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Euro Fed Lipid
european federation for the science and technology of lipids

SISSG
SOCIETÀ ITALIANA PER LO STUDIO DELLE SOSTANZE GRASSE

Congress SISSG 2022
"Edible oils and fats: innovation and sustainability in production and control"

"Solvent-saving sample preparation for high-sensitivity determination of MOSH and MOAH in vegetable oils"

Department of Agri-Food, Environmental and Animal Sciences

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Supervisor: Moret Sabrina

June 17th, 2022

AIMS OF MY PHD PROJECT

AIMS OF MY RESEARCH PROJECT

Problem:
contamination of olive oil by mineral oils

Stakeholders:
- *producers of the supply chain*: uncertainty about the sources of contamination ?
- *laboratories and instrument producers*: lack of validated, robust and sensitive sample preparation methods !

Two main parts

Implementation of analytical methods:
Validation and optimization of protocols with concentration factors appropriate to the required quantification limits (saponification, epoxidation and/or alternatives, Alox)

Monitoring along the supply chain:

sampling along the supply chain to collect data on the contamination in the different steps and found those more critical

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METHOD VALIDATION FOR MOAH DETERMINATION UP TO 0.5 mg/kg

PROPOSED REGULATION

There are currently no legal limits for the presence of mineral oils in olive oil.

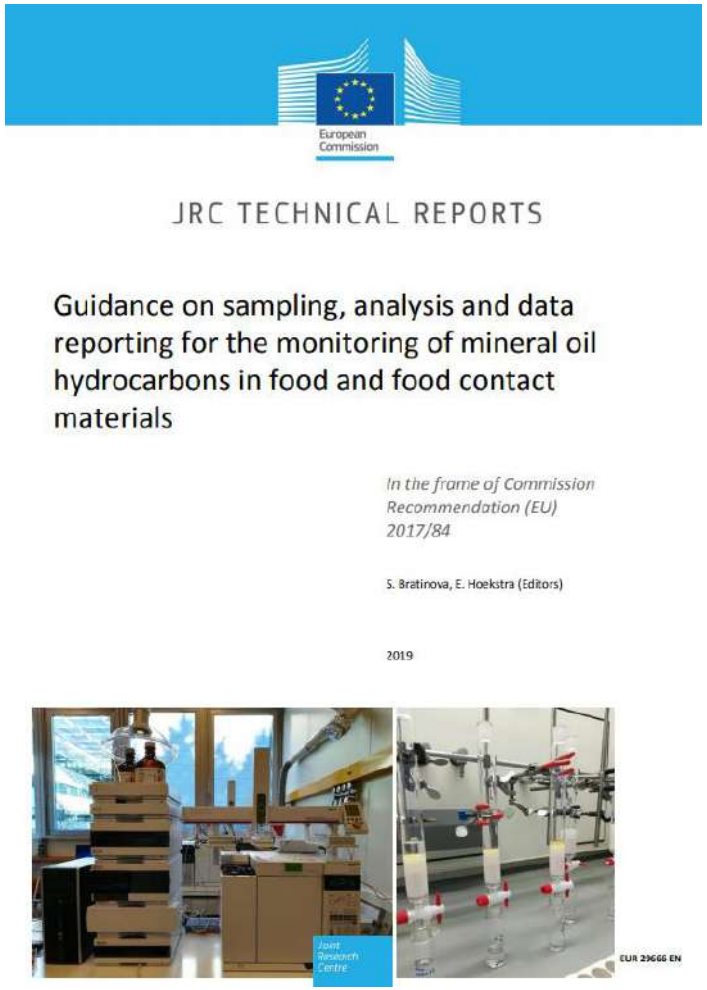
Year	Authority/document	Proposed limit in food (mg/kg)
2011	BMEL ordinance (I draft)	MOSH <0.6 MOAH nd (<0.15)
2011	BfR opinion	MOSH C_{10-16} <12 MOSH C_{16-20} <4
2013	BMEL ordinance (II draft)	MOAH nd (<0.15)
2014	BMEL ordinance (III draft)	MOSH C_{20-35} <2 MOAH C_{16-35} <0.5
2017	BMEL ordinance (IV draft)	MOAH <0.5
2017	FASFC advice on action thresholds	MOSH C_{16-35} 5-150 MOAH C_{16-35} <0.5
2020	BLL advice on benchmark levels	MOSH 4-13 MOAH nq (<LOQ)
2020	BMEL ordinance (V draft)	MOAH <0.5
2021	LAV and Food Federation Germany	MOSH 13 MOAH nq (<LOQ)

Never officially entered into force.

The limit of 0.5 mg/kg gives an indication of the levels now required by the large-scale retail trade to the olive oil producers.

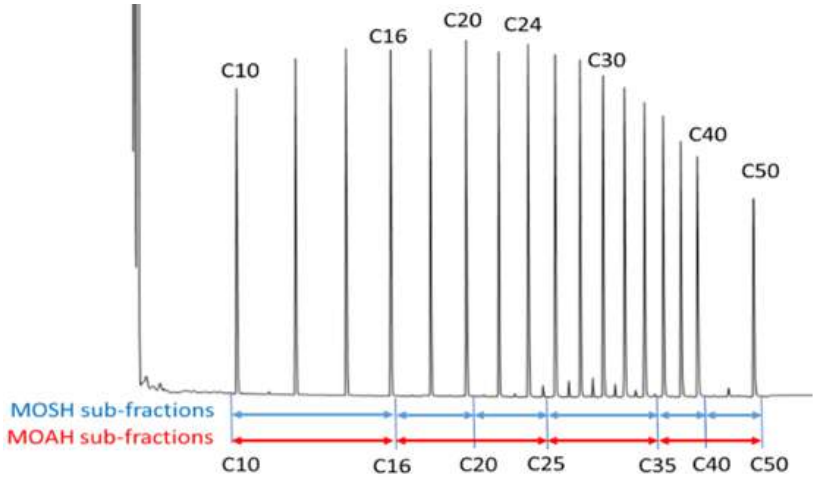
JRC GUIDANCE

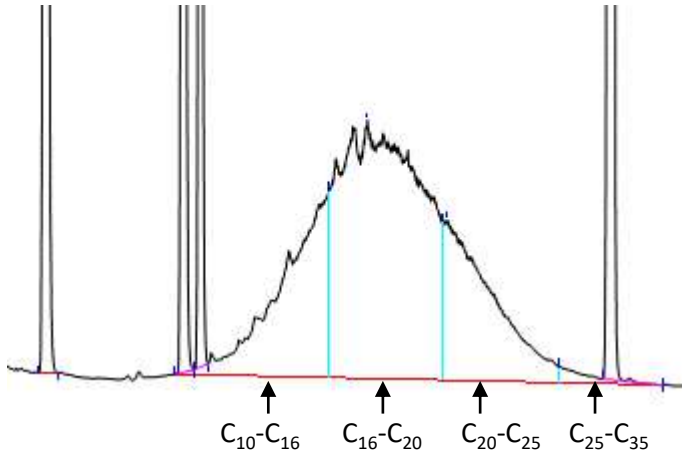
Minimum performance criteria of the methods applied in the analysis of MOSH and MOAH (Bratinova & Hoekstra, 2019).



Categories	Associated foods	LOQ - max (mg/kg)	LOQ - t (mg/kg)	R_{rec} (%)	Intermediate precision (%)
Fat/oils	animal fat (e.g. butter) vegetable oils	2	0.5	70-120	20

Target LOQ





LATEST UPDATES – LOQ AND DATA REPORTING

A common approach for total LOQ calculation and data reporting was lacking.

Example on 3 different EVOOs.

$LOQ_{C\text{-fraction}}$ of 0.2 mg/kg

$LOQ_{C\text{-fraction}}$ of 1.0 mg/kg (still acceptable following the JRC Guidance).

MOAH (mg/kg)					LOQ_{tot}	$LOQ_{C\text{-fraction}}$ L.B.	$LOQ_{C\text{-fraction}}$ U.B.	$LOQ_{C\text{-fraction}}$ L.B.	$LOQ_{C\text{-fraction}}$ U.B.
	$C_{10\text{-}16}$	$C_{16\text{-}25}$	$C_{25\text{-}35}$	$C_{35\text{-}50}$	$C_{10\text{-}50}$	$C_{10\text{-}50}$	$C_{10\text{-}50}$	$C_{10\text{-}50}$	$C_{10\text{-}50}$
EVOOa	<LOD	0.20	0.93	0.70	**1.8**	**1.8**	**2.0**	**<LOQ**	**4.0**
EVOOb	<LOD	0.14	0.85	0.49	**1.5**	**1.3**	**1.7**	**<LOQ**	**4.0**
EVOOc	<LOD	0.36	0.92	0.72	**2.0**	**2.0**	**2.2**	**<LOQ**	**4.0**

L.B. and U.B.: lower/upper bound approach

LOQ_{tot}: LOQ obtained considering the entire mineral oil hump

LATEST UPDATES

EUROPEAN COMMISSION

Health and Food Safety Directorate General

sante.ddg2.g.5(2022)3966048

Standing Committee on Plants, Animals, Food and Feed
Section *Novel Food and Toxicological Safety of the Food Chain*
21 April 2022

CIRCABC Link: *https://circabc.europa.eu/w/browse/39b13c55-0125-4bc0-886a-4dc8a1d6cdf2*

SUMMARY REPORT

A.01 Mineral oil hydrocarbons in food: follow-up to the December 2021 Foodwatch report.

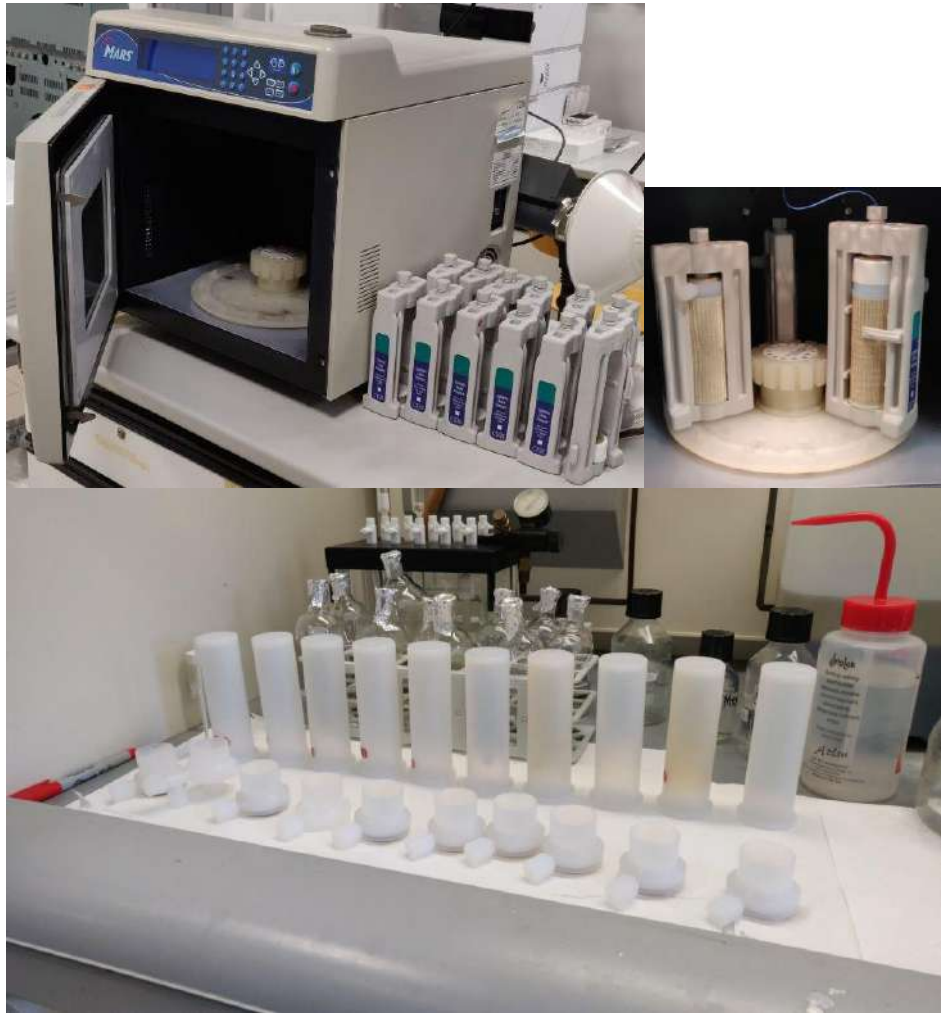
A Member State commented that the principle of determining LOQs per C-fraction should no longer be maintained, as there is no link between certain toxicological effects and specific C-fractions. The Commission confirmed that it is indeed the intention to amend the JRC Guidance accordingly. However the update of the Guidance will be

In order to ensure a uniform enforcement approach throughout the EU, the Member States agreed to withdraw and, if necessary, to recall products from the market, when the sum of the concentrations of MOAH in food are at or above the following maximum LOQs:

- *0.5 mg/kg for dry foods with a low fat/oil content (≤ 4% fat/oil)*
- *1 mg/kg for foods with a higher fat/oil content (> 4% fat/oil)*
- *2 mg/kg for fats/ oils*

Even with a limit of 2 mg/kg, the laboratories must aim to develop methods with lower limits of quantification, as in the INTERLABORATORY application the RSD% tends to increase.

