Application Note

Food Testing

Estimation of β-Sitosterol in Milk Fat (Ghee) Samples

Agilent 8890/5977B Single Quadrupole GC/MS System

Authors

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Abstract

This application note demonstrates the use of the Agilent 8890 GC and the Agilent 5977B GC/MS single quadrupole mass spectrometer in the detection and quantification of β-sitosterol in milk fat samples to check for vegetable oil adulteration.

The method provides the highest confidence for routine analysis of milk fat samples in the food industry, whether it is used in manufacturing, processing, commercial testing, or academia. Sample preparation for this method involved saponification, followed by extraction of unsaponifiable matter by liquid-liquid extraction (LLE) and derivatization of sitosterol to its trimethylsilyl derivative.

Introduction

Ghee (milk fat), also known as clarified butter, is commonly used in cuisine of the Indian subcontinent, traditional medicines, and religious rituals. Ghee is prepared by skimming the milk solids out of melted butter. Increasing demand for ghee in India has resulted in certain malpractices such as adulteration of ghee with vegetable oils.[1]

Plant sterols can be used as marker compounds for identification of vegetable oil adulteration in ghee. One of the common representatives of plant sterol is β-sitosterol.

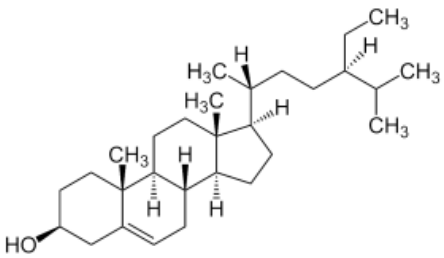

Figure 1. The structure of β-sitosterol.

This application note demonstrates detection of vegetable oil adulteration in ghee. The principle of detection of adulteration is based on the presence of β-sitosterol as a marker in the unsaponifiable matter of pure ghee and adulterated ghee samples.[3]

The method used for detection of the presence of any vegetable oil is based on gas chromatography coupled to mass spectrometry.

Experimental conditions

Chemicals required

20% ascorbic acid (AR grade) (2 g of ascorbic acid dissolved in 10 mL water, potassium hydroxide (10 M, 56 g of KOH dissolved in 100 mL of water)(AR grade), water (Millipore, Milli-Q) *n*-hexane (HPLC grade) β-sitosterol reference standard, BSTFA reagent (N,O-*bis*(trimethylsilyl) trifluro), pyridine (GC grade).[2]

All working solutions of β-sitosterol were prepared in *n*-hexane. 100 µL of standard was placed into a vial, before 50 µL of pyridine was added. The vials were kept at 80 °C for 40 minutes. The final extract was taken in vial inserts and injected into the GC/MS.

Instrument

Agilent 8890 GC system with S/SL inlet and Agilent 5977B GC/MS single quadrupole

Ghee sample preparation

1. Take 200 mg of fat sample in a 15 mL screw cap tube.
2. Add saponification mixture (200 µL or 20% ascorbic acid and 500 µL of 10 M KOH solution).
3. Vortex for 5 minutes.
4. Place in a water bath (45 °C/30 min), cooled.
5. Add H_2O:hexane mixture (1:5) and vortex for 10 minutes.
6. Remove the upper 1 mL of the hexane layer into another tube (centrifuge if necessary).
7. Add 0.5 mL H_2O and vortex again.
8. Take 100 µL of the upper extract in a vial and add 50 µL of BSTFA and 50 µL of pyridine.
9. Incubate at 80 °C for 40 minutes.
10. Transfer the extract in a vial insert and inject into the GC/MS.

Figure 2. Liquid-liquid extraction, sample preparation.

Table 1. GC method.

GC Conditions	
Column	Agilent J&W HP-5ms Ultra Inert, 30 m × 0.25 mm, 0.25 µm (p/n 19091s-433UI)
Inlet	Agilent split/splitless inlet 5190-2293, splitless liner Injection volume: 1 µL
Split Mode And Ratio	Split ratio 5:1
Inlet Temperature	280 °C
Oven	80 °C for 1 minute, at 15 °C/min to 290 °C, hold 30 minutes
Carrier Gas	99.9995% helium at 1.0 mL/ min, constant flow mode

Table 2. MS method.

MSD Conditions	
Quadrupole Temperature	150 °C
Ion Source Temperature	EI 230 °C
Transfer Line Temperature	290 °C
Acquisition Type	SIM mode; ions – 396, 486, 357, 381, 129
EMV Mode	Delta EMV: 0
Dwell Time for Each Mass	50

Results and discussion

With the method described, instrument LOQ was determined at 200 ppb for the reference standard. Figure 3 highlights the quantifier and qualifier EIC at LOQ level. Figure 4 describes the signal-to-noise for 200 and 500 ppb level standards. Figure 4 also showcases the specificity of the method by showing a blank injection.

