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Validated Ultra-High-Performance Liquid-Chromatography Hybrid High-Resolution Mass Spectrometry and Laser-Assisted Rapid Evaporative Ionization Mass Spectrometry for Salivary Metabolomics

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ABSTRACT: Whereas urine and blood are typically targeted in clinical research, saliva represents an interesting alternative as its intrinsic metabolome is chemically diverse and reflective for various biological processes. Moreover, saliva collection is easy and non-invasive, which is especially valuable for cohorts in which sample collection is challenging, *e.g.* infants and children. With this rationale, we established a validated UHPLC-HRMS method for salivary metabolic profiling and fingerprinting. Hereby, 450 µL of saliva was centrifuged and passed over a 0.45-µm polyamide membrane filter, after which the extract was subjected to chromatographic analysis (HSS T3 column) and Q-Exactive™ Orbitrap-MS. For the majority of the profiled metabolites, good linearity ($R^2 \geq 0.99$) and precision (coefficient of variance ≤ 15%) was achieved. The fingerprinting performance was evaluated based on the complete metabolome (11,385 components), whereby 76.8% was found compliant with the criteria for precision (coefficient of variance ≤ 30%) and 82.7% with linearity ($R^2 \geq 0.99$). In addition, the method was proven fit-for-purpose for a cohort of 140 adolescents (6-16 years, stratified according to weight), yielding relevant profiles (45 obesity-related metabolites) and discriminative fingerprints (Q^2 of 0.784 for supervised discriminant analysis). Alternatively, LA-REIMS was established for rapid fingerprinting of saliva, thereby using a Nd:YAG laser and Xevo G2-XS QToF-MS. With an acquisition time of 0.5 min per sample, LA-REIMS offers unique opportunities for point-of-care applications. In conclusion, this work presents a platform of UHPLC-HRMS and LA-REIMS, complementing each other to perform salivary metabolomics.

Metabolomics is one of the most recent omics technologies, whereby the metabolome is mapped as an accurate reflection of an individual's biological phenotype. Hereby, various host-related biological and external factors, including diet, microbial community, stress, lifestyle, *etc.* are integrated within the acquired metabolic fingerprint. This offers unique possibilities to characterize an individual's pathophysiological state, allowing the discovery of clinical biomarkers and elucidation of metabolic pathways.[2] As such, metabolomics has already uncovered biologically relevant alterations in *e.g.* type 2 diabetes mellitus, cystic fibrosis, and various types of cancers, including leukemia, breast, brain and prostate cancer.[1-3]

To date, high-resolution mass spectrometry (HRMS) is mostly used to perform metabolomics analyses, whereby accurate mass measurements (sub-ppm errors) allow for a highly selective and sensitive detection of low-abundant metabolites in complex biological samples. Moreover, hyphenation with ultra-high-performance liquid-chromatography (HPLC) provides additional selectivity and sensitivity, which enables LC-MS to reach high metabolome coverage in metabolic profiling and fingerprinting.[4,5] Whereas profiling typically aims for the identification and absolute quantification of a selection of pre-defined metabolites, the fingerprinting strategy represents a holistic top-down approach with the intention to monitor all metabolites present in the biological material under investigation.[6,7] Both strategies may be implemented for biomarker discovery and unraveling of mechanistic pathways, involved in health and disease.

When considering the translation of metabolomics into clinical practice, often involving large sample sets and point-of-care applications, conventional LC-MS and the associated extensive procedures are not very convenient. In this regard, ambient ionization constitutes an interesting alternative as sample preparation and chromatographic separation are omitted and MS-analysis is performed directly on the crude sample.[6] One type of ambient ionization concerns laser-assisted rapid

evaporative ionization MS (LA-REIMS), with the process of laser ablation being responsible for the release and ionization of metabolites from the sample. As the analysis time (including laser ablation and mass analysis) is typically less than one minute, this approach offers unique opportunities for rapid metabolic fingerprinting, as has already been demonstrated in surgical intervention, more specifically intersecting malignant tumors.[8] Nevertheless, data generated by LA-REIMS are not as comprehensive as for LC-MS. Indeed, as there is no chromatographic separation, LA-REIMS metabolite characterization is solely based on the accurate mass, which does not facilitate identification and absolute quantification. One element in this regard concerns the merger of isomeric and isobaric compounds, evidently compromising metabolome coverage. On the other hand, loss and conversion of metabolites is very likely to be reduced as no extraction and chromatography is performed.

To date, various biological fluids have been considered for metabolomics research in humans, for which especially blood plasma and serum, urine, and feces can be listed. Clinical applications have been established for all cited biofluids, *e.g.* fecal metabolomics to assess gastro-intestinal disorders (including irritable bowel syndrome and inflammatory bowel disease), colorectal cancer, and diet-microbiome-metabolome interactions, urinary metabolomics to diagnose inborn errors of metabolism, kidney dysfunctioning, liver disease, and type 2 diabetes, and blood metabolomics to research cancer, cardiovascular diseases, neurological and endocrine disorders, infections, *etc.*[9-12] However, with respect to the collection of blood samples, a number of substantial disadvantages can be noted; *i.e.* the invasive collection, need for trained personnel, and handling risks. In this context, urine and feces represent interesting alternatives, although there is still some inconvenience for repeated and on-the-spot sampling, as well as with respect to the extraction, which can be quite complex. As such, and despite existing diagnostic assays and self-test kits may circumvent a number of these issues[10], performing untargeted discovery-phase metabolomics or targeting extensive biomarker signatures in these biofluids still encounters practical difficulties. In this regard, the above-cited shortcomings are much less applicable for saliva, being the most accessible and readily available bio-fluid in humans. Although saliva is primarily composed of water, this matrix is chemically complex, containing mucus, digestive enzymes, growth factors, hormones, cytokines, immunoglobulins, anti-bacterial and anti-viral peptides, but also bacterial and viral cells including their metabolites[13]. Being produced by the salivary glands and the gingival sulcus, saliva has been recognized to play a vital role in food digestion, protection against harmful microorganisms and lubrication of the oral cavity. In addition, saliva is involved in taste-bud growth, wound healing and pain numbing. Moreover, as many metabolites can pass through blood passively or actively, saliva was also found to reflect processes from outside the oral cavity. Even more, saliva metabolites have been noted to parallel metabolic alterations that occur in blood, and may thus reflect many pathophysiological and nutritional changes, as well as exposure to medication and environmental factors.[14] In line with these observations, saliva has become a common tool for diagnosis of various cancers, diabetes, Cushing's disease, hepatitis, *etc.* [13,15-17]. Although saliva represents an interesting matrix, current studies mostly target specific metabolites or classes that are already known to be involved in a certain disease state or medication action, thus profiling only a small part of the saliva metabolome.[13,17-21] Therefore, untargeted fingerprinting will offer new perspectives in both fundamental and translational metabolomics.

