Comparison of protein precipitation methods for two-dimensional electrophoresis of dog salivary proteins

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ABSTRACT

Despite saliva being one fluid with growing interest as a source of biomarkers, both in humans and animal models, few studies have been reported that use proteomic approaches for canine saliva analyses. Two-dimensional electrophoresis (2-DE) is considerably used in biomarker research and its use for dog saliva study may had relevant knowledge about pathology/physiology. The quality of the results obtained using 2-DE greatly depends on sample preparation. Different protein precipitation methods are frequently used for removing interfering compounds and concentrating samples, but their efficiency varies according to sample characteristics. For dog saliva samples no information was found about the best precipitant and precipitation method for electrophoretic protein profiling.

In this study, six different protein precipitation methods were compared. Precipitation of dog salivary proteins with 20% (w/v) trichloroacetic acid (TCA) resulted in lower protein recovery rate than other methods tested, but allowed protein profiles highly correlated with the ones from original samples. Moreover, this protocol resulted in good protein separation in 2-DE, with the visualization of spots from salivary proteins not observed when samples were treated using other methods. Based on this, we propose the use of TCA for dog saliva whenever precipitation is needed for protein profile analysis.

Keywords: dog, saliva, proteins precipitation, two-dimensional electrophoresis

1. Introduction

Saliva is a clear fluid mainly composed of electrolytes, immunoglobulins, proteins and enzymes [1]. Whole saliva is mainly a mixture of the secretions from the major and minor salivary glands, and oral mucosa, periodontium and oral microflora, which also contribute to its final content. Therefore, whole saliva represents a complex balance among local and systemic sources. Mostly in humans, this has been allowing for the application of saliva in the diagnosis not only for salivary gland disorders but also for oral diseases and systemic conditions such as, among others [2-7]: periodontitis and dental caries; type-2-diabetes mellitus;

obesity; several infections; cancer in a number of tissues; human immunodeficiency virus (HIV).

The interest in the characterization of biological fluids protein composition has increased in the last few years, for both scientific and veterinary routine advancement [8]. The non-invasive and simple nature of saliva collection allows for repetition and multiple collection of biological material with minimally trained personnel and in a stress-free, painless, and economically viable manner [1,9]. However, saliva of different animal species, including the dog, is still little studied. Thus, protocol optimization for saliva analysis is firstly required.

Two-dimensional gel electrophoresis (2-DE) is one of the

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most popular techniques for the global analysis and initial profiling of salivary proteome for both humans and animals. 2-DE 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. One of the greatest advantages of 2-DE in salivary protein study relates to its capacity of separating proteins with different post-translational modifications (PTMs), allowing their separate quantification.

One of the constraints of using 2-DE in animal saliva samples is the difficulty to obtain saliva volumes sufficient to achieve the relatively high amount of proteins needed for performance of this technique. In dogs, in order to obtain high volumes of saliva may be not easy: thus different authors reported methods for stimulating saliva production, based on use of citric acid [10,11], soaking cotton rolls [12], or use of beef-flavoured cotton ropes [13]. However, these techniques could interfere with the salivary proteome and result in erroneous results and conclusions.

In 2-DE, sample preparation is an essential step for success. A high amount of charged molecules in solution will affect isoelectric focusing (IEF). In certain animal species, such as ruminants, this may be particularly relevant, since these animals have a high content of bicarbonate and phosphate ions in their saliva [14]. Moreover, charged detergents, lipids, phenolics and nucleic acids also need to be removed prior to IEF. Precipitation is frequently used to concentrate proteins from diluted samples and also to remove compounds that interfere with IEF [15,16]. Various methods for protein precipitation are applied, which rely on different chemical principles, and have different effectiveness according to the characteristics of samples. For human saliva, there are studies evaluating the efficiency and reliability of different protein precipitation protocols [17]. However, for dog saliva, which is different in composition from human saliva as based on biochemical studies [18-20], no such information was reported by now.

The objective of the present study was to compare and select the protein precipitant and precipitation method which will allow protein profiles with the highest similarity to original sample protein profile and, at the same time, efficient and reliable 2-DE analysis. For this, six of the protein precipitation methods already tested in human saliva [17] were compared using canine saliva samples.

2. Materials and Methods

2.1. Saliva collection

Saliva from seven healthy intact adult dogs (age range 2-8 years) of different breeds was collected: Portuguese Podengo (n=2), Border Collie (n=1), Bull Terrier (n=1), Boxer (n=1), Australian Cattle Dog (n=1) and mixed breed (n=1). Animals from different breeds and ages were used to constitute a pool representative of "general" dog saliva, instead of using saliva from only a particular breed and/or age. Saliva samples were taken by the same examiners using a cotton cylinder (Salivette®) [14]. The salivettes were inserted into the oral cavity of the dogs, under the tongue and for chew, until completely soaked with saliva [14]. Dogs did not eat for at least 12 hours prior to the salivary sampling but water were provided ad libitum. After collection, the salivettes were immediately placed in test tubes on ice, until laboratory arrival. The saliva was extracted from the cotton roll by centrifugation at 4°C, at 2650 g for 5 min. Samples were mixed in one pool, which was aliquoted and stored at -20°C until further analysis, less than two months in all cases.

