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Chemistry of Extra Virgin Olive Oil: Adulteration, Oxidative Stability, and Antioxidants

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Much analytical work has been published on the chemistry of extra virgin olive oil (EVOO) as a basis for the detection and quantitative analyses of the type and amount of adulteration with cheaper vegetable oils and deodorized olive oils. The analysis and authentication of EVOO represent very challenging analytical chemical problems. A significant amount of literature on EVOO adulteration has depended on sophisticated statistical approaches that require analyses of large numbers of samples. More effort is needed to exploit reliable chemical and instrumental methods that may not require so much statistical interpretation. Large assortments of methods have been used to determine lipid oxidation and oxidative stability and to evaluate the activity of the complex mixtures of phenolic antioxidants found in EVOO. More reliable chemical methods are required in this field to obviate excessive dependence on rapid antiradical methods that provide no information on the protective properties of antioxidants. The extensive literature on olive oil sensory tests, using many descriptors varying in different countries, should be supplemented by more precise gas chromatographic analyses of volatile compounds influencing the odor and flavors of EVOO.

KEYWORDS: Extra virgin olive oil; virgin olive oil and olive oil; methods; foods; adulteration; oxidative stability; volatiles; antioxidants; statistics; vegetable oils

INTRODUCTION

Extra virgin olive oils (EVOO) prepared by cold-pressing of olive flesh are important edible oils in the Mediterranean diet and are now recognized for their potential health benefits. Adulteration of the highly desirable and costly EVOO with cheaper vegetable oils and refined or processed olive oils has attracted much attention worldwide for both economic and health considerations. Detection of olive oil adulteration is a difficult and challenging analytical problem because EVOO consists of complex mixtures of triacylglycerols (TAGs), partial glycerides, hydrocarbons, tocopherols, pigments, sterols, alcohols, triterpene acids, volatile compounds, phenolic compounds, phospholipids, and proteins (*1*). Several international regulations have been developed to protect consumers with uniform definitions, labeling, and a multiplicity of analytical techniques to identify genuine quality standards in many countries. Although a large number of analytical methods have been developed in the past decades to determine adulteration of EVOO, the literature in this field is still controversial and confusing.

Many authors in this field are not fully exploiting the available powerful analytical methods to determine the authenticity of EVOO samples and rely too much on complex statistical methods requiring the analyses of a very large number of samples to obtain usually only qualitative or semiquantitative results.

DISCUSSION

Adulteration with Cheaper Vegetable Oils. The TAGs of EVOO contain mixtures of palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acids and traces of myristic, arachidic, heptadecanoic, and eicosanoic acids (*1*, *2*). Other constituents include partial glycerides [diacylglycerols (DAGs), monoacylglycerols (MAGs)], hydrocarbons (squalene, β-carotene, diterpenes, isoprenes, *n*-paraffins), tocopherols (α, β, γ, δ), pigments (chlorophylls and pheophytins, carotenoids), sterols, alcohols, triterpene acids, volatile compounds affecting aroma and flavors (*3*–*8*), phenolic compounds contributing to antioxidant activity and potential health effects (*9*), phospholipids, and proteins.

Much analytical work has been published in the past two decades together with extensive statistical interpretation for the detection and quantitative analyses of the type and amount of adulteration of EVOO. The analysis and authentication of EVOO represents one of the most challenging analytical problems to detect and determine its adulteration with cheaper vegetable oils and refined and not refined olive residue oils. Quality parameters defining olive oil categories, defects, identity characteristics, and fatty acid and sterols composition have been established by different international organizations. A recent book on olive oil (*9*) contains a chapter that includes five figures of unpublished data on high-resolution GC (HRGC) of DAG and non-glyceride components and HPLC of TAG profiles of a mixture of olive oil with 20% rapeseed oil. A GC method is described (*2*) that requires a high temperature of 350 °C to detect as little as 4% adulteration of EVOO with soybean oil by measuring trilinolein, which is not present in olive oil.

References selected from the extensive literature (**Table 1**) illustrate a wide assortment of analytical methods aimed at evaluating the authenticity and the presence of adulterants that can devalue EVOO. A rapid reversed-phase HPLC/differential refractometric detection system was used to determine an

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Table 1. Extra Virgin Olive Oil (EVOO) Adulteration[a]

methods	determinations	adulterants	exptl conditions	detection	threshold %	data processing	refs
RP-HPLC	TAGs	LO-rich vegetable oils	acetone/acetonitrile	differential refractometer	1	standard	*10*
NP-HPLC	tocopherols	palm oil	hexane THF solutions	fluorometric−photo diode	1−2	standard statistics	*11*
RP-HPLC	tocotrienols	grapeseed oil					
^{13}C NMR	unsaturated FA	seed oils	$CDCl_3$ solution	75.46 MHz	5	DA	*12*
HPLC-APCI MS	TAGs tocopherol, sterols	hazelnut	acetone/CH_3CN 64:36, v/v	mass spectral		ANOVA, PCA	*13*
^{13}C NMR	polar chromatogr fractions	seed oils, OO pomace oils	$CDCl_3$ solution	75.46 MHz	qualitative 97−98% validation	stepwise DA	*14*−*16*
FT-IR, ZnSe-ATR	pure vegetable oils	hazelnut + sunflower, vegetable oils	ambient	1400−900 cm^{-1}	2% sunflower oil, 25% hazenut oil	PLS, DA	*17*
visible and near-IR	spectral data	sunflower	neat samples	1100−2498 nm	0.8% SDE	PCA, HCA, SIMCA, PLS	*18*
GC, sterols, TAGs	pfficial EU methods	EVOO, 5 cultivars	cold pressing extraction	acidity, UV, GC, HPLC	correct classification	PCA, LDA, ANN	*19*
^{1}H NMR	unsat and acyl groups	edible oils	$CDCl_3$ solution	299.862 MHz IV	qualitative	standard	*20*
SPE-RP-HPLC	polar fraction	hazelnut oil	no treatment	UV	5%	standard	*21*
RP-HPLC-MS-positive APCI	TAGs, DAGs, sterols	EVOO	MeOH/H_2O HPLC	total ion intensity	qualitative 99% prediction	SDA	*22*
ESI-MS	polar phenolics FAs	edible oils	MeOH/H_2O extracts	fingerprints	qualitative	PCA	*23*
GC-MS	sterols	hazelnut oil	dimethyl-sterols	sterol fraction	<4%	standard	(*24*)
RP-HPLC-ELSD	TAGs	vegetable oils	reverse phase HPLC	ELSD FID	rel %: 5−10%	CAT-PCA	*25*
SPME-MDGC	filbertone	hazelnut oil	steam distillation	GC-MS	up to 7%	standard	*26*
synchronous fluorescence	270−720 nm	sunflower oil	hexane solutions	270−720 nm	3.4%	PLSR	*27*, *28*
	20−120 nm	vegetable oils		315−400 nm	2.6−13.8%		
GC-MS	FAMEs	edible oils	hexane solutions	areas of 23 FA + squalene	88−91% detection	SIMCA, KNN, PLSR	*29*
CEC	tocopherols tocotrienols	vegetable oils	99:1 MeOH/aqueous buffer, pH 8.0	205 and 295 nm	LOD: 1.50−2.30	qualitative	*30*
ESI-MS	rel peak intensities	olive oils	PrOH/MeOH + 40 mM KOH	rel peak intensities	5−11% prediction	LDA	*31*
^{1}H ^{31}P NMR	spectral analyses	geogr origin	EtOH/water extracts		87% prediction	CDA	*32*
Raman spectroscopy	spectral data	vegetable oils	neat	normailzed spectra	5% by vol	standard	*33*
GC, HPLC	FA, TAGs profiles	vegetable oils	IOOC global method	ECN	$R = r$ECN42/rECN44	Codex/IOOC	*34*
HPLC/APCI-MS	TAGs	plant oils	CH_3CN/2-propanol/hexane	statistical evaluation	1% sunflower oil	PCA	*35*
FT-IR spectra	spectral data	vegetable oils	neat samples	absorbance 4000−650 cm^{-1}	5%	PLS-DA	*36*, *37*

Table 1. Continued

methods	determinations	adulterants	exptl conditions	detection	threshold %	data processing	refs
gradient diffusion NMR	TAGs	plant oils	neat samples	*m*/*z* 50−1200	10% sunflower, soy, 30% hazelnut oils	DA	*38*
^{1}H NMR fingerprint	unsaponifiable matter	VOOs, vegetable oils	alcohol, sterol, hydrocarbon, tocopherol	0−5.44 ppm	geogr origin	pattern recognition	(*39*)

