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INVITED REVIEW

Gas chromatographic analysis of triglycerides - The reference method for testing purity of milk fat and perspectives on its use in India: Review

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Abstract Adulteration of milk fat is burning issue, as traditional parameters for testing its purity are becoming ineffective due to advances in practices employed for the adulteration. Gas chromatographic (GC) analysis of intact triglycerides appears as the most promising method for combating menace of the high tech adulteration of milk fat, since it is simple, rapid, robust and comprehensive. Unfortunately, use of this method in India may cause problem of false positive results due to its specifications of parameters suggested on purity of milk fat. Hence, for adopting the method in India, modifications are required in limits of specifications on some parameters. The modifications in specifications of the method require extensive survey to generate database on the parameters. Outcome these efforts will be helpful to recognise Indian milk fat as pure in international market, whenever tested by this method. Until the method gets official endorsement, Indian dairy industry may avail its unmatched advantages by using it as a screening tool. Since, no comprehensive publication on this method is available so far in the literature; an attempt is made in this paper to collate information on its genesis, development, adoption as reference/ standard, basic principle, operational steps, advantage, limitations and evaluation. This review paper serves a navigation tool for concerned scientists and users of the method to understand its fundamental aspects identify reasons for false positive results occur in its use and develop appropriate solutions to resolve the problems as well as improve its application.

Keywords: Milk fat, TG profile, GC analysis, S-values, Testing purity, ISO 17678

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Introduction

Milk fat is an important nutrient in human diet and testing of its purity is very important to ensure constant and well-defined quality of milk and milk products. In purchase of milk from producers or suppliers, its price is determined from %fat content. This pricing policy encourages mixing of cheaper oils and/or fats. Nuisance of adulteration in milk fat is acquiring new dimensions due to adoption of science-based advanced modus operandi for the adulteration (Kala et al. 2016, Sharma et al. 2018, Pathania et al. 2020). Therefore, now fight against the smart adulteration of milk fat requires parameters on its purity, which are very robust against manipulation and highly comprehensive in nature. Moreover, technique used for analysis of these parameters should be simple, rapid, safe, economical and environment friendly. A literature survey of 73 years (Achaya & Hilditch 1950, Si et al. 2023) reveals that the intact triglycerides-based parameters on purity of milk fat and their analysis by GC technique appear as the most promising tool to combat the nuisance (Bosque-Sendra et al. 2012, Cossignani 2019, Csapó et al. 2019). Unfortunately, in India potential of this method for testing purity of milk fat is not fully recognised (Prajapati and Aparnathi 2021) since use of this method may lead to false positive results in Asian countries (ISO/IDF 2010). Reasons for problem of the false positive results in country like India may be attributed to limits suggested in specifications of parameters on purity of milk fat (Pathania et al. 2020, Prajapati and Aparnathi 2021, Sharma et al. 2021). Hence, for implementing the method in India appropriate modifications in limits of some parameters are required.

Though India is the highest milk producer in the world science 1998 (Kalidas et al. 2021), it is very surprising to note that no attempt has been reported so far to find amicable solutions for the problems in limits of some parameters specified in the method. This situation has compelled some quality conscious Indian dairy organisations to adopt the method with only limited use of its specifications. In such implementation of the method only limited parameters are selected for implementation. The parameters and their limits selected in such a way that they are not likely to cause problems of false positive results. Some dairy organisations in India have adopted the method for testing purity of fat present in

supplies of incoming milk, cream and butter before their final acceptance by the dairy plants (Prajapati and Aparnathi 2021).

Although the method is recognised as a standard method by European Union (EU) since 1995 (EC 1995) and as reference method jointly by ISO and IDF since 2010 (ISO/IDF 2010), the survey of literature suggests that no effort is made so far to present information on various aspects of the method in a highly comprehensive form. Therefore, attempt has been made in this review to collate information on (1) problems faced in use of traditional methods for testing purity of milk fat and advanced techniques explored for the same as alternates, (2) significance of analysing intact triglyceride, developments in GC analysis of intact triglyceride to test purity of milk fat and its adoption as reference method and (3) basic principle in working, operational steps, advantage, limitations and evaluation of the method. Moreover, efforts are also made to find information about (a) secret behind stunning success of the method, (b) reasons for false positive results in the method and (c) perspectives on implementation of the method in India. Finally plan of actions is suggested for work required to adopt the method as official reference in India under dairy farming system presently followed in the country.

Traditional methods in testing purity of milk fat

Traditionally, parameters based on butyro refractometer reading at 40°C, Reichert-Meissl value, Polenske value, saponification value, iodine value, fatty acids profile and phytosterol or β-sitosterol test are suggested for testing purity of milk fat. Application of these parameters have number of lacunas and/or suffer from one or more drawbacks. Problems faced in use of the traditional methods are compiled from several sources (Fox et al. 1988, Naviglio & Raia 2003, Azadmard-Damirchi & Torbati 2015, Kala et al. 2016, Sherma et al. 2018, Nilchian et al. 2020, Pathania et al. 2020, Shinde et al. 2021) and summarised below.

- Highly time consuming and very tedious since most of them involve several unit operations and that too under highly precise conditions. As a result, they are not suitable for analysing substantial number of samples in routine.
- Usually necessitate isolation, purification and/or concentration of the compounds targeted as marker in analysis (*e.g.* β-sitosterol or other minor components).
- Manual operations and necessitate human intervention at various stages in analysis. Therefore, they always remain at risk of human bias and/or error
- Destructive in nature and involve use of several corrosive, toxic and/or expensive chemicals. These lead to health hazards, environmental pollution and/or excessive cost of the analysis.
- Difficult to obtain reproducible results, especially when foreign fat is present at low rates. So results remain questionable and situation become difficult to reach a conclusion.
- High limits of detection (LoD) due to large natural variations in parameters, owing to effect of feed given to lactating animals, season of the year and stage of lactation.
- Lack of comprehensive nature and each method detects only limited number of foreign fats. Hence, they necessitate number of different methods to get overall view about the purity.
- Standards on their parameters are mostly specified as minimum or maximum value. Such parameters become highly susceptible to manipulation, as it can be adjusted easily.

Add to all these, practice of adulteration is now becoming very smart because of knowledge about chemistry of milk fat and that of the foreign fats. Application of the science-based information and proficiency gained from experience, a mixture of foreign fats is formulated so precisely that its physical properties and chemical characteristics resembles very well with that of the pure milk fat. Hence, it becomes extremely difficult to detect such well-designed tailer made blend of foreign in milk fat by the conventional parameters on its purity. Consequently, day by day these parameters are becoming ineffective in testing authenticity of milk fat (Bhalerao & Kummerow 1953, Parodi 1969, Kala et al. 2016, Sharma et al. 2018, Pathania et al. 2020, Shinde et al. 2021).

Advanced techniques in testing purity of milk fat

In view of the problems faced in use of traditional methods, different instrumental techniques were explored as alternates for testing purity of milk fat ((Nazrim Marikkar 2022, Mahrous et al. 2023). These advanced analytical techniques are mostly belonged to three different types: (a) GC analysis (EC 2008, ISO/IDF 2010), (b) MIR spectroscopy (Upadhyay et al. 2016 & 2018, Antony et al. 2018a, Gandhi et al. 2022) and NIR spectroscopy (Antony et al. 2018b, Aparnathi et al. 2019) and (c) thermal analysis (Nurrulhidayah et al. 2015, Tomaszewska-Gras 2016, Upadhyay et al. 2017, Farah et al. 2018, Islam et al. 2022). Among the various advanced analytical techniques reported for testing purity of milk fat, the GC analysis of intact triglyceride molecules is the only technique which is developed most systematically, researched very extensively and recognised internationally as the reference method.

Significance of analysing intact triglyceride in testing purity of milk fat

Chemically triglycerides are esters formed by reaction of three fatty acids molecules with one molecule of glycerol. In common edible oils and fats of plants and animals origin triglycerides are present as their most abundant component. While analysis of intact triglycerides, natural distribution of fatty acids in their molecules is retained as such. Therefore, information obtained abut inherent characteristics of natural oils and fats is usually

much higher from analysing intact triglycerides compared to the destructive methods of analysis (Ulberth & Buchgraber 2000, de La Fuente & Juarez 2005, Ruiz-Samblás et al. 2015, Nilchian et al. 2020).

