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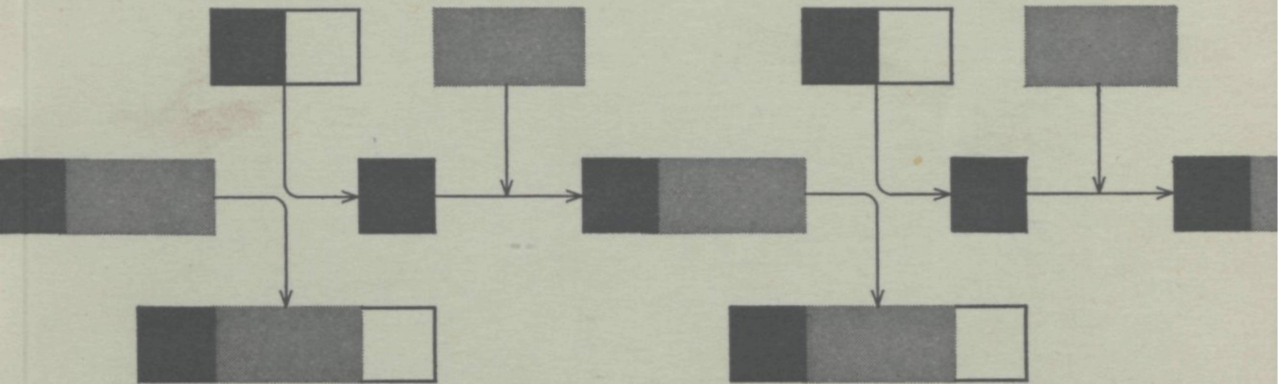
COLD-STORAGE DEFECTS IN BUTTER

AND THEIR RELATION TO THE AUTOXIDATION OF UNSATURATED FATTY ACIDS



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H. T. BADINGS

BIBLIOTHEEK
DER
LANDBOUWHOGESCHOOL
WAGENINGEN.

COLD-STORAGE DEFECTS IN BUTTER AND THEIR RELATION TO THE AUTOXIDATION OF UNSATURATED FATTY ACIDS

Dit proefschrift met stellingen van

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De Rector Magnificus van de Landbouwhogeschool
J. M. POLAK

Wageningen, 7 augustus 1970

COLD-STORAGE DEFECTS IN BUTTER AND THEIR RELATION TO THE AUTOXIDATION OF UNSATURATED FATTY ACIDS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN
OP GEZAG VAN DE RECTOR MAGNIFICUS, MR. J. M. POLAK,
HOOGLERAAR IN DE RECHTS- EN STAATSWETENSCHAPPEN
VAN DE WESTERSE GEBIEDEN,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP VRIJDAG 30 OKTOBER 1970 TE 16.00 UUR

DOOR

[illegible] BADINGS

H. VEENMAN & ZONEN N.V. - WAGENINGEN - 1970

STELLINGEN

I

Het gebrek tranig kan, in tegenstelling tot de gangbare opvatting, ook ontstaan in 'boter' die bereid is door melkvet, vrij van fosfolipiden, te reëmulgeren in melk-ultrafiltraat met een hoog kopergehalte.

F. D. TOLLENAAR, Proefschrift R.U. Utrecht (1953).
Dit proefschrift.

II

De autoxydatieve afbraak van linoleenzuur in boter is de belangrijkste oorzaak van het gebrek tranig.

Dit proefschrift.

III

De beschrijving die KHAN geeft van het reactiemechanisme van de autoxydatie van onverzadigde vetzuren, is niet in overeenstemming met de experimentele gegevens.

N. A. KHAN, Pakistan J. Sci. Res. 18 (1966) 34.
N. A. KHAN, Can. J. Chem. 37 (1959) 1029.
O. S. PRIVETT, E. C. NICKELL, Fette, Seifen, Anstrichmittel 61 (1959) 842.

IV

De Kovats retentie-index mag bij kwalitatieve gaschromatografische analyse alleen worden toegepast wanneer van de gebruikte kolom niet alleen de stationaire fase en de analysetemperatuur, maar ook het dragermateriaal en het beladingspercentage zijn vermeld.

V

De toepassing van het begrip 'odour unit' door GUADAGNI voor het bepalen van de bijdrage van afzonderlijke aromastoffen tot het totaalaroma van een produkt, is aanvechtbaar.

D. G. GUADAGNI, A.S.T.M. special tech. publ. nr. 440 (1968).

Proefschrift van H. T. BADINGS
Wageningen, 30 oktober 1970

To my father
and in memory of my mother

To my wife
and our children

VI

Bij het scheiden van lipiden door middel van dunnelaag-chromatografie wordt vaak onvoldoende rekening gehouden met het gevaar voor autoxydatieve veranderingen.

H. T. BADINGS, J. Chromatography 14 (1964) 265.

VII

Het gebruik van de methode van HURST voor het opvangen van fracties die bij gaschromatografische scheiding zijn verkregen, verdient geen aanbeveling.

R. E. HURST, Chem. & Ind. (1970) 90.
H. T. BADINGS, J. Chromatography 18 (1965) 159.

VIII

In vele voedingsmiddelen kan de vorming van 4-methyl-4-mercapto-pentaan-2-on uit zwavelwaterstof en mesityloxyde leiden tot ernstige smaakafwijkingen.

H. T. BADINGS, J. Dairy Sci. 50 (1967) 1347.
R. L. S. PATTERSON, Chem. & Ind. (1968) 548; (1969) 48.

IX

Het koelen van verse melk vóór het ontromen vormt een doeltreffend middel om het gebrek 'zwavelwaterstofsmaak' in gepasteuriseerde room en boter te voorkomen.

H. T. BADINGS, Proc. XVIIIth Int. Dairy Congr. (1970).

X

Het is noodzakelijk dat de constructie van roompasteurs wordt aangepast aan de verwerking van room met een hoog vetgehalte.

XI

Aan het feit dat, in een periode waarin het lijden aan hart- en vaatziekten in Nederland in verontrustende mate is toegenomen, juist de consumptie aan melkvet sterk is gedaald, wordt onvoldoende aandacht besteed.

XII

Een directe invloed van een aantal methyl- en ethylesters van alifatische vetzuren op het gedrag van *Trogoderma granarium* is door de onderzoekingen van IKAN *et al.* niet aangetoond.

R. IKAN, E. D. BERGMANN, U. YIONOV, A. SHULOV, Nature 223 (1969) 317.

XIII

De bepaling van zwavelwaterstof in kaas volgens de methode van LAWRENCE leidt tot onjuiste uitkomsten.

R. C. LAWRENCE, J. Dairy Res. 30 (1963) 235.

XIV

Het feit dat de mens nog steeds in onvoldoende mate de consequenties onder ogen ziet van de beperktheid van het aardoppervlak en de biosfeer, vormt een ernstige bedreiging voor het voortbestaan van vele levensvormen op aarde.

NATIONAL ACADEMY OF SCIENCES, Resources and Man,
W. H. Freeman and Cy. (1969).

Dit proefschrift verschijnt ook als
Verslag nr. 124 van het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede

PREFACE

On completing this thesis, I should like to take the opportunity of expressing my feelings of gratitude.

Sincere thanks are due to Professor Dr. H. J. den Hertog and Professor Dr. H. Mulder for their interest and invaluable criticism during the preparation of this thesis. I am also highly indebted to those who have contributed to my scientific education at the Technical University of Delft. Of them I wish to mention particularly the late Professor Dr. P. A. Roelofsen and Professor Dr. B. M. Wepster, who aroused my interest for autoxidation processes in lipids and for the theoretical aspects underlying the mechanism of autoxidation reactions.

My scientific education has in fact been made possible by the help of my father, who taught me the elements of mathematics and physics during the years of Japanese imprisonment. For this I am greatly indebted to him.

The investigations described in this thesis were carried out at the Netherlands Institute for Dairy Research (NIZO) at Ede. They were part of the regular research programme of the chemico-physical section. I am much obliged to the Board of the Institute, its chairman Professor Ir. E. A. Vos, and the Director of the Institute Ir. H. Lolkema, for their support and their permission to work up the investigations in this thesis, and for their personal interest.

I also wish to remember the late Professor Dr. J. W. Pette, former Director of the Institute, under whose guidance this study was started.

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The mass-spectrometric analyses were carried out at the Central Institute of Nutrition and Food Research (CIVO)-TNO at Zeist, for which my sincere thanks are due to Dr. R. J. C. Kleipool, Ir. M. C. ten Noever de Brauw, Mr. J. Th. Heins and Drs. R. Belz.

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Finally it is a pleasure to acknowledge the following collaborators for their help to bring this work in its final form: Mr. G. H. Stel (preparation of the manuscript, cover design and lay-out), Mr. H. J. van Brakel and Mr. J. H. Mondria (tracings and photographs), and Miss M. E. Seyd and Mrs. A. Schouwenaar-Klein (typewriting).

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CHAPTER I

INTRODUCTION

Butter sometimes deteriorates considerably in quality as a result of flavour defects which develop during cold storage. It is now well established that the auto-catalytic oxidation of unsaturated fatty acids is the main cause of these flavour defects, which usually develop in the order: metallic, fatty, oily, trainy* and tallowy. The most offensive flavour is reached in the trainy stage.

SUPPLEE (1919) attributed the trainy (fishy) flavour to the presence of trimethylamine or trimethylamine oxide. This conception was supported by SOMMER and SMIT (1923), DAVIES and MATTICK (1928) and DAVIES and GILL (1936). VAN DER WAARDEN (1944, 1947) presented conclusive evidence, however, that the trainy flavour in cold-stored butter was caused neither by trimethylamine nor by its oxide. First of all, extraction of cold-stored butter with dilute acid did not reduce the trainy flavour, whereas the above-mentioned compounds would be removed by this method. Secondly, nitrogen determinations indicated that the amount of these compounds, if present in butter, was far below their flavour thresholds. In the third place the addition of sufficient quantities of the compounds in question did not induce flavour defects which resembled those in butter with cold-storage defects.

VAN DER WAARDEN (1944, 1947) also studied the volatile components of trainy butter. With the techniques available in those days, however, it was not possible to identify the minute quantities of volatile compounds from trainy butter. However, these investigations led to the provisional conclusion that aliphatic carbonyl compounds ('possibly unsaturated aldehydes with the double bond not in conjunction with the carbonyl group') might cause the specific trainy flavour.

Since the investigations of VAN DER WAARDEN, several others have tried to identify the volatile compounds in trainy butter. The most extensive studies so far were carried out by PONT *et al.* (1960), FORSS *et al.* (1960a, b, c), STARK and FORSS (1962) and EL-NEGOUMY *et al.* (1961). Several carbonyl compounds, such as alkanals, 2-alkenals, 2,4-alkadienals, and in later publications 1-alken-3-ones and 1-alken-3-ols were detected.

It should be noted, however, that these investigations were carried out with butter fat, washed cream and butter made from sweet cream, which were subjected to oxidation at room temperature. Therefore the results cannot be regarded as representative of butter made from soured cream and stored at low temperature.

*The term *trainy* denotes a flavour reminiscent of cod-liver oil. In English publications the term *fishy* is sometimes used to describe this defect.

In addition, several objections can be raised against previous analyses. First of all, the methods of analysis were inadequate for obtaining a complete separation and identification of these compounds. Secondly, it was not taken into account that many of the aroma compounds are very unstable. Consequently they were lost or transformed into artefacts. This holds particularly for unsaturated carbonyl compounds with double bonds not in conjunction with the carbonyl group and for *cis*-isomers. The present work has indicated that these compounds contribute strongly to oxidation flavour defects.

The investigations described in this thesis were carried out with the purpose of analysing more exhaustively the flavour compounds which contribute to the trainy flavour of butter with cold-storage defects by the application of modern physico-chemical methods. It was also investigated whether the formation of these aroma compounds could be related to the autoxidative breakdown of unsaturated fatty acids, and therefore the autoxidation processes of such acids have also been studied.

CHAPTER II

AUTOXIDATION PROCESSES OF FATTY ACIDS*

1. Introduction

In this Chapter the mechanism of autoxidation of fatty acids is discussed. Attention is paid particularly to the unsaturated fatty acids because these compounds contribute most strongly to autoxidation off-flavours. The autoxidation of saturated fatty acids, which have a lower oxidation rate, is also considered briefly.

2. The mechanism of autoxidation of unsaturated fatty acids

2.1 General aspects

The mechanism of autoxidation processes of unsaturated fatty acids has been studied extensively during the last decades. Studies of the autoxidation of pure mono-olefins and unsaturated fatty acids have contributed considerably to our present knowledge of this matter. At first it was thought that during the autoxidation of these components peroxides were formed by a direct attack of the double bond by oxygen (Stephens, 1928). Subsequent studies by Hock (1937), Criegee *et al.* (1939) and by Farmer and Sundralingham (1942) established, however, that at moderate temperatures the autoxidation of mono-enes or non-conjugated poly-enes usually proceeds through an attack of the methylene group(s) adjacent to the double bond(s) (the 'α-methylene group(s)'), leading to the formation of allylic hydroperoxides:

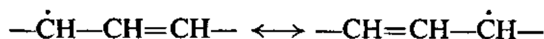
$$-CH_2-CH{=}CH- + O_2 \longrightarrow -CH(OOH)-CH{=}CH-$$

Special credit is due to workers at the British Rubber Producers Research Association for the elucidation of many fundamental aspects of autoxidation reactions of olefins and unsaturated fatty acids. Reviews on these studies have been presented by Bolland (1949) and Bateman (1954a). The results may be summarized as follows:

1. The majority of autoxidations proceed through free radical chain reactions. Peroxides are usually the primarily formed products.
2. There are three elementary reactions, as given in Figure 1:
   a. *Initiation.* Formation of free radicals which are necessary to start the propagation reaction.
   b. *Propagation.* This is the free radical chain reaction.
   If RH is the symbol for an olefin or an unsaturated fatty acid molecule,

* The subject-matter for this Chapter has been taken partly from a previous paper (Badings, 1960a).

H being a hydrogen atom of the methylene group adjacent to the double bond (α-methylene group), the propagation reaction proceeds as expressed in Figure 1. The α-methylene group is the preferential point of attack in the autoxidation reaction*, the intermediately formed α-methylene radical being stabilized by resonance:



c. *Termination.* Interruption of the free radical chain reaction by the formation of stable non-radical products from these free radicals.

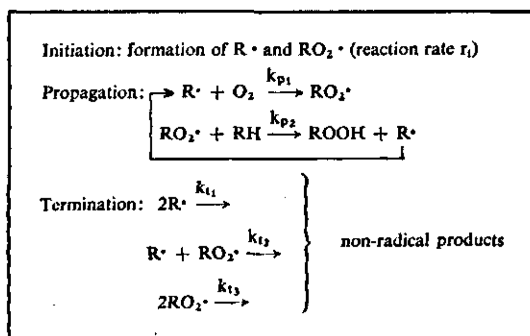


Fig. 1. Reaction scheme for olefin autoxidation.

3. The rate of autoxidation is dependent on the energy required for the rupture of the α-methylene C—H bond.
4. From the resonance formulae given in 2b, it can be predicted that isomeric hydroperoxides may be formed.
5. In line with their character, autoxidation reactions can be accelerated by light and by radical-forming products (such as benzoyl peroxide), initiating new chains. Conversely, these reactions may be inhibited by compounds (antioxidants) which react with free radicals to form non-radical products.
6. The outcome of the autoxidations is often complicated by numerous other reactions, such as the formation of polyperoxides by addition of peroxy-radicals to olefins, other polymerization reactions such as carbon-to-carbon polymerization, formation of epoxides, cyclic peroxides and secondary autoxidation products by dismutation of the hydroperoxides.
7. In contrast to the propagation reaction which is now fairly well understood, the initiation process is still by no means elucidated.

* This is true of the mono-unsaturated fatty acids and the poly-ene fatty acids with non-conjugated double bonds, but there is also some direct attack of the double bond occurring simultaneously. In fatty acids with conjugated double bonds this is even the main reaction mechanism, as will be discussed later.

The earlier theory proposed by FARMER (1946) and BOLLAND and GEE (1946b) implied either the direct attack of the double bond of the olefin by oxygen, or the abstraction of a hydrogen atom from the α-methylene group. The improbability of such reactions has been outlined by URI (1961). KHAN (1954a, b) proposed an initiation process, which is based on a direct olefin-oxygen reaction via a cyclic transition state leading to the formation of α-methylene hydroperoxides. Evidence for this concept was taken from the analyses of the hydroperoxides formed in the early stage of autoxidation. However, at observable rates of oxidation, the well-established free radical chain reaction being already dominant, no conclusions about the course of the initiation reaction can be drawn from the reaction products (BATEMAN, 1954b). For the same reason analyses of products formed in the initial stage of autoxidation (PRIVETT and BLANK, 1962) may not be relevant to the initiation reaction.

No doubt a very important part is played by trace amounts of heavy metals, which catalyse certain initiation reactions leading to the formation of free radicals (URI, 1961, 1967). If trace metals are absent, the initiation may be best explained by a termolecular reaction ($2RH + O_2 \longrightarrow 2R\cdot + H_2O_2$) proposed by DENISOV (1964).

It is clear that further research is necessary to establish which reactions are the basis of the initiation processes.

2.2 Autoxidation and hydroperoxide formation

2.2.1 *Classification*

According to their behaviour in autoxidation reactions, the unsaturated fatty acids can be classified into the following groups: mono-unsaturated fatty acids, poly-unsaturated fatty acids with non-conjugated double bonds, poly-unsaturated fatty acids with conjugated double bonds. The mechanism of autoxidation for each group will be discussed briefly.

2.2.2 *Mono-unsaturated fatty acids*

In these fatty acids there are usually two α-methylene groups which are the point of attack in the free radical chain reaction. The intermediately formed α-methylene free radicals are stabilized by resonance. From the resonance formulae it may be expected that isomeric hydroperoxides are formed.

In the autoxidation of oleic acid (see Figure 2) four isomeric hydroperoxides have indeed been found in approximately equal amounts (FARMER and SUTTON, 1943; ROSS *et al.*, 1949; PRIVETT and NICKELL, 1959). They seem to have predominantly the *trans*-configuration (SWERN *et al.*, 1953; FRANKEL, 1962). The energy required for dissociation of an α-methylene C—H bond being relatively high for mono-enes, their autoxidation rate, even at 50°C, is low (GUNSTONE and HILDITCH, 1945). A direct attack on the double bond by oxygen occurs simultaneously, particularly at higher temperatures (KHARASH *et al.*, 1953;

$$\mathrm{CH_3{-}(CH_2)_6{-}CH_2{-}CH{=}CH{-}CH_2{-}(CH_2)_6{-}COOH}$$

$$\nearrow\ \mathrm{{-}CH_2{-}CH{=}CH{-}\dot{C}H{-}} \longrightarrow \mathrm{CH_3{-}(CH_2)_7{-}CH{=}CH{-}CH(OOH){-}(CH_2)_6{-}COOH}$$

$$\updownarrow$$

$$\mathrm{{-}CH_2{-}\dot{C}H{-}CH{=}CH{-}} \longrightarrow \mathrm{CH_3{-}(CH_2)_7{-}CH(OOH){-}CH{=}CH{-}(CH_2)_6{-}COOH}$$

$$\searrow\ \mathrm{{-}CH{=}CH{-}\dot{C}H{-}CH_2{-}} \longrightarrow \mathrm{CH_3{-}(CH_2)_6{-}CH{=}CH{-}CH(OOH){-}(CH_2)_7{-}COOH}$$

$$\updownarrow$$

$$\mathrm{{-}\dot{C}H{-}CH{=}CH{-}CH_2{-}} \longrightarrow \mathrm{CH_3{-}(CH_2)_6{-}CH(OOH){-}CH{=}CH{-}(CH_2)_7{-}COOH}$$

Fig. 2. Hydroperoxide formation in the autoxidation of oleic acid.

SKELLON, 1948; ATHERTON *et al.*, 1944). Several other products (epoxides, polyperoxides, acids, etc.) are formed simultaneously (KNIGHT *et al.*, 1951; FEUEL and SKELLON, 1954; ELLIS, 1950).

2.2.3 *Poly-unsaturated fatty acids with non-conjugated double bonds*

In *linoleic acid* the C_{11}-methylene group is the preferential point of attack, because the formation of a C_{11}-radical requires less energy (larger resonance energy of the radical form R·) than in the case of attack at the C_8- or C_{14}-methylene group.

From Figure 3 it can be seen that there are three structures of R· contributing to the resonance hybrid, and the formation of three isomeric hydroperoxides would therefore be expected to take place. However, BERGSTRÖM (1945), CANNON *et al.* (1952), PRIVETT *et al.* (1953) and SEPHTON and SUTTON (1956) found that the greater part of the hydroperoxides formed consists of hydroperoxides with conjugated double bonds. From calculations by MOFFIT and COULSON (1948) it might be expected that the three R·-hybrid structures would contribute approximately to the same extent to hydroperoxide formation. Possibly the non-conjugated ROO·-radical is formed, but transforms rapidly into the more stable conjugated isomer (BATEMAN, 1954a):

```
—CH=CH—CH—CH=CH  →  —CH=CH—CH=CH—CH—
         \                          /
          O—O·                  ·O—O
```

The three structures contributing to the R·-resonance hybrid (in which coplanarity in the pentadienyl system of the carbon chain must be approached) are such that if conjugated diene hydroperoxides are formed, they will have predominantly a *cis, trans*-configuration.

```
 H   H  H   H            H   H  H   H            H   H  H   H
  \• /   \ /              \ /    \ /              \ /    \ •/
   C—C   C=C      ←→      C=C    C=C      ←→      C=C    C—C
  /   \ /   \            /   \ • /  \            /   \ //   \
CH2    C     CH2       CH2    C      CH2       CH2    C      CH2
       |                      |                       |
       H                      H                       H
```

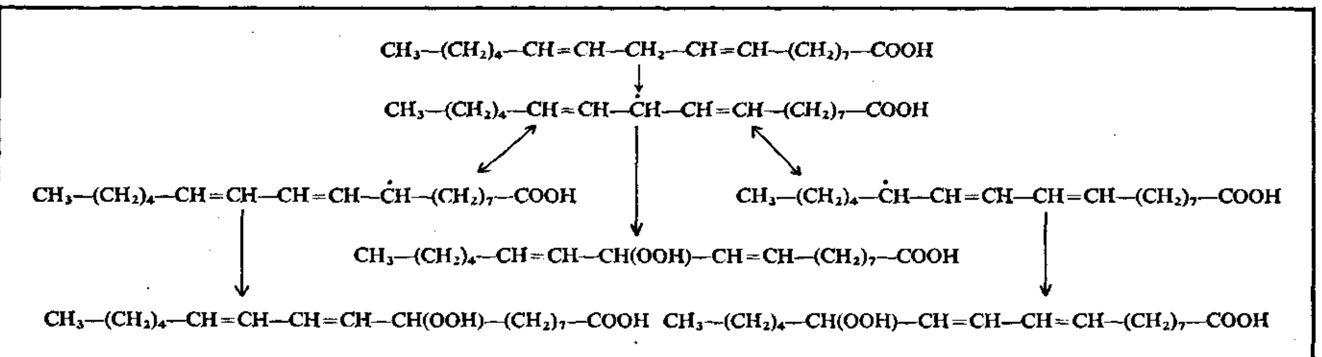


Fig. 3. Hydroperoxide formation in the autoxidation of linoleic acid.

with the number of double bonds. It is also evident that the free fatty acids oxidize more rapidly than do their methyl esters. The rate of autoxidation of methyl esters is comparable to that of the triglycerides if calculated per fatty acid moiety.

The effect of oxygen pressure on the rate of autoxidation of fatty acids and esters is negligible down to relatively low values of p_{O_2}. The higher the reactivity of RH, the higher this value will be, but even for ethyl linolenate the rate of autoxidation at 25°C decreases only at values of p_{O_2} below 40 mm Hg. Lowering the temperature will decrease this value even more.

The effect of temperature on the rate of autoxidation is somewhat greater than in many chemical reactions: not only is the chain propagation reaction accelerated, but also the decomposition of hydroperoxides, which leads to an increase in free radicals initiating new chains. Other factors influencing the rate of autoxidation will be discussed in Chapter III.

5. Secondary autoxidation products

5.1 Introduction

The autoxidation of saturated and unsaturated fatty acids and esters not only yields hydroperoxides but also other components such as hydroxy-, keto-, epoxy-compounds, polymers, cyclic peroxides, etc. For the present investigations the products which are formed by further degradation of the hydroperoxides of unsaturated fatty acids are of particular interest, because these secondary autoxidation products (saturated and unsaturated aldehydes, unsaturated ketones, secondary alcohols, etc.) contribute strongly to flavour defects in oxidized lipids.

A number of dismutation reactions for secondary hydroperoxides, as given by Bell *et al.* (1951), are presented in Figure 4.

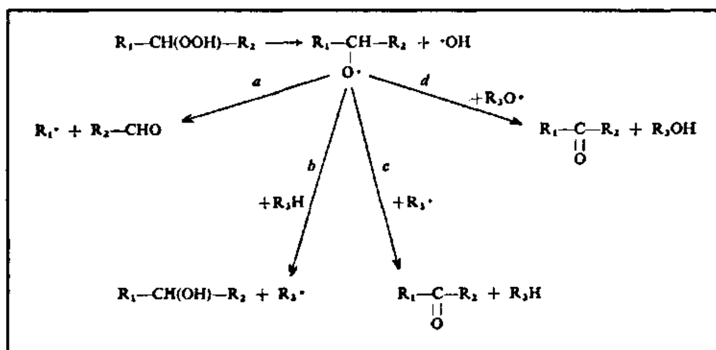


Fig. 4. Dismutation of secondary hydroperoxides.

5.2 Aldehydes

Aldehydes may be formed by dismutation of the fatty acid hydroperoxides according to reaction *(a)* of Figure 4. Table 2 indicates which hydroperoxides may be formed in the autoxidation of a number of fatty acids. Also the carbonyl compounds are listed which may be expected to result from dismutation of these hydroperoxides. It is assumed that the terminal α-methylene groups are also involved in the reaction and that the hydroperoxides with non-conjugated double bonds may also contribute in some way to the formation of carbonyl compounds.

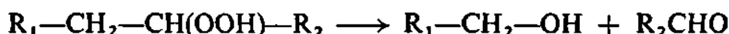
Finally, the secondary autoxidation products which were identified in previous investigations, are given in Table 2.

It can be seen that there is fair agreement between the list of aldehydes detected and the group of aldehydes that may be expected theoretically. However, the results of the identifications seem to be incomplete. Therefore further investigations are needed to determine whether other aldehydes which are to be expected theoretically (particularly those with non-conjugated double bond(s)) are present. The configuration of the double bonds in the aldehydes has also to be examined in more detail.

The identification of aldehydes other than those expected theoretically may be due to further degradation, migration of double bonds, etc. during the autoxidation processes.

5.3 Alkanols

Alkanols may be formed by the decomposition of secondary hydroperoxides according to the equation:

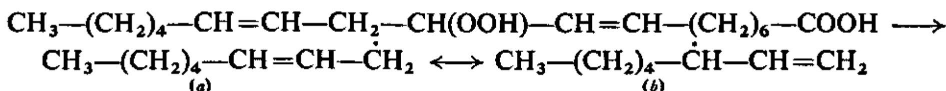


$$R_1—CH_2—CH(OOH)—R_2 \longrightarrow R_1—CH_2—OH + R_2CHO$$

STARK and FORSS (1966) have identified a number of 1-alkanols in oxidized butter fat in small quantities. Since these compounds have relatively high flavour threshold values, it is questionable whether the quantity present in oxidized butter fat (1 μmol/kg) is sufficient to contribute to flavour defects.

5.4. 1-Alken-3-ols*

HOFMANN (1962) and STARK and FORSS (1964) identified 1-octen-3-ol in oxidized linoleic acid and in butter fat. The formation of this compound can be explained by formation of 10-hydroperoxy-8,12-octadecadienoic acid from linoleic acid and degradation of this hydroperoxide by the reaction:



$$CH_3—(CH_2)_4—CH=CH—CH_2—CH(OOH)—CH=CH—(CH_2)_6—COOH \longrightarrow$$
$$\underset{(a)}{CH_3—(CH_2)_4—CH=CH—\dot{C}H_2} \longleftrightarrow \underset{(b)}{CH_3—(CH_2)_4—\dot{C}H—CH=CH_2}$$

* In the literature these compounds are sometimes given the name vinyl alkyl carbinols.

Table 2. Survey of the hydroperoxides and secondary autoxidation products (resulting from decomposition of these hydroperoxides) which might be expected and which have actually been found in the autoxidation of a number of unsaturated fatty acids.

fatty acid	methylene group involved (C-atom number)	isomeric hydroperoxides which may be formed from the structures contributing to the intermediate free radical resonance hybrid[1,3]
	8	8-OOH Δ^{9c} (+)
CH_3—$(CH_2)_7$—CH=CH—$(CH_2)_7$—COOH	8	10-OOH Δ^{8t} (+)
oleic acid	11	11-OOH Δ^{9c} (+)
	11	9-OOH Δ^{10t} (+)
	8	8-OOH $\Delta^{9c,12c}$
	8	10-OOH $\Delta^{8t,12c}$
	11	11-OOH $\Delta^{9c,12c}$
CH_3—$(CH_2)_3$—$(CH_2$—CH=CH$)_2$—$(CH_2)_7$—COOH	11	13-OOH $\Delta^{9c,11t}$ (+)
linoleic acid	11	9-OOH $\Delta^{10t,12c}$ (+)
	14	14-OOH $\Delta^{9c,12c}$
	14	12-OOH $\Delta^{9c,13t}$
	8	8-OOH $\Delta^{9c,12c,15c}$
	8	10-OOH $\Delta^{8t,12c,15c}$
	11	11-OOH $\Delta^{9c,12c,15c}$
	11	9-OOH $\Delta^{10t,12c,15c}$ (+)
CH_3—$(CH_2$—CH=CH$)_3$—$(CH_2)_7$—COOH	11	13-OOH $\Delta^{9c,11t,15c}$ (+)
linolenic acid	14	14-OOH $\Delta^{9c,12c,15c}$
	14	12-OOH $\Delta^{9c,13t,15c}$ (+)
	14	16-OOH $\Delta^{9c,12c,14t}$ (+)
	17	17-OOH $\Delta^{9c,12c,15c}$
	17	15-OOH $\Delta^{9c,12c,16t}$
	4	4-OOH $\Delta^{5c,8c,11c,14c}$
	4	6-OOH $\Delta^{4t,8c,11c,14c}$
	7	7-OOH $\Delta^{5c,8c,11c,14c}$
	7	5-OOH $\Delta^{6t,8c,11c,14c}$
	7	9-OOH $\Delta^{5c,7t,11c,14c}$
	10	10-OOH $\Delta^{5c,8c,11c,14c}$
CH_3—$(CH_2)_3$—$(CH_2$—CH=CH$)_4$—$(CH_2)_3$—COOH	10	8-OOH $\Delta^{5c,9t,11c,14c}$
arachidonic acid	10	12-OOH $\Delta^{5c,8c,10t,14c}$
	13	13-OOH $\Delta^{5c,8c,11c,14c}$
	13	11-OOH $\Delta^{5c,8c,12t,14c}$
	13	15-OOH $\Delta^{5c,8c,11c,13t}$
	16	16-OOH $\Delta^{5c,8c,11c,14c}$
	16	14-OOH $\Delta^{5c,8c,11c,15t}$

[1] In this column + indicates that this hydroperoxide has actually been identified.

[2] * indicates that these aldehydes are the major carbonyl compounds to be expected or found.

[3] Example of coding: 15-OOH = 15-hydroperoxy; $\Delta^{9c,11t}$ = 9 *cis*, 11 *trans*-diene.

[4] Example of coding: $C_8\Delta^{2c,5c}$-dienal = 2 *cis*, 5 *cis*-octadienal.

secondary autoxidation products which may be expected[2, 4]	secondary autoxidation products which have been identified			
	aldehydes (minor fractions not included)[2, 4]		other components	
$^{*}C_{11}\ \Delta^{2c}$-enal	$^{*}C_{11}\ \Delta^{2}$-enal	(1, 3, 10)		
$^{*}C_{9}$-al	$^{*}C_{9}$-al	(1, 3, 10)		
$^{*}C_{8}$-al	$^{*}C_{8}$-al	(1, 3, 10)		
$^{*}C_{10}\ \Delta^{2t}$-enal	$^{*}C_{10}\ \Delta^{2}$-enal	(1, 3, 10)		
	$C_{9}\ \Delta^{2}$-enal	(1, 3, 10)		
	C_{6}	(3, 10)		
	C_{7}	(1, 10)		
$C_{11}\ \Delta^{2c,\ 5c}$-dienal				
$C_{9}\ \Delta^{3c}$-enal	$C_{9}\ \Delta^{2}$-enal	(3, 4)		
$^{*}C_{8}\ \Delta^{2c}$-enal	$^{*}C_{8}\ \Delta^{2}$-enal	(2, 3)	1-octen-3-ol	(9, 13)
$^{*}C_{6}$-al	$^{*}C_{6}$-al	(2, 3, 4, 12)	1-octen-3-one	(4, 5)
$^{*}C_{10}\ \Delta^{2t,\ 4c}$-dienal	$^{*}C_{10}\ \Delta^{2,4}$-dienal	(2, 3, 4, 8, 12)		
C_{5}-al				
$C_{7}\ \Delta^{2t}$-enal	$C_{7}\ \Delta^{2}$-enal	(4, 5)		
	C_{2}-al	(3, 4)		
	C_{3}-al	(3, 4)		
$C_{11}\ \Delta^{2c,\ 5c,\ 8c}$-trienal				
$C_{9}\ \Delta^{3c,\ 6c}$-dienal				
$^{*}C_{8}\ \Delta^{2c,\ 5c}$-dienal			C_{1}–C_{5} hydrocarbons	(11)
$^{*}C_{10}\ \Delta^{2t,\ 4c,\ 7c}$-trienal				
$^{*}C_{6}\ \Delta^{3c}$-enal	$^{*}C_{6}\ \Delta^{3}$-enal (6);	$C_{6}\ \Delta^{2}$-enal (3, 4)		
$^{*}C_{5}\ \Delta^{2c}$-enal	$^{*}C_{5}\ \Delta^{2}$-enal	(3)	$C_{9}\ \Delta^{2,\ 6}$-dienal	(5)
$^{*}C_{7}\ \Delta^{2t,\ 4c}$-dienal	$^{*}C_{7}\ \Delta^{2,4}$-dienal	(3, 4, 7)		
$^{*}C_{3}$-al	$^{*}C_{3}$-al	(3, 4)		
C_{2}-al				
$C_{4}\ \Delta^{2t}$-enal	$C_{4}\ \Delta^{2}$-enal	(3)		
$C_{17}\ \Delta^{2c,\ 5c,\ 8c,\ 11c}$-tetraenal				
$C_{15}\ \Delta^{3c,\ 6c,\ 9c}$-trienal				
$^{*}C_{14}\ \Delta^{2c,\ 5c,\ 8c}$-trienal				
$^{*}C_{16}\ \Delta^{2t,\ 4c,\ 7c,\ 10c}$-tetraenal				
$^{*}C_{12}\ \Delta^{3c,\ 6c}$-dienal				
$^{*}C_{11}\ \Delta^{2c,\ 5c}$-dienal				
$^{*}C_{13}\ \Delta^{2t,\ 4c,\ 7c}$-trienal				
$^{*}C_{9}\ \Delta^{3c}$-enal				
$^{*}C_{8}\ \Delta^{2c}$-enal	$^{*}C_{8}\ \Delta^{2}$-enal	(14)		
$^{*}C_{10}\ \Delta^{2t,\ 4c}$-dienal	$^{*}C_{10}\ \Delta^{2,\ 4}$-dienal	(14)		
$^{*}C_{6}$-al	$^{*}C_{6}$-al	(14)		
C_{5}-al				
$C_{7}\ \Delta^{2t}$-enal				

Literature references

(1) Badings (1959a). (2) Badings (1959b). (3) Ellis *et al.* (1961). (4) El-Negoumy *et al.* (1962). (5) Hammond & Hill (1964). (6) Hofmann (1961a), (from linolenic acid containing oil). (7) Hofmann (1961b), (from linolenic acid containing oil). (8) Hofmann & Keppler (1960), (from linoleic acid containing oil). (9) Hofmann (1962). (10) Horikx & Schogt (1959). (11) Horvat *et al.* (1964). (12) Patton *et al.* (1959). (13) Stark & Forss (1964). (14) Ellis *et al.* (1966).

