Quantitative Determination of Hydroxy Pentacyclic Triterpene Acids in Vegetable Oils

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A simple and precise analytical method for the determination of hydroxy pentacyclic triterpene acids (HPTAs) in vegetable oils was developed. The acidic fraction was isolated by solid-phase extraction using bonded aminopropyl cartridges, and the extract was silylated and analyzed by gas chromatography. Repeatability and recovery of the method were determined. In virgin olive oils, similar amounts of oleanolic (3β-hydroxyolean-12-en-28-oic) and maslinic (2α,3β-dihydroxyolean-12-ene-28oic) acids and traces of ursolic (3β-hydroxyurs-12-en-28-oic) acid were found. The main factor affecting HPTA concentration was the oil quality since that increases as the quality decreases, while olive variety, olive ripeness, and oil extraction system had less influence. In crude olive pomace oils, the concentrations were very much higher than in virgin olive oils. During refining processes, total or significant losses of HPTAs were observed. Esterified derivatives of HPTAs were not found.

Keywords: *Hydroxy pentacyclic triterpene acids; solid-phase extraction−GC analysis; olive oil; vegetable oils*

INTRODUCTION

The presence of hydroxy pentacyclic triterpene acids (HPTAs) in olive fruits has long been known (Vioque and Maza, 1963; Vázquez et al., 1969). Oleanolic (3β-hydroxyolean-12-en-28-oic) and maslinic (2α,3β-dihydroxyolean-12-ene-28oic, also named crategolic) acids were found in the olive husk (Caglioti et al., 1961; Caputo et al., 1974). Both compounds, together with traces of ursolic (3β-hydroxyurs-12-en-28-oic) acid, were also located in the reticular lipid layer of olive skin (Frega et al., 1984, 1989; Frega and Lercker, 1986; Bianchi et al., 1992, 1994). Traces of betulinic (3β-hydroxylup-20-(29)-en-28-oic) acid have also been found in the skin of Coratina olive variety (Bianchi et al., 1992).

Minor amounts of oleanolic and maslinic acids were detected in olive oils (Vioque and Maza, 1963; Kotakis, 1967; Serverge, 1983) but significant concentrations (0.1% and 0.4%, respectively) were isolated from olive pomace oils (Vioque and Morris, 1961). These compounds seem to be responsible for the gelatinous precipitates in crude olive pomace oils (Vioque and Maza, 1963) and the turbidity observed in some physically refined olive oils (Kotakis, 1967).

HPTAs in vegetable oils were determined by direct oil analysis on silica gel TLC plates with color development (Vioque and Maza, 1963; Kotakis, 1967). In the case of olive pomace oils, the HPTAs were isolated by precipitation from a solution in petroleum ether, followed by fractionation on a silica gel column (Vioque and Morris, 1961). These methods are very inaccurate, and the development of quantitation procedures is necessary.

Because of the lack of data on HPTA concentrations in olive oils, determinations were carried out on oils of different quality from several olive varieties at various ripeness stages. The effect of the oil extraction system and refining processes is also studied. A simple and precise analytical method, using solid-phase extraction followed by gas chromatographic analysis, was developed to this end. Although the HPTAs in olive oils have always been found as the free forms (Vázquez and Janer, 1969), the presence of esterified derivatives was investigated since some triterpenic alcohols have been found both as free and as waxes with the 3-hydroxyl group esterified with fatty acids (Mariani et al., 1991).

EXPERIMENTAL PROCEDURES

Samples. Virgin olive oils from intact, clean fruits of Picual variety harvested at eight different times (September to December) were obtained using an Abencor system (Comercial Abengoa, S.A., Sevilla, Spain) (Martinez Suárez et al., 1975). This unit consists of three basic elements: olive crusher, thermobeater to mix the paste, and centrifuge to eliminate the solid residue. The oil was separated from wastewater by decanting.