In composition of milk fat triglycerides contribution about 98% (Amaral et al. 2018, Kapoor et al. 2023). Milk fat is most complex among the common edible oils and fats of plants and animals origin. The complexity is attributed to its fatty acid composition, because >400 structurally different fatty acids are identified as components of milk fat. Hence, it can form thousands of structurally different triglycerides (Buchgraber et al. 2004, Liu et al. 2020, Si et al. 2023). Liu et al. (2020) have identified 3454 triglyceride molecules in milk fat. If only 15 major fatty acids occurring @ >1% are considered and their position in triglyceride molecules is ignored, even then 680 compositionally different triglycerides are likely to be present in milk fat (MacGibbon & Taylor 2006). As a result, it is not feasible to exactly duplicate such a complex triglycerides composition of milk fat even by smart techniques. It indicates that, intact triglycerides analysis is a promising option in testing purity of milk fat.

Developments in GC analysis of triglyceride to test purity of milk fat

Significance of analysing intact triglyceride molecules in detecting blend of foreign fats in milk fat was recognized in early 1950s by Bhalerao and Kummerow (1953). However, its application in practice requires suitable technique for separation and quantification of triglyceride molecules and formation of parameters on purity of milk fat based on its triglyceride profile. The development of parameters from information of intact triglycerides was hampered due to problems in achieving their fine resolution. Doors were opened in early 1960s after getting success in separation and quantification of triglycerides by GC technique. The clear separation of triglycerides was achieved using stationary phase stable at high temperatures. The separation by this technique is based on differences in their number of carbon atoms content. The difference as low as two carbon atoms can be resolved successfully (Kuksis and McCarthy 1962). Chronology of developments in intact triglycerides profile-based parameters on purity of milk fat is summarised below. Perusal of this precious literature is very useful to understand fundamentals of the method and evolve constructive ideas to modify the exiting parameters as well as develop new parameters.

- Canadian researchers, Kuksis and McCarthy (1964) were pioneers to use GC analysis of intact triglycerides for detecting foreign fat in milk fat. They employed distortion in elution pattern of triglycerides during their fractionation by GC and relative proportion of specific triglyceride fractions (especially C52 & C54) as criterions in detection of the foreign fat.
- Pardi (1973) carried forward the idea suggested by Kuksis and McCarthy (1964). They worked out eleven ratios of triglyceride fractions in to two groups. Eight ratios comprise individual triglyceride fractions: (1) C54/C52, (2) C54/C36, (3) C52/C50, (4) C52/C42, (5) C52/C40, (6) C52/C38, (7) C52/C36 and (8) C50/C36, Whereas, three ratios comprise group of triglyceride fractions: (1) (C54+C52+C50)/C38, (2) (C54+C52+C50)/C40 and (3) (C54+C52+C50)/(C38+C40). They also worked out minimum and maximum values of the ratios for pure milk fat using authentic samples of Australian butterfat and suggested as criterions of butter fat purity.
- Timms (1980) suggested the following equation for multivariate analysis of three fractions of triglyceride (C40, C42 & C44) as characteristic for pure samples of Australian milk fat.

$$\mathbf{R = (14.197 \times C40) \ (36.396 \times C42) + (32.364 \times C44)}$$

Where, value of **R** should be 100 for the pure milk fat. As per this author, it detects 5% foreign fat with 99% confidence.

- Precht (1991) expanded the idea proposed by Timms (1980) on multiple regression analysis of triglyceride fractions. He standardised five equations to obtain five standardised values (S-values), which can serve as parameters on fat purity of milk fat. A very systematic and highly dedicated work of this author from Germany completed the search for intact triglyceride-based parameters to test purity of milk fat (Precht 1991, 1992a, 1992b). His technique is now used worldwide as reference method. Therefore, procedure followed by him in derivation of equations is shortly described here under.

Precht for his work gathered a required database on triglyceride composition of pure milk fat using carefully planned procedure and taking all relevant technical aspects in to consideration. The data were generated from analysis of 755 samples of milk fat from pure cow milk. The authentic samples of milk were collected from cows under different practices of feeding and stage of lactation as well as from different breeds and different regions of Germany over a period of five years. He made very extensive efforts to derive equations for calculation of S-values of milk fat by applying multiple linear regression in analysis of the database. As many as 32647 equations were derived, which comprise 3 to 15 different fractions of triglyceride from amongst the major fractions ranging (C26–C54). Each equation was examined for its efficacy and efficiency in detection of various foreign fats and five most promising equations were selected (Table 1). These five equations comprise 8 to 10 selected triglyceride fractions and coefficients specifically designed for each fraction of triglycerides in each equation. Precht also established limits of each S-value (*i.e.* S-limits) for pure cow milk fat using authentic samples of cow milk (Table 2). The five S-values are now also designated as S_1, S_2, S_3, S_4 and S_5 or S_{Total} (Povolo et al. 2008, Nilchian et al. 2020). Some Indian authors have designated them S_1 or S_{Total}, S_2, S_3, S_4 and S_5 (Kala 2013, Pathania et al. 2020, Sharma et al. 2021). Among the

two systems of designation the sequence reported as S_1, S_2, S_3, S_4 and S_5 or S_{Total} appears more logical, since it exactly coincides with the sequence of equations suggested for calculation of S-values by EC (2008) and ISO/IDF (2010, 2019). Among the five S-values, S_{Total} detects all type of foreign fats, whereas, rest of the S-values (*i.e.* S_1, S_2, S_3 & S_4) detect specific type of foreign fats.

After successfully developing the method Precht and his co-researchers continue the work on peripheral aspects of the method. Details of their work on some the important aspects and outcome of the work can be referred from their publications such as Precht (1993), Precht et al. (1998), Molkentin and Precht (1994, 1995, 2000), Molkentin (2006, 2007, 2013) as well as Molkentin and Crawford (2009).

Adoption of GC analysis of triglycerides as reference method

The technique developed by Precht for testing purity of milk fat using GC analysis of its triglycerides profile was recognised as a reference method at national and international level. German Institute for Standardization (Deutsches Institut für Normung) adopted it as standard for detection and determination of foreign fats in milk fats vide DIN No. 10336: 1994-09 (DIN, 1994). After considering results of six collaborative trials in member countries, EU adopted the technique as a reference for determination of milk fat purity, vide EC Regulation No. 454/95 (EC 1995) and revised it twice (EC 2001, EC 2008). International collaborative study of the technique was conducted by Molkentin & Crawford (2009). Based on their study ISO and IDF jointly adopted it as reference method for determination of milk fat purity vide ISO 17678: 2010 and IDF 202: 2010 respectively (ISO/IDF 2010). They published its updated version as second edition in 2019 (ISO/IDF 2019). In subsequent part of this article it will be referred as the method.

Basic principle of the method

In biosynthesis of triglycerides in plants and animals, spatial arrangement of fatty acids in their molecules is genetically controlled (Bhalerao & Kummerow 1956). Hence, all naturally occurring edible oils and fats have their unique profile of triglycerides (Buchgraber et al. 2004). Ruminant milk fat is unique in containing substantial amount of short chain fatty acids (C4:0–C14:0) along with most prominent medium chain (C16) and long chain (C18) fatty acids (Bear 1991, MacGibbon & Taylor 2006). Hence, in milk fat of cow and buffalo triglycerides profile ranges from C24 to C54 (Smiddy et al. 2012, Kala 2013, Hazra et al. 2017, Sharma et al. 2018, Pathania et al. 2021). The triglycerides profile of cow and buffalo milk fat has very distinct bimodal distribution pattern with first peak appears between C34 and C42, whereas, second peak appears between C46 and C54. On the other hand, most of the vegetable oil (Edem 2002, Povolo et al. 2008, Park et al. 2014, Dorni et al. 2018) and animal body fats (Wood et al. 2008, Park et al. 2014, Lisitsyn et al. 2017) comprise only medium chain and long chain fatty acids. As a result, in most of the vegetable oils and animal body fats fat major triglycerides generally range from C46 to C54 or C56. Therefore, triglycerides profile of plant oils and animal body fats have only monomodal distribution pattern with only one peak between C50 and C56 (Parodi 1973, Precht 1992a, Gutiérrez et al. 2009, Rohman et al. 2012, Kala 2013, Park et al. 2014). Although, coconut and palm kernel oils do contain short chain fatty acids in substantial amount (Krishna et al. 2010, Rahman et al. 2022), but their medium chain and long chain fatty acids content is very low compared to milk fat. Therefore, their triglycerides distribution pattern is also monomodal with broad

