Characterization of Phenolic Compounds in Virgin Olive Oil and Their Effect on the Formation of Carcinogenic/Mutagenic Heterocyclic Amines in a Model System

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Mutagenic heterocyclic amines (HAs) are formed at low levels during cooking of meat and fish, and some of them are considered to be possible human carcinogens. The formation of HAs may be affected by the presence of synthetic or naturally occurring antioxidants. In the present study the effect of virgin olive oil (VOO) phenolic compounds, identified and quantified by LC-MS, on the formation of HAs in a model system was evaluated. An aqueous solution of creatinine, glucose, and glycine was heated in the presence of two samples of VOO differing only in the composition of phenolic compounds. The addition of VOO to the model system inhibited the formation of 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) by between 30 and 50% compared with the control. Fresh-made olive oil, which contained a high amount of dihydroxyphenylethanol derivatives, inhibited HA formation more than a 1-year-old oil did. The inhibition of HA formation was also verified using phenolic compounds extracted from VOO.

Keywords: *Heterocyclic amines; phenolic compounds; virgin olive oil; LC-MS; Maillard reaction; mutagen; antioxidants*

INTRODUCTION

Heat-induced nonenzymatic browning (Maillard reaction) occurs in most foods, and such reactions often lead to the formation of antinutritional and genotoxic compounds. The finding of potent mutagenic activity in the charred surface of broiled fish (*1*) began intensive research to identify and quantify the mutagenic substances. They were later classified as heterocyclic amines (HAs) (*2*) and were shown to be mutagenic and carcinogenic in animal tests (*3*−*6*). Several studies have shown that human cells metabolize HAs, which result in DNA damage. These results, in combination with some epidemiological data showing a relationship between fried meat and cancer, suggest a carcinogenic effect of HAs for humans who are genetically susceptible or highly exposed to high levels of them (*7*, *8*). In the search for precursors and reaction pathways for HAs, model systems simulating mutagen-forming reactions have been useful tools (*9*−*12*). Chemical-based and meat-based model systems have been shown to yield similar results. Moreover, results generated in model systems agree with realistic cooking experiments in terms of yield and pattern of HAs (*5*, *13*, *14*). The most abundant HAs formed at normal cooking conditions are 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (*15*).

The Maillard reaction has been suggested to play an important role in the formation of some HAs (*9*), and amino acids, sugars, creatine, and creatinine have been shown to be their precursors (*15*). However, a free radical mechanism leading to the formation of HAs has been proposed. Pearson et al. (*16*) suggested that dialkylpyrazine free radicals and creatinine produce MeIQx and 4,8-DiMeIQx. Milic et al. (*17*) proposed a contribution of both the Maillard reaction and the free radical mechanism. Different antioxidants in spices, tea, and cherry tissues have been reported to reduce HA formation (*18*−*24*). This prompted us to investigate if antioxidant compounds present in virgin olive oil (VOO) could reduce the formation of HAs in a simple model system. VOO is commonly used for meat cooking in typical recipes of the Mediterranean diet.

The chemical composition of VOO is characterized by high amounts of unsaturated fatty acid (mainly oleic acid) and by specific phenolic compounds, which amount to up to 1200 mg/kg of oil (*25*, *26*). The phenolic fraction consists of the so-called secoiridoid derivatives, formed by *p*-hydroxyphenylethanol (p-HPEA) or by dihydroxyphenylethanol (DHPEA) linked to elenolic acid. Phenolic compounds, particularly *o*-dihydroxy derivatives, have strong antioxidative activity and are essential to preserve the fatty acid moiety of VOO from oxidative damage during processing and storage (*27*, *28*). Furthermore, *o*-dihydroxyphenolic compounds contribute to the stability of the oil, due to their ability to donate a hydrogen atom and form an intramolecular hydrogen bond between their hydroxyl group and phenoxyl radicals (*29*). The absolute concentration of phenolic compounds in olive oil is the result of complex interactions

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between several factors, including cultivar, ripening degree, climate, and extraction process (*30*−*35*). The amount of phenolic compounds is also affected by oxidative and hydrolytic modification during storage (*36*).

