Relevance of animal studies in the toxicological assessment of oil and wax hydrocarbons. Solving the puzzle for a new outlook in risk assessment

Juan-Carlos Carrillo\textsuperscript{a}, Dirk Danneels\textsuperscript{b} and Jan Woldhuis\textsuperscript{c}

\textsuperscript{a}Shell Health, Shell International B.V, The Hague, The Netherlands; \textsuperscript{b}European Wax Federation (AISBL), Brussels, Belgium; \textsuperscript{c}Paramelt B.V, Heerhugowaard, The Netherlands

\textbf{ABSTRACT}

Paraffin waxes and white mineral oils are distinct petroleum products separated from a common feedstock by crystallization, where only n-alkanes, iso- and cyclo-alkanes with a linear backbone of \textasciitilde20 carbon atoms long, selectively crystallize out from the oil to form the wax, which is solid at room temperature, whereas oils remain liquid. Up until the 90’s, these differences were reflected in separated regulatory assessments. A paradigm shift occurred when Fischer 344 rats (F-344) developed liver epithelioid granuloma following exposure to low and medium viscosity oils or waxes. This lesion was used as common denominator between these products to be jointly assessed under the common term “mineral hydrocarbons – MHC”, obviating compositional differences. This regulatory paradigm dominated for the next 30 years, exacerbated by the EFSA 2012 evaluation using the analytical term “MOSH” (mineral oil saturated hydrocarbons) which encompassed these products under single chromatography fraction. The reconstruction of historical developments, together with recent EFSA-sponsored studies of toxicity and accumulation and supporting literature, has allowed us to understand the etiology of the F-344 rat hepatic epithelioid granuloma, which is presented in an adverse outcome pathway (AOP). Considering chemical composition, it clearly demonstrates that the hepatic effects in F-344 rats caused by linear alkanes of waxes are irrelevant for humans. Waxes are thus not MOSH and should thus be evaluated on their own merit. The term MOSH should not include n-alkanes and be exclusively used to mineral oil fractions when considering their chemical makeup for a relevant human hazard assessment.

\textbf{Table of contents}

Introduction ........................................... 419  
What are mineral oils and waxes? Regulation, manufacturing and alkane composition ........................................... 419  
\textit{Regulation of waxes} .................................. 419  
\textit{Manufacturing of waxes} ................................ 420  
\textit{UVCB complexity of waxes determined by crystallization} ........................................... 420  
Relevance and limitations of physical parameters (e.g. viscosity) to infer composition ........................................... 424  
Safety evaluations of mineral oils and waxes ........................................... 425  
\textit{The CONTAM panel evaluation of 2012 and the MOSH paradigm} ........................................... 428  
Toxicological evaluations of hydrocarbon waxes ........................................... 429  
\textit{Animal studies – strain and species differences} ........................................... 429  
\textit{Shubik et al. 1962} ................................... 429  
\textit{Baldwin et al. 1992} ................................... 429  
\textit{Smith et al. 1995} ................................... 430  
\textit{CONCAWE 1993; Smith et al. 1996} .................. 431  
\textit{Firriolo et al. 1995} ................................... 432  
\textit{Griffis et al. 2010} ................................... 433  
\textit{Scotter et al. 2003} ................................... 434  
\textit{Hoglen et al. 1998} ................................... 436  
\textit{Conclusion of animal data 1962-2010} .................. 436  
\textit{EFSA study 2017 – new insights} ...................... 437  
\textit{Crystallization of n-alkanes in the F-344 liver and granuloma formation} ........................................... 439  
\textit{Metabolism vs crystallization of n-alkanes} ........................................... 439  
\textit{Granuloma formation and mode of action} ........................................... 441  
\textit{Granuloma formation} .................................. 441  
\textit{Mode of action} ........................................... 442  
\textit{Key event 1 – intestinal absorption of MOH} .......... 443  
\textit{Key event 2 – liver deposition and retention} .......... 443  
\textit{Key event 3 – inflammatory cell tissue infiltration in response to retained MOH in liver} .................. 443  
\textit{Proposed mode of action in an AOP} .................. 443  
\textit{Key event 1 (KE\textsubscript{1})} ......................... 444  
\textit{Key event relationship 2 (KER\textsubscript{2})} .............. 444  
\textit{Key event 2 (KE\textsubscript{2})} ......................... 444  
\textit{Key event relationship 3 (KER\textsubscript{3})} .............. 444  
\textit{Key event 3 (KE\textsubscript{3})} ......................... 444

\textbf{CONTACT} Juan-Carlos Carrillo \textsuperscript{a} \texttt{juan-carlos.carrillo@shell.com} Shell Health, Shell International B.V, The Hague, the Netherlands.

\textcopyright 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Relevance of F-344 rat granuloma mode of action to humans

a. Alkane metabolism in human livers

b. Crystallization of n-alkanes in humans

Synopsis – the etiology of the F-344 rat lesions

MOSH accumulation in humans

Conclusion and recommendations

Acknowledgements

Declaration of interest

References

Introduction

Mineral oils and petroleum waxes are complex hydrocarbon substances manufactured by the vacuum distillation of crude oil. They are considered “complex” because of the thousands of isomers found in them when assessing their composition. Under EU legislation, they are not “mixtures”, but substances that are legally defined as UVCB's (substances of Unknown or Variable composition, Complex reaction products or Biological materials) by distinct EINECS numbers (Rasmussen et al. 1999).

Mineral oils have varying proportions of paraffinic and aromatic constituents, with highly refined mineral oils (white oils) consisting almost entirely of paraffins of normal, iso- and/or cyclo-alkane configuration (aromatics may be present at trace levels). Petroleum waxes on the other hand, consist almost entirely of n-alkanes, with small amounts of iso- and cyclo-alkane constituents with minimal carbon backbone substitution that do not interfere with crystallization, resulting in waxes being solid at room temperature (more on this topic is discussed further on). Mineral oils which have little or no n-alkanes, are on the other hand liquid at room temperature.

It is these chemical structure differences between alkane constituents of oils and waxes which give them very distinct physical chemical properties and subsequently influence the outcome of toxicological evaluations in the selected animal model, as will be seen with studies carried out in the F-344 or the Sprague Dawley (SD) rat (and other rat strains and species).

The long history and use of petroleum-derived oils and waxes in the industry has generated a significant amount of data followed by a no less rigorous number of regulatory evaluations to ensure safe use. Although alkanes are relatively simple and non-reactive chemicals, their safety assessment as complex hydrocarbon products (i.e. oils and waxes) has not been without stumbling blocks and controversies, especially the discussion about the relevance of the F-344 rat for the assessment of mineral waxes and oils since the 90’s. This has led to the situation where acceptable daily intake – ADI – values have been set or revoked based on a complicated and puzzling data base of chemical compositions, toxicological endpoints, internal and external dose differences, rat strain differences and opinions on the relevance of these for human risk assessment. An extensive literature review on long-term toxicity of mineral waxes and oils has been recently published which provides a novel visualization of the data points and a general insight into two key points namely, hepatic granuloma and alkane accumulation (Pirow et al. 2019).

Although the review by Pirow and colleagues gives an excellent overview of the subject, in our paper, we go a step further in drawing conclusions of what the reviewed data means to paraffin waxes and the regulatory consequences that logically follow therefrom.

This paper will thus revisit the key pieces of the puzzle (Barp et al. 2014; Cravedi et al. 2017; Nygaard et al. 2019), that allowed us to postulate the etiology of the F-344 hepatic effects, namely by linking these lesions to specific alkane subclasses also found in waxes. What we demonstrate herein will provide the missing link that allows the disentangling of the confusion that has hampered risk assessment and effective setting of regulatory policies and ADI values.

What are mineral oils and waxes? Regulation, manufacturing and alkane composition

The terms mineral oil hydrocarbons (MOH) and mineral oil saturated hydrocarbons (MOSH) were coined to refer to fractions of hydrocarbons from petrogenic origin found in food or consumer products (Biedermann et al. 2009; Biedermann and Grob 2009a; Biedermann and Grob 2009b). While initially the term was used in the context of clear mineral oil contamination of food (Biedermann and Grob 2009a), the indiscriminate use of the term MOSH (mineral oil) as synonym of “petrogenic” created confusion because the term “oil” was used to refer to waxes, most prominently in the EFSA report of 2012 (EFSA 2012). Certainly, waxes can be of petrogenic origin, but definitively they are not oils. This basic distinction is crucial in understanding the results from toxicology studies in the F-344 rat that has been a blind spot in the hazard assessment of these products. The disentangling of the MOSH and F-344 paradigm starts by understanding the compositional differences that make waxes different from mineral oil, which is the subject of this section.

Waxes are crystalline solids at room temperature, contrary to most of the other products falling under the MOSH umbrella that typically are liquids. As crystalline solids, their manufacturing process involves in most cases two crystallization steps, resulting in a product composition that is much more narrowly defined than is typical for the MOSH (oil) family.

Regulation of waxes

From a regulatory point of view, the substances that will be the focus of the present paper are listed in Annex 1 of the Plastics Regulation (EU) 10/2011 as Food Contact Material (FCM) 93: an additive in plastic materials and articles intended to come into contact with food. The corresponding description listed in this Annex is: Waxes, paraffinic, refined, derived from petroleum-based or synthetic hydrocarbon feedstocks. The average molecular weight is not less than 350 Da and the viscosity at 100°C ranges from 2.5 to 11 mm²/s at 100°C.
The meaning of the term “paraffin” or “paraffinic” can be quite broad and context dependent. In a generic sense, it can be used as a synonym for alkanes, which are divided into three groups based on their structure: normal alkanes (normal paraffins or n-alkanes), branched alkanes (branched paraffins or iso-alkanes), and cyclo-alkanes (cyclo-paraffins or naphthenes). In some cases, paraffins refer to normal (linear) paraffins or iso-alkanes), and cyclo-alkanes (cyclo-paraffins or naphthenes). In other cases, the term “paraffin” refers to “paraffin waxes”. In this paper, we will use the term “paraffin waxes” as shorthand for the substances covered by the FCM 93 entry in Annex 1 of the EU Plastics Regulation 10/2011. Where it is relevant, we will use the term “hydrocarbon waxes” to cover the broader range of hydrocarbon waxes including both FCM 93 and FCM 94 (Waxes, paraffinic, refined, derived from petroleum-based or synthetic hydrocarbon feedstocks. The average molecular weight is not less than 500 Da and the viscosity at 100 °C is at least 11 mm²/s at 100 °C) and less refined waxes such as slack waxes explained in the next section.

Manufacturing of waxes

Understanding the similarities and differences in the chemical composition between mineral oils and waxes is important for the justification of not extending the term MOSH for waxes. Both mineral waxes and oils originate from the vacuum distillation columns of the lubricating oil manufacturing unit in a petroleum refinery. After crude oil is distilled at atmospheric pressure into light fractions the resulting residue is further refined to yield a non-carcinogenic “waxy raffinate”, which is the common feedstock of mineral oils and waxes and comprises of normal, iso- and cyclo-alkanes and predominantly alkylated aromatics, as the little alkylated and naked ring 3–7 PAC have been extracted (Carrillo et al. 2019). Before it is further processed under vacuum to yield a set of oil substances (base oils and white oils), the waxy raffinate must be dewaxed. The purpose of this dewaxing is to remove long chain paraffins from the product intended to become a lubricating oil. It is at this point that waxes and oils are separated from their common “ancestor” feedstock into distinct products: slack wax and base oil. The former becomes the starting material for paraffin waxes, where the latter are lubricating base oils, which after hydrogenation/acid treatment steps become white oils. The residue of the vacuum distillation of waxy raffinate is called “bright stock” and it will be the source for a heavy lubricating oil and “bright stock slack waxes” which can be further refined to yield microcrystalline waxes which have a higher molecular weight compared to paraffin wax. These products are considered related (feedstock related) but have their own separate characteristics and chemical composition.

Synthetic waxes on the other hand are directly manufactured via the Fischer-Tropsch process (i.e. without an oil counterpart) which yield UVCB products that have similar chemical properties as their petroleum-derived (mineral) analogs, but typically contain very low levels of aromatics. Thus, both mineral and synthetic waxes are covered under FCM 93 of Regulation (EU) 10/2011.

UVCB complexity of waxes determined by crystallization

Waxes are distinct petroleum-derived products that under REACH and CLP are characterized as UVCBs: substances of Unknown or Variable composition, Complex reaction products or Biological materials (Rasmussen et al. 1999; ECHA 2017). Typically, UVCBs cannot be sufficiently identified by their chemical composition, because the number of constituents is relatively large and/or the composition is, to a significant part, unknown and/or the variability of composition is relatively large or poorly predictable. For petroleum products, the refinery stream and the refinery processes during their manufacturing are very important elements in the identification of this type of UVCBs. In the case of waxes, the crystallization step(s) are the critical element to distinguish them from other products comparable carbon chain length, viscosity or boiling point ranges. Indeed, the presence or absence of constituents is selectively controlled via the crystallization steps during the manufacture of a wax from the waxy raffinate and subsequently from slack wax.

The crystallization that selectively separates waxes from oils is driven by two main alkane types:

- Fully linear and mostly linear structures that can form a wax crystalline matrix.
- Structures that, due to their isomeric forms, can only be liquid at room temperature and form the oil matrix.

To better understand the detailed composition of the hydrocarbon waxes covered under the definition of FCM 93 in Annex 1 of Regulation (EU) 10/2011, the European Wax Federation aisbl (EWF) undertook the characterization of seven waxes by two-dimensional gas chromatography (GC × GC). These waxes were selected because as a group their physical properties span the full range of FCM 93 waxes, but also because, individually, many of them have been test substances in the historical toxicological studies that will be discussed further. Table 1 provides a description of these waxes indicating their relevance in the context of the present paper. The point of the selectivity of the crystallization process in narrowing down the number of isomers is illustrated in Figure 1 and Table 2, both representing the GC x GC of WAX 1, a paraffin wax at the low end of the FCM 93 range. What is striking in this GC x GC is how few isomers in addition to the n-alkanes are present. This finding is not entirely new, already in the 60’s a study of paraffin waxes concluded that waxes are composed of “rather simple molecules”, and remarkably few of them (Levy et al. 1963). As we see in terms of composition, waxes are almost entirely formed by n-alkanes with an extremely low content of iso- and cyclo-alkanes. So, while waxes formally comply with the prerequisites to be considered UVCBs, their complexity is far less than the usual assumption that the number of constituents must increase exponentially with increasing carbon range following the molecular formula CₙH₂ₙ₊₂. For example, as can be seen in
Table 1. Description of the homologous series waxes tested by the EWF and mentioned in the present paper. Where relevant, reference is made to the toxicological studies they were used in.

| WAX | Description                                                                                           |
|-----|--------------------------------------------------------------------------------------------------------|
| 1   | Recent production sample of a commercial wax representing the low end of the FCM 93 carbon number range. |
| 2   | Historic retained wax sample tested in CONCAWE 1993/Smith et al. 1996, Hoglen et al. 1998, Scotter et al. 2003 and Griffis et al. 2010 and designated as "LMPW" in these articles. |
| 3   | Recent production sample of WAX 2 manufactured by the same plant producing the original WAX 2 sample |
| 4   | Recent production FCM 93 wax sample selected to reflect the average physical properties of the waxes identified as "Wax Nr 2" and "Nr 20" in Shubik et al. 1962. |
| 5   | Recent production sample of the intermediate melting point wax designated as "IMPW" in CONCAWE 1993/Smith et al. 1996 |
| 6   | Recent production sample of a not commercially available intermediate melting point wax representing the high end of the FCM 93 carbon number range. |
| 7   | Recent production sample of the synthetic wax identified as C80W in Scotter et al. 2003.                |

Figure 1. C18 to C36 section of the GC x GC -MS plot (Total Ion Chromatogram) of WAX 1. The number of isomers is quite limited and because of the resolving power of the two-dimensional GC technique, alkane isomers present in the C18 to C40 range can be individually observed. The exception are the 2-Methyl and 4-Methyl isomers that are still overlapping.

Table 2. GCxGC -FID identification of the individual alkane constituents of FCM 93 WAX 1 in Figure 1.

| Number of carbons | Normal | 2-Me/4-Me | 3-Me  | 1-Cyclopentyl | 1-Cyclo-hexyl | 1-Methyl cyclohexyl | TOTAL |
|-------------------|--------|-----------|-------|---------------|---------------|---------------------|-------|
| 18                | 0.01   |           |       |               |               |                     | 0.01  |
| 19                | 0.10   |           |       |               |               |                     | 0.10  |
| 20                | 0.59   |           |       |               |               |                     | 0.59  |
| 21                | 2.20   |           |       |               |               |                     | 2.20  |
| 22                | 4.82   | 0.02      |       |               |               |                     | 4.84  |
| 23                | 7.22   | 0.07      | 0.04  | 0.01          |               |                     | 7.33  |
| 24                | 9.60   | 0.11      | 0.20  | 0.03          |               |                     | 9.94  |
| 25                | 10.54  | 0.39      | 0.28  | 0.06          | 0.03          | 0.02                | 11.33 |
| 26                | 11.98  | 0.32      | 0.80  | 0.11          | 0.07          | 0.06                | 13.34 |
| 27                | 10.65  | 0.62      | 0.91  | 0.20          | 0.07          | 0.12                | 12.58 |
| 28                | 9.38   | 1.38      | 0.43  | 0.20          | 0.11          | 0.21                | 11.72 |
| 29                | 7.75   | 1.22      | 0.52  |               | 0.24          |                     | 9.73  |
| 30                | 5.77   | 1.32      | 0.18  |               |               |                     | 7.27  |
| 31                | 3.63   | 0.71      | 0.34  |               |               |                     | 4.67  |
| 32                | 1.93   | 0.43      | 0.20  |               |               |                     | 2.55  |
| 33                | 0.85   | 0.23      | 0.09  |               |               |                     | 1.17  |
| 34                | 0.35   | 0.05      | 0.08  |               |               |                     | 0.48  |
| 35                | 0.14   |           |       |               |               |                     | 0.14  |
| TOTAL             | 87.49  | 6.88      | 4.07  | 0.62          | 0.29          | 0.65                | 100.00|

The values shown here don’t add up exactly to those shown in Table 4. The data in this table were obtained from a different sample of WAX 1 and the chromatograms were ran under different conditions on different equipment. The 2-methyl and 4-methyl isomer peaks are still overlapping.

Table 3, based on simple theoretical considerations, with 20 carbons the number of alkane isomers is close to 400,000 but, due only to specific physical properties, there is not a single C20 iso-alkane found in the wax. With 25 carbons from the almost 37 million possible isomers, only eight potential isomers of C25 are found in the wax. With 30 carbons and with four billion possible isomers, less than 30 C30 isomers can possibly end up in the wax. This is not only remarkable, but highly illustrative that the "UVCB" nature of waxes is not some obscure and ambiguous descriptor, but rather quite constrained.

So, the question arises of what makes some iso-alkanes (or cyclo-alkanes) prefer the wax crystalline structure over the mineral oil matrix given the fact that, as we have seen, they
are all mineral hydrocarbons and related products. The answer to this lies in the crystallization process which is responsible for the high selectivity of those molecules coming out of the waxy raffinate and slack wax purification process.

The process of crystallization is tremendously sensitive, excluding alkanes which interfere with the n-alkane crystal arrangement. During this process, two almost perfectly linear alkanes will find their way into either the oil or the wax phase. This will be solely determined by the position of a single methyl group on the linear backbone of the alkane. If this methyl group is in a central position, the molecule is ejected to the oil phase, while a methyl at a “terminal” position may still be accommodated in the wax crystal. This is best exemplified by looking at the change on the melting point of a mono-branched alkane when its only methyl substituent in the carbon chain is gradually moved from a terminal position to a more internal one. This is shown by the melting points of the different mono-methyl substitutions of docosane (n-C\textsubscript{22}) as depicted in Figure 2. The impact on the melting point is quite dramatic and the shift from a 2- to a 4-position is already enough to push the melting point below room temperature. This is the highest carbon number for which melting point data on such a set of isomers is available, but it is not difficult to visualize how the presence of an internal methyl substituent will make the molecule unlikely to fit the n-alkane crystalline matrix leaving it in the oil phase during the wax crystallization process.

Thus, only those constituents that have a very specific structure and melting point can crystallize out from the oil, namely n-alkanes, and lightly branched iso-alkanes that have an uninterrupted linear alkane backbone allowing them to co-crystallize with the n-alkanes. Cyclo-alkanes can also co-crystallize provided they fulfill the “unbranched C\textsubscript{20} rule” for mono-methyl and cyclohexyl alkanes respectively. In the case of WAX-1 in Figure 1, this results in a composition of about 87% of n-alkanes and 11% of iso-alkanes with the methyl group in position 2 or 3, where the rest are small amounts of long chain carbon backbones with a cyclo-alkane in the first position.