Table 1: Equations suggested for calculation of S-values

S-values	Foreign fat(s) indicated & equation
S_1	Soybean, sunflower, rapeseed, linseed, maize, wheat germ, cottonseed & fish oil = (2.0983C30) + (0.728C34) + (0.6927C36) + (0.6353C38) + (3.7452C40) – (1.2929C42) + (1.3544C44) + (1.7013C46) + (2.5283C50)
S_2	Coconut & palm kernel fat = (3.7453C32) + (1.1134C36) + (1.3648C38) + (2.1544C42) + (0.4273C44) + (0.5809C46) + (1.2926C48) + (1.0306C50) + (0.9953C52) + (1.2396C54)
S_3	Palm oil & beef tallow = (3.6644C28) + (5.2297C30) – (12.5073C32) + (4.4285C34) – (0.2010C36) + (1.2791C38) + (6.7433C40 – (4.2714C42 + (6.3739C46)
S_4	Lard = (6.5125C26) + (1.2052C32) + (1.7336C34) + (1.7557C36) + (2.2325C42) + (2.8006C46) + (2.5432C52) + (0.9892C54)
S_5 (S_{total})	Total (*i.e.* all foreign fats in general) = – (2.7575C26) + (6.4077C28) + (5.5437C30) – (15.3247C32) + (6.2600C34) + (8.0108C40) – (5.336C42) + (0.6356C44) + (6.0171C46)

C26 to C54 represents percentage of each major triglycerides fraction containing respective number of carbon atoms in total triglycerides

Compiled from Precht (1991,1992a, 1992b), EC (2008), ISO/IDF (2019), Sharma et al. (2021)

peak between C32 and C44 (Huebner 1961, Pocklington & Hautfenne 1985, Precht 1992a, Krishna et al. 2010).

The difference in pattern of triglycerides profile of milk fat and that of the foreign fats is depicted in Figure 1. To include one foreign fat from each specific S-value (*i.e.* S_1, S_2, S_3 & S_4) soybean oil, coconut oil, bovine tallow and lard are selected as examples in diagrammatical presentation on triglyceride profile of milk fat and that of the foreign fats. In GC analysis of triglycerides for testing purity of milk fat, the differences exiting between triglyceride profile of milk fat and that of the foreign fats are exploited. The range of each S-value for pure milk fat remains in the vicinity of 100 (Table 2). When foreign fat is mixed with milk fat, it causes deviation in triglyceride profile of the milk fat beyond the natural variation, which in turn causes large shift in its S-value(s). Consequently, S-value(s) of adulterated milk fat cross the specified limit(s) and go below the lower limit or above the upper limit depending on type of foreign fat mixed (Precht 1992a, EC 2008, ISO/IDF 2019).

Operational steps involved in the method

Operation of the method involves eight sequential steps, Outline of these operational steps is compiled from different reports (Precht 1992a & 1992b, EC 2008, ISO/IDF 2010, Kala 2016, Sharma et al. 2021) and summarised below.

Extraction of fat from test sample

The fat from milk is first separated as cream and converted into butter, followed by thorough washing of the resulting butter grains. From butter fat is clarified by melting it at 50°C using a water bath, followed by filtration through folded fine-pored filter paper containing 0.5 to 1.0 g of sodium sulfate in oven maintained at 50°C. Care has to be taken that no serum is transferred. Use of Röse–Gottlieb gravimetric method or silica gel columns are suggested as an alternative method for the extraction of fat.

Preparation of fat solution

The clarified fat is completely dissolved in solvent (*n*-hexane or n-heptane). Amount of fat taken depends on type of column (packed or capillary) used and dimension of the column

Fractionation of triglycerides from the fat

The triglycerides in the fat solution separated into different fractions (i.e. groups) containing same number of carbon atoms in acyl chains of their constituent fatty acids. Fractionation of triglycerides is carried out by programable high temperature low resolution GC.

Quantification of the triglyceride fractions

Quantities of the triglyceride fractions are estimated from their respective peak areas in the chromatogram obtained from GC analysis. Mass of each triglyceride fraction is calculated as % m/m in total triglycerides plus cholesterol.

Calculation of S-values

S-values are calculated by inserting the estimated % mass of the appropriate triglyceride fractions into the standardised equations (Table 1). The results of the S-values to be expressed to two decimal places. Calculate all the S-values irrespective of the kind of foreign fat suspected. Though the S-values are calculated from percentages of triglyceride fractions, they do not represent a percentage themselves. Moreover, the S-values do not have a unit.

Comparison of the S-values with the specified limits

The five S-values obtained from the calculation are compared with their corresponding limits specified as standards (Table 2).

Interpretation of result

The results of the S-values are interpreted in accordance with their success or failure in complying the specified limits. When all five S-values calculated from analysis of the test sample fall within the specified limits, the sample is considered as pure. If one or more S-value(s) fall(s) outside the specified limits, the sample is considered as adulterated. Although individual S-values (*i.e.* S_1, S_2, S_3 & S_4) are more sensitive for certain foreign fats than the general S-value (S_{Total}), the positive result obtained in only one S-value does not allow to draw conclusion on type of foreign fat.

Table 2: Limits specified for S-values of pure fat from cow milk

S-value		Limits of the S-value
S_1	Vegetable oils[#] & Fish oil	98.05 to 101.95
S_2	Coconut fat & Palm kernel fat	99.42 to 100.58
S_3	Beef tallow & Palm oil	95.90 to 104.10
S_4	Lard	97.96 to 102.04
S_{total}	Total (General)	95.68 to 104.32

[#] Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ & Cottonseed

Compiled from Precht (1991, 1992a, 1992b), EC (2008), ISO/IDF (2010), Kala (2013), Povolo et al. (2008), Nilchian et al. (2020) and Sharma et al. (2021)

Quantification of foreign fat (if detected)

When presence of foreign fat is detected in milk fat (*i.e.* at least one of the S-value exceeds the specified limit), its quantity is estimated. Quantity of the foreign fat (% m/m) is calculated using the following formula.

$$\text{Foreign fat in milk fat (\%, m/m)} = \frac{(100 - S)}{(100 - S_f)} \times 100$$

Where, **S** is S-value of the test sample corresponding to the foreign fat detected and $\mathbf{S_f}$ is constant depending on type of foreign fat detected. In other words $\mathbf{S_f}$ is S-value of foreign fat detected in test sample. Its value may be established by analysing the foreign fat or may be taken from literature. In actual practice, particular foreign fat generally remains unknown, since most of the foreign fats are identified by the method as a group and not as a particular foreign fat (except lard). Same problem also arises when blend of foreign fats is mixed in milk fat. Therefore, when foreign fat is not known, $\mathbf{S_{total}}$ is taken **S** and value of $\mathbf{S_f}$ is taken as 7.46 in the formula for calculating quantity of foreign fat (EC 2008, ISO/IDF 2010 & 2019).

Advantages of the method

In testing purity of milk fat by using this method has number of unmatched advantages over the conventional destructive methods. These advantages of the method are compiled from several reports (Precht 1991, 1992a & 1992b, Povolo et al. 1999, Molkentin & Precht 2000, Kamm et al. 2001, de la Fuente & Juarez 2005, Fontecha et al. 2006, Molkentin 2007, Kala 2013, Amaral et al. 2018; Csapó et al. 2019, Cossignani 2019, ISO/IDF 2019, Shinde et al. 2020, Pathania et al. 2020 & 2021) and summarized here.

(1) Highly comprehensive: able to detect entire range of foreign fats including

(a) Plant oils and fats: soybean, sunflower, rapeseed, linseed, maize, wheat germ, cottonseed, coconut, palm kernel, palm and hydrogenated vegetable oil

(b) Animal body fats: Bovine tallow, lard and fish oil

(c) Blends of plant oils as well as fats and/or animal body fats in any combination

(2) Able to quantify the foreign fat or blend of foreign fat with fair degree of accuracy

(3) Better sensitivity: limit of detecting the foreign fat in milk fat ranges from

(a) 2.0 to 5.4%, when specific S-value (i.e. S_1, S_2, S_3 or S_4) employed
(b) 4.0 to 6.1%, when general S-value (i.e. S_{Total}) employed

(4) High reliability: limit of confidence in detection 99% and in quantification 95%

(5) Non-destructive in nature, hence information obtained is much higher

(6) Simple and rapid compared to conventional destructive methods

(7) Except solvent (for dissolving fat), no other chemical is required

(8) Applicable to some milk products e.g. cream, butter, ghee and milk powder

(9) Recognised and accepted internationally as a reference method

Pathania et al. (2021) clarified samples of butter from cow milk as well as buffalo milk at 110, 130 and 150°C and found that there was no significant difference in the content of different triglycerides in ghee on account of temperature of clarification both in case of cow ghee and buffalo ghee. They also stated that their finding can be extrapolated to the fact that temperature of clarification used to prepare ghee from butter will not affect S-limits of milk fat. Thus, it is very fortunate that the method may be applied to test purity of ghee irrespective of temperature employed in the process of clarification.

