Application Note

Food Analysis

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Ensuring System Suitability for *cis-trans* FAMEs Analysis by AOAC 2012.13 on an Agilent 8890 GC with Retention Time Locking

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Abstract

AOAC Method 2012.13 determines fatty acid content in milk products and infant formula by capillary gas chromatography with flame ionization detection. This method was implemented on an Agilent 8890 GC system with a 100 m CP-Sil 88 column, and, in addition to a common array of 37 fatty acid methyl esters, a more detailed separation efficiency of oleic, linoleic, and linolenic acids was probed using 19 mono- and polyunsaturated *cis-trans* isomers from the C18 family. The resolution between key *cis*- and *trans*- FAMEs critical pairs was evaluated using a qualitative standard. Finally, retention time locking (RTL) was also implemented to avoid the need for re-alignment after column maintenance.

Introduction

Dairy and vegetable fats contain triglycerides, which are esters of glycerol coupled to three fatty acid strands. Fatty acids consist of a terminal carboxyl group connected to a hydrocarbon chain that can be short or long, linear or branched, and saturated, mono-, or poly-unsaturated. The configuration and location of the double bond in unsaturated fats results in numerous positional and *cis-trans* isomers. Proper identification of the isomers is critical for accurate nutritional labeling.

Separation and identification of fatty acid homologues and isomers from a complex matrix is challenging. After esterification, the corresponding fatty acid methyl esters (FAMEs) can be reproducibly analyzed by gas chromatography with flame ionization detection. Isomer elution order and separation efficiency are highly dependent on column stationary phase type and length. Although no column can fully resolve the diverse array of FAMEs *cis-trans* isomers, a 100 meter capillary column coated with highly polar cyanopropyl stationary phase such as CP-Sil 88 enables reliable, detailed separation of most *cis* and *trans* FAMEs isomers. Unlike traditional wax columns, *trans* isomers are eluted before *cis* isomers on the highly polar cyanopropyl stationary phase.

Experimental

An Agilent 8890 gas chromatograph configured with a split/splitless inlet (SSL), a flame ionization detector (FID), and an Agilent 7693A automatic liquid sampler (ALS) was used to generate the data.

Chemicals

Analytical standards were purchased to evaluate FAMEs performance:

- A 37 component FAMEs mix (47885-U) containing C4 to C24 FAMEs in the concentration range of 100 to 600 µg/mL
- A four-component, 10 mg/mL total *cis-trans* linoleic acid methyl ester mix (CRM47791)
- An eight-component linolenic acid methyl ester isomer mix (L6031) containing weight % for each component in the range of 3 to 30%
- A *cis-trans* FAMEs column performance mix (40495-U) containing various FAMEs at 2.5 mg/mL

All above mixtures were purchased from MilliporeSigma (St. Louis, MO). An eight-component *cis-trans* FAMEs Mix (35079) was purchased from Restek (Bellefonte, PA).

Table 1. Instrument conditions.

Agilent 8890 GC Conditions – AOAC Official Method 2012.13	
Injection	
Syringe Size	10 µL, p/n G4513-80204
Injection Volume	1 µL
Inlet	SSL, split mode
Temperature	250 °C
Split Ratio	10:1
Septum Purge Flow	3 mL/min
Column	Agilent CP-Sil 88, p/n CP7489
Dimensions	100 m × 0.25 mm, 0.20 µm
Carrier Gas	He, 0.8 mL/min, constant flow
Oven	
Initial Temperature	60 °C
Initial Hold	5 minutes
Ramp	15 °C/min to 165 °C, hold 1 minute 2 °C/min to 225 °C, hold 20 minutes
Detector	
Type	FID
Temperature	250 °C
Air Flow	400 mL/min
H_2 Fuel Flow	40 mL/min
N_2 Make Up Flow	25 mL/min

Results and discussion

To evaluate the separation over the entire range (Figure 1), a mixture of 37 FAMEs from C4:0 to C24:0 in dichloromethane solvent was injected. The 60 °C initial oven temperature setpoint and hold time ensures separation of C4:0, an important indicator of milk quality, from the solvent front. The ramped oven temperature program allows for efficient separation of the *cis-trans* isomers and the long-chain polyunsaturated FAMEs, other than coelution between the C23:0 and C20:4n6 peaks at approximately 48 minutes. However, C23:0 is not commonly found in milk products; consequently, this is of little concern.

