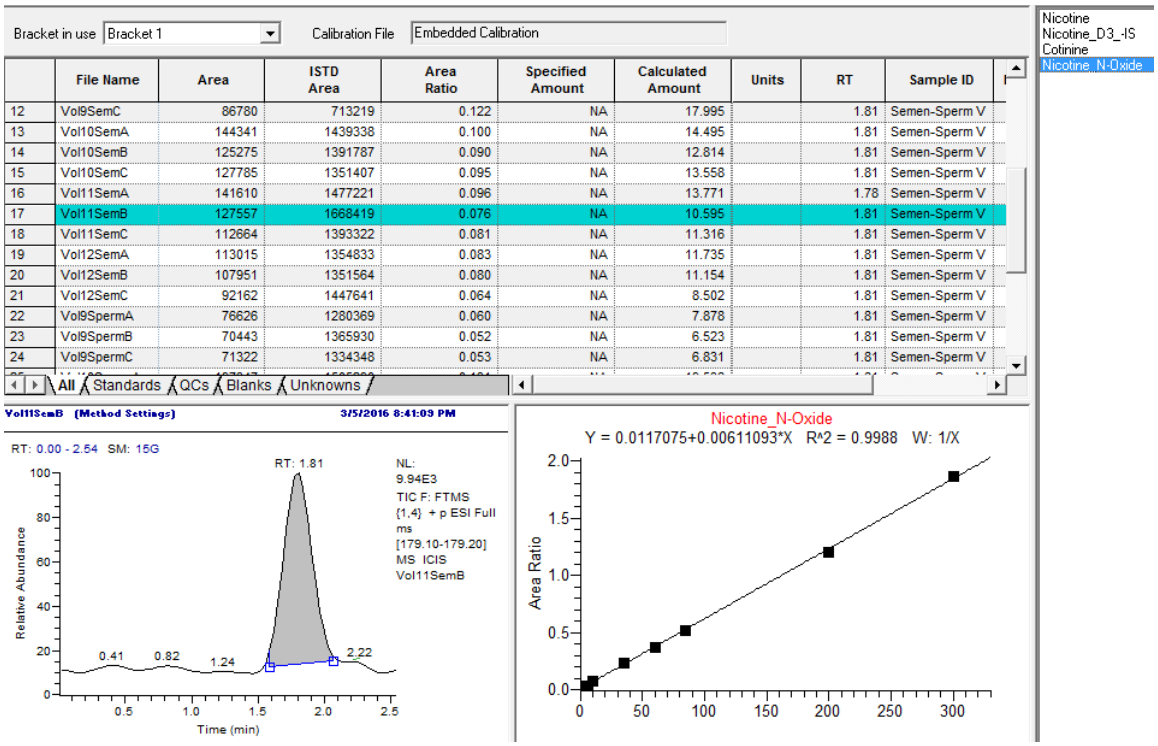
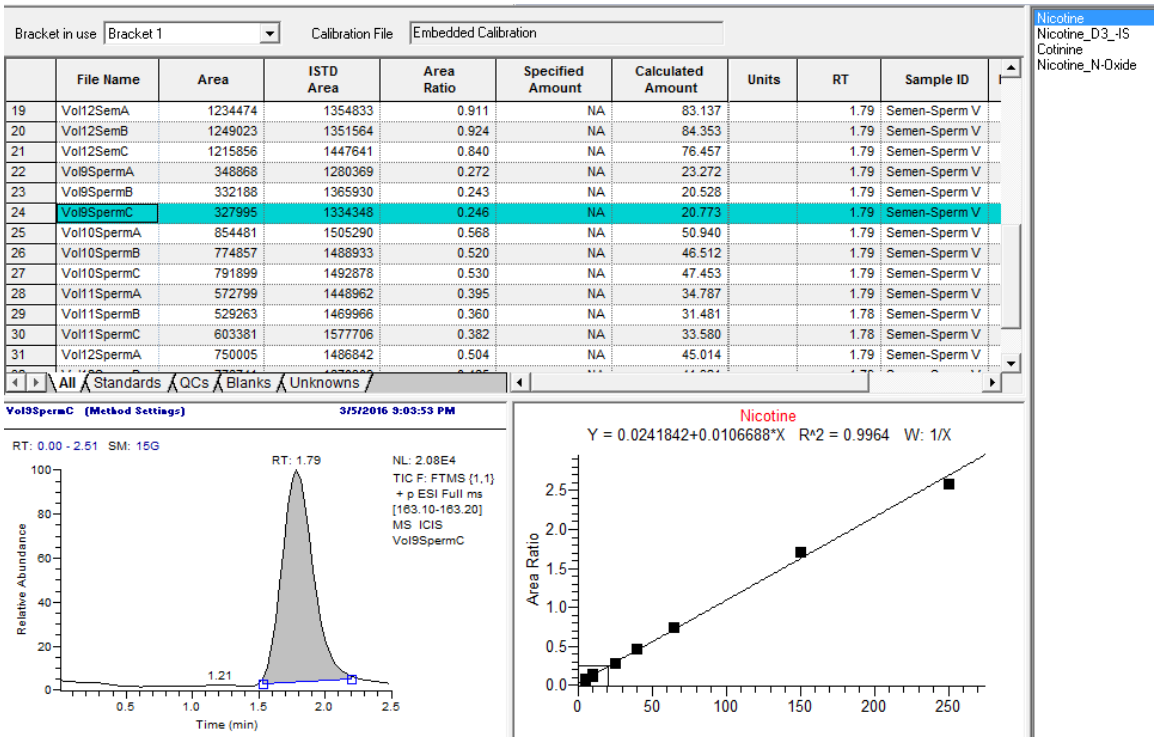
12	Vol9SemC	1585577	713219	2.223	NA	370.015		1.98	Semen-Sperm V
13	Vol10SemA	2187454	1439338	1.520	NA	250.741		1.98	Semen-Sperm V
14	Vol10SemB	1823821	1391787	1.310	NA	215.241		1.98	Semen-Sperm V
15	Vol10SemC	1633416	1351407	1.209	NA	197.989		1.98	Semen-Sperm V
16	Vol11SemA	1854166	1477221	1.255	NA	205.873		1.98	Semen-Sperm V
17	Vol11SemB	1676330	1668419	1.005	NA	163.406		1.98	Semen-Sperm V
18	Vol11SemC	1341525	1393322	0.963	NA	156.298		1.98	Semen-Sperm V
19	Vol12SemA	2971781	1354833	2.193	NA	364.984		1.98	Semen-Sperm V
20	Vol12SemB	2531984	1351564	1.873	NA	310.705		1.98	Semen-Sperm V
21	Vol12SemC	2790018	1447641	1.927	NA	319.847		1.95	Semen-Sperm V
22	Vol9SpermA	1579983	1280369	1.234	NA	202.284		1.98	Semen-Sperm V
23	Vol9SpermB	1689727	1365930	1.237	NA	202.801		1.95	Semen-Sperm V
24	Vol9SpermC	1666682	1334348	1.249	NA	204.837		1.98	Semen-Sperm V

