Quantitative Analysis of Tobacco Specific Nitrosamine in Human Urine Using Molecularly Imprinted Polymers as a Potential Tool for Cancer Risk Assessment

Kumar Shah
Virginia Commonwealth University

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QUANTITATIVE ANALYSIS OF TOBACCO SPECIFIC NITROSAMINE IN HUMAN URINE USING MOLECULARLY IMPRINTED POLYMERS AS A POTENTIAL TOOL FOR CANCER RISK ASSESSMENT

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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November, 2009
This work is dedicated to my wife and my parents for their unconditional love, constant support and encouragement.
I would like to take this opportunity to thank my major advisor Dr. H. Thomas Karnes for his constant support and encouragement. I feel deeply grateful and privileged to have been his student, not only because of his deep insights and often crucial advice, but also because of the never failing kindness and moral and intellectual honesty. His vision, generosity and guidance balanced by the freedom to express myself are highly appreciated. He has always stood by me with extreme patience.

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CSP</td>
<td>chiral stationary phase</td>
</tr>
<tr>
<td>DFN</td>
<td>deviation from nominal</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>ETS</td>
<td>environmental tobacco smoke</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>Gluc</td>
<td>glucuronide</td>
</tr>
<tr>
<td>HILIC</td>
<td>hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>iso-NNAC</td>
<td>4-(methylnitrosamino)-4-(3-pyridyl)butanoic acid</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LLOQ</td>
<td>lower limit of quantitation</td>
</tr>
<tr>
<td>LOQ</td>
<td>limit of quantitation</td>
</tr>
<tr>
<td>MIP</td>
<td>molecularly imprinted polymer</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NAB</td>
<td>N’-nitrosoanabasine</td>
</tr>
<tr>
<td>NAT</td>
<td>N’-nitrosoanatabine</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>NNK</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone</td>
</tr>
<tr>
<td>NNN</td>
<td>N’-Nitrosonornicotine</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>SFE</td>
<td>supercritical fluid extraction</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>TEA</td>
<td>thermal energy analysis</td>
</tr>
<tr>
<td>TSNA</td>
<td>tobacco specific nitrosamine</td>
</tr>
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</table>
Abstract

QUANTITATIVE ANALYSIS OF TOBACCO SPECIFIC NITROSAMINE IN HUMAN URINE USING MOLECULARLY IMPRINTED POLYMERS AS A POTENTIAL TOOL FOR CANCER RISK ASSESSMENT

By Kumar A. Shah, M.Tech.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009.

Major Director: H. Thomas Karnes, Ph.D.
Professor, Department of Pharmaceutics

Measuring urinary tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide conjugate may provide the best biomarker of tobacco smoke lung carcinogen metabolism. Existence of differences in the extent of NNAL metabolism rates may be potentially related to an individuals’ lung cancer susceptibility. Low concentrations of NNAL in smokers urine (<1 ng/mL) require sensitive and selective methods for analysis. Traditionally, this involves extensive, time-consuming sample preparation that limits throughput and adds to measurement variability. Molecularly imprinted polymers (MIPs) have been developed for the analysis of urinary NNAL by offline cartridge extraction combined with LC-MS/MS. This
method when reproduced demonstrated problems with matrix effects. In the first part of this work, investigation of matrix effects and related problems with sensitivity for the published offline extraction method has been conducted. In order to address the need to improve throughput and other analytical figures of merit for the original method, the second part of this work deals with development of a high-throughput online microfluidic method using capillary-columns packed with MIP beads for the analysis of urinary NNAL. The method was validated as per the FDA guidance, and enabled low volume, rapid analysis of urinary NNAL by direct injection on a microfluidic column packed with NNAL specific MIP beads. The method was used for analysis of urinary NNAL and NNAL-Gluc in smokers. Chemometric methods were used with this data to develop a potential cancer-risk-assessment tool based on pattern recognition in the concentrations of these compounds in urine. In the last part, method comparison approaches for the online and the offline sample extraction techniques were investigated. A ‘fixed’ range acceptance criterion based on combined considerations of method precision and accuracy, and the FDA bioanalytical guidance limits on precision and accuracy was proposed. Data simulations studies to evaluate the probabilities of successful transfers using the proposed criteria were performed. Various experimental designs were evaluated and a design comprised of 3 runs with 3 replicates each with an acceptance range of ±20% was found appropriate. The off-line and the on-line sample extraction methods for NNAL analysis were found comparable using the proposed fixed range acceptance criteria.
1.1. Introduction – Why Study Tobacco Specific Nitrosamines?

Cancer Facts and Figures published by the American Cancer Society, 2009 estimated that between the years 2000 to 2004, smoking accounted for loss of more than 5 million years of potential life in men and women combined. Different types of cancers such as cancers of the nasopharynx, nasal cavity and paranasal sinuses, lip, oral cavity, pharynx, larynx, lung, esophagus, pancreas, uterine cervix, kidney, bladder, stomach, along with acute myeloid leukemia have all been associated with smoking (US Department of Health and Human Services, 2004). Use of smokeless tobacco can also lead to a variety of health ill-effects such as pre-cancerous oral lesions, pancreatic and oral cancer as well as cardiovascular diseases (Hecht et al., 1986; International Agency for Research on Cancer, 1985; International Agency for Research on Cancer, 2007). Tobacco products contain nicotine which is addictive and toxic, but it is not carcinogenic. This addiction, however, causes people to continually use tobacco products and these products contain many carcinogenic compounds. Thus, carcinogens form the devastating link between nicotine addiction and cancer. A scheme linking nicotine addiction to cancer via carcinogens is shown in Figure 1.
Figure 1: Scheme linking nicotine addiction to cancer via carcinogens ( Adopted from Hecht, 1999).
Tobacco smoke contains almost 5000 different chemical compounds (Rodgman and Perfetti, 2009). Of these, there are more than 16 carcinogens in smokeless tobacco and more than 60 in tobacco smoke (Hecht, 2003). These carcinogenic compounds belong to a variety of chemical classes including polycyclic aromatic hydrocarbons (PAHs), nitrosamines, aromatic amines, aldehydes, phenols, volatile hydrocarbons, nitro compounds and other organic and inorganic compounds (Hecht, 1998a; Hoffmann et al., 1987; Hoffmann et al., 1995; Hoffmann et al., 2001; International Agency for Research on Cancer, 2002; Swauger et al., 2002). Tobacco and tobacco smoke contain three types of N-nitrosamines. These include volatile nitrosamines, nitrosamines derived from residues of agricultural chemicals on tobacco and tobacco specific nitrosamines (TSNA) (Spiegelhalder and Bartsch, 1996). TSNAs have emerged as a leading class of carcinogens in tobacco products (Hecht and Hoffmann, 1988; International Agency for Research on Cancer, 2007). Extensive research has indicated the role of TSNAs in cancer induction by tobacco products (Hecht, 1998b; Hecht, 1999). Thus far, seven TSNAs have been identified in cigarettes (Djordjevic et al., 1991; Fischer et al., 1989; Hoffmann et al., 1994; Tricker et al., 1991; Tricker et al., 1993) as well as in smokeless tobacco products in even greater concentrations (Hoffmann et al., 1994; Hoffmann et al., 1995; International Agency for Research on Cancer, 1995; Prokopczyk et al., 1995). These include NNK, NNN, NAB, NAT, NNAL, iso-NNAL and iso-NNAC.

The study of TSNA uptake will provide very useful insight into the mechanistic and epidemiologic role of these compounds in human cancer, especially since their origin is specific to tobacco (Hecht, 2003). A major challenge in this area is the ability to
quantitatively measure the amounts of these compounds and their metabolites in complex biological matrices, as a measure of exposure to these tobacco specific carcinogens.

1.2. Metabolism of Tobacco Specific Nitrosamines

Of all the TSNAs identified, NNK and NNN are the most prevalent strong carcinogens in tobacco products as documented by Hecht and Hoffmann, 1988, Spiegelhalder and Bartsch, 1996 and Hoffmann et al., 1995. Moreover, the International Agency for Research on Cancer, 2007 classifies NNK and NNN as the only TSNAs carcinogenic to humans. Thus, the discussion on the metabolism of TSNAs will be mainly focused on these two compounds.

The main routes of NNK and NNN metabolism are shown in Figure 2. NNK is rapidly metabolized in animals and humans via three main routes: carbonyl reduction, pyridine oxidation and α-hydroxylation (Hecht, 1998b). In humans, carbonyl reduction of NNK forms NNAL which is also a potent lung carcinogen having similar carcinogenicity and metabolic pathway as NNK. A major mode of metabolism of NNAL in vivo is UGT catalyzed glucuronidation, which is the most important detoxification pathway for NNK/NNAL metabolism (Chen et al., 2008b). The pyridine-N-oxidation pathway results in the formation of NNK-N-oxide and NNAL-N-oxide. Metabolic activation of NNK and NNAL to DNA adducts proceeds via α-hydroxylation pathways. The end products of this pathway are keto acid and hydroxy acid. NNN metabolism is similar to NNK metabolism (Hecht, 1998b) and is depicted in Figure 2.
Figure 2: Metabolism of NNK and NNN (Adopted from Carmella et al., 1997)
1.3. Biomarkers for Human Uptake and Metabolic Activation of TSNAs

Many types of TSNA related biomarkers have been discussed in the literature. An ideal biomarker would be able to provide a measure of the carcinogen dose in people who use tobacco products as well as in nonsmokers exposed to secondhand smoke. Apart from this, it should potentially identify differences and patterns in the uptake, metabolic activation, and detoxification of tobacco carcinogens. These data might ultimately lead to the prediction of the cancer risk that a tobacco user might be susceptible to. The two main types of TSNA biomarkers are TSNA-DNA/protein adducts found in lungs and urinary compounds (Hecht, 1998b). A description of the advantages and limitations in the measurement of these biomarkers is discussed below.

1.3.1. TSNA-DNA and Protein Adducts

Adducts of carcinogenic compounds with DNA might provide the most direct relationship to cancer risk as suggested by Phillips, 1996, Bartsch, 1996 and Poirier and Weston, 1996. Foiles et al., 1991 have successfully analyzed TSNA-DNA adducts in smokers and non-smokers. However, challenges such as the limited availability of sample tissues as well as low levels of their occurrence (example: 1 per $10^6$ to $10^8$ normal human bases, De Bont and van Larebeke, 2004) can often make their quantification extremely difficult and at times impractical. Measuring protein-adducts of TSNAs has also been proposed as an approach to understanding metabolic activation of these carcinogens. However, results from the studies by Schaffler et al., 1993 and Falter et al., 1994 have revealed the absence of any group-specific differences in the concentrations of
hemoglobin-TSNA adducts determined in smokers and non-smokers. Moreover, highly sensitive techniques such as $^{32}$P-postlabeling and immunoassays used in most of the studies involving DNA- and protein-carcinogen adducts measurements often lack chemical specificity. For immunoassays, antibodies raised against a particular DNA adduct often cross-react with other adducts of the same class. Moreover, the accuracy of quantification using both these techniques is uncertain. This difficulty is encountered particularly in the case of $^{32}$P-postlabelling as discussed by Poirier et al., 2000, Phillips, 1996 and Wild and Pisani, 1998. Besides, identification of adducts detected using $^{32}$P-postlabelling has seldom been achieved. Moreover, unknown adducts may become 5’-phosphorylated with varying efficiencies using the $^{32}$P-postlabelling technique, resulting in underestimation of adduct concentrations in a human samples.

1.3.2. Compounds Excreted in Urine

Considering the pitfalls that one might encounter in the measurement of DNA and protein adducts, measurement of urinary biomarkers of tobacco carcinogen uptake presents a more practical option (Hecht, 2002). Urine is very easy to obtain in sufficient quantities and this can allow for quantification of even trace amounts of TSNA compounds and their metabolites excreted in urine. Although measurement of urinary compounds may not provide the most direct link with the type and risk of a particular cancer, they can provide information on the carcinogen dose and as well as the ability of an individual to activate or detoxify these compounds. Moreover, Hecht et al., 2002 have shown that some of these TSNA compounds have long terminal half-lives. This, along with the fact that tobacco
products are habitually used, provides consistent concentrations of TSNA compounds for measurement in urine.

Measurement of urinary keto and hydroxy acids might be expected to be more appropriate since these are the end products of the DNA-adduct forming α-hydroxylation pathway of NNK and NNN metabolism. However, as described by Hecht, 2002, these compounds cannot be used to measure the extent of α-hydroxylation because they are formed from nicotine as well, which is a primary constituent of tobacco products. Recently nevertheless, Stepanov et al., 2008 have suggested the use of [pyridine-D₄]NNK as a biomarker to measure the deuterium-labeled keto and hydroxyl acid specifically formed from NNK. Biomarker strategies using stable isotope labeled compounds can be expensive and challenging however.

As depicted earlier in Figure 2, conversion of NNK to NNAL is an important metabolic pathway in humans with NNAL having similar carcinogenicity as NNK (Castonguay et al., 1983a, Hecht, 1998b). A characteristic feature of NNAL metabolism is the formation of the NNAL glucuronide conjugate (NNAL-Gluc), which is the most important detoxification product of the NNK-NNAL metabolic pathway in humans and animals as described by Hecht, 1998b, Hecht et al., 1993b and Morse et al., 1990. Additionally, NNAL and its glucuronide have a longer half-life (at least 10-15 days in smokers, and about 40-45 days in users of oral tobacco) in humans when compared with most other urinary metabolites. (Hecht, 2003, Hecht et al., 2002, Carmella et al., 2009). It has been hypothesized that the difference in half-lives of NNAL and its glucuronides in smokers and non-smokers arises from NNK/NNAL being sequestered in the lungs,
possibly in β2-adrenergic receptors found in the smooth muscle of the lungs (Hecht et al., 2002). Since the initial dose of NNK to lungs is greater in smokers compared to oral tobacco users, it results in a shorter half-life among smokers because the lung acts in part as a first-pass uptake tissue in smokers (Hecht et al., 2002). Existence of a receptor mediated role has also been hypothesized for NNK/NNAL metabolism. After binding to the β-adrenergic receptors of the lungs, NNAL is released from these receptors, and is converted to the corresponding glucuronide forms. NNAL is a β-adrenergic agonist and stimulates DNA synthesis in cells via receptor-mediated release of arachidonic acid. Increased release of arachidonic acid could lead to increased production of prostaglandin E2, which is implicated in lung tumorigenesis (Hecht et al., 2002).

NNN has a similar metabolic pathway to NNK as depicted in Figure 2. However, a problem from an analytical viewpoint is that the concentrations of NNN and its metabolites detected in urine of smokers is less than 15% of the total NNAL concentrations (Stepanov and Hecht, 2005), making its measurement much more difficult. Considering all of this, measuring urinary NNAL and NNAL-Gluc have evolved as one of the most prominently studied TSNA biomarkers.

1.3.3. Other TSNA Biomarkers

In addition to NNAL and NNAL-Gluc, other TSNAs such as NNN, NAB, and NAT along with their metabolites have also been studied (Gorrod and Jacob, 1999). These compounds have been detected and quantified in urine and saliva of tobacco users. More recently, TSNAs have been quantified in plasma, which is one of the most commonly
analyzed biological fluids in clinical studies. To further study the prevalence, etiology and mechanism of tobacco carcinogenesis, measurement of TSNAs has also been extended to amniotic fluid, cervical mucus, pancreatic juice, and toenails. Details of these studies along with appropriate references have been tabulated later in Table 1 and Table 2.

1.4. Predominant Analytical Methodologies in TSNA Bioanalysis

As described earlier, the study of TSNA uptake can be related to the mechanistic and epidemiologic role of these compounds in cancer. In fact, recent studies published by Yuan et al., 2009 and Church et al., 2009 have demonstrated the relationship of NNAL to lung cancer. It thus becomes essential to accurately and reproducibly quantify these compounds in biological matrices. It is highly desirable to develop a sensitive, selective and practical bioanalytical method to measure the levels of TSNAs in clinical studies.

A significant amount of research investigating in vivo disposition and excretion of TSNAs in laboratory animals such as rats, hamsters, mice, monkeys, etc. as well as humans has been carried out. Most animal studies use some form of radioactivity measurement to study the extent of metabolite formation in radioactive samples. Radioactivity is usually measured by liquid scintillation counting, autoradiography or radioflow detection (Brittebo and Tjalve, 1980; Brittebo and Tjalve, 1981; Castonguay et al., 1983b, Castonguay et al., 1984; Castonguay et al., 1985; Meger et al., 1999; Richter et al., 2009). The extent of TSNA metabolite formation is different in animals compared to humans as reviewed by Hecht, 1998b. For this reason, discussion in this dissertation will
be focused on the bioanalytical measurement of TSNA compounds and their metabolites (mainly urinary NNAL and its glucuronide metabolite) in humans.

Depending on the mode of exposure to tobacco products (direct user versus secondary exposure), human uptake of these compounds may vary significantly. Thus, different situations would demand different levels of assay sensitivity, different selectivity and validation. Three main aspects for consideration of a bioanalytical method include sample preparation, compound detection and throughput (Evans, 2004; Venn, 2000). Sample preparation involves extraction of the compound from the biological tissue, usually involving a concentration step to enhance assay sensitivity. Detection of the compound usually follows chromatographic separation from other components of the biological extract. The two most predominant techniques for TSNA bioanalysis are gas chromatography coupled with thermal energy analysis and liquid chromatography coupled with mass spectrometry employing various sample preparation techniques. The following two sections will discuss the progress and development in the analysis of TSNAs using both these techniques along with the most important analytical challenges.

1.4.1. Thermal Energy Analysis (TEA) Detection

1.4.1.1. Principle of Operation

Chemiluminescence detectors are one of the most selective of GC detectors available. One version of this detector which was originally manufactured by Thermedics Corporation (Chelmsford, MA, USA) is referred to as the thermal energy analyzer (TEA) which is a registered trademark (Beveridge, 1998). The TEA detection technique for N-
nitroso compounds was first reported more than three decades ago (Fine et al., 1973; Fine et al., 1974; Fine et al., 1975b). Subsequently, the technique was utilized for the analysis of seven TSNA compounds in tobacco and tobacco smoke (Adams et al., 1983; Brunnemann et al., 1987; Brunnemann and Hoffmann, 1981; Djordjevic et al., 1989; Hoffmann et al., 1979). The principle of the TEA detector has been discussed by Fine et al., 1975a. Briefly, the sample peak elutes from a GC column into a pyrolyzer in the TEA maintained at a temperature in excess of 275 °C which ruptures the compounds containing nitro or nitroso group to release the nitrosyl (NO\textsuperscript{1}) radical. Other organic compounds, solvents, and fragmentation products are removed using a cold trap. Nitrosyl radicals are then oxidized with ozone (formed by high voltage electric discharge of 7.5 kilo volts across a 1-mm glass dielectric in oxygen) in a reaction chamber to produce electronically excited NO\textsubscript{2}{*}. The NO\textsubscript{2}{*} decays back to the ground state emitting light in the near infrared region of the spectrum through the process of chemiluminescence. A photomultiplier then counts the corresponding photons which are proportional to the amount of NO moieties present.

**1.4.1.2. TSNA Analysis Using TEA Detection**

Some of the first reports of the occurrence of TSNAs in human biological matrices utilized either GC or LC hyphenated with TEA for analysis (Hoffmann and Adams, 1981; Nair et al., 1985). These methods measured the concentrations of NNN, NAB, NAT and NNK in the saliva of betel quid chewers and snuff dippers. Pioneering work that demonstrated the presence of metabolites of TSNAs in the urine of smokers also used GC coupled with TEA (Carmella et al., 1993). This study quantified the levels of NNAL and
its glucuronide in 24-hour urine samples of smokers. The concentrations of these metabolites were detected in quantities of 0.23-1.0 and 0.57-6.5 μg/24 hr respectively. Subsequently, the first report of the presence of NNAL and its glucuronide in the urine of non-smokers exposed to sidestream cigarette smoke was published (Hecht et al., 1993a). Both these methods were based on a modification of a previously published method for the analysis of TSNA in indoor air using GC coupled with TEA (Brunnemann et al., 1992). An outline of the sample preparation procedure for quantitation of NNAL and NNAL-Gluc that was first developed (Carmella et al., 1993) is depicted in Figure 3. As seen in Figure 3, the sample preparation was extensive, and required extraction of large volumes of urine (100 mL) with ethyl acetate. Artifactual nitrosamine formation was prevented by the addition of either ammonium sulfamate or sodium hydroxide to urine at the time of collection. Fraction 1 contained unconjugated NNAL. The aqueous portion of urine was then subjected to incubation with β-glucuronidase for 16 hour at 37°C to convert NNAL-Gluc to free NNAL. The NNAL released was further enriched by subsequent extraction steps. The procedure involved two liquid chromatographic steps for purification of the extracts. The secondary hydroxyl group was then transformed to a trimethylsilyl ether derivative. Silylation is commonly done to increase the volatility of the analytes and therefore improve their GC properties for more efficient separation (Blau and Halket, 1993). This derivative was finally subjected to GC analysis with TEA detection. For more than a decade following its publication, almost every study quantifying urinary TSNAs, mainly metabolites of NNK, i.e. NNAL and NNAL-Gluc utilized this method. Most of these studies were based on minor modifications of the method described above by
Carmella et al., 1993 and Hecht et al., 1993a. Some of these changes included a modified internal standard, decreased volume of urine analyzed, morning versus 24-hr urine samples, etc. The use of iso-NNAL instead of [5-\(^3\)H]NNAL or [5-\(^3\)H]NNAL-Gluc as the internal standard enabled quantification of both analyte and internal standard in the same chromatogram and eliminated the need for scintillation counting. Carmella et al., 1995 showed the absence of any significant difference in results in the quantification of NNAL and its glucuronide conjugate for different urine collection protocols (e.g. morning versus 24 hour samples).

Carmella et al., 1995 also attempted to use normal and reverse phase solid phase extraction cartridges to replace the HPLC purification steps. However, these attempts were unsuccessful because the GC-TEA chromatograms obtained on these samples had unacceptably high background. In a work published by Parsons et al., 1998, the sensitivity of the GC-TEA for urinary NNAL was improved nearly 20 fold through the use of capillary GC. Consequently, the urine aliquot size required for analysis was reduced to 50 mL. Anderson et al., 2001 were able to carry out the GC-TEA assay with an even reduced aliquot size of 20 mL for non-smokers and 5 mL for smokers for the analysis of urinary NNAL and NNAL-Gluc in women exposed to environmental tobacco smoke (ETS). Carmella et al., 2002 were successfully able to separately analyze NNAL-O-Gluc and NNAL-N-Gluc. However, the procedure was very time consuming and complex.
Figure 3: First developed extraction protocol for analysis of urinary NNAL and its glucuronide conjugate using GC-TEA

(Adopted from Carmella et al., 1993b)
The extensive sample preparation procedure for analysis of NNAL using GC with TEA detection was finally simplified by Carmella et al., 2003. The method was streamlined by introducing an acid partitioning step and omission of one of the HPLC purification steps. The pyridine ring on NNAL was protonated by adjusting the pH to 2-3. This allowed it to remain in the aqueous phase whereas relatively non-polar neutral and acidic organic compounds were extracted into CH₂Cl₂ and discarded. The traditional method for analysis of NNAL using GC-TEA published by Carmella et al., 1995 typically analyzed ten urine samples in an analytical run. Considering the extensive sample preparation, 24 hour enzyme hydrolysis and derivatization, followed by chromatographic run times of 20 minutes meant that the entire assay followed by data analysis could take up to a week to complete. In the simplified procedure developed by Carmella et al., 2003 described above, the authors reported that the speed of analysis was almost two fold faster than the traditional method. However, a disadvantage of the method was that NNAL and NNAL-Gluc were not distinguished in this assay. This was because the partition step using ethyl acetate to separate free and conjugated NNAL was omitted. Rather, the sample was directly hydrolyzed using β-glucuronidase. Free and conjugated NNAL can however be easily determined with this method by carrying out the analysis with and without the β-glucuronidase enzyme hydrolysis step, then determining NNAL-Gluc by the difference between total and free NNAL concentrations.

In order to study the tumorigenic contributions of the two enantiomers of NNAL, viz. (R)-NNAL and (S)-NNAL, Carmella et al., 1999 had used chiral stationary phase (CSP) GC-TEA for analysis. The assay procedure was similar to the traditional assay for
GC-TEA analysis of NNAL, except that GC column was replaced by a β-cyclodextrin chiral selector GC column. Using CSP-GC-TEA, baseline resolution of (S)- and (R)-NNAL-TMS standards, as well as (S)- and (R)-iso-NNAL internal standard, was achieved. The chromatographic run time of the assay was a long 100 minutes however. This procedure was successfully used by Hecht et al., 2002 to study the stereoselective receptor binding of NNAL.

Apart from urine, NNAL and NNK have also been quantified in other biological matrices such as plasma using GC combined with TEA analysis as suggested by Hecht et al., 1999. Analysis was done starting with a large volume of blood (5-10 mL). After alkalizing plasma with 0.1 N NaOH, it was neutralized and extracted three times with CH₂Cl₂. Further analysis of NNAL and NNK followed a similar procedure to the traditional urine method. A sample preparation technique using supercritical fluid extraction for determining NNK in cervical mucus and NNN, NNK and NNAL in pancreatic juice was published by Prokopczyk et al., 1997 and Prokopczyk et al., 2002 respectively. The method involved extraction using carbon dioxide containing 10% methanol followed by GC separation and nitrosamine selective detection using TEA. However, extraction of liquid samples (particularly biological fluids such as urine, blood and saliva) is often reported to be very difficult or impossible using supercritical fluid extraction (Zougagh et al., 2004).

A listing of the literature compiling the studies which analyzed NNAL and its metabolites along with other TSNA compounds in biological matrices using nitrosamine specific TEA detection and quantitation is provided in Table 1.
Table 1: Compilation of studies analyzing and quantifying TSNA compounds in biological matrices using TEA detection