A detailed separation of *cis-trans* isomers was probed using commercially available reference standard mixtures. The eight-component *cis-trans* FAMEs mix in dichloromethane from Restek was diluted 20x in dichloromethane, and injected into the GC. The method is well suited for resolution of the six *cis-trans* C18:1 FAMEs isomers (Figure 2).

Although there is minor coelution among the *trans* isomers, the group is well separated from the *cis*-C18 monounsaturated isomers, which is necessary since *trans* fatty acids are reported as a group in AOAC Official Method 2012.13.

To characterize the separation of the C18:2 isomers, a linoleic acid methyl ester FAMEs mix in dichloromethane (MilliporeSigma CRM47791) was diluted to a final concentration of 500 mg/L. Figure 3 shows that each of the linoleic FAMEs isomers is well resolved.

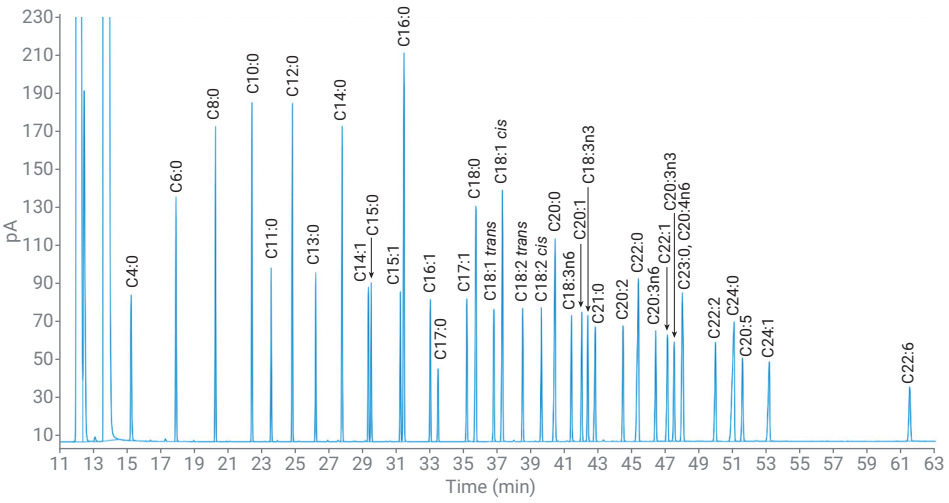


Figure 1. Chromatogram of 37 fatty acid methyl esters.

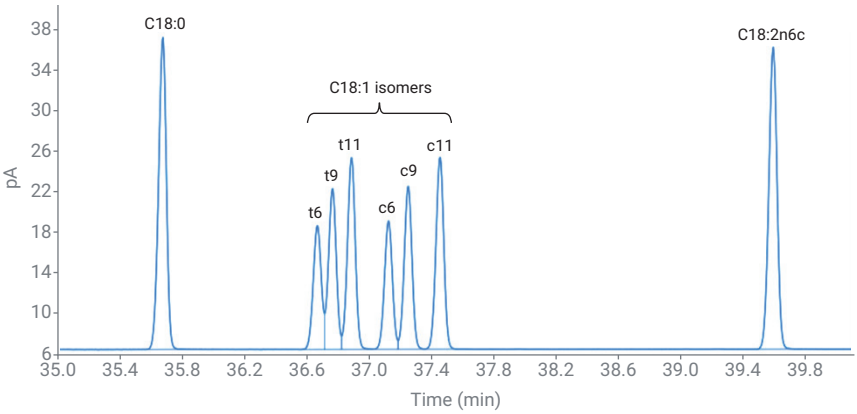


Figure 2. Enlarged chromatogram of C18:1 isomers by AOAC 2012.13.

