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Evaluation of different extraction procedures for salivary peptide analysis

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ABSTRACT

Sample preparation is a critical step for all kind of biological fluids analysis with serious implications in data retrieved. In this sense, efforts have been made to standardize biofluids' management procedures for diagnosis and research purposes. However, no agreement exists regarding saliva preparation. Aiming the delineation of an ideal preparation procedure for salivary peptidome analysis, the commonly used extraction methods such as selective precipitation with organic solvents, acid and addition of chaotropic agents in combination with filtration, were evaluated in the present study. Data concerning protein/peptide content, Tricine-gel electrophoresis and MALDI-TOF/TOF identification suggest that centrifugation, a generally used sample cleanup step, should be critically reconsidered based on the hydrophobic peptides that can be loss by aggregation with high molecular weight (MW) components. Although no individual method *per se* resulted in the identification of all MS identified peptides, the extraction method with bicarbonate/acetonitrile (ACN) followed by filtration resulted in the higher number of identified peptides.

1. Introduction

Saliva plays multifunctional roles in the oral cavity, including lubrication, digestion and maintenance of oral health ensured by the multitude of organic and inorganic species including protein and peptides [1–5]. Similarly to other bodily fluids, saliva contains several protein species of low molecular weight (MW) that in this case comprise around 40–50% of the total secreted protein content [5], mostly expressed by genes located at chromosomes 4, 12 and 20 [6]. These gene products have been grouped into six structurally related [7,8] major classes namely, histatins, basic proline-rich proteins (bPRPs), acidic proline-rich proteins (aPRP), glycosylated proline-rich proteins (gPRPs), statherin and cystatins [9–20]. In previous studies [21,22], a large range of peptides deriving from major peptide classes were identified in whole saliva (WS) emphasizing the high efficiency of proteolytic enzymes activity in oral cavity. In fact, several enzymes were identified when histatin, more specifically histatin 1 and 5 were used as substrate in a zymography-mass spectrometry approach [23]. These enzymes can be derived either from the major salivary glands, parotid (P), submandibular (SM) and sublingual (SL) and minor glands distributed in the oral cavity, or from bacteria, cellular debris, crevicular fluid and serum. Hence, oral fluid proteolysis is an important factor to be taken into account in saliva protein structure–function analyses [24,25] and in diagnostic exploitations. Furthermore, peptidome analysis allowed the identification of disease-specific proteases aiming the discovery of specific biomarkers [26]. Indeed, previous peptidome characterization of saliva from patients with head-and-neck cancer and type 1 diabetes allowed the identification of disease-related alterations in peptide profile evidencing decreased levels of statherin and histatin 1, respectively [27,28]. By virtue of its noninvasiveness, availability and low-cost storage, saliva can be elected as one of most promising bodily fluids for clinical trials [4].

In a similar way as to other bodily fluids such as serum, plasma and urine, the pre-analytical phase of saliva preparation is affected by a wide range of variables, both exogenous (instrument settings, sample collection and storage methods, freezing conditions, and the number of freeze-thaw cycles) and endogenous (pH, salts and proteins/glycoprotein concentration, and bacterial interferences), that can markedly influence the results of peptide profiling. Recently, de Jong et al. [29] evaluated some of these variables concluding that salivary peptidome is relatively resistant to fasting versus fed status of donors and sample degradation due to room temperature incubation. However, the influence of sample centrifugation, one of the most critical parameters in sample preparation, was not evaluated. In addition, the establishment of a standard extraction procedure for peptidome analysis was not achieved so far for saliva, as was for serum [30] and urine [31]. Hence, the aim of the present study was to compare different peptide extraction procedures in order to establish a standard pretreatment protocol for salivary peptidome analysis.

