



Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma



Development of a method for the quantification of whey allergen traces in mixed-fruit juices based on liquid chromatography with mass spectrometric detection

Linda Monaci, Arjon J. van Hengel*

European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg 111, B-2440 Geel, Belgium

ARTICLE INFO

Article history:

Received 17 January 2008
Received in revised form 11 March 2008
Accepted 14 March 2008
Available online xxx

Keywords:

Liquid chromatography
Mass spectrometry
Milk allergens
Whey proteins
Solid-phase extraction
Fruit juice

ABSTRACT

The availability of accurate and sensitive detection methods for food allergens is crucial for the food industry to ensure the correct labelling of their products in order to protect allergic consumers. For this purpose a method using solid-phase extraction and liquid chromatography coupled to mass spectrometry was developed to detect traces of three allergenic cow milk proteins (lactalbumin, lactoglobulins A and B) in mixed-fruit juice samples. Different sample pre-treatments were compared and the best recoveries were obtained with a method employing a solid-phase extraction cartridge. Recoveries ranging from 68% to 79% were achieved for 5 and 20 $\mu\text{g/ml}$ tested and the limit of detection was set at 1 $\mu\text{g/ml}$. Both full scan and multiple ion monitoring acquisition modes were investigated and compared. The method was utilized to analyse 15 mixed-fruit juices collected from the market and was found to be capable of positively identifying all three milk proteins. The developed method enables the unambiguous determination of allergenic whey proteins in mixed-fruit juices and can assist in the protection of milk allergic individuals.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Cow's milk represents a valuable ingredient in the human diet, thanks to its abundance in proteins, peptides, fatty acids and minerals. Major proteins include caseins, lactalbumin, lactoglobulin, immunoglobulin and bovine serum albumin [1] that are grouped into two main classes being: caseins and whey proteins. Whey proteins remain soluble in milk serum after acidic precipitation of caseins and are mainly represented by β -lactoglobulins (LGs) and α -lactalbumin (α -LA). Due to their wide ranging nutritional, biological and functional properties whey proteins are often utilized by the food industry in the preparation of foods or beverages [2]. They are used as skim milk replacers in ice cream and more recently as fat replacers in dairy and non-dairy products [3]. The high nutritional value of whey proteins as reflected by their essential amino acid profile, has promoted their use as a valuable ingredient in various protein fortified beverages such as fruit juices, sport drinks including those with long shelf life [4]. As a consequence many fortified soft drinks and fruit juices have entered the European market. Despite the high nutritional and functional properties of whey proteins, they are also recognized as powerful allergens, therefore the widespread use of milk proteins by the food industry poses a serious threat to the health of milk allergic consumers. Several soft drinks

and fresh juices that may contain undeclared milk have recently been recalled in Canada and the USA. The recalls of such products is of concern and highlights the possibility that undeclared milk traces represent a hazard to the health of individuals that are allergic to milk proteins.

Milk allergy is an immunological reaction to milk proteins and has been considered the most common food allergy with a highest incidence in children below 2 years [5]. Milk and whey proteins are key ingredients in a wide variety of infant formulae since they closely resemble components found in human breast milk. The frequent exposure to such formulae might be one of the reasons for the high incidence of milk allergy in young children. Studies carried out on a large population have proven that the most abundant milk proteins such as caseins, LGs and α -LA are the major allergens [6]. Very low doses of milk-derived products can induce allergic reactions and result in mild to fatal health problems. Unfortunately there are no safety threshold levels for allergenic foods. Since the risk that foods may trigger allergic reactions is considerable [7], it is necessary to provide consumers with all the necessary information on food composition on the label [8]. Both in the USA and in the European Union legislation has been put in place aimed at achieving a high level of health protection for allergic consumers [9]. Within the European Union, Directive 2003/89/EC [10] that amends Directive 2000/13/EC [11] states that the 12 most common food allergens, including milk have to be declared on the label when they have been intentionally introduced in a foodstuff. The availability of accurate and sensitive detection methods for food allergens is crucial for the

* Corresponding author. Tel.: +32 14 571803; fax: +32 14 571343.

E-mail address: adrianus.van-hengel@ec.europa.eu (A.J. van Hengel).

food industry to inspect and control their production processes, and to ensure the correct labelling of their products in the effort to protect allergic consumers.

Milk proteins are usually detected by bioassays, immunochemistry or electrophoresis [1]. Although these methods are relatively fast and suitable for routine screening, they are not amenable to protein identification. Besides this, immunochemical methods can suffer from cross-reactions with matrix proteins. For an unambiguous identification of allergenic ingredients confirmatory methods are required that usually employ a mass spectrometric (MS) approach [12,13]. A recent approach that is referred to as shotgun proteomics involves the digestion of complex protein samples that are subsequently subjected to MS/MS analysis and finally identified by database searching [14,15]. Recently a method based on nano liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of tryptic digested cleaned-up food matrices has been developed for the detection of milk allergens in cookies [16]. This type of method provides a good sensitivity and can be used as a confirmatory method but unfortunately quantification is hampered by the enzymatic step employed.

Here we describe a method based on liquid chromatography coupled to MS for the quantification of α -LA and lactoglobulins A (LG A) and B (LG B). This method benefits from the lack of an enzymatic digestion step, and the stability of the protonation pattern which had been assessed previously [17]. As a consequence, the most abundant ions of each whey protein can be selected as protein markers to investigate the presence of milk traces in fruit juices. The method has been applied to a number of fruit juices and enables the detection of 1 μ g/ml of the whey proteins α -LA, LG A and LG B.

