AOAC MIDYEAR MEETING

AOAC INTERNATIONAL 7th Annual Midyear Meeting, March 13-17, 2017

AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN)

Current state of MCPD and glycidol analysis in infant formulae and related foods

J. Kuhlmann

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2. Matrix

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b. Regulations

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2. Expected concentration for identity methods

Proposed Fitness for Purpose

I a 1. Background and History

History

Free 3-MCPD *(beside 2-MCPD, 1,3-DCP & 2,3-DCP) has been discovered as food contaminant in the 1970s.*

*The occurrence of **2- & 3-MCPD esters and glycidyl esters** in oils, fats and oil/fat containing foods is a newer topic discovered between 2004 and 2011:*

Svejkovska B. et al.: Esters of 3-Chloropropane-1,2-diol in Foodstuffs; *Czech J. Food Sci.* 22 (5), **2004**, 190-196

Divinova V. et al.: Determination of Free and Bound 3-Chloropropane-1,2-diol by Gas Chromatography with Mass Spectrometric Detection using Deuterated 3-Chloropropane-1,2-diol as Internal Standard; *Czech J. Food Sci.* 22 (5), **2004**, 182-189

Zelinkova Z., et al.: Fatty esters of 3-chloropropane-1,2-diol in edible oils. *Food Addit. Contam*., **2006**, 23, 1290-1298

Ilsi Europe Report Series: Summary Report of a Workshop held in February **2009**, Brussels, Belgium: “Dr. Seefelder reported on studies to investigate the formation of 2-MCPD during the deodorisation step of oils.”

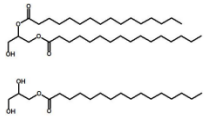
Weisshaar R., Perz R.:Fatty acid esters of glycidol in refined fats and oils; *Eur. J. Lipid. Sci. Technol*. 112, **2010**, 158-165

Kuhlmann J.: Determination of bound 2,3-epoxy-1-propanol (glycidol) and bound monochloropropanediol (MCPD) in refined oils; *Eur. J. Lipid. Sci. Technol*. 113, **2011**, 335-344

I a 1. Background and History

Background: what is 3-MCPD- & glycidyl esters in edible oils & fats?

Naturally occurring minor components e.g.:



Mono- & Diacylglycerides

Phospholipids

Free fatty acids

Colour compounds

Phytosterols

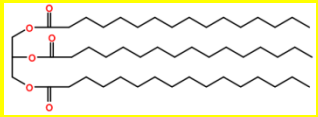
Trace elements

Volatiles

Mycotoxins

Major components:

Triacylglycerides



Contaminating minor components e.g.:

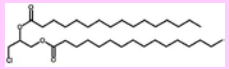
Pesticide residues

PAHs, Dioxins

Plasticisers

Mineral oils (MOSH/MOAH)