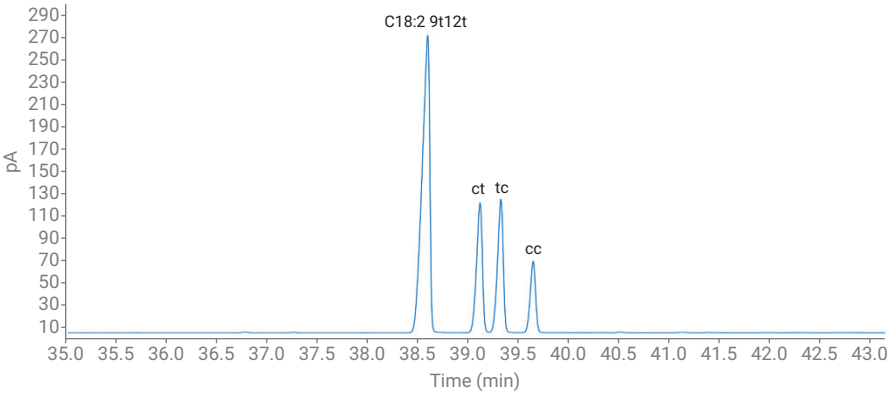


Figure 3. Enlarged chromatogram of C18:2 isomers by AOAC Method 2012.13.

Finally, method performance for the C18:3 isomers was examined by injecting a linolenic acid methyl ester mix (MilliporeSigma L6031), diluted to 2.5 mg/mL in dichloromethane (Figure 4). No column can completely resolve the linolenic acid isomers; however, for nutritional reporting purposes, the *trans*-containing isomers are well separated from *alpha*-linolenic acid methyl ester.

Method AOAC 2012.13 requires a performance evaluation check before calibration. Resolution is determined between *trans*-C18:1n13t/C18:1n14t and the *cis*-C18:1n9c/C18:1n10c peak (Figure 5, inset). Using Equation 1, acceptable resolution is achieved when the calculated R-value is equivalent to, or higher than, 1.00. Five replicates of a qualitative *cis-trans* FAME column performance mix (MilliporeSigma 40495-U) were injected to evaluate resolution and area/retention time repeatability.

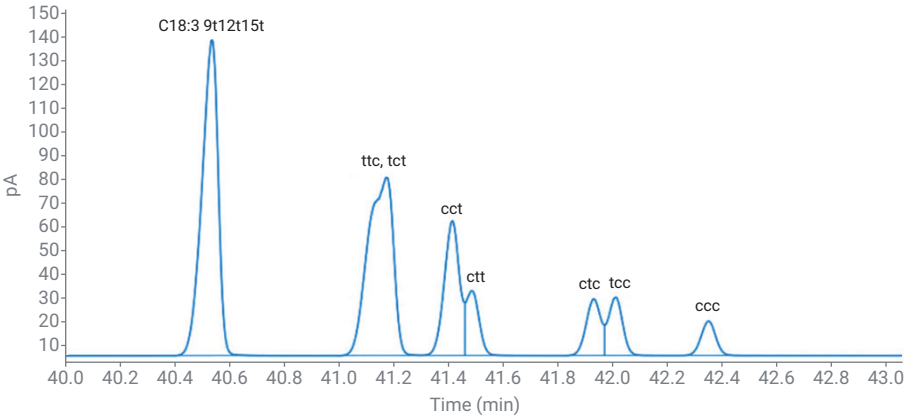


Figure 4. Enlarged chromatogram showing resolution of C18:3 isomers by AOAC Method 2012.13.

$$R = 1.18\left(\frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}}\right)$$

Equation 1. Peak resolution at half-height.

