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# GEL CHROMATOGRAPHIC SEPARATION OF UNSAPONIFIABLE FRACTIONS IN FATS AND OILS

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## SUMMARY

Unsaponifiable compounds from fats and oils were separated by gel chromatography in tetrahydrofuran on S-832 gel with an exclusion limit of 800 molecular weight units. The elution volumes and relative zone velocities of a number of standard compounds were determined, and unsaponifiable fractions of 10 types of edible fats and oils were separated. It was demonstrated that gel chromatography combined with thin-layer chromatography is a suitable method for the effective separation of unsaponifiable fractions in fats and oils.

## INTRODUCTION

The main components of unsaponifiable fractions in fats and oils are sterols, aliphatic and terpenic alcohols, terpenic hydrocarbons, tocopherols, carotenes and related compounds. Before analysis, the unsaponifiable compounds are usually separated by chromatographic methods into simple mixtures or pure compounds. Mordret[1] separated unsaponifiable compounds of vegetable oils on a thin layer of silica gel G into five to eight fractions. Animal fats yield four fractions on a thin layer of alumina: sterols, alcohols, tocopherols and hydrocarbons[2]. According to Karleskind[3], sterols and various types of alcohols can be determined in the unsaponifiable fraction by combining thin-layer chromatography (TLC) with gas chromatography (GC).

The sterol fraction of unsaponifiable fractions can be separated[4] on a thin layer of silica gel G by this procedure, and steroid derivatives that are formed by transformations of sterols during the industrial treatment of fats and oils, particularly bleaching, can also be determined[5,6]. The sterol fraction can, of course, also be analyzed by GC[7,8] or by combined TLC and GC[9]. In a number of cases, the sterols are advantageously transformed in advance into their acetyl derivatives[10,11].

Vitamins such as tocopherols, calciferols, ubiquinone and retinol, dissolved in fat and present in the unsaponifiable fraction, have been successfully separated by TLC on silica gel or alumina and identified by selective detection[12–14].

Adsorption column chromatography has also been used successfully for the

separation of unsaponifiable fractions of fats and oils. The unsaponifiable fraction of milk lipids was separated on a silica gel column[15]; recently, Naudet *et al.*[16] reported that a very good separation was obtained on a column of hydrated Florisil. Similarly, Sawicki[17] separated the unsaponifiable components of rape-seed oil into two hydrocarbon fractions, ketones, tocopherols, two fractions of triterpene alkaloids, sterols and polar components.

The specific properties of gel chromatographic separations according to the sizes and shapes of molecules were used in our earlier work on the quantitative analysis of inhibitors in edible fats and oils[18]. Some of the fractions obtained by gel chromatography contained tocopherols in admixture with monoglycerides, or sterols in admixture with free fatty acids. Owing to the relatively easy identification, attention has been devoted to the separation of unsaponifiable compounds by gel chromatography, thus supplementing the number of chromatographic procedures already in existence.

## EXPERIMENTAL

### *Materials*

The standards used were re-purified by chromatography on a thin layer of silica gel G and their purity was checked by gel chromatography. Toco Red was obtained from DL-$\alpha$-tocopherol by oxidation with iron(III) chloride[19]; the dimeric keto-ether was prepared by oxidation of DL-$\alpha$-tocopherol with potassium hexacyanoferrate (III)[20]. Brown condensation products were prepared by heating the methyl ester of arachidonic acid, oxidized to a peroxide number of 1200 $\mu$val $g^{-1}$, with egg albumin in the ratio 1:10 at 60° for 4 days in the presence of oxygen, extraction of the lipid fraction and isolation of the coloured fraction on a thin layer of silica gel G with chloroform–methanol–acetic acid (70:28:2) as solvent.

### *Methods*

The unsaponifiable compounds were isolated from natural fats and oils by using the light petroleum (boiling range 42–64°) method in a nitrogen atmosphere[21].

