Isolation of Unpleasant Flavor Compounds in the Avocado *(Persea americana)*

Brian I. Brown

Three long-chain C17 aliphatic compounds (a 4-keto-2-hydroxy-1-acetate, a 1,2-dihydroxy-4-acetoxy compound, and a 1,4-dihydroxy-2-acetoxy compound), each with a terminal acetylenic bond, which were isolated from immature avocado seed, flesh, and skin, appeared to be the main constituents of an unpleasant "bitter"-type flavor in each section. The last two compounds are considered to be the principal unpleasant flavor compounds. These compounds were shown to be present in immature fruit from several different avocado varieties, and in commercial samples of crude and rectified fresh oil of unknown origin.

The avocado is one of the few cultivated succulent fruits in which fatty oil is the characteristic and predominant dry constituent (Winton and Winton, 1935), and crude and rectified avocado oils are widely used in the cosmetic industry (Shannon, 1949). The oil content of the avocado has been taken as the most reliable index of maturity, although oil content for satisfactory flesh flavor may vary over a range of 15% or more for a particular variety and, further, the oil content of different varieties may be as low as 10% or as high as 30% for acceptable flavor and soft even consistency in flesh of mature ripened fruit (Hope, 1963).

It is well known that immature avocado flesh has an unpleasant "bitter"-type flavor, which, while not being particularly intense, nevertheless leaves a distinctive, prolonged aftertaste on the palate. In some varieties of avocado, such as Fuerte, Sharwil, and Hass, this unpleasant flavor is undetectable in mature, ripened flesh, while in other varieties, particularly Zutano, the strong unpleasant flavor in the flesh of the immature fruit is also readily detectable as an aftertaste in the flesh of fully mature fruit before and after postharvest ripening.

This apparent decline of the unpleasant flavor with maturity, particularly in varieties such as Fuerte, together with problems of heat-induced off-flavor development in the processing of ripened avocado flesh (Bates, 1970), suggested that if the chemistry of this unpleasant flavor was elucidated, further advances might be made in the maturity assessment and in the processing of the avocado, as well as in the development of improved hybrids, *e.g.*, from Zutano (which is noticeably blemish-resistant) and Fuerte (which is particularly prone to anthracnose).

Weatherby and Sorber (1931) reported a bitter-astringent principle in the avocado seed, and an anise-like odor in the seed from Mexican varieties. Unsuccessful attempts were made by Bilger *et al.* (1932) to isolate the bitter principle. Geissman and Dittmar (1965) isolated a proanthocyanidin of strong astringent flavor from avocado seed.

This paper reports the isolation and identification of compounds contributing to the unpleasant flavor in the Zutano avocado.

EXPERIMENTAL

Melting points were taken in a high-temperature oil bath or on a Yanagimoto micro-melting point apparatus. Optical rotation measurements were carried out using a Perkin-Elmer 141 automatic polarimeter and are of chloroform solutions (0.1–0.2%). Proton magnetic resonance spectra were taken on a Varian A60 spectrometer for 5–10% solutions of $CDCl_3$ with TMS ($\delta = 0$) as an internal standard. Molecular weight determinations were carried out using a Mechrolab 302 V.P.O. with benzene as solvent (0.01 *M* solution). Mass spectra were obtained on an E.A.I. Quadruple 160 mass spectrometer. Infrared spectra were obtained on a Unicam SP 1200 spectrometer. Elemental analyses were performed by the University of Queensland microanalytical laboratory.

Isolation Procedure. The general procedure for the isolation of substances from immature fruit of various varieties, in particular Zutano, is shown in Figure 1. The same procedure was followed in obtaining isolates from commercial samples of crude and rectified flesh oil (A. G. Hersom, Kingston upon Thames, England) of unknown origin.

