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Research Article

Analysis of major milk whey proteins by immunoaffinity capillary electrophoresis coupled with MALDI-MS

Two major milk whey proteins, β-lactoglobulin and α-lactalbumin, are among the main cow milk allergens and can cause allergy even at a very low concentrations. Therefore, these proteins are interesting targets in food analysis, not only for food quality control but also for highlighting the presence of allergens. Herein, a sensitive analysis for β-lactoglobulin and α-lactalbumin was developed using immunoaffinity capillary electrophoresis hyphenated with MALDI-MS. Magnetic beads functionalized with appropriate antibodies were used for β-lactoglobulin and α-lactalbumin immunocapture inside the capillary. After elution from the beads, analyte focusing and separation were performed by transient isotachophoresis followed by MALDI-MS analysis performed through an automated iontophoretic fraction collection interface. A LOD in the low nanomolar range was attained for both whey proteins. The method developed was further applied to the analysis of different milk samples including fortified soy milk.

Keywords:
Immunoaffinity capillary electrophoresis / Isotachophoresis / Magnetic beads / Matrix-assisted laser desorption/ionization-mass spectrometry / Milk allergy
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1 Introduction

Cow milk allergy is a widespread type of food hypersensitivity in human being. It is a serious health problem worldwide especially in childhood. Milk whey proteins, α-lactalbumin (α-lac) and β-lactoglobulin (β-lg), are among the main milk allergens. Prevention of adverse reactions for these compounds in clinics is largely based on milk-free diet and on avoidance of food products containing milk proteins [1]. To detect and precisely identify the presence of milk allergens in food, various analytical techniques were developed. Immunoassays like ELISA or immunoblotting are the methods of first choice in case of whey protein analysis as allergens due to their sensitivity and selectivity [2, 3]. Different types of HPLC methods are also well developed for milk protein detection. Reversed-phase [4, 5], size exclusion [6], and ion-exchange chromatography coupled to diode array detection [7, 8] or to MS detection [9, 10] are applied for whey proteins identification and characterization.

CE, due to its high resolution and fast separation performance, is a good alternative to HPLC for allergen detection [11]. It is widely used for analysis of milk whey proteins in various samples: milk [12–14], milk powders [15], cheese [16], in baby food [17], and in hypoallergenic formula [18]. A particular mode of CE, immunoaffinity capillary electrophoresis (IACE), as a combination of electrophoretic separation with the sensitivity and specificity of immunoassays, is nowadays a very powerful analytical tool. It is useful especially for the enrichment and quantification of low abundant analytes from complex matrices during drug, metabolite, or biomarker analysis [19, 20] and is suitable for allergen detection. In contrast with traditional immunoassays, IACE has the capability of performing characterization and quantification of several analytes simultaneously keeping high sensitivity and selectivity [21]. Besides the analyte enrichment on the immunoaffinity support inside the capillary, another step of sample preconcentration in IACE can also be realized by the creation of transient isotachophoresis (t-ITP) prior to CE separation. It allows further improving the sensitivity of IACE and decreasing the limit of detection. The effectiveness of IACE combination with t-ITP was already demonstrated for the analysis of β-lg and also for total IgE quantification in serum of allergic patients [22, 23].

Traditionally, UV- or laser-induced fluorescence detection are used in IACE analysis [20, 23]. However, UV has a

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Abbreviations: **α-lac**, α-lactalbumin; **β-lg**, β-lactoglobulin; **BCA**, bicinchoninic acid; **HPC**, hydroxypropylcellulose; **IACE**, immunoaffinity capillary electrophoresis; **MB**, magnetic beads; **t-ITP**, transient isotachophoresis; **UHT**, ultrahigh temperature

limited sensitivity due to the short path length of the capillary and fluorescence usually requires the fluorescent labeling for one of the components in analytical system to enable detection. In order to overcome these limitations in detection, IACE can be coupled with mass spectrometry. Meanwhile, ESI is the first choice for coupling CE [24, 25] and IACE [19] with MS, hyphenation of CE with MALDI-MS is also interesting [26, 27]. Coupling with MALDI is less straightforward, but it gives several advantages like a higher salt tolerance and the possibility to make on-plate sample modification [27–29].

In the current work, IACE was coupled with MALDI-TOF-MS in order to develop a sensitive quantitative analysis of two milk allergens: α-lac and β-lg. Magnetic beads (MBs) functionalized with appropriate antibodies were used for α-lac and β-lg immunocapture inside the coated fused silica capillary. A t-ITP step prior to CE separation was applied for additional analyte preconcentration after elution from the affinity support. The detection was performed either with a standard UV detector or with MALDI-MS analysis carried out using a home-built automated iontophoretic CE fraction collection interface [29]. The method developed was then tested on different milk samples.

2 Materials and methods

2.1 Chemicals

Tosyl-activated superparamagnetic beads of uniform size (1.08 μm diameter) were purchased from Dynabeads (Invitrogen, Oslo, Norway). Polyclonal rabbit anti-bovine β-lg (GTX77272) and polyclonal goat anti-bovine α-lac antibodies (GTX77275) were purchased from GeneTex (San Antonio, TX, USA). Bovine β-lg (A + B, 90%), bovine α-lac type I, cytochrome c from horse heart, BSA, and Tween 20 were purchased from Sigma-Aldrich (Buchs, Switzerland). Hydroxypropylcellulose (HPC) was purchased from Acros (Basel, Switzerland). Acetic acid (99.5%), TFA, sinapinic acid (≥ 99%) were purchased from Fluka (Buchs, Switzerland) and sodium borate, ammonium acetate from Merck (Darmstadt, Germany). All other chemicals were of analytical reagent grade. All the buffers and sample solutions were prepared with water produced by an alpha Q-Millipore System (Zug, Switzerland). Commercial ultra-high-temperature (UHT) treated bovine liquid milk (3.5% of fat), skimmed milk powder (35 g protein/100 g), and soy milk were purchased in a local supermarket.