Gel chromatography was carried out by elution with tetrahydrofuran at room temperature in five 8 × 1200 mm columns packed with the S-832 gel (Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) under similar conditions and in a similar arrangement to those described in a previous paper[18]. The flow-rate of the solvent was 30 ml/h. A Model R-403 differential refractometer (Waters Ass., Framingham, Mass., U.S.A.) connected in series with a flow UV spectrometer (Czechoslovak Academy of Sciences, Prague) was used as the detector. The responses of the detectors were recorded with a two-pen recorder (Philips PM-8010). The record from the UV detector is slightly shifted to the right compared with that from the differential refractometer, which was regarded as the decisive one, while the UV analyzer played only an auxiliary role. The gel packing was calibrated on the one hand with a mixture of defined styrene oligomers (number of basic structural repeat units $n = 1$–18) and defined ethylene glycol oligomers ($n = 1$–10), and on the other with standards given in Table I. In order to prevent undesirable effects of oxygen on the mixture under investigation and the formation of tetrahydrofuran peroxides, the whole apparatus was kept under an inert atmo-

sphere of pure nitrogen. Before use, tetrahydrofuran was freed from peroxides by shaking with iron(II) sulphate, dried with solid KOH and rectified under nitrogen on a column with 20 theoretical plates. The samples of standards were analyzed immediately after preparation or re-purification, in a similar manner to extracts from natural oils and fats.

## RESULTS AND DISCUSSION

The results of the gel chromatographic experiments with standard samples are summarized in Table I. Tocoquinone and its dimer and trimer are used as examples to demonstrate the very good reproducibility of the elution volume of standard compounds. A thin-layer chromatogram of a mixture of sterols and triterpenic hydrocarbons (Fig. 1) on silica gel G shows the good separation obtained when gel chromatography was unsuccessful. A gel chromatogram of a mixture of tocoquinone oligomers is shown in Fig. 2, and a chromatogram of a mixture of mono-, di- and triacyl glycerols in Fig. 3.

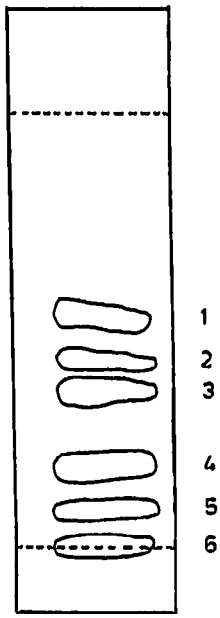
TABLE I

GEL CHROMATOGRAPHY OF STANDARDS

| *Compound* | *$V_e$ (counts)* | *Relative zone velocity, $R = V_0/V_e$ ($V_0 = 47.0$ counts)* |
|---|---|---|
| α-Tocopherol | 61.0 | 0.770 |
| Toco Red | 52.8 52.9 52.3 52.3 | 0.890 0.888 0.899 0.899 |
| Dimer | 49.0 49.0 49.2 49.0 | 0.959 0.959 0.955 0.959 |
| Trimer | 47.0 47.0 47.0 47.7 | 1.000 1.000 1.000 0.985 |
| Fatty acids, $C_{18}$ | 64.4 | 0.730 |
| Monoacylglycerols, $C_{18}$ | 61.2 59.8 | 0.768 0.786 |
| Diacylglycerols, $C_{18}$ | 53.8 | 0.874 |
| Triacylglycerols, $C_{18}$ | 51.5 | 0.913 |
| Cetyl alcohol | 66.1 | 0.711 |
| β-Carotene | 60.1 | 0.782 |
| Retinol | 68.5 | 0.686 |
| Cholesterol | 65.8 | 0.714 |
| β-Sitosterol | 65.0 | 0.723 |
| 3,4-Benzpyrene | 90.3 | 0.520 |

Gel chromatograms of light petroleum extracts of unsaponifiable compounds from vegetable oils (Figs. 4 and 5) and from animal fats (Fig. 6) illustrate the separation of individual groups of compounds according to the sizes of their molecules in a tetrahydrofuran solution.