Nox CC and function including chromatogram from semen analysis



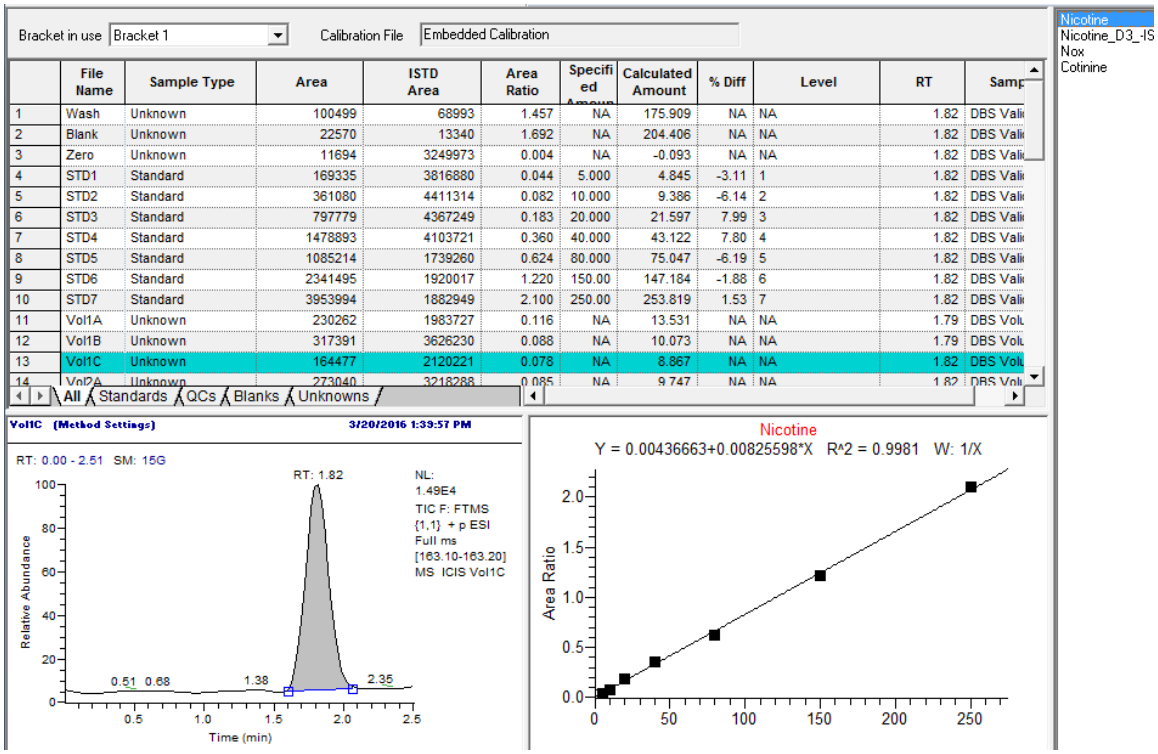
	File Name	Area	ISTD Area	Area Ratio	Specified Amount	Calculated Amount	Units	RT	Sample ID
12	Vol9SemC	86780	713219	0.122	NA	17.995		1.81	Semen-Sperm V
13	Vol10SemA	144341	1439338	0.100	NA	14.495		1.81	Semen-Sperm V
14	Vol10SemB	125275	1391787	0.090	NA	12.814		1.81	Semen-Sperm V
15	Vol10SemC	127785	1351407	0.095	NA	13.558		1.81	Semen-Sperm V
16	Vol11SemA	141610	1477221	0.096	NA	13.771		1.78	Semen-Sperm V
17	Vol11SemB	127557	1668419	0.076	NA	10.595		1.81	Semen-Sperm V
18	Vol11SemC	112664	1393322	0.081	NA	11.316		1.81	Semen-Sperm V
19	Vol12SemA	113015	1354833	0.083	NA	11.735		1.81	Semen-Sperm V
20	Vol12SemB	107951	1351564	0.080	NA	11.154		1.81	Semen-Sperm V
21	Vol12SemC	92162	1447641	0.064	NA	8.502		1.81	Semen-Sperm V
22	Vol9SpermA	76626	1280369	0.060	NA	7.878		1.81	Semen-Sperm V
23	Vol9SpermB	70443	1365930	0.052	NA	6.523		1.81	Semen-Sperm V
24	Vol9SpermC	71322	1334348	0.053	NA	6.831		1.81	Semen-Sperm V