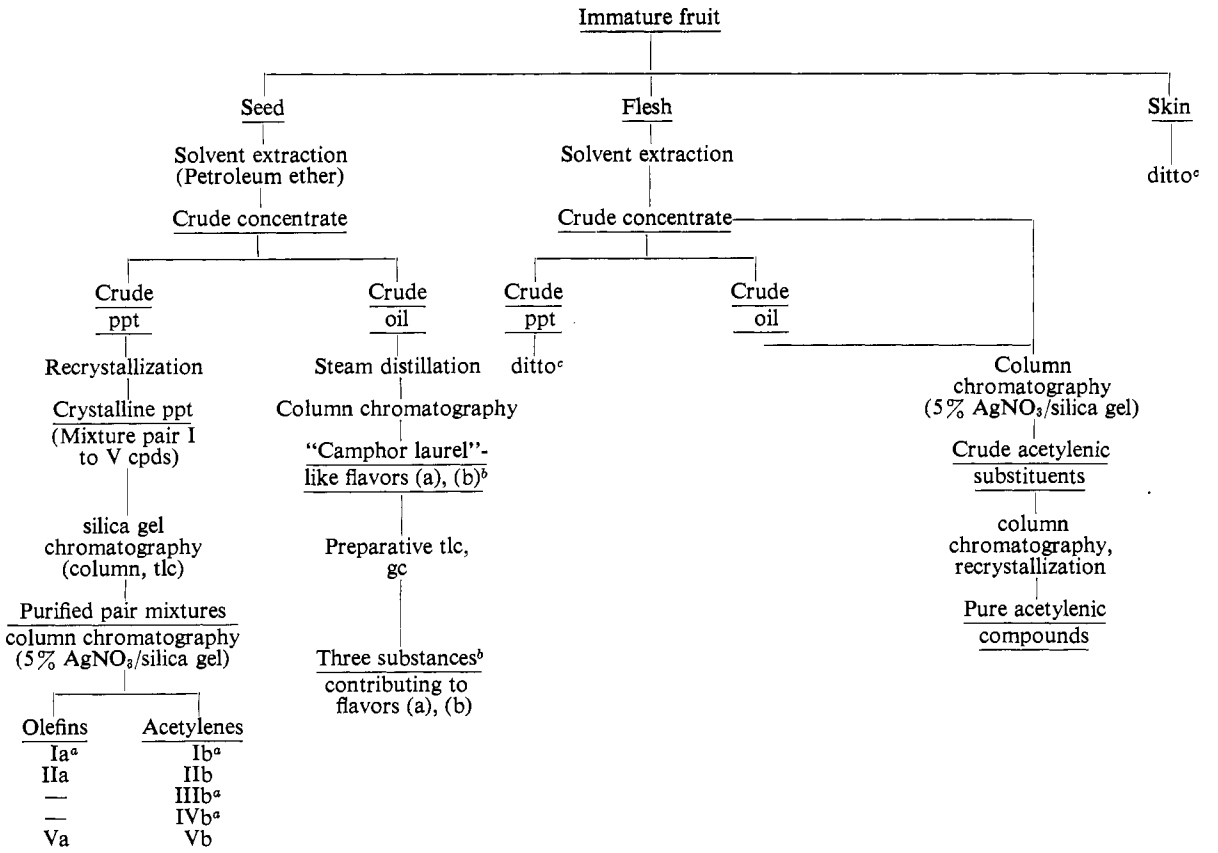
Extraction Procedure. Green immature fruit were sectioned (skin, flesh, seed), deep frozen (−30°C), and held at that temperature until assayed. The frozen material was immersed in petroleum ether (60–65°C), macerated in a Waring blender with three changes of solvent, and the extract vacuum filtered through acid-washed Kieselguhr. The filtrate was concentrated under reduced pressure on a steam bath, and allowed to stand at 5°C for precipitation of crystalline material.

Chromatographic Techniques. TLC. Silica gel (Woelm, Merck) slides were dried at 110°C for at least 2 hr. Approximately 5–10-μl samples were developed on slides in suitable solvents (30% acetone/hexane, 10% acetone/diethyl ether) in closed jars, and the substances visualized by H_2SO_4/anisaldehyde spray.

COLUMN CHROMATOGRAPHY. Florisil columns were used for rapid separation and isolation of pure compounds. Silica gel columns were used for gradient elution of crude samples, in which the sample was taken up in petroleum ether and eluted with petroleum ether containing acetone (3, 6, 9, 12, 14, 16, 20, 50%), and acetone. The olefinic and acetylenic compounds of each pair of substances were separated on 5% $AgNO_3$/silica gel columns eluted with 25% acetone/hexane. The acetylides were released from silica gel into diethyl ether by dropwise addition of 1:5 diluted HCl. (The method of Kashman *et al.* (1969b) was also used for isolation of acetylenes from flesh and skin oils.)

Hydrolysis. Three typical methods of alkaline hydrolysis were used. The sample (0.01 g) was dissolved in MeOH (1 ml) and 5% aqueous NaOH (1 ml), and allowed to stand for 1 hr. The product was taken up in diethyl ether, washed with diluted HCl and distilled water, dried, and the solution evaporated.

