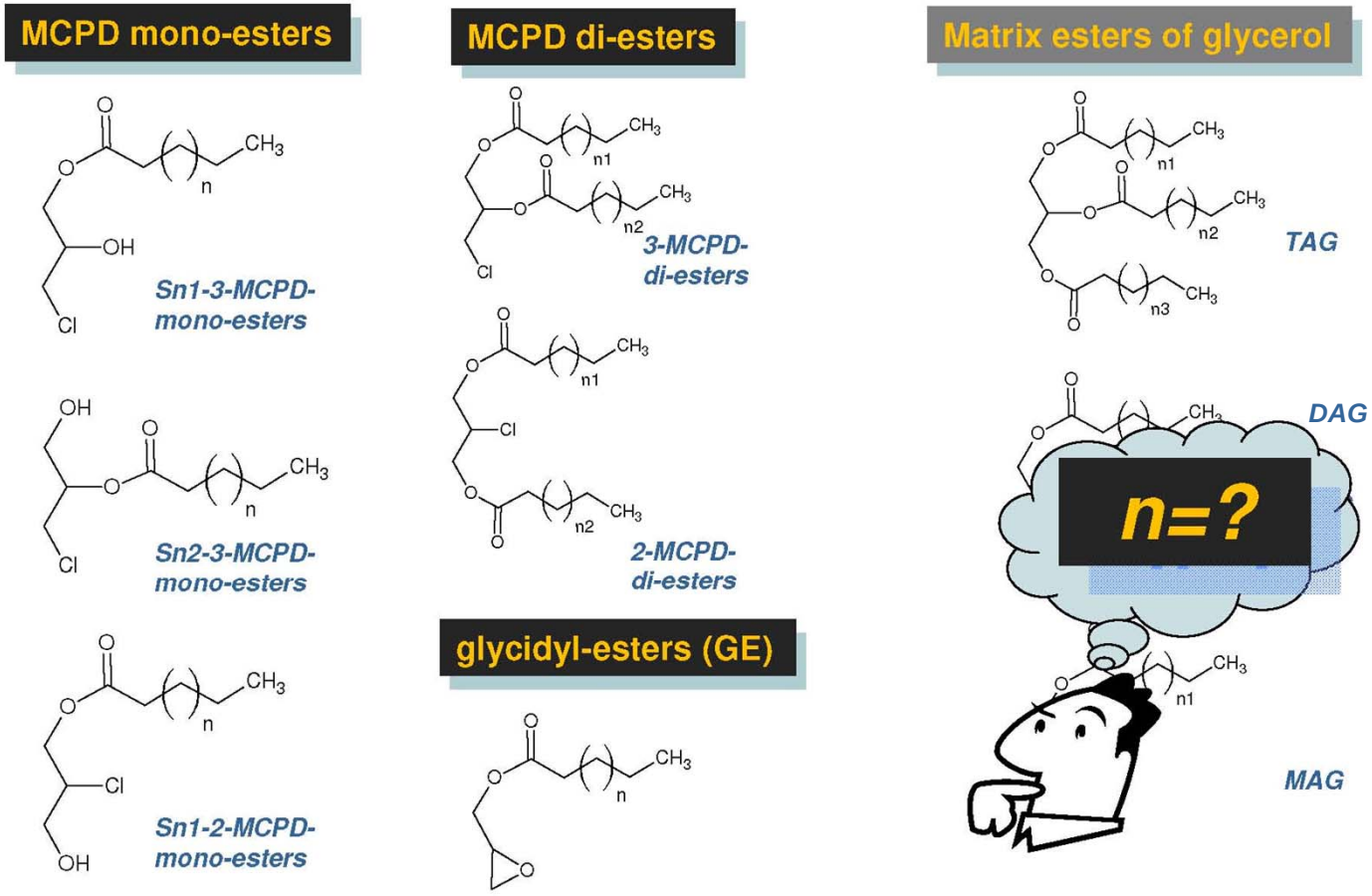
Development of a direct quantitation method of glycidyl esters in edible fats and oils via stable isotope dilution analysis

Michael Granvogl and Peter Schieberle

Technical University of Munich, Chair for Food Chemistry and German Research Centre for Food Chemistry, Lise-Meitner-Str. 34, Freising, Germany

Overview

MCPD and glycidyl esters are very similar in nature to TAG/DAG and MAG



Seefelder *et al.* Eur. J. Lipid Sci. Technol ***113*** *(3), 319-22 (2011)*

Dubois, AOCS, 2011

Occurence and toxicological relevance I

- 3-MCPD esters have been reported in refined fats and oils by several groups (*Svejkovská et al., 2004; Zelinková et al., 2006; Weißhaar, 2008*)
- ***free 3-MCPD***
  - EU Commission Regulation N° 466/2001 sets a MRL (*Maximum Residue Limit*) of 0.02 mg/kg in hydrolized vegetable protein (HVP) & HVP products
  - TDI (*Tolerably Daily Intake*) of 2 µg/kg body weight per day
  - **NEW**: IARC: group 2B „*Possibly carcinogenic to humans"*
- ***free glycidol***
  - International Agency for Research on Cancer (IARC): group 2A „*Probably carcinogenic to humans"*
- ***bound 3-MCPD***
  - BfR (*Federal Institute for Risk Assesment*): assumption that 3-MCPD is completely released from 3-MCPD esters during human digestion
- ***bound glycidol***
  - glycidyl and stearate glycidyl oleate: IARC: group 3 „*Not classifiable as to its carcinogenicity to humans"*
  - not much data available

Toxicological relevance II

BfR: initial results on metabolism

- ***free 3-MCPD***
  - diffusion across Caco-2 monolayer
  - 3-MCPD is not metabolized by Caco-2 cells
- ***3-MCPD-1-monoester***
  - no diffusion across Caco-2 monolayer
  - ester-hydrolysis via extracellular lipases/esterases
  - diffusion of liberated 3-MCPD across monolayer
- ***3-MCPD-1,2-diester***
  - no diffusion across Caco-2 monolayer
  - no liberation of free 3-MCPD

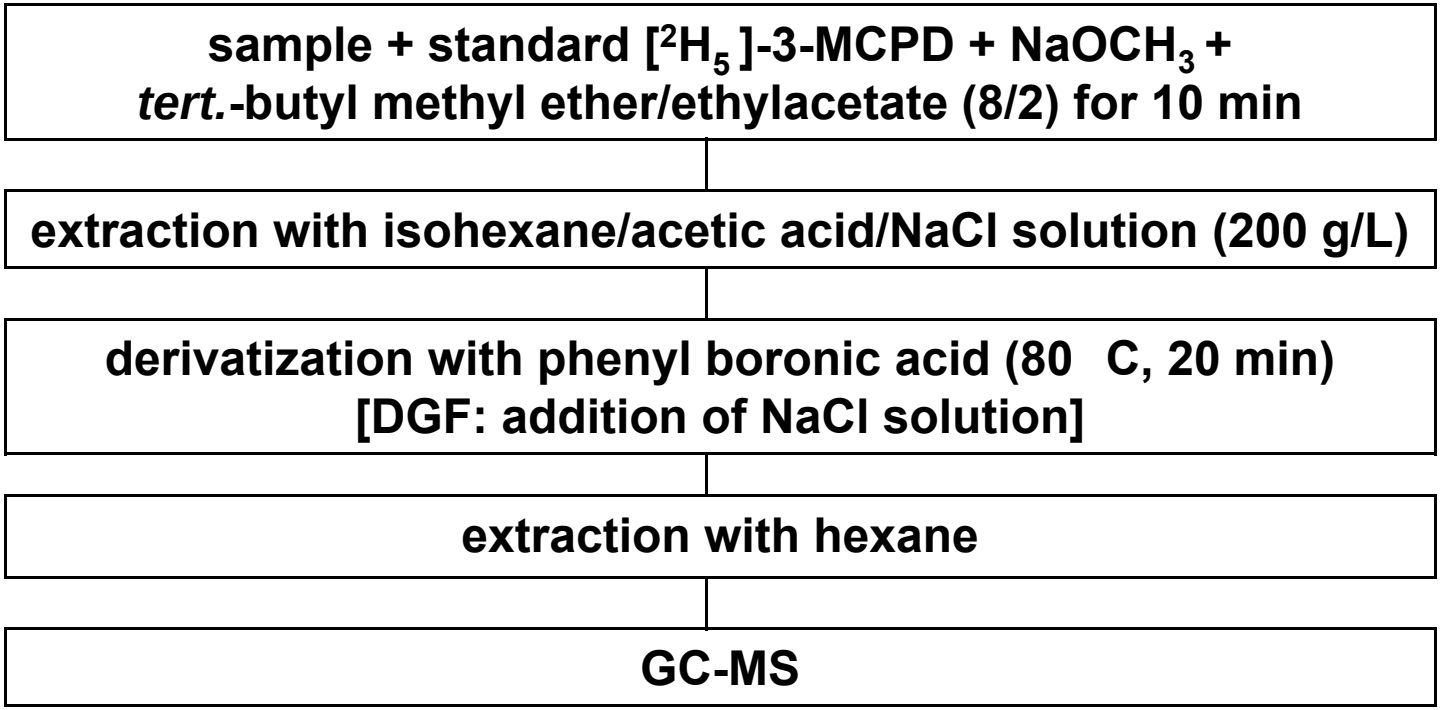
Toxicological relevance III

- **Barocelli et al. (University of Parma): Scientific Report submitted to EFSA (European Food Safety Authority)**
  - 3-Chloro-1,2-propanediol-dipalmitate exposure was associated with urinary excretion rates of both 3-MCPD and 3-MCPD mercapturate about 30 % lower as compared to the same urinary biomarkers observed after exposure to equimolar doses of 3-MCPD
  - = release of free 3-MCPD from 3-Chloro-1,2-propanediol-dipalmitate by about 70 % (90 day tox-study)

Toxicological relevance IV

- **Lampen et al. (BfR): ILSI Workshop, Brussels, November 2011**
  - radio-labeled: **2-[^{14}C]-glycidyl palmitate** and **9,10-[$^{3}H_2$]-glycidyl palmitate**
  - glycidyl palmitate cleavage at a very high degree
  - hemoglobin adduct formation of ester is comparable to that of free glycidol
  - non radio-labeled **3-chloro-1,2-propanediol-dipalmitate**
  - 3-MCPD ester cleavage is high: intestinal hydrolysis more or less complete
  - after approx. 3 h highest amounts of liberated 3-MCPD

