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Detection of the presence of soya protein in milk powder by sodium dodecyl sulfate capillary electrophoresis

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Abstract

Two different commercial kits for sodium dodecyl sulfate capillary electrophoresis (SDS-CE) were evaluated for the detection of the presence of soya protein in milk powder. The results obtained showed that SDS-CE allowed the separation of the basic subunits of glycinin and the α and α′ subunits of β-conglycinin from the main milk protein peaks. However, a detection limit lower than 10% (w/w) of soya protein in total protein could not be achieved. The use of a tetraborate–EDTA sample treatment minimized interferences from milk proteins, allowing the detection of at least 1% (w/w) of soya protein in total protein. The addition of soya protein hydrolysates could not be determined using SDS-CE.

Keywords: Milk powder; Food analysis; Proteins

1. Introduction

The compositional standards of most milk products require that they contain exclusively milk proteins, but the lower prices of some vegetable proteins could make it attractive to commit fraud. Soya protein is probably the most common non-milk protein used in milk replacers such as simulated yoghurts, coffee whiteners and frozen desserts and it is likely to be a major adulterant. Even though there has been remarkable progress in the field of the chemistry of the main soya proteins, glycinin (11S) and β-conglycinin (7S), their quantitative determination still presents problems, specially when mixed in low proportions with other products. In addition, different preparations of soya proteins are at present commercially available, as flours (42–52% protein), concentrates (62–29% protein), isolates (82–87% protein) and hydrolysates (around 20% protein) [1].

There is a long term history of applying immunological methods for the detection of soya protein in meat products, and the Association of Official Analytical Chemists in the USA has adopted an enzyme-linked immunosorbent assay (ELISA) procedure, which proved to be semiquantitative in a collaborative study [2]. It appears that immunological techniques applied to milk products also have a great potential for developing into sensitive, specific, rapid and reasonably quantitative methods [3], although selection of suitable antigens still remains the major problem [4].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most common electrophoretic technique used to study soya proteins and it has the advantage of not being very much affected by food processing, specifically heat treatment. However, results are difficult to quantify and the technique is rather time consuming. 5% (w/w) processed

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soya milk in pasteurized skim milk can be detected by SDS-PAGE, by virtue of four distinct bands characteristic of the soya milk containing samples [5]. Cattaneo et al. [6] increased the sensitivity of the SDS-PAGE method to 0.06% soya protein in total protein of melted cheese, by introducing a selective sample treatment which removed soluble casein from insoluble soya protein. By using an immunoblotting step after the electrophoretic separation, as little as 25 ng of soya protein per lane was detected in milk replacers [7].

In the last few years, the advent of capillary electrophoresis (CE) has resulted in the development of rapid and automated analyses with very high resolutions, requiring very small sample and buffer volumes and providing an accurate quantitation of the resulting data. Recently, using a hydrophilic coated capillary and a low pH buffer (2.5), CE was applied to the analysis of soya proteins and it was suggested that adulterations of dairy products with soya proteins could potentially be detected on the basis of their different CE patterns [8].

The aim of this work was the evaluation of SDS-CE methods for the detection of soya protein in milk powder.

2. Experimental

2.1. Samples

The following soya protein preparations were used: two soya protein isolates (Supro 500 E from Anvisa, Madrid, Spain and Soybean Protein Isolate from ICN Biomedicals, OH, USA), a soya protein hydrolysate (Peptigen DI-5043, MD Foods, Denmark), and a soya flour and a textured soya preparation, both purchased from a local market.

Fractions partially enriched with the 7S and 11S globulins (donated by M.L. Marina, Universidad de Alcalá de Henares, Madrid, Spain) were separated from the soya flour preparation by isoelectric precipitation with 0.03 *M* Tris–HCl (Fluka, Buchs, Switzerland) containing 0.01 *M* 2-mercaptoethanol (Merck, Darmstadt, Germany) [9].

The low heat skim milk powder used (NILAC) was from NIZO (Ede, Netherlands).