3-MCPD ester, 2-MCPD ester, glycidyl ester

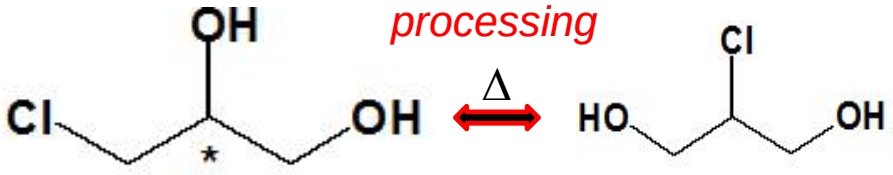


Heat-induced processing contaminants / Food-bourne contaminants

I a 2. Chemical Structures

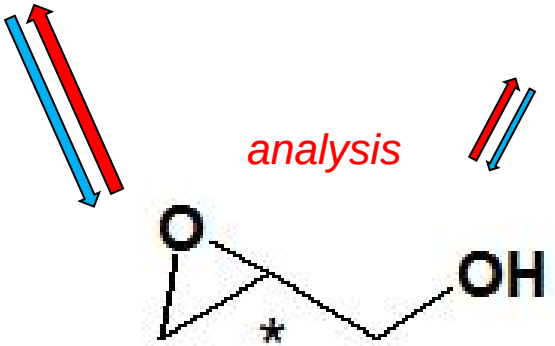
Chemical structures of the analytes

Free analytes



3-MCPD
3-*mono*-chloropropane-1,2-diol
3-Chloropropane-1,2-diol

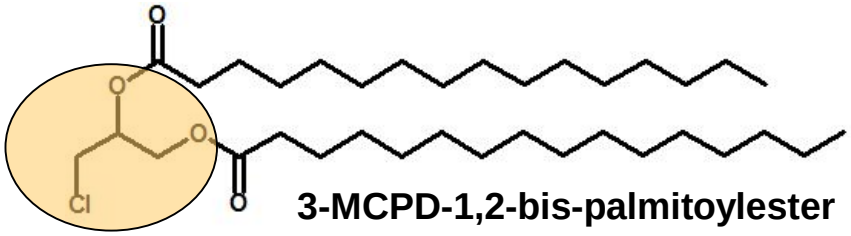
2-MCPD
2-*mono*-chloropropane-1,3-diol
2-Chloropropane-1,3-diol



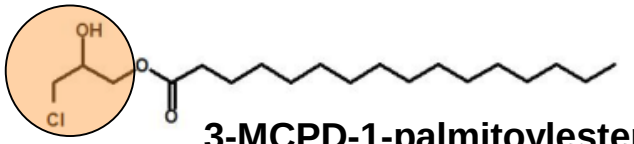
Glycidol
(2,3-Epoxi-1-propanol)

(Fatty acid) Bound analytes

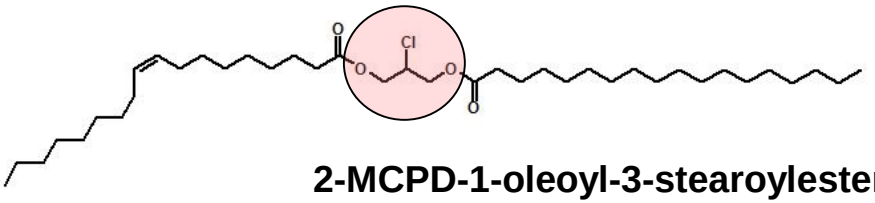
just examples, all fatty acids of an oil/fat might be present



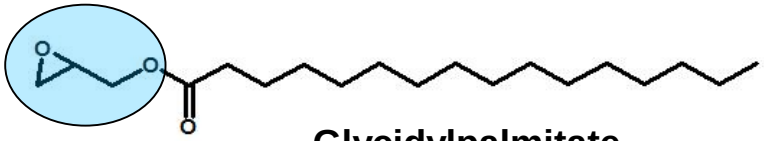
3-MCPD-1,2-bis-palmitoylester



3-MCPD-1-palmitoylester



2-MCPD-1-oleoyl-3-stearoylester

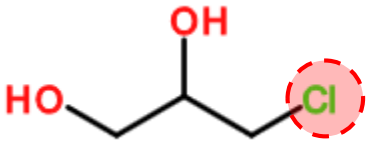


Glycidylpalmitate

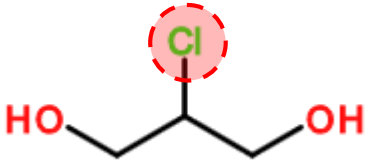
I a 3. Why it is an issue

Potential hazards of free 2-MCPD, 3-MCPD & glycidol

Toxicity of free 3-MCPD or glycidol is related to a **chloride** or an **epoxy group** at the molecular backbone.



3-MCPD



2-MCPD

Glycidol

glycidol: ***probably carcinogenic to humans 2A*** [1] **(genotoxic)**

3-MCPD: ***possibly carcinogenic to humans 2B*** [2]

2-MCPD: *No official classification available*

In regard to risk assessment (and product quality) **glycidol is the more problematic compound**!

No **MRL** or **TDI** applies; risk estimation is based on **MOE**!

