

Protein mapping of peanut extract with capillary electrophoresis[‡]

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Abbreviations: DS, dextran sulphate; PB, polybrene; PS, polysorbate; PTM, post-translation modifications; SML, successive multilayer ionic polymer layer; TETA, triethylenetetramine

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Abstract

Protein separation can be achieved with different modes of capillary electrophoresis, such as with capillary gel electrophoresis (CGE) or with capillary zone electrophoresis (CZE). CZE protein mapping of peanut extract was approached in four different ways, combining neutral-coated or multilayer-coated capillaries with pHs well over or under the isoelectric point range of the proteins of interest. At acidic pHs, the mobility ranges of the major peanut allergens Ara h1, Ara h2, Ara h3 and Ara h6 were identified. Although the pH is a major factor in CZE separation, buffers with different compositions but with the same pH and ionic strength showed significantly different resolutions. Different components of the electrolyte were studied in a multifactorial design of experiment. CE-SDS and CZE proved to be suitable for protein mapping and we were able to distinguish different batches of peanut extract and burned peanut extract.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Color online: See article online to view Figs. 1–5 in color.

Introduction

Peanuts (*Arachis hypogaea*) contain 44–56% lipids and 22–30% proteins. The flour extract of peanuts contains about 300 different proteins, among which proteins that cause an allergic reaction. There are at least 18 different allergen proteins. Although about 11 allergen proteins have been identified, they are not well characterized, as different sources give different descriptions (Table 1, [1–12]). Peanut allergens are water-soluble glycoproteins and are stable towards heat, acid and enzymatic digestion treatments [11]. Ara h1 is the most abundant protein and Ara h2 and Ara h6 are the proteins that are considered most important for food allergy. Each protein occurs in many isoforms. Ara h1 and Ara h3 are incorporated into high molecular weight protein complexes upon roasting [1].

Since the early days of CE, proteins have been in focus for analysis with CZE. Fundamentally, capillary zone electrophoresis (CZE) is a very suitable technique for the analysis of proteins. Proteins have lower diffusion coefficients than small molecules and lower diffusion coefficients result in higher efficiencies, provided the adsorption of protein to the capillary wall is prevented [13–15].

Early examples from industry showed the use of a 6-amino caproic acid buffer [16] that was further developed by Yan He into a well-used method for charge heterogeneity determinations of monoclonal antibodies [17]. However, this method is unsuitable for the proteins of this peanut extract as the pH of He's electrolysis solution is in the same range as the pIs of the proteins. If the pH is too close to the pI, the electrophoretic mobility is close to zero and peaks are very broad, preventing proper separation. If the pH = pI, the protein does not have a net-charge and therefore no electrophoretic mobility. pHs significantly above or below the pI result in sufficient electrophoretic mobilities and can also give different selectivities due to different functional groups being charged.

To prevent or reduce adsorption, there are a few approaches one can take. One approach is the use of dynamically or covalently coated capillaries. Another is selection of the pH such that the capillary wall and the proteins carry equally signed charges [14, 15]. However, these approaches by themselves are usually not sufficient, e.g., [18], so combination with additional measures are generally needed. Additional measures can include the use of high-concentration buffers, and the addition of (neutral) surfactants to the background electrolyte (BGE) solution [18, 19]. In these cases, care has to be taken that there is no excessive Joule heating due to high currents.

A simple scheme for a systematic approach for method development is depicted in Figure 1, making use of selectivity differences at pHs above and below the pI. These different pH regimes are combined with two different polymer wall coatings. The covalently neutral-coated capillaries are commercially available and result in a suppressed electro-osmotic flow (EOF). The SMIL2- and SMIL3-coated capillaries are treated with charged polymers that result in a two-layer coating with strong normal EOF, or a three-layer coating with strong reversed EOF, respectively [20, 21].

At a pH > pI, the net charge of the proteins is negative. In a covalently neutral coated capillary, there is no EOF. The proteins migrate towards the anode, the ones with the largest charge/size ratio migrate fastest. If at the same pH > pI in the same electrolyte solution a SMIL-2 coating is used, both

the capillary wall and the proteins are negatively charged. The negatively charged wall results in a strong EOF towards the cathode. The negatively charged proteins have an electrophoretic mobility towards the anode, but because of the strong cathodic EOF they ultimately migrate towards the cathode, where the proteins with the smallest charge/size ratio migrate out first. So at the same pH and in the same electrolyte, switching capillary coating in this case means mirroring the migration order.