<table>
<thead>
<tr>
<th>TSNA Analyzed</th>
<th>Study Population/Study Type</th>
<th>Sample Matrix</th>
<th>Analytical Method</th>
<th>Concentrations Measured</th>
<th>Sample Preparation and Remarks</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>NNN, NAT, NNK</td>
<td>Snuff dippers (4F)</td>
<td>Saliva</td>
<td>HPLC-TEA</td>
<td>5.0-125.0 ng/g (NNN); 2.1-201.0 ng/g (NNK); 6.6-147.0 ng/g (NAT)</td>
<td>~0.8 to 1.6 g saliva collected for analysis. No further assay description provided.</td>
<td>Hoffmann and Adams, 1981</td>
</tr>
<tr>
<td>NNN, NAT, NAB, NNK</td>
<td>Snuff dippers</td>
<td>Saliva</td>
<td>GC-TEA</td>
<td>Concentrations of TSNA measured ranged from 20-890 μg/kg</td>
<td>Assay description not provided</td>
<td>Hoffmann et al., 1982</td>
</tr>
<tr>
<td>NNN, NAT, NNK</td>
<td>Betel quid chewers</td>
<td>Saliva</td>
<td>GC-TEA</td>
<td>1.6-59.7 ng/g (NNN); 1.0-51.7 ng/g (NNK); 0.0-2.3 ng/g (NAT)</td>
<td>Assay description not provided</td>
<td>Nair et al., 1985</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>11 Smokers (9F) 7 Non-smokers</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>0.23-1.0 μg/24 hrs (NNAL); 0.57-6.5 μg/24 hrs (NNAL-Gluc). NNAL not detected in non-smokers. NNK not detected in urine.</td>
<td>Purification of extracts using two preparative HPLC steps, followed by derivatization of 2’ OH group of NNAL to trimethylsilyl ether, followed by GC-TEA analysis. Internal standard was [5-3H (NNAL)]. Conversion of NNAL-Gluc to the unconjugated form achieved by incubation with β-glucuronidase enzyme.</td>
<td>Carmella et al., 1993</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Non-Smokers exposed to sidestream cigarette smoke (5M)</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>33.9 ±20.0 ng/24 hrs (NNAL+NNAL-Gluc)</td>
<td>Sample preparation was based on Carmella et al., 1993 with a modified internal standard (iso-NNAL).</td>
<td>Hecht et al., 1993a</td>
</tr>
<tr>
<td>NNK, NNAL, NNAL-Gluc</td>
<td>Sudanese snuff-dippers (7M)</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>0.39 ±0.14 nmol/ml NNAL; 0.88±0.5 nmol/ml NNAL-Gluc</td>
<td>Method was based on Carmella et al., 1993, with minor modifications.</td>
<td>Murphy et al., 1994</td>
</tr>
<tr>
<td>NNAL, NNAL-O-Gluc</td>
<td>Smokers (30M, 31F). Study to investigate intra- and interindividual differences in</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>0.08-7.2 pmol/mg creatinine NNAL. 0.16-19.0 pmol/mg creatinine NNAL-Gluc</td>
<td>Method was based on Carmella et al., 1993, with minor modifications. 50-100 mL aliquot size. Internal standard was iso-NNAL. Attempts to use normal and reverse</td>
<td>Carmella et al., 1995</td>
</tr>
<tr>
<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured</td>
<td>Sample Preparation and Remarks</td>
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<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Smokers (5M, 6F). Study to investigate the effect of watercress consumption on metabolism of TSNAs</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Average baseline concentrations of NNAL+NNAL-Gluc were 3.28±1.88 nmol/24 hr; Average concentrations on the days of watercress consumption were 4.21±2.58 nmol/24 hrs</td>
<td>- Assay results demonstrated 6 month stability for NNAL and NNAL-Gluc stored at -20 °C. - No significant difference was found in urine collection protocols. (E.g. morning sample versus 24-hrs collection) - Detection limit of assay was reported as 1 ng per urine sample.</td>
<td>Hecht et al., 1995</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Non-tobacco users (8M). Non-smokers but smokeless tobacco users (39M). Study to investigate relations between urinary biomarkers and oral leukoplakia in smokeless tobacco users.</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>0.02-8.73 pmol/mg creatinine (NNAL); 0.14-30.3 pmol/mg creatinine (NNAL-Gluc)</td>
<td>- Method was based on Carmella et al., 1995 with minor modifications.</td>
<td>Kresty et al., 1996</td>
</tr>
<tr>
<td>NNK-N-Oxide, NNAL-N-Oxide</td>
<td>Smokers (18); Smokeless tobacco users (11)</td>
<td>Urine</td>
<td>i. GC-TEA ii. LC/ESI-MS/MS for analysis of NNAL-N-Oxide directly</td>
<td>NNK-N-Oxide not detected in urine; Concentrations of NNAL-N-oxide were 0.06-1.41 pmol/mg creatinine in smokers</td>
<td>i. 20 to 45 mL aliquot size. - Method involved solvent extraction and purification by HPLC to separate NNAL-N-Oxide and NNK-N-Oxide from endogenous NNAL and NNK. NNAL-N-Oxide and NNK-N-Oxide</td>
<td>Carmella et al., 1997</td>
</tr>
<tr>
<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured</td>
<td>Sample Preparation and Remarks</td>
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<tr>
<td>NNK</td>
<td>Smokers (15F), Non-smokers (10F). Study to identify NNK in cervical mucus.</td>
<td>Cervical mucus</td>
<td>Supercritical fluid extraction followed by: i. GC-TEA ii. Capillary GC-MS/MS</td>
<td>Mean concentrations of NNK in cervical mucus of smokers 46.9±32.5 ng/g; Mean concentrations of NNK in cervical mucus of non-smokers 13.0±9.3 ng/g; Supercritical fluid extraction of cervical mucus (31-615 mg) with the use of carbon-dioxide that contained 10% methanol. This was followed by analysis by GC-TEA and GC-MS/MS. d3-NNK was used as the internal standard.</td>
<td>being thermally unstable were not amenable to direct GC-TEA analysis. Thus, they were reduced to free NNAL and NNK respectively using Proteus mirabilis. NNAL and NNK were then analyzed by method suggested by Carmella et al., 1995. iso-NNAL-N-Oxide was used as the internal standard ii. For LC-ESI-MS/MS, 500 mL urine aliquot size was used. Following solvent extraction and HPLC purification, the samples were concentrated and analyzed using reverse phase LC-ESI-MS/MS.</td>
<td>Prokopczyk et al., 1997</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Smokers (13F) Study to investigate concentrations of NNK metabolites before and after indole-3-carbinol (I3C) treatment.</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Mean difference in NNAL+NNAL-Gluc concentrations before and after I3C treatment was -0.43±0.16 pmol/mg creatinine.</td>
<td>Method was based on Carmella et al., 1995</td>
<td>Taioli et al., 1997</td>
</tr>
<tr>
<td>NNAL, NNAL-</td>
<td>Smokers (34 black and 24 white). Study</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Black smokers: 1.22±1.44 pmol/mg</td>
<td>Method based on Carmella et al., 1995</td>
<td>Richie et al., 1997</td>
</tr>
<tr>
<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
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<td>Gluc</td>
<td>to investigate differences in NNK metabolites in black and white smokers.</td>
<td></td>
<td></td>
<td>creatinine (NNAL), 4.24±4.56 pmol/mg creatinine (NNAL-Gluc); White smokers: 0.603±0.345 pmol/mg creatinine (NNAL), 3.13±2.44 pmol/mg creatinine</td>
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<tr>
<td>NNAL-Gluc</td>
<td>Non-smokers (5M, 4F). Study to analyze concentrations of urinary NNAL-Gluc in non-smokers exposed to environmental tobacco smoke.</td>
<td>Urine</td>
<td>i. GC-TEA ii. GC-MS/MS (for identity confirmation)</td>
<td>Mean NNAL-Gluc concentrations 0.059±0.028 pmol/mL (23 pg/mL urine)</td>
<td>50 mL aliquot size GC-TEA Method was based on Carmella et al., 1995 with minor modifications. Use of capillary GC improved sensitivity 20 fold (LOD 4 fmol/mL). iso-NNAL was used as internal standard.</td>
<td>Parsons et al., 1998</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>27 Subjects (smokers) participating in a smoking cessation study</td>
<td>NNAL and NNAL-Gluc in urine, NNK and NNAL in plasma</td>
<td>i. GC-TEA ii. GC-MS/MS (for identity confirmation)</td>
<td>Urinary concentrations: Baseline levels - 0.6±0.366 pmol/mL (NNAL), 1.35±0.738 pmol/mL (NNAL-Gluc). 6 weeks after cessation, 7.6% of original NAL+NNAL-Gluc remained. Plasma concentrations: NNK was not detected in any of the samples. NNAL was detected in CH₂Cl₂ extracts of unhydrolyzed plasma from three of the four smokers analyzed. Concentrations of</td>
<td>Method to determine urinary NNAL and NNAL-Gluc was based on minor modifications of Carmella et al., 1995 and Parsons et al., 1998. For plasma analysis, 5-10 mL blood aliquots were drawn. RBC and plasma were separated. The RBC pellet was lysed with water, and extracted twice with hexane. The hexane extract was discarded and the aqueous phase was extracted thrice with CH₂Cl₂, and the combined extracts were analyzed for unconjugated NNAL and NNK as described for urine. The plasma phase treated with 0.1 N NaOH, with sonication for 1 hr at room temperature. After neutralization, it was extracted three times with equal volumes of CH₂Cl₂ and then analyzed for NNAL</td>
<td>Hecht et al., 1999</td>
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<tr>
<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured</td>
<td>Sample Preparation and Remarks</td>
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<td>NNAL, NNAL-Gluc</td>
<td>Newborns (31 from newborns whose mothers smoked and 17 from newborns whose mothers did not smoke)</td>
<td>Urine</td>
<td>i. GC-TEA ii. GC-MS/MS (for identity confirmation)</td>
<td>Mean concentration of 0.14 pmol/mL (NNAL+NNAL-Gluc) in newborns whose mothers smoked. Not detected in newborns whose mothers did not smoke.</td>
<td>Method was based on Carmella et al., 1993 and Parsons et al., 1998 with minor modifications</td>
<td>Lackmann et al., 1999</td>
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<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Pregnant mothers (21 smokers, 30 non-smokers)</td>
<td>Amniotic fluid (AF)</td>
<td>i. GC-TEA ii. GC-MS/MS (for identity confirmation)</td>
<td>Mean concentrations in AF of mothers who smoked: 0.025±0.029 pmol/mL (NNAL), 0.0032±0.01 pmol/mL (NNAL-Gluc); Mean concentrations in AF of non-smoker mothers: 0.0018±0.0074 pmol/mL (NNAL), 0.0069±0.03 pmol/mL (NNAL-Gluc);</td>
<td>Method based on Lackmann et al., 1999 with certain modifications. Further assay details were not provided.</td>
<td>Milunsky et al., 2000</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Heavy smokers (13F, 10 M). Study to determine effects of smoking cessation</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Average 25% reduction in total NNAL concentration observed at week 24 after smoking cessation</td>
<td>Method was based on Carmella et al., 1993 and Carmella et al., 1995</td>
<td>Hurt et al., 2000</td>
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<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured</td>
<td>Sample Preparation and Remarks</td>
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<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Non-smoker (23F whose partners smoked, 22F whose partners did not smoke). Study to measure environmental TSNA in women exposed to ETS</td>
<td>Urine</td>
<td>i. GC-TEA i. GC-MS/MS for identity confirmation</td>
<td>Geometric means in women whose husbands smoked: 0.013 pg/mg creatinine (NNAL), 0.027 pg/mg creatinine (NNAL-Gluc); Geometric means in women whose husbands did not smoked: 0.004 pg/mg creatinine (NNAL), 0.004 pg/mg creatinine (NNAL-Gluc)</td>
<td>Method based on Carmella et al., 1993; Hecht et al., 1993a and Parsons et al., 1998 with some modifications. 20 mL urine aliquots from non-smokers and 5 mL aliquots from smokers were sufficient for analysis.</td>
<td>Anderson et al., 2001</td>
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<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Children (38 – exposure to ETS reported, 35 – no exposure to ETS reported)</td>
<td>Urine</td>
<td>i. GC-TEA ii. GC-MS/MS for confirmation of NNAL-Gluc identity.</td>
<td>Mean concentration in children who reported exposure: 0.04 pmol/mL (NNAL+NNAL-Gluc); Mean concentration in children who did not report exposure: 0.008 pmol/mL (NNAL+NNAL-Gluc)</td>
<td>Method was based on Hecht et al., 1993a and Parsons et al., 1998 with some modifications. 20 mL aliquot size was sufficient for analysis. Detection limit of NNAL+NNAL-Gluc was reported as 0.003 pmol/mL.</td>
<td>Hecht et al., 2001</td>
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<td>NNAL, NNAL-Gluc</td>
<td>Non-smokers, smokeless tobacco users (13M). Participating in smokeless tobacco use cessation study</td>
<td>Urine, Plasma</td>
<td>i. GC-TEA ii. GC-MS/MS for identity confirmation</td>
<td></td>
<td>Method to measure NNAL and NNAL-Gluc in urine was based on Hecht et al., 1999. Enantiomers of NNAL and diastereomers of NNAL-Gluc were analyzed using CSP-GC-TEA. For plasma analysis, 5 mL aliquot size was used. Plasma analysis was based on method suggested by Hecht et al., 1999. Detection limit in plasma was 0.01 pmol/mL.</td>
<td>Hecht et al., 2002</td>
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<tr>
<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured</td>
<td>Sample Preparation and Remarks</td>
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<td>NNAL, NNAL-O-Gluc, NNAL-N-Gluc</td>
<td>Smokers (6M, 4F); Snuff dippers (10M); Toombak users (4M)</td>
<td>Urine</td>
<td>i. GC-TEA for analysis of smokers and snuff dippers urine ii. LC-ESI-MS/MS (for identity confirmation of NNAL-N-Gluc in urine of toombak users)</td>
<td>Mean concentrations in smokers: 0.462±0.214 pmol/mL (NNAL), 0.322±0.161 pmol/mL (NNAL-N-Gluc), 0.434±0.343 pmol/mL (NNAL-O-Gluc) Mean concentrations in snuff dippers: 1.48±1.13 pmol/mL (NNAL), 0.59±0.60 pmol/mL (NNAL-N-Gluc), 2.13±2.55 pmol/mL (NNAL-O-Gluc) Mean concentrations in toombak users: 354.8±187.2 pmol/mL (NNAL), 32.6±17.4 pmol/mL (NNAL-N-Gluc), 231.8±264.8 pmol/mL (NNAL-O-Gluc)</td>
<td>Aliquot size was 10 mL. Extraction using C18 SPE. NNAL-O-Gluc and NNAL-N-Gluc were eluted using 10% methanol, followed by β-glucuronidase treatment (for hydrolysis of O-an N-Gluc) or base treatment (for specific hydrolysis of N-Gluc). NNAL was eluted using 50% methanol as two separate fractions. After solvent partition and HPLC purification, NNAL was quantified by GC-TEA as described by Hecht et al., 2001. iso-NNAL used as internal standard was added to urine rather than ethyl acetate extracts as described in previous assay (Carmella et al., 1995) for better quantitation. NNAL-N-Gluc in toombak users was confirmed using C18-SPE followed by LC-ESI-MS/MS using graphite stationary phase for HPLC. 1 mL aliquots was used</td>
<td>Carmella et al., 2002</td>
</tr>
<tr>
<td>NNN, NNK, NNAL</td>
<td>smokers (18) and non-smokers (9)</td>
<td>Pancreatic juice</td>
<td>i. Supercritical fluid extraction followed by GC-MS/MS for quantitation of NNN and NNK i. Supercritical fluid extraction</td>
<td>Smokers: 0-68.1 ng/mL (NNN), 0-604 ng/mL (NNK) was detected; Non-Smokers: NNN was not detected, 0-96.8 ng/mL (NNK); NNAL was identified in 8 out of 15 smoker samples and 3 out of 9 non-smoker samples.</td>
<td>Aliquot size was ~300 μL. SFE with the use of carbon-dioxide that contained 10% methanol. This was followed by analysis by GC-MS/MS. d4-NNK was used as the internal standard. NNAL was identified but not quantified.</td>
<td>Prokopczyk et al., 2002</td>
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<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured</td>
<td>Sample Preparation and Remarks</td>
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<tr>
<td>Total NNAL (i.e. sum of NNAL and NNAL-Gluc)</td>
<td>Smokers (41), Snuff dippers (55), Non smokers exposed to ETS (18)</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Concentrations of total NNAL (pmol/mg creatinine) were 2.60±1.30 in smokers, 3.25±1.77 in snuff-dippers, and 0.042±0.020 in nonsmokers exposed to ETS</td>
<td>NNAL and NNAL-Gluc were not distinguished in this assay. Urine treated with β-glucuronidase, followed by solvent partitioning and further purification on a liquid-liquid extraction cartridge and by high-performance liquid chromatography. Total NNAL was silylated and finally quantified by GC-TEA. Acid partitioning step produced cleaner samples, Internal standard iso-NNAL was added directly to urine to improve accuracy. Detection limit for the assay was 0.1 pmol/mL starting with a 5 mL aliquot.</td>
<td>Carmella et al., 2003</td>
</tr>
<tr>
<td>Total NNAL (i.e. sum of NNAL and NNAL-Gluc)</td>
<td>Non-smokers exposed to ETS (13F, 3M)</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Mean concentrations of total NNAL prior to ETS exposure was 0.02±0.02 pmol/mg creatinine. After ETS exposure, the mean Total NNAL concentrations increased by 0.018 pmol/mg creatinine</td>
<td>Method based on Hecht et al., 1993a; Parsons et al., 1998 and Carmella et al., 1993 with some modification</td>
<td>Anderson et al., 2003</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Smokers (151). Study to determine effects of cigarette cessation.</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Subjects who reduced smoking by 70% achieved approximately 50% reduction in total NNAL concentration by</td>
<td>Method based on Carmella et al., 1995 and Hecht et al., 1999. Urine aliquots used for analysis were 4.5 mL.</td>
<td>Hecht et al., 2004b</td>
</tr>
<tr>
<td>TSNA Analyzed</td>
<td>Study Population/ Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured weeks 8-12.</td>
<td>Sample Preparation and Remarks</td>
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<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Cigarette smokers (74 M, 10 F). Study to investigate effect of cruciferous vegetable consumption on metabolism of NNK</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Range of NNAL concentration determined in urine was 0-3.62 pmol/mg creatinine while that of NNAL-Gluc was 0-5.07 pmol/mg creatinine. Association was shown between cruciferous vegetable intake and urinary NNAL, NNAL-Gluc and total NNAL concentrations</td>
<td>• Based on method described by Carmella et al., 1995.</td>
<td>Hecht et al., 2004a</td>
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<tr>
<td>Total NNAL</td>
<td>Cigarette smokers (38), Smokeless tobacco users (41). Investigation to study effects of reduced-exposure tobacco products or medicinal nicotine on tobacco associated carcinogens</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>Mean total NNAL concentration of the “reduced risk” cigarette group was 1.9 pmol of NNAL/mg of creatinine, while the nicotine patch group had concentration of 1.2 pmol of NNAL/mg of creatinine.</td>
<td>• Method as described by Carmella et al., 2003</td>
<td>Hatsukami et al., 2004</td>
</tr>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>46 Cigarette smokers. This study investigated the relationships of Urinary biomarkers of Tobacco and Carcinogen exposure in smokers</td>
<td>Urine</td>
<td>GC-TEA</td>
<td>The range of total NNAL measure was 0.9 to 54 pmol/mL with a mean value of 2.66±1.22 pmol/mL of urine.</td>
<td>• Based on method described by Hecht et al., 1999 and Carmella et al., 1995</td>
<td>Murphy et al., 2004</td>
</tr>
<tr>
<td>NNN, NNN-Gluc,</td>
<td>14 Smokers and 11 smokeless tobacco users.</td>
<td>Urine</td>
<td>i. GC-TEA ii. GC-MS/MS (for identity)</td>
<td>Mean concentrations of total NNN, NAT, and NAB in smokers were</td>
<td>• Total NNN (NNN plus NNN-N-Gluc) was assayed using 5-methyl-NNN as internal standard.</td>
<td>Stepanov and Hecht, 2005</td>
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<td>TSNA Analyzed</td>
<td>Study Population/ Study Type</td>
<td>Sample Matrix</td>
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</table>
| NAT, NAT-Gluc, NAB, NAB-Gluc, | First study to show presence of urinary NNN, NNN-Gluc, NAT, NAT-Gluc, NAB and NAB-Gluc in tobacco users. | | confirmation) | (pmol/mg creatinine) 0.18 ± 0.22, 0.19 ± 0.20, and 0.040 ± 0.039 respectively, whereas the corresponding amounts in the urine of 11 smokeless tobacco users were 0.64 ± 0.44, 1.43 ± 1.10, and 0.23 ± 0.19, respectively. | - 36 mL aliquot size was used.  
- Urine was treated with β-glucuronidase. Following solvent partitioning and and two SPE steps, total NNN was determined using GC-TEA.  
- Total NAT and total NAB were quantified in the same samples using GC-TEA.  
- Separate quantitation of NNN, NNN-N-Gluc, NAT, NAT-N-Gluc, NAB, and NAB-N-Gluc was accomplished by extraction of the urine with ethyl acetate before beta-glucuronidase hydrolysis. NNN, NAT and NAB was analyzed in the ethyl acetate extract. NNN, NAB and NAT released from the glucuronide conjugate was quantified in the extracted urine after enzyme treatment.  
- The detection limits of the method were 0.032 pmol/mL urine for NNN, 0.014 pmol/mL urine for NAT, and 0.018 pmol/mL urine for NAB. | Muscat et al., 2005 |
| NNAL, NNAL-Gluc and total NNAL | 69 Black and 93 White smokers. Study to investigate racial differences in exposure and glucuronidation of the NNK. | Urine | GC-TEA | The geometric mean concentrations of urinary NNAL, NNAL-Gluc and total NNAL (pmol/mg creatinine) detected were:  
i. 0.6, 1.9 and 2.6 in Black men and 0.45, 1.4 and 1.9 in White men respectively  
ii. 0.76, 1.9 and 2.7 in Black women and 0.7, 2.4 and 3.2 in White women respectively | Based on method described by Carmella et al., 1995 | Muscat et al., 2005 |
<table>
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<th>TSNA Analyzed</th>
<th>Study Population/Study Type</th>
<th>Sample Matrix</th>
<th>Analytical Method</th>
<th>Concentrations Measured</th>
<th>Sample Preparation and Remarks</th>
<th>Reference</th>
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</table>
| Total NNAL    | 20 Non-smokers (6M, 14F). Study to investigate concentration of total-NNAL in non-smokers exposed to smoke in restaurants and bars. | Urine       | GC-TEA           | Mean difference (SD) in total NNAL concentration before and after exposure to smoke was 0.033 (0.034) pmol/mL. | • Based on method described by Carmella et al., 2003  
• Total NNAL could not be determined in one subject recruited in the study because of co-eluting peaks, suggesting potential selectivity problems with the GC-TEA method.  
• Detection limit of the assay reported was 0.01-0.07 pmol/mL based on recovery. | Tulunay et al., 2005 |
| Total NNAL    | 400 participants in study to investigate relationships between cigarette consumption and biomarkers of tobacco toxin exposure. | Urine       | GC-TEA           | The range of total NNAL measurements was 0 to 23.9 pmol/mg creatinine. | • Based on method described by Hecht et al., 1999 and Carmella et al., 1995. | Joseph et al., 2005 |
| Total NNAL    | 144 infants between the ages of 3-12 months. Study to investigate concentrations of total NNAL in the urine of infants exposed to ETS. | Urine       | GC-TEA           | Mean concentrations of total NNAL in the 144 infants were 0.083 ± 0.200 pmol/mL. | • Total NNAL was analyzed as described in method by Carmella et al., 2003 except that HPLC purification step was replaced by purification on a mixed mode cation exchange SPE cartridge as described by Carmella et al., 2005.  
• 5-(methylnitrosamino)-1-(3-pyridyl)-1-pentanol was used as internal standard.  
• Detection limits for NNAL were calculated for each sample based on urine volume and recovery and ranged from 0.09-0.36pmol/mL | Hecht et al., 2006 |
| Total NNAL    | 80 children ages 5 to 10 years from areas of Moldova. The study | Urine       | GC-TEA           | Mean ± SD level of total NNAL was 0.09 ± 0.077 pmol/mL. | • Analysis was based on method described by Hecht et al., 2001 and Hecht et al., 1999 with some modifications.  
• C5-NNAL was used as the internal standard. | Stepanov et al., 2006b |
<table>
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<th>TSNA Analyzed</th>
<th>Study Population/Study Type</th>
<th>Sample Matrix</th>
<th>Analytical Method</th>
<th>Concentrations Measured</th>
<th>Sample Preparation and Remarks</th>
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<td>investigated uptake of NNAL by Moldovan children.</td>
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<td>standard</td>
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1.4.1.3. Problems with GC-Thermal Energy Analysis

Most of the studies conducted prior to 2003 used GC with TEA detection to quantify TSNAs. Although TEA has been highly instrumental in advancing our knowledge in the TSNA field, many laboratories cannot justify the use of such a highly specialized detector if they do not have a regular application. Secondly, the methods based on GC/TEA can require very high sample volumes. This can be a limitation, especially if urine or plasma is designated for multiple types of assays, and the quantity available is limited. Also, the sample preparation is highly complex including multiple extraction and purification steps, followed by 24-hour enzyme hydrolysis in the case of measuring conjugated compounds (note: this is a common step for LC/MS/MS based methods discussed later) and finally there is a derivatization step. Moreover, for most cases elution times are in excess of 15 minutes in order to get good separation of the target compounds. This significantly affects the throughput of the assays which can typically take almost a week to complete sample preparation, chromatography and data analysis for a batch of samples. It should be noted that with advancement in technology and experience over the years, the current state of the art has substantially improved throughput of GC-TEA based methods. Currently, however, there are no publications that have described validation of GC-TEA methods based on the widely used FDA guidelines (US Food and Drug Administration, 2001). The ability to validate these methods according to accepted standards becomes especially important with the recent legislation that gives the U.S. FDA authority to regulate tobacco (Curfman et al., 2009). Another major disadvantage of GC-TEA is its inability to distinguish co-eluted nitroso compounds even though it is nitroso...
specific as reported by Wu et al., 2008. Morcos and Wiklund, 2001 and Meulemans and Delsenne, 1994 have reported the presence of nitrates and nitrites in human urine which can be a potential source of interference as reported by Fine et al., 1975b. In fact, in the study published by Tulunay et al., 2005, total NNAL could not be determined in one subject recruited in the study because of co-eluting peaks. This suggests potential selectivity problems with the GC-TEA method. Moreover, for thermally unstable compounds, GC-TEA might not be the best method of choice. Consequently, there has been a recent trend towards the use of more widely available LC/MS/MS analytical technique for the analysis of TSNAs.

1.4.2. **LC-ESI-MS/MS**

One of the chief causes that precludes complete validation of GC-TEA based methods for analysis of tobacco specific nitrosamines is limited throughput due to extensive and complex sample extraction steps. The need for high throughput quantitative determination of analytes in biological matrices has been largely fulfilled with the modern development of liquid chromatography coupled with tandem mass spectrometry. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is one of the most prevalent hyphenated techniques that have been adopted for fast and cost-effective analytical methods. It has led to major breakthroughs in the field of quantitative bioanalysis for almost two decades due to its inherent selectivity, sensitivity, and speed (Xu et al., 2007). A mass detector is highly selective by virtue of its ability to isolate and quantify specific ions. It is now generally accepted as the preferred technique for quantitative analysis of
small molecule drugs, metabolites, and other xenobiotics in biological matrices such as plasma, blood, serum, urine, and tissue.

The basic principle of mass spectrometric analysis involves production of ions of the compound to be analyzed by inducing either a loss or gain of a charge from a neutral species. Once formed, the ions are electrostatically directed into the mass analyzer where they are separated and detected according to their mass-to-charge (m/z) ratio. The four main components of most mass spectrometers are a sample inlet, ionization source, mass analyzer and ion detector (Venn, 2000). Electrospray ionization (ESI) is one of the most widely used atmospheric pressure ionization source conducive to formation of singly charged small molecules as well as multiply charged large molecules. Thus far, all published methods for TSNA bioanalysis using LC-MS/MS have utilized an ESI source. ESI operates by formation of a fine spray of highly charged droplets from a liquid sample in the presence of an electric field. Dry gas and heat, are applied to the droplets at atmospheric pressure thus causing the solvent to evaporate from each droplet. As the size of the charged droplet decreases, the charge density on its surface increases. The mutual coulombic repulsion between like charges on this surface becomes so great that it exceeds the forces of surface tension, and ions are ejected. The ejected ions are then drawn into the vacuum of the mass analyzer through a series of lenses (Hoffmann and Stroobant, 2001). The most important advantage of the ESI source is its sensitivity, easy adaptability to liquid chromatography and amenability to tandem mass analyzers such as triple quadrupoles and ion traps. However, a common problem encountered in ESI is matrix effects which can affect the quantitative performance of an assay as well as compromise
method sensitivity (Van Eeckhaut et al., 2009). The issue of matrix effects commonly encountered in LC-ESI-MS/MS methods will be discussed in more detail in the following sections and chapters of this thesis.

It is now standard practice to validate bioanalytical methods according to the bioanalytical method validation guidelines as prescribed by the FDA (US Food and Drug Administration, 2001). Validation of the method serves to ensure reliability and reproducibility of a particular method used to quantitatively measure analytes in a biological matrix. Validation involves documenting the performance characteristics of the entire method in terms of accuracy, precision, selectivity, sensitivity, reproducibility and stability employing thorough laboratory investigations. Considering the extensive and complex sample preparation procedures, method validation may not be practical in the case of TEA based methods. There is currently no published GC-TEA method published for TSNA analysis which has been fully validated according to the FDA recommended bioanalytical method validation guidelines. Thus an increasing number of LC/MS/MS based methods are being published for bioanalysis of TSNAs.

1.4.2.1. TSNA Analysis Using LC-ESI-MS/MS

Properly employed LC-ESI-MS/MS methods possess high selectivity, sensitivity, and throughput. Byrd and Ogden, 2003 have published the first fully validated assay for the determination of NNAL in urine using LC/MS/MS and a single-step SPE. The SPE was performed on a mixed-mode cation exchange cartridge. d3-NNAL was used as an internal standard for the assay. The assay was validated according to the FDA criteria, and
was found to be accurate, precise and selective. The limit of quantitation of the assay was 20 pg/mL accomplished on a Micromass Quattro Ultima triple quadrupole mass spectrometer operated in the positive ESI mode. This was sufficient for analysis of free and total NNAL in smoker’s urine using a 15 mL aliquot.

NNAL and its glucuronide have also been quantified using LC-ESI-MS/MS in biological matrices other than urine such as plasma samples of smokers. Carmella et al., 2005 used a single step mixed-mode cation exchange SPE followed by separation on a reverse phase C18 HPLC column and MS/MS detection. Considering the polar nature of NNAL, Pan et al., 2004 developed a method for determination of NNAL concentrations in plasma based on a simple liquid/liquid extraction and hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS) analysis. While reverse phase chromatography is most widely used for analysis of polar analytes, it relies on separation by the analyte’s degree of hydrophobic interaction with the stationary phase. In reverse phase chromatography, a non-polar stationary phase and a polar mobile phase are used. HILIC on the other hand is useful for separation of polar compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the aqueous content in the mobile phase (Hsieh, 2008). The highly volatile organic mobile phases such as methanol and acetonitrile used in HILIC provide increased ionization efficiencies for MS/MS detection. Moreover, the lower viscosities of these solvents lead to lower column backpressure and possibly improved column life (Dejaegher et al., 2008). The throughput of the method developed by Pan et al., 2004 for the analysis of NNAL in plasma was substantially improved with chromatographic run
times of just 1 minute. Both, the reverse phase chromatography based method and the HILIC method described above used only 1 mL of plasma compared to 5-10 mL required in previously published GC-TEA based methods. The recent shortage in availability and skyrocketing in acetonitrile prices may pose a limitation for HILIC chromatography (Majors, 2009). Acetonitrile with a small amount of water content is the most typical mobile phase for HILIC methods.

More recently, mass spectrometry based assays have been developed by Stepanov and Hecht, 2008 and Stepanov et al., 2006a for analysis of NNAL and NNN in human toenail. Some of the advantages of biomarkers in the toenail include the potential for evaluation of long-term cumulative exposure to tobacco carcinogens, steady accumulation of biomarkers due to the slow growth rate of nails and enhanced sample stability. Toenail specimens are not typically collected in most epidemiological studies however.

A listing of the literature compiling the studies analyzing and quantifying NNAL and its metabolites along with other TSNA compounds in biological matrices using LC-MS/MS is provided in Table 2.

1.4.2.2. Matrix Effects - Challenge in LC-MS/MS Methods for TSNA Analysis

An important selectivity issue which is sometimes neglected in LC-MS/MS method development is matrix effects. These are caused by alteration of ionization efficiency by the presence of co-eluting substances. These effects can often have a detrimental impact on the method accuracy and sensitivity (Annesley, 2003). The recovery experiments carried out by Byrd and Ogden, 2003 revealed an almost 50% ion suppression of the NNAL signal
### Table 2: Compilation of studies analyzing and quantifying TSNA compounds in biological matrices using LC-MS/MS

<table>
<thead>
<tr>
<th>TSNA Analyzed</th>
<th>Study Population/ Study Type</th>
<th>Sample Matrix</th>
<th>Analytical Method</th>
<th>Concentrations Measured</th>
<th>Sample Preparation and Remarks</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>NNAL, NNAL-Gluc</td>
<td>Smokers (5M, 2F)</td>
<td>Urine</td>
<td>LC-MS/MS</td>
<td>Mean concentrations in smokers ranged from 126-961ng/24 hr (NNAL), 320-2033 ng/24 hr</td>
<td>First fully validated determination of NNAL in urine using LC/MS/MS and single step mixed-mode cation exchange SPE.</td>
<td>Byrd and Ogden, 2003</td>
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<td>Incurred plasma samples (n=9)</td>
<td>Plasma</td>
<td>HILIC-MS/MS</td>
<td>Free NNAL concentrations in incurred samples ranged from 5.5 to 16.4 pg/mL, total NNAL level ranged from 6.02 to 21.5 pg/mL</td>
<td>First LC-MS/MS method for analysis of NNAL in plasma. Method comprised of liquid/liquid extraction of NNAL from 1 mL plasma aliquots, followed by analysis by HILIC-MS/MS.</td>
<td>Pan et al., 2004</td>
</tr>
<tr>
<td>Total NNAL</td>
<td>16 smokers and 5 non-smokers.</td>
<td>Plasma</td>
<td>LC-ESI-MS/MS</td>
<td>Concentrations of total NNAL averaged 42 ± 22 (SD) and ranged 1.7 to 88 fmol/mL plasma in 16 smokers; NNAL was not detected in the plasma of five non-smokers</td>
<td>Aliquot size was 1 mL [Pyridine-D4] NNAL was used as internal standard. Samples were incubated with β-glucuronidase enzyme and then subjected to mixed mode cation exchange solid phase extraction. This was followed by LC-ESI-MS/MS. NNAL eluted at 16.3 minutes.</td>
<td>Carmella et al., 2005</td>
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<tr>
<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
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<tr>
<td>NNAL, Total-NNAL</td>
<td>41 smokers exposed to sidestream smoke</td>
<td>Urine</td>
<td>LC-ESI-MS/MS</td>
<td>Following exposure to sidestream smoke, the mean concentration of total NNAL post-exposure was 24.1 pg/mg of creatinine, with a mean difference of 20.6 pg/mg of creatinine.</td>
<td>The limit of quantitation of the assay was reported as ~8 fmol total NNAL/mL plasma. The assay was evaluated for accuracy and precision. Average recovery was 28±21%.</td>
<td>Xia et al., 2005</td>
</tr>
<tr>
<td>Total NNAL</td>
<td>16 Smokers. Assay was developed for combined analysis of r-1,t-2,3,c-4-tetrahydroxy-2,3,4-Tetrahydrophenanthrene and 4- (Methylnitrosamin)-1-(3-Pyridyl)-1-Butanol in Smokers’ Plasma</td>
<td>Plasma</td>
<td>LC-ESI-MS/MS</td>
<td>Concentrations of NNAL in plasma averaged 36 ± 21 fmol/mL, which are ~1% to 2% of the amounts found in urine</td>
<td>[D10]PheT and [pyridine-D4]NNAL were used as internal standards. Plasma was treated with β-glucuronidase to release conjugated PheT and NNAL. Analytes were enriched by SPE on a mixed mode cation exchange cartridge and the PheT fraction was further purified by HPLC. The appropriate fractions were analyzed by GC-negative ion chemical ionization-MS for PheT and LC-ESI-MS/MS for NNAL. Limits of quantitation for NNAL was 3 fmol/mL. The method was evaluated for accuracy and precision.</td>
<td>Carmella et al., 2006</td>
</tr>
<tr>
<td>NNAL</td>
<td>35 Smokers and six non-smokers. Assay</td>
<td>Toenail</td>
<td>LC-ESI-MS/MS</td>
<td>Mean NNAL in smokers was 50-80 mg toenails were used. Toenails were washed with CH₂Cl₂, and</td>
<td></td>
<td>Stepanov et al.,</td>
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<tr>
<td>TSNA Analyzed</td>
<td>Study Population/ Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
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<tr>
<td>NNAL, Total NNAL</td>
<td>266 Smokers (99M, 167F) This study investigated relations between machine-derived smoke yields and biomarkers in cigarette smokers in Germany</td>
<td>Urine</td>
<td>LC-ESI-MS/MS</td>
<td>Mean concentrations of total NNAL found in smokers $1.53 \pm 1.71\text{nmol}/24\text{hrs}$</td>
<td>digested overnight in NaOH. pH was adjusted to 6-8, the aqueous toenail digest was enriched by partitioning with CH$_2$Cl$_2$ on a ChemElut liquid-liquid extraction cartridge. Final enrichment was accomplished by a mixed mode cation exchange extraction on an Oasis MCX solid-phase extraction cartridge. The fraction containing NNAL was then directly analyzed by LC-ESI-MS/MS. $^{13}$C$_6$-NNAL was used as internal standard. The detection limit of the assay for NNAL in toenails from smokers was 0.02 pg/mg toenail. The assay was evaluated for accuracy and precision.</td>
<td>2006a Scherer et al., 2007</td>
</tr>
<tr>
<td>Total NNAL</td>
<td>420 smokers and 182 smokeless tobacco users. Study to compare relations between concentrations of urinary total NNAL in smokers versus smokeless tobacco users.</td>
<td>Urine</td>
<td>Study was conducted in six parts. GC-TEA was used for studies 1 and 2 while LC-ESI-MS/MS for studies 4-6</td>
<td>Concentrations of total NNAL in smokers ranged from 2.03 to 2.35 pmol/mL, while in smokeless tobacco users, concentrations of total NNAL ranged from 3.40 to 4.21 pmol/mL</td>
<td>Studies 1 and 2 were based on method described by Carmella et al., 1995. Studies 3-6 were based on method described by Carmella et al., 2003 and Carmella et al., 2005</td>
<td>Hecht et al., 2007</td>
</tr>
<tr>
<td>Total</td>
<td>212 smokeless tobacco</td>
<td>Urine</td>
<td>The study was</td>
<td>Mean concentrations of</td>
<td>The analysis was based on methods</td>
<td>Hecht et al., 2007</td>
</tr>
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<td>TSNA Analyzed</td>
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<tr>
<td>NNAL</td>
<td>users.</td>
<td></td>
<td>conducted in three parts and analyses were either done by GC-TEA and LC-ESI-MS/MS</td>
<td>total NNAL ranged from 2.47 to 5.21 pmol/mL as the duration of daily use of smokeless tobacco increased from 0 to more than 21 years</td>
<td>described by Carmella et al., 2005, Carmella et al., 2003 and Hecht et al., 1999.</td>
<td>al., 2008</td>
</tr>
<tr>
<td>NNN</td>
<td>17 Smokers. Assay was developed to measure NNN in human toenail.</td>
<td>Toenail</td>
<td>LC-ESI-MS/MS</td>
<td>Mean total NNN level in these samples was 4.63 ± 6.48 fmol/mg toenail.</td>
<td>40-100 mg toenails were used. Toenails were washed with CH₂Cl₂, and digested overnight in NaOH. The aqueous toenail digest was enriched by partitioning with CH₂Cl₂ on a ChemElut liquid-liquid extraction cartridge. Further enrichment was accomplished by a mixed mode cation exchange extraction on an Oasis MCX SPE cartridge. Final enrichment was achieved using Bond-Elut silica SPE cartridges. The fraction containing NNAL was then directly analyzed by LC-ESI-MS/MS. ¹³C₆-NNAL was used as internal standard. The detection limit of the assay for NNN in toenails from smokers was 0.02 pg/mg toenail. The assay was evaluated for accuracy and precision.</td>
<td>Stepanov and Hecht, 2008</td>
</tr>
<tr>
<td>Total NNAL</td>
<td>73 non-smokers who suffered from chronic obstructive pulmonary disease. The assay was developed subpicogram/mL determination of NNAL in human urine using</td>
<td>Urine</td>
<td>LC-ESI-MS/MS</td>
<td>Nonsmokers who suffered from chronic obstructive pulmonary disease had mean NNAL concentration of 5.5 pg/mL</td>
<td>The method involves liquid-liquid extraction followed by conversion of NNAL to the hexanoate ester derivative. (derivatization facilitated separation from interfering urinary constituents). LLOQ was 0.25 pg/mL for 5-mL urine samples. The method was evaluated for accuracy and precision.</td>
<td>Jacob et al., 2008</td>
</tr>
<tr>
<td>TSNA Analyzed</td>
<td>Study Population/ Study Type</td>
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<tr>
<td>NNAL, Total NNAL</td>
<td>Incurred urine samples from 10 smokers. Study investigated influence of ion suppression due to sample matrix effect on the LC-MS/MS determination of NNAL.</td>
<td>Urine</td>
<td>LC-ESI-MS/MS</td>
<td>Smokers had free NNAL concentrations ranging from below LOQ to 160.4 pg/mL, while total NNAL concentrations ranging from below LOQ to 392 pg/mL.</td>
<td>The method was a modification of the assay published by Xia et al., 2005 using SPE on a MIP column combined with ESI-MS/MS. LC conditions were modified to resolve the elution of the peak of interest from the region of ionization suppression. A 25-fold improvement in response was observed with the modified method.</td>
<td>Shah et al., 2009</td>
</tr>
<tr>
<td>Total NNAL, Total NNN, Total NAB, Total NAT</td>
<td>7 Smokers and 7 non-smokers. Assay was developed for simultaneous determination of four TSNA’s in human urine</td>
<td>Urine</td>
<td>LC-ESI-MS/MS</td>
<td>Mean concentrations of NNAL, NNN, NAB and NAT in smokers were 152.5, 7.2, 47.0 and 161.1 pg/mL. NNAL was detected in 3 out of 7 non-smokers (mean level 2.68 pg/mL), while other TSNAs were not detected.</td>
<td>Aliquot size was 6 ml. The method involved simultaneous determination of four TSNA in urine. Four corresponding deuterated internal standards were added to urine followed by treatment with β-glucuronidase. SPE was carried out on a TSNA-specific MIP cartridge followe by further enrichment by SPE on a cation-exchange resin followed by LC–ESI-MS/MS. The limits of detection (LOD) were 2.0, 0.8, 1.1 and 0.7 pg/ml for NNAL, NNN, NAB and NAT, respectively.</td>
<td>Kavvadias et al., 2009</td>
</tr>
<tr>
<td>Total NNN</td>
<td>16 smokers. Assay was used for investigation of respiratory retention of NNN</td>
<td>Urine</td>
<td>LC-ESI-MS/MS</td>
<td>After smoking, average NNN concentrations were found to reach 4.0 pg/mL within one day</td>
<td>20 mL aliquot size was used. NNN-d4 was used as internal standard. After pH adjustment urine was treated with β-glucuronidase. Sample enrichment was achieved by liquid extraction on an Extrelut diatomaceous earth cartridge followed by LLE and SPE on a mixed mode cation exchange cartridge. LC-ESI-MS/MS analysis consisted of two reverse phase analytical columns in series. Chromatographic run time was 15</td>
<td>Urban et al., 2009</td>
</tr>
<tr>
<td>TSNA Analyzed</td>
<td>Study Population/Study Type</td>
<td>Sample Matrix</td>
<td>Analytical Method</td>
<td>Concentrations Measured</td>
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<td></td>
<td></td>
<td>Method was validated. LLOQ was 2 pg/mL with calibration line linear upto 256 pg/mL.</td>
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</table>
by co-eluting species from the sample matrix when compared with samples in a simple water matrix.

