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High performance liquid chromatography with size exclusion column (HPLC-SEC) method for identifying the major whey proteins of whey protein products

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Abstract: This study was carried-out: a) to develop a high-performance liquid chromatography with size exclusion column (HPLC-SEC) method for the identification of the major whey proteins from selected whey protein products; and b) use the method to estimate the relative composition of the major whey proteins in different whey protein products. An HPLC Shimadzu (LC-10AD VP liquid chromatograph) with system controller (SCL-10A VP) equipped with a pump and auto-injector (SIL-10AD VP) and UV-vis detector (SPD-10AV) was used in the identification of whey proteins in standards and whey protein products. The size exclusion column (SEC) was a Yarra 3 µm, SEC-3000 Column, 7.8 mm I.D. x 30 cm with a security guard. The HPLC-SEC method was successful in identifying the major whey proteins of the different whey protein products. The β-LG contents had the highest level among the whey proteins for all the whey protein products studied, followed by the α-LA and then IgG in both WPC products. However, the α-LA and IgG of the Procream product had almost the same level which was probably due to a different process used in WPC. All the major whey proteins with the highest pump flowrate had the shortest elution times while the whey proteins with the lowest pump flowrate had the longest elution times. The optimal pump flowrate was 0.75 mL/min since it gave a faster analysis but differentiate the peaks of the different major whey proteins.

Keywords: Whey Proteins; HPLC-SEC; β-lactoglobulin; α-lactalbumin; Immunoglobulin G

1. Introduction

Whey is a co-product of cheese-making and casein manufacture in the dairy industry. After the casein curd separates from the milk, following coagulation of the casein proteins through the action of chymosin (rennet) or mineral/organic acid, the remaining watery and thin liquid is called whey (Zadow, 1994). Whey comprises 80-90% of the total volume of milk entering the process and contains about 50% of the nutrients in the original milk: soluble protein, lactose, vitamins, and minerals (Tetra Pak, 2015). Whey contains a multitude of biologically active proteins and peptides. Apart from the major whey proteins – β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and glycomacropeptide – whey contains several proteins with potent bioactivity – immunoglobulins, lactoferrin, lactoperoxidase and growth factors (Smithers, 2008).

According to Huffman and Harper (1999), a wide range of whey protein products are available for a variety of application. The three major classes are the whey protein concentrate (WPC), whey protein isolate (WPI) and Lactalbumin. WPC involves clarification of the whey, followed by ultrafiltration and several stages of diafiltration and eventually spray drying of the concentrate. WPI requires the microfiltration or ion exchange of whey, followed by similar operations as WPC. Lastly, Lactalbumin involves heat denaturation of whey, followed by precipitation/separation and several stages of washing and finally drying the washed material. Lactalbumin, not to be confused with α-LA, contains all the heat precipitable whey proteins, has a clean flavor, and is heat stable. WPC range in concentration from 34 to 85% proteins. The US produced the 34% WPC while the other dairy exporting countries developed the high protein products (75-85% WPC) with specific functionalities. Whey proteins of particular interest and currently commercially available include β-LG, α-LA, lactoferrin, lactoperoxidase, and immunoglobulins.

Modern membrane processing, including industrial applications of microfiltration, ultrafiltration/diafiltration, have helped to pioneer the development of high-protein and low-fat functional whey ingredients, such as whey protein concentrates (WPCs) (~35, 75 and 80% protein) and first-generation whey protein isolates (WPIs) (~85-90% protein), that have expanded the applications base for whey protein ingredients (Clark, 2005; Kelly et al., 2000; Saboya and Maubois, 2000). Microfiltration is a membrane separation process used for the reduction of bacteria in skim milk,

whey and brine, but also for defatting whey intended for WPC and for protein fractionation. Ultrafiltration is also a membrane separation process and typically used for concentration of milk proteins in milk and whey and for protein standardization of milk intended for cheese, yoghurt, and some other products. Diafiltration is a procedure in which water is added to the feed as filtration proceeds, to wash out low molecular components which will pass through the membranes, basically lactose and minerals. Procream or high fat retentate is obtained from microfiltration of whey retentate from the ultrafiltration of cheese whey which is a co-product obtained during the manufacture of WPI (Tetra Pak, 2015).