The main fraction of unsaponifiable compounds (up to 90%) in most fats and oils consists of sterols. As the differences in the sizes of the molecules of the individual sterols are very small, sterols are eluted as a single zone under the experimental conditions used; this zone, however, is clearly broader than those corresponding to pure compounds. For instance, the difference between the elution volumes of cholesterol and β-sitosterol was only 0.8 count (2.2 ml). The triterpenic alcohols, *e.g.*, cycloartenol, which are related to sterols, also had the same elution volumes, but by using chromatography on a thin layer of silica gel G (development with the mixture light



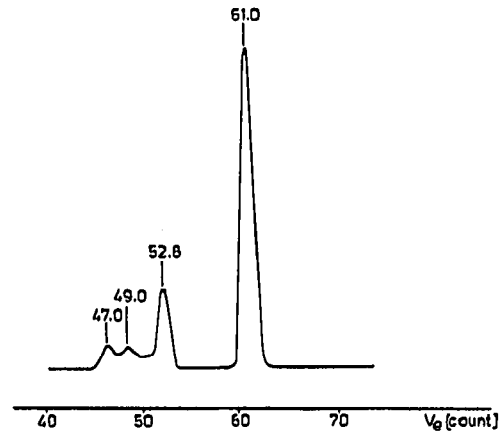




Fig. 1. Thin-layer chromatogram of a mixture of sterols and triterpenic alcohols on silica gel G during elution with the mixture light petroleum–diethyl ether–acetic acid (80:19:1). 1 = Dehydrosterols; 2 = triterpenoic alcohols (unidentified); 3 = triterpenoic alcohols (cycloartenol); 4 = β-sitosterol; 5 = hydroxylated sterol; 6 = unidentified oxidation products.

Fig. 2. Gel chromatogram of tocopherols, recorded with a differential refractometer. Peaks: 61.0, α-tocopherol; 52.8, Toco Red; 49.0, dimer; 47.0, trimer.

petroleum–diethyl ether–acetic acid, 80:19:1), sterols and triterpenic alcohols were successfully separated in unsaponifiable compounds of sunflower-seed oil (Fig. 1). Similarly, 4-methylsterols[22] or ketosteroids[23] could not be separated by gel chromatography. As in TLC[24], free fatty acids in gel permeation chromatographic analysis may interfere with sterols since their elution volumes are not sufficiently different from those of sterols (stearic acid in Table I).

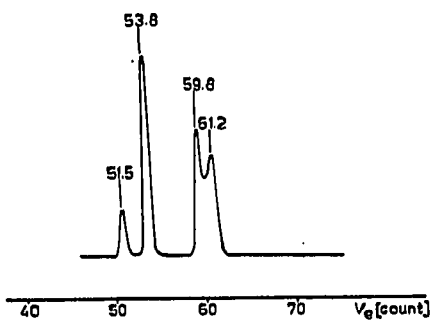



Fig. 3. Gel chromatogram of $C_{18}$ glycerides, recorded with a differential refractometer. Peaks: 61.2 and 59.8, 1- and 2-monoacylglycerol; 53.8, diacylglycerol; 51.5, triacylglycerol.

