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APPLICATION NOTE

Quick and confident determination of milk fat authenticity using triglyceride content and GC-FID

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Goal

The objective of this experiment is to develop and authenticate a simplified, quick and cost-effective GC-FID method that can be employed to detect adulterated milk samples by analyzing specific triglycerides (C24 to C54).

Introduction

Milk and milk-derived products are common nutritious foods, and they are included as important components of a healthy diet. In general, the gross composition of cow's milk is approximately 87.7% water, 4.9% lactose (carbohydrate), 3.4% fat, 3.3% protein, and 0.7% minerals (referred to as ash)[1]. About 98% or more of the cow's milk lipid content is triacylglycerol, phospholipids represent about 0.5 - 1% and sterols ~ 0.2 - 0.5% of total lipids[2] (analysis performed using gas chromatography with flame ionization detection (GC-FID)).

To assess the authenticity of milk sample, analyses of fatty acids, triacylglycerols, and cholesterol profiles of the mixtures of milk and non-milk fat are performed.

In nature, butyric (n-butanoic) acid (C4) occurs exclusively in milk fat. However, due to the large variation of C4, whose approximate content ranges from 3.1% - 3.8% mass fraction, it is difficult to provide qualitative and quantitative information for foreign fat to pure milk fat ratios of up to 20% mass fraction.[3] Here we are referring ISO 17678:2010 [IDF 202:2010] whereby using defined triglyceride equations, the integrity of milk fat is determined.[4]

The high price of milk fat makes it susceptible to get replaced by foreign fats of animal or plant origin.

Thermo Scientific™ TRACE™ 1110 GC

ThermoFisher SCIENTIFIC

Analysis of dairy products for their lipid content is usually done by GC which is an effective analytical technique for assessing the origin of milk to detect foreign fats. It is also recommended as an official method to evaluate the purity of cow's milk fat in many countries worldwide[5,6].

In this application note, fat extracted from milk or milk products was analyzed using GC-FID with a short capillary column to determine triglycerides (TGs), separated by total carbon numbers. By inserting the mass fraction, expressed as a percentage, of fat molecules of different sizes (C24 to C54, using even C numbers only) into suitable TG equations, S-values are calculated. The purity is determined using S-values, which are calculated from the content of triglycerides. Triglyceride mass fractions are expressed as percentages. If the S-values exceed the limits established with pure milk fat, the presence of foreign fat is detected. S-value is the sum of weighted TG mass fractions.

Experimental

Chemicals, apparatus, and consumables

- Micropipette
- Graduated pipette, capacity 5 mL, ISO 835 [2] class A
- Round-bottomed flask, capacity 50 mL
- Erlenmeyer flask, nominal capacity 250 mL
- Funnel
- Fine-pored filter paper
- Rotary evaporator
- Ampoules, nominal capacity 1 mL, fitted with a polytetrafluoroethylene-lined aluminum crimp cap or screw cap
- Analytical balance, capable of weighing to the nearest 1 mg, with readability of 0.1 mg
- Water (H_2O), HPLC grade
- Carrier gas, nitrogen with a purity of at least 99.995% volume fraction
- Fat standards, purity at least 99% mass fraction, for standardizing the milk fat standard
- Triglyceride standards, saturated; suitable products are available commercially
- Cholesterol standard
- Methanol (HPLC grade)
- Heptane (HPLC grade)
- N Hexane (HPLC grade)
- Hydrogen, purity at least 99.995% volume fraction, free from organic impurities
- Synthetic air/zero air, free from organic impurities
- Anhydrous sodium sulfate (Na_2SO_4)
- Extrelut column, capacity 1-3 mL, filled with silica gel, for the extraction of milk fat
- Oven, capable of operating at 50 °C ± 2 °C and 100 °C ± 2 °C

GC-FID Analysis

A gas chromatograph (TRACE 1110) was coupled to a Flame Ionization Detector. The GC-FID conditions are given below in Table 1.

