A fast and cost-effective GC-FID method for the determination of adulterated milk fat

Authors: Giulia Riccardino, Maria Mazzucotelli, and Daniela Cavagnino

Thermo Fisher Scientific, Milan, Italy

Subodh K. Budakoti

Thermo Scientific, Customer Solution Center, Ghaziabad, India

Keywords: Milk fat, adulteration, triglycerides, cholesterol, gas chromatography, GC, flame ionization detector, FID

Goal

The aim of this application note is to demonstrate the performance of the Thermo Scientific™ TRACE™ 1600 Series GC coupled to flame ionization detection for the assessment of milk fat authenticity according to the International Standard ISO 17678:2019 [IDF 202:2019] method.

Introduction

Fat is an important constituent of milk and dairy products.[1] Due to its high cost, lower-price vegetable oils or other cheaper animal fats are sometimes used in the manufacturing process.[1] Therefore, testing milk fat is essential to ensure that dairy products are sold as advertised and produced to a consistently high standard.

The International Organization for Standardization (ISO) and the International Dairy Federation (IDF) have published a reference method for the determination of milk fat purity (ISO 17678:2010 [IDF 202:2010])[2] based on the triglycerides fingerprint in milk fat. This method was revised in 2019 (ISO 17678:2019 [IDF 202:2019]).[3] In order to support analytical testing laboratories examining milk fat samples, the IDF has recently published additional guidelines[4] to ensure accurate testing of milk and dairy products. The integrity of the milk fat can be determined by comparing the triglycerides profile of samples with the triglycerides profile of pure milk fat by applying a set of mathematical equations. The method is suitable for the detection of both vegetable and animal fats such as beef tallow and lard.

In this study, gas chromatography (GC) coupled to flame ionization detection (FID) was used to assess the triglycerides content of commercially available clarified butter. The use of a programmable temperature vaporizing (PTV) injector in cold on-column (COC) mode allowed for direct transfer of analytes into the analytical column at low temperature, thus minimizing sample discrimination that can potentially occur during a hot injection. The triglycerides of interest (C24-C56) were separated by carbon number using a short metal capillary column, and their mass fractions were used to calculate the S-values to establish whether the presence of a foreign fat could be detected in the analyzed butter sample.[1]

Experimental

In all experiments, a TRACE 1610 GC equipped with a Thermo Scientific™ Instant Connect Programmable Temperature Vaporizing (iConnect PTV) injector, used in on-column mode, and a Thermo Scientific™ Instant Connect Flame Ionization Detector (iConnect FID), equipped with a wide bore jet (P/N 40402205), was coupled to a Thermo Scientific™ AS 1610 Series liquid autosampler. Chromatographic separation was achieved on a Thermo Scientific™ TraceGOLD™ TG-1MT capillary column, 6 m × 0.53 mm × 0.15 µm (P/N 26M99-4100). The low bleed of the TraceGOLD metal column combined with its high operating temperature (up to 400/430 °C) ensured compliance with the chromatographic requirements of the method for the analysis of triglycerides (TGs) and cholesterol. For this application, nitrogen represents a viable alternative to helium as carrier gas, as it can easily be produced in the lab with high purity using a nitrogen generator, making it very cost effective. Additional GC-FID and autosampler parameters are detailed in Table 1.

Table 1. GC-FID and autosampler experimental parameters used for the assessment of triglyceride fingerprint

AI/AS 1610 Autosampler parameters	
Injection type	Standard
Sample mode	Viscosity
Fill strokes	10
Sample depth	Bottom
Injection mode	Custom
Injection speed (µL/s)	100
Pre-injection delay time (min)	0
Post-injection delay time (s)	3
Pre-injection wash cycles	3
Post-injection wash cycles	5
Sample wash cycles	3
Injection volume (µL)	0.5
Syringe	5 µL, 50 mm, Ga 26* (P/N 36500505)

* The OC injection mode requires the use of a 26Ga needle syringe

iConnect PTV parameters	
Injection temperature (°C)	80
Liner	PTV Silcosteel liner Simile OC (P/N 45322052)
Inlet module and mode	PTV, simulated on-column
Injection time (min)	0.05
Transfer rate (°C/s)	0.7
Transfer temperature (°C)	380
Transfer time (min)	0.15
Post cycle temperature	Maintain
Split flow (mL/min)	10
Septum purge flow (mL/min)	1, constant
Carrier gas, flow (mL/min)	N_2, 5

