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Article

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Novel wastewater-based epidemiology approach based on liquid chromatography-tandem mass spectrometry for assessing population exposure to tobacco-specific toxicants and carcinogens

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Abstract

Tobacco smoking remains an important public health issue worldwide. Assessment of exposure to tobacco-related toxicants and carcinogens at the population level is thus an essential population health indicator. This can be achieved by wastewater-based epidemiology (WBE), which relies on the analysis of biomarkers in wastewater. However, required analytical methods for the simultaneous measurement of tobacco-related toxicants and carcinogens in wastewater are not available. In this study, a new analytical procedure was developed and validated to measure tobacco-related alkaloids, carcinogens and their metabolites in raw wastewater, including anabasine (ANABA), anatabine (ANATA), cotinine (COT), trans-3'-hydroxycotinine (COT-OH), *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), *N*-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), NNAL-*N*- β -glucuronide and NNAL-*O*- β -glucuronide. Different parameters were optimised for the solid-phase extraction procedure and instrumental analysis using liquid chromatography-tandem mass spectrometry. The optimised method was fully validated, resulting in acceptable within-run and between-run precision (<8% and <10% relative standard deviation, respectively) and accuracy (<9% and <13% bias, respectively). Method quantification limits were at 0.5-120 ng/L in wastewater. Target analytes were stable in wastewater at 4 °C and 20 °C over 24 h. The developed method was applied to wastewater samples from two Belgian cities. Average concentrations of COT, COT-OH, ANATA, ANABA, and NAT were 5200, 2600, 30, 10, and 0.6 ng/L, respectively, while NAB, NNN, NNK and NNAL were not detected in the samples. With the developed robust analytical method, our study provided the first insight into the population exposure to both toxicants and carcinogens resulting from tobacco use.

Keywords: tobacco-specific nitrosamines, nicotine, tobacco alkaloids, NNN, NNK, LC-MS/MS.

INTRODUCTION

Health issues associated with tobacco use are an enormous burden to modern society, with an estimated six million deaths each year.¹ Cigarette smoking has been linked to various types of cancer due to the intake of numerous toxicants and carcinogens occurring in mainstream smoke.² Tobacco-specific alkaloids (e.g. anatabine (ANATA), anabasine (ANABA)) and nitrosamines (e.g. 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN)) are among the most prevalent toxicants and carcinogens, respectively, found in tobacco products, which are also transferred to tobacco smoke.² Some of these compounds and their metabolites, some of which are also carcinogenic (e.g., 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), main metabolite of NNK), have been found in the urine of tobacco users and can serve as biomarkers to assess first and second hand exposure to tobacco smoke.³

The measurement of their levels in urine, together with metabolites of tobacco alkaloids (e.g. nicotine metabolites, cotinine and trans-3'-hydroxycotinine) is highly relevant from an epidemiological perspective, particularly to assess prevalence of tobacco use/exposure, as well as to plan and evaluate prevention campaigns and policies.⁴ For instance, urinary concentrations of nicotine metabolites and NNAL are monitored within the framework of the National Health and Nutrition Examination Survey (NHANES) and reported by the Centre for Disease Control (CDC).⁵ Yet, the collection of large sets of urine samples for assessing exposure to these chemicals at the population level is logistically demanding and is often confronted with ethical issues.⁶

An interdisciplinary approach, referred to as wastewater-based epidemiology (WBE), emerged in the last decade and has seen an astonishing development.⁷ The approach, based on the measurement of relevant urinary biomarkers in raw wastewater, uses the latter as a diluted pooled urine sample representative of the population served by the wastewater treatment plant (WWTP) from which samples are collected. Observed levels and patterns of the measured biomarkers provide timely geographical and temporal information about the lifestyle and health status of the investigated population.⁷ The first application of WBE focused on the measurement of illicit drug metabolites.⁸ Its added value to the existing methods has been nowadays recognised by the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) for monitoring illicit drug use across Europe.⁸ Beyond estimating illicit drug use, WBE can also be applied to obtain epidemiologically-relevant information, for example, the health status of a population, as initially proposed by Thomas et al.⁹ and Daughton.¹⁰ Recent applications focusing on the measurement of oxidative stress

biomarkers¹¹ and pesticide exposure biomarkers¹² have been reported, illustrating the potential of the approach to assess human health at general population scales.

There are a few studies to date that provide separated analytical methods for measuring two nicotine metabolites,^{13,14} two tobacco alkaloids¹⁵ and five tobacco nitrosamines and metabolites¹⁶ in wastewater. Three of these previous studies applied the method in the WBE context and were limited to only cotinine, trans-3'-hydroxycotinine, ANATA and ANABA¹³⁻¹⁵ while another study¹⁶ focused on assessing the fate of tobacco nitrosamines and metabolites during wastewater treatment. Furthermore, no studies have focused on optimising the sample preparation for the analysis of all tobacco-related compounds in wastewater. Also, tap water instead of wastewater matrix was used to estimate the method detection and quantification limits for the tobacco nitrosamines and metabolites.¹⁶ This approach thus often resulted in low but unrealistic values reported in the literature, which are not practical for studies involving wastewater as a matrix. Clearly, there is a lack of analytical methods to simultaneously analyse tobacco-specific carcinogens and metabolites in wastewater for WBE purposes. Moreover, no studies have investigated the occurrence of tobacco-specific carcinogens and their metabolites as well as the relationships between their levels and tobacco use from a WBE perspective. This will provide important and innovative evaluations of the population-wide exposure to tobacco-related toxicants and carcinogens as a whole.

The objective of this study was to develop a novel analytical procedure allowing the simultaneous measurement of various tobacco-related toxic and carcinogenic compounds in wastewater for WBE applications. The key aims were to (a) optimise and validate the sample preparation method with solid-phase extraction (SPE) and the instrumental analysis using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS); and subsequently (b) apply the developed method to real wastewater samples for evaluating the occurrence of tobacco-related toxic and carcinogenic compounds in different communities. To the best of our knowledge, this is the first report of a new analytical method to illustrate the co-presence of all tobacco-specific toxicants and carcinogens in wastewater as a way to examine population-wide exposure to tobacco.