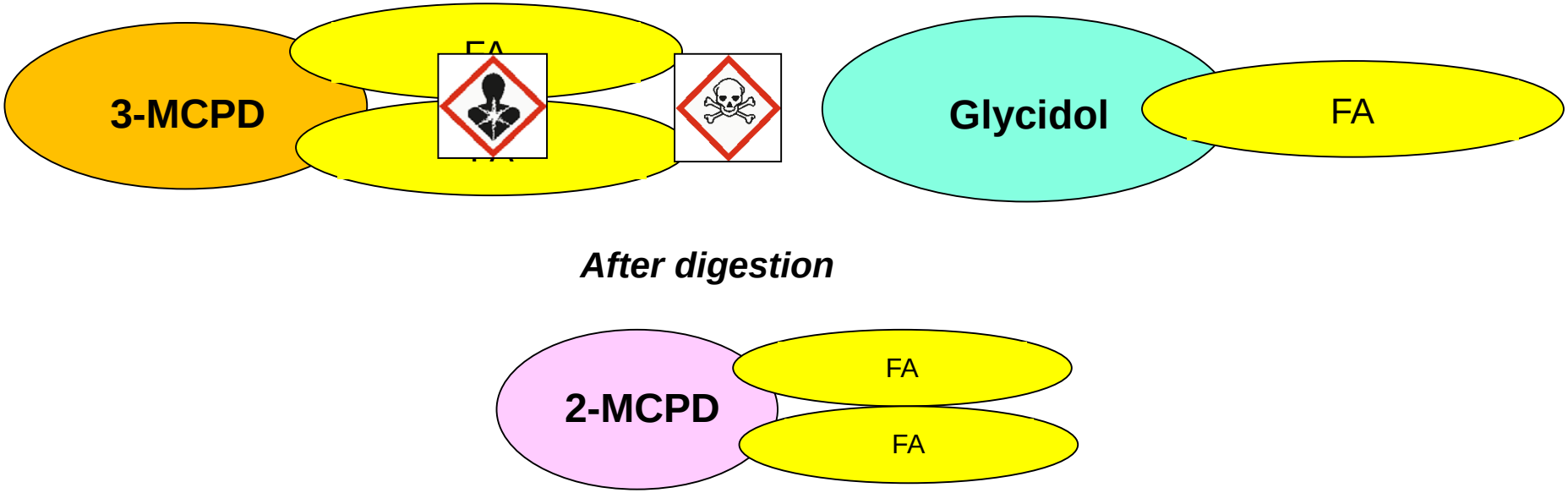
(Maximum Residue Limit) (Tolerable Daily Intake) (Margin of Exposure)

Consumers intake should be “as-low-as-reasonably-achievable” **ALARA**

[1] Opinion N° 007/2009, BfR, March, 10th, 2009, [2] Opinion N° 006/2013, BfR, April, 3rd, 2012

Potential hazards of ester-bound MCPD & glycidol

Do fatty acid esters of MCPD and glycidol are estimated to show toxic properties similar to the free anylates ?
What happens after intake?

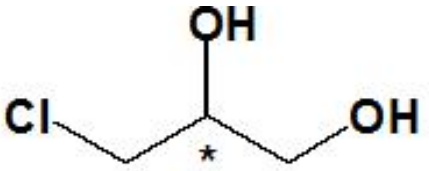


During digestion the toxicologically relevant core components are released by ester hydrolysis!

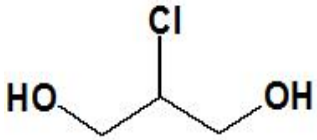
I c. Analytical challenges specific to: Analyte

What is the challenge?

Free analytes



3-MCPD

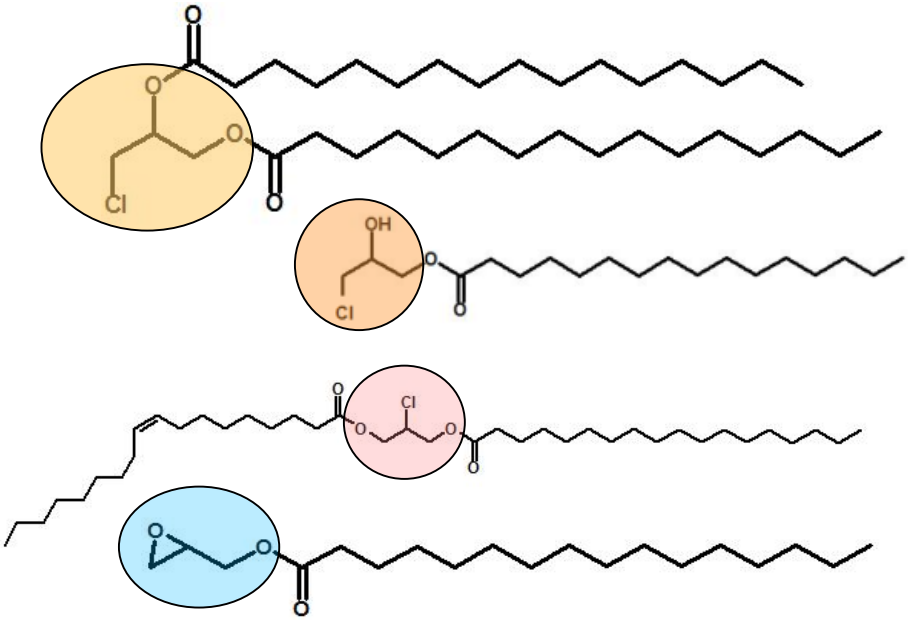


2-MCPD

Clear target structures!

