Unsaponifiable Matter, Total Sterol and Tocopherol Contents of Avocado Oil Varieties

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Four varieties (Zutano, Bacon, Fuerte, Lula) of avocado (*Persea americana*) have been investigated for their unsaponifiable matter (UM) in mature and immature fruits. The UM content in crude oil was always higher in immature fruits (15–40% *vs.* 4–9%). The UM was fractionated by high-performance liquid chromatography for the determination of total sterols and tocopherol. The sterol content in the oil was always higher in immature (1.1–6.2%) than in mature (0.8–2.0%) fruits. The tocopherol content differed with the varieties (10.2–25.0 mg/100 g UM), and the levels in the oil were higher in immature (20.1–45.6 mg/100 g oil) than in mature (5.7–10.3 mg/100 g oil) fruits.

KEY WORDS: Avocado oil, high-performance liquid chromatography, *Persea americana*, sterols, tocopherol, unsaponifiable matter.

Unsaponifiable matter (UM) of avocado oil, as that of soya, is widely used in various pharmaceutical applications for its therapeutic, dermatological and medical properties (1–4). A number of patents have been issued to the industry for extraction and use of avocado UM (5). Avocado oil is used as a raw material for extracting UM in various industrial ways: solvent extraction, low-pressure molecular distillation, fractional crystallization. It has been observed for some years that the UM content decreased in commercial crude avocado oil treated industrially. Although the extracting technology used in the industry has not changed for years, the level of UM obtained from commercial avocado oil seems to decrease with time. The reason was unknown until now. No information was available about the UM content of avocado oil as a function of the variety or of the stage of maturity of the fruits treated. Following our previous studies on triglyceride and fatty acid compositions during fruit development (6–8), we wanted to know how the UM content in oil and the composition of its sterol fractions varied with time. The traditional normalized methodology for sterol analysis in UM, AFNOR NF T 60-232 and NF T 60-239, required that the crude oil first be saponified to obtain the UM. Fractionation of UM was then needed by means of the tedious and time-consuming thin-layer chromatography (TLC). The fractions obtained were further subjected to gas chromatography (GC) analysis for individual sterol quantitation. Our proposed modification replaces the TLC step by high-performance liquid chromatography (HPLC) to fractionate UM obtained after the crude oil saponification step. This modification of the normalized methodology permits more results in a shorter time than is possible by TLC. These results include fast fractionation of UM into its different sterol fractions, direct quantitation on a mass basis of the total sterol content in crude oil, easily obtained purified sterol fractions for further GC analysis of their components and simultaneous quantitation on a mass basis of tocopherol content in the crude oil. This modified method is also used as a routine procedure to quantitate total sterol content when a large number of oil samples has to be studied.

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EXPERIMENTAL PROCEDURES

Materials. Four varieties (Lula, Zutano, Bacon, Fuerte) of avocado were collected from orchards located under Mediterranean agroecological conditions (Station de recherches agronomiques de Corse CIRAD-INRA) and in a tropical climate (Station de recherches agronomiques de Martinique CIRAD, France). Samples investigated were composed of randomly selected lots of 10–12 fruits. These fruits were considered representative of the lot, and they were analyzed individually to provide an averaged value of the parameter quantitated for the lot studied. Two stages of fruit development were identified, mature and immature (harvested about 15 wk before the mature stage). The fruits harvested at these two stages were analyzed within 48 h.

Sample preparation. The fruits were hand-peeled, the stones were removed and the remaining fresh mesocarps were freeze-dried. The oil was then extracted with hexane in a Soxhlet apparatus according to the procedure previously used (6,9). The oil samples were kept in capped glass vials under N_2 in a refrigerator until analysis.

Saponification procedure. The crude oil was then saponified with ethanolic KOH according to the normalized procedure AFNOR NF T60-205 (10) adapted as a micromethod. In 20 × 100-mm screw-cap tubes, 0.5 g of oil samples were saponified with 5 mL 1N ethanolic KOH. Betulin (Merck, Darmstadt, Germany) was used as an internal standard (IS). It was added to the weighed avocado oil samples before saponification at a level of 1 mL of 0.1% betulin ethanolic solution. The tubes were capped, then heated in a block heater for 1 h at 95°C. After cooling, 10 mL of water was added and mixed. The resulting solution was extracted three times with 10 mL of ether. The organic layers were combined and washed twice with water, once with KOH 0.5N, and then with water again until neutrality. The organic layer was then separated and dried over Na_2SO_4. After filtration of this solution, the solvent was evaporated to dryness under vacuum, and the UM was weighed. The dry UM was then dissolved with 200 μL chloroform and stored in capped injection vials until HPLC analysis. The use of the IS helped account for loss of components during the saponification of oil, extraction and chloroform dilution steps of the procedure.

