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Applicability of analytical and preparative monolithic columns to the separation and isolation of major whey proteins

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ABSTRACT

The separation and isolation of major whey proteins is already extensively covered in the literature although no study has been published in which monolithic columns were used. In our research we present, for the first time, the use of short convective interaction media (CIM) monolithic columns for the separation of all major whey proteins and isolation of β-lactoglobulin variant A and B (β-LgA and β-LgB) from a commercial product whey isolate (WI). Although our primary interest was directed towards finding a proper monolithic column and chromatographic conditions for the purification and isolation of β-LgA and β-LgB, three additional analytical LC methods, each having its own potential application target, were also developed in the course of our research. On the monolithic diethylaminoethyl convective interaction media analytical column (CIMac DEAE), the separation of major whey proteins was achieved by gradually lowering the pH of the mobile phase. The ever-so-hard obtainable linear external pH gradient was very linear in the range of pH 5.5–3 and the developed ion-exchange (IE) high-performance liquid chromatographic (HPLC) method was amenable to mass spectrometry (MS). A very fast baseline separation, with UV detection, of all major whey proteins was achieved on a prototype CIMac reversed-phase styrene-divinylbenzene (RP-SDVB) monolithic column in only 4 min and the performance of this column proved superior in comparison with the packed particle POROS perfusion column. The developed RP-HPLC–MS method is fast and, due to the MS detector, can offer low limits of detection and quantitation. Finally, in order to fulfill our primary interest, a scale-up method was developed, using a prototype 8 mL analogue of the CIMac RP-SDVB column, for the isolation of native and chemically unmodified β-LgA and β-LgB from WI with purities higher than 90% and 81%, respectively. The proteins were to be used in further protein–ligand binding studies. The developed methods excel in speed of the analysis, sensitivity, resolution, and simplicity. Thus, it is shown for the first time that short monolithic columns are applicable to the separation and isolation of major whey proteins and that their use has some obvious benefits.

1. Introduction

Whey is a liquid by-product in the cheese manufacturing process and was not long ago considered a waste [1]. The ever growing prices accompanying whey disposal and regulations, which prevented disposal of untreated whey, forced the experts to come up with an alternative use of whey. This eventually led to the birth of massive whey industry and much scorned whey rose from gutter to gold [1]. It mainly consists of water, lactose, proteins, vitamins, and minerals [2,3]. The most abundant whey proteins are β-LgA and β-LgB, α-lactalbumin (α-La) and bovine serum albumin (BSA); some minor constituents are immunoglobulins, β-casein fragments, membrane proteins, lactophorin, lactofferin, lactoperoxidase, and lysozyme [3–5].

These proteins cover all the essential amino acids and have the highest protein quality rating amongst other proteins. Based on various studies, one experiences numerous health benefits from whey and whey protein consumption [6,7]. Whey exhibits antiviral [6,8], antibacterial [9], antioxidant [10,11], antihypertensive [12,13], hypolipidemic [7], antimicrobial [6,14], and anticancer activity [6,15,16]. Moreover, whey is associated with improved muscle strength [6,17] and prevention of osteoporosis [18,19] and hepatitis B [7,20]. Its continuous intake can also reduce blood cholesterol levels [6]. Functional properties such as solubility, foaming, water sorption, viscosity, emulsification, and gelation make whey proteins an indispensable ingredient in food industry [3,21–24].

Major whey proteins have different chemical and physical properties [3–5,21]. For the most abundant whey protein,

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β-lactoglobulin, at least 9 variants (A, B, C, D, E, H, I, J, and W) are known with the most common being A and B variant [25]. β-LgA and β-LgB consist of 162 amino acid residues and are practically identical [26]; Asp64 and Val118 in β-LgA (MW = 18,363 Da) are substituted by Gly64 and Ala118 in β-LgB (MW = 18,276 Da). These properties make separating and isolating the pair individually, and from other constituents of whey, a challenging task. Despite an avalanche of research available the biological function of β-Lg is still unknown. The globular protein possesses one free cystein residue, is hydrophobic in nature, and belongs to the lipocalin family. The core structure of lipocalins consists of a characteristic eight-stranded antiparallel β-barrel that defines a calyx which represents a preferential binding site for various ligands [27–30]. β-Lg can exist as a monomer, dimer, and octamer depending on the pH of the solution [4]. Protein shows high stability and solubility at acidic conditions and also at the isoelectric point.

α-La is a globular protein composed of 123 amino acid residues (MW = 14,176 Da) and all 8 cystein residues form disulfide bonds, which stabilize the globular structure. Apo-α-La tightly binds different metal ions such as Ca^{2+}, Zn^{2+}, Mg^{2+}, Na^{+}, and others [4]. At pH < 4 the metal ions, which stabilize the holo-form of the protein, are probably progressively exchanged with protons; as a consequence the protein undergoes pronounced conformational rearrangement and faces irreversible denaturation and precipitation at low pH values [4,5]. α-La is known to take a crucial part in the process of lactose biosynthesis; a complex between lactose synthase and α-La catalyses the addition of glucose to galactose in the Golgi apparatus [4].

BSA is the largest single peptide chain whey protein with molecular mass of 69 kDa and comprises 582 amino acid residues [5]. It functions as a transport protein for fatty acids in the blood circulatory system [31]; the binding of fatty acids is also known to stabilize BSA and prevent its denaturation [5]. The denaturation of the protein proceeds at pH 4, probably due to the repulsion of positively charged amino groups alongside the polypeptide chain.

When a research on an individual major whey protein is about to be conducted their separation or isolation is a prerequisite. Often rapid separation methods are of the essence in the quality control of whey in dairy industry or in research. Today we notice an increased interest in the isolation methods for individual whey proteins from different sources due to their universal applicability; different isolation strategies and supports were evaluated [32,33]. Naturally, there are different approaches to whey protein separation, but if we restrict ourselves to chromatographic methods we can find numerous separations which are done on longer packed particle columns. In contrast, whey protein separation methods which utilize monolithic columns are not available; hitherto only one attempt has been made and it proved unsuccessful [34].