[a] Abbreviations: (analytical methods and instruments) APCI, atmospheric pressure chemical ionization; ATR, attenuated total reflectance; CEC, capillary electrochromatography; CEP, capillary electrophoresis; CI-MS, chemical ionization MS; CZE, capillary zone electrophoresis; DAD, diode array detection; DHS-HR-GC, dynamic headspace-high resolution GC; DTD, direct thermal desorption; ELSD, evaporative light scattering detector; EPR, electron paramagnetic resonance; ESI, electrospray interface; ES-MS, electron spin MS; ESR, electron spin resonance; FID, flame ionization detection; FT-IR, Fourier transform infrared; FT-NMR, Fourier transform NMR; GC-CIMS, GC-chemical ionization MS; GC-EIMS, GC-electron ionization MS; GC-IT-MS, gas chromatography−ion trap-mass spectrometry; GC-RI/MS, GC-refractive index/MS; HPLC, high-performance liquid chromatography; HPLC, HPLC-chemiluminescence; HPSEC, high-performance size exclusion chromatography; HR-GC, high-resolution GC; HS-MS, headspace mass spectroscopy; ^{1}H NMR, proton nuclear magnetic resonance; HS-SE, headspace-sorptive extraction; LC-MS, liquid chromatography-MS; LLE, liquid−liquid extraction; MDGC, multidimensional gas chromatography; MHz, megahertz; MS, mass spectrometry; NP-HPLC, normal phase high-performance liquid chromatography; OSI, oxygen stability index; PV, peroxide value; ROPO, refined olive pomace oil; RP-HPLC, reverse phase high-performance liquid chromatography; SHS, static headspace; SPE, solid phase extraction; SPME, solid phase microextraction; TLC, thin layer chromatography; (chemical names and products) CD, conjugateed dienes; CT, conjugated trienes; DHPEA, dihydroxyphenyletanol or hydroxytyrosol; DPPH, diphenyl-picrylhydrazyl; ECN, equivalent chain length; EDA, elenolic acid; EVOO, extra virgin olive oil; FAAES, FA alkyl esters; FAMEs, fatty acide methyl esters; HOSO, high-oleic safflower oil; LDL, low-density lipoprotein; LOOH, linoleate hydroperoxides; LOX, lipoxygenase; LnOOH, linolenate hydroperoxides; MAGs, monoacylglycerols; MDA, malonaldehyde; OPO, olive pomace oi; TAGs, triacylglycerols; TBARS, thiobarbituric acid reactive substances; THF, tetrahydrofuran; (statistical procedures) ANN, artificial neural network; ANOVA, analysis of variance; CAT-PCA, categorical principal component analysis; CDA, canonical discriminant analysis; DA, discriminant analysis; DCA, discriminant component analysis; HCA, hierarchical cluster analysis; KNN, K nearest neighbors; LDA, linear discriminant analysis; PCA, principal component analysis; PLS, regression analyses; PLSR, partial least-squares regression; PRESS, predicted residual error sum of squares; SDA, stepwise discrimination analysis; SDFA, stepwise discriminant function analysis; SIMCA, soft independent modeling of class analogy.

"authenticity" factor based on the equivalent carbon number (ECN) 42, ranging from 22.4 to 24.9 for vegetable oils high in linoleic acid (soybean, sunflower, and corn oils) compared to 1.0 for olive oil (*10*). Detection of adulteration with as little as 1% of vegetable oils was claimed possible by this approach. In another study, direct normal phase high-performance liquid chromatography (NP-HPLC) and reverse phase (RP)-HPLC/amperometric detection without saponification were used to obtain 97 and 102% recoveries of α-tocotrienols and γ-tocotrienols (*11*). No tocotrienols were detected in olive, hazelnut, sunflower, and soybean oils, but relatively high levels were found in palm and grapeseed oils. This method could detect 1% palm oil and 2% grapeseed oil in virgin and refined olive oils. A semiquantitative method using ^{13}C NMR in the olefinic region (127.5−130 ppm) was reported to detect the presence of seed oils (cottonseed, sunflower seed, soybean, and corn oils) in EVOO, which affected the intensities of 12 peaks and the α/β ratios of oleic acid (1.1) and linoleic acid (1.5) (*12*).

High-performance liquid chromatography−atmospheric pressure chemical ionization−mass spectrometry (HPLC-APCI-MS) was used to determine adulteration of EVOO with 10−50% hazelnut oil based on TAG composition and non-glyceride components (*13*). Discriminant analysis (DA) showed that hazelnut oil and mixtures with olive oil were clearly separated according to their TAG composition. In another study, ^{13}C NMR spectra were analyzed from 104 oils and mixtures from different geographical origins to distinguish among VOOs, high-oleic, and high-linoleic acid oils (*14*−*16*). Correct validation was achieved for 97.1% of the samples by using stepwise discriminant analysis (SDA). Determinations of minor components (DAGs, MAGs, and FFA) from the same oils allowed better than 98% correct validation by SDA.

Fourier transform infrared (FT-IR) was used to determine adulteration of EVOO with hazelnut, sunflower, and vegetable oils (*17*). This method together with discriminant analysis (DA) and partial least-squares analysis (PLS) allowed correct classification of olive oil and vegetable oils, with rapid detection level of 2% for sunflower oil, but only 25% and higher for hazelnut oil. Another study using visible and near-IR transflectance spectroscopy of 138 samples of EVOO adulterated with 1 and 5% sunflower oil allowed complete classification accuracy with a standard error of 0.8% by using the first-derivative treatment of spectral data between 1100 and 2498 nm (*18*). Successful discrimination between authentic EVOO and its adulterated mixtures was achieved at levels as low as 1% by using soft independent modeling of class analogy (SIMCA) and partial least-squares regression (PLSR). These relatively advanced statistical methods apparently required the analyses of a very large number of samples. Another study using 153 samples of EVOO from 5 Italian cultivars was based on Official Analytical Methods of fatty acid composition, sterols, trilinolein, and conjugated triene absorptivity at 270 nm (*19*). The authors concluded that 10 chemical indices (acidity, C16, C16:1, C18, C18:1, C18:2, C18:3, stigmasterol, sitostanol, and Δ^7-stigmasterol) are sufficient to obtain the correct classification by linear discriminant analysis (LDA).

^{1}NMR spectroscopy was a useful tool to simultaneously determine the proportions of C18:1, C18:2, and C18:3 acids in 66 samples of edible oils and of different acyl groups in 17 different vegetable oil samples (*20*). The method was based on the area of signals of spectra that is proportional to the number of hydrogen atoms and different acyl groups for each type of sample. However, correct identification of all samples required data on different cultivars and authenticity of the origin of samples. Polar components isolated by solid-phase extraction (SPE), followed by RP-HPLC with UV detection, were used to detect adulteration of EVOO with pressed hazelnut oil (*21*). Threshold levels as low as 5% were obtained with an accuracy of 90%, good precision [relative standard deviation (RSD) = 4.7%], and linearity (R^2 = 0.998) in the range of 5−40% adulteration. However, quantification of adulteration was prevented by the large variability of marker components in the hazelnut oils examined.

Correct discrimination of EVOO samples were obtained by HPLC-MS with direct injection and positive APCI detection without chemical derivatization and purification by using stepwise discriminant function analysis (SDFA) to select the variables and LDA (*22*). Correct classification and 99% prediction rate were obtained with samples from three Italian olive cultivars. The authors claimed qualitative detection of adulteration above 91 and 88% identification of the type of adulterant (sunflower, corn, peanut, and coconut oils).

Direct infusion electrospray ionization mass spectrometry (ESI-MS) was used to differentiate qualitatively unrefined olive oil from vegetable oils, to detect aging and adulteration of vegetable oils by analyzing the polar components extracted with methanol/water (1:1) from different oils and mixtures (*23*). Principal component analysis (PCA) was used to differentiate unique major diagnostic ions of olive oil from other vegetable oils (soybean, corn, canola, sunflower, and cottonseed). The corresponding ESI-MS fingerprints in the negative mode also differentiated olive oil from the other refined vegetable oils and oxidized soybean oil showing more additional ions than the fresh oil. Adulteration of virgin olive oils (VOOs) by hazelnut oil at levels of $<4\%$ was determined by GC-MS on the basis of characteristic dimethylsterols observed in significantly different relative proportions in sterol fractions (*24*). Hazelnut oils contained 86−91% 4-dimethylsterols compared to 51−57% in VOOs, whereas olive oils contained 32−40% of 4,4′-dimethylsterols. In another study, TAG profiles were determined in vegetable oils by HPLC separation using an evaporative light scattering detector (ELSD) (*25*). A total of 15 peaks were separated, identified, and quantified on the basis of the relative peak areas of 8 vegetable oils including olive oil in a total of 52 samples, using the statistical program categorical principal component analysis (CAT-PCA).

Different varieties and geographical origins of mixtures of VOOs with 10−20% hazelnut oils were analyzed by solid phase microextraction and multidimensional gas chromatography (SPME-MD-GC) (*26*). A method was developed to detect filbertone and establish adulteration of up to 7% with virgin hazelnut oils. However, this method could not be applied with some refined hazelnut oils containing very low concentrations of filbertone.

The potential application of total synchronous fluorescence spectra was combined with multivariate analysis to assess adulteration of VOO with sunflower oil (*27*). The extent of adulteration was quantified by using the PLSR model at a level of 3.4% in 2.5 min. The same technique was also used (*28*) to differentiate VOOs from olive pomace, corn, sunflower, soybean, rapeseed, and walnut oils by varying the excitation wavelength between 250 and 720 nm. Adulteration with the different vegetable oils in VOO could be detected at levels of 2.6−13.8%.