TRACE 1610 GC parameters	
Oven Temperature Program	
Temperature (°C)	80
Hold time (min)	0.5
Rate (°C/min)	50
Temperature 2 (°C)	190
Rate (°C/min)	6
Temperature 3 (°C)	370
Hold time (min)	5
GC run time (min)	37.70
FID	
Temperature (°C)	370
Air flow (mL/min)	350
H_2 flow (mL/min)	35
N_2 flow (mL/min)	40
Aquisition rate (Hz)	25
Column	
TraceGOLD TG-1MT	6 m, 0.53 mm, 0.15 µm (P/N 26M99-4100)

Standard and sample preparation

Small aliquots of two certified reference materials (CRM), BCR-632A and BCR-632B, were outsourced from one of the Thermo Scientific internal laboratories due to their current unavailability on the local market. According to the manufacturer's specification, the reference material should be injected on the day of opening and the opened ampoules should not be stored for future use.[6] Pure butter fat CRM (BCR-632A) was diluted to 1% with heptane (Fisher Scientific, >99%, P/N 10692752), injected in triplicate at the beginning of the sequence, and used to derive the response factor of each triglyceride and cholesterol as per the ISO / IDF method. Although not requested as part of the method validation, a CRM butter fat adulterated with coconut oil (BCR-632B)[6] was diluted to 1% with heptane and injected (n=5) to check the method suitability for detection of foreign fat adulteration.

Sample preparation

Commercially available clarified butter was purchased from a local retailer. Sample was melted at 50 °C and then diluted to 1% with heptane. An aliquot (1 mL) was then transferred to the GC vial for injection. This sample was used to check the consistency in the response and to evaluate the method precision (n=9).

Data acquisition, processing, and reporting

Data was acquired using the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.3. This single platform integrates instrument control, method development functionality, quantitation-focused workflows, reporting, and storage in compliance with the Title 21 CFR part 11, ensuring effective data management, ease of use, data integrity, and traceability.[5] Chromeleon CDS also offers the option to scale up the entire data handling from a single workstation to an enterprise environment.

Results and discussion

Chromatographic profile of triglycerides and cholesterol in clarified butter sample

Integration of multiple FID chromatographic peaks was performed as per the ISO / IDF method by applying a baseline integration from C24 to C56 and combining triglycerides with odd acyl-C numbers with the preceding even numbered. An example of a typical triglyceride fingerprint obtained for a clarified butter sample with baseline integration is reported in Figure 1.

Calculation of response factors for samples

As specified in the ISO method, CRM (BCR-632A) was injected in triplicate at the beginning of the sequence and used to calculate the response factors (fi) for TGs and cholesterol. The obtained mass fractions (%) were consistent with the expected certified values with deviations not exceeding 0.56%. C56 was not included into the calculations because of its low content as per the ISO method. Response factors were derived from the last injection of the pure butter fat CRM by applying Equation 1. Measured and expected mass fractions (%) as well as deviation from expected values and calculated fi are reported in Table 2.

Equation 1 $$fi = Wi * \sum Ai / \sum Wi * Ai$$

Where:

Wi = mass fraction (%) of each TG or cholesterol in the certified reference material

Ai = absolute peak area of each TG or cholesterol in the certified reference material

The calculated response factors were <1.25 for all the triglycerides and cholesterol therefore suitable to be applied to calculate the mass fractions of TGs and cholesterol in the analyzed sample.