MICROWAVE ASSISTED SAPONIFICATION (MAS)

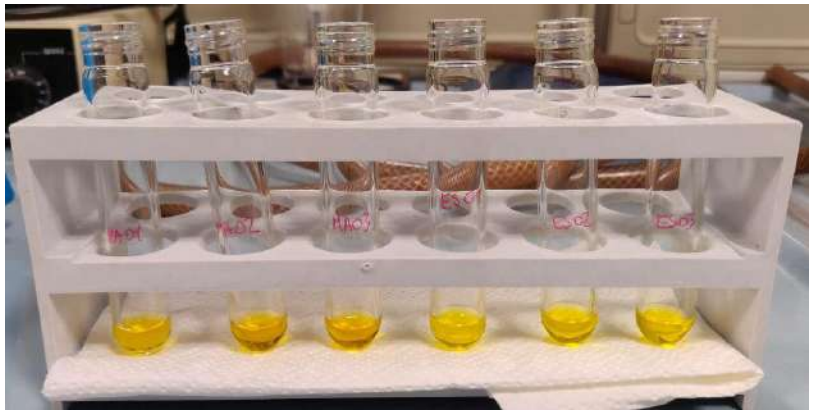


Sample preparation protocol:

- 1 g of olive oil inside the MAS Teflon vessel
- + 10 µL of MOSH/MOAH internal standard mix
- + 10 mL of *n*-hexane
- + 10 mL of methanolic KOH 1.5 N
- MAS cycle: 120 °C x 20 min
- + 40 mL of milliQ H_2O and 3 mL of MeOH
- rest for 30 min at -18 °C
- quantitative recovery of the organic phase and reconcentration to 4 mL
- + 3 mL of 2:1 MeOH/H_2O mixture, followed by agitation with vortex and centrifugation at 5000 rpm for 5 min
- quantitative recovery of the organic phase and reconcentration to 700 µL

EPOXIDATION

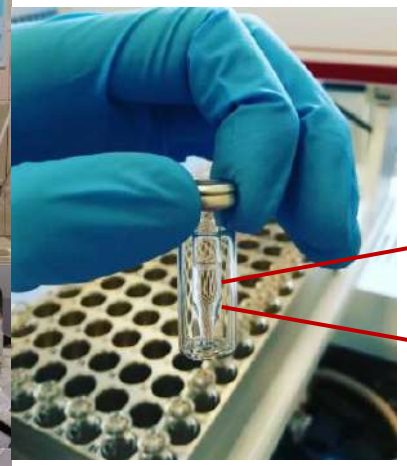
Epoxidation was performed on the saponified sample (Nestola & Schmidt, 2017).



Sample preparation protocol:

On the organic phase obtained from the previous step (reconcentrated to 700 µL)

- + 500 µL of 20% m-CPBA ethanolic solution
- agitation for 15 min at RT
- + 2 mL of 10% $Na_2S_2O_3$ acqueous solution and 500 µL of EtOH
- transfer of the hexane phase to a vial containing a spatula tip of Na_2SO_4



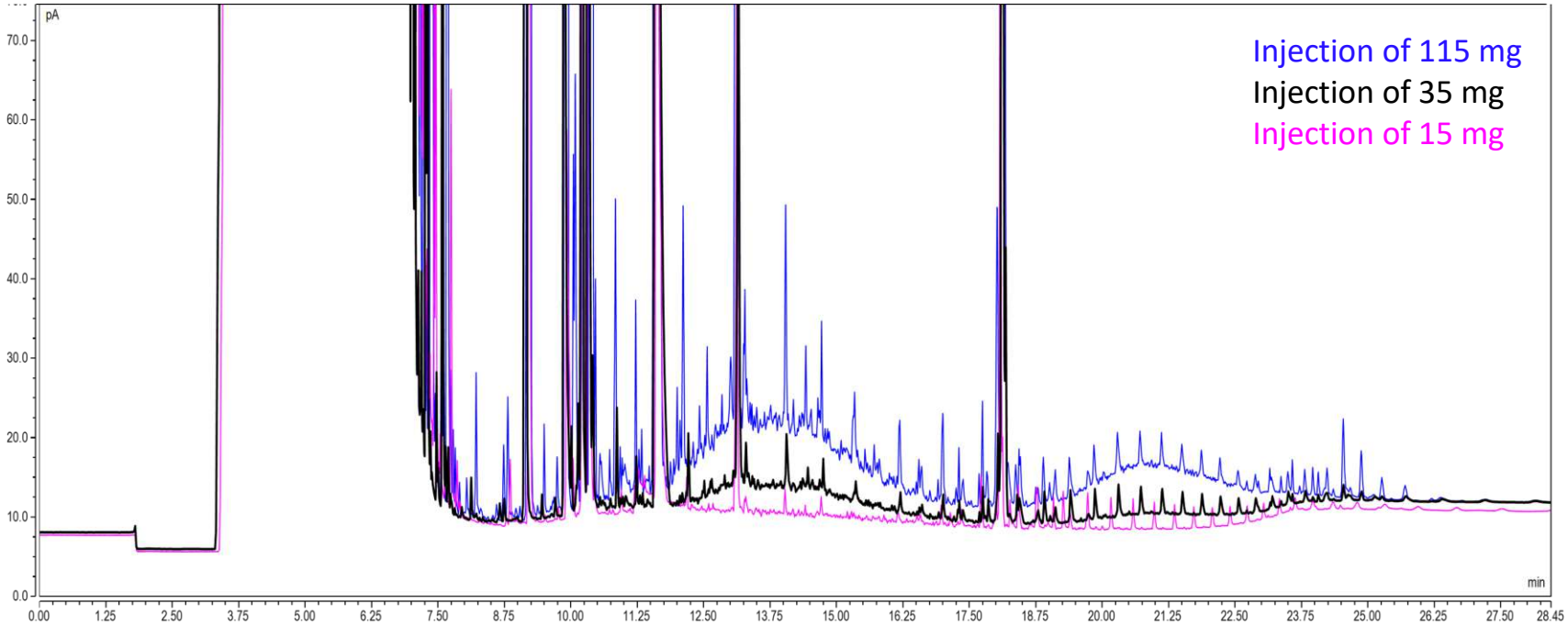
Instrumental analysis:

Injection into the LC-GC-FID system of:

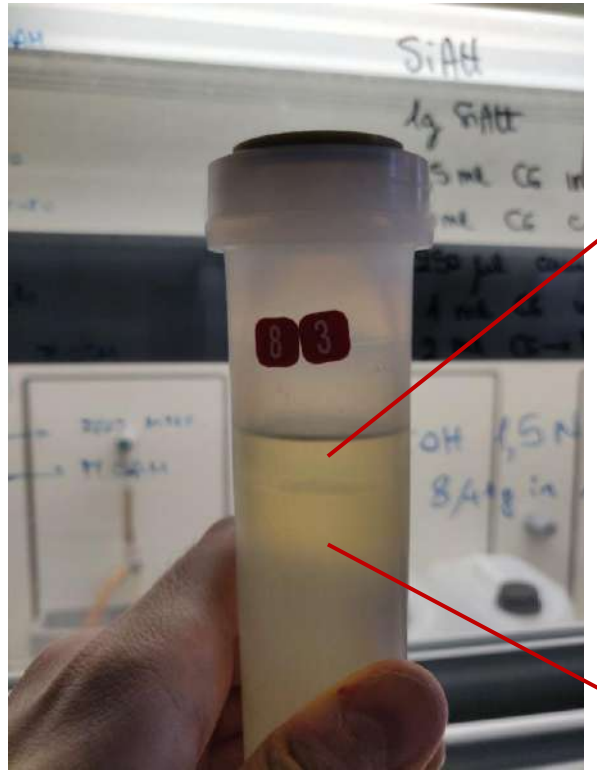
30 µL for the MOSH fraction

100 µL for the MOAH fraction

SENSITIVITY: THE EFFECT OF MAS ON THE MOAH FRACTION



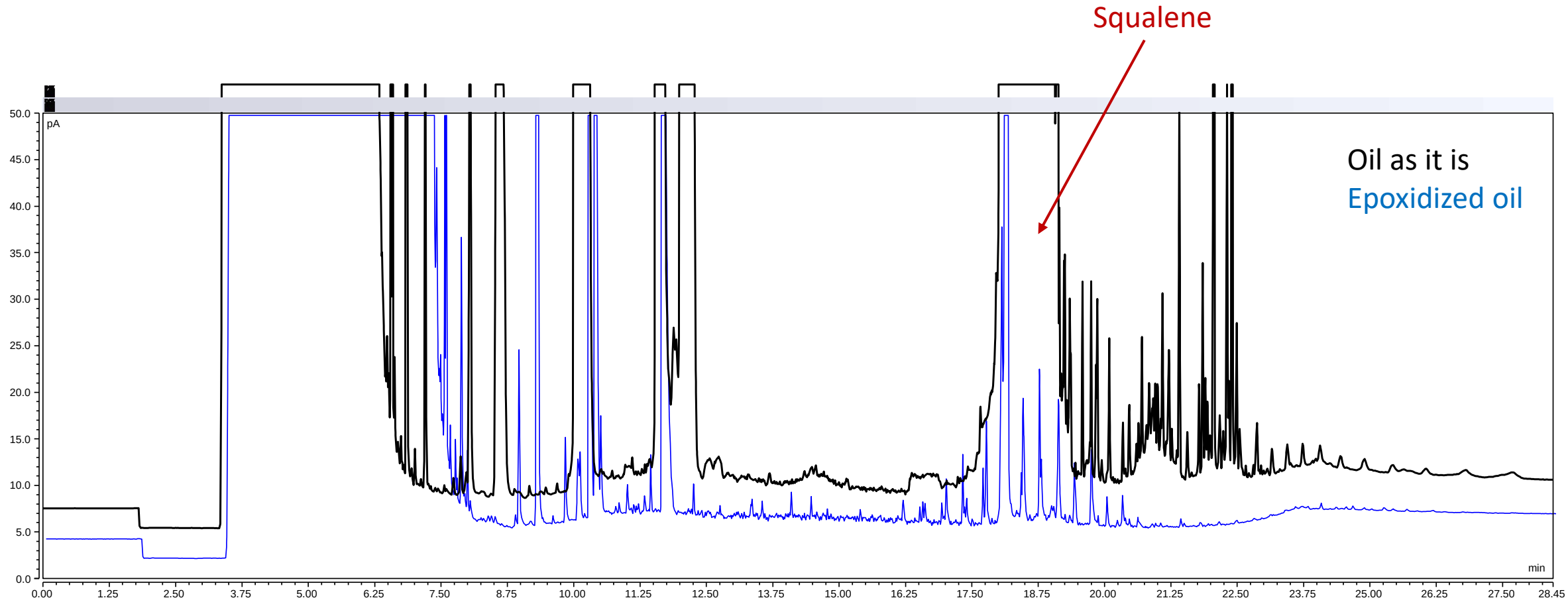
Overlay of MOAH chromatograms of the same oil after injection of different corresponding amount of sample.