Increasing interest in the beneficial effects of the Mediterranean diet on coronary heart disease and cancer led several scientists to study the effect of the VOO phenol fraction on human health. The VOO phenol fraction has been reported to contribute to lower low-density lipoprotein (LDL) oxidation in vitro and in vivo (*29*, *37*, *38*) and to protect caco-2 cells from oxidative damage (*39*). Furthermore, in persons with peripheral vascular disease, a VOO-rich diet increases the resistance of LDL to oxidation (*40*). The lack of methods for an unequivocal identification and quantification of each phenolic compound present in VOO is the bottleneck for in vitro and in vivo studies aiming to clarify the effects of VOO on human health. However, in a recent study, liquid chromatography−mass spectrometry (LC-MS) was successfully applied for identification and quantification of phenolic compounds in VOO (*41*, *42*).

One of the aims of this work was to chemically characterize phenolic compounds in freshly made and 1-year-old VOO. To achieve this goal, a high-performance liquid chromatography with diode array detection (HPLC-DAD) procedure and an LC-MS procedure for quantification of the main phenolic compounds have been developed. Another aim was to examine the effect of these phenolic compounds on the formation of HAs in a model system.

MATERIALS AND METHODS

Chemicals. All chemicals and solvents were of HPLC or analytical grade. 2-Amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) were purchased from Toronto Research Chemicals (Downview, Canada). Reference solutions were prepared with a final concentration of each HA of ~2 ng/μL. L-Glycine was purchased from Merck (Darmstadt, Germany), and glucose and creatinine were from Sigma (Kebo-lab, Sweden). Oleuropein was from Extrasynthese (Genay, France). The materials used for the extraction and purification of the HAs, diatomaceous earth (Extrelut), PRS columns (Isolute), and C_{18} columns (Varian), were purchased from Sorbent (Västra Frölunda, Sweden). Standard fatty acid methyl esters (FAMEs) were from Larodan (Malmö, Sweden).

VOO Samples. VOO was obtained from olives (*Olea europaea* var. Nostrale) harvested and extracted at Castel San Lorenzo (Salerno, Italy) in November 1998 in a continuous extraction plant, using a percolation−centrifugation system (Sinolea Rapanelli S.p.A., Foligno, Italy) with hammer crushers, centrifugal decanter, and separator. Unfiltered oils were stored in filled amber glass bottles at room temperature (between 14 and 25 °C) for 1 year. To evaluate the storage effect, chemical analyses were performed immediately after extraction and after 1 year of storage. Chemical characteristics of the two oil samples were monitored by measuring free acidity, peroxide value, and specific extinction K_{232} (conjugated dienes) and K_{270} (trienes and carbonyl compounds) values. All analyses were performed according to the official European methods for olive oils (*43*).

Fatty Acid Composition. FAMEs were determined by gas chromatography (GC) with flame ionization detection (FID) according to the method described by Márquez-Ruiz and Dobarganes (*44*) using a Quadrex Corp. (New Haven, CT) fused-silica capillary column (50 m × 0.25 mm i.d.) coated with cyanopropyl methyl silicone (0.25 μm film thickness) and a Shimadzu (GC-17A) gas chromatograph. The oven temperature was set at 80 °C for 5 min, then increased to 220 °C at a rate of 10 °C min^{-1}, and finally set at 220 °C for 6 min. The injector temperature was 220 °C and the detector temperature 250 °C. Individual FAMEs were identified by comparing their retention times with those of authentic standards.

