acid is affected by treatment with other proteins in exactly the same way as the tea metalloprotein (Table IV).

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Tocopherols in the Unsaponifiable Matter of Coffee Bean Oil

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The unsaponifiable matter of coffee oil was fractionated by column chromatography. Thin-layer chromatography of the fractions indicated for the first time the presence of α- and (β + γ)-tocopherol. They were identified by UV, IR, NMR, and mass spectrometry. In oil of green coffee beans of different origin α- and (β + γ)-tocopherol were found to be present in concentrations of 89–191 and 252–465 μg/g of oil, respectively.

The chemical composition of the lipids in green coffee beans has received considerable attention. Several GLC studies on the fatty acid composition have been reported (Carisano and Gariboldi, 1964; Van de Voort and Townsley, 1974; Folstar et al., 1975). In the unsaponifiable matter diterpene alcohols of the kaurene type have been found (Djerassi and Bendas, 1955; Haworth and Johnston, 1956; Djerassi et al., 1959; Wahlberg et al., 1975) and the occurrence of *n*-nonacosane (Neu, 1948) and squalene (Kaufmann and Sen Gupta, 1964) has been established. Moreover, the composition of 4,4-dimethylsterols, 4-methylsterols, and 4-demethylsterols has been studied in detail (Nagasampagi et al., 1971). This paper reports the fractionation of the unsaponifiable matter of coffee oil, the identification of tocopherols, and the determination of the amount of these compounds in different samples of beans.

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EXPERIMENTAL SECTION

Materials. Florisil (60–100 mesh) was obtained from the British Drug Houses Ltd., Poole, England. Commercially prepared silica gel plates and Al_2O_3 for column chromatography as well as 2,2′-bipyridine, squalane, and α-tocopherol were purchased from E. Merck AG, Darmstadt, Germany. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide was from Pierce, Rockford, Ill.

Isolation of the Unsaponifiable Matter. Green coffee beans (180 g) were coarsely ground in an Olland disk crusher. After cooling overnight in the refrigerator the material was ground more finely in a Retsch ZM I centrifugal mill, equipped with a sieve of 0.50-mm screen opening, and extracted for 24 h with petroleum ether (40–60 °C) in a Soxhlet. The solution was kept overnight in the refrigerator whereupon crystallized caffeine was filtered off by suction (0.2 μm membrane filter 11407, Sartorius GmbH, Göttingen, Germany). After evaporation a clear oil was obtained (20 g). According to the AOAC method (1965) the oil was saponified yielding 1.6 g of unsaponifiable matter.

Column Chromatographic Fractionation of the Unsaponifiable Matter. A slurry of 50 g of Florisil

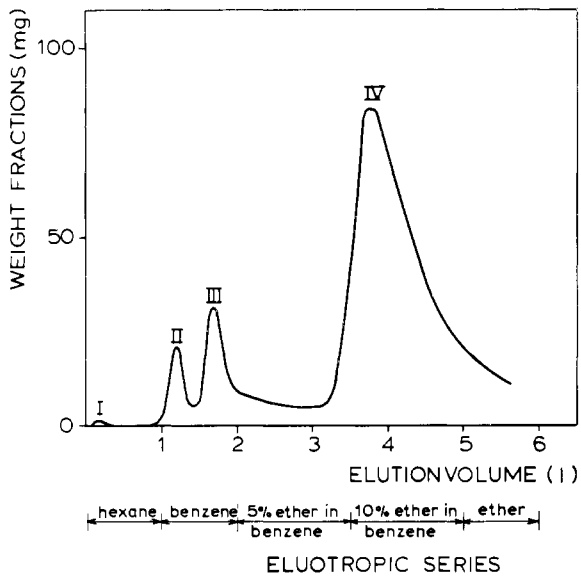

Figure 1. Column chromatographic separation of the unsaponifiable matter of coffee oil.

(60–100 mesh) in hexane was poured into a glass column to provide a bed of 34 × 2 cm. The unsaponifiable matter obtained as described above was separately adsorbed on 1 g of column material using ether and thereupon the solvent-free material was brought on top of the column. Elution was done with an eluotropic series as described in Figure 1. The amounts of material present in the eluates were determined gravimetrically. The fractions were analyzed by TLC using commercially prepared 20 × 20 cm silica gel 60 F254 plates with layer thickness of 0.25 mm.