2.2. Determination of total protein concentration

The total protein concentration of the dog saliva pool was determined using Bradford method protein assay [17] with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA). Standards and samples were run in triplicate, in 96 wells microplates. Absorbance was read at 600 nm in a microplate reader (Glomax, Promega).

2.3. Protein precipitation

Five different precipitant solutions and six precipitation methods were employed, which were already tested in human saliva [17]: method A (trichloroacetic acid-TCA only), method B (TCA/acetone), method C (TCA/acetone/dithiothreitol-DTT), method D (acetone only), method E (ethanol only) and method F (TCA/acetone fast method). Each of the methods was performed in triplicate.

TCA precipitation (method A)

For precipitation with TCA saliva sample was mixed with 20% w/v TCA (1:1). The mixture was vortexed to mix thoroughly and allowed to precipitate overnight at -20°C. This was followed by centrifugation at 23876 g, at 4°C for 30 min. The supernatant was discarded and the pellet obtained was washed twice with 200 μL cold acetone. For each wash, the pellet suspended was sonicated for 5 min or more until the whole pellet was fully broken to form a suspension (maximum 20 min). This suspension was then incubated at -20°C for 20 min and subsequently centrifuged at 4°C for 5 min at 23876 g . This wash procedure was repeated twice. At the end, the acetone-containing supernatant was decanted and the pellet obtained was dried at air to remove any residual acetone. The pellets prepared were stored at -20°C until further use.

TCA/Acetone precipitation (method B)

The procedures were performed as described for method A, except that the solution used for protein precipitation was 1 ml of TCA 20% (w/v) plus 9 ml acetone (90% v/v).

TCA/Acetone/DTT precipitation (method C)

The procedures were performed as described for method A, except that the solution was constituted by TCA 20% (v/v), acetone 90% (v/v) and 20 mM DTT (2.5 mL TCA+acetone and 0.0075 g of DTT); and that the first wash was done using acetone 90% (v/v) and 20 mM DTT and the second using acetone 80% (v/v) and 10 mM DTT.

Acetone precipitation (method D)

In this method, saliva was mixed with 90% (v/v) acetone at a proportion 1:3. The mixture was incubated overnight at -20°C followed by centrifugation at 4°C, for 30 min at 23876 g . The supernatant was discarded and the precipitated was dried at air and frozen until further analysis.

Ethanol precipitation (method E)

The method was carried out similar to method D except that precipitation was done with absolute ethanol.

TCA/Acetone precipitation (fast assay) (method F)

The method was carried out as described for method B, but instead incubation being overnight, it was performed during 1h at 4° C. The rest of procedures were similar.

2.4. Determination of the protein concentration after protein precipitation methods

A volume of saliva corresponding to 25 μg of total protein was precipitated following the methods described above. The precipitates obtained were re-suspended in 25 μL 2-DE rehydration buffer [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 60 mM DTT], since this is the buffer used in 2-DE. In order to avoid that compounds interfere with Bradford, the solution was diluted with 400 μL ultrapure water. Total protein concentration was determined following Bradford method as described in the section 2.2.

2.5. SDS PAGE and two-dimensional electrophoresis

Proteins were separated by SDS-PAGE electrophoresis in 14% acrylamide gels in a mini-protean apparatus (BioRad) [21]. A volume corresponding to 12 μg of total protein was precipitated by each of the methods described before. The precipitates were re-suspended in sample buffer [Tris–HCl 0.125 M pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol traces of bromophenol blue], heated at 95°C for 5 minutes and run at a constant voltage of 140V until the dye front reach the end of the gel. A volume of saliva correspondent to 12μg of total protein that was not previously precipitated was run for control. Gels were fixed in 40% methanol, 20% acetic acid, for one hour, stained with Coomassie Brilliant Blue (CBB) G-250 (0.125% CBB G-250,

20% ethanol) for two hours and destained in several washes with distilled water. The procedures were done in triplicate. Analysis was performed with GelAnalyzer software (http://www.gelanalyzer.com/).

For 2-DE, volumes of saliva correspondent to 150 μg of total protein were precipitated by the methods evaluated in this study, with exception of method F. The precipitates were mixed with 125 μL rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer, 60 mM DTT and traces of bromophenol blue], sonicated until total re-suspension and incubated during 1h at room temperature, being subsequently centrifuged for 5 min at 9390 g. IPG strips (7 cm, pH 3-10 NL; GE, Healthcare) were passively re-hydrated overnight with this solution. Focusing was performed in a Multiphor II (GE, Healthcare) for approximately 16kVh, at 20°C. Focused strips were equilibrated in two steps of 15 min each with equilibration buffer [50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol and 2 % (w/v) SDS], with the addition of 1% (w/v) DTT and 65 mM iodoacetamide in the first and second steps, respectively. After equilibration the strips were applied in the top of a SDS-PAGE gel 14% acrylamide and run at 150V constant voltage in a mini-protean system (BioRad). Staining with CBB-G250 and destaining were done through the same protocol described for SDS-PAGE gels. Gel images were acquired using a scanning Molecular Dynamics densitometer with internal calibration and LabScan software (GE, Healthcare), and images were analysed using ImageMaster 2D Platinum v7 software. Spot editing was performed manually and the match was done automatically and corrected manually.