Matrix effects can be minimized by a modification of the sample extraction methodology and/or improved chromatographic separation from co-eluting substances (Avery, 2003). In this respect, Jacob et al., 2008 modified the sample extraction protocol to develop a method for sub-picogram per milliliter determination of NNAL in human urine that avoided matrix effects. The method relied on the derivatization of the hydroxy group of NNAL to a relatively non-polar hexanoate ester. This facilitated the chromatographic separation from potentially interfering polar urinary constituents. More efficient separation from other ionizable urinary species by extraction and chromatography reduced the extent of ion suppression which commonly occurs in ESI. Further, the hexanoate ester derivative of NNAL would have a larger mass than the parent compound. Sterner et al., 2000 have shown that larger masses are less susceptible to suppression when compared to smaller molecules. The authors reported a limit of quantitation of 0.25 pg/mL for a 5 mL sample aliquot. This is the lowest reported LOQ for NNAL quantitation published to date. The authors state that a typical run consisting of 46 samples plus 26 standards and quality control samples required 3 days to carry out the enzyme incubation, extraction and derivatization steps. Further LC-MS/MS analysis with long chromatographic run times (20 minutes) means that a typical run can take a week to complete. This hinders sample throughput, and can be a problem economically when faster turnaround times are required.

It is highly desirable to develop novel sample preparation techniques and/or sorbents capable of highly selective sample extraction and at the same time be throughput
efficient. Molecularly imprinted polymers are one such type of synthetic sorbent having predetermined selectivity for an individual analyte or a class of compounds. These are fast gaining wide utility in the field of TSNA bioanalysis as discussed in the next section.

1.5. Molecularly Imprinted Polymers – New Sample Extraction Sorbents for TSNA Bioanalysis

Solid phase extraction (SPE) techniques are one of the most useful techniques for extraction of TSNAs from biological matrices prior to quantitative analysis. SPE has significant advantages compared to traditional liquid/liquid extraction methods. The wide range of sorbents available makes this technique applicable to most classes of compounds. Typical SPE sorbents have limited selectivity, however, since they retain analytes by nonselective hydrophobic or polar interactions. This constitutes a problem when a selective extraction from a complex biological matrix needs to be performed. The analyte is retained together with other matrix compounds, which can hinder its final determination (e.g., problem of matrix effects). Because of these limitations, synthetic molecular recognition sorbent materials which can recognize target analytes have been developed, called molecularly imprinted polymers (MIPs) (Mosbach and Mosbach, 1966).

The technique of molecular imprinting was further developed by Wulff and Sarhan, 1972. This technique has been shown to be capable of producing materials with ‘antibody-like’ selectivity (Andersson et al., 1995). Molecularly imprinted polymers are extensively cross-linked polymers containing specific recognition sites that act as artificial receptors
having predetermined selectivity for an analyte or class of analytes (Allender et al., 1999; Danielsson, 2008; Pichon, 2007; Sellergren and Andersson, 2000). The process of molecular imprinting consists of the following steps: (a) The imprint is obtained by arranging polymerisable functional monomers around a template representing the target molecule, and complexes are then formed through covalent or non-covalent or semi-covalent molecular interactions between the template and monomer functional groups; (b) The complexes are assembled in the liquid phase and undergo polymerization by cross-linking. (c) Subsequent removal of the template molecule through extraction or hydrolysis with appropriate solvents gives rise to imprints or cavities which are complementary to the analyte of interest both chemically and sterically. These binding sites thus offer high affinity for the target molecule. A schematic diagram of the imprinting process described above is shown in Figure 4.

The principle of selective extraction using MIPS is similar to that of immunosorbents, and typically involves a conditioning step, followed by sample loading, a washing step, and finally desorption of the target analyte. Synthesizing MIPs having high affinity and selectivity for target analytes is a challenge. Xia et al., 2005 were successfully able to design MIPs for selective extraction of NNAL in human urine followed by LC/ESI-MS/MS detection. These MIPs for NNAL are now commercially available. Further advancement in the use of MIPs for TSNA analysis came when Kavvadias et al., 2009 developed a method for simultaneous determination of urinary NNN, NNK, NAB and NAT using these artificial synthetic receptors. The sample preparation involved a two step SPE procedure – selective extraction on a commercially available MIP cartridge specific
Figure 4: Schematic representation of preparation of MIPs (Adopted from Lasakova et al., 2009)
for TSNAs, followed by extraction on a mixed-mode cation exchange cartridge. The
compounds were then subjected to analysis by LC-ESI-MS/MS. However, these published
methods did not investigate the issue of matrix effects which can affect the quantitative
performance as well as sensitivity of LC/MS-MS based assays.

1.6. Conclusions:

Considering the worldwide problem associated with tobacco use, study of TSNA
uptake will provide very useful insights into the mechanistic and epidemiologic role of
these compounds in human cancer, especially since their origin is specific to tobacco.
TSNA-DNA adducts might provide the most direct relationship to cancer risk. However,
there are significant measurement problems. Measurement of urinary NNAL and NNAL-
Gluc have evolved as one of the most prominently studied and practical TSNA biomarker.
The nitrosamine selective GC-TEA technique had been the method of choice for
bioanalysis of TSNAs for a long time. However, due to the extensive and complex sample
preparation steps for GC-TEA methods, recently there has been a shift to development of
methods for TSNA bioanalysis using the more widely available LC-MS/MS technique.
Molecularly imprinted polymers have recently been developed for selective sample
extraction of urinary NNAL combined with LC-MS/MS analysis. However, the important
issue of matrix effects that can potentially affect the quantitative performance of this assay
requires investigation. Further research that will enhance the throughput of sample
extraction using MIPs on a miniaturized scale presents an excellent potential for reduced
costs, fast analysis times and small sample size.
1.7. Dissertation Objectives:

The first part of the dissertation will investigate the presence of matrix effects and related problems with sensitivity for re-establishment of the method published by Xia et al., 2005. In this method, urinary NNAL will be analyzed based on an offline sample extraction using MIPs combined with LC/MS-MS. The method will be validated and used for subject sample analysis.

The second part of the dissertation will deal with the development of a high-throughput online sample extraction method using capillary micro-columns packed with MIP beads combined with tandem MS for the analysis of urinary NNAL. The method will be optimized and matrix effects will be evaluated and avoided. The method will enable low volume, rapid analysis of urinary NNAL by direct injection on a microfluidic column packed with molecularly imprinted beads specific to NNAL. The method will be validated and used for the analysis of NNAL and NNAL-Gluc concentrations in smokers’ urine. The ratio of NNAL-Gluc to NNAL will be used to investigate the extent of NNAL metabolism. Chemometric methods will be examined to develop potential cancer risk assessment tools based on recognition of patterns in the measured concentrations of these compounds.

In the third part of the dissertation, method comparison approaches for the online and the offline sample extraction techniques will be investigated. Currently there is no clear consensus on the most appropriate acceptance criteria or study design for transfer or comparison of bioanalytical methods. In this part of the dissertation, a ‘fixed’ range acceptance criterion based on a combined consideration of method precision and accuracy, and the FDA bioanalytical guidance limits on precision and accuracy will be proposed.
Data simulations to evaluate the probabilities of a successful transfer using the proposed criterion will be performed. Advantages and limitations of the proposed ‘fixed’ range criteria will be discussed.
2.1. Introduction

A number of clinical studies measuring tobacco specific nitrosamine NNAL and the NNAL-Glucuronide conjugate (NNAL-Gluc) in human urine have been carried out as seen from Table 1 and Table 2 in Chapter 1. These studies indicate that free and total NNAL (i.e. free NNAL along with its glucuronide detoxification products) concentrations in urine are often in the pico-molar range. Such low concentrations of NNAL in human urine require very sensitive and selective methods. At the same time, it is desired that these methods be easy to implement. GC-TEA and LC-MS/MS are the two most commonly used techniques for TSNA analysis. Considering the complex sample preparation procedure and limited throughput of GC-TEA based assays, LC-MS/MS is becoming the method of choice for the analysis of TSNA compounds.

The major disadvantage of conventional SPE sorbents, such as C18, ion-exchange and size-exclusion phases frequently used for sample preparation of LC-MS/MS methods is the lack of adequate selectivity, leading to co-extraction of matrix components with the target analytes. Hence additional sample preparation selectivity is usually needed before the final LC-MS/MS analysis. Due to the high selectivity and molecular recognition
ability, molecularly imprinted polymer (MIP) - solid phase extraction provides a powerful analytical tool which is capable of solving many problems occurring in the analysis of complex samples. The application of these synthetic polymers as sorbents allows not only pre-concentration and cleanup of the sample but also selective extraction of target analytes. This is important, particularly for complex biological sample matrices. MIPs are synthesized by two methods – covalent and non-covalent. The non-covalent approach is the most frequently used method of preparing MIPs. In this approach, a template molecule is mixed with monomers and cross-linkers in a suitable solvent prior to initiation of the polymerization. Frequently used functional monomers include methacrylic acid, 2- and 4-vinylpyridines, trifluoromethylacrylic acid, acrylamide and hydroxyethylmethacrylate. Common cross-linkers include ethylene glycol dimethacrylate, divinylbenzene and trimethylolpropane trimethacrylate. Maximal efficiency of imprint formation occurs when the polymerisation reaction is performed using non- to moderately polar and aprotic solvents such as toluene, dichloromethane, chloroform, etc. (Andersson, 2000). For the non-covalent approach, complexes mainly rely on electrostatic, hydrogen bonding, van der Waal forces, π–π and hydrophobic interactions. The polar nature of these interactions between the templates and monomers make it difficult to apply MIPs directly to aqueous samples. Recently a few studies have reported the use of polar and protic media, such as methanol, ethanol and even water, for the synthesis of MIPs targeted for compounds able to develop strong electrostatic interactions (Pichon, 2007). For aqueous matrices, it becomes important for initial removal of analyte from the matrix and binding to the MIP cavity. The affinity of MIP binding sites prepared using the non-covalent approach is
generally weaker and heterogeneous compared to those prepared using covalent methods which result in more homogenous binding sites. However, the ease of preparation and better kinetics make non-covalent imprinting the technique of choice for MIP synthesis (Pichon, 2007).

In order to improve the selectivity of solid phase extraction, Xia et al., 2005 have developed a method for the analysis of NNAL in urine by extraction on a molecularly imprinted polymeric (MIP) SPE cartridge followed by LC-MS/MS analysis. The MIPs were synthesized using non-covalent methodology. In order to avoid background interference for low-concentration analyses such as NNAL, the authors avoided the use of NNAL as the MIP template. Despite extensive washings, the MIPs may retain some template molecules, which may bleed during the extraction process causing background interference leading to false positive results. Instead, the authors used the commonly followed practice of utilizing structural analogues as MIP templates (identity of the structural analogue of NNAL used for MIP synthesis is not revealed in the publication, however). The choice of appropriate functional monomer is also essential in preparing a selective MIP as it provides specific non-covalent interaction complementary to particular chemical moieties of the functional group. It should also contain a polymerisable entity in its structure. Methacrylic acid which has a carboxylic acid group is capable of acting as a proton donor and a receiver. It is the most extensively used functional monomer in MIP production, and was also used by Xia et al., 2005 for the synthesis of MIPs specific to NNAL. It can therefore play the role of complementary group to the basic and acidic functional groups of the template molecule. Also, the authors used trimethylpropane
trimethacrylate as the cross-linking monomer. The extraction of NNAL on the MIP sorbent was comprised of a conditioning step, followed by loading of the urine sample, a wash step to remove interfering compounds and finally elution of NNAL by using a solvent capable of developing interactions with the sorbent to displace the analyte from the MIP bed.

Despite the enhanced selectivity offered by MIPs in sample extraction, it has been reported that matrix components may still be present after sample extraction (van Hout et al., 2003). The selectivity of the MS step may eliminate the signals of co-eluting components, but their presence in the eluate may still cause ion suppression or enhancement, also known as matrix effects. Matrix effects are proportional errors of slope which affect the calibration curve of a method (Karnes et al., 1991). This may result in the loss of reliability and accuracy of the obtained data. Matrix effects mainly occur when endogenous matrix components co-elute with the analyte of interest, thus altering its ionization efficiency. The exact mechanism of matrix effects is uncertain, but is likely due to competition reactions between an analyte of interest and the co-eluting matrix component (Matuszewski et al., 2003, Taylor, 2005). Mechanistically, matrix effects are generally thought to be caused by either a physical or chemical interaction of the analyte with some components of the matrix. Charge transfer or proton transfer reactions between analyte molecules and matrix components are such common forms of interactions (Figg and McLeod, 2004). These interactions are as shown below:

\[
\text{Charge Transfer: } A^+ + M \rightarrow M^+ + A
\]

\[
\text{Proton Transfer: } AH^+ + M \rightarrow MH^+ + A
\]
where $A^+$ and $AH^+$ represent charged and protonated analyte species, while $M^+$ and $MH^+$ represent charged and protonated matrix components. $A$ and $M$ represent the corresponding uncharged or unprotonated forms. These reactions lead to ion suppression matrix effects. Such an interaction is mediated through an interaction constant which results in a proportional relationship of matrix effects to concentrations. Other causes of ion suppression with electrospray ionization involve a decrease in evaporation of the solution or an increase in surface tension due to high concentrations of matrix compounds (Fu et al., 1998; King et al., 2000). An alternative pathway is binding to and/or co-precipitation of the analyte with nonvolatile matrix species (King et al., 2000). When ion suppression occurs, it can lead to decreased response of analyte measured in matrix compared to non matrix analysis. The sensitivity and lower limit of quantification of a method may in turn be adversely affected (Buhrman et al., 1996).

Although the FDA guidance does not specify any particular test for matrix effects, evaluating and reducing matrix effects in LC/MS/MS methods is an important aspect in the development of bioanalytical method. The two main techniques used to determine the degree of matrix effects are post-extraction addition and post-column infusion (PCI) (Matuszewski et al., 2003; Bonfiglio et al., 1999). The post-extraction addition technique involves comparing the signal from the analyte in a neat solution with the signal of analyte added to an extracted matrix sample. The difference in response between the post-extraction sample and the neat solution determines the degree of matrix effect. The main benefit of this technique is that it provides a quantitative assessment of matrix effects. However, a limitation of this technique is that it only provides information about matrix
effects at the point of elution of the analyte of interest, and not throughout the chromatographic run. On the other hand, the most important advantage of the PCI technique is that it enables investigation of the influence of the matrix on analyte response over the entire chromatographic run. The PCI technique uses an infusion pump to deliver a constant flow of analyte into the HPLC mobile phase at a point after the chromatographic column and before the mass spectrometer ionization source. A sample extract (without added analyte) is injected under the desired chromatographic conditions and the change in response from the infused analyte is recorded. Any endogenous compound that elutes from the column and causes a variation in ESI response of the infused analyte is seen as a suppression or enhancement in the response of the infused analyte. The limitation of this approach is that it only provides a semi-quantitative picture of the level of matrix effect observed for specific analytes. Another approach involves individual monitoring of suspected matrix components capable of causing unwanted matrix effects (Chambers et al., 2007, Ismaiel et al., 2007).

The use of stable isotope labeled internal standards is best suited to correct for matrix effects and to improve both the accuracy and precision of analytical methods employing LC-MS/MS. Stable isotope labeled internal standards are compounds in which several atoms in the analyte are replaced by their stable isotopes such as $^2$H (D), $^{13}$C, $^{15}$N or $^{17}$O. These compounds typically show identical behavior to the analyte of interest in terms of sample extraction, chromatography, as well as in ionization (Van Eeckhaut et al., 2009). Since the stable isotope labeled internal standard co-elutes with the analyte, the relative ionization efficiency of both the analyte and the internal standard remains unaffected.
Thus, matrix effects can be adequately compensated by the use of stable isotope labeled internal standards. However, use of internal standards may not fully solve the problems associated with matrix effects, especially if ion suppression significantly reduces the analyte or internal standard signal. In order to obtain a robust HPLC-MS/MS method, there is therefore a need to remove or minimize the presence of compounds that cause matrix effects.

An aim of the current work was to investigate ionization suppression in the method to determine urinary NNAL by solid phase extraction using molecularly imprinted polymers combined with LC-MS/MS. This important aspect of bioanalytical method development which can often compromise assay performance and sensitivity was not investigated by Xia et al., 2005. In spite of this, the authors were able to analyze low pg/mL concentrations of NNAL. When we tried to reproduce the method published by Xia et al., 2005 in our laboratory, we found that the sensitivity of the method was much lower, however. This did not allow detection of low pg/mL concentrations of NNAL. This indicated possible matrix effects. Matrix effect can vary with different lots and/or sources of biofluids (Matuszewski et al., 2003). Possibly matrix effects were not a problem in the original publication. However, ion suppression was present when we tried to reproduce the published method. The original method was modified to address ion suppression in order to achieve improved sensitivity of the assay. Also, sample throughput of the published method was limited by a tedious and time consuming solid phase extraction on a vacuum manifold. The HPLC column used in the method could maintain a stable back pressure for only a limited number of injections. Thus the ruggedness of the published method needed
improvement. Throughput was addressed by using centrifugation in the extraction procedure which significantly reduced extraction time. Finally, the method was validated according to the FDA guidance for bioanalytical method validations and used for real sample analysis.

2.2. Experimental

2.2.1. Chemicals and Reagents

NNAL (C_{10}H_{15}N_{3}O_{2}, M_w=209.25) (Figure 5) and 4-(methyl-d_3-nitrosamino)-1-(3-pyridyl)-1-butanol (i.e. d_3-NNAL) (C_{10}H_{12}D_3N_3O_2, M_w=212.16) (Figure 5) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). High purity water was obtained in-house using a Nanopure Diamond water system from Barnstead International (Dubuque, IA, USA). High purity methanol, acetonitrile, dichloromethane and toluene were obtained from Burdick and Jackson (Muskegon, MI, USA). Acetic acid was procured from Curtin Matheson Scientific Inc. (Houston, TX, USA). Formic acid was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). Blank as well as smokers human urine were obtained from BioChemed Services (Winchester, VA, USA), Type H1 β-glucuronidase and ammonium dihydrogen phosphate was obtained from Sigma Aldrich (St.Louis, MO, USA).
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

4-(methyl-d3-nitrosamino)-1-(3-pyridyl)-1-butanol

**Figure 5:** Chemical structures of (A) NNAL and (B) d3-NNAL
2.2.2. Materials

Urine samples were aliquoted into 15 mL flip-top plastic centrifuge tubes obtained from Nalge Nunc International (Rochester, NY, USA). SupelMIP – SPE NNAL MIP cartridges provided by Supelco (Bellefonte, PA, USA) were used for solid phase extraction using an Allegra X-15R centrifuge from Beckman Coulter (Fullerton, CA, USA). Solid phase extraction was carried out in the centrifuge using size 16 x 125 mm borosilicate glass culture tubes and 16 x 114 mm borosilicate conical centrifuge tubes with snap cap rims obtained from VWR International (West Chester, PA, USA). Extracted samples were evaporated using Turbomax LC Evaporator procured from Zymark Corp. A syringe pump (pump # 17) from Harvard Apparatus (Holliston, MA, USA) was used for infusing NNAL for post-column infusion studies.

2.2.3. Instrument and Analytical Conditions

A Shimadzu (Shimadzu, Kyoto, Japan) chromatographic system was used in the study. The HPLC system consisted of a Shimadzu system controller SCL-10A VP, pumps LC-10AD VP, solvent degasser DGU14A. The auto-sampler HTS PAL from CTC Analytics (Zwingen, Switzerland) and a CH-30 column heater from Eppendorf (Westbury, NY, USA) were used. The HPLC column was a Phenomenex (Torrance, CA) Gemini C-18 analytical column (100 mm x 2.0 mm I.D., 3.0 μm) with the adoption of an gradient elution mode (see Table 3) using two mobile phases, (A) 10 mM ammonium formate buffer (pH 6.1 adjusted with formic acid) and (B) acetonitrile. A Phenomenex C18 guard column (4.0 mm x 2.0 mm) was used to extend life of the analytical column. The mobile
Table 3: Modified gradient time-table for mobile phase

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase A (%)</th>
<th>Mobile Phase B (%)</th>
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<td>0.00</td>
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<td>10</td>
</tr>
<tr>
<td>6.50</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Mobile phase A: 10 mM ammonium formate buffer (pH 6.1 adjusted with formic acid)
Mobile phase B: Acetonitrile
phase was delivered at 0.5 mL/min and the column temperature was maintained at 55°C. The injection port was washed in between runs with 50% methanol. The mass spectrometer was Micromass Quattro LC triple quadrupole mass spectrometer from Waters Corporation (Milford, MA, USA) with data acquisition software MassLynx version 4.1 installed on an IBM Lenovo ThinkCenter computer. The data analysis was performed using the QuanLynx processing software that accompanies MassLynx.

2.2.4. Mass Spectrometric Conditions

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM). The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity. The desolvation temperature was maintained at 450 °C, and the source block temperature was maintained at 145 °C. The ion spray voltage and multiplier voltage were fixed at 3.20 kV and 700 V, respectively. The mass spectrometer was equipped with an Edwards E2M30 two stage rotary vacuum pump (Edwards, UK). Liquid nitrogen from Airgas National Welders (Charlotte, NC, USA) was used as nebulizer and desolvation gas source with flow rates of 95 L/hr and 600 L/hr, respectively. NNAL and NNAL-d3 eluted at approximately 1.85 minutes and were assayed by quantifying the MRM transition of \([M + H]^+\) ion of NNAL at m/z 210.1 \(\rightarrow\) 180.2 and d3-NNAL at m/z 213.0 \(\rightarrow\) 183.2 respectively. The transitions for the analyte and the I.S. were investigated for the presence of ion cross-talk. Absence of cross-talk was verified by infusing a 100 ng/mL methanolic solution of one compound and confirming the absence of the other transition, and vice versa.
2.2.5. Stock Solution Preparation and Stock Dilution

A standard stock solution of 10 mg/mL of NNAL was prepared in methanol and stored at -20 °C. A series of standard working solutions was then obtained by appropriately diluting the standard stock solution of NNAL with water. Internal standard stock solution at 1 mg/mL was prepared in methanol and stored at -20 °C. Internal standard working solution was prepared at a concentration of 50 ng/mL by appropriately diluting the internal standard stock solution in water. Both NNAL and internal standard working solutions were prepared immediately prior to spiking into the urine.

2.2.6. Preparation of Calibration Standards and Quality Control Samples

Three lots of analyte-free human urine (obtained from non-smokers) were thawed and pooled to provide the matrix for the study. Appropriate volumes of the working solutions of NNAL were spiked into the urine to obtain calibration curve standards spanning a range of 20 to 2500 pg/mL. Calibration standards were prepared freshly before each analytical run. In a similar fashion, quality control samples representing limit of quantitation (LOQ) quality control (QC), low (LQC), medium (MQC) and high (HQC) quality controls were prepared at 20, 60, 400 and 2000 pg/mL, respectively. A dilution QC sample above the upper limit of quantitation was prepared at a concentration of 10 ng/mL. All of the calibration standards as well as the QC samples contained less than or equal to 5% (v/v) of the working solution in order to simulate real samples as much as possible. The QC samples were divided into replicate aliquots of 5.5 mL each, and stored at -20 °C until analysis.
2.2.7. Sample Preparation

The sample preparation procedure was modified from the originally published method (Xia et al., 2005). A scheme depicting sample preparation steps is shown in Figure 6. Urine samples were thawed at room temperature. This method was used for the determination of both free and total NNAL. A 5 mL urine aliquot was required in either case to which 20 μL of internal standard working solution (50 ng/mL) was added. For total NNAL analysis, the urine aliquot fortified with internal standard was mixed with 5 mL of 50 mM pH 6.4 ammonium dihydrogen phosphate buffer (NH₄H₂PO₄·2H₂O). To this, 0.5 mL of 20,000 units/mL β-glucuronidase solution was added and the mixture was incubated at 37°C for 48 hours. For free NNAL analysis, the urine aliquot was adjusted to pH 6-7 using acetic acid. The samples were then centrifuged at 3000 r.p.m for 15 minutes. The solid phase extraction was performed in a centrifuge. The MIP cartridges were set onto 16 x 125 mm culture tubes which were used to collect the eluted liquid components. The cartridge-tube combinations were then loaded onto the centrifuge. Table 4 shows the optimized centrifugation conditions for the various extraction steps. The MIP cartridges were primed by applying 1.0 mL aliquots of dichloromethane, methanol and water in that order. Urine samples were then loaded onto the cartridge taking care that the packing material does not dry prior to loading the sample. The wash step to elute potential interferences while retaining the analytes comprised of two 1.0 mL aliquots of deionized water. This was followed by a drying step by centrifugation at a relatively high centrifugal force to remove residual moisture from the cartridge. The rinsing was continued with 1.0
5 mL urine aliquot

Add 20 µL internal standard

Free NNAL analysis

Incubate for 48 hrs at 37 °C

Adjust pH to 7.0

SPE carried out in centrifuge using MIP cartridges as per steps depicted in Table 4

Evaporate samples to dryness

Reconstitute with 100 µL mobile phase and inject 20 µL on to the MS

Add 20 µL internal standard

Total NNAL analysis

Add 5 mL pH 6.4 ammonium dihydrogen phosphate buffer + 0.5 mL of 20,000 units/mL β-glucuronidase solution

Figure 6: Scheme showing sample preparation steps of the modified method
<table>
<thead>
<tr>
<th>Step</th>
<th>Solvent</th>
<th>Aliquot size (mL)</th>
<th>RPM</th>
<th>Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Priming</td>
<td>Dichloromethane</td>
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<td>200</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>Water</td>
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<td>3</td>
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<td>Loading</td>
<td>Urine Sample</td>
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<td>200</td>
<td>10</td>
</tr>
<tr>
<td>Washing</td>
<td>Water</td>
<td>1</td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>1</td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Drying</td>
<td></td>
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<tr>
<td></td>
<td>Toluene</td>
<td>1</td>
<td>1500</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Toluene: dichloromethane (9:1 v/v)</td>
<td>1</td>
<td>700</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Toluene: dichloromethane (4:1 v/v)</td>
<td>1</td>
<td>500</td>
<td>3</td>
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<tr>
<td></td>
<td>Drying</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Elution</td>
<td>Dichloromethane: methanol (9:1 v/v)</td>
<td>1</td>
<td>350</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Dichloromethane: methanol (9:1 v/v)</td>
<td>1</td>
<td>200</td>
<td>5</td>
</tr>
</tbody>
</table>
mL aliquots of toluene, toluene: dichloromethane mixture (9:1 v/v) and toluene: dichloromethane (4:1 v/v), respectively. Low polarity solvents such as toluene and dichloromethane were used in order to suppress the nonspecific interactions without disrupting the selective interactions between the MIP cavity and the target analyte. Drying was repeated, and the collection tubes were appropriately replaced upon reaching volume capacity. Elution of the analytes was then performed using two 1 mL aliquots of 10% methanol in dichloromethane. The 16 x 125 mm culture tubes were replaced by 16 x 114 mm conical centrifuge tubes to collect the eluted analytes. Samples were evaporated to dryness using a TurboVap evaporator at 48°C for 17 minutes under a gentle stream of nitrogen at 20 psi. The residue was reconstituted in 100 μL of mobile phase A, and transferred to 150 μL silanized glass inserts for analysis. The injection volume was 20 μL.

2.2.8. Matrix Effects Analysis

In order to investigate matrix effects associated with the modified method, a post-column infusion study was conducted as described by Bonfiglio et al., 1999. A 100 ng/mL solution of NNAL was prepared in mobile phase A. It was continuously infused at 10 μL/min post HPLC column into the mass spectrometer using a “tee” connection. The post-column infusion setup is depicted in Figure 7. Upon stabilization of the baseline response, a processed blank urine sample was injected. The matrix effect was investigated from the resulting profile for any change in the ESI response of NNAL. The absolute matrix effect for the current method was also assessed using the method suggested by Matuszewski et al., 2003. This was done by comparing the peak areas of processed blank urine samples
Figure 7: Schematic diagram of the post-column infusion experimental arrangement
post-spiked with low, medium and high QC concentrations of NNAL to the peak areas of dilutions of aqueous NNAL stock solution prepared at equivalent concentrations.

2.2.9. Validation

The method validation was performed according to FDA guidelines for Bioanalytical Method Validation (US Food and Drug Administration, 2001). Validation runs containing calibration standards in duplicate, blank samples, blank sample spiked with internal standard, and replicates of QC samples were run on three separate days.

2.2.9.1. Linearity and LLOQ

Seven calibration standards having concentrations of 20, 50, 100, 250, 500, 1000 and 2500 pg/mL were prepared in duplicate in pooled blank human urine. For the determination of NNAL concentration, a 1/x weighing was employed for the linear regression of the ratio of the peak area response of NNAL and internal standard versus concentration. For each calibration curve, the back-calculated standard concentrations must be within 15% deviation from the nominal value (DFN) with RSD < 15% except at LOQ, where it can be within 20% DFN and RSD < 20%. The lower limit of quantitation (LLOQ) was the concentration of NNAL at which the analyte response was at least 5 times the signal to noise ratio of the blank response, with accuracy and precision as stated above.
2.2.9.2. Accuracy and Precision

Accuracy and precision were determined from the QC samples for three different validation runs. The concentrations of the QC samples were calculated from the calibration curves analyzed in the same run. A criterion of ±15% of the nominal concentration was used to assess accuracy (±20% for LOQ), while precision expressed as %RSD should not exceed 15% (20% for LOQ). Both intra- and inter-assay accuracy and precision were determined. Dilution controls at a level of 10 ng/mL were prepared to evaluate the capability of accurately diluting a sample having a concentration above the upper limit of quantitation (2500 pg/mL). These controls were analyzed in triplicate in each run with a 1:10 dilution in blank human urine to obtain an effective nominal concentration of 1000 ng/mL.

2.2.9.3. Selectivity

Six different lots of blank urine were used to assess selectivity. Each individual lot was processed and analyzed as per the procedure described earlier. For the method to be selective, areas of peaks co-eluting with NNAL should be less than 20% of the peak area of the LOQ sample of NNAL for all the six lots of blank urine. This would ensure that the endogenous urine components do not interfere with the assay.

2.2.9.4. Recovery and Carryover

Recovery or the extraction efficiency of NNAL from the urine after the extraction procedure was determined as follows. Urine samples were spiked with NNAL at the three QC concentrations (60, 400, 2000 pg/mL). These samples were compared with urine
samples spiked post-extraction with equivalent final concentrations of NNAL. The ratio of the peak area response of the pre-spiked and the post-spiked samples would then determine the recovery of NNAL from urine.

Sample carryover was assessed by injecting a reconstitution blank immediately following a HQC sample (2000 pg/mL) injection at the end of the run. The acceptance criterion for carryover was ≤20% of the LLOQ. Sample carryover during the run was also assessed by injecting a HQC sample immediately followed by a LQC sample six times and was evaluated for any bias. Lack of carryover was considered acceptable if the bias was within 15% of the LQC concentration.