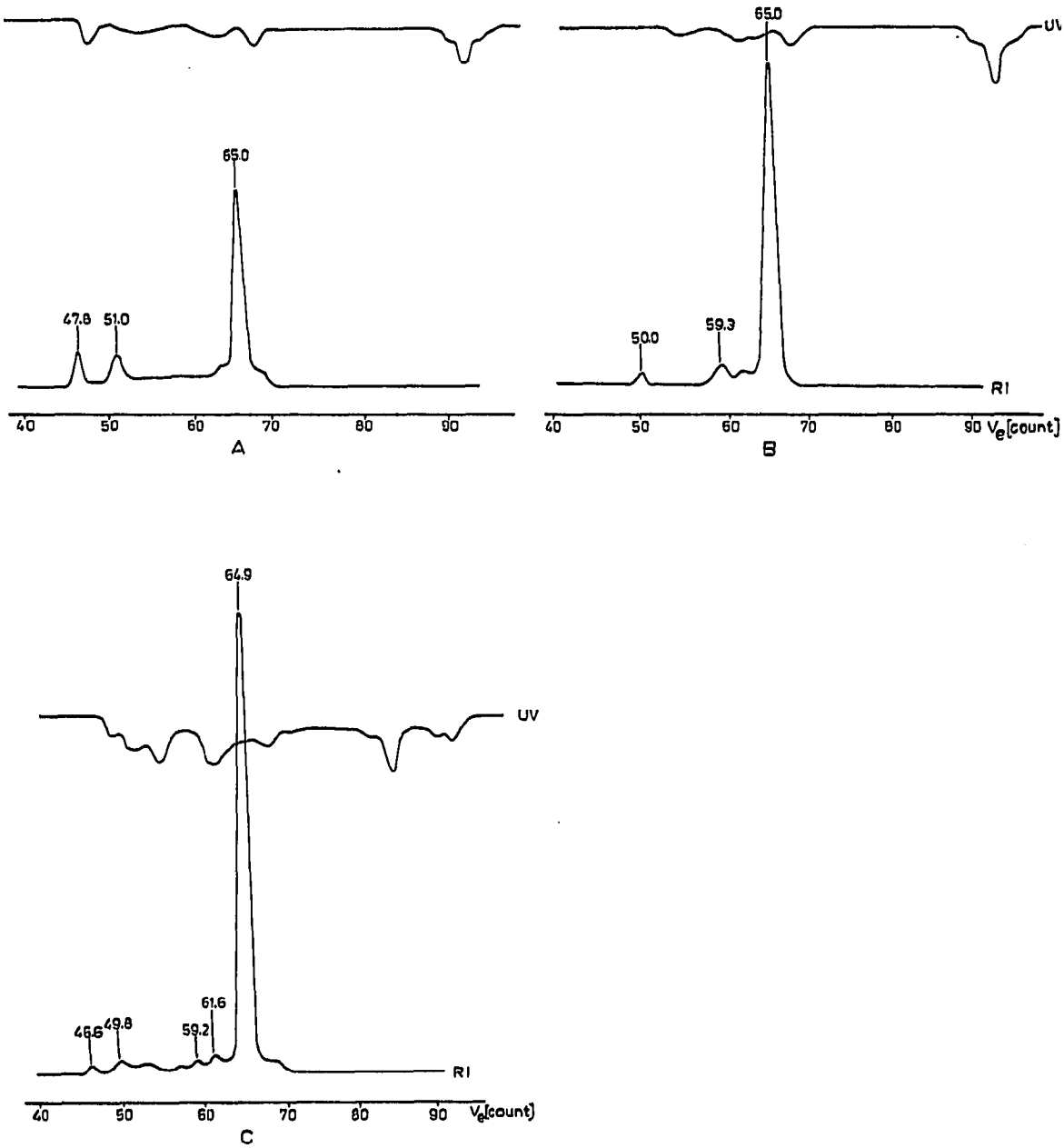



Fig. 4. Gel chromatograms of unsaponifiable fractions of vegetable oils: refractive index (RI), recorded with a differential refractometer; ultraviolet absorbance (UV), recorded with a UV detector at 254 nm. (A) Sunflower-seed oil: 47.8, oligomers of tocopherols, oxidized carotenes; 51.0, contamination of triacylglycerols; 65.0, phytosterols, triterpenic alcohols, alkanols; 90.0 (UV), 3,4-benzpyrene. (B) Rape seed oil: 50.0, tocopherol dimer; 59.3, unidentified compound; 65.0, sterols, triterpenic alcohols; 90.0 (UV), 3,4-benzpyrene. (C) Hydrogenated rape-seed oil: 46.6, oligomers of tocopherols; 49.8, tocopherol dimer; 59.2, unidentified compound; 61.2, tocopherols and their hydrogenated products; 64.9, sterols, triterpenic alcohols.

