Fatty Acid and FAME Analysis Using State-of-the-Art Gas Chromatography

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Agilent Technologies
Outline

• Analysis of Fats and Oils and Column Selection
• DB-FATWAX Ultra Inert
• DB-FastFAME
• DB-HeavyWAX
• Conclusions
The importance of fatty acid analysis

Quality

Labeling/Regulatory

Adulteration

EMA: Economically Motivated Adulteration

Process Monitoring/Product and assuring economics

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Fatty acids commonly found in food samples

- Milk fat (C4-C24 saturated/unsaturated FAMEs)
- Vegetable oil (hydrogenated and nonhydrogenated)
  - Palm oil, rapeseed oil, olive oil, soybean oil
  - Cooking oils
- Fish oil and meat fat
Fat and Oil Analysis - Triglycerides

- Used to identify composition and percentage of each TG present in the sample
- Very easy to prepare and analyze, typically, only a dilution of the oil is required
- Cold on-column injection is required due to the low volatility of the triglycerides
- A typical TG analysis is olive oil adulteration with cheaper seed oils
Triglycerides analysis of butter fat

Column: CP-TAP CB, 25m, 0.25mm, 0.10μm (p/n CP7483)
Inlet: on-column
Carrier: Hydrogen, 100kPa (1 bar, 15 psi)
Oven: 280 °C (1 min) to 355 °C at 3 °C/min
Detector: FID
Injection: 0.2 uL
Sample: 0.05% butter fat in hexane

Peak No
1. Cholesterol
2. PPP
3. PPS
4. PPO
5. PSS
6. PSO
7. POO
8. SSS
9. SSO
10. SOO
11. OOO

P: Palmitic acid, (hexadecanoic acid) C16:0
S: Stearic acid (octadecanoic acid) C18:0
O: Oleic acid (cis-9-octadecenoic acid) C18:1

Application Note: A0223
Fat and Oil Analysis – Free Fatty Acids

- Faster and easier sample preparation
- Traditionally small-chain or volatile fatty acids are analyzed in free form
- Usually difficult to analyze due to the carboxylic acid
- Typically requires specialized column such as acid-modified wax phases

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trans-Oleic acid
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cis-Oleic acid
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Fat → Hydrolysis → GC/FID
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Analysis of free fatty acids

Column: DB-FFAP, 30m, 0.25mm, 0.25μm (p/n 122-3232)
Carrier: Helium at 40 cm/sec, measured at 100 °C
Oven: 100 °C (5 min) to 250 °C (12 min) at 10 °C/min
Detector: FID, 300 °C
Carrier Helium at 40 cm/sec, measured at 100 °C
Oven: 100 °C (5 min) to 250 °C (12 min) at 10 °C/min
Detector: FID, 300 °C
Nitrogen makeup gas at 30 mL/min
Injector: Split 1:50, 250 °C

Peak No
1. Acetone
2. Formic acid
3. Acetic acid
4. Propionic acid
5. Isobutyric acid
6. Butyric acid
7. Isovaleric acid
8. Valeric acid
9. Isocaproic acid
10. Caproic acid
11. Heptanoic acid
12. Octanoic acid
13. Decanoic acid
14. Dodecanoic acid
15. Tetradecanoic acid
16. Hexadecanoic acid
17. Octadecanoic acid
18. Arachidic acid
Fat and Oil Analysis - FAME

- Transesterification of free fatty acids is the most preferred method for fat analysis
- Allows to distinguish slight differences among unsaturated fatty acids (e.g. positional geometric isomers)
- Generally, sample preparation requires a few complex steps

Chemical structure of a fatty acid methyl ester (FAME): ester group

Flowchart:

Fat → Hydrolysis → Methylated to FAMEs → GC/FID
Traditional FAME analysis on a CP-Sil 88

- Most common method, suggested by AOAC 996.06 and AOCS Ce 1j-07
- 100-m cyanopropyl columns are optimized for positional isomer separation of critical cis/trans FAMEs
- Analysis time typically in the 65-76 min range

