The composition of wax and oil in green coffee beans

Dit proefschrift met stellingen van Peter Folstar, landbouwkundig ingenieur, geboren te Amsterdam op 18 maart 1947, is goedgekeurd door de promotoren, dr. W. Pilnik, hoogleraar in de levensmiddelenleer en dr. H.C. van der Plas, hoogleraar in de organische chemie.

De Rector Magnificus van de Landbouwhogeschool,
J.P.H. van der Want

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P. Folstar

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
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Abstract

Methods for the isolation of wax and oil from green coffee beans were studied
and a method for the quantitative extraction of coffee oil from the beans
was introduced. Coffee wax, coffee oil and wax-free coffee oil as well as the
unsaponifiable matter prepared from each were fractionated by column
chromatography. The chemical composition of the fractions was studied by
thin-layer chromatography, gas-liquid chromatography, ultraviolet, infrared,
nuclear magnetic resonance and mass spectrometry. The results include quali-
tative and quantitative data on (1) the total fatty acid composition as well
as the composition of the fatty acids in triglycerides and diterpene alcohol
esters, (2) the positional distribution of the fatty acids in the triglyce-
rides, (3) the unsaponifiable matter and (4) N_β-alkanoyl-5-hydroxy-tryptamines
(C-5-HT) and other C-5-HT-like phenolic compounds.
    The unsaponifiable matter (3) was fractionated into diterpene alcohols,
sterols and hydrocarbons (including squalene and n-alkanes from C_{16} to C_{31}).
Moreover the presence of α-tocopherol and $(\beta + \gamma)$-tocopherol in
green coffee beans was described for the first time.
    In view of the results of this study the removal of coffee wax by
industrial processes, which are claimed to have a beneficial effect on the
digestibility of coffee, was discussed. The current literature on the lipids
of green coffee beans was extensively reviewed.

Stellingen

1. De afname van het percentage onverzeepbare bestanddelen in koffieolie welke Kaufmann & Hamsagar als gevolg van het branden van koffie meenden vast te stellen is aanvechtbaar.

   H.P. Kaufmann & R.S. Hamsagar, 1962. Fette Seifen AnstrMittel 64: 734-738.
   Dit proefschrift, hfdst. 5.

2. De beweerde verbetering van het verdragen van koffie door bepaalde personen ten gevolge van voorbehandelingen, anders dan decafeïneren, blijft een speculatief karakter behouden zolang geen verband is aangetoond tussen de bij deze voorbehandelingen optredende veranderingen in chemische samenstelling en de fysiologische werking van de betrokken verbindingen.

   S. Gal, 1974. Lebensm.Ernähr. 27: 218-220, 235-237.

3. Hartman et al. hebben bij de gekatalyseerde omestering van koffieolie de vorming van triglyceriden met een 'ad random' verdeling van de vetzuren onvoldoende aangetoond.

   L. Hartman et al., 1968. J.Am.Oil chem.Soc. 45: 577-579.

4. Voor het verkrijgen van een beter inzicht in de bij de fermentatie van koffie betrokken processen dient meer aandacht besteed te worden aan de samenstelling van de polysacchariden van de mesocarplaag.

   J. Castelein, 1974. Diss. Leuven.

5. Rotenberg et al. gaan bij de interpretatie van de door Vessey et al. en Jick et al. beschreven retrospectieve studies naar het verband tussen koffieconsumptie en het optreden van een hartinfarct voorbij aan de experimentele tekortkomingen van deze onderzoekingen.

   F.A. Rotenberg et al., 1976. Lancet: 140-141.
   M.P. Vessey et al., 1972. Lancet: 1278-1281.
   H. Jick et al., 1973. New Engl.J.Med. 289: 63-67.

6. Bij de bestudering van het effect van het onverzeepbare deel op het 'smeltpunt' van koffieolie hebben Hartman & Lago ten onrechte geen aandacht besteed aan verschillen in vetzuursamenstelling tussen triglyceriden en esters van diterpeenalcoholen en vetzuren.

   L. Hartman & R.C.A. Lago, 1973. J.Am.Oil chem.Soc. 50: 99-100.

7. Bij de gangbare methoden voor de bepaling van de totale hoeveelheid sulfiet in levens-
middelen dient meer aandacht besteed te worden aan irreversibele binding van sulfiet
aan bestanddelen van deze levensmiddelen.

G.J.M.W. Arkesteijn, 1975. Ir.-verslag, Sprenger Instituut en L.H.afd. Levensmiddelen-
chemie en -microbiologie.
K. Wucherpfennig, 1975. Flüss. Obst 92: 451-464.

8. Gutfinger & Letan tonen onvoldoende aan dat de waarden door hen opgegeven voor het
gehalte γ-tocoferol niet het totale gehalte van β- en γ-tocoferol omvatten.

T. Gutfinger & A. Letan, 1974. Lipids 9: 658-663.

9. De bij de reactie van 2,7-dimethylchromoon met hydroxylamine door Sayed et al.
beschreven vorming van hetzij 3-(4'-methyl-2'-hydroxyfenyl)-5-methylisoxazool, hetzij
van 3-methyl-5-(4'-methyl-2'-hydroxyfenyl)-isoxazool is onvoldoende aangetoond. Derhalve
is de door hen uitgesproken voorkeur voor eerstgenoemd reactieprodukt inconsequent en
ongegrond.

A.A. Sayed et al., 1975. Acta Chim.(Budapest) 87: 165-175.

10. Het opstellen van een balans van de bestanddelen van koffie bij branden en extractie
kan het inzicht in de effecten van genoemde processen op de chemische samenstelling van
koffie verruimen.

H. Streuli, 1973. 6th int.Coll.Coffee Chem. (ASIC, Paris), p. 61-72.

11. Subjectieve kwaliteitsnormen zijn voornamelijk bepaald door gewenning; derhalve moet
de invloed van het zich voortdurend bevinden binnen de gehoorsafstand van de 'lichte'
radiozender niet onderschat worden.

Proefschrift van P. Folstar
The composition of wax and oil in green coffee beans

Aan mijn ouders

Curriculum vitae

De auteur behaalde in 1964 het diploma HBS-B aan het Hervormd Lyceum te Amsterdam. Aansluitend begon hij zijn studie aan de Landbouwhogeschool te Wageningen waar hij in september 1969 het kandidaatsexamen aflegde in de levensmiddelentechnologie met chemisch-biologische specialisatie. In januari 1972 behaalde hij het doctoraal diploma met lof. Het vakkenpakket omvatte het hoofdvak kennis van levensmiddelen en de bijvakken levensmiddelenmicrobiologie en industriele bedrijfskunde.

Daartoe financieel in staat gesteld door Douwe Egberts-Jacobs International Research Company B.V. te Utrecht bewerkte hij na zijn afstuderen dit proefschrift als gastmedewerker op het Laboratorium voor levensmiddelenchemie en -microbiologie van de Landbouwhogeschool. Sinds 1 april 1975 is hij verbonden als wetenschappelijk medewerker aan dit laboratorium.

Acknowledgements

Contents

1 Introduction

Coffee beans are produced by plants belonging to the family of
Rubiaceae and the genus *Coffea*. Among the approximately 60 species that are
known (Purseglove, 1968) *Coffea arabica* L. (commonly known as Arabica cof-
fee) and *Coffea canephora* Pierre ex Froehner (commonly known as Robusta cof-
fee) are of main commercial importance. The chemical composition of green
coffee beans has been studied for over 100 years but the greatest progress
has been made during the last decades due to the development of the modern
separation and identification techniques. Recent reviews on the chemical
composition of coffee beans have been given by Streuli (1973), Clifford
(1975a, 1975b) and Vitzthum (1976). Table 1 shows the composition of green
beans for both Arabica and Robusta coffee according to Clifford (1975b).

More than a century ago it became known that the lipids of coffee beans
are composed of both cytoplasmatic oil and a small amount of wax located in
the outermost parts of the bean (Robiquet & Boutron, 1837). It is only since
the work of Meyer & Eckert (1910) that considerable research has been done
on coffee bean lipids.

Coffee oil has received most attention. Before World War II when coffee
prices were low Brazilian surplus coffees were used for coffee oil production

Table 1. Chemical composition (%) of green beans
for Arabica and Robusta coffee on dry basis
(source: Clifford, 1975b).

	Arabica	Robusta
Minerals	3.0 – 4.2	4.0 – 4.5
Caffeine	0.9 – 1.2	1.6 – 2.4
Trigonelline	1.0 – 1.2	0.6 – 0.75
Lipids	12.0 – 18.0	9.0 – 13.0
Chlorogenic acids	5.5 – 8.0	7.0 – 10.0
Aliphatic acids	1.5 – 2.0	1.5 – 2.0
Oligosaccharides	6.0 – 8.0	5.0 – 7.0
Polysaccharides	50.0 – 55.0	37.0 – 47.0
Amino acids	2.0	2.0
Proteins	11.0 – 13.0	11.0 – 13.0

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(Eckey, 1954). A much more important source of coffee oil is the spent grounds from the soluble coffee industry. Until now no profitable applications exist so that it is not worthwhile to exploit this source on commercial scale (Sivetz, 1963, p.175-183). As coffee oil has been found to possess strong aroma-binding properties, oil expelled or extracted from roasted coffee is used in the soluble coffee industry as aroma carrier for aromatizing soluble coffee powder (Sivetz, 1963, p.1-30). The oil of green and roasted beans and the spent grounds as well as crude oils from commercial sources have been used for analysis. Streuli (1970, p.19) pointed out that poor definition of coffee oil and insufficient standardization of the extraction procedure led to results which could hardly be compared. In this work coffee oil is considered to be that part obtained from ground green beans by extraction with petroleum ether (40-60°C).

A so-called decaffeination wax is obtained in large amounts as waste product of the decaffeination process. Its composition has been described incidentally (Haselmann, 1963; Dickhaut, 1966). According to Streuli (1970, p.21) it is questionable whether this decaffeination wax can be considered as representative for wax located in the outermost parts of the bean. In this study coffee wax is defined as the matter obtained from unground green beans which dissolves in chloroform.

More recently coffee wax compounds have been associated with gastro-intestinal disorders giving rise to complaints described as 'irritability' or 'indigestion' (Wurziger, 1971a; 1971b). Patents have been granted to produce a so-called wholesome ('reizarm' or 'bekömmlich') coffee which is characterized by a reduced wax content (Kurz & Vahland, 1971; Roselius et al., 1971). Only a few trials have been reported which claim to prove a causal relationship between gastro-intestinal disturbances and coffee which has been treated according to these patents. A review has been given by Wurziger (1973a). However it is not certain whether these experiments are really conclusive (Mühlens, 1972). Moreover it remains to be seen whether the phenomena observed are a measure of what is understood by 'indigestion'.

Another approach to the evaluation of the claimed effects of these patents is to obtain knowledge about the specific constituents of both wax and oil. The purpose of this study therefore was:
-to study the procedure of isolation for both wax and oil in order to obtain representative samples which are suitable for further analysis,
-to determine in detail the proportion of the main groups of lipids and their individual constituents in both wax and oil.

2 Production of green coffee
and special treatments of coffee beans

The fruit of the genus *Coffea* (also called 'coffee berry' or 'coffee cherry') is a stone fruit and normally contains two seeds ('beans') (Fig.1). The seeds have a half ellipsoidic form, the rounded edges being folded inwards forming a crease down the middle of the flat side. Within the fruit the flat sides are in juxtaposition. Each seed is surrounded by a rudimentary seed coat ('silverskin'). The silverskin penetrates the longitudinal crease. Around the seeds and seed coat a fibrous endocarp ('parchment') is found. The two seeds in parchment are lying in a soft yellowish pulp or mesocarp ('mucilage') which is surrounded by a smooth tough outer skin or exocarp.

The coffee seeds mainly consist of a tissue which has been found to be an endosperm originating from the embryo sac. Early studies claimed the endosperm to be a perisperm tissue formed from the nucellus but later this was shown to be incorrect (Melchior, 1970). The endosperm is a paremchymatic tissue with isodiametrical cells (25-100 µm) and characteristic thick cell walls (5-15 µm) which have numerous pits. There is a minute embryo at the base of the seed.

Within the cells of the seed droplets of oil can easily be observed by staining with for instance Sudan III or IV.

The epidermis of the seed is covered with a thin cuticle (Melchior, 1970). Dickhaut (1966) found that surface waxes, being a part of the cuticle, became intensively blue upon staining of microscopical sections with 2,6-dichloro-
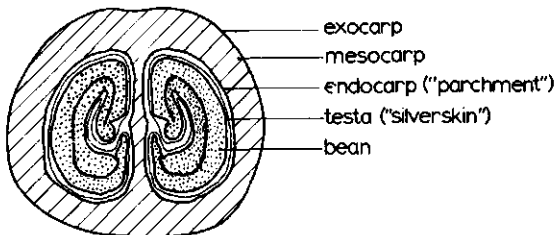

Fig. 1. Cross-section of a coffee fruit

quinone chlorimide under alkaline circumstances (Gibbs reagent). Upon de-
caffeination with solvents or a polishing treatment with sand the intensity
of the colour obtained in the reaction was found to be reduced (Wurziger &
Dickhaut, 1967). Bürgin (1969) confirmed these observations.

In the production of green coffee beans all the pericarp (which includes
exo-, meso- and endocarp) is removed as is the testa apart from the portion
in the endosperm cleft. There are two techniques for obtaining green coffee
beans:
1. *The dry method:* the fruit is allowed to remain on the tree past the fully
ripe stage and is partially dried before harvesting. Next the fruit is pick-
ed and sun-dried on a terrace until the moisture content is about 12%. Gen-
erally this requires a drying time of 8-10 days depending on the thickness
of the layer of the beans on the drying terrace and the weather conditions
(Sivetz & Foote, 1963, p.105). Next the outer layers including parchment and
parts of the silverskin are removed by hulling.
2. *The wet method:* red-ripe fruit is selectively harvested, classified and
pulped which results in the removal of the exocarp and parts of the mesocarp
leaving a slippery exposed layer of mucilage which cannot be dispersed in
water. This mucilage is broken down by fermentation. The necessary enzymes
are supplied by yeasts and bacteria which develop rapidly in the fermenting
tanks (Agate & Bhat, 1966; van Pee & Castelein, 1972). A literature review
by Castelein (1974) shows that there are contradictory reports about mucilage
decomposition by enzymes present in the fruit itself. Next the coffee is
washed and dried whereupon the parchment layer is separated from the seed by
the shrinkage of the latter. Washed coffee can be sun-dried but there is an
increasing tendency to use drying equipment, apart or in combination with
sun-drying. Drying with equipment is usually performed in stages of in-
creasing air temperatures. Ranges between $40^{o}C$ and $60^{o}C$ have been reported.
Generally drying times are between 8-36 h depending on the initial moisture
content and the type of dryer used (Sivetz & Foote, 1963, p.115-155; Rolz et
al., 1969). Finally the parchment and the silverskin are removed in a hulling
machine which can also polish the beans thus improving their appearance.

Certain persons seem to be susceptible to coffee. Thereby complaints des-
cribed as 'irritability' or 'indigestion' have been reported (Czok, 1966;
Vitzthum, 1976). These complaints are claimed to be reduced in the case of
coffee which has been subjected to special processes (Gal, 1974):

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-the Lendrich process. Green beans are treated for 1-3 h with saturated
steam at 1-3 atm. Next they are dried to the original moisture content
(Lendrich et al., 1933).
-the process for removal of coffee wax. Green beans are treated with chlorinated
hydrocarbons like dichloromethane to reduce the amount of coffee wax in the
beans. The solvent is then expelled from the beans by steaming under the con-
ditions of the Lendrich process (Kurz & Vahland, 1971; Roselius et al., 1971).
-the Kofrosta process. In contrast with the two processes just mentioned
roasted beans are used. After roasting the beans are immediately cooled in
liquid carbon dioxide. As a result the surface slightly crumbles and parts
of it fall off (Böhm & Ruf, 1972).
In all three processes the caffeine content in the beans remains unchanged.

In order to decaffeinate green coffee beans two main types of processes
are used (Vitzthum, 1976, p.34):
-green beans are treated with saturated steam so that they swell, then with
a low boiling solvent like dichloromethane. After extraction the beans are
steamed to expel the solvent. After evaporating off the solvent the residue
of the dichloromethane solution constitutes caffeine together with
decaffeination wax.
-green beans are extracted with water and caffeine is selectively transfer-
red from the aqueous solution to trichloroethylene by liquid/liquid counter-
current extraction. The decaffeinated aqueous solution is returned to the
countercurrent percolation system to achieve a continuous operation.

3 Literature

3.1 COFFEE OIL

3.1.1 Determination of the oil content

A review of the literature shows that the values reported for the oil content diverge widely. Eckey (1954) reported 10-15% and Clifford (1975a) .12-18% for Arabica coffee and 9-13% for Robusta. Apparently these wide ranges not only result from naturally occurring differences between, for instance, the species of *Coffea* but also from a lack of uniformity in the methods used for the determination.

