EXPLOITATION OF AGRICULTURAL BY-SIDE PRODUCTS FROM THE MEDITERRANEAN BASIN: OLIVE AND VINEYARD INDUSTRIES

M. D. LUQUE DE CASTRO, J. M. LUQUE-RODRÍGUEZ AND R. JAPÓN-LUJÁN

Department of Analytical Chemistry, Marie Curie Building, Campus of Rabanales, 14071, University of Córdoba, Córdoba, Spain. E-mail: qa1lucam@uco.es

SUMMARY: Research aimed at exploting the high potential of agrofood residues from olive and vineyard industries has been developed and is here reported. Thus, methods to extract valuable products from raw materials such as olive leaves and branches, alperujo (residue from olive oil production), vineshoots and senescent vine leaves, grape pomace and lees have been developed using auxiliary energies such as ultrasound, microwaves and high temperature+pressure. These methods dramatically reduce the time required for total extraction from hours to minutes. Additional studies focussed on discriminating variety and production zone of olive trees through the content of biophenols, methods for enrichment of edible oils with healthy olive phenols, as weel as the preventive and curative effects of these compounds have also been studied.

1. INTRODUCTION

One common cultural trait of the people settled around the Mediterranean Sea is their traditional diet, which has remained virtually unchanged for centuries. Obviously, economic, ethnic and even religious differences among regions influence the dietary intake. Thus, the Greek diet is dominated by the consumption of olive oil, vegetables and fruits, the Italian version by pasta and the Spanish version is especially rich in fish. However, a common pattern prevails in all variants including the following characteristics:

(a) A high consumption of legumes, cereals, fruits and vegetables. Legumes contain proteins of a high biological value and complex carbohydrates; cereals provide carbohydrates and contribute to the intake of vitamins in the B group; and fruits and vegetables are the primary sources of water-soluble vitamins (overall, vitamin C), provitamins (α- and β-carotene, β-cryptoxanthine), carotenoids, antioxidants, minerals and fibre.

(b) A low consumption of meat and meat products. Although animal foods are the main sources of B group vitamins, their intake is supplemented by cereals. Moreover, several Mediterranean countries consume preserved pork liver, which provides vitamin B_{12}. Liver is also a source of folates (also called vitamin B_9).

(c) A moderate consumption of milk and dairy products. The most commonly used dairy products are yoghurt and cottage cheese, which provide calcium without a high saturated fat intake. These are great sources of proteins of a high biological value, minerals and fat-soluble vitamins (A and D).

(d) A high monounsaturated/saturated fat ratio. The high consumption of olive oil is one of the most salient features of the Mediterranean diet and provides a large amount of oleic acid. On the other hand, fish and nuts provide also amounts of omega-3 fatty acids such as linoleic acid. Moreover, olive oil and nuts contain abundant vitamin E.

(e) A moderate consumption of wine, which is usually drunk in meals. This is the other essential feature of the Mediterranean diet. Wine, particularly of the red type, is an important source of polyphenols.

The health benefits of the traditional Mediterranean diet are extensively documented and include protective effects against cardiovascular diseases, diabetes, rheumatoid arthritis, intestinal diseases and several types of cancers. Some constituents of wine and olive oil (particularly polyphenols) play a key role in most of such healthy effects.

Studies on the healthy effects of wine began with the discovery of the “French Paradox” in the 1970s. This phenomenon is usually defined as a lower-than-expected coronary heart disease mortality rate in France, where classic risk factors are not less prevalent than in other industrialized countries and where, in addition, the diet has always been rich in saturated animal fat . A number of hypotheses have been put toward to explain the phenomenon, the most widely accepted of which ascribes it to dietary habits (particularly the wine consumption), the crucial role of which has later been confirmed by a number of epidemiological studies. Some studies have also associated alcohol or wine consumption with a reduced incidence of other health problems including dementia and Alzheimer’s disease, age-related macular degeneration, kidney stones, gallstones and cancer.

Although the exact origin of these effects remains unclear, alcohol (ethanol) and polyphenolic compounds are thought to play a key role in them. Thus, ethanol has been reported to increase the levels of "good" cholesterol (HDL) and to inhibit platelet aggregation, which protects against coronary heart disease and stroke. On the other hand, the health benefits of polyphenolic compounds are ascribed to their antioxidant and free radical scavenging properties.

Olive oil, which is the principal source of fat in the Mediterranean region, possesses a high content in monounsaturated fatty acids. Oleic acid, its main component, accounts for 55 − 83% (mean 75%) of total fatty acids in it.

The unsaponifiable fraction of olive oil contains a variety of minor components including tocopherols (mainly α-tocopherol, in amounts from 12 to 25 mg/100 g), phenols, flavour compounds, hydrocarbons and sterols. Olive biophenols (OBPs, which are inaccurately referred to as named polyphenols in some cases) are used as flavorants, colorants and antioxidants. The best known olive oil phenols are hydroxytyrosol, tyrosol, oleuropein, apigenin and luteolin, in both free and derivative forms.

Olive oil thus provides large amounts of stable, not easily oxidized fatty acids, as well as substantial quantities of powerful antioxidant molecules favouring a healthier ageing and increased longevity in its consumers.

In recent years, a number of healthy properties of olive oil have been demonstrated. Thus, the regular intake of olive oil reduces the major risk factors for cardiovascular diseases such as the lipoprotein profile, blood pressure, glucose metabolism and antithrombotic profile. Also, it facilitates the modulation of endothelial function, inflammation and oxidative stress.

Epidemiological studies suggest that olive oil exerts a protective effect against some types of malignant tumours (skin, breast, prostate, endometrium, digestive tract). Prevention of oxidative DNA damage or DNA strand breakage; protection against chronic liver disease and the bowel disorder known as Crohn's disease; and reduction of incidence of melanoma are but a few of the beneficial effects of olive oil on pre-cancerous lesions.

In addition to heart and cancer diseases, olive oil may also help prevent or delay the onset of diabetes; protects the immune system; reduces the risk of developing rheumatoid arthritis; and is extremely helpful in the treatment and prevention of disorders of the bile ducts, thereby improving hepato-biliary diseases. Also, it is recommended to fight pancreas diseases and facilitate calcium

absorption, thereby improving growth and preventing osteoporosis. These properties have boosted olive oil production to a world output exceeding 2.5 million tonnes in the 2005/06 campaign.

The scant exploitation of by-products from wine and olive oil industries and the excellence of the compounds they contain promp the authors to look for extraction methods based on present technology; thus endowed with rapidity, efficiency and availability for automation. Then, identification and characterization of the target compounds and their application were target goals partially developed and in present development in some proportion.

2. RESEARCH TO OBTAIN, IDENTIFICATE, QUANTITATE AND APPLY ANTIOXIDANT COMPOUNDS FROM OLIVE TREE, VINEYARD AND DERIVATIVE INDUSTRIES

The research has been developed by using vanguard techniques and technology, thus providing reliable and interesting results, as demonstrated by the scientific journals where they have been published —18 articles published so far in international journals of high impact-index and 2 pending patents; results which are commented below.

2.1. Development of fast and efficient methods for the extraction of antioxidants by using auxiliary energies

The aim of this research was the development of fast, efficient and automatic extraction methods easily transferred to the pilot-plant scale for subsequent development at the industrial scale. Water and ethanol–water mixtures have been used with a view to subsequent use of the extracts in food, pharmaceutical and/or cosmetic industries.

The raw materials used have been, on the one hand, olive leaves (isolated or together with to the small branches that join them); olive oil and alperujo (the waste from the two-phase decanter process for oil production), and, on the other, vineshoots, grape skin and seed (methods for the extraction of amino acids, aromas and other compounds from lees and vine leaves are under development).

The methods developed so far can be divided into 3 groups depending on the type of auxiliary energy used.

2.1.1. Acceleration of the extraction process by the use of superheated liquids

Superheated liquids are liquids at temperatures higher that their boiling point and pressure high enough to keep them in liquid state. Based on the use of superheated liquids and an extractor as that in Fig. 1, methods for extraction of biophenols from olive leaves and alperujo, and for extraction of polyphenols from vineshoots, anthocians and other polyphenols, dyes and aromas from grape skin and also oils and tanins from grape seeds have been developed. The energy necessary to superheate a liquid is lower than that required to evaporate it —particularly in the case of water— and the overpressure required to keep it as liquid facilitates its penetration inside the solid and thus, extraction. The extractor can work in static and dynamic regimes and in a sequence of static and dynamic steps. In the first instance the extract volume is smaller, but the process is not 100% efficient as a partition equilibrium is established between the solid raw material and the extractant; in the second case the extract volume is higher, but quantitative extraction of the target compounds is achieved as the solid is continuously put into contact with fresh extractant. The third possibility constitutes a compromise solution in between the two other: quantitative extraction and intermediate extract volume.

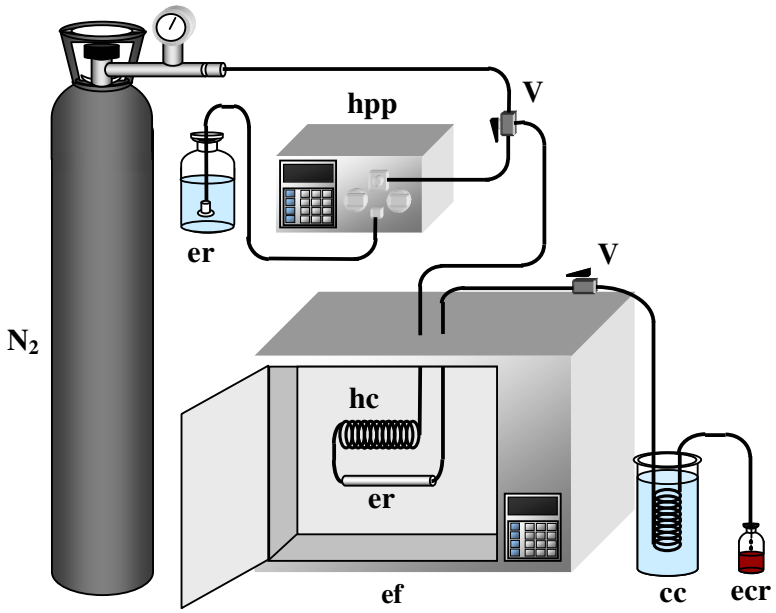


Figure 1. Extractor based on the use of superheated liquids. hpp, high-pressure pump; ec, extraction chamber; er, extractant reservoir; ecr, extract collection reservoir; ef, electrical furnace; N_2, nitrogen; hc y cc, heating and cooling coil, respetively.

