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ORIGINAL RESEARCH

A distinction of cow and buffalo ghee using principal component analysis of triglyceride composition

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Triglyceride (TG) profiling was explored to distinguish cow ghee from buffalo ghee to check their admixing. Cow and buffalo butter was clarified at 110, 130 and 150 °C to obtain ghee. Temperature of clarification did not show any significant effect on TG profile. Cow ghee showed maxima at TG C38 and C52. Buffalo ghee exhibited maxima at C38 and C50 unlike cow ghee. Cow ghee samples showed a higher TG content of C42 to C54, whereas buffalo ghee samples were associated with a greater content of C26 to C38. Principal component analysis (PCA) and hierarchical clustering showed two ghee types as distinct separate clusters.

Keywords Anhydrous milk fat, Ghee, Cow, Buffalo, Triglycerides.

INTRODUCTION

Ghee (heat clarified butter or anhydrous milk fat) has been used in India since 1500 BC (Achaya 1997). It is prepared by clarifying cream/butter at 110–130 °C, wherein ghee is obtained either from cream or from butter. Ghee differs slightly from butter oil in flavour on account of its preparation at a higher temperature of clarification. It is a very popular dairy product in the South Asian region (India, Bhutan, Sri Lanka and Nepal) and is the second largest dairy product (~28%) consumed in India (GAIN 2014). Ghee is also widely produced and consumed in Sudan, Ethiopia and the Middle East (Antony *et al*. 2018). A recent report suggested that the American continent, that is USA, Argentina and Paraguay, have increased the production of cow ghee to between 3000 and 12 000 tons per year (Pena-Serna and Restrepo-Betancur 2019). About 170 thousand metric tons of ghee was produced in India in the fiscal year 2020 (Jagmohan 2020). It has been suggested that the combined butter and ghee production in India will rise to 6.1 MMT against 5.8 MMT last year, indicating a strong consumption demand (GAIN 2020). The Indian ghee market reached a value of Indian rupees 2273 billion in 2019 and is expected to reach a value of Indian rupees 4653 billion by 2024 (IMARC 2020). India exported 94 000 tonnes of dairy products in 2018, valued at nearly US$290 million. Butter and other dairy fats (including ghee) make up the majority of exports, accounting for 65% in volume terms (Anon 2019).

The two most common types of ghee sold in the Indian subcontinent, are cow ghee and ghee. Cow ghee is prepared from cow cream or butter, whereas ghee is either prepared from buffalo cream or butter or from mixed cream or butter which is obtained from mixture of both cow and buffalo milk. These days cow ghee has gained more popularity and is being sold at a premium. However, the importance of buffalo milk cannot be over looked. Department of Animal Husbandry and Dairying (DAHD 2019) reported that an estimated 49 per cent of India's milk production originated from water buffalo. The demand for ghee from either cow or buffalo milk is still robust. As stated above, the demand of cow ghee is increasing, so incidences of buffalo ghee admixing with cow ghee have been reported. Hence, the industry is searching for some simple means to distinguish cow ghee from buffalo ghee. It has been reported that TG content varies among different species and also in different breeds of the same animal species (Fontecha *et al*. 1998). Goat milk fat did not show a bimodal distribution of triglycerides

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(TG) unlike cow milk fat, and maximum values were reported for TG having 38 and 40 carbons, that is C38 and C40 (Tolentino *et al*. 2015). In both cow and buffalo ghee, six TG having carbon numbers C36, C38, C40, C42, C50 and C52 were predominant which represented about sixty per cent of the total TG present in both cow and buffalo ghee (Hazra *et al*. 2017). Thus, the present investigation was carried out to evaluate the TG composition of cow and buffalo ghee to see the differences in TG fingerprints (ISO 2019). Further, the possibility to distinguish them by using chemometric tools, that is principal component analysis (PCA) and hierarchical clustering was investigated.