(Fatty acid) Bound analytes

just examples, all fatty acids of an oil/fat might be present

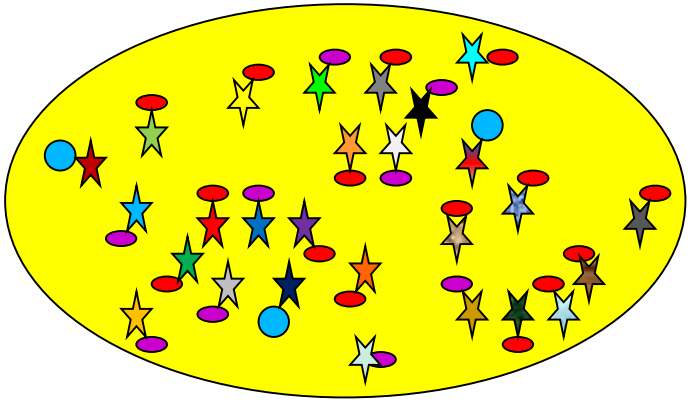


How do you receive sensitive and quantitative data about the core structures of an unknown and complex mixture of derivatives?

I b 1. Description of analytical methods

Direct analysis of the bound analytes: determination of the single original esters

- **glycidol**
- **3-MCPD**
- **2-MCPD**
- **Fatty acid(s)**



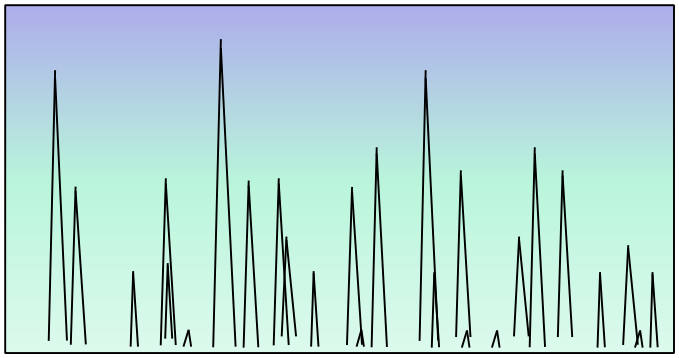
Hypothetic oil

Contains only 3 relevant fatty acids

This yields up to 27 analytes

3 Glycidyl ester
9-MCPD mono-ester
15 MCPD di-ester

Matrix removal in the majority of applications



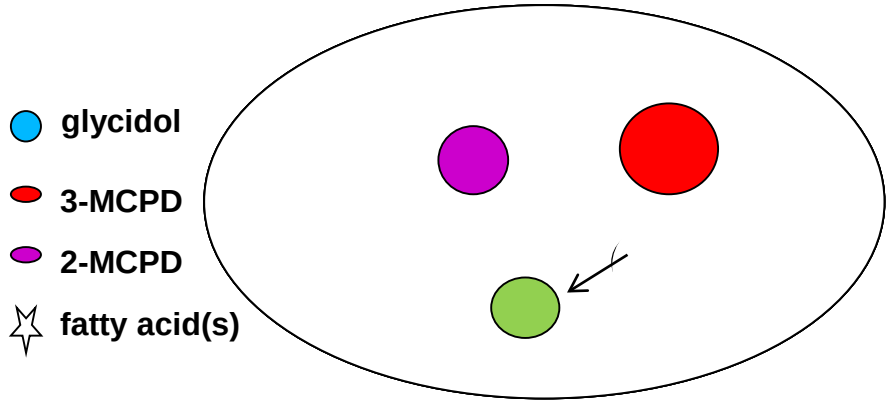
Chromatogram displays up to 27 analytes!

LC-MS / **LC-MS²** / LC-MS-TOF

Direct analysis – indirect quantification:
From every detected ester the amount of core analyte is calculated via molecular weights. Then the single 2-MCPD-, 3-MCPD- and glycidol contents are added up.