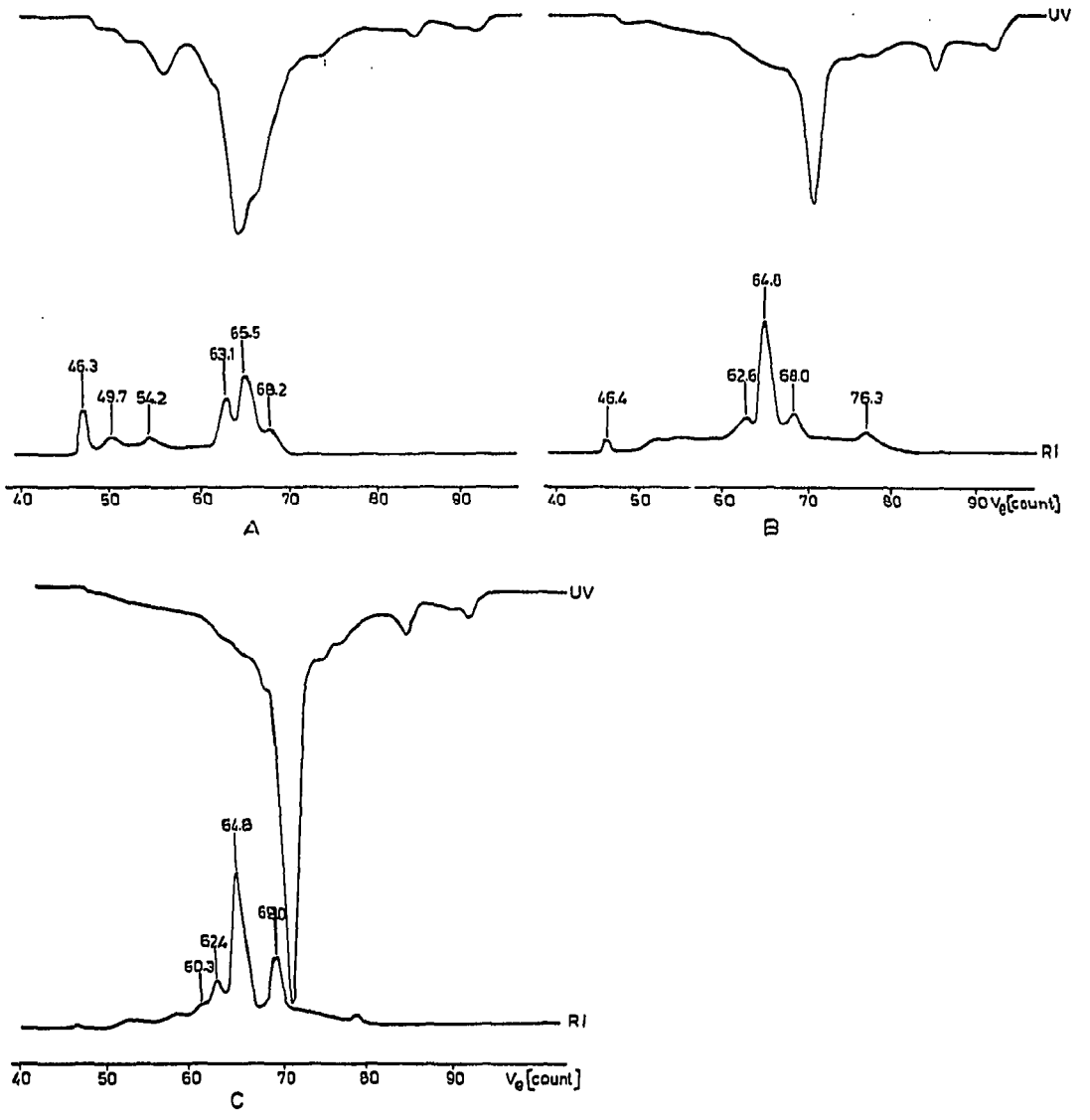



Fig. 5. Gel chromatograms of unsaponifiable fractions of vegetable oils (RI, recorded with a differential refractometer; UV, recorded with a UV detector at 254 nm). (A) Bleached palm oil: 46.3, 49.7 and 54.2, oxidation products of carotenes and tocopherols; 63.1, unidentified compound; 65.5, sterols and triterpenic alcohols; 68.2, probably 2-alkanones. (B) Palm-kernel oil: 46.4, oligomers of tocopherols; 62.6, free fatty acid; 64.8, sterols and triterpenic alcohols; 68.0, probably 2-alkanones; 76.3, unidentified compound. (C) Cocoa butter: 60.3, carotenes; 62.4, free fatty acids; 64.8, sterols and triterpenic alcohols; 69.0, component described in text.