Thus, only those constituents that have a very specific structure and melting point can crystallize out from the oil, namely n-alkanes, and lightly branched iso-alkanes that have an uninterrupted linear alkane backbone allowing them to co-crystallize with the n-alkanes. Cyclo-alkanes can also co-crystallize provided they fulfill the “unbranched C\textsubscript{20} rule” for mono-methyl and cyclohexyl alkanes respectively. In the case of WAX-1 in Figure 1, this results in a composition of about 87% of n-alkanes and 11% of iso-alkanes with the methyl group in position 2 or 3, where the rest are small amounts of long chain carbon backbones with a cyclo-alkane in the first position.

What we can conclude from the information of Figure 1, and Table 2, is that in the carbon range of WAX 1 (i.e. C\textsubscript{18} - C\textsubscript{35}), the first n-alkane (albeit faint) is found at a carbon number of 18, corresponding to a melting point of 28°C. From Table 3, we learn that in theory, the first iso-alkanes that can be found in the wax should appear only after C\textsubscript{20}. This is confirmed from the detailed composition shown in Table 2, where the first iso-alkane appears at C\textsubscript{22}. This is in line with the correlation between melting point and methyl-group position that would determine that only the 2-methyl C\textsubscript{22} but not the 3-methyl C\textsubscript{22} is found in the wax, because the latter has a melting point of 16°C which will make it liquid at room temperature and therefore partition to the mineral oil matrix. All information combined, leads to a rule of thumb that a molecule should contain a linear polymethylene fragment (i.e. an uninterrupted n-alkane section) of \textasciitilde C\textsubscript{20} carbon.

---

**Table 3.** Possible alkane isomers by carbon number.

| number of carbons | Isomers alkanes C\textsubscript{n}H\textsubscript{2n+2} (cycloalkanes not counted) | Mono-Methylalkanes potentially present in wax | Cyclohexyl alkanes potentially present in wax | Total Mono-Methyl and Cyclohexylalkanes |
|------------------|---------------------------------|--------------------------------------------|---------------------------------------------|--------------------------------------|
| 5                | 18                              | 0                                         | 0                                           | 0                                    |
| 20               | 366,319                         | 0                                         | 0                                           | 0                                    |
| 25               | 36,797,588                      | 4                                         | 0                                           | 4                                    |
| 30               | 4,111,846,763                   | 9                                         | 5                                           | 14                                   |
| 40               | 62,481,801,147,341              | 19                                        | 15                                          | 34                                   |
| 50               | 1,117,743,651,746,953,270       | 29                                        | 25                                          | 54                                   |

The 2nd column shows the number of possible alkane isomers for an alkane with molecular formula C\textsubscript{n}H\textsubscript{2n+2}. Data are taken from the literature (Rouvray 1988), and cycloalkanes are not included. The 3rd and 4th column show the number of isomers fulfilling the “unbranched C\textsubscript{20} rule” for mono-methyl and cyclohexyl alkanes respectively. The 5th column represents the total of columns 3 and 4.

---

**Figure 2.** Comparison of the melting points of monobranched docosane isomers in function of the position of the methyl-substituent.
A first fundamental observation is that three alkane structural subclasses constitute 99.1% of the complete composition of FCM 93 waxes: linear alkanes, mono-branched alkanes and mono-cyclic alkanes. In synthetic FCM 93 waxes, cyclic alkanes are virtually absent, and these waxes only comprise linear and mono-branched alkanes. In all cases, however, the molecules are essentially a linear chain between C_{16} and C_{50} and multibranched and polycyclic naphthenics are absent. This table furthermore allows to make several observations regarding the characteristics of FCM 93 waxes as a family:

1. For petroleum-based FCM 93 paraffin waxes, an increase in ACN is accompanied by a relative decrease of the presence of n-alkanes. At the same time, the levels of mono-branched alkanes and mono-cyclic alkanes increase.

2. When comparing the Carbon Chain Length Ranges present, one observes a shift from the predominant range being C_{26}-C_{30} in the waxes with an ACN of 26 to 28 to C_{41}-C_{45} for the waxes at the high end of the FCM 93 range. Inspection of the GC x GC chromatograms shows that basically the same alkane sub-classes are found in the different waxes with the carbon chains becoming longer with an increasing ACN of the wax.

3. The changes in the relative wax compositions, when moving through the FCM 93 ACN Range, are both gradual and systematic. The relative ratios of the three families of linear, mono-branched and mono-cyclic alkanes are constrained by the boundaries defined by the physical parameters controlled by the distillation and crystallization steps of the wax manufacturing process.

### Table 4. Composition of homologous series of FCM 93 waxes discussed herein.

| Composition by Alkane Structural Class (%) | WAX 1 Petroleum | WAX 2 Petroleum | WAX 3 Petroleum | WAX 4 Petroleum | WAX 5 Petroleum | WAX 6 Petroleum | WAX 7 Synthetic |
|------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Unbranched or Normal Alkanes             | 83.48           | 87.82           | 85.10           | 83.06           | 61.23           | 46.70           | 93.27           |
| Mono Branched Alkanes                    | 12.66           | 9.63            | 10.76           | 13.92           | 18.16           | 33.61           | 6.66            |
| Monoyclic Alkanes                        | 3.75            | 2.50            | 4.14            | 2.93            | 19.47           | 19.68           | 0.00            |
| 1-Alkenes                                | 0.09            | 0.00            | 0.00            | 0.00            | 0.00            | 0.00            | 0.00            |
| 1-Alcohols                               | 0.00            | 0.00            | 0.00            | 0.00            | 0.00            | 0.00            | 0.00            |
| Aromatic Hydrocarbons                    | 0.01            | 0.04            | 0.00            | 0.09            | 1.14            | 0.00            | 0.00            |
| Total                                    | 100.00          | 100.00          | 100.00          | 100.00          | 100.00          | 99.99           | 100.00          |
| Carbon Chain Length Ranges (%)           |                 |                 |                 |                 |                 |                 |                 |
| C_{16}-C_{20}                            | 0.78            | 0.48            | 0.93            | 0.04            | 0.06            | 0.01            | 0.31            |
| C_{21}-C_{25}                            | 34.36           | 40.76           | 42.21           | 21.54           | 2.68            | 0.46            | 0.93            |
| C_{26}-C_{30}                            | 53.37           | 48.02           | 48.28           | 59.12           | 21.53           | 2.51            | 2.08            |
| C_{31}-C_{35}                            | 10.00           | 8.85            | 7.36            | 18.34           | 39.42           | 5.42            | 9.76            |
| C_{36}-C_{40}                            | 0.56            | 1.53            | 0.99            | 0.57            | 23.54           | 14.36           | 24.62           |
| C_{41}-C_{45}                            | 0.08            | 0.26            | 0.18            | 0.23            | 8.85            | 41.44           | 27.81           |
| C_{46}-C_{50}                            | 0.00            | 0.05            | 0.04            | 0.06            | 2.19            | 29.13           | 21.98           |
| C_{51}-C_{55}                            | 0.00            | 0.01            | 0.01            | 0.01            | 0.58            | 6.27            | 12.18           |
| C_{56}-C_{60}                            | 0.00            | 0.00            | 0.00            | 0.00            | 0.00            | 0.40            | 0.28            |
| TOTAL                                    | 99.96           | 99.96           | 100.00          | 99.91           | 98.86           | 99.99           | 99.96           |
| Fraction (%) in C_{21}-C_{35} Range      |                  |                 |                 |                 |                 |                 |                 |
| Fraction (%) > C_{35}                     | 0.64            | 1.85            | 1.22            | 0.87            | 35.16           | 91.59           | 86.87           |
illustrate this with extreme example: A petroleum-based FCM 93 wax with 90% mono-cyclic alkanes is as impossible as one with 90% mono-branched alkanes. Or, a wax with an ACN of less than 30 will never contain more than about 5% of mono-cyclic alkanes.

4. In line with the toxicology studies discussed later (which confirm that higher molecular weight waxes are of lower toxicity), Table 4 shows why waxes with the compositions like WAX 1 to 4 can be considered a “worst case” for safety assessment: any effects that ever have been seen, were most severe for molecules with Carbon Numbers between 20 and 35 and inspection of the last row “Fraction (%) in the C_{21-35} Range” show that for the waxes toxicologically tested, these fractions comprise 98 to 99% of the entire substance. Furthermore, in a homologous series of the molecular weight range encountered in FCM 93 waxes, biological activity is typically inversely proportional to molecular weight. Therefore, FCM 93 waxes with ACN’s of approximately 25 to 30 are, from a toxicological point of view, true worst-case representatives for the FCM 93 series of products and form the basis for critical interpretation of the studies that are based on the low melting point wax-LMPW (e.g. WAX 2 used in (Griffis et al. 2010), (Smith et al. 1996), and (Scotter et al. 2003) see Table 1).

In summary, when considering alkane composition of paraffin waxes as UVCBs, there are two defining processes: the vacuum distillation that controls the carbon number range of the alkanes, and the crystallization steps that selectively allow certain alkane subclasses to be incorporated, that is, the essentially linear, largely unbranched ones that comprise waxes, versus the multi-branched and (multi) naphthenics that will constitute the hydrocarbon oil liquid phase.

The characterization of FCM 93 paraffin waxes in terms of molecular mass ranges and alkane structural subclasses provides a relevant background for the proper interpretation of historic toxicological studies, the core subject of the present paper. Often the relevance of these studies is put into question because of the insufficient characterization of the tested substances, especially in the light of recently developed techniques as for instance GC x GC. This element was taken into consideration when selecting the paraffin waxes that were included in the EWF FCM 93 characterization work (Table 1).

Of particular interest is WAX 2: It is a retained sample of the wax identified as LMPW in the sub-chronic toxicology studies conducted during the 90’s. It was serendipitously retrieved from the laboratory storage at the wax blending company Paramelt B.V. This is the original sample of the material identified as LMPW in the studies ran with the F-344 rat strain in the early 90’s and published in 1996 as Smith et al. Subsequently, a sample of the same batch was used in the study comparing the effect of LMPW on the F-344 and SD rat strains and published in 2010 by Griffis et al. To clearly establish a link between the material tested in the 90’s studies and products still commercially available today WAX 3 is included. WAX 3 is a sample of a commercial wax produced in 2020 by the refinery that originally manufactured the LMPW sample and commercially considered to be the same as WAX 2. It is known that waxes are extremely stable when stored in a dark cool place, and comparing the columns for WAXES 2 and 3 in Table 4 also illustrates that from a carbon chain and alkane type distribution the overall compositions remained unchanged over the last 30 years.

Another important study concerning waxes was the study published by Shubik in the 60’s (Shubik et al. 1962). A sample of the materials tested in those studies was not retrieved, but WAX 4 was included as representative for a product with the same overall physical characteristics as one of the waxes tested in this study.

It is important to stress that from the manufacturing point of view, the essential steps of the “wax from oil separation” processes have remained unchanged from the days the original toxicological studies were conducted. The solvent de-waxing to produce slack waxes and the solvent crystallization to remove the remaining oil from the slack wax were the established standard in the USA at the time of the Shubik study. In the meanwhile, they have evolved as the standard wax manufacturing processes worldwide. What did change over time are further improvements of the purification processes generally resulting in a further lowering of the presence of non-saturated alkane constituents in a wax.

The presence of high levels of n-alkanes in paraffin waxes at the lower end of the FCM 93 range is important in understanding why Low Melting Point Waxes (LMPW) have the strongest effects in the liver of F-344, as will be discussed further on. Furthermore, because toxicology studies on waxes used viscosity and not detailed composition as a key parameter, the relationship between these two characteristics will be addressed in the next section.

Relevance and limitations of physical parameters (e.g. viscosity) to infer composition

Mineral oils and waxes are UVCB substances and from an industrial application’s perspective a detailed chemical composition is not only nearly impossible but also impractical because the intended use is driven by performance and physical chemical properties. These are reflected in technical specifications that were introduced to manufacture different types of oils and waxes most suitable for an intended use, and for that purpose the exact composition is quite irrelevant.

From a hazard assessment perspective, a key specification is viscosity. With this parameter, the bulk composition, molecular weight and boiling range can be derived. For example, high viscosity products, have high molecular weight constituents, found at higher boiling points which consequently results in long carbon chains and a higher number of isomers. Figure 3 shows the correlation between molecular weight (expressed as Average Carbon Numbers) and the kinematic viscosity at 100 °C for FCM 93 paraffin waxes. A similar graph can be obtained for the corresponding mineral oils.

It is known that viscosity, via its connection to molecular weight, has for instance a strong effect on the absorption and bioavailability of hydrocarbons. For example, Albro and
Fischbein (Albro and Fishbein 1970) examined in a systematic study the relationship between carbon number of hydrocarbons and their absorption. A linear relationship was demonstrated: hydrocarbons with a carbon number of 14 were absorbed to ca. 60%, while no absorption of hydrocarbons with a carbon number > 30 was observed. This was confirmed by the studies with the F-344 rat strain. A GC-FID analysis of the hydrocarbons retained in the rat livers clearly indicated a cutoff at a carbon number of 35 for the IMPW tested. These results were documented in the relevant CONCAWE report (Freeman et al. 1993) but not retained for the peer reviewed publication (Smith et al. 1996). In the meanwhile, there is indeed ample evidence that the absorption and bioavailability of wax constituents with carbon numbers above 35 drops virtually to zero (Scotter et al. 2003; Cravedi et al. 2017).

Viscosity therefore became a crucial tool to interpret animal and human toxicological data as will be seen in the following sections. From a regulatory perspective, and particularly for food grade waxes, viscosity is the key parameter that has been used to regulate the substance and is directly linked to the available toxicity studies (SCF 1995). As was explained earlier, for example, FCM 93 (Waxes, paraffinic, refined, derived from petroleum based or synthetic hydrocarbon feedstocks, low viscosity) uses a viscosity at 100 °C of not less than 2,5 cSt (2,5 × 10⁻⁶ m²/s⁻¹) which is linked to an average molecular weight of not less than 350 Da. Compositonally such a wax should not have more than 40% of constituents with a carbon number <25, which means that the bulk of the material should be around C₂₅. This covers Low Melting Point Waxes (LMPW) and, to a lesser extent, Intermediate Melting Point Waxes (IMPW) that contain the critical carbon range that plays the key role in the formation of hepatic granuloma in toxicity tests when the F-344 rat was the experimental strain.

Therefore FCM 93 waxes represent a very homogenous and unified family of products but not all of them will have the same toxicological effect in the F-344 rat. The Average Carbon Number or its surrogate measure “viscosity” offers an easy way to locate them on the continuum that constitutes the FCM 93 range. From a hazard assessment perspective, the “low viscosity” in waxes is reflected in the critical carbon number of 20 to 35 with a peak at carbon numbers ~28 to 31 (corresponding to LMPW and the low end of IMPW) that will be discussed as the determining factor in the etiology of the F-344 granuloma.

Safety evaluations of mineral oils and waxes

Highly refined paraffin waxes including those currently listed under FCM 93 have been used for decades in food packaging, cosmetics and pharmaceutical products and up until 1995 they were separately evaluated from mineral oil, which had several assessments by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) with the following evolving views on establishing an acceptable daily intake (ADI) for waxes and oils:

- Mineral oils up to 1995:
  - the use of food grade mineral oil is self-limiting because of its laxative effect, no need to set a limit in food beyond that of good manufacturing practice (JECFA 1970);
  - No other recognized toxicological problems arising from the present technological uses of food-grade mineral oil; ADI "not limited" (JECFA 1974);
  - no demonstrable pathological consequences from the presence of (acid treated) mineral oils in human tissues resulting from ingestion, its storage is considered undesirable and exposure to mineral oils should be kept to a minimum. Due to the introduction of hydrogenation, there are
At the 44th meeting, mineral oil was jointly assessed (JECFA 1976). At the 30th meeting in 1989, for the first time, data on fied of oil (acid treated vs hydrogenated) to allow a toxico-
larities and differences in the composition of the two types
process, no ADI was thus established (JECFA 1987a, 1987b). The Committee deemed necessary a thorough review of the toxicological data and specifications. Synthetic waxes were not considered.

- At the 44th meeting of JECFA in 1995 (JECFA 1995) the situ-
ations changed completely when the data generated by the testing of paraffin waxes and mineral oils with the F-344 rat strain was evaluated. This resulted in the withdrawal of the ADI for paraffin wax, triggered by the study run in 1991-1992 and eventually published in 1996 (Smith et al. 1996). For the first time, viscosity specifications and descriptors derived from toxicology studies were introduced for listing of mineral waxes and oils.

Joint wax and mineral oil assessment of 1995 by JECFA and SCF

At the 44th report of JECFA in 1995 (JECFA 1995) the situation changed completely when the data generated by the testing of paraffin waxes and mineral oils with the F-344 rat strain was evaluated. This resulted in the withdrawal of the ADI for paraffin wax, triggered by the study run in 1991-1992 and eventually published in 1996 (Smith et al. 1996). For the first time, viscosity specifications and descriptors derived from toxicology studies were introduced for listing of mineral waxes and oils.

The JECFA assessment was based on a systematic series of sub-chronic 90-day feeding studies conducted in male and female F-344 rats on seven highly refined white mineral oils and five mineral waxes representative of those used for food applications (Freeman et al. 1993; Smith et al. 1996). The goal was to help clarify the mixed results found in other toxicity studies with laboratory animals and to assess physical chemical differences (e.g. viscosity), refinement methods (acid vs hydrotreatment) as well as compositional differences of paraffinic vs naphthenic crude types (see section on toxicological studies), but in essence it noted that higher molecular-sized hydrocarbons (microcrystalline waxes and the higher viscosity oils) were without biological effects. Paraffin
waxes and low to medium viscosity oils produced biological effects that were inversely related to molecular weight, viscosity, and melting point; oil type and processing did not appear to be determinants. Biological effects were more pronounced in females than in males. Effects occurred mainly in the liver and mesenteric lymph nodes (MLN) and included increased organ weights, microscopic inflammatory changes, and evidence for the presence of saturated mineral hydrocarbons in affected tissues. Inflammation of the cardiac mitral valve was also observed at high doses in rats treated with low viscosity paraffin waxes. This set the base to consider waxes and oils causing effects of a similar nature.

In regards to the effect on the mitral valve (only seen in waxes), it was noted that these lesions were occasionally seen in control rats, and that the significance and dose-response of this effect could be clarified by examination of the histological data on all groups (treated and controls) of recent and historical data.

In the same year, the European Scientific Committee for Food (SCF), following the similar approach of jointly evaluating waxes and oils based on their common effects in the F-344 rat (SCF 1995), confirmed the ADI for Microcrystalline Wax extending it to waxes meeting the same physical specifications:

1. Specifications and thus regulation via establishing or modifying ADIs based on “similarity” of products, the SCF went a step further by specifically defining physical chemical parameters (viscosity, carbon number and average molecular weight) which were considered sufficiently tightly drawn to ensure that only a small proportion of any product conform to these specifications will have carbon chain-lengths in the absorbable range. This approach also considered the question of whether the toxicity observed with some materials could be caused by very small proportions of unusual, highly toxic components, whose presence in products defined mainly by physical characteristics could be unpredictable and uncontrollable. Weighing the evidence on both mineral and synthetic hydrocarbons the SCF concluded that such a possibility is very unlikely. Rather it linked the toxicity to the amounts of lower molecular weight, shorter chain-length substances, which are absorbed and only slowly cleared from the body, that most probably determine the occurrence or absence of toxicity so that higher viscosity materials meeting certain specifications will not cause toxicity. These specifications defined those waxes and oils for which the established ADI applied, and clearly excluded, for example, LMPW and any other wax that did not comply with the following specifications:

- Highly refined waxes derived from petroleum-based or synthetic hydrocarbon feed stocks; Group ADI of 0–20 mg/kg bw, when
  - Viscosity not less than 11 mm²/s (cStokes) at 100 °C
  - Carbon number not less than 25 at the 5% boiling point
  - Average molecular weight not less than 500
- White paraffinic mineral oils derived from petroleum-based hydrocarbon feed stocks; temporary Group ADI of 0–4 mg/kg bw, when
  - Viscosity not less than 8.5 mm²/s (cStokes) at 100 °C
  - Carbon number not less than 25 at the 5% boiling point
  - Average molecular weight not less than 480
- Mineral hydrocarbons the SCF concluded that such a possibility is very unlikely. Rather it linked the toxicity to the amounts of lower molecular weight, shorter chain-length substances, which are absorbed and only slowly cleared from the body, that most probably determine the occurrence or absence of toxicity so that higher viscosity materials meeting certain specifications will not cause toxicity. These specifications defined those waxes and oils for which the established ADI applied, and clearly excluded, for example, LMPW and any other wax that did not comply with the following specifications:

1. Oils and waxes were no longer considered as separate substances, but rather on a common denominator based on idiopathic effects seen in the F-344 rat, granuloma formation in the mesenteric lymph nodes and liver.
2. Oils and waxes were not only united by a common effect but were jointly referred to as “mineral hydrocarbons”, effectively eliminating any product differentiation based on alkane sub-classes.
3. Specifications and thus regulation via establishing or withdrawing of ADIs were thus driven by the F-344 strain effects caused by “mineral hydrocarbons” MHC, which introduced a blind spot to both industry and regulators who focused solely on the association between “mineral hydrocarbons” and granuloma formation, but not on chemical differences between oils and waxes that
explained the type of hydrocarbon accumulation leading to, for example, epithelioid liver granuloma in the F-344 strain.

