Liquid Chromatographic Analysis of N^{β}-Alkanoyl-5-hydroxytryptamine (C-5-HT) in Green Coffee Beans

Peter Folstar,* Henk C. van der Plas, Walter Pilnik, Henk A. Schols, and Pim Melger

N^{β}-Alkanoyl-5-hydroxytryptamine (C-5-HT) was isolated from wax of green coffee beans and purified without preceding derivatization by polyamide column chromatography. C-5-HT was separated into four peaks by high-pressure liquid chromatography (LC) on a LiChrosorb 10 RP18 column; mass spectrometric analysis showed that these peaks were identical with four homologues of C-5-HT in which the alkanoyl group is a stearoyl (C_{18}-5-HT), arachidoyl (C_{20}-5-HT), behenoyl (C_{22}-5-HT), and lignoceroyl (C_{24}-5-HT) group. A method was given for the determination of the amount of C_{22}-5-HT in green coffee beans which can serve as control test for the amount of coffee wax present in green beans. The standard deviation of this determination was found to be below 3%; the recovery was 100%. A standard sample of C_{22}-5-HT was obtained from C-5-HT by LC on a preparative column; mass spectra, melting point, molar extinction coefficient, and results of an elementary analysis of the isolated sample of C_{22}-5-HT were found to be identical with those of a synthetic preparation of this compound.

Green coffee beans contain 0.2–0.3% of coffee wax, which is located in the outermost part of the bean (Folstar, 1976). The composition of this wax has received considerable attention (Wurziger and Dickhaut, 1967; Wurziger and Harms, 1969; Folstar et al., 1975). The main constituent was found to be N^{β}-alkanoyl-5-hydroxytryptamine (C-5-HT). Mass spectrometric analysis of C-5-HT indicated the presence of three compounds derived from 5-hydroxytryptamine in which one of the hydrogens of the primary amino group is replaced by an arachidoyl (C_{20}-5-HT), behenoyl (C_{22}-5-HT), or lignoceroyl (C_{24}-5-HT) group (Figure 1). The ratio of these amides was found by mass spectrometry to be 12:12:1, respectively (Harms and Wurziger, 1968). Hunziker and Miserez (1977) found a ratio of 24:68:8 by high-pressure liquid chromatography on a reversed phase column. Recently technical processes have been developed which have in common that the amount of wax in coffee beans is reduced substantially (Vitzthum, 1976). The amount of C-5-HT is used as analytical parameter for the amount of wax present in the beans.

Several methods have been published to determine the amount of C-5-HT. The original procedure, published by Harms and Wurziger (1969a,b), includes extraction of a grind of coffee beans with an organic solvent under reduced pressure, evaporation of the solvent, purification of the residue by column chromatography, and separation of the eluate by circular thin-layer chromatography on a Na_2CO_3-impregnated silica gel G plate. After spraying with Gibbs reagent (0.05% 2,6-dichloroquinonechlorimide in petroleum ether, 40–60 °C), the blue band which results from the reaction between reagent and C-5-HT is scraped off and eluted from the adsorbent using benzene, and the extinction of this solution was measured at 580 nm. Similar procedures have been published by Culmsee (1975), Kummer and Bürgin (1976), Hubert et al. (1977), and Van der Stegen and Noomen (1977). Recently Hunziker and Miserez (1977) published a method based on the analysis of C-5-HT by high-pressure liquid chromatography (LC) using a column with Spherisorb and chloroform (saturated with water) + 1% methanol as mobile phase; detection was carried out by measuring the extinction of the eluate at 278 nm. The amount of C-5-HT is usually determined by extrapolating the amount from a standard response curve. As a standard compound, Kummer and Bürgin (1976) used serotonine as well as C-5-HT isolated from coffee wax by silica gel column chromatography. The authors give, however, no specifications with regard to the purity of C-5-HT. Hubert et al. (1977) isolated C-5-HT from coffee wax after the method of Wurziger and Harms (1969): similar standard response curves were obtained for this sample as well as for a 12:12:1 mixture of three preparations of C_{20}-5-HT, C_{22}-5-HT, and C_{24}-5-HT that were synthesized by the authors. Hunziker and Miserez (1977) used C-5-HT with a melting point of 116 °C that was isolated from coffee wax by use of preparative thin-layer chromatography (Wurziger and Harms, 1969: 120 °C). For their C-5-HT analysis Van der Stegen and Noomen (1977) used a synthetic preparation of C_{22}-5-HT, melting at 123.5–124.5 °C and λ_{max} 278 nm (ϵ 6200) (Hubert et al., 1977: 117–118 °C).

