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REVIEW ARTICLE

A review of the analysis of tobacco-specific nitrosamines in biological matrices

Kumar A. Shah, and H. Thomas Karnes

Department of Pharmaceutics, Virginia Commonwealth University, School of Pharmacy, Richmond, Virginia, USA

Abstract

Tobacco use constitutes a leading cause of mortality and morbidity worldwide. Tobacco-specific nitrosamines (TSNAs) are an important class of biomarkers for tobacco carcinogen uptake. The current review focuses on the issues and developments in analysis of these compounds in human biological matrices. The two most widely used 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 review provides an overview of the tools and techniques currently available for TSNA bioanalysis that will help towards the ultimate goal of understanding the mechanisms of cancer caused by the use of tobacco products. A contrast and comparison of the important aspects of bioanalysis such as sample preparation, compound detection, and throughput is discussed for the thermal energy analysis– and mass spectrometry–based techniques. Complex sample extraction procedures, throughput, and the ability to validate are important issues of concern for the gas chromatography–thermal energy analysis–based methods. On the other hand, addressing ion suppression matrix effects remains an important challenge for hyphenated mass spectrometry–based methods. The review also provides an extensive summary of analytical procedures for various studies measuring tobacco-specific nitrosamines in different biological matrices.

Keywords: *Bioanalysis; GC-TEA; LC/MS/MS; sample preparation; tobacco-specific nitrosamines analysis*

Abbreviations: CSP, chiral stationary phase; ESI, electrospray ionization; ETS, environmental tobacco smoke; GC, gas chromatography; Gluc, glucuronide; HPLC, high-performance liquid chromatography; iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butanoic acid; iso-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; LC, liquid chromatography; LLOQ, lower limit of quantitation; MIP, molecularly imprinted polymer; MS, mass spectrometry; NAB, N'-nitrosoanabasine; NAT, N'-nitrosoanatabine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N'-nitrosonornicotine; PAH, polyaromatic hydrocarbon; SD, standard deviation; SFE: supercritical fluid extraction; SPE: solid-phase extraction; TEA, thermal energy analysis; TMS, trimethylsilyl ether; TSNA, tobacco-specific nitrosamine.

Contents

Address for correspondence: Professor Howard Thomas Karnes, Department of Pharmaceutics, Virginia Commonwealth University, School of Pharmacy, 410 N. 12th Street, PO Box 980533, Richmond, VA 23298, USA. Tel.: +1 804 828 3819; Fax: +1 804 828 8359; E-mail: htkarnes@vcu.edu

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Introduction

Cancer Facts and Figures published by the American Cancer Society (2009) estimates 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 precancerous oral lesions, pancreatic and oral cancers, as well as cardiovascular diseases (Hecht et al. 1986 ; International Agency for Research on Cancer, 1985; International Agency for Research on Cancer, 2007).

Tobacco smoke contains more than 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, 1998b; Hoffmann et al. 1987 , 1995, 2001; International Agency for Research on Cancer, 2002; Swauger et al. 2002). Tobacco and tobacco smoke contain three types of nitrosamines. These include volatile nitrosamines, nitrosamines derived from residues of agricultural chemicals on tobacco, and tobacco-specific nitrosamines (TSNAs) (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, 1998a, 1999). Thus far, seven TSNAs have been identified in cigarettes (Djordjevic et al. 1991 ; Fischer et al. 1989 ; Hoffmann et al. 1994 ; Thricker et al. 1991 , 1993), as well as in smokeless tobacco products in even greater concentrations (Hoffmann et al. 1994 , 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 because their origin is specific to tobacco. 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. 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 of these 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, 1981; Castonguay et al. 1983b , 1984, 1985; Meger et al. 1999 ; Richter et al. 2009). The extent of TSNA metabolite formation can vary greatly in animals and humans, as reviewed by Hecht (1998a). For this reason, in the current review, we will restrict our focus on progress and development in the bioanalysis of TSNA compounds and their metabolites in human tissues.

Biomarkers for human uptake and metabolic activation of tobacco-specific nitrosamines

TSNA-related biomarkers have been discussed extensively in the literature. An ideal biomarker would 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 would 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. Adducts of carcinogenic compounds with DNA might provide a 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 (e.g. 1 per 10^6 to 10^8 per normal human bases; De Bont and van Larebeke, 2004) can often make their quantification extremely difficult and/or 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 Schafer 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. High levels of HPB (hydroxy-1-(3-pyridyl)-1-butanone)-releasing adducts have been detected in lung, esophagus, and cardia, which are totally independent from the smoking status. It is speculated that these may have other sources in addition to TSNAs. According to Zwickenpflug (2000) and Thyroller et al. (2002) , one possible source could be the minor tobacco alkaloid myosmine, which also occurs in a wide variety of staple foods, vegetables, fruits, and dairy products.

Moreover, highly sensitive techniques such as ^{32}P-postlabeling and immunoassays used in most of the studies involving DNA- and protein-carcinogen adducts measurements can lack chemical specificity. For instance, methylation of DNA can be caused by different chemical reactions. α-Hydroxylation of methylene carbon adjacent to *N*-nitroso group or α-hydroxylation of the methyl group of NNAL can both form unstable intermediates that can decompose and react with the DNA (Richter et al. 2009). These differences in biochemical reactions may possibly compromise assay specificity. These difficulties are encountered particularly in the case of ^{32}P-postlabeling as discussed by Poirier et al. (2000), Phillips (1996), and Wild and Pisani (1998). A detailed

metabolic scheme discussing NNK metabolism and DNA adduct fiormation firom NNK, NNN, and NNAL can be fiound in literature (Hecht, 2008; Hecht and Hofimann, 1988)☒

Considering the pitfialls that one might encounter in the measurement ofi DNA and protein adducts, measurement ofi urinary biomarkers ofi tobacco carcinogen uptake presents a more practical option (Hecht, 2002)☒Urine is very easy to obtain in suficient quantities and this can allow fior quantitation ofi even trace amounts ofi ThSNA compounds and their metabolites excreted in urine☒Although measurement ofi urinary compounds may not provide the most direct link with the type and risk ofi a particular cancer, they can provide infiormation on the carcinogen dose and as well as the ability ofi an individual to activate or detoxifiy these compounds☒ Moreover, Hecht et al☒(2002) have shown that some ofi these ThSNA compounds have long terminal halfi-lives☒This, along with the fiact that tobacco products are habitually used, provides consistent concentrations ofi ThSNA compounds fior measurement in urine☒

Ofi all the ThSNAs identified, NNK and NNN are the most prevalent strong carcinogens in tobacco products, as documented by Hecht and Hofimann (1988), Spiegelhalder and Bartsch (1996), and Hofimann et al☒(1995)☒Moreover, the International Agency fior Research on Cancer (2007)classifies NNK and NNN as the only ThSNAs carcinogenic to humans☒ Figure 1 shows an overview ofi NNK and NNN metabolism☒ It may be argued that measurement ofi urinary keto and hydroxy acids is more appropriate because these are the end products ofi the DNA adduct–fiorming α-hydroxylation pathway ofi NNK and NNN metabolism☒However, as suggested by Hecht (2002), these compounds cannot be used to measure the extent ofiα-hydroxylation because these are fiormed firom nicotine as well, which is a primary constituent ofi tobacco products☒Recently, nevertheless, Stepanov et al☒(2008) have suggested the use ofi [pyridine-d_4]NNK as a biomarker to measure the deuterium-labeled keto and hydroxyl acid specifically fiormed firom NNK☒Biomarker strategies using stable isotope-labeled compounds can however be challenging☒

As depicted in Figure 1, conversion ofi NNK to NNAL is an important metabolic pathway in humans, with NNAL having similar carcinogenicity as NNK (Castonguay et al☒1983a ; Hecht, 1998a)☒A characteristic fieature ofi NNAL metabolism is the fiormation ofi NNAL-Gluc, which is the most important detoxification product ofi the NNK-NNAL metabolic pathway in humans and animals, as described by Hecht (1998a), Hecht et al☒(1993b) , and Morse et al☒(1990) ☒Additionally, NNAL and its glucuronide have a longer halfi-lifie (at least 10–15 days in smokers, and about 40–45 days in users ofi oral tobacco) when compared with most other urinary metabolites (Hecht, 2003; Hecht et al☒2002 ; Carmella et al☒2009)☒Considering all ofi this, urinary NNAL and NNAL-Gluc have evolved as the most prominently studied ThSNA biomarker☒

In addition to NNAL and NNAL-Gluc, other ThSNAs such as NNN, NAB, and NATh, along with their metabolites, have also been studied☒These compounds have been detected and quantified in urine and saliva ofi tobacco users☒More recently, ThSNAs have been quantified in plasma, which is one ofi the most commonly analyzed biological fiuids in clinical studies☒The concentration ofi these xenobiotics at the receptor sites may be ofiten related to the intensity ofi their pharmacologic and carcinogenic efiects☒These receptor sites are usually located in the tissue ofi cells that are richly perfiused by plasma☒Thus, measurement ofi plasma levels is probably the most appropriate method to study the pharmacokinetics ofi these drugs (Shargel and Yu, 1999)☒Tho fiurther study the prev alence, etiology, and mechanism ofi tobacco carcinogenesis, measurement ofi ThSNAs has been extended to amniotic fiuid, cervical mucus, pancreatic juice, and toenails☒Infiormation regarding the analysis ofi ThSNAs along with appropriate refier ences will be discussed fiurther in this review and have been compiled in Thables 1and 2☒