Whey proteins is of high nutritional value and has become an important source of functional ingredients in various health-promoting foods. For value-added industrial applications of whey proteins, the composition, and the changes in both the physicochemical and functional properties need to be accurately analyzed. Because whey proteins are a mixture of proteins, accurate analysis of whey proteins requires separation of the whey proteins (Kang et al., 2011). These include gel electrophoresis (Kinghorn et al., 1995; Bouaouina et al., 2006), capillary zone electrophoresis (Kinghorn et al., 1995; Liang et al., 2006) and various forms of high-performance liquid chromatography (HPLC) (Geberding and Byers, 1998; Elgar et al., 2000). The HPLC methods include reversed-phase (Garcia et al., 1998; Elgar et al., 2000), ion-exchange (Geberding and Byers, 1998; El-Sayed and Chase, 2010) and size-exclusion chromatography (SEC) (Bouaouina et al., 2006; El-Sayed and Chase., 2010).

Diosady et al. (1980) used a high-performance liquid chromatography size exclusion chromatography (HPLC SEC) method to determine the whey proteins using 2 Syncropak GPC columns in series. Gupta (1983) also used the HPLC SEC method to determine the native and denatured milk proteins but used a TSK 3000 SW column only. Downes and Silcock (2014) also reported the use of HPLC SEC method to analyze a range of whey proteins from pilot ultrafiltered dairy products using a Yarra 3 µm 2000 and Yarra 3 µm 4000 columns in series.

This study was carried-out: a) to develop a high-performance liquid chromatography with size exclusion column (HPLC-SEC) method for the identification of the major whey proteins from selected whey protein products; and b) use the method to estimate the relative composition of the major whey proteins in different whey protein products.

2. Materials and Methods

2.1. Materials

The Sodium Phosphate Dibasic Dodecahydrate was from Sigma Aldrich (St. Louis, MI, USA), Sodium Chloride from Fisher Chemical (Loughborough, UK) and Hydrochloric Acid (36%) from Ajax Finechem (Taren Point, Australia). The Bovine Whey Proteins standards such as β-lactoglobulin, α-lactalbumin, and Immunoglobulin G were procured from Sigma Aldrich (St. Louis, MI, USA). The Whey Protein Concentrate powders were procured from Westland Milk Products (JZ19) and Fonterra (CW29), New Zealand while the Procream powder was obtained from Mullins Whey (Mosinee, WI, USA).

2.2. Reconstitution of Whey Protein Powders

All the whey protein powders (WPC and Procream) were reconstituted to 5% solids by dissolving the powders in a lukewarm purified water (~40ºC) by stirring until all the powder particles were dissolved. The reconstituted products were left overnight in the chiller at 4ºC to fully hydrate and then stored until use. All the reconstituted products were used within one week.

2.3. Whey Proteins Detection Method

A high-performance liquid chromatography (HPLC) Shimadzu (LC-10AD VP liquid chromatograph) with system controller (SCL-10A VP) equipped with a pump and auto-injector (SIL-10AD VP) and UV-vis detector (SPD-10AV) was used in the detection of whey proteins in standards and whey products (permeate and retentate from ultrafiltration). The chromatographic column was a Yarra 3 µm, SEC-3000 Column, 7.8 mm I.D. x 30 cm with a security guard. The detector signal was analysed using the LC Solution software to obtain the integrated area of the peaks from the chromatogram. The mobile phase was a 50 mM sodium phosphate buffer containing 150 mM sodium chloride and buffered to pH = 7.0 using 1 M HCl solution and using an isochratic pump mode with a flowrate of 0.50 mL/min. HPLC measurements were done at room temperature of about 25ºC using a column heater (Thermasphere TS-130). The UV-vis detector was set with a wavelength of 280 nm for Channel 1 and 220 nm for Channel 2. The whey protein standards samples were prepared by dissolving various amounts of whey protein powders in 5-10 mL purified water. Then about 2 mL of sample was then filtered thru a 0.45 µm PTFE filter into glass vials for HPLC-SEC detection of the elution time for each whey protein standard. In the case of the whey products, about 13 mL of the sample was first centrifuged at 6500 rpm for 5 minutes (Eppendorf), then 2 mL of the supernatant liquid was obtained and centrifuged at 13,000 rpm for 5 minutes (Heraeus). Another supernatant liquid from this sample was obtained and then filtered thru a 0.45 µm PTFE filter into glass vials for HPLC detection of the elution time for each whey protein. A 50 µL of the filtered sample was automatically injected into the system for measurement. The running time for the HPLC detection was for 30 minutes. The elution time of the whey proteins were obtained from the print-out of the run.