Table 1. GC-FID instrument conditions

Parameter	Value
Gas Chromatograph Parameters	
Instrumentation	TRACE 1110 GC
Column	Thermo Scientific™ TraceGOLD™ TG-1MT column, 5 m x 0.53 mm ID x 0.1 µm film thickness (P/N 26M99-4130)
Injector	Thermo Scientific™ PTV Injector (P/N MSA0120153)
Liner	Thermo Scientific™ LinerGOLD™ PTV On Column (OC) injection, 1x88mm (P/N 208262932)
Injector Mode	PTV On-Column (OC) Injection mode (injector temperature to oven track mode)
Sampling Parameters	
Draw Speed	Slow
Pre injection washing cycle	3
Fill Strokes	5
Air volume	1.0 µL
Sample wash cycles	3
Sample depth	Bottom

Sampling Parameters contd.	
Pre injection delay time	1.0 Sec
Post injection washing cycle	5.0 sec
Post Injection delay time	5.0 sec
Injection Volume	0.5 µL
Injector Temp	80 °C, 0.5 min, 50 °C/min to 190 °C, 1.5 min, 6 °C/min to 350 °C, 12 min
Column Flow	4.0 mL/min
Carrier gas and purity	Nitrogen (99.99%)
Purge Flow	15.00 mL/min
Mode	Constant flow
GC Oven Program	80 °C, 0.5 min, 50 °C/min to 190 °C, 1.5 min, 6 °C/min to 350 °C, 12 min
Total Run time	41.37 min
Detector Parameters	
Detector	Flame ionization detector (TRACE™ 1110 FID MODULE P/N - MSA01320161)
Detector temp	370 °C
Airflow	400.0 mL/min
Hydrogen flow	40.0 mL/min
Makeup gas flow	15.0 mL/min
Detector acquisition	0-41.37 min

Sample preparation

a) **Fat extraction from raw milk**: Extraction of fat from milk sample was done as per extraction process recommended in the international standard ISO 17678:2010 [IDF 202:2010].[4]

b) **Fat/Ghee sample**: Samples were allowed to melt at 50 °C before dilutions were made with heptane. Prepared dilution of 1% fat concentration in heptane. Completely dissolved the fat in the solvent and transferred approximately 0.5 mL to 1 mL of the fat sample into the GC vial. The sample was injected through an on-column injector on a gas chromatograph mounted with capillary [0.53 mm internal diameter (ID), wide-bore] short length column.

Data acquisition and processing

The data acquisition and processing were carried out by using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software (v. 7.2), which allows instrument control, method development, quantitative/qualitative analysis, and customizable reporting all within one package. During the integration of peaks, triglycerides with an odd acyl-C number (2n + 1) were combined with the preceding even-numbered triglyceride (2n). C56 peak was not considered during integration due to poor response. Peaks were integrated using a base-to-base integration approach.

Results and discussion

Calibration with pure milk fat standard

Initially, a certified reference material containing standardized pure milk fat (BCR-632A) was analysed to calculate the response factor of each triglyceride and cholesterol. An example chromatogram obtained for this CRM and ghee sample is represented in Figure 2 and 3. For the calculation of the response factors, injection of CRM at the beginning of the sample batch was performed and this was used to determine the response factors, f_i (mass fraction divided by area fraction), of the TGs and cholesterol. Table 2. represents response factor obtained from the CRM which was used further for the calculation of test results. These values were subsequently applied to the real samples.

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

Where,

W_i is the mass fraction, expressed as a percentage, of each TG or cholesterol in the milk fat standard.

A_i is the numerical value of the peak area of each TG or cholesterol in the milk fat standard.

Express the response factors to two decimal places.