The overall study in each case consisted of multivariate optimization of the extraction step, a kinetics study to determine the optimum extraction time, identification and quantitation of the target compounds by gas chromatography–mass spectrometry or liquid chromatography with diode array spectrometric or mass detector, depending on the characteristics of the compounds under study. Tables 1–4 show the characteristics of the methods thus developed and those of the extracted compounds; meanwhile, and as examples, Figs. 2 to 4 show the chromatograms of some of the most representative extracts. It is worth noting that in the case of the grape skin grape the raw material used has been the grape pomace instead of that of direct separation from grapes, as is the case in research developed so far. This use shows that, after contact with wine, grape skin contains a high concentration of anthocianins, phenolics and flavonols; so, this is a raw material of interest from the point of view of residue exploitation.

Table 1. Optimization of the static–dynamic extraction of biophenolds from olive leaves
[1]Fractionated design; [2]factorial design; [3]response surface.

Variable	Range assayed			Optimum value
	First design[1]	Second design[2]	Third design[3]	
Temperature (ºC)	100–130	130–150	140	140
Extractant flow-rate (ml/min)	0.5–1.5	1	1	1
Ethanol (%)	60–80	70	70	70
Static extraction (min)	0–5	5–10	5–10	6
Dynamic extraction (min)	0–5	5–10	5–10	7

Table 1'. Characteristics of the method for determination of biophenols in the extracts and their concentration in olive leaves

Compound	LOD (mg/kg)	LOQ (mg/kg)	λ (nm)	Concentration (mg/kg ± SD)
Oleuropein	11.59	29.79	280	23046 ± 902
Verbacoside	2.57	6.66	330	665 ± 72
Apigenin-7-glucoside	1.67	4.01	340	1046 ± 70
Luteolin-7-glucoside	3.81	10.42	350	998 ± 81

LOD, limit of detection; LOQ, limit of quantitation; λ, maximum absorption wavelength used for determination; SD, standard deviation.

Table 2. Optimization of the static–dynamic extraction of biophenols from alperujo

[1]Factorial design; [2]surface response.

Variable	Range assayed		Optimum value
	First design[1]	Second design[2]	
Temperature (ºC)	150–200	200	200
Extractant flow-rate (ml/min)	0.5–1.5	1	1
Ethanol (%)	80–100	80	80
Static extraction (min)	5–10	5–15	12
Dynamic extraction (min)	5–15	5–15	15

Table 2'. Characteristics of the method for the determination of biophenols in the extracts and their concentration in alperujo

Compound	s_r (%)	s_{WR} (%)	LOD (mg/kg; ng)	LOQ (mg/kg; ng)	Concentration (mg/kg)
Hydroxytyrosol	8.33	12.10	1.42; 29	3.90; 77	2872
Tyrosol	5.60	8.35	1.94; 38	5.01; 100	1565
α-Taxifolin	5.13	7.38	1.61; 31	4.32; 85	147
Verbascoside	8.27	13.11	2.30; 46	6.41; 128	21
Apigenin-7-glucoside	2.28	18.90	3.28; 65	9.21; 185	30
Galic acid	3.72	4.21	1.88; 36	5.33; 106	56
Vanillinic acid	8.53	9.17	1.99; 39	5.24; 104	33

s_r, within-day variability expressed as relative standard deviation (%); s_{WR}, between-day variability expressed as relative standard deviation (%); LOD, limit of detection; LOQ, limit of quantitation.

Table 2". Comparison of biophenols yield obtained from alperujo by the proposed method and that by the conventional extraction method

Compound	Proposed method (mg/kg dry weigh)	Conventional method (%)
Hydroxytyrosol	2872	11
Tyrosol	1565	Por debajo del LOD
α-Taxifolin	147	100
Verbascoside	21	100
Apigenin-7-glucoside	30	100
Gallic acid gálico	56	5
Vanillinic acid	33	22

*The amount extracted by the proposed method, expressed as mg/kg (second column), was considered as 100% efficiency to be compared with the conventional method.

Table 3. Optimization of polyphenols extraction from vineshoots using superheated liquids

Variable	Range assayed		Optimum value
	First design	Second design	
Ethanol (%)	20–80	80–100	80
pH	3–11	11	11
Temperature (ºC)	120–180	180–240	240
Time (min)	20–60	60–90	60

Table 3'. Polyphenols concentration found in vineshoots extracts by the proposed method and the conventional solid–liquid extraction method (expressed as μg/g)

Compound	Proposed method		Conventional method	
	pH 3, 180 ºC	pH 11, 240 ºC	90 min, 50 ºC	24 h, 25 ºC
Gallic acid	508.2	71.4	3.4	3.0
Protocateuic acid	22.4	0	6.2	5.7
Vanillinic acid	95.2	70.0	1.9	1.7
Syringic acid	113.4	67.2	1.8	1.5
Vanillin	133.0	140.0	1.4	1.3
Syringaldehyde	107.8	126.0	0.9	0.8
Coniferaldehyde	133.0	113.4	0.6	0.6
Sinapaldehyde	162.4	126.0	0.5	0.5
Elagic acid	57.4	0	3.5	2.2
TPI	96.0	253.0	15.0	18.0

TPI, total polyphenol index.

Table 4. Optimization of the static–dynamic extraction of polyphenols from grape skin by using superheated liquids

Variable	First design[1]	Second design[2]	Optimum value
Ethanol (%)	60–100	40–60	50
HCl (%)	0.2–0.8	0.8	0.8
Temperature (°C)	60–90	90–120	120
Time (min)	20–40	40–60	30
Flow-rate (ml/min)	0.8–1.2	1.2	1.2
Sample (g)	1–3	1	1
Pressure (bar)	40–80	80	80

[1]Screening; [2]factorial design.

Table 4'. Comparison of the dynamic extraction yield by using superheated liquids (SEWE) and at ambient temperature and pressure (DNCE)

	SEWE	**DNCE[a]**
Spectrophotometric determination		
Total anthocians[b]	17510 ± 1571	5755
Total phenols[c]	126 ± 9	18
Total flavanols[d]	35 ± 1	3
HPLC-UV	**SEWE**	**DNCE[a]**
Total anthocians[b]	5967 ± 228	4424
Dp3G[e]	93.2 ± 3.8	43.4
Cy3G[e]	16.0 ± 0.8	2.0
Pt3G[b]	136.2 ± 4.6	79.2
Pn3G[e]	45.4 ± 1.7	11.2
Mv3G[e]	957.1 ± 32.4	635,4
Dp[e]	70.5 ± 1.9	-
Cy[e]	439.5 ± 25.2	12.7
Pt[e]	17.7 ± 2.4	-
Pn[e]	8.6 ± 1.4	-
Mv[e]	53.3 ± 5.4	-
Cf-Mv3G[b]	122.2 ± 8.3	101.6
Cm-Dp3G[f]	352.7 ± 23.4	337.1
Cm-Pt3G[b]	218.7 ± 10.7	346.2
Cm-Pn3G[f]	122.3 ± 3.3	109.6
Cm-Mv3G[b]	2519.1 ± 116.1	2571.7
4-VC-Mv3G (Pinotin A)[b]	19.6 ± 0.8	13.8
4-VC-Cm-Mv3G[b]	7.4 ± 0.7	3.7
Flavonols		
My[g]	121.1 ± 7.1	4.4
Qr[g]	236.1 ± 28.7	123.4
n.i.[g]	18.2 ± 1.9	8.6
Kp[g]	94.1 ± 6.5	46.8
Is[g]	32.5 ± 4.5	18.0

MyG[g]	23.7 ± 2.1	15.0
QrGluc[g]	86.7 ± 4.1	76.1
QrG[g]	12.4 ± 1.4	11.5
Other compounds		
Caffeic acid[e]	14.9 ± 0.9	12.5
p-Coumaric acid[e]	21.2 ± 2.6	12,0
Resveratrol[e]	9.6 ± 0.9	4.1

[a]Normal solid–liquid extraction conditions. Data expressed as [b]µg M3GE/g grape skin, [c]mg GAE/g grape skind, [d]mg CE/g grape skin. [e]Data obtained by the corresponding standard expressed as µg of the compound /g grape skin. [f]Data obtained with the corresponding glycoside standard and expressed as µg glycoside/g grape skin. [g]Data expressed as µg QrE/g grape skin. n.i. no identified. Abbreviations: Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pn, peonin; Mv, malvidin; VC, vinil catechol; My, miricetin; Qr, quercitin; Kp, canferol; G, glycoside; Gluc, glucuronide.