METHODS

Chemicals and materials

Reference standards (purity $\geq 99\%$) of the target analytes and the respective deuterated analogues (used as internal standards, IS) were purchased from Toronto Research Chemicals (TRC, Toronto, Ontario, Canada)

as neat crystals (1-5 mg) or solutions (1 mg/mL in methanol or acetonitrile). Working standard mixture solutions of native compounds (10 µg/mL) and deuterated analogues (1 µg/mL) were prepared in methanol (MeOH) and stored in amber bottles at -20 °C. Acetonitrile (ACN) and methanol (MeOH) were LC-grade quality, while ammonium hydroxide solution (NH₄OH, 25%), ammonium formate, hydrochloric acid (HCl, 37%), formic acid (99-100%) and acetic acid (99%) were of analytical-grade and were purchased from Merck (LiChrosolv®, Merck, Darmstadt, Germany) and Sigma Aldrich (Ph. Eur, Sigma Aldrich, Overijse, Belgium). Ultrapure water ($\rho \geq 18.2 \text{ M}\Omega$ at 25 °C) was obtained from an ELGA Purelab Flex water purification system (Veolia Water Technologies, Tienen, Belgium). Oasis® solid-phase extraction (SPE) cartridges (60 mg, 3 cc), including MAX, WAX, HLB and MCX, were obtained from Waters® (New Bedford, MA, USA). The enzyme solution (2 mL) of β -glucuronidase/aryl sulfatase (from *Helix pomatia*; β -glucuronidase 30 U/mL; arylsulfatase about 60 U/mL) was purchased from Merck (France). The SPE procedure was executed using a Supelco Visiprep™ SPE Vacuum Manifold with 24 ports and a self-cleaning vacuum system™. Glass microfiber filters (1.6 µm, GF/A, Whatman) were obtained from Sigma-Aldrich.

Target analytes

This study focused on biomarkers (parents and metabolites) used in the clinical studies as urinary analysis of smokers to evaluate tobacco use and assess the associated health outcomes, particularly risk of cancer development.^{2,17,18} Therefore, tobacco-specific nitrosamines (TSNAs), tobacco alkaloids and their metabolites were the key groups of target chemicals in this study. These included, the carcinogens (TSNAs): *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), NNN, NNK, the specific metabolites of NNK (namely NNAL, NNAL-*N*- β -glucuronide and NNAL-*O*- β -glucuronide); and the toxicants (alkaloids): ANABA, ANATA and the specific metabolites of nicotine (namely cotinine (COT) and trans-3'-hydroxycotinine (COT-OH)).

Sample collection

Raw 24-h composite wastewater samples were collected at the inlet of the WWTPs in Geraardsbergen (GER) and Ninove (NIV), two small cities in the centre of Belgium (Flanders) where high use of stimulants were observed in our previous WBE study.¹⁹ Also, these two locations were within the province of Oost-Vlaanderen, where smoking prevalence was higher compared to other Belgian provinces.²⁰ Wastewater sampling was conducted over eight consecutive days in March 2014 (NIV) and March 2015 (GER). The

sewer catchment covered approximately 29,000 persons in GER and 36,000 persons in NIV. Samples were collected between 12 AM and 12 AM next day using a refrigerated auto-sampler at 4 °C. Sampling was conducted time-proportionally (every 10 min). This sampling method was previously used to assess illicit drug and alcohol use at the population level through WBE.^{19,21} About 1 L composite sample was aliquoted to polypropylene bottles pre-cleaned with ultrapure water and MeOH. After collection, samples were immediately frozen and stored at -20 °C until analysis.

Enzymatic deconjugation

Clinical studies have showed that NNAL is the key metabolite of NNK and is excreted in urine in its free form (30% of the total NNAL), as well as NNAL-*N*- β -glucuronide (22% of the total NNAL) and NNAL-*O*- β -glucuronide (48% of the total NNAL).²² The latter glucuronides are considered detoxification products due to the absence of carcinogenic effects.² Thus, enzymatic deconjugation appears relevant to measure the total (free plus conjugated) load of target analytes. The current study assessed the performance of an enzymatic deconjugation step based on the optimised protocol developed only for COT and COT-OH by Rodríguez-Álvarez et al.¹⁴, but we further applied it to a larger range of compounds. In Rodríguez-Álvarez et al.'s study, different incubation times (2, 3 and 5h) and amounts of β -glucuronidase (100, 300, 600 and 1000 units) were tested for the deconjugation of COT and COT-OH in wastewater adjusted at pH 5 with 1M sodium acetate buffer. The optimised condition of 300 units for 5 h incubation, 600 units for 3 h incubation, and 1000 units for over 3 h incubation resulted in less than 1% of the conjugates remaining. Based on these optimised results, wastewater samples in this study were adjusted to pH 4.7 with acetate buffer (1 M) and added approximately 1500 units of β -glucuronidase followed by incubation for 3 h at 37 °C. To assess the performance of the deconjugation step, NNAL, NNAL-*N*- β -glucuronide and NNAL-*O*- β -glucuronide were added (each 10 ng) to the wastewater samples, which were then processed as described above. A three-fold increase in the NNAL signal was observed after deconjugation, thus confirming the efficacy of the deconjugation procedure.

Sample preparation

Prior to LC-MS/MS analysis, SPE was used as the technique for sample clean-up and enrichment of the analytes (NAB, NAT, NNN, NNK, NNAL, ANABA and ANATA). With the optimised and validated conditions, wastewater (100 mL) was filtered (1.6 μ m glass microfiber filters), adjusted to pH 1.5 using 6 M

HCl, and spiked with the deuterated IS (2 ng for each IS). The extraction was conducted using Oasis[®] MCX cartridges which were pre-conditioned with MeOH (2 mL) followed by acidified (pH 1.5) ultrapure water (3 mL). After sample loading, the cartridges were washed with acidified ultrapure water (2 mL) followed by MeOH (3 mL) and dried under vacuum for 30 min. Target analytes and deuterated IS were eluted with 5% NH₄OH/MeOH (7 mL) and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The dry residue was reconstituted in a mixture (100 µL) of ultrapure water:MeOH (95:5) with 5 mM ammonium formate. Being present in higher concentrations, COT and COT-OH were measured in wastewater samples (1 mL) after filtration, spiking with the deuterated IS (10 ng) and direct injection into the LC-MS/MS.