EVOO adulteration with sunflower, corn, peanut, and coconut oils was determined by sequential detection, identification, and quantitation by GC-MS (*29*). Chemometric models used were based on soft independent modeling of class analogy (SIMCA) and *K* nearest neighbors (KNN) able to predict $>91\%$ for adulterants and to identify the adulterants by 88%. Excellent precision was obtained with PLSR as shown by R^2 values of >0.90 for calibration and validation. Electrospray ionization MS was also used to predict olive oil quality according to European Union (EU) marketing standards (*30*). Samples diluted in an alkaline mixture of propanol/methanol were infused directly into the electrospray ionization source of an ion trap MS. Olive oil quality grade was predicted on the basis of ratios of peak abundance of free fatty acids (FFAs), followed by LDA. Binary mixtures of EVOO/VOO and EVOO/ROPO could be predicted with 5−11% average errors, by multiple linear regression and PLSR (*31*).

^{1}H and ^{31}P NMR spectroscopy were used to characterize monovarietal VOOs from 3three regions of southern Greece according to their contents of fatty acids, phenolics, diacylglycerols, total free sterols, and free acidity and iodine number (*32*). Canonical discriminant analysis (CDA) showed that 87% of the geographic source could be predicted. In another study, the intensity ratios of the cis (=C−H) and cis (C=C) bonds normalized by the band at 1441 cm^{-1} (CH_2), given in the form of two-dimensional charts, were used to determine the authenticity of olive oils (*33*). This method can distinguish more precisely and easily olive oils from the mixtures of olive oils containing $\geq 5\%$ by volume of other edible oils, such as soybean oil, rapeseed oil, sunflower seed oil, or corn oil.

Olive oils were classified by the fatty acids and TAGs calculated on the basis ECN42 (equivalent carbon number) and R = ECN42/ECN 44 (*34*). On the basis of the results of TAGs, experimental ECN48, ECN46, ECN50, ECN44, and ECN42 were obtained and the theoretical ECN42 and ECN44 were calculated. The values for R and ΔECN were proposed to identify adulteration, authenticity, and classification of olive oils. In another study, 93 oils including one olive oil composed of 355 TAGs by HPLC-MS were evaluated by PCA (*35*). Plant oils were authenticated by using model samples of olive oil adulterated with different concentrations of sunflower oil. Statistical multivariate data analysis enabled identification of adulterated oils from 1% of added sunflower oil.

Mid-IR and FT-IR spectroscopy combined with chemometrics [partial least-squares (PLS) and PCA model] were used to detect and quantify adulteration of EVOO with edible oils (*36*). The detection limit of adulteration was 5% for corn−sunflower binary mixtures and cottonseed and rapeseed oils. The model based on PLS analysis developed to detect adulteration was limited to 10%. In another study FT-IR was used to classify oil samples according to botanical origin and to determine the composition of binary mixtures of EVOO with cheaper oils (sunflower, corn, soybean, and hazelnut oils) (*37*). Absorbance peak areas were normalized within the FT-IR spectra as predictors of botanical origin by LDA. Multiple linear regression (MLR) models were used to determine binary mixtures as low as 5% of EVOO with other vegetable oils.

High-power gradient NMR diffusion coefficients (D) were determined to detect adulteration of EVOO for rapid screening of adulteration of olive oils with cheaper vegetable oils (*38*). Changes in D values could be detected with adulteration of 10% for sunflower and soybean oils and 30% for hazelnut and peanut oils. Correct validation of 100% was claimed for EVOO samples randomly adulterated with cheaper vegetable oils. The ^{1}H NMR fingerprint of the unsaponifiable fraction was used for geographical characterization of VOOs from Spain, Italy, Tunisia, Turkey, and Syria, based on NMR profiles of bulk oils and the corresponding non-glyceride fractions together with statistical pattern recognition techniques (*39*).

Many studies in **Table 1** are very much dependent on statistical evaluations requiring large numbers of samples, giving results expressed often in ranges that are not always precise. This table lists many statistical approaches that require special training and a multitude of samples. Too many authors appear to depend excessively on sophisticated statistical methods to determine degrees of adulteration of EVOO with cheaper oils, even though powerful analytical methods are not exploited to provide more precise and accurate chemical information. Although **Table 1** includes 16 different specialized statistical methods that were used in the analyses, more effort is needed in this field to use reliable chemical methods that would not require a large number of samples and obviate too much dependence on statistical interpretations.

Adulteration with Refined and Deodorized Olive Oils. Common adulteration practices consist of blending EVOO with low-quality and cheaper olive oils that have sensory defects and are referred to as "Lampante" oils (**Table 2**). To remove undesirable flavor volatiles derived from lipid oxidation, these oils are generally subjected to mild deodorization at lower temperatures than

Table 2. Adulteration with Refined and Deodorized Olive Oils ("Lampante" Oils)[a]

treatment	determinations	analyses	ref
steam-washing deodorization, <150−170 °C	dimer TAGs, polymers, stigmastadiene	short silica column, size exclusion HPLC	*40*
deodorization, physical refining, bleaching	stigmasta-3,5-diene	colum chromatography, HRGC	*41*
heating 160, 190 °C, 2 h	conjugated dienes	GC-MS, GC-MS/MS	*42*
neutralization, microfiltration	FFA, PV, FA composition, sterols, UV absorption	TLC, GC, polyphenols, sensory	*43*
deodorizer distillates	TAGS, steryl esters, hydrocarbons, alkyl esters, partial glycerides, FA, sterols	HPSEC, GC	*44*
esterification of FFA with low MW alcohols	fatty acid alkyl esters (FAAEs)	silica SPE cartridge GC	*45*
microwave, oven heating	FA alkyl esters, FFA, DAGs, FA composition	silica SPE cartridge, GC, volatiles, OFA, total polar compounds, tocopherols, OSI	*46*

[a] Abbreviations: see **Table 1** footnote.

conventionally practiced with vegetable oils. The level of dimer TAGs and stigmastadiene formed during deodorization was determined in olive oil and vegetable oils (*40*). A cleanup short silica gel column was used followed by size exclusion chromatography (SEC) with a refractive index detector to show the formation of dimer TAGs starting at 90 °C and increasing at deodorization temperatures.

The determination of non-glyceride components in olive oils was used to determine the effects of deodorization and physical refining. Stigma-3,5-diene produced by thermal dehydration of β-sitosterol was detected and quantified in refined oils in EVOO (*41*). A statistical study of processing parameters (N_2 flow, temperature, and oil load) was used to determine apparent kinetic constants for the formation of stigma-3,5-diene during deodorization. Another approach was based on the determination of conjugated 9,11-18:2 fatty esters produced at the elevated temperatures of deodorization (*42*). Although this study included the use advanced GC-MS and GC-MS/MS to identify one conjugated diene 9,11-18:2 fatty acid ester, formation of the corresponding conjugated 10,12-18:2 diene isomer would be expected. Both conjugated isomers can be readily determined quantitatively by standard spectrophotometric UV analysis (AOCS Standard method for specific extension in the UV: Ch 5-91).

A soft purification process for Lampante olive oils containing FFAs (3.67−7.2%) and oxidation products (PV 26−52 mequiv/kg) was based on deacidification with H_3PO_4 and oil conditioning to form micronic aggregates removed by microfiltration (*43*). The process lowered monoglycerides up to 78%, sterol components 36−50%, and phenolics 93%. In another study, the presence of refined olive oil in EVOO was determined on the basis of the complex volatile and nonvolatile compounds in deodorizer distillates. To improve GC analyses, samples were first separated by SEC into nonpolar compounds containing hydrocarbons, alkyl esters TAGs, and polar compounds including MAGs and DAGs, fatty acids, and sterols (*44*). Quantification of specific compounds in fractions was carried out by GC using internal standards.

The FFAs, MAGs, DAGs, and TAGs produced when olive fruits are stored before milling are readily converted into alkyl esters by microbial esterification with methanol and ethanol (*45*). The resulting methyl and ethyl esters can be isolated by a silica gel solid phase cartridge and analyzed by GC equipped with a programmed temperature vaporizer injector using a polar capillary column. GC analyses of 60 samples of Spanish olive oils showed wide ranges of FA methyl esters (5−23 ppm) and ethyl esters (3−40 ppm). In the Lampante olive oils, the corresponding esters ranged from 29 to 193 ppm and from 42 to 3636 ppm, respectively. Up to 800 mg/kg fatty ethyl esters was produced after soft deodorization for 4 h at 98 and 150 °C. The presence of fatty acid esters can therefore be considered a good marker of low-quality olive oil that has been subjected to soft deodorization.

The effects of hydrolysis and oxidation were also used to determine admixtures of mildly deodorized olive oil with EVOO by chromatographic and spectroscopic methods (*46*). To simulate home cooking or food catering, thermal treatments used were based on microwave and conventional heating at 180 °C for 90 min. Different mixtures of thermally stressed olive oils with EVOO were compared, but the results obtained under very artificial conditions of microwave and conventional heating may be questionable.

Oxidative Stability. Because EVOO is relatively stable to oxidation due to its relatively high levels of oleic acid, there has been apparently little or no control of shelf life in many retail markets. For these reasons, most bottles of imported EVOO found in many groceries are often not dated and generally stored for prolonged periods without controlling their shelf life at ambient temperatures. Unfortunately, when these olive oils oxidize, they eventually develop relatively high levels of rancidity, producing objectionable and undesirable flavors.