2.2. Sample preparation

The soya protein isolate Supro 500 E (82%, w/w, protein), was mixed in different proportions with low heat skim milk powder (35%, w/w, protein) reconstituted (10%, w/v) in distilled water, vigorously stirred for 30 min and lyophilized. Nitrogen contents were determined by the macro-Kjeldahl method and the conversion factors used to relate the percentage of nitrogen and the protein content were 6.38 for milk proteins and 6.25 for soya proteins. Lyophilized mixtures of soya protein and milk were directly dissolved in the SDS-CE sample buffer before injection, except in the case of milk samples containing low proportions of soya protein (0, 1, 2 and 5%, w/w, soya protein in total protein), where the sample preparation method proposed by Cattaneo et al. [6] was followed with some modifications. The lyophilized sample (75 mg) was extracted with 1 ml of tetraborate–EDTA buffer pH 8.3 (30 m*M* sodium tetraborate containing 40 m*M* $Na_2EDTA{\cdot}2H_2O$, both from Merck) by vortex-mixing (2×1.5 min, with a 5-min interval between each blending) and centrifugation at 6500 *g* for 30 min at 5°C. The supernatant was carefully removed with a pasteur pipette and the residue was extracted twice more with tetraborate–EDTA buffer. The final residue was treated with the SDS-CE sample buffer before injection.

2.3. Capillary electrophoresis

CE was carried out using a Beckman P/ACE System 2050, controlled by System Gold Software data system version 810 (Beckman Instruments, Fullerton, CA, USA). Two different commercial kits were used for the CE separations: the eCAP SDS 14-200 Kit (Beckman Instruments), with a capillary of 47 cm×100 μm I.D. (capillary 1) and the CE-SDS Protein kit (Bio-Rad Labs., Hercules, CA, USA), with a capillary of 27 cm×50 μm I.D. (capillary 2). The separation conditions used were those suggested by the manufacturers. Capillary 1 was rinsed with gel buffer (Beckman) for 5 min. Samples were injected for 30 s by hydrodynamic flow at 0.14 MPa, the voltage was 14.1 kV and the temperature 20°C. Orange G (Beckman) was used as reference marker. Capillary 2 was rinsed with 0.1 *M* NaOH (Panreac, Barcelona, Spain) for 5 min, 0.1 *M*

HCl (Panreac) for 3 min and gel buffer (Bio-Rad) for 7 min. Samples were electrokinetically injected at 10 kV for 7 s, the voltage was 21 kV and the temperature 20°C. Benzoic acid (Bio-Rad) was used as reference marker. Detection was at 214 nm in both cases. Samples were prepared by boiling for 10 min in SDS-CE sample buffer, consisting of 60 m*M* Tris–HCl (Fluka), 0.5% SDS (Merck), and 2.5% 2-mercaptoethanol (Merck). A molecular mass protein standard mixture (M_r from 14 100 to 200 000) was supplied with each SDS-CE kit.

3. Results and discussion

3.1. SDS-CE analyses of soya and milk proteins

Initially, SDS-CE electropherograms of different soya products: soya flour, textured soya, soya isolate and soya hydrolysate were compared and it was found that, except in the case of the hydrolysate, the overall protein patterns were very similar and the main peaks were present in all the preparations (results not shown). However, as expected, soya hydrolysate showed a completely different pattern with very few peaks, which was in accordance with the molecular mass distribution declared by the manufacturer (mean M_r 800–1100).

Soya protein electropherograms were quite complex, with many peaks in a wide range of M_r (Fig. 1c, Fig. 1g). The subunits of the purified major soya proteins, glycinin (with acid and basic polypeptides with M_r 31 000–38 000 and 18 000–20 000, respectively) and β-conglycinin (including α′, α, β and γ chains with M_r 82 220, 70 630, 48 420 and 46 000, respectively) are well characterized by SDS-PAGE [10,11]. Two protein fractions obtained by isoelectric precipitation [9] of a commercial soya flour preparation were used as standards for the assignment of SDS-CE peaks. As shown in Fig. 1, none of the fractions was 100% pure, but fraction 11S was considerably enriched with both acid and basic subunits of glycinin (Fig. 1a, Fig. 1e), while fraction 7S (Fig. 1b, Fig. 1f) was enriched with β-conglycinin subunits, including α- and β-type polypeptides. The preparation of soya globulins by isoelectric precipitation following the method of Thanh et al. [9] leads to protein fractions whose contents of glycinin and β-conglycinin vary between 50 to 100% [12].

