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The Use of Electrophoresis for the Study of Saliva Involvement in Ingestive Behavior

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1. Introduction

Whole saliva contains a wide variety of proteins and peptides, of glandular or blood origin, with diverse functions, namely proteins that participate in teeth and oral tissues protection, as well as proteins related to ingestive and digestive processes [1]. Several studies, in animals and humans, present evidences that saliva is involved in eating behaviour [e.g. [2–5]]. The link between saliva composition and oral perception is increasingly reported. Several studies refer that oral sensations, such as astringency, result from an interaction between food constituents (e.g. polyphenols) and salivary proteins [6]. Moreover, the involvement of saliva in taste sensitivity has been also considered and an example of this relationship is the correlation established between taste dysfunction and reduced levels of the salivary protein carbonic anhydrase VI [7]. Recently, some studies added evidences that protein saliva composition is involved in taste by associating salivary proteins to fat perception and liking [8], as well as to sensitivity for the bitter tastes of 6-n-propylthiouracil (PROP) [9] and caffeine [10]. Besides the involvement of saliva in oral perception, which can greatly influence food choices, the effect of this fluid in ingestive behaviour can also be considered by the role of certain salivary proteins in digestive processes and/or in the regulation of energy intake: one example is salivary α -amylase, which is involved in the digestion of starch in the mouth; another example is the presence in saliva of proteins involved in appetite/satiety regulation such as leptin [11] and ghrelin [12].

Electrophoresis has been applied for salivary protein separation for years and progresses have been made until now. Several studies report salivary protein separation according to their mass, isoelectric point and both parameters (two-dimensional electrophoresis – 2DE), both for human [e.g. [10]] and animal saliva [e.g. [13]]. The application of electrophoretic techniques to saliva samples, together with advances in mass spectrometry, for protein identification, resulted in an increased interest in this biological fluid as a source of biomarkers. In fact, saliva collection has the great advantage of being relatively easy, cheap and non-invasive to perform, presenting proteins also existent in other body fluids such as blood. Besides Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) and 2DE, salivary protein and peptide separation have been described to be achieved through capillary electrophoresis (CE). The possibility of work with small sample volumes and to perform high-resolution analysis is a great advantage of CE [14]. Although only few studies about salivary protein/peptide profiles related to ingestive behaviour have used CE, we believe that this area of research can gain from the use of this approach.

Electrophoresis has also the potential of being used in paraffin embedded tissue samples [15]. This possibility has the great benefit of allowing access to already acquired tissue samples which can be readily correlated with histological parameters. Moreover, it provides access to tissue that would be either difficult to collect prospectively in a timely manner or unlikely to be available as fresh samples. For salivary composition analysis it may have the potential of allowing the study of individual salivary glands composition, in situations where no individualized glandular saliva collection was possible. As such, this may have the potential of aiding in the comprehension of salivary gland regulation and differential contribution for total salivary composition.

This chapter gives an overview of the use of electrophoresis in studies about the involvement of saliva in ingestive behaviour. Studies about the role of salivary protein composition in oral sensations and taste sensitivity will be reviewed. The particularities in the use of electrophoresis in saliva samples will be discussed, as well as the advantages and limitations of the technique for the analysis of this body fluid. The major limitations in the electrophoretic study of salivary proteins, such as the presence of high abundant proteins (impairing the study of scarce ones), the high content of mucins, the high proteolytic activity, among others, will be critically commented based also on our experience. Moreover, the promising use of capillary electrophoresis, as well as electrophoresis of paraffin-embedded tissue samples, for the study of salivary secretion will be proposed. Finally, non-electrophoretic techniques that can give complementary information will be presented, accentuating the importance of integrated approaches in the study of saliva and salivary secretion.

2. The involvement of salivary proteins in food perception and choices

2.1. Taste sensitivity

Taste perception occurs when a sapid molecule (or tastant) activates a taste receptor of the tongue. Taste receptors are located mainly on cells of specialised structures, the taste buds,

which are themselves situated in the clefts of fungiform, circumvallate or foliate papillae. Five basic tastes are commonly admitted: sweetness, saltiness, bitterness, sourness and umami. In addition, discovery of receptors to free fatty acids on the human tongue [16,17] has prompted suggestions that fat could be a sixth taste.

The tongue is constantly lined and hydrated by the oral fluid made of secreted saliva, crevicular fluid, microorganisms etc. Therefore, saliva is intuitively associated to the gustatory function. This assumption applies even more prominently to saliva secreted by the von Ebner's glands (VEG). These minor salivary glands secrete their saliva at the bottom of the clefts of circumvallate and foliate papillae, i.e. in near vicinity to the taste buds. In other words, taste buds bathe in saliva from the VEG, a fact that attracted interest in this type of saliva as playing a role in taste perception. To our knowledge, systemic proteome analysis of VEG saliva has never been performed. However, by studying VEG from rats [18,19] and human subjects [20], two specific proteins have been identified. These are the VEG protein, which is also known as lipocalin 1, and Ebnerin. Lipocalin 1 was found in VEG saliva of human subjects [21]. This protein presents some sequence homology with transporters of hydrophobic molecules, and therefore some authors have proposed that it may help in concentrating and delivering hydrophobic tastants to the specialised gustatory cells [18]. However, in a sensory-based study, lipocalin 1, although measured, was not pointed as an important salivary factor correlated with detection threshold to the taste of oleic acid [22]. Bläker et al. (1993) [20] suggested another function for lipocalin 1, namely that it has a protective effect of taste structures against the detergent action of fatty acids. Concerning Ebnerin, it has been found only in rats, more specifically released into the clefts of circumvallate papillae [19]. The putative function attributed to this protein is to carry growth factors which would be expressed in VEG [19]. To conclude with VEG saliva, despite the proximity of VEG and taste buds, relatively little evidence is provided for involvement of VEG saliva proteins in taste perception.

