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Effect of an acid treatment on the phytochemical and antioxidant characteristics of extracts from selected Lamiaceae species

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Abstract

Aqueous-methanol extracts were prepared from basil (*Ocimum basilicum* L.), bay (*Laurus nobilis* L.), oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), savory (*Satureja hortensis* L.) and thyme (*Thymus vulgaris* L.) by maceration and their phytochemical and antioxidant characteristics were assessed. Each extract was submitted to an acid treatment (1.2 N HCl in 50% methanol) to determine whether such a process had an effect upon their chemistry and antioxidative efficacy. The treatment appeared to have improved the antioxidant potency of the extracts in general; however, there were some exceptions. The treatment did not increase the Fe(III) reductive activity of the extracts from basil or bay samples nor the ability of the oregano and sage extracts to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals at pH 7.4. The Folin-Ciocalteu phenol content of the basil extract was not increased with acid treatment.

Keywords: Lamiaceae (Labiatae); Basil; Bay; Oregano; Rosemary; Sage; Savory; Thyme; Iron reduction; 1,1- Diphenyl-2- picrylhydrazyl; Hydroxyl radical; Antioxidants; Acid hydrolysis

1. Introduction

Reactive oxygen-centred species are diverse compounds which are believed to play a cardinal role in the aetiology and pathogenesis of various chronic diseases, premature ageing and the oxidative deterioration of cosmetics, foodstuffs and pharmaceutical preparations. The development of synthetic compounds, capable of scavenging such species, has been a great success; however, it has been suggested that these substances may be inappropriate for chronic human consumption (Madhavi & Salunkhe, 1995). This has led to a search for and the use of naturally occurring antioxidants such as ascorbic acid, vitamin E and certain crude plant extracts. The importance of using naturally occurring antioxidants has further been clarified by different studies which have demonstrated that the consumption of foods and beverages rich in polyphenolic phytochemicals may exert a beneficial effect upon human health (Block, Peterson, & Subar, 1992; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993).

Plant secondary metabolites are an enormously variable group of phytochemicals in terms of their number, structural heterogeneity and distribution. Among the most studied diet-related phytochemicals are the hydroxycinnamic acids, which exist predominantly as ferulic and caffeic acids and their conjugates (Kroon & Williamson, 1999), and flavonoids, which exist predominantly as glycosides or polymers (Hammerstone, Lazarus, & Schmitz, 2000). Beneficial properties ascribed to such substances are many, including antioxidant (Kondo, Hirano, Matsumoto, Igarashi, & Itakura, 1996;

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Korkina & Afans'ev, 1997; Mazur, Bayle, Lab, Rock, & Rayssiguier, 1999) and cardioprotective (Hertog et al., 1993; Yochum, Kushi, Meyer, & Folsom, 1999) properties. It is thought that these components are principally responsible for the health-promoting properties of plants, fruits and vegetables.

The aim of this study was to analyse the effect an acid treatment (1.2 N HCl in 50% methanol) may have upon the phytochemical [total phenol content and qualitative–quantitative composition] and antioxidant [Fe(III) reduction and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) and hydroxyl radical-scavenging] characteristics of aqueous-methanol extracts isolated from commonly-used culinary herbs. Acid treatments are regularly used in phytochemical studies (Waterman & Mole, 1994); however, few studies have investigated the effect of such treatments on antioxidant activity (Wang et al., 2002; Mishra, Priyadarsini, Kumar, Unnikrishnan, & Mohan, 2003). Such a study may have an impact upon improving the antioxidative quality of ingredients used for the functionalisation of foods and beverages or the manufacture of nutraceuticals and thus further the health-promoting activity of functional foods and nutraceuticals.

2. Materials and methods

2.1. Plant material and reagents

Air-dried aerial plant material was obtained from Pimenta Oy (Finland). Chromatographic standards were purchased from Extrasynthese, France. Pycnogenol was obtained from Biolandes Arômes, France. Ultrapure water was used throughout and was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., MA, USA). All remaining reagents were of the highest purity available and obtained from the Sigma Chemical Company (St. Louis, MO, USA).

2.2. Preparation of plant and acid-treated extracts

Milled leaf material (10 g) was extracted with 100 ml methanol for 20 min at 60 °C in a water bath with shaking and was filtered. This procedure was repeated three times using the same batch of starting material and the resultant filtrates were combined and the solvent was removed in vacuo (40 °C). The resulting extract was dissolved in a small volume of methanol and eluted with 70% methanol through a pre-conditioned C_{18} reverse-phase solid phase extraction tube (Supelco, PA, USA). After the solvent had been removed, the residue was dissolved in methanol. An aliquot of each extract was refluxed for 2 h in 1.2 N HCl in 50% aqueous-methanol. Once cool, the mixture was filtered and the organic solvent was removed. Ethyl acetate was used to extract the resulting aglycones from the remaining aqueous phase. Thereafter, the ethyl acetate was removed and the residue was dissolved in methanol.

