Improving the Analysis of Fatty Acid Methyl Esters Using Automated Sample Preparation Techniques

Application Note

Food Testing and Agriculture

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Abstract

An automated method for esterifying fatty acids in canola oil samples is presented. Using the Agilent 7696A Sample Prep WorkBench, a side-by-side comparison was undertaken comparing a manual method employing automatic pipettors to a method developed for this automated system. Using the Agilent 7696A Sample Prep WorkBench, preparation of 10 samples resulted in 3% RSD for both an acid-catalyzed and base-catalyzed reaction. When comparing the automated acid-catalyzed method to the manual preparation, the RSD improved by a factor of two. Furthermore, by automating the fatty acid preparation the amount of reagents consumed was reduced up to 50-fold. Overall, the automated method resulted in better precision and accuracy with smaller amounts of reagents used and less time required from the operator to complete the task.

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Introduction

The analysis of fatty acids (FAs) is commonly performed in many industries. The food industry routinely performs FA analysis since lipids are a major component in oils, meats, seeds, and other products [1-5]. Furthermore, with the increased importance on fat as part of dietary health and its role in maintaining a healthy disposition, the determination of FA composition has become increasingly common [1-2]. Biomedical applications use FA profiles as a diagnostic tool since FA composition effects biological membranes [3-4,6-7]. Fatty acids are also found in many household products and are used industrially in cosmetics and surfactants, among other things [2,8].

Gas chromatography has been the predominant technique used for analyzing FAs since the 1950's across these industries [3-4,9]. While FAs can be separated and analyzed with the appropriate analytical conditions, they present a number of challenges due to their polar nature and high boiling points. This generally results in long retention times and poor peak shape. For that reason, most methods use derivatization reactions to convert FAs to fatty acid methyl esters (FAMEs), which are easier to separate and exhibit better peak shape.

Converting FAs to FAMEs, regardless of the matrix or application, can be achieved in a number of ways, often involving a two step process of saponification followed by methylation. Lipids can also be esterified in one step through a process known as alcoholysis [4], although many applications, specifically food applications, still use a two-step procedure [1,2,5]. Whether multi-step or single-step, the process of converting FAs to FAMEs can be achieved in a number of ways and different applications require different derivatization reagents [10]. A majority of the reactions can be categorized as using acid, base, or silylating reagents or diazomethane, each with their own advantages and disadvantages.

When performing acid-catalyzed reactions, the most common reagents are boron trifluoride (BF_3) in methanol, hydrochloric acid (HCl), and sulfuric acid (H_2SO_4). Procedures using HCl or H_2SO_4 often call for refluxing the acid for up to an hour, depending on the acid concentration and the sample, for example, free fatty acids, phosphoglycerides, or triglycerides, to achieve complete methylation [3-4,10]. This method is very effective, but it can be costly and requires specific glassware and training. Using BF_3 as the methylating reagent provides the fastest results, because it can be complete within two minutes when boiling. However, this can lead to degradation of labile fatty acids and has a limited shelf life at room temperature [3].

Base-catalyzed reactions use sodium hydroxide (NaOH) or potassium hydroxide (KOH) in methanol. This method has many advantages. It is quick, a simple one-step process, occurs at room temperature, and avoids the degredation of labile FAs. However, base-catalyzed reactions do not work on free fatty acids, and therefore can be limiting in their applicability [3-4,10].

Two additional reagents can be used, but are rarely employed. Diazomethane provides a rapid derivatization technique, but it can produce byproducts that interfere with the compounds of interest [3,10]. Its toxicity and potential for explosion make it a rarely used reagent in recent years. Silylating reagents are also rarely used because of their sensitivity to water although they react fairly quickly and at moderate temperatures [10].

Automation of these methods can be advantageous in many ways and recently there have been more automated and microscale methods for converting FAs to FAMEs [11-15]. Generally, automated methods use smaller amounts of reagents, reduce an operator's potential exposure to hazardous chemicals, can reduce the time required to complete a task, and provide intervention-free operation for hours. Automating the preparation of FAMEs is possible with the Agilent 7696A Sample Prep WorkBench (Figure 1). It features a 150-vial tray, two liquid dispensing modules, a single vial heater, mixer, and barcode reader. In addition, the individual vial racks can be heated or cooled. The liquid dispensing modules can be configured with either a standard syringe or a large volume (250 µL or 500 µL) syringe. Most applications use a standard syringe (10 µL or 25 µL) in one module for fine manipulations of liquids and a large volume syringe in the other module for dispensing larger volumes. With the two dispensing modules, mixer, and heater, the Agilent 7696A Sample Prep WorkBench is capable of sample dilutions, internal standard additions, derivatizations, liquid/liquid extraction, as well as many other tasks.