2.4. Analysis of IgG from Whey Protein Products

The IgG contents of the reconstituted whey protein products were analyzed at Cawthron Institute, Nelson, New Zealand using the method of Holland et al. (2011).

3. Results and Discussion

3.1. Detection of Whey Proteins Standards

The top 3 major whey proteins standards used in the analysis have the following approximate molecular weights as shown in Table 1.

The SEC used in the HPLC serves solely as a fractionation step to separate the various whey proteins based on its molecular weight with the heavier protein being eluted first and the lightest protein eluted last. Hence, it will be expected that the IgG will have the shortest retention time and the α lactalbumin will have the longest retention time.

Table 1. Approximate molecular weights of the major whey proteins

Whey Protein	Molecular Weight (Da)
Alpha Lactalbumin (α-LA)	14,175[a] – 16,250[b]
Beta Lactoglobulin (β-LG)	18,277[a] – 19,800[b]
Immunoglobulin G (IgG)	146,000[a] - >150,000[b]

[a] Madureira et al., (2007); [b]Ostertag et al. (2021)

Table 2 shows the results for the elution time of the 3 whey proteins dairy standards at the conditions specified in the previous section. As expected, IgG had the shortest elution time of about 16 minutes and the α Lactalbumin had the longest elution time of about 20 minutes. Figures 1, 2 and 3 are the chromatograms of IgG, β-LG and α-LA standards, respectively. There were 2 distinct peaks in the IgG chromatogram indicating that the whey standard was not very pure as compared with the β-LG and α-LA standards.

Table 2. Elution times of major whey proteins standards

Whey Protein Standard	Elution Time (minutes)
IgG	15.88-15.91
β-LG	18.49-18.51
α-LA	19.76-19.78

