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Toxicity of MOAH:

The Impact Of Molecular Structure

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Key points for discussion

- Mouse skin painting studies
- IP346
- Total aromatics (MOAH) and carcinogenicity
- Conclusions

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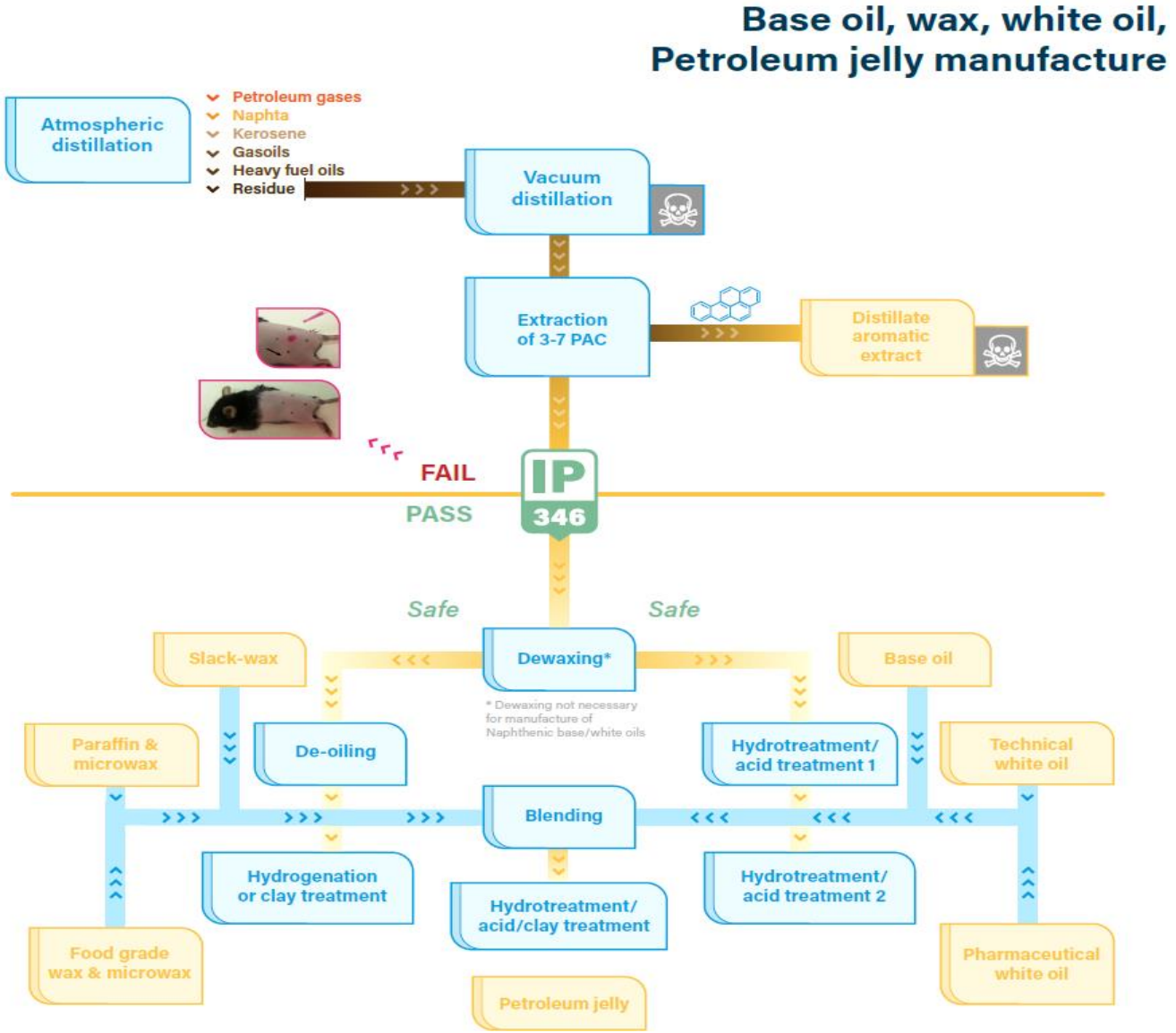
Mouse skin painting studies

The Mouse Skin Painting Bioassay And Manufacturing



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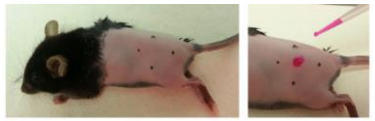
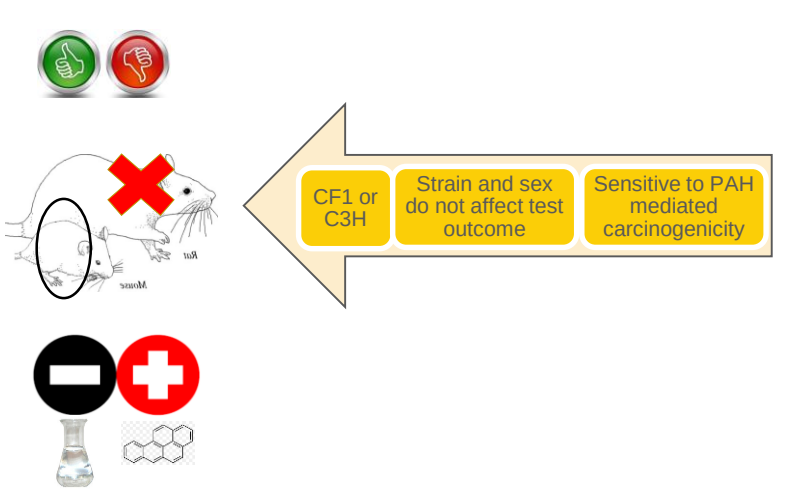
The Mouse Skin Painting Carcinogenicity Bioassay



