Food Research 7 (2) : 178 - 185 (April 2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Quantitative determination of α_{s1}–casein in goat's milk using reversed-phase high performance liquid chromatography (RP–HPLC)

[1]Simin, S., [1,*]Mohsin, A.Z., [1,2]Sukor, R. and [1]Wan Ibadullah, W.Z.

[1]Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Article history:
Received: 8 July 2022
Received in revised form: 23 August 2022
Accepted: 21 August 2022
Available Online: 4 April 2023

Keywords:
α_{s1}–casein,
Allergenic protein,
Goat's milk,
HPLC

DOI:
https://doi.org/10.26656/fr.2017.7(2).364

Abstract

The chemical, technological and allergy properties of goat's milk are significantly affected by the level of α_{s1}-casein. Despite indications that more than 90% of cow's milk protein allergy (CMPA) children reacted similarly to goat's milk in typical IgE-mediated forms, the availability of goat's milk without α_{s1}-casein appeared to be a potential alternative for patients with cow's milk allergy. In Malaysia, there is a lack of quantitative data on α_{s1}-casein in raw and commercial goat's milk. To address this problem, this study aimed to quantify α_{s1}-casein in goat's milk using reversed-phase high performance liquid chromatography (RP–HPLC). The procedure was developed using a total of twenty samples comprised of commercial and raw goat's milk. For the calibration curve, a linear relationship with $R^2 > 0.999$ was obtained using cow's α-casein standard and peak areas were observed over the concentration range, with a limit of detection (LOD) of 0.025 mg/mL and limit of quantification (LOQ) of 0.075 mg/mL, respectively. The amount of α_{s1}-casein in the twenty samples ranged from 0.08±0.01 to 1.45±0.17 mg/mL. Three samples which were claimed as goat's milk infant formula were not found to contain α_{s1}-casein. Repeatability and reproducibility were satisfactory for both retention times and peak areas. The RSD of peak areas ranged from 0.01 - 8.07% within an analytical day and from 0.61 - 1.77% across analytical days. This study will contribute as a reference method for analysis and surveillance data of α_{s1}-casein, in private or national laboratories, as well as milk and milk product manufacturers.

1. Introduction

Goat's milk industry is growing at a significant rate in the last decade and is considered an important agricultural commodity. From 1961 to 2017, there is a remarkable rising in global goat's milk production by over 27.9% in Asia alone (Miller and Lu, 2019). Despite the lack of officially recorded data in Malaysia about goat's milk production and consumption, in 2013, there were 8,195 heads of dairy goats in Peninsular Malaysia with 50% located in Johor (Shahudin *et al*., 2018). The significant growth in demand for goat's milk among the public is due to its health benefits contributed by the unique features of goat's milk compared to milk from other ruminants (Miller and Lu, 2019).

The casein profile of goat milk differs from that of other ruminants' milk, which is one of its distinguishing characteristics (Lima *et al.,* 2017). This mainly draws down to the concentration of α_{s1}–casein, a casein subunit encoded by the CSN1S1 gene in goat's milk (Mangia *et al.,* 2019). Twenty-three alleles of *CSN1S1* gene were classified into strong, intermediate, weak, and null alleles, producing four expression levels of α_{s1}-casein, i.e., 3.5, 1.1-1.8, 0.45-0.6 and 0 g/L, respectively (Mangia *et al.,* 2019). Previous studies have shown that a small presence of α_{s1}-casein in goat's milk contributed to lower allergenicity as compared to cow's milk (Bellioni-Businco *et al.,* 1999; Bevilacqua *et al.,* 2001; Lara-Villoslada *et al.,* 2005; Ah-Leung *et al.,* 2006; Ballabio *et al.,* 2011; Hodgkinson *et al.*, 2012). From these findings, goat's milk can serve as protein source for hypoallergenic formulas (Clark and Mora-García, 2017). The high variation of α_{s1}-casein in milk is not limited to allergenicity. It was also found to significantly impact the cheese-making process. Talach (2013) reported that a higher concentration of α_{s1}-casein caused lower pH of milk, which provided firmer curd and shorter coagulation process. Furthermore, it is crucial to have the data on the

*Corresponding author.
Email: *aliah_mohsin@upm.edu.my*

eISSN: 2550-2166
Published by Rynnye Lyan Resources

level of α_{s1}–casein as it can be used for breed characterization, diversity, and phylogenetic studies (Caroli *et al.,* 2009).

Due to the variation in α_{s1}-casein concentration, which can vary up to 10-folds, the need for an analytical tool with robust, sensitive, and good working range to measure α_{s1}-casein in goat's milk is vital. Reversed-phase high performance liquid chromatography (RP-HPLC) allows the quantification of the milk proteins (whey and casein) within a single run and without laborious sample preparation (Ostertag *et al.,* 2021). Hence, this study aimed to determine and quantify α_{s1}-casein in goat's milk using RP-HPLC. The data from this study would be valuable for many parties including health practitioners, consumers, and others with an interest in health outcomes especially in Malaysia as a scientific reference.