Figure 1. Agilent 7696A Sample Prep WorkBench.

Using the Agilent 7696A Sample Prep WorkBench, two methods of extracting and methylating FAs in canola oil were examined: a base-catalyzed reaction and an acid-catalyzed reaction. Both methods were adapted from a previously published manual method using 20-mL test tubes [5]. Recoveries between 93% and 107% with RSDs < 5% were achieved. In addition, when modifying the manual method for use on the Agilent 7696A Sample Prep WorkBench, the reaction time was reduced from 2 hours to 20 minutes with up to a 50-fold decrease in reagent and solvent usage.

Experimental

Materials

Hexane (reagent grade), isooctane, and methanol (HPLC grade) were purchased from Burdick and Jackson (Muskegon, Michigan). Boron trifluoride (BF_3) in methanol (14% w/v) was obtained from Aldrich (St. Louis, MO). A solution of sodium hydroxide (reagent grade, Sigma Aldrich, St. Louis, MO) in methanol (NaOH) was made to yield a 2N solution. Sodium chloride (certified ACS, Fisher Scientific, Atlanta, GA) was used to make a 1 M solution in Milipure water (H)O/NaCl). While some reports suggest using a saturated solution [2,16], such a concentrated solution was found to precipitate in the system and cause syringe errors.

Individual FAs were obtained from Alltech (Waukegan, IL) and consisted of caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, and behenic acid. These were used to generate a 1 mg/mL solution in hexane. FAME standards were made from a custom mix obtained from ChemService (West Chester, PA) consisting of methyl pentanoate, methyl hexanoate, methyl heptanoate, methyl octanoate, methyl decanoate, methyl laurate, methyl myristate, methyl palmitate, methyl stearate, methyl eicosanoate, and methyl behenate at 1 mg/mL. A 1 mg/mL solution of lauric acid in hexane was used as a surrogate standard. A 1 mg/mL solution of decane (99+% Sigma, St. Louis, MO), dodecane (99+% Aldrich, St. Louis, MO), tetradecane (99+% Fluka, St. Louis, MO), and hexadecane (99+% Aldrich, St. Louis, MO) in isooctane was used as the internal standard. The canola oil was obtained from the local supermarket.

Two wash solvents were used in the Agilent 7696A Sample Prep WorkBench: hexane and acetone (Laboratory grade, Fisher Scientific, Atlanta, GA). Acetone was used for a majority of wash steps since it provided a solvent in which all the reagents used were miscible.

Instrumentation

The Agilent 7696A Sample Prep WorkBench (Agilent Technologies, Santa Clara, CA) was used to prepare calibration curve standards, free FA samples, and canola oil samples. For this application, the liquid dispensing modules were configured with a 25-µL syringe in the back module and a 500-µL syringe in the front module for larger volumes.

All analyses were performed on an Agilent 7890A gas chromatography (GC) System (Agilent Technologies, Santa Clara CA) equipped with a split/splitless inlet, operated in split mode (10:1) and a flame ionization detector. The inlet was held at 300 °C with a constant column flow rate of 3 mL/min. An Agilent HP5-MS column (30 m × 0.25 mm, 0.25 µm, Agilent Technologies, Santa Clara) was used. A temperature program of 100 °C (5 min), 7 °C/min to 225 °C (5 min) was used to achieve separation of the FAME standards. The same temperature program was used for the canola oil samples as well, although baseline separation of all the compounds was not achieved. The detector was held at 300 °C and data collection was performed with Multi-Technique ChemStation.

The sample preparation method programming was performed with Easy SamplePrep, a drag and drop method editor developed for the Agilent 7696A Sample Prep WorkBench. Easy SamplePrep features a Resource Manager that allows users to allocate vials as chemical resources or empty vials. This way the user inputs all the solvents, reagents, standards, and empty vials needed for the sample preparation and the

Resource Manager keeps track of the vials as they are used throughout the program and sequence (Figure 2). With the resources configured in the Resource Manager, the sample preparation program is built. The Easy SamplePrep method editor allows the user to add steps in a manner similar to following a protocol or laboratory notebook and gives a textual display of the steps and the resources available (Figure 3).