2.6. Protein identification

Spots of interest were manually excised from gels and digested with trypsin following the protocol described before [21]. MALDI TOF-TOF mass spectrometry was used for protein identifications and this technique was performed by an external laboratory (MS Lab, ITQB-UNL; Portugal). Samples were diluted and concentrated using a reversal phase column (R2 pores-Applied Biosystems) and eluted with matrix α-cyano-4-hydroxycinnamic acid (CHCA; Fluka) 5mg/mL in 50% (v/v) CAN and 5% (v/v) formic acid. MS and MS/MS data were acquired in positive reflector mode through MALDI TOF/TOF (4800 Plus AB SCIEX) and through software 4000 Series Explorer, version 3.5.3.3 (Applied Biosystems). The mass of monoisotopic peptides was determined using the algorithm SNAP 2 in the analysis software (Bruker Daltonics) version 3.4. The external calibration was executed using CalMix5 (Protea). The 30 precursor ions most intense of the MS spectra were selected for analysis by MS/MS.

The monoisotopic masses of the peptides were used to search for the protein identification through the use of Protein Pilot v 4.5 (ABSciex) software with the Mascot search engine (MOWSE algorithm). Swiss Prot database,
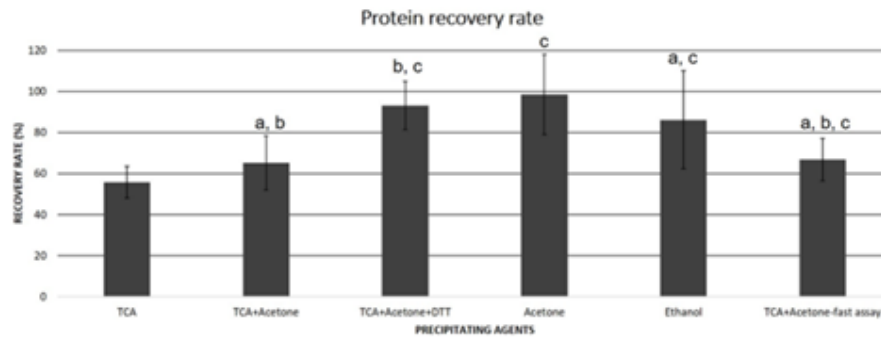
Figure 1. Protein recovery rates obtained for each of the precipitation methods studied. Different letters represent statistically significant differences among methods for p<0.05.

restricted to Canidae canidae (32092 sequences; 16404078 residues), was used for all the searches. A minimum mass accuracy of 50 ppm and a mass tolerance of 0.3 Da, two missed cleavages in peptide masses, carbamidomethylation of Cys and oxidation of Met, as fixed and variable amino acid modifications, respectively, were considered. Criteria used to accept the identification were homology scores higher than 58 and, at least, one fragmented peptide with individual significant score (P<0.05), in Mascot.

2.7. Statistical analysis

Data for protein recovery rate, percentage volume of SDS-PAGE bands and 2-DE spots were tested for normality and homocedasticity using Kolmogorov-Smirnov and Levene tests, respectively. One-way ANOVA was used for protein recovery rate, with Tukey test used for comparison of the different methods. The relationship between SDS-PAGE profiles (% volume of bands) from each precipitation method and the original sample was evaluated by calculating Pearson coefficient. Statistical significance was considered for p<0.05. All statistical analysis procedures were achieved using the SPSS 21.0 software package (SPSS Inc., Chicago, USA).

3. Results

3.1. Protein recovery rate

The different precipitation methods resulted in statistically

significant different rates of total protein recovery. The methods with higher losses of protein were TCA (method A) and TCA+acetone (methods B and F). On the other hand, the precipitation with acetone (method D) was the one for which higher protein recovery was achieved (Figure 1).

3.2. SDS-PAGE protein profiles

By relating the percentage of volume of the different profiles, strong positive correlations were obtained between original sample and methods A (r=0.717; p=0.003), B and F (r= 0.751; p=0.001, for overnight and r=0.715; p=0.003 for 1h incubation) and C (r=0.711; p=0.002). On the other hand, the precipitation methods with acetone (D) and with methanol (E) resulted in profiles not correlated with control (r=0.389; p=0.151 and r=0.339; p=0.216, respectively) (Figures 2 and 3).