UM fractionation and HPLC analysis. UM was fractionated by HPLC. A Spectra-Physics liquid chromatograph SP 8000B model (San Jose, CA) was equipped with two detectors mounted in series, a fluorimetric detector (FD) was first used to quantitate tocopherols, and a refractive index detector (RID), connected after the FD, was used to quantitate the separated sterol fractions of the UM.

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A 4.6×250-mm Lichrosorb Si-60, 5µm column (Merck) was used for fractionation of the UM into various tocopherol and sterol fractions. The elution was performed with a mixture of freshly redistilled solvents, hexane/isopropanol (99:1, vol/vol) at a flow rate of 2 mL/min at 35°C. For quantitation on an oil mass basis, betulin (Merck) was added as an IS to the avocado oil samples before the saponification procedure. The outlet flow of the chromatography column was first analyzed by the FD for tocopherol (vitamin E) quantitation. Only one peak is detected under these analysis conditions. The calibration was made with α-tocopherol standard solutions. The detector response was linear within the range studied (0–0.1% w/w standard solutions). The reproducibility of the standard solutions' analysis was better than 1%.

A Kratos Spectroflow 980 fluorimetric detector (Ramsey, NJ) was used. The excitation wavelength was 295 nm, and the emission wavelength was 330 nm. The photomultiplier PMT signal was set at 0.1 µA for an output of 10 mv f.s. with a range of 0.5.

The outlet cell flow of the FD was then analyzed by the RID for quantitation of the total Δ-5 sterol fraction. This fraction was hand-collected at the cell output for further GC analysis.

The differential refractive index detector Shodex RI.SE.11 model (Cunow, France) was used for the identification of the separated fractions according to the literature (11). The bands visualized on silica TLC after UM fractionation (standard procedure) corresponded to the HPLC peaks obtained after UM analysis (modified procedure).

TLC. UM samples of avocado oil dissolved in hexane were fractionated by TLC according to the normalized AFNOR NF T60-232 procedure (9). Elution was made on silica plates with a ternary eluent of hexane/ether/acetic acid (90:10:1, vol/vol/vol). Copper acetate saturated solution was sprayed on the plates. The visualization of the separated bands was made by heating 10 min in an oven at 150°C.

RESULTS AND DISCUSSION

The various horticultural varieties of avocado (*Persea americana* Mill., family Lauraceae) studied belonged to the main cultivars available at the agronomic stations of CIRAD on Corsica Island and West Indies Island. These cultivars were grown under the same agroclimatic conditions. The four cultivars, Zutano, Bacon, Fuerte and Lula, were produced under agroclimatic Mediterranean-like conditions and Lula was grown under tropical conditions the same year. The maturity of selected fruits was determined by the stage of development of these fruits expressed in weeks after flowering (WAF). The young immature fruits were generally collected 12–15 WAF. The mature fruits were those collected during the harvest season and had generally 34–36 WAF.

Sterol quantitation by HPLC. The usual fractionation technique of UM was carried out *via* the time-consuming TLC. HPLC separation was easier and quicker than TLC and could be completed within 15 min. From a single injection of a UM chloroform solution, both total tocopherol and total sterol amounts were quantitated by HPLC. The IS, added to the oil samples, allowed the true amount of tocopherol and of total sterols contained in the oil to be calculated from the HPLC peak areas. Some loss of UM was encountered during the saponification-extraction procedure. By adding betulin to the oil samples, a nonreactive molecule for the chemistry used during saponification, we noticed that the recovered amount calculated from the corresponding HPLC peak area was lower than the real quantity added. Recovery of betulin was always the same, indicating that losses took place mainly during the extraction steps. We assumed that all the UM components were subjected to the same loss as betulin. So, we corrected the amount of UM components by the recovery factor determined for betulin. A sample calculation for total sterol content (% w/w) in oil is given as

$$\%\ \text{(w/w) total sterol}_{oil} = (k_2 \cdot S_{st}/1000) \cdot (1/R) \cdot V \cdot (100/m_{oil}) \quad [1]$$

where S_{st} was the HPLC peak area for the total sterol fraction, k_2 was the response factor for β-sitosterol, R was the recovery factor for betulin, V was the volume of chloroform (200 µL) used to dilute UM according to the modified saponification procedure, m_{oil} was the amount of oil sample. The result is the total amount of sterol content in the oil sample.