Each sample prep step has a set of advanced parameters for a fully customizable program. In the Add Step, the Advanced Parameters allows the user to set parameters like wash volumes, draw and dispense speeds, and needle depths (Figure 4). The Mix Step can be customized with regard to speed and time, while the Heat Step allows the user to specify both time and temperature setpoints. The Flag as Result Step allows the user to select the vial that contains the finished sample for reporting purposes.

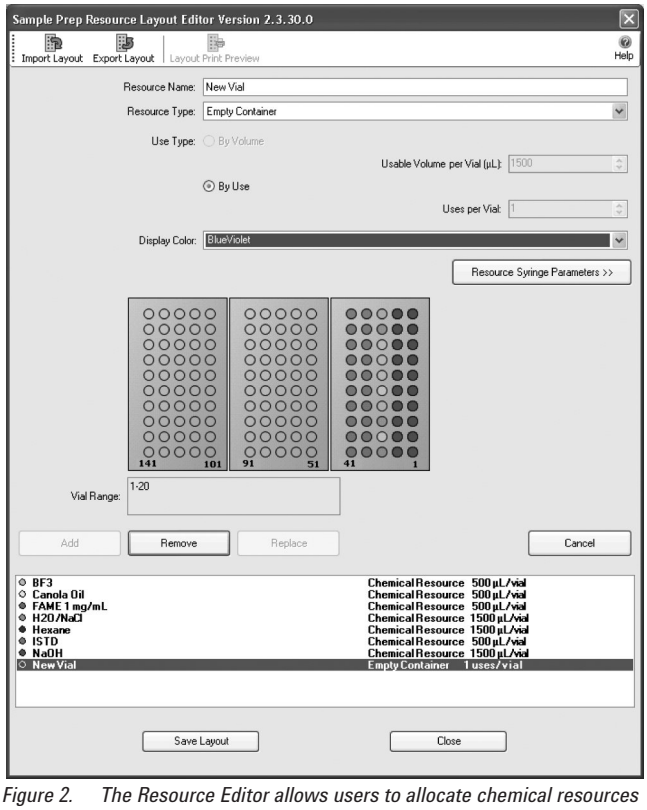

Figure 2. The Resource Editor allows users to allocate chemical resources and empty vials to be used during the sample preparation.

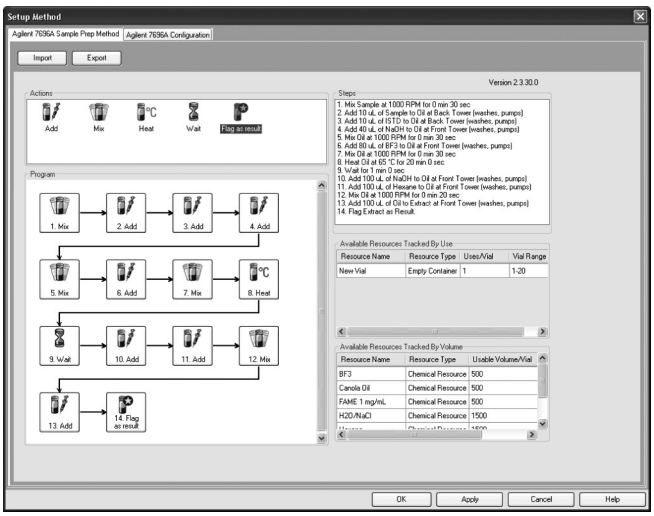

Figure3. SamplePrep Method Editor features drag and drop icons for easy step-wise programming. The steps used for the acid-catalyzed reaction are shown here.

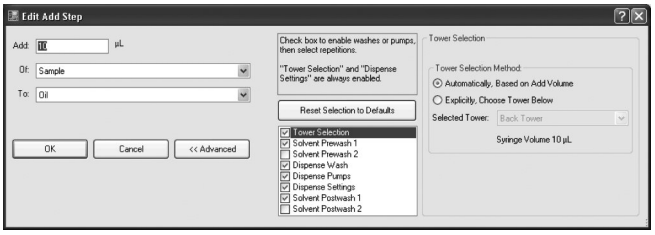

Figure4. Each sample prep step has advanced parameters, like the Add Step shown here, that allows the user to fully customize the operation.

Calibration Curve Generation

Prior to preparing samples with the Agilent 7696A Sample Prep WorkBench, a calibration curve was generated from the FAME standard with the instrument. An eight-level calibration curve consisting of the 11 FAMEs was generated spanning 1–500 ppm in approximately 100 µL.