2.2.9.5. Stability

The stock solution stability for NNAL was determined during storage and processing. The stock solutions were considered stable if the concentration of NNAL was within ±5% of the original concentration. Stability experiments in human urine were performed at least two QC concentrations (60 pg/mL and 2000 pg/mL) in triplicate. The stability in urine was assessed during storage and after three freeze-thaw cycles at -20 °C with at least 24 hours in between two cycles. These freeze-thaw QC samples were then run against freshly prepared calibration standards. The bench-top stability at room temperature was assessed in urine for 7 hours to cover for the processing time of the samples. The post preparative stability or the auto-sampler stability was assessed from re-injection reproducibility after storage of the samples in the auto sampler for 36 hours at 6°C. The
samples were considered to be stable in urine if the concentration of NNAL was within \( \pm 15\% \) of the nominal concentration for the QC samples tested.

### 2.2.9.6. Incurred Sample Analysis

Incurred urine samples from smokers were purchased from BioChemed Services (Winchester, VA, USA). Samples were obtained from ten male African American smokers. The average number of cigarettes smoked per day as reported by the smokers ranged from 20 to 50. The 24 hour urine samples were used for analysis.

### 2.3. Results and Discussion

#### 2.3.1. LC-MS/MS

Precursor and product ions were selected by infusing 500 ng/mL solutions of NNAL and internal standard into the mass spectrometer in the 5-500 m/z range. The best intensity for the \([M+H]^+\) ions for NNAL at m/z 210.1 and \([M+H]^+\) ions for NNAL-d3 at m/z 213.0 was found in the positive polarity mode using electrospray ionization. The analytical transitions at m/z 210.1 \( \rightarrow \) 180.2 for NNAL and m/z 213.0 \( \rightarrow \) 183.2 for NNAL-d3 were selected since they were the most abundant transitions as also reported by a previous study (Byrd and Ogden, 2003). Optimization of the desolvation temperature and the API gas flow was critical in achieving maximum sensitivity. It was observed that the sensitivity decreased as the gas flow dropped below 600 L/hr. The desolvation temperature was adjusted to 450°C. Minor changes in the ion spray voltage did not affect signal
intensity and was set at 0.32 kV. The dwell time was set at 0.2 seconds, and no cross-talk was found between the two MRM channels.

Chromatographic analysis of the analytes and internal standard was attempted on a variety of columns such as the XTerra MS C<sub>18</sub> (used in the original method), Ascentix Express C18, Polaris C8 and C-18, and Gemini C18. A gradient using a combination of ammonium formate buffer and acetonitrile was optimized to produce the best sensitivity, efficiency and peak shape. Column life was one of the biggest challenges encountered. The accumulation of strongly retained material on an HPLC column can dramatically reduce its lifetime. By modifying the packing surface, these retained materials can cause shifts in peak retention, loss of resolution, and efficiency, as well as degradation of peak shape. The Gemini C-18 analytical column (100 mm x 2.0 mm I.D., 3.0 μm) was found to have the best column life. It also provided good peak shape and response as seen in Figure 8. Whereas some of the other columns clogged leading to high back pressure with as few as 35 to 40 injections, the Gemini C18 column was stable after more than 350 injections. A Phenomenex C18 guard column (4.0 mm x 2.0 mm) was employed to extend the life of the analytical column. A mobile phase comprised of 10 mM ammonium formate (pH 6.1) and acetonitrile with the gradient conditions as depicted in Table 3 was found to be most suitable. The analyte and internal standard eluted at 1.8 minutes, with a run-time of 6.5 minutes.
Figure 8: Representative chromatograms of (A) blank urine (B) blank urine spiked with IS at 250 pg/mL and (C) Urine spiked with NNAL at LLOQ of 20 pg/mL and IS at 200 pg/mL. (MRM transitions 210.1→180.2 and 213→183.1 corresponds to NNAL and $d_3$-NNAL respectively).
2.3.2. Sample Preparation

The sample preparation was adapted from the previously reported method by Xia et al., 2005 that employed molecularly imprinted polymer column extraction and LC/MS/MS. The method was modified and improved for analysis using a relatively low sensitivity mass spectrometer, the Waters Quattro LC as compared to the PE Sciex API 4000 mass spectrometer used in the published paper. Considering the urine aliquot of 5 mL, it was not possible to use common automated SPE systems which are limited by their low volume handling capacities and would require multiple transfers. A vacuum manifold was initially evaluated for sample extraction although the limited number of sample ports on a typical manifold and highly uneven elution rates led to impaired throughput. We therefore utilized a centrifuge which could accommodate as many as 76 samples for the solid phase extraction. All the steps for extraction on the centrifuge are summarized in Table 4. The revolutions per minute (RPM) and the centrifugation times were adjusted to obtain optimal elution flow rates. The column drying step between the water and toluene wash was very important and required a very high RPM (1500 RPM) to ensure a completely dry column in order to prevent clogging due to the water-toluene immiscibility.

Xia et al., 2005 had used β-glucuronidase type IX-A from *E.coli* for conversion of the glucuronide conjugate to the free form. For the current assay, β-glucuronidase type H1 from *H.Pomatia* was used. This is another common source of β-glucuronidase employed in enzyme hydrolysis reactions, and was preferred over the *E.coli* source due to its lower cost. In order to ensure complete conversion of the glucuronide using β-glucuronidase type H1, a time course of the NNAL-Gluc hydrolysis reaction at 37°C was studied. The results of
the study are shown in Figure 9. The x-axis represents the time in hours for the various sampling intervals for which the reaction was carried out. The y-axis represents the percentage of maximum conversion. Three different lots of urine from smokers were subjected to the enzymatic reaction. As can be seen from the graph, the reaction appears to be complete by 24 hours, but was carried on for 48 hours in order to ensure maximum yield. The error bars in this graph represent the standard error of the mean measurement.

Some of the modifications in the extraction procedure are as listed in Table 4. An extra washing step comprised of toluene: dichloromethane (4:1) was introduced to yield cleaner extracts. The final reconstitution volume reported in the original method by Xia et al., 2005 was 20 μL with an injection volume of 10 μL. Experiments with other reconstitution and injection volumes were performed for this method. One would expect response to decrease as the sample is diluted. However, the response was almost three times greater when the sample was reconstituted in 100 μL of mobile phase with a 20 μL injection volume. This increase in response may be due to a dilution of the matrix effect as the final reconstitution volume is increased. Thus, in the current method, the residue was reconstituted after evaporation with 100 μL of the mobile phase with an injection volume of 20 μL. This also has an added advantage of providing enough sample for multiple injections if necessary.
Figure 9: Time course of NNAL-Gluc hydrolysis reaction
2.3.3. Matrix Effects

The results of the post column infusion study for the original method when reproduced in our laboratory and the current modified method are shown in Figure 10A and Figure 10B, respectively. Figure 10A(i) and Figure 10B(i) show the ion profiles when a 100 ng/mL solution of NNAL is infused into the mass spectrometer. Figure 10A(ii) and Figure 10B(ii) show the ion profiles upon injecting a processed blank sample. Figure 10A(iii) and Figure 10B(iii) show the retention time for the analytes (500 pg/mL NNAL spiked in urine). It can be observed that reproduction of the original method resulted in more than 30% ion suppression at the retention time of the analyte. The inherently more sensitive mass spectrometer used in the original publication allowed for the lower limits to be detectable despite the ion suppression.

In order to overcome the ion suppression, chromatographic conditions were modified and the additional wash step was included. The post column infusion experiments for the current method (Figure 10B) resulted in a relatively low amount (approximately 25%) of ionization enhancement at the retention time of the analyte. Comparison of the results for the two methods shows a 25 fold improvement in response for NNAL. This 25 fold improvement in response resulted from overcoming ion suppression as well as a possibly improved analyte desolvation and ionization leading to an enhanced response in MS detection with the modified chromatographic conditions.

Ionization enhancement was further confirmed using absolute matrix effect results by comparing the mean areas of post-extraction spiked samples to external solutions of the analyte. A positive matrix effect was observed at all three concentrations. The mean matrix
Figure 10: Results of post column infusion study for (A) method published by Xia et al., 2005 reproduced in the lab; (B) current modified method. (i) Ion profile when a 100 ng/mL solution of NNAL is infused into the mass spectrometer; (ii) Ion profile upon injecting a processed blank sample; (iii) Representative chromatograms showing the retention time for the analytes.
effects at the LQC, MQC and HQC were 123.3%, 124.8% and 119.3% with an overall mean of 122.5%. The ion enhancement did not affect the assay performance as seen in the validation results. The use of a stable isotope labeled internal standard compensated for any matrix effect variability.

2.3.4. Validation Results

2.3.4.1. Linearity

The peak area ratio of NNAL to internal standard in human urine was linear as a function of concentration over the range 20 to 2500 pg/mL. The calibration curves were well described with a mean correlation coefficient $\geq 0.998$. A weighing factor of $1/x$ was used since the back-calculated residuals demonstrated heteroscedasticity with a proportional change in residuals as the concentration changed. The data are presented in Table 5. Accuracy calculated in terms of the percent deviation from nominal (% DFN) for the mean back-calculated values of the calibration standards ranged from -8.0% to 7.2%, while precision measured in terms of percent relative standard deviation ranged from 1.9% to 7.4%. The LLOQ was established at 20 pg/mL of NNAL in human urine. The LLOQ was reproducible with accuracy and precision within the FDA guidance acceptance criteria with a signal-to-noise ratio of 10.
Table 5: Reverse predicted residuals for NNAL standard concentrations

<table>
<thead>
<tr>
<th>NNAL concentration (pg/ml)</th>
<th>intercept</th>
<th>slope</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>3</td>
<td>17.60</td>
<td>55.20</td>
<td>103.70</td>
</tr>
<tr>
<td></td>
<td>18.20</td>
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<td>107.80</td>
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<tr>
<td>Mean</td>
<td>18.40</td>
<td>53.58</td>
<td>106.55</td>
</tr>
<tr>
<td>SD</td>
<td>1.19</td>
<td>1.95</td>
<td>3.09</td>
</tr>
<tr>
<td>%RSD</td>
<td>6.49</td>
<td>3.64</td>
<td>2.90</td>
</tr>
<tr>
<td>%DFN</td>
<td>-8.00</td>
<td>7.16</td>
<td>6.55</td>
</tr>
</tbody>
</table>

Standard error of regression = 0.019
2.3.4.2. Accuracy and Precision

The intra- and inter-run precision and accuracy data are summarized in Table 6 and Table 7 respectively. The intra- and inter-run accuracies and precision were determined at LOQ QC, LQC, MQC and HQC. The intra-run accuracy was within ± 5.2% (maximum RSD of 7.2%) for all the concentrations including the LLOQ. Also, the inter-run accuracy was within ±1.5% (maximum RSD of 7.2%) for all the concentrations. Thus, the method was both accurate and precise according to established acceptance criteria. Analysis of the dilution QC samples revealed that concentrations above the upper limit of quantification can be safely diluted up to 10 fold. The dilution QC prepared at a concentration of 10000 pg/mL when diluted 10 fold demonstrated an intra-run accuracy of 0.11% DFN and a precision of 0.69% %RSD.

2.3.4.3. Selectivity

The selectivity of the method with regard to endogenous urine components was evaluated in six different lots of blank human urine. No endogenous peaks at the retention time of NNAL and the internal standard were observed for any of the urine lots. Figure 8 demonstrates the selectivity results with representative chromatograms of (A) blank urine sample, (B) blank urine lot spiked with internal standard, and (C) analyte at the LLOQ level (20 pg/mL).
Table 6: Intra-run accuracy and precision data for NNAL

<table>
<thead>
<tr>
<th>Run#</th>
<th>2000.00</th>
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<th>60.00</th>
<th>20.00</th>
</tr>
</thead>
<tbody>
<tr>
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<td>413.00</td>
<td>62.10</td>
<td>22.90</td>
</tr>
<tr>
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<td>1960.40</td>
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</tr>
<tr>
<td></td>
<td>1951.60</td>
<td>397.50</td>
<td>65.00</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>1964.40</td>
<td>400.70</td>
<td>62.00</td>
<td>20.70</td>
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<tr>
<td></td>
<td>1820.20</td>
<td>398.10</td>
<td>62.30</td>
<td>19.20</td>
</tr>
</tbody>
</table>

**Mean** 1938.15 400.92 63.12 20.72

**SD**

|       | 58.20 | 6.78 | 1.30 | 1.49 |

**%RSD**

|       | 3.00 | 1.69 | 2.07 | 7.19 |

**%DFN**

|       | -3.09 | 0.23 | 5.19 | 3.60 |

n.a. – not available since sample was lost due to vial breakage
Table 7: Inter-run accuracy and precision data for NNAL

<table>
<thead>
<tr>
<th>Run#</th>
<th>NNAL Concentration (pg/mL)</th>
<th>2000.00</th>
<th>400.00</th>
<th>60.00</th>
<th>20.00</th>
</tr>
</thead>
<tbody>
<tr>
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<td>413.00</td>
<td>62.10</td>
<td>22.90</td>
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<td></td>
<td>1972.90</td>
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<td>62.80</td>
<td>19.50</td>
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<tr>
<td></td>
<td>1960.40</td>
<td>403.00</td>
<td>64.50</td>
<td>21.30</td>
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<tr>
<td></td>
<td>1951.60</td>
<td>397.50</td>
<td>65.00</td>
<td>na</td>
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<td>62.00</td>
<td>20.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1820.20</td>
<td>398.10</td>
<td>62.30</td>
<td>19.20</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2024.10</td>
<td>366.30</td>
<td>60.50</td>
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<tr>
<td></td>
<td>2254.10</td>
<td>403.60</td>
<td>55.60</td>
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<tr>
<td></td>
<td>1869.80</td>
<td>431.10</td>
<td>55.50</td>
<td>20.80</td>
<td></td>
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<tr>
<td>3</td>
<td>2006.30</td>
<td>396.80</td>
<td>61.20</td>
<td>20.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2024.10</td>
<td>380.20</td>
<td>61.50</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2098.00</td>
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<td>57.90</td>
<td>18.00</td>
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</tr>
<tr>
<td>MEAN</td>
<td>1992.11</td>
<td>396.51</td>
<td>60.91</td>
<td>20.20</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>109.32</td>
<td>17.19</td>
<td>3.09</td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td>%RSD</td>
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<td>5.07</td>
<td>7.22</td>
<td></td>
</tr>
<tr>
<td>%DFN</td>
<td>-0.39</td>
<td>-0.87</td>
<td>1.51</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

n.a. – not available since sample was lost due to vial breakage
2.3.4.4. Recovery and Carryover

The mean recoveries of NNAL from human urine determined at the LQC, MQC and HQC concentrations were 32.7%, 30.14% and 43.09% respectively. The mean extraction recovery is 35.33% for the current extraction protocol. No detectable carryover in the analysis of NNAL was observed.

2.3.4.5. Stability

The stock solution of the analyte prepared in methanol and stored at -20°C was stable over a period of four months. The mean difference between the concentrations of NNAL analyzed over this time period was 4.73%. The stability tests performed indicated no significant degradation under the conditions of freeze-thaw test, bench-top stability and post-preparative stability. The results of stability analysis are provided in Table 8. This confirmed the overall stability of NNAL in the urine matrix under frozen conditions, assay processing and freeze-thaw conditions. The long term storage stability experiments were not performed as it is reported in the literature that NNAL is stable in frozen urine samples stored at -20 °C for up to four years as demonstrated by Yuan et al., 2009.

2.3.4.6. Incurred Sample Analysis

The results of the smoker’s urine analysis are summarized in Table 9. Two subjects had unconjugated NNAL concentrations below the LOQ while one subject had a total-NNAL level below the LOQ. The mean concentrations of NNAL and total-NNAL for the remaining subjects were 89.5 pg/mL and 101.2 pg/mL respectively. The ratio of total-NNAL to free NNAL ranged from 1.7 to 3.1 for these smokers. This outcome is comparable to values reported in the literature (Hecht et al., 1999).
Table 8: Post-preparative, freeze-thaw and bench-top stability data for NNAL

<table>
<thead>
<tr>
<th>Stability</th>
<th>NNAL Concentration (pg/mL)</th>
<th>(n=3)</th>
<th>2000.00</th>
<th>400.00</th>
<th>60.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-Preparative – 36 hours</td>
<td>Mean</td>
<td>2032.93</td>
<td>407.87</td>
<td>64.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>23.57</td>
<td>16.34</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>%RSD</td>
<td>1.16</td>
<td>4.01</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>%DFN</td>
<td>1.65</td>
<td>1.97</td>
<td>7.22</td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>2000.00</td>
<td>60.00</td>
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<tr>
<td>Freeze Thaw – 3 Cycles</td>
<td>Mean</td>
<td>1826.00</td>
<td>53.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>28.81</td>
<td>2.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%RSD</td>
<td>1.60</td>
<td>4.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%DFN</td>
<td>8.70</td>
<td>11.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>2000.00</td>
<td>60.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bench-Top – 7 hours</td>
<td>Mean</td>
<td>1910.42</td>
<td>59.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>39.69</td>
<td>6.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%RSD</td>
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<td>11.13</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>%DFN</td>
<td>-4.48</td>
<td>-0.44</td>
<td></td>
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</tr>
</tbody>
</table>
Table 9: NNAL and NNAL-Gluc measured in Smoker’s urine

<table>
<thead>
<tr>
<th>Subject</th>
<th>Self-reported Cig/Day</th>
<th>Age (years)</th>
<th>NNAL (pg/mL)</th>
<th>Total NNAL (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>48</td>
<td>160.4</td>
<td>392.2</td>
</tr>
<tr>
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<tr>
<td>6</td>
<td>30</td>
<td>39</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>35</td>
<td>64</td>
<td>200.3</td>
</tr>
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<td>8</td>
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<td>45</td>
<td>&lt;LOQ</td>
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</tr>
<tr>
<td>9</td>
<td>50</td>
<td>48</td>
<td>54.6</td>
<td>141.8</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>54</td>
<td>53.1</td>
<td>99.4</td>
</tr>
</tbody>
</table>
2.4. Summary:

The method published by Xia et al., 2005 described the first use of molecularly imprinted polymers for sample preparation in the analysis of urinary NNAL using LC/MS-MS. However, this method did not investigate the effect of matrix effects which can constitute a major concern in the analysis of biological samples using ESI MS. In the current work, we reproduced the original assay and concluded the presence of ion suppression matrix effect affected the sensitivity and likely the reproducibility of responses of the assay. The extraction steps and chromatographic separation were modified from the original publication to yield a 25 fold improvement in sensitivity. This allowed achievement of low pg/mL detection levels of urinary NNAL on the inherently low sensitivity Waters Quattro LC mass spectrometer available in our lab. The analysis in the original publication was carried out on a more sensitive PE Sciex API 4000 triple quadrupole mass spectrometer. Additionally, the Gemini C18 column used in the current method was stable even after as many as 350 injections as opposed to the XTerra MS C18 column described in the original publication which could not maintain a stable back pressure for more than 60 injections before clogging. The sample preparation time was cut down by almost half when the solid phase extraction was carried out by centrifugation, which also resulted in uniform elution rates. The selectivity of the method with regard to endogenous urine components was not evaluated in the original publication. Our modified method was demonstrated to be selective. Finally, the overall extraction efficiency (absolute recovery) was not reported in the original publication. Our results show that the recovery was 35%.
2.5. Conclusion

The determination of NNAL in human urine using MIPS coupled with LC-MS/MS has been accomplished using an extraction protocol and LC conditions that provided a more robust and sensitive method. Issues related to matrix effects and throughput have also been addressed and improved in the current method. This method can be used for quantitative analysis of low concentrations of free and total urinary NNAL in smokers. The validation data demonstrated excellent precision, accuracy and stability.
CHAPTER 3 Microfluidic Extraction of NNAL Using Molecularly Imprinted Polymers by Direct Injection of Urine Coupled On-Line with LC-MS/MS

3.1. Introduction

There is a constant need for reducing development and analysis times of bioanalytical assays. Ability to monitor biomarker concentrations is very important in understanding disease progression and drug development. In this sense, a large number of clinical trials are conducted, thereby increasing the number of samples requiring bioanalysis per unit time. Moreover, not only is the number of samples increasing, but also the time available for method development is decreasing. Apart from throughput requirements, most bioanalytical investigations also require analysis of biological samples at very low quantification levels (Evans, 2004). An important component of bioanalytical assays is sample extraction prior to analysis. Solid phase extraction (SPE) techniques have turned out to be the most preferred techniques for extraction of analytes of interest from biological fluids prior to quantitative analysis. SPE has many advantages compared to traditional liquid/liquid extraction methods, the most important being the availability of a wide range of sorbents which makes this technique applicable to most classes of compounds (Venn, 2000). Recently molecularly imprinted polymers (MIPs) have attracted much attention as SPE sorbents because they show promise as compound-selective or group-selective media (Lasakova and Jandera, 2009). Xia et al., 2005 have developed
MIPs for offline solid phase extraction of tobacco specific nitrosamine NNAL followed by LC-MS/MS analysis.

A drawback of off-line SPE procedures is that they can be time consuming and cumbersome to perform, often requiring several steps prior to reaching a concentrated extract suitable for instrumental analysis (Bones et al., 2006). The use of on-line SPE techniques can enable faster method development by reducing the sample preparation time and thus increasing the sample throughput. Conditioning, washing and elution steps can be automated and performed online. The need for high throughput can be met to a great extent by application of an automated integrated analytical system which also includes the sample extraction step. Other important advantages of online sample extraction include decreased risk of contamination of the sample, elimination of analyte degradation in evaporation steps, and improved precision and accuracy (Watabe et al., 2006). Higher sensitivity is achievable by concentrating the sample on-column. The analysis of the whole sample leads to lower detection limits, and consequently smaller sample volumes may be sufficient to obtain enough sensitivity. Additionally, the on-line sample extraction configuration has been shown to reduce solvent consumption, and is thereby environmentally friendly (Quintana et al., 2006). The SPE sorbent can be regenerated and can be used multiple times unlike the single use offline SPE cartridge format (Rodriguez-Mozaz et al., 2007). Thus, online SPE procedures are particularly attractive for high-throughput, low-cost, low sample volume automated analysis. Method development for determining drug or metabolite concentrations from urine samples can be substantially simplified with the implementation of online SPE. Because of its aqueous nature and lack of protein content,
urine samples can be directly injected and extracted online. Barrett et al., 2005 have developed a sensitive method for quantitation of urinary 6β-hydroxycortisol and cortisol by direct injection using online SPE and LC–MS/MS. The simplest configuration for online sample extraction coupled to LC-MS/MS, involves a single column approach in which the column plays a dual role: the extraction support as well as the analytical column (Veuthey et al., 2004). In this configuration the column is connected to the MS detector by means of a switching valve. The biological sample can be directly injected onto the extraction support with an aqueous mobile phase. After the extraction step, the valve is switched, and analytes are transferred to the detector with the eluting mobile phase. Finally, the extraction sorbent is re-equilibrated. Different extraction sorbents allowing direct injection of biological fluids can be used in the online sample extraction format. These extraction supports or sorbents include restricted access media (RAM), large-size particle, monolithic material, and disposable SPE cartridges (Xu et al., 2007). These sorbents are used by themselves, or in conjunction with other sorbents.

Recently, MIPs have been utilized as extraction supports for online sample extraction. Columns packed with MIPs have been directly coupled to liquid chromatography for analysis of 4-nitrophenol from river water. At least 70 river water samples of 10 mL volume were pre-concentrated online using the MIP column without any loss in performance (Masque et al., 2000). By coupling a MIP-SPE column online with a C18 column, triazine was separated from humic acid, reaching an enrichment factor up to 100-fold with satisfactory recoveries (Bjarnason et al., 1999). A similar online configuration of a MIP coupled with HPLC and electrochemical detection has been used
for the analysis of bisphenol A providing concentration factors up to 1000-fold (Ou et al., 2006). There is a significant challenge for the extraction of analytes using MIPs from aqueous matrices, however. When the analyte of interest is presented in an aqueous medium (e.g. urine), the analyte and other matrix compounds are retained nonspecifically on the polymer since the contribution of hydrophobic interactions increases (Qiao et al., 2006). Consequently, to achieve a selective extraction, a washing step with a non-polar solvent is introduced prior to the elution step. This will enhance the analyte binding to the MIP cavity while washing out the matrix components. Such an effective washing step can easily be incorporated when the extraction is to be performed in an off-line mode using non- to moderately polar solvents such as toluene or dichloromethane. However, when the extraction of aqueous samples is to be performed in an on-line format, incorporation of wash solvents such as toluene or dichloromethane may pose problems, since these may not always be compatible with the detector (e.g. mass spectrometer). Extraction of analytes using MIPs in an online mode may presents considerable challenge.

There is also a substantial interest in the development of miniaturized sample preparation methods for pre-treatment of complex biological samples. An online coupling of miniaturized sample preparation and micro-column separation enables the researcher to take advantage of features of the combined system such as high speed analysis with high efficiency, low cost of operation due to lower solvent consumption, and development of highly selective analysis through tailored systems designed for specific applications. MIPs are highly selective media, and present an excellent opportunity for development of miniaturized sample extraction in an online format. Because MIPs are man-made mimics
of antibodies (Sellergren, 2001) many of the principles applicable to immunoaffinity separation may be applicable to molecularly imprinted separations. Bead based immunoassays using immobilized antibodies packed in separation capillaries have been used for qualitative and quantitative bioanalysis. Peoples et al., 2008 have developed a capillary based microfluidic system to demonstrate direct capture immunoaffinity separation for C-Reactive protein. Peoples and Karnes, 2008 were able to use microfluidic capillaries packed with antibody coated silica beads to capture C-Reactive protein in human serum and cerebrospinal fluid. Microfluidic systems typically describe movement of liquids through chips or capillaries with internal dimensions smaller than 1.0 mm. Microchips, capillary columns and connecting tubing for microfluidic devices are often made of polymers, fused-silica or polyetheretherketone (PEEK). Analogous to immunoaffinity separations, the technology of packing microfluidic capillaries can potentially be extended to molecularly imprinted beads. According to Buranda et al., 2002 and Verpoorte, 2003, separation capillaries packed with beads offer the advantage of reduced diffusion distances for mass transport resulting in much shorter analysis times and the ability to concentrate samples online in a fluidic system. Alternative technologies using monolithic molecularly imprinted polymers (Huang et al., 2003) or in-situ polymerization (Matsui et al., 2000) may have potential for miniaturization in a microfluidic format, although these might suffer from problems of high back pressures and low efficiencies.

The aim of this work was to design a capillary microfluidic system employing MIP beads specific to the tobacco specific nitrosamine NNAL. The system was developed for online sample extraction combined with mass spectrometric detection to enable fast online
extraction and small volume injection with ability to detect low pg/mL concentrations of NNAL and its glucuronide conjugate metabolite in human urine. The system was optimized and evaluated with respect to matrix effects. The method was validated as per the FDA bioanalytical method validation guidance (US Food and Drug Administration, 2001) and used for analysis of urine samples from smokers. The measured concentrations of NNAL and NNAL-Glucuronide were used to develop a cancer risk assessment tool.

3.2. Experimental

3.2.1. Chemicals and Reagents

NNAL (C_{10}H_{15}N_{3}O_{2}, \text{MW}=209.25) (Figure 5) and 4-(methyl-d_3-nitrosamino)-1-(3-pyridyl)-1-butanol (i.e. d_3-NNAL) (C_{10}H_{12}D_3N_{3}O_{2}, \text{MW}=212.16) (Figure 5) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). High purity water was obtained in-house using a Nanopure Diamond water system from Barnstead International (Dubuque, IA, USA). High purity methanol was obtained from Burdick and Jackson (Muskegon, MI, USA). Acetic acid was procured from Curtin Matheson Scientific Inc. (Houston, TX, USA). Formic acid was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). Blank as well as smokers human urine were purchased from BioChemed Services (Winchester, VA, USA), Type H1 β-glucuronidase and ammonium dihydrogen phosphate was obtained from Sigma Aldrich (St.Louis, MO, USA).
3.2.2. Materials

Urine samples were aliquoted into 15 mL flip-top plastic centrifuge tubes obtained from Nalge Nunc International (Rochester, NY, USA). SupelMIP – SPE NNAL molecularly imprinted polymeric cartridges were obtained from Supelco (Bellefonte, PA, USA).

3.2.3. Instrument and Analytical Conditions

A basic schematic diagram of the instrumentation is displayed in Figure 11. A Shimadzu (Shimadzu, Kyoto, Japan) chromatographic system was used in the study. The HPLC system consisted of a Shimadzu system controller SCL-10A VP, pumps LC-10AD VP, solvent degasser DGU14A. The auto-sampler HTS PAL from CTC Analytics (Zwingen, Switzerland) and a CH-30 column heater from Eppendorf (Westbury, NY, USA) were used. Sample extraction was performed online on a capillary micro-column packed with molecularly imprinted polymeric beads specific to NNAL (description about the construction of the MIP micro-column will be discussed in a later section). A multi-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to divert the effluent from the micro-column during washing and elution steps. Mobile phase A was comprised of water which was used as the loading and wash solvent. Mobile phase B was comprised of methanol which was used as the elution solvent. The time-table for the mobile phase is shown in Table 10. The injection port and sample loop were washed in between runs three times with water containing 1% formic acid followed by three times with a 1:1 mixture of methanol and acetonitrile.
Figure 11: Schematic diagram of on-line sample extraction
Table 10: Time-table for mobile phase with on-line sample extraction

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase A mL/min</th>
<th>Mobile Phase B mL/min</th>
<th>Valve Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.25</td>
<td>0.75</td>
<td>A</td>
</tr>
<tr>
<td>3.20</td>
<td>0.25</td>
<td>0.75</td>
<td>B</td>
</tr>
<tr>
<td>4.51</td>
<td>0.25</td>
<td>0.75</td>
<td>A</td>
</tr>
<tr>
<td>6.00</td>
<td>0.25</td>
<td>0.75</td>
<td>A</td>
</tr>
</tbody>
</table>

Mobile phase A: Water
Mobile phase B: Methanol
Initial method development was performed on a Micromass QuattroMicro triple quadrupole mass spectrometer from Waters Corporation (Milford, MA, USA) with data acquisition software MassLynx version 4.1 installed on an IBM Lenovo ThinkCenter computer. The data analysis was performed using the QuanLynx processing software that accompanies MassLynx. Later method development, validation and real sample analysis was performed on a 4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer from Applied Biosystems (Foster City, CA, USA) with data acquisition and quantitation software Analyst version 1.5 installed on an Dell Precision T3400 computer.

3.2.4. Mass Spectrometric Conditions

The mass spectrometric conditions for Waters QuattroMicro mass spectrometer are as follows. It was operated in the positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM). The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity. The desolvation temperature was maintained at 500 °C, and the source block temperature was maintained at 135 °C. The capillary voltage and multiplier voltage were fixed at 0.25 kV and 650 V, respectively. The cone, extractor and RF lens voltages were optimized at 17.0 V, 3.0 V and 0.1 V respectively. The entrance potential, collision energy and exit potentials were set at values of 0, 10 and 1 respectively. The mass spectrometer was equipped with an Edwards E2M30 two stage rotary vacuum pump (Edwards, UK). Liquid nitrogen from Airgas National Welders (Charlotte, NC, USA) was used as nebulizer and desolvation gas source with flow rates of 90 L/hr and 700 L/hr, respectively.
The mass spectrometric conditions for Applied Biosystems 4000 QTrap mass spectrometer are as follows. It was also operated in the positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM). The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity. The desolvation temperature was maintained at 450 °C. The ion spray voltage and CEM multiplier voltage were fixed at 5.5 kV and 2.3 kV, respectively. The de-clustering, entrance and exit potentials were set at values of 49, 10 and 18 respectively. The collision gas was set at high. The mass spectrometer was equipped with a Varian MS40+ single stage rotary vane vacuum pump from Varian Vacuum Technologies (Lexington, MA, UK). Nitrogen was generated using LCMS-5000NA TriGas generator obtained from Parker Balston (Haverhill, MA, USA). The curtain gas was set at 15, while the ion source gas 1 and gas 2 flow rates were set at 62 and 20 respectively.

NNAL and NNAL-\textit{d}_3 were assayed by quantifying the MRM transition of \([\text{M + H}]^+\) ion of NNAL at m/z 210.1 \(\rightarrow\) 180.2 and \textit{d}_3-NNAL at m/z 213.0 \(\rightarrow\) 183.2.

3.2.5. **Stock Solution Preparation and Stock Dilution**

A standard stock solution of 10 mg/mL of NNAL was prepared in methanol and stored at -20 °C. A series of standard working solutions was then obtained by appropriately diluting the standard stock solution of NNAL with water. An internal standard stock solution at 1 mg/mL was prepared in methanol and stored at -20 °C. An internal standard working solution was prepared at a concentration of 10 ng/mL by appropriately diluting
the internal standard stock solution in water. Both NNAL and internal standard working solutions were prepared immediately prior to spiking into the urine.

3.2.6. Preparation of Calibration Standards and Quality Control Samples

Six lots of analyte-free human urine (obtained from non-smokers) were thawed and pooled to provide the matrix for the study. Appropriate volumes of the working solutions of NNAL were spiked into the urine to obtain calibration curve standards spanning a range of 20 to 2500 pg/mL. Calibration standards were prepared freshly before each analytical run. In a similar fashion, quality control (QC) samples representing limit of quantitation (LOQ) QC, low (LQC), medium (MQC) and high (HQC) quality controls were prepared at 20, 60, 400 and 2000 pg/mL, respectively. A dilution QC sample above the upper limit of quantitation was prepared at a concentration of 10 ng/mL. All of the calibration standards, as well as the QC samples contained less than or equal to 5% (v/v) of the working solution in order to simulate a real sample as much as possible. For total NNAL analysis, standards and QC samples were subjected to enzyme hydrolysis prior to analysis using β-glucuronidase enzyme as described later in section 0.

