Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 100 (2007) 998–1004
Food Chemistry
www.elsevier.com/locate/foodchem

Studies on oxidative stabilisation of lard by natural antioxidants recovered from olive-oil mill wastewater

Antonella De Leonardis [a,*], Vincenzo Macciola [a], Giuseppe Lembo [b], Alessandra Aretini [b], Ahindra Nag [c]

[a] Department of Agricultural, Food, Environmental and Microbiological Science and Technologies (DiSTAAM), University of Molise, via De Sanctis, 86100 Campobasso, Italy
[b] Department of AngioCardioNeurology, IRCCS Neuromed, Località Camerelle, 86077 Pozzilli (IS), Italy
[c] Department of Chemistry, Bioorganic Laboratory, Indian Institute of Technology (IIT), 721302 Kharagapur, India

Received 1 August 2005; received in revised form 28 October 2005; accepted 28 October 2005

Abstract

Free phenolic compounds was extracted by ethyl acetate from olive oil mill wastewater. Extraction yield was 50 mg of total phenol on 100 g of wastewaters. Phenolic compounds recovered, determined by HPLC, were hydroxytyrosol (as major compound) and tyrosol, caffeic acid and ferulic acid (as minor compounds). For determination of antioxidant effects by Rancimat, phenolic extract, dissolved in distilled water, was added in different lard samples in quantity ranged between 50 and 350 mg kg^{-1}. Antioxidant effectiveness was measured also at low temperature.

It was confirmed that natural antioxidants extracted from olive mill wastewater are highly effective for oxidative stabilization of lard. Cytotoxicity assay showed that wastewaters phenolic extract did not inhibit cell growth at the doses of 100–200 ppm. Thus, lard with olive phenols is a possible ''novel food'' for health benefit and contributes a significant utilization of olive waste water.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Olive-mill wastewater; Lard; Phenolic compounds; Antioxidant; Cytotoxicity

1. Introduction

Lard is an edible fat derived from pigs that is appreciated as an important ingredient for cooking. In 2000 the world production of lard was 5.4 million tons and the major manufacturer countries were China, the USA and Germany (FaoStat, 2000).

Generally, different qualities of lard are related to the parts of pig from which they originate, produced by steam-fusion or cut. Lard is prevalently composed of saturated fatty acids (40–45%), such as palmitic (25%), and stearic (15%) acids and also monounsaturated fatty acids (40–45%), especially oleic acid. It also contains a significant quantity of linoleic acid (10–15%).

Original lard is generally poor in antioxidants, with the exception of low quantities of tocopherols, ranging from 30 to 50 mg kg^{-1} (Gunstone, Hardwood, & Padley, 1994). In Europe and the USA, it is allowed to add several antioxidants, such as gallates and tocopherols for increasing the oxidative stabilisation of lard. We consider, here, how to improve oxidative stabilisation of lard with natural phenolic compounds extracted from olive mill wastewater.

It is well known that olives and their derivates are rich in phenolic substances, as important antioxidants with nutraceutical properties (Chimi, Rahmani, Cillard, & Cillard, 1990; De Leonardis & Macciola, 1998; Frega, Mozzon, Servidio, & Lercker, 1995). Several medicinal and pharmaceutical scientists have demonstrated that olive phenols, especially hydroxytyrosol (3,4-di-hydroxyphenyl-ethanol), were effective in preventing and curing some important diseases (Fedeli & Testolin, 1991; Tuck & Hayball, 2002; Visioli, Bellomo, & Galli, 1998; Visioli, Bellomo, Montedoro, & Galli, 1995).

* Corresponding author. Tel.: +39 0874 404641; fax: +39 0874 404652.
E-mail address: antomac@unimol.it (A. De Leonardis).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.10.057

The most abundant phenol in olives is oleuropein, formed from elenolic acid, glucose and hydroxytyrosol (Gariboldi, Jommi, & Verrotta, 1986; Panizzi, Scarpati, & Oriente, 1960; Servili et al., 1999; Soler-Rivas, Espın, & Wichers, 2000). Other complex phenols have been identified: demethyloleuropein and ligstroside, containing tyrosol and verbascoside, that contains hydroxytyrosol and caffeic acid and also flavonoids (Bianchi & Pozzi, 1994; Ragazzi, Veronese, & Guiotto, 1973).