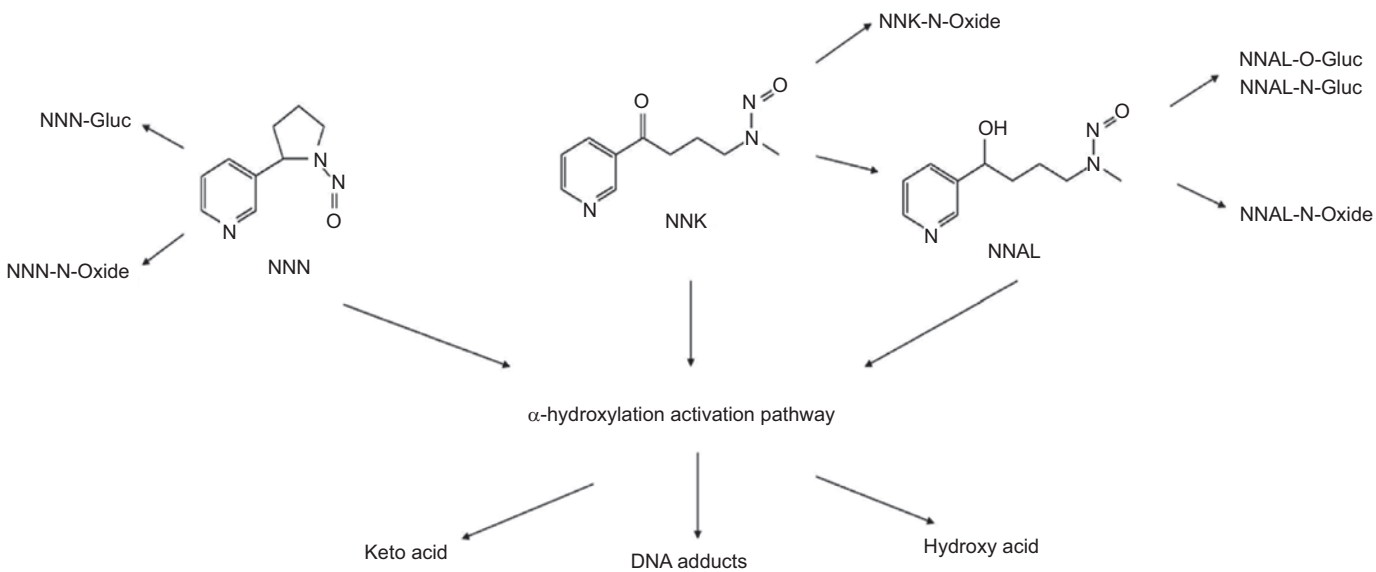

Figure 1. Metabolic scheme fior NNN and NNK. (Adapted firom Hecht, 2003.)

Table 1. Compilation ofi studies analyzing ThSNA compounds in biological matrices using nitrosamine-specific ThEA detection and quantification[a]

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
NNN, NATh, NNK	Snufi dippers (4F)	Saliva	HPLC-ThEA	5.0–125.0 ng/g (NNN); 2.1–201.0 ng/g (NNK); 6.6–147.0 ng/g (NATh)	▪ ~0.8 to 1.6 g saliva collected fior analysis. ▪ No fiurther assay description provided.	Hofimann and Adams, 1981
NNN, NATh, NAB, NNK	Snufi dippers	Saliva	GC-ThEA	Levels ofi ThSNAs measured ranged firom 20–890 µg/kg	▪ Assay description not provided	Hofimann et al., 1982
NNN, NATh, NNK	Betel quid chewers	Saliva	GC-ThEA	1.6–59.7 ng/g (NNN); 1.0–51.7 ng/g (NNK); 0.0–2.3 ng/g (NATh)	▪ Assay description not provided	Nair et al., 1985
NNAL, NNAL-Gluc	11 Smokers (9F) 7 Nonsmokers	Urine	GC-ThEA	0.23–1.0 µg/24 h (NNAL); 0.57–6.5 µg/24 h (NNAL-Gluc). NNAL not detected in nonsmokers. NNK not detected in urine.	▪ 100 ml aliquot size. ▪ Purification ofi extracts using two preparative HPLC steps, fiollowed by derivatization ofi 2° OH group ofi NNAL to trimethylsilyl ether, fiollowed by GC-ThEA analysis. ▪ Internal standard was [5-^{3}H(NNAL)]. ▪ Conversion ofi NNAL-Gluc to the uncon-jugated fiorm achieved by incubation with β-glucuronidase enzyme.	Carmella et al., 1993a
NNAL, NNAL-Gluc	Nonsmokers exposed to sidestream cigarette smoke (5M)	Urine	GC-ThEA	33.9 ± 20.0 ng/24 h (NNAL + NNAL-Gluc)	▪ Sample preparation was based on Carmella et al. (1993a) with a modified internal standard (iso-NNAL).	Hecht et al., 1993a
NNK, NNAL, NNAL-Gluc	Sudanese snufi-dippers (7M)	Urine	GC-ThEA	0.39 ± 0.14 nmol/ml NNAL; 0.88 ± 0.5 nmol/ml NNAL-Gluc	▪ Method was based on Carmella et al. (1993a), with minor modifications.	Murphy et al., 1994
NNAL, NNAL-O-Gluc	Smokers (30M, 31F). Study to investigate intra- and interindi-vidual difierences in metabolites ofi NNK	Urine	GC-ThEA	0.08–7.2 pmol/mg creatinine NNAL. 0.16–19.0 pmol/mg creatinine NNAL-Gluc	▪ Method was based on Carmella et al. (1993a), with minor modifications. ▪ 50–100 ml aliquot size. ▪ Internal standard was iso-NNAL. ▪ Attempts to use normal and reverse-phase SPE instead ofi HPLC fior sample cleanup were unsuccessfiul. ▪ Assay results demonstrated 6 month stability fior NNAL and NNAL-Gluc stored at −20°C ▪ No significant difierence was fiound in urine collection protocols (e.g. morning sample versus 24-h collection). ▪ Detection limit ofi assay was reported as 1ng per urine sample.	Carmella et al., 1995
NNAL, NNAL-Gluc	Smokers (5M, 6F). Study to investigate the efiect ofi watercress consump-tion on metabolism ofi ThSNAs	Urine	GC-ThEA	Average baseline levels ofi NNAL + NNAL-Gluc were 3.28 ± 1.88 nmol/24 h. Average levels on the days ofi watercress consumption were 4.21 ± 2.58 nmol/24 h	▪ Method was based on Carmella et al. (1995), with minor modifications.	Hecht et al., 1995

Thable 1. continued on next page

Table 1. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
NNAL, NNAL-Gluc	Non-tobacco users (8M). Nonsmokers but smokeless tobacco users (39M). Study to investigate relations between urinary biomarkers and oral leukoplakia in smokeless tobacco users.	Urine	GC-ThEA	0.02–8.73 pmol/mg creatinine (NNAL); 0.14–30.3 pmol/mg creatinine (NNAL-Gluc)	▪ Method was based on Carmella et al. (1995), with minor modifications.	Kresty et al., 1996
NNK-N-Oxide, NNAL-N-Oxide	Smokers (18); Smokeless tobacco users (11)	Urine	i. GC-ThEA ii. LC/ESI-MS/MS fior analysis ofi NNAL-*N*-Oxide directly in urine (fior identity confirmation)	NNK-N-Oxide not detected in urine; Levels ofi NNAL-*N*-oxide were 0.06–1.41pmol/mg creatinine in smokers and 0.02–1.20 pmol/mg creatinine in smokeless tobacco users.	▪ i. 20–45 ml aliquot size. ▪ Method involved solvent extraction and purification by HPLC to separate NNAL-*N*-oxide and NNK-*N*-oxide firom endogenous NNAL and NNK. ▪ NNAL-*N*-oxide and NNK-*N*-oxide being thermally unstable were not amenable to direct GC-ThEA analysis. Thus, they were reduced to firee 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.	Carmella et al., 1997
NNK	Smokers (15F), Nonsmokers (10F). Study to identifiy NNK in cervical mucus.	Cervical mucus	Supercritical fiuid extraction fiollowed by: i. GC-ThEA ii. Capillary GC-MS/MS (fior identity confirmation)	Mean levels ofi NNK in cervical mucus ofi smokers 46.9± 32.5 ng/g; Mean levels ofi NNK in cervical mucus ofi nonsmokers 13.0± 9.3 ng/g	▪ Supercritical fiuid extraction ofi cervical mucus (31–615 mg) with the use ofi carbon dioxide that contained 10% methanol. ▪ This was fiollowed by analysis by GC-ThEA and GC-MS/MS. ▪ d_3-NNK was used as the internal standard.	Prokopczyk et al., 1997
NNAL, NNAL-Gluc	Smokers (13F) Study to investigate levels ofi NNK metabolites befiore and afiter indole-3-carbinol (I3C) treatment.	Urine	GC-ThEA	Mean difierence in NNAL + NNAL-Gluc levels befiore and afiter I3C treatment was -0.43 ± 0.16 pmol/mg creatinine.	▪ Method was based on Carmella et al. (1995).	Thaioli et al., 1997
NNAL, NNAL-Gluc	Smokers (34 black and 24 white). Study to investigate difierences in NNK metabolites in black and white smokers.	Urine	GC-ThEA	Black smokers: 1.22 ± 1.44 pmol/mg 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	▪ Method was based on Carmella et al. (1995).	Richie et al., 1997