MATERIALS AND METHODS

Preparation of pure ghee samples

Milk samples of cows and buffaloes maintained under identical conditions of feeding and management in the Livestock Research Centre of the Institute were used for obtaining the cream. The forage/fodder given to each breed throughout year consisted of berseem, sorghum, Cabbage, makchari kabri and turnip in the winter season (November to February); berseem, oat, lucerne and wheat straw in summer season (April to May); and maize and sorghum in rainy season (June-July). In addition to these in June to August silage of Maize, Jowar and oats, while from November to March oat hay was also given to lactating cattle. The concentrate mixture of hay and silage comprised of mainly maize (>30%), barley, wheat, or oat, groundnut cake (22%), mustard cake (11%), wheat bran (25%), rice bran deoiled (8%), minerals mixture (2%) and common salt (1%). Pooled cow milk received in the experimental dairy of ICAR – National Dairy Research Institute, Karnal, was obtained from the herd of Karan Swiss, Karan Fries, Sahiwal and Tharparkar breeds on a bimonthly basis. The obtained milk was then separated to cream and skimmed milk by using a power-operated cream separator. Total eight samples of the cow cream were obtained during the study.

Pooled Murrah buffalo milk was collected from the Livestock Research Centre of the Institute on a bimonthly basis. This resulted into six samples. Cream was separated from the buffalo milk in the laboratory by using a laboratory-scale cream separator. Cream samples (cow and buffalo) thus obtained were pasteurised at 77 °C for 5 min and cooled to room temperature 28–29 °C. Ageing of the pasteurised cream was done at 7 °C for 3–5 h. The aged cream was churned into butter using hand-operated butter churn. Butter was then converted to ghee by heat clarification (De Sukumar 2019) at three different temperatures (110 °C, 130 °C and 150 °C). The prepared ghee samples were filtered through a muslin cloth (6–8 folds) followed by further filtration using Whatman No. 4 filter paper and were stored at refrigerated temperature (4–5 °C) until analysis.

Triglyceride mix, tristearin, cholesterol and anhydrous milk fat standards

(i) Standard TG mix (CRM18811) consisting of tricaprylin, tricaprin,trilaurin, trimyristin and tripalmitin; (ii) standard anhydrous milk fat (BCR-519); (iii) cholesterol; and (iv) standard tristearin were procured from Sigma-Aldrich Co, 3050 Spruce Street (St Louis, MO 63103, USA 314-771-5765). These standards were used to calibrate the Gas-Liquid Chromatography (GLC) conditions.

Standardisation of GLC conditions

GLC was calibrated as per the method specified by the International Organization for Standardization (ISO) for TG analysis of cow milk fat (ISO 2010). Shimadzu 2010 plus machine (Kyoto, Japan), with GC solution software, was used. Carrier gas used was nitrogen, and column was CP- SimDist Ultimetal CP7532 column [5.0 m (L) × 0.53 mm (ID) × 0.80 mm (OD), film thickness 0.17 μm] (Agilent Technologies. Inc, USA). Other conditions like carrier gas flow, oven temperature, detector and injector temperatures were as specified in the method (ISO 2010).

TG standard mix (5 TG mix- 100 mg neat mixture of 99% pure 20% each of tricaprylin, tricaprin, trilaurin, trimyristin and tripalmitin along with equal proportion, that is 20 mg each of cholesterol and tristearin were dissolved in 10 mL hexane and 0.5 μL was injected to the gas chromatograph.

Standard anhydrous milk fat (BCR-519)/ghee sample (1% volume fraction in hexane) was prepared, and 0.5 μL was injected.

The retention times and response factor (f_i) of these triglycerides (TGs) were determined as per the equation given below.

$$f_i = \frac{w_i \sum A_i}{\sum w_i A_i}$$

where w_i is the mass fraction, expressed as a percentage, of each TG or cholesterol in the standardised milk fat. A_i is the numerical value of the peak area of each TG or cholesterol in the standardised milk fat.

GLC analysis of ghee to determine TGs

Molten ghee sample 10 mL was passed through 0.5–1 g sodium sulphate to remove traces of moisture. 0.5 mL of the above said sample was transferred to 50 mL volumetric flask, and volume was made up by hexane to get a final concentration of 1% volume fraction sample solution. Contents of the flask were mixed gently for 1 min to have a uniform sample. To determine TGs, separated by total carbon numbers, 0.5 μL of the 1% volume fraction sample solution was injected into the gas chromatograph and GLC was operated using standardised conditions.

