MOSH and MOAH: occurrence and toxicological evaluation

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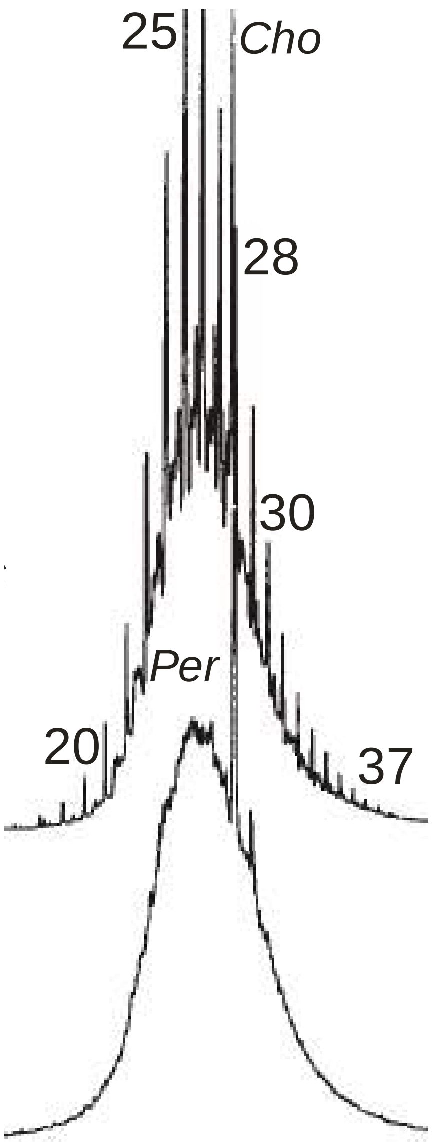
The issues

- MOSH/MOAH measurements are wrong
  - no standardized method available
- MOSH and MOAH are naturally formed by plants
- Environmental contamination is inevitable
- Olive pomace oils contain 250-400 mg/kg MOSH
  - and nobody complains
- Present MOSH and MOAH reference values have no toxicological base…
  - …and are far exaggerated

Measurements are wrong

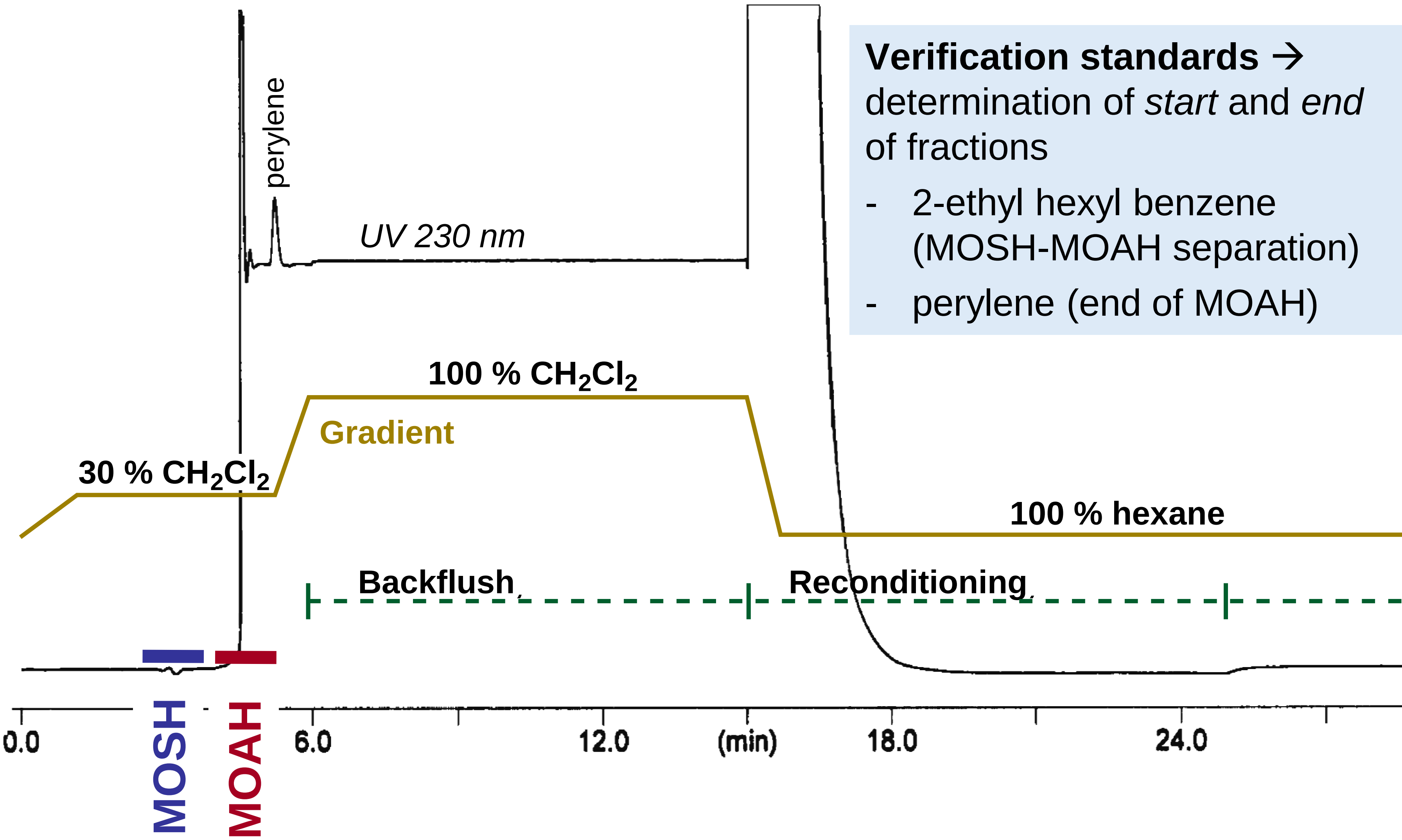
- MOSH/MOAH analysis is demanding!
  - battery of methods required
  - experience in interpretation of chromatograms
- Socialism in chemical analysis: Do the weakest laboratories determine whether the analysis is possible?
- Standardization ensures that all do the same - same errors?
  - interpretation of chromatograms cannot be standardized
- Is the quality of the data limited by the price the customer is willing to pay?
  - often additional steps would clarify – but are considered too expensive

Components of MOSH/MOAH analysis



- Extraction of the sample (solid samples!)
- HPLC preseparation
  - isolation from sample matrix, e.g. fat
  - separation of MOSH and MOAH
- GC-FID analysis (virtually equal response)
  - large volume GC injection/transfer
- Auxiliary methods
  - enrichment to achieve more reliable data
  - removal of natural n-alkanes (aluminum oxide)
  - removal of natural olefins (epoxidation)
  - GCxGC for confirmation, e.g. distinction from POSH
- Correct interpretation of chromatograms

HPLC isolation, MOSH/MOAH separation

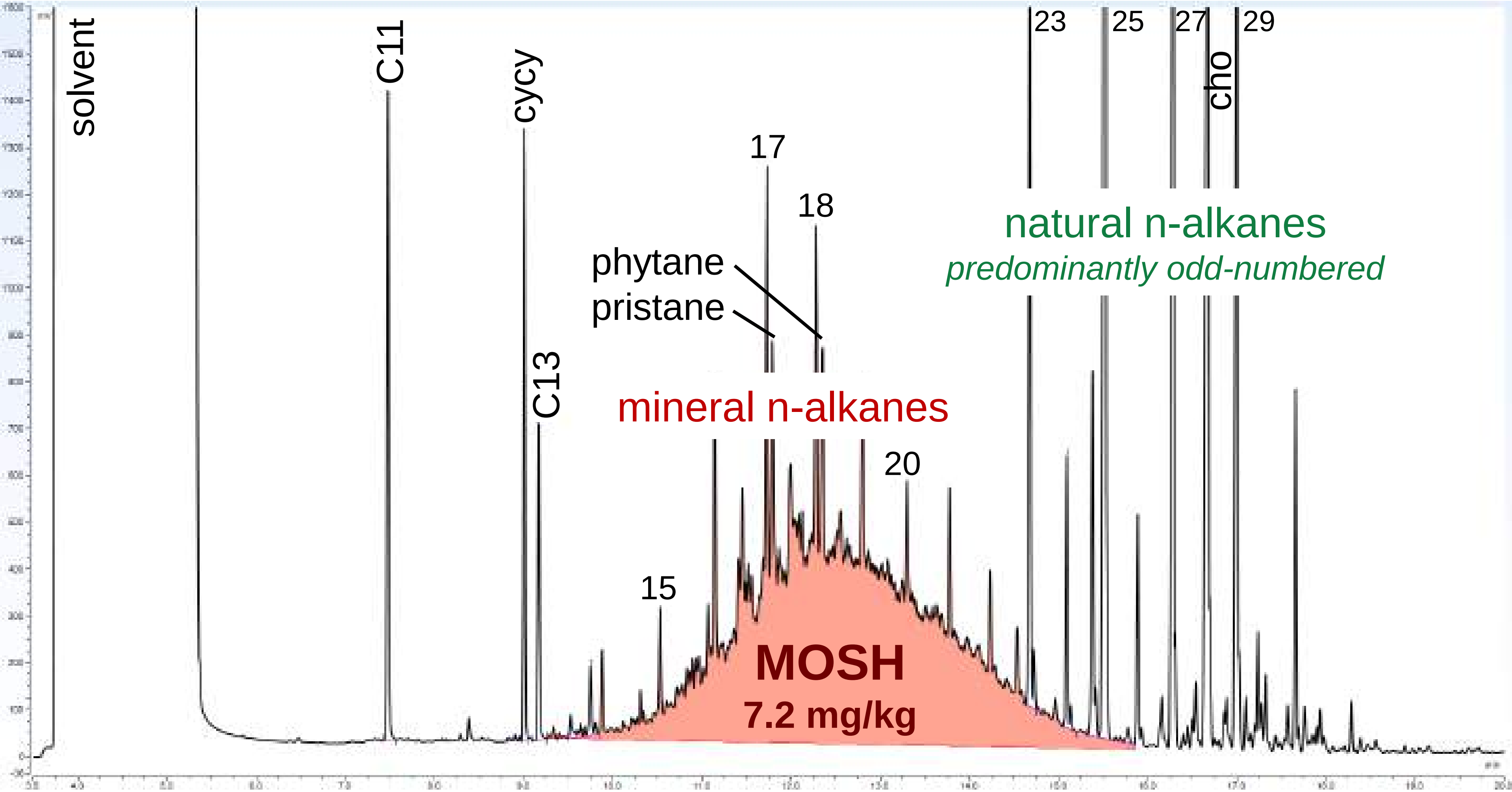


Verification standards → determination of *start* and *end* of fractions

- 2-ethyl hexyl benzene (MOSH-MOAH separation)
- perylene (end of MOAH)

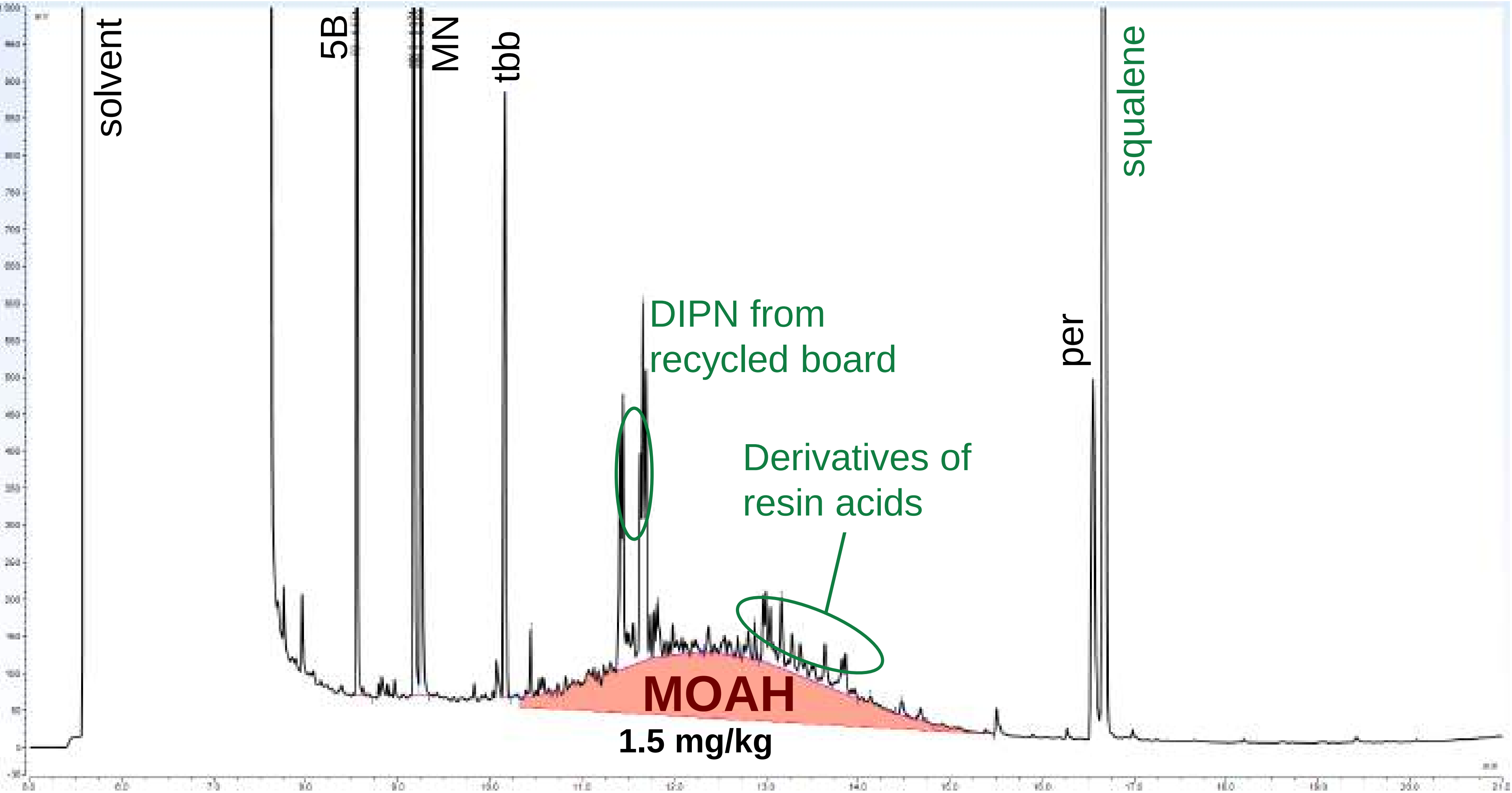
normal phase HPLC: e.g. Lichrospher Si 60 (250 x 2 mm i.d.)

Example for MOSH fraction: couscous



- homogeneous distribution of n-alkanes → MOSH
- natural waxes, terpenes → no MOSH

Example for MOAH fraction: couscous



- similar molecular mass distribution of MOSH and MOAH
- MOAH concertation smaller than MOSH

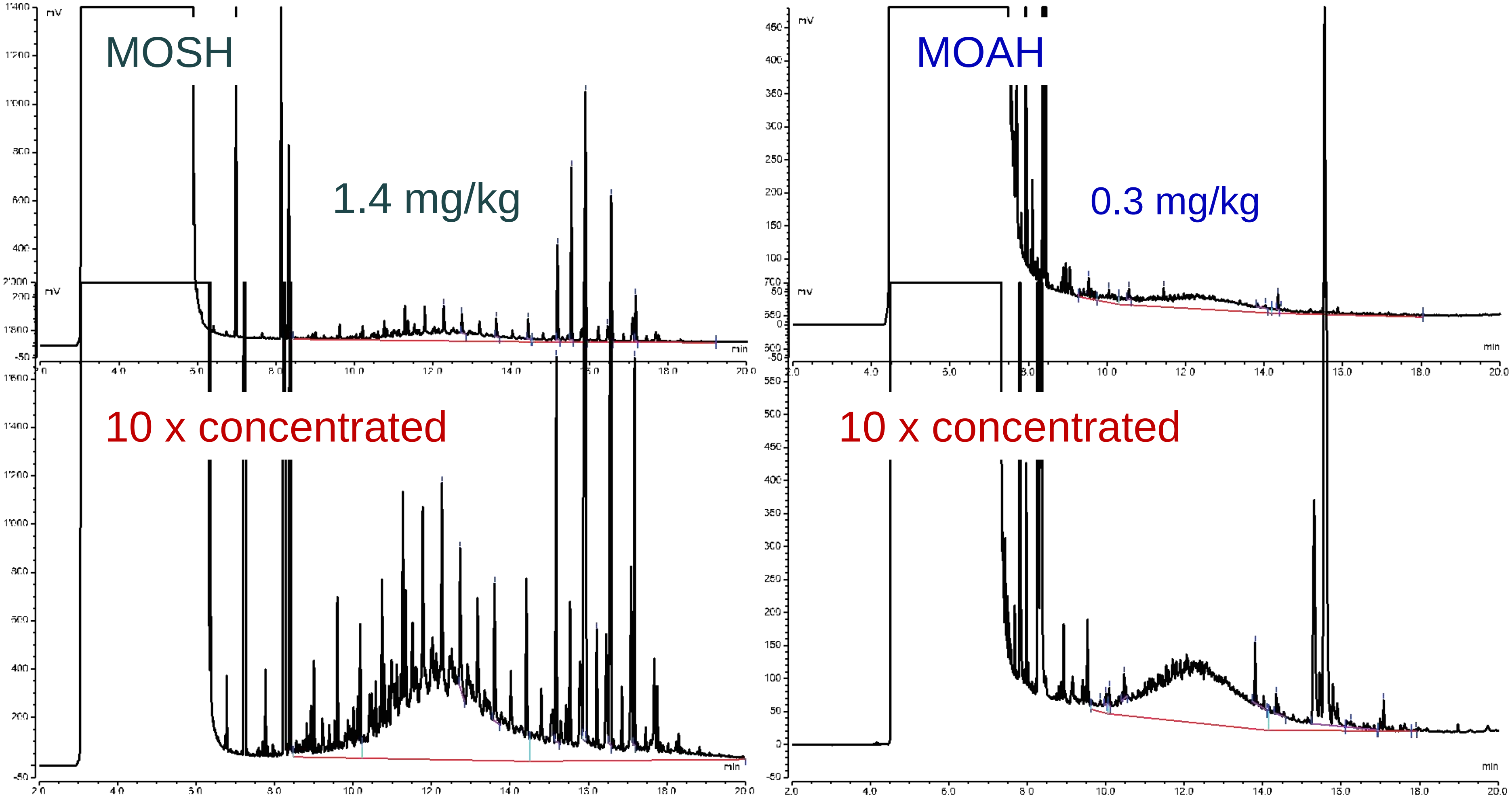
Limit of quantification without enrichment

Possible reconcentration of food extracts is limited by the capacity of the HPLC column for triglycerides: 20 mg