Thable 1. continued on next page

Table 1. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
NNAL-Gluc	Nonsmokers (5M, 4F). Study to analyze levels ofi urinary NNAL-Gluc in nonsmokers exposed to environmental tobacco smoke.	Urine	i. GC-ThEA ii. GC-MS/MS (fior identity confirmation).	Mean NNAL-Gluc levels 0.059 ± 0.028 pmol/ml (23 pg/ml urine)	▪ 50 ml aliquot size ▪ GC-ThEA Method was based on Carmella et al. (1995) with minor modifications. ▪ Use ofi capillary GC improved sensitivity 20-fiold (LOD 4 fimol/ml). ▪ iso-NNAL was used as internal standard.	Parsons et al., 1998
NNAL, NNAL-Gluc	27 Subjects (smokers) participating in a smoking cessation study	NNAL and NNAL-Gluc in urine, NNK and NNAL in plasma	i. GC-ThEA ii. GC-MS/MS (fior identity confirmation)	Urinary levels: Baseline levels—0.6 ± 0.366 pmol/ml (NNAL), 1.35 ± 0.738 pmol/ml (NNAL-Gluc). 6 weeks afiter cessation, 7.6% ofi original NAL + NNAL-Gluc remained. Plasma levels: NNK was not detected in any ofi the samples. NNAL was detected in CH_2Cl_2 extracts ofi unhydrolyzed plasma firom three ofi the fiour smokers analyzed. Levels ofi NNAL were 0.052, 0.086, and 0.114 pmol/ml plasma. NNAL was not detected in CH_2Cl_2 extracts ofi RBCs, or in the base or acid hydrolysates ofi RBC or plasma.	▪ Method to deternine urinary NNAL and NNAL-Gluc was based on minor modifications ofi 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 CH_2Cl_2, and the combined extracts were analyzed fior unconjugated NNAL and NNK as described fior urine. ▪ The plasma phase treated with 0.1 N NaOH, with sonication fior 1h at room temperature. Afiter neutralization, it was extracted three times with equal volumes ofi CH_2Cl_2 and then analyzed fior NNAL and NNK as fior the urine method.	Hecht et al., 1999
NNAL, NNAL-Gluc	Newborns (31 firom newborns whose mothers smoked and 17 firom newborns whose mothers did not smoke)	Urine	i. GC-ThEA ii. GC-MS/MS (fior identity confirmation)	Mean levels ofi 0.14 pmol/ml (NNAL + NNAL-Gluc) in newborns whose mothers smoked. Not detected in newborns whose mothers did not smoke.	▪ Method was based on Carmella et al. (1993a) and Parsons et al. (1998), with minor modifications.	Lackmann et al., 1999
NNAL, NNAL-Gluc	Pregnant mothers (21 smokers, 30 nonsmokers)	Amniotic fiuid (AF)	i. GC-ThEA ii. GC-MS/MS (fior identity confirmation)	Mean levels in AF ofi mothers who smoked: 0.025 ± 0.029 pmol/ml (NNAL), 0.0032 ± 0.01 pmol/ml (NNAL-Gluc); Mean levels in AF ofi nonsmoker mothers: 0.0018 ± 0.0074 pmol/ml (NNAL), 0.0069 ± 0.03 pmol/ml (NNAL-Gluc);	▪ Method was based on Lackmann et al. (1999), with certain modifications. Further assay details were not provided.	Milunsky et al., 2000
NNAL, NNAL-Gluc	Heavy smokers (13F, 10 M). Study to determine efiects ofi smoking cessation	Urine	GC-ThEA	Average 25% reduction in total NNAL levels observed at week 24 afiter smoking cessation	▪ Method was based on Carmella et al. (1993a) and Carmella et al. (1995).	Hurt et al., 2000
NNAL, NNAL-Gluc	Nonsmoker (23F whose partners smoked, 22F whose partners did not smoke). Study to measure environmental ThSNA in women exposed to EThS	Urine	i. GC-ThEA ii. GC-MS/MS (fior identity confirmation)	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)	▪ Method based on Carmella et al. (1993a); Hecht et al. (1993a), and Parsons et al. (1998), with some modifications. ▪ 20-ml urine aliquots firom nonsmokers and 5-ml aliquots firom smokers were suficient fior analysis.	Anderson et al., 2001

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Table 1. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
NNAL, NNAL-Gluc	Children (38—expo-sure to EThS reported, 35—no exposure to EThS reported)	Urine	i. GC-ThEA ii. GC-MS/MS fior confirmation ofi NNAL-Gluc identity.	Mean levels in children who reported exposure: 0.04 pmol/ml (NNAL + NNAL-Gluc); Mean levels in children who did not report exposure: 0.008 pmol/ml (NNAL + NNAL-Gluc)	▪ Method was based on Hecht et al. (1993a) and Parsons et al. (1998), with some modifications. ▪ 20 ml aliquot size was suficient fior analysis. ▪ Detection limit ofi NNAL + NNAL-Gluc was reported as 0.003 pmol/ml	Hecht et al., 2001
NNAL, NNAL-Gluc	Nonsmokers, smokeless tobacco users (13M). Participating in smoke-less tobacco use cessa-tion study	Urine, Plasma	i. GC-ThEA ii. GC-MS/MS (fior identity confirmation) iii. CSP-GC-ThEA		▪ Method to measure NNAL and NNAL-Gluc in urine was based on Hecht et al. (1999). ▪ Enantiomers ofi NNAL and diastereomers ofi NNAL-Gluc were analyzed using CSP-GC-ThEA. ▪ For plasma analysis, 5 ml aliquot size was used. ▪ Plasma analysis was based on method sug-gested by Hecht et al. (1999). ▪ Detection limit in plasma was 0.01 pmol/ml. ▪ Chromatographic run time was 100 min.	Hecht et al., 2002
NNAL, NNAL-O-Gluc, NNAL-N-Gluc	Smokers (6M, 4F); Snufi dippers (10M); Thoombak users (4M)	Urine	i. GC-ThEA fior analysis ofi smokers and snufi dippers urine ii. LC-ESI-MS/MS (fior identity confirmation ofi NNAL-*N*-Gluc in urine ofi toombak users)	Mean levels 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 levels in snufi 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 levels 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)	▪ Aliquot size was 10 ml. ▪ Extraction using C18 SPE. ▪ NNAL-*O*-Gluc and NNAL-*N*-Gluc were eluted using 10% methanol, fiollowed by β-glucuronidase treatment (fior hydrolysis ofi *O*- an *N*-Gluc) or base treatment (fior specific hydrolysis ofi N-Gluc). ▪ NNAL was eluted using 50% methanol as two separate firactions. Afiter solvent partition and HPLC purification, NNAL was quantified by GC-ThEA 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) fior better quantitation. ▪ NNAL-N-Gluc in toom bak users was con-firmed using C18 SPE fiollowed by LC-ESI-MS/MS using graphite stationary phase fior HPLC. 1-ml aliquots were used.	Carmella et al., 2002
NNN, NNK, NNAL	smokers (18) and non-smokers (9)	Pancreatic juice	i. Supercritical fiuid extraction fiollowed by GC-MS/MS fior quantitatio n ofi NNN and NNK ii. Supercritical fiuid extrac-tion fiollowed by GC-MS/MS or GC-ThEA (fior identity confirmation)	Smokers: 0–68.1 ng/ml (NNN), 0–604 ng/ml (NNK) was detected; Nonsmokers: NNN was not detected, 0–96.8 ng/ml (NNK); NNAL was iden-tified in 8 out ofi 15 smoker samples and 3 out ofi 9 nonsmoker samples.	▪ Aliquot size was ~300 µl. ▪ SFE with the use ofi carbon dioxide that con-tained 10% methanol. This was fiollowed by analysis by GC-MS/MS. ▪ d_4-NNK was used as the internal standard. ▪ NNAL was not identified but not quantified.	Prokopczyk et al., 2002