2.2 Apparatus

The IACE experiments were carried out with a HP^{3D}CE apparatus (Agilent, Waldbronn, Germany) equipped with a DAD. Fused silica capillaries (50 μm id, 375 μm od, 41.5 cm effective length, 50 cm total length) were obtained from BGB analytik AG (Böckten, Switzerland) and were coated with 5% HPC solution in the laboratory according to the procedure described by Shen et al. [30]. To capture the MBs, two permanent cylindrical magnets (Nd-Fe-B, 4 mm diameter, 12 mm length, Supermagnete, Zürich, Switzerland) were placed directly around the capillary at a distance of 14.5 cm from the inlet using a homemade Plexiglas holder. MALDI-MS experiments were carried out on a MALDI-TOF Microflex instrument (Bruker Daltonics, Bremen, Germany).

2.3 Magnetic bead derivatization

MBs were coated with anti-β-lg and anti-α-lac antibodies separately according to the manufacturer protocol with some minor modifications. A total of 2 μL of rinsed tosyl-activated MBs were mixed with 8 μL of coating buffer (100 mM sodium borate, pH 9.5), 8 μL of 3 M ammonium sulfate in coating buffer, and 8 μL of corresponding antibody solution with concentration of 5 mg/mL. The mixture was incubated for 24 h at 37°C under continuous moderate stirring to avoid MBs sedimentation. After incubation, beads were rinsed with 10 mM PBS buffer (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) and stored in same PBS buffer, but also containing 0.025% of Tween 20 and 0.02% of sodium azide. In order to estimate the amount of antibodies bound to the bead surface, a bicinchoninic acid (BCA) protein test (BCA kit, Pierce, Rockford, USA) was carried out following the standard manufacturer protocol. Prior to each experiment, MBs coated with anti-β-lg and anti-α-lac antibodies were rinsed with PBS buffer, mixed together in ratio 1:1, and diluted to a working concentration of 0.5 mg/mL for each type of beads.

2.4 IACE experiments

Throughout the experiments, the following buffers were used: leading electrolyte and washing buffer (50 mM ammonium acetate, pH = 8), sample buffer (10 mM PBS with 0.1% of Tween 20), elution buffer (10% acetic acid with 0.1% TFA, pH 1.5), separation buffer (10% acetic acid, pH 2). The concentration range for standard solutions of the two whey proteins (mixture 1:1), β-lg and α-lac, was 0.01–50 μg/mL.

The scheme of a typical IACE experiment was the following (Fig. 1):

– MBs mixture (0.5 mg/mL for each type of beads, in PBS buffer) injection for 300 s at 39 mbar and trapping by permanent magnets inside the HPC-coated capillary;
– washing with washing buffer, 250 s at 39 mbar;
– sample injection for 600 s at 39 mbar;
– washing for 650 s;
– elution via direct injection of elution buffer at 39 mbar;
– separation of the sample via application of 24 kV from anode to cathode with electrode compartments filled with separation buffer;
– detection by UV (at 200 nm) or MALDI-MS;
– MBs removal and capillary washing with high pressure, at 1000 mbar.

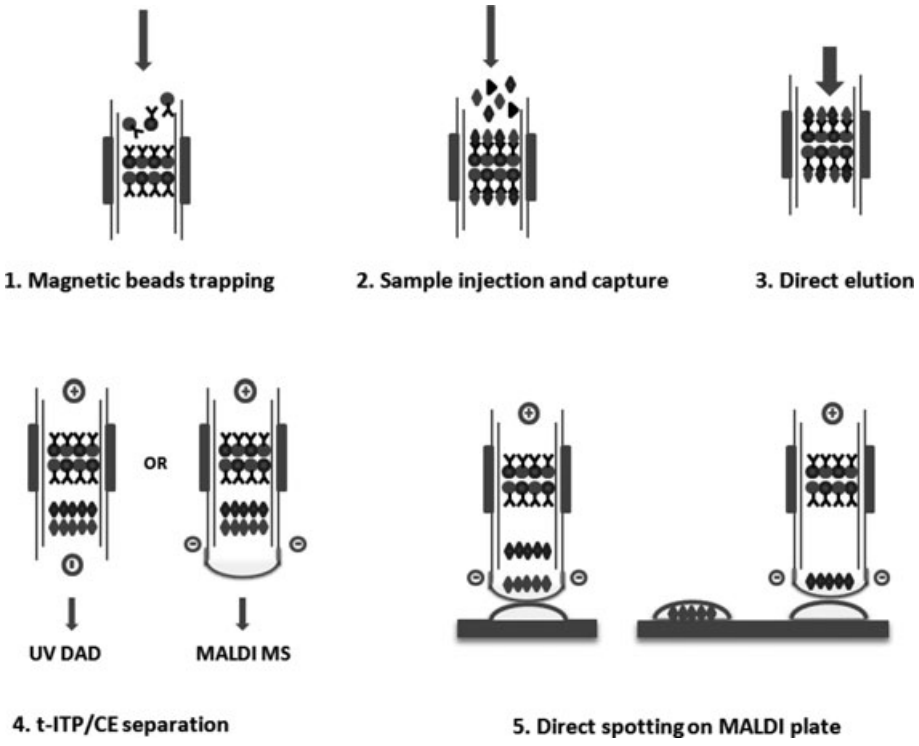

Figure 1. Schematic representation of a typical IACE-UV or IACE-MALDI-MS experiment.