2. Materials and methods

2.1. Materials and reagents

LG A, LG B (purity 92%) and α -LA (purity 98%) and formic acid (98–100% purity grade) (FA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium phosphate was obtained from Sigma–Aldrich. Trichloroacetic acid (TCA) was purchased from Seria Electrophoresis (Heidelberg, Germany).

Stock standard solutions of LG A and B (1 mg/ml) and α -LA (1 mg/ml) were prepared in water and stored for approximately 1 month at -20°C . Working solutions were freshly prepared by appropriate dilution of the stock solutions. 10 mM sodium phosphate solutions were prepared and adjusted with either 2 M NaOH aqueous solution to reach a final pH of 10, or with FA for a final pH of 3 or 7.

Solid-phase extraction (SPE) cartridges used were hydrophilic–lipophilic-balanced copolymer columns (Oasis HLB) with a volume of 3 ml (Waters, Milford, MA, USA) and C4 SPE columns with a volume of 3 ml (Supelco, Bellefonte, PA, USA).

Ultrafiltration was performed with Vivaspin devices with a volume of 2 ml and a 10 kDa cut-off (Vivascience, Hannover, Germany). The centrifuge used was a SIGMA 3–16K (Sigma, Osterode am Harz, Germany).

Filtering of juices was performed utilizing 0.45 μ m cellulose filters (Millipore, Bedford, MA, USA) before submission to ultrafiltration.

2.2. Samples

A total of 15 fruit juices containing from 1 to 14 fruit ingredients (apple, apricot, banana, passion fruit, guava, grape, kiwi, lemon, mango, orange, papaya, peach, pear, pineapple) were purchased from local retailers in Belgium and analysed. The samples consisted of 9 mixed-fruit juices and 5 orange-juices none of

which declared the presence of milk on the label, and one mixed-fruit juice declaring the presence of whey proteins (at unknown levels).

2.3. Sample clean-up

For ultrafiltration 1 ml of each sample was filtered through cellulose filters and the filtrate was loading in the ultracentrifugation device. After 30 min of centrifugation at 8000 rpm, 1 ml water was added to rinse the sample and the device was centrifuged for another 30 min. The retentate was collected, evaporated under vacuum and dissolved in 0.5 ml mobile phase before injection into the LC–MS system.

Protein precipitation with TCA in water at a final percentage of 10, 20 and 30% was employed to precipitate proteins. 1 ml of centrifuged fruit juice (at 6000 rpm in microcentrifuge) was mixed with 1 ml of a 60, 40 or 20% TCA solution and placed on ice for 30 min prior to centrifugation at 10 000 rpm for 5 min in the microcentrifuge. The resulting supernatant was discarded and the pellet was dissolved in 1 ml mobile phase and filtered with a 0.45 μ m cellulose filter before injection.

SPE was performed by using Oasis HLB cartridges with 30 mg mixed reversed phase (3 ml volume). The optimized method is as follows: 1 ml of fruit juice was acidified with 20 μ l FA to reach a final pH of 3, this was then directly loaded onto a SPE cartridge that was pre-conditioned with 2 ml of methanol followed by 2 ml water and 1 ml of water acidified with 0.1% FA. In case of viscous samples a filtration step using a 0.45 μ m cellulose filter is advised, while centrifugation is discouraged since a loss in protein content due to the co-precipitation of protein with the suspension was noticed. Subsequently, the column was washed with 1 ml water acidified with 0.1% FA to eliminate the most polar compounds. Elution was achieved with 1 ml acetonitrile/water (95/5, v/v+0.1% FA) solution. The extract was then evaporated under a gentle flow of nitrogen and reconstituted in 250 μ l mobile phase before injection into the LC–MS system. Alternatively, a 4-fold preconcentration step was introduced prior to injection.

2.4. Liquid chromatography

The analyses were carried out employing an on-line coupling between MS, UV and RP–HPLC. The chromatographic system consisted of a binary pump (Agilent HP1100) equipped with an autosampler (Agilent HP1100) and a diode array UV detection (DAD) system (Agilent HP 1100PDA). Chromatographic separation was performed on a Discovery C5 column, 100 $\times$ 2.1 mm, 3 μ m packing (Supelco). The mobile phase was composed of solvent A: a mixture of acetonitrile/water (5/95) + 0.1% FA, and solvent B: a mixture of acetonitrile/water (95/5) + 0.1% FA. The elution program is presented in Table 1. The flow rate was set at 0.25 ml/min and

Table 1
Elution program for HPLC analysis

Time (t) (min)	A (%)	B (%)
0	80	20
2	70	30
5	64.5	35.5
8	64.5	35.5
13	61.5	38.5
14	61.5	38.5
16	40	60
17	40	60
19	80	20
29	80	20

A = acetonitrile/water (5/95) + 0.1% FA; B = acetonitrile/water (95/5) + 0.1% FA.

the column temperature at 45 °C. The injection volume was set at 10 μ l.

2.5. Mass spectrometry

Mass measurements were carried out with an Ultima triple quadrupole mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an atmospheric pressure ionization source Z-spray. Solutions were sprayed through a capillary held at +3 kV, at a temperature of 120 °C and at a desolvation temperature of 220 °C, generating multiply charged ions. The cone voltage was set at 50 V. The cone gas flow was 220 L/h and desolvation gas flow 700 L/h. For direct injection experiments the flow rate of the syringe pump was set at 10 μ l/min.