4. Scientific publications (including those of industry) used the term MHC, which was used collectively for waxes and mineral oils and preventing product and alkane sub-class differentiation causing a specific effect in rats or humans (e.g. liver epithelioid granuloma vs lipogranuloma) (Fleming et al. 1998; Hoglen et al. 1998; Carlton et al. 2001; Scotter et al. 2003; Griffis et al. 2010).

The CONTAM panel evaluation of 2012 and the MOSH paradigm

The Zürich cantonal laboratory reported in 2009 that Ukrainian sunflower oil was adulterated with white mineral oil (Biedermann and Grob 2009b). The published chromatography methodology to isolate the characteristic mineral oil saturated hydrocarbon fraction (MOSH) and the complementary mineral oil aromatic hydrocarbons (MOAH) not only provided the tool for the assessment of mineral oil but it also coined the “MOSH” and “MOAH” term, which totally changed the jargon regarding the until then used literature terms (Biedermann et al. 2009; Biedermann and Grob 2009a). While “MOSH” and “MOAH” may have been intuitive for the context under which they were originally used, taken out of context introduced another layer of complexity (and confusion) in the interpretation of the data. For example, the MOAH fraction is a generic, unspecific analysis of a fraction containing aromatics (total content of aromatic compounds), thus it does not distinguish the harmless MOAH from the 3-7 ring polycyclic aromatic compounds (PAC) which are the species that are toxicologically relevant (Carrillo et al. 2019). Further, it has been recognized that MOSH not only encompasses “mineral oil” fractions, but also structurally similar molecules like poly olefins (Biedermann et al. 2015) and n-alkanes of natural origin (Cravedi et al. 2017). This latter distinction is extremely relevant for the interpretation of waxes causing hepatic epithelioid granuloma in the F-344 strain.

The limitations of the “MOSH and MOAH” paradigm was not fully understood by the time that the EFSA CONTAM panel wrote its opinion about “mineral oil hydrocarbons in food”, where MOSH and MOAH terms were used throughout the report (EFSA 2012). For MOAH, it was assumed that the fraction could pose a carcinogenic risk acknowledging that there is no suitable methodology to distinguish different aromatic subclasses including the 3–7 PAC fraction which is responsible for the mutagenicity of unrefined mineral oils. The consequence was that the focus shifted from the “toxicologically relevant” to a “catch all” method, which difficulties in interpretation and execution have been reviewed elsewhere (Weber et al. 2018; Pirow et al. 2019); but this is outside the scope of this paper. We will not address it further, because the issue of carcinogenicity of petroleum products has been recently addressed elsewhere (Carrillo et al. 2019; Pirow et al. 2019).

What is most relevant for this paper is that the generic reference to “MOSH” implied that the entire MOSH fraction was mineral oil and a contamination, although in reality “MOSH” also includes waxes and n-alkanes of natural origin. At the time of this opinion, these differences were not realized, and the interpretation outside expertise groups and at consumer level was one of confusion. The term MOSH was interpreted as a dangerous contaminant per se, of not only food, but also ironically of the mineral oil itself. It was not uncommon to receive customer requests demanding that the highly refined paraffin waxes and white oils used in the food industry would be “MOSH free”. This is because the applied analytical technique does not allow to distinguish between those hydrocarbons originating from waxes (both from natural or petrogenic origin) and those derived from mineral oils. Further, the catch all analysis of “MOSH” includes mineral oil from a contamination as well as those oils and waxes that can be legally used because of having been separately assessed as food additives by EFSA (EFSA 2009; EFSA 2013a; EFSA 2013b).

This joint assessment of waxes and oils as “MOSH” contamination, came to exacerbate the already blurred line between hydrocarbon waxes and oils as food additives generally considered as "mineral hydrocarbons". By amassing them under a chromatographic hump, and the explicit reference to “mineral”, “oil” and “saturated hydrocarbon” in the MOSH term, it eliminated any chemical or physical difference between waxes and oils and the distinction between contamination and food additive. For example, a synthetic wax produced by the Fischer-Tropsch process, although consisting of saturated hydrocarbons would still be analyzed as MOSH, even though it is not necessarily “mineral” (it can also be from biomass), and it is definitively not oil (it is a wax; solid at room temperature). One must thus consider that from an analytical perspective the term MOSH is a generic chromatographic hump that cannot distinguish origin; petrogenic vs biogenic or contamination vs lawful. However, when the context is known and the term is applied exclusively to the liquid product “mineral oil” or a fraction thereof, it may still be acceptable to interpret toxicology data if the wax constituents are excluded.

Bearing that in mind, and even though waxes and oils were jointly assessed as “MOSH”, the report however had important conclusions and recommendations:

1. Histiocytosis in the mesenteric lymph nodes was concluded as of low toxicological concern; thus, the epithelioid liver granuloma became the critical effect for assessment.
2. Previously established ADI (i.e. class II and III oils) based on the NOAEL from effects on MLN warrant revision; implicitly this would result in departing from the liver granuloma NOAEL that in comparison is 100x higher.
3. Toxicological evaluation of MOSH should focus on molecular mass range (carbon numbers) and alkane subclasses (n, iso, cyclic).
4. Data on accumulation of multi-branched and cyclic MOSH is needed.
5. Human relevance of the F-344 liver granuloma should be further investigated.
6. The most potent MOSH for the induction of liver granuloma is the LMPW.

In summary, the CONTAM panel assessment and introduction of the MOSH term in the already complex assessment of waxes and oils, “inverted” the evaluation from these products. Rather than assessing these as independent substances, it focused in the residuals thereof found in matrices including animal and human tissues, collectively measured as MOSH (with all its connotations) and identifying low melting point waxes (LMPW) as the “most potent MOSH”. This is the paradigm that is currently used in the EU to set policy (Bratina and Hoekstra 2019; Bratina et al. 2020). To question whether LMPW pose a risk to human health, it is important to reconstruct the history behind it; that is, by reviewing the key toxicology studies that lead us to this point. This will allow to examine a) whether waxes are the “most potent MOSH” in causing liver granuloma in the F-344, and b) its human relevance by proposing a mode of action.

Toxicological evaluations of hydrocarbon waxes

Animal studies – strain and species differences

While carcinogenicity assessment of mineral waxes and oils had been the key concern and was done using mouse skin painting studies discussed in detailed elsewhere (Doak et al. 1983; McKee et al. 1989; Chasey and McKee 1993; Carrillo et al. 2019); the assessment of systemic effects after oral exposure was carried out in rats in sub-chronic and chronic feeding (dietary) studies for the purposes of sensitive applications such as food contact. These toxicological evaluations aimed at assessing a wide range of waxes and oils with different viscosities and manufacturing history (e.g. hydrotreatment introduced in the early 80’s vs acid treatment) to produce highly refined waxes and white mineral oil (liquid paraffin). Initially assessed separately, the discussion of the following key studies explains how the issue of the F-344 granuloma effects came about as common denominator between waxes and oils and the studies that followed that now allow us explaining the human relevance of the F-344 liver granuloma, most prominently caused by waxes.

These papers have been reviewed and commented by others (Nash et al. 1996; Pirow et al. 2019), thus we will focus specifically on the F-344 epithelioid granuloma inflammation found in the liver, which is the current endpoint for risk assessment for mineral waxes and oils (EFSA 2012). The time of these publications lagged (for various reasons) several years from the actual experimental period, which makes it difficult at times to understand their study design. The following order tries to reconstruct the logic of these study designs and purpose which was also to address the regulatory assessments by JECFA and EFSA.

An overview of the studies is presented in Table 5.

Shubik et al. 1962

In this study, groups of 50 6- to 8-week old male and female SD rats were fed diets containing 10% ground wax for two years. The tested samples were two paraffin waxes and three microcrystalline waxes chosen to represent the range of PAH content of waxes in commercial use (0–0.64 ppm). In addition, 157 female and 140 male rats served as untreated controls. The rats were observed and weighed every other week, and all gross lesions were recorded. Rats were observed until spontaneous death or were killed in extremis; necropsies were performed on all animals and histologic examination was performed on all abnormal tissues. Survival rates and average weights of experimental groups did not differ significantly from those of control animals, and the incidence of tumors observed in experimental animals was consistent with the incidences of these tumors in control animals. No other wax-associated toxic effects were identified by histopathology (Shubik et al. 1962). Personal communications from two of the authors of this study (Shubik and Saffiotti) double checked and confirmed that findings in 90-day studies on mineral oils (Shubik 1992; Ekelman 1993) – deposition in the reticuloendothelial system and granulomas in the livers of rats – were not observed in the two-year oral toxicity study of waxes in rats. This 2-year study in SD rats resulted in a NOAEL of 10% (100,000 ppm) dietary concentration equivalent to approximately 5000 mg/kg bw/day.

Baldwin et al. 1992

While the focus of our arguments is primarily the relevance of the liver granuloma in the hazard assessment of hydrocarbon waxes, the following study on mineral oil is included because it was a game changer in the regulatory assessment of these substances as we have previously discussed.

In the decade of the 80’s, the introduction of hydrogenation started to play a significant role in the production of white mineral oils compared to the traditional method of acid treatment (also known as oleum treatment; using fuming sulfuric acid). Up to the early 90’s mineral oils had been evaluated in repeated dose studies without any remarkable outcome that would raise concern. Although the composition of mineral oils by either purification method was known to be equivalent (CONCAWE 1984), and no difference in dermal carcinogenicity was observed; questions were raised whether hydrogenation would give rise to systemic toxicological effects that would differ from studies based on acid treatment. Because there were no published toxicological studies in which laboratory species had been concurrently fed white oils processed by either of the two methods, the study by Baldwin was a project (initiated in the late 80’s and published in 1992) that was initially designed to specifically answer this question using two naphthenic crude derived pharmacopeia grade oils; an oleum-treated white oil (OTWO = N30A) or a hydrotreated white oil (HTWO = N70H). These oils were fed to male and female rats for 90 days followed by the monitoring of a full range of clinical, hematological, and pathological parameters (Baldwin et al. 1992).

In a subsequent study, aimed only at establishing a NOEL for each white oil, the parameters monitored were limited to the principal pathological effects in female rats, which showed a greater response to treatment than males.
Administered dietary doses were in ppm 10, 100, 500, 5000, 10 000, 20 000, equivalent to daily averages of approximately 1, 9, 45, 455, 925 and 1800 mg/kg bw.

The results of this study were a game changer that engaged toxicologists and regulators for the next 30 years. Firstly, it was the first study that used the F-344 as model. Although the lipid droplets observed in the mesenteric lymph nodes (MLN) and the liver that could be comparable to those reported in humans (Boitnott and Margolis 1966), this was the first study that reported treatment related effects in the MLN as granulomatous foci, and inflammation in the liver in the form of epithelioid granuloma (reported as lipogranuloma, discussed in the next sections) which would impact the risk assessment of mineral oils (and eventually also waxes).

Liver epithelioid granuloma was associated with increased absolute liver weight and blood alanine transaminase (ALT) levels, occasionally associated with hepatocellular degeneration and necrosis in rats with severe inflammatory response.

This unexpected result was dose-dependent, more pronounced for the N30A oil (OTWO) and in female rats. Considering that hydrotreatment and acid treated oils are not compositionally significantly different and because the oils were of different viscosity (N30A is less viscous with 26 mm²/sec at 40°C compared to N70H with 69 mm²/sec at 40°C); it was concluded that the observed toxicological effect would not be per se caused by treatment method, but rather by constituents present in the N30A which are not present in the N70H because of differences in viscosity (i.e. boiling range which determines viscosity). The lower viscosity N30A would contain constituents that have lower molecular weight and would thus be more bioavailable than those present in the N70H. The NOEL for granuloma in MLN is: 10 ppm (~1 mg/kg bw) and 500 ppm (~45 mg/kg bw) for the N30A and the N70H oils respectively. For the liver and based on the occurrence of Kupffer cell (KC) hypertrophy, the NOEL is 10 or 100 ppm for N30A and N70H, respectively, equivalent to mean daily intakes of ~1 or 9 mg/kg/day.

It was also observed that female rats accumulated ~5-fold more hydrocarbons in liver and MLN compared to males, where the N30A oil showed the highest accumulation probably related to bioavailability of shorter carbon chains.

The answer to which type of alkane constituents play the critical role in the development of liver epithelioid granuloma in the F-344 rat (and its link to viscosity) will only be finally elucidated in 2017 with the help of sophisticated analytical techniques (Barp et al. 2017a, 2017b; Cravedi et al. 2017). But to get there, several other pieces of the puzzle and the introduction of waxes into the assessment of mineral oil became the focus of the hazard assessment for the following three decades.

Smith et al. 1995
Because of the findings by Baldwin (Baldwin et al. 1992) an early study from the 70’s on mineral oil was published in order to compare the effects of four types of mineral oil fed to different strains of rats and dogs. In a 90-day study, Long-Evans (LE) rats (20/sex/dose) received on average dietary doses of ~25 or 125 mg/kg bw (300 or 1500 ppm respectively), while dogs (4/sex/dose) received approx. 10 or 50 mg/kg bw (300 or 1500 ppm respectively) of the following types of mineral oil for which the viscosity (in cSt) at 40°C has been used to describe them as approximately: P15A, P15H, P30H or P70H.

Because the study was designed to evaluate hydrocarbon deposits of oil or lipids in organs (liver, mesenteric lymph nodes- MLN, spleen, intestine, stomach and kidney) special staining techniques with Oil Red O were used in 5 rats/group and in all dogs from control and the high dose group.

What is particular about the histopathological re-evaluation is that the slides from this study were compared with those from the F-344 rat (Baldwin et al. 1992) and also those of another 90-day study which used a pharmacopoeia grade oil as vehicle control (N40A) via gavage at 4350 mg/kg bw (dietary 60,000 ppm) in the SD strain (McKee et al. 1989). For this comparison, the control and high-dose group of LE tissues (liver, spleen, MLN) were compared to those of the SD (liver and MLN) and F-344 (liver, spleen, MLN).

Treatment of LE rats with any of the white oils did not show systemic or biologically relevant adverse effects. (There were hematological changes in females, but these were either erratic or not considered biologically relevant). Contrary to the effects in F-344 rats, no dose–response pattern nor treatment-related effects on relative or absolute liver (or kidneys) were observed. Spleen and MLN were not examined. However, histopathological evaluation of all tissues of high-dose groups did not show changes associated to white oil exposure, neither did the special staining indicate significant organ deposition of hydrocarbons in liver, MLN, gastrointestinal tract and kidney.

Dogs exposed to the same oils had slight diarrhea as result of white oil laxative effect, more pronounced with the
higher viscosity oils. Like LE rats, no pattern of treatment related statistically significant or biologically relevant changes were observed for body (gain) weights, organ weights (including liver and gonads) or hematology. Histopathological evaluation of the high-dose groups and special tissue staining did not reveal effects related to treatment and significant oil deposition.

The comparative histopathological evaluation of LE and SD rats did not show significant macrophage or microgranuloma accumulation in liver or MLN at 125 mg/kg bw in the LE and as high as 4350 mg/kg bw in the SD strain.

In contrast, liver and MLN effects in the F-344 have a LOEL of 9 and 45 mg/kg bw for the P30H and N70A oils respectively. This difference in the F-344 response is better observed when oils of similar viscosities are compared. A viscosity of ~30 cSt (at 40 °C), the NOEL for liver granuloma in the LE is 125 mg/kg bw, compared to 45 mg/kg bw in the F-344 (for P30H and N30A oils respectively). At higher viscosity (~70 cSt at 40 °C) the NOEL is also 125 mg/kg bw in the LE and 45 mg/kg bw in the F-344 (for P70H and N70A oils respectively). Although the NOEL of different viscosity oils is equivalent, the severity in granuloma formation in the F-344 is most pronounced at a lower viscosity, indicative of compositional differences associated with the oil molecular weight.

Although this study gave some indication that there might be rat strain and species differences in systemic toxicity related to white mineral oil exposure, the strain (and species) related effects were difficult to interpret because of protocol differences; LE rats (and dogs) were exposed to paraffinic oils, whereas naphthenic oils were used in the Baldwin study (Baldwin et al. 1992). Also, different refinement methods were used for the tested materials (acid vs. hydrotreatment).

Until this point, waxes were not yet in the scope of this discussion (JECFA 1992).

**CONCAWE 1993; Smith et al. 1996**

Because of the differences in the protocols that made previous studies difficult to compare, CONCAWE conducted a large study on materials representative of the full range of materials typically used for food applications (Freeman et al. 1993). These studies were carried out using the same protocol which would account for previous differences in the dosing method (gavage versus feeding), strain of rat, dose-levels and white oil viscosity and processing type (acid treatment vs hydrogenation). The study included seven oil samples selected to cover the variables identified above: viscosity (from low to high), method of refining (acid vs hydrogenation) and oil type (paraffinic vs naphthenic). The tested oils included N10A, N15H, P15H, N70A, N70H, P70H and P100H.

In addition, five food grade waxes representative of those used in food applications were also included in the study as comparison, because Shubik and colleagues reported that 10% dietary feeding study of a range of petroleum waxes of varying viscosities tested in lifetime feeding studies in SD rats did not cause any adverse effects (Shubik et al. 1962). The tested waxes included LMPW (Low Melting Point Wax), IMPW (intermediate melting point wax), HMPW (high-melting-point wax), MP (1:1 mix of LMPW and HMPW), and a HSW (high sulfur wax; a microcrystalline wax refined by percolation).

Properties of these oils and waxes as well as details of the study design and outcome can be found elsewhere (Freeman et al. 1993; Smith et al. 1996).

Briefly, male and female F-344 rats were used throughout the study. Animals were exposed to dietary concentrations that were selected to cover the range of doses used in previous studies (20, 200, 2000 and 20 000 ppm, corresponding to ~2, 20, 200 and 2000 mg/kg bw/day in females). A recovery group was also included.

Toxicological evaluations included a range of parameters, including those that allowed direct comparison to previous studies: liver and MLN effects in organ weight, granuloma evaluation, hematology and alkane retention evaluated at the end of the study and in recovery groups. Alkane retention was reported as “total hydrocarbon”, thus no distinction between alkane sub-classes was made.

Results of these studies confirmed what was seen in previous studies namely:

a. Physical-chemical properties do play a direct role in biological response. Systemic effects were inversely related to MW, viscosity, boiling range and melting point (for waxes). Viscosity at 100 °C, a common denominator for oils and waxes, correlated well with the effects of both.

b. Oil type and processing history did not appear to influence biological response. This was already observed previously (Baldwin et al. 1992) but confirmed in this study by a side by side comparison with materials with similar physical chemical properties. It must be pointed out that purely paraffinic or naphthenic mineral oils do not exist, as these oils do contain both types of constituents but at varying proportions, so that an oil should be considered as “primarily” paraffinic or naphthenic based on the predominant alkane type. However, in respect to the qualitative effects in liver and MLN (e.g. liver epithelioid granuloma) no clear distinction between oil types or processing (acid vs hydrogenation) could be established.

c. Intestinal absorption of mineral hydrocarbons, from oils or waxes, caused liver epithelioid granuloma and MLN histiocytosis, where female F-344 appear to be the most sensitive sex. Liver effects were correlated to viscosity and more prominent in females, whereas in MLN, it was either males or females depending on oil viscosity. Low viscosity waxes had more pronounced effects than low viscosity oils.

d. Accumulation of total alkanes in tissues was observed for some oils and waxes, related to their viscosity. Statistically higher levels of total alkanes where accompanied with increased organ (liver, spleen and MLN) weights. In rats exposed to waxes, 2- to 5-fold higher levels were detected in the liver compared to levels in MLN and fat. LMPW showed highest levels in the liver accompanied by most severe liver epithelioid granuloma formation. All oils, except for the P100H, showed statistically significant alkane retention in the liver (and MLN) but retained alkane levels and severity of granuloma
was lower than in waxes. Except for P70H and P100H where no significant (or none) granuloma was observed in either liver or MLN, in all other oils significant granuloma formation in the liver was only observed at the highest tested dose and only in females whereas in the MLN granuloma was observed in both sexes with females being more sensitive.

e. Both oils and waxes with a viscosity of >8.5 cSt (mm²/s) at 100 °C do not elicit adverse effects in F-344 rats. It was demonstrated that although P70H oil did show hydrocarbon retention in liver and increased organ weight this was not accompanied with liver granuloma formation, nor with histiocytosis in MLN. For the waxes (HMPW and HSW) with these viscosities neither accumulation nor liver or MLN effects was seen, indicating poor bioavailability of the test material and an indication that accumulation of certain hydrocarbons has a relationship to liver granuloma formation.

f. Recovery groups indicated that alkane accumulation is reversible after cessation of exposure. No conclusions could be made on total elimination because of the short period for recovery.

g. Hematological parameters where not affected by treatment up to 1950 mg/kg bw/day. Effects seen in previous studies could thus not be replicated at even higher doses indicating that those affected blood parameters were incidental and had no toxicological meaning, exemplified by the fact that coconut oil (food control oil) caused a 2% statistically significant reduction in red blood cell counts.