With the RID, different peaks were observed, including the one of betulin (IS). The RID gave typical chromatograms as shown in Figures 1 and 2. The recording generally showed four different major groups of peaks, which were more or less resolved. These peaks corresponded to the separated fractions visualized by TLC. They were hydrocarbons, triglycerides, tocopherols, Δ-5 sterols and Δ-7 sterols. Peak III was well resolved and represented the Δ-5 sterol fraction contained in the UM sample (11,13). These HPLC peaks were composed of different molecules with similar chemical structures, as were the fractions from TLC. HPLC was not selective enough to separate in a single run all the fractions of UM together with the individual sterol molecules contained in each of these fractions. GC was a more suitable technique than HPLC for sterol quantitation of each of these separated fractions (12). The sterol fraction (peak III) was collected and kept in a refrigerator until GC analysis. The peaks eluting before peak III were not identified as to the chemical family of the organic molecules contained. As a tentative identification on the basis of data reported in the literature (13), fraction II could be attributed to 4-monomethylsterols. Because we were only interested in the Δ-5 sterol fraction, we did not try to develop new eluents (isocratic or gradient) for improving the separation of the peaks eluted before sterol peak III. The last eluted peak V was the IS betulin.

Response factors (RF) of sitosterol and betulin were calculated for the RID from their calibration plots. As shown in Table 1, the ratio of the respective RF of β-sitosterol and betulin was close to 1, even when the two products were mixed in different proportions before the HPLC analysis of the mixture. Assuming that all sterols were contained in the Δ-5 sterol fraction (peak III), we could quantitate from the HPLC analysis the total amount of sterols in the oil. Nevertheless, we should take into account the recovery of product during the saponification-extraction steps.

Various assays were undertaken to quantitate the loss of sterols during the saponification-extraction procedure. Aliquots of betulin and sitosterol standard solutions were

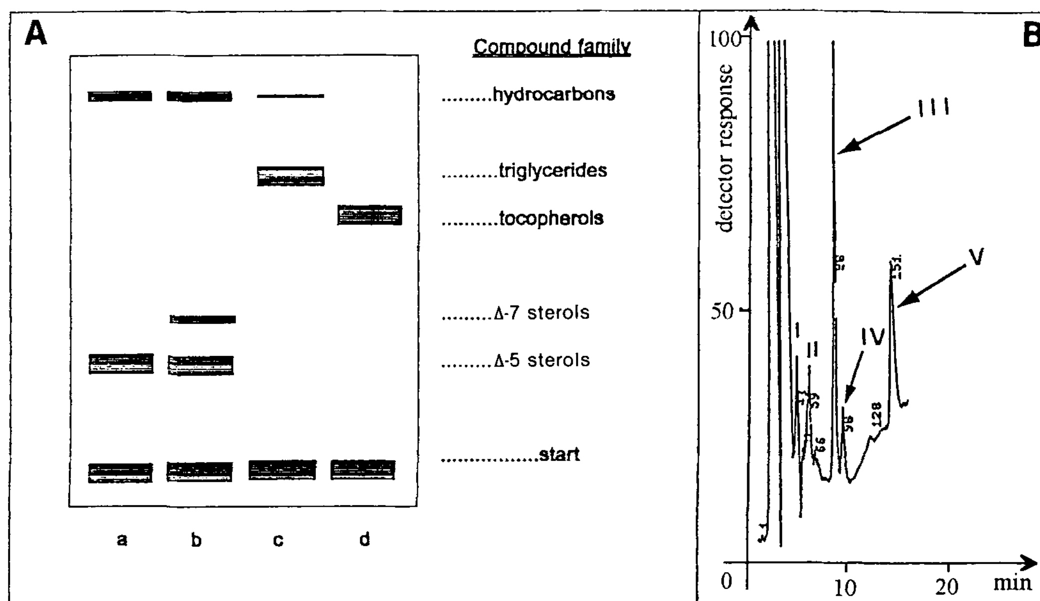

FIG. 1. Comparative chromatography of unsaponifiable matter (UM) of various oil samples: A, Thin-layer chromatography of UM extracted from various oils: avocado, sunflower; (a: avocado UM, b: sunflower UM, c: avocado oil, d: tocopherol). B, High-performance liquid chromatography of UM extracted from sunflower (refractive index detector). I, Peak fraction I; II, peak fraction II (4-monomethylsterols); III, peak collected for fraction III (Δ-5 sterols); IV, peak fraction IV (Δ-7 sterols); V, peak of betulin (internal standard).

