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Corresponding Author: Prof. Cristina Nerin, PhD

Corresponding Author's Institution: University of Zaragoza, Centro Politécnico Superior

First Author: Daniel Carrizo, PhD

Order of Authors: Daniel Carrizo, PhD; Isabel Nerin, PhD; Celia Domeño, PhD; Pilar Alfaro, Degree in Chemistry; Cristina Nerin, PhD

Abstract: A new screening method has been explored for direct analysis of tobacco smoke biomarkers in biological matrices (i.e. saliva and urine). Single run analysis using Atmospheric pressure Solid Analysis Probe (ASAP) and high resolution mass spectrometry with quadrupole and time of flight detector has been applied directly to some biological samples (i.e. urine and saliva), providing a fast, efficient and sensitive method of identification. The method has been applied to saliva and urine samples from heavy tobacco smokers for exposure studies. Nicotine itself, nicotine metabolites (i.e., cotinine, trans-3´-hydroxycotinine, nicotine-N-glucuronide) and other related tobacco smoke toxic compounds (i.e., NNK 4-[methyl(nitroso)amino]-1-(3-pyridinyl)-1-butanone, anatabine) were found in the analyzed samples. The identification of compounds was confirmed by ultrahigh performance liquid chromatography with MS-triple quadrupole detector after sample treatment. Different temporal trends and biomarkers behavior have been found in time series related samples. Both methods are compared for screening of these biological matrices.

Suggested Reviewers: Lourdes Ramos PhD
Prof. Research, Organic Chemistry, CSIC, Spain
l.ramos@iqog.csic.es
She is an expert in MS and has expertise as well in tobacco metabolites. She will have the criteria to evaluate the paper

DAvid Hammond PhD
dhammond@uwaterloo.ca
He works on tobacco and he will have the criteria to evaluate the paper.

P. Dhar
tate University of New York at New Paltz,, tate University of New York at New Paltz,
dharp@newpaltz.edu

She works in this area and she will have the criteria to evaluate the paper.

Cover Letter

Departamento de Química Analítica
Universidad Zaragoza

instituto de investigación en ingeniería de Aragón
Universidad de Zaragoza

Editor of
Journal of Pharmaceutical and Biomedical Analysis

Zaragoza, 22nd December, 2015

Dear Editor,

I'm sending you a new paper entitled **Direct screening of tobacco indicators in urine and saliva by Atmospheric Pressure Solid Analysis Probe coupled to Quadrupole-time of flight Mass Spectrometry (ASAP-MS-Q-TOF-)** by Daniel Carrizo[1], Isabel Nerín[2], Celia Domeño[1], Pilar Alfaro[1], Cristina Nerín[1*] for its publication in Journal of Pharmaceutical and Biomedical Analysis, if it is accepted.

This work deals with the direct analysis of contaminants from tobacco and their metabolites in several biological samples by ASAP-MS-Q-TOF, without any sample treatment. The samples are directly injected into the MS through a glass capillary and the MS conditions as well as the interpretation of the data obtained allow the identification of the contaminants and several metabolites. The relationship between the metabolites found and the tobacco exposure can be used to assess the health care program and the tobacco cessation strategies, as well as the policies to tobacco exposure. Using the direct analysis the monitoring of tobacco-related compounds and their metabolites turns into a fast and efficient procedure, which can be used for many purposes.
The main novelty of this work is the ASAP-MS-Q-TOF study for compounds and their metabolites in biological fluids, without previous sample treatment. This means that proteins, lipids and other components difficult to remove from the biological fluids, do not interfere in the analysis. The analysis itself can be done in only few minutes. Target analysis in which the specific compounds are searched for their characteristic masses become available, what facilitates the difficult task of analysis of biological fluids.
The procedure has been compared to UPLC-MS-TQ for the analysis of several biological samples of urine and saliva and time series samples from different smoking and non-smoking individuals. A deep discussion about the tobacco bioindicators found is included.

The corresponding author is Prof. Cristina Nerin, from the University of Zaragoza,
cnerin@unizar.es

This paper has not been sent to publish to any other journal.

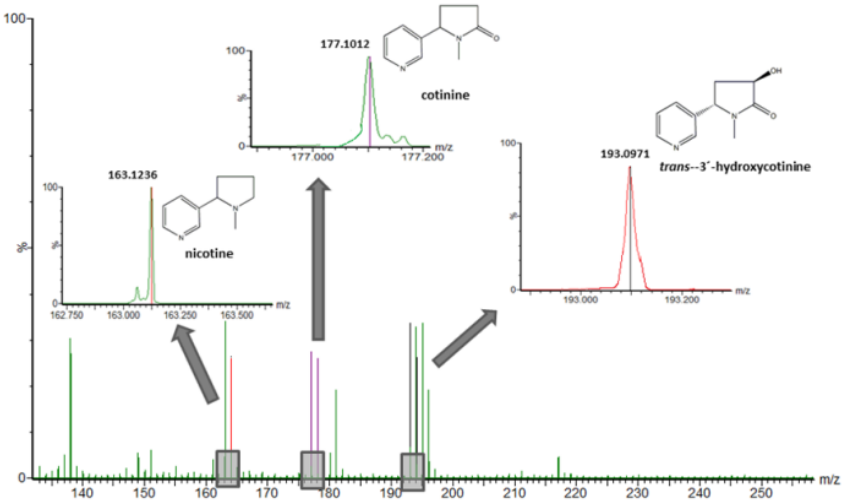
I hope that the paper can be accepted in its present version.
Best regards

Prof. Dra. Cristina Nerín
Catedrática de Universidad en Química Analítica
Directora del Grupo GUIA
Instituto de Investigación en Ingeniería de Aragón (I3A)
Escuela de Ingeniería y Arquitectura (EINA)
Depto. de Química Analítica, Campus Rio Ebro
Universidad de Zaragoza, Spain