The findings of this study did not only set a relationship between physical-chemical properties and toxicity but also served to set boundaries for these effects. Without consideration on chemical composition, it was assumed that the viscosity of ≥ 8.5 cSt (mm²/s) at 100 °C (ASTM D445; (ASTM D445 2019)) is the common denominator in predicting toxicity for both oils and waxes, where the viscosity at 40 °C is not. For example, N70H and P70H are both designated “70 oils” because of their respective viscosities at 40 °C are close to 70 cSt (68 and 69.5 respectively) and both underwent hydrotreatment. However, at 100 °C the difference in their viscosity becomes greater (7.65 and 8.56 cSt, respectively), which is clearly reflected in the formation of liver granuloma; for this endpoint the NOAEL for the N70H is 190 mg/kg bw/day, whereas for the P70H it is the highest tested dose of 1950 mg/kg bw/day.

For waxes, the criterium of >8.5 cSt (mm²/s) at 100 °C for liver granuloma effect would leave out all Low Melting Point Waxes (LMPW) and part of the intermediate melting point waxes (IMPW) that do not meet this criterium. All the microcrystalline waxes (e.g. HMPW or HSW) meet this criterium and thus do not show either accumulation or liver granuloma formation.

Hence, a relationship between physical-chemical properties and toxicity was established both for oils and waxes (no adverse effects at >8.5 cSt (mm²/s) at 100 °C). Also, a toxicity relationship was observed between high hepatic hydrocarbon levels and severe epithelioid granuloma formation for LMPW. Because of this new F-344-viscosity-granuloma framework, no further alkane sub-class distinction was made between oil and wax products since tissue accumulation was assessed as “total hydrocarbons”.

Consequently products that previously had been assessed separately were now tied up based on a common rat model (i.e. F-344), a physical-chemical parameter (i.e. viscosity at 100 °C) and a biological effect (i.e. liver epithelioid granuloma and MLN histiocytosis), effectively eliminating product, chemical, and compositional differences (alkane sub-classes).

Based on this framework, LMPW was the most potent wax together with oils with a viscosity <3.5 cSt at 100 °C (i.e. P15H, N15H, N10A). In addition, LMPW also caused cardiac mitral valve inflammation, not seen in earlier studies. For LMPW, the liver NOAEL was set at 200 ppm corresponding to ~20 mg/kg bw.

For the first time, specifications were set for waxes and oils when used in food contact materials, in order to define boundaries for hydrocarbon toxicity which were based on physical–chemical properties (SCF 1995).

As indicated earlier, for waxes this was: “Viscosity not less than 11 cSt (mm²/s) at 100 °C; carbon number not less than 25 at the 5% boiling point and average molecular weight not less than 500”.

And for mineral oils: “Viscosity not less than 8.5 cSt at 100 °C; carbon number not less than 25 at the 5% boiling point and average molecular weight not less than 480”.

Note that at this point of the regulation, waxes and oils were jointly assessed as “hydrocarbons” due to an (unstated) assumption that the cause of this similarity of effects (e.g. liver epithelioid granuloma), were some physical-chemical thresholds associated with “hydrocarbons”. The chemical distinction that waxes consist primarily of n-alkanes whereas oils are comprised of iso- and cycloalkanes was lost.

Nevertheless, questions remained open regarding the significance of the organ inflammatory effects in the F-344 rat and its significance to human health given the evidence that this was not observed in other rat strains or dogs (but following different protocols).

Firriolo et al. 1995

While other studies were available to confirm species and strain differences when exposed to white mineral oil, this was the first side-by-side comparison study between the F-344 and the SD (CRLCD type) rat exposed to the same type of oil following the same protocol (Firriolo et al. 1995).

In this comparative study, female F-344 and SD rats were exposed to a P15H oil at dietary concentrations of 200 or 20 000 ppm (~160 or 1600 mg/kg bw/day), in the same range as used in previous studies. Observations were focused on histopathological and organ weight changes in the liver and MLN, total hydrocarbon retention in these organs and clinical pathology. Animals were sacrificed at intervals 30, 61 and 92 days, providing an indication of a time course in the development of adverse effects. Retention of total hydrocarbons (alkanes) was, however, assessed at the last interval only.
Findings of this study were consistent with other studies using the F-344; namely the following:

1. Few, transient or minor changes in hematology and clinical parameters, not considered toxicologically relevant, but which were totally absent in the SD rat.

2. Treatment related organ weight effects were observed in the F-344, where liver and MLN weight increase (relative and absolute) was observed at all time intervals in the high dose. At study termination, liver weight increase (relative and absolute) was also observed in the low dose. None of the doses showed these effects in the SD rats (evaluated only at the end of the study).

3. Histopathology evaluation indicated a dose response relationship to the formation of hepatic epithelioid granuloma, which showed also a time dependent incidence. No hepatic epithelioid granuloma was observed at 31 days at any of the tested doses; at 61 days the incidence was >80% in the high dose but absent in the low dose; at 92 days incidence reached 95% in the top dose and 43% in the low dose. This indicates that hepatic epithelioid granuloma formation has both a time and dose dependency, which can be explained by considering the total accumulation of hydrocarbons. At the end of the study (the only time point available), higher levels of hydrocarbons were correlated with higher incidence of granuloma at the top dose; where at the lower dose, 1.5-fold lower level of retained hydrocarbons was associated with lower granuloma incidence. A similar trend was also observed for MLN (micro) granuloma formation and hydrocarbon retention although histiocytosis did not follow a dose response.

4. Contrary to the effects seen in the F-344, the SD strain did not show any histopathological changes in any organ (no granuloma formation in either liver or MLN), despite having retained comparable amounts of alkanes in the MLN indicative of similar absorption at the top dose. Alkanes (reported as “total mineral hydrocarbon”) levels in the liver were however 2- and 3-fold lower in the SD compared to the F-344 for the low and top dose, respectively, indicative of metabolic differences.

In summary, the results of this study clearly showed strain differences in the response to mineral oil exposure using the same toxicity study protocol. The F-344 had significant liver and MLN organ enlargement, dose and time dependent hepatic epithelioid granuloma formation and significantly higher retention of alkanes; effects which were not observed in the SD rat other than lower alkane retention even though it seemed that intestinal absorption might be comparable.

Still one could argue that, given the fact that hepatic granuloma formation (in the F-344) was shown to be related to exposure length and dose level, and because the SD rat can also accumulate alkanes (although at a lower rate), it could be argued that longer exposure time and/or higher exposure could also trigger the epithelioid granuloma formation in SD rats.

From the liver granuloma formation perspective, this argument considers therefore two points, a.) metabolism differences between strains that lead to b.) accumulation of hydrocarbons. While the former can be recognized as the SD having higher metabolic capacity, insight into the second was overlooked because the assessment of the retained material focused on “total mineral hydrocarbons” and less on the type of alkanes retained. As it would be discovered decades later and argued in the following sections of this paper, these two points were in fact two sides of the same coin: when differences in metabolism capacity and the type of accumulated alkanes are jointly recognized, it becomes clear why the F-344 is the wrong model to assess the safety of waxes.

Griffis et al. 2010
The study by Griffis was published more than a decade later than when it was conducted (Griffis et al. 2010). We have intentionally placed it after Firriolo because it was conducted around the same time and with a similar study design but using low melting point wax (LMPW).

Because previous studies with waxes were conducted only in F-344 rats (Smith et al. 1996) and due to the absence of similar liver effects in Long-Evans rat strains and Beagle dogs with mineral oils with similar carbon number range (Smith et al. 1995), it was necessary to determine whether the reported histopathological changes in F-344 rats with LMPW were also reproducible in other rat strains or unique to the F-344. In this respect, a feeding study was conducted to compare the effects of C_{19}-C_{42} paraffin wax (LMPW) in SD and F-344 rats at two dose levels (0.2 and 2%) for up to 90 days (Griffis et al. 2010), equivalent to ~160 and 1600 mg/kg bw/day for the SD rats and 157 and 1609 mg/kg bw/day for the F-344 rats. Evaluations included in life observations, gross necropsy, organ weight and histopathology focusing on hepatic and mesenteric lymph nodes (MLN) granuloma, hematology, and clinical chemistry. Determination of LMPW alkane residues in target tissues was also performed.

The immunohistochemical staining analysis of liver and MLN tissues assessed parameters for interpreting granuloma formation, including T-cell (CD3), T cytotoxic (CD8), T Helper (CD4) and B-Cell (CD45RA) lymphocyte presence. Also, resident macrophage (ED2 marker) responses and electron microscopy to determine KC activation and phagocytized material were evaluated.

The weights of mesenteric lymph nodes, liver and spleen were increased in F-344 rats but remained unaffected in SD rats.

Hematological parameters were affected in the F-344 but not in the SD rats. Neutrophils, aspartate transaminase (AST), alanine transaminase (ALT), and gamma-glutamyl transferase (GGT) were not different between low and high dose group but were significantly higher than controls in F-344 rats. Liver function was unaffected in SD rats.

Histopathological changes were dose- and time-dependently observed in the liver and mesenteric lymph nodes of F-344 rats but only treatment related slight effects were observed in the MLN of all SD rats. While granulomas were found in the livers of F-344 rats both in the low and high
In the F-344 rats, lymphoid cell infiltrates/aggregates were observed at the periphery of the granulomata, with a higher severity at the end of the study and little difference between the low- and high-dose group. In the high-dose group, foci of necrosis were associated with the epithelioid granuloma whose severity increased with exposure time. Also, time and dose-dependent increment in hepatocyte vacuolation over controls was observed in the F-344 rats.

Electron microscopy examinations of the granulomatus F-344 rat livers indicated that the size and number of activated KCs (larger size, ruffled borders, increased pinocytotic vesicles and lysosomes) were increased.

It also revealed the presence of variably sized polygonal crystalloid structures inside the KC lysosomes. These crystals were also seen in hepatocytes, very much like the observations reported before (Hoglen et al. 1998). This phenomenon was not seen in SD rats.

These observations are supported by the chemical measurements. Statistically significant levels of LMPW hydrocarbons were detected in the liver of F-344 rats in the highest dose group at 30, 60 and 90 days but only after 90 days in the lowest dose group. In the SD rats, no LMPW alkane residues were found in the liver in either dose group or at any of the time points measured (30, 60 and 90 days), suggesting a clear strain difference in the clearance of this type of hydrocarbons. In relationship to granuloma formation, already after 30 days exposure to 2% LMPW, F-344 rats developed hepatic granuloma corresponding to an alkane concentration of 1.86 mg/g liver. GC/MS analysis of liver extract of the LMPW (C<sub>19</sub>-C<sub>42</sub> paraffin wax) treated F-344 rats revealed that the retained alkane hepatic fraction corresponded to linear and branched alkanes in the C<sub>22</sub>-C<sub>31</sub> range. This finding is consistent to what was reported by other studies indicating that the F-344 rat has a preferential retention of alkanes (linear and branched) in the F-344 rats, especially alkane residues in the F-344 rats. It was also noted that most epithelioid macrophages forming the granuloma in F-344 rats were not resident macrophages (ED2 negative), suggestive of extrahepatic origin. In these macrophages, lysozyme production overall decreased with increasing size of the lesion.

Liver granuloma formation was further assessed by comparison of the resident macrophage population in unexposed rats. SD rat controls had a higher baseline population of resident KC compared to F-344 rats. Treatment related increase in KC hyperplasia and KC size hypertrophy was only noted in F-344 rats. It was also noted that most epithelioid macrophages forming the granuloma in F-344 rats were not resident macrophages (ED2 negative), suggestive of extrahepatic origin. In these macrophages, lysozyme production overall decreased with increasing size of the lesion.

T cells associated with the granuloma indicated that CD3, CD8 an CD4 numbers increased with dose and treatment duration; these lymphoid infiltrates were associated with epithelioid macrophages in the form of a peripheral lymphoid rim around the agglomeration (granuloma) with CD8+ presence relatively more frequent. In treated F-344 rats, the CD8+ were observed in the centriflobular and midzonal regions, indicative of movement from the sinusoids. B cells were a minor component in the granuloma formation and increased in numbers with dose and treatment duration.

None of the previous effects were observed in the treated SD rats.

Retention of alkanes in the mesenteric lymph nodes were also found in the F-344 and SD rats, with higher levels in the F-344 rats, where it was found in both doses but only after 90 days (thus not at other time points). Alkane residues in the SD rats were found only at the highest dose and 90 days of exposure. Both strains develop granuloma in the MLN, which was however not accompanied by a dose and time dependent increase in CD3, CD8, CD4 or B-cell populations. CD3-positive cells and B-cell numbers decreased with increased size and frequency of granuloma, whereas CD8 and CD4 populations were not affected by treatment in either strain. This decrease was associated with spatial displacement by macrophages, which had a treatment-related increase in lysozyme levels in both rat strains. Resident macrophages were more prominent in SD rats compared to F-344 rats, indicative of higher background populations in SD rats.

The specific retention of n-alkanes in the liver by the F-344 rats can be inferred by side by side comparison of the studies of Firriolo and Griffis, which share a similar design (Table 6). Although both studies generally refer to “mineral hydrocarbons”, based on product compositions, it is evident that the type of alkanes accumulated together with metabolic differences are responsible for the hepatic granuloma formation. If there was no difference in the type of hydrocarbon retained, but only metabolic differences between rat strains (slow vs fast) by simple triangulation one would expect that the SD rat would retain, proportionally to the P15H constituents, also 2–3 times less LMPW. As can be seen, while there is retention of oil constituents in both strains, n-alkanes from LMPW are absent in the livers of SD rats, indicating that there is something unique going on with the wax alkanes (linear and branched) in the F-344 rats, especially when we consider the formation of crystals in the phagosome as a result of LMPW exposure and the absence of such phenomena in the SD rat and human livers (see later).

**Scottet et al. 2003**

The Scottet study (Scottet et al. 2003) was designed as a follow up study based on the relationship established between physical-chemical properties and toxicity (Smith et al. 1996). Namely that LMPW was the most potent wax followed by oils with a viscosity < 3.5 cSt at 100 °C (i.e. P15H, N15H, N10A). In addition, LMPW also caused cardiac mitral valve inflammation, not caused by any of the oils tested.

The aim of this study was to generate tissue samples with accumulated hydrocarbon material that could be analyzed to better characterize the changes that occur following administration of selected oil and wax products to F-344 rats. This would allow a better understanding of both the mechanism of effect and relationship between composition and effect so that physical–chemical specifications could be reviewed and improved, if required.

Female F-344 rats were fed the test materials for up to 90 days at a dose level of 2%, equivalent to ~2500 mg/kg
cannot be concluded with absolute certainty because the bolic demand because of higher hydrocarbon uptake. This increased organ weight may be the result of higher meta-
length (Albro and Fishbein1970), so that the observed
gut bioavailability is inversely correlated to carbon chain length (Albro and Fishbein 1970), so that that the observed accumulation but did not induce the hepatic granulomata. Two waxes were also included, LMPW as the most potent hydrocarbon product inducing liver granuloma, and as comparison a synthetic wax (C80) of a similar viscosity (see wax 7 in Table 4) as the P70H oil at 100 °C with the aim of testing whether viscosity alone or hydrocarbon composition play a role in the formation of hepatic granulomata. An important step forward of this study regarding the analysis of the “mineral hydrocarbons” found in tissues, was the distinction of “total hydrocarbons” into n-alkanes and iso-
alcanes most extensively for waxes and to a limited extent for the oils which provided further insight into the assessment of accumulation by alkane types. At the end of the 28 and 90-day exposure period, the F-344 rat had significantly higher (15%) food intake for all oils compared to waxes. Body weights were not different from controls at the end of either observation period. However, after 90 days, the relative weights of lymph nodes, spleen and liver were statistically increased by exposure to LMPW and N15H oil. C80 wax and N70H oil did have an effect in increasing the (relative) weights of mesenteric lymph nodes (MLN) and spleen but not the liver. The P70H oil did not have any effect on any of the organs after 90 days. It is remarkable that the N15H oil produced a strong effect on liver and lymph nodes weight at 28 days (sustained at 90 days), at this time point, both waxes had a strong effect on the MLN weight but not yet on the liver, which was only seen later at 90 days. This observation might be the result of higher exposure because of higher bioavailability of the N15H constituents as a result of the low viscosity of the N15H oil. This may be explained by comparing it to the P70H oil. Although both the N15H and P70H oils had significantly higher food intake, which might result in higher exposures, it was indicated that in fecal extracts the N15H hydrocarbon constituents had a mass peak of C21-C22 compared to that of C31-C32 from the P70H oil. It is has been demonstrated that gut bioavailability is inversely correlated to carbon chain length (Albro and Fishbein 1970), so that that the observed increased organ weight may be the result of higher meta-
bolic demand because of higher hydrocarbon uptake. This cannot be concluded with absolute certainty because the quantitative analysis of “mineral hydrocarbons” in tissues (e.g. liver) was done only for the waxes, so that there is no way of linking organ weight increase to retained amount of hydro-
carbons against a baseline (the analysis of the tissues of the control group). As for the other organs (brain, kidney or heart) no weight increase was caused by any of the test materials at any time point of the study. Histopathological evaluation confirmed previous study findings, in that LMPW induced minimal to marked hepatic epithelioid granuloma formation. Only the LMPW showed focal necrosis with inflammation and generalized vacuolization. The LMPW also showed cardiac focal inflammation of the mitral valve and histiocytosis of the MLN. The higher vis-
cosity C80 wax, however had induced virtually no hepatic granuloma, although (periportal) vacuolization was present and histiocytosis was found in the lymph nodes. No effect on the cardiac mitral valve was observed for this wax. From the oils, only the N15H induced hepatic granuloma but at a lower incidence/severity compared to LMPW and was not accompanied by either vacuolization or focal necro-
sis with inflammation, indicative of “lower potency”. These effects were not seen with the other oils, although present in an earlier study but at 10-fold lower incidence (Smith et al. 1996). Histiocytosis in the MLN comparable to that caused by both waxes was observed with the N15H and N70H oils, but not with the P70H. Thus, the latter oil did not cause any pathological changes in the examined tissues. Insight into the observed systemic effects is obtained when combined with the profile of the alkane retention in these tissues. Despite analytical bias, especially with high molecular (>C40) weight hydrocarbons of the C80 wax being excluded due to low solubility, and the inaccurate determin-
ation of iso-alkanes carbon numbers relative to those of n-
alcanes, some general conclusions can be made. n-Alkanes and iso-alcanes are selectively retained in the F-344 rat, and the profile varies between tissues. In the liver of the LMPW group, n-alcanes show a prefer-
ential retention over iso-alcanes. This group also showed the highest severity in granuloma formation. As a comparison, the C80 wax accumulated material was slightly lower but clearly more iso-paraffinic and was associated with a 30-fold less severity of hepatic granuloma (no clear effect). Unfortunately, for comparison and, as indicated earlier, no alkane levels in the liver were analyzed for any of the oils. The carbon range of the retained n-alcanes in the liver showed a distribution of C23-C36 with a peak at C28 with the iso-alcanes showing a slightly narrower distribution but also with a peak at C28. Within this retention range the n-
alcanes > C28 and iso-alcanes > C22 were over-represented when compared to the test material. At about C33 either alkane was under-represented indicative of limited uptake due to chain length. In the MLN, n-alcanes from LMPW showed the highest retention and were associated with the highest incidence of histiocytosis. However, qualitatively compared to the liver, the n-alkane carbon number distribution was clearly shifted toward lower molecular weight constituents, with a range of

| Table 6. Liver hydrocarbon concentrations (mg/g) after 90-days of dietary con-
sumption of a low viscosity oil (P15H) and a low melting point wax (LMPW) in two rat strains, means and ± SD. |
|---|---|---|---|
| Dose group | Tested Dietary concentration (% w/w) | Mean dose (mg/kg bw/day) | P15H | LMPW |
| F-344 rat | 0.2 | 160 | 5.6 ± 1.2 | 13.3 ± 3.7 |
| | 2.0 | 1600 | 8.2 ± 1.0 | 19.8 ± 2.04 |
| SD rat | 0.2 | 160 | 1.7 ± 1.2 < LOQ |
| | 2.0 | 1600 | 4.1 ± 1.4 < LOQ |

*(Firriolo et al. 1995); *(Griffis et al. 2010).
C_{16} - C_{34} and the peak at C_{25}. The iso-alkanes again showed a narrower profile with a comparable peak (at C_{26}).

A similar shift toward shorter n-alkanes of LMPW was also observed in other tissues, indicating a selective retention in the liver for n-alkanes of a certain carbon chain length (\(\geq C_{23}\)) and effective elimination of those shorter chain alkanes found in other tissues which testify of their presence during exposure.

An important observation regarding the mitral valve focal inflammation and retained alkanes can be made. LMPW was the material causing this effect at the highest severity with n-alkanes as the most retained type of hydrocarbons. Higher hydrocarbon levels in the heart were observed for the N70H oil (almost 2-fold), without an effect on the mitral valve. Although no extensive quantitative hydrocarbon type (n-alkane versus iso-alkane) analysis was made for the oils, it seems that the total amount of retained hydrocarbon alone is not enough to trigger this type of effect. Differences in alkane type within the retained carbon number range thus must play a crucial role.

