Reversed-Phase High-Performance Liquid Chromatography Analysis of β-Lactoglobulin and α-Lactalbumin in Different Types of Milk

Oksana Rotkāja, Jelena Goluško, Pēters Mekšs

Abstract – The aim of this study was to detect major whey proteins α-lactalbumin and β-lactoglobulin by reversed-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC methods were developed using the column YMC Pack-C4, which enabled the separation of whey proteins within 30 min. Mobile phase was acetonitrile/water/0.1 % trifluoroacetic acid (TFA) with gradient elution, flow rate was 1.0 mL min−1, and the detection wavelength was 210 nm. Column temperature was 40 °C and injection volume was 20 μL. Milk samples contained α-lactalbumin and total β-lactoglobulin: No. 1 – 1500 mg·L−1 and 3600 mg·L−1; No. 2 – no α-lactalbumin and 450 mg·L−1 total β-lactoglobulin; No. 3 – 800 mg·L−1 and 94 mg·L−1, respectively.

Keywords – α-lactalbumin, β-lactoglobulin, RP-HPLC, milk.

I. INTRODUCTION

Whey proteins (α-lactalbumin and β-lactoglobulin) are strongly correlated with nutritional value and functional properties of milk [1]. Lactoglobulins can also cause the development of allergy to cow milk [1]. The most allergenic whey protein is β-lactoglobulin, which constitutes 50 % of whey proteins [1].

Quantitative determination of β-lactoglobulin has been proposed to distinguish between different categories of heat-treated milk [2].

Minimum content of β-lactoglobulin of 2600 mg·L−1 for pasteurized milk, 2000 mg·L−1 for high-pasteurized milk, and of 50 mg·L−1 for ultra-high temperature (UHT) milk is within the limits proposed by the International Dairy Federation [2].

The thermal process requires heating at 123–127 °C with a holding time of 1–5 s. Traditional high-temperature short time pasteurization is carried out at 72–75 °C for 15–30 s, and UHT milk is heated at a minimum of 135 °C for a few seconds [3].

Analytical determination methods employed for this purpose are: gel electrophoresis [4], capillary electrophoresis [5], immunochemical methods [6] or liquid chromatography [7], [8]. Reversed-phase high-performance liquid chromatography (RP-HPLC) in combination with simple ultraviolet detection (UV) is proposed by the International Dairy Federation to determine acid-soluble β-lactoglobulin in liquid milk [9].

RP-HPLC/UV methods have been used to evaluate the heat induced changes in the profile of major bovine whey proteins [10], [11].

HPLC methods, also, ultra-high performance/pressure liquid chromatography (UHPLC) have been used to determine whey proteins (α-lactalbumin, β-lactoglobulin genetic variants A + B) [12].

In our experiment, we used RP-HPLC with columns YMC Pack-C4 (150 mm × 4.6 mm I.D., 5 μm), Zorbax Extend300-C18 (50 × 2.1 mm I.D., 3.5 μm) and Zorbax SB300-Diphenyl (50 × 2.1 mm I.D., 1.8 μm) to develop a method that can be used to determine α-lactalbumin and β-lactoglobulin.

II. EXPERIMENTAL

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Acetonitrile of HPLC gradient grade, methanol of HPLC gradient grade, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, USA).

α-Lactalbumin (M~ 14100) and β-lactoglobulin (M~ 18000) were purchased from Sigma-Aldrich (St. Louis, USA).

Milk samples were: No. 1 – pasteurized cow milk (produced in Latvia), No. 2 – pasteurized goat milk (UHT, produced in Belgium), No. 3 – pasteurized cow milk (UHT, produced in Poland).

A HPLC analysis was performed using Agilent 1290 Infinity Quaternary liquid chromatography (LC) system (Agilent Technologies, Santa Clara, CA, USA). This instrument includes an UV detector, a binary pump, a thermostated column compartment, a column oven, and an auto sampler. Data acquisition, data handling, and instrument control were performed by ChromSword Auto 4.0 Professional software.

The following columns were used: YMC Pack-C4 (150 × 4.6 mm I.D., 5 μm), Zorbax Extend 300-C18 (50 × 2.1 mm I.D., 3.5 μm) and Zorbax SB300-Diphenyl (50 × 2.1 mm I.D., 1.8 μm). Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1 % TFA in water and solvent B was 0.1 % TFA in acetonitrile or methanol (Table I).

For YMC Pack-C4 column the flow rates used for analysis were: 1 mL·min−1 (basic method), 0.9 mL·min−1 and 1.1 mL·min−1, and elution was carried out at 40 °C (basic method), 35 °C and 45 °C. The detection was carried out at 210 nm.