2.3. Qualitative–quantitative chromatographic analysis

The liquid chromatographic apparatus (Waters 600) consisted of an in-line degasser, pump and controller, coupled to a 2996 photo diode array detector equipped with a Rheodyne injector (20 μl sample loop) interfaced to a PC running Millenium^{32} chromatography manager software (Waters Corp., MA, USA). Separations were performed on a 250×4.6 mm i.d., 5 μm particle size, reverse-phase Hypersil BDS-C18 analytical column (Agilent Technologies, MA, USA) operating at room temperature (22 °C) at a flow rate of 0.7 ml/min. Detection was carried out with a sensitivity of 0.1 aufs between the wavelengths of 200 to 550 nm. Elution was effected using a ternary non-linear gradient of the solvent mixtures MeOH:H_2O:CH_3COOH (10:88:2, v/v/v) (solvent A), MeOH:H_2O:CH_3COOH (90:8:2, v/v/v) (solvent B) and MeOH (solvent C). The composition of B was increased from 15% to 30% in 15 min, increased to 40% in 3 min and held for 12 min, increased to 100% in 5 min. Then the composition of C was increased to 15% in 2 min, increased to 30% in 11 min and then returned to initial conditions in 2 min. A 10 min equilibrium time was allowed between injections. Components were identified by comparison of their retention times to those of authentic standards under analysis conditions and UV spectra with an in-house PDA-library. All extracts and standards were dissolved in 70% aqueous-methanol at a concentration of 1 mg/ml and 10 mg/ml, respectively. The concentration used for the calibration of reference compounds was 0.01–0.10 mg/ml. All standard and sample solutions were injected in triplicate.

2.4. Total phenolics

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g extract (Singleton, Orthofer, & Lamuela-Raventós, 1999). To ca. 6.0 ml H_2O, 100 μl sample were added and transferred to a 10.0 ml volumetric flask, to which 500 μl undiluted Folin-Ciocalteu reagent was subsequently added. After 1 min, 1.5 ml of 20% (w/v) Na_2CO_3 were added and the volume was made up to 10.0 ml with H_2O. After 2 h of incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data were presented as the average of triplicate analyses.

2.5. Fe(III)–Fe(II) reduction activity

The ability of the extracts to reduce Fe(III) was assessed by the method of Oyaizu (1986). One ml of

each extract dissolved in H_2O, was mixed with 2.5 ml 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of a 1% (w/v) potassium hexacyanoferrate solution. After a 30 min incubation at 50 °C, 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) were added and the mixture was centrifuged for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml H_2O and 0.5 ml of 0.1% (w/v) $FeCl_3$ and the absorbance was recorded at 700 nm. The reductive activity of the extracts was expressed as ascorbic acid equivalents (AscAE) in mmol ascorbic acid/g sample (Dorman, Koşar, Kahlos, Holm, & Hiltunen, 2003a). The bigger the AscAE value, the greater is the reducing power of the sample. The data were presented as the averages of quadruplicate analyses.

2.6. 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity

The ability of the extracts to scavenge DPPH· was determined by the method of Gyamfi, Yonamine, and Aniya (1999). A 50 μl aliquot of each extract, in 50 mM Tris-HCl buffer (pH 7.4), was mixed with 450 μl of Tris-HCl buffer and 1.0 ml of 0.1 mM DPPH· in MeOH. After a 30 min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using Eq. (1). Estimated IC_{50} values were presented as the averages of quadruplicate analyses.

$$\text{Percentage Inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100. \quad (1)$$

2.7. Ascorbate-Fe(III)-catalysed hydroxyl radical-scavenging activity

The ability of the extracts to scavenge hydroxyl radicals was estimated by the method of Aruoma et al. (1997). Bovine brain extract (Folch type VII) was mixed with 10 mM phosphate-buffered saline (PBS, pH 7.4), and sonicated in an ice bath until an opalescent suspension was obtained containing 5 mg/ml of phospholipid liposomes. The liposomes (0.2 ml) were combined with 0.5 ml PBS buffer, 0.1 ml of 1 mM $FeCl_3$, and 0.1 ml of sample solution. The peroxidation was initiated by the addition of 0.1 ml of 1 mM ascorbate. The mixture was incubated at 37 °C for 60 min. After incubation, 50 μl of 2% (w/v) BHT in ethanol was added to each tube, followed by 1 ml of 2.8% (w/v) TCA and 1 ml of 1% (w/v) 2-thiobarbituric acid (TBA) in 50 mM NaOH. The solutions were heated in a water bath at 100 °C for 20 min. The resulting $(TBA)_2$-MDA chromogen was extracted into 2 ml of *n*-butanol and the extent of peroxidation was determined in the organic layer at 532 nm. Percentage inhibition was calculated using Eq. (1). Data are presented as the averaged estimated IC_{50} values of quadruplicate analyses.

