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UPLC-QTOF-ESI(+) MS AND DIRECT MS INJECTION USED TO FINGERPRINT RESTING AND STIMULATED SALIVA PROFILES: PRELIMINARY RESULTS

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ABSTRACT. A rapid and reliable profiling of resting and stimulated saliva by two advanced techniques, LC-QTOF-ESI (+) MS and direct injection mass spectrometry (DIMS) was performed. Male and female healthy volunteers (n=12) were randomly selected, their resting, and stimulated saliva being collected, before and after chewing stimulation with parafin. Base peak chromatograms of saliva methanolic extracts (BPC) were recorded, the main peaks were identifed and the MS data (m/z values) were used to identify specific biomarkers. The biostatistic analysis made by Principal Component Analysis was applied to discriminate between samples' profile. The comparative UPLC-QTOF-ESI(+)MS fingerprints, before and after storage at -20ºC showed similar data with DIMS analysis, but the later one identified a larger range of molecules, without a preliminary separation by UPLC. Around 10 major biomarkers were identified, mainly phosspholipid derivatives, showing quantitative differences among the resting and stimulated saliva. Such preliminary results will be used for early diagnosis and monitoring therapy's effects in dental pathology.

Keywords*: resting and stimulated saliva, metabolomics, UPLC-QTOF-MS, direct MS injection*

INTRODUCTION

Saliva is a complex mixture, of crevicular or gingival fluids, derived from the gingival sulcus, desquamated oral epithelial cells and microorganisms, i.e. viruses, fungi, bacteria and endotoxins [1-3] as well a large number of inorganic electrolytes and organic components [4]. Salivary glands produce

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90% of slight acidic (pH 6–7) secretions and 10% fluids from labial, buccal or palatal salivary glands [4-6]. Saliva components may represent a “mirror” of the body’s health or pathological condition, by its qualitative or quantitative composition [7-8] reflecting the organs function in the body [9-10]. Saliva proteins bind up to 80% of carbohydrates (i.e., MUC5B mucins), mainly sialic acid, but also galactose, mannose, aminosugars, glycolipids (i.e., neutral and sulphated glyceroglucolipids), neutral lipids (i.e. free fatty acids, cholesteryl esters, triglycerides and cholesterol), as well phospholipids (i.e. phosphatidyl-ethanolamine, phosphatidylcholine), [11-12] as well amylase, mucin, lysozyme, IgA, lactoferrin, peroxidase, metalloproteases, glycoproteins, and lipoproteins [13]. The nonproteic components of saliva are uric acid, bilirubin, creatinine, glucose, cholesterol, hormones and fatty acids [14-17] representing good diagnosis biomarkers.

Recently, the salivary biomolecules were identified by omics’ technologies, including genomics, transcriptomics, proteomics and metabolomics [18-21].

Saliva is an appropriate diagnostic fluid with interesting perspectives for personalized therapy [22-25]. The metabolic profiling of saliva in patients with primary Sjögren’s syndrome was recently reported by Mikkonen et al. [26]. By metabonomic analysis, saliva proved to be an adequate biofluid for chronic periodontitis signature as well [27, 28].

Saliva can be collected without exogenous stimulation (resting saliva) or by stimulation, which is influenced by olfactory stimulus, exposure to light, diurnal and seasonal factors [29]. Beside these factors, important differences have been reported in analyte levels, relating to collection and sample processing. It is therefore important to use appropriate methods in order to standardize the collection of saliva, use of specific inhibitors or additives after collection and storage [29].

Recently, the salivary metabolome was established based on a protein precipitation and UHPLC–IM–MS technique, before and after exercise-induced physiological stress [30]. Recently, a metabolic fingerprinting in saliva of smokers and nonsmokers was validated by GC-TOF-MS technique [31] identifying 13 altered metabolites in smokers, such as tyramine, adenosine, and glucose-6-phosphate, linked to detrimental perturbations of smoking.