From these data it can be concluded that there is a lack of consistency as far as the choice of the standard compound as well as its purity specifications concerns. Moreover, no evidence was obtained that the standard compound and the compound which is measured were fully identical. In this work it was investigated how far one of the homologues of C-5-HT can serve as analytical parameter for the amount of wax present in coffee beans. Since C_{22}-5-HT is the main homologue, its use as parameter was particularly considered. The work includes a procedure for the isolation of C-5-HT from wax of green coffee beans and its purification without preceding derivatization, as well as methods for the separation of C-5-HT into the homologues by reserved-phase liquid chromatography both on analytical and preparative scale and a description of the purity standards of a C_{22}-5-HT standard compound.

EXPERIMENTAL SECTION

Materials. Polyamide for column chromatography was obtained from Riedel de Haën AG, Seelze-Hannover, Germany. Commercially prepared silica gel plates, Al_2O_3 for column chromatography, and LiChroprep RP8 columns ("Lobar-Fertigsäule", size B) were from Merck AG, Darmstadt, Germany. LiChrosorb 10RP18 columns for LC were purchased from Chrompack BV, Middelburg, The Netherlands. Green coffee beans (Santos coffee) as well as a synthetic preparation of N^{β}-behenoyl-5-hydroxytryptamine were kindly supplied by D.E.J. International Research Comp. B.V., Utrecht, The Netherlands.

Isolation of C-5-HT. Coffee wax was obtained by refluxing and stirring 2 kg of unground green beans with

Laboratory of Food Chemistry (P.F., W.P., H.A.S.) and the Laboratory of Organic Chemistry (H.C.v.d.P., P.M.), Agricultural University, Wageningen, The Netherlands.

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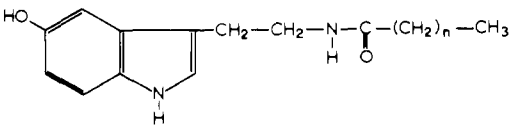

Figure 1. Structure of C_{20}-5-HT (n = 18), C_{22}-5-HT (n = 20), and C_{24}-5-HT (n = 22).

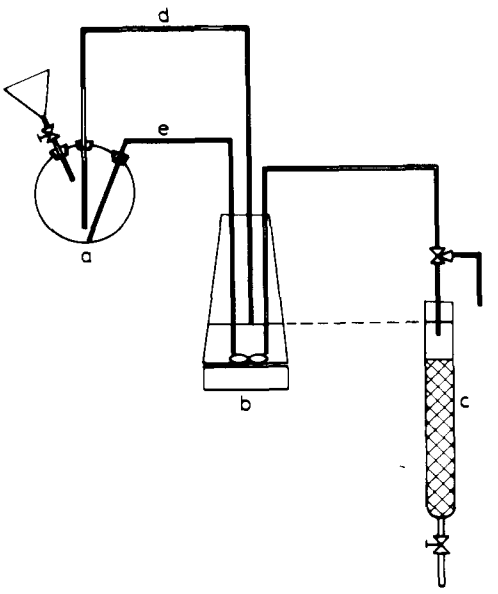

Figure 2. All-glass system for gradient elution with a solvent mixture of continuously increasing eluotropic strength (for explanation of a–e, see text).