During the olive ripening, oleuropein decreases, while its free components increase. In the ripe olive, hydroxytyrosol is present in quantities ranging from 1.0 to 3.0 g 100 g^{-1} of dried weight (Amiot, Fleuriet, & Macheix, 1986; Amiot, Fleuriet, & Macheix, 1989; Romero, Brenes, Garcia, & Garrido, 2002) *while oleuropein is fully transformed.*

Under mechanical processing, about 1% of the total phenols present in olives is in the oil (Di Giovacchino, Sestili, & Di Vincenzo, 2002). Most part of the olive phenols, in fact, remain in the wastewater and also in solid wastes (Niaounakis & Halvadakis, 2004). Several researches have evaluated the feasibility and economic processes for recovering olive phenols from olive oil mill wastewater or solid wastes (Capasso, Evidente, Avolio, & Solla, 1999; Felizon, Fernández-Bolaños, Heredia, & Gutllen, 2000; Fernández-Bolaños et al., 2002; Vásquez, Maestro Duràn, & Graciani, 1987). Principal systems proposed to recover the phenols from olive-mill wastewater are following: extraction with solvents; resin chromatography; selective concentration by ultra-filtration and inverse osmosis; solid–liquid or liquid–liquid extraction, supercritical fluid extraction. Finally, some possible uses of the phenols from olive oil mill wastewater are: food additives; antimicrobials, pesticides; cosmetics, integrator and pharmaceutical compounds (Visioli et al., 1999).

It is found that among the olive phenols, *ortho*-diphenols have the best antioxidant effect, especially hydroxytyrosol and caffeic acid (Baldioli, Servili, Perretti, & Montedoro, 1996; Papadopoulos & Boskou, 1991; Tsimidou, Papadopoulos, & Boskou, 1992). On the contrary, tyrosol and other mono-phenols, such as the acids *p*-hydroxybenzoic, *o*-coumaric and *p*-coumaric are not good antioxidants (Litridou et al., 1997).

We have obtained encouraging results, showing that olive phenols recovered by ethyl acetate extraction from olive oil mill wastewater are good antioxidants for lard. Lard enriched with olive phenols could be considered as a new food formula, agreeable to the modern consumer, demanding natural, safe and healthy food.

2. Materials and methods

2.1. Materials

Olive mill wastewater was directly drawn from a three-phase decanter in one mill situated in the Abruzzo region (middle Italy). Lard samples were purchased from a local market and were stored at 4 °C.

All reagents were of analytical grade from Carlo Erba (Rodano, MI, Italy).

Standard phenolic compounds were purchased from Acros Organics N.V., Geel, Belgium. Cholesterol, stigmasterol and tocopherol were from Sigma Chemical CO. (St. Louis, Mo, USA). Hydroxytyrosol was synthesised in the laboratory by acid hydrolysis of pure oleuropein, according to Fernández-Bolaños et al., 2002.

Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 (without red phenols), phosphate buffered saline (PBS), fetal bovine serum (FBS) and the antibiotic were from Invitrogen Corporation (Carlsbad, CA, USA), while [3-(4,5-dimethylthiazol-2-ile)-2,5-diphenyltetrazolium bromide] (MTT) was from Sigma Chemical CO. (St. Louis, Mo, USA). Cells were NIH/3T3 fibroblast.

Fatty acids were determined by a GC capillary column, Supelcowax 10 (Bellefonte, PA, USA) of 30 m × 0.32 mm, film 0.5 μm. For the determination of cholesterol and α-tocopherol, a GC capillary column Rtx-65TG (Restek International, Bellefonte, PA, USA) of 30 m × 0.32 mm, film 0.10 μm was used. To purify cholesterol and tocopherols, a Sep-Pak type Strata-NH_2 from Phenomenex (Torrance, USA) was used. The HPLC column to determine phenols was a Luna 5u phenyl-hexyl, 250 mm × 4.6 mm, purchased form Phenomenex (Torrance, USA).

2.2. Lard analysis

Free acidity and peroxide values were determined according to the official methods used for extra virgin olive oil (Union Euporea Regulation, 1996).

Spectrophotometric indices were determined with the following formulas: $E = A_\lambda / C{*}l$, where A_λ is the absorbance measured at 232 or 270 nm, C is the g of lard in 100 ml of *iso*-octane and finally l is the light thickness. R value was calculated as the ratio between E_{232} and E_{270} nm.

Fatty acids, cholesterol and α-tocopherol were determined by a gas-chromatography MOD-8000 connected to a PC in action with the Chrom-Card 1.2 software (Thermoquest Instrument, Rodano, MI, Italy). Experimental conditions to determine fatty acids were as follows: carrier gas He at 50 kPa; split injection system with a splitting ratio 1:40; FID detector system; injector and detector temperatures 250 °C; oven temperature 240 °C; injected quantity 1 μl; cold methylation with a solution of 2 N KOH in methanol. Cholesterol and α-tocopherol were determined by the Russo, De Leonardis, and Macciola (2005) method.

Induction time was determined by a Rancimat Methrom Instrument (AG, Herisau, Switzerland), Mod. 730, at 120 °C and 20 l h^{-1} air flow.