Wilbaux (1956) found that the species of *Coffea* can be classified on the basis of their percentage of ether soluble material. He reported values of 13.0-14.7% for Arabica, 10.6-12.6% for Robusta and 11.9-12.0% for Liberica coffee. Using petroleum ether (40-60°C) Subrahmanyam & Ackaya (1957) determined 11.8% 'fat' for Arabica and 10.0% for Robusta. According to the method of the Association of Official Agricultural Chemists (AOAC) Kröplien (1963) investigated about 40 samples from various origins. Arabica was found to contain 14.2-17.0% of material soluble in petroleum ether (35-50°C) and Robusta 7.2-11.0%. Carisano & Gariboldi (1964) extracted coffee oil with petroleum ether (30-50°C) and in addition colouring material and impurities were removed by treatment with 10% active carbon. They reported oil contents only on wet weight basis, being 9.07% for Indonesian Robusta and 11.78-12.97% for three Arabica coffees from different countries of origin. Chassevent et al. (1974) determined the percentage of hexane solubles for nine different species. Arabica was found to contain 14.8% and Robusta 10.3%.

Bressani et al. (1961) observed a correlation with 99% significance between the amount of ether soluble substances in green (and roasted) beans and the variety. No correlation was found between the amount of ether soluble substances in the beans and the processing method of the fruit. Before ether extraction the beans were ground to pass through a 30-mesh sieve. Later Menchu (1967) showed that this operation yielded incomplete extraction.

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For Guatemalan Arabica coffees of the varieties Typica and Bourbon, Menchu & Ibarra (1967) found that the percentage of material soluble in petroleum ether (35-50°C) correlates with the quality of the beans, the best quality ('strictly hard beans') from growing zones of high altitude having the highest percentage. The analysis was performed with 0.1 mm flaked grinds, percentages of 10.67-14.65% being found.

Kaufmann & Hamsagar (1962b) reported remarkably low values for three Arabica coffees from different geographical origin. After 20 h extraction with petroleum ether (35-50°C), they determined 6.65-7.15% oil.

Streuli et al. (1966) concluded that the percentages given by Kaufmann & Hamsagar (1962b) include only a part of the oil present in the bean. The first-named authors observed that the yield of extraction depends on the particle sizes of the grind. The official direct extraction procedures of both the AOAC (no.14.029, 1965) and the Deutschen Gesellschaft für Fettwissenschaft (DGF, no.B-Ib, 1952) were not found to be suitable to cope with this problem. The method of the AOAC specifies the particle sizes of the ground material (passing through a 30-mesh sieve). Further the method involves drying of the grind and an extraction time of 16 h with petroleum ether (35-50°C). According to the DGF method, which is closely related to the method of the International Union of Pure and Applied Chemistry (IUPAC, no.I.B.2, 1966), the ground material has to be dried before extraction at 105°C for 30-35 min if the moisture content exceeds 10%. Next the grind is extracted for 4 h with petroleum ether (40-55°C). Upon extraction the grind is additionally pulverized with sand in a mortar and extracted for another 2 h under similar conditions. None of these methods appeared to yield complete extraction. Another disadvantage is that traces of caffeine were found to be present in the extracts and particularly the samples prepared according to the DGF method were found to contain considerable amounts of sand.

According to Streuli et al. (1966), the Swiss official method (Schweiz. Lebensm.Buch, no. 35A/08, 1973) yields complete extraction. It includes grinding, not exceeding a particle size of 0.63 mm, and digestion with 4 N HCl followed by extraction with petroleum ether (b.p. < 60°C). Moreover caffeine does not interfere as it is removed before oil extraction by washing out with hot water. The authors found values of 15.9% for Santos, 10.3% for Congo and 9.2% for a Madagascar coffee.

Thaler & Gaigl (1962) obtained the following percentages on dry basis by digestion with 4 N HCl and extraction with petroleum ether (50-70°C): for Arabica 16.50-17.40%, for Robusta 11.05-11.33% and for Liberica 10.90-12.00%.

A recent report described the refractometric determination of oil in green coffee beans with α-bromonaphtalene as solvent (Padaryan, 1974). Values of 11.97-17.91% for Arabica and 9.44-12.87% for Robusta have been found. However it is questionable whether this technique is as precise as the gravimetrical methods (Pardun, 1969, p.414).

3.1.2 Isolation of coffee oil for analysis

For analytical purposes coffee oil is usually obtained by extraction of ground material with petroleum ether in a percolator. Incidentally other solvents like hexane (Chassevent et al., 1974; van de Voort & Townsley, 1974), ether (Calzolari & Cerma, 1963; Menchu, 1967) or chloroform (Janícek & Pokorný, 1970a; 1970b) have been used too. Kaufmann & Hamsagar (1962a) and Itoh et al. (1973a; 1973b) used crude oils from commercial sources for their experiments. In one report coffee beans were dewaxed with tetrachloroethane before oil extraction with petroleum ether but further specifications concerning dewaxing circumstances and the oil extraction procedure were omitted (Schuette et al., 1934).

In all these cases the way in which the oil has been obtained probably determines to some extent its characteristics.

Most reports on the coffee oil composition fail to give any specifications on the grinding procedure of the beans and especially the particle sizes of the grind. Thereby it remains uncertain whether complete extraction has been achieved (Section 3.1.1). The quoted literature does not give any information whether there is a relationship between the amount of oil which has been extracted from the grind and the composition of the oil. Because of its importance for coffee lipid analysis much attention is given to this question (Section 5.1; Folstar et al., 1975a).

3.1.3 The composition of coffee oil

Using silica gel column chromatography and elution with mixtures of ether in petroleum ether (35-45°C) of increasing eluotropic strength, Kaufmann & Hamsagar (1962a) investigated the composition of a commercial Brazilian coffee oil. By gravimetrical determination of the fractions they found 1.45% steroids, 75.2% triglycerides, 18.48% esters of diterpene alcohols and fatty acids, 1.2% diterpenes and 4.2% phosphatides and other unknown compounds. Similar experiments for oil obtained on analytical scale have not been carried out.

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3.1.3.1 The fatty acid composition

The presence of free fatty acids (FFA) has been described incidentally. Carisano & Gariboldi (1964) found 0.50-1.89% FFA in petroleum ether (30-50°C) extracts obtained from samples of different geographical origin. Hartman et al. (1968) reported 2.6% FFA in hexane extracts from 'good quality' beans and an unusual high value of 18.6% FFA in hexane extracts from 'low quality' beans. In both references the content of FFA has been expressed as percentage oleic acid. Calzolari & Cerma (1963) reported acid values in the range of 4.51-7.29 and Kaufmann & Hamsagar (1962b) found values of 3.14-4.16. Based on an average molecular weight of the fatty acids of 275, these acid values correspond with 1.5-3.6% FFA. In a chloroform extract of green coffee that was stored for 3 years, Pokorný et al. (1970) determined an acid value of 11.1.

The total fatty acid composition of coffee oil has been the subject of many investigations. Table 2 is a review of the results obtained by gas chromatography. The compositions as given by different authors closely agree with each other. Moreover the examinations of Carisano & Gariboldi (1964), Calzolari & Cerma (1963) and Chassevent et al. (1974) showed that no significant differences between Arabica and Robusta coffee exist as far as the total fatty acid composition is concerned.

Older reports on the fatty acid composition and especially those which

Table 2. Gas-chromatographic data on the fatty acid composition of oil from green coffee beans (%)[a].

		Ref.1	Ref.2	Ref.3	Ref.4	Ref.5
Myristic acid	$C_{14:0}$	traces	traces	0.06- 0.14		0.2
Palmitic acid	$C_{16:0}$	35.20-38.60	30.7-35.3	35.44-41.35	39	35.2-36.7
Palmitoleic acid	$C_{16:1}$	traces	traces			
Margaric acid	$C_{17:0}$	–	traces			
Stearic acid	$C_{18:0}$	6.60- 8.35	6.6- 9.0	7.53-10.60	6	7.2- 9.7
Oleic acid	$C_{18:1}$	7.55-10.90	7.6-10.1	8.07- 9.58	7	9.5-11.9
Linoleic acid	$C_{18:2}$	38.40-43.00	43.5-45.9	36.64-43.08	44	41.2-42.6
Linolenic acid	$C_{18:3}$	?	1.1- 1.7			1.3- 2.7
Arachidic acid	$C_{20:0}$	4.05- 4.75	2.7- 3.3	$C_{20:0}$ and	3	0.3- 1.5
Gadoleic acid	$C_{20:1}$	–	?	others:		
Behenic acid	$C_{22:0}$	0.65- 2.60	0.3- 0.5	4.28- 6.43		

a. Oil obtained by extraction with ether (ref.1), petroleum ether (30-50°C) (ref.2), or hexane (ref.3, 4 and 5);
Ref. 1: Calzolari & Cerma (1963); Ref. 2: Carisano & Gariboldi (1964); Ref. 3: Hartman et al. (1968); Ref. 4: van de Voort & Townsley (1974); Ref. 5: Chassevent et al. (1974).

Table 3. Partition between triglycerides and
diterpene alcohol esters of the fatty acids (%)
in coffee oil (source: Kaufmann & Hamsagar, 1962a).

	Coffee oil	Triglycerides	Diterpene alcohol esters
$C_{16:0}$	25.3	23.5	42.5
$C_{18:0}$	13.1	12.5	17.5
$C_{18:1}$	17.2	18.0	11.0
$C_{18:2}$	39.0	41.0	20.5
$C_{20:0}$	4.2	5.0	6.0
$C_{22:0}$	1.0	traces	2.5

made use of techniques other than gas chromatography show much more variation
in the percentages of the individual fatty acids (Subrahmanyam & Achaya, 1957;
Kaufmann & Hamsagar, 1962a).

Kaufmann & Hamsagar (1962a) also investigated the partition of the fatty
acids between triglycerides and the diterpene alcohol esters (Table 3). For
this purpose fractions, obtained by column chromatography of a crude oil as
described in Section 3.1.3, have been used. The fatty acids were separated
by paper chromatography on undecane-impregnated paper with acetic acid:
acetonitrile = 3:1 as solvent. Upon visualization with copper acetate/
rubidium hydride the concentration of the fatty acids was determined photo-
metrically. In spite of the inaccuracies of this method it can be concluded
that the saturated acids prefer to be esterified with diterpene alcohols.

3.1.3.2 The unsaponifiable matter

The substances which after saponification of an oil sample in alcoholic
alkali and extraction of the alkaline solution with ether or petroleum ether
are insoluble in water but soluble in the solvent used for the extraction
are called the unsaponifiable matter (IUPAC, no.II.D.5, 1966).

According to a survey of mainly older literature (Streuli, 1970, p.19)
the percentage of unsaponifiable material in coffee oil amounts to 2.0-12.1%.
This wide range is probably caused by differences in the solvent chosen for
the extraction of the unsaponifiable matter after saponification: for
ether Bauer & Neu (1938) determined 6.55% of unsaponifiables whereas extrac-
tion with petroleum ether yielded only 2.75% unsaponifiable matter. The low
percentage is due to the poor solubility of the diterpene alcohols
cafestol (I) and kahweol (II) and the sterols in petroleum ether (Wurziger,
1963). Using petroleum ether, Chassevent et al. (1974) found only 0.55% of

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unsaponifiable matter for Arabica coffee and 1.27% for Robusta. With
di-isopropyl ether Itoh et al. (1973a, 1973b) determined 3.4% of un-
saponifiable material for a crude oil from commercial source.

For an oil with a high percentage of unsaponifiable material the official
methods of the IUPAC (no. II.D.5, 1966) and the DGF (no.C-III.1, 1953) rec-
ommend ether instead of petroleum ether as solvent for extraction.

For Arabica coffee the ether method was reported to give 10.42-11.39% of
unsaponifiable matter (Kaufmann & Hamsagar, 1962b). Ravindranath et al.
(1972) found 11.4-12.7% for Indian Robusta.

There is no evidence in the literature of differences between the species
of coffee as far as the percentage of unsaponifiable matter is concerned.

The composition of the unsaponifiable matter received considerable
attention. Most prominent are the diterpene alcohols cafestol (I) and
kahweol (II) whose structures have been elucidated by Djerassi et al. (1955;
1958; 1959; 1960) and Haworth et al. (1955; 1956; 1957). In coffee oil I and
II mainly appear as monoester with fatty acids, only the primary hydroxyl
group at C16 being esterified (Kaufmann & Hamsagar, 1962a).

A diterpene alcohol, which was tentatively suggested as being abiet-15-
-en-13β,19 diol (Nagasampagi et al., 1971), has been identified as
kaur-16-en-19 ol (III) (Wahlberg et al., 1975).
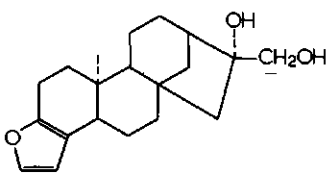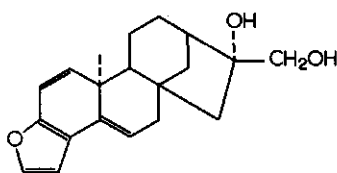
I   II
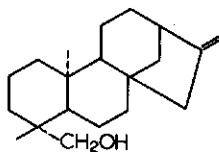
III

Sterols have been found both in free and esterified form (Nagasampagi
et al., 1971). The 4-desmethylsterols comprising 92.7% of the sterolic frac-
tion have been found to consist mainly of stigmasterol (IV), sitosterol (V)
and campesterol (VI) as well as traces of stigmastanol (VII), campestanol

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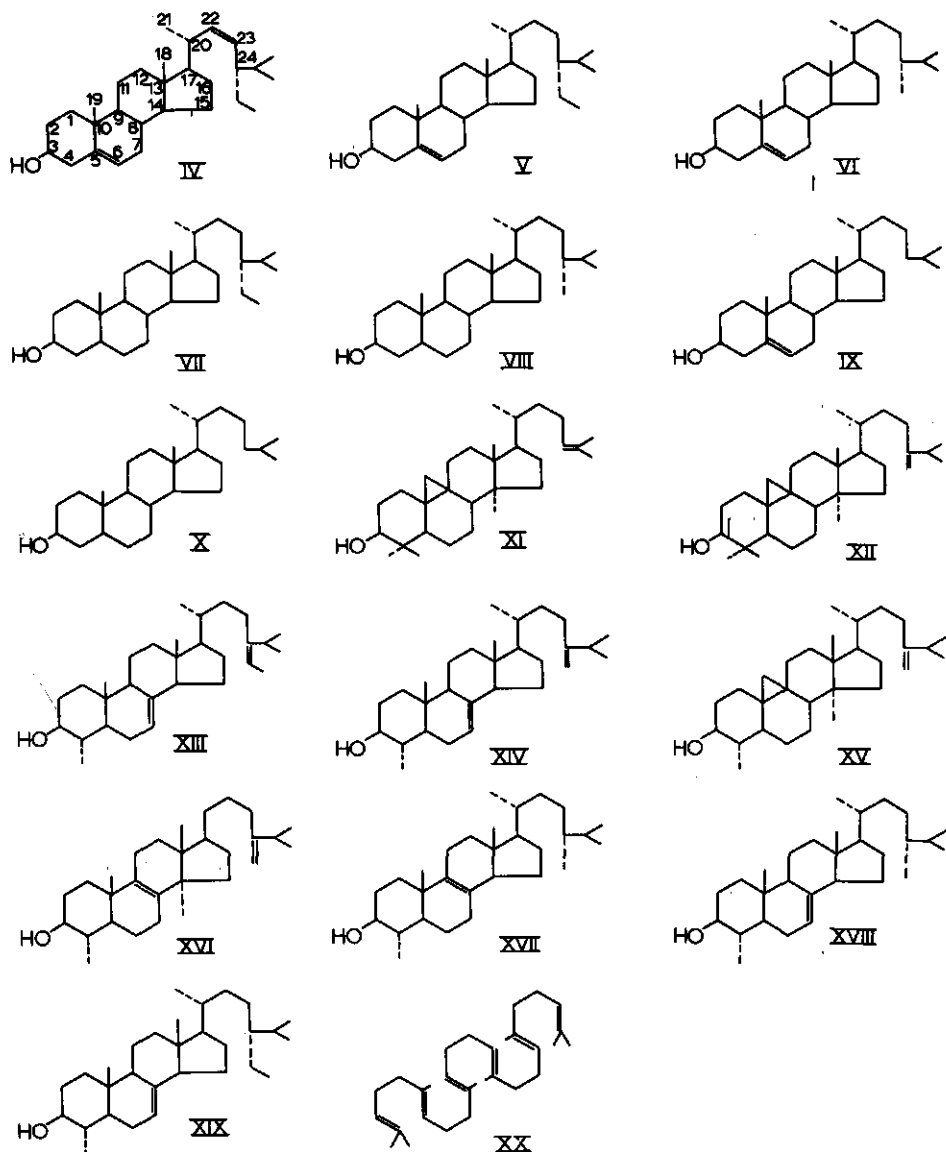
(VIII), cholesterol (IX) and cholestanol (X). Upon examination of the 4,4-dimethylsterols (5.1% of the sterolic fraction) cycloartenol (XI) and 24-methylenecycloartanol (XII) have been identified. Finally the 4-methylsterols (2% of the sterols) have been found to consist of citrostadienol (XIII), 24-methylenelophenol (XIV), cycloeucalenol (XV), obtusifoliol (XVI) and traces

of 4α,24R-dimethyl-5α-cholest-8-en-3βol (XVII), 4α,24R-dimethyl-5α-cholest-
-7-en-3βol (XVIII) and 4α-methyl-5α-stigmast-7-en-3β ol (XIX).