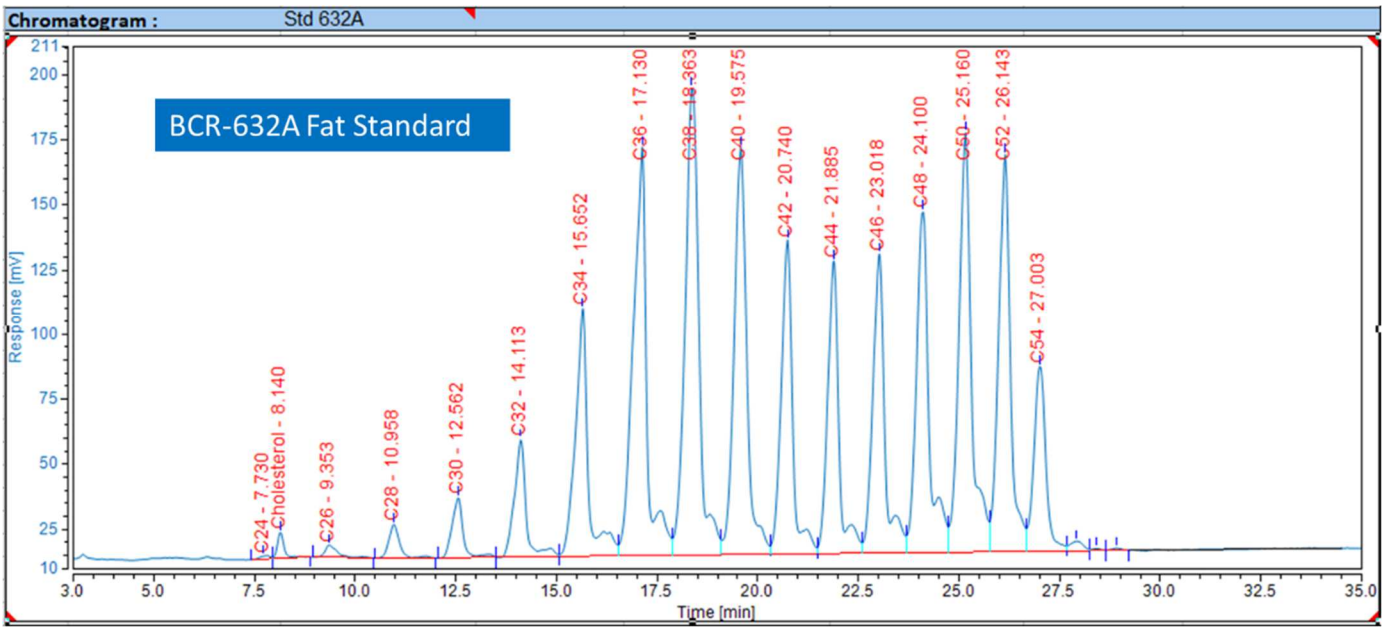


Figure 2. FID chromatogram of milk fat BCR-632A standard showing major TGs constituents

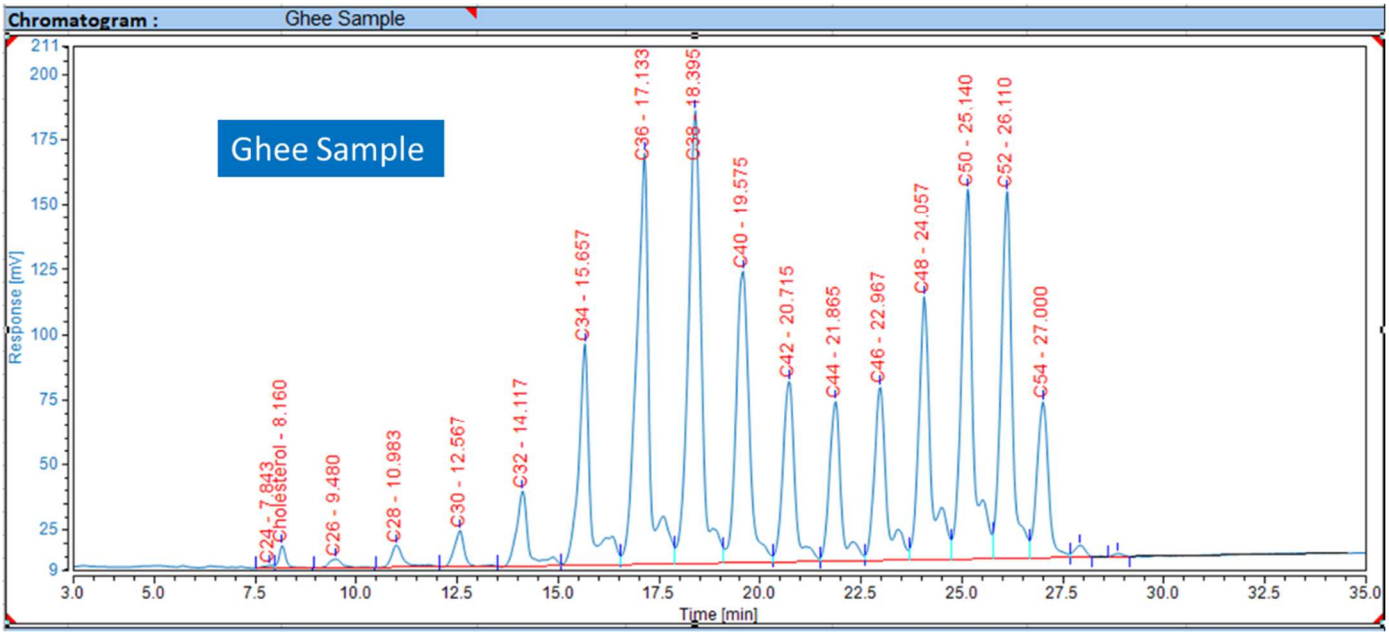


Figure 3. FID chromatogram of milk fat ghee sample showing major TGs constituents

