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

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Outline

- Introduction
  - Fatty acids and the challenges associated with their analysis
  - Methods of derivatization
  - Automated solutions
    - – 7696A Sample Prep WorkBench
- Development of an automated method for WorkBench
  - Calibration curves
  - Method validation
  - Oil sample analysis
- Conclusions

Analysis of Fatty Acids Is Common In Multiple Industries

- Fatty acids are found in many matrices
  - Food industry
    - Total lipid analysis (triglycerides, phospholipids, sterols): milk, eggs, meat, oils, seeds
    - Edible fat analysis (mainly fatty acids): oils
  - Biomedical applications
    - Fatty acid profiles as a diagnostic tool: blood, tissue
  - Chemical industry
    - Fatty acids found in cosmetics, surfactants, other household products

Fatty Acid Analysis Can Be Challenging

- Gas chromatography has been the predominant method of analysis
  - Underivatized fatty acids can be analyzed with polar columns but often have poor peak shape and long retention times
- Fatty acids are often derivatized to improve the peak shape and separation
  - More reproducible data

There Are Various Methods of Derivatization

- Acid catalyzed reactions forming methyl esters
  - Reagents include BF_3, HCl, or H_2SO_4
    - HCl and H_2SO_4 often need long reaction times and high reaction temperatures
    - BF_3 can methylate fatty acids within 2 minutes
    - Works on free fatty acids, phosphoglycerides, and triglycerides
- Base catalyzed reactions forming methyl esters
  - Reagents include NaOH or KOH in methanol
    - Very fast
    - Does not work on free fatty acids
- Methyl esters produced with diazomethane
    - Toxic and explosive; can produce byproducts
- Silylation reactions to form trimethylsilyl esters
    - Sensitive to water

Preparation of Fatty Acid Methyl Esters with an Acid Catalyzed Reaction

Step 1: 100 mg sample in 20 mL test tube

Step 2: Add 2 mL 2N NaOH in methanol

Step 3: Heat 80°C for 1 hour

Step 4: Add 2 mL 25% BF_3 in methanol

Step 5: Heat 80°C for 1 hour

Step 6: Add 5 mL 1M NaCl in H_2O

Step 7: Add 5 mL Hexane

Step 8: Shake

Transfer supernatant to autosampler vial

F. David, P. Sandra, P. Wylie, "Improving the Analysis of Fatty Acid Methyl Esters Using Retention Time Locked Method and Retention Time Databases," Application Note 5990-4822EN, Agilent Technologies publication 5988-5871EN (2003).
AOAC Official Methods of Analysis (1990), method 969.33.

Preparation of Fatty Acid Methyl Esters with a Base Catalyzed Reaction

Step 1: 100 mg sample in 20 mL test tube

Step 2: Dissolve in 10 mL hexane

Step 3: Add 100 mL 2N KOH in methanol

Step 4: Mix 30s

Step 5: Centrifuge

Transfer supernatant to autosampler vial

F. David, P. Sandra, P. Wylie, "Improving the Analysis of Fatty Acid Methyl Esters Using Retention Time Locked Method and Retention Time Databases," Application Note 5990-4822EN, Agilent Technologies publication 5988-5871EN (2003).

Automated Procedures Can Simplify FAME Preparation

- Tecan Freedom Evo
- Gerstal MPS PrepStation
- Agilent 7696 Sample Prep WorkBench
  - 2 syringe modules
  - 150 vial rack
  - Single vial vortex mixer
  - Single vial heater (80°C)
  - Individually heated (80°C) and cooled (5°C) racks
  - Sample tracking