*Graphical Abstract

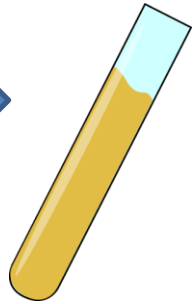
saliva

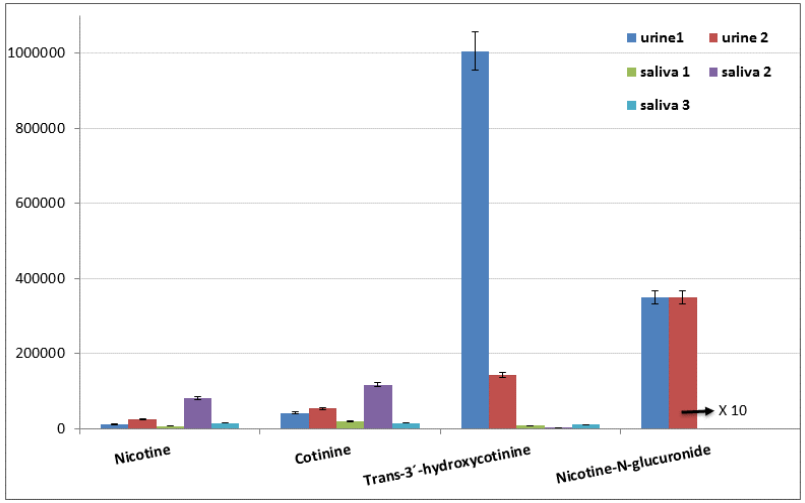
ASAP-MS-QTOF



UPLC-MS-TQ

urine





Highlights

- ASAP-MS-Q-TOF is optimized for direct screening of biological samples
- Urine and saliva from smokers and non-smokers are analyzed
- Time series of saliva and urine samples are studied
- ASAP is compared to UPLC-MS-TQ for quali and quantitative purposes
- Bioindicators of tobacco are found and discussed

Direct screening of tobacco indicators in urine and saliva by Atmospheric Pressure Solid Analysis Probe coupled to Quadrupole-time of flight Mass Spectrometry (ASAP-MS-Q-TOF-)

Daniel Carrizo[1#], Isabel Nerín[2], Celia Domeño[1], Pilar Alfaro[1], Cristina Nerín[1*]

[1]*Aragon Institute of Engineering Research (I3A), EINA, Department of Analytical Chemistry, University of Zaragoza, María de Luna 3, 50018 Zaragoza, Spain*

[2]*Smoking Cessation Unit, Department of Medicine, Psychiatry and Dermatology, Faculty of Medicine, University of Zaragoza, Domingo Miral s/n,50009 Zaragoza, Spain*

[#]. now at: Institute for Global Food Security, Queen's University, Belfast, United Kingdom

* Corresponding author cnerin@unizar.es

Abstract

A new screening method has been explored for direct analysis of tobacco smoke biomarkers in biological matrices (i.e. saliva and urine). Single run analysis using Atmospheric pressure Solid Analysis Probe (ASAP) and high resolution mass spectrometry with quadrupole and time of flight detector has been applied directly to some biological samples (i.e. urine and saliva), providing a fast, efficient and sensitive method of identification. The method has been applied to saliva and urine samples from heavy tobacco smokers for exposure studies. Nicotine itself, nicotine metabolites (i.e., cotinine, *trans*-3´-hydroxycotinine, nicotine-*N*-glucuronide) and other related tobacco smoke toxic compounds (i.e., NNK 4-[methyl(nitroso)amino]-1-(3-pyridinyl)-1-butanone, anatabine) were found in the analyzed samples. The identification of compounds was confirmed by ultrahigh performance liquid chromatography with MS-triple quadrupole detector after sample treatment. Different temporal trends and biomarkers behavior have been found in time series related samples. Both methods are compared for screening of these biological matrices.

Keywords: ASAP, direct analysis, tobacco exposure, cancer, biomarkers, screening.

Introduction

Tobacco use is an important cause of early death worldwide, causing a wide range of diseases and many types of cancer. Currently tobacco kills more than five million people and by 2030, the death toll will exceed eight million a year [1]. Recent predictions in China estimate a death toll higher than 2 million people in 2030 just because of tobacco. Cigarette smoke contains over 4000 different compounds, such as nicotine, hydrogen cyanide, carbon monoxide, nitrosamines and polyaromatic hydrocarbons [2]. Tobacco smoking is highly addictive, being nicotine, present in the cigarettes at relatively high concentration, the main responsible for that. Nicotine and other related alkaloids are absorbed in human beings through the skin and the lungs [3]. The primary precursors for the highly carcinogenic tobacco-specific nitrosamines are also at quite high concentration level and all together make these chemicals very important from a public health standpoint [4]. Tobacco also contains polycyclic aromatic hydrocarbons (PAHs), which are probably responsible as well for the cancer development in heavy smokers [5]. Nicotine and its major metabolite cotinine, used as tobacco biomarker, can be found after tobacco exposure in urine, blood and saliva samples [6-8] as well as in other non-conventional biological matrices like hair or meconium [9]. Although the concentration of these biomarkers can be very high in heavy smokers, the environmental and passive exposure to tobacco could be also measured in non-smokers with a sensitive analytical technique. Saliva and urine are

important alternative matrices to blood for monitoring tobacco exposure, since collection is simple, non-invasive and can be performed by non-medical personnel. It is worth to emphasize that the assessment of tobacco smoke exposure is a major topic medical science, with important implications in public health and government policies.

A wide variety of analytical techniques have been applied to the analysis of nicotine, cotinine, trans-3-hydroxycotinine (3-HC) and related tobacco smoke biomarkers in various biological fluids. Those analytical methods include immunoassays [10-11], gas chromatography (GC) coupled to either flame ionization (FID) [12] or mass spectrometric (MS) detection [13, 8, 14] and high-performance liquid chromatography (LC) coupled either to UV detector [15] or MS [16, 17]. All of them have a mandatory extraction step followed by extensive clean-up and fractionation steps prior to instrumental analysis. These steps are tedious, time consuming and expensive, due to the amount and type of chemicals and materials required. Thus the direct analysis of samples without any prior sample treatment is an important advantage for any laboratory performing routine analyses of these types of contaminants. A direct approach for detecting the presence of these compounds without investing time and money in the sample treatment is an attractive option that should be explored in detail. Atmospheric-pressure solid analysis probe (ASAP), is a new method for rapidly analyzing volatile or semi-volatile liquid or solid materials, which has only a few applications reported to date [18-21]. Two ambient mass spectrometry techniques, desorption electrospray ionization (DESI) [22] and the direct analysis in real time (DART) [23] originated the ASAP technique by McEwen in 2005 [18]. An important advantage of ASAP technique is that the whole sample can be introduced into the ionization chamber, instead of only the ionized vapor released by the sample (e.g.

