Food Bioprocess Technol (2012) 5:1384–1393
DOI 10.1007/s11947-010-0419-0

ORIGINAL PAPER

Hydro-Ethanolic Mixtures for the Recovery of Phenols from Mediterranean Plant Materials

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Received: 22 May 2010 / Accepted: 1 August 2010 / Published online: 12 August 2010

Abstract The optimization of phenol extraction in hydro-ethanolic mixtures was investigated by using dried samples of aromatic plants (*Salvia fruticosa* and *Origanum dictamnus* L.) and fruit-bearing tree leaves (*Olea europaeae* L. and *Citrus sinensis* L.) as substrates. Four extraction conditions were studied by monitoring the phenolic content and the antioxidant efficacy of the extracts. Temperature and time were the primary factors affecting the extraction yield, while their increase to 60 °C and 8 h, respectively, resulted in enhanced phenols recovery. Extraction equilibrium was obtained for all the determinations only in the case of dictamnus after 4 h. The sample/solvent ratio seem to affect conversely the phenol content and antioxidant efficacy (AE) yield, as the higher total phenols concentrations were followed by lower AE values. The ethanol content affected also the process, but the impact on yield was rather important (and negative) only by using absolute ethanol. Sage was the most phenol rich substrate with a maximum yield of 73.3 mg total phenols/g and AE value of 30.6 mg 2,2-diphenyl-1-picrylhydrazyl (DPPH)/g, which were obtained by using the conditions of 40 °C, 8 h, 40 g sample/L, and 70 mL ethanol/100 mL. Dictamnus, olive, and orange tree leaves followed with maximum yields of 43.9, 43.3, and 12.2 mg total phenols/g as well as 16.1, 19.3, and 1.2 mg DPPH/g, respectively (obtained with 40 °C, 2 h, 40 g/L, 70 mL/100 mL, 40 °C, 8 h, 40 g/L, 70 mL/100 mL, and 60 °C, 2 h, 40 g/L, 70 mL/100 mL, respectively). The aromatic plants extracts were proposed as additives in order to produce potable drinks with similar AE to established beverages, after a simple dilution with water.

Keywords Sage · Dictamnus · Olive · Orange · Leaves · Antioxidant

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Introduction

In recent years, there is a rapidly increasing interest to recover natural antioxidants due to the unknown safety of chemical food additives and the utilization of plant materials as initial substrates could be a cheap and feasible solution (Schieber et al. 2001; Moure et al. 2001; Avila-Sosa et al. 2010). Phenols constitute a major group of antioxidants that are widely distributed in the plant kingdom. They consist of a large number of structurally different compounds, including simple and complex configurations, in which at least one aromatic ring is present; in the aromatic ring, one or more hydrogen atoms are substituted by the hydroxyl group (Pereira et al. 2010). The most popular substrates with regard to the recovery of phenols include agricultural wastes, leaves, and aromatic plants (Boskou 2006). All these materials possess availability advantages, either due to their abundance in the Mediterranean region or because they represent a major disposal problem for the industry concerned (Boskou 2006; Mylonaki et al. 2008).

In particular, olive tree is massively cultivated in the Mediterranean region and is known to be the main carrier of

antioxidant phenols through the olive fruit and the by-products (wastewater and pruning leaves) of olive oil production (Niaounakis and Halvadakis 2004). For example, the corresponding leaves are known to contain high amounts of phenolic acids and alcohols, flavonoids, and secoiridoids (Niaounakis and Halvadakis 2004; Bouaziz and Sayadi 2005; Paiva-Martins and Pinto 2008; Mylonaki et al. 2008). Orange tree is also widely cultivated in the Mediterranean countries, but the references reporting the recovery of phenols from the corresponding leaves are scarce. Nevertheless, the occurrence of bound and free hydroxyl-cinnamic acids in the orange fruits (Peleg et al. 1991) indicates potential appearance of phenolic compounds in the other plant parts like leaves. On the other hand, aromatic plants have been studied as sources of different classes of natural antioxidants, and in some cases, grown wild or cultivated have been commercially utilized for various foods, cosmetics, and pharmaceutical preparations (Exarchou et al. 2002; Durling et al. 2007). Sage (*Salvia fruticosa*) and dictamnus (*Origanum dictamnus* L.) are two typical perennial aromatic plants that are native in the Mediterranean region, and their corresponding infusions in hot water are consumed as teas or decoctions. Sage essential oil and flavorings are known to be utilized as initial source for several possess antioxidant and anticarcinogenic properties (Gali-Muhtasib 2006), while dictamnus infusion is consumed to cure cough and sore throats and relieve stomachache and several pains (Kouri et al. 2007). These plants are known to contain flavones, flavonols, phenolic acids, and their derivative esters (Atoui et al. 2005; Boskou 2006).

