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Technical Report

Discovery® Ag-Ion SPE for FAME Fractionation and *Cis/Trans* Separation

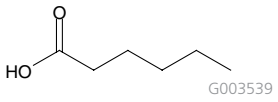
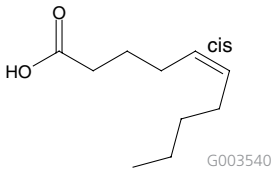
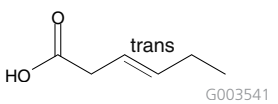
When silver ions are loaded and immobilized on to an SCX phase as counter-ions, they have the ability to form polar complexes with unsaturated fatty acid double bonds under normal-phase conditions. Discovery Ag-Ion SPE was developed for the fractionation of cis/trans isomers, and can also resolve FAMEs by degree of unsaturation in which retention strength increases with increasing number of double bonds. As a result, Discovery Ag-Ion SPE allows users to fractionate FAME samples prior to GC analysis, thereby simplifying analytical chromatography and improving method accuracy. In this report we demonstrate the utility of this technology against a variety of sample matrices including potato chips, butter cookies, popcorn oil, peanut butter, and poppy seed muffins.

Trans fats (trans unsaturated fatty acids) are fatty acids that contain double bonds that cause carbon atoms to bond in a straight configuration. As a result, they remain in a solid state at room temperature. Most naturally occurring unsaturated fatty acids are in the cis- orientation (bent), which allow for a liquid state at room temperature (Table 1).

Although small amounts of trans fat are produced in the GI tract of cattle and are found in dairy and beef fat, trans fats are predominately produced commercially in large quantities through a process called partial hydrogenation used to protect foods from spoilage (1).

Concerns have been raised for several decades that the consumption of trans fatty acids might have contributed to the 20th century epidemic of coronary heart disease (the raising of LDL or "bad" cholesterol). As a result, on July 9, 2003, the United States Food and Drug Administration (FDA) issued a regulation requiring manufacturers to list trans fat on the Nutrition Facts panel of foods and some dietary supplements. With this rule, consumers will have more information to make healthier food choices that could lower their consumption of trans fat as a part of a heart-healthy diet. As of January 1, 2006, food manufacturers are required to list trans fat on the nutrition label (2).

Table 1. Types of Fatty Acids

Structure	Common Sources	Health Effects
Saturated Fatty Acids (no double bonds)		
	Palm kernel, Palm oil, Coconut (tropical oils) Butter, Hydrogenated Oils and Shortenings	Raise LDL cholesterol and increase risk of cardiovascular disease
Mono and Polyunsaturated Fatty Acids (≥ 1 *cis* double bond)		
	Fluid/Liquid oils such as Soybean, Canola, Olive, Sunflower, and Corn Oils	Lower LDL cholesterol, associated with reduced risk of cardiovascular disease
Trans Fatty Acids (≥ 1 *trans* double bond)		
	Partially Hydrogenated Oils, Shortenings, Margarines, and Oils	Raise LDL cholesterol, like saturated fat, may also lower HDL. Associated with increased cardiovascular disease and possible type II diabetes

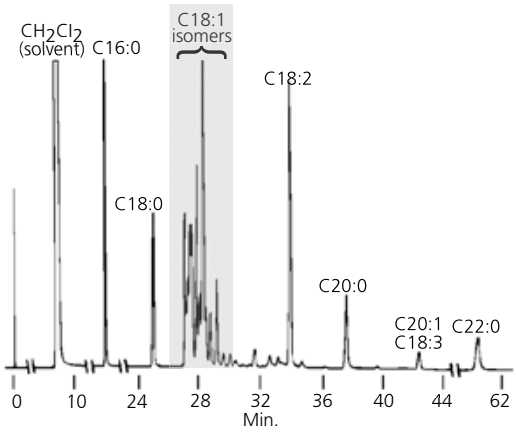
The Difficulty of *cis/trans* FAME Separation

Although there are a multitude of references available for the analysis of fatty acids, the analytical method specified for this new regulation is AOAC 996.06, "Fat (Total, Saturated, and Unsaturated) in Foods", and is suitable for the analysis of trans fats in a wide range of foods of varying fat content. In this method, fats are first extracted from food samples by hydrolytic methods (acidic and/or alkaline depending on food type) and petroleum ether followed by methylation to fatty acid methyl esters (FAMEs) using BF_3. FAMEs are further extracted into a small volume of hexane prior to GC analysis (3).