3.2.7. Micro-column Design

The micro-column design and construction procedure was based on a previously published method developed in our laboratory (Peoples et al., 2007). Micro-columns were constructed from polyetheretherketone (PEEK) capillary tubing of 500 μm inner diameter (ID) and 1/16 in outer diameter (OD) purchased from Upchurch Scientific (Oak Harbor, WA, USA). The choice of the PEEK tubing with 500 μm ID was governed by the 20-90
μm particle size distribution of the packing material. Also, 1/16 in OD is the most commonly used tubing size for standard, analytical LC and related techniques. The PEEK tubing has a pressure and temperature rating of 7000 psi and 100°C respectively. Stainless steel external column end-fittings and ferrules were purchased from Valco Instrument Co. Inc. (Houston, TX). A stainless steel 0.5 μm frit in a polymer ring (Upchurch) with dimensions 0.038 in x 0.030 in x 0.062 in was used to retain stationary phase material.

The PEEK tubing ends were cut and leveled with a PEEK tubing cutter to make them suitable for use with a HPLC system. After installing the frit, ferrule and fittings on the PEEK tubing, it was wrench-tightened. One set of end-fittings and frit was then removed, and the other open end of the micro-column was subjected to slurry packing under negative pressure (approximately 600 psi maximum) using a vacuum pump. The slurry consisted of a 10 mg/mL suspension of molecularly imprinted polymeric beads obtained from the commercially available SupelMIP – SPE NNAL molecularly imprinted polymeric cartridges in a 1:1 mixture of water and methanol. Approximately 5 to 10 μL drops were added each time using a microlitre pipette. To maintain the homogeneity of the slurry, it was continuously rotated on a suspension mixer. A jeweler engraver was intermittently tapped on the sides of the microcolumn to eliminate air voids and enable efficient packing. The procedure was continued till beads were seen at the top of the column. After leveling off the beads, the frit and end-fittings were reassembled by wrench tightening. The micro-column was then subjected to a 0.3 mL/min flow of a 1:1 mixture of methanol and water using the HPLC pump. This further compressed the packing material and ensured packing uniformity. Next, the fittings and frit at the end of the micro-column
Figure 12: Components of the packed micro-column and diagram of the packing procedure
originally used for packing were again de-assembled. More slurry was added at the top of
the column under vacuum to fill up any drop in packing level. Finally, the column fittings
and frit were reinstated. Column lengths of 19 mm (smallest possible size considering end-
fitting dimensions) were packed using this procedure. Approximately 100 μL of slurry
added drop-wise was sufficient to pack the micro-column. This corresponded to
approximately 1 mg of packing material (based on 10 mg/mL suspension of molecularly
imprinted polymeric beads as stated above). Thus, a 19 mm micro-column was able to
accommodate approximately 1 mg of the packing material and could be packed in
approximately 15 minutes. Figure 12 shows a diagram of the components of the PEEK
micro-column packed with MIP beads specific to NNAL and the packing procedure for the
same.

3.2.8. Sample Extraction

Sample extraction was performed on-line by direct injection of urine sample on the
molecularly imprinted polymer micro-column followed by LC-MS/MS analysis. Urine
samples were thawed at room temperature. The pH of the urine was checked with pH
paper, and adjusted between pH 5 and pH 7 using 10% v/v acetic acid solution. To a 1 mL
urine aliquot, 20 μL of internal standard working solution (10 ng/mL) was added. For total
NNAL analysis, the urine aliquot fortified with internal standard was mixed with 1 mL of
50 mM pH 6.4 ammonium dihydrogen phosphate buffer (NH₄H₂PO₄.2H₂O). To this, 0.1
mL of 20,000 units/mL β-glucuronidase solution was added and the mixture was incubated
at 37 °C for 48 hours. The samples were then centrifuged at 3000 r.p.m for 15 minutes, and
the clear supernatant was transferred to HPLC vials for direct injection. The injection volume was 20 μL.

3.2.9. **Method Optimization and Evaluation**

Studies were performed to optimize and evaluate the system in order to obtain maximum recovery of NNAL on the MIP micro-column. These studies included wash time optimization, wash step flow rate optimization, sample pH optimization, evaluation of column loadability and injection volume optimization.

3.2.9.1. **Wash Time Optimization**

To ensure that NNAL is not lost in the wash step, a wash time optimization study was performed. The matrix components were washed on the MIP micro-column by flowing water through the MIP micro-column for different time intervals at 1.2, 2.2, and 3.2 minutes respectively. The wash time was optimized to allow maximum removal of matrix components without any loss of NNAL in the wash step.

3.2.9.2. **Wash Step Flow Rate Optimization**

To ensure that NNAL is not lost in the wash step, a wash step flow rate optimization study was performed. Molecularly imprinted polymers are by nature artificial immunosorbents. Thus, flow rate may have an effect on the retention of the analytes on the MIP stationary phase just as shown for immunoaffinity separations by Phillips and Dickens, 2000. Thus, a wash step flow rate optimization study was performed in order to ensure balance between adequate recoveries of NNAL while allowing maximum removal
of matrix components. A 30 ng/mL urine sample spiked with NNAL was injected on to the MIP micro-column. The MS response for different wash step flow rates studied (0.05, 0.1, 0.25, 0.3, 0.4, 0.5 and 0.75 mL/min) was studied.

3.2.9.3. pH Optimization

Recognition of NNAL molecules on a MIP column depends on interactions based on hydrogen bonding and ionic and hydrophobic effects (Haginaka, 2009). Thus, the influence of pH on recovery of NNAL from the MIP columns was studied over a pH range of 2-10. The urine sample pH was adjusted with either 10% v/v acetic acid or 10% w/v sodium hydroxide.

3.2.9.4. Column Loadability Evaluation

The MIP micro-column loadability was evaluated in order to assess the maximum saturation binding. If the maximum saturation binding is reached before sufficient enrichment of NNAL on the MIP micro-column, the capacity of the column can be increased by packing longer columns. In order to evaluate maximum saturation binding, increasing concentrations of NNAL spiked in urine were injected on to the MIP micro-column. A 20 μL injection volume was used. The column loadability was investigated for a concentration range of 10 pg/mL to 100000000 pg/mL.
3.2.9.5. Injection Volume Study

In order to provide for low detection limits, the sample can be concentrated on-column. This can be achieved by increasing the injection volume. Initial studies on the recovery of NNAL with increasing injection volumes were evaluated at injection volumes of 20, 50, 100, 140 and 190 μL.

3.2.10. Evaluation of Matrix Effects

In order to investigate matrix effects, a post-column infusion study was conducted as described by Bonfiglio et al., 1999. A syringe pump (pump # 17) from Harvard Apparatus (Holliston, MA, USA) was used for infusing NNAL for post-column infusion studies. A 500 ng/mL solution of NNAL was prepared in methanol. It was continuously infused at 10 μL/min post HPLC column into the mass spectrometer using a “tee” connection. Upon stabilization of the baseline response, a blank urine sample was injected which was subjected to extraction in the on-line format on the MIP micro-column. The matrix effect was investigated from the resulting profile for any change in the ESI response of NNAL. A 100 ng/mL solution of NNAL in urine was injected as a reference.

3.2.11. Validation

The method validation was performed according to FDA guidelines for Bioanalytical Method Validation (US Food and Drug Administration, 2001). Validation runs containing calibration standards in duplicate, blank samples, blank sample spiked with internal standard, and replicates of QC samples were run on three separate days.
3.2.11.1. **Linearity and LLOQ**

Seven calibration standards having concentrations of 20, 50, 100, 250, 500, 1000 and 2500 pg/mL were prepared in duplicate in pooled blank human urine. For the determination of NNAL concentration, a 1/x weighing was employed for linear regression of the ratios of the peak area responses of NNAL and internal standard versus concentration. For each calibration curve, the back-calculated standard concentrations must be within 15% deviation from the nominal value (DFN) with RSD < 15% except at the LOQ, where it can be within 20% DFN with RSD < 15%. The lower limit of quantitation (LLOQ) was the lowest calibration standard concentration of NNAL at which the analyte response was at least 5 times the signal to noise ratio of the blank response, with accuracy and precision as stated above.

3.2.11.2. **Accuracy and Precision**

Accuracy and precision were determined from the QC samples for three different validation runs. The concentrations of the QC samples were calculated from the calibration curves analyzed in the same run. An acceptance criterion of ±15% of the nominal concentration was used to assess accuracy (±20% for LOQ). This describes the closeness of results obtained by the analytical method to the true concentration of the analyte. Precision expressed as %RSD should not exceed 15% (20% for LOQ). This describes the closeness of individual measures of the analyte when the method is applied repeatedly to multiple aliquots of the same sample. These acceptance criteria were selected based on the guidance for bioanalytical method validation as prescribed by the FDA (US
Food and Drug Administration, 2001). Both intra- and inter-assay accuracy and precision were determined. Dilution controls at a level of 10 ng/mL were prepared to evaluate the capability of accurately diluting a sample having a concentration above the upper limit of quantitation (2500 pg/mL). These controls were analyzed in triplicate in each run with a 1:10 dilution in blank human urine to obtain an effective concentration of 1000 ng/mL.

3.2.11.3. Selectivity

Six different lots of blank urine were used to assess selectivity. Each individual lot was processed and analyzed as per the procedure described earlier. In order to establish selectivity for urine samples subjected to enzymatic hydrolysis to measure total NNAL, six individual lots of blank urine samples were incubated with β-glucuronidase enzyme and processed as described in section 0. For the method to be selective, areas of peaks co-eluting with NNAL should be less than 20% of the peak area of the LOQ sample of NNAL for all the six lots of blank urine. This would ensure that the endogenous urine components do not interfere with the assay.

3.2.11.4. Recovery and Carryover

Recovery or the extraction efficiency of NNAL from the urine after the online extraction procedure was determined as follows. Urine samples were spiked with NNAL at the two QC concentrations (400, 2000 pg/mL) and analyzed using online extraction. These samples were compared with urine samples that were spiked with equivalent final concentrations of NNAL post extraction on a MIP cartridge in an offline format. The
washing and elution steps in the offline extraction were mimicked as in the online format. The ratio of the peak area response of the pre-spiked and the post-spiked samples were then compared to determine the recovery of NNAL from urine.

Sample carryover was assessed by injecting a reconstituted blank immediately following a HQC sample (2000 pg/mL) injection at the end of the run. The acceptance criterion for carryover was \( \leq 20\% \) of the LLOQ. Sample carryover during the run was also assessed by injecting a HQC sample immediately followed by a LQC sample six times and was evaluated for any bias. Lack of carryover was confirmed if the bias was within 15\% of the LQC concentration.

3.2.11.5. Stability

The stock solution stability for NNAL was determined during storage and processing. The stock solutions were considered stable if the concentration of NNAL was within ±5\% of the original concentration. Stability experiments in human urine were performed at least two QC concentrations (60 pg/mL and 2000 pg/mL) in triplicate. The stability in urine was assessed during storage and after three freeze-thaw cycles at -20 °C with at least 24 hours in between two cycles. These freeze-thaw QC samples were then run against freshly prepared calibration standards. The bench-top stability at room temperature was assessed in urine for 7 hours to cover for the processing time of the samples. The post preparative stability or the auto-sampler stability was assessed from re-injection reproducibility after storage of the samples in the auto sampler for 24 hours at 6 °C. The
samples were considered to be stable in urine if concentration of NNAL was within ±15% of the nominal concentration for the QC samples tested.

3.2.12. Real Sample Analysis

This method was applied to the analysis of free and total NNAL in urine samples from 43 smokers. The smoker’s urine samples were purchased from BioChemed Services (Winchester, VA, USA). These were informed consent 24 hour urine samples donated by healthy smoking volunteers. A product data sheet accompanied the urine samples citing demographic information such as age, gender, race, and cigarettes smoked per day (See appendix for copy). These demographic details are described in Error! Reference source not found..

Because these urine samples may be subject to differences in dilution resulting from each individual’s state of hydration, all results were normalized by expressing concentrations per milligram of urinary creatinine levels. Creatinine analyses were performed at the Department of Internal Medicine, Division of Nephrology, Virginia Commonwealth University Medical Center (Richmond, VA, USA) using the Nova Biomedical 16⁺ Electrolyte/Chemistry Analyzer obtained from Nova Biomedical Corp. (Waltham, MA, USA). This analyzer provided a rapid and simple method based on ion selective electrode technology for the measurement of urinary creatinine.
3.2.13. Real Sample Data Analysis – Pattern Recognition and Classification

Glucuronidation by the UDP-glucuronosyltransferase (UGT) superfamily of enzymes is a major mode of detoxification of NNAL in vivo (Ren et al., 2000; Wiener et al., 2004). Genetic variants have been identified in coding and non-coding sequences of

**Table 11: Demographic data for the smokers' urine samples**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Race</th>
<th>Age</th>
<th>Cig/day</th>
</tr>
</thead>
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<td>28</td>
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<tr>
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<td>50</td>
</tr>
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<td>20</td>
</tr>
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<td>White</td>
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<td>20</td>
</tr>
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<td>White</td>
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</tr>
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</tr>
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<td>50</td>
</tr>
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<td>Black</td>
<td>46</td>
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<tr>
<td>22</td>
<td>Male</td>
<td>Black</td>
<td>56</td>
<td>25</td>
</tr>
</tbody>
</table>
several UGT genes, and similar observations should be anticipated for all UGTs. As glucuronidation plays a critical part in the inactivation or elimination of NNK-NNAL pathway, genetic variants in this enzyme family can lead to altered expression or activity of UGTs and may likely have some impact on the cancer risk of an individual (Desai et al., 2003). In a study by Lazarus et al., 2005 it was shown that the polymorphic UGT2B17 gene deletion was associated with significant reductions in the rate of NNAL detoxification in vivo. This may likely increase an individuals’ susceptibility to tobacco related cancers. In another study, Chen et al., 2008a studied the association between UGT2B10 genotypes and NNAL-glucuronidation activity in human liver microsomes. They found that the UGT2B10<sup>67Tyr</sup> variant corresponding to the UGT2B10 haplotype C is a functional single nucleotide polymorphism. This polymorphism may be responsible for inter-individual variation in NNAL-N-glucuronidation activity and may increase susceptibility to smoking related cancers. Wiener et al., 2004 characterized NNAL-N-glucuronide formation in human liver and identified UGT1A4 as the major UGT responsible for this metabolic pathway. The authors speculate that variations in UGT1A4 expression or the presence of activity-altering UGT1A4 gene polymorphisms may be a reason for significant inter-individual variations in the formation of NNAL glucuronide.

As described earlier, glucuronide conjugates of NNAL have been identified in the urine of tobacco smokers (Carmella et al., 2002). The concentrations of free NNAL and NNAL-Gluc measured in the urine of smokers can be evaluated for the possible existence of patterns corresponding to the extent of metabolism for the NNK pathway. The existence of differences in the extent of NNK metabolism rates may in turn be potentially related to
an individuals’ susceptibility to lung cancer. In fact, findings of a recent study by Yuan et al., 2009 directly link NNK exposure to lung cancer development in humans. Ability of an individual to extensively metabolize NNK and NNAL to its glucuronide detoxification product may be correlated to a potentially reduced lung cancer risk. Conversely, the slight metabolism of NNK and NNAL to the glucuronide conjugate may possibly be related to a higher lung cancer risk of an individual. In other words, the higher the ratio of NNAL-Gluc concentrations to free NNAL concentrations, the lower may be an individuals’ vulnerability to lung cancer and vice versa. The most important benefit of knowing whether a smoker is at increased risk for lung cancer is that his or her doctor can screen the person regularly for abnormalities, in the hopes of diagnosing the cancer early. Lung cancer is, however, just one consequence of smoking. So this type of cancer susceptibility information may not be applicable to all forms of cancer. But as far as lung cancer goes, it may give people a better idea of when and how often to get screened. Even if the test outcome results in a low risk of developing lung cancer, an individual may be vulnerable to other forms of cancer caused by smoking.

The method of principal component analysis (PCA) which is one of the most widely used technique in chemometrics for the transformation and classification of multi-dimensional data sets in multivariate data analysis was used for the current data analysis (Otto, 1999; Massart, 1988). The main idea of PCA involves approximation of the original data matrix comprised of features and objects into a product of two smaller matrices – the score and loading matrices. The urine samples collected from the 43 smokers comprised the objects. The 3 features selected for the PCA were free NNAL concentrations, NNAL-
Gluc concentrations and ratio of NNAL-Gluc concentrations to free NNAL concentrations. The classification of the data was then done on the basis of the scores plot and the characteristics of each species were evaluated by the interpretation of loadings plots. Scores plots describe the relative position of each object in the principal component space. Each original feature has loadings that describe its contribution to each principal component. The corresponding loadings plots display relationships between features and can be used to identify which features contribute to the positioning of the objects on the scores plot and hence influence any observed separation in the dataset. PCA is an unsupervised classification method and any prior knowledge relating to the class membership is not used.

In order to further understand and improve the outcome of results of the PCA, a cluster analysis tool was used. The hierarchical cluster analysis is a commonly used exploratory data analysis tool used to solve classification problems (Azevedo et al., 2008, Wiedemann et al., 2005, Gonçalves et al., 2006). The principle of hierarchical cluster analysis is based on sorting of objects into groups or clusters in such a manner that the degree of association is strong between objects of the same cluster and weak between objects of different clusters. It is a statistical method for finding relatively homogeneous clusters of cases based on measured characteristics. It starts with each case in a separate cluster and then combines the clusters sequentially, reducing the number of clusters at each step until only one cluster is left (Massart, 1988).
The relationships between other demographic variables (age, cigarettes per day, gender and race) and each of the three features selected for PCA were also evaluated. This investigation was done by calculating the correlation matrices.

Computations for principal component analysis, correlation matrices and hierarchical cluster analysis were performed using the MATLAB software from MathWorks Inc. (version 7.7) (see appendix for MATLAB commands). Euclidean distance was used in the calculations for hierarchical cluster analysis. The features used in PCA as well as the demographic variables were normalized by autoscaling the data in order to eliminate the potential distortion of data that may be caused by different absolute values as well as different variances.

3.3. Results and Discussion

3.3.1. Sample Extraction

The initial results with online sample extraction demonstrated good selectivity with respect to interferences. Figure 13A shows chromatogram for a 500 ng/mL urine sample spiked with NNAL. Figure 13B shows the chromatogram for blank urine. The MS response of a 100% matrix sample spiked with NNAL was three orders of magnitude lower than a neat solution at a similar concentration, however. Also, based on the S/N ratio of a matrix sample, the sensitivity needed almost a 1000 fold improvement in order to determine low urinary concentrations of NNAL. The system thus needed optimization. The influence of matrix effects needed to be assessed.
Figure 13: Sample chromatograms after online sample extraction on a MIP micro-column of (A) Urine sample spiked with 500 ng/mL NNAL and, (B) Blank urine sample
3.3.2. Optimization Studies

3.3.2.1. Wash Time Optimization

Figure 14 shows the results of the wash time optimization study. The MS response was measured as a function of changing wash times. No significant decrease in response was observed with increasing wash times. Thus, a wash time of approximately 3.2 minutes was selected as an appropriate balance between an acceptable run time, and an opportunity to wash off the maximum amount of matrix components without loss of NNAL recovery.

3.3.2.2. Wash Step Flow Rate Optimization

Figure 15 shows the results of the wash step flow rate optimization study. The MS response was measured as a function of changing wash step flow rates. At lower flow-rates, the NNAL recovery was higher. This is possibly due to a slower mass transfer of NNAL on the molecularly imprinted polymer at lower flow rates, resulting in a better retention of NNAL. Thus, the molecularly imprinted polymer micro-column performance can be improved by decreasing the wash step flow rate. However, at very low flow rates, the variability in response was higher as seen in the graph. This may be possibly due to the limitations of the HPLC pump to perform accurately at lower flow rates. It was also observed that the response dropped as the flow rate was increased. These observations were in line with other published results employing molecularly imprinted polymers (Haginaka and Kagawa, 2002). Thus, a wash step flow rate of 0.25 mL/min was selected to maintain an adequate balance between sufficient recovery and consistency of results.
Figure 14: Results of wash-time optimization study

30 ng/mL NNAL in Urine; 20 μl Inj vol

n=3 replicates
**Figure 15:** Results of wash step flow rate optimization study
3.3.2.3. pH Optimization

Figure 16 shows the results of the pH optimization study. The MS response was measured as a function of changing pH. Optimum recoveries were obtained between pH ranges of 5 to 7. These results suggest development of electrostatic interaction between NNAL and the functional groups on the molecularly imprinted polymer in this pH range. The molecularly imprinted polymer for NNAL has acidic nature due to the presence of carboxylic acid functional group arising from the use of methacrylic acid as the functional monomer (Xia et al., 2005) during the synthesis process. The pK_a of this acid functions in the polymeric complex is estimated to be about 6.5 according to previously published studies (Baggiani et al., 1997). NNAL is a basic compound having pKa = 4.9 (calculated using pKa prediction software developed by Tetko et al., 2005), suggesting that it ionizable in the pH range of 5 to 7. Consequently, an ion exchange mechanism can take place between the hydrogen of carboxylic acid functions of the molecularly imprinted polymer and the basic function of NNAL. The maximum recovery between pH 5 to 7 suggests that this is the domain where both NNAL and the carboxylic acid functional groups are simultaneously ionized leading to the development of electrostatic interaction favoring NNAL rebinding to the molecularly imprinted polymer cavities. Thus, prior to analysis, the urine samples were checked to see if they lie within the pH ranges of 5 to 7 using a pH paper. If the measured pH was outside this range, buffered urine was adjusted appropriately using either 10% v/v acetic acid or 10% w/v aqueous solution of sodium hydroxide.
Figure 16: Results of pH optimization study
3.3.2.4. Column Loadability

A three parameter curve characterizing saturation binding was used to determine the binding capacity of the column based on neat solutions. The model followed the equation \( Y = \frac{B_{\text{max}} \times X^h}{K_d^h + X^h} \) and is sigmoidal in nature. The model used a logarithmic x-axis scale as shown in Figure 17A. In the above equation, \( X \) corresponds to the logarithmic concentration of free NNAL measured in ng/mL and \( Y \) represents the corresponding response. ‘\( B_{\text{max}} \)’ is the maximum specific binding (i.e. maximum number of binding sites), ‘\( k_d \)’ is the concentration required to achieve half-maximum binding and ‘\( h \)’ is the slope. A weighting factor of \((1/Y^2)\) was used to fit the data. The parameter estimates with standard error are: \( B_{\text{max}} = (1.75\pm0.05) \times 10^6, \; k_d = 695\pm48, \; h=0.939\pm0.007. \) The percent relative error (%RE) of each calibration point was plotted against the NNAL concentration as shown in Figure 17B. The difference between back-calculated values and nominal values was divided by the nominal values and multiplied by 100% to give %RE. The % RE data was less that 5% at all concentrations except at the lowest concentration of 10 pg/mL where it was less than 30%.

Based on the parameter estimates, the concentration required to achieve 90% binding is \( \sim 76000 \) ng/mL which corresponds to an on-column capacity of 1.52 \( \mu \)g of NNAL/mg of the packing material based on a 20 \( \mu \)L injection volume. Thus, the on-column capacity was more than five orders of magnitude greater than the expected levels of NNAL in urine indicating a great potential to concentrate the sample on-column without saturating the micro-column.
Figure 17: (A) Saturation binding study and, (B) Relative error plot of NNAL spiked in urine
3.3.2.5. Injection Volume Study

The results of the injection volume study are depicted in Figure 18A. The y-axis depicts the % increase or decrease in MS response as a function of increasing volumes plotted on the x-axis. The experiment was carried out with urine samples spiked with NNAL at a concentration of 100 ng/mL NNAL, and was repeated with a similar concentration of NNAL spiked in deionized water. It was observed that while the response increased proportionally with increasing injection volume for neat solutions, a similar situation is not observed in the case of urine matrix samples and it remained almost constant. In the presence of excess matrix, two situations might occur. Either NNAL does not bind as the binding sites might be depleted in the presence of matrix, or ionization suppression due to matrix effects is taking place.

In order to evaluate the possibility of matrix effects, a separate experiment was conducted. The change in MS response as a function of changing matrix/water compositions was evaluated. The injection volume was held constant at 20 μL for all matrix/water compositions. The results of this study are depicted in Figure 18B. Possible matrix effects due to ionization suppression may be indicated as depicted by the decreasing response for matrix samples for a same injection volume.

3.3.3. Evaluation of Matrix Effects

The results of the post column infusion study are shown in Figure 19. Figure 19A show the ion profiles when a 500 ng/mL methanolic solution of NNAL is infused into the mass spectrometer. Figure 19B shows the ion profiles upon injecting a blank urine sample.
Figure 18: (A) Percentage increase or decrease in MS response as a function of injection volume, (B) MS response as a function of varying percentage of urine matrix in a matrix/water mixture
Figure 19: Results of post-column infusion study for online extraction of NNAL on a MIP micro-column. (i) Ion profile when a 500 ng/mL solution of NNAL is infused into the mass spectrometer; (ii) Ion profile upon injecting a processed blank sample; (iii) Representative chromatogram showing retention time of the analyte.
Apart from injecting a blank urine sample in the latter case, no other experimental parameters were changed for the ion profiles represented by Figure 19A and Figure 19B. This ensured that a constant solvent background was being maintained for both the situations. Figure 19C shows a reference chromatogram for 100 ng/mL NNAL in urine. The results of the post column infusion study reveal the presence of ion suppression at the retention time of the analyte. Ion suppression occurred immediately as the multi-port valve was switched from the washing state to the elution state. This resulted in more than 90% drop in analyte response.

3.3.4. Addressing Matrix Effects

The two most prominent ways to eliminate or minimize matrix effects are either modification in the sample extraction procedure and/or chromatographically resolving the peak of interest from the region of suppression (Taylor, 2005; Van Eeckhaut et al., 2009). Matrix effects are generally related to an insufficient sample cleanup of the biological sample. Although molecularly imprinted polymers are highly selective sample extraction sorbents, optimization of the wash and elution step in the sample extraction procedure is very important, especially because non-specific binding of matrix components to the molecularly imprinted polymer cavities can be a potential problem. Analytes can also be retained chromatographically in such a manner that the impact of matrix effects can be minimized. In this regards, information obtained from a post column infusion experiment can be very useful. This data provides an idea where matrix effects are occurring in a
chromatographic run, and the analyte can be chromatographically resolved from the affected region in order to provide a more robust method.

3.3.4.1. Wash and Elution Step Evaluation

The wash step and the elution step were evaluated for their effects on ion suppression. The percentage of methanol, which is a stronger solvent, was increased in the wash step in a bid to remove matrix components in order to minimize matrix effects, while still providing adequate recovery for NNAL. The changing methanol composition in the wash step was studied as a function of: (a) Its effect on the ion suppression measured in terms of absolute matrix effects (Matuszewski et al., 2003), and (b) NNAL recovery measured in terms of the MS response. The composition of methanol which yields the biggest difference between the minimum matrix effects and maximum NNAL recovery can then be selected for the wash step. In order to calculate absolute matrix effects, 100 ng/mL solutions of NNAL in urine and water were used. Absolute matrix effect was calculated as $B/A \times 100\%$ where, $B$ corresponds to peak area obtained in matrix and $A$ corresponds to peak area obtained in neat solution.

The results of the wash step evaluation are shown in Figure 20A. Methanol concentration is plotted on the x-axis. The left y-axis represents NNAL recovery measured in terms of MS response as the methanol composition in the wash step is changed. The right y-axis represents absolute matrix effects as the methanol composition in the wash step is changed. For non-covalent MIPS, if the analyte is present in an aqueous medium, the contribution of non-specific hydrophobic interactions increases in the retention of the
analyte as well as other matrix components on the polymer (Qiao et al., 2006). To achieve selective extraction non-polar to moderately polar organic solvents are typically introduced in the wash step. The wash solvent should disrupt the non-specific interactions without disrupting the selective interactions between MIPs and the target molecule. Solvents such as toluene and dichloromethane are most widely used wash solvents in order to retain the target molecules (Haginaka, 2009). However, because of incompatibility with ESI-MS, these solvents were not selected. Consequently, changing concentrations of methanol were tested in the wash step. It was observed that an increasing percentage of methanol in the wash step resulted in a rapid decrease in the MS response indicating loss in NNAL recovery on the MIP micro-column. However, the changing methanol concentration did not affect absolute matrix effects. In other words, selective disruption of non-specific hydrophobic interactions was likely not achieved using this approach. Thus, the next step was to try and evaluate the elution step.

The percentage of methanol, which is a stronger solvent, was decreased in the elution step in a bid to retain back maximum matrix components on the MIP micro-column, yet still providing adequate recovery for NNAL. Similar to the previous case, the changing methanol composition in the elution step was studied as a function of: (a) Its effect on the ion suppression measured in terms of absolute matrix effects and (b) NNAL recovery measured in terms of the MS response. The composition of methanol which yields the biggest difference between the minimum matrix effects and maximum NNAL recovery can then be selected for the elution step.
The results of the elution step evaluation are shown in Figure 20B. Again, methanol concentration is plotted on the x-axis. The right y-axis represents the NNAL recovery measured in terms of MS response as the methanol composition in the elution step is decreased. The right y-axis represents the absolute matrix effects as the methanol composition in the wash step is decreased. It was observed that the MS response remained almost constant with decreasing concentration of methanol in the elution step, and decreased rapidly with less than 20% methanol. However, the changing methanol concentration did not affect absolute matrix effects. In other words, both the non-specific interactions between the matrix components and the polymer as well as the selective interactions between the analyte and the polymer were likely being disrupted using this approach. Consequently, the matrix effects did not improve as the methanol concentration in the wash step decreased.

3.3.4.2. Resolving Ion Suppression by Integration of an Analytical Column

As a next approach to address the ion suppression problem, a reverse phase HPLC column was introduced in-line after the MIP micro-column to separate matrix components from co-eluting with the analyte of interest. NNAL is a moderately polar organic compound. The relatively moderate polarity of NNAL would allow it to be more retained on a reverse phase HPLC column compared to the more polar interfering matrix species. This would be the mechanism for separation of NNAL from the matrix components which would potentially result in a reduced impact of ion suppression matrix effects commonly encountered in electrospray ionization of biological extracts.
Figure 20: NNAL recovery and matrix effects as a function of changing methanol concentration in (A) Wash Step and, (B) Elution step.
The schematic diagram of on-line sample extraction with the incorporation of analytical HPLC column is shown in Figure 21. The HPLC column was a Phenomenox Gemini C-18 column (100 mm x 2.0 mm I.D., 5.0 μm) with incorporation of an isocratic elution mode as shown in Table 12. A Phenomenex C18 guard column (4.0 mm x 2.0 mm) was used for extending life of the analytical column. The column temperature was maintained at 60°C. The wash solvent on the MIP micro-column comprised of water. Considering that NNAL recovery was not affected by decreasing methanol concentration in the elution step (see Figure 20B), the elution solvent was comprised of a 1:1 mixture of methanol water. This enabled retention of NNAL on the reverse phase HPLC column. A third solvent line comprised of 10 mM ammonium formate buffer (pH 6.1) was introduced via a tee connection in order to chromatographically separate the NNAL peak from the region of ion suppression. This was also necessary to ensure a relatively constant proportion of mobile phase introduced into the mass spectrometer and prevent any baseline shifts due sudden changes in solvent composition arising from switching of the valve going from the wash step to elution step.

A post column infusion study was repeated in order to evaluate the presence of matrix effect. Results of the post column infusion study after integration of the analytical HPLC column are shown in Figure 22. It can be observed that the region of ion suppression is now chromatographically resolved from the NNAL retention time.
Switching Valve Position A

Switching Valve Position B

**Figure 21:** Schematic diagram of on-line sample extraction with the incorporation of analytical HPLC column
Table 12: Time-table for mobile phase with on-line sample extraction after incorporation of analytical column

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase A mL/min</th>
<th>Mobile Phase B mL/min</th>
<th>Mobile Phase C mL/min</th>
<th>Valve Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.24</td>
<td>0.18</td>
<td>0.18</td>
<td>A</td>
</tr>
<tr>
<td>3.50</td>
<td>0.24</td>
<td>0.18</td>
<td>0.18</td>
<td>B</td>
</tr>
<tr>
<td>3.70</td>
<td>0.24</td>
<td>0.18</td>
<td>0.18</td>
<td>A</td>
</tr>
<tr>
<td>7.00</td>
<td>0.24</td>
<td>0.18</td>
<td>0.18</td>
<td>A</td>
</tr>
</tbody>
</table>

Mobile phase A: Water
Mobile phase B: Methanol:Water (1:1)
Mobile phase C: 10 mM Ammonium formate buffer (pH 6.1)
Figure 22: Results of post-column infusion study for online extraction of NNAL on a MIP micro-column after integration of analytical column. (i) Ion profile when a 500 ng/mL solution of NNAL is infused into the mass spectrometer; (ii) Ion profile upon injecting a processed blank sample; (iii) Representative chromatogram showing retention time of the analyte.
3.3.4.3. Optimization of Injection Volume after Resolution of Ion Suppression Region:

After integrating the analytical column in line with the MIP micro-column, the region of ion suppression was chromatographically resolved from the analyte. On-column concentration can now potentially be accomplished by increasing injection volumes in order to detect low concentration of urinary NNAL. However, the effectiveness of increasing injection volume will be limited because of an expected decrease in the resolution of NNAL from the ion suppression region due to the increased amount of matrix components injected. Lack of adequate resolution from the region of ion suppression may in turn compromise the assay precision. Thus, maintenance of an adequate balance between resolution of NNAL from the region of ion suppression (baseline resolution of at least 1.2) and the optimum injection volume required to achieve the necessary on-column concentration (target concentration of at least 20 pg/mL) is essential. On-column concentration is measured in terms of NNAL response. The graph in Figure 23 shows the relationship between the change in injection volume as a function of analyte response (measured in terms of signal-to-noise ratio) and the resolution of the analyte from the ion suppression region. It is observed that as the injection volume is increased, the s/n increases up to about 300 μL injection volume, and then begins to drop. Similarly, sufficient resolution of about 1.4 is observed between the region of ion suppression and the peak of interest up to about 200 μL injection volume. Further increases in injection volume compromises resolution drastically. So, to maintain sufficient balance between the two parameters, an injection volume of 200 μL was chosen.
Figure 23: Injection Volume as a function of a) s/n and b) resolution from region of ion suppression after integration of HPLC column in line with the MIP micro-column
3.3.5. Validation Results

3.3.5.1. Linearity

The peak area ratio of NNAL to internal standard in human urine was linear as a function of concentration over the range 20 to 2500 pg/mL. The calibration curves were well described with a mean correlation coefficient \( \geq 0.999 \). A weighing factor of \( 1/x \) was used since the back-calculated residuals demonstrated heteroscedasticity with a proportional change in residuals as the concentration changed. The data are presented in Table 13. Accuracy calculated in terms of the percent deviation from nominal (% DFN) for the mean back-calculated values of the calibration standards ranged from -3.5% to 5.9%, while precision measured in terms of percent relative standard deviation ranged from 0.95% to 7.39%. The LLOQ was established at 20 pg/mL of NNAL in human urine. The LLOQ was reproducible with accuracy and precision within the FDA guidance acceptance criteria with a s/n ratio of 10.