MAS

- reduced sample manipulation
- reduced solvent consumption
- sensitivity enhancement

INTERFERENCE: THE EFFECT OF THE EPOXIDATION ON THE MOAH FRACTION

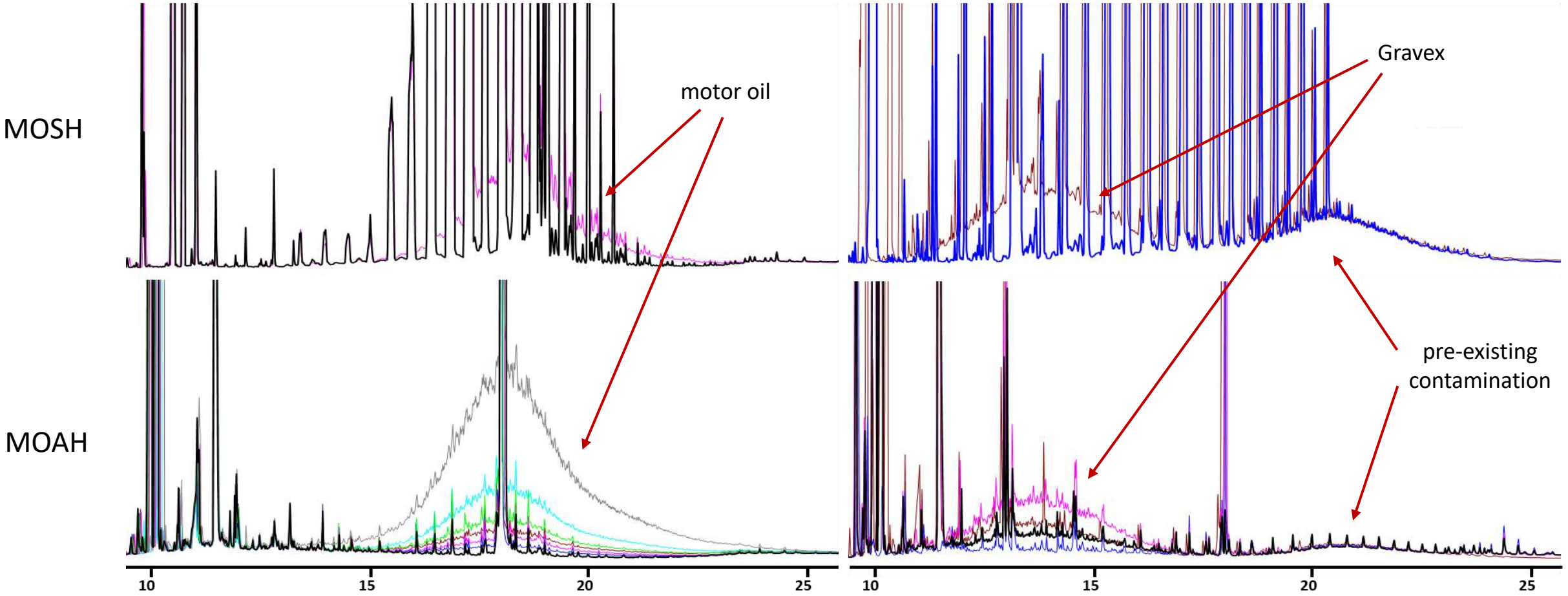


Overlay of MOAH chromatograms of the same sample injected before and after epoxidation.

Starting from the saponified sample, epoxidation worked well also when applied on an amount corresponding to 1 g of oil.

Combination of the two treatments: MAS + epoxidation → sensitivity enhancement + interference removal

VALIDATION

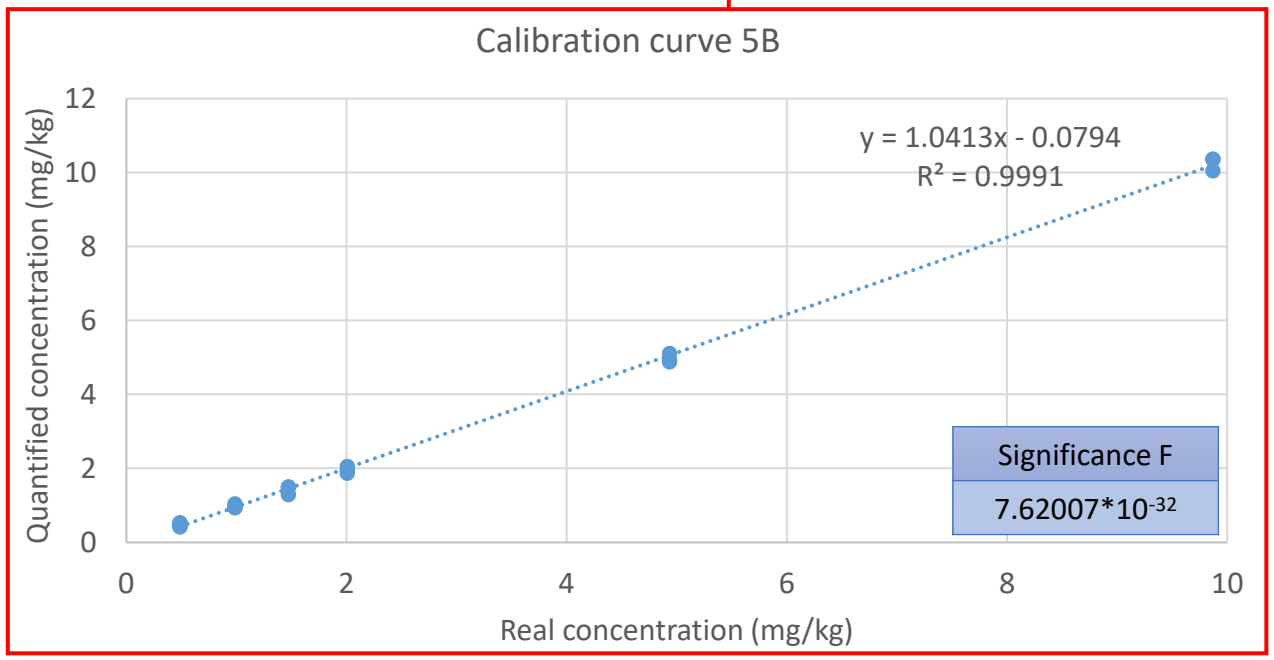


Fortification of different EVOOs with different mineral oils at different concentration levels and application of the protocol under validation (inter-day evaluation).

Based on the performance criteria in the JRC Guidance, recovery, repeatability, linearity and LOQ were evaluated.

LINEARITY

Fraction	Linearity range (mg/kg)	I.S.	Equation	R^2
MOSH	2.0 - 40.7	CyCy	y = 1.0718x - 0.4915	0.998
		C_{13}	y = 1.0168x - 0.3249	0.999
MOAH	0.5 - 9.9	5B	y = 1.0413x - 0.0794	0.999
		1-MN	y = 1.134x - 0.1181	0.999
		2-MN	y = 1.1421x - 0.1176	0.999
		TBB	y = 0.8834x - 0.0503	0.999



Method linearity was assessed for both MOSH and MOAH constructing a six-point calibration curve in matrix, covering the range of contaminations usually found in this type of oil.
Range considered:

- MOSH: 2.0 – 40.7 mg/kg
- MOAH: 0.5 – 9.9 mg/kg

Based on the regression analysis, linearity was confirmed for both MOSH and MOAH.

RECOVERY

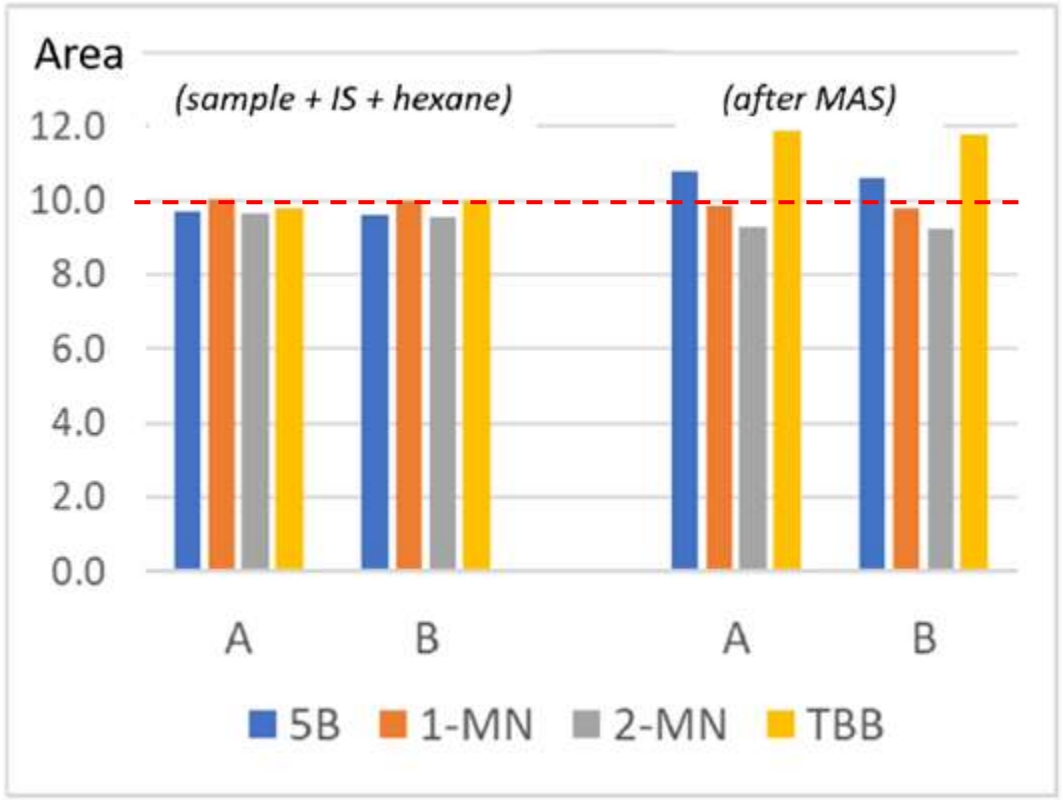
Sample	Type of mineral oil	Number of replicates	MOSH added (mg/kg)	Recovery % (mean)		RSD (%)		MOAH added (mg/kg)	Recovery % (mean)				RSD (%)			
				C_{13}	CyCy	C_{13}	CyCy		5B	1-MN	2-MN	TBB	5B	1-MN	2-MN	TBB
EVOO1	motor oil	6	**2.0**	95.5	98.7	4.0	4.6	**0.5**	96.4	104.7	105.8	81.9	9.4	9.4	9.2	9.0
		4	**4.1**	95.2	98.2	8.2	9.3	**1.0**	99.2	105.2	106.0	84.8	5.1	3.5	3.3	4.4
		4	**6.1**	94.7	96.6	5.5	7.0	**1.5**	96.6	102.9	103.8	83.4	8.1	7.0	6.6	7.6
		4	**8.2**	95.3	97.6	4.8	6.7	**2.0**	97.3	104.3	105.2	83.8	4.8	2.6	2.2	5.2
		4	**20.4**	97.9	100.9	3.3	3.9	**4.9**	100.7	106.7	107.4	87.2	2.4	3.0	3.3	1.8
		4	**40.7**	103.1	106.6	2.4	3.3	**9.9**	102.4	109.8	110.4	87.8	2.6	4.7	5.0	1.7
EVOO2	Gravex	6	**2.2**	99.6	103.1	3.3	2.6	**0.8**	93.4	99.7	101.7	79.4	5.0	4.2	4.8	5.0
		6	**3.7**	94.9	100.4	4.9	3.9	**1.4**	94.6	102.4	102.6	79.8	2.2	2.7	2.9	2.6
		6	**7.4**	98.1	104.3	4.5	6.3	**2.8**	100.6	106.0	108.0	83.3	5.8	5.7	6.1	4.4
MEAN RECOVERY*				**97.2**	**100.7**				**97.9**	**104.6**	**105.7**	**83.5**				