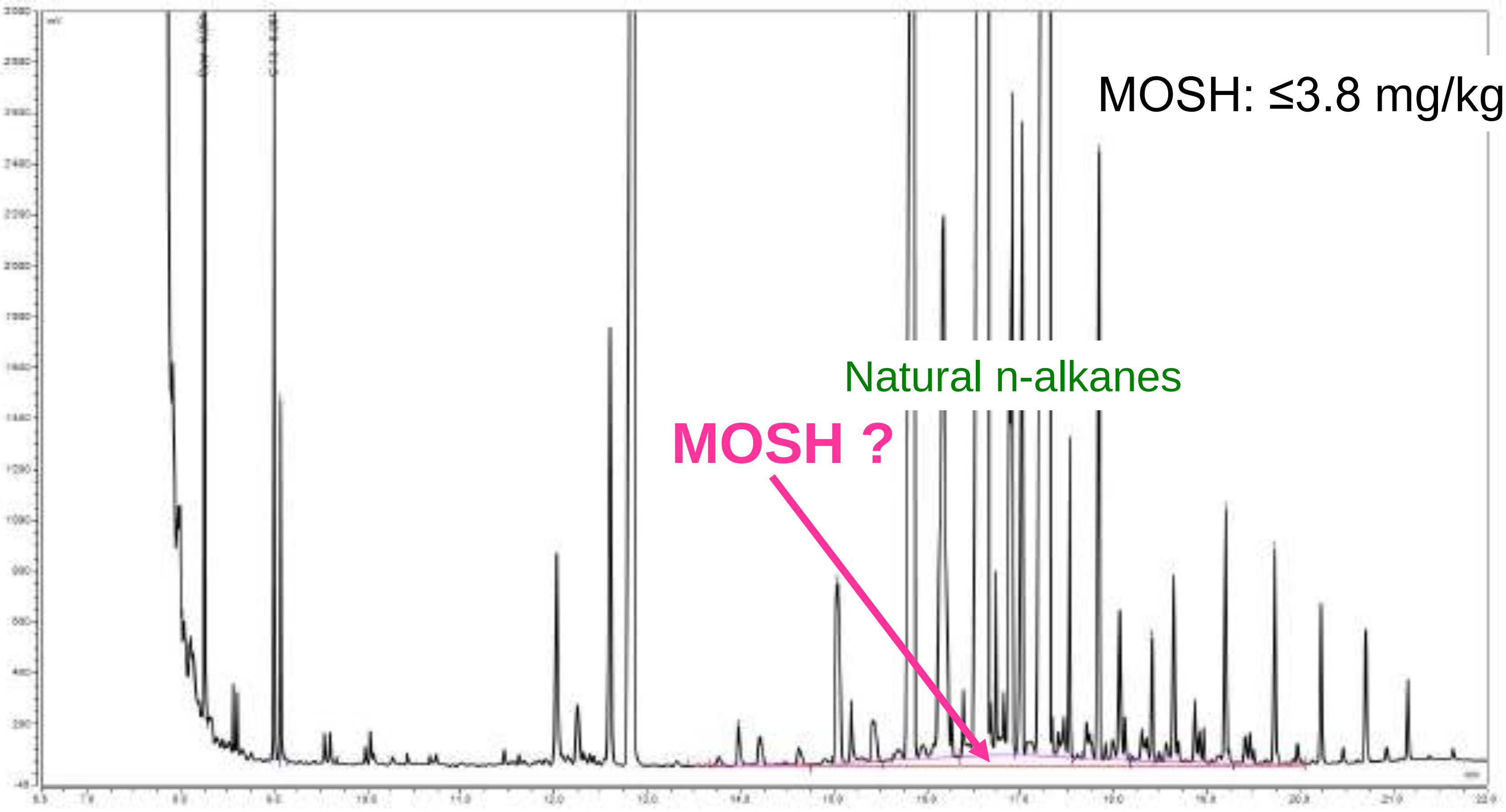
Referring to amount of sample; 100 µl injections into HPLC:

- low fat (≤4 %) samples (e.g. rice, corn, noodles)
  - 10 times (10 g food to 1 mL hexane) → LOQ ca. **0.1 mg/kg**
- medium fat (~20 %) samples (e.g. cereals, muesli, biscuits)
  - no reconcentration (1 g to 1 mL) → LOQ ca. **0.5 mg/kg**
- high fat (~40 %) samples (e.g. chocolate)
  - only half amount/concentration (0.5 g to 1 mL) → LOQ ca. **1 mg/kg**
- vegetable oils
  - 20 % solutions → LOQ ca. **2.5 mg/kg**

Reconcentration of extracts

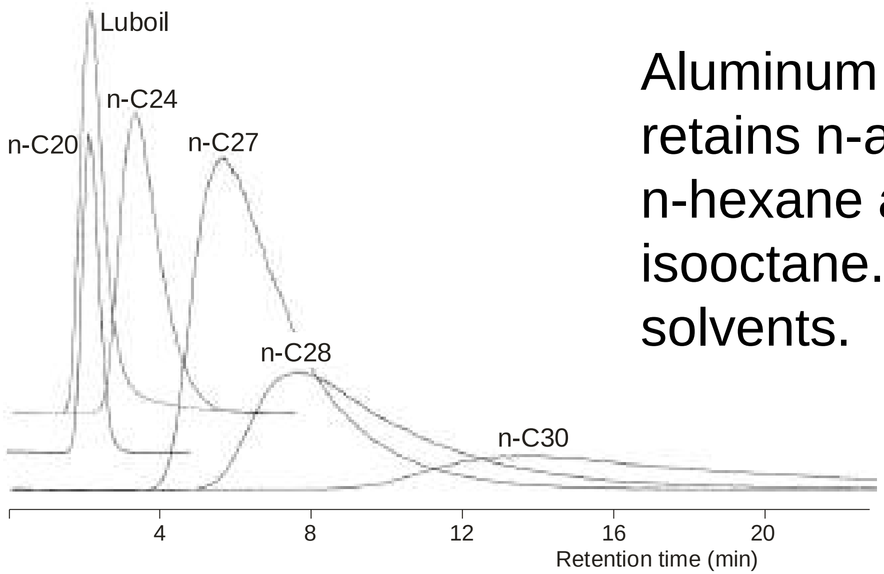


Interferences: MOSH in sunflower oil



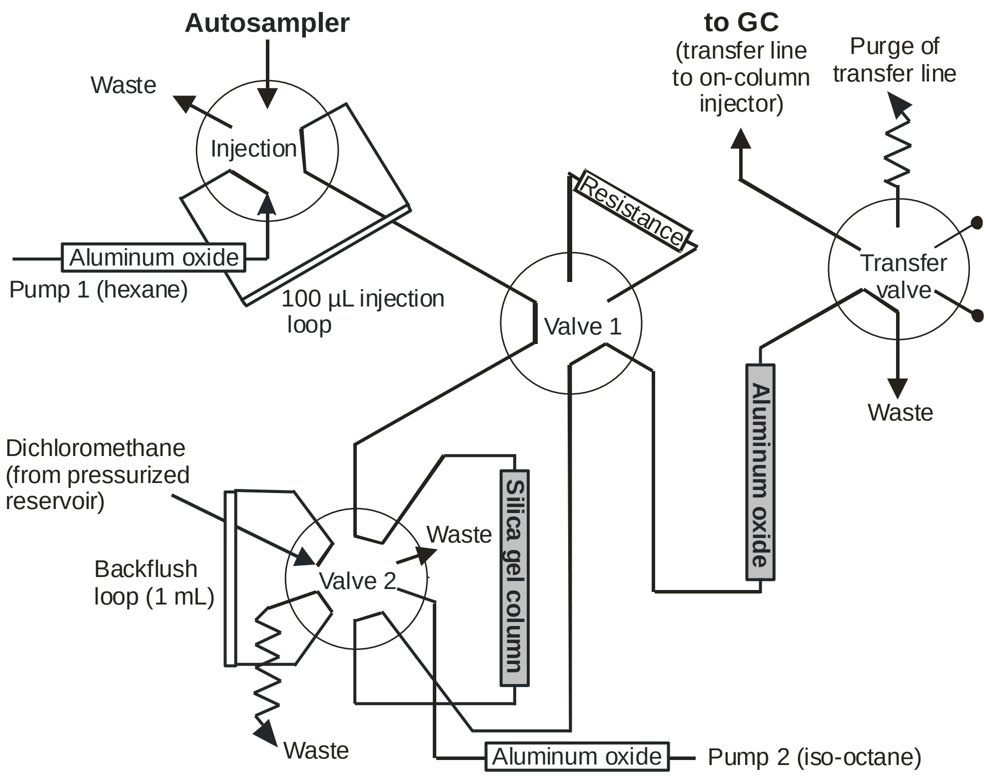
→ Enrichment and removal of long-chain n-alkanes

Removal of natural n-alkanes



Aluminum oxide activated at about 400 °C retains n-alkanes above about C24 using n-hexane as eluent – no retention with isooctane. Prerequisite: no humidity or polar solvents.

Performed off-line (SPE-style) or on-line LC (SiO_2) – LC (alox), with backflush of the alox by isooctane

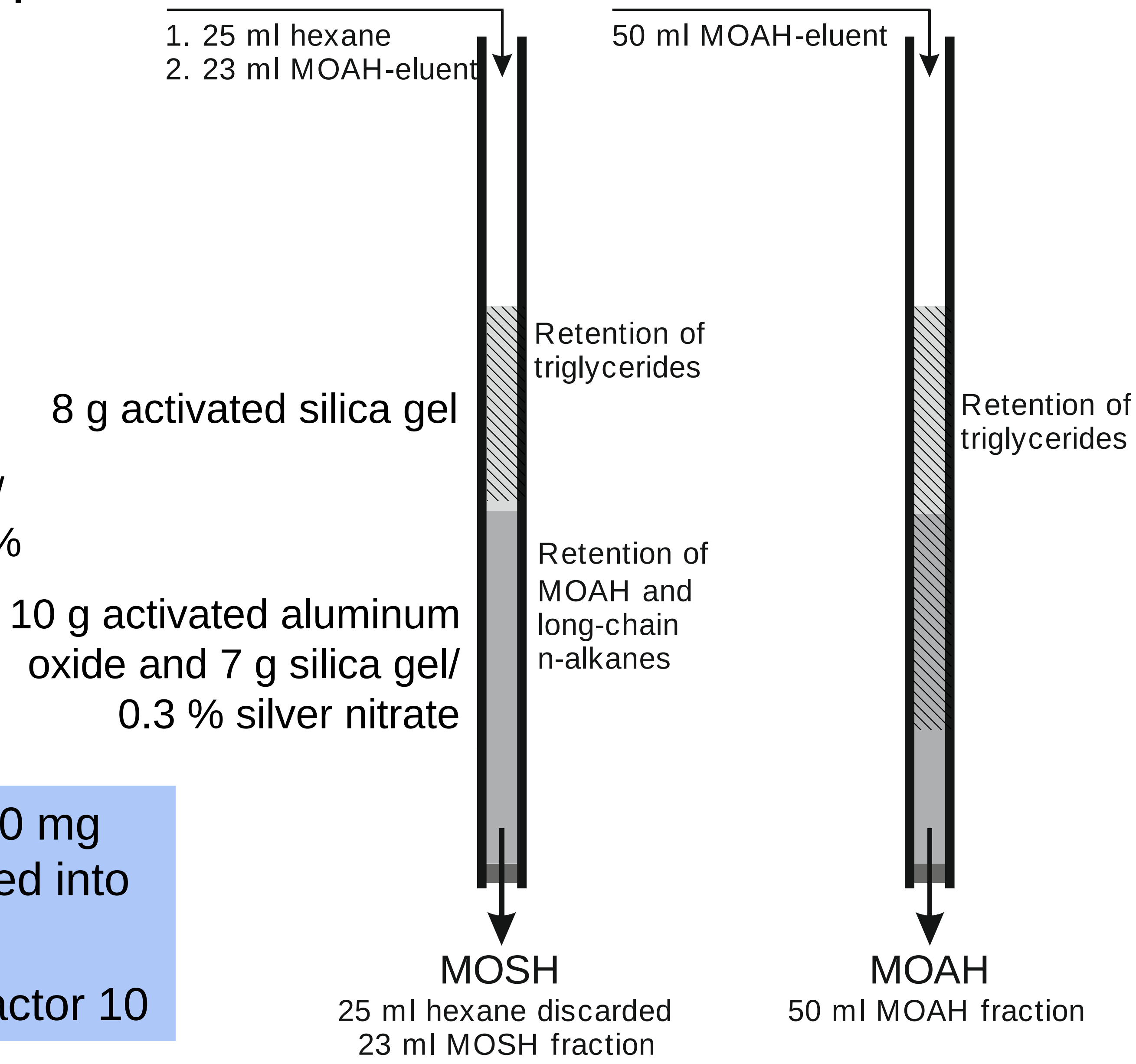


Activated aluminum oxide selectively retaining long chain n-alkanes. Part I, description of the retention properties.
K. Fiselier, D. Fiorini, K. Grob. Anal. Chim. Acta 634 (2009) 96–101

Activated aluminum oxide selectively retaining long chain *n*-alkanes. Part II, integration into an on-line HPLC-LC-GC-FID method to remove plant paraffins for the determination of mineral paraffins in foods and environmental samples
K. Fiselier, D. Fiorini, K. Grob. Anal. Chim. Acta 634 (2009) 102–109.

Enrichment + removal of n-alkanes

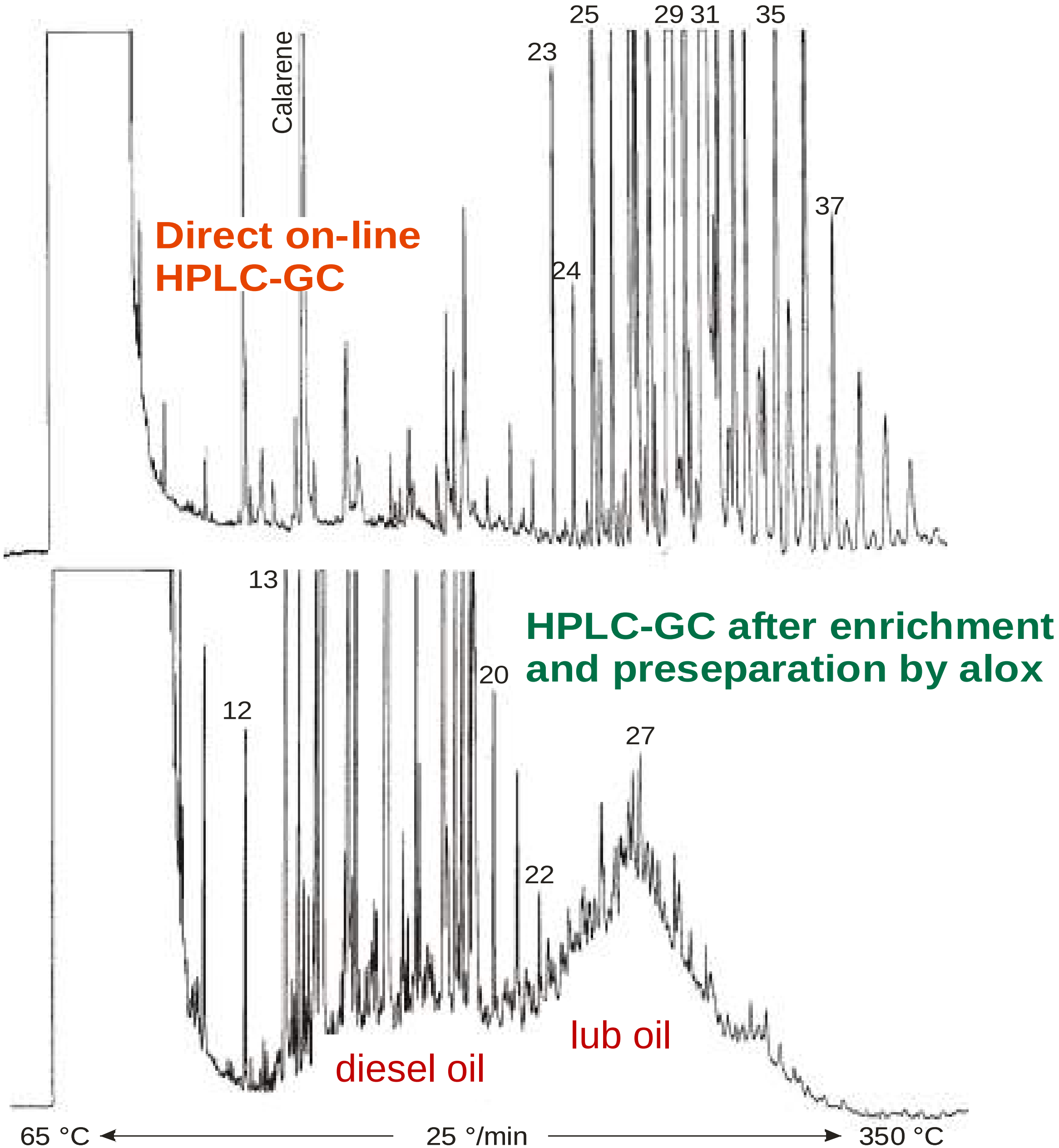
MOAH eluent:
25 % dichloromethane/ 0.25 % toluene/74.75 % hexane



