Protein Electrophoresis in Saliva Study

Elsa Lamy, Ana R. Costa, Célia M. Antunes,
Rui Vitorino and Francisco Amado

Additional information is available at the end of the chapter

1. Introduction

Saliva started for been less studied than other body fluids, but in the last years it has being receiving an increased attention. Until now, more than 2000 different proteins and peptides have been identified in whole saliva and salivary glandular secretions [1]. From these, more than 90% derive from the secretion of the three pairs of "major" salivary glands (parotid, submandibular and sublingual glands). The remaining 10% derives from "minor" salivary glands and from extra-glandular sources, namely gingival crevicular fluid, mucosal transudations, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, and food debris [2].

Saliva secretion is mainly under autonomic nervous system regulation. Sympathetic and parasympathetic stimulation have different effects on the flow rate and composition of saliva secreted. Whereas parasympathetic stimulation results in the production of a high volume of saliva with low protein concentration, stimulation of the sympathetic branch of the autonomic nervous system is responsible for the secretion of a small amount of saliva with increased protein concentration. Besides this distinctive characteristic, and inversely to what is observed for the majority of body systems, the effects of parasympathetic and sympathetic innervations are not antagonic but rather exert relatively independent effects in which the activity of one branch may synergistically augment the effect of the other [3,4]. Despite the thought of an exclusive nervous regulation, recent in vivo animal experiments indicate a short-term endocrine regulation of salivary glandular activities as well [5-9].

The primordial function of saliva is to aid in the moistening and preprocessing of food, aiding in deglutition. Besides this, other important functions exist for saliva, which can generally be grouped in digestive (and ingestive) and protection [10]. For digestive (and ingestive) purposes, saliva contains enzymes, including proteases, lipases and glycohydrolases, which initiate partial break-down of food components. Among these

enzymes, alpha-amylase is by far the enzyme present in higher amounts. There are also salivary proteins involved in food perception, such as: salivary PRPs (proline-rich proteins), which bind dietary polyphenols (mainly tannins) and are involved in astringency perception [11]; carbonic anhydrase VI, suggested to influence bitter taste sensitivity [12]; and alpha-amylase, involved in sweet taste sensitivity [13]. Additionally, recent studies suggest changes in saliva composition induced by taste [14-16], reinforcing the potential of saliva in food perception and ingestive choices. Concerning protection role, several different salivary proteins have been identified, namely mucins, acidic PRPs, statherins, among many others. For example, salivary proteins adsorbed to the enamel surface form the enamel pellicle, which helps to protect teeth [17]. It is also relevant to point the presence of proteins with more than one function, and the sharing of the same function by different families of proteins. This functional redundancy may help to ensure that a given function is always present under a broader range of physiological conditions [18, 19]. An in-depth analysis of saliva proteome, including the posttranslational modifications can therefore provide a valuable resource for saliva function research.

The high potential of saliva as a source of biomarkers was one of the main responsible for the great interest in this fluid. Several analytes are present in saliva in amounts that relates to blood, with the great advantage of being collected using simple and non-invasive methods. Proteomic techniques such as two-dimensional electrophoresis (2-DE), 2D-liquid chromatography/mass spectrometry (2D-LC/MS), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS), and surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF/MS), have been used in saliva studies. Based on those techniques, potential salivary biomarkers for diseases such as Sjögren syndrome [20], diabetes mellitus [21] and some different cancers [22] have been suggested. 2-DE has been one technique of choice for the global analysis and initial profiling of salivary proteins, being used as a first step for protein separation, followed by MS or tandem MS (MS/MS) [23].

Whereas the salivary proteins from humans have deserved substantial attention, both in terms of identification and characterization, as well as functional properties, animal saliva has been much less studied. However, interest for the latter is being increasing, due to the convenience on the use of animal models for diverse pathological and physiological conditions and due to the potential of this fluid for disease diagnostic and for understanding behavioral and physiological processes, important in animal production. Moreover, a multidisciplinary approach that integrates knowledge about salivary proteins in the animal kingdom (most important in mammals) and draws comparisons to possible functions in humans would be valuable.

The following sections will give an overview about the use of electrophoresis in saliva studies. Methodological issues and the major advantages and limitations for the use of this technique in human and animal saliva studies will be presented. We will finish the chapter by presenting alternatives to electrophoresis for the study of salivary proteome.

2. Applications of electrophoresis for saliva proteome characterization

Even before the advent of proteomics, electrophoresis was frequently used for salivary protein separation. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [24], PAGE in non-denaturing conditions [25], isoelectric focusing [26], two-dimensional electrophoresis (2-DE) [27], capillary electrophoresis (CE) [28,29] and free flow electrophoresis [30] have all been used in saliva studies, with different purposes.

One-dimensional gel electrophoresis under denaturing conditions has several advantages: virtually all proteins are soluble in SDS, allowing their separation; it covers a relatively high range of molecular masses (from 10000 to 300000 Da) and allows the possibility of extremely acidic and basic proteins to be visualized [31]. Moreover, SDS-PAGE has the advantage of having a low sensitivity to salt concentration. However, by separating proteins only based in their molecular masses, only limited information is obtained, since each of the bands present in the gels is frequently constituted by different proteins. Limitation in the number of proteins separated also occur using IEF, which separates proteins only based in their charges.

2-DE takes advantages of the two different properties of proteins (molecular masses and isoelectric points), allowing the separation and visualization, in a gel matrix, of a considerable number of different proteins. This technique, which originates from the work of O'Farrell and Klose in the 1970's [32,33] became very useful for the study of complex protein mixtures, such as saliva. Besides the very high separation capability of 2-DE, ensuring well-resolved protein maps with more than 2000 protein spots, this technique has also the advantage of mapping posttranslational modifications. Coupled to mass spectrometry, for protein identification, 2-DE has been considerably used in several different samples, including saliva [27,34]. Using this approach, the protein spots observed in 2-DE gels are subsequently digested using a protease (usually trypsin), with the resultant digest products analyzed by mass spectrometry. 2-DE can be used to compare expression levels of proteins in related samples, such as those from altered experimental conditions, allowing the response of classes of proteins to be determined. This approach has been suceffuly used in a number of saliva studies [e.g. 35].

Salivary peptides and proteins have been analyzed by a variety of CE approaches (reviewed in [36]. The term CE, although often used as shorthand for capillary zone electrophoresis, refers to a family of related techniques, all based on the performance of the separations in narrow-bore capillaries across which an electric field is applied. The types of CE that have the greatest potential in proteomics (commonly used together with mass spectrometry) are capillary zone electrophoresis and capillary isoelectric focusing [37]. The use of CE to analyze saliva proteome, have been described and allowed the profiling and identification of several salivary proteins [38]. Recently this technique was successfully used for the identification of salivary profiles in cancer diagnosis [29].

The existence of different electrophoretic methodologies, allowing separations based on different protein characteristics is of great utility in proteomics, contributing for the

resolution of complex samples such as saliva. The electrophoretic methods described are complementary of each other and since all have advantages and limitations, the choice for each one will depend on the objectives of the study and on the salivary proteins of interest.

3. Methodological issues related to saliva proteome analysis

The concept of salivary proteome is related to the creation of a salivary protein catalogue, where information that can be further used for several different purposes (e.g diagnostics, physiological status) can be placed [39]. In this context, it is important that the results obtained from different laboratories can be compared. Accurate examination of salivary components requires optimal collection, processing and storage conditions. Moreover, most of the studies aim comparisons among pathological/physiological conditions, and as such it is essential that the differences obtained are not due to external factors. To avoid changes in the protein and peptide composition from salivary secretions, standardized salivary sampling protocols, processing and storage conditions need to be applied [23,39,40].