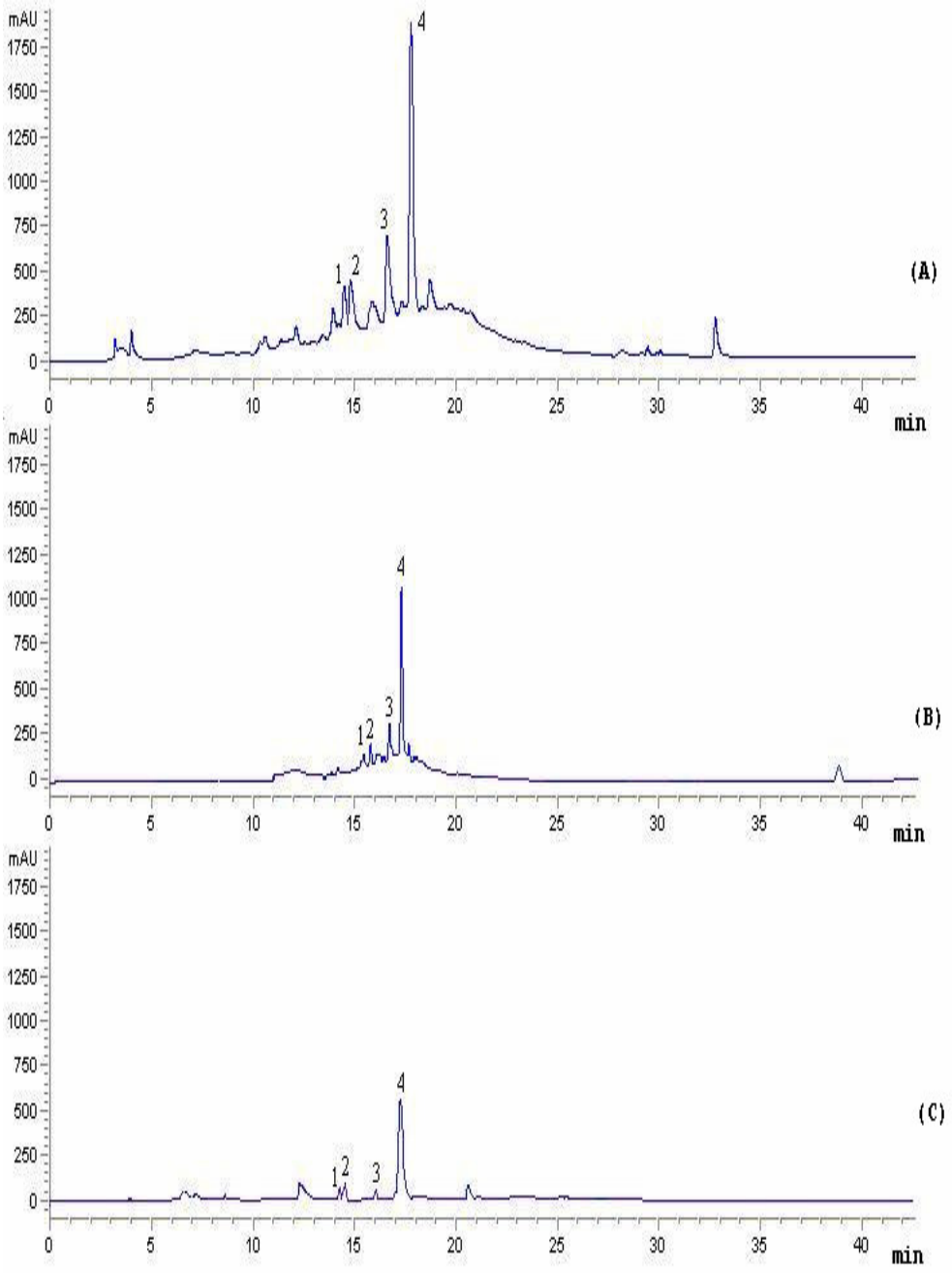


Fig. 2. Chromatograms at 280 nm of an olive leaf extract obtained under optimum working conditions. (A) Direct injection of the extract into the liquid chromatograph; (B) injection after liquid–solid extraction using C18 Hydra cartridges; (C) injection after liquid–liquid extraction (the cleanliness of the extracts obtained by the proposed methods is thus demonstrated). Peaks identification: 1, verbascoside; 2, luteolin-7-glucoside; 3, apigenin-7-glucoside; 4, oleuropein.

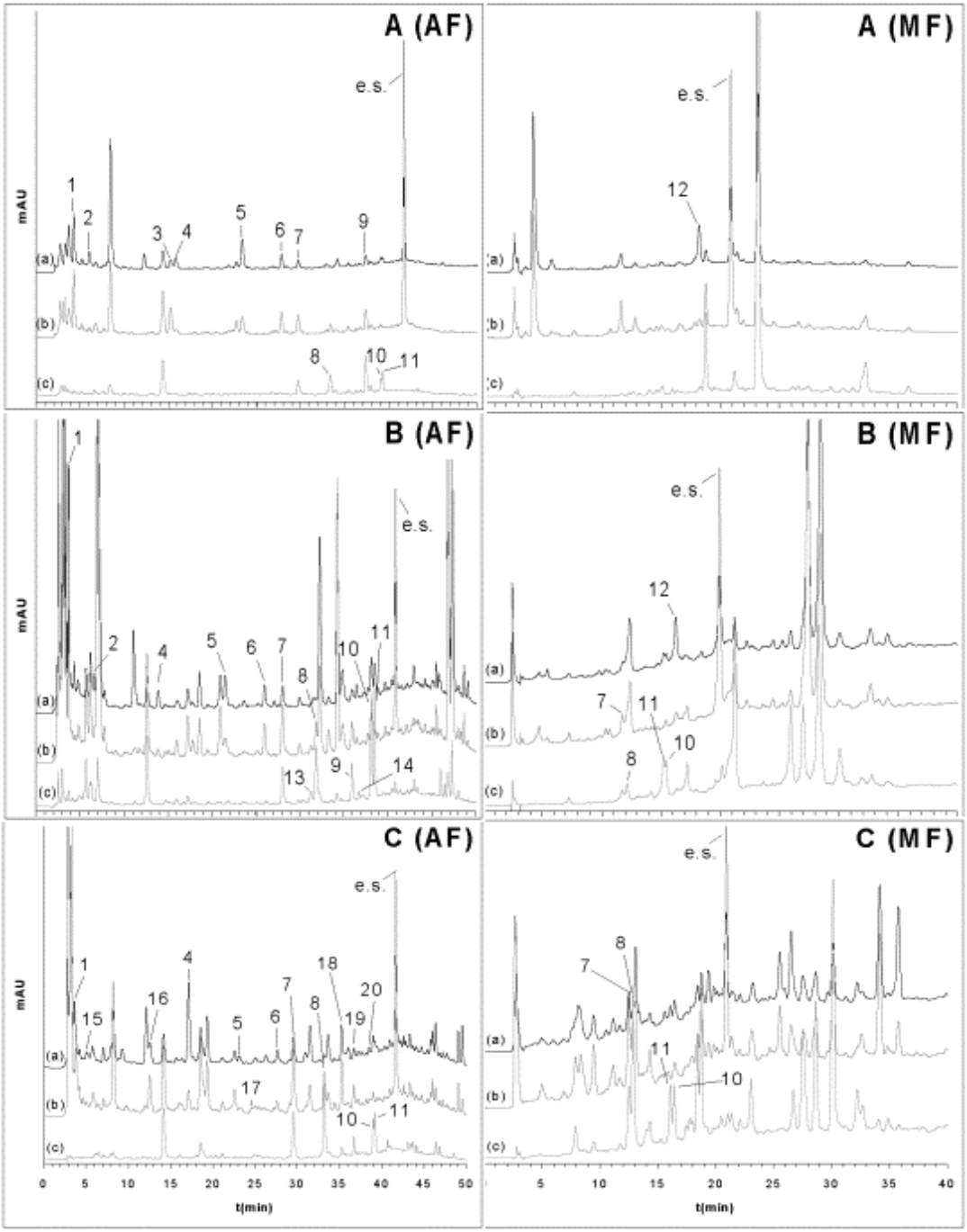


Fig. 3. Chromatograms obtained at 260 (a), 280 (b) and 320 nm (c) of the aqueous fraction (AF) and methanolic fraction (MF) of the vineshoot extracts obtained under the following working conditions: 80% ethanol, ambient temperature (25ºC), 24 h (**A**); 80 % ethanol, pH = 3, 180 ºC, 60 min (B); 80 % ethanol, pH = 11, 240 ºC, 60 min (**C**). Compounds: 1**.** gallic acid; 2**.** protocateuic acid; 3**.** catequin; 4**.** p-hydroxybenzoic acid; 5**.** vanillinic acid; 6**.** syringic acid; 7**.** vanillin; 8**.** syringaldehyde; 9**.** ferulic acid; 10**.** coniferaldehyde; 11**.** sinapaldehyde; 12**.** ellagic acid; 13**.** p-cumaric acid; 14**.** synapic acid; 15. pyrogalol; 16. furfural; 17. 5-methylfurfural; 18. acetovanillona; 19. acetosyringona; 20. siringol; e.s**.** p-cresol.

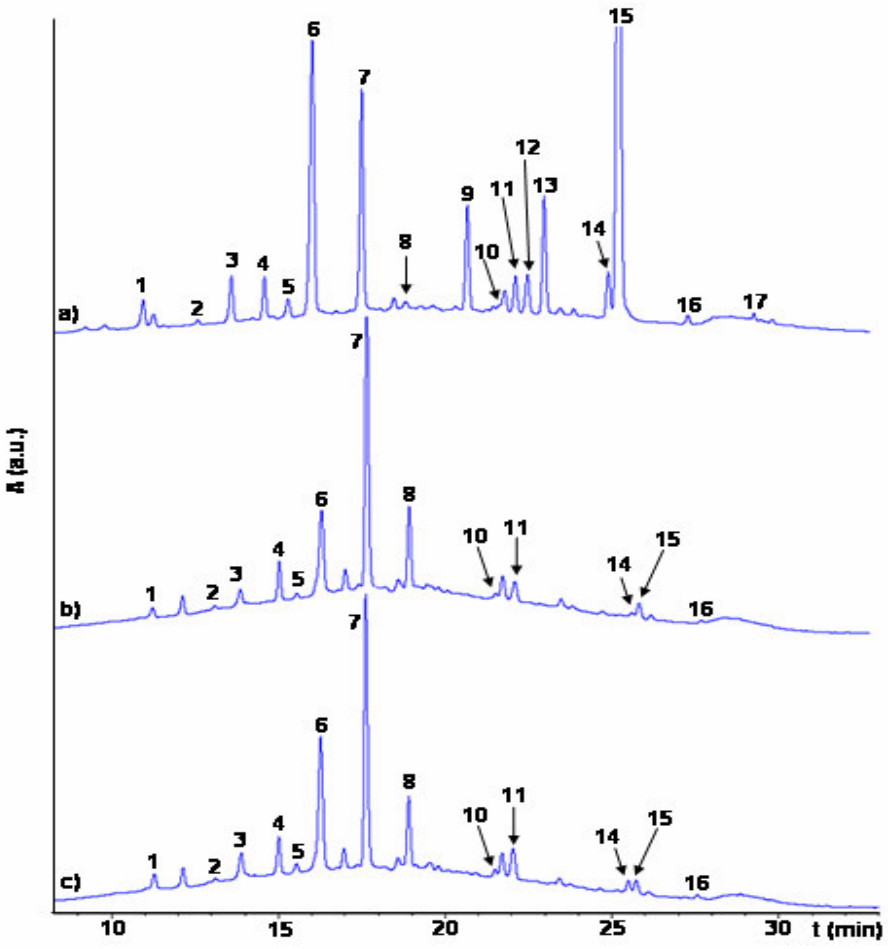


Fig. 4. Chromatograms of grape skin extracts obtained at 530 nm from (a) SEWE; (b) GSTA (5-times diluted); (c) GST (5-times diluted). Compounds: 1**.** Dp3G; 2. Cy3G; 3. Pt3G; 4**.** Dp; 5**.** Pn3G; 6**.** Mv3G; 7**.** Cy; 8**.** Pn; 9**.** Cm-Dp3G; 10**.** Pt; 11**.** Mv; 12**.** Cf-Mv3G; 13**.** Cm-Pt3G; 14**.** Cm-Pn3G; 15**.** Cm-Mv3G; 16**.** 4-VC-Mv3G; 17**.** 4-VC-Cm-Mv3G (see Table 4' for identification).