From the resonance structure *(b)*, it may be expected that 1-octen-3-ol is formed by reaction with an OH-radical. However, it may also form the hydroperoxide CH_3—$(CH_2)_4$—CH(OOH)—CH=CH_2. When this hydroperoxide decomposes according to reaction *(b)* in Figure 4, 1-octen-3-ol may also be formed. Other 1-alken-3-ols (e.g. 1-penten-3-ol) have been identified by IWATA *et al.* (1965) and STARK *et al.* (1967). Their formation may be explained in a similar way.

5.5 1-Alken-3-ones*

STARK and FORSS (1962) identified 1-octen-3-one in oxidized butter fat. HAMMOND and HILL (1964) detected this compound in oxidized trilinolein. HILL and HAMMOND (1965) found 1-penten-3-one in oxidized lipids and STARK *et al.* (1967) identified this compound in oxidized cream.

1-Octen-3-one may be formed via the formation of 3-hydroperoxy oct-1-ene (see Section 5.4), followed by dismutation according to reaction *(c)* in Figure 4.

5.6 Hydrocarbons

In the autoxidation of lipids, hydrocarbons are also formed. These components were identified by HORVAT *et al.* (1964, 1965) (autoxidation of methyl linoleate), FORSS *et al.* (1967) (copper-catalysed oxidation of butter fat), LÜCK *et al.* (1966b) (oxidation with UV-radiation), MERRIT *et al.* (1965) and LÜCK *et al.* (1966a) (oxidation with γ-radiation).

SMOUSE *et al.* (1965) identified 1-decyne in some autoxidized vegetable oils. Its flavour threshold value is very low.

The formation of hydrocarbons is explained by the fact that alkyl radicals are formed during the autoxidation processes, which may transform into alkanes, alkenes, alkynes, etc. Because these compounds are of less importance for the present investigations, their formation will not be discussed in more detail.

* In the literature these compounds are sometimes given the name vinyl alkyl ketones.

CHAPTER III

FLAVOUR DEFECTS IN AUTOXIDIZED FOOD LIPIDS

1. Introduction

Autoxidative deterioration is one of the main causes of flavour defects in lipid-containing food products. The unsaturated fatty acids are particularly susceptible to autoxidation and will primarily form hydroperoxides, which are mostly odourless and tasteless (Henick *et al.*, 1954). Their dismutation products, however, induce flavour defects, even if they are present in very small quantities, sometimes 1 part per 10^9.

In this chapter, the formation of odorous components as a result of autoxidation processes in lipids and in lipid-containing food products will be discussed.

2. Organoleptic properties of compounds formed by autoxidation of lipids

Compounds which contribute to autoxidation off-flavours belong to the following classes: alkanals, 2-alkenals, 2,4-alkadienals, non-conjugated enals and dienals, 1-alken-3-ols, 1-alken-3-ones, saturated and unsaturated hydrocarbons. The flavour threshold values of a number of these compounds, as compiled from the literature and from own experiments, are presented in Table 3. It also contains the terms that are generally used to describe the flavours caused by the various compounds.

In general the data found here are in fairly good agreement with those found by Meijboom (1964) and by others. The threshold values found by Lea and Swoboda (1958) are considerably higher. Aliphatic carbonyl compounds with more than twelve carbon atoms in the chain have much higher threshold values and are of less importance.

Mixtures of the compounds in Table 3 can give detectable flavours at subthreshold concentrations (Day *et al.*, 1963). On the other hand Meijboom (1964) found that on mixing 3 *cis*-hexenal and 2 *trans*, 4 *trans*-heptadienal at concentrations of 13.2 p.p.m. and 12.5 p.p.m. respectively, an almost tasteless mixture was obtained. It therefore seems that in certain cases extinguishing effects are possible.

n-Alkanals have threshold values which lie between 0.025 p.p.m. (butanal) and 0.7 p.p.m. (decanal). The lower members have a sharp and irritating flavour; the medium ones (C_5 to C_9) have the most offensive flavour (green, oily, tallowy) and the higher members (C_{10} to C_{12}) rather resemble the flavour of orange peels.

Table 3. Flavours and flavour threshold values of volatile components which contribute to autoxidation off-flavours (determination by the method of Patton and Josephson, 1957).

compound: type	compound: name	threshold value (in p.p.m.) for taste in paraffin oil: literature		threshold value (in p.p.m.) for taste in paraffin oil: own experiments	description of flavour
alkanals	propanal	1.6	(1)	–	sharp, irritating
	butanal	0.024	(1)	0.025	sharp, irritating
	pentanal	0.15	(1)	0.07	sharp
	hexanal	0.15	(1)	0.08	green
	heptanal	0.042	(1)	0.055	oily, putty
	octanal	0.068	(1)	0.040	fatty
	nonanal	0.32	(1)	0.20	tallowy
	decanal	1.07	(1)	0.70	orange peels
	undecanal	0.1	(1)	0.09	citrus
	dodecanal	0.046	(1)	0.045	fatty, citrus
2-alkenals	2 *trans*-pentenal	0.32	(1)	1.0	sharp, paint, green
	2 *cis*-pentenal			0.8	sharp, fatty, green
	2 *trans*-hexenal	2.5	(1)	0.6	green
	2 *cis*-hexenal			0.5	green
	2 *trans*-heptenal	0.63	(1)	0.2	putty, fatty
	2 *cis*-heptenal			0.15	putty
	2 *trans*-octenal	1.0	(1)	0.15	fatty, woodbugs
	2 *cis*-octenal			0.05	fatty, wallnuts
	2 *trans*-nonenal	0.1	(1)	0.04	tallowy, cucumbers
	2 *cis*-nonenal			0.04	washing, cucumbers
	2 *trans*-decenal	5.5	(1)	0.15	tallowy
	2 *cis*-decenal			0.10	washing, fatty
	2 *trans*-undecenal	4.2	(1)		
	2 *cis*-dodecenal	6.3	(1)		
other alkenals	3 *trans*-hexenal	1.2	(1)	0.95	green leaves
	3 *cis*-hexenal	0.11	(1)	0.09	fresh green leaves
	4 *trans*-heptenal	0.32	(4)	0.1	putty
	4 *cis*-heptenal	0.0016	(4)	0.0005	creamy, putty
	4 *trans*-decenal			0.01	tallowy, cucumbers
2,4-alkadienals	2 *trans*, 4 *trans*-hexadienal	0.036	(1)	0.040	fatty, green
	2 *trans*, 4 *trans*-heptadienal	0.46	(1)	0.10	fatty, oily
	2 *trans*, 4 *cis*-heptadienal	0.055	(1)	0.040	frying odour
	2 *trans*, 4 *trans*-octadienal	0.15	(1)		cardboard
	2 *trans*, 4 *trans*-nonadienal	0.46	(1)		fatty, oily
	2 *trans*, 4 *trans*-decadienal	0.28	(1)	0.10	'deep-fried'
	2 *trans*, 4 *cis*-decadienal			0.020	frying odour
other alkadienals	2 *trans*, 6 *trans*-nonadienal	0.018	(1)	0.020	cucumbers, tallowy
	2 *trans*, 6 *cis*-nonadienal	0.002	(1)	0.0015	fresh cucumbers
1-alken-3-ones	1-buten-3-one			0.25	sharp, irritating
	1-penten-3-one			0.003	sharp, fishy
	1-octen-3-one	0.0001	(2)	0.0001	metallic
1-alken-3-ols	1-penten-3-ol			4.2	sharp, irritating
	1-octen-3-ol	0.01	(3)	0.0075	mushroom
other compounds	3,5-octadien-2-one			0.30	fruity, fatty
	3,5-undecadien-2-one			1.6	fatty, fried
	1-decyne	0.1	(5)		raw mango

Literature references
(1) Meijboom (1964).
(2) Stark and Forss (1962)
(3) Stark and Forss (1964)
(4) Hofmann and Meijboom (1968)
(5) Smouse *et al.* (1965)

2-Alkenals have much higher threshold values than the corresponding saturated aldehydes. Exceptions are 2-nonenal and 2-decenal. The flavours of 2 *trans*-enals vary from green (C_5, C_6) to putty (C_7), woodbugs (C_8) and tallowy/cucumbers (C_9, C_{10}). The threshold values of 2 *cis*- and 2 *trans*-enals of the same chain length are fairly close to each other. However, the odour of a 2 *cis*-enal is generally more pleasant than that of the corresponding 2 *trans*-isomer.
2,4-Alkadienals have threshold values which are usually a great deal lower than those of the corresponding 2-alkenals. Values of 2 *trans*, 4 *trans*-dienals fall in the range from 0.036 p.p.m. (2,4-hexadienal) to 0.46 p.p.m. (2,4-nonadienal). The values of 2 *trans*, 4 *cis*-dienals are lower than those of the corresponding 2 *trans*, 4 *trans*-dienals. Also, the flavour of the former components is more pleasant. All 2,4-dienals contribute to fatty or 'fried' flavours. The lower terms (2,4-hexadienal) also have a green note; the higher terms give a strong fatty/oily sensation, which is most noticeable with 2 *trans*, 4 *trans*-decadienal (PATTON *et al.*, 1959).
Non-conjugated alkenals are highly potential odorous compounds. 3 *Cis*-hexenal has a flavour that is reminiscent of freshly crushed green leaves, and has a threshold value of approximately 0.1 p.p.m. As for the 2,4-alkadienals, here also the *trans*-isomer has a higher threshold value and a less pleasant odour. The threshold value of 3 *trans*-hexenal is ten times higher (approximately 1 p.p.m.) than that of the *cis*-isomer. Its odour is also 'green' but less 'fresh'. Non-conjugated C_6–C_{10} alkenals possess flavours reminiscent of putty, cardboard or train-oil (VAN DUIN, 1960). 4 *Cis*-heptenal deserves special mention, because its threshold value is at an exceptionally low level (0.5 p.p.b.). HAVERKAMP BEGEMANN and KOSTER (1964) found that low concentrations of this component (1.5 p.p.b.) cause a pleasant cream-like flavour. At slightly higher levels (10 p.p.b.), however, an unpleasant flavour, reminiscent of oxidative deteriorated butter, appears (BADINGS, 1965).
Non-conjugated alkadienals possess strong flavours that resemble cucumbers and sliced beans; upon dilution these flavours become beany and fishy (VAN DUIN, 1960). 2,6-Nonadienal, a compound with a typical cucumber-like flavour, is the best known. The 2 *trans*, 6 *cis*-isomer has a very low threshold value (approximately 2 p.p.b.). The 2 *trans*, 6 *trans*-isomer has a threshold value that is about ten times higher.
2-Alkanones, found in the flavour concentrate of oxidized milk lipids, often must be attributed to the formation of artefacts during the isolation procedure. It is known that these compounds may be formed when milk fat is heated in the presence of small quantities of water (LANGLER and DAY, 1964).

Since 2-alkanones are the outcome of non-oxidative reactions, their organoleptic properties will not be discussed. It should be mentioned, however, that their threshold values are considerably higher than those of the corresponding alkanals.

1-Alken-3-ols are highly potential odorous compounds. 1-octen-3-ol, whose threshold value is very low (8 to 10 p.p.b.), has a mushroom-like flavour.
1-Alken-3-ones. A component with a metallic flavour is 1-octen-3-one (STARK and FORSS, 1962); its threshold value is exceptionally low (0.1 p.p.b.). The threshold values of 1-buten-3-one and 1-penten-3-one are 0.25 p.p.m. and 3 p.p.b. respectively. These compounds possess a very sharp, fishy flavour.
Hydrocarbons have been identified in the volatile fraction from oxidized lipids, but little is known about their contribution to (oxidation) flavour defects. It seems, however, that among the unsaturated hydrocarbons, compounds with low threshold values are present. An example is 1-decyne (SMOUSE *et al.*, 1965).
1-Alkanols have been found by STARK and FORSS (1966) in oxidized butter. These compounds are formed by autoxidation and dismutation of unsaturated fatty acid hydroperoxides. Because of their high threshold values they are of only secondary consideration in the field of oxidation flavours.
Other compounds, not belonging to the groups mentioned above, may also contribute to oxidation flavours. For example, it must be expected (see Table 2) that trienals and tetraenals will also be formed by the oxidation of unsaturated fatty acids. Indications of the formation of such compounds have been obtained by ELLIS *et al.* (1966), but their organoleptic properties and threshold values are not known at present.

Another component which has been identified in the volatile fraction of oxidized butter is 3,5-octadien-2-one (BADINGS and VAN DUIN, 1967). It has a fruity flavour, and a threshold value of 0.30 p.p.m.

It may be concluded that the oxidation of lipids leads to the formation of a large number of volatile components, many of which have very low flavour threshold values and possess offensive odours. Therefore even a limited autoxidation is often sufficient to cause flavour defects.

3. CONTRIBUTION OF VARIOUS FATTY ACIDS TO AUTOXIDATION OFF-FLAVOURS

The contribution of a number of fatty acids to autoxidation off-flavours was determined experimentally. Small quantities of the esters were spread on filter paper which were placed in conical flasks. After autoxidation for some time the odour of the autoxidized esters was evaluated. The experimental details and the organoleptic observations made are recorded in Table 4.

It is of interest to note that the observed odours are in good agreement with those of the compounds which may be expected on account of the dismutation reactions given in Table 2 (BADINGS, 1960a). From Table 4 it is also clear that linolenic, arachidonic and linoleic acid in particular seem to contribute strongly to autoxidation off-flavours. Of this group of fatty acids, linolenic acid causes flavour defects which are most reminiscent of those in trainy butter.

Recently a number of octadecadienoic acids have been identified in butter fat (DE JONG and VAN DER WEL, 1964, 1967a, b). Some of these fatty acids have been found to be precursors to the oxidative formation of 4 *cis*-heptenal, which may contribute strongly to oxidation flavours (BADINGS, 1965).

Preliminary investigations have been carried out on the monocarbonyl compounds formed by autoxidation of methyl eicosa-pentaenoate, methyl docosahexaenoate and cod-liver oil (FISCHER and WISHNER, 1968), but only alkanals, 2-enals and 2,4-dienals have been identified.

Table 4. Organoleptic evaluation of a number of autoxidized fatty acids.

substrate	autoxidation temperature (°C)	period of autoxidation	flavour
tristearin	40	6 months	similar to candle grease
triolein	20	2 months	tallowy
trilinolein	20	4 hours	linseed oil, 'deep-fried'
methyl linolenate	20	2 hours	trainy, fishy
methyl arachidonate	20	1 hour	oily, raw liver

The extent to which many fatty acids contribute to autoxidation off-flavours is as yet unknown. Saturated and mono-enoic fatty acids may do so to a limited extent only, but in the prolonged oxidation they may also contribute significantly. Conversely, poly-enoic acids may be potential precursors to autoxidation flavour defects. This subject will be further discussed in Chapter VII.

4. OXIDATION DEFECTS IN LIPID-CONTAINING FOODS

The way in which oxidative deterioration develops in fats, oils and lipid-containing food products depends on numerous factors. Most important are: the presence of oxygen, the composition of the lipid fraction, the presence of pro- and anti-oxidants, the physical structure of the product, the storage temperature, etc.

In liquid milk products (milk, cream, buttermilk) and in butter, the main substrate for oxidation is the phospholipid fraction. The position of the phospholipids at the fat/water interface, where they may come into contact with pro-oxidants such as copper, renders them highly susceptible to oxidation. In dried milk products, the effect of water-soluble pro- and anti-oxidants is of minor importance. The fat seems to be attacked directly by oxygen in the same way as is done with pure oils and fats. It is noteworthy that in these products the presence of phospholipids in the lipid phase displays a certain anti-oxidant activity (SMITH *et al.*, 1958). In many products which contain milk fat, distinct oxidized flavours occur even at peroxide levels lower than 1.0 mæq/kg.

Poly-unsaturated fatty acids play a predominant role in autoxidative deterioration. Their rapid autoxidation increases the oxidation susceptibility of lipid-containing products as a whole. Their autoxidation products also contain strong off-flavours.

Flavour defects which occur in the initial stage of autoxidative deterioration will be due mainly to volatile (carbonyl) compounds originating from polyene fatty acids. An example is the reversion flavour in vegetable oils (DUTTON *et al.*, 1951; HOFFMANN, 1961a; SMOUSE and CHANG, 1967). Flavour defects at a later stage of autoxidative deterioration may be caused by volatile compounds which originate from the less unsaturated fatty acids and by compounds resulting from a further degradation of the compounds formed in the early stages of autoxidation.

It is clear that the autoxidation processes are of a dynamic nature. During the progress of the reactions, the mixture of components which is formed changes continuously (qualitatively and quantitatively). This is the main cause of changes in oxidation flavour (metallic, green, fishy, tallowy) and of the order in which oxidation defects develop during deterioration of food products.

Although the reaction rate of autoxidation is lower as the temperature decreases, this is by no means a general rule. BETTS and URI (1963) found that the reaction rates for the metal-catalysed oxidation of 1-docosene (m.p. 41 °C) at 25 °C and 50 °C are almost identical. Conversely, the reaction rate of the metal-catalysed oxidation of 1-hexadecene (m.p. 4 °C) at 50 °C is equal to that of 1-docosene, but is almost ten times lower at 25 °C. This anomalous aspect of autoxidation can be explained as follows. In the solid phase there is a sharp reduction of the diffusion-controlled rate of termination by free radical combination ($RO\cdot_2 + RO\cdot_2 \longrightarrow$ non-radical products). The rate-determining propagation reaction ($RO\cdot_2 + RH \longrightarrow ROOH + R\cdot$) is expected to proceed at only a slightly reduced rate in the solid phase.

5. COLD-STORAGE DEFECTS IN BUTTER

As has been outlined before, the typical flavour defects of cold-stored ripened cream butter are usually caused by oxidative processes. Before discussing these processes, attention will be paid to the lipid fraction in milk and butter.

5.1 Lipids in milk

The fat globules in milk contain the majority of the milk lipids. These consist almost entirely (approximately 99 %) of triglycerides of fatty acids with carbon chain lengths between C_4 and C_{26}). Minor constituents are free fatty acids, mono- and diglycerides, phospholipids, cerebrosides, sterols, carotenoids, fat-soluble vitamins, water, etc.

The fat globules derive their stability from the presence of an enveloping

membrane, the fat globule membrane. It consists mainly of phospholipids, proteins and (possibly) triglycerides (PALMER *et al.*, 1933, 1935, 1939). A phospholipid protein complex (lipoprotein) seems to be the principal constituent of this membrane. Since the surface layers of the fat globules are readily damaged (by stirring, churning, foaming, etc.), the composition of the layers may change with the treatment to which the milk is subjected (MULDER, 1957).

The milk phospholipids consist mainly of lecithin, cephalin and sphyngomyelin in the ratio 30:45:25 (KOOPS, 1958). Short-chain fatty acids are not present in milk phospholipids. They contain more unsaturated fatty acids (particularly in the cephalin fraction) than does milk fat. Major fatty acids are palmitic acid 15.9%, stearic acid 16.3%, oleic acid 40.3% and linoleic acid 6.1% (BADINGS, 1962; SMITH and LOWRY, 1962; MATTSON, 1962). Other constituents of the lipid moiety of the membrane material are cholesterol (partly esterified) (MULDER and ZUIDHOF, 1958), mono-, di- and triglycerides, squalene, carotenoids, etc. (THOMPSON *et al.*, 1961). It is noticeable that the triglyceride fraction (prepared from membrane material obtained from washed cream) is composed principally of high melting triglycerides (melting point approximately 50°C). It contains mainly myristic acid (10%), palmitic acid (60%) and stearic acid (17%) (PATTON and KEENEY, 1958; BADINGS, 1960b; THOMPSON *et al.*, 1961). The quantity of protein present on the surface of the fat globule membrane is 0.1 to 3 g (average 0.8 g) per 100 g of fat globules (MULDER and MENGER, 1958).

The natural iron content of milk lies between 100 and 250 µg/kg (MULDER, MEIJERS and MENGER, 1964a). Approximately 25% of this iron is present in the fat globule membrane (MULDER and KOPPEJAN, 1953). The natural copper content of normal milk lies between 20 and 40 µg/kg (MULDER *et al.*, 1964b), of which approximately 18% is present in the fat globule membrane (MENGER, 1961; KOOPS, 1963). As will be discussed in the following sections, the contamination of milk with copper may cause a rapid oxidation of milk lipids.

5.2 The structure of the fat globule membrane

Although a great deal of information is available concerning the chemical composition of the membranous material, comparatively little is known about its physical structure. It is clear that intermolecular associations (lipid/protein, etc.) will participate in this structure. KING (1955) has suggested a structure for the milk fat globule membrane which consists in principle of a layer of phospholipid molecules (and minor constituents), which is oriented radially around the milk fat globule, and a second layer of proteins.

It is questionable whether KING's model agrees with the process of formation of milk fat globules in the udder of the cow. It has to be taken into account that the fat globules and milk plasma are very probably formed separately in different parts of the secretory cells of the udder (MULDER, 1947, 1957). Consequently it is reasonable to suppose that the fat globule is covered by a layer of cyto-

plasmic components. When this fat globule then comes into contact with the milk plasma, a second layer of milk plasma material may be formed around the first layer.

MORTON (1954) isolated microsomes from washed cream buttermilk. He postulated that these particles were adsorbed to a protein layer surrounding the fat globule. Recent investigations by HAYASHI *et al.* (1965a, b) appear to support Morton's conception to some extent. The treatment of intact milk fat globules with sodium desoxycholate was found to release lipoprotein particles accounting for 45% of the membrane material. After this treatment, the other part of the membranous material was still associated with the fat globule, which indicates that this fraction occurs as an adsorbed layer around the globule.

Other concepts of the structure of the fat globule membrane have been given by GREENBANK *et al.* (1961) (adsorption of lipoprotein particles to a highly surface-active protein layer which envelopes the fat globule) and by BAUD (1953) (patch-wise distribution of the phospholipids in a continuous protein 'carpet').

Of particular interest are the studies of BARGMAN (1959, 1962) and BARGMAN *et al.* (1959, 1961) on the mammary tissue of lactating rats. From electron-microscopic examinations it was concluded that fat globules formed in the secretory cells are enveloped by a layer of cytoplasmic components, possibly encapsulated by a cellular membrane.

It must be concluded that, despite numerous investigations, the knowledge of the structure of the fat globule membrane is still limited. BRUNNER (1965) has summarized many of the above theories without suggesting which model would be most acceptable. COPIUS PEEREBOOM (1969), however, concluded that a model in accordance with the findings of MORTON (1954) and HAYASHI *et al.* (1965a, b) would be the most acceptable. This model seems to support the view of MULDER (1947) that the fat globule membrane is of a more or less dynamic nature, which is demonstrated by the effect of various dairy technological processes on the composition of this layer.

5.3 The structure of butter

In the conventional buttermaking process, cream is churned and worked to obtain butter and buttermilk. The processes which take place during churning have been studied extensively (VAN DAM and HOLWERDA, 1934; MULDER, 1947; KING, 1953).

During the churning and working of the butter granules, a certain proportion of the fat globules is disintegrated. Consequently the structure of the resulting butter consists of a continuous butter fat phase, fat globules, water droplets, air bubbles, etc. The continuous fat phase is intersected by a network of small water-containing channels which connect water droplets and surface layers of the fat globules (MULDER, 1947).

According to KING (1947), the amount of fat occurring in the fat globules may be estimated at 40 to 70%, while approximately 30 to 60% represent the continuous fat phase. The diameter of the fat globules varies from 1.6 to 8 μ (average 4 μ), and the number is approximately 10^{10} per ml of butter.

5.4 The development of cold-storage defects in butter

5.4.1 *Mechanism of the development of oxidation defects in cold-stored butter*

VAN DER WAARDEN (1947) was the first to present conclusive evidence that cold-storage defects in butter are caused by oxidative degradation of lipid components. These oxidation processes were thought to occur particularly in the serum phase or at the interface between serum and fat. The pronounced effect of the acidity of the serum, of copper and of water-soluble antioxidants was put forward by MULDER *et al.* (1947a, b) as strong evidence that oxidation processes do indeed start at the serum/fat interface.

From his experiments, TOLLENAAR (1953) also came to this conclusion. When oxidative deterioration occurs in butter, the oxidation continues in the fat phase. This causes an increase in the peroxide value of the fat, parallel to an increase in the intensity of cold-storage defects. If, however, a fat-soluble antioxidant (dodecyl gallate) is added, the fat oxidation is retarded, although the flavour defects develop normally. The investigations of the antioxidant activity of ascorbyl palmitate by KOOPS (1964) have furnished further evidence. Ascorbyl palmitate is partly hydrophilic (ascorbyl moiety) and partly lipophilic (palmitate moiety). This component will therefore most probably accumulate in the fat/water interface where it effectively retards the oxidation processes.

The indication that cold-storage defects are primarily caused by oxidation of the phospholipids is strengthened by the finding that, whereas the fat of trainy butter is still in its induction period, the phospholipids are already in a stage of active oxygen absorption (VAN DUIN, 1958).

From determinations of TBA-values, similar conclusions may be drawn (BADINGS, 1967b). Table 5 presents TBA-values of butter which had been

Table 5. TBA-values of butter, stored at –10°C and of the constituents (fat and serum); copper content of the butter 80 μg/kg.

products which were analysed	period of storage at –10°C (in months)			
	0	1	3	6
10 g of butter	0.060	0.250	0.480	0.570
fat phase of 10 g of butter	0.015	0.050	0.062	0.068
serum phase of 10 g of butter	0.045	0.220	0.415	0.528
per g of butter fat (A)	0.002	0.006	0.007	0.008
per g of phospholipid (B)	2.250	11.000	20.750	26.400
value for $\frac{B}{A}$	1125	1833	2964	3300

stored at –10°C for some months. The TBA-values of this butter as well as those of the constituents (butter fat and butter serum) were determined by the method of KOOPS (1960). To prevent further oxidation during heating, the determination was carried out in a closed vessel under nitrogen. From these determinations, the TBA-values per gram of butter fat and per gram of serum phospholipids were calculated. These figures indicate that in butter with cold-storage defects the rate of oxidation of phospholipids is 2000 to 3000 times that of butter fat.

All these results strongly suggest that the autoxidative deterioration of cold-stored butter is primarily due to the oxidation of the phospholipids. However, there is no absolute proof that the off-flavours are in fact derived only from the oxidative degradation of the unsaturated fatty acids in the phospholipids. The much higher oxidation rate of the phospholipids cannot provide conclusive evidence that the oxidation defects are caused exclusively by the oxidation of this fraction (EL-NEGOUMY and KU, 1968). TOLLENAAR (1953) stated that the initiation of the oxidative deterioration of butter starts at the fat/serum interface. Coupled to this process, the oxidation of butter fat can start as a result of the diffusion of hydroperoxides (from the interface) into the fat. This implies that the off-flavours may result from oxidation of the unsaturated fatty acids of the phospholipids, or of the butter fat, or of both.

5.4.2 *Experimental work*

Some recent experiments (BADINGS and VAN DER POL, 1968a) have shown that earlier theories, which suggested that trainy flavour in butter would develop only when the fat globule membrane material was present, are incorrect. Artificial butter, which did not contain fat globule membrane material or phospholipids, became trainy within a few months of storage at –10°C, provided that the serum phase had a very high copper content. The experiments which led to these results were carried out in the following way:

Fresh butter was melted at 38°C, after which fat and serum were separated by centrifugation. Then 400 g of the melted fat (free from phospholipids) was added dropwise to 150 ml of dialysate obtained by dialysing 200 ml distilled water against 30 l skim milk which had been soured to pH 4.6 by means of lactic starter bacteria.

During addition of the butter fat, the mixture was stirred vigorously by means of an Ultra turrax. The emulsion, stirred continuously, was cooled quickly until it became solid. It was then cooled to 0°C for 15 minutes and worked with a spatula. During these manipulations the phase inversion to a water-in-fat emulsion was achieved. After the excess of serum had been removed, an artificial butter was obtained containing 15 to 20% dialysate.

By this method, six batches of artificial butter were made which differed in the composition of the serum phase. In some of the preparations the following

Table 6. Results of the evaluations of artificial butter.

batch No.	additions to the serum phase		fresh butter			after 1 month at −10 °C		after 2 months at −10 °C		after 3 months at −10 °C		after 5 months at −10 °C	
	casein	cupric sulphate	$Cu^{\cdot\cdot}$ (μg/kg)	TBA	flavour	TBA	flavour	TBA	flavour	TBA	flavour	TBA	flavour
1	−	−	42	0.034	n	0.080	n	0.153	sl.f	0.175	sl.f	0.212	sl.f
2	−	+	446	0.052	n	0.180	sl.tr	0.355	tr	0.450	tr	0.560	tr
3	−	+	14000	0.140	sl.f	0.392	sl.tr	0.565	tr	0.640	tr	0.750	st.tr
4	+	−	46	0.040	n	0.090	n	0.170	sl.f	0.235	sl.f	0.275	f
5	+	+	511	0.069	n	0.305	sl.tr	0.428	tr	0.540	tr	0.725	tr
6	+	+	14550	0.180	sl.f	0.470	tr	0.750	st.tr	0.875	st.tr	1.086	st.tr

Abbreviations used: n = normal, f = fatty, tr = trainy, sl.f = slightly fatty, sl.tr = slightly trainy, st.tr = strongly trainy

additions were made to 100 ml of skim milk dialysate, prior to stirring it with the butter fat: total casein free from phospholipids (4 g) and/or cupric sulphate (0.25 to 8 mg $Cu^{··}$). These batches were stored at -10 °C. The results of the analyses and organoleptic evaluations are summarized in Table 6, from which it can be concluded that a very high copper content (Batches 2, 3, 5 and 6) leads to the development of typical cold-storage defects. If the copper content is low (Batches 1 and 4), a trainy flavour does not develop, even after five months storage at -10 °C. It can also be seen that the oxidative deterioration proceeds more rapidly when copper and casein are present in the serum phase than when copper alone is present.

These results indicate that in artificial butter without fat globule membrane material or phospholipids, the oxidation of butter fat itself may cause a trainy flavour, provided that a strong pro-oxidant is present at the lipid/water interface.

It must therefore be concluded that it is uncertain whether the cold-storage defects in butter are caused by the oxidation of the unsaturated fatty acids of the phospholipids, or of the butter fat, or of both fractions.

5.4.3 *Other observations*

Investigations have been carried out to determine whether the development of cold-storage defects in butter is accompanied by changes in the fatty acid composition of the butter fat or phospholipid fractions.

Such differences, however, were not detectable by means of iodine-value determinations and alkali isomerization techniques (KOOPS, 1963; PONT and HOLLOWAY, 1967). Also, analysis of the fatty acid composition of these fractions by means of gas chromatography did not show detectable differences (BADINGS, 1960b).

It should be noted that these results do not necessarily conflict with the theory of oxidative breakdown of unsaturated fatty acids during development of cold-storage defects in butter. Volatile secondary autoxidation products can already cause flavour defects in butter when they are present in quantities of only 1 p.p.m. to 1 p.p.b. To obtain such concentrations, only a very limited degree of autoxidation of the polyenoic fatty acids is sufficient. It is very likely that the amount of polyenoic acids which must be oxidized to obtain this concentration of volatiles is so low that it is within the limits of accuracy of the methods of analysis mentioned above.

5.4.4 *Factors which affect the keeping quality of cold-stored butter*

The keeping quality of cold-stored butter depends mainly on the extent to which milk, cream or butter is contaminated with copper, and on the pH of the butter serum. Since the beginning of this century the detrimental effect of copper contamination has been recognized (GOLDING and FEILMAN, 1905; ROGERS,

1909, 1914). However, it was not until 1949 that MULDER and co-workers made the first precise experiments on the effect of copper, iron and manganese salts on the keeping quality of cold-stored butter. Addition of small amounts of copper caused oxidative deterioration. The addition of iron or manganese salts, however, did not affect the keeping quality of butter, nor did it accelerate the formation of peroxides.

MULDER and KLEIKAMP (1947a) supplied experimental evidence that high acidity of the cream strongly promotes the development of oxidation defects in cream and butter. In line with this, KOOPS (1963) found that low pH and added copper strongly increase the rate of oxidation of milk phospholipids and of the lipoprotein complex of the fat globule membrane.

MENGER (1961) investigated to what extent the natural copper content of milk and added copper influenced the development of cold-storage defects in butter. These studies demonstrated that butter which contained natural copper only (even at high levels of 100 µg/kg) was still of good quality after nine months of cold storage. If, however, small quantities of copper salts were added during buttermaking, cold-storage defects usually developed within a few months. Addition of copper salt which increased the copper content of the butter by only 25 µg/kg, was already sufficient to induce oxidation defects.