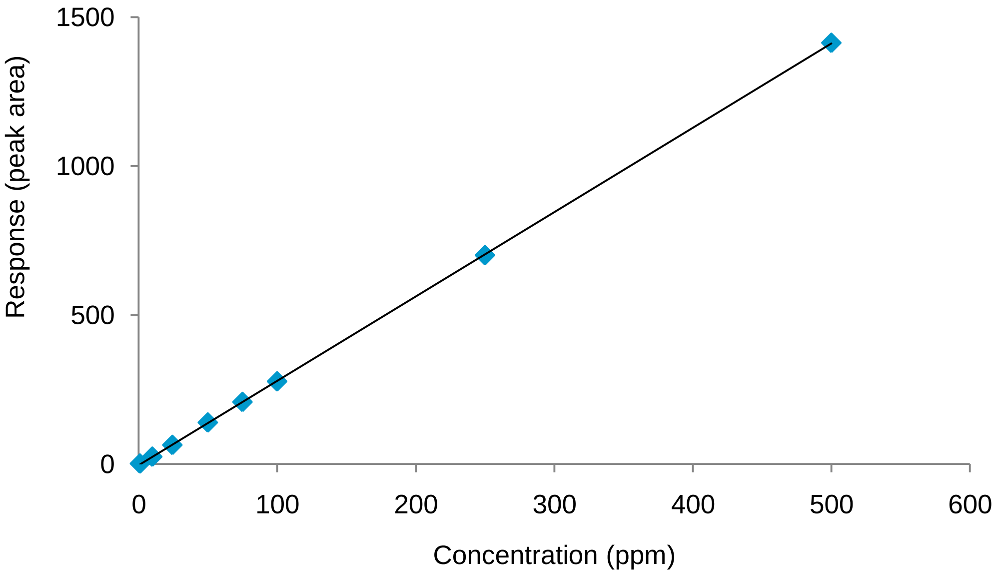
R. Perkins, K. Summerhill, and J. Angove, *Chromatography Today*, Sept/Oct, 17-19 (2008).
M. Athar Masood and N. Salem Jr., *Lipids*, **43**, 171-180 (2008).

Step-Wise Approach to Developing a Method for WorkBench

- Make calibration curve standards
- Validate the automated method
  - Translate manual AOAC method to WorkBench appropriate volumes
  - Verify WorkBench method gives the same (or better) results as a manual method with a fatty acid standard
- Acid reaction
  - Canola oil sample
- Base reaction
  - Canola oil sample

Calibration Curve Standard Preparation Is Fast and Yields Excellent Results

- Eight level calibration curve: 1 to 500 ppm
- Linear dilutions in ~100 µL of hexane
- Complete in 40 minutes
- Excellent linearity (R^2=0.999)



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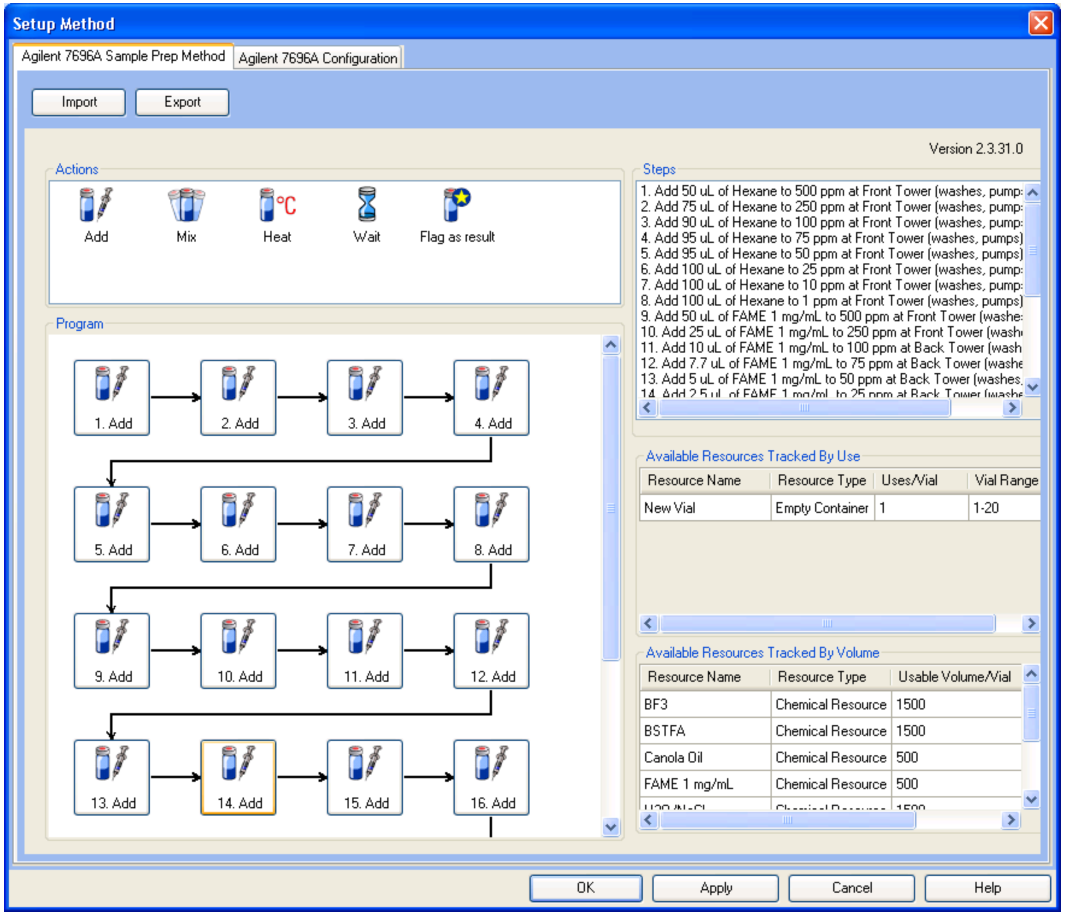