→ Equivalent of 200 mg oil/fat can be injected into LC-GC

→ enrichment by factor 10

Example: sunflower oil

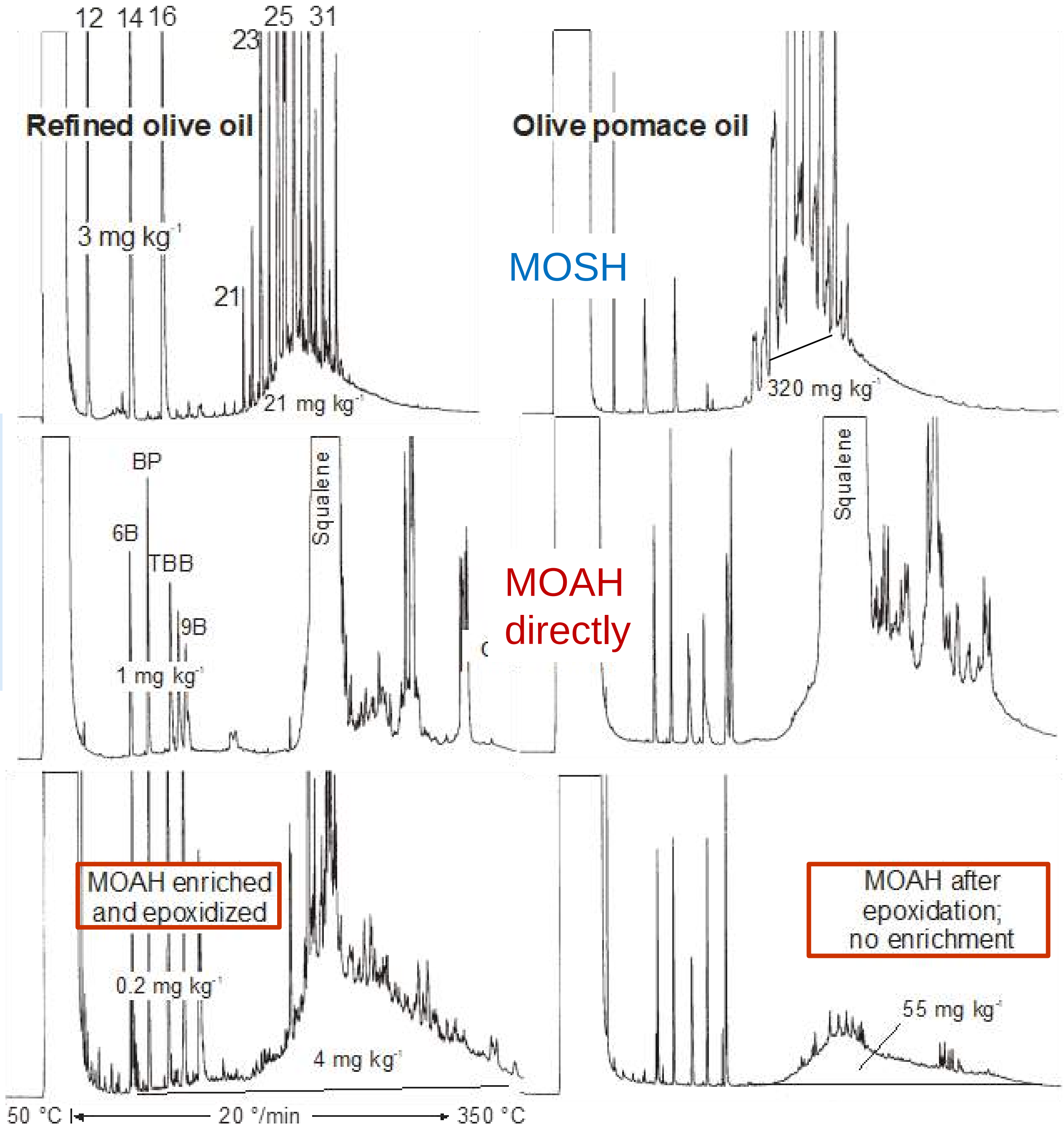


oil extracted from manually collected seeds

total MOSH content: 1.4 mg/kg

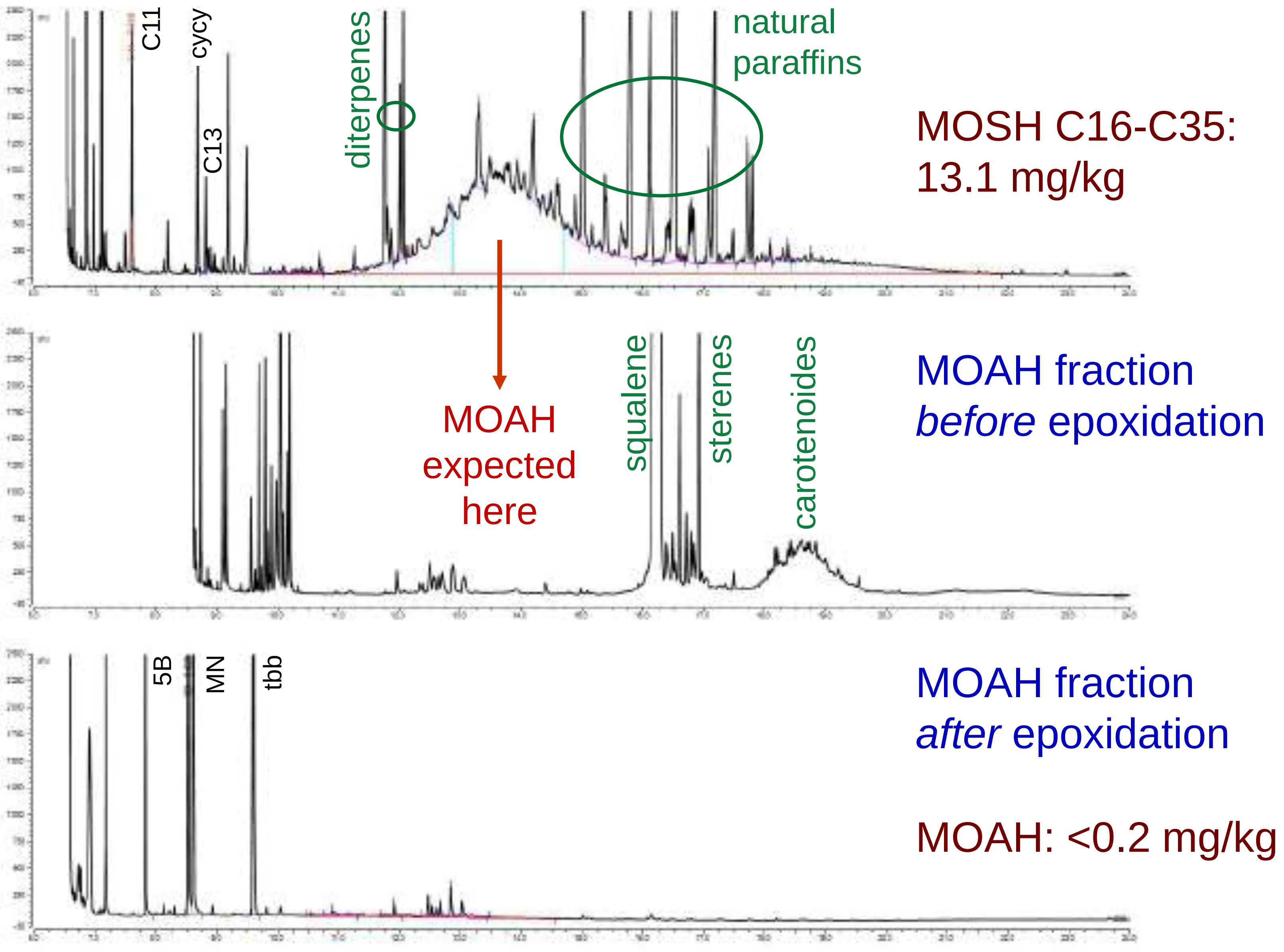
Examples needing epoxidation

Epoxidation renders olefins more polar → retention on LC beyond MOAH



Aromatic hydrocarbons of mineral oil origin in foods: method for determining the total concentration and first results
M. Biedermann, K. Fiselier and K. Grob
J. Agric. Food Chem. 57 (2009) 8711-8721

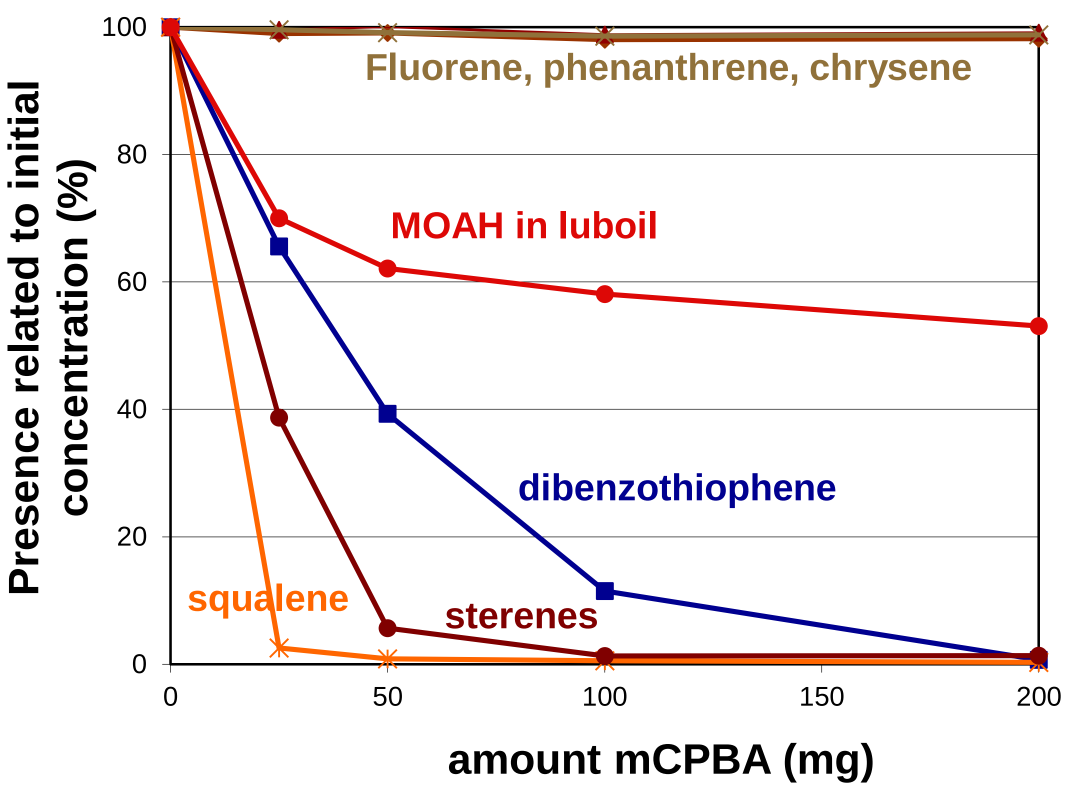
Epoxidation not always needed: Panettone



Epoxidation: the best presently available

Epoxidation of olefins is faster than that of most MOAH, but

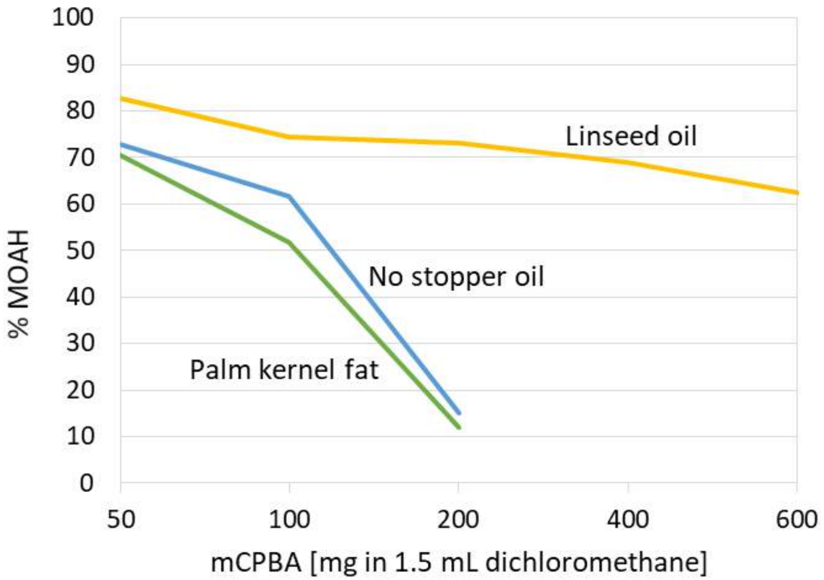
- partial loss of MOAH
- removal of interferences may remain incomplete

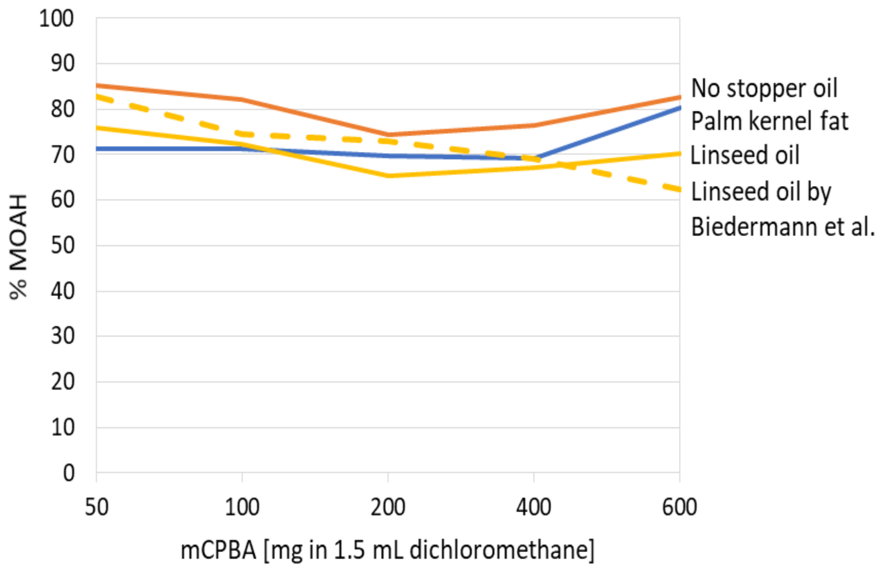


The two methods for epoxidation

Biedermann et al. (2009)
- Reaction in dichloromethane
- Fast → requires cooling
- Reaction stopped by polyunsaturated fats/oils

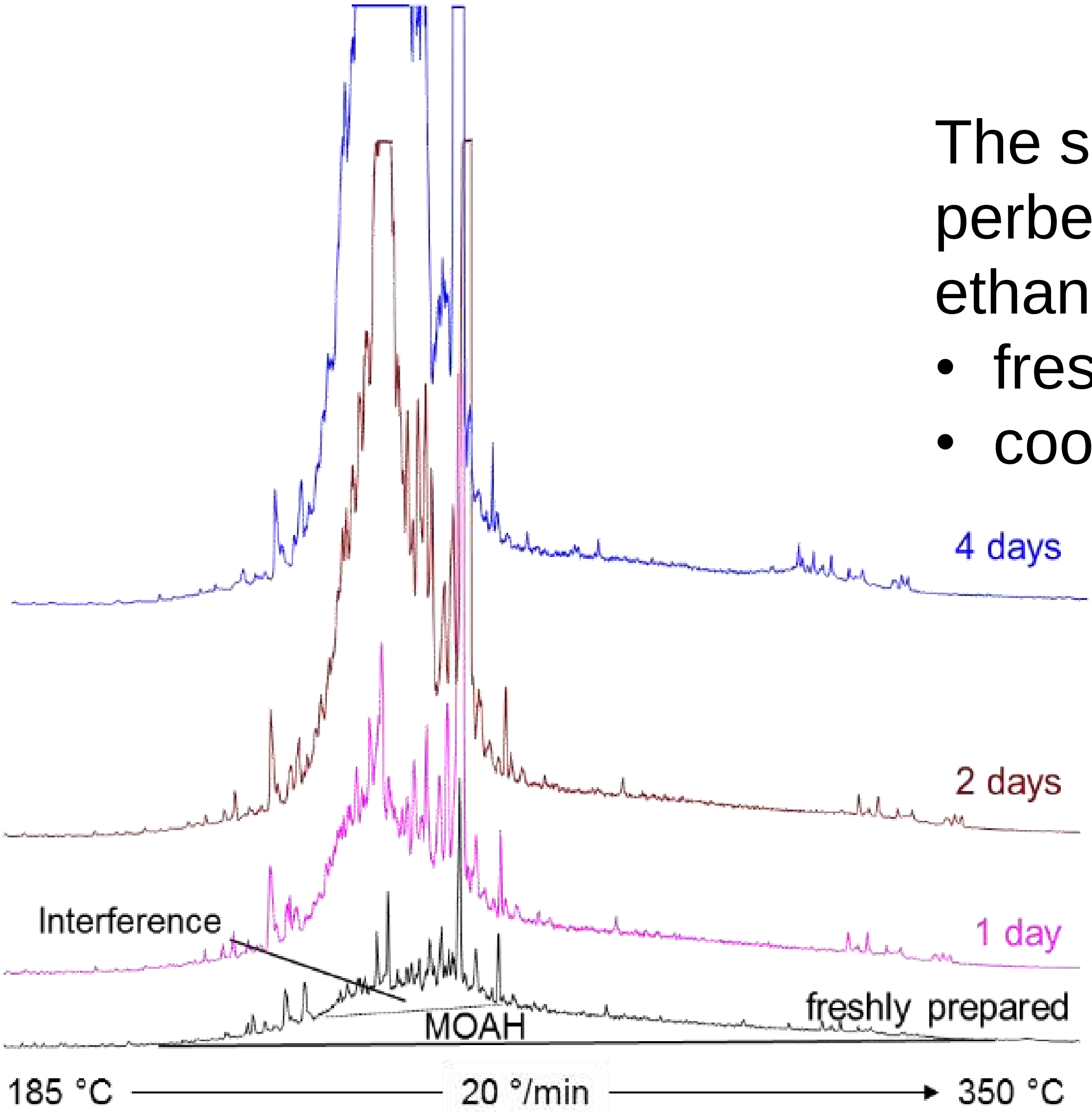
Nestola/Schmidt (2017)
- Reaction in ethanol: far slower!
- No cooling required (autosampler!)
- Reaction kinetically stopped
- No evaporation step





We prefer the Nestola/Schmidt method:
- more convenient, particularly for automated preparation
- MOAH losses are same
- peracid is not stable in ethanol: fresh solutions