Table 3 summarizes selected studies that evaluate lipid oxidation and oxidative stability of olive oils using a large assortment of methods and their limitations. The quality of olives and olive oil was evaluated by storage stability and sensory tests (*47*). The titratable acidity of the oil was maintained below 5% after storage at 5 °C. The initial PV of 4 increased sharply to a maximum PV of 14 at 8 °C and to a maximum PV of 8 at 5 °C. In another study, the oxidative stability of commercial EVOO stored at 60 °C in the dark and under fluorescent light was compared before and after its chlorophyll and tocopherol constituents had been stripped by column chromatography (*48*). Chlorophyll was an important component that accelerated photooxidation of EVOO. Other minor components, including tocopherols, phenolic compounds

Table 3. Methods To Evaluate Lipid Oxidation and Oxidative Stability of Olive Oils[a]

methods	conditions	results	limitations	refs
acidity, PV, CD, CT, stability	storage: ambient, 5 and 8 °C	quality and stability of fruits and oils	sensory evaluations	*47*
PV, CD, TBARS	oxidized at 60 °C, dark, fluorescent light	natural vs stripped EVOO	TBARS is questionable	*48*
PV, CD, α-tocopherol, HPLC	ambient storage, 40 and 60 °C, Rancimat	OSI, 120 °C, polar phenols, squalene, β-carotene, lutein, chlorophyll, pheo	Rancimat (OSI) is questionable	*49*
α-tocopherol, polyphenols, PV, CD	effect of α-tocopherol on olive oil stability	PUFA hydroperoxides, complex polyphenols, tocopherol vs polyphenols	effect of high temperature on tocopherol stability	*50*
PV, CD, CT, FA, total polar, α-tocopherol, squalene	oxidized at 60 °C, OSI at 100 °C	tocopherol, total phenolics, *o*-diphenols, β-carotene, lutein, chlorophylls, squalene	Rancimat (OSI) is questionable	*51*
PV, UV, HPLC, ES-MS, DPPH sensory	screening stability/instability by EU methods	model developed on 10 samples: PV, CD, and lipid oxidation status	questionable DPPH antiradical test	*52*
EPR, spin trapping, Rancimat	15 samples of EVOO tested by EPR	polyphenols and tocopherols correlated with Rancimat	questionable Rancimat stability	*53*
PV, total phenols, hexanal, DPPH Rancimat	calorimetric analyses at 3, 25, 40, 60 °C	melting thermograms of EVOO, liquid fraction, viscosity, PV	questionable DPPH, Rancimat stability	*54*
OSI, GC, GC-MS	oxidative stability, OSI at 110, 98 °C model systems, total sterols	sterol composition and identification by capillary GC-MS, peanut oil used as model systems	questionable OSI stability	*55*
PV, FFA, fotal phenolics, α-tocopherol	room temperature storage for 21 months	α-tocopherol, total phenols, OH tyrosol and tyrosol complexes	changes in storage stability	*56*
OSI, PV, UV, oxidized TAG	oxidative stability, OSI at 100, 110, 120 °C, SO, HOSO, EVOO	FFA, K_{270}, unsaponifiables, FA profiles, tocopherols, TG monomers, dimers, oligomers	questionable OSI stability	*57*
PV, phenolics, tocopherols, FA profile	increase in PV, K_{232}, K_{270}, decrease in PUFAs, Arrhenius plots	rate constants, times required to reach EU standards at 25, 40, 50, 60 °C	questionable Rancimat stability	*58*
HPLC, tocopherol, FA composition	increase in PV, K_{232}, K_{270}, phenolic extract	storage at 60 °C, LLE of phenolic compounds decrease in OSI stability	questionable OSI	*59*

[a] Abbreviations: see **Table 1** footnote.

and carotenoids in stripped olive oil, influenced the oxidative stability in the dark. In another stability study, changes in VOO samples stored for 24 months in the dark at ambient temperatures were followed by PV, K_{232}, and K_{270} measurements and other HPLC analyses (*49*). Unfortunately, in this study and several listed in **Table 3**, the authors used the questionable Rancimat test at 120 °C, causing significant losses of α-tocopherol of 21−50% after 24 months of storage in the dark. In another study, the thermal oxidation of EVOO in bulk and in thin layer was compared with storage tests (*50*). As expected, the decrease in α-tocopherol concentration correlated with increases in PV, CD, and complex polyphenols after storage at room temperature for 7 months. Significant increases in CD and PV were observed at a threshold concentration of α-tocopherol of 60−70 mg/kg. Most of the α-tocopherol was lost after 72 h at 37 °C in thin layer and after 96 h at 75 °C in bulk phase.

Under accelerated storage at 60 °C, three samples of EVOO exhibited induction periods of 40, 46, and 88 days and similar trends by the OSI stability test at 100 °C and development of CD (K_{232}) (*51*). These differences in stability were attributed to the changes in oleic/linoleic acid ratio, the contents of *o*-diphenols, and loss of α-tocopherol. Carotenoids and chlorophylls showed similar trends, whereas squalene showed much higher stability. In another study (*52*), multivariate statistical analyses of EVOO showed that a combination of only three parameters, acidity, oleic acid content, and bitter taste values, could predict oil stability expressed by the PV, CD at K_{232} UV index, and oxidative status of fatty acids on 10 samples. Other parameters on oxidative status included cis−trans and trans−trans-CD, conjugated triene K_{270}, OH tyrosol, tyrosol, total aglycon, minor polar content, and sensory characteristics. The conclusion was that this simple model of predicting the future stability of EVOO, containing complex mixtures of fatty acids and phenolic compounds, based only on three parameters may not be justified.

Using a different approach, the oxidative stability of EVOO was evaluated by electron paramagnetic resonance (EPR) and spin trapping with *N-tert*-butyl-α-phenylnitrone to measure induction time at 70 °C (*53*). The EPR results correlated with oxidative stability data based on the Rancimat at 110 °C and with radical scavenging activity toward another artificial galvinoxyl radical by EPR and the contents of polyphenols and tocopherols. This study provides another example of using an expensive

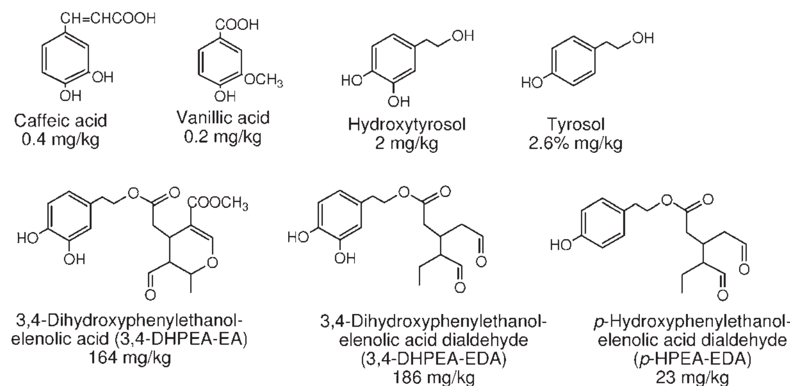

Figure 1. Phenolic compounds in extra virgin olive oil and typical average values from 116 oil samples (*78*).

sophisticated method based on measuring the tendency to form radicals during early stages of oxidation. Although the results of this method correlated with the questionable stability Rancimat test, the polyphenolic antioxidants in EVOO are known to be decomposed significantly at the high temperature used in this test. The EPR method (*60*) measures free radicals formed by initial products of lipid oxidation rather than breakdown products that are more relevant to rancidity and flavor deterioration.

The physical state of EVOO based on storage from 3 to 60 °C and oxidation products were used to predict oxidative stability (*54*). The deviation of zero-order rate constants for PV increases at 3 25, 40, and 60 °C was attributed to the increase in concentration of unsaturated TAGs and decrease in polyphenol content. Unfortunately, these authors used the questionable DPPH antiradical and Rancimat tests. Additional questionable stability tests were reported of the influence of total sterols on antioxidant activity of EVOO based on OSI testing at 98 and 110 °C with crude and refined peanut oil model systems (*55*). Although the addition of polyphenol extracts and EVOO to refined peanut oil significantly increased the OSI stability, the amounts of sterols added did not correlate with OSI stability.

Another oxidative stability study of VOOs reported significant changes in α-tocopherol from 12 to 23% and of total phenols from 43 to 73% after storage at room temperature for 21 months (*56*). Although the PV did not exceed the upper limit of 20 mequiv/kg during this storage period, the respective linoleate and linolenate contents decreased by 2.1−3.5 and 5.8−10%. Phenolic compounds were not very stable even at room temperature. Hydroxytyrosol and tyrosol levels increased linearly in most samples by the hydrolysis of the corresponding secoiridoids (**Figure 1**). In a further stability study (*57*), the formation of oxidation products from sunflower and olive oils was compared with the depletion of α-tocopherol under questionable high-temperature OSI conditions. In VOO, α-tocopherol was completely depleted after 25 h at 100 °C, after 14 h at 110 °C, and after 4 h at 120 °C. These results provide further evidence that the oxidative stability of EVOO cannot be tested reliably under the elevated temperatures of the OSI test because the phenolic antioxidants would be destroyed.