Phenols are normally recovered from olive tree leaves, sage, and *Origanum* species by solvent extraction and yield as well as antioxidant activity of the extracts are strongly dependent on the solvent, due to the polarity diversity of active compounds. The usual solvents include methanol, ethanol, acetone, petroleum ether, diethyl ether, and ethyl acetate (Exarchou et al. 2002; Pizzale et al. 2002; Tsimogiannis et al. 2006; Kouri et al. 2007; Paiva-Martins and Pinto 2008). The selected method, the extraction temperature, and the time are also important parameters for the recovery of phenols (Moure et al. 2001; Oreopoulou 2003). With regard to olive leaves treatment, hydro-alcoholic solvents such as methanol or ethanol mixtures with several concentrations have been recently investigated due to their ability to extract both lipophilic and hydrophilic phenols (Japón-Luján et al. 2006a, b; Japón-Luján and Luque de Castro 2008; Mylonaki et al. 2008; Boudhrioua et al. 2009). Hydro-ethanolic mixtures have also been utilized for the recovery of phenols from dried sage (*Salvia officinalis*) (Durling et al. 2007). The main disadvantage of methanol containing mixtures is that they are toxic for human consumption and thus undesirable for industrial use (Japón-Luján et al. 2006a, b).

Ethanol possesses a lot of advantages: it is cheap, reusable, as well as nontoxic, and the corresponding extracts could be utilized directly in beverages, foods, and cosmetics (Japón-Luján and Luque de Castro 2008; Mylonaki et al. 2008; Galanakis et al. 2010c). Likewise, ethanol (85 mL/100 mL) has been referred to preserve the phenolic characteristics of the extracts recovered from olive mill wastewater and indeed to increase their radical scavenging ability during 18 weeks storage (Galanakis et al. 2010c). Extraction of phenols with varying concentration of ethanol can fractionate phenolics on the basis of polarity (Durling et al. 2007). Such an approach possesses a definite advantage: hydrophilic and lipophilic active compounds are recovered together in different proportions and synergistic interactions occur inside the medium (McDonald et al. 2001). Thereby, fractions of particular high activity could be identified and studied further, i.e., for the recovery of individual components or designing a specific application.

Despite the obvious usefulness of hydro-ethanolic mixtures, studies referring the optimization of the corresponding extraction processes from natural dried plant substrates are still rather limited. Thereby, the objective of the current study is to investigate the recovery of phenols from Mediterranean plant materials like aromatic plants (*S. fruticosa* and *O. dictamnus* L) and fruit-bearing (olive—*Olea europaeae* L. and orange—*Citrus sinensis* L.) tree leaves. Thus, plant substrates were treated with hydro-ethanolic mixtures, and the effect of extraction parameters was studied by monitoring the phenolic content and the scavenging ability against DPPH radical.

Experimental

Materials and Equipment

Reagents were of analytical grade. Sage (*S. fruticosa*) and dictamnus (*O. dictamnus*) aboveground samples were purchased prepacked from the local market in Chania, Greece. Fresh leaf samples were directly harvested from one olive (*O. europaea*) and one orange (*C. sinensis* L.) tree, which are cultivated in Chania, Greece. Spectrophotometric analysis was performed with a Shimadzu UV-mini-1240 spectrophotometer (Tokyo, Japan) with cuvettes of 1 cm optical path.

Experimental Plan and Extraction Experiments

Phenol extraction was based on the dispersion of the aforementioned plant materials in hydro-ethanolic with several proportions and up to different concentrations. Four different experimental series were conducted for each plant material in order to study the following factors of extraction

process: (1) temperature, (2) time, (3) ratio of sample per solvent, and (4) ethanol content of the final mixture. Table 1 shows the assayed combinations of sample per solvent ratio, ethanol content of final mixture, extraction temperature, and time. For each experimental series, the three parameters remained constant, and one of them was assayed in different conditions. The recovered hydro-ethanolic extracts were assayed for the classification of phenols (total, *o*-diphenols, hydroxycinnamic acid derivatives, and flavonols) and the determination of corresponding antioxidant efficacy. Results are represented for each experimental series separately. All the experiments as well as the analytical determinations were performed in triplicates.

Samples were treated in four different batches according to the assayed experimental series. Particularly, each sample batch was washed with a wet paper and cut up in squares of 5×5 mm. Cutting samples were dried at 102 °C for 3 h in an oven. After drying, aliquots (2, 4, or 8 g) of samples were placed together with 100 mL of hydro-ethanolic solution (25, 50, 75, or 100 mL ethanol/100 mL) into a 250-mL glass bottle and closed with a plastic lid. The bottles were placed into a circulated water bath and incubated at different temperatures (25, 40, or 60 °C) for 1, 2, 4, or 8 h. After incubation, samples were quickly cooled to room temperature and filtered through a G2 glass filter. The recovered extracts were assayed to the aforementioned determinations within the same day.

Phenols Determination and Classification

The determination and classification of the phenols in the extracts were measured colorimetrically using different methods. Total phenolic content was determined using the Folin–Ciocalteau reagent (Galanakis et al. 2010a, b). In particular, an appropriately diluted sample was mixed with 0.25 mL Folin–Ciocalteau reagent. One milliliter of saturated sodium carbonate solution (35 g/100 mL) was added after 3 min stirring, and the final solution was left in the dark for 1 h. The absorbance of the solution was measured at 725 nm. A standard curve was prepared using 0–50 mg/L solutions of gallic acid in methanol/water. The standard solutions were prepared with several dilutions of a mother solution in water. Mother solution was prepared as follows: 1 g of gallic acid was solubilized in 100 mL of methanol and then 1 mL of the resulted solution was diluted to 100 mL of water. Total phenol values were expressed as gallic acid equivalents (mg/g).