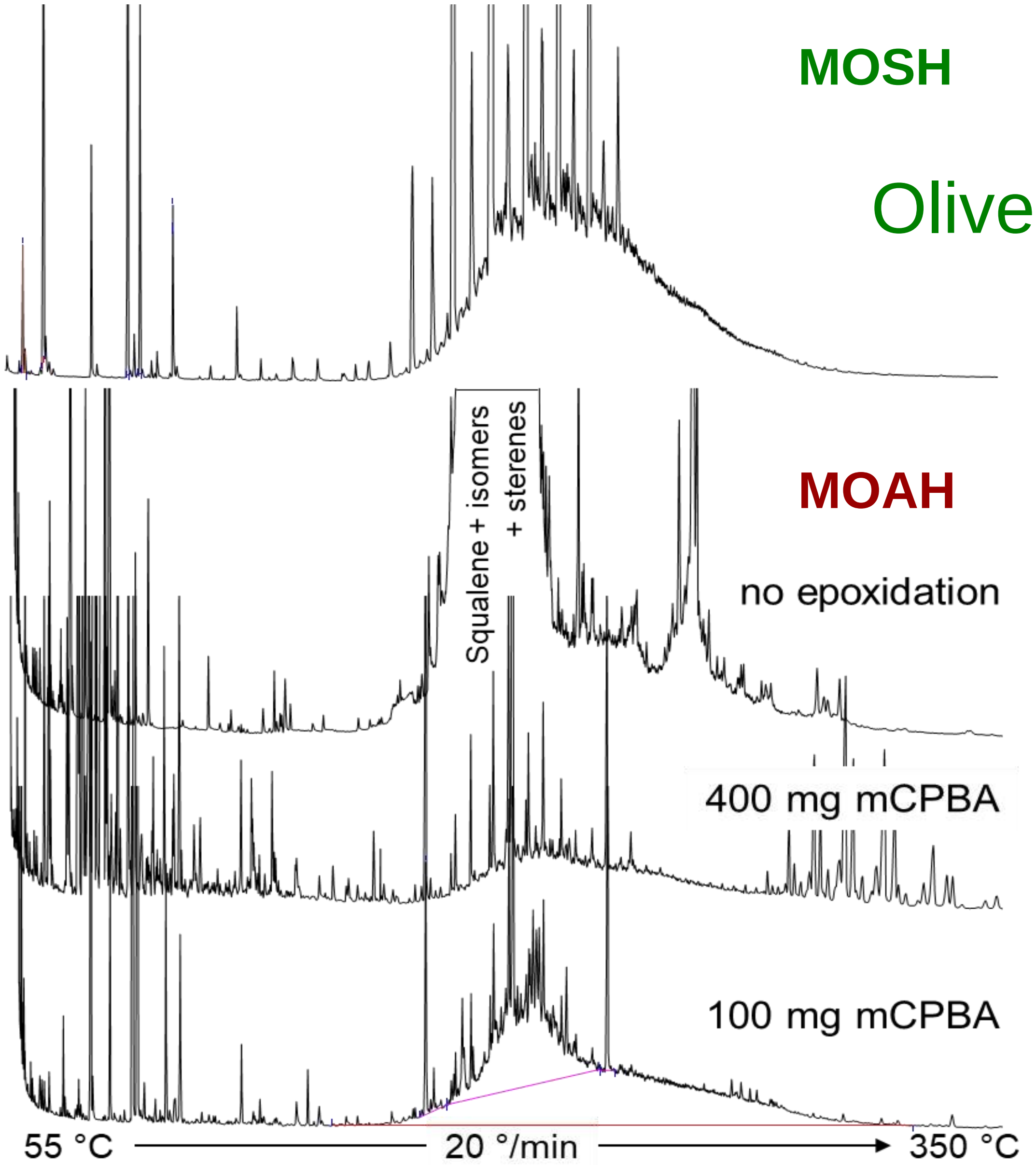
The peracid is not stable in ethanol



The solution of 3-chloro-perbenzoic acid in ethanol needs being

- fresh
- cooled

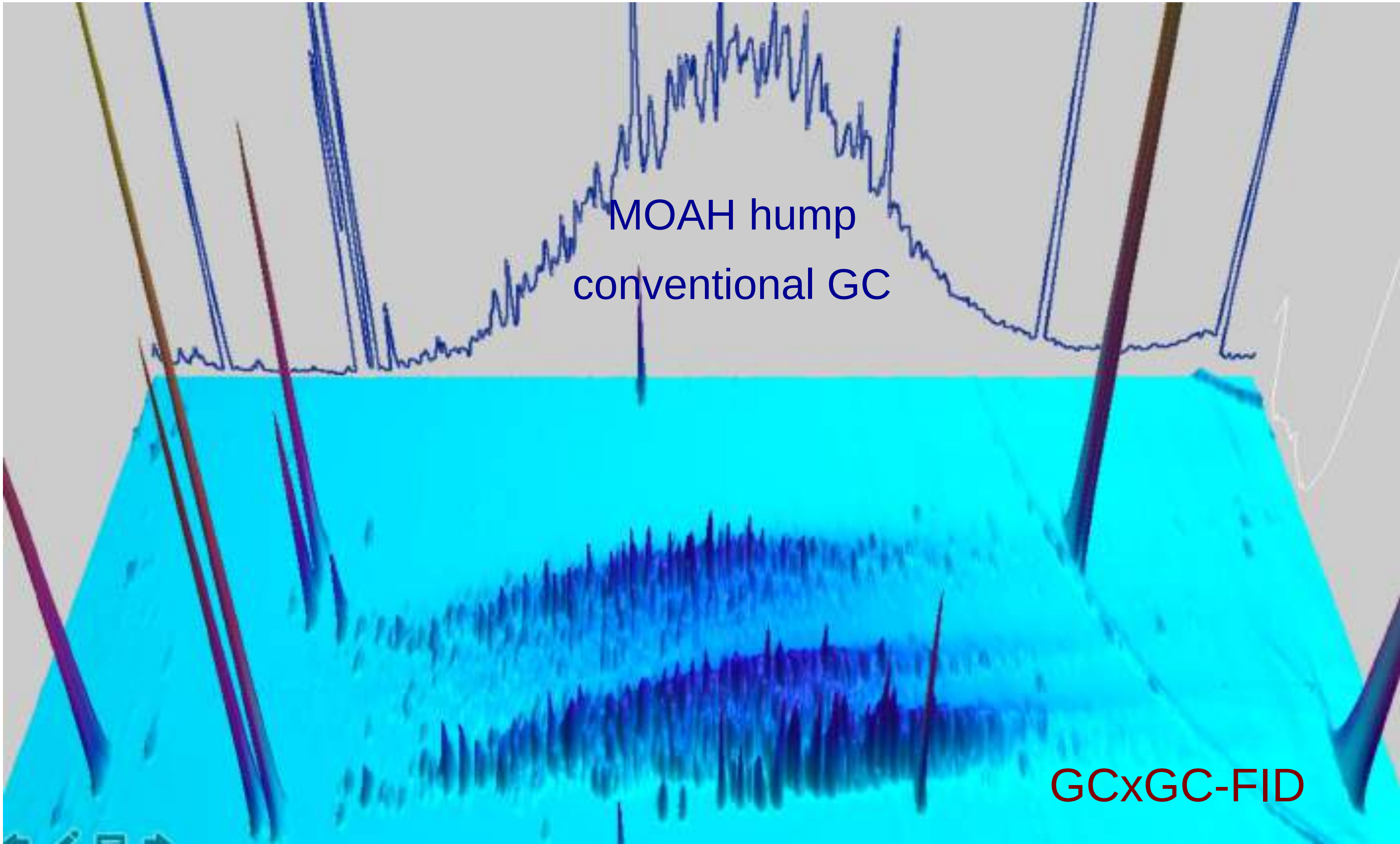
Incomplete removal of interferences



Olive pomace (sansa) oil

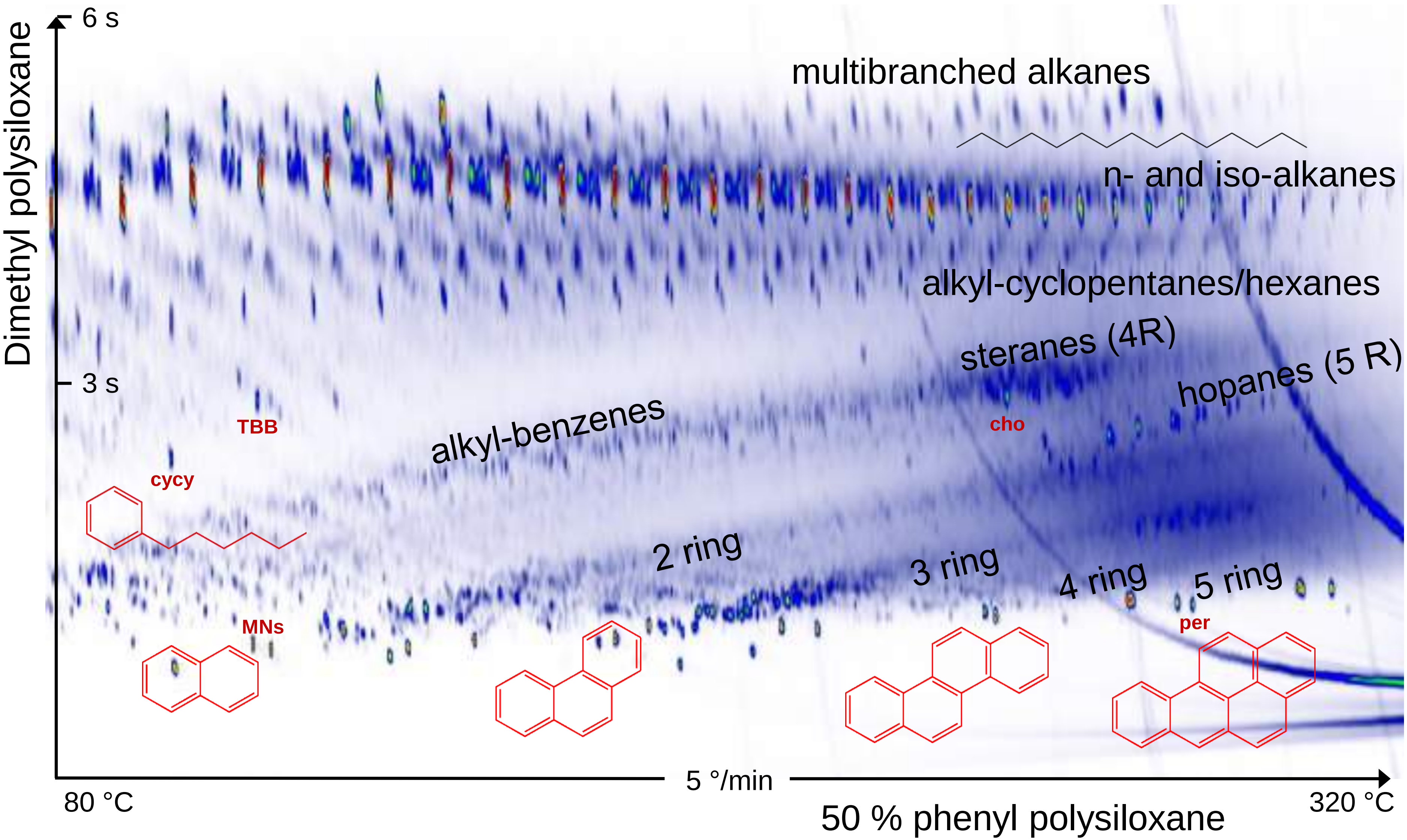
Safe recognition of interference may be more important than efficient removal!

Characterization by comprehensive two-dimensional GC (GCxGC)

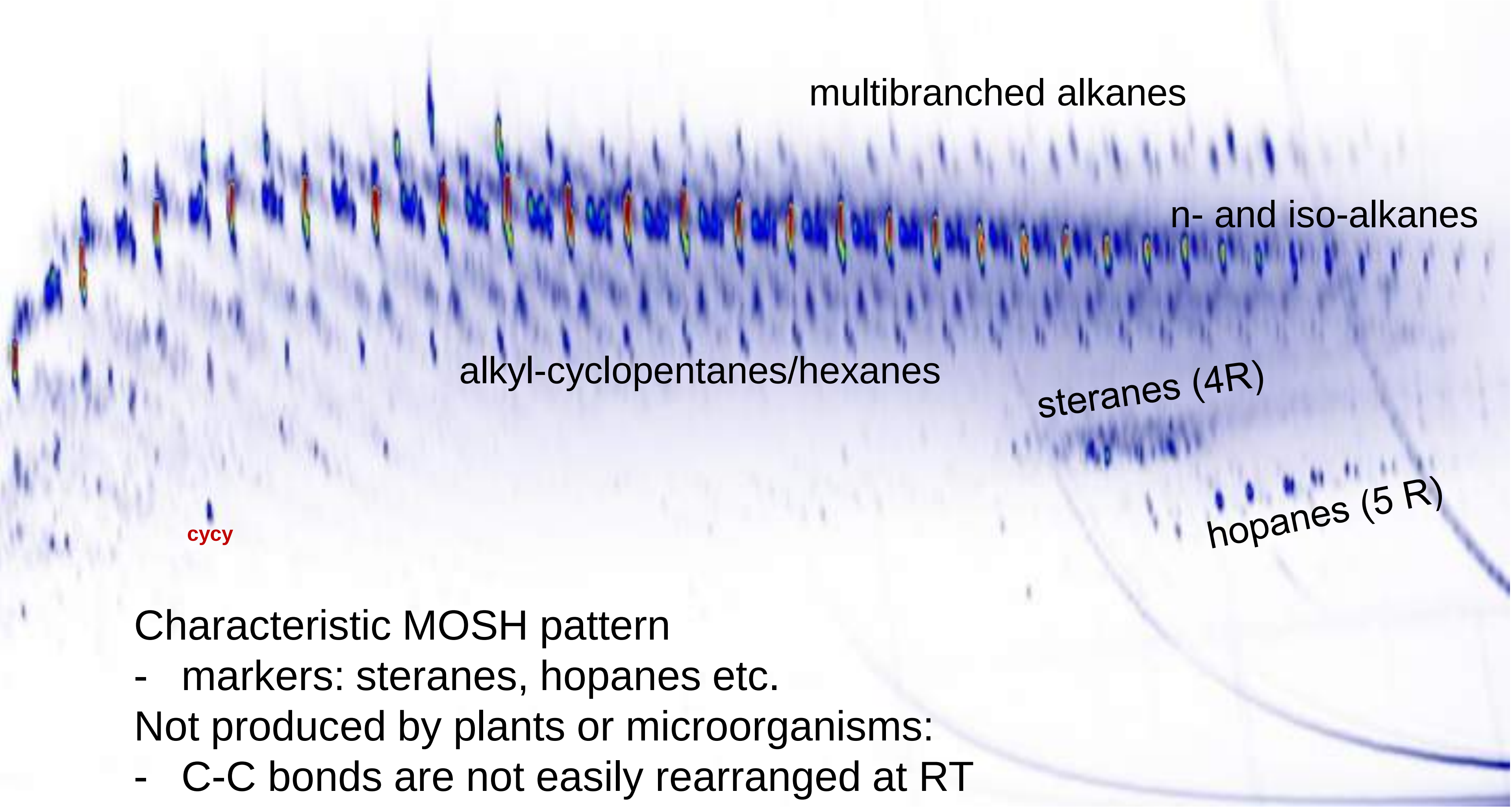


GCxGC-FID of mineral oil hydrocarbons

Mixture of crude mineral oil fractions + 16 EPA PAHs, column set: polar – apolar, FID



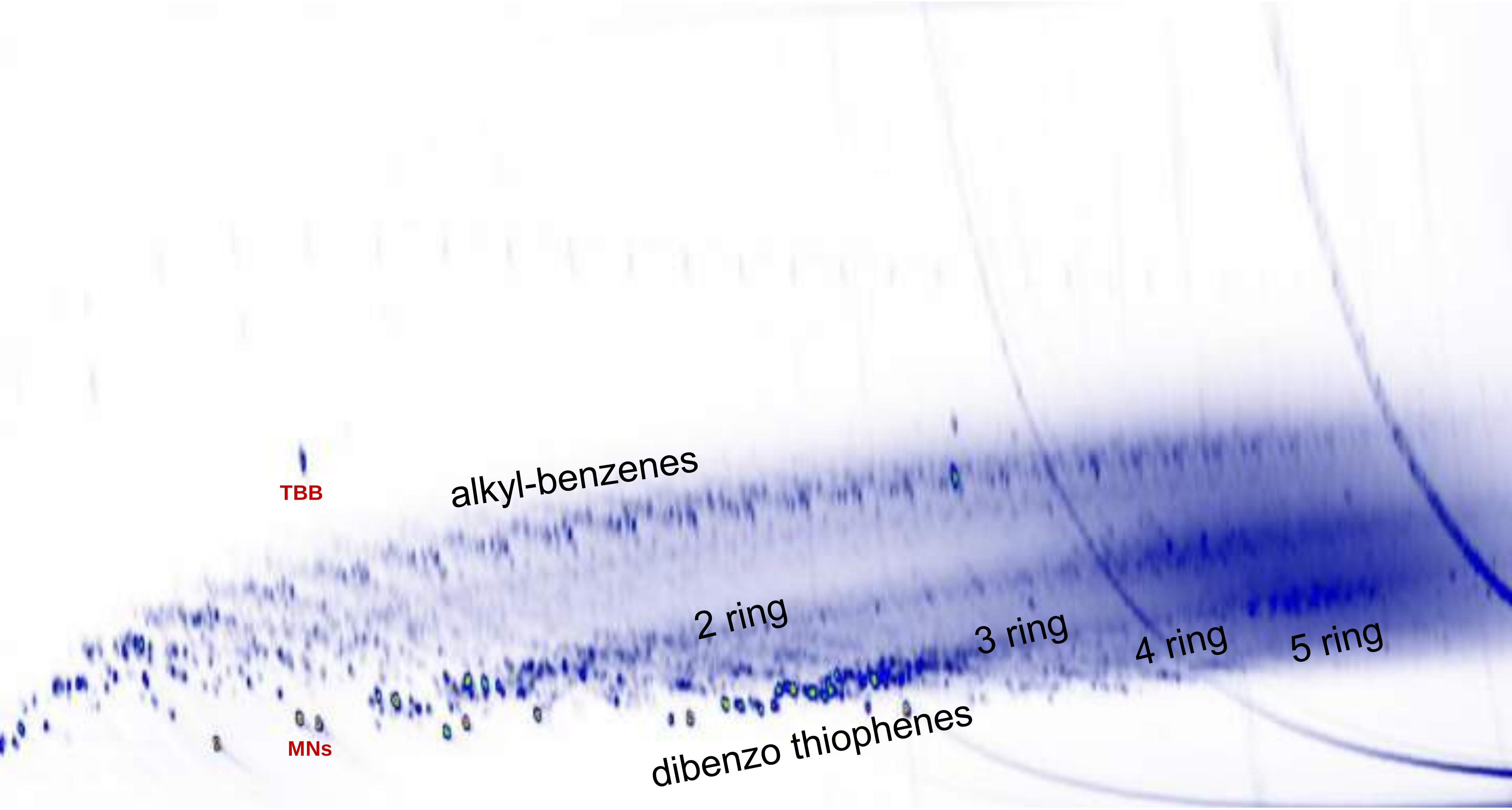
Plants produce MOSH and MOAH???



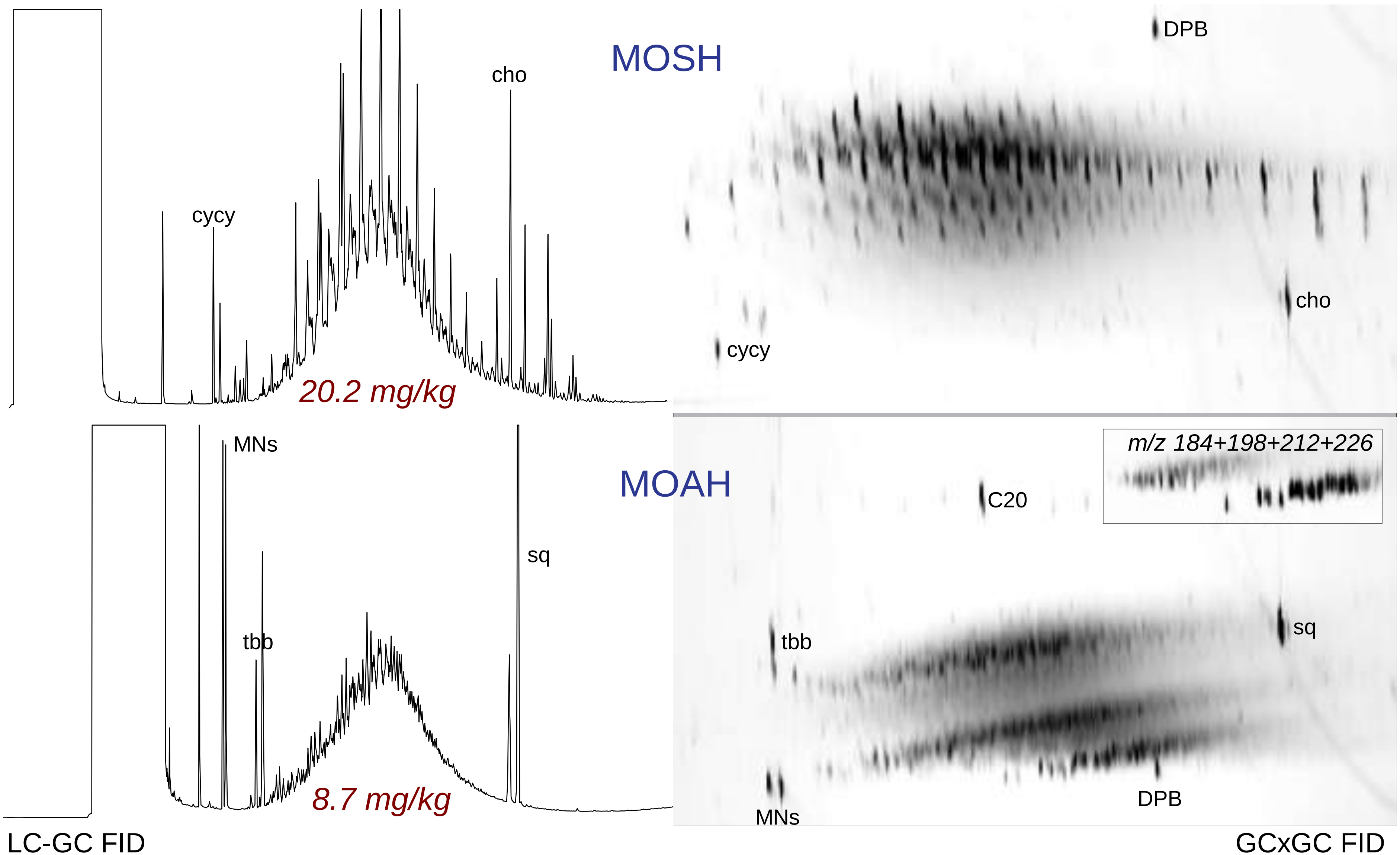
Characteristic MOSH pattern
- markers: steranes, hopanes etc.

