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# Capillary isoelectric focusing with whole column imaging detection (iCIEF): A new approach to the characterization and quantification of salivary α-amylase

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ABSTRACT

Saliva is an easily collected biological fluid with potentially important diagnostic value. While gel electrophoresis is generally used for salivary analysis, we employed the capillary isoelectric focusing technique to allow for a rapid, automated mode of electrophoresis. Capillary isoelectric focusing coupled with UV whole column imaging detection (iCIEF) was used to develop a robust protocol to characterize salivary α-amylase collected from various glands. Notably, three sample preparation methods were examined: ultrafiltration, gel-filtration, and starch affinity interaction with salivary amylase. Salivary α-amylase separated into two major peaks before sample treatment; while both filtration methods and starch affinity interaction of salivary amylase enhanced the resolution of isozymes, desalting with gel-filtration displayed the best recovery and the highest resolution of isozymes. Good agreement existed between the observed isoelectric points and the values reported in the literature. In addition, a high level of precision was apparent, and the relative standard deviation for replicates was less than 0.5% for pIs (peak positions) and below 10% for peak area. Furthermore, saliva secreted from the parotid gland proved to have a higher amylase content compared to either secretions from the submandibular/sublingual complex, or whole saliva, as well as amylase enhancement under stimulation. The results suggest that the iCIEF technique can be used to accurately resolve and quantitate amylase isozymes in a rapid and automated fashion, and that gel-filtration should be applied to saliva samples beforehand to allow for optimal purification and characterization.



## 1. Introduction

Amylase is the major enzyme in human saliva, and has several isozymes that assist in the early stages of digestion [1]. In addition, α-amylase, which is also the most abundant salivary protein, is gaining acceptance as a stress biomarker that is released in response to both physiological and psychological stressors [2–5]. As well, salivary α-amylase can convey diagnostic information; for example, amylase concentration is affected by the presence of dental caries [6] and certain amylase isozymes have been shown to increase in proportion with dental caries [7]. As a result, a rapid, quantitative technique that accurately measures amylase isozymes should provide clinical benefit.

A number of methods, each with unique strengths and weaknesses, have been applied to the separation and analysis of amylase isozymes [8]. For example, gel electrophoresis is commonly used to separate salivary proteins [9], and, more specifically, to investigate amylase isozymes [10]. However, due to the wide variety of electrophoretic methods and procedures, interpreting data based on direct comparisons of the absolute, or relative electrophoretic mobility of the species is difficult. In addition, accurately quantifying the amylase isozymes in a stained gel can prove challenging [11]. In contrast, the isoelectric focusing (IEF) mode of electrophoresis separates proteins on the basis of their isoelectric point (pI), and yields exquisitely resolved bands. Gel isoelectric focusing (gel-IEF), in particular, has been used to separate human salivary amylase into one major isozyme with an isoelectric point of pH 6.5, and two minor isozymes with isoelectric points of pH 6.0 and 6.9 [12].

The isoelectric focusing technique in gel format has become the most commonly used tool for salivary amylase analysis, how-

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ever, despite its relative advantages, gel-IEF is semi-quantitative, labor-intensive, and difficult to automate. Notably, imaging capillary isoelectric focusing (iCIEF) using a UV source at the 280 nm wavelength, a newly developed analytical technique, can provide fast and accurate protein separation and quantitation. For example, the imaging CIEF method has been used to analyze proteins, such as myoglobin, cytochrome c, and IgG [13], to measure protein diffusion coefficients [14] and molecular weights [15], and to investigate drug-protein interactions [16]. As well, iCIEF has been successfully used for the quantitative determination of human hemoglobin variants [17].

Due to its numerous advantages, the capillary isoelectric focusing technique has been adopted for saliva analysis [18]. Furthermore, the largest catalogue of salivary proteins has been established by employing a CIEF-based multidimensional separation platform coupled with electrospray ionization tandem mass spectrometry (MS) [18]. Although MS provides a very sensitive research tool, the lower price and complexity of UV detection may make this a more desirable technique for the determination of isoenzymic forms of proteins [19].

Saliva is a complex matrix and contains numerous compounds that make the electropherogram crowded with a great number of peaks; as a result, sample treatment is required prior to separation. Additionally, α-amylase has a number of adjacent isozymes with close pI values that necessitate further sample cleanup and preconcentration. Isolation of α-amylase on cross-linked starch, and by adsorption on starch crystallites, are traditionally used purification methods based on enzyme-substrate specific interaction [20,21].

In this report, we investigate sample preparation and iCIEF separation and quantitation of human salivary α-amylase isozymes. While the capillary isoelectric focusing method has been already used for measuring α-amylase activity in rice using single point UV detection, [22] conventional single point detection encounters several problems, including protein precipitation, a long analysis time and distortion of the pH gradient due to the mobilization process [23]. Here, we use the UV whole column imaging detection technology that eliminates the mobilization process. We also demonstrate that CIEF with whole column imaging detection provides relatively, higher detection sensitivity, better reliability, and higher analytical throughput for the characterization of human salivary proteins.

## 2. Materials and methods

### 2.1. Chemicals

Human salivary α-amylase, bovine serum albumin, and potato starch were purchased from Sigma-Aldrich. The isoelectric focusing (IEF) solution, pI markers, methylcellulose, and electrolytes were supplied by Advanced Electrophoresis Solutions Ltd. (AES; Cambridge, ON, Canada).