THE gold standard for studying carcinogenesis



Shaving of dosing site before dosing

Undiluted oil, single dose

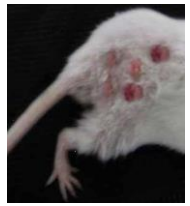
Repeat 2-3 times/week

controls
n = 40-96

undiluted oil treatment
n = 40-50

78-104 weeks

Tumor latency and regressions recorded

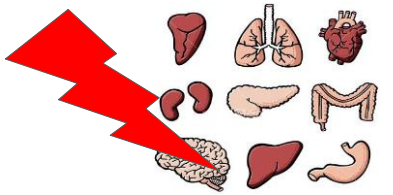


Necropsies on all mice and all macroscopic observations recorded

All tumours (benign & malignant) and skin lesions are recorded

Histopathology of skin and major organs

Skin tumor classification: benign or malignant

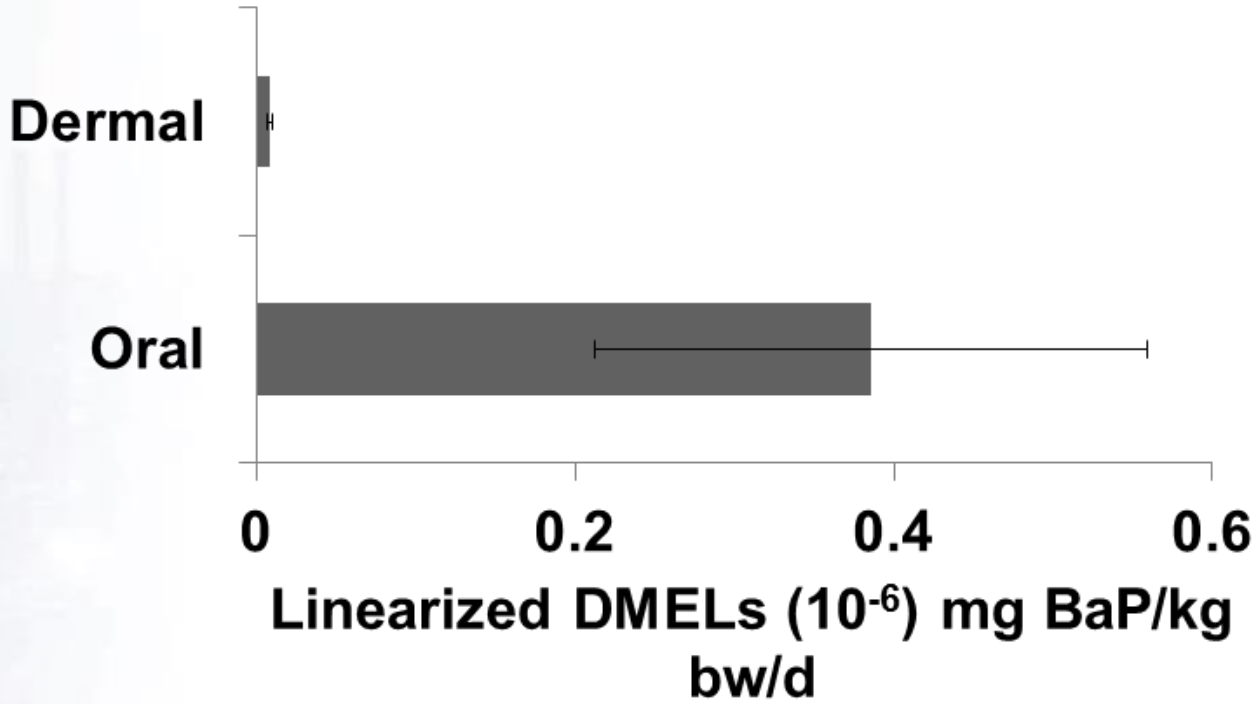


Systemic metastasis is common in mice with skin malignant tumours

http://www.bio-protocol.org/attached/image/20160911/20160911234503_5045.jpg
http://www.ratbehavior.org/RatsMice.htm
http://www.phytojournal.com/vol2Issue2/images/40.1.png
https://www.euromabnet.com/img/antibody/507-TEJ.RATON.SKIN.PAPILLOMA.HUGO291.copia.jpg

Tissue Sensitive to B[a]P-induced Tumors is Reflected in Average Rodent DMEL Values

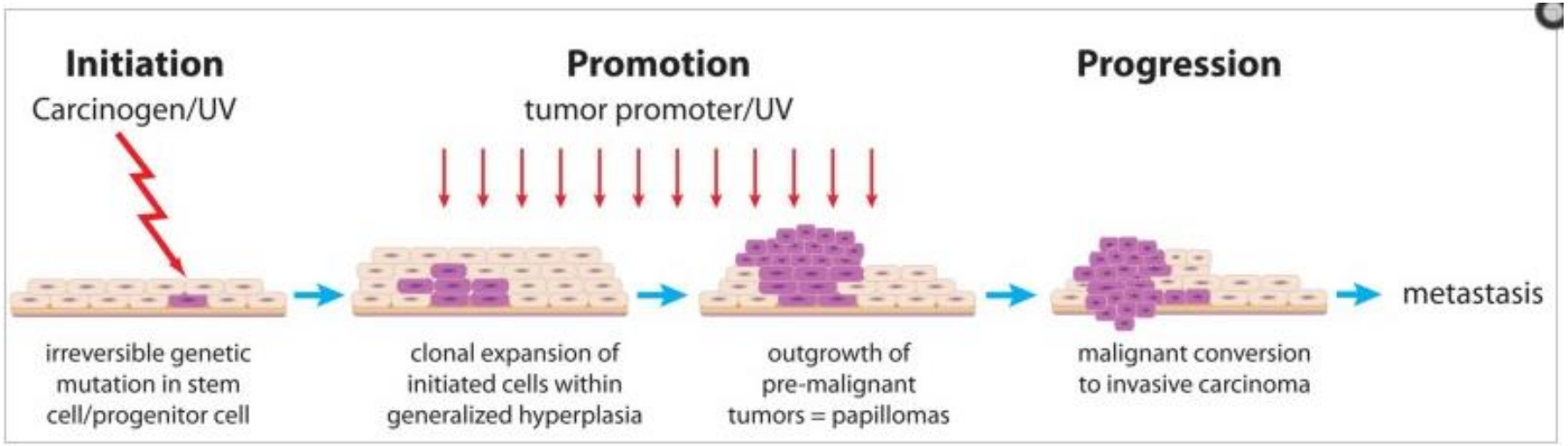
Dermal route is the worst case scenario for PAH (PAC) mediated carcinogenicity.



Slide credits: D. Adenuga - ExxonMobil

Why the Mouse Skin Painting Model?

- Extensively used as a holistic multistage carcinogenesis model[1]



- Principles studied in this model are relevant to other epithelial tissues
- Carcinogenicity and potency of naked ring and alkylated PAH and isomerism has been studied in this model[2, 3]
- Model of choice to study the impact of petroleum refining on modulating carcinogenicity of petroleum substances[4]
- Main route of exposure for petroleum products

1. Rundhaug et al., 2010. Cancers; 2(2): 436–482.
2. La Voie el al., 1985. Carcinogenesis; 6(10): 1483-1488.
3. Luch A., 2009. Mol. Clin. Env. Tox. (1): 151-179
4. Bingham et al., 1980. J. Env Path Tox; (3)483-563.

B[a]P Rodent Tumor Mode of Action Dictates Tumor Location

Key Event 1
Bioactivation to reactive metabolites

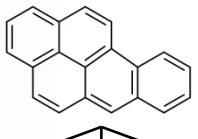
Key Event 2
Reactive DNA damage, formation of DNA adducts

Key Event 3
Fixing of DNA mutations

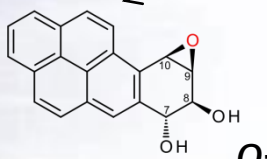
Key Event 4
Clonal expansion – proliferation of initiated tumor cells

Apical Event
Tumors

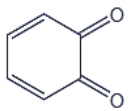
B[a]P



CYP1A1/1B1/1A2

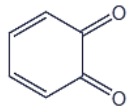


Epoxide



o-quinone

cation



Tissues rich in CYP activity – skin, liver etc.