DART and DESI). As vaporization and ionization with ASAP occur at atmospheric pressure a mass spectrum can be acquired in seconds from solid and liquid volatile or semi-volatile compounds. The non-volatile compounds which are not volatilized at about 500ºC cannot be analyzed using ASAP, as also occurs with DART and DESI. Thus, the ASAP technique extends the power of the analysis to unknown complex matrices. When ASAP is coupled to the high resolution -Q-TOF-MS technique the accurate mass of the fragments obtained facilitates the identification of the molecular structure of the compounds. This is an important advantage, especially when complex matrices (i.e., saliva or urine) without any prior treatment are involved. The identification of unknown compounds can be reached with the help of specific software tools such as MassLynx and ChemSpider chemical databases.

The aim of this work was to explore a direct method for the screening of nicotine and their major metabolites as well as other highly toxic tobacco biomarkers in biological fluids. The results will be compared to the conventional sample treatment followed by UPLC-MS-TQ. From this study additional biomarkers will be proposed for studying the tobacco exposure and evaluate the risk for consumers. This article represents the first study using direct analysis of this kind of compounds in one single run through ASAP-MS-Q-TOF in biological matrices. This way, fast identification of toxic compounds and new markers from tobacco could be possible.

Materials & Methods

Chemicals and Reagents

Nicotine (>98%) cotinine (98%), 1-hydroxypyrene (98%), 9-hydroxyphenanthrene (technical grade), ammonium acetate and formic acid

(98%), methanol (reagent grade) and acetonitrile (LC-MS quality) were purchased to Sigma (Madrid, Spain). Stock solutions of nicotine and cotinine at a concentration of 1.0 mg/mL, were prepared separately in methanol. Stock solutions of 1-hydroxypyrene and 9-hydroxxphenanthrene at a concentration of 1.0 mg/ml were prepared separately in acetonitrile. All stock solutions were stored at -20ºC until analysis.

ASAP- Q-TOF-MS analysis

Samples were directly introduced into the ASAP-Q_TOF_MS Xevo G2 QTOF (Waters Corporation, Manchester, UK) dipping a solid glass capillary in the liquid samples. Then, the samples wet the exterior of the glass capillary. Two dips were used for each analysis. Nitrogen was used as a desolvation gas at 450 Lh^{-1} flow. No cone flow was needed for this technique. Optimization of key ion source parameters, corona current (µA), sample cone (V) and desolvation gas temperature (ºC) were carried out using nicotine as a reference standard. The voltage of the sampling cone was varied from 30 V to 80 V and the voltage of the extraction cone was fixed at 0.1 V. Target samples were analyzed in continuous mode (3 min.) with a cone voltage ramp (30 V to 80 V) and desolvation gas temperature ramp (200 ºC to 500 ºC). Atmospheric Pressure Ionization (API) in positive polarity was selected, source temperature was 120ºC. The parameters of the XEVO G2 QTOF were: scan time 1s and the mass range considered was m/z 130-1000. Each sample was analyzed in triplicate. A blank sample was also analyzed under the same experimental conditions.

In addition to the high resolution mass achieved, isotopic ratios (C^{12}/C^{13}, N^{14}/N^{15}, O^{16}/O^{18}) and software tools were used to confirm the target compounds. MassLynx software from Waters was used, which considers the isotopic model and the elemental composition. The first one generates

an isotopic model for a specific compound of interest, while the elemental composition gives an idea of an elemental composition, which is *a priori* known. Another used tool is ChemSpider (www.chemspider.com), which was used to confirm and support the obtained mass spectra.

After the identification, quantitative analysis was performed using saliva and urine spiked samples with two pure standards, 1-PYR and 9-PHE, and analytical features were obtained.

UPLC-MS/MS analysis

The conventional method was carried out using an Acquity UPLC-MS-TQ (triple quadrupole) system from Waters (Milford, MA, USA). Chromatographic separation was performed on a Waters Acquity UPLC@ BEH C18 (1.7 µm, 2.1 mm × 100 mm) at 28 ºC. A 0.2 µm precolumn filter (Acquity UPLC stainless steel in-line filter). Mobile phases were: eluent A (acetonitrile with 0.3% formic acid) and eluent B (water with 0.3% formic acid). Flow rate was 0.25 ml min^{-1} and injection volume 10 µl. The time program for multi-step gradient was 0–6 min, 35% A–65% B to 60% A–40% B, 6–9 min, 60% A–40% B to 100% A- 0% B;, 9–10 min, 100% A–0% B to 35% A–65% B. Run time was 10 min and sample temperature was set at 7°C.

The MS equipment consisted of a Waters Micromass Quattro Micro™ triple-quadrupole system (Manchester, UK). The MS system was controlled by MassLynx Software, Version 4.0. The APCI+ (positive Atmospheric Pressure Chemical Ionization) interface consisted of a heated nebulizer probe and a standard atmospheric pressure source equipped with a corona discharge pin. The source and probe temperatures were set to 100 ◦C and 550 ◦C, respectively. The corona current was 6.0 µA; the cone voltage was 35 V; the extractor voltage was 5 V, and the RF lens voltage

was set to 0.1V. The desolvation and cone flow gases were 600 and 40 L/h, respectively. Analysis was performed in selected ion recording (SIR), selected m/z was 194.23 with a transition to m/z 165.34 for 9-phenantrol and for 1-hydroxypyrene m/z 218.25 with a transition to m/z 189.333.

The analytical features included intra-day precision, dynamic range and accuracy for quantitative purposes. A representative family of analytes (i.e., hydroxy-PAHs), thus, two hydroxy-PAHs, as representative compounds, 9-phenantrol (9-PHE) and 1-hydroxypyrene (1-PYR) were used.

Calibration curves were obtained by plotting the experimental concentration of the 9-PHE and 1-PYR against the theoretical concentration of each compound, using a least-square regression. The limit of detection (LOD) was determined as the concentration corresponding to a peak height that was three times the baseline noise. A 10:1 ratio of peak height to baseline noise was used to determine the limit of quantification (LOQ).

To test the reproducibility of this analytical method, quality control samples were analyzed. The samples used were urine diluted 10 times, spiked) with two OH-PAHs at two concentration levels: 0.5 µg/ml and 5 µg/ml each. The intra-day precision was measured as the relative standard deviation (RSD) of the measured concentration of the two OH-PAHs to the theoretical concentration. Five injections of the sample at two concentration levels were done. The accuracy (%) was assessed by comparison of the calculated mean concentration (n = 3) to nominal concentration for each compound.