LC-MS/MS analysis

Analysis of target analytes was conducted using HPLC (Agilent Infinity 1200, Santa Clara, CA, USA) coupled to a triple quadrupole mass spectrometer (Agilent 6460 MS) with an electrospray ionisation (ESI) interface. The HPLC system was equipped with a binary mixing pump, vacuum membrane degasser, thermostatic column compartment and an auto-sampler. Under the optimised conditions, three different chromatographic methods were used for the analysis the target analytes.

In method 1, separation of COT, COT-OH, ANATA, ANABA, NAT, NAB, and NNK was performed on a Waters Atlantis T3 column (150 x 2 mm, 3 µm, Waters, Zellik, Belgium) using a mobile phase consisting of ultrapure water (A) and MeOH (B), both with 0.1% formic acid and 5 mM ammonium formate. The following gradient was used: 0-0.5 min, 5% B; 12 min, 17% B; 16-20 min, 98% B; 20.7-25 min, 5% B.

Method 2, used for the separation of NNN and NNAL, differed from method 1 only in the mobile phase used (i.e., use of only 5mM of ammonium formate in (A) and (B)) and the gradient: 0-0.5 min, 5% B; 4 min, 75% B; 4.5-7.5 min, 95% B; 8-12 min, 5% B.

Method 3 was used for the separation of the NNAL glucuronides, using a Hilicon iHILIC[®]-Fusion column (150 x 2.1 mm, 3.5 µm, 100Å, Hilicon, Umeå, Sweden) using ultrapure water (A) and ACN (B), both with 10 mM ammonium formate, and the following gradient: 0-0.5 min, 90% B; 8.5-11.5 min, 25% B; 12-15.5 min, 90% B. The column temperature was set at 40 °C and the flow rate of the mobile phase was maintained at 0.3 mL/min. For analysis, 2 µL of the sample extracts were injected into the LC-MS/MS system.

The ESI interface was operated in positive mode for each of the three methods. Nitrogen was used as the nebulising, sheath and collision gas. Source parameters were optimised for each method, including ion spray

voltage, temperature, nebulising gas flow and pressure. The MS was operated in multiple-reaction monitoring (MRM). The MRM parameters, including fragmentor voltage, collision energy and cell accelerator voltage, were optimised for each analyte (Table 1). The two most intense MRM transitions per analyte were selected for confirmation and quantification in the samples. The dwell time for each MRM transition was set to 70 ms. Acquisition and data processing were conducted using MassHunter software (Agilent).

Data quantification

Together with the six-point calibration curves (0.5-100 ng/mL, IS 20 ng/mL for SPE and IS 10 ng/mL for direct injection), concentrations of the target analytes in the sample extracts were measured using LC-MS/MS and quantified through the isotope dilution approach. Analyte identification and confirmation was based on (a) analyte retention time in the sample compared to neat calibration standards and its corresponding deuterated IS (± 0.1 min); (b) consistent concentrations measured in both MRM transitions of the analyte ($\leq 20\%$, relative standard deviation (RSD %)); and (c) ion ratio of the MRM transitions in the samples comparable to the calibration standards ($\leq 20\%$), as described elsewhere.²³⁻²⁵ With the isotope dilution approach, concentrations of analytes in the wastewater samples were quantified taking into account the respective IS recovery. This allows to compensate for the loss of analytes during SPE, as well as for potential matrix effects during instrumental analyses.

Method validation

The optimised method was validated on the basis of the European Medicines Agency guideline on bioanalytical method validations.²⁶ However, since blank raw wastewater samples are not available, slight modifications were made following other WBE studies.^{14,27,28} The within-run and between-run precision (RSD %) and accuracy (bias %), extraction efficiency, matrix effect, limits of detection and quantification, linearity and carry-over were evaluated.

For the examination of precision and accuracy, three different levels (low, medium and high) within the calibration range and five replicates at the low level were evaluated on three different days. The precision of the developed method was assessed based on the variation (RSD %) of the repeated low level (n=5) within a day (i.e. within-run precision) and the variation of each level on three different days (i.e. between-run precision). The accuracy of the method was evaluated based on the deviation (bias %) of the repeated low

level ($n=5$) from the nominal value within a day (i.e. within-run accuracy) and the deviation of each level from the respective nominal value on three different days (i.e. between-run accuracy). The within-run and between-run precision of wastewater extraction and analysis was assessed using spiked wastewater samples.^{14,27,28} Accordingly, both tap water and wastewater samples were used for each SPE batch of validation per day, consisting of (a) tap water samples spiked at three different levels (6.25, 37.5, and 75 ng/L) of the target analytes, (b) five tap water replicates spiked at the lowest level, and (c) duplicate wastewater samples spiked at 10 ng/L (at least 30% of the expected level). All samples for SPE were spiked with IS at 20 ng/L. For direct injection analysis (i.e. without SPE), the setup included (a) tap water samples spiked at three different levels (1, 5 and 10 $\mu\text{g/L}$) of COT and COT-OH, (b) five tap water replicates spiked at the lowest level, and (c) duplicate wastewater samples spiked at 10 $\mu\text{g/L}$. All samples for direct injection were spiked with IS at 10 $\mu\text{g/L}$. The criteria of acceptance were assigned as a RSD <15% and a bias <15%.

Extraction efficiency was determined based on the difference between the spiked and non-spiked wastewater sample and relative to the blank sample spiked at the same level. Method detection and quantification limits (MDLs and MQLs) were estimated at a signal-to-noise (S/N) ratio of 3 and 10, respectively, using the spiked wastewater and/or wastewater samples with low analyte concentrations.^{14,27,28} The estimated MQL is included as the lowest calibration point that can be determined within an acceptable precision and accuracy (i.e., RSD and bias < 20%).