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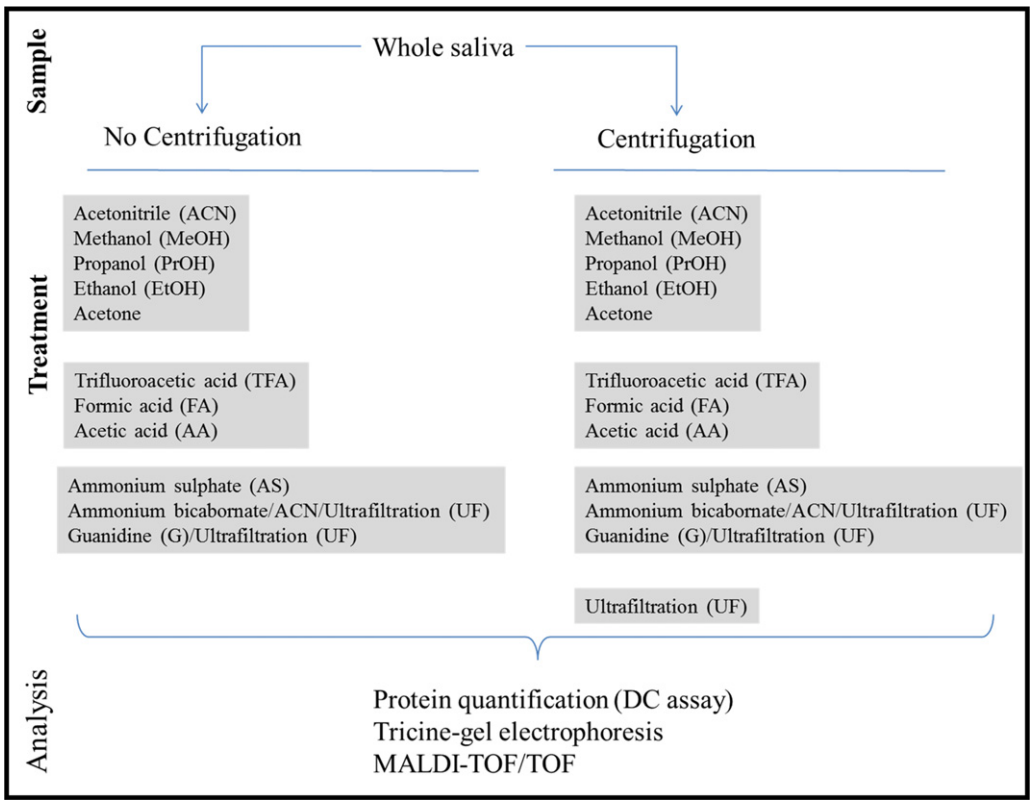

Fig. 1. Schematization of sample preparation methods evaluated.

2. Experimental

2.1. Chemical and reagents

All HPLC solvents (acetonitrile (ACN), 2-propanol, ethanol, acetone, methanol) were grade quality. General chemical reagents such as ammonium sulphate, ammonium bicarbonate, trifluoroacetic acid (TFA), formic acid (FA), acetic acid (AA), protease inhibitor cocktail, α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma (Karlsruhe, Germany).

2.2. Whole saliva collection

Three healthy subjects (3 male aged 22, 28 and 32 years old) showing no evidence of oral pathologies or inflammatory processes participated in this study, which was approved by the local ethics committee. The unstimulated whole saliva was collected at 10 a.m. by direct draining into a saliva collection tube from all subjects, who had refrained from eating and drinking for at least 2 h. To each mL of saliva it was added 10 μL of PMSF 0.1 M, 1 μL of pepstatin 1 mM and 20 μL of anti-protease cocktail (Sigma P2714) including aprotinin, E-64, EDTA, AEBSF and leupeptin. Saliva samples were pooled and divided into two sets: centrifuged and non-centrifuged saliva. Equal fractions of 300 μL were further processing as schematized in Fig. 1 and described in the next sections. Unless when is referred, all procedures were performed at room temperature (25 °C). The amount of peptide/protein material in all obtained extracts was determined by DC protein assay kit (Bio Rad, Hercules, CA, USA).

2.3. Organic solvent precipitation

Saliva samples were precipitated with 4 volumes of each organic solvent (acetonitrile, 2-propanol, acetone, ethanol and methanol) and incubated on ice for 15 min with sporadic vortexing. After incubation, each sample was centrifuged at 14,000 × *g* for 15 min. The supernatant and pellet were separated, and the supernatant was dried under vacuum. The dried supernatant was re-suspended in the HPLC mobile phase for protein quantification, Tricine-gel electrophoresis and peptide analysis by LC–MS/MS.

2.4. Ammonium sulfate precipitation

Saliva was precipitated using 4 volumes of saturated ammonium sulfate on ice for 15 min, followed by centrifugation at 15,000 × *g* for 15 min. The supernatant and pellet were collected into separate tubes. The supernatant was stored for protein quantification, Tricine-gel electrophoresis and peptide analysis by LC–MS/MS.

2.5. Acid precipitation

Saliva samples were acidified with trifluoroacetic acid (0.2%), formic acid (0.2%) and acetic acid (0.4%) in the proportion of 1:1, incubated in ice for 5 min and centrifuged at 12,000 × *g* for 30 min (4 °C). The supernatant and pellet were separated, and the supernatant was stored for protein quantification, Tricine-gel electrophoresis and peptide analysis by LC–MS/MS.