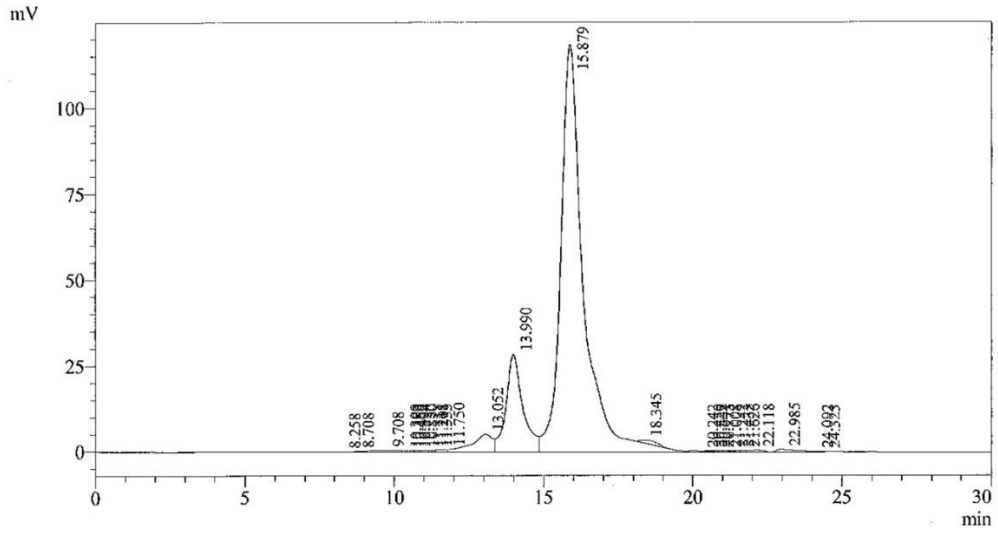

Fig 1. Chromatogram of the IgG standard

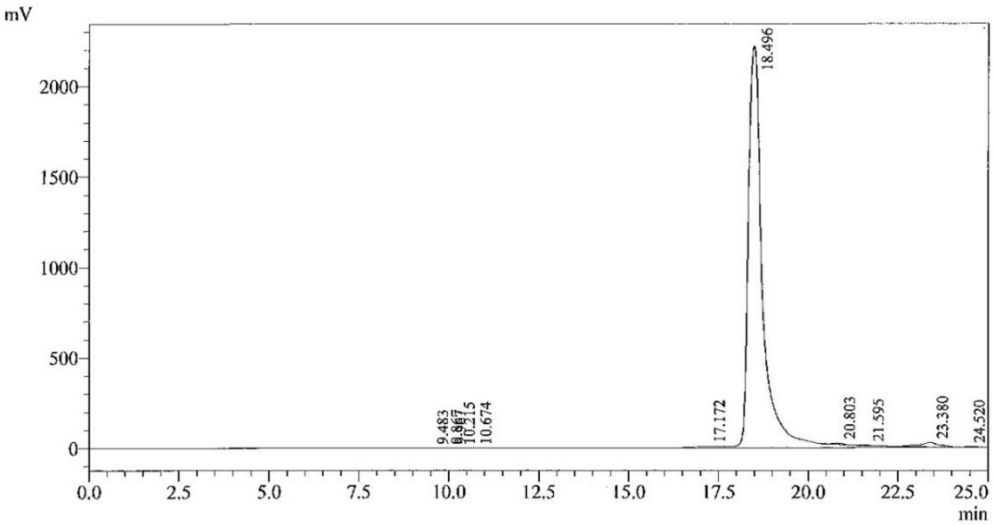

Fig 2. Chromatogram of the β-LG standard

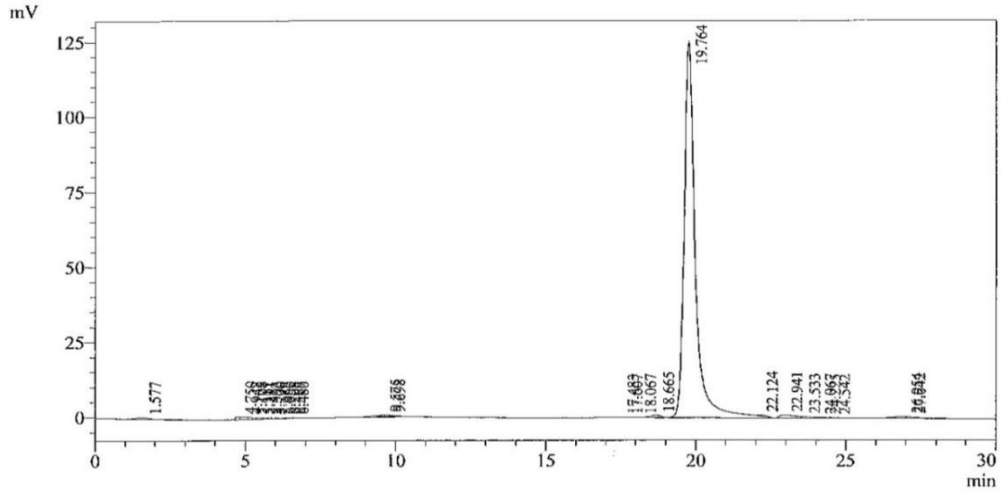

Fig 3. Chromatogram of the α-LA standard

3.2. Elution Times for the Whey Proteins in Various Whey Products

Table 3 shows the elution times of the various whey proteins found in the various whey protein products studied. There were some variations from the elution times of whey protein standards probably due to the impurities in the whey protein products. Figures 4, 5 and 6 are the chromatograms of WPC JZ19, WPC CW29 and Procream products, respectively. The other peaks in the chromatograms were not identified since these were not part of the objective of the study. Table 4 presents the percentage of the total area of the chromatogram for the major whey proteins in the various whey protein products. The percentage of the total area can be used as an estimate of the relative composition of the major whey proteins in the various whey protein products. The results showed that the β-LG contents were the highest among the whey proteins, followed by α-LA and then IgG in both WPC products. De Wit (1998) and Pires et al. (2021) reported that β-LG was the highest, followed by α-LA and then IgG in bovine whey proteins. However, the α-LA and IgG of the Procream product was almost the same which was probably due to a different process used in WPC. Procream was from a high fat retentate obtained from microfiltration of the whey retentate from the ultrafiltration of cheese whey, which is co-product obtained during the manufacture of WPI (Tetra Pak, 2015). The Procream gave the highest IgG content, followed by WPC JZ19 and then WPC CW29. This is the same order also of IgG contents of 221, 67 and 18 mg/g for Procream, WPC JZ19 and WPC CW29, respectively from the analysis using the Protein G Affinity Chromatography method (Holland et al., 2011).

