ELSEVIER

Food Chemistry 67 (1999) 399–414

Food Chemistry

www.elsevier.com/locate/foodchem

Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago officinalis* L.) seeds

Mahinda Wettasinghe, Fereidoon Shahidi*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, NF, A1B 3X9, Canada

Received 22 February 1999; accepted 5 May 1999

Abstract

Borage meal exerted a concentration-dependent antioxidant activity in a meat model system. At 2% (w/w), it inhibited ($p \leq 0.05$) 2-thiobarbituric acid-reactive substances (TBARS), hexanal and total volatile formation in meat by 26.5, 30.5 and 18.6%, respectively. Antioxidant compounds in the meal were concentrated at optimum extraction conditions (in 52% ethanol at 74°C for 62 min) predicted by response surface methodology (RSM). The resulting extract inhibited ($p \leqslant 0.05$) the coupled oxidation of β-carotene and linoleate in a β-carotene-linoleate system. The system containing extract at a level providing 200 ppm phenolics retained 81% of the initial β-carotene after 2 h of assay whereas the control retained only 11%. Inhibition ($p \leqslant 0.05$) of TBARS, hexanal and total volatile formation in a meat system containing 200 ppm extract ranged from 18.9 to 88.3%, depending upon the concentration being tested. The extract inhibited ($p \leqslant 0.05$) conjugated diene, hexanal and total volatile formation in bulk corn oil (8.3–49.6% inhibition) and corn-oil-in water emulsion (5.2–32.2% inhibition). Hydrogen peroxide, hydroxyl radical and superoxide radical-scavenging properties of the extract were somewhat less than, but comparable to, those observed for *trans*-sinapic acid at similar concentrations of phenolics. At 200 ppm, a 100% quenching of the hydroxyl radical and superoxide radical was evident. The extract scavenged 29–75% of the hydrogen peroxide in assay media after 10 min of assay as compared to 3% reduction in the control.

1. Introduction

Lipid oxidation is one of the major causes of quality deterioration in lipid-containing foods. It affects the colour, flavour, texture, and nutritive value of foods (Hettiarachchy, Glenn, Gnanasambandam & Johnson, 1996; Shahidi & Wanasundara, 1992). In a peroxide-free system, lipid peroxidation is initiated when a hydrogen atom is abstracted from a diallylic methylene (-CH_2-) group of an unsaturated fatty acid (Halliwell & Gutteridge, 1985). Oxygen-derived free radicals such as superoxide radical (O_2^{-}), hydroxyl radical ($^{\cdot}OH$), hydroperoxyl radical (HO_2) and nitric oxide radical ($^{\cdot}NO$) can abstract hydrogen atoms from fatty acid chains of lipid molecules (Packer & Glazer, 1990). Singlet oxygen ($^1O_2$), an electronically excited species of oxygen, and hydrogen peroxide (H_2O_2), another reactive oxygen species, can initiate lipid oxidation through ene reaction where olefenic groups of fatty acids are converted to their corresponding allyl hydroperoxides and $^{\cdot}OH$ generation, respectively (Halliwell & Gutteridge, 1985). Incorporation of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tertiary*-butyl hydroquinone (TBHQ), and propyl gallate (PG) into foods can retard lipid oxidation. However, use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (Hettiarachchy et al.). This situation created a necessity for scrutinizing naturally occurring antioxidant substances which may be used in foods in place of synthetic antioxidants. A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Ramarathnam, Osawa, Ochi & Kawakishi, 1995). Among natural antioxidants, phenolic antioxidants are in the forefront as they are widely distributed in the plant kingdom. For example, phenolic compounds occur in oilseeds as the hydroxylated derivatives of benzoic and cinnamic acids,

* Corresponding author. Fax: + 1-709-737-4000.
E-mail address: fshahidi@morgan.ucs.mun.ca (F. Shahidi)

0308-8146/99/$ - see front matter
PII: S0308-8146(99)00137-5

coumarins, flavonoid compounds, and lignins (Oomah, Kenaschuk & Mazza, 1995). Phenolic antioxidants are reported to quench oxygen-derived free radicals as well as the substrate-derived free radicals by donating a hydrogen atom or an electron to the free radical (Wanasundara & Shahidi, 1996; Yuting Rongliang, Zhongjian & Yong, 1990). Furthermore, phenolic extracts of plant materials have been shown to neutralize free radicals in various model systems (Ruch, Cheng & Klaunig, 1989; Silva, Darman, Fernandez, & Mitiavila, 1991; Zhang et al., 1996). Several authors have reported that the extracts of various oilseed meals possess antioxidant properties which in some cases exerted better antioxidant properties than those observed for synthetic antioxidants at the same concentrations (Amarowicz, Wanasundara, Wanasundara & Shahidi, 1993; Fukuda, Osowa, Namiki & Ozaki, 1985; Naczk, Shahidi & Sullivan, 1992; Oomah, et al., 1995; Shahidi, Wanasundara & Amarowicz, 1994; Wanasundara, Amorowicz & Shahidi, 1994).