2. Materials and methods

2.1 Sampling

Sample collection was carried out according to method ISO 707:2008/IDF 50:2008 (ISO, 2008). A total of twenty samples were used for the analysis. Details of the samples are shown in Table 1. Commercial goat's milk refers to goat's milk purchased from commercial online or physical stores that have been pasteurized prior to commercialization. Raw goat's milk refers to goat's milk purchased directly from the goat's farm right after the milking session (without pasteurization). For liquid commercial and raw goat's milk, the samples were transferred to the laboratory in a cooler bag with a temperature of 2-4°C. Upon arrival at the lab, the samples were aliquoted at 500 µL and kept frozen at -20° C until further analysis. Powdered samples were kept at room temperature until further analysis.

Table 1. Details of the 20 goat's milk samples used in the study

Sample Code	Sample Type	Place of sample collection
A	Liquid	Ranau, Sabah
B		Kota Belud, Sabah
C		Putatan, Sabah
D		Kuala Terengganu, Terengganu
E		Sungai Buloh, Selangor
F		Seremban, Negeri Sembilan
G		Serdang, Selangor
H		Johor Bahru, Johor
I		Serdang, Selangor
J		Ipoh, Perak
K	Powder	Physical store, Selangor
L		Physical store, Selangor
M		Physical store, Selangor
N		Physical store, Sabah
O		Physical store, Selangor
P		Physical store, Selangor
Q		Online store, Selangor
R		Online store, Selangor
S		Physical store, Selangor
T	Liquid	Serdang, Selangor

Sample A to S were obtained from commercial sources while sample T was raw milk obtained from a farm in Selangor

2.2 Materials and reagents

HPLC-grade acetonitrile, BisTris buffer, dithiothreitol (DTT), guanidine hydrochloride (GdnHCl), sodium citrate, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical grade. Alpha-casein from cow's milk standard was purchased from Sigma Aldrich (St. Louis, MO, USA). All buffers and solutions were prepared using ultrapure water (18.2 MΩ-cm) from the ELGA water purification system (High Wycombe, UK).

2.3 Milk samples preparation

With modifications, milk samples were prepared according to Bobe *et al.* (1998) and Montalbano *et al.* (2014). A solution containing 0.1 M BisTris buffer (pH 6.8), 6 M GdnHCl, 5.37 mM sodium citrate, and 19.5 mM DTT (pH 7) was added directly to frozen aliquots milk sample in a 1:1 ratio (*v:v*) and allowed to thaw at room temperature (28°C). After thawing, each sample was shaken for 10 s, incubated for 1 hr at room temperature, and centrifuged for 10 mins at 8 000×*g*. The supernatant was diluted at a ratio of 1:3 (*v:v*) with a solution containing 4.5 M GdnHCl and solvent A. All samples were filtered through a 0.22 µm nylon filter.

2.4 Construction of calibration curve

Since there was no commercial standard available for α_{s1}-casein from goat's milk, lyophilized commercial α–casein cow's standard was used as the standard. The standard solution was solubilized in a solution containing 4.5 M GndHCl and solvent A at 0.025, 0.05, 0.10, 0.50, 1.00, and 5.00 mg/mL, filtered and further used for the construction of the calibration curve. The calibration curve was used for protein quantification, where the limit of detection (LOD) and the limit of quantitation (LOQ) were determined by 3:1 and 10:1 signal to noise (S/N), respectively (Shrivastava and Gupta, 2011).

2.5 HPLC analysis

The HPLC system consisted of an Agilent 1200 Series chromatography (Santa Clara, CA, USA) equipped with a quaternary pump (Agilent 1200 Series, G1322A). Two mobile phases were used in the gradient elution system, whereby mobile phase A consisted of 10% acetonitrile and 0.1% TFA (*v/v*) (pH 2), and pH was

https://doi.org/10.26656/fr.2017.7(2).364

adjusted with 0.1 N of HCl. Mobile phase B consisted of 90% of acetonitrile and 1% TFA (*v/v*). α_{s1}-casein were detected at 204 nm variable wavelength with a diode array detector (Agilent 1200 Series, G1311A). The equipment was controlled by Agilent ChemStation software. Separation was performed on a C8 reversed-phase analytical column (Zorbax 300SB-C8, Agilent Technologies) with a silica-based packing (3.5 µm, 300Ă, 4.6 × 150 mm). The sample was injected via an auto-sampler (Agilent 1200 series, G1329A) using an injection loop of 100 µL and an injection volume of 5 µL. Samples were chromatographed in a gradient elution system, according to Bonfatti *et al*. (2008) for 45 mins at a flow rate of 0.5 mL/min.