The instrumental detection and quantification limits (IDLs and IQLs) were estimated from low concentrations giving a S/N ratio of 3 and 10, respectively. The within-run and between-run precision and accuracy of the analysis were assessed using the lowest calibration point. Instrumental linearity (R^2) was evaluated based on three-time analyses of the calibration curve, in which each calibration point illustrated the back-calculated concentration within 15% of the nominal level but within 20% for the lowest calibration point. Carry-over during analysis was evaluated by injection of blank tap water samples after the highest calibration point and was determined with the criterion of the analyte's response less than 20% of the IQL.

Additionally, the developed method was tested using the provided urine sample containing NNAL in an inter-laboratory exercise (<http://www.g-equas.de>). Urine samples (1 mL) were diluted with tap water to 100 mL, followed by enzymatic deconjugation, SPE and LC-MS/MS analysis, as described above for wastewater samples.

Stability assessment

1 The stability of target compounds in wastewater was assessed in darkness at (i) room temperature (20 °C)
2 and (ii) refrigerated (4 °C). Real wastewater samples (pH 7.5) were spiked at 30 ng/L of the target
3 compounds. Stability was assessed over 24 h by collecting triplicate aliquots at regular intervals (t = 0 h, 0.5
4 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, and 24 h). Immediately after collection, and before analysis, aliquots were spiked
5 with deuterated IS and centrifuged (5 min at 10000 rpm) using 0.45 µm cellulose acetate centrifuge tube
6 filters (Costar® Spin-X®, Sigma-Aldrich, Overijse, Belgium). The experimental design (spiking levels,
7 sampling intervals and durations) was based on the recommended practice for such stability study in WBE²⁹
8 and has been applied in our previous studies.^{30,31}

19 **Estimation of chemical mass loads in the studied populations**

21 The daily mass load (mg/day) of the analyte was computed through multiplying the analyte concentration
22 (ng/L) measured in the sample by the corresponding daily influent wastewater flow (L/day) recorded in the
23 WWTP. Population normalised loads were then obtained from dividing the mass loads by the number of
24 inhabitants served by the WWTP, and therefore providing estimates of the average exposure to tobacco-
25 related compounds in the investigated populations.

RESULTS AND DISCUSSION

Method optimisation

LC-MS/MS method

The MS parameters of the target analytes were optimised for the most abundant signal by direct injection of individual standard solutions (1 ng/μL) into the MS. Both positive and negative electrospray ionisation (ESI) modes were tested. The former showed higher analyte responses as the presence of a nitrogen moiety facilitated the protonation to form $[M+H]^+$ ions. Two most abundant product ions were selected for quantification and confirmation (Table 1).

Different combinations of mobile phases and LC columns were investigated in order to optimise the separation and the sensitivity of the target analytes (Figure S-1). Chromatographic separation was attempted using an Atlantis® T3 column, a reversed-phase (RP) C18 column compatible with 100% aqueous mobile phases, allowing a balanced retention of both polar and hydrophobic compounds. Mobile phases consisting of water/ACN and water/MeOH were tested together with different acidic modifiers (0.1% acetic acid, 0.1% formic acid, and 0.1% formic acid with 5 mM ammonium formate), which can promote the protonation of basic molecules, and therefore increase signals in the positive ESI mode. Highest intensities for COT, COT-OH, ANATA, ANABA, and NNK were found using water/MeOH with 0.1% formic acid and 5 mM ammonium formate. NAT and NAB showed lower sensitivities with this mobile phase, yet better peak shapes were obtained compared to water/ACN with acidic modifiers. Using the selected mobile phase, further tests were carried out on other RP analytical columns, including Kinetex C18 (100 x 2.1 mm, 2.6 μm, 100Å, Phenomenex), Synergi Fusion-RP (100 x 2 mm, 2.5 μm, 100Å, Phenomenex) and Biphenyl (100 x 2.1 mm, 2.6 μm, 100Å, Phenomenex). However, none provided improved peak retention and sensitivity compared to the Atlantis® T3 column. Chromatographic separation of COT, COT-OH, ANATA, ANABA, NAT, NAB, and NNK was thus achieved on the Atlantis® T3 column using a mobile phase consisted of water/MeOH with 0.1% formic acid and 5 mM ammonium formate (Figure 1A).

Due to several pKa for the molecules under acidic conditions, poor sensitivity and peak shape of NNN and NNAL were observed. Increased pH to about 6.5 provided improved results and the optimal condition was obtained using only 5 mM ammonium formate in combination with the Atlantis® T3 column (Figure 1B).

For NNAL glucuronides, further optimisation of the chromatographic conditions was required due to the high polarity of the two compounds. Adequate separation and retention were only possible using a

hydrophilic interaction chromatographic column (HILIC, iHILIC[®]-Fusion, Hilicon) with water/ACN mobile phase with ammonium formate buffer (Figure 1C). Two levels (5 and 10 mM) of ammonium formate buffer were tested and the best result, in terms of separation and sensitivity, was obtained with 10 mM.

Sample preparation method

Solid-phase extraction sorbent types. Various types of SPE sorbents were tested to isolate the target analytes from wastewater samples (Table 2). Hydrophilic-lipophilic balance (Oasis[®] HLB) and mixed-mode cation exchange (Oasis[®] MCX) cartridges (3 cc, 60 mg) were evaluated. Both cartridges are capable of capturing the analytes through various molecular interactions, such as hydrophilic, hydrophobic and π - π forces. MCX cartridges further allow better extraction of cations through ionic interactions with positively charged compounds. Sample acidification thus promotes protonation of the amine moiety of our analytes for better attachment to the MCX sorbents. Samples were adjusted to pH 7 for HLB and to pH 1.5 for MCX. Absolute recoveries and matrix effects were evaluated for selecting the most suitable SPE cartridges (Table 2). The recovery was calculated by comparing the analyte area between wastewater samples spiked with deuterated analytes before and after SPE. The matrix effect was assessed by comparing wastewater extracts spiked with deuterated analytes after SPE to neat standards spiked at the same concentration level.

