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Application News

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Gas Chromatography

Modifying AOAC Method 996.06 for FAME Analysis in Foods: Faster Throughput Using Hydrogen Carrier Gas

■ Abstract

As the cost of helium continues to rise, more labs are converting to hydrogen carrier gas for their gas chromatographs. This change, coupled with a method that increases sample throughput, can lead to a significant increase in company profits. A Shimadzu Nexis™ GC-2030 was used for FAME analysis. It was shown that a modification to AOAC method 996.06 for FAME analysis in food products can reduce the analysis time by greater than 50% while yielding baseline resolution for all analyte peaks in a 37-component mixture. A four-point, quadratic calibration curve that was generated using the modified H2 method gave an R^2 value greater than 0.9995 for each analyte. Reproducibility studies were able to provide low area count relative standard deviation values below 2.1%. This study shows that the modified method using hydrogen carrier gas provides results consistent with the industry standard.

■ Introduction

The US Food and Drug Administration (FDA) requires that nutrition labels reflect the fat content within food products. This regulation is in effect because foods that are high in saturated and *trans* fats have negative impacts on the health of the consumer. These types of fats have high melting points and therefore have a greater potential to be solid in the blood stream. This could lead to plaque buildup in the arteries that can result in heart disease, the leading cause of death in the United States.

Gas chromatography can be used to identify and quantify the fatty acid content in foods. Due to the acidity and high polarity of the carboxylic acid functional group in fatty acids, it is necessary to convert them to fatty acid methyl esters (FAMEs) prior to analysis to make them compatible with the stationary phase of the column being used. A peer reviewed method for this conversion to the esterified form, as well as the analytical conditions of the gas chromatograph consistent with this analysis, has been published by the Association of Official Analytical Chemists (AOAC) in method 996.06[1].

Many adhering to AOAC 996.06 have expressed discontent with the time required to complete this method and its general operating costs. A faster method that can maintain or increase the baseline resolution of the peaks would result in an increased throughput of samples. Converting the carrier gas from helium to hydrogen would greatly reduce overhead given that the price of helium has increased exponentially as this non-renewable resource has become scarcer. Hydrogen gas is cheap and can be created through the electrolysis of water using a hydrogen generator. As per the Golay curve (Figure 1), the use of hydrogen carrier gas offers greater chromatographic efficiency, at increased linear velocities, than other carrier gas options. Therefore, the goal of this study was to modify the AOAC method such that the analysis time was significantly reduced, while being more budget-friendly, by using hydrogen carrier gas.

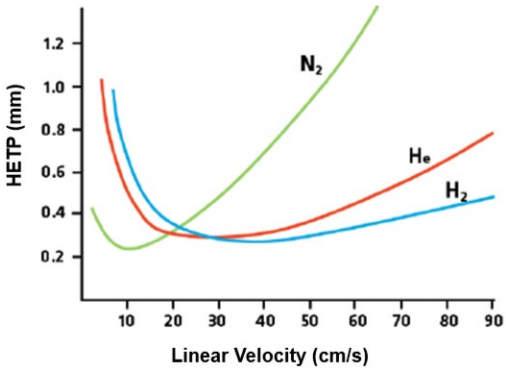

Figure 1: Golay plot to indicate the separation efficiency of common carrier gases in relation to their linear velocity. The separation efficiency is best when the height equivalent to theoretical plates (HETP) is at a minimum such that the lowest point of each curve indicates the optimum linear velocity for the analysis. Hydrogen carrier gas possesses a broad curve indicating that it is suitable at a wide range of increased linear velocities.

■ Samples and Analytical Conditions/Experimental

Reagents

A 37-component food industry FAME mix was purchased from Restek (cat. # 35077) as a representative sample of the most common FAME content in food products (Table 1). Hexane (> 95% from Sigma-Aldrich) was used as the diluent in a serial dilution of the standard.

This was performed to give additional calibration levels that were 0.50, 0.25, and 0.10 times the concentration of the individual analytes in the sourced standard for a total of four calibration standards. Each standard was analyzed in triplicate for the hydrogen carrier method.