The aim of this study was to apply two rapid and reliable screening protocols, to find metabolic biomarkers in resting and stimulated saliva of healthy subjects. The UPLC-QTOF-ESI(+)MS technique was applied in parallel with a direct injection mass spectrometry (DIMS) to fingerprint the methanolic saliva extracts and their stability, after 1 year storage. The principal component analysis (PCA) was applied to evaluate qualitative and quantitative modifications of saliva biomarkers, considering comparatively the statistical buckets of resting *versus* stimulated saliva.

RESULTS AND DISCUSSION

The “omics” technology applied to saliva proved to reflect a complete set of small metabolites using liquid- or gas- chromatography coupled with mass spectrometry (LC-MS, GC-MS) [32] to be used in translational and clinical applications, including personalized dentistry and medicine [32-36].

Most attention was given to separation and identification protocols to find appropriate saliva biomarkers of diagnosis and disease monitoring [13] the low concentrations (picograms to nanograms) of different metabolites in saliva need sensitive equipments and protocols [37-39].

1. Comparative UPLC-QTOF-ESI(+)MS fingerprints based on Base Peak chromatograms, registered before and after storage

Fig. 1 shows comparatively the Base Peak Chromatograms (BPC) of a resting saliva extract compared with a stimulated saliva based on the UPLC-QTOF ESI(+)MS analysis. Around 32 minor and major peaks with high similarity

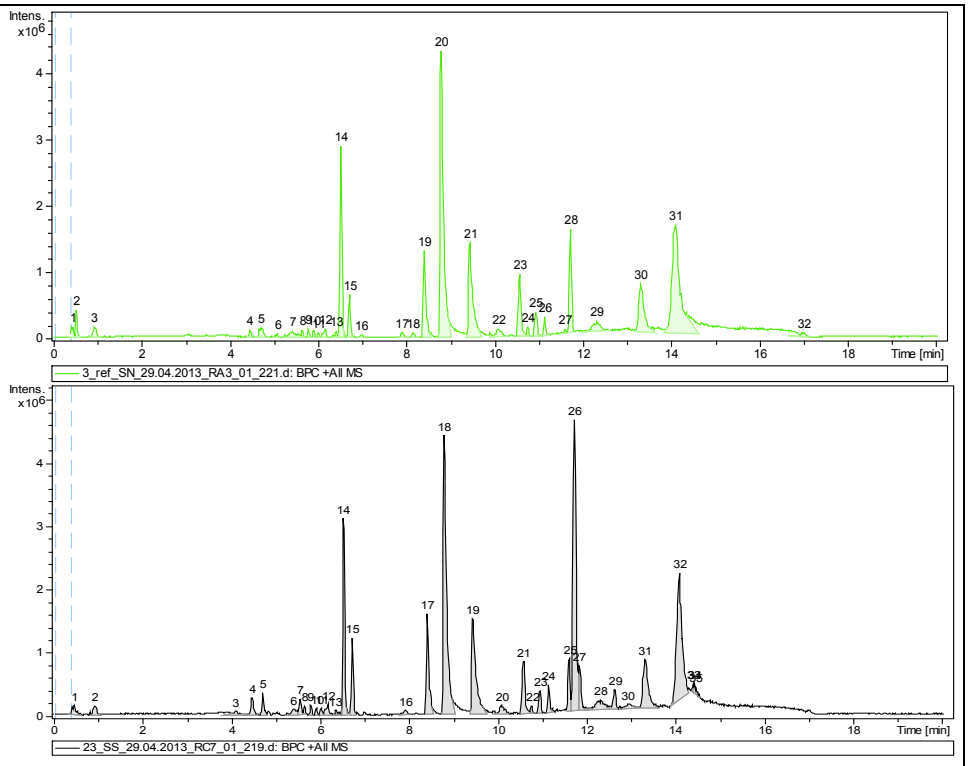

Fig. 1. The profiles of Base Peak Chromatograms (BPC) of a resting sample (up) compared with a stimulated sample (down), based on the UPLC-QTOF ESI(+) MS analysis

were identified, as follows: at t_R= 2-6.5 min includes minor peaks followed by 2 major peaks from 6.5-6.7 min (nr. 14 and 15). Between t_R= 8.4-9.4 3 major peaks (nr. 19, 20, 21) followed by many peaks between 10.6 to 13.0 min. (21, 26 for resting saliva and 23, 28 for stimulated saliva. Only 2 peaks (30, 31) were observed after 13.3 min. The identification of these peaks is presented in Table 1.