chloroform for 30 min. After evaporation of chloroform, the petroleum ether (40–60 °C) soluble substances were extracted from the wax by refluxing with the solvent for four times 15 min each. The residue constitutes 3.2 g of petroleum ether insoluble substances of the wax. These substances were dissolved into 20 mL of a mixture of benzene–methanol (1:1) and 3.2 g of polyamide was added to this solution. Next the solvent was evaporated in a rotating vacuum evaporator and the polyamide with adsorbed material was brought on top of a polyamide column. The column was prepared by pouring a slurry of 125 g of polyamide in benzene into a glass column which provided a bed of 30 × 4.2 cm; the column was eluted with 500 mL of benzene which was continuously mixed with successively 4 L of ether, 2 L of ether–acetone (3:1), 2 L of ether–acetone (2:1), 2 L of ether–acetone (1:1), 5 L of acetone, 2 L of acetone–ethanol (19:1), and 2 L of ethanol using the system in Figure 2.

The apparatus is a simplified form of the gradient mixer described by Wurziger and Dickhaut (1967). Benzene (500 mL) is put in a 1-L flask (b), which is connected with the column (c) by a siphon system. (a) is a closed system equipped with ground-glass joints. When the column is eluted the solvent in b and c drops below the end of the air-inlet tube d of a. Thereupon the more polar solvent, which was previously introduced into a, streams from a into b through e until the original level is reached again. The solvents are continuously mixed in b with a magnetic stirrer. In this way an elution gradient with continuously increasing eluotropic strength was obtained. The amounts of material present in the eluate were determined gravimetrically, collecting fractions of 250 mL.

High-Pressure Liquid Chromatography of C-5-HT. On analytical scale C-5-HT was separated into the homologues by LC using a Spectra Physics 3500B liquid chromatograph equipped with UV vis 770 variable wavelength detector. Conditions: 10 μL of 0.1% of C-5-HT in methanol was injected on a 25 × 0.46 cm column with LiChrosorb 10RP18 and methanol as mobile phase at 1 mL/min; detection was carried out by measuring the extinction of the eluate at 278 nm. For quantitative determinations the height of the peaks was used as measure for the amount of the homologues of C-5-HT in a sample. Separations on preparative scale were carried out by use of a 25 × 0.9 cm column with LiChrosorb 10RP18 and methanol as mobile phase at 3 mL/min.

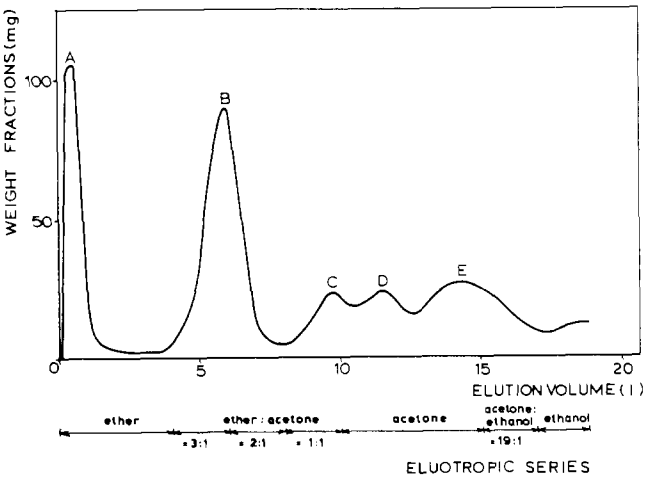

Figure 3. Fractionation of the petroleum ether insoluble substances in coffee wax on a polyamide column eluted with a continuous solvent gradient.

Isolation of a Standard Compound of C_{22}-5-HT. The isolation was carried out either by preparative LC as described before or by use of a low-pressure chromatographic system. Therefore 50 mg of C-5-HT, dissolved in 5 mL of chloroform–methanol (1:4), was injected on a LiChroprep RP8 column. The outlet of this column was connected with the inlet of a second column which is identical with the first one. In this way a system of two columns in series was obtained. Using a CFG-Duramat pump, methanol was pumped through the system at a rate of 2.5 mL/3 min. Fractions of 2.5 mL were collected by use of a LKB 7000 Ultrorac fraction collector. The fractions were analyzed by LC as mentioned before.