Tumor promotion:
Local irritation, cytotoxicity, inflammatory response

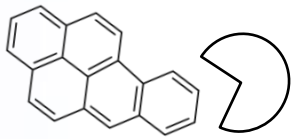
Most sensitive tumor sites – dermal, oral (forestomach, oral cavity, GIT)

Slide credits: D. Adenuga - ExxonMobil

MOAH Molecular Structure Determines Carcinogenicity
Steric Hindrance

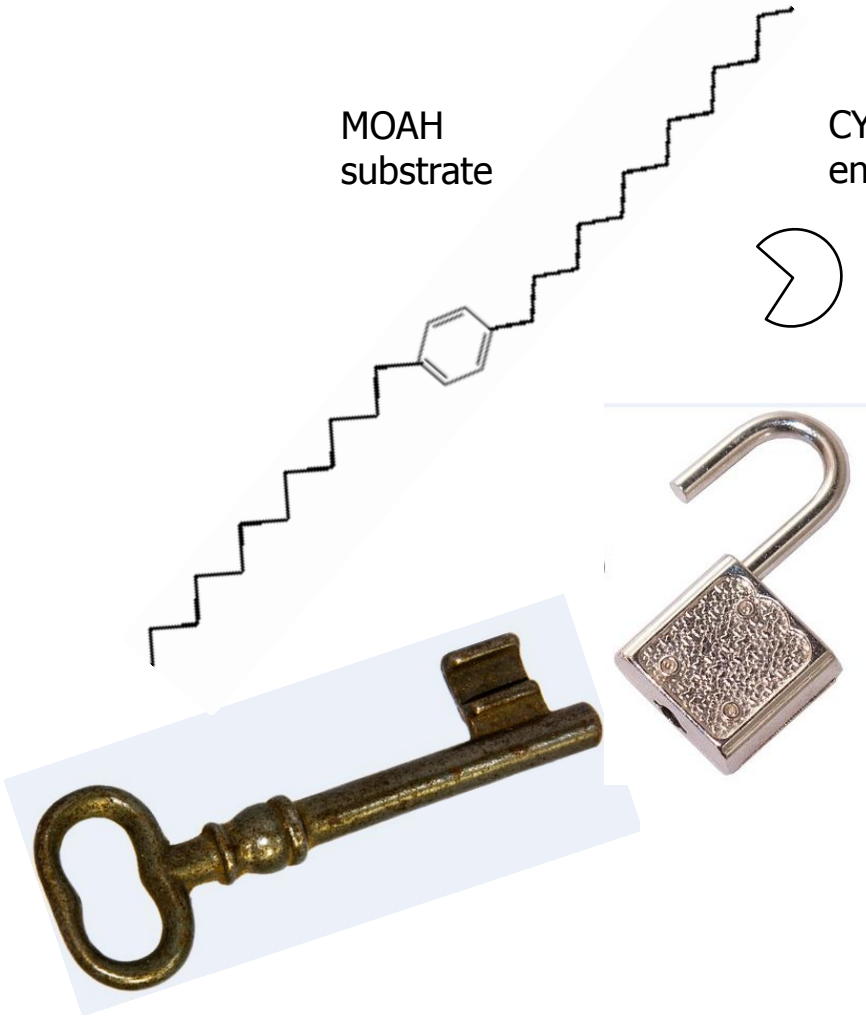
MOAH substrate

CYP enzymes



MOAH substrate

CYP enzymes



Tumour initiation and promotion model – Testing of "MOAH" fractions

What type of aromatics in an oil are carcinogenic?
Fractionation of a carcinogenic oil demonstrates where the hazard is[1]:

Substance or fraction	Live animals after 40 weeks	Re-treatment of live animals with a promotor
Carcinogenic oil	Tumours in all animals	-
Fraction I+II+III	Tumours in all animals	-
Fraction I (PAH "free")	No tumours	No tumours
Fraction II (2 and 3 rings)	No tumours	No tumours
Fraction III (> 3 rings)	No tumours	Tumours in all animals

What can we learn from this experiment?

- Fractions of a carcinogenic oil were tested with or without a promotor.
- There is a type of MOAH that is NOT carcinogenic with or without promotor.
- It is the > 3 ring MOAH fraction which is potentially carcinogenic.
- The interaction of ALL fractions causes the carcinogenic effect. Therefore, especially with UVCB's, it is imperative to test SUBSTANCE (the actual oil), and NOT the fractions thereof.

- There are refined aromatic oils, with high level of aromatics which are not carcinogenic[2]

1. Agarwal et al., 1988; 2.Doak et al.,1985

Take Home Message for Mouse Skin Painting Studies in Mineral Oils

- Toxicologists focus on PAC* not MOAH
- Mouse model is the gold standard to assess PAC carcinogenicity
- Principles studied on mouse skin are relevant to other epithelial tissues and humans
- Industry dermal mouse studies and protocols are fit for purpose and reliable
- Dermal route is the worst case scenario compared to oral route
- Carcinogenicity potential depends on molecular structure and route of exposure
- It is the >3 ring PAC which are the potentially carcinogenic species
- There are MOAH structures that have no carcinogenic potential !

**PAC = polycyclic aromatic compounds (PAH) + Sulphur, Nitrogen atoms in polyaromatic-ring structures*

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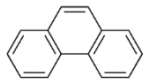
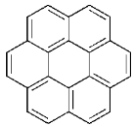
IP346

The Necessity For A Carcinogenicity Screening Method

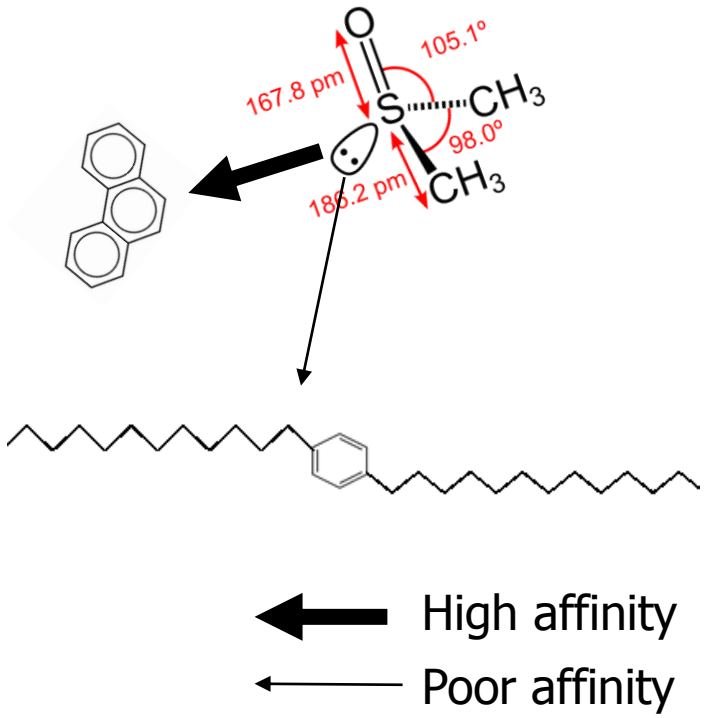
- Mouse skin painting studies are the gold standard, but
  - time consuming
  - limit manufacturing flexibility
  - animal and cost intensive
- Carcinogenicity screening method should be fit for purpose to mineral oils
  - rapid, reliable, specific, simple, low cost
  - reflect variability in feedstock and manufacturing conditions
  - animal free test
- Reflect toxicological hypothesis
  - potentially hazardous are the 3-7 PAC
  - PAC are bare or with few and short alkyl substituents
  - highly correlated to mouse skin painting data