At a $\text{pH} < \text{pI}$, the net charge of the proteins is positive. In a neutral coated capillary, the proteins migrate towards the cathode and the ones with the largest charge/size ratio migrate fastest. In a SMIL-3 coated capillary with the same electrolyte, the wall is positively charged resulting in a strong anodic EOF. The proteins net migrate towards the anode and the proteins with the smallest charge/size ratio migrate out first. So again, switching capillary coating mirrors the migration order.

Capillary electrophoresis (CE) can effectively resolve peanut proteins and peptides, as demonstrated by Basha et al. [22–24]. Acidic and basic defatted seed extracts were analyzed with CGE and CZE, illustrating that patterns changed upon maturation of the seeds and that burning of the seeds resulted in less acid-soluble peptides and small proteins. Unfortunately, no proteins were identified in these works.

The purpose of the current study was to investigate the applicability of different CE modes for the profiling of proteins in peanut flour extracts and to set-up a strategy for CZE method development for protein mapping, with special attention for proteins Ara h1, Ara h2 and Ara h6, in order to develop a method for batch-to-batch comparison. The results highlight that the function of the BGE is not only to determine the charge-to-size ratio of the proteins, but that at the same pH and ionic strength, different BGE components can affect the separation significantly.

Materials and methods

Reagents

CE-SDS sample buffer, 10 kDa internal standard and CE-SDS separation gel buffer were obtained from Sciex (Brea, CA, USA). Polysorbate 80 (PS-80), triethylenetetramine (TETA), oxalic acid, α -Lactose monohydrate, sucrose, maltose, maltotriose, sodium chloride, calcium chloride, 4 amino-butanol, sorbitol, trehalose, maltose monohydrate, dextran sulfate sodium (DS), Polybrene (PB), bupivacaine and benzoic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Tris base was from Promega (Fitchburg, WI, USA). Polysorbate 20, mercaptoethanol, and malonic acid were from Alfa Aesar (Ward Hill, MA, USA). Orthophosphoric acid, hydrochloric acid 1N, acetonitrile, trifluoroacetic acid and propan-2-ol were obtained from Carlo Erba (Milan, Italy). Glycerol was from VWR. Polysorbate 40 and 60 and methanesulfonic acid were obtained from Acros Organics (Geel, Belgium). Sodium hydroxide 1 M was from Fisher scientific (Pittsburgh, PA, USA) and water was of MilliQ quality (Millipore, Bedford, MA, USA).

Ara h1, h2, h3 and h6 protein standards were obtained from Indoor Biotechnologies Ltd (Cardiff, UK). Freeze-dried peanut extract: Protein seeds were milled into protein flour and defatted with acetone. The material was then air dried at ambient temperature to exhaust the acetone and sieved

through ≤ 2.0 mm sieve. The sieved material was dried at 35–41°C for 12–24 hours to reduce moisture to $\leq 11.3\%$ (Greer allergy immunotherapy, Lenoir, NC, USA). The protein flour raw material was defatted inhouse by hexane extraction. Subsequently the proteins were extracted with a slightly alkaline aqueous buffer and filtered. The resulting protein buffer solution was freeze-dried into lyophilized powder.

CE-SDS

The CE-SDS analyses were performed on a MDQ P/ACE system (Beckman Coulter, Fullerton, CA, USA), that was equipped with a UV detector. The software used with the apparatus was 32 Karat (SCIEX, Brea, CA, USA). The fused silica capillary was 30 cm total length and 20 cm effective length and the polyimide was removed from the ends.

About 2 mg of freeze-dried peanut extract was weighed accurately in a centrifugation tube and then dissolved in 2 mL of CE-SDS sample buffer (pH 9) [25]. The resulting solution was centrifuged for 20 min at 13.2 rpm and the supernatant collected. For the sample under reduced conditions, 95 μ L of the supernatant was mixed with 2 μ L of internal standard 10 kDa protein and 5 μ L of mercaptoethanol. The solution was mixed with a vortex mixer, heated for 3 min at 100°C and cooled for 5 min at room temperature before injection.