The advantages of monolithic columns over packed particle columns in the field of large biomolecule separations are widely known [35–44]. Due to the discontinuous structure of packed particle columns the mobile phase flows preferentially between the particles, hence, eddies are formed. These create dispersion and deteriorate the resolution of the separation on account of the dilution of the analytes. What is more, eddies create flow dependant sheer forces which can damage large labile biomolecules. Monolithic columns overcome these problems as a consequence of the continuous structure of monolith media. In contrast to packed particle columns where both convective (laminar and turbulent) and diffusive mass transport are present, only laminar convective mass transport is characteristic for monoliths. Hence, monoliths provide faster separation times at no loss of resolution. Other advantages of monolithic columns over packed particle ones are higher dynamic binding capacity, ease of scale up, and high recovery rates. New scaled up chromatographic parameters are usually easily determined by the following equation:

$$t_{g2} = t_{g1}\left(\frac{V_2}{V_1}\right)\left(\frac{F_1}{F_2}\right)\left(\frac{L_1}{L_2}\right) \quad (1)$$

where t_g denotes gradient time, V the column void volume, F the flow rate, L the length of the column, and subscripts 1 and 2 denote smaller and larger column size.

Unlike RP chromatography, coupling IE chromatography of proteins with MS is always a challenging task due to numerous restrictions. The use of salt solutions as an eluent is strongly discouraged as they permanently damage the MS. What is more, only volatile buffers in low enough concentrations are allowed to avoid unwanted ion suppression in the ion source. With this in mind, we are left with but a few organic acids and bases. Only one publication regarding whey protein separation can hitherto be found where an IE column is directly hyphenated to the MS; in that instance 7 proteins were separated in a tedious 70 min chromatographic run [45]. In such cases the use of monoliths should prove useful in order to increase the speed of the analysis.

In our research we studied the applicability of short monolithic columns to the separation and isolation of major whey proteins. To the best of our knowledge no such separations have been attempted to this date or the results were discouraging, respectively [34]. Thus, our primary goal was to develop a method for the isolation of β-LgA and β-LgB from a commercially available product WI and to examine the usefulness of CIM and CIMac columns for this purpose; the first step represented the development of a rapid HPLC method for the separation of major whey proteins on the basis of which the preparative scale-up would be performed. Different CIMac columns, with very small bed volumes (0.1–0.3 mL) and different chemistries, such as CIMac DEAE, CIMac RP-SDVB were tested. We were also interested in developing a rapid LC separation method which could be coupled to MS for possible quality control of whey or other dairy products in dairy industry. The performance of the prototype CIMac RP-SDVB monolithic column was briefly compared with the performance of a packed particle column with the same chemistry.

2. Experimental

2.1. Chemicals and standards

Synthetic grade acetic acid (AcOH), ammonium acetate (NH_4OAc), and ammonia (NH_3) were purchased from Merck (Darmstadt, Germany). Analytical grade formic acid (HCOOH) was purchased from Kemika (Zagreb, Croatia) and ethanol (EtOH) from Sigma–Aldrich (Steinheim, Germany). BSA (from bovine serum, >96%), α-La (from bovine milk, >85%), β-Lg (from bovine milk, 90% – a mixture of β-LgA and β-LgB), β-LgA (from bovine milk, 90%), and β-LgB (from bovine milk, 90%) were purchased from Sigma (Steinheim, Germany). HPLC gradient grade acetonitrile (MeCN) and methanol (MeOH) were purchased from J.T. Baker (Deventer, The Netherlands) and trifluoroacetic acid (TFA, 99%) from Riedel-de Haën (Seelze, Germany). Bidistilled water was used. WI was purchased from T.H.E. d.o.o. (Ribnica, Slovenia); declared amount of proteins in the product is 90%.

Unless stated otherwise a standard protein mixture was prepared daily by dissolving BSA (2 mg), α-La (2 mg), and β-Lg (2 mg) in the appropriate buffer solution (1 mL) – initial binding buffer used for the chromatographic separation. For the confirmation of the chromatographic peak identity individual protein solutions (2 mg/mL) were prepared. A solution of WI (20 mg/mL) was prepared by suspending WI powder in water and sonicating it for 5 min; the suspension was then filtered through 0.45 μm

Millipore Millex-HV hydrophilic poly(vinylidene difluoride)-PVDF membrane filter (Middlesex, USA).

2.2. IE-HPLC–UV and IE-HPLC–MS analysis on CIMac DEAE monolithic column

Both, IE-HPLC–UV and IE-HPLC–MS, techniques utilized an analytical CIMac DEAE weak anion exchange column (4.95 mm × 5.2 mm i.d., BIA Separations) as a separation media.

2.2.1. IE-HPLC–UV analysis

Separations of major whey proteins were carried out using Surveyor Plus HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a thermostated autosampler Surveyor Autosampler Plus (Thermo Finnigan) with a 100 μL loop, a quaternary pump Surveyor LC Pump Plus (Thermo Finnigan) and with a diode-array detector Surveyor PDA Plus (Thermo Finnigan). ChromQuest 4.2 was used for evaluation of the collected data. In each run 20 μL of standard protein mixture were injected. Flow rate was set to 1 mL/min, the temperature was maintained at 25 °C and the acquisition wavelength was set to 280 nm. Mobile phase consisted of two buffers: buffer A was NH_4OAc (40 mM, pH 6.5) and buffer B was AcOH (100 mM). The following gradient was applied: 10–50% B (0–9 min), 10% B (9–10 min).

2.2.2. IE-HPLC–MS analysis

The LCQ (Thermo Finnigan) HPLC–MS system was equipped with a Surveyor LC pump version 1.3 SP2 (Thermo Finnigan), Surveyor autosampler version 1.3 SP2 (Thermo Finnigan) with a fixed 25 μL loop and a 3D quadrupole ion trap as a detector. Electrospray ionization (ESI) source in positive mode was employed for the ionization of compounds. Mass spectra were collected using full scan mode in the 1300–2000 or 1300–4000 scan range, respectively (see text for more information). The capillary temperature was set to 260 °C, spray voltage to 4.5 kV, capillary voltage to 33 V, sheath gas to 35 a.u., and auxiliary gas to 15 a.u., automatic gain control (AGC) was on. Xcalibur 1.3 was used for evaluation of the collected data.

In each run 2 μL of standard protein mixture were injected. Flow rate was set to 1 mL/min and the temperature was maintained at 25 °C. Mobile phase consisted of two buffers: buffer A was NH_4OAc (60 mM, pH 6.5) and buffer B was AcOH (60 mM). The following gradient was applied: 10–100% B (0–9 min), 10% B (9–12 min). Two T-links were incorporated in the system at the column exit. The first one split the effluent in 19:1 ratio and at the second one 1% HCOOH in MeOH was added at 20 μL/min; pH of the effluent at the column exit was monitored with pH indicator strips (Merck).