2.3. Preparation of phenol extract from olive-oil mill wastewater

About 100–150 ml of wastewater were first boiled for 30 min. After centrifugation at 3500 rpm for 30 min, the supernatant was paper-filtered and it was twice washed

with 10 ml of *n*-hexane. The water phase was concentrated by rotovapor and then, extracted thrice with 25 ml of ethyl acetate. Solvent was evaporated by rotovapor and the residue was dissolved in 25 ml of distilled water. Final extract was called PE-OMWW.

2.4. Phenolic extract analysis

Total phenols were estimated by the Folin-Ciocalteu spectrophotometric method, using a propyl gallate calibration curve. Phenolic composition was determined by an HPLC instrument Model ProStar 230 (Varian, Mulgrave, AUS) attached to a UV–Visible spectrophotometer. Operating conditions were as follows: methanol mobile phase A; 2% acetic acid mobile phase B; elution programme: *A* (%)/*B* (%): 0 min 95/5; 10 min 75/25; 20 min 50/50; 30 min 100/0; 40 min 5/95; eluent flow 1 ml min^{-1}; inject quantity 20 μl; fixed wavelength 280 nm.

2.5. Evaluation of antioxidant effect

Antioxidant effect of the PE-OMW extract was compared with that of propyl gallate. All phenols were added to lards dissolved in water. The dose of phenol added was expressed as mg kg^{-1} of lard, as propyl gallate. The doses added ranged between 50 and 350 mg kg^{-1}. Antioxidant effects were evaluated by the Rancimat test under the above described operative conditions. Protection factor was calculated as the ratio between induction time of lard with and without the antioxidant.

In our experiment at low temperature, a lard was prepared in two ways: one with 275 mg kg^{-1} of PE-OMWW and another without antioxidant. Then, the samples were stored at 4 °C for 60 days. After 30 and 60 days, peroxide values, spectrophotometric indices and induction time were determined. The decrement percentage of induction time was calculated by the following formula:

$$D_{IT}\% = \left(\frac{IT_x days}{IT_{0days}} - 1\right) * 100$$

In all experiments, in the controls, water was added in a quantity equal to the sample.

2.6. Evaluation of cytotoxicity

NIH/3T3 were grown in DMEM, containing 10% FBS, penicillin (60 mg l^{-1}), streptomycin (100 mg l^{-1}) on 100 mm plates and were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. When cells reached 80% of confluence they were treated with 0.25% trypsin and 1 mM EDTA. They were counted and 10,000 cells were seeded in 96-wells and serum-deprived for 24 h. Cells were stimulated with PE-OMWW and propyl gallate (0.1–10.0 mM) for 12 h. The cytotoxic effect of PE-OMWW and propyl gallate in NIH/3T3 was measured by MTT. This assay can determine cellular viability by measuring conversion of the tetrazolium MTT salt to a blue formazan produced by mitochondrial dehydrogenase in viable cells. 0.5 mg ml^{-1} of MTT solution was added to each well and cells were further incubated at 37 °C for 4 h. Then, isopropanol/HCl solution was added to each well. MTT conversion was measured with a microplate spectrophotometer at a wavelength of 570 nm. Each condition was assayed in triplicate.

2.7. Data elaboration

Generally, analytical determinations were carried out in three replicates, expressing the results as means ± standard deviation. Statistical analysis was performed by SPSS software (Chicago, IL, USA), validating the statistical significance with a Student *t* test at $P < 0.05$.

3. Results and discussion

3.1. Chemical characteristics of lard samples

Chemical characteristics of lard samples are listed in Table 1.

In all lard samples, free acids and peroxide values are very low (Table 1). In particular cases, peroxide values ranged from 1.9 to 3.6 meq O_2 kg^{-1} and these low peroxide

Table 1
Chemical characteristics of the lard samples (means ± standard deviation; $n = 3$)

	Lard samples		
	L1	L2	L3
Free acids (% as oleic acid)	0.5 ± 0.02	0.1 ± 0.01	0.1 ± 0.01
Peroxide values (meq O_2 kg^{-1} oil)	2.1 ± 0.16	1.9 ± 0.02	2.8 ± 0.16
Water content (%)	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
Spectrophotometric indices			
E_{232}	2.4 ± 0.02	3.9 ± 0.04	2.6 ± 0.01
E_{270}	0.1 ± 0.01	1.3 ± 0.03	0.2 ± 0.01
R	32 ± 0.80	3.2 ± 0.04	37.0 ± 0.01
α-Tocopherol (mg kg^{-1})	150 ± 8	245 ± 10	50 ± 3
Cholesterol (mg 100 g^{-1})	103 ± 6	88 ± 5	124 ± 6
Fatty acids (%)			
$C_{12:0}$	0.1	0.6	0.1
$C_{14:0}$	1.5	1.7	1.4
$C_{16:0}$	26.1	24.4	24.3
$C_{16:1}$	2.1	2.4	2.3
$C_{17:0}$	0.3	0.3	0.3
$C_{17:1}$	0.2	0.3	0.3
$C_{18:0}$	15.8	13.2	13.8
$C_{18:1}$	41.6	43.6	44.4
$C_{18:2}$	10.7	11.7	11.5
$C_{18:3}$	0.5	0.6	0.5
$C_{20:0}$	0.2	0.2	0.2
$C_{20:1}$	0.9	1.0	1.0
Saturated	44.0	40.4	40.0
Monounsaturated	44.8	47.2	48.0
Polyunsaturated	11.2	12.4	12.0
Unsaturated/saturated	1.3	1.5	1.5
Monounsaturated/polyunsaturated	4.0	3.8	4.0
Induction time (120 °C)	1.37 ± 0.01	2.74 ± 0.01	0.69 ± 0.03