From the investigations by KOOPS (1963, 1969) it has become clear that the copper content of the fat globule membrane appears to be the key factor in the development of cold-storage defects in butter. If natural copper only is present, the copper content of the fat globules is relatively low (11 µg/100 g of fat globules). It was found that 15 to 20% of the natural copper is bound to the surface layers of the fat globules; the remainder is almost completely bound to the proteins of the butter serum.

Acidification to pH 4.6 does not affect the partitioning of natural copper. Therefore, butter made from ripened cream (pH 4.6) which contains natural copper only, usually has a good keeping quality. If, however, milk is strongly acidified (pH 1.8) and subsequently neutralized, part of the natural copper migrates from the plasma proteins to the fat globule membrane. As a result of the much higher copper content of the fat globule membrane, the keeping quality of sour-cream butter (pH 4.6) made from this milk is poor.

Added copper is partitioned in quite a different way; only 1 to 2% is bound to the surface layers and the rest to the plasma proteins. Acidification of milk to pH 4.6 is sufficient to cause 30 to 40% of the added copper to migrate from the plasma proteins to the fat globule membrane.

Apart from the migration of copper, the lowering of the pH to approximately 4.6 also increases the interaction between the cephalin from the membrane phospholipids, which is highly susceptible to oxidation, and the copper-containing membrane protein. As a result, the rate of oxidation of the lipoprotein complex increases considerably.

Other factors which in practice may have an adverse effect on the keeping quality of cold-stored butter, are the presence of lipid peroxides and free fatty acids. It is well established that these compounds exert a pro-oxidative influence.

High-temperature pasteurization of the cream (30 seconds at 90–96 °C) improves the keeping quality of cold-stored butter. This effect has been ascribed to the formation of 'active sulphydryl groups' which would retard the oxidation processes in butter (TOWNLEY and GOULD, 1943). From recent investigations by BAIJENS (1964) and VAN DUIN and BRONS (1967), however, it has become clear that the copper content of sour-cream butter made from low-pasteurized cream ($\leqslant$78 °C) is relatively high, while high pasteurization ($\geqslant$82 °C (summer period) or $\geqslant$86 °C (winter period)) reduces the copper content.

It may be concluded that the factors which mainly influence the keeping quality of cold-stored butter are: the extent to which milk, cream and butter are contaminated with copper, and the pH of the butter serum.

CHAPTER IV

TECHNIQUES USED FOR THE ISOLATION AND IDENTIFICATION OF VOLATILE FLAVOUR COMPOUNDS IN OXIDIZED LIPIDS

1. Introduction

In the analysis of food odours specific difficulties are encountered, which are related to their characteristic properties (see Table 7).

Food odours are often present in very low concentrations (sometimes 1 p.p.m., 1 p.p.b. or less). This makes it necessary to work up large amounts of the food product. During the analysis procedures, the contact with relatively large surface areas of glassware (columns, flasks, pipettes, etc.) and considerable amounts of solvents may easily lead to severe contamination of the fractions to be identified, which makes impossible their characterization by spectrometric methods. It is therefore absolutely necessary to apply rigorous cleaning procedures for the glassware and solvents.

Table 7. Characteristic properties of food odours[1].

Characteristic properties of food odours are:

a. the complexity of the mixture of odorous components present in most food products;
b. the low concentration in which these components are generally present;
c. the chemical reactivity (and lability) of the components;
d. the polar character of many aroma compounds;
e. the wide range of boiling points of the components;
f. the large differences in the relative amount of the individual components;
g. the presence of large quantities of volatile non-aroma compounds (water, etc.);
h. the strict relation between the quantitative composition of the mixture and the organoleptic properties;
j. differences in flavour potencies of different components.

[1] List according to Weurman (1967), but with additions.

The isolation of food odours must be carried out in such a way that changes in the composition of the odorous fraction as a result of chemical reactions (ultimately catalysed by enzymes) are minimized. After concentration of the odorous fraction, many of the labile components are present in such high concentrations that rapid polymerization, condensation etc. may occur. It was found that a concentrate of volatiles from cold-stored butter can be kept for twelve hours at –40 °C without changes. At room temperature, however, the composition of the odorous fraction is considerably altered.

It is also necessary to take account of the lability and the polar character of many aroma compounds when they are separated by gas chromatography. Therefore glass columns and direct 'on column' injection is preferably used. The support material should not have catalytic or adsorptive properties. The use of polar stationary phases (or the addition of a small quantity of a polar phase to a non-polar stationary phase) and an inert carrier gas of high purity is also important. Temperature-programmed gas chromatography is often used to separate mixtures of low boiling and high boiling components.

The qualitative and quantitative composition of the mixture of odorous components should not be altered by the concentration techniques, because this would also alter the organoleptic properties of this fraction when it is diluted to the original concentration in the food product. If large quantities of volatile non-aroma compounds are present, special concentration techniques are necessary (adsorption, extraction etc.).

The concentration of an odorous compound is in no way indicative of its contribution to the flavour of a food product: the threshold values for taste and smell of odorous components vary widely.

There is no relation between the response of a gas-chromatographic detector and the olfactory response (WEURMAN, 1963). For propanal a G.C.-flame ionization detector is approximately one hundred times more sensitive than the human olfactory organ. Conversely, for several other compounds (mercaptans, aldehydes etc.) this detector is ten or more times less sensitive.

2. ISOLATION OF VOLATILE FLAVOUR COMPOUNDS

Methods for the isolation and concentration of volatile flavour compounds from food products usually consist of vacuum distillation, fractional evaporation, extraction, freeze concentration, adsorption, etc. Recently LEA *et al.* (1967) and WEURMAN (1969) have given a survey of techniques which are used at the present time.

In butter, the volatile compounds present in the aqueous phase are of no importance with regard to cold-storage defects. VAN DER WAARDEN (1944, 1947) already presented proof that the typical cold-storage defects in butter are caused entirely by the volatile compounds in the fat phase. In the present investigations the butter fat was therefore separated (melting of the butter fat at 40 °C followed by centrifugation at approximately 900 g) prior to further isolation of the volatile compounds.

For products of low water content (i.e. oils, lipids, dehydrated food products, etc.) the techniques for the isolation of volatile flavours are mostly based on high-vacuum degassing and cold-finger molecular distillation (DE BRUYN and SCHOGT, 1961; LEA and SWOBODA, 1962; ANGELINI *et al.*, 1967, FORSS and HOLLOWAY, 1967).

The methods mentioned all suffer from the disadvantage that only limited quantities of oil can be degassed. As the volatile flavour compounds in cold-stored butter are present in extremely low concentrations, these methods will not yield sufficient quantities of the volatiles to permit unambiguous physical and chemical identification.

A method which permits large quantities of oil to be worked up has been designed by CHANG (1961). The continuous process consists of a counter-current contact of the oil with steam, in an Oldershaw column under reduced pressure at 80°C. Clearly this method has some disadvantages: the oil is heated in the apparatus in the presence of water vapour for 12 minutes, which inevitably promotes hydrolysis and decomposition reactions (*inter alia* 2-alkanone formation in the case of butter fat).

For the present work a high-vacuum degassing apparatus was used in which the volatiles are stripped from a thin film of butter fat passing through a molecular still at 55°C. Details are given in Figure 5 and Section 5.4.

The efficiency of recovery of volatile carbonyl compounds with this degassing unit was determined by degassing 750 g of butter fat to which alkanals C_6 to C_{10} inclusive had been added in concentrations of 0.5 to 1 p.p.m. The results are given in Table 8.

Table 8. Recovery of a number of alkanals from butter fat by high-vacuum degassing.

aldehyde	concentration (p.p.m.)	recovery (%)
pentanal	0.4	80
hexanal	0.4	85
heptanal	0.6	89
octanal	0.7	87
nonanal	0.8	85
decanal	0.8	72

Although the isolation of these compounds was far from quantitative, the working up of large amounts of butter fat by the present method made possible the collection of sufficient quantities of all odorous compounds in order to permit their identification.

To identify the compounds which are formed in the autoxidation of specific unsaturated fatty acids, small quantities of material (1 to 2 g) had to be degassed. A batch process high-vacuum distillation was carried out, similar to the technique used by DE BRUYN and SCHOGT (1961). The equipment has been described previously (BADINGS, 1967a, 1968; BADINGS *et al.*, 1968b).

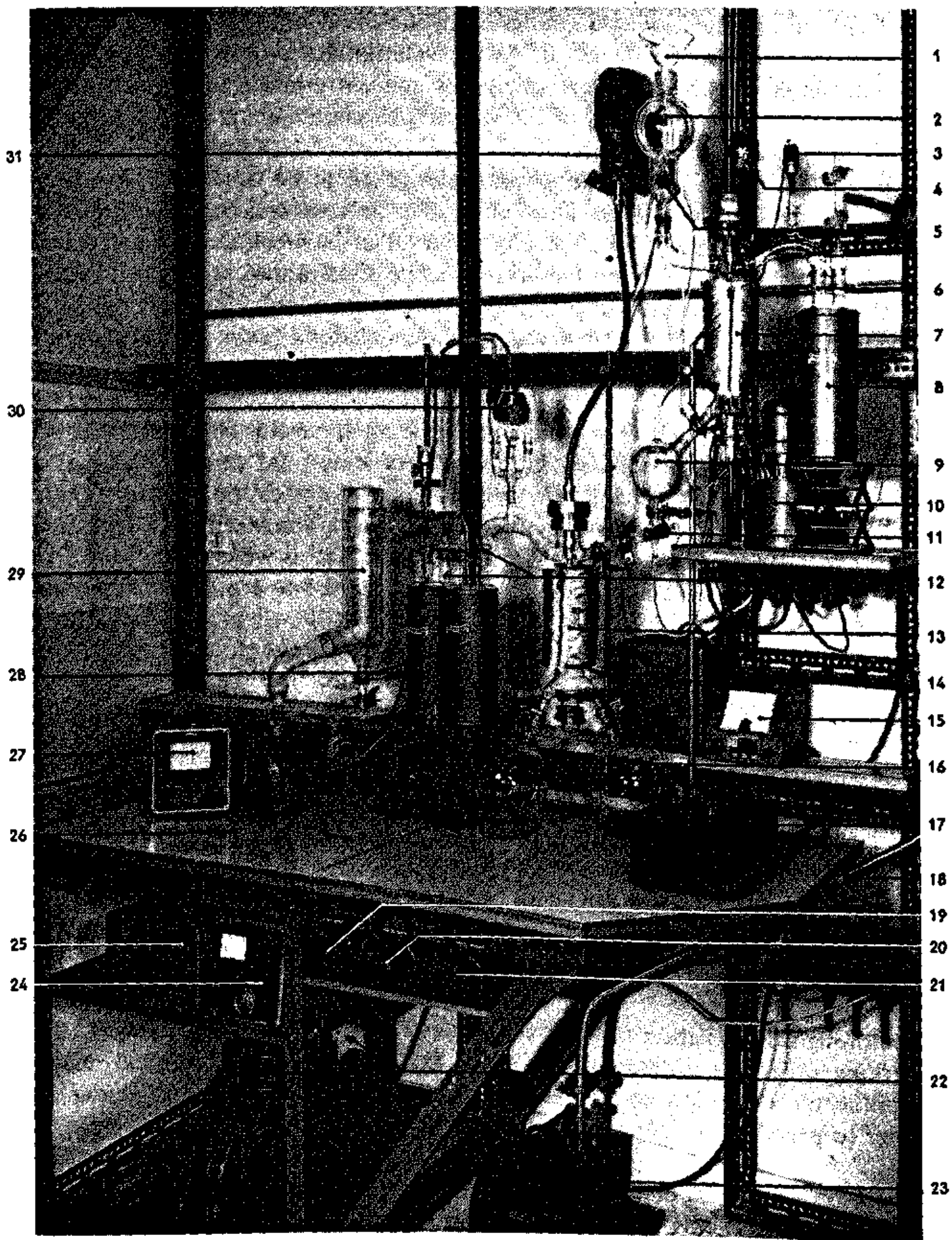

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Fig. 5. Two-stage high-vacuum degassing apparatus used for the collection of volatile compounds from butter fat.

1. introduction of butter fat;
2. storage vessel (surrounded by a warm-water jacket);
3. thermocouple vacuum gauge for measuring pressure in the first degassing unit (Arthur Smith Inc., (ASCO), New York, U.S.A.);
4. gearmotor for rotation of teflon wipers in the still (7);
5. stopcock for regulation of the flow of butter fat into the first degassing unit;
6. U-tube for collecting volatile compounds from the butter which is degassed at (7);
7. ASCO '50' rota-film molecular still for degassing of butter fat (first unit);
8. Dewar flask with liquid nitrogen for cooling U-tube (6);
9. storage vessel (surrounded by a warm-water jacket) for collection of the butter fat from the first degassing unit;
10. warm-water bath with circulation pump for heating the jackets of vessels (2) and (9);
11. high-vacuum stopcock for introduction and regulation of the flow of butter fat in the second degassing unit;
12. U-tubes for collecting volatile compounds from the butter fat which is degassed at (13);
13. Asco '50' rota-film molecular still for high-vacuum degassing of butter fat (second unit);
14. regulator for motor (31);
15. read-out of vacuum measured at (3) (ASCO);
16. receiver flasks (capacity 7 litres) for collection of butter fat which is degassed at (13);
17. read-out of temperatures measured in the rota-film stills (7) and (13);
18. vacuum line between pump (23) and first degassing unit;
19. regulator for motor (4);
20. regulator for heating jacket (7);
21. regulator for heating jacket (13);
22. pumping equipment (vacuum pump D-2 and high-vacuum oil diffusion pump DO-30 (Leybold, Cologne, Western Germany)) for maintaining high-vacuum in second degassing unit;
23. vacuum pump (Speedivac ISC 30 rotary oil vacuum pump Edwards, Crawley, England) for maintaining low pressure in first degassing unit;
24. leak detector (Leybold);
25. switch panel;
26. membrane valve (Leybold);
27. read-out of vacuum measured at (30) (Leybold);
28. Dewar flasks filled with liquid nitrogen for cooling of U-tubes (12);
29. cold-finger safety trap;
30. Penning and thermotron vacuum gauges for measurement of pressure in the second degassing unit (Leybold);
31. gear-motor for rotation of teflon wipers in the still (13).

3. The separation and identification of flavour compounds in oxidized lipids

3.1 Introduction

The volatile flavour fraction from butter with cold-storage defects and from other oxidized lipids is of such a complicated composition that separation into the individual components is only possible by using a combination of separation techniques. It was found, for example, that separation by capillary column gas chromatography only is inadequate to obtain satisfactory identification. The best method for the identification was found to be a combination of gas chromatography, liquid chromatography (column or thin-layer) of derivatives, and ultraviolet, infrared and mass spectrometry. In the following sections these techniques and their combination will be discussed. The experimental details are given in Section 5.

3.2 Gas chromatography

The volatile compounds from autoxidized lipids were first separated by temperature-programmed gas chromatography. A column filling was chosen to minimize the risk of artefact formation during gas chromatography of labile compounds. Winter *et al.* (1962) have demonstrated that in the gas chromatography of 3 *cis*-hexenal (a very labile carbonyl compound), the use of a non-polar stationary phase leads to the formation of artefacts. According to this investigator this problem was largely overcome by using a non-polar stationary phase which was deactivated by the addition of 0.2 to 0.5% of a polar phase (Carbowax). The low proportion of Carbowax does not influence the non-polar character of the column to any extent.

In the present work a column filling coated with 20% silicone oil and 0.4% Carbowax 4000 was used. Further experimental details are given in Section 5.6.

Gas chromatography was used in the present work primarily for the *separation* of the flavour compounds. However, the retention times (or retention indices) of peaks in the gas chromatogram may also serve as an aid in identification. For this purpose retention times were determined for a number of reference compounds (alkanals, 2-alkenals, 2,4-alkadienals etc.) under several conditions of isothermal or programmed-temperature gas chromatography. From these determinations the retention times (relative to hexanal) (Table 9) were calculated. The data given in Table 9 are in accordance with the required linear relation between log retention time and $1/T$ (T in °K) (Littlewood *et al.*, 1955; Ambrose *et al.*, 1958). Very useful in compound identification by means of isothermal retention data is the retention index proposed by Kovats (1958) (Wehrli and Kovats, 1959).

Table 9. Log retention times (relative to hexanal) for a number of compounds in isothermal gas chromatography at 70 °C, 100 °C and 130 °C, using silicone oil as stationary phase.

compound	log retention time (relative to hexanal)		
	70 °C	100 °C	130 °C
pentanal	0.670–1	0.720–1	0.760–1
hexanal	0	0	0
heptanal	0.325	0.280	0.240
octanal	0.660	0.552	0.465
nonanal	0.980	0.830	0.700
decanal	1.307	1.102	0.930
undecanal	1.635	1.380	1.160
2 *trans*-pentenal	0.840–1	0.870–1	0.895–1
2 *trans*-hexenal	0.170	0.150	0.134
2 *trans*-heptenal	0.495	0.426	0.362
2 *trans*-octenal	0.820	0.697	0.595
2 *trans*-nonenal	1.145	0.972	0.825
2 *trans*-decenal	–	1.247	1.055
2 *cis*-pentenal	0.802–1	0.840–1	–
2 *cis*-hexenal	0.130	0.120	0.113
2 *cis*-heptenal	0.461	0.393	0.335
2 *cis*-octenal	0.780	0.661	0.563
2 *cis*-nonenal	1.100	0.935	0.792
2 *cis*-decenal	–	1.209	1.019
2-pentanone	0.640–1	0.698–1	0.748–1
2-hexanone	0.970–1	0.980–1	0.990–1
2-heptanone	0.294	0.230	0.223
2-octanone	0.620	0.525	0.443
2-nonanone	0.945	0.800	0.677
2-decanone	1.273	1.075	0.905
2 *trans*, 4 *trans*-hexadienal	0.367	0.310	0.263
2 *trans*, 4 *trans*-heptadienal	0.690	0.575	0.477
2 *trans*, 4 *trans*-octadienal	1.010	0.850	0.710
2 *trans*, 4 *trans*-nonadienal	1.334	1.120	0.940
2 *trans*, 4 *trans*-decadienal	1.673	1.400	1.170
2 *trans*, 4 *cis*-heptadienal	0.649	0.537	0.441
2 *trans*, 4 *cis*-decadienal	–	1.349	1.124
2 *trans*, 6 *trans*-nonadienal	–	0.950	0.801
2 *trans*, 6 *cis*-nonadienal	–	0.967	0.817
1-penten-3-one	0.620–1	0.688–1	0.744–1
1-hexen-3-one	0.940–1	0.940–1	0.940–1
1-hepten-3-one	0.267	0.215	0.170
1-octen-3-one	0.583	0.485	0.403
1-nonen-3-one	0.910	0.765	0.640
1-hexen-3-ol	0.945–1	0.945–1	0.947–1
1-hepten-3-ol	0.275	0.223	0.180
1-octen-3-ol	0.602	0.498	0.413
3 *cis*-hexenal[1]	0.995–1	0.997–1	0.999–1
3 *cis*-heptenal[1]	0.315	0.270	0.230
3 *cis*-octenal[1]	0.645	0.542	0.454
3 *cis*-nonenal[1]	0.970	0.821	0.688

[1] The corresponding 3 *trans*-enals, 4 *cis*-enals and 4 *trans*-enals have identical retention values.

The Kovats retention index (I) for a compound is a number indicating on a logarithmic scale its retention volume relative to that of the series of n-alkanes.

$$(I_x)_T = 100 \times \frac{\log \frac{R_x}{R_n}}{\log \frac{R_{n+1}}{R_n}} + 100 \times \text{n}$$

in which:

$(I_x)_T$ = retention index of compound X for isothermal gas chromatography at T°C;

R_x = retention volume (or time) of compound X;

R_n = retention volume (or time) of the alkane C_nH_{2n+2} which comes closest to R_x ($R_n \leqslant R_x$);

R_{n+1} = retention volume (or time) of the alkane with one carbon atom more than C_nH_{2n+2}.

From the data in Table 9 the Kovats retention indices for a number of compounds have been calculated. These are given in Table 10.

Retention indices have also been found to be applicable to temperature-programmed gas chromatography. According to GUIOCHON (1964), VAN DEN DOOL and KRATZ (1963) and HABGOOD and HARRIS (1964, 1966) the logarithmic interpolation between retention volumes must be replaced by a linear interpolation between retention temperatures:

$$(I_x)_{TP} = 100 \times \frac{Tr_x - Tr_n}{Tr_{n+1} - Tr_n} + 100 \times n$$

in which:

$(I_x)_{TP}$ = retention index of compound X for temperature-programmed gas chromatography;

Tr_x = retention temperature of compound X;

Tr_n = retention temperature for the alkane C_nH_{2n+2} which comes closest to that of X ($Tr_n \leqslant Tr_x$);

Tr_{n+1} = retention temperature for the alkane with one carbon atom more than C_nH_{2n+2}.

This formula is valid only when the retention temperatures of the alkanes increase linearly with the number of C-atoms. Otherwise the retention indices should be determined by graphical interpolation, as explained by KAISER (1966). This method was used because of the limited possibility for exact linear temperature programming with the instrument used for the present work. The values which have been found for a number of model compounds are given in Table 11.

Table 10. Retention indices for a number of compounds in isothermal gas chromatography at 70 °C, 100 °C and 130 °C, using silicone oil as stationary phase.

compound	retention index		
	70 °C	100 °C	130 °C
pentanal	683	684	687
hexanal	783	784	787
heptanal	882	884	887
octanal	983	984	989
nonanal	1082	1085	1089
decanal	1182	1185	1190
undecanal	1283	1286	1290
2 *trans*-pentenal	735	736	738
2 *trans*-hexenal	834	837	842
2 *trans*-heptenal	935	937	942
2 *trans*-octenal	1032	1037	1044
2 *trans*-nonenal	1132	1137	1144
2 *trans*-decenal	–	1237	1244
2 *cis*-pentenal	724	726	–
2 *cis*-hexenal	822	827	833
2 *cis*-heptenal	923	925	930
2 *cis*-octenal	1023	1024	1031
2 *cis*-nonenal	1122	1124	1130
2 *cis*-decenal	–	1225	1230
2-hexanone	772	776	780
2-heptanone	872	875	880
2-octanone	971	975	979
2-nonanone	1071	1075	1080
2-decanone	1172	1176	1180
2 *trans*, 4 *trans*-hexadienal	892	893	896
2 *trans*, 4 *trans*-heptadienal	992	993	995
2 *trans*, 4 *trans*-octadienal	1091	1093	1095
2 *trans*, 4 *trans*-nonadienal	1191	1193	1195
2 *trans*, 4 *trans*-decadienal	1291	1293	1296
2 *trans*, 4 *cis*-heptadienal	978	978	977
2 *trans*, 4 *cis*-decadienal	–	1275	1276
2 *trans*, 6 *trans*-nonadienal	–	1130	1134
2 *trans*, 6 *cis*-nonadienal	–	1136	1141
1-penten-3-one	666	–	–
1-hexen-3-one	765	764	764
1-hepten-3-one	864	864	864
1-octen-3-one	960	964	964
1-nonen-3-one	1060	1065	1065
1-hexen-3-ol	766	764	761
1-hepten-3-ol	866	864	862
1-octen-3-ol	966	964	965
3 *cis*-hexenal[1]	780	782	784
3 *cis*-heptenal[1]	879	881	883
3 *cis*-octenal[1]	979	981	984
3 *cis*-nonenal[1]	1079	1082	1084

[1] The corresponding 3 *trans*-enals, 4 *cis*-enals and 4 *trans*-enals have identical retention indices.

Table 11. Retention indices and retention temperatures for temperature-programmed gas chromatography, using silicone oil as stationary phase (further details in text).

compound	retention index	retention temperature
hexane	600	58
1-penten-3-one	666	58
pentanal	682	58
heptane	700	58
2 *cis*-pentenal	723	58
2 *trans*-pentenal	734	58
1-hexen-3-one	765	58
1-hexen-3-ol	766	58
2-hexanone	772	58
3 *cis*-hexenal	780	58
hexanal	783	58
octane	800	58
2 *cis*-hexenal	821	60.5
2 *trans*-hexenal	835	62
1-hepten-3-one	864	65
1-hepten-3-ol	865	65.5
2-heptanone	872	66
3 *cis*-heptenal	880	67
heptanal	882	67
2 *trans*, 4 *trans*-hexadienal	892	67.5
nonane	900	69.5
2 *cis*-heptenal	923	72
2 *trans*-heptenal	935	73
1-octen-3-one	961	76
1-octen-3-ol	965	76.5
2-octanone	972	77
2 *trans*, 4 *cis*-heptadienal	978	77.5
3 *cis*-octenal	980	78
octanal	983	78.5
2 *trans*, 4 *trans*-heptadienal	992	79.5
decane	1000	80
2 *cis*-octenal	1023	82.5
2 *trans*-octenal	1035	84
1-nonen-3-one	1064	87
2-nonanone	1075	88.5
3 *cis*-nonenal	1081	89
nonanal	1084	90
2 *trans*, 4 *trans*-octadienal	1093	90.5
undecane	1100	91
2 cis-nonenal	1124	94
2 *trans*, 6 *trans*-nonadienal	1130	95
2 *trans*, 6 *cis*-nonadienal	1136	95.5
2 *trans*-nonenal	1137	95.5
2-decanone	1176	100
decanal	1185	101
2 *trans*, 4 *trans*-nonadienal	1193	102.5
dodecane	1200	103.5
2 *cis*-decenal	1225	106.5
2 *trans*-decenal	1237	108
2 *trans*, 4 *cis*-decadienal	1275	112.5
undecanal	1288	114
2 *trans*, 4 *trans*-decadienal	1295	115
tridecane	1300	115.5
tetradecane	1400	126.5

3.3 Conversion of carbonyl compounds into the 2,4-dinitrophenylhydrazones

For the analysis of carbonyl compounds it is often useful to use their DNPH*-derivatives, because these have several favourable characteristics. They are relatively stable, have good properties for separation by liquid chromatography and for identification by their melting points and by spectrometric analysis (ultraviolet, infrared and mass spectrometry). The amount of a DNPH which is needed for these analyses is fairly small. A few μg are sufficient for ultraviolet and mass-spectrometric analysis. For infrared spectrometry 5 to 10 μg are needed in KBr-disc analysis and 10 to 20 μg for analysis in CCl_4-solution.

It is also necessary that the original carbonyl compounds can be liberated from the DNPH's to evaluate their organoleptic properties. However, there are some points which have to be taken into account to prevent erroneous conclusions. The presence of a $>C=N-$ bond in a DNPH implies the possibility of stereoisomerism. Carbonyl compounds with an asymmetrical structure may form *syn*- and *anti*-isomers (van Duin, 1954, 1961). These isomers tend to migrate as separate zones in many methods of chromatography. However, in adsorption chromatography the *syn*- and *anti*-isomers of aliphatic carbonyl compounds usually move as one zone. This has been found also for silver nitrate complex chromatography.

A suitable method for the conversion of carbonyl compounds into the corresponding DNPH's must satisfy certain conditions. The conversion percentage should be sufficiently high, even if the concentration of carbonyl compound is very low. The conversion reaction should not cause the formation of products other than the DNPH of the parent carbonyl compound. Formation of secondary products by isomerization, cyclization, etc. must be avoided. Carbonyl-free solvents must be used.

Methods for micro-scale conversion of carbonyl compounds into the DNPH's have been published by Haverkamp Begemann and de Jong (1959), Schwartz and Parks (1961), Forss *et al.* (1955) and Linow (1966).

All these methods can be used for the conversion of normal saturated aliphatic aldehydes and ketones. However, from the experiments of van Duin and Poll (1967a) it has become clear that one meets difficulties when trying to convert more labile carbonyl compounds into the 2,4-dinitrophenylhydrazones: compounds such as 3 *cis*-enals and 2 *cis*-enals were largely or completely converted into the DNPH's of the corresponding 2 *trans*-enals. Moreover it was found that 1-buten-3-one and 1-penten-3-one could not be converted into the DNPH's, while the conversion percentage for 1-octen-3-one was very low. In these cases good results were obtained when the reagent proposed by Henick *et al.* (1954) was used (a solution of 2,4-dinitrophenylhydrazine in benzene which contains 1% of trichloroacetic acid), provided that lower temperatures

* Abbreviation for 2,4-dinitrophenylhydrazone.

and shorter reaction times are used. For example, 1-penten-3-one in a concentration of 70 μg per ml gave a yield of 85% at room temperature in 15 minutes and a yield of 95% in 60 minutes without the formation of detectable quantities of artefacts. The rate of conversion for aldehydes was higher, as well as the conversion percentage. In a solution of 3 *cis*-hexenal which contained 70 μg/ml of the aldehyde, the conversion into the DNPH was more than 95% in 2 minutes.

By this method, VAN DUIN and POLL (1967a) were able to prepare for the first time DNPH's of aliphatic 2 *cis*-enals. BADINGS, WASSINK and POLL (1967) identified a 2 *cis*-enal as an autoxidation product of unsaturated fatty acids.

The use of the benzene trichloroacetic acid reagent in the conversion reaction is subject to certain conditions in order to prevent the formation of artefacts: the TCA (abbreviation for trichloroacetic acid) must be recrystallized twice from light petroleum. The use of freshly prepared benzene/TCA-reagent is recommended (the contact between 2,4-dinitrophenylhydrazine and TCA should be kept short). Finally, contact between the reagent solution and alkaline solutions must be avoided.

3.4 Separation of 2,4-dinitrophenylhydrazones by liquid chromatography

As complete separation of components by gas chromatography was not possible, a further resolution was necessary. Because gas chromatography using a silicone-oil-impregnated column results primarily in separation of carbonyl compounds according to chain length, any further resolution should be according to the type and degree of unsaturation. For this purpose liquid chromatography using silver-nitrate-impregnated columns or plates is most useful. To all the fractions obtained by gas chromatography the DNPH-reagent was added in order to convert the carbonyl compounds into the DNPH's. These were further separated by silver nitrate complex column chromatography according to VAN DUIN (1962).

It is also possible to use thin layer chromatography on silver-nitrate-impregnated plates (BADINGS and WASSINK, 1963). Although the latter method has the advantage of being more rapid, it was not applied in the present work, because of the larger risk of contamination.

In the column chromatography method, silica gel impregnated with an aqueous solution of silver nitrate is used as the immobile phase and mixtures of petroleum ether and benzene as the mobile phase.

In general, the larger the number of double bonds in the carbonyl moiety, the more slowly the DNPH will move through the column. Thus 2,4-hexadienal DNPH moves more slowly than 2-hexenal DNPH and the latter compound moves more slowly than hexanal DNPH. The mobility is also affected by the configuration and the position of the double bond: in general *cis*-isomers move more slowly than the corresponding *trans*-isomers, 4-enals more slowly than the corresponding 3-enals, and these in turn are slower than the 2-enals.

Because of these effects, the mobility of a certain DNPH on the 'silver nitrate column' gives some information about its structure. In Figure 6 the separation on a silver-nitrate-impregnated silica gel column is presented schematically.

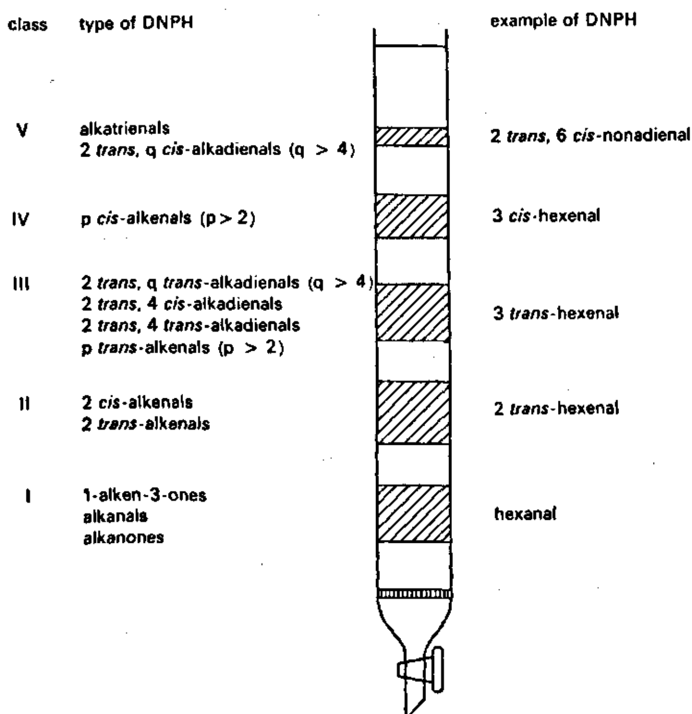


Fig. 6. Separation of different types of 2,4- dinitrophenylhydrazones on a column of silica gel impregnated with silver nitrate.

Five groups (I to V) can be distinguished:

Group I (most rapidly moving DNPH's) contains the DNPH's of 1-alken-3-ones, alkanals and alkanones; Group II contains the DNPH's of the 2 *cis*- and 2 *trans*-alkenals. The 2 *trans*-isomer moves only slightly faster than the 2 *cis*-isomer. Group III contains the DNPH's of the p *trans*-alkenals (p>2), the 2 *trans*, 4 *trans*-, the 2 *trans*, 4 *cis*- and the 2 *trans*, q *trans*-alkadienals (q>4). Group IV represents the DNPH's of p *cis*-enals (p>2). The most slowly moving DNPH's are present in Group V, which consists of DNPH's of non-conjugated *trans*, *cis*-dienals, trienals etc.

It should be noted that the method described here is only valid for the separation of DNPH's of corresponding chain length. Therefore, the use of gas chromatography for separation according to chain length is necessary, prior to silver nitrate column chromatography.

3.5 Regeneration of carbonyl compounds from the 2,4-dinitrophenylhydrazones

For organoleptic evaluations, the carbonyl compounds had to be regenerated from the DNPH's. This regeneration should not produce isomerization or artefacts of the parent carbonyl compound. The regeneration of very small quantities of DNPH's must be possible. Finally the reagents used must be odourless. A blank regeneration should have only a minimum of odour.

Two methods were found to be in agreement with these conditions (VAN DUIN and POLL, 1967b). The first method consists in treating a few μg of the DNPH with a concentrated aqueous solution of α-ketoglutaric acid. After heating, the odour of the reaction mixture is evaluated. In the second method a few μg of the DNPH in petroleum ether is treated with a small quantity of MnO_2. The carbonyl compound is liberated from the DNPH and its odour evaluated after evaporation of a few drops of the petroleum ether solution on filter paper.

