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white oil and waxes - summary of 90-day studies

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ABSTRACT

CONCAWE has conducted a study on white mineral oils and waxes which are representative of those used for food applications. The aim was to help clarify the mixed results found in other toxicity studies with laboratory animals and the implications from a human health viewpoint.

It was found that microcrystalline waxes and 100 cSt white oil elicited no adverse effects in the 90-day feeding studies carried out. Biological effects were inversely related to molecular weight, viscosity and melting point, but oil type and processing did not appear to be determinants.

The absorption of mineral hydrocarbons and the nature of the occurrences of the biological responses were confirmed and a new effect for paraffin wax has been identified. Further studies are required to elucidate more fully the mechanism for the responses observed.

KEYWORDS

Absorption, animal, health effects, rat, research, toxicity, wax, white oil

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1. INTRODUCTION

While it has been generally accepted that humans absorb some mineral hydrocarbons from food, this has not given cause for concern from a health viewpoint. However, toxicity studies in laboratory animals have yielded mixed results and as a consequence the safety of food grade mineral hydrocarbons has recently come under scrutiny.

The effect of the repeated ingestion of wax was reported by Shubik et al [1] who conducted lifetime feeding studies in rats with a range of petroleum waxes. The authors concluded that waxes did not cause any untoward effects.

With regard to white oil, in one series of tests, no significant effects were reported in rats or dogs following oral intakes for 13 weeks. [2] On the other hand, another laboratory reported evidence of some absorption of mineral oils and inflammatory responses in the liver and lymph nodes of rats following 13 weeks of exposure. [3]

The results of these two series of studies have been previously compared and reviewed. [2] It is of interest that there were significant differences in the protocols used by the two laboratories. For example, there were differences in the dosing regime (gavage versus feeding), strain of rat, dose-levels, etc. Thus, firm conclusions as to the significance of the results could not be made. Nevertheless, the results raised questions of the influence on biological effect of factors such as oil type (naphthenic or paraffinic), refining method (acid treatment or hydrogenation) and viscosity.

CONCAWE therefore conducted a study on materials representative of the full range of materials typically used for food applications. The study included six oil samples selected to cover the variables identified above viz: viscosity, method of refining and oil type. Three waxes representative of those being used in food applications were also included in the study. Follow-up studies included a seventh oil and two additional waxes intended to investigate gaps in the molecular weight ranges of the initial samples.

Samples of liver tissues were taken from the CONCAWE and follow-up studies and were examined in more detail for their hydrocarbon contents.

The purpose of this report is to summarize and review the CONCAWE and follow-up studies. The complete report of the study to examine hydrocarbons in the liver tissue is included as an **Appendix**.

2. MATERIAL CHARACTERIZATION

2.1 WHITE MINERAL OIL

White mineral oils are complex mixtures of saturated hydrocarbons derived from petroleum by distillation and purification to remove aromatics and other impurities. They are composed of two hydrocarbon types: paraffinics - predominantly branched chain alkanes and naphthenics - alkanes containing one or more saturated cyclic structures. The manufacturing process of white oils removes nearly all linear alkanes which are used instead to produce waxes. The chemical composition, that is, the ratio of paraffinics to naphthenics, and the molecular weight determines physical properties of white oils. Many of these properties are important in the applications in which these products are used, and are measured for the purpose of controlling manufacturing processes.

Seven white mineral oils covering the spectrum of products available in the marketplace were included in the 90-day feeding studies summarized in this report. The reference nomenclature, nominal viscosity at 40°C, oil type, and refining method for the oils used in the studies are shown in **Table 1**.

Table 1: Identification of oil samples

CONCAWE REF. NO.*	VISCOCITY AT 40°C (cSt)**	OIL TYPE	METHOD OF REFINING
N10A	10	Naphthenic	Acid Treatment
N15H	15	Naphthenic	Hydrogenation
P15H	15	Paraffinic	Hydrogenation
N70A	70	Naphthenic	Acid Treatment
N70H	70	Naphthenic	Hydrogenation
P70H	70	Paraffinic	Hydrogenation
P100H	100	Paraffinic	Hydrogenation

* This nomenclature reflects the viscosity of the product
** cSt = mm^2/s

The properties of these oils are shown in **Table 2**. Of interest are the spread of viscosity, boiling range and average molecular weight, and the consistently low levels of impurities such as sulfur, and heavy metals. This work and earlier, more detailed chemical investigations carried out by CONCAWE [4] on other samples show that the differences between the various grades of medicinal white oil and their physical properties lie in the molecular weight and type of saturated hydrocarbons present.

Table 2: Properties of tested white mineral oils

TEST	METHOD	N10 (A)	N15 (H)	P15 (H)	N70 (A)	N70 (H)	P70 (H)	P100 (H)
Viscosity at 40°C (mm^2/s)	ASTM D 445	13.3	16.6	15.0	76.4	68.0	69.5	99.8
Viscosity at 100°C (mm^2/s)	ASTM D 445	3.08	3.45	3.52	7.88	7.65	8.56	11
Density at 15°C	ASTM D 4052	857	860	851	894	877	870	874
Pour Point (°C)	ISO 3016	-36	-39	-9	-42	-45	-21	-15
Distillation Range (°C)								
5%	ASTM D 2887	265	295	305	365	380	422	430
50%		365	375	395	425	435	481	495
95%		445	445	450	485	505	541	555
Refractive Index at 20°C	ASTM D 1218	1.4688	1.4700	1.4669	1.4852	1.4787	1.4760	1.4781
Average Molecular Weight	ASTM D 2502	320	330	350	410	420	485	510
Carbon Type, % CN/CP	ASTM D 2140	39/61	40/60	34/66	48/52	38/62	33/67	32/68
Flash Point (°C)	ISO 2592	174	179	186	216	228	264	261
Sulphur Content (mg/kg)	DIN 51400 T7	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Heavy Metals, Total (mg/kg) (Cd, Pb, Ni, Mo, Cr, As, Zn)	ICP Method	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Purity Requirements	European Pharm. II	Pass	Pass	Pass	Pass	Pass	Pass	Pass

The boiling point distribution of the samples was determined by gas chromatographic (GC) analysis (ASTM 2887). These data are presented in Figure 1. The boiling points of saturated hydrocarbons are directly related to molecular weight, thus, the boiling range data provide a good indication of the molecular weight distribution of each sample. Figure 2 depicts the relationship between oil viscosity, boiling range, and molecular weight. This figure shows that the higher viscosity oils boil at higher temperatures than lower viscosity oils and higher viscosity oils have higher molecular weights. It is also known that oil type (paraffinic or naphthenic) influences the viscosity:temperature relationship. Thus, for a given viscosity, a paraffinic oil will have a higher boiling range and molecular weight than a naphthenic oil (compare N70A and N70H with P70H).

Figure 1 Boiling point distribution curves for white oils - part 1

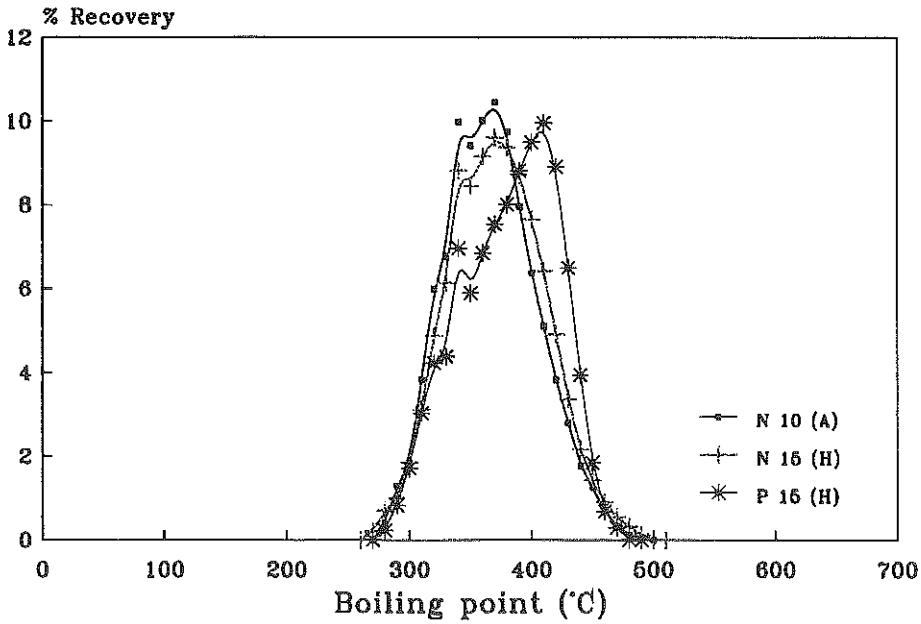


Figure 1 Boiling point distribution curves for white oils - part 2

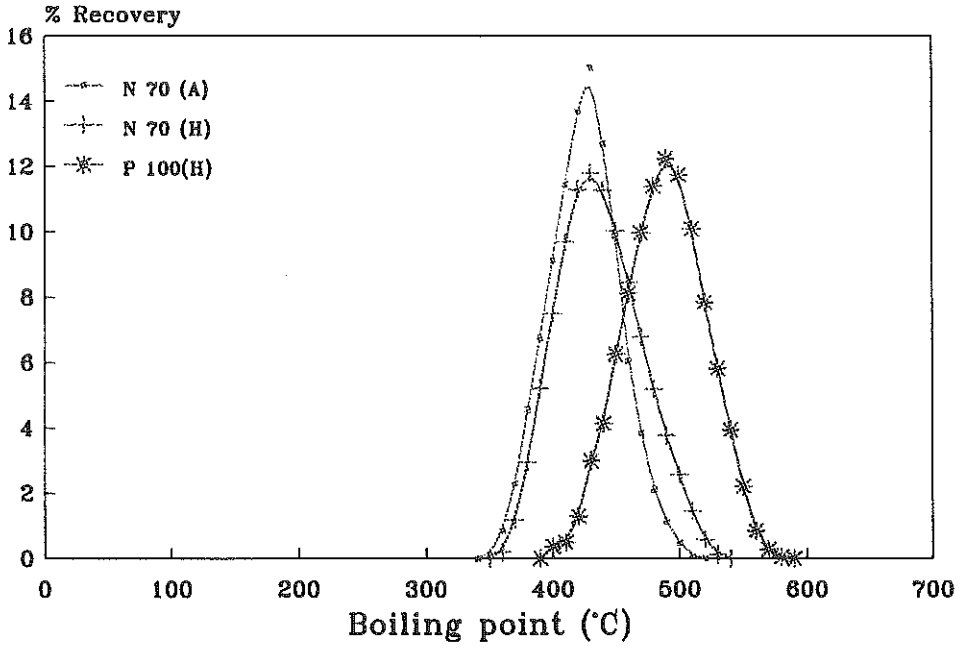
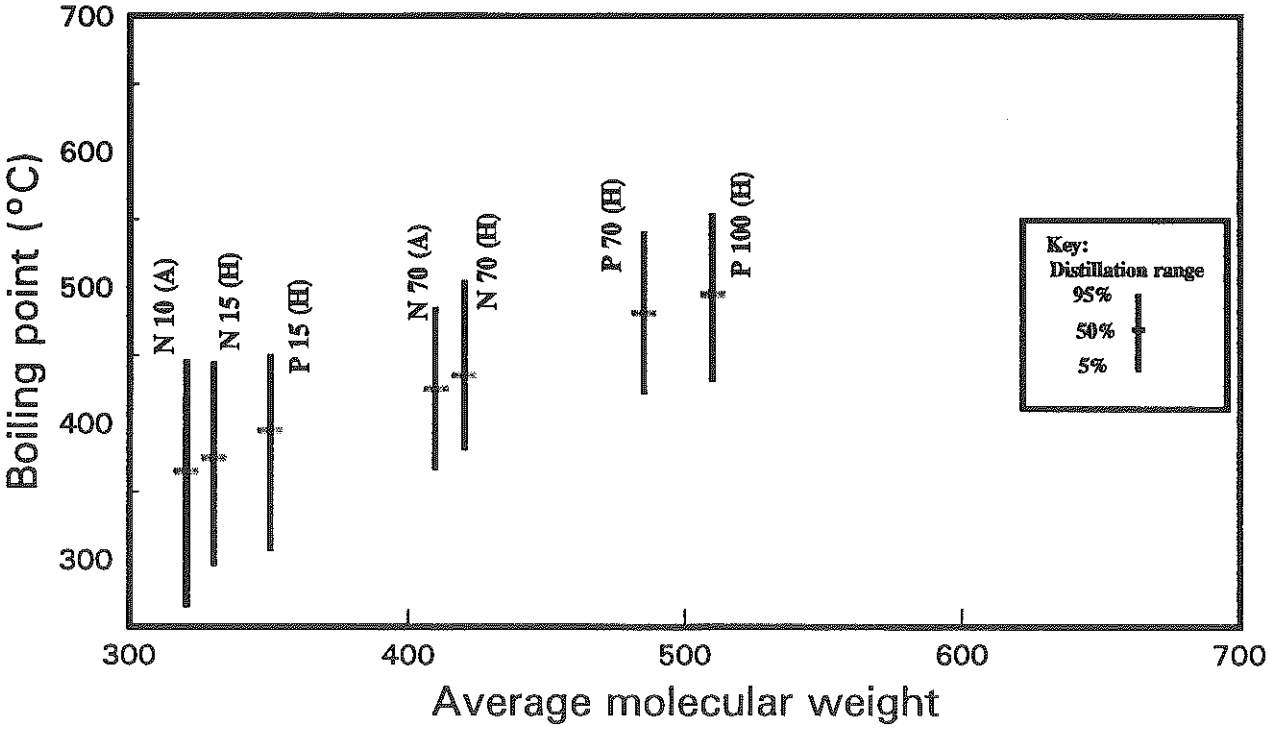


