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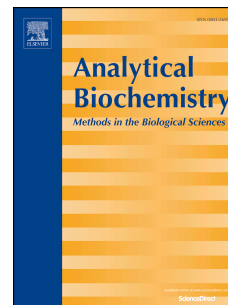
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Author contributions:

## **Sample optimization for saliva $^1\text{H-NMR}$ metabolic profiling**

Eleonora Quartieri - data acquisition

Emanuela Casali - design, data interpretation, drafted and critically revised the manuscript

Elena Ferrari – design, data interpretation, drafted and critically revised the manuscript

Benedetta Ghezzi - sample collection

Mariana Gallo - data interpretation, statistical analysis, drafted and critically revised the manuscript.

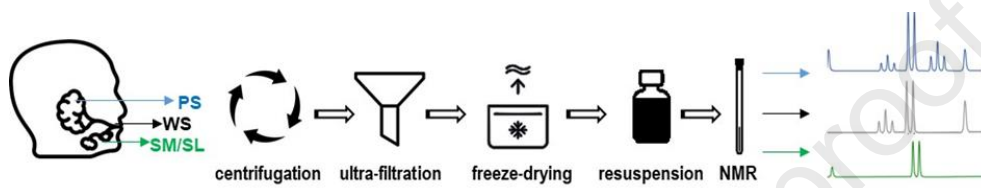
Alberto Spisni – critically revised the manuscript.

Marco Meleti – critically revised the manuscript.

Thelma A. Pertinhez - Conceptualization, data interpretation, and critically revised the manuscript.

**Sample optimization for saliva  $^1\text{H}$ -NMR metabolomics**

Eleonora Quartieri, Emanuela Casali, Elena Ferrari\*, Benedetta Ghezzi, Mariana Gallo, Alberto Spisni, Marco Meleti, Thelma A. Pertinhez

**Graphical abstract**

## Sample optimization for saliva <sup>1</sup>H-NMR metabolic profiling

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### Abstract

Nuclear Magnetic Resonance (NMR) based metabolomic analysis of whole saliva has provided potential diagnostic biomarkers for numerous human diseases contributing to a better understanding of their mechanisms. However, a comprehensive interpretation of the significance of metabolites in whole, parotid, and submandibular/sublingual saliva subtypes is still missing. Precision and reproducibility of sample preparation is an essential step. Here, we present a simple and efficient protocol for saliva <sup>1</sup>H-NMR metabolic profiling. This procedure has been specifically designed and optimized for the identification and quantification of low concentration metabolites (as low as 1.1 μM) and is suitable for all the saliva subtypes.

### Keywords:

<sup>1</sup>H-NMR metabolic profile, whole saliva, parotid saliva, submandibular/sublingual saliva, sample preparation

Hydrogen Nuclear Magnetic Resonance (<sup>1</sup>H-NMR) has been extensively used for metabolomic studies on biofluids. Initially, metabolomic research on biofluids focused primarily on blood and urine. In recent years, saliva has been the subject of an increasing number of metabolomic investigations. In fact, even though saliva composition is complex and variable, it is sensitive to metabolic changes, and therefore has a significant potential as a diagnostic fluid [1-3]. Furthermore, saliva exhibits some advantages including simple, inexpensive, and non-invasive collection, ease of multiple sampling and is more respectful of privacy than urine. Those features are driving the search of salivary biomarkers of diseases and the development of saliva based diagnostic tests [6].

Saliva contains electrolytes, immunoglobulins, proteins, enzymes, nucleic acids, and metabolites [7-9]. It is known that protein concentration in salivary fluid is approximately 10 times lower than in serum (salivary protein concentration ranges from 0.72 to 2.45 mg/mL, according ref. [10]). However, an investigation regarding the total metabolites concentration in the different saliva subtypes is still missing.