With HLB cartridges, methanol (2 mL) followed by ultrapure water (3 mL) were used to condition the sorbent. After sample loading, the sorbent was washed with ultrapure water (5 mL) to remove residual salts, dried under vacuum and eluted with 5% formic acid in methanol (7 mL). A similar procedure was used for MCX cartridges, except using acidified (pH 1.5) ultrapure water as the condition and wash solutions and 5% ammonia hydroxide in methanol as the elution solution. Both SPE sorbents provided acceptable recoveries and similar matrix effects (Table 2). The MCX sorbent was eventually considered for further applications, as it allows more flexibility for addressing matrix effects using organic solvents without substantial reduction in recoveries. This is in contrast to the study of Wu et al.¹⁶ which reported difficulties in dealing with matrix effects with the analysis of tobacco-related compounds in wastewater using HLB. The findings obtained here illustrate that improved sample preparation can be achieved.

Since NNAL-*N*- β -glucuronide and NNAL-*O*- β -glucuronide resulted in very low recovery (<10%) using the abovementioned methods, three additional cartridges were tested, including the mixed-mode polymeric (Oasis[®] MAX, 60 mg, 3 cc), polymeric reversed-phase (Oasis[®] WAX, 60 mg, 3 cc) and a molecular-imprinted polymer (MIP) specific for NNAL (SupelMIP-NNAL, 25 mg, 10 cc, Sigma-Aldrich, Overijse,

Belgium). For MAX and WAX cartridges, various sample pH values were evaluated (i.e., 4.5, 7 and 9) as well as different elution media (i.e., methanol with 2, 5, 10 and 20% ammonium hydroxide for WAX and 2% formic acid for MAX). For the MIP, manufacturer conditioning (1 mL dichloromethane followed by 1 mL methanol and 1 mL ultrapure water), washing (2 mL ultrapure water, 5 min vacuum drying, 2 mL toluene and finally 2 mL of toluene:dichloromethane, 9:1, v/v) and elution (2 x 2 mL of dichloromethane) media were used. None of the tested extraction procedures provided satisfactory results. The two glucuronides were either not recovered (i.e., SupelMIP) or only one of the two was found at very low recovery (<10%). Consequently, it was decided to proceed with the extraction using MCX cartridges, as described above, after enzymatic deconjugation to measure total NNAL in the samples.

Washing and elution solutions. With the MCX cartridges, alternative washing solutions proposed in the literature³² were modified for our study (Table 2). First, a sequence of acidified (pH 1.5) ultrapure water (3 mL) followed by methanol (3 mL) was tested, allowing to remove inorganic ions and neutral hydrophobic interferences, respectively. Second, a mixture of water-MeOH-NH₄OH (96:2:2) (1 mL) was tested for removing cationic interferences. In both cases, our analytes were eluted with 5% NH₄OH in MeOH (7 mL). The acidified water-MeOH wash sequence provided reduced matrix effects for some analytes while maintaining acceptable recoveries for the target analytes (Table 2). The second wash sequence showed neither improvement of matrix effects nor recoveries (Table 2). Therefore, the acidified water-MeOH wash sequence was adopted for this study using MCX cartridges.

Furthermore, elution with 10% NH₄OH/MeOH was tested, but did not improve recoveries nor matrix effects. The initial approach using 5% NH₄OH/MeOH was thus used for this study.

Pre-concentration steps. Collection and evaporation of SPE eluates using different recipients (glass versus plastic) and temperature were investigated and optimised to assess potential analyte losses during sample preparation (Figure 2). This was done by spiking the elution solution (5% NH₄OH/MeOH, 7 mL) with target analytes in glass and polypropylene tubes, followed by evaporation under a gentle stream of nitrogen at room temperature and with heating (40 °C). Recoveries were calculated with respect to a neat standard which did not undergo any evaporation step. Slightly better results were obtained with polypropylene tubes and heating, except for ANATA and ANABA, which provided higher recoveries without temperature (Figure 2). For all other analytes, evaporation at room temperature yielded lower recoveries (Figure 2), likely because of longer evaporation times and the sorption of analytes onto the tube walls, particularly for glass. Overall, the results showed that the analytes were not susceptible to heat and

higher recoveries were obtained using polypropylene tubes. Thus, pre-concentration was performed at 35 °C using polypropylene tubes.

Method validation

The developed method was validated by examining the within-run and between-run precision (RSD %) and accuracy (bias %), extraction efficiency, sensitivity and linearity (Tables 1 and 3). For the SPE-LC-MS/MS procedure, the within-run precision (n=5) of the low concentration level was 2.5-4.6% (Table 3). The between-run precision in three different days was 4.0-5.2%, 1.7-4.6% and 1.7-4.4% for three different levels (low, medium, high), respectively. The within-run accuracy for measuring the low level was 3.0-4.3% and the between-run accuracy was 3.6-6.6%, 1.7-10% and 3.6-13% for the low, medium and high level, respectively. The within-run and between-run precision of the method in wastewater was at 2.0-8.4% and 4.1-9.3%, respectively. The extraction recovery of the analytes in wastewater was 97-106%. The matrix effect was minimal for ANATA, NAT and NAB but enhancement was observed for ANABA and suppression for NNK, NNAL and NNN (Table 2). The MDLs and MQLs were estimated respectively at 0.1-2.7 ng/L and 0.5-9.1 ng/L. The sensitivity herein is reported for the first time when it comes to measuring NAB, NAT, NNN, NNK and NNAL in wastewater. Another study¹⁶ reported the MDLs (0.02-0.1 ng/L) for NAB, NAT, NNN, NNK and NNAL using tap water which are often substantially lower due to the absence of matrix induced by wastewater constituents. Our method showed a similar range of MDLs (0.02-0.3 ng/L) for these analytes in tap water analyses. For ANABA and ANATA, our method showed the MDLs up to six-times lower than another study (20 ng/L) (Tscharke et al. 2015).