Figure 2 Relationship between viscosity, boiling range & molecular weight for white oils



2.2 PETROLEUM WAXES

COMPOSITION

Food grade waxes are solid hydrocarbons comprising mixtures of linear and branched chain alkanes with lesser amounts of saturated cyclic structures (naphthenics). Similar to white oils, waxes are also derived from petroleum distillation fractions. Wax is separated from these fractions by crystallization. Food grade waxes are produced by further purification of these crystalline components.

Waxes can be classified in order of increasing boiling range or molecular weight range into three broad categories; i.e., paraffin waxes, intermediate waxes, and microcrystalline waxes. As the molecular weight range increases the relative proportion of branched chain alkanes and cyclo alkanes increases. Viscosity also increases with boiling range and molecular weight.

Paraffin Waxes

- Paraffin waxes are derived from light lube oil distillates. They consist predominately of linear alkanes, with varying proportions of branched alkanes and small amounts of cyclo alkanes. Viscosities at 100°C vary from about 3 - 6 cSt.

Intermediate Waxes

- Intermediate waxes are derived from higher boiling distillation fractions and thus have higher average molecular weights than paraffin waxes. They consist of linear and branched chain alkanes with lesser amounts of cyclo alkanes. Viscosities at 100°C vary from about 6 - 10 cSt.

Microcrystalline Waxes

- Microcrystalline waxes are derived from the highest boiling petroleum fractions (vacuum residuum) and have the highest average molecular weights. They have the highest branched and cyclo alkane content of the three wax categories. Viscosities at 100°C vary from about 10 - 30 cSt.

The initial 90-day feeding study included a low melting point paraffin wax produced by hydrogenation (designated LMPW), a high melting point microcrystalline wax produced by hydrogenation (designated HMPW), and a microcrystalline wax produced by percolation. This latter wax had a higher sulfur content than the waxes produced by hydrogenation and was designated HSW. A follow up 90-day feeding study included an intermediate wax produced by hydrogenation (designated IMPW), and a 1:1 blend of the LMPW and HMPW used in the earlier study (designated BLEND). The wax test article designations are summarized in **Table 3**. Properties of the waxes are shown in **Table 4**. Boiling point distribution curves are shown in **Figure 3** and the relationship between molecular weight, and boiling point distribution is shown in **Figure 4**.

Table 3: Identification of wax samples

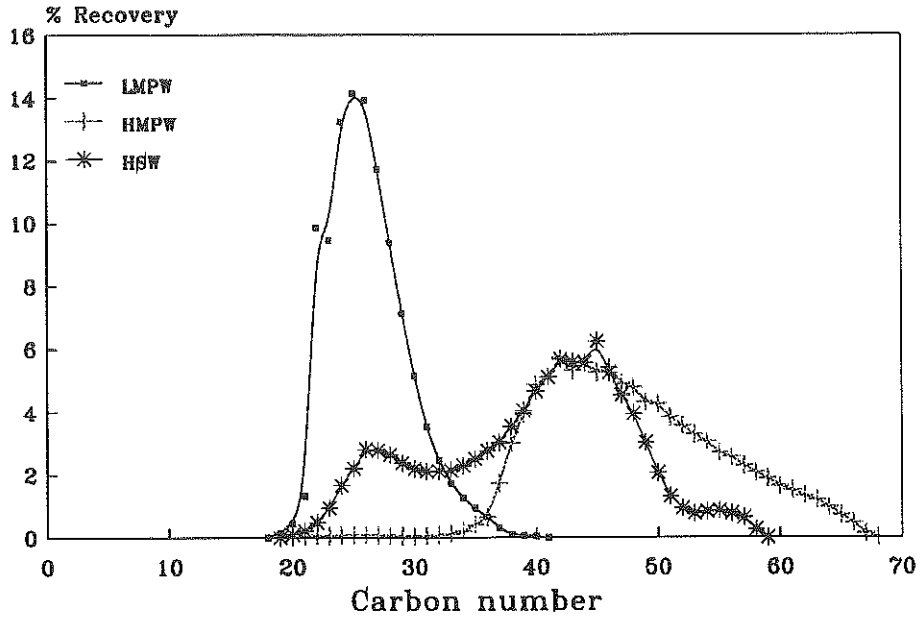
CONCAWE REF. DESIGNATION	WAX TYPE	REFINING METHOD
LMPW	Low melting point, paraffin	Hydrogenation
HSW	Percolated, microcrystalline	Percolation
HMPW	High melting point, microcrystalline	Hydrogenation
IMPW	Intermediate, paraffin	Hydrogenation
BLEND	1:1 LMPW : HMPW blend	Hydrogenation

Table 4: Properties of tested waxes

CONCAWE REF. NO.			LMPW	HSW	HMPW	IMPW	BLEND
PROPERTIES	UNIT	METHOD					
Colour		ASTM D-1500	L0.5	L0.5	L0.5	L0.5	L0.5
Penetration at 25°C	0.1 mm	ASTM D-1321	18	27	13	19	15
Penetration at 40°C	0.1 mm	ASTM D-1321	83	101	29	58	66
Congealing point	°C	ASTM D-938	53.5	74.5	85.0	64.0	76
Drop melting point	°C	ASTM D-127	55.6	82.0	91.4	65.1	83.6
Oil content	%	ASTM D-721	0.1	1.8	1.3	0.5	0.7
Distillation Ranges (°C)		ASTM D-86					
5%			369	411	510	426	373
50%			414	551	564	463	450
95%			467	698	721	546	617
Viscosity at 100°C	mm^2/s	ASTM D-445	3.3	13.7	15.4	6.3	8
Density at 100°C	kg/m^3	ASTM D-1298	751.5	794.4	789.2	773.0	NA
Ash content	%	ASTM D-482	< 0.01	0.01	< 0.01	< 0.001	< 0.01
Refractive index at 100°C		ASTM D-1747	1.4230	1.4404	1.4393	1.4292	NA
Sulphur content	ppm	ASTM D-2622	5	2100	77	10	41
Acidity/alkalinity		USP-XX111	Pass	Pass	Pass	Pass	Pass
UV absorbance		FDA-172.806					
280-289 nm			Pass	Pass	Pass	Pass	Pass
290-299 nm			Pass	Pass	Pass	Pass	Pass
300-359 nm			Pass	Pass	Pass	Pass	Pass
360-400 nm			Pass	Pass	Pass	Pass	Pass
Arsenic	ppm	AAS	< 1	< 1	< 1	< 1	<1
Chromium	ppm	AAS	< 1	< 1	< 1	< 1	<1
Cadmium	ppm	AAS	< 1	< 1	< 1	< 1	<1
Lead	ppm	AAS	< 1	< 1	< 1	< 1	<1
Carbon no. distribution of n-alkane content		EWF GC-method	C19-C42	C20-C74	C22-C80	C21-C49	C19 -C80
Non-normal paraffin content	%	EWF GC-method	11	52	28	44	NA