2.1.2. Use of ultrasound to accelerate extraction

When transmitted through a liquid, ultrasound —waves of frequency above 16 kHz, that is, higher than the audible frequency— the phenomenon known as cavitation is produced. It consists of molecular expansion and compresion cycles, which, at a microcospic scale, give place to temperatures above 5000 ºC and pressures up to 2000 atm without appreciable liquid heating, which enormously facilitate solid–liquid extraction. The extractor designed in this case (see Fig. 5) is of the dynamic type, in which the extractant circulated through the solid in a programmed way by changing the circulation direction a programmed times, thus favouring extraction without generating large extract volumes and without creating overpressure in the system due to increased compactness of the raw material in the extraction chamber.

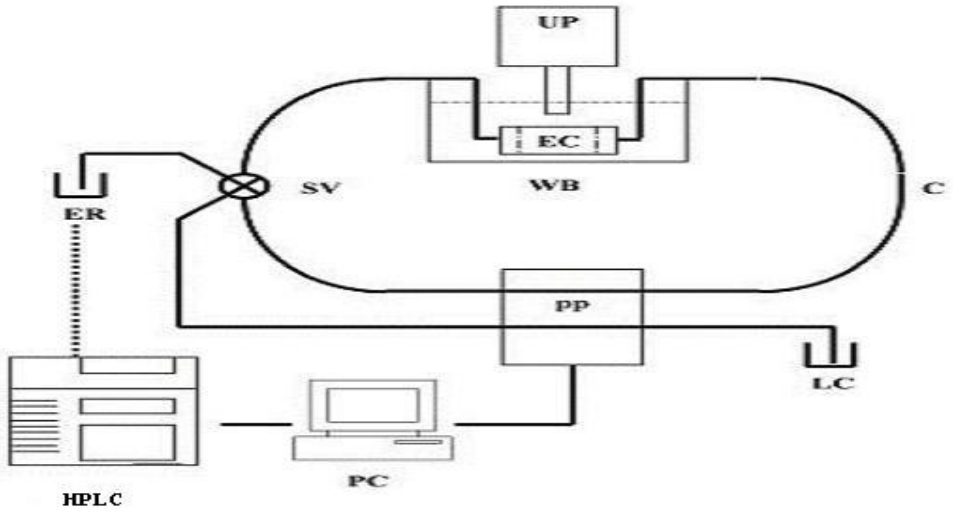


Fig. 5. Extractor assisted by an ultrasonic probe (UP) and with iterative change of the flow direction. C, extraction coil; EC, extraction chamber; ER, extraction reservoir; HPLC, high-performance liquid chromatograph; PC, personal computer; PP, peristaltic pump (low pressure); SV, switching valve; WB, water bath.

As in the previous methods, optimization based on multivariate designs was carried out, as well as kinetics studies, identification and quantitation of the extracted compounds using the same instrumentation; so, the chromatograms obtained with these extracts were also similar. Table 5 shows the characteristics of the methods in which, obviously, the compounds extracted from olive leaves, also quantitatively, were the same as in the previous studies with this raw material, as degradation was not observed in any case.

Table 5. Optimization of the ultrasound-assisted dynamic extraction method for biophenols from olive leaves

Variable	Range assayed		Optimum value
	Plackett-Burman design	Factorial design	
Radiation amplitude (%)	10–50	30	30
Duty cycle (%)	30–70	70	70
Irradiation time (min)	6–18	18–30	25
Extractant flow-rate (ml/min)	4–6	5	5
Ethanol (%)	70–90	50–70	59
Probe distance (cm)	0–4	4	4
Temperature (ºC)	25–40	40	40

Table 5'. Characteristics of the method for determination of biophenols in the olive leaf extracts obtained by ultrasound-assisted extraction

Compound	Dynamic linear range[1]	LOD (mg/kg)	LOQ (mg/kg)	λ (nm)	Concentration (mg/kg ± SD)
Oleuropein	30000	11.46	30.67	280	22610 ± 632
Verbacoside	700	2.59	6.47	330	488 ± 21
Apigenin-7-glucoside	1200	1.57	4.21	340	1072 ± 38
Luteolin-7-glucoside	1000	3.92	10.63	350	970 ± 43

[1]The linear ranges are between de LOQ and the upper limit in the table. LOD: limit of detection; LOQ: limit of quantitation; SD: standard deviation.

2.1.3. Microwave-assisted extraction

Microwaves consist of non-ionizant electromagnetic radiation which cause molecular mouvement by ion migration and dipole rotation without alteration of the molecular structure; so, this energy is tremendously appropriate for polar liquids. In this case the commercial device shown in Fig. 6 was used and extraction was performed in a static regime. The results, obtained in a way similar to as in the two previous cases, are shown in Table 6.

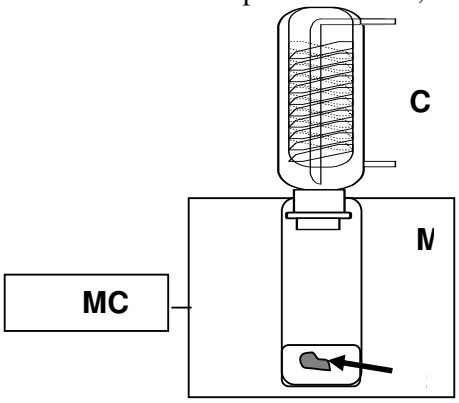


Fig. 6. Extractor working in a static regime for the extraction of biophenols from olive leaves and from alperujo. C, cooler; MC, magnetron; MD, microwave digester; S, sample.

Table 6. Optimization of microwave-assisted extraction of biophenols from olive leaves

Variable	Range assayed	Optimum value
Irradiation power (W)	100–200	200
Irradiation time (min)	5–15	8
Ethanol (%)	80–100	80

Table 6'. Characteristics of the method for the determination of biophenols of the extracts from olive leaves obtained by microwave-assisted extraction and concentration of these compounds in the raw material.

Compound	Linear dynamic range[1]	LOD (mg/kg)	LOQ (mg/kg)	λ (nm)	Concentration (mg/kg ± SD)
Oleuropein	30000	11.04	29.59	280	23149 ± 852
Verbacoside	700	2.68	6.77	330	631 ± 43
Apigenin-7-glucoside	1200	1.49	3.98	340	1076 ± 65
Luteolin-7-glucoside	1500	3.91	10.58	350	1016 ± 60

[1]The linear ranges are within the LOQ and the upper limit in the table. LOD, limit of detection; LOQ, limit of quantitation; SD, standard deviation.

2.2. Comparison of the methods

By comparing the results obtained by the 3 methods, for example, in the case of olive leaves, Table 7 shows that:

Table 7. Comparison of the methods for extraction of biophenols from olive leaves

Variable	With superheated extractant	Ultrasound assisted	Microwave assisted
Extraction time (min)	13	25	8
Extract volume (ml)	11	15	24
Ethanol–water ratio	70:30	59:41	80:20

(1) The shorter time for complete extraction is provided by microwaves, 8 min.
(2) The smaller extract volume is achieved by superheated extractant, 11 ml/5 g of raw material.
(3) The lower ethanol–water mixture ratio used as optimum extractant was that of the ultrasound-assisted method (≈ 1:1 ratio).
(4) The extraction times are very short in all instances; therefore, all the proposed methods could be used at an industrial scale in campaign periods without storage of the raw material. Thus, one of the objectives of this research —development of fast, simple and quantitative extraction methods— has been achieve in all instances.

2.3. Identification, quantitation and comparison of biophenols in olive tree and its products

2.3.1. Biophenols in leaves and branches

In the studies on biophenols of olive leaves published so far, a mixture of leaves and small branches (fibrous softwood) have been used but not specified. Research developed by the authors using separately both raw materials demonstrates that they contain common and uncommon biophenols and that the former are in different concentration and ratio in both materials, as shown in the results listed in Table 8.

Table 8. Concentration of biophenols in small branches of different olive varieties, expressed as mg/kg (the concentration of oleuropein and verbascoside found in leaves of each olive variety is given in brackets)

Variety	Oleuropein	Verbascoside	Hydroxtyrosol	Tyrosol	α-Taxifolin
Alameño	4365 (5310)	52 (371)	369	1254	569
Arbequina	18856 (24800)	1044 (10303)	685	1915	852
Azulillo	2957 (4922)	558 (2240)	524	1741	587
Chorna	1602 (2127)	156 (722)	600	1845	847
Hojiblanca	4213 (5584)	69 (475)	471	1423	695
Lechín	8541 (10554)	362 (1853)	474	1659	753
Manzanillo	2451 (3302)	101 (779)	214	1201	412
Negrillo	8369 (10542)	120 (1173)	469	1833	455
Nevadillo	6897 (8002)	230 (1362)	698	1697	502
Ocal	4993 (5972)	65 (284)	453	1660	554
Pierra	6541 (8089)	164 (1091)	475	1477	741
Sevillano	8741 (10913)	841 (6702)	740	1635	854
Tempranillo	5429 (7000)	83 (427)	580	1507	699

This study shows that extracts with different type and concentration of these compounds can be obtained depending on the raw material used: leaves, branches or a mixture of both.