Reference method
(sum of 3-MCPD and/or glycidyl esters)



(Concentration A)

Weisshaar (2008, Eur. J. Lipid Sci. Technol.)/(DGF)

Reference method (3-MCPD esters)

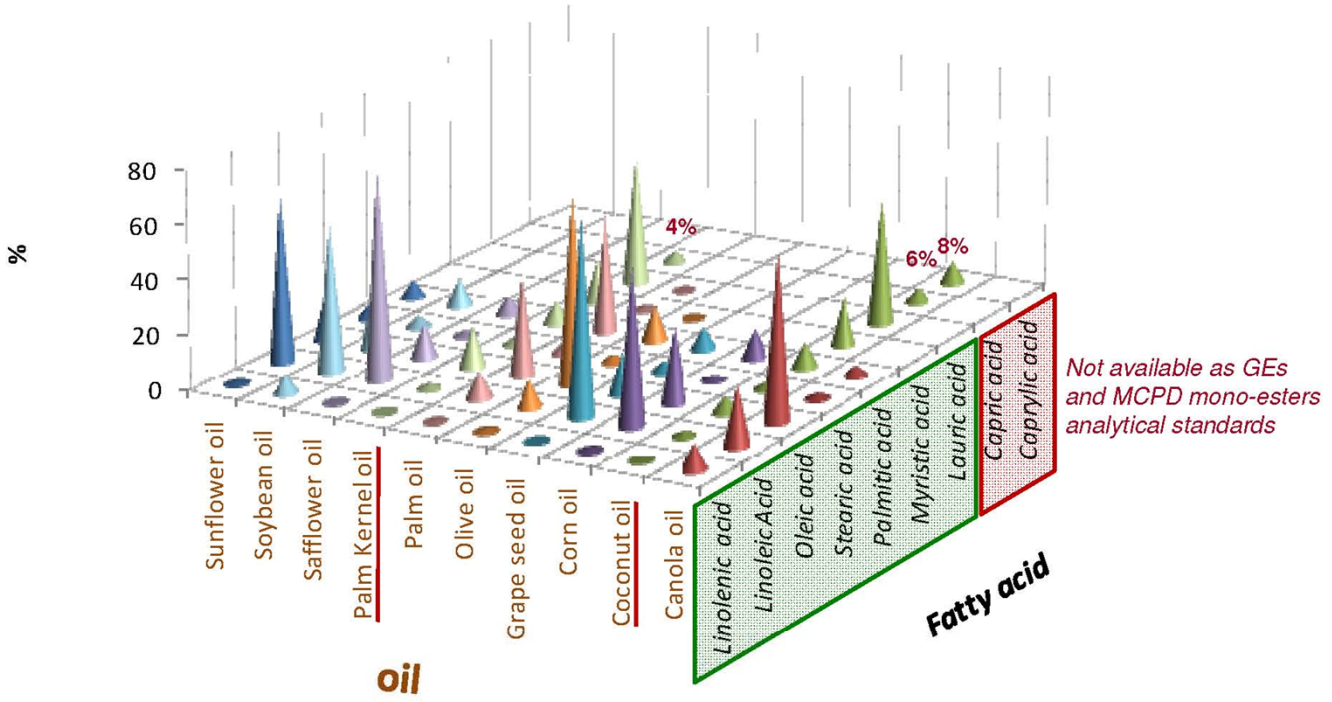
In a **second trial**, the **sample** is treated with a mixture of **propanol/sulphuric acid** under mild conditions. The **epoxide ring of the glycidyl ester is opened** and **glycidol is quantitatively removed** forming different reaction products. After this treatment **the content of ester-bound 3-MCPD in the sample is determined again (Concentration B)**. Concentration B **corresponds** to the **original content of ester-bound 3-MCPD** in the sample.

Provided that the difference of the two determinations is most exclusively due to the occurrence of glycidol, the difference is used to calculate the content of ester-bound glycidol using a stoichiometric conversion factor: 0,67 * (Concentration A – Concentration B).

Weisshaar (2008, Eur. J. Lipid Sci. Technol.)/(DGF)

DEVELOPMENT OF A DIRECT QUANTITATION METHOD

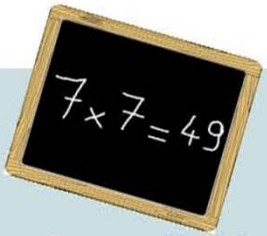
Fatty acid composition of edible fats and oils



Rational approach for glycidyl- and MCPD-mono-esters

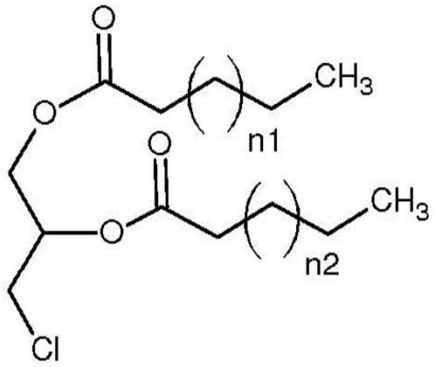
Dubois, AOCS, 2011

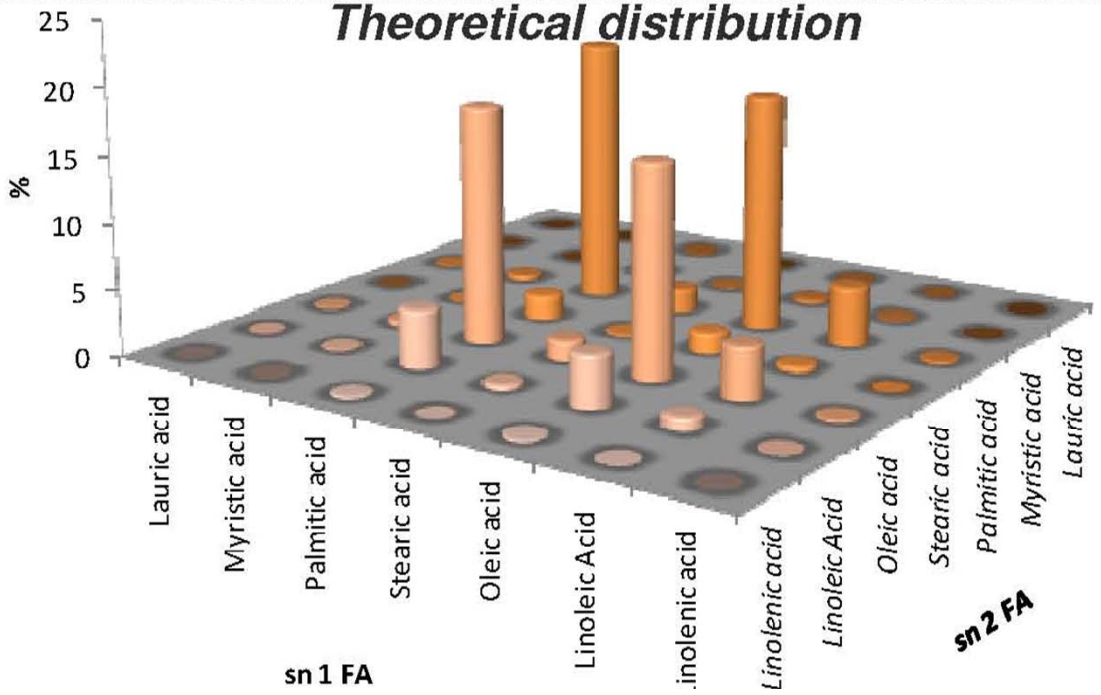
Theoretical example of palm oil:



- 3-MCPD-di-esters chemical standards to cover all possibilities:

  49 standards → 100% 3-MCPD di-ester content (Theory)

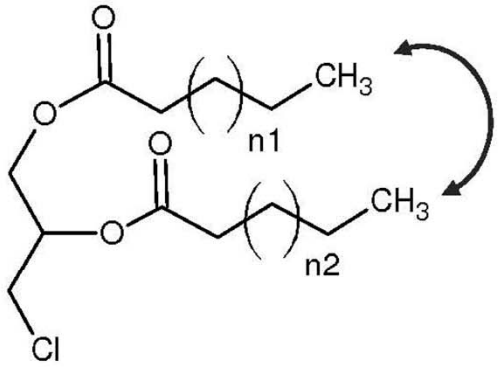


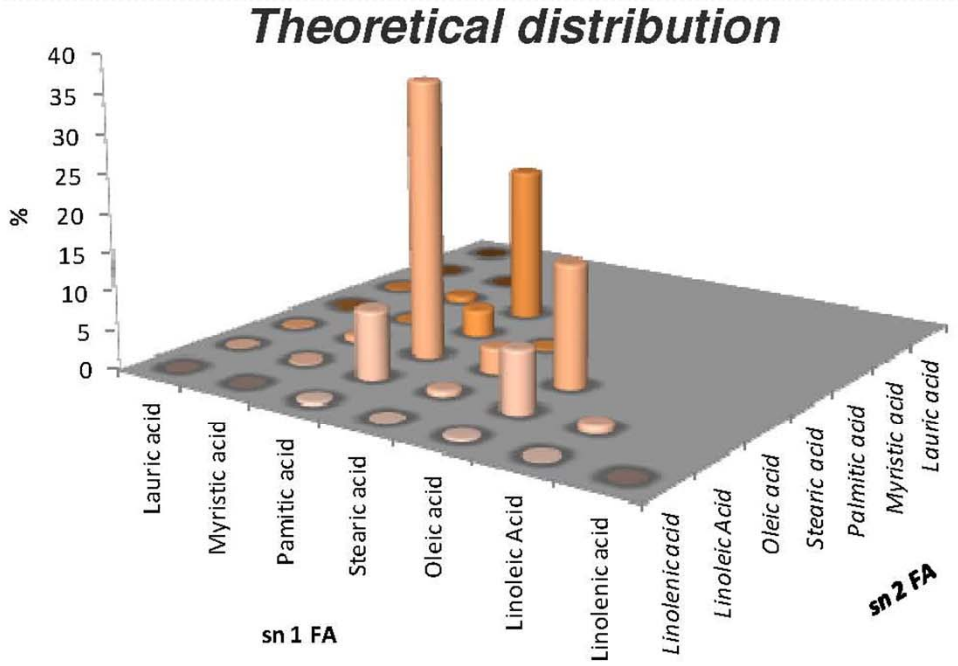


Dubois, AOCS, 2011

Theoretical example of palm oil:

- 3-MCPD-di-esters chemical standards to cover all possibilities:
  - 49 standards → 100% 3-MCPD di-ester content (Theory)
  - **28 standards → 100% 3-MCPD di-ester content (Experimental)**



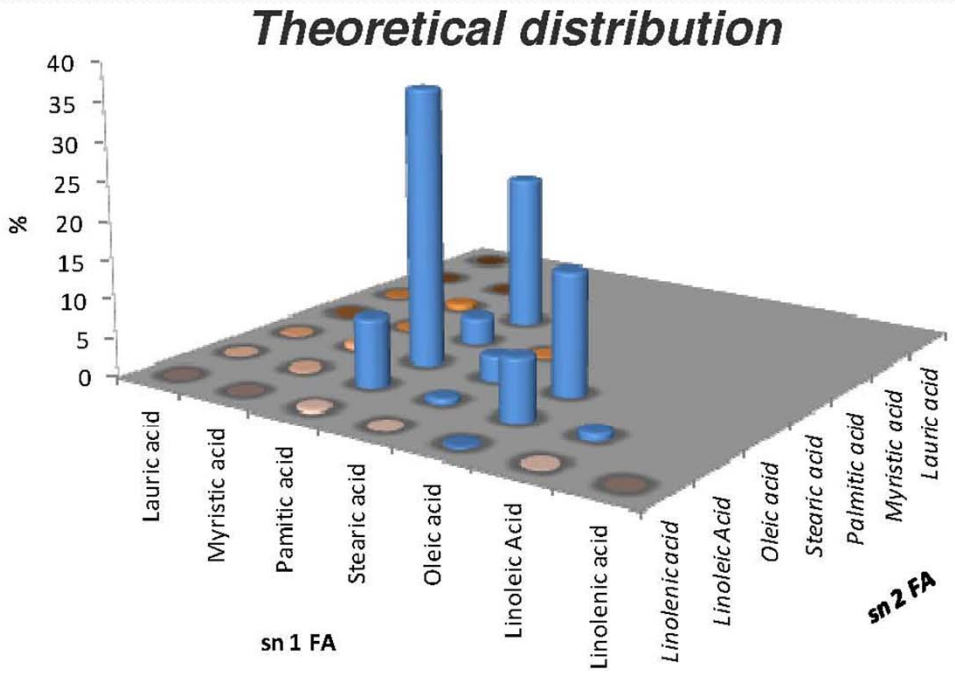


Dubois, AOCS, 2011

Theoretical example of palm oil:

- 3-MCPD-di-esters chemical standards to cover all possibilities:
  - 49 standards → 100% 3-MCPD di-ester content (Theory)
  - 28 standards → 100% 3-MCPD di-ester content (Experimental)
  - **10 standards → 98% 3-MCPD di-ester content (Pragmatic)**

Oil	3-MCPD di-esters coverage (10 standards included)
canola oil	93%
corn oil	98%
cotton seed Oil	96%
grape seed oil	99%
olive oil	95%
palm oil	98%
palm Olein	96%
safflower oil	99%
soybean oil	90%



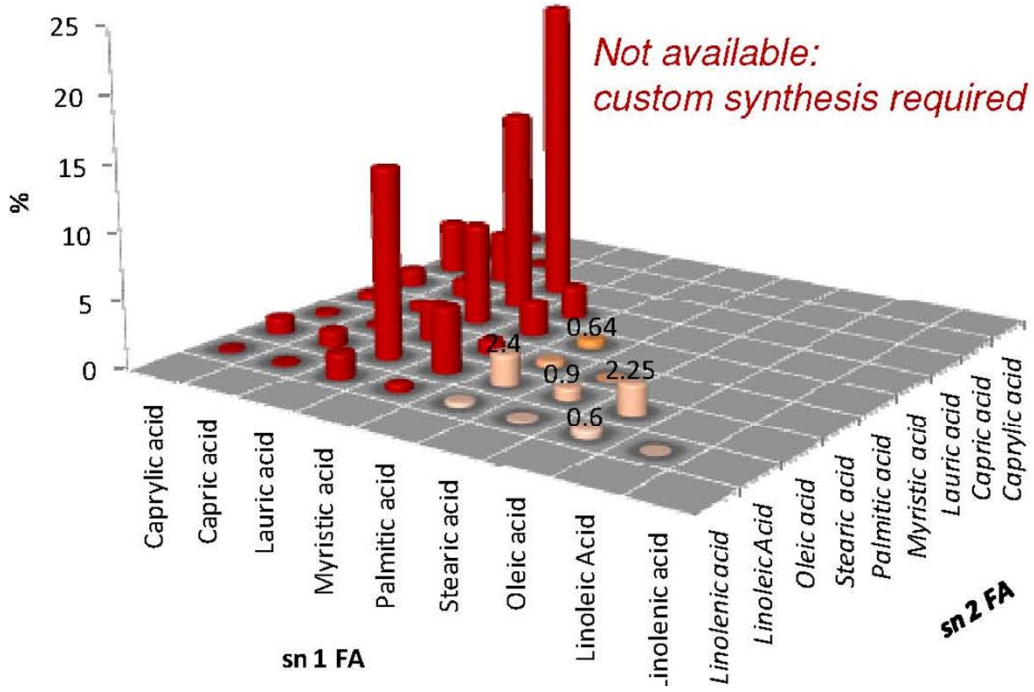
Dubois, AOCS, 2011

Issues with palm kernel and coconut oil

Current available standards do not fit with:

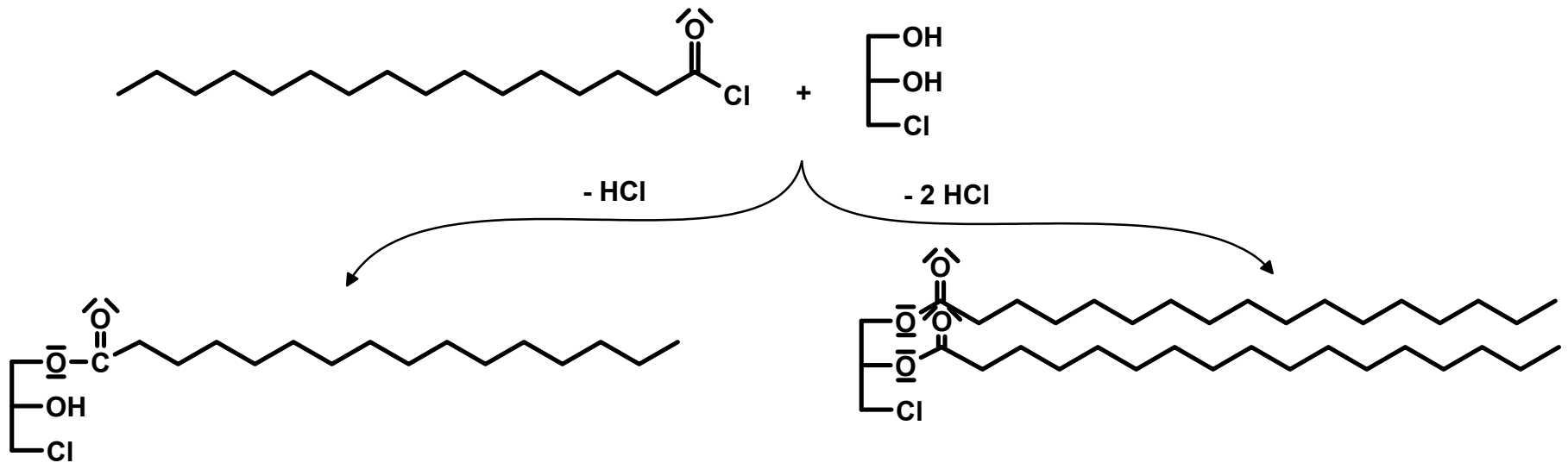
- Palm kernel oil → 7.8% 3-MCPD di-ester coverage
- Coconut oil → 3.9% 3-MCPD di-ester coverage

Theoretical distribution in palm kernel oil



Dubois, AOCS, 2011

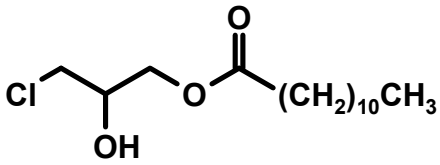
Synthesis of 3-MCPD-1-mono and 1,2-diesters