A series of samples of virgin olive oils from Picual, Lechin, and Arbequina varieties were obtained using industrial centrifugation equipment. Olives were crushed in a hammer crusher; the olive paste was mixed for 90 min and then centrifuged by "two-" and "three-phase" centrifuging modes. In the former, little or no water is added, giving rise only to oil and a plastic paste. In the latter, a significant flow of water is added, yielding separately oil, residual water, and solid waste.

Other virgin olive oil samples, supplied by various oil mills, were obtained from different olive varieties and with different acidity values. Crude and refined olive pomace oil, crude palm oil, and crude seed oils were obtained from industrial plant.

Finally, chemical and physical refining processes were carried out in an industrial plant, and the oils obtained in the different steps were analyzed. The chemical process involves neutralization with caustic soda, decoloration with bleaching earth, and deodorization at 220 °C under vacuum. The physical refining procedure consists of degumming with aqueous phosphoric acid solution, decoloration with bleaching earth, and deodorization at 240 °C under vacuum.

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Materials, Reagents, and Methods. All reagents were of analytical reagent grade. Oleanolic, ursolic, and betulinic acids were obtained from Sigma (St. Louis, MO).

The solid-phase extraction cartridges (3 mL), packed with bonded aminopropyl phase, were from Supelco (Bellefonte, PA).

The silylating reagent was prepared by adding 3 mL of hexamethyldisilazane and 1 mL of trimethylchlorosilane to 9 mL of anhydrous pyridine.

Acidity was determined by titration of a solution of oil in a solvent (ethanol:diethyl ether 1:1) previously neutralized with 0.1 N ethanolic potash using phenolphthalein indicator (Regulation EEC/2568/91). Results are expressed as percentage of oleic acid.

Analytical Procedure. *Standard and Sample Preparation.* A solution of betulinic acid in chloroform (1 mg/mL) was used as the internal standard.

For virgin olive oil and crude and refined vegetable oils, 0.2 ± 0.001 g of oil was weighed in a 3-mL vial, and 25 μL of a standard solution was added. The chloroform was evaporated under nitrogen flow, and the residue was diluted with 1 mL of hexane. For crude olive pomace oils, 25 μL of standard solution was introduced into a 3-mL vial, the solvent was evaporated under nitrogen flow, and then a 1-mL aliquot of a solution of the oil in hexane (0.2 ± 0.001 g in 5 mL) was added.

Solid-Phase Extraction. A bonded aminopropyl phase cartridge was placed in a vacuum elution apparatus, conditioned by the consecutive passing of 3 mL of methanol and 3 mL of acetone, and then dried under vacuum. The column was washed with 4 mL of hexane, and the vacuum was released to prevent the column from becoming dry. The oil solution in hexane (1 mL) was applied to the column, and the solvent was pulled through, leaving the sample and the standard on the column. The sample container was washed with two 0.5-mL portions of hexane that were run out the cartridge. Next, the column was eluted with 6 mL of the admixture hexane: methylene chloride 90:10. The vial was washed with two 1-mL portions of the admixture hexane:ethyl acetate 40:60 that were run out the cartridge, and the column was eluted with 5 mL of the same admixture. These eluates were rejected, and a new collection flask was placed. Finally, the sample vial was washed with two 1-mL portions of the admixture diethyl ether: acetic acid 98:2 that were run through the cartridge, and the column was eluted with 4 mL of the same eluent. The eluate was evaporated in a rotary evaporator at room temperature under vacuum until dryness. The residue was treated with 200 μL of the silylating reagent and left at room temperature for at least 10 min. A volume (1 μL) of the final solution was injected into the gas chromatograph. The SPE cartridges were reused six times.