The most common trans fats in food are monounsaturated octadecenoic (C18:1) fatty acids (2), and are also the most difficult to resolve on polar capillary GC columns such as SP-2560, 100 m x 0.25 mm I.D., 0.20 µm (Figure 1). To simplify analysis, we discuss the use of silver-ion solid phase extraction (Ag-Ion SPE) to fractionate cis/trans-isomers and other FAMEs prior to capillary GC analysis.

Figure 1. Difficult Separation of C18:1 Fatty Acid Isomers

column: SP-2560, 100 m x 0.25 mm I.D., 0.20 µm (24056)
oven: 175 °C
det.: FID, 200 °C
carrier gas: helium, 20 cm/sec. at 175 °C
injection: 1 µL of positional *cis/trans* standard (5.0 mg/mL FAME isomers in methylene chloride), split 100:1, 200 °C



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Ag-Ion Chromatography

Silver-ion (argentation) chromatography was originally pioneered by Morris who demonstrated the first practical applications by separating lipids based on degree of unsaturation using TLC (4). Since then it has been adapted for HPLC by binding silver ions to an ion-exchange support (5). William Christie was among the first to demonstrate silver ion-chromatography using SPE (6) in which he treated packed bed SCX SPE tubes with silver nitrate. The bonded phase's silver counter-ions interact with the sample to offer good separation of monoenes, diene, and triene FAMEs.

How Does Ag-Ion Work?

In Ag-Ion SPE, silver ions are anchored onto SCX SPE functional group through electrostatic interaction. As the FAME sample passes through the cartridge, the SCX-silver counter-ions form specific polar complexes with double bonds of unsaturated FAMEs. More specifically, pi electrons of the fatty acid double bonds act as an electron donor and silver acts as an electron acceptor. Strength of the interaction increases with the number of double bonds, and saturated fatty acids (no double bonds) are poorly retained (7). Cis double bonds offer more steric accessibility than their trans counter part, and therefore form stronger polar complexes (Figure 2). As a result, cis fatty acids are more strongly retained on Ag-Ion SPE than trans fatty acids. Differences in retention strength between classes of FAMEs and silver counter-ions can be exploited allowing for FAME fractionation prior to GC analysis.

Discovery Ag-Ion SPE was specifically developed for the fractionation of FAMEs based on degree of unsaturation, and for the resolution of cis/trans isomers. The phase is loaded with silver using a proprietary procedure to offer optimal resolution, performance, and capacity. Each lot is tested and quality controlled for cis/trans FAME resolution.

Figure 2. Schematic Representation Ag-Ion SPE Interacting with cis-FAME

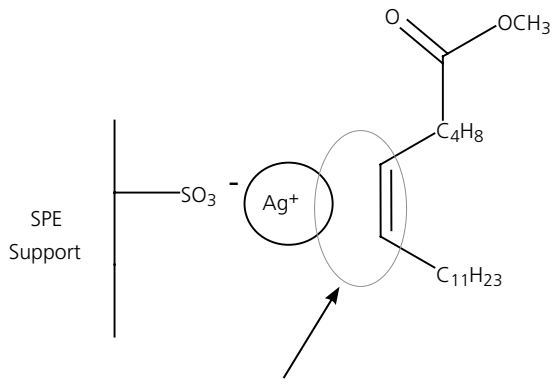

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Table 2. Recommended Discovery Ag-Ion SPE Protocol

Sample Extraction:

Food samples can be initially extracted using hydrolytic methods such as in AOAC 996.06 (3), or directly with petroleum ether. Fatty acids are then methylated to FAMEs using BF_3 in methanol. FAMES are further extracted into hexane prior to SPE fractionation and capillary GC analysis. Detailed procedures are described in individual applications discussed in this report.

Alternatively, extraction and methylation procedures described in AOAC 996.06 (3) and AOCS Official Method Ce 2-66 can be used prior to SPE fractionation (8). FAMEs should be dissolved in hexane prior to SPE fractionation.