Nic CC and function including chromatogram from sperm analysis



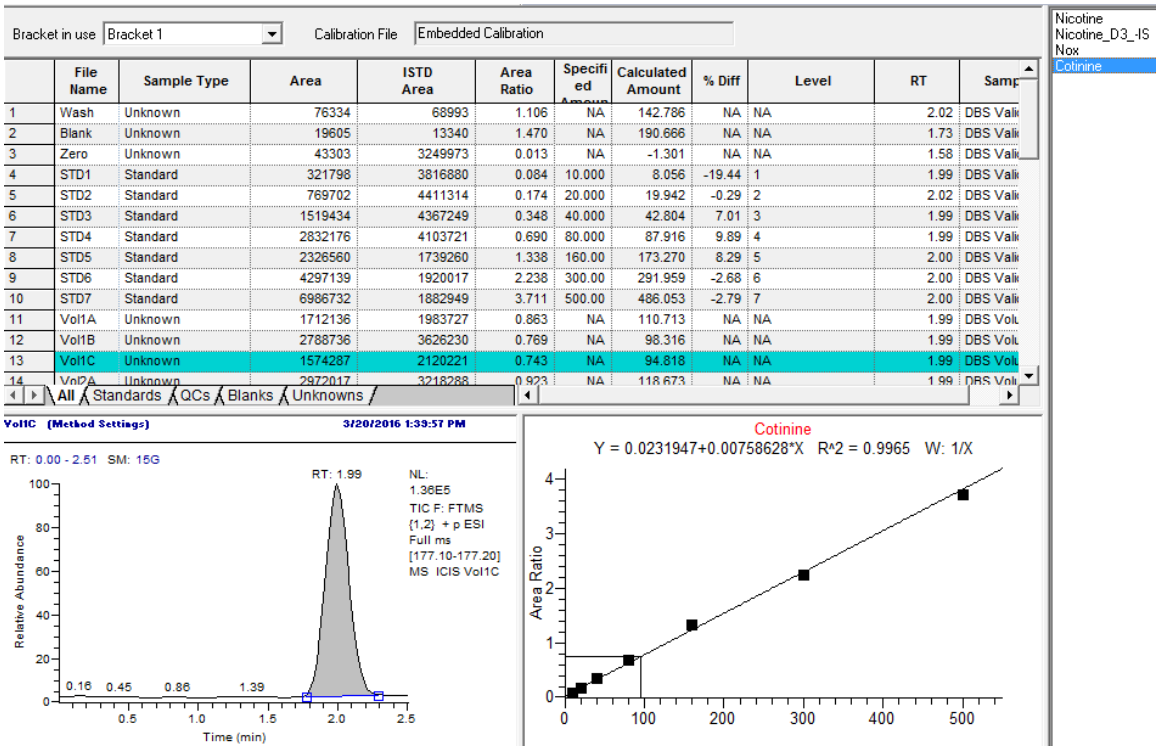
	File Name	Area	ISTD Area	Area Ratio	Specified Amount	Calculated Amount	Units	RT	Sample ID
19	Vol12SemA	1234474	1354833	0.911	NA	83.137		1.79	Semen-Sperm V
20	Vol12SemB	1249023	1351564	0.924	NA	84.353		1.79	Semen-Sperm V
21	Vol12SemC	1215856	1447641	0.840	NA	76.457		1.79	Semen-Sperm V
22	Vol9SpermA	348868	1280369	0.272	NA	23.272		1.79	Semen-Sperm V
23	Vol9SpermB	332188	1365930	0.243	NA	20.528		1.79	Semen-Sperm V
24	Vol9SpermC	327995	1334348	0.246	NA	20.773		1.79	Semen-Sperm V
25	Vol10SpermA	854481	1505290	0.568	NA	50.940		1.79	Semen-Sperm V
26	Vol10SpermB	774857	1488933	0.520	NA	46.512		1.79	Semen-Sperm V
27	Vol10SpermC	791899	1492878	0.530	NA	47.453		1.79	Semen-Sperm V
28	Vol11SpermA	572799	1448962	0.395	NA	34.787		1.79	Semen-Sperm V
29	Vol11SpermB	529263	1469966	0.360	NA	31.481		1.78	Semen-Sperm V
30	Vol11SpermC	603381	1577706	0.382	NA	33.580		1.78	Semen-Sperm V
31	Vol12SpermA	750005	1486842	0.504	NA	45.014		1.79	Semen-Sperm V