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Flow rate was 0.2 mL min\(^{-1}\) for Zorbax 300Extend-C18 and 0.3 mL min\(^{-1}\) for Zorbax 300SB-Diphenyl, elution was carried out at 40 °C, and detection was carried out at 210 nm.

Preparation of milk samples was performed as described in source [8]. 2 M hydrochloric acid was added to the milk sample until pH 4.6 was reached in order to precipitate caseins and denatured whey proteins. After centrifugation (4000 × g, 4 °C, 30 min), the obtained clear acid-whey containing the acid-soluble whey proteins was diluted (1:5) with 0.1 mM sodium phosphate buffer (pH 6.7), filtered through a 0.45 μm cellulose membrane, and directly used for chromatographic analysis. The injection volume was 20 μl.

### TABLE I

| Gradient profile, B% (acetonitrile) | Gradient profile, B% (methanol) | Gradient profile, B% (acetonitrile) | Gradient profile, B% (acetonitrile) |
|------------------------------------|---------------------------------|------------------------------------|------------------------------------|
| YMC Pack-C4                        | Zorbax 300Extend-C18            | Zorbax 300SB-Diphenyl              |                                    |
| 0                                  | 0                               | 0                                  | 35                                 |
| 36                                 | 10.9                            | 31                                 | 15.4                               |
| 43                                 | 29.9                            | 76                                 | 20.6                               |
|                                    |                                 |                                    | 43                                 |

### III. RESULTS AND DISCUSSION

Full spectrum scanning was used to select the largest absorption wavelength of whey proteins and 210 nm was selected as the detection wavelength (Fig. 1).

The chromatogram detection of α-lactalbumin and β-lactoglobulin standard with Zorbax 300Extend-C18 and Zorbax 300SB-Diphenyl is shown in Fig. 2. The elution gradient of a C18 column was: 0–15.4 min 31–76 % B, 15.4–20.6 min 76 % B. The elution gradient of Diphenyl column was: 0–18.1 min 35–77 % B, 18.1–25.1 min 77 % B. Retention time of both UHPLC columns was less than 6 min.

Resolution for β-lactoglobulin B and A variants on C18 was \( R_s = 1.29 \) and on Diphenyl – \( R_s = 0.74 \), α-Lactalbumin and β-lactoglobulin retention times for Diphenyl column with methanol organic solvent in the same condition as C18 were 12.7 min and 14.8 min and resolution for β-lactoglobulin B and A genetic variants was 0.5. β-Lactoglobulin B and A shape separation on Diphenyl column was weak. β-Lactoglobulin B and A genetic variant separation to the baseline on C18 and Diphenyl columns was not obtained.

The following conditions for milk protein separation were obtained: phase A was composed of 0.1 % (V/V) TFA in ultrapure water and phase B (organic phase) was composed of 0.1 % TFA in acetonitrile; the flow rate was 1 mL·min\(^{-1}\); the detection wavelength was 210 nm, the injection volume was 20 μL and column temperature was 40 °C. The elution gradient of C4 column was set this way: 0–10.9 min 0–36 % B, 10.9–29.9 min 36–43 % B, 29.9–35 min 43 % B. The chromatogram detection of bovine milk with C4 column is shown in Fig. 3 (milk sample No. 1) and Fig. 4, (milk sample No. 3). All peaks were separated and resolution is shown Table II (basic method).

Milk samples from the supermarket were determined with RP-HPLC C4 column, and each sample replicated 3 times. Results are shown in Table III. α-Lactalbumin and β-lactoglobulin contents in milk sample No. 1 were 1500 mg·L\(^{-1}\) and 3600 mg·L\(^{-1}\) with C4; this was obviously higher in milk samples No. 2 and sample No. 3. Goat milk sample No. 2 did not contain α-lactalbumin. Milk samples No. 2 and No. 3 were pasteurized at ultra-high temperature. Whey proteins of milk were lost in the pasteurization process at ultra-high temperature. Milk sample No. 3 had the least amount of whey protein (Table III).

The following flow rates were chosen: 0.9 mL·min\(^{-1}\), 1.0 mL·min\(^{-1}\) and 1.1 mL·min\(^{-1}\). Results revealed that the highest peak was at 1.0 mL·min\(^{-1}\), but under other conditions of flow rate the peaks were accordingly narrower. Flow rate affected the peak separation – peak separation was better at 1.0 mL·min\(^{-1}\) (Table II).

The chosen temperatures for measurements were 35 °C, 40 °C and 45°C, respectively, but the results revealed that the temperature did not have any significant effect on the separation.
Fig. 2. Chromatogram of milk protein standard. Protein separation on Zorbax Extend-C18 and Zorbax 300-SB Diphenyl column, mobile phase: methanol/water/0.1 % TFA, gradient profile – see Table I. 1 – α-lactalbumin, 2 – β-lactoglobulin B and 3 – β-lactoglobulin A.