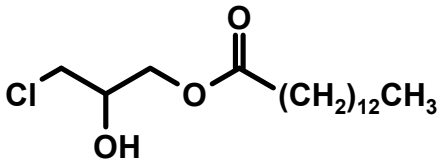
1-palmitoyl-3-chloropropandiol

1,2-dipalmitoyl-3-chloropropandiol

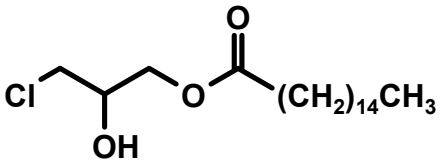
3-MCPD-1-monoesters



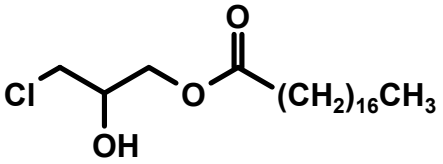
1-lauroyl-3-chloropropandiol



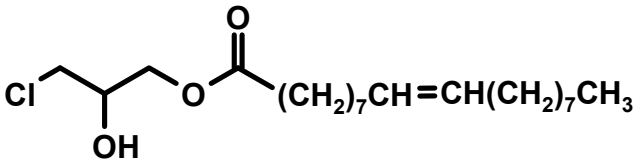
1-myristoyl-3-chloropropandiol



1-palmitoyl-3-chloropropandiol

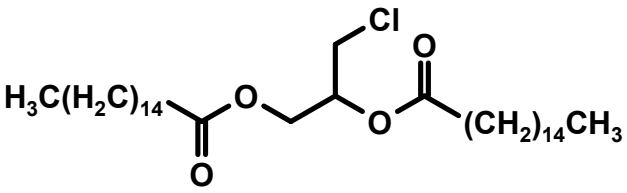


1-stearoyl-3-chloropropandiol

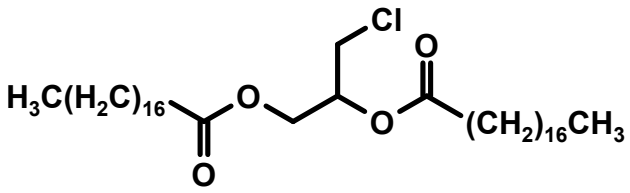


1-oleoyl-3-chloropropandiol

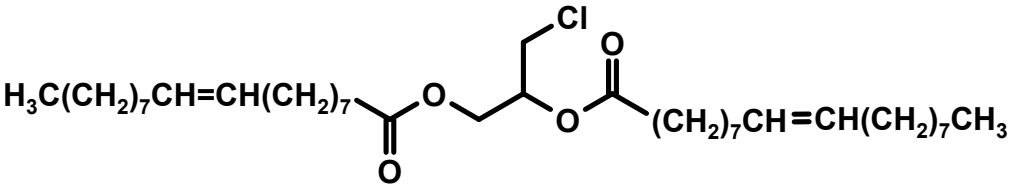
3-MCPD-1,2-diesters



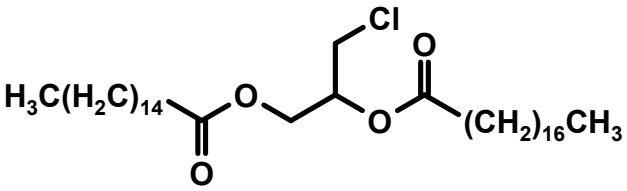
1,2-dipalmitoyl-3-chloropropandiol



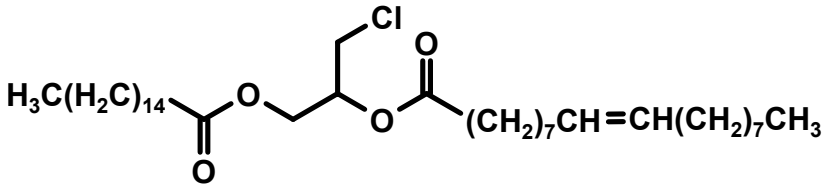
1,2-distearoyl-3-chloropropandiol



1,2-dioleoyl-3-chloropropandiol

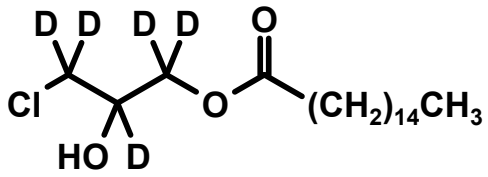


1-palmitoyl-2-stearoyl-3-chloropropandiol

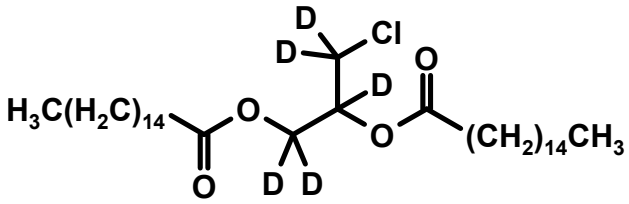


1-palmitoyl-2-oleoyl-3-chloropropandiol

Labeled standards (3-MCPD esters)

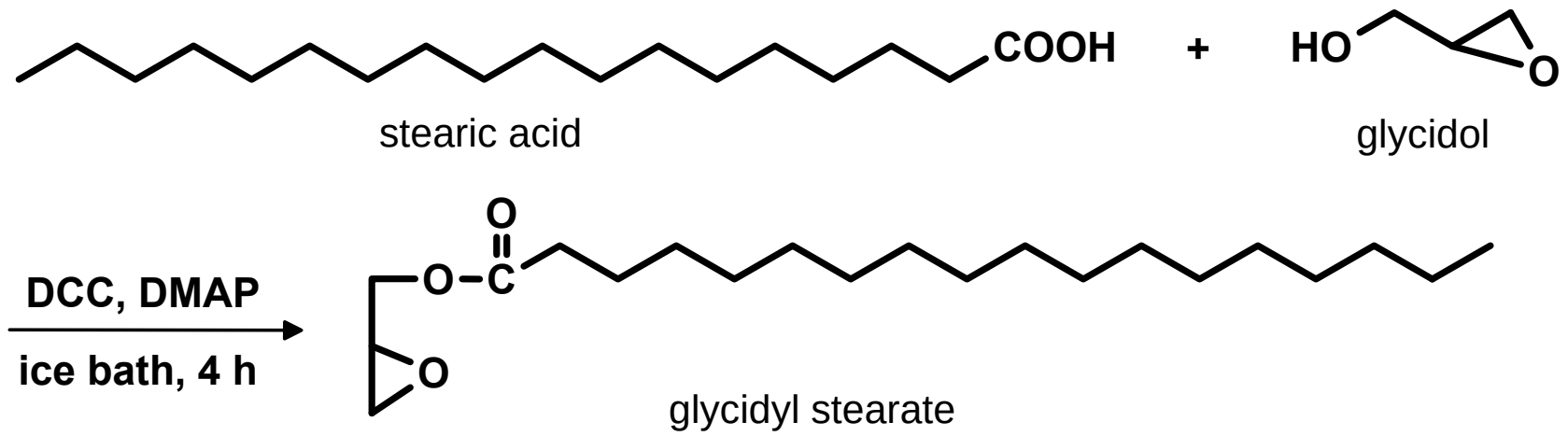


[$^2H_5$]-1-palmitoyl-3-chloropropandiol



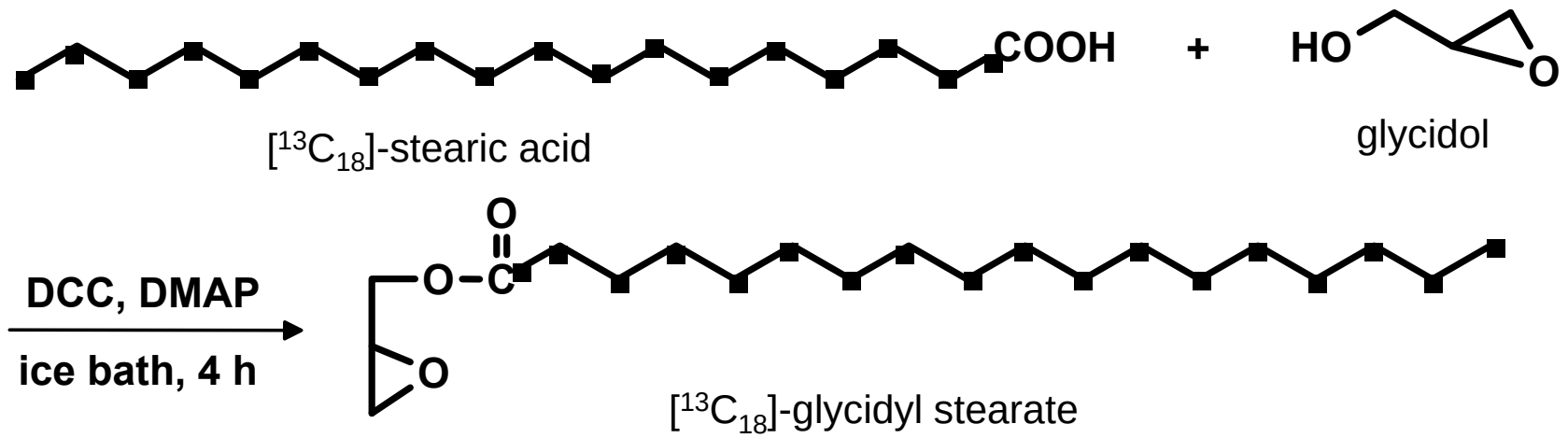
[$^2H_5$]-1,2-dipalmitoyl-3-chloropropandiol