Table 3. Elution times of whey proteins from whey products

Whey Protein	Elution Time (minutes)		
	WPC JZ19	WPC CW29	Procream
IgG	15.75	15.80	15.75
β LG	18.45	18.44	18.51
α LA	19.75	19.70	19.73

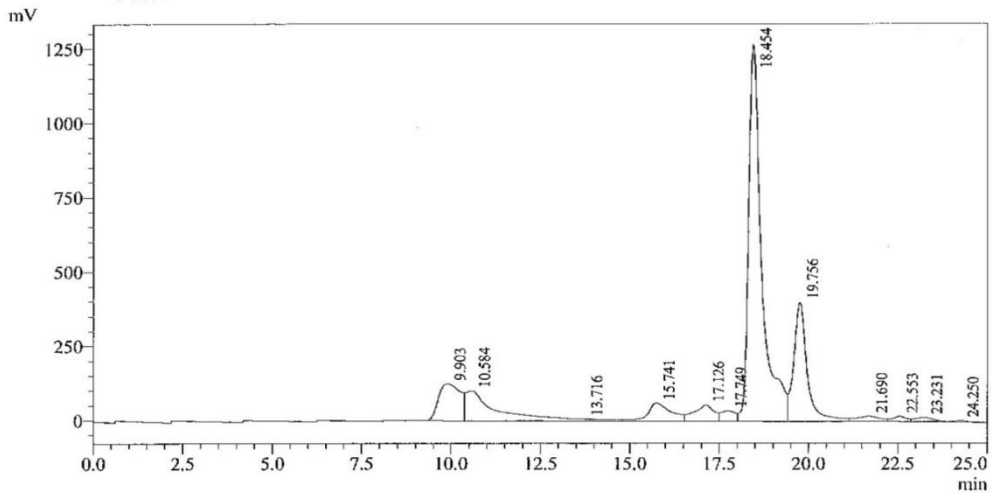

Fig 4. Chromatogram of the WPC JZ19 product

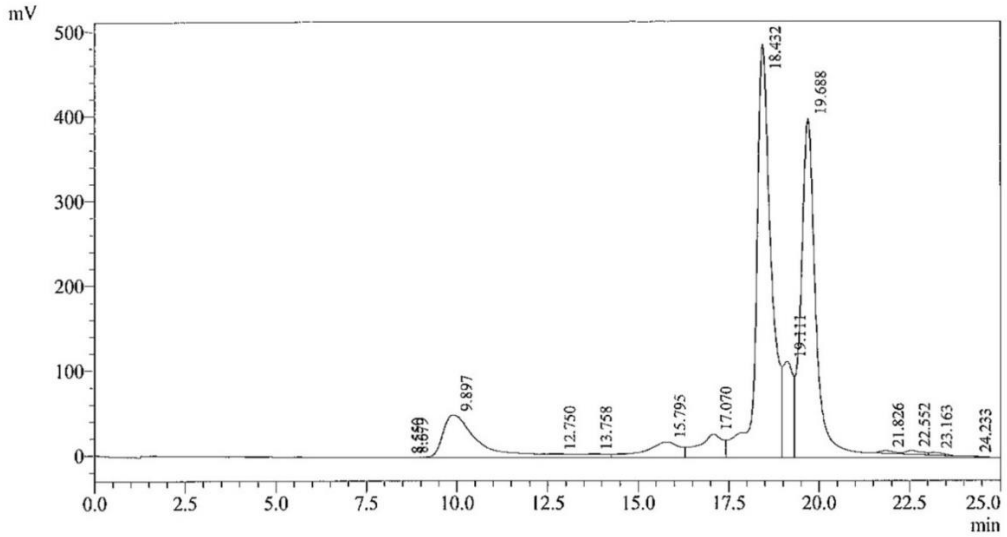

Fig 5. Chromatogram of the WPC CW29 product

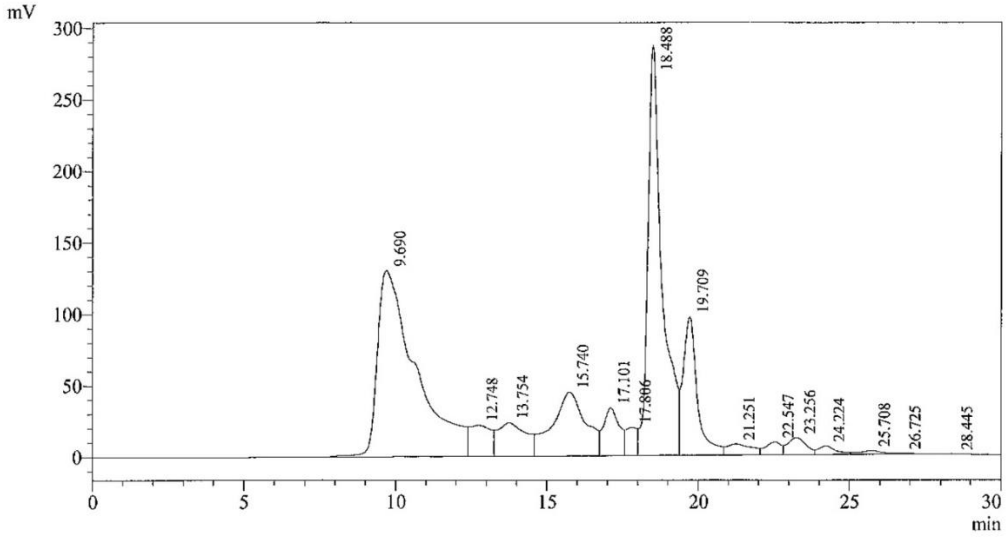

Fig 6. Chromatogram of the Procream product.

Table 4. Percentages of the total area of the chromatogram for the top 3 major whey proteins in various whey protein products

Sample	α-LA(%)	β-LG(%)	IgG(%)
WPC JZ19	17.0	52.1	4.3
WPC CW29	34.7	40.2	3.6
Procream	11.1	33.0	11.3

3.3. Effect of HPLC Pump Flowrate on the Elution Times for the Selected Whey Proteins Standards

Table 5 shows the elution time of the major whey proteins standards at the conditions specified earlier but with different HPLC pump flowrates. As expected, all the whey proteins with the highest pump flowrate had the shortest elution times while the whey proteins with the lowest pump flowrate had the longest elution times. At higher pump flowrate, the speed of whey proteins elution will be faster, hence shorter elution time.

Table 5. Elution times of major whey protein standards with different pump flowrates

Whey Protein (minutes)	Pump Flowrate (mL/min)/Elution Time		
	0.50	0.75	1.00
IgG	15.90	10.63	7.80
β-LG	18.50	12.34	9.29
α-LA	19.77	13.20	9.93

3.4. Effect of HPLC Pump Flowrate on the Elution Times for the Selected Whey Proteins from WPC Products