NA: Not Available

Figure 3 Carbon number distributions for waxes



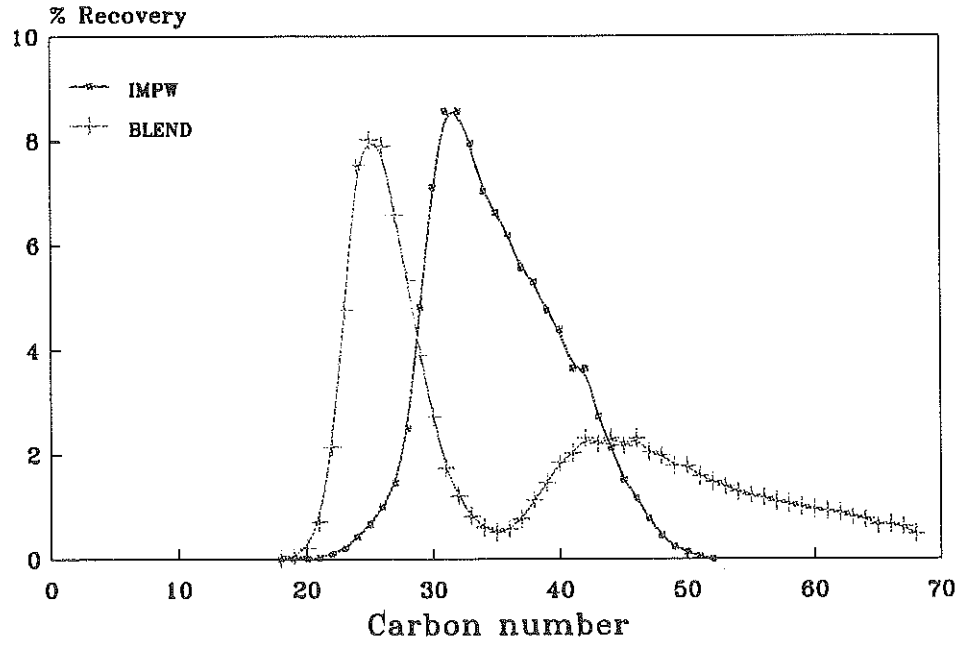
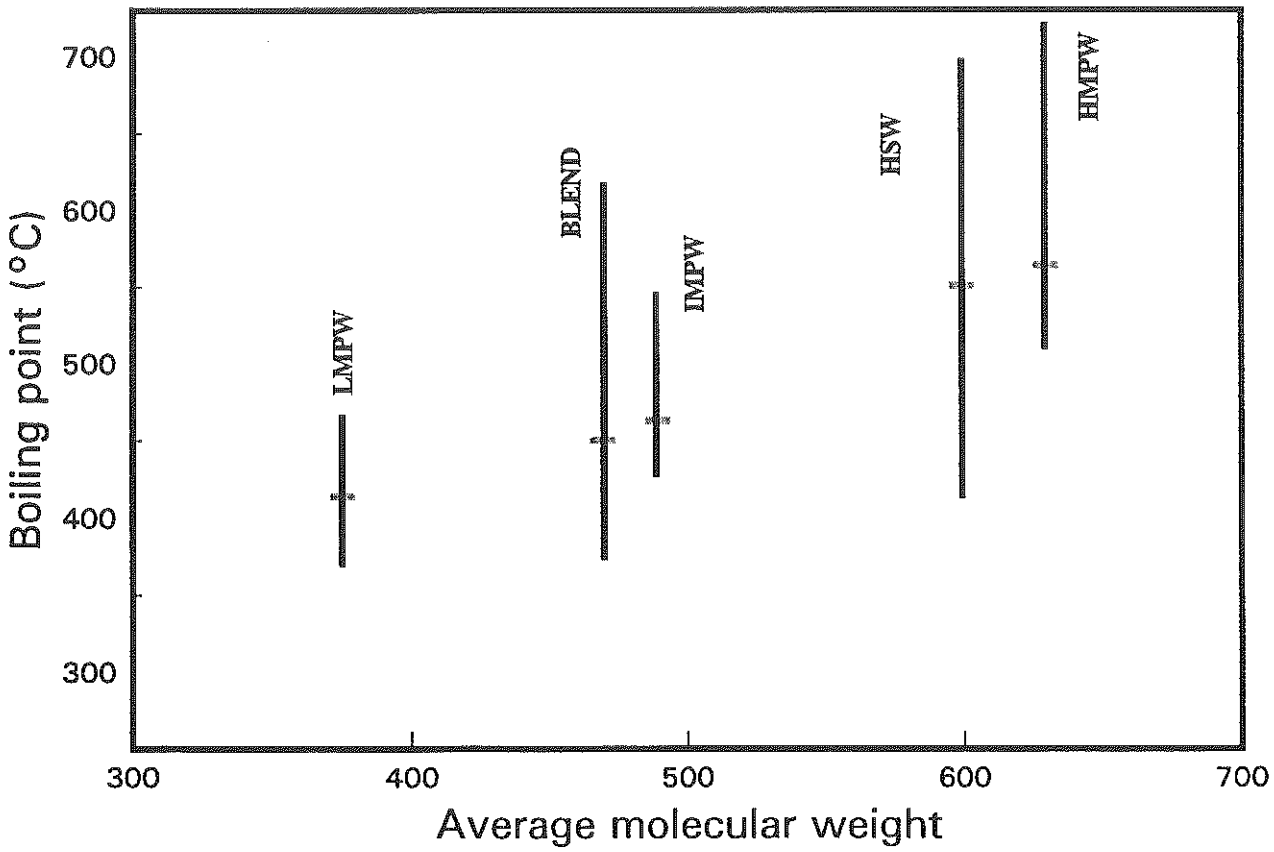


Figure 4 Relationship between viscosity, boiling range & molecular weight for waxes



3. STUDY OUTLINE

The seven white oil and four wax samples were tested in a series of three studies conducted at BIBRA Toxicology International (UK). [5,6,7] All three studies used similar protocols (Table 5).

Table 5: Outline of BIBRA study protocols

STUDY	SAMPLES	DOSE LEVELS	EXPERIMENTAL PARAMETERS
1	N10(A), N15(H), P15(H), N70(A), N70(H), P100(H), LMPW, HSW, HMPW,	0.002%, 0.02% 0.2%, 2.0%	Organ weights, histopathology, haematology clinical chemistry, serum vitamin E levels, tissue hydrocarbon levels, 30-day reversibility
2	P70(H)	0.002%, 0.02%, 0.2%, 2.0%	Organ weights, histopathology, haematology clinical chemsitry, tissue hydrocarbon levels, no reversibility
3	IMPW, BLEND	0.02%, 0.2% 2.0%	Organ weights, histopathology, haematology, clinical chemistry, tissue hydrocarbon levels, 90-day reversibility

The dietary concentrations (ranging from 20-20 000 ppm) were selected on the basis of those used in previous studies. [3] The diets were prepared by mixing test material with diet to yield a 100 000 ppm oil mixture. This was then blended with further diet to give a 20 000 ppm dietary mixture. The lower dietary concentrations of 2000, 200 and 20 ppm were produced by further serial dilution with fresh diet. The mineral hydrocarbon concentrations in each dilution were confirmed analytically. Fresh batches were prepared weekly.

Four week old male and female Fischer 344 rats were used for the study. This rat strain was chosen because effects were noted in it in previous studies [3] of white mineral oils. The study consisted of a 90-day treatment period. Group sizes for each tested hydrocarbon were 20 animals/sex/group (60/sex for the control group of Study #1). Several supplementary groups were also included in the study design. At 90 days, selected tissues were collected from groups of 5 animals/sex/group for analysis for mineral hydrocarbons. Studies of reversibility included 10 animals/sex/group (30 control animals/sex in Study #1) which were removed from diet containing white oil/wax and maintained on control diet for an additional period of time (30 days in Study #1, 90 days in Study #3). Tissue analyses were also conducted at the end of the reversibility period in groups of 5 animal/sex/group (including control).

All animals were examined by necropsy and organ weights were determined for liver, mesenteric lymph nodes, heart, kidney, ovaries, testes, spleen, thymus, adrenal glands, brain and the caecum (with and without contents). Haematology and clinical chemistry analyses were conducted. Serum vitamin E levels were determined in the high dose groups of Study #1. In those animals selected for tissue analyses, mineral hydrocarbon concentrations were determined in liver, mesenteric lymph nodes, spleen and kidneys. Microscopic examinations were performed on an extensive list of haematoxylin and eosin stained tissue sections, including those organs identified in previous studies [3] to be target organs: liver, mesenteric lymph nodes, spleen and kidney.

Samples of liver tissue were collected from a limited number of animals, homogenized and analyzed by gas chromatography. The data were converted to boiling point distributions, and in the case of waxes, separated into relative contents of normal and non-normal paraffins.

4. RESULTS OF STUDY

No deaths or other overt clinical effects were noted in any of the treatment groups. Growth, food consumption, behavior and appearance were all normal. Three of the test compounds, P100(H), HMPW and HSW, did not elicit any treatment-related histopathological effects nor consistent patterns of other effects. P70(H) elicited no treatment-related histopathological effects, although there was some evidence of mineral hydrocarbons in liver and lymph nodes, with associated changes in organ weight.

The histopathologic effects noted following treatment with the other waxes and white oils were qualitatively similar to one another, for the most part, but differed in magnitude depending on the nature of the materials. The rank order for the white oils was: low viscosity oils (10-15 cSt) > intermediate viscosity (70 cSt) naphthenic oils. The rank order for the waxes was: LMPW > IMPW and BLEND. LMPW elicited the most marked effects of any of the oil or wax samples. Generally, the effects were more severe in female than in male animals. Treatment-related effects in the liver and mesenteric lymph nodes were similar to those previously reported.

When observed, the liver effects consisted of increased organ weight, small increases in serum liver enzyme levels, the presence of mineral hydrocarbons, and the development of granulomatous changes. Granulomatous changes were observed at the 2% dietary level in the N10 (A), N15 (H), P15 (H), N70 (A), and N70 (H) white oil groups and at the 0.2% level in the LMPW, IMPW and BLEND groups. (**Table 6**). When observed, effects found in the mesenteric lymph nodes were characterized by an increase in tissue weight, the presence of mineral hydrocarbons and an increased incidence of histiocytosis. This increased incidence of histiocytosis was observed at the 0.02% dose level for N10 (A), P15 (H), N70 (H), IMPW, BLEND, and at the 0.002% level for N15 (H) and LMPW (**Table 6**).

The high doses of some of the test compounds resulted in increased kidney and spleen weight. No histopathological findings were associated with the increased organ weight.

An unusual inflammatory lesion of the mitral valve of the heart was observed in the groups given LMPW, IMPW, and BLEND. There was no significant incidence of this lesion in animals receiving any of the other treatments. The lesion was confined to the mitral valve and was not associated with the myocardium.

A spectrum of additional treatment-related effects were often noted in groups with histopathological changes in the liver and mesenteric lymph nodes. These effects were usually in the higher dose groups and often relatively small in magnitude. Haematological changes included a small decrease in red blood cell count in female animals, sometimes accompanied by slight reticulocytosis, and an increase in white blood cell count in both sexes. The clinical chemistry results showed raised serum enzyme levels (i.e., alanine amino transferase, aspartate amino transferase, and gamma glutamyl transferase). There was a slight but statistically significant decrease in alkaline phosphatase in a number of treatment groups in both the males and females. Glucose levels were significantly higher than the controls in all but the highest dose of many of the female treatment groups. Males showed a more limited range of differences than females. Many of these findings appear to be consistent with a relatively mild inflammatory reaction.

Serum concentrations of vitamin E were assessed in the high dose groups of Study #1. Vitamin E levels were significantly decreased in all the white oil treatment groups and the male HMPW group. In contrast, vitamin E levels were significantly increased in both males and females receiving LMPW.

Over the 30 day recovery period in Study #1, only a slight reduction in the severity of the histopathological changes in the lymph nodes and liver were found following the administration of four compounds (LMPW, and white oils N10 (A), P15 (H), and N15 (H)). The histopathological results obtained following the administration of the other compounds were either unchanged or increased at the end of the reversal period. A decrease in the hepatic mineral hydrocarbon material was found following the one month reversal period. Little reduction was found in the mesenteric lymph nodes or in the kidneys. The other treatment related effects also showed no consistent pattern of change over this period. In Study #3, the 90 day recovery period resulted in a marked decrease in the severity of the histopathologic effects with associated reductions in hematological and clinical chemistry findings. The level of hydrocarbons present in the tissues was also reduced.