3.3.5.2. Accuracy and Precision

The intra- and inter-run precision and accuracy data are summarized in Table 14 and Table 15 respectively. The intra- and inter-run accuracies and precision were determined at LOQ QC, LQC, MQC and HQC. The intra-run accuracy was within \( \pm 14.8\% \) (maximum RSD of 11.3%) for all the concentrations including the LLOQ. Also, the inter-run accuracy was within \( \pm 9.6\% \) (maximum RSD of 11.4%) for all concentrations. The method was both accurate and precise according to established acceptance criteria. Analysis of the dilution QC samples revealed that concentrations above the upper limit of
Table 13: Reverse predicted residuals for NNAL standard concentrations

<table>
<thead>
<tr>
<th>NNAL concentration (pg/ml)</th>
<th>intercept</th>
<th>slope</th>
<th>r²</th>
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<tbody>
<tr>
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<td>50.00</td>
<td>100.00</td>
</tr>
<tr>
<td>1</td>
<td>20.80</td>
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<td>50.20</td>
<td>98.90</td>
</tr>
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<td></td>
<td>19.40</td>
<td>44.70</td>
<td>n.a.</td>
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<td>3</td>
<td>20.50</td>
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<tr>
<td>Mean</td>
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<td>96.85</td>
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<tr>
<td>%RSD</td>
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<td>3.23</td>
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<tr>
<td>%DFN</td>
<td>5.90</td>
<td>-2.68</td>
<td>-3.15</td>
</tr>
</tbody>
</table>

Standard error of regression = 0.028

n.a. – not available because of broken vial
Table 14: Intra-run accuracy and precision data for NNAL

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<th>60.00</th>
<th>20.00</th>
</tr>
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<td>58.80</td>
<td>15.30</td>
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<tr>
<td></td>
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<td>335.00</td>
<td>54.30</td>
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<td></td>
<td>1780.00</td>
<td>366.00</td>
<td>49.90</td>
<td>20.00</td>
</tr>
<tr>
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<td>1830.00</td>
<td>322.00</td>
<td>52.50</td>
<td>22.30</td>
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<td>16.90</td>
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<table>
<thead>
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<th></th>
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<th>19.65</th>
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<tr>
<td>SD</td>
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<td>20.03</td>
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<tr>
<td>%RSD</td>
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<tr>
<td>%DFN</td>
<td>8.88</td>
<td>14.75</td>
<td>13.17</td>
<td>1.75</td>
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</table>
Table 15: Inter-run accuracy and precision data for NNAL

<table>
<thead>
<tr>
<th>Run#</th>
<th>NNAL Concentration (pg/mL)</th>
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<th>60.00</th>
<th>20.00</th>
</tr>
</thead>
<tbody>
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<td>58.80</td>
<td>15.30</td>
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<tr>
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<td>335.00</td>
<td>54.30</td>
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<td>366.00</td>
<td>49.90</td>
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<td>322.00</td>
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<tr>
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<td>56.50</td>
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<td>405.00</td>
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<td>MEAN</td>
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<tr>
<td>SD</td>
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<td>43.36</td>
<td>2.90</td>
<td>1.99</td>
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<tr>
<td>%RSD</td>
<td>1.93</td>
<td>11.43</td>
<td>5.29</td>
<td>10.23</td>
<td></td>
</tr>
<tr>
<td>%DFN</td>
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<td>5.17</td>
<td>8.68</td>
<td>2.83</td>
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</tr>
</tbody>
</table>
quantitation can be accurately and precisely diluted up to 10 fold. The dilution QC prepared at a concentration of 10000 pg/mL when diluted 10 fold demonstrated an intra-run accuracy of -0.24% DFN and a precision of 3.47% %RSD.

3.3.5.3. Selectivity

The selectivity of the method with regard to endogenous urine components was evaluated in six different lots of blank human urine. No endogenous peaks at the retention time of NNAL and the internal standard were observed for any of the urine lots. Figure 24 demonstrates the selectivity results with representative chromatograms of (A) blank urine sample, (B) blank urine lot spiked with internal standard, and (C) analyte at the LLOQ level (20 pg/mL). The method also showed adequate selectivity for urine samples spiked with β-glucuronidase enzyme.

3.3.5.4. Recovery and Carryover

The mean recoveries of NNAL from human urine determined at the MQC and HQC concentrations were 30.3%, and 31.7% respectively. The mean extraction recovery is 31.0% for the current extraction protocol. No detectable carryover in the analysis of NNAL was observed.

3.3.5.5. Stability

The stability tests performed indicated no significant degradation under the conditions of freeze-thaw test, bench-top stability and post-preparative stability. The
accuracy was within ±11.6% (maximum RSD of 10.1%) for all conditions which was
within the FDA prescribed limits for accuracy and precision. The results of stability
analysis are provided in Table 16. This confirmed the overall stability of NNAL in the
urine matrix under frozen conditions, assay processing and freeze-thaw conditions. The
long term storage stability experiments were not performed as it is reported in the literature
that NNAL is stable in frozen urine samples stored at -20 °C for up to four years as
demonstrated by Yuan et al., 2009.

3.3.6. Capillary Micro-column Ruggedness

After validating the method, the capillary micro-column packing reproducibility
was evaluated. Three capillary micro-columns were packed on separate occasions and
tested at low, medium and high QC concentrations. Additionally, three different lots of
MIP beads were packed in capillary micro-columns and evaluated at similar
concentrations. Results of column packing uniformity as well as lot-to-lot uniformity study
are shown in Figure 25. Excellent column packing reproducibility was obtained at all QC
concentrations as seen in Figure 25A. The lot-to-lot recovery was slightly lower for one lot
of MIPs at high QC concentration as seen in Figure 25B. This may potentially be due to
reduced number of binding sites for that particular lot. A significant advantage of the
online capillary micro-column was the ability to re-use the micro-column for multiple
injections. More than 300 injections were obtained on a single micro-column without loss
of performance.
Figure 24: Representative chromatograms of (A) blank urine (B) blank urine spiked with IS at 250 pg/mL and (C) Urine spiked with NNAL at LLOQ of 20 pg/mL and IS at 200 pg/mL. (MRM transitions 210.1 → 180.2 (upper channel) and 213 → 183.1 (lower channel) corresponds to NNAL and NNAL-d3 respectively.)
Table 16: Post-preparative, freeze-thaw and bench-top stability data for NNAL

<table>
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<tr>
<th>Stability</th>
<th>NNAL Concentration (pg/mL)</th>
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</tr>
<tr>
<td>%DFN</td>
<td>8.70</td>
<td>11.57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td>2000.00</td>
<td>60.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bench-Top – 7 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1816.67</td>
<td>54.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>32.15</td>
<td>1.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%RSD</td>
<td>1.77</td>
<td>2.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%DFN</td>
<td>9.17</td>
<td>9.72</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 25: Capillary micro-column reproducibility study - (A) Column packing reproducibility study. (B) Lot-to-lot reproducibility study.
3.3.7. **Real Sample Analysis**

The mean age of smokers whose urine samples were analyzed in the study was 37.9 years (median age = 40 years). The self-reported average number of cigarettes smoked per day was 26.8 cigarettes (median cigarettes per day = 25). The ratio of males to females in the study was 65:35, while the ratio of black smokers to white smokers was 77:23. Urine samples were stored at -20 °C until analysis. The concentrations of free NNAL measured ranged from BLOQ to 1.26 pmol/mg creatinine while NNAL-Gluc concentrations ranged from BLOQ to 0.50 pmol/mg creatinine.

Table 17 shows the concentrations of NNAL and NNAL-Gluc measured in smokers. The ratios of NNAL-Gluc concentrations to NNAL concentrations are also depicted in Table 17.

3.3.8. **Subject Sample Data Analysis – Pattern Recognition and Classification**

Before carrying out the multivariate analysis, the relationship between the demographic variables (gender, age, race and cigarettes/day) and the feature variables (NNAL concentrations, NNAL-Gluc concentrations and ratio of NNAL-Gluc to NNAL concentrations) was studied. Each feature variable \(x\) was compared with the demographic variable \(y\), and the corresponding correlation matrix \(r\) was calculated to decide if a correlation existed between these two variables. This was is an important preliminary study to identify if the feature variables selected for the multivariate analysis showed any systematic dependence to the demographic variables. Maximal absolute correlation exists when the absolute value of the correlation coefficient \(|r(x,y)| = 1\), and it can be concluded
Table 17: Concentrations of NNAL and NNAL-Gluc measured in urine of smokers

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>NNAL concentrations</th>
<th>NNAL-Gluc concentrations</th>
<th>Ratio</th>
<th>Subject ID</th>
<th>NNAL concentrations</th>
<th>NNAL-Gluc concentrations</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.194</td>
<td>0.080</td>
<td>0.412</td>
<td>23</td>
<td>0.378</td>
<td>0.353</td>
<td>0.934</td>
</tr>
<tr>
<td>2</td>
<td>0.153</td>
<td>0.096</td>
<td>0.630</td>
<td>24</td>
<td>0.311</td>
<td>0.187</td>
<td>0.603</td>
</tr>
<tr>
<td>3</td>
<td>0.120</td>
<td>0.176</td>
<td>1.466</td>
<td>25</td>
<td>0.319</td>
<td>0.155</td>
<td>0.485</td>
</tr>
<tr>
<td>4</td>
<td>0.389</td>
<td>0.229</td>
<td>0.589</td>
<td>26</td>
<td>0.574</td>
<td>0.399</td>
<td>0.695</td>
</tr>
<tr>
<td>5</td>
<td>0.350</td>
<td>0.166</td>
<td>0.474</td>
<td>27</td>
<td>1.259</td>
<td>0.165</td>
<td>0.131</td>
</tr>
<tr>
<td>6</td>
<td>0.235</td>
<td>0.054</td>
<td>0.229</td>
<td>28</td>
<td>0.310</td>
<td>0.113</td>
<td>0.363</td>
</tr>
<tr>
<td>7</td>
<td>0.000</td>
<td>0.221</td>
<td>0.000</td>
<td>29</td>
<td>0.326</td>
<td>0.188</td>
<td>0.576</td>
</tr>
<tr>
<td>8</td>
<td>0.205</td>
<td>0.344</td>
<td>1.682</td>
<td>30</td>
<td>0.465</td>
<td>0.291</td>
<td>0.626</td>
</tr>
<tr>
<td>9</td>
<td>0.000</td>
<td>0.113</td>
<td>0.000</td>
<td>31</td>
<td>0.289</td>
<td>0.275</td>
<td>0.952</td>
</tr>
<tr>
<td>10</td>
<td>0.230</td>
<td>0.157</td>
<td>0.684</td>
<td>32</td>
<td>0.386</td>
<td>0.207</td>
<td>0.536</td>
</tr>
<tr>
<td>11</td>
<td>0.112</td>
<td>0.233</td>
<td>2.091</td>
<td>33</td>
<td>0.694</td>
<td>0.501</td>
<td>0.722</td>
</tr>
<tr>
<td>12</td>
<td>0.236</td>
<td>0.148</td>
<td>0.626</td>
<td>34</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>13</td>
<td>0.000</td>
<td>0.125</td>
<td>0.000</td>
<td>35</td>
<td>0.360</td>
<td>0.132</td>
<td>0.368</td>
</tr>
<tr>
<td>14</td>
<td>0.243</td>
<td>0.100</td>
<td>0.410</td>
<td>36</td>
<td>0.327</td>
<td>0.300</td>
<td>0.917</td>
</tr>
<tr>
<td>15</td>
<td>0.445</td>
<td>0.213</td>
<td>0.479</td>
<td>37</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>16</td>
<td>0.396</td>
<td>0.198</td>
<td>0.500</td>
<td>38</td>
<td>0.210</td>
<td>0.113</td>
<td>0.540</td>
</tr>
<tr>
<td>17</td>
<td>0.307</td>
<td>0.283</td>
<td>0.921</td>
<td>39</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>18</td>
<td>0.472</td>
<td>0.401</td>
<td>0.849</td>
<td>40</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>19</td>
<td>0.384</td>
<td>0.192</td>
<td>0.501</td>
<td>41</td>
<td>0.250</td>
<td>0.414</td>
<td>1.651</td>
</tr>
<tr>
<td>20</td>
<td>0.168</td>
<td>0.072</td>
<td>0.429</td>
<td>42</td>
<td>0.379</td>
<td>0.355</td>
<td>0.935</td>
</tr>
<tr>
<td>21</td>
<td>0.234</td>
<td>0.411</td>
<td>1.754</td>
<td>43</td>
<td>1.054</td>
<td>0.234</td>
<td>0.222</td>
</tr>
<tr>
<td>22</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: NNAL and NNAL-Gluc concentrations are reported as pmol/mg creatinine
NNAL-Gluc concentrations determined from difference between free and total NNAL concentrations
Ratio is the Ratio of NNAL-Gluc to NNAL concentrations
that the feature variables are perfectly co-related with the corresponding demographic variable. When the two variables are not correlated, the value of $|r(x,y)|$ approaches close to zero. The results of the correlation matrices of the data are depicted as correlation coefficients. A 95% confidence interval for the correlation coefficient was calculated. As seen from the Table 18, all the 95% confidence intervals included zero indicating that none of the feature variables bear any significant correlation to the demographic variables. This conclusion is important in the interpretation of the pattern recognition discussed below, confirming that any difference in classification may not be attributable to demographic variables.

Multivariate data analysis was carried out after normalization of the feature data that were preprocessed by autoscaling before PCA transformation. Autoscaling is a scaling method used to eliminate differences in the metric properties of the feature variables. It is used to give equal weighting to all portions of experimentally measured data. The resulting columns of the variables are said to be scaled to unit variance. Autoscaling is performed as shown in the equation below:

$$z_{ij} = \frac{x_{ij} - x^m_j}{s_j}$$

where $x_{ij}$ is the value of object $i$ of feature variable $j$, $x^m_j$ is the mean for feature variable $j$, and $s_j$ is the standard deviation of feature variable $j$ (Massart, 1988; Otto, 1999). The $z_{ij}$ values were then used for PCA transformation.

A new system of co-ordinates corresponding to principal components was used to study the relationship between objects and features in a more efficient way. The percentage of explained variance approach was applied in calculating the number of principal
components used to represent the data (Otto, 1999). According to this, each subsequent principal component describes a maximum of variance that is not modeled by the earlier components. The use of all principal components is usually not justified in order to separate the pure components from the noise components. Table 19 shows the percentage of explained variance of the autoscaled data. More than 92% of the explained variance of the autoscaled data can be explained by the first two principal components which were used for analysis. Accordingly, sample patterns were detected using the first two principal components (PC1 and PC2 with 60.66% and 32.06% of explained variance respectively). Interpretation of the PCA outcome to evaluate the existence of patterns was carried out by visual inspection (Otto, 2007). As seen in the scores plot (Figure 26), the data revealed a visual differentiation among the objects and offered valuable evidence for potential classification. Two classes of smokers were adequately separated.

Class 1: Positioned at the upper right quartile (pink ellipse). These were subjects with measured with high concentrations of free NNAL and having low NNAL-Gluc to NNAL ratios indicating poor metabolic deactivation, and consequently may be at a higher lung cancer risk.

Class 2: Positioned at the bottom right quartile (purple ellipse). These were subjects with having high NNAL-Gluc to NNAL ratios indicating strong metabolic deactivation, and consequently may be at a lower lung cancer risk.

The majority of the objects (non-classified) were positioned between classes 1 and 2, near the center of the PCA axes (green ellipse). The discrimination of more classes within the non-classified group seemed difficult indicating similarities in the extent of metabolism of
Table 18: Correlation coefficients obtained for feature variables w.r.t. demographic variables as calculated from the correlation matrices using MATLAB software (ver. 7.7)

<table>
<thead>
<tr>
<th>Variables (x, y)</th>
<th>Age</th>
<th>Gender</th>
<th>Race</th>
<th>Cigarettes/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNAL Concentrations</td>
<td>-0.1141</td>
<td>0.2276</td>
<td>-0.0529</td>
<td>-0.1803</td>
</tr>
<tr>
<td></td>
<td>[-0.40, 0.19]</td>
<td>[-0.08, 0.49]</td>
<td>[-0.35, 0.25]</td>
<td>[-0.46, 0.13]</td>
</tr>
<tr>
<td>NNAL-Gluc Concentrations</td>
<td>-0.0088</td>
<td>-0.0451</td>
<td>-0.1497</td>
<td>-0.1508</td>
</tr>
<tr>
<td></td>
<td>[-0.31, 0.29]</td>
<td>[-0.34, 0.26]</td>
<td>[-0.43, 0.16]</td>
<td>[-0.43, 0.16]</td>
</tr>
<tr>
<td>Ratio (NNAL-Gluc: NNAL Concentrations)</td>
<td>0.0707</td>
<td>-0.1945</td>
<td>-0.1737</td>
<td>-0.0837</td>
</tr>
<tr>
<td></td>
<td>[-0.23, 0.36]</td>
<td>[-0.47, 0.11]</td>
<td>[-0.45, 0.13]</td>
<td>[-0.37, 0.22]</td>
</tr>
</tbody>
</table>

Note: Numbers in the parentheses show the 95% confidence interval of the respective correlation coefficient.
<table>
<thead>
<tr>
<th>Component</th>
<th>Eigenvalue (autoscaled)</th>
<th>Explained variance %</th>
<th>Cumulative variance %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76.43</td>
<td>60.66</td>
<td>60.66</td>
</tr>
<tr>
<td>2</td>
<td>40.4</td>
<td>32.06</td>
<td>92.72</td>
</tr>
<tr>
<td>3</td>
<td>9.17</td>
<td>7.28</td>
<td>100.00</td>
</tr>
</tbody>
</table>
Figure 26: Scores plot from the PCA for the first and second principal components (Numbers on the scores plot correspond to the subject I.D.)
these subjects, although having different concentrations of free and conjugated NNAL. A closer examination of the non-classified class revealed a small sub-class positioned at the extreme right along the PCA axis where the concentrations of NNAL and/or total NNAL were BLOQ (orange ellipse).

Correlation and the importance of feature variables are decided from the loading plots. Figure 27 shows the loading plots for the first two components of the nitrosamine data. As seen in the plot, there is mild correlation between the NNAL-Gluc concentrations with NNAL concentrations and the ratio of NNAL-Gluc to NNAL concentrations (described by the cosine of angles between these vectors). On the other hand, correlation between NNAL concentrations and the ratio of NNAL-Gluc to NNAL concentrations is less. Also, all features are important in describing the first principal component. The second component is mainly characterized by NNAL concentrations and the ratio of NNAL-Gluc to NNAL concentrations, with almost not contribution from NNAL-Gluc concentrations.

In order to better understand and verify discrimination among the objects by PCA, a statistical approach consisting of cluster analysis was performed. This approach sorts a set of objects described by several features, into ‘homogeneous’ clusters. Similarity index values are then calculated by the following equation: similarity_{ab} = 1 - \frac{d_{ab}}{d_{max}}, where d_{ab} is the Euclidian distance of objects a and b and d_{max} the largest Euclidian distance in the data set (Wiedemann et al., 2005). The resultant dendrogram is shown in Figure 28. The links between objects in the dendrogram are represented as upside-down U-shaped lines. The height of the U indicates the Euclidian distance between the objects. Upon examination of
Figure 27: Loading plot from the PCA for the first and second principal components (Numbers on the loading plot correspond to the features – 1: NNAL concentrations; 2: NNAL-Gluc concentrations; 3: Ratio of NNAL-Gluc to NNAL concentrations)
Figure 28: Dendrogram showing results of hierarchical cluster analysis
the dendrogram, the data set can be classified into three clusters when the similarity index was fixed at about 0.45. The clusters observed in the dendrogram correspond well with the PCA classification of objects as seen in the scores plot.

The characteristics of the three clusters were as follows:

First cluster (purple): Corresponds to class 1 from PCA analysis

Second cluster (pink): Corresponds to class 2 from PCA analysis

Third cluster (green+orange): Corresponds to the non-classified class from PCA

The sub-group obtained in the PCA scores plot can be observed as a sub-cluster in the third cluster of the dendrogram obtained from hierarchical cluster analysis. This sub-cluster (orange color) can be observed in the third cluster when the similarity index is fixed at about 0.65. This sub-group corresponded to NNAL and/or NNAL-Gluc concentrations that were BLOQ.

Thus, measurement of urinary NNAL and its glucuronide conjugate might provide a valuable tool to assess the cancer risk of an individual subject. In order to validate this tool, individual subjects should be monitored for possible progression towards disease. Correlation of the actual disease outcome should then be performed with the outcome from the NNAL data analysis. In order to make the cancer risk-assessment tool more robust, real sample analysis should be performed under controlled conditions and on increased number of subjects. Lack of important information and no control on variables such as type of diet, brand and type of cigarette smoked, active years of smoking, other disease conditions or impairments, concurrent medication, etc. are all important factors that can affect an individuals’ susceptibility to cancer. Nevertheless, under controlled conditions,
measurement of NNAL and its glucuronide metabolite in human urine can be used to investigate the extent of NNK metabolism, thus giving potential insights in the assessment of lung cancer risk.

3.4. Conclusions

The determination of NNAL in human urine using on-line extraction on a microfluidic capillary column packed with MIPs coupled with LC-MS/MS has been accomplished. The method was optimized to achieve maximum analyte recovery. Issues related to matrix effects were addressed in the current method, and the method was validated as per the FDA bioanalytical method validation recommendations. The validation data demonstrated excellent precision, accuracy and stability. This method can be used for routine assay of low concentrations of free and total urinary NNAL in smokers. A distinct advantage of the on-line format compared with the off-line format (discussed in Chapter 2) in the extraction of urinary NNAL is the micro-column re-usability. While the MIP cartridges used for off-line extraction are only single-use cartridges, as many as 300 urine samples were successfully analyzed on a single MIP capillary micro-column in the on-line format. The capillary micro-column contains approximately 1 mg of the MIP sorbent beads compared to a 25 mg MIP bed-weight for the off-line cartridges. Considering this, a cost saving of approximately 7500 fold is perceivable just by substituting the cartridge format with the capillary micro-column format and performing the extraction in the on-line mode. This cost saving becomes all the more significant when one takes into account that a single non-
reusable MIP cartridge used in the off-line format costs approximately $11.50. However, it should also be realized that an extra HPLC pump would be required to perform the extraction in the on-line format which may potentially add to the cost. Finally, chemometric methods were employed to analyze the low concentrations of NNAL and NNAL-Glucuronide measured in smokers, and a possible cancer risk assessment tool was developed based on the ratio of the concentrations of these compounds in human urine.
CHAPTER 4 A Proposed Fixed Range Decision Criteria for Bioanalytical Method Transfers and Comparison

Drawn from manuscript published in *J. Chromatogr. B* (2009) 877: 2270-2274

4.1. Introduction

Quantitative bioanalytical methods are used for the analysis of drugs and their metabolites in biological matrices such as blood, plasma, serum or urine. Procedures such as gas chromatography (GC), high-pressure liquid chromatography (LC), combined GC and LC mass spectrometric (MS) procedures such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS are routinely performed for determination of small and large molecules. After development, the method is validated to establish that it is shown to provide accurate, precise, and reproducible data. Bioanalytical method validation plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetics, and toxicokinetics studies involving bioanalytical data. Bioanalytical methods are also routinely transferred and redeveloped for a number of reasons including: a change in analytical technology (e.g., changes in detection system), change in the relevant concentration range (e.g., need for more sensitivity), transfer to new laboratories to improve capacity, addition of metabolites, new species or matrices, etc. (US Food and Drug Administration, 2001). In the current work, an offline sample extraction method for the analysis of urinary tobacco specific nitrosamine NNAL was developed using
molecularly imprinted polymer combined with LC-MS/MS. Subsequently, the method was redeveloped by adapting a capillary microfluidic extraction system coupled online with mass spectrometric detection for analysis of urinary NNAL using molecularly imprinted polymers. Apart from physically transferring or redeveloping a validated procedure, transfer or redevelopment of the method requires assurance that the “test” laboratory or method can obtain reliable and equivalent results as the “reference” laboratory or method. In the current case, the original validated off-line sample extraction method serves as the reference method, while the revised on-line microfluidic sample extraction method will serve as the test method. Comparability of between-study data is helpful to ensure proper interpretations of bioavailability, bioequivalence, pharmacokinetics, and toxicokinetics data. Cross validation and/or transfer of bioanalytical methods encompasses comparison of control data for two or more bioanalytical circumstances used to generate data within the same studies or across different studies (US Food and Drug Administration, 2001). Inter-laboratory and cross validation studies as well as incurred sample reanalysis are generally evaluated using spiked matrix controls.

In order to evaluate whether the on-line microfluidic sample extraction approach for the analysis of NNAL using molecularly imprinted polymers (chapter 3) is capable of producing comparable results as the off-line sample extraction approach (chapter 2), the standard of practice is to cross-validate the method. Cross validation of bioanalytical methods is an area that requires consideration of method transfers concepts. These concepts include (a) an ability to demonstrate that two laboratories or methods are capable of producing equivalent results through appropriate experimental design, (b) to identify the
source of any differences and (c) to resolve the differences. At present, however, several approaches have been used for method comparisons and there is no clear consensus on the most appropriate acceptance criteria or study design in such bioanalytical method data comparisons.

Relatively complex “true” statistical approaches are not easily carried out and/or understood in many bioanalytical laboratories. The standard of practice has been use of fixed criteria established by the FDA. However FDA has not as yet established any regulatory guidance on transfer of bioanalytical methods. Several approaches have been described for the evaluation of method transfer (International Society for Pharmaceutical Engineering, 2003; Dewé et al., 2007; Hartmann et al., 2002; Feng et al., 2006; Vial et al., 1998; de Fontenay, 2008; DeStefano, 2006). Some groups which are active within the pharmaceutical industry have also proposed their own transfer methodologies (Schepers and Watzig, 2005; International Society for Pharmaceutical Engineering, 2003) for different circumstances. Use of multiple approaches in different laboratories can often make it difficult to determine whether the test laboratory meets the needs of a particular project. More recently, Dewé et al., 2007 have proposed the use of total error as a decision criteria in analytical method transfer, and Rozet et al., 2008 have used these criteria in bioanalytical applications. The total error approach is based on a complete consideration of statistics and is more robust. However, this approach may not be readily applicable in laboratories lacking knowledge in statistics. In these cases, it may be useful to investigate “fixed” criteria that are based in part on statistical science. Although not completely correct statistically, fixed criteria based on statistical concepts are more appropriate than
fixed criteria which do not consider statistics. Such criteria would be effective for evaluation of the major risks associated with the transfer of methods and, at the same time, would be more acceptable among non-statistician bioanalysts.

In the present chapter, the predominant approaches used for method transfer evaluation will be discussed addressing the advantages and limitations in each case. A fixed range decision criteria based on standard error of the mean will be proposed. Computer simulations will be evaluated in order to gauge the probability of successful transfers for various experimental designs using the proposed criteria. The on-line and the off-line sample extraction methods for NNAL analysis will be then evaluated for method comparability using the proposed criteria.

4.2. Predominant Approaches used in Bioanalytical Method Transfers

One of the most important questions that need to be addressed when transferring a method is whether or not one can assure comparability of data. There is generally a trade-off between the following two situations. Acceptance criteria that are not very restrictive might fail to adequately demonstrate the equivalence of two methods. In this case, the acceptance criteria are too wide and can lead to acceptance of non-equivalent results from the two participating laboratories (also referred to as β-error or false negative error). Conversely, a criterion that is too narrow might lead to generation of unnecessary data and may lead to rejection of equivalent results from the two participating laboratories (also referred to as α-error or false positive error). The definitions of false positive and false negative errors are summarized in Table 20. The goal of a bioanalytical method transfer
should be to limit the number of false positive as well as false negative errors and ensure that the method performs equivalently at each site. Currently, the predominant approaches used for evaluation of control data for bioanalytical method transfer include i) The independent validation approach, ii) Statistical difference testing using a Student’s t-test and iii) Statistical equivalence testing, iv) The total error based approach.

4.2.1. Independent Validation Approach

According to the independent validation approach, both the reference and the test method must be shown to meet the validation criteria for accuracy (± 15% of the nominal concentration) and precision (≤ 15% coefficient of variation) as prescribed by the US Food and Drug Administration, 2001. The means of the two methods (e.g. x1 and x2 respectively) are compared against the true reference value (μ) which is taken as the nominal spiked concentration. As depicted in Figure 29, the maximum allowable difference between the means of the two laboratories would be 30% (i.e. ± 15% of the nominal value for each mean) and the maximum allowable imprecision would be 15% coefficient of variation (%CV) in order to fulfill the current FDA criteria on bioanalytical method validation. The school of thought with this approach is that if both methods are valid to within FDA criteria using the same control, then the methods are also comparable within FDA established criteria.
Table 20: α- and β-errors in bioanalytical method transfer

<table>
<thead>
<tr>
<th>Statistical test results</th>
<th>Actual equivalence of reference and transferred method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test shows bias present</td>
<td>Bias present: Truly non-equivalent methods</td>
</tr>
<tr>
<td></td>
<td>No bias present: False positive (α-error)</td>
</tr>
<tr>
<td>Test shows no bias present</td>
<td>Bias present: False negative (β-error)</td>
</tr>
<tr>
<td></td>
<td>No bias present: Truly equivalent methods</td>
</tr>
</tbody>
</table>
Figure 29: Normal distributions around means measured at the extremes of the maximum acceptable difference of ±30% based on the existing FDA criteria for independent validation.
It has been very well established that such acceptance criteria based on ad-hoc rules are subject to unknown and uncontrolled risks of accepting unsuitable bioanalytical methods and rejecting suitable bioanalytical methods (Kringle and Khan-Malek, 1994). The non-statistical approach of simply comparing the observed bias and precision between two laboratories to pre-set acceptance limits can result in both, rejection of results that are truly equivalent, and acceptance of results that are truly non-equivalent. For example, Kringle et al., 2001 have discussed that when the true relative bias is at the extreme of the acceptance criteria, there is a 50% chance of falsely concluding that the relative bias is acceptable. This is evident in Figure 29 where half of the area of the distribution around each mean measurement is inside of the acceptance region. This independent validation approach based on FDA acceptance limits might lead to a relatively high acceptance of bioanalytical method transfers when the maximum allowable difference between two laboratories is close to 30% as depicted in Figure 29. This is because such an approach fails to control both, the risk of accepting transfers when they are not truly acceptable, and the risk of rejecting transfers when they are truly acceptable. Such a situation is not desirable and should be avoided.

4.2.2. Difference Approach using Student’s t-test

A common statistical approach used within bioanalytical laboratories is the “difference approach” using the Student’s t-test. A t-test is applied to test the hypothesis that the transferred method yields identical results as the reference method. The t-test asks
the question: “Are the means the same?” The null and alternate hypotheses for a t-test can be stated as:

\[ H_0: X_1 = X_2 \]
\[ H_1: X_1 \neq X_2 \]

The t-test controls the risk of false positives, i.e. the risk of concluding that there is bias when there is none (\(\alpha\)-error). Figure 30A depicts the distribution of measurements around the means of the reference and the test methods (one mean is inside the acceptance range - \(x_1\), and one is outside - \(x_2\)). The FDA allows a ±15% acceptance range for accuracy. A pre-selected t-test \(\alpha\) value therefore allows control of the probability of concluding there is a clinically significant bias between the two methods, when in fact they are equivalent (note: only positive bias is shown in the figure. The risk of committing a false positive or a false negative error can be minimized in the following way. The most obvious approach is to suitably modify the test method (e.g. change technology) so that the means are closer together or the variance around the mean is reduced. This might not be the most efficient approach since many bioanalytical methods are very complex and modification can be a hit or miss proposition.. A second approach would be to adjust the acceptance range for a successful transfer. This would allow control over the risk of committing a false positive or a false negative error. However, there is an inverse relationship between these two types of errors. As shown in Figure 30B for example, when one tries to control the risk of falsely concluding that there is a bias (false positive) by increasing the acceptance range, the risk of wrongly concluding the absence of bias increases correspondingly (false negative). Narrowing the spread of observations around
the means of the reference and the test method can also be accomplished by increasing the number of replicate analyses, and thus improving the estimate of the means. Figure 31A and Figure 31B show that an increase in the sample size will reduce the variance of the mean, thus reducing both, the risk of false positives and false negative errors. Kringle et al., 2001 have demonstrated in the case of analytical method transfers, when the true bias between the reference and the test laboratory is exactly equal to the acceptance limit, the chance of a false negative conclusion (β-error) can be as high as 38% for a three day sampling period. As the sampling period increases to five days, the false negative error reduces to 12%. In both sampling situations, the false positive conclusion is constant at 50% because the true relative bias is exactly at the acceptance boundary. However, when the true relative bias is small and within acceptance limits, the authors were able to show that the probability of correctly concluding that the two methods are equivalent decreases with increasing sample size. This phenomenon is a result of the way the null and alternative hypothesis are set up, and makes the difference test a scientifically illogical approach when the objective is to demonstrate that two methods are sufficiently similar. Such a situation is undesirable and based on these limitations, there is a need to modify the \textit{t-test} approach so that bias and precision can be combined as total error, which is the sum of the systemic and random errors.
Figure 30: Normal distribution of measurements around the means of hypothetical reference and the test methods (x1, x2 respectively). Figures A and B show that decreasing the risk of false positives increases the risk of false negatives.
Figure 31: Normal distribution of measurements around the means of hypothetical reference and the test methods (x1, x2 respectively). Figures A (high variance) and B (low variance) show that the risk of false positives and false negatives are reduced by increasing sample size.
4.2.3. **Statistical Equivalence Test**

The statistical equivalence test is based on reversing the null (H0) and the alternate (H1) hypotheses as follows: H0: | μ_T – μ_R | ≥ δ and H1: | μ_T – μ_R | < δ where μ_T and μ_R are the mean results of the test and reference formulations, and δ is the acceptance limit pre-specified by the analyst. If the null hypotheses is rejected, the test and reference methods will be considered comparable (i.e. μ_T = μ_R). This statistical test is rigorous and scientifically correct. It was originally proposed for bioequivalence studies by Schuirmann, 1987 and has also been extended to analytical method comparisons by Hartmann et al., 2002. In the case of bioanalytical method transfers, a two-sided (1-2α)100% confidence interval will be constructed for μ_T – μ_R that will then be compared to the FDA acceptance criteria of -15% to +15%.