3.1. Sample collection, processing and storage

One of the particularities of saliva is its capacity of rapidly adapt to different conditions. This is related to the fact of salivary secretion being mainly regulated by the two branches of the autonomic nervous system (both sympathetic and parasympathetic), with only a minor regulation from hormonal origin. Such type of regulation results in variations in composition according to the stimulus. Circadian rhythm [41], gender [42], drugs [43], exercise [44], among others, are factors that change salivary flow rate and saliva composition. Additionally to variations in the composition from each salivary gland cell type, the contribution of each individual salivary gland to the total fluid is not the same. For example, minor salivary glands and submandibular glands have an important contribution at rest, whereas in response to strong stimuli during feeding is the parotid contribution that becomes dominant [45]. Concerning daytime, the flow rate of resting and stimulated saliva is higher in the afternoon than in the morning, with the peak occurring in the middle of the afternoon. The salivary protein concentration also follows this diurnal pattern [2]. Eating is another strong stimulus for the secretion of saliva, and as such the interval between feeding and saliva collection influences salivary flow rate, viscosity and protein composition.

The points referred above are important for the selection of the collection method: with or without stimulation. For unstimulated saliva collection the draining method is usually choose. Accordingly, saliva is allowed to drip off the lower lip to a tube maintained on ice. On the other hand, stimulated saliva is frequently obtained after parafilm mastication, or after sour taste stimulation [46].

Advantages and disadvantages exist for both approaches. Unstimulated saliva collection is several times preferred, as stimulated saliva contains a diluted concentration of several proteins, which may be of interest. However, it is difficult to have saliva completely free of stimulation: due to the high range of stimulus influencing salivary secretion, even small

variations in collection conditions, among different saliva donors (e,g light intensity, temperature, emotional status, or others) may be sufficient to induce differences in results [39].

Stimulated saliva collection is generally used to have higher volumes of sample. In some pathological conditions (e.g Sjögren's syndrome, xerostomia) or post-radiation, stimulation of salivary secretion may be the only way of obtaining adequate amounts of saliva samples for analysis. However, difficulties may exist to make uniform the intensity and duration of the stimulus and the secretion of certain proteins may be affected by the duration of stimulation. For example, with prolonged stimulation of salivary flow, certain glycoproteins may be incompletely glycosylated [39].

Another issue in regard with saliva sampling is the origin of the collected secretions, i.e. whether it is glandular or whole saliva. Glandular fluid can be obtained through the use of adapted collection devices, for both parotid and submandibular/sublingual secretions [47]. Through this, changes in salivary proteins by exogenous enzymes are avoided, since the fluid is collected before it reaches the mouth.

Most of the studies on saliva in general have been obtained from human saliva. Concerning animal saliva less is known and, although methodologies of collection, processing and storage are based on the ones reported for humans, in most cases it is difficult to obtain unstimulated saliva from animals. Only in domestic ruminants, which produce considerably high volumes of saliva daily, in a continuous flow, it is possible to obtain whole saliva samples by simply collecting the mouth flow into a large beaker [48]. Moreover, this method only works with domesticated animals that are used to be handled by man.

For all the animals that do not produce large amounts of saliva, or for specific investigation purposes, stimulation may be necessary. Under controlled experimental conditions, the collection of stimulated saliva can be initiated either mechanically or chemically. Cotton rolls (e.g. Sallivetes) are currently used for animal saliva collection for being both practical and efficient in getting this fluid. Animals chew for some time, being saliva production stimulated by mastication. The cotton roll moistened by the saliva is further centrifuged to release the fluid. There is the possibility of some salivary proteins such as mucins and/or other potential biomarkers irreversibly adsorb to the cotton roll, resulting in their loss. However, to our knowledge, studies to elucidate this aspect are lacking. Chemical (pharmacological) stimulation is sometimes used for saliva collection in animals with low amounts of saliva. For example, in small rodents, it is very difficult to collect sufficient amounts for analysis without stimulation and the use of parasympathetic agonists (e.g. pilocarpine) appears to be effective [49]. This type of stimulant is referred to increase the volume production without changing the proportion of the proteins secreted. Others types of chemical stimulants may be used. For example, sympathetic agonists, such as isoproterenol, induce the synthesis and secretion of granular proteins from salivary glands, thereby increasing salivary protein concentration and changing the salivary protein profile [50].

While whole saliva can be collected using the already mentioned cotton-rolls, or through direct aspiration from the mouth [49], glandular saliva collection can be achieved using

catheters inserted into the ducts of the gland of interest. Although this method is invasive, the level of invasion is minimal, and once the collection period is finished, the catheter can easily be removed without causing any damage to the animal. This method was used to study sheep and goat parotid salivary proteomes [34,51]. Collection of glandular saliva has the advantage of accessing the secretion product of certain glands, as well as obtaining a clear saliva sample with virtually no interfering compounds, such as food debris or microorganisms. Glandular collection is most important for studies that aim on unraveling the effects of particular factors on the secretion from individual glands [e.g. effects of dietary constituents on parotid saliva [35]. However, this approach, if not carried properly, might have the disadvantage of faulty catheter insertion which causes leakage of plasma proteins into the salivary secretions due to disruption of epithelial integrity.

Wherever in humans or animals, the collection method of choice, as well as the origin of fluid selected, will affect the outcomes and thus should depend on the objectives of the study and on the specific group of proteins of interest.

In complex protein mixtures, such as saliva, sample preparation and fractionation constitute one of the most crucial processes for proteome study. None of the currently available proteomic techniques allow the analysis of the entire proteome in a single step. In body fluids such as saliva, proteins have different physicochemical characteristics and a wide concentration dynamic range and, as such, fractionation is essential. In this context, electrophoresis may also be useful for that purpose. Preparative isoelectric focusing using free flow electrophoresis is an example of the methods available for sample fractionation and which has been used in saliva [30]. Before 2-DE separation, free flow electrophoresis has the advantage of generating different fractions, which can be independently run. For example, a first separation using isoelectric free flow electrophoresis results in fractions with different pI ranges. Each of the resulting fractions can be further separated according to charge, by using narrow pH ranges, in the first dimension allowing a more detailed picture of the protein profile [30].

Saliva contains proteins present in high levels, and numerous low abundant proteins, for which analysis may be of interest. The observation of the latter in electrophoretic gels is obscured by the presence of the high-abundant proteins. When salivary proteome is used for disease diagnostic purposes, the analysis of low abundant proteins is almost always necessary, since most of potential salivary biomarkers are present in relative low amounts. The major components of saliva are mucins, proline-rich glycoproteins, amylase and some antimicrobial proteins that include agglutinin, lisozyme, lactoferrin, immunoglobulins, histatins and defensins [27]. The protein alpha-amylase contributes to almost 60% of total salivary proteome [52] and its depletion allows the analysis of less abundant proteins. Salivary alpha amylase depletion can be achieved through elution of samples from starch columns to deplete this protein specifically [53].

One of the main concerns when working with protein sample is to avoid undesirable alterations during the several steps that go from collection to final analysis. One characteristic of saliva is that many salivary proteins enter post-translational modifications

(PTMs), namely glycosylation, phosphorylation, sulfation and proteolysis. From the considerable amount of glycoproteins present in saliva, mucins represent an important group, which is responsible for bacterial agglutination and lubrication of the oral cavity tissues. Phosphoproteins also exist in a considerable amount of salivary proteins, with several diverse roles [54]. Such modifications start in the acinar cells of salivary glands, and continue when saliva enters the mouth, mainly due to the presence of host- and bacteria-derived enzymes, what results in additional protein modifications [55].