2.6 Precision

Repeatability and reproducibility were carried out to determine the precision of the method. The repeatability was established by three consecutive extractions of each sample within a day. The reproducibility, known as day-to-day repeatability, was determined by analyzing each sample on different days.

2.7 Statistical analysis

A calibration curve was established using Microsoft Excel Version 1908/2019. The statistical analyses were performed by one-way analysis of variance (ANOVA) using Minitab 18.0 Statistical Software (2017) (State College, PA, USA). Means were compared using the least significant difference (LSD) test at $p<0.05$.

3. Results and discussion

3.1 Sample separation and extraction

Since the commercial standard of α_{s1}-casein was not available as a single protein, this study utilized cow's α-casein standard, which was composed of α_{s1}- and α_{s2}-caseins. A clear separation of α_{s1}-casein and cow's α_{s2}-casein in standard is shown in Figure 1(a). The α_{s2}-casein showed multiple peaks due to the phosphorylated form of α_{s2}-casein (Bordin *et al.,* 2001). At 0.10 mg/mL of α-casein, the peaks of α_{s2}-casein were non-identified, which supported the basis of a 4:1 proportion of α_{s2}-casein in milk (Campbell and Marshall, 2016). Figure 1 (b) shows the calibration curve of peak area versus concentration of α_{s1}-casein with $R^2 > 0.99$. The values of peak area were derived from the chromatographic profiles of the cow's α-casein standard at different concentrations (Figure 1a).

For extraction of α_{s1}-casein from goat's milk, the duration of storage after solubilization of the sample with a solution containing 4.5 M GdnHCl and Solvent A was not discussed in Bobe *et al.* (1998) and Montalbano *et al.* (2014). In this study, no peak was observed after the sample was stored for 1 hr after the addition of that solution which could be contributed to the instability of the milk proteins. To address this, the extraction of α_{s1}-casein where the addition of 4.5 M GdnHCl and Solvent A solution in the samples was done freshly before separating the HPLC column. This modification resulted in an improved resolution of α-casein.

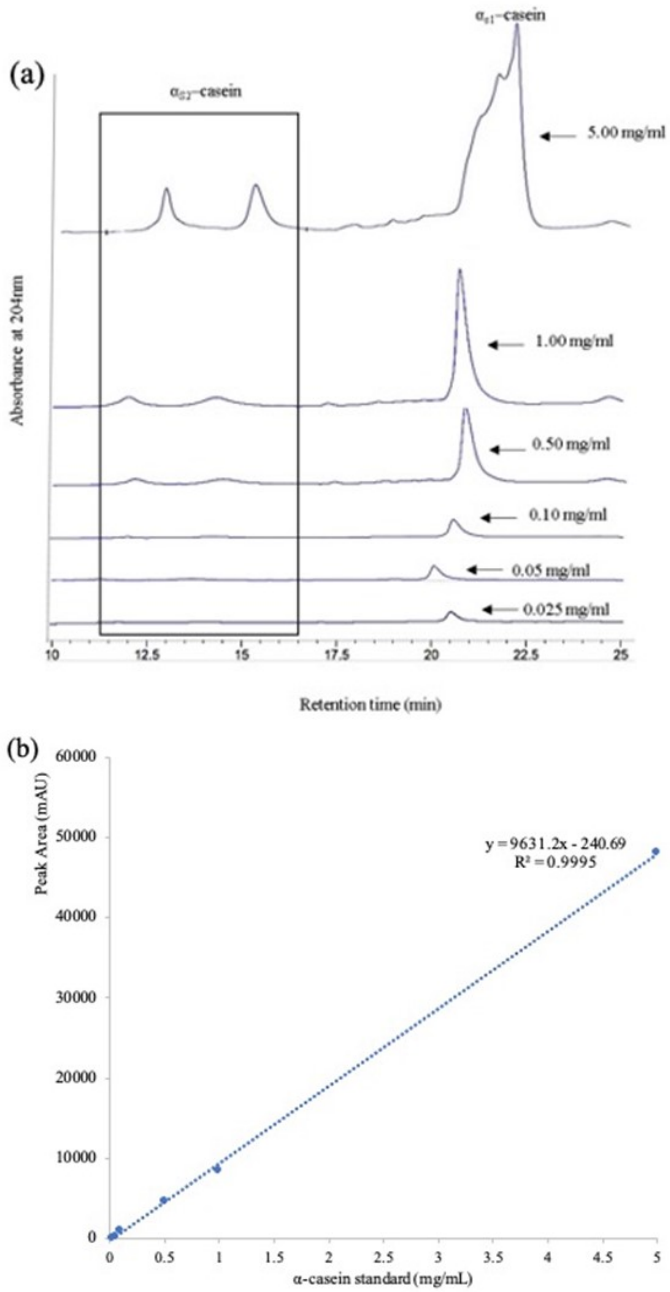

Figure 1. (a) Chromatographic profiles of different concentrations (0.025, 0.05, 0.10, 0.50, 1.00, and 5.00 mg/mL) of cow's α-casein standard for calibration curve; (b) calibration curves of peak area versus concentration of α_{s1}-casein from cow's α-casein standard .