### 2.2. Sample collection and preparation

Saliva was collected by having four apparently healthy volunteers (three women, 20–30 years old, and a 45-year-old man) expectorate ~1 mL of saliva into a tube. Saliva collections were done in the morning (10–11 am), and the participants were instructed to avoid eating and drinking for at least one hour prior to sampling and to complete an oral rinse with water 10 min before sample collection (to prevent saliva dilution).

Whole saliva was collected with, and without, stimulation and the flow rates were calculated. In this study, whole saliva was always collected in an unstimulated manner, unless noted otherwise. In the resting condition, participants were asked to tilt their heads forward and drool down the straw to collect non-stimulated saliva in the provided cryovial. For the stimulated condition, a piece of Parafilm laboratory film was chewed and saliva held in the front of the mouth and then expectorated into the cryovial. In addition to whole saliva, samples were collected from the major salivary glands: a pair in front of the ears (parotid glands) and two pairs under the cavity beneath the tongue (submandibular and sublingual glands). Secretions were absorbed onto cotton balls placed bilaterally by the parotid ducts, and the saturated cotton rolls were then placed into a vial and the saliva obtained by centrifugation. Saliva from the sublingual and submandibular glands was collected by pipette suction from under the tongue while the parotid ducts were covered by gauze. Collected samples were centrifuged at room temperature for 15 min at 14,000 × *g* to remove food debris and foam, and supernatants used for analysis.

### 2.3. Sample purification and pre-concentration

Ultrafiltration and gel-filtration were used to pre-concentrate α-amylase based on the size-sieving effect. Following centrifugation, 500 μL of each cleared sample was added to an Ultra-0.5 mL centrifugal filter with a 50 kDa molecular weight cut-off (based on the molecular weight of amylase; Millipore) and spun at 14,000 × *g* for 15 min at room temperature. The filtrate and concentrate volumes were approximately 450 μL and 50 μL, respectively; the filtrate represented the amylase-free portion, while the concentrate contained α-amylase, along with other compounds larger than the filter cut-off weight, and represented the amylase-rich portion. To recover the concentrated solute, the filter was placed upside down in a clean micro-centrifuge tube and spun at 1000 × g for 2 min at room temperature. The pre-concentration factor was calculated as the ratio of the initial sample volume to the volume of the amylase-rich portion.

For separation by gel-filtration with a spin column, size exclusion media (Sephadex G10) was used wherein the media was composed of inert porous spheres. The media was initially equilibrated with IEF solution to fill the space between spheres, and then spun at 10,000 × *g* to remove the solution. Next, 0.2 mL of a saliva sample was introduced to the filter and allowed to elute from the column by spinning at 14,000 × *g* for 1 min. Small molecules were trapped in the spheres, and larger molecules, such as proteins, were eluted.

The α-amylase was removed from whole saliva by affinity adsorption to potato starch, and the collected filtrate was used as an α-amylase free sample. We have adopted the design of the amylase removing device used by Deutsch et al. [24] Potato starch (0.5 g) was mixed with 2 mL of the centrifuged saliva sample, and left at room temperature for 1 h. Next, 200 μL of the IEF solution was added to dilute the mixture before transfer to the centrifugal tube. The amylase-rich saliva was separated from the starch powder; the mixture was then passed through the filter during a 1 min centrifugation at 14,000 × *g*. After centrifugation, the supernatant containing α-amylase was collected for further analysis.

### 2.4. Instrumentation and method development

After cleaning via centrifugation, saliva samples were mixed with IEF solution in a one to one (v/v) ratio (the IEF solution consists of carrier ampholyte that establishes the pH gradient). Then, 2 μL of the two pI markers were added to the sample; in this study, the employed markers had pIs of 4.22, 5.85, 7.65, and 9.33. The mixture was then centrifuged at 14,000 × *g* for 1 min prior to being injected into a silica capillary tube (AES).

The CIEF experiments were conducted using the CEInfinite (AES; Cambridge, ON, Canada). The system was equipped with a scientific Complementary metal–oxide–semiconductor (CMOS) camera and UV detection at a 280 nm wavelength. The exposure time was

optimized and set to 160 μs. Each final image was an average of four scans to increase the signal to noise ratio. Separations were performed on commercial cartridges with silica capillary tubes (AES), with an effective length of 5 cm, and an internal diameter of 100 μm. Focusing was carried out within a total focusing time of 6 min; 1 kV for 1 min, 2 kV for 1 min, and 3 kV for the remainder of the analysis. The catholyte and anolyte contained 1% PVP with 100 mM NaOH and 100 mM of $H_3PO_4$, respectively. The sample, which was mixed with 0.5% methylcellulose and 4% ampholyte (pH 3–10), was injected by the hydrodynamic method with 0.5 mL air pressure from a syringe. The capillary was rinsed with water and dynamically coated with 0.5% methylcellulose before sample injection to suppress the electroosmotic flow.

## 3. Results and discussion

Purification of amylase was accomplished by either filtration, or affinity adsorption, and this was followed by separation of salivary proteins based on isoelectric focusing. The amylase isozymes were characterized by pI calibration. Afterwards, the concentration of the isozymes was determined using a calibration curve that employed standard human salivary α-amylase. The matrix effect was investigated by the standard addition method.

### 3.1. Identification and characterization of amylase isozymes

The CIEF profile of whole saliva was demonstrated, and the peaks associated with α-amylase were identified, using two approaches: 1) starch-amylase affinity interaction and 2) comparison with standard human salivary α-amylase.