The objective of this study was to establish a validated analytical platform of comprehensive UHPLC-HRMS and rapid LA-REIMS for salivary metabolomics, inclosing both targeted profiling and untargeted fingerprinting. With respect to the profiling, it was mainly intended to cover an increased number of chemical classes compared to the currently available salivary profiling methods.[13,17-21] The abilities of salivary metabolomics were demonstrated in a cohort of adolescents that were stratified according to weight, whereby the collected saliva samples were subjected to UHPLC-HRMS and LA-REIMS analysis to define the pathophysiological state and relevant metabolic alterations. As such, presenting an analytical platform that is validated and fit-for-purpose, salivary metabolomics may find its way in clinical practice, for both in-depth mechanistic elucidations as well as point-of-care applications.

MATERIALS AND METHODS

Analytical Standards and Reagents

Analytical and internal standards that were used for LC-MS and LA-REIMS analysis are listed in Table S1. Stock solutions were prepared at a concentration of 1 $mg.mL^{-1}$, using either methanol or ultrapure water (0.055 $\mu S \cdot cm^{-1}$, delivered through a purified water system (VWR International, Belgium)). These and the derived working solutions were stored at -20 °C in amber glass vials. Solvents used for analysis were of LC-MS grade and from VWR International (Belgium) or Fisher Scientific (USA).

Biological Samples

In this study, saliva was obtained from adolescents (6-16 years) through a standardized protocol, whereby samples were collected via Salivette synthetic swabs and Cryovials (Sarstedt, Germany) at Ghent University Hospital in the presence of trained staff members. Saliva was collected between 4.30 and 5.30 p.m., with the participants being restrained from any food or drinks (except water) for at least 3 h prior to collection and allowed to brush their teeth in the morning only. Smoking or alcohol use was not permitted during the whole day. Samples were stored at -80 °C. The study was approved by the Ethical Committee of Ghent University Hospital EC UZG 2017/0527.

Salivary metabolomics by UHPLC-HRMS

Optimization of Salivary Extraction. Generic extraction was optimized through a design of experiments, for which JMP 12 software was used (SAS, UK). First, a Chakravarty fractional factorial screening design (FFD) was established with 21 experiments (3 center points) to assess four factors: centrifugation (no centrifugation, 4,200 or 17,000 x g), volume of saliva (300 to 1500 µL), dilution factor (0.1 to 1), and filtration (no filtration or usage of a polyvinylidene difluoride (PVDF) membrane filter, 13 mm diameter, 0.22 µm pore size (Merck, Ireland)). The effect of each factor was statistically evaluated based on the metabolome coverage (*i.e.* total number of detected components) as well as the summarized normalized peak area of those metabolites that were detected upon targeted profiling (n = 81) (Table S1). With respect to the latter, identification at Tier 1 was achieved based on the accurate mass (*m/z*-value, considering the molecular ion and ^{13}C isotope) and the retention time relative to that of an internal standard, all with

reference to an authentic standard.[22] In a second phase, response surface modeling (RSM) with 16 experiments (3 center points) was applied to optimize the significant quantitative factors, *i.e.* the centrifugation speed (4,200 to 17,000 x g), volume of saliva (300 to 500 µL), and dilution factor (0.1 to 1). Statistical evaluation was based on the metabolome coverage and summarized normalized peak area of detected target metabolites (n = 54) (Table S1). In addition, also aspects of practical feasibility were taken into account, *i.e.* a filtration step appeared inevitable to prevent clogging of the LC-column. In this regard, the PVDF filter was tested against a polyamide membrane filter (25 mm diameter, 0.45 µm pore size, Macherey-Nagel, Germany) and Amicon® ultra-centrifugal filter (30 kDa, Merck, Ireland).

Final Salivary Extraction Protocol. The optimized protocol for polar metabolomics used 450 µL of saliva, which was pipetted into a 1.5-mL Eppendorf tube. After adding 10 µL of internal standard mixture (25 ng.µL^{-1} D-valine-d_8 and L-alanine-d_8), the solution was shortly vortexed and centrifuged for 5 min at 17,000 x g (room temperature). Next, the supernatant was collected using a 1-mL syringe with needle and passed over a polyamide membrane filter (25 mm diameter, 0.45 µm pore size, Macherey-Nagel, Germany). Finally, 150 µL of the undiluted extract was transferred to an LC-vial with glass insert.

UHPLC-HRMS Analysis. UHPLC-HRMS analysis of saliva extract was based on the validated methods of Vanden Bussche *et al.* (2015) for feces and De Paepe *et al.* (2018) for urine and plasma.[4,23] For chromatographic separation, a Dionex Ultimate 3000 XRS UHPLC system (Thermo Fisher Scientific, USA) was used, which was equipped with an Acquity HSS T3 C18 column (1.8 µm, 150 x 2.1 mm) (Waters, UK) that was kept at 45 °C. The binary solvent system consisted of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% formic acid. Using a flow rate of 0.4 mL·min^{-1}, the following gradient was applied (solvent A, v/v): 0-1.5 min at 98%, 1.5-7.0 min from 98% to 75%, 7.0-8.0 min from 75% to 40%, 8.0-12.0 min from 40% to 5%, 12.0-14.0 min at 5%, 14.0-14.1 min from 5% to 98%, followed by a re-equilibration step of 4.0 min. The sample injection volume was 10 µL. For mass analysis, a Q-Exactive™ mass spectrometer was employed (Thermo Fisher Scientific, USA), which was equipped with a heated electrospray ionization (HESI-II) source that was operated in polarity switching mode and positioned in 0/B/1. Instrumental parameters included a sheath, auxiliary and sweep gas flow rate of, respectively, 50, 25 and 5 arbitrary units (au), heater and capillary temperature of, respectively, 350 and 250 °C, S-lens RF level of 50%, and a spray voltage of 3 and 2 kV for positive and negative ionization mode, respectively. The *m/z* scan range was set from 53 to 800 Da for both ionization modes, the automatic gain control target was 1 x 10^6 ions, the maximum injection time was 70 ms and the mass resolution was 140,000 FWHM (1 Hz).