The appearance of IV, V, VI, IX, XI, XII, XIII, XIV and XVI has also been
described by Itoh et al. (1973a; 1973b). The presence of lanosterol, di-
hydrolanosterol or 'coffeasterol', as reported by Kaufmann & Sen Gupta (1964),
could not be confirmed by Alcaide et al. (1971) as well as Nagasampagi et al.
(1971).

Kaufmann & Sen Gupta (1964) identified squalene (XX) in the unsaponifiable
part of coffee oil and confirmed the presence of n-nonacosane which had
previously been found by Neu (1948).

From older literature there has been some indication that the vitamins A,
D and E are present in coffee oil too (Sivetz, 1963, p.176) but until now
this has not been investigated thoroughly.

3.2 COFFEE WAX

Most reports on coffee wax refer to the composition of the decaffeination
wax. This has been found to consist of 54% of material soluble in petroleum
ether (Meyer & Eckert, 1910). According to these authors the composition of
the petroleum ether extract from this wax is identical with the composition
of coffee bean oil but no further details were reported. Also Wagner (1938a;
1938b; 1939) reported that 50% of decaffeination wax is soluble in petroleum
ether. It has been found to contain palmitic acid, oleic acid, linoleic acid
and unsaponifiable matter. In the part of the decaffeination wax insoluble
in petroleum ether a 'resin-like' material has been observed.

Haselmann (1963) fractionated decaffeination wax according to the solu-
bility properties in successively petroleum ether, 80% ethanol, 96% ethanol
and benzene and by preparative chromatography. Infrared spectroscopy, melting
range, elementary analyses and specific colour tests showed n-alkanes, fatty
acids, diglycerides, sterols, esters of fatty acids and sterols, 'phlobaphenes',
an unsaturated secondary alcohol ($C_{14}H_{25}OH$) and caffeine to be present.

It remains to be seen whether some of the compounds, as found in the
decaffeination wax, have not been formed as a result of decaffeination.

Dickhaut (1966) analysed both decaffeination wax and wax obtained by
polishing green beans with sand and subsequent extraction of the sand with
methanol. By use of an Al_2O_3 (activity III) column with a solvent gradient
of continuously increasing eluotropic strength, both waxes were found to
yield different elution patterns.

Lindner (1955; 1958) stated that the composition of both coffee wax and the unsaponifiable matter in coffee oil are more or less identical. According to Lindner the oil in the inner part of the bean which is soluble in petroleum ether consists of about 2% of unsaponifiable matter. If another solvent (like alcohol, acetone, benzene, chloroform, tetrachloroethane or glacial acetic acid) is used for oil extraction, coffee wax is removed together with the oil. Lindner pointed out that this leads to an increase of the percentage of unsaponifiable matter up to 20%.

With reference to Lindner, Dickhaut (1966) suggested that the determination of the percentage of unsaponifiable matter and especially its concentration of diterpene alcohols, as described by Kaufmann & Schickel (1963), could serve as assay for the determination of the amount of coffee wax present in the bean. However it is very unlikely that only 2% of unsaponifiable matter can be found in the oil soluble in petroleum ether (Section 3.1.3.2) and it is even more difficult to explain that extraction of the wax, which only amounts to 0.2% of the bean, together with 10-15% oil results in an increase from 2 up to 20% unsaponifiable matter.

In this context it is curious that Schuette et al. (1934) found 12.63% of unsaponifiable matter in oil obtained by extraction with petroleum ether from green beans which had previously been dewaxed with tetrachloroethane. The fatty acid composition and the physical and chemical characteristics of the oil were also determined. Since a similar analysis for oil obtained from the same beans before wax removal was not reported, a comparison is impossible. Schuette et al. (1934) found 0.24% of wax in green beans.

Wurziger & Günther (1960) observed that alcoholic extracts from unground green or roasted beans became blue with 2,6-dichloroquinone chlorimide under alkaline circumstances (Gibbs test). This was ascribed to the appearance of phenolic compounds in the outermost parts of the beans. This test was negative for decaffeinated coffee. Microscopical examinations confirmed these phenomena (Chapter 2).

According to Dickhaut (1966) and Wurziger & Dickhaut (1967) the phenolic compounds are insoluble in petroleum ether, partially soluble in benzene and ether and soluble in chloroform, acetone, ethanol, methanol and benzene: methanol = 1:1. Thin-layer chromatography on silica gel G (0.25 mm) with benzene:methanol = 88:12 as solvent indicated the presence of eight Gibbs-positive compounds. The one showing the highest Rf value was called the main component. They were found to be present in stored as well as fresh green

beans, fresh coffee in parchment and beans from unripe coffee fruits.

The main phenolic component was found to have antioxidative properties. Its activity corresponded with that of 'butylated hydroxyanisole' (BHA) or nordihydroguaiaretic acid (NDGA). It was susceptible to light, oxygen and heat. It was demonstrated by thin-layer chromatography that reaction products were formed in a 0.1% solution in benzene:methanol = 1:1 upon exposure to daylight. At high temperatures under acid or alkaline circumstances the main phenolic compound tends to resinify.

The main component has been isolated as its acetyl derivate by Florisil column chromatography with chloroform as solvent (Harms, 1968; Harms & Wurziger, 1968). By hydrolysis with 0.5 N alcoholic KOH deacetylation occurs. Thereupon the solution was diluted with water, adjusted at pH = 3 and extracted with ether. After evaporation of the solvent a residue was obtained which was recrystallized from benzene, the white crystals showing a m.p. of $120^{o}C$. Structural analysis indicated the presence of three compounds derived from a combination of the primary amino group of 5-hydroxytryptamine and arachidic acid (n = 18), behenic acid (n = 20) or lignoceric acid (n = 22) in a ratio of 12:12:1 (XXI) respectively (C-5-HT). According to the authors
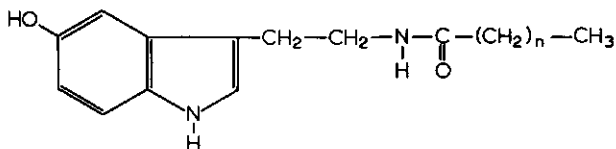
N_{β} - alkanoyl - 5 - hydroxytryptamine (XXI)

C-5-HT is only present in the surface wax of the bean. It is therefore used as an indicator for the amount of coffee wax present in green or roasted beans. Until now the structure of the other Gibbs-positive compounds is unknown.

Harms & Wurziger (1969) reported a method for the quantitative determination of C-5-HT. The procedure includes extraction of the grind with methanol under reduced pressure, purification by column chromatography, circular thin-layer chromatography of the eluate on a Na_2CO_3-impregnated silica gel G plate, with chloroform:ethanol (96%) = 9:1 as solvent and spraying with a 2,6-dichloroquinone chlorimide solution. The blue band which results from the reaction between reagent and C-5-HT is scraped off, eluted from the adsorbent and measured at 580 nm. In this way 500-1000 ppm C-5-HT was found in various samples of green beans. Values of 800-1800 ppm have been found for the total

group of Gibbs-positive substances in the wax of green beans. No large differences exist between Arabica and Robusta coffee. Harms & Wurziger (1969) indicated that the coffee fruit processing conditions as well as the storage time of the green beans are of importance for the amount of C-5-HT. No C-5-HT has been found in the mesocarp. Small amounts appeared in the parchment layer.

C-5-HT has been detected in other oil seeds too (Wurziger & Harms, 1973). Considerable C-5-HT contents occur in Brazil nuts (*Bertholletia excelsa* Humb. & Bonpl.). Amounts of 240-790 ppm C-5-HT have been found in the seed kernel of Brazil nuts. As for coffee, C-5-HT is located in the outermost parts of the seed too. Only traces could be detected in the seed coat. C-5-HT in Brazil nuts is mainly composed of the derivates with the palmitic and stearic acid: the derivates of arachidic, behenic and lignoceric acid occur in smaller quantities. According to the authors the shell of cocao beans (*Theobroma cacao* L.) contains 18-57 ppm C-5-HT. After removal of the shell only traces were reported in the seed itself. C-5-HT is mainly located on the inner surface of the shell. In cocao beans only the behenic and lignoceric acid derivates have been detected. Small amounts of C-5-HT have also been found on the inner surface of the shell of both sunflower seed (*Helianthus annuus* L.) and walnuts (*Juglans regia* L.). It has been assumed that the phenolic compounds in the outermost floral parts provide a protective factor against oxidative deterioration (Harms & Wurziger, 1969).

3.3 DISCUSSION AND LAYOUT OF THE STUDY

In the preceding sections the lipids of green coffee beans have been subdivided into coffee oil and coffee wax. The chemical composition of coffee oil has been investigated thoroughly. However quantitative measurements on percentage oil and the oil characteristics as reported in the literature quoted show a lack of consistency. Only a few studies have been made on coffee wax. Until now only C-5-HT has been reported as a specific constituent.

According to reviews by Kolattukudy (1970a; 1970b), Martin & Juniper (1970) and Hamilton & Hamilton (1972), plant waxes are known as cuticular constituents in many floral parts like leaves and fruits. In seeds a cuticle can develop between the testa and the remains of the nucellus or the endosperm. The wax is embedded within the cuticle of certain plant parts and sometimes exudes over the surface of the cuticle (epicuticular wax).

Although waxes occur primarily as cuticular constituents, they have been

found in other plant parts too (Kolattukudy, 1970a). For instance jojoba seeds contain 40-50% of a wax which is characterized by a high content of mono unsaturated C_{20} and C_{22} acids and alcohols (Miwa, 1971).

The term wax is used to denote a class of substances which are defined by physical properties such as plasticity rather than by a precise chemical constitution. Waxes are complex mixtures of aliphatic hydrocarbons, primary and secondary alcohols and α, ω-diols, aldehydes, ketones and β-diketones, monocarboxylic and dicarboxylic acids and long chain esters with about 50 C-atoms. Less common are diterpenes and triterpenes, flavones and aromatic hydrocarbons.

In the more classical chemical concept waxes were considered to be esters, composed of long-chain primary alcohols and long-chain fatty acids (Pardun, 1969, p.918). According to this concept, Kaufmann & Hamsagar (1962b) pointed out that the esters of diterpene alcohols and fatty acids in coffee oil can be considered as waxes too.

A reduction of the material of the bean soluble in petroleum ether from 14.8% to 12.5% was found as a result of wax removal by polishing with sand (polishing loss = 6.2%) (Dickhaut, 1966). According to these figures Dickhaut suggested that certain compounds soluble in petroleum ether might be specific coffee wax constituents.

The main purpose of this study was to gain more knowledge about the proportion of the main groups of coffee lipids and their individual constituents in coffee wax and coffee oil.

With the literature discussed in this chapter taken into account this study was concentrated on the following points:
1. Method of isolation of coffee oil in its relation to the chemical composition of the oil (Section 5.1).
2. Method of isolation of coffee wax (Section 5.2).
3. Extraction of the petroleum ether soluble substances from coffee wax and comparison of its composition with that of coffee oil after wax removal (wax-free coffee oil) (Chapter 6).
4. Investigation of the petroleum ether insoluble matter of coffee wax (Chapter 7).

4 Materials and methods

4.1 ISOLATION

Isolation of coffee wax (a in Fig. 2) Unground green beans[1] were extracted
with chloroform[2] either by stepwise extraction in a Soxhlet to study the
composition of five successive wax isolates (total extraction time amounted
to 1000 min) or by refluxing and stirring for 30 min, conditions resembling
those as described for the removal of coffee wax on technical scale (Chap-
ter 3). After evaporation of chloroform the *petroleum ether (40-60°C) sol-
uble substances* were extracted from the wax by refluxing with the solvent
for 4 times 15 min (b). The residue constitutes the *petroleum ether insol-
uble substances* of the wax.

Grinding procedure (c) Coarse grinds were obtained from green beans using
an "Olland" disc crusher. Finely ground material (finer than 0.5 mm) was
prepared from coarse grinds with a "Retsch ZM 1" centrifugal mill, equipped
with a sieve of 0.50 mm screen opening.

Isolation of coffee oil (direct extraction procedure) (d)
material were extracted with petroleum ether (40-60°C) in a Soxhlet, si-
phoning 6-7 times per h during 24 h unless mentioned otherwise, using
Schleicher and Schüll extraction thimbles 28 x 80 mm No. 603. The solution
was kept overnight in the refrigerator and then crystallized caffeine was
filtered off by suction (0.2 µm membrane filter no. 11407, Sartorius GmbH,
Göttingen). The filter was rinsed with cold petroleum ether (40-60°C) twice.

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1. If not mentioned otherwise green Columbian Arabica coffee beans, supplied
by D.E.J. International Research Company B.V., Utrecht, the Netherlands,
were used.

2. All solvents used in this work are analytical grade.

Unground beans
|
Extraction
with
chloroform (a)

Coffee wax   Wax-free beans
    Extraction with   Grinding (c) and   Grinding (c) and
    petroleum ether   extraction with   extraction with
    (40-60°C)(b) petroleum ether   petroleum ether
                          (40-60°C)(d) (40-60°C)(d)

Petroleum ether Petroleum ether Wax-free coffee Coffee oil
insoluble part   soluble part   oil

    Fractionation
    on a polyamide
    column (g)

                     Fractionation   Saponification   Saponification
                     on a Florisil   and   and extraction
                     column (e)   methylation(h)   with ether (i)

Caffeine,C-5-HT
and others

        Triglycerides and   Fatty acids   Unsaponifiable
        esters of fatty   matter
        acids and diterpene
        alcohols

                                                  Fractionation
                                                  on a Florisil
                                                  column (f)

                                               Hydrocarbons,
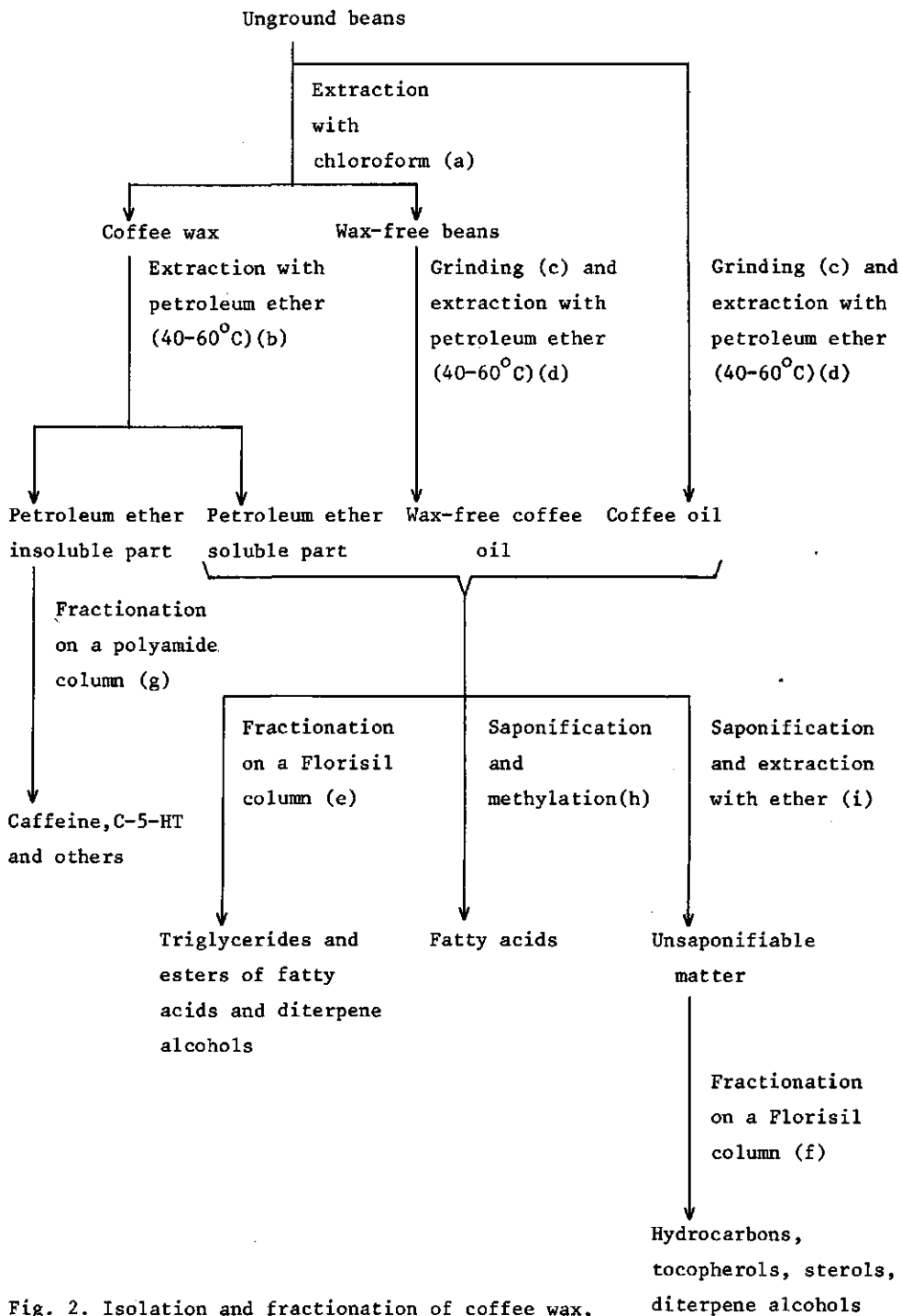   tocopherols, sterols,
Fig. 2. Isolation and fractionation of coffee wax,   diterpene alcohols
coffee oil and wax-free coffee oil.