The starch-saliva reaction was able to effectively remove α-amylase; Fig. 1 displays electropherograms of standard human salivary α-amylase, α-amylase found in whole saliva, and the amylase-free fraction present after interaction of a saliva sample with starch. Since the electropherogram from the whole saliva sample did not show interfering species in the region of the presumptive amylase peaks, amylase measurement within saliva could be done without any additional sample pre-treatment step.

According to the supplier information, the standard human salivary α-amylase was chromatographically purified and should have contained only the two major isozymes matching the amylase peaks observed in the whole saliva sample. The isoelectric points of the amylase peaks were found to be 6.3 and 6.5 with relative standard deviations below 0.5% for three replicates.

Sample preparation by gel filtration prior to the iCIEF provided improved resolution compared to no-treatment condition, and resulted in the emergence of low abundant isozymes. Given its relative advantages, the characterization of α-amylase isozymes according to their isoelectric points was performed by iCIEF following desalting with Sephadex G10 filtration (Fig. 2). The isoelectric points were calibrated by pI markers bracketing the region of interest, and the accuracy of the pIs was validated through comparison of the capillary isoelectric focusing results with literature values from gel-IEF; the identified values are in good agreement with those reported from the gel-IEF method [12]. The highly resolved α-amylase isozymes seen in Fig. 2b demonstrate two major peaks at pH 6.3 and 6.5 and two minor peaks at pH 6 and 6.9, whereas, with gel-IEF, salivary amylase is separated into one major isozyme with a pI of 6.5 and two minor isozymes with pIs of 6 and 6.9.

Importantly, more than three quarters of the salivary amylase appeared to be distributed in the two major isozymes. The iso-

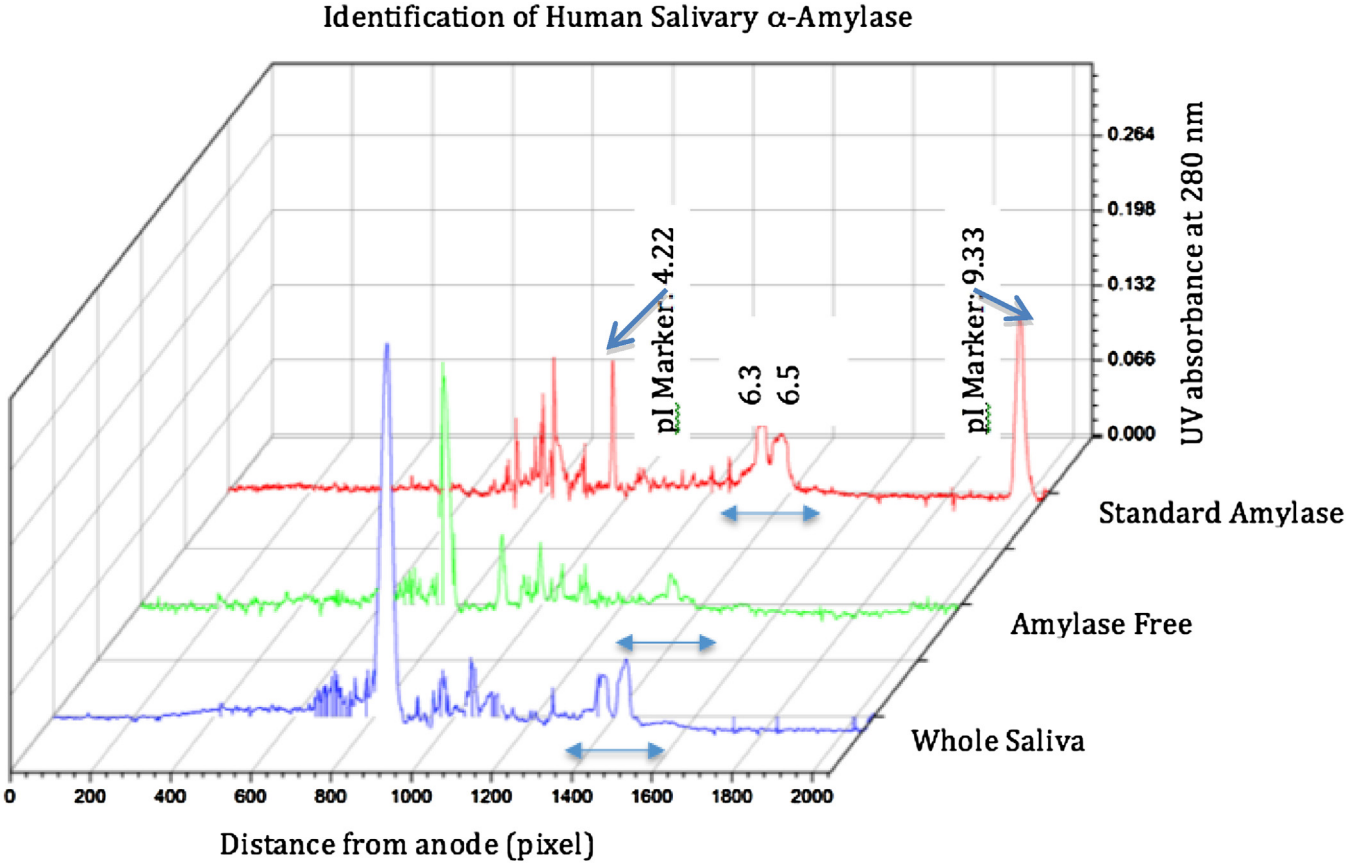


**Fig. 1.** Identification of salivary α-amylase peaks in (a) standard human salivary α-amylase, (b) an amylase free sample (amylase removed by starch affinity interaction), and (c) a whole saliva sample.

