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Article

DEVELOPMENT AND VALIDATION OF A METHODOLOGY FOR QUANTIFICATION OF WHEY PROTEIN FRACTIONS IN MICROPARTICULATE INGREDIENTS

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Microparticulated whey protein can be used as a fat substitute in foods to enhance their nutritional value and improve their sensory and rheological characteristics. Microparticulation denatures whey proteins through heat treatment, followed by shear rate control to determine the aggregate sizes formed. The processing method used during microparticulation can modify the protein denaturation rate, which may enable the application of new proteins. This study sought to develop a methodology for quantifying whey protein fractions in commercial samples before and after microparticulation at different concentrations and physical states. The developed methodology proved to be applicable because of its selectivity, quantification, detection limits, and precision. The analyzed samples exhibited a higher denaturation rate for β-lactoglobulin than α-lactoalbumin; the denaturation rate varied among samples due to differences in the microparticulation process.

Keywords: HPLC; WPC; microparticulation; validation; denaturation protein.

INTRODUCTION

Whey is obtained from the coagulation of milk used in cheese production. It comprises water, proteins, lactose, minerals, and fats.[1] Whey is widely used in the food industry because it contains all essential amino acids, improves blood pressure, prevents cardiovascular diseases, and has antioxidant properties.[2,3] Whey protein can be divided into β-lactoglobulin (ranging from 48 to 58%), α-lactoalbumin (13 to 19%), caseinomacropeptide (12 to 20%), immunoglobulins (8 to 12%), bovine serum albumin (6 to 7%), lactoferrin (1 to 3%), and other proteins (0.5 to 1%).[4]

One of the main applications of whey proteins in food products is whey protein concentrates (WPC).[5] However, these proteins are thermally destabilized due to their low denaturation temperatures, this denaturation can lead to product defects, as phase separation and loss of sensory characteristics may occur. For example, α-lactoalbumin has a denaturation temperature ranging from 30 to 60 °C, depending on its variant.[6,7]

Microparticulation is an alternative method to enhance the thermal stability of whey proteins.[8] In this process, heat is applied to denature and aggregate the proteins, followed by high-shear steps to break the aggregated proteins into smaller, more uniform particles.[9]

With this process, microparticulated proteins combine denatured and native proteins with controlled particle sizes ranging from 0.1 to 100 µm.[10,11] This native and aggregated protein combination increases thermal treatment resistance because it contains fewer free thiol groups, which are responsible for protein denaturation.[12]

Microparticulation modifies the whey protein structures, resulting in products with functional characteristics distinct from native whey protein,[13] such as emulsifying activity and gelation properties.[14] These products can provide textural sensations similar to fat and are used as fat substitutes in food products, including concentrated fermented milk, ice cream, desserts, and processed cheeses.[15,16]

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Several studies have quantified the WPC proteins used as ingredients, which are the main focus of this study. One study[17] quantified WPC to understand how pasteurization, membrane concentration, and spray drying modified the protein concentration in the final powder obtained. Another[18] sought to understand how heat treatment can modify the microstructure, denaturation, and functional properties of goat whey proteins. Finally, Buggy *et al.*[19] studied how protein concentration and pH interfere with WPI (isolated why protein) protein denaturation under heat treatment. However, a methodology that quantifies β-lactoglobulin and α-lactoalbumin via high-performance liquid chromatography (HPLC) before and after microparticulation has yet to be reported.

Based on this premise, this study sought to develop an HPLC methodology to quantify the two main WPC protein fractions in liquid and powdered form before and after microparticulation and at various protein concentrations. Additionally, we calculated the denaturation percentage of each protein fraction.

EXPERIMENTAL

Reagents, chemicals, standards

Analytical standards of β-lactoglobulin (CAS number: 9045-3-2) and α-lactoalbumin (CAS number: 9051-29-0) were obtained from Sigma-Aldrich (São Paulo, Brazil), trifluoroacetic acid (TFA) and acetonitrile from Merck (São Paulo, Brazil). Water was purified using the Milli-Q system (Millipore, USA).