2.5 CE-MALDI interface and MALDI-TOF-MS

The hyphenation of IACE with MALDI-TOF-MS was realized through a homemade automated fraction collection interface [29]. One extremity of the separation capillary was painted with silver ink from Ercon (Wareham, MA, USA) over a length about 10 cm (from the outlet). The ink was cured at 80°C during 90 min in the oven. This capillary placed in a CE-MS cassette was installed in the CE instrument with its painted extremity fixed in a ceramic holder outside the apparatus. The holder is an integrated part of the robotic system, which is capable to move the capillary in all three axes above the MALDI plate (Anchor Chip MALDI target, Bruker, Bremen, Germany) positioned on the stage. Movements of the capillary and fractionation time scale were controlled via a Labview program (National Instruments, Austin, USA). During the CE separation, the painted extremity of the capillary was grounded and dipped into a droplet (6 μL) of separation buffer placed directly on the MALDI plate at a certain position. After delivery of the separated species on one position, the capillary was quickly lifted by the robot without current interruption and moved to the next position. After direct sample collection on the MALDI plate, the separation buffer droplets were allowed to dry, 2 μL of matrix solution (2 mg/mL sinapinic acid in 70% ACN, 0.1% TFA) were spotted above each sample droplet and also dried at room temperature. For analyte quantification, an internal calibration was performed using a matrix solution containing 10 μg/mL of cytochrome c from horse heart (MW = 12 384 Da). Positively charged ions were detected from each spot as the average spectrum from 800 laser shots at different spot locations.

2.6 Milk sample preparation

Before IACE-UV and IACE-MALDI-MS analysis, 10 mL of UHT milk or skimmed milk powder solution (20 mg/mL) were centrifuged (centrifuge Heraeus Biofuge Stratos, Kendro Laboratory Products, Osterode, Germany) for 5 min at 15 000 × *g* in order to remove the fat. A total of 2 mL of defatted milk were adjusted to pH 4.5 with 1M HCl solution and were centrifuged for 5 min at 15 000 × *g* to remove the precipitated casein (based on [31] with modifications). The supernatant was diluted 50 times with sample buffer before analysis.

Soy milk initially free of bovine proteins was fortified with mixture of standard solutions of bovine β-lg and α-lac (ratio 1:1) to obtain the following final concentrations: (i) 50 μg/mL, (ii) 25 μg/mL, and (iii) 5 μg/mL for each protein. Then, fortified soy milk samples were centrifuged for 5 min at 15 000 × *g* and diluted 70 times with sample buffer prior to IACE-MALDI-MS analysis.

3 Results and discussion

3.1 Magnetic bead derivatization

MBs coated with appropriate antibodies were chosen as immunoaffinity support for IACE analysis. The use of MBs is the easiest way to create a concentrator-microreactor inside the capillary, which does not require complicated and time-consuming procedures such as frits fabrication or membrane/monolithic structure integration. Another advantage is

the possibility to renew the affinity support prior to a next experiment by simply refilling the capillary with a fresh plug of MBs.

After covalent binding of anti-bovine β-lg and anti-bovine α-lac antibodies on tosyl-activated MBs surface according to the standard manufacturer protocol, a BCA protein assay was carried out in order to roughly estimate the amount of antibodies attached to the beads: 26 μg/mg for anti-β-lg and 20 μg/mg for anti-α-lac antibodies, while maximal active chemical functionality of beads reported by manufacturer is 6–9 mg/mg (see more details in Supporting Information). Detailed discussion about magnets configuration and positioning for MBs trapping is provided in Supporting Information.

3.2 IACE experiments

In the present work, IACE for allergen detection was developed using t-ITP step as additional sample preconcentration induced by the separation medium with one stacker as leading electrolyte (ammonium acetate) and the coions of BGE (acetic acid) being the terminating electrolyte. Several parameters of IACE experiments were optimized using UV detection prior to the coupling of CE with MALDI-TOF-MS. An ammonium acetate solution (50 mM, pH 8) was used as leading electrolyte due to its compatibility with MS analysis. The concentration of this solution was chosen according to the fact that a high ionic strength (e.g. 100 mM) can cause the overstacking of the samples in t-ITP step [32].

Acetic acid (10% solution, pH 2) is a well-known t-ITP-CE separation buffer for proteins, which is also compatible with MS detection. Its low pH provides an effective breaking of the antigen–antibody complex on the MBs and recovery of the trapped proteins. TFA (0, 0.1, or 0.2%) was added to the acetic acid elution solution to further decrease the eluent pH in order to ensure a complete analyte elution from the MBs. Acetic acid solution containing 0.1% TFA (pH 1.5) provided the best analytes recovery and was used for further experiments (see more details in Supporting Information). Absolute electrophoretic mobility of TFA ions (-42.7 cm^2/(V·s)) is very close to the mobility of acetate ions (-42.4 cm^2/(V·s)) and cannot influence the t-ITP phenomenon in the system. HPC-coated capillaries were used for all IACE experiments to minimize protein adsorption on the capillary walls and to suppress EOF [33] that could lead to significant band broadening.