values indicated that all the samples were adequate for testing the antioxidants.

Water content for all samples was enclosed between 0.1% and 0.2%.

Spectrophotometric indices at 232 and 270 nm, respectively, showed the presence of dienes and trienes (conjugated), that form during lard processing. *R* value measures the quality of a lard: high values are typical of high-quality lard, while low values are typical of low-quality or refined lard. Generally, high-quality lards have a *R* equal to 25.

The samples L1 and L3 (from spectrophotometric indices) were high-quality lard, while the sample L2 was of lower quality. In fact, in the sample L2, spectrometric indices E_{232} and E_{270} were equal to 3.9 and 1.3, respectively, and these values were significantly higher than those registered in the samples L1 and L3. Probably, lard L2 was refined, as confirmed from the low value of *R* (3.2).

Our purchased lard samples, in any case, did not declare the quality and category. Moreover, only the L2 label declared that tocopherols were added. In this sample, α-tocopherol value was estimated at 245 mg kg^{-1} while, in the samples L1 and L3, it was equal to 150 and 50 mg kg^{-1}, respectively. Cholesterol was slightly variable and equal to 105 mg 100 g^{-1} on average. Also, regarding the fatty acid composition, lard samples were very similar. In the case of saturated and monounsaturated acids, values were quite equivalent and generally, the second was higher than the first. Oleic acid was the most abundant fatty acid, present at 41.6%, 43.6% and 44.4% of samples L1, L2 and L3, respectively. The total saturated fatty acids represented around 40% in L2 and L3 and 44% in L1. Principal saturated fatty acids were palmitic and stearic acids. A significant quantity of linoleic acid (11.3% on average) was also registered, while linolenic acid was less than 1%.

At high temperature, α-tocopherol content affected the lard oxidation resistance more than fatty acids composition (Table 1). In fact, induction time measured at 120 °C by Rancimat, was correlated with lard α-tocopherol content and it decreased in the following order: L2 (2.74 h) > L1 (1.37 h) > L3 (0.69 h). These results confirmed that lard, even if it is of high-quality, is slightly oxidation resistant at high temperature; lard oxidation resistance can be improved by addition of antioxidants.

3.2. Composition of phenolic extract from olive mill wastewater

During boiling of wastewater, all macro-compounds, such as protein, pigment, gums and impurities, were precipitated. In fact, after the treatment of wastewater at 100 °C for 30 min, a spontaneous solid precipitate was formed and, consequently, supernatant water phase was limpid. By *n*-hexane treatment, the water phase was cleaned of residue oil. Finally, phenols were recovered by ethyl acetate extractions, dried by rotovapor and dissolved in 25 ml of distilled water. This final solution was PE-OMWW.

In PE-OMWW, total phenols were determined by the Folin-Ciocalteu method, using propyl gallate as standard, because this phenol was also compared in the Rancimat experiments. Propyl gallate is a very good synthetic phenol food additive at high temperatures, and permitted in the European Union as an antioxidant.

In the final extract, total phenols were equal to 2 mg ml^{-1}. The extraction yield was equal to 50 mg 100 g^{-1} of wastewater, corresponding to around 0.05% of wastewater and to 10% of total phenols originally present. Other researchers have reported that phenolic extraction yield was equal to 1–2% of wastewater (Niaounakis & Halvadakis, 2004).

On the basis of the results, it is possible to calculate that 1 kg of olives during processing with the three-phase system produces about 70 kg of wastewater. According to the method used in this research, about 39 g of phenols can be recovered from 70 kg of wastewater, with which it is theoretically possible to stabilise around 200 kg of fat at a 0.02% dose or 390 kg at 0.01% dose. In conclusion, phenol recovery from olive mill wastewater can be considered profitable.

Finally, HPLC analysis revealed the phenol composition of PE-OMWW (Fig. 1).