For the direct injection of COT-OH and COT, the within-run precision of the low level was respectively 4.2% and 2.4% (Table 3). The between-run precision of measuring COT-OH and COT in three different days was respectively 7.1% and 4.6% at the low level, 9.8% and 3.1% at the medium level, and 2.7% and 2.3% at the high level. The within-run accuracy was 7.2% for COT-OH and 8.6% for COT. The between-run accuracy was 8.0%, 10% and 6.2% for COT-OH and 12%, 8.4% and 5.5% for COT at the low, medium and high level, respectively. The within-run and between-run precision of the method in wastewater analysis was 5.3% and 3.9% for COT-OH and 1.9% and 6.1% for COT, respectively. Recovery of these two analytes in the spiked samples was about 90-100% with minimal matrix effect (-5.4%, suppression). The MDLs and MQLs were, respectively, estimated at 23-100 ng/L and 37-120 ng/L.

The instrumental within-run and between-run precision at the lowest calibration standard was at 0.4-5.8% and 1.2-4.4%, respectively (Table 1). The between-run accuracy was 0.8-11%. The IDLs of the analytes were <0.2 ng/mL and the IQLs were estimated 0.01-0.6 ng/mL. Linearity with the weighting of 1/x for all target analytes was in the range of 0.9988-0.9995 (Table 1). The back-calculated level of each calibration point, except the lowest one, was found within 0.7-9.3% of the nominal value. For the lowest calibration point, the back-calculated level was within 0.8-11% of the nominal value with a within-run and between-run precision of 0.4-6% and 1.2-4.4%, respectively (Table 1). The results were fit to the acceptable criteria of the guideline. There was no carry over during the analysis observed as all analytes showed negligible responses in the blank tap water samples following an injection of the highest calibration point.

Furthermore, when the developed method was implemented for the analysis of NNAL in the inter-laboratory urine sample, the deviation from the reference value was less than 12%. The average IS recovery was 75% with the variation 1.8% (RSD %) and matrix effect -90%. These values were similar to the validation results obtained for wastewater (Table 3).

Overall, the method performance was within acceptable variations and accuracy as defined in the guideline. Therefore, the developed method is reliable, repeatable and reproducible for the measurement of tobacco-related toxicants and carcinogens in wastewater.

Stability tests

The stability experiment conducted at 20 °C was used to evaluate the potential stability of target analytes in wastewater during in-sewer transportation. Generally, compared to the response measured at t₀, a decrease of 10-20% was found for all analytes during the first hour (Figure 3). No substantial decline was observed thereafter. The decrease was considered minimum and thus suggested that the analytes are relatively stable in such conditions. However, the complexity of the real sewer system, e.g., the presence of biofilms, could potentially promote chemical degradation.^{29,30} Our study followed common approaches^{13-15,27} for a preliminary evaluation of chemical stability in the context of WBE studies.²⁹ The stability test at 4 °C was used to assess whether the analytes are stable during the sample collection and storage under refrigeration. All target analytes appeared to be stable at 4 °C over 24 h (Figure 3).

Real sample applications

1 The occurrence and level of the target analytes in wastewater samples of two Belgian small cities (GER
2 and NIV) were evaluated using the developed analytical method (Table 4). Among the nine analytes, COT-
3 OH, COT, ANATA, ANABA, and NAT were found in each sample of the sampling week in GER. The same
4 profile was observed in NIV, except that NAT was detected on only two days. NAB, NNN, NNK, and
5 NNAL were not quantifiable in any of the investigated samples. In both locations, COT-OH (GER: 4800-
6 5500 ng/L; NIV: 4000-6100 ng/L) and COT (2300-2800 ng/L; 1900-3200 ng/L) were the most abundant
7 compounds, followed by ANATA (26-38 ng/L; 33-40 ng/L) and ANABA (11-16 ng/L; 11-15 ng/L), whereas
8 NAT (0.5-1.4 ng/L; <0.5-0.5 ng/L) was found only in trace levels. Examples of the chromatograms of
9 ANATA, ANABA and NAT in wastewater extracts are showed in Figure S-2. There were no weekly
10 patterns observed for the target analytes, suggesting a stable consumption of tobacco and a constant overall
11 exposure to these compounds in the two studied cities.

12 The higher concentration of COT-OH than COT observed in this study was consistent with results from
13 China³³ and Spain.¹⁴ The average ratio of COT-OH to COT was 1.9 in the investigated Belgian cities, similar
14 to findings from Spain (1.8), yet higher than in China (1.2 measured across 11 cities). These ratios are
15 similar to data from human metabolism of nicotine, which approximately 44% is excreted as COT-OH and
16 30% as COT (COT-OH/COT: 1.5).¹³

17 The average population-normalised mass load (range) of ANATA and ANABA were measured at 9.5
18 (7.9-11) and 4.1 (3.4-4.6) mg/day/1000 people for GER and 8.7 (7.3-13) and 3.2 (2.3-5.0) mg/day/1000
19 people for NIV, respectively. The level of these two analytes and their ratio (2.5) are similar to those
20 measured in Adelaide, Australia.¹⁵ The results implied similar tobacco use per capita between the small cities
21 in Belgium and the major urban city in Australia, although survey data of the World Health Organisation
22 showed higher prevalence of tobacco smoking in the general population in Belgium compared to Australia.¹

23 Compared to tobacco alkaloids, TSNAs were often not detected in wastewater or were measured in very
24 low levels. The average concentration and population-normalised mass load of NAT was about 0.7 ng/L and
25 0.2 mg/day/1000 people for GER, and 0.5 ng/L and 0.1 mg/day/1000 people for NIV, respectively. Our
26 findings were similar to the study of Wu et al. (2014)¹⁶, in which NAB and NNN were not detected and NAT
27 was found in 50% of the samples with levels of 0.02-0.07 ng/L. NNK (0.4-1 ng/L) and NNAL (2.6-5.4 ng/L)
28 were also measured in low levels by Wu et al. (2014).¹⁶

29 The rank order of the tobacco alkaloids, carcinogens and metabolites detected in wastewater
30 corresponded to the urinary excretion of these compounds reported in previous clinical studies.^{34,35} These

found that the amount (average $\pm$ standard deviation) of urinary excretion was in the μg per day level for the alkaloids (total excretion over 24 h: COT 3360 ± 2070 ; ANATA 24.1 ± 25.3 ; ANABA 15 ± 11.5) but in the ng per day level for the carcinogens (NAT 490 ± 420 ; NAB 120 ± 90 ; NNN 12.3 ± 8.59 ; NNAL 289 ± 179). The particularly low levels of carcinogens measured in urine would thus reasonably explain the low amounts found in wastewater. Furthermore, the matched pattern between the chemical levels measured herein and in the clinical studies as well as the similar ratio of tobacco alkaloids measured in our study and in a previous WBE study¹⁵ suggest that the target compounds are likely due to human intake of tobacco.