Table 6: Lowest observed effect levels for key findings from CONCAWE 90-days toxicity study of white oils and waxes in Fischer 344 rats

	LIVER			MESENTERIC LYMPH NODE			
Test Article	Granulomatous	Organ Wt	MHC [1,2]	Histicytosis	Organ Wt [2]	MHC [1,2]	Heart Mitral Valve
N10(A)	2.0 (F) [3]	0.2 (M,F)	2.0 (M,F)	0.2 (M) 0.02 (F)	2.0 (M,F)	2.0 (M,F)	--
N15(H)	2.0 (F)	2.0 (M) 0.2 (F)	2.0 (M,F)	0.002 (M) 0.02 (F)	2.0 (M,F)	2.0 (M,F)	--
P15(H)	2.0 (F) [4]	0.2 (M) 0.02 (F)	2.0 (M,F)	0.02 (F) 0.002 (M)	2.0 (M,F)	2.0 (M,F)	--
N70(A)	2.0 (F)	0.02 (F)	2.0 (M,F)	0.2 (M) 0.02 (F)	2.0	2.0 (M,F)	--
N70(H)	2.0 (F)	2.0 (F)	2.0 (M,F)	0.2 (M) 0.02 (F)	2.0	2.0 (M,F)	--
P70(H)	--	0.2 (F)	2.0 (M,F)	--	--	2.0 (M,F)	--
P100(H)	--	--	2.0 (M,F) [6]	--	--	2.0 (M,F) [6]	--
LMPW	0.2 (M,F)	0.2 (M,F)	2.0 (M,F)	0.02 (M) 0.002 (F)	2.0 (M,F)	2.0 (M,F)	2.0 (M) 0.2 (F)
IMPW	0.2 (M,F)	0.2 (M,F)	2.0 (M,F)	0.02 (M,F)	0.02 (M,F)	2.0 (M,F)	-- [5]
BLEND	0.2 (M,F)	0.2 (F) 2.0 (M)	2.0 (M,F)	0.02 (M,F)	0.2 (M,F)	2.0 (M,F)	2.0 (M,F)
HMPW	--	--	--	--	--	--	--
HSW	--	--	--	--	--	--	--

-- No effect observed
(1) Mineral hydrocarbons measurable in tissues
(2) Evaluated only at 2.0% dietary level
(3) Dietary level (and sex) associated with effect
(4) Observed in reversal group only
(5) Present but not statistically significant ($P > 0.05$)
(6) Statistically significant ($P \leq 0.05$) but numbers were highly variable and therefore uncertain

5. DISCUSSION

The purpose of the studies was to examine the effects to both sexes of Fischer 344 rats of dietary administration of a range of mineral oils and waxes. The samples varied by oil type, processing method and viscosity. The waxes were selected to allow a comparison between high or low melting point paraffin waxes and hydrogenated versus clay-treated microcrystalline waxes.

The mineral hydrocarbons examined in this study varied widely in their biological effects. Three materials, the 100 cSt oil and the two microcrystalline waxes showed no effects. The results of the study were insensitive to some variables (acid treatment versus hydrogenation, crude type). However, the effects were inversely related to the original study parameters: oil viscosity and wax melting point. Average molecular weight and viscosity at 100°C also were well correlated to observed effects.

The correlation with average molecular weight is supported by the results of the study with P70(H), a paraffinic 70 cSt hydrotreated oil. [6] This oil had a viscosity at 40°C similar to that of N70(A) and N70(H) but had a higher average molecular weight then either of these oils (485 vs 410 or 420). The P70 (H) did not cause either liver granuloma or a dose-dependent increase in histiocytosis in the mesenteric lymph nodes at any of the dose levels tested up to and including a dietary concentration of 2%. The molecular weight relationship was also evident in the follow-up study with IMPW and BLEND. [7] Thus, IMPW and BLEND, with higher average molecular weights (489 and 500) than LMPW (375), elicited fewer and less marked effects than LMPW.

Viscosity at 100°C is a measure common to both oil and wax and is affected by molecular weight as well as molecular size and shape. Viscosity at 100°C is highly correlated with effect. No biological response occurred with material with 100°C viscosities of 8.5 cSt or greater, and little or no mineral hydrocarbon was detected with materials with viscosities at 100°C of 11 cSt or greater.

Analysis of hydrocarbon residues in rat livers indicate that different size hydrocarbons are detected to different degrees in the livers of F344 rats. These analyses, conducted as adjuncts to Studies #1 and #3 (attached as an **Appendix**) suggest that the larger hydrocarbons are not well absorbed from the intestines; hydrocarbons with carbon chain sizes of $\geq C_{35}$ were not identified in rat liver. Also, the smaller hydrocarbons appear to be metabolized and eliminated from liver tissue. This is evidenced with waxes by the low persistence in liver of hydrocarbons with carbon chain lengths of less than approximately C_{23}. These liver analyses also suggest that n-alkanes and iso/cyclo-alkanes have different absorption/distribution/metabolism kinetics. This is evidenced by changes in hydrocarbon distributions in rat liver compared to the parent hydrocarbon material. However, this requires further study.

In general, most of the findings appear to be consistent with a mild inflammatory reaction in the target organs.

The effects noted with some white oils and waxes in Fischer 344 rats is not believed to be significant for human health. These materials have been used in food applications for many years without apparent adverse effects on human health. At Toxicology Forums held in Oxford UK (September 1992) and Washington D.C. (February 1993) it was noted that mineral hydrocarbons and nonreactive granulomatous lesions have been found in human tissues. However, these are believed to be nonsignificant to human health. [8,9] The mitral valve effect is unusual and documented to occur only in the Fischer 344 rat. None of these effects are thought to be precancerous. Neither cancer nor other noteworthy effects have been noted in chronic toxicity studies of refined mineral

hydrocarbons. [1] Significant species/strain differences in response may exist, as Sprague-Dawley and Long-Evans rats and beagle dogs appear to be less sensitive than the Fischer rat. [2,7] A recent, yet unpublished, study of P15(H) in Fischer 344 and Sprague-Dawley rats demonstrated that in the Fischer rat greater levels of mineral hydrocarbon in tissues were detected and this ellicited a greater response than was observed in the Sprague-Dawley. [10] Thus, the findings in the Fischer rat may not be a true predictor of human response. Further studies may help elucidate this.

6. CONCLUSIONS

- Microcrystalline waxes and 100 cSt white oil elicited no adverse effects in 90-day feeding studies in Fischer 344 rats. With a 70 cSt paraffinic white oil, there was evidence of mineral hydrocarbon in tissues, but no histopathologic effects.

- Biological effects were inversely related to molecular weight, viscosity, boiling range and melting point. Viscosity at 100°C correlated well with the effects of both oils and waxes.

- Oil type (naphthenic/paraffinic) and processing history (acid treatment/hydrogenation) did not appear to be determinants of this biological response.

- The absorption of mineral hydrocarbons and occurrence of inflammatory responses in the mesenteric lymph nodes (histiocytosis) and liver (granuloma) were confirmed to occur in Fischer 344 rats.

- An inflammatory effect on the heart mitral valve by paraffin wax represents a new finding.

- Further studies are required to elucidate more fully the mechanism for the responses observed. Such studies would allow the identification of these molecular types responsible for the biological responses observed.

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5. BIBRA (1992) A 90-day feeding study in the rat with six different mineral oils (N15(H), N70(H), N70(A), P15(H), N10(A) and P100(H), three different mineral waxes (a low melting point wax, a high melting point wax and a high sulphur wax) and coconut oil. BIBRA Project No: 3.1010. Carshalton, Surrey: Bibra Toxicology International

6. BIBRA (1993) A 90-day feeding study in the rat with P70(H). BIBRA Project No: 3.1195. Carshalton, Surrey: Bibra Toxicology International

7. BIBRA (1993) A 90-day feeding study in the rat with two mineral waxes identified as paraffin wax 64 (OFH-064) and micro/paraffin wax mixture. BIBRA Project No: 3.1205. Carshalton, Surrey: Bibra Toxicology International

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9. Toxicology Forum (1993) 1993 Annual Winter Meeting. February 15-17, 1993, Capitol Hilton, Washington DC. Washington DC: The Toxicology Forum. p 166-196

10. EXXON Biomedical Sciences (1993) 90-Day dietary study in the rat. Test article P 15(H) White Oil (EC 7404) MRD-92-458. Project No. 145870A. East Millstone NJ: EXXON Biomedical Sciences

APPENDIX

Analysis of Hydrocarbon Residues in Rat Livers

BIBRA first study (1)
(white oils and waxes)

Concawe/EWF

BIBRA second study (2)
(waxes)

EWF

September 1993

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CONTENTS :

INTRODUCTION

Two 90-days feeding studies in the rat at BIBRA Toxicology International showed a variety of biological effects for the different oils and waxes. The effects were more pronounced with the low melting point wax and oils with lower viscosities. It has been postulated that a preferential uptake of mineral hydrocarbons (MHC) within a certain molecular weight range might explain these differences. In all cases the effects in female rats were more pronounced and showed at lower diet levels.

The purpose of this study was to determine differences in the molecular weight distribution of the hydrocarbons, extracted from the female rat livers (referred to as the "main"-group) and the original test samples, by means of a gaschromatographic method.

Also, an analysis was carried out on the livers of rats that were subjected to a recovery period (28 days for the first Bibra study), following the 90 day feeding period. The composition of the "reversal" group extracts were compared with those of the original materials.

In the second 90-days feeding study, other waxes were tested. The purpose of this study was to follow the reversibility over a longer (90 days) withdrawal period and to study the effects of the two waxes in comparison to the earlier tested ones.

METHODS

1. Tissue Extraction Procedure:

1.1 BIBRA (First study)

Extraction was carried out by BIBRA Toxicology International, Surrey, England. Project Number No. 3.1010, Report Number 1010/3/92.

STEP 1
Tissues were homogenised in 70% KOH using an Ultraturrax blade homogeniser. Approximately 1 gram of tissue was cut from the periphery of the liver, homogenised and used for the tissue analysis. If repeat analysis was necessary, a further portion was cut away, homogenised and analysed. The samples supplied for GLC analysis were prepared from tissue remaining after storage at -10 °C since necropsy.

STEP 2
The homogenates or aliquots of homogenate from step 1 were extracted into 20 ml carbontetrachloride at 60°C in an ultrasonic bath for 30 minutes. After centrifugation, 8 ml aliquots were passed through columns of partially deactivated Florisil, collecting to a final volume of 10 ml. This extract was then analysed by FTIR for mineral hydrocarbon content.

STEP 3
The extracts obtained in step 2 were then evaporated to dryness under nitrogen in the containers for transport.

1.2 BIBRA (second study)

Extraction was carried out by BIBRA Toxicology International, Surrey, England. Project Number No. 1205/1/93.

STEP 1
The entire liver-sample was homogenised in 70% KOH using an Ultraturrax blade homogeniser, using 3 ml KOH for each whole gramme of tissue. The volume of the homogenate was measured and a 3 ml aliquot taken for extraction, column clean-up, analysis and to provide the samples for GLC analysis.

STEP 2
The homogenates or aliquots of homogenate from step 1 were extracted into 20 ml carbontetrachloride at 60°C in an ultrasonic bath for 30 minutes. After centrifugation, 8 ml aliquots were passed through columns of partially deactivated Florisil, collecting to a final volume of 10 ml. This extract was then analysed by FTIR for mineral hydrocarbon content.

STEP 3
The extracts obtained in step 2 were pooled in groups of five animals and evaporated in total.

2. Samples and their data

2.1 Treatment groups

All samples were obtained from female animals in the designated tissue level groups, receiving either control diet or 2% w/w test article in diet for 90 days. (Reversal group received control diet for the reversal period).

2.2 Test articles

See Concawe report for description of test articles in both studies.

2.3 Sample data

From the BIBRA-studies extracts of liver tissue were analysed. The samples were received from BIBRA with the following data:

BIBRA study Nr.:	Animal-Number:	Group:	[MHC] in liver /animal (μg/g):	Test Article:
1	1010/1971	Main	319	Control
1	1010/1979	Reversal (28 d)	408	Control
1	1010/1981	Main	4017	N15(H)
1	1010/1989	Reversal (28 d)	3055	N15(H)
1	1010/1993	Main	3789	N70(H)
1	1010/1996	Reversal (28 d)	3181	N70(H)
1	1010/2004	Main	8474	N70(A)
1	1010/2006	Reversal (28 d)	2002	N70(A)
1	1010/2011	Main	5937	P15(H)
1	1010/2017	Reversal (28 d)	2812	P15(H)
1	1010/2022	Main	5090	N10(A)
1	1010/2028	Reversal (28 d)	1923	N10(A)
1	1010/2034	Main	1253	P100(H)
1	1010/2039	Reversal (28 d)	595	P100(H)
1	1010/2041	Main	14846	LMPW
1	1010/2047	Reversal (28 d)	5083	LMPW
1	1010/2055	Main	193	HMPW
1	1010/2057	Reversal (28 d)	199	HMPW
1	1010/2064	Main	195	HSW
1	1010/2069	Reversal (28 d)	564	HSW
2	1205/741-745	Main	63	Control
2	1205/401-405	Reversal (90 d)	36	Control
2	1205/356-360	Main	3680	LMPW
2	1205/431-435	Reversal (90 d)	539	LMPW
2	1205/346-350	Main	2231	IMPW
2	1205/411-415	Reversal (90 d)	402	IMPW
2	1205/351-355	Main	2140	MP
2	1205/421-425	Reversal (90 d)	177	MP

3. Gaschromatographic Analysis

3.1 Sample treatment

The samples received from BIBRA were stored at -20°C. For chromatographic analysis the residues were dissolved in iso-octane p.a.