Since these forms of PTMs are responsible for many functions of this fluid, a particular attention should be directed to saliva collection processing and storage, in order to minimize proteolysis, de-glycosylation and de-phosphorylation. Different research groups have employed different methods for avoiding proteolysis, de-glycosylation and de-phosphorylation. The addition of 0,2% trifluoroacetic acid to saliva after collection has been one protocol used [56]. In a recent study, it was observed that the addition of protease inhibitors to saliva may allow its storage at 4°C for approximately two weeks, without significant degradation [53]. Nonetheless, some authors report that not even an inhibitor cocktail can prevent all protein degradation [57]. Additionally, it was suggested to be possible to keep the samples at room temperature (for a period of about two weeks), without considerable changes in salivary proteome occurred, only by adding ethanol, [53]. Nevertheless, working on ice for no longer than one hour and subsequent storage of samples at -80°C has been considered a safe and practical handling protocol [57-59]. Nonetheless, long time storage, as well as freeze-thaw cycles can induce protein precipitation, in particular from low molecular mass components [57]. In any case, little research has been directed on ways of minimizing degradative processes, and this is clearly needed [39].

3.2. Staining procedures and PTMs in-gel analysis

Protein visualization is necessary for quantitative and qualitative analysis. In electrophoresis this is achieved through reversible or irreversible binding of a colored organic or inorganic chemical to the protein. An ideal staining procedure would be the one with a very low detection limit, an optimal signal to noise ratio, a wide dynamic range and a wide linear relationship between the quantity of protein and the staining intensity, and compatible to mass spectrometry [60]. However, such an ideal stain does not exist.

Silver, Coomassie Brilliant Blue (CBB) and fluorescent staining are the most frequently used methodologies. Silver staining presents a high sensitivity, making possible the visualization of proteins present in amounts as low as 1 ng [61]. Silver staining techniques are based upon saturating gels with silver ions, washing the less tightly bound metal ions out of the gel matrix and reducing the protein-bound metal ions to form metallic silver. In 2-DE, Silver staining is regularly used due to its potential for the visualization of low intensity protein spots. However, it presents a narrow dynamic range and the tendency of the dye to stain differently based on amino acid composition and PTMs. Moreover, by detecting low levels of protein, each of the stained spots may have not sufficient amounts o protein for subsequent analysis by mass spectrometry.

CBB is a disulfonated triphenylmethane textile dye. CBB staining presents a linear dynamic range and a moderately sensitivity. As such, CBB dyes are suitable for protein quantitative analysis, which is necessary in proteomics analysis. Moreover, this staining technique is compatible with mass spectrometry. Two modifications of CBB exist: R-250 and G-250. In acidic solutions the dye sticks to the amino groups of the proteins by electrostatic and hydrophobic interactions. Inversely to silver stain, CBB is not so extremely sensitive, thus being necessary to load higher amounts of proteins in the gel. Consequently, CBB stained spots contains considerable amounts of proteins, suitable for mass spectrometry analysis.

Saliva samples present particularities that should be considered for silver and CBB staining. Parotid saliva contains a considerable amount of proline-rich proteins (PRPs), which are difficult to stain with silver since they present low amounts of amino acids containing sulfur, necessary for the binding of silver ions [61]. On the other hand, when stained with CBB R-250 they usually present a violet-pink stain, which allows differentiating them from all the other salivary proteins that stain blue. A destain protocol of 10% acetic acid, instead of the common 10% acetic acid/ 10% methanol is generally used for this purpose [62].

The fluorescent dyes were more recently developed, presenting a high sensitivity and a linear dynamic range, and in this way, being advantageous relatively to common silver staining. For example, SYPRO staining technique has been used in several salivary proteomic studies [e.g. 63]. This is a novel, ruthenium-based fluorescent dye. SYPRO Red and Orange, bind to the detergent coat surrounding proteins in SDS denaturing gels, thus, staining in such gels is not strongly selective for particular polypeptides [64]. SYPRO stains are compatible with mass spectrometry and can be used in combination with other staining techniques, for detection of PTMs, such as glycosylations or phosphorylations.

DIGE (Difference Gel Electrophoresis) technology represented an improvement in 2-DE based proteomics, allowing more accurate comparisons among samples. Its convenience is also true for saliva samples [65]. DIGE is based on the modification of proteins, before electrophoresis, by attaching a fluorescent labeling. Cyanine-bases dyes (Cy2, Cy3 and Cy5) are used with this purpose. These dyes label the amine group of protein lysines specifically and covalently to form an amide. A different cyanine-based dye is added to each individual sample. The dyes are designed to have the same molecular mass and charge to ensure that proteins common to both samples have the same relative 2-DE mobility. The samples are mixed and resolved in a single 2-DE gel. The proteins from the different dyes are visualized by alternatively illuminating the gel at different wavelengths. With this technique, it is possible to avoid some inter-gel variation.

PTMs, such as glycosylation and phosphorylation, can be accessed in electrophoretic gels through staining procedures. As it was referred before, several different salivary proteins suffer PTMs, which confer their characteristic functions. Consequently, their visualization in electrophoretic gels is valuable. Detection of phosphoproteins can be achieved using different procedures. Recently, Pro-Q Diamond was developed for phosphoproteins staining. This affords wide specificity and high sensitivity. Phosphoserine, phosphothreonine and phosphotyrosine containing proteins are detected [66].

O- and N- glycosylated proteins are abundant in saliva. Some of the most well studied salivary glycoproteins are mucins (MUC5B and Muc7) and proline-rich glycoproteins. The most classical procedure for glycoproteins staining is periodic-acid Schiff (PAS) with the protocol that allows detection of glycoproteins in gels being adapted from protocols of histochemistry. However, it does not present a high sensitivity, resulting in the need of high levels of protein load [67]. Another possible method is the more recently developed stain Pro-Q Emerald that allows glycoprotein detection, presenting approximately 50-fold more sensitivity than PAS [68] very useful when amount of sample is limited.

3.3. Protein identification

Proteome studies may be performed with two main different but somewhat complementary purposes: to characterize a particular sample (e.g organism, cell, fluid), or to compare samples from different experimental conditions. Independently of which of these two is the focus, protein identification is a central aspect. In proteomic studies, electrophoresis is frequently coupled to mass spectrometry technologies. Proteins selected in the gels are excised and subjected to enzymatic in-gel digestion. In this process it is important that each of the excised spots (or bands) present the amount of protein sufficient for MS analysis. So, the choice of the staining methodology should take this into consideration, as well as the need for the use of dyes compatible with MS (referred in previous section).

After proteins being identified, MS identifications need further to be validated, by Western blotting, through the use of antibodies to the proteins of interest. This is only possible for proteins for which commercial antibodies are available. There are some particularities of saliva samples that may be considered for Western blotting. Using this technique, only the protein spots (or bands) that react with the antibody are visualized. When comparison among different samples is to be made, it is important to have the same load of protein in all lanes or 2-DE gels. Different loads may result in erroneous results. The existence of internal controls, i.e., proteins for which the levels are proportional to the amount of total protein loaded in the gels, is important and is commonly used to circumvent putative differences in protein loads [e.g 69]. The simultaneous use of a primary antibody for such internal control and the use of a primary antibody for the protein of interest may allow adjustments: by comparing the intensity of these internal controls, the relative amount of protein run for each sample can thus be estimated. For saliva studies such strategy is not possible, since it is not yet known one salivary protein which relative amount to total protein content remains constant. One way to circumvent this limitation is through the staining of the membrane, allowing the relative evaluation of protein content, before incubation with the primary antibody [70]. With this procedure, it is possible to visualize the several lanes and consequently the expression of the protein of interest may be compared as a percentage of the total band intensity. The reversible Ponceau red staining [71] is the standard procedure, despite its low sensitivity (detection limit in the range of 1 μg/spot).

