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SELDI-TOF-MS of saliva: Methodology and pre-treatment effects☆

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Abstract

Interest in saliva as a diagnostic fluid for monitoring general health and for early diagnosis of disease has increased in the last few years. In particular, efforts have focused on the generation of protein maps of saliva using advanced proteomics technology. Surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is a novel high throughput and extremely sensitive proteomic approach that allows protein expression profiling of large sets of complex biological specimens. In this study, large scale profiling of salivary proteins and peptides, ranging from 2 to 100 kDa was demonstrated using SELDI-TOF-MS. Various methodological aspects and pre-analytical variables were analysed with respect to their effects on saliva SELDI-TOF-MS profiling. Results show that chip surface type and sample type (unstimulated versus stimulated) critically affect the amount and composition of detected salivary proteins. Factors that influenced normal saliva protein profiling were matrix composition, sample dilution and binding buffer properties. Delayed processing time experiments show certain new peptides evolving 3 h post-saliva donation, and quantitative analyses indicate relative intensity of other proteins and peptides changing with time. The addition of protease inhibitors partly counteracted the destabilization of certain protein/peptide mass spectra over time suggesting that some proteins in saliva are subject to digestion by intrinsic salivary proteases. SELDI-TOF-MS profiles also changed by varying storage time and storage temperature whereas centrifugation speed and freeze–thaw cycles had minimal impact. In conclusion, SELDI-TOF-MS offers a high throughput platform for saliva protein and peptide profiling, however, (pre-)analytical conditions must be taken into account for valid interpretation of the acquired data.

Keywords: Salivary proteome; Specimen sampling and handling; Surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry

1. Introduction

Human saliva is a complex biological fluid, which contains a large array of proteins and peptides that have important biological functions including the maintenance of oral health. In addition, blood concentrations of many identifiable components are reflected in saliva, since saliva contains an ultrafiltrate of the blood. The relatively easy non-invasive nature of collection and the relationship of saliva with plasma levels make saliva an attractive diagnostic tool. It is of special interest to identify "salivary biomarkers" to monitor general health and for the early diagnosis of diseases [1,2].

In whole saliva, the major sources of proteins are the contralateral major (parotid, submandibular, sublingual) and minor (von Ebner) salivary glands but also blood, oral tissues and microrganisms can be contributors to the salivary proteome. The protein composition of whole saliva also depends on circadian rhythm, diet, age, gender and physiological status [3]. With respect to protein content, saliva is mainly constituted by glycoproteins (e.g., mucins, proline-rich glycoproteins), enzymes (e.g., α-amylase, carbonic anhydrase) and a wide range of peptides (cystatins, statherin, histatins, proline-rich proteins).

The biological functions of most of saliva proteins are still poorly understood, although protein components in saliva have been partially revealed by conventional biochemical strategies

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focused on individual molecules or specific group of salivary proteins. A promising new approach to study saliva is the global analysis of salivary proteins using proteomic techniques. Such exploration of the salivary proteome will not only improve our knowledge of oral physiology, but can also allow the identification of novel proteins and the examination of changes in protein levels under different physiological or pathologic conditions.

Nowadays, state-of-the art proteomic methods are applied to the analysis of salivary peptides/proteins [4]. Several investigators have used two-dimensional gel electrophoresis (2-DE) to separate the protein components followed by mass spectrometry to subsequently identify the peptides produced from in-gel digests of the proteins of interest (2D-MS) [5–8]. With this approach up to 19 salivary-specific proteins and 18 serum-derived proteins were identified in whole saliva. Although 2D-MS is a very powerful approach to protein separation, it has limitations when dealing with small molecular weight proteins, highly acidic or basic proteins, very hydrophobic proteins, or proteins in low-abundance. In addition, the technique requires relatively large amount of sample, is labour-intensive, and high gel-to-gel reproducibility is hard to achieve. An alternative approach is the combination of liquid chromatography (LC) as the separation step, with the mass spectrometer (LC–MS). Using this approach, Wilmarth et al. [9] was able to identify 102 proteins including most known salivary proteins and 67 serum proteins. This technique has the disadvantage that it is still labour-intensive, has limited throughput and provides little information about the relative abundance of the detected proteins. The whole salivary proteome was greatly extended by combining LC–MS and 2D-MS identifying up to 309 proteins [10]. Recently, surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) ProteinChip has been introduced [11]. The technology has been used successfully in the field of diagnostic proteomics to detect several disease-associated proteins and protein expression patterns in a variety of biological tissues and body fluids [12,13].

The objective of this study was to explore the methodology of SELDI-TOF-MS for profiling salivary proteins and peptides. We analysed various conditions (chip surface type, matrix composition, sample dilution, the use of chaotropics/detergents) necessary for generation of optimized and reproducible spectra. In addition, we have studied several pre-analytical steps that can alter the analysis of saliva including sample type, centrifugation speed, proteolytic degradation and stability during storage.

2. Experimental

2.1. Chemicals

All chemicals were obtained from Merck (Darmstadt, Germany), unless stated otherwise. ProteinChips, 3,5-dimethoxy-4-hydroxycinnamic acid, and calibrants were purchased from Ciphergen Biosystems (Ciphergen, Freemont, CA, USA). Ammonium acetate was purchased from ICN Biomedicals (Aurora, Ohio, USA).