Quality Assurance. To ensure accurate mass measurements (*i.e.* mass deviations ≤ 3 ppm), instrument calibration was performed with ready-to-use calibration solutions according to the manufacturer's guidelines (Thermo Fisher Scientific, USA). In addition, the operational conditions of the device were evaluated by injecting a standard mixture of 300 target metabolites (1 ng·µL^{-1}) (Table S1) at the beginning and end of every sequence. For analysis of large batches, quality control (QC) samples were included, which were made from a pool of all biological samples. QC extracts were repeatedly injected at the beginning of the analytical sequence for conditioning of the system and in between the analyzed biological samples (two QCs after every ten samples) to allow correcting for instrumental drift.

Method Validation. Linearity and precision were assessed in accordance to Naz *et al.* (2014), FDA guidelines (2018), and Ulaszewska (2019)[5,24,25], through a targeted and untargeted evaluation. For the targeted approach, 32 endogenously present metabolites were carefully chosen in order to attain adequate representation of various chemical classes (Table S2).

Linearity was assessed by serial dilution of a QC extract with artificial saliva (20 mM sodium dihydrogen phosphate, pH 7.4), thereby establishing a 9-point calibration curve with dilution factors ranging from 1 (no dilution) to 100. Evaluation of the linearity was based on the determination coefficient (R^2). For the untargeted approach, 9-point calibration curves (n=3) in ultrapure water were used, thereby performing calculations only for components recovered across all dilutions.

Precision analysis comprised evaluation of the instrumental, intra- and inter-day assay precision, which were all expressed as the coefficient of variance (CV, %). The instrumental precision was evaluated by the sequential injection of a same QC extract ten times (assay performed in triplicate). The intra-day assay precision was assessed by considering a QC sample that was extracted nine times in parallel under repeatable experimental conditions, by the same analyst. For the intra-laboratory reproducibility, a QC sample was extracted 18 times, half of which were extracted by a second analyst on a different day.

Data Processing. Targeted processing of the HRMS full-scan data, including metabolite identification and quantification, was performed using XCalibur 3.0 software (Thermo Fisher Scientific, USA). Identification was based on three parameters, whereby congruence with the analytical standard was required, *i.e.* the *m/z*-value of the molecular ion (mass deviation ≤ 5 ppm), the C isotope pattern ($^{13}C/^{12}C$ isotope ratio, compliant with CD 2002/657/EC) and a retention time relative to that of the internal standard (maximum deviation of 2.5%).[25] With respect to the untargeted data analysis, SIEVE™ 2.2 (Thermo Fisher Scientific, USA) was applied to process data as acquired during method development and validation. Compound Discoverer™ 2.1 (Thermo Fisher Scientific, USA) was used for analysis of full-scan data from the clinical study, allowing to process the data from the positive and negative ionization mode together. Both software programs applied automated peak alignment, peak extraction, deconvolution, blank peak filtering, and noise removal. Detected features were characterized by the *m/z*-value, retention time and peak intensity. Clusters of related features (*i.e.* components) were established, considering amongst others the presence of various ionization adducts and isotopes. The main software settings are presented in Table S3.

Salivary metabolomics by LA-REIMS

Final Protocol for Saliva Pre-treatment. Prior to LA-REIMS analysis, 450 µL of saliva was pipetted in a 1.5-mL Eppendorf tube and centrifuged for 5 min at 17,000 x g (room temperature). Next, the supernatant was collected using a 1-mL syringe with needle and passed over a polyamide membrane filter (25 mm diameter, 0.45 µm pore size, Macherey-Nagel, Germany). A volume of 50 µL filtrate was transferred to a glass microscope slide for LA-REIMS analysis. Although it was

intended to limit sample pre-treatment to the absolute minimum for LA-REIMS applications, inclusion of the filtration step was needed to address some practical issues (*i.e.* clotting within the saliva sample upon laser ablation, most likely due to protein precipitation).

Optimization of LA-REIMS Analytical Method. Relevant parameters for LA-REIMS analysis were optimized by a design of experiments, using the software program JMP 12 (SAS, UK). First, a D-optimal FFD (36 runs, 6 center points) was performed in duplicate for both the positive and negative ionization mode to assess the significance of various factors (with indication of the tested value range); the laser pulse time (165 to 220 µs), cone voltage (20 to 100 V), heater bias voltage (10 to 50 V), scan time (0.1 to 1 $s \cdot scan^{-1}$), and isopropanol solvent flow rate (0.05 to 0.20 $mL \cdot min^{-1}$). The effect of each factor was evaluated based on the absolute metabolome coverage (*i.e.* number of detected ions) and detection sensitivity (total ion abundance, TIC), thereby considering separate *m/z* intervals (*i.e.* from 50 to 250 Da, from 250 to 500 Da, from 500 to 750 Da, from 750 to 1000 Da, and from 1000 to 1200 Da) to warrant the holistic nature of the analytical method. Following the FFD-based screening, a central composite RSM design was applied to define the optimal settings for the significant factors. For both ionization modes, all factors were incorporated in the RSM design (29 runs, 3 center points), whereby different ranges were tested for each mode; laser pulse time (165 to 220 µs for negative ionization, 165 to 195 µs for positive ionization mode), cone voltage (15 to 100 V for negative mode, 20 to 70 V for positive mode), heater bias voltage (10 to 70 V for both modes), scan time (0.1 to 0.8 $s \cdot scan^{-1}$ for negative mode, 0.5 to 1 $s \cdot scan^{-1}$ for positive mode), and isopropanol solvent flow rate (0.05 to 0.30 $mL \cdot min^{-1}$ for negative mode, 0.05 to 0.15 $mL \cdot min^{-1}$ for positive mode). Evaluation of the RSM design was based on the metabolome coverage and sensitivity, considering various *m/z*-intervals. Additional tests (full factorial designs, ≥ three technical replicates) were conducted to further optimize the heater bias.

Final LA-REIMS Analytical Method. The system used for laser ablation was an Opolette™ HE2940 pump laser (OPOTEK LLC, USA) that consisted of a Nd:YAG laser, steering optics, optical parametric oscillator (fixed at 2940 nm), and wavelength separation optics. Transmission of the laser energy to the sample was achieved through free space optics, including a series of metallic-coated mirrors (OptoSigma, France) and a plano-convex lens (Thorlabs *GmbH*, Germany). Main operational settings for the laser pumping system were a pulse time of 180 and 165 µs for negative and positive ionization mode, respectively, and a repetition rate of 20 Hz. The total exposure time per burn (*i.e.* the period of laser ablation) was about 3 s and at least two burns per sample were generated. The aerosol produced was transferred to the REIMS platform by a 2.5-m polytetrafluorethylene tubing (1.6 mm internal diameter and 3.2 mm outer diameter), which was connected to the electrospray REI-interface by means of a T-shaped piece. A flow of isopropanol, containing the internal standards palmitic acid-d_{31} (5 $ng \cdot \mu L^{-1}$, $[M-H]^-$) and 1,2-dimyristoyl-d_{54}-sn-glycero-3-phosphocholine (100 $ng \cdot \mu L^{-1}$, $[M+H]^+$), was provided to ameliorate ionization. The flow rate was set at 0.2 $mL.min^{-1}$ for negative and 0.12 $mL \cdot min^{-1}$ for positive ionization. Full-scan mass analysis was carried out using a Xevo G2-XS Quadrupole Time-of-Flight (QToF) mass spectrometer (Waters Corporation, UK), applying an *m/z* scan range from 50 to 1200 Da. Main settings included a cone voltage of 45 V for negative and 20 V for positive ionization, a heater voltage of 70 V for negative and 75 V for positive ionization, and a scan time of 0.5 $s \cdot scan^{-1}$ for both ionization modes. The acquisition time was 0.50 $min \cdot sample^{-1}$.