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For the experiments reported in Section 5.1 the filtrate was diluted to 250 ml with the solvent. From 200 ml of this solution the solvent was evaporated. The residue was used for further analysis. The remaining 50 ml was evaporated separately, the residue was dried to constant weight (2-3 h) at 105°C and the percentage of oil was calculated on dry basis. A comparison with the percentage of oil determined after the acid-digestion procedure indicated the yield of direct oil extraction. For the experiments reported in Chapter 6 the filtrate was used for further analysis.

If oil was extracted from beans from which coffee wax had been removed according to one of the two wax isolation procedures mentioned before, this oil is called 'wax-free' oil.

4.2 FRACTIONATION

Fractionation of petroleum ether soluble substances (e)   150 mg of petroleum ether soluble substances were chromatographed on a column of 15 g of Florisil 60-100 mesh (BDH 15025) (24 x 1.4 cm), deactivated with 6% (w/w) distilled water and a stepwise elution gradient of ether in hexane. The eluate was collected in fractions of 25 ml. The amount of material present in the fractions was studied gravimetrically. The composition of the fractions was studied by t.l.c. on commercially prepared silica gel 60 plates (Merck 5715) with benzene:ether = 4:1 as solvent. Triglycerides were detected by spraying with 0.1% 2',7'-dichlorofluorescein (Merck 9677) and judging under u.v. light of 254 nm. The diterpene alcohol esters readily become brown upon spraying with 3.5% phosphomolydic acid (Merck 531) according to Kaufmann & Hamsagar (1962a). Both reagents are non-specific and only give an indication for the presence of the compounds mentioned above. Next the fatty acid compositions of the triglycerides and the diterpene alcohol esters were determined by g.l.c. (Section 4.3).

Fractionation of the unsaponifiable matter (f)   2-2.5 g of unsaponifiable matter was chromatographed on a column of 50 g of Florisil 60-100 mesh (34 x 2 cm) with a stepwise eluotropic series of hexane, benzene, 5% ether in benzene, 10% ether in benzene and ether. The eluate was collected in fractions of 50 ml. The amounts of material present in the fractions were determined gravimetrically. The composition of the fractions was generally studied by t.l.c. The following colour tests were used:
- Liebermann-Burchard reagent. Plates are successively sprayed with chloro-

form:acetic acid anhydride = 1:1 and with concentrated sulfuric acid. The
plates are heated for 5-15 min at 110°C. Upon spraying with this reagent
sterolic compounds show the following colour sequence: red, violet, blue,
and blue-green (Brieskorn & Hofmann, 1964; Holloway & Challen, 1966). Other
compounds like unsaturated acids turn brown-yellow with this reagent.
- Antimony trichloride. Plates are sprayed with a solution of 20 g of $SbCl_3$
in 20 ml of glacial acetic acid + 60 ml of chloroform. The plates are heated
for 5-15 min at 100°C (Stahl, 1967). This reagent reacts with many constit-
uents of fats and oils (Holloway & Challen, 1966; Richter, 1965). Sterolic
compounds become orange, red or violet (Lisboa, 1969).
- Phosphomolybdic acid (Merck 531). This reagent is also non-specific
(Stahl, 1967). According to Kaufmann & Hamsagar (1962a) cafestol and kahweol
become red to red-brown with this reagent.
- 2,2'-bipyridyl-$FeCl_3$. Plates are sprayed with equal volumes of 0.5% of
$FeCl_3$ in ethanol and 0.5% of bipyridyl (Merck 3098) in ethanol. With toco-
pherols a red reaction is observed (Stahl, 1967). This reagent was also
found to react with other phenolic substances.

Fractionation of the petroleum ether insoluble substances (g) These sub-
stances were fractionated by chromatography on a column of polyamide (Woelm
03282) eluted with an eluotropic series of continuously increasing strength.
The amounts of material present in the eluate were determined gravimetrical-
ly, collecting fractions of 250 ml.

4.3 ANALYTICAL METHODS

Sieve analysis This was done with a 'Haver & Boecker' laboratory sieve
device EML with control sieves 'Metaalgaas Twente' standard N480 screen
openings ranging from 0.15 to 1.70 mm.

Percentage of oil in green beans (acid-digestion procedure) Measured as the
percentage of petroleum ether (40-60°C) soluble material in grinds of green
beans (< 0.63 mm) which were previously digested with 4 N HCl according to
the Swiss official method (Schweiz.Lebensm.Buch, method 35A/08, 1973). The
percentage was calculated on dry weight basis. All determinations were done
in duplicate unless mentioned otherwise.

Moisture content of the grinds This was defined and determined in duplicate as the loss on drying to constant weight (2-3 h) at 105°C.

Fatty acid analysis (h) Fatty acid methyl esters were prepared according to the Dutch official method (NNI, 1972) which includes saponification of the sample and methylation of total amount of fatty acids with 2% methanolic KOH and 14% BF_3-methanol. The methyl esters were analysed by gas chromatography, using a Hewlett Packard Research Chromatograph 5750G with flame ionization detector (FID) coupled with an Infotronics integrator CRS-208. Conditions: 6 ft $\frac{1}{4}$" i.d. stainless steel column packed with 10% Silar-5CP (Appl.Science phase ref. 89) on Gaschrom Q 100-120 mesh (Appl.Science 02203). Temperature programme: 175°C-225°C with 1°C/min (dual column system). Carrier gas (N_2): 19 ml/min. The fatty acid methyl esters were identified by comparison of their retention times with those of reference compounds (Appl.Science mixtures K108 and H104). Their percentages were calculated as percentage of the total peak area. According to Ackman (1969) the peak area of the fatty acid methyl esters is directly proportional to the weight percentage within a narrow range of chain lengths and for molecules with 0-3 double bonds. Determinations were done in duplicate.

Unsaponifiable matter This was measured as percentage of the oil after the AOAC (method 26.071, 1965). All determinations were done in duplicate unless mentioned otherwise. In this work the procedure was used for quantitative determinations and for preparative purposes (i) as well.

Carbon number of the triglycerides This was determined by g.l.c. using a Hewlett Packard Research Chromatograph 5750G with FID equipped with a dual column system of two 3 ft 1/8" i.d. stainless steel columns, packed with 3% SE-30 GC-grade on Gaschrom Q 100-120 mesh. Column temperature programme: 200-350°C with 8°C/min. Carrier gas (N_2): 20 ml/min. The peaks were identified by comparison of the elution temperatures with those of known references (Appl.Science 18902; Supelco 04-4351 and 04-4358).

The positional distribution of the fatty acids in the triglycerides
This was studied by partial deacylation with pancreatic lipase according to the method of Hollstein et al. (1973) with some minor modifications: in a glass stoppered flask (10 ml) 100 mg of triglycerides together with 2 ml of a 1 M Tris-HAc buffer (pH = 8), 0.5 ml of a 0.1% solution of bile acids (Merck 4054) and 0.2 ml of a 22% $CaCl_2$ solution were heated for 3 min at 50°C

in a water bath. Next the mixture was emulsified in a Gallenkamp orbital shaker for 3 min at 38°C (300 rev/min). Thereupon 50 mg of pancreatic lipase (Steapsin, Sigma L 3127) was added and shaking was continued for 6 min at 38°C (300 rev/min). The reaction was stopped by adding 5 ml of ether. The mixture was shaken vigorously and the ether was separated from the solution of hydrolysis in a small separation funnel. The solution was extracted for another time with ether and the solvent was evaporated from the combined ether extracts. The resulting mixture of glycerides was separated on a column of 15 g of Florisil (60-100 mesh), deactivated with 6% distilled water under similar conditions as described before. Triglycerides and diglycerides are first eluted with 15% and 50% ether in hexane respectively, whereas the monoglyceride fraction is obtained with 100% ether. The composition of the fractions was studied on thin layer plates which were prepared from a suspension of 30 g silica gel H (Merck 7736) in 60 ml 0.4 M boric acid. After drying at room temperature the plates were activated at 105°C for 2 h. As solvent chloroform:acetone:alcohol (96%) = 91:8:1 was used. The spots were detected upon spraying with 2',7'-dichlorofluorescein under u.v. light (254 nm). The fatty acid composition of the monoglycerides was determined by g.l.c. as described before.

Gas chromatography of the hydrocarbons was done on a dual column operated Hewlett Packard Research Chromatograph 5750G with FID equipped with two 6 ft 1/8" i.d. stainless steel columns with 5% SE-30 GC grade on Chromosorb W-AW 80-100 mesh. Column temperature programme: 125-300°C with 4°C/min. Carrier gas (N_2): 20 ml/min. The elution temperatures of the peaks were compared with those of known references of *n*-alkanes (supplied by W.Ch.Melger, Laboratory of Organic Chemistry, Agricultural University, Wageningen, the Netherlands). Using the same apparatus, *squalene* as well as the *methylsterols* and *4,4-desmethylsterols* were analysed on a 6 ft 1/8" i.d. stainless steel column packed with 10% UCCW-982 on Chromosorb WAW-DMCS 80-100 mesh at a column temperature of 250°C. Carrier gas (N_2): 18 ml/min. Upon derivatization with *N*-methyl-*N*-TMS-trifluoroacetamide (Pierce 48910) cafestol was successfully chromatographed under these conditions as well. The following reference samples were available: squalene (Sigma S 3626); sitosterol (Merck 3741); stigmasterol (Merck 3743); campesterol (Appl.Science 19549); cycloartenol and 24-methylenecycloartanol (from H.J. Nicolas, St. Louis University School of Medicine, USA); citostadienol (from J.W. Rowe, Forest Products Laboratory, U.S. Department of Agriculture, Madison, Wisconsin, USA).

Spectral analysis The u.v. spectra in hexane were run on a Varian Cary 118 and the i.r. spectra in chloroform were recorded with a Hitachi EPI-G3. The n.m.r. spectra were obtained with a Varian XL-100 in deuterochloroform with tetramethylsilane as internal standard. The mass spectra were determined with an A.E.I. M.S. 902.

Quantitative tocopherol analysis From 2 g of finely ground material (finer than 0.5 mm) a tocopherol rich unsaponifiable fraction was prepared according to the general procedure of Slover et al. (1969), which includes extraction with ethanol at room temperature, saponification with KOH/ethanol under N_2 and recovering of the unsaponifiable matter with petroleum ether (40-60°C). Pyrogallol was added during extraction and saponification to minimize oxidation. A solution of the unsaponifiable fraction in ether was quantitatively applied across the bottom of a commercially prepared 20 x 20 cm silica gel $60F_{254}$ plate (Merck 5744) 0.5 mm in thickness, together with two spots of a tocopherol rich reference along both sides of the plate. Plates were developed with chloroform twice. After development the sides were sprayed with the 2,2'-bipyridyl-$FeCl_3$ reagent whereupon tocopherols appeared as red spots. Upon examination of the plate under short wave u.v. light the corresponding bands were marked and scraped off. After elution with ether and removal of the solvent under N_2, 0.3 ml of an internal standard (13.6 mg of squalane/100 ml of hexane; squalane, Merck 9766) was added and the solvent was evaporated again. Then derivates were prepared by addition of 150 µl of *N*-methyl-*N*-TMS-trifluoroacetamide (Pierce 48910) to the mixture in closed reaction vials (Pierce 13222) under N_2. The sample was analysed by g.l.c. with a Hewlett Packard Research Chromatograph 5750G with FID coupled with an Infotronics integrator CRS 208. Conditions: 6 ft 1/8" i.d. stainless steel column with 10% UCCW 982 on Chromosorb WAW-DMCS. Column temperature = 250°C. Carrier gas (N_2) : 20 ml/min. Determinations were done in duplicate.

For the construction of a standard curve α-tocopherol (Merck 8283) was purified by chromatography on a 10 x 2.1 cm Al_2O_3 column (activity II-III, Merck 1097) eluted with successively 200 ml of 4% benzene in petroleum ether (40-60°C), 200 ml of a 10% mixture and 200 ml of a 15% mixture. The last 200 ml consisted of pure α-tocopherol as indicated by t.l.c. A standard solution of 141 mg of α-tocopherol/100 ml of hexane was prepared and a standard curve was constructed using a fixed amount of 0.3 ml of the internal standard solution mentioned before and various amounts, within the range of 0.05-0.5 ml, of the α-tocopherol solution. The area ratio was plotted against the weight ratio.

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C-5-HT content of the beans Determined in ppm of the bean weight on dry basis after the method of Harms & Wurziger (1969) with some modifications (Noomen, P.J. & G.D.H. van der Stegen, 1975, pers. comm.). After extraction of the grind with methanol under reduced pressure and purification by Al_2O_3 column chromatography the solution containing C-5-HT was separated by t.l.c. on silica gel plates impregnated with Na_2CO_3. Benzene:ethylacetate = 1:1 was used as solvent. After development the plates were sprayed with 0.05% of 2,6-dichloroquinone chlorimide in petroleum ether (40-60°C) whereupon C-5-HT and the other phenolic compounds in coffee wax appeared as blue spots. The amount of C-5-HT was measured by comparison with a reference of known concentration using the Vitatron TLD 100 densitometer.

Electron microscopy Apparatus: Philips transmission electron microscope, type EM 300.
A small section of the outermost part of the bean was prepared for electron microscopy as follows:
- pre-fixation in a 12.5% solution of glutaraldehyde in 0.2 M cacodylate buffer (pH = 7.4) for 6 h
- washing overnight in the same buffer with 7% saccharose
- post-fixation for 2 h in a solution of 1% osmium tetraoxide in a veronal/ acetate buffer (pH = 7.4)
- staining for 1 h in 2% uranyl acetate in distilled water
- dehydration in ethanol
- embedding in epon-araldite.

5 Isolation and composition

5.1 COFFEE OIL (Folstar et al., 1975a)

As pointed out in Section 3.1.1, for the determination of the amount of
oil in green beans it is preferable to use finely ground material (particle
size < 0.63 mm) treated with 4 N HCl, followed by an extraction with petrol-
eum ether (b.p. < 60°C) as described by the Swiss official method (Schweiz.
Lebensm.Buch, method 35A/08, 1973). However, this procedure is restricted to
oil content determinations and is not suitable for the extraction of an oil
to be used for the study of its chemical composition. Then acid digestion
should be avoided and direct solvent extraction should be employed.

The yield of the extraction depends on the particle sizes of the grind.
Table 4 shows that for beans which have been coarsely ground with an
'Olland' disc crusher, the extraction of the oil is incomplete: even after
16 and 24 h extraction no significant improvement of the yield of extraction
was observed. This is in agreement with the results of Streuli et al. (1966).
On the other hand the data for beans, which have additionally been ground
with a 'Retsch ZM 1' centrifugal mill (< 0.50 mm), indicate a complete
extraction.

For the direct extraction of fine grinds, substantial amounts of caffeine
had to be removed from the oil as indicated in Section 4.1. Thin-layer
chromatography of the residue on the membrane filter used showed that no
other components were present. In spite of these precautions the oil still

Table 4. Oil content on dry basis of grounds obtained from green coffee
beans by two different grinding methods in relation to the extraction time
(all determinations were performed in quadruplicate).

	Oil (%) direct extraction with petroleum ether (40-60°C)			Oil (%) according to Schweiz. Lebensm.Buch (method 35A/08, 1973)
	6h	16h	24h	
Coarse (Olland)	8.33	10.64	10.65	-
Fine (Retsch)	15.52	15.50	15.52	15.53

Table 5. Percentage unsaponifiable matter and fatty acid composition in cof-
fee oil after different grinding methods of green coffee beans and 6h direct
extraction with petroleum ether (40–60°C) (all determinations were performed
in quadruplicate).

	Fine (Retsch)	Coarse (Olland)	Coarse, sieve fraction < 0.6 mm
Oil (% on dry basis)	15.62	8.33	–
Unsaponifiables (% in the oil)	9.00	9.81	–
Fatty acids: $C_{16:0}$	31.06	35.55	33.68
$C_{18:0}$	9.56	7.45	8.43
$C_{18:1}$	9.57	7.57	8.34
$C_{18:2}$	43.12	45.05	44.45
$C_{18:3}$	1.77	0.93	1.06
$C_{20:0}$	4.07	2.70	3.05
$C_{22:0}$	0.87	0.76	1.00

contained traces of caffeine which caused a slight inaccuracy in the yield
calculation.