There were identified peaks corresponding to Phospholipids (LysoPC (18:2) (11), (LysoPE 16:0) (15), Oleoyl glycine (14), Oleamide (28 /NSS; 27/ SS), N-Lauroyl-glycine (21/ NSS; 19/ SS), Heptanoylcarnitine (19/NSS; 17/SS), peptides like asparaginyl-proline or prolyl-asparagine (/ 20/NSS;18/SS) and tyrosyl-arginine or argynil-tyrosine (31/NSS; 32/ SS), as well Hydroxyglutaric acid (23/NSS; 21/SS) and 12-ketodeoxycholic acid (30/NSS; 31/SS).

When the BPC from the same patient saliva (NSS *vs* SS) were compared, quantitative but not qualitative differences, were seen (data not shown). In SS samples there were noticed increased peak areas for 26 and 27 (Fig.1; table 1) corresponding to oleamide and monoacylglicerol C16:0, respectively.

Table 1. Tentative identification of peaks identified in resting (NSS) and simulated saliva (SS) by LC-QTOF-ESI(+) MS analysis, in the t_R range from 6 to 14.1 min. Minor peaks (mP) and bolded marks for main peaks are represented.

t_R (min.)	NSS		SS		Tentative identifications by Mass Spectrometry
	Peak nr.	m/z [M+1]	Peak nr.	m/z [M+1]	
6.00	**11**	**520.3555**	**11**	**520.3573**	**Lyso PC 18:2(9Z,12Z)**
6.40	mP	742.4768	mP	742.4768	PE(18:2(9Z,12Z)/18:1(9Z); PE (18:0/18:3(9Z,12Z,15Z)) PC(15:0/18:3(6Z,9Z,12Z))
6.50	14	340.2771	14	340.2777	Oleoyl glycine
6.70	15	453.3653	15	453.3662	Lyso PE 16:0(9Z,12Z)
7.00	mP	171.1573	mP	171.1575	2-Undecen-1-ol
7.90	mP	213.1565	mP	213.1566	Methyl (E)-2-dodecenoate
8.22	mP	227.1363	mP	227.1367	Ammonium citrate, dibasic
8.42	19	274.2883	17	274.2886	Heptanoylcarnitine
8.81	20	230.2607	18	230.2607	Asparaginyl-Proline or Prolyl-Asparagine
9.43	21	258.2929	19	258.2933	N-Lauroylglycine
10.11	mP	286.325	mP	286.3252	Myristoylglycine
10.60	23	149.0308	21	149.0309	L-2-Hydroxyglutaric acid
11.28	mP	331.268	**27**	**331.268**	**MG (16:0)**
11.71	28	282.294	**26**	**282.2946**	**Oleamide**
11.95	mP	353.269	**mP**	**353.269**	**MG (18:3)**
13.30	30	391.3047	31	391.3048	12-Keto-deoxycholic acid
14.10	31	338.3598	32	338.3599	Tyrosyl-Arginine or Arginyl-Tyrosine

Fig. 2 presents the BPC fingerprints of SS samples after 1 year storage of saliva at -20ºC (A1), or stored as methanol extract (A2).

These modifications shows a general decrease of components in the stored methanol extract comparing with saliva storage, up to 2 times, dependent on the individual molecules, as it is visible in Fig.2 and Fig.3.