Sandy Trout Food Preservation Research Laboratory, Queensland Department of Primary Industries, Brisbane, Queensland, Australia.



[a] Identified as unpleasant flavo r compounds by tasting chromatographic fractions
[b] Identified by tasting chromatographic fractions.
[c] This underwent a process similar to the other two.

Figure 1. Isolation of substances from avocado

The sample (0.1 g) was refluxed for 5 min with MeOH (5 ml) and 5% aqueous NaOH (5 ml).

The sample (0.05 g) was left to stand overnight in the presence of methanolic KOH (2%, 5 ml).

The compound IIb (0.25 g) underwent acid treatment by dissolving it in a solution of toluene sulfonic acid (1% by wt) in MeOH containing 1% H_2O, and heating at 70°C in a water bath for 2 hr.

Hydrogenation. The pair II mixture (0.075 g) was taken up in EtOH (3 ml), Adam's catalyst (0.10 g) was added, and then the mixture was hydrogenated in the usual way. The acetylenic compound IIb (0.115 g) was partially hydrogenated to the olefin IId with the catalyst (0.1 g) of Lindlar and Dubuis (1969) in quinoline (0.5 ml) and acetone (10 ml).

Acetylation. A solution of AcOH (2 ml), cooled in ice with one drop of perchloric acid, was added to the sample (0.015 g) and the reaction mixture was cooled in ice for 3 min. The mixture was treated with 10 ml of ice-cold saturated Na_2CO_3, and worked up in the usual way.

RESULTS AND DISCUSSION

Seed Flavors. After maceration of sliced frozen seed with distilled (60–65°C) petroleum ether in a Waring blender and evaporation of the extract to dryness, the best separation of flavors in the crude extract was obtained by gradient elution. By individual tasting of each chromatographic extract, four distinct flavors were detected: (a) a "chalky" flavor eluted with 3% acetone in petroleum ether; (b) a "camphor laurel" or "citrus-like" odor and flavor, eluted with 6% acetone in petroleum ether; (c) an unpleasant flavor, eluted with 12% acetone in petroleum ether; and (d) an unpleasant flavor, eluted with 16% acetone in petroleum ether.

The presence of these flavors was not authenticated by a replicated taste panel; however, they were all intense enough to be readily detected and characterized in the appropriate chromatographic fractions by several experienced tasters.

Flavors (a) and (b). These volatile flavors were detected in a mixture of terpenoids obtained by steam distillation for 24 hr (27% yield) of the crude petroleum ether extract. Preparative tlc (silica gel, hexane, followed by development of the tlc residue in 10% acetone/hexane) and gas chromatography (Aerograph 200, 100 ft Apiezon M capillary column at 130°C) of the steam volatile oil revealed the presence of three substances which contributed to the flavors (a) and (b). One of these appeared to account for approximately 90% of the total steam volatile oil. However, these substances have not been further characterized.

Flavors (c) and (d). These nonvolatile unpleasant flavors were the principal substances of interest in this chemical investigation. It was noted (Bates, 1970) that apparently similar unpleasant flavor fractions have been reported in heated Fuerte avocado flesh.

By allowing the crude petroleum ether extract to stand overnight at 5°C, a substantial quantity of precipitate formed. After recrystallization from hexane, the precipitate (although varying in composition with different samples of fruit) contained at least ten components with R_f values from approximately 0.75 (component I) to 0.15 (component X). Subsequently it became apparent that these components occurred

in pairs, of which the pairs II and V were major components, pairs I and IV, which had an unpleasant flavor, were present in moderate amounts, and the pair III was a minor constituent.

THE PAIR II COMPONENTS. The pair II components were the most abundant and could be isolated as such from the mixture by recrystallization from hexane. The flavorless pair II mixture, an optically active colorless crystalline substance of mp 62–64°C, was studied in detail first, since it was thought that a knowledge of the identity of the pair II components would help to eludidate the structures of the other eight components. The infrared spectrum of the mixture revealed the presence of hydroxyl (3400, 1139, 1092 cm^{-1}), terminal acetylenic (3260, 2100 cm^{-1}), terminal olefinic (3050, 1630, 990, 905 cm^{-1}), and acetyl (1715, 1265, 1048 cm^{-1}) functional groups, as well as the likelihood of a long-chain aliphatic structure. The pmr spectrum of the mixture revealed peaks at δ 1.3 ($-(CH_2)_n-$), δ 2.0 ($-OCOCH_3$), and a broad triplet at δ 3.95 ($-CH_2OCOCH_3$ and $-CHOH$). However, the pmr spectrum was not sufficiently resolved for further structural identification. Microanalysis and molecular weight determination (mol wt 328) of the mixture corresponded to $C_{19}H_{35}O_4$.