HPLC equipment

The analytes were separated and quantified using HPLC (Waters, model 1252, Milford, USA), a UV-Vis detector (DAD, diode array detector), and a binary pump. The chromatographic separation employed a Phenomenex column Luna (5 µm, 250 mm × 4.6 mm), kept at 40 °C. The injection volume was 20 µL; β-lactoglobulin and α-lactoalbumin were detected at a wavelength of 214 nm.

Chromatographic conditions

Gradient elution was performed using a mixture of two solvents at a flow rate of 0.5 mL min^{-1}. The mobile phase comprised of 0.1% TFA in ultrapure water (solvent A) and 0.09% TFA in 90% acetonitrile:10% ultrapure water (solvent B). A gradient program was initiated as follows: 0-3 min, 20-40% B; 3-5 min, 45-45% B; 5-7 min, 45-50% B; 7-9 min, 50-80% B; 9-14 min, 80-20% B; and 14-15 min, 20-20% B.

Validation procedure

For method validation, the selectivity, linearity, precision (intra- and inter-assay), accuracy, limit of detection (LOD), and limit of quantification (LOQ) were evaluated following the criteria of RDC No. 166, from July 24, 2017, Brazil.[20] Selectivity was proven by the retention time equivalence for β-lactoglobulin and α-lactoalbumin as external standards and by the peak area increase in fortified samples. Selectivity was also evaluated for the solutions used in sample preparation and mobile phase; the absence of analytical response at the retention time of β-lactoglobulin and α-lactoglobulin proved it. The analytical curves were constructed using five points, each measured three times, to a concentration ranging from 100 to 300 µg mL^{-1}. The LOD and LOQ were calculated based on the standard deviation of the response and slope using the following formulas:

$$\mathrm{LOD} = 3.3\frac{\sigma}{S} \qquad (1)$$

$$\mathrm{LOQ} = 10\frac{\sigma}{S} \qquad (2)$$

where σ is the standard deviation of the Y-axis intercept, and S is the slope of the calibration curve.

The sample dilution integrity was validated in the ratio of 1:5 to a final concentration of 200 µg mL^{-1} of β-lactoglobulin and α-lactoalbumin. This parameter was validated according to ICH guideline M10.[21]

Samples

WPC samples of various concentrations (WPC45%, WPC60%, and WPC80%) were analyzed before and after microparticulation. Liquid and powder samples were obtained from a partnering company. The samples analyzed are listed in Table 1.

Table 1. Number of WPC samples analyzed before and after microparticulation

Form	Protein concentration / %	Number of samples
Solution	WPC45	2
	WPC60	2
	WPC80	1
Powder	WPC45	8
	WPC60	5
	WPC80	2

WCP: whey protein concentrates.

Sample preparation for RP-HPLC analysis

The liquid samples were centrifuged (22,000 × g) using a Sigma 2K15 (Sigma, Barueri, São Paulo) for 10 min at 5 °C. Solutions of the powdered samples were prepared at 2% concentration (m v^{-1}) and then centrifuged under the same conditions as the liquid samples. After centrifugation, all samples were filtered through 0.22 µm pore syringe filters for subsequent HPLC analysis. Depending on the protein fraction content determined by analysis, the samples were diluted when necessary to meet the working range of the validated analytical curve.

Percentage calculation of additional denaturation from microparticulation process

To determine the denaturation percentage for β-lactoglobulin and α-lactoalbumin, the Equation 3 was applied:[5]

$$\%\ \text{denaturation} = 1 - \frac{\text{microparticulated whey protein}}{\text{whey protein}} \times 100 \qquad (3)$$

The additional denaturation percentage caused by microparticulation is a relationship between each protein fraction concentration in whey and microparticulated whey. The protein concentration in the whey represents the native protein amount in that product. Whey exhibits some protein denaturation percentage due to pre-processing; this pre-existing denaturation was not considered in the calculation. The protein concentrations in the microparticulate are the native protein amounts remaining after processing.