2.2. Pre-analytical procedures

The influences of different pre-analytical procedures on saliva SELDI-TOF-MS profiling were examined as outlined in Fig. 1.

2.2.1. Sample collection

Whole saliva (oral fluid) was obtained from healthy non-smoking subjects in the morning at least 2 h after eating and after rinsing mouth with water. For non-stimulated sampling,

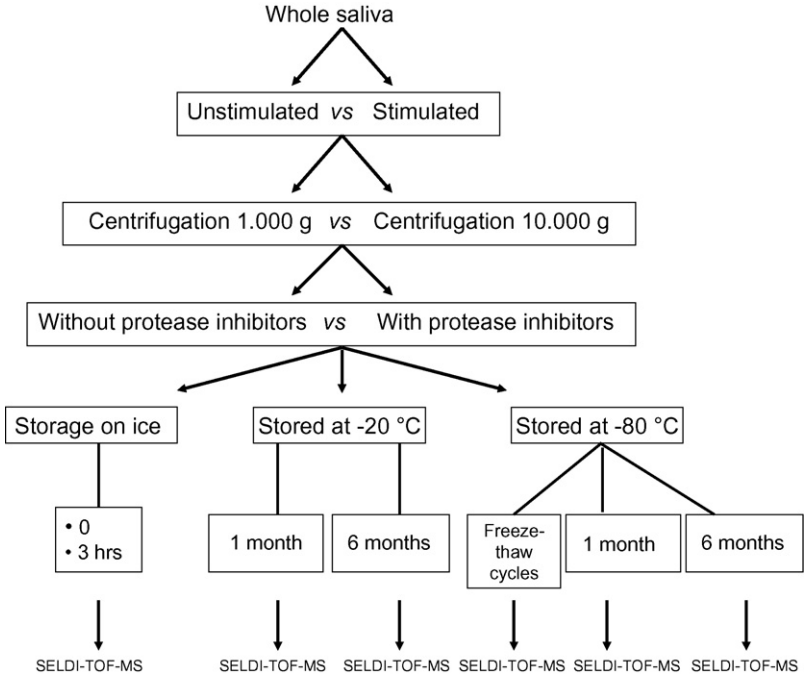

Fig. 1. Outline of the pre-treatments studied for their effect on saliva SELDI-TOF-MS profiling.

saliva was collected by means of a Salivette® collection device (Sarstedt B.V., Etten-Leur, The Netherlands). When saturated, the cotton wool roll was removed from the mouth, and placed into the Salivette tube. For a stimulated specimen, the subject first chewed for 2 min on a piece of parafilm before drooling into a universal container. Both collected unstimulated and stimulated saliva specimens were centrifuged for 5 min at 1000 × *g* or at 10,000 × *g* at 4 °C. Supernatants were sampled and used for further studies.

2.2.2. Storage time and temperature

The samples were divided in 250 μl aliquots and stored either on ice, at −20 °C or at −80 °C. From the aliquots stored on ice, samples were collected after 0 or 3 h. After collection, samples were immediately processed for SELDI-TOF-MS analysis. Frozen aliquots were thawed (a) immediately (for studying the effect of freeze–thaw cycles); (b) after 1 month or; (c) after 6 months, and further processed for SELDI-TOF-MS analysis.

2.2.3. Protease inhibition studies

To study proteolytic changes during storage, a protease inhibitor cocktail (PIC; P8340, Sigma, St. Louis, MI, USA) was added to saliva samples before further processing. The final inhibitor concentrations in the saliva samples were 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 800 μM aprotinin, leupeptin (20 μM), bestatin (40 μM), pepstatin A (15 μM) and E-64 (14 μM).

2.3. SELDI-TOF-MS analysis

The SELDI-TOF-MS technology (Ciphergen Biosystems, Fremont, CA, USA) consists of three major components: the ProteinChip array, a mass spectrometer and the data analysis software. The arrays are composed of different chromatographic surface types that unlike conventional HPLC or GC are designed to retain and not elute proteins of interest.

2.3.1. Chip surface types

Four types were used: a normal phase (NP20), strong anion exchanger (Q10), weak cation exchanger (CM10), reversed phase (H4) and immobilized metal affinity capture array with copper surface (IMAC-Cu). The NP20 chips were used to determine the effect of sample dilution on general SELDI-TOF-MS profiling. Spots were incubated with 100 μl of 1/10, 1/100 or 1/1000 dilutions of saliva in binding buffer (PBS, pH 7.4 and 0.1% Triton-X100). The binding/washing buffer for the anionic exchange arrays (Q10) contained 0.1% Triton X-100 with 0.1 M Tris–HCl (pH 8.0) or 0.1 M sodium acetate (pH 6.0) and that for the cationic exchange arrays (CM10) contained 0.1% Triton-X100 with 0.1 mM ammonium acetate (pH 4.5 or pH 6). H4 chips were pre-treated with 50% ACN and 400 mM NaCl in PBS, pH 7.4. Subsequently, chips were washed twice with binding/washing buffer (10% or 30% ACN, PBS pH 7.4, 250 mM NaCl) just before and after application of sample. IMAC-Cu chips were loaded with 50 μl 0.1 M copper sulphate by vigorous shaking at room temperature for 10 min. After a short wash with water the chip surface was neutralised using 150 μl sodium acetate buffer pH 4 followed by a short wash with water and pre-incubation with binding buffer (0.1 M Tris–HCl, pH 7.4) containing 0.1% Triton X-100 and 100 mM or 500 mM NaCl.

