Experience Sharing Seminar for Food Testing Laboratories

Testing of other harmful substances listed in the Harmful Substances in Food (Amendment) Regulation 2021 (Cap. 132AF)

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Harmful substances in The Harmful Substances in Food (Amendment) Regulation 2021 (Cap. 132AF) mentioned in this presentation include

- Benzo[a]pyrene
- Erucic acid
- 3-MCPD
- Glycidyl fatty acid esters
- Partially hydrogenated oil
- (PHO)

Other information on testing methods are also available on CFS's website:

https://www.cfs.gov.hk/harmfulsubstance/

Benzo[a]pyrene

Scope:
Oil, fat, infant and follow-up formula

For reference:
GB5009.265-2021

Oil and fat

- Weigh sample
- Add d_{12}-BaP and cyclohexane
- Proceed to MIP-SPE cleanup

Infant formula

- Weigh sample
- Add d_{12}-BaP, EtOH, water and 25% NH_3

Fat extraction

- Warm at 65 °C.
- Cool to r.t. Add EtOH and transfer to Mojonnier flask
- Add **Et_2O** and **petroleum ether**, shake
- Centrifuge and collect the upper layer
- Repeat extraction
- Combine the organic layers and evaporate solvent
- Reconstitute with cyclohexane
- Proceed to MIP-SPE cleanup

Molecularly-imprinted polymer (MIP)-SPE cleanup

- Condition **MIP-SPE** with cyclohexane
- Load sample onto MIP-SPE column
- Wash with cyclohexane
- Elute with EA
- Evaporate eluate and reconstitute with pentane

Further cleanup

- Load sample onto **deactivated silica gel column**
- Collect eluate
- Elute with pentane
- Evaporate eluate to about 0.2 mL
- Add d_{12}-perylene as injection standard

GC-MS/MS analysis

Benzo[a]pyrene

GC-MS/MS analysis

GC settings

Column	:	DB-35ms (30 m × 0.25 mm, 0.25 μm)
Injector temperature	:	280 °C
Injector mode	:	Splitless
Injection volume	:	1 μL
Column flow	:	Helium at 1.2 mL/min
Oven programme	:	150 °C (hold for 1 min), 20 °C/min to 320 °C (hold for 15 min)

MS settings

MS system	:	TSQ 8000 Evo
Ionization mode	:	EI
Transfer line temperature	:	280 °C
Ion source temperature	:	300 °C

Analytes	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
d_{12}-BaP	12.9	264	260	25
BaP	13.0	252	250	30
			251	15
d_{12}-Perylene	13.2	264	260	25

Erucic acid

Scope:
Oil, fat and food to which oil and/or fat has been added

Oil and fat

- Weigh sample into RB flask

Transmethylation

- Add **methanolic NaOH**
- Reflux until fat droplets disappear
- Add **12.5% (w/v) BF_3 in MeOH**, boil
- Add heptane and boil
- Remove from heat
- Add sat. NaCl until the heptane layer float to the neck of flask
- Transfer out the heptane layer and dry with anhy. Na_2SO_4

GC-FID analysis

For reference:
AOAC 922.06 (fat extraction);
ISO 12966-2:2017, AOAC 963.22, AOAC 969.33

Erucic acid

GC-FID analysis

Column	:	HP-DB-FFAP (30 m × 0.53 mm, 1 μm)
Injector temperature	:	250 °C
Injection mode	:	Splitless
Injection volume	:	1 μL
Column flow	:	Helium at 7 mL/min
Column temperature	:	170 °C (hold for 1 min), 10 °C/min to 210 °C (hold for 6 min), 10 °C/min to 240 °C (hold for 9 min).
Detector temperature	:	250 °C
Make up gas and flow	:	Helium, 15 mL/min
H_2 flow	:	40 mL/min
Air flow	:	400 mL/min

For reference:
ISO 12966-4:2015

3-MCPD

Scope:
Solid and liquid condiments containing acid hydrolysed vegetable proteins

For reference:
BS EN ISO 14573:2004

Condiments

- Add d_5-3-MCPD into sample

Removal of fat

- Add **heptane** and 5 M NaCl
- Mix and separate the layers
- Discard the heptane layer