The unsaponifiable compounds usually contain a small amount of aliphatic alcohols with 16–30 carbon atoms and trace amounts of fatty acids, which contaminate the unsaponifiable compounds and which are difficult to separate. Aliphatic alcohols are also difficult to separate from terpenic alcohols by TLC[25]; the separation of these

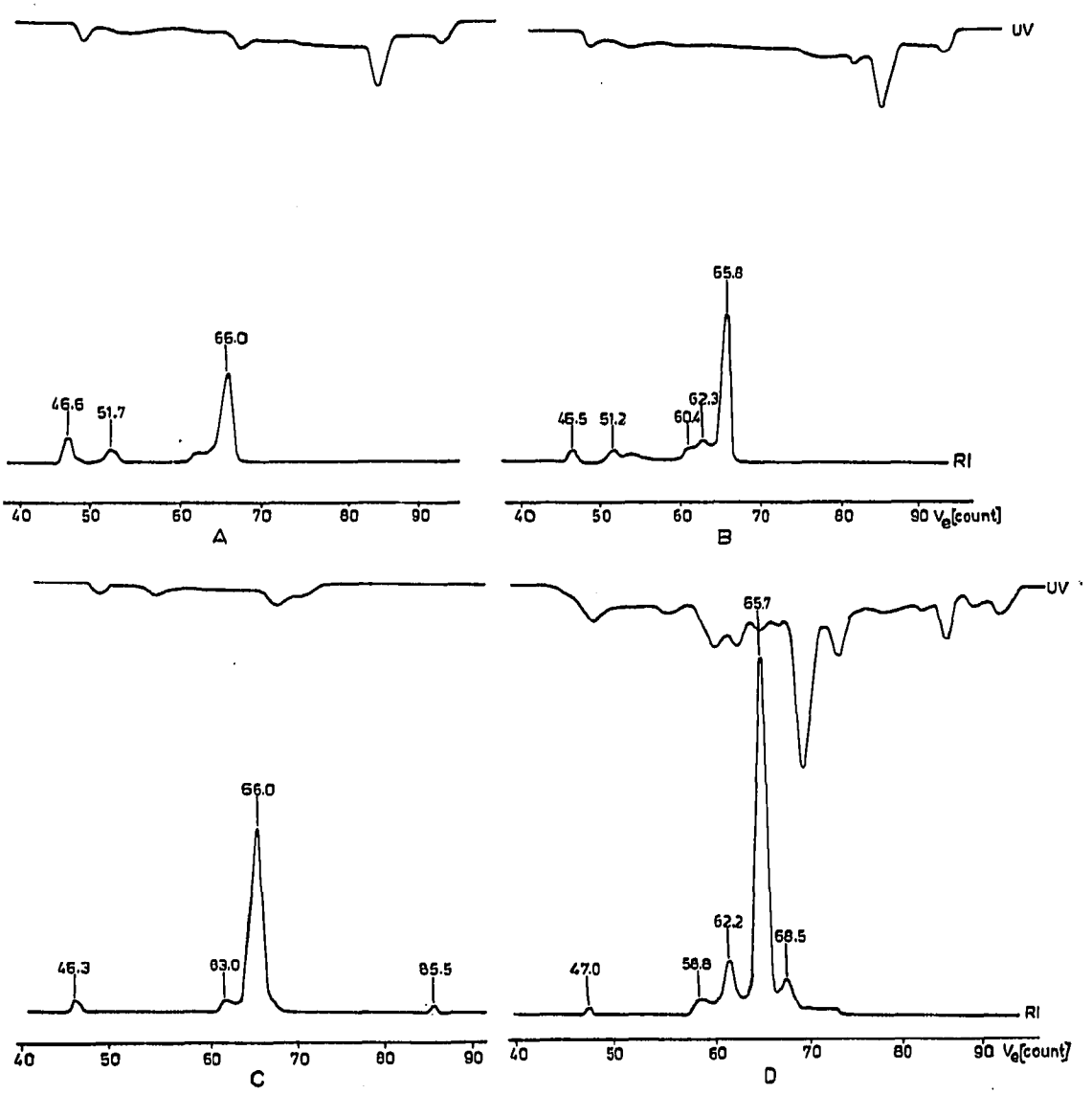



Fig. 6. Gel chromatograms of unsaponifiable fractions of animal fats (RI, recorded with a differential refractometer; UV, recorded with a UV detector at 254 nm). (A) Lard: 46.6, "brown residue" (see text); 51.7, residues of unsaponifiable triacylglycerols; 66.0, cholesterol. (B) Beef talow: 46.5, "Brown residue" (see text); 51.2, residues of unsaponifiable triacylglycerols; 60.4, tocopherol; 62.3, free fatty acids; 65.8, cholesterol. (C) Butter fat: 46.3, polymer components; 63.0, unidentified compound; 66.0, cholesterol; 85.5, components of aromates. (D) Cod liver oil: 47.0, polymer products; 58.8 and 62.2, unidentified products; 65.7, cholesterol; 68.5, retinol.

alcohols by gel permeation chromatography is no more effective (cetyl alcohol, $V_e = 66.1$; $R = 0.711$).

The content of aliphatic hydrocarbons in the unsaponifiable fractions amounts to only a few percent[26–28], so that they are hardly reflected in the chromatograms.

The content of squalene in sunflower-seed or rape-seed oil is only 1–4%[29,30], and in cod-liver oil 3.3%[31]. Under the experimental conditions used, the hydrocarbons will be eluted in the proximity of the elution zone of sterols.

Comparatively important components of unsaponifiable compounds obtained from vegetable oils are tocopherols[32], which are present in the animal fats only in trace amounts[33]. They could not be separated from each other by gel chromatography, as the sizes of their molecules differ only slightly. On the other hand, by the TLC method of Jáky[34] we obtained two or three components from the tocopherol fractions corresponding to the $\alpha$-, $\beta$- + $\gamma$- and $\delta$-tocopherols. Tocotrienols[35] can be expected to be eluted in the same zone. In oils stored for a long time tocopherols undergo oxidation to a certain extent, giving rise to tocoquinones[36], which immediately polymerize to yield dimers and trimers. The oxidation products of tocoquinones can readily be separated by gel chromatography (Fig. 2). As can be seen from the chromatograms, oligomeric products prevail over the content of tocopherols themselves, especially in older samples.