Plants produce phenolic compounds to deal with reactive oxygen species (ROS) and substrate derived-free radicals produced during the process of photosynthesis (Lu & Foo, 1995). ROS play an important role in tissue damage in humans (Pryor, 1966; Silva et al., 1991). Reaction of ROS with biomolecules such as membrane lipids, proteins and deoxyribonucleic acid (DNA) can provoke irreversible changes in their structure (Iuliano, Colavita, Leo, Pratico & Violi, 1997). Membrane lipid peroxidation has been known particularly to associate with many tissue injuries and disease conditions (Chiarpotto et al., 1997; Weber et al., 1997). Plant phenolics can delay the onset of lipid oxidation and decomposition of hydroperoxides in food products as well as in living tissues. Yanishlieva and Marinova (1995) have shown that phenolic acids such as ferulic, sinapic, and caffeic acids are involved in various stages of chain initiation and propagation reactions during the oxidation of pure triacylglycerols and methyl esters of sunflower oil.

Borage (*Borago officinalis* L.) oil is of great interest among medical and nutritional research groups due to its high content of γ-linolenic acid (GLA) (Huang, Lin, Redden & Horrobin, 1995; Rahmatullah, Shukla & Mukherjee, 1994a,b; Wolff & Sébédio, 1994). Oils containing GLA have been used in treating a number of clinical conditions caused by GLA deficiency in humans (Chapkin & Carmichael, 1990; Engler, Engler & Paul, 1992; Engler, Karanian & Salen, 1991; Gibson, Lines & Newmann, 1992; Rahmatullah et al., 1994a,b; Redden, Lin, Fahey & Horrobin, 1995). Although borage oil is rich in polyunsaturated fatty acids (PUFA) such as GLA, it is highly resistant to oxidation in intact seed tissues. This resistance may arise from the presence of tocopherols and several other phenolic compounds in oil-bearing tissues (Lu & Foo, 1995). When oil is extracted, antioxidants such as tocopherols are also coextracted with the oil and play a major role in the prevention of rancidity in bulk oil. However, the borage meal, like any other oilseed meal, may retain a significant amount of phenolic compounds following the oil expulsion. These antioxidants can be concentrated either as crude extracts or individual phenolic compounds to be used in highly unsaturated oils such as marine oils. Extraction of phenolic compounds from oilseed meals, however, is dependent on several factors or variables such as the extraction medium, temperature and time.

Response surface methodology (RSM) is a widely used tool to analyze responses which are affected by multiple variables and their interactions (Box & Wilson, 1951; Wanasundara & Shahidi, 1996). When a multitude of variables affects a particular response, a common problem associated with experimentation is the difficulty of testing all possible treatment combinations at different levels. An appropriate experimental design can reduce the number of experiments or observations and a mathematical model can be fitted to the data acquired from a selected number of variable combinations (Mead, 1988). Values for a given response at any treatment combination can be predicted using the mathematical model. Experiments are carried out within a predetermined range, usually; ranges for variables are determined based on the data obtained from a preliminary study.

The objectives of this study were to evaluate antioxidant activity of borage meal in a meat model system, optimize the extraction conditions in order to obtain an extract with high antioxidant activity and to evaluate antioxidant-and ROS-scavenging properties of the extract.

2. Materials and methods

Borage seeds were obtained from Bioriginal Food and Science Co., Saskatoon, SK, Canada. Seeds were stored in vacuum packaged polyethylene pouches at −20°C until used. Seeds were ground for 15 min and the defatting was done by blending ground seeds with hexane (1:5 w/v, 5 min, three times) in a Waring Blendor at ambient temperatures. Defatted seeds were air dried for 12 h and stored in vacuum packaged polyethylene pouches at −20°C until used. Fresh pork shoulder meat (one day after slaughtering) was acquired from a local supermarket and most of its surface fat removed. The meat was ground twice in a meat grinder (Omega, Type 12) using a 0.79 and then a 0.48 cm plate. Ground pork was vacuum-packaged in polyethylene pouches and stored in a freezer (Ultra Low, Revco, Inc., West Columbia, SC) at −60°C until used. Bulk corn oil, stripped of its natural antioxidants, was purchased from Fisher Scientific (Nepean, ON).