3.2 Gaschromatographic conditions:

	Oil Analysis	Wax Analysis
Gaschrom.	Perkin Elmer 8700	Carlo-Erba HRGC-5300
Inj.method	Split/splitless (1:10)	Cold-on-Column
Carrier gas	Nitrogen 5 psi	Helium 2ml/min
Sample size	0,1 μl , 2%	0,1 μl , 0,2%
Detector	FID	FID
Column	WCOT Fused Sillica 10 mtr x 0,32 mm ID Coating: SIMDIST DF: 0,1	WCOT Fused Sillica 25 mtr x 0,25 mm ID Coating: CP-SIL 5CB DF: 0,25
Injector temp.	320°C.	110°C.
Detector temp.	400°C.	370°C.
Temp.-Program	70-350°C: 10°C/min	110-220°C: 20°C/min 220-320°C: 10°C/min 320-370°C: 5°C/min
Final Time	25 minutes	30 minutes
Rt Internal Std.	Octadecane	Octadecane

3.3 Conversion of chromatograms

In the case of oils, raw gaschromatographic data were converted to boiling point distributions (BPD), using ASTM D-2887.

The waxes were analysed following the EWF-method (determination of n- and iso-paraffins content and their carbon number spread by cold-on-column capillary gas chromatographic method).
In this method, the total carbon distribution of the wax is expressed as normal- and iso-percentage (iso has to be read as non-normal, mainly branched paraffins). For comparison's sake the carbon number distribution of the waxes was in some cases converted to BPD, using the (expanded) ASTM D-2887 conversion table (appendix).

RESULTS

Chromatograms of liver extracts of the control(s), reversal P100 (H), main and reversal HMPW as well as main and reversal HSW, showed no wax or oil profile (figure 8: two typical examples) and represent very low residual weights compared to the others. For this reason these chromatograms were not converted to BPD's or used to correct the other BPD's for background level. All the other chromatograms were converted to BPD's or carbon distributions and can be found in the following figures:

FIRST STUDY:

Fig.1-6	Oils	:	For each oil the BPD is compared with the BPD of the hydrocarbon residue in the liver of the main and reversal group
Fig. 7	LMPW	:	BPD is compared with the BPD of the hydrocarbon residue in the liver of the main and reversal group
Fig. 8		:	Chromatograms of control and HMPW (main), showing no oil or wax profile
Fig.9-10		:	Deviation plots main minus original makes differences in composition more visible. Negative values mean underrepresentation of fractions and positive values overrepresentation. The correlation with carbon numbers is given

In case of wax, chromatograms give more detailed information than just a boiling point distribution (as can be seen in the following figures):

Fig. 11	LMPW	:	Carbon distribution
Fig. 12	LMPW	:	Deviation plot main minus original, which shows not only changes in carbon distribution but also in ratio normal/iso paraffins
Fig. 13	LMPW	:	Deviation plot reversal minus original and boiling point correlation

SECOND STUDY:

Fig. 14	LMPW	:	Carbon distribution
Fig. 15	LMPW	:	Deviation plot main minus original
Fig. 16	LMPW	:	Deviation plot reversal minus original
Fig. 17	IMPW	:	Carbon distribution
Fig. 18	IMPW	:	Deviation plot main minus original
Fig. 19	IMPW	:	Deviation plot reversal minus original
Fig. 20	MP	:	Carbon distribution
Fig. 21	MP	:	Deviation plot main minus original
Fig. 22	MP	:	Deviation plot reversal minus original

SUMMARY AND CONCLUSIONS

Based on analysis of liver tissue from single individual animals (BIBRA 1) and single samples of pooled liver extracts of five animals (BIBRA 2), the following qualitative observations are made:

1. No hydrocarbons are deposited in livers of animals fed with microcrystalline waxes.
2. Lower and/or higher molecular weight fractions are underrepresented in the liver extracts. Moreover it is concluded that no hydrocarbons with carbon numbers over C35 (boiling point 491°C) are passing the intestinal membranes.
3. In liver extracts from the 28 day-reversal group a substantial reduction in quantity of the deposited hydrocarbons was observed.
   After 90 days reversal period the deposits have further decreased.
   In these residues after 28 days and, more pronounced, after 90 days, the higher molecular fractions are overrepresented in comparison with the main group.
4. For wax:
   the isoparaffin content of the residues is relatively high compared to the original test material. This suggests a difference in kinetics for normal and non-normal alkanes in absorption, distribution or metabolism in the rat liver.

Mineral Hydrocarbon Study

White Oil P15(H)

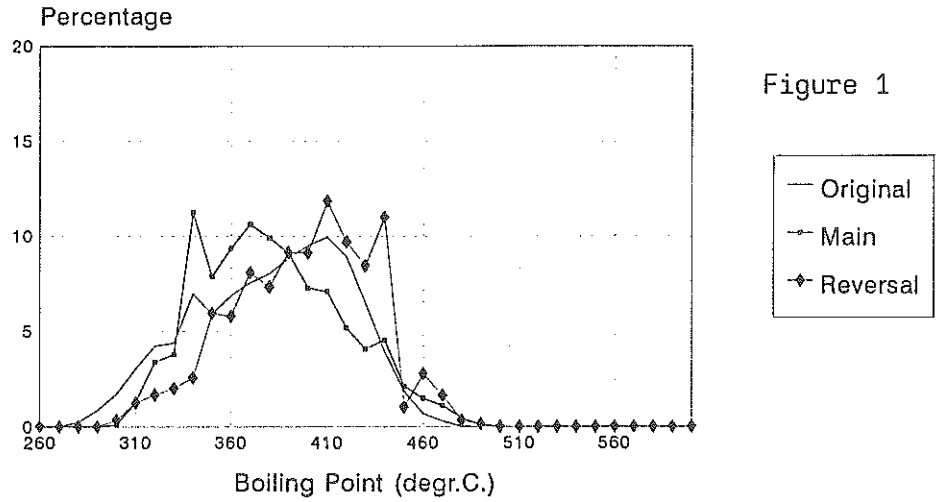


Figure 1

White Oil N10(A)

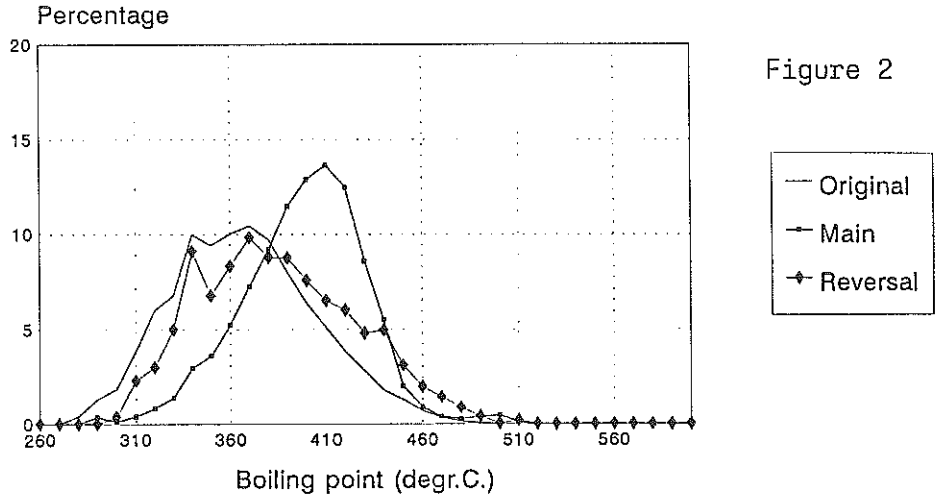


Figure 2

White Oil N15(H)

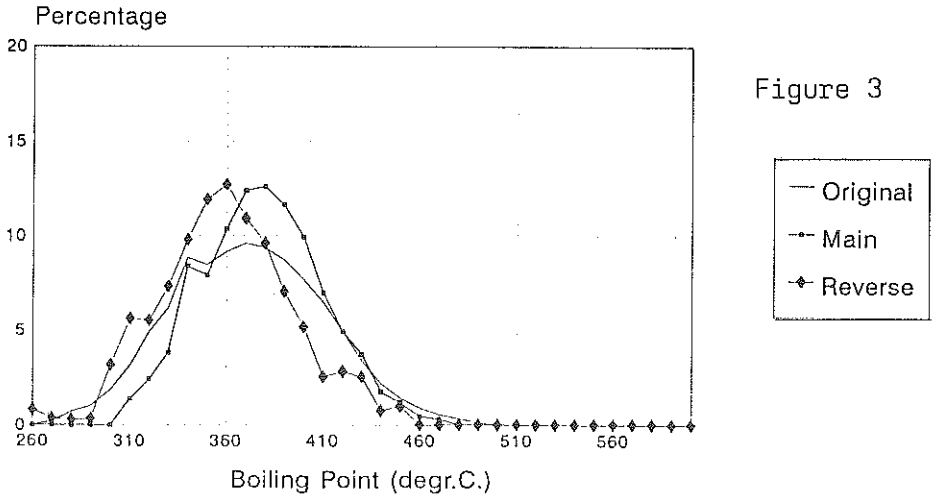


Figure 3

Mineral Hydrocarbon Study

White Oil N70(H)

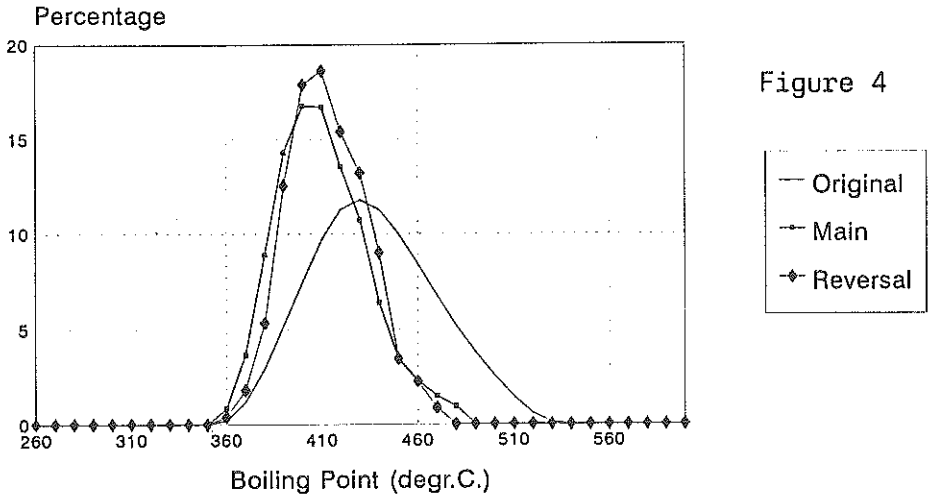


Figure 4

White Oil N70(A)