I b 1. Description of analytical methods

Indirect analysis of free and bound analytes: determination of the released core components



ester cleavage (***alkaline***, ***acidic***, ***enzymatic***)

↓

matrix clean-up *(e.g. l/l-extraction)*

↓

glycidol conversion *(into MXPD)*

↓

Derivatisation*
(e.g. HFBA/acetone/PBA)

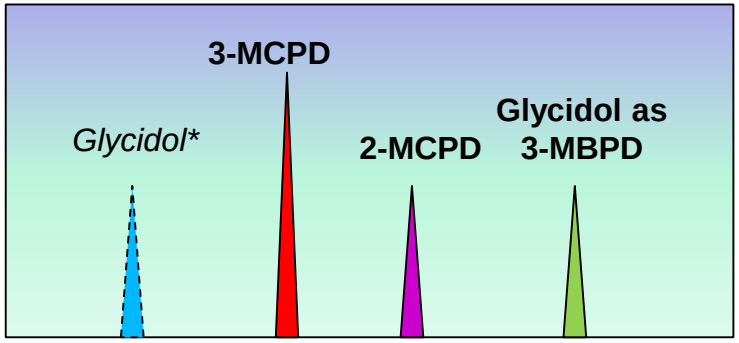
↓

GC-MS

Hypothetic oil

Contains only 3 relevant fatty acids

Indirect analysis – direct quantification:
The target analytes can be quantified directly via internal standards



I b 1. Description of analytical methods

Some of the analytical approaches available for the bound analytes in oils and fats.

Indirect determination
(ester cleavage releases the 3 core analytes, GC-MS)

alkaline

Early **DGF C-III 18 (09)**
Σ 3-MCPD + glycidol
DGF C-VI 17 (10); fast

Late **DGF C-III 18 (09) A,B**
A: Σ 3-M + g, B: 3-MCPD
Withdrawn by DGF

BfR method 09
3-MCPD
fast

DGF C-VI 18 (10) A, B
A: Σ 3-M + g, B: 3-MCPD
AOCS Cd 29c-13; fast

Küsters et al. 2010
3-MCPD, Glycidol
fast

SGS **"3-in-1"** Kuhlmann 2011
3-MCPD, 2-MCPD, Glycidol
AOCS Cd 29b-13; slow

acidic

Divinova et al. 2004
Zelinkova et al. 2006
3-MCPD; slow

BfR method 08
3-MCPD
slow

"Unilever"
Ermacora et al. 2013
3-MCPD, 2-MCPD, Glycidol
AOCS Cd 29a-13; slow

Myasaki et al. 2012
3-MCPD, 2-MCPD, Glycidol
fast

Koyama et al. 2015
3-MCPD, Glycidol
fast

enzymic

Validated methods

Validated Methods covering MCPD & glycidol

EU commission recommends to use the AOCS Official Methods **Cd 29a,b,c-13** not only for analysis of bound 2- & 3-MCPD and glycidol in oils and fats but also in oil- & fat containing foods.
LOQ = 0.1 mg/kg in the oil/fat fraction
LOQ ≤ 0.01 mg/kg in foods with ≤ 10 % of fat.

Direct determination
(determination of a selected number of contaminant esters)

Dilute & shoot

Blumhorst et al. 2011
GE
LC-MS²

Haines et al. 2011
3-MCPD-E, GE
LC-MS²

SPE or SPE²

Masukawa et al. 2010/11
GE
SPE²; LC-MS:
AOCS Cd 28-10

Granvogl et al. 2011
GE
SPE; LC-MS²

Dubois et al. 2011
3-MCPD-E, 2-MCPD-E, GE
SPE²; LC-MS²

Steenbergen et al. 2013
GE
l/l; LC; GC/MS

MacMahon et al. 2013
3-MCPD-E, 2-MCPD-E, GE
2 x SPE²; 2 x LC-MS²

I b 1. Description of analytical methods

General approaches for the analysis of *complex composed foods*

Two principal ways might be used for routine analysis of complex matrices:

Fat extraction
prior to analysis with an AOCS method.
Extraction suitable?
Impact on ruggedness/trueness?
Free MCPD included?

No fat extraction:
takin[illegible] sam[illegible] to an A[illegible]od.
Impact on ruggedness/trueness?
Free MCPD is included.

This approach applies for easy to extract foods but not for infant formula

Some points have to be checked!