The determination of *o*-diphenols was performed following a modification of the method described by Mateos et al. (2001). One milliliter of diluted extract was vigorously mixed with 1 mL of sodium molybdate dihydrate solution (5 g/100 mL in 50 mL ethanol/100 mL) into a 10-mL volumetric flask, and the volume was made up to 10 mL with 50 mL ethanol/100 mL. After 15 min, the absorbance was measured at 370 nm. A blank was prepared by mixing the sodium molybdate dihydrate solution with 50 mL ethanol/100 mL. A standard curve was prepared using solutions of caffeic acid (0–200 mg/L) in 50 mL ethanol/100 mL. Results were expressed as caffeic acid equivalents (mg/g).

The determination of different phenolic classes was processed according to Obied et al. (2005). One milliliter of diluted extract was mixed with 1 mL of HCl–ethanol solution (0.1 mL HCl/100 mL in 95 mL ethanol/100 mL) into a 10-mL volumetric flask, and the volume was made up to 10 mL with 2 mL HCl/100 mL. After mixing, the absorbance was measured at 280, 320, and 360 nm to determine total phenols, hydroxycinnamic acid derivatives, and flavonols, respectively. A blank was prepared by mixing the HCl–ethanol solution with 2 mL HCl/100 mL. The corresponding standard curves to the above determinations were prepared using standard solutions (10 mL ethanol/100 mL water) of gallic acid (0–200 mg/L), caffeic acid (0–100 mg/L), and quercetin (0–150 mg/L), respectively. The main difference between the two methods for the analysis of total phenols is that this protocol has been referred to determine phenols with higher molecular weights compared to Folin–Ciocalteau method (Galanakis et al. 2010b, c).

Determination of Antiradical Efficacy

Antiradical efficacy (AE) of the extracts was determined by their scavenging activity against DPPH radical, following a modification of the method described by Kulisic et al.

Table 1 Extraction conditions for each experimental series

Experimental series	Condition (unit)			
	Temperature (°C)	Time (h)	Ratio sample/solvent (g/L)	Ethanol content (mL/100mL)
1	25, 40, 60	2	40	70
2	40	1, 2, 4, 8	40	70
3	40	2	20, 40, 80	70
4	40	2	40	25, 50, 70, 100

(2004). Particularly, 100 μL of each extract in different dilutions were vigorously mixed with 1.5 mL methanolic solution of DPPH radical (32 mg/L) in 2-mL plastic tubes. The absorbance at 517 nm of the resulting mixtures was measured after 1 h, while pure methanol was utilized to zero the spectrophotometer. A blank solution of the DPPH radical without antioxidant was used as control sample. The percentage inhibition of the DPPH radical by the samples was calculated according to the equation:

$$\%\ \text{inhibition} = \left(\left(A_{C(0)} - A_{A(t)}\right)/A_{C(0)}\right) \times 100 \quad (1)$$

where $A_{C(0)}$ is the absorbance of the control at t=0 min and $A_{A(t)}$ (mg DPPH/g antioxidant) is the absorbance of the antioxidant at t=1 h. Results were expressed in AE values that were calculated according to the equation:

$$AE = 1/EC_{50} \quad (2)$$

where EC_{50} is the effective concentration of the antioxidant extract that resulted in 50% scavenging of DPPH radical.

Statistical Analysis

Each variable was performed for three replicates, and the mean ± standard deviation values were calculated. Data were analyzed using Student's t test (pair wise comparisons, MS Office Excel 2007). Significant differences between samples were detected when the acceptable level of probability was 5% ($P \leq 0.05$) for all the comparisons.

Results

Results are represented in Tables 2, 3, 4, and 5. Each table is separated with lines to four "row blocks" that refer to the different assayed experimental series.

Recovery of Phenols from Aromatic Plants

The phenolic characteristics and AE of the extracts recovered from sage are shown in Table 2. The extract with the highest AE (30.6 mg DPPH/g) was obtained with an extraction mixture of 70 mL ethanol/100 mL and 40 g sample/L, after 8 h treatment at 40 °C (second row block). Moreover, concentrations of phenolic classes decreased according to the following order: *o*-diphenols > hydroxycinnamic acid derivatives > flavonols. All the tested parameters were increased by enhancing the extraction temperature from 25 to 60 °C, although the values were not always significantly different (first row block). Besides, all the tested parameters were significantly increased by extending the extraction time from 1 to 8 h, except for the values of total phenols (at 280 nm) and *o*-diphenols obtained between 2 and 4 h, respectively (second row block). On the other hand, phenolic concentrations and AE showed a different behavior at each tested sample per solvent ratio. For example, *o*-diphenols concentrations possessed the highest value at 20 g/L and the lowest at 40 g/L, while AE value showed a conversely behavior, as it was more than 6-fold enhanced by increasing the ratio from 20 to 80 g/L, respectively (third row block). Finally, the highest values of phenolic concentrations and AE were generally obtained for the mixtures possessing 50 and 70 mL ethanol/100 mL, while the lowest values were obtained for absolute ethanol (fourth row block).