Extraction of Phenolic Compounds. Phenolic compounds were extracted from the different oils as described by Vasquez-Roncero (*45*). In brief, olive oil (50 g) was dissolved in hexane (50 mL), and polar compounds were extracted with methanol/water (3:2, v/v, 3 × 30 mL). Each extract was washed with hexane (50 mL). The extracts were then combined, and the solvent was evaporated under reduced pressure at 40 °C. The residue obtained was weighed, dissolved in methanol, filtered through 0.2 μm filters (Amicon), and used for analysis and model experiments. The amount of phenolic compounds expressed as gallic acid equivalents (milligrams per kilogram) was also determined using Folin−Ciocalteau reagent (*35*). The antioxidative capacity of the crude phenolic extract was measured using the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) colorimetric method as described by Pellegrini et al. (*46*).

Analytical and Preparative Separation of VOO Phenolic Extracts. Chromatographic separation of VOO phenolic compounds was performed by HPLC using a Shimadzu instrument equipped with SCL-10A VP pumps and an SPD-M10A diode array detector. Chromatography was achieved on a Spherisorb S5 ODS-2 reversed-phase column (particle size = 5 μm; 4.6 mm i.d. × 250 mm) and on a Supelco LC18 column (particle size = 5 μm; 10 mm i.d. × 250 mm) for analytical and preparative separation, respectively. Solvent gradient and peak collection were performed as described by Fogliano et al. (*34*).

LC-MS Analysis of VOO Phenol Extracts. LC-MS analysis was carried out on an API-100 single-quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments) equipped with an atmospheric pressure chemical ionization (APCI) ion source. The instrument used nitrogen as curtain gas and air to dry the ion spray. Preliminary experiments were also performed using electrospray ion source (ESI). The HPLC equipment was connected to the MS detector, and chromatographic separation was carried out as described above with UV detection at 240 nm and a flow rate of 0.8 mL/min. Ionization was achieved at a temperature of 70 °C using a probe voltage of 4.8 kV and a declustering potential of 70 V. The mass-to-charge ratio scale was calibrated with the ions of ammonium adduct of polypropylene glycol. Full-scan spectra were acquired from 100 to 800 atomic mass units (amu) using a step size of 0.5 amu and a dwell time of 2 ms. Single ion monitoring (SIM) was performed simultaneously for four ions: 305, 321, 363, and 379 amu corresponding to the molecular ions (MH^+) of the main phenolic compounds. In these experiments a 500 ms dwell time was selected.

HA Model System. Model experiments were carried out according to the method of Johansson et al. (*47*) with slight modifications. Briefly, precursors creatinine (0.9 mmol), glycine (0.9 mmol), and glucose (0.45 mmol) were dissolved in Milli-Q water (2.0 mL) in stainless steel test tubes to which olive oil (500 mg) or phenolic compounds (between 0.25 and 25 mg, i.e., a final concentration between 100 and 10000 mg/kg) had been added. The final volume was brought to 2.5 mL by the addition of methanol. Test tubes were closed and vortexed for 1 min and then heated in an oil bath at 180 °C for 30 min. Oil temperature was carefully controlled (±0.1 °C) by a regulator equipped with a thermocouple (*13*). After heating, test tubes were immediately cooled in an ice bath. All experiments were performed in duplicate or triplicate. Control samples were heated without the addition of oils or phenolic compounds.

Solid-Phase Extraction, Identification, and Quantification of HAs. Heated samples were extracted using the solid-phase extraction method of Gross and Grüter (*48*), with modifications from Johansson et al. (*47*). After evaporation, samples were dissolved in methanol (200 μL) and analyzed by HPLC as previously described (*13*). HAs were identified by comparing retention times and UV spectra with those of reference compounds. Inhibition of HA formation was calcu-

Table 1. Main Analytical Parameters of the Two Samples of VOO Used in the HA Inhibition Assays[a]

oil sample	free acidity	peroxide no. (mequiv of O_2/kg)	K_{232}	K_{270}	ΔK	total amount of phenols (mg/kg)	antioxidative activity (TEAC)
fresh	0.19[a]	4.56[a]	1.440[a]	0.096[a]	−0.0030[a]	810[a]	36.5[a]
1-year-old	0.42[b]	9.12[b]	1.936[b]	0.159[b]	−0.0020[b]	780[a]	27.1[b]

[a] Different letters, within each parameter, indicate significant differences.

