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RESEARCH ARTICLE

A Comparative study on the S- values of cow and buffalo ghee calculated using equations specified in ISO (17678) method of determining the milk fat purity by gas chromatographic analysis of triglycerides

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Abstract: Cow and buffalo ghee samples were prepared from the milk collected from the locations in eastern, western, southern, and northern parts of the country. Ghee prepared so was subjected to triglyceride analysis using gas-liquid chromatography and S- limits were calculated using the equations specified in the ISO method. All the five S- limits, as specified in the standard for cow milk, got deviated on both the lower and upper side of the limits in the case of the cow as well as buffalo ghee samples of all four regions. Buffalo ghee samples were found to have a higher upper S- total (ST) limit ranging from 109.34 to 118.21 in the samples from all four regions, whereas lower value was slightly less (94.06 to 94.59) than the lower range specified in the standard for buffalo ghee samples from eastern, northern and southern region samples. A similar trend was observed in the case of s- limit (S4) specified for the detection of Palm oil and beef tallow. In cow ghee also the S- values showed a trend of deviation from the standard.

Keywords: Ghee, cow, buffalo, ISO, triglycerides, S- values

Introduction

Ghee is one of the very important constituents of the India Dairy products basket. As per the definition Milk fat, ghee, butter oil, anhydrous milk fat and anhydrous butter oil are fatty products derived exclusively from milk or products obtained from milk, or both, by means of processes which result in almost total removal of water and milk solids-not-fat. Ghee has especially developed flavor and physical structure as a result of its method of manufacturing (FSSR, 2019). It is generally prepared by clarifying cream/ butter at 110 – 130˚C, wherein butter is obtained either from cream or from curd (traditional practice). Ghee 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). 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). According to a report, the Indian ghee market reached a value of Indian rupees 2,273 billion in 2019 and is expected to reach a value of Indian rupees 4,653 billion by 2024 (IMARC, 2020). India's Export of Dairy products was 51,421.85 MT to the world for the worth of Rs. 1,341.03 Crores/ 186.71 USD Millions during the year 2019-20 (APEDA, 2020). The most common types of ghee available in the Indian subcontinent are cow ghee and ghee. International Organization for Standardization has specified a reference method (ISO, 2019) to ensure the purity of only cow milk fat based upon the profiling of triglycerides with 24- 54 carbons (C24-C54) using Gas-liquid chromatography and thereby calculating standard – values (S-values) for the cow milk fat. The detection of different vegetable oils and animal fats in cow milk fat is represented by S-limits (S2, S3, S4 and S5). The ISO/IDF reference values for cow milk fat are ST: 95.68 – 104.32; S2: 98.05 – 101.95; S3: 99.42 – 100.58; S4: 95.90 – 104.10; S5: 97.96 – 102.4.

The scope of the above said method has already mentioned a likelihood of obtaining the false-positive result in fat obtained from bovine milk other than cow's milk. Note 3 of the method also stated that sometimes false positive results reporting for milk from certain Asian regions. This was attributed to special feeding practices such as the feeding of a high proportion of vegetable oils, serious underfeeding (ISO, 2019). Literature also suggested that the presence of phospholipids overlaps with the short-chain triglycerides and might distort the results (Precht 1992). During heating, especially after most of the moisture has

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evaporated, antioxidants are produced from phospholipids, which in turn are believed to be absorbed by the fat (Tamime, 2009). Literature also suggested that there is a variation in the interval of concentration i.e. range of triglycerides in the cow milk fat of various countries (Tolentino et al. 2007). Reports also suggested that goat milk fat did not show a bimodal distribution of triglycerides unlike cow milk fat and maximum values were reported for triglycerides C38 and C40 (Tolentino et al. 2015). Therefore, there is a possibility that the said standard may not be applicable as such to anhydrous milk fat extracted from buffalo milk and also the ghee which is prepared by heat clarifying the butter at a higher temperature like 110- 130°C. There are only limited reports available on the S- values of ghee (Amrutha Kala, 2013; Kala et al. 2016; Sharma et al. 2018). Hence, the present work was carried out to investigate the deviation in S- limits of cow and buffalo ghee. The results obtained will be useful in checking the suitability of the standard (ISO, 2019) to check the purity of cow and buffalo ghee. This will also be helpful in filling the knowledge gap in the S- limits of buffalo ghee.