3.6 Ultraviolet spectrophotometry

For the qualitative and quantitative analysis of DNPH's, valuable information can be obtained by ultraviolet spectrometric analyses. This is obvious from the data of BRAUDE and JONES (1945), presented in Table 12. The ultraviolet spectra of DNPH's give an indication of the number of double bonds which are present in conjunction with the chromophoric group.

Table 12. Maxima in the UV-absorption spectra of DNPH's of aliphatic aldehydes and ketones (Braude and Jones, 1945).

type of DNPH	λ_{max} in ethanol (mμ)	λ_{max} in chloroform (mμ)	E_{max} $\times 10^{-3}$
formaldehyde	348	348	20–30
other alkanals	356–360	358–361[1]	
alkanones	362–365	365–368[2]	
acrolein	366	367	25–35
other 2-alkenals	373	373	
2,4-alkadienals conjugated dienones	379–395	388–407	30–40
2,4,6-alkatrienals conjugated trienones	395–410	400–415	40–50

[1] More recent analyses have indicated that this maximum lies at 356–358 mμ.
[2] More recent analyses have indicated that this maximum lies at 362–364 mμ.

It was found that the ultraviolet absorption spectra in chloroform can be used to distinguish between different groups of DNPH's in the following way:

Class A: Absorption maximum 355–358 mμ. Saturated aliphatic aldehyde DNPH's and unsaturated aliphatic aldehyde DNPH's, provided that the double bond(s) in the carbon chain is (are) not in conjunction with the chromophoric group.

Class B: Absorption maximum 362–364 mμ. Saturated aliphatic ketone DNPH's (2-alkanones, symm. ketones) and unsaturated aliphatic ketone DNPH's, provided that the double bond(s) in the carbon chain is (are) not in conjunction with the chromophoric group.

Class C: Absorption maximum 370 mμ. 1-alken-3-one DNPH's, such as 1-buten-3-one DNPH, 1-penten-3-one DNPH and 1-octen-3-one DNPH.

Class D: Absorption maximum 373–374 mμ. Aliphatic aldehyde DNPH's with one double bond in conjunction with the chromophoric group or with more double bonds in the chain, provided that these double bonds are not in conjunction with the 2-enal DNPH-group.

Class E: Absorption maximum 395 mμ (second maximum at 303 mμ). 3,5-octadien-2-one DNPH and higher terms of the homologous series.

Class F: Absorption maximum 388 mμ (second maximum at 303 mμ). Aliphatic aldehyde DNPH's with two double bonds in conjunction with the chromophoric group (2,4-dienal DNPH's) or with more double bonds in the chain, provided that these double bonds are not in conjunction with the 2,4-dienal DNPH-group.

Class G: Absorption maximum 405 mμ (second maximum at 322 mμ). Aliphatic aldehyde DNPH's with three double bonds in conjunction with the chromophoric group (2,4, 6-trienal DNPH's) or with more double bonds in the chain, provided that these double bonds are not in conjunction with the 2, 4, 6-trienal DNPH-group.

3.7 Infrared spectrophotometry

The analysis of the infrared spectra of a great number of DNPH's of saturated and unsaturated aliphatic carbonyl compounds in this laboratory has led to the following results:

1. Within a homologous series, the ratio between the extinction at 3.4–3.5 μ (CH-bonds) and at 3.0–3.1 μ (NH-bond), increases parallel with the chain length. At constant chain length this ratio decreases with the increase in the number of double bonds.
2. The DNPH's of ketones show a slightly more differentiated infrared spectrum than do the DNPH's of aldehydes. This is particularly the case in the 9 μ-region. For example, the infrared spectrum of 2-heptanone DNPH in CCl_4-solution, has bands at 8.8 and 9.0 μ, whereas heptanal DNPH has only one band at 8.8 μ. Similar phenomena are found in the infrared spectra of these compounds in KBr; here 2-heptanone DNPH shows additional peaks in the region 7.7–8.0 μ.
3. The DNPH's of unsaturated aldehydes, in which the double bond is *not* in conjunction with the carbonyl group, have the following characteristics. In the *trans*-configuration there is a distinct band at 10.3 μ when the compound is analysed in CCl_4-solution and at 10.4 μ in KBr. In the *cis*-configu-

ration, the spectrum of the DNPH in CCl_4-solution or in KBr is similar to that of the corresponding saturated aldehyde DNPH, although the ratio of the absorption at 3.4–3.5 μ to that at 3.0–3.1 μ is distinctly lower.

4. The infrared spectra of 2-alkenal DNPH's have the following characteristics. The 2 *trans*-enal DNPH's in CCl_4-solution show a small characteristic band at 6.1 μ, which is visible on the flank of the strong phenyl band at 6.2 μ. Another characteristic band is present in the 10.25 μ region. When making recordings of spectra in KBr, the characteristic bands appear at 6.05–6.10 μ and at 10.15 μ. The 2 *cis*-enal DNPH's lack these characteristics. Their infrared spectra are similar to those of the alkanals, but the bands between 7.4 and 8.0 μ are somewhat differently indented.
5. The infrared spectra of 2,x-dienal DNPH's (x>4), e.g. 2 *trans*, 6 *trans*-nonadienal DNPH, and 2 *trans*, 6 *cis*-nonadienal DNPH have the following characteristics. The infrared spectra of 2 *trans*, x *cis*-dienal DNPH's (x>4) in KBr are similar to those of the 2 *trans*-enal DNPH's, but there are some additional bands in the regions 9.2–9.6 μ and 10.5–11.0 μ. The infrared spectra of 2 *trans*, x *trans*-dienal DNPH's (x>4) in KBr show two CH-*trans*-bands* at approximately 10.15 μ and 10.30 μ.
6. The 2,4-alkadienal DNPH's do not show the small peak at 6.1 μ that is characteristic of the 2 *trans*-enal DNPH's.
   For the 2 *trans*, 4 *trans*-dienal DNPH's there is a distinct CH-*trans*-band at approximately 10 μ (10.1 μ for CCl_4 recording, 9.95–10.05 μ for KBr recording). The infrared spectra of 2 *trans*, 4 *cis*-dienal DNPH's (e.g. 2 *trans*, 4 *cis*-heptadienal DNPH) are slightly different. The latter compound in CCl_4-solution shows bands at 10.15 μ and 10.53 μ and in KBr at 10.03, 11.5 and 11.8 μ, and weak bands at 10.27 μ and 10.60 μ. The CH-*trans*-bands at 10.15 μ and 10.03 μ are distinctly less strong than in the corresponding 2 *trans*, 4 *trans*-isomer.
7. The solubility of 2 *trans*, 4 *trans*, 6 *trans*-octatrienal DNPH in CCl_4 is too low to permit the recording of a clear infrared spectrum. In KBr, the infrared spectrum of this compound bears a close resemblance to that of the 2 *trans*, 4 *trans*-dienal DNPH's. The position of the CH-*trans*-band is the same; its intensity is slightly stronger.
8. The DNPH's of ketones with a *trans*-double bond in conjunction with the carbonyl group have a CH-*trans*-band in the area 10.30–10.40 μ (recording in CCl_4-solution), except for the 1-alken-3-one DNPH's in which the absorption maximum is shifted to 10.13 μ. When analysed in KBr, the values are 10.25–10.30 μ for conjugated alkenone DNPH's (not incl. 1-alken-3-ones) and 10.00–10.15 μ for 1-alken-3-one DNPH's. The latter derivatives have also more detailed bands in the region 7.4–8.4 μ.

* The term CH-*trans*-band denotes the absorption band(s) from the CH-deformation vibration(s) of the $R_1CH{=}CHR_2$ group in the *trans*-configuration.

9. The infrared spectrum of 3 *trans*, 5 *trans*-octadien-2-one DNPH shows small but distinct differences if compared with that of 2 *trans*, 4 *trans*-dienal DNPH's. In CCl_4-solution there is a shift of the 9.25 band to 9.15 μ. In addition, the CH-*trans*-band shifts from 10.07 μ to 10.13 μ. When analysed in KBr, the 9.28 μ band shifts to 9.18 μ, and the 9.97 μ band to 10.07 μ. There are also slight differences in the 7.2–8.0 μ region.

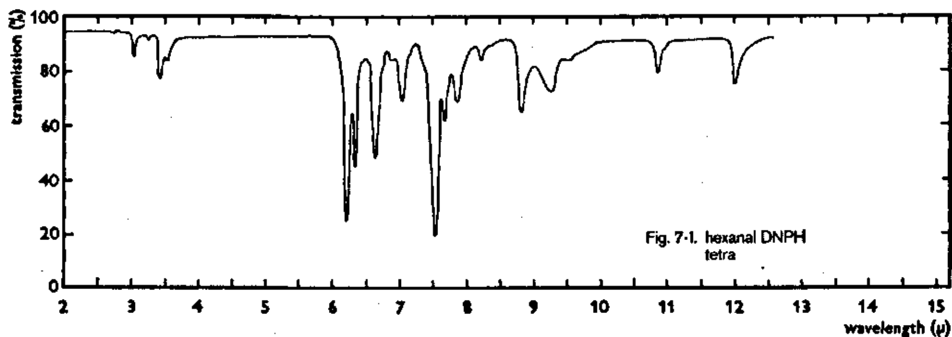
The data from this laboratory summarized above are in general well in line with the results of other investigators (see Ross, 1953; Jones *et al.*, 1956; Stitt *et al.*, 1961; Mehlitz *et al.*, 1965; Forss *et al.*, 1962). It should be noted, however, that the rules mentioned may not be applied to the first and second term of each homologous series. These usually show certain differences. With this exception the infrared spectra presented in Figures 7–1 to 7–31 incl. show the characteristics of the different homologous series.

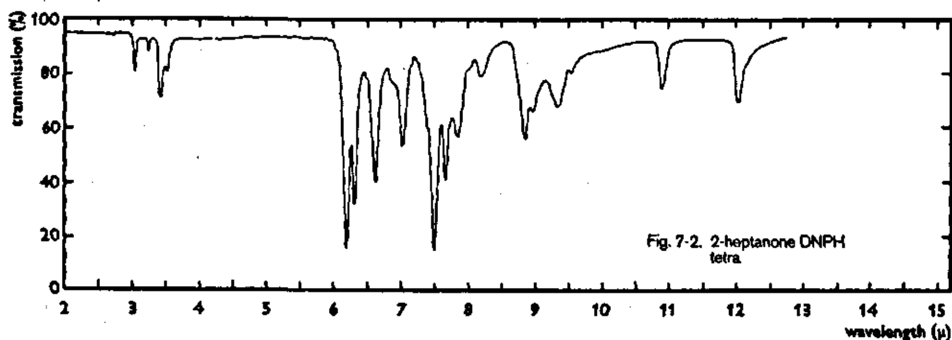
As an aid to the classification of DNPH's according to their infrared spectra, the data mentioned above have been summarized in Table 13. From this table it can be seen that a classification is possible which is based on the absorption bands in the regions *9.9–10.4* μ and *6.05–6.10* μ.

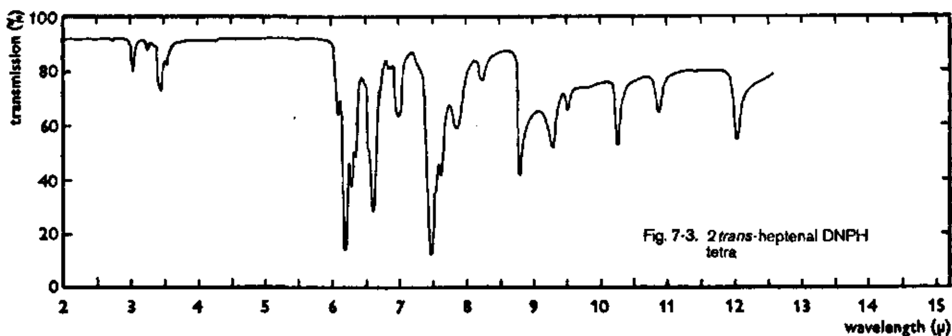
Table 13. Classification of 2,4-dinitrophenylhydrazones of carbonyl compounds, according to their characteristic infrared absorption bands in the 9.9–10.4 μ and 6.05–6.10 μ range.

classi-fication	type of DNPH	absorption[1] at 9.9–10.4 μ		absorption[1] at 6.05–6.10 μ
		CCl_4-spectrum	KBr-spectrum	
a	alkanals alkanones *cis*-alkenals *cis*-alkenones	–	–	–
b	2 *trans*-alkenals	10.25 μ	10.15 μ	+
c	x *trans*-alkenals[2] (x>2)	10.30 μ	10.40 μ	–
d	*trans*-alkenones[3 4]	10.30–10.40 μ	10.25–10.30 μ	–
e	2,4-alkadienals 2,4,6-alkatrienals alkadienones[3] 1-alken-3-ones	10.10–10.15 μ	9.95–10.05 μ	–

[1] – no absorption band present; + absorption band present.
[2] Also *trans*-enones in which the double bonds are not in conjunction with the carbonyl group.
[3] Provided that the double bond(s) is (are) in conjunction with the carbonyl group.
[4] 1-alken-3-ones not included.







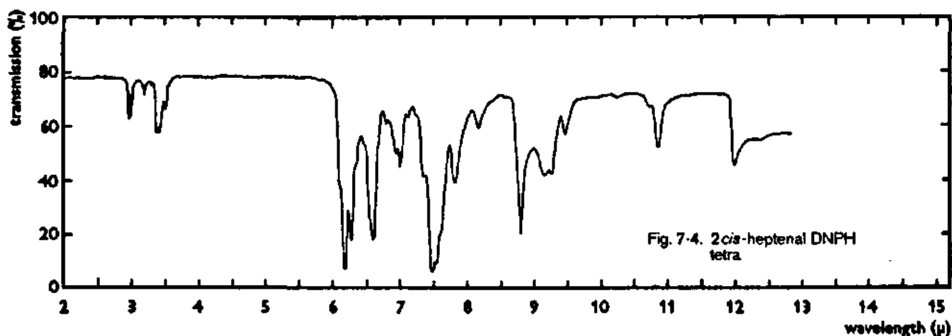
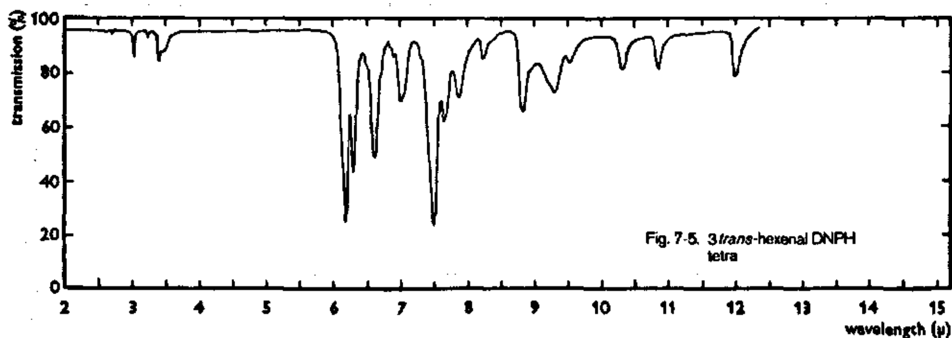
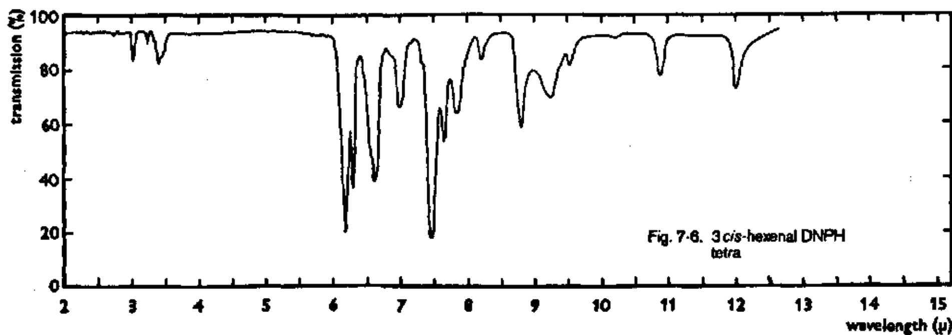
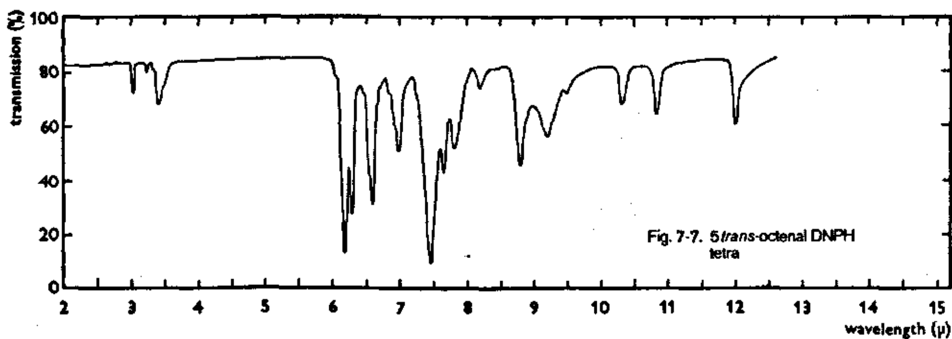


Fig. 7. Infrared spectra of 2,4-dinitrophenylhydrazones of a number of carbonyl compounds.







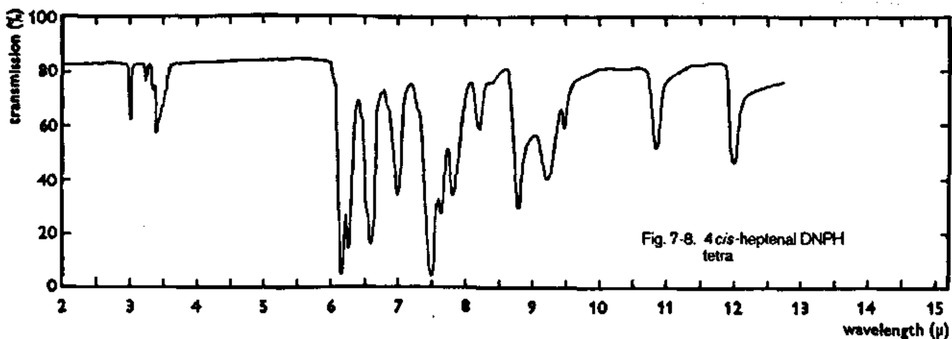


Fig. 7. (continued).

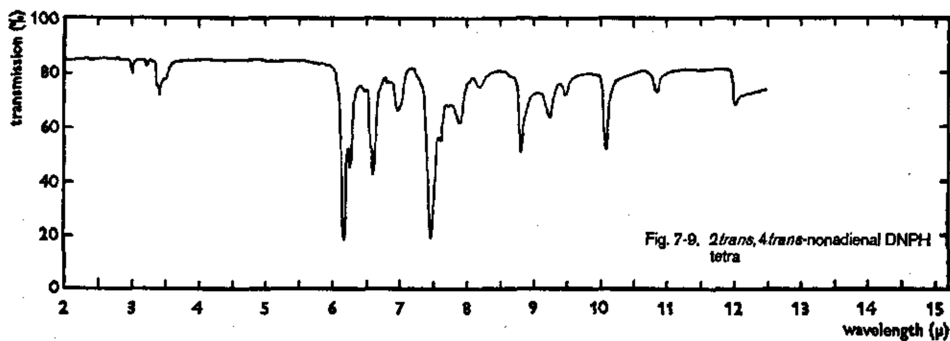


Fig. 7-9. 2*trans*,4*trans*-nonadienal DNPH tetra

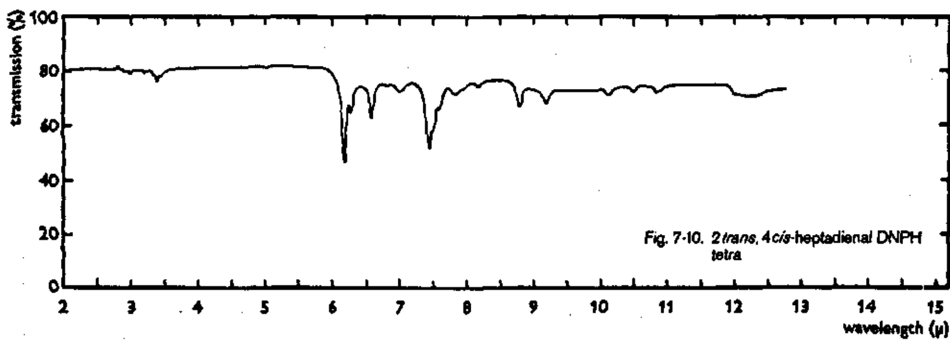


Fig. 7-10. 2*trans*,4*cis*-heptadienal DNPH tetra

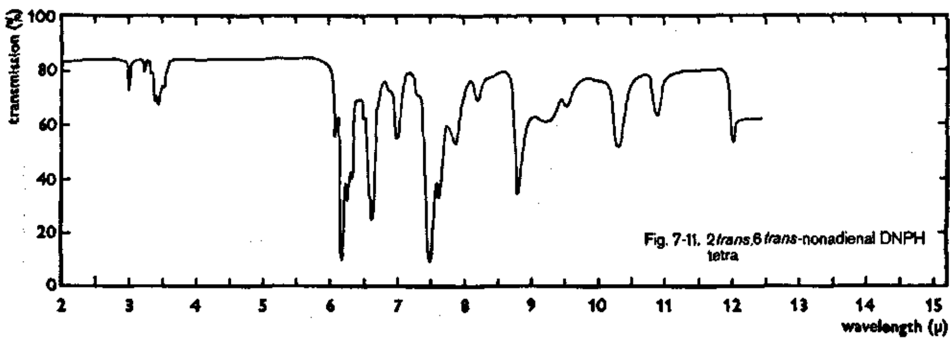


Fig. 7-11. 2*trans*,6*trans*-nonadienal DNPH tetra

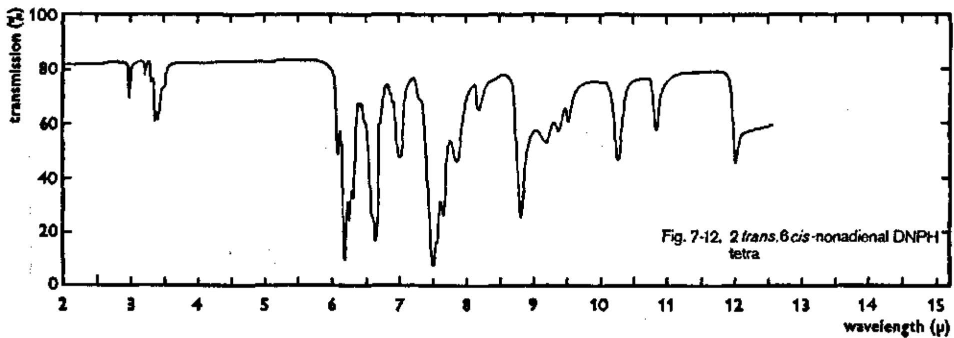
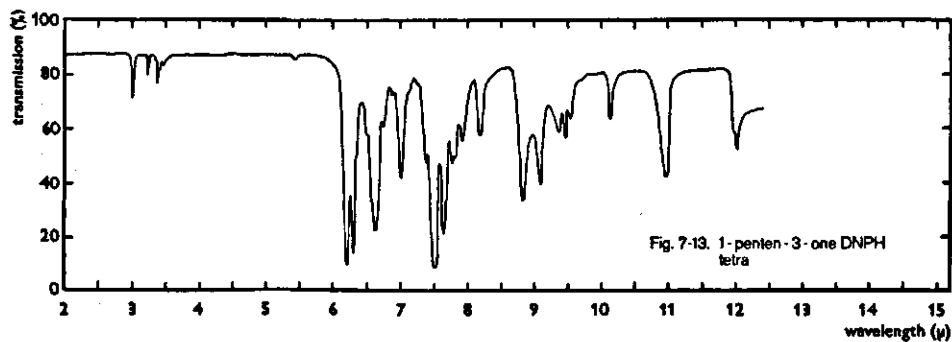
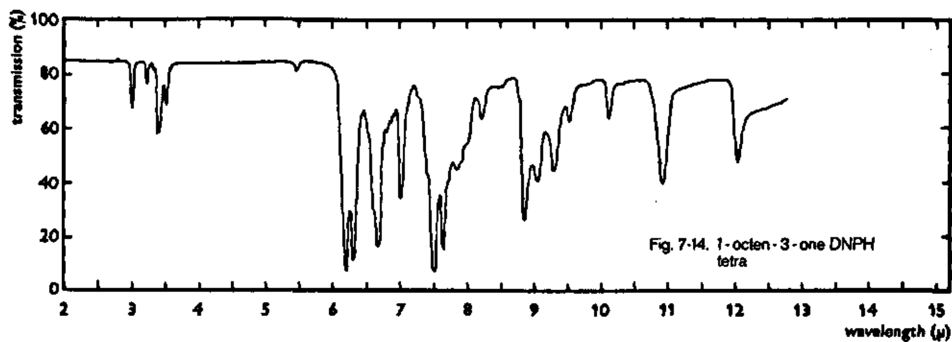
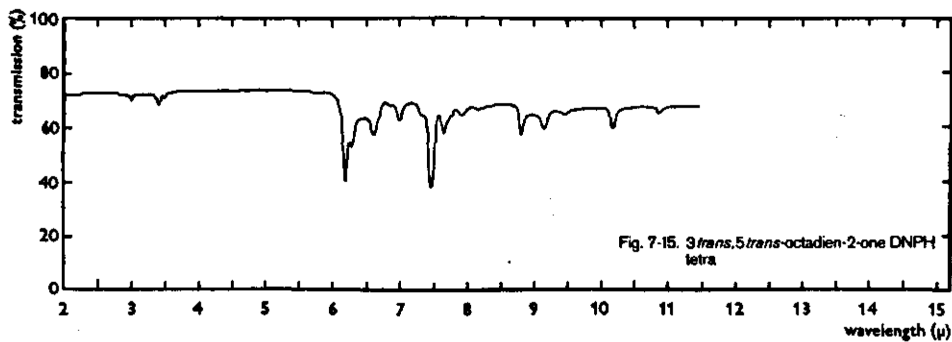


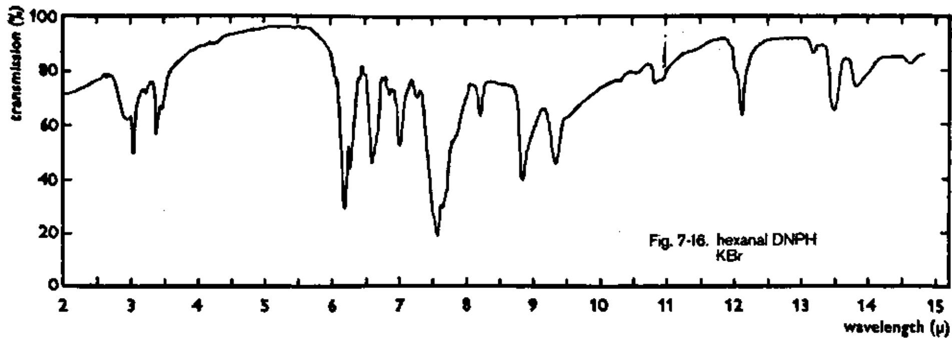
Fig. 7-12. 2*trans*,6*cis*-nonadienal DNPH tetra

Fig. 7. (continued).









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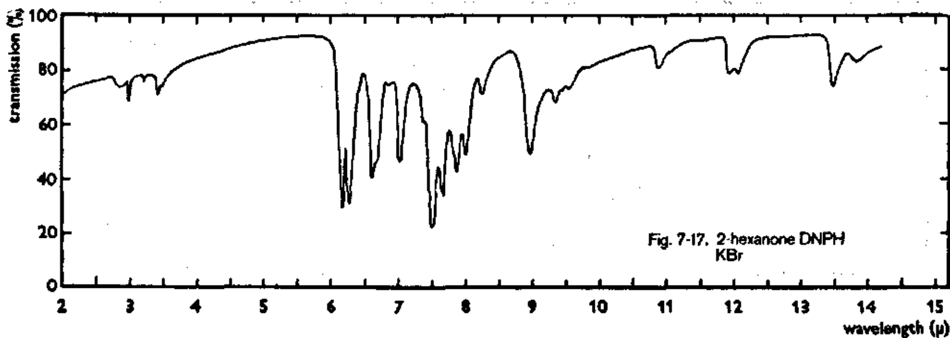


Fig. 7-17. 2-hexanone DNPH
KBr

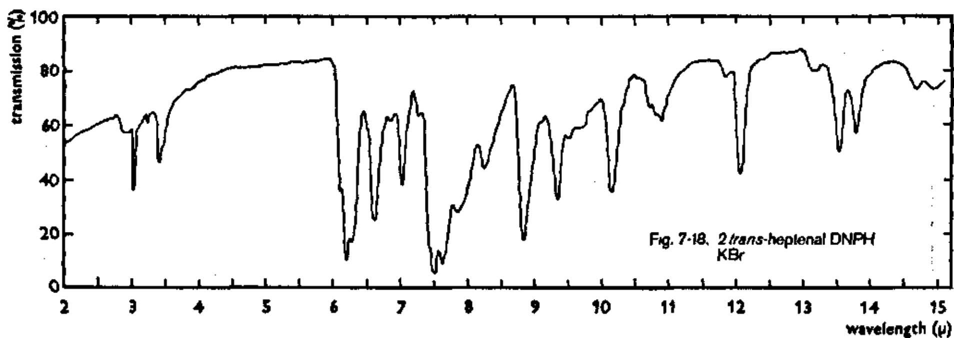


Fig. 7-18. 2 *trans*-heptenal DNPH
KBr

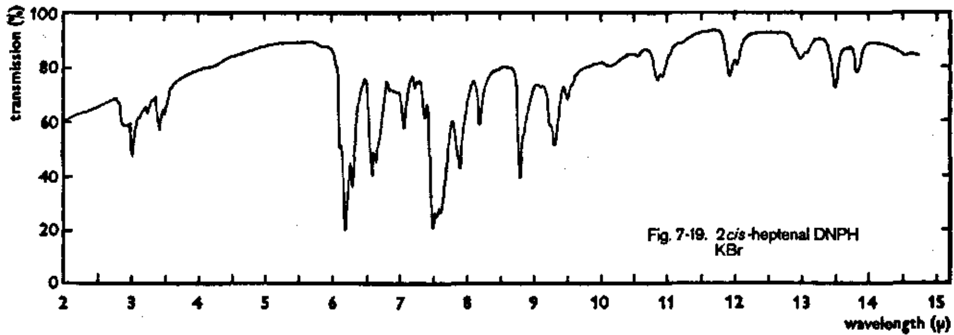


Fig. 7-19. 2 *cis*-heptenal DNPH
KBr

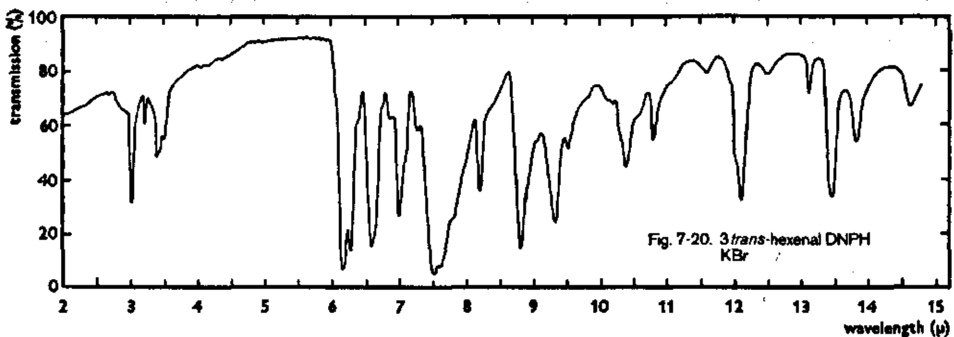
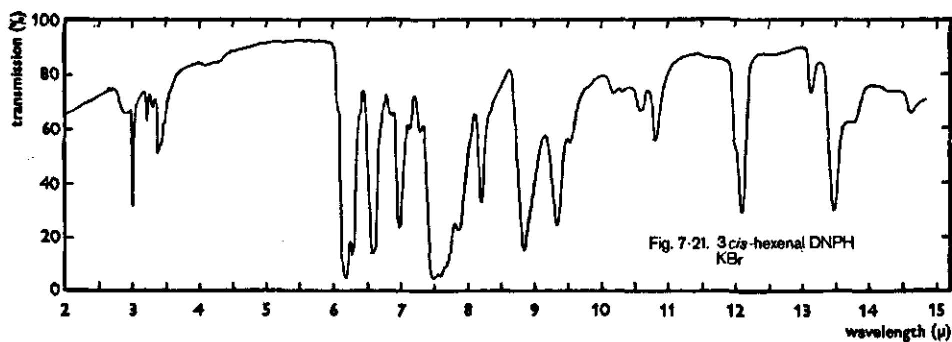
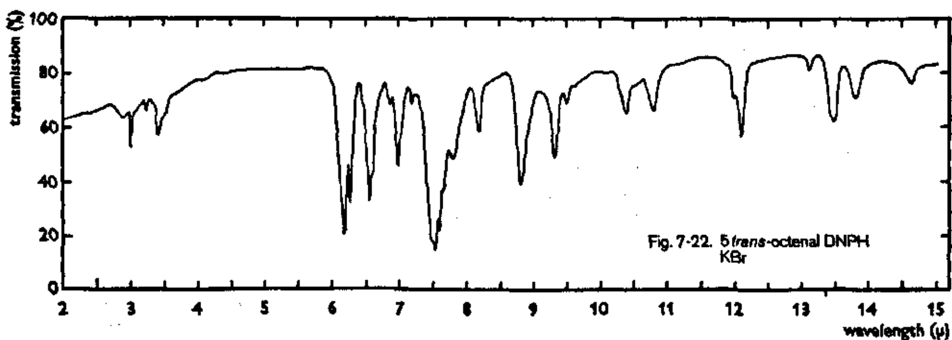
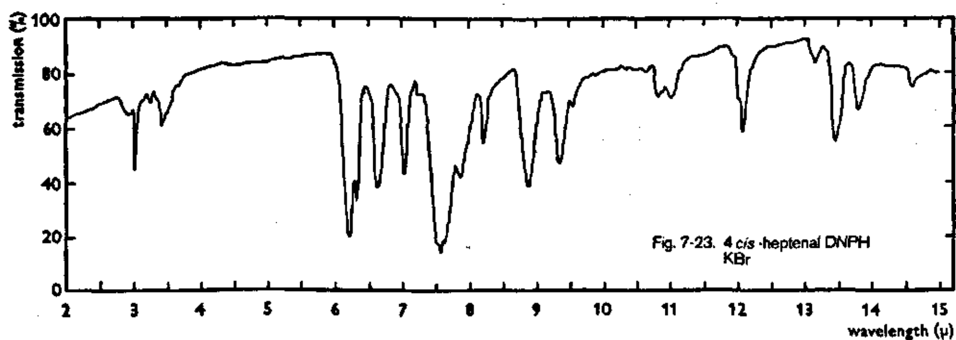


Fig. 7-20. 3 *trans*-hexenal DNPH
KBr

Fig. 7. (continued).