**all replicates at different spiking levels*

Recoveries resulted always within the acceptability range indicated in the JRC Guidance (70-120%)
RSD resulted always below 10% (limit of 20%), also at the level of 0.5 mg/kg → **LOQ_{tot}**=0.5 mg/kg

→ Different recovery for different IS

INTERNAL STANDARDS BEHAVIOUR



Different recoveries for different IS are due to:

- reconcentration of the IS in the residual hexane phase due to incomplete recovery of the added hexane
- different partition coefficients for the different IS

The behaviour was reproducible → data correction for recovery

REPEATABILITY

Sample	Number of replicates	Type of mineral oil	MOAH added or present (mg/kg)	C-fraction	Mean concentration (mg/kg)*				RSD%			
					5B	1-MN	2-MN	TBB	5B	1-MN	2-MN	TBB
EVOO1	6	motor oil	0.5	*n*-C_{16-25}	0.1	0.1	0.1	0.1	21.0	21.0	20.8	21.0
				n-C_{25-35}	0.3	0.3	0.3	0.3	6.7	6.8	6.6	5.8
				n-C_{35-50}	0.1	0.1	0.1	0.1	16.9	16.6	16.4	17.2
	4		1.0	*n*-C_{16-25}	0.2	0.2	0.2	0.2	9.3	10.4	10.5	9.8
				n-C_{25-35}	0.7	0.7	0.7	0.7	8.0	6.0	5.8	7.1
				n-C_{35-50}	0.1	0.1	0.1	0.1	13.3	14.4	14.5	13.8
	4		1.5	*n*-C_{16-25}	0.3	0.3	0.3	0.3	11.0	10.8	10.6	10.2
				n-C_{25-35}	1.0	1.0	1.0	1.0	4.3	3.9	3.7	3.7
				n-C_{35-50}	0.2	0.2	0.2	0.2	19.6	17.6	17.1	19.4
	4		2.0	*n*-C_{16-25}	0.4	0.4	0.4	0.4	5.3	3.1	2.9	6.0
				n-C_{25-35}	1.3	1.3	1.3	1.3	2.9	2.0	1.9	2.7
				n-C_{35-50}	0.3	0.3	0.3	0.3	15.9	13.5	13.1	16.4
	4		4.9	*n*-C_{16-25}	1.0	1.0	1.0	1.0	6.4	5.3	5.4	5.9
				n-C_{25-35}	3.4	3.3	3.3	3.4	2.4	4.2	4.6	1.7
				n-C_{35-50}	0.7	0.7	0.7	0.7	4.9	2.6	2.2	5.3
	4		9.9	*n*-C_{16-25}	2.0	2.1	2.0	2.1	2.8	4.3	4.6	2.9
				n-C_{25-35}	6.8	6.8	6.8	6.9	3.3	5.3	5.5	2.2
				n-C_{35-50}	1.5	1.5	1.5	1.5	3.9	5.6	5.7	3.3

Sample	Number of replicates	Type of mineral oil	MOAH added or present (mg/kg)	C-fraction	Mean concentration (mg/kg)*				RSD%			
					5B	1-MN	2-MN	TBB	5B	1-MN	2-MN	TBB
EVOO2	6	Gravex	0.8	*n*-C_{10-16}	0.1	0.1	0.1	0.1	26.6	26.4	26.9	26.9
				n-C_{16-25}	0.7	0.7	0.7	0.7	3.7	2.6	3.0	3.5
	6		1.4	*n*-C_{10-16}	0.2	0.2	0.2	0.2	2.3	2.8	2.9	3.0
				n-C_{16-25}	1.1	1.1	1.1	1.1	2.2	2.8	2.9	2.6
	6		2.8	*n*-C_{10-16}	0.4	0.4	0.4	0.4	8.3	6.6	6.5	7.5
				n-C_{16-25}	2.4	2.4	2.4	2.3	6.3	6.4	6.8	4.9
	18	pre-existing contamination	0.9	*n*-C_{25-35}	0.3	0.3	0.3	0.3	6.4	5.9	5.5	5.9
				n-C_{35-50}	0.6	0.6	0.6	0.6	4.0	4.2	5.0	4.4

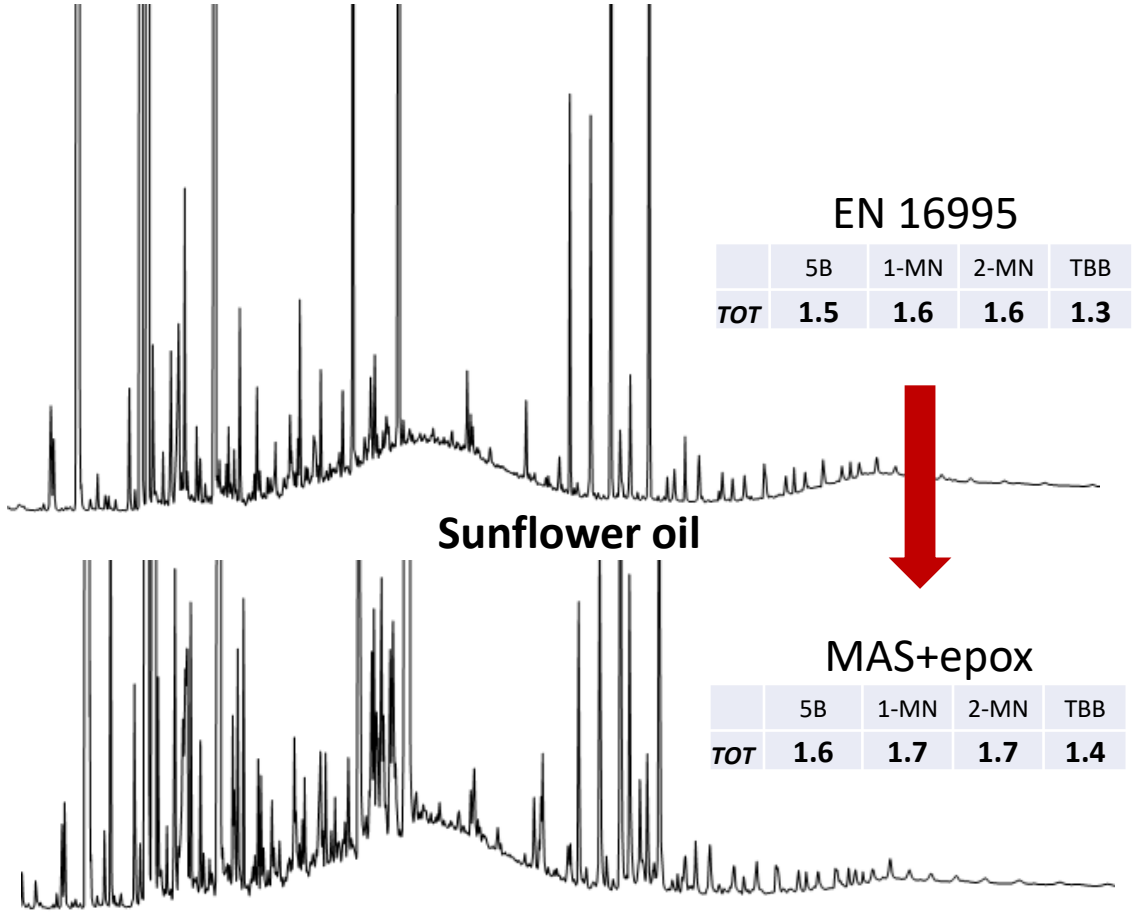
*data corrected for recovery

RSD on the C-fraction was below the limit of the JRC Guidance (limit of 20%) up to 0.2 mg/kg → **$LOQ_{C\text{-}fraction}$**=0.2 mg/kg

LOQ_{tot}=0.5 mg/kg

COMPARISON ON SAMPLES FROM A COLLABORATIVE TRIAL

Data obtained using the “MAS + epox” protocol were found to be in line with those obtained as part of the participation in the collaborative study for the revision of the EN 16995 method.