It was deduced that the mixture contained two compounds whose only structural difference appeared to be a terminal double bond in one and a terminal triple bond in the other. Hydrogenation (EtOH, Adam's catalyst) and recrystallization yielded a single product (IIc) of mp 66.0–67.0°C (Found: C, 69.2; H, 11.7. Calcd. for $C_{19}H_{38}O_4$: C, 69.0; H, 11.6). Partial hydrogenation of the pair II mixture (quinoline/acetone, Lindlar catalyst) yielded a single product (IId) of mp 57.5–58.0°C (Found: C, 69.4; H, 11.1. Calcd. for $C_{19}H_{36}O_4$: C, 69.5; H, 11.1). This verified that except for terminal unsaturation, the two compounds in the pair II were identical.

The components of the pair II were then separated on a 5% $AgNO_3$/silica gel column to give an olefinic compound (IIa) of mp 57.5–58.0°C, $[\alpha]^{25}D$ −4.5°C (Found: C, 69.5; H, 11.1. Calcd. for $C_{19}H_{36}O_4$: C, 69.5; H, 11.1), and an acetylenic compound (IIb) of mp 71.5–72.5°C, $[\alpha]^{25}D$ −4.5°C (Found: C, 70.0; H, 10.5. Calcd. for $C_{19}H_{34}O_4$: C, 69.9; H, 10.5). Mixture melting points of IIa and IId verified that they were identical, and the infrared spectra of compounds IIa, IIb, and IId were consistent in every way with the spectrum of the pair II mixture.

Alkaline hydrolysis of IIc and recrystallization of the single reaction product from hexane yielded a compound (IIe) of mp 75.5–76.6°C (Found: C, 70.9; H, 12.7. Calcd. for $C_{17}H_{36}O_3$: C, 70.8; H, 12.6). This, together with infrared and pmr spectra, revealed the presence of an acetyl group in Pair II.

Acetylation of IIa yielded a triacetate as an oil (Found: C, 66.8; H, 9.7. Calcd. for $C_{23}H_{40}O_6$: C, 66.9; H, 9.8). Therefore, it appeared likely that the structures for IIa and IIb were tentatively as indicated in Figure 2.

THE PAIR V COMPONENTS. The components of the colorless and flavorless pair V mixture were separated as their silver salts to give an olefinic compound (Va) of mp 67.5–68.0°C and an acetylenic compound (Vb) of mp 75.5–76.0°C. The infrared spectra of the compounds revealed the presence of a broad hydroxyl band (3350, 1139, 1070 cm^{-1}) but no other functional groups.

Alkaline hydrolysis of the pair II mixture yielded a pair of reaction products, which were separated as their silver salts to yield an olefin (IIf) of mp 67.5–68.0°C (Found: C, 71.0; H, 11.8. Calcd. for $C_{17}H_{34}O_3$: C, 71.3; H, 12.0), and an acetylene (IIg) of mp 75.5–76.0°C (Found: C, 71.9; H, 11.5. Calcd. for $C_{17}H_{32}O_3$: C, 71.8; H, 11.3).