The system was operated in positive ion mode. For data acquisition two different acquisition modes were used. In full scan mode the second quadrupole was scanned over the range 800–1800 m/z with a step size of 0.5 m/z and a dwell time of 0.5 s. Protein spectra were obtained as an average of the spectra of multiple scans. Protein molecular mass was determined by using MaxEnt software (Waters-Micromass) from the measured m/z values of the protonated molecules. For multiple ion monitoring mode the acquisition was split in three segments as follows:

α -LA from 10 to 14 min monitoring the masses m/z = 1419.7, and 1577.3.

LG A from 17 to 20 min monitoring the masses m/z = 1082.2, 1149.7, 1226.2, 1313.6.

LG B from 15 to 18 min monitoring the masses m/z = 1077.1, 1144.4, 1220.5, 1307.5.

The current relative to those specific ions was registered.

3. Results

3.1. Separation and detection of LGs and α -LA by LC-MS

All liquid chromatographic parameters such as gradient composition, flow rate, and column temperature were optimized for the separation of α -LA, LG A and LG B by employing a C5 chromatographic column. The MS system was tuned upon injection of each standard protein directly into the source in full scan mode in order to find the best conditions for a good resolution and maximal signal for all three proteins. The spectra of each protein obtained by injection of a standard dissolved in a mixture of acetonitrile/water (50/50, v/v) acidified with 0.1% FA directly into the MS source are shown in Fig. 1. Each protein displays an envelope of multi-charged ions corresponding to several protonated states of the same protein that represents a type of fingerprint of the protein. This specific distribution pattern resulting from the electrospray ionization might be exploited to confirm the identity of the protein in complex matrices. For electrospray ionization mass spectrometry (ESI-MS) quantification two different data acquisition scanning modes were compared with the aim to achieve the highest sensitivity for whey protein detection.

The full scan mode (FS) was employed in the mass range 800–1800, and the peak areas of total ion chromatograms were used for the construction of calibration curves. Multiple ion monitoring (MIM) acquisition was based on recording specific masses selected of the most abundant mass-to-charge ions for each protein of interest (as described in Section 2).

Fig. 2 depicts chromatograms acquired in both the FS and MIM modes after injection of 10 μ l of a standard solution containing a mixture of the three proteins (1 μ g/ml each) that represents the minimum amount of protein detectable.

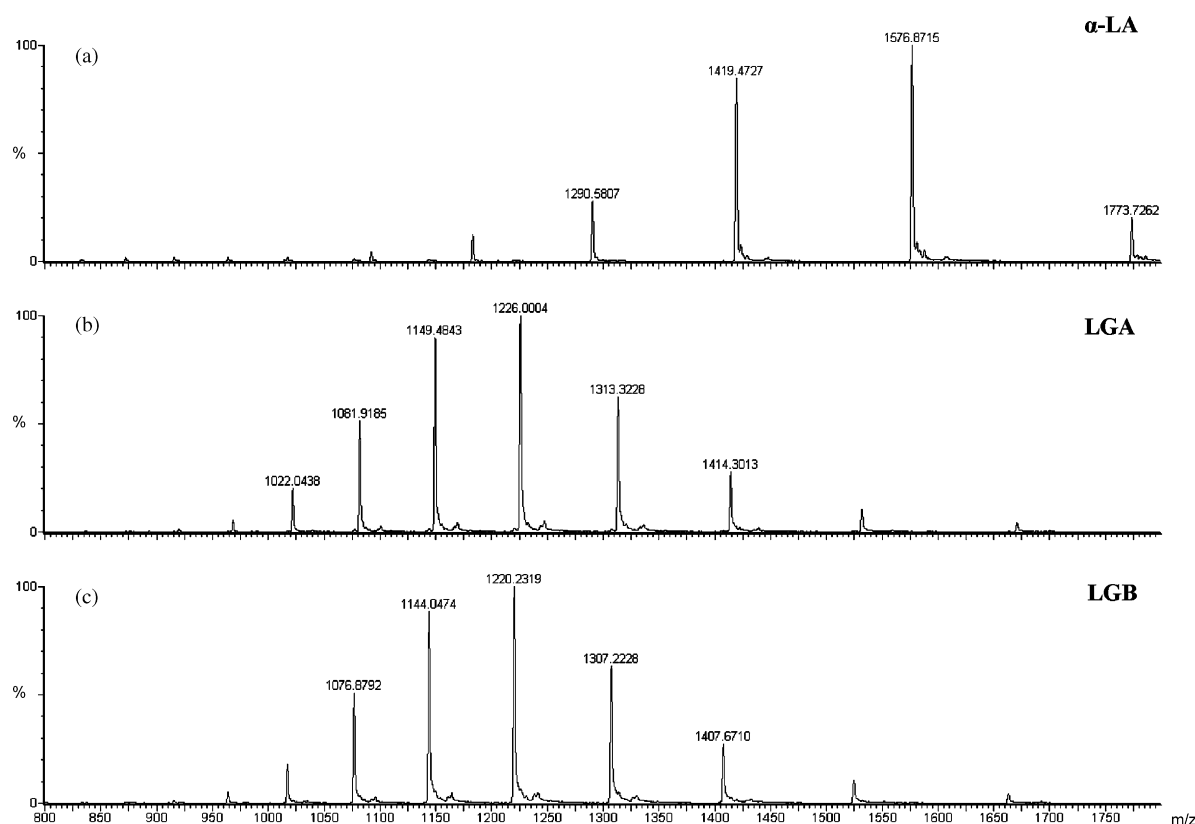


Fig. 1. Spectra obtained in direct injection mode of a 20 μ g/ml standard solution of α -LA, LG A and LG B. Acquisition range: 800–1800 m/z .