Translating the Acid Catalyzed FAME Preparation

Step 1: Add 10 µL sample

Step 2: Add 10 µL internal standard

Step 3: Add 40 µL 2N NaOH in methanol

Step 4: Mix 30s

Step 5: Add 80 µL 14% BF_3 in methanol

Step 6: Mix 30s

Step 7: Heat at 65°C for 20 min

Step 8: Cool 2 min

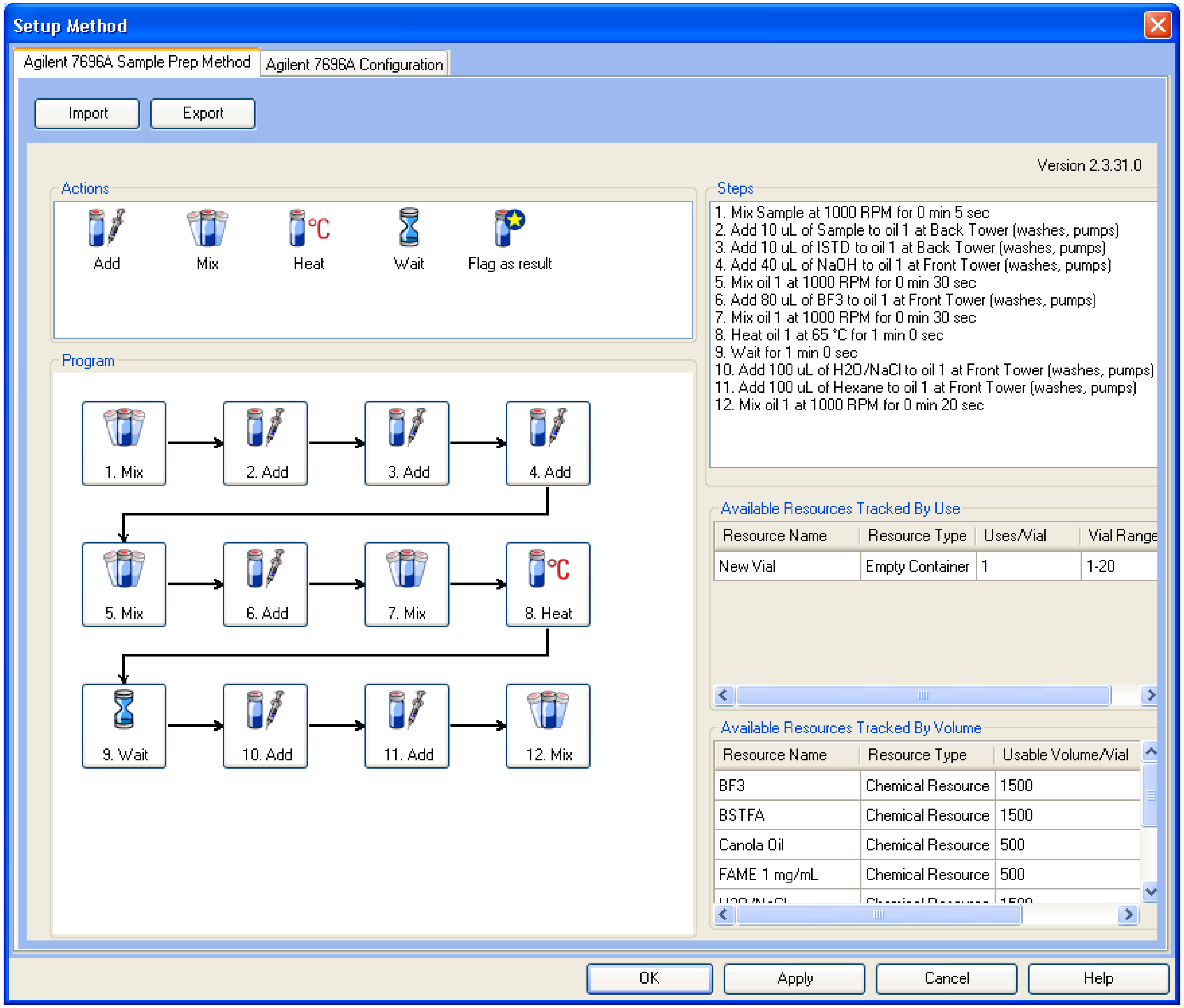
Step 9: Add 100 µL 1M H_2O/NaCl

Step 10: Add 100 µL hexane

Step 11: Mix 20s

Step 12: Transfer top 100 µL to a new vial

Translating the Acid Catalyzed FAME Preparation



WorkBench Method Validation with a Fatty Acid Standard

- WorkBench made 5 samples per day for 3 days
  - Determine day to day reproducibility
  - Sample was a 1 mg/mL fatty acid standard
  - 1 mg/mL alkane internal standard
- For any given day, the RSD for 5 samples was <2% and recovery was 103%
- For the 15 samples made across 3 days, the average RSD was 1.2% and recovery was 103%

Manually Prepared Samples Were Not as Reproducible

- 5 samples made on 3 days alongside the WorkBench made samples
- For a given day, the average RSD was >4.5% with an average recovery of >110%