Sample Preparation: Acid-Catalyzed Reaction

The original manual method followed the Association of Official Analytical Chemists Official Methods of Analysis and started with a 50-mg sample of canola oil in 20-mL test tubes and included two heating steps at 80 °C for 60 minutes each [5,17].

When converting the AOAC method to an automated one, the scale of the reaction was necessarily reduced since the Agilent 7696A Sample Prep WorkBench accepts only 2-mL autosampler vials. The manual method was reduced approximately 50-fold and applied to both the oil sample and a free FA sample.

Initially the oil, surrogate standard (lauric acid), and internal standard (alkanes) were added in separate steps. However, because of difficulty in achieving acceptable reproducibility when dispensing the oil, a solution consisting of 0.4 mL of the oil sample and 0.4 mL of the surrogate standard was made, greatly improving the reproducibility of the method.

An empty, 2-mL autosampler vial was capped and 10 µL of sample (either the free FA sample in hexane or oil/surrogate standard solution) and 10 µL of the internal standard were added. To the sample, 40 µL of NaOH was added and the mixture was vortexed at 1000 rpm for 30 sec. After saponification, 80 µL of BF_3 was added and the mixture was again vortexed at 1000 rpm for 30 sec. The mixture was then heated for 20 minutes at 65 °C to facilitate the reaction. After heating, the mixture was allowed to sit at room temperature for 2 minutes to let it cool slightly. To the cooled mixture, 100 µL of H_2O/NaCl and 100 µL of hexane was added to extract the newly formed FAMEs into the organic layer. The sample was mixed a final time for 20 sec at 1000 rpm and the top layer (100 µL) was transferred to a new, empty, capped 2-mL autosampler vial and taken to the GC for analysis.

Sample Preparation: Base-Catalyzed Reaction

As with the acid-catalyzed reaction, the manual preparation for the base-catalyzed reaction was too large to be prepared in a 2-mL autosampler vial since it started with a 100-mg oil sample in a 20-mL test tube [5]. For this reaction to work on the Agilent 7696A Sample Prep WorkBench, it was reduced approximately 10-fold.

Since the base-catalyzed reaction does not convert free fatty acids, the surrogate standard was omitted. A solution of 0.4 mL of oil and 0.4 mL of internal standard was again used to improve the reproducibility of the method. To an empty, capped 2-mL autosampler vial, 10 µL of sample (oil/internal standard solution) was added. To methylate the FAs and extract the newly formed FAMEs 100 µL of NaOH and 500 µL of hexane was added and the mixture was vortexed at 1000 rpm for 30 sec. After waiting 2 minutes, the top layer (100 µL) was transferred to a new, empty, capped 2-mL autosampler vial and taken to the GC for analysis. Unlike the acid-catalyzed reaction, this base-catalyzed reaction occurs in a single step and is complete within minutes.

Validation of the Acid-Catalyzed Reaction

Because the acid-catalyzed reaction works as well on lipid bound fatty acids as it does on free fatty acids, the method was performed on the free FA sample in hexane. Five samples were prepared on three different days to determine repeatability between samples as well as day-to-day reproducibility.

The same procedure was followed when performing the reaction manually. Volumes were added using adjustable pipettors and the reaction took place in a heated block for comparison. The manual procedure was performed alongside the automated procedure to give an accurate comparison between the manual and automated preparations.

Results and Discussion

Calibration

Excellent linearity was achieved for the eight standards made with the Agilent 7696A Sample Prep WorkBench. The calibration data are given in Table 1. Because the standards were made with a selection of saturated FAMEs, those were the only compounds that were identified and quantified in the oil and FA standard samples.

Table 1. Instrument Calibration Data for FAME Standards Prepared with the Agilent 7696A Sample Prep WorkBench

Analyte	R2	Linear regression
Methyl pentanoate	0.9997	y = 1.33x + 4.1175
Methyl hexanoate	0.9998	y = 1.4876x + 8.9684
Methyl heptanoate	0.9998	y = 1.5671x + 7.7412
Methyl octanoate	0.9998	y = 1.6669x + 8.2446
Methyl decanoate	0.9998	y = 1.7825x + 9.0499
Methyl laurate	0.9998	y = 1.8786x + 9.7365
Methyl myristate	0.9998	y = 1.9727x + 10.264
Methyl palmitate	0.9998	y = 1.9623x + 10.369
Methyl stearate	0.9998	y = 1.9828x + 10.64
Methyl eicosanoate	0.9998	y = 2.0155x + 10.826
Methyl behenate	0.9998	y = 2.087x + 11.266

Method Validation

Before the oil samples were examined, a free FA sample was prepared with the automated method and the manual method described above to validate the use of the Agilent 7696A Sample Prep WorkBench.