2.3.2. Sample dilution and matrix composition

Saliva samples were diluted 1–100 in binding buffer (total volume of 100 μl) and applied, at random and in duplicate, to the chip and incubated for 1 h shaking on a mixer, unless otherwise stated. Spots were then washed six times with 150 μl binding buffer for 10 min (three times with and three times without 0.1% Triton X-100). Before application of the matrix another short wash with water was performed with HPLC-grade water and air-dried for 10 min. Sinapinic acid (SPA; Ciphergen) was prepared in 50% acetonitrile/0.1% trifluoroacetic acid according to the manufacturer's instructions. Matrix was applied twice (0.8 μl each time and 1 min apart) and air-dried prior to reading on a ProteinChip Reader IIC instrument (Ciphergen Biosystems).

2.3.3. Instrument settings

The following settings were used: detector sensitivity 9; detector voltage 2900; positions 20–80 were read with an increment of 10 (resulting in seven different sampling positions); 50 laser shots were collected on each position (total shots collected and averaged: 350/sample); two warming shots were fired at each position, which were not included in the collection; lag time focus of 241 ns. Laser intensity was optimised for each chip type. Calibration was done with a mixture of proteins with masses ranging from 7 to 30 kDa. After baseline substraction, peak labelling was performed with CiphergenExpress Software (version 3.0) for peaks with a signal-to-noise (S/N) ratio of $\geq$5 in the *m/z* range from 1.5 to 30 kDa, and then normalized by total ion current.

2.3.4. Assay and sample variability

The intra-assay variability of the SELDI assay was evaluated by applying samples of the same saliva aliquot to eight different spots. In another experiment, the same sample was tested in three repeated SELDI assays (inter-assay variability). Forty peaks common to all spectra were selected and compared with regard to their peak intensity by calculating the coefficient of variation. To estimate the variability between samples from the same person (intra-individual variation) and samples from different persons (inter-individual variation) we collected unstimulated saliva from four individuals every 15 min for 2.5 h. Each saliva sample was snap-frozen in liquid nitrogen immediately after collection and stored at −80 °C before processing. After a few days, all samples were thawed and analysed by SELDI-TOF-MS using the IMAC-Cu chip.

3. Results

3.1. SELDI-TOF-MS analysis

3.1.1. Comparison of the different chip surface types

In order to comprehensively analyse the applicability (and quality) of salivary protein and peptide profiling by means of

SELDI-TOF-MS, we tested different ProteinChips with anionic (CM10), cationic (Q10), hydrophobic (H4) and metal (IMAC-Cu) treated surface.

On CM10 chips, best results were achieved with binding buffer of pH 6.0 showing 70 different peaks (39 in the range 2–10 kDa, 17 from 10 to 20 kDa and 14 from 20 to 100 kDa). Q10 chips displayed a total of 108 peaks of which most peaks were observed in the range below 10 kDa (80 from 2 to 10 kDa, 21 from 10 to 20 kDa and 7 from 20 to 100 kDa). Lowering the pH from 8 to 6 dramatically decreased the intensity of peaks in the whole spectrum but induced a few new peaks between 6 and 8 kDa and increased the intensity of some peaks around 15 kDa. Using H4 chips, the numbers of peaks detected overall were similar to those detected on Q10, both in terms of numbers of peaks detected (81 from 2 to 10 kDa, 15 from 10 to 20 kDa and 3 from 20 to 100 kDa) and their intensities. Increasing the percentage of ACN from 10 to 30% decreased both number of peaks and their intensities. Using IMAC-Cu chips, the highest total number (116) of peaks was detected (78 from 2 to 10 kDa, 19 from 10 to 20 kDa and 19 from 20 to 100 kDa), but the intensities of the peaks were considerably less than using the other chips. Increase of salt concentration to 500 mM had only very little effect on the spectra.

Representative protein profiles in the range of 2–20 kDa from the different chip types are shown in Fig. 2.

3.1.2. Sample dilution and matrix composition

To investigate the effect of sample dilution on SELDI-TOF-MS profiling, one sample of saliva was diluted 10, 100 or 1000 times in binding buffer and loaded onto NP20 chips. Optimal results for both number and intensities of peaks were achieved using a 1/100 dilution, which corresponds to the application of 1 μl of non-diluted saliva (Fig. 3A). More diluted saliva gave lower intensity of the peaks, whereas more concentrated saliva gave a higher intensity of some peaks but lower intensity of most of the peaks.

