Searching and finding unusual fatty acids and compounds of the unsaponifiable matter

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polyhalogenated compounds
halogenated natural products
halogenated flame retardants
stable isotope mass spectrometry (IRMS)
countercurrent chromatography
lipid analysis
polyhalogenated compounds
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stable isotope mass spectrometry (IRMS)
countercurrent chromatography
lipid analysis
methyl-branched fatty acids
furan fatty acids
unsaponifiable matter
enantiomer separations
standard compounds
GC/MS
food authenticity
Gas chromatograms of fatty acids (as methyl esters) in food samples

**Sesame Oil**
- 16:0
- 18:0
- 18:1n-9
- 18:2n-6
- 18:3n-3

**Fish Oil**
- 14:0
- 16:0
- 16:1n-7
- 18:0
- 18:1n-9
- 18:2n-6
- 18:4n-3
- 20:3n-9
- 20:4n-6
- EPA
- DHA

**Linseed Oil**
- 16:0
- 18:0
- 18:1n-9
- 18:2n-6
- 18:3n-3

**Milk Fat**
- 10:0
- 12:0
- 14:0
- 15:0
- 16:0
- 16:1n-7
- 18:0
- 18:1n-9
- a15:0
- a17:0
anteiso-fatty acids acids

- methyl substituent at \((n-2)\) carbon \(\Rightarrow\) anteiso-fatty acid

- typical chain length: \(C_{14}\) and \(C_{16}\), odd carbon number (due to methyl group)
- usually found in ruminants and fish (barely in plants)
- usually occurring together with iso-fatty acids (methyl-branch on second last carbon)

12-methyl tetradecanoic acid (a15:0)

13-methyl tetradecanoic acid (i15:0)
Bacterial lipids

- anteiso- and iso-fatty acids dominate (in gram-positive bacteria)
  \[ \Rightarrow \text{up to } 80\% \text{ contribution to the total fatty acids} \]

- occurrence of anteiso-fatty acids in food is mostly linked with the presence of bacteria
  \[ \Rightarrow \text{in gnotobiologic (germ-free) rats only present at traces} \ [1] \]

Stable isotope analysis: GC-IRMS method

• eluate from the GC column led to combustion unit
• organic carbon is transferred into CO₂
• exact determination of the $^{13}$C/$^{12}$C ratio is difficult
  ⇒ instead, measurement relative to reference standard

$$\delta^{13}C \ [\%o] = \frac{\left[^{13}C/^{12}C\right]_{sample} - \left[^{13}C/^{12}C\right]_{standard}}{\left[^{13}C/^{12}C\right]_{standard}} \times 1000$$

• typical $\delta^{13}C$ values in the range -10 to -40‰

**δ^{13}C values [%] of fatty acids in suet**

<table>
<thead>
<tr>
<th>fatty acid*</th>
<th>δ^{13}C value [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>-26.6</td>
</tr>
<tr>
<td>14:0</td>
<td>-27.2</td>
</tr>
<tr>
<td>i15:0</td>
<td>-36.7</td>
</tr>
<tr>
<td>a15:0</td>
<td>-35.9</td>
</tr>
<tr>
<td>15:0</td>
<td>-30.5</td>
</tr>
<tr>
<td>i16:0</td>
<td>-37.6</td>
</tr>
<tr>
<td>16:0</td>
<td>-27.1</td>
</tr>
<tr>
<td>i17:0</td>
<td>-36.2</td>
</tr>
<tr>
<td>a17:0</td>
<td>-35.5</td>
</tr>
</tbody>
</table>

* measured as methyl ester

**δ^{13}C values verify:**

- methyl-branched fatty acids are depleted in $^{13}$C compared to straight-chain fatty acids ⇒ different sources!

Properties of methyl-branched fatty acids

- inertness towards oxidation
- low melting point
- changing physiological properties of lipids
  ⇒ increase of the membrane fluidity
- positive effect on the penetration of other compounds into the skin

1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC)

Differential scanning calorimetry (DSC): DSC thermograms of lipids

A thought during a student lecture

- Amino acids – building blocks of proteins – are chiral (typically L-form)

- Sugars – building blocks of carbohydrates – are chiral (typically D-form)

- Fatty acids – building blocks of lipids – are non-chiral

⇒ one notable exception: anteiso-fatty acids
Chirality of \textit{anteiso}-fatty acids