2.3. RP-HPLC–UV and RP-HPLC–MS analysis on CIMac RP-SDVB monolithic column

For the separation of major whey proteins under RP conditions a prototype analytical CIMac RP-SDVB (14.85 mm × 5.2 mm i.d., BIA Separations) column was used.

2.3.1. RP-HPLC–UV analysis

HPLC–UV system used was the same as in the case of the analyses done on the DEAE column (see Section 2.2.1). In each run 10 μL of standard protein mixture or 1 μL of WI solution were injected. Flow rate was set to 1.5 mL/min, the temperature was maintained at 30 °C and the acquisition wavelength was set to 280 nm. Mobile phase consisted of two buffers: buffer A was 0.6% TFA (v/v) and buffer B was 0.6% TFA in MeCN (v/v). The following gradient was applied: 35–39% B (0–1 min), 39–40.5% B (1–5.3 min), 40.5–70% B (5.3–5.7 min), 70–35% B (5.7–6 min), 35% B (6–8.6 min).

2.3.2. RP-HPLC–MS analysis

The LC–MS separation of the proteins was achieved on an Accela U-HPLC system (Thermo Finnigan) which was coupled to the LTQ Velos mass spectrometer (Thermo Finnigan) with a linear ion trap. The Accela U-HPLC system was equipped with a thermostated autosampler Accela Autosampler (Thermo Finnigan) with a 25 μL loop, a quaternary pump Accela Pump (Thermo Finnigan) and with a diode-array detector Accela PDA Detector (Thermo Finnigan). Xcalibur 2.1 was used for evaluation of the collected data.

In each run 0.5 μL of standard protein mixture were injected, which was diluted 20-fold with buffer A prior to the analysis. Flow rate was set to 1.5 mL/min and the temperature was maintained at 30 °C. Mobile phase consisted of two buffers: buffer A was 1% HCOOH (v/v) and buffer B was 1% HCOOH in MeCN (v/v). The following gradient was applied: 25–45% B (0–10 min). At run completion the column was let to equilibrate at starting conditions for 2 min. Between the outlet of the LC system and the inlet of the MS system a T-link was incorporated which split the effluent in 9:1 ratio.

ESI source in positive mode was employed for the ionization of compounds. Mass spectra were collected using full scan mode in the 1000–3000 scan range. The capillary temperature was set to 275 °C, heater temperature to 150 °C, spray voltage to 2.5 kV, sheath gas to 40 a.u., auxiliary gas to 5 a.u., sweep gas to 0 a.u., and AGC was on. Acquisition of the data by the MS was delayed for 2.1 min in each run.

2.3.3. Monolithic vs. packed particle column

The performance of the CIMac RP-SDVB column was compared with that of the POROS R1/10 μm perfusion (100 mm × 2.1 mm i.d.) packed particle column from Applied Biosystems, Inc. (Foster City, CA, USA). HPLC–UV system used was the same as in the case of the analyses done on the DEAE column (see Section 2.2.1). For the separations done on POROS column, 10 μL of the standard protein mixture were injected. Flow rate was set to 0.9 mL/min, the temperature was maintained at 30 °C and the acquisition wavelength was set to 280 nm. Mobile phase consisted of two buffers: buffer A was 0.6% TFA (v/v) and buffer B was 0.6% TFA in MeCN (v/v). The following gradient was applied: 36–39% B (0–1.5 min), 39–41% B (1.5–7.5 min), 41–70% B (7.5–8.2 min), 70–36% B (8.2–9 min), 36% B (9–13 min).

2.4. Isolation of β-LgA and β-LgB from WI using CIMac RP-SDVB monolithic column (0.3 mL)

Isolation of β-LgA and β-LgB from WI, using an analytical CIMac RP-SDVB monolithic column, was achieved on the same system and with the same conditions as in the case of RP-HPLC–UV analysis on RP-SDVB column (see Section 2.3.1). Ten consecutive runs were made and in each run 5 μL of WI solution (20 mg/mL) were injected onto the column and the fractions were collected at the detector outlet. The fractions were pooled and were then either lyophilised (see Section 2.7) or submitted to the solid-phase extraction (SPE) procedure (see Section 2.6). When SPE procedure was employed the solutions containing β-LgA and β-LgB, respectively, were lyophilised afterwards. White voluminous powder was obtained in both cases.

2.5. Scale-up for the isolation of β-LgA and β-LgB from WI

A scale-up for the isolation of β-LgA and β-LgB from WI was achieved using a prototype 8 mL CIM RP-SDVB (56 mm l., 6.5 mm i.d., 15 mm o.d., BIA Separations) monolithic column. Analyses were done on Smartline system (Knauer, Berlin, Germany) equipped with a thermostated autosampler Midas (Spark Holland B.V., Emmen, The Netherlands) with a 100 μL loop, two Smartline Pump 1000 pumps (Knauer), a Smartline Manager 5000 (Knauer) and with a

Smartline UV Detector 2500 detector (Knauer). Chromatography data were processed using ChromGate 3.1 software.

Flow rate was set to 8 mL/min, the acquisition wavelength was set to 280 nm, and the analyses were conducted at ambient temperature. Mobile phase consisted of two buffers: buffer A was 0.6% TFA (v/v) and buffer B was 0.6% TFA in MeCN (v/v). The following gradient was applied: 35–39% B (0–2.5 min), 39–40% B (2.5–13 min), 40–70% B (13–13.5 min), 70% B (13.5–14.5 min), 70–35% B (14.5–15 min), 35% (15–20 min). Sixteen consecutive runs were made and in each run 60 μL of WI solution were injected onto the column and the fractions were collected at the detector outlet. Fractions were pooled and submitted to the SPE procedure (see Section 2.6); afterwards, the solutions containing β-LgA and β-LgB were lyophilised and white voluminous powder was obtained (see Section 2.7).