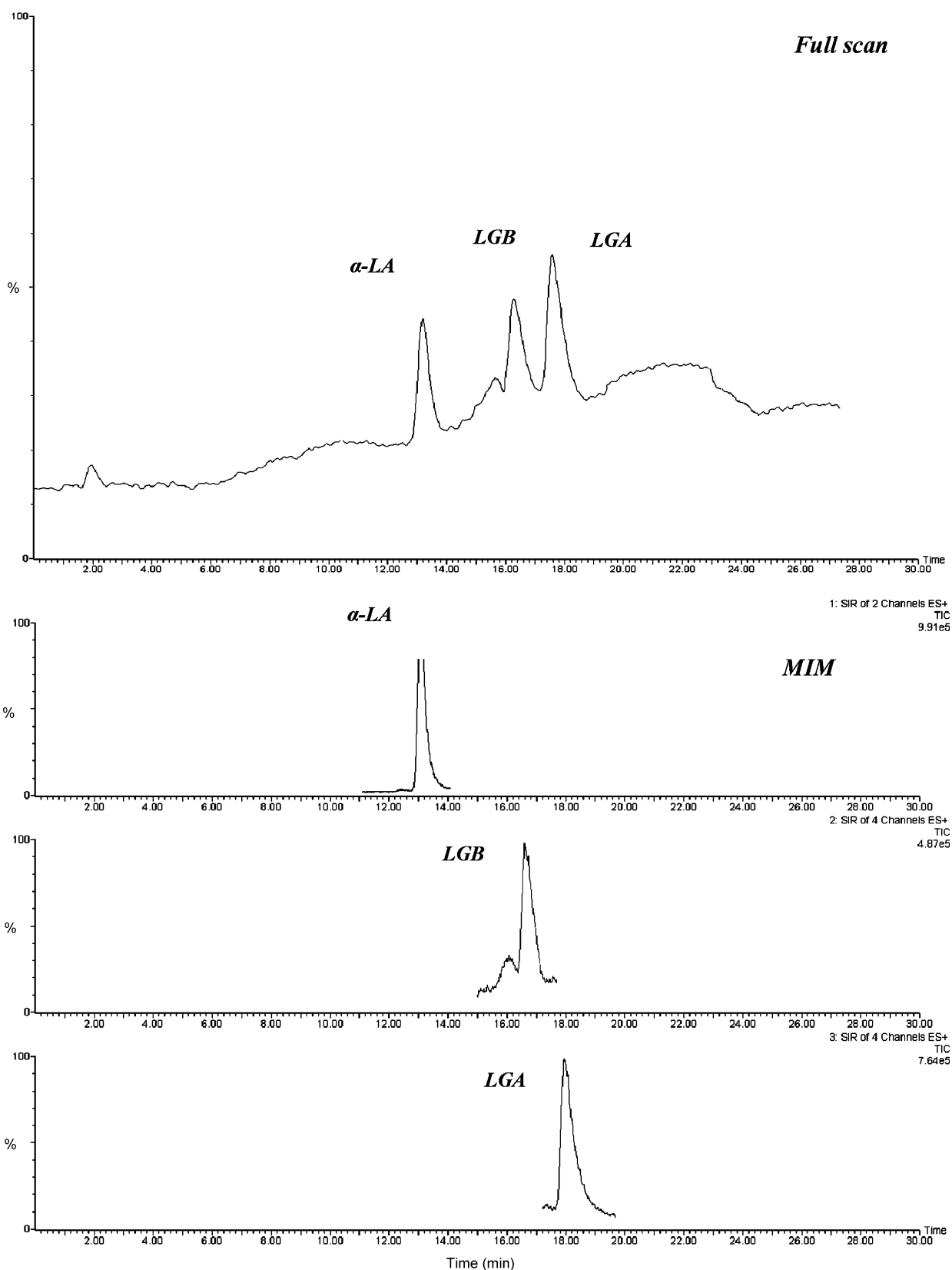


Fig. 2. Comparison between full scan and MIM acquisition modes after injection of 10 μ l standard solution containing 1 μ g/ml α -LA, LG A and LG B.

In order to check the reproducibility of this method the intra-day and inter-day variation were evaluated by repetitive analyses of the same standard mixture stored at 4 °C at the concentration of 10 μ g/ μ l. Analyses were performed daily in triplicate over

a period of five days and the peak area of each protein was calculated. One-way analysis of variance (ANOVA) was used to compare the variances of the means per each day of investigation. Results showed no significant difference between the

Table 2

Recoveries and their standard deviations (SD) as obtained by utilizing different sample pre-treatment procedures

UF	α -LA ($\pm$ SD)	LG A ($\pm$ SD)	LG B ($\pm$ SD)
10 kDa	48 $\pm$ 1	27 $\pm$ 10	27 $\pm$ 10
TCA (%)	α -LA ($\pm$ SD)	LG A ($\pm$ SD)	LG B ($\pm$ SD)
20%	33 $\pm$ 2	22 $\pm$ 4	21 $\pm$ 3
40%	20 $\pm$ 2	9 $\pm$ 3	12 $\pm$ 2
60%	18 $\pm$ 1	3 $\pm$ 0.2	5 $\pm$ 0.4

intra-day and in between-day RSD at a confidence level of 95% ($\alpha = 0.05$).

3.2. Sample preparation procedure and optimization

Several approaches were investigated in order to find the most suitable sample preparation method in terms of highest recovery and ease of use. For this purpose a fruit juice sample ($n = 3$) was fortified with 20 $\mu\text{g}/\text{ml}$ of each protein and the effects of different pre-treatment procedures were carefully checked.