Discovery Ag-Ion SPE:

SPE: Discovery Ag-Ion SPE Tube, 750 mg/6 mL (54225-U); OR

Discovery Ag-Ion Rezorian▲ SPE Cartridge, 750 mg/1 mL (54226-U)

Note: For all SPE steps, flow rate should be controlled at 5 mL/min., or 2-3 drops per second, and the sample/mobile phase should be pulled completely through the cartridge. However, excessive drying of the sorbent should be avoided.

SPE Step	Description	Comments
1. Condition	Condition SPE with 4 mL acetone.	Moisture adsorbed on to the SPE phase can affect normal-phase performance. Acetone conditioning removes any residual moisture from the SPE phase.
2. Equilibrate	Equilibrate cartridge with 4 mL hexane	
3. Sample Load	Load 1 mL of 1 mg/mL FAMEs in hexane derived from sample extraction.	Discovery Ag-Ion 750 mg cartridges have a maximum capacity of 1 mg FAMEs. Exceeding the capacity will reduce resolution efficiency of the cartridge.
4. Fraction 1	Elute fraction 1 with 6 mL hexane:acetone 96:4	Fraction 1 will target: • Saturated fatty acids • Trans monoenes • Cis/cis and trans/trans conjugated linoleic acids (CLAs)
5. Fraction 2	Elute fraction 2 with 4 mL hexane:acetone 90:10	Fraction 2 will target: • Cis monoenes • Trans/trans dienes • Cis/trans and trans/cis CLAs
6. Fraction 3	Elute fraction 3 with 4 mL acetone	Fraction 3 will target: • Cis/cis dienes • Other dienes • Most trienes
7. Evaporation/Reconstitution	Evaporate all fractions at 40 °C under N_2 sparge. Reconstitute in 1 mL hexane prior to GC analysis.	

▲Rezorian cartridges are small polypropylene barrels that are capped at both ends with Luer-Lock® fittings. The hardware is designed for low positive pressure applications. The cartridges can also be adapted for use with vacuum manifolds when used in conjunction with the proper Luer-Lock connectors. Please contact Supelco Technical Service for more information (800-359-3041/814-359-3041 or techservice@sial.com).

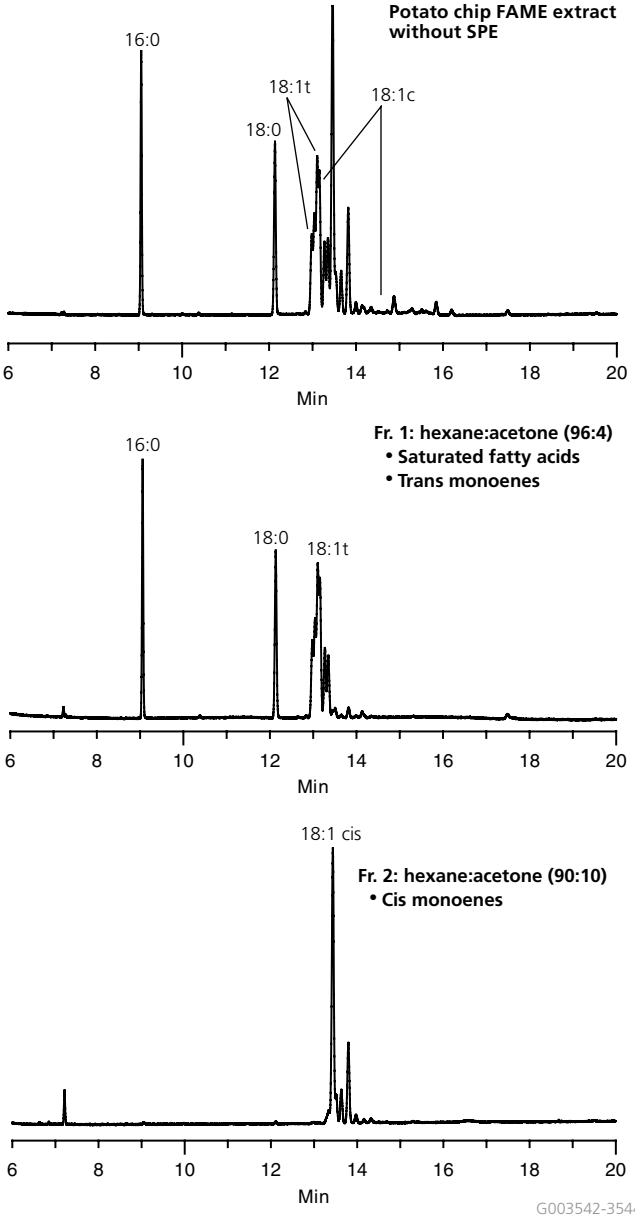
Table 3. GC Conditions for FAME Analysis