Supported Liquid Extraction (SLE) Cleanup

- Mix aqueous layer with **diatomaceous earth-based solid phase**
- Load into column packed with the same solid phase
- Elute with **Et_2O**
- Evaporate to ~2 mL, dry with an. Na_2SO_4
- Transfer out the dried eluate, add 0.1 mL isooctane
- Evaporate solvent to about 0.1 mL

Derivatisation

- Add isooctane and heptafluorobutyric imidazole (**HFBI**)
- Incubate at 70 °C
- Add water, mix
- Separate the isooctane layer, dry with anhy. Na_2SO_4
- Collect the isooctane layer

GC-MS analysis

3-MCPD

GC-MS analysis

GC settings

Column	:	HP-5MS (30 m × 0.25 mm, 0.25 μm)
Injector temperature	:	250 °C
Injector mode	:	Splitless
Injection volume	:	1 μL
Column flow	:	Helium at 1 mL/min (constant flow)
Oven programme	:	58 °C (hold for 3 min), 1 °C/min to 65 °C, 2 °C/min to 77 °C, 50 °C/min to 300 °C (hold for 10 min).

3-MCPD

GC-MS analysis

MS settings

MS system	:	Shimadzu QP2020 NX
Ionization mode	:	EI
Transfer line temperature	:	280 °C
Source temperature	:	230 °C

MS system	:	Q Exactive GC Orbitrap
Ionization mode	:	EI (70 eV)
Transfer line temperature	:	280 °C
Ion source temperature	:	280 °C
Resolution	:	120,000
AGC target	:	5e6
Maximum injection time	:	Auto
Microscan	:	1
Scan range	:	m/z 50 - 500

Analytes (derivatised product)	Retention time (min)	Ions monitored		
		Quantitation ion	Qualifier ions	
d_5-3-MCPD (+ 2HFB)	14.4	257.03451	-	-
3-MCPD (+ 2HFB)	14.6	274.97067	452.98023	288.98616
2-MCPD (+ 2HFB)	14.9	74.99960	76.99665	288.98616

Glycidyl fatty acid esters

Glycidyl fatty acid esters expressed as glycidol

Scope:
Infant formula and follow-up formula

For reference:
AOAC 2018.03

Solid and liquid infant formula

- Add d_5-glycidyl ester

Fat extraction

- Add **EA** and water
- Sonicate at 50 °C
- Separate and collect the EA layer
- Repeat EA extraction
- Combine EA layers. Evaporate to dryness
- Reconstitute with THF

Conversion of GE to 3-MBPDE

- Add **acidic NaBr**
- Incubate at 50 °C
- Add 0.6% (w/v) Na_2CO_3
- Extract with heptane
- Evaporate the heptane layer
- Reconstitute with THF

Methanolysis of 3-MBPDE to 3-MBPD

- Add **1.8% (v/v) H_2SO_4 in MeOH**
- Incubate at 40 °C
- Add sat. Na_2CO_3, vortex.
- Evaporate solvent to 1 mL
- Add 20% (w/v) Na_2SO_4 and heptane
- Vortex. Remove the organic layer
- Extract the aqueous layer with EA twice

Derivatisation

- Add 0.1 mL isooctane to the combined EA layer
- Evaporate to 0.1 mL
- Add isooctane, **HFBI** and anhy. Na_2SO_4
- Incubate at 70 °C
- Add water, vortex
- Collect the isooctane layer

GC-MS analysis

Glycidyl fatty acid esters

Glycidyl fatty acid esters expressed as glycidol

GC-MS analysis

GC settings

Same as the analysis of 3-MCPD.