Table 3 shows the phenolic characteristics and AE of the extracts recovered from dictamnus. The extract with the highest AE (16.1 mg DPPH/g) was obtained with an extraction mixture of 70 mL ethanol/100 mL and 40 g sample/L, after 2 h treatment at 60 °C (first row block). Concentrations of phenolic classes were again decreased for all the experimental series according to the order obtained in the case of sage. Parameters were enhanced by increasing the extraction temperature from 25 to 60 °C (first row block), although the corresponding values were not always significantly different. A similar tendency was observed by comparing the data obtained at different extraction times (second row block): all the tested parameters were increased by extending the extraction time from 1 to 4 h, but the values were not always significant different. Nevertheless, no significant differences were observed by comparing all the parameters of the samples recovered after 4 and 8 h, respectively. At this case, *o*-diphenols concentrations possessed the highest value at 40 g/L and the lowest at 20 g/L, while AE value was more than 3-fold enhanced by increasing ratio from 20 to 80 g/L, respectively. With regard to the different hydro-ethanolic mixture contents (fourth row block), all the assayed parameters possessed the highest values for the mixtures of 25 and 50 mL ethanol/100 mL, while the lowest values were obtained for absolute ethanol.

Recovery of Phenols from Fruit-Bearing Tree Leaves

The phenolic characteristics and AE of the extracts recovered from olive tree leaves are shown in Table 4. In particular, the extract with the highest AE (19.3 mg DPPH/g) was obtained as in the case of sage with an extraction mixture of 70 mL ethanol/100 mL and 40 g sample/L, after 8 h treatment at 40 °C (second row block). Concentrations of *o*-diphenols were higher compared to these of hydroxycinnamic acid derivatives and flavonols for all the experimental series. As in the case of dictamnus, all the assayed parameters were enhanced by increasing the extraction temperature from 25 to 60 °C (first row block), while all the values obtained at 40 and 60 °C were significantly different between them. Likewise, all the tested parameters were significantly

Table 2 Phenolic content and antioxidant efficacy of ethanolic extracts, recovered from sage (*Salvia fruticosa*) under different extraction conditions

Assayed extraction parameter (unit)	Value	Phenolic[a] content					Antioxidant efficacy (AE)[a]
		Total[b] (725nm) mg/g	Total[b] (280nm)	Hydroxycinnamic acids[c] (320nm)	Flavonols[d] (360nm)	*o*-diphenols[c] (517nm)	mg DPPH[e]/g
Temperature (°C)	25	41.6±2.1a	34.0±2.5a	18.7±2.3a	15.8±1.7a	26.4±9.6a	13.8±1.4a
	40	63.3±3.6b	48.0±3.1b	26.2±1.0b	24.1±1.8b	42.9±4.8b	20.4±0.5b
	60	67.6±3.9b	51.2±2.9b	27.9±0.8c	28.6±1.3c	57.2±3.6c	24.5±1.4c
Time (h)	1	49.7±3.8a	35.9±0.9a	20.1±0.2a	18.4±0.4a	27.5±2.6a	18.0±0.6a
	2	64.1±2.2b	49.1±1.9b	26.5±0.4b	24.5±0.7b	45.3±3.2b	21.8±0.3b
	4	65.3±0.8b	54.0±3.0c	28.4±1.5c	27.1±1.5c	51.6±6.6b	24.8±1.3c
	8	73.3±3.4c	70.9±4.4d	38.8±3.3d	38.0±3.1d	62.1±7.6c	30.6±3.3d
Ratio sample/solvent (g/L)	20	65.2±9.8a	57.7±6.3a	29.9±4.1a	28.2±4.0a	51.8±4.3a	5.3±0.3a
	40	61.1±2.6a	45.8±1.6b	25.6±1.8a	23.5±4.2a	40.4±2.3b	18.9±1.7b
	80	47.1±2.2b	48.2±3.8b	26.5±1.9a	25.6±2.0a	46.3±2.5c	34.5±0.1c
Ethanol content (mL/100 mL)	25	34.9±3.5a	26.8±2.7a	14.3±1.5a	13.4±1.4a	30.7±3.6a	11.3±1.3a
	50	53.0±2.4b	44.9±1.7b	25.2±1.3b	24.0±0.8b	43.9±3.0b	17.0±2.1b
	70	64.1±2.2c	49.5±4.5b	28.1±2.9b	24.5±0.7b	43.4±1.5b	21.4±0.9c
	100	15.8±1.2d	14.7±0.3c	11.9±1.1c	7.0±0.2c	9.0±0.8c	1.2±0.3d

[a] Values represent mean ± standard deviation (n=3). Values these possessing the same letters (at least one) within a column block are not significantly different ($p \leq 0.05$)

[b] Expressed as mg gallic acid/g dry matter

[c] "Hydroxycinnamic acids" for "hydroxycinnamic acid derivatives." Results expressed as mg caffeic acid/g dry matter

[d] Expressed as mg quercetin acid/g dry matter

[e] "DPPH" for "2,2-diphenyl-1-picrylhydrazyl." Results expressed as mg DPPH/g extract

increased by extending the extraction time from 1 to 8 h (second row block), except for the values of flavonols obtained between 2 and 4 h, respectively. Total phenols (both at 725 and 280 nm) and hydroxycinnamic acid derivatives possessed the highest values at 20 g/L and the lowest at 40 g/L, while flavonols and AE values showed a conversely behavior (third row block). With regard to the assayed contents of hydro-ethanolic mixture (fourth row block), the values obtained with 70 mL ethanol/100 mL were significantly higher compared to the other combinations for all the tested parameters. The corresponding lowest values were observed either with 100 or 25 mL ethanol/100 mL, but absolute ethanol possessed more than 3-fold lower AE value.