The method was modified because casein exists in milk protein as large, colloidal particles and due to the aggregation nature of the protein, it is necessary to fully dissociate and denature the casein before being subjected to HPLC (Gaspard and Brodkorb, 2019). Pre-treating samples with denaturing or reducing agents can disrupt

https://doi.org/10.26656/fr.2017.7(2).364

the non-covalent interactions and disulfide bonds. In this study, there were few reducing agents used, i.e., guanidine hydrochloride and DTT. However, the amphipathic nature of casein molecules has made casein displays a strong tendency to associate (Melnikova *et al.*, 2019). This can cause a formation of a gel-like structure in α-casein which was shown to be the reversible action. During the gel formation, disulfide bonds were not involved. There is only minimal effect on the intermolecular interaction between α-casein molecules when disulfide bonds were disrupted as it only changes the local electrostatics (Melnikova *et al.,* 2019). Thus, this shows α-casein tends to self-association. Therefore, as storage time increased, α-casein could not be detected, which might be due to the casein being self-associated to form a micelle. A similar condition was reported by Dumpler *et al.* (2017), in which the milk protein was unstable especially when whey protein was dissolved in guanidine buffer and left at room temperature for several hours. This was due to the incomplete denaturation of whey proteins or subsequent refolding at lower guanidine concentrations (Dumpler *et al.,* 2017).

3.2 HPLC analysis

As shown in Figure 2, retention times of α_{s1}-casein from commercial goat's milk of liquid type (sample A), raw goat's milk of liquid type (sample T), and commercial cow's milk of liquid type (sample S) were confirmed with the retention times of the cow's α-casein standard. All peaks were set at a fixed time,±0.47 min (calculation not showed) based on the cow's α-casein standard.

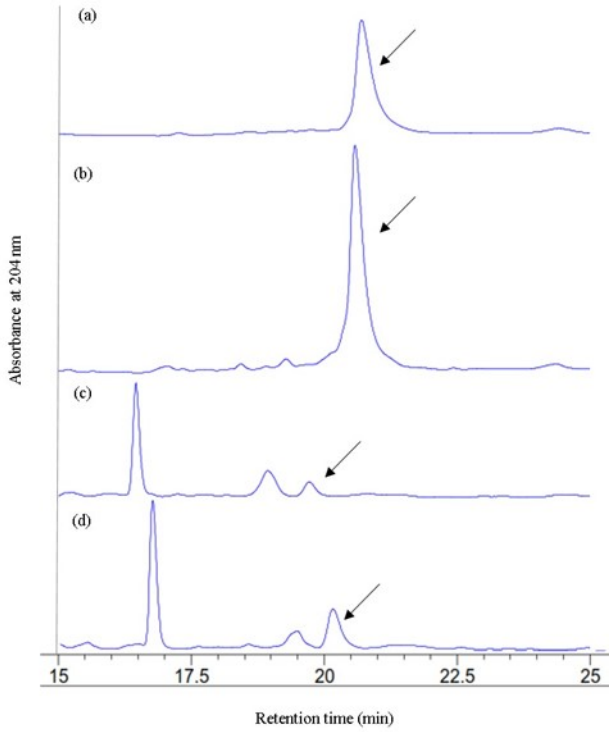

Figure 2. Chromatographic profiles of (a) cow's α-casein standard at 0.5 mg/mL; (b) commercial cow's milk (Sample S); (c) raw goat's milk (Sample T), and (d) commercial goat's milk (Sample A) obtained by HPLC at 204 nm. Peak identified as αs1-casein at 19.84 - 20.78 mins of retention time

In Figure 2, samples A, S, and T had similar retention time with cow's α–casein standard. This result was supported by Montalbano *et al.* (2016) who reported that in homozygous condition, it was possible to identify and observe not only major peaks but some minor peaks in the goat's milk chromatogram. Therefore, cow's standards can be used to identify casein in goat's milk. An in-depth insight into α-casein structure was elaborated by Ingham *et al.* (2018), in which goat's milk had a strong similarity in internal structure with cow's milk according to the resonant soft X-ray scattering (RSoXS) and small-angle X-ray scattering (SAXS). In our study, the detection wavelength was set at 204 nm because it showed the most significant peak during the screening of standard α –casein using a UV/Vis spectrophotometer.