2.3.2. Evaluation of biophenols in all raw materials from olive tree

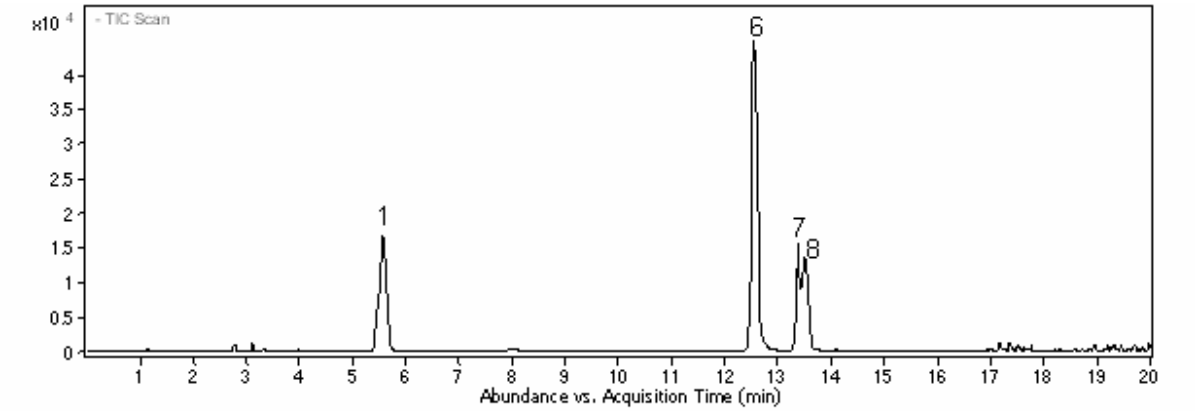
A liquid chromatograph coupled to a triple quadropole mass spectrometer by a negative mode ionization interface and with multiple reaction monitoring for optimal selection of the transitions with a view to evaluating all biophenols existing in olive tree has been used. This equipment has provided the detection and quantitation limits shown in Table 9.

Table 9. Analytical characterization of the method

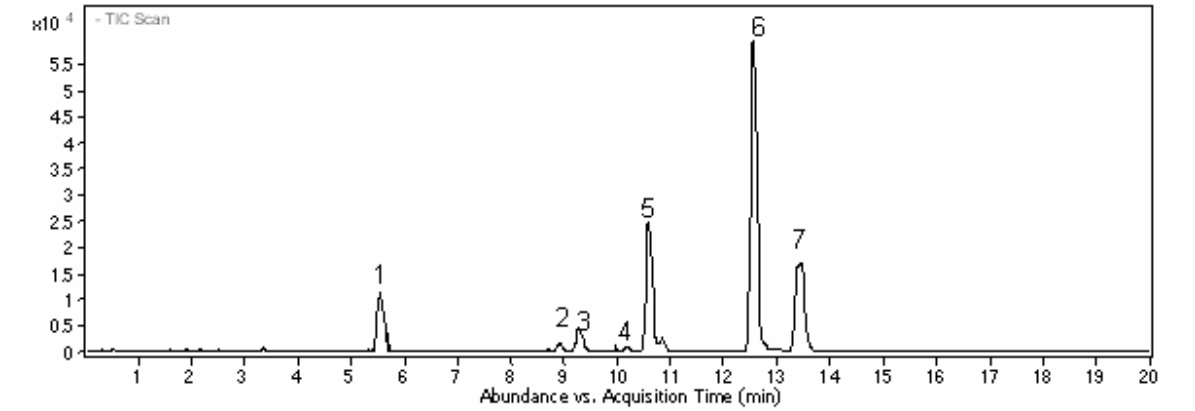
Compound	LOD (ng/ml)	LOQ (ng/ml)
Hydroxytyrosol	5.10	16.87
Verbascoside	5.55	18.22
Luteolin-7-glucoside	6.03	19.90
Apigenin-7-glucoside	5.89	17.65
Oleuropein	11.65	38.40
Luteolin	6.77	22.01
Apigenin	6.20	20.46
Diosmetin	5.15	16.99

Application of the method to extracts from all materials from olive tree (namely, oil, alperujo, leaves, small branches and seed bones) has provided the chromatograms in Fig. 7. Table 10 lists the concentrations found of each biophenol in the differnt fractions; data which allow to establish the following conclusions:

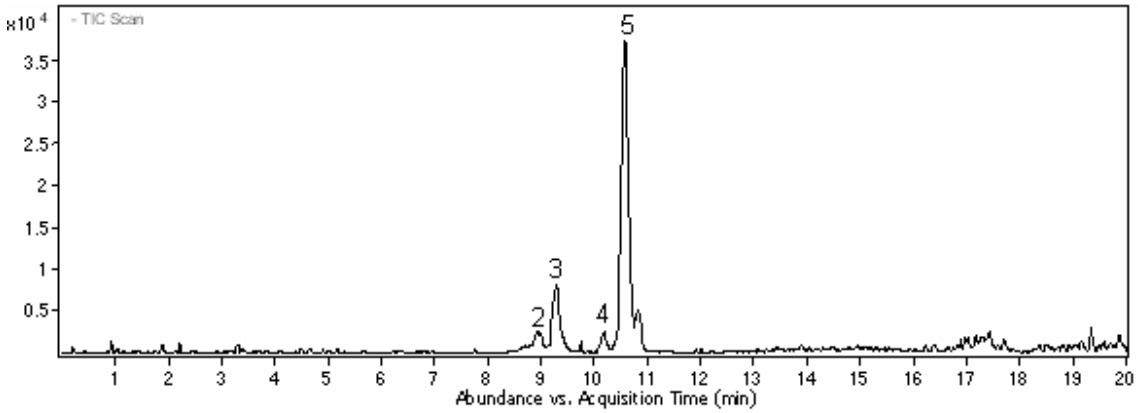
(A) Extra virgin olive oil



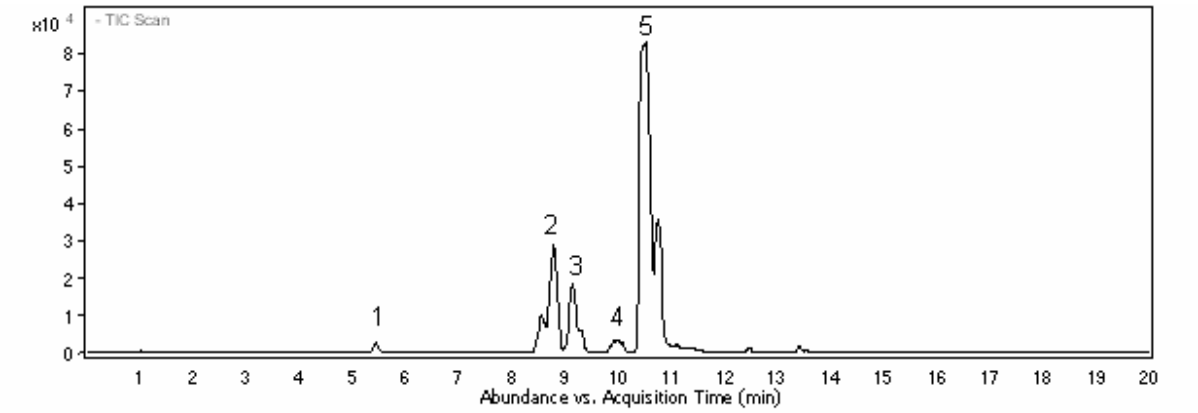
(B) Alperujo



(C) Olive leaves



(D) Small olive branches



(E) Seed bones

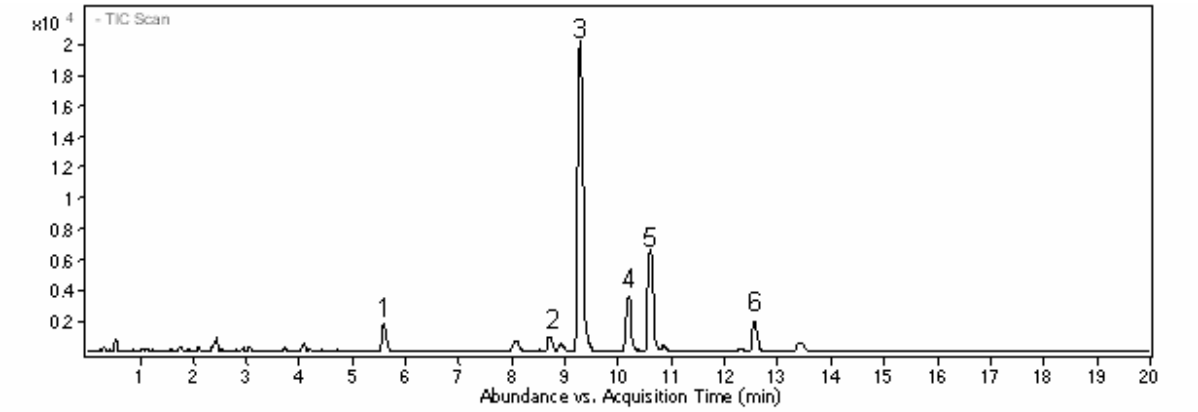


Fig. 7. Chromatogram obtained with the LC–MS–MS method for the determination of biophenols: (1) hydroxytyrosol; (2) verbascoside; (3) luteolin-7-glucoside; (4) apigenin-7-glucoside; (5) oleuropein; (6) luteolin; (7) apigenin; (8) diosmetin.

Table 10. Concentration of biophenols found in different raw materials from olive tree using LC–MS–MS (n = 3, concentration ± standar deviation, mg/kg)

N.d.: not detected.

Sample/Biophenol (mg/kg)	Hydroxytyrosol	Luteolin-7-glucoside	Apigenin-7-glucoside	Verbascoside	Oleuropein	Apigenin	Luteolin	Diosmetin
Olive oil	3.01 ± 0.22	N.d.	N.d.	0.08 ± 0.02	N.d.	0.65 ± 0.04	8.60 ± 0.89	0.62 ± 0.08
Alperujo	831.41 ± 21.76	14.32 ± 2.34	6.25 ± 0.95	20.22 ± 2.80	37.11 ± 3.78	22.45 ± 3.01	22.42 ± 3.11	N.d.
Olive leaves	N.d.	154.99 ± 9.80	206.80 ± 10.01	1428.00 ± 45.98	19049.32 ± 879.90	N.d.	N.d.	N.d.
Small olive branches	22.20 ± 1.98	175.30 ± 7.98	10.91 ± 0.76	1560.41 ± 50.09	672.63 ± 33.98	N.d.	N.d.	N.d.
Seed bones	18.1 ± 1.87	6.22 ± 0.76	0.09 ± 0.02	0.15 ± 0.03	0.06 ± 0.02	N.d.	1.18 ± 0.23	N.d.

(1) All raw materials contain higher contrations of biophenols than extra virgin olive oil, which is the oil with the highest contents of these compounds.