2.8. Statistical analysis

Data were presented as mean values ± standard errors. Statistical differences between treatments were identified by the paired (two-tailed) Student's *t*-test procedure, with analysis of variance performed using ANOVA procedures and significant differences between controls and sample means determined using Dunnett's multiple comparison test at a level of $P < 0.05$. IC_{50} values were estimated using a non-linear regression algorithm.

3. Results

3.1. Extract yield

The amount of extractable components (extract yield) obtained from the non-treated extracts ranged from 100 to 199 mg/g dry plant material. The amount of extractable components obtained for the acid-treated extracts ranged from 79 to 153 mg/g dry plant material.

3.2. Qualitative–quantitative chromatographic analysis

The qualitative–quantitative data from the chromatographic analyses are presented in Table 1, with representative chromatograms for the non-treated and treated samples presented in Fig. 1. The components, *p*-hydroxybenzoic acid, caffeic acid, luteolin-7-*O*-glycoside, naringenin-7-*O*-glucoside, apigenin-7-*O*-glucoside, rosmarinic acid, eriodictyol, luteolin and apigenin, were identified within the samples. Remaining analytes were tentatively identified by chemical class, namely, hydroxybenzoates, hydroxycinnamates and flavonoids, based upon their chromatographic behaviour and UV spectral characteristics.

The non-treated sage and thyme extracts contained the greatest amounts of extractable phenolic components while the non-treated bay extract contained the least amount. Overall, the acid treatment appeared to increase the amount of analytes from between 16.7% to 65.3%. Caffeic acid was not identified in the non-treated bay, oregano, rosemary or thyme extracts, but was identified in the basil, sage and savory extracts (0.90 ± 0.04, 0.97 ± 0.04 and 1.38 ± 0.04 mg/g, respectively). Except for the bay extract, rosmarinic acid was identified as the major component in all the non-treated extracts and was present in the range of 36.3 ± 0.07 to 145 ± 1.42 mg/g. In accordance with the literature, caffeic acid was present at lower concentrations than rosmarinic acid when identified (Janicsák, Máthe, Miklóssy-Vári, & Blunden, 1999). After the