Quality Assurance. Prior to LA-REIMS analysis, the instrument was calibrated (≤ 1 ppm mass deviation, also warranted by lock mass correction) in sensitivity mode according to the manufacturer's guidelines (Waters Corporation, UK), thereby using a solution of 0.5 mM sodium formate in isopropanol and ultrapure water (90/10, v/v). During analysis, instrument performance was monitored based on the internal standards' signal intensities and/or incorporation of quality control (QC) samples, which were pooled saliva extracts from at least seven persons. For the clinical cohort, ten external QC samples were included at the beginning and end of the analytical batch and two internal QC samples were inserted after every forty runs.

Data processing. Data from the FFD and RSM designs were processed by Abstract Model Builder 1.0.1581.0 (Waters Corporation, UK), thereby creating mass bins of 0.1 Da. For the clinical study, data were processed using MassLynx® 4.1 Progenesis Bridge (Waters Corporation, UK), whereby adaptive background subtraction, lock mass correction, creation of extracted chromatograms for each burn, and separation of multiple burns was carried out. Subsequently, the data file from the most intense burn was selected and subjected to Progenesis® QI 2.3 peak picking (Waters Corporation, UK), applying the automatic sensitivity mode with a default value of 3.

Multivariate analysis

Multivariate statistical analysis of UHPLC-HRMS and LA-REIMS data was achieved using SIMCA 14.1 software (Umetrics AB, Sweden), for which data were pre-processed by TIC and QC-normalization, log-transformation, and pareto-scaling. Principal Component Analysis (PCA-X) was applied to visualize natural patterning of samples and identify outliers. Supervised modelling was performed, constructing Orthogonal Partial Least-Squares Discriminant Analysis (OPLS-DA) models. The validity of these models was assessed by cross-validated analysis of variance (CV-ANOVA, *p*-value), permutation testing (n = 100) and the model characteristics $R^2(X)$ and $R^2(Y)$ for model fit and $Q^2(Y)$ for model predictivity. Selection of discriminative components or features was based on the S-plot (covariance p and correlation corr(p)), Variable Importance in Projection (VIP) score, and the Jack-knifed confidence interval.

Salivary Metabolomics in a Clinical Context

To demonstrate the potential of UHPLC-HRMS and LA-REIMS salivary metabolomics, the established methods were implemented in a clinical context of healthy versus unhealthy weight (*i.e.* overweight and obesity). This study was approved by the Ethics Committee of Ghent University Hospital (EC UZG 2017/0527) whereby 140 adolescents (6 to 16 years, 51% girls) were recruited for assembling a biobank of saliva samples. A written consent was obtained from the parents and children (from the age of 12 years) while verbal assent was obtained for younger children. Exclusion criteria included the presence of known endocrine or metabolic diseases other than overweight or obesity. Furthermore, data on drug use, age, tanner stage, sex and physical activity were collected. Adiposity classifications were made using BMI z-scores (adjusted BMI

for age and sex) following Roelants *et al.* (2012)[26] and extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity by Cole *et al.* (2012)[27]. Weight and height were measured by trained staff using a bio-impedance instrument (TANITA BC-420 SMA, Tanita Corporation, Japan) and stadiometer (Seca 225, SECA, USA), respectively. As such, the following classification structure was obtained: 7 adolescents with underweight, 92 with normal weight, 28 with overweight and 13 obese.

RESULTS AND DISCUSSION

Salivary UHPLC-HRMS Profiling and Fingerprinting.

Optimization of Salivary Extraction. The saliva extraction protocol was optimized through a sequential strategy of experimental designs, *i.e.* FFD and RSM, for which the results are presented in Table S4. Based on the FFD, centrifugation was assigned a significant positive effect (p-value < 0.05) in both the targeted and untargeted evaluation, whereby no differences were observed between the two centrifugation speeds. Implementation of a centrifugation step is typically incorporated to reduce clogging of the LC-column, which may be caused by the presence of mucus, large proteins, and food residues.[28] Accordingly, saliva extraction protocols often also include filtration for the removal of proteins.[13,29] The outcome of the FFD indicated no significant effect of the filtration on the extraction efficiency in metabolic profiling nor on the metabolome coverage in fingerprinting, which allowed inclusion of a filtration step to avoid clogging of the LC-column. With respect to the saliva starting volume, no significant differences were observed, although a higher volume tended to have a positive impact. On the other hand, dilution of the extract was associated with a borderline non-significant negative effect (p-value of 0.056) for the summarized normalized peak areas of the 81 endogenous metabolites, selected for targeted evaluation.

Additional optimization of the quantitative factors was performed by RSM, which allowed to determine the optimal centrifugation speed, saliva volume and dilution factor. Usage of undiluted saliva had a significant positive effect (p-value ≤ 0.002) on the extraction efficiency, independently from the saliva start volume and for both the targeted and untargeted approach. This observation was expected as metabolites, especially those not directly originating from saliva, but from blood or other matrices are usually present in saliva in low concentrations.[13] With the volume of saliva not having a significant effect in the RSM design, it was reasonable to limit the amount used. Indeed, as collected samples are always precious for multiple testing, it was opted to start the protocol with 450 µL saliva, which yields a minimum of 150 µL extract for UHPLC-HRMS analysis. Similar to the results from the FFD, the centrifugation speed had no significant effect on the RSM outcome, although the effect tended to be positive towards the highest speed (17,000 x g). A final element of the extraction protocol concerned the kind of filter used. After usage of the PVDF filter (0.22 µm pore size), it was experienced that the LC-column still tended to clog after the consecutive analysis of more than 20 samples. Other filters were tested, including a polyamide membrane filter (0.45 µm pore size) and a 30 kDa Amicon® ultra-filtering centrifugal filter. The polyamide membrane filter had a significant positive impact (p-value of 0.014) on the saliva extraction profiling and fingerprinting approach. It was presumed that the Amicon® filter specifically binds large proteins, which may hamper the passage of small molecules. The polyamide filter with a pore size of 0.45 µm was assumed to mainly retain mucus and debris coming from food. This implied that the final extract still contained proteins that might be subject of protease activity, with potential introduction of artefacts. In this regard, low handling temperatures (collection of samples on ice, storage at -80 °C, thawing at 4 °C) and short processing times (ca. 5 min) were considered essential to limit the formation of these artefacts, as protease activity is limited at low temperatures.