MS settings

MS system	:	Q Exactive GC Orbitrap
Ionization mode	:	EI (70 eV)
Transfer line temperature	:	280 °C
Ion source temperature	:	280 °C
Resolution	:	120,000
AGC target	:	5e6
Maximum injection time	:	Auto
Microscan	:	1
Scan range	:	m/z 50 - 500

Analytes (derivatised product)	Retention time (min)	Ions monitored		
		Quantitation ion	Qualifier ions	
d_5-3-MBPD (+ 2HFB)	18.6	257.03451	-	-
3-MBPD (+ 2HFB)	18.7	120.94747	118.94925	253.00940

Melamine

Scope:
Infant formula and follow-up formula, milk and food

Without SPE cleanup

- Weigh sample
- Make up with 49:49:2 (v/v/v) MeCN / water / 1 M HCl
- Transfer out 5 mL
- Wash with CH_2Cl_2
- Collect the upper aqueous layer
- Back wash the CH_2Cl_2 layer with 0.1 M HCl
- Collect and combine the aqueous layers
- Make up with water
- Dilute with MeCN, filter
- Add $^{13}C_3{}^{15}N_3$-melamine

LC-MS/MS analysis

With SPE cleanup

- Add $^{13}C_3{}^{15}N_3$-melamine into sample
- Make up with 49:49:2 (v/v/v) MeCN / water / 1 M HCl
- Transfer out 5 mL
- Wash with CH_2Cl_2
- Collect the upper aqueous layer
- Back wash the CH_2Cl_2 layer with 0.1 M HCl
- Collect and combine the aqueous layers
- Make up with water

SPE cleanup

- Condition **MCX column** with MeOH and water
- Load aqueous sample
- Wash with 0.1 M HCl and MeOH
- Dry the column
- Elute with 5% ammonia in MeOH
- Remove solvent and reconstitute with 95:5 (v/v).0.1% formic acid in MeCN / 20 mM NH_4OAc

LC-MS/MS analysis

For reference:
https://www.govtlab.gov.hk/doc/our_work/texchange/melamine.pdf

Melamine

LC-MS/MS analysis

LC settings

Column	:	Waters Altantis HILIC column (100 mm x 2.1 mm, 3 μm particle size)
Mobile phase	:	A: 20 mM Ammonium acetate in water B: Acetonitrile
Flow rate	:	0.5 mL/min
Injection volume	:	5 μL
Solvent programme	:	

Time (min)	Solvent A (% v/v)	Solvent B (% v/v)
0.0	5	95
3.8	95	5
6.0	95	5
6.5	5	95
13.5	5	95

Melamine

LC-MS/MS analysis

MS settings

MS/MS system	:	Sciex Qtrap 5500
Ionization mode	:	+ ESI
Temperature (TEM)	:	550 °C
Curtain Gas (CUR)	:	25 psi
Collision Gas (CAD)	:	Medium
Ion Spray Voltage (IS)	:	5500 V
Ion Source Gas 1 (GS1)	:	55 psi
Ion Source Gas 2 (GS2)	:	45 psi

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Energy (CXP)
Melamine	2.94	127	85	81 V	10 V	25 V	22 V
			68	81 V	10 V	39 V	30 V
			60	81 V	10 V	27 V	14 V
Melamine-$^{13}C_3{}^{15}N_3$	2.94	133	89	96 V	10 V	25 V	16 V

Partially hydrogenated oil (PHO)

Cap132AF Amendment Regulation

Partially hydrogenated oil (PHO) means any oil or fat that has undergone the process of hydrogenation but is not fully saturated as a result of that process.

部分氫化油指有經過氫化的過程但並無因為該過程而完全飽和的油或脂肪。

- PHO is the major source of industrially produced trans fatty acids (IP-TFA).
  部分氫化油是工業生產的反式脂肪酸的主要來源。

- Natural sources of dietary trans fatty acids are available from ruminal source and refined oils.
  天然的膳食反式脂肪酸可於反芻動物和精煉油中獲得。