Table 1
Qualitative–quantitative analysis of the non-acid-treated and acid-treated extracts by HPLC-PDA

Sample[c]	Identified components[a]									Unidentified components[b]			
	p-HBA (12.8)[d]	CA (15.4)	Lut-glu (29.4)	Nar-glu (30.4)	Ap-glu (33.1)	RA (33.3)	Erio (36.1)	Lut (44.1)	Ap (45.5)	∑HBA	∑HCA	∑Fl	∑
Crude non-acid treated extracts													
(1)	nd[e]	0.90 ± 0.04	nd	nd	nd	36.3 ± 0.07	nd	nd	0.89 ± 0.09	nd	1.32 ± 0.01	14.4 ± 0.05	53.9 ± 0.13
(2)	nd	nd	nd	nd	nd	nd	nd	nd	nd	9.92 ± 0.01	0.10 ± 0.00	28.96 ± 2.93	38.9 ± 2.93
(3)	4.29 ± 0.18	nd	15.1 ± 0.94	0.68 ± 0.11	16.1 ± 0.17	44.6 ± 0.39	2.59 ± 0.05	2.39 ± 0.01	1.51 ± 0.02	7.00 ± 0.23	5.85 ± 0.03	22.0 ± 0.52	122 ± 1.20
(4)	nd	nd	2.90 ± 0.57	7.16 ± 0.08	nd	51.5 ± 0.61	nd	2.45 ± 0.02	1.80 ± 0.02	6.95 ± 0.10	6.85 ± 0.10	35.0 ± 1.50	115 ± 1.72
(5)	nd	0.97 ± 0.01	49.3 ± 0.70	nd	nd	97.9 ± 0.88	nd	3.62 ± 0.37	nd	nd	1.73 ± 0.01	106 ± 2.34	259 ± 2.62
(6)	nd	1.38 ± 0.04	57.2 ± 0.69	4.05 ± 0.13	3.00 ± 0.14	103 ± 1.06	0.88 ± 0.01	nd	5.78 ± 0.08	3.10 ± 0.01	4.09 ± 0.02	6.33 ± 0.04	188 ± 1.28
(7)	nd	n.d.	24.2 ± 0.14	nd	nd	145 ± 1.42	3.70 ± 0.04	0.60 ± 0.03	5.28 ± 0.01	31.7 ± 0.02	20.4 ± 0.02	28.7 ± 0.38	259 ± 1.48
Acid treated extracts													
(1)	nd	nd	nd	nd	nd	22.2 ± 0.09	nd	0.57 ± 0.01	1.45 ± 0.02	32.6 ± 0.05	6.33 ± 0.01	7.28 ± 0.03	70.4 ± 0.11
(2)	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.70 ± 0.01	0.11 ± 0.01	47.61 ± 0.21	53.4 ± 0.21
(3)	nd	nd	nd	nd	nd	23.4 ± 0.24	nd	15.6 ± 0.91	7.43 ± 0.16	22.1 ± 0.01	20.8 ± 0.10	57.1 ± 0.05	147 ± 0.96
(4)	nd	1.84 ± 0.07	nd	nd	nd	105 ± 0.55	nd	23.6 ± 0.04	15.7 ± 0.08	57.0 ± 0.03	57.0 ± 0.03	88.5 ± 0.06	348 ± 0.57
(5)	nd	3.67 ± 0.18	nd	nd	nd	123 ± 0.90	nd	38.7 ± 0.45	36.3 ± 0.34	146. ± 0.02	38.8 ± 0.05	164 ± 0.15	550 ± 1.09
(6)	nd	0.54 ± 0.04	nd	nd	nd	27.6 ± 0.08	nd	39.7 ± 0.81	17.7 ± 0.18	157 ± 0.03	45.4 ± 0.02	45.8 ± 0.80	334 ± 1.16
(7)	nd	n.d.	nd	nd	nd	125 ± 0.69	nd	25.9 ± 0.07	3.03 ± 0.03	87.7 ± 0.11	50.6 ± 0.02	88.7 ± 0.50	380 ± 0.86