A kinetic study of EVOO oxidation was carried out at 25−60 °C in the dark (*58*). PV and increase of K_{232} followed apparent pseudo-zero-order kinetics, whereas the increase of K_{270} followed apparent pseudo-first-order kinetics according to the linear Arrhenius equation. From the loss of polyunsaturated fatty acids (PUFA), which correlated with increase of K_{232}, an accelerated stability test was set up at temperatures below 60 °C to estimate the potential shelf life under normal storage temperature conditions. In another study using accelerated storage of EVOO at 60 °C, the addition of its phenolic extract resulted in a decrease of antioxidant levels followed by an increase in oxidized products (*59*). The increase in quality parameters (K_{232} and K_{270}) corresponded with a decrease in tocopherols levels after 3 weeks in EVOO supplemented with phenols and after 2 weeks in EVOO without added phenols. Some of the phenolic compounds were also modified into secoiridoids and oxidized products, including carboxylic acid derivatives and loss of phenolic and acidic groups. These changes could be used as quality markers for EVOO.

A majority of the studies (*49,51,53−55,57−59*) in **Table 3**, like many others in the literature on the oxidative stability of olive oil, employed unfortunately questionable high-temperature Rancimat or OSI tests that cannot be used reliably to predict storage shelf life at room temperature (*60*). These tests are not reliable because the mechanism of lipid oxidation changes at the elevated temperatures at which they are run. Although at ambient temperatures, the rate of lipid oxidation is independent of O_2 pressure, at elevated temperatures, this rate becomes dependent on O_2 pressures because the solubility of O_2 decreases and the O_2 concentration becomes a significant limiting factor that increases with the degree of oxidation. In oxidative stability tests, it is therefore important to use several storage temperatures and in a range as low as practical and preferably at or below 60 °C (*60*). Polymerization and cyclization of PUFA that become important at elevated temperatures are not significant at room temperatures. Because volatile acids that are measured by the Rancimat and OSI methods are produced only at elevated temperatures, they are not relevant to normal storage conditions. The results of the Rancimat and OSI stability tests can be especially misleading in evaluating the effectiveness of antioxidants (see section E on Antioxidants). Other effects of temperature (25−80 °C) have been reported on autoxidation and changes in antioxidant levels of olive oils (*61*), including artificial and severe thermal stress conditions used to monitor oxidation by heating at 100, 150, and 190 °C (*62*) or at 150 °C (*63*) and microwave heating for extended times (*64*).

Volatile Compounds. Considerable attention has been devoted to the interpretation of sensory data of olive oils by capillary GC of volatile compounds that influence odor and flavors of EVOO. Two comprehensive reviews of VOO aromas analyzed by GC-MS listed approximately 180 compounds composed of volatile carbonyls, esters, alcohols, and hydrocarbons (*7, 8*). Important pathways of hydroperoxide decomposition by homolytic cleavage into aroma volatiles included C_5 alcohols, aldehyde, and ketone, C_6 aldehydes, alcohols, and esters, and C_8 alcohols and ketones. The most common techniques for quantitative analysis of olive oil volatiles were evaluated as “very good” for dynamic headspace and stable isotope dilution, “quite good” for solid phase microextraction (SPME) and supercritical fluid extraction, “good” for distillation-extraction, and “poor” for both static

Table 4. Potent Odorants in Different Virgin Olive Oil Samples[a]

compound	concn (μg/kg)	FD factor Italy	OAV (rn)	origin[b]	rel FD
hexanal	1770	8	24	18:2-OOH	2
(*E*)-3-hexenal	36	16	30	18:3-OOH	3
(*E*)-2-hexenal	6770	32	26	18:3-OOH	4
octanal	382	32	6.8	18:1-OOH	4
(*Z*)-2-nonenal	28	32	47	18:2-OOH	4
(*E*)- 2-nonenal	91	16	1.4	18:2-OOH	3
(*E,E*)-2,4-nonadienal	49	8	<1	18:3-OOH	2
(*E,Z*)-2,4-decadienal	255	<8	13	18:2-OOH	1
(*E,E*)-2,4-decadienal	422	16	10	18:2-OOH	3

[a] From ref *65*. FD, flavor dilution factor = extent of dilution required for detection; OAV, odor activity values = ratio of concentration to odor threshold. [b] From ref *60*. 18:1-OOH, 18:2-OOH, 18:3-OOH = hydroperoxides of oleate, linoleate, and linolenate.

headspace and direct injection GC. The level of rancidity was attributed to 2-heptenal or the ratio of hexanal/nonanal. The sensory effects of ethanol and ethyl acetate were described as "winey," and "moldy" and "earthy" defects were attributed to benzaldehyde.

Basic evaluations of the quality of VOO on the basis of flavor profiles were developed early by isolating, analyzing, identifying, and evaluating flavor-significant volatiles by high-resolution gas chromatography and gas chromatography−olfactometry of headspace samples (*65*). Flavor-significant potent volatiles of VOOs from Italy, Spain, and Morocco were isolated and quantified by "odor activity values" (OAV) based on the ratio between their concentrations and their nasally and retronasally odor threshold values when added to a bland sunflower oil. Lipid oxidation derived aldehydes of high OAVs included propanal, hexanal, (*E,Z*)-2,4-decadienal, (*Z*)-3-hexenal, (*E*)-2-hexenal, and (*Z*)-2-nonenal. **Table 4** summarizes a list of potent aldehydes, referred to as "odorants," from VOOs derived by lipid oxidation. The relative sensory impact of these volatiles was calculated by their flavor dilution (FD) factors. The most potent aldehydes in **Table 4**, based on their FD factor, include in decreasing order of flavor impact (*E,Z*)-2,4-decadienal (1), hexanal (2), (*E,E*)-2,4-nonadienal (2), (*Z*)-3-hexenal (3), (*E*)-2-nonenal (3), (*E,E*)-2,4-decadienal (3), (*E*)-2-hexenal (4), octanal (4), and (*Z*)-2-nonenal (4). Although the concentrations of linoleate are much higher (2.8−21%) than those of linolenate (0.4−1.9%) in olive oils (*9*), higher relative concentrations of aldehydes are derived from linolenate hydroperoxides (6855 μg/kg) than from linoleate hydroperoxides (2948 μg/kg), as expected, because linolenate is known to oxidize twice as quickly as linoleate (*60*).

Table 5 summarizes selected methods used to evaluate volatile compounds in olive oils. An early study conducted detailed analyses by dynamic headspace GC of 65 volatile compounds from VOOs that were related to sensory attributes by 5 European panels (*66*). By using a GC−sniffing method to assess aroma notes, the GC volatile peaks were correlated to 52 sensory attributes by PCA and a "sensory wheel." Unfortunately, too many attributes represented vague descriptors that may be difficult to reproduce by panels from different countries. Another study identified 60 volatiles in EVOOs by dynamic headspace GC−olfactometry and characterized 9 compounds to be responsible for the pleasant sensory characteristics of the oil and 51 compounds as possibly responsible for off-flavors (*67*). After heating at 100 °C and purging with O_2 for 55 h, the early formation of nonanal (regression coefficient, R = 0.98) was suggested as an appropriate method to detect initial oxidation. At later stages of oxidation, the formation of hexanal (R = 0.94) and the ratio of hexanal/nonanal were used to differentiate between oxidized and good-quality VOO samples. Although the maximum limit for PV of <20 is accepted by EC regulation (EC, 1995), the sensory panel rejected the oxidized oil when the PV was <4.

Volatile compounds were also analyzed by headspace mass spectroscopy (HS-MS) in olive oils mixed with different proportions of sunflower and olive pomace oil (*68*). The MS signal intensities of all the ions from total volatiles without separation were compared between nonadulterated and adulterated olive oils. Correct classification of 100% and prediction of adulteration were claimed by using LDA chemometric techniques with 121 samples. Unfortunately, no useful chemical information of unique volatile compounds was reported to identify and quantify more precisely any adulterants with fewer samples without using LDA.

Several headspace techniques were compared with French VOOs, including static headspace (SHS), headspace solid phase microextraction (HS-SPME), and direct thermal desorption (DTD) (*69*). The static HS (run at 110 °C and equilibrated for 120 min) was considered to be not sufficiently sensitive and unsuitable to characterize olive oil volatile compounds. However, to evaluate oxidized lipids, a much lower temperature (40 °C) is required for equilibration by HS-GC to avoid excessive decomposition of hydroperoxides (*60*).

The analysis of oxidation products in VOOs by GLC can provide useful markers to compare with sensory panel of flavor evaluations. Although the HS-GC approach is more precise than sensory methods, the results vary with different unsaturated vegetable oils, antioxidants, and metal chelators. Headspace analysis by the SPME method provides useful information on the origin of volatile oxidation products and individual flavor precursors. Total and individual volatiles can be correlated between oxidation time and flavor scores, but their flavor significance is not clearly established. As a marker of oxidative status, nonanal showed the highest rate of increment during oxidation of VOOs and was considered to be the most suitable index of degree of oxidation of olive oils (*70*, *71*). Another study based on headspace SPME showed that 2-hexenal was the main compound that characterized the oil samples (*72*). Other volatile compounds identified included mainly hexanal, 3-hexenol, 2-hexenol, and hexanol. During storage over several months, 2-hexenal decreased and C_6 alcohols and C_5 ketones increased. Another study using HS-GC at a relatively lower temperature of 50 °C for 30 min showed that octanal, nonanal, 2-decenal, and 2-undecenal were the most abundant volatiles in oxidized olive oils (*73*). These aldehydes were shown previously (*60*) to be derived by homolytic decomposition of the corresponding 11-, 10-, 9-, and 8-hydroperoxides produced by oleate oxidation.