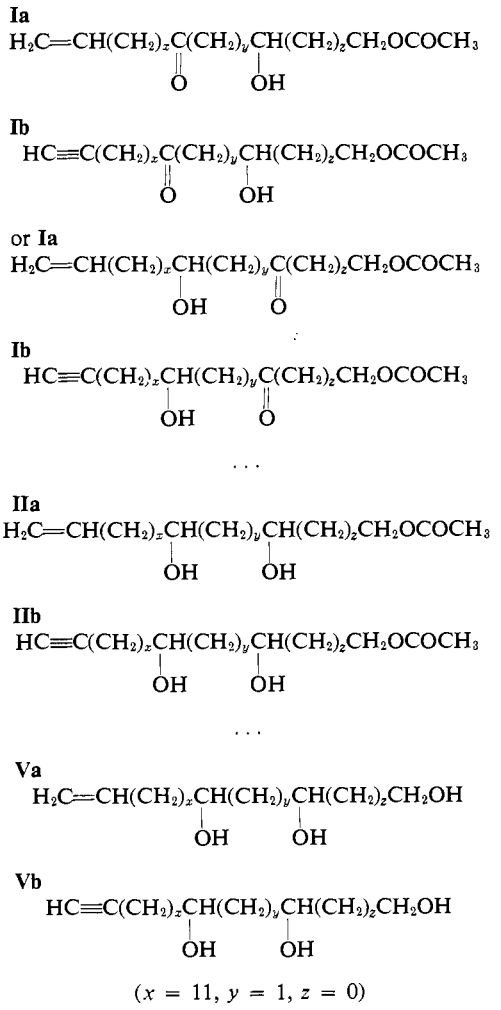


Figure 2. Structures of crystalline compounds from avocado

These two compounds had R_f values on tlc silica gel (30% acetone/hexane) identical with those of the two compounds in the pair V. Infrared spectra and mixture melting points further verified that compounds IIf and IIg were identical with the olefinic (Va) and acetylenic (Vb) compounds respectively from the pair V. Tentative structures for Va and Vb are shown in Figure 2.

THE PAIR I COMPONENTS. By recrystallization of the pair I mixture, colorless crystals, mp 43–44°C, of unpleasant flavor were obtained. The infrared spectrum revealed hydroxyl (3400, 1135, 1085 cm^{-1}), terminal acetylenic (3260, 2100 cm^{-1}), terminal olefinic (3060, 1640, 990, 910 cm^{-1}), acetyl (1735, 1255, 1045 cm^{-1}), and a possible keto (1710 cm^{-1}) functional group. The pmr spectrum of the pair I mixture showed peaks at δ 1.3 ($-CH_2)_n-$), δ 1.5–1.8 ($AcOCCH_2-$), δ 2.0 ($=CHCH_2-$), δ 2.15 (CH_3CO-), δ 3.5 ($-CHOH$), and δ 4.1 ($-CH_2OCOR$). Microanalysis of the mixture corresponded to $C_{19}H_{33}O_4$.

The components of the pair I were then separated as their silver salts. The acetylenic compound (Ib) had a moderately intense unpleasant flavor, while that from the olefin (Ia) was weaker. The compound Ib was hydrolyzed and the infrared spectrum of the product clearly indicated the absence of an acetyl group but presence of a carbonyl band at 1710 cm^{-1}. Tentative structures for compounds Ia and Ib are shown in Figure 2.

THE PLANE STRUCTURES OF THE COMPOUNDS. It was then proposed to define or limit the values of x, y, and z by oxidative degradation of the pair II by the method of Murray (1959), but at this juncture two publications (Kashman *et al.*, 1969a,b) were received, in which a series of long-chain oxygenated compounds in the avocado were reported. From this work it appeared that the compounds in the pairs I, II, and V were identical with substances reported by Kashman *et al.*, in which x = 11, y = 1, z = 0, and the two compounds in the Pair I were presumably identical with compounds (Kashman *et al.*, 1969b) which had the 4-keto structure. A further research paper (Kashman *et al.*, 1970) reported on the configuration of the compounds, which have two asymmetric active centers at C-2 and C-4. The structures of two further pairs of compounds were also reported.

The work of Kashman *et al.* made full use of high-resolution instrumental techniques rather than techniques of chemical degradation, and the incomplete results of the present investigations appeared to be in agreement with their results. It seemed worthwhile to continue with the characterization of these compounds, and to obtain a chemical proof of their plane structure.

The compound IIe (200 mg) was oxidized (Murray, 1959) by refluxing it with excess $KMnO_4$ in acetone, and the neutral and acidic products were separated.

The sole neutral product gave a single gas chromatographic peak (Aerograph 600, 13% B.D.S. at 135°C) which showed precise agreement with that for authentic pentadecanone-2 prepared from myristic acid. The ketone was further verified as being identical with pentadecanone-2 by the melting point and mixture melting point of pentadecanone-2 and the ketone being 38.0–38.5°C (lit. 38.5–39.0°C), the 2,4-dinitrophenylhydrazone derivative from each having melting point and mixture melting point 69.5–70.5°C, and the semicarbazone from each having melting point and mixture melting point 120.0–120.5°C (lit. 120.0–120.5°C).