Samples

After the optimization of the instrumental parameters a random set of samples (i.e. urine and saliva) from heavy smokers (i.e. more than 20 cg/day) were used for this study. Six volunteers were recruited within a health program for heavily smokers monitoring under a smoking cessation program [24]. Biological samples were taken using clean medical specific containers. The collected samples were kept at -20 º C prior laboratory analyses. Target biological samples for each person were as follows:

Urine: two urine samples were taken, one in the early morning on an empty stomach (fasting) and the other one at the end of the day, before going to sleep.

Saliva: three samples were taken, the first one in the early morning on an empty stomach (fasting), a second one immediately after the first cigarette and a third one 1 hour after the first cigarette.

Sample preparation

For the UPLC-MS-TQ method, a sample treatment step was applied as follows:. 300 µL of urine/saliva were extracted by applying a modification of the Bligh-Dyer method [25]. A volume of 1 mL of chloroform-methanol (1:2, v/v) was mixed with 30 µL of the IS solution and then added to the sample. The mixture was then centrifuged at 9000 rpm (7500g) for 10 min in a Microfuge® 18 Centrifuge, Beckman Coulter™ (Brea, CA, USA). The lower organic phase was recovered using a Pasteur pipet and transferred to a 2-mL vial. The extract was then accurately weighed and 1 mL of methanol was added. The resulting extract was filtered through a 0.22-µm Nylon syringe filter.

Results and Discussion

Optimization of Ion source parameters in ASAP

The ion source parameters have a strong influence on the observed spectrum. The key parameters explored were the corona current (µA), cone voltage (V) and desolvation gas temperature (ºC). The ramp of desolvation gas temperature allowed progressively to volatilize the ligther compounds before the heavier ones, facilitating the further identification. The desolvation gas flow was found to have a minor influence for the ASAP generated spectrum, so a fixed value was used for all the analytes. Cone voltage was varied from 30 to 80 volt. The aim was to have the maximum number of intact molecular ions reaching the MS detector and diminishing as much as possible the in- source fragmentation. In order to achieve this, one key parameter was changed while maintaining the other fixed. Ambient ionization MS, in this case ASAP, is a soft ionization technique, thus providing a spectra rich in molecular ions with minimal fragmentation. The obtained spectra were formed by the protonated molecular ion ($MH^{+\bullet}$). Table 1 shows all the analyzed compounds, molecular structure, chemical formula and theoretical and measured mass in the ASAP-MS-Q-TOF method. As can be seen the error (ppm) between the theoretical and the measured mass varies between - 9 ppm (e.g. cotinine) to 3.7 ppm for the anatabine. Lower values were found for nicotine, muconic acid or 1-hydroxiphenanthrene.

ASAP-Q-TOF-MS

Analytical features of the analytes in ASAP-Q-TOF-MS can be seen in Table 2. Good linearities (regression coefficient between 0.998 and 0.999) were obtained for the calibration curves with five concentrations (0.1, 1,

10, 100, 1000 µg/ml). The linear range was 1-1000 µg/mL, for both compounds. LOD and LOQ values for 1-PYR were better than for 9-PHE, and 1-PYR being 0.1 µg/ml and 1 µg/ml for LOD and LOQ and 0.51 µg/ml and 3 µg/ml for LOD and LOQ respectively. Intra-day precision values of 9-PHE were -7.7% and -3.9 % and -3.2 % RSD, for the two levels of concentrations (0.5 and 5 µg/mL) respectively. The intra-day accuracy varied from 92.1 % to 111.2% for both compounds at these concentrations.

Analytical features of the UPLC-MS-MS method

The data for method performance can be seen in Table 2. Good linearities (regression coefficient between 0.998 and 0.999) were obtained for the calibration curves with five concentrations (0.1, 0.5, 1, 5, 10 µg/ml). The linear range was 0.01-10 µg/ml for both compounds. LOD and LOQ values for 1-PYR (0.01 and 0.1 µg/ml) were better than for 9-PHE (0.05and 0.5 µg/ml), respectively. Intra-day precision values of 9-PHE and 1-PYR were 5.7% and 4.9% RSD and 5.3 % RSD respectively, for the two levels of concentrations (0.5 and 5.0 µg/ml). The intra-day accuracy varied from 105.7% to 94.6 % for both compounds at these concentrations.

ASAP-Q-TOF-MS versus UPLC-MS/MS comparison

Comparison of both techniques has been done using two real urine samples, the first one early in the morning and the last one at the end of the day before going to sleep and using OH-PAH quantification (9-PHE and 1-PYR). Both techniques show a similar trend, increasing the concentration for both compounds in the second urine sample. As can be seen in Table 2, 9-PHE was found at higher level by both techniques in both samples (urine 1, and urine 2. When comparing the concentration found by both techniques, ASAP always give higher values than UPLC-MS-TQ. This

could be expected, as ASAP is a direct technique without sample treatment, while UPLC-MS-TQ requires an extraction and elimination of the matrix. These results suggest that matrix effect is not present even though the whole sample containing proteins and fat matter was injected. When applying a sample treatment the 100% recovery is really difficult, what drives to a lower concentration values.

The better sensitivity of the UPLC-MS/MS method is not surprising due to the application of selected ion monitoring (SIM) where only a few target ions were monitored. In contrast, ASAP-Q-TOF-MS technique used full-scan mode, from 130 to 1000 m/z. If only a few ions were monitored (parent and daughter ions) by UPLC-MS/MS, sensitivity increased, thus a lower LOD/LOQ for the target compounds could be achieved. A clear advantage of using ASAP-Q-TOF-MS for screening purposes is the direct injection of the sample without any prior extraction method or sample treatment. Also the scan acquisition allowed us to analyze hundreds of ions in a few minutes as chromatographic step was not involved. Thus for a fast target screening purposes with a high number of samples to analyze, the use of ASAP-Q-TOF-MS technique is recommended. Meanwhile, for quantitative purposes or for untarget analysis the UPLC-MS/MS method using target ions in order to confirm and improve the LOD/LOQ limits for quantification purposes is needed.