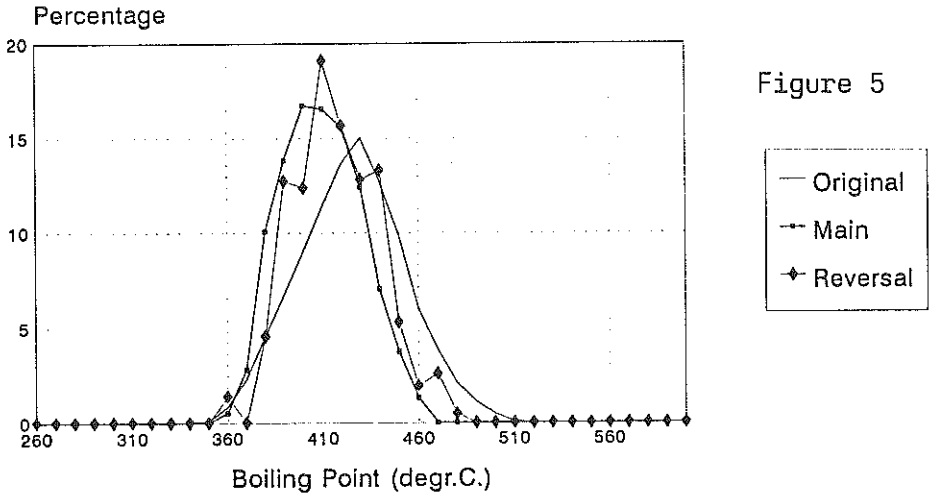


Figure 5

White Oil P100(H)

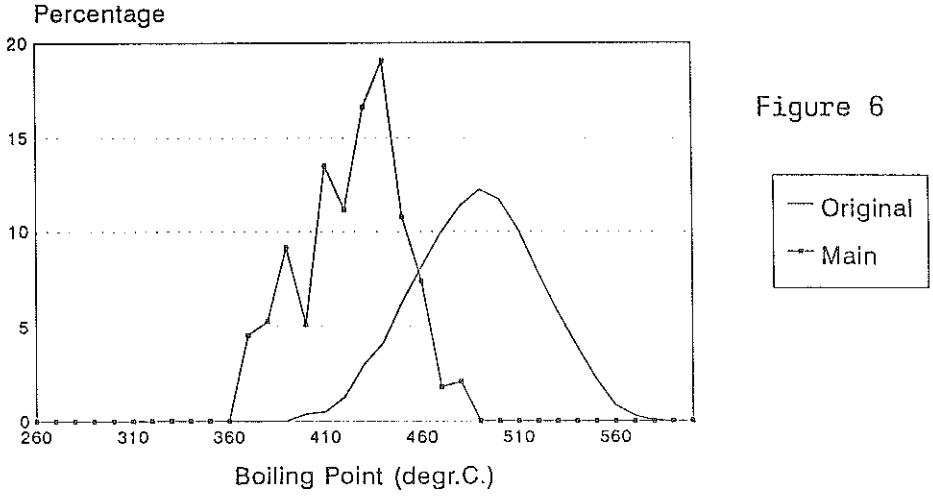


Figure 6

Mineral Hydrocarbon Study

LMPW

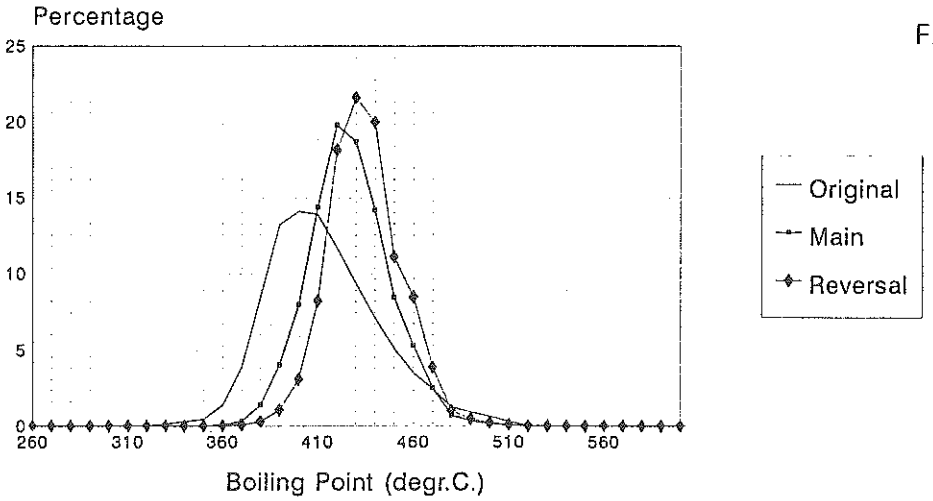


Figure 7

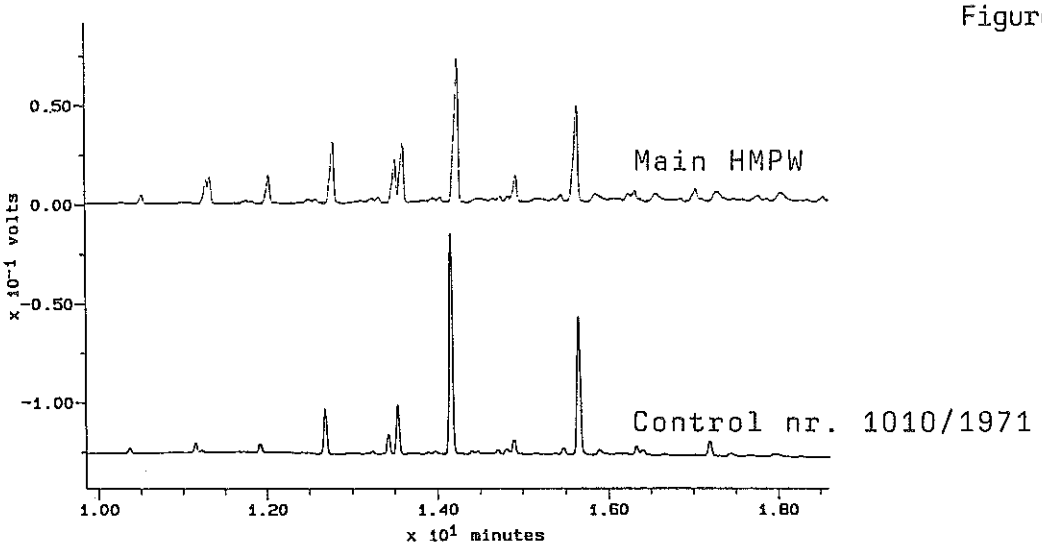
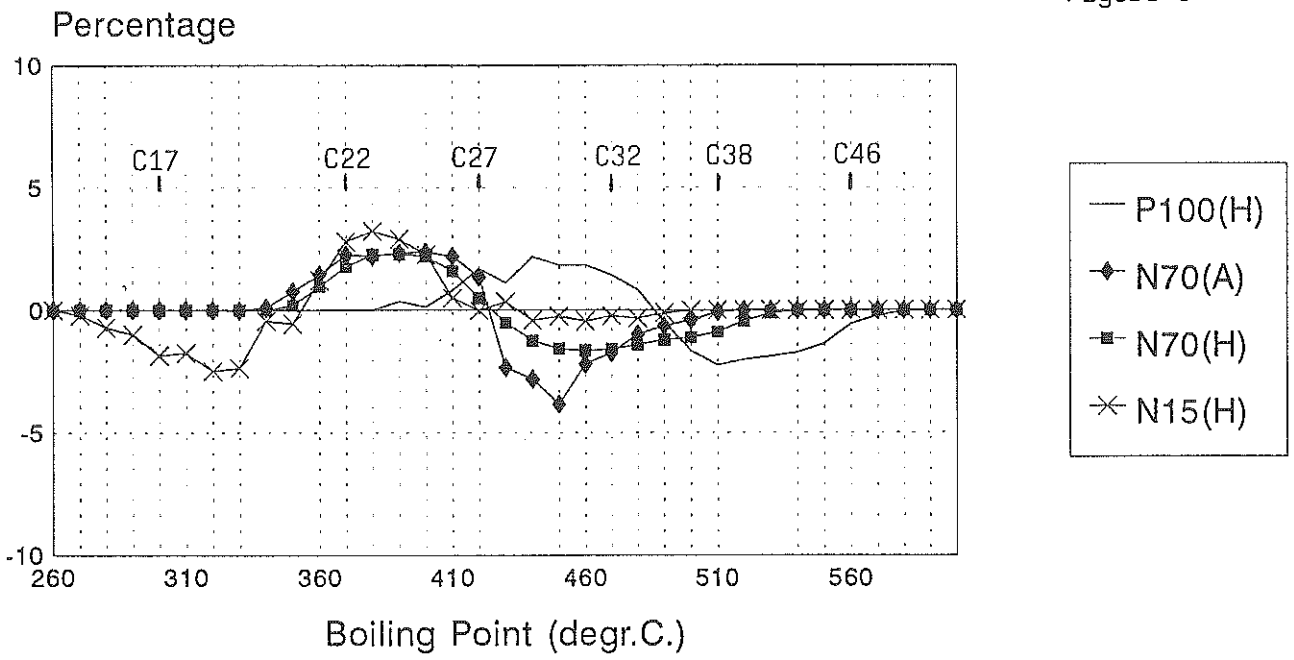