Calculation and expression of test sample results

Calculate the mass fraction of each TG (for i = C24, C26, C28, C30, C32, C34, C36, C38, C40, C42, C44, C46, C48, C50, C52 to C54) plus cholesterol, w_i, expressed as a percentage, of the total TG content of the test sample using below equation:

$$w_i = \frac{A_i \ f_i}{\sum (A_i \ f_i)} \times 100$$

Where,

A_i is the numerical value of the peak area of each TG in the test sample

f_i is the response factor of each TG determined by calibration.

Express the results to two decimal places.

Table 2. Response factor calculated from pure milk fat standard for the calculation of test sample results

Standard 632 A (Calibration STD)			
Standard	**Area (A_i)**	**Area% (W_i)**	**Response factor**
C 24	0.45	0.07	0.9082
Cholesterol	1.78	0.29	0.9587
C 26	1.94	0.33	0.9964
C 28	4.05	0.74	1.0737
C 30	7.74	1.37	1.0399
C 32	16.07	2.83	1.0344
C 34	36.48	6.09	0.9803
C 36	63.87	10.7	0.9837
C 38	75.69	12.5	0.9698
C 40	60.46	10.05	0.9762
C 42	42.56	7.07	0.9755
C 44	39.46	6.67	0.9926
C 46	43.31	7.36	0.9980
C 48	51.55	8.74	0.9956
C 50	61.40	10.74	1.0272
C 52	54.68	9.8	1.0524
C 54	26.05	4.7	1.0596
Total	**587.526**	**100.1**	

Refer to international standard ISO 17678:2010 [IDF 202:2010] document and proceed in accordance to point with 8.3.3.1 and 8.3.3.2. Area% of standard Milk Fat mix (632A) taken from COA of the standard mixture.

Calculation of milk purity using S-values

The w_i value of each TG (for i = C24, C26, C28, C30, C32, C34, C36, C38, C40, C42, C44, C46, C48, C50, C52 to C54) plus cholesterol from the test sample, was involved into the calculation of S values.

The following equations 1 to 5 were checked irrespective of the kind of foreign fat suspected.

Note: Although the S-values are calculated from TG percentages, they do not represent a percentage themselves and do not have a unit.

Equation 1: Adulteration due to soya bean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed and fish oil

S = 2.0983 wC30 + 0.7288 wC34 + 0.6927 wC36 + 0.6353 wC38 + 3.7452 wC40 – 1.2929 wC42 + 1.3544 wC44 + 1.7013 wC46 + 2.5283 wC50

Equation 2: Adulteration due to coconut and palm kernel fat

S = 3.7453 wC32 + 1.1134 wC36 + 1.3648 wC38 + 2.1544 wC42 + 0.4273 wC44 + 0.5809 wC46 + 1.2926 wC48 + 1.0306 wC50 + 0.9953 wC52 + 1.2396 wC54

Equation 3: Adulteration due to palm oil and beef tallow

S = 3.6644 wC28 + 5.2297 wC30 – 12.5073 wC32 + 4.4285 wC34 – 0.2010 wC36 + 1.2791 wC38 + 6.7433 wC40 – 4.271 4 wC42 + 6.373 9 wC46

Equation 4: Adulteration due to Lard

S = 6.5125 wC26 + 1.2052 wC32 + 1.7336 wC34 + 1.7557 wC36 + 2.2325 wC42 + 2.8006 wC46 + 2.5432 wC52 + 0.9892 wC54

Equation 5: Total result failure

S = –2.7575 wC26 + 6.4077 wC28 + 5.5437 wC30 – 15.3247 wC32 + 6.2600 wC34 + 8.0108 wC40 –5.0336 wC42 + 0.6356 wC44 + 6.0171 wC46

Based on the above equations, the S value limits were given in the Table 3 (Ref. ISO 17678:2010).[4]