SDS-CE electropherograms of a sample of low heat milk powder are also shown (Fig. 1d, Fig. 1h). The main whey proteins, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), are fairly well separated, but the casein fraction does not appear well resolved as compared with the separation performance obtained with conventional SDS-PAGE [13].

Standard curves for molecular mass estimation were constructed by plotting the log of the M_r of the standard proteins included in the molecular mass calibration kit as a function of the reciprocal of their relative migration time (migration time of the protein divided into the migration time of the reference marker). A linear relationship was demonstrated in both capillaries (r^2=0.996 and 0.980 for capillaries 1 and 2, respectively), but when those curves were used for relative mass estimation of the main soya and milk proteins, a better accuracy was obtained with capillary 2, since capillary 1 provided more than 10% error in the calculation of the M_r of most proteins (results not shown). In general terms, the M_r of many proteins, calculated from their relative migration times in CE, vary from that calculated using conventional SDS-PAGE because of the use of different polymeric sieving matrices in both methods as well as different field strengths [14].

In addition, when comparing both commercial SDS-CE kits, repeatability of the relative migration times (R.S.D.) were 0.6–0.9 and 2.5–5.5%, and repeatability of the peak areas (R.S.D.) were 3.5–8.1 and 9.4–18%, for capillaries 1 and 2, respectively, as calculated from eight injections performed on different days. In capillary 2, electrokinetic injection is required because of the high viscosity of the gel buffer and this probably contributed to a higher injection imprecision and a poorer run-to-run repeatability [15]. The ability of both kits to quantify soya proteins was examined and a linear calibration graph was found at least in the range 1.2–10.2 mg/ml of soya protein in SDS-CE sample buffer (r^2=0.967 and 0.934 for capillaries 1 and 2, respectively).