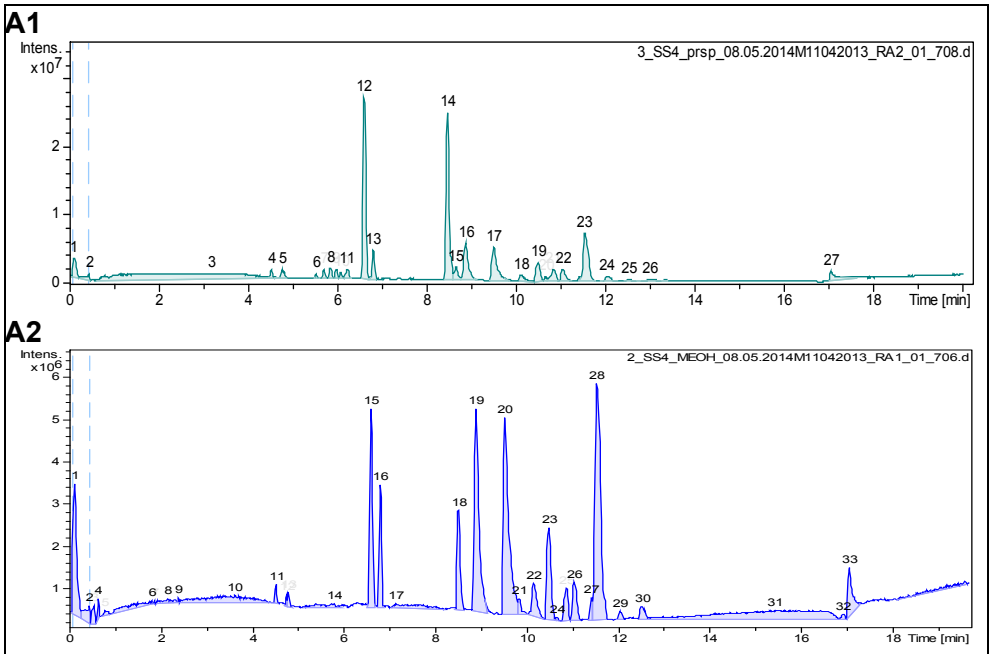

Fig. 2. Comparative BPC fingerprints of SS samples analyzed after 1 year storage at -20ºC, as saliva (up, A1) or as methanolic saliva extract (down, A2).

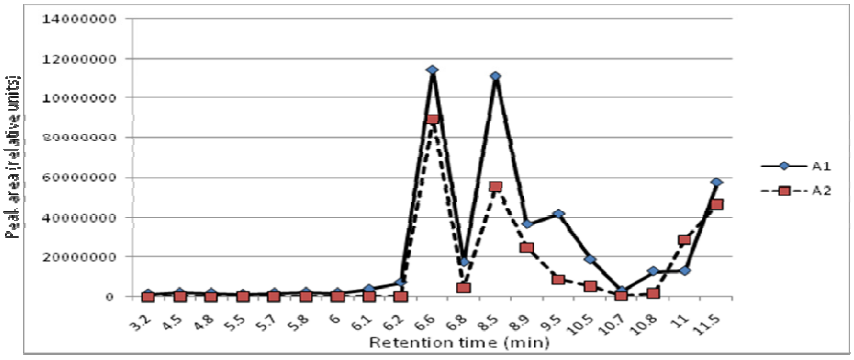

Fig. 3. Comparative evolution of the BPC peak areas for samples A1 *vs* A2, which show differences after 1 year storage of methanolic extract (A2) *vs* saliva storage (A1).

2. Principal Component Analysis (PCA) to discriminate differences between saliva groups

According to Fig. 4, PCA scores and loadings considering the 5 principal components from each group, with a statistical relevance of > 70%, were determined for the t_R range from 10 to 12.3 min.

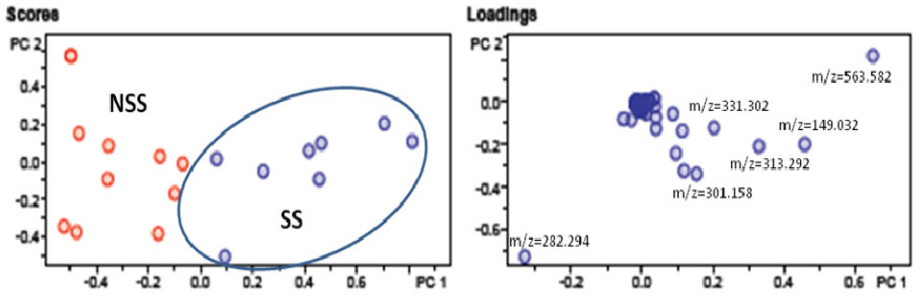

Fig. 4. PCA analysis of NSS *vs* SS samples, considering 5 principal components separated at t_R from 10 to 12.3 min. The scores (left) shows the good clustering of NSS samples and SS samples. The loadings (right) shows the m/z values of responsible molecules for discriminations between NSS and SS samples.