FAMES by AOAC 996.06 using 100-m CP-Sil 88

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C4:0</td>
</tr>
<tr>
<td>2.</td>
<td>C6:0</td>
</tr>
<tr>
<td>3.</td>
<td>C8:0</td>
</tr>
<tr>
<td>4.</td>
<td>C10:0</td>
</tr>
<tr>
<td>5.</td>
<td>C11:0</td>
</tr>
<tr>
<td>6.</td>
<td>C12:0</td>
</tr>
<tr>
<td>7.</td>
<td>C13:0</td>
</tr>
<tr>
<td>8.</td>
<td>C14:0</td>
</tr>
<tr>
<td>9.</td>
<td>C14:1 (c9)</td>
</tr>
<tr>
<td>10.</td>
<td>C15:0</td>
</tr>
<tr>
<td>11.</td>
<td>C15:1</td>
</tr>
<tr>
<td>12.</td>
<td>C16:0</td>
</tr>
<tr>
<td>13.</td>
<td>C16:1 (c9)</td>
</tr>
<tr>
<td>14.</td>
<td>C17:0</td>
</tr>
<tr>
<td>15.</td>
<td>C17:1 (c10)</td>
</tr>
<tr>
<td>16.</td>
<td>C18:0</td>
</tr>
<tr>
<td>17.</td>
<td>C18:1 (t9)</td>
</tr>
<tr>
<td>18.</td>
<td>C18:1 (c9)</td>
</tr>
<tr>
<td>19.</td>
<td>C18:2 (t9, t12)</td>
</tr>
<tr>
<td>20.</td>
<td>C18:2 (c9, c12)</td>
</tr>
<tr>
<td>21.</td>
<td>C20:0</td>
</tr>
<tr>
<td>22.</td>
<td>C20:1</td>
</tr>
<tr>
<td>23.</td>
<td>C20:2</td>
</tr>
<tr>
<td>24.</td>
<td>C20:3</td>
</tr>
<tr>
<td>25.</td>
<td>C20:4</td>
</tr>
<tr>
<td>26.</td>
<td>C20:5</td>
</tr>
</tbody>
</table>

In red, the only two pairs of cis/trans isomers

65-75 min analysis time
Detailed Analysis of cis/trans FAMEs C18:1 positional isomers

Column: Select FAME, 200-m, 0.25 mm i.d. (P/N CP7421)
Inlet: 250°C, split mode, split ratio 1:20
Carrier: Helium, 520 kPa
Oven: 185°C
FID: 250°C
Injection: 0.5 μL

Peak No
1. C18:0
2. C18:1 7 trans
3. C18:1 8 trans
4. C18:1 9 trans
5. C18:1 10 trans
6. C18:1 11 trans
7. C18:1 12 trans
8. C18:1 trans + ?
9. C18:1 9 cis
10. C18:1 10 cis
11. C18:1 11 cis
12. C18:1 12 cis
13. C18:1 13 cis
14. C18:1 14 cis
15. C18:1 15 cis
Comprehensive Portfolio for Fatty Acids, FAME and Oils Analysis

- **DB-FFAP**
  - Traditional phase for free fatty acids
  - Modified with nitroterephthalic acid
  - Consistent results for fatty acids

- **DB-FATWAX UI**
  - Free fatty acids, C4-C16
  - Simple mixtures
  - Omega 3 and 6 analysis, chain length/degree of unsaturation separation
  - Good reproducibility
  - Superior inertness for difficult samples (food matrix)
  - Simple, reliable, and multi-purpose

- **DB-FastFAME**
  - Fast Separation of cis/trans isomers
  - No cis/trans overlap with right conditions
  - 22-24 min analysis
  - Faster than conventional CP-Sil 88
  - Faster than traditional methods

- **CP-Sil 88 for FAME/HP-88**
  - Full resolution of cis/trans FAMEs
  - As suggested in AOAC 999.06
  - A0CS approved

- **Select FAME**
  - Highly detailed analysis of cis/trans FAME Isomers
  - Best GC column for GC/MS applications
  - The ultimate in high efficiency

- **CP-TAP CB**
  - Mono-, di- and triglycerides analysis
  - Optimal selectivity for triglycerides
  - High-temp applications
DB-FATWAX Ultra Inert

For the analysis of:

- FAMEs including Omega 3 and Omega 6
- Small organic acids and free fatty acids
- Applications in fish oil and animal fat analysis
37-FAME mix analysis with DB-FATWAX Ultra Inert

- Best option for saturated and polyunsaturated FAMEs, including Omega 3 and Omega 6
- WAX-type selectivity not ideal for cis/trans separation

GC System: Agilent 7890B
Column: DB-FATWAX UI, 30m, 0.25mm i.d., 0.25μm (G3903-63007)
Inlet: 250°C, split/splitless mode, split ratio 50:1
Carrier: Helium, constant flow, 40cm/s@50°C
Oven: 50°C(2min), 50°C/min to 174°C(14min), 2°C/min to 215°C(25min)
FID: 280°C, Hydrogen:40mL/min, Air:400mL/min, make-up
Injection: 1μL

**: Co-elution C18:1c/C18:1t
**: Separation C18:2c/C18:2t

**Critic separation**
Comparison study of the C24:0/C22:6n3 (DHA) critical pair

- DB-FATWAX UI fully resolves the C24:0/DHA critical pair
- It remains stable with no change in selectivity or retention time even after 40 h at MAOT.