Table 3. S-limits for pure milk fats

Foreign fat	Equation	S-limits*
Soybean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed, fish oil	1	98.05 to 101.95
Coconut and palm kernel fat	2	99.42 to 100.58
Palm oil and beef tallow	3	95.90 to 104.10
Lard	4	97.96 to 102.04
Total	5	95.68 to 104.32

*Calculated on a 99% confidence level, so that foreign fat addition is only indicated if the detection limits of the relevant equation are exceeded

Identification of foreign fat/ fat purity

Based on the five equations given above, the calculated S-values range/limits were provided in Table 3. Here, the pure milk fat sample was analyzed and the S values were calculated by using the above five equations. If all the S values observed from the five equations fall within the defined limits (Table 3.) the sample was considered to be pure milk fat. If observed S value falls outside these limits the sample will be considered as adulterated milk fat sample. To assess the performance of the method, two certified samples were assessed. After review, the data for pure CRM standard, the data was passing S-limit criteria satisfactorily for all five equations. These results indicated that the pure milk fat standard does not have any foreign fat content.

Chromeleon Chromatography Data System (CDS) software offers the automated calculation and report generation which was represented in Figure 4. This approach offered a simple, fast user friendly and cost-effective solution for the lab. To assess the method applicability few commercially available fat/ghee samples were analysed to identify adulteration or any foreign fat. The results for ghee samples represented in Table 4. which were within the acceptance criteria as per ISO 17678 method.

To check the precision of method CRM STD 632-A was injected in six replicates which offered excellent repeatability (%RSD< 5%) except for C24 (%RSD 7.1%). The details for repeatability data are presented in Table 5 and Figure 5 represents the overlay chromatogram of CRM STD A with (n=7) injection analysis. The optimized method has demonstrated excellent accuracy and precision through the CRM STD 632-A.[7]

Table 4. Performance check for S-limit criteria of four injections of commercial market milk fat (Ghee) sample

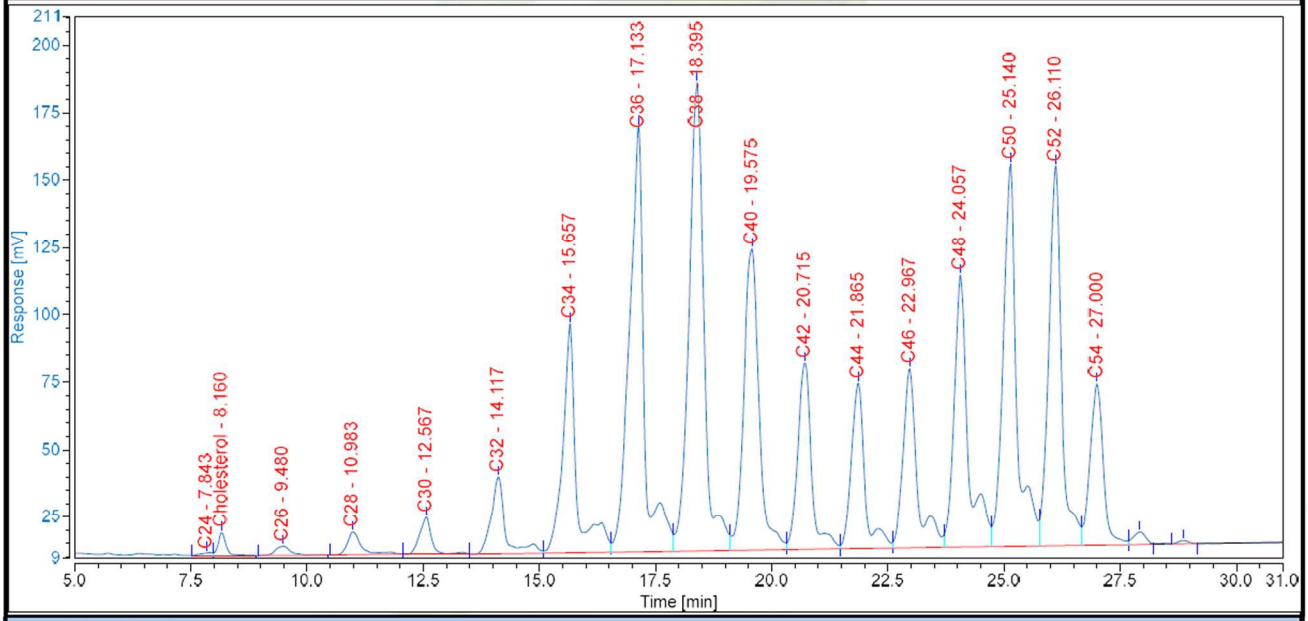
Foreign fat	S-Value (Sample Injection 1)	S-Value (Sample Injection 2)	S-Value (Sample Injection 3)	S-Value (Sample Injection 4)	S-limits	Average	% RSD
Soybean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed, fish oil	100.32	100.57	100.44	100.46	98.05-101.95	100.45	0.1
Coconut and palm kernel fat	99.78	99.88	99.74	99.63	99.42-100.58	99.76	0.1
Palm oil and beef tallow	101.02	101.14	101.07	101.56	95.90-104.10	101.20	0.2
Lard	100.11	99.39	99.79	99.86	97.96-102.04	99.79	0.3
Total	100.16	100.60	100.67	101.07	95.68-104.32	100.62	0.4