Materials and Methods

Milk collection and preparation of ghee samples: To prepare ghee, milk samples were collected from four regions of the country viz, Karnal (Northern), Bengaluru (Southern), Mehsana (Western) and Patna (Eastern). Samples from Northern region (Karnal District) were collected on monthly basis (eight months), whereas, samples were collected after every two months from other regions. These samples were brought to the laboratory at NDRI- Karnal in frozen state. Samples were then thawed and warmed to 40°C and cream was separated using mechanical cream separator. Cream obtained was then heated on a direct flame in a stainless steel vessel and clarified into ghee with continuous stirring at a temperature of 120°C/flash. Ghee was then filtered through muslin (6-8 folds) cloth followed by further filtration using Whatman No.4 filter paper. These samples were then subjected to triglyceride (TG) analysis.

Triglyceride mix, Tristearin, and Anhydrous milk fat standards

Standard triglyceride mix (CRM18811) consisting of Tricaprylin, Tricaprin, Trilaurin, Trimyrstin, Tripalmitin, and standard anhydrous milk fat (BCR-519) were procured from Sigma - Aldrich Co, 3050 Spruce Street (St Louis, MO 63103, USA 314-771-5765). These standards were used to calibrate the GLC conditions.

Gas Chromatographic (GLC) analysis of triglycerides

Triglyceride analysis of ghee samples was carried out as per the method specified for triglyceride analysis of the cow milk fat (ISO 17678: 2010). Shimadzu 2010 plus machine (Kyoto, Japan), with GC solution software and CP7532 CP-SimDist Ultimetal capillary column (5 m X 0.53 mm X 0.17 µm) was used

Calculations of S- limits

S- limits for the pure ghee samples were calculated by substituting the respective triglyceride values in the equations specified in ISO 17678 standard method (ISO 2019)

Statistical analysis

Mean and the standard deviation was calculated using Graphpad Prism 5 software. Upper and lower limits were calculated as per the method described by Kroemer, 2006 for widely scattered data. The following equation was used to calculate the upper and lower limit of S- values:

$$pmax = m+(kmax*S)$$

$$pmin = m+(kmin*S)$$

where

m= mean value
S= standard deviation
k= factor selected from the Table, which is positive sign for above mean and negative for below mean.
One- way ANOVA was performed using Graph pad Prism 5 software and two- way ANOVA by SPSS, to check the significant differences in S- values of cow and buffalo ghee as well as regional variations.

Results and Discussion

Standardization of GLC conditions

Conditions of GLC were standardized as per the requirements of the ISO methodology, that baseline drift should be minimum, no splitting of peaks, response factors close to 1.0 and not higher than 1.250. It is evident from the chromatograms (Figure 1) that all the standard triglycerides in the standard mix (CRM18811) have been separated distinctly and drift in the baseline is also negligible. Similarly, in the case of standard anhydrous milk fat (BCR-519), the baseline is stable and peaks of all major triglycerides are also clear without any splitting (Figure 1). Similarly, in the chromatograms of pure cow and buffalo ghee (Figure 1), the baseline is stable and peaks of all major triglycerides are well resolved. Response factors calculated using standard anhydrous milk fat (BCR-519) were also in the range of 0.92-1.1 for different triglycerides having carbon numbers C24- C54. These results demonstrated that the GLC machine's conditions were as desired to have accurate triglyceride analysis of ghee samples.

Regional variation in the S- limits of ghee

Cow ghee

S- total (ST) represents the S- limit for total milk fat. It is evident from the data (Table 1) that the upper limit of ST in the case of