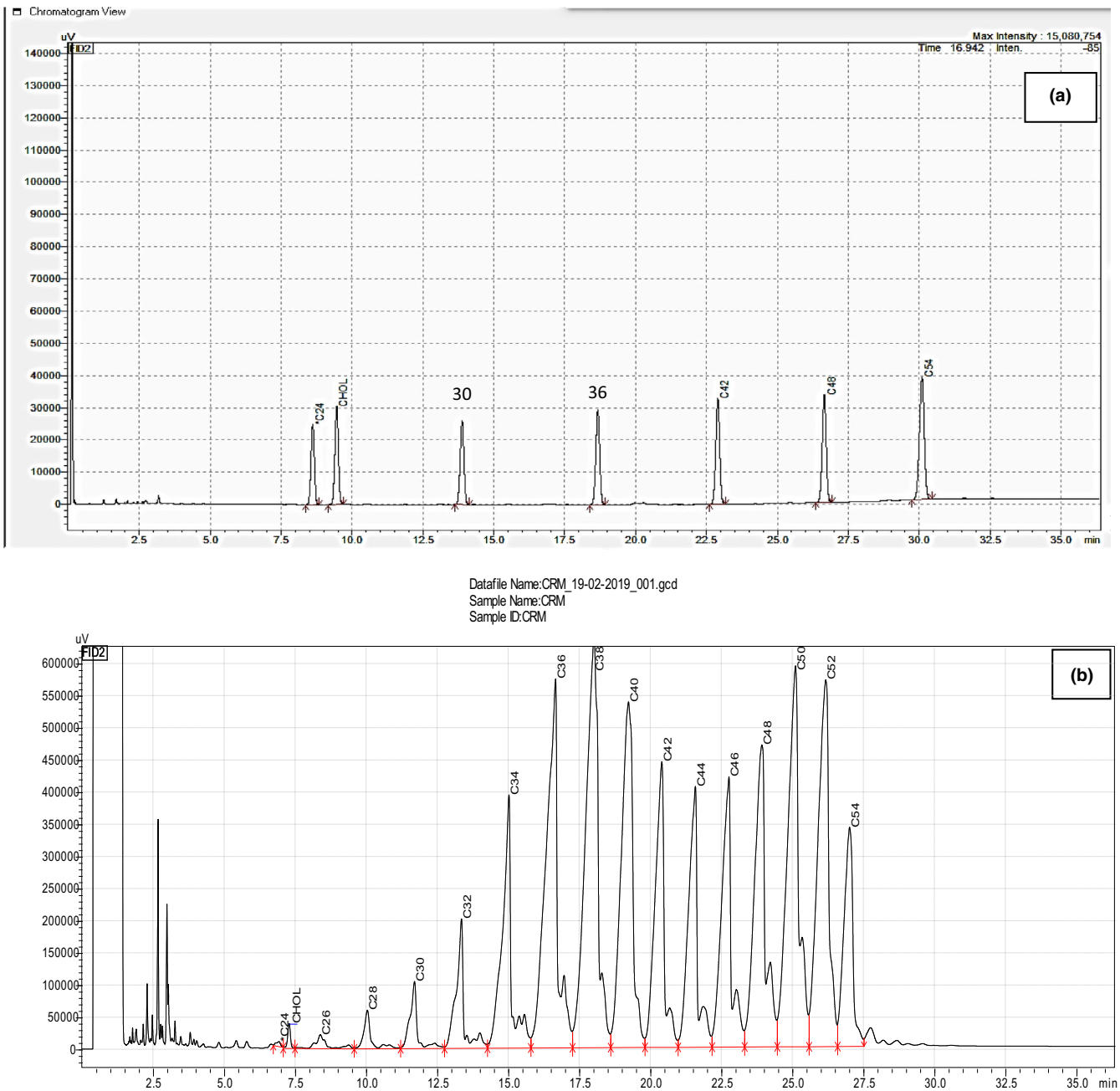

Figure 1 Chromatograms of (a) Triglyceride mix and (b) Anhydrous milk fat certified reference material (BCR-519).