I b 1. Description of analytical methods

Methods applied to infant formulae

BfR

Addition of internal standards & extraction:

•Accelerated Solvent Extraction (ASE)
petrolether / acetone / *iso*-hexane (2+2+1, v/v/v)
103 bar – 125˚ C*
changed to:
acetone : *iso*-hexane 4:1 (v,v)
1500 psi – 100˚ C

Analysis of bound analytes:

•Early DGF* or Divinova
changed to:
• „Unilever method“
AOCS Cd29a-13
changed to:
•„SGS 3-in-1“ method
AOCS Cd29b-13

** BfR-method 22: validated for bd. 2- & 3-MCPD in infant formula etc. 2010-2012*

EC-JRC

Extraction:

•Pressurised Liquid Extraction (PSE)
Ambient pressure, 40˚ C
tert-butylmethyl ether for all foods
changed to:
petrolether / acetone / *iso*-hexane (2+1+2, v/v/v) for **Infant formula**
changed to additional application of:
•Solid-Phase-Extraction (SPE)*
n-hexane : ethylacetate (85+15, v/v)

Addition of internal standards & analysis of bound analytes:

• „Unilever method“
AOCS Cd29a-13

** Method validation trial 2017*

Addition of internal standards & extraction:

•Liquid/Solid Extraction
n-hexane / acetone / (1+1, v/v)
30 s shaking, 5 min ultra-sonic bath

Analysis of free analytes:

• GC-MS analysis of PBA-derivatives, comparable to AOCS methods Cd 29b,c-13

I b 1. Description of analytical methods

Methods applied to infant formulae

FDA

Extraction:

•Liquid/Liquid Extraction
ethyl acetate / water (1+1, v/v)
35˚ C + 500 RPM 1,5 h , 2 times repeated for 30 min
1st extraction: 2 x 20 min
centrifugation @ 14.500 rpm
2nd/3rd extraction: 1 x 20 min
centrifugation @ 14.500 rpm

Addition of internal standards & analysis of bound analytes:

•Direct analysis:
MacMahon et al. 2013
2 x SPE²; 2 x LC-MS²

Applied for analysis of ester-bound 3-MCPD & ester-bound glycidol in infant formula.
Method directly applicable to liquid samples

SGS „5-in-2“

Addition of internal standards & extraction:

•Heat-Ultrasonic-Pressure-supported-Solvent Extraction (HUPsSE)
methanol 15 min ultrasonic bath @ 65˚ C
(mini-ultra turrax in case of agglutination)
methanol/*tert*-butylmethyl ether (1+1, v,v) 15 min ultrasonic bath @ 65˚ C
t*ert*-butylmethyl ether 15 min ultrasonic bath @ 65˚ C

• Liquid/Liquid separation of bound & free analytes

Analysis of bound analytes:

• „SGS 3-in-1“ method *modified*
AOCS Cd29b-13 *modified*

Analysis of free analytes:

• GC-MS analysis similar to AOCS method Cd 29b-13

** Method applied on behalf of the Federal German Ministry of Food and Agriculture for investigating the occurrence of free and ester bound 2- and 3-MCPD and ester-bound glycidol in various foods, including 220 infant formulae from the German market in 2016. Results were in parallel reported to EFSA. Also the Svedish Authorities requested the „5-in-2“ methodology for analysis of infant nutrition. Same request from the Danish Authorities is currently in progress.*

2. Limitations / problems of methods

A general view on limitations / problems

Indirect analysis:

- The analytes easily can be converted into each other. Indirect methods must include techniques to suppress and/or control these interconversions.
- No information on original ester structures
- The **„Unilever-method“** AOCS Cd 29a-13 might give glycidol-overestimations when applied to aged or extracted oils and fats or to foods.

Direct analysis:

- So far not sufficient reference compounds/internal standards for poly-unsaturated, medium and short length fatty acid MCPD or glycidol derivatives.
- The high number of isomeric analytes results in chromatographic challenges.
- Larger costs for reference and standard compounds.

Extraction:

- Some implemented extraction techniques have not been tested for the fate of eventually occurring **free MCPD**.
- For infant formulae a strong extraction efficiency is required (next page).

I c. Analytical challenges specific to: 2 Matrix

What is the challenge with analyte extraction from infant formula?

1.: The extraction of analytes is much harder to achieve – in comparison to other foods.

Consequence: Sample spiking with the analytes does not serve for determination of method performance criteria like recoveries, precision, trueness…!