column:	SP-2560, 75 m x 0.18 mm I.D., 0.14 µm (23348-U)
oven:	180 °C, isothermal
inj.:	220 °C
det.:	FID, 220 °C
carrier gas:	hydrogen, 40 cm/sec. at 180 °C
injection:	0.5 µL, 100:1 split
liner:	4 mm I.D., split, cup design

Extraction and Fractionation of FAMES in Potato Chips

2.1 g of Middleswarth brand potato chips was ground to a fine consistency and extracted with 4 x 4 mL petroleum ether. For each ether extraction, the sample was centrifuged, and the supernatant was decanted, combined, and evaporated. The remaining fat residue was reconstituted in 16 mL toluene. A 1 mL aliquot of the toluene extract was transferred to a conical reaction vial, and 2 mL 7% BF_3 in methanol was added to facilitate methylation of extracted fatty acids. The reaction was incubated at 80 °C for 15 minutes using a heating block, and was allowed to cool to room temperature. 1 mL DI H_2O was added to quench the reaction, and 2 x 1 mL hexane (two fold extraction) was added to extract FAMEs. For each extraction, the upper hexane layer was transferred to a fresh vial, combined, evaporated, and reconstituted with 5 mL hexane and 50 mg anhydrous Na_2SO_4 prior to SPE fractionation using Discovery Ag-Ion SPE, 750 mg/6 mL. GC results are illustrated in Figure 3.

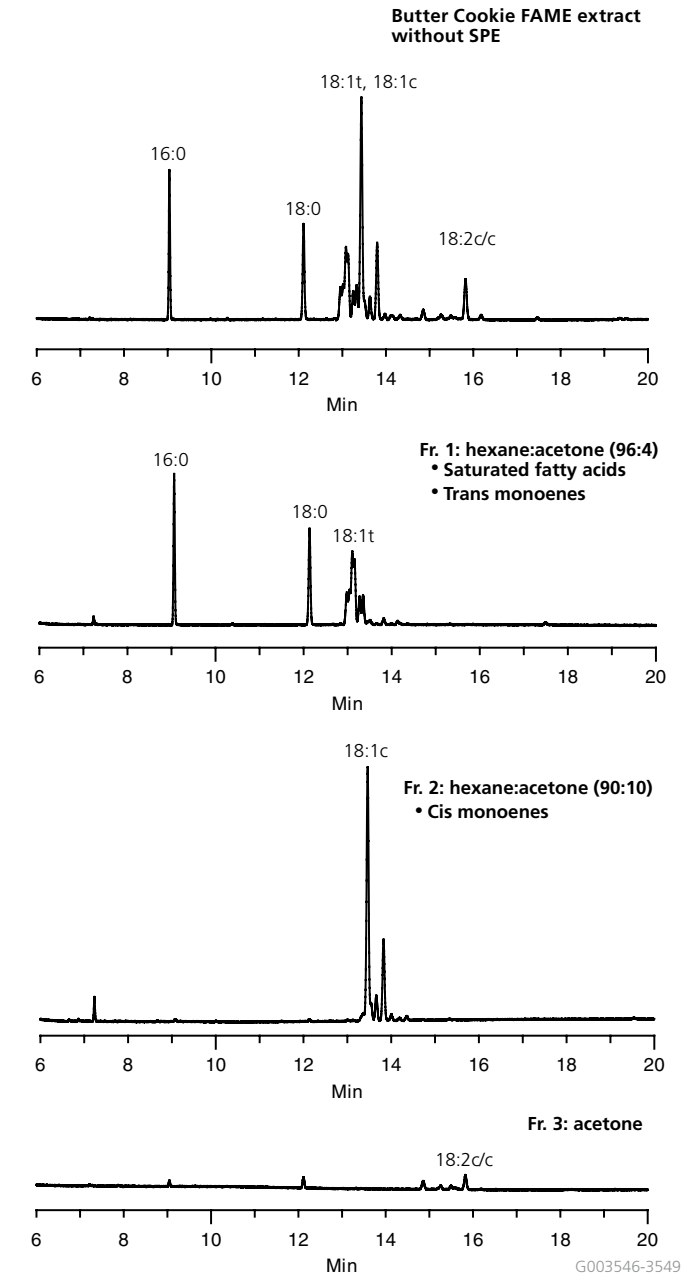
Figure 3. GC Results of Cis/Trans Fractionation of Potato Chips



