Journal of proteome research

Article

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Untargeted Metabolomic Profiling in Saliva of Smokers and Nonsmokers by a Validated GC-TOF-MS Method

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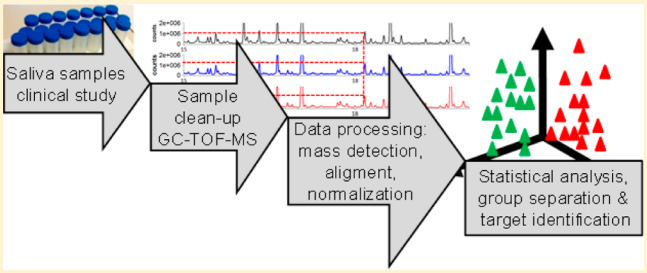
ABSTRACT: A GC-TOF-MS method was developed and validated for a metabolic fingerprinting in saliva of smokers and nonsmokers. We validated the method by spiking 37 different metabolites and 6 internal standards to saliva between 0.1 μM and 2 mM. Intraday coefficients of variation (CVs) (accuracies) were on average, 11.9% (85.8%), 8.2% (88.9%), and 10.0% (106.7%) for the spiked levels 25, 50, and 200 μM, respectively (N = 5). Interday CVs (accuracies) were 12.4% (97%), 18.8% (95.5%), and 17.2% (105.9%) for the respective levels of 25, 50, and 200 μM (N = 5). The method was applied to saliva of smokers and nonsmokers, obtained from a 24 h diet-controlled clinical study, in order to identify biomarkers of endogenous origin, which could be linked to smoking related diseases. Automated peak picking, integration, and statistical analysis were conducted by the software tools MZmine, Metaboanalyst, and PSPP. We could identify 13 significantly altered metabolites in smokers (p < 0.05) by matching them against MS libraries and authentic standard compounds. Most of the identified metabolites, including tyramine, adenosine, and glucose-6-phosphate, could be linked to smoking-related perturbations and may be associated with established detrimental effects of smoking.



KEYWORDS: *untargeted metabolomics, human saliva, gas chromatography, time-of-flight mass spectrometry, validation*

INTRODUCTION

It is well-known, that smoking causes diseases such as lung cancer,[1,2] cardiovascular diseases (CVDs),[3] and chronic obstructive pulmonary disease (COPD).[4−6] More than 5000 chemical compounds have been identified in cigarette smoke,[7,8] of which over 70 are classified as established (class 1), probable (class 2A), or possible (class 2B) human carcinogens, according to the International Agency for Reasearch on Cancer (IARC) (refer to the IARC monographs, http://monographs.iarc.fr/ENG/Monographs/PDFs/index.php). Some mechanisms for diseases developing are well investigated. For instance, the metabolic activation of polycyclic aromatic hydrocarbons or of the tobacco specific nitrosamine NNK leads to DNA adducts during the detoxification process. These adducts can cause gene mutations and cancer due to miscoding, if they evade molecular repair mechanisms.[9]

Endogenous metabolites as intermediates and end products of biological processes in humans are most predictable for the phenotype of an organism.[10] However, endogenous alteration of metabolic pathways evoked by smoking are less well investigated,[11] since systematic research of smoking related effects in appropriately designed studies are still missing. Research on smoking-related changes at the molecular level could contribute to the understanding of the underlying physiological mechanisms and might, therefore, lead to the identification of potential biomarkers of effect. Discovering exposure-related perturbation of metabolic pathways in human body fluids is a challenging task, although advancements in analytical technologies in the past years have been emerging to measure a broad spectrum of metabolites.[12,13] One example for this is gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS). Since this technology combines the separation power of GC with structural information obtained by high-resolution mass spectrometry, it is suitable for measuring and possibly also identifying numerous classes of analytes in different matrices. By using GC-TOF-MS for untargeted metabolic fingerprinting,[13] it is also possible to explore differences on the metabolite levels among groups, due to genetic or environmental impacts.[10,12,14] One has to admit that an inherent disadvantage of GC compared to liquid chromatography (LC) is certainly the fact that derivatization is required for analyzing the full metabolome in body fluids.

There are only few studies which investigated the metabolome in human body fluids of smokers (S) compared to nonsmokers (NS), most of them in plasma[15] and urine,[16,17] and almost none used an untargeted profiling approach.[18] Xu and colleagues described recently phospholipids and other metabolites significantly altered in smokers' plasma.[19] We previously found a shift in the total fatty acid profile to more monounsaturated fatty acids, phosphatidylcholine and phosphatidylethanolamine in the plasma of smokers as compared to

Received: November 7, 2013
Published: December 19, 2013

nonsmokers (published in a separate manuscript). Hsu[20] and co-workers used ultra performance liquid chromatography coupled to quadrupole with time-of-tlight mass spectrometry (UPLC-QTOF-MS) for a metabolomics approach in smokers' plasma but published mainly metabolites which are known constituents of tobacco smoke. Saliva can be used as an alternative matrix for metabolomic investigations,[18,21−23] since it is easily and noninvasively accessible without any health risks and stress for the subjects. Up to now, NMR has been used for metabolite profiling in smokers saliva samples.[18] However for the time being, MS based investigations are still rare.

Untargeted metabolic investigations require a controlled study design, to minimize the influences of factors other than that of interest on metabolite levels, such as diet,[21,24] age, gender, race and many others. Most of the conducted studies including smokers and nonsmokers lack a controlled study design or are limited to insufficient food questionnaire data.[18,21,24]

Hence, we conducted a study under strictly controlled conditions including the food and beverage intake as well as matching the body mass index (BMI) and age between the two groups (smokers and nonsmokers). Furthermore, we developed and validated a GC-TOF-MS method for an untargeted metabolic fingerprinting of saliva samples. The method was applied to 25 smokers and 25 nonsmokers from a 24 h in-house clinical smoking study.

MATERIAL AND METHODS

Chemicals and Solutions

Calibration standards, pyridine (anhydrous, 99.8%), *N,O*-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA + 1% TMCS, 99%), methoxyamine HCl (MOX, 98%), C7−C30 saturated alkane standard mix (each at 1 mg/mL in hexane, analytical grade), and cotinine were supplied by Sigma-Aldrich/Fluka (Taufkirchen, Germany). L-Proline, *trans,trans*-muconic acid (ttMA), pyruvic acid, and water were purchased from Merck (Darmstadt, Germany). Thymidine was obtained from Roth (Karlsruhe, Germany). $^{13}C,^{15}N_2$-urea was supplied by Cambridge Isotopic Laboratories (Tewksbury, MA). $^{13}C_6$-glucose was purchased from Cortecnet (Voisins-Le-Bretonneux, France) and d_4-ttMA, *trans*-3-hydroxycotinine (OH-cotinine), *trans*-3-hydroxycotinine-methyl-d_3 (OH-cotinine-d_3), and cotinine-methyl-d_3 were obtained from Toronto Research Chemicals (Toronto, Canada). Methanol (UPLC grade) was purchased from Promochem (Wesel, Germany).