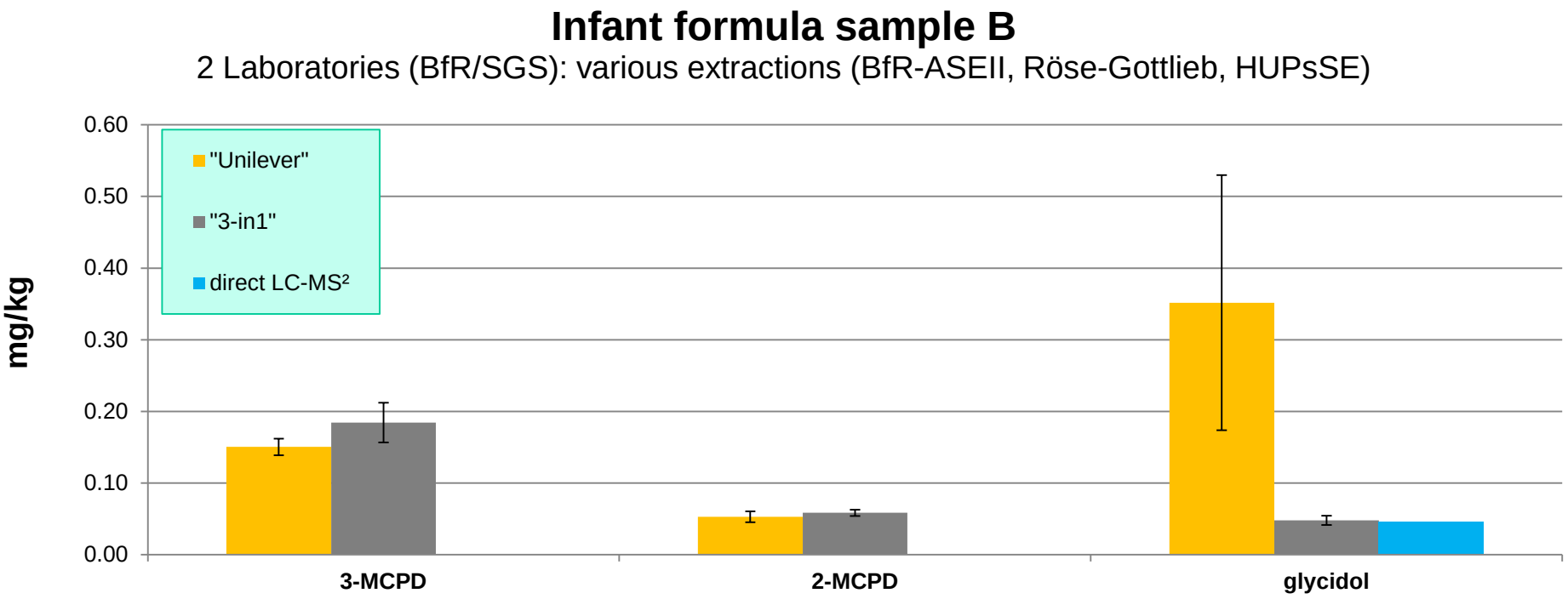
2.: Infant formulae can show very different extractability: What suits for the one product may not serve for another one!

Consequence: Method validation should be carried out by comparing new extraction techniques with well established approaches like the extraction according to Röse-Gottlieb. Validation should also include a representative set of different samples!

I c. Analytical challenges

Practical experiences, example

2014-2015 results from an inofficial interlaboratory comparison focusing on fats extracted from infant formula: „Unilever“ vs. „3-in-1“ & direct LC-MS² method.



The “Unilever-method” gave inconsistent glycidol values in fat extracted from aged infant formula.

Low extraction yields (< 20 %) were observed using PSE-US (tBME) for infant formula (data not shown).

II. Regulatory Information

a. Regulatory organizations

Some organisations being active in the fiele with direct or indirect impact on regulations for MCPD/glycidol:

European Commission (EC) formerly: **Commission of the European Communities**

European Food Safety Authority (EFSA) formerly: **Scientific Committee for Food (SCF)**

Part of EFSA: **The Scientific Panel on Contaminants in the Food Chain (CONTAM)**

German Federal Institute for Risk Assessment (BfR)

Joint FAO/WHO Expert Committee on Food Additives (JECFA)

U.S. Food & Drug Administration (FDA)

Health Canada

II. Regulatory Information

b. Recommendations *in the EU*

COMMISSION RECOMMENDATION of 10 September 2014 on the monitoring of the presence of 2 and 3-monochloropropane-1,2-diol (2 and 3-MCPD), 2- and 3-MCPD fatty acid esters and glycidyl fatty acid esters in food (Text with EEA relevance) (2014/661/EU)