The term "whole saliva" (WS) refers to a clear mixture of fluids produced by major and minor salivary glands as well as gingival crevicular fluid. Salivary glands contribute to WS in different percentages: 20% parotid, 73% submandibular and sublingual, and less than 10% is due to minor glands secretions [8]. Most of the current metabolomics studies deal with WS because of its easy sampling. Few studies are available on parotid saliva (PS), while submandibular and sublingual saliva (SM/SL) metabolomic investigations turn out to be virtually absent in the literature, likely due to the very low metabolites concentration. Notably, since gland contamination by exogenous and microbial molecules is less probable, most of metabolites in PS and SM/SL are host derived. Recognizing the high scientific and diagnostic impact of the salivary metabolomic research, the knowledge of an accurate qualitative and quantitative metabolic profile of each saliva subtype will be valuable for biochemical studies and effective biomarkers identification.

$^1\text{H-NMR}$  based metabolomics analysis of WS has provided reliable biomarkers for numerous diseases. Those studies include systemic conditions, such as head and neck cancer [11], dementia [12], glioblastoma [13], as well as oral states in paediatric oral health [14] and in chronic periodontitis [15]. However, the use of  $^1\text{H-NMR}$  for the identification of salivary gland metabolites as biomarkers requires the development of a reproducible sample preparation protocol specifically designed for the identification and quantification of low concentration metabolites. Bertram et al., without including practically any treatment to saliva, could identify only 16 metabolites in their samples [16]. Duarte and collaborators, using 1D and 2D NMR experiments, identified ca. 50 metabolites, after sample centrifugation. Nevertheless, the authors highlight the presence of broad signals of proteins that makes metabolites quantification difficult [17]. Finally, Gardner and co-workers have evaluated the effect of salivary sample centrifugation in combination with a freeze-thaw cycle, validating a successful protocol for metabolite quantification in WS [18]. In the present study, we improved that protocol with the introduction of ultra-filtration and freeze-drying, before the final sample dissolution, to deplete proteins and increase metabolites concentration. Here we detail this procedure, highlighting the quality improvement of  $^1\text{H-NMR}$  spectra and the progressive metabolites enrichment of the sample, two features that synergically contribute to enhance the detection of low concentration metabolites. Serum is a widely used biofluid for NMR metabolomics studies and serum sample preparation methods are well validated [19,20]. Therefore, we evaluated the metabolites content of the three saliva subtypes of 20 healthy donors and compared the results with those obtained for serum samples of the same subjects, used as a reference in terms of the NMR spectra quality and of the number of metabolites.

### ***Saliva preliminary treatment***

To illustrate the steps of the proposed saliva preparation method, we select SM/SL from a single subject, since it is the subtype with the lowest metabolites content.

Non-stimulated saliva were collected to avoid metabolite's dilution [21].

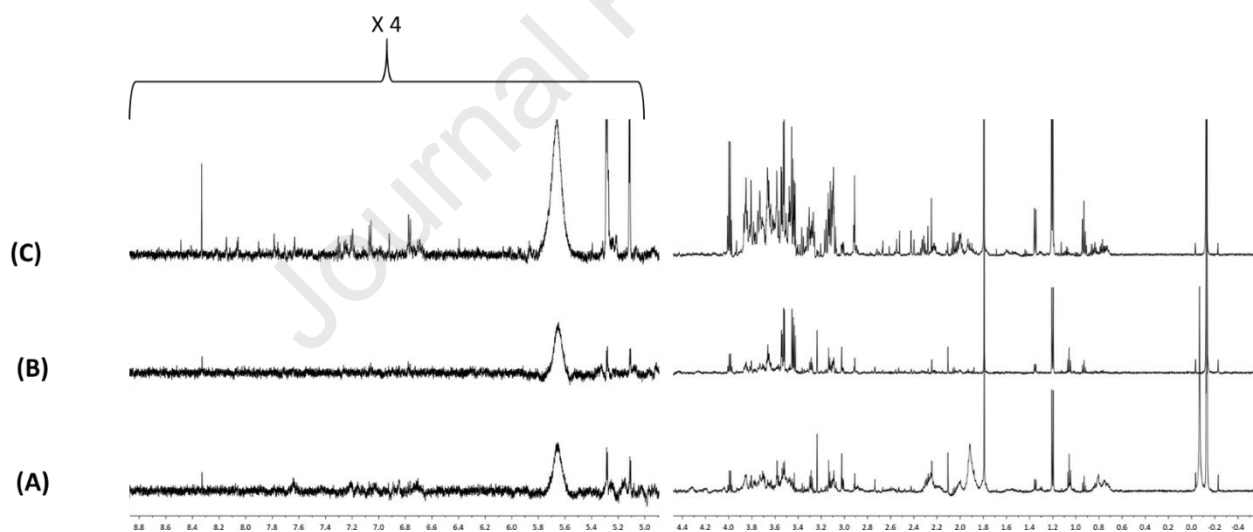
During collection, the salivary sample was gradually transferred to cryovials containing  $\text{NaN}_3$  (0.05% final concentration) and kept on ice. The final volume of saliva was 3.6 mL. According to most of the protocols