- 1950ies: one-time investigation of suet and shark liver oils \cite{1,2}
  \[ \Rightarrow \sim 10 \text{ kg fat/oil converted into methyl esters} \]
  \[ \Rightarrow \text{fractionation via distillation} \]
  \[ \Rightarrow \text{repeated crystallization} \]
  \[ \Rightarrow \text{x-ray analysis, optical rotation} \]

\textbf{result:}
- all samples exclusively featured the (+)-enantiomer

Ref.: \cite{1} R. P. Hansen, F. B. Shorland, N. J. Cooke, \textit{Biochem. J.} 52 (1952) 203-207
\cite{2} I. M. Morice, F. B. Shorland, \textit{Biochem. J.} 64 (1956) 461-464
GC enantiomer separation of anteiso-fatty acids

- not reported when we started
- only of $\alpha$-, $\beta$- and $\gamma$-methyl-substituted acids resolved [1][2]
- the more remote the methyl group from the head group, the more difficult the enantiomer separation is [1][2]

Small problems had to be solved...

- **synthesis of enantiopure standards** [1]
  - Wittig reaction (olefination of chiral aldehydes via ylides)
  - hydrogenation of the resulting double bond

- **searching for a chiral stationary GC phase** [2]
  - testing of ~20 chiral stationary phases
  - improvement of the only promising one

- **development of sensitive and selective method** [3]
  - development of a GC/MS-SIM method
  - enrichment/isolation of anteiso-fatty acids by hydrogenation, urea complexation and/or (Ag⁺) HPLC fractionation

Fatty acid analysis

• important routine task in food science (and life sciences)
• classic method using GC/FID after formation of fatty acid methyl esters (FAME)

⇒ peak abundance correlates with amount
⇒ determination of relative contributions (“100% method“)

disadvantages of GC/ FID

• low selectivity
• co-elutions may be overlooked
• problems with low abundant fatty acids

GC: gas chromatography
FID: flame ionisation detector
Coelution of monoenoic and *anteiso*-fatty acids

- 17:0-ME: $M^+ = m/z$ 284
- 16:1-ME: $M^+ = m/z$ 268

- co-elutions can hardly be omitted on 50 m columns

GC/MS chromatogram of a milk fat sample

(GC column: 50 m x 0.25 mm i.d. x 0.2 µm 100% cyanopropyl polysiloxane)
Why GC/MS in SIM mode?

(Selected ion monitoring (SIM))

- more sensitive and selective than full scan

$m/z$ 284 extracted from full scan

$m/z$ 284 measured in SIM mode


GC column: 50 m x 0.25 mm i.d. x 0.2 µm 100% cyanopropyl polysiloxane
Determination of fatty acid methyl esters by GC/MS-SIM

- sensitivity and selectivity

Quantitative determination of individual fatty acids

- quantification requires use of internal standards (IS) not present in the sample

(1) IS for sample cleanup
(addition before/after the extraction)

DC-11:0

(2) syringe standard
(addition to GC/MS solution)

14:0-EE

House method

lipid extraction using accelerated solvent extraction (ASE) for dry samples or microwave-assisted extraction (MAE) of aqueous samples

gc/ms-sim analysis

Microwave-assisted extraction (focused-open vessel; FOV-MAE)

- connection tool with water trap and nitrogen inlet

No commercial system, self-constructed, base instrument for acid digestions
Concentrations [g/ 100 g fat] of methyl-branched fatty acids in food

<table>
<thead>
<tr>
<th>FAME</th>
<th>mozzarella (cow) [g/ 100 g]</th>
<th>feta (cow) [g/ 100 g]</th>
<th>feta cheese [g/ 100 g]</th>
<th>human milk [g/ 100 g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>i14:0</td>
<td>0.08 ± 0.00</td>
<td>0.08 ± 0.00</td>
<td>0.08 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>i15:0</td>
<td>0.20 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.06 ± 0.00</td>
</tr>
<tr>
<td>a15:0</td>
<td>0.30 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.34 ± 0.03</td>
<td>0.08 ± 0.00</td>
</tr>
<tr>
<td>i16:0</td>
<td>0.12 ± 0.00</td>
<td>0.09 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>i17:0</td>
<td>0.47 ± 0.01</td>
<td>0.56 ± 0.02</td>
<td>0.37 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>a17:0</td>
<td>0.39 ± 0.01</td>
<td>0.42 ± 0.02</td>
<td>0.37 ± 0.01</td>
<td>0.14 ± 0.00</td>
</tr>
</tbody>
</table>

- only these fatty acids were quantified using the corresponding ethyl esters as IS

Birth goo (Vernix caseosa)