Table 5 shows the phenolic characteristics and AE of the extracts recovered from orange tree leaves. Generally, all the parameters possessed much lower values compared to the extracts recovered from the previous sources, while the corresponding AE values were very low (0.2–1.2 mg DPPH/g). The extract with the highest AE (2.3 mg DPPH/g) was obtained with an extraction mixture of 70 mL ethanol/100 mL and 80 g sample/L, after 2 h treatment at 40 °C (third row block). Likewise, all the tested parameters were significantly enhanced by increasing the extraction temperature from 25 to 60 °C (first row block). The same tendency was observed by extending the extraction time from 1 to 8 h (second row block), but the values obtained after 2 and 4 h extraction were not significantly different between them for each tested parameter. As in the case of aromatic plants, AE value possessed the highest value at the ratio of 80 g/L (third row block). On the other hand, total phenols (at 725 and 280 nm), hydroxycinnamic derivatives and flavonols possessed higher values at 40 g/L. In the fourth row block, all the assayed parameters possessed the highest values for the mixtures of 50 and 75 mL ethanol/100 mL, but the differences were not always significant.

Discussion

The preparation of extracts with improved antioxidant potency should be based on the estimation both of total phenols and specific classes, since the overall AE may be defined by the relative amounts of the most active compounds (Makris et al. 2007). The recovery of several phenols in the hydro-ethanolic medium involves extraction from plant cytoplasmatic vacuoles and cell walls matrixes, as well as simultaneous solubilization of hydrophilic and lipophilic compounds. The driving force for the extraction is the solvent concentration

Table 3 Phenolic content and antioxidant efficacy of ethanolic extracts, recovered from dictamnus (*Origanum dictamnus* L.) under different extraction conditions

Assayed extraction parameter (unit)	Value	Phenolic[a] content					Antioxidant efficacy (AE)[a]
		Total[b] (725nm) mg/g	Total[b] (280nm)	Hydroxycinnamic acids[c] (320nm)	Flavonols[d] (360nm)	*O*-diphenols[c] (517nm)	mg DPPH[e]/g
Temperature (°C)	25	20.2±2.2a	21.8±2.3a	10.3±1.3a	7.1±0.5a	11.7±0.7a	7.6±0.4a
	40	32.0±1.6b	20.7±1.0a	10.8±0.6a	7.5±1.2a	21.9±5.0b	10.4±0.3b
	60	43.9±0.9c	38.5±1.6b	19.7±0.7b	15.7±0.6b	32.1±0.9c	16.1±1.2c
Time (h)	1	23.8±1.4a	19.8±0.6a	10.4±0.2a	7.2±0.3a	8.7±2.0a	9.0±0.6a
	2	33.1±2.3b	20.9±1.7a	11.1±0.9a	8.0±0.8a	20.2±0.6b	10.9±0.5b
	4	35.3±2.4b	28.7±3.5b	14.2±0.7b	10.9±1.5b	22.8±0.8c	13.6±1.6c
	8	36.8±1.7b	30.3±1.0b	16.3±1.7b	11.5±0.2b	22.4±1.1c	13.9±1.0c
Ratio sample/solvent (g/L)	20	28.8±4.1a	21.5±3.7a	11.2±2.1a	7.5±1.6a	15.0±1.3a	4.3±0.6a
	40	31.2±0.9a	18.1±0.3b	10.1±1.7a	7.2±2.0a	19.7±2.9b	9.9±0.9b
	80	23.1±1.1b	23.2±1.8c	11.8±0.9a	8.6±0.7a	16.5±0.6a, b	14.4±0.7c
Ethanol content (mL/100 mL)	25	41.7±2.8a	31.8±1.0a	18.7±0.6a	15.8±0.5a	34.3±1.2a	15.5±1.3a
	50	43.8±0.7a	34.8±1.6b	21.4±0.8b	17.2±0.5b	33.9±1.8a	16.5±0.3a
	70	31.5±0.9b	19.5±1.7c	10.1±0.7c	7.1±1.3c	20.2±2.1b	9.7±0.8b
	100	4.2±0.6c	6.2±0.4d	2.0±0.5d	1.9±0.2d	2.2±0.2c	0.7±0.2c

[a] Values represent mean ± standard deviation (n=3). Values these possessing the same letters (at least one) within a column block are not significantly different ($p \leq 0.05$)

[b] Expressed as mg gallic acid/g dry matter

[c] "Hydroxycinnamic acids" for "hydroxycinnamic acid derivatives." Results expressed as mg caffeic acid/g dry matter

[d] Expressed as mg quercetin acid/g dry matter

[e] "DPPH" for "2,2-diphenyl-1-picrylhydrazyl." Results expressed as mg DPPH/g extract

gradient within the material particles, while each compound develops equilibrium between concentrations of the plant tissue and the solvent, respectively (Gertenbach 2001). An increase in extraction temperature enhances diffusivity, softens the plant tissue, and promotes elution of attached phenols inside the hydro-ethanolic mixture. Nevertheless, the temperature affects also the compound stability due to chemical and enzymic degradation or losses by thermal decomposition (Moure et al. 2001).