Although in human saliva, protein identification can be generally performed with success, for animal saliva this is not always the case. One of the major limitations in animal

proteomic studies is the lack of complete and annotated genome and protein sequences for a great number of species, making salivary protein identification challenging. As consequence there is the need for search in other related species databases, at the cost of eventually producing high number of false positive results [72]. Moreover, the inexistence of commercial antibodies for most of animal proteins makes validation of identifications more difficult. Nevertheless, recent advances in sequencing the genomes of various domestic animals (cattle, pigs and sheep) are increasing the ability to identify salivary proteins in these animal species.

4. Advantages and limitations of electrophoresis for saliva proteome analysis

Electrophoresis has been widely used in saliva characterization. Despite the advances in the knowledge of salivary protein composition that SDS-PAGE and IEF allowed, the development of 2-DE and its application to the study of saliva contributed for a great advance in the comprehension of this body fluid. 2-DE has played a major role in the birth and developments of proteomics, although it is no longer the exclusive separation tool used in this field. Nevertheless, 2-DE continues to be essential in proteomics studies. Apart from the great advantage arising from its efficiency in resolving a high number of proteins in complex samples such as saliva and allowing the visualization of PTMs, 2-DE method has also the advantage of being a method relatively inexpensive (at least comparatively to most of the other techniques used in proteomics).

Although the great advantages of electrophoresis for the study of salivary protein composition, saliva presents several particularities that need to be consider, and that will limit the use of this technique. One of these particularities is the considerable ionic content that saliva presents, which is important to account for when IEF or 2-DE is to be used in protein separation; since separation according to electrical charge will occur, the existence of charged compounds, or salts, in the sample may cause interference. Lowering of ions and salt content may be achieved using ultracentrifugation membranes [e.g. 51], or through protein acid precipitation (e.g. 10% TCA/90% acetone), in which the precipitate is subsequently re-suspended in a buffer. Both of these procedures will also increase the protein concentration, which may constitute an advantage since saliva samples may sometimes be too diluted (for example, after parasympathetic stimulation). In any case some protein loss will inevitably occur.

Another characteristic of saliva that limits its study by electrophoresis is the considerable amount of mucins present. These are glycoproteins with high molecular masses, which present particular physicochemical characteristics that difficult analysis using electrophoretic separation. Moreover, the presence of mucins also limits the study of other salivary proteins, since mucins form complexes with several proteins, interfering in the analysis of the latter. Human salivary glands secrete two types mucins: oligomeric mucin (MG1) with molecular mass above 1 MDa and monomeric mucin (MG2) with molecular mass of 200–250 kDa, which together represent about 26% of total proteins from saliva [73].

The high molecular mass of these molecules impede them to migrate through the polyacrylamide matrix, resulting in their deposition in the top of the gel [74]. The exact mucin content is dependent on the proportion of contribution from the different major salivary glands for the total saliva. As such, the type of stimulation will influence the amount of salivary mucins present. Stimuli that increase sublingual and submandibular saliva result in higher amount of these proteins than when parotid glands are the major contributors.

The presence of high amounts of mucins confers a high viscosity to saliva, what has practical drawbacks in sample preparation for analysis, such as difficulties in sample pipetting. Usage of denaturing conditions, such as buffers containing 4-6M guanidine hydrochloride (GdmCl), or the reducing agent dithiothreitol (DTT) can diminish the viscosity of mucous salivary secretions [75]. However, this effect is achieved at expense of effects in the structure of proteins, and should be avoided in studies where the maintenance of such structure is necessary (for example when studying salivary complexes). The centrifugation of saliva samples, which is one of the first approach usually performed in saliva preparation, also aids in the removing of mucins. However, mucins and other glycoproteins are frequently involved in protein complexes with other salivary proteins, namely amylase, statherin and PRPs, resulting in particular protein losses, when the pellet is discharged.

Another limitation for the study of saliva using electrophoresis, which is common to the majority of body fluids, is the high diversity in the levels of the different protein species. As it was mentioned in section 3.1., a few salivary proteins are present in high amounts, whereas many others appear in low levels. Depletion methods are needed, in order to visualize the low abundant proteins, which may be of particular importance. If considering the important application of saliva as source of disease biomarkers, and knowing that many of those potential biomarkers are not secreted by the salivary glands, it is essential to have access to the low abundant proteins, such as metabolic enzymes with antimicrobial activity (e.g. lysozyme and lactoperoxidase).

Finally, saliva contains several low-molecular mass components that have important functions, namely important bactericidal activity (e-g- histatins, defensins) [76]. The fact that electrophoresis does not allow the separation of these compounds constitutes another limitation in the application of electrophoresis to saliva characterization.

5. Alternatives to electrophoresis for saliva proteome analysis

Through this chapter the use of electrophoresis in saliva proteome analysis has been emphasized. 2-DE and protein MS represent an integrated technology by which several thousand proteins can be separated, quantified and identified. And, as has been being referred, this approach has been considerably used in saliva proteome studies. However, although the advantages of 2-DE, it does not allow the study of the complete proteome. Moreover, as it was stated before, 2-DE have drawbacks which include poor gel-to-gel reproducibility, and the requirement of relatively large amounts of sample, as well as extensive labor and a considerable time required. As such, other proteomic techniques are valuable for the study of saliva proteome.

With the introduction of high-throughput LC coupled to tandem MS (MS/MS), the study of complex systems moved towards a bottom-up proteomics analysis, where complex protein samples are digested and the generated peptides, which are separated by high pressure liquid chromatography (HPLC), are introduced into a mass spectrometer for fragmentation and sequencing to identify and quantify the parent proteins. However, LC-MS analysis of highly complex proteomic samples remains a challenging endeavor [77]. With this approach, tryptic cleavage generates multiple peptides per protein so that proteomic samples typically consist of hundreds of thousands of peptides. To date, no separation method is capable of resolving so many components in a single analytical dimension prior to the MS analysis. Thus, many research efforts have focused on the development of a more sensitive multidimensional liquid chromatography (MudPIT) with higher peptide separation power [21,22]. Initially, large scale shotgun proteomics was defined as an ion exchange chromatography (specifically strong cation exchange-SCX) coupled to reverse phase (RP) and mass spectrometry [78]. Nowadays, alternative configurations to SCX with RP have also been investigated and include the use of anion exchange chromatography and RP, affinity chromatography (AC) and RP, isoelectric focusing (IEF) and RP, capillary electrophoresis (CE) [79,80]. Recently, a RPRP system was proposed for proteomics where the first RP column uses a pH of 10 and the second RP column uses a pH of 2.6 [81,82]. This later approach yielded higher proteome coverage when compared with the SCX-RP approach [81,83].