2.6. Ultrafiltration

Vivaspin® 500–50 KDa (Sartorius Stedim Biotech) ultrafiltration membranes were used to extract the salivary peptides from centrifuged saliva, from saliva incubated with guanidine and saliva incubated with ammonium bicarbonate/acetonitrile. In the first approach, saliva supernatant were directly loaded into the device and filtered under centrifugation at 10,000 × *g*. The filtrate was stored for protein quantification, Tricine-gel electrophoresis and LC–MS/MS analysis. In the second approach, a solution of guanidine (6 M) was added to the saliva samples in the ratio 1:3, incubated for

5 min and filtrate in similar conditions. In the third approach saliva was diluted to a final concentration of 50 mM ammonium bicarbonate (pH 8.3) with 20% (v/v) acetonitrile, incubated for 5 min and filtrated as described previously.

2.7. Fractionation and analysis of salivary peptides by LC–MS/MS.

Twenty microliters of each sample, corresponding to 2 μg of protein were separated using an Ultimate 3000 (Dionex) using a capillary column (Pepmap100 C18; 3 μm particle size; 0.75 μm internal diameter, 15 cm in length). A gradient of solvent A, (water/acetonitrile/trifluoroacetic acid (98:2:0.05, v/v/v)) to solvent B (water/acetonitrile/trifluoroacetic acid (10:90:0.045, v/v/v)) was used. The separation was performed using a linear gradient (5–55% B for 30 min, 55–80% B for 10 min and 70–5% A for 5 min) with a flow rate of 0.3 μL/min. The eluted peptides were applied directly on a MALDI plate in 15 s fractions using an automatic fraction collector Probot (Dionex, Amsterdam). The MALDI spots were analyzed using an ABI 4800 MALDI TOF/TOF analyzer operated with 4000 Series Explorer software. The MS acquisition was in positive ion reflector mode with 800 laser shots per spectrum performed. The 16 strongest precursors per spot were chosen for MS/MS, and the MALDI spot was interrogated until at least 12 peaks in the MS/MS, spectra achieved a signal/noise (S/N) > 40. The resulting MS/MS spectra were analyzed using which uses internal Mascot software (v.2.1.0.4, Matrix Science Ltd, U.K.) for protein/peptide identification based on peptide mass fingerprints and MS/MS data. Searches were performed against the SwissProt protein database (March 2009, 428650 entries) for *Homo sapiens*. A MS tolerance of 30 ppm was found for precursor ions and 0.3 Da for fragment ions. Confidence levels upper to 99% were used as positive protein identification criteria. In order to estimate the false discovery rate (FDR) a reverse decoy database was created for all SwissProt resulting in 5% of FDR (false positive peptides/(false positive peptides + total peptides)) × 100. Unique peptides retrieved from FDR search were considered for analysis.

2.8. Gel electrophoresis

Each extract was analyzed using Tricine-PAGE according to Schägger [32]. Briefly, equal amounts based on protein estimation were lyophilized, dissolved in PAGE sample buffer (50 mM Tris–HCl, pH 6.8, containing 50 mM DTT, 0.5% SDS and 10% glycerol) and incubated at 45 °C for 30 min. Peptide/protein separation was carried at 100 V for 2 h. After electrophoresis, the gels were incubated in fixing solution 50% methanol, 10% acetic acid, 100 mM ammonium acetate) and stained with a solution of 0.025% Coomassie dye in 10% acetic acid. Once stained, the gels were destained in the same solution without the Coomassie Blue and then scanned using the Molecular Imager Gel Doc XR+ System (Bio-Rad) and analyzed with QuantityOne software version 4.6.3 (Bio-Rad, Hercules, CA).

2.9. Statistical data analysis

A CSV (comma separated values) dataset containing relevant information pertaining 1738 peptides was analyzed to extract meaningful information. An in-house developed C# program with LINQ (language integrated query) (Microsoft Visual Studio 2010)© was used for data-mining the dataset. The output of the program has given several statistics which were them used by R language scripts [33] to produce box plots and heat maps, in order to facilitate the dataset analysis. The recovered statistics were: (1) the peptide counts as a function of extraction procedure (Fig. 3), represented by a false color surface and dendrograms for both peptides and treatments in order to analyze their similarity/dissimilarity, (2) total peptide count as a function of the extraction procedure (Figs. 3 and 4) distribution of molecular weight and gravy as a function of the extraction procedure (Figs. 5 and 6).