The magnitude and efficiency of the t-ITP preconcentration step also depend strongly on the length of the leading electrolyte (50 mM ammonium acetate) zone [22, 34]. To find the optimal conditions for the analysis of the mixture of β-lg and α-lac (50 μg/mL for each protein), the elution solution (10% acetic acid, 0.1% TFA) injection time was varied (Fig. 2a). Here, the elution solution is the terminating electrolyte. The more terminating electrolyte is injected, the smaller the zone of leading electrolyte becomes and thus t-ITP is weaker and shorter. When the elution solution injection time is only 210 s, a long t-ITP is observed; proteins are stacked by t-ITP for a longer time, but both proteins are not separated. A 220 s of injection results in moderate t-ITP, which efficiently preconcentrates both whey proteins and allows a good CE separation: the β-lg peak appears at 13.2 min and the α-lac peak appears at 13.7 min. An elution solution injection during 230 s leads to a very short t-ITP step, which is not sufficient to preconcentrate the sample. Proteins are separated just like in normal CZE, which decreases the sensitivity of the analysis compared to the moderate t-ITP (see more details in Supporting Information).

According to the results obtained, the best injection time for the elution solution was determined to be 220 s, because in this case, proteins are preconcentrated and at the same time are well separated, which is necessary for further coupling of IACE with MALDI-TOF-MS. The optimal value for the injection time of the elution solution can vary slightly (±10s) from one capillary to another due to differences in the quality of capillary coatings. This value should be identified every time a new capillary is used. Normally, an HPC-coated capillary handled with care provides reproducible results during 20–25 runs of IACE experiments.

Another important feature of IACE analysis as a technique based on immunoreaction is its specificity. To reduce nonspecific interactions during the analyte reaction with the grafted MBs, 0.1% of Tween 20 was added to the sample solution [22, 23]. The efficiency of this measure was tested on a mixture of β-lg and α-lac (25 μg/mL per protein) with BSA (10 μg/mL) (Fig. 2b). In the presence of Tween 20 in the sample buffer, only two peaks corresponding to β-lg and α-lac appear on the UV spectrum, while without Tween 20, an additional peak of BSA can also be observed (see more details in Supporting Information).

For quantification, standard mixtures of β-lg and α-lac (ratio 1:1) were analyzed in the following concentrations: 0.5, 1, 5, 10, 25, 50 μg/mL per protein. Calibration curves obtained are presented in Fig. 2c. These curves are linear ($R^2 \geq 0.989$) in chosen concentration range. The reproducibility of protein migration times for one capillary RSD = 1% ($n = 5$), from capillary to capillary RSD = 2% ($n = 5$), and average RSD = 6% ($n = 3$, i.e. the average of the RSD for five concentration levels analyzed by triplicates) for the reproducibility of the peak area were calculated. Due to the t-ITP stacking effect, a relatively low LOD calculated for signal-to-noise ratio of 3 was achieved for both proteins: 0.55 μg/mL or 30 nM for β-lg and 0.54 μg/mL or 38 nM for α-lac. However, the efficient analysis of trace amounts of allergens in food matrices requires a higher sensitivity (in the low nanomolar range), which is difficult to be achieved with direct UV detection. This level of sensitivity can be obtained with MS detection.

3.3 IACE-MS analysis

The hyphenation of IACE with MALDI-TOF-MS was performed via a homemade automated iontophoretic fraction collection robotized system. All the parameters of IACE