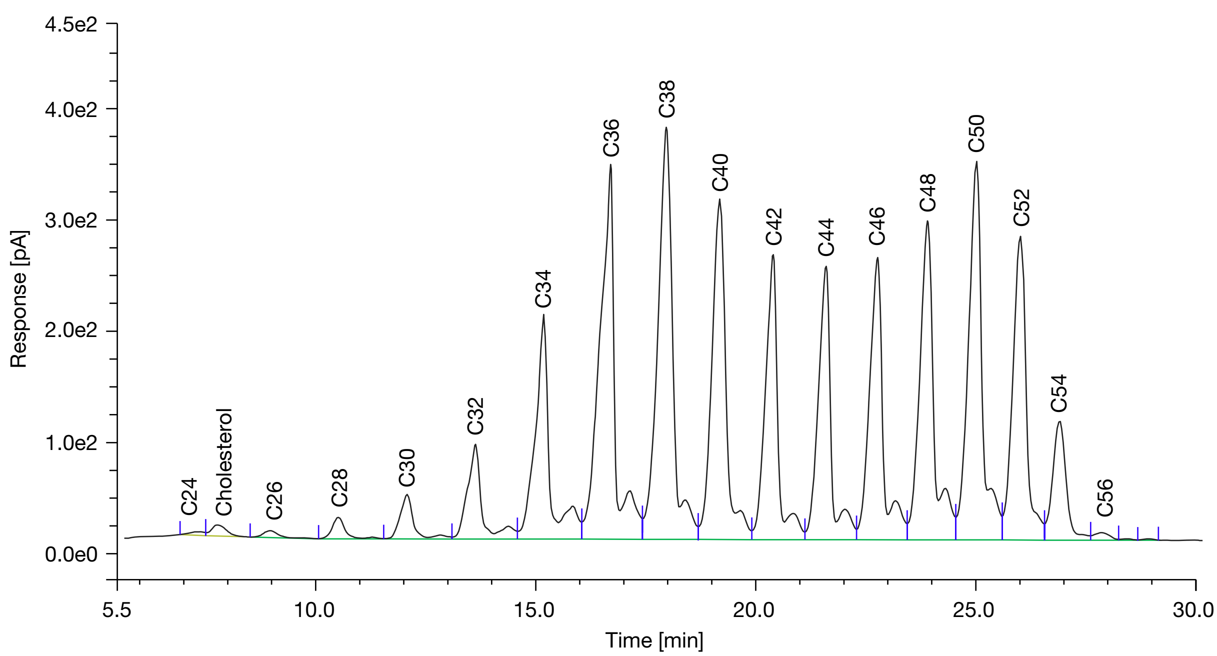

Figure 1. Baseline integration of TGs and cholesterol in clarified butter sample as per the ISO 17678:2019 method

Table 2. Mass fractions (%) obtained for a pure butter standard within 0.005–0.56% of the certified values, with calculated fi < 1.25 for triglycerides and cholesterols. Response factors are expressed with four digits after the decimal point, as per the method requirement.

Triglyceride	RT (min)	BCR-632 A average mass fraction (%, n=3)		Deviation from expected value (%)	Determined response factor (*fi*)
		Measured	Expected		
C24	7.20	0.05	0.07	0.02	1.0541
Cholesterol	7.64	0.284	0.289	0.005	0.9999
C26	8.81	0.26	0.33	0.07	0.9936
C28	10.37	0.64	0.74	0.10	0.9995
C30	11.95	1.26	1.37	0.11	1.0036
C32	13.50	2.79	2.83	0.04	0.9996
C34	15.06	6.24	6.09	0.15	0.9999
C36	16.57	11.11	10.70	0.41	0.9995
C38	17.85	13.06	12.50	0.56	1.0003
C40	19.03	10.44	10.05	0.39	0.9998
C42	20.27	7.25	7.07	0.18	1.0003
C44	21.46	6.62	6.68	0.06	1.0003
C46	22.64	7.11	7.36	0.25	0.9995
C48	23.78	8.46	8.74	0.28	0.9999
C50	24.88	10.29	10.74	0.45	1.0002
C52	25.89	9.35	9.80	0.45	0.9994
C54	26.77	4.43	4.70	0.27	1.0008

Triglyceride composition in samples

Clarified butter sample was prepared as detailed in the sample preparation section. The sample was injected multiple times (n=9) to evaluate the consistency of results and precision. In addition, an adulterated butter fat (BCR-632B) was run (n=5) to check the method suitability for detection of adulteration. The mass fraction of each TG and cholesterol was calculated by applying Equation 2.