Salivary proteins or peptides have been related to taste perception, acting at different levels (Figure 1). Some of these proteins/peptides found in whole saliva have been suggested to act as factors implicated in the growth and renewal of taste buds. For example, carbonic anhydrase 6 (CA6 or gustin) levels are reduced in hypogeusic patients [23] and such patients also present taste bud anatomical abnormalities [24]. CA6 was therefore considered as a trophic factor involved in taste bud growth and development. Leinonen et al. [25] later suggested that CA6 may also have an anti-apoptotic action on taste buds by locally regulating pH. More recently, the perception of the bitter compound 6-n-propylthiouracil (PROP) was also associated to the protein CA6. However, it was not the quantitative expression of the protein that was different between PROP tasters or nontasters, but rather the ability of CA6 to bind zinc which conditions its functionality. This ability differed according to genetic polymorphism [26], although some controversy appears to exist, with a recent study lacking to obtain similar results [27]. Another protein, metalloproteinase MMP-3, is under-expressed in saliva from subjects with taste disorders and may serve in continuous regeneration of taste buds [28]. Finally, the epidermal growth factor (EGF) secreted by the parotid and submandibular glands might assist in maintaining the morphological integrity of taste buds. In rats, for instance, removal of major salivary glands alters greatly the appearance of the taste buds of fungiform papillae but oral

administration of EGF restores a normal morphology [29]. Whether salivary EGF is linked to taste in humans has not been demonstrated to our knowledge.

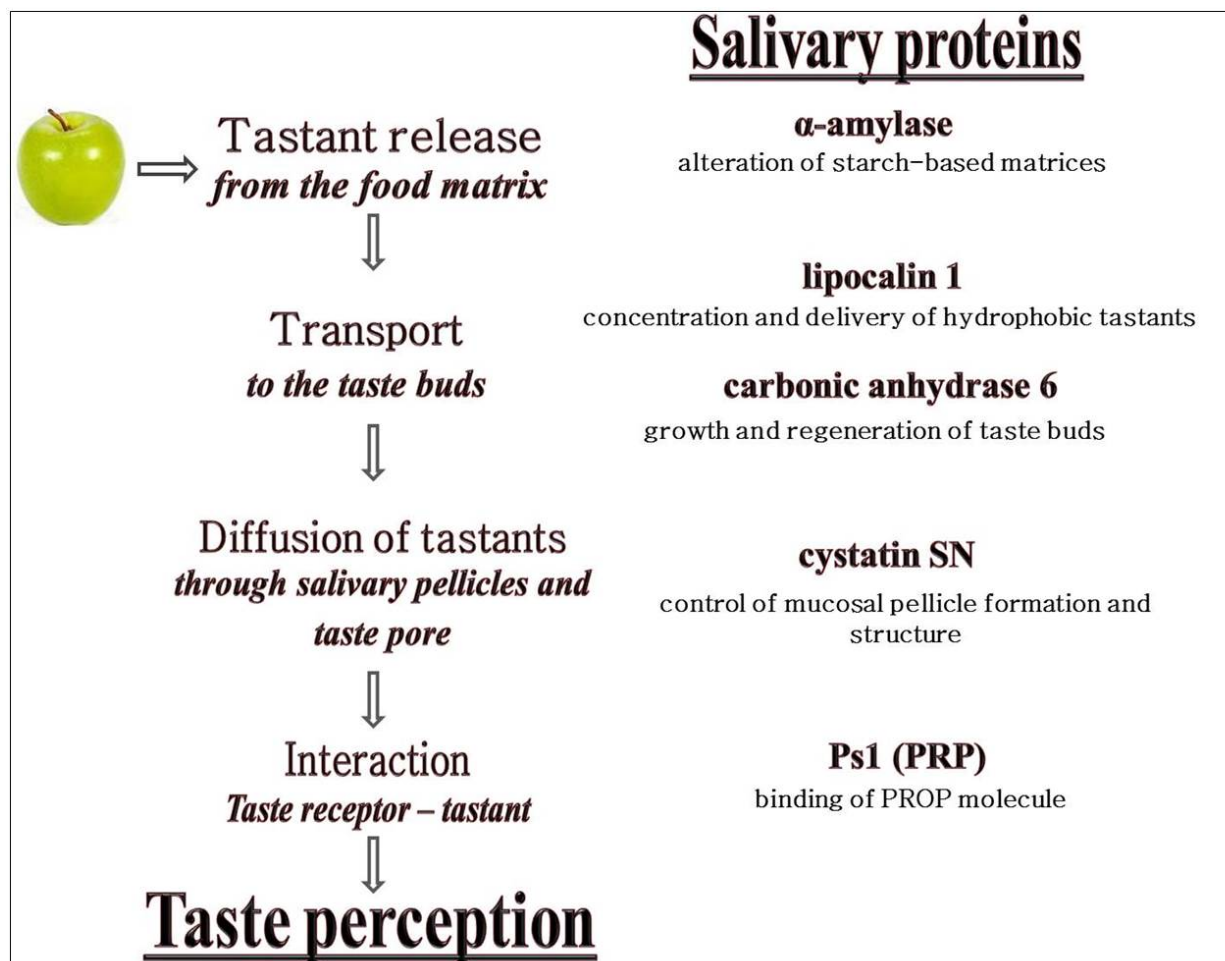


Figure 1. From food to taste perception: examples of salivary proteins involved in taste function (right – examples of salivary proteins potentially involved in each of the phases of taste perception described on the left)