Values (mg/g) are presented as means ± SE ($n = 3$).

[a] *p*-HBA, *p*-hydroxybenzoic acid; CA, caffeic acid; Lut-glu, luteolin-glucoside; Nar-glu, naringenin-glucoside; Ap-glu, apigenin-glucoside; RA, rosmarinic acid; Erio, eriodictyol; Lut, luteolin and Ap, apigenin.

[b] HBA, hydroxybenzoic acid derivatives quantitated using *p*-hydroxybenzoic acid; HCA, hydroxycinnamic acid derivatives quantitated using caffeic acid; Fl, flavonoids quantitated using apigenin.

[c] (1) Basil, (2) Bay, (3) Oregano, (4) Rosemary, (5) Sage, (6) Savory and (7) Thyme.

[d] Retention time (min).

[e] nd, not detected.

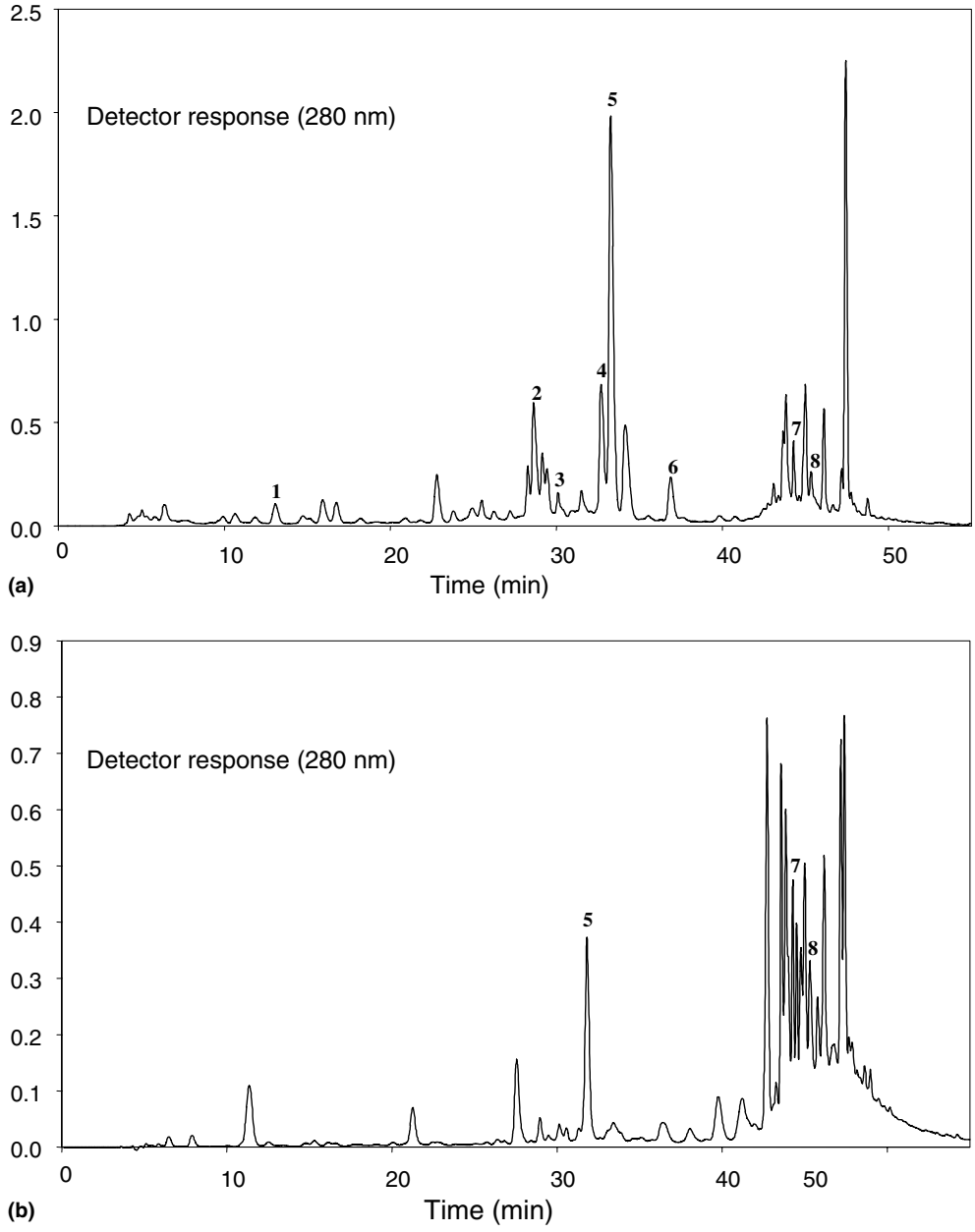

Fig. 1. HPLC-PDA analyses of oregano prior to (a) and post (b) acid treatment of samples with detector responses at 280 nm. Identified analytes: **1**, *p*-hydroxybenzoic acid; **2**, luteolin-7-*O*-glucoside; **3**, naringenin-7-*O*-glucoside; **4**, apigenin-7-*O*-glucoside; **5**, rosmarinic acid; **6**, eriodictyol; **7**, luteolin and **8**, apigenin.

acid treatment, the contents of rosmarinic and caffeic acids in the rosemary and sage extracts increased, whereas the rosmarinic acid content in the basil, oregano, savory and thyme extracts appeared to decrease as did the caffeic acid content of the basil and savory extracts. Luteolin-7-*O*-glucoside was the second major identified component in the non-treated extracts except in oregano, where it occurred as abundantly as the apigenin glucoside, in rosemary, where the naringenin glucoside was more abundant, and in basil, where caffeic acid was the second major identified component. Luteolin was the second most abundant identified component in the treated extracts, except in basil where apigenin was present in greater amounts. After acid treatment, the unidentified components appeared to make a greater contribution to the composition of the extracts overall.

3.3. Total phenols

The phenol content for the crude and hydrolysed extracts ranged from 121 ± 1.2 to 241 ± 0.3 mg/g extract and 79.3 ± 0.5 to 464 ± 6.3 mg/g extract, respectively. In all cases, except basil, the effect of the acid treatment