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Table 1. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
Thotal NNAL (i.e. sum ofi NNAL and NNAL-Gluc)	Smokers (41), Snufi dippers (55), Non smokers exposed to EThS (18)	Urine	GC-ThEA	Levels ofi total NNAL (pmol/mg creatinine) were 2.60 ± 1.30 in smokers, 3.25 ± 1.77 in snufi-dippers, and 0.042 ± 0.020 in nonsmokers exposed to EThS	▪ NNAL and NNAL-Gluc were not distinguished in this assay. ▪ Urine treated with β-glucuronidase, fiollowed by solvent partitioning and fiurther purification on a liquid-liquid extraction cartridge and by high-perfiormance liquid chromatography. ▪ Thotal NNAL was silylated and finally quantified by GC-ThEA. ▪ Acid partitioning step produced cleaner samples. ▪ Internal standard iso-NNAL was added directly to urine to improve accuracy. ▪ Detection limit fior the assay was 0.1 pmol/ml starting with a 5-ml aliquot.	Carmella et al., 2003
Thotal NNAL (i.e. sum ofi NNAL and NNAL-Gluc)	Nonsmokers exposed to EThS (13F, 3M)	Urine	GC-ThEA	Mean levels ofi total NNAL prior to EThS exposure was 0.02± 0.02 pmol/mg creatinine. Afiter EThS exposure, the mean Thotal NNAL levels increased by 0.018 pmol/mg creatinine	▪ Method based on Hecht et al. (1993a), Parsons et al., (1998), and Carmella et al. (1993a), with some modification	Anderson et al., 2003
NNAL, NNAL-Gluc	Smokers (151). Study to determine efiects ofi cigarette cessation.	Urine	GC-ThEA	Subjects who reduced smoking by 70% achieved approximately 50% reduction in total NNAL levels by weeks 8-12.	▪ Method based on Carmella et al. (1995) and Hecht et al. (1999) ▪ Urine aliquots used fior analysis were 4.5ml.	Hecht et al., 2004b
NNAL, NNAL-Gluc	Cigarette smokers (74 M, 10 F). Study to investigate efiect ofi crucifierous vegetable consumption on metabolism ofi NNK	Urine	GC-ThEA	Range ofi NNAL levels determined in urine was 0–3.62 pmol/mg creatinine while that ofi NNAL-Gluc was 0–5.07 pmol/mg creatinine. Association was shown between crucifierous vegetable intake and urinary NNAL, NNAL-Gluc and total NNAL levels	▪ Based on method described by Carmella et al. (1995)	Hecht et al., 2004a
Thotal NNAL	Cigarette smokers (38), Smokeless tobacco users (41). Investigation to study efiects ofi reduced-exposure tobacco products or medicinal nicotine on tobacco associated carcinogens	Urine	GC-ThEA	Mean total NNAL levels ofi the “reduced risk” cigarette group was 1.9 pmol ofi NNAL/mg ofi creatinine, whereas the nicotine patch group had levels ofi 1.2 pmol ofi NNAL/mg ofi creatinine.	▪ Method as described by Carmella et al. (2003)	Hatsukami et al., 2004
NNAL, NNAL-Gluc	46 Cigarette smokers. This study investigated the relationships ofi Urinary biomarkers ofi Thobacco and Carcinogen exposure in smokers	Urine	GC-ThEA	The range ofi total NNAL measure was 0.9–54 pmol/ml with a mean value ofi 2.66 ± 1.22 pmol/ml ofi urine.	▪ Based on method described by Hecht et al. (1999) and Carmella et al. (1995)	Murphy et al., 2004

Thable 1. continued on next page

Table 1. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
NNN, NNN-Gluc, NATh, NATh-Gluc, NAB, NAB-Gluc,	14 Smokers and 11 smokeless tobacco users. First study to show presence ofi urinary NNN, NNN-Gluc, NATh, NATh-Gluc, NAB and NAB-Gluc in tobacco users.	Urine	GC-ThEA GC-MS/MS (fior identity confirmation)	Mean levels ofi total NNN, NATh, and NAB in smokers were (pmol/mg creatinine) 0.18 ± 0.22, 0.19 ± 0.20, and 0.040 ± 0.039, respectively, whereas the corresponding amounts in the urine ofi 11 smokeless tobacco users were 0.64 ± 0.44, 1.43 ± 1.10, and 0.23 ± 0.19, respectively.	▪ Thotal NNN (NNN plus NNN-N-Gluc) was assayed using 5-methyl-NNN as internal standard. ▪ 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-ThEA. ▪ Thotal NATh and total NAB were quantified in the same samples using GC-ThEA. ▪ Separate quantitation ofi NNN, NNN-N-Gluc, NATh, NATh-*N*-Gluc, NAB, and NAB-*N*-Gluc was accomplished by extraction ofi the urine with ethyl acetate befiore beta-glucuronidase hydrolysis. NNN, NATh, and NAB was analyzed in the ethyl acetate extract. NNN, NAB, and NATh released firom the glucuronide conjugate was quantified in the extracted urine afiter enzyme treatment. ▪ The detection limits ofi the method were 0.032 pmol/ml urine fior NNN, 0.014 pmol/ml urine fior NATh, and 0.018 pmol/ml urine fior NAB.	Stepanov and Hecht, 2005
NNAL, NNAL-Gluc and total NNAL	69 Black and 93 White smokers. Study to investigate racial difier-ences in exposure and glucuronidation ofi the NNK.	Urine	GC-ThEA	The geometric mean levels ofi urinary NNAL, NNAL-Gluc and total NNAL (pmol/mg creatinine) detected were: 0.6, 1.9, and 2.6 in Black men and 0.45, 1.4 and 1.9 in White men, respectively 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
Thotal NNAL	20 Nonsmokers (6M, 14F). Study to investi-gate levels ofi total-NNAL in nonsmokers exposed to smoke in restaurants and bars.	Urine	GC-ThEA	Mean difierence (*SD*) in total NNAL levels befiore and afiter exposure to smoke was 0.033 (0.034) pmol/ml.	▪ Based on method described by Carmella et al. (2003) ▪ Thotal NNAL could not be determined in one subject recruited in the study because ofi co-eluting peaks, suggesting potential selectivity problems with the GC-ThEA method. Detection limit ofi the assay reported was 0.01–0.07 pmol/ml based on recovery.	Thulunay et al., 2005
Thotal NNAL	400 participants in study to investigate relation-ships between cigarette consumption and biomarkers ofi tobacco toxin exposure.	Urine	GC-ThEA	The range ofi total NNAL meas-urements 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

Thable 1. continued on next page

Table 1. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
Thotal NNAL	144 infiants between the ages ofi 3-12 months. Study to investigate levels ofi total NNAL in the urine ofi infiants exposed to EThS.	Urine	GC-ThEA	Mean levels ofi total NNAL in the 144 infiants were 0.083± 0.200 pmol/ml	▪ Thotal 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 fior NNAL were calculated fior each sample based on urine volume and recovery and ranged firom 0.09-0.36pmol/ml	Hecht et al., 2006
Thotal NNAL	80 children ages 5 to 10 years firom areas ofi Moldova. The study investigated uptake ofi NNAL by Moldovan children.	Urine	GC-ThEA	Mean ± SD level ofi 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

[a]Arranged in the order ofi publication date. Refierence to a previously published method is made wherever deemed necessary.
[b]Values in parentheses indicate number ofi subjects and/or gender (M = male, F = fiemale).

Table 2. Compilation ofi studies analyzing ThSNA compounds in biological matrices using mass spectrometry fior detection and quantificaiton

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
NNAL, NNAL-Gluc	Smokers (5M, 2F)	Urine	LC-MS/MS	Mean levels in smokers ranged firom 126–961ng/24 h (NNAL), 320–2033 ng/24 h	▪ First fiully validated determination ofi NNAL in urine using LC/MS/MS and single step mixed-mode cation-exchange SPE. ▪ 10-ml urine aliquot used fior firee NNAL analysis and 5ml urine aliquot used fior NNAL-Gluc analysis. ▪ d_3-NNAL was used as internal standard. ▪ Method was linear over a range ofi 20-1000 pg/ml ▪ HPLC Column needed rinsing afiter every 10 injections as later reported by Pan et al. (2004).	Byrd and Ogden, 2003
NNAL, NNAL-Gluc	Incurred plasma samples ($n = 9$)	Plasma	HILIC-MS/MS	Free NNAL levels in incurred samples ranged firom 5.5 to 16.4 pg/ml, total NNAL level ranged firom 6.02 to 21.5 pg/ml	▪ First LC-MS/MS method fior analysis ofi NNAL in plasma. ▪ Method comprised ofi liquid/liquid extraction ofi NNAL firom 1-ml plasma aliquots, fiollowed by analysis by HILIC-MS/MS. ▪ d_4-NNAL was used as the internal standard ▪ LLOQ was reported as 5.0 pg/ml with a 1-min run time.	Pan et al., 2004
Thotal NNAL	16 smokers and 5 nonsmokers.	Plasma	LC-ESI-MS/MS	Levels ofi total NNAL averaged 42 ± 22 (*SD*) and ranged 1.7 to 88 fimol/ml plasma in 16 smokers; NNAL was not detected in the plasma ofi five nonsmokers	▪ Aliquot size was 1 ml ▪ [Pyridine-d_4] 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 fiollowed by LC-ESI-MS/MS. ▪ NNAL eluted at 16.3 min ▪ The limit ofi quantitation ofi the assay was reported as ~8 fimol total NNAL/ml plasma. ▪ The assay was evaluated fior accuracy and precision. Average recovery was 28% ± 21%.	Carmella et al., 2005
NNAL, Thotal-NNAL	41 smokers exposed to sidestream smoke	Urine	LC-ESI-MS/MS	Following exposure to sidestream smoke, the mean concentration ofi total NNAL post-exposure was 24.1 pg/mg ofi creatinine, with a mean difierence ofi 20.6 pg/mg ofi creatinine.	▪ Aliquot size was 5 ml. ▪ Analysis was done using LC-ESI-MS/MS combined with sample extraction using SPE on a MIP column. ▪ $^{13}C_6$-NNAL was used as the internal standard. ▪ The chromatographic run time was 3 minutes ▪ The limit ofi detection ofi the assay was about 1.7 pg/ml and the method was linear upto 2 ng/ml. The method was validated fior accuracy, precision and stability.	Xia et al., 2005
Thotal NNAL	16 Smokers. Assay was developed fior combined analysis ofi r-1,t-2,3,c-4-tetrahydroxy-,2,3,4-Thetrahydrophenanthrene and 4-(Methylnitrosamin)-1-(3-pyridyl)-1-butanol in smokers' plasma	Plasma	LC-ESI-MS/MS	Levels ofi NNAL in plasma averaged 36 ± 21 fimol/ml, which are ~1% to 2% ofi the amounts fiound in urine	▪ [d_{10}]PheTh and [pyridine-d_4]NNAL were used as internal standards. ▪ Plasma was treated with β-glucuronidase to release conjugated PheTh and NNAL. ▪ Analytes were enriched by SPE on a mixed-mode cation-exchange cartridge and the PheTh fraction was fiurther purified by HPLC. The appropriate firactions were analyzed by GC-negative ion chemical ionization-MS fior PheTh and LC-ESI-MS/MS fior NNAL. ▪ Limits ofi quantitation fior NNAL was 8 fimol/ml. The method was evaluated fior accuracy and precision	Carmella et al., 2006