3.2. Detection of soya protein in milk powder

The comparison between the patterns of the milk

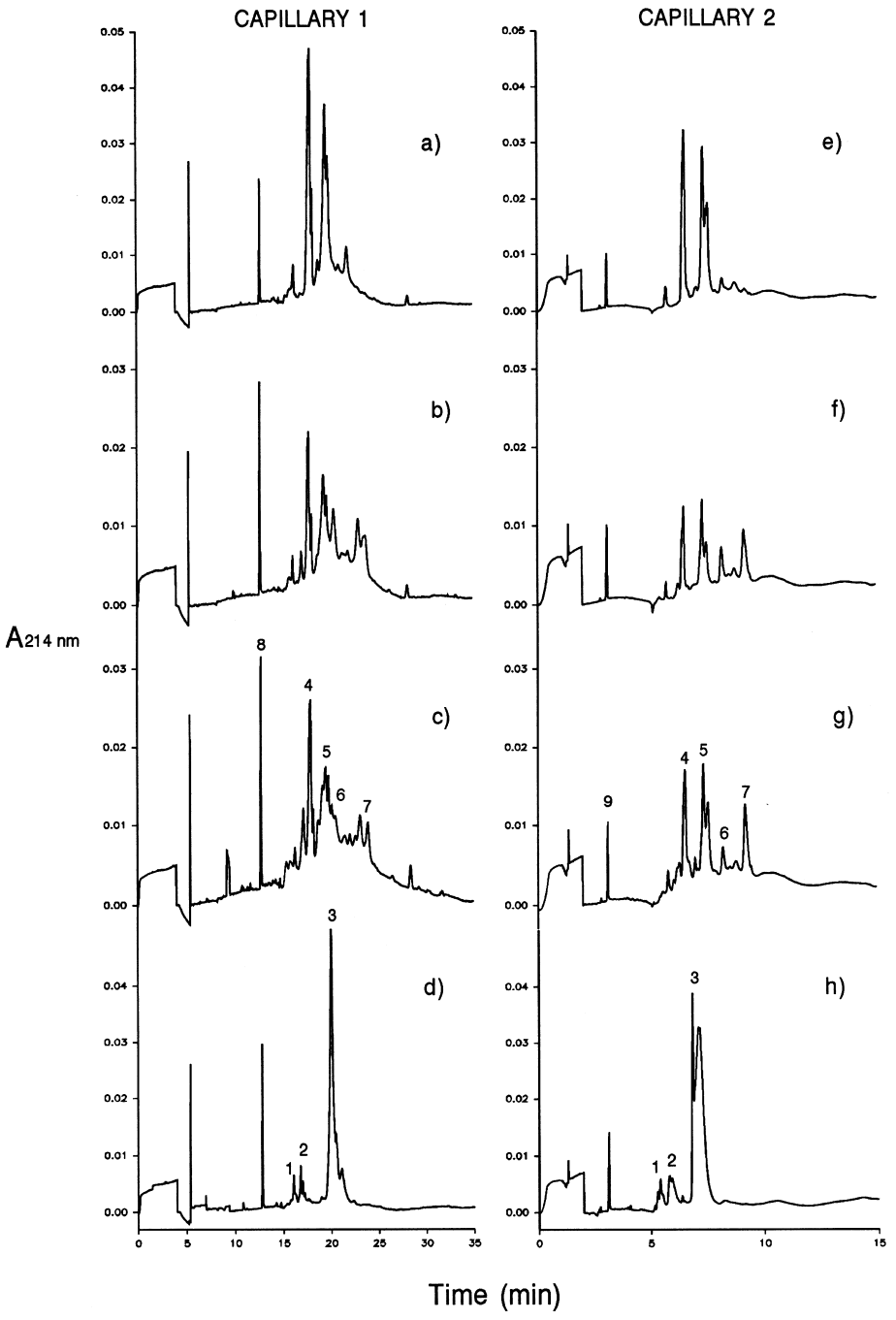

Fig. 1. SDS-CE electropherograms of 11S fraction obtained from soya flour by isoelectric precipitation (a, e), 7S fraction obtained from soya flour by isoelectric precipitation (b, f), soya flour (c, g) and low heat milk powder (d, h). Electropherograms a, b, c and d were obtained with capillary 1 (Beckman Instruments) and e, f, g and h with capillary 2 (Bio-Rad). Peaks: 1=α-Lactalbumin, 2=β-lactoglobulin, 3=caseins, 4=basic subunits of glycinin, 5=acid subunits of glycinin, 6=chain β of β-conglycinin, 7=α and α′ chains of β-conglycinin, 8=orange G, 9=benzoic acid.