Method Validation. The results from the method validation are presented in detail in Table S2 and summarized in Table 1.

Linearity. For the targeted approach, excellent linearity ($R^2 \geq 0.99$)[24,25] was achieved for 31 endogenous compounds (Table S2) whereby this was achieved across the total dilution range for 22 metabolites while for 9 metabolites the strongest dilutions had to be excluded because of limited sensitivity. This was particularly observed for a number of carbohydrates, amino acids, and one steroid, whereby their low detection response and/or low abundance in saliva are plausible reasons. In contrast, one metabolite (L-carnosine) exhibited limited linearity (R^2 of 0.984), probably due to saturation effects. Untargeted evaluation suggested good linearity performance, with 82.7% of the 8,134 components having a $R^2 \geq 0.90$.

Precision. With respect to the precision, the FDA guidelines recommend a CV ≤ 15%, except when operating close to the limit of detection (CV ≤ 20%). For the targeted analyses (Table S2), 25 of the 32 representative compounds had an instrumental precision with a CV ≤ 15%, six had a CV ≤ 20% and only one metabolite, D-gluconic acid, had a CV above the cited thresholds (*i.e.* CV of 23.6%). Evaluation of the intra-day repeatability resulted in 28 metabolites with a CV ≤ 15%, three with a CV ≤ 20%, and one with a CV of 25.3% (*i.e.* L-carnosine). For the inter-day repeatability, all metabolites had a CV ≤ 15%, with the exception of vanillic acid (*i.e.* CV of 17.7%). As a result, only for the class of the hybrid peptides (represented by L-carnosine), the guidelines for precision were not completely met (*i.e.* a CV-value > 20% was observed). One possible explanation may relate to the saturation effects that were noted for this metabolite during the linearity evaluation. For untargeted fingerprinting, a CV ≤ 30% is generally recommended for acceptable precision.[22,23,28] For a total of 11,385 salivary components, the instrumental precision, intra-assay and inter-day precision were calculated and evaluated towards the cited threshold. This resulted in, respectively, acceptable precision for 86.6%, 83.0% and 76.8%, of all salivary components. These results are very promising as the method is capable to obtain a high saliva metabolome coverage with a high level of precision.

Table 1. Summary of the UHPLC-HRMS validation for successful targeted profiling and untargeted fingerprinting.

	Targeted	Untargeted
Linearity	29/32 ($R^2 \geq 0.99$)	82.7% ($R^2 \geq 0.90$)
Precision		
Instrumental	31/32 (CV < 20%), 25/32 (CV < 15%)	86.6% (< 30%)
Intra-day	31/32, (CV < 20%), 28/32 (CV < 15%)	83.0% (< 30%)

Inter-day	32/32 (CV < 20%), 31/32 (CV < 15%)	76.8% (< 30%)

Salivary Metabolomics in a Clinical Context using UHPLC-HRMS. Saliva has only recently been implemented in the research of various pathophysiological states such as CMV infection, cancer, Cushing's disease, and cystic fibrosis.[30,31] The non-invasive character and the easiness of collection, even multiple times a day makes saliva an interesting matrix for research, diagnosis and prognosis. In this regard, this study presents a validated UHPLC-HRMS method for salivary metabolomics, for which its potential for metabolic profiling and fingerprinting was demonstrated in health and disease, *i.e.* obesity.

Profiling of saliva to study obesity. In first instance, it was investigated whether our validated salivary profiling approach was able to define metabolic perturbations in saliva, underlying overweight and obesity in adolescents. Whereas our in-house library allowed the profiling of 129 metabolite compounds (Table S1) from 18 different classes, 45 of these compounds have been reported in literature to be involved in obesity metabolism (Table S5). One prominent class encloses the amino acids, for which both branched-chain amino acids (leucine, isoleucine and valine) and aromatic amino acids (phenylalanine, tryptophan, tyrosine and histidine) have been linked to weight increase, obesity and even type 2 diabetes. More specifically, the phenylalanine-tyrosine-dopamine pathway (as defined for saliva) plays an important role in the desire to eat and has been linked directly to obesity. In addition, salivary tryptophan and kynurenine (Table S5) are involved in the serotonin pathway, mediating serotonin levels and as a consequence satiety, mood disturbance and depression, all being physiological markers of obesity. Another class concerns that of the carbohydrates, with their salivary concentration levels often being linked to food intake but with mannose being hypothesized to play a role in insulin resistance, as a result of a defect glycosylation. Also the class of fatty acid esters has been described to be involved in obesity and insulin resistance, with *e.g.* methylbutyrylcarnitine being assigned a role in childhood obesity. Hereby, a link with an unbalanced mitochondrial fatty acid oxidation has been suggested. As such, metabolic profiling of saliva offers substantial potential for studies on obesity in young children, adolescents and adults, which may lead to the discovery of potential biomarkers for obesity and its co-morbidities.

Fingerprinting of saliva to study obesity. To demonstrate the potential of UHPLC-HRMS for metabolic fingerprinting, we analyzed saliva from 41 obese/overweight and 41 healthy weight adolescents. Fingerprints covered 9,044 components (Figure 1). The associated data matrix was pre-processed by TIC- and QC-normalization, whereby only components were retained that exhibited a CV ≤ 30% in the set of QC-samples. This rendered 6,639 components (not per se representing actual metabolites), which were subjected to multivariate statistics.

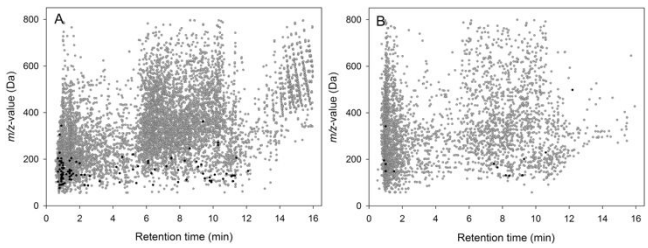

Figure 1. Fingerprint of positive (A) and negative (B) component ions upon UHPLC-HRMS analysis of saliva from adolescents (n = 140). Identified metabolites (in-house library) are indicated in bold.