Nic CC and function including chromatogram from DBS analysis



	File Name	Sample Type	Area	ISTD Area	Area Ratio	Specified Amount	Calculated Amount	% Diff	Level	RT	Samp
1	Wash	Unknown	100499	68993	1.457	NA	175.909	NA	NA	1.82	DBS Vali
2	Blank	Unknown	22570	13340	1.692	NA	204.406	NA	NA	1.82	DBS Vali
3	Zero	Unknown	11694	3249973	0.004	NA	-0.093	NA	NA	1.82	DBS Vali
4	STD1	Standard	169335	3816880	0.044	5.000	4.845	-3.11	1	1.82	DBS Vali
5	STD2	Standard	361080	4411314	0.082	10.000	9.386	-6.14	2	1.82	DBS Vali
6	STD3	Standard	797779	4367249	0.183	20.000	21.597	7.99	3	1.82	DBS Vali
7	STD4	Standard	1478893	4103721	0.360	40.000	43.122	7.80	4	1.82	DBS Vali
8	STD5	Standard	1085214	1739260	0.624	80.000	75.047	-6.19	5	1.82	DBS Vali
9	STD6	Standard	2341495	1920017	1.220	150.00	147.184	-1.88	6	1.82	DBS Vali
10	STD7	Standard	3953994	1882949	2.100	250.00	253.819	1.53	7	1.82	DBS Vali
11	Vol1A	Unknown	230262	1983727	0.116	NA	13.531	NA	NA	1.79	DBS Vol
12	Vol1B	Unknown	317391	3626230	0.088	NA	10.073	NA	NA	1.79	DBS Vol
13	Vol1C	Unknown	164477	2120221	0.078	NA	8.867	NA	NA	1.82	DBS Vol
14	Vol2A	Unknown	273040	3218288	0.085	NA	9.747	NA	NA	1.82	DBS Vol

Cot CC and function including chromatogram from DBS analysis



	File Name	Sample Type	Area	ISTD Area	Area Ratio	Specified Amount	Calculated Amount	% Diff	Level	RT	Samp
1	Wash	Unknown	76334	68993	1.106	NA	142.786	NA	NA	2.02	DBS Vali
2	Blank	Unknown	19605	13340	1.470	NA	190.666	NA	NA	1.73	DBS Vali
3	Zero	Unknown	43303	3249973	0.013	NA	-1.301	NA	NA	1.58	DBS Vali
4	STD1	Standard	321798	3816880	0.084	10.000	8.056	-19.44	1	1.99	DBS Vali
5	STD2	Standard	769702	4411314	0.174	20.000	19.942	-0.29	2	2.02	DBS Vali
6	STD3	Standard	1519434	4367249	0.348	40.000	42.804	7.01	3	1.99	DBS Vali
7	STD4	Standard	2832176	4103721	0.690	80.000	87.916	9.89	4	1.99	DBS Vali
8	STD5	Standard	2326560	1739260	1.338	160.00	173.270	8.29	5	2.00	DBS Vali
9	STD6	Standard	4297139	1920017	2.238	300.00	291.959	-2.68	6	2.00	DBS Vali
10	STD7	Standard	6986732	1882949	3.711	500.00	486.053	-2.79	7	2.00	DBS Vali
11	Vol1A	Unknown	1712136	1983727	0.863	NA	110.713	NA	NA	1.99	DBS Vol
12	Vol1B	Unknown	2788736	3626230	0.769	NA	98.316	NA	NA	1.99	DBS Vol
13	Vol1C	Unknown	1574287	2120221	0.743	NA	94.818	NA	NA	1.99	DBS Vol
14	Vol2A	Unknown	2972017	3218288	0.923	NA	118.673	NA	NA	1.99	DBS Vol