In Section 3.1.2 it has been discussed whether the composition of an oil
obtained by incomplete extraction is representative for the total amount of
oil present in the bean. To answer this question oils from the two grinds
described above were tested for differences in both fatty acid composition
and percentage unsaponifiable matter (Table 5). Obviously differences exist
in the fatty acid composition. These differences are only due to the particle-
size distribution and not to the way of grinding or the use of a particular
crushing device: the particle-size distribution (Fig. 3) and the fatty acid
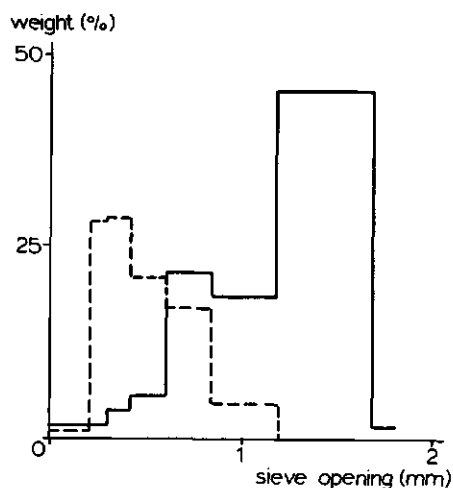

Fig. 3. Sieve analysis of coarse-
ly (———) and finely (– – –) ground
green coffee beans.

Table 6. Percentages oil and unsaponifiable matter in green and roasted coffee beans.

		Own experiments[1]	Literature[2]		
			a	b	c
Oil (%)	: green	16.21	6.65- 7.04	14.5-15.7	9.07-12.97
	roasted	16.91	15.56-16.16	16.3-16.5	11.27-16.05
Unsaponi-	: green	9.96	10.42-11.39	11.4-12.6	6.40- 6.62
fiables (%)	roasted	10.12	9.04- 9.53	10.0-12.0	5.65- 6.25

1. Oil (%) according to Schweiz.Lebensm.Buch (method 35A/08, 1973) on dry basis.
2. a: Kaufmann & Hamsagar (1962b). b: Ravindranath et al. (1972).
   c: Carisano & Gariboldi (1964).

composition of a sieve fraction, smaller than 0.60 mm, which has been obtained from the coarse grind, lie between those of coarsely and finely ground material. It can also be seen that the oil extracted from a coarse grind has a higher percentage of unsaponifiable matter.

Literature data summarized in Table 6 indicate that the percentage of unsaponifiable matter decreases as a result of the roasting process while the percentage oil increases. However my own data for an oil extracted from fine grinds of green and roasted coffee do not confirm these literature data. There was no loss of unsaponifiable matter upon roasting: the relatively high percentage in green beans as reported in the literature was apparently due to incomplete oil extraction. This proves the importance of a reliable extraction procedure.

Comparison of the percentages of oil obtained by direct extraction of several sieve fractions with those determined after the official total oil assay leads to the conclusion that the sieve fraction of 0.15-0.42 mm is suitable for a total coffee oil analysis (Table 7). After 6 h direct ex-

Table 7. Oil content on dry basis of three sieve fractions from coarsely ground green coffee beans.

Sieve openings (mm)	Oil (%) direct extraction with petroleum ether (40-60°C) for 6h	Oil (%) according to Schweiz. Lebensm.Buch (method 35A/08, 1973)
0.15-0.42	15.51	15.54
0.42-0.60	13.10	15.66
0.60-0.85	9.36	14.06

traction only 0.35% of oil was found in the residual grind according to the Swiss assay. In all cases pre-drying of the grind was omitted.

5.2 COFFEE WAX

Like most plant waxes, coffee wax readily dissolves in chloroform. Without contamination by cytoplasmatic constituents surface waxes can be isolated by short-time immersions in this solvent at room temperature (Martin & Juniper, 1970). In a preliminary experiment this technique was found to give only a partial reduction of the wax content in coffee beans as indicated by the C-5-HT concentration: dipping of unground beans for six successive times of 10 s each in chloroform resulted in a reduction of the C-5-HT content from 1250 ppm to only 1020 ppm. According to the technical process, dealing with wax removal (Chapter 2), the extraction has to be performed with hot solvent and stirring benefits the extraction.

In order to study the procedure of wax isolation five thimbles, each containing 50 g of unground beans, were extracted with chloroform in a Soxhlet, siphoning 6-7 times per h. Total time of extraction was 1000 min. At five successive times during the extraction the composition of the wax according to its solubility in petroleum ether (40-60°C) was determined (Fig. 4). Apparently this composition depends on the time of extraction: the longer
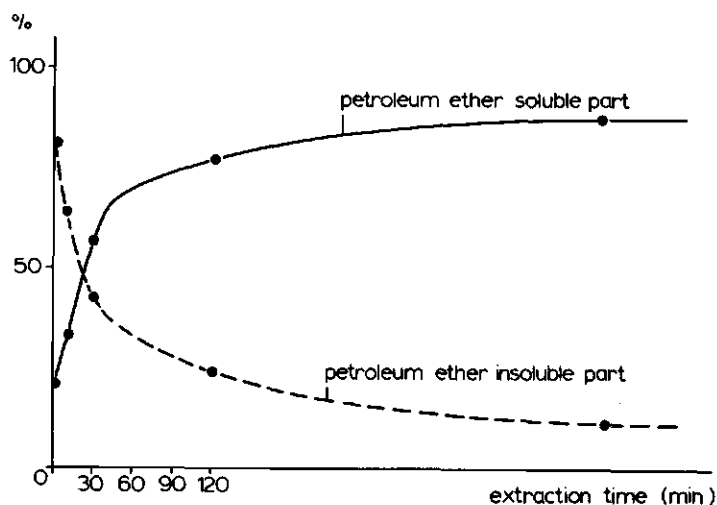

Fig. 4. Changes in composition of coffee wax according to the solubility in petroleum ether (40-60°C) during stepwise extraction of unground beans with chloroform in a Soxhlet.

the extraction lasts, the higher the ratio between the amounts of petroleum
ether soluble and insoluble substances in the wax. At the same time the ab-
solute quantity of C-5-HT in the bean decreases from 1250 to 100 ppm.

Next the wax-free coffee beans were ground and extracted with chloroform.
The chloroform was evaporated and the residue extracted with petroleum ether
(40-60°C). Thin-layer chromatography indicated that, apart from traces of
C-5-HT and other Gibbs positive compounds, the petroleum ether insoluble
fraction in these wax-free beans only consists of caffeine.

These results show that the petroleum ether insoluble substances in
coffee wax are important in view of the claims of the technical process for
reduction of the amount of wax in the beans (Chapter 2) since these sub-
stances, apart from caffeine, are almost quantitatively extracted from the
bean during coffee wax removal.

However the question remains to be answered whether the petroleum ether
soluble substances in coffee wax are also important in view of these claims.
According to the results shown in Table 8 the amount of petroleum ether
soluble substances in coffee wax is about 1% of the percentage of petroleum
ether soluble substances in the bean, expressed as percentage of the weight
of the bean on dry basis. Moreover the percentage of unsaponifiable matter
in coffee oil was almost the same before and after wax removal. On the basis
of these data the petroleum ether soluble substances in coffee wax could be
considered as being of only minor importance. However a conclusive answer
to the question can only be obtained by comparing the chemical composition
of the petroleum ether soluble substances in the wax with that of wax-free
oil (see Chapter 6).

In another experiment the amount of coffee wax and its composition were
determined for a wax, obtained by stirring and reluxing 300 g of green
beans for 30 min with 300 ml of chloroform, conditions resembling the

Table 8. Effect of wax removal[1] on the percentages coffee oil and unsaponi-
fiable matter in the oil.

	Oil (%)[2]	Unsaponifiables (%)
Before removal	15.60	9.92
After removal	14.76	10.00

1. Unground beans were extracted for 1000 min in a Soxhlet with chloroform.
This corresponds to a C-5-HT reduction from 1250 to 100 ppm.
2. Determined after Folstar et al. (1975a).

Table 9. Composition (%) of coffee wax obtained from unground green beans by stirring and refluxing for 30 min with chloroform.

Amount of wax (% of green bean on dry basis)	0.23	
Petroleum ether soluble part in the wax :	36.8	
Unsaponifiable matter		10.1
Fatty acids		23.9
Rest (mainly glycerol)		2.8
Petroleum ether insoluble part in the wax :	63.3	
C-5-HT		25.3
Rest		38.0

technical process for reduction of the amount of wax in the bean (Chapter 2). The results are shown in Table 9.

In the literature it was found that C-5-HT and the other compounds showing a positive reaction with Gibbs reagent occur as a surface wax on the epidermis of the green bean (Chapter 2). An electron micrograph of a section through the outermost part of the bean shows a part of an epidermical cell (Fig. 5). The outermost osmophilic layer is insoluble in chloroform. Under the light microscope the surface turns red with Sudan IV. Comparison with the literature (Noel & van Staden, 1976) showed that this layer can be considered as a thin cuticle (1.5-2 μm in thickness). However more experiments, which do not lie within the scope of this work, have to be done to obtain more conclusive results about the location and structure of the cuticular wax.

Fig. 5. Electron micrograph of a section through the outermost part of a green coffee bean (x 8.300) (made by the Department of Electronmicroscopy, Technical and Physical Research Service (TFDL), Wageningen, the Netherlands).

6 Petroleum ether soluble substances

The composition of coffee wax depends on the extraction time of unground green beans with chloroform: the longer the extraction lasts, the higher the ratio between the amounts of petroleum ether soluble and insoluble materials (Section 5.2). It is likely that the petroleum ether soluble substances in the wax are at least partially derived from cytoplasmatic coffee oil which is removed as a result of the long extraction time of the unground beans with chloroform. But certain petroleum ether soluble substances may specifically occur in coffee wax.

In this chapter the compositions of both the petroleum ether soluble substances in coffee wax and coffee oil are studied. This study is subdivided into the investigation of the composition of the total fatty acids (Section 6.1), the positional distribution of the fatty acids in the triglycerides (Section 6.2) and the composition of the unsaponifiable matter (Section 6.3).

6.1 THE FATTY ACID COMPOSITION (Folstar et al., 1975b)

300 g of unground beans were extracted in a Soxhlet with five successive portions of chloroform (see also Section 5.2). The chloroform was evaporated off and the residues were extracted with boiling petroleum ether $(40-60^{o}C)$ for 4 times 15 min. The wax-free beans were ground finer than 0.5 mm and extracted for 24 h with petroleum ether $(40-60^{o}C)$. The fatty acid composition of the petroleum ether extracts from the wax and the oil obtained from wax-free beans is given in Table 10. It can be seen that in the wax relatively large amounts of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ are found whereas the percentages of $C_{16:0}$ and the unsaturated acids are relatively small.

These figures caused me to examine the distribution of the fatty acids between wax and wax-free oil for both the triglycerides and the diterpene alcohol esters. Therefore the petroleum ether extracts, which were obtained as described before, were subjected to a fractionation on a column of Florisil (60-100 mesh) deactivated with 6% distilled water and a stepwise elution

Table 10. Fatty acid composition (%) of the petroleum ether soluble material in coffee wax during stepwise extraction of unground beans and in wax-free oil

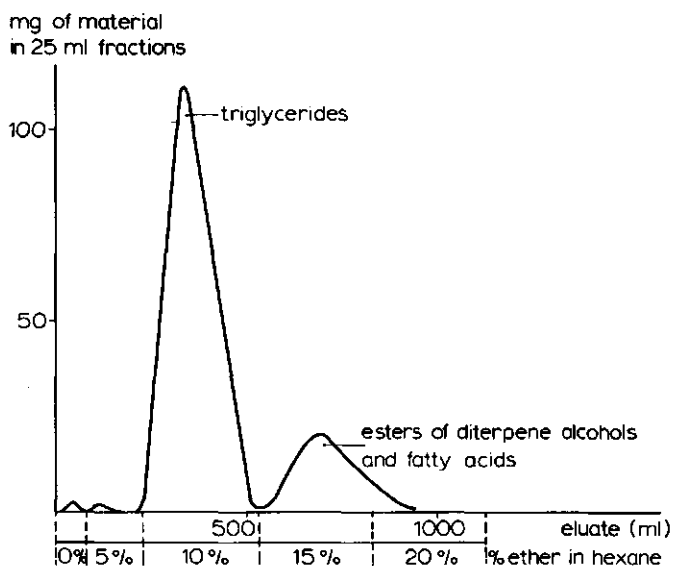|	Wax					Wax-free oil
	Time of extraction with chloroform (min)					
	1	15	30	120	1000	
$C_{14:0}$	traces	traces	traces	traces	traces	traces
$C_{16:0}$	21.8	29.4	33.2	36.0	37.1	34.4
$C_{18:0}$	6.6	7.7	8.2	8.1	8.3	8.4
$C_{18:1}$	3.7	5.7	6.7	7.1	7.4	8.6
$C_{18:2}$	15.1	28.7	34.8	39.5	40.2	42.9
$C_{18:3}$	0.3	1.0	1.4	1.3	1.6	1.6
$C_{20:0}$	17.8	10.4	7.3	5.1	4.2	3.4
$C_{22:0}$	29.7	14.4	6.8	2.4	1.1	0.7
$C_{24:0}$	5.0	2.8	1.6	0.4	-	-

Fig. 6. Fractionation of the main components of the petroleum ether soluble part of coffee wax as well as wax-free coffee oil on a Florisil (60-100 mesh) column, deactivated with 6% distilled water. Elution with a gradient of ether in hexane.

gradient of ether in hexane (Fig.6). For both the triglycerides and the diterpene alcohol esters the fatty acid composition was determined.

For the triglycerides the percentages of $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ in the wax and in the wax-free oil are almost the same (Table 11). It is of considerable interest that coffee wax exhibits a lower value for $C_{18:2}$ and higher

Table 11. Fatty acid composition (%) of triglycerides in coffee wax obtained by stepwise extraction of unground beans and in wax-free oil.

	Wax					Wax-free oil
	Time of extraction with chloroform (min)					
	1	15	30	120	1000	
$C_{14:0}$	0.9	0.4	0.3	0.3	0.2	0.2
$C_{16:0}$	29.0	32.5	31.4	31.2	30.5	33.3
$C_{18:0}$	7.6	7.7	8.2	8.7	8.2	7.3
$C_{18:1}$	5.4	5.8	6.5	7.3	8.6	6.6
$C_{18:2}$	37.2	43.1	45.8	45.8	47.2	47.7
$C_{18:3}$	1.1	1.7	1.5	1.6	1.9	1.7
$C_{20:0}$	10.2	5.6	4.1	3.8	2.5	2.5
$C_{22:0}$	7.3	2.5	1.2	1.0	0.6	0.5
$C_{24:0}$	1.4	0.6	0.5	0.4	0.3	0.3

Table 12. Fatty acid composition (%) of diterpene alcohol esters in coffee wax obtained by stepwise extraction of unground beans and in wax-free oil.

	Wax					Wax-free oil
	Time of extraction with chloroform (min)					
	1	15	30	120	1000	
$C_{14:0}$	traces	traces	traces	traces	1.8	1.5
$C_{16:0}$	16.7	18.3	38.4	47.8	51.8	50.2
$C_{18:0}$	4.0	4.0	7.7	9.0	8.7	8.9
$C_{18:1}$	3.2	3.3	7.0	7.9	6.7	7.2
$C_{18:2}$	9.9	12.9	21.9	24.4	24.3	25.8
$C_{18:3}$	traces	traces	0.7	0.9	0.6	0.8
$C_{20:0}$	26.7	24.0	10.7	6.4 ·	4.8	4.5
$C_{22:0}$	34.4	32.6	11.5	3.0	1.2	1.1
$C_{24:0}$	5.1	4.9	2.2	0.7	traces	traces

values for $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ than wax-free oil.

The figures for the diterpene alcohol esters are even more striking (Table 12). An increase of the percentages of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ with extraction time up to its final concentration in wax-free oil and a sharp reduction of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ can be observed.

Since green beans contain only 0.2-0.3% of wax but about 10-16% of wax-free oil the absolute amount of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ in the bean after wax removal is still considerable. Gas-liquid chromatography with margaric acid

($C_{17:0}$) as internal standard indicated a reduction of only 5% of $C_{22:0}$ as a result of 1000 min wax extraction in the Soxhlet with chloroform.

From these results it can be concluded that a concentration gradient of fatty acids exists in the green bean in such a way that the saturated acids $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ are preferably located in the outermost parts of the bean and $C_{16:0}$ and $C_{18:0}$ as well as the unsaturated acids in the inner part.

Kaufmann & Hamsagar (1962a) found that esters of cafestol and $C_{18:0}$, $C_{20:0}$ and $C_{22:0}$ possess waxy properties melting above $30^{\circ}C$ in a range of about $3-4^{\circ}C$ whereas the esters formed with $C_{14:0}$, $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ are oils at room temperature.