Screening Method Is In Line With Manufacturing

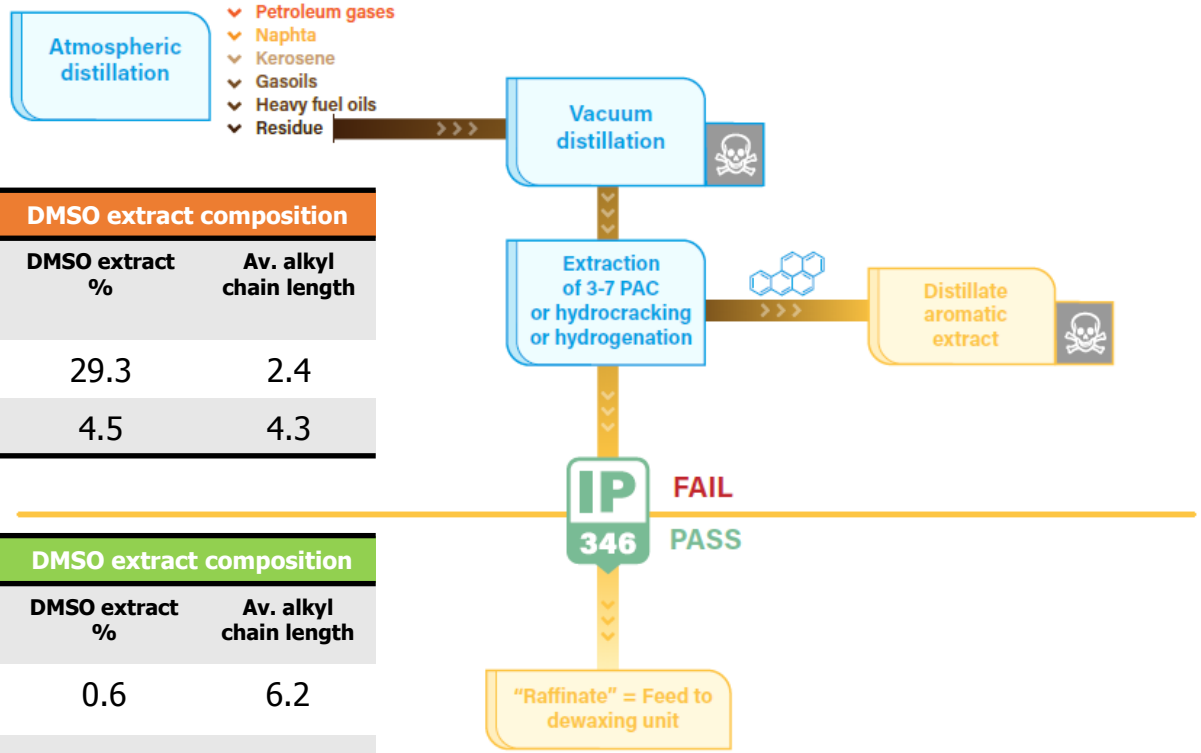
- Boiling Point < 300 ^{0}C at 1bar → substituted benzenes and naphthalenes
- Mineral Oils boiling range > 300 ^{0}C at vacuum
- The PAC found in mineral oil are found in the 340-565^{0}C boiling point range (BP)
- BP 340-565^{0}C (vacuum) → 3-7 PAC
  - Phenanthrene BP 340^{0}C 
  - Coronene BP 535^{0}C 
- The higher the alkylation, the higher the BP
- Method should collective assess of all isomers and alkylation levels within given BP range
- Thus method links PAC to boundaries set by manufacturing and toxicological relevance

Screening Method Should Be Selective To Toxicologically Relevant PAC

- Screening method based on DMSO
- DMSO shows special interaction with PAH (PAC) Pi-system
  - Can be modulated by alkyl substitution and halogenation
  - Can be interrupted by polar and non-polar solvents
  - Allow selective extraction based steric hindrance of highly alkylated PAC
  - Extraction efficiency drops with decreasing number of fused aromatic rings per molecule, and length of alkyl chains
- DMSO-extract composition
  - reflect refinement efficacy in PAC removal
  - Mechanistic explanation of biological process: bioavailability and bio-activation of the type of DMSO-extracted PAC
  - relevant in the prediction of mouse skin painting studies

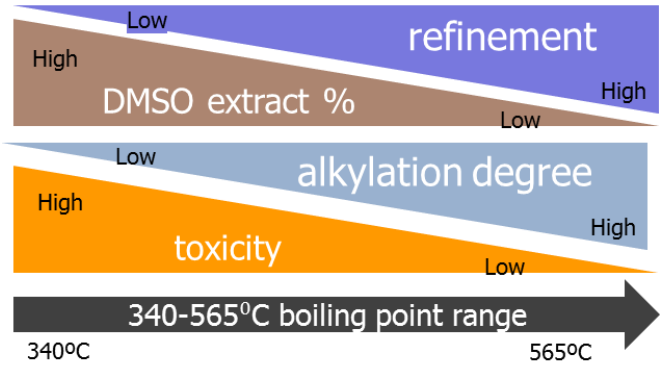


DMSO extract aromatic composition
Relationship to Refinement



DMSO extract composition		
Aromatic extracts*	**DMSO extract %**	**Av. alkyl chain length**
Extract A	29.3	2.4
Extract B	4.5	4.3

DMSO extract composition		
Base Oil*	**DMSO extract %**	**Av. alkyl chain length**
A. Low viscosity	0.6	6.2
B. High viscosity	0.1	12.5

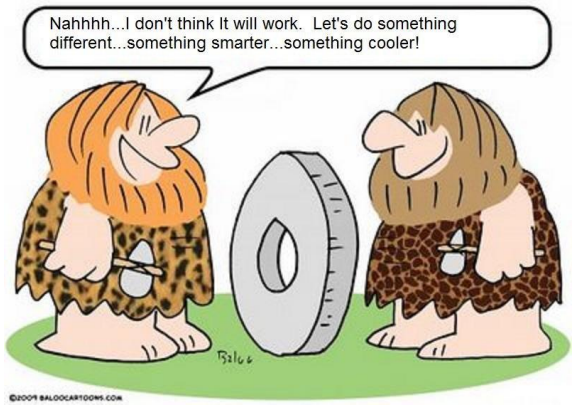


*Note:
At equivalent cut extraction

Screening Method By Chromatography

- PLC-MS Chromatography was considered in 1970:
- Not selective
  - Only PAH are determined,
  - S and N containing PAC are not accurately reflected
- No distinction of alkylation degree – blow up effect
- Does not correlate with tox studies
  - Carcinogenic Oil N2 has lower "MOAH" than non-carcinogenic Oil B
  - MOAH chromatographic values don't reflect manufacturing!
- Complex, time consuming, expensive and not simple to transfer or implement
- PLC-MS is the 1970's equivalent approach to today's "MOAH" !

	PAC Analysis (1970)			
Oil type	Chromato graphy	DMSO extract %	Av. alkyl chain length	Cancer
Oil N2 Low viscosity	2.9	6.8	3.4	YES
Oil B. High viscosity	5.7	0.1	12.5	NO