The impact of matrix composition on the saliva protein profile was determined by loading IMAC-Cu chips with different dilutions of SPA, i.e., original (100%), two times (50%) and four times (25%) diluted with the otherwise unchanged protocol stated above. SPA increased the number and intensity of detected spectra in a concentration-dependent manner for the majority of the bands from 2 to 10 kDa, as shown in Fig. 3B.

3.1.3. Assay and sample variability

Intra-assay variability ($n = 8$) was 18% (SD 5.6%, for 22 peaks) in the range of 2–10 kDa and 31% (SD 11%, for 18 peaks) in the range from 10 to 50 kDa. Inter-assay variability ($n = 3$) ranged from 3 to 25%.

Saliva samples from 4 individuals had 91 common peaks (61 from 2–10 kDa, 30 from 10–20 kDa, and 10 from 20–100 kDa) although their relative intensities varied among individuals. Differences in the range 2 to 10 kDa between individuals are shown in Fig. 4A. Variability between the samples of the same individual was much lower than among the 4 individuals as demonstrated by the clustering and ranking of all 8 samples from each individual (Fig. 4B).

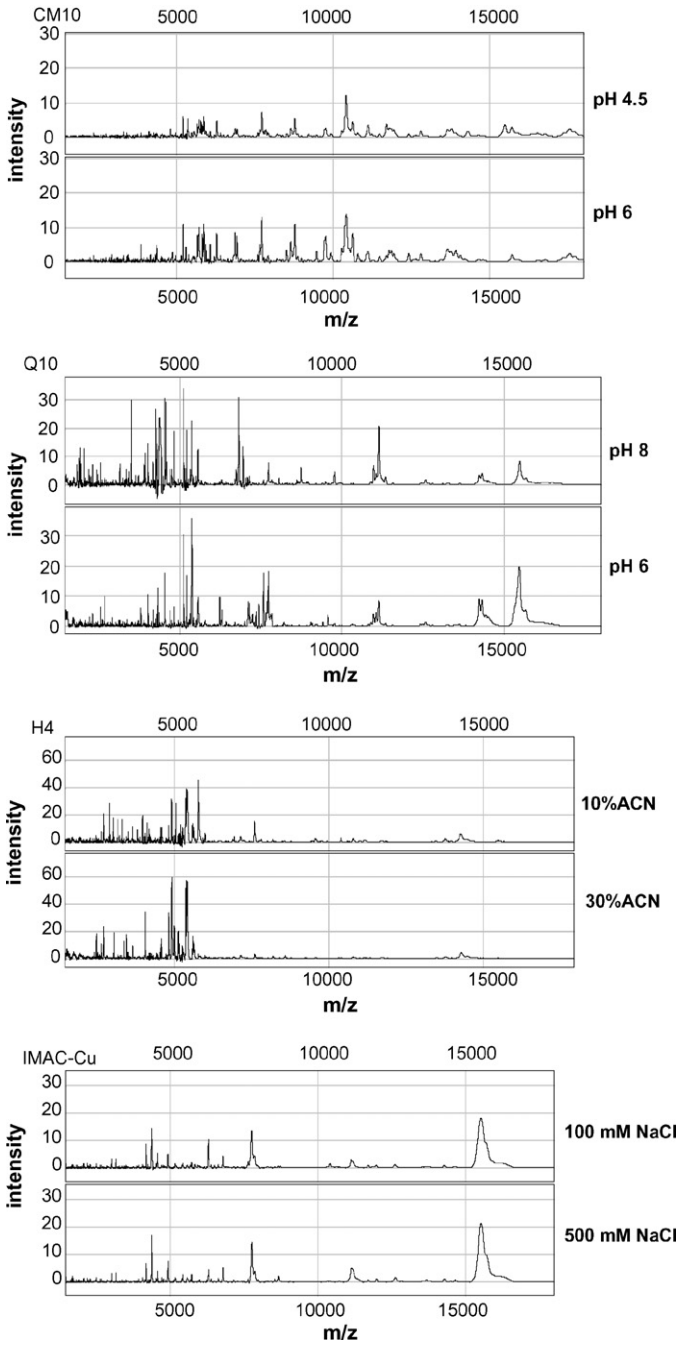

Fig. 2. Comparison of results obtained with the different chips. Results shown were representative profiles from the same saliva sample in range of 2000–10,000 *m/z* on CM10, Q10, H4 and IMAC-Cu chips.

3.2. Pre-analytical variables

The IMAC-Cu chip was used to investigate pre-analytical variables, i.e., centrifugation speed, storage temperature, exposure to freeze–thaw cycles and protease inhibition. We examined these pre-analytical aspects for both unstimulated and stimulated saliva. Results are summarized in Table 1.