(2) The concentrations of biophenols in alperujo is very superior to that in extra virgin olive oil; a foreseeable result as alperujo is a residue with a high humidity (between 65 and 70%) and the target compounds have a polar character (hydrophilic phenols), so the afinity justifies that they are mainly in the residue.

(3) Oleuropein is massively in olive leaves (concentrations close to 2% m/m), thus constituting an excellent source to obtain this compound, of special interest taking into account the huge amount of this raw material generated from pruning and drupes harvest period, both with poor or nil exploitation. Oleuropein hydrolysis produces hydroxytyrosol, a compound of higher commercial interest than oleuropein (up to 12 times more expensive).

(4) Verbascoside, other hydroxytyrosol precursor, is present in leaves and branches at concentrations around 1500 mg/kg; therefore, leaves and branches are not only key sources of oleuropein and verbascoside, but also indirect sources of hydroxytyrosol.

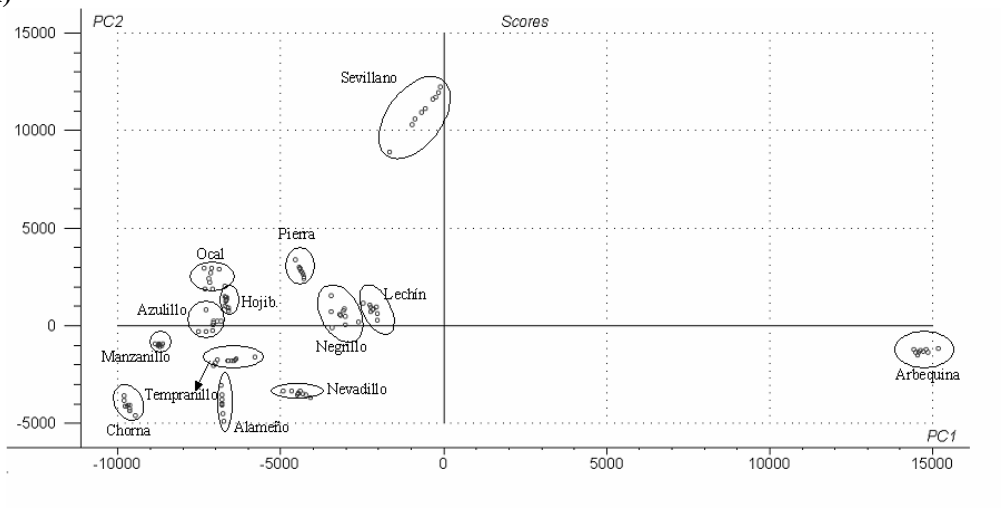
(5) Alperujo is the raw material with higher concentration of hydroxytyrosol; wich is a compehensible fact taking into account the polar character of this residue and thus, the high alperujo–oil partition coefficient. The high mixture content of alperujo and the use of water during olive oil production by the two-phase system favour the hydrolysis of oleuropein and verbascoside with subsequent increase of hydroxytyrosol concentration. Small branches and bones are also materials with significant contents of hydroxytyrosol (around 20 mg/kg).

(6) Leaves and branches have also been found highly concentrated in glucosilated flavones such as luteolin-7-glucoside and apigenin-7- glucoside; however, the aglycone flavones are not detected in both raw materials. Similarly, hydroxytyrosol, luteolin and apigenin are present in alperujo, which can be ascribed to hydrolysis of glucoside derivatives in the aqueous medium provided by alperujo. However, a flavone such as diometin was only detected in virgin olive oil, probably due to its lower polarity as compared to luteolin and apigenin. In addition, the methoxide group of diosmetin can be hydrolysed in contact with water.

2.3.3. Identification of olive tree varieties and cultivation zones through the biophenols content in leaves

Thirteen olive tree varieties and 6 cultivation zones were used in this study in which the biophenols from leaves were extracted with the help of microwaves and determined by liquid chromatography–mass spectrometry. After application of models based on PCA, HCA, KNN and SIMCA, the best models for both identifications were those based on PCA, as can be seen en Figs. 8 and 9, which show the discrimination capacity of these compounds.

(A)



(B)

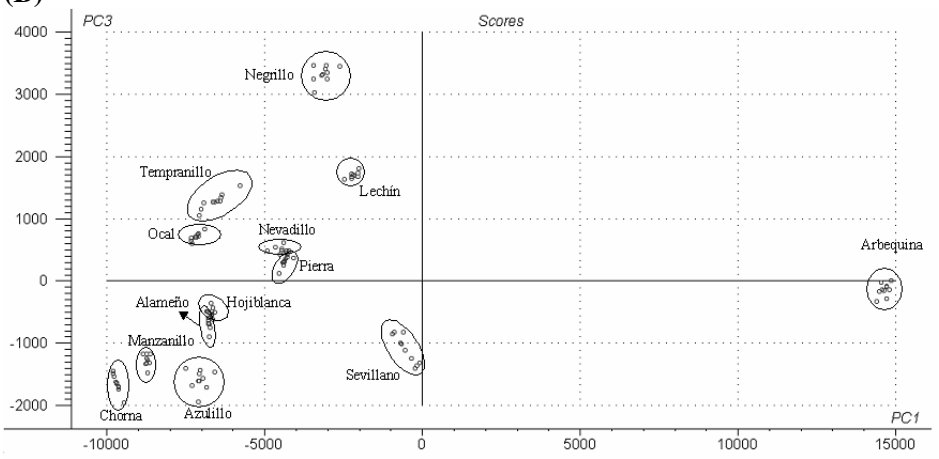
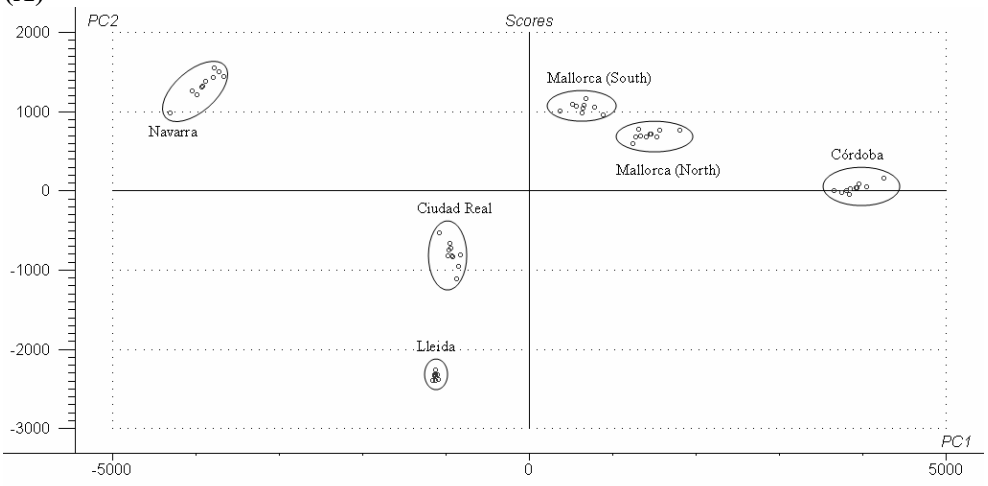


Fig. 8. PC1–PC2 (A) and PC1–PC3 (B) plots of the 13 olive varieties studied through leaf biophenols.

(A)



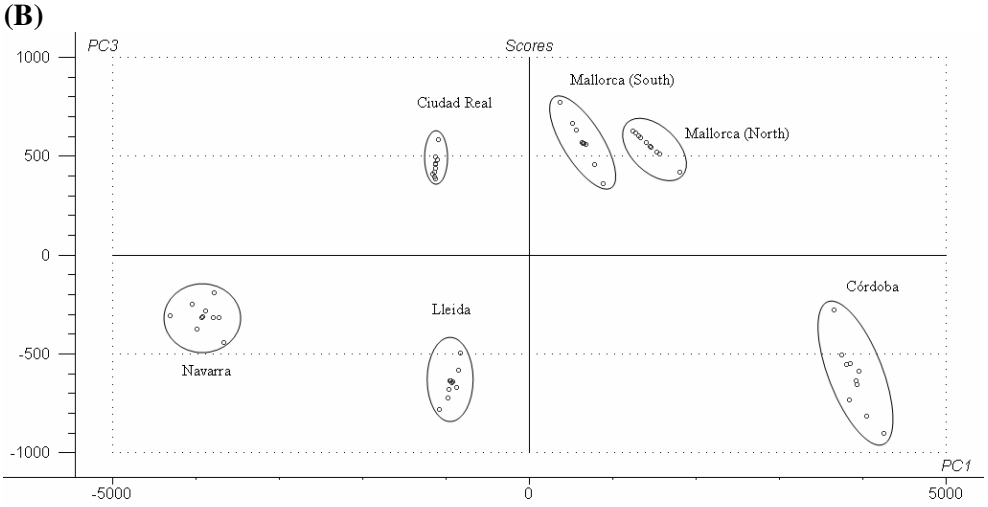


Fig. 9. Identification of production zones of arbequina olive tree through biophenols extracted from leaves.

3. USE OF BIOPHENOLS EXTRACTS FOR THE ENRICHMENT OF FOOD WITH POOR OR NIL CONTENTS OF THESE COMPOUNDS

Biophenols extracts from leaves+branches and from alperujo have been used to enrich food with these compounds. A food with low afinity to biophenols due to the difference in polarity (oil) was selected. The enriched oils have been from olive, soya and sunflower and enrichment monitoring has been performed through oleuropein as representative biophenol. Extracts with different concentrations (doubling the content from 248 mg/l to 5472 mg/l in oleuropein) have been used in the case of olive leaf extracts, the results of which are shown in Table 11.

Table 11. Enrichment of edible oils in biophenols by extracts from olive leaves

Oil	Concentration of oleuropein in leaf extracts (mg/l)	Concentration of oleuropein in oil after liquid–liquid extraction for 25 min (mg/l)	Partition coefficient
Olive	248	7.99 ± 0.46	0.033±0.001
	596	21.12 ± 0.72	
	1293	40.37 ± 0.67	
	2686	93.29 ± 1.43	
	5472	184.10 ± 3.19	
Sunflower	248	3.04 ± 0.48	0.013±0.001
	596	7.48 ± 0.86	
	1293	17.29 ± 1.46	
	2686	35.50 ± 0.46	
	5472	66.40 ± 3.21	
Soya	248	3.34 ± 0.57	0.013±0.001
	596	7.55 ± 0.87	
	1293	15.14 ± 0.79	
	2686	36.87 ± 1.59	
	5472	67.19 ± 3.31	

As can be seen in the table, the partition coefficient is constant for each type of oil. It is similar for sunflower and soya oils (0.013±0.001) and higher for olive oil (0.033±0.001). From these results it can be concluded that a given enrichment can be achieved by using the needed concentration in the extract, which can be deduced from the partition coefficient. Concentrated extracts as required can be obtained eliminating the extractant by conventional evaporation or in rotary evaporator.