At the current study, temperature increase (from 25 to 60 °C) was shown to affect positively the extraction of phenols from all the tested plant sources, as concentrations of total and separated classes were gradually and in most cases significantly increased. The temperature induced acceleration of the extraction was impressed in enhanced antioxidant activity of the extracts, too. Besides, there was no indication that the maximum extraction had been reached at 60 °C; thus, recovery of phenols could eventually increase by further increase of the temperature. These results are in accordance to other plant materials. For example, the optimum extraction temperature with hydro-ethanolic mixtures from dried sage (*S. officinalis*) was equal to 60 °C for total phenols recovery, although the observed enhanced extraction (from 40 to 60 °C) has basically been attributed to the solubilization of non-active compounds (Durling et al. 2007).

The solid–liquid extraction depends also on the dissolution rate of phenols and how fast they reach the equilibrium concentration in the liquid (Gertenbach 2001). Particularly, results indicated that 4 h were adequate to reach the equilibrium in the case of dictamnus, while extraction process from sage and fruit-bearing tree leaves does not seem to reach the equilibrium after 8 h of treatment. These results are in debate to previous studies. Mylonaki et al. (2008) referred that the optimum extraction time from olive tree leaves with hydro-ethanolic extracts was equal to 5 h; however, longer time intervals were not examined and equilibrium time was not detected. On the other hand, Durling et al. (2007) referred that the extraction of total phenols from dried sage (*S. officinalis*) with similar conditions (40 °C and 80 mL ethanol/100 mL) reached the equilibrium after 3 h. This equilibrium had been attributed to the hypothesis that other than phenols oligomer compounds detected in total phenol fractions were unstable (Lu and Foo 1999). Nevertheless, this phenomenon does not seem to occur for the same type of sage (*S. fruticosa*).

Another way to enhance the diffusivity is to decrease the viscosity or the sample per solvent ratio, which increases

Table 4 Phenolic content and antioxidant efficacy of ethanolic extracts, recovered from olive (*Olea europaea* L.) tree leaves under different extraction conditions

Assayed extraction parameter (unit)	Value	Phenolic[a] content					Antioxidant efficacy (AE)[a]
		Total[b] (725nm) mg/g	Total[b] (280nm)	Hydroxycinnamic acids[c] (320nm)	Flavonols[d] (360nm)	*O*-diphenols[c] (517nm)	mg DPPH[e]/g
Temperature (°C)	25	20.3±1.3a	15.4±1.9a	4.1±1.1a	1.4±0.1a	7.7±0.8a	10.3±1.2a
	40	22.3±3.1a	14.7±1.8a	5.3±0.6a	5.8±0.4b	10.1±1.2b	13.2±1.9b
	60	39.1±1.8b	22.3±3.8b	8.7±0.4b	8.1±0.9c	16.0±1.1c	19.8±1.6c
Time (h)	1	12.5±2.0a	10.4±1.4a	3.7±0.4a	2.9±0.5a	6.1±0.9a	7.9±0.7a
	2	21.7±2.1b	14.2±1.1b	5.1±0.3b	5.5±0.3b	9.8±0.7b	11.4±1.2b
	4	36.3±0.2c	22.2±1.0c	7.0±0.3c	5.7±0.4b	15.3±1.8c	18.3±1.4c
	8	43.3±4.4d	27.2±0.9d	9.9±0.5d	11.3±1.1c	19.6±0.8d	19.3±2.3d
Ratio sample/solvent (g/L)	20	27.3±1.4a	19.1±1.4a	7.7±0.7a	3.8±0.3a	11.8±1.1a	4.7±0.1a
	40	21.8±0.6b	14.9±0.5b	5.4±0.5b	6.2±0.5b	10.9±0.7a	13.9±1.2b
	80	24.2±0.9c	16.9±0.2c	6.2±0.1c	4.8±0.6c	12.6±1.6a	9.7±0.2c
Ethanol content (mL/100 mL)	25	6.7±0.6a	4.7±0.1a	1.4±0.1a	2.5±0.1a	2.6±0.3a	3.5±0.6a
	50	11.7±0.2b	7.8±0.2b	2.5±0.1b	3.4±1.2a.c	5.4±0.4b	5.3±0.3b
	70	21.8±2.5c	14.2±1.0c	5.3±0.5c	5.7±0.6b	9.5±2.5c	12.6±2.4c
	100	5.4±0.5d	6.7±0.5d	2.1±0.3d	3.8±0.2c	2.8±0.4a	1.1±0.3d