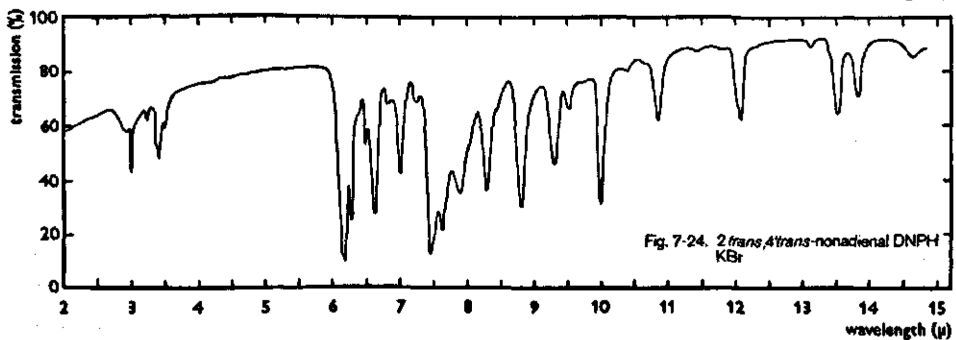
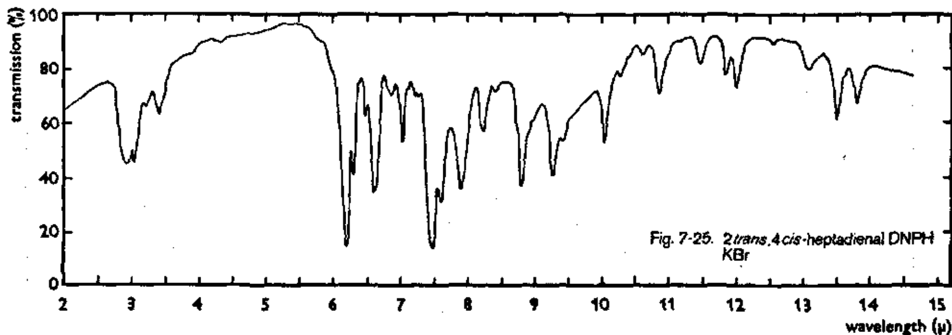
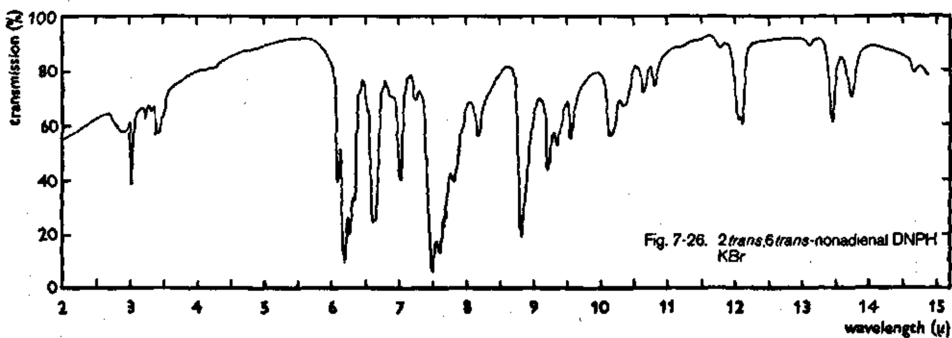
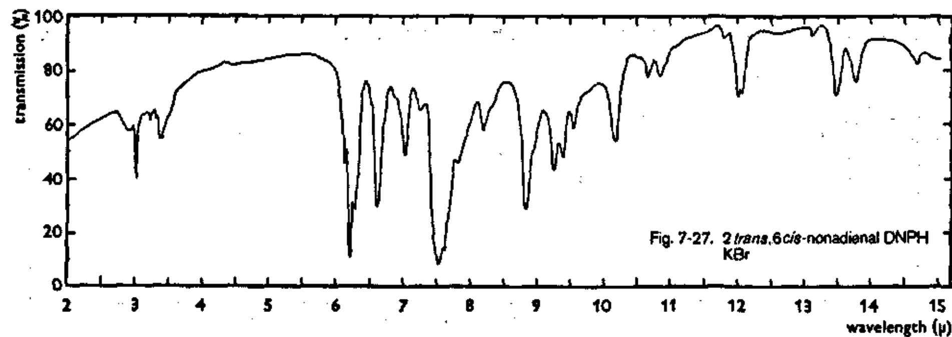


Fig. 7. (continued).







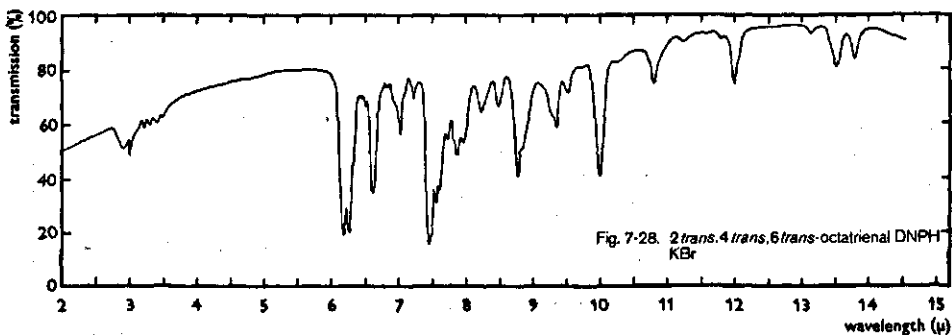
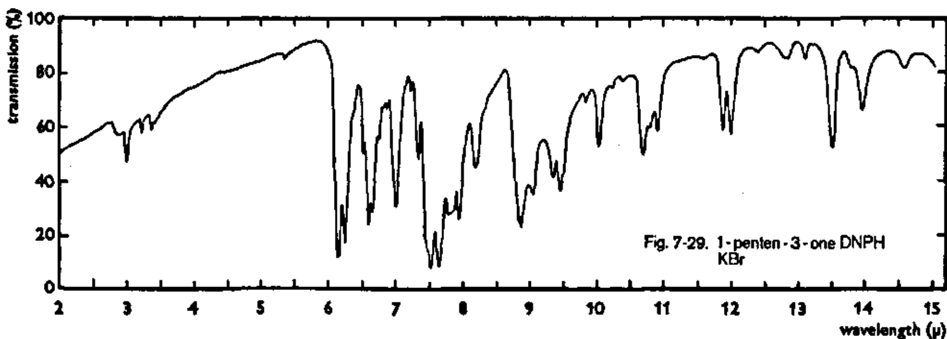
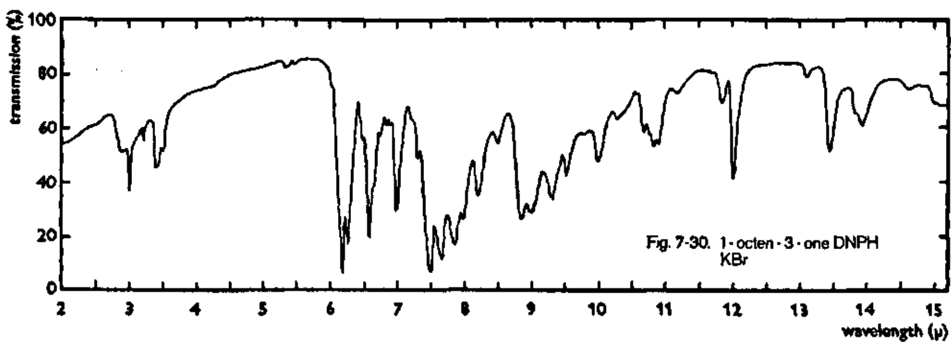


Fig. 7. (continued).





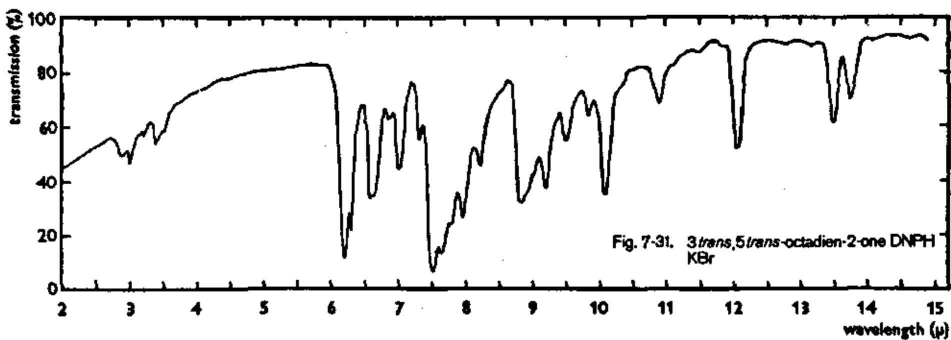


Fig. 7. (continued).

3.8 Mass spectrometry

Mass spectrometry has been most valuable for the identification of aroma compounds in the present investigations. This method of analysis was used particularly for the identification of aliphatic aldehydes and ketones. KLEIPOOL and HEINS (1964) found that mass spectrometry of the free compound is less suitable because of the very rapid breakdown of the parent compound to low molecular weight fragments. Contrary to this, it has been found (KLEIPOOL and HEINS, 1964; KIENITZ, 1968) that the DNPH's of carbonyl compounds lend themselves extremely well to identification by mass spectrometry. In particular the intense molecular ion peak and the strong peaks in the high mass range are very helpful for the elucidation of the structure of the parent carbonyl compounds.

Although the relations between mass spectrum and structure of DNPH's have been only partly elucidated, the recording of spectra of several DNPH-model compounds by KLEIPOOL and HEINS (1964), and KLEIPOOL, HEINS, TEN NOEVER DE BRAUW and BELZ (1968) has made possible the identification of many of the carbonyl compounds which were isolated in the present investigations.

In the mass-spectrometric analysis of DNPH's, the fragmentation of these compounds seems to be characteristic of each homologous series. Not only the *m/e*-values of the different peaks, but also their intensity relative to each other and to the parent peak, are of diagnostic value for their identification. Characteristic peaks in the mass spectra of DNPH's of aliphatic carbonyl compounds are: *m/e* = M, M–17, M–35, M–46, M– (saturated alkyl group) and values of *m/e* 178, 180, 181, 183, 189, etc.

Alkanal DNPH's. Pentanal and the higher alkanal DNPH's display an intense peak at *m/e* 224, which is the result of β-fission of the alkyl chain, accompanied by hydrogen rearrangement (KLEIPOOL and HEINS, 1964; DJERASSI and SAMPLE, 1965) and at *m/e* 206, resulting from further loss of one molecule of H_2O.

2-alkanone DNPH's. 2-hexanone DNPH and the higher terms display an intense peak at *m/e* 238. This peak is the result of a breakdown similar to that explained for the alkanal DNPH's. A prominent peak at *m/e* 178 is also present.

3-alkanone DNPH's. For 3-heptanone DNPH and higher homologues, strong peaks at *m/e* 252 and *m/e* 178 are present.

2-Alkenal DNPH's. Terms in this homologous series show prominent peaks at *m/e* 235 and *m/e* 231, which have the composition $C_9H_7N_4O_4$ and $C_{10}H_7N_4O_3$ respectively. It has also been found that the relative intensities of the breakdown peaks are different for the *cis*- and *trans*-isomers.

Other alkenal DNPH's, i.e. those with the double bond not in conjunction with the carbonyl group. These have mass spectra which are similar to those of the 2-enal DNPH's but which differ in the relative intensity of the breakdown peaks.

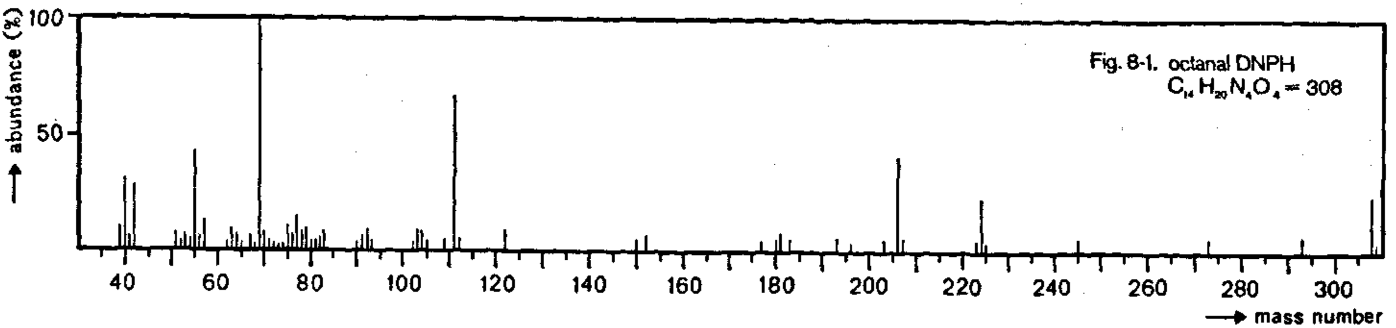
2,4-Alkadienal DNPH's and 2,4,6-alkatrienal DNPH's. The terms of this homologous series have prominent peaks at *m/e* 261 ($C_{11}H_9N_4O_4$) and 214 ($C_{11}H_8N_3O_2$).

Non-conjugated DNPH's. From the analysis of 2, 6-nonadienal DNPH it has been found that a prominent peak at *m/e* 249 is present.

1-Alken-3-one DNPH's. These compounds have been found to show prominent peaks in their mass spectra at *m/e* 202–203 and 216–217.

3,5-Alkadien-2-one DNPH's. From the terms available (C_8 and C_{11}) it has been found that a prominent peak at *m/e* 275 is present.

The mass spectra of DNPH's of a number of aliphatic aldehydes and ketones are presented in Figures 8–1 to 8–12 incl. The structure of unknown DNPH's which belong to other classes and which were isolated in the present investigations, could be identified when a comparison with a reference compound (or a homologue) was possible.



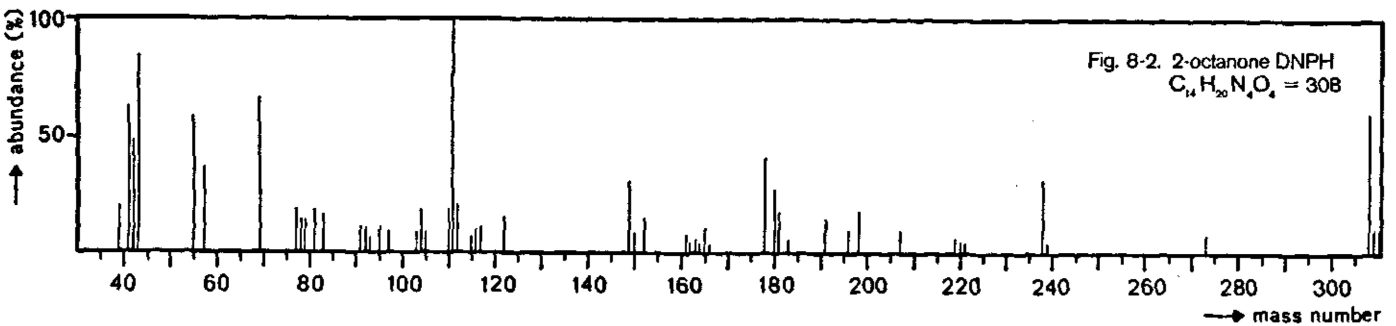


Fig. 8. Mass spectra of 2,4-dinitrophenylhydrazones of a number of carbonyl compounds.

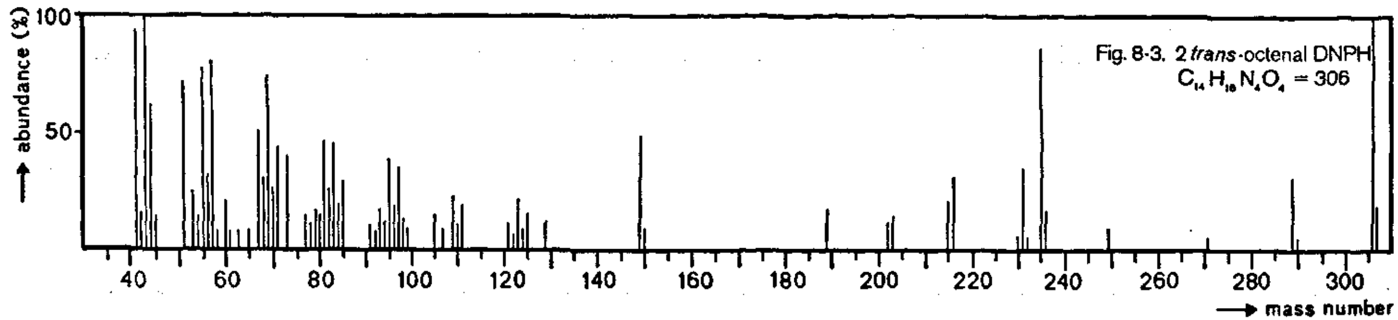


Fig. 8-3. 2 *trans*-octenal DNPH
$C_{14}H_{18}N_4O_4 = 306$

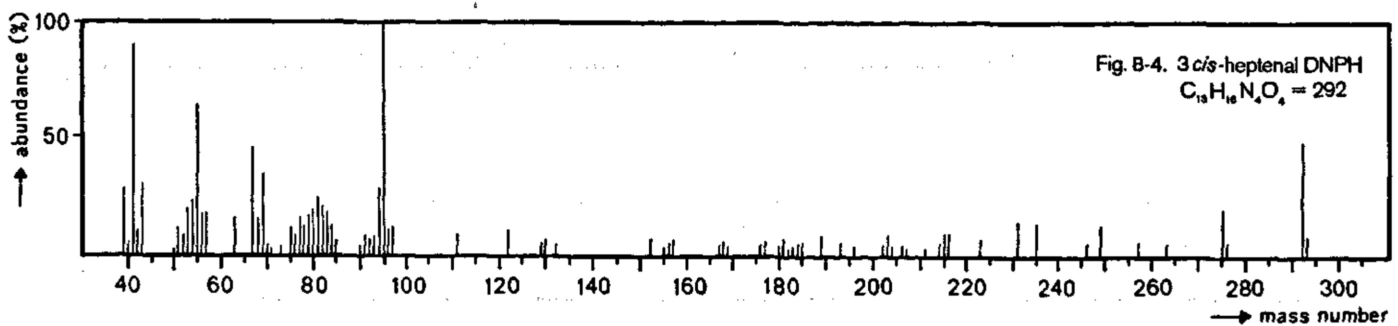
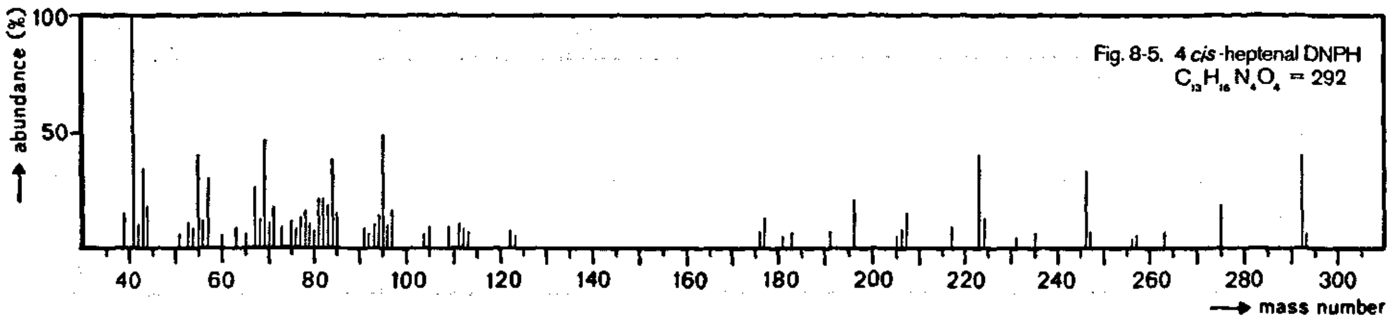


Fig. 8-4. 3 *cis*-heptenal DNPH
$C_{13}H_{16}N_4O_4 = 292$

Fig. 8. (continued).



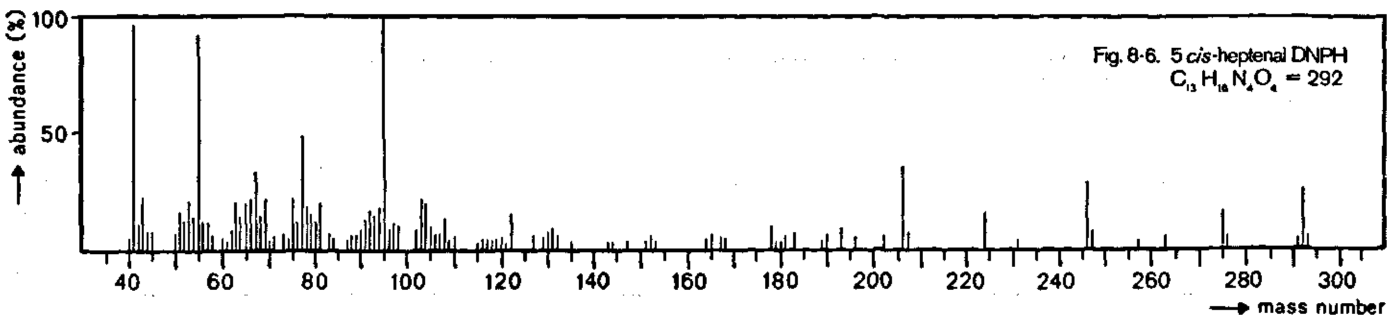
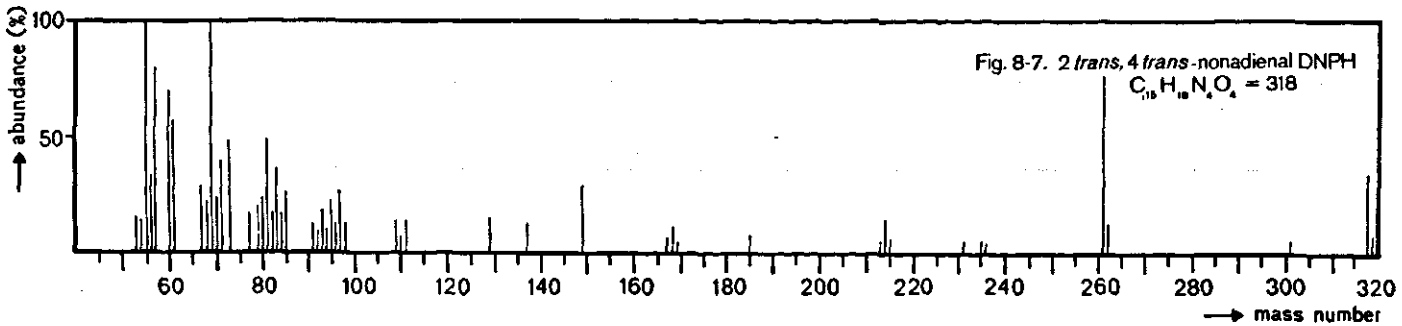


Fig. 8. (continued).



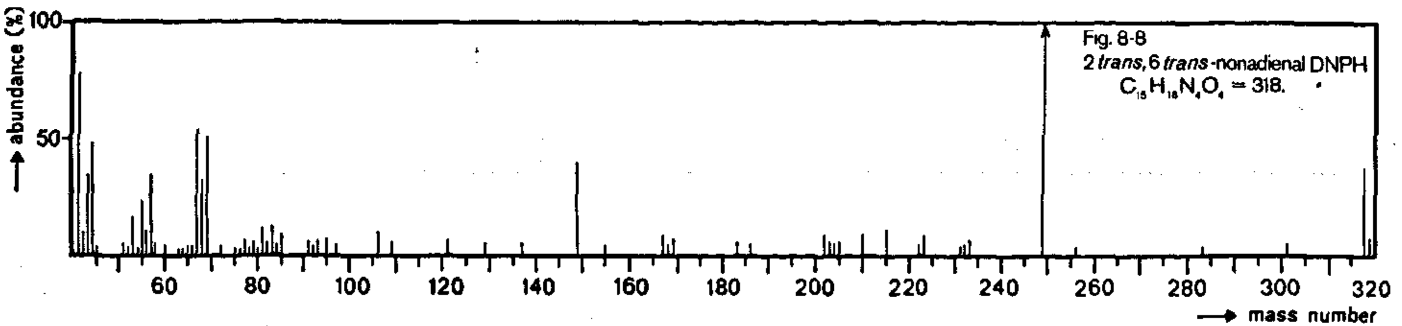
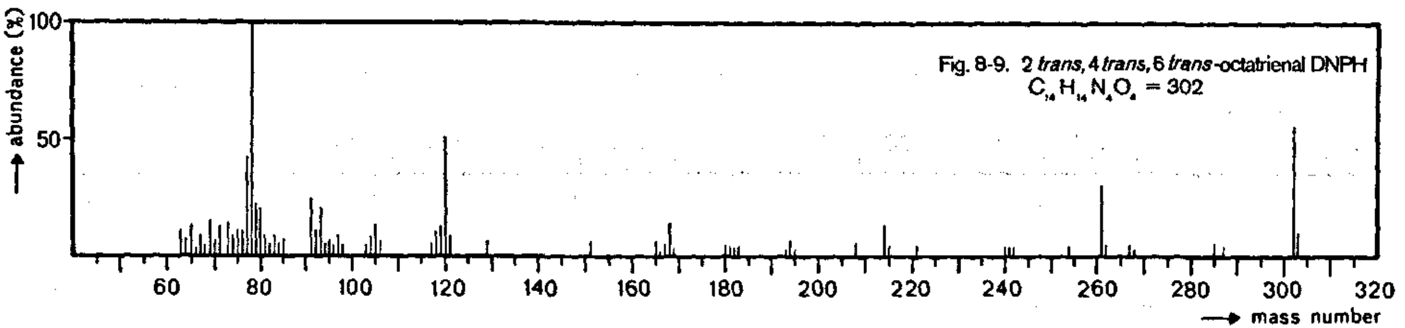


Fig. 8. (continued).



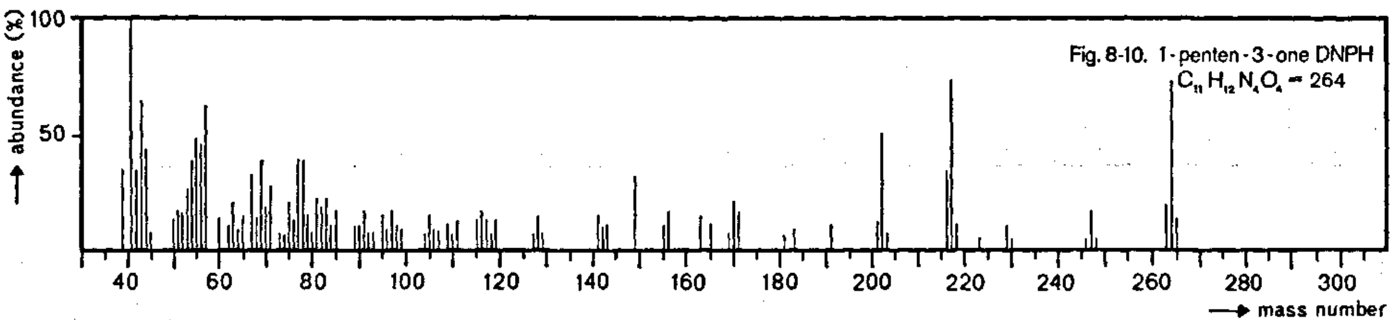


Fig. 8. (continued).

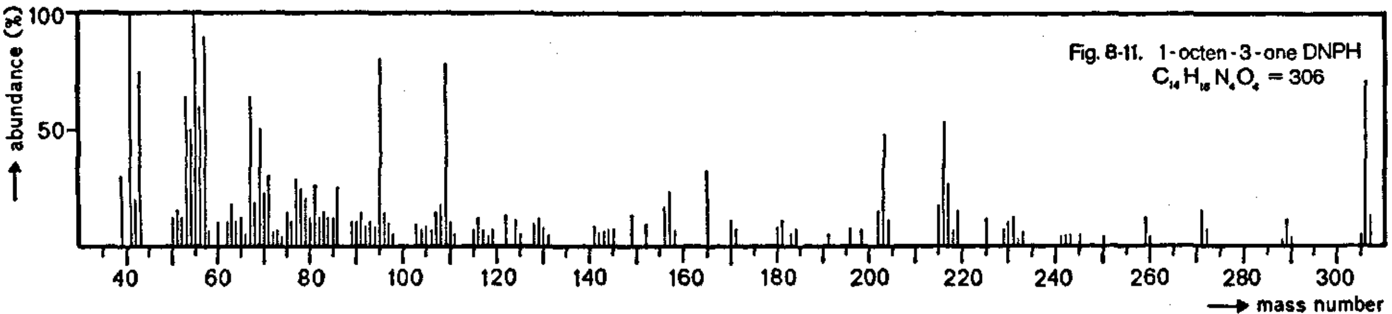


Fig. 8-11. 1-octen-3-one DNPH
$C_{14}H_{18}N_4O_4 = 306$

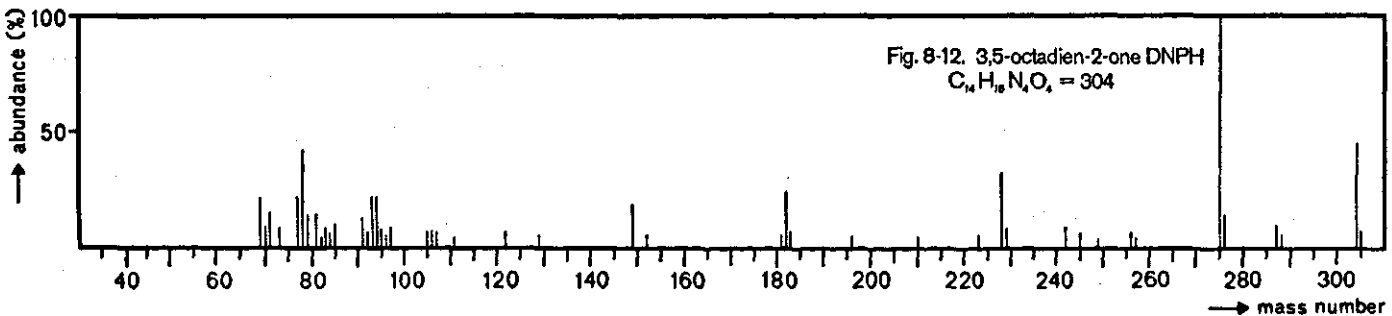


Fig. 8-12. 3,5-octadien-2-one DNPH
$C_{14}H_{16}N_4O_4 = 304$

Fig. 8. (continued).

4. Combination of techniques

A diagram of the combination of techniques used for the identification of volatile compounds obtained from oxidized lipids is represented in Figure 9.

The volatile compounds from oxidized lipids were obtained by high-vacuum distillation. This concentrate was further separated by temperature-programmed gas chromatography. The fractions obtained were evaluated for their contribution to flavour. Only those fractions which possessed distinct odours were investigated further. In a few cases direct recording of the infrared spectrum of a fraction was carried out, provided that this fraction contained only one component. The other fractions were treated with 2,4-dinitrophenylhydrazine reagent for conversion of the carbonyl compounds into DNPH's. The individual DNPH's obtained after further separation by column chromatography using silver-nitrate-impregnated silica gel, were analysed by ultraviolet spectrophotometry, infrared spectrophotometry and mass spectrometry.

Of the above-mentioned techniques, the following have been used for the final characterization of the volatile flavour compounds: gas chromatography (retention indices), silver nitrate complex column chromatography (mobility of DNPH's), ultraviolet, infrared and mass spectrometry. The flavour evaluation has also been of great value.

5. Experimental procedures

5.1 Cleaning of glassware

Rinse with chloroform, wash with an aqueous solution of a suitable detergent (P_3-tritex, Henkel, Düsseldorf, Western Germany) and rinse with distilled water. This preliminary cleaning procedure is followed by a second, to remove traces of silicones, fat etc. Two procedures are possible:

Procedure a. Treat the glassware for two minutes with a solution containing 5% hydrofluoric acid, 33% nitric acid, 2% Teepol and 60% water (Crawley, 1954);

Procedure b. Treat the glassware for three days with a solution of 10% NaOH and 5% borax.

After one of these procedures has been used, the glassware is rinsed several times with tap water, 2% sodium bicarbonate solution, tap water and finally with double-distilled water.

5.2 Purification of solvents

Petroleum ether. Petroleum ether (Chemproha, Zwijndrecht, Neth.; chemisch zuiver, b.p. 60–70 °C) is shaken twice with 10% of its volume of concentrated sulphuric acid. The solvent is then washed with distilled water, a 10% sodium carbonate solution and finally with distilled water. It is dried over anhydrous calcium chloride. It is then distilled from sodium wire, the fraction b.p. 60–70 °C being taken. Finally the petroleum ether is percolated through a column containing Al_2O_3 (Woelm, Eschwege, Western Germany; neutral alumina, activity I).

Benzene. Benzene, free from thiophene (Baker, Deventer, Neth., for Chromatography) is saturated with 2, 4-dinitrophenylhydrazine. The solution is then percolated through a column containing Al_2O_3 (Woelm, neutral alumina, activity I). The benzene is finally distilled and the fraction b.p. 80–81 °C is collected.