Figure 8

Mineral Hydrocarbon Study

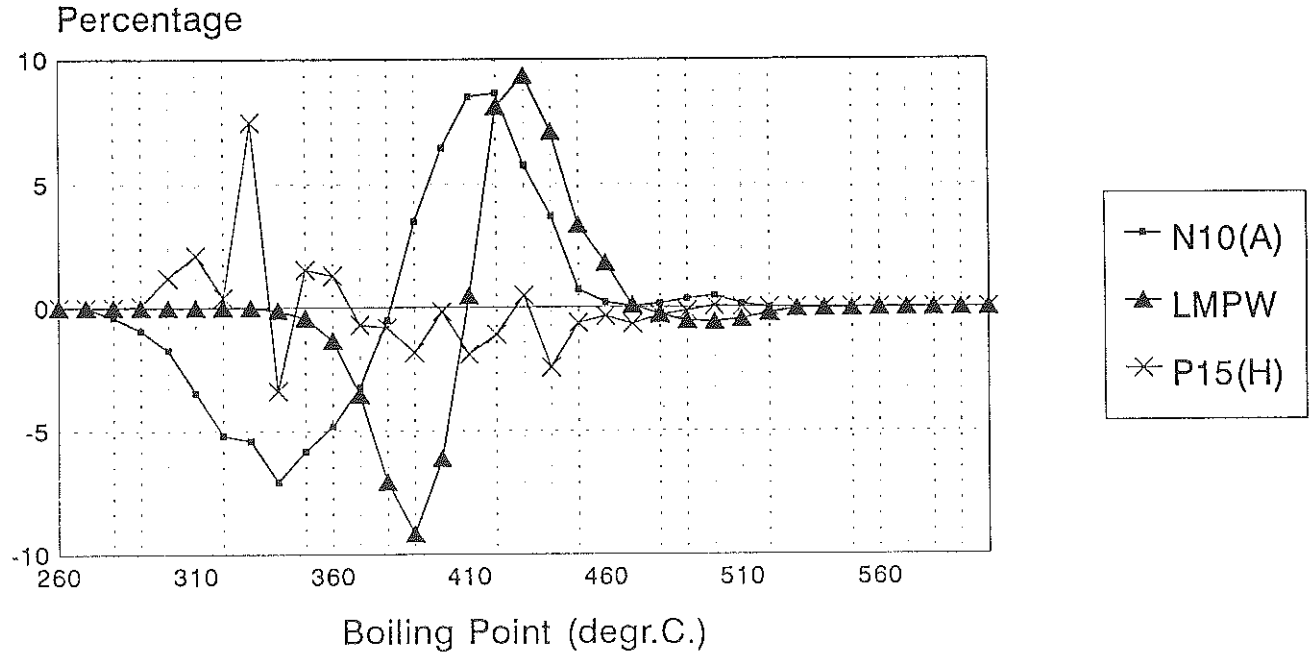
Deviation plot Main minus Original

Figure 9

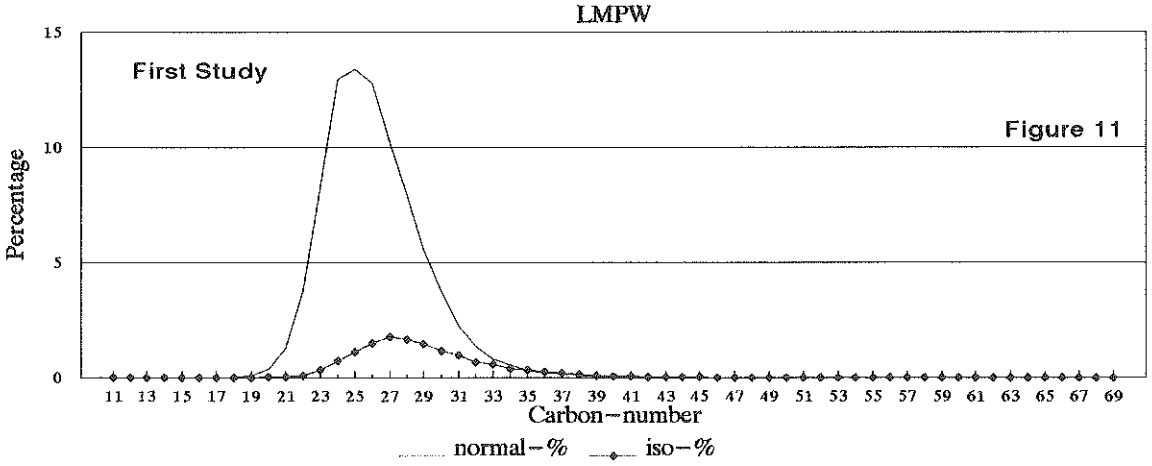


Deviation plot Main minus Original

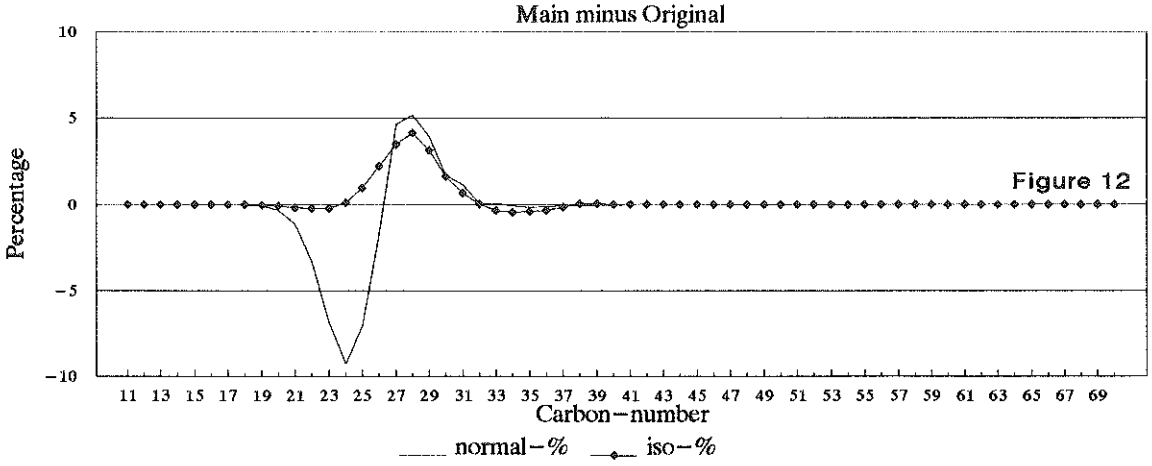
Figure 10



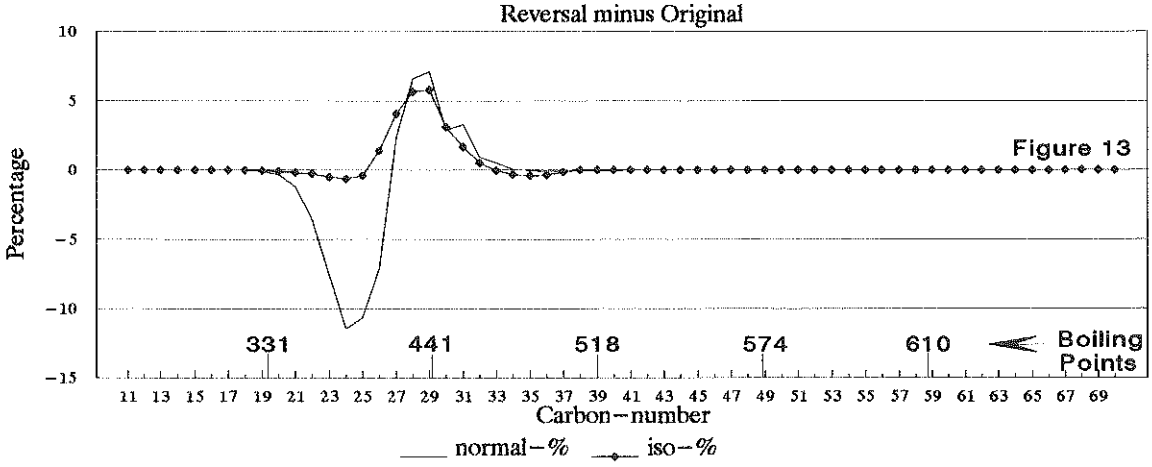
Normal/Iso – % Carbon Distribution



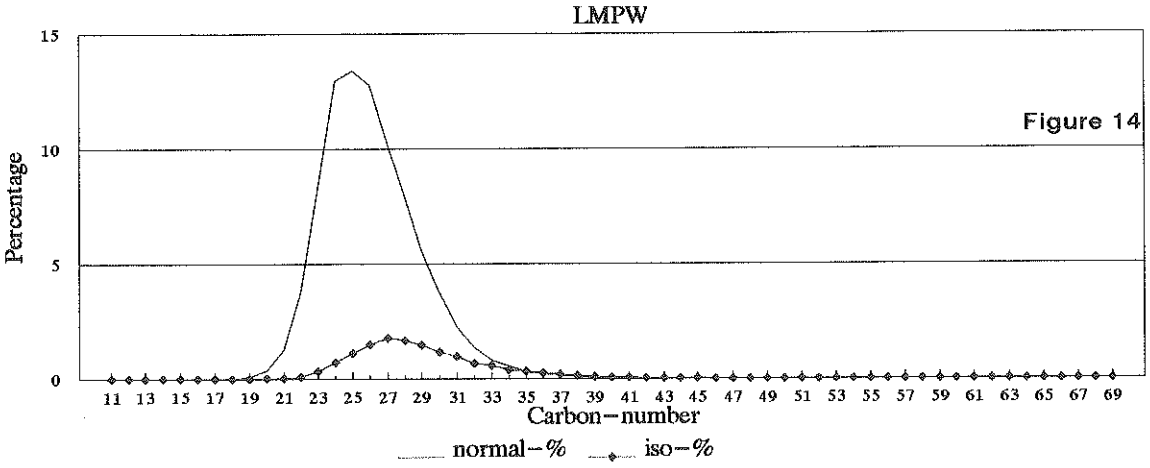
Deviation Plot



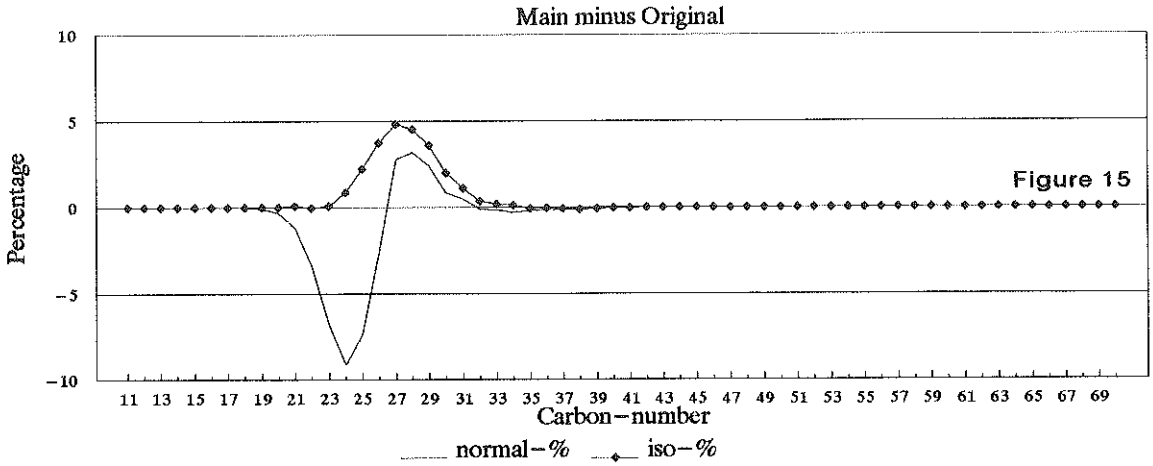
Deviation Plot



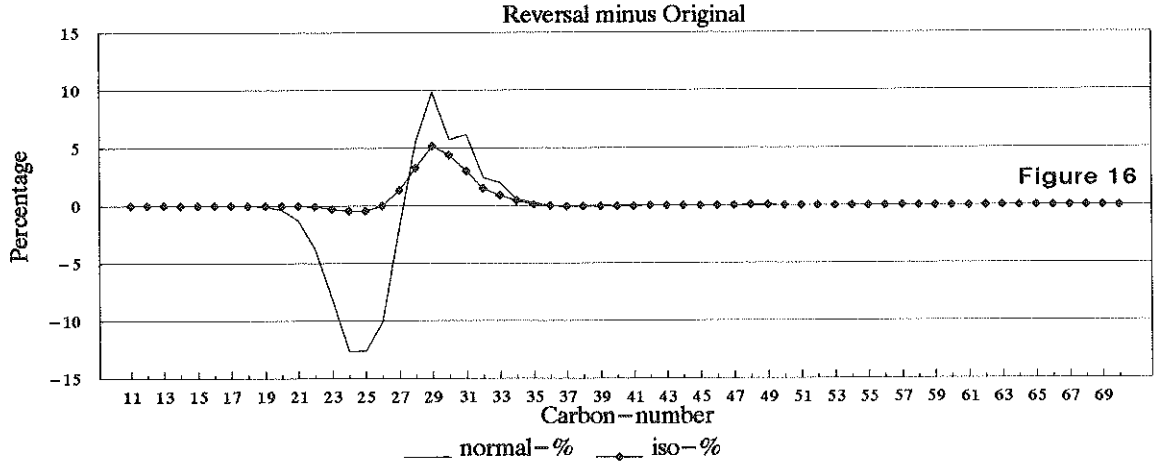
Normal/Iso – % Carbon Distribution



Deviation Plot



Deviation Plot



Normal/Iso Carbon – % Distribution

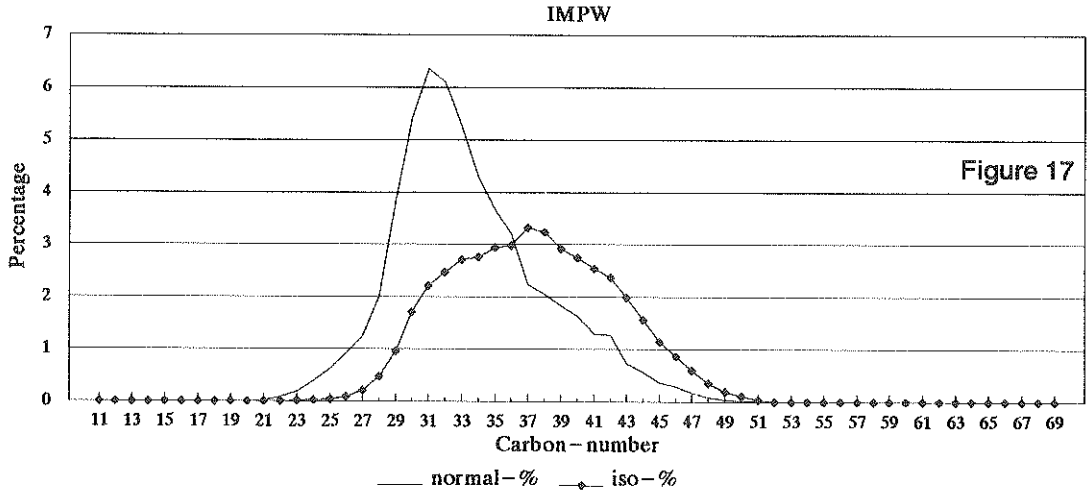


Figure 17

Deviation Plot

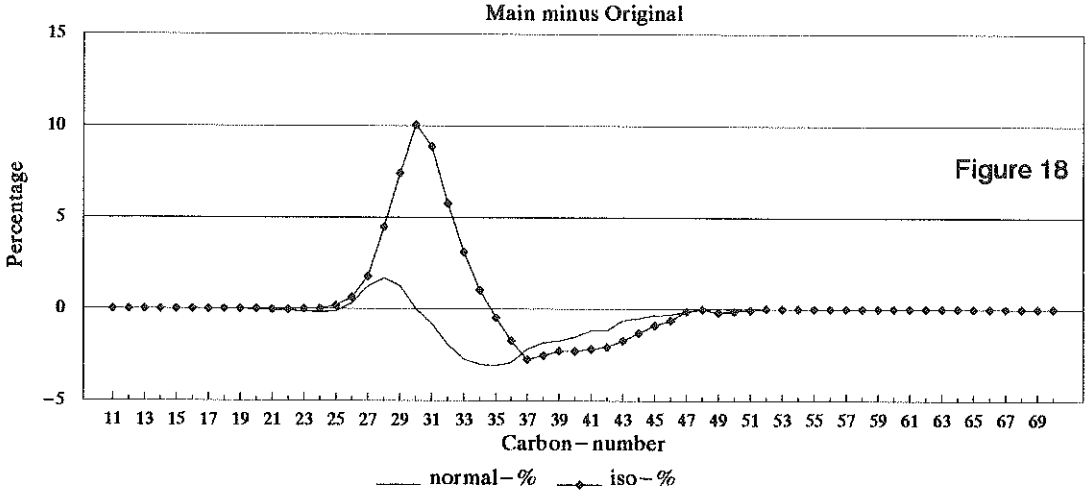


Figure 18

Deviation Plot

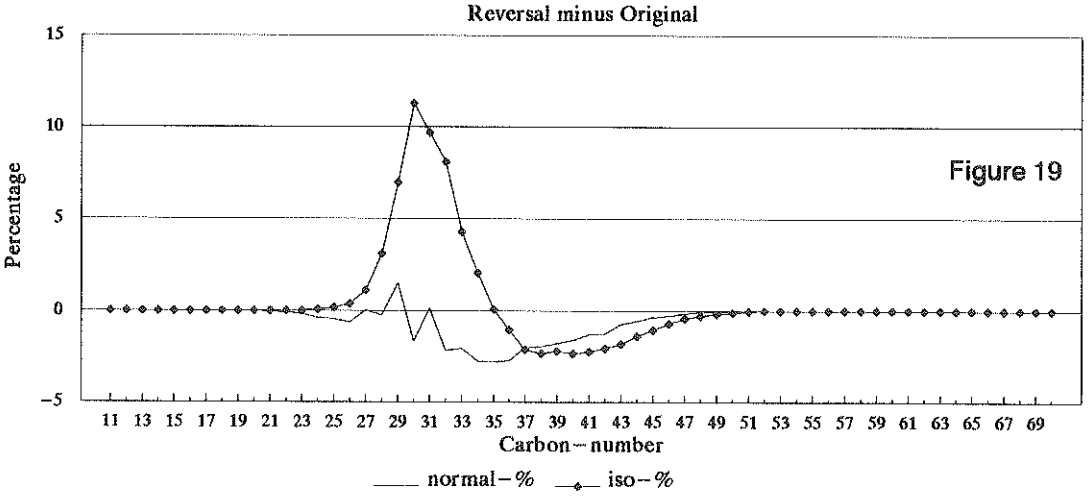


Figure 19

Normal/Iso−% Carbon Distribution