Gas Chromatography Analysis. Chromatographic analysis of HPTAs was performed using a Chrompack (Middelburg, The Netherlands) CP9000 gas chromatograph fitted with a flame ionization detector and a split injection system (split ratio 1:30). Separations were carried out on a SGL5 capillary column (25 m × 0.25 mm i.d.) coated with 5% phenylmethylsilicone, 0.25 μm thickness (Sugelabor, Madrid, Spain). The operating conditions were the following: oven temperature 260 °C for 4 min and then increased at 2 °C/min up to 320 °C; injector and detector temperatures: 320 °C. Hydrogen was used as carrier gas at a column head pressure of 110 psi. Data acquisition and processing were carried out using a Chrom-Card Data System (Fisons, Altrincham, U.K.). HPTAs were quantified assuming the same response factor for all triterpenic acids.

Repeatability and Recovery. For determination of recovery, a stock solution of oleanolic and maslinic acids was prepared by dissolving in chloroform the HPTA fraction obtained from several oil samples. To obtain oil samples with increasing quantities of these compounds, different amounts of the stock solution were added to a refined olive oil (not containing HPTAs). Other series of samples were prepared by taking different volumes of the stock solution. The oil samples were processed through an SPE cartridge according to the analytical procedure, and the HPTA fractions were collected. Both series of solutions were spiked with a standard solution of betulinic acid and evaporated. The residues were treated with silylating reagent and injected into the gas chromatograph. For each concentration, recovery was calculated by comparing the HPTA concentrations in the two series of samples. Three oil samples of each concentration were analyzed.

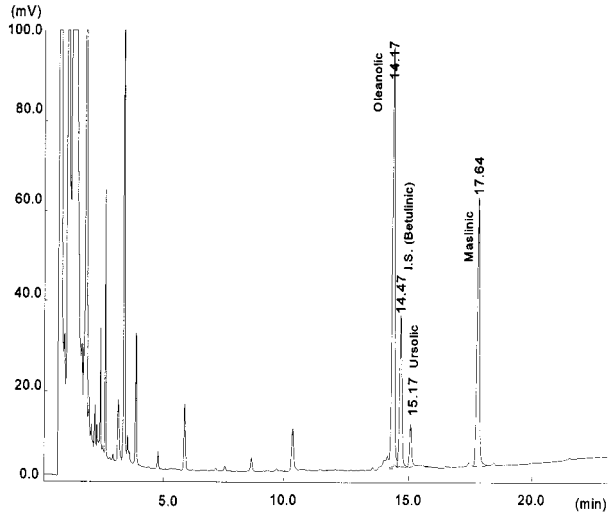

Figure 1. Gas chromatogram of the silyl derivatives of HPTAs isolated from a virgin olive oil.

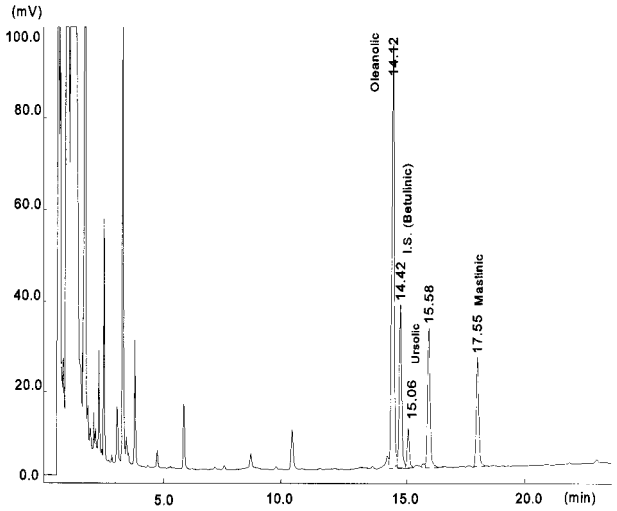

Figure 2. Gas chromatogram of the silyl derivatives of HPTAs injected just after the addition of the silylating agent. Peak appearing at RT 15.58 min is due to partially silylated maslinic acid.