It has been opined that as on date GC analysis of triglycerides as suggested by EC (2008) and ISO/IDF (2010) is highly effective, most reliable and irreplaceable analytical tool to verify authenticity of fat present in milk as well as in some of the milk products (Bosque-Sendra et al. 2012, Cossignani 2019, Csapó et al. 2019).

Limitations of the method

Though the method is very versatile, its applicability is restricted in specific circumstances due to possibilities for occurrence of false positive results. The circumstances which give rise to false positive results are compiled from different reports (Precht 1992a & 1992b, Battelli & Pellegrino 1994, Precht et al.1998, Kamm et al. 2001, Molkentin 2006, 2007 & 2013, EC 2008, Amaral et al. 2018, ISO/IDF 2019, Pathania et al. 2020, Sharma et al. 2021) and listed here.

(1) Since false positive result may occur, one or more limits of S-values specified in the method may not be applicable to fat obtained from:

(a) Milk of animal species other than cows (*e.g.*, buffalo, goat, sheep, etc.)

(b) Milk of single cow

(c) Milk of cows receiving oilseeds in diet (*e.g.*, cottonseeds)

(d) Milk of cows suffering from acute underfeeding (*i.e*., starvation or malnutrition)

(e) Post-parturient milk (*i.e*. colostrum)

(f) Milk subjected to treatment for removal of cholesterol

(g) Fractionation for alteration in specific properties (*e.g*., softening, melting, etc.)

(h) Skim milk, buttermilk or whey and

(i) Products undergone extensive lipolysis (fat acidity >8 mmol/100 g of fat)

(2) Since phospholipids and partial glycerides overlaps with triglycerides containing short chain fatty acids, hence their presence in large amounts may interfere with the results. Therefore, procedure used for extraction of fat from the test sample is another restriction in applicability of the method.

It is recommended that the fat for testing its purity to be obtained from butter by melting it at 50°C. Therefore, hardly any phospholipids remain present in the test samples of fat. If other extraction methods are used, sufficient care has to be exercised so that the amounts of phospholipids and partial glycerides remain to a minimum (Precht et al.1998, Molkentin 2006 & 2008).

Among the above listed circumstances responsible for false positive results, feeding of lactating milch animals and species of the milch animals are most relevant to some of the Asian countries in general and India in particular. Hence their role in causing the false positive results is covered in depth under separate sub-title 'major causes of false positive results in method'.

Evaluation of the Method

Kala (2013) undertook a study to test the applicability of the method in detection of selected foreign fats in ghee. Adulterated samples of ghee were prepared from control ghee made in the laboratory from mixed milk. The adulterated samples were prepared by mixing beef tallow (@ 2.0 & 6.56%), partially hydrogenated vegetable oil (@ 2.0 & 5.0%), lard (@ 2.0 & 6.27%) and coconut oil (@ 2.0%) in control samples of ghee. The data reported on S-values of adulterated ghee samples suggest that not only the samples containing foreign fats at higher rates, but those containing 2.0% of foreign fat also failed to comply two or more limits of S-values. It proves capability of the method to detect adulteration at the rate as low as 2.0%.

Kala et al (2016) evaluated purity of fat in ghee based sweets using the method, taking *Mysore pak* as one of example of the sweets. Eight market sample of the ghee based sweets were analyzed and compared with laboratory prepared control samples of the sweet. The control samples of sweet were prepared from ghee procured from dairy and also from ghee prepared in laboratory. Fat was extracted from the samples of sweet gravimetrically after removing added spices and nuts. The extracted fat was analysed by the method to get value of its S_{Total}. Examination of the data reported on value S_{Total} suggest that the fat extracted from control samples of sweets complied the limits specified for S_{Total}. Whereas, in case of fat extracted from eight market samples of sweet, seven samples failed in complying the limits of S_{Total}. These results suggest that the method can also be employed in testing purity of ghee used in preparation of tradition Indian sweets like *Mysore pak*.

Shinde et al. (2020) evaluated the method to detect adulteration in ghee. Samples of pure ghee were adulterated with coconut, soya bean, groundnut and sunflower oil @ 1, 2.5, 5, and 10%. The authors found that the method was capable to detect adulteration @ 5% for all the oils, except groundnut which was detected @ 10%. The probable reason for high limit of detection in case of ground oil may be attributed to selection of S-value employed for its detection. In the detection ground oil the authors have employed S_{total}. According to report from Pocklington and Hautfenne (1985), the triglyceride profile of groundnut oil closely resembles to that of the soya bean oil. Therefore, looking to the similarity in triglyceride profile of groundnut oil to that of the soya bean oil, S-value corresponding to soya bean oil might be better option to detection of groundnut oil rather than S_{total}.

Pathania et al. (2020) conducted a study on use of the method to detect highly manipulated fat (*i.e.* designer fat) mixed in ghee as an adulterant. Using limits of S-values specified by ISO/IDF (2010) for cow milk fat. The minimum level for detection of the highly manipulated fat was 7.5%. The authors stated due to lack of the limits specified for S-values for buffalo milk fat, detection of the adulterant fat in buffalo ghee was not possible. Their finding has proved the capability of the method to detect highly complex admixture of foreign fats in cow milk fat with a fairly high degree of sensitivity. Therefore, this report serves as a testimony on robustness of the method.

Nilchian et al. (2020) opined that the detection of animal body fats such as tallow in butter by conventional parameters of purity is more difficult compared to vegetable fats. They mixed tallow in butter @ 0 to 15% and analysed the samples for fatty acids, triacylglycerols and conventional physicochemical parameters. The found that physicochemical parameters and fatty acids could not indicate the adulteration up to 5% level. However, S_1-, S_3-, S_{Total} could detect the adulteration at 5% level. The use of fatty acids and triacylglycerols in combination resulted in the capability to detect the adulteration above 1%.

Secret behind the success of the method

The unmatched advantages of the method may be attributed to way in which (1) idea conceived to use information laying in intact triglycerides, (2) designed of S-values decided, (3) procedure devised to get the S-values, (4) specifications on limits of S-values fixed and (5) natural variations in triglyceride profile of milk fat taken care of. These virtues of the method are compiled from various reports (Precht 992a, Povolo et al. 1999, Molkentin & Precht 2000, Kamm et al. 2001, de la Fuente & Juarez 2005, Fontecha et al. 2006, Molkentin 2007, EC 2008, Amaral et al. 2018; Csapó et al. 2019, Cossignani 2019 and ISO/IDF 2010, 2019) and briefly summarised below.

(1) The idea conceived for utilising inherent information present in intact triglycerides of milk fat is so meticulous that it gives highly robust criterions for testing purity of milk fat. The equations of S-values are designed so carefully that even on mixing different foreign fats with milk fat in any combination, the S-values of adulterated milk fat vary only in one direction. Hence, manipulation of S-values becomes extremely difficult, which makes method highly robust against manipulation.

(2) The designs of five S-values are decided so tactfully that the method capable to detect entire range of foreign fats both from plants and animals origin as well as their blend with high degree of confidence. Even complex admixture of foreign fats can be detected with fair degree of sensitivity. As a result the method can serve as highly comprehensive tool, since it single handedly detects wide variety of foreign fats in milk fat.

(3) Analytical procedure devised to get S-values of test sample is so simple and rapid that all the five S-values can be obtained just from a single run of the GC machine within 30 minutes. The sample of fat extracted from milk or milk product to be just dissolved in solvent and injected in GC machine. Thus, the method is non-destructive, simple, convenient and rapid.

(4) Specifications on limits of all the S-values are fixed in form of a range. Consequently, for each S-value both minimum value and maximum value to be complied, rather than only minimum value or maximum value. The five S-values, each with two limits (lower and upper) leads to formation of ten (5x2) criterions within the single method for testing purity of milk fat.

(5) Effect of natural variations in triglyceride profile of milk fat on efficiency of the method is almost eliminated due to appropriate care taken in designs of the equations employed in calculation of the S-values. It appears that the designs of equations automatically compensating the natural variations occurring in triglyceride profile of milk fat due to normal variations in feed given to the lactating cows, season of the year, breed of the cows and stage of their lactation as well as regional variations. This became possible due to use of very sound database generated for development of the equations. While gathering the database over a period of five years effect of all these factors was very well taken into conidiation.