Not produced by plants or microorganisms:
- C-C bonds are not easily rearranged at RT
- fermentation of olive pomace did not produce MOSH or MOAH

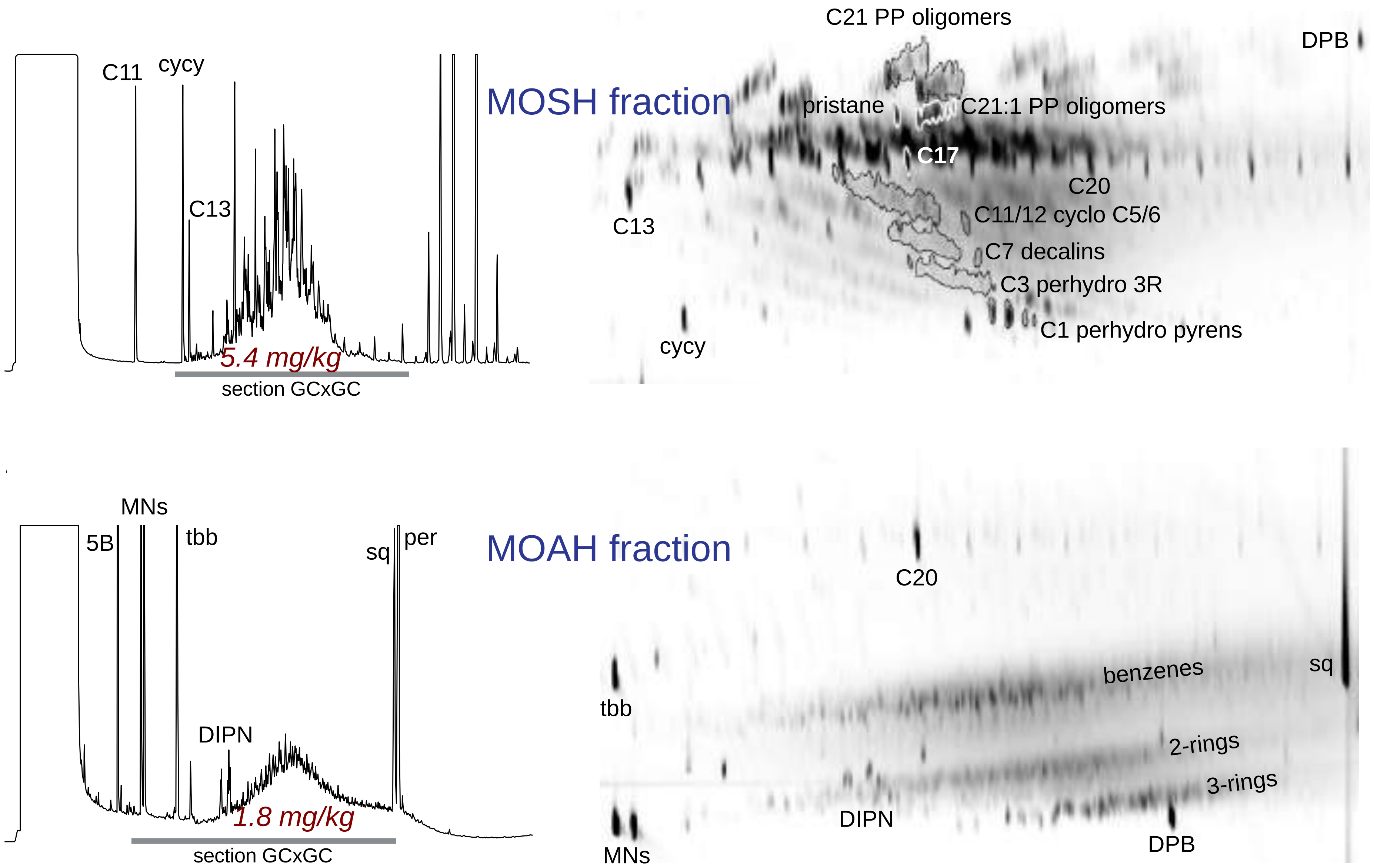
MOAH fraction, FID



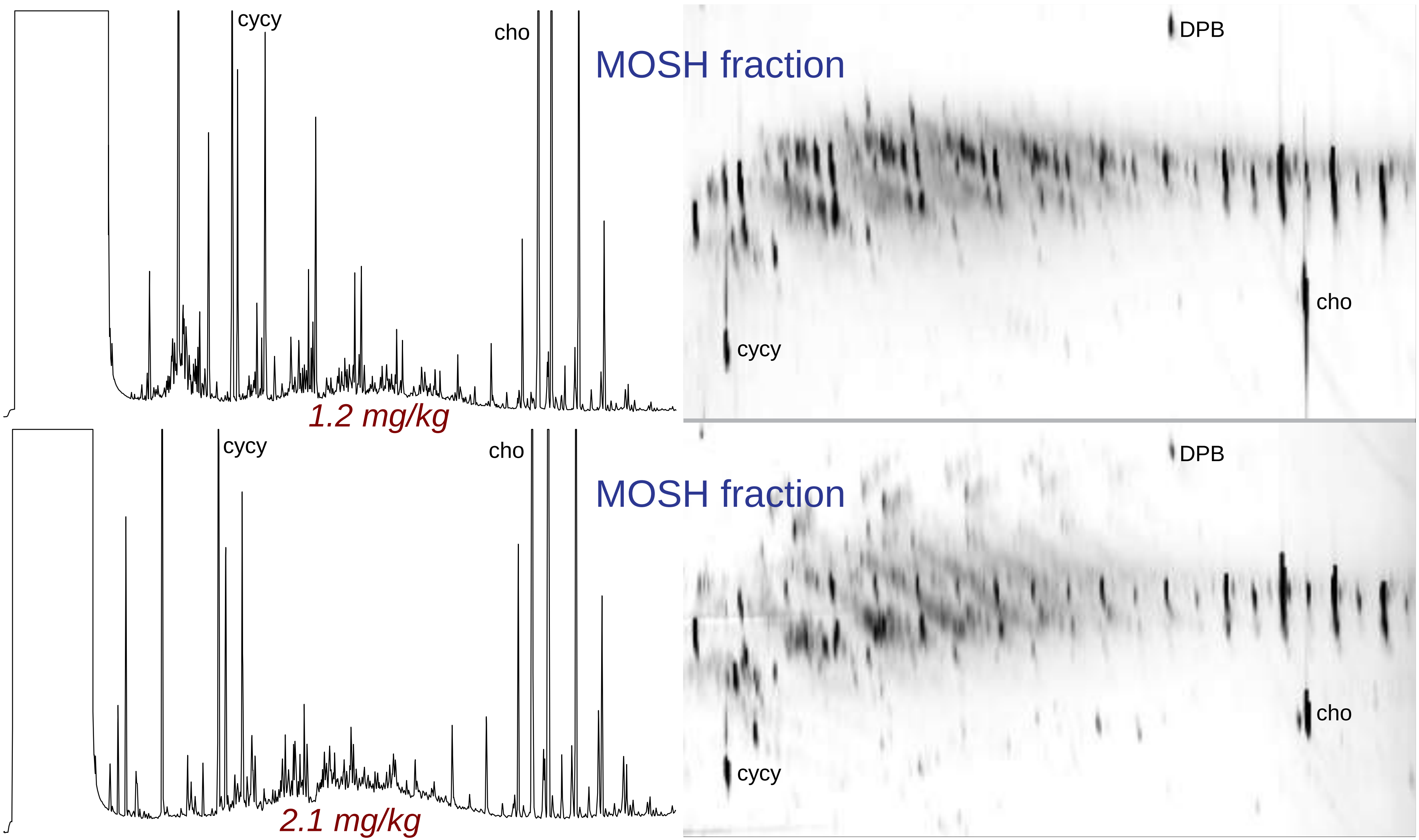
Rice 1: contamination with batching oil



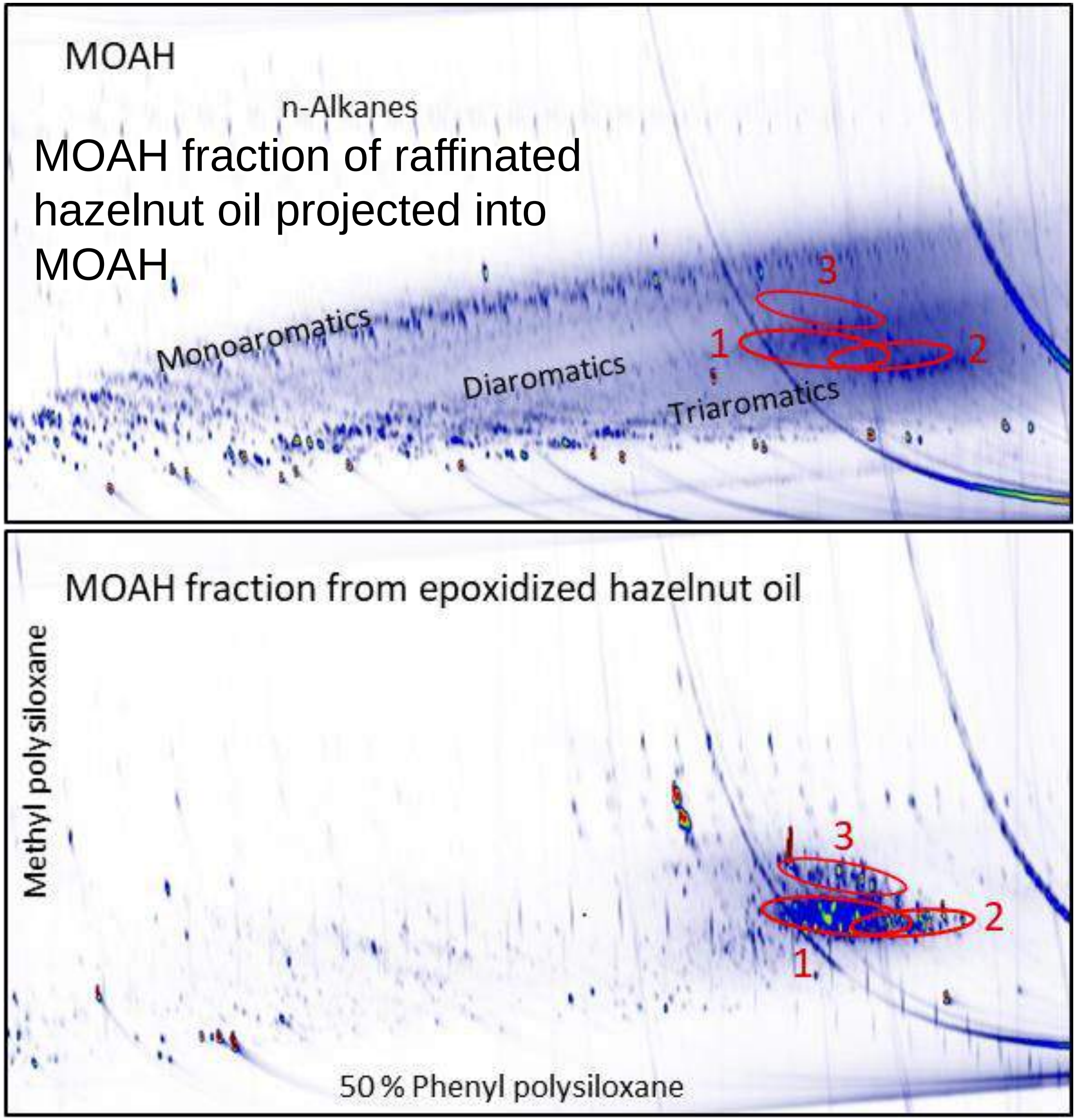
Rice 2: MOH and POSH



Rice 3 and 4: LDPE/LLDPE oligomers (POSH)



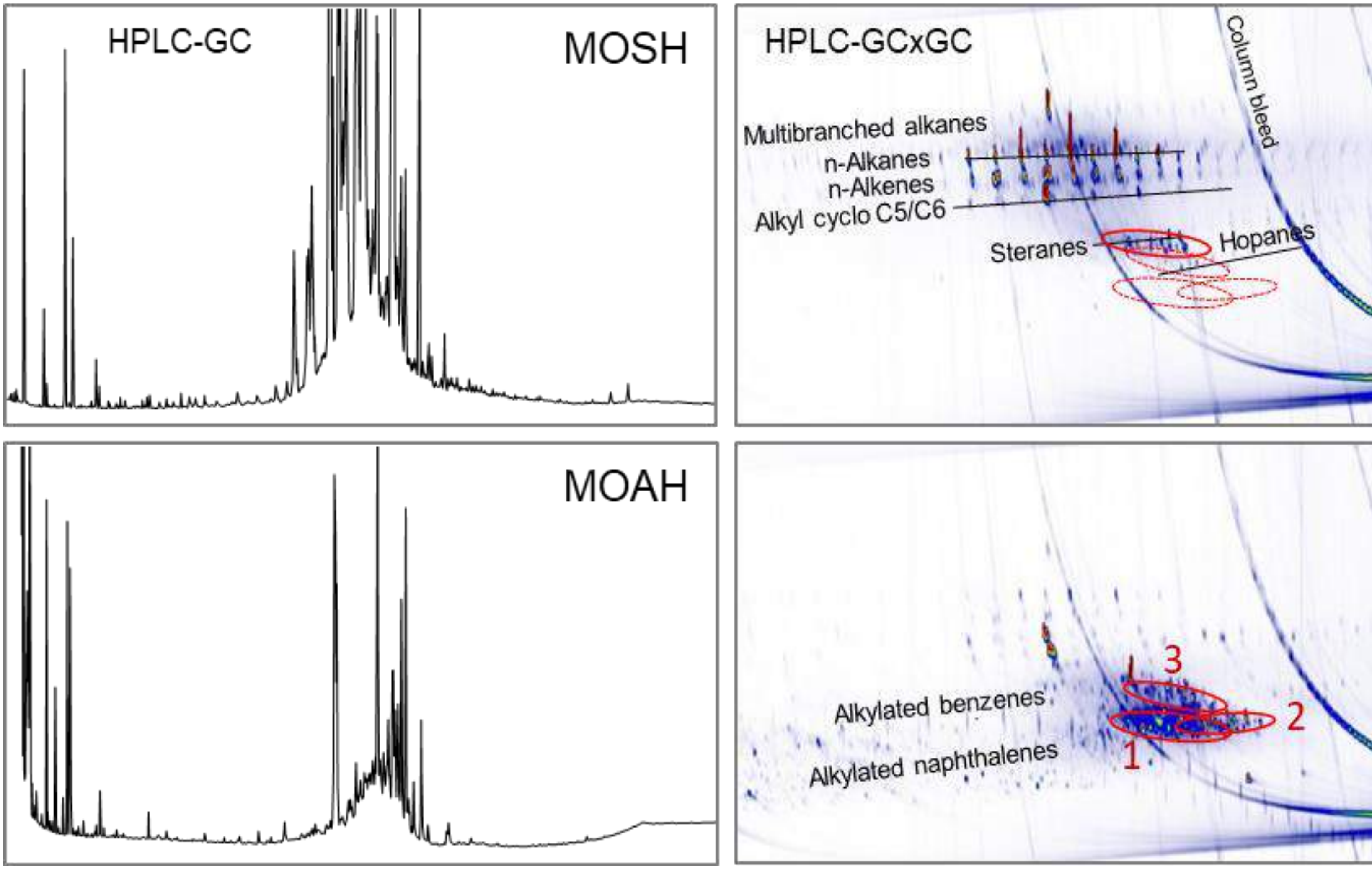
Recognition of interferences in MOAH fraction



- No interferences from carotenes
- Hump at the position of squalene/sterenes
- Hump more narrow than for MOAH

- Characteristic fields in GCxGC

Refined hazelnut oil

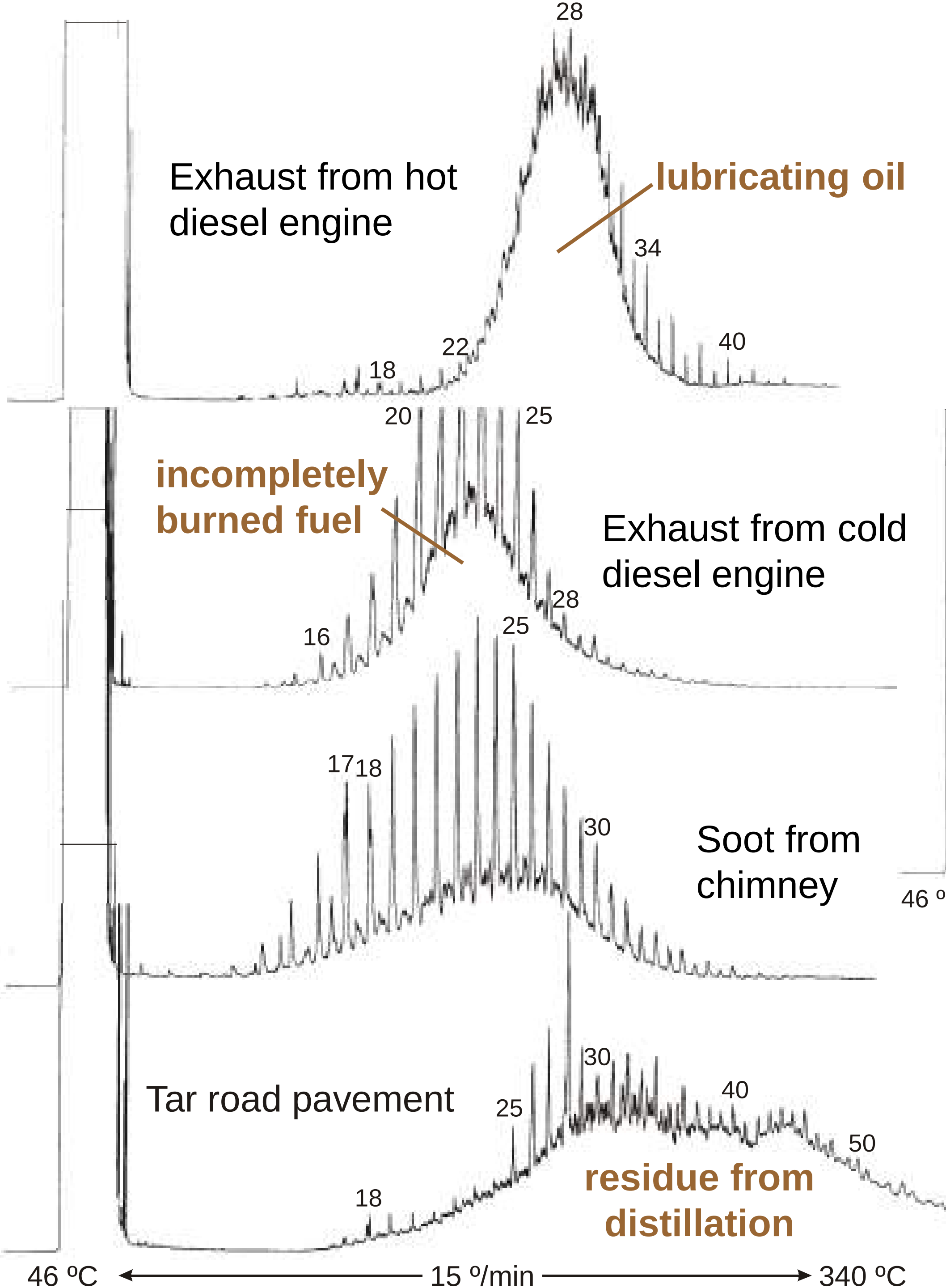


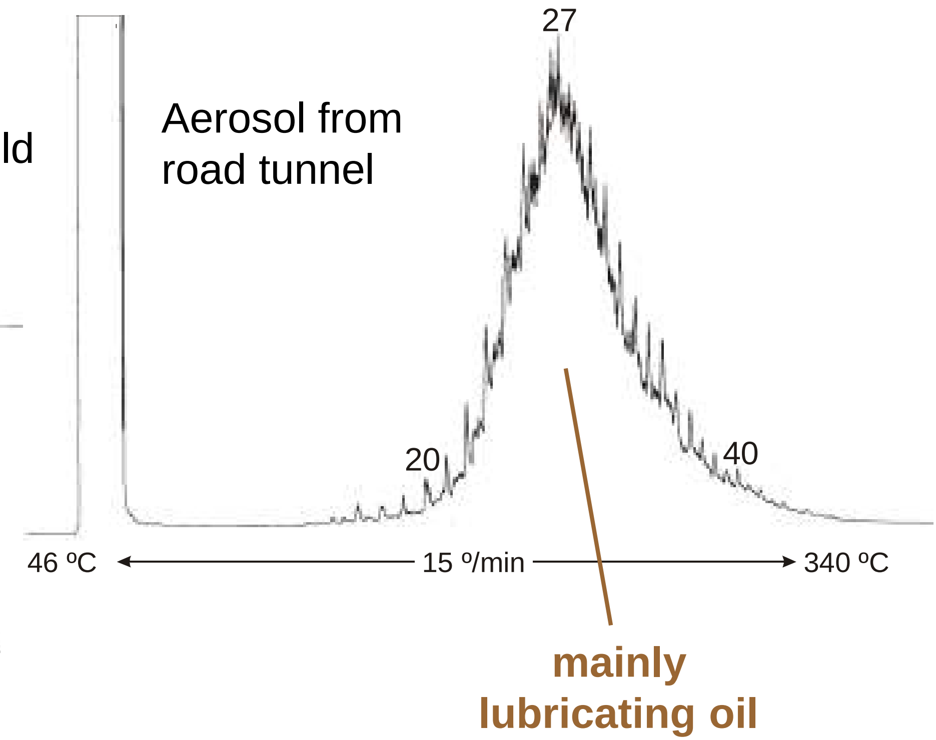
Environmental input

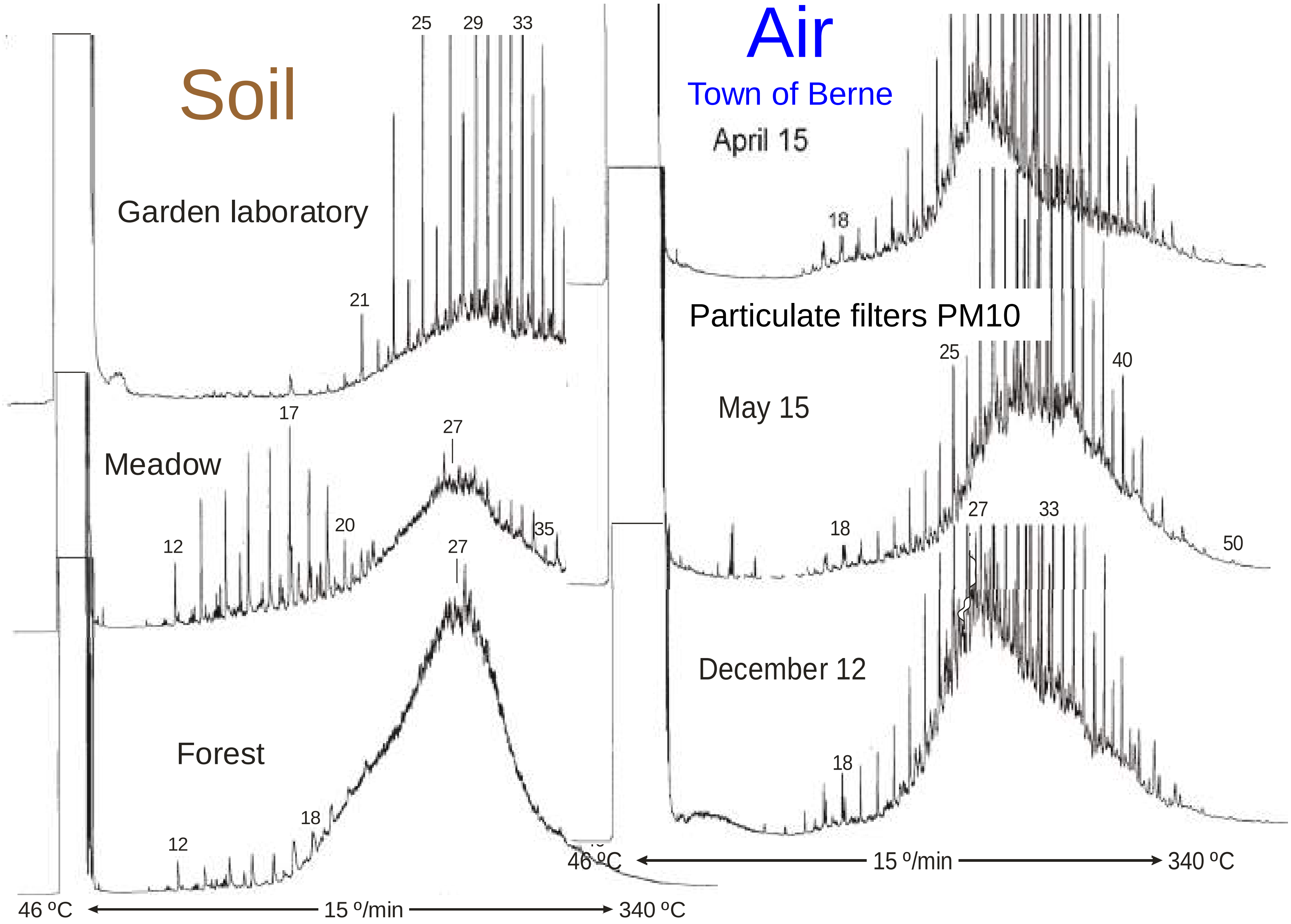
White shirts get gray when left outdoor for some days – plants are left outdoor for more than a few days!