3. Results and discussion

While in the case of salivary protein analysis there are few works addressing the evaluation of variables affecting saliva sample handling and treatment [34–37], in the case of peptidome analysis this number is even lower [29,38,39]. So, this study was addressed to compare the peptide extraction efficacy from salivary samples between the most common strategies used nowadays for peptidome analysis such as ultrafiltration, acid and organic solvent precipitation techniques. One of the first approaches generally performed in saliva preparation is centrifugation in order to cleanup bacteria and cell debris. However, with this approach large aggregates are pelleted and discharged and so namely the peptides that are strongly bound to glycoprotein/carrier proteins are loss. Indeed, the presence in saliva of protein complexes comprising the high molecular glycoproteins MUC5B and MUC7, and other salivary proteins and peptides, such as amylase, statherin and PRPs is widely recognized [40], which is a challenge in sample handling. Thus, in the present study we evaluate not only the effect of centrifugation (typically the first saliva sample preparation step) in the peptide extraction ratio but also different extraction procedures. The influence of organic solvents, acids and chaotropic solutions in combination with ultrafiltration in the peptides extraction yield was evaluated. Organic solvents such as acetonitrile have been widely used in peptides extraction from serum since in its presence proteins with molecular weight above 20 KDa tend to aggregate and precipitate while peptides remain soluble. Furthermore, ACN is miscible in several buffers making ideal its use for proteomics studies at a peptide level. [41]. Other approach largely used for protein precipitation involves the addition of acids such as FA, AA and TFA. For example the acid precipitation with TFA has been adopted by Castagnola and co workers [42] as a standard procedure for salivary peptide extraction. Finally, ultrafiltration has been considered one of the most suitable approaches for peptide isolation from minute amount samples aiming subsequent HPLC analysis [43].

In the present study, we evaluate the protein recovery for each strategy tested using a commercial method (DC protein assay). Although more suitable for protein than peptide quantitation, this method allow the establishing of reproducibility considering the contribution of protein >10 KDa. Starting with the same volume of saliva (300 μL), the extracted protein amount obtained was in the range of 0.03–0.75 μg/μL (Table 1). Comparing the protein recoveries for all methods tested based on DC assay (Table 1), a higher amount of protein recovered was noticed when acid was added to the salivary sample before centrifugation. Acetonitrile, guanidine and ammonium sulphate yielded the lower amount of protein recovered. It should be pointed that in general, higher amount of recovered protein was obtained from non-centrifuged saliva in all tested methods, being more pronounced when ethanol and acetone were used. However, sample acidification with TFA or sample treatment with acetonitrile combined with ultrafiltration resulted in similar recovered protein amounts from centrifuged and non-centrifuged saliva. Overall, these data emphasize the potential protein loss associated to saliva centrifugation.

The distinct extraction methods were also compared by peptide analysis assessed by Tricine–SDS-PAGE (Fig. 2). With this approach, all extracts were analyzed in terms of optical density (OD) over a wide range of molecular weights (3.5–80 KDa). From the comparison of extraction procedures involving organic solvents we noticed a higher depletion of high molecular components when acetonitrile (lane 1 and 2) and acetone (lane 9 and 10) were used, in opposition

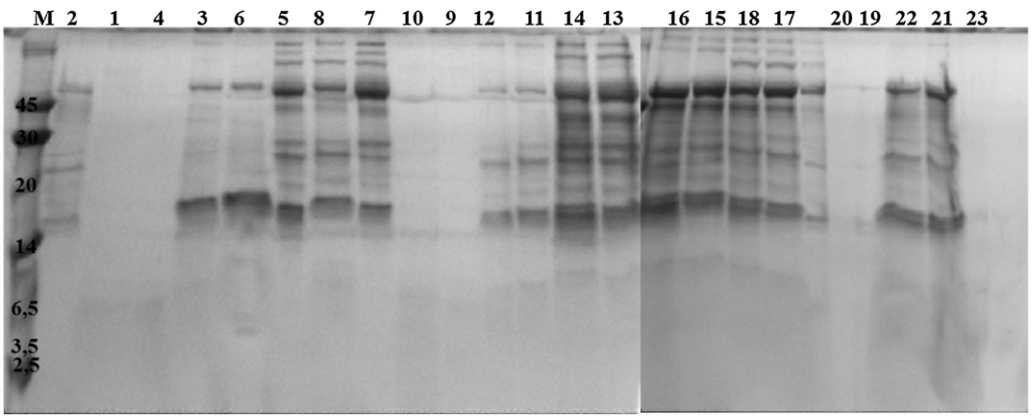

Fig. 2. Representative Tricine-gel of samples obtained from the extraction methods tested (numbers refers to sample number in Table 1). *M* refers to the low-range rainbow molecular weight markers (molecular weight presented in KDa).