Reagents, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, β-carotene, linoleic acid, Tween 40, butylated hydroxyanisole (BHA), sodium carbonate, mono- and dibasic sodium phosphate, 5,5-dimethyl-1 pyroline *N*-oxide, nitro blue tetrazolium, hypoxanthine, xanthine oxidase, 2-heptanone, hexanal standard, *trans*-sinapic acid standard and Folin–Denis reagent, were obtained from Sigma Chemical Co. (St. Louis, MO). Hexane, methanol, ethanol, butanol, acetone, chloroform, hydrogen peroxide, ferrous sulphate, and trichloroacetic acid (TCA) were obtained from Fisher Scientific (Nepean, ON). Helium, hydrogen, and compressed air were obtained from Canadian Liquid Air Ltd. (St. John's, NF).

2.1. Response surface methodology (RSM)

Before the acquisition of data to be analyzed by RSM, one should know the range of each variable where the maximum response would occur. These ranges may be acquired from literature data or by performing preliminary experiments (Mason, Gunst & Hess, 1989). When this information is available, three levels from each variable are selected. Preliminary studies can also help in selecting a proper general regression model (linear, quadratic, cubic, etc.). The following sections explain the protocols applied to determine the variable levels and the experimental design.

2.2. Determination of levels for independent variables

Three levels for each independent variable were chosen based upon the data acquired from experiments which were carried out with a series of levels of the independent variable under investigation while keeping the other two variables at a constant level. A series of extraction media with different polarities were prepared using aqueous organic solvents, namely, methanol, ethanol and acetone at 0, 30, 50, 60, 70, 80, 90 and 100% (solvent:water, v/v). One hundred millilitres of an extraction medium were added to a 6 g sample of defatted seeds in a 250 ml round-bottom flask. Extractions were carried out under reflux in a thermostated water bath for 20 min at 80°C. Samples were extracted repeatedly for three times (under the same conditions) and the contents were centrifuged for 5 min at $4000\times g$ (ICE Centra M5, International Equipment Co., Needham Heights, MA). Supernatants were pooled and mixed, followed by the removal of solvent under vacuum at 40°C. The resulting concentrated solution was lyophilized for 72 h at −49°C and 62×10^3 mbar (Freezone 6, Model 77530, Labconco Co., Kansas City, MO). Extract yield (g/100 g meal) and total phenolics (mg *trans*-sinapic acid eq/g) in the extract were determined (the method for determining total phenolics is explained in a later section). The response studied was the antioxidant index calculated for the extract (at 200 ppm total phenolics as *trans*-sinapic acid eq.) in a β-carotene-linoleate model system (the method is given in a later section).

Three levels for the temperature variable were chosen from a series of experiments carried out at 25, 40, 55, 70 and 85°C while holding the other two variables at a constant level (extraction medium: 50% ethanol; extraction time: 20 min). Variable levels for the extraction time were also determined. Extractions were carried out for 15, 30, 45, 60, 75, 90 and 105 min of total extraction time while maintaining the other two variables at a constant level (extraction medium: 50% v/v, ethanol; extraction temperature: 80°C). Extract yields were recorded and the total phenolics extracted was expressed as mg *trans*-sinapic acid eq/g extract. All experiments were carried out in triplicate. Response (antioxidant index) at different variable combinations was recorded.

2.3. Experimental design

The experimental design adopted for this study was a three-factor three-level face-centered cube design (partial factorial design) with 15 individual design points (Gao & Mazza, 1996; Mason et al., 1989; Snedecor & Cochran, 1980). Three independent variables or factors studied were solvent content in the extraction medium (% v/v, X_1) extraction temperature (°C, X_2), and extraction time (min, X_3) (Table 1) for uncoded variable levels. Response (Y) at each design point was recorded. Duplicate extractions were carried out at all design points except for the centre point (0, 0, 0) where triplicate extractions were carried out.