Another impact of salivary proteins on taste perception resides in their direct physico-chemical interaction with taste molecules, which modifies the availability of tastants to the taste receptors. For example, Wada et al. (2010) [30] have shown that histatin 5 concentration was significantly lower in subjects hypersensitive to the taste of quinine, and the authors further demonstrated that histatin 5 could bind quinine. For a given quantity of quinine, subjects with lower levels of histatin 5 had therefore more free quinine which could interact with their taste receptors, thereby increasing their sensitivity. Another example of salivary protein – tastant interaction has been suggested by [31] based on the demonstration by ^1H -NMR that the PROP molecule could interact with the arginine and lysine amino-acids. This investigation followed a study where top-down analysis of saliva of supertasters *vs* nontasters of PROP revealed that saliva of supertasters had higher levels of basic proline-rich proteins (PRPs), in particular the Ps1 protein which contains a high proportion of arginine and lysine [9]. In this particular case,

higher proportion of Ps 1 and the consequent binding of PROP would confer to the molecule a new conformation, which would facilitate its access to its taste receptor. Finally, the interaction of oleic acid with the salivary protein zinc- α -2glycoprotein (ZAG) has been suggested, on the basis that this protein was over-expressed in a group of subjects hypersensitive to the taste of this fatty acid [32]. This protein may, for example, act as a solubilizer of oleic acid in saliva.

In addition to these two mechanisms (growth or protection of taste buds/physico-chemical interactions with tastants), it is also possible that the structured biological layer covering the tongue surface, and made mainly of salivary proteins, may contribute to modulate accessibility of tastants to the taste receptors. This hypothesis has been formulated by Dsamou et al. (2012) [10] and deserves further investigations. In this context, one should focus in particular on the “taste pore material” which is the biological layer covering directly the taste buds, described for example by Matsuo (2000) [33].

2.2. Mechanical sensations (astringency)

Physical properties of foods are also important factors in consumer appreciation and enjoyment of food products. Salivary proteins are known to modulate food tactile characteristics by interacting with food constituents. The involvement of salivary proteins in food perception started to be studied in terms of their effect in astringency development. Astringency is described as “the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins” by the American Society for Testing of Materials [34]. Astringent molecules are mainly plant-based products, namely polyphenols, among which tannins [35], but also acids and metal alums [6] and other dehydrating agents such as alcohols [36], which can also create this subjective oral feeling.

Astringency has been considered as an aversive quality responsible for the lowering acceptability of some plant food products [37,38]. Two possible mechanisms were proposed to explain this oral sensation. The oldest belief is that astringency is due to the precipitation of salivary proteins by astringent substances, with such precipitates increasing friction between mouth surfaces and stimulating mechanoreceptors [39]. On the other hand, a number of studies suggest that astringency is the result of modifications in the viscous elastic properties of glycosylated proteins, which result in the rupture of the lubricating saliva film that lines the oral cavity [40–43]. Nayak and Carpenter [43] proposed a more integrative view, suggesting a two-step interaction between salivary proteins and polyphenols (and/or other astringent substances). According to this model, saliva is considered to be composed of two different phases, a thin dynamic film coating the internal oral surfaces and an adsorbed layer of proteins on the hard and soft tissues. In the first step of interaction, the astringent molecules may bind the proteins from the dynamic film with the highest binding affinity. In the second step, the remaining astringent molecules, not bound in the first step, can interact with the adsorbed glycoprotein layer, with the consequent oral cavity loss of lubrication and astringency development.

Different families of salivary proteins have been considered as having a specific role in astringency of polyphenols, due to its high affinity for these compounds: PRPs [44], histatins [45], statherins and cystatins [46]. These salivary proteins represent a considerable part of the

saliva total protein content and have important biological functions, in this oral fluid, associated with calcium binding to enamel, maintenance of ionic calcium concentration (PRPs and statherin), antimicrobial action (histatins and cystatins), or protection of oral tissues against degradation by proteolytic activity (cystatins) [47]. The nature of the interaction between these salivary proteins and polyphenols depends on several factors, among which protein characteristics and the type of polyphenol. Salivary proteins such as acidic PRPs and statherins present lower selectivity towards polyphenol structures, comparatively to histatins and cystatins [46]. Among these, salivary PRPs were by far the most studied, being generally considered as the main family of salivary proteins involved in astringency. PRPs have an extended structure allowing them to have a high affinity to bind tannins [48]. Basic PRPs have been suggested as having a primary role in the prevention against the negative antinutritive and/or toxic effects of these polyphenols [37,49]. The induction in the secretion of basic PRPs by the regular consumption of tannins reinforced this hypothesis [50]. Additionally, glycosylated PRPs, which are thought to contribute to salivary lubrication [51] also interact with tannins [52,53] and, consequently, these are proteins with potential impact in astringency.

Mucins also seem to have a role in astringency, although some controversy exists among studies. These proteins are the main responsible molecules for the viscoelastic properties of saliva. Some authors reported the binding of mucins to polyphenols, and consequently an effect on astringency development [54]. On the other hand, other authors [e.g. [36]], observed that these proteins are precipitated by alum and acid, but not by polyphenols, suggesting different involvement according to the type of astringent molecule.

From all these different studies, with different suggestions about astringency mechanism, what appears to be unequivocal is the complexity of this oral sensation, with multiple mechanisms potentially involved in its development, and the participation of salivary proteins.