RESULTS AND DISCUSSION

Separation, identification, and quantification of serum proteins

Selectivity was the first parameter evaluated during method validation by comparing the retention time of the diluent solution chromatograms with that of the fortified samples, ensuring that the peak response was exclusively for the compounds of interest, as shown in Figure 1.

Linearity of the analytical standards β-lactoglobulin and α-lactoalbumin was determined by constructing analytical curves, from which the respective coefficients of determination (R^2) were obtained (Table 2). All analyses were performed in triplicate.

The LOD and LOQ were calculated according to Equations 1 and 2 and determined by a method based on the response of three replicates of an analytical curve (Table 2).

Precision parameters were evaluated using intra- and inter-assays. The intra-assay was undertaken by analyses of six analytical standard replicates at identical concentrations of 125, 225, and 275 µg mL^{-1} on the same day under the same experimental conditions. For the inter-assay, solutions with identical concentrations were analyzed on two different days. The data obtained were expressed by estimating the relative standard deviation (RSD) to be less than 5%. The accuracy was in the range of 90-110% associated with precision values, as shown in Table 3.

Table 4 shows the effect of sample dilution on the theoretical concentration compared to the analyzed concentration. The European Medicines Agency[21] stipulates that the correlation coefficient should be < 15%. The results for both analytes were below the specified values, indicating that the dilution effect produced no interference in the samples.

Quantification of β-lactoglobulin and α-lactoalbumin in WPC samples

WPCs in solid and liquid forms were analyzed before and after microparticulation at protein concentrations of 45, 60, and 80% (m m^{-1}).

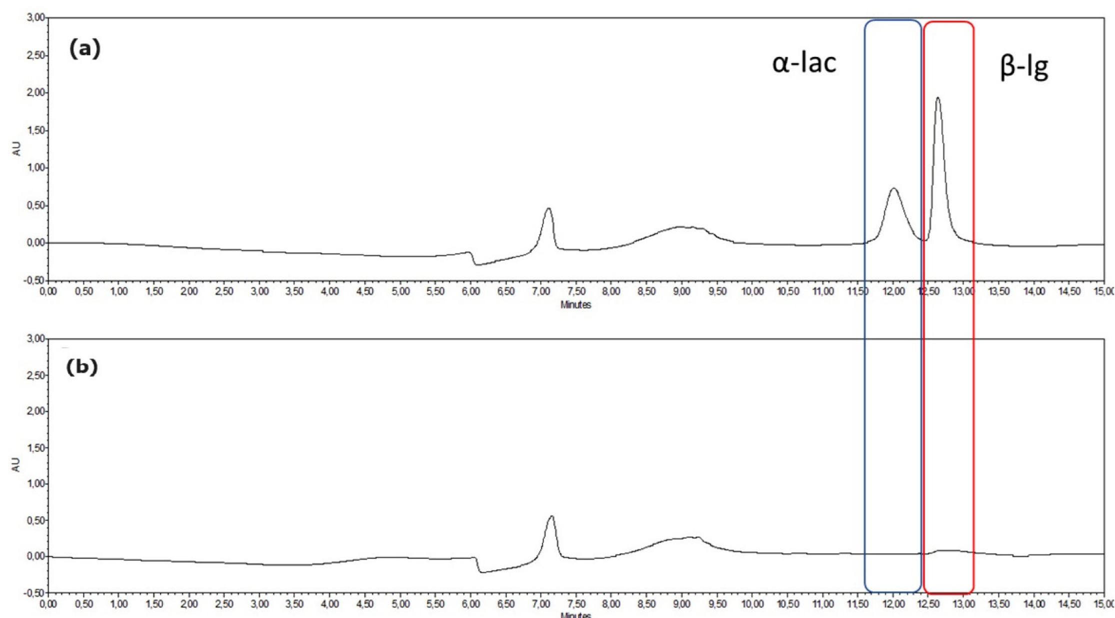

Figure 1. Chromatogram of solution containing α-lactoalbumin (α-lac) and β-lactoglobulin (β-lg) standards (a) and chromatogram of blank showing absence of interferents (b)

Table 2. Parameters determined from analytical curve

Analyte linear range	Linear equation	R^2	LOD / (μg mL^{-1})	LOQ / (μg mL^{-1})
β-Lactoglobulin	y = 32054x – 610871	0.9953	18.4	55.9
α-Lactoalbumin	y = 46826x – 638918	0.9971	7.2	22.1

R^2: coefficient of determination; LOD: limit of detection; LOQ: limit of quantification.