First, ultrafiltration devices with a cut-off of 10 kDa were employed for this purpose. 1 ml of fruit juice was filtered using a cellulose filter before being submitted to ultrafiltration. Then the tube was filled up with 1 ml water and centrifuged for another 30 min. The retentate was collected and evaporated in a spin-vac to be reconstituted in 0.5 ml mobile phase prior to injection into the LC–MS apparatus. Recoveries of this procedure ranged from 48% $\pm$ 8 (SD) for α -LA, to 27% $\pm$ 10 (SD) for the LGs (Table 2).

The recoveries obtained were not considered adequate, therefore another procedure was investigated with the aim to increase protein yield. TCA is used extensively for protein precipitation. Therefore, we investigated the use of TCA, at different percentages, for sample preparation. Briefly: 1 ml centrifuged fruit juice was combined with 1 ml of either 60%, 40% or 30% TCA, the mixture was placed on ice to precipitate proteins. After centrifugation, the pellet was reconstituted in 1 ml mobile phase and filtered through a cellulose filter before injection into the LC–MS apparatus. Table 2 shows the recoveries obtained by using TCA precipitation. Since the highest recovery was only 33% (as shown for α -LA) another method was investigated.

The final approach used was SPE clean-up and the parameters involved in the performance of the SPE process such as the composition of wash and elution solutions, the breakthrough volume, etc., were carefully analysed and optimized in order to obtain a high recovery.

In this study α -LA, LG A, and LG B were simultaneously extracted by employing two different sorbent chemistries: mixed hydrophilic–lipophilic-balanced (HLB) SPE columns and C4 SPE columns. Test solutions containing all three proteins at 20 $\mu\text{g}/\text{ml}$ were prepared and adjusted either with a 2 M NaOH aqueous solution to reach a final pH of 10, or with FA for a final pH of 3 or 7 and 1 ml of each was loaded onto the conditioned cartridge. The liquid loaded on the column was collected and analysed by LC–MS for any protein loss. Although the C4 SPE columns did not show significant protein loss during loading, protein adsorbed to the cartridge was very difficult to elute although solutions of different polarity and ionic strength were tested. Therefore, HLB cartridges were chosen for further experiments. The pH value providing the highest retention of the proteins on HLB cartridges was found to be near 3 and therefore this pH was used for subsequent analyses. The breakthrough volume was estimated by loading six times 1 ml of standard test solution (20 $\mu\text{g}/\text{ml}$ of each protein) on the same column and the maximum amount of proteins that can be loaded on the cartridge was calculated. Results of this study are shown in Fig. 3

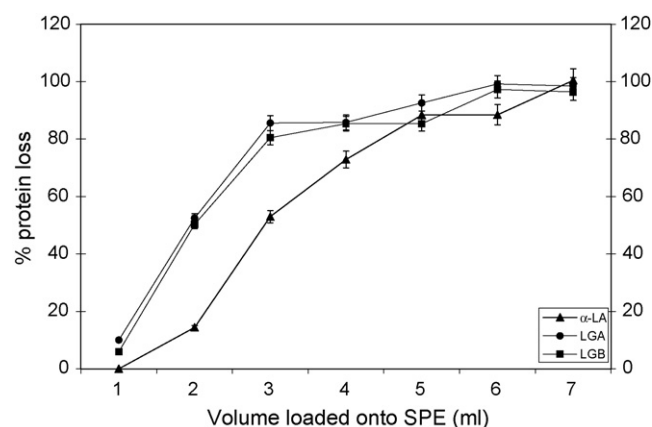


Fig. 3. Protein loss on SPE columns.

which indicates that loading a volume of 2 ml of standard (=40 μg of each protein) resulted in a nearly 50% loss of LG protein, whereas this was 3 ml (=60 μg of each protein) in the case of α -LA. This difference in behaviour is likely to be correlated with the different chemistry of the two proteins causing them to interact differently with the sorbent.

To find the most suitable washing solution that minimized protein loss but at the same time allowed interfering compounds to be washed out, 1 ml standard solution that had previously been acidified with FA (pH 3) was loaded onto the SPE column. Subsequently, washing solutions with different water percentages were pushed through the column by using a manifold extraction system. The solutions investigated were: water acidified with 0.1% FA (W), water/acetonitrile (95/5, v/v) (W/ACN, 95/5), water/acetonitrile (90/10, v/v) (W/ACN, 90/10), and water/acetonitrile 80/20 (W/ACN, 80/20). As shown in Fig. 4 the best washing solutions were acidified water or a mixture of water/acetonitrile 95/5 that resulted in minimal protein loss. α -LA demonstrated a good retention on the cartridge and even the most apolar washing solution did not significantly modify its adsorption on the stationary phase. In contrast, both LG variants showed to be more sensitive to the polarity of the washing solution employed.

Ultimately, the elution solution was optimized in order to maximize the final recovery. Different percentages of acetonitrile/water mixtures were tested. The best results in terms of the lowest volume requested for the elution and the highest recovery was obtained with a solution containing acetonitrile/water (95/5, v/v) acidified with 0.1% FA.

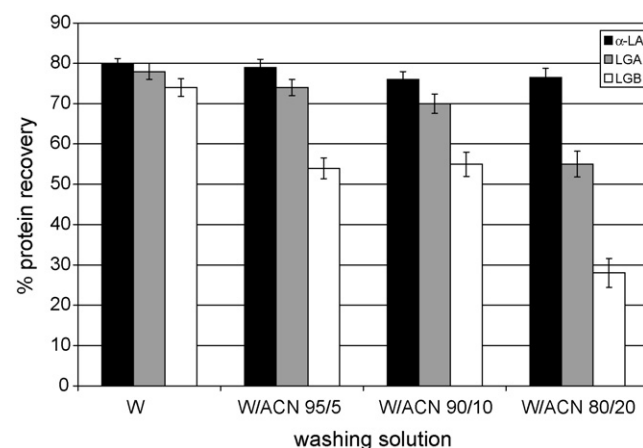


Fig. 4. Determination of protein recovery as a consequence of employing different washing solutions.