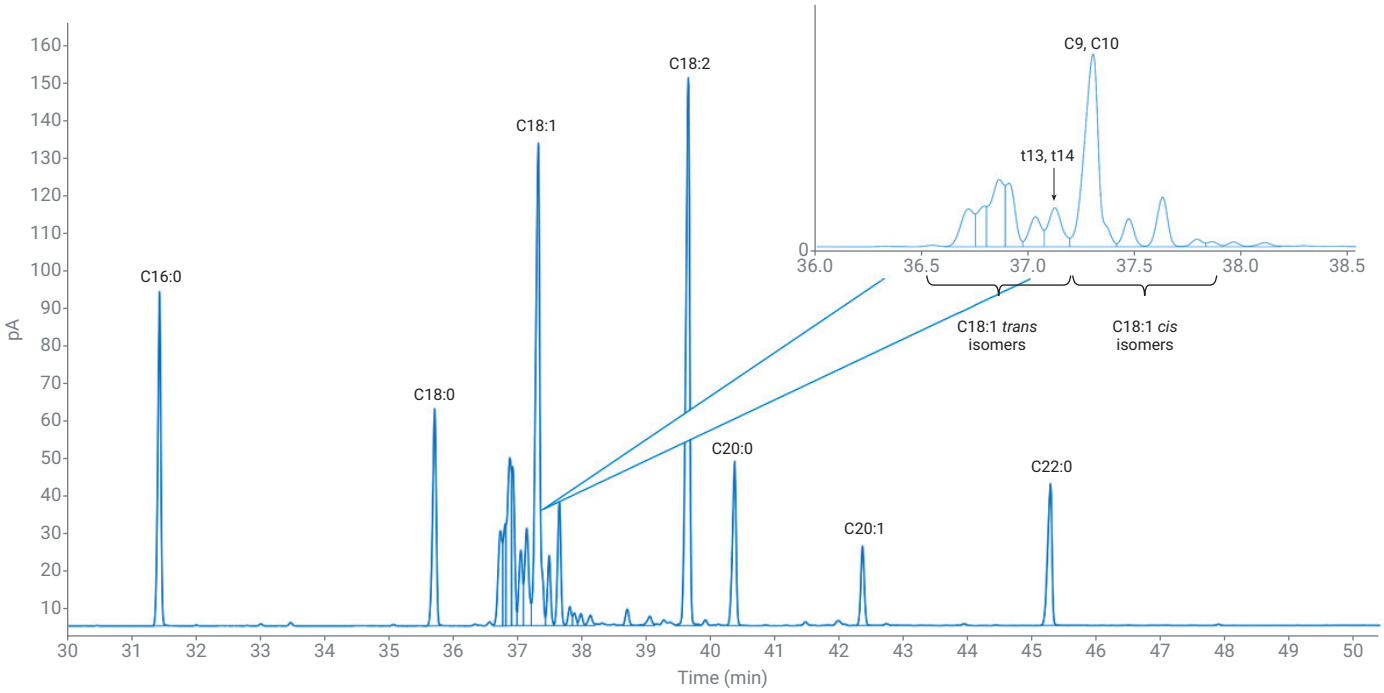


Figure 5. Enlarged chromatogram of qualitative column performance standard.

Table 2 summarizes the results for the critical pair used in the resolution check: retention time repeatability for both isomers is excellent, at 0.007% relative standard deviation (RSD). Peak area precision is less than 2.5% RSD for each isomer. The average peak resolution at half-height of 1.494 exceeds the minimum method resolution requirement.

Retention Time Locking

The array of FAMEs determined by AOAC 2012.13 requires both temperature and flow precision for proper identification; consequently, it is an excellent candidate for RTL. The Agilent RTL feature provides long-term repeatability on a given instrument, and eliminates the need to adjust retention times after column maintenance. It can maintain the same retention times after moving the method to a different GC, facilitating easier method transfer, and simplifying interlaboratory comparisons. RTL correctly matches and locks retention times for a specified compound by studying the relationship between inlet parameters and retention time during a series of reference runs, calibrating the system using the results, and storing the relationship in the method file.