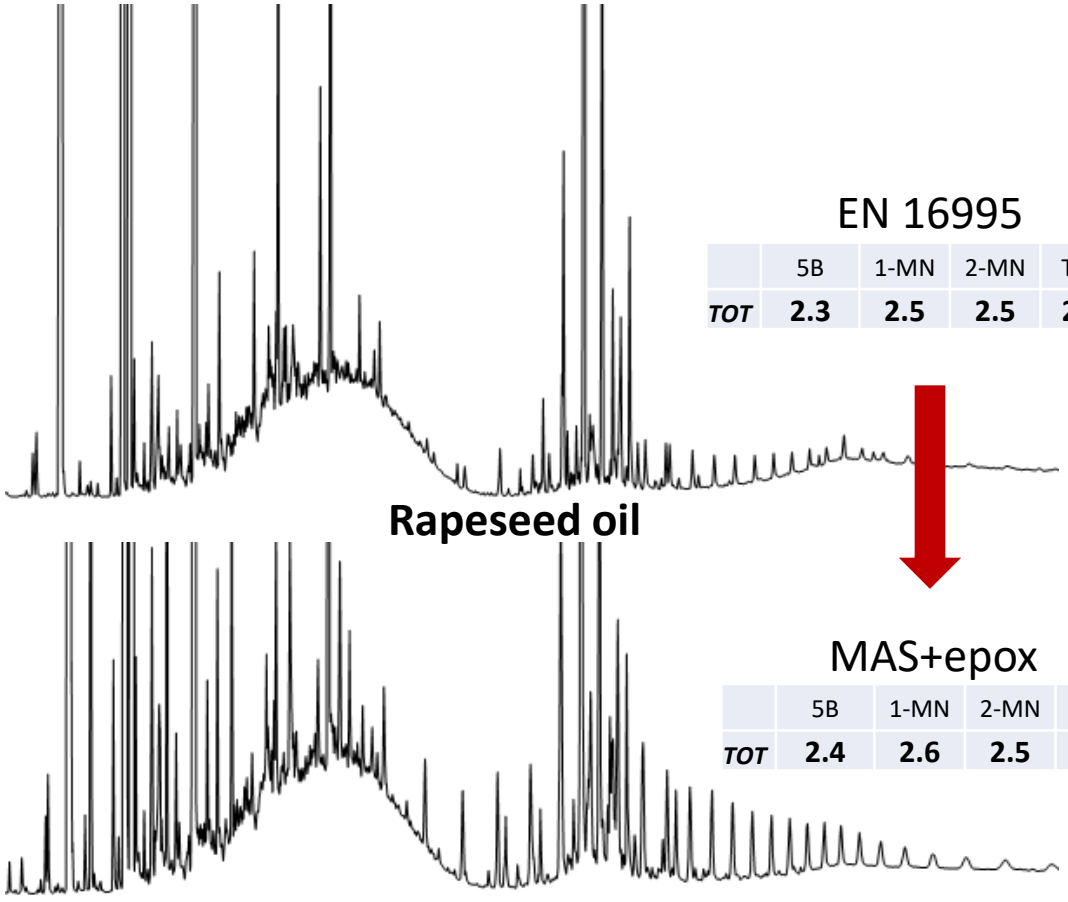
Sunflower oil

EN 16995

	5B	1-MN	2-MN	TBB
TOT	**1.5**	**1.6**	**1.6**	**1.3**

MAS+epox

	5B	1-MN	2-MN	TBB
TOT	**1.6**	**1.7**	**1.7**	**1.4**



Rapeseed oil

EN 16995

	5B	1-MN	2-MN	TBB
TOT	**2.3**	**2.5**	**2.5**	**2.0**

MAS+epox

	5B	1-MN	2-MN	TBB
TOT	**2.4**	**2.6**	**2.5**	**2.0**

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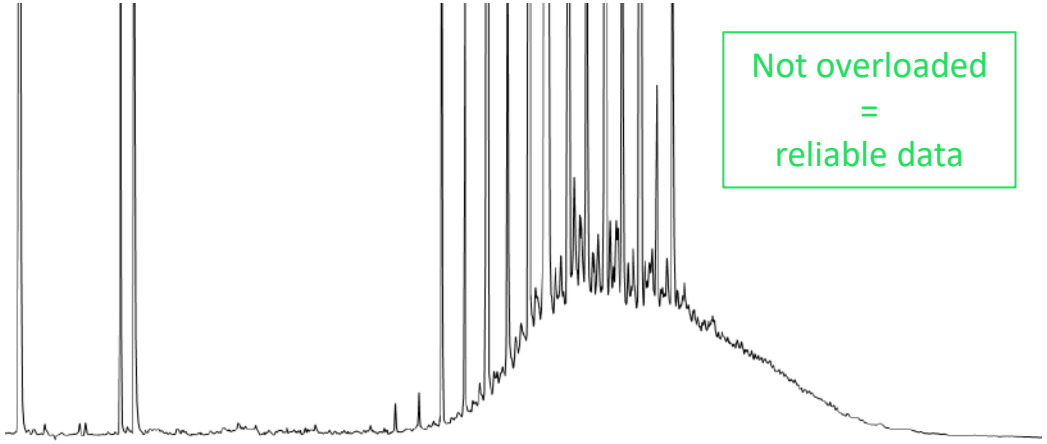
ALOX

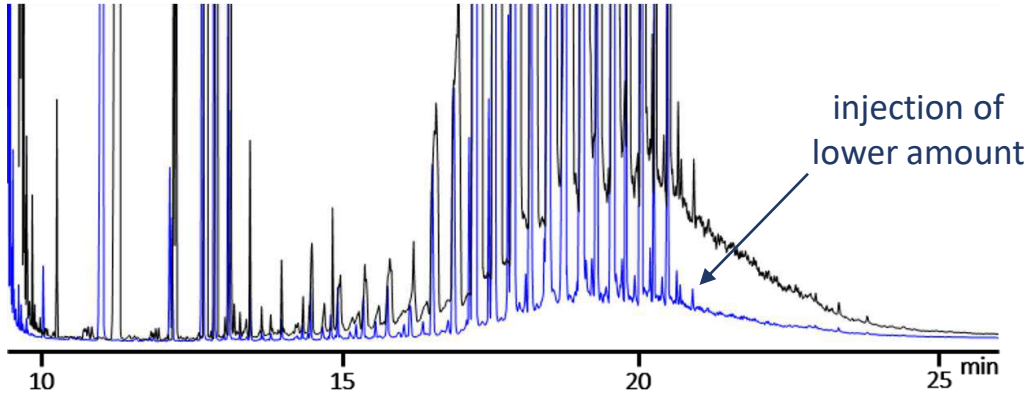
THE INTERFERENCE FROM ENDOGENOUS *n*-ALKANES

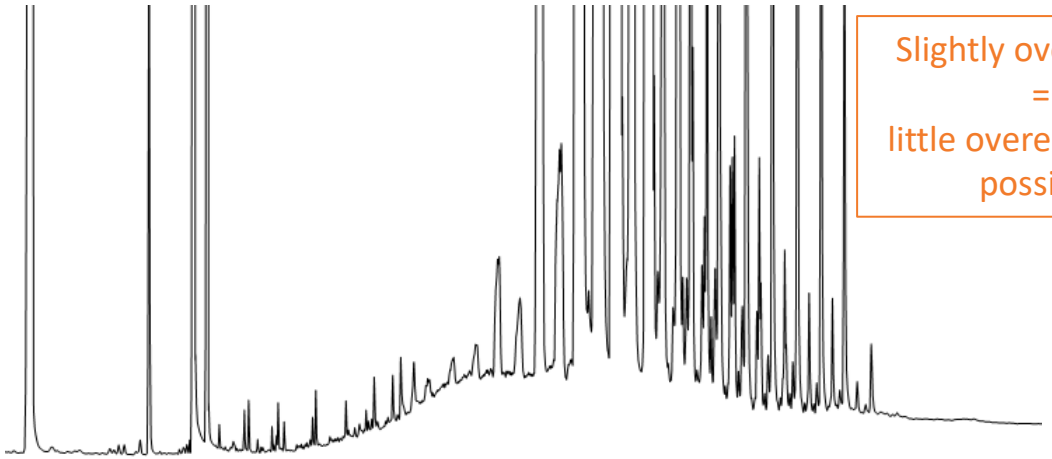
Different possible scenarios:



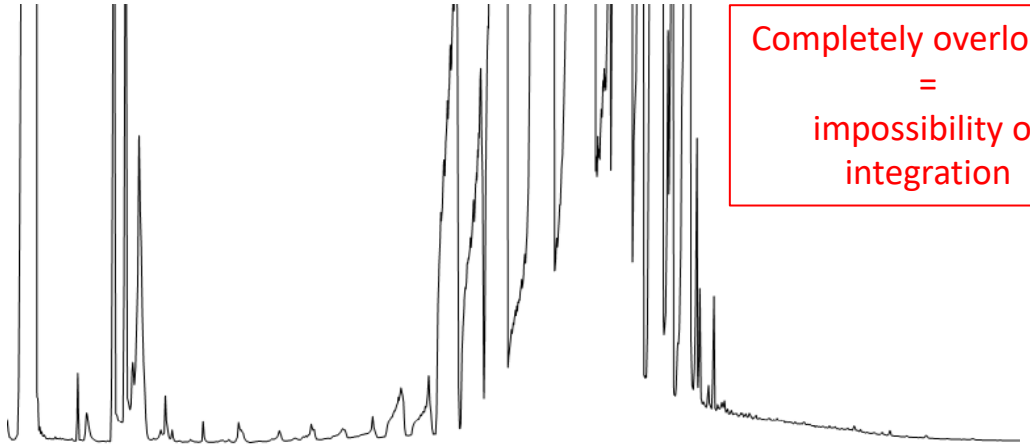
Not overloaded
=
reliable data

Interference by endogenous *n*-alkanes is not always a problem, if their amount is not excessive and the MOH level allows to find a compromise between the injectable amount and the sensitivity.





Slightly overloaded
=
little overestimation
possible



Completely overloaded
=
impossibility of
integration

EXISTING PROTOCOLS

Wagner *et al*., 2001
- 100 mg of oil dissolved in 0.5 mL of *n*-hexane
- loading on a single phase cartridge of 3.5 g of Alox, not conditioned
- elution with *n*-hexane, collecting the first 2 mL
- reconcentration and injection

Fiselier & Grob, 2009
- 1 g of oil dissolved in 2 mL of *n*-hexane
- loading on a double phase cartridge (20 g Alox + 7 g act. Si), previously conditioned with 25 mL of *n*-hexane
- elution with 40 mL of *n*-hexane
- reconcentration and injection

Zurfluh *et al*., 2014
- 1 g of oil dissolved in 1 mL of *n*-hexane
- loading on a double phase cartridge (17 g mixture of Alox and SiAg + 8 g act. Si), previously conditioned with 50 mL of *n*-hexane
- elution with 25 mL of *n*-hexane and then with 23 mL of a mixture DCM/toluene/*n*-hexane
- reconcentration and injection

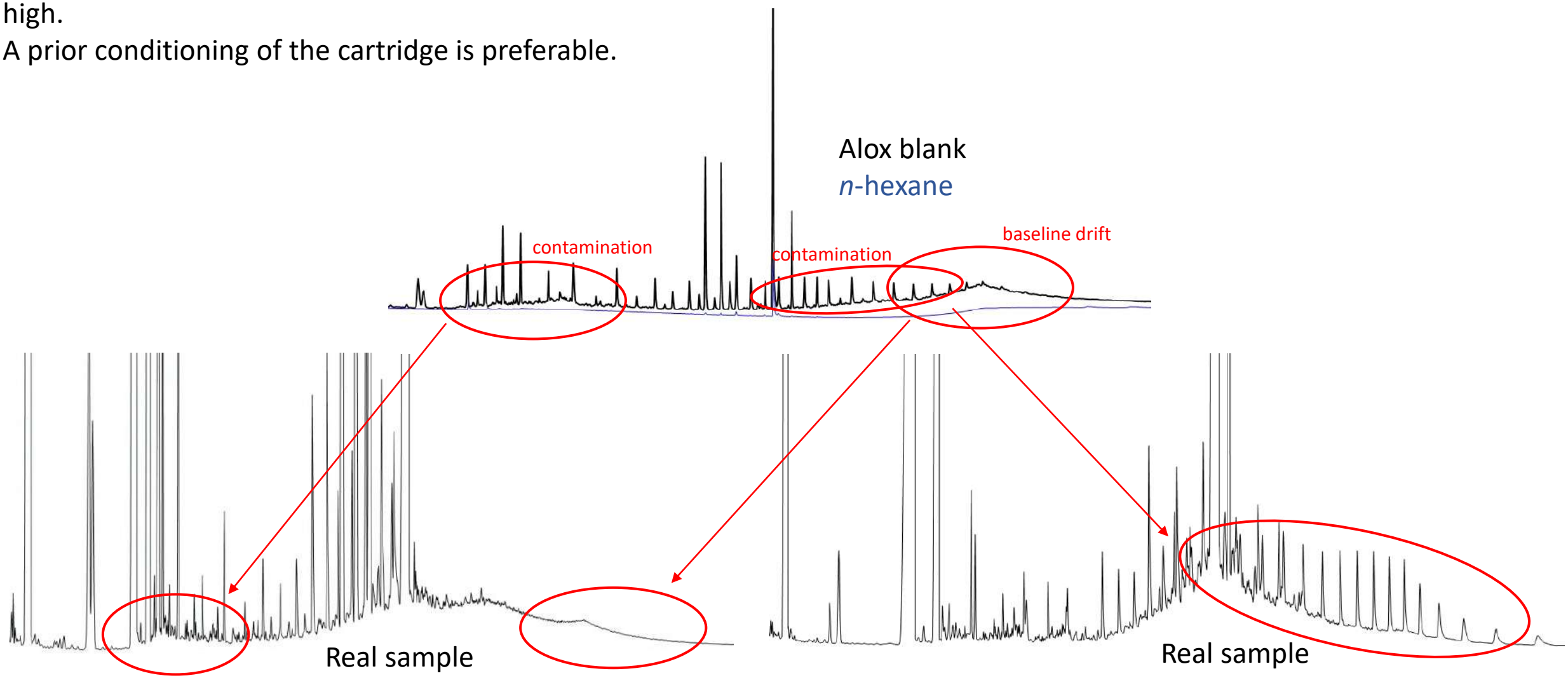
EN 16995:2017
- 300 mg of oil dissolved in 2 mL of *n*-hexane
- loading on a double phase cartridge (10 g Alox + 3 g act. Si), previously conditioned with 20 mL of *n*-hexane
- elution with 25 mL of *n*-hexane
- reconcentration and injection

Revision of EN 16995:2017
- 15 mL of *n*-hexane from saponification of ±900 mg of oil
- loading on a double phase cartridge (10 g Alox + 3 g act. Si + 1 g Na_2SO_4), previously conditioned with 20 mL of *n*-hexane
- elution with 25 mL of *n*-hexane
- reconcentration and injection