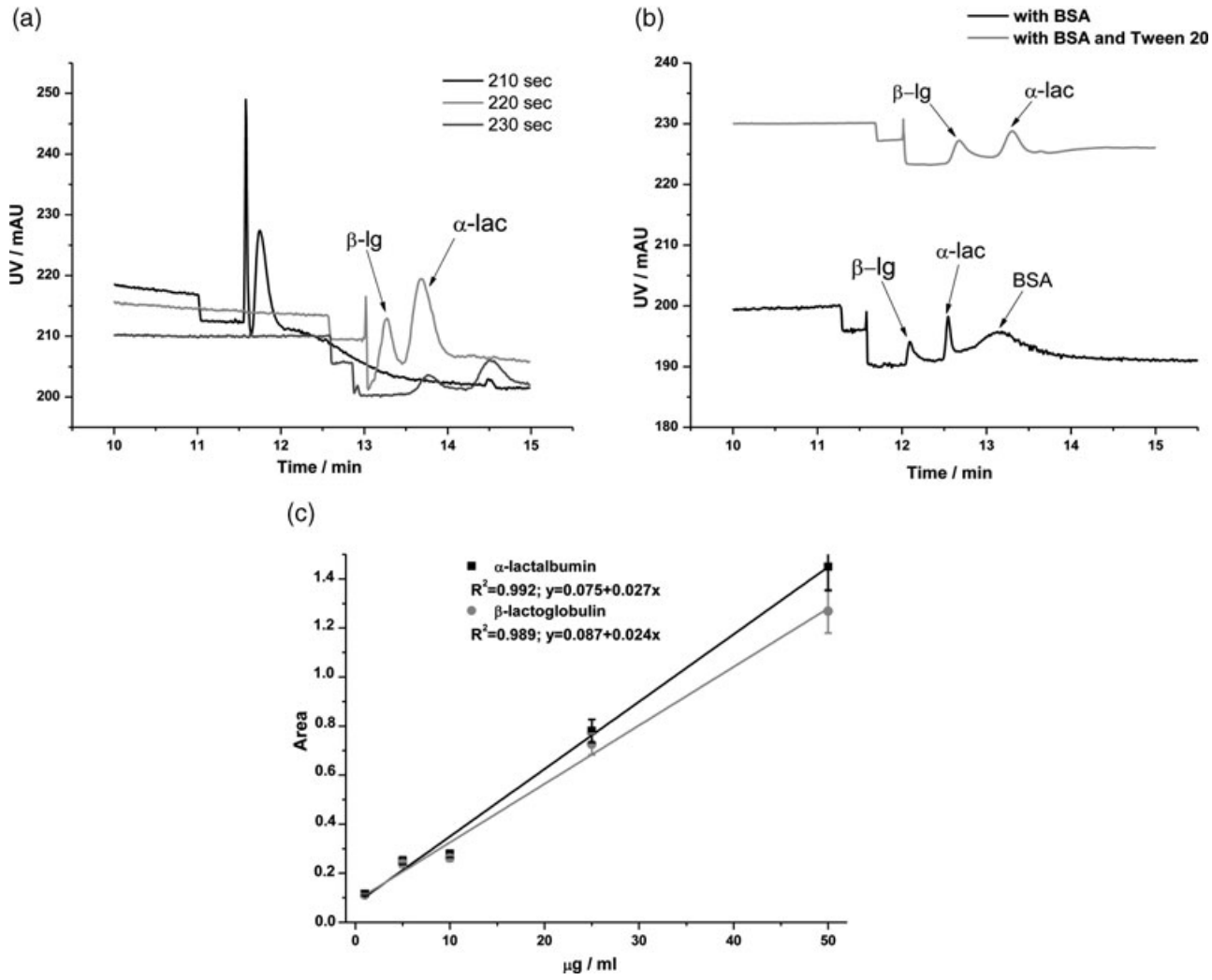

Figure 2. (a) Evolution of the t-ITP magnitude depending on the terminating electrolyte (i.e. elution solution) injection time. Conditions: CE-UV spectra at 200 nm, sample mixture of β-lactoglobulin and α-lactalbumin, 50 μg/mL both; typical IACE protocol with direct elution solution injection at 39 mbar during: 210 s—long t-ITP; 220s—moderate t-ITP, good quality CE separation; 230s—very short t-ITP, normal CE separation. (b) Nonspecific interactions study in IACE experiments. Conditions: UV spectra at 200 nm, typical IACE protocol with direct elution solution injection during 220 s at 39 mbar. Sample: β-lactoglobulin (25 μg/mL), α-lactalbumin (25 μg/mL), and BSA (10 μg/mL) mixture without (black line) and with addition (gray line) of 0.1% Tween 20 to the sample buffer. α-lac: α-lactalbumin, β-lg: β-lactoglobulin. Full electropherograms are presented in Supporting Information. (c) Calibration curves for IACE-UV quantification of two milk whey proteins: α-lactalbumin (black line) and β-lactoglobulin (gray line). Error bars: ±SD, $n = 3$.

analysis were the same as for normal UV detection, while sample spotting on MALDI plate was synchronized with CE separation without hindering it [29].

Two main fractions (spotting during 30 s per fraction) were directly collected on the MALDI plate: β-lg at 17.3 min and α-lac at 17.8 min after the voltage application. The time for spotting was calculated based on the results of UV detection taking into account the additional time needed for proteins to migrate from the detection window to the outlet of the capillary.

After drying of the collected fractions and matrix application, MALDI-TOF-MS detection was performed. The quantification was carried out using a cytochrome c (MW = 12 384 Da) added to the sinapinic acid matrix solution as internal standard. This protein was chosen for internal calibration because of its molecular mass close to the masses of the analytes. The intensities of β-lg and α-lac peaks were calculated relative to the intensities of cytochrome c peaks. As a quantification technique, MALDI-TOF-MS has some general limitations: the lack of repeatability due to baseline variation and significant variation in signal intensities. These limitations arise mainly from sample/matrix spot heterogeneity [35, 36]. In the present work, a dried-droplet preparation technique of sample mixing with matrix solution was applied in order to decrease the inhomogeneity of spot crystallization [37, 38]. Influence of the heterogeneous crystallization was also reduced by the application of high number of laser shots per sample spot (800 shots).

The average detected masses of the β-lg and α-lac were 18 360 Da and 14 180 Da, respectively. Examples of typical MALDI-TOF-MS spectra obtained for IACE-MALDI-MS analysis of target proteins (e.g. initial sample concentration 0.05 μg/mL for each protein) are shown in Fig. 3a. The measurements of β-lg and α-lac standard mixtures (ratio 1:1) by IACE-MALDI-MS technique were made in the following concentrations: 0.01, 0.05, 0.1, 1, 5, 10 μg/mL per protein. MS detection is more sensitive than UV, therefore in this case, the analysis was focused on lower analyte concentrations. Obtained calibration curves presented on Fig. 3b are linear ($R^2 \geq 0.994$) in chosen concentration range. Low LODs calculated for signal-to-noise ratio of 3 were achieved for both proteins: 0.02 μg/mL or 1.1 nM for β-lg and 0.03 μg/mL or 2.1 nM for α-lac. Average RSD = 10% ($n = 3$, i.e. the average of the RSD for five concentration levels analyzed by triplicates) for the reproducibility of the relative peak intensities was calculated. Reached LODs for both whey proteins are in the low nanomolar range (≤2.1 nM) suitable for sensitive allergen detection that requires the sensitivity at the ppm level (0.1–100 mg allergen per kg food) [2, 3]. Sensitivity of elaborated technique is comparable with the sensitivity of some classical immunoassays [2], though not as sensitive as the one described by Makinen-Kiljunen [39] and Mariager [40]. It is also two times better than HPLC-ESI-MS [9], five times better than CE-LIF [18], and between 5 and 62 times better than CIEF-MS techniques [41]. At the same time, obtained LODs are two times higher than those obtained by CE-LIF [17] and between 6 and 20 times higher than LODs obtained by HPLC-ESI-MS with marker peptides [42]. However, developed IACE-MALDI-MS analysis bypasses laborious fluorescent labeling of the analytical system components and time-consuming whey proteins tryptic digestion to obtain marker peptides. Further optimizations of the IACE-MALDI-MS method by,