**Table 1**
Comparison of three sample preparation techniques used prior to CIEF analysis in regards to their ability to isolate and pre-concentrate salivary amylase isozymes. The initial amount of salivary amylase in the whole saliva samples before treatment was used to calculate the percent recovery.

| Purification Method | Gel-filtration | Ultrafiltration | Affinity Interaction |
|---|---|---|---|
| Recovery | 90.83% | 83.03% | 78.54% |
| [a]RSD% (N = 3) | 8.76% | 13.95% | 12.12% |
| Resolved Isozymes | I, II, III, IV | II, III, IV | II, III |

[a] The reported RSDs are the relative standard deviation of amylase peak area integration from amylase residuals in amylase free solution.

electric points and percentage distribution in whole saliva after gel-filtration were: isozyme I, pH 6.0, 10%; isozyme II, pH 6.3, 39%; isozyme III, pH 6.5, 37%; isozyme IV, pH 6.9, 14%.

### 3.2. Pre-concentration and sample cleanup

In the CIEF technique, separation and focusing of the protein bands based on their isoelectric points results in clean-up and pre-concentration of the salivary proteins. However, since the concentration of proteins in saliva is often low, the further enrichment of the analytes by removing the bulk water content is often needed. Common amylase purification procedures have included techniques such as gel permeation and ion-exchange chromatography, and precipitation with starch, or glycogen [8]. In this study, we examined three methods to purify amylase; specifically, ultrafiltration, gel-filtration, and an enzyme-substrate affinity reaction. Each purification technique was observed to have a unique balance of resolution, speed, and recovery.

In terms of α-amylase isozyme analysis, the choice of purification method was important. The starch affinity adsorption method resulted in the production of two major peaks (Fig. 3b), which are the same as those observed in the commercially available, chromatographically purified amylase (Fig. 3a). In the sample purified with the ultrafiltration method, a third isozyme was detectable (Fig. 3c), while the gel-filtration method helped to resolve four isozymes (Fig. 3d). Notably, in the electropherogram from the sample prepared by gel-filtration all the isozyme peaks, except the first one, show an unresolved shoulder that could be separated as an individual minor isozyme by improving the resolution (e.g., via establishing a narrower range pH gradient [25]).

Recovery with each of the sample preparation techniques was calculated by measuring the amylase concentration in each whole saliva sample and the matching amylase free solution:

$$R\% = \frac{(WS - AF)}{WS} \times 100$$

Table 1 demonstrates the variable recovery rates of α-amylase from whole saliva using the three preparative techniques examined; in addition, the table also notes the number of isozymes apparent with each method. Although affinity interaction and ultrafiltration were able to enrich amylase to a high degree, gel-filtration was the most effective pre-concentration method, and was able to resolve the greatest number of isozymes. The improved resolution is the result of gel-filtration's superior ability to remove interfering compounds.

From a methodological point of view, the filtration techniques are more straightforward than affinity interaction with starch. Ultrafiltration and gel-filtration also require less time to complete (approximately 15 min versus 1 h), and they are commercially available. Importantly, when concentrating the α-amylase with ultrafiltration, two minor isozymes emerge in the electropherogram and the major isozymes are better resolved compared to the affinity isolation (Fig. 3c). After desalting by gel-filtration prior to CIEF, the α-amylase isozymes in human saliva were further separated into two major peaks with a shoulder emerging on both peaks, and two minor isozymes with a shoulder on the more basic (6.9) one. In sum, the filtration techniques were more successful at removing amylase than the starch-amylase reaction (Table 1). However, depending on the substrate used, the affinity interaction method can be improved significantly; for example, Peng et al. found that a glycogen-amylase reaction was able to purify salivary amylase more efficiently than the ultrafiltration technique [26].

### 3.3. Quantitative analysis

In order to quantify the amount of α-amylase found in saliva, a calibration curve was created using known concentrations of standard human salivary α-amylase. The external standard calibration curve showed linearity with $R^2 = 0.997$ over the tested standard human salivary α-amylase concentration range. The concentration range was set according to the average reported values for salivary α-amylase in the literature; notably, α-amylase accounts for about 50–60% of the total protein in whole saliva with an approximate concentration of 0.5 mg/mL [24].

The salivary amylase concentration was determined to be 0.69 mg/mL, using external calibration curve, with a relative standard deviation of 3.13% for three replicates. To verify the matrix effect on amylase measurement, standard addition method was examined (Fig. 4). A series of six samples were prepared ranging from 0 to 0.8 mg/mL by spiking varying amounts (25 μL increments) of known concentration of standard human salivary α-amylase stock solution into the same amount of whole saliva as an unknown sample. The concentration of the amylase in WS sample was determined to be 0.65 mg/mL, which was calculated from the extrapolated line that crossed the concentration axis at 0.39 mg/mL, after multiplication by the dilution factor.

The comparison between the results obtained from the external standard calibration method and the standard addition method reveals that the matrix effect influences the measurements and overestimates the concentration of salivary amylase. However, the associated relative error is 6.1% and therefore the standard calibration curve can be employed for quantitation of the salivary amylase.

### 3.4. Applications

In addition to the whole saliva, secretions from different salivary glands, the submandibular/sublingual and parotid glands, were collected. Notably, saliva from the parotid gland was found to contain an amylase concentration that was approximately two-fold greater than that collected from the submandibular/sublingual glands, and more than four times greater than whole saliva (Table 2).