For the non-reduced sample, 95 μ L of the supernatant was mixed with 2 μ L of internal standard and 5 μ L of a 250 mM solution of iodoacetamide. The solution was then mixed with a vortex mixer, heated for 3 min at 70°C and cooled for 5 min before injection. The capillary conditioning and separation conditions were as described by the vendor [25].

Capillary zone electrophoresis

CZE capillary electrophoresis analyses were performed on a G1600AX capillary electrophoresis instrument (Agilent Technologies, Santa Clara, CA, USA) that was equipped with a Diode Array Detector (DAD). The software used with the apparatus was 3D CE Chemstation v.10.1 (Agilent Technologies, Santa Clara, CA, USA).

Different capillaries that were used were fused silica capillaries of 50 μ m internal diameter (TSP050375, Polymicro Technologies (Phoenix, AZ, USA)), and PVA-coated capillaries (G1600-61240, Agilent Technologies). The capillaries were manually cut to size. The UV detection windows of fused silica capillaries were made with a Window Maker (Microsolv, Leland, NC, USA). The capillaries had an effective length of 24.5 cm and a total length of 33 cm. For the fused silica capillaries, the polyimide was removed at the ends. The sample was injected by hydrodynamic injection by applying a pressure of 50 mbar for 5 s followed by a BGE injection. The applied voltage was 15 kV in positive or negative mode (see Figure 1) for 20 min with 1 min ramping. Between each injection, the capillary was rinsed with the electrolyte solution. UV detection was recorded at 200 nm. The cassette temperature was set at temperatures as indicated.

The successive multilayer ionic polymer layer (SMIL) coating SMIL-2 was made by flushing (1 bar) the fused silica capillary for 30 min with 1 M NaOH, 10 min with water, 10 min with 10% PB, 5 min with water, 10 min with 0.5% DS and 5 min with water. The SMIL-3 coating was made by flushing the fused silica capillary for 30 min with 1 M NaOH, 10 min with water, 10 min with 10% PB, 5 min with water, 10 min with 0.5% DS, 5 min with water, 10 min with 10% PB and 5 min with water [21].

About 5 mg of freeze-dried peanut extract was weighed accurately in a centrifugation tube and then dissolved in 1 ml of Tris-HCl buffer pH 8.2 or water. The solution was mixed with a vortex mixer (Fisher Scientific, Pittsburgh, PA, USA) and centrifuged for one minute at 13.2 rpm using a 5414 D centrifuge (Eppendorf, Hamburg, Germany). The liquid phase is collected and used as the analytical sample solution.

The BGE solutions were made freshly from stock solutions by accurately pipetting and making up to volume. The pHs were not adjusted, but measured as control.

Tentative identification of the Ara h1, Ara h2, Ara h3, and Ara h6 protein peak groups was performed on a BGE consisting of 100 mM glycine, 100 mM phosphoric acid and 3 mg/mL PS-80. The standards showed heterogenic peak patterns somewhat different from the samples, however, migration windows could be identified. The difference between samples and standards was confirmed with LC analysis (data not shown).

Results and discussion

CE-SDS

CE-SDS has been used for purity and stability characterization of therapeutic monoclonal antibodies since the 1990s and is a well-established technique within the biopharmaceutical industry [19]. Separation is based on size, as the proteins are denatured with SDS and have uniform charge/size ratios. The CE-SDS kit was optimized for resolution of the non-glycosylated IgG heavy chain and the glycosylated IgG heavy chain and the separation range is about 10–200 kDa. Although well-established for monoclonal antibodies and their derivatives, there are few reports on the use of CE-SDS for the pharmaceutical analysis of other protein mixtures, e.g., [13, 26, 27]. Analysis of the peanut extract under non-reduced and reduced CE-SDS conditions gave around 20 distinct peaks each (Figure 2). Under reduced conditions, smaller sized proteins were observed than under non-reduced conditions, in line with expectations. Although without proper standards it is hard to distinguish which peaks belongs to which Ara h proteins, patterns like this can be used for batch-to-batch comparison in early pharmaceutical development.