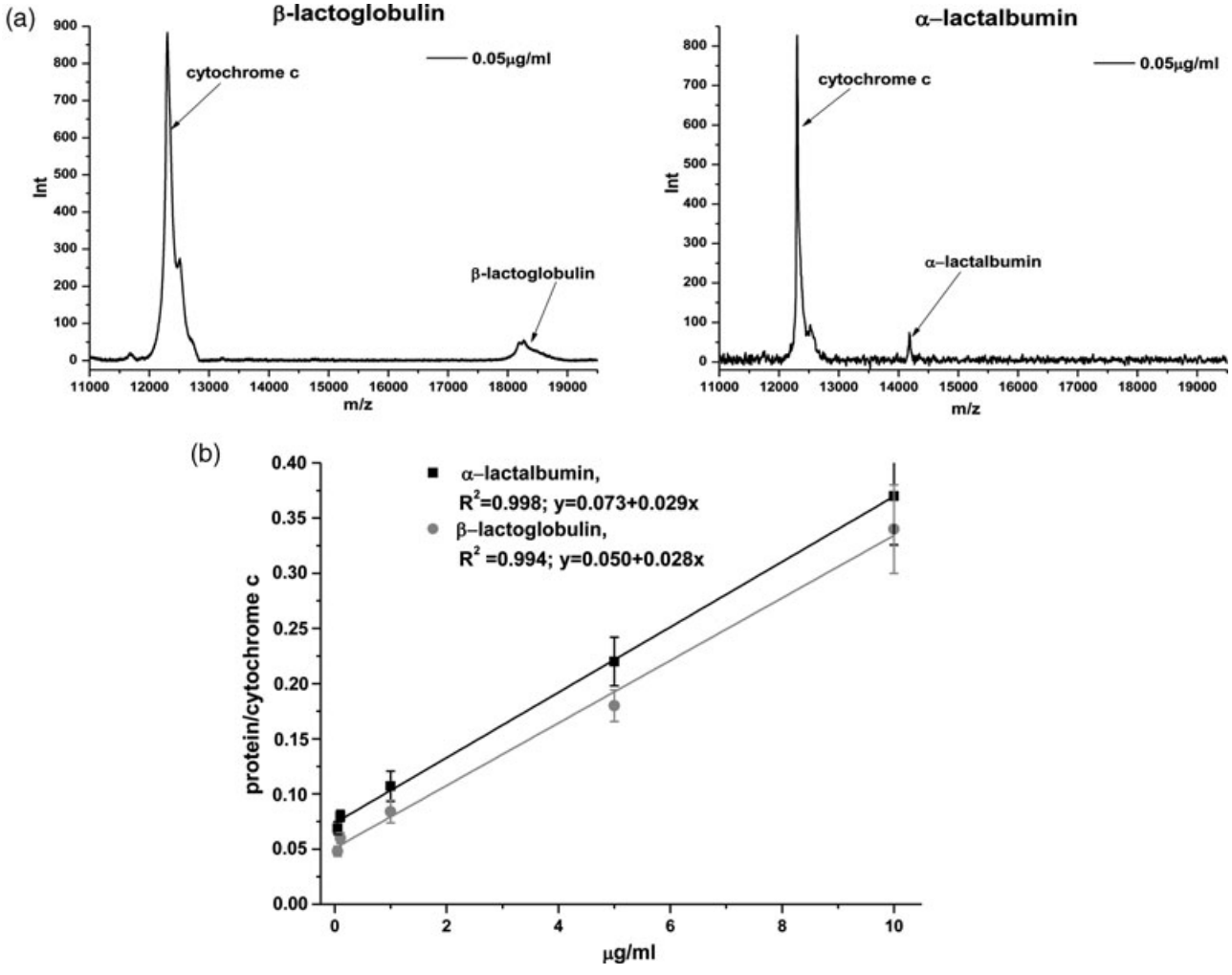

Figure 3. (a) MALDI-TOF-MS spectra of two milk whey proteins fractions collected during the CE separation step of typical IACE experiment (direct elution solution injection during 220 s at 39 mbar): (left panel) β-lactoglobulin, 0.05 μg/mL, eluted at 17.3 min, and (right panel) α-lactalbumin, 0.05 μg/mL, eluted at 17.8 min. Matrix solution: 2 mg/mL sinapinic acid in 70% ACN, 0.1% TFA with 10 μg/mL cytochrome c as internal standard. (b) Calibration curves for IACE-MALDI-MS quantification of two milk whey proteins: α-lactalbumin (black line) and β-lactoglobulin (gray line) with cytochrome c as internal standard (10 μg/mL) added to the MALDI matrix solution. Error bars: ±SD, $n = 3$.