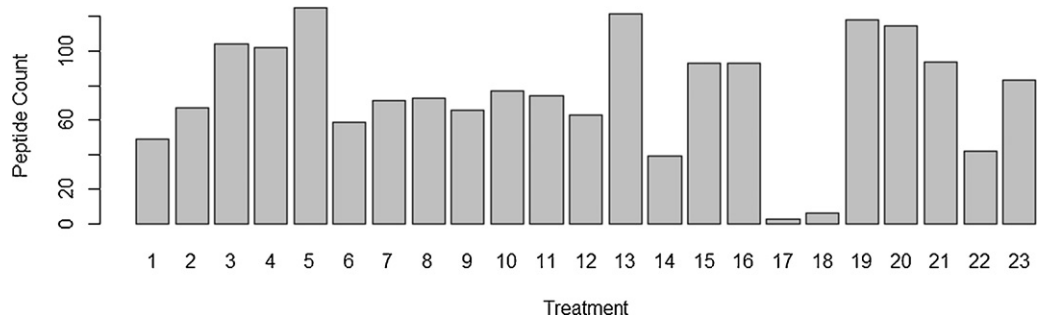

Fig. 3. Number of peptides identified by LC–MALDI-TOF/TOF in each extraction procedure (numbers refers to sample number in Table 1).

to ethanol (lane 5 and 6). With the exception of ACN treatment, more protein bands and with higher OD intensity were observed in centrifuged saliva. Regarding acid extraction, TFA (lane 9 and 10) yielded the higher amount of high molecular protein precipitated in opposition to AA (lane 15 and 16). Although significant protein depletion was observed in TFA method, some species remained soluble as can be depicted in Tricine-gel where bands with molecular weight above 10 KDa are observed. Data from Tricine-gel also suggest that filtration by itself or in tandem with ACN (lane 19, 20 and 23) is the most effective approach in depleting high molecular components. Although no peptides were detected by Tricine electrophoresis followed by Coomassie Blue staining in filtrated samples (Fig. 2), by MALDI mass spectrometry an average of 120 peptides were identified (supplemental data). The lower individual amount of each peptide might justify that no bands were detected in the Tricine-gel.

Chaoptropic agents such as guanidine have been added to samples like saliva to promote protein–protein complex disruption [40] allowing a better separation of lower molecular components. Although increments in the amount of salivary peptides were previously observed after guanidine addition to saliva [39], many other species with molecular weight in the range of 10–50 KDa seem to co-elute with guanidine and pass through the filter membrane, as observed in Fig. 2 (lane 21 and 22).

All samples obtained from each of the extraction methods tested were analyzed by nLC–MALDI-TOF/TOF. All obtained spectra for all extracts obtained from the different extraction methods tested were submitted to the same procedure in terms of noise and background subtract (material and methods) in order to assign all the ion species presenting a S/N > 15, which are common or unique to all extracts. Retention time was also included in this analysis. From this procedure, an inclusion list with all the species was submitted for MS/MS analysis relying in the identification of a total of 2497 peptides. Of these, 1738 peptide sequences belonging to the categorized salivary peptides classes and 372 unique peptides were identified (supplemental data). Most of these unique peptides are clustered in histatin 1, aPRP, bPRP2 and SMR3B.

Table 1
Total protein content and percentage of total protein extracted for each extraction procedure evaluated (data is presented as medium ± standard deviation).

Sample	Treatment	Total protein (μg/μL)	(%) of total protein extracted
1	WS + ACN	85 ± 36	15 ± 2
2	WSC + ACN	67 ± 5	12 ± 1
3	WS + MeOH	111 ± 10	19 ± 1
4	WSC + MeOH	101 ± 7	18 ± 0
5	WS + Propanol	165 ± 18	29 ± 3
6	WSC + Propanol	129 ± 26	22 ± 3
7	WS + EtOH	171 ± 31	30 ± 5
8	WSC + EtOH	84 ± 5	15 ± 1
9	WS + Acetone	112 ± 19	20 ± 3
10	WSC + Acetone	84 ± 16	15 ± 7
11	WS + 0.2%TFA	224 ± 20	39 ± 3
12	WSC + 0.2% TFA	221 ± 21	39 ± 3
13	WS + 0.25% Hac	195 ± 31	34 ± 5
14	WSC + 0.25%Hac	178 ± 14	31 ± 2
15	WS + 0.2% FA	213 ± 21	37 ± 4
16	WSC + 0.2% FA	182 ± 2	32 ± 7
17	WS + AS	26 ± 5	5 ± 6
18	WSC + AS	27 ± 3	5 ± 4
19	WS + 80% ABC + 20% ACN + UF	106 ± 3	18 ± 4
20	WSC + 80% ABC + 20% ACN + UF	111 ± 6	19 ± 5
21	WS + G	10 ± 1	2 ± 13
22	WSC + G	13 ± 5	2 ± 16
23	WSC + UF	188 ± 7	33 ± 2