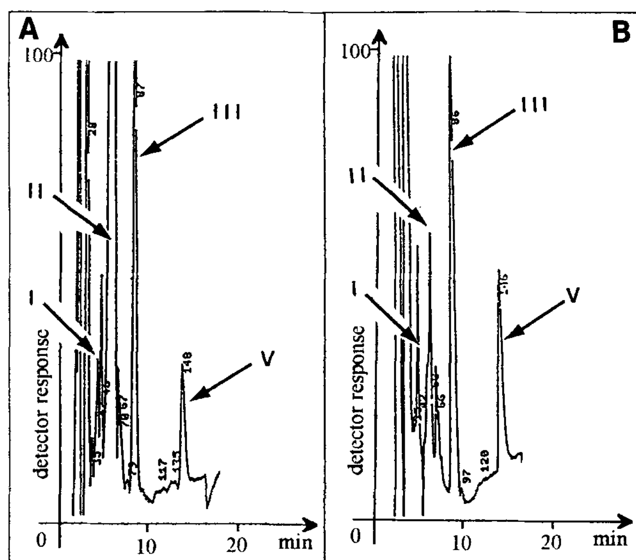

FIG. 2. Chromatography of unsaponifiable matter (UM) of avocado oil samples: A, High-performance liquid chromatography (HPLC) chromatogram of UM extracted from immature avocado fruits; B, HPLC chromatogram of UM extracted from mature avocado fruits. (refractive index detector). For peak number identification, see Figure 1 caption.

treated as samples. They were analyzed by HPLC before and after being subjected to the saponification and extraction procedures and to the extraction procedure without saponification. Normally, neither betulin nor sitosterol would react during those chemical treatments. Nevertheless, we noticed some losses of product during

TABLE 1

High-Performance Liquid Chromatography Response Factor Ratios for Different Concentrations of Betulin and β-Sitosterol Ethanolic Solutions Before Injection

Compound concentration (w/w) in the mixtures injected (20 μL)		$K = \frac{k_2}{k_1} = \frac{C_{sito}\ S_{betul}}{S_{sito}\ C_{betul}}$[a]
Betulin (%)	β-Sitosterol (%)	
0.5	3	1.03
0.4	2	1.09
0.3	1.5	0.99
0.2	1	.98
0.1	0.5	.94

[a]C_{Sito}: concentration of sitol in the injected solution; S_{Sito}: area of sitol peak; $k_1 = C_{betul}/S_{betul}$: response factor for betulin; $K = k_2/k_1$: ratio of response factors for β-sitosterol and betulin.

the liquid-liquid extraction. Moreover, total recovery of UM during the end-step dilution with chloroform was not complete. The results reported in Table 2 showed the percentages of recovery of betulin under these different experimental conditions. We noticed that the IS was recovered up to 76% when it was submitted to the complete saponification-extraction procedure. The same percentage (78%) was found for sitosterol, a representative molecule for sterol fraction III of the UM, when treated similarly. Added to a crude avocado oil sample, IS was recovered to the same extent (76%) as in the previous saponification-extraction assays. By using an IS, the amount of sterols found in the oil by HPLC of the UM could be corrected for losses during sample preparation to yield the true levels of sterols contained in the oil.

HPLC fractionation on a silica column gave more peaks than the number of bands observed with traditional TLC

TABLE 2

Percent Recovery of Betulin (internal standard) and Sterol (as β-sitosterol) During the Saponification—Extraction Procedure

Sample tested	Chemistry applied	Recovery (%)	
		Betulin	β-Sitosterol
Betulin alone[a]	None	100	—
Betulin alone	Saponification + extraction	77	—
β-Sitosterol alone[b]	None	—	100
β-Sitosterol alone	Saponification + extraction	—	78
Betulin + avocado oil[c]	Saponification + extraction	76	n.a.[e]
Betulin + β-sitosterol[d]	Saponification + extraction	76	75

[a]1 mL solution at 0.1%
[b]100 μL solution at 2%.
[c]1 mL solution at 0.1% + 0.5 g oil.
[d]100 μL solution at 0.1% + 100 μL solution at 2%.
[e]n.a., Not applicable.