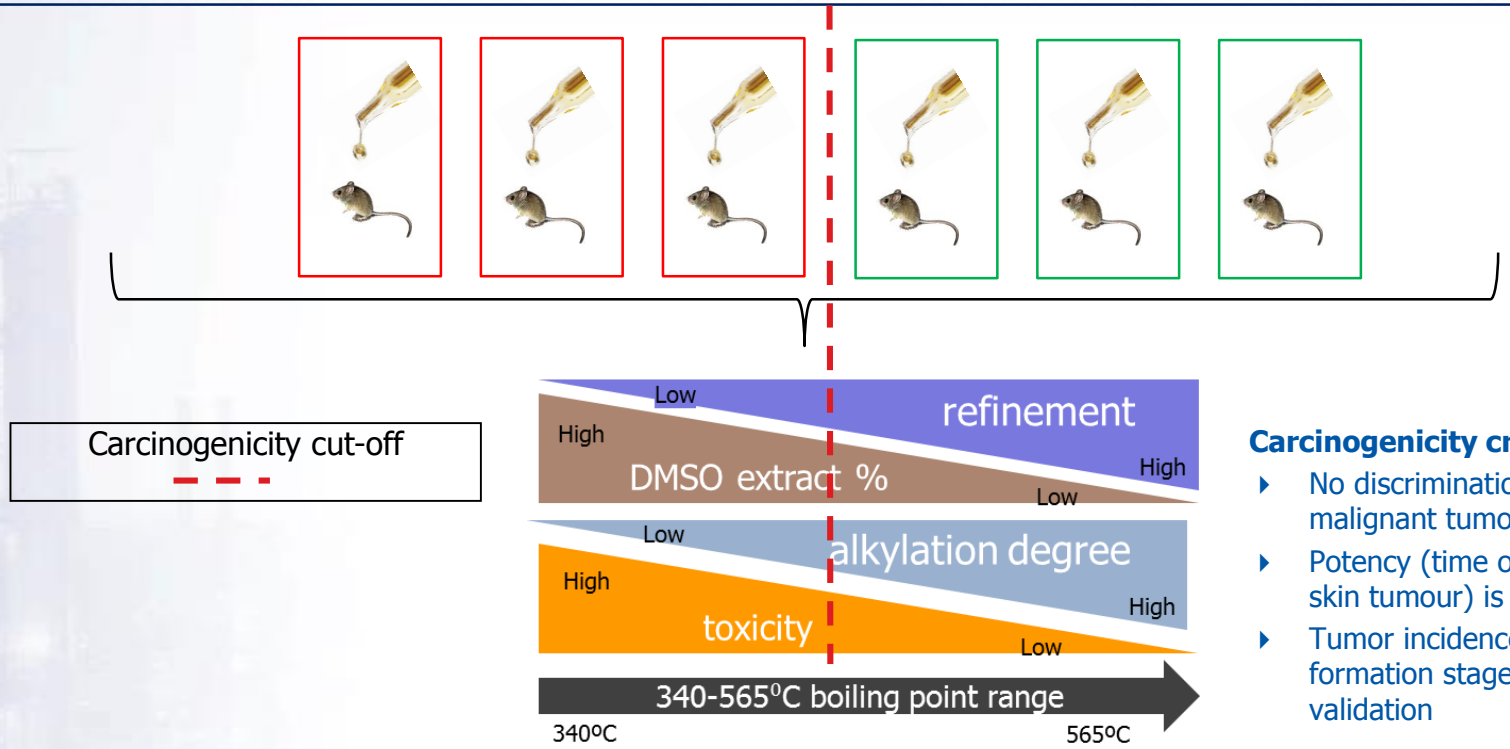


Conclusions On Screening Method Based On DMSO Extraction

- DMSO based screening method is selective towards toxicologically relevant PACs
  - There will be aromatics in refined oils but these are not carcinogenic
- The method considers
  - manufacturing boiling range
  - refinement process which determine PAC levels and alkylation degree
  - composition of mineral oils
  - carcinogenic potential
- Steric hindrance will heavily influence DMSO extract efficiency, reflecting the enzyme-substrate behavior of highly alkylated PAC
- Toxicological data is aligned with DMSO extract carcinogenic potential
- Thus, it is not surprising that mineral oil refinement level and mouse skin painting studies show high correlation

- This is the basis of the regulatory standard: **IP346**

IP346 – The Carcinogenicity Screening Method



Carcinogenicity criteria for validation:

- No discrimination between benign or malignant tumors
- Potency (time of appearance of first skin tumour) is not considered
- Tumor incidence (4%) and not tumour formation stages used for IP346 validation

IP346 validation:

- DMSO-based screening method validated with animal data
- 1:1 relationship same oil mouse skin painting studies and its own DMSO-extract
- Determine a "cut-off": % DMSO extract that is correlated to non-carcinogenic oil
- Cut-off solely on a hazard basis:
  - Pass/fail in carcinogenicity assessment
  - Pass/fail is binary. "*Pass*" means **safe** (and not safer, safest, etc…)

IP346 And Mouse Skin Painting Studies – Data Base

- Completely eliminated carcinogenicity testing on animals
- Adopted in the 90's in the EU and in other countries (e.g. Australia, Malaysia) as regulatory standard for carcinogenicity assessment

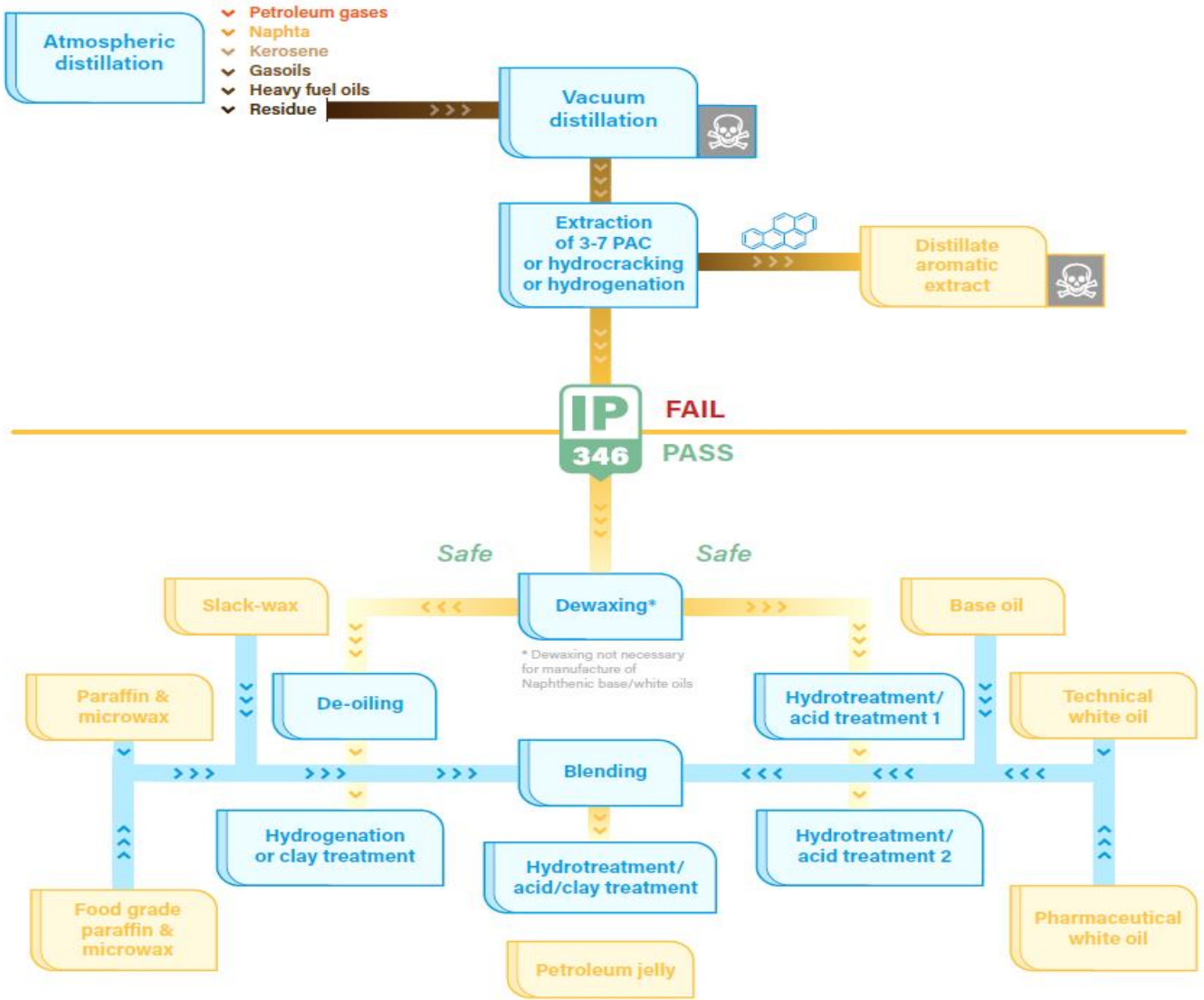
  - IP 346 < 3% oil is not carcinogenic
  - IP 346 ≥ 3% is carcinogenic