FAMES Extraction and Fractionation of Butter Cookies

1.6 g of a butter cookie was ground to a fine consistency and extracted with 4 x 4 mL petroleum ether. The ether extract was centrifuged to remove particulates, and the ether supernatant was combined, evaporated, and reconstituted in 16 mL toluene. 1 mL of the toluene extract was transferred to a conical reaction vial to which 2 mL 7% BF_3 in methanol was added. The reaction vial was incubated at 80 °C for 15 minutes using a heating block and subsequently cooled to room temperature. 1 mL DI H_2O was added, and FAMEs were extracted with 2 x 1 mL hexane. The upper hexane layer was transferred combined into a fresh vial for each extraction where it was evaporated and reconstituted with 5 mL hexane and 50 mg anhydrous Na_2SO_4 prior to SPE fractionation using Discovery Ag-Ion SPE, 750 mg/6 mL. GC results are illustrated in Figure 4.

Figure 4. GC Results of Cis/Trans Fractionation of Butter Cookies



FAMEs Extraction and Fractionation of Microwave Popcorn Oil

1.0 g of popcorn oil was scraped from a popped microwave oven popcorn bag and mixed with 8 mL DI H_2O. The oil-water extract was further extracted with petroleum ether and methylated using the procedure described previously for both potato chips and butter cookies. GC results and recovery data are described in Figure 5 and Table 4, respectively.