Table 1: Instrument Configuration and Analysis Conditions

Elution Order	Analyte	Structural Nomenclature	Conc. of Standard (µg/mL)	Elution Order	Analyte	Structural Nomenclature	Conc. of Standard (µg/mL)
1	Methyl butyrate	C4:0	40	20	Methyl linoleate	C18:2 (c9, c12)	20
2	Methyl caproate	C6:0	40	21	Methyl archidate	C20:0	40
3	Methyl octanoate	C8:0	40	22	Methyl linolenate	C18:3 (c6,c9,c12)	20
4	Methyl decanoate	C10:0	40	23	Methyl eicosenoate	C20:1 (c11)	20
5	Methyl undecanoate	C11:0	20	24	Methyl linolenate	C18:3 (c9,c12,c15)	20
6	Methyl dodecanoate	C12:0	40	25	Methyl heneicosanoate	C21:0	20
7	Methyl tridecanoate	C13:0	20	26	Methyl eicosadienoate	C20:2 (c11,c14)	20
8	Methyl myristate	C14:0	40	27	Methyl behenate	C22:0	40
9	Methyl myristoleate	C14:1 (c9)	20	28	Methyl eicosatrienoate	C20:3 (c8,c11,c14)	20
10	Methyl pentadecanoate	C15:0	20	29	Methyl erucate	C22:1 (c13)	20
11	Methyl pentadecenoate	C15:1 (c10)	20	30	Methyl eicosatrienoate	C20:3 (c11,c14,c17)	20
12	Methyl palmitate	C16:0	60	31	Methyl archidonate	C20:4 (c5,c8,c11,c14)	20
13	Methyl palmitoleate	C16:1 (c9)	20	32	Methyl tricosanoate	C23:0	20
14	Methyl heptadecanoate	C17:0	20	33	Methyl docosadienoate	C22:2 (c13,c16)	20
15	Methyl heptadecenoate	C17:1 (c10)	20	34	Methyl lignocerate	C24:0	40
16	Methyl stearate	C18:0	40	35	Methyl eicosapentaenoate	C20:5 (c5,c8,c11,c14,c17)	20
17	Methyl octadecenoate	C18:1 (t9)	20	36	Methyl nervonate	C24:1 (c15)	20
18	Methyl oleate	C18:1 (c9)	40	37	Methyl docosahexaenoate	C22:6 (c4,c7,c10,c13,c16,c19)	20
19	Methyl linolelaidate	C18:2 (t9,t12)	20				

Instrumentation and Method Conditions

A Shimadzu Nexis™ GC-2030 equipped with a split/splitless (SPL) injector port, flame ionization detector (FID), and AOC-20i+s autosampler was used for this application. A hydrogen sensor was installed on the GC as a safety measure because of the flammable nature of hydrogen. Shimadzu's new automated Gas Selector was also installed on the unit to allow method development without the need to manually replumb any gases. All gases were passed through in-line filters.

The 37-component FAME mix was initially analyzed according to the AOAC method 996.06 which requires the use of helium carrier gas. The GC parameters for this analysis, as well as the modified fast method using hydrogen carrier gas, are included in Table 2.

Table 2: Method conditions for the AOAC method 996.06 and Shimadzu's faster method using hydrogen carrier gas, at an increased linear velocity, with a modified oven temperature program.

	Original AOAC Method 996.06	Shimadzu Nexis GC-2030 Fast Hydrogen Method
Inlet	1 µL Split Injection; 225 °C; Split Ratio 200:1	
Column	Rt-2560 100 m × 0.25 mm ID × 0.20 µm film thickness	
Carrier	Helium; Constant Linear Velocity 18 cm/s	Hydrogen; Constant Linear Velocity 35 cm/s
Oven	100 °C (4 min hold); 3 °C/min to 240 °C (15 min hold)	150 °C (2 min hold); 4 °C/min to 220 °C; 2 °C/min to 240 °C (8 min hold)
FID	285 °C; H2 32 mL/min; Air 200 mL/min; Make-up (N2) 24 mL/min	

■ Results and Discussion

The AOAC method 996.06 outlines the analysis of total, saturated, and unsaturated fat in food products. Although the method does suggest that the linear velocity of the carrier gas should be set to 18 cm/s and the column flow rate should be 0.75 mL/min, it does not indicate which parameter (inlet pressure, linear velocity, or column flow) should be held constant throughout the analysis. Because the Golay plot indicates that the separation efficiency has a direct correlation with the linear velocity parameter, the linear velocity of the carrier gas was held constant for all analyses.