For the automated method, five samples prepared on any day resulted in an average RSD of < 2%. When comparing the five samples made on three different days with the Agilent 7696A Sample Prep WorkBench, good reproducibility was again achieved for all the compounds with retention times greater than methyl octanoate. The mean, standard deviation, relative

standard deviation, and recoveries are given in Table 2. Methyl octanoate is significanly lower than the other analytes due to its volatility and proximity to the solvent peak [3,4]. This data comprises 15 samples prepared over three days with triplicate injections.

Table 2. Results from the Free Fatty Acid Sample Acid-Catalyzed Reaction using an External Standard to Determine Concentration and Recovery

Analyte	Amount (ppm)	Standard Deviation (ppm)	Relative Standard Deviation (%)	Recovery (%)
Decane	81.8	0.968	1.18	97.3
Dodecane	84.6	0.517	0.611	102.4
Tetradecane	88.0	0.869	0.967	105.7
Hexadecane	92.2	1.02	1.11	111.4
Methyl octanoate	63.5	0.570	0.898	75.1
Methyl decanoate	101.2	0.175	0.173	97.5
Methyl laurate	110.1	0.681	0.619	104.8
Methyl myristate	97.0	0.713	0.735	102.6
Methyl palmitate	115.2	0.688	0.597	106.6
Methyl stearate	98.0	0.266	0.271	106.9
Methyl eicosanoate	80.6	0.394	0.489	104.0
Methyl behenate	90.0	1.12	1.25	99.9

The results from the automated method were then compared to those obtained using a manual method. The reproducibility was much worse for the manual method on any of the three days tested. Treating the manual data in the same manner as the automated data, the mean, standard deviation, relative standard deviation, and recoveries are given in Table 3. Recoveries are routinely higher for the manually prepared samples than those achieved with the automated preparation

Table 3. Results from the Free Fatty Acid Sample Prepared Manually with the Acid-Catalyzed Preparation using an External Standard to Determine Concentration and Recovery

Analyte	Amount (ppm)	Standard Deviation (ppm)	Relative Standard Deviation (%)	Recovery (%)
Decane	88.2	13.2	14.9	104.9
Dodecane	98.1	7.92	8.08	118.8
Tetradecane	104.2	6.81	6.53	125.0
Hexadecane	109.8	6.66	6.06	132.8
Methyl octanoate	94.6	14.1	14.9	111.9
Methyl decanoate	126.4	13.8	10.9	121.8
Methyl laurate	130.0	12.1	9.33	123.8
Methyl myristate	113.1	10.0	8.84	119.6
Methyl palmitate	134.4	12.0	8.93	124.3
Methyl stearate	114.0	9.89	8.67	124.3
Methyl eicosanoate	93.6	8.41	8.98	120.8
Methyl behenate	104.7	9.83	9.39	116.3

due to either a greater amount of standard added or slightly less hexane added, the later being more likely the case.

While the results shown in Table 2 and 3 were determined using the external calibration, the data was also examined using an internal standard. The peak areas were normalized to methyl laurate which produced better overall results than the absolute peak areas. Normalizing to methyl laurate, used here as the internal standard, provides results indifferent to the dilution. In doing so, RSDs generally improved. Using normalized peak areas, the relative standard deviation for the samples made both manually and with the automated method across the three days are presented in Table 4. Comparing the manual and automated results, it was clear that the automated method provided a viable solution for derivatizing fatty acids and could improve the reproducibility and recovery.

Table 4. Results from the Free Fatty Acid Sample Normalized to Methyl Laurate Prepared Both Manually and with the Automated Acid-Catalyzed Reaction

Analyte	Relative Standard Deviation-automated (%)	Relative Standard Deviation-manual (%)
Methyl octanoate	1.31	7.65
Methyl decanoate	0.452	2.63
Methyl laurate	–	–
Methyl myristate	0.425	1.05
Methyl palmitate	0.779	1.80
Methyl stearate	1.10	1.93
Methyl eicosanoate	1.59	1.72
Methyl behenate	2.62	1.77

Canola Oil analysis

After validating the automated method using the free FA sample, the Agilent 7696A Sample Prep WorkBench was used to prepare oil samples. As stated above, dispensing canola oil proved to be more difficult than originally thought due to its high viscosity. However, by mixing the oil sample with the surrogate standard, the viscosity of the solution was much closer to that of hexane and therefore easier to dispense reproducibly. The results are given in Table 5 with a representative chromatogram presented in Figure 5.