By using dynamic headspace high-resolution GC−olfactometry, the most prominent volatile compounds responsible for the main sensory defects of olive oils (*74*) included "mustiness-humidity," attributed to 1-octene-3-ol, "fusty" to ethyl butanoate and propanoic and butanoic acids, "winey-vinegary" to acetic acid, 3-methylbutanol, and ethyl acetate, and "rancid" to several saturated and unsaturated aldehydes and acids. Virgin olive oils of 39 varieties from 8 countries cultivated together under the same agronomic conditions were characterized by 64 volatile compounds analyzed by dynamic headspace GC (*75*). GC volatiles were characterized by sensory attributes determined by olfactometry. This study showed a wide variability in the chemical and sensory characteristics of the VOOs as expected by the diversity of the varieties analyzed.

New Tunisian olive cultivars were extracted, and the oil volatiles were analyzed by SPME, showing 46 compounds characterized by GC-MS (*76*). New previously unreported volatiles included

Table 5. Methods To Evaluate Volatile Compounds in Olive Oils[a]

methods	conditions, identifications	limitations	attributes	refs
DHS-HR-GC	samples heated at 40 °C, swept with N_2, Tenax trap, desorbed at 220 °C, capillary GC	GC sniffing of 65 compounds, 9 unidentified	arbitrary and vague, statistical sensory wheel	*66*
DHS-GC, GC-MS, HRGC−olfactometry	60 volatiles identified by HRGC−olfactometry fresh and oxidized at 100 °C for 55 h	excessive thermal oxidation	samples evaluated by 4 trained assessors initially and after aging	*67*
volatile methodology, LOX pathways	static HS compared with enrichment step dynamic HS	more emphasis on lipoxygenase than chemical pathways	improved sensitivity of static and dynamic headspace GC	*4*
HS-MS	volatile compounds from mixtures of olive oils with sunflower and olive pomace oil, 121 samples	excessive heating 120 °C, qualitative threshold	correct classification and prediction by LDA chemometric techniques	*68*
SHS, HS-SPME, HSSE, DTD	>60 compounds identified by GC-RI, GC-MS	SHS not sensitive enough	SPME and HSSE were more successful than SHS	*69*
SPME-MS-FID	best fiber coating (DVB-CAR-PDMS), >100 compounds identified	determination of response factors necessary	qualitative and quantitative analyses of VOO samples	*70*
SPME, PV, UV, PUFA loss	HS-SPME volatiles compared with standard methods	pentanal from 13-LOOH coeluted with 3-pentanone	determination of oxidation reactions	*71*
HS-SPME GC-RI/MS	41 compounds isolated representing 85.3−92.7% of total	olive quality limited to (*E*)-hex-2-enal	(*E*)-hex-2-enal decreased, C_6 alcohols and C_5 ketones increased on storage	*72*
GC, SPME-MS	relationship between FA profiles and volatiles, MVA statistical analyses	correlation data are not = cause and effect	good relationship between hydroperoxide precursors and volatiles	*73*
dynamic HS-HR-GC-FID olfactometry	relationship between volatiles and sensory defects	dynamic HS involves high-temp desorption	systematic comparison GC olfactomtry and sensory	*74*
dynamic HS-GC, sensory, olfactometry	64 volatiles analyzed in 39 varieties of VOO for volatiles	origin of samples determined by statistical SLA	comparison GC−olfactometry and sensory for VOOs	*75*
SPME, GC-MS	45 compounds isolated and characterized by GC-MS = 85−98% of total amount	Rancimat used at 101.6 °C	identification of genetic new varieties of olives and VOOs	*76*
SPME, GC-EIMS, GC-CIMS, sensory	phenols and *o*-diphenols, hexanal/(*E*)-2-hexenal is important indicator of degree of oxidation	Rancimat and DPPH, ABTS antiradical methods	total polyphenols and SAT/PUFA-18:1/18:2 ratio = major parameters for antioxidant stability	*77*

[a] Abbreviations: see **Table 1** footnote.

(*E*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, trocosane, and *β*-selinene. "Oleaster" virgin olive oils were thus regarded as distinctly different from European and Tunisian oils. Although claims were made that these oils provide a rich source of natural antioxidants, only levels of chlorophylls (1.9−4.9 mg/kg) and carotenoids (1.68−4.19 mg/kg) were reported. In another study, the hexanal/(*E*)-2-hexenal ratio was considered to be a very important indicator to estimate the degree of oxidation and of the freshness of Tunisian olive oils (*77*). Ratios of total polyphenols and saturated to PUFAs (and/or ratio of oleic to linoleic acid) were shown statistically to be the major parameters in oil antioxidant stability, unfortunately, by the questionable high-temperature OSI test and artificial antiradical DPPH and ABTS tests.

Antioxidants. Olive oil has been generally considered to be nutritionally desirable for its health properties and oxidative stability not only because of its relatively high content of oleic acid but also due to the abundance of its natural phenolic antioxidants. Several reviews on VOO included the effect of extraction and processing conditions on the prevalent classes of hydrophilic phenols found, their special fatty acid composition rich in oleic acid (56−84%) and linoleic acid (3-21%) (*9*, *78*), and their oxidative stability and antioxidant activity of phenolic compounds (*79*). A variety of minor constituents found in the unsaponifiable fraction of olive oil depends on cultivars, drupe ripening, climate and environment, time of harvesting, and storage and oil processing techniques. The highest concentration of these compounds is found in EVOO obtained from the first cold pressing of the olive paste.

Table 6 presents selected studies based on methods used to evaluate antioxidants in olive oils and their limitations. Commercial olive oil samples were evaluated to determine the factors influencing their oxidative stability (*80*). Different trends in antioxidant activity were observed when tested at 60 °C according

Table 6. Methods To Evaluate Antioxidants in Olive Oils[a]

methods	conditions, identifications	limitations	antioxidants	refs
PV, hexanal	oxidized at 60 °C, in bulk oil, shaking	relatively slow oxidation	phenol extract, *o*-diphenols, α-tocopherol	*80*
SPE, RP-HPLC	polar fraction	oxid stability at 80 °C, O_2 flow	total phenols, *o*-diphenols, tyrosol esters	*81*
APCI-MS	methanolic extracts crude olive oil	qualitative, partially quantified	tyrosol, OH tyrosol, ligstroside, oleuropein/aglycon	*82*
SPE and RP- HPLC	Rancimat at 100 °C	Rancimat is questionable	*o*-diphenols, hydroxytyrosol, and α-tocopherol	*83*, *84*
PV, UV, FFA FA profile RV-HPLC	storage in diffuse and dark light, total phenols, α-tocopherol	incomplete characterization of HPLC fractions	tyrosol, OH tyrosol, vanillic, syringic, coumaric, OH tyrosol, complex phenols,	*85*
LLE−SPE−HPLC, electrophoretic methods	effect of storage at 37 and 75 °C on phenolic fractions	incomplete recoveries	α-tocopherol, tyrosol, OH tyrosol, aglycons	*86*
HPLC, CE, CZE	liquid−liquid and solid−liquid extraction	DPPH, OSI are questionable	simple phenols, secoiridoid derivative lignans	*87*
SPE-GC-MS, HPLC	phenolic extracts, TMS ethers, oxidized products	oxidation products identified by MW only	oleuropein and ligstroside aglycons and oxidation products.	*88*
radical scavenging, antioxidant activities	antiradical DPPH and antioxidant activity in liposome and Me linoleate (MeLo)	antiradical DPPH test and use of MeLo are questionable	flavonoids, OH tyrosol, tyrosol, dialdehyde derivatives, oleuropein aglycon	*89*
HPLC-DAD LDL oxidation	HPLC separation and identification of OO phenolics, inhibition of LDL oxidation	MDA is not reliable end point of LDL oxidation	OH tyrosol, tyrosol, elenolic acid derivs, diacetoxyoleuropein aglycon, oleocanthal	*90*
direct injection HPLC, fluorometric detection	RV-HPLC of reference compounds, NMR identification	incomplete separation by HPLC	ohenyl alcohols, acids, secoiridoids, oleuropein, lignans, flavonoids	*91*
polyphenols from olive leaves	different phenolic extracts tested by HPLC tested in bulk oil and emulsions	limited polyphenol composition tested	limited polyphenol composition of olive leaves	*92*
simple, complex phenols, lignans, phenolic acids	HPLC-UV/MS CE-UV heating at 180 °C	excessive thermal conditions, OSI stability	OH tyrosol, elenolic ac, oleuropein derivatives, OH tyrosol acetate, ligstroside aglycon	*93*
minor polar compounds, storage shelf life	HPLC-DAD, HPLC-MS, LDL oxidation, TBARS, DPPH	questionable TBARS and DPPH tests	OH tyrosol, tyrosol, elenolic ac derivs, oleuropein derivs	*94*
LC-MS tocopherols	total phenols, standard AOCS method: tocopherols	PLS regression analyses	α-tocopherol, OO samples from France and Spain	*95*
synthetic tyrosol esters	Rancimat, FRAP, ABTS assays in MeOH	use of Rancimat at 80 °C, and antiradical tests	tyrosyl C_4, C_{12}, C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$	*96*
3,4-DHPEA-EDA	Bis-Me acetal of oleuropein acidified to yield the dialdehyde form	use of questionable antiradical DPPH method	strong antioxidant in oil, emulsions and LDL	*97*
phenolic profile by HPLC, LC-MS of pure standards	analyses before and after 12 months ofstorage	bleaching of β-carotene, antiradical ABTS	total and individual phenols	*98*
CE-ESI-MS	polar fraction containing phenolic acids optimized by SPE	focus limited only to phenolic acids	hydroxyphenyl acetic, *p*-coumaric, ferulic, and vanillic acids	*99*
β-carotene−linoleate bleaching, ABTS/ met+H_2O_2	β-carotene bleaching, storage at room temperature, 12 months	not specific test with emulsified linoleic acid, interference by redox agents	crude phenolic extracts of Italian EVOOs	*100*
GC-MS	extraction with MeOH/water (80:20 v/v)	claim of di-OH-phenylacetic acid as initial oxidation product	several new minor phenols (0.2−1.5 mg/kg)	*101*