The analysis of the FAME mixture using AOAC method 996.06 is shown in Figure 2. This analysis had a run time over 65 minutes and had problems with the resolution of some peaks. There was poor resolution between the peaks eluting at 51.770 minutes [C18:3(c9,c12,c15)] and 51.902 minutes [C21:0] with a resolution of 1.346 (USP calculation method). Although there appeared to be one peak at 55.890 minutes, it actually corresponded to both C20:3(c11,c14,c17) and C20:4(c5,c8,c11,c14) due to coelution. It is also important to note that the large split ratio in the AOAC method resulted in the waste of expensive helium gas at a rate of over 300 mL/min. These problem areas provided room for improvement in the fast hydrogen method.

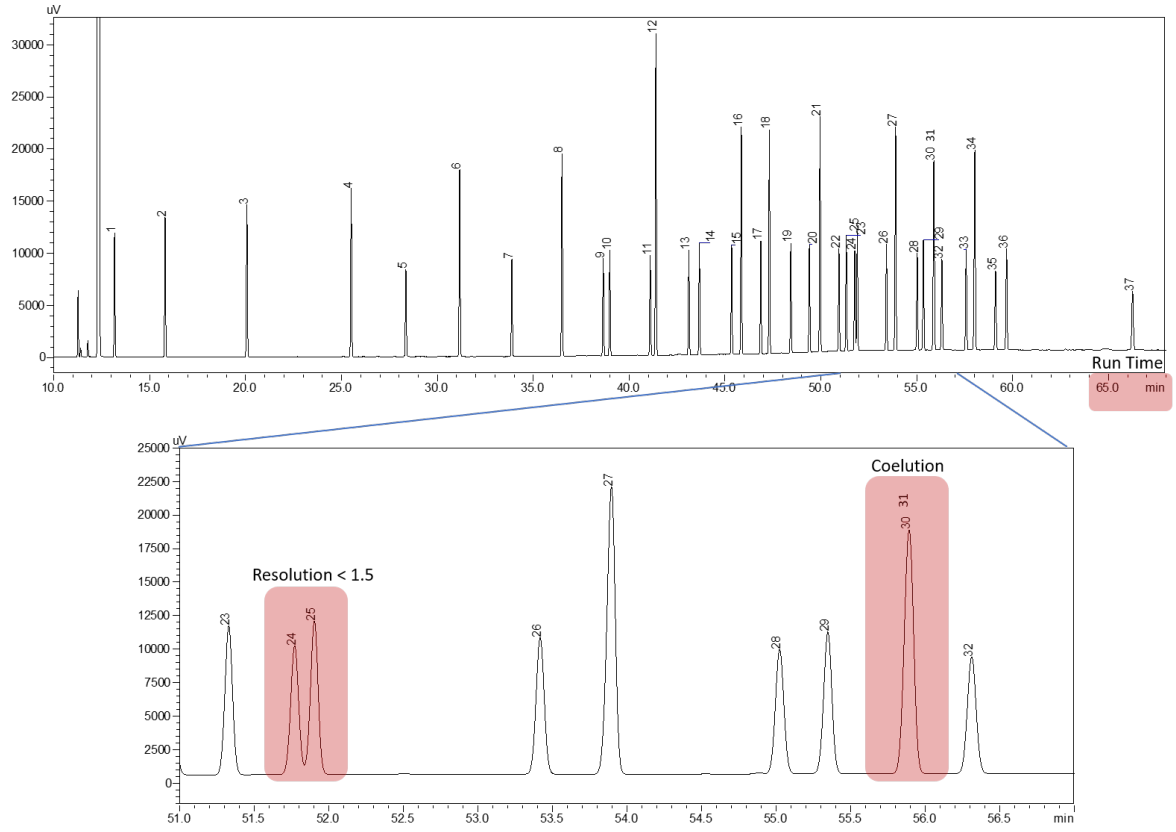

Figure 2: Chromatogram of the 37-component FAME mix using AOAC method conditions. The dead time has been removed and the problem areas are highlighted in red. The long run time and coelution of peaks warrants the modification of this method to yield better results. The analysis segment from 51 to 57 minutes has been expanded to show the unresolved peaks.