4. DIRECT ENRICHMENT OF FOOD IN BIOPHENOLS BY CONTACT WITH THE RAW MATERIAL AND ULTRASOUND ASSISTANCE

This enrichment study has been carried out using the continuous system in Fig. 5, in which olive leaves have been placed in the chamber, which was subject to ultrasound action meanwhile different types or oil (olive, soya or sunflower) circulated through the dynamic system. The enrichment in biophenols of these oils under the optimal working conditions and for an irradiation time of 20 min depends on the type of oil, as can be seen in Table 12, and ranges between 14.45–9.92 μg/ml for oleuropein, 2.29–2.12 μg/ml for verbascoside, 1.91–1.51 μg/ml for apigenin-7-glucoside and 1.60–1.42 μg/ml for luteolin-7-glucoside have been obtained .

It is worth emphasizing that the enrichment process is carried out at ambient temperature in the absence of any solvent or extractant different to the given oil, thus converting the enrichment process in a cheap, simple and non-contaminant process by which a more healthy oil can be obtained in a short time and using as source of the enrichment compounds a material with scant or nil value.

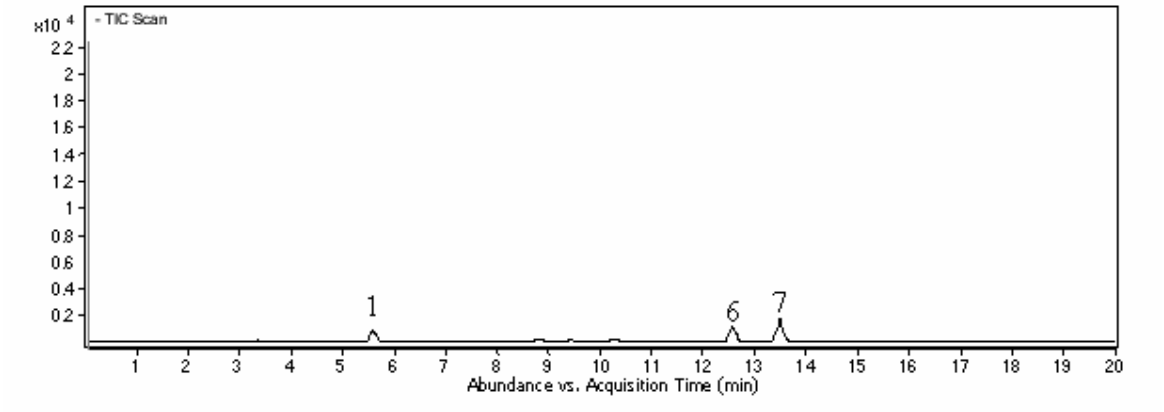
Table 12. Enrichment of edible oils using the proposed method and two reference methods

Biophenol	Olive oil				Sunflower oil			Soya oil		
	Blank	Proposed method	RM 1	RM 2	Proposed method	RM 1	RM 2	Proposed method	RM 1	RM 2
Oleuropein	ULOD	14.45 ± 3.32	3.44 ± 0.90	ULOD	10.21 ± 2.65	2.27 ± 0.64	ULOD	9.92 ± 2.72	2.20 ± 1.69	ULOD
Apigein-7-glucoside	ULOD	1.91 ± 0.21	ULOQ	ULOD	1.32 ± 0.43	ULOQ	ULOD	1.51 ± 0.10	ULOQ	ULOD
Luteolin-7-glucoside	ULOD	1.60 ± 0.20	ULOQ	ULOD	1.42 ± 0.19	ULOQ	ULOD	1.39 ± 0.43	ULOQ	ULOD
Verbascoside	ULOD	2.12 ± 0.45	ULOQ	ULOD	2.29 ± 0.43	ULOQ	ULOD	2.25 ± 0.39	ULOQ	ULOD
Hydroxytyrosol	1.54 ± 0.23	1.59 ± 0.34	1.67 ± 0.30	1.43 ± 0.29						
Apigenin	2.98 ± 0.76	2.78 ± 0.54	2.65 ± 0.67	3.00 ± 0.66						
Luteolin	2.43 ± 0.65	2.30 ± 0.33	2.29 ± 0.21	2.67 ± 0.43						

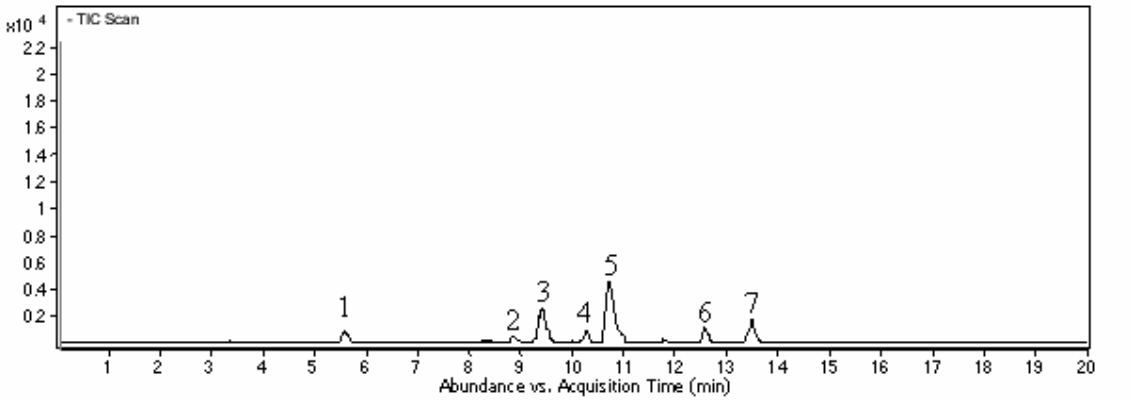
The standard deviation was calculated by 3 replicates in all instances. Hydroxytyrosol, apigenin and luteolin do not exist in sunflower and soya oils.
RM1 = reference method 1 (similar to that proposed, but in the absence of ultrasound).
RM2 = reference method 2 (stirring for 24 h at 25 ºC).
ULOD = under the detection limit.
ULOQ = under the quantitation limit.

The chromatograms in Fig. 10 show comparatively the contents of biophenols in the original olive oil, that enriched after stirring of the oil–leaves system for 24 h and that after 20 min of ultrasonic irradiation, thus demonstrating the efficiency of the last method (the other oils assayed — soya and sunflower— show a similar behaviour). Biophenols or oil degradation products have not appeared in any case.

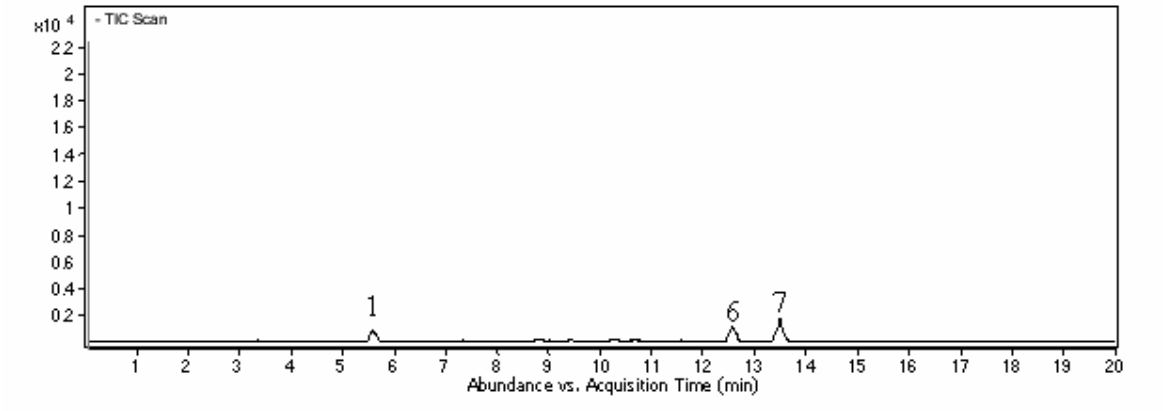
(A)



(B)



(C)



(D)

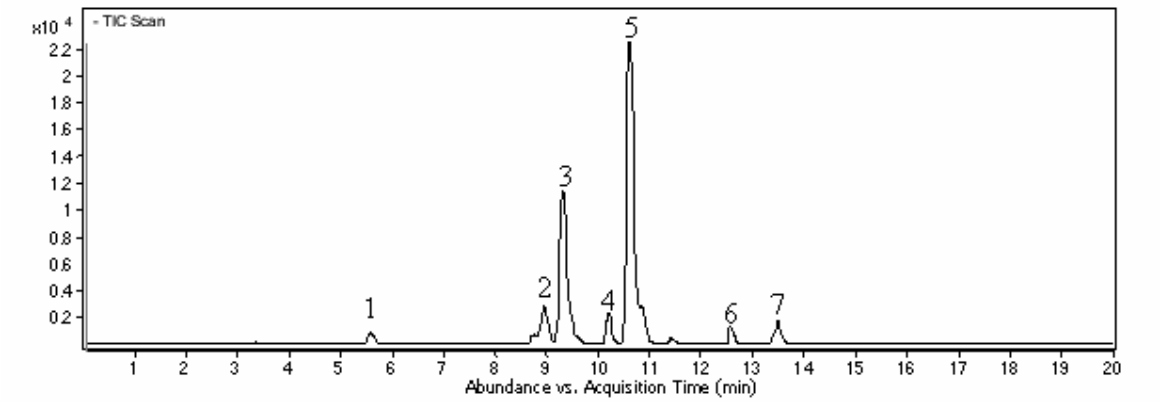


Fig. 10. TIC chromatograms obtained by isolation of the precursor ion for each biophenol in the analysis of olive oil: (A) blank; (B) after application of the reference method 2; (C) after application of the reference method 1; (D) after application of the proposed method. Peak identification: 1, hydroxytyrosol; 2, verbascoside; 3, luteolin-7-glucoside; 4, apigenin-7-glucoside; 5, oleuropein; 6, luteolin; 7, apigenin.