Nicotine metabolism in samples

Many nicotine metabolites were found in both saliva and urine samples. **Table 3** shows the main tobacco smoke biomarkers identified in this work. As can be seen nicotine, cotinine and *trans*-3´-hydroxycotinine (3-HC) were found in saliva samples (figure 1), while all of them plus nicotine-*N*-glucuronide were found in urine samples. Higher values of Nicotine and

cotinine were found in the saliva sample 2 that corresponds to the sample taken immediately after smoking the first cigarrete, whereas 3-HC was much higher (10 times or more) in urine samples than in saliva samples. The differences in nicotine metabolites found in saliva compared to those found in urine samples were in agreement with the nicotine pathway of metabolism degradation and half-life of this type of compounds in human body [26-27]. Recent reports found that nicotine has a short life of 1-2 hr, cotinine 18-20 hr and *trans*-3´-hydroxycotinine with 4-8 hr [17]. In urine samples one of the glucuronide conjugates of nicotine (i.e. nicotine-*N*-glucuronide) was found, which is originated from the liver metabolism of nicotine. These results are in agreement with other studies found in the literature [27, 14, 28, 29].

Other Tobacco smoke biomarkers

In order to explore the possibilities of the ASAP-MS-QTOF technique a wide screening was performed with many other tobacco smoke biomarkers, such as: hydroxyl metabolites of polycyclic aromatic compounds (e.g. hydroxypyrene or hydroxyphenanthrene), other specific biomarkers for tobacco smoke (i.e. anatabine or methyladenine), specific nitrosamines (i.e. NNK, NNN), benzene exposure (i.e. muconic acid) or acrolein exposure (i.e. 3-HPMA). The chemical structure of the compounds is shown in Table 1. Figure 2 and 3 show some of the MS spectra of these tobacco biomarkers (e.g. NNK) identified in saliva and urine samples respectively analyzed by ASAP-MS-QTOF. As can be seen, the identification was achieved by extracting the exact mass of the molecular ion and further searching for the best molecular structure that matched with the mass value. Two hydroxy-PAHs were found in the samples (i.e. saliva and urine), hydroxypyrene and hydroxyphenanthrene and higher values were found for the last one, which

reflects the higher proportion of this compound in the tobacco smoke [5]. To confirm these values, sample treatment of urine samples and further analysis by HPLC-MS-TQ were carried out. This trend is in agreement with the data obtained through UPLC-MS-TQ analysis of the same urine samples (fasting and at the end of the day). Specific nitrosamines, which are highly carcinogenic compounds, were found in all the analyzed samples, but with lower values than the other biomarkers. Anatabine, which is an alkaloid present in the tobacco smoke, was detected in both saliva samples (in a small amount) and in both urine samples, and even higher in the second urine sample. This behavior could be expected: diluted through swallow in the mouth within the saliva, accumulated during the day within the human body and excreted in the urine. Specific biomarker of benzene exposure (muconic acid) and the specific biomarker of acrolein exposure (3-hydroxypropyl-mercapturic acid) were found in both urine samples, with higher amount in the early morning sample, which can reflect the human metabolism of the parental compounds during the day (figure 4). Benzene, a volatile and very toxic compound, is also related to the compounds coming from the tobacco, although environmental pollution can be also considered as a source of this compound. However, when analyzing the urine from non-smokers the muconic acid was not detected, what suggests that the source of this metabolite is also the smoking fume. This fact emphasizes the risk for health of smoking, which involves the intake of a very toxic and carcinogenic compound such as benzene.

Exposure differences

To investigate any difference in exposure and time trends for the analyzed compounds, a study over time (24 h) was performed with a set of six samples. The samples analyzed started with saliva 1 (early in the morning

just after waking up), saliva 2 (just after smoking the first cigarette of the day), saliva 3 (1 h after smoking the first cigarette) and two urine samples, the first one early in the morning and the last one at the end of the day before going to sleep. Figure 1 and Table 3 show the data for these samples. Different trends of the analyzed compounds in the different matrices can be seen. Higher values of Nicotine were found at higher concentrations in the saliva sample immediately after smoking (saliva 2), as expected. Cotinine, which is a metabolite from nicotine and a component of tobacco itself, is higher in saliva 2 (immediately after smoking) followed by the urine samples. Higher values in saliva samples versus urine samples were also found by Jacob, 2010 [28]. Once the nicotine enters the human body the metabolization occurs and cotinine is finally excreted in the urine. However, cotinine concentration is highly diluted in the urine and for this reason the value of this compound found in saliva is higher than in urine. As expected, the major nicotine metabolite (i.e. *trans*-3-hydroxycotinine) is present in the urine samples and at very low concentrations in saliva samples (1 and 3). The high values in the early morning urine agrees with an accumulation of the product from the nicotine metabolism in the human body.

Conclusions

The screening method by ASAP allows us to determine nicotine and it´s major metabolites as well as a wide variety of other important tobacco smoke biomarkers of exposure (e.g. hydroxypyrene, NNK, anatabine, muconic acid or 3-hydroxypropylmercapturic acid). Different trends were found in time series samples of saliva and urine, which may reflect different chemical behavior comparing saliva versus urine or metabolic pathways in the human body when urine 1 vs urine 2 were compared.

ASAP as direct technique has been demonstrated to be very efficient for direct analysis of biological samples, without sample treatment and consequently able to provide a fast analysis. Using this technique, the monitoring of many samples for tobacco exposure purposes is possible, which could be used as a way to improve the tobacco cessation policies.

It is important to emphasize the relevance of this direct technique for biological samples, which contain proteins fat matter and metabolites. Even with these complex matrices ASAP has been shown as an attractive, fast and useful technique well recommended for screening and target analysis of biological samples.

Acknowledgments

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References

1. WHO Report on the Global Tobacco Epidemic, 2008: The MPOWER package. Geneva, World Health Organization, 2008.

2. U.S. Department of Health and Human Services. How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2010.

3. Shakelya DM, Huestis MA (2009) Optimization and validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of nicotine, cotinine, *trans*-3′-hydroxycotinine and norcotinine in human oral fluid. Anal Bioanal Chem 395:2349- 2357.

4. Wu W, Ashley DL, Watson CH (2002) Determination of nicotine and other minor alkaloids in international cigarettes by solid-phase microextraction and gas chromatography/mass spectrometry. Anal Chem 74:4878-4884.

5. Jenkins RA, Guerin MR, Tomkins BA (2000) The chemistry of environmental tobacco smoke: composition and measurement.

(Eisenberg, M. Eds) CRC press, Boca Raton, Florida, USA.

6. Inoue R, Yagi K, Saito K (2009) Determination of nicotine, cotinine, and related alkaloids in human urine and saliva by automated in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. J Pharm Biomed Anal 49:108-114.