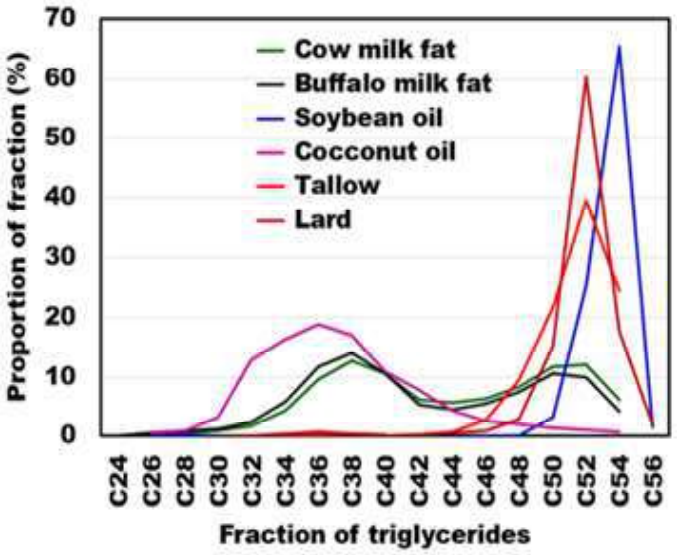

Fig. 1 Pattern of triglyceride profile in milk fat and foreign fats

Data on triglycerides of cow milk fat and buffalo milk fat are compiled from Kala (2013), Hazra et al. (2017), Sharma et al. (2018) and Pathania et al. (2021). Whereas, data on triglycerides of foreign fats are compiled from Parodi (1973), Pocklington & Hautfenne (1985), Precht (1992a), Gutiérrez et al. (2009), Kala (2013) and Park et al. (2014).

Common reasons of false positive results in the method

The S-values of milk fat are calculated from percentage of different triglyceride fractions present in it. In oils and fats fatty acids being integral parts of triglycerides, chain length of the constituent fatty acids determines the number of carbon atoms in their triglyceride molecules. Therefore, in milk fat percentage of short chain (C4 to C14), medium chain (C16:0 & C16:1) and long chain (C18:0, C18:1 and C18:2) fatty acids dictates percentage of its triglyceride fractions and hereby in its S-value. Consequently, wide variation in content of these fatty acids in milk fat leads wide variation in its S-value and there by causes false positive results in the method. Hence, to get idea about causes of the false positive results, it is necessary to understand factors responsible for variation in fatty acids composition of milk fat during its synthesis in mammary gland. To simplify explanation of this complex biological phenomenon, sources for supply of different fatty acids in biosynthesis of milk fat is schematically presented in Figure 2.

For biosynthesis of milk fat in ruminants there are two basic sources of fatty acids: (1) *de novo* synthesis in mammary gland

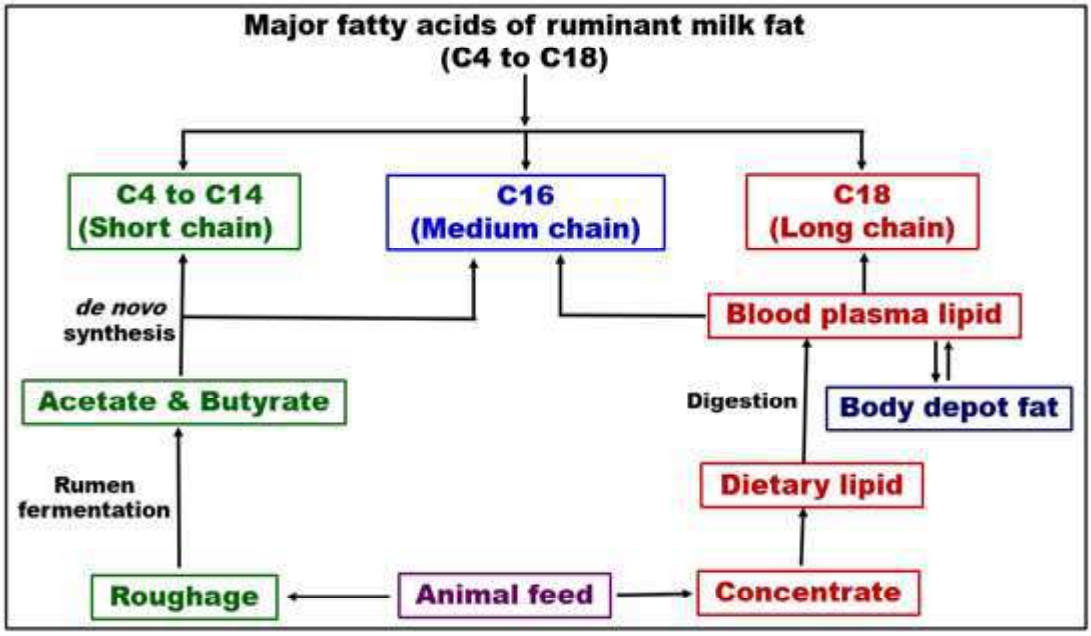

Fig. 2 Sources of different fatty acids for biosynthesis of milk fat in ruminants

Conceptualised from description given by Hawke & Taylor (1983), Grummer (1991), Bear (1991), Palmquis (2006) and Fox et al. (2015)

from acetate and butyrate supplied by rumen fermentation of roughage and (2) uptake from circulating blood plasma lipids which arises from dietary lipid or depot body fat. There is a vast difference in chain length of fatty acids obtained from these two sources. Short chain fatty acids are derived exclusively from the *de novo* synthesis. Whereas, preformed long chain fatty acids are derived exclusively by uptake from circulating blood plasma lipids. However, medium chain fatty acids are derived partly from the *de novo* synthesis and partly from the circulating blood plasma lipids. Proportion of fatty acids from these two sources in biosynthesis of milk fat is greatly influenced by type of feed given to lactating milch animals in their diet and also by species of the milch animals (Hawke & Taylor 1983, Grummer 1991, Bear 1991, Palmquis, 2006). Therefore, variation in type of feed given to lactating milch animals and change in species of the milch animals are generally responsible for occurrence of false positive results in the method.

Type of feed given to lactating ruminants

Table 3: Effect of cottonseed feeding on fatty acid profile of cow milk fat

Sr. No.	Major fatty acids of milk fat	Fatty acids (%)		Difference (CSD – NSD)
		CSD	NSD	
1	Short chain (C4 to C14)	22.76	25.99	–3.23
2	Medium chain (C16:0)	26.81	29.33	–2.52
3	Long chain (C18:0, C18:1, C18:2 & C18:3)	41.62	34.38	+7.24
4	Others (?)	7.93	9.20	–1.27

CSD = Milk fat from cotton seed supplemented diet
NSD = Milk fat from non-supplemented diet (*i.e.* normal or control diet)
Computed from Contarini et al. (1996)

Table 4: Effect of cottonseed feeding on triglyceride profile of cow milk fat

Sr. No.	Major triglyceride fractions of milk fat	Triglyceride fraction (%)		Difference (CSD – NSD)
		CSD	NSD	
1	C28 to C48 (except C40)	58.41	65.37	–6.96
2	C40 + (C50 to C54)	41.56	34.63	+6.93

CSD = Milk fat from cotton seeds supplemented diet
NSD = Milk fat from non-supplemented diet (*i.e.* normal or control diet)
Computed from Contarini et al. (1996)

Most of the plants oils and all animals body fats comprising of long chain fatty acids, followed by medium chain fatty acids and no or negligible short chain fatty acids. Therefore, supplementing any of these oils or fats in feed of lactating ruminant increases the level of long chain fatty acids in blood plasma and their uptake by lactating mammary gland. Elevated level of long chain fatty acids in mammary gland inhibits the *de novo* synthesis of short chain and medium chain fatty acids. As a consequence content of short chain and medium chain fatty acids in milk fat decreases, with simultaneous increase in its long chain fatty acids content (Hawke & Taylor 1983, DePeters et.al. 2001, Onetti et al. 2002, Palmquist 2006). Similar phenomenon also occurs during starvation or acute under feeding due to mobilisation of depot body fat from adipose tissues into blood plasma for compensating shortage of short fatty acids in biosynthesis of milk fat (Precht1991). Such changes in fatty acids composition of milk fat leads to corresponding changes in triglyceride profile of the milk fat.