Synthesis and Determination of Esterified Oleanolic Acid. The 12-en-28-carboxy-3β-oleanyl oleate was prepared by reaction of oleanolic acid with oleyl chloride according to the Mattson and Volpenhein method with slight modifications. Briefly, nitrogen was bubbled through a solution of both compounds (2:1 molar ratio) in pyridine−chloroform in a reflux apparatus for 5 h at room temperature. The solvent was evaporated with nitrogen steam, and the residue was redissolved. The solution mixture of oleanolic acid and 12-en-28-carboxy-3β-oleanyl oleate was fractionated by SPE as indicated in the analytical procedure by passing the reaction mixture through the aminopropyl cartridge. The diethyl ether acetic acid (98:2) fraction was evaporated, the residue was silylated, and the solution was analyzed using a new gas chromatography system on a high-temperature column (Figure 4).

GC Analysis. Silylated samples of esterified HPTAs were analyzed in a Chrompack (Middelburg, The Netherlands) CP9000 gas chromatograph fitted with a flame ionization detector and split injection system (split ratio 1:30). Separations were carried out on a high-temperature fused-silica capillary column (25 m × 0.25 mm i.d.) coated with 65% methylphenylsilicone to a thickness of 0.1 μm (Quadrex, New

Table 1. Recovery and Precision Data of Pentacyclic Hydroxy Triterpene Acid Analysis by SPE-GC[a]

	oleanolic acid			maslinic acid		
sample	mean (mg/kg)	recovery (%)	RSD (%) ($n = 3$)	mean (mg/kg)	recovery (%)	RSD (%) ($n = 3$)
1	161.4	95.7	0.5	147.5	93.1	1.2
2	123.4	97.5	0.7	106.0	85.9	2.5
3	81.0	95.0	0.4	70.0	85.3	2.2
4	42.6	98.7	0.7	35.8	84.3	2.2
5	21.4	98.5	2.8	17.1	82.9	2.7
6	8.8	97.5	3.7	6.2	82.8	4.7

[a] n = number of replicates.

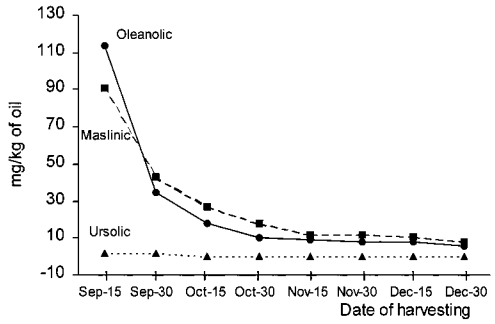

Figure 3. HPTA concentrations in virgin olive oils obtained from olive fruits at several ripening stages.

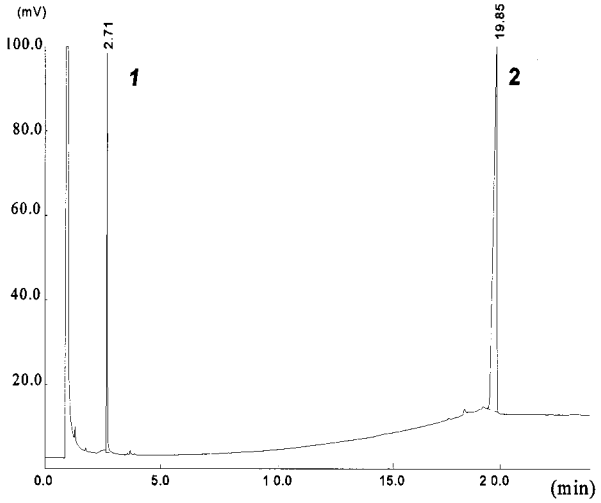

Figure 4. Gas chromatogram of silyl derivatives of oleanolic acid (**1**) and 12-en-28- carboxy-3β-oleanoyl oleate (**2**).