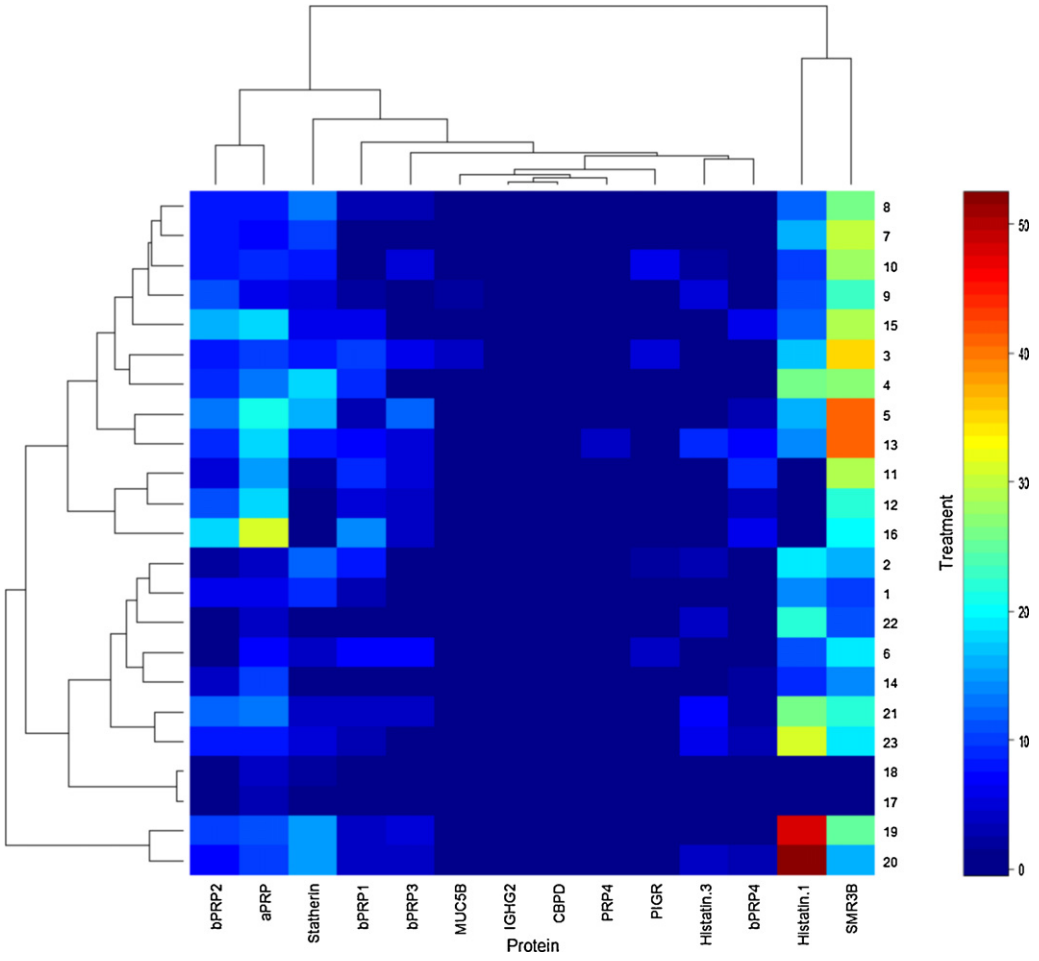

Fig. 4. Heatmap with dendrogram of identified peptides as function of protein class. In the right side is presented the color scale (from blue to red) based on the number of identified peptides. In *X*-axis are presented all the protein classes analyzed and in the *Y*-axis the treatments evaluated, which number has correspondence in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

With the exception of ACN, EtOH and acetone treatments, an increment of the number of peptides was observable when the extraction was performed before centrifugation, in opposition to the observed in Tricine-gel (Fig. 2). The combination of organic solvent extraction with bicarbonate solution followed by ultrafiltration (treatment 22 and 23 in Fig. 3) yielded the best results. As can be seen in Fig. 4, two main clusters of extraction procedures were noticed regarding the peptide number identified by MS/MS, grouping ACN with procedures involving ultrafiltration in one cluster and the remaining treatments in the other cluster. The protein classes more affected by the extraction procedure were histatin 1, aPRP, bPRP2, statherin and SMR3B, being noticed increments in the number of identified peptides when no centrifugation was performed (Fig. 4). For instance, more histatin 1 peptides were observed in non-centrifuged saliva treated with EtOH (treatments 7 and 8). Protein cluster association presented in Fig. 4 also reinforce the idea previously reported about the interaction of histatin and PRP peptides with high molecular weight glycoproteins or other carrier proteins [40,44], which seem to be trapped and removed by centrifugation.