The extended dead time and excessive resolution in the beginning of the helium analysis suggests that the starting temperature of the oven and the initial temperature ramp could be increased without having a negative impact on the chromatography. This will decrease the dead time and compress the early eluting peaks together, resulting in a shortened run time.

Also, because a Golay plot shows that the optimal linear velocity for hydrogen carrier gas is higher than that of helium, pushing the carrier gas through the column at a higher linear velocity would also result in a reduced analysis time without sacrificing separation efficiency.

The analytical conditions for the modified fast method using hydrogen carrier gas can be found in Table 2 and the resulting chromatogram is shown in Figure 3.

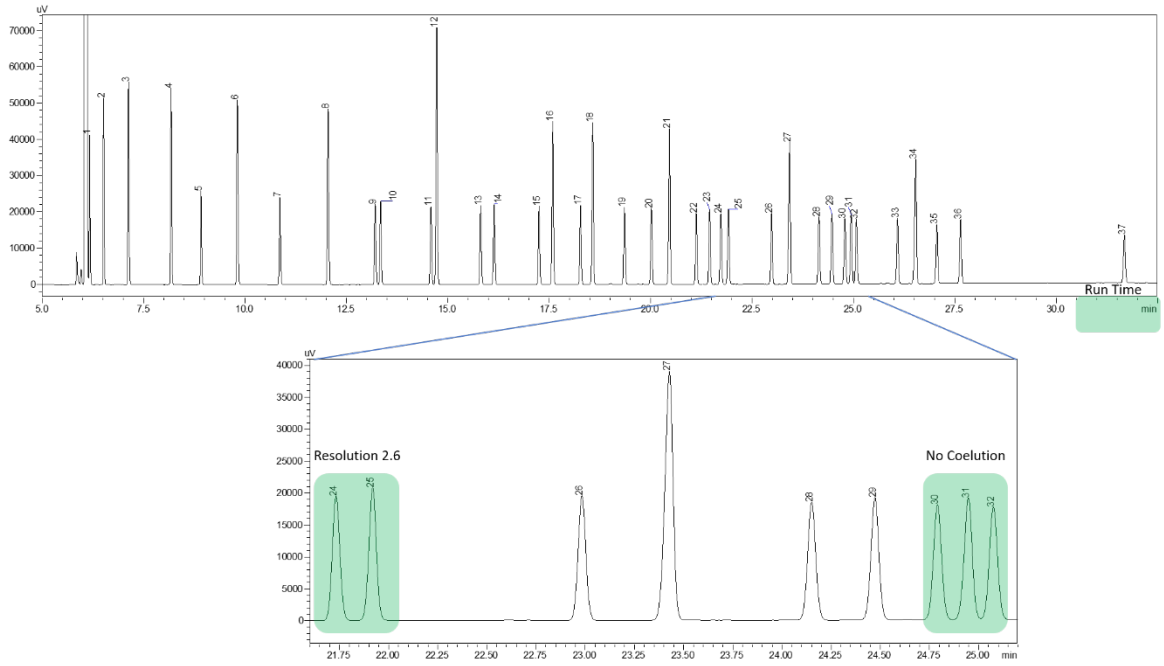

Figure 3: Chromatogram of the 37-component FAME mix using the modified fast method with hydrogen carrier gas. The dead time has been removed and the positive improvements over the AOAC method have been highlighted in green. The AOAC run time has been reduced by greater 50% and there is baseline resolution of all peaks of interest.