The generalized second order polynomial model used in the response surface analysis was:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum\sum_{i<j=1}^{3}\beta_{ij}x_i x_j$$

where, β_0, β_i , β_{ii} and β_{ij} are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; x_i and x_j are independent variables. Data was

Table 1
Inhibition[a] of TBARS, hexanal and total volatiles formation of cooked lean pork containing borage meal on day 7 of storage at 4°C[b]

Borage meal (%, w/w)	% Inhibition		
	TBARS[c]	Hexanal[c]	Total volatiles[c]
1	9.5 ± 0.6a	12.5 ± 0.7a	8.2 ± 0.4a
2	26.5 ± 1.2b	30.5 ± 1.8b	18.6 ± 1.1b

[a] % Inhibition = 100 (value for sample/value for control)×100.

[b] Results are mean value of three determinations ± standard deviation.

[c] Means sharing the same letter in a cloumn are not significantly ($p > 0.05$) different from one another.

analyzed using the GLM and RSREG procedures of SAS Institute Inc. (1990) and the estimated regression coefficients were substituted in the quadratic polynomial equation. Response surface and contour plots were obtained using the fitted model, by keeping the least effective independent variable at a constant value while changing the other two variables simultaneously. Verification experiments were carried out using combinations of variables at different levels (within the experimental range) to determine the adequacy of the model.

2.4. Determination of total, hydrophilic and hydrophobic phenolic contents

A spectrophotometric method explained by Swain and Hill's (1959) was adopted. The extract was dissolved in methanol at a concentration of 0.5 mg/ml. Folin–Denis reagent (0.5 ml) was added to a centrifuge tube containing the extract (0.5 ml). The contents were mixed and a saturated sodium carbonate solution (1 ml) was added into the tube. The volume was adjusted to 10 ml by the addition of 8 ml of distilled water and the contents were mixed vigorously. Tubes were allowed to stand at ambient temperatures for 25 min and then centrifuged for 5 min at $4000\times g$. Absorbance of the supernatant was measured at 725 nm. Blank samples for each extract were used for background subtraction. Total phenolic content in each extract was determined using a standard curve prepared for *trans*-sinapic acid. 'Total phenolics' extracted was expressed as mg *trans*-sinapic acid eq/g extract. The extract was fractionated into its hydrophilic and hydrophobic components by mixing 5 g of it with 100 ml of deionozed water and 100 ml of butanol in a separatory funnel. The mixture was allowed to stand at 4°C for 12 h; separated layers were removed and desolventized using a Rotavapor (Buchi, Flawil, Switzerland) set at 40°C. The resulting concentrated liquids were lyophilized for 72 h at −49°C and 62×10^{-3} mbar. The weight of each fraction was recorded and the content of phenolics was determined.

The two concentrations (100 and 200 ppm) of the extract used in the experiments involving the determination of antioxidant efficacy and ROS scavenging activity were based on the weight of extract required to obtain 100 and 200 ppm total phenolics (as *trans*-sinapic acid equivalents) in the model system.

2.5. β-Carotene-linoleate system

A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two millilitres of this solution were pipetted into a 100 ml round-bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40°C, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into a series of tubes containing 100 or 200 μl of the extract (in methanol) so that the final concentrations of the extract in the assay media were 100 and 200 ppm. The total volume of the systems was adjusted to 5 ml with methanol. BHA and *trans*-sinapic acid were used for comparative purposes. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a Hewlett–Packard diode array spectrophotometer (Model 8452A, Hewlett–Packard Co., Mississauga, ON). Subsequent absorbance readings were recorded over a 2-h period at 15 min intervals by keeping the samples in a water bath at 50°C. Blank samples, devoid of β-carotene, were prepared for background subtraction (Miller, 1971). Antioxidant index (AI) was calculated using the following equation: AI = (β-carotene content after 2 h of assay/initial β-carotene content)×100.