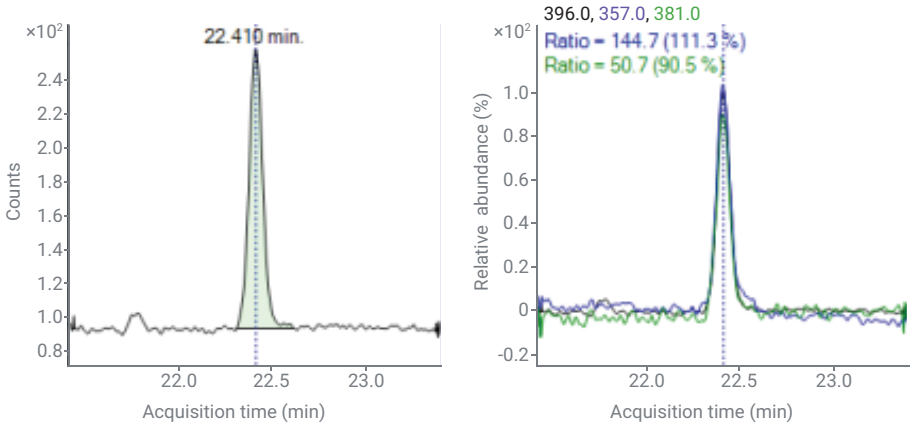

Figure 3. Qualifier and quantifier peaks of sitosterol at LOQ (200 ppb).

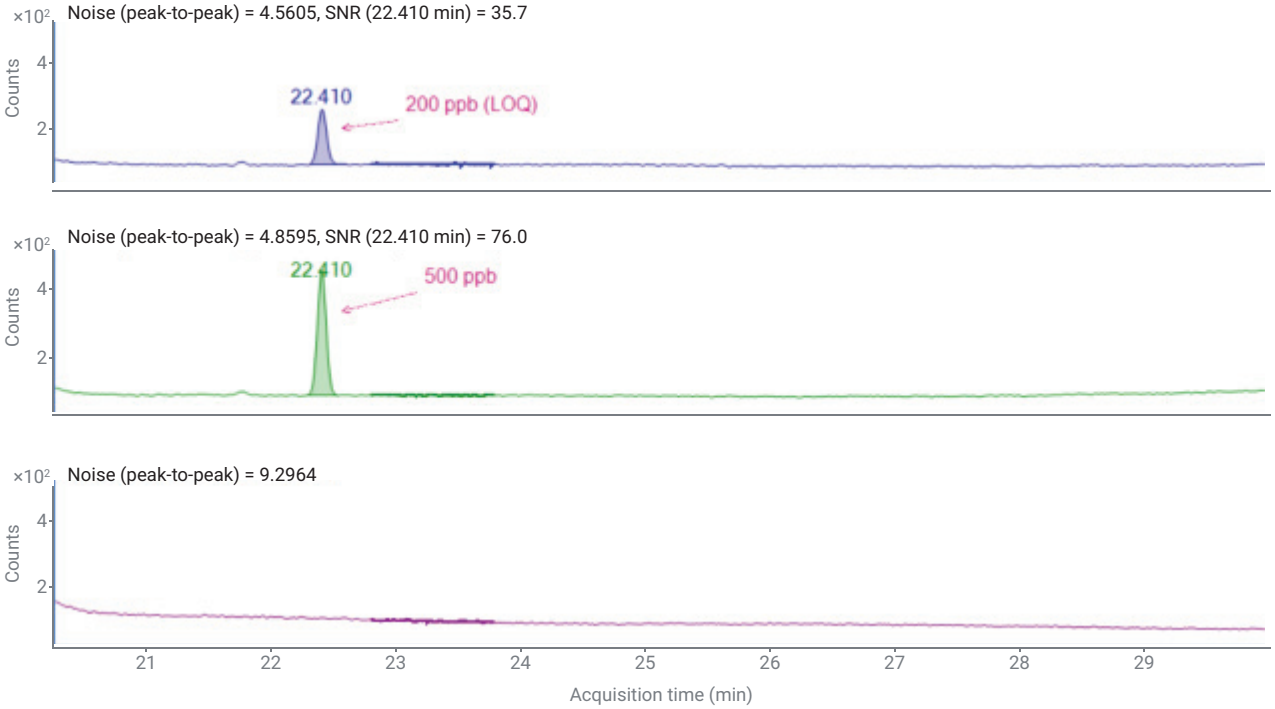

Figure 4. Sensitivity of β-sitosterol at 200 ppb (LOQ), 500 ppb, and blank.