[a] Values represent mean ± standard deviation (n=3). Values these possessing the same letters (at least one) within a column block are not significantly different ($p \leq 0.05$)

[b] Expressed as mg gallic acid/g dry matter

[c] "Hydroxycinnamic acids" for "hydroxycinnamic acid derivatives." Results expressed as mg caffeic acid/g dry matter

[d] Expressed as mg quercetin acid/g dry matter

[e] "DPPH" for "2,2-diphenyl-1-picrylhydrazyl." Results expressed as mg DPPH/g extract

the concentration gradient inside the plant material (Cacace and Mazza 2003). Increased ratios derive extracts with higher content of solubilized materials and thus more viscous. At the present study, the extraction with several ratios showed a different behavior among the assayed materials. For example, extracts recovered from sage and olive tree leaves showed higher phenol content values for the lower and the highest assayed ratios (20 and 80 g/L, respectively). The obtained variance is probably related to the different nature of the tested plant tissues, i.e., the phenol extract yield with hydro-ethanolic mixtures from sage (*S. officinalis*) has been shown to increase by decreasing the sample per solvent ratio (Durling et al. 2007). Moreover, the viscosity of the extracts does not seem to be affected so much by the assayed ratios, as there is no clear indication that lower ratio was followed by higher extraction yield from all the tested materials. On the other hand, the assayed ratios showed generally a reverse effect on the AE values of the extracts, i.e., the higher AE of the extracts from the sage and olive tree leaves were observed for the highest (80 g/L) and the middle (40 g/L) ratio, respectively. This result indicates that the different phenol classes of the plant materials may possess antagonistic interactions above certain concentrations.

The interactions between phenols of different polarity were herein observed by monitoring the concentrations of phenolic classes and AE values of the extracts recovered with different ethanol contents, although these mixtures are generally considered as hydrophilic. Solvent composition affects the physical properties of the mixture and particularly density, dynamic viscosity and dielectric that reflects diffusion and extraction rate (Cacace and Mazza 2003). In particular, results indicate that all the assayed plant materials contain polar phenols that are more easily extracted in hydro-ethanolic mixtures (25–75 mL ethanol/100 mL) instead of absolute ethanol. This hypothesis was confirmed by the fact that firstly the *o*-diphenols and secondly the hydroxycinnamic acid derivatives dominated inside the extracts recovered from either aromatic plants or olive tree leaves. Orthodiphenols possess two hydroxyl groups and thus are rather polar compounds. Hydroxycinnamic acid derivatives are similar to phenolic acids that contain a carboxylic group and a hydrophobic glycosidated benzene ring. Glycosides have been considered as covalent polar molecules with an intermediate interaction that do not prefer solvents with higher ethanol levels (i.e., >60 mL/100 mL) (Khiari et al. 2009). Besides, flavonols are generally known to possess very low solubility in water (Cacace and Mazza 2003).

Table 5 Phenolic content and antioxidant efficacy of ethanolic extracts, recovered from orange (*Citrus sinensis* L.) tree leaves under different extraction conditions

Assayed extraction parameter (unit)	Value	Phenolic[a] content					Antioxidant efficacy (AE)[a]
		Total[b] (725nm) mg/g	Total[b] (280nm)	Hydroxycinnamic acids[c] (320nm)	Flavonols[d] (360nm)	*O*-diphenols[c] (517nm)	mg DPPH[e]/g
Temperature (°C)	25	3.1±0.1a	4.0±0.2a	1.4±0.1a	1.4±0.1a	1.8±0.2a	0.3±0.1a
	40	7.2±0.3b	11.9±0.8b	5.1±0.3b	5.2±0.2b	6.7±0.3b	0.6±0.1b
	60	12.2±0.7c	17.1±1.5c	7.1±0.8c	8.1±0.9c	11.7±0.5c	1.2±0.2c
Time (h)	1	3.3±0.4a	6.5±1.1a	2.7±0.4a	2.9±0.5a	3.1±0.6a	0.3±0.1a
	2	7.0±0.7b	11.1±1.2b	4.7±0.8b	5.1±0.9b	6.2±0.9b	0.5±0.1b
	4	7.3±0.9b	12.1±2.2b	5.3±0.5b	5.3±1.0b	6.7±0.1b	0.7±0.1b
	8	11.9±0.3c	22.9±1.9c	9.9±0.7c	11.3±1.1c	13.8±1.0c	1.2±0.2c
Ratio sample/solvent (g/L)	20	5.6±0.5a	7.0±0.7a	3.3±0.1a	3.8±0.3a	7.3±0.8a	0.4±0.1a
	40	6.9±0.4b	11.4±1.0b	4.9±0.5b	5.8±1.0b	7.0±0.5a	0.6±0.1a
	80	4.5±0.8a	8.5±1.2c	4.3±0.5c	4.8±0.6b	5.7±0.9b	2.3±0.3b
Ethanol content (mL/100 mL)	25	4.5±0.5a	6.4±0.4a, b	3.3±0.2a	2.5±0.1a	2.6±0.8a	0.6±0.1a
	50	5.3±0.1b	7.6±1.5a, c	3.6±1.1a, c	3.4±1.2a, c	5.3±1.2b, c	0.8±0.1b
	70	7.4±0.5c	12.1±1.4b	5.4±0.5b	5.6±0.6b	6.9±0.8b	0.5±0.1c
	100	3.1±0.9d	7.0±0.4c	3.0±0.1c	3.8±0.2c	4.7±0.2c	0.2±0.1d