From sections 3 and 4 it can be concluded that the extracts from leaves or alperujo, and also directly leaves as such, can be used to enrich any type of food, as the research has been carried out with the type of food with less affinity to biophenols. Therefore, the food can be "tailored"enriched in such as way that keeping the diet typical of any country or region, the consumers can enjoy the benefits of the Mediterranean diet.

5. APPLICATION OF THE EXTRACTS TO PREVENTIVE AND CURATIVE MEDICINE, CELLULAR BIOLOGY AND BIOCHEMISTRY

Multidisciplinary uses of the extracts obtained by the proposed methods have involved collaboration with biochemistry, cellular biology and medicine teams in some of the areas in which the effects of these biophenols have not been studied so far. The effects of the extracts have been compared with those of the individual biophenols at similar concentrations. The preliminary results obtained so far have been spectacular, and some of them are commented below:

5.1. Antigenotoxic and antiproliferative effect of olive biophenols on cellular biology

The objectives in this field were as follows:

1. To study the absence of toxicity and the protective effects of olive biophenols from both olive leaves and alperujo on a cellular eukaryotic system in somatic proliferation.
2. To identify molecules with potential antiproliferative effect in human tumoural lines of leukaemic type (HL-60).

5.1.1.Results

(a) Determination of toxicity absence, antigienotoxic and protective activity of the genetic material by olive leaf and alperujo extracts.

The results obtained so far show low or medium toxicity (see Table 13). There was not effect on chronic treatments; therefore, the security for ingestion of these extracts is good, as toxicity of only 20% has been found at very high concentrations.

Table 13. Toxicity of the different olive leaf extracts on the eukaryotic system *D. melanogaster*

	Control	3.75 µl/ml	7.5 µl/ml	15 µl/ml	30 µl/ml	Media
H-E[a]	1	0,98	0,70	1,03	0,65	0,84
H-EC	1	0,71	1,12	0,80	0,91	0,88
HC-EC	1	0,88	0,67	1,03	0,78	0,84
Average	1	0,92	0,83	0,95	0,78	

[a]H-E, leaves and extracts kept at ambient conditions; H-EC, frozen extract; HC-EC, frozen leaves and extracts.

The results of toxicity for two separated biophenols (hydroxytyrosol and oleuropein) are listed in Tables 14 and 15 at concentrations similar to those in the extracts. Similarly to the extracts, there is not significant toxicity or dose effect for these two biophenols.

Table 14. Hydroxytyrosol toxicity on the eukaryotic system *D. melanogaster*

Control	0.000001%	0.00001%	0.0001%	0.001%	0.01%	Average
1	0,89	0,77	1,72	0,43	1,26	1,01

Table 15. Oleuropein toxicity on the eukaryotic system D. melanogaster

Control	0.0017 mg/ml	0.004 mg/ml	0.018 mg/ml	0.075mg/ml	0.3 mg/ml	Average
1	0,53	2,94	0,74	0,80	1,01	1.20

(b) Determination of the antiproliferative potential: inhibition of the tumoural growing in HL-60 cells The inhibition of the tumoural growing, expressed as growing relative to non-treated control has been determined. As shown in Figs. 11 to13, olive leaf extracts inhibit tumoural growing on human promielocitic leukaemic cells HL-60, independent of the preservation mode of both leaves and extracts.

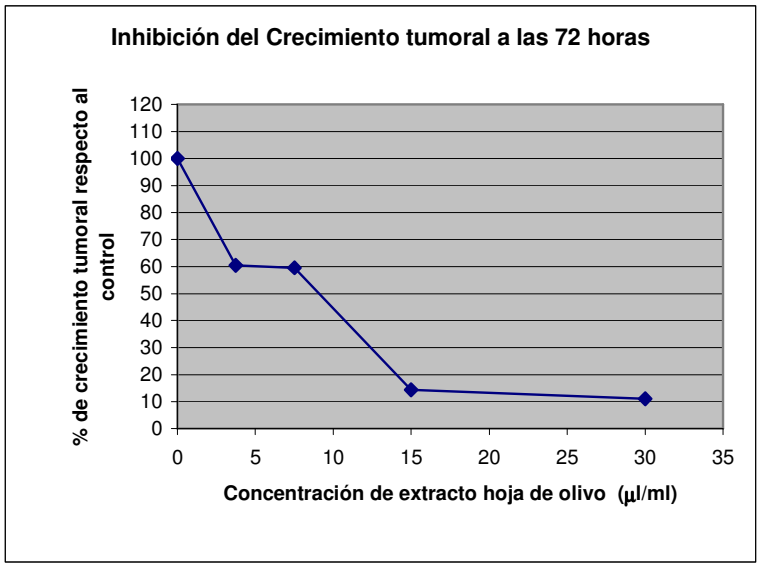


Fig. 11. Inhibition of the tumoural growing on HL-60 cells by non-frozen olive leaf extract.

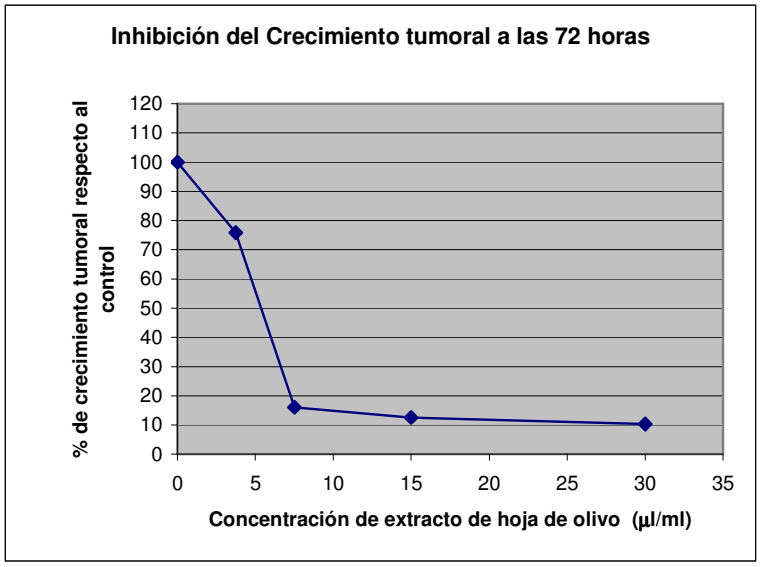


Fig. 12. Inhibition of the tumoural growing on HL-60 cells by previously frozen olive leaf extract.

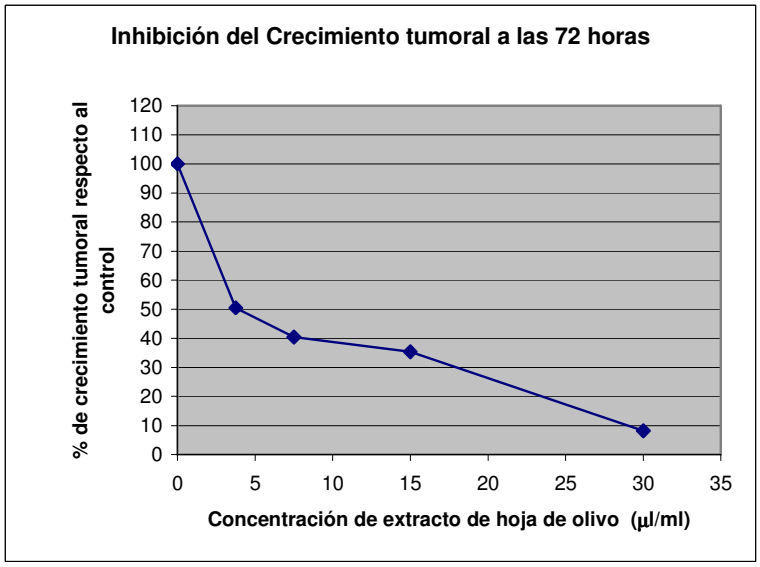


Figura 13. Inhibition of the tumoural growing on HL-60 cells by previously-frozen olive leaf extract.

5.1.3. Conclusions

(1) Olive leaf or alperujo extracts are not toxic to normal Drosophila cells.
(2) Biophenols are less toxic than water.
(3) Both extracts and individual biophenols are tumouricide.
(4) Hydroxytyrosol shows tumouricide capacity 10 times higher than oleuropein.

5.2. Effects of olive phenols on osteoblast stem-cells

Ageing produces decrease on osteoblasts —cells forming bone— and increase adipocytes in the spinal cord, thus contributing to bone loss and development of osteoporosis as a result.

Mesenquimal stem cells (MSC) are the precursors of both types of cells. There is the hypothesis that the oxidative stress and ageing are cooperant factors of degenerative processes taking place during ageing. Oxidative stress favours differenciation of stem cells to adipocytes, instead of to osteoblasts, resulting in loss of bone mass.

Consumption of compounds with high antioxidant capacity helps to prevention and/or retardation of physiological processes characteristic of ageing. Oleuropein is among these compounds, and it posses a high activity as anti-inflammatory and antioxidant both *in vitro* and *ex vivo*. A decrease of bone loss produced by strogen defficiency in rats has been observed when oleuropein was in their diet.

Studies on mesenquimal stem cells from spinal cord, in the presence of different concentrations of oleuropein (between 10^{-5} – 10^{-6} M) during differentiation to either osteoblasts or adipocites, showed that within these concentrations of the target biophenol differentiation to osteoblastos was favoured (potentiation of osteogenesis marker genes such as alkaline phosphatase, osteoprotegerine of transcription runx2 factor). On the contrary, in cells induced to adipocites gene expression related to adipogenesis such as the transcription factor ppar-γ2 and lipoprotein lipase were inhibited.

These results show that consumption of products from olive tree, rich in oleuropein, have a beneficial effect on bone, decreasing loss and the risk of osteoporosis associated to ageing as a result.

6. POSSIBLE USE OF THE RESIDUES FROM THE EXTRACTION

The residues from the extraction, particularly in the case of alperujo, lose the toxic and bad odour character after antioxidants extraction; so they can be stored *sine die* without environmental contamination problems. The difficulty to compost this material also disappears.

Nevertheless, with a view to obtaining a higher added-value research focussed on biodiesel or bioethanol production, depending on the residue, has been started.

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