Spectral Analysis. UV spectra in hexane were run on a Varian Cary 118 and the IR spectra in chloroform were recorded with a Hitachi EPI-G3. The NMR spectra were obtained with a Varian XL-100 in deuteriochloroform with tetramethylsilane as internal standard. The mass spectra were determined with an AEI MS 902.

Quantitative Tocopherol Analysis. In order to prepare an unsaponifiable fraction which is suitable for the determination of the tocopherol content the general procedure of Slover et al. (1969) was followed. This includes extraction of 2 g of finely ground material (<0.50 mm) with ethanol at room temperature, saponification with KOH/ethanol, and recovery of the unsaponifiables with petroleum ether (40–60 °C). Pyrogallol was added during extraction and saponification to minimize oxidation. A solution of the unsaponifiable matter in ether was quantitatively applied across the bottom of a commercially prepared 20 × 20 cm silica gel 60 F254 plate 0.5 mm in thickness together with two spots of a tocopherol rich reference along both sides of the plate. Plates were developed with chloroform twice. After development the sides were sprayed with the 2,2′-bipyridine–$FeCl_3$ reagent (Stahl, 1967), whereupon tocopherols appeared as red spots. Upon examination of the plate under short-wave UV light the corresponding bands were marked and scraped off. After elution with ether and removal of the solvent under N_2, 0.3 ml of an internal standard (13.6 mg of squalane/100 ml of hexane) was added and the solvent was evaporated again. Then derivates were prepared by addition of 150 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide to the mixture in closed reaction vials under N_2.

The sample was analyzed by GLC with a Hewlett-Packard Research Chromatograph 5750G with flame ionization detector coupled with an Infotronics integrator CRS 208. Conditions were: 6 ft × 1/8 in. i.d. stainless steel column packed with 10% UCCW 982 on Chromosorb WAW-DMCS. The oven temperature was 250 °C and the temperature of both injection port and detector was 300 °C. N_2 was used as carrier gas at a flow rate of 20 ml/min and a pressure of 4 kgf/cm^2. Determinations were done in duplicate. Under these circumstances the retention times of squalane, (β + γ)-tocopherol, and α-tocopherol amounted to 6.92, 15.04, and 21.20 min, respectively.

Table I. Mass Spectral Data of Two Isolates

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

For the construction of a standard curve 450 mg of α-tocopherol was purified by chromatography on a 10 × 2.1 cm Al_2O_3 column (activity II–III) eluted with, successively, 200 ml of 4% benzene in petroleum ether (40–60 °C), 200 ml of a 10% mixture, and 200 ml of a 15% mixture. The last 200-ml fraction consisted of pure α-tocopherol as indicated by TLC. Next, a standard solution of 141 mg of purified α-tocopherol in 100 ml of hexane was prepared and a standard curve was constructed using various amounts, within the range of 0.05–0.50 ml of the α-tocopherol solution and a fixed amount of 0.3 ml of the internal standard solution mentioned before. The area ratio was plotted against the weight ratio. It was assumed that detector response was similar for the various tocopherols and that the standard curve based on α-tocopherol could be used for other tocopherols as well.

Percentage Oil. Determination was done following the method of Schweizerisches Lebensmittelbuch (1973). The moisture content was determined as the loss of volatile material upon drying to constant weight at 105 °C.

RESULTS AND DISCUSSION

Fractionation and Identification. The unsaponifiable matter of coffee oil was separated into four fractions by column chromatography as illustrated in Figure 1. The recovery of this fractionation was found to be 89%.

The IR spectrum of fraction I showed absorptions near 2940, 2860, 1470, and 1380 cm^{-1}. Lack of any functional groups indicated that fraction I consists of saturated hydrocarbons.

TLC of fraction II with chloroform as solvent showed three main spots: at R_f 0.42 and 0.28 giving a red color with the 2,2′-bipyridine–$FeCl_3$ reagent which is indicative for tocopherols and at R_f 0.19. Comparison of the retention times and mass spectra with those of authentic samples showed that the spot at R_f 0.19 consists of 4,4-dimethylsterols and 4-methylsterols which have been found in coffee beans before (Nagasampagi et al., 1971).