*MAOT: maximum allowable operating temperature
Analysis of real samples

Column: DB-FATWAX UI, 30m, 0.25mm i.d., 0.25μm (G3903-63007)
Inlet: 250°C, split/splitless mode, split ratio 100:1
Carrier: Helium, constant flow, 30cm/s@180°C
Oven: 180°C(2min), 2°C/min to 210°C(35min)
FID: 280°C, Hydrogen:40mL/min, Air:400mL/min, make-up gas:25mL/min
Injection: 1μL
Sample: PUFA No.3 (diluted)

Menhaden Oil analysis following AOAC method

Baseline resolution of EPA and DHA

1. C14:0
2. C16:0
3. C16:1n7
4. C16:2n4
5. C16:3n4
6. C16:4n1
7. C18:0
8. C18:1n9
9. C18:1n7
10. C18:2n6
11. C18:3n3
12. C18:3n4
13. C18:4n3
14. C20:1n9
15. C20:4n6
16. C20:4n3
17. C20:5n3 (EPA)
18. C22:5n3
19. C22:6n3 (DHA)
Analysis of real samples

Column: DB-FATWAX UI, 20m*0.18mm I.D, 0.18um
Inlet: 250°C, split/splitless mode, split ratio 100:1
Carrier: Hydrogen, constant flow, 2mL/min
Oven: 140°C, 20°C/min to 190°C(3min), 5°C/min to 220°C(15min)
FID: 250°C, Hydrogen:40mL/min, Air:400mL/min, make-up gas:25mL/min
Injection: 1uL

Optimize separation of Menhaden Oil using a fast GC method

Baseline resolution of EPA and DHA, 13 minutes analysis time

1. C14:0
2. C16:0
3. C16:1n7
4. C16:2n4
5. C16:3n4
6. C16:4n1
7. C18:0
8. C18:1n9
9. C18:1n7
10. C18:2n6
11. C18:3n3
12. C18:3n4
13. C18:4n3
14. C20:1n9
15. C20:4n6
16. C20:4n3
17. C20:5n3
18. C22:5n3
19. C22:6n3
Great need in the food, forensic and cosmetic industries to monitor the content of free and natural occurring fatty acids.

Analysis of underivatized organic acids and free acids is desirable to eliminate the problems associated with derivatization, extraction, and cleanup procedures.

Volatile organic acids and fatty acids are difficult to quantify accurately by standard WAX columns. These acids often elute as tailing or poorly resolved peaks. For some acids, adsorption can become irreversible.

Normally:
- Fatty acids are derivatized to the methyl ester (FAME)
- Free fatty acids are analyzed using acid-deactivated wax columns. The acid modifier, nitroterephthalic acid, however, reduces thermal stability, operating temperature and reacts with humidity, reducing column life time

DB-FATWAX UI provides the desire inertness and thermal stability to separate challenge organic acids and fatty acids
Separation of Short-chain volatile organic acids in water using a competitor WAX column and DB-FATWAX Ultra Inert

- Standard WAX columns don’t have the inertness to separate most organic acids
Comparison between acid-modified WAX and DB-FATWAX UI after 50h at 250 °C

DB-FATWAX Ultra Inert shows superior inertness and thermal stability than acid-modified WAX
Analysis of Fatty acids (FA)

- Analysis of most FA and FAMEs without the need of an acid-modified WAX phase

1. Acetone  
2. Acetic acid  
3. Propionic acid  
4. Isobutyric acid  
5. Butyric acid  
6. Isovaleric acid  
7. Valeric acid  
8. 4-Methylvaleric acid

9. Hexanoic acid  
10. Heptanoic acid  
11. Octanoic acid  
12. Nonanoic acid  
13. Decanoic acid  
14. Lauric acid  
15. Myristic acid  
16. Palmitic acid

Column: Agilent DB-FATWAX UI, 30m, 0.25mm, 0.25μm (G3903-63007)
Carrier gas: Helium, 40cm/s @ 100°C
Oven: 100°C, to 250°C @10°C/min; 250°C (10min)
Inlet: 250°C, split ratio= 50:1,  
FID: 280°C  
Injection vol.: 1ul
Fatty acids and naturally occurring fatty acid ethyl esters analysis in alcoholic beverages