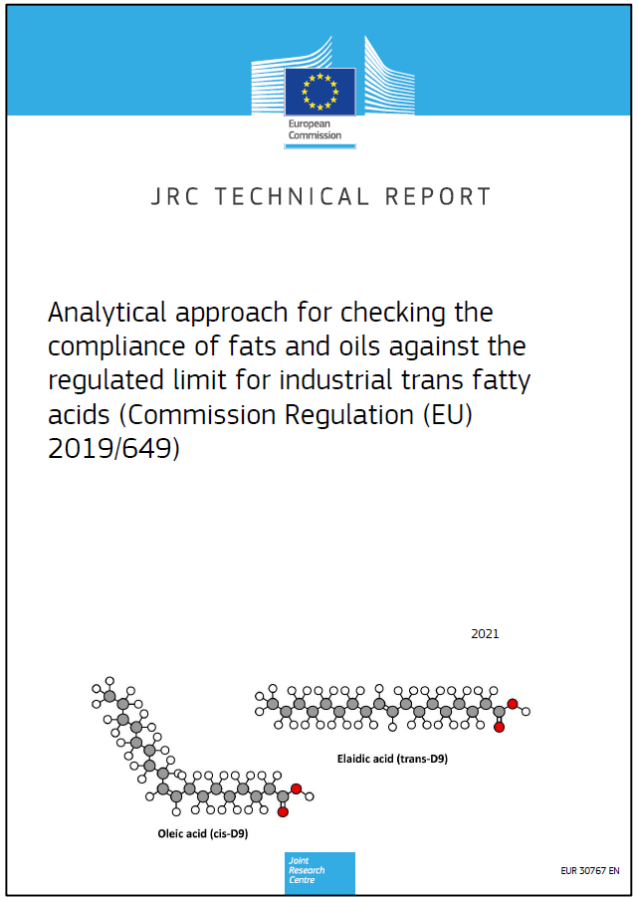
- Need to differentiate whether the detected TFA is from industrial source.
  需要區分檢測到的反式脂肪酸是否來自工業來源。

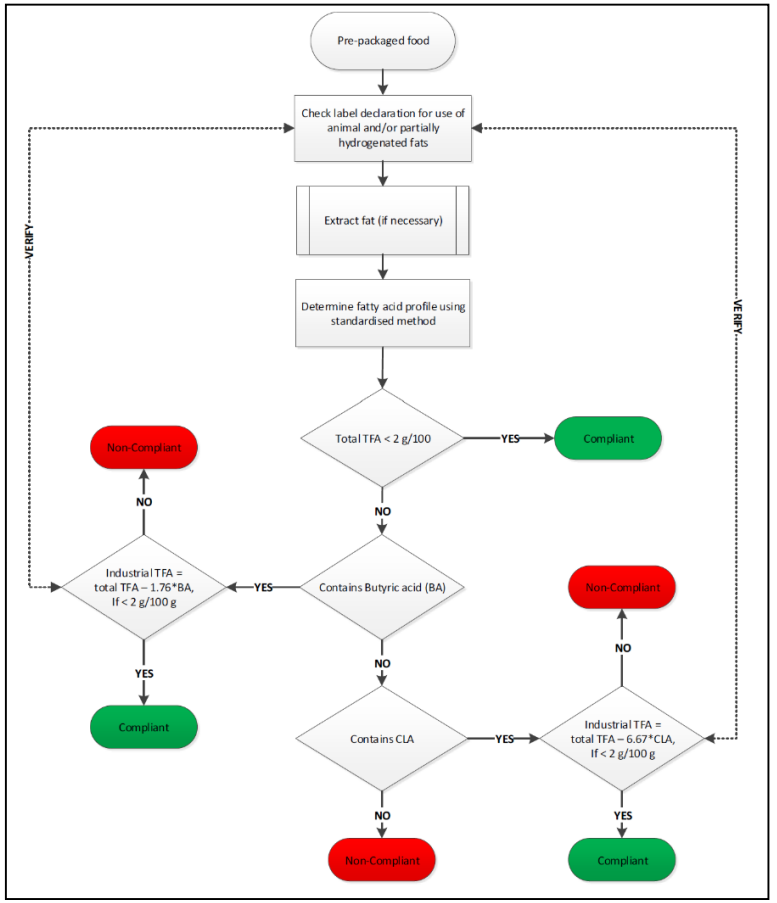
IP-TFA

EU's analytical approach

(Commission Regulation (EU) 2019/649)