EXISTING PROTOCOLS

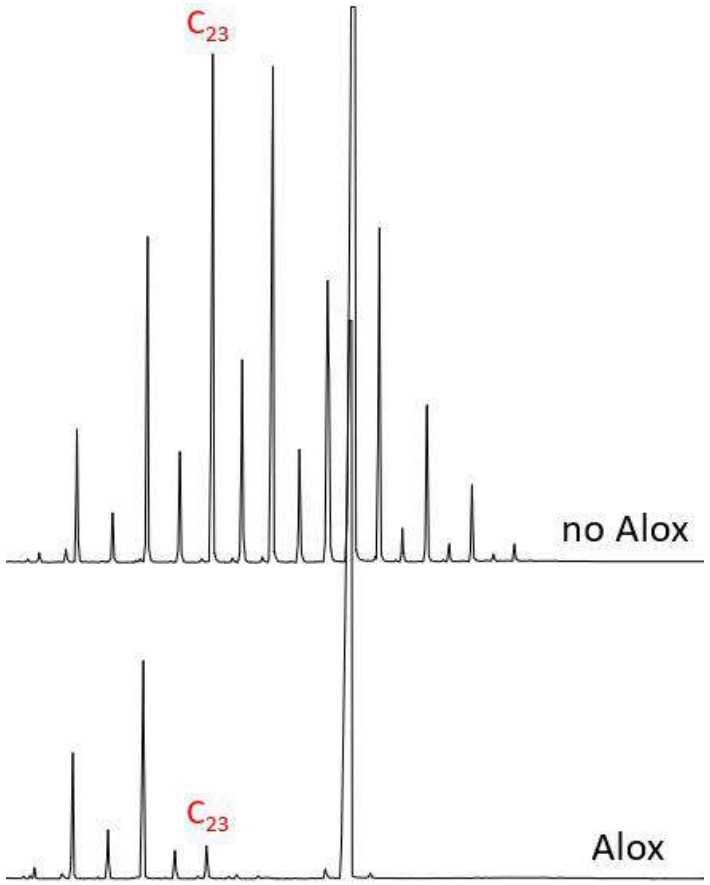
With a high reconcentration factor to achieve high sensitivity, but working with a dry SPE, the risk of obtaining dirty blanks is high.

A prior conditioning of the cartridge is preferable.



SMALL SCALE ALOX

The reduction of the amounts of adsorbents and the volumes of solvents, while maintaining high sensitivity levels, is feasible.



Weigh 150 mg of oil, add 3 µL of IS and dissolve in 500 µL of *n*-hexane

↓

Prepare a double bed SPE cartridge consisting of an underlying phase of 2.5 g of activated aluminum oxide and an upper one of 1 g of activated silica

↓

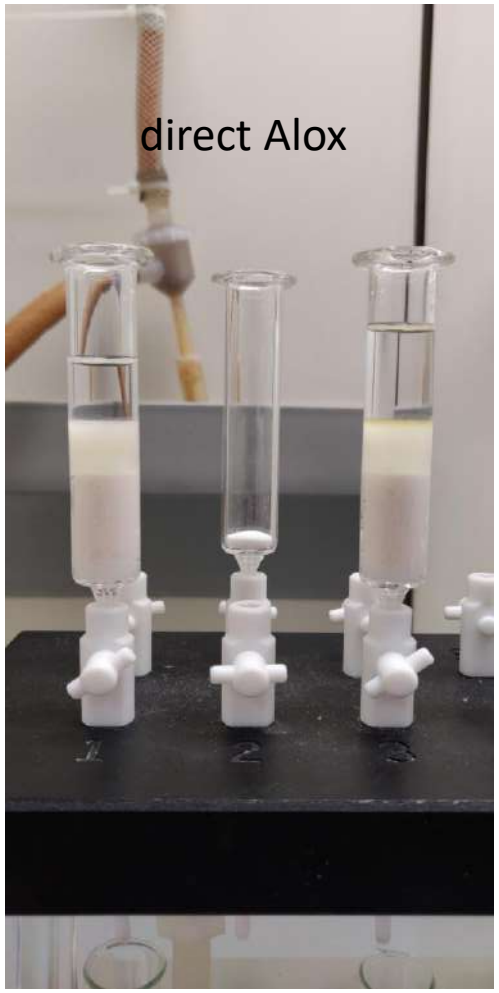
Condition the column with 6 mL of *n*-hexane and then load the sample

↓

Elute the MOSH fraction with 6 mL of *n*-hexane

↓

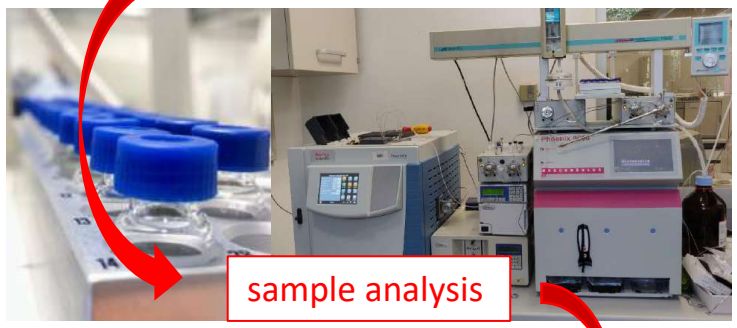
Evaporate the eluate to 150 µL and inject 100 µL into the LC-GC-FID

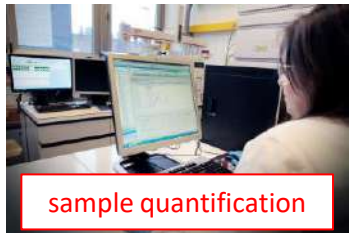


→ 0.5 mg/kg

SAVING EVEN MORE

When applied to MAS+epox samples, there is no need for activated silica, obtaining a further reduction in solvents and adsorbents. Moreover, the analyst can first process the sample and then decide whether Alox is needed or not.

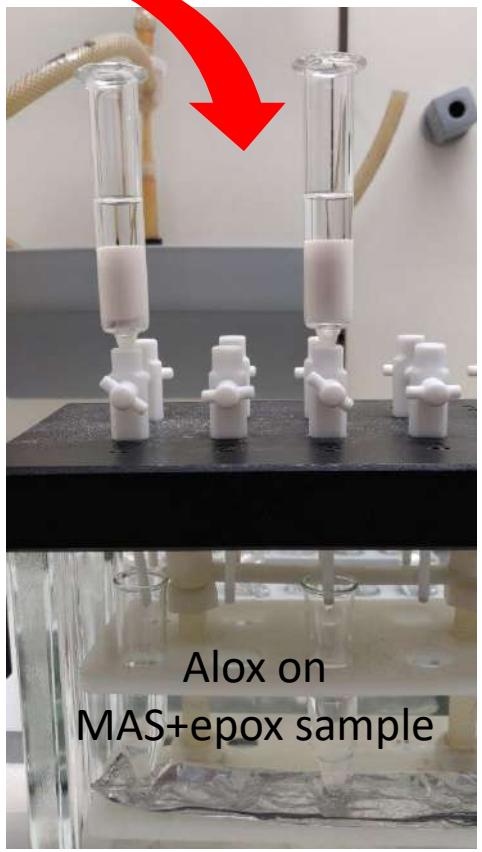




Is MOSH quantification reliable?

NO

YES



FINAL RESULT

Prepare a single phase SPE cartridge consisting of 2.5 g of activated aluminum oxide

Condition the column with 5 mL of *n*-hexane and then load 150 µL of MAS+epox sample, previously diluted to 500 µL

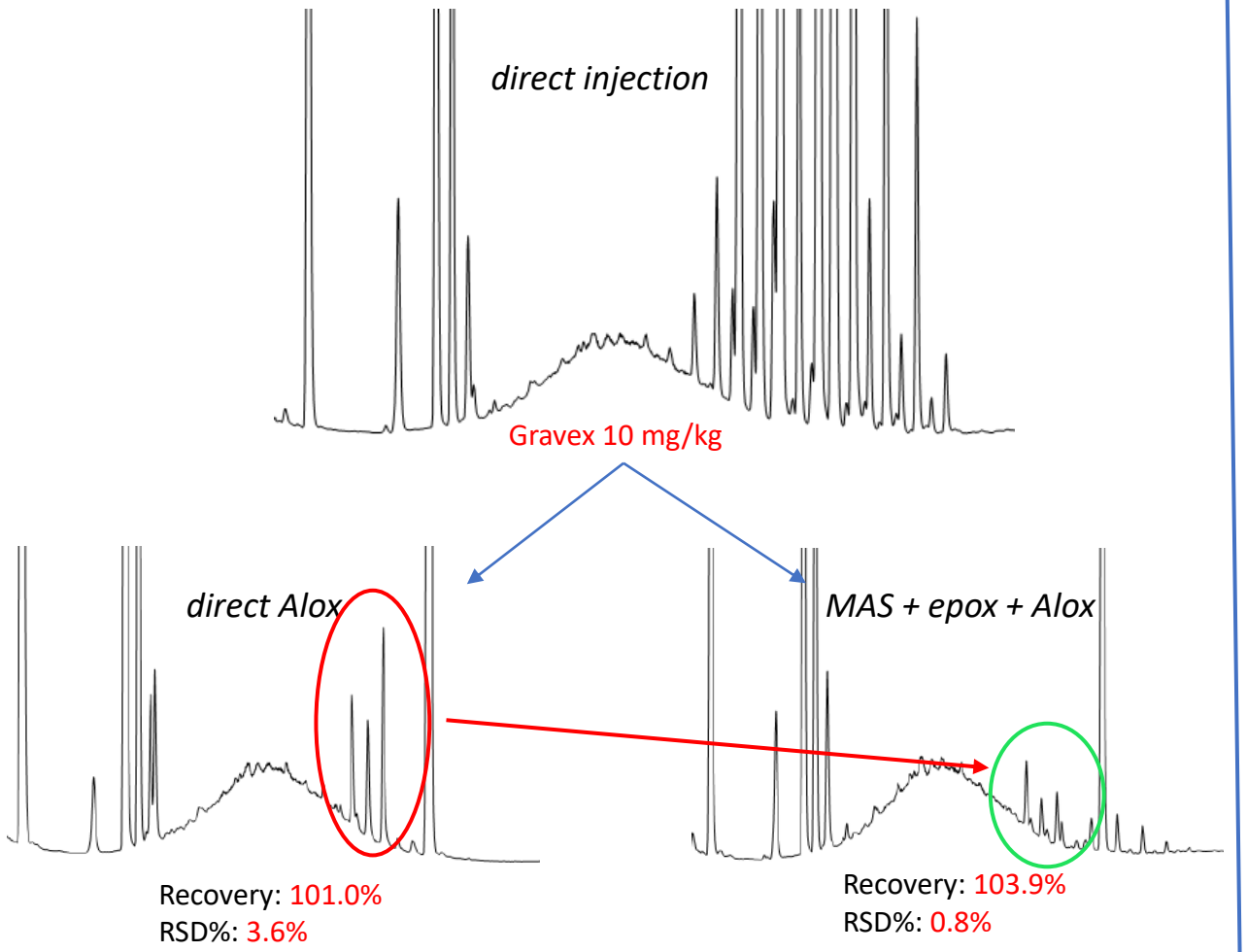
Elute the MOSH fraction with 5 mL of *n*-hexane