Statistical analysis

To evaluate the effect of temperature of clarification on TGs, data related to individual TGs were subjected to two-way repeated measure analysis of variance (RM-ANOVA) for evaluating the significant difference of either of the factors and their interaction at 95% confidence interval with GraphPad Prism software (version 5.01 for windows). To ascertain the differences in cow and buffalo ghee, data pertaining to TG irrespective of clarification temperature were pooled and subjected to one-way ANOVA. Principal component analysis (PCA) and hierarchical clustering of TG data were carried out using software JMP version 10.0 from SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, USA.

RESULTS AND DISCUSSION

Standardisation of GLC conditions

Conditions of analysis using GLC were standardised as per the requirements of the ISO methodology that baseline drift

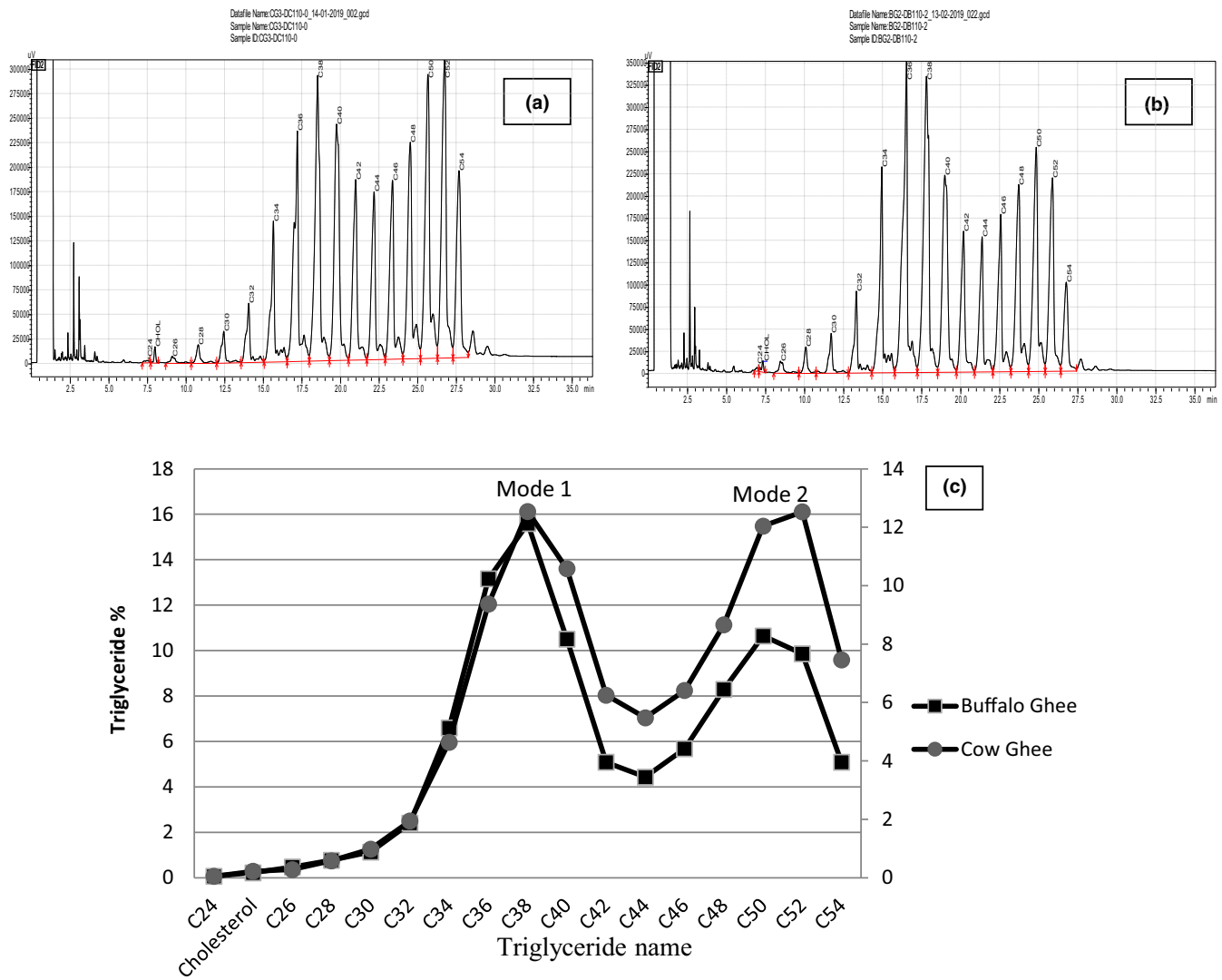

Figure 2 Chromatograms of (a) Cow ghee, (b) Buffalo ghee and (c) Bimodal profile of triglycerides of cow and buffalo ghee.

should be minimum, no splitting of peaks, and response factors close to 1.0 and not higher than 1.250. It is evident from the chromatograms (Figure 1a) that all the standard TGs in the standard mix (CRM18811) including cholesterol and tristearin separated distinctly and drift in the base line were also negligible. Similarly, in case of standard anhydrous milk fat (BCR-519) the baseline was stable and the peaks were also easily distinguishable without any splitting (Figure 1b). Similar observations were recorded from the chromatograms of both pure cow and buffalo ghee (Figure 2a,b). Response factors calculated using standard anhydrous milk fat (BCR-519) were also in the range of 0.92–1.1 for different TGs having carbon numbers C24 - C54. These results demonstrated that the conditions of GLC analysis were perfect, resulting in accurate TG analysis of ghee samples.

Effect of temperature of clarification on TG profile of cow and buffalo ghee

Butter was heat clarified to ghee at 110, 130 and 150 °C to evaluate the variation in TG composition of cow and buffalo ghee separately with temperature. It is evident from the data that there was no significant difference in the content of different TGs on account of temperature of clarification both in the cow and buffalo ghee samples (Table 1). This finding can be extrapolated to the fact that temperature of clarification used to prepare ghee from the butter will not affect the *S*- limits (ISO 2019) generally used to evaluate the quality of milk fat.

Difference in TG profile of cow and buffalo ghee

It is evident from the preceding discussion that heat of clarification did not alter the TG composition; hence to elucidate

Table 1 Effect of heat of clarification on the triglyceride (%w/w) profile of cow ghee and buffalo ghee.