PCA-X analysis revealed narrow clustering of the QC samples (Figure 2) and thus indicated good instrumental stability and reliable data acquisition. Moreover, no outliers were observed, which was verified based on the Hotelling's T^2 99% confidence interval as calculated for the biological samples only (*i.e.* excl. QC samples). However, no patterning according to weight status was uncovered, which means that the largest variations in the salivary fingerprints were due to aspects that cannot directly be related to adiposity classification, such as for example diet, gender, tanner stage, and environment. Also, subsequent OPLS-DA modelling was not able to reveal significant metabolic differences, as the model was not compliant with the set validation criteria ($Q^2(Y) < 0.5$, p-value > 0.05, and inacceptable permutation testing). This may relate to the relative low number of cases per study population, which hampers the statistical power and the ability to uncover the subtle metabolic differences that are underlying the clinical condition under investigation. Nevertheless, pre-filtering of the data based on the VIP-score (≥ 1) and Jack-knifed confidence interval (not across zero) yielded a subset of 295 components (of which 7 were identified using our in-house library, Table S1) for which significant differentiation according to weight status could be established upon PCA-X and OPLS-DA modelling (Figure 2). Hereby, a valid OPLS-DA model was obtained with an $R^2(X)$ of 0.322, $R^2(Y)$ of 0.846, $Q^2(Y)$ of 0.726, p-value < 0.001, and good permutation testing.

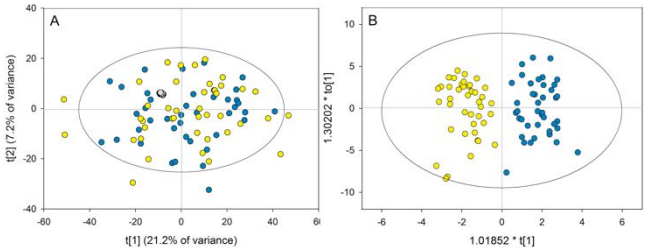

Figure 2. PCA-X (A) and OPLS-DA (B) score plot as obtained upon UHPLC-HRMS fingerprinting of saliva from normal weight (n = 41, blue) and obese/overweight (n = 41, yellow) individuals. Grey circles in Figure A represent QC-samples.

Salivary metabolomics by LA-REIMS

Optimization of LA-REIMS Analytical Method. LA-REIMS for rapid fingerprinting of saliva was optimized by means of an experimental design, which allowed to efficiently define the optimal settings for the various methodological parameters. The FFD revealed that the main effects of the solvent and pulse time were significant (p-value < 0.01) for both ionization modes, whereas the main effect of the cone voltage was also significant for the negative ionization mode (p-value < 0.01) (Table S6). In addition, evaluation of the first-degree interaction effects showed that also the cone voltage (positive ionization) and scan time (negative and positive ionization) had a significant impact on the metabolome coverage and detection sensitivity (p-values < 0.05). Taking into consideration these findings, it was opted to evaluate all five factors by the subsequent RSM modelling, thereby adjusting the tested range of settings according to the preliminary results from the FFD. For the negative ionization mode, the RSM indicated significant main effects (p-value < 0.001) for all factors across the tested ranges, with exception of the heater bias (Table S6). However, the latter factor showed

significance when evaluating the first-degree interactive effects (p-value < 0.05). Optimal values for the scan time (0.5 s·scan^{-1}), pulse time (180 µs), and cone voltage (45 V) were clearly expressed through the RSM prediction profiler but the optimal setting for the flow rate was noted to vary according to the *m/z* interval. Indeed, lower flow rates (0.05 mL·min^{-1}) seemed to improve the ionization efficiency of the low-weight molecules (*m/z* from 50 to 500 Da) whereas higher flow rates (0.25 mL·min^{-1}) tended to ameliorate ionization of the high-weight molecules (*m/z* from 500 to 1200 Da). A compromise value of 0.2 mL min^{-1} was set. Using these final settings, the heater bias voltage was further optimized based on a full factorial design (testing values of 10, 30, 50, 70, and 90 V). Although no significant differences were observed, 70 V seemed to result in the best detection sensitivity. For the positive ionization mode, the main effect was significant (p-value < 0.05) for all factors with exception of the heater bias voltage. Optimal values for the scan time (0.5 s·scan^{-1}), pulse time (165 µs), and cone voltage (20 V) were clearly expressed by the RMS prediction profiler. Also here, the optimal setting for the solvent flow rate varied according to the *m/z* interval whereby a compromise value of 0.12 mL·min^{-1} was selected. However, lower values (0.05 mL·min^{-1}) seemed to improve the ionization efficiency of the low-weight molecules (*m/z* from 50 to 500 Da). The heater bias was further optimized through a full factorial design (testing values of 60, 70, 80, and 90 V), whereby a value of 75 V rendered the highest signal intensities, although not being significant.

Fingerprinting by LA-REIMS. Metabolic fingerprints as acquired by LA-REIMS analysis are depicted in Figure 3, whereby the burn TIC signal as well as the underlying acquired mass spectrum are presented for each ionization mode. The total number of covered ions (features) amounted 13,231, of which 50.2% were obtained in positive ionization mode. Clustering of ions (features) was not performed (*i.e.* grouping of related isotopes and adducts) as the lack of orthogonal separation data (*e.g.* from LC) would result in too many false positive matches. It may be noted that LA-REIMS analysis in positive ionization seemed to be more sensitive for the low-molecular weight metabolites or/and the saliva metabolome contained more low-molecular weight metabolites that were proton acceptor (*e.g.* H^+).

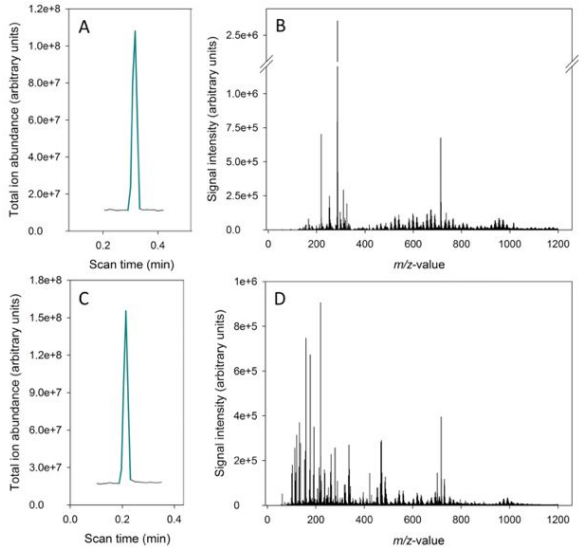

Figure 3. TIC signal ("burn") as obtained upon LA-REIMS analysis of saliva in negative (A) and positive (C) ionization mode. Mass spectra for negative and positive ionization (B and D, respectively).