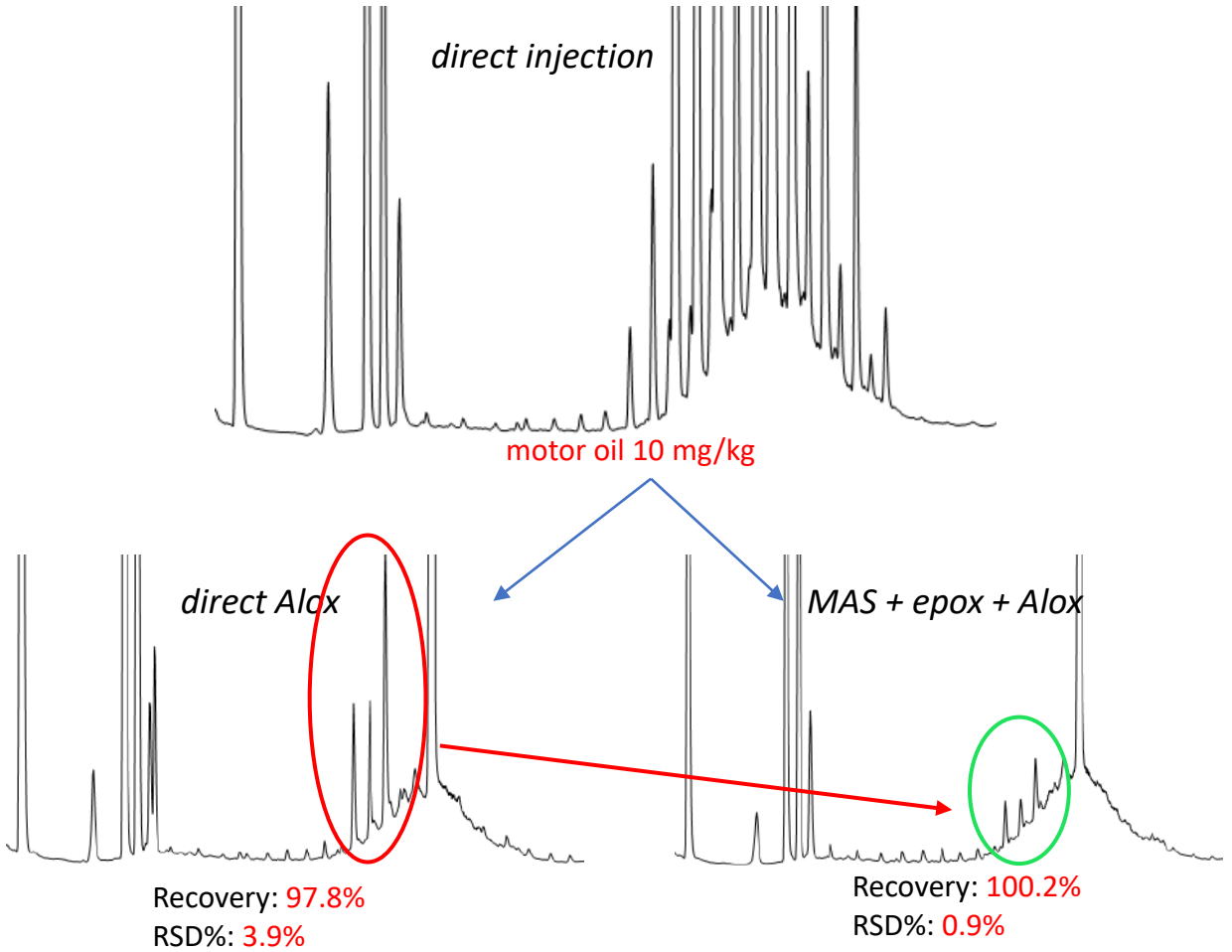
Evaporate the eluate to 150 µL and inject 100 µL into the LC-GC-FID

0.5 mg/kg

RECOVERY

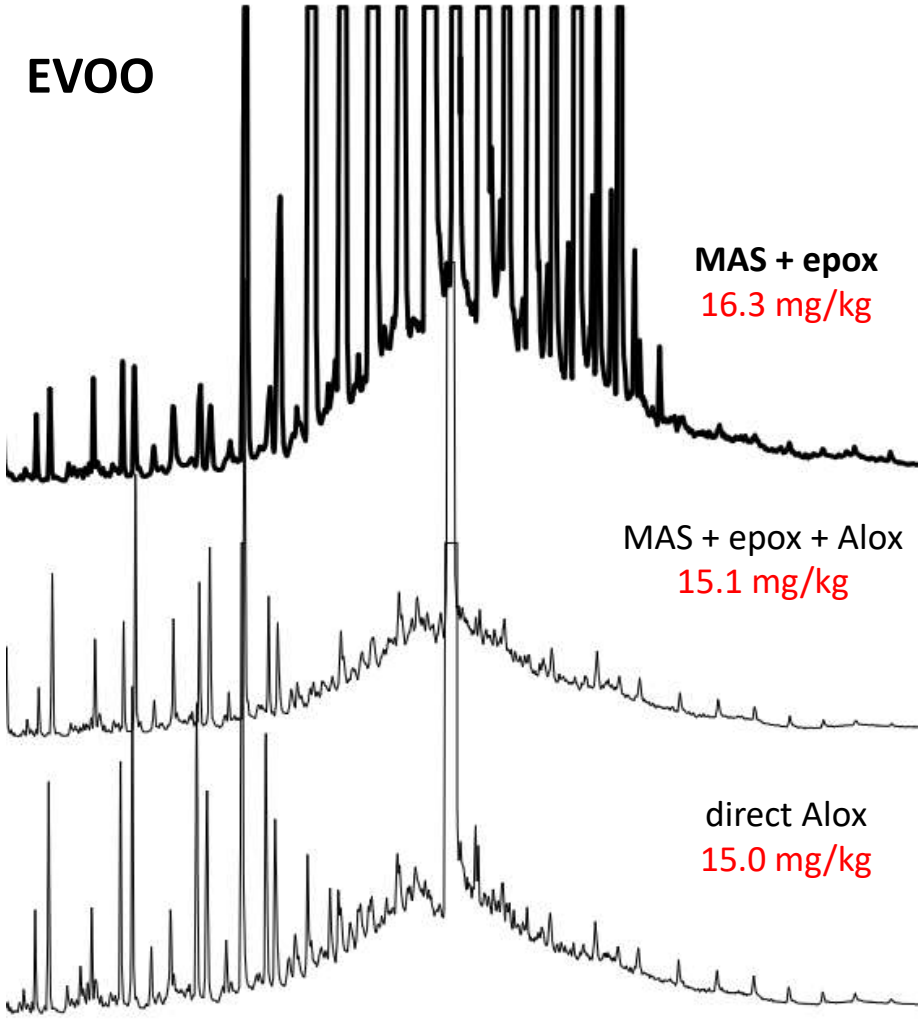
Fortification experiments with Gravex and motor oil





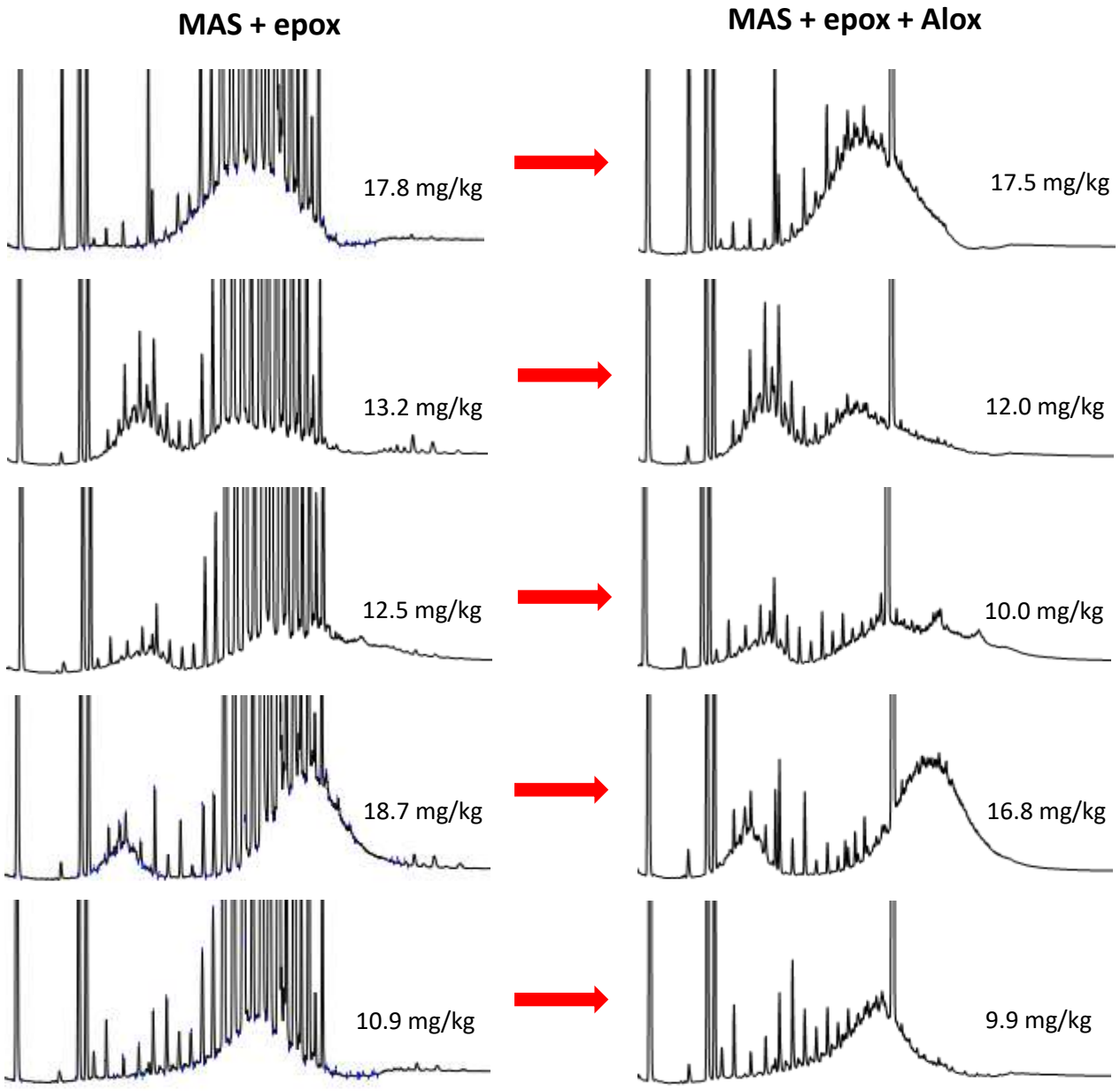
n-alkanes removal is generally better in the absence of matrix

SOME REAL SAMPLES



Higher quantification before Alox due to a slight overload due to *n*-alkanes, but maybe also to the retention of a little fraction of isoalkanes into the SPE.