	Cow ghee			*Buffalo ghee*		
	Clarification temperature (°C)					
Triglycerides with carbon numbers	*110 °C*	*130 °C*	*150 °C*	*110 °C*	*130 °C*	*150 °C*
C24	0.05 ± 0.02[a]	0.05 ± 0.02[a]	0.05 ± 0.01[a]	0.07 ± 0.03[a]	0.07 ± 0.02[a]	0.07 ± 0.02[a]
C26	0.26 ± 0.03[a]	0.26 ± 0.03[a]	0.27 ± 0.03[a]	0.47 ± 0.05[a]	0.50 ± 0.04[a]	0.46 ± 0.06[a]
C28	0.55 ± 0.05[a]	0.57 ± 0.03[a]	0.57 ± 0.03[a]	0.77 ± 0.08[a]	0.78 ± 0.09[a]	0.79 ± 0.08[a]
C30	0.95 ± 0.07[a]	0.94 ± 0.06[a]	0.95 ± 0.06[a]	1.12 ± 0.19[a]	1.11 ± 0.22[a]	1.12 ± 0.20[a]
C32	1.85 ± 0.14[a]	1.88 ± 0.12[a]	1.88 ± 0.13[a]	2.39 ± 0.39[a]	2.41 ± 0.39[a]	2.37 ± 0.43[a]
C34	4.48 ± 0.16[a]	4.50 ± 0.14[a]	4.50 ± 0.15[a]	6.53 ± 0.83[a]	6.58 ± 0.86[a]	6.53 ± 0.89[a]
C36	9.26 ± 0.04[a]	9.30 ± 0.09[a]	9.31 ± 0.10[a]	13.01 ± 0.94[a]	13.12 ± 1.09[a]	13.09 ± 1.04[a]
C38	12.58 ± 0.13[a]	12.64 ± 0.22[a]	12.64 ± 0.21[a]	15.48 ± 0.20[a]	15.55 ± 0.34[a]	15.59 ± 0.23[a]
C40	10.60 ± 0.21[a]	10.66 ± 0.23[a]	10.66 ± 0.23[a]	10.49 ± 0.20[a]	10.49 ± 0.17[a]	10.54 ± 0.16[a]
C42	6.11 ± 0.31[a]	6.16 ± 0.34[a]	6.16 ± 0.34[a]	5.06 ± 0.48[a]	5.04 ± 0.40[a]	5.05 ± 0.48[a]
C44	5.31 ± 0.31[a]	5.35 ± 0.36[a]	5.35 ± 0.35[a]	4.42 ± 0.59[a]	4.41 ± 0.49[a]	4.39 ± 0.57[a]
C46	6.29 ± 0.22[a]	6.29 ± 0.28[a]	6.29 ± 0.27[a]	5.68 ± 0.54[a]	5.70 ± 0.41[a]	5.64 ± 0.51[a]
C48	8.61 ± 0.17[a]	8.57 ± 0.17[a]	8.58 ± 0.17[a]	8.32 ± 0.17[a]	8.35 ± 0.06[a]	8.26 ± 0.14[a]
C50	12.22 ± 0.40[a]	12.15 ± 0.33[a]	12.15 ± 0.34[a]	10.71 ± 0.76[a]	10.69 ± 0.80[a]	10.63 ± 0.78[a]
C52	12.94 ± 0.60[a]	12.85 ± 0.57[a]	12.84 ± 0.56[a]	10.02 ± 1.74[a]	9.91 ± 1.75[a]	9.96 ± 1.82[a]
C54	7.73 ± 0.40[a]	7.61 ± 0.31[a]	7.60 ± 0.32[a]	5.24 ± 1.72[a]	5.08 ± 1.68[a]	5.28 ± 1.86[a]

Data are represented as Mean ± SD ($n = 8$) in cow ghee and Mean ± SD ($n = 6$) in buffalo ghee; Mean within rows with different superscripts are significantly different ($P < 0.05$) from each other.

the difference in cow and buffalo ghee in terms of different TG, the data for TG profile irrespective of clarification temperature were pooled separately for cow and buffalo ghee samples and analysed statistically for any significant variation in TGs with different carbon numbers. It was evident from the data (Table 2) that TGs having carbon numbers 24, 26, 28, 30, 32, 34, 36 and 38 were significantly higher ($P < 0.01$) in buffalo ghee than cow ghee, whereas TGs having carbon numbers (C) 42, 44, 46, 48, 50, 52 and 54 were significantly higher ($P < 0.01$) in cow ghee. The TG profile of cow and buffalo ghee showed a bimodal behaviour (Figure 2c). In the case of cow ghee, the first mode appeared in C38 and the second in C52. However, in case of buffalo ghee first maxima appeared in C38 and the second maxima was in C50 instead of C52, unlike cow ghee. These findings were in accordance with the earlier findings wherein bimodal behaviour of cow milk fat having two clear maxima located at TG C38 and C52 and unimodal in goat milk fat having one maxima at TG C42 was reported (Fontecha *et al*. 1998; Tolentino *et al*. 2015). Findings akin to the present investigation that cow ghee contained a higher amount of TG C42 to C54 and buffalo ghee had more C26 to C36 were also reported earlier (Amrutha Kala 2013). However, based on simple descriptive statistics it was not possible to distinguish cow ghee from buffalo ghee. Therefore, to ascertain the possibility of determination of similarities and differences in TG content of cow and

Table 2 Triglyceride (%w/w) profile of cow and buffalo ghee.