6.3 List of abbreviations

(AS) autosampler

(BMI) body mass index

(CC) calibration curve

(CDER) center for drug evaluation and research

(CV %) coefficient of variation

(Cot) cotinine

(CYP) cytochrome p450

(Da) Dalton

(nicotine-d3) deuterated nicotine

(DBS) dried blood spot

(DM) drug metabolism

(DMPK) Drug Metabolism and Pharmacokinetics

(ESI) electrospray ionization

(EMA) European Medicine Agency

(FDA) food and drug administration

(FT) fourier transform

(FTICR) fourier transform ion cyclotron resonance

(FWHM) full width at half maximum

(GC-MS) gas chromatography mass spectrometry

(GE) General Electronics

(H-ESI) heated-electrospray ionization source

(HPLC-UV) high performance liquid chromatography with ultraviolet detection

(QqTOF) hybrid tandem quadrupole time of flight

(3HC) 3-hydroxy-Cot

(IS) internal standard

(IS-NMF) internal standard-normalized MF

(JCPR) Jordan center for pharmaceutical research

(LL) liquid-liquid

(LC-MS/MS) liquid chromatography coupled to tandem mass spectrometry

(LLOQ) lower limit of quantification

(m) mass

(Δm) mass difference

($\Delta m_{50\%}$) mass difference at the half maximum for two equivalent peaks height

(MS) mass spectrometry

(MF) matrix factor

(Nic) nicotine

(Nox) nicotine N-oxide

(NRT) nicotine replacement therapy

(pk) pharmacokinetic

(PDA) photodiode array

(QTOF) quadrupole time-of-flight

(QC) quality control

(RSD %) relative standard deviation

(RP) reversed phase

(SPE) solid phase extraction

(TDM) therapeutic drug monitoring

(TOF) time of flight

(TRC) Toronto Research Chemical

(TIC) total ions count

(TK) toxicokinetic

(TCA) trichloroacetic acid solution

(US) United State

(ULOQ) upper limit of quantification

6.4 List of figures

6.5 List of tables

6.6 Publication list

1 Determination of nicotine and cotinine in human blood by dried blood spot/ LC-Orbitrap MS technique.
Ahmad Abu-awwad, Tawfiq Arafat, Oliver J. Schmitz*
Journal of Analytical & Pharmaceutical Research. 5 (2016) 1-7.

2 Study the Influence of Licorice and Pomegranate Drinks on Nicotine Metabolism in Human Urine by LC-Orbitrap MS.
Ahmad Abu-awwad, Tawfiq Arafat, Oliver J. Schmitz*
Journal of Pharmaceutical and Biomedical Analysis. 132 **(2016)** 60–65.

3 Simultaneous determination of nicotine, cotinine and nicotine N-oxide in human plasma, semen and sperm by LC-Orbitrap MS.
Ahmad Abu-awwad, Tawfiq Arafat, Oliver J. Schmitz*
Analytical and Bioanalytical Chemistry, 408 **(2016)** 6473–6481.

4 Determination of Fenofibric Acid in Human Plasma by LC–MS/MS and LC–UV.
Tawfiq Arafat, Basil Arafat, **Ahmad Abu Awwad***, Oliver J. Schmitz.
Chromatographia, 79 **(2016)**,685–692.

5 Relative Bioavailability of Ciprofloxacin Doses (750 and 1000) mg in Healthy Male Volunteers by Using HPLC Method.
Mallah E, Arafat, Al khawaja , Abu Dayyih, **Abu Awwad**, Hroub H, Hamad M3 and Arafat.
Journal of Bioequivalence Studies, 1 **(2015)** 1-6.