2.6. SPE procedure for the removal of TFA from β-LgA and β-LgB

Collected β-LgA and β-LgB, respectively, HPLC fractions were pooled and diluted 2-fold with water. Strata SDB-L Styrene-Divinylbenzene cartridges (100 μm, 260A, 500 mg/3 mL) from Phenomenex (Torrance, CA, USA) were utilized for the purpose of obtaining pure proteins. The following procedure was applied: solvation of the cartridge was attained with 60% MeCN (50 mL), equilibration with 10% MeCN (50 mL) followed, and afterwards the sample was loaded onto the cartridge. Washing step was done with 10% MeCN (50 mL) and β-LgA and β-LgB, respectively, were eluted with 60% MeCN (10 mL).

2.7. Lyophilisation of β-LgA and β-LgB solutions after SPE

After the protein was eluted from the SPE cartridge the solution containing β-LgA and β-LgB, respectively, was diluted with water so that the content of MeCN was <10%. The solutions were lyophilised by means of ModulyoD Freeze Dryer (Thermo Electron Corporation).

2.8. Direct inlet MS analyses

Mass spectrometry was used to determine the state of the lyophilised isolated proteins before and after SPE. In both cases a small amount (<0.1 mg) of the lyophilised protein was dissolved in 0.02% HCOOH (1 mL) and was being continuously injected into the LTQ Velos MS system at 5 μL/min. ESI source in positive mode was employed for the ionization of compounds. Mass spectra were collected using full scan mode in the 1000–3000 scan range. The capillary temperature was set to 275 °C, heater temperature to 55 °C, spray voltage to 2.5 kV, sheath gas to 30 a.u., auxiliary gas to 5 a.u., sweep gas to 0 a.u., and AGC was on.

2.9. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The purity and molecular masses of isolated β-LgA and β-LgB were determined by SDS-PAGE on a slab gel prepared with 15% (resolving gel) and 4% (stacking gel) acrylamide by the Laemmli method [46]. The WI, isolated protein fractions, and β-Lg standard were applied in parallel with the protein standard marker #SM1811 (kit for molecular masses, 10,000–250,000, Fermentas, Burlington, Canada). After electrophoresis, the gels were subjected to coomassie blue staining.

3. Results and discussion

In our research we studied the applicability of short monolithic columns to the separation and isolation of major whey proteins. No such study was done hitherto although some advantages of monolithic columns over packed particle columns are universally known. Our primary interest was directed towards purification and isolation of β-LgA and β-LgB from WI using monoliths and the first step was to obtain a satisfactory HPLC separation of major whey proteins.

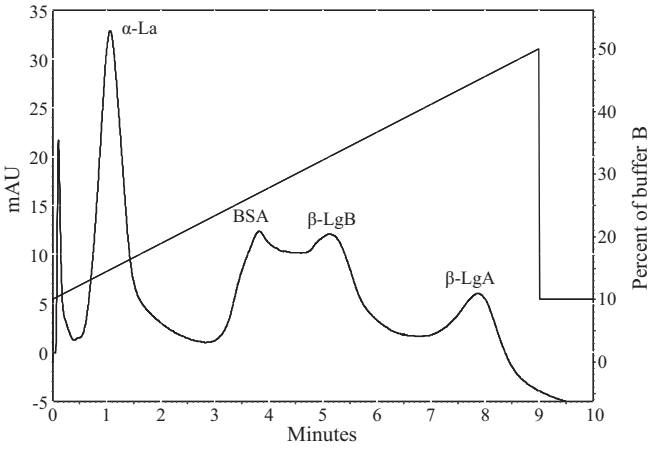

Fig. 1. IE-HPLC–UV analysis of standard protein mixture utilizing CIMac DEAE column. The proteins elute approximately at the following pH values: α-La (5.5), BSA (5), β-LgB (4.5), and β-LgA (4).

3.1. Separation of major whey proteins on CIMac DEAE monolithic column

The conditions of the separation were varied to obtain the best resolution possible; the proteins were eluted from the column by either an external pH gradient or by increasing the ionic strength of the binding buffer. Even under optimised conditions all major whey proteins could not be fully resolved as BSA and β-LgB partially overlapped (Fig. 1). The binding buffer (buffer A) applied was NH_4OAc and the eluting buffer (buffer B) was AcOH. Although an appropriate separation of whey proteins could not be established and the preparative scale-up was not feasible, these results gave birth to a side-quest.

Every endeavour was made to apply this method, developed on a CIMac DEAE column, to the LC–MS system. Some modifications were necessary to achieve a satisfactory sensitivity of the MS; two T-links were incorporated in the system after the separation has already taken place. The first one reduced the noise level by splitting the effluent (19:1) and the second one (addition of 1% HCOOH in MeOH) was crucial for the multiple charging of the proteins and their detection.

In our first attempt we got some unexpected results. Instead of four, only two peaks, which represented β-LgA and β-LgB, appeared in the chromatogram (Fig. 2A). An MS signal for the BSA could not be obtained already in the MS system tuning phase so the absence of BSA in the chromatogram is understandable. However, this was not the case with α-La. In the chromatographic analysis the protein probably was not charged enough to fall into the selected scan range, which was set to 1300–2000. If this be the case then selecting a broader scan range should have provided us with a better chromatogram. Indeed, setting the scan range to 1300–4000 resulted in the appearance of the α-La peak (Fig. 2B). On the other hand, due to the broader scan range the overall S/N ratio was seriously compromised which is evident from the MS signal drop for β-LgA and β-LgB in the latter case.

The pH of the effluent at the column exit was monitored to determine the linearity of the pH gradient. For this purpose pH indicator strips were used because a pH flow cell was not at our disposal