3.3 Quantitative analysis of α_{s1}–casein

Table 2 shows the mean of retention time, area, and calculated concentration of α_{s1}–casein in the twenty samples of goat's milk. The concentrations ranged from 0.08±0.01 mg/mL to 1.45±0.17 mg/mL. In contrast, a total of six samples showed a concentration of α_{s1}–casein lower than the LOQ, while α_{s1}–casein was not detected in three samples. There was a significant variation in the concentration of α_{s1}-casein in all goat's milk samples obtained in this study. Based on the theory discussed earlier, different concentrations of α_{s1}–casein could be correlated with genetic polymorphisms, however, the genotypes were not analyzed in this study (Mangia *et al.,* 2019). A clear vision of this can be observed in samples B and C. Both samples were a mixture of goat's milk from Saanen and Toggenburg breeds but derived from different farms. However, both samples showed significant differences ($p<0.05$) in terms of concentration of α_{s1}–casein. This can be explained further by Mohsin *et al.* (2019), where variation in chemical composition can occur even when similar breed and environmental settings were applied. Other factors that can influence the chemical composition of milk are breed, individuality, stage of lactation, diet, parity, feeding regime, management practices, environment, locality, health and nutritional status of the animal (Sonu and Basavaprabhu, 2020).

The α_{s1}-casein in samples P, Q, and R was considered as not detected because their chromatographic profiles showed no visible peak of α_{s1}-casein (Figure 3). All three samples were commercial goat's milk powder and from the labelling, they were

https://doi.org/10.26656/fr.2017.7(2).364

Table 2. Retention time, peak area, and calculated concentration of α_{s1}-casein in goat's milk samples

Sample	RT (min)	Peak Area (mAU)	Calculated concentration (mg/mL)
A	20.19±0.04	1199.80±17.10	0.15±0.002[f]
B	20.12±0.01	1846.80±36.60	0.22±0.004[e]
C	20.89±0.03	50.14±1.72	<LOQ
D	20.06±0.24	407.00±114.90	0.08±0.012[h]
E	21.03±0.01	257.60±41.50	<LOQ
F	19.90±0.29	435.50±48.90	0.07±0.005[h]
G	19.99±0.87	4702.00±291.00	0.51±0.030[c]
H	20.31±0.01	640.90±5.09	0.10±0.001[g]
I	20.62±0.10	191.15±11.38	<LOQ
J	20.57±0.34	745.30±21.40	0.10±0.002[g]
K	19.92±0.03	208.30±6.08	<LOQ
L	19.96±0.01	3973.00±679.00	0.44±0.071[c]
M	20.32±0.11	51.58±8.32	<LOQ
N	20.54±0.01	3055.00±144.00	0.34±0.015[d]
O	20.70±0.12	13707.00±1598.00	1.45±0.166[a]
P	nd	nd	nd
Q	nd	nd	nd
R	nd	nd	nd
S	20.45±0.184	8731.00±275.00	0.93±0.029[b]
T	20.47±0.584	122.10±23.30	<LOQ

Values are presented as mean±SD of triplicates (n = 3). Values with different superscript within the same column are significantly different (p<0.05). nd: not detected, LOQ: Limit of quantification.

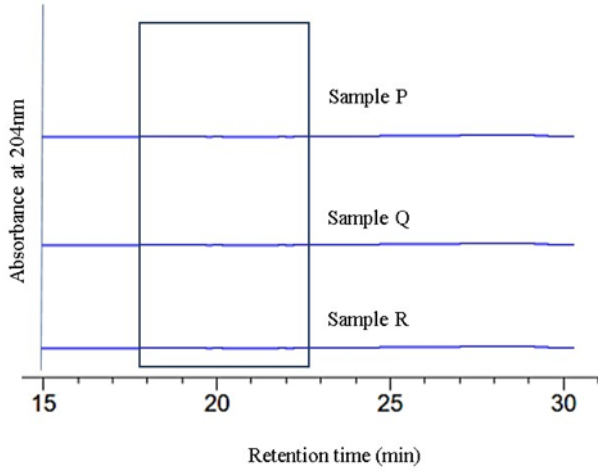

Figure 3. Peak of α_{s1}–casein was not shown in samples P, Q, and R within the range of 20.31±0.47 mins

intended for infant formula. Furthermore, the manufacturer for sample R had claimed that their sample was absent from α_{s1}-casein. Although these samples might be derived from goat's milk with a null variant of α_{s1}-casein, however, to postulate that genetic polymorphism affects the quantification of α_{s1}-casein in these samples was inconclusive. As referred by Geiselhart *et al.* (2021), processing technologies have been applied to prevent and eliminate milk allergies. This correlated to the three samples, in dried powder form and the processing of milk was involved. A few processing technologies which were proven to mitigate allergenicity include thermal processing (pasteurization, sterilization, ultrahigh temperature, spray drying) and nonthermal processing (high pressure, homogenization, ultrasonic, enzymatic hydrolysis, fermentation) (Geiselhart *et al.,* 2021). Due to a lack of information on the type of processing used for the samples as well as the genetic polymorphism of the goat's breed, it is difficult to determine the factors which influenced the concentration of α_{s1}–casein in these samples.