7. Dhar P (2004) Measuring tobacco smoke exposure: quantifying nicotine/cotinine concentration in biological samples by colorimetry, chromatography and immunoassay methods. J Pharm Biomed Anal 35:155-168.

8. Massadeh AM, Garaibeh AA, Omari KW (2009) A single-step extraction method for the determination of nicotine and cotinine in Jordanian smokers' blood and urine samples by RP-HPLC and GC-MS. J. Chromatogr. Sci 47:170-177.

9. Llaquet H, Pichini S, Joya X, Papaseit E, Vall O, Klein J, Garcia-Algar O. (2010) Biological matrices for the evaluation of exposure to environmental tobacco smoke during prenatal life and childhood. Anal Bioanal Chem 396:379-399.

10. Park S, Lee DH, Park JG, Lee YT, Chung J (2010) A sensitive enzyme immunoassay for measuring cotinine in passive smokers. *Clin. Chim. Acta* 411:1238-1242.

11. Miller EI, Norris HR, Rollins DE, Tiffany ST, Moore CM,

Vincent MJ, Agrawal A, Wilkins DG (2010) Identification and quantification of nicotine biomarkers in human oral fluid from individuals receiving low-dose transdermal nicotine: a preliminary study. J. Anal. Toxicol 34:357-366.

12. Kardani, F., Daneshfar, A., Sahrai, R. Determination of nicotine, anabasine, and cotinine in urine and saliva samples using single-drop microextraction. *J. Chromatogr. B.* 2010, 878, 2857-2862.

13. Kim I, Darwin WD, Huestis MA (2005) Simultaneous determination of nicotine, cotinine, norcotinine, and trans-3'-hydroxycotinine in human oral fluid using solid phase extraction and gas chromatography-mass spectrometry. J. Chromatogr. B 814:233-240.

14. Toraño JS, van Kan HM. (2003) Simultaneous determination of the tobacco smoke uptake parameters nicotine, cotinine and thiocyanate in urine, saliva and hair, using gas chromatography-mass spectrometry for characterization of smoking status of recently exposed subjects. Analyst 128:838-843.

15. Rabbaa-Khabbaz L, Abi Daoud R, Karam-Sarkis D (2006) A simple, sensitive, and rapid method for the determination of cotinine in urine by high-performance liquid chromatography with UV detection. J. Chromatogr. Sci. 44:535-538.

16. Shakleya DM, Huestis MA (2009) Simultaneous and sensitive measurement of nicotine, cotinine, trans-3'-hydroxycotinine and

norcotinine in human plasma by liquid chromatography-tandem mass spectrometry. J. Chromatogr. B 877:3537-2542.

17. Miller EI, Norris HR, Rollins DE, Tiffany ST, Wilkins DG (2010) A novel validated procedure for the determination of nicotine, eight nicotine metabolites and two minor tobacco alkaloids in human plasma or urine by solid-phase extraction coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry. J. Chromatogr. B 878:725-737.

18. McEwen CN, McKay RG, Larsen BS (2005) Analysis of Solids, Liquids, and Biological Tissues Using Solids Probe Introduction at Atmospheric Pressure on Commercial LC/MS Instruments. Anal. Chem 77:7826-7831.

19. Fussell RJ, Chan D, Sharmann M (2010) An assessment of atmospheric-pressure solids-analysis probes for the direction of chemical food. *TRAC* 29:1326-1335.

20. Smith MJP, Cameron NR, Mosely JA (2012) Evaluating Atmospheric pressure Solids Analysis Probe (ASAP) mass spectrometry for the analysis of low molecular weight synthetic polymers. Analyst 137:4524- 4530.

21. Carrizo D, Domeño C, Nerín I, Alfaro P, Nerín C (2015) Atmospheric pressure Solid Analysis Probe coupled to Quadrupole-time of Flight Mass Spectrometry as a tool for fast screening and semi-quantitative

approach of Polycyclic Aromatic Hydrocarbons, Nitro-Polycyclic Aromatic Hydrocarbons and Oxo-Polycyclic Aromatic Hydrocarbons in complex matrices J. Mass Spectrom. Talanta 131: 175-184.

22. Cody RB, Laramee JA, Durst HD (2005) Versatile new ion source for analysis of materials in open air under ambient conditions. Anal. Chem. 77:2297-2302.

23. Takáts Z, Wiseman JM, Gologan B, Crooks G (2004) Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. Science 306:471-473.

24. Nerin I, Novella P, Beamonte A, Garagallo P, Jiménez‐Muro A, Marqueta A. Results of smoking cessation therapy in a specialist unit. Arch Bronconeumol 2007; 43 (12):669‐73

25. Bligh E.G., Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Physiol. Pharmacol.. 37:911-917.

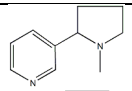
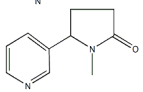
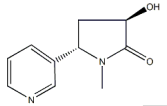
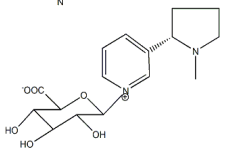
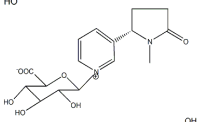
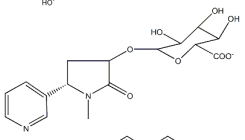
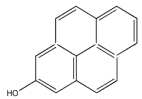
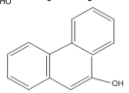
26. Benowitz, N.L., Jacob, P. III. Metabolism of nicotine to cotinine studied by a dual stable isotope method. Clin. Pharmacol. Ther. 1994. 56:483-493.

27. Benowitz NL, Jacob P III (2001) Trans-3′-hydroxycotinine: Disposition kinetics, effects and plasma levels during cigarette smoking. Br J Clin Pharmacol 51:53–59.

28. Jacob P III, Yu L, Duan M, Ramos L, Yturralde O, Benowitz NL (2011) Determination of the nicotine metabolites cotinine and trans-3'-hydroxycotinine in biologic fluids of smokers and non-smokers using liquid chromatography-tandem mass spectrometry: biomarkers for tobacco smoke exposure and for phenotyping cytochrome P450 2A6 activity. J Chromatogr B Analyt Technol Biomed Life Sci 879: 267-276.

29. Shepperd JC, Eldridge AC, Mariner DC, McEwan M, Errington G, Dixon M (2009) A study to estimate and correlate cigarette smoke exposure in smokers in Germany as determined by filter analysis and biomarkers of exposure. Regulatory Toxicology and Pharmacology. 55:97-109.