Eleven oil samples were prepared across two days. Because a fresh solution of oil and lauric acid was made each day, an average RSD for all 11 samples cannot be given. However, good reproducibility was still found. The average RSD for the six samples prepared on day one was 3.6%. The average RSD for the five samples prepared on day two was slightly lower at 2.5%. Using methyl laurate as an internal standard to normalize the FAMEs, the average RSD for all eleven samples decreases, as seen in Table 5. The average recovery for these samples was 101%.

Table 5. Results Using Both an External Standard (ES) and an Internal Standard (IS) for the Canola Oil Samples Prepared with the Agilent 7696A Sample Prep WorkBench and the Acid-Catalyzed Reaction

Analyte	Amount (ppm)	Standard Deviation (%)	Relative Standard Deviation-ES (%)	Relative Standard Deviation-IS (%)	Recovery (%)
Methyl laurate	51.0	1.91	3.74	–	97.1
Methyl palmitate	1499.6	57.8	3.85	0.778	–
Methyl stearate	306.8	12.9	4.20	0.928	–
Methyl eicosanoate	226.8	9.44	4.16	1.10	–
Methyl behenate	111.6	4.73	4.24	0.861	–

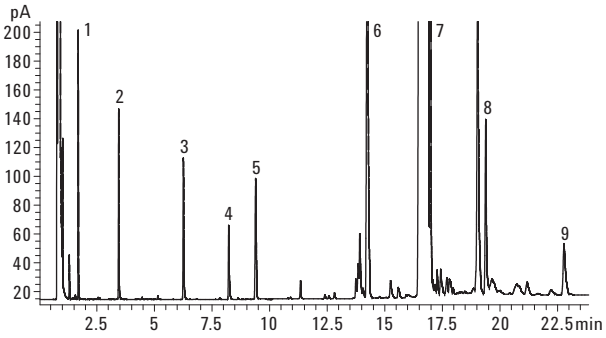

Figure 5. A typical chromatogram of a canola oil sample after automated acid-catalyzed sample preparation and analysis by GC. Compounds identified and quantified were 1) decane, 2) dodecane 3) tetrade cane, 4) methyl laurate, 5) hexadecane, 6) methyl palmitate, 7) methyl stearate, 8) methyl eicosanoate, and 9) methyl behenate. Other unidentified, uncalibrated peaks are various unsaturated FAMEs.

The base-catalyzed reaction provided excellent results as well. A total of 10 samples were prepared in one day and yielded similar reproducibility (Table 6). The average RSD for the 10 samples was 3.2%. Using hexadecane as the internal standard to normalize the peak areas did not sufficiently lower the RSDs as it did with the acid catalyzed reaction. The average recovery was found to be 94%.

Table 6. Results Using Both an External Standard (ES) and an Internal Standard (IS) for the Canola Oil Sample Prepared with the Agilent 7696A Sample Prep WorkBench and the Base-Catalyzed Reaction

Analyte	Amount (ppm)	Standard Deviation	Relative Standard Deviation-ES	Relative Standard Deviation-IS (%)	Recovery (%)
Hexadecane	9.66	0.215	2.23	–	99.2
Methyl palmitate	312.5	13.01	4.16	2.69	–
Methyl stearate	49.95	3.39	6.80	4.89	–
Methyl eicosanoate	40.98	1.66	4.04	2.15	–
Methyl behenate	18.07	0.945	5.23	2.83	–

Benefits of automated sample preparation

Automating the sample preparation procedure proves to be advantageous in many ways. By adapting this method to an automated one, the scale of the reaction was reduced. In doing so, the level of chemical exposure is reduced as well as the amount of solvent and reagent used. This increases the safety of the method and reduces the cost of the analysis.

More importantly, automating this method resulted in better recoveries and reproducibility. Automating this method resulted in reproducibilities at least twice as good as the compared manual method.

Conclusions

Two automated methods for derivatizing fatty acids to fatty acid methyl esters were described in this Application Note. Using the Agilent 7696A Sample Prep WorkBench, derivatization reactions were easily converted to automated methods with an increase in reproducibility. Furthermore, smaller volumes of solvents and reagents were used, which significantly reduced the cost per analysis. Excellent reproducibility and recovery were achieved for most compounds in both a fatty acid standard and a canola oil sample. These results show that methods such as these can be easily be adapted for use on the Agilent 7696A Sample Prep WorkBench with many advantages.

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