Calibration and linearity

A linearity plot was generated for response (peak area) across concentration levels from 200 ppb to 5 ppm (figure 6). Calibration was performed at five levels, 200 ppb, 500 ppb, 1 ppm, 2 ppm, and 5 ppm. Linearity with R^2 >0.999 was observed. The calibration table with one quantifier ion and two qualifier ions is shown in Figure 7, in accordance with the regulations.

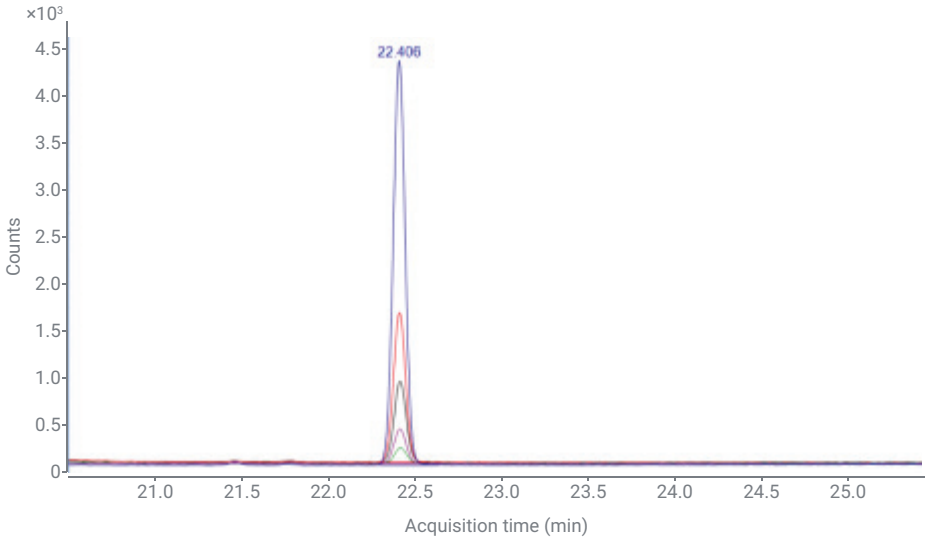

Figure 5. Overlay of various concentrations of β-sitosterol.

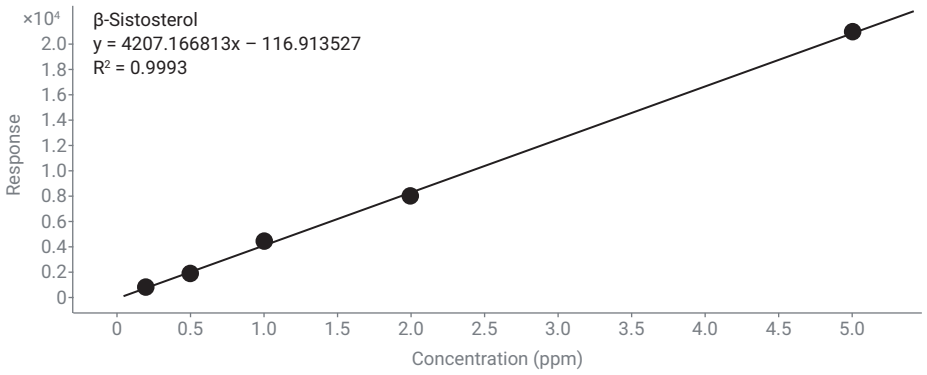

Figure 6. Calibration plot for β-sitosterol standards (concentration (ppm) versus response (peak area)).

Sample			b-Sitosterol...	b-Sitosterol Results				Qualifier (357...	Qualifier (381...
Name	Type	Level	Exp. Conc.	RT	Resp.	Final Conc.	Accuracy	Ratio	Ratio
STD-200ppb	Cal	1	0.2000	22.410	853	0.2305	115.2	144.7	50.7
STD-500ppb	Cal	2	0.5000	22.410	1852	0.4681	93.6	135.2	51.1
STD-1 ppm	Cal	3	1.0000	22.410	4323	1.0554	105.5	131.4	51.2
STD-2 ppm	Cal	4	2.0000	22.406	7983	1.9252	96.3	132.5	50.7
STD-5 ppm	Cal	5	5.0000	22.406	21006	5.0208	100.4	131.7	51.0
STD-1 ppm_rep1	Sample			22.402	4794	1.1672		127.1	53.2
STD-1 ppm_rep2	Sample			22.401	4894	1.1909		124.6	53.6
STD-1 ppm_rep3	Sample			22.397	4971	1.2094		128.8	52.4
STD-1 ppm_rep4	Sample			22.397	5082	1.2357		126.8	50.4
STD-1 ppm_rep5	Sample			22.393	5006	1.2178		121.0	51.3
STD-1 ppm_rep6	Sample			22.393	5132	1.2477		134.3	53.1

Figure 7. Calibration table for β-sitosterol from 200 ppb to 5 ppm and repeatability at 1 ppm.