Table 1. ASAP-MS-Q-TOF analysis of Tobacco and nicotine biomarkers in urine and saliva samples (theoretical mass: M+H, n.d: not detected)

Compounds	Formula	Molecular structure	Measured m/z	Theoretical m/z	Error (ppm)
Nicotine	$C_{10}H_{14}N_2$		163.1236	163.1235	0.6
Cotinine	$C_{10}H_{12}N_2O$		177.1012	177.1028	-9
Trans-3´-hydroxycotinine	$C_{10}H_{12}N_2O_2$		193.0971	193.0977	-3
Nicotine-N-glucuronide	$C_{16}H_{22}N_2O_6$		339.1541	339.1556	-4
Cotinine-N-glucuronide	$C_{16}H_{20}N_2O_7$		n.d	353.1349	
Trans-3-hydroxy-Cotinine-*N*-glucuronide	$C_{16}H_{20}N_2O_8$		n.d	368.1220	
1-hydroxypyrene	$C_{10}H_{16}O$		153.1270	153.1279	-6
1-hydroxyphenanthrene	$C_{14}H_{10}O$		195.0811	195.0810	0.5

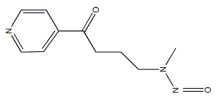
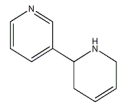
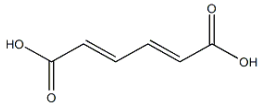
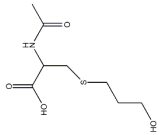
4-[Methyl(nitroso)amino]-1-(3-pyridinyl)-1-butanone - NNK	$C_{10}H_{13}N_3O_2$		208.1080	208.1086	-2
Anatabine	$C_{10}H_{12}N_2$		161.1085	161.1079	3.7
Methyladenine (M^+)	$C_6H_7N_5$		149.0693	149.0701	-5
Muconic acid	$C_6H_6O_4$		143.0345	143.0344	0.7
3-Hydroxypropylmercapturic acid	$C_8H_{15}NO_4S$		222.0808	222.0800	3.6

Table 2. Analytical features for method validation and comparison of the selected compounds (hydroxy-PAHs) by UPLC-MS/MS and ASAP-Q-TOF-MS

UPLC-MS/MS	**Linearity**	**R^2**	**LOD (µg/ml)**	**LOQ (µg/ml)**	**Intra-day precision (RSD%)**		**Intra-day accuracy (%)**		**Real samples**	
Compound					0.5 µg/ml	5 µg/ml	0.5 µg/ml	5 µg/ml	Urine 1	Urine 2
9-hydroxy-phenanthrene	y=14.156x+3.2259	0.999	0.05	0.5	-5.7	4. 9	105.7 ±9.2	95.1±3.7	0.18	1.54
1-hydroxy-pyrene	y=108.53x+18.189	0.998	0.01	0.1	4.9	5.3	95.0±1.2	94.6±3.1	0.08	0.35
ASAP-Q-TOF-MS	**Linearity**	**R^2**	**LOD (µg/ml)**	**LOQ (µg/ml)**	**Intra-day precision (RSD%)**		**Intra-day accuracy (%)**		**Real samples**	
Compound					0.7 µg/ml	5 µg/ml	0.7 µg/ml	5 µg/ml	Urine 1	Urine 2
9-hydroxy-phenanthrene	y=1449.8x+4176.5	0.999	0.5	3	-7.7	5. 4	111.2 ±9.3	95.6±3.4	0.58	3.15
1-hydroxy-pyrene	y=837.7x+1253	0.998	0.1	1	-3.9	-3.2	103.3±7.2	92.1±3.4	0.21	1.10

Table 3. Nicotine metabolism biomarkers and other tobacco smoke compounds found (X) in urine and saliva samples by ASAP-Q-TOF-MS

Compounds	Saliva 1	Saliva 2	Urine 1	Urine 2
Nicotine	x	x	x	x
Cotinine	x	x	x	x
Trans-3´-hydroxycotinine		x	x	x
Nicotine-N-glucuronide			x	
Cotinine-N-glucuronide				
Trans-3-hydroxy-Cotinine-N-glucuronide				
1-hydroxypyrene	x		x	x
9-hydroxyphenanthrene	x	x	x	x
4-[Methyl(nitroso)amino]-1-(3-pyridinyl)-1-butanone -NNK	x	x	x	x
Anatabine	x	x	x	x
Muconic acid			x	x
3-Hydroxypropylmercapturic acid			x	x

Figure 1. Exposure trends and behavior of nicotine and degradation compounds in analyzed samples

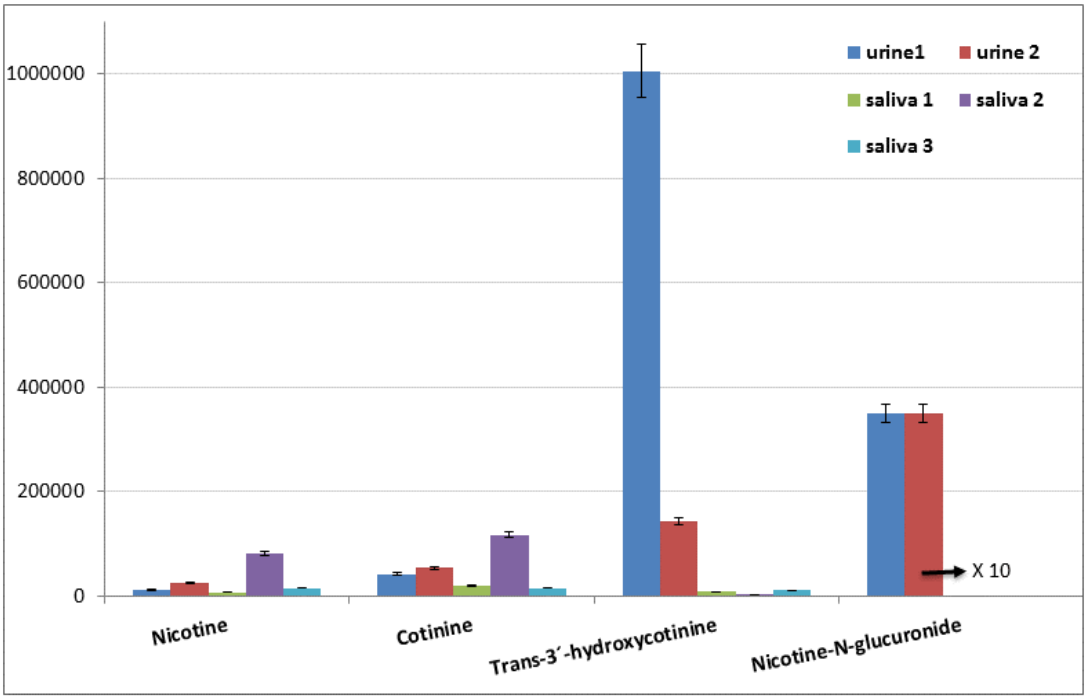


Figure 2. Overlapped mass spectra of an analyzed saliva sample, with the identification through the theoretical mass spectra of nicotine, cotinine and *trans*-3´-hydroxycotinine.