Reference	Data points (2 year studies)
CONCAWE 6/16 **CONCAWE 94/51**	**133 *** **104**
Chasey et al., 1993	94
McKee et al., 1989	9
Doak et al., 1983 and (1985)	12 (6)
Blackburn et al., 1996	120
Roy et al., 1988	39

Negative predictivity = 95%
Accuracy = 89% (because of false positives)

*Including all studies cited, without repetitions

Base oil, wax, white oil, petroleum jelly manufacture



IP346 – Take Home Message

- Completely eliminated carcinogenicity animal testing
- Critical and rapid quality control tool to ensure ***in-situ*** refinement efficacy
  - **Vital specification** to ensure product safety for its release downstream
- The extracted 3-7 PAC material is related to intrinsic carcinogenicity of the undiluted oil
- Not just an analytical method but rather a fit for purpose tool to assess carcinogenicity

Thus, IP 346 is NOT to measure PAC content, but rather indicative of the relationship between the refinement history and the PAC biological activity of the oil in mouse skin painting assays (are the PAC active or not?)

- The IP346 (DMSO extract) encompass PAC (also low alkylated PAC), and other substances that *per se* may not be biologically active, but together in the oil may decrease/potentiate carcinogenic activity
- IP 346 is not intended for risk assessment purposes – only hazard assessment
- Thus, IP346 is a gatekeeper and the method for assessing refinement effectiveness: **"green light" for further processing** in order to meet other regulatory standards (e.g. pharmacopeia)

It is the only validated analytical method with biological significance

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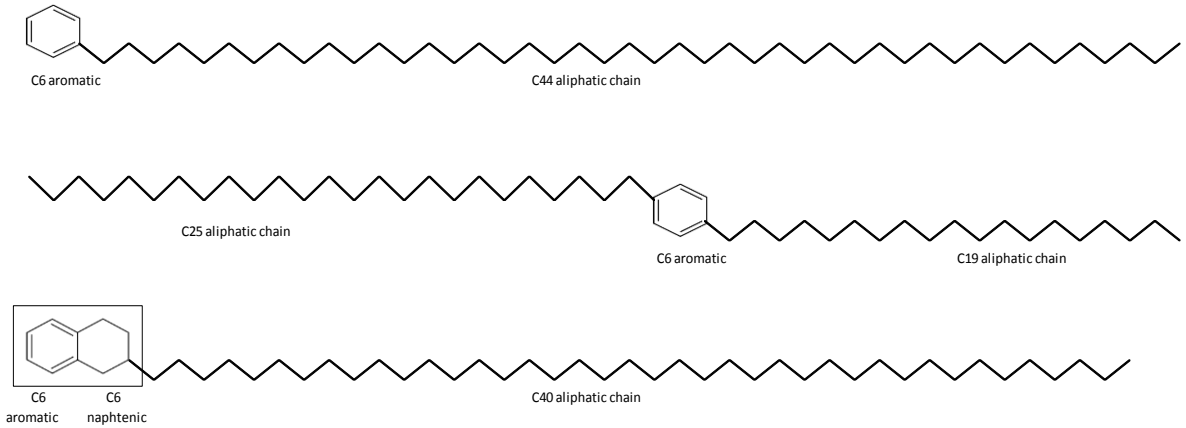
Total Aromatics (MOAH) and Carcinogenicity

Why Is MOAH "High"? – The MOAH Paradox
Example Microcrystalline Wax

- ***MOAH (HPLC-GC FID) typical levels:***
  - 1-5 %.
- ***MOAH content < C35***
  - virtually absent
- ***Content of aromatic protons (NMR):***
  - ~ 0,1 – 0,5 %
- ***Typical av. mol weight microwax:***
  - 700 (C50H102)
- ***3-7 rings aromatics:***
  - trace levels (specific UV test / Grimmer etc.)

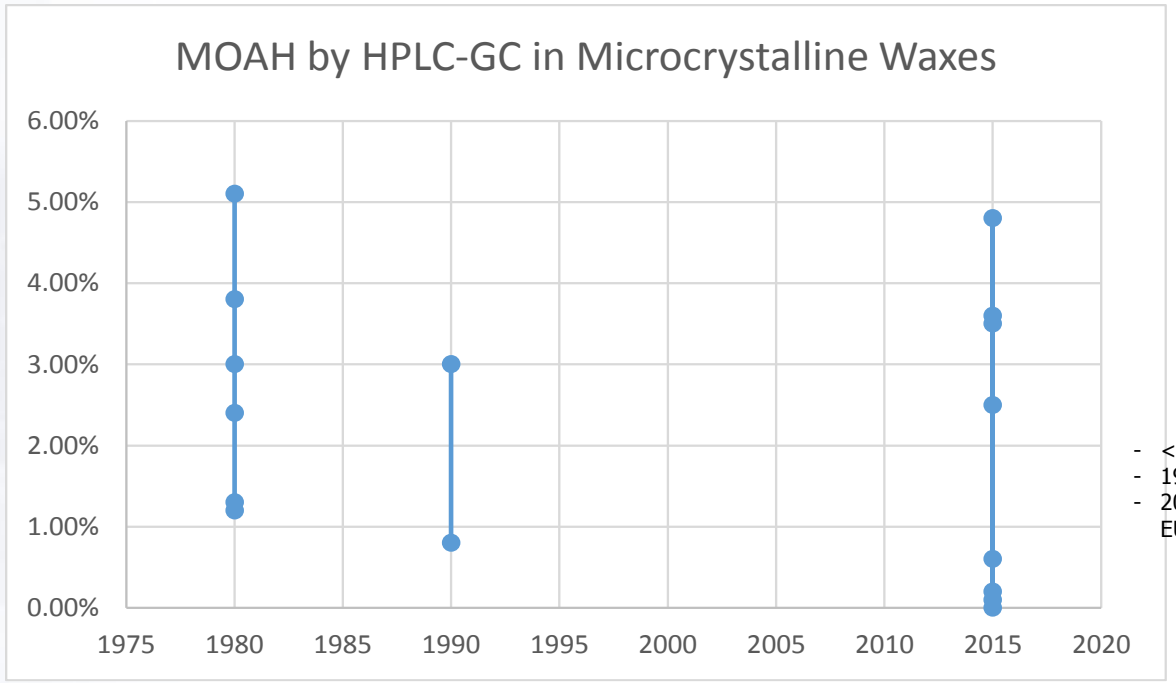


- High alkylation of a small number of aromatic carbons leads to high MOAH values (everything is interpreted as aromatic)
- The higher the MW the greater the MOAH

MOAH paradox: the more aliphatic, the more "aromatic"

Former Material Is Representative For Today – Decades Long Consistency In Manufacturing

- Recent HPLC-GC measurements on old and new production samples of several (EU) manufacturers (2015) confirm that MOAH was always present – nothing new!
- Historic concentrations used for fundamental toxicological studies were at least as high or even higher than those in products presently on the market



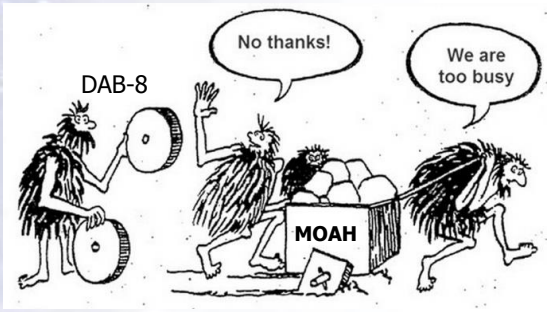
Do We Need A Sophisticated MOAH Method?