PE-OMWW was composed of four compounds recognised as: hydroxytyrosol, tyrosol, caffeic acid and ferulic acid. Hydroxytyrosol was the best compound and it alone represented 66.8%, followed by tyrosol (16.6%), caffeic acid (8.3%) and ferulic acid (8.3%). *In PE-OMWW oleuropein was present only in traces. This fact was foreseeable, as a very small amount of oleuropein is generally present in olive mill wastewaters, because enzymatic reactions degrade during olive ripening or processing.*

3.3. Oxidative stabilisation of lard

The same doses (150 mg kg^{-1}) of PE-OMWW and propyl gallate were added to lard samples. Lard, with and without antioxidant, was placed in the Rancimat at 120 °C and 20 l h^{-1} air flow. The results are shown in Table 2.

In all samples, propyl gallate was more effective than PE-OMWW. Nevertheless, the PE-OMWW antioxidant effect was satisfactory, especially in the high-quality lard. In fact, in L1 and L3, the PE-OMWW protection factor were 7.7 and 14.9, respectively. In L2, instead, the PE-OMWW protection factor was 2.7. Also, the propyl gallate protection factor, in L2 was lower (5.5) than those in L1 (12.5) and L3 (21.3). Antioxidant effect of phenols added was directly correlated with the qualities of the lard and indirectly correlated with α-tocopherol content. In fact, the antioxidant effect of both PE-OMWW and propyl gallate decreased with α-tocopherol amount. The relationship between phenols and tocopherols, in reciprocal antioxidant action, is worthy of major studies.

PE-OMWW antioxidant effect was evaluated as function of increasing doses ranging between 50 and 350 mg kg^{-1} (Fig. 2). It was found that there was a good

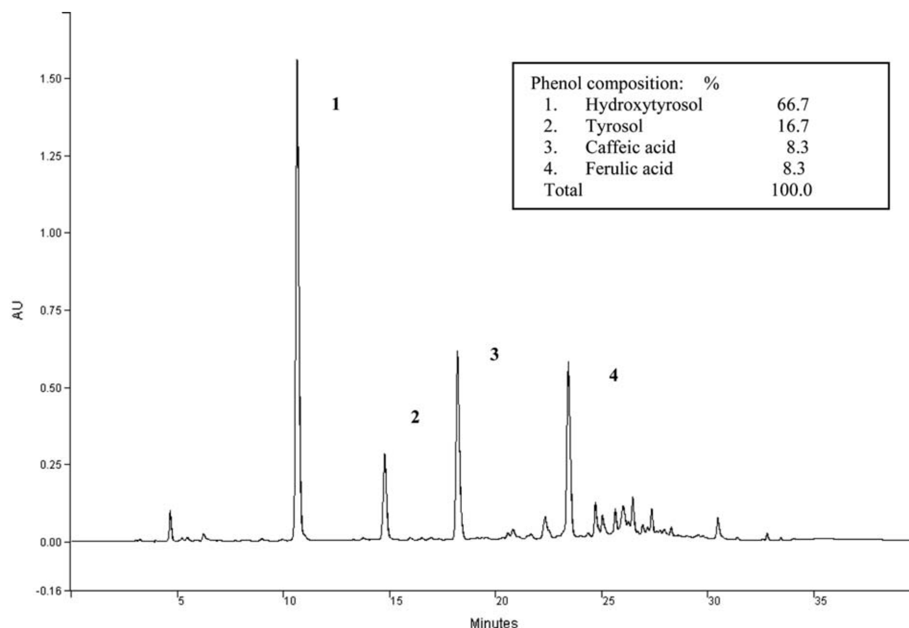

Fig. 1. HPLC chromatogram of phenol composition of the extract obtained from olive-oil mill wastewater.

Table 2
Antioxidant effects on different lards of 150 mg kg^{-1} PE-OMWW and propyl gallate, measured by Rancimat, under 120 °C temperature and 20 l h^{-1} air flow conditions

	Lard 1	Lard 2	Lard 3
Induction time (IT) (h)			
Control	1.37 ± 0.07^a	2.74 ± 0.05^a	0.69 ± 0.01^a
PE-OMWW	10.51 ± 0.12^b	7.33 ± 0.09^b	10.33 ± 0.10^b
Propyl gallate	17.06 ± 0.24^c	15.09 ± 0.15^c	14.72 ± 0.18^c
Protection factor (PF)[a]			
PE-OMWW	7.7	2.7	14.9
Propyl gallate	12.5	5.5	21.3

[a] PF, $IT_{with\ antioxidant}/IT_{control}$.