These methodologies have been applied to saliva proteome characterization aiming the extension of salivary proteins catalogue and further comparison within other biological fluids. For instance, the use of a classical MudPit approach through combination of SCX-RP, allowed the identification of more than 100 proteins [84]. In other experiments a shotgun approach using only LC-MS/MS resulted in the identification of more than 300 proteins [85]. Different chromatographic combinations have been succeeded [30,80,86] which in conjugation to instrumentation advances conducted to the identification of more than 3000 different components in saliva. Other approaches were also performed aiming the reduction of saliva sample complexity through the utilization of combinatorial chemistry derived hexapeptide libraries (Proteominer from BioRad) which lead to the identification of more 2300 different proteins [1]. This has been later used for PTM characterization mainly N-linked salivary glycoproteins and their glycosylation sites [87]. Other methodologies aimed at characterizing salivary glycoproteins consist in enrichment procedures based on affinity chromatography with lectins [88] acting as a first dimension. In fact, saliva is a rich source of both N- and O-linked glycoproteins, which play an important role in the maintenance of oral health and protection of teeth [68]. In line with the characterization of the most abundant PTMs in salivary proteins, namely phosphorylation, other systems were also developed. For instance, Salih et al [89] developed an enrichment procedure based on chemical derivatization using dithiothreitol (DTT) leading to the identification of 65 phosphoproteins. In a different approach, combining hexapeptide libraries, immobilized metal ion affinity chromatography, SCX and RP, 217 unique phosphopeptides sites were positively identified representing 85 distinct phosphoproteins [54].

As stated above, and similarly to other bodily fluids, saliva contains several protein species of low molecular weight which comprise around 40-50% of the total secreted protein content [90]. Albeit particular functions can be attributed to the major peptide classes, several questions about their precise role in oral cavity remain unclear. A strategy based on acidic precipitation or passing the saliva supernatant through a defined cut-off filters and LC-MS/MS have been widely adopted to perform the characterization of this low molecular weight fraction [91-107]. Behind the identification of several fragments deriving from those major peptide classes, several PTMs were also assigned. For instance, novel N- and O-glycosylation sites were identified in PRPs [108] as well as S-Glutathionyl, S-cysteinyl and S-S 2-mer recently identified in cystatin B [109].

More than identification of proteins, many studies aim the evaluation of protein expression under different purposes and pathophysiological conditions. In fact, quantitative proteome profiling is key for comparative analysis of proteins from normal and diseased patients, as similar proteins may be present in both states but at significantly different concentrations. Without quantitative information, the value of these differentially abundant proteins as biomarkers may be overlooked. In the pursuit of these goals, gel-free approaches using stable-isotope tagging or label free (based on spectral counting) have been used for comparative analysis involving salivary samples. In these approaches, the typical flowchart starts by protein digestion being, in case of isotope labeling, derivatized with respective isotope, mixed and analysed simultaneously. Depending on the approach, up to 8 different samples (iTRAQ-8plex, Absciex) can be compared at same time. As an example, Streckfus et al. [110] evaluated the salivary protein expression in patients with breast cancer using a iTRAQ approach identifying 55 proteins that were common to both cancer stages in comparison to each other and healthy controls while there were 20 proteins unique to Stage IIa and 28 proteins that were unique to Stage IIb. In case of label free, eluted peptides are aligned in terms of retention time and comparative analysis will be based on spectral counting [111]. For instance, Ambatipudi et al. [112] by MudPit and label free evaluated the aging effect in the abundance of human female parotid salivary proteins where extensive age associated changes in the abundance of many of salivary proteins were noted, especially for proteins associated with host defense mechanisms.

6. Concluding remarks

The proteome of human saliva received considerable attention in the last years. However, the improvement of the methods to study and characterize salivary proteins and/or the changes in its profile is still an issue since the identification of potential biomarkers for several pathological and physiological conditions is yet to be established. Moreover, a complete knowledge on the importance of each salivary protein in oral environment is not completely understood. Additionally, the growing interest in animal saliva, both due to their value as models for humans, as well as for veterinarian and production purposes, justifies the efforts to develop new protocols adapted to the particularities of these samples.

Despite the existence of limitations, electrophoresis continues to be an essential tool in the study of salivary proteome. It constitutes the bases for the separation of several different components, allowing a summary characterization and also providing a purification step prior the application of more selective and commonly more expensive methods. Nevertheless, enhanced methodologies for sample fractionation and processing might be useful to circumvent some of the limitations for the study of this fluid by electrophoresis. Profiling such a fluid that rapidly changes according stimulus and where some of its constituents interact with each other is challenging. Improved approaches will be necessary to cope with the challenges in understanding of these interactions, their functions and health consequences – the so called interactome – which will be the future in saliva characterization and biomarker identification.

Author details

Elsa Lamy[*]
*ICAAM – Institute of Mediterranean Agricultural and Environmental Sciences,
University of Évora, Évora, Portugal
QOPNA, Mass Spectrometry Center, Department of Chemistry, University of Aveiro,
Aveiro, Portugal*

Ana R. Costa
*ICAAM – Institute of Mediterranean Agricultural and Environmental Sciences,
University of Évora, Évora, Portugal
Department of Chemistry, University of Évora, Évora, Portugal*

Célia M. Antunes
*ICAAM – Institute of Mediterranean Agricultural and Environmental Sciences,
University of Évora, Évora, Portugal
Department of Chemistry, University of Évora, Évora, Portugal
Center for Neuroscience and Cell Biology, University of Coimbra, Portugal*

Rui Vitorino and Francisco Amado
*QOPNA, Mass Spectrometry Center, Department of Chemistry,
University of Aveiro, Aveiro, Portugal*

Acknowledgement

* Corresponding Author

7. References

[1] Bandhakavi S, Stone MD, Onsongo G, Van Riper SK, Griffin TJ (2009) A dynamic range compression and three-dimensional peptide fractionation analysis platform expands proteome coverage and the diagnostic potential of whole saliva. J. Proteome Res. 8: 5590–5600.

[2] Castagnola M, Cabras T, Iavarone F, Fanali C, Nemolato S, Peluso G, Bosello SL, Faa G, Ferraccioli G, Messana I (2012) The human salivary proteome: a critical overview of the results obtained by different proteomic platforms. Expert Rev Proteomics.9: 33-46.

[3] Emmelin N (1987) Nerve interactions in salivary glands. J Dent Res. 66: 509-517.

[4] Huang AY, Castle AM, Hinon BT, Castle D 2001 Resting (basal) secretion of proteins is provided by the minor regulated and constitutive-like pathways and not granule exocytosis in parotid acinar cells. J Biol Chem. 276: 22296-22306.

[5] Cevik-Aras H, Ekstrom J (2006) Pentagastrin-induced proteinsynthesis in the parotid gland of the anaesthetized rat, and its dependence on CCK-A and -B receptors and nitric oxide generation. Exp Physiol. 91: 673-679.

[6] Cevik-Aras H, Ekstrom J (2006) Cholecystokinin- and gastrin induced protein and amylase secretion from the parotid gland of the anaesthetized rat. Regul Pept. 134: 89-69.

[7] Cevik-Aras H, Ekstrom J (2008) Melatonin-evoked in vivo secretion of protein and amylase from the parotid gland of the anaesthetised rat. J Pineal Res. 45: 413-421.

[8] Cevik-Aras H, Godoy T, Ekstrom J (2011) Melatonin-induced protein synthesis in the rat parotid gland. J Physiol Pharmacol. 62: 95-99.

[9] Loy F, Diana M, Isola R, Solinas P, Isola M, Conti G, Lantini MS, Cossu M, Riva A, Ekström J (2012) Morphological evidence that pentagastrin regulates secretion in the human parotid gland. J Anat. doi: 10.1111/j.1469-7580.2012.01489.x

[10] Ruhl S (2012) The scientific exploration of saliva in the post-proteomic era: from database back to basic function. Expert Rev Proteomics. 9: 85-96.