Because of the limitations in the analysis (e.g. not all tissues have the same qualitative information), no clear picture can be drawn from the chemical analysis in understanding the role alkane types play in an effect, but the following points can be made:

- Although the excess in fed material is defecated in basically the exact composition, the absorbed material shows a tissue dependent narrower profile, with a distinct “tissue” carbon distribution.
- Accumulation of n-and iso-alkanes are within a “focused” range. This critical range is the result of a selective retention/elimination dynamic where constituents of either oils or waxes accumulate resulting in a profile different from that of the original test material.
- The liver of LMPW rats had preferential retention of n-alkanes with a peak at C_{28} and associated with the strongest response in developing liver granuloma.
- Although accumulation of hydrocarbons and effects may be associated, accumulation per se is not enough to predict or interpret an effect. Distinction of alkane types are needed to associate chemical profiles to biological effects. More elaborated analytical characterization was needed to expand this point to oils.
- Beyond alkane type, molecular weight considerations must play a role in explaining the effects observed. LMPW and C80 waxes consist primarily of n-alkanes; however, carbon chain length and consequently differences in retention profiles may help explain the lack of liver granuloma in the C80 wax group.

More insight over alkane sub-class effect accumulation and liver effects were obtained from subsequent studies.

Hoglen et al. 1998
Differences in the inflammatory response to LMPW were assessed in female F-344 and SD rats in a 60-day dietary study (Hoglen et al. 1998). After exposure to 2% LMPW (equivalent to 1600 mg/kg/day) tissues were taken to assess both general liver morphology and KC function. Other parameters addressed included final body weight (after 60 days exposure), hematology (total white blood cell and neutrophil counts), serum clinical chemistry AST, ALT, and GGT. Furthermore, liver samples were collected to determine LMPW content. Isolated KC from livers were also investigated because they play a central role in granuloma formation.

Granuloma formation with lymphoid cell aggregation and some areas of necrosis were observed only in the livers of the F-344 rats. Similar lesions were not observed in the SD rats. Liver enzymes were elevated in the F-344 but not in the SD rats. Similarly, white blood cells and neutrophil counts were only elevated in the LMPW-treated F-344 rats.

LMPW-related hydrocarbons were detected in the livers of F-344 but not of SD rats, which did not have detectable levels after 60 days of exposure. Of extreme importance is the observation that the KC of LMPW treated F-344 rats had membrane associated irregularly shaped vacuoles suggesting their content was phagocytosed foreign material. This was not observed in F-344 controls or SD rats, and because the material was highly lipophilic and formed crystal-like bodies, it is not unreasonable to deduct that this was unmetabolized crystalized LMPW. This is also supported by the fact that no differences in the basal activity of KCs across both strains were observed, indicating that the immunological differences observed in the study are directly related to reaction to the foreign body (LMPW crystals in this case) and not potential differences in immunological susceptibility. This was accompanied by increased superoxide anion and nitrite production measured in isolated KC. One concludes that KC play a direct role in the formation of granuloma caused by accumulation of formed wax crystals, consistent with observations in other studies of hepatic epithelioid granuloma formation following accumulation of degradation resistant injected particles (Fujita et al. 1983; Yamashita et al. 1985).

The authors argued that the differential LMPW accumulation in the rat strains could be due to higher bioavailability in the F-344 rat because no detectable amount of LMPW was found in the SD rat livers, compared to significant amounts in the livers of the F-344 rats which resulted in higher KC exposure and thus higher relative phagocytic activity. However, we believe that there is another possible explanation, namely that F-344 rats are incapable of metabolizing these type of molecules as we will explain later.

Conclusion of animal data 1962-2010
From the studies carried out on waxes and their comparison to oils, we can highlight the following general observations using the liver epithelioid granuloma as critical endpoint:

- The F-344 is the only rat strain to have developed liver epithelioid granuloma after exposure to low viscosity waxes and mineral oils.
Liver epithelioid granuloma severity is correlated with the retention of alkanes with a certain profile.

- Although viscosity used as a surrogate of molecular weight (and therefore of carbon number) initially appeared to correlate with liver granuloma formation and accumulation, in reality in the liver there is a critical range of \( \sim C_{20} - C_{35} \) with a “peak” at about \( C_{28} \) of accumulated hydrocarbons.
- The most potent substance to induce epithelioid granuloma is LMPW comprised virtually of only these type of alkanes \(< C_{35} \). This corresponds to \( n \)-alkanes and isomers that crystallize when they have an un-interrupted linear backbone of at least 20 carbon atoms (Table 4).
- The formation of epithelioid granuloma is thus associated with a focused range of accumulated alkanes of a specific type and carbon chain length.
- Severe liver granuloma formation is associated with an increase in white blood cells, specifically neutrophils, eosinophils, monocytes and lymphocytes. Although consistent in LMPW, for those oils that induce liver granuloma this effect is not always reproducible in the type of white blood cells that are elevated, indicating that because the granuloma formation in oils is less strong compared to LMPW, concomitant increases in specific types of white blood cells might be less sensitive.
- Severe liver granuloma formation is associated with an increase in markers for liver damage, specifically ALT, AST and GGT.
- Liver enlargement (as in relative organ weight increase) is observed in cases with and without liver granuloma formation, indicative of increased metabolic demand due to elevated hydrocarbon tissue levels and thus non-adverse (Hall et al. 2012).
- Comparison of the F-344 and SD rat indicate that while both may accumulate hydrocarbons in their livers, the SD rat efficiently eliminates \( n \)-alkanes and related wax components (that is those that co-crystallize with \( n \)-alkanes). Although accumulated hydrocarbons from oil in the liver of SD rats is a factor of about 2-3 lower compared to that found in the livers of F-344 rats, that proportion is not the case for hydrocarbons from LMPW where virtually none are found in the liver of the SD; indicating clear differences in the type of hydrocarbons that the F-344 rat and the SD rat tends to retain, as outlined in Table 6.
- The virtual absence of LMPW alkanes in the livers of SD rats is not due because of lack of exposure, as these are readily found in their mesenteric lymph nodes indicating the efficient removal of these hydrocarbons from the liver, which is not the case for the F-344 rat.

Overall, the data described above and summarized in Tables 5 and 6 indicate that there are clear strain differences in the adverse tissue response to paraffin waxes; with the F-344 rat being more sensitive than the SD rat. While adverse effects in the form of liver epithelioid granulomas have been shown in F-344 rats (approximate NOAEL of 20 mg/kg/day) with LMPW, at least two identical studies in SD rats have not shown liver effects (NOAEL 1644 mg/kg/day, the highest dose tested). This difference in liver effects is correlated with barely detectable levels of mineral hydrocarbons in liver tissues of SD rats, where they were detected in high amounts in liver tissues of F-344 rats. In a 2-year study in SD rats, a NOAEL of 5000 mg/kg/day was established. To judge whether the F-344 rat is either the most sensitive or an inadequate model for the assessment of waxes becomes possible when the studies on human liver samples and F-344 rats that included improved analytical techniques are considered, which is presented in the following section.

**EFSA study 2017 – new insights**

Because previous industry studies were focused in comparing specific products (mineral oils vs. waxes) in their causing hepatic granuloma, immunological activity, reversibility of effects and the measurement of total hydrocarbon retention (“mineral hydrocarbons”) as opposed to measuring sub-types of alkanes, there was no clear understanding whether the retention of a specific sub-type of alkanes could explain these differences.

Therefore, a large study to address the effect of different fractions of mineral oil and waxes causing liver granuloma was sponsored by EFSA, which was subsequently reported in a series of papers (Barp et al. 2017a, 2017b; Cravedi et al. 2017; Nygaard et al. 2019), which followed the EFSA CONTAM panel recommendation that toxicological evaluation of mineral oil hydrocarbons should focus on the molecular mass range and structural sub-classes rather than chemical–physical properties such as viscosity (EFSA 2012). Therefore, in this study F-344 rats were not exposed to specific products as commonly placed on the market, but rather to mixtures of mineral oil and wax that represent the range of mineral hydrocarbons that humans are orally exposed to. These mixtures were subsequently fractionated to produce “MOSH” test materials with a wide and a narrow “cut” of molecular mass ranges. Of interest to the present discussion are the following three MOSH test materials that due to their narrow range allowed a clear interpretation of the chemical composition behind the granuloma effect:

- S-C25, denoting short chain hydrocarbons eluting \(< C_{25} \) of a low viscosity oil.
- L-C25, denoting long chain hydrocarbons eluting \( > C_{25} \) of a high viscosity oil
- Mixture of L-C25 and a medium viscosity paraffin wax on a 1:1 (L-C25W)

It must be pointed out that S-C25 and L-C25 are oils and not “waxes” as wrongly quoted in the materials and section method, which might have been obvious to the authors but confusing to the reader not familiar with these substances (Barp et al. 2017a, 2017b; Cravedi et al. 2017; Nygaard et al. 2019).

When female F-344 rats were exposed to either of these MOSH test materials at three dietary concentrations, the results were quite like what was already known from
literature regarding granuloma formation due to low viscosity oil or low-medium wax exposure. However, additional analysis by GCxGC-FID of the residues found in the liver revealed what alkane subtypes and molecular weight cause the liver granuloma in the F-344 rat, for example, after 120-day exposure to 400, 1000 and 4000 mg/kg food (Figure 4). The following outcomes were noted:

1. Exposure to S-C25 led to the formation of granuloma only at the highest dose tested. Compositionally this oil contained also n-alkanes and low branched alkanes in the C18-C30 range. The n-alkane signal at C30 was already faint and negligible.

2. Exposure to L-C25, however did not lead to granuloma at any of the tested doses. Compositionally this oil did not contain significant amounts of n-alkanes, in fact these were virtually absent. Rather it consisted of an unresolved cloud of highly branched and cycloalkanes (naphthenes) in the >C25 range with strong signals at >C30.

3. When L-C25 (negative for liver granuloma) was mixed on a 1:1 basis with the wax, granuloma was observed in every single dose group. Compositionally the wax n-alkanes were clearly superimposed over the L-C25 hydrocarbon “cloud” in the chromatograms.

These results would not have been any different to those from similar studies (Smith et al. 1996; Scotter et al. 2003), namely that waxes are the most potent inducers of liver granuloma as clearly seen from the fact that the L-C25 “MOSH” alone did not have any effect unless mixed with a wax which clearly triggered the hepatic granuloma formation. The value of this study would have been limited if it was not for a set of measurements that completely turns around the EFSA CONTAM conclusion that waxes are the “most potent MOSH”, namely the qualitative analysis of the livers (Figure 4, middle row) but also the insight obtained of analyzing the n-alkane content in control feed and its residues in the tissues of control animals (Table 7).

From the comparison of the two sets of chromatograms in Figure 4, it becomes clear that what is found in the liver (middle row) is compositionally different to what was originally contained in the feed (top row).

1. In the S-C25 group, n-alkanes below C25 are virtually absent, indicating an effective metabolism by the
liver, while those > C25 are still present with clear signals at C25−C29 surrounded by a narrower cloud (compared to the original mixture) of unresolved cyclo- and highly branched alkanes. This might not be too surprising as the original material did have all of these components albeit at a broader distribution.

2. The L-C25 is however intriguing. The liver shows high accumulation of n-alkanes in the C29−C31 range although these were not present in the original material. These are surrounded by a narrower cloud of cyclo- and highly alkylated alkanes centered around C30 that have the L-C25 as origin.

3. The L-C25W indicates a strong accumulation of a narrower series of n-alkanes from C29−C35 compared to the original mixture in the feed and is surrounded by an attenuated cloud of cyclo- and highly alkylated alkanes centered around C30. Between the n-alkanes, accumulated iso-alkanes are also visible, corresponding to mainly 2- and 3-methyl on a n-alkane backbone (Biedermann et al. 2015).

The preferential liver accumulation of a narrow range C29−C35 of n-alkanes in all groups and especially in the L-C25 group which was originally n-alkane free, can be explained by looking into the n-alkane content in the control feed (MOSH free) and its residues in the livers of the control animals fed this diet (Table 7).

Remarkably, the strong accumulation of predominantly uneven n-alkanes in the control liver corresponds to the range of n-alkanes from biogenic origin (thus not from a mineral wax), because the odd/even carbon number ratio of petrogenic origin n-alkanes is more balanced when compared to biogenic ratios where the odd predominates (Lester 1979). This indicates that the F-344 model is not even able to eliminate those n-alkanes that humans regularly ingest from a diet rich in vegetables and fruits, which puts a question mark on safety evaluations that use this model concluding that waxes are the most potent MOSH.

Already at zero level exposure, the F-344 controls show a 35-fold hepatic accumulation of n-alkanes from natural origin, when considering 0.22 ppm and 7.72 ppm of the C29−C31 n-alkane concentration in control feed and liver, respectively.

The authors of this investigation argued that while retention of n-alkanes was observed in both in control and L-C25W groups, only the latter develop granuloma because crystallization of the retained, encapsulated material protected it from metabolism; while “part of the n-alkanes were removed from equilibration within the body, particularly with adipose tissue” page 71 in (Cravedi et al. 2017).

Crystallization of unmetabolized n-alkanes has been observed in previous studies (Hoglen et al. 1998; Griffis et al. 2010); therefore, the first part of the statement is supported by experimental data. The latter we interpret as re-distribution of n-alkanes from the liver to the adipose tissue, which prevents granuloma formation. While redistribution of n-alkanes in tissues is certainly plausible, there is however no reason to believe that crystallization in the liver of F-344 controls would not result from “biogenic” n-alkanes, but only from “mineral” n-alkanes; of course, n-alkanes are always n-alkanes regardless of petroleum or biogenic origin.

We argue that the reason that the control group does not develop liver epithelioid granuloma from biogenic n-alkanes is not due to their “encapsulation” but because the dose is too low. At a level of 0.22 mg/kg feed (Table 7), the received daily dose of natural n-alkanes is about 0.02 mg/kg bw/day, while the NOAEL for waxes containing these range of n-alkanes is 20 mg/kg bw/day (Smith et al. 1996). This is either too low, or the produced crystals are too small, to be able to trigger an immunological response by resident macrophages, but clearly demonstrate the F-344’s inability to efficiently remove n-alkanes from biogenic/petrogenic origin.

Therefore, the fundamental question about the F-344 as a toxicology model is the liver retention of n-alkane and their eventual crystallization that is associated with hepatic granuloma: namely “because n-alkanes crystallize, they can’t be metabolized” or “because n-alkanes are not metabolized, they crystallize?” and thus induce the formation of hepatic granuloma. Which one is it? We are convinced that the weight of the evidence indicates that because the F-344 is extremely poor in metabolizing n-alkanes above a certain carbon range, they eventually crystallize.

**Crystallization of n-alkanes in the F-344 liver and granuloma formation**

**Metabolism vs crystallization of n-alkanes**

The fact that F-344 rats are poor metabolizers of hydrocarbons was demonstrated in vitro with liver microsomes of three rat strains, Wistar, SD and F-344 (Cravedi et al. 2011) using n-octadecene (C18) and n-heptadecane (C17) as model compounds. While the results of this study are limited, it was shown the rate of metabolism is Wistar > SD > F-344 rats.

This observation is however limited to comparing strains and n-alkanes with carbon numbers below C20 which is not representative to those n-alkanes that crystallize at room temperature, or those that trigger liver granuloma formation in the F-344 rat.

The capability of the F-344 rat strain to “enrich” in their livers the n-alkanes with melting points above the rat’s body temperature is shown very clearly in Figure 5, from an experiment that compared the GC-FID profiles of a low viscosity oil (P7H) fed to F-344 rats and its liver residues after 90 days (McKee et al. 2012). It should be noted these types of oils may contain low levels of n-alkanes.

As can be seen, hydrocarbons with molecular weights ≤C20 are underrepresented in the liver, and the corresponding n-alkanes are no longer visible in the chromatogram of

**Table 7. Concentrations (mg/kg) of natural origin n-alkanes in the control feed (MOSH free) the liver and adipose tissue of the control animals feed this diet (adapted from table 2S; (Cravedi et al. 2017)).**

| Feed | Liver | Adipose tissue |
|------|-------|----------------|
| n-C29 | 0.05  | 1.64           | 0.42           |
| n-C30 | 0.02  | 0.37           | 0.06           |
| n-C31 | 0.12  | 4.57           | 0.39           |
| n-C32 | 0.02  | 0.38           | 0.03           |
| n-C33 | 0.02  | 0.75           | 0.00           |
| Total | 0.22  | 7.72           | 0.89           |
the liver extract indicating effective elimination. Conversely, n-alkanes with molecular weights above C_{22} (melting point 42°C) are hardly visible in the chromatogram of the original P7H oil while they clearly show their presence in the chromatogram of the liver extract. Interestingly, n-heneicosane (n-C_{21}) with a melting point of 40.5°C does not appear conspicuously in either chromatogram, suggesting a transition phase between liquid and solid. The above observation does not imply that (all) the n-alkane peaks seen above C_{22} were necessarily present in the P7(H) oil that was tested by McKee et al. Given the earlier mentioned "concentration" of n-alkanes from the feed in livers of F-344 rats, and the fact that some vegetable oils, notably olive oil contain n-alkanes below C_{27}, they could also come from the feed. From the publication by McKee et al. it cannot be discerned whether n-alkanes were also found in the livers of the control group. Only the highest dietary dose (2%) showed limited evidence of liver granuloma formation at a lesser severity than studies with higher carbon numbers in the low viscosity range (Firriolo et al. 1995; Smith et al. 1996). There was no assessment of crystallized material in the liver but given the clear accumulation of n-alkanes in the critical range it is plausible that should the n-alkane concentration be high enough crystallization would then occur. Given our limited knowledge of metabolism efficiency of alkanes > C_{20} in the F-344 rat, we come thus back to the key question: does the apparent slow metabolism of these n-alkanes result in their accumulation and eventual crystallization, or do they enrich to form crystals and cannot thus be removed by metabolism?

To answer this question, it is also worth noting that in the chromatogram of the liver extract, discrete peaks are visible between those of the n-alkanes. This observation was specifically addressed in the L-C25 (high viscosity oil) tested in the EFSA study (Cravedi et al. 2017, p. 76), indicating that these peaks correspond to a type of iso-alkanes with minimal branching, which were also retained in the liver. These prone to enrichment iso-alkanes also showed a very slow metabolism and are also retained as their n-alkanes counterparts as shown in Figure 6.

As indicated earlier, this high viscosity L-C25 oil was virtually n-alkane free, so that the accumulated n-alkanes must come from the feed and thus are of "biogenic" origin because of their typical n-alkane odd/even ratio where the odd predominate (Lester 1979).

These "biogenic" n-alkanes that strongly accumulate are in the C_{29}-C_{31} range (Table 7), so that by comparison to their "mineral" origin counterparts (iso-alkanes with minimal branching found in the L-C25 oil), we can determine that the liver retains n- and iso-alkanes with similar carbon numbers and structures (the fact that they elute close to one another), indicating that these type of "mineral" iso-alkanes are also subject to the same metabolic slowdown as the "biogenic" n-alkanes from the feed.

As we learned from the first part of this paper, these minimally branched iso-alkanes can only originate from mineral oils so that they can never crystallize at body temperature and are therefore always liquid. What this means is that if the crystallization of n-alkanes would block their metabolism, one would expect that the "iso-alkane" homolog series, which do not crystallize, would still undergo metabolism. But this is not the case as both are similarly accumulated by the

--

**Figure 5.** GC-FID analysis of P7H oil (red) and liver extract (blue) of F-344 rats exposed to P7H oil. The corresponding melting point is shown above each n-alkane peak, for n-C_{20} it is 37.5°C which corresponds to the rat’s body temperature ~37°C. The dotted red line on n-C_{21} illustrates those n-alkanes that are liquid (left arrow) and those that are solid (right arrow) if they are below or above the rat’s body temperature (adapted from McKee et al. 2012). The arrow indicates the switch taking place in the liver at which constituents start accumulating.
alkanes are identical to those of n-alkanes (Figure A2). But accumulation in the liver, the terminal ends of these retained iso-alkanes are identical to those of n-alkanes (Figure A2). But the single internal methyl group, which is far away from the terminal ends, prevents this molecule from crystalizing making it always liquid.

Generally, metabolism of normal and iso alkanes may undergo terminal (ω) or sub-terminal (ω-1) oxidation with a strong preference for the latter one (Le Bon et al. 1988; Ortiz de Montellano 2010).

From the perspective of ω versus ω-1 preference, the oxidation of this type of iso-alkane will be identical to that of a normal alkane. Therefore, the decreased metabolic capacity of the F-344 rat, will not be able to deal with either one which will lead to their accumulation.

This evidently demonstrates that the initial trigger of the n-alkane deposition in the F-344 rat livers is not the crystalline solid nature of the enriched substance, but the slowdown in metabolism. Hence, n-alkanes are not slowly metabolized in F-344 rats because they crystallize, but rather they crystallize because they are slowly metabolized.