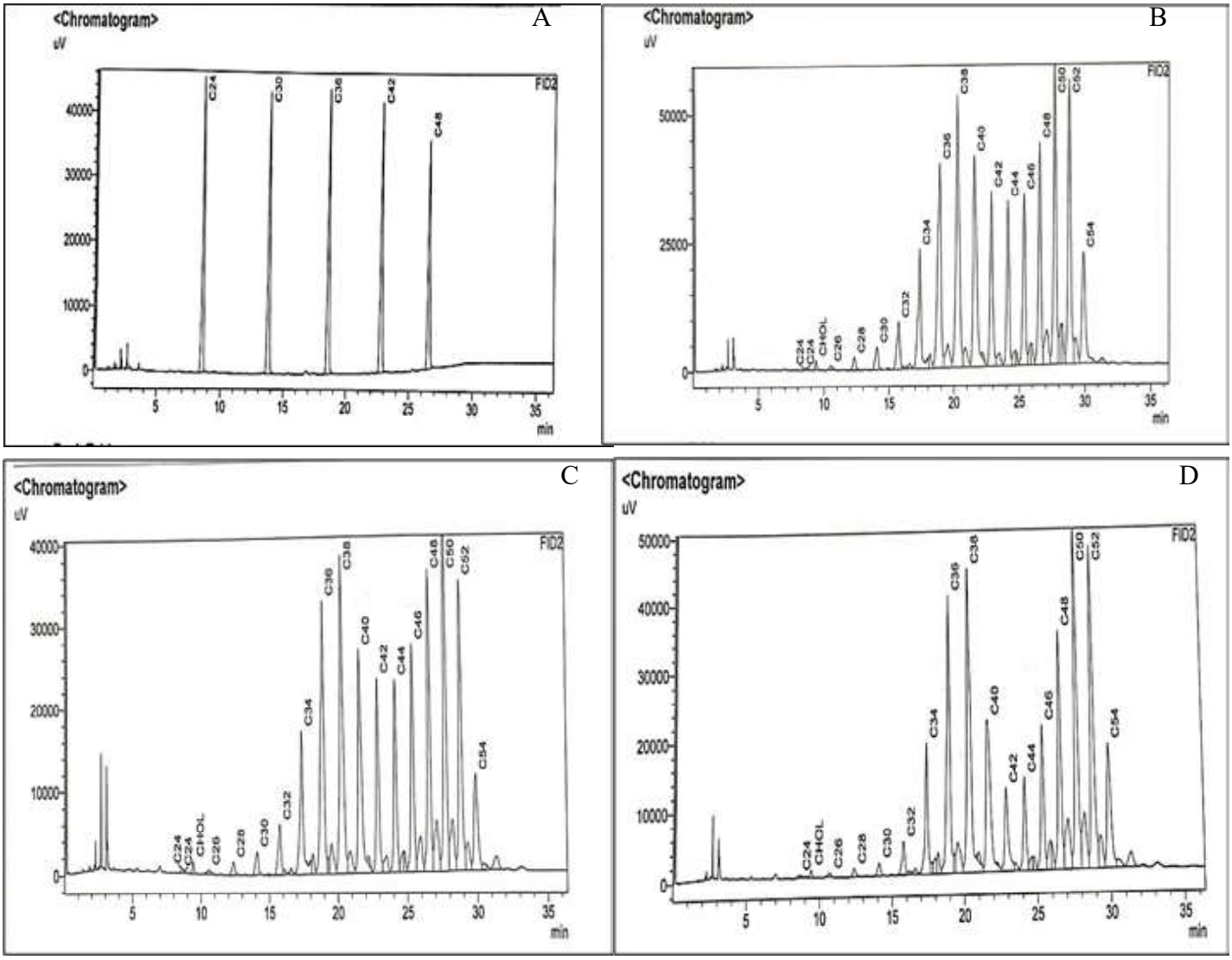

Fig. 1 Chromatograms of Triglyceride in **(A)** Standard tryglyceride mix (CRM18811) **(B)** Anhydrous milk fat (BCR-519) **(C)** Cow ghee **(D)** Buffalo ghee

northern cow ghee samples was within the upper limit (104.32) specified in the standard, whereas in other regions the observed upper limit was more than the upper limit specified in the ISO/IDF standard of cow milk fat. Similarly, the lower limit for ST observed in eastern, northern, and southern regions was less than the standard lower limit (95.68) specified for cow milk fat (ISO, 2019). Only the western region's samples could meet the lower limit. The perusal of the data (Table 2) also revealed that the ST of cow ghee from the western region was significantly ($p<0.05$) different from the ST of other religions.

S-limits for the detection of Soybean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cottonseed, fish oil in ghee is represented by (S2). For pure milk fat the S2 should be 98.05 – 101.95 as per the standard specified for cow milk fat (ISO, 2019). If the values obtained in the tested milk fat samples are not within the range, then those samples are considered to be adulterated with the above said oils/ fats. It is evident from the range of S2 (Table 1) observed in the tested samples of cow ghee from different regions that some samples had S2 lower than the specified standard, whereas some had more than the specified in the standard. A perusal of the data (Table 2) also revealed that samples from the eastern and western regions had significantly (<0.05) different S2 than the samples from eastern and western regions.