Although saliva is produced in the salivary glands, its flow rate is controlled by the nervous system, and will be increased with stimulation. Secretion rate patterns for total protein and α-amylase mirrored those of flow rate. The total protein and α-amylase concentrations of the saliva, and the specific activity of α-amylase are influenced by the type of stimulus. [27] To investigate the effect of stimulation (flow rate) on α-amylase secretion, whole saliva was collected under resting conditions and while chewing Parafilm laboratory film. The results demonstrate an increase of 10.5 percent in the salivary α-amylase content after stimulation (Table 2). The relative standard deviation of the measurements was below 10% for determination of the concentration.

As discussed earlier, the level of salivary α-amylase isozymes has diagnostic value, in this study we employed the iCIEF technique for quantitative analysis of the isozymes. The highest resolution was obtained by gel-filtration. Thus, the concentration of the salivary amylase isozymes was determined after purification by gel-filtration followed by CIEF separation (Fig. 5). The quantitative results presented in Table 3, show promise for the fast and precise

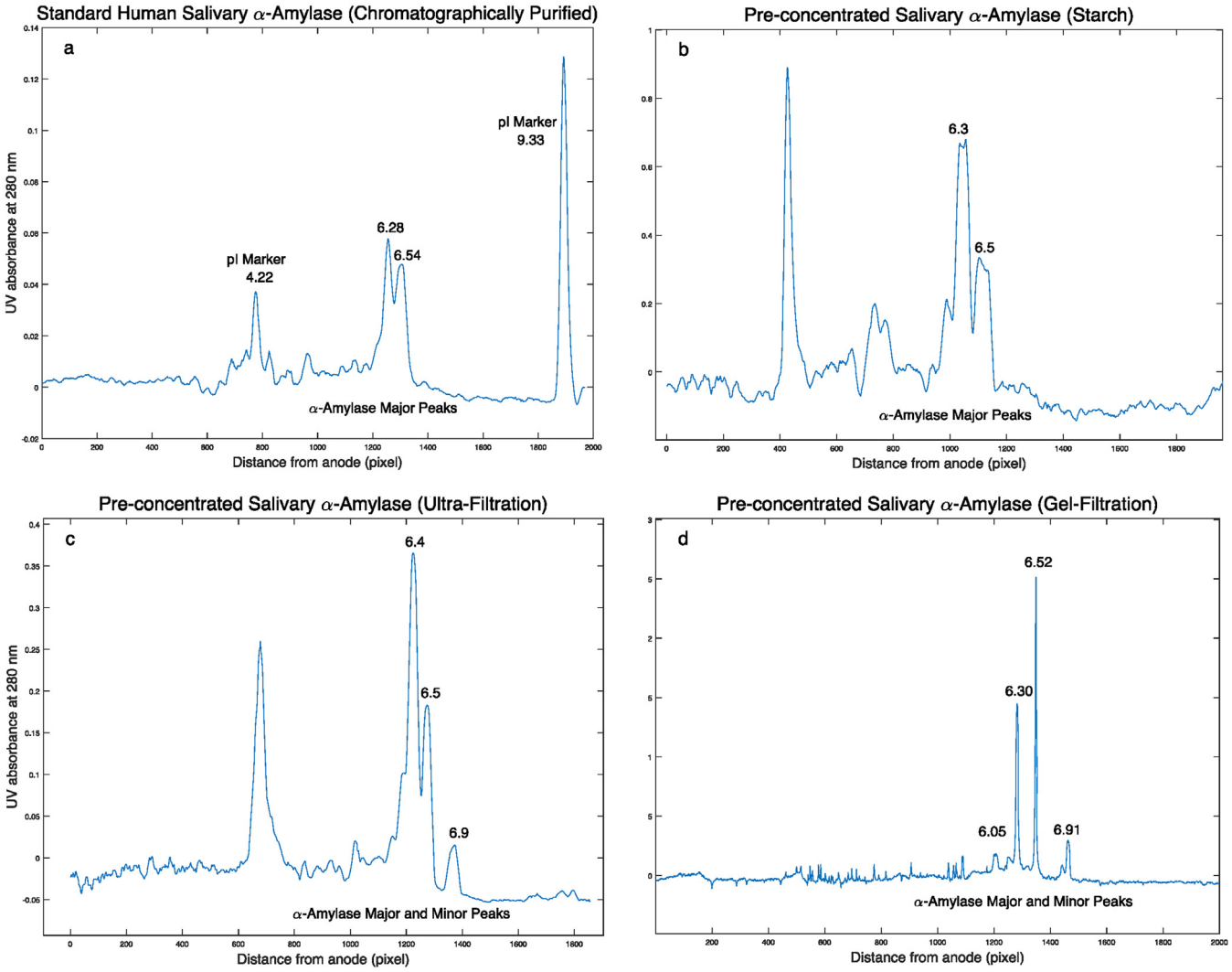


**Fig. 3.** Pre-concentration and purification of human salivary α-amylase isozymes; a) commercial standard amylase, and whole saliva after cleanup by (b) starch-amylase interaction, (c) Ultrafiltration 50 kD, (d) Sephadex G10 filtration.