- detection of ~60 methyl-branched fatty acids (although germ-free)

Direct GC enantiomer separation of anteiso-fatty acids

- application of modified cyclodextrins

\[ \alpha\text{-cyclodextrin} \quad \beta\text{-cyclodextrin} \quad \gamma\text{-cyclodextrin} \]
(6 glucose units) (7 glucose units) (8 glucose units)

- \(O\)-derivatisation at C-2, C-3 and C-6 provides a range of modified cyclodextrins suitable as chiral stationary GC phases
6-\textit{O-}\textit{ tert.}-butyldimethylsilyl-2,3-di-\textit{O-}methyl-\(\beta\)-cyclodextrin (\(\beta\)-TBDM)

- tests of >20 chiral stationary phases only partly successful with \(\beta\)-TBDM
  - \(\Rightarrow\) improvement of the initial phase
    (collaboration with G. Hottinger, BGB-Analytik)

10\% \(\beta\)-TBDM thin film
- lowers elution temperature
- resolution decreases

50\% \(\beta\)-TBDM standard film
- better interaction
- better resolution

\(\beta\)-TBDM:
\(R_1 = R_2 =\) methyl

Enantioselective determination of a17:0 on $\beta$-TBDM

racemates partly resolved

racemates spiked with $S$-enantiomer
elution order: $R < S$

pure $S$-enantiomer
$\Rightarrow$ required GC run time
8–10 h elution time

Sample preparing for enantiomer separation:
(1) urea complexation
(2) silver ion chromatography

urea complexation => separation of major saturated fatty acids

Ag⁺-HPLC => separation of unsaturated fatty acids

Results: Chirality of anteiso-fatty acids

- Yet, up to 10% $R$-anteiso-fatty acids detected in milk fat and fish
- $R$-anteiso-fatty acids cannot be synthesized by the classical biosynthesis via isoleucine as the primer
  $\Rightarrow$ A hitherto mostly unknown biosynthesis pathway must exist
Results: Chirality of anteiso-fatty acids

- Yet, up to 10% R-anteiso-fatty acids were detected in milk fat and fish.
- R-anteiso-fatty acids cannot be synthesized by the classical biosynthesis via isoleucine as the primer.


4-alkyl: R-enantiomers
anteiso: S-enantiomers

Surprisingly, 4-alkylbranched fatty acids (principal flavor compounds of goat and sheep (4-Me-8:0 and 4-Et-8:0) were recently shown to be R-enantiopure.

Enantiopure 4-alkylbranched standards were produced via repeated enantioselective esterification.
Two dimensional HPLC/ GC chromatogram of fish oil fatty acids (as methyl esters)

- excel-programmed 2D evaluation

- similar approach as GCxGC

**disadvantage**
- time consuming
  (~2 days per sample)

**advantage**
- good orthogonality
- higher amounts (post analysis possible)

HPLC/ GC - 2D Konturplot

PUFAs in the HPLC/GC plot of a fish oil

- many, many PUFAs

- non-aqueous RP-HPLC with three C₁₈ columns

HPLC/ GC plots of branched chain fatty acids

- HPLC fractionation allowed to detect traces of a16:0
- most likely produced by α-oxidation of a17:0
Valuable minor fatty acids in milk

<table>
<thead>
<tr>
<th>parameter</th>
<th>organic milk*</th>
</tr>
</thead>
<tbody>
<tr>
<td>phytanic acid</td>
<td>+</td>
</tr>
<tr>
<td>PUFA (ALA, EPA)</td>
<td>+</td>
</tr>
<tr>
<td>CLA</td>
<td>+</td>
</tr>
<tr>
<td>furan fatty acids</td>
<td>+</td>
</tr>
</tbody>
</table>

“+” higher content in organic milk than in conventional milk due to green feed
Features of furan fatty acids

- structural feature: furan moiety in the carbon chain
- substituted with one ("M") or two ("D") methyl groups

- short terms: 9M5 9D5

- "D"-furan fatty acids are more widespread but much less stable
  ⇒ partly absent in processed or stored samples
- excellent antioxidants (very effective protectors of PUFAs)
- degradation products responsible for off flavor of soy (among other)

Calculated daily intake of furan fatty acids per capita [mg], in Germany

Calculated daily intake of furan fatty acids per capita [mg], in Germany

- yesterday, John Bowden of NIST presented data from >20 labs on lipidomics (fish, krill oil)
- SRM 1950 (human plasma), krill oil, fish
- some 1500 lipids reported
- on my question, he confirmed that none of the labs had detected furan fatty acids