Table 3. Precision parameters expressed through intra- and inter-assay for α-lactoalbumin (α-lac) and β-lactoglobulin (β-lg)

Analyte	Concentration / (μg mL^{-1})	Intra-assay RSD / %	Intra-assay accuracy / %	Inter-assay RSD / %	Inter-assay accuracy / %
β-Lactoglobulin	125	3.63	100.23-107.37	3.40	100.23-108.16
	225	3.83	93.84-100.84	2.44	96.23-100.95
	275	4.16	90.69-97.40	4.25	90.69-102.50
α-Lactoalbumin	125	2.60	99.56-104.31	3.96	95.65-104.31
	225	1.11	99.46-101.54	2.25	96.34-101.54
	275	1.54	95.65-98.48	1.85	95.65-100.79

RSD: relative standard deviation.

Table 4. Dilution integrity for β-lactoglobulin and α-lactoalbumin

Analyte	Theoretical concentration / (μg mL^{-1})	Real concentration / (μg mL^{-1})	Dilution integrity / %
β-Lactoglobulin	200	205.96 ± 9.15	4.44
α-Lactoalbumin	200	220.29 ± 3.94	1.79

The number of specific samples for each concentration is presented in Table 1 and described in "Sample preparation for RP-HPLC analysis" sub-section. Table 5 shows the protein fraction concentrations for each analyzed sample and their denaturation percentages.

After powder sample reconstitution (2% m v^{-1}), higher protein fraction concentrations were observed compared with the liquid products. The β-lactoglobulin concentration (48-58%) in the samples was higher than that of α-lactoalbumin (13-19%).[6,7]

The concentration of each protein fraction in the samples after microparticulation decreased because of protein denaturation.[22] Differences in the sample concentrations before microparticulation can be attributed to the raw materials used. However, the difference in the sample protein concentrations after microparticulation may have resulted from variations in the methods employed, as the collaborating industry did not provide these details.

The denaturation percentage was calculated using the values obtained for each protein fraction. In general, β-lactoglobulin showed a higher denaturation percentage than α-lactoalbumin, except in the WPC45%-1 sample. The denaturation values for α-lactoalbumin ranged from 5.24 to 56.20% and for β-lactoglobulin from 1.44 to 87.50%. This significant variation in the denaturation percentage may be related to microparticulation process factors, such as pH, protein and salt concentrations, heat treatment, and shear rate.[23] Therefore, our development of an analytical method to quantify α-lactoalbumin and β-lactoglobulin can contribute as a guide to the microparticulation process.

CONCLUSIONS

We developed and validated a methodology for quantifying α-lactoalbumin and β-lactoglobulin in commercial and laboratory-produced microparticulated WPC samples. Statistical analyses

Table 5. Concentration of protein fractions before and after microparticulation and percentage of additional protein denaturation from processing in samples calculated by Equation 3