Equation 2 $$wi = [Ai * fi / \sum(Ai * fi)] * 100$$

Where:

Ai = absolute peak area of each TG in the test sample

fi = response factor of each TG determined by using the CRM BCR-632A

The integrity of milk fat was determined by using defined triglyceride equations (S-values, Appendix 1) based on the mass fractions of fat molecules ranging from C24 to C54 using even C numbers only, and results were compared with the S-values limits provided in the ISO / IDF method.

The use of a defined triglycerides content formulae based on the normalized weighted sum of individual triglyceride peaks allowed for easy comparison of triglyceride fingerprints in pure and adulterated samples. S-values calculated for the clarified butter sample fell within the S-limits, therefore it can be considered as a pure milk fat sample. On the contrary, in the adulterated fat sample the S-value related to coconut and palm kernel fat failed. The sample can be considered to contain a foreign fat, thus confirming the suitability of the method in detecting possible fraud, as reported in Figure 2.

The results obtained for both test samples were consistent across the replicated injections with %RSD < 0.3 (Appendix 1). Absolute peak area %RSDs were evaluated on the clarified butter sample over n=9 replicates, obtaining an average of 1.1% as reported in Figure 3. In addition, the customizable report options of Chromeleon CDS ensured quick data review and easy interpretation for possible fat adulteration, as reported in Figure 4.