Fig. 3. Chromatogram of milk sample No. 1. Protein separation on YMC Pack-C4 column, mobile phase: acetonitrile/water/0.1 % TFA, gradient profile – see Table I. 1 and 2 – unknown peaks, 3 – α-lactalbumin, 5 – β-lactoglobulin B and 6 – β-lactoglobulin A.

Fig. 4. Chromatogram of milk sample No. 3. Protein separation on YMC Pack-C4 column, mobile phase: acetonitrile/water/0.1 % TFA, gradient profile – see Table I. 1, 2 and 4 – unknown peaks, 3 – α-lactalbumin, 5 – β-lactoglobulin B and 6 – β-lactoglobulin A.

It was revealed that the decrease of organic solvent concentration by approximately 2% in the mobile phase had no significant effect on the separation. But the increase of organic solvent concentration by approximately 2% in the mobile phase decreased the resolution (Table II).

### TABLE II

| Components | 65 °C | 75 °C | +2% | -2% | -0.1 mL min⁻¹ | +0.1 mL min⁻¹ | Basic method |
|------------|------|------|-----|-----|---------------|---------------|--------------|
| Resolution (Rs) |      |      |     |     |               |               |              |
| 2/3        | 1.86 | 1.92 | 1.89 | 2.03 | 1.87          | 1.70          | 1.95         |
| 3/4        | 3.23 | 3.33 | 2.82 | 3.25 | 3.30          | 3.05          | 3.28         |
| 4/5        | 7.50 | 7.57 | 6.01 | 7.59 | 7.23          | 7.12          | 7.58         |
| 5/6        | 2.32 | 2.24 | 2.14 | 2.29 | 2.26          | 2.24          | 2.30         |

### TABLE III

| Protein Concentration in Milk Samples |
|--------------------------------------|
| Milk No. 1                           |
| mg/L                                 |
| LA | LG-B | LG-A | LG* |
| Average | 1500 | 1700 | 1900 | 3600 |
| RSN | 1.2 | 2.1 | 1.8 | 0.46 |
| Milk No. 2                           |
| mg/L                                 |
| LA | LG-B | LG-A | LG* |
| Average | – | 450 | – | 450 |
| RSN | – | 1.7 | – | 1.7 |
| Milk No. 3                           |
| mg/L                                 |
| LA | LG-B | LG-A | LG* |
| Average | 800 | 49 | 45 | 94 |
| RSN | 1.3 | 2.6 | 2.3 | 1.3 |

LA – α-lactalbumin; LG-B – β-lactoglobulin B; LG-A – β-lactoglobulin A; LG* – total β-lactoglobulin, RSN – relative standard deviation.
### IV. CONCLUSION

YMC Pack C4 column was used to determine α-lactalbumin and β-lactoglobulin in milk samples. The results revealed that the best peak form was obtained at 1.0 mL·min⁻¹, and that the temperature did not have a significant effect on the separation or composition effect on peak separation in the mobile phase.

Milk samples contained α-lactalbumin and total β-lactoglobulin: No. 1 – 1500 mg·L⁻¹ and 3600 mg·L⁻¹; No. 2 contained no α-lactalbumin and 450 mg·L⁻¹ total β-lactoglobulin; No. 3 – 800 mg·L⁻¹ and 94 mg·L⁻¹, respectively.

The retention time of both UHPLC C18 and Diphenyl columns were less than 6 min, but β-lactoglobulin B and A genetic variant separation to the baseline was not obtained. These methods can be used to determine the fastest milk pasteurization type. Whey proteins were lost in milk samples which were pasteurized at ultra-high temperature.

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Oksana Rotkāja, Jeļena Goluško, Pēters Meksis, Dažādu piena parādu analīze apgriežās fāzes augstas efektivitātes šķidruma hromatogrāfijas apstākļos, β-Laktoglobulinu var izmantot kā īndikatoru, lai noskaidrotu kāds pasteurizācijas veids ir izmantots pienam. Šī darba ietvaros apstākļos ik nesakrīt viens un otrs hromatogrāfiskie apstākļi olbaltumvielu atdalīšanu. Tika izvēlēta kolonna C4, ar kuru tika veikta sākotnējā hromatogrāfisko apstākļu optimizācija, lai iegūtu pilnīgu olbaltumvielu atdalīšanu. Tika izstrādāta metode uz C4 kolonnas par kustīgo fāzi lietojot acetonitrīla/ūdens/0,1 % TFA maisījumu, analīzes laiks ir 30 min.