Figure 5. GC Results of Cis/Trans Fractionation of Microwave Popcorn Oil

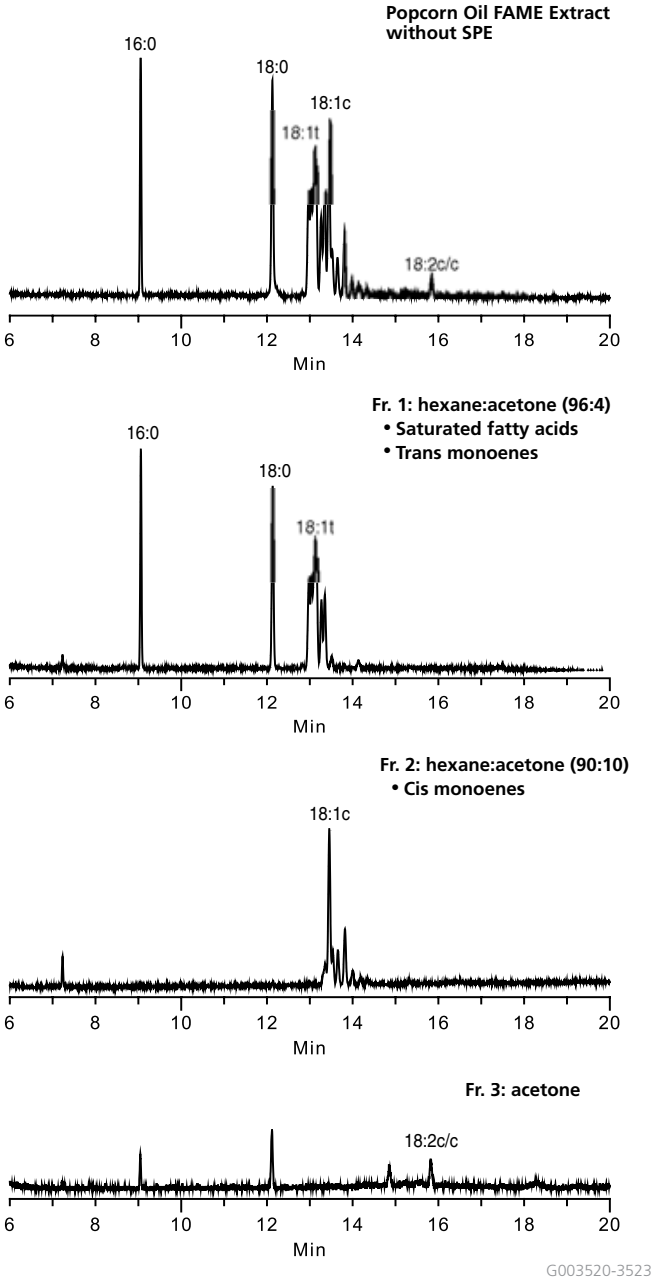


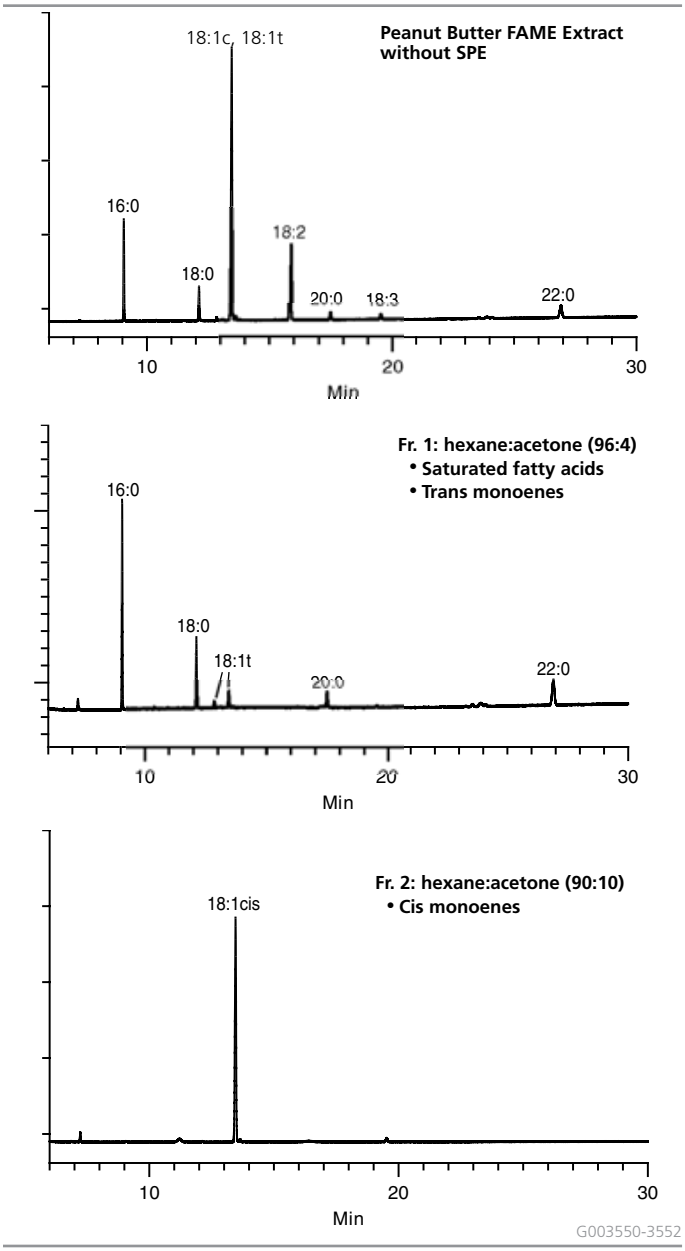
Table 4. Recovery Distribution of FAMEs Fractions of Microwave Popcorn Using Discovery Ag-Ion SPE

Fraction No.	Eluent (v/v)	Vol (mL)	18:0	Trans 18:1	Cis 18:1	Cis/Cis 18:2
1	hexane:acetone 96:4	6	100%	100%	2%	—
2	hexane:acetone 90:10	4	—	—	98%	—
3	acetone	4	—	—	—	100%

FAMEs Extraction and Fractionation of Peanut Butter

1.0 g of peanut butter was mixed with 8 mL DI H_2O and extracted with 4 x 4 mL petroleum ether and methylated using the procedure described previously for microwave popcorn oil. GC results are illustrated in Figure 6.