Hydrocarbons ≤C24 mainly in gas phase
Hydrocarbons ≥C24 mainly in the particulates

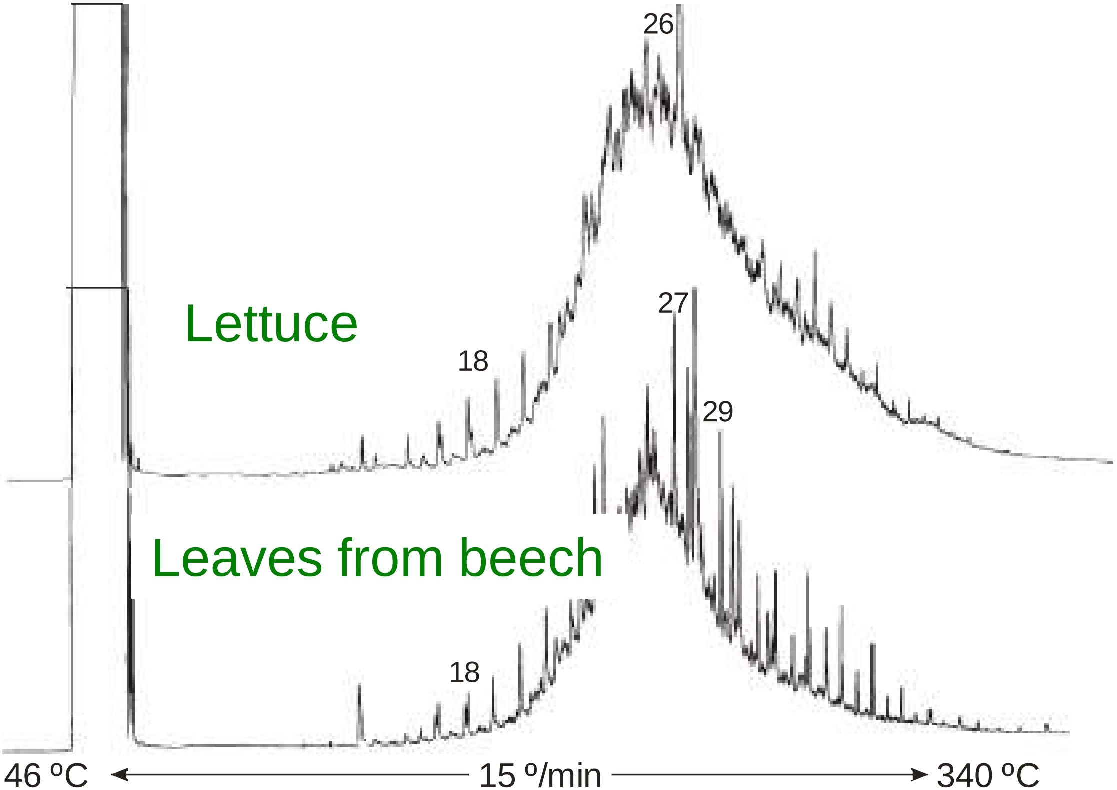
Environmental contributions







Soil
Garden laboratory
25
29
33
21
Meadow
17
27
12
20
35
Forest
27
12
18
46 ºC
15 º/min
340 ºC
Air
Town of Berne
April 15
18
Particulate filters PM10
May 15
25
40
18
50
27
33
December 12
18
46 ºC
15 º/min
340 ºC

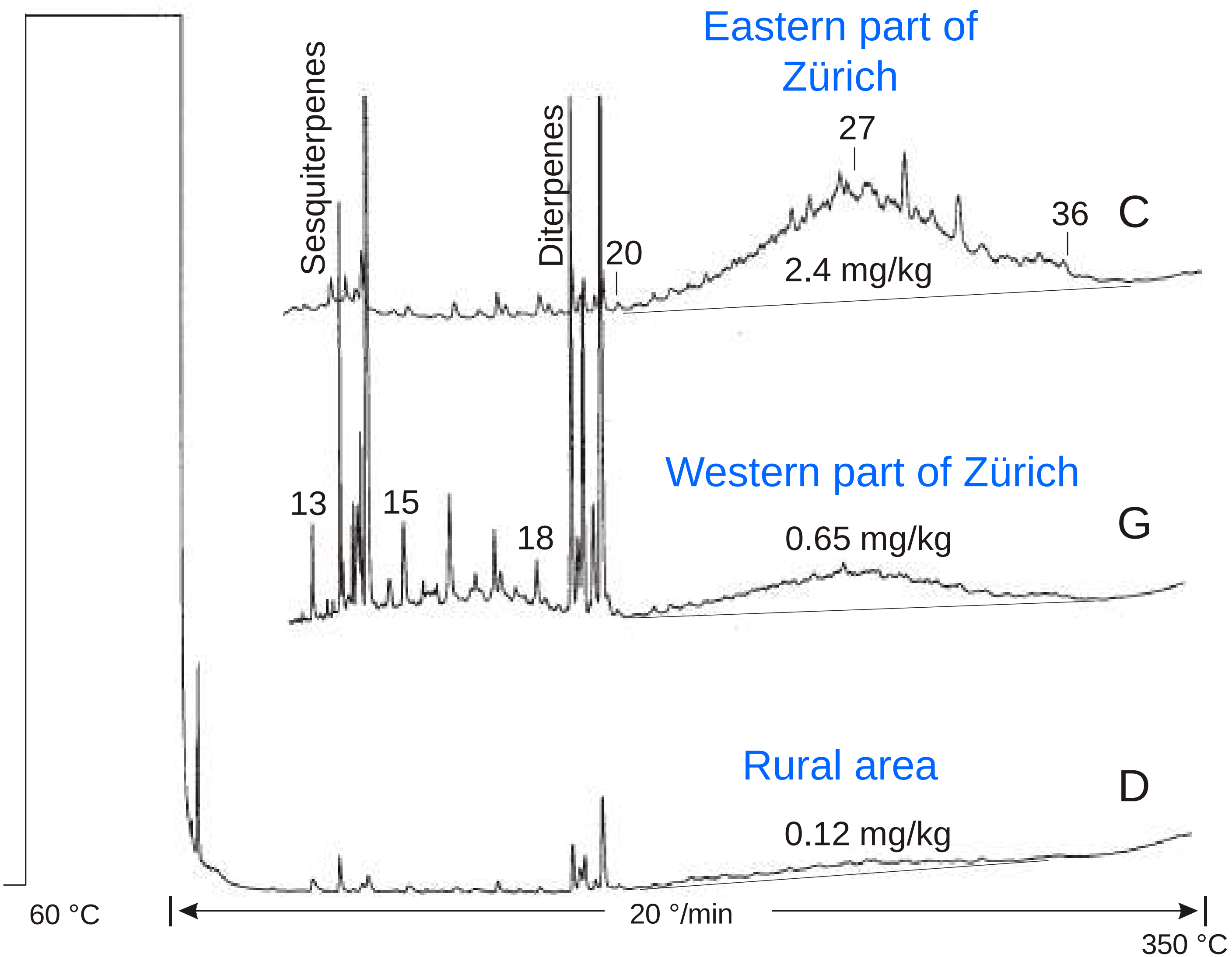


Leaves from beech in Zürich over seasons:

Date	Concentration (mg/kg dry weight)
8 May 2001 (leaves few days old)	4.7
1 July	4.0
28 September	4.5
12 November (leaves freshly dropped)	4.8

MOSH in sunflower oil

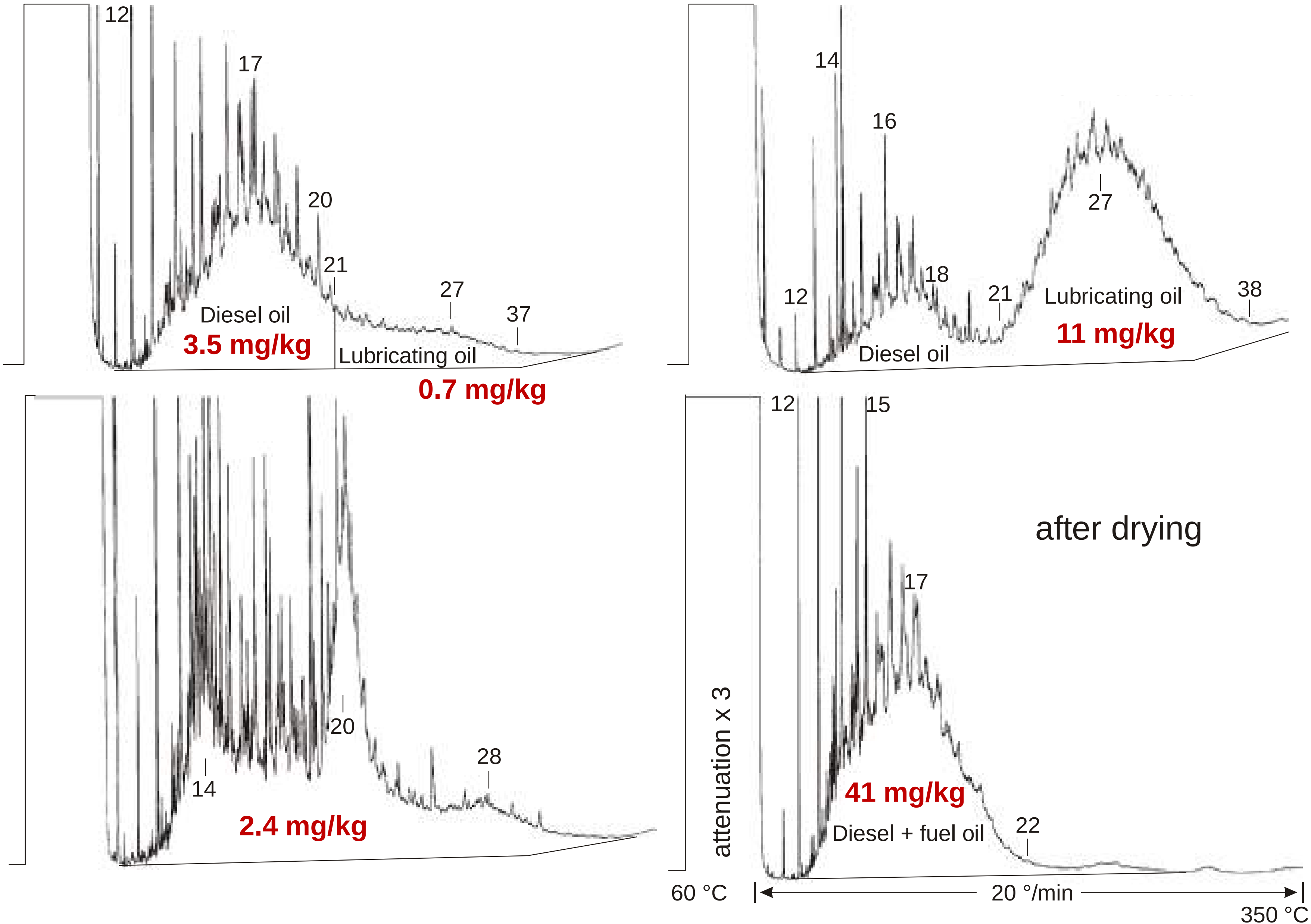
Enrichment + removal of n-alkanes



Manually picked kernels; concentrations related to oil

On-line HPLC-GC-FID after enrichment and removal of natural n-alkanes

Oil from mechanically harvested kernels



Toxicological evaluation

History of errors

Difficulties in better evaluation

The “old” toxicological evaluation

- Based on experiments with entire mineral oil products
  - mixtures with little information about composition (mainly viscosity)

  → no information about which components produced which effect
- End points considered pivotal:
  - granuloma formation for MOSH
  - MOAH are genotoxic
- Increased organ weights in rats
  - repeatedly observed…
  - …but not adequately investigated, probably since human exposure was grossly underestimated
- Measured half-life in rats seemed moderate…
  - …but accumulation of minor parts cannot be excluded in this way

→ SCF and JECFA 2002: very high tolerance for MOSH >C25

2002 JECFA classification of white mineral oils

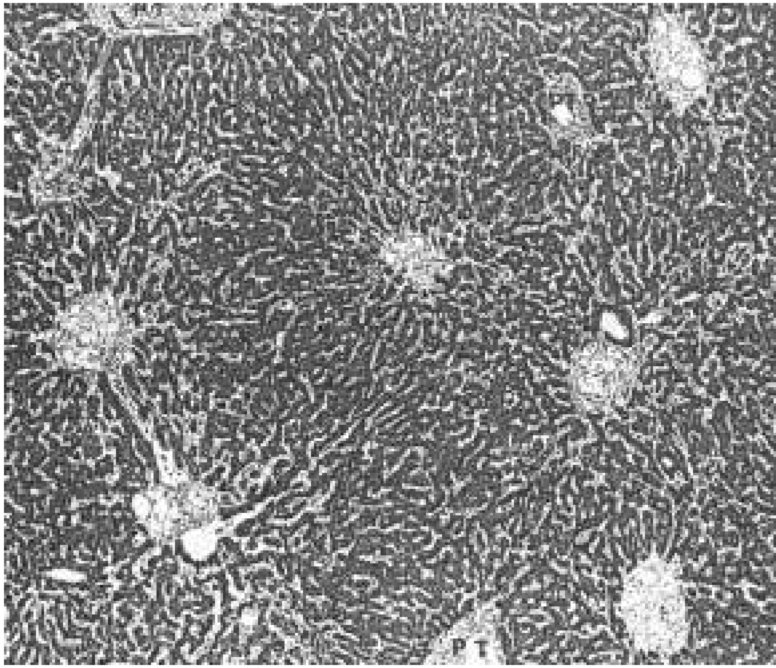
Name	ADI (mg/kg bw)[a]	Viscosity at 100 °C (mm^2/s)	Average relative relative molecular mass	Carbon number at 5% distillation-point
Microcrystalline wax	0–20[a,b]	≥ 11	≥ 500	≥ 25
High-melting-point wax		Not included in the present studies		
Low-melting-point wax	Withdrawn[c]		No specification	
Low-melting-point wax		3.3	380	22
Mineral oil (high viscosity)	0–20[a]	> 11	> 500	≥ 28
P100		11	520	29
Mineral oil (medium and low viscosity) class I	0–10[d]	8.5–11	480–500	≥ 25
P70		9.0	480	27
Medium-viscosity liquid petroleum		8.7	480	25
P70(H)		8.6	480	27
Mineral oil (medium and low viscosity) class II	0–0.01[e,f]	7.0–8.5	400–480	≥ 22
N70(H)		7.7	420	23
Mineral oil (medium and low viscosity) class III	0–0.01[e,f]	3.0–7.0	300–400	≥ 17
P15(H)		3.5	350	17
N15(H)		3.5	330	17

C34=478 Da

http://whqlibdoc.who.int/trs/WHO_TRS_913.pdf

However…

- Frequent occurrence of MOH granulomas in human tissues, reported in about 1950-1990
  - remained unexplained
- 2003, Scotter et al.: strong accumulation of MOSH >C25 (those with the very high ADI!) in rat tissues
  - no follow-up
- Long-term accumulation of MOSH not adequately investigated
  - little data on tissue concentrations (demanding analysis!)