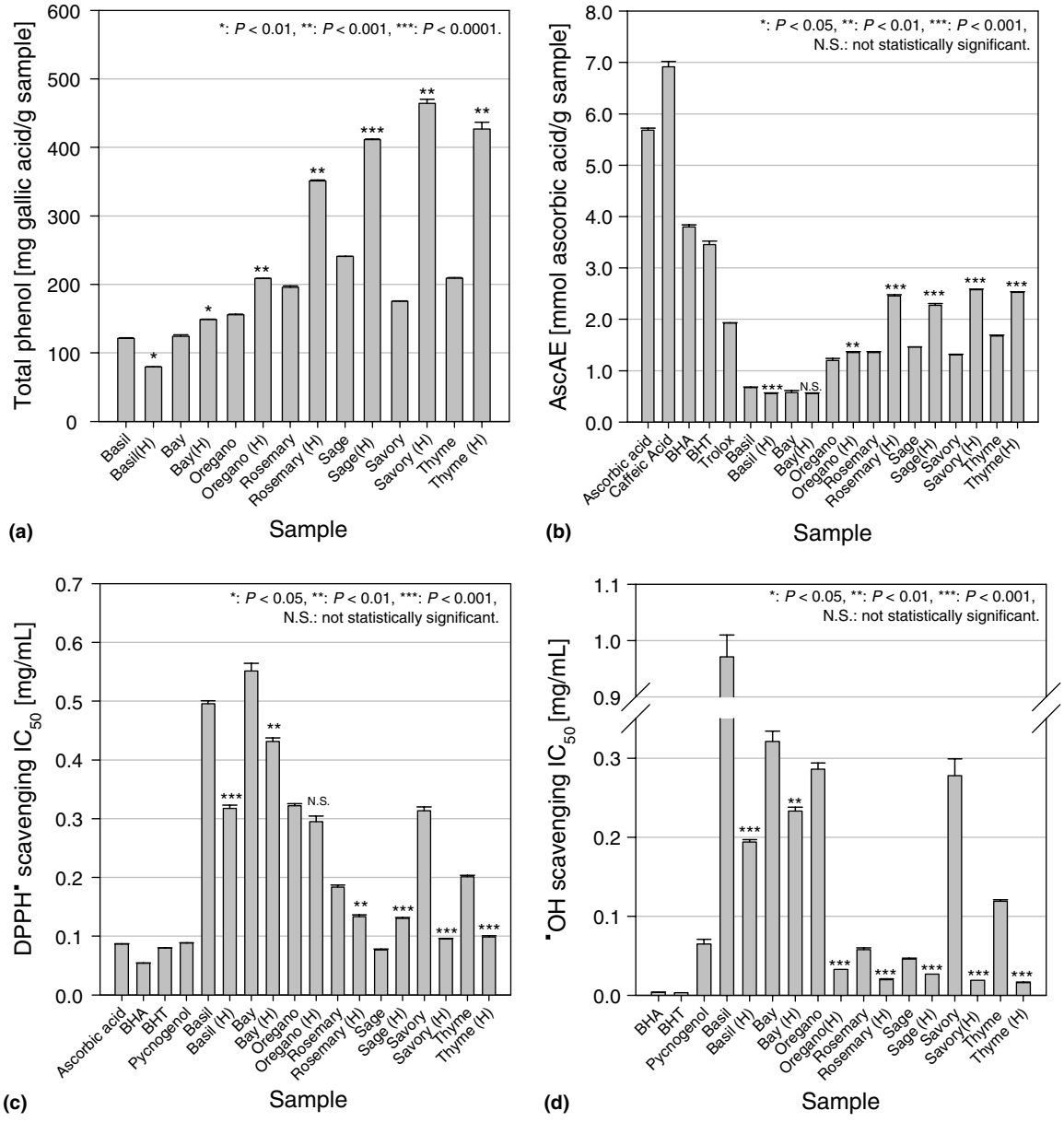

Fig. 2. The (a) total phenolic content, (b) AscAE values, (c) DPPH•-scavenging and (d) hydroxyl radical-scavenging performance for the positive controls, crude and acid-treated (H) extracts. Significance between crude and hydrolysed samples was determined using a paired (two-tailed) Student's *t*-test.

was to significantly increase the total phenolic content (Fig. 2a).

3.4. Fe(III)–Fe(II) reduction activity

Each extract was capable of reducing Fe(III)–Fe(II) and did so in a linear concentration-dependent fashion. As can be seen in Fig. 2b, the acid treatment increased the reductive potency of the rosemary, sage, savory and thyme non-treated extracts; there was a statistically significant ($P < 0.05$) increase in the calculated AscAE values for these extracts. Moreover, their performance in this assay was statistically superior to that demonstrated by Trolox, the synthetic water-soluble α-tocopherol analogue. Despite this, the acid treatment had no significant ($P = 0.720$) effect upon the Fe(III) reductive efficacy of the bay extract and significantly ($P < 0.001$) decreased the reductive power of the basil extract. Despite the activity of the non-treated and acid treated extracts, none were as effective as the reference substance ascorbic acid or the positive controls.

3.5. 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity

The extracts were able to scavenge the synthetic nitrogen-centred free radical, DPPH•, to varying degrees as can be seen from the estimated concentrations required to scavenge 50% of the available DPPH• species

(Fig. 2c). The non-treated basil and bay extracts were the least effective samples, followed by those of oregano and savory. Rosemary, sage and thyme extracts were considerably better DPPH· scavengers. Moreover, the non-treated sage extract out-performed both ascorbic acid and pycnogenol and was equivalent to the synthetic antioxidant BHT. The acid treatment clearly had an effect on DPPH· scavenging except in the case of the oregano extract, where no statistically significant ($P = 0.120$) difference occurred. The increased scavenging activity observed for the basil, savory and thyme extracts was statistically significant ($P < 0.001$) and only just less so for the bay and rosemary ($P < 0.01$) extracts. The efficacy of the non-treated sage extract appeared to decrease after the acid treatment.