Synthesis of glycidyl esters I



DCC: dicyclohexylcarbodiimide; DMAP: dimethylaminopyridine

Synthesis of glycidyl esters II



■: ^{13}C-labeling; DCC: dicyclohexylcarbodiimide; DMAP: dimethylamino-pyridine

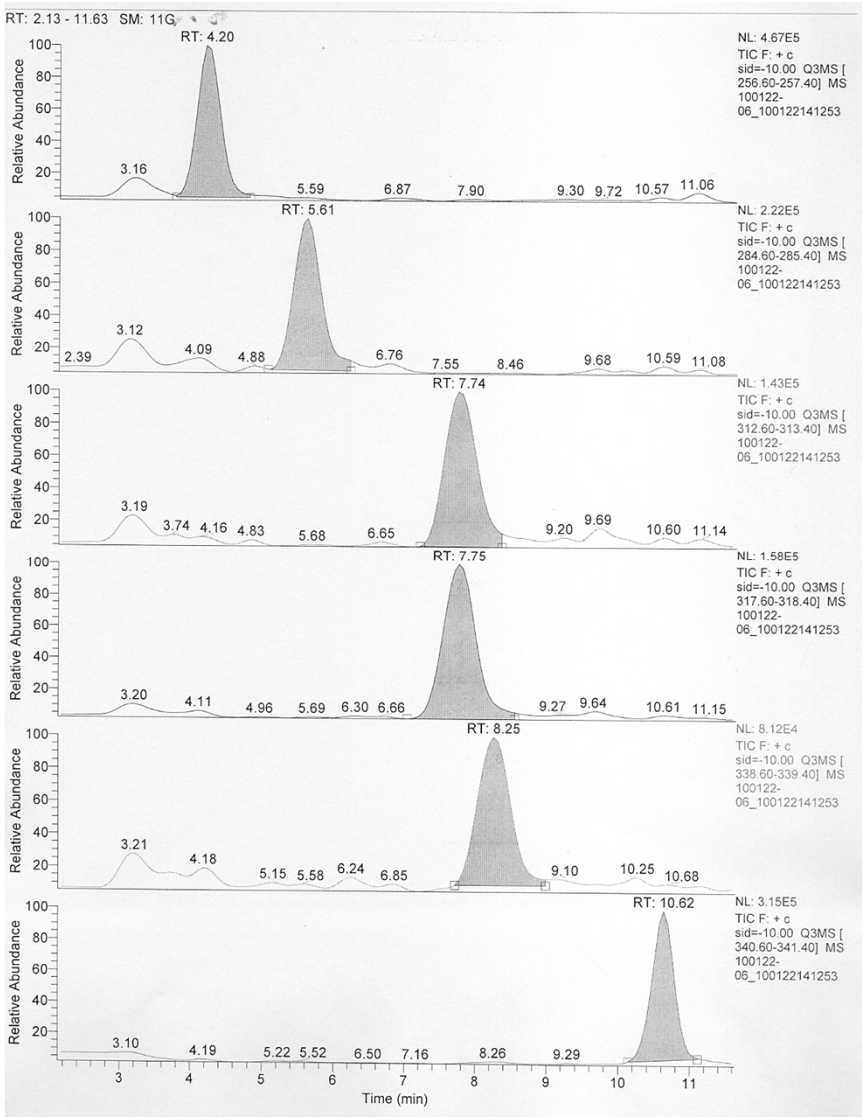
Synthesized glycidyl esters

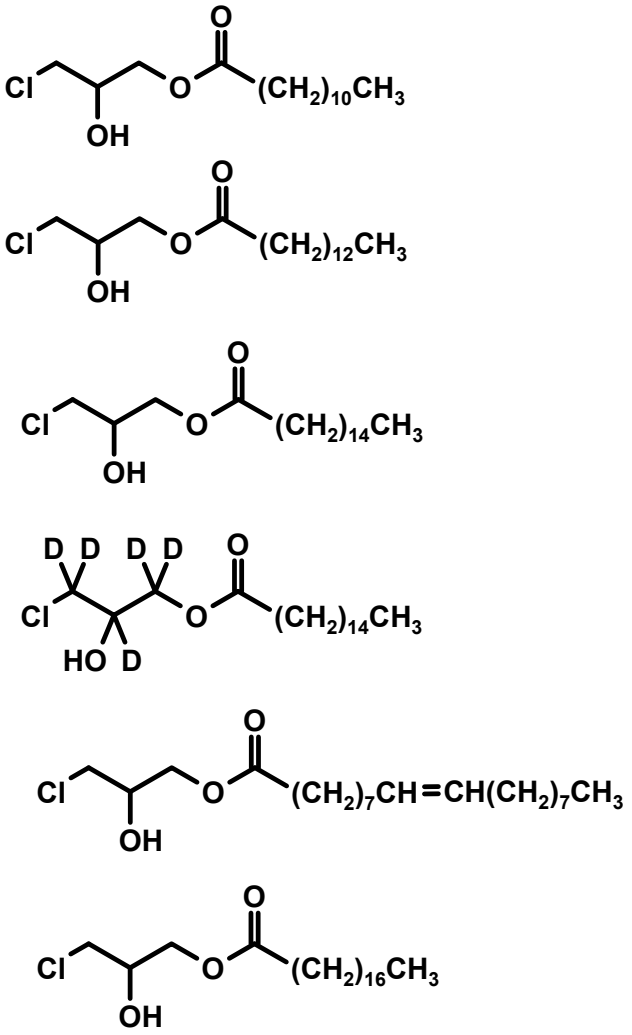
analyte	standard
glycidyl laurate	$[^{2}H_{3}]$-glycidyl laurate
glycidyl myristate	$[^{2}H_{5}]$-glycidyl myristate
glycidylpalmitate	$[^{13}C_{4}]$-glycidylpalmitate
glycidylstearate	$[^{13}C_{18}]$-glycidylstearate
glycidyloleate	$[^{13}C_{18}]$-glycidyloleate
glycidyllinolate	$[^{13}C_{18}]$-glycidyllinolate
glycidyllinolenat	$[^{13}C_{18}]$-glycidyllinolenate

LC-MS (APCI$^+$) I

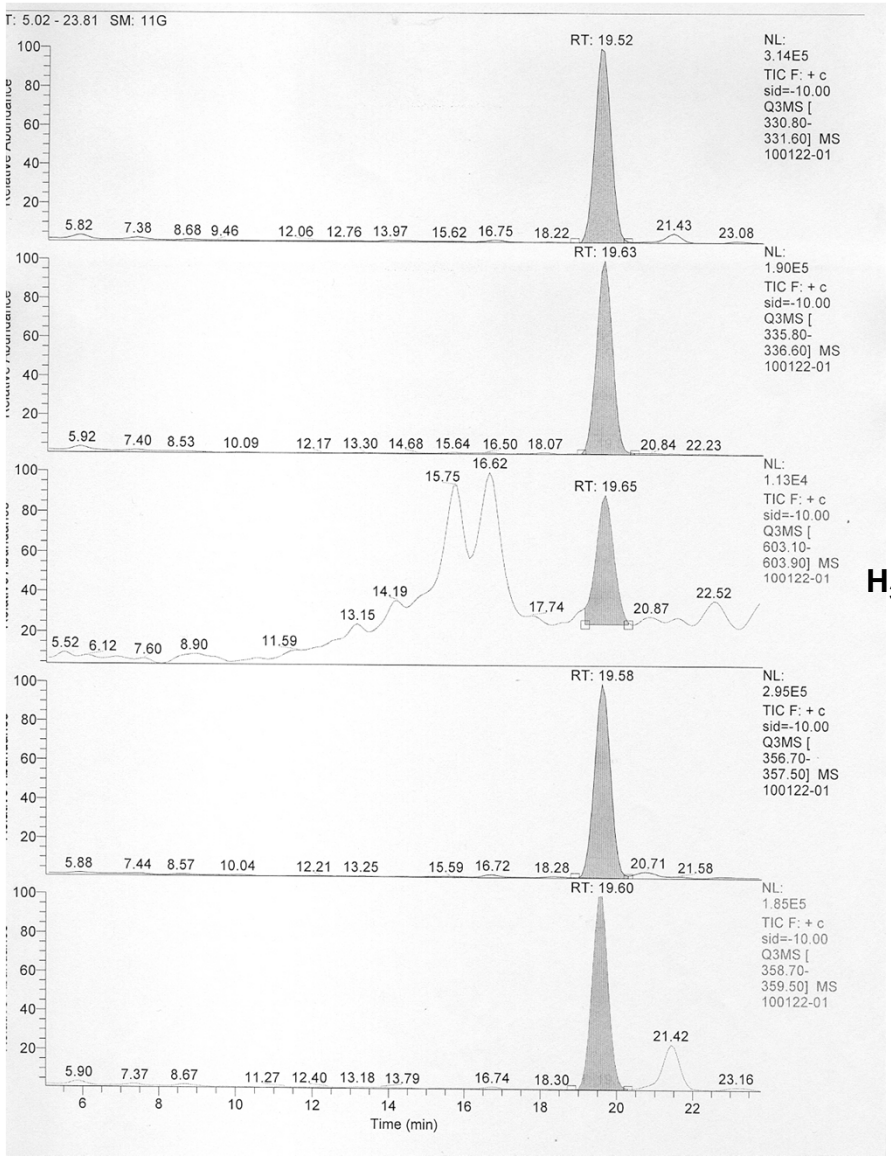
- **High pressure liquid chromatography**
- Surveyor chromatography system
- thermostated (20 °C) autosampler
- column: 150 × 2.0 mm i.d., 3 µm Luna 3u PFP(2) 100 Å (Phenomenex); kept at 20 °C; connected to a 4 × 2.0 mm i.d. precolumn
- solvent system: formic acid in water (0.1%, A) and formic acid in acetonitrile (0.1%, B)
- linear gradient: increasing the concentration of B from 80 to 90 % within 15 min, then within 2 min to 100 % B, afterwards further 20 min at 100 % B
- flow rate: 0.2 mL/min
- **Mass spectrometry**
- triple-quadrupole tandem mass spectrometer (TSQ Quantum Discovery)
- APCI$^+$ mode