Quantitative C_{22}-5-HT Analysis. Ten grams of unground green coffee beans were extracted with methylene chloride for 4 h in a Soxhlet apparatus, siphoning 5–6 times/h. Before extraction the Soxhlet apparatus was flushed with nitrogen. Next the solvent was evaporated in a rotating vacuum evaporator at 30 °C. The evaporator was also briefly flushed with nitrogen before evaporation. After evaporation the dry residue was immediately dissolved in 2–3 mL of toluene–methanol (4:1) and purified over Al_2O_3 neutral, activity 1 with toluene–methanol (4:1) according to Van der Stegen and Noomen (1977). The eluate (25 mL) was analyzed by LC as mentioned before.

Spectral Analysis. The IR spectra in chloroform were recorded with a Hitachi EPI-G3. The mass spectra were determined with an A.E.I. MS 902.

Moisture Content of the Beans. This was defined and determined in duplicate as the loss of drying to constant weight (72 h) at 105 °C.

RESULTS AND DISCUSSION

Isolation and Purification of C-5-HT. The fractionation pattern of the petroleum ether insoluble substances of coffee wax on a polyamide column is shown in Figure 3. The recovery as determined from the total weight of the fractions was found to be 75%. Fraction A

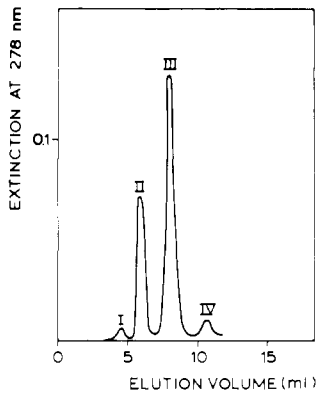

Figure 4. Fractionation of C-5-HT by high-pressure liquid chromatography on an analytical LiChrosorb 10RP18 column.

Table I. Mass Spectral Data of Four LC Peaks and a Synthetic Preparation of C_{22}-5-HT

m/e intensity, %									
peak I		peak II		peak III		peak IV		C_{22}-5-HT synth.	
443	3	471	2	499	1	527	2	499	2
442	6	470	6	498	4	526	4	498	4
441	2	469	1	497	1	525	1	497	1
284	2	312	1	340	2	368	3	340	3
267	1	295	1	323	1	351	1	323	2
160	34	160	33	160	19	160	21	160	20
159	100	159	100	159	100	159	100	159	100
146	29	146	27	146	15	146	23	146	15

consists of caffeine as indicated by thin-layer chromatographic comparison with a reference compound. In fraction B only one spot was found by TLC on silica gel $60F_{254}$ with benzene–ethyl acetate–acetic acid (5:5:1) as solvent and detection under short-wave UV light. Both the IR spectrum and the mass fragmentation pattern are fully consistent with those of C-5-HT. The structure of the compounds in the fractions C, D, and E have not yet been elucidated. Preliminary experiments indicate a close similarity with the structure of C-5-HT (Folstar, 1976).