The derivatization solvent MOX was dissolved in pyridine, yielding a final concentration of 20 mg/mL. A surrogate stock solution containing the 37 standard metabolites was prepared at a concentration of 2 mM in water for each analyte, respectively. Two internal standard (IS) solutions were prepared separately by dissolving $^{13}C_9,^{15}$N-L-tyrosine, $^{13}C,^{15}N_2$-urea, d_4-ttMA, d_4-succinic acid, $^{13}C_6$-glucose at a concentration of 1 mM in water (IS 1), and 2 mM heptadecanoic acid in methanol (IS 2). All solutions were stored at −20 °C prior to use (for up to 3 months). For cotinine and OH-cotinine analysis in saliva, the respective labeled internal standards were dissolved and diluted in water, to obtain a final concentration of 5.2 mM for OH-cotinine-d_3 and 5.7 mM for cotinine-methyl-d_3.

The stability of the untargeted metabolomics method was assured and controlled during the analysis to obtain consistent and comparable results. For this purpose, we randomly placed six quality control (QC) samples in the batch. QCs were prepared by pooling the saliva of the 50 subjects and spiking the pool with 50 μM of each of the 37 components from the validation measurements.

Subjects and Clinical Study Design

A clinical study was conducted on 50 healthy male caucasian subjects at the CRS Clinical Research Services Mönchengladbach GmbH. The participants were separated into two groups comprising 25 smokers and 25 nonsmokers (never smokers). At baseline, a standard medical examination proved that no subject showed any kind of disease traits. Inclusion criteria for smokers was a cigarette consumption of at least 5 cigarettes per day over a period of 1 year. The study was performed according to the Helsinki declaration and was approved by the ethic committee of the Medical Chamber of Nordrhein-Westfalen, Germany. During application screening, subjects underwent a standard medical examination, where the carbon monoxide in exhaled breath (COex) was measured to verify their smoking status. The cutoff for COex between S and NS was defined at 5 ppm. Each individual completed a questionnaire providing different lifestyle parameters such as leisure time activities, sporting activities, dietary habits and smoking history. The participants were matched in 5 year age groups ranging from 20 to 50 years. Meals were served on Day 1 at 9 am, 12 noon and 7 pm. All subjects were served the same food quantities normalized to their bodyweight (Table 1), by fitting the quantities of the lunch when the same breakfast and dinner quantities were served. Caloric intake comprised 72% carbohydrates, 14% fat, and 14% proteins. Grilled, fried or otherwise extensively heated or smoked food was prohibited throughout the study, to avoid the dietary uptake of smoking related pyrolysis products, such as polycyclic aromatic hydrocarbons and nitrosamines. All participants had to drink at least 3 L of water during the study and no other beverages were allowed. The clinical study comprised a 24 h stay of each subject in the clinic, starting on Day 1 at 8 am. No food consumption was allowed since 11 pm of the day before Day 1. The smokers were allowed to smoke ad libitum prior and until 11 pm of Day 1. Each subject smoked their own cigarettes throughout the entire study. Manufactured as well as roll-your-own cigarettes were permitted for smoking. To avoid the exposure to environmental tobacco smoke of the nonsmoking group, smoking took place in a designated room, where nonsmokers were not permitted. From 11 pm (Day 1) to 8 pm (Day 2), only drinking was allowed (no smoking or eating).

Table 1. Standardized Caloric Intake by Weight of the Participants

body weight range (kg)	<65	<75	<85	<95	<105
caloric intake (kcal)	1801	2036	2239	2436	2561

Biological Samples

Saliva collection took place at five different time points: on Day 1 at 10 am, 2 pm, 6 pm, 10 pm and on day 2 at 8 pm (Figure 1). In order to avoid systematic errors provoked by the collection method,[25] saliva samples were collected by means of a modified unstimulated spitting method as described by Navazesh.[26] Briefly, subjects did not eat, smoke or tooth brush for at least 20 min before sampling. Five minutes prior to sampling, the participants rinsed their mouth with water three times for 20 s. Saliva collection was conducted in sitting position. Saliva was allowed to accumulate on the floor of the

dx.doi.org/10.1021/pr401099r | J. Proteome Res. 2014, 13, 1602−1613

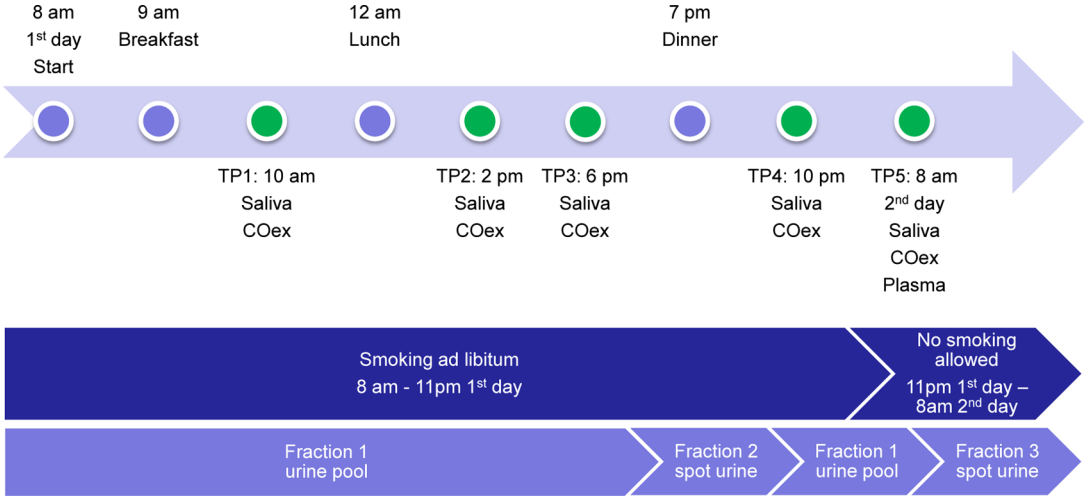


Figure 1. Timetable of the clinical study. Saliva were collected at five different time points (TP). Furthermore, EDTA-plasma was collected after 24 h, and a 24 h urine was collected for each subject in different fractions to obtain spot urines after 12 and 24 h of controlled diet.

mouth for 30 s and the subjects spitted it out into a 50 mL sterile polypropylene tube (Greiner Bio-One, Frickenhausen, Germany). This procedure was repeated until about 2 mL of saliva accumulated. The sampling procedure took about 5 min and the samples were frozen at −20 °C immediately after collection. Right after saliva collection COex was measured using a Bedfont "smoke analyzer" (Harrietsham, England). Breath sampling and COex measurements were conducted according to the instructions of the manufacturer. Prior to the last saliva collection on Day 2, blood was drawn, and after centrifugation aliquots of the obtained EDTA-plasma were frozen on dry ice and stored at −20 °C. Furthermore, a 24 h urine sample was collected in three fractions for each subject, comprising the time period from 8 am on Day 1 until 8 am on Day 2. We will focus in the following only on saliva metabolomics; the results for the metabolic profiling of plasma and urine samples are being published elsewhere.