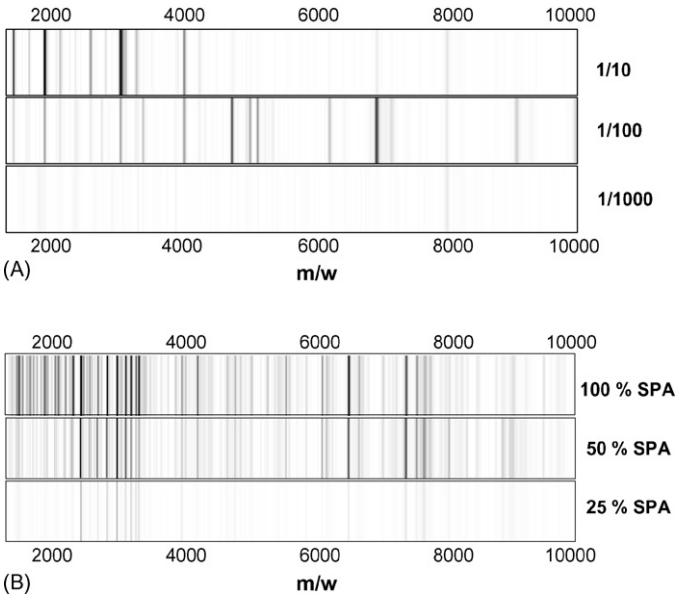

Fig. 3. Effect of sample dilution (A) and matrix composition (B) on profiles of NP20 chip bound proteins.

3.2.1. Effect of storage time and temperature, centrifugation speed and freeze–thawing

To examine the change with delayed sample processing, spectra of fresh saliva were compared with those from saliva kept for 3 h on ice (see Table 1 and Fig. 5A and B). With chilled storage, a few peaks (*m/z* 2620, 3155, 3370 and 15500) decreased and one peak increased (*m/z* 5310*)* in intensity irrespectively of saliva type (unstimulated or stimulated) or centrifugation speed ($1000 \times g$ or $10{,}000 \times g$). Compared to unstimulated, stimulated saliva showed two extra peaks: one of *m/z* 4920 and one of *m/z* 5310 that decreased and increased, respectively after storage for 3 h on ice. Centrifugation speed affected peak intensities but did not affect the changes observed with delayed sample processing, except for peaks of *m/z* 3484 and 5690. Protein pattern stability of both fresh non-stimulated and stimulated saliva was assessed by comparing aliquots of the same samples that had been stored at −20 °C and −80 °C for 1 month or 6 months (see Fig. 5C and D). For non-stimulated saliva, the intensity of two peaks (*m/z* 3009 and 3156) was markedly reduced after storage. After storage at −20 °C the intensity of several peaks was reduced whereas after storage at −80 °C similar results as obtained before freezing were found with some exceptions (decrease of *m/z* 4366 and increase of *m/z* 5689). Stimulated saliva gave similar results to unstimulated with the exception that storage at −20 °C gave a series of new peaks appeared in the range of 6–8 kDa.

The effect of freeze–thawing was studied by thawing 300 μl of saliva for 10 min and freezing it again in liquid nitrogen. Almost the same spectra could be generated after four freeze–thaw cycles (results not shown).

3.2.1.1. Protease inhibition studies. The use of a 1% (1/100 dilution in saliva) of cocktail of protease inhibitors did prevent the changes in some peaks but not all. Some of the peaks (between *m/z* 6100 and 6700) were derived from the inhibitors themselves as shown by control experiment in which only protease inhibitor cocktail diluted in water was applied to the chip (results not shown).

Table 1
Effect of pre-analytical processing (centrifugation speed, protease inhibition, time interval between sampling and SELDI analysis) on peak profiles generated from unstimulated and stimulated saliva

Pre-analytical variables									
Centrifugation speed		Intensities $1000 \times g$				Intensities $10{,}000 \times g$			
Protease inhibition		−PIC		+PIC		−PIC		+PIC	
Time (h) interval		$t=0$	$t=3$	$t=0$	$t=3$	$t=0$	$t=3$	$t=0$	$t=3$
	Peak, *m/z*								
Unstimulated	2,620	16	11	14	10	21	8	20	10
	3,155	28	21	23	24	35	17	31	21
	3,370	13	10	19	8	8	5	8	6
	3,480	21	16	17	15	5	6	9	5
	5,420	13	20	14	29	35	46	40	53
	5,690	16	15	11	9	14	13	11	10
	15,550	54	50	53	45	57	49	53	50
Stimulated	2,620	53	50	47	66	60	35	82	70
	3,155	74	67	67	73	80	64	101	80
	3,370	70	63	64	73	57	46	72	66
	3,480	61	58	58	60	53	45	64	61
	5,420	11	18	13	14	22	34	17	19
	5,690	25	28	23	14	19	16	16	19
	15,550	26	19	27	19	26	22	25	18

Results shown are the peaks (IMAC-Cu chip, range *m/z* 1.5–50 kDa) that were affected by the pre-analytical procedures. + and − PIC: with or without protease inhibitor cocktail.