The measurement of Total Aromatics (MOAH) is nothing new

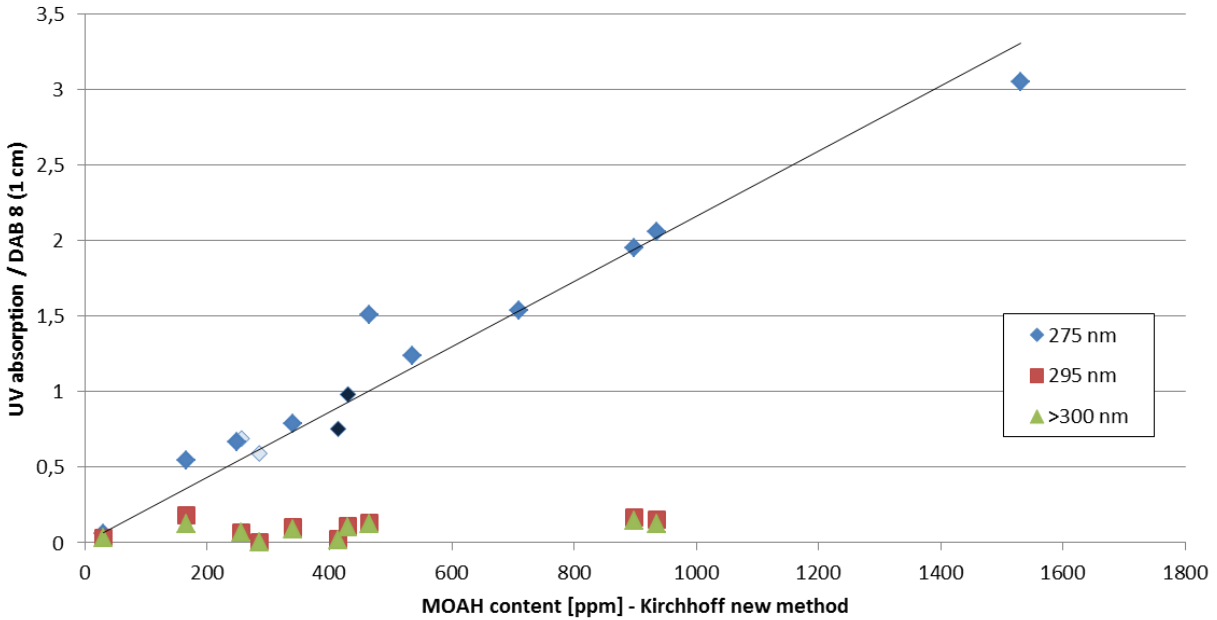
- **DAB 8 UV-method** did the same

Best correlation with Oils

- Oils have shorter MOAH`s
- Longer MOAH chains are not toxicologically relevant
- Replaced by UV-methods including DMSO extraction to focus on PAH
- not biased by MW



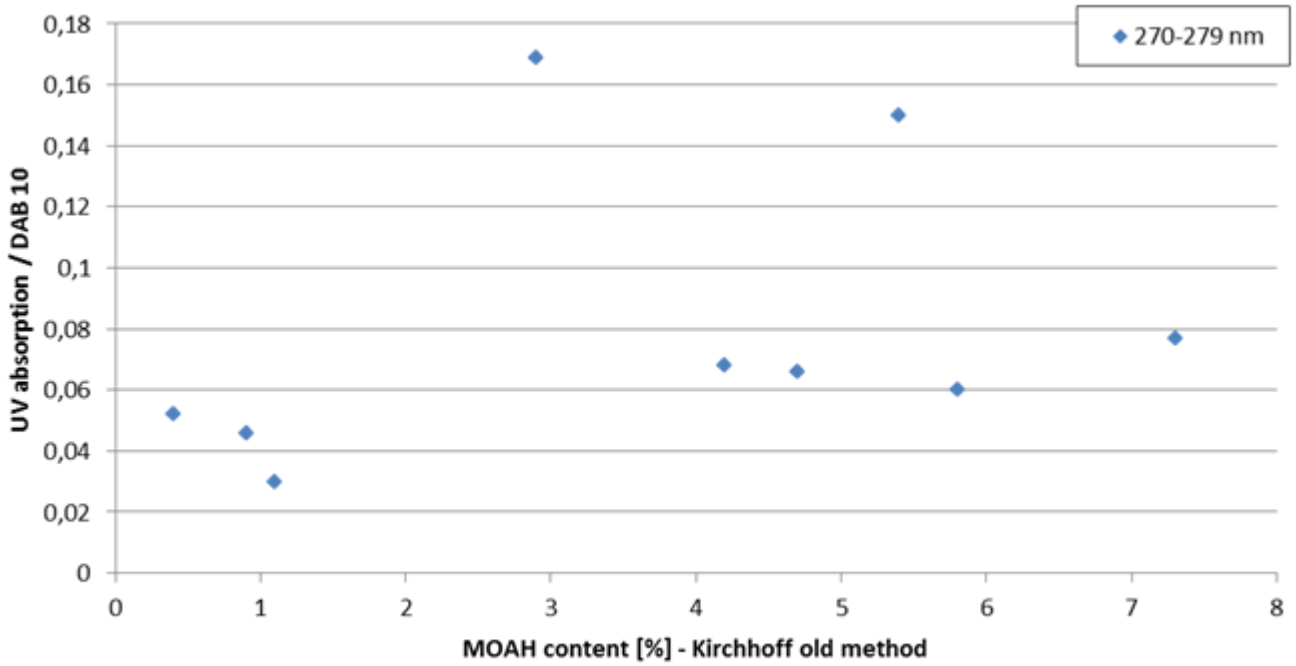
Data presented in collaboration with the company H&R



MOAH HPLC Does Not Correlate With DMSO-PAC Measurements

Data presented in collaboration with the company H&R

- No correlation between MOAH content* and UV absorption according to the pharmacopoeia PAC test
- Amount of PAHs found in products is independent of measured MOAH content