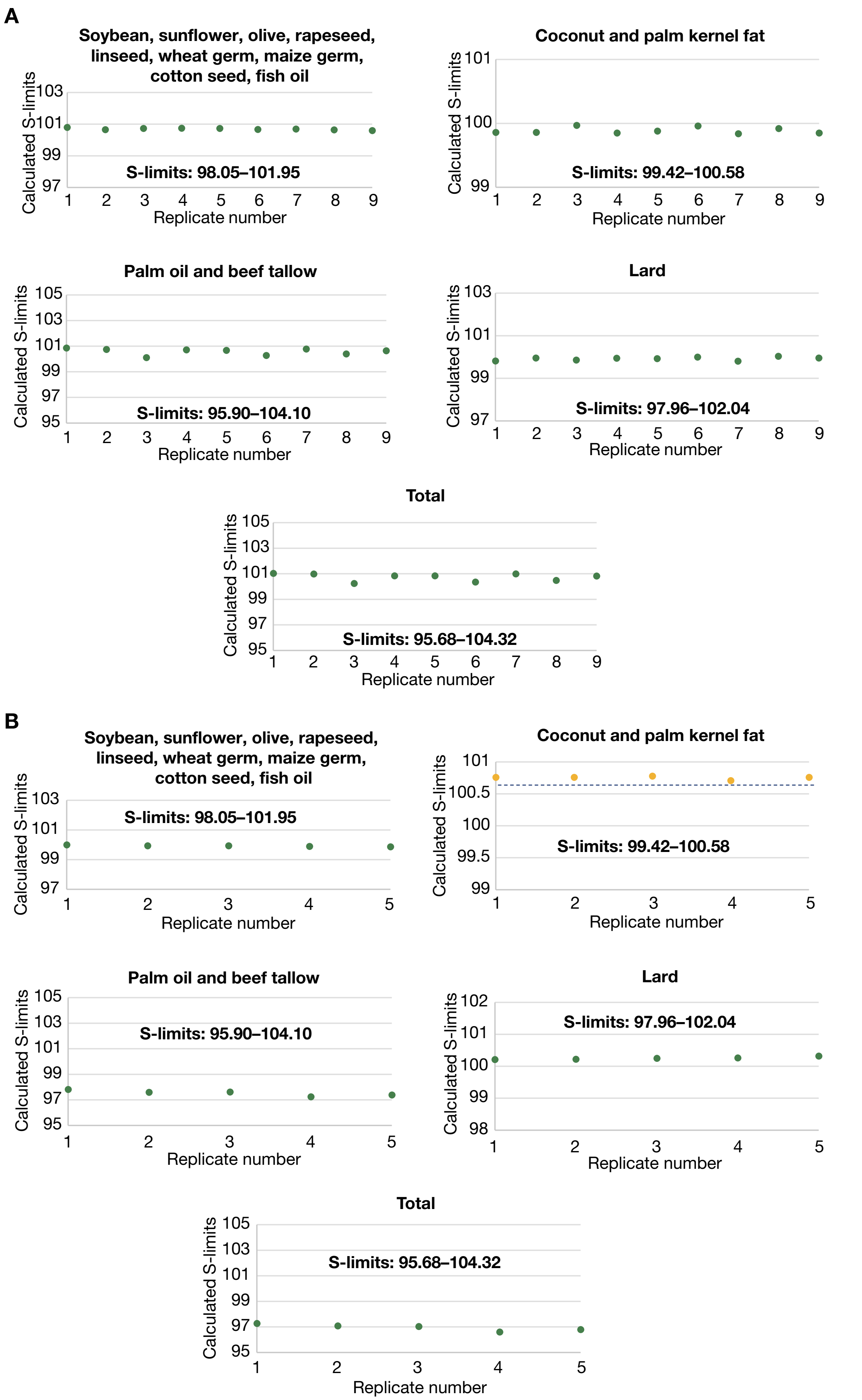

Figure 2. Calculated S-values for replicated injections of a clarified butter (n=9) (A) and adulterated butter fat (n=5) (B) samples. S-values for the clarified butter sample fell within the S-limits, therefore it can be considered as a pure milk fat sample. On the contrary, the S-value related to coconut and palm kernel fat failed in the adulterated fat sample. S-limits are annotated (refer to Appendix 2 for the calculated S-values of each injection).

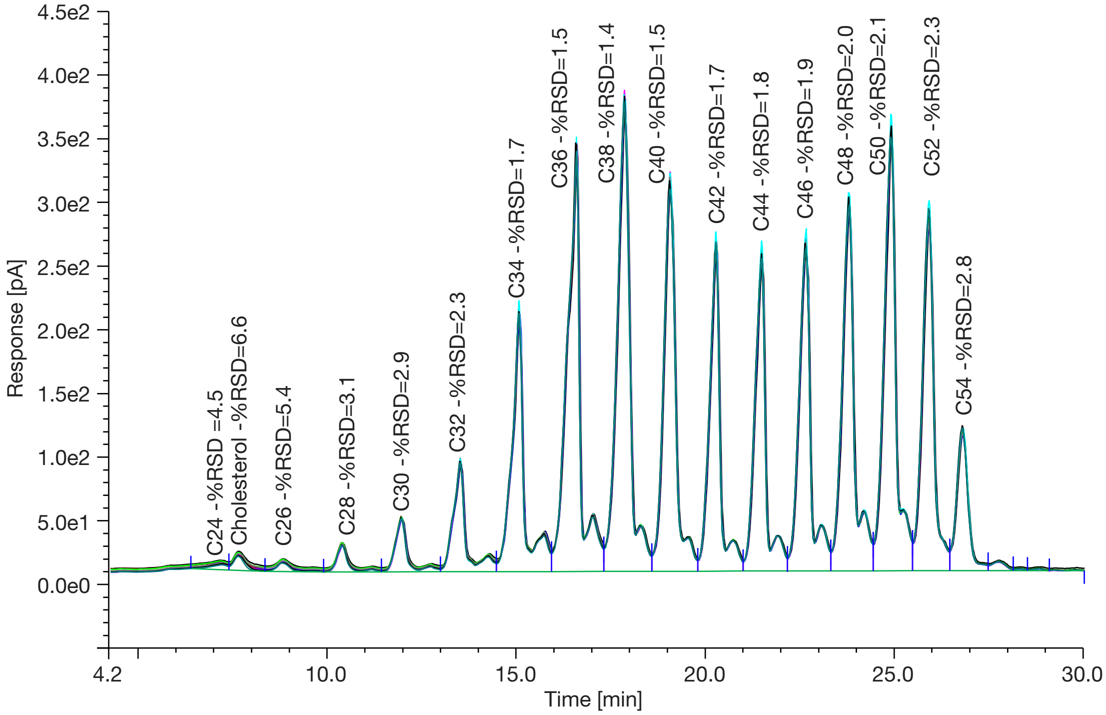

Figure 3. Clarified butter sample overlaid chromatograms (n=9) with absolute peak area %RSDs calculated from n=9 repeat injections (annotated)