Considering this, the test method can be considered equivalent to the reference method at a 95% confidence level if the entire two sided 90% confidence interval is completely included in the acceptance range. Figure shows the normal distributions (± 15% CV) of the difference (d) between the reference and test method means around the extremes of the FDA guidance accuracy criterion (± 15%) to yield an acceptance criterion of 30%. Applying the reverse hypothesis t-test, as shown in Figure 32, *situation 1* would lead to a conclusion that there is no significant difference since the estimated mean difference along with the 90% CI is entirely within the acceptance criteria. *Situation 2* would lead to a conclusion that there is a significant difference and in the case of *situation 3*, the transferred method is out of specification when compared with the reference method. By stating the hypothesis in such a manner, we gain control over the risk of falsely
concluding that the test method is equivalent to the reference method, when in fact they are not. In this case, the probability of accepting a method that shows a relevant bias of 30% is limited to a fixed $\beta$-error.

The statistical equivalence test is especially appropriate when the objective is to demonstrate sufficient equivalence beyond a reasonable doubt. This test asks the question “Do the means differ more than a certain amount?” Although the statistical equivalence test controls the false negative error at a fixed level a limitation of this test is that the false positive error may be unacceptably high, and like the Student’s $t$-test, is controlled only with a sufficiently large number of replicates.

### 4.2.4. Total Error Based Approach

In order to avoid limitations observed with the above described approaches, it has been proposed to combine the estimates of systematic and random errors into one single decision criterion – the total error defined as the sum of the systematic and random errors. The concept of total error is shown in Figure 33 (Westgard and Barry, 1986). The total error is the sum of random and systematic errors. The random error can either be positive or negative. It can thus potentially add to systematic errors. Combination of accuracy and precision criteria allows acceptance of situations where there is no bias but a random variation higher than the acceptance limit. In such a case, the absence of systematic error may compensate for random error and one can still guarantee that the results will be close enough to their true value. The opposite situation also exists when a systematic difference is not acceptable by the systematic error criterion but the total error criterion is met because the random variation is small. The total error approach simultaneously controls the
Figure 32: Normal distributions (± 15% CV) of the difference between the reference and test method means ($d$) around the extremes of the FDA guidance accuracy criterion (± 15%) to yield a total error criterion of 30%. Solid lines across the measured situational mean represent a 90% confidence interval, which correspond to a one-sided 95% confidence interval. For the test method to be acceptable the entire CI should be within the acceptance limits. (Situation 1: No significant difference; Situation 2: Significant difference; Situation 3: Cannot conclude with confidence that there is no significant difference)
Figure 33: Total error concept
risk of both false positive and false negative errors, and is statistically the most correct approach (Dewé et al., 2007).

Dewé et al., 2007 have developed the statistical method based on total error concepts applicable for analytical method transfers. The principle of this method is to calculate a tolerance interval \([L_R, U_R]\) from the experimental results for the test laboratories (or receiver laboratory as per the authors’ nomenclature), in which we expect to find a large proportion (\(\beta\)) of the measurement results. This interval is then compared with respect to the acceptance limit interval \([\mu^U(1-\lambda), \mu^L(1+\lambda)]\), where \(\mu^L\) and \(\mu^U\) are the upper and lower limits of a confidence interval (usually a 95% confidence interval) for the reference laboratory mean measurement, and \(\lambda\) is the is a tolerance value chosen in accordance with the performance of the measurement method of interest (\(\lambda\) is selected as 15% in case of bioanalytical method transfers). The transfer is accepted if the \(\beta\)-expectation tolerance interval for the test laboratory falls within this acceptance limit.

The \(\beta\)-expectation tolerance interval for the test (receiver) laboratory \([L_R, U_R]\) to estimate the interval in which a proportion \(\beta\) of the measured population is expected to belong can be calculated as:

\[
[L_R, U_R] = [\hat{\mu}_R - k \hat{\sigma}_{RI}; \hat{\mu}_R + k \hat{\sigma}_{RI}]
\]

where \(L\) and \(U\) are the lower and upper limits of the tolerance interval, sub-script \(R\) indicates test (receiver) laboratory, \(\mu\) is the mean concentration value, \(\sigma\) is the standard deviation, sub-script \(I\) indicates the inter-run precision estimate, and \(k\) is calculated as
follows in order to have an expected proportion $\beta$ of the population within the tolerance interval:

$$k = t\left(f, \frac{1 + \beta}{2}\right) \sqrt{1 + \frac{J\hat{R} + 1}{N(\hat{R} + 1)}} \quad \text{with} \quad J = \frac{(\hat{R} + 1)^2}{\left(\hat{R} + \frac{1}{2}\right) + \left(1 - \frac{1}{N}\right)}$$

where $t(f, \gamma)$ is the $\gamma$th percentile of a student $t(f)$ distribution and $R$ is the ratio between the run-to-run variance and the within-run variance. $I$ indicates the number of runs with $J$ replicates.

This is a sophisticated statistical approach to judge method transfers which considers a full risk analysis. However, such a sophisticated approach may not always be easily applicable within laboratories lacking statisticians. Thus, there may be some benefit in a compromise between a completely statistically derived approach, and a fixed range approach derived from statistical considerations.

4.3. Proposed Fixed Criterion

As described earlier, bioanalysts have been historically more prepared to accept criteria which are fixed and easy to use. The “4/6/15” rule is one such example in method validation. According to this rule as defined in FDA Bioanalytical Method Validation guidance, “at least four of every six QC samples should be within 15% of their respective nominal value. Two of the six QC samples may be outside the 15% of their respective
nominal value, but not both at the same concentration” (US Food and Drug Administration, 2001). A criticism of this criterion is that it lacks a consistent statistical foundation. In the absence of an accepted guidance criterion for method transfer, many laboratories choose to use the fixed accuracy criteria of ±15% to compare the difference between means of the reference and the test method (DeStefano, 2006). Since this involves a comparison of the means, it is logical to allow for random variation although no allowance is generally made. Failure to account for imprecision in such a comparison would lead to method transfers being judged unsuccessful, when the method is truly valid under both conditions. At the same time, failure to evaluate imprecision could also lead to the acceptability of method transfer when the transfer is truly not acceptable.

It would be desirable to employ approaches based on statistical considerations to establish fixed criteria rather than using fixed criteria based on a consensus opinion without statistical considerations. Such a proposal, like any other “fixed” criterion, may have some drawbacks and not be entirely correct statistically, but can have practical and conceptual advantages over a straight comparison of means approach.

If μT is defined as the test method mean result, and μR as the reference method mean result, the proposed acceptance criterion would be more accurately comprised of the sum of the FDA guidance accuracy limit of ±15% and the standard error of the mean of μT. The standard error of the mean is defined as the ratio of the standard deviation “s” of the test method to the square root of the number of replicate measurements ‘n’ (i.e., s/√n). The standard error of the mean provides a gauge for how variable the mean can be expected to be when performing n replicate analyses (Massart, 1988). Considering the
FDA guidance on precision of 15% CV (US Food and Drug Administration, 2001), fixed acceptance limits for bioanalytical method transfer based on the FDA guidance acceptance limit maxima can be set as ± (15% + 15%/√n). Figure 34 depicts the proposed acceptance criterion that takes into account method imprecision. In this context, the x-axis shows the estimated mean difference (d) between the two methods. A risk analysis can then be employed to determine the probability that the test laboratory would observe results outside the stated acceptance limits of ± (15% + √n). This provides a tool to determine a reasonable fixed criterion for bioanalytical method transfer. Such a tool would depend on the number of samples, the number of times the samples are run, number of quality control samples, etc.

4.4. Experimental

4.4.1. Computer Simulations

Computer simulations were performed in order to evaluate the probability of a successful transfer for different experimental designs. The probability of success for the proposed fixed range total error approach was compared with the independent validation criteria. The transfer simulations were performed using Crystal Ball (ver. 7.3.1) software developed by Oracle (www.crystalball.com). The software uses Monte Carlo simulations to forecast a range of results possible for a given situation. It also shows confidence intervals to gauge the probability of a given event taking place. A one-way random effects (ANOVA I) model described in the equation below was used to generate data for the reference and test laboratories.
Figure 34: Normal distributions (± 15% CV) of the difference between the reference and test method means (d) around the extremes of the FDA guidance accuracy criterion (± 15%), the broadness of which is determined by the standard error of the means. The acceptable difference (d) between reference and test method is based on the maximum allowable error of the mean
$Y_{ij}^R = \mu + \varepsilon_B^R + \varepsilon_W^R$ and $Y_{ij}^T = \mu^T + \varepsilon_B^T + \varepsilon_W^T$

where $Y_{ij}$ is the $j$th ($j = 1, 2, \ldots, J$) replicate observation from the $i$th ($i = 1, 2, \ldots, I$) run, $\mu$ is the true analytical mean, $\varepsilon_B$ and $\varepsilon_W$ are the errors associated with between- and within-run variability. The super-scripts R and T represent the reference and the test laboratories respectively. The random errors $\varepsilon_B$ and $\varepsilon_W$ are assumed to be normally and independently distributed with means zero, and variances $\sigma_B^2$ and $\sigma_W^2$ respectively. The total analytical variance $\sigma_{TOT}^2 = \sigma_B^2 + \sigma_W^2$. The variance estimates used in the data simulations are shown in Table 21. The data were simulated in a manner so that the total analytical variance of the reference method would not exceed the FDA criterion of 15% CV.

The variance of the reference and the test method were assumed to be the same for the various simulations performed. Parameters used for data simulation are depicted in Table 22. Various combinations of experimental designs were simulated. The number of replicates simulated were $J = 3$ and 6, and the number of runs simulated were $I = 3$ and 6. In each case, 10000 simulations were run. The limits of acceptance based on the proposed fixed range approach were set as $\pm (15\% + \frac{15\%}{\sqrt{n}})$ where $n$ is the number of samples evaluated. Since an actual experiment would involve comparing mean values between the results of the two laboratories, a standard error can be expected when performing $n$ replicate analyses of the same sample. This acceptance criterion is logically more correct, since it provides an estimate of the confidence in the mean value. The quality of the mean results would be expected to improve as the number of replicates increases, and consequently the acceptance limits would become narrower. For various combinations of these simulations, the probability of the test method results falling within acceptance limits
Table 21: Estimate of variances for one-way random effects model

<table>
<thead>
<tr>
<th>Variance component</th>
<th>Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma^2_w$</td>
<td>$\sum_{i=1}^{I} \sum_{j=1}^{J} (Y_{ij} - \bar{Y}_j)^2 / (I-1)$</td>
</tr>
<tr>
<td>$\sigma^2_b$</td>
<td>$\left{ \frac{J \sum_{i=1}^{I} (Y_i - \bar{Y})^2}{(I-1)} - \sigma^2_w \right} / J$</td>
</tr>
<tr>
<td>$\sigma^2_{TOT}$</td>
<td>$\sigma^2_w + \sigma^2_b$</td>
</tr>
</tbody>
</table>
**Table 22: Parameters used for computer simulations**

<table>
<thead>
<tr>
<th>Variance component</th>
<th>Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ng/mL)</td>
<td>0.5, 4, 80</td>
</tr>
<tr>
<td>Within-run %RSD</td>
<td>4, 7, 10</td>
</tr>
<tr>
<td>Variance Ratio (R = \sigma^2_B / \sigma^2_W)</td>
<td>0.5, 1</td>
</tr>
<tr>
<td>Bias (%) between the averages of reference and test laboratories</td>
<td>14, 15, 16, 18, 20, 22, 25, 30</td>
</tr>
</tbody>
</table>
was evaluated. The probability of a successful transfer for the proposed fixed range acceptance criterion was then compared with the ±15% difference between the means criterion without any allowance for imprecision.

4.4.2. Application of Criteria to Real Data for comparison of Offline and Online Sample Extraction Procedure for the analysis of NNAL

The proposed fixed range acceptance criterion for method comparison was evaluated through application to data for the offline and an online sample extraction method for the extraction of tobacco specific nitrosamine NNAL in human urine. Initially, an offline sample extraction method using molecularly imprinted polymers specific to NNAL in SPE format was developed. The sample extraction method was based on offline SPE due to commercially available molecularly imprinted polymer cartridges for NNAL in SPE format. Subsequently, a capillary microfluidic system developed in our laboratory for immunoaffinity separations (Peoples and Karnes, 2008; Peoples et al., 2007; Peoples et al., 2008) was adapted to extraction of NNAL from human urine using molecularly imprinted polymers. This method was developed in an online format in order to improve the assay. Both the methods were based on LC-MS/MS detection operated in the positive ESI mode suitable for analysis of free and total NNAL as described in Chapter 2 and Chapter 3. Some of the other advantages of the online sample extraction method included low sample volume, high throughput, reusable microfluidic columns, elimination of the evaporation step for sample concentration and low cost per sample.
This was a typical example of method transfer in which a validated offline sample extraction method for analysis of NNAL is adapted to an online format. Method comparison was indicated such that the modified method generates “equivalent” results to the reference method. For both the offline and online method, the inter- and intra-assay precision and accuracy were calculated from quality control samples at four concentration levels (LOQ quality control – 20 pg/mL, low quality control 60 pg/mL, medium quality control 400 pg/mL and high quality control 2000 pg/mL) over three validation days. The means of the test online method were then compared with the reference offline method. The success or failure of the transfer was then evaluated using the proposed fixed range decision criteria based on a consideration of standard error of the means. At the same time, success or failure of the transfer was also evaluated by the comparison of the means approach employing an acceptance limit of ±15 %, without accounting for imprecision and the total error approach suggested by Dewé et al., 2007. The β-expectation tolerance interval calculations for the total error based approach were performed using the E-Noval Software (ver. 3.0) designed by Arlenda Laboratory Solutions (Liege, Belgium).

4.5. Results and Discussion

4.5.1. Computer Simulations

Figure 35 to Figure 38 shows the probability of successful transfer for the test method based on the two separate decision criteria for simulated results involving various experimental scenarios. As seen in these figures and as expected, there is a clear difference in the probabilities of a successful transfer for the two criteria. For example, considering
Figure 35, if the true relative bias between the means of the two methods is 15%, the ±15% criterion without accounting for imprecision demonstrates a probability of rejecting a transfer of 50% for all combinations of runs and replicates. Such a large probability of rejection for a level of imprecision that is acceptable according to the FDA criterion may not be reasonable. Conversely, if the limits of acceptance are established to be ± (15% + 15%/√n) we can conclude from the simulation that the probability of an unsuccessful transfer is less than or equal to 5% for all experimental designs, thereby saving the labor and cost of generating unnecessary data.

If one compares the results represented by Figure 35 and Figure 36, it can be observed that for both criteria, when the relative bias is within the acceptance criterion, the probability of accepting a transfer is greater with more precise methods. Conversely, when the relative bias is outside the acceptance criteria, the probability of rejecting a transfer increases with more precise methods. There is a better correlation for the proposed fixed range acceptance criteria in this regard as seen by the steeper slopes of the probabilities of successful transfer for simulations with a 4% RSD as compared to simulations with a 10% RSD. Thus, as the precision of a method improves, the chance that a correct decision is made is better controlled with the ± (15% + 15%/√n) criteria as compared to the ±15% criteria.

Another interesting observation is seen when the true bias between the means of the two methods is exactly at the boundary of the FDA acceptance limit for accuracy (±15%) (US Food and Drug Administration, 2001). Looking at Figure 35, a node is observed when one views the probability of successful transfer for various experimental designs. This
indicates that at the boundary of acceptance, the probability of a successful transfer does not change even as the experiments become more rigorous. The failure rate of a 3-replicate 3-run design is about 50% and is the same for a 6-replicate 6-run simulation. Such an outcome negates the benefit of rigorous experimental designs in their ability to more accurately assess transfers. No such node is observed in the case of the proposed fixed criteria. In fact, the acceptance limits changes in this case as the experimental design changes. For instance, the probability of accepting a transfer with a 3 run 3 replicate design is about 50% at the acceptance boundary of ± 20% [i.e. ± (15% + 15%/9)]; however, the probability of accepting a transfer increases to approximately 75% with a 6 run 6 replicate design at the acceptance boundary of 17.5% [i.e. ± (15% + 15%/36)] as observed in Figure 35.

Figure 37 shows simulations for a concentration of 0.5 ng/mL with an RSD of 10% and a variance ratio R = 0.5. When comparing Figure 36 and Figure 37, the probabilities of accepting a comparison are almost constant over a broad range of concentrations using both the criteria for the various experimental designs. Figure 38 shows simulations for 0.5 ng/mL with an RSD of 10% and a variance ratio (R) = 1. When comparing Figure 37 and Figure 38, it can be observed that the difference in variance ratio did not affect the outcome of accepting a transfer for both criteria. In other words, the probability of accepting a comparison is constant for varying values of R (i.e. \( \sigma^2_B / \sigma^2_W \)) using both criteria for the various experimental designs.
Figure 35: Probability of successful transfer as a function of % relative bias between test and reference methods for simulated results and various experimental scenarios (Concentration = 80 ng/mL; within-run RSD = 4%; R = 0.5). Dotted lines represent the ±15% criterion without accounting for imprecision. Continuous lines represent the proposed ±(15%+15%/√n) criterion.
Figure 36: Probability of successful transfer as a function of % relative bias between test and reference methods for simulated results and various experimental scenarios (Concentration = 80 ng/mL; within-run RSD = 10%; R = 0.5). Dotted lines represent the ±15% criterion without accounting for imprecision. Continuous lines represent the proposed ±(15%+15%/√n) criterion.
Figure 37: Probability of successful transfer as a function of % relative bias between test and reference methods for simulated results and various experimental scenarios (Concentration = 0.5 ng/mL; within-run RSD = 4%; R = 0.5). Dotted lines represent the ±15% criterion without accounting for imprecision. Continuous lines represent the proposed ±(15%+15%/√n) criterion.
Figure 38: Probability of successful transfer as a function of % relative bias between test and reference methods for simulated results and various experimental scenarios (Concentration = 80 ng/mL; within-run RSD = 4%; R = 0.5). Dotted lines represent the ±15% criterion without accounting for imprecision. Continuous lines represent the proposed ±(15%+15%/√n) criterion.
Based on the simulations, the simplest experimental design of 3-runs and 3-replicates provide reasonable results using the proposed fixed range criterion. Experimental designs that employ a greater number of runs and/or replicates provide an increased probability of success for systematic error differences of 15% or less. It is more efficient in terms of throughput, however, to use experimental design with fewer replicates and runs in order to detect differences. Similarly, for systemic errors outside the acceptance range, the results can be made more reliable with an increase in the number of replicates and runs, resulting in an increased rejection rate.

Based on a 3-replicates and 3-runs experimental design, a fixed range acceptance criterion can be proposed as $\pm(15\% + \frac{15\%}{\sqrt{9}}) = \pm20\%$. Since the criterion is derived based on a consideration of one standard deviation, the probability that the true mean would be expected to be within the defined range would be 67%. Each concentration would require individual evaluation and must conform to the acceptance criterion for a method to be considered equivalent. A 3 run, 3 replicates design would thus result in a consistent, reliable and easy to remember rule of thumb which we will refer to as the 3/3/20 rule based on the number of runs (3), the number of replicates (3) with a maximum allowable relative bias of the mean test result of $\pm20\%$. Similarly, for a 3 run 6 replicate experimental design, the fixed range acceptance criteria can be proposed as $\pm (15\% + \frac{15\%}{\sqrt{18}}) = \pm18.5\% (= 18\% approximately) of the reference method mean. A 3/6/18 rule can be suggested in this case resulting in a more rigorous experimental design and an improved ability to detect differences at the expense of requiring a larger number of experiments.
Although the proposed acceptance criteria controls the risk of α errors more efficiently when compared to the ±15% criterion without an allowance for precision, it increases the risk of committing a β error. This is evident from the results of simulations depicted in Figure 35 to Figure 38. The proposed criterion is a “fixed range criterion” however, and a drawback of any fixed criteria is that it is not possible to simultaneously control both α and β errors. Individual laboratories might require tighter control of β errors and rigorous statistical approaches are acknowledged to be more correct in providing this. Fixed criteria are universally used and accepted in regulated environments however, and a fixed criterion which better controls β errors can be proposed as follows.

An alternative approach to limit β error, would be to reduce the accuracy criteria of ±15% prescribed by the FDA by a factor equal to 15%/\sqrt{n}. In this alternate approach, the proposed fixed criterion would then be a maximum allowable acceptance limit of ±15% and would still account for imprecision. The proposed acceptance limit would then be a sum of the more conservative accuracy criterion of ± (15% - 15%/\sqrt{n}) and the maximum allowable standard error of the mean of the test method i.e., 15%/\sqrt{n}. This would result in a maximum acceptance limit of ± 15% for method comparison that would depend on the experimental design. Figure 39 and Figure 40 show representative simulation results for the modified criterion. The parameters simulated were similar to those previously described. The systematic bias simulated was in the range of 9% to 30%. It can be observed from these results that the probabilities have been reversed when compared with the earlier described ±(15% + 15%/\sqrt{n}) criterion. For example, when the proposed criterion was fixed at ±(15% + 15%/\sqrt{n}), the risk of making a false negative error for a 6 run 6
replicate design at the acceptance boundary of 17.5% was approximately 70%. This is evident from Figure 35. This risk of committing a false negative error at the boundary of acceptance, however, reduces to about 50% for the same experimental design when the acceptance criterion is fixed at ±15% as seen in Figure 39. Figure 39 shows simulation results for a RSD of 4%, while Figure 40 shows simulation results for a RSD of 10%. Comparing the simulation results of Figure 39 and Figure 40, it can be observed that more precise methods have better control over falsely rejecting truly comparable methods as well as falsely accepting truly non-equivalent methods. Simulation outcomes based solely on the accuracy criterion (shown by dotted lines in Figure 39 and Figure 40) would result in a greater incidence of rejecting truly comparable methods. Thus, a trade-off exists between the type of error that needs a more stringent control, and the proposed acceptance criteria present a good compromise. Based on the simulation results, an experimental design of 6 runs and 6 replicates provides greater control over the β-error when compared to an experimental design with lesser number of runs or replicates. Based on a 6-replicates and 6-runs experimental design, a fixed range acceptance criterion can be proposed as ±(15% - 15%/√36 + 15%/√36) = ±15%. A 6/6/15 rule can be suggested in this case.
Figure 39: Probability of successful transfer using a more conservative accuracy criterion as a function of % relative bias between test and reference methods for simulated results and various experimental scenarios. (Concentration = 80 ng/mL; within-run RSD = 4%; R = 0.5). Dotted lines represent the ±15% criterion without accounting for imprecision. Continuous lines represent the proposed ±(15%+15%/√n) criterion.
Figure 40: Probability of successful transfer using a more conservative accuracy criterion as a function of % relative bias between test and reference methods for simulated results and various experimental scenarios. (Concentration = 80 ng/mL; within-run RSD = 10%; R = 0.5). Dotted lines represent the ±15% criterion without accounting for imprecision. Continuous lines represent the proposed ±(15%+15%/√n) criterion.
4.5.2. Application of Criteria to Real Data for comparison of Offline and Online Sample Extraction Procedure for the analysis of NNAL

Table 23 represents a summary of the results obtained for the offline and the online sample extraction methods for the analysis of NNAL. Precision (measured as the percent relative standard deviation based on the pooled variance, %RSD) and accuracy (measured as the percent difference from nominal, %DFN) were calculated from quality control samples at four quality control concentrations over three validation days. The overall inter and intra-assay precision was $\leq 7.8\%$ and $7.2\%$ respectively for the offline mode. The inter- and intra-assay accuracy for the offline method was $\leq 1.3\%$ and $5.2\%$ respectively. Similarly, in the case of the online sample extraction method, the overall inter and intra-assay precision was $\leq 11.7\%$ and $11.3\%$ respectively, while the inter- and intra-assay accuracy was $\leq 9.3\%$ and $14.8\%$, respectively. Comparing the difference between the means of the two methods (Table 23), the method transfer is successful according to the $\pm 15\%$ criteria as shown in the table.

Table 23 also represents the transfer outcome using the proposed fixed range acceptance criteria based on standard error of the mean. The acceptance criteria in this case as discussed earlier was fixed at $\pm 20\%$ of the reference method mean when 3 independent runs with 3 replicates were used. As we can observe from the outcome of the results, using the proposed criteria, the transfer is successful in this case also.

Table 24 represents the transfer outcome using the total error based tolerance interval approach suggested by Dewé et al., 2007 (see appendix for software output used in the generation of results). The tolerance value $\lambda$ was set as $15\%$ keeping in mind the FDA
regulations (US Food and Drug Administration, 2001). A 95% confidence interval was calculated around the reference method mean, results of which are depicted in Table 24. The upper and lower limits of this confidence interval were multiplied by $(1-\lambda)$ and $(1+\lambda)$ respectively, in order to set the acceptance interval. A tolerance interval for the test method was then calculated. The maximum risk tolerated $1-\beta$ was fixed at 20%. The lower and upper limits of this tolerance interval are shown in Table 24. It is observed that the tolerance interval is included in the acceptance limits and the effective risk is smaller than the maximum tolerated risk $(1-\beta)$. In other words, it can be said with 95% confidence that at least 80% of the future results of NNAL obtained with the test method will be within 15% of the reference method mean. This implies that the total error based total error approach resulted in a successful transfer of the method under the specified conditions.

Although the transfers were successful using all the three approaches, these examples do not imply that the three approaches are equivalent. They only imply that all the three approaches result in judging the example comparison to be successful in this case.
Table 23: Summary of results obtained for the offline method and the online method for analysis of NNAL. The transfer outcomes are shown for (i) ±15% difference between the means acceptance criteria without accounting for precision and (ii) ±(15% + 15%/√n) total error based fixed acceptance criteria. The results are based on an outcome for 3 runs and 3 replicates.

<table>
<thead>
<tr>
<th>Spiked QC conc. (pg/mL)</th>
<th>Offline Method (Reference Method)</th>
<th>Online Method (Test Method)</th>
<th>% Difference between Means</th>
<th>Transfer Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td></td>
<td></td>
<td>±15% criteria without accounting for precision</td>
</tr>
<tr>
<td>Spiked</td>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QC conc. (pg/mL)</td>
<td>20  60  400  2000</td>
<td>20  60  400  2000</td>
<td>20  60  400  2000</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>20.26  60.18  395.76  2018.79</td>
<td>19.63  54.94  381.78  1813.33</td>
<td>3.07  8.70  3.53  10.18</td>
<td>Successful</td>
</tr>
<tr>
<td>SD</td>
<td>1.58  3.17  20.08  107.88</td>
<td>1.67  2.33  44.79  36.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%RSD</td>
<td>7.80  5.26  5.07  5.34</td>
<td>8.51  4.25  11.73  2.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%DFN</td>
<td>1.28  0.30  -1.06  0.94</td>
<td>-1.83  -8.43  -4.56  -9.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD – Standard deviation
RSD – Relative standard deviation based on pooled variance
DFN – Deviation from nominal
Table 24: Summary of results obtained for the offline method and the online method for analysis of NNAL. The transfer outcomes are shown for the total error based tolerance interval approach. The results are based on an outcome for 3 runs and 3 replicates (See appendix for software output used in the generation of results).

<table>
<thead>
<tr>
<th>Conc. (pg/mL)</th>
<th>Offline Method (Reference Method)</th>
<th>Online Method (Test Method)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower limit 95% CI*</td>
<td>Upper limit 95% CI*</td>
</tr>
<tr>
<td>20</td>
<td>17.38</td>
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<td>60</td>
<td>56.23</td>
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<td>400</td>
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<td>410.80</td>
</tr>
<tr>
<td>2000</td>
<td>1939.02</td>
<td>2106.01</td>
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</table>

*CI – Confidence Interval

(1-\(\beta\)) = 20%
\(\lambda\)=15%
4.6. Conclusion

Several approaches have been suggested in the literature to address the issue of analytical method transfer and method comparisons. The statistically rigorous total error approach proposed by Dewé et al., 2007 is perhaps the most robust and scientifically correct. However, this may require adequate knowledge of statistics as well and an evaluation of the variability of the methods being tested. A ±15% difference between the means criteria, without allowance for precision, does not provide a reasonable approach to address the risk of false conclusions regarding method comparisons. A user-friendly “fixed” range acceptance criteria has been proposed based on a consideration of method accuracy and precision. This approach combines a consideration of the maximum allowable FDA bioanalytical guidance limits on precision and accuracy and the ease of application of a fixed range approach. Various experimental designs were evaluated and although there are many options, an experiment with 3 runs with 3 replicates each at 3 concentrations with an acceptance range of ±20% is proposed. This general approach could be applied to individual dosed subject samples or prepared controls. A 3/3/20 criterion for method comparisons turns out to be reasonable in terms of its ease of use, the number of required experiments and the control of false positive results. In instances where a greater control over false negative results is required, a reduced accuracy based fixed criterion has been suggested. The proposed fixed range approach could also be extended to other validation situations in which data comparisons are involved. For example, in the case of incurred sample reanalysis, an experimental design could be anticipated that would involve comparing observations with n=1 for each comparison. An acceptance criterion consistent
with this approach and the established guidance would then be \( \pm (15\% + \sqrt{15}\%) = \pm 30\% \).

The off-line and the on-line sample extraction methods for NNAL analysis discussed in chapters 2 and 3 respectively were deemed comparable using the proposed fixed range acceptance criteria.
CHAPTER 5 Overall Summary

Tobacco products have been estimated to cause approximately 30% of deaths in developed countries (American Cancer Society, 2009). Cancers of the lung, larynx, oral cavity, esophagus, pancreas, kidney, liver, bladder, stomach and colon are all associated with tobacco use (Hecht, 2003). Several types of tobacco carcinogen related biomarkers have been studied extensively. Although measurement of DNA and protein adducts may provide a direct link to cancer risk, there have been significant analytical challenges in the measurement of these compounds (Poirier et al., 2000).

Urinary metabolites are probably the most practical biomarkers for cancer risk and provide important information about carcinogen dose and metabolism (Hecht, 2002; Hecht, 2003). Tobacco specific nitrosamines (TSNA) are a prominent class of carcinogens found only in tobacco products that play an important role in cancer induction (Hecht, 1999). Of the several TSNAs that have been identified in tobacco products, NNK is one of only two carcinogens considered to be carcinogenic to humans. It is a systemic lung carcinogen (International Agency for Research on Cancer, 2007).

The three main routes of NNK metabolism in humans include carbonyl reduction, pyridine oxidation and α-hydroxylation (Hecht, 2002). NNK undergoes extensive carbonyl
reduction to form NNAL, which is also a potent lung carcinogen having similar
carcinogenicity and a similar metabolic pathway to NNK. One feature of NNAL
metabolism is the formation of NNAL-Gluc, which is an important detoxification pathway
for NNK and NNAL (Chen et al., 2008b). Both NNAL and its glucuronides are excreted in
human urine, although unchanged NNK has not been detected in human urine. The half-
lives of NNAL and NNAL-Gluc are long as compared to other urinary metabolites making
them ideal candidates as biomarkers (Stepanov et al., 2008). The pyridine-N-oxidation
pathway results in the formation of NNK-N-oxide and NNAL-N-oxide (Hecht et al.,
1993b). This is, however, a minor metabolic pathway for NNK. Metabolic activation of
NNK and NNAL to DNA adducts proceeds via the α-hydroxylation pathways. The end
products of this pathway are keto acid and hydroxy acid. Both of these compounds can be
measured in human urine. However, these cannot be used to measure the extent of α-
hydroxylation because they are formed from nicotine as well (Hecht, 2002). This renders
measurement of the end-products of the α-hydroxylation pathways non-specific as markers
and suggests that measurement of urinary NNAL and its glucuronide conjugate may
provide the best biomarker of tobacco smoke lung carcinogen metabolism.