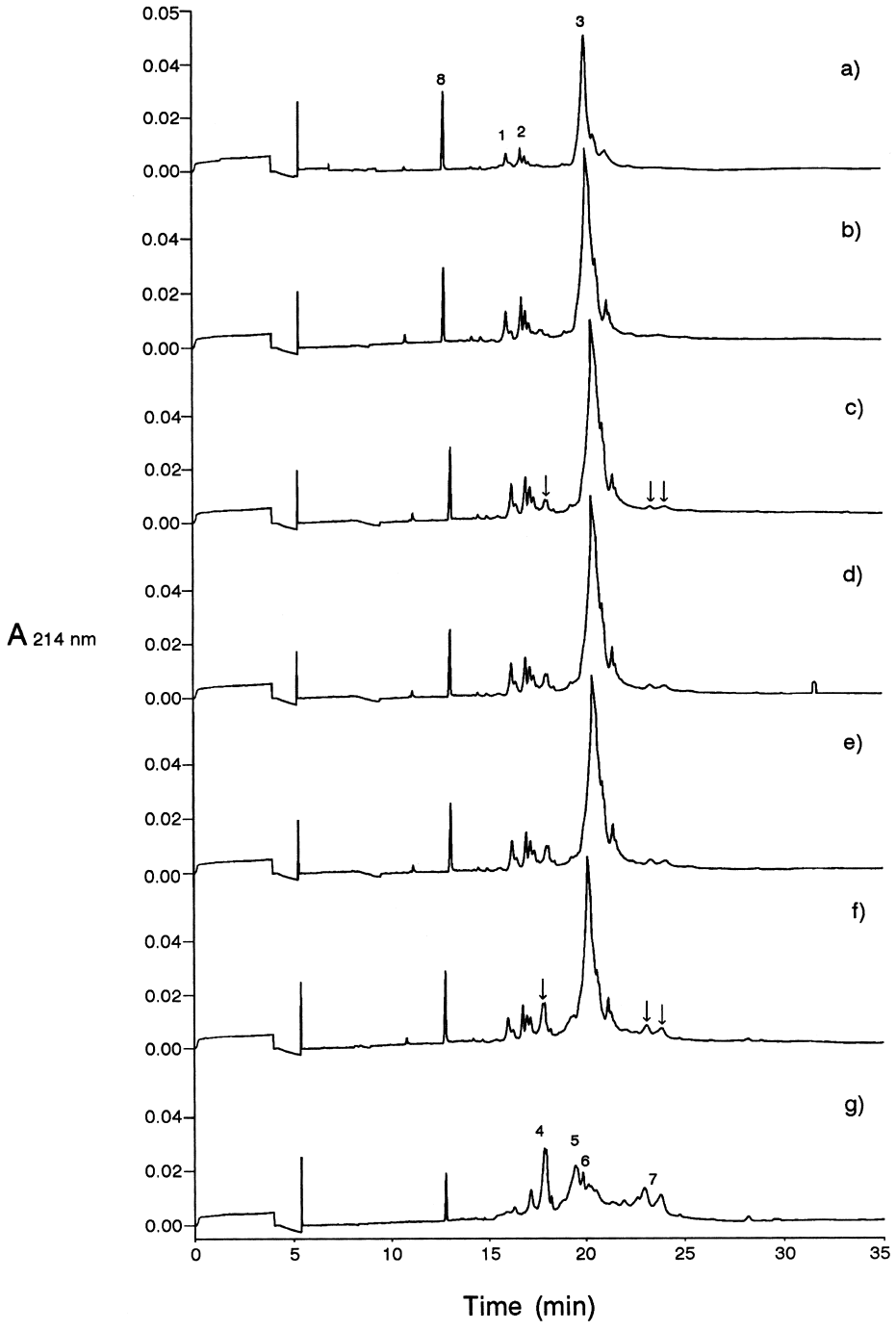

Fig. 2. SDS-CE electropherograms of samples of milk powder containing 0% (a), 7% (b), 17% (c), 25% (d), 36% (e) and 44% of soya protein in total protein and soya protein isolate (g). Electropherograms were obtained with capillary 1 (Beckman Instruments). Peaks: 1=α-Lactalbumin, 2=β-lactoglobulin, 3=caseins, 4=basic subunits of glycinin, 5=acid subunits of glycinin, 6=chain β of β-conglycinin, 7=α and α′ chains of β-conglycinin, 8=orange G. Arrows (↓) point out the indicators of the presence of soya protein in milk powder.