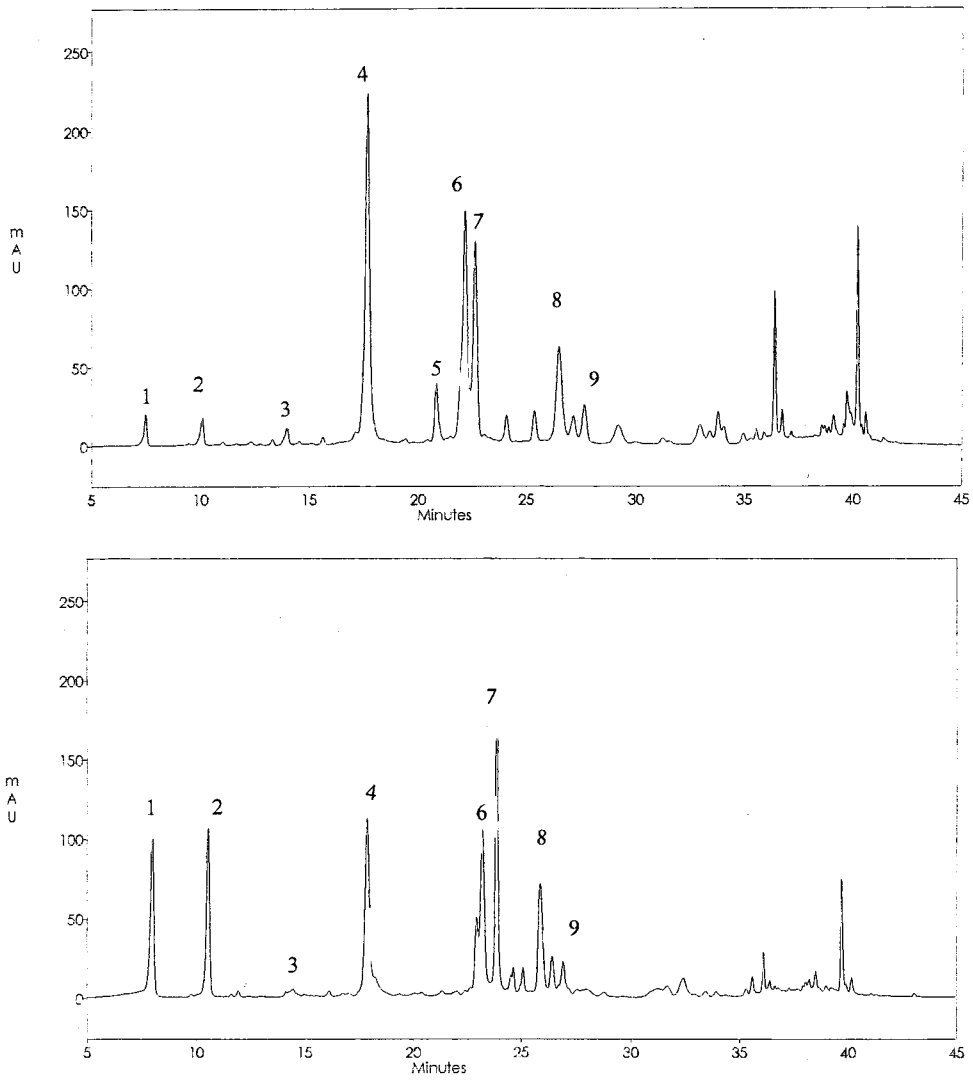

Figure 1. UV chromatograms at 280 nm of the phenolic fraction extracted from (A) fresh and (B) 1-year-old oil samples. Peaks: 1, 3,4-DHPEA; 2, p-HPEA; 3, *p*-coumaric acid; 4, 3,4-DHPEA-EDA; 5, unknown compound; 6, p-HPEA-EDA; 7, pinoresinol (**58**); 8, 3,4-DHPEA-EA; 9, p-HPEA-EA.

lated by comparison of the amounts of IQx, MeIQx, and DiMeIQx in samples with and without the addition of phenolic compounds. Control experiments were performed for each trial, and extraction recovery rates were determined as previously reported (*49*).