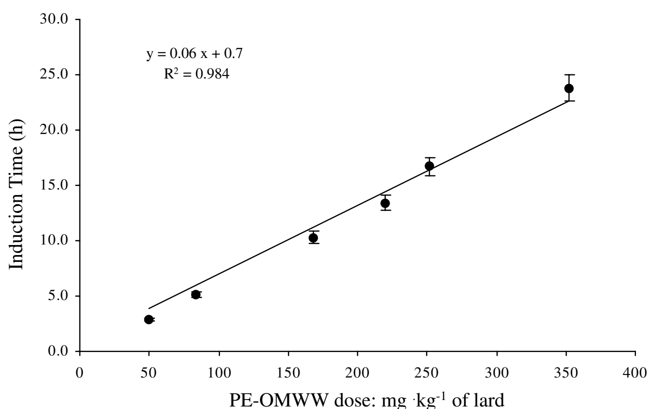

Fig. 2. Oxidative stabilisation of lard as a function of different concentration of PE-OMWW measured in Rancimat at 120 °C temperature and 20 l h^{-1} air flow (means ± standard deviation; $n = 3$).

linear correlation between antioxidant effect and dose of PE-OMWW added.

In the conclusive experiment, 25 g of Lard 1 was separately treated with water (1% v/w) as control and 275 mg kg^{-1} of PE-OMWW for the sample-test. Control and test sample were stored at 4 °C for 60 days. At subsequent interval of 0, 30 and 60 days peroxide values, spectrophotometric indices and induction times were determined. Results are shown in Table 3.

Peroxide values were significantly increased in the control from 2.1 (initial value) to 9.0 meq O_2 kg^{-1} (final value) after 60 days of storage. In the sample-test, peroxide value was unchanged during storage-time, indicating the antioxidant effectiveness of PE-OMWW.

Equally, in the control and in the sample-test, spectrophotometric indices changes were not significant. On the contrary, oxidative resistances, measured by Rancimat, were greatly modified. In the control, from the initial value, oxidative resistance at 120 °C was reduced to 17.5% and 62.8% after 30 and 60 days, respectively. In the sample-test, the same oxidative resistance was reduced to 12.1% after 30 days and only to 19.9% after 60 days. Protection factor values, in addition, indicated the advantages of adding PE-OMWW to the lard. In fact, the PE-OMWW protection factor was equal to 10.4 in the first 30 days, while it was practically doubled after 60 days.

3.4. Cytotoxicity assay

The effect of PE-OMWW and propyl gallate on cellular growth was tested in vitro. The results are shown in Fig. 3.

The tested concentrations (0.1, 1.0 and 10.0 mM), are equivalent to the doses of 20, 200 and 2000 mg kg^{-1} if expressed as the measurement unit that are generally used for food additives. *In PE-OMWW, molar concentration was calculated, using molecular weight of the hydroxytyrosol.*

After 12 h of exposure at concentrations of 0.1 and 1.0 mM, the cell number was similar to the control for both

Table 3
Oxidative state of lard, with PE-OMWW (275 mg kg^{-1}) and without (control), during storage at 4 °C for 60 days

	Peroxide values (meq O_2 kg^{-1})	Spectrophotometric indices		Induction time (120 °C, 20 l h^{-1} air flow)	Percentage decrease of induction time	Protection factor
		E_{232}	E_{270}			
0 days						
Control	2.1 ± 0.16	2.40 ± 0.02	0.10 ± 0.01	1.37 ± 0.01		
PE-OMWW	nd[a]	nd[a]	nd[a]	13.39 ± 0.01		9.8
30 days						
Control	4.5 ± 0.02	2.61 ± 0.04	0.24 ± 0.01	1.13 ± 0.01	−17.5	
PE-OMWW	2.9 ± 0.02	2.58 ± 0.04	0.17 ± 0.01	11.77 ± 0.01	−12.1	10.4
60 days						
Control	9.0 ± 0.16	2.67 ± 0.01	0.16 ± 0.01	0.51 ± 0.01	−62.8	
PE-OMWW	2.8 ± 0.16	2.61 ± 0.01	0.19 ± 0.01	10.72 ± 0.03	−19.9	21.0

[a] nd, not determined.

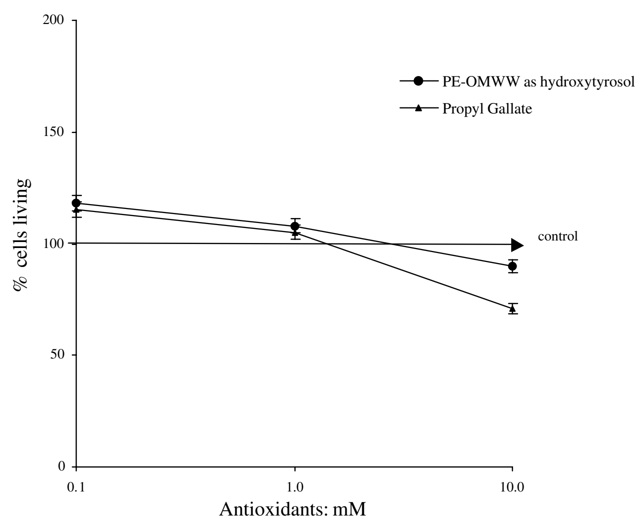

Fig. 3. PE-OMWW and propyl gallate cytotoxic effects measured on NIH/3T3 cells after an incubation time of 12 h (means ± standard deviation; $n = 3$).

phenolic solutions. At a concentration of 10 mM, both phenolic solutions showed toxic effects (more evident than the propyl gallate). Actually, propyl gallate at 10 mM concentration was less soluble than the PE-OMWW.