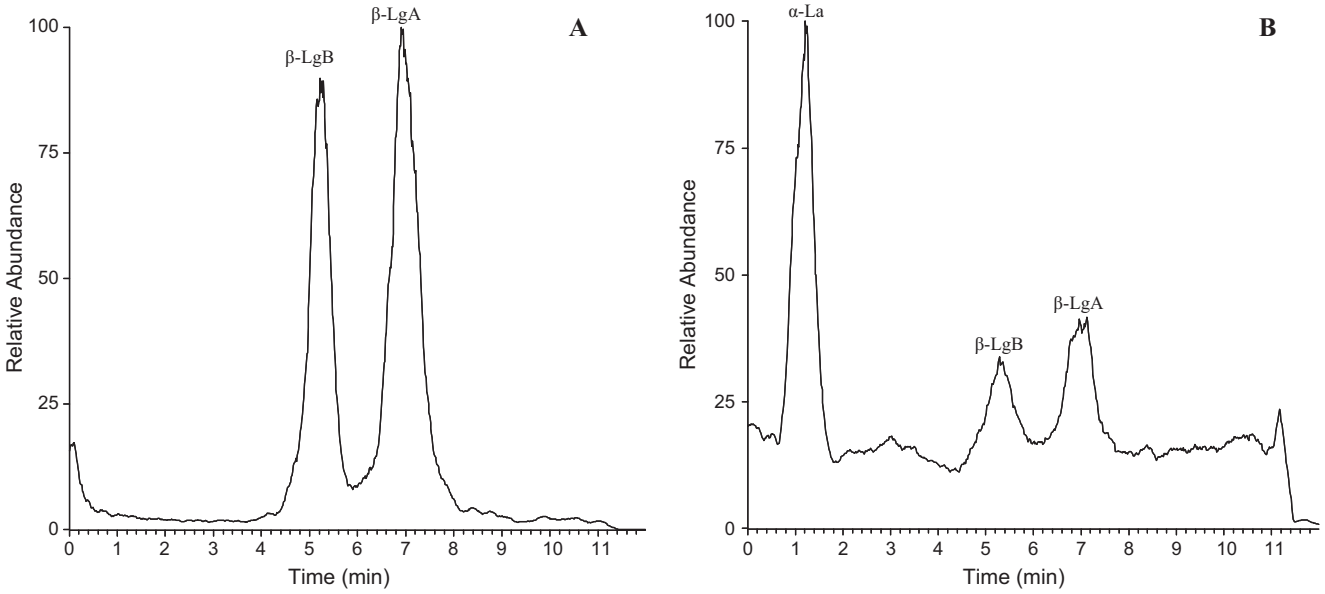

Fig. 2. IE-HPLC–MS analyses of standard protein mixture utilizing CIMac DEAE column. (A) Scan range m/z = 1300–2000. (B) Scan range m/z = 1300–4000.

and the collected effluent fractions would have been too small to measure their pH with a conventional glass electrode in reasonable time increments due to the fast chromatographic run. The pH gradient is very linear from approximately pH 5.5–3 (Fig. 3). The linearity of the external pH gradient is most probably the reflection of a very small bed volume of the column (0.10 mL), thus, the column buffering capacity is not pronounced. For the same reason, short monolithic columns are not amenable to chromatofocusing; in these experiments there was a sudden drop of pH as the internal pH gradient could not be formed inside the column (data not shown).

Longer packed particle columns often deteriorate the linearity of the external pH gradient due to considerable column buffering capacity and are, thus, not applicable in IE-HPLC if the proteins are to be eluted from the column by gradually lowering the pH of the mobile phase; as a result the majority of reported protein separation methods are developed employing a salt gradient. The somewhat compromised sensitivity and the lack of BSA peak put aside, the developed IE-HPLC–MS method is rapid, simple and, given the excellent linearity of the external pH gradient, the method could additionally be of interest to similar protein separations where their resolution is not possible under RP conditions. Thus, the developed IE-HPLC method combines rapid separation, very good pH gradient linearity, and MS compatibility; these attributes are normally extremely hard to achieve individually, let alone all together, incorporated in a single method.

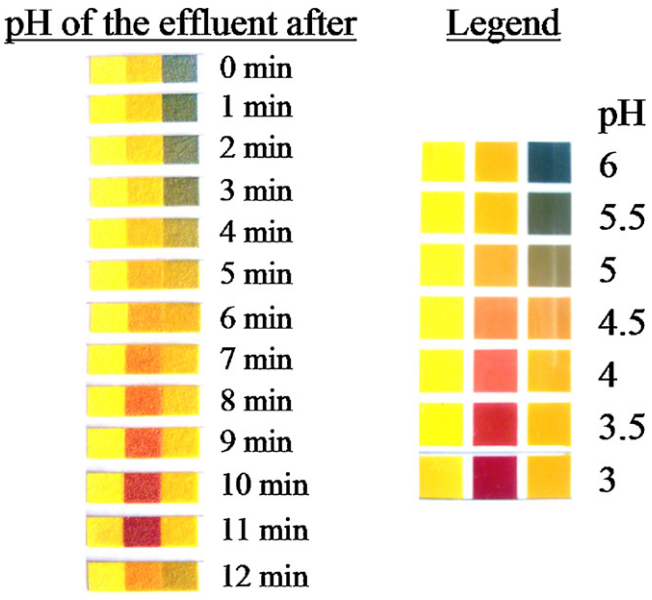

Fig. 3. Obtained pH gradient in the IE-HPLC–MS analyses of standard protein mixture utilizing CIMac DEAE column (left) and a graphic pH scale included in the pH indicator strips package (right).

3.2. Separation of major whey proteins on CIMac RP-SDVB monolithic column

Since the baseline resolution of all major whey proteins was not attained using IE monolithic column we decided to bring a RP monolithic column into use. Again, the separation was subjected to scrupulous method development. In all cases water-organic modifier gradients were applied where MeOH, EtOH, or MeCN were used. Different organic solvents did not show significant differences in the selectivity and neither did various temperatures used (20, 30, 40 °C). At higher pH levels of the mobile phase the selectivity between β-LgA and β-LgB worsened, that is why different acids (HCOOH, AcOH, and TFA) were added to the mobile phase in turn. In the end, under optimised conditions, a baseline separation of all major whey proteins was obtained in under 5 min (Fig. 4A).