3. Usefulness of electrophoresis for ingestive behaviour analysis using saliva

3.1. Methodological issues related to the use of electrophoresis in saliva study

Accurate examination of salivary components requires optimal collection, processing and storage conditions and, as such, there is a need for standardized protocols [55,56]. Salivary secretion is mainly regulated by the two branches of the autonomic nervous system (both sympathetic and parasympathetic), and as such it presents some plasticity, changing in amount and composition according to the nature of the stimulus. Factors such as circadian rhythm [57], gender [58], drugs [59], exercise [60], eating [61], among others, result in variations in saliva composition. Based on that, it is important to define the protocol of collection method. Firstly, it must be determined if the collection of saliva will be performed without or with stimulation. According to the first, the most used procedure consists in allowing saliva to drip off the lower lip into a tube maintained on ice. Concerning stimulated saliva, it is frequently obtained after parafilm mastication, or after sour taste

stimulation [62]. Advantages and disadvantages of each approach have been mentioned previously [63]. Another issue to be considered is the origin of collected secretions, i.e., whether it is glandular or whole saliva, since considerable differences exist in composition. Reported storage conditions of saliva samples have varied widely among studies, with no current consensus on optimal procedures. Saliva contains many salivary proteins that are processed by post-translational modifications (PTMs), namely glycosylation, phosphorylation, sulfation and proteolysis, these modifications being responsible for many of this fluid functions. Consequently, de-glycosylation, de-phosphorylation and proteolysis should be minimized, and different research groups have employed different methods with this purpose. For example, the addition of ethanol apparently allows the storage of saliva samples at room temperature (for a period of about two weeks) without considerable changes [64]. The use of protease inhibitors to prevent proteolysis was also referred as allowing saliva storage at 4°C, during approximately 2 weeks, without significant degradation [64]. Nonetheless, it is known that not even an inhibitor cocktail can prevent all protein degradation [65]. It is important to note that the ideal handling and storage procedures will greatly depend on the peptides/proteins of interest. Whereas working on ice for no longer than one hour, with subsequent storage of samples at -80°C, has been considered a safe and practical handling protocol [65,66], recovery of salivary immunoglobulin A (sIgA) has been suggested to be greater for short storage times at room temperature than when samples are stored at low temperatures [67]. Freeze-thaw cycles are also not desirable, inducing protein precipitation, in particular from low molecular mass components, resulting in loss of some proteins expression and particularly enzymatic activity [65]. In any case, little research has been directed on ways of minimizing degradative processes, and this is clearly needed [56].

Another methodological issue, in electrophoresis of salivary proteins, concerns the presence of particular proteins in high levels, obscuring the low abundant ones, for which analysis may be of interest. This is even more pertinent considering studies aimed at detecting physiological biomarkers, since most of them are present in saliva at low amounts. The protein α -amylase contributes to almost 60% of total salivary proteome [68] and, many times, its depletion is necessary. Salivary α -amylase depletion can be achieved through elution of samples from starch columns to reduce this protein amounts specifically [64,69].

Saliva contains a diverse array of proteins and particularities of each type should be considered when choosing the staining procedure. PRPs, which exist in considerable amounts in parotid saliva, contain reduced amounts of amino acids containing sulphur. Consequently, these proteins are not easily stained with silver [70]. On the other hand, these proteins stain violet-pink with Coomassie Brilliant Blue R-250, particularly when a destain protocol consisting in 10% acetic acid, instead of 10% acetic acid/10% methanolis used [71]. Phosphoserine, phosphothreonine and phosphotyrosine containing proteins are detected at low levels by using Pro-Q-Diamond [72]. Periodic-acid Schiff (PAS) is used for glycoprotein staining. Nevertheless this procedure presents the limitation of needing high levels of protein load [73]. When the amount of sample is limited and higher sensitivity is needed, Pro-Q-Emerald can be a good

option for glycoprotein detection, since it can be approximately 50-fold more sensitive than PAS [74].

Some particular characteristics of saliva should also be taken into consideration for Western blotting. It is important to be sure that samples are being compared for an equal amount of total protein. Since by using this technique only the protein that react with the antibody is visualized, the existence of a control of the quantity of protein loaded is important. In some types of samples there are proteins which levels are proportional to the amount of protein loaded, and as such, they can function as internal controls. In these cases, the simultaneous use of primary antibodies for these proteins and the protein of interest may allow adjustments [e.g. 75]. Nevertheless, in saliva a protein which relative amount to total protein remains constant is not known. One way to circumvent this limitation is through the staining of the membrane with a reversible stain (e.g. Ponceau), before incubation with the primary antibody, in order to visualize the several bands present in each lane [76, 77].

Although needing studies to elucidate that, it is possible that some salivary mucins and/or other proteins can irreversibly adsorb to cotton roll, and, consequently, being lost. Another possible drawback, when working with animal saliva is the low amount produced by some species. For example, for small rodents, which are frequently used as human and animal models, saliva collection is not easy without stimulation. The use of parasympathetic agonists, such as pilocarpine, is often used, since it induces the increase in the volume secreted without changing the relative amount of each protein. On the other way, the use of sympathetic agonists (e.g. pilocarpine) is also frequent, but in this case, when the amount of protein concentration is wanted. This type of stimulation induces the synthesis and secretion of proteins from salivary glands, but a relatively low volume [79].

Most of the gel-based approaches need further protein identification, most of the times by mass spectrometry. Although advances have emerged, in the last years, for some animal species, salivary proteins identification continues to be challenging, due to the lack of complete and annotated genome and protein sequences [81]. When this happens, the search in other related species databases is needed, increasing the possibility of to rise the number of false positive results.

3.2. The use of SDS PAGE and 2-DE in salivary proteome study related to ingestive behaviour

For both humans and animals, it has been observed that 2DE is the most popular technique for the global analysis and initial profiling of saliva, being used as a first step for protein separation, followed by mass spectrometry (MS) or tandem MS (MS/MS) [82]. 2DE simultaneously separates proteins according to their isoelectric points (*pI*) and molecular masses, enabling the visualisation and identification of several thousand proteins on a single gel. It is particularly useful for screening and comparing complex mixtures such as saliva samples. By opposition to the study of several different diseases, the use of saliva for biomarker discover in the field of nutrition only more recently started to emerge. One of the greatest advantages of 2DE in salivary protein study relates to its capacity of separating proteins with different PTMs, allowing their separate quantification. Many salivary proteins present different

isoforms [e.g. [83]], with only some of them potentially related to taste sensitivity, making this individual isoform comparison essential.