When comparing the different 16 protein bands consistently present in SDS-PAGE gels, the profile obtained without sample protein precipitation differs from profiles using protein precipitation methods for 3 protein bands: B, H and N. The differences are mainly in relation to the profiles obtained through precipitation with acetone or ethanol (Table 1).

#### 3.3. Two-dimensional electrophoretic protein profiles

2-DE was run only for precipitated samples, since the volume of original sample needed exceeded the maximum of 25 μL allowed for the dry-gel strips used. Figure 4 shows the

Table 1. Expression levels (% volume) of the protein bands mainly affected by protein precipitation protocols. A – TCA; B – TCA/acetone; C - TCA/acetone/DTT; D – acetone; E – ethanol; F – TCA + acetone fast assay.

Band	TCA (method A)	TCA+acet (method B)	TCA+acet+DTT (method C)	Acetone (method D)	Ethanol (method E)	TCA+acet fast assay (method F)	Control	P value
B	4.54 ± 1.17	6.54 ± 0.32	9.62 ± 1.05	2.40 ± 0.00*	3.57 ± 0.86*	6.79 ± 0.17	6.90 ± 0.65	0.010
H	7.50 ± 0.45*	5.62 ± 0.31*	1.97 ± 0.00	5.39 ± 0.61*	3.96 ± 1.13	5.31 ± 0.70*	1.97 ± 0.00	0.014
N	8.19 ± 0.82	8.50 ± 0.38	2.43 ± 0.00	12.04 ± 3.25*	12.04 ± 1.26*	9.49 ± 2.09*	3.24 ± 0.82	0.016

* Differences comparatively to control group (P<0.05)
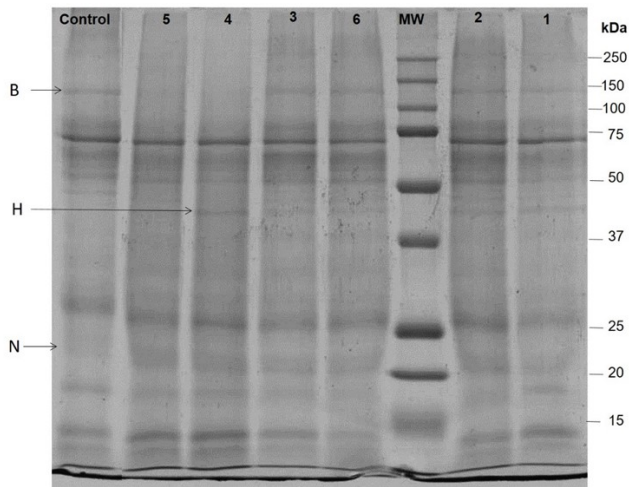
Figure 2. Representative SDS-PAGE profiles for each of the precipitation methods tested. Numbers in each lane indicate the precipitation method used (1 – TCA; 2 – TCA/acetone; 3- TCA/acetone/DTT; 4 – acetone; 5 – ethanol; 6 – TCA + acetone plus assay); MW – molecular mass marker. Precipitation methods 1 and 2 were the ones presenting higher differences from control protein profiles.

representative 2-DE of dog saliva, after each of the precipitation methods under study.

The highest number of resolved spots were obtained for the precipitation with TCA (83 spots), decreasing for the methods acetone (69 spots), ethanol (64 spots), TCA+acetone+DTT (59 spots) and TCA+acetone (55 spots).

The profile in which protein spots were less well resolved was the one obtained for precipitation with absolute ethanol (method E). On the other hand, when precipitations were performed with methods A and D, the profiles did appear enriched in proteins from the alkaline range of the pH gradient. Even in the other pH gradient regions, these two profiles were the ones allowing better focusing of the proteins, with well resolved protein spots. It is particularly interesting the group of protein spots from the alkaline region of the gel that were only observed in the gels obtained following these two precipitation methods (methods A and D - Figure 4).

The six spots observed only in the precipitation methods

TCA (A) and acetone (D) were identified by mass spectrometry (Table 2).

These spots correspond to proteins of keratin family or to serine-type endopeptidase inhibitor family. Although some of them are present in databases as uncharacterized proteins, one has been already identified as a submandibular gland secreted protein.

4. Discussion

The present study compared six different protein precipitation methods for the gel-based proteomic analysis of dog saliva. It characterizes the precipitation efficiency on the amount of total protein that can be loosed at the end, as well as on a protein-specific level. Although there are studies in which different protocols for preparation of human saliva samples for 2-DE were compared [17], the protein composition of dog saliva is not well known. As such, there are no guarantees that the most appropriate protocols for preparing human saliva samples are also the ideal ones for the preparation of dog saliva samples.

The proteome is a dynamic structure, so the sample itself and the conditions of its storage for research directly affect the result of proteomic analysis [22]. Moreover, the ideal sample preparation procedure should reproducibly capture the most comprehensive repertoire of proteins without any artefactual modification, proteins loss, degradation or non-proteinaceous contamination [14,15,20-26]. Besides the need for removing sample components that may interfere with isoelectric focusing, saliva analysis by electrophoresis requires a concentration step, due to the dilute nature of these samples, where protein precipitation methods can be useful.