Table 13 shows the fatty acid composition in coffee oil from untreated beans and in coffee wax, obtained under the conditions of the technical process for the reduction of the amount of wax in the beans (Chapter 2). It can be seen that the percentage saturated higher acids amounts to 45.3% in coffee wax but only 14.6% in coffee oil. From a comparison between the Tables 11, 12 and 13 and Fig. 6 the conclusion can be drawn that especially the diterpene alcohol esters contribute to the high value observed for coffee wax.

From a summary of the characteristic differences in the fatty acid composition between oil and wax as reported it is possible to give one explanation for the differences in consistency, coffee wax having a semi-solid state and coffee oil being a fluid at room temperature.

Table 13. Composition of the fatty acids (%) in coffee oil and in the petroleum ether soluble part of the coffee wax. The wax was obtained from unground beans by refluxing and stirring for 30 min with chloroform.

	Coffee wax	Coffee oil
$C_{14:0}$	1.5	traces
$C_{16:0}$	24.9	31.1
$C_{18:0}$	6.5	9.6
$C_{18:1}$	4.8	9.6
$C_{18:2}$	23.8	43.1
$C_{18:3}$	traces	1.8
$C_{20:0}$	14.1	4.1
$C_{22:0}$	21.0	0.9
$C_{24:0}$	3.7	traces
saturated acids $C_{18:0} - C_{24:0}$	45.3	14.6

6.2 THE POSITIONAL DISTRIBUTION OF THE FATTY ACIDS IN THE TRIGLYCERIDES

In Section 6.1 the fatty acid composition of the triglycerides was studied. Then it was interesting to determine the positional distribution of the acids in the glycerol molecule.

Thus triglycerides in both the petroleum ether soluble part of coffee wax as well as in coffee oil were studied. Coffee wax was obtained by refluxing and stirring unground beans for 30 min with chloroform. After evaporation of the solvent the waxy residue was extracted with petroleum ether (40-60°C) for 4 times 15 min. Coffee oil was obtained as described in Section 4.1. The triglycerides were isolated by Florisil column chromatography as described in Sections 4.2 and 6.1 (Fig. 6).

The triglycerides were separated according to their carbon number by g.l.c. on short silicone columns with a programmed column temperature. The carbon number is defined as the total number of carbon atoms in the acyl chains of the triglycerides (Litchfield, 1972, p.105). So saturated and unsaturated triglycerides with similar carbon numbers will all elute together. In Table 14 the results are presented. The peaks were identified by comparison of the elution temperatures with those of known references. Although coffee wax and coffee oil were found to contain 24.9% and 31.1% of $C_{16:0}$ respectively (Table 13), only traces of C_{48} could be demonstrated. Apparently coffee beans do not contain significant amounts of tripalmitin. The high percentages of C_{50} and C_{52} in the triglycerides indicate the presence of mixed forms of triglycerides with both saturated and unsaturated acids in the same molecule.

By partial deacylation with pancreatic lipase the positional distribution pattern was further studied. The enzyme specifically catalyses the hydrolysis

Table 14. Composition of the triglycerides (%) according to their carbon number for both coffee oil and the petroleum ether soluble part of coffee wax.

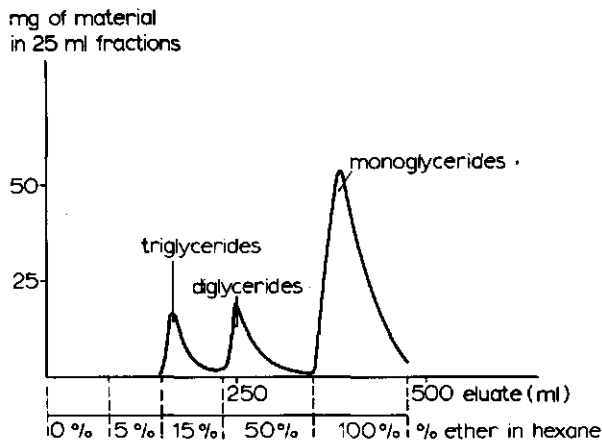|	Coffee oil	Coffee wax
C_{48}	traces	traces
C_{50}	31.5	32.2
C_{52}	49.8	44.7
C_{54}	18.1	19.4
C_{56}	0.6	3.6

mg of material
in 25 ml fractions

Fig. 7. Fractionation of a mixture of triglycerides, diglycerides and mono-
glycerides on a Florisil (60-100 mesh) column, deactivated with 6% distilled
water. Elution with a gradient of ether in hexane.

of the primary ester linkages in triglycerides to produce sn-1,2(2,3)-digly-
cerides, sn-2-monoglycerides and free fatty acids. In this work the method
of Hollstein et al. (1973) was followed. The monoglycerides were isolated by
fractionation on a Florisil column with a stepwise elution gradient of ether
in hexane (Section 4.3 and Fig. 7). Thin-layer chromatography on silica gel
H plates impregnated with boric acid and with chloroform:acetone:alcohol (96%) =
= 91:8:1 as solvent indicated that mainly the sn-2-isomer was present in the
monoglyceride fraction. Traces of sn-1(3)-monoglycerides arise almost entirely
from isomerization of the sn-2-isomer under the reaction conditions used for
the hydrolysis. The fatty acid composition of both the triglycerides before
enzymatic hydrolysis and of the monoglycerides after hydrolysis was deter-
mined (Table 15). Coffee oil and coffee wax were obtained as described in the
first part of this Section 6.2. For the triglycerides in coffee oil it is
apparent that $C_{16:0}$ and $C_{18:0}$ are almost exclusively esterified at the pri-
mary hydroxyl positions whereas $C_{18:2}$ and to a lesser extent also $C_{18:1}$ and
$C_{18:3}$ show some preference for being esterified at the secondary hydroxyl
position. These data generally fit in the regular fatty acid distribution
patterns observed in plant triglycerides (Litchfield, 1973). In the mono-
glycerides of coffee wax a higher percentage of saturated acids can be found
than expected from these distribution patterns. A possible explanation might
be that fats with a high melting point (above $50^{\circ}C$) emulsify less well. As a
result it has been found that lipase preferably splits off unsaturated acids.

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Table 15. Fatty acid composition (%) in triglycerides and in the monoglycerides obtained by deacylation with pancreatic lipase.

	Coffee oil		Coffee wax	
	triglycerides	monoglycerides	triglycerides	monoglycerides
$C_{14:0}$	-	-	1.1	traces
$C_{16:0}$	38.1	1.9	30.4	5.3
$C_{18:0}$	6.2	0.3	6.8	5.4
$C_{18:1}$	6.1	12.8	5.2	7.7
$C_{18:2}$	47.0	79.7	37.7	77.3
$C_{18:3}$	1.1	4.6	traces	-
$C_{20:0}$	1.5	1.5	9.5	4.3
$C_{22:0}$	-	-	8.0	traces
$C_{24:0}$	-	-	1.3	traces

Therefore the monoglycerides which are obtained in this way cannot be considered as representative (Hollstein et al., 1973).

Finally the triglyceride composition of coffee oil was calculated from the results reported in Table 15. This calculation was based on the 1,3-random--2-random distribution hypothesis according to Coleman (1961) and van der Wal (1960). This hypothesis is fairly accurate for predicting the triglyceride composition of seed oils containing only common acids (Gunstone, 1962; Mattson & Volpenheim, 1963; Litchfield, 1973; Sengupta et al., 1974).

The percentages of the fatty acids in both the triglycerides and the monoglycerides (Table 15) were recalculated to a mole per cent basis. Next the amount of each component triglyceride (sn-XYZ) was calculated from the equation:

% sn-XYZ = (mole % X at 1,3) (mole % Y at 2) (mole % Z at 1,3) (10^{-4})

Table 16. Triglyceride composition (mole %) of coffee oil calculated by the 1,3-random-2-random hypothesis[a].

PPP	0.7	β-SLS	0.7	β-POL	5.8
β-PPS	0.2	β-SLP	8.6	β-PLO	2.7
β-PSP	0.6	β-PLP	28.1	β-PLL	27.5
β-PSL	0.6	β-LSL	0.1	β-OOL	0.3
β-SPL	0.1	β-LPL	0.2	LLL	6.7
β-PPL	0.7	β-SOL	0.9	β-LLO	1.3
β-SOS	0.1	β-SLO	0.4	β-LOL	1.4
β-SOP	1.8	β-SLL	4.2		
β-POP	5.9	β-POO	0.6		

a. P = 16:0; S = 18:0 + 20:0; O = 18:1 + 18:3; L = 18:2

The results are reported in Table 16. The prefix β in β-XYZ means that the fatty acid esterified at sn-2 is known, but the positions of the other two acids are not. In the calculation $C_{18:0}$ and $C_{20:0}$ as well as $C_{18:1}$ and $C_{18:3}$ were considered as one group. The triglyceride composition is in agreement with the results reported in Table 14.

6.3 THE UNSAPONIFIABLE MATTER

6.3.1 Isolation and fractionation

The unsaponifiable matter was obtained from the petroleum ether soluble part of coffee wax and from wax-free coffee oil by the following procedures:

Wax 10 kg of unground green beans were stirred and refluxed for 30 min with chloroform. After evaporation of the solvent the residue was extracted for 4 times 15 min with petroleum ether (40-60°C). Caffeine was then removed from the petroleum ether solution, the solvent was evaporated off and the unsaponifiable part was prepared from the residue in the usual way to yield 2.48 g.

Wax-free oil 180 g of green beans, which had been dewaxed as just described,
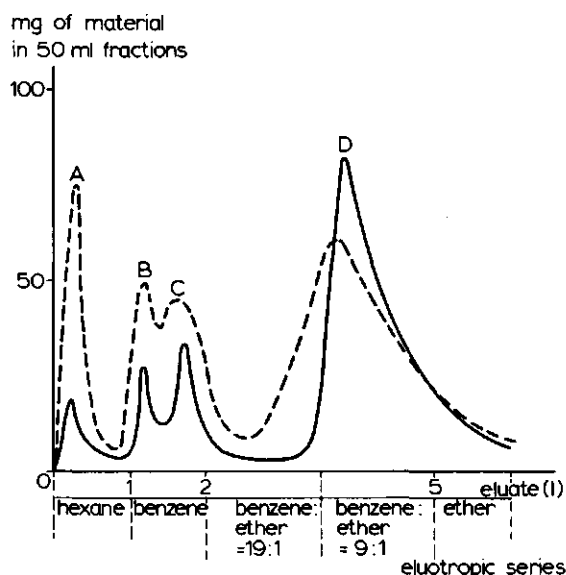

Fig. 8. Fractionation of the unsaponifiable matter from the petroleum ether soluble part of coffee wax (---) as well as wax-free coffee oil (——) on a Florisil (60-100 mesh) column.

were ground finer than 0.5 mm and extracted for 24 h with petroleum ether (40-60°C) in a Soxhlet. The petroleum ether solution was further treated as above. Thereby 1.60 g of unsaponifiable matter was obtained.

The unsaponifiable matter of both coffee wax and wax-free oil was chromatographed on Florisil (60-100 mesh) with a stepwise eluotropic series as illustrated in Fig. 8. The amounts of material present in the eluates were determined gravimetrically. The recovery of the fractionations was found to be 85%. Both samples could be separated into four fractions (A-D). Their composition is discussed in Section 6.3.2. To study the composition of the hydrocarbons in the beans it was found necessary to distil all solvents twice as non-volatile components in the solvents (0.002%) disturb the final g.l.c. analysis of the hydrocarbons.

6.3.2 Analysis of the fractions

Fraction A The i.r. spectrum shows absorptions near 2940, 2860, 1470 and 1380 cm^{-1}. Lack of absorptions due to functional groups indicated that Fraction A consists of saturated hydrocarbons.

The composition of the hydrocarbons was analysed by g.l.c. using known references of *n*-alkanes for comparison (Table 17). Both odd and even chain

Table 17. Composition of the hydrocarbons (%) in coffee wax and wax-free oil (obtained by the methods described in Section 6.3.1).

Chain length	Coffee wax	Wax-free oil
16	2.4	4.5
17	8.8	8.2
18	9.3	11.2
19	6.4	8.6
20	4.3	7.5
21	9.5	12.0
22	2.2	5.6
23	1.9	5.6
24	3.0	4.5
25	5.2	3.7
26	2.0	4.5
27	6.0	4.5
28	12.8	15.0
29	26.2	4.5
30	traces	traces
31	traces	traces

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length alkanes were found to be present in a bimodal distribution with maxima around C_{17}-C_{21} and C_{28}-C_{29}.

In the petroleum ether soluble part of coffee wax 0.5-1% saturated hydrocarbons were found, in wax-free oil 0.1%. Consequently saturated hydrocarbons cannot be considered as specific coffee wax constituents (like C-5-HT).

Hamilton & Hamilton (1972) showed that surface waxes consist of 10-100% hydrocarbons. Those with odd chain length of C_{27}, C_{29} and C_{31} are mostly predominant.

However hydrocarbons from internal leaf lipids or seeds show a uniformity in odd and even chain length distribution and often exhibit a bimodal distribution pattern (Brown et al., 1975). This is observed in coffee beans too.

Table 17 illustrates the differences between coffee wax and wax-free oil. It is of interest to note that the percentage C_{29} is considerably higher in coffee wax than in wax-free oil.

Fraction B Thin-layer chromatography on silica gel 60 with chloroform as solvent and detection with 2',7'-dichlorofluorescein under both short-wave and long-wave u.v. light showed 4 spots with R_f values of 0.47, 0.42, 0.28 and 0.19, respectively.

The spot at R_f = 0.47 was isolated on a preparative scale using the t.l.c. system just mentioned. Both its retention time and mass spectral data were identical with those of a reference sample of squalene (XX). Squalene has already been found in coffee by Kaufmann & Sen Gupta (1964).
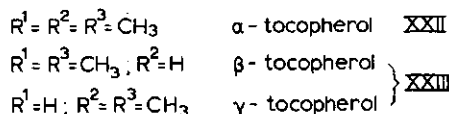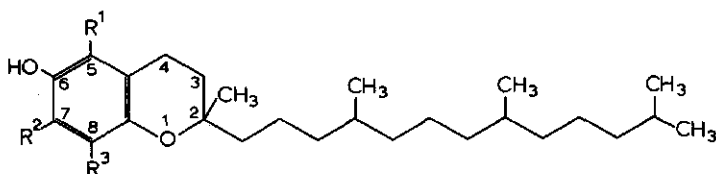
The spots at R_f = 0.42 and 0.28 turned red with the 2,2'-bipyridyl-FeCl$_3$ reagent which is indicative for tocopherols. Isolation was performed by preparative t.l.c. on silica gel 60 using chloroform for development and ether for elution of the substances from the silica gel.

Mass spectral data are listed in Table 18. The base peak at m/e = 430 and 416 constitutes the molecular ions of α-tocopherol and (β + γ)-tocopherol (XXII; XXIII). In agreement with the literature (Scheppele et al., 1972) a sidechain radical ($C_{19}H_{37}$) is split off whereupon mainly tropylium ions of m/e = 165 and 151 respectively (XXIV) are formed. Cleavage of the non-aromatic ring in which no hydrogen transfer is involved results in the formation of m/e = 164 and 150 (XXV).

N.m.r. spectral analysis (in CDCl$_3$) of XXII gives signals at δ 0.88 (doublet, broadened; CH$_3$ side chain); δ 1.28 (CH$_3$ at C$_2$; CH$_2$ at C$_3$; CH$_2$ side chain); δ 2.12 (CH$_3$ aromatic ring) and δ 2.60 (triplet; H at C$_4$). The spectrum of XXII was found to be consistent with that reported in the literature

Table 18. Mass spectral data of two isolates from Fraction B.

Rf = 0.42		Rf = 0.28	
m/e	% intensity	m/e	% intensity
431	32	417	32
430	100	416	100
428	2	191	15
205	9	189	14
203	11	152	14
166	15	151	81
165	100	150	37
164	35		

$R^1 = R^2 = R^3 = CH_3$ α - tocopherol XXII

$R^1 = R^3 = CH_3$, $R^2 = H$ β - tocopherol ⎫
                                                  ⎬ XXIII
$R^1 = H$; $R^2 = R^3 = CH_3$ γ - tocopherol ⎭

(Varian 1962, No. 366). Similar signals were observed for XXIII but additionally a signal at δ 6.51 (H aromatic ring) was found. The n.m.r. spectrum does not tell us whether β- or γ-tocopherol is present or whether there is a mixture of both compounds.

The i.r. spectrum is identical with those reported in the literature for tocopherols (Green et al., 1959).

λ_{max} = 297 nm (log ε = 3.54) is consistent with the u.v. absorption maximum found in the literature (Handbook of chemistry and physics, 1971). As the individual tocopherols have not been detected before in green coffee beans, the amounts of tocopherols were also determined (Section 6.3.3).

The spot at R_f = 0.19 was purple-brown with the Liebermann-Burchard reagent and red-brown with antimony trichloride in glacial acetic acid which indicates the presence of sterolic substances. The spot was isolated by preparative t.l.c. using the system mentioned before. Comparison of the retention times and mass spectra with those of authentic samples showed the appearance of the main methylsterols present in coffee beans: cycloartenol (XI), 24-methylenecycloartanol (XII) and citrostadienol (XIII).