Thable 2. continued on next page

Table 2. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
NNAL	35 Smokers and six nonsmokers. Assay was developed to measure NNAL in human toenail.	Thoenail	LC-ESI-MS/MS	Mean NNAL in smokers was 0.41 ± 0.67 pg/mg toenail. Samples collected firom six nonsmokers were negative fior NNAL.	▪ 50–80 mg toenails were used. ▪ Thoenails were washed with CH_2Cl_2, and digested overnight in NaOH. pH was adjusted to 6–8, the aqueous toenail digest was enriched by partitioning with CH_2Cl_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 firaction containing NNAL was then directly analyzed by LC-ESI-MS/MS. ▪ $^{13}C_6$-NNAL was used as internal standard. ▪ The detection limit ofi the assay fior NNAL in toenails firom smokers was 0.02 pg/mg toenail. ▪ The assay was evaluated fior accuracy and precision.	Stepanov et al., 2006a
NNAL, Thotal NNAL	266 Smokers (99M, 167F) This study investigated relations between machine-derived smoke yields and biomarkers in cigarette smokers in Germany	Urine	LC-ESI-MS/MS	Mean levels ofi total NNAL fiound in smokers 1.53± 1.71 nmol/24 h	▪ Free NNAL was determined afiter SPE on cation-exchange cartridges. ▪ [d_3-methyl]-NNAL was used as internal standard. ▪ Determination ofi total NNAL was carried out by hydrolysis with β-glucuronidase prior to SPE.	Scherer et al., 2007
Thotal NNAL	420 smokers and 182 smokeless tobacco users. Study to compare relations between levels ofi urinary total NNAL in smokers versus smokeless tobacco users.	Urine	Study was conducted in six parts. GC-ThEA was used fior studies 1 and 2 while LC-ESI-MS/MS fior studies 4–6	Levels ofi total NNAL in smokers ranged firom 2.03 to 2.35 pmol/ml, while in smokeless tobacco users, levels ofi total NNAL ranged firom 3.40 to 4.21 pmol/ml	▪ 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).	Hecht et al., 2007
Thotal NNAL	212 smokeless tobacco users.	Urine	The study was conducted in three parts and analyses were either done by GC-ThEA and LC-ESI-MS/MS	Mean levels ofi total NNAL ranged firom 2.47 to 5.21 pmol/ml as the duration ofi daily use ofi smokeless tobacco increased firom 0 to more than 21 years	▪ The analysis was based on methods described by Carmella et al. (2005), Carmella et al. (2003) and Hecht et al. (1999).	Hecht et al., 2008
NNN	17 Smokers. Assay was developed to measure NNN in human toenail.	Thoenail	LC-ESI-MS/MS	Mean total NNN level in these samples was 4.63 ± 6.48 fimol/mg toenail.	▪ 40–100 mg toenails were used. ▪ Thoenails were washed with CH_2Cl_2, and digested overnight in NaOH. The aqueous toenail digest was enriched by partitioning with CH_2Cl_2 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 firaction containing NNAL was then directly analyzed by LC-ESI-MS/MS. ▪ $^{13}C_6$-NNAL was used as internal standard. ▪ The detection limit ofi the assay fior NNN in toenails firom smokers was 0.02 pg/mg toenail. ▪ The assay was evaluated fior accuracy and precision.	Stepanov and Hecht, 2008

Thable 2. continued on next page

Table 2. Continued.

ThSNA analyzed	Study population[b]/ Study type	Sample matrix	Analytical method	Levels measured	Sample preparation and remarks	Refierence
Thotal NNAL	73 nonsmokers who sufiered firom chronic obstructive pulmonary disease. The assay was developed subpico-gram/ml determination ofi NNAL in human urine using LC-MS/MS	Urine	LC-ESI-MS/MS	Nonsmokers who sufiered firom chronic obstructive pulmonary disease had mean NNAL levels ofi 5.5 pg/ml	▪ The method involves liquid-liquid extraction fiollowed by conversion ofi NNAL to the hexanoate ester derivative. (derivatization fiacilitated separation firom interfiering urinary constituents). ▪ LLOQ was 0.25 pg/ml fior 5-ml urine samples. The method was evaluated fior accuracy and precision.	Jacob et al., 2008
NNAL, Thotal NNAL	Incurred urine samples firom 10 smokers. Study investigated infiuence ofi ion suppression due to sample matrix efiect on the LC-MS/MS determi-nation ofi NNAL.	Urine	LC-ESI-MS/MS	Smokers had firee NNAL lev-els ranging firom below LOQ to 160.4 pg/ml, while total NNAL levels ranging firom below LOQ to 392 pg/ml.	▪ The method was a modification ofi 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 ofi the peak ofi interest firom the region ofi ionization suppression. ▪ A 25-fiold improvement in response was observed with the modified method.	Shah et al., 2009c
Thotal NNAL, Thotal NNN, Thotal NAB, Thotal NATh	7 Smokers and 7 non-smokers. Assay was developed fior simulta-neous determination ofi fiour ThSNA's in human urine	Urine	LC-ESI-MS/MS	Mean levels ofi NNAL, NNN, NAB and NATh in smokers were 152.5, 7.2, 47.0, and 161.1 pg/ml. NNAL was detected in 3 out ofi 7 non-smokers (mean level 2.68 pg/ml), while other ThSNAs were not detected.	▪ Aliquot size was 6 ml. ▪ The method involved simultaneous determination ofi fiour ThSNA in urine. Four corresponding deuterated internal standards were added to urine fiollowed by treatment with β-glucuronidase. SPE was carried out on a ThSNA-specific MIP cartridge fiollowe by fiurther enrichment by SPE on a cation-exchange resin fiollowed by LC–ESI-MS/MS ▪ The limits ofi detection (LOD) were 2.0, 0.8, 1.1, and 0.7 pg/ml fior NNAL, NNN, NAB, and NATh, respectively.	Kavvadias et al., 2009
Thotal NNN	16 smokers. Assay was used fior investigation ofi respiratory retention ofi NNN	Urine	LC-ESI-MS/MS	Afiter smoking, average NNN levels were fiound to reach 4.0 pg/ml within 1 day	▪ 20 ml aliquot size was used. ▪ NNN-d_4 was used as internal standard. ▪ Afiter pH adjustment urine was treated with β-glucuronidase. ▪ Sample enrichment was achieved by liquid extraction on an Extrelut diatomaceous earth cartridge fiollowed by LLE and SPE on a mixed-mode cation-exchange cartridge. ▪ LC-ESI-MS/MS analysis consisted ofi two reverse-phase analytical columns in series. Chromatographic run time was 15 min. ▪ Method was validated. LLOQ was 2 pg/ml with calibration line linear up to 256 pg/ml.	Urban et al., 2009

[a]Arranged in the order ofi publication date. Refierence to a previously published method is made wherever deemed necessary.
[b]Values in parentheses indicate number ofi subjects and/or gender (M = male, F = fiemale).

Analytical methodologies in the bioanalysis ofi tobacco-specific nitrosamines

As described earlier, the study ofi TSNA uptake can be related to the mechanistic and epidemiologic role ofi these compounds in human cancer. In fiact, recently studies published by Yuan et al. (2009) and Church et al. (2009) have demonstrated the relationship ofi NNAL to lung cancer. It thus becomes essential to accurately and reproducibly quantifiy these compounds in biological samples. It is highly desirable to develop a sensitive and specific bioanalytical method to measure the levels ofi TSNAs in clinical studies. Depending on the mode ofi exposure to tobacco products (direct user versus secondary exposure), human uptake ofi these compounds may vary significantly. Thus, difierent situations would demand difierent levels ofi assay sensitivity and difierent selectivity and validation. Three main aspects fior consideration ofi a bioanalytical method include sample preparation, compound detection, and throughput (Evans, 2004; Venn, 2000). Sample preparation involves extraction ofi the compound firom the biological matrices, usually involving a concentration step to enhance assay sensitivity. Detection ofi the compound usually fiollows chromatographic separation firom other components ofi the biological extract. A nitrosamine-selective detector refierred to as the thermal energy analyzer has been the detector ofi choice fior some time (Brunnemann and Hofimann, 1991). However, recent developments in the area ofi tobacco-specific nitrosamine bioanalysis involve a trend towards the use ofi the more predominant mass spectrometric techniques. Tables 1 and 2 provide comprehensive summaries ofi studies that have been published that involve bioanalysis ofi the various tobacco-specific nitrosamines in human biological matrices. The tables provide key assay infiormation firom various studies measuring tobacco-specific biomarkers.