Precipitation methods based in acetone and/or TCA were used by several authors in several animal tissues like ovaries, pericardium, brain, muscle and animal fluids like saliva, cerebrospinal fluid and blood [14,15,20-26]. One of the limitations of precipitation protocols is the protein loss, usually resulting from a poor re-suspension of the precipitates [27]. Among the protein precipitation methods tested in the present work, it was the acetone precipitation

Table 2. MS Identification of differentially expressed salivary proteins.

Spot	Protein	Uniprot entry reference	ID score	Estimated/ Theoretical MW (kDa)	Estimated/ Theoretical pI	Function/Family
1	Keratin, type I cytoskeletal 10	Q6EIZ0	207	14.0/57.7	8.1/5.1	Structural molecular activity
2	Keratin, type II cytoskeletal 8	F1PW98	111	16.0/55.0	8.1/5.7	Structural molecular activity/ Keratin family
3	Double-headed protease inhibitor, submandibular gland	P01002	96	16.0/12.8	7.9/8.3	serine-type endopeptidase inhibitor
4	Uncharacterized protein	L7N097	168	15.5/51.9	6.0/6.0	Structural molecular activity/Keratin family
5	Uncharacterized protein	F1PR78	121	14.0/12.7	5.0/8.4	serine-type endopeptidase inhibitor
6	Keratin, type I cytoskeletal 10	Q6EIZ0	192	14.0/57.7	7.9/5.0	Structural molecular activity
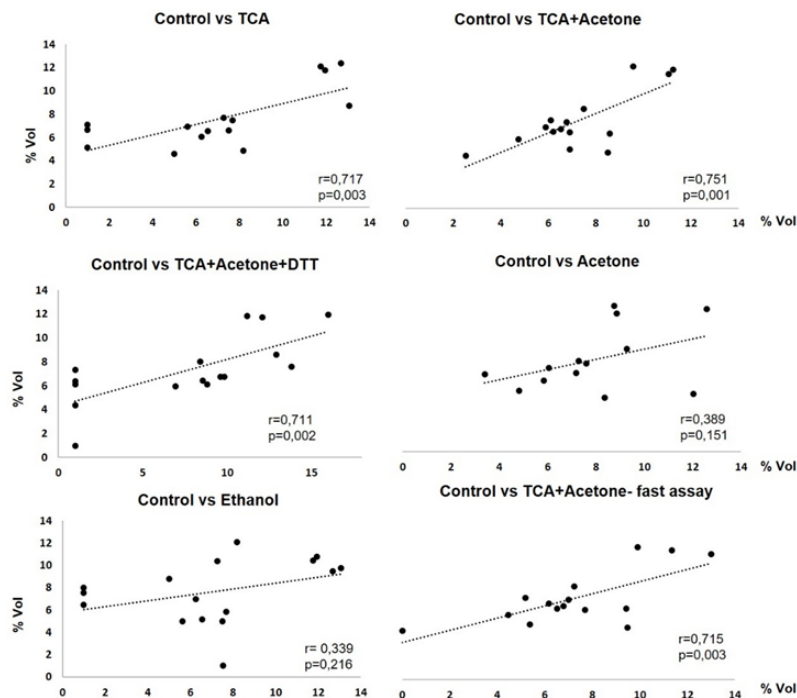
Figure 3. Correlation between the SDS-PAGE profiles of each of the precipitation methods tested and control. Correlations are significant for $p < 0.05$.

the one that presented almost no losses, with protein recovery rates near 99%. For human saliva and rat brain samples, protein recovery rates of about 70% and 52.2% with acetone precipitation were reported [17,27], respectively, what suggests that the efficacy of this method depends on the type of biological material. In addition to the reduced losses in total protein, the other great advantage of this protocol is its simple execution, although requiring a high volume of organic solvent.

In our study, TCA-containing precipitation protocols resulted in lower rates of protein recovery (56%), similar to that already observed for human saliva by Jessie et al. (2008) [17], where TCA was observed as one of the precipitating agents leading to higher total protein losses (46.5%). The recovery of the protein depends on the re-solubilisation of the sample, being observed that after protein precipitation with TCA the recovery rate is considerably higher if the samples are sonicated (24% and 77%, without and with sonication, respectively) [27]. In the present work, besides the use of vortex, for resuspension of the precipitate, sonication of the samples was also carried out but even so, no high recovery rates were obtained. However, when acetone and DTT was added to the mixture (method C) higher recovery rates were observed, similarly to what has been reported to human saliva samples [17].