Before leaving the discussion of the role of the crystal formation in the etiology of liver epithelioid granulomas in the F-344 rat livers, an observation on the n-C29/iso-C30 in the adipose tissue is given. Here the situation has a different dynamic where the shorter chain n-alkanes are more efficiently stored in the adipose tissue than the analogue “iso-alkanes”. The reason for this is that, with an internal and more central methyl group on the carbon backbone (as discussed below, see also Appendix 1), these “iso-alkanes” do not integrate as well within the fatty acid structures of the adipose tissue as do linear n-alkanes chains.

**Granuloma formation and mode of action**

**Granuloma formation**

The inability of the F-344 rat to effectively metabolize n-alkanes > C20 results in their gradual build-up in liver tissue including those of natural origin (McKee et al. 2012; Cravedi et al. 2017; Nygaard et al. 2019). From studies that used LMPW as test material, we learn that the rat can be exposed up to 20 mg/kg bw without developing an adverse reaction (Freeman et al. 1993; Smith et al. 1996), with epithelioid granuloma already present at 200 mg/kg bw.

Once these n-alkanes start forming crystals in the liver, these are phagocytized by resident macrophages (Hoglen et al. 1998; Griffis et al. 2010) which then form granulomata. Throughout the literature that report the F-344 rat hepatic inflammation resulting from the accumulation of alkanes we find that several terms are used, such as “microgranuloma”, “granuloma” (Smith et al. 1995; Scotter et al. 2003) “granulomatous foci” and “lipogranuloma” (Baldwin et al. 1992) and even the use of “lipogranuloma” as synonymous of “microgranuloma” (EFSA 2012; page 96 footnote #2) just to name a few examples of varying terms that render this evaluation difficult.

The term “lipogranuloma” has been used to describe the deposition of mineral oil in human livers with non-pathological outcome (Christoffersen et al. 1971; Fleming et al. 1998), and because these are morphologically different to the granuloma lesions seen in the F-344 rat liver which are composed of epithelioid macrophages (e.g. Kupffer Cells - KC) that progress to necrosis and fibrosis. Hence, because “lipogranuloma” is not epithelioid, it should not be used to describe the lesion seen in the F-344 rat (Fleming and Carrillo 2018).

Microgranuloma has been described as a small collection of macrophages (~5 or less), with few lymphocytes in the periphery, which gives way to a larger size granuloma as the dose and duration of treatment increase. Necrosis and variable fibrosis may follow (Smith et al. 1996). This larger granuloma has the key feature that resident macrophages (KC) have become epithelioid. Epithelioid macrophages are...
enlarged macrophages with eosinophilic cytoplasm, indistinct (ruffled) cell borders and vesicular eccentric nuclei, resembling epithelial cells (Fleming et al. 1998). They fuse to become multinucleated cells and in their periphery are associated with CD8 and CD4 lymphocytes (Griffis et al. 2010). These epithelioid KC clearly show crystallized phagocytized material which correlates with the test substance (Hoglen et al. 1998; Griffis et al. 2010). The term “activated” refers to macrophages that have undergone morphological change to become epithelioid.

Thus, for the present discussion we use the term “epithelioid granuloma” as it better describes the compact structure and morphological transformation that KC have undergone after phagocytizing foreign material. The term “microgranuloma” simply refers to an early or mild stage of the process.

There are however different types of epithelioid granuloma (Pagán and Ramakrishnan 2018).

The type of concern here is the “non-infectious type”, which results from the presence of insoluble, degradation resistant foreign particles that activate resident macrophages (i.e. KC), such as latex particles (Yamashita et al. 1985) or Indian ink (Fujita et al. 1983). When degradation resistant particles reach the liver, KC try to isolate foreign material by pinocytosis and store it in large or small cytoplasmic vacuoles in close contact with lysosomes, which may fuse to destroy it. As a response to persistent and extended presence of foreign material KC are induced to become epithelioid, form a compact aggregate (granuloma). Some KC in the granuloma may fuse to form multinucleated giant cells (MGC) containing numerous vacuoles filled with the persistent foreign material. Large granulomata are in close contact with lymphocytes. It was observed that these MGC remain for an extended period after exposure to the foreign material ceased, potentially sustained by newly recruited macrophages from the liver or from influx of monocytes that differentiate at the granuloma location. The formation of the granulomata can be considered as a mechanism of isolating and disposing of foreign material from the functional liver tissue, as a vital protection of the organ from indigestible material. The balance between protection or pathological (when followed by necrosis and fibrosis) in the case of reaction of foreign material has been suggested to depend on the specific features (like geometry) of the sequestered material in the granuloma (Pagán and Ramakrishnan 2018).

The above description of granuloma formation is the same type of response that is seen after exposure to e.g., LMPW, where accumulation of (phagocytized) lysosomal poly­­onal crystalloid material was observed inside isolated KC. Additionally vacuolated hepatocytes also showed variably sized polygonal crystalloid material. Increased level of vacuolization was concomitant with increased levels in markers of liver damage (Hoglen et al. 1998; Griffis et al. 2010). Increased vacuolization of hepatocytes and concomitant granuloma formation has also been reported elsewhere (Smith et al. 1996).

After pinocytosis of the crystallized LMPW, the progression of epithelioid KC to forming MGC, is also accompanied by the recruitment of T cells, to form a compact aggregate of activated KC, with peripheral CD8 and CD4 lymphocytes, but with an apparent preponderance of CD8 surrounding the granuloma and part of a periphery lymphoid rim. As the persistence of the crystallized LMPW continues, the epithelioid granuloma grows by further recruiting nonresident (extrahe­­pheric) macrophages, monocytes that undergo differentiation once in the liver.

An important factor of this extrahepatic recruitment can be supported by two observations. Firstly, by the fact that while unexposed F-344 and SD rats show no differences in KC basal activity (Hoglen et al. 1998); the former have lower populations of resident KC (Griffis et al. 2010), and secondly by a concomitant increase in WBC when granuloma formation is observed (Smith et al. 1996; Hoglen et al. 1998; Griffis et al. 2010). Therefore, because of the relative lower amount of resident KC in F-344 rats, an increased level and presence of LMPW crystallized material would lead to “overload” of the local KC phagocytic capacity, which in turns triggers the recruitment of additional white blood cells (neutrophils and eosinophils) and monocytes that differentiate to macrophages at the granuloma formation site (Yamashita et al. 1985) to help eliminating the foreign body.

The formation of epithelioid granuloma is accompanied by an increase in ALT, AST and GGT liver enzymes levels, indicative of liver injury. Elevated levels of these enzymes are indirectly related to decreased metabolism of n-alkanes of a critical carbon number (C25-C35) in hepatocytes. That is, failure to metabolize certain LMPW alkanes result in their accumulation and eventual crystallization that plays a physical cytotoxic effect in hepatocytes, which in turn rupture and release these enzymes and the crystalloid material that is subsequently phagocytized by KC. It may also be part of the explanation of the larger granuloma developing a necrotic core (Carlton et al. 2001), associated with hepatocellular degeneration (Smith et al. 1996).

In any case, the formation and progression of epithelioid granuloma and localized necrosis has an element of associated T-cell response as both, cytotoxic T-cells (CD8+) and T-helper (CD4+) cells were observed around the granuloma. This may trigger macrophages to undergo apoptosis and be replaced by recruited monocytes that differentiate into macrophages at the granuloma site. It is not possible to know whether the CD4 Th cells polarized down to a Th1 or Th2 response, but it seems that not all foreign body granuloma triggers T-cell responses indicating that size, geometry and chemical composition may play an important role in determining this additional feature of the type of granuloma developing as a reaction of persistent foreign body material (Fleming et al. 1998; Pagán and Ramakrishnan 2018).

Mode of action

There has been a previous attempt to establish a mode of action that would explain the etiology of the liver epithelioid granuloma in the F-344 rat (Adenuga et al. 2017). While the framework that was used allows a systematic comparison between human and animal data, by using the generic term “mineral hydrocarbons” the authors did not make any distinction between the alkane types that make up waxes and oils.
and thus could not explain the specific mechanism for the development of hepatic epithelioid granulomas in the F-344 rat (key event 3 of their proposed mode of action) or its corollary why waxes are more potent than oils.

The proposed mode of action by Adenuga et al., had the 3 key events to which the following comments are given.

**Key event 1 – intestinal absorption of MOH.** Mineral Oil Hydrocarbons (MOH) was the generic term used but as explained earlier, the distinction between sub-classes of alkanes is essential in explaining the crystallization of wax in the liver of F-344 rats. Consequently, when looking at the intestinal absorption of alkane subtypes this distinction should also be examined. It has been determined that n-alkanes are less well absorbed than their iso- or cyclo-alkane analogues, the latter showing the highest absorption, at least when present in a complex hydrocarbon matrix (Low et al. 1992). While for hydrocarbon constituents some differences have been observed between SD and F-344 rats (Halladay et al. 2002), for n-alkanes there seems to be no difference. After an oral dose of n-octadecane no differences in GI absorption between SD and F-344 strains were observed; however, F-344 rats showed higher concentrations of the test material (Lonardo et al. 1998) indicating that differences seen between SD and F-344 rats are not due to absorption but rather of metabolism which is consistent with other reports (Cravedi et al. 2011).

Thus, for Key event 1 differences in the types of alkane absorption must be assumed, namely that, despite n-alkanes showing the lowest intestinal absorption, the formation of epithelioid granuloma is related to the ineffective metabolism and subsequent retention of this specific type of alkane. We thus, consider that absorption per se should not be part of the mode of action.

**Key event 2 – liver deposition and retention.** As discussed previously, there is a critical range of accumulated n-alkanes in the C26-C35 range in the liver of the F-344 rat. While there is ample evidence that LMPW is the most potent wax in the induction of liver granuloma, one realizes that this is not surprising when considering that in this wax virtually only n-alkanes < C35 are present (and traces of iso-alkanes with minimal subterminal branching that can co-crystallize).

The experiments by Cravedi and colleagues (Barp et al. 2017b; Cravedi et al. 2017), used an intermediate melting point wax, which consists of predominantly n-alkanes in the range of ~C26-C44 and provided two important conclusions that are relevant for this key event, a.) the same critical range of wax alkanes are retained in the liver and b.) those above C35 are absent, indicating that there is no absorption above this carbon number, reinforcing the fact that the retained critical range is not the result of experimental shortcomings.

As we have seen in Figures 4 and 7, the mere retention of n-alkanes does not necessarily lead to granuloma formation. Therefore, the most important aspect of this key event is not the liver deposition of n-alkanes per se, but rather the crystals that form as a result n-alkane accumulation because of ineffective metabolism at this critical carbon range. If there are no crystals formed, no granuloma is formed.

**Key event 3 – inflammatory cell tissue infiltration in response to retained MOH in liver.** The use of MOH – users of the term “mineral oil hydrocarbons” may not fully appreciate that differences in alkane subtypes do make a difference in the type of response observed in the liver, best exemplified by comparing the liver inflammatory responses of L-C25 and L-C25W illustrated in Figure 4. Both materials accumulate at a critical carbon range of C25-C35, however that which has the highest number of n-alkanes induces the inflammatory granuloma response. L-C25 is retained as an unresolved cloud of mostly polycyclic and highly branched alkanes, but that type of alkane accumulation does not cause an inflammatory response. This is supported by studies that fed oils with similar composition (virtually n-alkane free) that resulted in liver accumulation of hydrocarbons, significant liver enlargement but without formation of epithelioid granuloma (Trimmer et al. 2004).

Thus, the presence of foreign material, in the form of wax crystals is essential to trigger an inflammatory response orchestrated by resident KCs phagocytizing these particles. The hypothesis given by Adenuga and colleagues is that the immunological response may be due to the known potent adjuvant properties that MOH poses (Satoh and Reeves 1994). We do not believe this is correct. While pristane (the actual substance used in the cited experiments) may be a constituent of mineral oil, this is a C19 iso-alkane, which is not in the critical range of the accumulated hydrocarbons. Further, pristane is readily metabolized by the F-344 rat as clearly shown by GCxGC chromatograms of F-344 rat livers where pristane is virtually absent versus its presence in the fed MOSH mixtures and adipose tissue (testifying exposure), indicating effective elimination of this iso-alkane chain length by the F344 rats (Barp et al. 2017a). In contrast, higher molecular weight, highly branched iso-alkanes do accumulate in the F-344 rat liver, but do not trigger granuloma formation (Barp et al. 2017a, 2017b). Finally, no association was found in orally administered pristane, or alkane mixtures with immunological responses similar to that of an adjuvant (Andreasen et al. 2017).

Thus, based on the studies by Hoglen and Griffis (Hoglen et al. 1998; Griffis et al. 2010), the immunological response that lead to granuloma formation is more akin to wax (n-alkane) crystal formation, and not to adjuvant properties of the retained hydrocarbons.

**Proposed mode of action in an AOP.** The current proposal has used the Adverse Outcome Pathway (AOP) framework to better organize and present the data (OECD 2018). It is not intended to give extensive details of every key event but rather the critical steps that would allow us evaluating its human relevance. The information organized in an AOP consists typically of a molecular initiating event (MIE) followed by key events (KE) interconnected by key event relationships (KER) leading to an adverse outcome (AO). The distinction between a MIE and a KE is that the MIE is a specialized KE
where evidence is required that the event can be triggered by a chemical (or other stressors) and a list of stressors. In our proposal we acknowledge that KE1 could very well be a MI E. We have chosen to keep it as KE to better juxtapose and build upon what Adenuga and colleagues proposed but with our corrections that consider the different elements of the etiology of the liver granuloma formation in the F-344 rat. The following key events and mode of action are proposed (Figure 7).

**Key event 1 (KE1)**

Decreased metabolism (of stressor); The F-344 rat strain is not able to effectively metabolize and eliminate the chemical stressor which are the n-alkanes and similar linear alkanes that follow the rule of thumb (see previous sections) of an “uninterrupted linear alkane backbone of at least 20 carbon atoms” which show a critical range of C25-C35. (Freeman et al. 1993; Smith et al. 1996; Scotter et al. 2003; Cravedi et al. 2011; McKee et al. 2012; Cravedi et al. 2017).

We don’t know the reason for this, although it is known that other rat strains and animals efficiently metabolize n-alkanes including those in the critical range (McCarthy 1964; Tulliez and Bories 1975; Tulliez and Bories 1978; Lester 1979; Cravedi et al. 2011). Nonacosane (n-C29) is oxidized to the corresponding fatty acid, which undergoes successive β-oxidation until the carbon chain length becomes similar to those fatty acids that are commonly present in the organism such as C16 and C18 (Kolattukudy and Hankin 1966). Given the wide exposure to n-alkanes through a vegetable diet it would be expected that these types of molecules are efficiently metabolized. There are no data on the CYP isofrom differences between the F-344 and other rat strains (or human), but we do not consider it essential to draw our conclusions.

**Key event relationship 2 (KER2)**

Overwhelmed metabolic capacity; where the unmetabolized compounds accumulate with increasing dose and length of exposure. The evidence for this related event can be inferred from the same literature as KE1.

**Key event 2 (KE2)**

Crystallization (of stressor); Accumulated n-alkanes and linear molecules that co-crystallize form wax crystals inside hepatocytes which rupture to release them after a critical shape and size is achieved. Liver enzymes (ALT, AST, GGT) denoting damage increase (Smith et al. 1996; Hoglen et al. 1998; Griffis et al. 2010). Wax crystals become foreign material that will trigger an immune reaction (Fujita et al. 1983; Yamashita et al. 1985; Pagán and Ramakrishnan 2018).

**Key event relationship 3 (KER3)**

Reduced phagocytosis; the F-344 strain has an inherent lower population of resident KCs which may be overloaded when the presence of foreign material is too high (Hoglen et al. 1998; Griffis et al. 2010). The persistence of the foreign material triggers a positive feedback loop for the recruitment of extra hepatic phagocytic capacity.

**Key event 3 (KE3)**

Granuloma formation (to isolate/eliminate wax crystals); The presence of wax crystals triggers the movement of resident KCs to phagocytize the foreign material. KCs phagocytize wax crystals, aggregate and undergo epithelioid activation forming microgranuloma. These subsequently fuse to form multinucleated giant cells. Extrhepatic monocytes are recruited, along with neutrophils and eosinophils, which all form large granulomata. T-cells are recruited to form a rim around large granulomata (Smith et al. 1996; Hoglen et al. 1998; Griffis et al. 2010).

Necrosis: continuous presence of wax crystals, along with sustained inflammation and constant turnaround of macrophages (apoptosis), results in an increase of the necrotic core of the granuloma (Smith et al. 1996; Hoglen et al. 1998; Carlton et al. 2001; Griffis et al. 2010).

Therefore, granuloma forms as a protection mechanism to isolate/dispose the sequestered persistent foreign material; the balance between benign or pathological depends on the physical features of the foreign body. Given the course of the granuloma in the F-344 rat to become epithelioid and then necrotic, we infer that the geometry of the wax crystals is such that it tips the pathway balance toward an adverse outcome (AO).

The F-344 rat pre-condition and the subsequent key events for the granuloma formation raise and important question about human relevance, namely whether key event 1 or 2 is ever encountered in humans that would then cascade down to a full blown epithelioid granulomatous response. This is explored in the following section.

**Relevance of F-344 rat granuloma mode of action to humans**

It is well documented that humans can develop epithelioid granuloma due to various insults (Pagán and Ramakrishnan 2018). So, the question here is not whether humans develop...
this type of lesion but rather, considering the AOP, whether in humans a.) metabolism of n-alkanes is ineffective, and b.) whether crystallization would ever occur as an absolute prerequisite for the subsequent development of epithelioid granuloma as adverse outcome (AO).

**a. Alkane metabolism in human livers**

The question of ineffective alkane metabolism is to address whether humans, like F-344 rats, can accumulate n-alkanes as a prerequisite to form wax crystals. To approach this question and acknowledging that both F-344 and SD rats retain alkanes in the liver, we are thus interested in knowing which types of alkanes are found as residues in the human liver, including n-alkanes.

The presence of alkanes in human tissues is well documented and known for quite some time. Boitnott and Margolis determined the alkane content in 60 livers and 34 spleens obtained from 63 patients (4 children and 59 adults) at time of autopsy (Boitnott and Margolis 1970) to investigate the link between alkane deposition in these tissues and the presence of oil droplets in their histological evaluations. The organs were extracted with two different methods. By using molecular sieves which retain n-alkanes and exclude branched and cyclic compounds, the authors concluded that branched-chain and/or cyclo-alkanes predominated over n-alkanes in all samples examined. An average of 71% of the alkanes found in the liver were non-linear (i.e. branched and cyclic), and the proportion of the n-alkanes were the highest in the liver with the lowest total alkane content, suggesting transient levels of n-alkanes. Mass spectrometric analysis was done on the alkanes, isolated by column chromatography on two cases selected to represent the quantitative extremes of the alkane content. This mass spectrometric analysis indicated the presence of complex mixtures of alkanes like that of mineral oil, including high proportions of polycycloalkanes. When the retained alkanes exceeded a critical concentration in the liver (>200 mg/kg tissue) oil droplets were observed. Thus, what was found to accumulate in the human livers were non-linear alkanes (i.e. iso- and cyclo-alkanes) associated with the formation of oil droplets. We need to emphasize that accumulated n-alkanes form wax crystals in the F-344 rat, while these are not accumulated in human livers which rather retain the non-linear alkanes constituents of mineral oil, and thus form oil droplets.

A similar experiment was performed on a total of 3 kg liver (wet weight) obtained from healthy persons who died accidentally (Schlunegger 1972). From the 350 mg of hydrocarbons found, the greater part were branched-chain alkanes, and only 30 mg of n-alkanes were isolated by molecular sieving. This would amount to 107 of nonlinear and 10 mg/kg liver, for n-alkanes. Therefore, both studies indicate that the bulk of the hydrocarbons found in human livers are of branched and/or polycyclo-alkane nature, with a low n-alkane background concentration attributed to the exposure of n-alkanes from vegetable or animal origin and representing a “work-in-progress” background of the metabolic processes taking place in the liver.

A fundamental clue about the chemical composition of the retained alkanes in human livers is obtained from a human study (Barp et al. 2014) that involved the measurement of saturated hydrocarbons in subcutaneous abdominal fat, mesenteric lymph nodes, spleen, liver and lung taken at autopsy from 37 subjects aged 25–91 years (mean 67 years). When discussing the chromatogram obtained of hydrocarbons present in abdominal fat tissue, the authors described the presence of odd numbered n-alkanes as distinct peaks at regular intervals on the downslope of the hump of unresolved highly isomerized branched and cyclic alkanes likely from mineral oil. After identifying these peaks as odd numbered n-alkanes typical of plant origin, such as n-C_{30} to n-C_{33}, the authors attributed also a transient presence of these hydrocarbons, reasoning that in view of the expected exposure to n-alkanes in vegetables and vegetable oils, a continuous accumulation would have led to far higher concentrations. This is supported by comparing the alkane profile of the fat tissue to that of the liver of the same individual(s), where the peaks representing the odd numbered n-alkanes of plant origin are absent from the liver, but clearly present in the fat. In view of the well-documented metabolic conversion of n-alkanes to fatty alcohols and fatty acids, (McCarthy 1964; Kolattukudy and Hankin 1966; Albro and Thomas 1974) (Tulliez and Bories 1978; Tulliez and Bories 1979), this is indeed what one would have expected, which is in contrast to what we see in the livers of F-344 rats (Figure 8).