Form	Sample	β-Lactoglobulin			α-Lactoglobulin		
		WPC / (g 100 g^{-1})	MWP / (g 100 g^{-1})	Protein additional denaturation / %	WPC / (g 100 g^{-1})	MWP / (g 100 g^{-1})	Protein additional denaturation / %
Solution	WPC45%-1	2.51 ± 0.01	0.72 ± 0.01	71.50	0.77 ± 0.02	0.38 ± 0.01	50.74
	WPC45%-2	3.96 ± 0.20	1.89 ± 0.02	52.31	0.76 ± 0.02	0.52 ± 0.01	31.35
	WPC60%-1	8.52 ± 0.33	1.06 ± 0.02	87.60	1.08 ± 0.02	0.65 ± 0.03	40.06
	WPC60%-2	10.02 ± 0.27	1.48 ± 0.03	85.19	1.26 ± 0.02	1.02 ± 0.09	18.48
	WPC80%-1	5.97 ± 0.01	1.19 ± 0.03	80.11	1.67 ± 0.12	0.87 ± 0.04	47.59
Powder	WPC45%-1	19.66 ± 0.14	8.10 ± 0.27	58.80	5.57 ± 0.11	4.59 ± 0.15	17.63
	WPC45%-2	19.66 ± 0.14	5.81 ± 0.14	70.47	5.57 ± 0.11	3.68 ± 0.07	33.97
	WPC45%-3	21.58 ± 0.49	8.27 ± 1.22	61.66	3.87 ± 0.12	3.67 ± 0.13	5.24
	WPC45%-4	21.58 ± 0.49	7.03 ± 0.25	67.41	3.87 ± 0.12	3.12 ± 0.13	19.35
	WPC45%-5	23.37 ± 0.34	5.46 ± 0.27	76.65	4.92 ± 0.31	2.28 ± 0.16	53.66
	WPC45%-6	21.39 ± 0.35	4.70 ± 0.30	78.02	4.26 ± 0.18	2.19 ± 0.25	48.63
	WPC45%-7	21.39 ± 0.35	4.68 ± 0.06	78.13	4.26 ± 0.18	2.27 ± 0.01	46.55
	WPC45%-8	24.58 ± 3.74	24.22 ± 1.79	1.44	5.34 ± 0.43	4.85 ± 0.08	9.02
	WPC60%-1	27.80 ± 2.66	6.03 ± 0.34	78.31	8.23 ± 0.24	5.24 ± 0.18	36.27
	WPC60%-2	30.76 ± 0.50	5.11 ± 0.30	83.38	7.03 ± 0.65	4.09 ± 0.02	41.89
	WPC60%-3	17.26 ± 1.08	5.77 ± 0.18	66.57	6.13 ± 0.09	4.08 ± 0.09	33.42
	WPC60%-4	21.46 ± 0.30	6.42 ± 0.04	70.07	6.25 ± 0.09	3.68 ± 0.14	41.21
	WPC60%-5	26.98 ± 1.05	9.00 ± 0.66	66.63	7.42 ± 0.19	3.84 ± 0.09	48.32
	WPC80%-1	31.77 ± 0.34	14.28 ± 0.75	55.06	8.69 ± 0.21	5.75 ± 0.03	33.87
	WPC80%-2	31.77 ± 0.34	7.27 ± 0.16	77.11	8.69 ± 0.21	3.81 ± 0.02	56.20

WPC: whey protein concentrates; MWP: microparticulated whey protein. The % of protein additional denaturation is related to the amount of protein that was denatured by the microparticulation process, which can be calculated by Equation 3.

demonstrated that the method was accurate, selective, linear, and precise, with a recovery rate exceeding 90%.

The commercial samples exhibited significant variations in the denaturation percentage during microparticulation, which can be attributed to the microparticulation parameters employed.