Table 2. Pentacyclic Hydroxy Triterpene Acids in Extra Virgin Olive Oils from Different Varieties Obtained by Two- and Three-Phase Centrifugation Modes[a]

	extraction system			
	oleanolic acid (mg/kg)		maslinic acid (mg/kg)	
sample	2 phases	3 phases	2 phases	3 phases
Picual 1	74	89	35	52
Picual 2	45	53	37	38
Picual 3	16	20	31	40
Picual 4	17	21	36	41
Lechin 1	77	96	22	38
Lechin 2	73	97	23	59
Arbequina 1	108	114	42	45
Arbequina 2	95	121	35	38

[a] Each value is the mean of three determinations (CV values < 5%).

Haven, CT). The operating conditions were as follows: oven temperature 310 °C for 4 min and then increased at 3 °C/min to 360 °C and held at 360 °C for 10 min; injector temperature 310 °C; detector temperature 360 °C. Carrier gas hydrogen at 100 kPa. Data acquisition and processing were carried out using a Chrom-Card Data System (Fisons, Altrincham, U.K.).

Saponification. The fractions eluted from the cartridge with hexane:methylene chloride 90:10 and hexane:ethyl acetate 40:60 were combined and evaporated at room temperature under vacuum until dryness. Standard solution was added to the residue, and the solvent was removed by a nitrogen flow. Then, 3 mL of 2 N methanolic potash was added, and the mixture was gently boiled for 30 min. When cold, the solution was transferred to a separating funnel with the addition of two portions of 2 mL of water and two portions of 2 mL of diethyl ether. The mixture was shaken and left to stratify. The aqueous phase was transferred to a second separating funnel, and the organic phase was reextracted with 3 mL of water. The combined aqueous extracts were acidified with 12 N ClH until pH < 3 and extracted three times with diethyl ether. The organic extracts were evaporated at room temperature under vacuum, and the residue, when silylated, was analyzed by gas chromatography.

The diethyl ether:acetic acid fraction was evaporated at room temperature under vacuum until dryness. The residue was saponified with 3 mL of 2 N methanolic potash, acidified,

Table 3. Quantitative Determination of Pentacyclic Hydroxy Terpenic Acid in Extra Virgin Olive Oils Obtained from Different Olive Varieties[a]

variety	country[b]	acidity (%)	oleanolic acid (mg/kg)	maslinic acid (mg/kg)	ursolic acid (mg/kg)
Carolea	I	0.42	50	36	nd[c]
Galega	P	0.38	58	42	nd
Cobrançosa	P	0.35	30	19	nd
Lechin	S	0.20	71	71	3
Hojiblanca	S	0.25	52	62	4
Picual	S	0.22	35	62	nd
Cornicabra	S	0.39	46	38	nd
Verdial	S	0.23	54	54	nd
Arbequina	S	0.35	85	98	nd
Chamlali	T	0.25	17	21	nd
Picholine Marrocaine	M	0.48	35	22	3
Picholine Languedoc	M	0.77	42	20	3

[a] Each value is the mean of three determinations (CV values < 5%). [b] I, Italy; S, Spain; P, Portugal; T, Tunisia; M, Morocco. [c] nd, not determined.

Table 4. Pentacyclic Hydroxy Triterpene Acids in Virgin Olive Oils with High Acidity Values[a]

sample	acidity (%)	variety	oleanolic (mg/kg)	maslinic (mg/kg)	ursolic (mg/kg)
1	1.1	Picual	167	145	nd[b]
2	1.5	Hojiblanca	198	169	2
3	1.9	Cornicabra	215	166	nd
4	2.2	Verdial	344	205	nd
5	5.3	Farga	212	251	6
6	8.9	Picual	216	194	4
7	9.3	Picual	356	227	nd

[a] Each value is the mean of three determinations (CV values < 5%). [b] nd, not determined.

Table 5. Pentacyclic Hydroxy Triterpene Acids in Crude Olive Pomace Oils[a]

sample	oleanolic (mg/kg)	maslinic (mg/kg)	ursolic (mg/kg)
1	8603	1485	nd[b]
2	6425	856	nd
3	4630	691	nd
4	3605	801	nd
5	2525	283	nd
6	2173	212	nd

[a] Each value is the mean of three determinations (CV values < 5%). [b] nd, not determined.