Some vegetable oils, *e.g.*, sunflower-seed or palm-kernel oil, contain insignificant traces of polycyclic aromatic hydrocarbons, mainly 3,4-benzpyrene[37,38], which, owing to the high extinction coefficient, can be detected by using a UV detector. Trace amounts of intensely absorbing components at an elution volume corresponding to 3,4-benzpyrene were detected in unsaponifiable compounds of all fats studied with the exception of butter.

Carotenes are an important component of unsaponifiable compounds of some crude oils (particularly palm oil); however, in refined oils they are usually present in trace amounts only. Carotenes are readily oxidized and polymerized, so that their decomposition occurs mainly during the isolation of unsaponifiable compounds. A characteristic feature of the oxidation products of carotenes (Fig. 7) is their considerably smaller elution volumes; they were detected even in our samples of the unsaponifiable compounds from palm oil, which is characterised by its high natural content of carotenes. In all unsaponifiable fractions of oils and fats, we detected a certain proportion of higher molecular-weight fractions (molecular weight 600–1000), which, in addition to the oxidation products of carotenes and dimers and trimers of tocopherol, also contained some other components. The high-molecular-weight fractions detected in lard and beef tallow are also very interesting; even if carefully treated in an inert atmosphere, they are brown in colour and exhibit an intense UV absorption (Fig. 8). Loury and Forney[39] have described the presence of such components. It is probable that these compounds are condensation products of the oxidation products of lipids with amino acids or with other amino derivatives[40] which have been detected in dry-rendered fat[41,42]. The brown products of the interaction of oxidized lipids with proteins had similar elution characteristics (Fig. 8).