Quality assurance. The analytical performance of LA-REIMS was primarily evaluated in terms of the precision, which was studied based on the batch of clinical samples (total analysis time of about 5 h, covering 246 runs). It should be noted that these samples were analyzed in a single continuous batch, performing only minimal cleaning, *i.e.* using a cotton swab that was wetted with ethanol to remove saliva splashes from the lens (~ every 5 samples). Hereby, the internal and external QC samples were subjected to CV-analysis. The external QC samples allowed to define the instrumental precision, whereby ten sequentially analyzed samples at the end of the sequence showed acceptable repeatability, *i.e.* 87.0% of the negative and 55.8% of the positive ions (features) showed a CV ≤ 30%. With respect to the internal QC samples (indicating the intra-day precision), acceptable CV (*i.e.* ≤ 30%) was obtained for 69.0% and 59.5% of the negative and positive ions (features), respectively. Based on these findings and the PCA-X score plots (Figure 2), which indicated narrow clustering of the internal QC samples, LA-REIMS showed excellent ruggedness and was proven suited for reliable fingerprinting, especially in negative ionization mode, which is in line with Bodai *et al.* (2018).[31]

Salivary Metabolomics by LA-REIMS in a Clinical Context. The performance of LA-REIMS to achieve rapid discriminative fingerprinting was evaluated within the clinical cohort, thereby considering a balanced selection of normal weight (n = 35) and overweight/obese (n = 35) individuals. In comparison with UHPLC-HRMS, a lower number of samples was included due to reasons of depletion. PCA-X modelling (Figure 4) indicated narrow clustering of the internal QC-samples and thus stable analytical performance. However, no differentiation according to weight was observed, which was confirmed by the subsequent OPLS-DA modelling approach (Q^2 < 0.5 and CV-ANOVA p-value > 0.05). Pre-filtering of the data based on the Jack-knifed confidence intervals (not across zero) and VIP-score (≥ 1) rendered 505 ions (features), for which valid OPLS-DA models (1 predictive and 2 orthogonal principal components) could be constructed (R^2(X) of 0.227, R^2(Y) of 0.946, Q^2 of 0.808; p-value of 9.01 e^{-21}; good permutation testing) (Figure 4). Performing a fivefold 20%-leave-out validation strategy, the general classification accuracy was 97.1% (sensitivity of 100% and specificity of 94.3%). As such, LA-REIMS fingerprinting allowed to establish predictive models that are able to appoint metabolic perturbations according to pathophysiological state (*i.e.* weight state), also offering potential to evaluate *e.g.* the contribution of etiological factors and intervention efficacy.

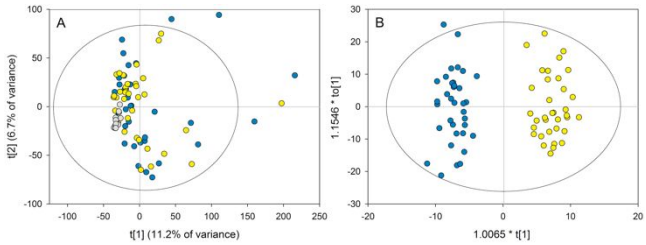

Figure 4. PCA-X (A) and OPLS-DA (B) score plot as obtained upon LA-REIMS fingerprinting of saliva from normal weight (n =

35, blue) and overweight/obese (n = 35, yellow) individuals. Grey circles in Figure A represent QC-samples.

Conclusion

Saliva represents an interesting matrix for clinical and epidemiological research because saliva is chemically rich, its collection is non-invasive and easy, multiple samples can be collected per day, and no highly trained personnel is needed. However, current methods for analysis of saliva are typically elaborated for targeted profiling and lacking thorough validation. With this study, we present a UHPLC-HRMS method for salivary metabolomics that was validated and demonstrated fit-for-purpose for profiling and fingerprinting. In a clinical context of overweight in adolescents, our methodology was able to define biologically relevant metabolite profiles (129 metabolites were identified, of which 45 have been ascribed a role in obesity) and discriminative fingerprints (> 6,500 metabolites detected), offering potential for biomarker discovery and pathway elucidation. In light of these findings, saliva may serve as a valuable matrix for point-of-care applications and precision medicine. Therefore, to translate metabolomics towards a clinical environment, we proposed the concept of LA-REIMS for salivary metabolomics. With the metabolome coverage being comparable to UHPLC-HRMS, this ambient ionization technique also achieved differentiation according to weight status. In conclusion, this work presents a complementary platform of validated UHPLC-HRMS and robust LA-REIMS for salivary metabolomics, offering opportunities for in-depth mechanistic studies and rapid discriminative fingerprinting, respectively.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information (Supplementary Table S1 – S6) is available free of charge on the ACS Publications website. Table S1: Analytical standards. Table S2: Analytical performance UHPLC-HRMS. Table S3: Settings for automatic feature detection. Table S4: Outcomes from the FFD and RSM designs for UHPLC-HRMS. Table S5: Obesity-related markers. Table S6: Outcomes from the FFD and RSM designs for LA-REIMS.

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Notes

The authors declare no competing financial interest.

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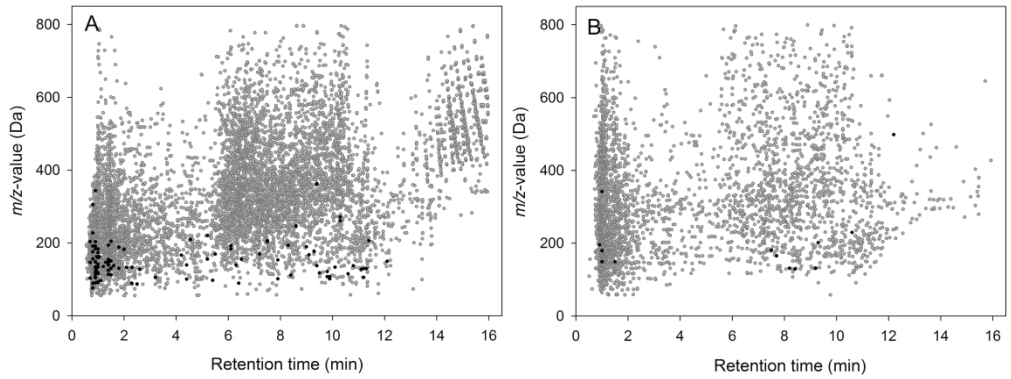

Figure 1. Fingerprint of positive (A) and negative (B) component ions upon UHPLC-HRMS analysis of saliva from adolescents (n = 140). Identified metabolites (in-house library) are indicated in bold.