[11] Soares S, Vitorino R, Osório H, Fernandes A, Venâncio A, Mateus N, Amado F, de Freitas V (2011) Reactivity of human salivary proteins families toward food polyphenols. J Agric Food Chem. 59: 5535-5547.

[12] Padiglia A, Zonza A, Atzori E, Chillotti C, Calò C, Tepper BJ, Barbarossa IT (2010) Sensitivity to 6-n-propylthiouracil is associated with gustin (carbonic anhydrase VI) gene polymorphism, salivary zinc, and body mass index in humans. Am J Clin Nutr. 92: 539-545.

[13] Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S, Breslin PA (2010) Individual differences in AMY1 gene copy number, salivary α-amylase levels, and the perception of oral starch. PLoS One. 5: e13352.

[14] Dsamou M, Palicki O, Septier C, Chabanet C, Lucchi G, Ducoroy P, Chagnon MC, Morzel M (2012) Salivary protein profiles and sensitivity to the bitter taste of caffeine. Chem Senses. 37: 87-95.

[15] Neyraud E, Sayd T, Morzel M, Dransfield E (2006) Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. J Proteome Res. 5: 2474-2480.

[16] Harthoorn LF, Brattinga C, Van Kekem K, Neyraud E, Dransfield E (2009) Effects of sucrose on salivary flow and composition: differences between real and sham intake. Int J Food Sci Nutr. 60: 637-46.

[17] Vitorino R, Calheiros-Lobo MJ, Duarte JA, Domingues P, Amado F (2006) Salivary clinical data and dental caries susceptibility: is there a relationship? Bull Group Int Rech Sci Stomatol Odontol. 47: 27-33.

[18] Nieuw Amerongen AV, Veerman EC (2002) Saliva - the defender of the oral cavity. Oral Dis. 8: 12-22.

[19] Huq, NL, Cross KJ, Ung M, Myroforidis H, Veith PD, Chen D, Staton D, He H, Ward BR, Reynols EC (2007) A review of the salivary proteome and peptidome and saliva-derived peptide therapeutics. Int J Res Ther. 13: 547-564.

[20] Baldini C, Giusti L, Ciregia F, Da Valle Y, Giacomelli C, Donadio E, Ferro F, Galimberti S, Donati V, Bazzichi L, Bombardieri S, Lucacchini A (2011) Correspondence between salivary proteomic pattern and clinical course in primary Sjögren syndrome and non-Hodgkin's lymphoma: a case report. J Transl Med. 9: 188.

[21] Caseiro A, Vitorino R, Barros AS, Ferreira R, Calheiros-Lobo MJ, Carvalho D, Duarte JA, Amado F (2012) Salivary peptidome in type 1 diabetes mellitus. Biomed Chromatogr. 26: 571-82. doi: 10.1002/bmc.1677

[22] Xiao H, Zhang L, Zhou H, Lee JM, Garon EB, Wong DT (2012) Proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry. Mol Cell Proteomics. 11: M111.012112

[23] Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S (2011) Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review. OMICS. 15: 353-361

[24] da Costa G, Lamy E, Capela e Silva F, Andersen J, Sales Baptista E, Coelho AV (2008) Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins. J Chem Ecol. 34: 376-387

[25] Fontanini D, Capocchi A, Saviozzi F, Galleschi L (2007) Simplified electrophoretic assay for human salivary alpha-amylase inhibitor detection in cereal seed flours. J Agric Food Chem. 55: 4334-4339.

[26] Chisholm DM, Beeley JA, Mason DK (1973) Salivary proteins in Sjögren's syndrome: separation by isoelectric focusing in acrylamide gels. Oral Surg Oral Med Oral Pathol. 35: 620-630,

[27] Vitorino R, Lobo MJ, Ferrer-Correira AJ, Dubin JR, Tomer KB, Domingues PM, Amado FM (2004) Identification of human whole saliva protein components using proteomics. Proteomics. 4: 1109-1115.

[28] Pobozy E, Czarkowska W, Trojanowicz M (2007) Determination of amino acids in saliva using capillary electrophoresis with fluorimetric detection. J Biochem Biophys Methods. 69: XIII-XXIII.

[29] Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M (2010) Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. Metabolomics. 6:78-95.

[30] Xie H, Rhodus NL, Griffin RJ, Carlis JV, Griffin TJ (2005) A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol Cell Proteomics. 4: 1826-1830.

[31] Pandey A, Mann M (2000) Proteomics to study genes and genomes. Nature 405: 837-846.

[32] Klose J 1975 Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26: 231–243.

[33] O'Farrell, PH 1975 High resolution two-dimensional electrophoresis of proteins. J Biol Chem. 250: 4007–4021.

[34] Lamy E, da Costa G, Santos R, Capela E Silva F, Potes J, Pereira A, Coelho AV, Sales Baptista E (2009) Sheep and goat saliva proteome analysis: a useful tool for ingestive behavior research? Physiol Behav. 98: 393-401.

[35] Lamy E, da Costa G, Santos R, Capela e Silva F, Potes J, Pereira A, Coelho AV, Baptista ES (2011) Effect of condensed tannin ingestion in sheep and goat parotid saliva proteome. J Anim Physiol Anim Nutr (Berl). 95: 304-312.

[36] Lloyd DK (2008) Capillary electrophoresis analysis of biofluids with a focus on less commonly analyzed matrices. J Chromatogr B Analyt Technol Biomed Life Sci. 866: 154-166.

[37] Simpson DC, Smith RD (2005) Combining capillary electrophoresis with mass spectrometry for applications in proteomics. Electrophoresis. 26: 1291-1305.

[38] Guo T, Lee CS, Wang W, DeVoe DL, Balgley BM (2006) Capillary separations enabling tissue proteomics-based biomarker discovery. Electrophoresis. 27: 3523-3532.

[39] Siqueira WL, Dawes C (2011) The salivary proteome: challenges and perspectives. Proteomics Clin Appl. 5: 575-579.

[40] Castagnola M, Picciotti PM, Messana I, Fanali C, Fiorita A, Cabras T, Calò L, Pisano E, Passali GC, Iavarone F, Paludetti G, Scarano E (2011) Potential applications of human saliva as diagnostic fluid. Acta Otorhinolaryngol Ital. 31: 347-357.

[41] Dawes C (1972) Circadian rhythms in human salivary flow rate and composition. J Physiol 220:529-545.

[42] Inoue H, Ono K, Masuda W, Morimoto Y, Tanaka T, Yokota M, Inenaga K (2006) Gender difference in unstimulated whole saliva flow rate and salivary gland sizes. Arch Oral Biol. 51: 1055-1060.

[43] Scully C (2003) Drug effects on salivary glands: dry mouth. Oral Dis. 9: 165-176.

[44] Usui T, Yoshikawa T, Orita K, Ueda SY, Katsura Y, Fujimoto S, Yoshimura M. Changes in salivary antimicrobial peptides, immunoglobulin A and cortisol after prolonged strenuous exercise. Eur J Appl Physiol. 111: 2005-2014.

[45] Humphrey SP, Williamson RT (2001) A review of saliva: Normal composition, flow and function. J Prosthet Dent. 85:162-169.

[46] Navazesh M (1993) Methods for collecting saliva. Ann N Y Acad Sci. 694:72-77.