The unsaponifiable compounds of palm-kernel oil contain a low-molecular-weight compound that absorbs strongly in the UV region at 254 nm. As with coconut oil, palm-kernel oil also contains fatty acids with 10–14 carbon atoms, which, by treating them with enzymes, are readily transformed into the corresponding 2-alkanones, mainly 2-undecanone arising from lauric acid[43]. During isolation, these 2-alkanones pass into the unsaponifiable fraction and can be detected after gel chromatographic separation (Fig. 5, $V_e$ = 68.0 counts). The compounds that absorb in the UV region have been detected previously in cocoa butter. They also include alkaloids

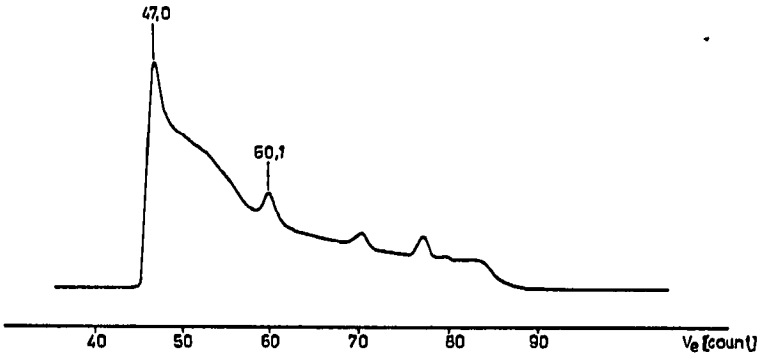



Fig. 7. Gel chromatogram of the oxidation products of carotene, recorded with a UV detector.

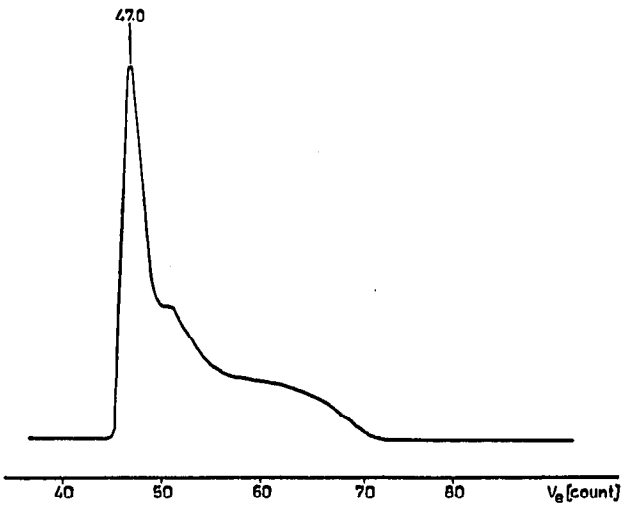



Fig. 8. Gel chromatogram of the "brown residue", recorded with a UV detector.

and vanillin[44]; the high extinction coefficients may be the result of the presence of oxidation products. The absorbing components can be partly removed by extraction with alkali. However, in samples of poorer quality, a certain proportion of these compounds remains in the mixture[45].

Small amounts of triglycerides or partial esters of glycerol can also be present in the unsaponifiable compounds as contaminants. These fractions are reflected in the chromatogram of the unsaponifiable compounds by characteristic zones (Fig. 3).

It can be seen from this study that gel chromatography permits a good separation of compounds present in the unsaponifiable fractions of oils and fats. In most cases, gel chromatography must be combined with TLC, liquid–liquid chromatography or GC in order to achieve complete separation, thus making use of the specific advantages of the individual chromatographic procedures. A perfect gel chromatographic separation of some important fractions (sterols, glycerides, *etc.*) of the mixture can considerably facilitate the subsequent analytical determination by other methods.

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