Table 6. Pentacyclic Hydroxy Triterpene Acids in Refined Olive Oils after Different Steps of the Refining Processes (mg/kg)[a]

		sample A		sample B		sample C	
refining process	refining step	oleanolic	maslinic	oleanolic	maslinic	oleanolic	maslinic
	initial samples	212	251	215	166	67	91
chemical refining	neutralized	7	0	10	0		
	decolored	0	0	0	0		
	deodorized	0	0	0	0		
physical refining	decolored	212	237	216	150	64	85
	deodorized	99	102	41	31	34	37

[a] Each value is the mean of three determinations (CV values < 5%).

and extracted with diethyl ether. The solvent was evaporated, and the residue was silylated and analyzed by gas chromatography.

RESULTS AND DISCUSSION

SPE Isolation of HPTA Fraction. The solid-phase extraction (SPE) using bonded aminopropyl phase columns has been described for the separation of fatty compounds in biological extracts (Kaluzny et al., 1985). In the present work, the acids were strongly retained on the activated phase and were removed by elution with diethyl ether:acetic acid mixtures. The eluent and the volume optimization were chosen by monitoring the olive oil fractions using GC. Hydrocarbons, waxes, and triacylglycerols were eluted with hexane and hexane: methylene chloride 90:10; alcohols, sterols, and diacylglycerols were eluted with hexane:ethyl acetate 40:60. The elution with diethyl ether acetic acid 98:2 desorbs the HPTAs together with the free fatty acids. Due to the low solubility of the HPTAs in hexane, the container must be consecutively washed with the different solvents to guarantee the complete introduction of HPTAs into the cartridge.

A representative chromatogram of the silyl derivatives of HPTAs is shown in Figure 1. As can be seen, the gas chromatogram does not show interfering groups, since the fatty acids appear in the solvent peak tail. If the GC is performed just after reagent addition, a new chromatography peak appears (Figure 2). This peak disappears and the maslinic acid increases when the GC injection is delayed 10 min, suggesting a partial silylation of maslinic acid. For quantitative determinations, betulinic acid was used as the internal standard, since it was not detected in the olive oil samples. The gas chromatographic response is the same for oleanolic, ursolic, and betulinic acids. The maslinic acid concentrations were calculated assuming a response equal to that of the other acids.

Recovery and Precision of SPE-GC. Recovery and precision data for different concentrations of oleanolic and maslinic acids are given in Table 1. It can be seen that oleanolic acid showed a better recovery than maslinic acid (97% and 88%, respectively). The repeatability data, expressed as relative standard deviation (%), range from 0.5 to 3.7 for oleanolic and from 0.2 to 4.7 for maslinic acids and are good for both acids.

Olive Oil Studies. *Olive Ripening.* In oils extracted by the Abencor system from Picual olives harvested during fruit development, similar amounts of oleanolic and maslinic acids were found (Figure 3). The high concentrations (ca. 100 mg/kg) detected in oils from very unripe green olives fell with ripening, reaching an almost constant value (ca. 10 mg/kg) in oils from ripe olives. In the sample of September, 1.5 mg/kg of ursolic acid was also detected, but the content was negligible in the other samples. In the olive fruit, a similar decrease in HPTA concentration with ripeness has been observed (Vázquez et al., 1965). These three HPTAs maintained similar proportions to those reported in the olive skin (Bianchi et al., 1992, 1994), although the concentrations in the oil were very much lower than in the fruit (ca. 2000 mg/kg). These facts indicate that HPTA concentration in olive oils is related to that in the olive fruit. However, the HPTA concentrations found during the usual harvesting time (November−December) were practically constant, suggesting a scarce influence of the fruit ripeness.