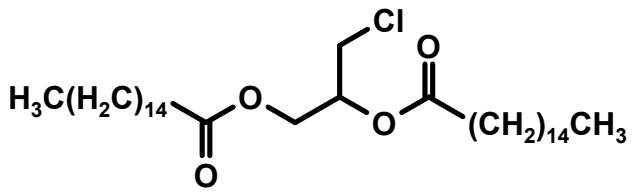
LC-MS (APCI$^+$) – 3-MCPD-1-monoesters

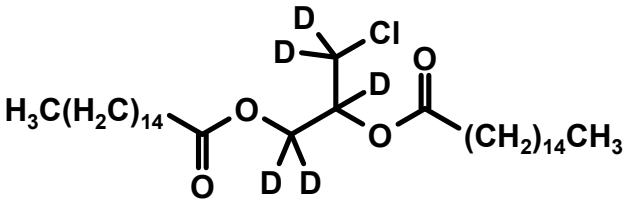


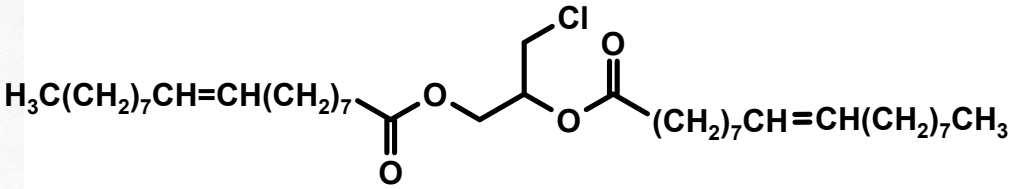


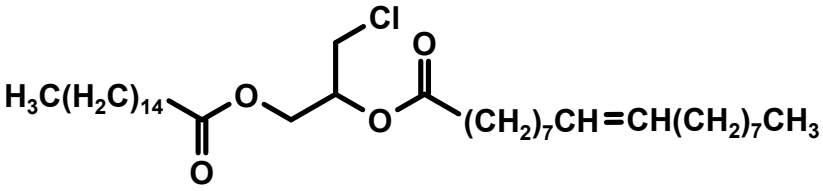
LC-MS (APCI$^+$) – 3-MCPD-1,2-diesters

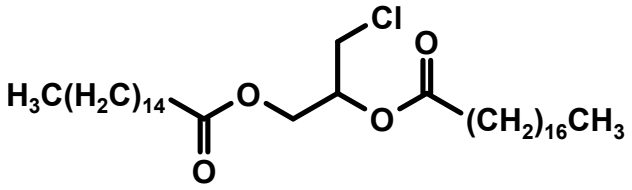




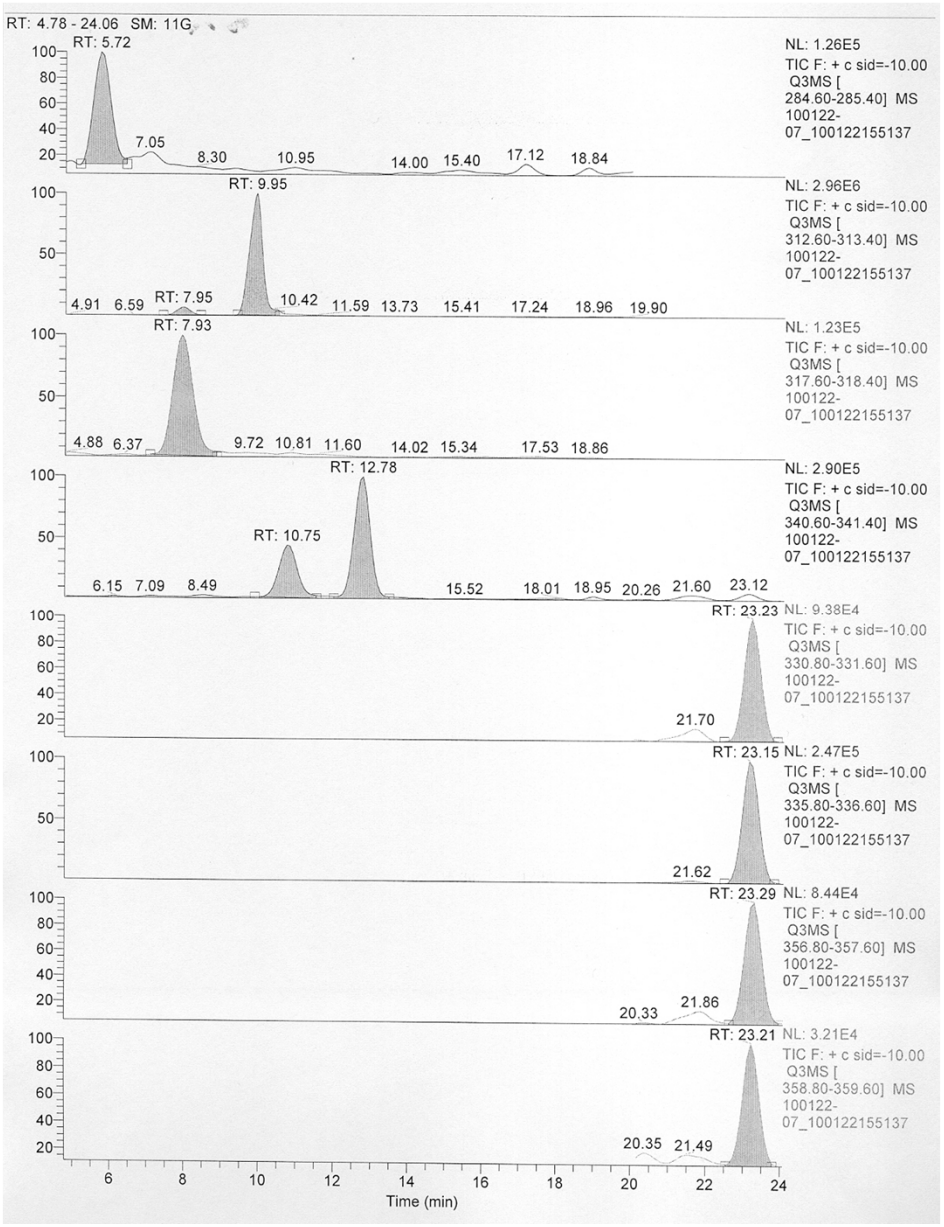


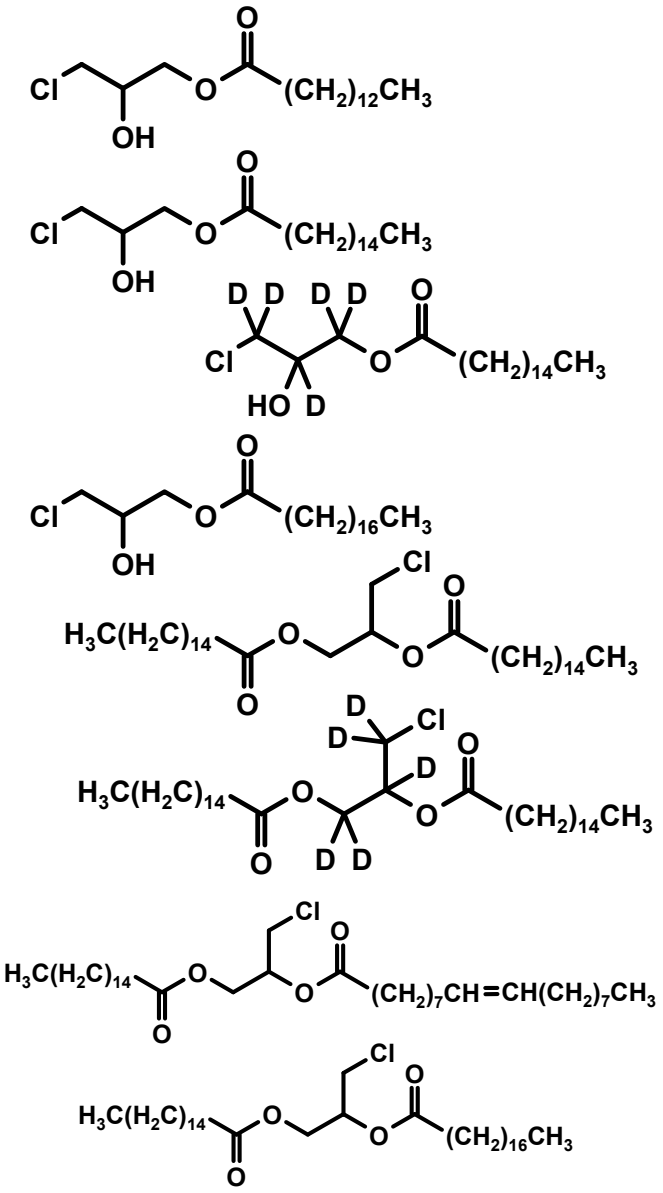




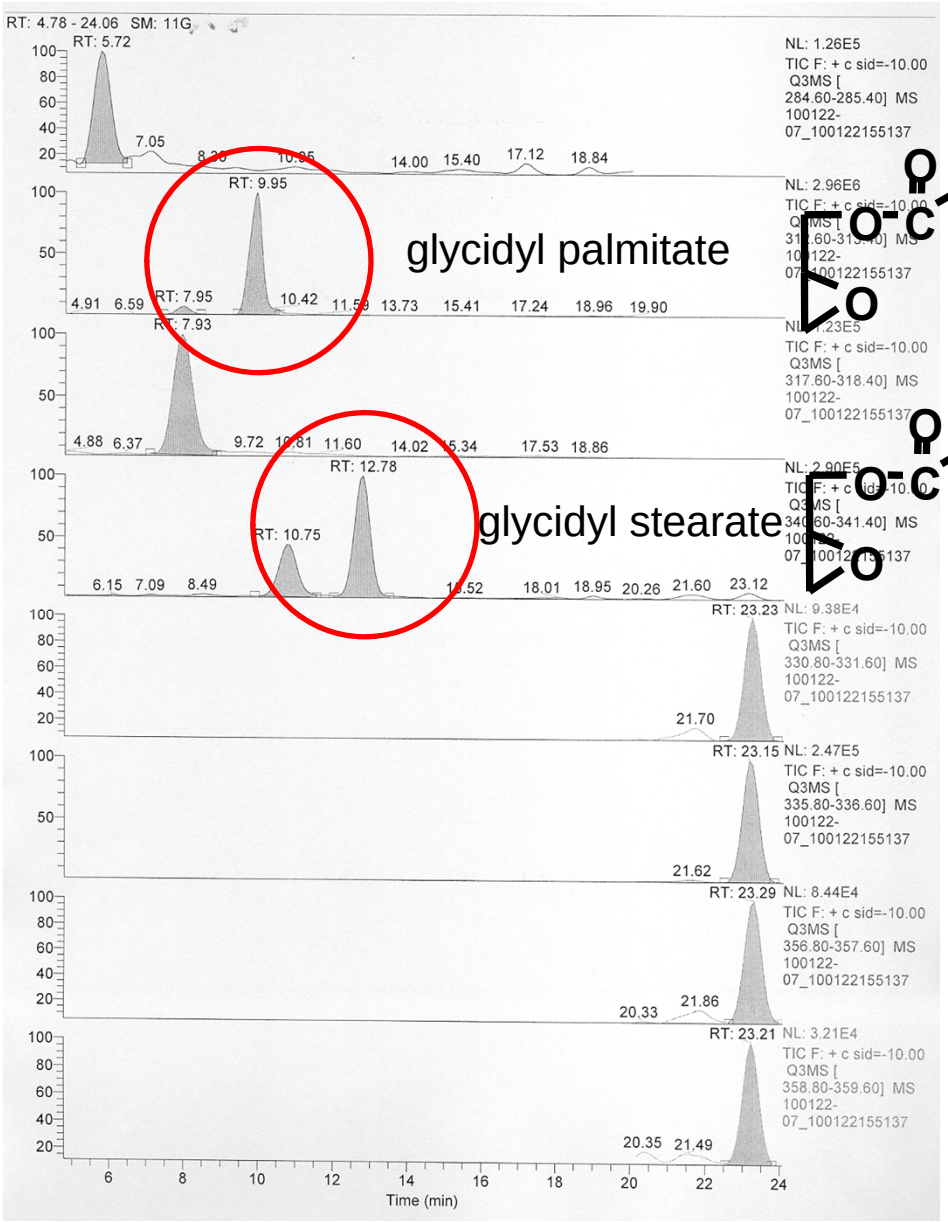


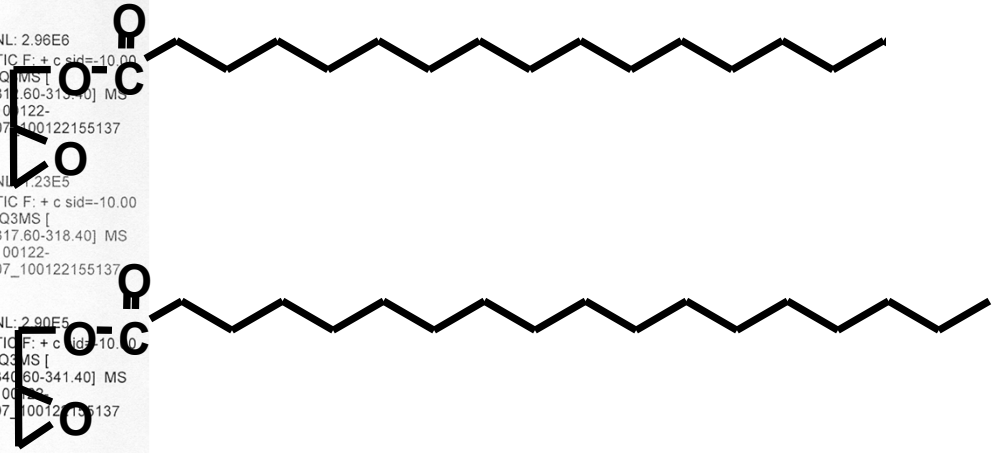
LC-MS (APCI$^+$) – 3-MCPD-1-mono and 1,2-diesters as well as glycidyl esters



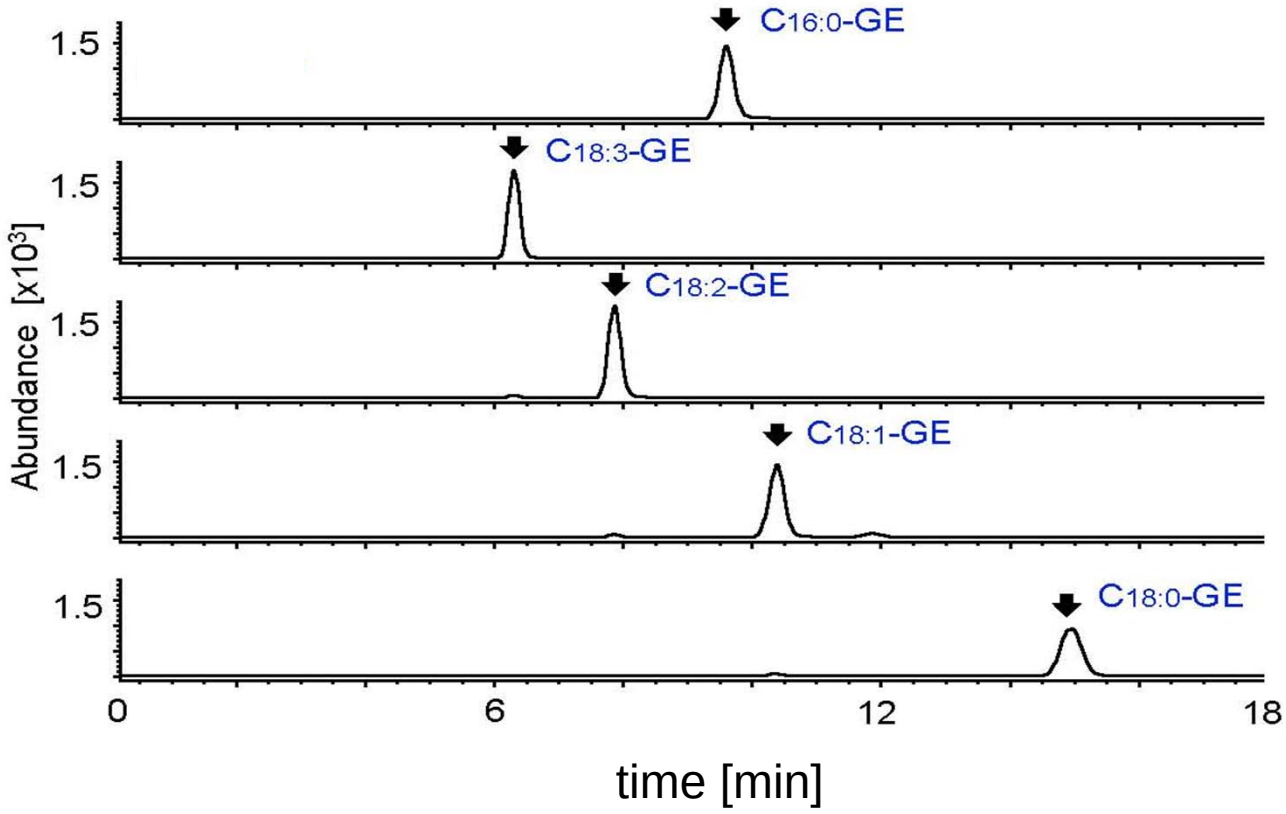


LC-MS (APCI$^+$) – 3-MCPD-1-mono and 1,2-diesters as well as glycidyl esters





LC-MS (APCI$^+$) – glycidyl esters



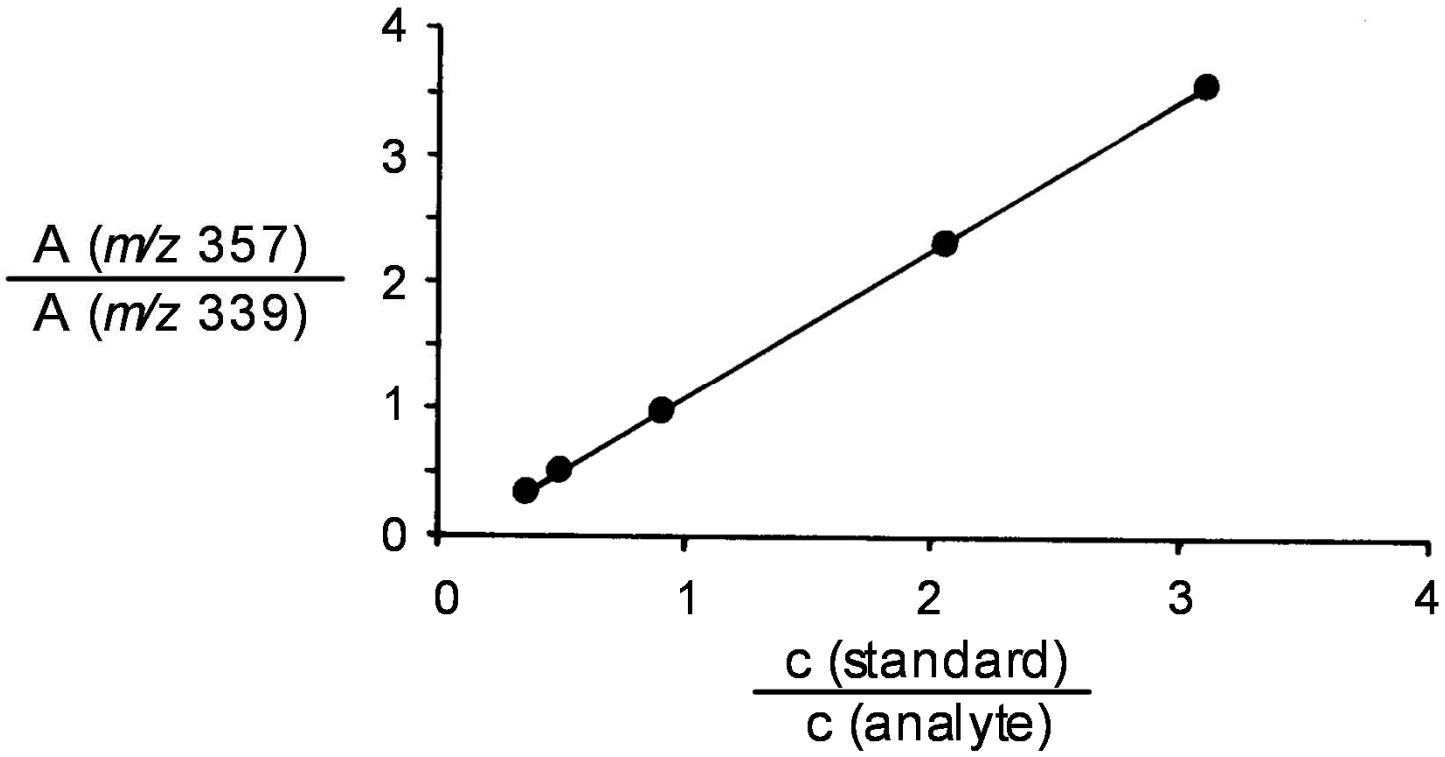
O I L S A M P L E S

Work-up procedure: glycidyl esters

- Samples (750 mg) are dissolved in pentane/diethyl ether (2 mL; 95/5, v/v)
- Addition of the labeled standards and equilibration (stirring for 15 min)
- Application onto a silica gel column (150 x 20 mm; 16 g of silica gel 60, 0.063-0.2 mm; containing 7 % of water)
- Stepwise elution with pentane/diethyl ether (250 mL; 95/5, v/v):
  - Fraction 1: 150 mL waste
  - Fraction 2: **100 mL** **glycidyl esters**
- Evaporation of the solvent (vacuum; 30 C) and solving of the residue in acetonitrile (1.5 mL)
- An aliquot (10 µL) of this solution was used for LC-MS

LC-MS (APCI$^+$) - Calibration curve

Calibration curve based on 5 mixtures with known concentrations of analyte and labeled standard in a ratio of 5+1 to 1+5 (glycidyl oleate)