Fractionation of C-5-HT. C-5-HT, which was obtained as described before, was separated by LC on an analytical LiChrosorb 10RP18 column. As shown in the chromatogram in Figure 4 four peaks were found with $k'_{I} = 0.7$, $k'_{II} = 1.3$, $k'_{III} = 1.9$, and $k'_{IV} = 2.9$. This separation was repeated on a preparative column with the same column filling material and the peaks I–IV were collected. After evaporation of the solvent, the residues were investigated by mass spectrometry (Table I). The spectra I–IV are characteristic for those reported for N^3-alkanoyl-5-hydroxytryptamine with base peak at *m/e* 159 ($M^+ - NH_2CO(CH_2)_nCH_3$) and another fragmentation peak at *m/e* 146 ($M^+ - CH_2NHCO(CH_2)_nCH_3$) (Wurziger and Harms, 1969). The peaks at *m/e* 442 (spectrum I), 470 (spectrum II), 498 (spectrum III), and 526 (spectrum IV) constitute the molecular ions of C_{18}-5-HT, C_{20}-5-HT, C_{22}-5-HT, and C_{24}-5-HT, respectively. In agreement with the literature (Hubert et al., 1977), fragmentation peaks at *m/e* 284 (I), 312 (II), 340 (III), and 368 (IV) can be attributed to a $CH_3(CH_2)_nCONH_3^+$ ion with $n = 16$ (I), $n = 18$ (II), $n = 20$ (III), and $n = 22$ (IV); peaks at *m/e* 267 (I), 295 (II), 323 (III), and 351 (IV) are a result of a $CH_3(CH_2)_nCO^+$ ion with $n = 16$ (I), $n = 18$ (II), $n = 20$ (III), and $n = 22$ (IV) again. From the height of the peaks in Figure 4 it was found that the ratio C_{18}-5-HT:C_{20}-5-HT:C_{22}-5-HT:C_{24}-5-HT amounts to 1:34:61:4%. The presence of C_{18}-5-HT in coffee wax has not been described before. The ratio of the homologues is different from those reported in the literature. Preliminary experiments indicated that this must mainly be attributed to the origin of the coffee beans.

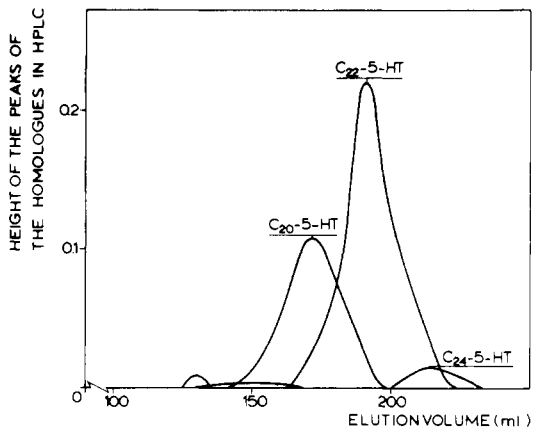

Figure 5. Fractionation of C-5-HT on two columns of LiChroprep RP8 in series.

Table II. C_{22}-5-HT Analysis in Unground Green Beans (ppm on Dry Basis)

sample	content, ppm	standard deviation[a]	recovery, %
without standard	266	±7 ppm (= 2.6%)	
with 1.38 mg of C_{22}-5-HT	404	±11 ppm (= 2.7%)	100

[a] $n = 6$.

C_{22}-5-HT Standard Compound for Quantitative Analysis. Since C_{22}-5-HT was found to be the main homologue of C-5-HT it was chosen as parameter for quantitative analysis. As standard compound a sample of C_{22}-5-HT, which was obtained by preparative LC as described before, was used. The purity of this sample was compared to the purity of a synthetic preparation of C_{22}-5-HT. Table I shows that the mass spectra are fully identical. For both the isolated compound and the synthetic preparation λ_{max} 278.5 nm ($\log \epsilon = 3.77$) was found. The isolated as well as the synthetic compound sharply melted at 122.5–123 °C; the mixed melting point was fully identical. The thermometer of the microscope was calibrated before use. Elementary analysis gave 76.26% C, 10.67% H for the isolated compound and 76.71% C, 10.65% H for the synthetic preparation (calculated 77.05% C, 10.91% H).

If no preparative column for LC is available, the isolation of a standard can also be done using a system of two LiChroprep RP8 columns in series. In one separation 50 mg of C-5-HT was largely separated into C_{20}-5-HT, C_{22}-5-HT, and C_{24}-5-HT (Figure 5).