Furan fatty acids

- minor fatty acids, low concentrations (typically <0.1% contribution to the total fatty acids)
- but: widely spread in virtually all plants and animals

- discovered, studied in the 1970s (R.L. Glass, H. Schlenk, F.D. Gunstone)
- mostly forgotten, studied again ~1985-1995 (G. Spiteller, W. Grosch)
- almost forgotten since the 2000s
- no reference standards commercially available
  ⇒ research only possible with house-prepared standards
Compound isolation using countercurrent chromatography (CCC)

- CCC is a well-established method in natural product isolation [1][2]
- all liquid based chromatographic method (no solid support) [3]
- allows injection and isolation of gram-amounts of analytes [3]
- barely used in field of lipid compounds [1]

Applications of countercurrent chromatography (CCC) according to SciFinder (1981-2015)

- currently >300 CCC papers/year, number is increasing
- ~3% in the field of lipid compounds
CCC instrumentation

- CCC systems same setup as HPLC instruments except the column
  ⇒ instead: CCC centrifuge with multilayer coils

Diagram:
- Stationary phase
- Mobile phase
- Pump
- Injection valve
- CCC centrifuge with multilayer bobbins
- Detector
- Fraction collector
CCC method development

- CCC is different to liquid-liquid extractions as it aims to distribute the analyte evenly between both phases

- determination of the partitioning factor

\[ K_{U/L} = \frac{\text{[concentration in upper phase]}}{\text{[concentration in lower phase]}} \approx 0.4 - 2.5 \]

- even distribution: \( K_{U/L} = 1 \); acceptable range: \( K_{U/L} = 0.4 - 2.5 \)

⇒ the goal challenge is to find a biphasic solvent system in which the analytes are ~ evenly distributed (and resolved)

⇒ see tutorial on CCC in the AOCS Lipid Library
Isolation of the valuable furan fatty acid 11D5

11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid (11D5)

- excellent antioxidant
- no standards available
- limited research

**Injection:** 1 g

**Yield:** 19 mg 11D5

**Purity:** 99%

**Solvent consumption:** 100 mL

**Time per mg 11D5:** 3.4 min

CCC isolation of fatty acid methyl esters

hexane/methanol/water (350/175/2) for fatty acid methyl esters (FAME)

- **equivalent chain length (ECL) rule:**
  \[ \Rightarrow 2 \text{ carbons} \sim 1 \text{ DB} \]

- no problem with 16:4 (ECL: 12:2 / 10:1 / 8:0)

Sample fractionation and analyte isolation via countercurrent chromatography (CCC)

1. isolation of lipid compounds for use as standards/in biotests
   • examples:  - isolation of uncommon fatty acids
                - isolation of phytosterols, tocopherols, carotenes etc.

2. fractionation of lipids or lipid fractions by CCC
   • detection of minor compounds usually “invisible“ without fractionation
   • examples:  - detection of 430 fatty acids in one butter sample
                - discovery of aromatic fatty acids in milk fat
Detailed analysis of a butter sample

- 21 g butter
  - 16.9 g butter oil
    - 17.1 g FAME
    - 4.7 g residue
    - 0.2 g filtrate

- CCC #1 from FAME fraction (major fatty acids)
- CCC #2 from filtrate of urea complexation (rare trace fatty acids)
- 430 different fatty acids
- >100 PUFAs
- several rare fatty acids

Detailed analysis of a butter sample

• potential formation from oleic acid (lipid oxidation)

Aromatic fatty acids in butter

- previously not known to occur in milk (and other food)
- potential formation from phenylalanine as the primer

Cyclic fatty acids

- previously known to occur in milk
- potential formation with aromatic fatty acids by hydrogenation?

Very nonpolar lipid compounds

- log $K_{ow}$ 7 -20
- main interest in this study: log $K_{ow}$ >15

- except sitosterol no polar groups (-OH)

- sitosterol
  
- tricaprylin
  
- tripalmitin (PPP)
  
- cholesteryl stearate (18:0-CE)

- huge difference between triacylglycerols

- log $K_{ow}$~14.6
- log $K_{ow}$~10
- log $K_{ow}$~15
- log $K_{ow}$~21
- log $K_{ow}$~9.7

- log $K_{ow}$ 7 -20
- main interest in this study: log $K_{ow}$ >15
Introduction of benzotrifluoride as modifier in solvent systems

- bridging solvents between the biphasic system [1]