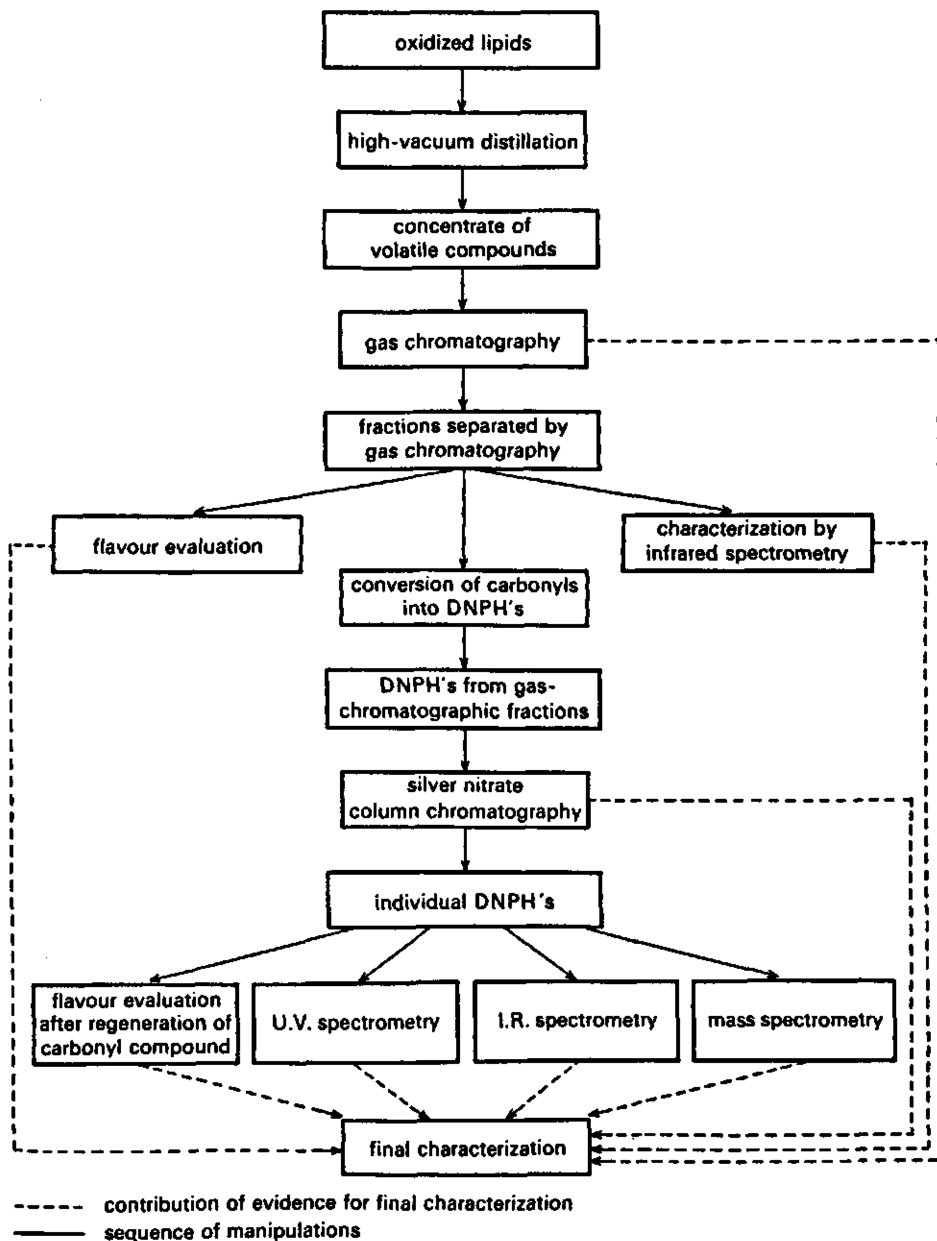


Fig. 9. Combination of techniques, used for the characterization of volatile compounds from oxidized lipids.

Nitromethane. Nitromethane (Fluka, Buchs, Switzerland; puriss) is dried for three days over anhydrous calcium chloride. After decantation a little HgO is added to the solvent after which it is distilled. The nitromethane is then refluxed with 2,4-dinitrophenylhydrazine (2,5 g/l) for two hours and finally distilled under reduced pressure.
Pyridine. Pyridine (BDH-Analar, Poole, England) is refluxed over sodium hydroxide pellets and subsequently distilled with exclusion of moisture (b.p. 115 °C/76 cm Hg).
Chloroform. Chloroform (Lamers & Indemans, 's-Hertogenbosch, Neth.; chemisch zuiver) is shaken twice with 5 % by volume of concentrated sulphuric acid and then three times with distilled water. The solvent is then dried over anhydrous calcium chloride and distilled from P_2O_5 (b.p. 61 °C/76 cm Hg). After the addition of 0.5 % of ethanol, the chloroform is again distilled. To the distillate another 0.5 % of ethanol is added.

In order to be certain that no contamination remains in the solvents, they are checked by infrared spectrophotometry. A 100 ml portion of the solvent is distilled off under vacuum and the residue (if any) is taken up in the minimum of carbon tetrachloride and is analysed in an IR-microcell.

5.3 Purification of reagents

2,4-Dinitrophenylhydrazine. 2,4-dinitrophenylhydrazine (BDH-Analar) is recrystallized from nitromethane solution.
Trichloroacetic acid. Trichloroacetic acid (Hoechst, Frankfort, Western Germany; DAB 6.) is recrystallized twice from petroleum ether.

5.4 Isolation of volatile components

Degassing of butter fat. The high-vacuum degassing apparatus for butter fat is shown in Figure 5. For each analysis some 10 litres of butter fat were used. The molten butter fat is introduced at (1) into a vessel (2) which is surrounded by a jacket through which water at 40 °C is circulating in order to keep the butter fat fluid. By means of the stopcock (5) a steady flow of butter fat is maintained at approximately 250 ml/h. The butter fat then enters the first degassing unit, which consists essentially of an ASCO '50' rota-film molecular still (7) (Arthur Smith Inc., New York, U.S.A.) slightly modified to fit in the unit as a whole. Teflon wipers driven by the gearmotor (4) operate at 40 rpm along the inner side of the cylindrical glass still body, thus spreading the butter fat evenly. Because of the grooves at the surface of the teflon wipers, the film of butter fat is gently moved downwards. During the process the butter fat is heated by an electrical heating mantle which surrounds the glass still body. The temperature of this mantle is held at 70 °C (temperature of the butter fat $\leq$55 °C) in order to prevent formation of thermal artefacts. The retention time of the butter fat in each degassing unit is 60 sec: film thickness is 0.15 mm. The pressure in the first degassing unit is kept at 0.1 mm Hg by means of a vacuum pump (Speedivac ISC 30 vacuum pump, Edwards, Crawley, England) (23). Vacuum is measured by a thermocouple vacuum gauge (ASCO) (3) and is visualized at (15). A U-tube (6) cooled with liquid nitrogen is placed in the vacuum line between the degassing unit and the pump. Volatiles liberated from the butter fat are thus collected in this U-tube.

In the first degassing unit, only the more volatile components (water, diacetyl, low-boiling carbonyl compounds, etc.) are separated. After passing this unit, the butter fat is collected in a flask (9) which is also surrounded by a warm water jacket. Via a high-vacuum stopcock (11) the butter fat is introduced dropwise into the second degassing unit which is held at a much lower pressure (10^{-4} mm Hg). Its construction is similar to the first unit: the butter fat enters a second ASCO-'50' molecular still (13) which operates under the same conditions as the first. The volatiles are collected in the two U-tubes (12) which are cooled in liquid nitrogen. The working pressure is measured at (30) with thermotron and Penning vacuum gauges (Leybold, Cologne, Western Germany) and visualized at (27).

Vacuum is applied by a high-vacuum oil diffusion pump (Leybold Do-30) and a vacuum-pump (Leybold D-2) (22). A safety trap (29) (cold-finger filled with liquid nitrogen) is placed between the pumps and the collecting tubes (12) in order to prevent any volatile material from the pumps contaminating the flavour concentrate. The butter fat which has passed the second still is collected in the two receiving flasks (16). After collecting 7 l of butter fat, they can be replaced by new flasks.

All joints in the apparatus are lubricated with Apiezon N (Shell, England) (joints used at room temperature) or with Apiezon T (joints at elevated temperature).

Degassing of small samples of oxidized lipids. 1 g of an autoxidized fatty acid is brought into a 100 ml vessel with standard tapered joints. This vessel is connected to a vacuum system consisting of a U-tube for collecting the volatiles, a second U-tube as a safety trap, and a vacuum pump system made up of a high-vacuum oil diffusion pump (Leybold Do-30) and a vacuum pump (Leybold D-2). The U-tubes are cooled with liquid nitrogen immediately after the oil pump has been started. A vacuum valve is located between the degassing unit and the pumping system. This valve is opened only occasionally in order to maintain the required vacuum. Distillation is carried out for 3 hours at 10^{-3} mm Hg, 3 hours at 10^{-4} mm Hg and, after standing in vacuum overnight, another 4 hours at 10^{-4} mm Hg. The lipid fraction is maintained at 20 °C for the first period and at 40 °C for the last 4 hours of the degassing.

5.5 Preparation of the condensate for gas chromatography

After slowly letting in pure nitrogen gas to the vacuum system until atmospheric pressure has been reached, the U-tube which contains the volatiles from 10 kg of butter fat or from 1 g of an autoxidized fatty acid, is brought to approximately 0 °C. This tube is then washed with four 0.5 ml portions of light petroleum b.p. 30–40 °C.

After each extraction the U-tube is cooled to −20 °C in order to solidify the water droplets in the tube so that the petroleum ether can be taken out essentially free from water. The petroleum ether solution is shaken for a few minutes with a small quantity of sodium carbonate/sodium bicarbonate (1:1) (BDH-Analar), and finally with a small quantity of anhydrous sodium sulphate (BDH-Analar).

5.6 Gas chromatography

A Becker gas chromatograph (J. Becker, Delft, Neth.) Type 1458 K was used with a 2 M glass column (i.d. 4 mm) filled with 60–80 mesh Gaschrom A (Applied Sci. Lab., State College, Pa., U.S.A.) coated with 20% silicone oil (May & Baker Inc., Dagenham, England) and 0,4% Carbowax 4000 (Union Carbide Inc., New York, U.S.A.). Carrier gas: purified helium. Detector: katharometer. The petroleum ether solution of the condensate (approximately 2 ml) is introduced by direct 'on-column' injection by means of a 2 ml Agla syringe (Burroughs, Wellcome, England). Temperatures of injection port and katharometer detector are 130 °C. After isothermal analysis at 58 °C for a period of time equivalent to the retention time of n-octane, the column is subjected to programmed-temperature heating (rate 0.6 °C/min).

5.7 Collection of fractions

Special glass capillary traps cooled in liquid nitrogen are used, which give efficient trapping. The traps are connected to the ball joint attached to the outlet of the katharometer. Details have been published earlier (BADINGS and WASSINK, 1965).

5.8 Conversion of carbonyl compounds into DNPH's

After bringing the collection traps to room temperature, they are rinsed with small portions of benzene up to 1 ml in volume. These portions are collected in a 100 ml vessel. To this solution are added 10 ml of a benzene solution saturated with 2,4-dinitrophenylhydrazine and 1 ml of a 12% trichloroacetic acid solution in benzene.

The reaction mixture is held at room temperature. The reaction time is dependent on the carbonyl compound to be expected, ketones 1 hour, other carbonyl compounds 5 minutes. After the required time the reaction mixture is washed four times with 10 ml portions of double-distilled water.

5.9 Separation of DNPH's by liquid chromatography

Silver nitrate complex chromatography. Silica gel (neutral) is prepared according to NIJKAMP (1951, 1954). After drying, the fraction of 120–200 mesh is separated by sieving. This fraction is then heated for two hours at 650 °C in order to remove traces of organic material. For each analysis, a conventional glass chromatography column (height 30 cm, diameter 1.3 cm) is used. 4 g of the prepared silica gel is taken for each column. It is loaded with 6.0 ml of 50% silver nitrate solution in double-distilled water. To the loaded gel is added sufficient petroleum ether (saturated with 50% silver nitrate solution) to obtain a thin slurry, which is poured into the column. After settling of the gel the column is eluted with a few ml of petroleum ether containing 10% benzene.

The benzene solution containing the DNPH's (see Section 5.8) is evaporated under vacuum. The residue is extracted with successive portions of petroleum ether containing 10% benzene (total approximately 3 ml). This solution is brought on to the top of the column, after which chromatographic separation is carried out in the usual way. During elution the concentration of benzene in the eluent is gradually increased to 50%. The separation which can be achieved in this way has been discussed in Chapter IV, Section 3.4.

In those cases where very slowly moving bands are observed the elution is carried out in a slightly different way. Soon after starting the elution the concentration of benzene in the petroleum ether is rapidly increased to 50%. In this way the more rapidly moving bands are eluted as one fraction, whereas the slower moving bands are eluted separately within a reasonable time. The more rapidly moving bands are then further analysed separately by the method described above.

The solutions of the separated DNPH-bands are washed twice with double-distilled water, to remove traces of silica gel and silver nitrate.

Partition chromatography. Separation according to chain length is achieved by partition chromatography, according to VAN DUIN (1961).

Adsorption chromatography. For separation of alkanals and 2-alkanones, adsorption chromatography using zinc carbonate (VAN DUIN, 1961) was used.

5.10 Regeneration of carbonyl compounds from the DNPH's

Method 1. The DNPH in petroleum ether solution is collected in a small test tube (length 10 cm, inner diameter 0.8 cm). The solvent is evaporated carefully. Then 50 to 100 mg of α-ketoglutaric acid (BDH-Analar) is added to the DNPH-residue, followed by one drop of distilled water. The test tube is now heated in a water-bath at 60 °C for 20 seconds, during which time the contents of the tube are shaken. Then 2 to 3 ml of distilled water is added and the mixture is poured into a small beaker, after which the odour is evaluated.

Method 2. MnO_2 prepared according to ATTENBUROW *et al.* (1952), is suspended in distilled water and then neutralized (pH 7) by addition of N acetic acid. It is then filtered, washed with distilled water and dried at 120 °C. A volume of 0.5 to 1.0 ml of a petroleum ether solution of the DNPH is brought in a small test tube (length 10 cm, inner diameter 0.8 cm). 10 to 50 mg of the prepared MnO_2 is added. After agitating the tube for a few seconds, part of the solution is poured on a piece of filter paper. During evaporation of the petroleum ether, the odour of the filter paper is evaluated. If no odour is detectable, the tube is heated for a few seconds in a water-bath at 50 °C, after which the odour evaluation is repeated. This procedure is particularly suitable for the regeneration of 2 *cis*- and 3 *cis*-enals and of 1-alken-3-ones.

5.11 Ultraviolet spectrophotometry

Recording of ultraviolet spectra is made with a Perkin Elmer 4000-A double-beam recording spectrophotometer (Perkin Elmer, Norwalk, Conn. U.S.A.). Quartz cuvettes (1 cm light path) are used. The recordings are generally made with chloroform solutions.

5.12 Infrared spectrophotometry

Recording of infrared spectra is made with a Perkin Elmer model 221 infrared spectrophotometer. For the recording of spectra of compounds in carbon tetrachloride solution, the following cells are used: cell M 05N (0.2 mm light path cell, manufactured by Research and Industrial Instruments Company, R.I.I.C., London, England) and a variable-path cell SR 05 N (also from R.I.I.C.). Carbon tetrachloride (BDH-special for spectroscopy) is used.

For the recording of spectra of compounds in KBr, 0.5 mm or 1.5 mm tablets are pressed. The required amount of KBr (Merck, für Infrarotspektroskopie) is transferred to a mortar and the DNPH in a minimum of benzene is added to it. After evaporation of the solvent, the mixture is ground and transferred to a small test tube (3 cm length, 0.3 cm inner diameter). This test tube is placed in a flask which is evacuated by means of a vacuum pump, in order to remove traces of solvent. The KBr is then pressed to a pellet by means of a hydraulic press Model H30–1 (R.I.I.C.). The infrared spectrogram is recorded with the aid of a Beam Condenser, Model C21, (R.I.I.C.) with a Perkin Elmer attenuator (venetian blind type) in the reference beam.

5.13 Mass spectrometry

An Atlas CH4-mass spectrometer (Varian-Atlas, Bremen, Western Germany), equipped with a TO4-ion source and an ionization chamber, interchangeable via a vacuum lock, is used.

An elegant technique, developed by HEINS and TEN NOEVER DE BRAUW (1966) is used for introduction of the sample. The DNPH, dissolved in a few μl benzene is applied to a small piece (3 × 5 mm) of glass paper (Glasfaser-papier nr. 6, Carl Schleicher & Schull, Dassel, Western Germany.). It is very important to remove traces of extraneous material from the glass paper before it is used. This is done by heating the pieces of glass paper at 600 °C in a small oven for a few hours. The piece of glass paper to which the DNPH has been applied is brought into the heating device.

With DNPH's of molecular weight less than about 340, no extra heating was usually necessary to obtain a good mass spectrum. The temperature of the sample did not exceed 70 °C when special cathode filaments were used. If this temperature was not sufficient, the sample was heated very carefully until sufficient vapour pressure of the DNPH had been reached to record a clear mass spectrum.

Using this method it has not only been found that the sample is used very efficiently, but also that the risk of thermal or catalytic decomposition in the supply line is minimal (BRUNNÉE and VOSHAGE, 1964).

In a few cases the exact composition of the ions was determined with an Atlas SM-1 double-focussing mass spectrometer.

IDENTIFICATION OF AROMA COMPOUNDS WHICH CONTRIBUTE TO THE COLD-STORAGE DEFECTS IN BUTTER

1. Introduction

In this Chapter the results of the analyses of volatile aroma compounds which contribute to the cold-storage defects of butter are presented. The analyses have been focussed particularly on the identification of carbonyl compounds, because these are the main cause of flavour defects in cold-stored butter. Data obtained by different identification techniques, described in Chapter IV, have contributed to the characterization of various aroma components. In order to present these data in a convenient form, they have been summarized in tables. In these tables the information obtained by the different analyses is given in a number of codes which refer to characteristics measured in the different identification methods. These codes are explained in Table 14. They have been used only when the characteristics measured were in accordance with the system of classification given in this Table.

Table 14. System of classification of DNPH's in the various identification techniques (further details are given in the corresponding sections of Chapter IV).

Silver nitrate complex chromatography of DNPH's	
class I :	alkanones; alkanals; 1-alken-3-ones;
class II :	2 *cis*-alkenals; 2 *trans*-alkenals;
class III:	p *trans*-alkenals $(p>2)$; 2 *trans*, 4 *trans*-alkadienals; 2 *trans*, 4 *cis*-alkadienals; 2 *trans*, q *trans*-alkadienals $(q>4)$; 3,5-alkadien-2-ones;
class IV:	p *cis*-alkenals $(p>2)$;
class V :	2 *trans*, q *cis*-alkadienals $(q>4)$; alkatrienals, etc.
Ultraviolet spectrophotometry of DNPH's	
class A:	alkanals; p-alkenals $(p>2)$; p, q-alkadienals $(p>2, q>4)$;
class B:	alkanones; non-conjugated alkenones;
class C:	1-alken-3-ones;
class D:	2-alkenals; 2, q-alkadienals $(q>4)$;
class E:	3,5-alkadien-2-ones;
class F:	2,4-alkadienals; 2,4, r-alkatrienals $(r>6)$;
class G:	2,4,6-alkatrienals; 2,4,6, s-alkatetraenals $(s>8)$.
Infrared spectrophotometry of DNPH's	
class a:	alkanals; alkanones; *cis*-alkenals; *cis*-alkenones;
class b:	2 *trans*-alkenals;
class c:	x *trans*-alkenals $(x>2)$; non-conjugated *trans*-enones;
class d:	conjugated *trans*-alkenones (except 1-alken-3-ones);
class e:	2,4-alkadienals; 2,4,6-alkatrienals; conjugated alkadienones; 1-alken-3-ones.

2. Aroma compounds which contribute to the trainy flavour of butter with cold-storage defects

Butter with a relatively high copper content (approximately 100 μg per kg of butter) was prepared in the summer period from fresh milk to which cupric sulphate had been added. After storage for 10 months at -10 °C this preparation of butter had become distinctly trainy.

The cold-stored butter was melted carefully at 37 °C, after which the fat phase was separated from the serum by centrifugation. 10 kg of the butter fat was subjected to high-vacuum degassing. The volatiles obtained were separated and the odorous fractions further analysed as described in Chapter IV. Several analyses of butter fat from trainy butter were carried out. An example of the results of such an analysis is given in Table 15.

The identification of a number of fractions needs some more explanation. Since fraction 1 could not be split up into its individual components by silver nitrate complex chromatography, it was further analysed by liq./liq. partition chromatography according to van Duin (1961) using the system petroleum ether (mobile phase)/methanol (immobile phase). Two DNPH-bands were obtained. The first (1-1), was found to be 3-methylbutanal DNPH, and the second (1-2) was identified as pentanal DNPH. However, this fraction also contained a small quantity of a DNPH with a molecular weight of 264 and with breakdown peaks at m/e 217 and 202. Because of these observations and because of the typical fishy flavour of this fraction after regeneration of the carbonyl compound, it could be concluded that a small quantity of 1-penten-3-one DNPH was also present.

Fraction 3-1 was identified as heptanal DNPH. However, from the mass-spectrometric analysis the presence of a small quantity of 2-heptanone DNPH could be established.

Fraction 4-3 was difficult to identify at first because it did not fit into the system of classification for aliphatic carbonyl compounds. Its ultraviolet spectrum showed a maximum absorption at 376 mμ, which is slightly higher than that of a 2-alkenal DNPH. From the infrared- and mass-spectrometric analysis it could be concluded that this component was identical with benzaldehyde DNPH.

Fraction 4-4 was identified as 2,4-heptadienal DNPH. On the basis of the infrared-spectrometric analysis it could be concluded that this fraction contained both the 2 *trans*, 4 *trans*- and the 2 *trans*, 4 *cis*-isomer. The ratio between these isomers was estimated after further separation by silver nitrate complex chromatography.

The mass spectrometric analysis of fraction 4-4 also showed small peaks at m/e 306 and 203. This finding together with the observation of a distinct metallic odour after regeneration of the carbonyl compound (different from the odour of the 2,4-heptadienals), may indicate the presence of a small quantity of 1-octen

Table 15. Summary of results of the analysis of the volatile carbonyl compounds from a sample of train cold-stored butter.

gas chromatography		silver nitrate complex chromatography of DNPH's		organoleptic evaluation after regeneration		ultraviolet spectro-photometry of DNPH[2]
fraction number[1]	retention temperature °C	fraction number[1]	mobility of fraction[2]	aroma	intensity[3]	
1	58	1–1	I	sharp	++	A
		1–2	I	sharp, fishy	++	(A)
2	58	2–1	I	green	+++	A
		2–2	II	green, paint	++	D
3	58–69	3–1	I	oily, putty	++	(A)
		3–2	II	green	+	D
		3–3	III	putty	++++	A
		3–4	IV	creamy, putty	++++	A
4	69–81	4–1	I	fatty	++	A
		4–2	II	putty, fatty	+++	D
		4–3	II→III	weak	±	[4]
		4–4	III	metallic, fried	++++	F
5	81–92	5–1	I	tallowy	++	(A)
		5–2	II	woodbugs, fatty	++	D
		5–3	III	fatty, fruity	+	E
		5–4	III	fatty, fruity	++	E
		5–5	V	fatty, beans	+++	D
6	92–104	6–1	I	orange peels	++	A
		6–2	II	tallowy, cucumbers	++++	D
		6–3	III	fatty, oily	++	F
		6–4	III	cucumbers	+++	D
		6–5	V	fresh cucumbers	+++	D
7	104–119	7–1	I	fruity, citrus	++	[4]
		7–2	II	tallowy	+++	D
		7–3	III	deep-fried	++	F
		7–4	III	frying odour	+++	F
		7–5	V	fatty, fishy	+++	G
		7–6	V	fatty, fishy	+++	G
		7–7	V	sliced beans	+++	F
		7–8	V	sliced beans	+++	F
8	119–132	8–1	I	citrus, fatty	+	A
		8–2	II	fatty, tallowy	++	D
		8–3	III	fatty, fried	+	E
		8–4	III	fried, fatty	+++	F

[1] Only those fractions which possessed a distinct aroma are mentioned.
[2] Symbols refer to the system of classification of DNPH in the various identification techniques (Table 14).

infrared spectro-photometry of DNPH[2]	mass spectrometry of DNPH		results of identification	
	molecular weight	structure	identity of components	quantity µg/kg of trainy butter (p.p.b.)
a	266	3-methylbutanal	3-methylbutanal[4]	25
(a)	266	pentanal,	pentanal,	40
	(264)	1-penten-3-one[4]	1-penten-3-one[4]	1
a	280	hexanal	hexanal	180
b	264	2 *trans*-pentenal	2 *trans*-pentenal	20
a	294	heptanal,	heptanal[4],	230
		2-heptanone[4]	2-heptanone[4]	10
b	278	2 *trans*-hexenal	2 *trans*-hexenal	30
c	292	4-heptenal	4 *trans*-heptenal	5
a	292	4-heptenal	4 *cis*-heptenal	5
a	308	octanal	octanal	30
b	292	2 *trans*-heptenal	2 *trans*-heptenal	20
[4]	286	benzaldehyde[4]	benzaldehyde[4]	5
e	290	2,4-heptadienal,	2 *trans*, 4 *trans*-heptadienal[4],	240
			2 *trans*, 4 *cis*-heptadienal[4],	80
	(306)	1-octen-3-one[4]	1-octen-3-one[4]	1
a	322	nonanal,	nonanal,	100
		2-nonanone[4]	2-nonanone[4]	5
b	306	2 *trans*-octenal	2 *trans*-octenal	20
e	304	3,5-octadien-2-one	3,5-octadien-2-one[4]	50
e	304	3,5-octadien-2-one	3,5-octadien-2-one[4]	180
[4]	304	octadienal[4]	2,x-octadienal ($x > 4$)[4]	5
a	336	decanal	decanal	1
b	320	2 *trans*-nonenal	2 *trans*-nonenal	130
e (*t*, *t*)	318	2,4-nonadienal	2 *trans*, 4 *trans*-nonadienal	10
[4]	318	2,6-nonadienal	2 *trans*, 6 *trans*-nonadienal[4]	10
[4]	318	2,6-nonadienal	2 *trans*, 6 *cis*-nonadienal[4]	15
[4]	350	undecanal,	undecanal,	5
		2-undecanone	2-undecanone[4]	5
b	334	2 *trans*-decenal	2 *trans*-decenal	15
e (*t*, *t*)	332	2,4-decadienal	2 *trans*, 4 *trans*-decadienal	30
e (*t*, c)	332	2,4-decadienal	2 *trans*, 4 *cis*-decadienal	45
e	316	2,4,6-nonatrienal	2 *trans*, 4 *trans*, 6 *trans*-nonatrienal[4]	20
e	316	2,4,6-nonatrienal	2,4,6-nonatrienal[4]	5
e	330	2,4,7-decatrienal	2,4,7-decatrienal[4]	75
e	330	2,4,7-decatrienal	2,4,7-decatrienal[4]	70
a	364	dodecanal	dodecanal	25
b	348	2 *trans*-undecenal	2 *trans*-undecenal	5
e	346	3,5-undecadien-2-one	3,5-undecadien-2-one	5
e (*t*, *t*)	346	2,4-undecadienal	2 *trans*, 4 *trans*-undecadienal	10

[3] Intensity: + = faint; ++ = moderate; +++ = strong; ++++ = very strong.
[4] For further explanation of this identification, see text.

-3-one DNPH. Because of the small quantity, no additional proof could be presented by other identification techniques.

Fraction 5–1 was identified as nonanal DNPH, but on the basis of mass-spectrometric analysis it was also found to contain a small quantity of 2-nonanone DNPH.

Fractions 5–3 and 5–4 were both found to be 3,5-octadien-2-one DNPH. However, by silver nitrate complex chromatography of the DNPH's, it was found that these two fractions had distinctly different mobilities. These two fractions were considered to be *cis/trans*-stereo isomers. This hypothesis was supported by the fact that the infrared spectra of 5–3 and 5–4 showed similar differences as those found for the 2 *trans*, 4 *trans*- and 2 *trans*, 4 *cis*-alkadienal DNPH's. On account of their mobilities in silver nitrate complex chromatography it is supposed (cf. Chapter IV, Section 3.4) that fraction 5–3 may be 3 *trans*, 5 *trans*-octadien-2-one DNPH and fraction 5–4 3 *trans*, 5 *cis*-octadien-2-one DNPH.

On account of the mass-spectrometric analysis, fraction 5–5 was found to be an octadienal DNPH. Only one double bond was in conjunction with the DNPH group (ultraviolet spectrum). The recorded spectra did not permit a definite elucidation of the structure of this compound. However, it had several characteristics in common with a fraction from autoxidized linolenic acid, which was identified as 2,5-octadienal.

Fractions 6–4 and 6–5 were found to be stereo-isomeric 2,6-nonadienal DNPH's. From their infrared spectra it could be concluded that 6–4 is the 2 *trans*, 6 *trans*-isomer and 6–5 the 2 *trans*, 6 *cis*-isomer.

Table 16. Summary of results of the analysis of the volatile carbonyl compounds from a sample of fresh butter without defects.

gas chromatography		silver nitrate complex chromatography of DNPH's		organoleptic evaluation after regeneration		ultraviolet spectrophotometry of DNPH's
fraction number[1]	retention temperature °C	fraction number[1]	mobility of fraction[2]	aroma	intensity[3]	
1	58	1-1	I	sharp, fishy	++	[4]
2	58–62	2-1	I	green	+++	A
3	63–70	3-1	I	oily, soapy	++	[4]
5	76–80	5-1	I	fatty	+	A
7	86–91	7-1	I	fruity, soapy	++	[4]
8	91–98	8-1	II	tallowy, cucumbers	+++	D
10	110–117	10-1	I	fruity, soapy	+	B

For footnotes reference is made to Table 15.

Fraction 7–1 was found to contain undecanal DNPH and 2-undecanone DNPH. Therefore a further separation was achieved by zinc carbonate adsorption chromatography according to VAN DUIN (1961).

Fractions 7–5 and 7–6 had the mass spectrum of a 2, 4, 6-nonatrienal DNPH. These two fractions proved to be different components because they had distinctly different mobilities in silver nitrate complex chromatography, and would therefore be expected to be stereo-isomeric 2,4,6-nonatrienals. As fraction 7–5 had an infrared spectrum similar to that of synthetic 2 *trans*, 4 *trans*, 6 *trans*-octatrienal, it could be concluded that this fraction contained 2 *trans*, 4 *trans*, 6 *trans*-nonatrienal.

Fractions 7–7 and 7–8 were found to be decatrienal DNPH's (molecular weight 330). On the basis of ultraviolet spectrophotometry, it could be established that only two double bonds were in conjunction with the carbonyl group. In the mass spectrum, peaks at *m/e* 261 and 214 also indicated the presence of the 2,4-dienal configuration. Breakdown peaks at 287 and 301 indicated the presence of a third double bond at the 6- or 7-position. Because the 6-position must be ruled out on account of the ultraviolet spectrum, it could be concluded that 7–7 and 7–8 were stereo-isomeric 2,4,7-decatrienal DNPH's.

In the analysis fractions with retention indices higher than those of fraction 8 were also observed. These components were not further investigated because their organoleptic contributions were insignificant.

Because 1-octen-3-ol was detected as a strongly odorous compound in autoxidized fatty acids (see Chapter VI) it was of interest to determine whether it was also present in the aroma fraction of trainy butter. For this purpose the

infrared spectro-photometry of DNPH[2]	mass spectrometry of DNPH		results of identification	
	molecular weight	structure	identity of components	quantity µg/kg of butter (p.p.b.)
(a)	266	3-methylbutanal	3-methylbutanal[4]	5
	(264)	1-penten-3-one[4]	1-penten-3-one[4]	1
a	280	hexanal	hexanal	20
a	294	heptanal,	heptanal,	30
		2-heptanone[4]	2-heptanone[4]	5
a	308	octanal	octanal	5
a	322	2-nonanone,	2-nonanone,	25
		nonanal	nonanal	10
b	320	2 *trans*-nonenal	2 *trans*-nonenal	5
a	350	2-undecanone	2-undecanone	20

volatiles from trainy butter were separated by temperature-programmed gas chromatography with silicone oil as the stationary phase, as described previously. The fraction with retention temperature 76–77 °C (retention temperature of 1-octen-3-ol) was collected and further separated by isothermal gas chromatography on a column with diethylene glycol succinate as the stationary phase. A peak was observed with the same retention time as 1-octen-3-ol. This peak had a distinct mushroom-like odour. The quantity of this compound was insufficient to obtain a clear infrared spectrum.

As a result of gas-chromatographic retention time measurements on two types of column and on account of odour evaluation, it could be concluded that a small quantity of 1-octen-3-ol was present in trainy butter. Its quantity was estimated to be approximately 10 μg/kg of trainy butter.

3. Aroma compounds from butter without flavour defects

In order to determine which changes in the content of volatile aroma components are associated with the typical cold-storage defects in butter, it is necessary to make a comparison with the volatile constituents of butter without defects.

For this purpose freshly prepared butter without flavour defects, which was prepared in the summer period from fresh milk (copper content 18 μg per kg of butter) was used. 10 kg of the butter fat was subjected to high-vacuum degassing, and the volatiles obtained were further analysed in the same way as described for trainy butter in Section 2. Several analyses of butter fat from butter without flavour defects were carried out. An example of the results of the qualitative and quantitative analyses is given in Table 16.

The identification of a number of fractions needs some more explanation:

The mass spectrum of 1–1 indicated the presence of 3-methylbutanal DNPH. However, this fraction also contained a small quantity of a DNPH with molecular weight 264, in relation to breakdown peaks 217 and 202. These observations, together with the organoleptic evaluation of this fraction after regeneration (typical fishy odour), make it very tempting to assume that 1–1 did contain a small quantity of 1-penten-3-one DNPH.

Fraction 3–1 was found to be a mixture of an alkanal DNPH and a 2-alkanone DNPH. This fraction was therefore further separated by adsorption chromatography on zinc carbonate.

With fraction 7–1 the same observations were made as for 3–1. This fraction was also further separated by adsorption chromatography on zinc carbonate.

In fresh butter no indications were found of the presence of 1-octen-3-one or 1-octen-3-ol.

CHAPTER VI

IDENTIFICATION OF AROMA COMPOUNDS WHICH ARE FORMED IN THE AUTOXIDATION OF UNSATURATED FATTY ACIDS

1. Introduction

As the carbonyl compounds which contribute to the cold-storage defects in butter are clearly related to the autoxidative breakdown products of unsaturated fatty acids, extensive analyses of the odorous compounds which are formed in the autoxidation of a number of unsaturated fatty acids, were carried out. The results are presented in Tables 17 to 20 and in the explanatory text of the following sections.