Besides the interest in having a precipitation protocol that allows a high protein recovery rate, it is important that it imposes the minimum of modifications in the protein content of samples, namely in the relative abundance of these proteins. In order to control this aspect, the protein profiles obtained after each precipitation method were

compared with the one from the original sample (without precipitation). This was possible to be done only for SDS-PAGE, since in 2-DE the volume of sample necessary to have the amount of protein needed for spot visualization is too high to be run without concentration. Moreover, the use of Centricon devices for protein concentration did not work in these dog saliva samples, with the filters clogged during centrifugation step (data not shown), with the need of further research to identify the dog saliva components responsible for that. Despite the low protein recovery rate, referred above, a high correlation between the protein profiles from TCA precipitation method and the protein profiles from original sample was observed.

Interestingly, 20% (w/v) TCA solution, representing a concentration of 10% of this acid in sample, induces no major changes in dog saliva. Some previous studies in rats reported that salivary proline rich proteins present the particular characteristic of being soluble in 10% TCA solutions [28], suggesting that this precipitation method would lead to a selective loss of these proteins. In the present work, the high correlation found between the profiles obtained from TCA protocols and the original sample profile suggest that the protein losses resulting from the precipitation occur in the same proportion for the different proteins, with no evidence of some proteins being more affected relatively to others. One hypothesis for explaining these results is that dog saliva may not contain these types of proteins. In fact, some authors report the absence of these proteins in animal species' saliva such as dogs and cats due to their eating patterns [30].

The 90% (v/v) acetone and absolute ethanol protocols
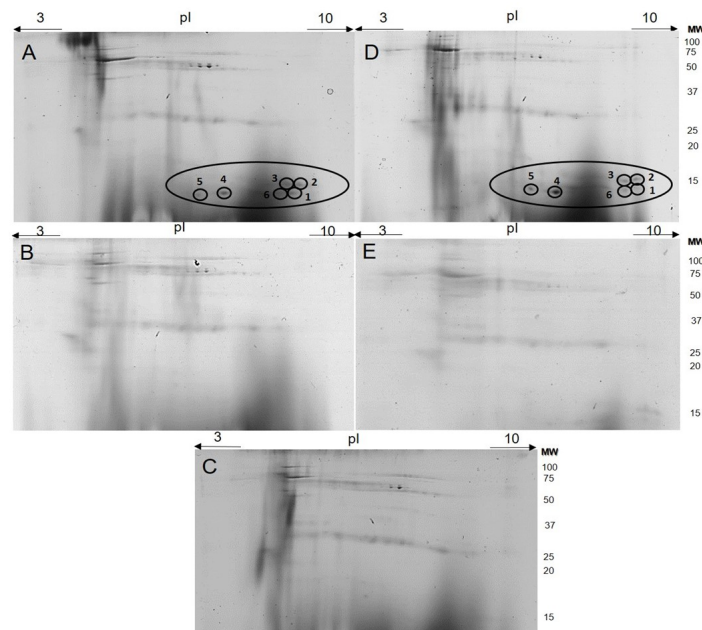
Figure 4. 2-DE protein profiles from dog saliva after various precipitation methods. (A) TCA; (B) TCA+acetone; (C) TCA+acetone+DTT; (D) acetone; (E) ethanol. Gels were stained with CBB-G250 .

were those that gave profiles less correlated with the control profiles. However, in these protocols protein recovery rates were relatively higher. The low correlation between these protocols and the control in SDS-PAGE profile may be due to higher losses of some types of proteins relative to others. Although not identified in the present study (since each band can be constituted by several proteins), proteins with molecular masses around 150 kDa are less intense in acetone and ethanol precipitation methods, whereas proteins with molecular masses around ⬜kDa and 25 kDa are highly expressed in acetone and ⬜cetone and ethanol methods, respectively. The further identification of these proteins are of interest to understand the main changes induced by these protocols.

In 2-DE profiles, precipitations of proteins with TCA or acetone were the methods that allowed the observation of the higher number of spots, with better resolution. It was also only in the gels obtained using these two protocols that spots in the alkaline region were observed. The enrichment in alkaline proteins was already reported for precipitation with TCA [27].

The protein spots observed in the gels obtained following the two methods referred were identified as belonging to two families of dog proteins: keratins and serine-type endopeptidases inhibitors. In the case of keratin 1, which is a constituents of the intermediate filament cytoskeleton in epithelial cells, its presence in saliva from different mammalian species, including dogs, has been reported [31]. It was previously reported, for humans, that the amounts of keratins present in saliva may be a marker of gingival damage [32]. Since, in this study, dog saliva has been collected through cotton rolls, some level of gingival damage may occur, resulting in the identification of this protein in saliva.

Other protein spots, differently expressed among the precipitation methods tested, were identified as protease inhibitors and, interestingly, one of them was identified as a protein secreted by submandibular glands. The exact role of this protease inhibitor in saliva is not known and further studies would be needed to explore and explain why this protein behaves differentially with different precipitation methods.