Salivary Cotinine and OH-Cotinine

Cotinine and OH-cotinine in saliva were determined by means of a validated LC-MS/MS method.[27] Briefly, 100 μL of saliva were spiked with 25 μL of an IS-mix. Protein precipitation was conducted by adding 400 μL of methanol followed by vortexing and freezing the mixture for 1 h at −20 °C. The mixture was then vortexed for another 10 min, centrifuged at 15 330*g*, and 200 μL of the supernatant was transferred into a glass vial. For analysis, 5 μL was injected in the HPLC-system (Agilent 1100 series, Waldbronn, Germany) equipped with a Synergi MAX-RP 80 column (150 mm × 4.6 mm) with 4 μm particles (Phenomenex, Torrance, CA). Column temperature was set to 45 °C. Isocratic separation was performed using a mobile phase consisting of 20% water with 10 mM ammonium acetate and 80% methanol at a flow rate of 1 mL/min. MS/MS analysis was performed in positive ionization mode on a triple quadrupole MS (API 4000, AB Sciex, Darmstadt, Germany) equipped with an APCI ionization source (Turbo V Ion Source, AB Sciex, Darmstadt, Germany). Calibration was performed with the standard addition method by spiking increasing amounts of authentic standard compounds into matrix samples. The multiple reaction monitoring (MRM) of 177.2−80.1 *m*/*z* for cotinine and 193.1−79.9 *m*/*z* for OH-cotinine were normalized to the areas of the corresponding deuterated internal standards (MRMs for OH-cotinine-d_3 196.2−79.9 *m*/*z* and for cotinine-methyl-d_3 180.2−80.0 *m*/*z*) and then divided by the calibration slope in order to get absolute concentrations. The calibration ranged from 10 nmol/L to 10 μmol/L.

GC-TOF-MS Analysis of Saliva Samples

Saliva samples were thawed at room temperature and 10 μL of the IS 1 solution and 5 μL of the IS 2 solution were added to 100 μL of each sample. For protein precipitation, 460 μL of methanol/acetonitrile (70/30 v/v) was added, thoroughly vortexed and kept at −20 °C for 1 h. The samples were then centrifuged with 15 330*g* at 4 °C for 15 min and the supernatant was transferred to a glass vial. A second extraction was conducted by adding 100 μL methanol/water (8:1 v/v) to the residue. The mixture was vortexed, kept for 20 min at −20 °C and then centrifuged for 15 min at 15 330*g* (4 °C). The organic phases were combined and evaporated in a SpeedVac (RC10.22, Thermo Scientific, Dreieich, Germany) to almost dryness. Then, 50 μL of pyridine was added and the mixture was dried under a gentle stream of nitrogen. For derivatization purposes, 60 μL of the MOX solution in pyridine was added, sonicated for 3 min, and incubated for 30 min at 60 °C. Afterward, 60 μL of BSTFA containing 1% TMCS was added, sonicated for 3 min, incubated for further 30 min at 60 °C, and 2 μL of a saturated alkane standard solution was added. Finally, the mixture was centrifuged for 15 min at 3300*g* (10 °C). The supernatants were transferred to GC vials and placed in an autosampler (MPS2, Gerstel, Mülheim an der Ruhr, Germany) at 10 °C until analysis. Analysis of the samples was performed by injection of 1 μL of the derivatized saliva sample with a 1:5 split ratio into the GC system (GC 6980, Agilent Technologies, Santa Clara, CA) coupled to a time-of-flight mass spectrometer (BenchTOF-dx, Almsco, LLantrisant, United Kingdom). A Rxi-1 ms 30 m × 250 μm ID fused silica column (Restek, Bad Homburg, Germany) with dimethyl polysiloxane as stationary phase (0.25 μm film thickness) was used as analytical column. Helium served as carrier gas at a flow rate of 1.1 mL/min. A CAS 4 (Gerstel, Mühlheim an der Ruhr, Germany) was used for injection equipped with a Siltek deactivated baffled glass liner (Gerstel, Mühlheim an der Ruhr, Germany). The injector temperature was set to 50 °C for 1 s followed by a linear increase to 300 °C with 12 °C/s and a hold step for 6 min at 300 °C. The oven temperature was kept at 50 °C for 1 min, increased to 300 °C with 10 °C/min and held at 300 °C for 10

dx.doi.org/10.1021/pr401099r | J. Proteome Res. 2014, 13, 1602−1613

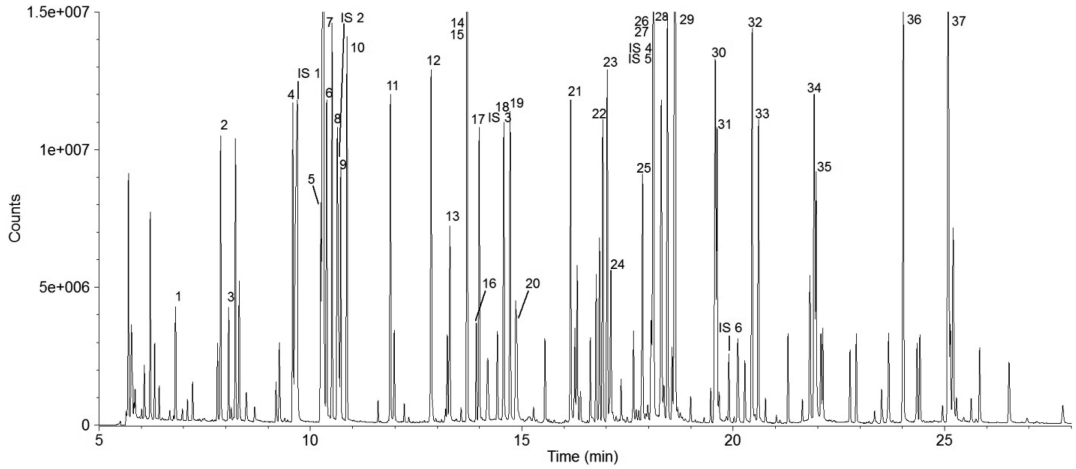


Figure 2. TIC of saliva spiked with 750 μM of standard compounds: (1) pyruvate, (2) L-alanine, (3) oxalic acid, (4) L-valine, (5) nicotinic acid, (6) L-leucine, (7) glycerol, (8) L-proline, (9) succinic acid, (10) L-glycine, (11) thymine, (12) mandelic acid, (13) L-glutamine, (14) γ-aminobutyric acid (GABA), (15) L-4-hydroxyproline, (16) creatinine, (17) 2-ketoglutarate, (18) ttMA, (19) *p*-hydroxyphenylacetic acid, (20) cotinine, (21) homovanillic acid, (22) gly-gly, (23) citric acid, (24) 2-methylhippuric acid, (25) 4-methylhippuric acid, (26) L-tyrosine, (27) D-(+)-glucose, (28) glucuronic acid, (29) sorbitol, (30) dopamine, (31) uric acid, (32) 5-HIAA, (33) L-tryptophan, (34) G6P, (35) thymidine, (36) adenosine, (37) β-lactose, (IS 1) $^{13}C,^{15}N_2$—urea, (IS 2) d_4-succinic acid, (IS 3) d_4-ttMA, (IS 4) $^{13}C_9,^{15}N$-tyrosine, (IS 5) $^{13}C_6$-glucose, (IS 6) heptadecanoic acid.