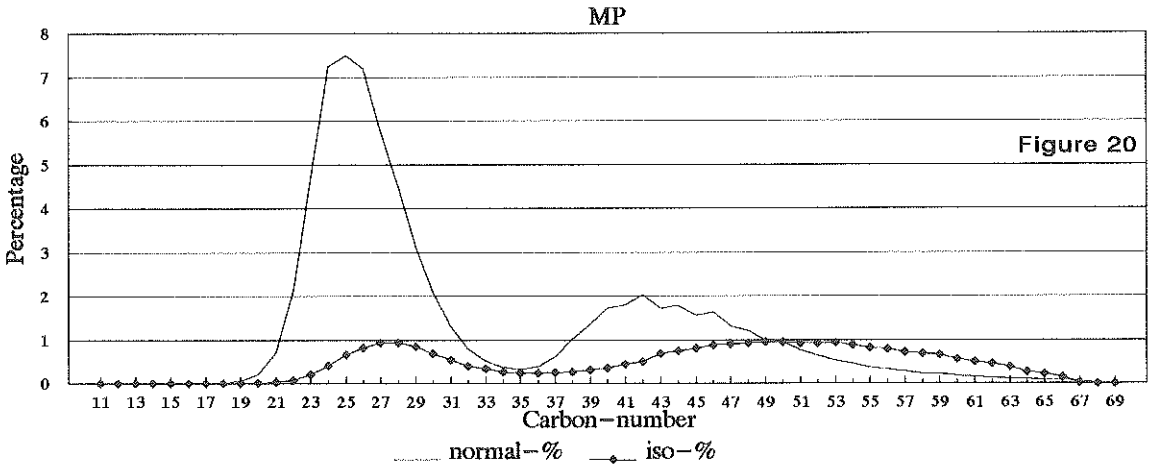


Figure 20

Deviation Plot

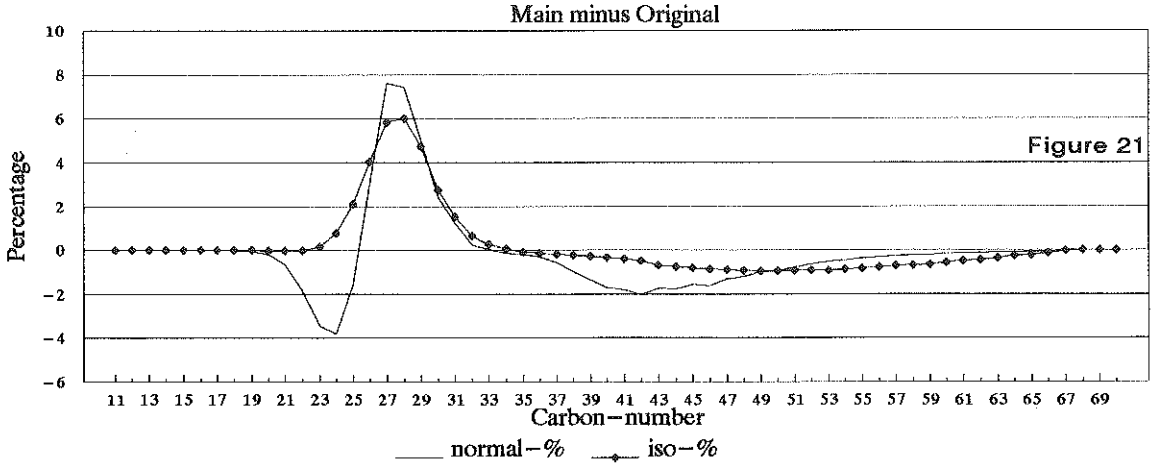


Figure 21

Deviation Plot

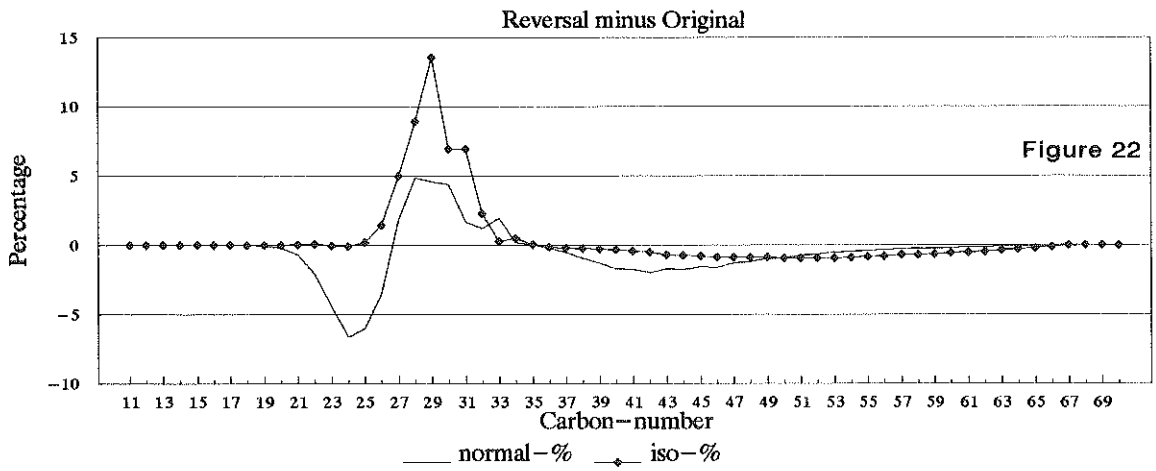


Figure 22

BOILING POINTS OF NORMAL PARAFFINS

[C−nr..]	BP	[C−nr..]	BP	[C−nr..]	BP
11	196	34	483	57	602
12	216	35	491	58	606
13	235	36	498	59	610
14	253	37	505	60	615
15	271	38	512	61	618
16	287	39	518	62	621
17	302	40	525	63	624
18	317	41	531	64	627
19	331	42	537	65	630
20	344	43	543	66	633
21	356	44	548	67	636
22	369	45	553	68	639
23	380	46	559	69	643
24	391	47	564	70	646
25	402	48	569	71	649
26	412	49	574	72	652
27	422	50	575	73	655
28	432	51	579	74	658
29	441	52	583	75	661
30	450	53	587	76	664
31	459	54	590	77	667
32	468	55	594	78	670
33	476	56	598		

SOURCES:

- From C2 to C44 : ASTM D−2887
- C50, C−60 and C−78 : Witco, Amsterdam
- The underlined figures are interpolated