6 Comparative bioavailability of two different quetiapine doses (50 and 200 mg) in healthy volunteers by using LC/MS method.
Eyad Mallah, Basel Arafat, **Ahmad Abu Awwad**, Wael Abu Dayyih, Mohammed Mima and Tawfiq Arafat.
Journal of Chemical and Pharmaceutical Research, 12 **(2014)** 559-569.

7 Determination of loperamide in human plasma and saliva by liquid chromatography-tandem mass spectrometry.
Tawfiq Arafat, Basil Arafat, Riad awad, **Ahmad Abu awwad***
Journal of Chromatography B, 972 **(2014)** 81–88.

8 High Performance Liquid Chromatographic Determination of Montelukast Sodium in Human Plasma: Application to Bioequivalence Study
Ashok K. Shakya*, Tawfiq A. Arafat, Nancy M. Hakooz, **Ahmad N. Abu-awwad**, Hamza Al-Hroub, Munther Melhim
Acta Chromatographica Journal, 26 **(2014)** 457–472.

9 Study of food effect on Cyclobenzaprine absorption in human body by using liquid chromatography tandem mass spectrometer technique.
Ahmad Abu awwad, Mahmoud Yacoub, Mahmoud Alawi, Tawfiq Arafat
International Journal of Research in Pharmaceutical and Biomedical Sciences, 4 **(2013)** 2229-3701.

10 Simultaneous Determination of Amlodipine and Atorvastatin with Its Metabolites; Ortho and Para Hydroxy Atorvastatin; in Human Plasma by Liquid Chromatography Ion Trap Mass Spectrometry.
Mahmoud Yacoub, **Ahmad Abu awwad**, Mahmoud Alawi*, Tawfiq Arafat.
Journal of Chromatography B, 15 **(2013)** 36-47.

11 Tamsulosin quantification in human plasma by high performance liquid Chromatography coupled to electrospray tandem mass spectrometry.
Ashok K. Shakya, Nancy M. Hakooz, Tawfiq A. Arafat, **Ahmed N. A. Abu-awwad**, Jafar Abd El-Ghani and Munther Melhim.
Acta Chromatographica Journal, 22 **(2010)** 99-114.

12 Simple and rapid HPLC method for the determination of alfuzosin in human plasma.
Ashok K. Shakya, Tawfiq A. Arafat, **Ahmad Abu-awwad**, Hamza Al-Hroub, Munther Melhim.
Jordan Journal of Pharmaceutical Sciences, 3 **(2010)** 25-36.

13 Simultaneous determination of triprolidine and pseudoephedrine in human plasma by liquid chromatography-ion trap mass spectrometry
Ashok K. Shakya, Tawfiq A. Arafat, **Ahmad N. Abu-awwad**, Munther Melhim, Jafar Al-Ghani, Mahmoud J. Yacoub,
Journal of Chromatography B. 877 **(2009)** 4071-4078.

14 Validated liquid chromatographic–ultraviolet method for the quantitation of tadalafil in human plasma using liquid–liquid extraction.
Ashok K. Shakya, **Ahmed N.A. Abu-awwad**, Tawfiq A. Arafat, Munther Melhim.
Journal of Chromatography B. 852 **(2007)** 403-408.

6.7 "The biography is not included in the online version for reasons of data protection"

6.8 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel:

"Determination of Nicotine, Cotinine and Nicotine N-Oxide in Human Blood, Plasma, Urine, Semen and Sperm by LC-Orbitrap MS: Application to Clinical Study"

selbst verfasst und keine außer den angegebenen Hilfsmittel und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keener anderen Universität eingereicht wurde.

Essen, im Oktober 2016

Ahmad Abu-awwad

6.9 Acknowledgment

I would like to thank my supervisor Prof. Oliver J. Schmitz for hosting my PhD research and his valuable instructions in the all achievement steps for my PhD in his research facility of applied analytical chemistry.

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