S-limits for the detection of Coconut and palm kernel fat in cow milk fat is represented by (S3). For pure cow milk fat, the S3 should be 99.42 – 100.58 as per the standard specified for cow milk fat (ISO, 2019). It is visible from the data (Table 1) barring samples from the northern region in all other regions the lower values observed were slightly less than the lower limit specified

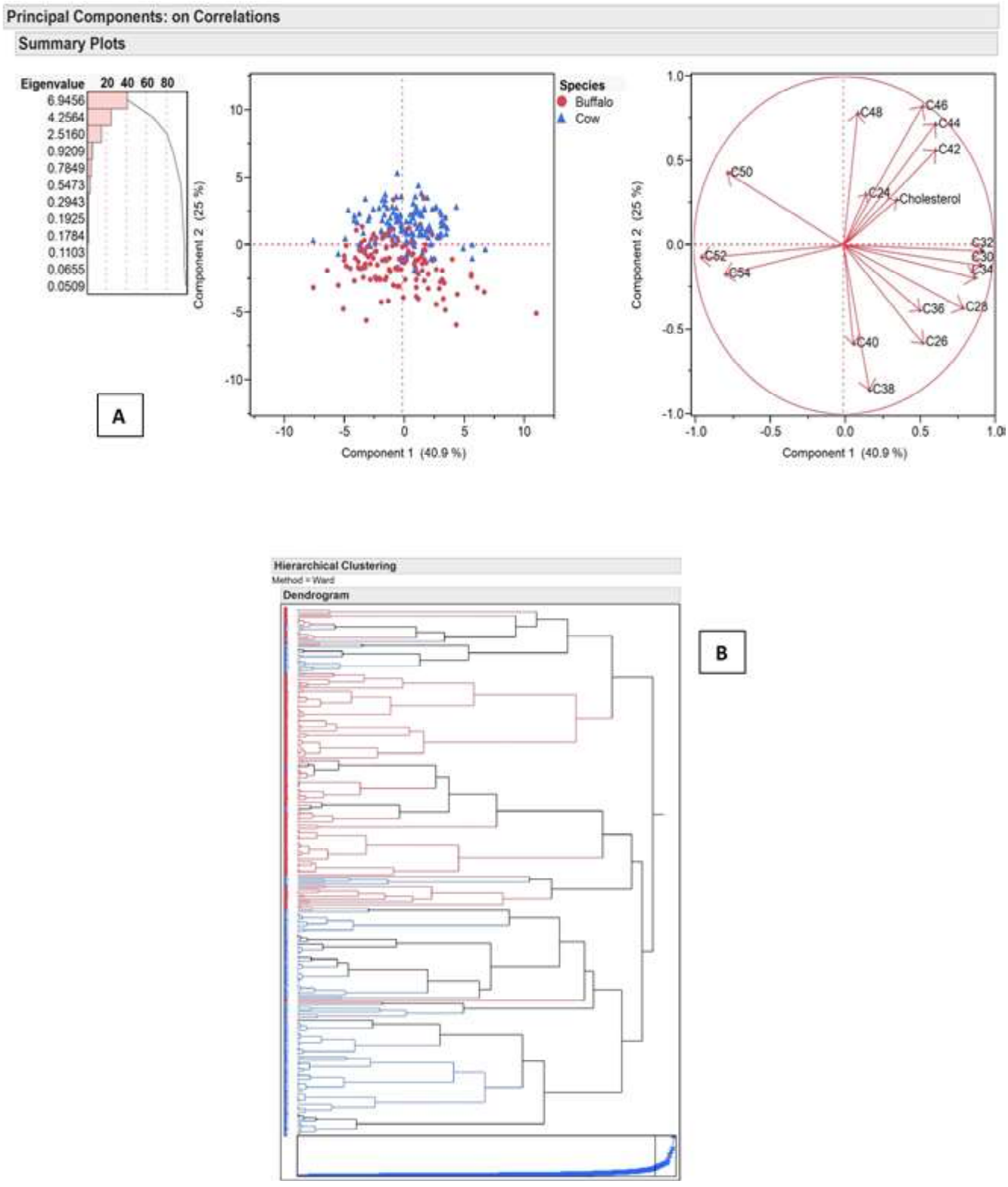

Fig. 2 Scores and loading plots of PCA model for triglycerides of Cow and Buffalo ghee (A) Dendrogram showing separate clusters of cow (blue) buffalo(red) ghee (B)