**ternary phase diagram**

- well suited composition and properties:
  - Hex/ACN/BTF: 10 / 6.5 / 3.5
  - settling time: < 20 sec

Isolation of carotenoids from carrot juice using hexane / acetonitrile / benzotrifluoride (BTF)

\[ \text{β-carotene} \]
\[ \text{α-carotene} \]
\[ \text{lutein} \]

- **β-carotene**: Purity ≥95%
- **α-carotene**: Purity ≥95%
- **Lutein**: Purity ≥97%

**CCC/vis-chromatogram**
- **β-carotene**
- **α-carotene**
- **Lutein**

**HPLC/UV chromatograms (450 nm)**
- **β-carotene**: 51 mg, purity ≥95%
- **α-carotene**: 32 mg, purity ≥95%
- **Lutein**: 4 mg, purity ≥97%

### Difference between “Hex / ACN“ and the “Hex / ACN / BTF“ solvent system

Phase composition of solvent systems determined by GC/ FID [1]

<table>
<thead>
<tr>
<th>solvent system</th>
<th>lower</th>
<th>upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexane / ACN [1]</td>
<td>1.2 / 98.8</td>
<td>99.5 / 0.5</td>
</tr>
<tr>
<td>hexane / ACN / BTF [2]</td>
<td>29.0 / 56.7 / 14.3</td>
<td>76.0 / 12.5 / 11.6</td>
</tr>
</tbody>
</table>

- >1/4th hexane partitions into lower phase
- difference in polarity decreased

Ref.:  
**K\textsubscript{U/L} of lipid compounds in the BTF system**

(Hex/ACN/BTF, 10:6.5:3.5)

<table>
<thead>
<tr>
<th>lipid compound</th>
<th>log $K_{\text{OW}}$</th>
<th>HEMWAT -7 $K_{\text{U/L}}$</th>
<th>BTF $K_{\text{U/L}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>oleic acid</td>
<td>7.7</td>
<td>29</td>
<td>0.57</td>
</tr>
<tr>
<td>oleic acid methyl ester</td>
<td>7.5</td>
<td>41</td>
<td>2.4</td>
</tr>
<tr>
<td>sitosterol</td>
<td>9.7</td>
<td>16</td>
<td>2.2</td>
</tr>
<tr>
<td>squalane</td>
<td>14.6</td>
<td>550</td>
<td>62</td>
</tr>
<tr>
<td>cholesteryl stearate</td>
<td>15</td>
<td>1260</td>
<td>80</td>
</tr>
<tr>
<td>tripalmitin (PPP)</td>
<td>21</td>
<td>1490</td>
<td>60</td>
</tr>
</tbody>
</table>

Excellent for FAMEs, sterols, tocopherols

BTF system in co-current* CCC mode

* introduced by Sutherland et al., theory by Berthod et al.

- both phases are moved
- accelerates elution of analytes with high $K_{U/L}$
- exponential increase leads to co-elution of analytes with high $K_{U/L}$, even if $\Delta K_{U/L}$ is high

Co-current CCC mode with the BTF system

- elution of lipid compounds spreading from log \( K_{ow} \) 3 – 30 within acceptable run time
- no separation of extremely nonpolar lipid compounds

![Diagram showing elution range of triacylglycerols, standard mix, and elution of lipid compounds spreading from log \( K_{ow} \) 3 – 30 within acceptable run time.](image)
Co-current CCC mode with the BTF system

bubble blot of the co-current CCC separation of 0.5 g rice bran oil


CCC isolation of vitamin E compounds

- dietary supplementary capsule made from palm oil
  - each isolated at 10-65 mg/run (purity 99%)

Tocotrienol artefacts

- 170 non-natural vitamin E compounds in a palm-oil based dietary supplementary oil
- ~80 tocotrienol isomers
- tocotetra- & pentaenols
- ...
- formed during inadequate sample processing

Summary

- analysis of minor lipid compounds is a fascination and varied research field
- the actual relevance of minor fatty acids may currently be underrated
- unavailability of standards frequently hampers progress in the field
  (no standard = no research = no knowledge)
- lipid standards can be isolated by countercurrent chromatography
- our work is a mixture of basic research and applications
- and sometimes …
  … it’s like a road movie (the journey is the goal)
Thousand thanks!

• to my former and previous ph. d. students, master and bachelor students, especially those pictured in this presentation (people first)!

• to our research partners here, there and everywhere!

• to our funders (without money, no research)!

• to Analytical Division of AOCS for honoring our research with the Herbert J. Dutton Award

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• to YOU for your attention