There were identified differences between NSS (circles) and SS (triangle) samples, at t_R=11.28 and 11.95 min, corresponding to MG(16:0/0:0/0:0) and MG(0:0/18:3/0:0), respectively. The scores (left) shows the good discriminations between the NSS and SS groups. The loadings (right) shows the m/z values of the molecules responsible for the discriminations, the most significant differences being noticed for m/z values of 563.582, 282.294, 301.158, 313.292, 331.302 and 149.032 (right).

3. Evaluation and identification of saliva (NSS or SS) molecules by direct, shotgun DIMS analysis

Fig. 5 represents the comparative MS spectra of saliva samples, obtained by DIMS for m/z ranges from 100 to 1000 (upper left), 75-250 (upper right), 250-430 (lower left) and 350-555 (lower right).

The DIMS analysis can identify more molecules than UPLC-QTOF-ESI(+)MS, and m/z values higher than 391, up to 742.47 corresponding mainly to polar PA, PC and PE, SM, DG lipids. Meanwhile, one should consider that UPLC-QTOF-ESI(+)MS analysis was done on methanolic saliva extract, which contain more polar molecules having smaller m/z values, e.g. lyso derivatives of phospholipids, and monoglycerides (MG).

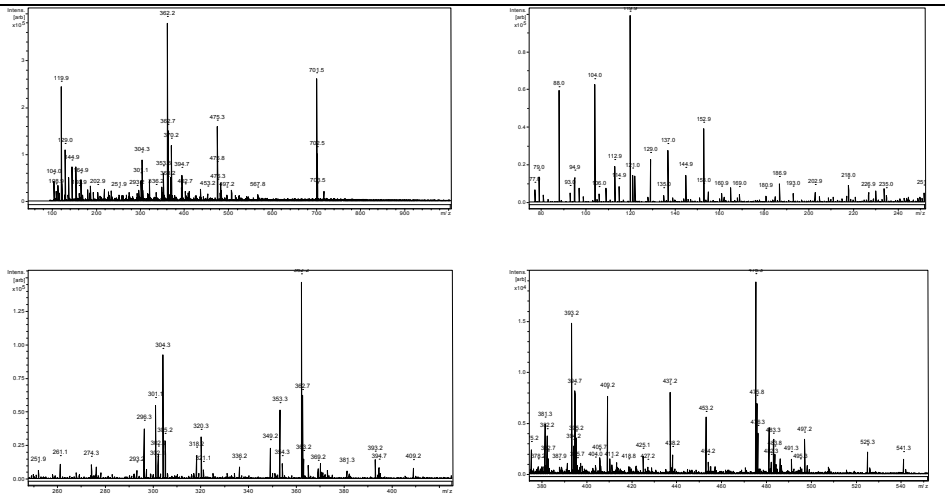

Fig. 5. Comparative mass spectra of saliva samples, obtained by DIMS for the whole range m/z = 100-1000 (upper left), and for different m/z ranges: 75-250 (upper right), 250-430 (lower left) and 350-555 (lower right).

Finally this metabolomics fingerprinting proved to provide rapid and accurate measurements of saliva, in agreement with other authors [40].

CONCLUSIONS

Considering the objectives and results of these experiments, using in parallel two advanced technologies (UPLC-QTOF-ESI(+)MS and shotgun DIMS analysis) we can conclude that metabolomic fingerprinting of resting *vs* stimulated saliva can be achieved fast and in a reliable manner, supporting the identification of main biomarkers, which can be confirmed the Human Metabolomic Databases.

The DIMS analysis allows a larger and more detailed identification of the most relevant small metabolites, offering a fast and reliable picture of the saliva samples, without preliminary separation. By both methods, quantitative, more than qualitative differences were noticed between samples.