Sample P, Q, and R were powdered goat's milk that has been claimed as infant formula, in which the target market is children who could not tolerate cow's milk. However, substituting cow's milk with goat's milk for children with cow's milk allergy should be avoided without any investigation by the paediatrician as proteins in both types of milk share high homology attributes, which could elicit similar types of allergic reactions. Cross-reactivity of goat's milk in children was investigated by Bellioni-Businco *et al.* (1999), who reported that 24 of the 26 children tested reacted to goat's milk, however, a 5-fold concentration of goat's milk is needed to induce a reaction. Goh *et al*. (2019) also found that it was unusual to have patients with isolated goat's milk allergy without cow's milk allergy. This was further supported by Pham and Wang (2017), who reported that goat's milk and cow's milk had a high protein identity with more than 84%. Hence, cross-reactivity between both milk is expected even though goat's milk may have low levels of α_{s1}–casein.

3.4 Repeatability and reproducibility

https://doi.org/10.26656/fr.2017.7(2).364

Table 3. Precision of retention times and peak areas of α_{s1}–casein from cow's α–casein standard

Repeatability[a]			Reproducibility[b]		
Concentration (mg/mL)	Retention time, RSD (%)	Peak area, RSD (%)	Concentration (mg/mL)	Retention time, RSD (%)	Peak area, RSD (%)
0.10	0.01	0.68	0.05	1.10	12.24
0.50	0.96	8.07	0.50	1.77	15.01
5.00	0.34	5.21	1.00	0.61	1.79

[a] Three aliquots of three consecutive extraction in each sample (n = 9)

[b] Three aliquots of three consecutive extraction in each sample in different days. Concentration of 0.5 mg/mL was used as a daily indicator for reproducibility

Repeatability and reproducibility of HPLC analysis were assessed to determine the method precision (Montalbano *et al.,* 2014). The RSD values for the retention times and peak areas are given in Table 2. Montalbano *et al.* (2014) reported that RSD values for retention times were below 0.22% (repeatability) and 0.60% (reproducibility), while RSD for peak areas were below 0.77% (repeatability) and 5.00% (reproducibility). Based on Table 3, the RSD values for retention times were below 0.96% (repeatability) and 1.77% (reproducibility). The RSD values for peak areas were below 8.07% (repeatability) and 15.01% (reproducibility). The RSD values for both retention times and peak areas showed a higher percentage than Montalbano *et al*. (2014). Pre-column (guard column) conditions might have affected this variation, which was not used in our study. The significant effect of guard column application in the HPLC system was reported by Scott (1992). Nevertheless, injecting 1.00 mg/mL of cow's α–casein standards in a two-days interval assisted in the improvement of reproducibility and quantitative precision. A blank injection was also used between each sample to have a better reproducibility of quantification as described in Montalbano *et al.* (2014). According to Cox *et al.* (2011) and European Medicines Agency (2012), RSD values less than 20% were considered satisfactory for HPLC analysis. Therefore, it can be concluded that the method used in this provides good precision to determine α_{s1}–casein in goat's milk.

4. Conclusion

In the present work, we have quantified α_{s1}–casein in 20 samples of goat's milk with a quantification range from 0.08±0.01 to 1.45±0.17 mg/mL. Three samples of commercial goat's milk infant formula were not found to contain any α_{s1}-casein. Repeatability and reproducibility were satisfactory for both retention times and peak areas with RSD < 20%. HPLC analysis showed good sensitivity, repeatability, and reproducibility for the detection and quantification of α_{s1}-casein in goat's milk. This method can be applied in further studies to quantify α_{s1}-casein in other types of milk with considerations of precaution steps highlighted in this study. Overall, the data on the level of α_{s1}-casein in goat's milk from this study can be used as a scientific reference and would be beneficial for many including testing laboratories, health practitioners and milk product manufacturers. More importantly, the general consumer can have a better understanding and awareness of the incidence and level of α_{s1}-casein in raw and commercial goat's milk for better management of allergenicity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

The research was funded by Universiti Putra Malaysia under Putra Grants (GP/2020/9693800 and GP-IPS/2016/9507900).