Milk Fat Analysis Report

Injection Details

Injection Name:	Ghee Sample	***Run Time (min):***	***36.00***
Vial Number:	6	***Injection Volume:***	***0.50***
Injection Type:	Unknown	***Channel:***	***Detector3***
Calibration Level:		***Wavelength:***	***n.a.***
Instrument Method:	ONCOLUMN WITH AUTOSAMPLER range 0 5 mtr col N hepta	***Bandwidth:***	***n.a.***
Processing Method:	Quantitative GGMMF	***Dilution Factor:***	***1.0000***
Injection Date/Time:	21/Sep/20 18:53	***Sample Weight:***	***1.0000***

Chromatogram : Ghee Sample



Integration Results

No.	Peak Name	Retention Time min	Area mV*min	Rel.Area %	Rel.Area (IDF) %	Rel.Height (IDF) %	Rel.Amount (TOTAL) %
1	C24	7.843	0.27	0.06	0.06	0.08	0.06
2	Cholesterol	8.160	1.67	0.39	0.39	0.69	0.32
3	C26	9.480	1.30	0.30	0.30	0.27	0.34
4	C28	10.983	2.92	0.67	0.68	0.69	0.69
5	C30	12.567	3.97	0.91	0.92	1.12	0.96
6	C32	14.117	9.97	2.30	2.31	2.33	2.38
7	C34	15.657	29.20	6.72	6.75	6.88	6.57
8	C36	17.133	58.72	13.52	13.57	12.80	13.09
9	C38	18.395	65.39	15.05	15.12	14.10	14.67
10	C40	19.575	42.57	9.80	9.84	9.06	9.57
11	C42	20.715	23.86	5.49	5.52	5.61	5.48
12	C44	21.865	20.69	4.76	4.78	4.98	4.84
13	C46	22.967	23.75	5.47	5.49	5.40	5.62
14	C48	24.057	35.36	8.14	8.17	8.17	8.25
15	C50	25.140	48.26	11.11	11.15	11.52	11.36
16	C52	26.110	44.86	10.33	10.37	11.43	10.88
17	C54	27.000	19.85	4.57	4.59	4.86	4.90
Total:			**432.617**	**99.600**	**100.00**	**100.00**	**100**

Foreign Fats	S Value	S Limit	Judgement
Soybean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed, fish oil	98.81	98.05-101.95	Confirms
Coconut and palm kernel	99.97	99.42-100.58	Confirms
Palm oil and beef tallow	99.97	95.90-104.10	Confirms
Lard	99.95	97.96-102.04	Confirms
Total	99.46	95.68-104.32	Confirms

Analyzed By: Reviewed By:

Figure 4. Automated report for ghee sample generated through software

Table 5. Repeatability data for CRM STD 632 A (N=6) injections

Repeatability data for Area % (N=6) injections							
Inj=1	Inj=2	Inj=3	Inj=4	Inj=5	Inj=6	Avg Area %	% RSD
0.08	0.08	0.08	0.07	0.07	0.07	0.07	7.1
0.31	0.31	0.31	0.30	0.30	0.31	0.31	1.0
0.29	0.28	0.29	0.31	0.32	0.31	0.30	5.0
0.70	0.73	0.73	0.69	0.68	0.69	0.70	3.1
1.29	1.26	1.25	1.28	1.29	1.26	1.27	1.4
2.72	2.70	2.72	2.69	2.68	2.68	2.70	0.7
6.06	6.07	6.04	6.02	6.04	6.01	6.05	0.4
10.63	10.69	10.67	10.65	10.65	10.65	10.66	0.2
12.82	12.75	12.75	12.67	12.75	12.68	12.75	0.4
10.20	10.21	10.20	10.20	10.24	10.21	10.21	0.1
7.23	7.22	7.22	7.22	7.21	7.27	7.22	0.3
6.80	6.77	6.78	6.80	6.74	6.82	6.78	0.4
7.35	7.34	7.37	7.36	7.33	7.38	7.35	0.2
8.69	8.68	8.71	8.72	8.71	8.68	8.70	0.2
10.57	10.60	10.60	10.67	10.67	10.61	10.62	0.4
9.63	9.67	9.66	9.72	9.64	9.67	9.66	0.3
4.64	4.63	4.61	4.64	4.68	4.71	4.64	0.9

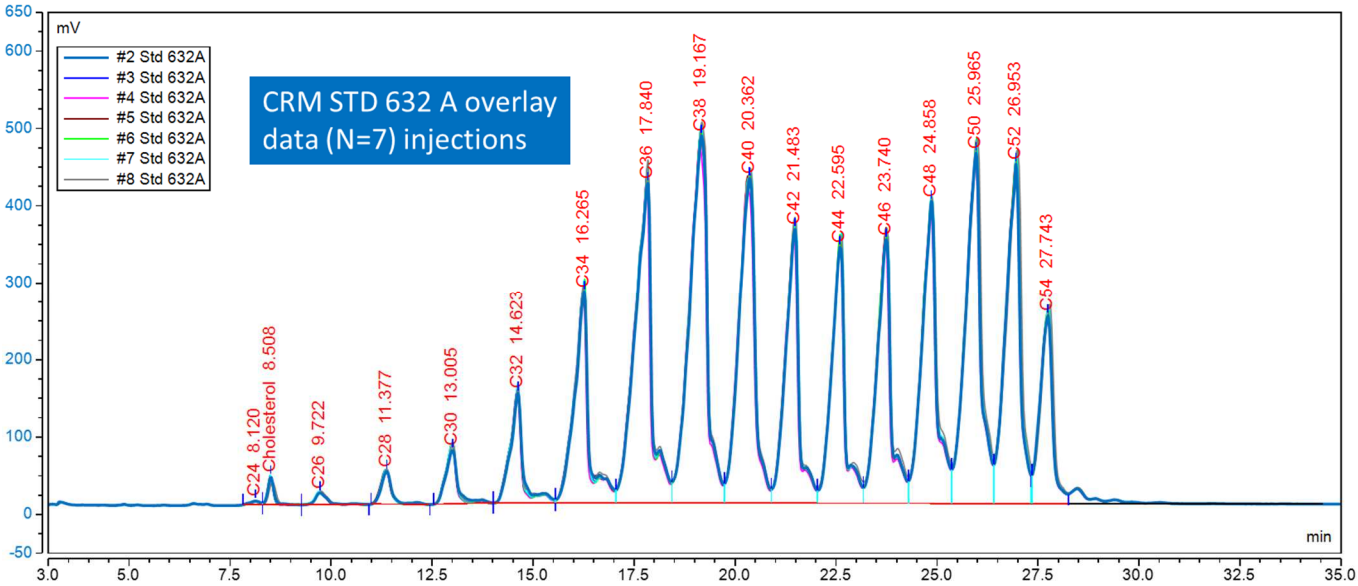


Figure 5. Comparison of chromatogram for overlay of CRM STD 632 A (N=7) injections.

Conclusion

- The optimized method performance was demonstrated using CRM (BCR-632A)
- All standards and samples could be effectively analyzed in accordance with the ISO 17678:2010 [IDF 202:2010] document.
- CRM was verified using the said conditions as mentioned in the certificate of analysis and results found satisfactory
- Overall, the optimized method offered excellent repeatability with % RSD below 1.0 % for the S-values calculated for standards as well as real samples
- The ISO 17678: 2010 method requirement was successfully demonstrated on Thermo Scientific TRACE 1110 GC (Make in India).
- This analysis provides a cost-effective solution by using nitrogen as carrier gas instead of helium gas which has challenge for availability and cost (~10 times higher) as compared to nitrogen gas.
- Chromeleon Chromatography Data System (CDS) software offered automated calculations and results interpretations is provided in predefined report template which saves time and avoid chances of data transcription errors.

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