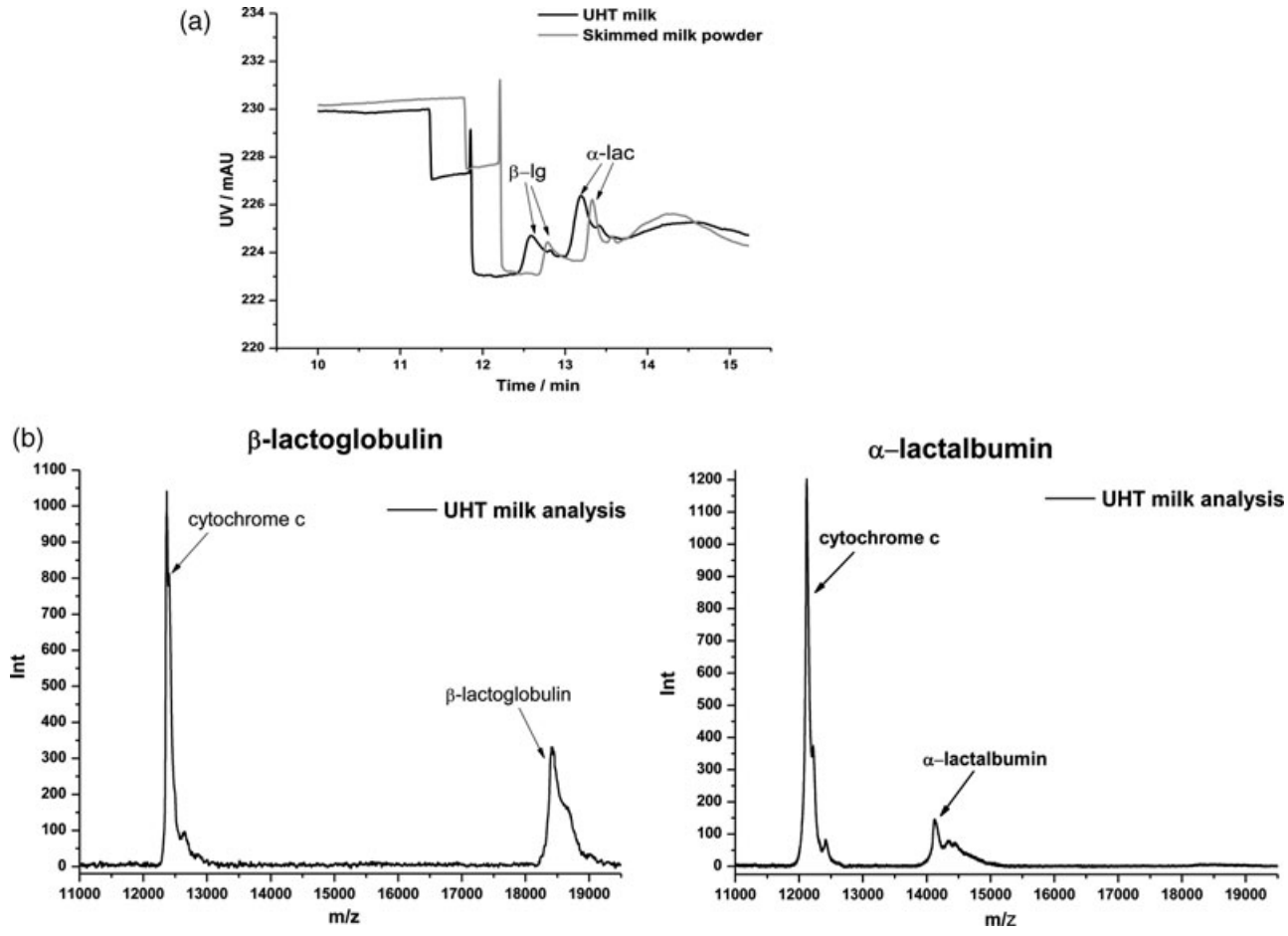

Figure 4. (a) Examples of IACE-UV electropherograms obtained during milk samples analysis. Conditions: UV spectra at 200 nm, typical IACE protocol, direct elution solution injection during 220 s at 39 mbar. Samples: UHT milk (black line) and skimmed milk powder solution (gray line), diluted 50 times after fat and casein removal. α-lac: α-lactalbumin, β-lg: β-lactoglobulin. Full electropherograms are presented in Supporting Information. (b) Examples of MALDI-TOF-MS spectra obtained during IACE-MALDI-MS analysis of milk samples. Conditions: typical IACE-MALDI-MS protocol, direct elution solution injection during 220 s at 39 mbar. Sample: UHT milk diluted 50 times after fat and casein removal.

for example, increasing the sample loading time or improving CE separation conditions, might allow reaching a better sensitivity. Meanwhile, application of MALDI-MS for detection not only allows sensitive analyte identification and quantification, but also gives the possibility to perform on-plate sample modification, for example, tryptic digestion prior to structural characterization of separated analytes.

3.4 Milk sample analysis

The IACE-UV and IACE-MALDI-MS techniques developed for β-lg and α-lac analysis were applied to a UHT and skimmed milk samples analysis. Prior to any manipulations, milk samples were defatted by centrifugation, casein was removed, and supernatant was diluted with sample buffer to decrease nonspecific interactions inside the capillary. Supernatant dilutions 25 and 50 times with sample buffer were tested. Diluting 50 times provided an optimal reduction of negative matrix effect and was further used for milk samples pretreatment (see more details in Supporting Information). Analysis of each milk sample was performed in three repetitions. Some examples of electropherograms and MALDI-TOF-MS spectra obtained are presented on Fig. 4a and 4b. The concentrations of β-lg and α-lac calculated from both types of analysis are summarized in Table 1. MALDI-TOF-MS detection gives values for proteins concentrations in UHT milk and in skimmed milk powder similar to UV detection method. These results are in the same order of magnitude as the ones found in the literature about the concentrations of whey proteins in native form for such kind of samples [22, 43]. Normally significant variations in whey protein concentrations can be observed for different UHT milk samples due to large variation of whey protein concentrations in raw milk samples used for production of UHT milk. The amount of whey proteins in raw milk significantly varies with environmental factors and genetic polymorphism [44]. As can be concluded from the obtained results, it is possible to use

Table 1. Concentration (±SD) of proteins in UHT milk and skimmed milk powder defined by IACE-UV ($n = 3$) and IACE-MALDI-MS analysis ($n = 3$).