References

Ah-Leung, S., Bernard, H., Bidat, E., Paty, E., Rance, F., Scheinmann, P. and Wal, J.M. (2006). Allergy to goat and sheep milk without allergy to cow's milk. *Allergy,* 61(11), 1358-1365. https://doi.org/ 10.1111/ j.1398-9995.2006.01193.x

Ballabio, C., Chessa, S., Rignanese, D., Gigliotti, C., Pagnacco, G., Terracciano, L., Fiocchi, A., Restani, P. and Caroli, A.M. (2011). Goat milk allergenicity as a function of α_{S1}-casein genetic polymorphism. *Journal of Dairy Science,* 94(2), 998-1004. https:// doi.org/10.3168/JDS.2010-3545

Bellioni-Businco, B., Paganelli, R., Lucenti, P., Giampietro, P.G., Perbornc, H. and Businco, L. (1999). Allergenicity of goat's milk in children with cow's milk allergy. *Journal of Allergy and Clinical Immunology,* 103(6), 1191-1194. https:// doi.org/10.1016/S0091-6749(99)70198-3

Bevilacqua, C., Martin, P., Candalh, C., Fauquant, J., Piot, M., Roucayrol, A.M., Pilla, F. and Heyman, M. (2001). Goats' milk of defective α_{s1}-casein genotype decreases intestinal and systemic sensitization to β-lactoglobulin in guinea pigs. *Journal of Dairy Research*, 68(2), 217-227. https://doi.org/ 10.1017/ S0022029901004861

https://doi.org/10.26656/fr.2017.7(2).364

Bobe, G., Beitz, D.C., Freeman, A.E. and Lindberg, G.L. (1998). Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry,* 46(2), 458-463. https://doi.org/10.1021/jf970499p

Bonfatti, V., Grigoletto, L., Cecchinato, A., Gallo, L. and Carnier, P. (2008). Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. *Journal of Chromatography A*, 1195(1–2), 101-106. https://doi.org/10.1016/j.chroma.2008.04.075

Bordin, G., Cordeiro-Raposo, F., de la Calle, B. and Rodriguez, A.R. (2001). Identification and quantification of major bovine milk proteins by liquid chromatography. *Journal of Chromatography A*, 928(1), 63-76. https://doi.org/10.1016/S0021-9673(01)01097-4

Campbell, J.R. and Marshall, R.T. (2016). Dairy Production and Processing: The Science of Milk and Milk Products. Long Grove, Illinois, USA: Waveland Press.

Clark, S. and Mora-García, M.B. (2017). A 100-year review: Advances in goat milk research. *Journal of Dairy Science*, 100(12), 10026-10044. https://doi.org/10.3168/jds.2017-13287.

Caroli, A.M., Chessa, S. and Erhardt, G.J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science,* 92(11), 5335–5352. https://doi.org/ 10.3168/JDS.2009-2461

Cox, K.L., Devanarayan, V., Kriauciunas, A., Manetta, J., Montrose, C. and Sittampalam, S. (2011). Immunoassay methods. In Sittampalam, G.S., Grossman, A., Brimacome, K. (Eds.). Assay Guidance Mannual, p. 1-38. Indianapolis, USA: Eli Lilly and Company; National Center for Advancing Translational Sciences.

Dumpler, J., Wohlschläger, H. and Kulozik, U. (2017). Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection. *Dairy Science and Technology,* 96 (6), 807–826. https://doi.org/10.1007/s13594-016-0304-3

European Medicines Agency (2012). Guideline on Bioanalytical Method Validation. Retrieved on January 31, 2021 from Europa website: www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf

Gaspard, S.J. and Brodkorb, A. (2019). The use of high performance liquid chromatography for the characterization of the unfolding and aggregation of dairy proteins. In Protein Self-Assembly, p. 103-115. Totowa, New Jersey, USA: Humana Press.

Geiselhart, S., Podzhilkova, A. and Hoffmann-Sommergruber, K. (2021). Cow's milk processing—friend or foe in food allergy? *Foods*, 10(3), 572-589. doi.org/10.3390/foods10030572

Goh, S.H., Chong, K.W., Loh, W. and Goh, A.E.N. (2019). Goat's milk anaphylaxis in a cow's milk tolerant child. *Asia Pacific Allergy*, 9(4), 1-3. https://doi.org/ 10.5415/apallergy.2019.9.e34

Hodgkinson, A.J., McDonald, N.A., Kivits, L.J., Hurford, D.R., Fahey, S. and Prosser, C. (2012). Allergic responses induced by goat milk α_{S1}-casein in a murine model of gastrointestinal atopy. *Journal of Dairy Science*, 95(1), 83-90. https://doi.org/10.3168/jds.2011-4829

Ingham, B., Smialowska, A., Kirby, N.M., Wang, C. and Carr, A.J. (2018). A structural comparison of casein micelles in cow, goat and sheep milk using X-ray scattering. *Soft Matter*, 14(17), 3336-3343. https://doi.org/10.1039/c8sm00458g

ISO (International Standards of Organization) (2008). Milk and milk products-Guidance on sampling (ISO 707:2008; IDF 50:2008). Switzerland: ISO.