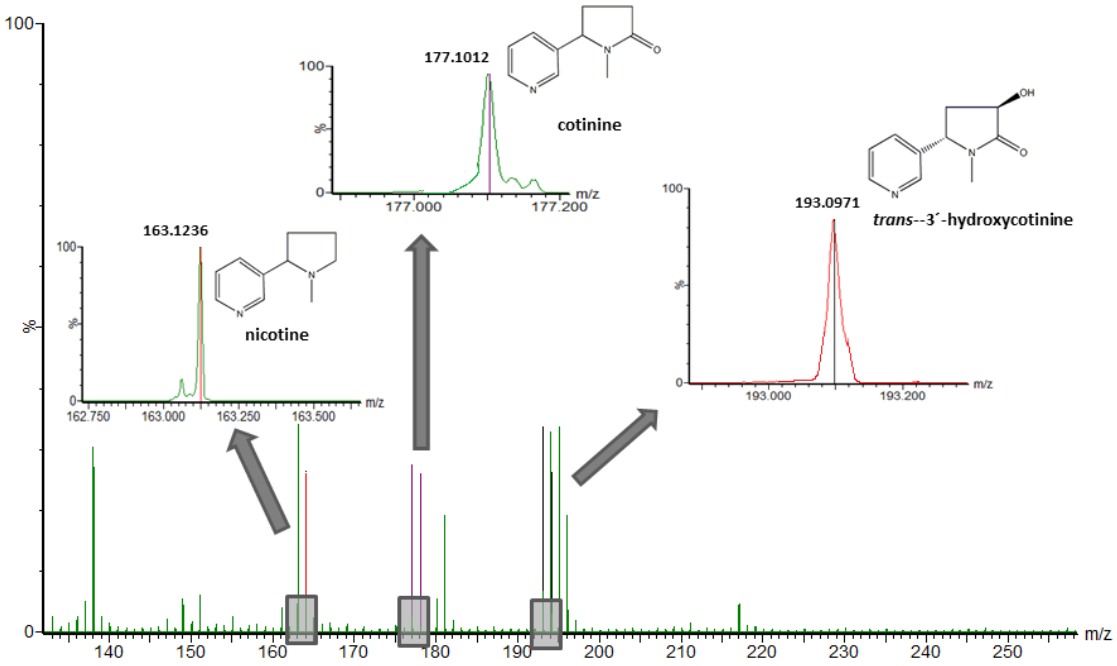


Figure 3. Overlapped mass spectra of nicotine and NNK theoretical mass spectra in a urine sample.

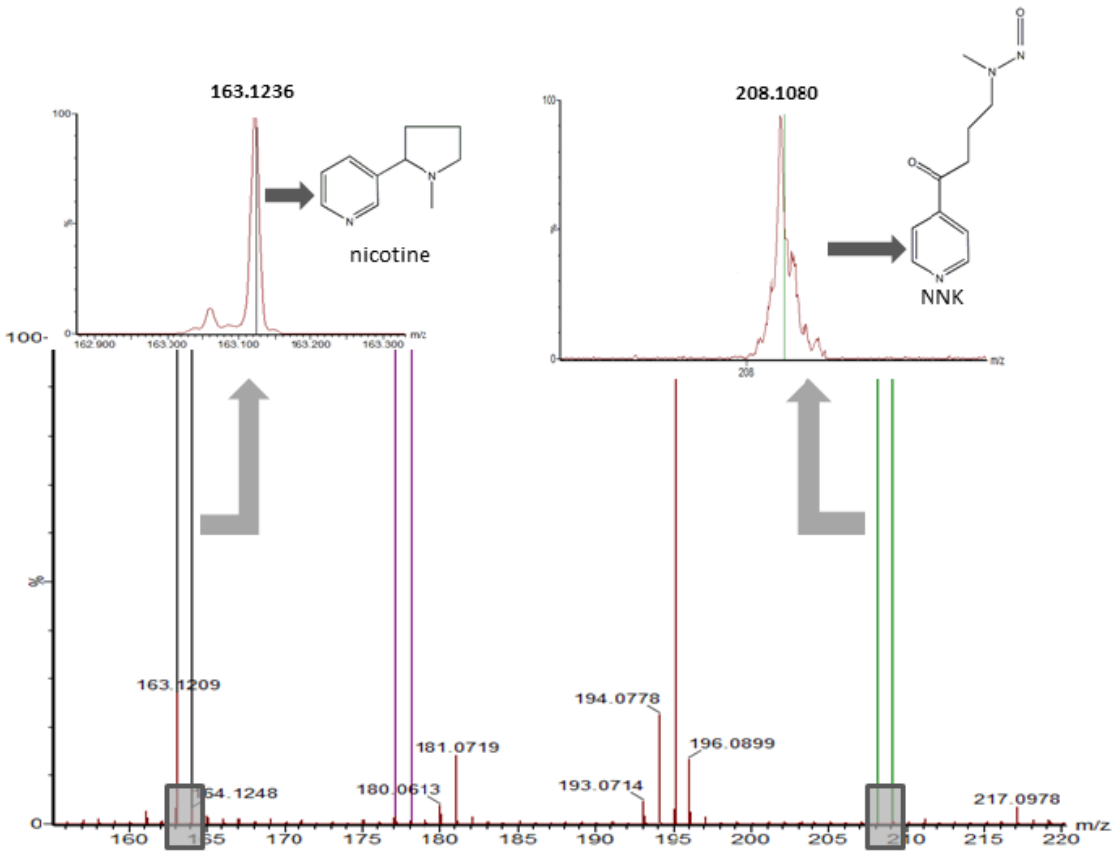


Figure 4. Time trends in saliva and urine samples (24 hs) analysis of other tobacco biomarkers found in this work