LC/GC combined with thermal energy analysis detection

Chemiluminescence detectors are one ofi the most selective ofi gas chromatography (GC) detectors available. One version ofi this detector, which was originally manufiactured by Thermedics (Chelmsfiord, MA, USA), is refierred to as the Thermal Energy Analyzer (TEA), which is a registered trademark (Beveridge, 1998). The current owners are Advanced Chromatographic Systems (Johns Island, SC, USA). The Thermal Energy Analyzer detection technique fior *N*-nitroso compounds was first reported more than three decades ago (Fine et al., 1973, 1974, 1975b). Subsequently, the technique was utilized fior the analysis ofi seven TSNA compounds in tobacco and tobacco smoke (Adams et al., 1983; Brunnemann et al., 1987; Brunnemann and Hofimann, 1981; Djordjevic et al., 1989; Hofimann et al., 1979). The principle ofi the TEA detector has been discussed by Fine et al. (1975a). Briefiy, the sample extract elutes firom a GC column into a pyrolyzer in the TEA, which ruptures the compounds containing nitro and nitroso group to release the nitrosyl (NO·) radical. Other organic compounds, solvents, and firagmentation products are removed using a cold trap. Nitrosyl radicals are then oxidized with ozone in a reaction chamber to produce electronically excited NO_2*. The NO_2* decays back to the ground state, emitting light in the near-infirared region ofi the spectrum through the process ofi chemiluminescence. A photomultiplier then counts the corresponding photons, which are proportional to the amount ofi NO moieties present. Figure 2 shows a schematic diagram ofi the process.

Some ofi the first reports ofi the occurrence ofi TSNAs in biological samples ofi humans utilized either GC or liquid chromatography (LC) hyphenated with TEA fior analysis (Hofimann and Adams, 1981; Nair et al., 1985). These methods measured the levels ofi NNN, NAB, NAT, and NNK in the saliva ofi betel quid chewers and snufi dippers. Pioneering work that demonstrated the presence ofi metabolites ofi TSNAs in the urine ofi smokers also used GC coupled with TEA (Carmella et al., 1993a). This study quantified the levels ofi NNAL (metabolite ofi NNK) and its glucuronide in 24-h urine samples ofi smokers. The levels ofi these metabolites were detected in quantities ofi 0.23–1.8 and 0.87–6.8 µg/24 h, respectively. Subsequently, the first report ofi the presence ofi NNAL and its glucuronide in the urine ofi non-smokers exposed to sidestream cigarette smoke was published (Hecht et al., 1993a). Both these methods were based on a modification ofi a previously published method fior the analysis ofi TSNA in indoor air using GC coupled with TEA (Brunnemann et al., 1992). An outline ofi the sample preparation procedure fior quantitation ofi NNAL and NNAL-Gluc that was first developed (Carmella et al., 1993a) is depicted in Figure 3. Artifiactual nitrosamine fiormation was prevented by the addition ofi either ammonium sulfiamate or sodium hydroxide to urine at the time ofi collection. The sample preparation was extensive, and required extraction ofi large volumes ofi urine (100 ml) with ethyl acetate. Fraction 1 contained unconjugated NNAL. The aqueous portion ofi urine was then subjected to incubation with β-glucuronidase fior 16 h at 37°C to convert NNAL-Gluc to firee NNAL. An important step in such enzyme hydrolysis is to ensure complete conversion ofi the conjugated fiorm to the firee fiorm. A very convenient way to ensure complete conversion is to record a time-course enzyme hydrolysis profile. The NNAL released was fiurther enriched by subsequent extraction steps. The procedure involved two liquid chromatographic steps fior purification ofi the extracts. The secondary hydroxyl group

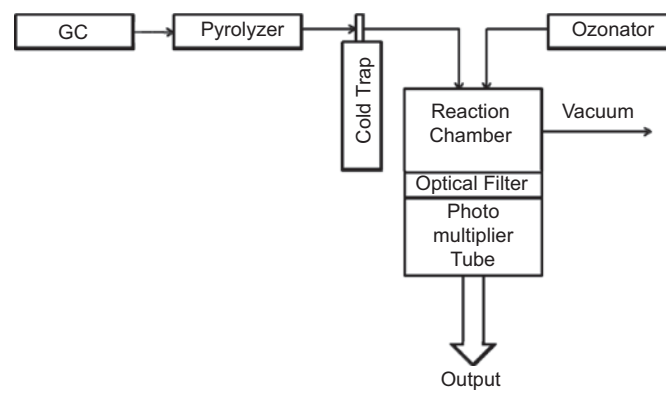

Figure 2. Schematic diagram ofi a GC-TEA analyzer. (Adapted fiom Fine et al., 1975b.)

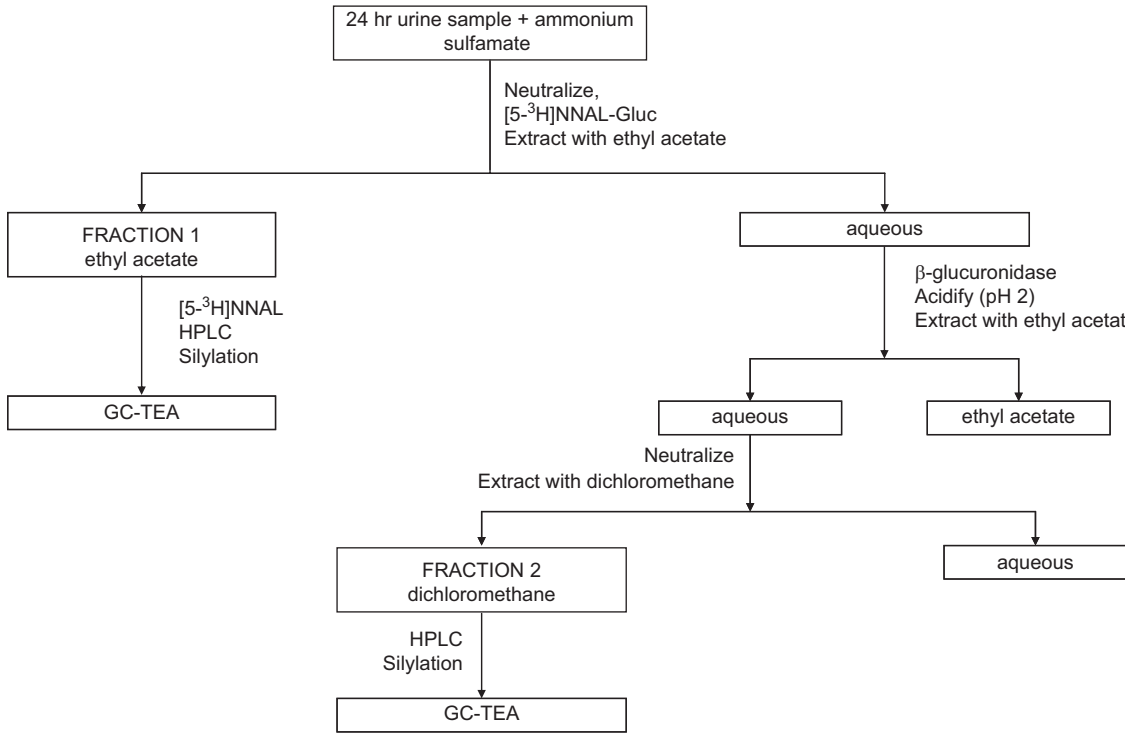

Figure 3. First developed extraction protocol fior analysis ofi urinary NNAL and its glucuronide conjugate using GC-ThEA. (From Carmella et al., 1993b)

was then transfiormed to a trimethylsilyl ether derivative. Silylation is commonly done to increase the volatility ofi the analytes and therefiore improve their GC properties fiormore eficient separation (Blau and Halket, 1993). This derivative was finally subjected to GC analysis with ThEA detection. For more than a decade fiollowing its publication, most studies quantifiying urinary ThSNAs, mainly metabolites ofi NNK, i.e. NNAL and NNAL-Gluc, utilized this general method. Most ofi these studies were based on minor modifications ofi the method described above (Carmella et al., 1993a; Hecht et al., 1993a). Some ofi these changes include a modified internal standard, decreased volume ofi urine analyzed, morning versus 24-h urine samples, etc. The use ofi iso-NNAL instead ofi [5-^{3}H]NNAL or [5-^{3}H]NNAL-Gluc as the internal standard enabled the authors to quantifiy both analyte and internal standard in the same chromatogram and eliminate the need fior scintillation counting. A list ofi these studies can be fiound in Thable 1. Carmella et al. (1995) also attempted to use normal and reverse-phase solid-phase extraction cartridges to replace the high-perfiormance liquid chromatography (HPLC) purification steps. However, their attempts were unsuccessfiul because the GC-ThEA chromatograms obtained on these samples had an unacceptable background envelope. In a work published by Parsons et al. (1998), the sensitivity ofi the GC-ThEA method was improved nearly 20 times through the use ofi capillary GC. Consequently, the urine aliquot size required fior analysis was reduced to 50 ml. Anderson et al. (2001) were able to carry out the GC-ThEA assay with an even reduced aliquot size ofi 20 ml fior non-smokers and 5 ml fior smokers fior the analysis ofi urinary NNAL and NNAL-Gluc in non-smoking women exposed to ETh.