- For the 15 samples made over 3 days, the average RSD was 6.8% with an average recovery of 125%

- Conclusion: WorkBench made samples are as good as manually prepared samples

Dispensing Reagents with WorkBench Is Reproducible and Accurate

- Dispensing of the reagents was measured gravimetrically to determine the accuracy and precision of WorkBench as a liquid handling system

Dispensed Volume	RSD	Accuracy
10 µL sample	0.84%	10%
40 µL 2N NaOH in methanol	0.33	2.1%
100 µL 2N NaOH in methanol	0.48%	1.1%
80 µL 14% BF_3 in methanol	0.30%	0.93%
100 µL 1M H_2O/NaCl	0.55%	1.0%
100 µL hexane	0.54%	1.9%
500 µL hexane	0.27%	0.30%

WorkBench Prepared Samples – Acid Prep

Analyte	Amount (ppm)	RSD (%)	Recovery (%)
Methyl laurate	51	-	97
Methyl palmitate	1500	0.78	-
Methyl stearate	307	0.93	-
Methyl eicosanoate	227	1.1	-
Methyl behenate	112	0.86	-

- 11 samples prepared across 2 days
- Sample was mixture of the initial oil sample and a lauric acid standard
- RSD was calculated using methyl laurate as the internal standard
    - Average RSD=0.92%
  - Using an internal standard takes dilution inaccuracy into account
  - Using an external standard, the RSD was 4.0%