RESULTS AND DISCUSSION

VOO Characterization. The two oil samples were characterized by determining free acidity, peroxide value, conjugated dienes and trienes (K_{232}, K_{270}, and ΔK), and fatty acid composition (grams per 100 g of oil): palmitic acid (11.42); palmitoleic (0.73); stearic (2.30); oleic (75.49); vaccenic (2.19); linoleic (5.63); and linolenic (0.64). Total amount of phenolic compounds and antioxidative activity were also measured. The main data are summarized in Table 1. All analytical parameters fulfill the requisite of the virgin olive oil (*43*). As expected, the peroxide value and other oxidation parameters were lower for the freshly made oil. The freshly made oil had the highest antioxidative capacity. A 25% decrease of its Trolox equivalent antioxidant capacity (TEAC) value was recorded after 1 year of storage.

HPLC-UV chromatograms displaying the phenolic profile of the two oils are shown in Figure 1. Compounds eluting before 15 min are simple phenols and phenolic acid derivatives of hydroxycinnamic and hydroxybenzoic acid, accounting for roughly 10% of the total amount of phenolic compounds in the oils. Their antioxidative capacity is low (*27*, *34*). Compounds eluting after 15 min are complex phenols formed by elenolic acid linked to simple phenols (*50*). It is evident that hydrolysis of complex phenolic compounds occurs during storage, with a corresponding increase of simple phenols, which corresponds with results from other studies (*36*). Identification and quantification of the complex phenolic