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$\underline{\text{XXV}}$

$\underline{\text{XXIV}}$
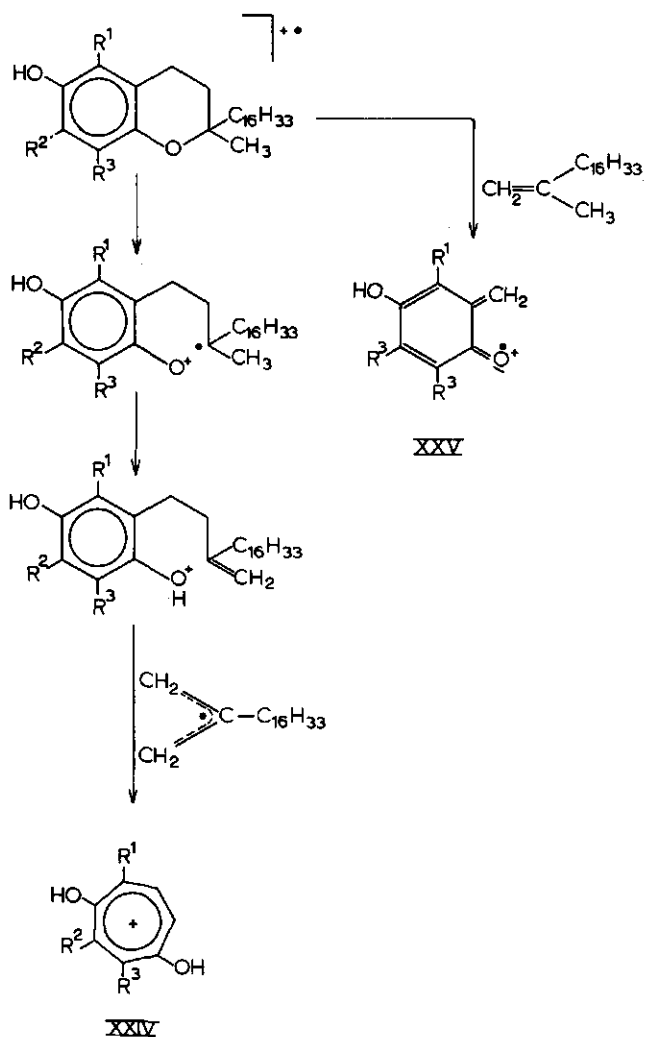
Fraction C On silica gel 60 with chloroform:ethyl acetate = 1:1 as solvent one spot at R_f = 0.49 was found. This spot was purple-brown with the Liebermann-Burchard reagent and purple with antimony trichloride in glacial acetic acid, which indicates the presence of sterolic substances again. The spot was isolated on a preparative scale. Gas-liquid chromatography yielded three peaks which were found to correspond with known references of stigmasterol (IV), sitosterol (V) and campesterol (VI) according to their retention times and mass spectra.

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Fraction D By use of t.l.c. on silica gel 60 with ethyl acetate as solvent
two spots were found at R_f = 0.26 and 0.13, the first one being the main spot.
Both spots were red-brown with phosphomolybdic acid. After isolation by prepar-
ative t.l.c. the main spot was derivatized with *N*-methyl-*N*-TMS-trifluoro-
acetamide and yielded one peak by g.l.c. Its mass spectrum was found to
correspond with that of the TMS derivate of pure cafestol (I). The reference
sample was obtained from coffee beans according to the procedure of Kaufmann
& Hamsagar (1962a); it melted at 158-160°C (Kaufmann & Hamsagar, 1962a;
158-160°C). The u.v. spectrum of the main spot (R_f = 0.26) showed absorp-
tion maxima at 223 and 290 nm, the one at 290 nm being due to traces of
kahweol (II). The spot at R_f = 0.13 showed a maximum in the u.v. spectrum at
260 nm. This compound was not studied in this work but will be the subject
of future investigations.

Although some minor differences have been observed between the unsaponi-
fiable matter in the petroleum ether soluble part of coffee wax on the one
hand and in wax-free coffee oil on the other, the composition of both the
samples of wax and wax-free oil was almost the same: all compounds described
for the fractions A-D were detected in both samples. Moreover the proportion
of the individual fractions in the total unsaponifiable matter was almost the
same for wax and wax-free oil (Fig. 8).

6.3.3 *Quantitative determination of the tocopherols*

Section 6.3.2 describes the chemical composition of the unsaponifiable
matter in the petroleum ether soluble part of coffee wax and in wax-free oil.
Thereby the presence of tocopherols was established. These compounds are
known to occur in most oil seeds. In vegetable oils total tocopherol contents
range from about 1500 µg/g of oil in soya bean oil to only traces in coconut
oil. The relative percentages of α-, (β + γ)- and δ-tocopherol in several
seed oils vary considerably (Gutfinger & Letan, 1974). The absence of data
on the percentages of the individual tocopherols in green coffee beans
prompted this investigation.

As tocopherols are readily oxidized, especially under alkaline conditions
in the presence of light, special precautions were taken by adding pyrogallol
and working under N_2 to obtain quantitative recoveries during the assay pro-
cedure (Christie et al., 1973). The general procedure of extraction and
preparation of a tocopherol-rich unsaponifiable fraction as described by
Slover et al. (1969) was found suitable for green coffee beans. The toco-

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pherols were quantitatively obtained by thin-layer chromatography on silica
gel $60F_{254}$ plates (0.5 mm in thickness). The plates were developed with
chloroform twice. The quantity of the individual tocopherols was measured
by gaschromatography of the TMS-derivates and calculated according to:

$$weight_{tocopherol} = \frac{area_{tocopherol} \times weight_{internal\ standard}}{area_{internal\ standard} \times response\ factor}$$

The response factor was determined separately from the ratio between the
response of the internal standard (squalane) and that of a reference sample
of α-tocopherol. It was assumed that the response of the flame ionization
detector was the same for the various tocopherols and that the response fac-
tor based on α-tocopherol could be used for the other tocopherols as well.
From the area ratio plotted against the weight ratio at various amounts of
α-tocopherol and a fixed amount of the internal standard, response linearity
was established in the range of the determination (Fig. 9). As internal
standard squalane was chosen. In their work on cocoa lipids Erickson et al.
(1973) used sitosterol as internal standard. In this work this was not found
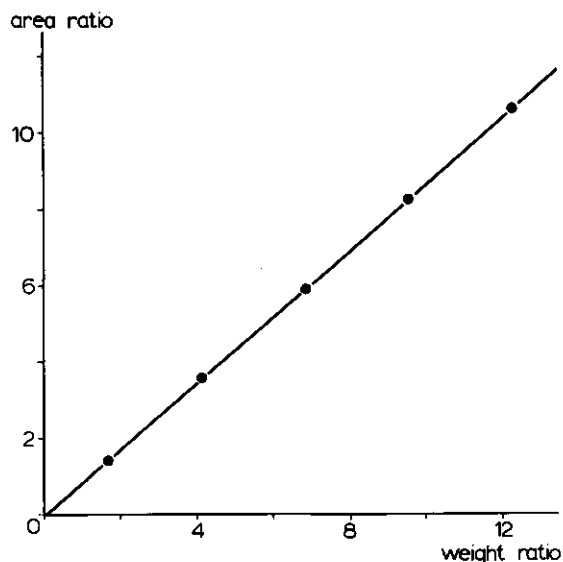

Fig. 9. Response linearity of α-tocopherol: area ratio (α-tocopherol/squalane)
plotted against weight ratio (α-tocopherol/squalane) with various amounts of
α-tocopherol and a fixed amount of squalane.

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satisfactory as traces of sitosterol from the unsaponifiable matter were incidentally observed in the $(\beta + \gamma)$-tocopherol samples after the t.l.c. fractionation procedure. Under the conditions of this experiment the retention times of squalane, $(\beta + \gamma)$-tocopherol and α-tocopherol amounted to 6.92, 15.04 and 21.2 min respectively. Since t.l.c. and g.l.c. failed to completely separate β- and γ-tocopherol, these compounds were considered as one group as in most tocopherol assays.

Parallel to each tocopherol determination an oil determination was made in an aliquot coffee sample to calculate the percentage tocopherol as µg of tocopherol/g of oil.

In this experiment samples of green beans, which differed in species, storage time, geographical origin and processing conditions, were analysed (Table 19). In the same table literature values were included for comparison.

Generally large differences between the samples were not observed. Remarkably the content of $(\beta + \gamma)$-tocopherol exceeds that of α-tocopherol. This has been observed in other seed oils like soya bean and corn oils too (Juillet, 1975; Slover et al., 1969). This result is of particular interest as $(\beta + \gamma)$-tocopherols possess much better antioxidant activity than α-

Table 19. Percentage oil[a] on dry basis and tocopherol concentrations in oil of green coffee beans as compared with values of other seed oils reported by Slover et al. (1969).

	Oil (%)	µg of tocopherol/g of oil		
		α-toco-pherol	$(\beta + \gamma)$-toco-pherol	δ-toco-pherol
Colombian Arabica fresh, fermented	15.98	140	465	-
Colombian Arabica old, fermented	15.73	132	355	-
Santos, 3 years old, non-fermented	15.47	89	334	-
Robusta Ivory Coast, 1-2 years old, non-fermented	11.23	191	252	-
Soyabean oil	-	94	630	232
Cotton seed oil	-	320	313	-
Peanut oil	-	186	138	-
Olive oil	-	8	-	-

a. Schweiz.Lebensm.Buch (method 35A/08, 1973)

tocopherol (Chow & Draper, 1974).

Wax removal, according to the technical process for the reduction of the amount of wax in the beans (Chapter 2), had no significant effect on the content of the tocopherols in the bean as a whole.

The question whether tocopherol contributes to the antioxidant properties of the coffee wax on the unground bean or not remains to be studied.

7 Petroleum ether insoluble substances

In Table 9 (Section 5.2) it was shown that coffee wax obtained under the conditions of the technical process for reduction of the amount of wax in the beans consists of about 25% of C-5-HT. Expressed as percentage of the amount of petroleum ether insoluble substances in the wax C-5-HT comprises about 40%. The remaining 60% is largely unknown but consists at least for a part of the so-called "Begleitsubstanzen" (Wurziger & Harms, 1969) which react with Gibbs reagent like C-5-HT (Section 3.2).

According to the literature the fractionation of the petroleum ether insoluble substances on columns of silica gel and Al_2O_3 is difficult because these substances were found to be unstable with these column materials (Dickhaut, 1966). The affinity between the substances and the column materials mentioned and their instability on the column could be reduced by acetylation of the phenolic hydroxyl group. In this way Harms (1968) succeeded in isolating C-5-HT as its acetyl derivate.

Since polyamide is particularly suitable for the fractionation of phenolic compounds (Zawta & Hölzel, 1968) in my experiments polyamide was used to separate the petroleum ether insoluble substances in coffee wax without preceding derivatization.

Coffee wax was isolated from 2 kg of unground beans by stirring and refluxing for 30 min with chloroform. By the usual methods 3.2 g of petroleum ether insoluble material was obtained from the wax. It was separately adsorbed on 3.2 g of polyamide using about 20 ml of a mixture of benzene:methanol = = 1:1 and the solvent-free material was brought on top of a polyamide column. The column was prepared by pouring a slurry of 125 g of polyamide in benzene into a glass column which provided a bed of 30 x 4.2 cm. The column was eluted with 500 ml of benzene which was continuously mixed with successively 4 l of ether, 2 l of ether:acetone = 3:1, 2 l of ether:acetone = 2:1, 2 l of ether:acetone = 1:1, 5 l of acetone, 2 l of acetone:ethanol = 19:1 and 2 l of ethanol using the system illustrated in Fig. 10.

The apparatus is a simplified form of the gradient mixer described by Dickhaut (1966): 500 ml of benzene is put in a 1 l flask (2) which is con-
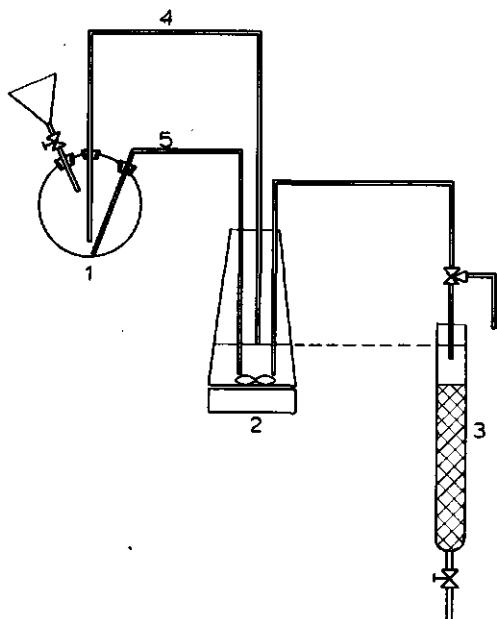
Fig. 10. All-glass system for gradient elution with a solvent mixture of continuously increasing eluotropic strength (for explanation 1-5 is referred to the text).

nected with the column (3) by a siphon system. 1 is a closed system equipped with ground-glass joints. When the column is eluted the solvent in 2 and 3 drops below the end of the air-inlet tube (4) of 1. Thereupon the more polar solvent, which was previously introduced into 1, streams from 1 into 2 through 5 until the original level is reached again. The solvents are continuously mixed in 2 with a magnetic stirrer. The fractionation pattern is illustrated in Fig. 11. The recovery as determined from the total weight of the fractions was found to be 75%.

Fraction A It consists mainly of caffeine as indicated by thin-layer chromatographic comparison with a reference compound.

Fraction B One spot is found by t.l.c. on silica gel $60F_{254}$ with benzene: :ethyl acetate:acetic acid = 5:5:1 as solvent.

Both the i.r. spectrum and the mass fragmentation pattern are consistent with those of C-5-HT (XXI). After recrystallization of this fraction from acetone a white powder melting at 117-117.5°C was obtained (reference compound, N_{β}-behenoyl-5-hydroxytryptamine[1]: 117.5-118°C; Harms (1968): 120°C).

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1. Obtained from D.E.J. International Research Company B.V., Utrecht, the Netherlands.
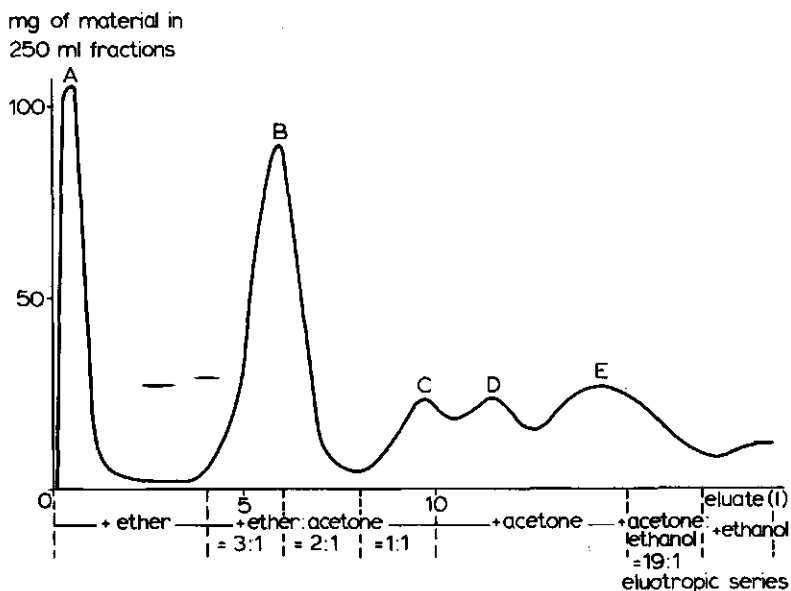
mg of material in 250 ml fractions

Fig.11. Fractionation of the petroleum ether insoluble substances in coffee wax on a polyamide column eluted with a continuous solvent gradient.

Fraction C and D On silica gel 60F$_{254}$ both fractions could be separated into three spots each. In both cases the plates were developed twice with benzene:acetone:methanol = 20:10:0.5 as solvent for Fraction C and benzene: :ethyl acetate:acetic acid = 10:10:1 for Fraction D. They all show a positive Gibbs test.

In preliminary experiments mass spectra of the constituents of Fractions C and D were found to be only slightly different from that of C-5-HT. They all show fragmentation peaks at m/e = 159 and 146, being identical with those found for C-5-HT (Harms, 1968). Therefore it can be assumed that the structure of C-5-HT and the other phenolic compounds are closely related.

Fraction E Two dimensional t.l.c. with successively benzene:acetone:methanol = = 15:15:1 and benzene:acetone:methanol = 20:10:0.5 indicated the presence of one main spot and at least four other spots, again all with positive Gibbs test. Further studies on the structure of these phenolic compounds seem desirable.

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8 Discussion and conclusions

The main purpose of this study was to gain more knowledge about the identity and quantity of coffee wax constituents as removed from green beans during the technical process for the reduction of the amount of wax in the beans (Chapter 2) in view of the claimed effects of decreased irritability for coffee treated in this way.