Table 3

Recovery estimations for a fruit juice sample spiked with all three proteins at a final concentration of 5 or 20 $\mu\text{g/ml}$

Protein spike ($\mu\text{g/ml}$)	α -LA ($\pm\text{SD}$)	LG A ($\pm\text{SD}$)	LG B ($\pm\text{SD}$)
5	73 $\pm$ 5	68 $\pm$ 4	75 $\pm$ 5
20	79 $\pm$ 3	74 $\pm$ 4	78 $\pm$ 5

3.3. Application of the method to fruit juice

Matrix components within samples can influence analyte detection resulting in enhancement of background noise and, or suppression of the analyte response. In order to obtain a matrix-matched calibration curve, a blank mixed-fruit juice extract was fortified with standards at four different concentrations (5, 10, 20 and 50 $\mu\text{g/ml}$). Analyses were performed in full scan mode and peak areas were plotted against concentration.

The linearity was satisfactory with squared regression coefficients better than 0.995. To estimate the matrix effect, the calibration curves obtained were compared to calibration curves based on protein standard solutions. For all three proteins differences in slope values were observed between matrix-matched calibration curves and non-matrix-matched standard curves demonstrating an effect of the matrix on method sensitivity. For this reason only matrix-matched calibration curves were utilised as reference curves during this study.

The mean recovery was estimated for a spiked analytes orange juice matrix at the 5 $\mu\text{g/ml}$ or 20 $\mu\text{g/ml}$ level. Three replicate samples per concentration were analysed and recoveries were calculated and are reported in Table 3. A chromatogram obtained after analysis of a sample spiked at 5 $\mu\text{g/ml}$ is shown in Fig. 5. Comparison of the blank sample (upper part of Fig. 5) and the spiked sample clearly shows the absence of major interferences in the range of interest; we therefore conclude that the clean-up procedure employed is suitable for the analysis of the three whey proteins in this matrix. The developed LC–MS method allows the estimation of very low amounts of α -LA and LGs in mixed-fruit samples. The limit of detection (LOD) was calculated to be around 1 $\mu\text{g/ml}$ ($S/N=3$) for each protein in mixed-fruit samples, corresponding to a total amount of 10 ng protein injected while the limit of quantification (LOQ) was 4 $\mu\text{g/ml}$ ($S/N>10$). The method demonstrated to be linear in a range of 5–40 $\mu\text{g/ml}$.

3.4. Analysis of commercial juices

To test the performance of the developed method 15 fruit juices were purchased from local retailers in Belgium. All juices were analysed as described to determine whether traces of allergenic whey proteins could be detected. The potential of mass spectrometric detection was utilised for protein identification by analysis of the spectrum at the expected retention time of each protein. The identification of the targeted whey proteins was confirmed by applying MaxEnt software for the deconvolution of multiply charged electrospray data and for state charge attribution. Amongst the 15 samples a single product was declared to contain whey proteins on its label albeit at unknown levels. The LC–MS method was applied blindly to all samples, so during analysis it was not known which sample was expected to contain whey proteins. Only one out of 15 samples tested positive for whey proteins and contained all three proteins, as determined by FS and MIM acquisition and analysis of the charge-state-distribution. This sample was identified as the one that declared whey proteins on its label. The analysis allowed us to calculate the concentrations of α -LA, LG A and LG B from the calibration curve and the sample was shown to contain 150 $\mu\text{g/ml}$, 240 $\mu\text{g/ml}$ and 342 $\mu\text{g/ml}$ of the three proteins respectively. This is

comparable with the relative amounts of LG and α -LA as present in cow's milk [1].

In order to investigate the performance of the method in a wider variety of fruit juices each of the collected samples was fortified with the three whey proteins at a final concentration of 20 $\mu\text{g/ml}$ for each whey protein (α -LA, LG A and LG B) and subsequently submitted to LC–MS analysis. Recoveries were 75% $\pm$ 10 for α -LA, 72% $\pm$ 12 for LG A, and 68% $\pm$ 12 for LG B which is in the same range as the recoveries determined during method development (Table 3).

4. Discussion

Liquid chromatographic and capillary electrophoretic methods have been exploited for the separation of milk proteins in complex samples as reported by several research groups [18–22] that usually have employed UV or fluorescence detection for quantitative analyses. The reported LODs of the developed methods range from 0.1 to 2 μg of total protein injected, depending on the protein under investigation [1]. MS analysis offers the potential to univocally identify a protein. Studies based on LC–MS methods for the analysis of milk proteins described the use of ESI–MS for in depth analysis of intact milk proteins and conjugates formed during heat treatments, mainly aimed at qualitative assessment and protein characterization [23,24]. Siciliano and co-workers utilized ESI–MS to study intact proteins and MALDI–MS to analyse the proteolytic digest for an investigation on structural modifications induced by industrial treatments of milk proteins [25]. Such proteomic investigation into the identification of proteins is usually achieved via a four-step process consisting of protein separation, protein digestion, MS analysis and comparison of the observed peptides with those in a database and allows obtaining both mass and sequencing information of the proteins under investigation. For the detection of milk traces in food products Weber et al. exploited the use of capillary LC coupled to a hybrid analyser, quadrupole time-of-flight (Q–TOF) which provided protein identification and sequence information [16]. Two candidate peptides were identified as potential milk biomarkers and milk traces down to a level of 1.25 $\mu\text{g/ml}$ could be detected in spiked cookies' extract. Although the MS methods described so far are amenable for elucidation and characterization studies and can be used for the identification of milk allergens in food products, their capacity to provide quantitative information is limited due to the unknown efficiency of the enzymatic protein digestion step involved.