Table 3 shows a comparison of the retention times between the AOAC method and the faster method using hydrogen carrier gas. Notably, the analysis time for the hydrogen method is reduced to less than half of the time for the AOAC method.

Each of the standards used for the fast hydrogen method were analyzed in triplicate and the calibration curves were generated using an external standard method with a quadratic fit that was not forced through zero. The R^2 value for each curve was above 0.9995 as shown in Table 3.

Sample calibration curves for peaks 30 and 31 have been included in Figure 4. Note that these peaks coeluted when using the AOAC method; therefore, it was important to highlight the quality of these calibration curves when baseline resolved using the fast hydrogen method.

Table 3: Retention time comparison of each analyte using the AOAC method with helium versus the Fast Method with hydrogen. The included area %RSD and R^2 value for the quadratic calibration curve for the Fast H2 Method reflects the quality of the data obtained.

Elution Order	Analyte	Retention Time for AOAC Method (min)	Avg. Retention Time for Fast H_2 Method (min)	Area % RSD for Conc. Standard Fast H_2 Method (n = 3)	R^2 Value for the Quadratic Fit Calibration Curve Fast H_2 Method
1	Methyl butyrate	13.174	6.164	1.606	0.999535
2	Methyl caproate	15.803	6.511	1.560	0.999995
3	Methyl octanoate	20.074	7.126	1.516	0.999998
4	Methyl decanoate	25.511	8.178	1.571	0.999975
5	Methyl undecanoate	28.357	8.919	1.546	0.999944
6	Methyl dodecanoate	31.166	9.818	1.772	0.999945
7	Methyl tridecanoate	33.883	10.869	1.687	0.999918
8	Methyl myristate	36.501	12.059	1.695	0.999928
9	Methyl myristoleate	38.655	13.222	1.470	0.999917
10	Methyl pentadecanoate	38.996	13.358	1.401	0.999968
11	Methyl pentadecenoate	41.099	14.598	1.864	0.999966
12	Methyl palmitate	41.396	14.738	1.813	0.999983
13	Methyl palmitoleate	43.116	15.827	1.784	0.999984
14	Methyl heptadecanoate	43.665	16.158	1.744	0.999983
15	Methyl heptadecenoate	45.350	17.269	1.518	0.999981
16	Methyl stearate	45.855	17.602	1.746	0.999997
17	Methyl octadecenoate	46.864	18.291	1.837	0.999986
18	Methyl oleate	47.309	18.589	1.821	0.999995
19	Methyl linolelaidate	48.431	19.375	1.654	0.999984
20	Methyl linoleate	49.402	20.040	1.737	0.999998
21	Methyl archidate	49.957	20.478	1.798	0.999998
22	Methyl linolenate	50.945	21.147	1.865	0.999997
23	Methyl eicosenoate	51.328	21.473	1.751	0.999991
24	Methyl linolenate	51.770	21.754	1.662	0.999999
25	Methyl heneicosanoate	51.902	21.937	1.576	0.999996
26	Methyl eicosadienoate	53.416	23.006	1.680	0.999999
27	Methyl behenate	53.895	23.439	1.675	0.999998
28	Methyl eicosatrienoate	55.023	24.175	1.815	0.999995
29	Methyl erucate	55.346	24.493	1.480	0.999989
30	Methyl eicosatrienoate	55.890*	24.814	1.594	0.999999
31	Methyl archidonate	55.890*	24.966	1.792	0.999998
32	Methyl tricosanoate	56.310	25.099	1.532	0.999996
33	Methyl docosadienoate	57.579	26.114	1.750	0.999989
34	Methyl lignocerate	58.032	26.548	1.841	0.999998
35	Methyl eicosapentaenoate	59.113	27.085	1.714	0.999999
36	Methyl nervonate	59.679	27.667	1.822	0.999981
37	Methyl docosahexaenoate	66.249	31.718	2.080	0.999994