Separation of proteins from mixed saliva, by SDS PAGE and 2DE, coupled to MS for protein identification, allowed a better understanding about the relation between saliva and taste. In 2DE profiles, the expression of some protein spots were observed to be differently changed following taste stimulation, these differences being dependent on the type of taste stimuli [84]. This suggests different adaptations of saliva to the five basic tastes. Concerning bitter taste, the use of these techniques also added a great contribution to advances of knowledge in this field. Until recently, only salivary CA6 was reported to influence taste sensitivity, whereas now it is known that other salivary proteins may also be involved. For example, by using 2DE based approaches, cystatin SN was identified as being differently expressed among groups of sensitivity to the bitter taste of caffeine [10]. In addition, recently, using SDS PAGE for separation of proteins from mixed saliva of 3-month-old-infants, differences in salivary profiles related to bitterness acceptance were reported: higher abundance of zinc-alpha-2-glycoprotein and CA6 was associated to a lower acceptance of this taste, whereas higher abundance of lactoperoxidase, S-type cystatins and prolactin inducible protein was associated to a higher acceptance [32,85].

Electrophoresis also proved to be useful in studies aimed at understanding astringency, namely in the search for the salivary proteins involved in the development of this oral sensation, as well as for assessing the astringency of some food compounds. Several different studies used SDS PAGE and 2DE to compare salivary protein profiles before and after tannin ingestion, and to identify salivary proteins interacting with these plant secondary metabolites [e.g. [86,87]]. Dinella et al. [88,89], by separating proteins through SDS-PAGE, presented evidences that differences among individuals in sensitivity for astringent stimuli relates to the differences they present in salivary protein composition after repeated stimulation, i.e., individuals in which tannin stimulation results in higher changes in salivary protein profiles are the ones who strongly detect astringency. The evaluation of polyphenol astringency was also demonstrated to be possible by using SDS PAGE to separate the salivary proteins present in human saliva after mixture with these plant secondary metabolites [54].

3.3. New perspectives for electrophoresis in saliva study

3.3.1. Capillary electrophoresis: potential and use in saliva study

Capillary electrophoresis (CE) is a separation method, performed in capillaries, in which analytes migrate through electrolyte solutions under the influence of an electric field. The electrophoretic velocity of the analyte will depend upon the magnitude of the electric field and its electrophoretic mobility, and upon the rate of electroosmotic flow.

In the last years, some studies surged highlighting the potential of CE in several different areas of research [e.g. [14]]. A great advantage of this over the conventional electrophoretic techniques is that, although also based on the movement of molecules in an electric field, CE is not restricted to the separation of large molecules based on size or charge. As such, it allows also the separation of molecules that have low molecular mass and/or neutral charge. Moreover,

CE has the advantage of working with considerable small volumes of sample (from picoliters to nanoliters) [14], what in saliva study may be important.

The commonly separation modes by CE are capillary zone electrophoresis, capillary gel electrophoresis, capillary isoelectric focusing, micellar electrokinetic chromatography and capillary isotachopheresis. Capillary zone electrophoresis, also known as free solution capillary electrophoresis, is the simplest form of CE. The application of the sample occurs in a narrow zone or band, with the separation buffer surrounding it. With the application of the electric field, the migration of each component in the sample occurs according to its own mobility. In this method, non-charged molecules cannot be separated, since this will move at the velocity of electroosmotic flow.

Capillary gel electrophoresis is based on the use of a polymeric gel medium, inside the capillary, which acts as a molecular sieve in which smaller molecules migrate faster than larger ones. This method has the advantage of separating molecules that have similar charge-to-mass ratios, which in the absence of the gel medium (i.e. in free solution) would have similar electrophoretic migration rates. Capillary isoelectric focusing is a type of CE that offers significant advantages over isoelectric electrophoresis slab gels in terms of automation, separation speed and quantitation. Although at the beginning capillary isoelectric focusing was not readily accepted, due to the difficulties in isolating the focused zones for the detector, actually this separation technique presents the highest resolution of all charge based separation techniques, due to nowadays systems that allow the monitorization of the whole column at once [90]. Micellar electrokinetic capillary chromatography is an electrophoretic technique developed in the early 90's that extends the applicability to non-charged molecules, which cannot be separated using simple free solution CE [91]. This technique is based on the combined effects of: 1) the differential partitioning of molecules between the aqueous buffer and the micellar phase; 2) the differential migration of ionic species. It consists in the separation as the result of the combined effect of the differential partitioning of molecules between the aqueous buffer and the micellar phase, as well as any differential migration of ionic species. The sample is inserted between a leading electrolyte, which as a higher mobility than ions in sample zone, and a terminating electrolyte, with lower mobility, relatively to these last. The separation achieved through capillary isotachopheresis based on differences in the velocities of analyte ions within the sample zone.

CE can help to circumvent some limitations in the study of saliva. One constraint of searching for salivary protein biomarkers is that they may be massively diluted, requiring highly sensitive analytical approaches, often exceeding the dynamic range of currently available proteomic platforms. Moreover, and although saliva collection is non-invasive, sometimes it is not possible to have access to large volumes. Different CE-based approaches have been described in the study of salivary protein components. Conventional electrophoretic analysis of parotid saliva by SDS-PAGE is hampered by the fact that a number of proteins, such is the case of PRPs, are only poorly stained by currently used stains (as it was described in section 3.1). In this context, CE was suggested to offer advantages for the quantitation of the main components of parotid saliva, including α -amylase and a number of PRPs [92]. Analysis of salivary peptides, have also been report-

ed, namely separation of histatins [93], which are a group of neutral to basic low molecular-weight proteins of human saliva with relevant antifungal properties. Moreover, substance P, a peptide present in saliva at trace levels, in the picomolar to nanomolar range, that functions as a neurotransmitter and/or as neuromodulator, has been analysed in this fluid by a CE method [94], circumventing the problems of detection of such small amounts. Another challenging approach has been presented in the case of secretory immunoglobulin A (sIgA) that was analysed in saliva by CE in association with immuno-fluorescence-labelling detection, which was considered an enormous advantage over radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) usually used to assess the amounts of this salivary protein [95]. For proteome study, capillary isoelectric focusing has been used in combination with nano-reversed phase HPLC and mass spectrometry to profile and identify over a thousand salivary proteins [96]. The use of transient capillary isotachopheresis/capillary zone electrophoresis had been used for allowing the reduction of high-abundance proteins such as amylases, mucins, PRPs, and sIgA complex, what provides unparalleled advantages toward the identification of low-abundance proteins [97].