Despite a lack of pharmacokinetic data for most of these tobacco-related compounds, temporal variations in mass loads of the target chemicals should reflect changes in the use/exposure of these tobacco-specific chemicals in the populations, and therefore population health status with respect to tobacco exposure over time. These results provide relevant information from the epidemiological perspective, in particular to monitor the long-term effectiveness of interventions and policy changes. Similar limitation can be also seen in other WBE studies in which pharmacokinetic data on the studied chemicals are not yet available, such as exposure to pesticides²⁸ and new psychoactive substances.^{24,36} In fact, mass loads measured in wastewater could be further converted to any estimates of interest in the future, particularly when pharmacokinetic data becomes available. Also, future studies with more and different types of communities are required to explore which analytes could be appropriate WBE markers to assess population exposure and health with regard to tobacco use. Yet, as observed via WBE in this preliminary study, it appears that tobacco smoking in the studied populations did not lead to a substantial exposure to its related carcinogens. However, exposure to these carcinogens over time remains a concern for public health. Future monitoring studies of these compounds in wastewater should be considered for a continuous evaluation at the population level as well as in different international communities.

CONCLUSIONS

Our study provided a comprehensive analytical procedure for the simultaneous determination of TSNAs, tobacco alkaloids and their metabolites in raw wastewater. Excellent validation parameters confirmed the robustness of the developed method, which was then applied to assess the occurrence and levels of these compounds in wastewater from two Belgian small cities. Both locations showed similar profiles and levels of the target analytes. Tobacco alkaloids and metabolites were noticed in all samples with high levels, whereas TSNAs were not found except for NAT which was observed in most samples at low levels. The results also

showed stable tobacco use throughout the week and illustrated for the first time an approach with the combined monitoring of toxicants and carcinogens to evaluate the exposure to tobacco at the population level in the context of WBE.

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ASSOCIATED CONTENT

Supporting Information

This material containing Figures S-1 and S-2 is available free of charge via the Internet at <http://pubs.acs.org>.

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Tables and Figures

Table 1: Instrumental parameters and performance for the analysis of the target analytes and internal standards.

Analytes	Q1 > Q3 (quantifier; qualifier) (m/z)	FV	CE	CAV	Linearity (R ²) ^a	Precision ^b (within-run ^c ; between-run ^d) (RSD %)	Accuracy ^b (between- run ^d) (% bias)	IDL (ng/mL)	IQL (ng/mL)
COT-OH	193.1 > 80.1; 134.0	70	30; 15	0; 4	0.9988	0.37; 2.44	10.5	0.17	0.57
COT	177.1 > 146.1; 80.1	70	15; 25	4; 0	0.9989	0.60; 1.86	6.49	0.16	0.52
COT-D ₃	180.1 > 80.1	70	25	0	-	-	-	-	-
ANATA	161.1 > 144.1; 106.1	80	8; 6	7; 7	0.9992	0.35; 1.53	8.49	0.05	0.18
ANATA-D ₄	165.1 > 148.1	60	10	7	-	-	-	-	-
ANABA	163.1 > 120.1; 80.1	70	12; 20	7; 0	0.9993	5.81; 4.39	2.67	0.08	0.28
ANABA-D ₄	167.1 > 84.0	60	26	0	-	-	-	-	-
NAT	190.1 > 160.1; 106.1	50	6; 14	0; 0	0.9992	2.21; 2.60	3.49	0.04	0.15
NAT-D ₄	194.1 > 164.1	50	6	4	-	-	-	-	-
NAB	192.1 > 162.1; 133.1	50	6; 20	4; 0	0.9995	3.19; 3.89	3.77	0.03	0.09
NAB-D ₄	196.1 > 166.1	40	6	4	-	-	-	-	-
NNK	208.2 > 122.0; 106.1	40	8; 20	0; 0	0.9994	1.64; 1.23	0.81	0.02	0.07
NNK-D ₄	212.2 > 126.1	80	14	0	-	-	-	-	-
NNAL	210.1 > 180.1; 149.1	50	6; 6	0; 0	0.9988	2.90; 3.14	5.27	0.04	0.12
NNAL-D ₅	215.1 > 185.1	60	6	0	-	-	-	-	-
NNN	178.1 > 148.1; 120.1	60	6; 14	4; 4	0.9995	2.00; 2.26	2.17	0.003	0.01
NNN-D ₄	182.2 > 152.1	40	6	4	-	-	-	-	-

Q1: parent ion; Q3: product ions; FV: fragmentor voltage; CE: collision energy; CAV: accelerator voltage; ^a1/x weighting with three-time analyses; ^bat the lowest calibration level; ^cn=2; ^d3 days; IDL: instrumental detection limit; IQL: instrumental quantification limit.

Table 2: Comparisons of absolute recovery and matrix effect with different cartridges and washing solutions.

Analytes	HLB		MCX		MCX ^a		MCX ^b	
	Recovery (%)	Matrix effect (%)	Recovery (%)	Matrix effect (%)	Recovery (%)	Matrix effect (%)	Recovery (%)	Matrix effect (%)
ANATA	91	-66	103	-80	81	-8.0	65	-20
ANABA	95	-38	101	-56	87	63	73	4.0
NAT	85	62	97	-62	79	3.0	64	19
NAB	88	45	98	-59	75	5.0	68	20
NNK	90	-89	106	-93	73	-89	65	-84
NNAL	106	-99	94	-97	85	-92	67	-96
NNN	98	-97	115	-98	84	-93	58	-95

Elution solution: 0.1% formic acid in methanol for HLB and 5%NH₄OH in methanol for MCX; ^awash solution: (1) pH 1.5 water (3 mL) then (2) methanol (3 mL); ^bwash solution: (1) pH 1.5 water (3 mL), (2) methanol (3 mL) then (3) water-MeOH-NH₄OH (96:2:2, 1mL). Matrix effect in -ve as suppression and +ve as enhancement. Bold: the optimum condition selected for this study.