For analytical purposes the performance of the CIMac RP-SDVB column was compared with that of the POROS perfusion packed particle column. POROS column is a perfusion type of column, thus, the particles are porous. But in contrast to the conventional particle type, where the analyte diffuses into and out of the pores, here, the particles possess large pores through which the analyte (protein) "perfuses" from one end of the particle to the other. Under optimised chromatographic conditions the POROS column gave similar results (Fig. 4B) with almost identical selectivity, what was to be expected from two columns possessing the same chemistry (styrene-divinylbenzene). However, when the two chromatograms were more thoroughly examined it was obvious that with CIMac RP-SDVB the separation of the proteins was achieved in almost half the time needed with the POROS column. The runtime could be

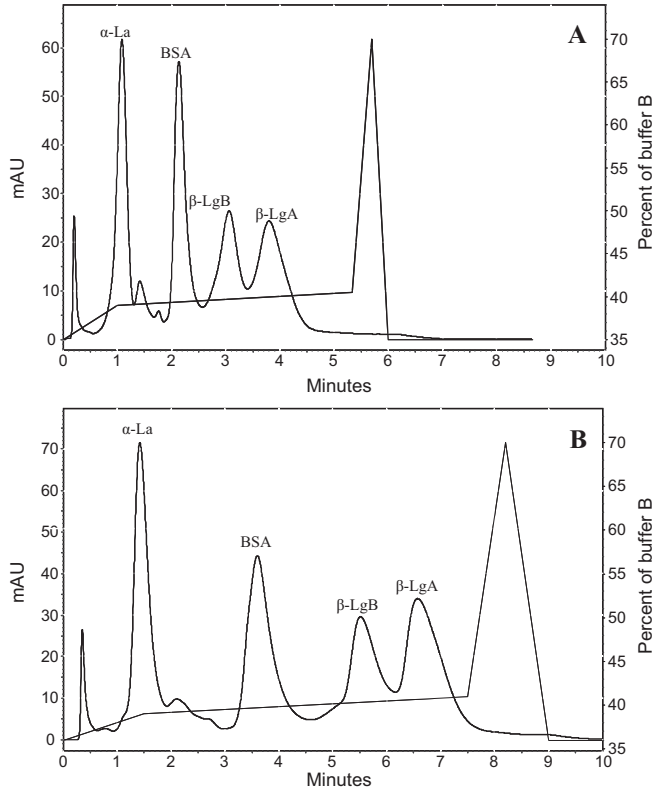

Fig. 4. RP-HPLC–UV analyses of standard protein mixture utilizing CIMac RP-SDVB (A) and POROS R1/10 μm (B) column.

even further reduced in the case of CIMac RP-SDVB, by increasing the flow rate of the mobile phase, but not in the case of POROS column as the analysis was already running at the column maximum pressure limit. Also, the resolution is somewhat increased when using the monolithic column as can be seen for some impurities around 1.5 min (Fig. 4A). Rapidness of the developed RP-HPLC–UV method employing the CIMac RP-SDVB column is noteworthy as it ranks right next to the fastest existing methods for the separation of major whey proteins [47]. These results unequivocally demonstrate some advantages of the monolithic columns over the packed particle ones.

Additionally, with the use of CIMac RP-SDVB, a rapid RP-HPLC–MS method was developed (Fig. 5). TFA in the mobile phase was exchanged for the HCOOH because TFA is known to cause ion

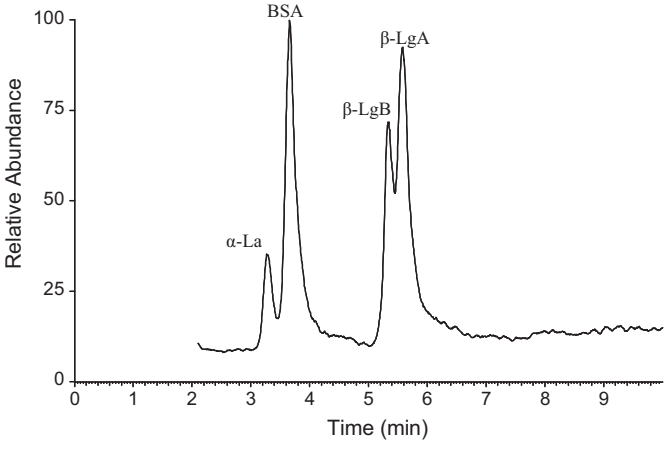

Fig. 5. RP-HPLC–MS analysis of standard protein mixture utilizing CIMac RP-SDVB column.

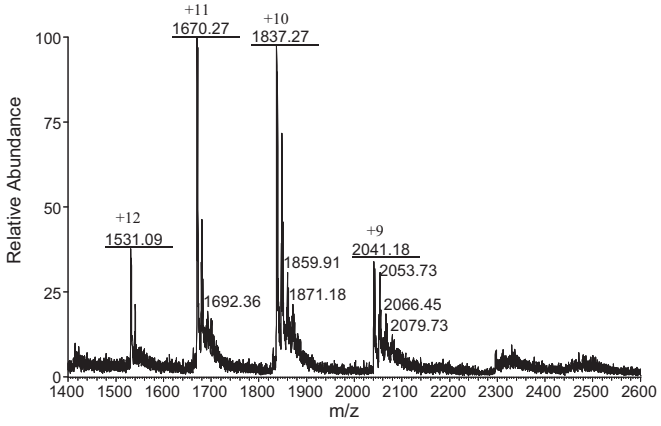

Fig. 6. Direct inlet MS analysis of isolated β-LgA from WI prior to SPE. Underlined values depict m/z values of peaks for multiple charged β-LgA molecules and other values depict m/z values of peaks for protein-TFA adducts.

suppression in the ion source and as a result such methods suffer from sensitivity issues. We would like to emphasize that the separation of proteins was not as efficient as with TFA due to less acidic mobile phase, but the developed method is rapid, simple, and the use of the MS as a detector can offer considerably lower limits of detection and quantitation compared to the UV detector.

3.3. Isolation of β-LgA and β-LgB from WI using CIMac RP-SDVB monolithic column (0.3 mL)

The developed RP-HPLC–UV method (see Section 3.2) was employed for the isolation of β-LgA and β-LgB from WI. The proteins were needed in pure, native, and chemically unmodified state for further protein–ligand binding studies. WI was applied to the CIMac RP-SDVB column and β-LgA and β-LgB fractions were collected at the detector exit. The collected fractions were pooled, diluted with water, and lyophilised. The state of the isolated proteins was determined by direct inlet MS. For the sake of simplicity, in this section only the results obtained for β-LgA will be presented although the same procedure was employed for β-LgB and the results were analogous.

The MS spectrum of β-LgA showed high intensity peaks which corresponded to the multiple charged protein molecules, but there were also some additional peaks present (Fig. 6). Our worst fear was that these might be chemically modified protein molecules with oxidized methionine residues for instance, or similar. These turned out to be multiple adducts of β-LgA and TFA, which is known to bind tightly to numerous proteins, cause unpleasantness in protein purification processes, and is therefore often avoided. Increasing source fragmentation voltage decreased additional adduct peaks in the MS spectra significantly until only peaks for pure protein were observed. Apart from the corresponding m/z value, this also indicated that the additional peaks were indeed adducts between β-LgA and TFA. Since pure isolated proteins were needed another purification step was applied to remove TFA from the protein.