A

No.	Peak Name	Area pA*min	Height pA	Relative Area %	Soybean	Coconut	Palm	Lard	Total
1	C24	0.616	2.107	0.05					
2	Cholesterol	3.010	8.750	0.25					
3	C26	2.598	6.649	0.21				1.74	-0.74
4	C28	6.984	21.483	0.58			2.49		4.35
5	C30	14.955	42.765	1.24	3.00		7.49		7.94
6	C32	33.792	88.840	2.80		12.73	-42.53	4.10	-52.11
7	C34	78.146	212.500	6.47	5.05		30.70	12.02	43.40
8	C36	138.110	341.074	11.43	8.09	13.00	-2.35	20.50	
9	C38	149.935	374.412	12.41	8.36	17.95	16.83		
10	C40	115.712	312.612	9.57	38.58		69.47		82.53
11	C42	87.823	266.447	7.27	-9.36	15.60	-30.93	16.16	-36.44
12	C44	86.339	259.312	7.14	8.95	2.82			4.20
13	C46	95.973	268.741	7.94	12.04	4.11	45.13	19.83	42.60
14	C48	113.941	296.931	9.43		10.75			
15	C50	131.869	358.448	10.91	24.92	10.16			
16	C52	103.834	290.536	8.59		8.64		22.09	
17	C54	41.052	113.403	3.40		4.99		3.98	
Total:		**1204.688**	**3265.008**	**99.68**	99.65	100.76	96.30	100.42	95.74
					Passed	Passed	Passed	Passed	Passed

B

No.	Peak Name	Area pA*min	Height pA	Relative Area %	Soybean	Coconut	Palm	Lard	Total
1	C24	0.751	2.513	0.06					
2	Cholesterol	3.252	9.085	0.28					
3	C26	3.165	8.115	0.27				1.74	-0.74
4	C28	7.994	23.682	0.68			2.49		4.35
5	C30	16.776	44.866	1.42	3.00		7.49		7.94
6	C32	39.995	93.847	3.39		12.73	-42.53	4.10	-52.11
7	C34	81.535	199.834	6.91	5.05		30.70	12.02	43.40
8	C36	137.387	306.076	11.65	8.09	13.00	-2.35	20.50	
9	C38	154.628	365.900	13.11	8.36	17.95	16.83		
10	C40	121.159	313.349	10.27	38.58		69.47		82.53
11	C42	85.106	255.749	7.22	-9.36	15.60	-30.93	16.16	-36.44
12	C44	77.706	224.163	6.59	8.95	2.82			4.20
13	C46	83.290	229.642	7.06	12.04	4.11	45.13	19.83	42.60
14	C48	97.794	252.572	8.29		10.75			
15	C50	115.869	299.323	9.82	24.92	10.16			
16	C52	102.173	277.878	8.66		8.64		22.09	
17	C54	47.252	133.185	4.01		4.99		3.98	
Total:		**1175.829**	**3039.778**	**99.68**	99.65	100.76	96.30	100.42	95.74
					Passed	Failed	Passed	Passed	Passed

Figure 4. Example of customized report in Chromeleon CDS browser for a clarified butter sample (A) and adulterated butter fat (B)

Conclusions

The results obtained in the performed experiments demonstrate that the TRACE 1600 series GC in combination with FID and PTV cold on-column injection and equipped with the AI/AS 1610 liquid autosampler represents a cost-effective (with nitrogen as carrier gas) and reliable analytical configuration for triglyceride profiling in milk and dairy products, allowing for identification of foreign fats added with fraudulent purposes.

- The use of PTV injection in on-column mode ensures efficient injection of analytes into the column, eliminating possible compound discrimination.
- The low bleed of the TraceGOLD metal column combined with its high operating temperature ensures suitable chromatographic separation in compliance with the ISO / IDF method.
- The robustness of the AI/AS 1610 liquid autosampler, offering a sample capacity of 155 vials, ensures reliable unattended operations. Its high repeatability in sample handling combined with the high inertness of the system allows for reliable and consistent results with average absolute peak area %RSD of 1.1 for n=9 repeated injections of clarified butter sample.
- Chromeleon CDS (compliant with the FDA 21 CFR part 11 requirements) ensures data integrity, traceability, and effective data management, allowing for easy and fast data processing, quantitation, and reporting.