Lipogranuloma in non-fatty human livers.
A mineral oil induced environmental disease.
Dincsoy et al., Am. Soc. of Clinical Pathol. 1981

Presently used reference values from German Ministry

- German values were originally derived from JECFA evaluation from 2002 for class III oils: ADI of 0.01 mg/kg body weight
  - 60 kg person, 1 kg food/d → 0.6 mg/kg food
  - 25 % MOAH → 0.15 mg/kg food
- This ADI was withdrawn in 2012
- Limits later increased to 2 mg/kg/0.5 mg/kg
  - no toxicological justification

More recent evaluations

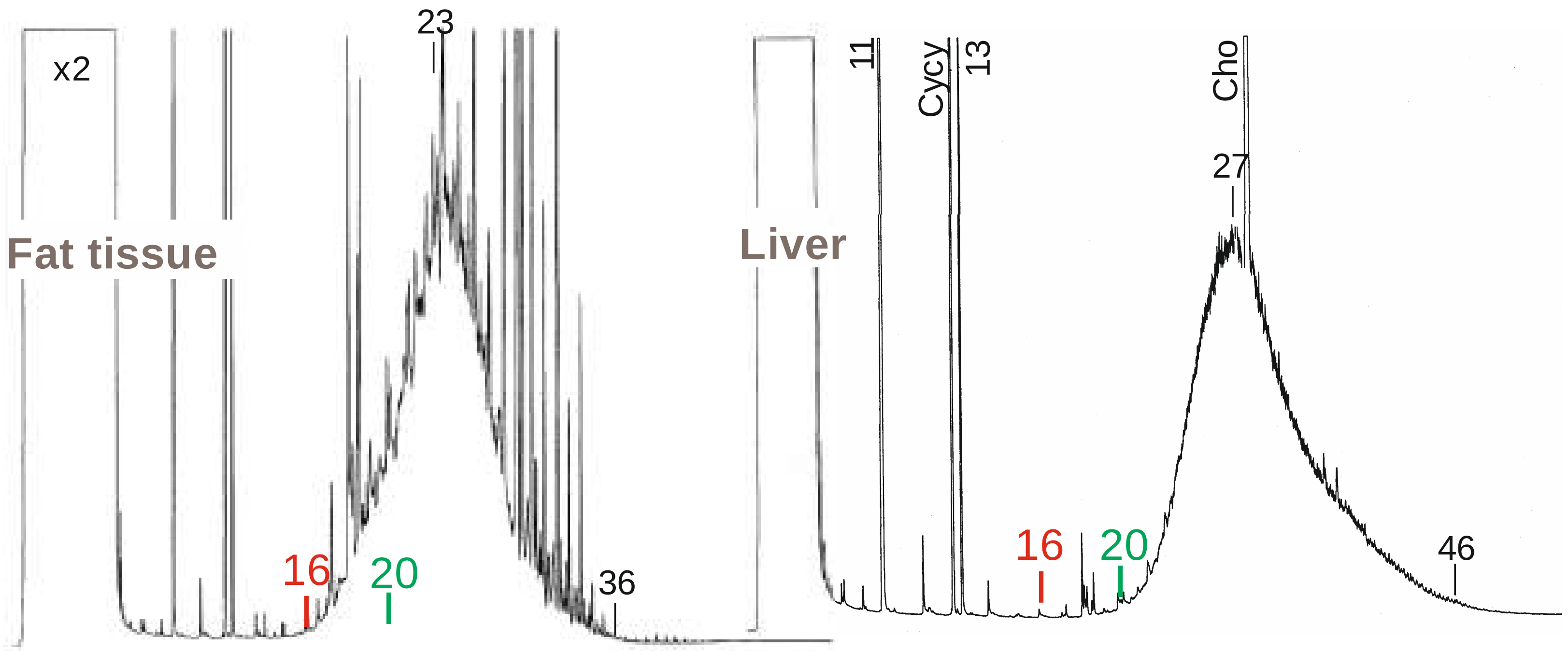
- 2003, 2008: MOSH in human milk and body fat
  - milk: mean ~ 50 ppm, maximum 1300 ppm/fat
- 2012: Evaluation by EFSA
  - no ADI or TDI owing to lack of data primarily on accumulation
  - present exposure to MOSH considered «of potential concern»
    - based on the old (inadequate!) data (low melting wax)
  - “MOAH with three or more, non- or simple-alkylated, aromatic rings may be mutagenic and carcinogenic, and therefore of potential concern ”
- 2012: WHO/JECFA withdraws ADI for Class II/III oils
- 2014: Measurement in human tissues reveals
  - strong accumulation of a probably small part of the MOSH
  - no accumulation of MOAH
- 2017: EFSA project with Fischer 344 rats
  - source of granuloma formation, increased organ weights

2011: BfR evaluation by accumulation

- BfR: potential adverse effects are from the accumulated MOSH → limits related to accumulation
  - 12 mg/kg C10-C16 (not accumulated)
  - 4 mg/kg C16-C20 (low accumulation)

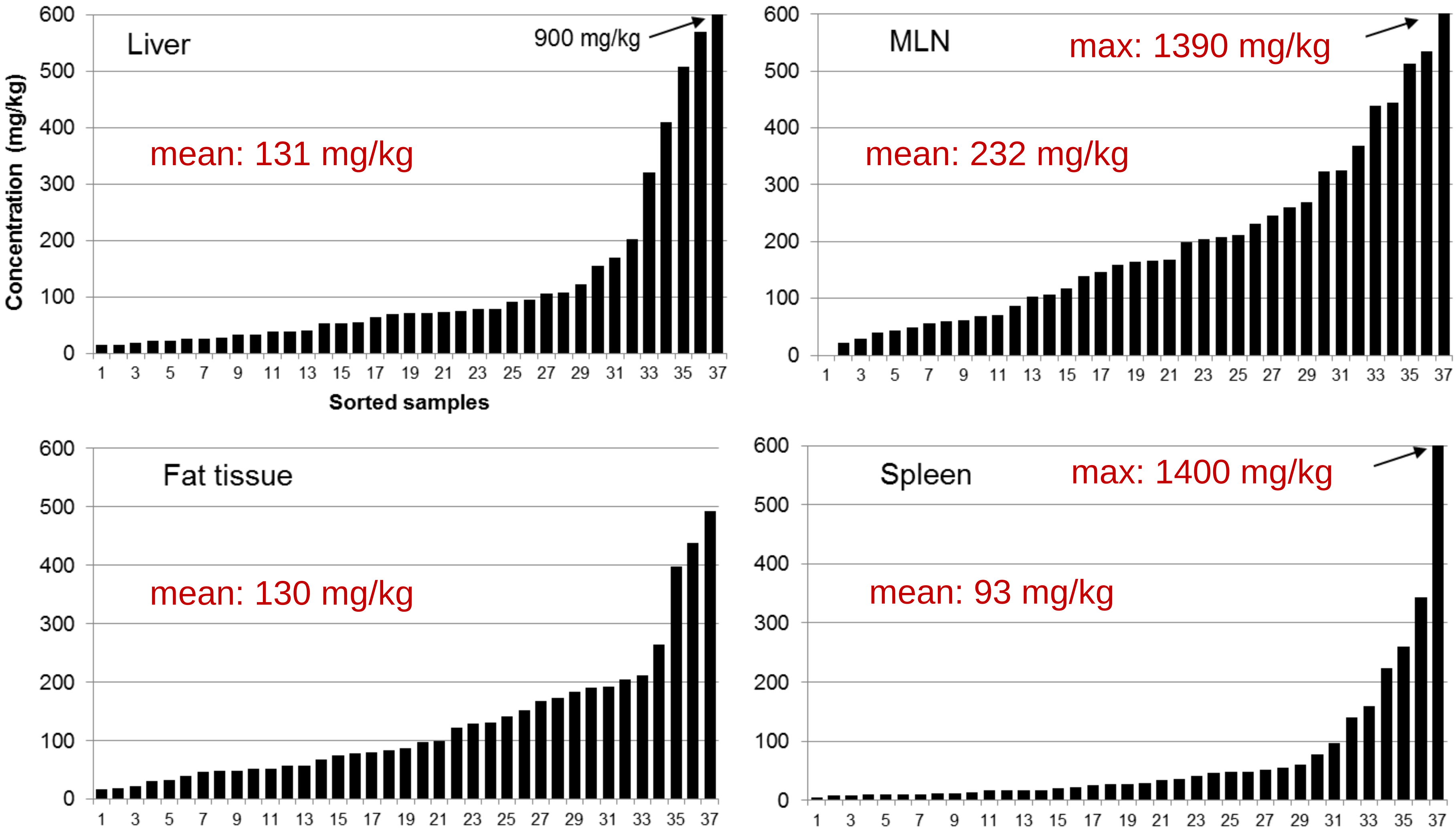
  → anticipated lower limit for >C20

In conflict with JECFA evaluation!



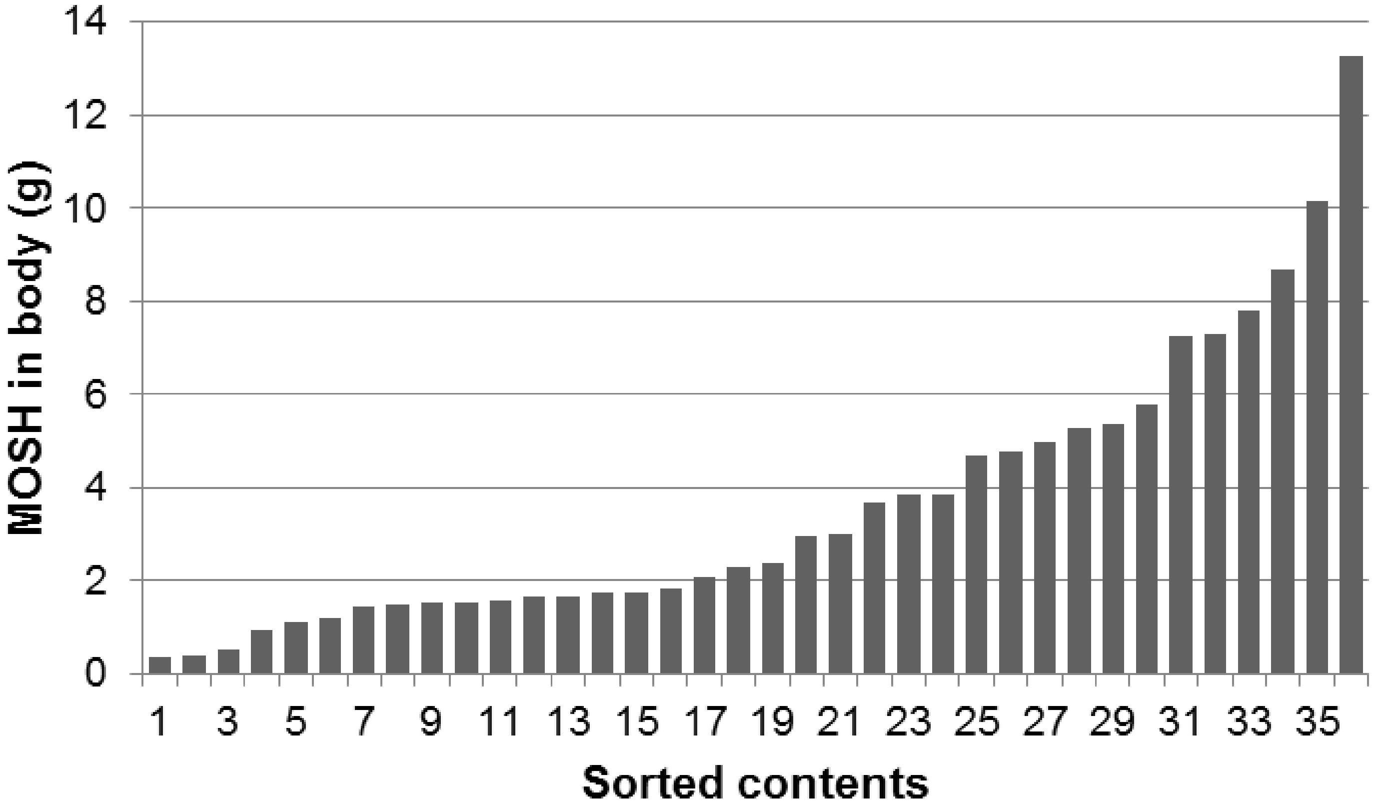
2014: Concentrations in human tissues

Samples from Pathology Wien, 37 subjects, mean age: 67 y



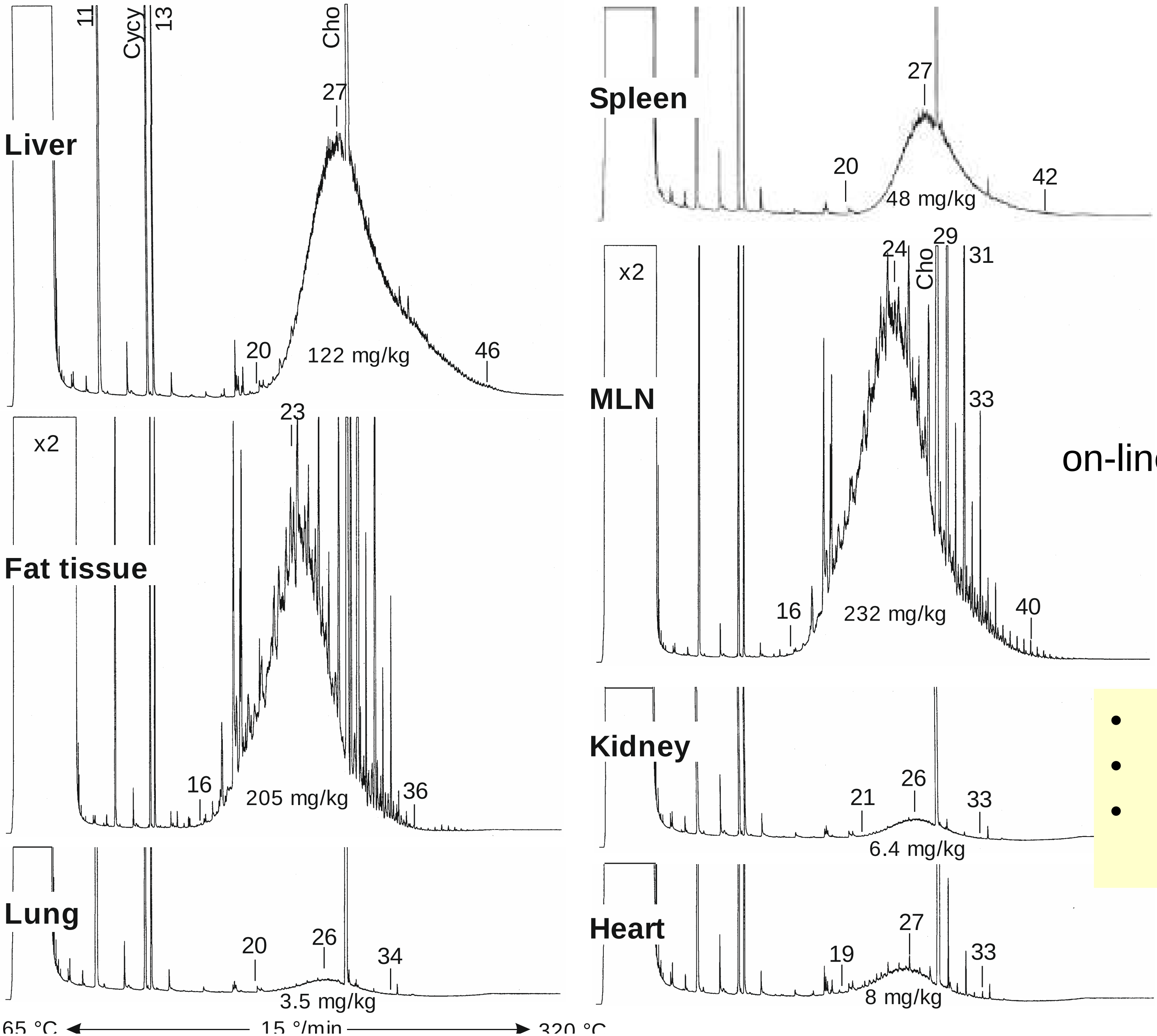
Mineral oil in human tissues, Part I: concentrations and molecular mass distributions. L. Barp, C. Kornauth, T. Würger, M. Rudas, M. Biedermann, A. Reiner, N. Concin, K. Grob. Food Chem. Tox. 72 (2014) 312–321.

Calculated human body burden



Quarter of subjects: >5 g MOSH

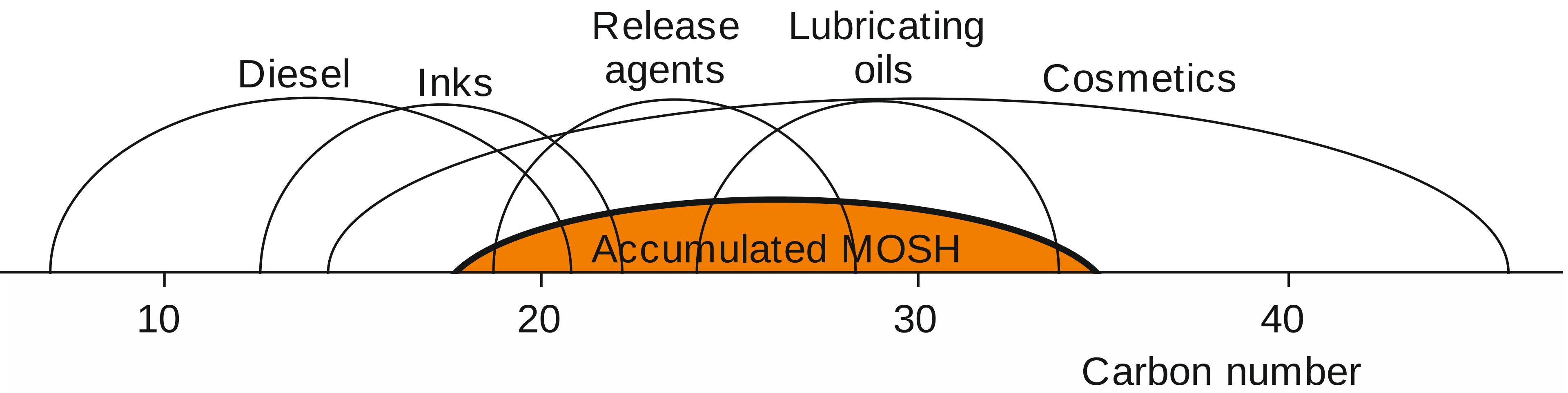
Human tissues



on-line HPLC-GC-FID

- Fat tissue ≈ MLN
- Liver ≈ spleen
- Composition in all subjects ± equal

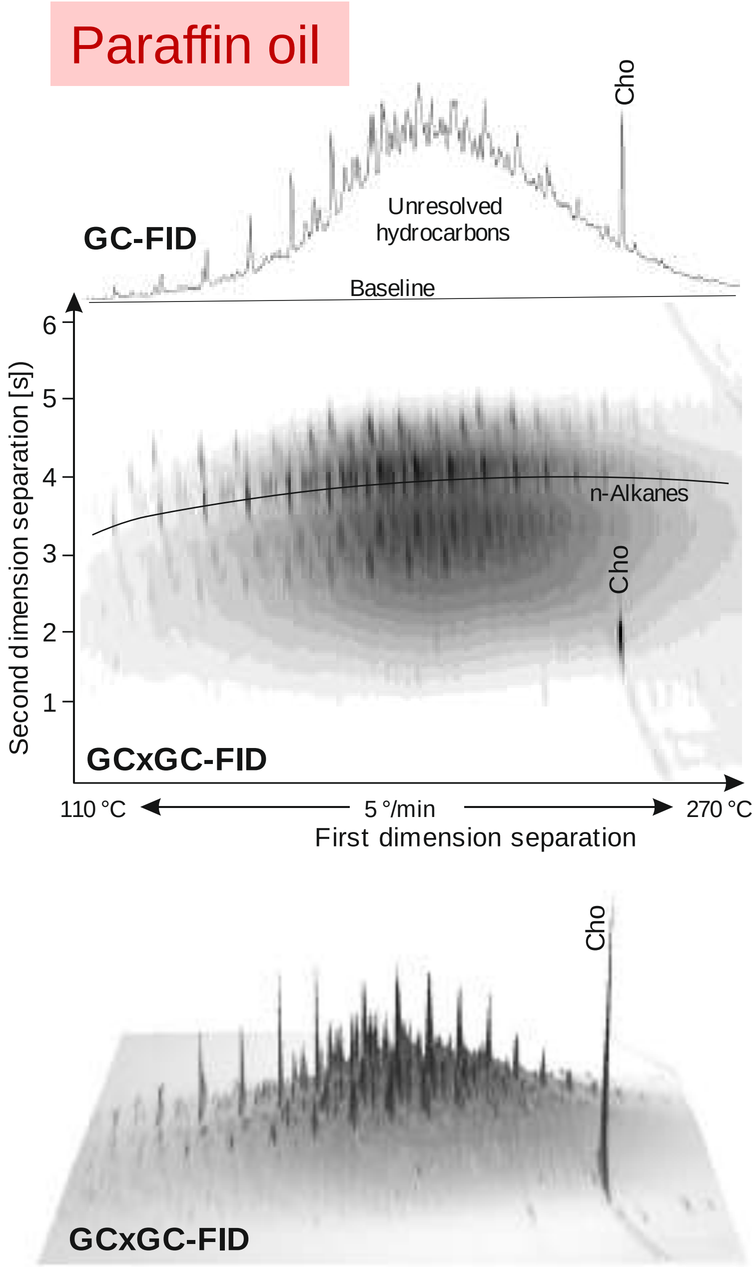
Selective accumulation



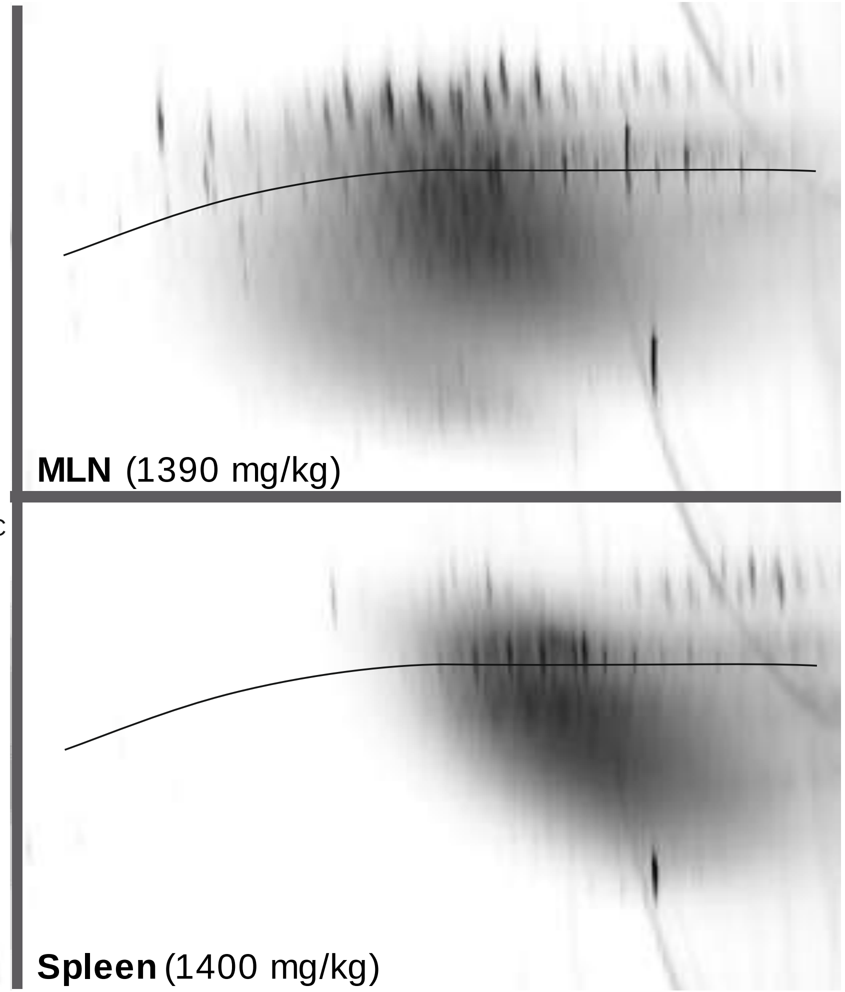
- Only C18-C35 efficiently accumulated
  - more volatiles exhaled
  - higher masses not absorbed (or no exposure?)
- Metabolisation within this range
- Human milk transfers most accumulating MOSH to babies