The acidic products from IIe were methylated with diazomethane in ether for gas chromatography (Aerograph 600, 13% B.D.S. at 135°C), and were shown to consist of myristic acid (C_{14}), with the series of lower homologs present in progressively lower concentrations. Oxidation of myristic acid and methylation of the acidic products for gas chromatography gave a series of methyl esters with precisely the same retention times. In order to produce such an oxidation of IIe, one hydroxy group must have been located at C-4. The second hydroxyl in the chain could then only be located at C-2. Therefore, the plane structure of the compound IIe could only be satisfied by x = 11, y = 1, z = 0, in agreement with Kashman *et al.* (1969b).

THE PAIR III AND PAIR IV COMPONENTS. Investigations were continued on the acetylenic compounds of the pairs III and IV, because the compound IVb was believed to be the principal unpleasant flavor, and Kashman *et al.* did not report at all on flavor in their work. While no detailed sensory evaluation of flavors has been carried out in this investigation, it has been possible to determine those of the pure compounds which have unpleasant flavor and those which are tasteless.

The acetylenic compound IVb of mp 56.0–56.5°C was found to have a stronger unpleasant flavor than compound Ib. The pure olefin (IVa) has not been isolated but it is thought to contribute to the overall unpleasant flavor.

The infrared spectrum of IVb was very similar to that of IIb in having hydroxyl, acetyl, and acetylenic peaks. However, when the spectrum of IVb was taken as a melt, the carbonyl band appeared as a sharp peak at 1710 cm^{-1}, with a shoulder at 1725 cm^{-1}. In Nujol, the band appeared as a split peak (1705, 1720 cm^{-1}), in which either peak could be slightly more intense than the other. This was considered to be indicative of hydrogen bonding between the ester carbonyl and an adjacent hydroxyl group (Jones and Sandorfy, 1956), the degree of bonding being dependent on the physical state of the sample.

The mass spectra of IIb and IVb had no detectable molecular ions, but both had base peaks at *m/e* 43 ($CH_3CO\cdot^+$). A scan of low mass fragments (100 μA, 70–75°C, magnification 0.2, 0–200 mass nos., center mass 100) and high mass fragments (100 μA, 100°C, magnification 2.0, 30–400 mass nos., center mass 200) for IIb and IVb showed that structurally they were indistinguishable by their mass spectra and were consistent with the mass spectrum reported by Kashman *et al.* (1969b) for the compound IIc.

The pmr spectrum of IVb taken on a JEOL MH 100 spectrometer showed peaks at δ 1.25 ($-(CH_2)_n-$), δ 1.75 ($HC\equiv C-$), δ 2.0 ($-OCOCH_3$), δ 2.1 ($\equiv CCH_2-$), δ 3.5 ($-CH_2OH$), δ 3.7 (–C(–OH)H–), and δ 4.92 (–C(–OAC)H–). Apart from the acetylenic absorptions, this spectrum was in agreement with that reported by Kashman *et al.* (1970) for the saturated hydrogenation product of IVb.

The close chemical relationship between compounds IIb, IIIb, and IVb was further demonstrated by the alkaline hydrolysis (KOH/dioxan) of the triacetate from IIb which yielded IIIb, IVb, and Vb, and the acid treatment (TsOH, MeOH) of IIb, which yielded IIIb, IVb, and Vb. During preparative tlc, IVb partially converted to IIIb and IIb in the presence of silica gel and hot acetone. As observed by Kashman *et al.* (1970), IIb partially converted to IIIb and IVb during recrystallization from ethanol. The compound IIIb, a colorless oil of unpleasant flavor, could not be obtained in a pure state free from IIb and IVb. Therefore, compounds IIb, IIIb, and IVb appeared to be interconvertible by transesterification as reported by Kashman *et al.* (1970). The compounds IIIb and IVb, both of unpleasant flavor, appeared to be identical with the 1,4-dihydroxy-2-acetoxy compound X, and the 1,2-dihydroxy-4-acetoxy compound IX (Kashman *et al.*, 1970), respectively. The olefin IIIa may also contribute to the unpleasant flavor but it was not isolated in a pure state to verify this.

By extraction of seed from the immature avocado varieties Fuerte, Rincon, Ryan, and Edranol, it was demonstrated on tlc that this same range of five pairs of compounds was present in all of these varieties.