min. The transfer line between GC and MS was kept at 250 °C. The mass spectrometer operated in electron ionization mode at 70 eV. The filament delay was set to 5.6 min. Data acquisition was performed in the full scan mode from m/z 40 to 650 with a sampling rate of 5 Hz.

Validation of the GC-TOF-MS Method

A detailed analytical evaluation of the GC-TOF-MS method was conducted by determining the analytical performance including reproducibility, linearity, reinjection, repeatability and stability across the 37 compounds. These compounds covered a broad range of chemical classes, potentially occurring in saliva and representing both exogenous and endogenous metabolites. The chemicals of interest were spiked into a saliva pool at concentration levels ranging from 0.1 μM to 2 mM, prior to sample preparation. For quantification purposes, only the most characteristic fragment ions for each compound were considered.

Calibration

Calibration was performed by the standard addition method in matrix samples. Therefore, increasing amounts of the 37 authentic standard compounds were spiked at different concentration levels into saliva samples, covering a calibration range from 0.1 μM to 2 mM. Each level was analyzed as duplicate. Calibration curves were determined by plotting the response (peak area analyte/peak area of corresponding IS) of the fragment ions against the spiked concentrations.

Precision and Accuracy

To assess precision of the analytical workflow, saliva samples at three different concentration levels (low, medium, high) were analyzed five times in a row and on 5 consecutive days to determine intraday and interday variations, respectively. The different concentration levels were prepared by spiking the 37 standard compounds to saliva at a concentration of 25, 50, and 200 μM, respectively. To obtain instrument precision, a reinjection analysis was performed. Therefore, three saliva samples spiked with 25, 50, and 200 μM of the standard compounds were injected 5 times and the respective CV of the instrument response was calculated to determine the instrument precision.

Stability of Derivatives

One GC run of the described method takes about 40 min (including cool down time of the GC). Therefore the analysis time for a batch of 60 samples (25 smokers, 25 nonsmokers, 6 quality control samples and 4 blanks) lasts about 40 h. For this reason, we tested the stability of the derivatized samples by injecting 3 saliva samples spiked with 25, 50 and 200 μM of the surrogate solution at 0, 1, 2, 3, and 5 days after derivatization. Between the measurements, the samples were stored at 10 °C (autosampler conditions). The deviation of the analytes compared to the reference measurements on day 0 was determined.

Data Analysis

Data processing and background compensation of MS spectra were carried out by the ProtoTOF software package (Almsco, Llantrisant, United Kingdom). For the validation experiments, the raw data were exported to Chemstation 2.0 (Agilent, Santa Clara, CA) and manually integrated for all analytes. For the metabolics fingerprinting, the raw data were background corrected by ProtoTof and the files were converted to Common Data Format (CDF) files. Next, automated peak picking, integration, retention time adjustment by alkane standards and alignment were conducted with the open source software package MZmine 2.10 (http://mzmine.sourceforge.net/).[28] The peaklist generated by MZmine contains all features, consisting of a signal, characterized by its m/z value, retention time and the peak area. The peaklist was exported to Excel 2007 (Microsoft, Redmond, WA) and normalized to the IS d_4-ttMA. Known signals derived from IS or alkane standards were removed and univariate analysis in order to find differences between the two groups (smokers and nonsmokers) was performed by applying the Mann–Whitney U test using PSPP (http://www.gnu.org/software/pspp/). Signals with $p >$ 0.3 and signals derived from tobacco specific biomarkers such as nicotine and its metabolites were manually removed, since we were more interested on endogenous metabolites significantly discriminating smokers from nonsmokers. After-

dx.doi.org/10.1021/pr401099r | J. Proteome Res. 2014, 13, 1602–1613

ward, the data were transformed by the generalized logarithm (glog)[29] and a partial least-squares discriminant analysis (PLS-DA) was performed by using the public online platform Metaboanalyst (www.metaboanalyst.ca).[30] Model performance was evaluated by 10-fold cross validation using the R^2 and Q^2 parameters. R^2 provides a measure of how much variation is represented by the model. How accurate the data are classified in the model is predicted by the Q^2 value. Both values are between 0 and 1 and are commonly represented as percentages.[31] Significant hits obtained from the PLS-DA and Mann−Whitney U test (Variable Importance in Projection (VIP) > 0.8 and $p < 0.05$) were further processed for identification using the NIST MS library (National Institute of Standards and Technology, Gaithersburg, MD), equipped with the NIST MS Search Program 2.0 and mass spectral databases (NIST 05, GOLM 2010[32]) and the deconvolution software AMDIS, Version 2.71 (Automated Mass Spectral Deconvolution and Identification System, www.amdis.net).[33,34] Further identification and confirmation of the results was performed with authentic reference compounds, if available.

RESULTS AND DISCUSSION

Validation

As shown in Figure 2 almost all standard compounds and internal standards were sufficiently separated and detectable by our newly developed GC-TOF-MS method. The coeluting compounds γ-aminobutyric acid and L-Hyp as well as glucose and L-Tyr could be unambiguously identified by analyzing different fragment ions. The linear response of the detector signal ranged from 2.5 μM to 2 mM across all analytes (Table 2). We achieved mean intraday precisions of 11.9%, 8.2%, and 10.0% for spiked concentrations of 25, 50, and 200 μM, respectively. The average accuracies were found to be 85.8% (25 μM), 88.9% (50 μM), and 106.7% (200 μM). Interday accuracies (CV) were 97.0% (12.4%), 95.5% (18.8%), and 105% (17.2%) over the entire calibration range. Although we used a quite simple and straightforward sample preparation (only derivatization was performed with no further sample purification) and only 6 IS were used for the quantification of 37 compounds (Figure 2), satisfactory validation results in terms of precision, accuracy, and linearity could be achieved with some exceptions. The higher CVs and narrower linear ranges could be explained by the relatively high endogenous metabolite levels in saliva, limiting particularly the lower end of the calibration range. Additionally, accuracies of the low abundant metabolites were also affected by the high endogenous levels. For instance, the background for uric acid was so high that we were not able to determine precision and accuracy for this metabolite. The stabilities of the analytes, except for 2-methylhippuric acid (50 μM), L-leu (50 μM), and gly-gly (50 μM), showed excellent accuracies with a deviation <30%. There is no obvious reason for this remarkably deviation at the various concentration levels investigated.