Effect of supplementing whole cottonseeds in diet of lactating cows on fatty acid composition of their milk fat and associated changes in its triglyceride profile can be realised from excellent work reported by Contarini et al. (1996). Differences in short chain, medium chain and long chain fatty acids contents of milk fat from cows received cottonseed supplemented diet and that from cows received non-supplemented (normal) diet are computed from this repot and presented in Table 3. In the same way differences in contents of different triglycerides fractions also computed and presented in Table 4. The changes in fatty acid profile suggest that on supplementing the cottonseed in diet of lactating cows, contents of short chain and medium chain fatty acids decrease substantially with concomitant increase in long chain fatty acids in their milk fat. Similarly, the changes in triglyceride profile indicate that on supplementing the cottonseed in diet of lactating cows, contents of triglycerides containing lower number of carbon atoms (C28-C48, except C40) decrease considerably with simultaneous increase in triglycerides containing higher number of carbon atoms (C50-C54, together with C40) in their milk fat. The authors concluded that the ratios of different fatty acids cannot be used as indices of purity for butter made from milk from cows fed whole cottonseeds. Therefore, same logic obviously to be hold true in use of triglycerides ratios and S-values in testing purity of milk fat. Because changes in S-values of milk fat is but natural on supplementing the cottonseed other oilseed in diet lactation ruminant.

Contarini et al. (2014) also evaluated efficacy of the EU reference method in testing purity of milk fat from cows kept on mountain feeding. All the samples showed characteristic fatty acid composition of milk fat from mountain pasture-fed cows: high content of linolenic acid, vaccenic acid, *cis*-9, *trans*-11 conjugated linoleic acid and low concentration of saturated fatty acids. All the samples produced false positive results for at least one of the five S-values. The authors suggested that in order to avoid false charges of adulteration, this behaviour should be considered by competent authorities while applying the method to test purity of milk fat.

Alonso et al. (2022) studied fatty acids and triglycerides profile of milk fat from cow receiving ecological v/s conventional pasture in northern of Spain. The author found that among different fatty acids, medium chain fatty acid (C16:0) was significantly lower in milk fat from cow fed ecological pasture, compared to conventional pasture. On the other hand, the long chain fatty acid (C18:1, C18.2, C18.3 & C18:2 c9*t*11) were significantly higher in milk fat from cow fed ecological pasture, compared to conventional pasture. Concomitant significant changes occurred in triglyceride profile of milk fat. Triglycerides containing C40 was significantly lower and those containing C44, C46, C48, C50, C52 and C54 were significant higher in milk fat from cow fed ecological pasture, compared to conventional pasture.

The above reports clearly indicate that following special feeding practices in feeding of ruminants such as feeding oilseeds or their by-products, mountain grasing or feeding ecological pasture result into drastic change in triglyceride profile of their milk fat thereby in its S-value(s), which ultimately leads to false positive results. Thus, the false positive results on testing pure fat from milk lactating ruminants receiving cottonseed or other oilseeds or their by-products like meal and cake in diet is but obvious. This limitation of the method is very clearly reported by ISO/IDF (2010, 2019).

Species of lactating ruminants other than cow

Table 5. Comparative appraisal on triglyceride profile of buffalo and cow milk fat

Triglyceride fraction	Triglycerides fraction (%)[#]		Difference (B – C)
	Buffalo milk fat (B)	Cow milk fat (C)	
C24	0.11	0.10	+0.01
C26	0.55	0.34	+0.21
C28	0.82	0.53	+0.30
C30	1.12	0.90	+0.22
C32	2.40	1.77	+0.63
C34	5.81	4.41	+1.39
C36	11.77	9.55	+2.22
C38	14.02	12.76	+1.26
C40	10.55	10.53	+0.03
C42	5.27	6.20	–0.94
C44	4.51	5.64	–1.13
C46	5.44	6.36	–0.92
C48	7.49	8.29	–0.81
C50	10.64	11.70	–1.06
C52	9.90	12.04	–2.14
C54	4.12	6.07	–1.95

[#] Calculated from Kala (2013), Hazra et al. (2017), Sharma et al. (2018) & Pathania et al. (2021)

In biosynthesis of milk fat proportion of fatty acids used from *de novo* synthesis and that from blood plasma lipids also depends on species of the lactating ruminants (Hawke & Taylor 1983). As a result triglyceride composition of fat from milk of different species will vary and lead to variation in S-values of their milk fat. Differences in triglyceride composition of milk fat from different species and in their S-values are briefly summarised below.

Differences in triglyceride profile of milk fat in ruminants species

Triglyceride composition of fat from milk of various mammalian species (cow, buffalo, sheep, goat, donkey, camel, and/or horse) are reported by Achaya and Hilditch (1950), Breckenridge and Kuksis (1967), Addeo and Kuzdzal-Savoie (1980), Fontecha et al. (1998), Povolo et al. (2008), Goudjil et al. (2003), Smiddy et al. (2012), Tolentino et al. (2015), Bononi et al. (2017) Cossignani (2019) and/or Peyma et al. (2022). This literature suggests wide variation in triglyceride composition of milk fat obtained from different mammalian species.

Triglyceride composition of fat from Indian buffalo milk and that from cow milk has been reported by Kala (2013), Hazra et al. (2017), Sharma et al. (2018) and Pathania et al. (2021). An average triglyceride composition of cow milk fat and that of buffalo milk fat is computed from those reported in publications by these authors. Differences in triglyceride composition of cow milk fat and buffalo milk fat are worked out from their respective average composition derived from the computation and presented in Table 5. The noticeable difference in triglyceride profile of fat of cow milk and that of the buffalo milk is clearly evident. The differences triglyceride profile confirm that contents of triglycerides possessing C24 to C40 is higher buffalo milk fat, whereas, contents of triglycerides possessing C42 to C54 is higher cow milk fat. Therefore, occurrence of differences in S-values of their milk fat is but obvious.

De la Fuente and Juarez (2005) also opined that due to the differences in triglyceride profiles of goat and ewe milk fat compared that of the cow milk fat, the equations proposed by EU regulations are not suitable to monitor purity of goat or ewe milk fat. Therefore, to detect foreign fat in goat and ewe milk fat new multiple regression equations based on their triglyceride profile were developed. Fontecha et al.(1998) proposed two multiple regression equations for detecting mixtures of non-milk fats in goat milk Similar work was carried for ewe milk fat by Goudjil et al. (2003).

Differences in S-values of milk fat from different ruminants species

Romano et al. (2004) carried out work to determine suitability of the official EU method to buffalo milk fat. Their results show that it is necessary to modify the limits of S-values specified in Precht method to determine the purity of buffalo milk fat. Povolo et al. (2008) also carried out investigation on sheep, goat and buffalo milk fat and confirmed that the S ranges, calculated for cow milk fat, are not applicable to the evaluation of genuineness of non-

Table 6: S-values of ghee prepared out of milk collected from farm of the institute

S-value		S-value of ghee from		
		Cow milk[#]	Mixed milk[#]	Buffalo milk[#]
S_1	Vegetable oils[*] & fish oil	100.96	101.47	101.97
S_2	Coconut & Palm kernel fat	99.62	99.10	98.58
S_3	Beef tallow & Palm oil	104.08	107.69	111.29
S_4	Lard	99.43	98.68	97.92
S_{Total}	Total	104.11	107.58	111.05

[*] Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ & Cottonseed
[#] Compiled from Pathania et al. (2020)
[#] Calculated from values of cow & buffalo by average of their respective values

Table 7: S-values of ghee prepared out of milk collected from different regions of India

S-value		S-value of ghee from			
		Cow milk		Buffalo milk	
		Four regions*	Western region	Four regions*	Western region
S_1	Vegetable oils[#] & fish oil	92.82-103.08	97.17-101.77	96.12-103.27	97.20-102.70
S_2	Coconut & Palm kernel fat	99.15-101.41	99.15-100.19	97.75-101.35	98.60-00.78
S_3	Beef tallow & Palm oil	89.53-107.25	97.32-106.48	95.60-117.37	99.58-14.32
S_4	Lard	96.52-105.88	98.16-101.84	94.47-104.81	96.75-101.13
S_{Total}	Total	90.59-106.34	97.27-105.93	94.06-118.21	96.83-112.37

[#] Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ & Cottonseed
*Four regions = Eastern, Western, Northern and Southern
Compiled from Sharma et al. (2021)

bovine milk fats. In analysis of buffalo milk fat these authors found the values of S_1, S_2, S_3, S_4 and S_{Total} as 100.18 (±0.36), 98.88 (±0.13), 107.39 (±0.24), 99.56 (±0.36) and107.46 (±0.55) respectively. Their results indicate that buffalo milk fat failed to comply lower limit of S_2 and upper limit of S_3 as well as S_{Total}.