Table 3: Method performance of the optimised analytical method.

Analytes	Tap water								Wastewater				
	Within-run precision ^a (RSD %)	Between-run precision ^b (RSD %)			Within-run accuracy ^a (bias %)	Between-run accuracy ^b (bias %)			Within-run precision ^c (RSD %)	Between-run precision ^d (RSD %)	Recovery ^e %	MDL (ng/L)	MQL (ng/L)
		Low	Med	High		Low	Med	High					
COT-OH	4.24	7.10	9.83	2.72	7.18	8.04	10.3	6.21	5.25	3.91	89	23	37
COT	2.44	4.61	3.14	2.30	8.57	11.7	8.43	5.46	1.90	6.11	100	100	120
ANATA	3.26	4.03	1.69	1.65	3.05	3.62	10.0	13.2	1.95	4.96	102	2.7	9.1
ANABA	3.89	4.99	2.49	1.83	3.53	6.58	2.17	7.00	8.41	9.25	105	1.0	3.5
NAT	3.80	4.65	3.41	2.69	3.55	5.20	2.29	3.58	3.86	4.05	98	0.1	0.5
NAB	3.40	4.74	4.55	2.19	2.79	4.46	3.27	4.17	7.04	7.37	106	0.1	0.5
NNK	4.55	4.24	2.57	1.88	4.29	4.87	2.23	5.55	5.46	7.93	97	0.4	1.4
NNAL	2.54	5.22	2.37	4.36	2.97	6.04	1.67	4.66	7.94	9.16	101	1.8	6.0
NNN	4.05	4.23	3.12	2.37	3.19	3.86	2.35	5.16	2.61	4.12	103	0.4	1.5

^an =5; ^b3 different days; ^cn=2 ; ^d2 different days. ^en=2; MDL: method detection limit; MQL: method quantification limit.

Table 4: Daily concentrations, mass loads and population-normalised mass loads of the analytes found in the samples.

GER		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Concentration (ng/L)	COT-OH	4840	4840	4960	5540	5500	4930	4830	5140
	COT	2450	2300	2350	2830	2750	2550	2500	2730
	ANATA	29.6	26.1	29.9	31.2	37.5	35.8	32.8	37
	ANABA	14.8	11.2	12.1	14.8	14.2	12.9	15.6	15.5
	NAT	0.66	0.55	0.55	1.40	0.55	0.50	0.60	0.73
Mass load (g/day)	COT-OH	43.2	42.4	43.8	47.2	46.6	41.0	39.1	41.1
	COT	21.9	20.1	20.7	24.1	23.3	21.2	20.2	21.8
	ANATA	0.26	0.23	0.26	0.27	0.32	0.30	0.27	0.30
	ANABA	0.13	0.10	0.11	0.13	0.12	0.11	0.13	0.12
	NAT	0.006	0.005	0.005	0.012	0.005	0.004	0.005	0.006
Population-normalised mass load (mg/day/1000 people)	COT-OH	1490	1460	1510	1620	1610	1410	1350	1420
	COT	754	693	713	828	803	729	697	751
	ANATA	9.10	7.86	9.09	9.13	10.9	10.3	9.12	10.1
	ANABA	4.55	3.39	3.67	4.33	4.14	3.68	4.34	4.26
	NAT	0.20	0.17	0.17	0.41	0.16	0.13	0.17	0.20
NIV		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Concentration (ng/L)	COT-OH	4750	4770	4960	5650	6040	4020	5870	6080
	COT	2490	2470	2590	3010	3210	1920	3060	3040
	ANATA	32.7	35.9	39.6	34.6	35.2	35.6	34.7	36.0
	ANABA	11.2	11.3	13.4	13.6	15.1	11.3	13.1	14.9
	NAT	0.50	0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
Mass load (g/day)	COT-OH	39.3	41.1	48.3	75.9	49.1	29.7	44.9	45.7
	COT	20.6	21.2	25.2	40.4	26.1	14.2	23.4	22.8
	ANATA	0.27	0.31	0.39	0.46	0.29	0.26	0.27	0.27
	ANABA	0.09	0.10	0.13	0.18	0.12	0.08	0.10	0.11
	NAT	0.004	0.004	na	na	na	na	na	na
Population-normalised mass load (mg/day/1000 people)	COT-OH	1090	1140	1330	2100	1360	822	1240	1260
	COT	570	587	696	1120	721	393	648	631
	ANATA	7.47	8.54	10.7	12.8	7.91	7.28	7.35	7.47
	ANABA	2.57	2.69	3.61	5.03	3.39	2.31	2.77	3.10
	NAT	0.11	0.12	na	na	na	na	na	na
na: not available when concentrations <MQLs. Daily influent wastewater flow over the monitoring period, range (mean ± standard deviation): GER, 8-9 ML/day (8.5 ± 0.34 ML/day) and NIV, 7.4-13 ML (8.8 ± 2.0 ML/day).

Figure 1: Chromatograms of a mixture standard of seven analytes (each 0.1 ng injected) in method 1 (A); NNAL and NNN (each 0.1 ng injected) in method 2 (B); and NNAL-*N*- β -glucuronide and NNAL-*O*- β -glucuronide (each 5 pg injected) in method 3 (C).

Figure 2: Absolute recovery (%; n=3) of the target analytes during the pre-concentration step using different recipients (polypropylene (PP) and glass tubes (Glass)) at room temperature (RT) and with heating (T) at 40 °C.

Figure 3: Stability of target analytes in wastewater at 20 °C (top) and at 4 °C (bottom) over 24 h.

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