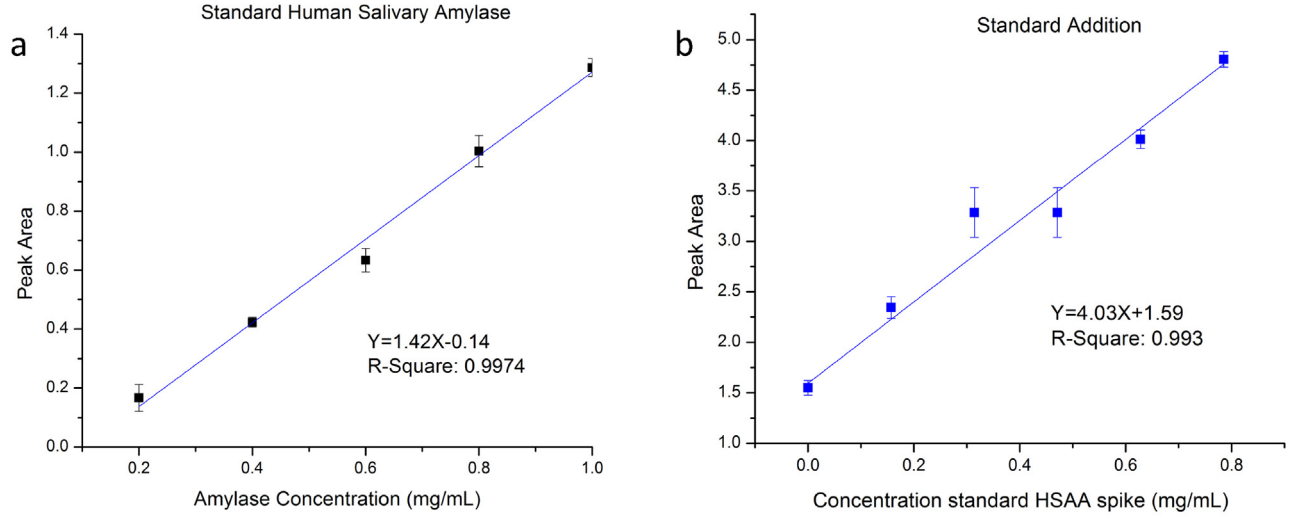


**Fig. 4.** The calibration curves are plotted based on the standard human salivary α-amylase (two major isozymes); (a) external calibration and (b) standard addition methods. Whole saliva samples were tested and the peak areas of two major isozymes were integrated. The equation of the linear regression fit and the regression coefficient is shown for the mean of the three replicates.

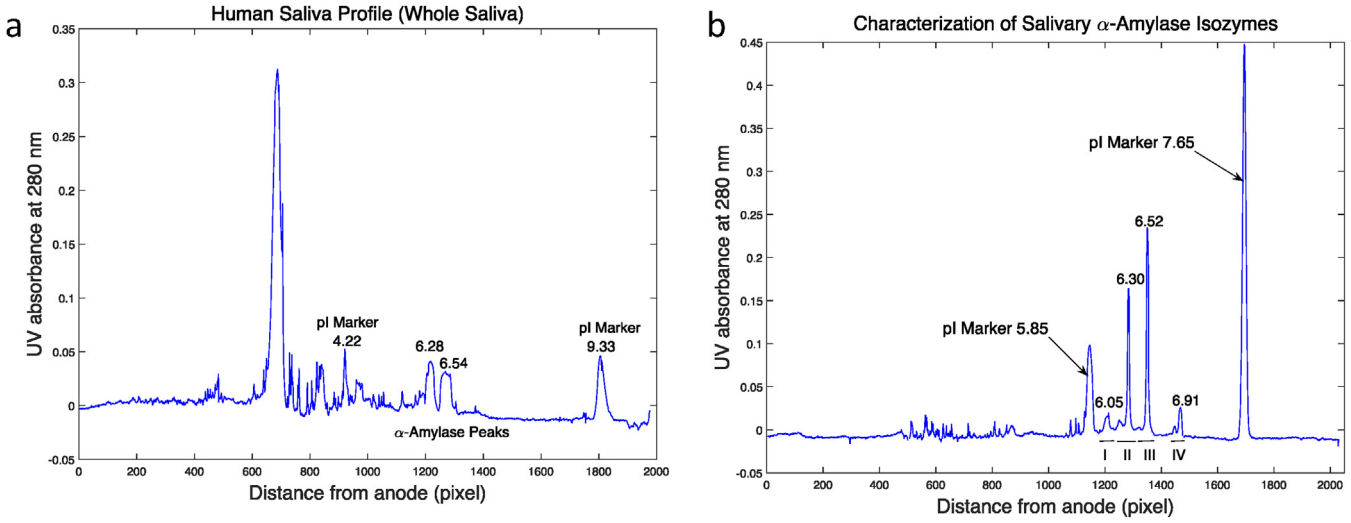


**Fig. 2.** High-resolution electropherogram of salivary α-amylase isozymes; (a) without treatment calibrated by 4.22 and 9.33 pI markers, and (b) after desalting with Sephadex G10 calibrated by 5.85 and 7.65 pI markers.