Pathania et al. (2020) reported work on novel approach to detect highly manipulated fat adulterant in ghee (clarified butter) through signature peaks by GC analysis of its triglycerides. The S-values of pure ghee from cow and buffalo milk are compiled from the results reported by these authors and presented in Table 6. The data suggest that all the five S-values of cow ghee were very well within the limits specified as standards by ISO/IDF (2010). However, in case of pure buffalo ghee, all the five S-values fall out sides the range specified in the standards. Thus, all the five S-values of ghee from buffalo milk failed to comply the specified limits. The authors opined that the limits specified for S-values of cow milk fat are not suitable for the detection of adulterants in ghee made from buffalo milk.

Sharma et al. (2021) conducted a comparative study on the S-values of ghee from cow milk and buffalo milk. The samples of milk were collected from eastern, western, southern, and northern regions of India. The authors reported that in all the four regions all the five S-values of buffalo ghee as well as cow ghee deviated widely from the limits specified by ISO/IDF (2010, 2019). Variation in S-values of cow ghee and buffalo ghee in western region and overall in pooled data of all the four regions are compiled and presented in Table 7. The variations S-value are very large in the pooled data. The rate of compliance by the results to the specification of S-values is most important aspect in this type of study. Such information is required to get correct idea about prospects of the method for its implementation in India and about modifications required in particular limits of specific S-values. However, the rate of compliance by the results not reported by these authors.

Prajapati and Aparnathi (2021) conducted a project work for evaluation of database on S-values of milk fat collected by GCMMF, Anand (Gujarat). The database was collected by analysing 21552 samples of bulk mixed milk over a period of two years. The samples were generally drawn from road tankers containing 10000 to 200000 liters of mixed milk. The main aim in evaluation of the database was to find rate of success in compliance by fat in the milk samples to limits specified by ISO**:** 17678 in the method. The rates of compliance found in samples of low fat milk (≤ 4.0%), medium fat milk (4.01 to 5.99%) and high fat milk (≥6%) are presented in Table 8. The data revealed that the best rates of compliance (>96-100%) are found in samples of low fat milk, which may be attributed to high proportion of its cow milk content. In samples of medium fat milk and high fat milk the rates of compliance for S_1, S_2 and S_4 remained almost ≥ 95%. The rate of compliance for S_{Total} in samples of medium fat milk and high fat milk remained around 93%. In samples of medium fat milk and high fat milk only S_3 has the lowest rate of compliance, which remains in vicinity of 80%. Thus, except S_3 rates of compliance in other four S-values are very promising for their use in application of the method in testing purity of fat in bulk mixed milk in Gujarat.

The rates of compliance observed by Prajapati and Aparnathi (2021), in samples of low fat milk while evaluating the large database, are completely in agreement with S-values of cow ghee and buffalo ghee as reported by Pathania et al. (2020) who obtained the milk samples from institute farm and Sharma et al. (2021) who obtained the obtained the milk samples from western region of India. Similarly, the rates of compliance in medium fat milk and high fat milk are corroborating very well with those reported by Povolo et al. (2008) for S-values of buffalo milk fat.

One of the typical reasons for wide variation in S-values of milk fat reported within the same study or reported between different studies might be attributed to differences in bulk of milk at the time of sampling. The method is basically meant for testing purity of bulk milk, since Prech developed this method using the

Table 8: Compliance rate of milk fat to its specifications on S-values in large database collected by industry

S-value		Samples of milk fat under each category (%)								
		Low fat milk			Medium fat milk			High fat milk		
		BLL	WBLs	AUL	BLL	WBLs	AUL	BLL	WBLs	AUL
S_1	Vegetable oils[#] & Fish oil	0.56	99.44	0	1.10	98.89	0.01	5.02	94.97	0.01
S_2	Coconut & Palm kernel	1.12	98.88	0	4.03	95.78	0.19	3.35	96.19	0.28
S_3	Palm oil & Beef tallow	0	96.07	3.93	0.04	77.80	22.16	0	80.16	19.84
S_4	Lard	0	100	0	0.05	99.93	0.02	0.15	99.72	0.13
S_{Total}	Total	0	98.31	1.69	0.14	92.60	7.26	0.19	93.29	6.52
Total number of samples (21552)			178			11772			9602	

BLL = below lower limit (Fail), WBLs = within both limits (Pass) and AUL = Above upper limit (Fail)

[#]Soybean, Sunflower, Olive, Rapeseed, Linseed, Wheat germ, Maize germ and Cottonseed

Compiled from Aparnathi and Prajapati (2021)

database obtained wherein samples were drawn from bulk milk (Prech 1991, 1992a). Therefore, samples of milk obtained from single cow may leads to false positive results (ISO/IDF 2010, 2019). Similar phenomenon of false positive result may likely to occur when sample is drawn from a very limited bulk of milk. Sharma et al. (2021) also expressed the similar views for variations in S-values of milk fat. The authors stated that it is likely that cow ghee produced commercially by dairy plants meets the limits of S-value specified in the method. In dairy industry ghee is produced from pooled milk collected from large area in chain of milk collection, this nullifies the variations arising from milk of individual cows or a particular route in milk collection.

The forgoing resume of various reports clearly reveals that differences in triglycerides composition of fat from cow milk and that from buffalo milk do exists. These inherent differences in triglycerides composition of their milk fat may create differences in their S-values. Therefore, when specification on S-values meant for cow milk fat are applied to buffalo milk fat may causes false positive results. This limitation of the specification of the method is very clearly and categorically admitted in the method (ISO/IDF 2010, 2019).

From careful and keen examination of the data on (1) S-values of fat in buffalo milk reported by Povolo et al. (2008), (2) S-values of ghee prepared from cow milk and that from buffalo milk reported by Pathania et al. (2020), (3) S-values of ghee from mixed milk computed by averaging respective vales of buffalo ghee and cow ghee reported by Pathania et al. (2020), (4) S-values of ghee prepared from cow milk and that from buffalo milk in western region of India reported by Sharma et al. (2021) and (5) rate in complying S-values of milk fat to limits specified in the method as observed by Prajapati & Aparnathi (2021), the following inferences may be drawn regarding applicability of the limits on various S-values specified in the method for testing purity of fat.

- Major changes in upper limits of S_3 (palm & beef tallow) and S_{Total} (General) are required for application of the method.
- Minor changes in lower limits of S_1 and S_2 (Soy bean oil & other oils) and S_2 (coconut fat & palm kernel fat) are required for application of the method.

After establishing the above referred changes in limits of S-values method may be used very well for testing purity of fat in bulk milk prevailing in Gujarat. However, the problem of false positive results on feeding cottonseed to lactating cows and buffalo in application of limits on S-values specified in the method remains unresolved and to be tackled separately.

Mixing milk of other ruminants species with cow milk

It is also reported that even mixing of milk from other species to cow milk may also lead to a false positive result (Molkentin 2007). Unfortunately, data on S-values of pure fat from mixed milk or pure ghee prepared from mixed milk are not reported in literature. Hence, direct comparison between S-values of fat from cow milk and that from mixed milk is not possible. As an alternate, S-values of ghee from mixed milk are computed from S-values of ghee prepared from buffalo milk and that from cow ghee as reported by Pathania et al. (2020). S-values of ghee from mixed milk are derived by averaging respective S-values of cow ghee and buffalo ghee, assuming their proportion as 1:1 in mixed milk (Table 6). The computed S-values of ghee from mixed milk suggest that among the five values, it failed to comply the specifications on lower limit of S_2 and upper limits of S_3 and S_{total}. These observations are in complete agreement with the opinion expressed by Molkentin (2007).

Perspectives on use of the method in India

In view of practices presently followed in Indian dairy farming system, there are three factual problems of serious concerned in implementing specifications on limits of S-values suggested in the method. These problems include (1) role of buffalo as a major contributor in Indian milk production, (2) practice of mixing of buffalo milk with cow milk and (3) feeding cottonseeds to lactating animals. Each of the three situations may give rise to possibility of false positive results. Therefore, question arises that how to make use of this versatile method in India?