This led to the conclusion that “virtually no mineral n-alkanes were detectable”. It was argued that the lack of n-alkanes in the tissues is likely reflective of a process that is dependent on highly selective uptake, elimination and metabolic degradation (which may proceed at a faster rate in n-alkanes than highly branched and cyclic alkanes).

Inspection of the individual chromatograms of all 37 subjects showed the same pattern as the set of chromatograms of Figure 8 (personal communication with Grob, one of the authors of the study quoted (Barp et al. 2014)). That is, n-alkanes from vegetable origin are present in the adipose tissue (and MLN, but not shown) but none in the liver or other tissues (i.e. spleen, heart, lung, kidney). The presence of the n-alkanes in the adipose tissue and MLN are important in this context because they demonstrate that their absence in the liver is not caused by lack of exposure. Thus, exposed individuals eliminate n-alkanes retained in different tissues through metabolism in the liver.

To round up and confirming the observations of Boitnott and Margolis on the type of hydrocarbons retained in human livers (Boitnott and Margolis 1970), the hydrocarbon composition of the tissues collected by Barp et al. 2014 was characterized using comprehensive two-dimensional chromatography (GCxGC-FID), presently the best technique for characterizing the composition of saturated hydrocarbons (Biedermann et al. 2015). The absence of n-alkanes (both those from vegetable origin and mineral oil derived products) was noted in the liver. Furthermore, all hydrocarbon components forming distinct signals are essentially removed including lightly branched, pristane and phytane, which are clearly present in the fat. What is left behind in the liver is an
unresolved “gray cloud” of hydrocarbons mainly represented by highly isomerized and polycyclic compounds, as shown in Figure 9.

By comparing between the GCxGC chromatogram of the saturated hydrocarbons in a human liver with one obtained from a pure typical paraffin wax sample, one realizes that what is in the wax is not in the liver, and what is in the liver is not in the wax (Figure 9). As explained earlier, wax crystallization effectively leaves out mineral oil constituents. The red frame encompassing the wax is superimposed over a human liver sample, with the vertical line indicating the position of C30 for comparison. The horizontal black line in the human samples indicates the position of the n-alkanes. As it can be clearly seen the background of the wax chromatogram is white in comparison to a gray background in the mineral oil samples. A narrower gray cloud appears in the human liver sample, which lacks virtually all the signals typical of a wax.

Thus state of the art analysis confirm that the lack of n-alkanes is consistent with the older studies (Boitnott and Margolis 1970; Salvayre et al. 1988) indicating that normal paraffins are most likely rapidly eliminated in tissues and hence are not retained in tissues long enough to cause crystallization that would lead to any significant adverse effects.

b. Crystallization of n-alkanes in humans

The second point to address is whether crystallization of n-alkanes would ever occur in the human liver as an absolute prerequisite for the subsequent development of hepatic epithelioid granuloma.

While there are no extensive studies addressing this question there is empirical evidence indicating that although rare, humans may develop n-alkane crystals in their organs, including the liver.

The evidence comes from the case of a 55-year old man who died because of ventricular fibrillation due to coronary failure associated with the patient’s risk factors such as familial antecedents, smoking, hypertension and excess weight (Rocchiccioli et al. 1987; Salvayre et al. 1988; Duboucher et al. 1989). At autopsy, the authors reported an abnormal accumulation of alkanes in the lung lymph nodes, which was not associated to cause of death. It was noted that the man used to eat approximately one kilogram of unpeeled apples every day for 18 years, which would correspond to an intake of about 10 mg of natural long chain n-alkanes per day. Chest X-ray radiography showed the presence of micronodules in the axillary and apical lung areas. Their histological examination revealed the presence of granulomas with giant macrophagic cells containing crystalline “needle like” inclusions of material contained in membrane-surrounded organelles clearly identified as secondary lysosomes (indicative of phagocytosis), as seen in Figure 10. The same crystals were also numerous in the abdominal lymph nodes. The liver had a lesser incidence of this type of crystals engulfed by KCs but importantly, no inflammatory cells were present (Rocchiccioli et al. 1987). The presence of crystals in these organs was not linked to the man’s death, but it was argued that the man...
may have had an enzyme deficiency which allowed for the accumulation of plant wax n-alkanes.

Compared to control tissues, lipid analyses indicated high concentrations of C_{29}, C_{31} and C_{33} n-alkanes in the lung granuloma (4x higher than in the whole lung); but interestingly, one order of magnitude lower in the liver. High concentrations of n-alkanes were also measured in the lumbo-aortic lymph nodes and adrenal gland, see Table 8. It was concluded that these alkanes are not of mineral oil origin but rather typical of vegetable cuticular wax. For comparison apple peel wax shows an n-alkane range of C_{19}-C_{35} with peak at C_{29}, and a wax concentration of 54–85 mg/g cuticle (Lester 1979).

Therefore, although n-alkanes were present in the liver and other organs to form wax crystals, under the same conditions their high incidence was observed in the lung where n-alkane concentrations are significantly higher because they cannot readily be metabolically removed. Here, they triggered the formation of granulomata that although not epithelioid it included lymphocytes, macrophages and eosinophils, surrounded by fibrosis which was regarded as prejudicial. In the liver, where 10 times lower n-alkane levels were observed, the same wax crystals did not elicit this response. Rather, these crystals were isolated by KCs without the adverse reaction seen in the lung. As indicated earlier, the presence in the liver of a foreign body that is difficult to remove (e.g. wax crystals) triggers the formation of granuloma that can follow two possible paths, benign or pathological (Pagán and Ramakrishnan 2018). In the presence of the same stressor, humans seem to develop the benign type, which encapsulates and isolates wax crystals without any adverse outcome. The F-344 rat on the other hand goes down the pathway of a pathological epithelioid granuloma which we have extensively discussed.

This clearly demonstrates that even in extreme conditions, where sensitive individuals with a high n-alkane intake via the diet can experience their crystallization in the organs, the human liver is still capable of metabolizing a large proportion of these dietary n-alkanes and isolate those that crystallize. By extension the same would happen with constituents present in a mineral hydrocarbon wax because n-alkanes are always n-alkanes regardless whether they are of biogenic or petrogenic origin. This is the opposite case of the F-344 rat where n-alkanes from both biogenic and petrogenic origin accumulate in the liver (Barp et al. 2017b; Cravedi et al. 2017) a requirement for crystal formation and epithelioid granuloma formation (Hoglen et al. 1998; Griffis et al. 2010), thus denying the F-344 rat any value as a model to extrapolate wax systemic toxicity effects to humans.

In summary, we have demonstrated that even in extreme conditions where the key events 1 or 2 are found in humans, key event 3 does not take place. Therefore, the liver granuloma inflammatory response seen in the F-344 rat after exposure to paraffin waxes is not relevant to humans. Consequently, the F-344 rat is the wrong model to assess the intrinsic hazard of paraffin waxes, which makes the SD rat the more appropriate model to assess waxes.

**Synopsis – the etiology of the F-344 rat lesions**

Highly refined Paraffin Waxes have been used for decades in food packaging, cosmetics and pharmaceutical products, and it was generally thought that such products posed no harm to human health. However, in the early 90’s, studies using the F-344 rat strain to test the systemic toxicity of white oils and waxes indicated that some of them, and most significant low melting point wax LMPW, induced granuloma formation in the lymph nodes and livers, the latter further progressing
to epithelioid granuloma (Baldwin et al. 1992; Smith et al. 1996). Several follow-up studies were launched by industry attempting to explain the differences in species/strain response to alkanes originating from these products (Miller et al. 1996; Hoglen et al. 1998; Griffis et al. 2010) but no final conclusion could be drawn regarding the root cause for the atypical response of the F-344 rat strain. Already then, retention of alkanes was correlated to the liver effects observed. The absorption and subsequent toxicity of oils and waxes appeared to be linked to lower molecular weight components and lower viscosities which facilitated absorption and subsequent accumulation in the tissues. However, other factors such as carbon number and alkane sub-types (normal, branched or cyclic) that may influence accumulation were not specifically addressed because for UVCB substances, such as paraffin waxes and mineral oils, the paradigm consisted of testing finished products and not fractions thereof. Ironically, while there was an emphasis of Industry to regard paraffin waxes and mineral oils as distinct products, when it came down to assess accumulation both industry and assessors focused on waxes and oils as being “mineral hydrocarbons” (MHC), assuming all alkane sub-types would have the same effect, even though there was knowledge indicating that branched (iso-) chain alkanes are cleared from the liver more slowly than straight (normal-) chain alkanes (Boitnott and Margolis 1970). Therefore, at tissue level, accumulated MHC (encompassing constituents of waxes and oils) had the liver “micro-granuloma” as a common denominator and became the paradigm for the next three decades.

All the following safety assessments which we have reconstructed were based on this paradigm, resulting in recommendations based on viscosity for both paraffin waxes and oils, which were pragmatic but focused on an apical endpoint which had lost its connection to chemical composition of the regulated products (waxes and oils). The ambiguity of the term MHC was exacerbated with the introduction of the “mineral oil saturated hydrocarbons – MOSH” chromatography term which applied to all hydrocarbon waxes and mineral oils, led the popular belief that waxes, and mineral oils were equivalent. This lead EFSA to denominate LMPW as the “most potent MOSH” (EFSA 2012), for the apical endpoint of liver granuloma, and the ultimate entanglement of hazard assessment of two very different products.

In hindsight, it might be clear that the differences in chemical structure and physical properties between paraffin waxes and mineral oils, should have been fully appreciated. What is even more striking is, that when LMPW was
characterized as “the most potent” in the F-344 rat strain, this did not raise the question whether this rat strain would show a similar response when exposed to n-alkanes of biogenic origin.

Based on previous blind-spots in the assessment of paraffin waxes and the fact that n-alkanes found in a vegetable diet are also found as the main constituents of paraffin waxes, we have tried to compile all the pieces of the puzzle that would help to disentangle the confusion between paraffin waxes and mineral oils and their safety assessment.

To understand the etiology of the F-344 rat hepatic granuloma, we have explained the differences between paraffin waxes and mineral oil. These are related products because both share a common refinery ancestor, but they are chemically and physically different, which is the key to understand the reason why LMPW is the most potent inducer of liver granuloma in the F-344 rat. Paraffin waxes are solid at room temperature, which is the result of a very selective process of the n-alkanes crystallizing out of an oily matrix (e.g. slack wax). To be able to form a crystalloid structure, specific structural requirements are essential, namely that there is an uninterrupted linear alkane backbone of at least 20 carbon atoms. This is logically met by n-alkanes, which can be up ~90% of a paraffin wax (Table 2), but also by iso and cyclo-alkanes that fulfill this requirement, and thus co-crystallize with the n-alkanes. Having a saturated ring in the linear segment is no impediment for metabolism as has been experimentally demonstrated by experiments using eicosanyl-cyclohexane, a C30 linear molecule with a C6 saturated ring attached to it (Halladay et al. 2002). We have also indicated that the reason LMPW is the most potent substance inducing hepatic granuloma is because compositionally its carbon range covers exactly the most critical range for the granuloma formation, C20-C35. Carbon numbers below C20 are well metabolized (McKee et al. 2012), whereas those higher than C35 are not absorbed in the gut, which is proven by testing an intermediate wax whose constituents fall partially in the critical range to induce granuloma (C25-C35), while the other part consists of those above ~C35 which are not intestinally absorbed (Cravedi et al. 2017). What is a logical deduction and experimentally proven is that because intermediate point waxes have less constituents in the critical range, they are less potent than LMPW (Smith et al. 1996; Pirow et al. 2019).

These observations, however, do not yet allow disentangling oils from waxes from the hepatic granuloma as the common denominator because under this paradigm the differences in the type of accumulated alkanes that cause this response was not known. What was well known is that high viscosity mineral oils do not cause liver granuloma, although a certain fraction is clearly (reversibly) retained in the liver of rats (Trimmer et al. 2004). Despite liver accumulation, the lack of liver effects was attributed solely to high molecular weight constituents, rather than the overlooked fact that these oils are well depaaffinized (virtually n-alkane free) and thus lack these constituents within the critical range.

The fundamental clue demonstrating that it was the accumulation of n-alkanes the root cause of the granuloma formation in F-344 rats came from the EFSA sponsored study (Barp et al. 2017a, 2017b; Cravedi et al. 2017; Nygaard et al. 2019), in which control rat livers where also analyzed for background alkane type concentrations, an angle overlooked by previous industry studies. It became very clear that the control rats accumulate exactly those n-alkanes that are ubiquitous in a vegetable rich diet, which also clearly appeared in the livers of the dose group which had received a well depaaffinated oil, similar to that used by Trimmer et al. (Figure 4 and Table 7). This issue is not unimportant, because the fact that the F-344 rat accumulates both n-alkanes from both biogenic and petrogenic origin leads one to postulate that a) the F-344 rat is the wrong animal model to assess the systemic toxicity of n-alkanes, and then, by excluding the hepatic epithelioid granuloma formation unique to this rat strain, all natural, petrogenic or synthetic n-alkanes can be demonstrated to be safe, or b) the F-344 rat is the right model, and then one should be seriously concerned about the consumption of dietary n-alkanes that constitute an estimated 0.1% dry weight of a human diet (Lester 1979).

Based on review of the data and the proposed mode of action (albeit not fully described as yet), one can understand why the F-344 rat is the wrong model to assess the systemic toxicity of paraffin waxes. The lack of metabolic capacity of the F-344 rat in removing n-alkanes >C20 from the liver results in their crystallization that trigger the formation of epithelioid granuloma. Polycyclo- and iso-alkanes that aren’t metabolized do accumulate but because of their oil character they do not form crystals and thus do not cause hepatic granuloma. The issue of the F-344 rat granuloma must be viewed therefore from a perspective of causality due to distinct types of alkanes, and not from the paradigm of “mineral hydrocarbons” or “MOSH” that may lead to the unorthodox view that n-alkanes from “natural” sources would have somehow a different mode of action than those from “petrogenic” or “synthetic” origin in causing hepatic granuloma. It is intriguing, therefore that one of the papers reporting on the EFSA study (Nygaard et al. 2019) conclude that “induction of liver granuloma appeared to be related to n-alkanes > C25″ (consistent with the type of wax tested and the expected results) but then indicate that “MOSH levels and compositions appear to be similar to those observed in humans”, which might be misinterpreted as indicating that humans and the F-344 rat accumulate the same type of alkanes, which we know that for n-alkanes is not generally the case as demonstrated by analyzing human livers (Boitnott and Margolis 1970; Barp et al. 2014).

Because many of the n-alkanes found especially in LMPW are also found to be present in animal and plant tissues (Lester 1979), by virtue of their ubiquity they comprise a small but definite part of the normal diets of humans, thus the apical endpoint of liver granuloma in the F-344 rat caused by n-alkanes is no longer viable. It is important to highlight that one of the coauthors reporting on the EFSA study (Nygaard et al. 2019), states in a personal opinion that because n-alkanes are absent in human livers waxes should therefore be evaluated separately (Grob 2018).
MOSH accumulation in humans

Although not in the scope of this review, it is important to mention that for the human situation, the MOSH assessment must consider what is known about mineral oil exposure. It is known that a fraction of mineral oil can be retained in human livers (and other tissues), which may result in the formation of so-called "lipogranuloma" (Christoffersen et al. 1971; Fleming et al. 1998). In the liver these deposits appear as oil droplets in vacuoles that are intra or extracellular. Smaller vacuoles are within macrophages. Commonly the extracellular vacuoles are in the organ parenchyma, unaccompanied by any tissue response (Carlton et al. 2001). These lesions are largely portal, with associated inflammatory cells such as lymphocytes and plasma cells at varying amounts. Lipogranuloma do also occur in other organs, such as spleen, lymph nodes and bone marrow (Cruickshank and Thomas 1984). Although lipogranulomatous associated with mineral oil ingestion are commonly found in human livers and spleen with varying frequencies (from nil to 50%) (Kelsall and Blackwell 1969; Cruickshank 1984); crucially, in all reports, there has been no evidence of any clinical significance associated with the presence of these lesions and thus MOSH deposition in tissues, including the liver. Again, note that these lesions are morphologically quite different from the epithelioid granulomas seen in the F-344 rats (Fleming et al. 1998; Carlton et al. 2001; Fleming and Carrillo 2018). The composition of the MOSH oil droplets in lipogranuloma is of iso-alkanes and high proportions non-condensed and condensed polycycloalkanes (naphthenes) of 2–5 rings, which remain liquid at the temperature used to prepare the frozen liver sections (-23°C) (Boitnott and Margolis 1970). The retention of MOSH tough, does not necessarily lead the formation of lipogranuloma. MOSH material similar to that reported by Boitnott and Margolis was found in livers of individuals (Barp et al. 2014), who did not develop lipogranuloma as confirmed by one of the authors in a related publication (Grob 2018). Together with the proposed adverse outcome pathway (AOP) applicable to waxes the reevaluation of mineral oils of across viscosities is warranted.

This calls for the definitive separation of paraffin waxes from mineral oils for systemic hazard assessment so that the effects and accumulation of mineral oils should be evaluated on their own data set considering what is also observed in humans. This will help disentangle the confusion caused by jointly considering paraffin waxes and mineral oils as "MOSH" and the consequences that this has for regulatory policies such as the reevaluation of the ADI of paraffin waxes based on "low viscosity, carbon number and molecular weight" (SCF 1995).

Conclusion and recommendations

1. Paraffin waxes under FCM93 are of low order of toxicity, safe to human exposure and should not be called MOSH.
2. The F-344 rat has the inherent inefficiency to metabolize n-alkanes and similarly linearly structured alkanes within the critical range of C20-C35, with the consequence that these accumulate and eventually crystallize triggering a foreign body type of inflammatory reaction.
3. The inflammatory reaction to wax crystals has several stages, the ultimate being the formation of epithelioid granuloma, which is not observed in humans.
4. Because humans are efficient metabolizers of n-alkanes, crystallization does not occur and therefore no
The subsequent formation of epithelioid granuloma due to wax dietary exposure is observed.

5. The F-344 rat had been considered “the most sensitive” strain for hazard assessment but based on its discrepancies in n-alkane metabolism compared to humans, the hepatic granuloma should be considered the inadequate model for systemic toxicity assessment.

6. Excluding the wax constituents, the hepatic accumulation of mineral oil constituents (highly isomerized iso-alkanes and cycloalkanes) in F-344 and SD-rat strains is comparable to that in humans.

7. The generalized approach to assess waxes and mineral oils together as “mineral hydrocarbons” or “MOSH” should be updated by assessing waxes separately, and mineral oils on their own data set.

8. Consequently, the chromatographic analysis of MOSH by current state of the art methods should always discard n-alkanes, regardless of their biogenic or petrogenic origin.

As a last point, one must reflect on the challenge that toxicologists have in extrapolating animal data to humans. While allometric scaling reflects the knowledge that two species are different, with this current issue one realizes that two rat strains respond differently to the same chemical challenge. It was through an interdisciplinary team of toxicologists, pathologists and analytical chemists that the etiology of the liver granuloma in the F-344 rat could be elucidated by combining decades of published literature. Humans unlike F-344 rats have no inherent problem in metabolizing n-alkanes ingested through a diet rich in fruits and vegetables. Should one insist in extrapolating the F-344 rat liver granuloma results to humans, one should then also modify the old wisdom into “a peeled apple a day keeps the doctor away” (Figure 11).

Notes

1. ADI “not specified” means that, on the basis of the available data (chemical, biochemical, toxicological, and other), the total daily intake of the substance, arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an acceptable daily intake (ADI) expressed in numerical form is not deemed necessary.

2. Class I, medium viscosity P70H; Class II, medium viscosity N70H, N70A; Class III, low viscosity P15H, N15H and N10A.

Acknowledgements

The authors thank the anonymous reviewers who provided detailed and extensive comments, suggestions and corrections to the present manuscript which resulted in a significant improvement of this paper.

Also, we acknowledge the following colleagues who helped in the review of the manuscript during its preparation:

Peter Boogaard, Shell, The Netherlands
Trinidad Espinosa Castilla, Cepsa, Spain
Allison L. Isola, ExxonMobil Biomedical Sciences, USA
Geoff Granville, GCGranville Consulting Corp., Canada
Olaf Kretschmer, Sasol, Germany
Anna Steneholm, Nynas, Sweden

Declaration of interest

Juan-Carlos Carrillo is a senior toxicologist and fulltime employee at Shell International B.V., whose day-to-day job includes the writing of scientific opinions. Dirk Danneels is the Secretary General of the European Wax Federation aisbl (EWF), and Jan Woldhuis a retired employee of Paramount B.V. and past Chairman of the EWF.

The European Wax Federation aisbl (EWF) represents the European wax producers, refiners and blenders (EU Transparency Register 35124162688-86). www.wax.org

The EWF requested EFSA for the reevaluation of Waxes, paraffinic, refined, derived from petroleum based or synthetic hydrocarbon feedstocks (FCM 93, Ref. 95858).

Shell is not a member of the EWF, but is involved in the manufacturing and commercialization of waxes.