reported in the literature, we froze at - 80 °C the sample immediately after collection. For the analysis, the saliva sample was thawed at room temperature and centrifuged at 15000 x g for 10 min at 4 °C; supernatant was separated from the pellet and kept on ice until use. Centrifugation allows a substantial removal of cellular debris, bacteria, mucins, and high molecular weight aggregates [18].

#### **Saliva supernatant sample produces low quality NMR spectrum**

For NMR analysis, saliva supernatant sample was prepared as follows: 10  $\mu$ L of 1 M potassium phosphate buffer (pH 7.4) and 15  $\mu$ L of 1% 3-trimethylsilyl propionic acid (TSP) in D<sub>2</sub>O were added to 575  $\mu$ L of the salivary supernatant, reaching a final volume of 600  $\mu$ L (1.45 mM TSP, 2.5% D<sub>2</sub>O in 16 mM phosphate buffer). TSP was used as a reference for chemical shift (0.00 ppm) and quantitative internal standard.

One dimensional <sup>1</sup>H-NMR spectra were acquired at 25 °C with a JEOL 600 MHz ECZ600R spectrometer using the first increment of the 1DNOESY pulse sequence, 128 scans, sweep window of 20 ppm, 128 k points and relaxation delay of 5 seconds [22]. The spectra were processed and analysed with Chenomx NMR suite 8.3 software (Chenomx Inc., Edmonton, Alberta, Canada), zero-filling to 256 k points, and line broadening 0.5 Hz. The saliva supernatant spectrum turned out to be noisy and contained broad signals attributable to residual salivary proteins (Figure 1A). The broadened signals severely interfere with metabolites identification and quantification. In fact, in this sample, we were able to quantify only 13 metabolites.



**Figure 1.** <sup>1</sup>H-NMR spectra obtained with (A) supernatant, (B) ultra-filtered, and (C) ultra-filtered and lyophilised SM/SL saliva samples originated from a single subject. The aromatic region (left panel) is enhanced by a factor of 4.

#### **Improving NMR spectra quality: the ultra-filtration step**

To eliminate the interfering proteins, we introduced an ultra-filtration step, previously suggested by Dame et al. [23]. Thus, the saliva supernatant was ultra-filtered using Amicon Ultra-4 Centrifugal filters (3000 MWCO, Merck Millipore) at 4000 x g at 10 °C. Filters were previously washed several times with water at 4000 x g to remove glycerol from the filter membrane. In Figure 1B is-reported the <sup>1</sup>H-NMR spectrum of the ultra-filtered SM/SL sample prepared as described above. The disappearance the proteins broad signals

improves the quality of the NMR spectrum enabling the identification and quantification of 22 metabolites, ranging from 6.0  $\mu\text{M}$  to 2750.0  $\mu\text{M}$  (vs. 13 metabolites identified in the supernatant saliva sample). For example, we could identify and measure the concentration of glutamate and citrate (42.6  $\mu\text{M}$  and 25.2  $\mu\text{M}$ ), which were undetectable in the supernatant sample because of the masking effect of broad protein signals.