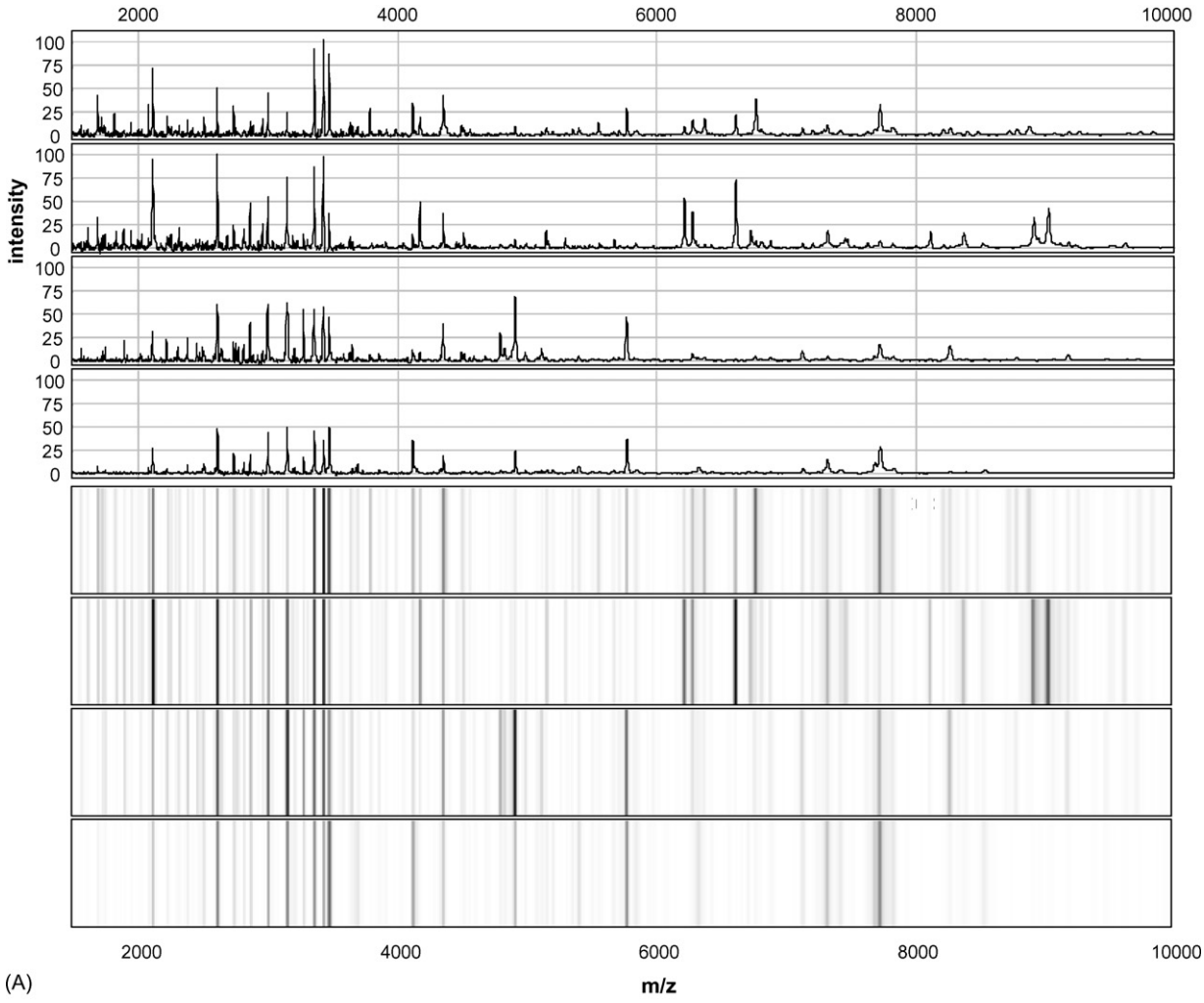

Fig. 4. (A) Examples of profiles showing the variability among four individuals on IMAC-Cu chips with comparable gel-views below the profiles. (B) Dendrogram showing how samples are clustered based on the similarities between the eight consecutive saliva donations (represented by numbers 1–8) of the four individuals (represented by a different grey scaled colours).

4. Discussion

During the last years there has been an increasing interest in exploring human saliva as diagnostic fluid. The interest to identify "salivary biomarkers" is based on several advantages of saliva versus other body fluids, for example, serum or urine. Saliva collection is a straightforward, safe, non-invasive and stress-free procedure that can be applied to a large group of sub-