[a] Abbreviations: see **Table 1** footnote.

to whether measurements were made of either PV for hydroperoxide formation or hexanal for hydroperoxide decomposition. Although the samples of VOOs contained higher levels of total phenolic compounds, ranging from 63 to 534 ppm gallic acid equivalents (GAE), than did the refined, bleached, and deodorized (RBD) olive oil (8 ppm GAE) used as control, their oxidative stability was decreased by their relatively high initial PVs, ranging from 11 to 33 mequiv/kg compared to a PV of 0.4 for the RBD olive oil. Phenolic compounds extracted from VOOs increased the oxidative stability when added to the control RBD olive oil. The antioxidant activity of a mixture of phenolic compounds extracted from VOO was compared with that of pure caffeic acid, *p*-coumaric acid, cinnamic acid, vanillic acid, and α-tocopherol added to the RBD olive oil. The phenolic extract had the best antioxidant activity at 50 ppm GAE on the basis of PV, but better antioxidant activity was observed at 100 and 200 ppm GAE on the basis of hexanal formation by static headspace GC. α-Tocopherol behaved as a prooxidant at high concentrations (>250 ppm GAE) based on PV for hydroperoxide formation, but was more effective than the other phenolic antioxidants in inhibiting hexanal formation in RBD olive oil. This study emphasized (a) the need to measure at least two oxidation parameters to better evaluate antioxidants at different concentrations and the oxidative stability of olive oils and (b) the fact that the antioxidant effectiveness of phenolic compounds in VOOs can be diminished in oxidized oils.

Virgin olive oil was separated into fractions by SPE and analyzed by RP-HPLC before and after hydrolysis (*81*). Fractions analyzed for total phenols and *o*-diphenols were tested for antioxidant activity at 80 °C and O_2 flow. Three HPLC fractions were identified as mixtures of hydroxytyrosol, tyrosol, and syringic acid, and one was tentatively identified as an ester of tyrosol. In another study (*82*), phenolic profiles were identified by APCI-MS in methanolic extracts of crude olive oil, including tyrosol, hydroxytyrosol, elenolic acid, deacetoxyl-ligstroside and deacetoxyl-oleuropein aglycons, ligstroside and oleuropein aglycons, hydroxyl-oleuropein, and isomers of oleuropein (*9*). Other studies separated VOO phenols quantitatively into flavones and lignans by SPE, followed by RP-HPLC and colorimetric determination of *o*-diphenols and by NMR to confirm the aldehydic structure of the ligstroside aglycon (*83*, *84*). At the same millimolar concentrations, squalene, various phenolic compounds, and different *o*-diphenols showed similar oxidative stability by the questionable Rancimat test at 100 °C and were more stable than α-tocopherol and tyrosol. These results of oxidative stability are questionable because the polyphenolic antioxidants would be expected to decompose significantly at the elevated temperature used for the Rancimat test.

The effects of changes in the concentrations of α-tocopherol, total phenols, and complex phenols were studied during storage of VOO (*85*). After 4 months of storage under diffused light, α-tocopherol was decomposed by 79% and the phenols by <45%. Complex phenols were the least stable. After storage in the dark, α-tocopherol was decomposed by 39−45% after 6 months and total phenols by 50−62% after 12 months. When the olive oil reached a PV of 20 mequiv/kg, the remaining levels of these compounds ranged between 50 and 73% under diffused light and between 40 and 62% in the dark. In another study, the effects of α-tocopherol and complex polyphenols on the oxidative stability of EVOO were compared at room temperature in glass bottles and at 37 and 75 °C as thin layer (*86*). Depletion of α-tocopherol showed an inverse correlation with increase in CD, and the loss of total phenols was much lower than that of α-tocopherol. The phenolic fraction isolated by HPLC from VOO was extracted by liquid−liquid (LLE) and SPE and analyzed by MS as mixtures of tyrosol, hydroxytyrosol, oleuropein aglycon, ligstroside aglycon, and decarboxymethyl oleuropein aglycon.

In another study using HPLC and capillary zone electrophoresis (CZE) (*87*), simple phenols (tyrosol, hydroxytyrosol, and vanillic acid), a secoiridoid derivative (diacetoxy oleuropein aglycon), and two lignans (pinoresinol and acetoxypinoresinol) were detected as the main components in EVOO. The concentration of phenols decreased with ripeness of olive fruits. Unfortunately, the use in this study of the antiradical method DPPH and antioxidant activity test OSI is questionable (*79*).

Phenolic compounds in Spanish VOO were extracted with an SPE cartridge and analyzed by GC-MS as trimethylsilyl (TMS) ether derivatives (*88*). Identification of 21 compounds included hydroxytyrosol, tyrosol, tyrosyl and hydroxytyrosol acetate, and aldehydic and dialdehydic forms of elenolic acid linked to tyrosol and hydroxytyrosol (**Figure 1**) as the most abundant compounds. Oxidation products from the aldehydic and dialdehydic forms of elenolic acid and of ligstroside and oleuropein aglycons were detected and their structures confirmed by HPLC-APCI-MS. The radical scavenging and antioxidant activities of phenolic compounds from olive pulp and olive oils were due mainly to a 3,4-dihydroxy moiety linked to an aromatic ring (*89*). Glucosides and complex phenolics were more active antioxidants in liposomes and aglycons in bulk lipids. The antioxidant activity of lignans was attributed to their chelating properties toward copper acetate. The antioxidant activity of VOO was mainly due to the dialdehyde of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA).

Phenolic compounds from two Italian VOOs were separated by HPLC with diode array detection into tyrosol, hydroxytyrosols, oleocanthal, elenolic acid and derivative, diacetoxy-oleuropein aglycon, oleuropein aglycon, secoiridoid derivative, lignan derivative, and luteolin (*90*). The oil extracts inhibited the Cu-catalyzed oxidation of human LDL based on malonaldehyde (MDA) and conjugated dienes, and their antioxidant potency correlated with their total polar compounds. Unfortunately, MDA is a notoriously unreliable marker of lipid oxidation in foods and biological systems (*60*). The further claim that the Italian olive oils influenced biological activities is unsupported by most recent studies on absorption and bioavailability (*91*). In another study, the hydrophilic phenols from VOO were analyzed by direct injection HPLC using a fluorescence detector and compared with traditional LLE followed by HPLC (*92*). Better efficiency and quantitation were obtained for phenyl alcohols and 3,4-DHPEA-EA, but lower efficiency and quantitation were found for 3,4-DHPEA-EDA and p-HPEA-EDA (*9*) (**Figure 1**). Unfortunately, the HPLC chromatograms showed incomplete separation of many phenolic components analyzed.

Refined olive oil was supplemented with polyphenol extracts of olive leaf to obtain the same stability as VOO (*93*). Refined olive oil extracted from leaves was evaluated for oxidative stability and factors including metal concentration, cultivar, and time of year of the collection of leaves. A different study (*94*) aimed at the thermal decomposition of EVOO at frying temperatures showed that after heating at 180 °C for 30 min the levels of hydroxytyrosol (61.5%), elenolic acid (56%), decarboxymethyl oleuropein aglycon (28.3%), and oleuropein aglycon (25.3%) were rapidly depleted, but tyrosol (19.9%), hydroxytyrosol acetate (5%), and the lignan aglycon (3%) were more thermally resistant. Unfortunately, heating olive oils without foods at frying temperatures is known to cause excessive thermal damage without the tempering influence of steam produced by the moisture in foods (*60*). A stability study of bottled EVOO reported changes in minor polar compounds after storage for 18 months at 18 °C in the dark (*95*). Concentrations of tyrosol and 5-hydroxytyrosol

were relatively stable for the first 12 months and then rapidly decreased between 12 and 18 months of storage. Similarly, the secoiridoids and diacetoxy-oleuropein aglycone were depleted by 34−55 and 38−61%, respectively, after the same storage period. Unfortunately, "antioxidant" activity was based on LDL oxidation measured by the questionable TBARS test and antiradical DPPH test, without a suitable lipid substrate (*60*). A stability study of bottled EVOO reported changes in minor polar compounds after storage for 18 months at 18 °C in the dark (*95*).