Since the standard compounds covered a broad range of chemical classes, we assume that most of the silylated products in the samples are stable for the time, which is required to measure the 50 study derived samples from smokers and nonsmokers. In general, one has to consider that metabolite fingerprinting including a variety of different chemical classes has to be a compromise between several analytical parameters (solubility, stability, influence of the matrix, derivatization etc.). Comprehensiveness and overall dynamic range, for a large number of analytes and chemical classes, are considered as more important than achieving very low detection limits for single metabolites.[10]

Metabolomics Fingerprinting in Saliva of Smokers and Nonsmokers

Mean ages (±standard deviation) of the participants were 36.5 ± 9.1 and 36.8 ± 9.7 years for smokers and nonsmokers, respectively. The BMIs of the subjects were between 19.3 and 29.1 kg/cm^2 (nonsmokers, 24.8 ± 2.8 kg/cm^2; smokers, 25.0 ± 2.7 kg/cm^2). All COex levels for nonsmokers were lower than 5 ppm. The COex concentrations measured during the screening phase (about 1 week before start of the study) also confirmed the self-reported smoking status (smoker or nonsmoker) of the subjects. During the study, on average 13 cigarettes (9−19 cig) were smoked which was reflected by the smokers' COex levels on Day 2 (8.9 ± 3.4 ppm), the cotinine (1.37 ± 0.50 μM, 240.8 ± 88.3 ng/mL) and the OH-cotinine (0.28 ± 0.17 μM, 53.2 ± 32.2 ng/mL) levels, which were in the common range of light to moderate cigarette smokers.[27] Nonsmokers' cotinine and OH-cotinine levels in saliva were below the lower limit of quantification (LLOQ: cotinine = 7 nM, OH-cotinine = 10 nM), confirming that the nonsmokers had not smoked, were not significantly exposed to tobacco smoke or had not used any other tobacco or nicotine products during and shortly before the clinical study. To obtain the stability of the metabolomics analysis method, the CVs for each of the 37 components in the 6 QC samples were calculated from raw areas without IS correction. The CVs were 11.4% on average, which approved the excellent method performance, considering the fact that no internal standard was used for correction and an automated peak picking and integration procedure was applied.

The peaklist processed with MZmine, from the data analysis of the 25 smokers and 25 nonsmokers samples, contained over 14 000 features. A p-value was calculated between smokers and nonsmokers for each feature. The box plots of the glog transformed response of some significant fragments are shown in Figure 3. Not unexpectedly, we found significantly elevated levels ($p < 0.001$) of fragment ions resulting from cotinine and OH-cotinine in saliva of smokers, which confirms the applicability of our metabolomics workflow. The levels of these signals found for nonsmokers are in the range of the unspecific background. Since this was an untargeted analytical approach, such coeluting compounds generating same fragments cannot be excluded for any analyte. Next, the tobacco specific metabolite peaks from cotinine and OH-cotinine, as well as peaks showing no significant difference between the two groups ($p > 0.3$) and fragments derived from added compounds such as IS, alkane standards and derivatizaton agents were excluded. The remaining peaks were used for a PLS-DA analysis. Using three components, the groups could be separated as shown in Figure 4. There is an outlier of one smoking subject in the score plot. Theoretically, the reason for this could have a biological or technical origin. A technical problem seems to be unlikely, since the IS signal intensities are comparable across all 50 subjects. Therefore we kept this subject part of the data processing and statistical evaluation. Further, the outlier is not noticeable in terms of cigarette consumption, measured biomarkers of exposure (such as nicotine and 9 of its metabolites (Nic+9, data not shown) or leisure time activity.

The highest ranked features obtained after the PLS-DA and Mann−Whitney-U test were tentatively identified using mass

Table 2. Analytical Parameters of the Validation for the GC-TOF-MS Method in Saliva for 37 Compounds

compd	corresponding IS	*m/z* of the evaluated ion	retention time, min	R^2 duplicates, $N = 4-10$	linear range (duplicates), $N = 4-10$ μM	spiked level, μM	reinjection CV %	intraday $N = 5$		interday $N = 5$		stability D5/D1[a], %
								accuracy %	CV %	accuracy %	CV %	
L-Gly	^{13}C. $^{15}N_2$-urea	174	11.86	0.9891	50−500	200	1.4	84.1	15.5	99.0	13.9	96
						50	2.4	81.6	20.3	105.3	34.0	101
						25	–	–	–	–	–	–
L-Pro	$^{13}C_9$. ^{15}N-L-tyrosine	142	10.63	0.9948	75−750	200	1.2	104.1	8.1	123.6	23.2	124
						50	–	–	–	–	–	–
						25	–	–	–	–	–	–
L-Hyp	$^{13}C_9$. ^{15}N-L-tyrosine	230	13.7	0.9992	7.5−100	200	–	–	–	–	–	–
						50	5.6	90.4	1.2	91.6	9.6	86
						25	9.0	78.3	11.2	91.5	5.2	92
L-Leu	$^{13}C_9$. ^{15}N-L-tyrosine	158	10.38	0.9997	25−750	200	2.1	94.9	5.0	101.4	9.3	98
						50	10.4	89.1	1.8	96.3	19.5	60
						25	12.9	50.1	35.2	83.9	16.5	74
L-Val	^{13}C. $^{15}N_2$-urea	144	9.58	0.9996	5−200	200	1.4	91.5	6.3	98.9	7.6	102
						50	10.0	95.0	7.2	92.7	19.0	74
						25	9.5	69.1	20.3	86.8	16.1	83
L-Ala	^{13}C. $^{15}N_2$-urea	116	7.87	0.9944	5−200	200	2.2	92.0	8.8	102.3	9.7	103
						50	8.1	96.1	9.9	96.3	18.5	77
						25	7.4	63.3	31.9	99.1	16.4	94
Gaba	d_4-ttMA	174	13.7	0.9985	2.5−100	200	–	–	–	–	–	
						50	0.2	93.2	2.2	98.7	9.4	102
						25	0.7	88.0	7.2	94.9	4.6	100
L-Trp	$^{13}C_9$. ^{15}N-L-tyrosine	202	20.59	0.9956	5−100	200	–	–	–	–	–	
						50	13.1	80.8	2.1	81.4	40.8	95
						25	10.9	73.8	11.5	74.1	31.7	82
L-Tyr	$^{13}C_9$. ^{15}N-L-tyrosine	218	18.1	0.9985	50−1000	200	0.34	97.3	3.9	103.1	13.4	99
						50	0.6	66.6	4.7	74.8	18.4	100
						25	–	–	–	–	–	
L-glutamine	d_4-ttMA	156	13.29	0.9966	50−1000	200	1.9	84.4	11.2	102.8	24.2	108
						50	1.5	31.4	31.1	80.9	64.1	102
						25	–	–	–	–	–	
nicotinic acid	d_4-succinic acid	180	10.25	0.9989	10−750	200	1.3	93.9	8.8	97.3	10.1	100
						50	1.2	88.1	1.6	94.9	15.2	100
						25	1.5	88.6	6.9	92.6	4.9	97
α-ketoglutarate	d_4-ttMA	198	13.98	0.9995	25−1000	200	0.4	96.9	3.9	101.3	12.1	101
						50	1.2	100.1	2.8	103.0	4.0	101
						25	0.8	117.1	5.0	121.7	4.1	101
oxalic acid	13C. 15N2-urea	190	8.06	0.9989	2.5−200	200	1.7	100.8	14.3	112.2	21.6	105