[47] Veerman EC, van den Keybus PA, Vissink A, Nieuw Amerongen AV (1996) Human glandular salivas: their separate collection and analysis. Eur J Oral Sci. 104: 346-352.

[48] Mau M, Kaiser TM, Südekum KH (2010) Carbonic anhydrase II is secreted from bovine parotid glands. Histol Histopathol. 25:321-329.

[49] Lamy E, Graça G, da Costa G, Franco C, E Silva FC, Baptista ES, Coelho AV (2010) Changes in mouse whole saliva soluble proteome induced by tannin-enriched diet. Proteome Sci. 8:65.

[50] Gorr SU, Venkatesh SG, Darling DS (2005) Parotid secretory granules: crossroads of secretory pathways and protein storage. J Dent Res. 84: 500-509.

[51] Lamy E, da Costa G, Capela e Silva F, Potes J, Coelho AV, Baptista ES (2008) Comparison of electrophoretic protein profiles from sheep and goat parotid saliva. J Chem Ecol. 34:388-397.

[52] Deutsch O, Fleissig Y, Zaks B, Krief G, Aframian DJ, Palmon A (2008) An approach to remove alpha amylase for proteomic analysis of low abundance biomarkers in human saliva. Electrophoresis 29: 4150-4157.

[53] Xiao H, Wong DT (2012) Method development for proteome stabilization in human saliva. Anal Chim Acta. 722: 63-69.

[54] Stone MD, Chen X, McGowan T, Bandhakavi S, Cheng B, Rhodus NL, Griffin TJ (2011) Large-scale phosphoproteomics analysis of whole saliva reveals a distinct phosphorylation pattern. J Proteome Res. 10: 1728-1736.

[55] Helmerhorst EJ, Oppenheim FG (2007) Saliva: a dynamic proteome. J Dent Res. 86: 680-693.

[56] Castagnola M, Inzitari R, Fanali C, Iavarone F, Vitali A, Desiderio C, Vento G, Tirone C, Romagnoli C, Cabras T, Manconi B, Sanna MT, Boi R, Pisano E, Olianas A, Pellegrini M, Nemolato S, Heizmann CW, Faa G, Messana I (2011) The surprising composition of the salivary proteome of preterm human newborn. Mol Cell Proteomics. 10: 1-14.

[57] Thomadaki K, Helmerhorst EJ, Tian N, Sun X, Siqueira WL, Walt DR, Oppenheim FG (2011) Whole-saliva proteolysis and its impact on salivary diagnostics. J Dent Res. 90: 1325-1330.

[58] Schipper R, Loof A, de Groot J, Harthoorn L, Dransfield E, van Heerde W (2007) SELDI-TOF-MS of saliva: methodology and pre-treatment effects. J Chromatogr B Analyt Technol Biomed Life Sci 847:45-53.

[59] Schipper RG, Silletti E, Vingerhoeds MH (2007) Saliva as research material: Biochemical, physicochemical and practical aspects. Arch Oral Biol. 52: 1114-1135.

[60] Westermeier R, Marouga R (20059 Protein Detection Methods in Proteomics Research. Bioscience Reports 25; DOI: 10.1007/s10540-005-2845-1

[61] Patton WF (2002) Detection technologies in proteome analysis. J Chromatogr B Analyt Technol Biomed Life Sci. 771(1-2):3-31.

[62] Beeley JA, Sweeney D, Lindsay JC, Buchanan ML, Sarna L, Khoo KS (1991) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human parotid salivary proteins. Electrophoresis12: 1032-1041.

[63] Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG (2003) Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J Biol Chem. 278: 5300-5308.

[64] Steinberg TH, Jones LJ, Haugland RP, Singer VL (1996) SYPRO orange and SYPRO red protein gel stains: one-step fluorescent staining of denaturing gels for detection of nanogram levels of protein. Anal Biochem. 239: 223-237.

[65] Zhang L, Xiao H, Karlan S, Zhou H, Gross J, Elashoff D, Akin D, Yan X, Chia D, Karlan B, Wong DT (2010) Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. PLoS One. 5: e15573

[66] Steinberg TH (2009) Protein gel staining methods: an introduction and overview. Methods Enzymol. 463: 541-563.

[67] Miller I, Crawford J, Gianazza E (2006) Protein stains for proteomic applications: which, when, why? Proteomics. 6: 5385-5408.

[68] Sondej M, Denny PA, Xie Y, Ramachandran P, Si Y, Takashima J, Shi W, Wong DT, Loo JA, Denny PC (2009) Glycoprofiling of the Human Salivary Proteome. Clin Proteomics. 5: 52-68.

[69] Costa AR, Capela e Silva F, Antunes CM, Cruz-Morais J (2011) Key role of AMPK in glucose-evoked Na,K-ATPase modulation. Diabetologia. 54 [Supplement1]; S196.

[70] Nakamura K, Tanaka T, Kuwahara A, Takeo K (1985) Microassay for proteins on nitrocellulose filter using protein dye-staining procedure. Anal Biochem. 148: 311-319.

[71] Salinovich O, Montelaro RC (1986) Comparison of glycoproteins by two-dimensional mapping of glycosylated peptides. Anal Biochem. 157: 19-27.

[72] Johnson RS, Davis MT, Taylor JA, Patterson SD (2005) Informatics for protein identification by mass spectrometry. Methods 35: 223-236.

[73] Zalewska A, Zwierz K, Zółkowski K, Gindzieński A (2000) Structure and biosynthesis of human salivary mucins. Acta Biochim Pol. 47: 1067-1079.

[74] Tabak LA 1990 Structure and function of human salivary mucins. Crit Rev Oral Biol Med. 1:229-234.

[75] Schipper RG, Silletti E, Vingerhoeds MH (2007) Saliva as research material: biochemical, physicochemical and practical aspects. Arch Oral Biol. 52:1114-1135.

[76] Amado F, Lobo MJ, Domingues P, Duarte JA, Vitorino R (2010) Salivary peptidomics. Expert Rev Proteomics. 7: 709-721.

[77] Boersema PJ, Mohammed S, Heck AJ (2008) Hydrophilic interaction liquid chromatography (HILIC) in proteomics. Anal Bioanal Chem. 391: 151-159.

[78] Nagele E, Vollmer M, Horth P, Vad C (2004) 2D-LC/MS techniques for the identification of proteins in highly complex mixtures. Expert Rev Proteomics. 1: 37-46.

[79] Zhou F, Sikorski TW, Ficarro SB, Webber JT, Marto JA (2011) Online nanoflow reversed phase-strong anion exchange-reversed phase liquid chromatography-tandem mass spectrometry platform for efficient and in-depth proteome sequence analysis of complex organisms. Anal Chem. 83: 6996-7005.

[80] Guo T, Rudnick PA, Wang W, Lee CS, Devoe DL, Balgley BM (2006) Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. J Proteome Res. 5: 1469-1478.

[81] Manadas B, Mendes VM, English J, Dunn MJ (2010) Peptide fractionation in proteomics approaches. Expert Rev Proteomics. 7: 655-663.

[82] Stephanowitz H, Lange S, Lang D, Freund C, Krause E (2012) Improved Two-Dimensional Reversed Phase-Reversed Phase LC-MS/MS Approach for Identification of Peptide-Protein Interactions. J Proteome Res. 11: 1175-1183.

[83] Manadas B, English JA, Wynne KJ, Cotter DR, Dunn MJ (2009) Comparative analysis of OFFGel, strong cation exchange with pH gradient, and RP at high pH for first-dimensional separation of peptides from a membrane-enriched protein fraction. Proteomics. 9: 5194-5198.