Lara-Villoslada, F., Olivares, M. and Xaus, J. (2005). The balance between caseins and whey proteins in cow's milk determines its allergenicity. *Journal of Dairy Science*, 88(5), 1654-1660. https://doi.org/10.3168/jds.S0022-0302(05)72837-X

Lima, M.J.R., Teixeira-Lemos, E., Oliveira, J., Teixeira-Lemos, L.P., Monteiro, A. and Costa, J.M. (2018). Nutritional and health profile of goat products: focus on health benefits of goat milk. In Kukovics, S. (Ed). Goat Science, p. 189-232. Intech Open E-Book. https://doi.org/10.5772/intechopen.70321

Mangia, N.P., Saliba, L., Zoumpopoulou, G., Chessa, S., Anastasiou, R., Karayiannis, J., Sgouras, D., Tsakalidou, E. and Nudda, A. (2019). Goat Milk with different Alpha-s1 casein genotype (CSN1S1) fermented by selected *Lactobacillus paracasei* as potential functional food. *Fermentation,* 5(3), 55. https://doi.org/10.3390/fermentation5030055

Melnikova, D.L., Skirda, V.D. and Nesmelova, I.V. (2019). Effect of reducing agent tcep on translational diffusion and supramolecular assembly in aqueous solutions of α-casein. *Journal of Physical Chemistry B*, 123(10), 2305-2315. https://doi.org/10.1021/acs.jpcb.8b12511

Miller, B.A. and Lu, C.D. (2019). Current status of global dairy goat production: An overview. *Asian-*

Australasian Journal of Animal Science, 32(8), 1219 -1232. https://doi.org/10.5713/ajas.19.0253

Mohsin, A.Z., Sukor, R., Selamat, J., Shobirin, A., Hussin, M. and Ismail, I.H. (2019). Chemical and mineral composition of raw goat milk as affected by breed varieties available in Malaysia. *International Journal of Food Properties,* 22(1), 815-824. https://doi.org/10.1080/10942912.2019.1610431

Montalbano, M., Segreto, R., Gerlando, R. Di, Mastrangelo, S. and Sardina, M.T. (2016). Quantitative determination of casein genetic variants in goat milk: Application in Girgentana dairy goat breed. *Food Chemistry*, 192, 760-764. https://doi.org/10.1016/j.foodchem.2015.07.075

Montalbano, M., Tortorici, L., Mastrangelo, S., Sardina, M.T. and Portolano, B. (2014). Development and validation of RP-HPLC method for the quantitative estimation of α_{s1}-genetic variants in goat milk. *Food Chemistry,* 156, 165-169. https://doi.org/10.1016/j.foodchem.2014.01.091

Ostertag, F., Schmidt, C.M., Berensmeier, S. and Hinrichs, J. (2021). Development and validation of an RP-HPLC DAD method for the simultaneous quantification of minor and major whey proteins. *Food Chemistry*, 342, 128176. https://doi.org/10.1016/j.foodchem.2020.128176

Pham, M.N. and Wang, J. (2017). Mammalian milk allergy: case presentation and review of prevalence, diagnosis, and treatment. *Annals of Allergy, Asthma and Immunology,* 118(4), 406-410. https://doi.org/10.1016/j.anai.2017.02.018

Scott, K.J. (1992). Observations on some of the problems associated with the analysis of carotenoids in foods by HPLC. *Food Chemistry,* 45(5), 357-364.

Shahudin, M.S., Ghani, A.A.A., Zamri-Saad, M., Zuki, A.B., Abdullah, F.F.J., Wahid, H. and Hassim, H.A. (2018). The necessity of a herd health management programme for dairy goat farms in Malaysia. *Pertanika Journal of Tropical Agricultural Science,* 41(1), 1-18.

Shrivastava, A. and Gupta, V.B. (2011). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientist*, 2(1), 21-25. https://doi.org/10.4103/2229-5186.79345

Sonu, K.S. and Basavaprabhu, H.N. (2020). Compositional and therapeutic signatures of goat milk: A review. *International Journal of Chemical Studies*, 8(2), 1013-1019. https://doi.org/10.22271/chemi.2020.v8.i2p.8902

Talach, A. (2013). Determination of relation between α_{S1} casein concentration and coagulation properties of goat milk. Sweden: Swedish University of Agricultural Sciences, MSc Thesis.