In chapter 1 of the thesis, a listing of the literature compiling the issues and
developments in the analysis of tobacco specific nitrosamines in human biological matrices
is provided. The two most commonly used approaches for TSNA analysis in biological
matrices are based on GC-thermal energy analysis (TEA) and LC-ESI-MS/MS. The
advantages as well as the limitations for both approaches have been discussed. The
nitrosamine selective GC-TEA technique had been the method of choice for bioanalysis of TSNAs until the early 2000’s. However, due to the extensive and complex sample preparation steps for GC-TEA methods, recently there has been a shift to development of methods for TSNA bioanalysis using the more widely available LC-MS/MS technique. LC-MS/MS based methods are also more amenable to thorough validation. Addressing ion suppression matrix effects remains an important challenge for hyphenated mass spectrometry based methods, however.

Recently, molecularly imprinted polymers have been developed with applications in the area of bioanalysis to provide high selectivity and molecular recognition ability (Haginaka, 2009). The application of these synthetic polymers as sorbents allows pre-concentration and selective as well as selective extraction of target analytes. Xia et al., 2005 had published a method for the off-line analysis of NNAL in human urine by extraction on a molecularly imprinted polymeric (MIP) cartridge followed by LC-MS/MS analysis. Chapter 2 of this thesis investigated the presence of matrix effects and related problems with sensitivity for modification of the method by Xia et al., 2005. Issues related to throughput were also addressed and improved in the modified method. In this method, urinary NNAL was analyzed based on an “off-line” sample extraction using MIPs combined with LC/MS-MS. The method was validated and the validation data demonstrated excellent precision, accuracy and stability complying with the FDA bioanalytical method validation guidance. This method was used for quantitative analysis of free and total urinary NNAL concentrations in incurred samples obtained from smokers.
With the goal of further addressing throughput and efficiency, chapter 3 of this dissertation involved development of a high-throughput “on-line microfluidic” sample extraction method using capillary micro-columns packed with MIP beads hyphenated with tandem MS for the analysis of urinary NNAL. The method was optimized and matrix effects were evaluated and resolved. The method enabled low volume, rapid analysis of urinary NNAL by direct injection on the microfluidic column packed with molecularly imprinted beads specific to NNAL. The method was validated as per the FDA bioanalytical method validation guidance. It was used for the analysis of NNAL and NNAL-Gluc concentrations in smokers’ urine. The ratio of NNAL-Gluc to NNAL was used to investigate the extent of NNAL metabolism. Chemometric methods were examined to develop a potential cancer risk assessment tool based on pattern recognition in the measured concentrations of these compounds.

In chapter 4 of this dissertation, method comparison approaches for the on-line and the off-line sample extraction techniques were investigated for generating comparable results. Currently there is no clear consensus on the most appropriate acceptance criteria or study design for transfer or comparison of bioanalytical methods. In this part of the dissertation, a ‘fixed’ range acceptance criterion based on a combined consideration of method precision and accuracy, and the FDA bioanalytical guidance limits on precision and accuracy was proposed. Considering the FDA guidance on precision and accuracy, fixed acceptance limits for bioanalytical method transfer based on the FDA guidance acceptance limit maxima were set as ±(15% + 15%/√n). Data simulations to evaluate the probabilities of a successful transfer using the proposed criterion were performed.
Alternate fixed range criterion providing better control over false negative errors (β-error) was also proposed. The advantages and disadvantages of the proposed fixed range criteria have also been discussed.

In conclusion, the high-throughput, sensitive, selective and reliable methods developed in the current work for the measurement of free and total urinary NNAL will provide improved tools and approaches that will continue to provide understanding of the mechanisms by which tobacco carcinogens cause cancer and may enable the prediction of cancer risk. This ultimately would lead to advances and improvements in chemoprevention strategies.
References:


determination of tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-
1-butanol in human urine by solid phase extraction using a molecularly imprinted
polymer and liquid chromatography tandem mass spectrometry. *J Chromatogr B


Stepanov, I. and Hecht, S. S., (2005). Tobacco-specific nitrosamines and their pyridine-N-
glucuronides in the urine of smokers and smokeless tobacco users. *Cancer

spectrometric quantitation of nicotine, cotinine, and 4-(methylnitrosamino)-1-(3-
15(12):2378-2383.

Stepanov, I., Hecht, S. S., Duca, G., and Mardari, I., (2006b). Uptake of the tobacco-
specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanolone by

in human toenails by liquid chromatography-electrospray ionization-tandem mass

Stepanov, I., Upadhyaya, P., Carmella, S. G., Feuer, R., Jensen, J., Hatsukami, D. K., and


Yuan, J. M., Koh, W. P., Murphy, S. E., Fan, Y., Wang, R., Carmella, S. G., Han, S., Wickham, K., Gao, Y. T., Yu, M. C., and Hecht, S. S., (2009). Urinary levels of

Appendix
Commands Used in MATLAB:

\[
[n,p]=\text{size}(\text{TSNA});
\]
\[
\text{meanx}=\text{mean}(\text{TSNA});
\]
\[
\text{stdevx}=\text{std}(\text{TSNA});
\]
\[
\text{xas}=\text{(TSNA-ones(n,1)*meanx)./(ones(n,1)*stdevx)};
\]
\[
\text{covariance\_matrix}=\text{cov}(\text{TSNA})
\]
\[
\text{correlation\_matrix}=\text{corrcorr}(\text{TSNA})
\]
\[
[u, s, v] = \text{svd}(\text{covariance\_matrix},0)
\]
\[
\text{eigen\_val\_covariance\_matrix}=s*s
\]
\[
[u1, s1, v1] = \text{svd}(\text{xas},0)
\]
\[
\text{plot}(u1(:,1)*s1(1,1),u1(:,2)*s1(2,2),',')
\]
\[
\text{for}\ m=1:n;\text{text}(u1(m,1)*s1(1,1)),(u1(m,2)*s1(2,2)),16023823366\text{end};
\]
\[
\text{title('SVD-mode Scores Plot')}\
\]
\[
\text{xlabel('PC #1')}
\]
\[
\text{ylabel('PC #2')}
\]
\[
\text{plot}(v1(:,1),v1(:,2),'*m')
\]
\[
\text{for}\ m=1:p;\text{text}(v1(m,1),v1(m,2),\text{num2str}(m));\text{end};
\]
\[
\text{title('SVD-mode Loadings Plot')}
\]
\[
\text{xlabel('PC #1')}
\]
\[
\text{ylabel('PC #2')}
\]
\[
\text{X = xas;}
\]
\[
\text{Y = pdist(X);} \]
\[
\text{Z = linkage(Y);} \]
\[
\text{dendrogram(Z);} \]
\[
\text{dendrogram(Z,43);} \]
\[
\text{set(H,'LineWidth',2);} \]
\[
\text{ylabel('Distance between Objects');}
\]
\[
\text{xlabel('Objects (Smokers)' );}
\]
PRODUCT DATA SHEET

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<th>Normal Human Urine – INDIVIDUAL 24HOUR DONATIONS FROM HEAVY SMOKERS – Donors deny a history of renal, endocrine, hepatic, autoimmune or neoplastic disease. Urine collected from healthy adult donors, and maintained as small individual pools. Urine is non-sterile in present containers. Note: Each individual donor smokes more than 25 cigarettes daily from ages 21 to 60.</th>
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<td>VOLUME PER UNIT:</td>
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<td>Although donors were tested and found negative for certain serologies (see &quot;Testing&quot; below), please handle these biologicals as if they are capable of transmitting disease.</td>
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<td>STORAGE:</td>
<td>-20 °C or below (Frozen)</td>
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<td>THE INDIVIDUAL DONORS IN THIS URINE ORDER WERE TESTED BY FDA-APPROVED METHODS AND FOUND NEGATIVE FOR HIV-1 PCR, HIV-1/HIV-250, HBsAg, AND HCV PCR</td>
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THIS PRODUCT IS BEING PROVIDED FOR RESEARCH AND/OR MANUFACTURING PURPOSES ONLY. THE USER ASSUMES ALL RESPONSIBILITIES FOR ITS PROPER USAGE AND DISPOSAL IN ACCORDANCE WITH ALL APPLICABLE REGULATIONS. DO NOT USE IN HUMANS OR ANIMALS

Date: May 6, 2009
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Software output [E-Noval (ver. 3.0)] used in the generation of results for the total error approach for method comparison:

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7 Accuracy
8 Linearity of results
9 Limit of detection (LOD), limits of quantitation (LOQ) and dosing range
10 Selectivity
11 Conclusion
12 Bibliographic references
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Appendix 3 Back-calculated concentrations from the selected model
Appendix 4 Diagnosis
Appendix 5 Statistics
Appendix 6 Summary tables

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Table 3.1. Sorting of the calibration models
Table 3.2. Regression parameters
Table 4.1. Trueness
Table 5.1. Relative Intermediate Precision and Repeatability
Table 5.2. Absolute Intermediate Precision and Repeatability
Table 5.3. 95% Upper Confidence Limits
Table 5.4. By series Recovery of the Samples
Table 6.1. Uncertainty
Table 7.1. Method accuracy obtained by considering Weighted (1/σ) Linear Regression
Table A-1.1. Calibration standards
Table A-1.2. Validation standards
Table A-2.1. Data alignment
Table A-2.2. Method trueness and accuracy obtained by considering Weighted (1/σ) Linear Regression
Table A-2.3. Method trueness and accuracy obtained by considering Linear Regression
NNAL-OFFLINE

1 INTRODUCTION

The aim of validation is to establish that the analytical method is suitable for its intended use and consequently to prove the reliability of the results obtained within well defined limits. Several widely recognized validation criteria should be tested in order to ensure the reliability of the developed method.

The validation criteria presented in this report are:

- response function (calibration curve)
- linearity
- precision (repeatability and intermediate precision)
- accuracy
- sensitivity
- limits of detection (LOD) and of quantitation (LOQ)
- range

The applied rounding rule is: values greater or equal to 100 will be reported with scientific notation with four decimals, values greater or equal to 1000 will be reported as an integer, values smaller than 1000 and greater or equal to 1 will be reported with four numbers, four significant decimals will be used for reporting values lower than 1, values strictly inferior to 10^{-1} will be reported as 0. This rule applies to absolute values, the ± sign doesn’t account as significant figure.

However, this rule is not applied to the data introduced by the user.

2 EXPERIMENTAL DESIGN

In order to validate the analytical method, two kinds of samples were prepared in an independent way: calibration standards and validation standards.

The calibration standards are samples with or without matrix, containing known concentrations of the analyte of interest and are only used for calibration.

The matrix is: Urine

In Table 2.1 are reported the number of calibration standards by concentration level, the concentration levels envisaged and the different series that were performed.

Table 2.1. Calibration standards

<table>
<thead>
<tr>
<th>Series</th>
<th>Concentration levels (pg/mL)</th>
<th>No. of repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>2</td>
</tr>
</tbody>
</table>

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10/19/2009
1 | 2.0 | 2
---|---|---
1 | 3.0 | 2
1 | 4.0 | 2
1 | 5.0 | 2
1 | 6.0 | 2
1 | 7.0 | 2
2 | 1.0 | 2
2 | 2.0 | 2
2 | 3.0 | 2
2 | 4.0 | 2
2 | 5.0 | 2
2 | 6.0 | 2
2 | 7.0 | 2
3 | 1.0 | 2
3 | 2.0 | 2
3 | 3.0 | 2
3 | 4.0 | 2
3 | 5.0 | 2
3 | 6.0 | 2
3 | 7.0 | 2

The total number of observations is 42.

The validation standards are reconstituted samples within the matrix containing known concentrations of the analyte of interest which are considered as true values by consensus.

In Table 2.2 are reported the number of validation standards by concentration level, the concentration levels envisaged and the different series that were performed.

<table>
<thead>
<tr>
<th>Series</th>
<th>Concentration levels (pg/mL)</th>
<th>No. of independent repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>4.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>5.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>6.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>7.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>6.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>3</td>
</tr>
</tbody>
</table>

The total number of observations is 36.

All calibration and validation standards are presented in Appendix 1.

Enter your comments here (Optional):

---

3 RESPONSE FUNCTION

The response function of an analytical method is, within the range, the existing relationship between the response (signal) and the concentration (quantity) of the analyte sample. The calibration curve is the most appropriate response function.
Table 3.1 presents all selected regression models that have been sorted according to the Accuracy Index (cf. Appendix 2).

**Table 3.1: Sorting of the calibration models**

<table>
<thead>
<tr>
<th>Model</th>
<th>Accuracy Index</th>
<th>Lower and upper limits of quantitation (LOQ) (pg/mL)</th>
<th>Dosing Range Index</th>
<th>Precision Index</th>
<th>Trueness Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weighted (1/X) Linear Regression</td>
<td>0.7699</td>
<td>[51.00, 2000]</td>
<td>0.9039</td>
<td>0.4725</td>
<td>0.940</td>
</tr>
<tr>
<td>Weighted (1/X)^2 Linear Regression</td>
<td>0.7579</td>
<td>[22.08, 2000]</td>
<td>0.9988</td>
<td>0.4674</td>
<td>0.9327</td>
</tr>
<tr>
<td>Linear Regression</td>
<td>0.0000</td>
<td>[253.2, 2000]</td>
<td>0.8317</td>
<td>0.5515</td>
<td>0</td>
</tr>
</tbody>
</table>

Explanation of Accuracy Index, Dosing Range Index, Precision Index and Trueness Index can be found in Appendix E.

The selected calibration model is: **Weighted (1/X) Linear Regression**

Enter your comments here (Optional):

The calibration curves obtained from this regression model (cf. Table 3.2 and Figure 3.1) are represented by the following equation:

\[ Y = a + bX \]

where \( Y \) = Analytical response (in Area Ratio) and \( X \) = Introduced concentration (in pg/mL)

**Table 3.2: Regression parameters**

<table>
<thead>
<tr>
<th>Series</th>
<th>Intercept</th>
<th>Slope</th>
<th>( r^2 )</th>
<th>Residual d.f.</th>
<th>RSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0027930</td>
<td>0.0016484</td>
<td>0.99959</td>
<td>12</td>
<td>0.034022</td>
</tr>
<tr>
<td>2</td>
<td>0.003729</td>
<td>0.001658</td>
<td>0.99811</td>
<td>12</td>
<td>0.05047</td>
</tr>
<tr>
<td>3</td>
<td>0.011058</td>
<td>0.001695</td>
<td>0.99514</td>
<td>12</td>
<td>0.1071</td>
</tr>
</tbody>
</table>

\( r^2 \) = coefficient of determination, d.f. = degrees of freedom; RSS = residual sum of squares

**Figure 3.1: Calibration curves**

---


---

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4 TRUENESS

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value and a mean experimental one. It gives information on systematic error.

As shown in Table 4.1, trueness is expressed in terms of absolute bias (in pg/mL), relative bias (%) or recovery (%) at each concentration.
level of the validation standards.

If, for a concentration level \( \hat{\mu} \) is the mean of the introduced concentrations and \( \bar{x} \) is the estimate of the mean concentration obtained from calculated concentrations then we have:

Absolute bias = \( \bar{x} - \hat{\mu} \)

Relative bias (%) = \( 100 \times \frac{\bar{x} - \hat{\mu}}{\hat{\mu}} \)

Recovery (%) = \( 100 \times \frac{\bar{x}}{\hat{\mu}} \)

Table 4:1. Trueness

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Mean back-calculated concentration (pg/mL)</th>
<th>Absolute bias (pg/mL)</th>
<th>Relative bias (%)</th>
<th>Recovery (%)</th>
<th>95% Confidence Interval of Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>20.00</td>
<td>19.72</td>
<td>-0.27</td>
<td>-1.39%</td>
<td>96.81</td>
<td>[95.72, 100.2]</td>
</tr>
<tr>
<td>2.0</td>
<td>60.00</td>
<td>59.87</td>
<td>-1.27</td>
<td>-1.97%</td>
<td>98.82</td>
<td>[94.91, 102.7]</td>
</tr>
<tr>
<td>3.0</td>
<td>400.0</td>
<td>395.2</td>
<td>-4.86</td>
<td>-1.23%</td>
<td>98.79</td>
<td>[94.81, 102.7]</td>
</tr>
<tr>
<td>4.0</td>
<td>2000</td>
<td>2022</td>
<td>22.22</td>
<td>1.11%</td>
<td>99.11</td>
<td>[96.35, 106.3]</td>
</tr>
</tbody>
</table>

5 PRECISION

Precision is the closeness of agreement among measurements from multiple sampling of a homogenous sample under the recommended conditions. It gives some information on random errors and it can be evaluated at three levels: repeatability and intermediate precision.

As can be seen in Table 5:1 and Table 5:2, precision is expressed in terms of standard deviation (SD) and relative standard deviation (RSD) values for repeatability and intermediate precision.

The estimates of variance components are obtained by the iterative approach of restricted maximum likelihood (REML).

Table 5:1. Relative Intermediate Precision and Repeatability

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Repeatability (RSD%) (^1)</th>
<th>Intermediate precision (RSD%) (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>20.00</td>
<td>6.322</td>
<td>9.313</td>
</tr>
<tr>
<td>2.0</td>
<td>40.00</td>
<td>5.360</td>
<td>6.283</td>
</tr>
<tr>
<td>3.0</td>
<td>400.0</td>
<td>5.042</td>
<td>5.042</td>
</tr>
<tr>
<td>4.0</td>
<td>2000</td>
<td>5.419</td>
<td>5.419</td>
</tr>
</tbody>
</table>

\(^1\) The RSD% for Repeatability and Intermediate precision has been obtained by dividing the corresponding SD by the "Mean Introduced Concentration".

Table 5:2. Absolute Intermediate Precision and Repeatability

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Repeatability (SD - pg/mL)</th>
<th>Between-series (SD - pg/mL)</th>
<th>Ratio of Variance components (between / within)</th>
<th>Intermediate precision (SD - pg/mL)</th>
</tr>
</thead>
</table>

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Report customization

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4. Precision
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NNAL-ONLINE

1. Introduction

The aim of validation is to establish that the analytical method is suitable for its intended use and consequently to prove the reliability of the results obtained within well-defined limits. Several widely recognized validation criteria should be tested in order to ensure the reliability of the developed method.

The validation criteria presented in this report are:
- response function (calibration curve)
- linearity
- precision (repeatability and intermediate precision)
- accuracy
- limits of detection (LOD) and of quantitation (LOQ)
- range

The applied rounding rule is: values greater or equal to 1000 will be reported with scientific notation with four decimals, values greater or equal to 100 will be reported as an integer, values smaller than 100 and greater or equal to 1 will be reported with four numbers, four significant decimals will be used for reporting values lower than 1, values strictly inferior to $10^{-3}$ will be reported as 0. This rule applies to absolute values, the - sign doesn't account as significant figure.

However, this rule is not applied to the data introduced by the user.

2. Experimental design

In order to validate the analytical method, two kinds of samples were prepared in an independent way: calibration standards and validation standards.

The calibration standards are samples with or without matrix, containing known concentrations of the analyte of interest and are only used for calibration.

The matrix is: Urine

In Table 1 are reported the number of calibration standards by concentration level, the concentration levels envisaged and the different series that were performed.

<table>
<thead>
<tr>
<th>Series</th>
<th>Concentration levels (pg/mL)</th>
<th>No. of repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>2</td>
</tr>
</tbody>
</table>

https://www.arlenda.be/enoval3.0/design.jsp?type=2&matrix=0&model=report.xml&com... 10/19/2009
The total number of observations is 42.

The validation standards are reconstituted samples within the matrix containing known concentration of the analyte of interest, which are considered as true values by consensus.

In Table II are reported the number of validation standards by concentration level, the concentration levels envisaged and the different series that were performed.

<table>
<thead>
<tr>
<th>Series</th>
<th>Concentration levels (pg/ml)</th>
<th>No. of independent repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>4.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>3</td>
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<tr>
<td>2</td>
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<tr>
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<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>3</td>
</tr>
</tbody>
</table>

The total number of observations is 36.

All calibration and validation standards are presented in Appendix 1.

Enter your comments here (optional):

3. Response function

The response function of an analytical method is, within the range, the existing relationship between the response (signal) and the concentration (quantity) of the analyte sample. The calibration curve is the most appropriate response function.

Table III presents all selected regression models that have been sorted according to the Accuracy Index (cf. Appendix 2).

Table III - Sorting of the calibration models

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<table>
<thead>
<tr>
<th>Model</th>
<th>Accuracy Index</th>
<th>Lower and upper limits of quantitation (LOQ) (pg/mL)</th>
<th>Dosing Range Index</th>
<th>Precision Index</th>
<th>Trueness Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weighted (1/Y)</td>
<td>0.654</td>
<td>[55.62, 1278]</td>
<td>0.6177</td>
<td>0.5777</td>
<td>0.7043</td>
</tr>
<tr>
<td>Linear Regression</td>
<td>0.6479</td>
<td>[98.98, 1989]</td>
<td>0.7522</td>
<td>0.4806</td>
<td>0.7523</td>
</tr>
<tr>
<td>Weighted (1/Y^2)</td>
<td>0.5203</td>
<td>[49.92, 1200]</td>
<td>0.6210</td>
<td>0.5602</td>
<td>0.5512</td>
</tr>
</tbody>
</table>

Explanation of Accuracy Index, Dosing Range Index, Precision Index and Trueness Index can be found in Appendix E.

The selected calibration model is: Weighted (1/Y) Linear Regression

Enter your comments here (Optional)

The calibration curves obtained from this regression model (cf. Table IV and Figure 1) are represented by the following equation:

\[ Y = a + bx \]

where \( Y \) = Analytical response (in Area Ratio) and \( x \) = Introduced concentration (in pg/mL)

**Table IV - Regression parameters**

<table>
<thead>
<tr>
<th>Series</th>
<th>Intercept</th>
<th>Slope</th>
<th>( r^2 )</th>
<th>Residual d.f.</th>
<th>RSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.03002</td>
<td>0.003505</td>
<td>0.9995</td>
<td>12</td>
<td>0.05141</td>
</tr>
<tr>
<td>2</td>
<td>-0.01112</td>
<td>0.003259</td>
<td>0.9997</td>
<td>12</td>
<td>0.05496</td>
</tr>
<tr>
<td>3</td>
<td>-0.000820</td>
<td>0.003129</td>
<td>0.9997</td>
<td>12</td>
<td>0.00023</td>
</tr>
</tbody>
</table>

\( r^2 \) = coefficient of determination; d.f. = degrees of freedom; RSS = residual sum of squares

**Figure 1 - Calibration curves**

[Graph showing calibration curves for Series 1]
4. Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value and a mean experimental one. It gives information on systematic error.
As shown in Table V, trueness is expressed in terms of absolute bias (in pg/mL), relative bias (%) or recovery (%) at each concentration level of the validation standards.

If, for a concentration level, $\hat{\mu}$ is the mean of the introduced concentrations and $\bar{x}$ is the estimate of the mean concentration obtained from calculated concentrations then we have:

Absolute bias = $\bar{x} - \hat{\mu}$

Relative bias (%) = $100 \times \frac{\bar{x} - \hat{\mu}}{\hat{\mu}}$

Recovery (%) = $100 \times \frac{\bar{x}}{\hat{\mu}}$

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Mean back-calculated concentration (pg/mL)</th>
<th>Absolute bias (pg/mL)</th>
<th>Relative bias (%)</th>
<th>Recovery (%)</th>
<th>95% confidence interval of recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>10.00</td>
<td>19.41</td>
<td>-3.8993</td>
<td>-2.946</td>
<td>97.05</td>
<td>[89.71, 104.40]</td>
</tr>
<tr>
<td>2.0</td>
<td>60.00</td>
<td>65.48</td>
<td>-5.18</td>
<td>-7.524</td>
<td>92.47</td>
<td>[88.70, 96.21]</td>
</tr>
<tr>
<td>3.0</td>
<td>400.0</td>
<td>396.6</td>
<td>-1.401</td>
<td>-0.3503</td>
<td>99.65</td>
<td>[94.08, 105.22]</td>
</tr>
<tr>
<td>4.0</td>
<td>2000</td>
<td>1773</td>
<td>-236.8</td>
<td>-11.34</td>
<td>88.44</td>
<td>[86.01, 91.33]</td>
</tr>
</tbody>
</table>

### 5. Precision

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions. It gives some indication of random errors and it can be evaluated at two levels: repeatability and intermediate precision.

As can be seen in Table VI and Table VII, precision is expressed in terms of standard deviation (SD) and relative standard deviation (RSD) values for repeatability and intermediate precision.

The estimates of variance components are obtained by the iterative approach of restricted maximum likelihood (REML).

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Repeatability (RSD%) 1</th>
<th>Intermediate precision (RSD%) 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>10.00</td>
<td>2.000</td>
<td>2.911</td>
</tr>
<tr>
<td>2.0</td>
<td>60.00</td>
<td>4.859</td>
<td>4.859</td>
</tr>
<tr>
<td>3.0</td>
<td>400.0</td>
<td>5.974</td>
<td>7.617</td>
</tr>
<tr>
<td>4.0</td>
<td>2000</td>
<td>3.065</td>
<td>3.573</td>
</tr>
</tbody>
</table>

1 The RSD% for Repeatability and Intermediate precision has been obtained by dividing the corresponding SD by the "Mean Introduced concentration".

### Table VI - Absolute Intermediate Precision and Repeatability

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Repeatability (SD - pg/mL)</th>
<th>Between-series (SD - pg/mL)</th>
<th>Ratio of Variance components (between / within)</th>
<th>Intermediate precision (SD - pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>20.00</td>
<td>1.911</td>
<td>0</td>
<td>0</td>
<td>1.911</td>
</tr>
<tr>
<td>2.0</td>
<td>60.00</td>
<td>2.915</td>
<td>0</td>
<td>0</td>
<td>2.915</td>
</tr>
</tbody>
</table>

https://www.arlenda.be/enoval3.0/design.jsp?type=2&matrix=0&model=report.xml&com... 10/19/2009
Table VIII - 95% Upper confidence limit

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>95% Upper Confidence Limit Repeatability (SD - pg/mL)</th>
<th>95% Upper Confidence Limit Intermediate Precision (SD - pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>23.00</td>
<td>3.270</td>
<td>3.270</td>
</tr>
<tr>
<td>2.0</td>
<td>60.00</td>
<td>4.908</td>
<td>4.908</td>
</tr>
<tr>
<td>3.0</td>
<td>400.0</td>
<td>46.77</td>
<td>196.4</td>
</tr>
<tr>
<td>4.0</td>
<td>2000</td>
<td>117.4</td>
<td>231.7</td>
</tr>
</tbody>
</table>

In addition, the recovery of each series as well as for all the series is reported in Table IX.

Table IX - Recovery of the Samples

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Series</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Back-calculated concentration (pg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1</td>
<td>23.00</td>
<td>18.81</td>
<td>94.68</td>
</tr>
<tr>
<td>1.0</td>
<td>2</td>
<td>28.00</td>
<td>29.17</td>
<td>100.0</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>26.00</td>
<td>19.25</td>
<td>96.26</td>
</tr>
<tr>
<td>2.0</td>
<td>Mean of all series</td>
<td>20.00</td>
<td>19.41</td>
<td>97.05</td>
</tr>
<tr>
<td>2.0</td>
<td>1</td>
<td>56.00</td>
<td>54.34</td>
<td>95.56</td>
</tr>
<tr>
<td>2.0</td>
<td>2</td>
<td>56.00</td>
<td>55.93</td>
<td>94.90</td>
</tr>
<tr>
<td>2.0</td>
<td>3</td>
<td>56.00</td>
<td>55.10</td>
<td>91.97</td>
</tr>
<tr>
<td>2.0</td>
<td>Mean of all series</td>
<td>60.00</td>
<td>55.48</td>
<td>92.47</td>
</tr>
<tr>
<td>3.0</td>
<td>1</td>
<td>400.0</td>
<td>372.4</td>
<td>93.11</td>
</tr>
<tr>
<td>3.0</td>
<td>2</td>
<td>400.0</td>
<td>417.6</td>
<td>104.4</td>
</tr>
<tr>
<td>3.0</td>
<td>3</td>
<td>400.0</td>
<td>425.8</td>
<td>101.4</td>
</tr>
<tr>
<td>3.0</td>
<td>Mean of all series</td>
<td>400.0</td>
<td>398.6</td>
<td>99.65</td>
</tr>
<tr>
<td>4.0</td>
<td>1</td>
<td>2000</td>
<td>1717</td>
<td>85.84</td>
</tr>
<tr>
<td>4.0</td>
<td>2</td>
<td>2000</td>
<td>1277</td>
<td>63.33</td>
</tr>
<tr>
<td>4.0</td>
<td>3</td>
<td>2000</td>
<td>1836</td>
<td>90.81</td>
</tr>
<tr>
<td>4.0</td>
<td>Mean of all series</td>
<td>2000</td>
<td>1773</td>
<td>88.66</td>
</tr>
</tbody>
</table>

Enter your comments here (Optional)

6. Uncertainty of measurements

The uncertainty is a parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measured.

Table X - Uncertainty

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration (pg/mL)</th>
<th>Uncertainty of the bias (pg/mL)</th>
<th>Uncertainty (pg/mL)</th>
<th>Expanded Uncertainty (pg/mL)</th>
<th>Relative Expanded Uncertainty (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>20.00</td>
<td>0.0370</td>
<td>2.014</td>
<td>4.629</td>
<td>20.14</td>
</tr>
</tbody>
</table>

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7. Accuracy

Accuracy refers to the closeness of agreement between the test result and the accepted reference value, namely the conventionally true value. The Accuracy takes into account the total error, i.e. systematic and random errors, related to the test result. It is assessed from the Accuracy Profile illustrated in Figure 2.

The acceptance limits have been set at ± 15%, selected according to the intended use of the analytical procedure.

An accuracy profile is obtained by linking on one hand the lower bounds and on the other hand the upper bounds of the β-expected tolerance intervals calculated at each concentration level. The formula for calculating these β-expected tolerance intervals is:

\[ \text{bias} (\%) \pm k \times \text{RSD}_{\text{exp}} (\%) \]

Explanation about k and RSD_{exp} can be found in Appendix 5.

The method is considered as valid within the range for which the Accuracy Profile is within the acceptance limits. This approach gives the guarantee that each further measurement of unknown samples is included within the tolerance limits at the 95.0 % level.

The plan red line is the relative bias, the dashed lines are the β-expected tolerance limits and the dotted lines represent the acceptance limits. The dots represent the relative error of the back-calculated concentrations and are plotted with respect to their targeted concentration.

The upper and lower β-expected tolerance limits expressed in relative error are also presented in Table XII as a function of the introduced concentrations. Risk of measurements at each level are only estimated when there are at least two replicates per series.

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Figure 3 - Risk profile obtained by considering weighted (1/Y) linear regression

The dotted line represents the maximum risk level chosen: 20.0 %.

Table III - Method accuracy obtained by considering weighted (1/Y) linear regression

<table>
<thead>
<tr>
<th>Concentration level (pg/mL)</th>
<th>Mean introduced concentration(^1) (pg/mL)</th>
<th>Beta-expectation tolerance limits (pg/mL)</th>
<th>Relative Beta-expectation tolerance limits (%)</th>
<th>Risk(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>20.00</td>
<td>[16.53, 22.23]</td>
<td>[-17.04, 11.17]</td>
<td>19.06</td>
</tr>
<tr>
<td>2.0</td>
<td>60.06</td>
<td>[51.18, 69.70]</td>
<td>[-14.71, -0.05]</td>
<td>9.331</td>
</tr>
<tr>
<td>3.0</td>
<td>180.0</td>
<td>[149.9, 240.7]</td>
<td>[-20.67, 11.97]</td>
<td>13.25</td>
</tr>
<tr>
<td>4.0</td>
<td>360.0</td>
<td>[166.2, 180.8]</td>
<td>[-16.92, -5.76]</td>
<td>19.07</td>
</tr>
</tbody>
</table>

\(^1\) Introduced concentration and associated responses may have been subjected to transformation to align all the data to an average concentration value by concentration level (cf. Appendix 1).

\(^2\) Risk of having measurements falling outside of the acceptance limits.

Enter your comments here (Optional)?

8. Linearity of results

The linearity of an analytical method is the ability within a definite range to obtain results directly proportional to the concentration (quantity) of the analyte in the sample.

A linear regression model (cf. Figure 4) is fitted on the back-calculated concentrations as a function of the introduced concentrations in order to obtain the following equation:

\[
Y = 14.81 + 0.8820 \times X
\]

where \(Y\) = back-calculated concentrations (pg/mL) and \(X\) = introduced concentration (pg/mL). The coefficient of determination (\(R^2\)) is equal to 0.9999. The residual sum of squares (RSS) is equal to 5.571E-04.

The back-calculated concentrations are presented in Appendix 3.

Figure 4 - Relationship between the introduced and the back-calculated concentrations

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Vita

Kumar A. Shah was born on July 01, 1981 in Mumbai, India. He received a Bachelor of Technology degree in Pharmaceuticals and Fine Chemicals from the Institute of Chemical Technology, Mumbai, India in 2003. He received a Master of Technology degree in Pharmaceuticals and Fine Chemicals from Institute of Chemical Technology, Mumbai, India in 2005, after which he joined the PhD program in Pharmaceutics at Virginia Commonwealth University, Richmond, VA. Kumar has authored 5 accepted manuscripts to date from his research at the Institute of Chemical Technology, Mumbai, India and Virginia Commonwealth University, Richmond. These have been published in journals such as *Journal of Chromatography B, Critical Reviews in Toxicology, International Journal of Pharmaceutics* and *Journal of Biomedical Nanotechnology*. Kumar has also co-authored four invited presentations and several posters related to his graduate research. Kumar was recipient of the VCU Leadership and Service Award in 2008. He was also recipient of the AAPS-APQ Graduate Symposium Award in 2009. Apart from this, he has also been awarded the AAPS Travelship award in 2007-2009. Kumar has served as an appointed Graduate Student Representative in the executive committee of the AAPS – Analysis and Pharmaceutical Quality Section (APQ) as well as the AAPS – Membership Strategic Organization Committee from 2007-2009. He has also served in the capacity of President and Vice-President in the VCU-Pharmaceutics Graduate Student Association as well as the VCU-AAPS Student Chapter.