Saliva study based on the use of CE was not limited to the separation of proteins and peptides. Fluorescein-labeled amines were separated and detected in saliva using a microchip CE apparatus [98]. Additionally, some researchers have used this separation technique for the analysis of inorganic ions in saliva. The UV absorbing anions nitrate, nitrite and thiocyanate were determined in saliva by micellar electrokinetic capillary chromatography employing N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (zwittergent-3-14) as a surfactant additive to the BGE [99]. Other slightly different micellar electrokinetic capillary chromatography procedures were optimized for these ions and are described elsewhere [e.g. [100–102]].

In salivary studies related to taste sensitivity and ingestive behaviour, the use of CE-based approaches may therefore offer advantages. Besides the already reported advantages for the study of saliva, some of the results obtained so far suggest the potential involvement of peptides in taste sensitivity and ingestive behaviour [e.g. [10]]. Hormones and peptides related to ingestive behaviour, known to be present in saliva are present in relatively low amounts (e.g. PYY, leptin and ghrelin) [12,103], and in this context their analysis can also benefit from some of the advantages in CE. Nonetheless, this is an issue deserving further attention.

3.3.2. Electrophoresis in formalin-fixed and paraffin-embedded tissue samples

The fixation of tissues in formalin followed by embedding in paraffin is the standard tissue fixation and storage method adopted by most health institutions of pathology and histology departments, for research on biomarkers and molecular mechanisms of diseases. A great deal of information on proteins involved in many biological aspects is encased within these formalin-fixed paraffin-embedded (FFPE) tissue repositories, which offer an extensive resource for conducting retrospective and prospective studies.

The FFPE tissues are highly stable, even stored at room temperature, with cell or tissue structure being mainly preserved. The stability of proteins is achieved through cross-linking, with multiple reports showing that protein modifications, such as phosphorylation are

maintained and can be determined years later by immunohistochemistry [104]. As such the FFPE tissues have the potential to constitute a valuable source of samples for proteome analysis using electrophoresis [105,106]. Protein extraction from FFPE tissue samples allows the generation of protein profiles by high-throughput techniques, providing an improvement over other traditional methods such as immunohistochemistry [107,108].

However, many questions remain in terms of quantification of the expression levels of proteins extracted from FFPE tissue samples and this approach has proven to be a daunting task with success varying according to the biomarker of interest and the tissue type [109,110]. In fact, the protein extraction efficiency is reduced by the extensive molecular crosslinking that occurs upon formalin fixation, with possible interferences in immunoreactivity or even in protein identification by mass spectrometry [111], imposing the development of efficient extraction methods [109,110,112,113]. The achievement of protein recovery with minimal losses in immunoreactivity is particularly relevant in studies needing immunoblotting. Several studies report methods of collecting and processing tissues directly from selected areas of histological sections using different tissue types [see extensive review in [113]], including salivary glands [114–116]. Most studies revealed comparability between protein extracts from FFPE and from unfixed fresh frozen tissue samples by different validation methods including SDS–PAGE and Western blotting [105,109,114,116,117].

The use of alternative fixatives, their advantages and disadvantages and potential for performing both morphological and molecular analyses on paraffin-embedded tissue sample, for proteomic applications and genome-wide expression analysis have been extensively investigated [114,117–120]. Another alternative for overcoming the problems posed by fixation with formalin passes through the development of robust long-term room temperature biospecimen tissue storage technologies that provide high quality nucleic acids or proteins [121].

Functional and morphological/histological changes of salivary glands are associated with various diet-related diseases, such as diabetes [122,123], hyperlipidemia [124], and obesity [125]. Moreover, the physical and chemical properties of foods affect salivary glands morphology and histology [126–129]. Although the regulation of the secretory activity of salivary glands has been almost exclusively attributed to the autonomic nervous system [130], recent evidences suggest that during variations in appetite and food intake the glands are probably also regulated by gastric (gastrin) and intestinal (cholecystokinin and melatonin) hormones [131–133]. Additionally to the peptide-hormones involved in short-term regulation of food-intake, the action of leptin in the salivary gland should not be discarded considering that the the presence of leptin receptors in salivary glands suggests *in situ* effects of this hormone [134,135]. As such, not only research in saliva fluid, but also in salivary glands, is valuable in studies related to ingestive behaviour.