[a] Values represent mean ± standard deviation (n=3). Values these possessing the same letters (at least one) within a column block are not significantly different ($p \leq 0.05$)

[b] Expressed as mg gallic acid/g dry matter

[c] "Hydroxycinnamic acids" for "hydroxycinnamic acid derivatives." Results expressed as mg caffeic acid/g dry matter

[d] Expressed as mg quercetin acid/g dry matter

[e] "DPPH" for "2,2-diphenyl-1-picrylhydrazyl." Results expressed as mg DPPH/g extract

In the case of sage, the extracts of 50 and 70 mL ethanol/100 mL possessed insignificant different values of phenolic classes, but the second sample possessed significantly higher values of AE. This observation could be attributed to the higher phenol combination of the second extract, but the polarity variation between these two mixtures should be minor. Moreover, this result is in contrast to Pasias et al. (2009) study referring that microwave-assisted extraction of phenols from sage (*S. fruticosa*) was enhanced by increasing ethanol content from 50 mL to 100 mL/100 mL. An explanation could be the simultaneous solubilization of other phytochemicals detected at the current study. The hypothesis was consolidated by the fact that the second extract possessed higher total phenols concentration as analyzed with Folin–Ciocalteau method, which is known to determine any reducing compound of the samples (Niaounakis and Halvadakis 2004). Dictamnus was shown to contain the more hydrophilic phenol fragments among the assayed plant materials, as the corresponding extraction yield was maximum for the lower contents of ethanol (25 and 50 mL/100 mL). On the other hand, phenol recovery from olive tree leaves was optimum for the extract of 70 mL ethanol/100 mL. This result is in accordance to previous studies that refer maximum recovery from olive tree leaves with ultrasound-, microwave-, or mixing-assisted extraction and contiguous ethanol contents (60–80 mL/100 mL) (Japón-Luján et al. 2006a, b; Mylonaki et al. 2008).

As a general overview, sage was shown to be the most phenol rich substrate with a maximum yield of total phenols (determined at 725 nm) equal to 73.3 mg/g and a respective AE value of the extract equal to 30.6 mg DPPH/g (Table 2). This total phenol yield corresponds to ~33 mg/g (g/Kg) extract (data are not shown), which is lower compared to the yields of a previous study. For example, Pizzale et al. (2002) referred that the yield of methanolic extracts recovered from sages (*S. fruticosa* and *S. officinalis*) were equal to 75.0 and 81.3 g/Kg, respectively. Nevertheless, the aforementioned maximum AE value of the extract recovered at the current study is more than 5-fold higher compared to traditional antioxidant beverages like Chinese green tea, which has been referred to possess 6.6 mg DPPH/g (Atoui et al. 2005). This means that the sage extract (70 mL ethanol/100 mL) could provide a potable drink with similar antioxidant activity to green tea after a simple dilution with water (i.e., up to 20 mL ethanol/100 mL). Dictamnus and olive tree leaves followed sage with lower but satisfactory maximum yields: 43.9 and 43.3 mg total phenols/g, respectively (Tables 3 and 4). Likewise, the AE of the corresponding two extracts were also similar: 16.1 and

19.3 mg DPPH/g. These yields are more than 2-fold higher compared to a numerous of natural herbs hydro-alcoholic (80 mL methanol/100 mL) extracts that possessed a range of values from 9.1 to 23.3 mg total phenols/g dry weight (expressed as gallic acid equivalents) (Kähkönen et al. 1999). Thereby, dictamnus extract (70 mL ethanol/100 mL) could also be diluted with water and utilized as a drink with advanced antioxidant activity. On the other hand, olive tree leaves are not known as traditional tea substrates and thus the corresponding edibility of such a drink cannot be ensured. Finally, extracts from orange tree leaves contained more than 6-fold lower total phenols (Table 5, up to 7.4 mg/g) compared to the other assayed substrates and thus is not suggested as a potential initial source.

Conclusion

The optimization of phenol recovery from aromatic and fruit-bearing tree leaves using hydro-ethanolic mixtures showed that the extraction temperature and time were the primary factors affecting the yield of the process. The increase of these conditions resulted in enhanced recovery of phenols inside the mixtures, while extraction equilibrium for all the phenolic and antioxidant determinations was obtained only in the case of dictamnus treatment (after 4 h). As a superficial rule, the sample per solvent ratio seems to affect conversely the phenol content and AE of the resulted extracts, as the higher values of total phenol were followed by lower AE of the extracts and the reverse. The ethanol content of the extraction mixture affected also the process, but the impact was rather important only for absolute ethanol, which resulted in much lower phenol recovery yields. With regard to the antioxidant potential of the assayed Mediterranean plant materials, sage followed by dictamnus and olive tree leaves were shown to be the most phenol rich substrates, while the aromatic plants extracts with the highest yield were proposed as beverage additives in order to produce drinks with advanced antioxidant activity.

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