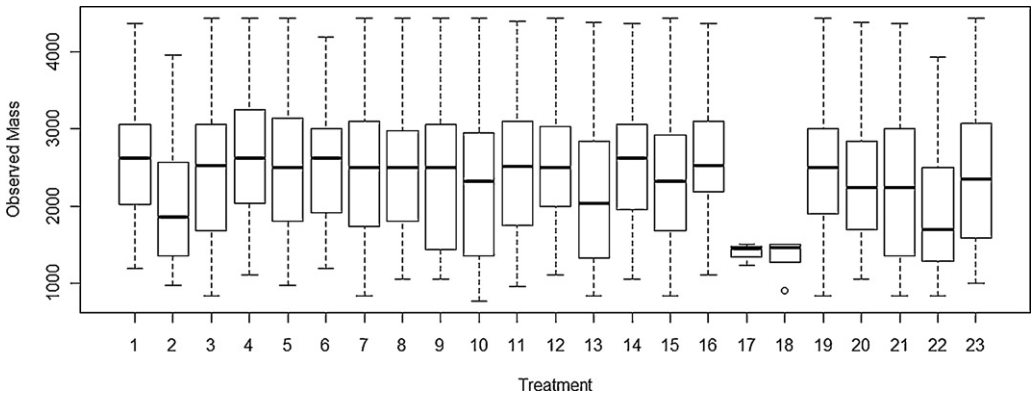

Fig. 5. Box plot of molecular weight distribution of identified peptides as a function of sample treatment (numbers in the *X*-axis correspond to sample number in Table 1).

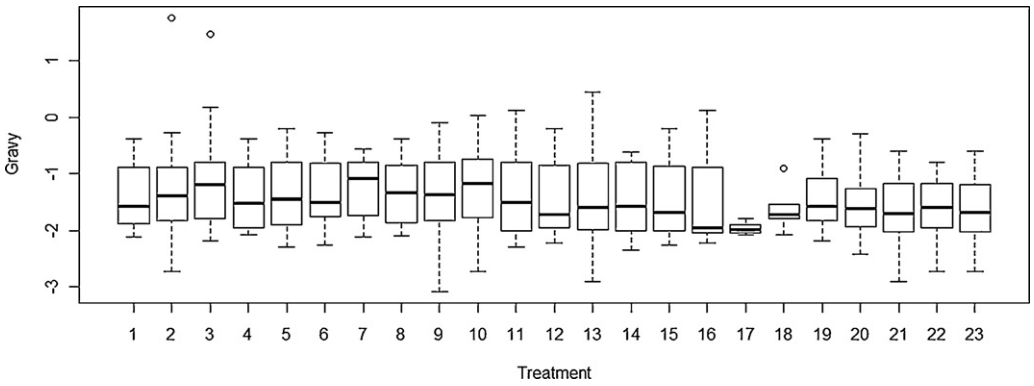

Fig. 6. Box plot of gravy score distribution as a function of sample treatment (numbers in the *X*-axis correspond to sample number in Table 1).

The identified peptides were also evaluated in terms of molecular weight (MW) and GRAVY (determined by the ProtParam sequence analysis tool available at http://us.expasy.org/tools/) score distribution. Regarding the molecular weight (Fig. 5), most of the identified peptides were comprised in the range 1000–3500 Da. Although no clear enrichment of peptides with similar molecular weight was associated to a specific treatment, precipitation with ACN as well as with AA and FA showed more notorious alterations between centrifuged and non-centrifuged saliva. In opposition to AA and FA, ACN precipitation allowed the extraction of peptides from non-centrifuged saliva with an average higher molecular weight. In general, positive (+) GRAVY scores are associated with peptides with overall hydrophobic character, whereas negative (−) GRAVY scores indicate hydrophilic character. As observed in Fig. 6, most of the identified peptides presented a GRAVY score ranging from −0.8 to −2.0 which seems normal for an aqueous fluid like saliva. Regarding the effect of the extraction method on GRAVY score of all identified peptides (Fig. 6), no evidences of a specific