in the standard. Similarly, except for the western region, the upper values observed were slightly higher than the upper limit specified in the standard. This again indicated that the limits observed in the present study were not the same as specified in the standard for cow milk fat. Average values (Table 2) revealed that the samples from the western region had significant ($p<0.05$) difference from the S3 values of other regions.

S-limits for the detection of palm oil and beef tallow in cow milk fat are represented by (S4). For pure cow milk fat the S4 should be 95.90 – 104.10 as per the standard specified for cow milk fat (ISO, 2019). It is evident from the data (Table 1) except for western region samples, the lower limit observed in the cow ghee samples was lower than the value specified in the standard. On the contrary, the observed upper limit in the samples of the eastern, western, and southern regions was higher than the upper limit specified in the said standard (ISO, 2019). In the case of northern region samples, the observed upper value was within the upper limit specified in the standard. On account of S4 also it can be inferred that the values determined in the present study were not following the range specified in the standard for cow milk fat. The regional difference was significant as evident in the data presented in Table 2.

S-limits for the detection of lard in cow milk fat are represented by (S5). For pure cow milk fat, the S5 should be 97.96 – 102.4 as per the standard specified for cow milk fat (ISO, 2019). In the present study, it was observed that cow ghee samples of eastern and southern regions had observed lower range values slightly less than the lower limit specified in the standard. On the contrary, the samples from northern and southern regions also showed upper values higher than the upper limit specified in the standard. S5 also indicated clearly that the range obtained in the present study was showing a deviation from the limits specified in the standard of pure cow milk fat. A perusal of the data on the basis of average S5 values of cow ghee from different regions it was found that there was a significant (<0.05) difference in the S5 of cow ghee samples of eastern and southern regions.

This can be attributed to the fact that in countries like India, wherein organized dairy farming is still not fully developed and milk is poured into the pool by a variety of farmers including the poor, and marginal. At the same time, there is regional diversity with respect to the feed/ fodder and breeds of cattle, which might be the factor contributing to such differences among the cow ghee samples from different regions as well as deviation from the standard (ISO, 2019) specified for cow milk fat.

Buffalo ghee

S- limits have not been specified for buffalo milk fat in the standard (ISO, 2010 & 2019). Therefore it is difficult to compare the findings of the present investigation with reference to any standard. However, it was observed that the profile of buffalo ghee with reference to the major triglycerides was akin to the triglyceride profile of cow ghee. The only difference was that the concentration of some of the triglycerides (C34, C36, C38, C52, and C54) was higher in buffalo ghee and some of the triglycerides (C42, C44, C46, C48) were less than their concentration in cow ghee. Therefore, the concentration value of these triglycerides was substituted in the different equations, and S- values were determined. It is evident from the data (Table 1) that in all four regions the variation in the upper range for (ST) as determined in the present investigation was higher (109.34 – 118.21). It is evident from the ST data that both lower and upper limits were higher than the values observed in cow ghee samples in the present investigation. The observed variation may be attributed to the species difference in the fatty acids composition of milk fat. Similarly, the lower range also varied from 94.06 – 96.83.

On perusal of the average ST values (Table 2), it was inferred that barring samples from the western region there was a significant (<0.05) difference in the ST- values of Cow and buffalo ghee samples. This could be attributed to the variation in the triglyceride concentration of cow and buffalo ghee as the literature suggests that buffalo and cow ghee has different saturated fatty acids (SFA) profiles (Carolina and Luis Fernando, 2020). Similarly, other researchers also reported a variation in the triglyceride containing different carbon numbers from C24- C54 (Smidy et al. 2012; Hazra et al. 2017; Sharma et al. 2018 and Amrutha Kala, 2013) in the milk fat of different species.

In the case of S2 (Table 1) the range in different regions varied between 96.12 to 103.27. On comparing the average (Table 2) it was observed that the regional difference was not significant. However, the difference in the S2 of cow and buffalo ghee samples was statistically significant ($p<0.05$) in the southern region.