[84] Wilmarth PA, Riviere MA, Rustvold DL, Lauten JD, Madden TE, David LL (2004) Two-dimensional liquid chromatography study of the human whole saliva proteome. J Proteome Res. 3: 1017-1023.

[85] Hu S, Xie Y, Ramachandran P, Ogorzalek Loo RR, Li Y, Loo JA, Wong DT (2005) Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics. 5: 1714-1728.

[86] Denny P, Hagen FK, Hardt M, Liao L, Yan W, Arellanno M, Bassilian S, Bedi GS, Boontheung P, Cociorva D, Delahunty CM, Denny T, Dunsmore J, Faull KF, Gilligan J, Gonzalez-Begne M, Halgand F, Hall SC, Han X, Henson B, Hewel J, Hu S, Jeffrey S, Jiang J, Loo JA, Ogorzalek Loo RR, Malamud D, Melvin JE, Miroshnychenko O, Navazesh M, Niles R, Park SK, Prakobphol A, Ramachandran P, Richert M, Robinson S, Sondej M, Souda P, Sullivan MA, Takashima J, Than S, Wang J, Whitelegge JP, Witkowska HE, Wolinsky L, Xie Y, Xu T, Yu W, Ytterberg J, Wong DT, Yates JR 3rd, Fisher SJ (2008) The Proteomes of Human Parotid and Submandibular/Sublingual Gland Salivas Collected as the Ductal Secretions. J Proteome Res. 7: 1994-2006.

[87] Bandhakavi S, Van Riper SK, Tawfik PN, Stone MD, Haddad T, Rhodus NL, Carlis JV, Griffin TJ (2011) Hexapeptide libraries for enhanced protein PTM identification and relative abundance profiling in whole human saliva. J Proteome Res. 10(3): 1052-1061.

[88] Ferreira JA, Daniel-da-Silva AL, Alves RM, Duarte D, Vieira I, Santos LL, Vitorino R, Amado F (2011) Synthesis and Optimization of Lectin Functionalized Nanoprobes for the Selective Recovery of Glycoproteins from Human Body Fluids. Anal Chem. 2011;83: 7035-7043.

[89] Salih E, Siqueira WL, Helmerhorst EJ, Oppenheim FG (2010) Large-scale phosphoproteome of human whole saliva using disulfide-thiol interchange covalent chromatography and mass spectrometry. Anal Biochem. 407: 19-33.

[90] Amado FM, Vitorino RM, Domingues PM, Lobo MJ, Duarte JA (2005) Analysis of the human saliva proteome. Expert Rev Proteomics. 2: 521-539.

[91] Huq NL, Cross KJ, Ung M, Myroforidis H, Veith PD, Chen D, et al. A review of the salivary proteome and peptidome and saliva-derived peptide therapeutics. Int J Pept Res Ther. 2007;13:547-64.

[92] Hu S, Loo JA, Wong DT (2006) Human body fluid proteome analysis. Proteomics. 6: 6326-6353.

[93] Schlesinger DH, Hay DI, Levine MJ (1989). Complete primary structure of statherin, a potent inhibitor of calcium phosphate precipitation, from the saliva of the monkey, Macaca arctoides. Int J Pept Protein Res. 34: 374-380.

[94] Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, Troxler RF (1988) Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. J Biol Chem. 263: 7472-7477.

[95] Hay DI, Bennick A, Schlesinger DH, Minaguchi K, Madapallimattam G, Schluckebier SK (1988) The primary structures of six human salivary acidic proline-rich proteins (PRP-1, PRP-2, PRP-3, PRP-4, PIF-s and PIF-f). Biochem J. 255: 15-21.

[96] Shomers JP, Tabak LA, Levine MJ, Mandel ID, Ellison SA (1982) Characterization of cysteine-containing phosphoproteins from human submandibular-sublingual saliva. J Dent Res. 61:764-767.

[97] Kauffman D, Wong R, Bennick A, Keller P (1982) Basic proline-rich proteins from human parotid saliva: complete covalent structure of protein IB-9 and partial structure of protein IB-6, members of a polymorphic pair. Biochemistry.;21: 6558-62.

[98] Isemura S, Saitoh E, Sanada K (1982) Fractionation and characterization of basic proline-rich peptides of human parotid saliva and the amino acid sequence of proline-rich peptide P-E. J Biochem. 91: 2067-2075.

[99] Isemura S, Saitoh E, Sanada K (1980) The amino acid sequence of a salivary proline-rich peptide, P-C, and its relation to a salivary proline-rich phosphoprotein, protein C. J Biochem. 87: 1071-1077.

[100] Bennick A (1977) Chemical and physical characterization of a phosphoprotein, Protein C, from human saliva and comparison with a related protein A. Biochem J. 163: 229-239.

[101] Bennick A (1975) Chemical and physical characteristics of a phosphoprotein from human parotid saliva. Biochem J. 145: 557-567.

[102] Hay DI, Oppenheim FG (1974) The isolation from human parotid saliva of a further group of proline-rich proteins. Arch Oral Biol. 19: 627-632.

[103] Azen EA, Oppenheim FG (1973) Genetic polymorphism of proline-rich human salivary proteins. Science. 180: 1067-1069.

[104] Oppenheim FG, Hay DI, Franzblau C (1971) Proline-rich proteins from human parotid saliva. I. Isolation and partial characterization. Biochemistry. 10: 4233-4238.

[105] Vitorino R, Barros A, Caseiro A, Domingues P, Duarte J, Amado F (2009) Towards defining the whole salivary peptidome. Proteom Clin Appl. 3: 528-540.

[106] Helmerhorst EJ, Sun X, Salih E, Oppenheim FG (2008) Identification of Lys-Pro-Gln as a novel cleavage site specificity of saliva-associated proteases. J Biol Chem. 283: 19957-19966.

[107] Vitorino R, Barros AS, Caseiro A, Ferreira R, Amado F (*in press*) Evaluation of different extraction procedures for salivary peptide analysis. Talanta.

[108] Vitorino R, Alves R, Barros A, Caseiro A, Ferreira R, Lobo MC, Bastos A, Duarte J, Carvalho D, Santos LL, Amado FL (2010) Finding new posttranslational modifications in salivary proline-rich proteins. Proteomics. 10: 3732-3742.

[109] Cabras T, Manconi B, Iavarone F, Fanali C, Nemolato S, Fiorita A, Scarano E, Passali GC, Manni A, Cordaro M, Paludetti G, Faa G, Messana I, Castagnola M (2012) RP-HPLC-ESI-MS evidenced that salivary cystatin B is detectable in adult human whole saliva mostly as S-modified derivatives: S-Glutathionyl, S-cysteinyl and S-S 2-mer. J Proteomics. 75: 908-913.

[110] Streckfus CF, Storthz KA, Bigler L, Dubinsky WP (2009) A Comparison of the Proteomic Expression in Pooled Saliva Specimens from Individuals Diagnosed with Ductal Carcinoma of the Breast with and without Lymph Node Involvement. J Oncol. 2009:737619.

[111] Matros A, Kaspar S, Witzel K, Mock HP (2009) Recent progress in liquid chromatography-based separation and label-free quantitative plant proteomics. Phytochemistry. 72: 963-974.

[112] Ambatipudi KS, Lu B, Hagen FK, Melvin JE, Yates JR (2009) Quantitative analysis of age specific variation in the abundance of human female parotid salivary proteins. J Proteome Res. 8: 5093-5102.