Quatitative Determination of C_{22}-5-HT in Green Coffee by LC. A standard response curve of the height of the C_{22}-5-HT peak in the chromatogram against the concentration of C_{22}-5-HT was established. At an injection volume of 10 μL this curve was found to be linear between concentrations of 0.1 and 15 mg of C_{22}-5-HT/25 mL of solvent. Using 10 g of green coffee beans for an analysis this means that the response curve is linear for amounts between 10 and 1500 ppm in coffee. Next the standard deviation and recovery of the method were studied. Therefore the analysis was done with and without addition of C_{22}-5-HT standard compound. C_{22}-5-HT (1.38 mg) was added to the flask of the Soxhlet apparatus before extraction. In both series six determinations were made. The results are given in Table II. The standard deviation was found to be below 3% and the recovery was 100%. After collecting the C_{22}-5-HT peak on semi-preparative scale

TLC of the residue upon evaporation indicated that no other spots but the C_{22}-5-HT spot were apparent in this LC peak. Further work is required for the development of this method in roasted and ground coffee samples.

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Fatty Acid Profiles of Three Sweet Orange Cultivars during Maturation

Harold E. Nordby* and Steven Nagy

Total lipid fatty acid profiles for Hamlin (early ripening), Pineapple (midseason ripening), and Valencia (late ripening) oranges were examined monthly over an 11-month growing period. Purified total lipid extracts were separated into neutral lipid, glycolipid, and polar lipid fractions, and each fraction, in turn, was analyzed for fatty acids. Fatty acid profiles were also obtained for the triglyceride and sterol ester fractions for the months of September (immature fruit) and April (mature fruit). Fatty acid analyses by GLC revealed three periods, or stages, in the maturation of the cultivars. The period of greatest change occurred between July and the end of October; and the rate of change was greatest for Pineapple, next for Hamlin, and lastly, Valencia. Following this early rapid-change period was a 3- to 5-month middle period of relative inactivity (rates of changes were noticeably lower). The late (senescent) period, which then ensued, showed accelerated rate changes for the major fatty acids. Each of the three orange cultivars showed different fatty acid profiles at their respective maturation dates.

In previous publications we studied the lipids of citrus fruits with two main objectives. The first objective was to observe whether any relationship existed between lipids and off-flavor production during high-temperature storage of citrus products (Nagy and Nordby, 1970). The second objective was concerned with the possibility of utilizing lipids as chemotaxonomic markers (Nordby and Nagy, 1974). Our most recent objective is concerned with the relationship of lipids to the maturity of citrus fruits. To this end, we initiated studies on hydrocarbon changes in juice sacs during sweet orange maturation (Nordby and Nagy, 1977) and, more recently, showed that quantitative changes occurred in the neutral lipid, glycolipid, and polar lipid fractions of sweet oranges during an 11-month maturation period (Nagy et al., 1978).

Information on changes in fatty acids during fruit maturation is quite limited. Galliard (1968) presented profiles for apples during pre- and postharvest periods. Other fruits studied at two or three maturation periods include banana (Goldstein and Wick, 1969), pear (Romani et al., 1965), tomato (Kalra and Brooks, 1973), and mango (Gholap and Bandyopadhyay, 1975).

We undertook to determine the fatty acid profiles of sweet oranges during their maturation. Citrus fruits are distinct from the above fruits in that they will only ripen on the tree. Also, various citrus cultivars within the same species mature at different periods during the year, e.g., Hamlin, November–December; Pineapple, Janurary–February; and Valencia, March–April in Florida. In our study with hydrocarbons (Nordby and Nagy, 1977) "transition periods" corresponding to these cultivars ripening months were observed. Our objective of this study was to determine whether fatty acid profiles showed similar "transition periods".

EXPERIMENTAL SECTION

Fruit which set in February or March 1971 were collected monthly from July 1971 to May 1972. Cultivars examined were Hamlin (early season, November–December), Pineapple (midseason, January–February), and Valencia (late season, March–April) at the USDA Whitmore Experimental Farm (Crops Research Division, Orlando, Fla.). The preparation and storage of the freeze-dried juice powders, extraction–purification of the lipids, and fractionation of these extracts into three lipid classes were presented previously (Nordby and Nagy, 1977; Nagy

U.S. Citrus and Subtropical Products Laboratory, Science and Education Administration, Federal Research, U.S. Department of Agriculture, Winter Haven, Florida 33880.