Nonetheless, with optimized extraction methods, FFPE tissue samples can be a valuable source of protein, allowing reproducible and biologically relevant proteomic profiles for research on biomarkers and biological molecular mechanisms [109,110,112,136], with particular interest in systematic analysis of saliva and salivary gland feed-back regulation. However, despite the number of studies taking advantage of electrophoresis in FFPE tissue samples, currently, there is no consensus on the optimal protocol for protein extraction, neither accepted standards for

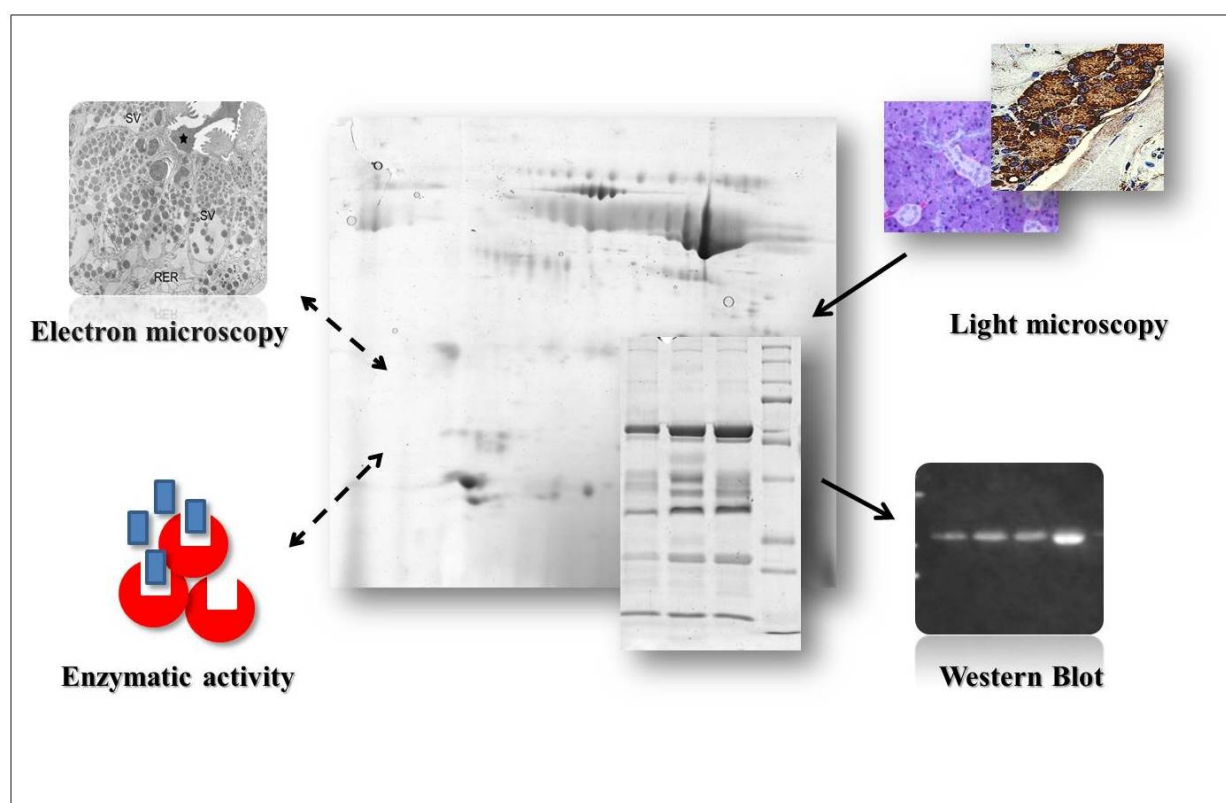


Figure 2. An integrative approach for studying saliva: techniques that can complement electrophoresis.

quantitative evaluation of the extracts. As such, further research is recommended to develop standardized methods ensuring quantitative and qualitative reproducibility in the protein recovery.

3.4. Complementarity of techniques — Contribution to a best case scenario

The potential of the different electrophoretic techniques, for the study of salivary secretion related to food perception and ingestive behaviour, alone or in combination, have been presented so far. However, the knowledge in this research area can be increased by combining different techniques that can give information which cannot be obtained through electrophoresis (Figure 2).

3.4.1. Enzymatic activity analysis

Some of the most abundant proteins in saliva are enzymes. These are the cases of: α -amylase, which, among several functions in saliva, begins the process of carbohydrate digestion in the mouth; and CA6, responsible for the maintenance of pH in oral cavity. Both of these enzymes are described to play a role in oral food perception and/or taste sensitivity, since they may contribute to changes in the concentration of simple sugars in the mouth and affect the viscosity of starch based products, in the case of α -amylase [137], or influence the conditions of the medium surrounding taste buds, in the case of CA6 [7].

The knowledge about the expression of salivary enzymes is important and can be achieved through electrophoretic techniques. However, proteome by its own does not represent, necessarily, the functionality of these proteins. The complementarity between enzymatic activity data acquisition and electrophoretic protein profiles is also valuable for enzymes present in several different isoforms, being α -amylase and CA6 two examples [83]. In most of the cases the different isoforms are not all involved in the same physiological pathways and frequently express differences in activity and regulation motifs. Enzyme activity is dependent namely on the allosteric and fine-tuned phosphorylation/dephosphorylation regulation processes, thus reflecting an average of the activity of several isoenzymes present in the sample in a specific environment. For example, α -amylase activity depends on the presence of calcium and chloride [138], and changes in the amounts of these ions result in variations in enzyme activity.

Moreover, although two-dimensional electrophoresis allows the separation, visualization, and even the quantification, of the different isoforms (based both in different molecular masses and pI s), as well the discrimination of isoforms resulting from different PTMs, the major drawback of this technique resides in the fact that, by being an at least partially denaturing method, it does not allow the identification of the native structure of an enzyme but its subunits only. In this context, enzyme activity analysis offers functional information that is complementary to proteome analysis by electrophoresis. The acquisition of electrophoretic profiles and enzymatic activity data, simultaneously, can greatly amplify the physiological interpretation of results, being valuable in the analysis of the interaction saliva-food perception. Previous results, from studies in obesity, obtained in our laboratory, present evidences of the meaning of such complementarity, showing that minor changes in protein expression, may be associated with significant differences in enzymatic activity [139]. For instances, a higher α -amylase activity was not accompanied by changes in the expression of this protein, evidenced by western blot analysis. Despite the unchanged expression, it was hypothesised that this increase in enzymatic activity could be related to changes in sweet food perception and acceptance, reported by other authors for obese individuals [140]. Moreover, the evaluation of enzymatic activity has the advantages of being relatively inexpensive, in comparison with some electrophoretic techniques and of offering the possibility of being almost fully automated. The most common methodologies applied are dependent on spectrophotometry or fluorimetry techniques. The use of the multi-well plate readers allows the analysis of a high number of samples, with minimal reagent consumption.