In conclusion, the cytotoxicity assay showed that, at the usual dose of 100–200 mg kg^{-1}, PE-OMWW did not inhibit cell growth.

PE-OMWW was a mixture of at least four phenols and this fact affects the evaluation of cytotoxicity. It is known that the degree of toxicity of a phenol varies in relation to its concentration and the chemical structure. Babich and Visioli (2003) have measured, in vitro, in human cells, the cytotoxicity of several phenols typically present in virgin olive oil, in the following order of toxicity: oleuropein aglycone > oleuropein glycoside > caffeic acid > *o*-coumaric acid > cinnamic acid ≫ tyrosol and syringic, protocatechuic and vanillic acids.

On the otherhand, low doses (0.1 and 0.5 mM) of olive polyphenols showed an antioxidant effect on intestinal human epithelial cells (Manna et al., 1997) and a cytostatic action on some tumour cells (Owen et al., 2000).

4. Conclusion

In this study, lard oxidative stabilisation by phenols recovered from olive-oil mill by-products is considered. It was found that (from olive-oil wastewater) a lot of free phenols could be recovered by a simple liquid–liquid extraction process. Final phenolic extract, dissolved in water, was composed of hydroxytyrosol, in major quantity, and also tyrosol, caffeic acid and ferulic acid in minor quantities. It is known by scientific literature that among these phenolic compounds only hydroxytyrosol and caffeic acid are good antioxidant properties, but in all cases, tyrosol and ferulic acid, are considered to be nutraceutically positive.

It was found that phenols from olive-oil mill wastewater significantly increased the oxidative stability of lard and the applied doses were not cytotoxic.

In conclusion, a profitable use of olive phenol emerges as food a additive. Lard enriched with olive phenols has two advantages: improved oxidative stability and increased nutritional value.

The lard with olive phenol can be considered as a "novel food" that satisfies the modern consumer's demand for natural, safe and healthy food. Moreover by this application, a significant utilisation of the olive-mill wastewater is proposed.

References

Amiot, M. T., Fleuriet, A., & Macheix, J. T. (1986). Importance and evolution of phenolic compounds in olive during growth and maturation. *Journal of Agricultural and Food Chemistry, 34*, 823–826.

Amiot, M. T., Fleuriet, A., & Macheix, J. T. (1989). Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry, 28*, 67–69.

Babich, H., & Visioli, F. (2003). In vitro cytotoxicity to human cells in culture of some phenols from olive oil. *Il Farmaco, 58*, 403–407.

Baldioli, M., Servili, M., Perretti, G., & Montedoro, G. F. (1996). Antioxidant activity of tocopherols and phenolic compounds in virgin olive oil. *Journal of the American Oil Chemists' Society, 73*, 1589–1593.

Bianchi, A., & Pozzi, N. (1994). Di-hydroxyphenylglycol, a major C_6–C_2 phenols in *Olea Europeae* fruits. *Phytochemistry, 35*, 1335–1337.

Capasso, R., Evidente, A., Avolio, S., & Solla, F. (1999). A highly convenient synthesis of hydroxytyrosol and its recovery from

agricultural waste waters. *Journal of Agricultural and Food Chemistry, 47*, 1745–1748.

Chimi, H., Rahmani, M., Cillard, J., & Cillard, P. (1990). Autooxidation des huiles d'olive: rôle des composés phénoliques. *Revue Fraçaise des corps gras, 37*, 363–367.

De Leonardis, A., & Macciola, V. (1998). Evaluation of the shelf-life of virgin olive oils. *Rivista Italiana Sostanze Grasse, 75*, 391–396.

Di Giovacchino, L., Sestili, S., & Di Vincenzo, D. (2002). Influence of olive processing on virgin olive oil quality. *European Journal of Lipid Science and Technology, 104*, 587–601.

FaoStat, 2000. Available from www.FAOSTAT.com.

Fedeli, E., & Testolin, G. (1991). Edible fats and oils. In G. A. Spiller (Ed.), *The Mediterranean diet in health and disease* (pp. 125–134). New York: Van Nostrand Reinhold.