	Ghee type	
TG	*Cow ghee*	*Buffalo ghee*
C24	0.05 ± 0.00[a]	0.08 ± 0.01[b]
Cholesterol	0.22 ± 0.00[a]	0.23 ± 0.01[a]
C26	0.26 ± 0.01[a]	0.49 ± 0.01[b]
C28	0.56 ± 0.01[a]	0.80 ± 0.02[b]
C30	0.95 ± 0.01[a]	1.17 ± 0.06[b]
C32	1.87 ± 0.03[a]	2.51 ± 0.12[b]
C34	4.49 ± 0.03[a]	6.81 ± 0.26[b]
C36	9.29 ± 0.02[a]	13.38 ± 0.31[b]
C38	12.62 ± 0.04[a]	15.62 ± 0.08[b]
C40	10.64 ± 0.04[a]	10.47 ± 0.05[b]
C42	6.14 ± 0.07[a]	5.19 ± 0.14[b]
C44	5.34 ± 0.07[a]	4.58 ± 0.17[b]
C46	6.29 ± 0.05[a]	5.82 ± 0.15[b]
C48	8.59 ± 0.03[a]	8.34 ± 0.04[b]
C50	12.17 ± 0.07[a]	10.43 ± 0.24[b]
C52	12.87 ± 0.11[a]	9.41 ± 0.54[b]
C54	7.65 ± 0.07[a]	4.66 ± 0.54[b]

Data are represented as Mean ± SD ($n = 24$) in cow ghee and Mean ± SD ($n = 18$) in buffalo ghee; Mean within rows with different superscripts are significantly different ($P < 0.05$) from each other.