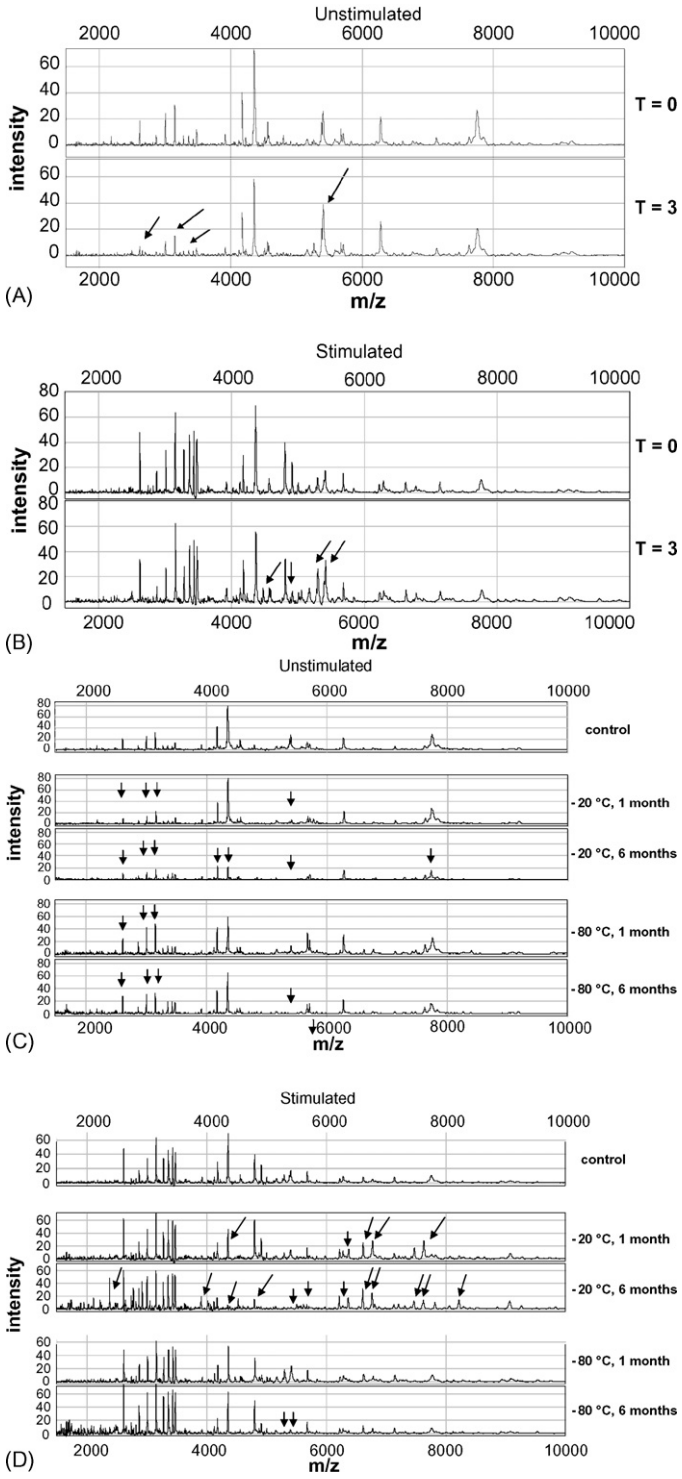

Fig. 5. Effect of delayed processing time (A and B) and storage temperature (C and D) of unstimulated (A and C) and stimulated (B and D) saliva on IMAC-Cu chips. Profiles are for the same saliva sample stored for 0 and 3 h on ice or stored at −20 °C or −80 °C for 1 month or 6 months. Some of the more marked changes are indicated (arrows).

jects. Moreover, sufficient quantities for analysis can be obtained in a continuous manner, which can be stored and transported at lower costs than those for blood or urine.

Despite being an important body fluid, interest in the large-scale determination of salivary proteins exist only very recently and was prompted by recent advancements in proteomic analysis. One advancement in proteomic analysis is the development of surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), a high throughput and sensitive technique. It has revealed a large number of new biomarkers for a wide variety of cancers. Its versatility has also been demonstrated by its application as a tool for mining a wide variety of biological tissues and fluids [13].

In the present study, we examined the usefulness of SELDI-TOF-MS, to detect distinctive proteomic patterns for whole saliva. Results demonstrate that SELDI-TOF-MS offers many advantages for protein profiling in saliva. First, only 1 μl of sample is needed for one analysis. Second, due to the simple chip preparation, many samples can be analysed quickly (a single operator can obtain spectra up to 150 different samples per day). Third, the washing step removes most of the salts, which otherwise interfere with mass spectrometric analysis. And fourth, the impact of different chromatographic chemistries can be analysed, which may allow one to find optimal purification conditions for a protein of interest in a short time with small amounts of sample.

Our results show that all tested types of chips are suitable for profiling salivary peptides but document differences in retention behaviour. Chips with anionic (CM10) treated surfaces were effective in binding salivary proteins in the range from 6000 to 16,000 *m/z*). The cationic (Q10) and hydrophobic (H4) chips were found to be most valuable for the characterisation of saliva peptides and proteins in the low molecular (from 1000 to 6000 *m/z*) range. The IMAC-Cu chip produced the largest number of peaks in a wide molecular range.

The SELDI-TOF-MS results show that most peaks are found in the low-molecular weight range. Studies using other techniques have shown that whole saliva is rich in peptides in the range of 1 to 6 kDa [14–16], many of which have important biological functions, e.g., histatins, cystatin, defensins with antimicrobial activities [2,17,18].

The peak analysis (*m/z*) shows a considerable overlap between the tested array surfaces. For example, in the range of 50 kDa, 18 peaks of the IMAC-Cu are also found on the CM10 chip. On the contrary, all four chips show peaks that are unique for its particular array surface. Analysis of this aspect could be used to determine the optimal (combination of) array type(s), in terms of the number and resolution of peaks, to be adopted for saliva profiling strategy. For our strategy to study the pre-treatment effects on saliva profiling, the IMAC-Cu chip was the optimal chip since this surface array produced the largest number of peaks in a wide molecular range.