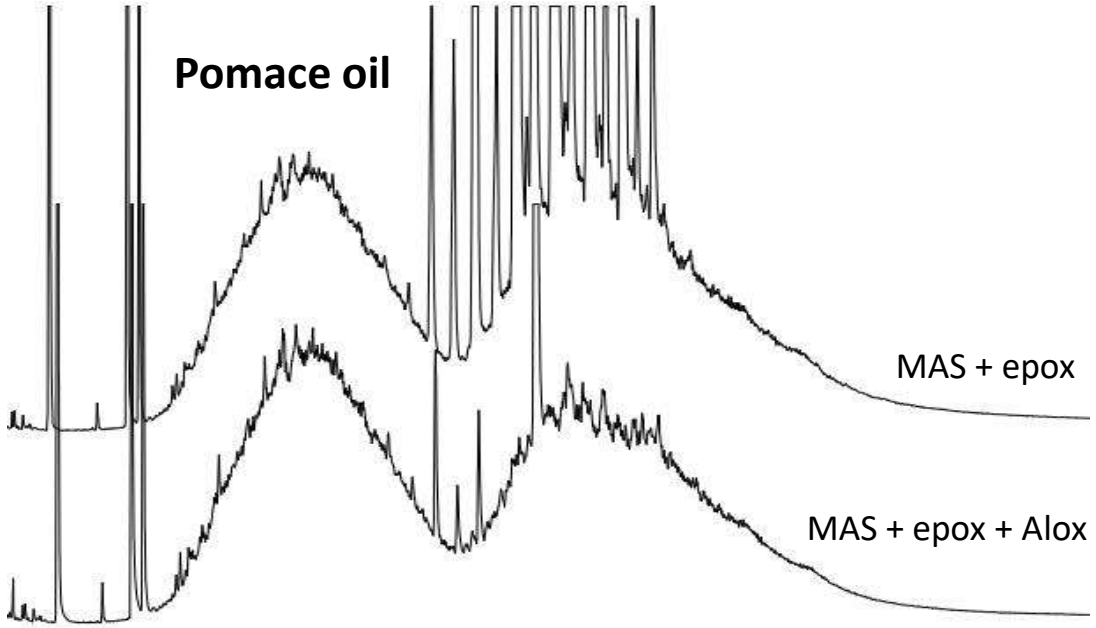
EVOOs

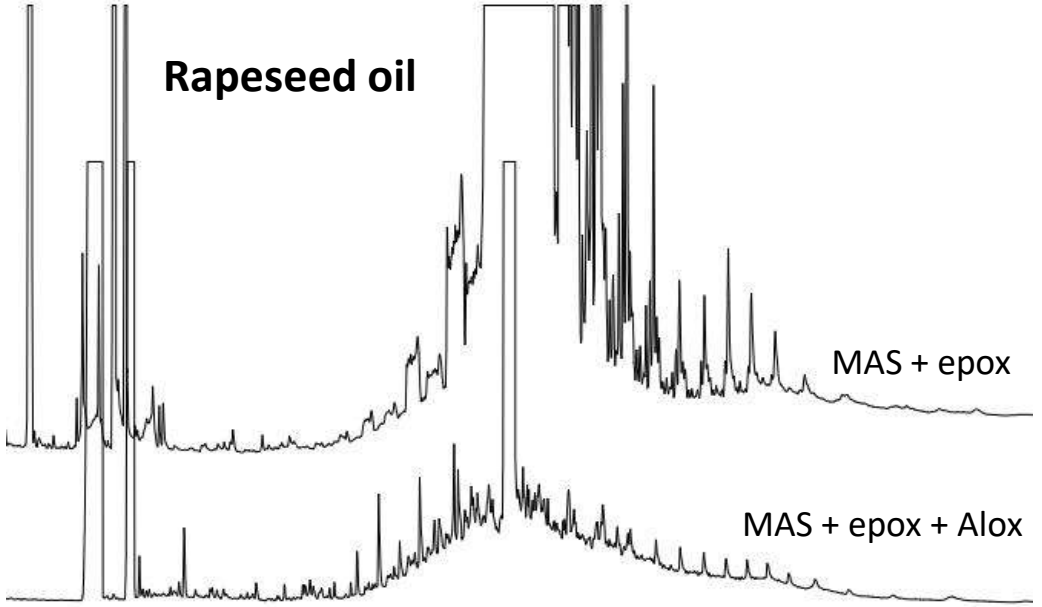


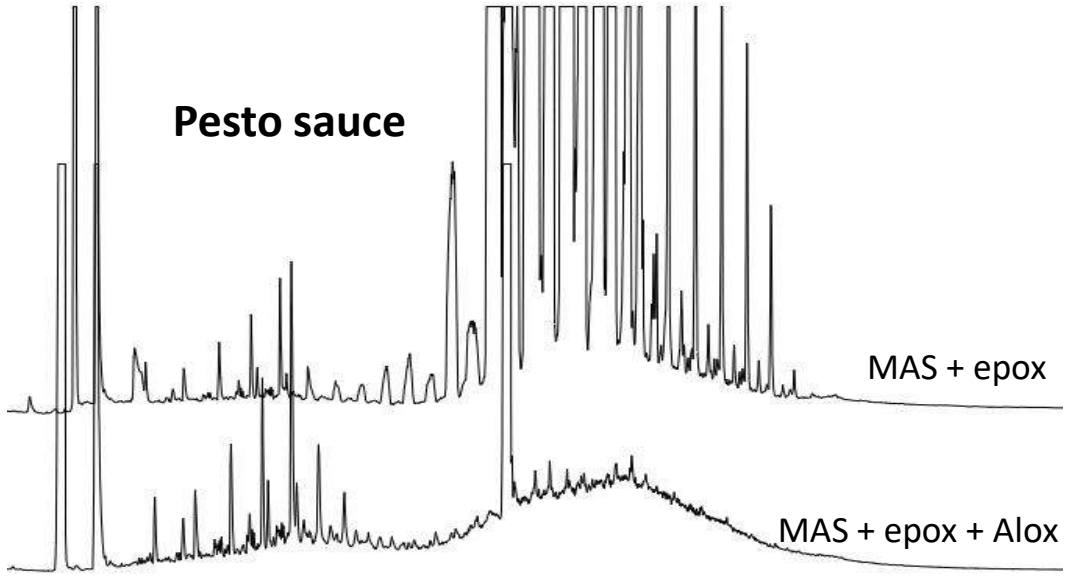
SOME REAL SAMPLES

High efficiency even in samples rich in endogenous *n*-alkanes

Robustness







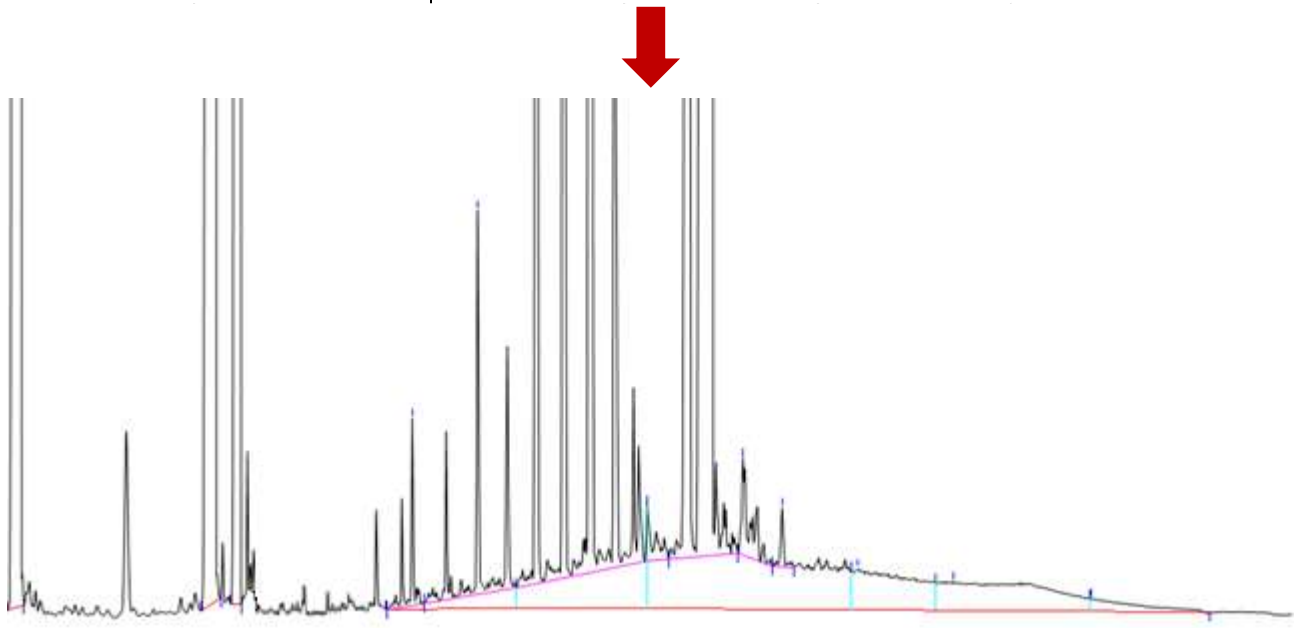
WHY REACHING A LOQ OF 0.5 mg/kg?

Vegetable oils on the market are generally contaminated with levels around 10-20 mg/kg of MOSH on average, so there should't be an objective reason to push on sensitivity that much.

However, the evaluation of background levels, e.g. olives hand-picked from the tree, requires large corresponding amounts of sample to be injected into the LC-GC-FID. Moreover, the LOQ of the single laboratory needs to be lower than the requirements to be sure to meet them when the method is applied INTERLABORATORY.

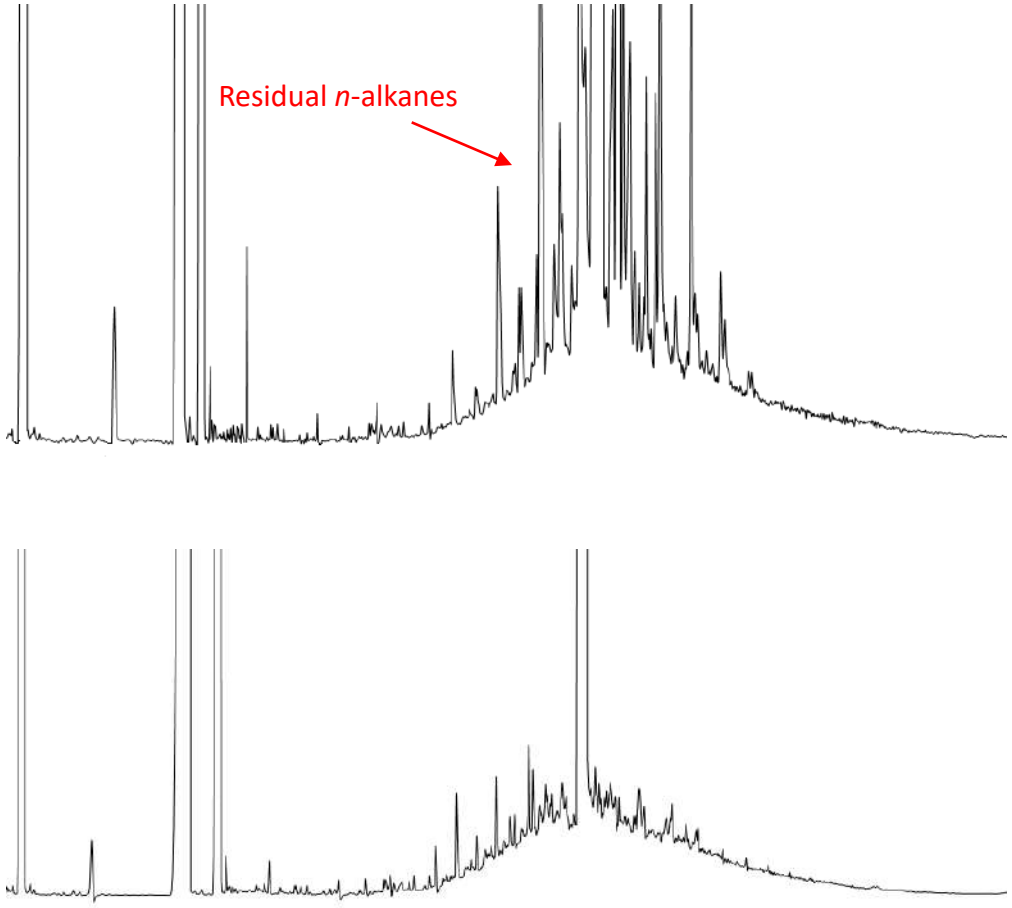
- Olives from the trees
- Olives after harvesting
- Olives after transport
- Olives before washing
- Olives after washing
- Olive paste after milling
- Olive paste after malaxation
- Olive pomace after the separator
- Olive oil after the separator
- Olive oil after the vertical centrifuge

	Buje	Vosteni	Parenzo
Leccino	1.3	0.9	1.0
Bianchera	0.6	0.6	0.7
Pendolino	1.2	1.4	1.1
Rosignola	-	1.0	1.0



REPEATABILITY: THE ENVIRONMENTAL CONDITIONS

Even when using freshly activated silica and aluminum oxide, the humidity of the matrix and of the laboratory environment can affect the effectiveness of the SPE in the retention of *n*-alkanes.



+ drying of the glass cartridges in the oven
+ reactivation of drying salts of the desiccator
+ 500 mg of Na_2SO_4 on the top of the SPE

Anyway, a complete removal is usually not necessary. An incomplete removal that allows *n*-alkanes to be resolved and not overloaded is enough to perform a reliable quantification.

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- Chelab S.r.l.