*Kirchhoff method, July 2015

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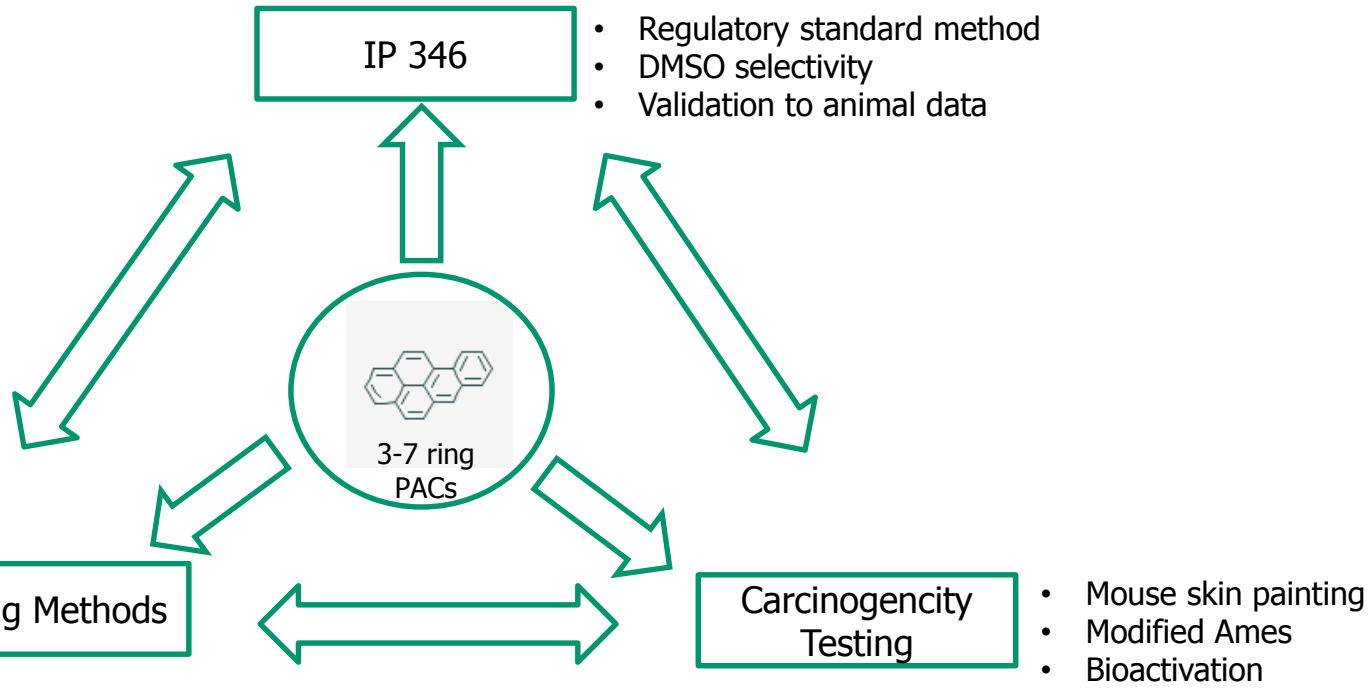
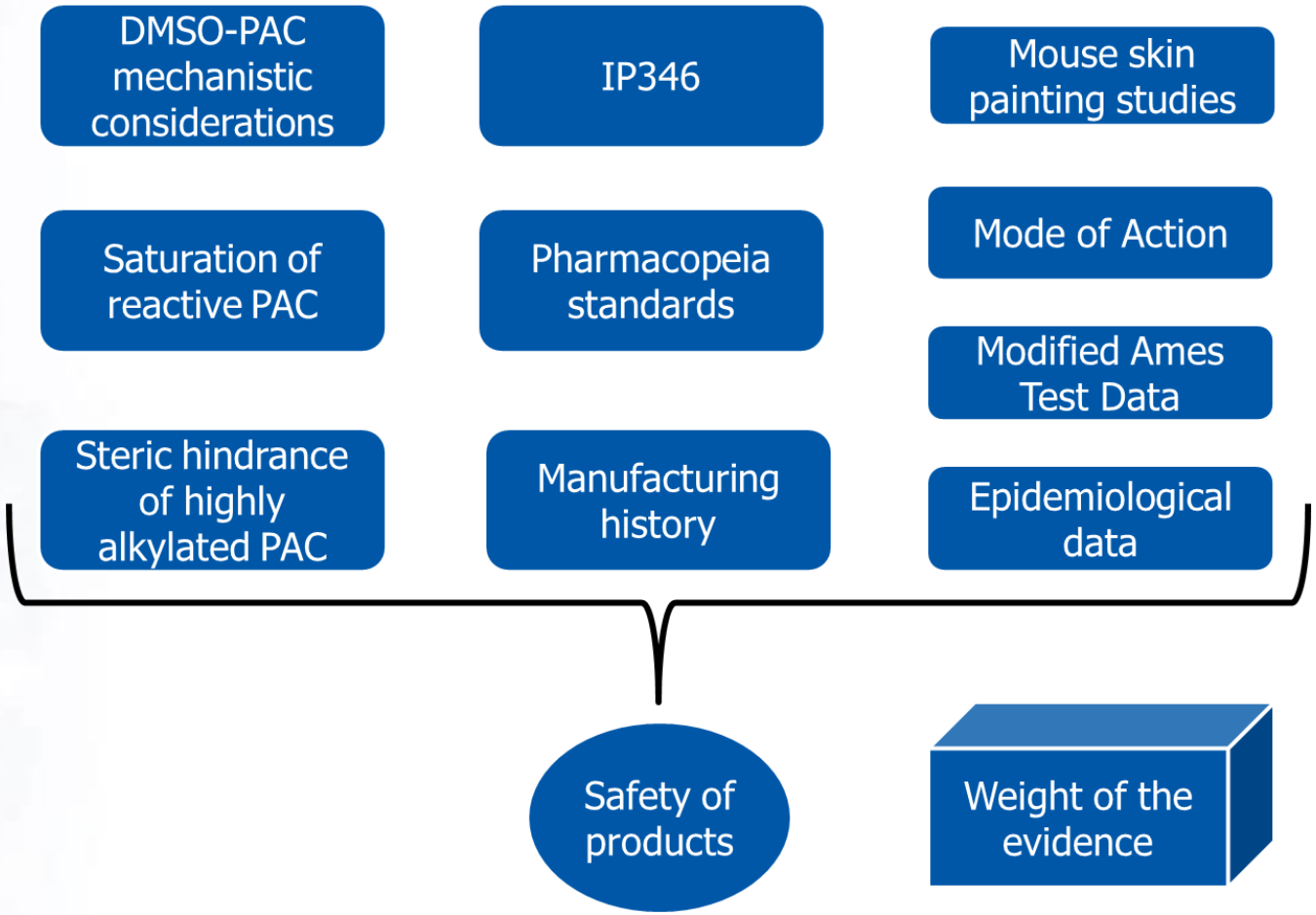
Conclusions

Can't See The Forest For The Trees



The Carcinogenicity Weight of Evidence



MOAH Take Home Message I

- MOAH is an integral part of the substance, can't exist in isolation
- MOAH as "catch all" term is confusing for substance assessment – no toxicological context
- Toxicologists focus on what matters: 3-7 PAC
- Only manufacture determines type of MOAH in mineral oil products i.e. the MW of the intended final product

- Bad MOAH: 3-7 PAC (eliminated through refinement)
- Harmless MOAH: highly alkylated aromatics (what is left after 3-7 PAC elimination)

MOAH Take Home Message II

- If refinement history is known, MOAH can be put into context, its logical but not the other way around
  - compliance focuses on regulating 3-7 PAH
  - therefore MOAH from unknown sources should target the bad MOAH (3-7 ring PAH)
- Harmless MOAH levels vary at each refinement step and increase with MW
- Always been present, at same levels, nothing new
  - Can be measured with a simple UV test (e.g. DAB 8)
- PAC by DMSO extraction are better descriptors of cancer hazard
  - White Oil purity with a simple UV test (e.g. EuPharm 9)
- **Refined mineral oil** products are safe even if MOAH is present
- This includes all process oils e.g. printing ink oil, lubricating oils

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Thank You