CZE

The four different approaches from Figure 1 were tested on the peanut extract. At high pH, the peak patterns were similar to Basha [22]. Although no reproducible results suitable for pharmaceutical analysis were obtained at basic pHs, the use of phosphate in the BGE resulted in more peaks and better resolutions (data not shown).

The Ara h proteins are stable at low pH. Phosphate is a favorite buffer at low pH, as it has little to no UV absorbance even at low wavelengths and protein-phosphate complexes are thought to adsorb less to the wall because of lower net charge and less electrostatic attraction [28]. First a simple 100 mM phosphoric acid – 70 mM tris buffer (pH 2.5) was tested. All electrolytes were made by precise weighing and dilution of specific concentrations, and not by adjusting the pH. The pH was calculated with help of the Henderson-Hasselbalch equation or with PeakMaster, and the preparation was controlled by measuring the pH. The phosphate buffer was made with tris instead of NaOH, as tris⁺ has a lower mobility than Na⁺, and therefore use of the tris-phosphate electrolyte results in lower

currents. In addition, the use of tris^+ instead of Na^+ usually means better mobility matching as in general the mobility of tris^+ is closer to the mobilities of the analytes than the fast-migrating Na^+ . Better mobility matching means reduced electromigration dispersion and thus the use of tris-phosphate generally results in better peak shapes. Tris was also a component from the formulation buffer, stabilizing the product.

Because of the low pH-range, a PVA-coated neutral capillary was selected. The vendor information for the PVA-coated capillary says that this capillary is stable down to pH 2.5 in 10 mM phosphate buffers. We have used this capillary down to pH 2.0 with 100 mM phosphate buffers and we noted no issues of the coating stability due to the use of lower pH and higher buffer concentrations. The only issues we observed were contributed to, as expected, the nature of our samples. In low pH tris-phosphate buffer, multiple groups of peaks were observed, especially after addition of PS-80 to the BGE (Figure 3A). The addition of PS-80 reduced adsorption and precipitation of the proteins and resulted in better repeatabilities and reproducibilities. The same tris-phosphate buffer pH 2.5 also gave good resolution on the SMIL-3 coated capillary (Figure 3C). The peak groups were tentatively identified with Ara h standards. As can be seen from the marking of the peak groups, indeed the migration order mirrors changing capillary coating. So one might opt for a neutral coated capillary or for a SMIL-3 coated capillary, each giving a different focus on specific peak groups, depending on the proteins of interest.

When lowering the pH, the proteins become more charged and will have higher electrophoretic mobilities. For the SMIL-3 coated capillary, with the strong anodic EOF, increased electrophoretic mobilities meant that the proteins migrated slower. At pH 2.2 (100 mM phosphoric acid, 50 mM tris, 3% PS-80, Figure 3D), the reduced net mobilities resulted in peak broadening, and resolution was lost. Lowering the pH in the PVA coated capillary, on the contrary, resulted in a wider protein separation window (Figure 3B).

In order to have higher buffering capacity, tris was replaced by glycine. A solution with 100 mM phosphoric acid and 100 mM Gly ($\text{pK}_{\text{a}}\text{s}$ 2.3 and 9.8) has a pH of 2.3. Interestingly, on a SMIL-3 coated capillary, a BGE made with phosphoric acid and glycine showed better separation for the early migrating peaks of the peanut extract, while a BGE of exactly the same pH and ionic strength but made of phosphoric acid and tris showed better resolution and peak shape of the later migrating peaks (Figure 4A).

To investigate this further, solutions with varying concentrations tris and glycine in phosphoric acid with the same pHs and ionic strengths were compared. This confirmed that glycine had a positive effect on the early migrating proteins and tris on the later migrating proteins (Figure 4A). These results suggest that Gly^+ and tris^+ interact differently with the proteins themselves, and that finding an appropriate BGE buffer solution comprises not only finding the right pH and ionic strength, but also finding the right co- and counter-ions in the solution. Unfortunately, this example also underlines once more that there is no such thing as one-size-fits-all in protein analysis, and a certain amount of method development will always be needed. Inspiration for appropriate buffer components for proteins can be found from formulation development.