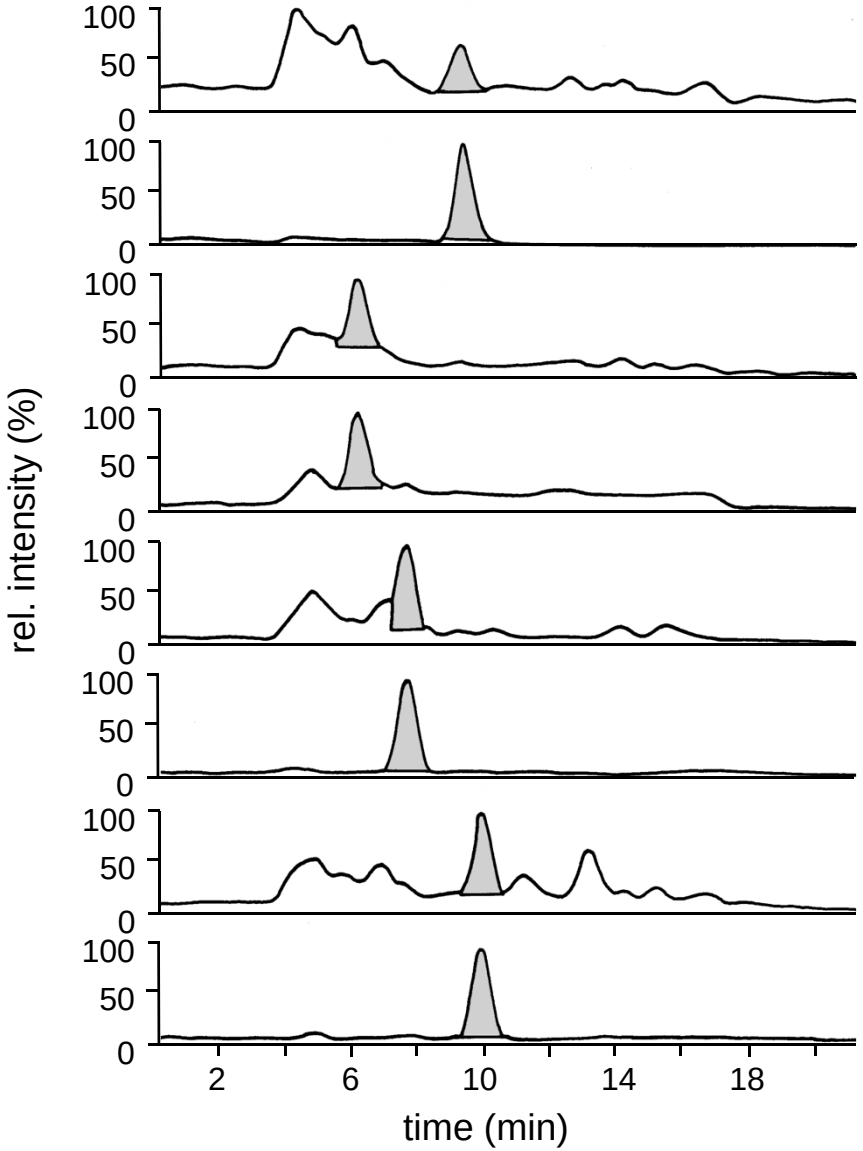


Commercial oil samples – glycidyl esters

oil	glycidyl ester concentration [mg/kg] of						
	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
sunflower 1	n.d.[b]	n.d.[b]	0.05	0.03	0.34	1.68	n.d.[b]
sunflower 2	n.d.[b]	n.d.[b]	0.08	n.d.[b]	0.31	1.27	n.d.[b]
sunflower 3	n.d.[b]	n.d.[b]	0.04	n.d.[b]	0.29	1.11	n.d.[b]
sunflower 1[a]	n.d.[b]	n.d.[b]	6.3	4.0	26.7	63.0	n.d.[b]
rapeseed 1	n.d.[b]	n.d.[b]	0.03	n.d.[b]	0.11	0.10	0.05
rapeseed 2	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	0.17	0.15	0.04
rapeseed 3	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	0.15	0.05	n.d.[b]
rapeseed 3[a]	n.d.[b]	n.d.[b]	4.7	1.6	65.0	19.8	8.9

[a]: fatty acid composition [%]. [b]: not detectable.

Rapeseed oil



glycidyl palmitate

[$^{13}C_4$]-glycidyl palmitate

glycidyl linolenate

[$^{13}C_{18}$]-glycidyl linolenate

glycidyl linolate

[$^{13}C_{18}$]-glycidyl linolate

glycidyl oleate

[$^{13}C_{18}$]-glycidyl oleate

Comparison of analytical methods

Glycidol	M = 74	factor
Glycidyl laureate	M = 256	3.46
Glycidyl myristate	M = 284	3.84
Glycidyl palmitate	M = 312	4.22
Glycidyl stearate	M = 340	4.59
Glycidyl oleate	M = 338	4.57
Glycidyl linolate	M = 336	4.54
Glycidyl linolenate	M = 334	4.51

Oil samples – glycidyl esters

oil	glycidyl ester concentration [mg/kg] of						
	C16:0	C18:0	C18:1	C18:2	C18:3	sum	corr.
avocado1	0.90	0.08	4.78	1.93	0.09	7.78	1.72
avocado2	0.67	0.04	2.78	1.03	0.12	4.64	1.03
avocado3	0.64	0.06	4.21	1.53	0.06	6.50	1.43
avocado4	0.05	0.03	1.79	0.58	0.03	2.48	0.54
avocado1-4[a]	16.7	0.9	62.5	12.6	0.4		
olive1	0.34	0.13	2.10	0.79	0.04	3.40	0.75
olive2	0.62	0.32	2.41	0.68	n.d.[b]	4.05	0.89
olive1/2[a]	12.5	2.7	71.0	11.6	0.66		
olive3	0.11	0.07	1.04	0.18	0.03	1.43	0.31
olive3[a]	10.5	3.4	78.0	6.6	0.54		

[a]: fatty acid composition [%]. [b]: not detectable.

Oil samples – glycidyl esters

oil	glycidyl ester concentration [mg/kg] of						
	C16:0	C18:0	C18:1	C18:2	C18:3	sum	corr.
soy bean	0.03	n.d.[b]	0.29	0.14	0.03	0.49	0.11
soy bean[a]	4.7	1.6	61.7	20.2	11.0		
palm	2.82	0.39	8.75	4.61	n.d.[b]	16.6	3.68
palm[a]	36.5	3.9	45.1	12.4	n.d.[b]		
oil rich of TG	5.10	0.50	15.4	10.2	0.80	32.0	7.12
oil rich of TG[a]	17.4	1.8	42.8	36.0	1.0		
oil rich of DG	5.34	0.41	111.4	235.2	27.2	380	83.9
oil rich of DG[a]	2.8	1.1	37.7	50.0	8.3		

[a]: fatty acid composition [%]. [b]: not detectable.

LC-MS vs LC-MS-MS

LC-MS: APCI$^+$ mode

TSQ Quantum Discovery; triple-quadrupole tandem mass spectrometer (Thermo Scientific)

LC-MS-MS: ESI$^+$ mode

Sciex 4000; QTRAP (Applied Biosystems)

Comparison of the results for glycidyl oleate:

LC-MS: 7.22 mg/kg

LC-MS-MS:
m/z 339-135 (analyte) and *m/z* 357-145 (standard): 6.93 mg/kg
m/z 339-121 (analyte) and *m/z* 357-130 (standard): 7.08 mg/kg

Comparison of the newly developed method with existing sum (indirect) methods

sample	concentration of glycidyl esters as sum [mg/kg]		
	DFA	Weisshaar[1]	Kuhlmann (“3 in 1“)
avocado oil A	1.38	1.49	1.46
avocado oil B	0.98	n.d.	0.97
sunflower oil	0.35	n.d.	0.43
palm oil	3.63	2.92	3.32
oil (rich of TG)	6.01	5.6	7.99
oil A (rich of DG)	70.3	68.5	70.9
oil B (rich of DG)	2.40	0.43	2.00

[1]: concentration B is sometimes too high resulting in too high amounts of 3-MCPD esters and in too low amounts of glycidyl esters (concentration A – concentration B)!

DGF Standard method C-III 18 (09) withdrawn

Comparison of the newly developed method with existing direct and indirect methods II

palm oils	concentration of glycidyl esters as sum [mg/kg]		
	DFA	Nestlé (direct)	Kuhlmann (“3 in 1“)
1	< LoQ[1]	< LoQ[1]	< LoQ[1]
2	0.01	0.067	< LoQ[1]
3	0.01	0.022	< LoQ[1]
4	0.53	0.44	0.68
5	0.83	0.81	1.05
6	1.02	1.03	1.44
7	2.54	2.14	2.66
8	3.39	2.98	3.45
9	4.13	3.26	4.10
10	4.12	3.53	4.32

[1]: Limit of quantitation.

Take home messages...

- synthesis of the most important glycidyl esters as reference compounds and as the corresponding stable isotopically labeled internal standard
- development of stable isotope dilution assays for the direct quantitation of each single glycidyl ester
- application of the newly developed method on various edible fats and oils
- good accordance of the data obtained by the newly developed method with an actual as reliable rated indirect sum method (SGS method) as well as with another actual as reliable rated direct method (Nestlé method)
- concentrations of glycidyl esters are correlated to the fatty acid composition
- concentrations of glycidyl esters varying within one type of oil – dependence on the refining process, the growing conditions or genera

Outlook – available standards

- glycidyl esters: 7 analytes and 7 standards
  (but only two are 13C-labeled)
- 3-MCPD-1-monoester: 7 analytes and 4 standards
- 3-MCPD-2-monoester: 7 analytes and 7 standards
- 3-MCPD-1,2-diester: 14 analytes and 12 standards
- 2-MCPD-monoester: 3 analytes and 3 standards
- 2-MCPD-1,3-diester: 4 analytes and 3 standards

Many thanks for the financial support!

DFA Lebensmittelchemie

Bundesministerium für Wirtschaft und Technologie

WIRTSCHAFT.
WACHSTUM.
WOHLSTAND.

FEI

FORSCHUNGSKREIS DER ERNÄHRUNGSINDUSTRIE E.V.

AIF

ALLIANZ INDUSTRIE FORSCHUNG