5. Concluding Remarks

2-DE is a popular technique for the global analysis and initial profiling of saliva prior to further fractionation and identification with other high throughput techniques such as mass spectrometry. This technique needs a sample preparation that, at the same time, allows cleaning of interfering compounds and protein concentration. Among the several precipitation methods studied in the present work, precipitation using 20% (v/v) TCA (method A) showed best results with dog saliva under studied conditions. Although this method results in considerable amount of total protein loss, the protein profiles are highly correlated with the ones from the original samples, with the advantage of allowing good resolution of spots in 2-DE and the visualization of spots from proteins that may be of interest.

6. Supplementary material

Ethical statement: The study did not involve the manipulation of experimental animals. Saliva was collected from healthy, normal weight, adult dogs of different breeds belonging to staff members of the University of Évora and to two kennels, who gave their informed consent for its

collection. The saliva collection and all animal procedures were carried out by a researcher accredited by the Federation of European Animal Science Associations (FELASA), and conformed to Portuguese law (Decreto-lei nº 113/2013, 7 August), which transposed the directive 2010/63/EU of the European Parliament of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocols were also reviewed by the Évora University animal welfare commission.

Acknowledgements

References

1] E. Scarano, A. Fiorita, P.M. Picciotti, G.C. Passali, L. Calò, T. Cabras, R. Inzitari, C. Fanali, I. Messana, M. Castagnola, G., Paludetti Proteomics of saliva: personal experience, Acta Otorhinolaryngol Italica 30 (2010) 125-130.

2] S. Al Kawas, Z.H. Rahim, D.B. Ferguson, Potential uses of human salivary protein and peptide analysis in the diagnosis of disease, Arch Oral Biol 57 (2012)1-9.

3] S.J. Farnaud, O. Kosti, S.J. Getting, D. Renshaw, Saliva: physiology and diagnostic potential in health and disease, Scientific World Journal 10 (2010) 434-456. DOI: 10.1100/tsw.2010.38

4] D. Belstrøm, R.R. Jersie-Christensen, D. Lyon, C. Damgaard, L.J. Jensen, P. Holmstrup, J.V. Olsen, Metaproteomics of saliva identifies human protein markers specific for individuals with periodontitis and dental caries compared to orally healthy controls, PeerJ 4 (2016) e2433. DOI:10.7717/peerj.2433

5] L. Delmonico, M. Bravo, R.T. Silvestre, M.H. Ornellas, C.M. De Azevedo, G.Alves, Proteomic profile of saliva and plasma from women with impalpable breast lesions, Oncol Lett 12 (2016) 2145-2152. DOI: 10.3892/ol.2016.4828

6] N. Nowroozilarki, S. Jamshidi, T.Z. Salehi, S. Kolahian, Identification of Helicobacter and Wolinella spp. in oral cavity of Toy breed dogs with periodontal disease, Topics in Compan An Med (2017). DOI: 10.1053/j.tcam.2017.07.004

7] A. Tvarijonaviciute, C. Castillo, J.J. Ceron, S. Martinez-Subiela, F. Tecles, P.López-Jornet, Leptin and NGF in saliva of patients with diabetes mellitus type 2: A pilot study, J Oral Pathol Med 46 2017 853-855. DOI.org/10.1111/jop.12587

8] I. Miller, A. Preßlmayer-Hartler, R. Wait, K. Hummel, C. Sensi, I. Eberini, E. Razzazi-Fazeli, E. Gianazza, In between - Proteomics of dog biological fluids, J Proteomics 106 (2014) 30-45. DOI: 10.1016/j.jprot.2014.04.016

9] E. Lamy, M. Mau, Saliva proteomics as an emerging, non-invasive tool to study livestock physiology, nutrition and diseases, J Proteomics 75 (2012) 4251-4258. DOI: 10.1016/j.jprot.2012.05.007

10] R. Bergeron, S.L. Scott, J.P. Emond, F. Mercier, N.J. Cook, A.L., Schaefer Physiology and behavior of dogs during air transport, Can J Vet Res 66 (2002) 211-216.

11] B. Beerda, M.B.H. Schilder, J. van Hooff, H.W. de Vries, J.A. Mol, Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs, Appl Anim Behav Sci 58 (1998) 365-381.

12] A.J. Kobelt, P.H. Hemsworth, J.L. Barnett, K.L.Butler, Sources of sampling variation in saliva cortisol in dogs, Res Vet Sci 75 (2003) 157-161. DOI: 10.1016/S0034-5288(03)00080-8

13] N.A. Dreschel, D.A. Granger, Methods of collection for salivary cortisol measurement in dogs, Horm Behav 55 (2009) 163-168. DOI: 10.1016/j.yhbeh.2008.09.010

14] E. Lamy, G. da Costa, R. Santos, F. Capela E Silva, J. Potes, A. Pereira, A.V. Coelho, E. Sales Baptista, Sheep and goat saliva proteome analysis: A useful tool for ingestive behavior research?, Physiol Behav, 98 (2009) 393-401. DOI: 10.1016/j.physbeh.2009.07.002