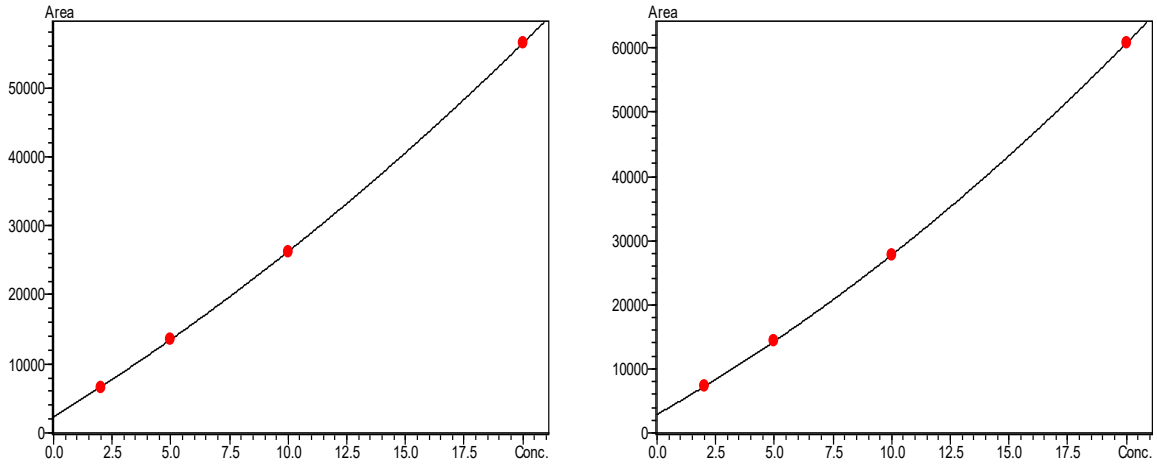

Figure 4: Representative calibration curves for analytes 30 (left) and 31 (right) using LabSolutions. A quadratic regression line was applied and was not forced through zero. The R^2 values for each fit have been included in table 3.

The relative standard deviation, with respect to area counts, was below 2% for all analytes in the sourced standard except for the last peak. It is common in GC that the area % RSD increases in analytes toward the end of the analysis due to longitudinal diffusion leading to peak broadening. Despite the slight increase in the area relative standard deviation, the % RSD values are well below the industry standard, exemplifying the quality and sensitivity of the FID detector.

■ Conclusion

The motivation to modify the AOAC method was to decrease the 65+ minute analysis time (increasing throughput) while maintaining or improving the baseline resolution of all peaks such that they were baseline resolved with a resolution above 1.5. By increasing the linear velocity of the carrier gas and adjusting the oven temperature program, the analysis time was reduced by greater than 50% with the last analyte eluting before 32 minutes; all peaks were baseline resolved. The use of hydrogen carrier gas significantly decreased the operating costs for this analysis. Low relative standard deviation values, with respect to area counts, and calibration curves with impressive R^2 values proved that the Shimadzu Nexis™ GC-2030 performs exceptionally well for this application.

■ Reference

1. *Official Methods of Analysis* (1995) Method 996.06, Fat (Total, Saturated, and Monounsaturated) in Foods, AOAC INTERNATIONAL, Gaithersburg, MD

■ Consumables

Part Number	Item Description	
220-97331-31	1.5 mL screw vial kit including vials, caps, and septa (100/pack)	Sample Preparation
220-97331-62	200 µL inserts for 1.5 mL screw vials (100/pack)	
220-91521-10	4 mL screw vial kit including vials, caps, and septa (100/pack)	
221-74469-00	10 µL syringe with PTFE tipped plunger for AOC-20i	
227-35007-01	SPL-2030 inlet liner with wool for split injection (5/pack)	Injection Port
036-11203-84	O-ring for glass liner (5/pack)	
227-35004-01	Premium light green septa rated to 350 °C (50/pack)	
221-81162-01	ClickTek ferrules for 0.25 mm ID columns (6/pack)	Column
227-36311-01	Rt-2560 Column; 100 m x 0.25 mm x 0.20 µm	
225-50730-00	FID 3-position gas filter kit for hydrogen, nitrogen, and air	Gas Filtration

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