dx.doi.org/10.1021/pr401099t | J. Proteome Res. 2014, 13, 1602−1613

Table 2. continued

compd	corresponding IS	m/z of the evaluated ion	retention time, min	R^2 duplicates, $N = 4-10$	linear range (duplicates), $N = 4-10$ μM	spiked level, μM	reinjection CV %	intraday $N = 5$		interday $N = 5$		stability D5/D1[a], %
								accuracy %	CV %	accuracy %	CV %	
						50	5.4	101.1	7.2	102.7	5.1	99
						25	3.5	90.6	14.0	102.1	7.1	99
succinic acid	d_4-succinic acid	247	10.71	0.9986	5−100	200	–	–	–	–	–	
						50	0.4	87.0	2.7	103.9	16.1	101
						25	0.8	99.1	9.9	117.4	29.8	99
mandelic acid	d_4-ttMA	179	12.85	0.9984	5−100	200	–	–	–	–	–	
						50	0.7	88.0	3.4	94.6	14.5	102
						25	0.8	85.1	7.1	91.2	5.0	99
pyruvate	d_4-succinic acid	174	6.8	0.9945	5−100	200	–	–	–	–	–	
						50	1.9	100.7	4.8	109.6	16.1	107
						25	3.1	105.1	11.1	118.5	27.8	105
citric acid	d_4-succinic acid	273	17.01	0.9985	5−100	200	–	–	–	–	–	
						50	1.9	83.9	4.2	93.3	15.3	103
						25	1.5	88.7	8.8	100.7	15.3	104
ttMA	d_4-ttMA	271	14.56	0.9995	25−750	200	0.8	97.6	3.3	102.6	11.3	100
						50	0.6	90.8	3.1	93.0	7.8	100
						25	0.6	77.6	8.6	82.0	4.5	101
OH-phenylacetic acid	d_4-ttMA	179	14.71	0.9982	10−100	200	–	–	–	–	–	
						50	0.2	84.9	1.5	93.6	14.9	102
						25	1.2	85.9	6.3	92.8	4.5	100
D-glucose	13C6-glucose	319	18.11	0.9997	25−1000	200	0.20	94.7	3.8	102.2	14.6	92
						50	1.2	101.3	3.8	110.1	11.5	104
						25	3.6	93.4	4.8	124.5	29.8	98
glucuronic acid	13C6-glucose	333	18.44	0.9974	7.5−200	200	1.4	99.1	5.8	109.5	16.2	100
						50	0.5	94.5	5.4	94.8	4.8	103
						25	0.8	79.9	8.1	93.9	6.8	101
β-lactose	$^{13}C_6$-glucose	204	25.08	0.9977	7.5−100	200	–	–	–	–	–	
						50	0.7	110.1	6.5	109.0	21.1	103
						25	1.4	75.3	5.3	108.6	27.1	102
G6P	C_{17}COOH	299	21.91	0.9993	50−750	200	1.4	80.3	14.3	100.4	6.8	100
						50	1.1	57.7	2.9	99.0	9.7	100
						25	3.8	86.0	8.9	165.5	9.2	100
glycerol	d_4-succinic acid	205	10.5	0.9990	75−750	200	1.0	110.8	17.0	106.1	9.5	98
						50	–	–	–	–	–	
						25	–	–	–	–	–	
homovanillic acid	d_4-ttMA	326	16.14	0.9976	50−1000	200	0.9	98.7	14.2	102.0	13.8	103
						50	0.8	63.2	5.4	72.9	23.8	102
						25	–	–	–	–	–	
sorbitol	$^{13}C_6$-glucose	319	18.61	0.9999	25−2000	200	0.7	98.6	7.2	101.8	7.5	99
						50	0.8	88.7	2.8	89.9	11.1	103

dx.doi.org/10.1021/pr401099t | J. Proteome Res. 2014, 13, 1602−1613

Table 2. continued

compd	corresponding IS	m/z of the evaluated ion	retention time, min	R^2 duplicates, N = 4−10	linear range (duplicates), N = 4−10 μM	spiked level, μM	reinjection CV %	intraday N = 5		interday N = 5		stability D5/D1[a], %
								accuracy %	CV %	accuracy %	CV %	
						25	0.9	107.5	8.7	100.3	12.1	101
cotinine	C_{17}COOH	98	14.9	0.9983	25−500	200	0.8	90.5	10.4	104.8	9.4	98
						50	1.0	88.0	12.6	84.3	8.7	98
						25	1.6	89.7	16.8	103.3	11.0	100
creatinine HCl	d_4-ttMA	115	13.92	0.9964	25−1000	200	1.0	71.6	28.1	89.9	24.0	98
						50	0.7	59.1	10.3	91.1	36.7	98
						25	0.9	52.8	15.3	87.7	20.5	90
5-HIAA	13C9. 15N-L-tyrosine	290	20.43	0.9993	5−200	200	0.9	100.2	5.0	108.0	15.0	106
						50	1.3	93.2	1.5	104.0	10.7	93
						25	6.0	89.0	9.7	103.8	22.2	108
dopamine	$^{13}C_9$. ^{15}N-L-tyrosine	174	19.57	0.9988	5−100	200	–	–	–	–	–	
						50	1.6	92.7	1.9	101.4	7.4	90
						25	5.5	95.1	8.6	105.1	18.8	102
Gly-Gly	$^{13}C_9$. ^{15}N-L-tyrosine	174	16.91	0.9992	2.5−50	200	–	–	–	–	–	
						50	6.7	121.7	12.3	104.2	27.4	227
						25	9.1	97.3	26.7	99.0	14.5	140
4-methylhippuric acid	$^{13}C_9$. ^{15}N-L-tyrosine	119	17.84	0.9997	10−200	200	1.3	99.1	4.4	107.9	14.4	101
						50	2.0	89.1	2.3	99.1	8.8	88
						25	6.6	92.9	7.3	103.2	16.3	98
2-methylhippuric acid	$^{13}C_9$. ^{15}N-L-tyrosine	119	17.10	0.9985	100−2000	200	4.0	100.9	10.5	101.7	24.5	99
						50	5.2	139.8	2.2	156.7	8.4	54
						25	–	–	–	–	–	
thymine	^{13}C. $^{15}N_2$-urea	255	11.89	0.9988	7.5−75	200	–	–	–	–	–	
						50	2.6	91.9	7.1	95.7	7.2	102
						25	2.1	89.1	7.5	96.1	3.5	102
thymidine	$^{13}C_9$. ^{15}N-L-tyrosine	103	21.94	0.9983	50−750	200	2.2	95.7	6.6	103.8	22.0	105
						50	2.8	103.7	4.3	108.6	14.5	85
						25	–	–	–	–	–	
adenosine	$^{13}C_9$. ^{15}N-L-tyrosine	230	24.01	0.9996	10−1000	200	0.7	97.0	4.3	102.2	11.7	102
						50	2.1	91.7	1.7	100.2	8.8	85
						25	5.4	94.0	7.6	104.7	13.9	89
uric acid	^{13}C. $^{15}N_2$-urea	456	19.62	0.9950	500−2000	200	–	–	–	–	–	
						50	–	–	–	–	–	
						25	–	–	–	–	–	