Whey protein	Concentration[a)] detected by	
	UV	MALDI-MS
UHT milk		
α-Lactalbumin	0.15 ± 0.01	0.13 ± 0.02
β-Lactoglobulin	0.52 ± 0.03	0.47 ± 0.05
Skimmed milk powder		
α-lactalbumin	3.7 ± 0.2	3.4 ± 0.3
β-lactoglobulin	12.9 ± 0.8	13.1 ± 1.3

a) Concentration measured in mg/mL for UHT milk and mg/g for skimmed milk powder.

developed technique also for β-lg and α-lac detection in case of cow's milk containing products characterization and quality control where target proteins are presented in relatively high concentrations.

To test the efficiency of the IACE-MALDI-MS analysis for allergen detection in food products where the target analyte is presented in small amounts, soy milk samples were used. Soy milk samples were fortified with bovine β-lg and α-lac in order to create a model dairy-free product contaminated with milk allergens. Prior to IACE-MALDI-MS experiment, fortified samples were diluted with sample buffer to decrease possible negative matrix effect. Fortified sample dilutions 50 and 70 times were evaluated. Diluting 70 times with sample buffer insured effective reduction of matrix effect and was chosen for fortified samples pretreatment (see more details in Supporting Information). The calculated percentages of both whey proteins recovery in the fortified soy milk samples after analysis are the following:

(i) initial fortified concentration 50 μg/mL, $n = 3$ (±SD): 89 (±5.4)% for β-lg (A + B) and 87 (±6.3)% for α-lac were recovered;
(ii) initial fortified concentration 25 μg/mL, $n = 3$ (±SD): 87 (±5.8)% for β-lg (A + B) and 84 (±6.9)% for α-lac were recovered;
(iii) initial fortified concentration 5 μg/mL, $n = 3$ (±SD): 98 (±7.8)% for β-lg (A + B) was recovered.

For β-lg, detection and quantification were successfully performed in all fortified samples providing high percentage of analyte recovery. In case of α-lac, the analyte was detected and quantified in samples fortified at 50 and 25 μg/mL, while for soy milk fortified at 5 μg/mL, the analyte quantification was not possible due to the fact that after sample pretreatment concentration to measure, 0.071 μg/mL, was below calculated LOQ = 0.1 μg/mL. During analysis of this sample, MALDI-MS spectra obtained for α-lac possessed signal-to-noise ratio higher than 10 (S/N ~20–30, $n = 3$, RSD = 7.4%). So for α-lac, concentration 0.071 μg/mL could be regarded as experimentally defined LOQ. Taking this fact into account, the α-lac recovery for sample fortified at 5 μg/mL was calculated as 99 (±7.4)%. Meanwhile, for the correct performance of the quantitative analysis, it is necessary to use calculated LOQ = 0.1 μg/mL and only α-lac detection is considered as successfully performed in case of this fortified sample analysis.

However, obtained high percentage of target proteins recovery in fortified soy milk samples proves effectiveness of the developed technique. The present IACE-MALDI-MS analysis of β-lg and α-lac combines sensitivity and selectivity of immunoassays with the complex analytes electrophoretic separation and mass spectrometric detection. It is effective for real sample analysis and could be applied to bovine β-lg and α-lac identification as major components for food characterization and quality control or as low-abundant analytes for allergen detection.

4 Concluding remarks

IACE technique based on t-ITP step was developed for the analysis of two major milk whey proteins: β-lg and α-lac. MBs coated with appropriate antibodies were trapped inside a neutrally coated CE capillary as immunosupport for specific protein extraction and isolation prior to t-ITP preconcentration and CE separation. Then, IACE was coupled with MALDI-TOF-MS detection in order to improve the sensitivity of analysis: a LOD in the low nanomolar range (≤2.1 nM) was obtained for both whey proteins. The technique was tested on real cow milk and fortified soy milk samples to check the effectiveness of β-lg and α-lac analysis when analytes are presented in high and low concentrations, respectively. Obtained results proved that IACE-MALDI-MS analysis is suitable and efficient for both types of samples. While classical immunoassays and HPLC methods that are mainly used for allergen detection and whey proteins analysis have their own advantages and drawbacks, developed technique unites the advantages of these methods: high sensitivity and selectivity of immunoassays with simultaneous separation and characterization of several analytes. Preconcentration by t-ITP allows improving more the sensitivity of the analysis. Developed IACE-MALDI-MS method can be applied to allergen detection. Various food products can be analyzed for the presence of milk allergens: target milk proteins can be specifically isolated, separated by IACE, and analyzed via MALDI-TOF-MS. The direct spotting of the separated CE fractions on the MALDI plate also opens a possibility to characterize the structure of the allergens detected, for example, to perform on-plate sample modification like tryptic digestion, for proteomic studies of target analytes.

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