3.6. Ascorbate-Fe(III)-generated hydroxyl radical-scavenging activity

The non-treated and acid treated extracts were capable of inhibiting the formation of TBARS and, therefore, were capable of inhibiting Fenton chemistry-catalysed hydroxyl radical-mediated degradation of bovine brain phospholipids (Fig. 2d). The non-treated rosemary and sage extracts were very potent hydroxyl radical scavengers in this model system. Contrary to the variable performance of the extracts in the Fe(III) reduction and DPPH· assays after acid treatment, the performance of the extracts increased significantly after acid treatment. The estimated IC_{50} concentrations for the basil, oregano and savory extracts decreased significantly. Both sage samples and the oregano, rosemary, savory and thyme acid-treated samples were significantly better ($P < 0.05$) inhibitors of hydroxyl radical-mediated deterioration of phospholipids than the positive control, pycnogenol, in this assay.

4. Discussion

Many dietary phytochemicals with ascribed beneficial properties predominantly occur as glycosides. These compounds are susceptible to enzymatic action and hydrolytic reactions, resulting in the release of a sugar moiety and the corresponding aglycone. These reactions can affect a compound's chemical and biological properties. Glycosylation has been reported to affect the antioxidant (Fukumoto & Mazza, 2000; Ratty & Das, 1988; Wang et al., 2002) and antibacterial (Basile, Giordano, López-Sáez, & Cobianchi, 1999) performance of phenolic compounds. Therefore, the authors set forth to determine whether an acid treatment capable of cleaving glycosidic bonds could positively affect the Folin-Ciocalteu reactive content and antioxidative potency of crude, polyphenol-enriched plant extracts. In order to determine an affect, we decided upon the use of three model systems to characterise the antioxidant performance, namely, Fe(III) reduction, DPPH·-scavenging and inhibition of Fenton chemistry-catalysed hydroxyl radical-mediated phospholipid degradation. Furthermore, the extent of compositional change within each sample was monitored qualitatively and quantitatively using reverse phase high performance liquid chromatography coupled with photodiode array detection.

Lamiaceae species are known to produce a diverse array of secondary metabolites, such as volatile and non-volatile terpenes, hydroxybenzoates, hydroxycinnamates and flavonoids, among others. These phytochemicals can occur either as aglycones or glycosides. The qualitative–quantitative analysis of the content of the extracts used in this study prior to and post acid treatment is presented in Table 1, with representative chromatograms presented in Fig. 1. The acid treatment changed the composition of the extracts extensively. Luteolin, naringenin and apigenin glycosides were no longer detected in the treated samples, while the levels of their respective aglycones increased with the exception of apigenin in the thyme sample. Contrary to expectations, the concentration of the aglycone flavonoid eriodictyol decreased, as did the concentration of rosmarinic acid in the treated basil, oregano, savory and thyme extracts, with the treatment. Interestingly, the abundance of rosmarinic and caffeic acids increased in the rosemary and sage extracts after the acid treatment. Lu and Foo (1999) reported that sage contains a variety of rosmarinic acid derivatives, such as salvianolic and sagerinic acids, which is consistent with the findings of Peterson and Simmonds (2003) who reported the existence of rosmarinic acid–caffeic acid conjugates in lamiaceae plants. If such complex components are present in the rosemary and sage extracts, this may explain the increased contents of both of these organic acids post treatment, as the bridging bond may have been broken under the experimental conditions used. It is apparent that dramatic changes in the levels of unidentified components occurred after the acidic treatment. The sum of unidentified hydroxybenzoic and hydroxycinnamic acids increased dramatically, except in the bay sample, as did the sum of unidentified flavonoids. These major changes were reflected in the levels of total components, which increased across-the-board.

Fe(III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003b; Yildirim, Mavi, & Kara, 2001), and can be strongly correlated with other antioxidant properties (Dorman et al., 2003a; Yildirim et al., 2001). In this study, Fe(III) reduction was determined from the formation of the chromogenic complex known as Prussian blue $[Fe_4(Fe(CN)_6)_3]$ and the activity of the samples was ranked in terms of ascorbic acid equivalents (AscAE). As seen in Fig. 2b,

the crude extracts contained compounds which were capable of participating in redox reactions. With the exception of basil and bay, the acid treatment appeared to increase the redox-active properties of the extracts. An explanation of this may be the increased number of phenolic groups available to participate in such reactions. Thus, the data suggest that the acid treatment improved electron-donating efficacy. This should improve the ability of treated samples to convert unstable reactive species into more stable non-reactive species.