TABLE 3

Unsaponifiable Matter (UM), Total Sterol and Tocopherol Content in the Extracted Crude Oil of Avocado Fruits of Different Varieties and Different Stages of Maturity

Variety	Maturity	Moisture[a] (%)	Lipids[a] (%)	μM (% lipids)	Total sterol[b] (% lipids)	Total sterol (% UM)	Tocopherol[b] (mg/100 g oil)
Lula[c]	Immature	84.7	2.7	40.0	6.2	15.5	45.6
Lula[c]	Mature	69.2	14.3	5.0	2.0	40.0	10.3
Lula[d]	Immature	84.5	4.8	19.0	2.6	13.7	n.a.[e]
Lula[d]	Mature	77.7	10.5	6.5	1.4	21.5	n.a.
Bacon[d]	Immature	83.4	6.7	15.0	1.2	8.0	32.5
Bacon[d]	Mature	71.2	19.1	4.0	0.8	20.0	8.7
Fuerte[d]	Immature	88.3	5.8	21.0	1.1	5.2	20.1
Fuerte[d]	Mature	71.6	19.1	8.8	0.9	10.2	5.7
Zutano[d]	Immature	83.6	6.6	17.0	1.7	10.0	23.6
Zutano[d]	Mature	72.8	18.7	6.0	1.5	25.0	6.9

[a]Fresh avocado pulp (wt%).
[b]Corrected values for internal standards or sterol factor recovery (see Table 2).
[c]Fruits grown under tropical agroecological conditions.
[d]Fruits grown under Mediterranean agroecological conditions.
[e]n.a., Not applicable.

(Figs. 1A and 1B). This HPLC fractionation was also more selective. Moreover, smaller quantities of UM could be used with HPLC than with TLC. This point is important when only a small amount of oil (0.5 g) was available from each avocado sample for UM content analysis. The HPLC profiles represented UM obtained from immature (Fig. 2A) and from mature (Fig. 2B) fruits. They were roughly similar in the number of peaks separated but the proportion varied according to the maturity of the fruit.

Fraction II was generally more important in oil samples extracted from immature fruits than in the oil extracted from mature ones. In other words, the composition of UM of avocado oil is dependent on the stage of development of the fruits. For all mature fruits, one should notice that peak III (total sterol) represented a big area compared to the cumulative areas of fraction peaks I, II and III or compared to the total peak area of the HPLC chromatogram. This led to the conclusion that the total Δ-5 sterol content represented the largest content of the components contained in the UM sample extracted from mature fruits. However, immature fruits contained substantially more peak II than peak III, although peak III was not negligible. Nevertheless, we never noticed another peak representative of the Δ-7 sterol fraction, as we normally did in fractionated samples of sunflower UM. In our chromatographic conditions, this peak normally eluted just after the Δ-5 sterol fraction (Fig. 1B). The avocado oil samples investigated here did not show Δ-7 sterols as components of the UM at a significant level, as some authors found with other varieties of avocado fruits (14,15).

As shown also in Table 3, the UM content in the oil varied with the nature of the cultivar observed and with the stage of development of the fruits. This content was high for very young fruits. For mature fruits, the UM values, corrected by the recovery factor of betulin, were about of the same order as those given in the literature, which ranged from 1 to 7.5% of the crude avocado oils (16–20).

The total sterol contents in UM were higher in mature than in immature fruits. The Lula variety, grown in a tropical climate, showed a higher Δ-5 sterol content in UM for both mature and immature fruits, compared to the respective fruits of varieties grown in a Mediterranean climate. We noticed that the variation of the sterol contents in crude oil from immature to mature fruits was reversed from the variation for the sterol contents in UM. There was less sterol content in the crude oil of mature than of immature fruits. This is a direct consequence of

the tremendous drop of UM content in the oil between the two stages of maturity of the fruits.

Depending on what the industry is looking for, UM or Δ-5 sterol fraction, the choice should be made to process either mature (for UM as raw material) or immature (for sterols as active substances) fruits. Tocopherol, considered as a protective agent (natural antioxidant) or as a vitamin substance (vitamin E), showed generally lower contents in the oil extracted from mature (5.7–10.3 mg/100 g oil) than from immature (20.1–45.6 mg/100 g oil) fruits. We did not find significant variation for the varieties studied or the agroclimatic conditions of fruit production.

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