In the present study we have developed a method based on LC coupled to quadrupole mass spectrometry that enables quantification of whey proteins in juice samples. This marks a step forward compared to UV methods published so far [18–22]. Not only in terms of specificity, since both the retention time as well as the specific envelope of multi-charged ions can be employed for protein identification, but also in terms of sensitivity. Since the instrument utilised in this study was coupled to a UV detector as well as to a mass spectrometer the same samples were analysed with both detectors. Utilizing UV detection we found the lowest concentration detectable for all three proteins to be in the range of 10–15 $\mu\text{g/ml}$ (data not shown), while mass spectrometric detection increased the sensitivity of the method with nearly 1 order of magnitude. The low limit of detection of 1 $\mu\text{g/ml}$ that can be achieved with this method represents an important issue since it has been reported that even little amounts of milk/whey proteins in the range of only a few $\mu\text{g/ml}$ can trigger severe allergic reactions in sensitive consumers. Therefore the availability of sensitive analytical methods for the quantification of milk allergens is of paramount importance for establishing threshold values [26] since this estimation is strictly dependant on the availability of reliable methods that can quantify the allergen content in foods. The LC–MS based method developed

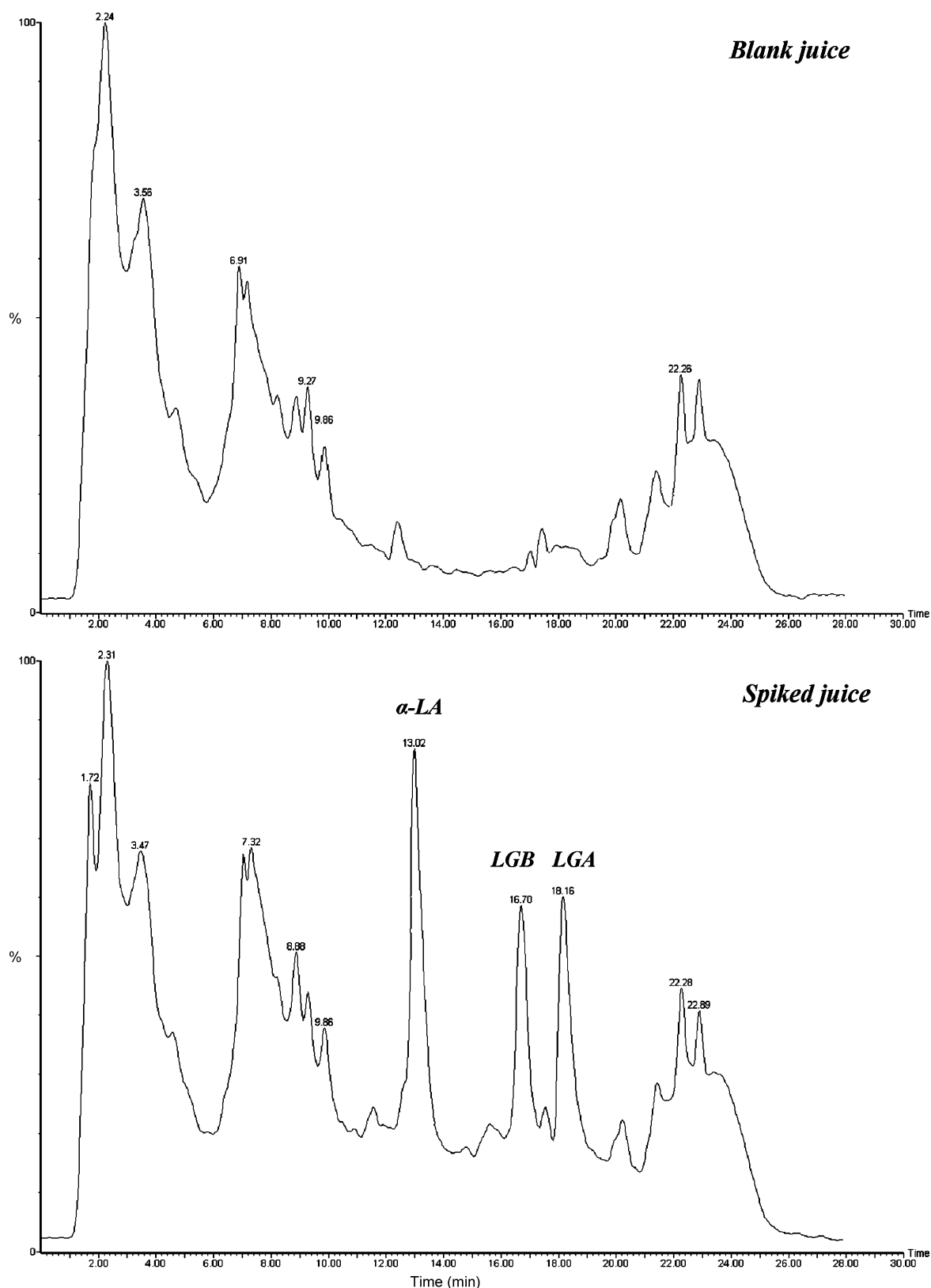


Fig. 5. Chromatograms of an unspiked juice sample, and a juice sample spiked with α -LA, LG A and LG B, each at 5 μ g/ml.

in this study allows the identification and quantification of whey proteins which can be readily deduced from the spectrum recalled at the retention time of the α -LA, LG A and LG B peaks. This is of importance especially since the features of the MS/MS acquisition mode could not be exploited for the analysis of intact proteins due

to the fact that the fragmentation of a macromolecule with such a high molecular weight might generate a multitude of fragments that are difficult to be resolved and to interpret.