According to our studies, saliva investigations have several advantages considering the simple and noninvasive collection, easily handled, low risk for hazardous results, easy to be stored and processed, with lower costs. The saliva analysis is simple, low cost and rapid, easy to storend reliable in time, keeping constant its composition. Such investigations can have good relevance for the utilization of saliva as a diagnostic fluid, for clinical application.

Future research will focus on the identification and validation of saliva biomarkers for systemic diseases, to change the perception that saliva is only useful for the diagnosis of oral diseases, but a mirror of the whole body health.

EXPERIMENTAL SECTION

Collection of saliva. Male and female healthy volunteers were randomly selected from patients of the Prevention Department of the University of Medicine and Pharmacy „Iuliu Hatieganu" in Cluj-Napoca (period April-May 2013) The study was approved by the university Ethics Committe, the inclusion criterion was the clinicaly healthy patient, mean age of 23.1, including 8 females and 4 males.

The sample collection was made in the morning, the subjects did not eat within 60 minutes prior to sample collection. For saliva recovery, alcohol, caffeine, and dairy products were not avoided.

The stimulated saliva (SS) was collected after chewing stimulation with parafin. The whole saliva was collected by drooling it into a vial, allowing to accumulate in the mouth and then expectorate it into a special cup used for saliva testing. A volume of 1 ml saliva was introduced in an Eppendorf vial containing 1 ml of Natrium azide solution 1%, in order to avoid microbial development. All saliva-azide samples were homogenized by a vortex mixer for 1 min. and stored at -20ºC before analysis.

Sample preparation. Aliquots of 1 ml saliva (NSS or SS) were mixed with 1 ml methanol (HPLC grade, Merck) and kept 15 min at -20ºC, for protein precipitation. After centrifugation at 10.000×g, for 10 min., the supernatant was filtered through nylon filters (0.25 µm) to cut-off molecules with molecular weight > 1000 Da. The methanolic extracts were kept at -20ºC before analysis. To check the stability and reproductibility of the samples and their fingerprints, the UPLC-QTOF-MS analysis was repeted 1 year after storage of raw saliva (A1) or methanolic extract (A2).

UPLC–QTOF(ESI+)MS analysis. Aliquots of 5 µl of NSS and SS methanolic extracts were subjected to chromatographic separation on a Thermo Scientific UPLC UltiMate 3000 system equipped with a quaternary pump delivery system Dionex UltiMate 3000 and autosampler. The separation was made with the Thermo Scientific Acclaim C18 column (3µm, 2.1x 250 mm) using a gradient elution program. The column temperature was set at 40°C. The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.5 $mL \cdot min^{-1}$. The elution program consisted on a linear gradient from 1% B to 15% B (0 - 3 min), 15% to 50% B (3-6 min), 50% to 95% B (6-9 min) and isocratic 95% B for more 6 min,

returning to initial conditions at min. 15, then kept isocratic for more 5 min with 1% B. The molecules released succesively from UPLC column were introduced automatically into the mass spectrometer using electrospray injection.

The mass spectrometry was performed on a Bruker Daltonics MaXis Impact Q-TOF operating in positive ion mode (ESI+). The mass range was set between 50-1000 m/z. The nebulizing gas pressure was set at 0.4 bar, the drying gas flow at 4 L/min, the drying gas temperature at 200 ºC. Before each chromatographic run, a calibration solution of sodium formate was injected.

Shotgun Direct Infusion Mass Spectrometry (DIMS). The saliva were directly infused into the same mass spectrometer using a KD Scientific syringe pump (Holliston, USA). The flow was set at 3 μl / min, infusion time of 2 min per sample. The results were expressed as MS peaks intensities (x 10^5) at different m/z ranges.

Statistical Analysis. The control of the UPLC-QTOF-MS instrument was done using TofControl 3.2 and Data Analysis 4.1 (Bruker Daltonics). The biostatistic processing used Profile Analysis 5.1 (Bruker Daltonics) which provided Principal Component Analysis (PCA).

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