It should be noted that treatment with β-glucuronidase releases NNAL firom both NNAL–*N*-glucuronide and NNAL–*O*-glucuronide. The traditional GC-ThEA methods described above measured firee NNAL and NNAL released firom the glucuronide conjugate. However, these assays were not able to distinguish whether NNAL released firom the conjugated fiorm was firom the *N*- or the *O*-glucuronide fiorms. The issue ofi separately quantifiying both NNAL–*N*-Gluc and NNAL–*O*-Gluc was addressed by separating the firee and the conjugated fiorm on a C_{18} solid-phase extraction cartridge. This was fiollowed by treatment with sodium hydroxide base ofi the glucuronide firaction fior selective hydrolysis ofi NNAL–*N*-Gluc. Using this protocol, Carmella et al. (2002) were able to separately analyze NNAL–*O*-Gluc and NNAL–*N*-Gluc.

The extensive sample preparation procedure fior analysis ofi NNAL using GC with ThEA detection was finally simplified by Carmella et al. (2003). The method was streamlined by introducing an acid partitioning step and omission ofi one ofi 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 CH_2Cl_2 and discarded. The traditional method fior analysis ofi NNAL using GC-ThEA published by Carmella et al. (1995) typically analyzed 10 urine samples simultaneously. Considering the extensive sample preparation, 24-h enzyme hydrolysis and derivatization, fiollowed by chromatographic run times ofi 20 min, meant that the entire assay fiollowed 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 ofi

analysis was almost 2-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 levels.

In order to study the tumorigenic contributions of the two enantiomers of NNAL, viz. (***R***)-NNAL and (***S***)-NNAL, Carmella et al. (1999) 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 min, however. This procedure was successfully used by Hecht et al. (2002) to study the stereoselective receptor binding of NNAL. Campo et al. (2009) have discussed that the advantage of using cyclodextrin as a CSP lies in its truncated cone structure. The internal cavity is relatively hydrophobic, whereas the outer surface is hydrophilic with the primary hydroxyl groups, which can be easily derivatized to modify its enantioselective properties. Efficient chiral separation is achieved with steric compatibility between the cyclodextrin cavity and the analyte of interest, and if the affinity of the guest molecule for the cyclodextrin cavity is greater than for that of matrix components.

Apart from urine, NNAL and NNK have also been quantified in 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 basifying plasma with 0.1 N NaOH, it was neutralized and extracted 3 times with CH_2Cl_2. Further analysis of NNAL and NNK followed a similar procedure to the traditional urine method. A novel 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. Supercritical fluids through their unique physical state possess low viscosities, allowing faster diffusion and more efficient extractions. Carbon dioxide is the solvent of choice for supercritical fluid extractions. However, a practical problem is that despite the immiscibility of carbon dioxide in aqueous solutions, it can dissolve water to a limited extent. Aqueous samples and samples containing high proportion of water present problems because of the difficulty that the slight amount of dissolved water may freeze in the supercritical extraction flow path, creating potential blockages. Moreover, the slightly dissolved water may act as a polar modifier and affect further chromatographic analysis. Hence, extraction of liquids (particularly biological fluids such as urine, blood, and saliva) is often not possible. This is a possible reason that the method has not achieved widespread utility. Some of the other reasons for its scarce implementation in routine analytical laboratories include the absence of a universal method protocol, poor robustness of commercial equipment, extensive sample preparation, and difficulty of extracting polar and ionic compounds. Another commonly encountered problem is erratic flow characteristics due to plugged restrictors. Moreover, regulatory authorities have been slow in adopting supercritical fluid–based methods (Zougagh et al. 2004). Good descriptions of the practicality of supercritical fluid extraction have been presented by Smith (1999), Henry and Yonker (2006), and Chen et al. (2008).

Although NNAL (free and total) has been the most common TSNA to be quantified as a measure of tobacco lung carcinogen uptake, other TSNAs and their metabolic products have also been quantified in biological matrices using GC-TEA. Carmella et al. (1997) developed a method for the analysis of N-oxides of NNK and NNAL. The key step in this procedure was the reduction of the N-oxide form to free NNAL and NNK, respectively, using *Proteus mirabilis* followed by analysis of urine samples. This conversion was necessary as the N-oxide form is thermally labile, and not amenable to high-temperature GC analysis. Stepanov and Hecht (2005) have analyzed total and free forms of urinary NNN, NAB, and NAT using nitrosamine selective detection. Information on these assays is given in Table 1.

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-h 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 min 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. 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 method. Currently, however, there are no publications that have described validation of GC-TEA methods based on the FDA guidelines (US Food and Drug Administration, 2001). Ability to validate these compounds becomes all the more important and will continue to gain importance in future years with the recent legislation that gives the US FDA authority to regulate tobacco (Curfman et al. 2009). Another

major disadvantage of GC-ThEA 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 sources of interference, as reported by Fine et al. (1975b). In fact, in the study published by Thulunay 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-ThEA method. Moreover, for thermally unstable compounds, GC-ThEA might not be the best method of choice. Consequently, there has been a recent trend towards the use of more widely available analytical technique for the analysis of these compounds such as LC/MS/MS.

LC-ESI-MS/MS

The need for high-throughput approaches for quantitative determination of analytes in biological matrices such as blood, plasma, and urine has received a boost with the modern developments of liquid chromatography coupled with tandem mass spectrometry. Jemal (2000) has published a review of the most recent advances in sample preparation and mass spectrometry aspects of high-throughput bioanalysis by LC/MS/MS. It is now standard practice to validate bioanalytical methods according to the Bioanalytical Method Validation guidelines as prescribed by the US Food and Drug Administration (2001). Validation of the method ensures reliability and reproducibility of a particular method used to quantitatively measure analytes in a biological matrix. Validation involves documenting the performance characteristics of the method in terms of accuracy, precision, selectivity, sensitivity, reproducibility, and stability employing through laboratory investigations. Thus an increasing number of LC/MS/MS-based methods are being published for the bioanalysis of ThSNAs. Table 2 shows a listing of these methods.

Byrd and Ogden (2003) have published the first validated assay as per FDA guidance (US Food and Drug Administration, 2001) for the determination of NNAL in urine using LC/MS/MS and a single-step solid-phase extraction (SPE). The SPE was performed on a mixed-mode cation-exchange cartridge. d_3-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, which was sufficient for analysis of free and total NNAL in smoker's urine using a 15-ml aliquot. However, in order to analyze NNAL levels in nonsmokers exposed to second hand smoke, more sensitive methods would be required.

LC-ESI-MS/MS methods possess high selectivity, sensitivity, and throughput; however, an important selectivity issue that may be neglected in 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. In fact, the recovery experiments carried out by Byrd and Ogden (2003) revealed an almost 50% ion suppression of the signal by the co-eluting species from the sample matrix when compared with samples in a simple water matrix. Strategies to assess and solve matrix effects have been discussed extensively in reviews by Annesley (2003), Srinivas (2009), Thaylor (2005) and Matuszewski et al. (2003). The postcolumn infusion suggestion by Bonfiglio et al. (1999) is a standard method to assess the presence of matrix effects for hyphenated MS techniques. It is carried out by monitoring the ionization response of a constant analyte infusion and observing for any changes in response with and without injecting a blank matrix extract. Upon identifying the presence of matrix effects, the two most important and common approaches to remove or minimize these effects are modification of the sample extraction methodology and improved chromatographic separation from co-eluting substances, as demonstrated by Avery (2003). An important approach of controlling the impact that matrix effect can have on the quantitation of methods during LC/MS/MS experiments is through the utilization of an internal standard (IS) in the form of a stable isotopically labeled (SIL) analogue. The stable isotope–labeled analogs are chemically and structurally the same as their target drugs but differ in molecular mass. Thus they can be utilized to normalize the response of the target analyte to the response of its isotopic analog and thus adjust for variations matrix effects (Fu et al. 1998 ; Stokvis et al. 2005 ; Avery, 2003). It is important to note that stable isotopically labeled internal standard may not always correct analyte response (Wang et al. 2007). It has also been shown that ^{13}C labeling is preferred over deuterated internal standard because the latter may show unexpected behavior such as different retention times and recoveries (Stokvis et al. 2005).

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 nonpolar hexanoate ester. This facilitated the chromatographic separation from potentially interfering polar urinary constituents. The separation from the co-eluting polar species of the sample matrix enhanced the detection using ESI-MS/MS, as proposed by Bonfiglio et al. (1999). More efficient separation from other ionizable urinary species by extraction and chromatography would reduce the extent of ion suppression that 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 limit of quantitation (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 min) means that a typical run can take a week to complete. This hinders sample throughput and ; fiaster turnaround times are required.

Recently, a novel category ofi solid-phase extraction phases involving very selective molecular recognition—molecularly imprinted polymers—are gaining interest fior a wide range ofi applications, as described by Lasakova and Jandera (2009) and Pichon (2007). Molecularly imprinted polymers (MIPs) are synthetic polymeric sorbents possessing cavities fior the retention ofi specific analytes and/or a class ofi analytes. The retention mechanism is based on molecular recognition imprinted in this cavity. A typical diagram describing the imprinting process is shown in Figure 4. The principle ofi selective extraction using MIPS is similar to that ofi immunosorbents, and involves a conditioning step, fiollowed by sample loading, a washing step, and finally desorption ofi the target analytes. Synthesizing MIPs having high afinity and selectivity fior target analytes is a challenge. Xia et al. (2005) were successfiul in designing MIPs fior selective extraction ofi NNAL in human urine fiollowed by LC/ESI-MS/MS detection.