#### ***Increasing sensitivity: the freeze-drying step***

Low NMR sensitivity represents a limit when used to analyse diluted solutions such as saliva, especially SM/SL. To reduce this limitation, we added a freeze-drying step immediately after ultra-filtration. Briefly, 3.0 mL of ultra-filtered SM/SL were frozen and lyophilised, and finally dissolved in 600  $\mu\text{L}$  of 50 mM phosphate buffer (pH 7.4), containing 1.45 mM TSP and 2.5%  $\text{D}_2\text{O}$ . The result is a five-fold increase in metabolites concentration. Indeed, the spectrum in Figure 1C now allows the quantification of 47 metabolites, with corresponding concentrations as low as 1.1  $\mu\text{M}$  in the original SM/SL. Overall, this step increases the metabolites identification by a factor of two with respect to the ultra-filtered saliva and enables the quantification of metabolites undetectable in the ultra-filtered sample, such as the aromatic amino acids phenylalanine and tyrosine. It must be acknowledged that volatile metabolites (such as methanol, ethanol, and acetone) are inevitably lost during the lyophilisation process. Besides this limit, the whole procedure turned out to be conservative and did not introduce additional peaks nor produced side reactions within metabolites.

Furthermore, as reported in Supplementary Material Section S1, the calculated precision and accuracy demonstrate that the protocol is reproducible and accurate, while the LOQ and LOD values evidence its sensitivity.

#### ***Evaluation of spectra quality and total metabolite content of the three salivary subtypes***

WS, PS, and SM/SL were collected separately from 20 healthy subjects (Supplementary Material Section S2), as previously described [24]. Blood samples from the same donors were also collected and prepared as reported in ref. [25].

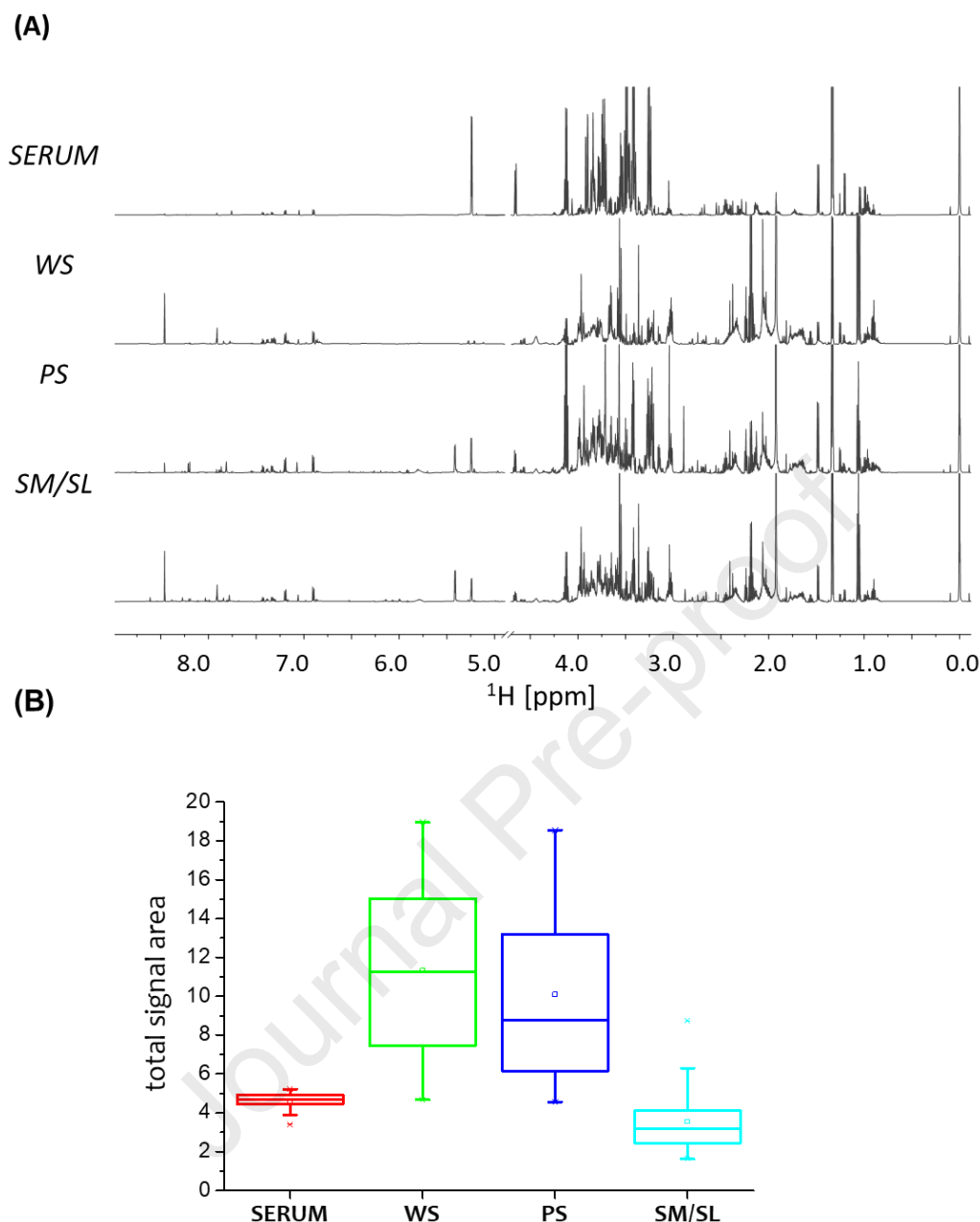
Following the complete protocol here presented, we prepared WS, PS, and SM/SL saliva samples for all the donors. Figure 2A illustrates the NMR spectra of the three saliva subtypes and serum from one of them. It can be appreciated that the quality of the saliva spectra is comparable to the one of serum. The analysis of the 20 subjects allowed the identification and quantification of  $58 \pm 2$ ,  $57 \pm 2$ , and  $48 \pm 2$  (mean  $\pm$  SD) metabolites in WS, PS, and SM/SL samples, respectively (Supplementary Material Section S3), while  $52 \pm 1$  metabolites were detected in the corresponding serum samples (data not shown).