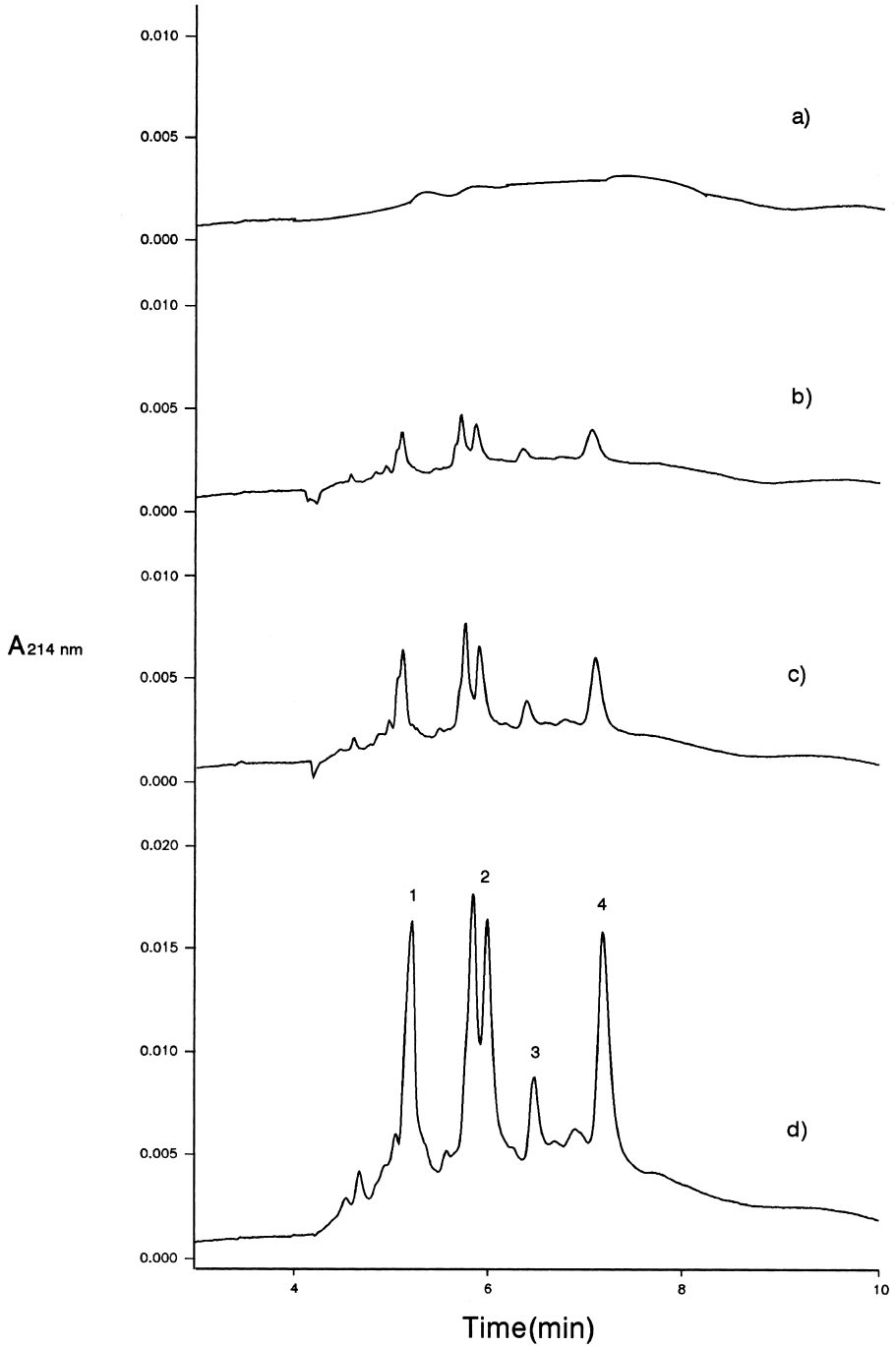

Fig. 3. SDS-CE electropherograms of samples of milk powder containing 0% (a), 1% (b), 2% (c) and 5% (d) of soya protein in total protein, once the milk proteins were removed by treatment with tetraborate–EDTA buffer. Electropherograms were obtained with capillary 2 (Bio-Rad). Peaks: 1=Basic subunits of glycinin, 2=acid subunits of glycinin, 3=chain β of β-conglycinin, 4=α and α′ chains of β-conglycinin.

and soya proteins indicated that SDS-CE methods could be applied to the detection of soya protein in bovine milk powder, on the basis of the basic subunits of glycinin and the α and α′ subunits of β-conglycinin which, by virtue of their different molecular masses, could be separated from the main protein peaks of the milk sample. The acid subunits of glycinin and the γ and β subunits of β conglycinin coincide with the main casein peaks (Fig. 1).