The usefulness of saliva determinations by SELDI-TOF-MS depends on the application of pre-analytical procedures adequate for the assay in saliva. Once the saliva sample is collected, it is important that the handling procedures do not affect the nature and the content of the saliva. Most workers centrifuge

the sample at 1000 × *g*, while others recommend higher centrifugation speed to remove cellular debris, bacteria and high molecular weight mucoproteins [4]. Another point where opinions differ is the temperature to store samples if analysis is not performed immediately after sampling. In addition, slow freezing, long storage time [19,20], as well as freeze–thawing cycles [21] may cause problems since some protein-induced precipitation may occur.

We have examined the effect of these pre-analytical variables on saliva profiling by SELDI-TOF-MS. Table 1 shows which peaks are affected by the different pre-treatments. This might be important for future studies that may identify one or more of these peaks as biomarkers.

Centrifugation speed had no effect on the number but a small effect on intensities of the peaks. Delayed processing time induces both decreases and increases of peaks suggesting a proteolytic breakdown of some proteins. Human whole saliva contains a number of proteolytic enzymes from white blood cells, oral bacteria and the salivary glands [22]. Proteolytic processing is an important posttranslational modification of some salivary proteins, e.g., histatins, statherins [23], proline-rich proteins [24] and cystatins [25]. So, these proteolytic systems may be sufficiently active at low temperature to change the SELDI-TOF-MS spectra in time.

Addition of a protease inhibitor cocktail (PIC), which inhibits serine-, cysteine-, aspartic-, and metallo-proteases reduced but did not completely prevent the destabilisation of the protein/peptide mass spectra over time. Possibly, degradation of some proteins in saliva already occurs during the centrifugation step (5 min at 4 °C). Otherwise, the destabilization of these proteins may not be due to proteolytic breakdown, at least not by the enzymes sensitive for PIC.

According to our study, the best method to store saliva before testing them for SELDI-TOF-MS protein profiling is at −80 °C. Storage at this temperature provides the same spectra as fresh samples. Storage at −20 °C resulted in spectral changes, especially in stimulated saliva, which was not prevented by the PIC.

Saliva protein profiling by SELDI-TOF-MS has some drawbacks. First, high-molecular weight salivary proteins and glycoproteins such as mucins were not detected. However, more mass peaks in the high-molecular weight range may be obtained by applying more appropriate buffers or varying the stringency of the washing steps. For example, Streckfus et al. [26] showed that lowering of the pH in the binding buffer produced additional peaks in the 125–250 *m/z* molecular weight range, which might represent mucins. Second, competitive binding of high abundance non-informative proteins (like the mucins) on the chip surface may reduce the intensity of peptides/proteins of interest, particularly the low abundance ones. This can be overcome by pre-fractionation of the saliva sample or the use of membranes with a specific cut-off, as suggested by Rawel et al. [27]. However, pre-treatment using a membrane with a cut-off of 10 kDa had little or no effect on the spectra (results not shown), from 0 to 10 kDa indicating that the high-molecular weight proteins do not interfere with the profiles using IMAC-Cu chips.

Finally, SELDI-TOF-MS has low-mass resolution, which together with a lack of tandem MS capabilities, limits the identification of salivary proteins.

The proteins present in whole saliva are derived mainly from the salivary glands. Some proteins, however, originate from oral microorganisms, epithelial cells, crevicular fluid, leucocytes or dietary components. The relative contribution of sources other than the salivary glands to the composition of saliva will vary depending on e.g., the physiological status, method of stimulation, and the type of protein. We have compared SELDI spectra (results not shown) of whole saliva with that of the parotid gland and found that a large portion of the proteins in whole saliva was derived from the parotid gland.

An advantage of SELDI-TOF-MS over MALDI-TOF-MS is its ability to perform a miniaturized on-chip pre-fractionation of complex biological samples. There is still a great potential to obtain further protein specificity on these chips by applying more appropriate buffers or varying the stringency of the washing steps. Such experiments can provide useful starting points when designing purification schemes to isolate specific salivary proteins with classical chromatographical methods.

The value of SELDI-TOF-MS lies in the ability to obtain and compare spectra from a significant number of samples in a relatively short time with very little sample preparation or sophisticated chromatography. The analysis of a large number of samples will ideally reveal biomarkers, i.e., protein signals that are unique to, or differently expressed in, one sample set when compared with a different sample set.

First examples of the use of SELDI-TOF-MS for detecting salivary biomarkers have been published recently [26,28]. Ryu et al. [28] compared parotid saliva samples of subjects suffering Sjögren's syndrome (SS) with non-SS subjects and found ten biomarkers of which three have not been associated previously with SS. An explorative study comparing protein profiles in salivary samples from a group of breast cancer patients and known healthy controls is described by Streckfus et al. [26]. These authors identified 5 mass peaks that were increased more than two-fold in the cancer patients.

In conclusion, the obtained results show that SELDI-TOF-MS provides a simple and high throughput technique to rapidly identify a large number of differently expressed peptides/proteins in saliva samples, especially low mass proteins (<10 kDa) that have been difficult to assay effectively with other methods. We anticipate that this proteomic technique will be a valuable tool in projects ranging from identification of diagnostic biomarkers for a variety of diseases or description of whole body physiological changes as well as to the study of modification and biomolecular interactions of salivary peptides and proteins.

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