1. **Total fat**
2. **Butyric acid** ($C_{4:0}$) (g/100 g fat)
3. **Total TFA** (g/100 g fat): Sum of four non-conjugated TFAs –
   - hexadecenoic acid (trans-$C_{16:1}$)
   - octadecenoic acid (trans-$C_{18:1}$)
   - octadecadienoic acid (trans-$C_{18:2}$)
   - octadecatrienoic acid (trans-$C_{18:3}$)
4. **Conjugated linoleic acid (CLA)** (9-cis,11-trans-$C_{18:2}$)





IP-TFA

Analysis of total fat

General foods

- Add EtOH to sample. Mix well

Acid hydrolysis

- Add 8.3 M **HCl**. Mix well
- Shake in water bath at 70–80 ˚C
- Add EtOH and cool to r.t.

Extraction of fat

- Transfer content to Mojonnier flask
- Add **Et_2O** and shake
- Add **petroleum ether** and shake
- Centrifuge
- Collect the ether layer
- Repeat the extraction with Et_2O and petroleum ether twice
- Combine the ether layer and remove solvent
- Dry in oven at 100 ˚C. Stand in desiccator

Weighing

For reference:
AOAC 922.06

IP-TFA

Analysis of fatty acids

- Weigh sample
- Add pyrogallic acid and triundecanoin (C11:0 triglyceride)
- Add EtOH. Mix well.

For dairy products

- Add H_2O. Mix.
- Add **NH_4OH** (58% w/w). Mix.
- Shake in water bath at 70–80 °C
- Keep solution basic to phenolphthalein indicator using NH_4OH.
- Proceed to fat extraction.

For cheese and formula products

- Add H_2O. Mix.
- Add **NH_4OH**. Mix.
- Shake in water bath at 70–80 ˚C.
- Add 12 M HCl and keep in boiling water bath.
- Proceed to fat extraction.

For other foods

- Add 8.3 M **HCl**. Mix.
- Shake in water bath at 70–80 ˚C.
- Proceed to fat extraction.

Extraction of fat

- Remove sample from water bath. Cool to r.t.
- Transfer content to Mojonnier flask
- Add **Et_2O** and shake
- Add **petroleum ether** and shake
- Centrifuge. Collect the ether layer.
- Repeat the extraction with Et_2O and petroleum ether twice.
- Filter and dry with Na_2SO_4.
- Evaporate solvent to about 2 mL

Methylation

- Add **7% BF_3 in methanol** and toluene to sample.
- Heat at 100 ˚C
- Cool to r.t. and add H_2O, hexane and Na_2SO_4. Shake.
- Collect the top layer and dry with Na_2SO_4.

GC-FID analysis

For reference:
AOAC 996.06

IP-TFA

GC-FID analysis

Column	:	SP-2560 (100 m × 0.25 mm, 0.2 μm)
Injector temperature	:	260 °C
Injection mode	:	Split (50:1)
Injection volume	:	1 μL
Column flow	:	Helium at 0.7 mL/min
Column temperature	:	100 °C (hold for 4 min), 3 °C/min to 240 °C (hold for 20 min).
Detector temperature	:	250 °C
Make up gas and flow	:	Helium, 45 mL/min
H_2 flow	:	40 mL/min
Air flow	:	400 mL/min

Reference materials

Certified Reference Materials

Benzo[a]pyrene	– NMIJ
Erucic acid	– Sigma-Aldrich (as mixed standard)
Melamine	– Supelco

Reference Materials

Erucic acid	– Santa Cruz Biotechnology, Sigma-Adrich
3-MCPD	– Sigma-Aldrich, TCI
2-MCPD	– Santa Cruz Biotechnology, TRC
Glycidyl palmitate	– TRC, Wako
Fatty acids	– Nu-Chek-Prep, Sigma-Aldrich

Isotope Labelled Internal Standards

d_{12}-BaP	– Cambridge Isotope Laboratory
d_{12}-Perylene	– Cambridge Isotope Laboratory
d_5-3-MCPD	– Cambridge Isotope Laboratory
d_5-glycidyl palmitate	– Medical Isotopes
$^{13}C_3{}^{15}N_3$-melamine	– Cambridge Isotope Laboratory
Triundecanoin	– Nu-Chek-Prep

The End

Thank you