Initially, experiments were conducted with NILAC milk powder containing 44, 36, 25, 17 and 7% (w/w) soya protein in total protein. Fig. 2 shows the SDS-CE separation with capillary 1. As expected, the basic subunits of glycinin and the subunits α and α′ of β-conglycinin (marked with arrows in Fig. 2) separated from caseins and whey proteins. However, these bands are hardly visible in samples containing less than 10% soya protein. In addition, bands corresponding to α and α′ subunits of the β-conglycinin have a M_r similar to that of bovine serum albumin (67 000) [10,13], so they might not be good indicators of the presence of soya proteins in milk powder. Furthermore, milk proteolysis products such as γ-casein could appear near the basic subunits of glycinin complicating their determination. Therefore, once the potential soya protein indicators were pre-defined, a sample pretreatment consisting of the selective removal of milk proteins with tetraborate–EDTA buffer [6] was assayed to enhance detection.

Extraction with tetraborate–EDTA buffer of lyophilized milk powder preparations containing low proportions of soya isolate was found to avoid completely the interferences from milk proteins and detection of as low as 1% soya protein in total protein was feasible (Fig. 3). No protein peaks were found in the electropherograms of the milk samples devoid of soya proteins (Fig. 3a), while no soya proteins were found solubilized in the tetraborate–EDTA buffer. In fact, the electrophoretic pattern of the precipitated soya proteins after extraction with tetraborate–EDTA buffer was qualitatively and

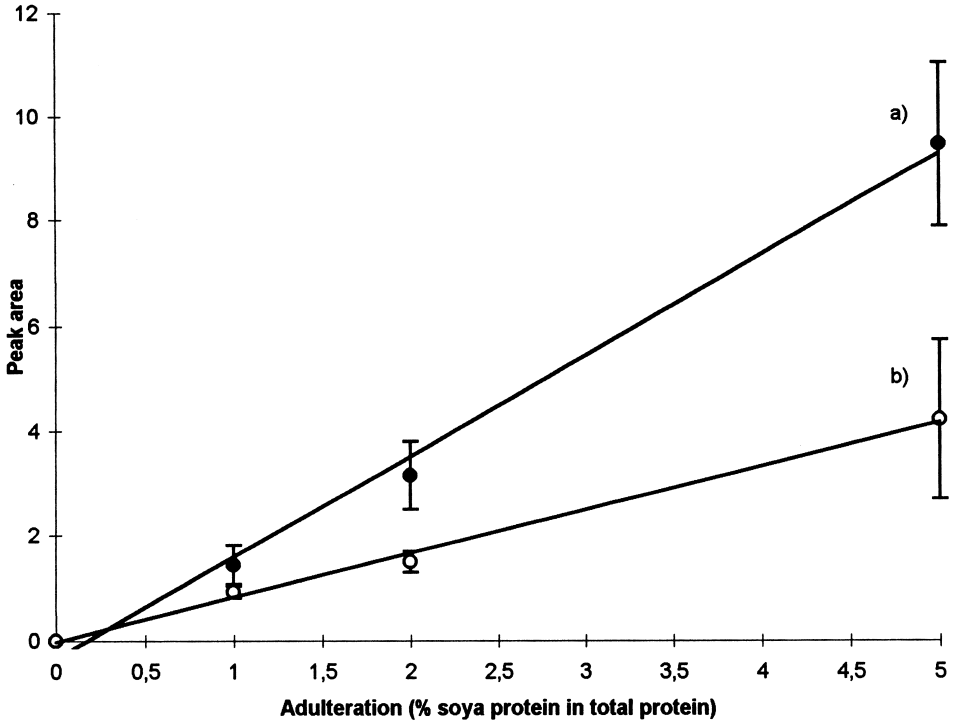

Fig. 4. Calibration graphs for peak areas of soya proteins versus % soya protein in total protein of adulterated milk powder, obtained after milk proteins were removed by treatment with tetraborate–EDTA buffer. The means (±SD) of eight independent extractions are shown. (a) Capillary 1 (Beckman Instruments) $r^2=0.9942$, (b) capillary 2 (Bio-Rad) $r^2=0.9960$.