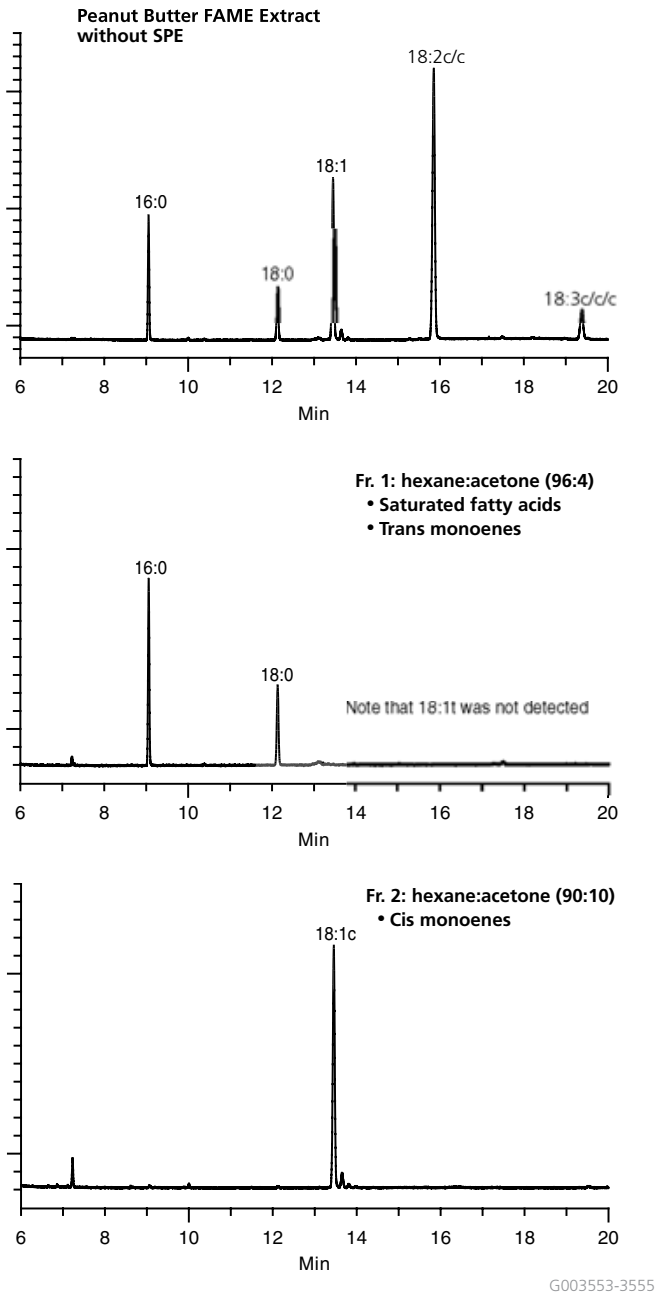
Figure 6. GC Results of Cis/Trans Fractionation of Peanut Butter



FAMEs Extraction and Fractionation of Poppy Seed Muffin

1.6 g of poppy seed muffin was chopped and ground to a fine consistency and extracted with 4 x 4 mL petroleum ether and methylated using the procedure described previously for microwave popcorn oil. GC results are illustrated in Figure 7. Note that no 18:1 trans fatty acids were detected under the described analytical conditions.

Figure 7. GC Results of Cis/Trans Fractionation of Poppy Seed Muffin



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Fractionation of FAMEs Based on Degree of Unsaturation

NuChek 68A FAMEs standard text mix (NuChek Prep, Inc., Elysian, MN) was diluted to 0.5 mg/mL in hexane. 0.2 mL of the diluted FAMEs standard was applied to a Discovery Ag-Ion Rezorian SPE cartridge, 750 mg/1 mL, pre-conditioned with 4 mL acetone and 4 mL hexane sequentially. Retained FAMEs were fractionally eluted with 6 solvent mixtures of increasing polarity. The fractions were analyzed via GC-FID (Table 5), and the results were tabulated for recovery and resolution efficiency (Table 6).