[a]D5 (Day 5), D1 (Day 1).

dx.doi.org/10.1021/pr401099t | J. Proteome Res. 2014, 13, 1602−1613

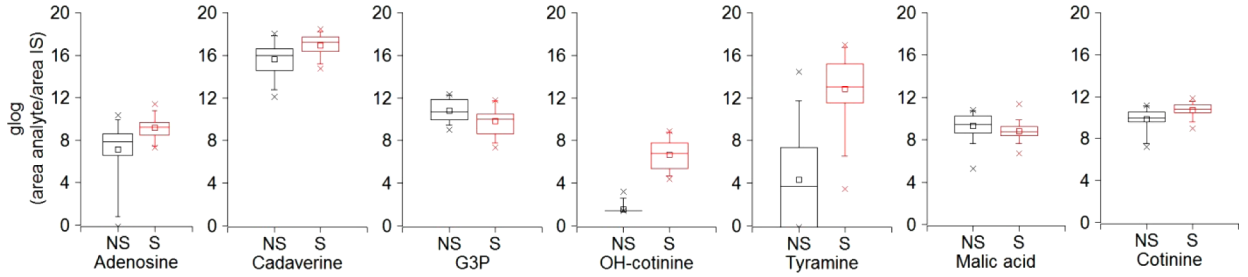


Figure 3. Boxplots of fragments from some significantly altered metabolites ($p < 0.05$) in saliva of S compared to NS. The boxes represent the 25% and 75% percentiles. Whiskers represent 5% and 95% percentiles. Crosses stand for single data. The middle line of the boxes indicates the median while the little squares show the arithmetic mean of the distribution.

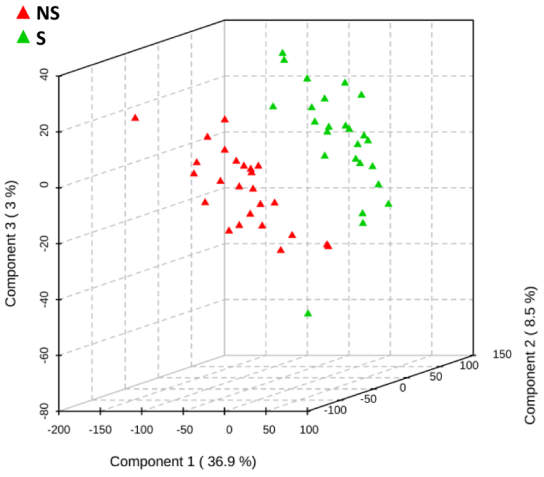


Figure 4. Three-dimensional PLS-DA score plot for GC-TOF-MS analysis of saliva samples of 25 smokers (S) and 25 nonsmokers (NS). Q^2 = 69.9%. R^2 = 92.3%.

spectral libraries and authentic reference substances. The origin of the identified compounds (exogenous or endogenous) along with their relation to endogenous metabolic pathways are shown in Table 3. In general, one has to consider that cigarette smoke contains more than 5000 compounds.[7] Hence, the differentiation between exogenous and endogenous origin is challenging. At least compounds in smokers' saliva showing decreased levels can definitely not be obtained from tobacco smoke uptake, but were rather likely of endogenous origin.

In this study, we found several compounds significantly increased in smokers' saliva compared to nonsmokers which occur in tobacco smoke. Thus, the elevated levels might be due to an uptake from tobacco smoke (for example, hexanoic acid or 4-hydroxyphenylethanol). Other metabolites found to be different in smokers and nonsmokers, such as adenine, adenosine, uridine or cadaverine are constituents of tobacco leaves, however, have not been reported as constituents of tobacco smoke,[7] to the best of our knowledge. These metabolites are most likely of endogenous origin, and a smoking-related alteration of the metabolism can be assumed. Adenosine is increased in patients suffering from COPD,[37] a disease which is linked to tobacco smoke. It is also released upon metabolic stress, cell damage, trauma, ischemia, or hypoxia to modulate homeostasis.[38] Adenosine, although unspecific of the biological end point of disease, might be a promising biomarker candidate of effect for early stage perturbations due to smoking. Further research is certainly required to elucidate the role of adenosine in saliva as a biomarker of smoking induced biological effect. Glucose 6-phosphate (G6P), benzenepropanoic acid, malic acid and glycerol-3-phosphate (G3P) were significantly decreased in

Table 3. Identified compounds in Saliva, Which Are Significantly Altered in Smokers Compared to Nonsmokers[a]

compd	m/z of analyzed fragment	p-value (S vs NS)	S vs NS[b]	identification	possible origin of increased metabolites	endogenous pathway
tyramine	174	2.0×10^{-7}	↑	RF	TS,[7] D[35]	tyrosine metabolism[36]
4-hydroxyphenylethanol	179	2.7×10^{-5}	↑	DB	derivate of benzeneethanol TS[7]	
adenosine	230	3.7×10^{-4}	↑	RF	T[7]	purine metabolism
hexanoic acid	173	4.6×10^{-4}	↑	DB	TS[7]	
adenine	264	2.4×10^{-3}	↑	DB	T,[7] D	purine metabolism
cadaverine	174	2.4×10^{-3}	↑	DB	T,[7] D	lysine metabolism[35]
G6P	387	5.1×10^{-3}	↓	RF		energy metabolism
G3P	357	7.6×10^{-3}	↓	DB		lipid/energy metabolism
guanosine or guanosine derivate	324	1.1×10^{-2}	↑	DB		purine metabolism
N-acetylglucosamine	205	3.5×10^{-2}	↑	DB		amino sugar metabolism
uridine	103	4.1×10^{-2}	↑	DB	T[7]	pyrimidine metabolism
benzenepropanoic acid	104	4.3×10^{-2}	↓	DB		tyrosine metabolism
malic acid	233	4.7×10^{-2}	↓	DB		energy metabolism