Oil Extraction System. In relation to the extraction process, other series of olive oils were obtained from the same olive cultivars (Picual, Lechin, and Arbequina) using industrial equipments by two- and three-phase centrifugation modes. In all the varieties, oils obtained by the three-phase mode showed higher HPTAs than those obtained by the two-phase mode (Table 2). The increases of oleanolic and maslinic acids were similar. Mixing time showed no influence on the terpenic acid concentrations. These results indicate that the oil extraction system affects sligthly the amounts of HPTAs passing from the olive fruit to the oil.

Olive Oil Quality. HPTA concentrations in extra virgin olive oils (acidity lower than 1%) obtained from different olive varieties did not reach 200 mg/kg (Table 3), but in oils with acidity higher than 1%, HPTAs exceeded 300 mg/kg (Table 4) regardless of olive variety and extraction system. These facts suggest that hydrolytic processes taking place in the olive fruit facilitates the liberation of HPTAs from the olive skin, since these compounds constitute the cuticular layer of the olive skin (Bianchi et al., 1992). Nevertheless, there was no linear relationship between free fatty acids and terpenic acids because other variables, such as the olive ripening and the oil extraction system, also have some influence. Therefore, the HPTA concentrations in oils are related mainly to the quality of the fruit before crushing.

Crude Olive Pomace Oils. Crude olive pomace oils showed very large amounts of HPTAs, the oleanolic acid concentrations being much higher than those of maslinic (Table 5) acid. These results confirm that degradation of the raw material increases the HPTA content.

Refined Olive Oils. The HPTA concentrations in refined olive oils depend on the type of refining process (Table 6). In chemical refining, HPTAs are neutralized during the alkali treatment and then eliminated together with the soaps by centrifuging and washing. Maslinic acid is lost completely during the neutralization step, but minor amounts of oleanolic acid remain in the oil. These residues disappear during decoloration.

In physical refining, significant amounts of HPTAs are lost during the deodorizing step (Table 6). Several oil samples of physical refined oils shown HPTA con-

centration ranging from 200 to 0 mg/kg. These facts suggest that the HPTA content of physical refined oils depends mainly on the operating conditions of the refining process. Refined olive pomace oils are always obtained by chemical refining of the crude oils and, thus, do not contain HPTAs.

Other Vegetable Oils. In the case of crude oils obtained from seeds, such as sunflower, high oleic sunflower, walnut, safflower, and rapeseed, neither esterified nor free HPTAs were found.

Esterified HPTAs. In olive leaves, olive fruits, and olive oils, HPTAs were found only as free forms (Vioque and Morri, 1961). However, some triterpenic alcohols (cycloartenol, 24-methylencycloartanol, and erythrodiol) are present, both free and esterified with fatty acids in olive oils (Mariani et al., 1991). Therefore, the formation of HPTA derivatives by reaction of the 3-hydroxyl groups with fatty acids would be possible and has been investigated.

The analysis of virgin olive oils having high acidity and crude olive pomace oils showed no gas chromatographic peaks at retention times near that of the synthesized 12-en-28carboxy-3β-oleanoyl oleate. In addition, the acidic fraction obtained from cartridge elution was saponified, then acidified, extracted with diethyl ether, and evaporated. The residue was silylated and analyzed by gas chromatography. The amounts of HPTAs were similar to those found by direct analysis of the oil. These facts indicate that 3β-esterified HPTAs are absent in olive oils.

The carboxyl group of HPTAs might react with alcohols (methanol, ethanol, fatty alcohols) present in virgin olive oils of low quality, yielding derivatives with hydroxy and ester functional groups. None of these compounds is retained in the SPE cartridges, and all would elute in the hexane:methylene chloride and hexane:ethyl acetate fractions. Saponification of these fractions would lead to free HPTAs, which would be detected by the analytical method. HPTAs were not detected in the saponified hexane fractions obtained from lampant virgin olive oils and crude olive pomace oils. All these results confirm that HPTAs are present only in the free form.

The methodology proposed is rapid, simple, and reproducible and enables the quantitative determination of free HPTAs in vegetable oils. Refining processes involving alkali treatment eliminate the HPTAs.

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