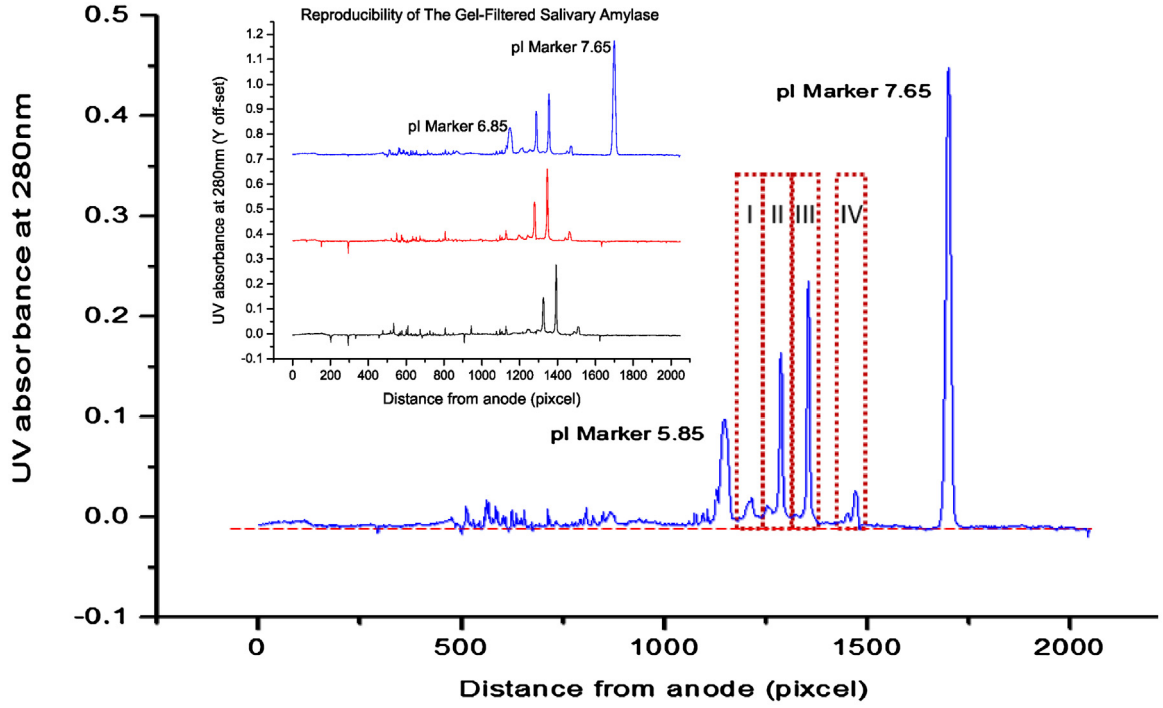


**Fig. 5.** The electropherogram of salivary α-amylase isozymes after gel-filtration and separation by CIEF. The inset demonstrates the reproducibility of the peaks' pattern and position for three replicates.

**Table 2**
Concentration of human salivary α-amylase from different glands; submandibular/sublingual complex and parotid secretion (N = 3). Stimulated and un-stimulated whole saliva flow rates are 6 and 0.8 mL/min, respectively.

| Sample Collection | Submandibular & Sublingual | Parotid | Whole Saliva | |
|---|---|---|---|---|
| | | | Stimulated | Un-stimulated |
| α-amylase (mg/mL) | 1.07(±0.1) | 2.34(±0.1) | 0.63(±0.04) | 0. 57(±0.05) |

determination of amylase isozyme concentrations. Relative standard deviations of the salivary amylase isozymes concentrations were approximately 5% for the minor isozymes.

In general, the salivary amylase content depends on its biological root, and, in our experiments, the salivary amylase (from whole saliva) of four individual participants with different backgrounds ranged from 0.49 (±0.02) to 1.2 (± 0.09) mg/mL. The samples were collected without stimulation and measured by the external calibration method. The largest relative standard deviation of the

**Table 3**
Salivary amylase isozymes concentration measurements from external calibration curve by integration of the isozyme peak areas from four trials.

| Amylase | Isozyme I | Isozyme II | Isozyme III | Isozyme IV | WS |
|---|---|---|---|---|---|
| Conc. (mg/mL) | 0.06 | 0.22 | 0.27 | 0.05 | 0.67 |
| RSD% | 4.91 | 4.16 | 2.06 | 4.99 | 1.71 |

measurements was 7.5%, which shows the relative precision of the method.

## 4. Conclusion

The iCIEF technique provides information about the pI of salivary proteins, and the direct measurement of their concentration; in particular, salivary α-amylase was successfully analyzed without any treatment, and following three sample preparation techniques. Ultrafiltration, starch-amylase affinity reaction, and gel-filtration were used to purify and isolate α-amylase from other proteins in saliva, prior to characterization by high-resolution iCIEF. The gel-filtration method provided the highest purification of amylase as well as the greatest ease of operation. As a result, the preconcentration of protein can improve the characterization salivary proteins and should be considered when examining putative salivary biomarkers.

The described iCIEF technology appeared to provide a powerful tool for quantitative analysis of salivary amylase in a high throughput fashion (analysis time of 6 min). Compared to MS, the UV detector offers a more cost-effective and straightforward detection method that should meet the needs of clinical laboratories. The instrument proved especially useful for determination of salivary α-amylase isozyme distributions that can not only provide information on post-translational modifications, but may also be of diagnostic importance. The high performance and simplicity of the technique, together with the low sample consumption, makes this analytical method a viable choice for a point of care clinical/diagnostic device.