The lower and upper S3 limits (Table 1) varied between 97.75 to 99.26 and 100.78 to 101.35, respectively. It is also evident from the data (Table 2) that there were a regional difference in the S3, eastern and northern samples were found to have statistically ($p<0.05$) higher values than the samples of western and southern regions. On perusal of the data, it was also observed that cow ghee samples from the southern region had significantly ($p<0.05$) higher S3 than the buffalo ghee samples of the same region.

The lower and upper S4 limits of buffalo ghee from different regions (Table 1) varied between 95.60 to 99.58 and 110.60 to 117.37, respectively. It is evident from the data depicted in Table 2 that there was a significant ($p<0.05$) difference in the average S4 of buffalo ghee among the different regions. Northern region samples of buffalo ghee were found to have statistically lower average S4 than the samples of the western and southern region.

Similarly, the lower and upper S5 values in buffalo ghee samples from different religions varied from 94.47 to 97.11 and 101.01 to 104.81, respectively. The perusal of the average values (Table 2)

Table 1 Range of S- values in pure Ghee from different regions

S- limits	Type of ghee	Eastern (E)	Western (W)	Northern (N)	Southern (S)	No of samples analyzed			
						E	W	N	S
ST	Cow	91.59- 105.24	97.27 -105.93	90.60 -102.36	93.00 - 106.34	36	40	50	24
	Buffalo	94.30 - 110.10	96.83 - 112.37	94.06 -109.34	94.59 -118.21	32	39	32	19
S2	Cow	96.28 - 102.53	97.17 -101.77	95.42 - 100.48	92.82 - 103.08	36	40	50	24
	Buffalo	96.39 - 102.09	97.20 - 102.70	96.12 -101.52	96.23 -103.27	32	39	32	19
S3	Cow	99.21 - 101.36	99.15 - 100.19	99.79 -101.41	99.16 -101.24	36	40	50	24
	Buffalo	98.90 - 101.30	98.60 -100.78	99.26 - 101.34	97.75 -101.35	32	39	32	19
S4	Cow	92.53 - 106.37	97.32 - 106.48	92.22 - 102.80	89.53 -107.25	36	40	50	24
	Buffalo	95.65 - 112.35	99.58 -114.32	95.60 -110.60	96.23 -117.37	32	39	32	19
S5	Cow	97.03 - 102.31	98.16 - 101.84	98.36 - 102.84	96.52 -105.88	36	40	50	24
	Buffalo	97.11 - 101.01	96.75 - 101.13	96.77 - 101.81	94.47 -104.81	32	39	32	19

ST-S- total; S2-Soybean, sunflower, olive, rape seed, linseed, wheat germ, maize germ, cotton seed, fish oil; S3- Coconut and palm kernel fat; S4- Palm oil and beef tallow; S5- Lard

(ISO/IDF Reference values for cow milk fat: ST: 95.68 – 104.32; S2: 98.05 – 101.95; S3: 99.42 – 100.58; S4: 95.90 – 104.10; S5: 97.96 – 102.4)

Table 2 S- values (Average ± SD) in pure Ghee from different regions

S- limits	Type of ghee	Eastern (E)	Western (W)	Northern (N)	Southern (S)	No of samples analyzed			
						E	W	N	S
ST	Cow	98.42± 5.33^{aA}	101.6± 3.38^{bA}	96.48±4.59^{aA}	99.67± 5.21^{aA}	36	40	50	24
	Buffalo	102.2±6.17^{aB}	104.6±6.07^{abA}	101.7±5.97^{aB}	106.4±9.23^{bB}	32	39	32	19
S2	Cow	99.41± 2.44^{aA}	99.47± 1.8^{aA}	97.95±1.99^{bA}	97.95±4.02^{bA}	36	40	50	24
	Buffalo	99.24±2.23^{aA}	99.95±2.15^{aA}	98.82±2.11^{aA}	99.75±2.75^{aB}	32	39	32	19
S3	Cow	100.2± 0.91^{aA}	99.67± 0.41^{bA}	100.6±0.63^{cA}	100.2±0.81^{acA}	36	40	50	24
	Buffalo	100.1±0.94^{aA}	99.69±0.85^{bA}	100.3±0.81^{aA}	99.55±1.41^{bB}	32	39	32	19
S4	Cow	99.45± 5.41^{abA}	101.9± 3.56^{aA}	97.51± 4.13^{bA}	98.39±6.92^{bA}	36	40	50	24
	Buffalo	104.0±6.5^{abB}	106.5±5.76^{aB}	103.1±5.86^{bB}	106.8±8.27^{aB}	32	39	32	19
S5	Cow	99.67±2.06^{aA}	100.00±1.44^{abA}	100.60±1.75^{abA}	101.20±3.66^{bA}	36	40	50	24
	Buffalo	99.06±1.52^{aA}	98.94±1.71^{aA}	99.29±1.97^{aB}	99.64±4.04^{aB}	32	39	32	19