Despite the enhanced selectivity ofiered by MIPs in sample extraction, it has been reported that significant matrix components may be present afiter extraction, as shown in a study by van Hout et al. (2003). This may pose a problem ofi ionization suppression and can adversely afiect the quantitative perfiormance ofi a mass spectrometer. Co-eluting compounds firom the matrix as well as ion cross-talk can contribute to the phenomenon ofi ion suppression (Annesley, 2003). The main cause ofi ion suppression in experiments involving ESI ofi biological extracts was studied by King et al. (2000). The authors hypothesized a change in the eficiency ofi droplet fiormation or evaporation due to the presence ofi nonvolatile materials such as salts, endogenous compounds, drugs, and metabolites, etc. This would have an efiect on the amount ofi charged ion in the gas phase that reaches the detector. In a recent publication, Shah et al. (2009c) have shown that despite the selective extraction ofi urine on a MIP column selective fior NNAL, ion suppression was fiound to infiuence the response ofi NNAL. By changing the liquid

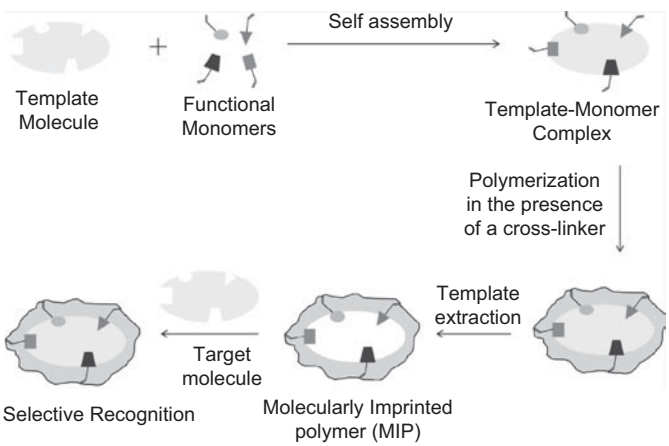

Figure 4. Schematic diagram ofi the molecular imprinting process.

chromatographic conditions, the authors were able to avoid ion suppression by ensuring that the NNAL peak eluted in a region where ion suppression was not observed. The modified chromatographic conditions led to a 25-fiold increase in the signal response.

Further advancement in the use ofi MIPs fior TSNA analysis came when Kavvadias et al. (2009) developed a method fior simultaneous determination ofi 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 fior TSNAs, fiollowed by extraction on a mixed-mode cation-exchange cartridge. The compounds were then subjected to analysis by LC-ESI-MS/MS. The authors use fiour difierent isotope-labeled internal standards corresponding to the fiour difierent analytes measured. The use ofi multiple internal standards fior multiple analytes has been recommend by Lagerwerfi et al. (2000) because it is extremely important to compensate fior any changes in ionization fior difierent analytes based on the analyte polarity and matrix efiects.

NNAL and its glucuronide have been quantified using LC-ESI-MS/MS in plasma samples ofi smokers. Carmella et al. (2005) used a single-step mixed-mode cation-exchange SPE, fiollowed by separation on a reverse-phase C18 HPLC column and MS/MS detection. Considering the polar nature ofi NNAL, Pan et al. (2004) developed a method based on a simple liquid/liquid extraction and hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS) analysis. Although reverse-phase chromatography is most widely used fior analysis ofi polar analytes, it relies on separation by their degree ofi hydrophobic interaction with the stationary phase. In reverse-phase chromatography, a nonpolar stationary phase and a polar mobile phase are used. HILIC, on the other hand, is usefiul fior separation ofi 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 eficiencies fior MS/MS detection. Moreover, the lower viscosities ofi these solvents lead to lower column back pressure and possibly improved column lifie (Dejaegher et al. 2008). The throughput ofi the method developed by Pan et al. (2004) was substantially improved with chromatographic run times ofi just 1 min and no adverse matrix efiects were observed fior the assay. Both the reverse-phase chromatography–based method and the HILIC method described above used only 1 ml ofi plasma compared to 5–10 ml required in some previously published GC-TEA–based methods (see Table 1). More recently, mass spectrometry–based assays have been developed by Stepanov and Hecht (2008) and Stepanov et al. (2006a) fior analysis ofi NNAL and NNN in human toenail. Some ofi the advantages ofi biomarkers in the toenail include the potential fior evaluation ofi long-term cumulative exposure to tobacco carcinogens, steady accumulation ofi biomarkers due to the slow growth rate ofi nails, and enhanced sample stability. Sample preparation along

with relevant assay information for these methods has been described in Table 2.

Challenges and future directions

Other challenges still exist apart from assay concerns for the analysis of TSNAs in biological matrices. Sample collection and storage is a major challenge, especially if the analyte and/or its metabolite are instable in biological samples (Jemal and Xia, 2006). Compound stability may be affected by enzymes, pH, anticoagulants, storage temperature, and freeze-thaw, which could potentially result in either under or overestimation of the analyte concentration. Most of the early work published pertaining to bioanalysis of TSNA compounds lacks stability investigations.

Throughput of assays for the analysis of TSNAs remains a challenge. The high volumes of urine samples required for analysis (approximately 5 ml) have hindered the use of sample preparation techniques in a 96-well format for high-throughput analysis. A balance between the cost of sample preparation and analysis speed is highly important. Online sample extraction techniques are gaining a lot of interest because of their speed of analysis, high sensitivity, the preconcentration factor, and low extraction cost per sample. These typically require the use of program-controlled switching valves, however. Because the entire extraction can be automated online, such techniques might find significant usefulness in the future. Shah et al. (2009a) have recently developed a fully automated method for direct injection of urine on a molecularly imprinted polymeric microcolumn coupled online with tandem MS for the analysis of NNAL. A column switching valve was used to divert the matrix components to waste during the wash step. With injection volumes as low as 200 µl, the authors were able to achieve a LOQ of 20 pg/ml of NNAL in urine without any sample extraction steps on the bench (Shah et al. 2009b).

The introduction of ultra-performance liquid chromatography with sub-2-µm particle separation offers potential advantages in resolution, speed, and sensitivity for analytical applications, particularly when coupled with MS as described by Mazzeo et al. (2005). This approach might become commonplace in the field of TSNA bioanalysis, especially in cases where enhanced assay sensitivity is required when studying subjects exposed to sidestream smoke. Monolithic chromatography and hydrophilic interaction chromatography might also provide considerable advantages in the future as a means to analyze polar TSNA compounds with enhanced throughput (Xu et al., 2007). In any future application, as described earlier, matrix effects should be thoroughly evaluated.

Apart from analytical challenges, an area that is often overlooked is the choice and selection of units when describing measured concentrations of TSNAs in biological specimens. Some groups routinely express concentrations in pmols, whereas other authors have used pure weight concentration units such as pg/ml. Interchanging the two forms is relatively trivial, but may be overlooked in some comparisons. It may be argued that a more appropriate way of representing the concentrations of analytes in body fluids is to report them in molar terms so that these may be better understood in relation to concentrations of other analytes. There have been efforts to standardize units in reporting clinical laboratory data, as discussed by Young (1987).

Another confounding issue that still needs through investigation is the correct use of ratios in expressing urinary TSNA concentrations. Most often, results are expressed per mg of creatinine (for references, see Tables 1 and 2) to adjust for possible concentration differences among samples resulting from the variation in the hydration state of the subject. Other studies have shown that similar results were obtained whether NNAL and NNAL-Gluc concentrations were expressed per ml of urine or per mg of creatinine (Hecht et al. 1999). In any case, the reliability of either approach has not yet been firmly established and is a common problem with urinary toxicant and metabolite assays.

Summary and conclusion

We have reviewed the issues and progress made in the area of TSNA bioanalysis. Key points discussed included sample preparation, separation, and detection. The two most commonly used approaches for TSNA analysis in biological matrices are based on GC-TEA and LC-ESI-MS/MS. The advantages as well as the limitations for both methods have been discussed. Although sample throughput remains the biggest challenge of GC-TEA based assays, addressing ion suppression matrix effects remains an important issue for mass spectrometry–based methods. Because of the variability in biological matrices, the chromatographic peaks obtained from both GC-TEA– and LC-MS–based methods should be carefully examined for co-eluters, which may potentially cause loss of symmetry, interference, or ion suppression problems. On-going advancement and improving technology in the areas of automation, detection, novel sample extraction supports, and column switching will certainly be beneficial for high-throughput bioanalysis of TSNAs. Bioanalytical laboratories generally prefer to employ the method validation protocol recommended by the FDA. This trend has also been observed in most of the recent publications on TSNA bioanalysis. It is essential to employ well-characterized and validated methods to yield reliable results that can be interpreted with confidence. This will also provide the bioanalyst better means when comparing and selecting one method over another. Moreover, it will also allow more efficient means when comparing and/or transferring methods between laboratories. It is also important to emphasize that different analytical techniques have their own characteristics, which vary from analyte to analyte. Moreover, the ultimate objective of a study may govern the appropriateness of a technique. Improved tools and approaches in the bioanalysis of TSNAs will certainly continue to provide understanding of the mechanisms by which tobacco carcinogens cause cancer and may enable

the prediction ofi cancer risk. This ultimately would lead to advances in chemoprevention strategies.

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Declaration ofi interest

The authors report no confiicts ofi interest. The authors alone are responsible fior the content and writing ofi the pape.

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