To estimate the total metabolite content of saliva and to compare it with the corresponding serum, we used the spectral area of the whole NMR spectrum, which is related to the total observed metabolite concentrations. We used MestReNova 11.0 software (Mestrelab Research, Santiago de Compostela, Spain) to calculate the integral of each spectrum and normalised it by the signal area of the internal standard TSP. The box plots in Figure 2B show the signal area distributions obtained for each salivary subtype and for serum



of the 20 donors. WS and PS distributions are indicative of a similar total metabolite content and a comparable large variability. The metabolite content of the SM/SL sample is less variable, though markedly lower than WS and PS, with an estimated content ratio of 1:1:0.3 (WS:PS:SM/SL). It is worth noting that the use of our protocol leads to a total metabolite content of WS and PS higher than the one of serum. Even if in SM/SL samples the metabolite total content remains lower than serum, our protocol allows the identification of a significant number of metabolites ( $48 \pm 2$ ).

In this study, we present a method for salivary samples preparation suitable for  $^1\text{H-NMR}$  metabolic investigations. The introduction of an ultra-filtration and a freeze-drying step overcomes the limited metabolites content of all saliva subtypes. The application of this protocol allowed the characterisation of the metabolic profile of the three saliva subtypes (WS, PS, and, for the first time, SM/SL), for a group of healthy subjects [24]. In this way, we intend to contribute to the definition of a standardised protocol that may enable the comparison of data obtained from saliva samples prepared in different laboratories. We expect that the possibility to reliably identify and quantify low concentration metabolites will favour the discover of biomarkers with diagnostic value.



**Figure 2.** (A)  $^1\text{H}$ -NMR spectra of serum, WS, PS, and SM/SL samples obtained from the same subject using the complete proposed protocol. (B) Total signal area distribution of the  $^1\text{H}$ -NMR spectra ( $n=20$ ) of WS, PS, and SM/SL ultra-filtered and lyophilised samples. Serum signals area distribution is included as a reference.

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The present study was approved by the Ethical Committee of the “Area Vasta Emilia Nord” (AVEN) (protocol number: 808/2018/SPER/UNIPR METASAL3). Written consent was obtained from all volunteers who participated in this study, according to the Declaration of Helsinki.

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**Highlights:**

- Saliva is being the subject of an increasing number of metabolomic studies
- Definition of a standardized protocol for saliva metabolomics
- Quantification of low concentrated metabolites in saliva sub-types

Journal Pre-proof