Table 5. GC Conditions for FAMEs Analysis of Discovery Ag-Ion Fractions

column:	SP-2380, 30 m x 0.32 mm I.D., 0.20 µm (24116-U)
oven:	100 °C, 3 °C/min. to 205 °C
inj.:	240 °C
det.:	250 °C
carrier gas:	helium
injection:	1.0 µL

Table 6. Fractionation of FAMEs Based on Degree of Unsaturation■

	% from total FAMEs present in each fraction						
Fatty Acid	**% composition**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
14:0	4.2	100					
14:1	4.0		100				
16:0	13.4	98	2				
16:1	6.5		100				
18:0	6.5	98	2				
18:1	6.7		100				
18:2	6.7			100			
18:3 ω 3	6.6				100		
20:0	4.7	100					
20:1 ω 9	5.0		100				
20:2 ω 6	5.0			100			
20:3 ω 6	5.1				100		
20:4 ω 6 / 20:3 ω 3	10.4				23	77	
22:0	3.3	100					
22:1	3.5		100				
22:6 ω 3	3.7						100
24:0	2.4	100					
24:1 ω 9	2.3		100				

F1 = saturated; 3 mL hexane
F2 = monoenes; 4 mL hexane:acetone 90:10
F3 = dienes; 5 mL acetone
F4 = trienes; 5 mL acetone:acetonitrile 97:3
F5 = tetraenes; 5 mL acetone:acetonitrile 94:6
F6 = hexanese; 5 mL acetone:acetonitrile 60:40

■Data provided by Mr. Richard Adlof of the USDA, Peoria, IL

Conclusion

It is well known that the double bonds of unsaturated fatty acids can form polar complexes with transitional metals such as silver. When silver is immobilized as a counter-ion on an SCX SPE phase, FAMEs can be resolved/fractionated on the basis of degree of unsaturation. Discovery Ag-Ion SPE also offers sufficient selectivity to resolve structural isomers such as cis/trans fatty acids. With the new trans fat labeling regulation mandated by the FDA, Discovery Ag-Ion SPE serves as a powerful tool for simplifying GC analysis and improving method accuracy.

Acknowledgements

We would like to thank Mr. Richard Adlof, USDA, Peoria, IL, for permission to use the method and data described in Tables 5 and 6 of this report.

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Ordering Information & Related Products

Description	Qty.	Cat. No.
Discovery Ag-Ion SPE		
750 mg/6 mL SPE Tube	30	**54225-U**
750 mg/1 mL Rezorian™ Cartridge	10	**54226-U**
SP-2560		
75 m x 0.18 mm I.D., 0.14 µm	1	**23348-U**
100 m x 0.25 mm I.D., 0.20 µm	1	**24056**
SP-2380		
30 m x 0.32 mm I.D., 0.20 µm	1	**24116-U**
30 m x 0.25 mm I.D., 0.20 µm	1	**24110-U**
Supelco 37 Component FAME Mix		
	1	**47885-U**
Linoleic Acid Methyl Ester Mix, cis/trans		
10 mg/mL (total weight) in methylene chloride, 1 mL C18:2 D 9c, 12c (10 % w/w) C18:2 D 9t, 12c (20 % w/w) C18:2 D 9c, 12t (20 % w/w) C18:2 D 9t, 12t (50 % w/w)	1	**47791**
Linolenic Acid Methyl Ester Isomer Mix		
10 mg/mL (total weight) in methylene chloride, 1 mL C18:3 D 9c, 12c, 15c (~3 % w/w) C18:3 D 9t, 12c, 15c (~7 % w/w) C18:3 D 9c, 12c, 15t (~7 % w/w) C18:3 D 9t, 12c, 15t (~15 % w/w) C18:3 D 9c, 12t, 15c (~7 % w/w) C18:3 D 9t, 12t, 15c (~15 % w/w) C18:3 D 9c, 12t, 15t (~15 % w/w) C18:3 D 9t, 12t, 15t (~30 % w/w)	1	**47792**
cis-9-Octadecenoic methyl ester		
10 mg/mL in heptane, 1 mL	1	**46902-U**
trans-9-Octadecenoic methyl ester		
10 mg/mL in heptane, 1 mL	1	**46903**

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