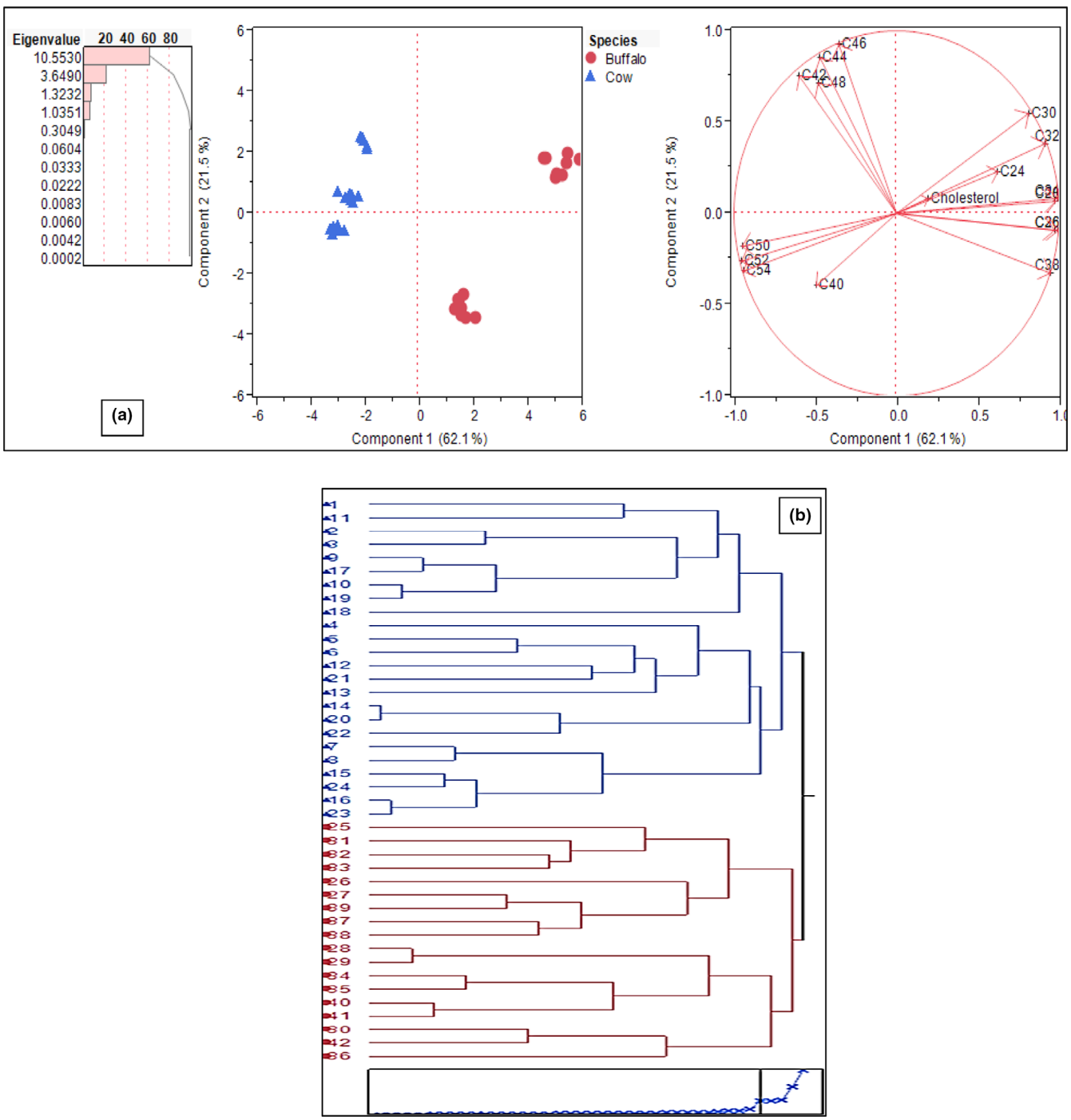

Figure 3 (a) Scores and loading plots of PCA model for triglycerides of cow ghee (blue) and buffalo (red). (b) Dendrogram showing separate clusters of cow ghee (blue) and buffalo ghee (red).

buffalo ghee principal component analysis (PCA) was performed. It is evident from the PCA (Figure 3) that cow and buffalo ghee could be distinguished from each other on the basis of variation in TG composition. The loading plot of the PCA of TGs composition (Figure 3a) showed that cow ghee samples were associated with a higher content of C42 to C54, whereas buffalo ghee samples were associated with a greater content of C26 to C38. It is also evident from the dendrogram (Figure 3b) that cow and buffalo ghee samples formed two separate hierarchical clusters and were grouped separately and not overlapped with each other. This further confirmed that cow and buffalo ghee have dissimilarities

based on certain TG, which could be attributed to differences in fatty acid concentration in the two fats. These findings further led to the assumption that *S*- limits of cow and buffalo ghee will not be the same. These findings proved that there is a likelihood of erroneous results if the standard (ISO 2019) specified for cow milk fat is applied in totality for buffalo ghee.

CONCLUSION