The cardinal mode of action of natural antioxidants is their ability to scavenge free radicals before they can initiate free radical chain reactions in cellular membranes or lipid-rich matrices, as found in cosmetics, foodstuffs and pharmaceutical preparations. The elimination of such species, once formed, is considered to have a practical affect on the stability of susceptible products and may have a beneficial effect on human health. Therefore, a simple and commonly-used free radical assay was employed to determine whether the acid treatment used in this study would have an advantageous effect on the free radical-scavenging potency of the isolated extracts. The radical used was the DPPH$^{\cdot}$ species. In this assay, the radical serves as the oxidisable substrate and reaction indicator molecule: as the radical is reduced by an antioxidant to its hydrazine form, its adsorption at 517 nm decreases. All the samples were capable of scavenging this radical in a concentration-dependent fashion. The extracts possessed varying degrees of efficacy and, overall, the acid treatment significantly increased their radical-scavenging properties (Fig. 2c). There was an increase in the oregano extract's scavenging activity but this change was not statistically significant ($P > 0.05$). The activity of the sage extract, however, appeared to decrease significantly ($P < 0.001$) after the treatment. Despite this, the data do suggest that the treatment increased the free radical-scavenging potential of the crude extracts.

Fe(III) reduction and DPPH$^{\cdot}$-scavenging assays are often used to characterise the antioxidative properties of plant extracts; however, concerns have been expressed as to the suitability of such assays for identifying antioxidants, particularly for applications in heterogeneous media or physiological systems (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002; Frankel & Meyer, 2000). It has been suggested that it is important to establish the potential antioxidant activity of dietary compounds using phospholipids, microsomal extracts or other lipid model systems (Aruoma, 2003). It is thought that free radical-mediated damage to phospholipids is responsible for the oxidative deterioration and off-flavour development of foods (Wu & Sheldon, 1988). Consequently, it was considered necessary to determine whether the acid treatment enhanced the performance of the samples in a complex, lipid-rich food/biologically relevant matrix, i.e. a bovine brain-derived phospholipid liposome system at pH 7.4, using Fe(III) and ascorbate as radical-generating catalysts.

Phosphate-buffered phospholipids rapidly undergo hydroxyl radical-mediated degradation in the presence of Fe(III) and ascorbate at 37 °C, producing byproducts which react with 2-thiobarbituric acid to produce a chromogenic complex (Aruoma et al., 1997). Any substance capable of preventing the formation of this complex is capable of protecting phospholipids from hydroxyl radical attack. The non-treated and acid-treated extracts were capable of inhibiting the formation 2-thiobarbituric acid-reactive substances in a concentration-dependent fashion and, therefore, were capable of protecting phospholipids from oxidative degradation by Fenton chemistry-generated hydroxyl radicals. From the IC_{50} values estimated from the dose-response curves for the samples, it can be seen that all the extracts showed variable degrees of efficacy (Fig. 2c). However, the acid treatment appeared to significantly improve the ability of all the non-treated extracts to protect the phospholipids in this model system. Therefore, one would expect the use of an acid treatment to improve the ability of the original extracts to prevent hydroxyl radical-mediated deleterious reactions if added to a susceptible matrix such as a foodstuff or biological system.

5. Conclusion

Crude plant-derived extracts are increasingly added to products susceptible to oxidative degradation as an alternative to synthetic preservatives. Furthermore, dietary phytochemicals appear to be able to exert health-promoting effects upon human biology. This ability appears to be mediated primarily through their antioxidant action, i.e. their ability to quench reactive species, reduce local oxygen concentrations, terminate free radical reactions and chelate and deactivate transition metals before they can participate in hydroperoxide decomposition or Fenton chemistry-type reactions. In this study, the authors have investigated whether the in vitro antioxidant efficacy of crude extracts could be improved by refluxing the isolated extracts in 1.2 M HCl in 50% aqueous methanol. We proposed that such a process would change glycosides to aglycones and increase the phenolic yield. The increase in the number of phenolic groups would make the extracts more potent participants in redox reactions. From the observed performance, the non-treated extracts would be expected to participate in free radical termination reactions and delay the initiation and progression of free radical-mediated deleterious reactions in susceptible matrices. Yet, in a number of cases, it was possible to further increase their protective ability by using the acid treatment. It appears, therefore that, by treating crude polyphenol-enriched extracts, it may be possible to improve their

usefulness as antioxidative preservative ingredients. The usefulness of this process in the functionalisation of foods, or in the production of nutraceuticals, requires further research. In order to confidently evaluate in vivo antioxidant capacity, it is not sufficient to solely determine the reactivity of natural products toward specific radicals; factors such as bioavailability, distribution, localisation, fate of antioxidant-derived radicals, interactions with cellular components and metabolism have to be characterised.

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