A paper on phenolic extracts from 29 monocultivar olive oil samples reported that French olive oils had lower total phenol content than Spanish samples but similar individual phenolic compounds by LC-MS except for lower pinoresinol in the French oil (*96*). Amounts of α-tocopherol were generally different among the five cultivars examined. In another study, new synthetic lipophilic esters of tyrosol were less active by the Rancimat test run at 80 °C than hydroxytyrosol and its analogues, including BHT and α-tocopherol (*97*). In contrast, the hydroxytyrosol esters were more active than hydroxytyrosol in methanol solution by the FRAP and ABTS methods used without a suitable lipid substrate. Unfortunately, more sensitive and reliable methods (*60*) were needed to evaluate antioxidant activities of the tyrosyl esters.

In another advanced structural study by ^{1}H and ^{13}C NMR spectra of complex hydroxytyrosol derivatives (*98*), one of the most concentrated polyphenols in olive leaves including the bis-methylacetal of oleuropein aglycone secoiridoid (*9*) was shown to have a much stronger antiradical activity by the questionable antiradical DPPH test (*60*) than the corresponding dialdehyde form of elenolic acid or α-tocopherol. A "new" analytical method based on capillary electrophoresis−electrospray interface-MS (CE-ESI-MS) was used to identify and determine seven selected antioxidants (cinnamic and benzoic acids) and three isomeric coumaric acids (*99*). The presence in substantial amounts of hydroxyphenyl acetic, *p*-coumaric, ferulic, and vanillic acids was confirmed in Spanish VOO and EVOO samples.

A different approach was aimed at determining chemical changes in Italian EVOO samples during storage at room temperature for 12 months (*100*). The antioxidant activity in the aqueous phase was tested by the radical scavenging of the artificial ABTS [(2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)] radical cation and in the lipid phase by the β-carotene−linoleate bleaching method. Although the phenolic content was strongly correlated with β-carotene bleaching test ($R = 0.839$), it was weakly correlated with the scavenging activity by the ABTS radical cation ($R = 0.550$). The authors concluded that the phenolic compounds were not able to scavenge free radicals. This study represents one more example of a doubtful conclusion made on the basis of two invalid tests for antioxidant activity. The β-carotene−linoleate bleaching test measuring the loss of β-carotene in an emulsified aqueous system in the presence of O_2 at 50 °C is nonspecific and influenced by the micelle properties of an inappropriate emulsified linoleic acid substrate (*60*). ABTS/metmyoglobin + H_2O_2 monitors the decay of ABTS radical, using Trolox as reference, measures reactivity toward the artificial ABTS radical, and does not test antioxidants for their inhibition of lipid oxidation (*79*).

Several new minor phenolic compounds were detected in methanol/water extracts of 34 VOOs by GC-MS (*101*), including 4-hydroxyphenylacetaldehyde (0.034 mg/kg), *trans*-isoeugenol [*trans*-2-methoxy-4-(1-propenyl)phenol] (0.026 mg/kg), 1,4-dihydroxy-2,6-dimethoxybenzene (0.005 mg/kg), 3,4-dihydroxybenzyl alcohol (0.023 mg/kg), and 3,4-dihydroxyphenylacetic acid (0.17 mg/kg). The authors suggested that these compounds could be used to characterize olive oils. 3,4-Dihydroxybenzylacetic acid was reported as a naturally occurring oxidation product of hydroxytyrosol and may indicate initial autoxidation processes in olive oils. However, the evidence presented referred more specifically to a naturally occurring oxidation product of hydroxytyrosol, which, like other *o*-diphenols, proved to be a more powerful antioxidant than hydroxytyrosol (*102*).

The results of many studies listed in **Tables 3** (*49*, *51*, *53*, *54*, *58*, *59*) and **6** (*83*, *84*, *87*, *94*, *97*) confirm the misleading application of the Rancimat and OSI tests for oxidative stability and especially to evaluate the antioxidant activity of olive oil phenolic compounds. The activation energy of lipid oxidation is higher in the presence of antioxidants (∼20−25 kcal/mol) than in their absence (∼18 kcal/mol), because antioxidants lower the rates of oxidation by increasing the overall energy of activation. An Arrhenius plot of log induction period versus the reciprocal of absolute temperature shows that the effectiveness of antioxidants increases as temperature decreases (*60*). The temperature coefficients are different for a fat according to the relative concentration and effectiveness of natural or added antioxidants. Therefore, any testing for oxidative stability at lower temperatures requires testing at several different temperatures. Other problems of the frequent use of the Rancimat and OSI tests to evaluate antioxidants in olive oils include the loss of volatile low molecular weight phenolic compounds by distillation and the continuous stream of air bubbled through the oil at high temperatures. Furthermore, the activities of these natural antioxidants are markedly influenced by the effect of elevated oxidation temperatures. For these reasons the results are often widely diverging when antioxidants are compared at temperatures below and above 60 °C (*91*).

The literature reviewed on olive oil adulteration, oxidation products, oxidative stability, and antioxidants is extensive and often difficult to interpret. A wide variation in test methods is used to determine adulteration, the end-point of lipid oxidation, and oxidative stability and to evaluate phenolic antioxidants (*9*). For many oxidative stability studies of EVOO, drastic conditions are often used to accelerate oxidation. Although olive oils are generally stable to oxidation because of their relatively high oleic acid content and natural phenolic antioxidants, they are still susceptible to oxidation due to their PUFAs (5−9%) and minor constituents including chlorophylls (9−20 mg/kg), carotenoids (>10 mg/kg), and metal impurities (Fe, 0.5−3; Cu, 0.001−0.2 mg/kg) (*9*, *79*). The quality of stored commercial EVOOs can vary widely because their initial PV can range between 0.4 and 33 mequiv/kg (*80*). Interactions between minor components in EVOO and trace metals can produce prooxidant effects. The choice of methods and conditions to evaluate oxidative stability and antioxidants is therefore critical (*60*, *79*, *91*). Oxidative stability evaluations of EVOOs from Greece, Italy, and Spain showed induction periods varying from 40 to 88 days at 60 °C, with PV ranging from 6 to 37 mequiv/kg after ambient storage between 11 and 24 months (*79*). Storage under different conditions resulted in losses of polar phenolic antioxidants of 18−38%.

Several potential problems become apparent from the extensive literature published in the past several decades on different kinds of olive oils. Many studies on the adulteration of EVOO with cheaper vegetable oils were based on advanced sophisticated statistical methods that require the analyses of large numbers of samples (footnote of **Table 1**). Powerful analytical methods are now available to provide more precise and accurate chemical information on olive oils that may obviate too much dependence on statistics.

For the adulteration of EVOO with cheaper soybean and canola oils that are most available in the United States and Canada, many potential problems may be caused by high-temperature GC to analyze olive oil directly for adulteration. Linolenic acid ranging from 7 to 10% in soybean and canola oils

may undergo excessive thermal decomposition at the elevated temperatures (> 350 °C) used for GC separation of TAGs (*103*). The quantitation of olive oil samples adulterated with these vegetable oils would also be compromised because the TAGs containing linolenic acid are distributed among several peaks in the gas chromatograms. The use of high-temperature GC may also influence the quantitative analyses of non-glyceride components by thermal decomposition. More reliable and quantitative analytical results may be achieved by exploiting many HPLC methods available for TAGs and minor constituents of vegetable oils.

Questionable methods were used in several studies listed in **Tables 3** and **6**, including the Rancimat and OSI tests for oxidative stability and the antiradical methods DPPH and ABTS for antioxidant activity (*79*). At the elevated temperatures of the Rancimat and OSI tests the phenolic antioxidants in EVOO are significantly destroyed and the mechanism of lipid oxidation changes. These antioxidants would not be destroyed by using oxidative stability tests run at 60 °C (*80*) or lower. The use of the antiradical methods DPPH and ABTS to determine antioxidant activity of EVOO are also questionable because they measure free radicals by using completely artificial scavengers in the absence of suitable lipid substrates (*79*).

Other invalid methods used for olive oils include the β-carotene−linoleate bleaching test, measuring the loss of β-carotene in an emulsified aqueous system, and the ABTS/metmyoglobin + H_2O_2, monitoring the reactivity toward the artificial ABTS radical, without testing for inhibition of lipid oxidation. Another questionable and relatively expensive method used to measure lipid oxidation is based on measuring the formation of free radicals during the early stages of oxidation by EPR or ESR using artificial free radical scavengers (*60*). These methods use completely artificial synthetic free radical scavengers that perturb the kinetics of lipid oxidation and do not evaluate breakdown decomposition products causing rancidity in olive and other oils.

Much literature on olive oil sensory tests has been based on panels trained to recognize and evaluate many attributes representing vague descriptors that may be difficult to reproduce by panels from different countries. More precise and diagnostic chemical information may be expected by supplementing the sensory tests with capillary GC analyses of olive oil volatiles. Although some detailed GC−/olfactometry methods (*66*, *73*, *74*) have been used to characterize samples of EVOOs, more detailed information is needed to relate volatile profiles with sensory attributes and defects. Useful information of unique volatile compounds could also be exploited to identify and quantify more precisely the adulteration of EVOO with vegetable oils.

Many claims have been made regarding the nutritional benefits of using olive oils in the Mediterranean diet. Such claims may be exaggerated, however, and unsupported by the most recent studies on absorption and bioavailability (*91*). A wide variety of biological protocols have been used to evaluate the nutritional benefits of natural antioxidants in EVOOs. This subject will be reviewed in a separate publication on the nutritional and biological properties of EVOOs and their antioxidants.

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