Agilent OpenLab CDS 2 Acquisition features an RTL wizard to guide the operator through the process by selecting an acquisition method for locking, along with a target compound chosen from a previously acquired data file. The RTL wizard then sets up a series of pressure calibration runs based on the parameters specified by the designated acquisition method. One run is made using the original pressure setpoint, and two runs are made deviating the pressure by 15% above/below the method setpoint, respectively. The deviation in pressure setpoints simulates changes in column length commonly encountered after maintenance, for example, after column trimming. Locked methods do not require retention time re-alignment after column maintenance or replacement; rather, the original instrument retention times can be matched through a simple relocking standard. If the relocking standard also serves as the column performance evaluation standard, users can satisfy the performance evaluation requirements specified by AOAC Method 2012.13 in a single step.

Table 2. Results from five replicate injections of a qualitative column performance FAMEs standard mixture.

Value	Description	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	RSD
t_{R1}	Retention time in min, t13/t14 peak	37.124	37.125	37.128	37.128	37.13	0.007%
t_{R2}	Retention 2 in min, c9/c10 peak	37.304	37.305	37.308	37.308	37.31	0.007%
	Area t13/t14 peak	115.605	113.463	117.747	115.944	120.812	2.356%
	Area c9/c10 peak	599.259	588.91	610.828	602.276	628.644	2.458%
$W_{0.5h1}$	Width at half height, t13/t14 peak	0.073	0.072	0.073	0.073	0.074	0.969%
$W_{0.5h2}$	Width at half height, c9/c10 peak	0.069	0.069	0.069	0.069	0.07	0.646%
R	Peak resolution at half-height	1.496	1.506	1.496	1.496	1.475	0.77%