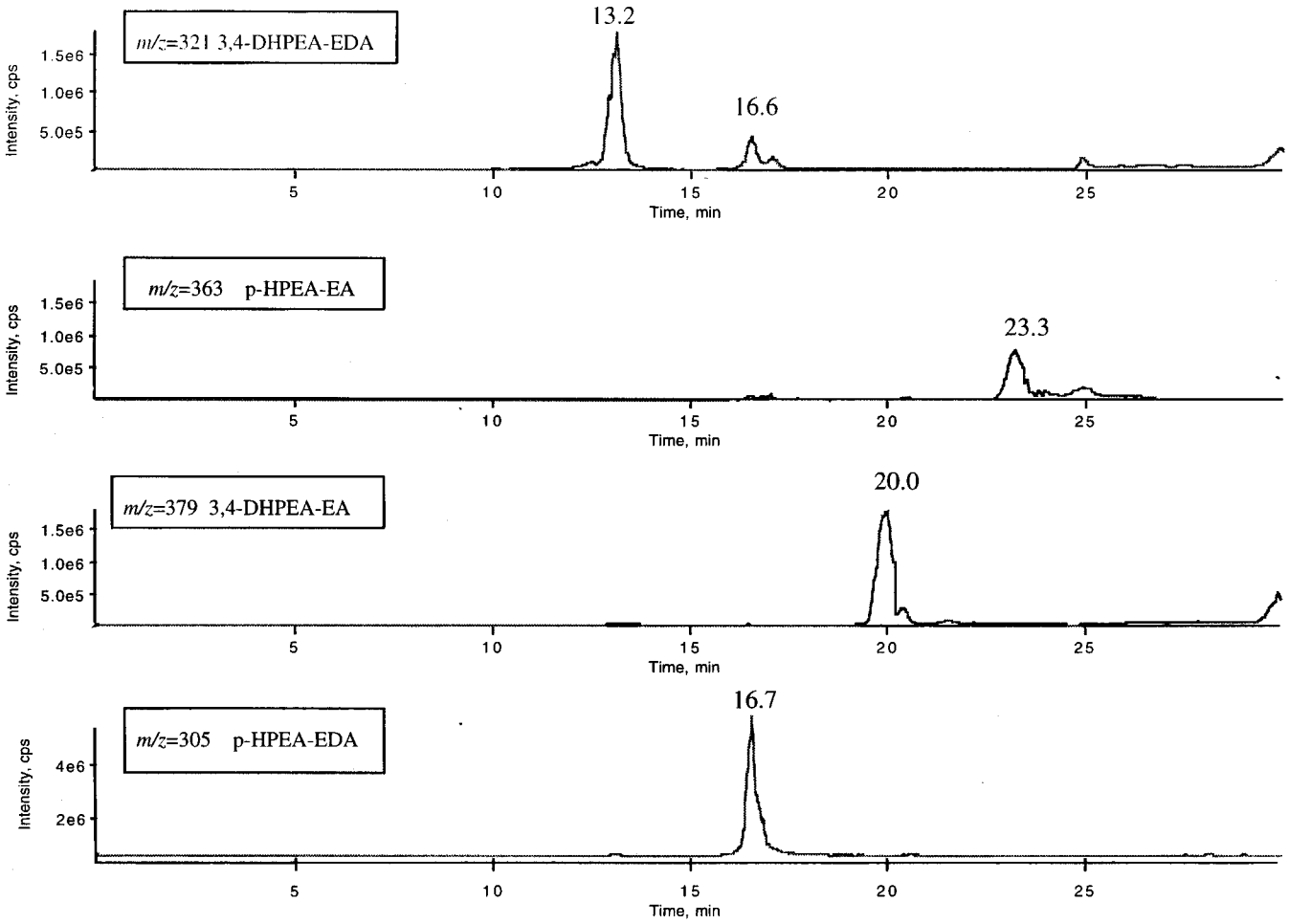

Figure 2. HPLC-MS chromatographic analysis of phenolic extract from VOO. Detection was performed in SIM mode monitoring the *m*/*z* indicated.

Table 2. Amount (Milligrams per Kilogram of Oil) of the Main Complex Phenols in the Two Virgin Olive Oils[a]

oil sample	3,4-DHPEA-EDA	SD	p-HPEA-EDA	SD	3,4-DHPEA-EA	SD	p-HPEA-EA	SD
fresh	350[a]	± 25	240[a]	± 25	40[a]	± 4	45[a]	± 8
1-year-old	230[b]	± 20	200[a]	± 16	10[b]	± 1	35[a]	± 5

[a] Different letters, within each parameter, indicate significant differences.

compounds present in the second part of the chromatograms are hampered by the lack of commercially available reference compounds. However, their identification is easily achieved using mass spectrometry, which showed for each chromatographic peak both molecular ion and fragmentation pattern (*41*). Preparative HPLC and peak collection allowed us to obtain sufficient pure material to develop calibration curves for the following phenolic compounds: 3,4-DHPEA-EDA, p-HPEA-EDA, 3,4-DHPEA-EA, and p-HPEA-EA. The curves were obtained using SIM to enhance detection sensitivity, which was between 50- and 200-fold higher than UV detection. A good linearity ($R^2 > 0.99$) was obtained in the range between 1 and 500 ppm. It is worth noting that when ESI was used instead of the APCI source, as reported by Ryan and co-workers (*42*, *51*), the signal was lower and fairly reproducible. Figure 2 shows a chromatogram from LC-MS analysis (SIM) of a phenolic extract from VOO. In a single run the four phenolic compounds were simultaneously quantified, and the results are reported in Table 2. The precision of the method expressed as coefficient of variation was between 7 and 15% for the four compounds. Comparison between the two samples clearly shows that 3,4-DHPEA-EDA and 3,4-DHPEA-EA undergo severe degradation during oil storage (decreases of 35 and 75%), whereas p-HPEA derivatives were less affected (decreases of 16 and 22%). This result is in agreement with the reported decrease of VOO antioxidative activity during storage (*52*), confirming that there is a strong correlation between the presence of oleuropein derivatives and the antioxidative activity of the VOO.