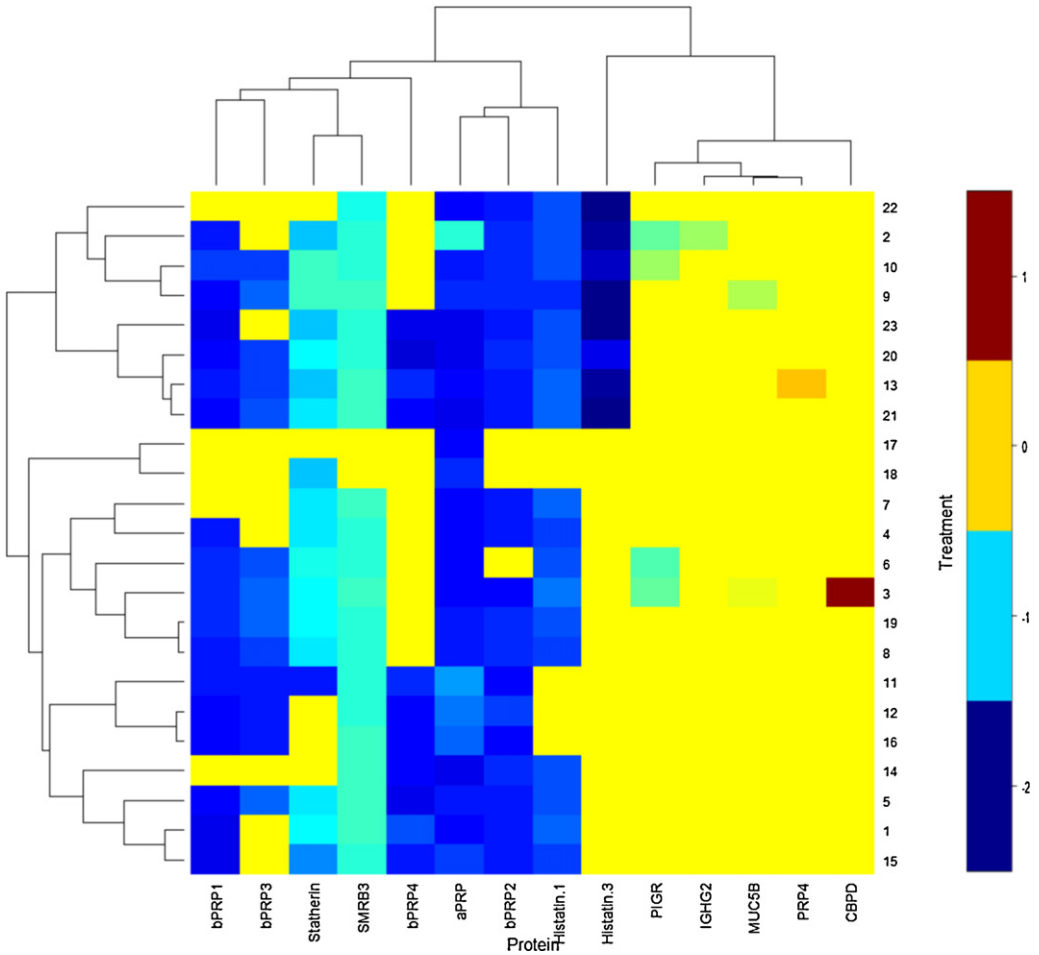

Fig. 7. Heatmap and dendrogram of gravy score as a function of sample treatment and protein class. In the right side is presented the color scale for gravy score (blue: from −2.5 to −1.5; light blue: from −1.5 to −0.5; yellow: from −0.5 to 0.5; brown: from 0.5 to 1.5). In *X*-axis are presented all the protein classes analyzed and in the *Y*-axis the treatments evaluated, which number has correspondence in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

enrichment was noticed. Nevertheless, extraction with HAc presented the larger window of GRAVY score (Fig. 6), which together with the high peptide count and broad range of molecular weight observed (Figs. 3 and 5), makes this procedure an interesting option for the analysis of salivary peptidome. However, the large relative content of proteins with high molecular weight observed in Tricine-gel (Fig. 2) makes this procedure less attractive for peptide profiling. When the association between GRAVY score and identified peptides *per* protein class was analyzed, the predominance of hydrophilic peptides from histatin 3 was notorious when extraction with guanidine, acetone or organic solvent with bicarbonate solution followed by ultrafiltration was performed. In addition, a predominance of hydrophilic peptides from aPRP, bPRP1, bPRP2, and histatin 1 classes was found in all sample treatments when no centrifugation was performed (Fig. 7). These data also suggest that most of the peptides that aggregate with glycoproteins present a hydrophobic character given by a positive GRAVY score. For instance, the peptide sequence ^{7}SVALLAFSS^{15} belonging to aPRP was one of those sequences identified in extracts from non-centrifuged saliva.

4. Conclusion

In conclusion, our data highlights the advantages and limitations of several straightforward methods used in saliva peptidome analysis, such as selective precipitation with organic solvent, acid and addition of chaotropic agents on combination with ultrafiltration. Albeit the high number of peptides identified in all tested methods, the most suitable one involves the use of a bicarbonate/acetonitrile solution followed by filtration. Centrifugation, a usual clearance step should be critically considered in saliva analysis since the hydrophobic peptides that tend to be aggregated with high molecular components salivary peptides can be lost, ultimately resulting in the incorrect identification of potential biomarkers in biomedical studies involving saliva peptidomics.

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The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.03.023.

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