15] R.L. Chen, E.A. Sage, M.J. Dunn, R. Wait, J.E. Preston, Optimising ovine cerebrospinal fluid preparation for two-dimensional gel electrophoresis, Proteomics 6 (2006) 3170-3175. DOI: 10.1002/pmic.200500623

16] U. Jankowska, A. Latosinska, B. Skupien-Rabian, B. Swiderska, M. Dziedzicka-Wasylewska, S. Kedracka-Krok, Optimized procedure of extraction, purification and proteomic analysis of nuclear proteins from mouse brain, J Neurosci Methods 261 (2016) 1-9. DOI: 10.1016/j.jneumeth.2015.12.002

17] K. Jessie, O.H. Hashim, Z.H. Rahim, Protein precipitation method for salivary proteins and rehydration buffer for two-dimensional electrophoresis, Biotechnology 7 (2008) 686-693. DOI: 10.3923/biotech.2008.686.693

18] H. Nakanishi, T. Ohmori, M. Hara, K. Yoneyama, A. Takada, K. Saito, Identification of canine saliva using mRNA-based assay, Int J Legal Med 131 (2017) 39-43. DOI: 10.1007/s00414-016-1391-7

19] C. Boehlke, O. Zierau, C. Hannig, Salivary amylase – The enzyme of unspecialized euryphagous animals, Arch Oral Biol 60 (2015) 1162-1176. DOI: 10.4239%2Fwjd.v7.i6.112

20] E. Lavy, D. Goldberger, M. Friedman, D. Steinberg, pH values and mineral content of saliva in different breeds of dogs, Israel J Vet Med 67 (2012) 244-248.

21] E. Lamy, C. Simões, L. Rodrigues, A.R. Costa, R. Vitorino, F. Amado, C. Antunes, I. do Carmo, Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not, J Physiol Biochem 71 (2015) 691-702. DOI 10.1007/s13105-015-0434-8

22] P. Pomastowski, B. Buszewski, Two-dimensional gel electrophoresis in the light of new developments, Trends Analyt Chem 53 (2014) 167-177. DOI: 10.1016%2Fj.trac.2013.09.010

23] M. Sandri, A. Colussi, M.G. Perrotta, B. Stefanon, Salivary cortisol concentration in healthy dogs is affected by size, sex, and housing context, J Vet Behav 10 (2015) 302-306. DOI: 10.1016/j.jveb.2015.03.011

24] F. Panchout, J. Letendre, F. Bultelle, X. Denier, B. Rocher, P. Chan, D. Vaudry, F. Durand, Comparison of protein-extraction methods for gills of the shore crab, Carcinus maenas (L.), and application to 2DE, J Biomol Tech 24 (2013) 218-223. DOI: 10.7171/jbt.13-2404-002

25] R. Hao, C. Adoligbe, B. Jiang, X. Zhao, L. Gui, K. Qu, S. Wu, L. Zan, An Optimized Trichloroacetic Acid/Acetone Precipitation Method for Two-Dimensional Gel Electrophoresis Analysis of Qinchuan Cattle Longissimus Dorsi Muscle Containing High Proportion of Marbling, PLoS ONE 10(4) (2015) e0124723. DOI:10.1371/journal.pone.0124723

26] P. Yiou, A. Shaoli, L. Kebin, W. Tao, F. Kui, Z. Hua, S. Yu, Y. Xun, X. Jinghui, Evaluation of extraction procedures for 2-DE analysis of aphid proteins, J Sep Sci 36 (2013) 532-539. DOI: 10.1002/jssc.201200642

27] E. Fic, S. Kedracka-Krok, U. Jankowska, A. Pirog, M. Dziedzicka-Wasylewska, Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis, Electrophoresis 31 (2010) 3573-3579. DOI: 10.1002/elps.201000197

28] H. Mehansho, A. Hagerman, S. Clements, L. Butler, J. Rogler, D.M. Carlson, Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels, Proc Natl Acad Sci 80 (1983) 3948-3952.

29] J.C. Suares, D.K. Shori, G.B., Proctor Expression of proline-rich proteins in the parotid & submandibular glands of the untreated rat, Biochem Soc Trans 25 (1997) 31S.

30] J.B. Harborne, in: Plant Ecology (Ed.), Crawley MJ, Oxford, UK, 1996, pp.132-155.

31] P. de Sousa-Pereira, M. Cova, J. Abrantes, R. Ferreira, F. Trindade, A. Barros, P. Gomes, B. Colaço, F. Amado, P.J. Esteves, R. Vitorino, Cross⊠species comparison of mammalian saliva using an LC-MALDI based proteomic approach, Proteomics 15 (2015) 1598-1607. DOI: 10.1002/pmic.201400083

32] W.S. McLaughlin, J. Kirkham, M.J. Kowolik, C. Robinson, Human gingival crevicular fluid keratin at healthy, chronic gingivitis and chronic adult periodontitis sites, J Clin Periodontol 23 (1996) 331-135.