The authors are involved in proceedings related to the use of waxes in food, cosmetics and other regulated applications and did not receive any funding or material compensation for writing this paper.

References

Adenuga D, Goyak K, Lewis RJ. 2017. Evaluating the MoA/human relevance framework for F-344 rat liver epithelioid granulomas with mineral oil hydrocarbons. Crit Rev Toxicol. 47(9):750–766.

Albro PW, Fishbein L. 1970. Absorption of aliphatic hydrocarbons by rats. Biochimica et Biophysica Acta (BBA)-Biomembranes. 219(2):437–446.

Albro PW, Thomas RO. 1974. Metabolism of phytane in rats. Biochimica et Biophysica Acta (BBA)-General Subjects. 372(1):1–14.

Andreassen M, Hjertholm H, Cravedi JP, Grob K, Alexander J, Nygaard UC. 2017. Effect of dietary pristane and other saturated mineral oils (MOSH) on autoimmune arthritis in rats. Toxicol Rep. 4:104–112.

ASTM D445 2019. Standard test method for kinematic viscosity of transparent and opaque liquids (and Calculation of Dynamic Viscosity). ASTM Conshohocken, PA.

Baldwin MK, Berry PH, Esdaile DJ, Linnett SL, Martin JG, Periastis GC, Priston RA, Simpson BJ, Smith JD. 1992. Feeding studies in rats with mineral hydrocarbon food grade white oils. Toxicol Pathol. 20(3 Pt 1): 426–435.

Barp L, Biedermann M, Grob K, Blas-Y-Estrada F, Nygaard UC, Alexander J, Cravedi J-P. 2017a. Accumulation of mineral oil saturated hydrocarbons (MOSH) in female Fischer 344 rats: comparison with human data and consequences for risk assessment. Sci Total Environ. 575:1263–1278.

Barp L, Biedermann M, Grob K, Blas-Y-Estrada F, Nygaard UC, Alexander J, Cravedi J-P. 2017b. Mineral oil saturated hydrocarbons (MOSH) in female Fischer 344 rats; accumulation of wax components; implications for risk assessment. Sci Total Environ. 583:319–333.

Barp L, Kornauth C, Würgler T, Rudas M, Biedermann M, Reiner A, Concin N, Grob K. 2014. Mineral oil in human tissues, Part I: concentrations and molecular mass distributions. Food and chemical toxicology: an international journal published for the. Food Chem Toxicol. 72:312–321.

Biedermann M, Barp L, Kornauth C, Würgler T, Rudas M, Biedermann M, Reiner A, Concin N, Grob K. 2015. Mineral oil in human tissues, part II: characterization of the accumulated hydrocarbons by comprehensive two-dimensional gas chromatography. Sci Total Environ. 506-507:644–655.

Biedermann M, Fiselier K, Grob K. 2009. Aromatic hydrocarbons of mineral oil origin in foods: method for determining the total concentration and first results. J Agric Food Chem. 57(19):8711–8721.

Biedermann M, Grob K. 2009a. Comprehensive two-dimensional GC after HPLC preseparation for the characterization of aromatic hydrocarbons of mineral oil origin in contaminated sunflower oil. J Sep Sci. 32(21): 3726–3737.

Biedermann M, Grob K. 2009b. How “white” was the mineral oil in the contaminated Ukrainian sunflower oils? Eur J Lipid Sci Technol. 111(4): 313–319.

Boitnott J, Margolis S. 1966. Mineral oil in human tissues. 2. Oil droplets in lymph nodes of porta hepatitis. Bulletin of the Johns Hopkins Hospital. 118(S):414.
Boitnott J, Margolis S. 1970. Saturated hydrocarbons in human tissues. 3. Oil droplets in the liver and spleen. Johns Hopkins Med J. 127(2):65–78.

Boogaard PJ, Goyak KO, Biles RW, van Stee LLP, Miller MS, Miller MJ. 2012. Comparative toxicokinetics of low-viscosity mineral oil in Fischer 344 rats, Sprague-Dawley rats, and humans-implications for an Acceptable Daily Intake (ADI). J. Regul Toxicol Pharmacol. 63(1):69–77.

Bratinova S, Hoekstra E. 2019. Guidance on sampling, analysis and data reporting for the monitoring of mineral oil hydrocarbons in food and food contact materials. Luxembourg: Publications Office of the European Union.

Bratinova S, Hoekstra E, Emons H, Hutzler C, Kappenstein O, Biedermann Bratinova S, Hoekstra E. 2019. Guidance on sampling, analysis and data reporting for the monitoring of mineral oil hydrocarbons in food and food contact materials. Luxembourg: Publications Office of the European Union.

Carlton WW, Boitnott JK, Dungworth DL, Ernst H, Hayashi Y, Mohr U, Parodi AL, Pattengale PK, Rittinghausen S, Ward JM. 2001. Assessment of the morphology and significance of the lymph nodal and hepatic lesions produced in rats by the feeding of certain mineral oils and waxes. Proceedings of a pathology workshop held at the Fraunhofer Institute of Toxicology and Aerosol Research Hannover, Germany, May 7-9, 2001. Exp Toxicol Pathol. 53(4):247–255.

Carrillo JC, van der Wiel A, Danneels D, Kral O, Boogaard PJ. 2019. The selective determination of potentially carcinogenic polycyclic aromatic compounds in lubricant base oils by the DMSO extraction method IP346 and its correlation to mouse skin painting carcinogenicity assays. Regul Toxicol Pharmacol. 106:316–333.

Chasey KL, McKee RH. 1993. Evaluation of the dermal carcinogenicity of lubricant base oils by the mouse skin painting bioassay and other proposed methods. J Appl Toxicol. 13(1):57–65.

Christoffersen P, Braendstrup O, Juhl E, Poulsen H. 1971. Lipogranulomas of the spleen, liver and lymph nodes in mouse after long-term feeding of refined mineral oil. Pathologic and chemical findings in a case. Archives of Pathology & Laboratory Medicine. 113(4):423–436.

CONCAWE 1984. Assessment and comparison of the composition of food-grade white oils and waxes manufactured from petroleum by catalytic hydrogenation versus conventional treatment. Report no 84/60. CONCAWE, Den Haag.

Craveldi JP, Grob K, Nygaard UC, Alexander J. 2017. Bioaccumulation and ecotoxicity of medium viscosity white mineral oils with a kinematic viscosity between 8.5–11 mm²/s at 100 °C for the proposed uses as a food additive. Panel on Food additives Nutrient Sources added to Food. Efsa J. 11(1):3073.

Ekelman K. 1993. Microcrystalline wax and paraffin wax. Toxicological Evaluation of Certain Food Additives and Naturally Occurring Toxicants. 30:253–260.

Firriolo JM, Morris CF, Trimmer GW, Twitty LD, Smith JH, Freeman JJ. 1995. Comparative 90-day feeding study with low-viscosity white mineral oil in Fischer-344 and Sprague-Dawley-derived CRL:CD rats. Toxicol Pathol. 23(1):26–33.

Fleming KA, Carrillo JC. 2018. MOH accumulation in F344 rats. Sci Total Environ. 615:1095–1098.

Fleming KA, Zimmerman H, Shubik P. 1998. Granulomas in the livers of humans and fischer rats associated with the ingestion of mineral hydrocarbons: a comparison. Regul Toxicol Pharmacol. 27(1 Pt 1):75–81.

Freeman J, Simpson BJ, Tietze P. 1993. White Oil and Waxes: Summary of 90-day Studies. CONCAWE report 93/56. CONCAWE.

Fujita H, Kamawata S, Yamashita K. 1983. Electron microscopic studies on multicentric foreign body giant cells derived from Kupffer cells in mice given Indian ink intravenously. Virchows Arch B Cell Pathol Incl Mol Pathol. 42(1):33–42.

Griffis LC, Twedt DE, Francke-Carroll S, Biles RW, Schroeder RE, Bolte H, Faust H, Hall WC, Rojko J. 2010. Comparative 90-day dietary study of paraffin wax in Fischer-344 and Sprague-Dawley rats. Food Chem Toxicol. 48(1):363–372.

Groß K. 2018. Toxicological assessment of mineral hydrocarbons in foods: state of present discussions. J Agric Food Chem. 66(27):6969–6974.

Gupta A, Severin D. 1997. Characterization of petroleum waxes by high temperature gas chromatography–chromatophysics with physical properties. Pet Sci Technol. 15(9-10):943–957.

Hall AP, Elcombe CR, Foster JR, Harada T, Kauffmann W, Knipple A, Küttler K, Malareck DE, Maronpot RR, Nishikawa A, et al. 2012. Liver hypertrophy: a review of adaptive (inverse and non-adverse) changes-conclusions from the 3rd International ESTP Expert Workshop. Toxicol Pathol. 40(7):971–994.

Halladay JS, Mackerer CR, Twedt DE, Sipes IG. 2002. Comparative pharmacokinetic and disposition studies of [1-14C]-ecosanoclyclohexane, a surrogate mineral hydrocarbon, in female Fischer-344 and Sprague-Dawley rats. Drug Metab Dispos. 30(12):1470–1477.

Hoglen NC, Regan SP, Hensel JL, Younis HS, Sauer JM, Steup DR, Miller MJ, Waterman SJ, Twedt DE, Sipes IG. 1998. Alteration of Kupffer cell function and morphology by low melt point paraffin wax in female Fischer-344 but not Sprague-Dawley rats. Toxicol Sci. 46(1):176–184.

JECAF 1970. Toxicological evaluation of some extraction solvents and certain other substances: Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives, FAO Nutrition Meetings Report Series, Geneva, 24 June -2 July 1970. FAO Nutrition Meetings Report Series No 48A WHO/FOOD ADD/7039. http://www.inchem.org/documents/jeccaf/jecmono/v48aje08.htm.

JECAF 1974. Toxicological evaluation of certain food additives with a review of general principles and of specifications: seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives, Geneva, 25 June-4 July 1973. Wild Hlth Org tech Rep Ser, 1974, No 539; FAO Nutrition Meetings Report Series, 1974, No 53 WHO FOOD ADDITIVES SERIES NO. 5. http://www.inchem.org/documents/jeccaf/jecmono/v05je84.htm.

JECAF 1976. Twentieth Report of the Joint FAO/WHO Expert Committee on Food Additives, Geneva, 1976. WHO Technical Report Series No 599. FAO Food and Nutrition Series No. 1. WHO Technical Report Series Geneva: World Health Organization. http://www.inchem.org/documents/jeccaf/jecmono/v10je08.htm.

JECAF 1987a. Evaluation of certain food additives and contaminants The 30th Report of the Joint FAO/WHO Expert Committee on Food Additives (JECAF), Geneva 1987 WHO Technical Report Series 751. http://apps.who.int/iris/bitstream/handle/10665/41306/WHO_TRS_751.pdf?sequence=1&isAllowed=y.

JECAF 1987b. Toxicological evaluation of certain food additives and contaminants The 30th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECAF), Geneva 1987 WHO Food Additives Series
JECFA 1989b. Evaluation of certain food additives and contaminants. World Health OrganizationTechnical Report Series Geneva: 776. https://apps.who.int/iris/bitstream/handle/10665/39252/WHO_TRS_776.pdf?sequence=1. (The 33rd meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Geneva, 1989).

JECFA 1992. Evaluation of certain food additives and naturally occurring substances, micro-organisms, petroleum products, soaps and detergents, and metallic compounds. Toxicol Environ Chem. 69(3-4):403–416.

Rocchiccioli F, Lageron A, Duboucher C. 1987. Abnormal n-nonacosane storage in humans: detection by gas chromatography/mass spectrometry of tissue extracts. Biomed Environ Mass Spectrom. 14(9):481–485.

Roussy D. 1988. The challenge of characterizing branching in molecular species. Discrete Appl Math. 19(1-3):317–338.

Salvayre R, Nègre A, Rocchiccioli F, Duboucher C, Maret A, Vieu C, Lageron A, Polonovski J, Douste-Blazy L. 1988. A new human patho-

J. V. Kelsall G, Blackwell J. 1969. The occurrence and significance of lipophage clusters in lymph nodes and spleen. Pathology. 1(3):211–220.

Kissin Y, Feulner G. 1986. Gas chromatographic analysis of alkyl-substituted paraffins. J Chromatogr Sci. 24(2):53–59.

Kollattukudy P, Hankin L. 1966. Metabolism of a plant wax paraffin (n-nonacosane) in the rat. J Nutr. 90(2):167–174.

Kumar S, Nautiyal S, Khan H, Agrawal K, Dimri J. 2005. Composition and properties of some petroleum waxes. Pet Sci Technol. 23(7-8):939–951.

Le Bon A, Cravedi JP, Tulliez J. 1988. Disposition and metabolism of pristane in rat. Lipids. 23(5):424–429.

Lester D. 1979. Normal paraffins in living matter—occurrence, metabolism and pharmacokinetic studies of radiolabelled normal paraffinic, isoparaffins and branched hydrocarbons in BALB/c mice by intraperitoneal injection of pristane. J Exp Med. 180(6):2341–2346.

SCF 1995. Opinion of the Scientific Committee for Food on: Mineral and synthetic hydrocarbons: In Food: SCF (ed) SCF reports: 37th series 25 Sept 1995. http://aeipittedu/40846/1/37th_foodpdf.volhttp://aei.pitt.edu/40846/1/37th_food.pdf.

Schlenegger UP. 1972. Distribution patterns of n-alkanes in human liver, urine and sweat. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism. 260(3):339–344.

Scotter M, Castle L, Massey R, Branton P, Cunninghame M. 2003. A study of the toxicity of five mineral hydrocarbon waxes and oils in the F344 rat, with histological examination and tissue-specific chemical characterisation of accumulated hydrocarbon material. Food Chem Toxicol. 41(4):489–521.

Shubik P. 1992. Toxicology Forum. Special Meeting on Mineral Hydrocarbons. September 21-23, 1992. Toxicology studies prior to 1975. Green College, Oxford, United Kingdom. p. 42–44.

Shubik P, Saffiotti U, Lijinsky W, Pietra G, Rappaport H, Toth B, Raha CR, Tomatis L, Feldman R, Ramahi H. 1962. Studies on the toxicity of petroleum waxes. Toxicol Appl Pharmacol. 4(Suppl):1–62.

Smith J, Bird M, Lewis S, Freeman J, Hogan G, Scala R. 1995. Subchronic feeding study of four white mineral oils in dogs and rats. Drug Chem Toxicol. 18(1):83–103.

Smith JH, Mallett AK, Priston RA, Brantom PG, Worrell NR, Sexsmith C, Simpson BJ. 1996. Ninety-day feeding study in Fischer-344 rats of highly refined petroleum-derived food-grade white oils and waxes. Toxicol Pathol. 24(2):214–230.

Trimmer GW, Freeman JJ, Priston R, Urbanus J. 2004. Results of chronic dietary toxicity studies of high viscosity (P70H and P100H) white mineral oils in Fischer 344 rats. Toxicol Pathol. 32(4):439–447.
Tulliez J, Bories G. 1978. Metabolism of a n-paraffin, heptadecane, in rats. Lipids. 13(2):110–115.

Tulliez J, Bories G. 1975. Metabolism of paraffinic and naphthalenic hydrocarbons in higher animals. I. Retention of paraffins (normal, cyclo and branched) in rats. In: Annales de la Nutrition et de L’alimentation. 29:201–211.

Tulliez JE, Bories GF. 1979. Metabolism of naphthenic hydrocarbons. Utilization of a monocyclic paraffin, dodecyclohexahane, by rat. Lipids. 14(3):292–297.

Weber S, Schrag K, Mildau G, Kuballa T, Walch SG, Lachenmeier DW. 2018. Analytical methods for the determination of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH)-A Short Review. Anal Chem Insights. 13:117739011877757.

Yamashita K, Fujita H, Kawamata S. 1985. Fine structural and cytochemical aspects of granuloma formation derived from Kupffer cells in mice injected with latex particles. Arch Histol Jpn. 48(3):315–326.

Appendix 1

In the section “Metabolism vs Crystallization of n-alkanes” of the present paper, we argued that the ultimate cause of the granuloma formation in the F-344 livers is not that n-alkanes are not metabolized because they crystallize, but rather that due to the absence of an efficient metabolism, their concentration increases, leading to concentrations sufficiently high to trigger crystallization. A key element in making that determination is the identification of a series of iso-alkanes in a comparable mass range of the accumulated n-alkanes. The presence of these iso-alkanes, in oils but not in waxes, was observed by the authors of the EFSA study with the F-344 rat strain (Cravedi et al. 2017). The elucidation of the structural features of these is-alkanes remained, however, inconclusive. Our subsequent discussion and proposal for a possible structure of these iso-alkanes is entirely based on information gleaned from Cravedi et al. 2017, notably on pages 76 and 77 of their report. A first indication of the possible structure is revealed by the author’s observation that “in the first dimension they are eluted earlier than their n-alkanes homologues by an equivalent of 0.8 carbon atoms, which is more than by mono-methyl branching in the positions 2 or 3.” Kissin and Feulmer extensively studied the gas chromatographic retention times various alkyl-substituted paraffins in the C10 to C30 range (Kissin and Feulmer 1986). They noticed that as a single methyl substituent moves more internally in the chain, the molecules elute closer to the (n-1) homologue. In their experiments, for a C27 chain with an “internal methyl substituent, they report a downward shift of 0.7 while for a methyl in the two and 3 position, this is in the 0.25 to 0.35 range respectively.

Especially important in identifying the structure of the accumulating iso-alkane is the mass spectrum of the C27 iso-alkane that was isolated from a F-344 liver exposed to a high viscosity oil and published in Cravedi et al. 2017. We fully subscribe to the author’s assertion that the fragmentation pattern shows no indication of substitution at the end of the chains. Based on the presence of the loss of an ethyl, the authors concluded the absence of branching in position 2 and they also provide convincing arguments to exclude branching at the 3 position. What is very striking, however, is their observation that the mass spectra of the subject iso-alkanes show the pronounced presence of even numbered fragments in the intermediate mass range. The mass spectrum of the C27 iso-alkane and taken from Cravedi et al. 2017 is reproduced in Figure A1.

As can be seen in this figure, there is a peak at mass to charge ratio (m/z) 224, that is slightly more intense than the one at m/z 225.

As it turns out, the presence of even mass numbered fragments in the mass spectra of branched alkanes has a high diagnostic value because important information to interpret the spectrum and is instrumental in identifying the position of the internal methyl. Long chain, internally branched methylalkanes are characterized by the typical cleavage at the branched position and by a fragment at M-15, the molecular ion is often not being visible (Nelson et al. 1972). But in 1968, a study on fragmentation patterns of saturated aliphatic hydrocarbon found that internally branched alkanes may undergo additional fragmentation pathways, for instance involving the equivalent of an hydrogen radical transfer (McCarthy et al. 1968). It was noticed that this hydrogen transfer results in a CnH2n peak whose intensity is greater than that of the corresponding CnH2n-1 peak, when the CnH2n fragment ion contains seven or more carbon atoms in the chain.

This paper proposed a mechanism for the formation of this even mass fragment, and the application of this mechanism to an internally methyl substituted C27 alkane is depicted in Figure A2. As can be seen in this figure rather, than only the straight split between carbons 11 and 12, leading to a fragment with m/z of 225, the transfer of secondary hydrogen from carbon 17 to 11 leads to the formation of n-undecane and the 1-methyl-2-nonylcyclohexane ion. The fact that this 1,2-dialkylcyclohexane ion has an m/z of 224, leads to the conclusion that in the

Figure A1. Mass spectrum (GCxGC-MS) of the C27 iso-alkane belonging to the series of iso-alkanes in liver extract of rats exposed to L-C25, 4000 mg/kg food. (Figure 51; from Cravedi et al. 2017). The inset figure at the upper right is an enlarged part of the main figure. The arrow indicates the peak with the even m/z (mass to charge ratio) of 224. The peaks at m/z -15 and -29 correspond to the loss of respectively methyl or ethyl fragments and in the original report the absence of 2- or 3-methyl branching was concluded on their abundance.
investigated iso-alkane the methyl branching must be located on the 12 position.

While the NIST Mass Spectral database does not contain the mass spectrum of 12-methylhexacosane, the mass spectra of 11-methylheptacosane and 13-methylheptacosane (C28H58) are included. In the spectra of both products, even m/z peaks can be clearly identified.

Therefore, the presence of these even m/z fragments in the mass spectra of the accumulating iso-alkanes provides evidence that these are essentially internally methyl substituted linear alkanes without further methyl groups at either end. It is clear from Figure A2, that an internal methyl group significantly lowers the melting point of a corresponding iso-alkane, and from this perspective it is not surprising that these substances would remain in the oil phase during the de-waxing process. The fact, however, that they reveal to the same lack of metabolism as n-alkanes, while not being prone to crystallization, provides evidence that crystallization is not the ultimate trigger to the accumulation of alkanes in F-344 livers.

Figure A2. Hydrogen transfer mechanism proposed based on 12-methylhexacosane (McCarthy et al. 1968). The curvy line indicates the fragmentation position, the hydrogen transfer occurs from the original carbon atom 17 and the dashed line the ring closure resulting in 1-methyl-2-nonyl cyclohexane and n-undecane.