Felizon, B., Fernández-Bolaňos, J., Heredia, A., & Gutllen, A. (2000). Steam-explosion pre-treatment of olive cake. *Journal of American Oil Chemists' Society, 77*, 15–22.

Fernández-Bolaňos, J., Rodríguez, G., Rodríguez, R., Heredia, A., Guillén, R., & Jiménez, A. (2002). Production in large quantities of highly purified hydroxytyrosol from liquid–solid waste of two-phase olive oil processing or "Alperujio". *Journal of Agricultural and Food Chemistry, 50*, 6804–6811.

Frega, N., Mozzon, M., Servidio, G., & Lercker, G. (1995). Studio della resistenza all'ossidazione forzata degli oli extra vergini mediante Rancimat test. *Rivista Italiana Sostanze Grasse, 72*, 493–496.

Gariboldi, P., Jommi, G., & Verrotta, L. (1986). Secoiridoids from *Olea Europeae*. *Phytochemistry, 25*, 865–869.

Gunstone, F. D., Hardwood, J. L., & Padley, F. B. (1994). Animal fats, lard and animal tallow. In *The lipid handbook* (pp. 156–166). London: Chapman & Hall.

Litridou, M., Linssen, J., Schols, H., Bergmans, M., Posthumus, M., Tsimodou, M., et al. (1997). Phenolic compounds in virgin olive oils: fraction by solid phase extraction and antioxidant activity assessment. *Journal of the Science of the Food and Agricultural, 74*, 169–174.

Manna, C., Galletti, P., Cucciolla, V., Moltedo, O., Leone, A., & Zappia, V. (1997). The protective effect of the olive oil polyphenols (3-4-dyhydroxyphenyl)-ethanol counteracts reactive oxygen metabolite induced cytoxicity in caco-2-cells. *Journal of Nutrition, 127*, 286–292.

Niaounakis, M., & Halvadakis, C. P. (2004). In *Olive-mill waste management. Literature review and patent survey*. Atene: Typothito-George Dardanos.

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Spiegelhalder, B., & Bartsch, H. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer, 36*, 1235–1247.

Papadopoulos, G., & Boskou, D. (1991). Antioxidant effect of natural phenols on olive oil. *Journal of American Oil Chemists' Society, 68*, 669–671.

Panizzi, L., Scarpati, M. L., & Oriente, E. G. (1960). Structure of oleuropein bitter glycoside with hypotensive action of olive oil. Note II. *Gazzetta Chimica Italiana, 90*, 1449–1485.

Ragazzi, E., Veronese, G., & Guiotto, A. (1973). Demethyloleuropein: a new glycoside isolated from ripe olives. *Annali di Chimica, 63*, 13–20.

Romero, C., Brenes, M., Garcia, P., & Garrido, A. (2002). Hydroxytyrosol-4-β-D-glucoside, an important phenolic compound in olive fruits and derived products. *Journal of Agricultural and Food Chemistry, 50*, 3835–3839.

Russo, V., De Leonardis, A., & Macciola, V. (2005). Gas chromatographic determination of cholesterol in animal fats by purification with solid phase extraction. *Journal of Food Composition and Analysis, 18*, 617–624.

Servili, M., Baldioli, M., Selvaggini, R., Miniati, E., Maccioni, A., & Montedoro, G. (1999). HPLC evaluation of phenols in olive fruit, virgin olive oil, vegetation waters and pomace, and 1D- and 2D-NMR characterization. *Journal of American Oil Chemists' Society, 76*, 873–882.

Soler-Rivas, C., Espín, J. C., & Wichers, H. J. (2000). Oleuropein and related compounds. *Journal of the Science of Food and Agricultural, 80*, 1013–1023.

Tsimidou, M., Papadopoulos, G., & Boskou, D. (1992). Phenolic compounds and stability of virgin olive oil. *Food Chemistry, 45*, 141–144.

Tuck, K. L., & Hayball, P. J. (2002). Major phenolic compounds in olive oil: metabolism and health effects. *The Journal of Nutritional Biochemistry, 13*, 636–644.

Union Euporea Regulation, no. 1263/96, 1996.

Vásquez, R., Maestro Duràn, R., & Graciani, C. (1987). Phenols components of olive oil mill waste water. *Grasas y aceites, 25*, 341–345.

Visioli, F., Bellomo, G., & Galli, C. (1998). Free radical-scavenging properties of olive oil polyphenols. *Biochemical and Biophysical Research Communications, 247*, 60–64.

Visioli, F., Bellomo, G., Montedoro, G. F., & Galli, C. (1995). Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis, 117*, 25–32.

Visioli, F., Romani, A., Mulinacci, N., Zarini, S., Conte, D., Vincieri, F. F., et al. (1999). Antioxidant and other biological activities in olive oil mill waste water. *Journal of Agricultural and Food Chemistry, 47*, 3397–3401.