Chinese liquor sample

62% aqueous sample
Good resolution and peak shape
DB-FastFAME

For the analysis of:

- Ultra fast FAME analysis

- < 10 min analysis time for complex FAME mixtures
DB-FastFAME (30 m x 0.25 mm x 0.25 μm)  
Technical note 5991-8706EN

Stronger interaction of cis-isomers with the cyano-dipole causes trans-isomers to elute before the cis-isomers.
**DB-FastFAME**
**20m x 0.18mm x 0.20μm**

<table>
<thead>
<tr>
<th>Column</th>
<th>Agilent J&amp;W DB-FastFAME, 20 m x 0.18 mm, 0.20 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas</td>
<td>Hydrogen, 28 psi, constant pressure mode</td>
</tr>
<tr>
<td>Inlet</td>
<td>Split/splitless, 250 °C, split ratio 50:1</td>
</tr>
<tr>
<td>Oven</td>
<td>80 °C (0.5 min), <strong>65 °C/min</strong> to 175 °C, 10 °C/min to 185 °C (0.5 min), 7 °C/min to 230 °C</td>
</tr>
<tr>
<td>FID</td>
<td>280 °C, Hydrogen: 40 mL/min; Air: 400 mL/min; make-up gas: 25 mL/min.</td>
</tr>
<tr>
<td>Injection</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

**Strong interaction between cis isomers and the dipoles of the cyano propyl ligands. That allows the trans to elute after the cis isomers.**

\[ R_s \geq 1.95 \text{ for cis/trans isomers} \]

- **C18:1 cis**
- **C21:0, C20:3n6, C20:4n6**
- **R_s \geq 1.52**

**7 min analysis time**
Agilent Intuvo 9000

- New design
- New column connection
- Guard chip
- New column heating
- Application to FAME analysis

Intelligent, Intuitive, Innovative. Intuvo.
Innovating a New Path to GC Productivity

- Disposable Guard chip
- Modular Intuvo flow chips
- Ferrule-free click-and-run connections
- No-trim column
- Direct heating
Fast Analysis of a 36-FAME mix (AOAC 2012.13)
With the Intuvo 20-m DB-FastFAME

GC System: Agilent Intuvo 9000 GC system
Column  DB-FastFAME, 20m*0.18mm I.D, 0.20um, Intuvo module
Inlet  250°C, split/splitless mode, split ratio 100:1
Intuvo Guard chip  200°C
Carrier  Hydrogen, constant pressure, 28psi
Oven  50°C(0.3min), 200°C/min to 200°C(0.4min), 20°C/min to 240°C(1min)
FID  260°C, Hydrogen:40mL/min, Air:400mL/min, make-up gas:25mL/min
Injection  1uL

4 min analysis time!
How about more complex FAME samples like PHVO?

New DB-FastFAME GC column dimensions coming soon…
Analysis of 57-FAMEs using a 90m DB-FastFAME

GC System: Agilent 7890B
Column DB-FastFAME, 90m*0.25mm I.D, 0.25um
Inlet 260°C, split/splitless mode, split ratio 50:1
Carrier Helium, constant pressure, 46psi
Oven 70°C(1min), 40°C/min to 200°C(5 min), 2°C/min to 205°C(11.5 min); 15°C/min to 245°C(15 min)
FID 280°C, Hydrogen:40mL/min, Air:400mL/min, make-up gas:25mL/min
Injection 1uL

C18 isomers
See next slide
Expanded view of 57-FAME Chromatogram
90-m DB-FastFAME

26 min analysis time!
Fast Analysis of a 54-FAME mix
With the Intuvo 60-m DB-FastFAME (coming soon!)

GC System: Agilent intuvo GC/FID
Column DB-FastFAME intuvo GC column, 60m*0.25mm I.D, 0.25um
Inlet 260°C, split/splitless mode, split ratio 100:1
Guard chip 200°C
Carrier Helium, constant pressure, 30psi
Oven 70°C(1min), at 200°C/min to 175°C(2min); at 5°C/min to 210°C(8min);
at15°C/min to 240°C(15min)
FID 260°C, Hydrogen:40mL/min, Air:400mL/min, make-up gas:25mL/min
Injection 1uL

List of Trans-FAMEs added to the 37-FAME mix

Complex FAME separation < 30 min
Expanded view of C18:1 cis/tran isomers (from GC/FID Chromatogram of 54-FAMEs )
DB-HeavyWAX

For the analysis of:

- High temp applications for polar analytes
- Essential oils and flavor & fragrances
- Other high temp. requirements (GCxGC)
Introducing DB-HeavyWAX

- WAX column with increased MAOT compared to existing columns on the market
  - 280°C isothermal and 290°C programmed
- Provides increased thermal stability
- Has a low bleed level
- Advantages

<table>
<thead>
<tr>
<th>General GC</th>
<th>GC/MS</th>
<th>GC×GC</th>
</tr>
</thead>
</table>
| • Shorter runtimes when late eluters are present  
  • Better S/N ratio, improved detection  
  • Better thermal stability  
  • Faster column bake-out | • Desire for “zero” bleed  
  • Avoid MS contamination by column bleed for longer system uptime and column lifetime  
  • Improve detection limit | • Extended scope of compounds |
Bleed Summary at 280°C Over 100 Hours

- WAX A at 100 Hours
- WAX B at 100 Hours
- WAX B at 0 Hours
- WAX A at 0 Hours
- DB-HeavyWAX at 100 hours
- DB-HeavyWAX at 0 hours

Response Units (%) vs. Acquisition Time (min)
Bleed reductions for FAMEs in Biodiesel (IP-585) using a DB-HeavyWAX, 60m x 0.25mm x 0.50 µm

<table>
<thead>
<tr>
<th>Peak</th>
<th>FAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C16:0</td>
</tr>
<tr>
<td>2</td>
<td>C17:0-d33 (IS)</td>
</tr>
<tr>
<td>3</td>
<td>C17:0</td>
</tr>
<tr>
<td>4</td>
<td>C18:0</td>
</tr>
<tr>
<td>5</td>
<td>C18:1</td>
</tr>
<tr>
<td>6</td>
<td>C18:2</td>
</tr>
<tr>
<td>7</td>
<td>C18:3</td>
</tr>
</tbody>
</table>

Counts (%) vs. Acquisition Time (min)

Agilent J&W DB-HeavyWAX

Bleed reductions:
- C16:0: 3.1 pA
- C17:0: 8.8 pA

Agilent Confidential

June 20, 2019
Retention time stability on a DB-HeavyWAX

IP-585: FAMEs in aviation fuel

➢ Less bleeds reduces noise level
➢ Enhance sensitivity without changing the detector
➢ Increase thermal stability → Better stability in the SIM window → Less service time

<table>
<thead>
<tr>
<th>Compound</th>
<th>Operating Hours at 260°C</th>
<th>Average</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>C16:0</td>
<td>36.58</td>
<td>36.47</td>
<td>36.45</td>
</tr>
<tr>
<td>C17:0 d33 (ISTD)</td>
<td>39.57</td>
<td>39.47</td>
<td>39.45</td>
</tr>
<tr>
<td>C17:0</td>
<td>41.09</td>
<td>40.99</td>
<td>40.98</td>
</tr>
<tr>
<td>C18:0</td>
<td>45.26</td>
<td>45.16</td>
<td>45.15</td>
</tr>
<tr>
<td>C18:1</td>
<td>46.25</td>
<td>46.15</td>
<td>46.14</td>
</tr>
<tr>
<td>C18:2</td>
<td>48.20</td>
<td>48.10</td>
<td>48.09</td>
</tr>
<tr>
<td>C18:3</td>
<td>51.01</td>
<td>50.89</td>
<td>50.87</td>
</tr>
</tbody>
</table>

GC Conditions

<table>
<thead>
<tr>
<th>Component</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>60m x 0.25 mm x 0.5 μm</td>
</tr>
<tr>
<td>Carrier</td>
<td>Helium, constant flow, 1.2 mL/min</td>
</tr>
<tr>
<td>Oven</td>
<td>150°C (5.0 min), Ramp 12°C/min to 200°C (17.0 min), Ramp 3°C/min to 252°C (10 min)</td>
</tr>
</tbody>
</table>
Conclusion

• New column technologies for faster & high performance FAME analysis, complements our comprehensive portfolio of GC columns for Fat & Oil analysis

  – DB-FATWAX UI is our new GC Column specially tested for FAMEs in Fish Oil and Animal Fat. Ultra Inert technology makes it possible to analyze fatty acids as well as FAMEs and FAEEs with DB-FATWAX UI, simplifying column selection

  – DB-FastFAME is a new high-content cyanopropyl phase that allows the fast analysis of FAMEs without sacrificing in resolution.

  – DB-HeavyWAX, our new addition to our WAX GC columns portfolio, is a new high temperature WAX phase, ideal for analysis of FAMEs in biodiesel
Thank you!