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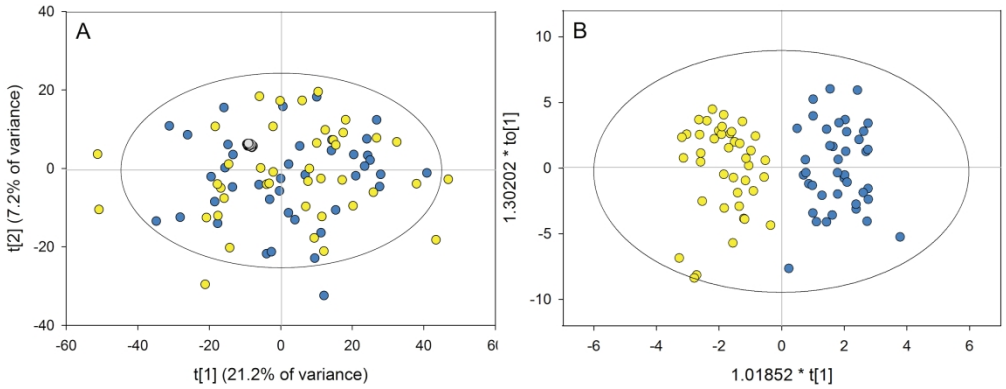

Figure 2. PCA-X (A) and OPLS-DA (B) score plot as obtained upon UHPLC-HRMS fingerprinting of saliva from normal weight (n = 41, blue) and obese/overweight (n = 41, yellow) individuals. Grey circles in Figure A represent QC-samples.

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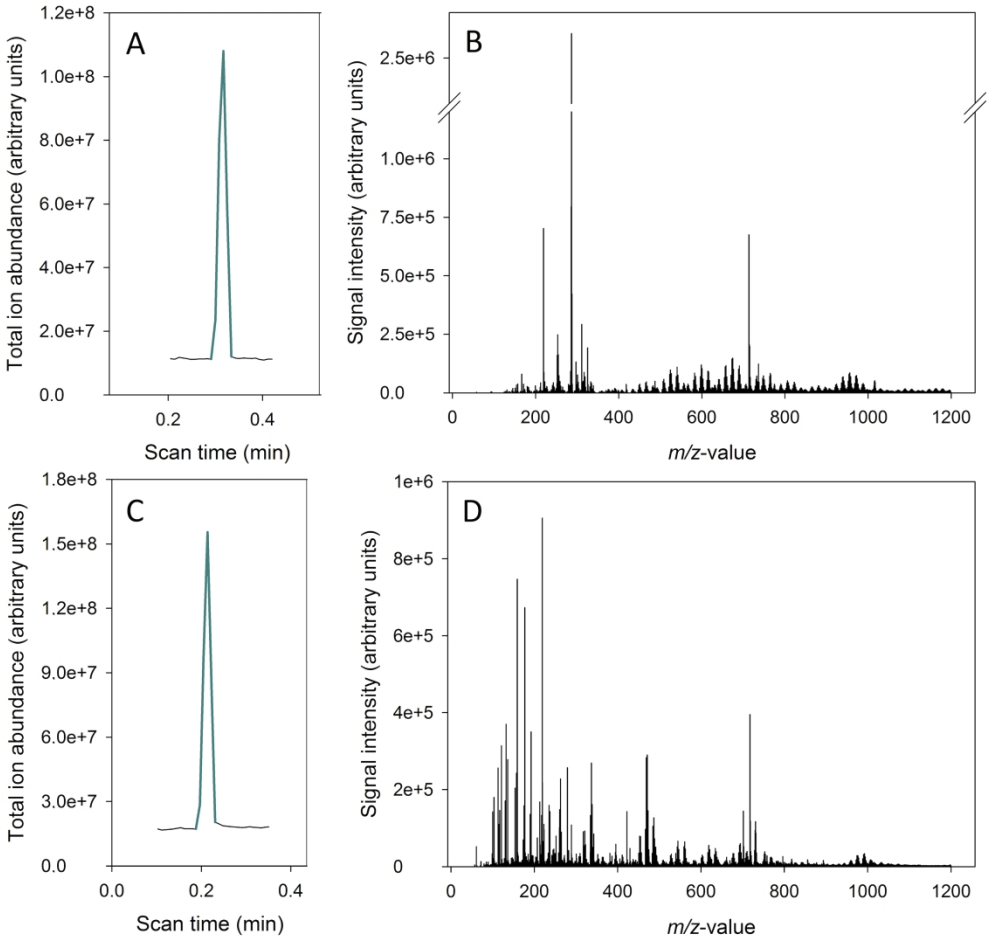

Figure 3. TIC signal ("burn") as obtained upon LA-REIMS analysis of saliva in negative (A) and positive (C) ionization mode. Mass spectra for negative and positive ionization (B and D, respectively).

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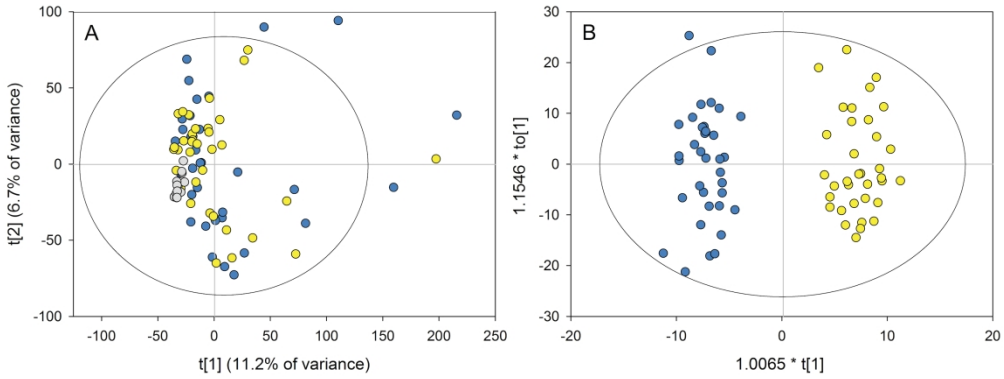

Figure 4. PCA-X (A) and OPLS-DA (B) score plot as obtained upon LA-REIMS fingerprinting of saliva from normal weight (n = 35, blue) and overweight/obese (n = 35, yellow) individuals. Grey circles in Figure A represent QC-samples.

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