The approach which was followed in this work comprised:

A. Isolation of coffee wax and investigation of its composition.

B. Analysis of the chemical composition of the beans after wax removal.

C. Comparison of the results obtained under A and B to answer the question which constituents are specifically removed as a result of the technical process just mentioned.

8.1 ISOLATION

The investigation of the wax composition (A) was preceded by a study of the wax isolation method. In Section 5.2 it was established that the composition of coffee wax depends on the time of extraction of unground beans with chloroform: the longer the extraction lasts, the higher becomes the ratio between the amounts of the percentages of petroleum ether soluble and insoluble substances in the wax (Fig. 4). The amount of coffee wax was 0.23% under the conditions which resemble those of the technical process mentioned earlier. This wax consists of approximately 0.08% petroleum ether solubles and 0.12% petroleum ether insolubles, the petroleum ether insoluble part containing 0.05% C-5-HT and the remaining 0.07% being caffeine and unknown substances.

Wax-free coffee beans were ground and extracted with chloroform. The chloroform was evaporated and the residue extracted with petroleum ether (40-60°C). In the petroleum ether insoluble part only caffeine was found. Therefore the investigation of the beans after wax removal (B) was restricted to the petroleum ether soluble matter (= wax-free coffee oil) only.

In order to obtain correct analyses it was necessary to develop a reliable

extraction procedure for the isolation of coffee oil. In Section 5.1 it was
shown that the yield of the extraction of oil from ground green beans de-
pends on the particle sizes of the grind. This is in agreement with the
literature (Section 3.1.1). Moreover it was demonstrated that incomplete
extraction of the grind results in samples of oil which are not representa-
tive for the total amount of oil present in the bean, at least as far as the
fatty acid composition and the percentage of unsaponifiable matter are
concerned. Therefore the literature on the analysis of oil in green coffee
beans should be considered critically if the oil isolation procedure has not
been described completely. For instance the relative high percentage of un-
saponifiable matter in the oil of green beans reported by Kaufmann & Hamsagar
(1962b) can be explained by incomplete extraction of the grinds.

8.2 ANALYSIS

The petroleum ether soluble part of coffee wax and wax-free oil (some-
times also total coffee oil) were investigated in groups of total fatty
acids, triglycerides and unsaponifiable matter.

In Section 6.1 it is reported that interesting differences exist
between the fatty acid composition of wax and oil: (a) in wax a high percen-
tage of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ is found whereas (b) in wax-free oil (and
also in total coffee oil) the percentage of particularly $C_{18:2}$ is very high.
In both cases the esters of diterpene alcohols and fatty acids are preferably
formed with the saturated acids. The differences observed between the fatty
acid compositions in wax and in oil were found to provide a possible expla-
nation for the differences in consistency between coffee wax and coffee oil.

In this context it should be noticed that many plant waxes are found to
contain saturated fatty acids, commonly with chain length of $C_{24:0}$, $C_{26:0}$
and $C_{28:0}$.

On the basis of the fatty acid composition of the triglycerides and that
of the monoglycerides after cleavage of the sn-1,3-acylgroups with pancreatic
lipase, the triglyceride composition of coffee oil could be calculated
(Section 6.2). The data as obtained for this composition generally fit in
the 1,3-random-2-random distribution theory: $C_{16:0}$ and $C_{18:0}$ being almost
exclusively esterified at the sn-1,3 position and $C_{18:2}$ showing a preference
for the sn-2 position. Exceptionally also $C_{18:1}$ was found to have some
preference for the sn-2 position, whereas in the distribution theory
$C_{18:1}$ is thought to be equally distributed at all three positions available.

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Results obtained from the gas-chromatographic determination of the carbon number of the triglycerides were found to be consistent with those for the fatty acid composition of the triglycerides as well as with those for the distribution of the acids in the triglyceride molecule. The percentage of triglycerides with a carbon number of 56 was found to be higher in wax than in oil because of the presence of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ in the wax. However the absence of triglycerides with a carbon number > 56 indicated that no tri-saturated glycerides of $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$ are present in coffee wax. The same result has been found for tri-saturated glycerides of $C_{16:0}$ and $C_{18:0}$ in the oil.

In Section 6.3 the unsaponifiable matter of the petroleum ether soluble part of coffee wax as well as wax-free oil was investigated. In addition to hydrocarbons, squalene, methylsterols, 4,4-desmethylsterols and diterpene alcohols which were already known from the literature (Section 3.1.3.2), the presence and the quantity of α-tocopherol and (β + γ)-tocopherol was described for the first time in coffee beans in detail. Between the composition of the unsaponifiable matter in wax and in wax-free oil no large differences were found. Only the hydrocarbon fractions were found to yield different gas-chromatographic pictures: the percentage of n-nonacosane in wax slightly exceeds its percentage in wax-free oil. The hydrocarbons in coffee wax amount to only 0.5- -1% of the wax which is rather low when compared with the literature values for other waxes (10.9% in cranberry wax, found by Croteau & Fagerson, 1971; 36% in cabbage leaf wax, cited by Kolattukudy, 1970a).

In Chapter 7 the composition of the petroleum ether insoluble substances is discussed. A detailed fractionation procedure was worked out using a polyamide column eluted with a continuous solvent gradient. Thereby preceding derivatization of the substances (Harms, 1968) is superfluous and C-5-HT can be obtained, separately from the other phenolic substances rather easily. The petroleum ether insoluble matter consists of about 40% of C-5-HT, 30% of a fraction which mainly contains caffeine and 30% of other phenolic compounds which react with Gibbs reagent. The structures of these phenolic compounds have not yet been elucidated. However preliminary experiments indicated a close similarity with the structure of C-5-HT.

Finally the effect of the technical process for the reduction of the amount of wax in the beans on the chemical composition of the bean should be summarized. This process comprises both the removal of coffee wax and a steam treatment which is used to expel traces of the solvent from the bean. Steaming of green coffee is applied on large scale in the production of so-

called mild coffee (Lendrich process). However in the context of this work the interest is mainly concerned with the wax removal in view of the claimed beneficial effects.

It has been shown that the differences between the chemical composition of the petroleum ether soluble part of coffee wax and wax-free coffee oil are obvious as far as the fatty acid composition is concerned. However the effect of wax removal on the total content of petroleum ether soluble substances in the bean is only very small: the percentage of oil amounts to 10-16% of the bean whereas the percentage of petroleum ether soluble substances in the wax was found to be only 0.08% of the bean. From the literature it is known that the composition of the petroleum ether soluble substances before and after roasting is similar (Vitzthum, 1976). Moreover only 0.1-0.8% of the petroleum ether soluble substances, expressed as percentage of the bean weight on dry basis was found in the coffee drink (Vitzthum, 1976; Kaufmann & Schickel, 1965). As a result it can hardly be imagined that the petroleum ether soluble substances in the wax are of importance for the claimed beneficial effects of the technical process mentioned before.

The petroleum ether insoluble substances in the wax (notably C-5-HT and the other phenolic compounds which react with Gibbs reagent) are removed almost quantitatively during this process. However these substances are also insoluble in water. In instant coffee Würziger (1972) found only 40-60 ppm C-5-HT (expressed as ppm of the amount of instant coffee) in spite of the drastic extraction conditions.

It must be realized that this study was done on green coffee. It certainly seems worthwhile to extend these investigations to roasted coffee as the influence of roasting on the degradation of the important wax constituents is not sufficiently known.

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Summary

In this work the lipids in green coffee beans are studied in detail. These substances have recently attracted attention since commercial processes have been developed to remove coffee wax from green beans in order to obtain a so-called 'wholesome' coffee which is claimed to cause less complaints commonly described as 'irritability' or 'indigestion'.

Special attention is paid to differences between the chemical compositions of coffee wax and coffee oil. Coffee wax was considered to be the group of substances which is isolated by extracting unground beans with chloroform (yield = 0.2-0.3%) and coffee oil was defined as the petroleum ether soluble part of ground green beans and amounts to 10-16%.

In Chapter 3 the literature on coffee lipids is reviewed. The methods for the determination of the percentage of coffee oil are summarized in Section 3.1.1 and the problems encountered, when coffee oil is isolated for analysis are discussed in Section 3.1.2. A review of the literature on the fatty acid composition and the composition of the unsaponifiable matter is presented in Section 3.1.3.

Only a few reports on coffee wax were available. Most reports dealt with the chemical composition of the so-called 'decaffeination wax', which is a waste product derived from decaffeination (Section 3.2).

In Chapter 4 the materials used and the methods applied in this study are given.

In Section 5.1 the isolation method of coffee oil is described. The yield of oil extraction from ground green beans was seen to depend on the particle sizes of the ground material. Moreover the chemical composition of the oil as far as the percentage of unsaponifiable matter and the fatty acid composition are concerned were found to be dependent on the yield of oil extraction. A method for the quantitative extraction of the oil from the beans is introduced.

The composition of coffee wax is influenced by the length of extraction time of the wax from unground beans: the longer the extraction time with chloroform, the higher the ratio between the amounts of the petroleum ether

soluble and insoluble substances in the wax (Section 5.2). Coffee wax as obtained under the conditions of the commercial process mentioned in the first part of this summary consists of 63% of petroleum ether insoluble and 37% of petroleum ether soluble material. The petroleum ether insoluble part consists for about 40% of C-5-HT.

In Chapter 6 the chemical composition of both the petroleum ether soluble ·substances in the wax as well as that for coffee oil obtained from normal and dewaxed beans are reported. Interesting phenomena were observed for the fatty acid composition. Wax showed a high percentage of saturated higher fatty acids whereas oil was seen to be rich in unsaturated acids (Section 6.1.). These differences were more prominent for the diterpene alcohol esters than for the triglycerides.

The distribution pattern of the fatty acids in the triglycerides was also investigated (Section 6.2). From results obtained by pancreatic lipase hydrolysis, the triglyceride composition was calculated. Generally the fatty acid distribution pattern was found to fit into the 1,3-random-2-random distribution hypothesis. The experimental data for the total chain length of the triglycerides as determined by gas chromatography were in agreement with these results. In Section 6.3 the fractionation of the unsaponifiable matter is described. The fractions were analysed by t.l.c., g.l.c. and spectral methods. For the first time the occurrence of α-tocopherol and $(\beta + \gamma)$-tocopherol in green beans and their amounts were described in detail. Moreover the presence of hydrocarbons (n-alkanes ranging from C_{16} to C_{31} and squalene), methylsterols, 4,4-desmethylsterols and diterpene alcohols was confirmed. No large differences were observed between the composition of the unsaponifiable matter in the petroleum ether soluble part of coffee wax and in wax-free coffee oil.

In Chapter 7 a column-chromatographic method was developed to fractionate the petroleum ether insoluble substances. Apart from caffeine these substances consist of C-5-HT and other C-5-HT-like phenolic substances. Both C-5-HT and the other phenolic substances specifically occur in coffee wax.

In Chapter 8 the differences between the chemical compositions of coffee wax and coffee oil are evaluated. Then the relation between coffee wax removal and the claimed effects of decreased irritability resulting from this removal is critically discussed.

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Samenvatting

Het doel van deze studie was de chemische samenstelling van de was van groene koffiebonen, alsmede het effect van de verwijdering van de was op de hoeveelheid en de samenstelling van de, in koffiebonen aanwezige, lipiden te bestuderen.

De samenstelling van koffiewas staat sinds enige jaren in de belangstelling in verband met octrooien volgens welke koffie met een gereduceerd wasgehalte door bepaalde personen beter verdragen zou worden dan niet behandelde koffie. Volgens de, in deze octrooien beschreven, processen wordt het wasgehalte verminderd door behandeling van ongemalen groene bonen met gechloreerde koolwaterstoffen; vervolgens worden resten van het oplosmiddel verwijderd met stoom en de bonen worden na drogen gebrand. De chemische samenstelling van de was en de betere verdraagbaarheid van zetsels uit behandelde koffie werden tot nu toe niet uitgebreid onderzocht. In deze studie wordt uitsluitend op de chemische aspecten ingegaan.

De opzet van het onderzoek was als volgt:

A. Isolatie van de was uit ongemalen groene bonen en onderzoek naar de chemische samenstelling

B. Isolatie van de lipiden uit gemalen groene koffie waaruit de was voordien is verwijderd als beschreven onder A en onderzoek naar de chemische samenstelling

C. Vergelijking van de resultaten verkregen onder A en B met als doel de beantwoording van de vraag welke verbindingen specifiek verwijderd worden bij de produktie van groene koffie met een gereduceerd wasgehalte.

In hoofdstuk 2 worden de produktie van groene koffie uit de koffievrucht alsmede de speciale voorbehandelingen besproken. In hoofdstuk 3 wordt een literatuuroverzicht betreffende lipiden in groene koffie gegeven.

Hoofdstuk 4 behandelt de gebruikte isolatie-, scheidings- en analysemethoden. De hoofdstukken 5, 6 en 7 omvatten het experimentele gedeelte.

In paragraaf 5.2 wordt aangetoond, dat de samenstelling van de was afhangt van de tijd gedurende welke ongemalen bonen met chloroform geëxtraheerd worden: hoe langer de extractietijd met chloroform, des te groter is de ver-

houding tussen de hoeveelheden van de in petroleumether (40-60°C) oplosbare en onoplosbare bestanddelen in de was. Was, verkregen onder de condities van de eerder beschreven octrooien, bestond uit 37% petroleumether-oplosbare en 63% petroleumether-onoplosbare bestanddelen.

Na verwijdering van de was werden de bonen gemalen en opnieuw met chloroform geëxtraheerd. Het petroleumether-onoplosbare deel van het residu dat na verwijdering van de chloroform achterbleef, bleek uitsluitend uit cafeïne te bestaan. Dientengevolge konden de bewerkingen, genoemd onder B, beperkt worden tot de wasvrije koffieolie, zijnde het petroleumether-oplosbare deel van wasvrije bonen.

In paragraaf 5.1 wordt de isolatiemethode van (wasvrije) olie uit gemalen groene koffie beschreven. De opbrengst bij extractie van de gemalen bonen met petroleumether (40-60°C) bleek af te hangen van de maalgrofteverdeling. Eveneens bleken de vetzuursamenstelling en het percentage onverzeepbaar hiervan af te hangen. Er wordt een methode aangegeven aan de hand waarvan representatieve monsters, omvattende vrijwel de totale hoeveelheid in de boon aanwezige olie, verkregen kunnen worden.

In hoofdstuk 6 worden de chemische samenstelling van het petroleumether--oplosbare deel van de was, van de wasvrije olie, en van de totale olie beschreven.

In paragraaf 6.1 wordt aangetoond dat was een hoger percentage verzadigde hogere vetzuren ($C_{20:0}$, $C_{22:0}$ en $C_{24:0}$) bevat dan wasvrije olie. Deze olie bevat daarentegen een veel hoger percentage onverzadigde vetzuren (met name $C_{18:2}$). De genoemde verschillen tussen de vetzuursamenstelling van was en wasvrije olie komen sterker bij de diterpeenalcoholesters dan bij de triglyceriden naar voren.

De verdeling van de vetzuren in de triglyceriden werd eveneens onderzocht (paragraaf 6.2). Op basis van resultaten verkregen door partiele hydrolyse met pancreaslipase werd de triglyceridensamenstelling berekend. De gevonden samenstelling sluit aan bij de 1,3-random-2-random verdelingstheorie. Deze samenstelling stemt overeen met de triglyceridensamenstelling naar totale ketenlengte (het C-getal), zoals verkregen door middel van gaschromatografie.

Paragraaf 6.3 beschrijft de fractionering van het onverzeepbare gedeelte. De fracties werden geanalyseerd met behulp van dunne-laag-chromatografie, gaschromatografie en spectrometrische methoden. Voor de eerste maal wordt de aanwezigheid van α-tocoferol en (β + γ)-tocoferol in groene koffie en de hoeveelheid van beide verbindingen in verschillende koffiemonsters beschreven. De aanwezigheid van koolwaterstoffen (n-alkanen van C_{16}-C_{31} en squaleen)

alsmede van methylsterolen, 4,4-desmethylsterolen en diterpeenalcoholen in het onverzeepbare gedeelte werd bevestigd. Er werden geen grote verschillen vastgesteld tussen de samenstelling van het onverzeepbare gedeelte in het petroleumether-oplosbare deel van de was en in de wasvrije olie.

In hoofdstuk 7 wordt beschreven op welke wijze het petroleumether-onoplosbare deel van de was gefractioneerd kan worden. Door middel van kolomchromatografie over polyamide werden 5 fracties verkregen bestaande uit respectievelijk cafeïne, N_β-alkanoyl-5-hydrotryptaminen (C-5-HT) en 3 fracties welke verbindingen bevatten, waarvan de structuur, op grond van eerste aanwijzingen, in belangrijke mate met die van C-5-HT overeenkomt. Iets minder dan de helft van het petroleumether-onoplosbare deel van de was bestaat uit C-5-HT.

Hoofdstuk 8 bevat de algemene discussie en de conclusies. Geconcludeerd wordt dat aan de petroleumether-onoplosbare wasbestanddelen het meeste belang moet worden gehecht in verband met de verschillen tussen koffie met een gereduceerd wasgehalte en niet behandelde koffie.

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