The results demonstrated that the clarification temperature used to heat clarify the butter for ghee manufacture will not affect the TG composition of ghee. However, cow and buffalo ghee can be distinguished on the basis of their TG profiling. TGs having carbon numbers (C) 24, 26, 28, 30, 32, 34, 36 and 38 were significantly higher ($P < 0.01$) in buffalo ghee than cow ghee, whereas TGs having carbon numbers (C) 42, 44, 46, 48, 50, 52 and 54 were significantly higher ($P < 0.01$) in cow ghee. On account of the differences in the TG composition of cow and buffalo ghee, there is a possibility of a deviation in the *S*- limits in buffalo ghee *vis'-a-vis'* the standard specified for cow milk fat. Hence, the likelihood of erroneous results in buffalo ghee is always there if the standard (ISO 2019) specified for cow milk fat is applied in totality for buffalo ghee. Moreover, India is having more diversity in live-stock population than the Western counterparts; hence, there is a scope to work further to develop a new standard for buffalo ghee and validate the standard for cow ghee by collecting the samples of both the types of ghee from the length and breadth of India.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Parul Pathania: Formal analysis; methodology. **Vivek Sharma**: Conceptualization; data curation; investigation; methodology; supervision; writing-original draft. **Priyanka Singh Rao**: Investigation; methodology; writing-review & editing. **Narender Raju Panjagari**: Formal analysis.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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