Thus, collected HPLC protein fractions were further manually purified on a SDB-L SPE cartridge, which proved very fruitful for this occasion. After the SPE procedure the protein solutions were lyophilised and white powder was obtained (<100 μg). Once again, MS was chosen as a tool for the determination of the state of the purified β-LgA (Fig. 7). After SPE no protein-TFA adducts were observed in the spectrum.

The proteins unfolded to some extent during the RP-HPLC separations (they were eluted at approx. 40% MeCN), but they folded back to their native state when they were put in an aqueous media once again. At 40% MeCN the charge distribution of lactoglobulin

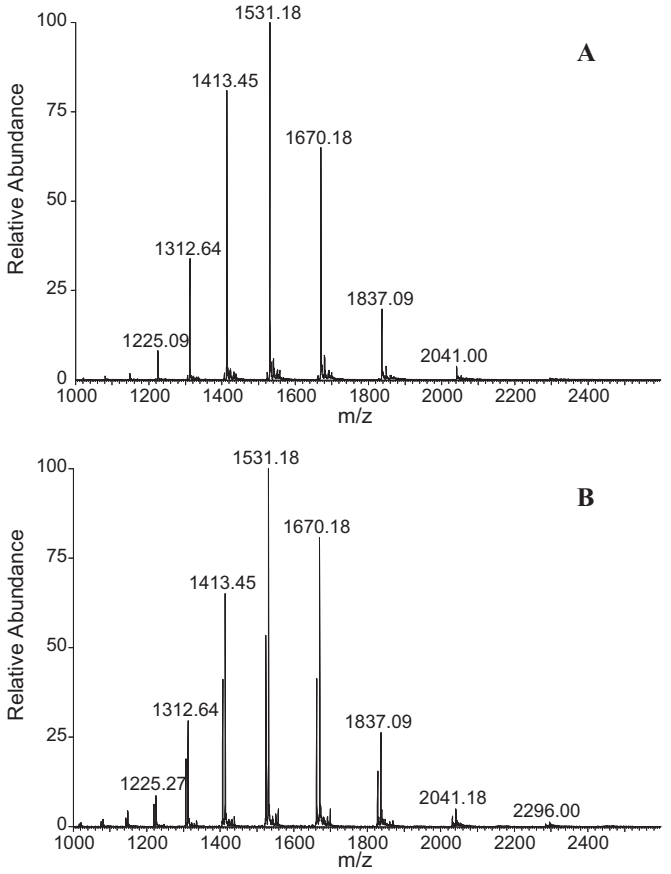

Fig. 7. Direct inlet MS analysis. (A) MS spectrum of isolated β-LgA from WI after SPE; (B) MS spectrum of β-Lg standard (mixture of β-LgA and β-LgB, hence double peaks).

signals in the MS spectra was centered on the 15+ charged molecule, which was indicative of an unfolding process in contrast to the MS spectrum of lactoglobulin in the absence of MeCN, where 12+ signal predominated (data not shown). The reversible unfolding/folding is supported by Fig. 7 which shows that the native protein standard (not subjected to the RP-HPLC and SPE analysis) and the isolated protein had the same charge distribution profile in the MS spectrum. This indicated the same protein structure.

The overall isolation procedure yielded chemically unmodified proteins in the native state with purity higher than 80% for β-LgA and β-LgB (determined by RP-HPLC–MS) with no salts present. Therefore, the developed method is useful for fast, simple, and relatively inexpensive isolation of β-LgA and β-LgB and it has its advantages over other methods. Existing methods are either tedious, cannot distinguish between β-LgA and β-LgB (β-Lg is being isolated as a mixture of the two), can induce β-Lg denaturation, or produce low yields with other proteins or salts as contaminants, making them useless in some cases and expensive and time consuming in others [48–53]. For the separation of closely related substances (such as β-LgA and β-LgB) displacement chromatography seems to be the most suitable choice [33,54], but the cleaning step that removes the displacer from the column is sometimes proving to be a nuisance; therefore the reuse of the stationary phase is questionable. The only drawback of our developed method is a low throughput. We eliminated this inconvenience somewhat by developing a scale-up method utilizing a larger column with the same stationary phase as CIMac RP-SDVB column.

3.4. Isolation of β-LgA and β-LgB from WI using CIM RP-SDVB monolithic column (8 mL)

The same overall mode of proceeding was applied for the scale-up with some minor alterations in the chromatographic conditions. By transferring the method from disk to tube format the chromatographic conditions were changed and determined by trial and error. The equation (Eq. (1)) normally used for upscaling was not very efficient, probably due to the very shallow MeCN gradient and a lack of a thermostated oven in the case of 8 mL monolith.

The collected β-LgA and β-LgB fractions from the WI injections were pooled and diluted with water and then subjected to the SPE purification step. After TFA removal, the protein containing solutions were diluted with water yet again and lyophilised to yield 1.88 mg and 1.68 mg of β-LgA and β-LgB, respectively. The RP-HPLC–UV analysis of WI showed that 59% of the proteins present are represented by β-Lg (29% β-LgA and 30% β-LgB). The yield of the isolated proteins was 36% and 33% for β-LgA and β-LgB, respectively, with purity higher than 90% and 81% for β-LgA and β-LgB, respectively (determined by RP-HPLC–MS). The yields, although significantly higher in comparison with the 0.3 mL column, were still the weak point of the method; the expected amount of individual lactoglobulin was targeted at approximately 20 mg. In the final stage, the purity and nativeness of the lyophilised proteins were also authenticated by direct inlet MS (Fig. 8) and SDS-PAGE (Fig. 9).