3.4.2. Microscopy techniques

Changes in salivary proteome related to food perception and ingestion can be better understood if complemented with microscopy imaging techniques. These may improve the knowledge about the secretory mechanisms and cell type contributing for the secretion of salivary proteins, its expression and localization, i.e. information not possible to obtain through electrophoretic salivary protein profiles alone. Different studies have used different microscopy based techniques in salivary gland research. Some examples are: the observation of subcellular distribution of lysozyme in the mouse major salivary glands, the

intragranular compartmentation of this secretion enzyme and its relationship with α -amylase using light and electron microscopy [141]; the study of contraction of myoepithelial cells in the human submandibular gland using confocal microscopy [142]. Moreover, the combination of electrophoretic and immunohistochemical techniques have been used to evaluate the distribution and expression of several proteins, such as CA6 in minor and major salivary glands of humans [25,143], leptin and the functional leptin receptor in major salivary glands of humans [135], muscarinic receptor subtypes in salivary glands of rats, sheep and man [144], aquaporin water channels in rat major salivary glands [145,146], and cytoskeleton proteins in rat parotid glands [147].

The salivary glands are a good model for the study of exocrine secretion. The proteins that will be secreted are synthesized in the endoplasmic reticulum and transported through the Golgi apparatus to the trans-Golgi network where they are stored in secretory granules (SCGs) after which they are released into the cytoplasm and transported to the periphery of the cell [148]. Electron microscopy studies have shown that the secretory cells of salivary glands are ultrastructurally equipped to produce and store large amounts of secretory proteins in secretory granules (SGs) [149]. The volume and number of SGs per cell are a morphological evidence of the storage capacity of the secretory cells. Regarding the secretory cell activity, the number and development of organelles, as well as membrane cell specializations, are ultrastructural traits associated with the mechanism of secretory production. A recent study reported differences in secretory activity and volume of saliva secreted by goat parotid glands (PGs) between liquid and solid diets: milk-suckling kids (MSKs) and diet-fed goats (DFGs) [150]. Such differences were correlated with the ultrastructure of the secretory cells. In PGs of DFGs a well-developed system of membrane specializations was identified, which was not prominent in PGs of MSKs. Such a characteristic is typical of cells in phase of intense secretory activity [151]. Moreover, the presence of well-developed smooth muscular cells around the secretory endpieces of the PGs of DFGs, but not in MSKs, is a morphological evidence of the release of large amounts of secretion into the acinar lumen. This is also supported by the low number of SGs observed in the apical cytoplasm of the secretory cells of DFGs as compared with that in MSKs.

The ultrastructure of the salivary glands can also reflect the chemical composition of the secretion and the subcellular localization of the salivary proteins of interest. Secretory cells of the three major types of salivary glands possess SGs with variable appearance and density under electron microscopy. Presumably, these morphological features of the SGs are indicative of constituent differences in secretory protein content [152]. Immunohistochemistry coupled to electron microscopy proved also to be useful by providing an increased knowledge of localization and distribution of the different salivary proteins in the parenchyma of major and minor salivary glands [e.g. [153,154]]. From the different studies, evidences arrived that different proteins can be differently distributed among secretory granules and that different mechanisms can be involved in the packaging of the different salivary proteins. In conclusion, the electron microscopy coupled to the cytochemistry may provide valuable knowledge on cell biology of the salivary glands. This information is a basis to know the morphological and functional diversity of these glands, which allows understand-

ing the modes of production and secretion, as well as the types of secretion correlated with ingestive behavior.

4. Concluding remarks

Saliva study received considerable attention in the last years, particularly due to the potential of this fluid as a non-invasive source of biomarkers. Despite the focus had been put mainly in pathological conditions, it is known that salivary proteins can be useful for the understanding of physiological mechanisms. Research in the area of ingestive behaviour can be improved with the study of saliva. Salivary proteins, besides participating, by different manners, in food perception, may also be markers of taste sensitivity and/or food choices with potential application in the management of dietary programs.

Despite the existence of limitations, electrophoresis continues to be an essential tool in the study of salivary proteome. It constitutes the basis 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. Several human and animal salivary proteins, involved in taste perception and food choices, have been separated through electrophoretic techniques, for further identification. Nevertheless, enhanced methodologies for sample fractionation and processing might be useful to circumvent some of the limitations in the study of this fluid by electrophoresis. The possibility of using electrophoresis in samples obtained from formalin-fixed paraffin embedded tissues, for different purposes, has been presented as a great advantage for the study of long-term stored samples. However, this approach has rarely been applied to non-malignant salivary tissue samples, being mainly used in quantitation of protein biomarkers in clinical tissue specimens. Non-electrophoretic techniques, such as microscopy and/or studies of enzymatic activity, can greatly add in saliva research related to ingestive behaviour. Knowledge in secretion induced by dietary compounds can benefit from structural and ultra-structural information. Moreover, protein expression is not necessarily concordant with enzymatic activity.

In conclusion, new and improved approaches will be valuable to cope with the challenges in understanding the influence of saliva in food perception and ingestive behaviour. The study of saliva appears to be promising in biomarker discovery contributing for the understanding of food choices, hence having major impact in human and animal health, and major fundamental relevance for animal production. As such further integrated and systematic analysis of saliva, combining old and new approaches, will profit from complementarity of different methodologies.

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