The different effects of tris and glycine were confirmed on the PVA-coated capillary. Changing from 100 mM phosphoric acid and 55 mM tris (pH 2.3) to 100 mM phosphoric acid, 50 mM tris and 10 mM glycine (pH 2.3) meant that the largest peak resolved into two (Figure 4B). Further varying the

amounts of tris and glycine while maintaining the pH and ionic strength resulted in major shifts in the late migrating peaks. Strikingly, again the early migrating peaks are best resolved in 100 mM phosphoric acid and 100 mM glycine, without addition of tris. Only with the PVA-coated capillary, the migration order was reversed so the proteins migrating early on the PVA-coated capillary migrated late on the SMIL-3 coated capillary.

Seeing the strong effects of tris and glycine, even the role of phosphoric acid was investigated. However, replacing phosphoric acid (pK_a 2.16) by oxalic acid (pK_a s 1.27, 4.27), trifluoroacetic acid (pK_a 0.6), methane sulfonic acid (pK_a -2), maleic acid (pK_a s 1.92, 6.23), or malonic acid (pK_a s 2.85, 5.70) did not give good results, so further experiments were done with phosphoric acid.

For the BGE containing phosphoric acid, tris, glycine, and polysorbate 80, the composition was varied and also the effect of the temperature was investigated. Best results on a PVA-coated capillary were obtained with 100 mM phosphoric acid, 50 mM tris, 10 mM glycine, 6 mg/ml polysorbate 80, at 40°C. Other additives were tested, such as other polysorbates, acetonitrile, methanol, isopropanol, urea, sucrose, lactose, sorbitol, trehalose, maltotriose, TETA, butanolamine [29], NaCl, CaCl₂, or MgCl₂. Some minor effects were observed for butanolamine or sorbitol. Consequently, a Design of Experiments was performed varying the concentrations of phosphoric acid (80–120 mM), glycine (5–25 mM), tris (5–60 mM), butanolamine (0–30 mM), polysorbate 80 (2–6 mg/ml) and sorbitol (10–50 mM) resulting in solutions in the pH range of 1.6–3.2. As both tris and butanolamine act as strong bases in the tested range, they were coupled factors and tested by total concentration of base and by the percentage tris of the total base concentration. Again, mobilities of different proteins varied independently from each other, and very diverse protein maps were obtained, underlining the importance for optimization of different applications (see Supporting Information).

For such a complex mixture as the peanut extract, more enhanced conditioning than usual between injections was needed. For the SMIL-3 coating, conditioning between injections was studied. Initially, the capillary was rinsed with only BGE between injections, but this was not sufficient and migration times drifted during a sequence and resolution reduced. Different steps were investigated, such as prolonging the BGE rinse, rinsing with the coating solutions, with phosphoric acid, varying the concentration of polysorbate 80 in solutions, all followed by long sequences of injections to investigate the effects. The best results were obtained with rinsing for 3 min with 0.1 M NaOH followed by rinsing for 3 min with BGE. This gave very stable migration times and resolutions for a sequence with at least 40 injections. The PVA-coated capillaries proved very stable even only rinsing with BGE. The vendor's instructions allowed for BGEs in the pH range of pH 2.5–9.5. However, we used the PVA capillaries from pH 1.8 and higher in phosphate/tris/glycine buffers without noticing any difference on the stability of the PVA coating. Different batches of peanut extract and a sample of burned peanut extract were compared and CZE proved suitable for batch-to-batch comparison (Figure 5).

Concluding remarks

Protein mapping can be done both with CGE, (i)CIEF, as well as CZE. For very complex mixtures like the peanut extracts with likely > 300 different proteins, (i)CIEF is less useful as it is very sensitive for protein heterogeneity. The advantage of CZE over CGE is that it takes both charge and size into

account, not just size. For CZE, the strategy sketches four different situations depending on whether the pH is above or below the pI, whether capillaries with or without EOF are used. The best approach in a certain situation depends on the proteins of interest, the complexity of the sample, the presence of well-characterized standards and all physical-chemical knowledge of the proteins available. The use PVA-coated capillaries or SMIL-coated capillaries means that one can zoom in on different categories of proteins in a protein extract. In our case of acidic proteins, a pH below the isoelectric point range of the proteins proved most successful. More generally, selecting a pH above or below the pI (range) also focuses on different functional groups and properties of the proteins of interest. Although the most important role for the BGE as separation medium in CZE is supposed to be the pH and the theory of CZE does not take sample-electrolyte interactions into account, the analysis of proteins demonstrates that the composition of the buffer co-ions and counter-ions can play an important role, even if the pH and the ionic strength remains the same. For the peanut extracts, the multifactorial design of experiments identified several parameters for further method optimization. However, for the protein extract this was impeded by the lack of well-characterized standards. Nonetheless, different CE techniques such as reduced and non-reduced CE SDS and CZE, especially in combination, proved to be useful for the protein mapping of vegetal extracts such as the peanut extract, and for batch-to-batch comparisons.