Effects of VOO on Formation of HAs. The two oil samples with known phenolic composition were assayed in a model system established as resembling well the conditions for formation of HAs in real meat (*15*). Heating an aqueous solution of creatinine, glucose, and glycine without VOO yielded IQx, MeIQx, and DiMeIQx at a level of ~10 nmol/mmol of creatinine, which is similar to results obtained earlier (*47*). The addition of VOO inhibited the formation of IQx derivatives compared with the control sample heated without oil (Figure 3A). Fresh oil inhibited the formation of IQx, MeIQx, and DiMeIQx by 45, 50, and 59%, respectively, whereas 1-year-old oil inhibited the formation by 27, 13, and 42%, respectively. The possibility of an oil matrix effect

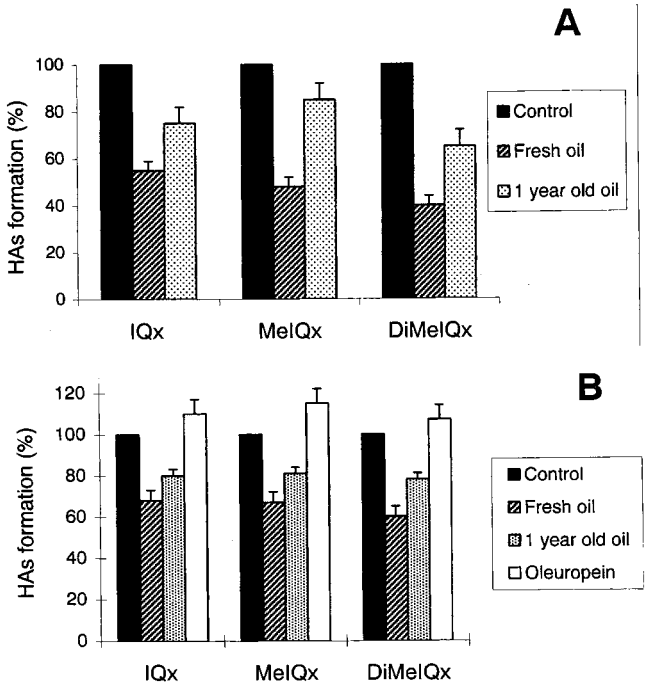

Figure 3. Effect of VOO (500 mg) (A) and effect of phenolic compounds from VOO and pure oleuropein (1100 ppm) (B) on IQx, MeIQx, and DiMeIQx formation.

leading to a reduction of MeIQx formation was ruled out because in previous experiments (*47*) an increased level of MeIQx was observed after the model system had been heated with corn oil and olive oil, different from VOO. The promoting effect of the oils on HA formation was attributed to lipid oxidation occurring during heating. The results obtained in the present study suggest that lipid oxidation is counteracted by the VOO phenol compounds, which were not present in the other oils.

Lipids enhance the formation of certain Maillard reaction products such as pyrazines and aldehydes (*53*), molecules that contribute to the formation of the quinoxaline part of the imidazoquinoxalines (*54*). Furthermore, formation of free radicals from thermal oxidation of lipids can occur during cooking. This phenomenon favors condensation reactions, which lead to the formation of HAs. The phenolic antioxidants of VOO not only interfere with the radical mechanism of HA formation but also prevent lipid oxidation in the system studied, as observed also during heating and frying (*37*).

The freshness of the oil influences the antioxidative capacity: the fresher, the better. This has been confirmed in this study, in which the freshly made olive oil inhibited HA formation more than the same oil after 1 year of storage. The inhibition of HA formation seems to be related to the presence of the 3,4-DHPEA derivatives (Table 2), which hydrolyze during storage (*29*, *36*). Previous studies have demonstrated that this compound is the main constituent responsible for the antioxidative capacity of the oil (*34*) and for oil resistance to accelerated oxidation (*52*).