Flesh Flavors. Investigations on tlc (silica gel, 30% acetone/hexane) and 5% $AgNO_3$/silica gel columns clearly indicated that at least eight acetylenic compounds could be isolated from the crude petroleum ether extract from immature flesh and skin, and five of these had identical R_f values on tlc with the crystalline seed acetylenic compounds.

Gradient elution (silica gel, hexane/acetone solvent) of the acetylenes from the flesh oil extract yielded two fractions of unpleasant flavor. One colorless crystalline fraction was shown by preparative tlc, mixture melting points, and infrared spectra to contain IIb and IVb (as well as IIIb which could not be isolated in a pure state), and the other oily crystalline fraction was shown to contain Ib and IIb. The unpleasant flavors in the two fractions were contributed by Ib, IIIb, and IVb.

By allowing the acetylene-free flesh oil to stand for several days at 5°C, a crude olefinic precipitate was obtained, from which IIa was isolated and characterized.

Tlc examination of the petroleum ether extracts of immature

flesh and skin from the varieties Fuerte, Rincon, Ryan, and Edranol indicated the presence in all extracts of the same range of acetylenic compounds, including Ib, IIIb, and IVb.

Commercial samples (1 lb each) of crude and rectified avocado flesh oil (A.G. Hersom, Kingston upon Thames, England) as used for cosmetic purposes were treated to isolate crude acetylenic fractions (crude oil, 0.3% yield, rectified oil, 0.2% yield). The tlc examination showed that they contained at least eight acetylenic substances, of identical R_f value to those from immature Zutano skin and flesh oil. By preparative tlc, melting points, and infrared spectra, it was demonstrated that the acetylenic fractions from crude and rectified commercial oil contained flavor compounds Ib, IIIb, and IVb. These commercial oils, of unknown origin, were very likely extracted from avocadoes of differing variety and maturity. The flavor compounds Ib, IIIb, and IVb therefore appear to be widespread among avocado varieties, and a relationship may exist between the content of the compounds and maturity of the avocado.

GAS CHROMATOGRAPHY OF THE COMPOUNDS IN THE PAIRS I, II, IV, AND V. Attempts were made to develop a gas chromatographic method for qualitative and quantitative estimation of the compounds. The natural substances, injected as such into various columns (DEGS, SE 30, QFI), decomposed at the oven temperatures required for short retention times. The compounds were then silylated and injected into various columns (SE 30, DEGS, Apiezon M, QFI). Although a single peak of short retention time could be obtained from all of the compounds, none of the columns used was selective enough to differentiate between the derivatives of compounds known to be structurally different. The compounds were apparently too closely related chemically to enable satisfactory separations to be obtained. It might, however, be possible to develop a total estimation of all the compounds, or of the olefinic or acetylenic groups of compounds.

This aspect is currently being investigated further, so that the relationship between content of these compounds and the maturity and processing characteristics of various avocado varieties can be studied.

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Oats and Their Dry-Milled Fractions: Protein Isolation and Properties of Four Varieties

Y. Victor Wu,* Kenneth R. Sexson, James F. Cavins, and George E. Inglett

Four varieties oats were dry-milled into break flour, reduction flour, shorts flour, shorts, bran, and hulls. The three flour fractions have protein contents not much different from those of the whole oats, but the shorts and bran fractions have about double the protein content of whole oats. The 1 *M* NaCl extract accounts for a large percentage of total nitrogen from all fractions, whereas, the 0.1 *N* acetic acid extract represents a major part of the protein from the three flour fractions. Wyndmere whole oats, as well as its break flour, reduction flour, and shorts flour, have high lysine content (4.5–4.8 g/16 g of N) and almost the same amino acid composition. The water-soluble protein has high lysine (8.1 g/16 g of N) and half cystine (5.4 g/16 g of N), and the residue has high lysine and methionine (4.1 g/16 g of N).

Although cereal grains in general have only moderate protein content and are deficient in some essential amino acids, oats have both a high protein content and a good quality protein (Cremer, 1951; Hischke *et al.*, 1968; Jones *et al.*, 1948; Murlin *et al.*, 1938; Robbins *et al.*, 1971; Smuts and Malan, 1938). Practically no work has been done on individual oat protein fractions or dry-milled fractions of oats.

Oat proteins from dry-milled fractions were isolated by solubility methods. Water-, sodium chloride-, ethanol-, acetic acid-, and sodium hydroxide-soluble proteins were obtained. The amino acid composition and the protein content of the mill fractions and the isolated proteins were

Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois 61604.