The authors have declared no conflict of interest.

Data availability statement

The data that support the findings of this study are stored at Sanofi Montpellier. Restrictions apply to the availability of these data, which were used under license for this study.

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Supporting information

Supporting information file: Selected results from the multivariate DoE investigation of the concentrations of phosphoric acid, tris, glycine, PS-80, butanolamine and sorbitol on the protein mapping of peanut extract.

Tables

Table 1: Allergen proteins from *Arachis hypogaea* characteristics [1–12]

Allergens	Mw (kDa)	pI	Estimated % of the total protein in peanut extract	Additional information
Ara h1	65	4.55	12–16	Homotrimer (180 kDa), dimer; Extensive PTM and glycosylation; No disulphide bonds
Ara h2	17–19	5.8 5.5 5.2	5.9–9.3	2 Isoforms; No PTM except for disulphide bonds, glycosylation disputed
Ara h3	14–60	4.6 [1] 5.5 [10]		Hexamer (360 kDa) [1], trimeric [2]. Ara h3 and Ara h4 are regarded as isoforms of each other and are therefore considered to be the same allergen.
Ara h6	15	5.5	2.5–4	No PTM except for disulphide bonds, except for Asn-containing form (subunits 9.2 and 5.4 kDa)

Figures

Figure 1:

Four approaches for the separation of complex protein extracts with CZE.

Figure 2:

CE-SDS separation of peanut extract under non-reduced and reduced conditions.

Figure 3:

Initial results for the peanut extract at $pH < pI$. Conditions: UV detection at 200 nm (4 nm bandwidth), $T = 25^{\circ}C$, sample injection 50 mbar x 5 s. A. PVA-coated capillary, 100 mM phosphoric acid, 70 mM tris, 3 mg/ml PB-80 (pH 2.5), $V = 15$ kV. B. PVA-coated capillary, 100 mM phosphoric acid, 50 mM tris, 3 mg/ml PB-80 (pH 2.2), $V = 15$ kV. C. SMIL 3 coated capillary, 100 mM phosphoric acid, 70 mM tris, 3 mg/ml PB-80 (pH 2.5), $V = -15$ kV. D. PVA-coated capillary, 100 mM phosphoric acid, 50 mM tris, 3 mg/ml PB-80 (pH 2.2), $V = 15$ kV.

Figure 4:

Analysis of peanut extract proteins at pH 2.3. BGE pH 2.3 with 3 mg/ml PS-80 and buffer as indicated in the picture. Other conditions: SMIL-3 coated capillary 33.0 (24.5) cm x 50 μm , $25^{\circ}C$, detection at 200 nm (4 nm bandwidth), sample injection 50 mbar x 5 s. A. SMIL 3 coated capillary, $V = -15$ kV with 1 min ramping. B. PVA-coated capillary, $V = 15$ kV with 1 min ramping.

Figure 5:

Peanut extract batch comparison. Conditions: PVA-coated capillary 33.0 (24.5) cm x 50 μm ; BGE 100 mM phosphoric acid, 50 mM tris, 10 mM glycine, 6 mg/ml PS-80 (pH 2.29), $T = 40^{\circ}C$, $V = 15$ kV, sample injection 50 mbar x 5 s, detection at 200 nm (4 nm bandwidth).