2. Aroma compounds which are formed in the autoxidation of oleic acid

A quantity of 1 gram of oleic acid (Hormel Institute, Austin, Minnesota, U.S.A.; purity >99%, checked by gas chromatography) was submitted to autoxidation at 20 °C in a 100 ml round-bottomed flask. The contents of the flask were stirred magnetically. In order to have sufficient oxygen present in the flask for the autoxidation, it was opened occasionally. The autoxidation was continued for a period of time sufficient for an uptake of approximately 0.5 mole of oxygen per mole of fatty acid. The volatile components formed were then isolated by high-vacuum degassing and the aroma compounds were further separated and identified as described in Chapter IV. The results of the qualitative and quantitative analyses are given in Table 17.

3. Aroma compounds which are formed in the autoxidation of linoleic acid

A quantity of 1 gram of linoleic acid (Hormel Institute, Austin, Minnesota, U.S.A.; purity >99%, checked by gas chromatography) was submitted to autoxidation at 20 °C in the same way as described in Section 2 for oleic acid. The autoxidation was continued for a period of time sufficient for an uptake of approximately 0.5 mole of oxygen per mole of fatty acid. The volatile components formed were isolated by high-vacuum degassing and the aroma compounds were further separated and identified as described in Chapter IV. The results of the qualitative and quantitative analyses are given in Table 18. The identification of a number of fractions as presented in Table 18 needs some more explanation.

Table 17. Summary of results of the analysis of the volatile carbonyl compounds from a sample of aut oxidized oleic acid.

gas chromatography		silver nitrate complex chromatography of DNPH's		organoleptic evaluation after regeneration		ultraviolet spectro-photometry of DNPH[2]
fraction number[1]	retention temperature °C	fraction number[1]	mobility of fraction[2]	aroma	intensity[3]	
2	65–69	2–1	I	oily, putty	+++	A
4	78–81	4–1	I	fatty	++	A
6	87–91	6–1	I	tallowy	+++	A
8	99–103	8–1	I	orange peels	++	A
10	107–111	10–1	II	tallowy	++	D
11	117–122	11–1	II	tallowy	++	D

For footnotes reference is made to Table 15.

Table 18. Summary of results of the analysis of the volatile carbonyl compounds from a sample of aut oxidized linoleic acid.

gas chromatography		silver nitrate complex chromatography of DNPH's		organoleptic evaluation after regeneration		ultraviolet spectro-photometry of DNPH[2]
fraction number[1]	retention temperature °C	fraction number[1]	mobility of fraction[2]	aroma	intensity[3]	
1	58	1–1	I	sharp	++	A
2	58	2–1	I	green	++++	A
3	58–69	3–1	I	oily, putty	++	A
4	69–75	4–1	II	fatty, putty	+++	D
5	75–79	5–1	I	fatty, metallic	+++	A
6	79–83	6–1	II	fatty, wallnuts	++++	D
		6–2	IV	fatty, bast	+	A
7	83–86	7–1	II	fatty, woodbugs	+++	D
8	86–91	8–1	I→II	oily	++	B
		8–2	III	washing, cucumbers	++++	A
		8–3	IV	fresh cucumbers	++++	A
		8–4	V	green, cucumbers	+++	[4]
9	91–99	9–1	II	tallowy, cucumbers	+++	D
10	99–108	10–1	II	fatty, washing	+++	D
		10–2	III	fatty, oily	+++	F
11	108–114	11–1	III	frying odour	+++	F
12	114–119	12–1	III	'deep-fried'	+++	F
13	119–129	13–1	V	fatty, fried, cucumbers	++	[4]

For footnotes reference is made to Table 15.

infrared spectro-photometry of DNPH[2]	mass spectrometry of DNPH		results of identification	
	molecular weight	structure	identity of components	quantity µg/g of oxidized fatty acid (p.p.m.)
a	294	heptanal	heptanal	50
a	308	octanal	octanal	320
a	322	nonanal	nonanal	370
a	336	decanal	decanal	80
b	334	2 *trans*-decenal	2 *trans*-decenal	70
b	348	2 *trans*-undecenal	2 *trans*-undecenal	85

infrared spectro-photometry of DNPH[2]	mass spectrometry of DNPH		results of identification	
	molecular weight	structure	identity of components	quantity µg/g of oxidized fatty acid (p.p.m.)
a	266	pentanal	pentanal	55
a	280	hexanal	hexanal	5100
a	294	heptanal	heptanal	50
b	292	2 *trans*-heptenal	2 *trans*-heptenal	450
(a)	308	octanal,	octanal,	45
	(306)	1-octen-3-one[4]	1-octen-3-one[4]	2
a	306	2 *cis*-octenal	2 *cis*-octenal	990
a	306	octenal[4]	non-conjugated *cis*-octenal[4]	25
b	306	2 *trans*-octenal	2 *trans*-octenal	420
[4]		[4]	[4]	25
c	320	3-nonenal	3 *trans*-nonenal	30
a	320	3-nonenal	3 *cis*-nonenal	30
[4]	322	[4]	[4]	540
b	320	2 *trans*-nonenal	2 *trans*-nonenal	30
a	334	2 *cis*-decenal	2 *cis*-decenal	20
e (*t*, *t*)	318	2,4-nonadienal	2 *trans*, 4 *trans*-nonadienal	30
e (*t*, *c*)	332	2,4-decadienal	2 *trans*, 4 *cis*-decadienal	250
e (*t*, *t*)	332	2,4-decadienal	2 *trans*, 4 *trans*-decadienal	150
[4]	336	[4]	[4]	1200

Fraction 5–1 was found to contain mainly octanal DNPH. From the mass spectrum of this fraction, however, it could be concluded that a small quantity of 1-octen-3-one DNPH was also present. This is in accordance with the organoleptic properties (metallic) of this fraction.

Direct organoleptic evaluation of the gas-chromatographic fraction 5 showed a much stronger metallic/mushroom aroma than did the compounds regenerated from the DNPH's. Therefore fraction 5 was also analysed in a different way: after collection, this fraction was again subjected to gas chromatography using diethylene glycol succinate as stationary phase. In this way the fraction was split up into a number of components, one of which had a distinct mushroom odour. On account of its retention time and its infrared spectrum it could be proved that this compound was 1-octen-3-ol. This result confirms the finding of HOFMANN (1962), who also identified this compound in oxidized linoleate. The quantity of 1-octen-3-ol was found to be 430 p.p.m.

Fraction 6–2 could not be fully identified. From the mass spectrum it could be concluded that it was an octenal DNPH. The ultraviolet spectrum indicated that the double bond was not in conjunction with the DNPH-group. The infrared spectrum resembled most that of an alkanal DNPH or a *cis*-alkenal DNPH. From these results it may be concluded that fraction 6–2 contained a non-conjugated *cis*-octenal DNPH. The position of the double bond could not be established, because reference compounds were not available.

Fraction 8–1 could not be identified. Because of impurities the infrared spectrum and the mass spectrum could not be interpreted. This fraction was not studied further, because the odour of the compound(s) regenerated from the DNPH was of minor importance.

Fraction 8–4 showed a mass spectrum of a DNPH with a molecular weight of 322. It could not be nonanal DNPH, because the fragmentation peak at $m/e = 224$ was much too small. From further analysis by high-resolution mass spectrometry the molecular weight was determined (M = 322, 1273). Closest to this came the formula $C_{14}H_{18}N_4O_5$ (M = 322, 1277). This would mean that fraction 8–4 might contain a hydroxyoctenal DNPH. The infrared spectrum also displayed a distinct OH-band. The ultraviolet absorption maximum was at 346 mμ. It is known that α-hydroxyalkanal DNPH's have their absorption maximum in this region.

It is possible that fraction 8–4 contained a hydroxyoctenal DNPH, but this could not be established, because reference compounds were not available.

It should be noted that SCHAUENSTEIN (1967) has found 4-hydroxyoct-2-enal after the autoxidation of methyl linoleate in water emulsion. Because the DNPH of 4-hydroxyoctenal has an ultraviolet absorption maximum at 374 mμ, fraction 8–4 cannot be identical with this compound.

Fraction 13–1 resembled very much fraction 8–4, and hence may be a homologue. On account of its molecular weight it might be a hydroxynonenal DNPH.

4. Aroma compounds which are formed in the autoxidation of linolenic acid

A quantity of 1 gram of linolenic acid (Hormel Institute, Austin, Minnesota, U.S.A.; purity >99%, checked by gas chromatography) was submitted to autoxidation at 20°C in the same way as described in Section 2 for oleic acid. The autoxidation was continued for a period of time sufficient for an uptake of approximately 0.5 mole of oxygen per mole of fatty acid. The volatile components formed were then isolated by high-vacuum degassing and the aroma compounds were further separated as described in Chapter IV. The results of the qualitative and quantitative analyses are given in Table 19. The identification of a number of fractions needs some more explanation.

Fraction 2–1 was identified as 1-penten-3-one DNPH. The mass spectrum showed that a small quantity of 2-butenal DNPH was also present.

Fraction 6–2 was found to have an ultraviolet absorption maximum of an alkanal DNPH. Both infrared and mass spectrometry indicated the presence of acetaldehyde DNPH. This, however, is not in agreement with the retention temperature and the odour of the original compound. This discrepancy calls for further investigation.

Fraction 7–1 was found to be 2 *trans*, 4 *cis*-heptadienal DNPH. This could be confirmed by treatment of the component with 90% sulphuric acid for 24 hours. After this treatment it was found to have the infrared spectrum of the 2 *trans*, 4 *trans*-isomer.

The mass spectrum of fraction 9–1 indicated the presence of an octadienal DNPH. From its ultraviolet spectrum it could be concluded that only one double bond was in conjunction with the carbonyl group. The infrared spectrum did not show *trans*-absorption bands. The breakdown peaks in the mass spectrum at 261 and 275 indicated that the second double bond was in the 4- or 5-position. On account of the ultraviolet spectrum, the second double bond could not be in the 4-position. It must therefore be concluded that fraction 9–1 contained the DNPH of 2 *cis*, 5 *cis*-octadienal.

Fraction 10–1 was found to be 3,5-octadien-2-one DNPH. However, its infrared spectrum did not show the *trans*-bands as pronouncedly as for the 3 *trans*, 5 *trans*-isomer. It may be that 10–1 consisted of a *trans*, *cis*-isomer.

Fraction 11–1 was found to be a nonadienal DNPH on account of its mass spectrum. From the ultraviolet spectrum no indications were found for the 2,x-dienal (x>4) or 2,4-dienal configuration. The infrared spectrum did not show *trans*-bands. Fraction 11–1 could therefore be an x *cis*, y *cis*-nonadienal DNPH (x>2; y>4). Possibly this component is 3 *cis*, 6 *cis*-nonadienal DNPH, which can be expected theoretically (see Table 2).

Fraction 12–1 was found to be 2, 6-nonadienal DNPH on account of its mass spectrum. From the infrared spectrum it could be concluded that 12–1 was 2 *trans*, 6 *cis*-nonadienal DNPH.

Table 19. Summary of results of the analysis of the volatile carbonyl compounds from a sample of autoxidized linolenic acid.

gas chromatography		silver nitrate complex chromatography of DNPH's		organoleptic evaluation after regeneration		ultraviolet spectrophotometry of DNPH[2]
fraction number[1]	retention temperature °C	fraction number[1]	mobility of fraction[2]	aroma	intensity[3]	
2	58	2–1	I→II	sharp, fishy	++++	(C)
3	58	3–1	II	paint, green	++	D
		3–2	II	fatty, green	++	D
4	58–65	4–1	I	green	+	A
		4–2	II	green	++	D
		4–3	III	green leaves	+++	A
		4–4	IV	fresh green leaves	++++	A
6	70–75	6–1	II	fatty	+	D
		6–2	II→III	green	+++	A
7	75–78	7–1	III	frying odour	++	F
8	78–80	8–1	III	fatty, oily	++	F
9	80–83	9–1	V	fatty, melon	+++	D
10	83–86	10–1	III	fruity, fatty	+	E
11	86–90	11–1	V	fresh cucumbers	+++	A
12	90–97	12–1	V	fresh cucumbers	+++	D
13	97–105	13–1	III	fatty, fried	+++	(F)
14	105–115	14–1	V	sliced beans	+++	F
15	115–120	15–1	V	sliced beans	+++	F

For footnotes reference is made to Table 15.

Table 20. Summary of results of the analysis of the volatile carbonyl compounds from a sample of autoxidized arachidonic acid.

gas chromatography		silver nitrate complex chromatography of DNPH's		organoleptic evaluation after regeneration		ultraviolet spectrophotometry of DNPH[2]
fraction number[1]	retention temperature °C	fraction number[1]	mobility of fraction[2]	aroma	intensity[3]	
1	58	1–1	I	green	+++	A
3	71–74	3–2	II	fatty, putty	++	D
4	74–78	4–1	I	metallic	++++	C
		4–2	V	fatty	++	A
5	80–83	5–1	I→II	fatty	++	D
		5–2	II	fatty, wallnuts	+++	D
6	83–85	6–1	II	fatty, woodbugs	+	D
7	87–90	7–1	IV	fresh cucumbers	+++	A
9	97–103	9–1	IV	tallowy	+++	A
11	108–114	11–1	III	frying odour	+++	F
12	114–117	12–1	III	deep-fried	+++	F
13	117–130	13–1	III	fatty, fried	+++	E
14	130–146	14–1	V	raw liver	+++	D
15	146–150	15–1	V	stale eggs	++++	F
16	150–155	16–1	V	stale eggs	++	F

For footnotes reference is made to Table 15.

infrared spectrophotometry of DNPH[2]	mass spectrometry of DNPH		results of identification	
	molecular weight	structure	identity of components	quantity μg/g of oxidized fatty acid (p.p.m.)
(e)	264	1-penten-3-one,	1-penten-3-one[4],	30
	(250)	2 *trans*-butenal[4]	2 *trans*-butenal[4]	10
b	264	2 *trans*-pentenal	2 *trans*-pentenal	35
a	264	2 *cis*-pentenal	2 *cis*-pentenal	45
a	280	hexanal	hexanal	5
b	278	2 *trans*-hexenal	2 *trans*-hexenal	10
c	278	3-hexenal	3 *trans*-hexenal	15
a	278	3-hexenal	3 *cis*-hexenal	90
b	292	2 *trans*-heptenal	2 *trans*-heptenal	5
[4]	224	[4]	[4]	70
e (*t*, *c*)	290	2,4-heptadienal	2 *trans*, 4 *cis*-heptadienal[4]	320
e (*t*, *t*)	290	2,4-heptadienal	2 *trans*, 4 *trans*-heptadienal	70
[4]	304	2,5-octadienal	2 *cis*, 5 *cis*-octadienal[4]	20
e	304	3,5-octadien-2-one	3,5-octadien-2-one[4]	30
a	318	nonadienal	x *cis*, y *cis*-nonadienal, $(x>2, y>4)$[4]	30
[4]	318	2,6-nonadienal	2 *trans*, 6 *cis*-nonadienal[4]	10
(e)	318	2,4-nonadienal[4]	[4]	25
e	330	2,4,7-decatrienal[4]	2,4,7-decatrienal[4]	55
e	330	2.4,7–decatrienal[4]	2,4,7-decatrienal[4]	30

infrared spectrophotometry of DNPH[2]	mass spectrometry of DNPH		results of identification	
	molecular weight	structure	identity of components	quantity μg/g of oxidized fatty acid (p.p.m.)
a	266	pentanal[4],	pentanal[4],	45
	280	hexanal	hexanal[4]	420
b	292	2 *trans*-heptenal	2 *trans*-heptenal	140
e	306	1-octen-3-one	1-octen-3-one	35
[4]	322	[4]	[4]	15
[4]	306	3-octen-2-one	3-octen-2-one[4]	10
a	306	2 *cis*-octenal	2 *cis*-octenal	50
b	306	2 *trans*-octenal	2 *trans*-octenal	15
a	320	3-nonenal	3 *cis*-nonenal	20
a	334	4-decenal[4]	4 *cis*-decenal[4]	30
e (*t*, *c*)	332	2,4-decadienal	2 *trans*, 4 *cis*-decadienal	220
e (*t*, *t*)	332	2,4-decadienal	2 *trans*, 4 *trans*-decadienal	90
e[4]	346	3,5-undecadien-2-one	3,5-undecadien-2-one[4]	65
[4]	360	[4]	2 *trans*, 6 *cis*-dodecadienal[4]	30
[4]	372	[4]	2,4,7-tridecatrienal[4]	150
[4]	[4]	[4]	[4]	75

Fraction 13–1 had several characteristics which resembled those of 2,4-nonadienal DNPH. However, there were slight deviations in the ultraviolet and infrared spectra. Therefore the identification of 13–1 can only be tentative.

Fractions 14–1 and 15–1 were found to have exactly the same characteristics as fractions 7–7 and 7–8 from trainy butter. It could therefore be concluded that 14–1 and 15–1 were stereo-isomeric 2,4,7-decatrienal DNPH's.

From gas-chromatographic analysis, it was indicated that ethanal and butanal were present. Further identification and quantitative determination were not carried out, because these compounds did not contribute significantly to the autoxidation flavour of linolenic acid.

5. Aroma compounds which are formed in the autoxidation of arachidonic acid

A quantity of 1 gram of arachidonic acid (Hormel Institute, Austin, Minnesota, U.S.A.; purity >90%, checked by gas chromatography) was submitted to autoxidation at 20°C in the same way as described in Section 2 for oleic acid. The autoxidation was continued for a period of time sufficient for an uptake of approximately 0.5 mole of oxygen per mole of fatty acid. The volatile components formed were isolated by high-vacuum degassing and the aroma compounds were further separated and identified as described in Chapter IV. The results of the qualitative and quantitative analyses are given in Table 20. The identification of several compounds needs some more explanation.

Fraction 1–1 was found to consist chiefly of hexanal DNPH. From its mass spectrum, however, it could be concluded that it also contained a certain quantity (approximately 10%) of pentanal DNPH.

Fraction 4–2 could not be identified. It did not consist of one single component. The main fraction had a molecular weight of 322, but its mass spectrum was different from that of nonanal DNPH. The characteristics of this component were also different from those of fraction 8–4 from linoleic acid, which was a hydroxyoctenal DNPH. Because of its minor contribution to the flavour of autoxidized arachidonic acid, fraction 4–2 was not investigated further.

Fraction 5–1 had a molecular weight of 306. This might indicate an octenal DNPH. Its characteristics differed from those of 2-octenal DNPH. It was found that 5–1 was identical to 3-octen-2-one DNPH, which was available as a model compound.

Fraction 9–1 consisted of a DNPH with a molecular weight of 334 (decenal DNPH). The compound was found to have the same characteristics as synthetic 4 *trans*-decenal DNPH, except that its infrared spectrum did not show a *trans*-band. Thus 9–1 was identified as 4 *cis*-decenal DNPH.

Fraction 13–1 was found to be identical with synthetic 3 *trans*, 5 *trans*-undecadien-2-one DNPH. The infrared spectrum, however, did not show the *trans*-

bands as pronouncedly as for this DNPH. Fraction 13–1 may therefore have been a *trans, cis*-isomer.

The mass spectrum of fraction 14–1 demonstrated the presence of a dodecadienal DNPH with analogies to 2,6-nonadienal DNPH. It was found to have an ultraviolet spectrum representative of a 2-enal DNPH, or a 2,x-dienal DNPH ($x>4$). The infrared spectrum resembled that of 2 *trans*, 6 *cis*-nonadienal DNPH. Therefore 14–1 may be 2 *trans*, 6 *cis*-dodecadienal DNPH.

The mass spectrum of fraction 15–1 indicated the presence of a tridecatrienal DNPH. From the ultraviolet spectrum, it could be established that only two double bonds were in conjunction with the carbonyl group. In the mass spectrum, peaks at m/e 261 and 214 also indicated the presence of the 2,4-dienal configuration. A breakdown peak at 301 indicated the presence of a third double bond at the 7-position. Thus it may be concluded that 15–1 was a 2,4,7-tridecatrienal DNPH.

Fraction 16–1 was difficult to identify, because it contained impurities. As this fraction had several characteristics in common with fraction 15–1, it may be that 16–1 is an isomer of fraction 15–1.

From odour evaluations of the fractions separated by gas chromatography it could be established that a component with a mushroom odour was present in the volatile fraction from autoxidized arachidonic acid. This compound displayed the same infrared spectrum and gas-chromatographic retention times (on a polar and a non-polar column) as 1-octen-3-ol. It could therefore be concluded that this compound was also present in autoxidized arachidonic acid. The quantity of 1-octen-3-ol was found to be 27 p.p.m.

CHAPTER VII

GENERAL DISCUSSION

1. Introduction

In this final chapter, the results obtained in the present investigations will be discussed and summarized. In the first place it will be considered whether the odorous compounds identified in a number of autoxidized unsaturated fatty acids are in agreement with the components that can be expected from the reaction mechanisms explained in Chapter II. Secondly, the volatile compounds which have been identified in trainy butter will be compared with those formed in the autoxidation of unsaturated fatty acids, in order to determine which fatty acid(s) may play a particular role as precursors in the development of the trainy flavour. Thirdly, it will be discussed how the changes in flavour during deterioration of cold-stored butter (fatty, trainy, tallowy) may be explained.

2. Formation of aroma compounds in the autoxidation of unsaturated fatty acids

2.1 Introduction

Comparisons between the odorous compounds identified in autoxidized unsaturated fatty acids and those which can be predicted by the reaction mechanisms explained in Chapter II are presented in the following sections. A number of the identified compounds correspond with those which are expected from the reaction mechanisms. Others are closely related to the expected compounds and thus their formation is also explicable. Finally, there are a few compounds of which the formation cannot be explained in a simple manner.

2.2 Odorous autoxidation products from oleic acid

A survey is given in Table 21. The formation of octanal, nonanal, and 2 *trans*-decenal is well in line with the reaction mechanisms of autoxidation. The formation of 2 *cis*-undecenal was to be expected, but the 2 *trans*-isomer was identified. During the prolonged autoxidation of oleic acid, the conditions are favourable for isomerization of 2 *cis*-undecenal to 2 *trans*-undecenal.

The formation of heptanal and decanal may be the result of further oxidation or intramolecular rearrangement. Butanal, pentanal and hexanal, as found by Horikx (1965) in the autoxidation of oleic acid at 85 °C, were not found in the present work when a temperature of 20 °C was used.

Table 21. Odorous compounds from autoxidized oleic acid, in comparison with those which can be expected theoretically.

class	compounds expected	compounds identified
alkanals		C_7
	C_8	*C_8
	C_9	*C_9
		C_{10}
2-alkenals	$C_{10}\Delta^{2t}$	*$C_{10}\Delta^{2t}$
	$C_{11}\Delta^{2c}$	
		**$C_{11}\Delta^{2t}$

* Identified compounds which are in accordance with those which can be expected theoretically.
** Identified compounds whose formation can be explained by further isomerization or by other reactions.

Table 22. Odorous compounds from autoxidized linoleic acid, in comparison with those which can be expected theoretically.

class	compounds expected	compounds found	
		identified	tentative
alkanals	C_5	*C_5	
	C_6	*C_6	
		C_7	
		C_8	
2-alkenals	$C_7\Delta^{2t}$	*$C_7\Delta^{2t}$	
		**$C_8\Delta^{2t}$	
	$C_8\Delta^{2c}$	*$C_8\Delta^{2c}$	
		**$C_9\Delta^{2t}$	
		$C_{10}\Delta^{2c}$	
other alkenals			$C_8\Delta^{x}$ $(x>2)$
		**$C_9\Delta^{3t}$	
	$C_9\Delta^{3c}$	*$C_9\Delta^{3c}$	
2,4-alkadienals		$C_9\Delta^{2t,4t}$	
		**$C_{10}\Delta^{2t,4t}$	
	$C_{10}\Delta^{2t,4c}$	*$C_{10}\Delta^{2t,4c}$	
other alkadienals	$C_{11}\Delta^{2c,5c}$		
miscellaneous			hydroxy $C_8\Delta^{x}$
			hydroxy $C_9\Delta^{x}$
		**1-octen-3-one	
		**1-octen-3-ol	

For explanation of symbols reference is made to Table 21.

2.3 Odorous autoxidation products from linoleic acid

A survey is given in Table 22. It appears that the formation of pentanal, hexanal, 2 *trans*-heptenal, 2 *cis*-octenal, 3 *cis*-nonenal and 2 *trans*, 4 *cis*-decadienal is in agreement with the reaction mechanisms of autoxidation. Only 2 *cis*, 5 *cis*-undecadienal was not found.

The formation can also be explained of 2 *trans*-octenal (isomerization of 2 *cis*-octenal), 2 *trans*-nonenal and 3 *trans*-nonenal (isomerization of 3 *cis*-nonenal), 2 *trans*, 4 *trans*-decadienal (isomerization of 2 *trans*, 4 *cis*-decadienal), 1-octen-3-ol and 1-octen-3-one (mechanism of formation explained in Sections 5.4 and 5.5 of Chapter II).

To explain the formation of heptanal, octanal, 2 *trans*, 4 *trans*-nonadienal, a non-conjugated octenal and two hydroxy-alkenals, further investigations are needed.

2.4 Odorous autoxidation products from linolenic acid

A survey is given in Table 23. Here again many of the identified compounds, (ethanal, propanal, 2 *trans*-butenal, 2 *cis*-pentenal, 3 *cis*-hexenal, 2 *trans*, 4 *cis*-heptenal, 2 *cis*, 5 *cis*-octadienal and 2,4,7-decatrienal) are well in accordance with those which are expected from the reaction mechanisms of autoxidation.

The formation can also be explained of 2 *trans*-pentenal (isomerization of 2 *cis*-pentenal), 2 *trans*-hexenal and 3 *trans*-hexenal (isomerization of 3 *cis*-hexenal), 2 *trans*, 4 *trans*-heptadienal (isomerization of 2 *trans*, 4 *cis*-heptadienal), 2 *trans*, 6 *cis*-nonadienal (isomerization of 3 *cis*, 6 *cis*-nonadienal) and 1-penten-3-one (mechanism of formation explained in Section 5.5 of Chapter II).

The detected x,y-nonadienal ($x>2$, $y>4$) may be 3 *cis*, 6 *cis*-nonadienal, which is expected from the reaction mechanisms of autoxidation.

The identification of 3,5-octadien-2-one indicates the presence of a hitherto unknown class of carbonyl compounds which can contribute to oxidation odours. The formation of 3,5-octadien-2-one in the autoxidation of linolenic acid can be explained as follows:

a. Formation of 12 hydroperoxy-9 *cis*, 13 *trans*, 15 *cis*-octadecatrienoic acid as shown in Table 2.

$$CH_3-CH_2-CH\overset{c}{=}CH-CH\overset{t}{=}CH-CH(OOH)-CH_2-CH\overset{c}{=}CH-(CH_2)_7-COOH$$

b. Dismutation of this hydroperoxide to the corresponding keto-acid according to reaction *d* of Figure 4, and further breakdown

$$CH_3-CH_2-CH\overset{c}{=}CH-CH\overset{t}{=}CH-\underset{\underset{O}{\|}}{C}-CH_2-\vdots-CH\overset{c}{=}CH-(CH_2)_7-COOH$$

$$\xrightarrow{H\cdot} CH_3-CH_2-CH\overset{c}{=}CH-CH\overset{t}{=}CH-\underset{\underset{O}{\|}}{C}-CH_3$$

Table 23. Odorous compounds from autoxidized linolenic acid, in comparison with those which can be expected theoretically.

class	compounds expected	compounds found		
		identified	tentative	speculative
alkanals	C_2	*C_2		
	C_3	*C_3		
		C_6		
2-alkenals	$C_4\Delta^{2t}$	*$C_4\Delta^{2t}$		
		**$C_5\Delta^{2t}$		
	$C_5\Delta^{2c}$	*$C_5\Delta^{2c}$		
		**$C_6\Delta^{2t}$		
		$C_7\Delta^{2t}$		
other alkenals		**$C_6\Delta^{3t}$		
	$C_6\Delta^{3c}$	*$C_6\Delta^{3c}$		
2,4-alkadienals		**$C_7\Delta^{2t,4t}$		
	$C_7\Delta^{2t,4c}$	*$C_7\Delta^{2t,4c}$		
			$C_9\Delta^{2,4}$	
other dienals	$C_8\Delta^{2c,5c}$	*$C_8\Delta^{2c,5c}$		
	$C_9\Delta^{3c,6c}$		$C_9\Delta^{x,y}(x>2, y>4)$	*$C_9\Delta^{3c,6c}$
		**$C_9\Delta^{2t,6c}$		
trienals	$C_{10}\Delta^{2t,4c,7c}$	$C_{10}\Delta^{2,4,7}$		*$C_{10}\Delta^{2t,4c,7c}$
		$C_{10}\Delta^{2,4,7}$		**$C_{10}\Delta^{2t,4t,7c}$
miscellaneous		**1-penten-3-one		
		**3,5-octadien-2-one		**$C_8\Delta^{3t,5c}$-2-one

For explanation of symbols reference is made to Table 21.

From this reaction, the 3 *trans*, 5 *cis*-isomer would be expected. Accordingly, the DNPH of 3,5-octadien-2-one isolated from autoxidized linolenic acid showed a weaker *trans*-band in its infrared spectrum than synthetic 3 *trans*, 5 *trans*-octadien-2-one DNPH.

No reference compounds were available to add further proof to the presumption that one of the detected 2,4,7-decatrienals might be the 2 *trans*, 4 *cis*, 7 *cis*-isomer which may be expected from the reaction mechanisms of autoxidation, while the other might be the 2 *trans*, 4 *trans*, 7 *cis*-isomer.

To explain the formation of 2 *trans*-heptenal and 2, 4-nonadienal (tentative), further investigations are needed.

2.5 Odorous autoxidation products from arachidonic acid

A survey is given in Table 24. Many of the identified compounds (pentanal) hexanal, 2 *trans*-heptenal, 2 *cis*-octenal, 3 *cis*-nonenal, 2 *trans*, 4 *cis*-decadienal, are again found to be in accordance with those which are expected from the reaction mechanisms of autoxidation.

The formation can also be explained of 2 *trans*-octenal (isomerization of 2 *cis*-octenal), 2 *trans*, 4 *trans*-decadienal (isomerization of 2 *trans*, 4 *cis*-

decadienal), 2 *trans*, 6 *cis*-dodecadienal (isomerization of 3 *cis*, 6 *cis*-dodecadienal), 1-octen-3-ol and 1-octen-3-one (mechanism of formation explained in Sections 5.4 and 5.5 of Chapter II).

The formation of 3,5-undecadien-2-one in the autoxidation of arachidonic acid can be explained in the same way as has been done for octa-3,5-dien-2-one in the autoxidation of linolenic acid (see preceding section).

The detection of two isomeric 2,4,7-tridecatrienals may indicate the presence of 2 *trans*, 4 *cis*, 7 *cis*-tridecatrienal and 2 *trans*, 4 *trans*, 7 *cis*-tridecatrienal, which are expected from the mechanisms of autoxidation.

Table 24. Odorous compounds from autoxidized arachidonic acid, in comparison to those which can be expected theoretically.

class	compounds expected ($<C_{13}$)	compounds found		
		identified	tentative	speculative
alkanals	C_5	*C_5		
	C_6	*C_6		
2-alkenals	$C_7\Delta^{2t}$	*$C_7\Delta^{2t}$		
		**$C_8\Delta^{2t}$		
	$C_8\Delta^{2c}$	*$C_8\Delta^{2c}$		
other alkenals	$C_9\Delta^{3c}$	*$C_9\Delta^{3c}$		
		$C_{10}\Delta^{4c}$		
2,4-alkadienals		**$C_{10}\Delta^{2t,4t}$		
	$C_{10}\Delta^{2t,4c}$	*$C_{10}\Delta^{2t,4c}$		
other dienals	$C_{11}\Delta^{2c,5c}$			
	$C_{12}\Delta^{3c,6c}$			
		**$C_{12}\Delta^{2t,6c}$		
trienals	$C_{13}\Delta^{2t,4c,7c}$	$C_{13}\Delta^{2,4,7}$		*$C_{13}\Delta^{2t,4c,7c}$
			$C_{13}\Delta^{2,4,7}$	**$C_{13}\Delta^{2t,4t,7c}$
miscellaneous		**1-octen-3-one		
		**1-octen-3-ol		
		3-octen-2-one		
		**3,5-undecadien-2-one		

For explanation of symbols reference is made to Table 21.

The formation of 4 *cis*-decenal and 3-octen-2-one is more difficult to explain, although the structures of these compounds have certain characteristics in common with the structure of arachidonic acid.

It should be noted that autoxidation products with more than thirteen carbon atoms were not isolated by the techniques of degassing used in the present work.

3. Aroma compounds which contribute to the trainy flavour of butter with cold-storage defects

3.1 Autoxidation products from trainy butter compared with those from fatty acids

When comparing the results from the identification of odorous compounds formed in the autoxidation of unsaturated fatty acids with those obtained from the analysis of trainy butter, it is possible to indicate which fatty acids may have acted as precursors to the odorous compounds in trainy butter.

This is an outcome of the present investigations which is of particular interest. To facilitate the making of such a comparison, Table 25 and Figure 10 have been constructed. Here are presented the results of the identifications of odorous compounds from trainy butter, their organoleptic properties and their concentration compared with the flavour threshold values of these compounds. The fatty acids which may have acted as precursors to the formation of the odorous compounds in trainy butter, are also given in Table 25. Only the fatty acids which were studied are mentioned in this Table. It is clear, however, that other fatty acids, with the same alkyl-terminal structure of the carbon chain, may also produce these autoxidation products.