Effects of Phenolic Compounds Extracted from VOO on the Formation of HAs. To evaluate the effect of VOO phenolic compounds on HA formation in absence of the lipid matrix, the crude phenolic extracts from the two VOO samples were assayed. Results obtained using a final phenolic concentration of 1100 ppm are shown in Figure 3B. Phenolic compounds from fresh oil inhibited the formation of IQx, MeIQx, and DiMeIQx by 32, 32, and 38%, respectively, whereas those from 1-year-old oil led to decreases of 21, 21, and 20%, respectively. Phenolic compounds from fresh oil, which was richer in *o*-diphenolic compounds, inhibited the formation of IQx derivatives more than phenolic compounds from 1-year-old oil did. Addition of oleuropein to the model system resulted in a slight increase of the formation of IQ compounds (Figure 3B). Oleuropein has the same reactive groups (i.e., the *o*-diphenols) as 3,4-DHPEA-EDA but is more hydrophilic due to the carbohydrate moiety. It is known that the relative polarity of the environment and of the antioxidant compounds is a fundamental matter when the antioxidative capacity is studied (*55*, *56*). Our data point out that also for HA formation the polarity of the antioxidant play a pivotal role to determine its effect.

The dose-dependent effect of phenolic compounds on HA formation was also investigated. When the concentration was decreased from 1100 to 110 ppm, only a slight decrease of HA formation was observed. On the other hand, when the concentration was increased to 11100 ppm, HA formation increased above that of the control. This effect may be related to the pro-oxidant action observed for many antioxidant compounds at high concentration (*57*). In previous studies (*20*, *22*), in which different antioxidants were used to prevent HA formation in similar model systems, inhibition effects were observed only at very high concentrations (>10000 ppm).

Besides the results obtained with whole VOO with respect to isolated phenolic compounds (cf. Figure 3A,B), it is also interesting that a concentration of phenolic compounds between 100 and 1000 ppm, which gave the more efficient HA inhibition, is of the same order of magnitude as the phenolic concentration present in a good quality VOO.

In conclusion, using antioxidants from olive oil, an inhibition of up to 60% of the formation of the different HAs was observed, which supports the hypothesis that HA formation partially involves free radical reactions. A reaction between phenolic compounds and key intermediates of HA formation may also contribute to reduce the levels of HAs. To the best of our knowledge, this is the first study in which phenolic compounds in VOO have been characterized and shown to reduce the formation of HAs in a model system.

Some epidemiological studies have shown an association between the intake of fried meat and cancer in the large intestine, breast, and prostate, although other studies have shown no such relationship. Cancers in these organs are typically associated with a "Western style" diet; however, the risk imposed to human health by HAs is not clear. The putative risk depends on the level of exposure, dietary factors that influence their uptake and biotransformation, and the capacity of the individual to handle HAs. Our results suggest that phenolic compounds in VOO can reduce the formation of HAs during domestic cooking, and research is now ongoing to examine the effect in real cooking experiments. Using "functional VOO" designed to contain a very high level of phenolic compounds could be one way of minimizing the formation of HAs. Besides the beneficial effects for human health reported for this type of fat, the capacity of VOO to decrease the formation of HAs represents a further additional value to the Mediterranean diet in which VOO is traditionally used also for marinating raw meat before grilling.

ABBREVIATIONS USED

VOO, virgin olive oil; p-HPEA, 4-hydroxyphenylethanol; 3,4-DHPEA, 3,4-dihydroxyphenylethanol; 3,4-DHPEA-EA, oleuropein aglycon; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked with 3,4-DHPEA-EA; p-HPEA-EA, ligstroside aglycon; p-HPEA-EDA, dialdehydic form of elenolic acid linked with p-HPEA; IQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 7,8-DiMeIQx, 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline; SIM, single ion monitoring; LC-MS, liquid chromatography−mass spectrometry; HAs, heterocyclic amines; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; LDL, low-density lipoprotein.

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