Repeatability

A repeatable response was obtained by injecting 1 ppm β-sitosterol standard. As shown in Table 3, the % RSD data of β-sitosterol are calculated from peak areas of six replicate injections at 1 ppm concentration.

Table 3. Percentage RSD (CV) for peak area at 1 ppm β-sitosterol.

Area Inj-1	Area Inj-2	Area Inj-3	Area Inj-4	Area Inj-5	Area Inj-6	%RSD
4794	4894	4971	5082	5006	5132	2.48

Quantitation in ghee samples

The suggested method was extended to a ghee sample. The ghee sample was purchased from a market for the analysis and recovery study.

As shown in Figure 8, 2.24 ppm β-sitosterol was found in the ghee sample on which the study was performed. A chromatogram of ghee sample is shown in Figure 9.

Sample		b-Sitosterol Results			Qualifier (357.0)...	Qualifier (381.0)...
Data File	Type	RT	Resp.	Final Conc.	Ratio	Ratio
Ghee spike 5 ppm.D	Sample	22.381	1341	8.5195	106.1	42.8
Ghee spike 25 ppm.D	Sample	22.397	3815	23.1042	94.4	41.7
Ghee Sample.D	Sample	22.381	276	2.2418	107.6	

Figure 8. Quantitation in ghee samples.

Recovery study

Spiking was done at 5 and 25 ppm respectively. Table 4 shows recovery in the spiked samples after blank subtraction.

Table 4. Recovery in ghee samples.

Spiked Amount (ppm)	Observed Amount (ppm)	Final Amount (ppm)	Recovery (%)
5	8.52	6.28	125.6
25	23.1	20.86	83.4

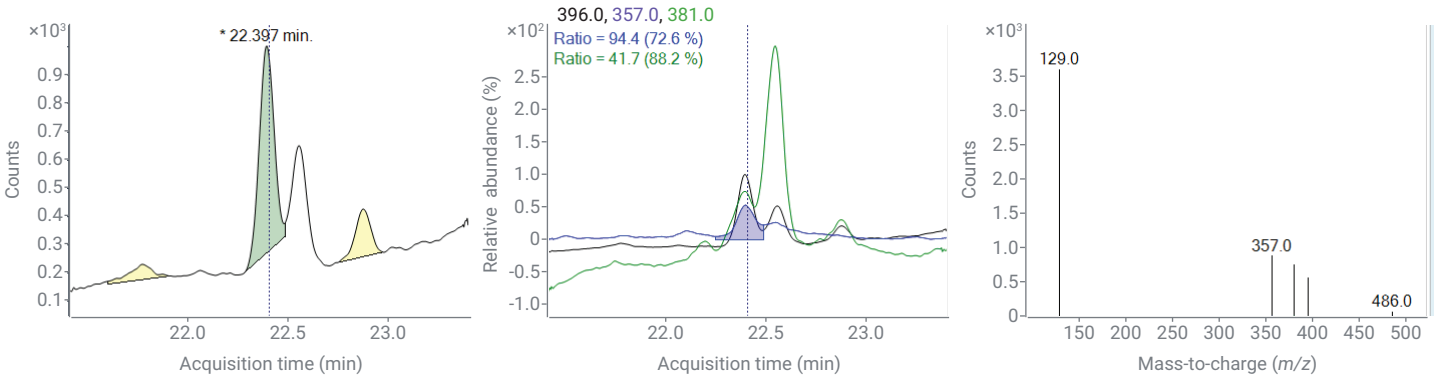

Figure 9. Chromatogram for ghee sample spiked at 25 ppm level for sitosterol.

Conclusion

An accurate and rugged method was developed for analysis of β-sitosterol in ghee samples for the identification of adulteration by vegetable oils. The saponification/LLE/derivatization based sample preparation method used easy and less time-consuming steps. The lowest calibration level was 200 ppb for the standard. Repeatable results were found for six replicates of standard. Satisfactory recovery was found at 5 and 25 ppm spiked concentration in ghee samples.

References

1. FSSAI releases Methods of Detecting Adulteration in Ghee, Food Safety Mantra, May 27, **2019**. https://www.foodsafetymantra.com/2019/05/fssai-releases-methods-of-detecting-adulteration-in-ghee.
2. Jyotika. Profiling of milk fat of different species of milch animals. Publisher: NDRI, Karnal. Citation: P-1452, **2016**.
3. Rani, A. *et al.* A Rapid Reversed-Phase Thin Layer Chromatographic Protocol for Detection of Adulteration in Ghee (Clarified Milk Fat) with Vegetable Oils. *Journal of Food Science and Technology* **2015**, *52(4)*, 2434–2439.

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