References

1. IDF issues guidance to help determine milkfat purity, https://fil-idf.org/news_insights/updated-idf-guide-to-good-animal-welfare-in-dairy-production-released-2-2-2-4-2/
2. ISO 17678:2010(E) [IDF 202:2010(E)] Milk and products – Determination of milk fat purity by gas chromatographic analysis of triglycerides (Reference method), https://www.iso.org/standard/43899.html
3. ISO 17678:2019 [IDF 202:2019] Milk and products – Determination of milk fat purity by gas chromatographic analysis of triglycerides (Reference method), https://www.iso.org/standard/72284.html
4. Bulletin of the IDF N° 499/ 2019: Guidance on the Practical Application of IDF/ISO Standard on the Determination of Milkfat Purity, https://fil-idf.org/publications/bulletin/bulletin-of-the-idf-n-499-2019-guidance-on-the-practical-application-of-idf-iso-standard-on-the-determination-of-milkfat-purity/
5. Thermo Fisher Scientific, Chromeleon CDS Enterprise – Compliance, Connectivity, Confidence, BR72617-EN0718S.
6. Zeleny R., Bernreuther A., Linsinger T., Schimmel H, Sejerøe-Olsen B., Kramer G.N., Pauwels J., European Commission Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, The method-specific certification of the cholesterol and triglyceride contents of a pure and an adulterated butter fat reference material BCR-632A and BCR-632B Report EUR 21316 EN, 2005.

Appendix 1

Calculation of milk fat purity using S-values:

Equation 1

Adulteration due to soybean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed, fish oil:

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

S-limits: 98.05–101.95

Equation 2

Adulteration due to coconut and palm kernel fat:

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

S-limits: 99.42–100.58

Equation 3

Adulteration due to palm oil and beef tallow:

$$S = 3.6644 * wC28 + 5.2297 * wC30 - 12.5073 * wC32 + 4.4285 * wC34 - 0.2010 * wC36 + 1.2791 * wC38 + 6.7433 * wC40 - 4.2714 * wC42 + 6.3739 * wC46$$

S-limits: 95.90–104.10

Equation 4

Adulteration due to lard:

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

S-limits: 97.96–102.04

Equation 5

Total result failure:

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

S-limits: 95.68–104.32

thermo scientific

Appendix 2

Calculated S-values for clarified butter sample and adulterated butter fat

Replicate number	Soybean, sunflower, olive, rapeseed, linseed, wheat germ, maize germ, cotton seed, fish oil S-limits: 98.05–101.95	Coconut and palm kernel fat S-limits: 99.42–100.58	Palm oil and beef tallow S-limits: 95.90–104.10	Lard S-limits: 97.96–102.04	Total S-limits: 95.68–104.32
Calculated S-values in clarified butter sample					
Replicate 1	100.80	99.86	100.86	99.82	101.03
Replicate 2	100.66	99.86	100.75	99.96	100.98
Replicate 3	100.74	99.97	100.11	99.86	100.24
Replicate 4	100.75	99.85	100.72	99.95	100.84
Replicate 5	100.74	99.88	100.68	99.93	100.84
Replicate 6	100.67	99.96	100.28	100.00	100.35
Replicate 7	100.70	99.84	110.78	99.81	100.99
Replicate 8	100.65	99.92	100.40	100.04	100.48
Replicate 9	100.60	99.85	100.65	99.96	100.82
%RSD	**0.05**	**0.05**	**0.25**	**0.08**	**0.29**
Calculated S-values in adulterated butter fat					
Replicate 1	100.00	100.76	97.82	100.21	97.27
Replicate 2	99.94	100.76	97.60	100.22	97.08
Replicate 3	99.94	100.78	97.62	100.25	97.03
Replicate 4	99.90	100.71	97.25	100.26	96.60
Replicate 5	99.87	100.76	97.39	100.32	96.79
%RSD	**0.05**	**0.03**	**0.23**	**0.04**	**0.27**

Find out more at **thermofisher.com/tracegc**

AN000136-NA-EN 0122S

ThermoFisher SCIENTIFIC