[a]For increased metabolites, their possible exogenous origins are stated. [b]Decreased metabolites cannot be due to tobacco smoke uptake. For increased metabolites their possible exogenous origins are stated. T (Tobacco), TS (Tobacco smoke), D (Diet). DB (spectral database), RF (reference compound).

dx.doi.org/10.1021/pr401099r | J. Proteome Res. 2014, 13, 1602−1613

saliva of smokers. The latter compound is an intermediate metabolite in the glycolysis pathway and enzymatically converted to glyceraldehyde 3-phosphate. The enzyme which further degrades glyceraldehyde 3-phosphate (glyceraldehyde 3-phosphate dehydrogenase) has been shown to be up-regulated as a consequence of tobacco smoke exposure.[39,40] Another explanation for significantly decreased levels of G3P is its conversion to phospholipids or triglycerides by re-esterefication with fatty acids. This pathway has been shown to be accelerated in smokers.[41] Furthermore, smoking is known to increase malate dehydrogenase and malate enzyme 1 expression in humans, which might lead to enhanced decomposition of malic acid.[40,42] Our finding of decreased salivary malic acid levels is in agreement with this observation. G6P takes part in the glucose metabolism, which reroutes to the pentose phosphat pathway in smokers.[39] Therefore, decreased levels of G6P in smokers might be an effect of activation and increased expression of the enzyme glucose-6-phosphate dehydrogenase which decomposes G6P in the pentose phosphat pathway.[39] Tyramine showed the highest significance in terms of differences between smokers and nonsmokers in our analysis of saliva samples. Tyramine came up as a high ranking metabolite both from the PLS-DA and the Mann−Whitney-U test. It is generated by endogenous decarboxylation of tyrosine,[36] but it can also be taken up from the diet, particularly by consuming fermented food such as cheese.[43] Since our study was strictly diet controlled, we assume that the observed differences between smokers and nonsmokers are solely related to an altered endogenous metabolism. Tyramine was described as a constituent of tobacco and tobacco smoke;[7,44] however, up to now, no quantitative data are available. It appears unlikely that the 22-fold increase of tyramine levels in smokers are caused by an absorption of tyramine from tobacco smoke, as the smokers have not smoked for about 9 h prior to saliva sampling and the metabolic decomposition of tyramine in the human body is rather fast with an elimination half-life of about 0.5 h after oral administration.[45] A more likely explanation for higher levels in smokers is probably the fact that the decomposition of tyramine by monoamine oxidase A and B is inhibited by smoking, as shown by Fowler and co-workers.[46,47] Tyramine is of physiological relevance in the human bodies, since it acts as an indirect sympathomimetic agent by inhibiting the neural cell uptake of catecholamines including dopamine, noradrenaline and adrenaline, thus prolonging the activity of these neurotransmitters.[48] Tyramine-related physiological effects, includes migraine, hypertension, increased heart rate or elevated blood sugar levels.[45,48,49] However, new studies have indicated that the altered vasoconstriction is not an indirect sympathomimetic effect rather than mediated by trace amine-associated receptors.[49,50] Some of the physiological effects have been reported to be implicated with smoking.[4,51] Further studies are required to decipher the role of tyramine and smoking related induction of the mentioned effects.

We were interested in endogenous alterations of the human metabolome induced by smoking. Therefore, we were concerned about residues from tobacco smoke constituents and nutrition in the oral cavity of smokers. Thus, we specified a 9 h interval of fasting and nonsmoking along with this intense rinse technique prior to saliva sampling. Nicotine, the major constituent of tobacco smoke, was not found significantly different between the two groups with our metabolomic approach, which provides strong evidence of the effectiveness of the applied procedure. Interestingly, we found cotinine and OH-cotinine significantly elevated in smokers' saliva. Cotinine is the most frequently used biomarker for smoking and its levels in plasma and saliva are highly correlated.[52] Nicotine metabolism most likely takes place in the liver and the lungs. Since there is evidence that metabolites detectable and quantifiable in saliva likely originate from the bloodstream,[53] we believe that most of the metabolites which we detected in saliva originate from the bloodstream from where it is transported by passive and acitve mechanisms. However, it is well-known, that the ratios of metabolite levels between blood and saliva can be highly variable and are determined by a number of factors including physicochemical properties of the compounds, pH and flow rate of the saliva and probably other factors.[54,55] Nevertheless, we cannot certainly allocate in which organs or regions of the human body altered metabolism occurs. Admittedly, extensive metabolism takes place within the mouth, since there are over 700 bacterial species[56] and proteins in the oral cavity. It is well-known that smoking causes various changes in the flora of the oral cavity.[57] Therefore, alterations from the cavity and a transition from metabolites to the blood and reuptake is theoretically possible.

■ CONCLUSION

In summary, we developed and validated a GC-TOF-MS method for a metabolic fingerprinting in saliva to find new biomarkers of effect related to smoking. We could demonstrate that saliva, as a noninvasively accessible body fluid, is a suitable biological matrix for investigating the human metabolome. We could proof that saliva samples, obtained from a strictly diet-controlled clinical smoking study, were well suited to identify 13 metabolites, significantly different between smokers and nonsmokers. Possible mechanisms of how smoking can lead to an alteration of the metabolite levels as well as implicated biological effects were discussed. Our method, using open source software packages such as MZmine and Metaboanalyst, is hypotheses generating and seems to be applicable to further understand which metabolites might serve as biomarkers of early biological effects caused by smoking. Nevertheless, further research in this direction is required to confirm and extend our findings.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the Imperical Tobacco Group PLC and the Analytisches-Forschungslabor München GmbH. There has not been any influence on the work or publication by the sponsor Imperial Tobacco.

ABBREVIATIONS

BMI, body mass index; BSTFA, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide; COex, carbon monoxide in exhaled breath; COPD, chronic obstructive pulmonary disease; G3P, glycerol-3-phosphate; G6P, glucose 6-phosphate; GC-TOF-MS, gas chromatography coupled to time-of-flight mass spectrometry; glog, generalized logaritm; IS, internal standard; LLOQ, lower limit of quantification; MOX, methoxyamine HCl; MRM, multiple reaction monitoring; NS, nonsmoker; OH-cotinine, trans-3-hydroxycotinine; OH-cotinine-d_3, trans-3-hydroxycotinine-methyl-d_3; PLS-DA, partial least-squares discriminant analysis; QC, quality control; S, smoker; TMCS, trimethylchlorosilane; ttMA, trans,trans-muconic acid; UPLC, ultra performance liquid chromatography; VIP, variable importance in projection

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