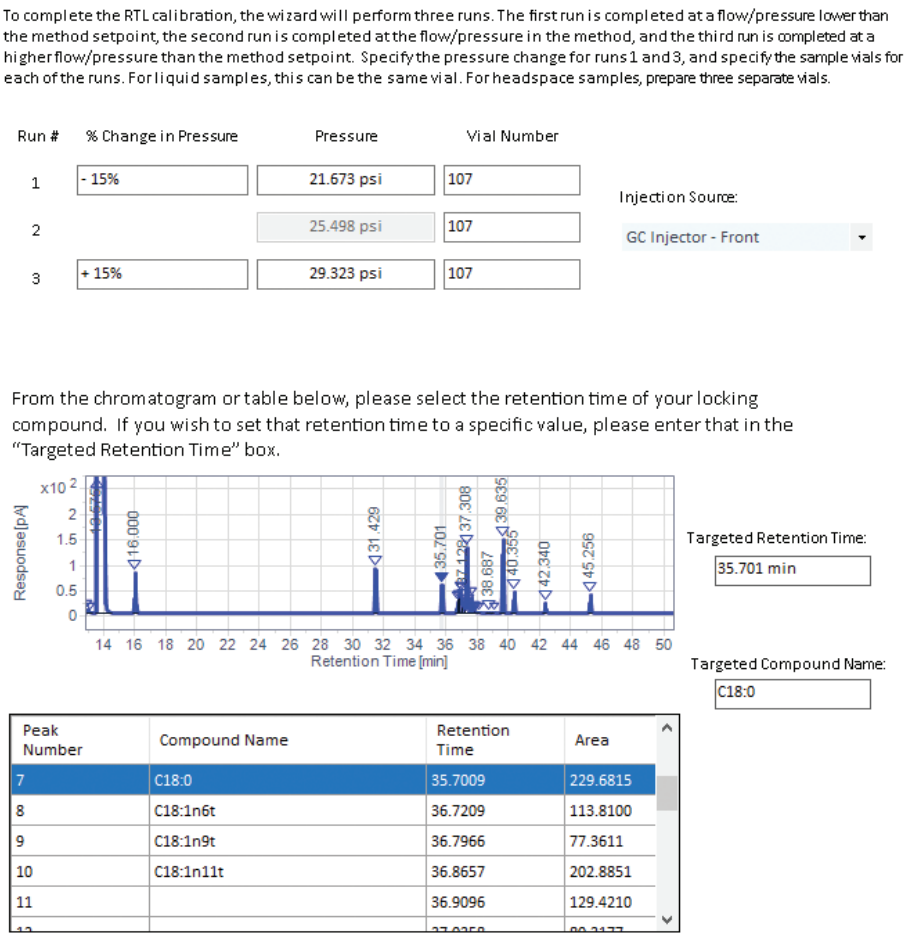


Figure 6. Retention time locking wizard setup screen.

The *cis-trans* FAMEs Column Performance Mix standard was used to retention lock the method. An acquisition method, processing method, and a processed results file from a previously acquired injection of the performance mix standard were selected. C18:0 was chosen as the target compound for RTL. As shown in Figure 6, the RTL Wizard guided the pressure calibration process using the C18:0 peak with a targeted retention time of 35.701 minutes. The pressure calibration curve achieved from the series of three runs automatically scheduled by the RTL wizard demonstrated excellent correlation (Figure 7). The analytical method was then locked at the indicated pressure of 25.498 psi. Afterwards, column maintenance was performed by trimming approximately 0.5 m from the head of the CP-Sil 88 100 m column.

To evaluate the extent of retention loss from the change in column length, the *cis-trans* FAMEs Column Performance Mix standard was reanalyzed post-maintenance; the retention time of the target C18:0 peak shifted forward by approximately 0.232 minutes. Had the system not been retention locked, the operator would have been required to analyze a standard containing the full suite of desired FAMEs, and manually re-enter analyte retention times in the data processing method.

Rather than manually re-enter the new analyte retention times, the analytical method was then relocked using the RTL Wizard, and a new pressure setpoint of 25.253 psi was implemented. This new pressure setpoint accommodates the change in column length to ascertain the expected retention times, thereby facilitating faster system readiness. The *cis-trans* FAMEs Column Performance Mix standard was re-injected to ascertain if the new pressure setpoints resulted in the expected retention times. Figure 8 shows a summary of the results, along with a chromatographic overlay. The relocked, postcolumn trim retention times closely matched the original, pretrim retention times, with changes in retention times ranging from 0.000 to 0.009 minutes for each of the key analytes on the 100 m column. Note the actual C18:0 retention time of 35.700 versus the initially targeted retention time of 35.701 minutes—a relative percent difference of 0.0028%.