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REFERENCES

1. Schuck, P.; Bouhallab, S.; Durupt, D.; Vareille, P.; Humbert, J. P.; Marin, M.; *Lait* **2004**, *84*, 243. [Crossref]
2. Corrochano, A. R.; Ferraretto, A.; Arranz, E.; Stuknytė, M.; Bottani, M.; O'Connor, P. M.; Kelly, P. M.; De Noni, I.; Buckin, V.; Giblin, L.; *Food Chem.* **2019**, *288*, 306. [Crossref]
3. Pal, S.; McKay, J.; Jane, M.; Ho, S. In *Nutrition in the Prevention and Treatment of Abdominal Obesity*; Watson, R. R., ed.; Academic Press: Cambridge, 2019, ch. 20. [Crossref]
4. Tunick, M. H. In *Whey Processing, Functionality and Health Benefits*; Onwulata, C. I.; Huth, P. J., eds.; Wiley-Blackwell: Iowa, 2008, p. 1. [Crossref]
5. Çakir-Fuller, E.; *Food Hydrocolloids* **2015**, *47*, 41. [Crossref]
6. Bonnaillie, L. M.; Tomasula, P. M. In *Whey Processing, Functionality and Health Benefits*; Onwulata, C. I.; Huth, P. J., eds.; Wiley-Blackwell: Iowa, 2008, p. 15. [Crossref]
7. Tavares, G. M. In *Química e Tecnologia do Soro de Leite*; de Carvalho, A. F.; Perrone, I. T.; Stephani, R.; Tavares, G. M.; Renhe, I. R. T., orgs.; Innóvite: Juiz de Fora, 2020, ch. 1.
8. Kerche, F.; Weterings, M.; Beyrer, M.; *Int. Dairy J.* **2016**, *60*, 32. [Crossref]
9. Shewan, H. M.; Stokes, J. R.; *J. Food Eng.* **2013**, *119*, 781. [Crossref]
10. Renard, D.; Lavenant, L.; Sanchez, C.; Hemar, Y.; Horne, D.; *Colloids Surf., B* **2002**, *24*, 73. [Crossref]
11. Sun, C.; Liu, R.; Wu, T.; Liang, B.; Shi, C.; Zhang, M.; *Food Sci. Biotechnol.* **2015**, *24*, 1637. [Crossref]
12. Dissanayake, M.; Vasiljevic, T.; *J. Dairy Sci.* **2009**, *92*, 1387. [Crossref]
13. Considine, T.; Patel, H. A.; Anema, S. G.; Singh, H.; Creamer, L. K.; *Innovative Food Sci. Emerging Technol.* **2007**, *8*, 1. [Crossref]
14. Chung, C.; Degner, B.; McClements, D. J.; *Food Res. Int.* **2014**, *56*, 136. [Crossref]
15. Sandoval-Castilla, O.; Lobato-Calleros, C.; Aguirre-Mandujano, E.; Vernon-Carter, E. J.; *Int. Dairy J.* **2004**, *14*, 151. [Crossref]
16. Torres, I. C.; Janhøj, T.; Mikkelsen, B. Ø.; Ipsen, R.; *Int. Dairy J.* **2011**, *21*, 645. [Crossref]
17. Muuronen, K.; Partanen, R.; Heidebrecht, H. J.; Kulozik, U.; *Int. Dairy J.* **2021**, *116*, 104959. [Crossref]
18. Zhao, X.; Cheng, M.; Zhang, X.; Li, X.; Chen, D.; Qin, Y.; Wang, J.; Wang, C.; *J. Dairy Sci.* **2020**, *103*, 1289. [Crossref]
19. Buggy, A. K.; McManus, J. J.; Brodkorb, A.; Hogan, S. A.; Fenelon, M. A.; *J. Dairy Sci.* **2018**, *101*, 10819. [Crossref]
20. Agência Nacional de Vigilância Sanitária (ANVISA); Resolução da Diretoria Colegiada (RDC) No. 166, de 24 de julho de 2017, *Dispõe sobre a Validação de Métodos Analíticos e dá Outras Providências*;

Diário Oficial da União (DOU), Brasília, No. 141, de 25/07/2017. [Link] accessed in July 2024
21. European Medicines Agency; *ICH Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis*; Amsterdam, 2022. [Link] accessed in July 2024
22. Sánchez-Obando, J. D.; Cabrera-Trujillo, M. A.; Olivares-Tenorio, M. L.; Klotz, B.; *LWT--Food Sci. Technol.* **2020**, *120*, 108933. [Crossref]
23. Toro-Sierra, J.; Schumann, J.; Kulozik, U.; *Dairy Sci. Technol.* **2013**, *93*, 487. [Crossref]

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