The calibration curves obtained in full scan mode showed a good linearity and coefficient of correlation for all three proteins inves-

tigated, and demonstrated therefore that the developed method is a valid tool for the quantification of intact milk proteins. Previously, we demonstrated the stability of the multi-charged pattern of whey proteins by LC–MS analysis [17] which prompted us to perform a MIM acquisition to increase the selectivity of the method. This acquisition mode might be exploited further for more complex matrices that require an increase in selectivity. For MIM acquisition, m/z ions specific for each protein were chosen amongst the most intense peaks in the spectrum. Cleaner chromatograms were obtained by using the MIM acquisition mode which resulted in an improvement of method selectivity. By introducing a final preconcentration step it was even possible to increase the sensitivity of the overall method. The application of the LC–MS method to analyse fruit juices required the development and optimization of a new extraction method. Sample clean up by means of solid phase extraction, as can be performed with columns like Oasis HLB–SPE, provided good recoveries and several parameters were individually optimized to achieve the best recovery for fruit juices. After in-house validation of the method we tested a total of 15 fruit juices purchased at local supermarkets. The aim of the study was to evaluate the potential content of the method to detect whey proteins in such samples and to determine the level of α -LA, LG A and LG B in the samples. The single sample that tested positive turned out to be the only product that listed whey amongst its ingredients. Recovery rates that were determined by spiking a variety of mixed-fruit juices were found to be similar, underlining the precision of the method. We conclude that the method developed here enables an unambiguous detection of intact whey proteins at the very low level of 1 $\mu\text{g/ml}$ in fruit juices. This marks a step forward in sensitive LC–MS methods that can be applied for the detection of milk allergens in beverages and thereby can assist in protecting the health of allergic consumers.

Acknowledgments

The authors are grateful to Dr. E. Anklam for supporting this project and to Dr. Franz Ulberth for valuable comments on the manuscript.

References

- [1] L. Monaci, V. Tregoat, A. van Hengel, E. Anklam, *Eur. Food Res. Technol.* 23 (2006) 149.
- [2] E.A. Foegeding, P.J. Luck, J.P. Davis, *Food Hydrocolloids* 20 (2006) 284.
- [3] A. Kilara, D. Penyam, *Crit. Rev. Food Sci. Nutr.* 43 (2003) 607.
- [4] D.E.W. Chatterton, G. Smithers, P. Roupas, A. Brodkorb, *Int. Dairy J.* (2006) 1229.
- [5] J.M. Wal, *Ann. Allergy, Asthma Immunol.* 93 (2004) S2.
- [6] M. Natale, C. Bisson, G. Monti, A. Peltran, L.P. Garoffo, S. Valentini, C. Fabris, E. Bertino, A. Coscia, A. Conti, *Mol. Nutr. Food Res.* 48 (2004) 363.
- [7] J.O' B. Hourihane, A.C. Knulst, *Toxicol. Appl. Pharmacol.* 207 (2005) S152.
- [8] A. van Hengel, *Trends Food Sci. Technol.* 18 (2007) 96.
- [9] A. van Hengel, *Anal. Bioanal. Chem.* 389 (2007) 111.
- [10] European Parliament and Council, *Off. J. Eur. Commun.* L308 (2003) 15.
- [11] European Parliament and Council, *Off. J. Eur. Commun.* L109 (2000) 29.
- [12] T.D. Veenstra, *J. Chromatogr. B* 847 (2007) 3.
- [13] M. Careri, A. Mangia, *J. Chromatogr. A* 1000 (2003) 609.
- [14] M. Ye, X. Jiang, S. Feng, R. Tian, H. Zou, *Trends Anal. Chem.* 26 (2007) 80.
- [15] P.R. Goodwin, *J. AOAC Int.* 87 (2004) 1383.
- [16] D. Weber, P. Raymond, S. Ben-Rejeb, B. Lau, *J. Agric. Food Chem.* 54 (2006) 1604.
- [17] L. Monaci, A.J. van Hengel, *J. Agric. Food Chem.* 55 (2007) 2985.
- [18] M.C. García, M.L. Marina, M. Torre, *Anal. Chem.* 69 (1997) 2217.
- [19] M.C. García, M.L. Marina, M. Torre, *J. Chromatogr. A* 822 (1998) 225.
- [20] G. Bordin, F. Cordeiro Raposo, B. de la Calle, A.R. Rodríguez, *J. Chromatogr. A* 928 (2001) 63.
- [21] E. Bramanti, C. Sortino, G. Raspi, *J. Chromatogr. A* 958 (2002) 157.
- [22] I.M. Ferreira, H. Cacote, *J. Chromatogr. A* 1015 (2003) 111.
- [23] R. O' Donnel, J.W. Holland, H.C. Deeth, P. Alewood, *Int. Dairy J.* 14 (2004) 1013.
- [24] J. Leonil, D. Molle, J. Fauquant, J.L. Maubois, R.J. Pearce, S. Bouhallab, *J. Dairy Sci.* 80 (1997) 2270.
- [25] R. Siciliano, B. Rega, A. Amoresano, P. Pucci, *Anal. Chem.* 72 (2000) 408.
- [26] C. Bindslev-Jensen, D. Briggs, M. Osterballe, *Allergy* 57 (2002) 741.