Some attention should also be paid to sample loading as it had a noteworthy effect on the performance. When a large amount of sample was loaded onto the column, the retention times of the

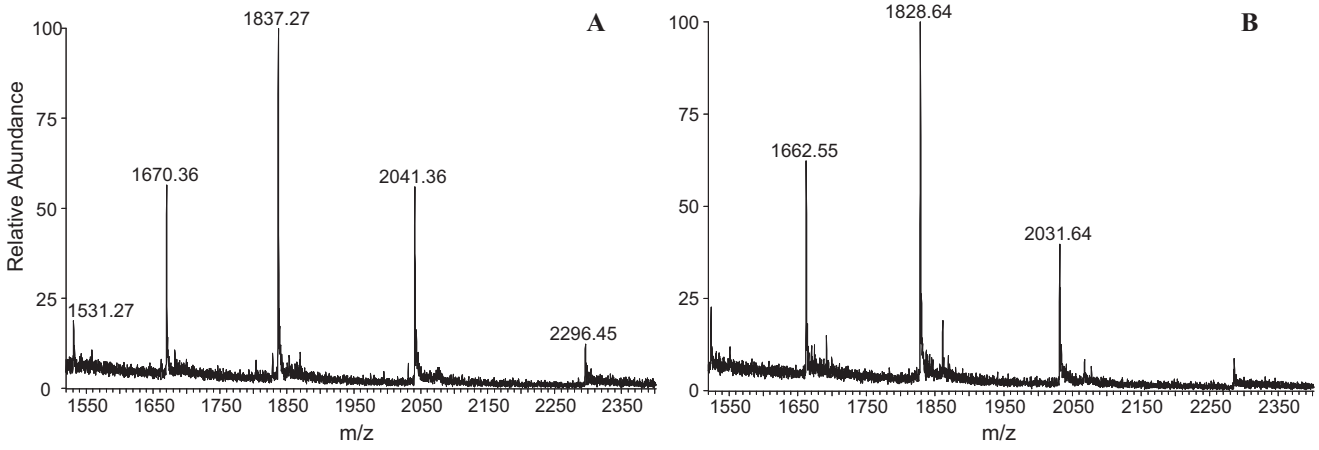

Fig. 8. Direct inlet MS analysis. (A) MS spectrum of isolated β-LgA from WI after SPE; (B) MS spectrum of isolated β-LgB from WI after SPE.

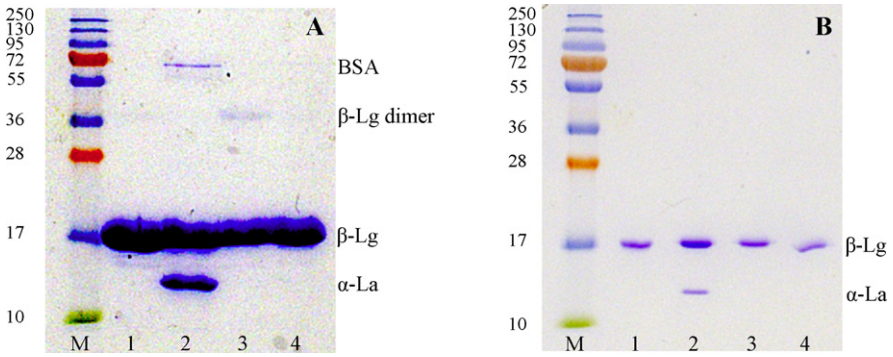

Fig. 9. SDS-PAGE analyses with coomassie blue staining. (A) Lanes: (M) standard protein marker (values in kDa at left-hand side); (1) β-Lg standard; (2) WI; (3) isolated and purified β-LgA; (4) isolated and purified β-LgB. (B) same as A with 10-fold lower sample concentrations.

analytes decreased proportionally to the sample load and the resolution for β-lactoglobulins was impaired. Hence, the smaller the sample load the better the performance. In all the preparative runs (small and larger scale) the loading used, which still provided good performance required for the isolation of β-LgA and β-LgB individually, was the largest possible (0.1 mg and 1.2 mg, respectively) and is described in Section 2. If β-lactoglobulins were to be isolated as a mixture, a higher sample loading capacity would of course be achievable since the resolution between β-LgA and β-LgB would not play a crucial role anymore. POROS column exhibited similar loading related performances as CIMac RP-SDVB column.

β-Lg and α-La represent the majority of the WI proteins and low amount of BSA is also present (Fig. 9A). By isolation and purification of β-LgA and β-LgB all other proteins were removed although β-LgA dimer was observed in trace amounts beside the main compound – β-LgA monomer (Fig. 9A).

Depending on the purpose of isolated β-LgA and β-LgB, the isolation yields of both proteins could be greatly improved at the cost of lower purity; on the contrary, even higher purities could be obtained on account of lower yields. The developed method produces very high purities with only β-LgB being the contaminant of β-LgA and vice versa. What is more, the speed of the HPLC fractionation could be considerably improved by using higher flow rates of the mobile phase (up to 400 mL/min) instead of the flow rate we used (8 mL/min) due to the hardware limitations.

4. Conclusions

Hitherto short monolithic columns have not been successfully employed in the field of major whey protein separation and isolation. In our study we show that monolithic columns can be used for the separation and isolation of major whey proteins. The property which they have in common is the high speed of separation. Although our primary interest lay in finding a proper monolithic column and chromatographic conditions for the purification and isolation of β-LgA and β-LgB, three additional analytical LC methods were also developed during the course of our research.

The developed IE-HPLC method using CIMac DEAE column combines rapid separation, very good pH gradient linearity, and MS compatibility. This is the first IE-HPLC–MS method for the separation of major whey proteins that meets the above demands. The excellent linearity of the pH gradient of the developed IE-HPLC–MS method could also be diligently used in numerous other protein separations. A baseline separation of all major whey proteins was achieved in under 5 min employing a prototype CIMac RP-SDVB monolithic column. In the light of column performance, a brief comparison between monolithic CIMac RP-SDVB and packed particle POROS perfusion column was also made, where the monolith gave better results. In addition, the developed RP-HPLC–MS method offers prompt and sensitive separation although the resolution is slightly compromised for the pair β-LgA and β-LgB. Finally, the RP-HPLC–UV method was used to obtain chemically unmodified β-LgA and β-LgB in native conformational state from WI. A scale-up method was successfully developed, which produced larger quantities of isolated β-LgA and β-LgB with purity higher than 90% and 81%, respectively. Using monoliths, the scale-up process, transferring the method from 0.3 mL to 8 mL column, was relatively fast and simple and could even be applied to much larger industrial monolithic columns (8 L), even further increasing the throughput.

Our results show that monolithic columns are applicable for the separation and isolation of major whey proteins. What is more, we show that the developed methods on monoliths are comparable to the state-of-the-art separation and isolation methods and can even surpass them in some aspects discussed above; this fact should render the monoliths at least as an alternative chromatographic stationary phase to the packed particle columns.

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