Using the same techniques in fractions III and IV, 4-demethylsterols and diterpene alcohols, respectively, were found to be present. They have been described in the literature too.

In view of this work the spots in fraction II with R_f 0.42 and 0.28 were isolated in pure form on preparative scale using the TLC system mentioned before. After development the bands were scraped off and eluted from the silica gel with ether.

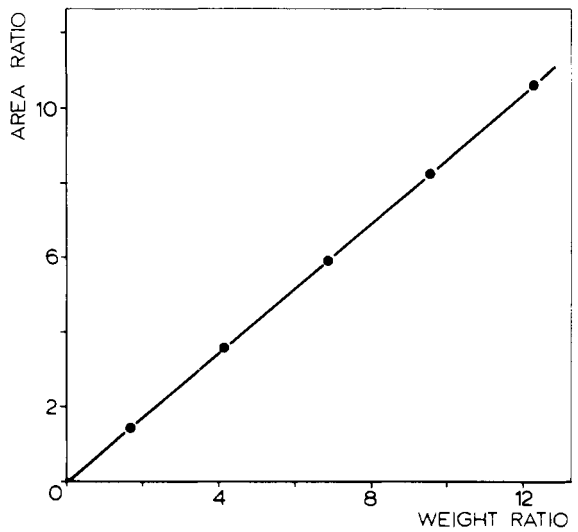

Figure 2. Response linearity of α-tocopherol: area ratio (α-tocopherol/squalane) plotted against weight ratio (α-tocopherol/squalane) with various amounts of α-tocopherol and a fixed amount of squalane.

Mass spectral data are listed in Table I. The base peaks at m/e 430 and 416 constitute the molecular ions of α-tocopherol and (β + γ)-tocopherol, respectively. In agreement with the literature (Scheppele et al., 1972) a side-chain radical ($C_{19}H_{37}$) is split off whereupon mainly tropylium ions of m/e 165 and 151, respectively, are formed. Cleavage of the nonaromatic ring in which no hydrogen transfer is involved results in the formation of m/e 164 and 150.

NMR spectral analysis (in $CDCl_3$) gives signals at δ 0.88 (doublet, broadened, CH_3 side chain), 1.28 (CH_3 at C_2; CH_2 at C_3; CH_2 side chain), 2.12 (CH_3 aromatic ring), and 2.60 (triplet, H at C_4). The spectrum of the compound isolated from the spot at R_f 0.42 was found to be consistent with that reported in the literature for α-tocopherol (Varian, 1962, no. 366). Similar signals were observed for the compound from R_f 0.28 but an additional signal at δ 6.51 (H aromatic ring) was found. The NMR spectrum does not tell us whether β- or γ-tocopherol is present or whether there is a mixture of both compounds.

The IR spectra are identical with those reported in the literature for tocopherols (Green et al., 1959). λ_{max} 297 nm ($\log \epsilon = 3.54$) is consistent with the literature ("Handbook of Chemistry and Physics", 1971).

Quantitative Determination. From the standard curve shown in Figure 2 response linearity was established in the range of the determination. For the determination of the tocopherol concentration in coffee oil samples which differed in species, storage time, geographical origin, and processing conditions were taken. The results are listed in Table II. Since TLC and GLC failed to completely separate β- and γ-tocopherol these compounds were considered as one group as in most tocopherol assays (Christie et al., 1973). The results do not allow conclusions concerning significant differences in tocopherol contents between the samples. Remarkably, the content of (β + γ)-tocopherol exceeds that of α-tocopherol. This has been found in other seed oils like soybean and corn oils also (Juillet, 1975). This result is of particular interest as (β + γ)-tocopherols possess much better antioxidant activity than α-tocopherol (Chow and Draper, 1974).

Table II. Percentage Oil[a] and Tocopherol Concentrations[b] in Oil of Green Coffee Beans of Different Origin

Sample	Oil, %	α-Tocopherol	(β + γ)-Tocopherol
Colombian Arabica, fresh, fermented	15.98	140	465
Colombian Arabica, 5 years old, fermented	15.73	132	355
Santos, 3 years old nonfermented	15.47	89	334
Robusta Ivory Coast 1-2 years old, nonfermented	11.23	191	252

[a] On dry matter. [b] Micrograms of tocopherol/gram of oil.

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