The following conclusions can be drawn from Table 25 and Figure 10:

a. Odorous compounds which are present in trainy butter in quantities higher than their flavour threshold are hexanal; heptanal; 2 *trans*-nonenal; 4 *cis*-heptenal; 2 *trans*, 4 *trans*-heptadienal; 2 *trans*, 4 *cis*-heptadienal; 2 *trans*, 4 *cis*-decadienal; 2 *trans*, 5 *cis*-octadienal (tentative); 2 *trans*, 6 *cis*-nonadienal; 2 *trans*, 4 *trans*, 7 *cis*-decatrienal (tentative); 2 *trans*, 4 *cis*, 7 *cis*-decatrienal (tentative); 3 *trans*, 5 *cis*-octadien-2-one (tentative); 1-octen-3-ol and 1-octen-3-one (tentative).

b. Precursors to the compounds mentioned in *b* are in particular fatty acids with the alkyl-terminal structure of linoleic, linolenic and arachidonic acid. From these precursors, however, it seems that in particular linolenic acid (and fatty acids with the same alkyl-terminal structure) contribute most to the trainy flavour. Not only is the number of odorous compounds which seem to be formed by autoxidative breakdown of linolenic acid large, but also their types of odour and flavour threshold values seem to indicate an important contribution to the trainy flavour.

c. Many of the odorous compounds in trainy butter are present in quantities below their flavour threshold values. However, this does not mean that these components do not contribute to the trainy flavour. From the investigations made by Day, Lillard and Montgomery (1963) it is clear that an additive interaction of compounds at sub-threshold concentrations to give a detect-

Table 25.
Odorous compounds from a sample of trainy butter, in comparison with their possible fatty acid precursors

class	identified	tentative	speculation	flavour
alkanals	C_5			sharp
	C_6			green
	C_7			oily, putty
	C_8			fatty
	C_9			tallowy
	C_{10}			orange peels
	C_{11}			citrus
	C_{12}			fatty, citrus
	3-methyl-C_4			sharp
2-alkanones	C_7			blue cheese
	C_9			blue cheese
	C_{11}			fruity
2-alkenals	$C_5\Delta^{2t}$			green, paint
	$C_6\Delta^{2t}$			green
	$C_7\Delta^{2t}$			putty, fatty
	$C_8\Delta^{2t}$			woodbugs, fatty
	$C_9\Delta^{2t}$			tallowy, cucumbers
	$C_{10}\Delta^{2t}$			tallowy
	$C_{11}\Delta^{2t}$			fatty, tallowy
other alkenals	$C_7\Delta^{4t}$			putty
	$C_7\Delta^{4c}$			creamy, putty
2,4-alkadienals	$C_7\Delta^{2t,4t}$			fatty, fried
	$C_7\Delta^{2t,4c}$			frying odour
	$C_9\Delta^{2t,4t}$			fatty, oily
	$C_{10}\Delta^{2t,4t}$			deep-fried
	$C_{10}\Delta^{2t,4c}$			frying odour
	$C_{11}\Delta^{2t,4t}$			fried, fatty
other alkadienals		$C_8\Delta^{2,5}$	$C_8\Delta^{2t,5c}$	fatty, beans
	$C_9\Delta^{2t,6t}$			cucumbers
	$C_9\Delta^{2t,6c}$			fresh cucumbers
alkatrienals	$C_9\Delta^{2t,4t,6t}$ } [5]			fatty, fishy
	$C_9\Delta^{2,4,6}$ }			fatty, fishy
	$C_{10}\Delta^{2,4,7}$		$C_{10}\Delta^{2t,4t,7c}$	sliced beans
	$C_{10}\Delta^{2,4,7}$		$C_{10}\Delta^{2t,4c,7c}$	sliced beans
miscellaneous	1-penten-3-one			sharp, fishy
	benzaldehyde			weak
	$C_8\Delta^{3,5}$-2-one } [5]		$C_8\Delta^{3t,5t}$-2-one	fatty, fruity
	$C_8\Delta^{3,5}$-2-one }		$C_8\Delta^{3t,5c}$-2-one	fatty, fruity
	$C_{11}\Delta^{3,5}$-2-one			fatty, fried
	1-octen-3-ol			mushroom
		1-octen-3-one		metallic

Legend

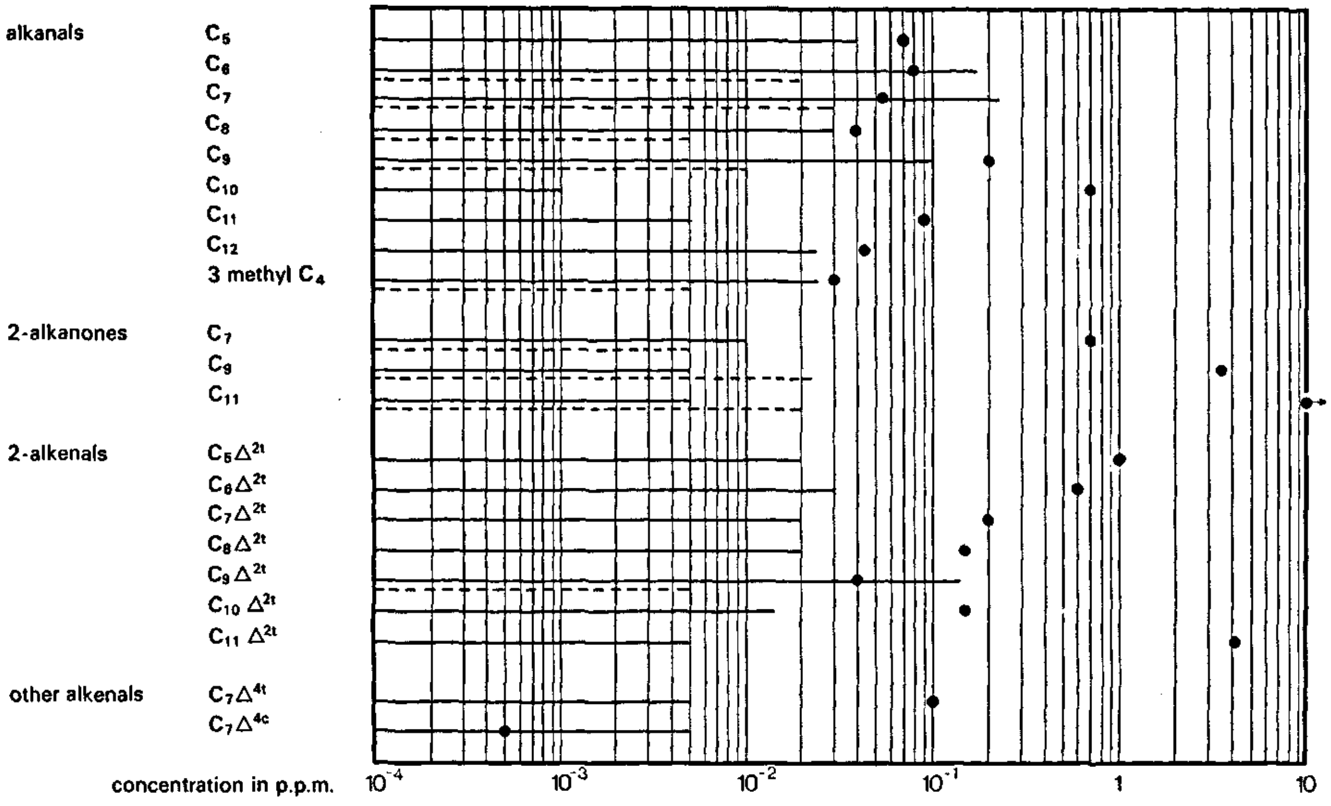
[1] Figures in parentheses indicate approximated values.

[2] Abbreviations: ol = oleic acid; lo = linoleic acid; le = linolenic acid; ar = arachidonic acid.

[3] * Components found after autoxidation of this fatty acid and in accordance with the theory.
** Components found after autoxidation of this fatty acid and in accordance with the theory, but as a result of further isomerization or by other reactions.
Components that are not indicated by an asterisk were found after autoxidation of this fatty acid, without direct theoretical explanation.

quantity in trainy butter p.p.m. (a)	threshold value p.p.m. (b)	quotient[1] $\left(\frac{a}{b}\right)$	fatty acid precursors, examples[2] [3] [4]
0.040	0.07	0.6	*lo *ar
0.180	0.08	2.3	*lo le *ar
0.230	0.055	4.2	ol lo
0.030	0.040	0.8	*ol lo
0.100	0.20	0.5	*ol
0.001	0.70	0.001	ol
0.005	0.09	0.06	
0.025	0.045	0.6	
0.025	0.030	0.8	
0.010	0.7	0.01	
0.005	3.5	0.001	
0.005	15.5	0.0003	
0.020	1.0	0.02	**le
0.030	0.6	0.05	**le
0.020	0.2	0.1	*lo le *ar
0.020	0.15	0.1	**lo **ar
0.130	0.04	3.3	**lo **ar
0.015	0.15	0.1	*ol lo
0.005	4.2	0.001	**ol
0.005	0.10	0.05	**$C_{18}\Delta^{10c,15c}$, $C_{18}\Delta^{11c,15c}$
0.005	0.0005	10	**$C_{18}\Delta^{10c,15c}$, $C_{18}\Delta^{11c,15c}$
0.240	0.10	2.4	**le
0.080	0.04	2.0	*le
0.010	0.46	0.02	lo
0.030	0.10	0.3	**lo **ar
0.045	0.02	2.3	*lo *ar
0.010	–	(<1)	
0.005	(0.002)	(2.5)	*le
0.010	0.020	0.5	**le
0.015	0.0015	10.0	**le
0.020			
0.005			
0.075	(0.038)	(2.0)	**le
0.070	(0.024)	(3.0)	*le
0.001	0.003	0.3	**le
0.005	3.6	0.001	
0.050	0.30	0.2	**le
0.180	(0.2)	(≈1)	**le
0.005	1.6	0.003	**ar
0.010	0.0075	1.3	**lo **ar
0.001	0.0001	10	**lo **ar

[4] $C_{18}\Delta^{10c,15c}$: 10 *cis*, 15 *cis*-octadecadienoic acid.
[5] *cis/trans*-stereo isomers.



alkanals
C_5
C_6
C_7
C_8
C_9
C_{10}
C_{11}
C_{12}
3 methyl C_4
2-alkanones
C_7
C_9
C_{11}
2-alkenals
$C_5\Delta^{2t}$
$C_6\Delta^{2t}$
$C_7\Delta^{2t}$
$C_8\Delta^{2t}$
$C_9\Delta^{2t}$
$C_{10}\Delta^{2t}$
$C_{11}\Delta^{2t}$
other alkenals
$C_7\Delta^{4t}$
$C_7\Delta^{4c}$
concentration in p.p.m.
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
10

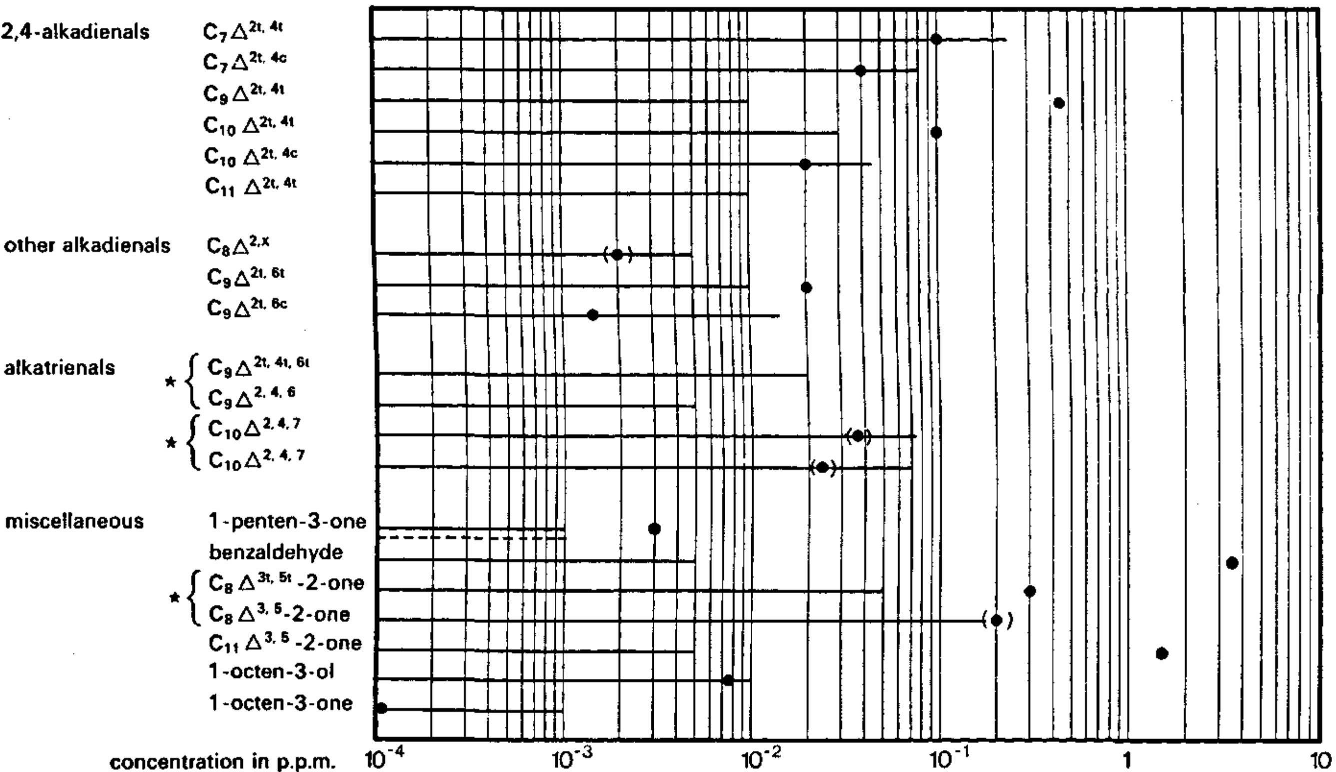


Fig. 10. Results of the qualitative and quantative analyses of odorous compounds in fresh and in trainy butter.

Legend: - - - - - quantity of compound present in fresh butter; (●) approximate flavour threshold value;
——— quantity of compound present in trainy butter; C_n number of carbon atoms of compound;
● flavour threshold value; *) isomers.

able flavour, can occur. Conversely, extinguishing effects may also occur, as was demonstrated by MEYBOOM (1964).

d. It is very remarkable that only 2 *trans*-alkenals occur in trainy butter, and that the 2 *cis*-isomers are absent. It seems likely that, during the prolonged period of cold storage of butter, the more labile 2 *cis*-alkenals are converted into the 2 *trans*-isomers, as was also found in the autoxidation of oleic acid.

e. Comparison of the composition of the mixture of odorous compounds from trainy butter and from fresh butter without defects shows large differences, both qualitative and quantitative (see Figure 10). Most significant is the sharp rise in the quantity of the alkenals, -dienals, -trienals, -enones, -dienones and 1-octen-3-ol listed in Figure 10. Of these classes, only 2 *trans*-nonenal and 1-penten-3-one are present in fresh butter in detectable quantities.

f. Most of the compounds listed in Table 10 are formed as a result of autoxidation processes. Exceptions are, at least, 3-methylbutanal and the 2-alkanones. These compounds occur in fresh and in oxidized butter, but not in autoxidized fatty acids. It is known that 3-methylbutanal is formed by certain lactic streptococci from the starter culture (MACLEOD and MORGAN, 1958). 2-Alkanones are known to be formed by hydrolysis and decarboxylation of esterified β-keto acids which are present in low quantities (about 0.03%) in butter fat (VAN DER VEN *et al.*, 1963). It should be noted that the conditions of degassing were chosen in such a way (temperature $\leqslant 50\,^{\circ}C$) that 2-alkanones could not be formed during the analyses (VAN DUIN, 1965).

In the present studies the class of carbonyl compounds was investigated particularly. Not only does a major pathway in the autoxidative breakdown of unsaturated fatty acids lead to their formation, but in particular these carbonyl compounds often have very low flavour thresholds and thus contribute strongly to oxidation flavour defects.

When unsaturated fatty acids are autoxidized, as described in Chapter VI, and the reaction mixture is subsequently shaken with a solution of 2N sulphuric acid saturated with dinitrophenylhydrazine, the autoxidation odours largely disappear. With linoleic and arachidonic acid a mushroom odour remains, which can be attributed to the presence of 1-octen-3-ol.

The same observation was made with a concentrate of volatiles from cold-stored butter.

3.2 Development of cold-storage defects

The factors which influence the development of oxidation-flavour defects in food lipids have been discussed extensively in Chapter III. These factors will also undoubtedly be responsible for the different modes of the development of oxidation flavours in various milk products, such as butter, milk, milk powder,

buttermilk, etc. The degree of oxidation also has influence on the type of oxidation flavour. For example, cold-storage defects in butter are known to develop in a certain order. In the first stage a metallic or fatty off-flavour can be observed. On prolonged storage the flavour defect changes in the order: oily, trainy and (finally) tallowy.

It may be expected that off-flavours in the first stage are caused mainly by the oxidation of the (highly susceptible) poly-enoic acids. In accordance with this, it was found that a quantity of 2.5 mg of autoxidized arachidonic acid (oxygen uptake approx. 0.5 mol. per mol. acid) added to 100 g of fresh butter, created a distinct fatty-metallic flavour.

To imitate the trainy flavour, various autoxidized fatty acids and mixtures of these were added to fresh butter. Autoxidized linolenic acid in particular gave rise to a distinct trainy flavour (see Chapter III, Section 3). The best imitation of this flavour defect was obtained when 15 mg of autoxidized linolenic acid (oxygen uptake approx. 0.5 mol. per mol. of acid) and 2.5 mg of autoxidized arachidonic acid (oxygen uptake approx. 0.5 mol. per mol. of acid) were added to 100 g of fresh butter.

The tallowy flavour was most difficult to reproduce. It must be expected that in this final stage of the oxidative deterioration of cold-stored butter not only the poly-unsaturated fatty acids but also mono-ene fatty acids (and possibly also saturated fatty acids) will to some extent be involved in the oxidation processes. Mixtures of autoxidized oleic acid, linoleic acid, linolenic acid and arachidonic acid were added to butter fat, but they resulted in flavours mainly reminiscent of candle grease. The best results were obtained by the prolonged autoxidation of a concentrate of unsaturated fatty acids from milk fat, which is in accordance with earlier investigations of RAGHUVEER and HAMMOND (1967). This result indicates that a continued oxidation of many fatty acids is necessary to cause the tallowy flavour of cold-stored butter. In accordance with this, it was found by FORSS *et al.* (1960c) that the total quantity of volatile carbonyl compounds in tallowy butter is about ten times greater than in trainy butter fat and that there is a relative increase in heptanal, octanal, nonanal, 2-heptanone, 2-heptenal and 2-nonenal in tallowy butter fat.

3.3 Conclusion

The investigations described in this thesis have led to the identification of odorous compounds which contribute to the trainy flavour of butter with cold-storage defects. Undoubtedly not all of the flavour compounds have been detected, but the identification of the greater part of the odorous compounds which are essential for the trainy flavour has been achieved.

By comparison of the composition of the mixture of odorous compounds from trainy butter with those from autoxidized fatty acids it has been possible to determine that the autoxidation of unsaturated fatty acids and particularly

the autoxidation of linolenic acid (and fatty acids with the same alkyl-terminal structure) chiefly contribute to the development of a trainy flavour. This could be confirmed experimentally; the addition of autoxidized linolenic acid (with a small quantity of arachidonic acid) to butter without flavour defects produced a trainy flavour.

The analyses of the mixtures of aroma compounds which result from the autoxidation of a number of pure unsaturated fatty acids, have established that the components formed agree well with those that can be expected from the mechanisms of autoxidation of unsaturated fatty acids and the dismutation of the hydroperoxides.

SUMMARY

In this thesis investigations are described of the identification of aroma compounds which are formed as a result of oxidative deterioration of butter during cold storage, producing a typical trainy (fishy)* off-flavour. As these flavour defects are caused chiefly by autoxidative breakdown of unsaturated fatty acids, it was also studied which fatty acids may act as precursors in the formation of these off-flavours. For this purpose the volatile odorous compounds formed in the autoxidation of pure unsaturated fatty acids were identified and compared with the compounds formed in butter with cold-storage defects.

In the introductory chapter (I), a survey is given of results obtained in previous investigations concerning the aroma compounds in butter with cold-storage defects. Aliphatic carbonyl compounds in particular are considered to be responsible for these flavour defects, but these components were isolated only from washed cream and sweet-cream butter, and not from cold-stored butter made from soured cream. In addition, several classes of compounds such as carbonyl compounds with double bonds not in conjunction with the carbonyl group and *cis/trans*-isomers have been overlooked in previous investigations, because of inadequate analytical techniques.

In Chapter II a survey is given of the mechanisms of autoxidation of fatty acids. The mechanisms are discussed of the radical chain reactions leading to the formation of hydroperoxides, and of the dismutation of these compounds to secondary autoxidation products. The latter reactions are of particular interest, as they result in the formation of aroma compounds which may cause flavour defects.

In Table 2 a survey is given of the hydroperoxides and the secondary oxidation products that may be expected in the autoxidation of a number of unsaturated fatty acids, and of those which were actually found in previous investigations.

Chapter III deals with flavour defects observed in autoxidized food lipids in general and cold-storage defects of butter in particular.

First of all, the organoleptic properties and flavour threshold values are given of many secondary autoxidation products (see Table 3) which have very low flavour threshold values and highly repugnant odours.

Secondly, autoxidation experiments are described which indicate the contribution of various unsaturated fatty acids to autoxidation off-flavours.

Thirdly, the generation of oxidation defects in lipid-containing foods, and the effect of several factors on this type of deterioration, are discussed.

* A flavour reminiscent of cod-liver oil.

Finally, special attention is paid to the development of cold-storage defects in butter. The main factors which influence the keeping quality of cold-stored butter are: the extent to which milk, cream and butter are contaminated with copper, and the pH of the butter serum.

It is known that the oxidation processes in butter, which may cause *inter alia* a trainy flavour, start at the fat/serum interface. Experiments were carried out which provided further evidence that the oxidative deterioration of cold-stored butter is due primarily to oxidation of the phospholipids. Further experiments show, however, that 'artificial butter' made from butter fat and milk serum (without fat globule membrane material or phospholipids) may also develop a trainy flavour, provided that a strong pro-oxidant (copper) is present at the lipid/water interface. It must therefore be concluded that it is uncertain whether the off-flavours are formed by oxidation of the unsaturated fatty acids of the phospholipids, or of the butter fat, or even of both.

Chapter IV consists of a description of the techniques which were used for the isolation and identification of volatile flavour compounds in oxidized lipids. The special problems concerning the isolation and separation of volatile flavour compounds are outlined.

For the isolation of volatile flavour compounds in butter fat, a semi-continuous high-vacuum distillation apparatus was designed. Aroma compounds were isolated from small samples of oxidized lipids by means of a batch-wise high-vacuum distillation technique.

The aroma components were first separated by temperature-programmed gas chromatography, using a type of column which minimized the risk of artefact formation.

For further separation and identification, the carbonyl compounds from the fractions obtained by gas chromatography were converted into their DNPH*-derivatives, because the latter have favourable properties for liquid-chromatographic and spectrometric analyses. A special conversion reaction was used to avoid isomerization and artefact formation. A method of silver nitrate complex chromatography was used for the further separation of mixtures of DNPH's according to type and degree of unsaturation.

After these manipulations, the DNPH's were in general separated into the individual compounds. For their further identification ultraviolet, infrared and mass spectrometry were used. Many reference DNPH's have enabled specific characteristics to be determined (absorption wavelengths in ultraviolet and infrared spectrometry, and m/e-values in mass spectrometry).

In a few cases odorous compounds other than carbonyl compounds had to be identified. These compounds were separated by gas chromatography (using a second column, if necessary) and subsequently used for spectrometric analysis.

* Abbreviation for 2,4-dinitrophenylhydrazone.

Figure 9 is a diagram which visualizes the combination of techniques used for identification of volatile compounds obtained from oxidized lipids. It could be established that even very labile compounds such as 2 *cis*-enals, 3 *cis*-enals, etc. were not modified and could be identified correctly by means of this combination of techniques.

In Chapter V the results are presented of investigations on the identification of volatile compounds contributing to the trainy flavour of cold-stored butter. For the sake of comparison, the aroma compounds from fresh butter without flavour defects have also been analysed.

In Chapter VI the results are given of the identification of aroma compounds which are formed in the autoxidation of a number of unsaturated fatty acids.

Many compounds have been identified, which have not been detected previously in butter with oxidation defects or in autoxidized fatty acids.

In Chapter VII the results of the present investigations are discussed and summarized. From the analysis of trainy butter and butter without flavour defects it can be seen that the odorous fraction shows large changes as a result of the oxidative deterioration. Many of the aroma compounds in trainy butter are the same as those found in autoxidized fatty acids.

By comparing the composition of the mixture of odorous compounds from trainy butter with that of compounds from autoxidized fatty acids (see Table 25 and Figure 10) it has been possible to determine that the autoxidation of unsaturated fatty acids and particularly the autoxidation of linolenic acid (and fatty acids with the same alkyl-terminal structure) chiefly contributes to the development of a trainy flavour. This conclusion was confirmed experimentally; the addition of autoxidized linolenic acid (with a small quantity of arachidonic acid) to butter without flavour defects caused a distinctly trainy flavour.

The analyses of aroma compounds which result from the autoxidation of a number of pure unsaturated fatty acids have established that the aroma compounds formed are well in line with those that can be expected from the mechanisms of autoxidation of unsaturated fatty acids and the dismutation of the hydroperoxides (see Tables 21 to 24).

It was established that the changes in flavour in the first stages of oxidative deterioration of cold-stored butter can be ascribed to the rapid oxidation of the highly unsaturated fatty acids, which play the main part in the development of a fatty-metallic off-flavour. The tallowy flavour in the last stage of oxidative deterioration of cold-stored butter can be ascribed to the continued oxidation of several fatty acids.

This thesis, although dealing in particular with cold-storage defects in butter, also presents general information on the development of oxidation flavours in lipid-containing food products.

SAMENVATTING

In dit proefschrift worden onderzoekingen beschreven over de identificatie van aromastoffen die ontstaan bij het oxydatief bederf van koelhuisboter en die een smaakafwijking veroorzaken, die wel met de term tranig* wordt aangegeven. Aangezien bekend is dat deze smaakgebreken in het bijzonder worden veroorzaakt door autoxydatieve afbraak van onverzadigde vetzuren, was het doel van dit onderzoek ook, na te gaan welke vetzuren als precursors voor het ontstaan van deze aromastoffen fungeren. Daartoe werden de vluchtige geurstoffen die bij autoxydatie van zuivere onverzadigde vetzuren ontstaan, geïdentificeerd en vergeleken met de geurstoffen van boter met koelhuisgebreken.

In het inleidende hoofdstuk (I), wordt een overzicht gegeven van de resultaten van vroegere onderzoekingen over de aromastoffen van boter met koelhuisgebreken. Hieruit blijkt dat deze gebreken vooral worden veroorzaakt door alifatische carbonylverbindingen, maar deze conclusie berust alleen op analyses van gewassen room en zoete boter, en niet van koelhuisboter bereid uit gezuurde room. Uit het hier beschreven onderzoek is bovendien gebleken dat in vroegere onderzoekingen bepaalde typen van vluchtige verbindingen, zoals carbonylverbindingen met niet-geconjugeerde dubbele bindingen en *cis/trans*-isomeren, over het hoofd werden gezien ten gevolge van ontoereikende analysemethoden.

In hoofdstuk II wordt een beschouwing gegeven over het mechanisme van de autoxydatie-reacties van vetzuren. Het mechanisme van de radicaal-kettingreacties die tot de vorming van hydroperoxyden leiden en de dismutatie van deze stoffen tot secundaire autoxydatieprodukten, wordt in het kort besproken. Laatstgenoemde reacties zijn van bijzonder belang, omdat zij leiden tot het ontstaan van aromastoffen die smaakgebreken kunnen geven. Tabel 2 geeft een overzicht van de hydroperoxyden en secundaire oxydatieprodukten die op grond van de theorie kunnen worden verwacht bij de autoxydatie van verschillende onverzadigde vetzuren, en die welke bij vroegere onderzoekingen konden worden geïdentificeerd.

Hoofdstuk III heeft betrekking op de smaakgebreken van geoxydeerde voedingsvetten in het algemeen en koelhuisgebreken van boter in het bijzonder.
Allereerst worden de organoleptische eigenschappen en smaakdrempelwaarden besproken van een groot aantal secundaire autoxydatie-produkten

* Deze afwijking doet sterk denken aan de smaak van levertraan.

(zie tabel 3). Vele van deze stoffen vertonen zeer lage smaakdrempelwaarden en bezitten een onaangenaam aroma.

In de tweede plaats worden autoxydatie-experimenten beschreven, waaruit blijkt welke bijdrage verschillende onverzadigde vetzuren tot autoxydatieve smaakgebreken kunnen geven.

In de derde plaats wordt de ontwikkeling van oxydatiegebreken in vethoudende voedingsmiddelen en de invloed die verschillende factoren daarop hebben, besproken.

Ten slotte wordt speciale aandacht besteed aan het ontstaan van koelhuisgebreken in boter. De factoren die de houdbaarheid van boter bij opslag in het koelhuis in hoofdzaak beïnvloeden zijn: de mate waarin melk, room en boter met koper zijn besmet, alsmede de pH van het boterserum.

Het is bekend dat de oxydatie van boter, die o.a. tot een tranige smaak kan leiden, begint in het grensvlak tussen vet en serum. Dat het oxydatief bederf van boter in eerste aanleg het gevolg is van de oxydatie van de fosfolipiden uit deze grenslaag wordt verder ondersteund door enkele uitgevoerde experimenten. Hieruit blijkt echter ook dat in 'kunstboter' die uit botervet en melkserum is bereid, eveneens een tranige smaak kan ontstaan (hoewel geen oppervlaktelaagjes of fosfolipiden aanwezig zijn), mits een sterk pro-oxydant (koper) in het serum aanwezig is. Het is dus niet zeker of de aromastoffen in koelhuisboter worden gevormd uit de onverzadigde vetzuren van de fosfolipiden of van het botervet, of van beide.

In hoofdstuk IV worden de technieken beschreven die voor de isolatie en identificatie van de vluchtige geurstoffen in geoxydeerde lipiden zijn toegepast. De speciale problemen die zich voordoen bij het isoleren en scheiden van vluchtige geurstoffen worden toegelicht.

Voor het isoleren van vluchtige aromastoffen uit botervet werd een semicontinu hoogvacuümdestillatieapparaat geconstrueerd. Voor het isoleren van aromastoffen uit kleine monsters geoxydeerde lipiden werd een batch-gewijze hoogvacuümdestillatietechniek toegepast.

De aromastoffen werden allereerst gescheiden met behulp van door temperatuur geprogrammeerde gaschromatografie, waarbij een zodanig type kolom werd gebruikt dat het risico van artefact-vorming zo gering mogelijk was.

Voor het verder scheiden en identificeren van carbonylverbindingen uit de fracties die werden verkregen bij de gaschromatografie, werden ze eerst in hun DNPH-derivaten* omgezet, omdat deze gunstige eigenschappen hebben voor vloeistofchromatografie en spectrometrische analyses. Een speciale omzettingsreactie werd toegepast om isomerisatie en vorming van artefacten te vermijden. Zilvernitraat-complexchromatografie werd toegepast voor het verder scheiden van de DNPH's naar type en graad van onverzadigdheid.

* DNPH = 2,4-dinitrofenylhydrazon.

Na deze bewerkingen waren de DNPH's meestal gescheiden in de individuele componenten. Voor de verdere identificatie werd gebruik gemaakt van ultraviolet-, infrarood- en massaspectrometrie. Verschillende DNPH-modelstoffen zijn gebruikt voor het bepalen van specifieke karakteristieken (absorptiegolflengten bij UV- en IR-spectrometrie en *m/e*-waarden in massaspectrometrie).
In enkele gevallen was de identificatie van vluchtige geurstoffen die niet behoorden tot de carbonylverbindingen, noodzakelijk. Hiertoe werden deze componenten gezuiverd door gaschromatografie (eventueel na gebruik van een tweede kolom), waarna spectrometrische analyse volgde.
Figuur 9 is een schema dat aangeeft op welke wijze de verschillende technieken voor de identificatie van vluchtige verbindingen uit geoxydeerde lipiden werden gecombineerd. Er kon worden vastgesteld dat zelfs zeer labiele verbindingen zoals 2 *cis*-enalen, 3 *cis*-enalen etc. niet waren veranderd en goed konden worden geïdentificeerd door deze combinatie van technieken.

In hoofdstuk V zijn de resultaten vermeld van de identificaties van vluchtige geurstoffen die bijdragen tot de tranige smaak van koelhuisboter. Ter vergelijking werden ook de vluchtige geurstoffen uit verse boter zonder smaakgebreken geanalyseerd.

In hoofdstuk VI worden de resultaten beschreven van de identificatie van aromastoffen die ontstaan bij autoxydatie van onverzadigde vetzuren.
Vele geurstoffen die nog niet eerder waren gevonden in boter met oxydatiegebreken en in geautoxydeerde vetzuren konden worden geïdentificeerd.

In hoofdstuk VII zijn de resultaten van de onderzoekingen besproken en samengevat. Uit de analyses van tranige boter en van boter zonder smaakgebreken blijkt dat het oxydatief bederf van koelhuisboter leidt tot grote veranderingen in de fractie van de geurstoffen. Vele van de aromastoffen uit tranige boter zijn dezelfde als die welke geïdentificeerd werden in geautoxydeerde vetzuren.

Door de geurstoffen van tranige boter te vergelijken met die van geautoxydeerde vetzuren (zie tabel 25 en figuur 10), was het mogelijk vast te stellen dat de autoxydatie van onverzadigde vetzuren en in het bijzonder linoleenzuur (en vetzuren met dezelfde alkyl-terminale structuur) bijdraagt tot het ontstaan van een tranige smaak. Deze conclusie kon experimenteel worden bevestigd; een toevoeging van geautoxydeerd linoleenzuur (met een weinig arachidonzuur) aan verse boter leidde tot een uitgesproken tranige smaak.
De analyses van aromastoffen die ontstaan bij de autoxydatie van een aantal zuivere, onverzadigde vetzuren, hebben aangetoond dat de gevormde aromastoffen zeer goed overeenkomen met die welke op grond van het mechanisme

van de autoxydatie van onverzadigde vetzuren en de dismutatie van de hydroperoxiden (zie tabellen 21 t/m 24) kunnen worden verwacht.

Vastgesteld kon worden dat vooral de snel oxyderende poly-onverzadigde vetzuren aanleiding geven tot het ontstaan van een vettig-metalige smaakafwijking. Daarentegen zal de talkige smaak van koelhuisboter in het eindstadium van bederf vooral moeten worden toegeschreven aan de voortgaande oxydatie van vele vetzuren. Een en ander kon ook experimenteel worden bevestigd.

Hoewel dit proefschrift in het bijzonder betrekking heeft op koelhuisgebreken in boter geeft het onderzoek ook een algemeen inzicht in de ontwikkeling van oxydatieve smaakgebreken in vethoudende voedingsmiddelen.

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