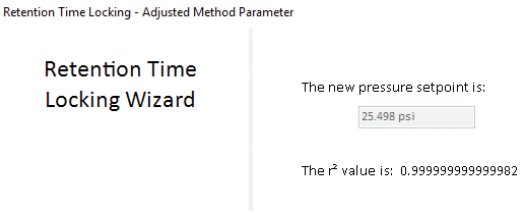
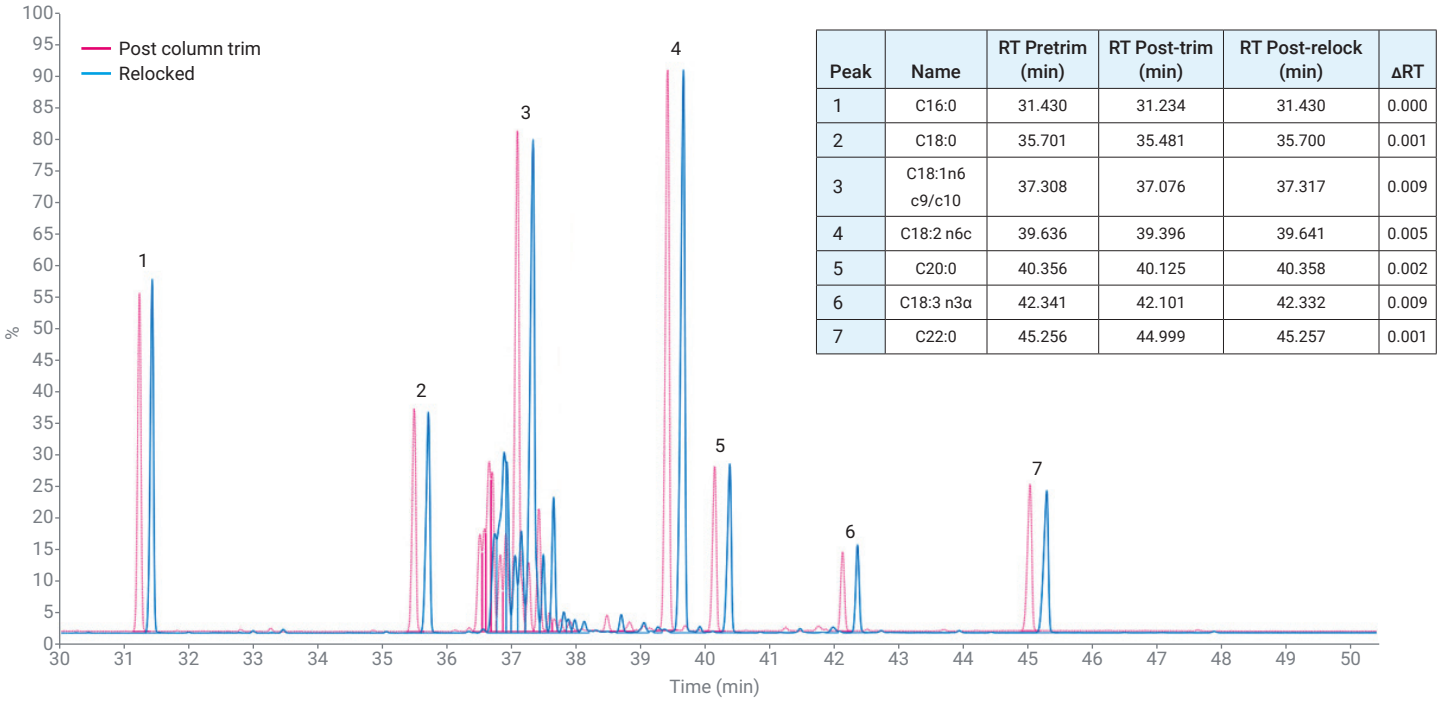


Figure 7. Retention time locking pressure calibration results.



Peak	Name	RT Pretrim (min)	RT Post-trim (min)	RT Post-relock (min)	ΔRT
1	C16:0	31.430	31.234	31.430	0.000
2	C18:0	35.701	35.481	35.700	0.001
3	C18:1n6 c9/c10	37.308	37.076	37.317	0.009
4	C18:2 n6c	39.636	39.396	39.641	0.005
5	C20:0	40.356	40.125	40.358	0.002
6	C18:3 n3α	42.341	42.101	42.332	0.009
7	C22:0	45.256	44.999	45.257	0.001

Figure 8. Results and enlarged chromatographic overlay of column performance standard before and after relocking.

Conclusion

Separation of FAMEs isomers using an Agilent CP-Sil 88 100 m column on an Agilent 8890 GC system enables reliable resolution that exceeds the minimum performance evaluation requirements specified by AOAC Method 2012.13. Excellent injection repeatability of a specialized FAMEs column performance standard was obtained, with retention time RSDs of 0.007% for two key C18 isomers. Finally, using a column performance standard in tandem with Agilent's unique RTL feature enables consistently correct identification of FAMEs isomers, faster uptime after instrument maintenance, and easier lab-to-lab comparisons.

References

1. Official Methods of Analysis AOAC International, Method 2012.13, **2012**.
2. David, F.; Sandra, P.; Vickers, A. K. Column Selection for the Analysis of Fatty Acid Methyl Esters. *Agilent Technologies Application Note*, publication number 5989-3760EN, **2005**.
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