quantitatively similar to that of the original soya isolate, indicating that losses were negligible. Extractions were performed eight times at each concentration and the repeatability was of the same order as that previously mentioned for soya proteins not treated with tetraborate–EDTA buffer. The regression curves representing peak area versus % of soya protein in total protein, after treatment with tetraborate–EDTA buffer are shown in Fig. 4.

4. Conclusions

The present results show that SDS-CE affords less resolution than SDS-PAGE for the separation of soya and milk proteins, but presents the advantage of providing shorter analysis times, offering the possibility of screening more samples in a similar period of time, and allows a more accurate quantitation of the resulting data [16].

SDS-CE methods can be applied to investigate the presence of soya proteins in milk products. The use of a tetraborate–EDTA pretreatment to enhance detection, by minimizing interferences from milk proteins, allowed the determination of, at least, 1% (w/w) soy protein in total protein. When different commercial soya protein preparations were analyzed, similar electrophoretic patterns were found, except in the case of hydrolysed products. This might suggest that the main soya protein indicators can be detected by SDS-CE independently of the type of ingredient, its composition and processing conditions used. Nevertheless, a study on the composition of glycinins isolated from the seeds of 18 cultivars of soybean by PAGE under various conditions, revealed differences in the number and relative proportion of the protein subunits [17]. Furthermore, depending on the nature of the soya product, the losses due to tetraborate–EDTA washing may vary [6]. Therefore, we are currently investigating whether the precision in the quantitative measurement can be limited by variations in the response of different types of soya preparations.

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References

[1] M.C. García, M. Torre, M.L. Marina, F. Laborda, CRC Crit. Rev. Food Sci. Nutr. 37 (1997) 361–391.
[2] J.E. McNeal, J. Assoc. Off. Anal. Chem. 71 (1988) 443.
[3] M.M. Hewedy, C.J. Smith, Food Hydrocolloids 3 (1990) 485–490.
[4] H. Meisel, Int. Dairy J. 3 (1993) 149–161.
[5] M.M. Hewedy, C.J. Smith, Food Hydrocolloids 3 (1989) 399–405.
[6] T.M.P. Cattaneo, A. Feroldi, P.M. Toppino, C. Olieman, Neth. Milk Dairy J. 48 (1994) 225–234.
[7] B.L. Ventling, W.L. Hurley, J. Food Sci. 54 (1988) 766–767.
[8] M. Kanning, M. Casella, C. Olieman, LC·GC Int. 6 (1993) 701–705.
[9] V.H. Thanh, K. Okubo, K. Shibashaki, Plant. Physiol. 56 (1975) 19–22.
[10] S.K. Sathe, G.G. Lilley, A.C. Mason, C.M. Weaver, Cereal Chem. 64 (1987) 380–384.
[11] W.J. Wolf, Cereal Chem. 72 (1995) 115–121.
[12] S. Iwabuchi, F. Yamauchi, J. Agric. Food Chem. 35 (1987) 200–209.
[13] J.J. Basch, F.W. Douglas Jr., L.G. Procino, V.H. Holsinger, H.M. Farrell Jr., J. Dairy Sci. 68 (1985) 23–31.
[14] A. Guttman, J. Nolan, Anal. Biochem. 221 (1994) 285–289.
[15] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, San Diego, CA, 1993.
[16] A. Guttman, J.A. Nolan, N. Cooke, J. Chromatogr. 632 (1993) 171–175.
[17] T. Mori, S. Utsumi, H. Inaba, K. Kitamura, K. Harada, J. Agric. Food Chem. 29 (1981) 20–23.