Base Catalyzed Preparation

Step 1: Add 10 μL sample

Step 2: Add 500 μL hexane

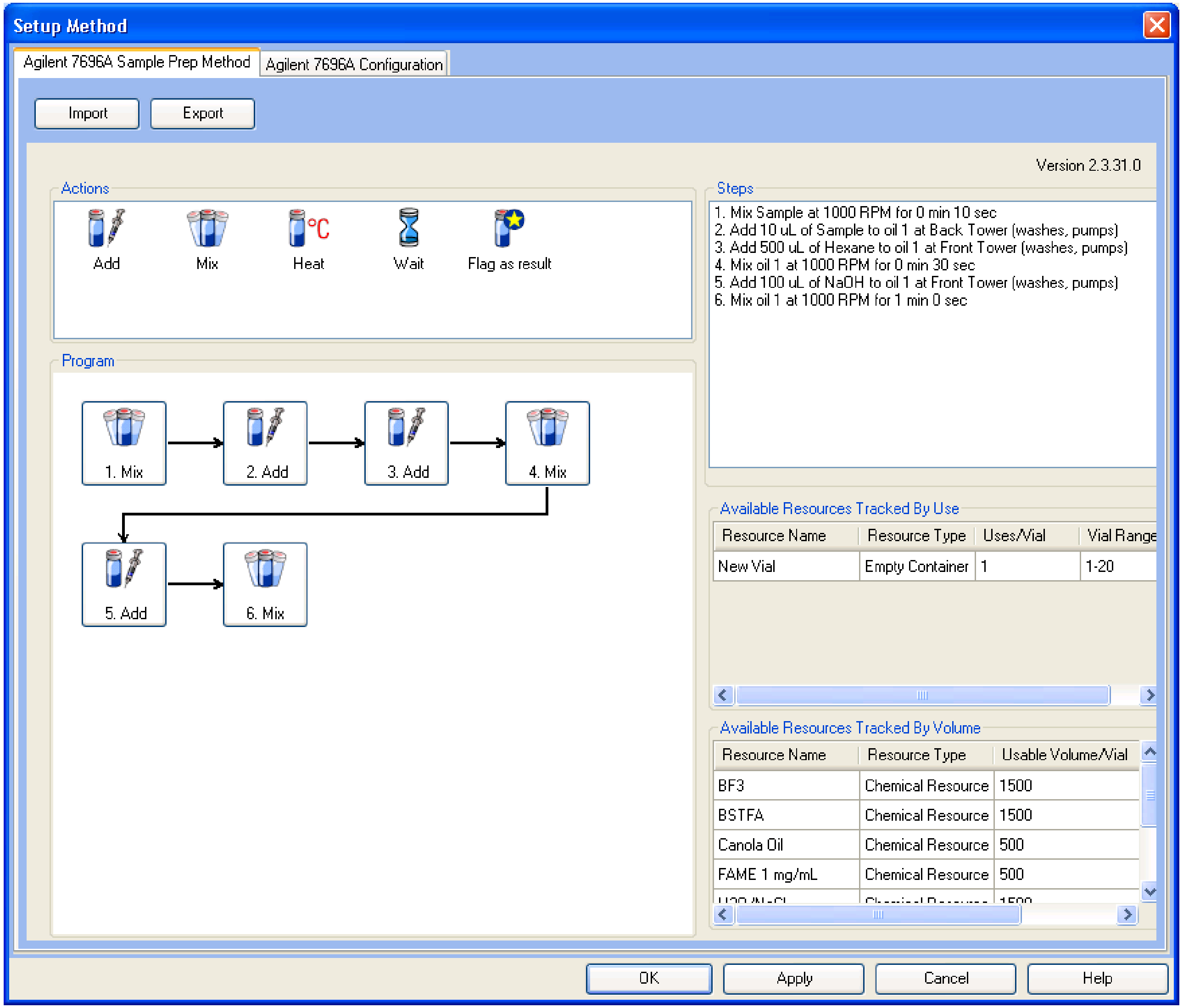
Step 3: Mix 30s

Step 5: Add 100 μL 2N NaOH in methanol

Step 6: Mix 60s

Step 7: Transfer top 100 μL to a new vial

Base Catalyzed Preparation



WorkBench Prepared Samples – Base Prep

Analyte	Amount (ppm)	RSD (%)	Recovery (%)
Hexadecane	9.7	-	99
Methyl palmitate	313	2.7	-
Methyl stearate	50	4.9	-
Methyl eicosanoate	41	2.2	-
Methyl behenate	18	2.8	-

- 10 samples prepared in 1 day
- Sample was mixture of the initial oil sample and an alkane standard
  - The base catalyzed reaction does not esterify free fatty acids
- RSD was calculated using hexadecane as the internal standard
    - Average RSD=3.2%
  - Using an external standard, the RSD was 4.5%

Conclusions

- FAME preparations can be easily translated to an automated system
  - Method development on WorkBench enabled a shorter reaction time
- Samples prepared with WorkBench are more reproducible than those prepared manually
- WorkBench can prepare FAMEs via acid or base catalyzed reactions
    - Acid catalyzed reactions result in an average RSD <1% with 97% recovery
    - Base catalyzed reactions result in an average RSD 3% with 99% recovery

Conclusions

- Preparing oil samples with WorkBench reduces the volume of reagents needed
  - Acid catalyzed reaction was reduced ~50-fold
  - Base catalyzed reaction was reduced ~10-fold
- Operator exposure is reduced
  - Reagents and samples are in capped vials
- Automated preps frees the operator to work on other tasks beyond sample preparation

THANK YOU