Selective accumulation also by structure

HPLC GCxGC-FID



78 y old female, 56 kg bw

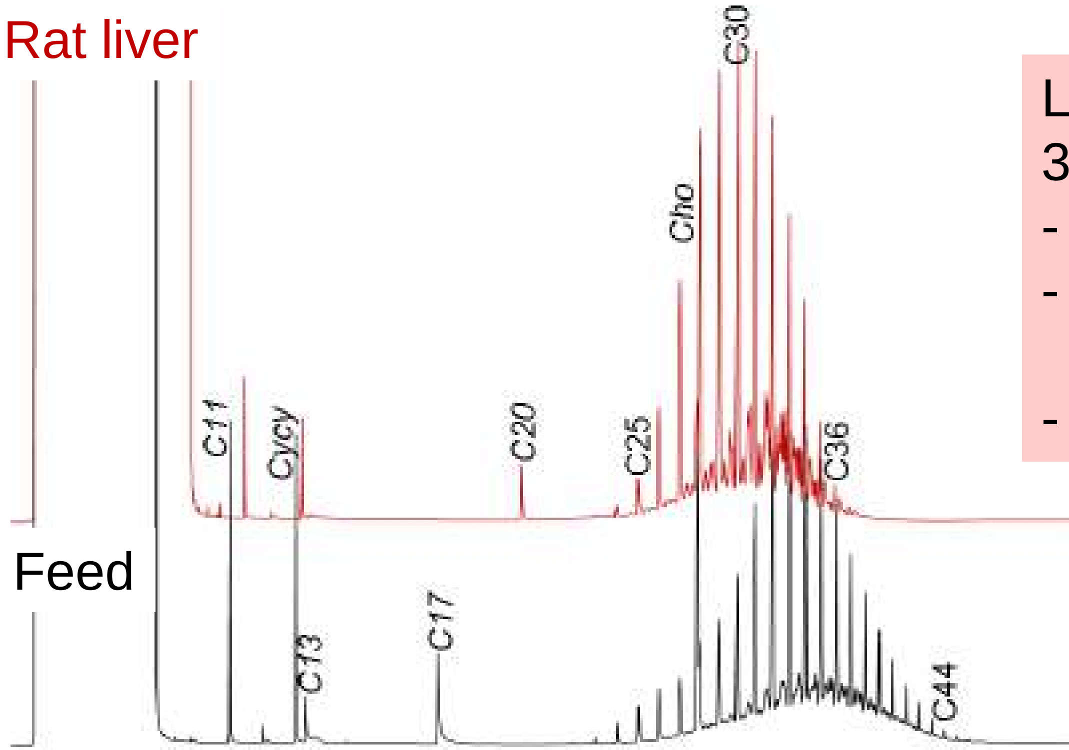


EFSA project on MOSH 2014-2017

- Data gaps identified in EFSA-Opinion from 2012 :
  - Classification of MOSH according to composition rather than products
  - Effect of MOSH accumulation: comparison of animal data with human tissue data (internal exposure)
- ♀ Fischer 344 rats (considered as most sensitive)
- Phase 1: broad MOSH-mixture (C14-C50)
  - 40, 400, 4000 mg/kg added to feed, 30-120 days
- Phase 2: specific MOSH mixtures:
  - Oil mostly <C25 (S-C25; “bad” MOSH according to JECFA)
  - Oil mostly >C25 (L-C25; “good” MOSH according to JECFA)
  - Oil (L-C25) + wax 1:1 (L-C25W)
  - 400, 1000, 4000 mg/kg feed, 120 days

Accumulation of n-alkanes in F344 rats

n-Alkanes are generally considered as readily metabolized, but some are strongly accumulated by Fischer 344 rats.



Liver and spleen of Fischer 344 rats:

- n-alkanes <C24 eliminated
- strong accumulation centered at C30
- none >C38 (not absorbed).

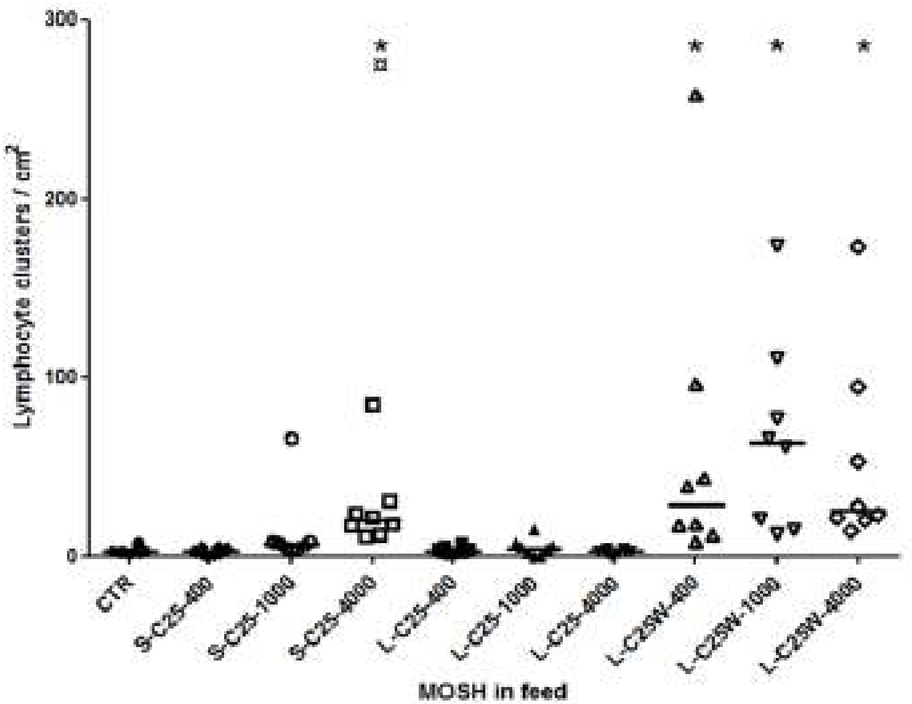
Crystallization prevents metabolization?
Melting point of n-C25: 54 °C

F344 rats: granulomas from n-alkanes

Occurrence of granulomas in livers of Fischer 344 rats:

- MOSH largely <C25: some granulomas at high dose
  - test mixture contained some n-alkanes C25-C30
- MOSH >C25: hardly any granulomas
  - no n-alkanes C25-C30
- MOSH >C25 + wax: very many granulomas, even at low dose

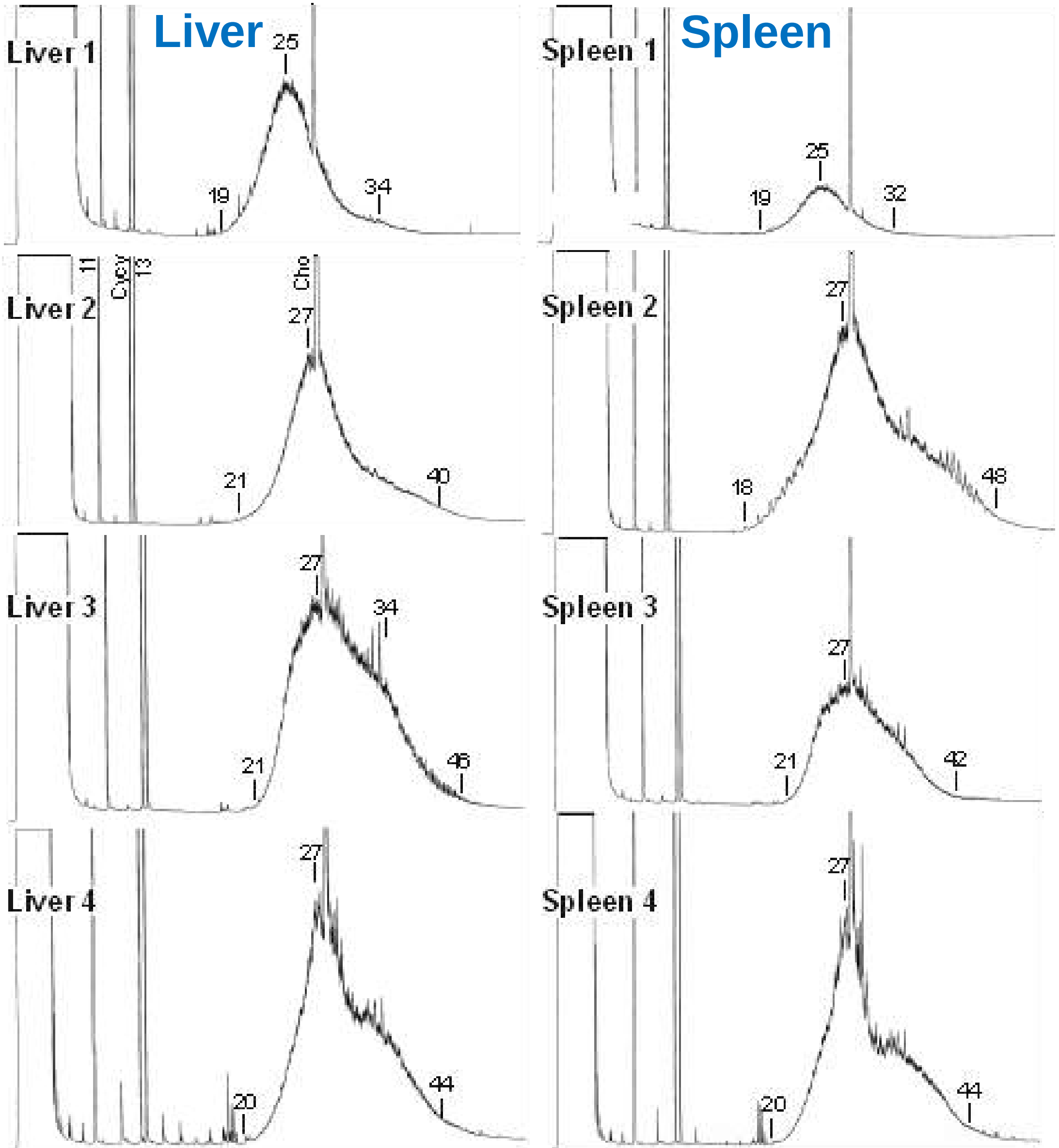
→ Granuloma formation correlated with wax components



Crystal formation triggers granuloma formation?

J.-P. Cravedi, K. Grob, U.C. Nygaard, J. Alexander, EFSA, External Scientific Report, http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2017.EN-1090/pdf

Accumulation of n-alkanes in humans?



Human liver and spleen contain hardly any n-alkanes

Potential explanations:

- efficient elimination
- negligible exposure

Exposure to mineral waxes is low…

…but exposure to plant waxes (odd-numbered n-alkanes) is high (single apple ≈ 25 mg!)

Humans probably readily eliminate n-alkanes

1. n-Alkanes

If granuloma formation in F344 rats is due to crystallization of n-alkanes, granuloma formation should not be of concern for humans.

however:

- What caused then the wide-spread granulomas in human tissues in the past?
  - was the exposure to MOSH that high that even oils precipitated and formed granulomas?
- Open questions:
  - are there still granulomas in human tissues?
  - uptake of MOSH depends on physical and matrix properties
    - are crystalline waxes (e.g. from apples) not absorbed?

Extrapolation from animal data

Rat, 120 days			
Dose (mg/kg feed	Concentration (mg/kg)		
	Liver	Spleen	Fat tissue
40	220	32	13
400	1604	202	95
4000	5511	383	274

Human exposure 1998-2010 according to EFSA (2012):
0.03-0.3 mg/kg body weigh/day
≈ 1.8-18 mg/day
≈ 1.8-18 mg/kg food (mean of all)

Rats feed ~10 times more per body weight than humans eat

Extrapolation from 4000 mg/kg dose)

Humans	Concentration (mg/kg)		
	Liver	Spleen	Fat tissue
Measured data (n=37)			
Mean	131	93	130
Maximum	901	1400	493
Extrapolated from animal data			
1.8 mg/d	2.5	0.2	0.1
18 mg/kg	24.8	1.7	1.2

Liver: maximum (n=37) >100 times higher than extrapolated

Spleen:

- Maximum >1000 times higher
- Higher than in rats

Fat tissue: 100-1000 times higher

Reasons for the underestimation

1. Certain MOSH accumulate over very long periods
   – possibly decades instead of, e.g. 120 days in rats (factor >100)
2. Rats: tissue concentrations do not increase linearly with dose: higher absorption at low concentration

Rats, 120 days		linear extrapolation				
Dose (mg/kg feed	Concentration (mg/kg)					
	Liver		Spleen		Fat tissue	
40	220	**51**	32	**3.8**	13	**2.7**
400	1604	**551**	202	**38**	95	**27**
4000	5511		383		274	

3. Humans exposed to pre-digested MOSH (enriched accumulating MOSH)?

Basic safety assessment

- Standard safety assessment based on No Observed Adverse Effect Level (NOAEL) in animals:
  - most sensitive animal, except effect is known to be irrelevant for humans
- Standard safety margin for extrapolating animal tox data to humans; for solid data set: factor 100
  - factor 10 for inter-species differences
  - factor of 10 for variable sensitivity within species
- In case of accumulation: comparison of internal doses (tissue concentrations) rather than external doses (exposures)

Safety margin for MOSH

- Human tissues (n=37)
- Concentrations in Fischer 344 rats at maximum dose (4000 mg/kg feed), mixture >C25 free of n-alkanes (no granulomas)

	Rat	**Man** (mg/kg)		**Margin**	
	(mg/kg)	Mean	Max	**Mean**	**Max**
Liver	3805	131	901	**29**	**4.2**
Spleen	419	93	1400	**4.5**	**0.3**
Adipose tissue	36	130	493	**0.3**	**0.1**

→ Margin far <100; human tissue concentrations may even exceed those in test animals (red)

Increased weight of liver and spleen in rats

Is the maximum tissue concentration in F344 rats really a NOAEL?

Increased organ weights indicate struggling with an extra-task

Data from EFSA project:

	Dose	Weight after 120 days (g)			
	(mg/kg)	Body	Liver	Liver/body	Spleen
Control	**0**	**211**	**6.8**	**0.032**	**0.62**
MOSH largely <C25	400	209	6.8	0.033	0.63
	4000	205	6.9	0.034	0.64
MOSH largely >C25	400	213	7.4	0.035	0.7
	4000	211	8.4	0.040	**1.17**
MOSH largely >C25	400	203	8.1	0.040	**1.07**
+ wax 1:1	4000	210	9.1	0.043	**1.25**

→ Affected organ performance as relevant end point?

- doubled spleen weight already at an internal dose 3 times below maximum in human spleen?

New evidence from EFSA Project 2017

1. Granuloma formation in Fischer 344 rats is correlated with n-alkanes C25-C35
   – n-alkanes probably not accumulated in humans → might be an exceptional feature of Fischer 344 rats
2. MOSH concentrations in human tissues are far higher than extrapolated from animal experiments
   – maximum concentrations (n=37): 1.4 ‰ (spleen and lymph nodes)
   – insoluble in water → concentrations in lipids (membranes?) many %!

   → safety margins far smaller than assumed (or inexistent)
3. Increased organ weight as most relevant end point?
4. Maximum accumulation in human liver and spleen: C27-C28
   – JECFA classification is fundamentally wrong

Mislead evaluations for MOSH

- SCF/JECFA 2002: focused on granulomas, not noting that these are due to accumulation of n-alkanes (only) in F344 rats
  - oils >C25 are well deparaffinated → no n-alkanes → no granuloma formation → oils >C25 considered of little concern → high ADI
  - oils of lower mass tested contained n-alkanes → granulomas → considered of concern → very low ADI of Classes II and III oils and waxes
  - underestimated human exposure → no other end points considered
- EFSA 2012
  - margin of exposure (MoE) still based on granuloma formation
    - lowest NOAEL from a low melting wax (high content of n-alkanes)
  - "old" classification by molecular mass distribution no longer confirmed
    - but not corrected
  - insufficient safety margin not noted (unknown human data)
  - no comment on increased organ weights

MOAH

- EFSA: “of potential concern” for MOAH with >2 aromatic rings
  - known genotoxic MOAH have >2 aromatic rings
  - fraction <2 aromatic rings was Ames-negative
- Most mineral oils used in the context of food contain virtually no MOAH >2 aromatic rings
  - exception: jute and sisal bags
- Analytical method should separate ≤2 from >2 aromatic rings
- Environmental contaminants ± free of MOAH
  - apparently degraded (to what?)

→ **MOAH might not be of main concern**

  - MOAH are automatically low if MOSH are regulated adequately

Outlook

- JECFA 2002 and EFSA 2012 evaluations need to be revised
  - high limits must be withdrawn
- Classification (Class I with <5 % below C25) is perverse
  - MOSH <C20 hardly accumulated and probably not of concern
  - main part in Class I (C25-C35) is of most concern
- Granulomas are not the pivotal end point
  - to be investigated, see increased organ weights
- Insufficient safety margin: exposure must be strongly reduced
  - high exposure according to EFSA 2000-2010: 18 mg/kg food

  → maximum MOSH concentration across all foods 10 times lower?

Conclusions

- Oils and waxes should be evaluated separately
  - Oils contain strongly accumulating constituents, waxes probably not
- Tox evaluation must be based on human tissue data
  - what are the levels from present exposure?
  - is only oral exposure relevant? Were the highest tissue concentrations only from contaminated food?
  - **problem: relationship exposure – concentrations in human tissues**
- Environmental contribution, almost exclusively of (predigested!) MOSH, is already in the range of the limit and difficult to avoid
- Use of mineral oil products should not longer be authorized
  - at least until adequate safety assessment is achieved
- Synthetic hydrocarbons (e.g. polyolefin oligomers) should be considered more critically

Publications

- Mineral oil in human tissues, part I: concentrations and molecular mass distributions. L. Barp, C. Kornauth, T. Würger, M. Rudas, M. Biedermann, A. Reiner, N. Concin, K. Grob. Food and Chemical Toxicology 72 (2014) 312–321.
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- Accumulation of mineral oil saturated hydrocarbons (MOSH) in female Fischer 344 rats: Comparison with human data and consequences for risk assessment
  L. Barp, M. Biedermann, K. Grob, F. Blas-Y-Estrada, U.C. Nygaard, J. Alexander, J.-P. Cravedi
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  Science of the Total Environment 583 (2017) 319–333.
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- Toxic effects of MOSH and relation to accumulation in rat liver
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  Food and Chemical Toxicology 123 (2019) 431-442.
- Toxicological Assessment of Mineral Hydrocarbons in Foods: State of Present Discussions. Perspective.
  K. Grob
  J. Agric. Food Chem. 66 (2018) 66 6968–6974