Role of buffalo as a major contributor in Indian milk production

It has been already discussed that substantial difference triglycerides profile of buffalo milk fat and that of the cow milk fat, which causes concomitant differences in S-values of their milk fat and ultimately leads to false positive results. In present system of Indian dairy farming, buffalo is a major milk producing animal. Contribution wise buffalo ranked second in the world milk production with an annual growth rate of 3.5%, compared to 2.1% in the cow milk production. Buffalo has contributed >13% in world, >35% in Asian and >50% in Indian milk production (Balhara et al. 2017). Therefore, possibility of false positive results in the samples of genuine buffalo milk fat is the foremost concern in implementing specification on S-values of the method in some Asin countries in general and in India in particular. Therefore, this fact needs consideration in implementing the method in India for testing purity buffalo milk fat.

Mixing of buffalo milk with cow milk

As mentioned in earlier section, not only buffalo milk, but its mixing with cow milk may also leads problem of false positive results. In present system followed by organized dairy sector for milk collection and its transportation, mixing of buffalo milk with cow milk has become very usual practice. At village milk collection centres cow milk and buffalo milk are generally pooled after collection and stored in bulk milk chilling (BMC) unit. The milk from BMC units of nearby villages is pooled in a road tanker for

transporting it to dairy plant. Therefore, under present system used in collection and transportation, milk received by the organized Indian dairy sector is mostly in form mixed milk, containing buffalo milk and cow milk in different proportion, depending on population of the two species existing in different areas of milk collection. Consequently, possibilities of false positive result in fat from genuine mixed milk is also a matter of serious concerned in India and requires due consideration in implementing the method.

Feeding cottonseeds to lactating cows and buffaloes

It has been established that the feeding cottonseed to lactating cows completely change the of fatty acid profile of their milk fat, thereby concomitant changes in its triglyceride profile of their milk fat. Hence, limits of S-values specified for pure milk in the method may not be applied to milk fat obtained from cows whose diet contain high amount of cottonseed, since it may lead to false positive results. In many parts of India, feeding cottonseeds and/or their by-products such as meal and cake to lactating cows and buffaloes is very common practice. Therefore, the practice of feeding cottonseeds or their by-products is also a serious concern in implementing the specifications of the method on S-values. Even issue of this feeding practise will create a serious problem while forming Indian specifications on limits of S-values. The effect of feeding cottonseed requires consideration in implementing the method in India for testing purity milk fat, obtained from cows and buffalo receiving cottonseed or their byproducts in diet.

It appears from the literature survey that effect of feeding cottonseeds to lactating ruminants on S-values of their milk fat been not reported so far. However, its effect on fatty acid composition of milk fat and consequent changes in physicochemical parameters of the milk fat are given due considerations by AGMARK as well as FSSAI while formation of specification on parameters used in purity of Ghee. This fact is evident from the specification formed on relevant parameters of ghee by AGMARK (Table 9) and that by FSSAI (Table 10). Now FSSAI specifications of ghee on RM value and BR reading are made uniform for the entire country (FSSAI, 2021), considering variations in these parameters in different parts. Somewhat similar considerations are also required while forming Indian specifications on S-values of milk fat for adopting the method in India as an official reference.

Use of the method under present Indian situation

Formation of Indian specifications on S-values of pure milk fat and their adoption as legal standard is very long process and may take several years. Therefore, under present Indian situation an immediate solution for use of the method could be its adoption as a screening tool, by using the specification partially and/or modified as per the local requirement. For modification in the specification of S-value, its limits may be changed as per requirements to suite the pure milk received from the supply. The changes required in limits of S-values may be established by testing genuine samples of milk received from concerned area of milk collection by dairy organisation.

In the use of specifications in partial or modified from it is advisable that doubtful samples found in analysis to be counterchecked through testing it by suitable complimentary analytical technique(s) such as fatty acid profile, β-sitosterol test or any other technique as deemed suitable. Such counter verification of positive samples by suitable complimentary analytical technique(s) may also be adopted to resolve disputes about positive samples. ISO/IDF has also advocates such counter verification of doubtful cases by using suitable complimentary method(s) of analysis (ISO/IDF 2010, 2019).

In cases, where the method is adopted just as a screening or specifications on S-values are not implemented in total, it is very essential to continuously update the database, perform its

Table 9: Agmark specifications on BR reading, RM value & Polenske value of ghee

Parameter	Areas other than cotton tract	Cotton tract area	
		Winter	Summer
BR reading at 40°C	40.0 to 43.0	41.5 to 44.0	42.5 to 45.0
RM value (minimum)	28	23	21
Polenske value	1 to 2	0.5 to 1.2	0.5 to 1.0

Compiled from Agmark (1991)

Table 10: FSSAI specifications on BR reading and RM value of ghee

Parameter	FSSAI (2017)		FSSAI (2021)
	Area other than cotton tract[#]	Cotton tract area	
BR reading at 40°C	40.0-43.0, 40.0-43.5, 40.0-44.0 or 41.0-44.0	41.5 to 45.0	41.0 to 44.0
RM value (min)	28, 26 or 24	21	24

[#] In areas other than cotton tract BR reading & RM value vary from area to area

Compiled from FSSAI, (2017, 2021)

statistical analysis and have a perusal of results. This exercise at regular interval of every one year may provide very important clues for improving use of the method. The changes in S-values over the years may also give indications about possibles weaknesses existing in adopting only partial specifications on limits of S-values. If any weakness is noticed, appropriate measures to be taken to prevent the misuse of the weakness.

Work required to adopt the method as official reference in India

The limits of S-values specified in the method were exclusively developed on the basis of variations found in triglyceride composition of fat from cow milk, that too only from European countries (Molkentin 2007, Povolo et al. 2008, Cottenet et al. 2011). Even in international collaborative study on validation of the method, carried out by Molkentin and Crawford (2009), samples of milk fat from Asian countries were not included. Thus, limits of S-values specified in the method are meant for testing purity of cow milk fat only. Hence, before official adoption the method in India it is essential to (1) ascertain limits of S-values for pure fat from milk of Indian cows and buffaloes as well as from their admixture (*i.e.* mixed milk) thoroughly in entire country and (2) make suitable amendments in the specification on limits of S-values in accordance with the limits of S-values ascertained for the pure fat of Indian cows and buffaloes as well as mixed milk.

In India only limited work is carried out so far to check applicability of the specifications on limits of S-values suggested by ISO/IDF (2010). Hence, very extensive survey is required to ascertain S-values of pure milk fat in India and to form their specification. The survey should cover the following aspects in sufficient length, breadth and depth.

- Different agroclimatic regions of all states and union territories
- Milk from cow and buffalo as well as their mixture in different proportions
- Practices followed in feeding lactating cows and buffaloes, including cottonseed feeding
- Seasonal variations occurring round the year
- Sufficient number of repetitions (replications): data from minimum three years

After collection, compilation and classification of data the specification to be formed by applying appropriate statistical tools. In case of any technical problem faced in formation of specifications on limits of one or more S-values, work may be undertaken for entire revamping of equation(s) of those S-value(s) using the same database generated from the survey. As discussed earlier such exercise on revamping of the equations was carried out for goat milk fat by Fontecha et al. (1998) and for ewe milk by Goudjil et al. (2003). Somewhat similar exercise may also be required for some of the S-values of buffalo milk fat.

The work reported by Prajapati and Aparnathi (2021) for evaluation of large database on S-values of milk fat collected from industry may serve as a useful guide for systematic planning of the work on survey and subsequently for proper classification as well as processing of the data collected from the survey.

The database generated from the exercise on limits of S-values of Indian milk fat should be made available on public domain by publishing it suitably in open access mode. The database to be also submitted to concerned international organisations to consider Indian milk fat as pure on testing its purity by the method and values found in accordance with the Indian standards. It will help to recognised Indian milk fat as pure, in spite of its deviation from the specification suggested by ISO/IDF (2019).

Conclusions

The GC analysis of triglycerides is simple, rapid, robust and internationally approved reference method for testing purity of milk fat. However, specifications suggested in the method for pure milk fat are formed from European cow milk fat only and may cause false positive results in pure fat from buffalo milk and also that from mixed milk. Therefore, it is necessary to establish limits of S-values for pure fat from different types of Indian milk and amend the specifications on S-values accordingly. If technical hitch arises in amending the specifications on some of the S-values, work on redevelopment of equations for those S-values may be undertaken, because application of this versatile method in India is a need of the hour to curb nuisance of mixing cheaper oils and fats in milk fat. Until suitable specifications on milk fat are formed and the method is officially adopted as reference in India, its unmatched advantages may be availed by using it as a screening test.

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