$^{a, b}$ means within a row and ^{AB} means with in a Colum with different superscripts are significantly different ($p<0.05$) from each other

ST-S- total; S2-Soybean, sunflower, olive, rape seed, linseed, wheat germ, maize germ, cotton seed, fish oil; S3- Coconut and palm kernel fat; S4- Palm oil and beef tallow; S5- Lard

(ISO/IDF Reference values for cow milk fat: ST: 95.68 – 104.32; S2: 98.05 – 101.95; S3: 99.42 – 100.58; S4: 95.90 – 104.10; S5: 97.96 – 102.4)

revealed that there was no significant difference between these values on account of regional variations in the sample of buffalo ghee.

However, the difference in cow and buffalo ghee samples was evident in S5 also irrespective of the region. These differences between cow and buffalo ghee S- limits can be attributed to the variation in the concentration of certain triglyceride moieties. The said justification is further supported by the PCA analysis of the triglyceride data of both the ghee types (Fig 2A&B) carried out in the present investigation. The loading plot of the PCA of triglycerides composition (Figure 2A) showed that the majority of the cow and buffalo ghee samples were distinct from each other in terms of triglycerides having 26, 28, 36, 42, 44, 46, and 50 acyl carbons. It is also evident from the dendrogram (Figure 2B) that majority of the cow and buffalo ghee samples were clustered into two separate hierarchical clusters barring a few samples overlapping with each other. 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). It has been reported that triglyceride content varies among different species and also in different breeds of the same animal species (Fontecha et al. 1998). This further confirmed that cow and buffalo ghee have dissimilarities based on certain triglycerides, which could be

attributed to differences in fatty acid concentration in the two fats. Hence it can be concluded that cow and buffalo ghee are two distinct types of ghee and the standard specified for cow milk fat cannot be applied to buffalo milk fat.

A perusal of the data (Table 2) revealed that the average S- values of cow ghee samples were within the limits of the standard specified for cow milk fat (ISO, 2019). This led to an interesting extrapolation of the findings that there is a likelihood that the cow ghee which is produced commercially by the Dairy industry shall meet the S- limits specified in the standard of cow milk fat (ISO, 2019). The very reason for the above mentioned observation is that the milk is procured by the Dairy processing plants from the large area of their milk collection chain which nullifies the deviations arising from the milk of individual farmers or a particular milk route.

However, in the case of buffalo ghee the average S- total and S4, were on the higher side of the limits and even more than the upper limits in western and southern buffalo ghee samples. This led to the observation that there is a definite need to develop a separate standard for buffalo ghee.

Conclusions

Cow and buffalo ghee were found to have different concentrations of certain triglycerides. These variations led to the differences between the S- values of cow and buffalo ghee. Though the average S- values of cow ghee were within the limits of the standard but S- limits had a deviation from the S- limits of cow milk fat specified in the standard (ISO, 2019). Buffalo ghee showed a greater deviation in all the S- limits. Even the average S- total and S4- (Palm oil and beef tallow) in buffalo ghee was higher than the upper limit specified for theses in cow milk fat standard. Regional differences were also found in both cow and buffalo ghee samples. On the basis of this limited study, there seems to be a need to develop a standard of S- limits for buffalo ghee and relook into the standard of cow ghee of Indian origin. This could be achieved by collecting the milk samples throughout year from the organized dairy farms and stakeholders in the dairy supply chain from the length and breadth of India and thereby preparing the ghee samples from such milk and analyzing the samples. The setting up the S-values breed-wise is also one of the challenging task. This will only be possible by the concerted efforts of the Dairy Industry and Academia.

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