Table 6 shows the elution times of the major whey proteins from two WPC products. There were minimal variations from the elution times of the major whey proteins from each other at the same pump flowrates. Again, the whey proteins with the highest pump flowrate had the shortest elution times while the whey proteins with the lowest pump flowrate had the longest elution times. The purpose of this

investigation was to determine the optimum pump flowrate that would give faster analysis but would still differentiate the peaks of the different whey proteins for each product. The results show that a pump flowrate of 0.75 mL/min would be the optimum.

Table 6. Elution times of major whey proteins from WPC products with different pump flowrates

Sample/Whey Protein	Pump Flowrate (mL/min) / Elution Time (minutes)		
	0.50	0.75	1.00
WPC JZ19			
IgG	15.75	10.55	7.80
β-LG	18.45	12.32	9.27
α-LA	19.75	13.19	9.92
WPC CW29			
IgG	15.80	10.57	7.95
β-LG	18.44	12.33	9.27
α-LA	19.70	13.17	9.90

4. Conclusion

The HPLC-SEC method was successful in identifying the major whey proteins of the different whey protein products. The β-LG contents had the highest level among the whey proteins for all the whey protein products studied, followed by the α-LA and then IgG in both WPC products. However, the α-LA and IgG of the Procream product had almost the same level which was probably due to a different process used in WPC.

All the major whey proteins with the highest pump flowrate had the shortest elution times while the whey proteins with the lowest pump flowrate had the longest elution times. The optimal pump flowrate was 0.75 mL/min since it gave a faster analysis but differentiate the peaks of the different major whey proteins.

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Conflict of interest disclosure:

The author declares no conflict of interest on the written article.

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