1. *Member States should, with the active involvement of feed and food business operators, perform monitoring for the presence of 2 and 3-MCPD, 2 and 3-MCPD fatty acid esters and glycidyl fatty acid esters in food, and particularly in:*

   (a) …,
   (b) foods for particular nutritional uses as defined in Directive 2009/39/EC of the European Parliament and of the Council (1) and intended for infants and young children, **including infant- and follow on formulae** as defined in Commission Directive 2006/141/EC (2) and dietary foods for special medical purposes as defined in Commission Directive 1999/21/EC (3) intended for use by infants,
   (c) - (f)…..

II. Regulatory Information

b. Regulations (MCPD)

SCF opinion 1994: TDI Free 3-MCPD = **2 µg/kg x bw x d**; http://ec.europa.eu/food/fs/sc/scf/out91_en.pdf

EU-Regulations

free 3-MCPD ≤ 20 µg/kg in soy sauce (or HVP); **EU 466/2001**

free 3-MCPD ≤ 100 µg/kg in glycerol used as food additive; **EU 232/2012**

BfR 2007: TDI Bound 3-MCPD = **2 µg/kg x bw x d**; BfR opinion 047-2007

May 2016: EFSA opinion on 2- & 3-MCPD and glycidol; j.efsa.2016.4426:

- From toxicological perspective the free and bound analytes are considered to be equivalent on molar base.
- "*The CONTAM Panel established for 3-MCPD a Tolerable Daily Intake (TDI) of 0.8 µg/kg bw per day and concluded that this TDI constitutes a group TDI for 3-MCPD and its fatty acid esters (expressed as MCPD equivalents). …* "

Nov. 2016: JECFA (JECFA/83/SC) defined a TDI for 3-MCPD to be **4 µg 3-MCPD/kg x bw x d**
MOE for glycidol remains unchanged.

II. Regulatory Information

b. Safe levels - special focus on infant formula

Glycidol:

BfR 2009: In order not to fall below a MoE of 10.000 the content of glycidol in infant formula should not exceed 67 µg/kg in the fat phase. BfR opinion 007-2009

≈18 µg/kg product with fat content of 25 %

3-MCPD

The BfR assumes for infants a maximum daily uptake of 6 g fat/kg x bw.

	Infant formula: max 3-MCPD content in order to be below the TDI	
TDI 3-MCPD	fat phase	dry product (25 % fat)
µg/kg bw d	µg/kg	µg/kg
4	667	167
2	333	83
0.8	133	33

II. Regulatory Information

Do we expect MRLs?

Draft for MRLs in the EU based on a TDI (3-MCPD) of 0.8 µg/kg x bw x d

Sum of Free 3-monochloropropane-diol (3-MCPD) and 3-MCPD fatty acid esters, expressed as 3-MCPD	**Maximum level (µg/kg)**
Infant formula and follow-on formula (powder)	125 until 30th June 2019 50 as from 1st July 2019
Infant formula and follow-on formula (liquid)	15 until 30th June 2019 6 as from 1st July 2019
Glycidyl fatty acid esters expressed as glycidol	**Maximum level (µg/kg)**
Infant formula and follow-on formula (powder)	75 until 30th June 2019 30 as from 1st July 2019
Infant formula and follow-on formula (liquid)	10 until 30th June 2019 4 as from 1st July 2019

Due to the nonconformity of TDIs the EC advised EFSA to review the calculation of TDI for 3-MCPD. It is expected that the implementation of maximum levels for glycidol remains unchanged.

Proposed Fitness for Purpose

The method should be applicable to the quantitative determination of free 2- and 3-MCPD, 2- and 3- MCPD esters (expressed as 2- and 3-MCPD, respectively) and glycidyl esters (expressed as glycidol) in infant formula, follow-on formula, and – if applicable – also adult nutritionals

- The extraction should be sufficient to isolate the analytes in a satisfying manner from all merchantable products.
- The applied analytical approach should be classified as “state of the art” and should have received international acceptance.
- The method should be sensitive in a way that recent and future maximum residue limits of the analytes are covered.
- Needless to say that the method should suit for routine analysis. This means that beside the required scientific characteristics also turn-around-time, costs, practicability, sustainability etc. should be considered as important criteria.

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Thank you for your kind attention!