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## References

[1] N. Ramasubbu, V. Paloth, Y. Luo, G.D. Brayer, M.J. Levine, Structure of human salivary alpha-amylase at 1.6 A resolution: implications for its role in the oral cavity, Acta Crystallogr. D Biol. Crystallogr. 52 (1996) 435–446.
[2] N. Takai, M. Yamaguchi, T. Aragaki, K. Eto, K. Uchihashi, Y. Nishikawa, Effect of psychological stress on the salivary cortisol and amylase levels in healthy young adults, Arch. Oral Biol. 49 (2004) 963–968.
[3] U.M. Nater, N. Rohleder, W. Schlotz, U. Ehlert, C. Kirschbaum, Determinants of the diurnal course of salivary alpha-amylase, Psychoneuroendocrinology 32 (2007) 392–401.
[4] U.M. Nater, N. Rohleder, J. Gaab, S. Berger, A. Jud, C. Kirschbaum, U. Ehlert, Human salivary alpha-amylase reactivity in a psychosocial stress paradigm, Int. J. Psychophysiol. 55 (2005) 333–342.
[5] N. Rohleder, U.M. Nater, Determinants of salivary alpha-amylase in humans and methodological considerations, Psychoneuroendocrinology 34 (2009) 469–485.
[6] S. Singh, A. Sharma, P.B. Sood, A. Sood, I. Zaidi, A. Sinha, Saliva as a prediction tool for dental caries: an in vivo study, J. Oral Biol. Craniofacial Res. 5 (2015) 59–64.
[7] H. Liang, Y. Wang, Q. Wang, M.S. Ruan, Hydrophobic interaction chromatography and capillary zone electrophoresis to explore the correlation between the isoenzymes of salivary alpha-amylase and dental caries, J. Chromatogr. B Biomed. Sci. Appl. 724 (1999) 381–388.
[8] J.J. Zakowski, D.E. Bruns, Biochemistry of human alpha Amylase isoenzymes, Crit. Rev. Clin. Lab. Sci. 21 (1985) 283–322.
[9] E. Lamy, A.R. Costa, C.M. Antunes, Protein electrophoresis in saliva study, in: K. Ghowsi (Ed.), Electrophoresis, InTech, 2012, pp. 63–84.
[10] R.O. Wolf, L.L. Taylor, Isoamylases of human parotid saliva, Nature 18 (1967) 1128–1129.
[11] C. Scully, P.D. Eckersall, R.T.D. Emond, P. Boyle, J.A. Beeley, Serum a-amylase isozymes in mumps: estimation of salivary and pancreatic isozymes by isoelectric focusing, Clin. Chim. Acta 113 (1981) 281–291.
[12] T. Takeuchi, T. Matsushima, T. Sugimura, Separation of human a-Amylase isozymes by electro-focusing and their immunological properties, Clin. Chim. Acta 60 (1975) 207–213.
[13] Q. Mao, J. Pawliszyn, Capillary isoelectric focusing with whole column imaging detection for analysis of proteins and peptides, J. Biochem. Biophys. Methods 39 (1999) 93–110.
[14] A.S. Zarabadi, J. Pawliszyn, Accurate determination of the diffusion coefficient of proteins by fourier analysis with whole column imaging detection, Anal. Chem. 87 (2015) 2100–2106.
[15] Z. Liu, T. Lemma, J. Pawliszyn, Capillary isoelectric focusing coupled with dynamic imaging detection: a one-dimensional separation for two-dimensional protein characterization, J. Proteome Res. 5 (2006) 1246–1251.
[16] T. Lemma, J. Pawliszyn, Human serum albumin interaction with oxaliplatin studied by capillary isoelectric focusing with the whole column imaging detection and spectroscopic method, J. Pharm. Biomed. Anal. 50 (2009) 570–575.
[17] J. Wu, J. Pawliszyn, Application of capillary isoelectric focusing with absorption imaging detection to the quantitative determination of human hemoglobin variants, Electrophoresis 16 (1995) 670–673.
[18] T. Guo, P.A. Rudnick, W. Wang, C.S. Lee, D.L. Devoe, B.M. Balgley, Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS, J. Proteome Res. 5 (2006) 1469–1478.
[19] J.R. Petersen, A.O. Okorodudu, A. Mohammad, D.A. Payne, Capillary electrophoresis and its application in the clinical laboratory, Clin. Chim. Acta 330 (2003) 1–30.
[20] W.A.C. Somers, P.H.M. Koenen, H.J. Rozie, J. Visser, F.M. Rombouts, K. van 't Riet, Isolation of α-amylase on crosslinked starch, enzyme microb, Technol 17 (1995) 56–62.
[21] P. Colonna, V.M. Leloup, S.G. Ring, a-Amylase adsorption on starch crystallites, Biotechnol. Bioeng. 38 (1991) 127–134.
[22] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, A novel method for measurement of a-amylase activity in rice koji by capillary isoelectric focusing, J. Ferment. Bioeng. 85 (1998) 451–453.
[23] X.-Z. Wu, T. Huang, Z. Liu, J. Pawliszyn, Whole-column imaging-detection techniques and their analytical applications, TrAC Trends Anal. Chem. 24 (2005) 369–382.
[24] O. Deutsch, Y. Fleissig, B. Zaks, G. Krief, D.J. Aframian, A. Palmon, An approach to remove alpha amylase for proteomic analysis of low abundance biomarkers in human saliva, Electrophoresis 29 (2008) 4150–4157.
[25] J. Lin, Q. Tan, S. Wang, A high-resolution capillary isoelectric focusing method for the determination of therapeutic recombinant monoclonal antibody, J. Sep. Sci. 34 (2011) 1696–1702.
[26] Y. Peng, X. Chen, T. Sato, S.A. Rankin, R.F. Tsuji, Y. Ge, Purification and high-resolution top-down mass spectrometric characterization of human salivary α-Amylase, Anal. Chem. 84 (2012) 3339–3346.
[27] D.A. Froehlich, R.M. Pangborn, J.R. Whitaker, The effect of oral stimulation on human parotid salivary flow rate and alpha-amylase secretion, Physiol. Behav. 41 (1987) 209–217.

## Glossary

*iCIEF:* Imaging capillary isoelectric focusing
*IEF:* Isoelectric focusing