Protein mapping of peanut extract with capillary electrophoresis[‡]

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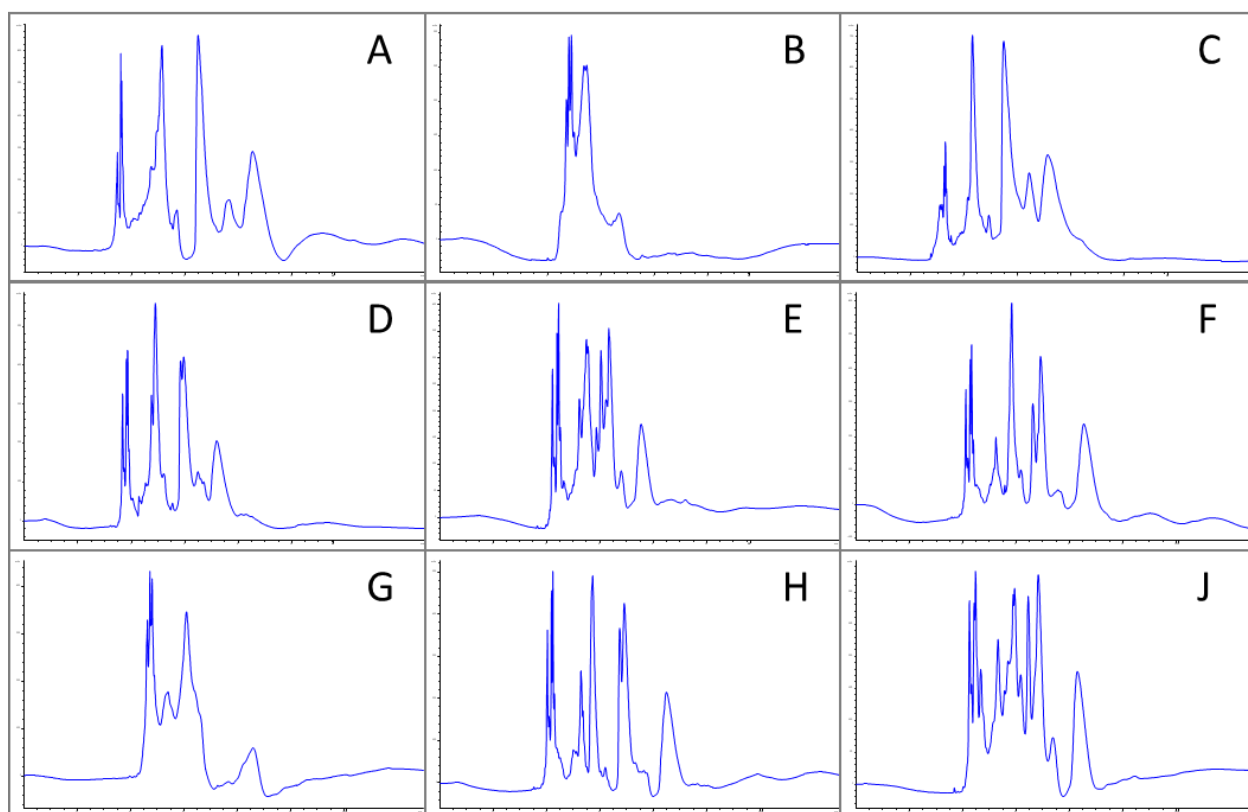
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Supporting information

Supporting information file: Selected results from the multivariate DoE investigation of the concentrations of phosphoric acid, tris, glycine, PS-80, butanolamine and sorbitol on the protein mapping of peanut extract.



Egram	H ₃ PO ₄ (mM)	Tris (mM)	Gly (mM)	PS80 (mg/ml)	Butanolamine (mM)	Sorbitol (mM)	pH
A	120	10	25	6	0	10	1.79
B	80	60	25	2	0	10	3.16
C	80	5	5	2	5	50	1.79
D	100	26	15	2	9	30	3.16
E	120	60	25	2	0	50	2.36
F	120	30	25	6	30	10	2.37
G	80	60	5	6	0	50	2.91
H	100	50	10	6	0	0	2.29
J	120	60	25	6	0	50	2.55

Supporting information: Some results of a multivariate design of experiments with factors phosphoric acid concentration, tris concentration, glycine concentration, PS-80 concentration, butanolamine concentration and sorbitol concentration. The pH of the solution was measured. Other experimental conditions: PVA-coated capillary, 33.0 (24.5) cm x 50 µm, T = 40 °C, V = 15 kV, sample injection 50 mbar x 5 s, detection at 200 nm (4 nm bandwidth).