2.6. Meat model system

Ground pork was mixed with 20% by weight of deionized water in Mason jars (height 10 cm, internal diameter 6 cm). Borage meal (1 and 2%, w/w) or its extracts (100 and 200 ppm) was added directly to meat. A control sample containing no meal/extract was also prepared. Meat systems were homogenized in a Waring Blendor (Model 33BL73) for 30 s, transferred into plastic pans and then stored for 7 days at 4°C. Samples were analyzed for TBA-reactive substances (TBARS) according to the methods of Siu and Draper (1978) as described by Shahidi and Hong (1991). Meat samples (2 g) in centrifuge tubes were mixed with 5 ml of 10% trichloroacetic acid and vortexed for 2 min at high speed. TBA reagent (5 ml) was added to the tube and vortexed for 0.5 min. Samples were centrifuged at $4000 \times g$ for 10 min and the supernatants were filtered through Whatman No. 3 filter paper. The tubes were placed in a boiling water bath for 45 min, cooled to room temperature in ice, and the absorbance value of TBA-malonaldehyde adduct was read at 532 nm. TBARS values were calculated by multiplying absorbance readings by a factor of 3.4. This factor was determined from a standard curve prepared for1,1,3,3-tetramethoxypropane, a precursor of malonaldehyde (Wettasinghe & Shahidi, 1996).

2.7. Static headspace gas chromatographic analysis

A Perkin–Elmer 8500 gas chromatograph and an HS-6 headspace sampler (Perkin–Elmer Corp., Montreal, PQ) were used for analysis of volatiles in cooked pork samples. A high polarity Supelcowax 10 fused silica capillary column (30 m x 0.32 internal diameter, 0.10 μm film, Supelco Canada Ltd., Oakville, ON) was used. Helium was the carrier gas employed at an inlet column pressure of 120.6 kPa and a split ratio of 7:1. The oven

temperature was maintained at 40°C for 5 min, raised to 200°C at 20°C/min, and held at 200°C for 5 min. The injector and flame ionization detector (FID) temperature were adjusted to 280 (C and held at this temperature throughout the analysis (Shahidi & Pegg, 1993; Wettasinghe & Shahidi, 1996).

For headspace (HS) analysis, 4 g portions of homogenized pork samples were transferred to 10 ml glass vials, capped with Teflon-lined septa, crimped and then frozen at −60°C until used. To avoid heat shock after removal from storage, frozen vials were tempered at room temperature for 30 min and then preheated in the HS-6 magazine assembly at 90°C for a 45 min equilibration. Pressurisation time of the vial was 6 s, and the volume of the vapour phase drawn was approximately 1.5 ml. Chromatogram peak areas were expressed as integrator count units. Individual volatile compounds were tentatively identified by comparing relative retention times of GC peaks with those of commercially-available standards. Quantitative determination of hexanal and total volatiles were accomplished using 2-heptanone as an internal standard (Shahidi & Pegg, 1993; Wettasinghe & Shahidi, 1996).

2.8. Bulk stripped corn oil model system

Extract (100 and 200 ppm) and oil (5 g) were mixed well in 70 ml capped glass tubes (20 cm×2 cm internal diameter). Systems were stored in a forced-air convection oven set at 60°C for 7 days. Samples for conjugated diene (0.2 g) and headspace (0.2 g) analyses were drawn at days 0, 1, 3, 5 and 7.

2.9. Stripped corn oil in-water emulsion system

Corn oil (10%, w/w), deionized water (88%, w/w) and Tween 40 emulsifier (2%, w/w) were mixed in a beaker and sonicated for 30 min. The resulting emulsion (20 ml) was transferred into 70 ml capped glass tubes (20 cm× 2 cm internal diameter) containing 100 or 200 ppm extract. Aliquots for conjugated diene (2 ml) and headspace (2 ml) analyses were drawn at days 0, 1, 3, 5 and 7. Oil was extracted with hexane and desolventised by fluxing nitrogen. The weight of the retrieved oil was recorded.

2.10. Determination of hydrogen peroxide-scavenging properties of the extract

The extract (100 and 200 ppm,) was dissolved in 3.4 ml of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 μl of 43 mM solution of hydrogen peroxide (prepared in the same buffer). The absorbance value (at 230 nm) of the reaction mixture was recorded at 0 min and then at every 10 min up to 40 min. For each concentration, a separate blank sample (devoid of hydrogen peroxide) was used for background subtraction (Ruch et al., 1989). The concentration (mM) of hydrogen peroxide in assay media was determined using a standard curve.

2.11. Determination of hydroxyl radical-scavenging properties of the extract

The hydroxyl radical was generated via iron-catalyzed Haber–Weiss reaction (Fenton driven Haber–Weiss reaction) and spin trapped with 5,5-dimethyl-1 pyrroline *N*-oxide (DMPO). The resultant DMPO-OH adduct was detected using an electron paramagnetic resonance (EPR) spectrometer. The extract was dissolved in 0.1 M phosphate buffer (pH 7.4) so that a 200 μl aliquot will

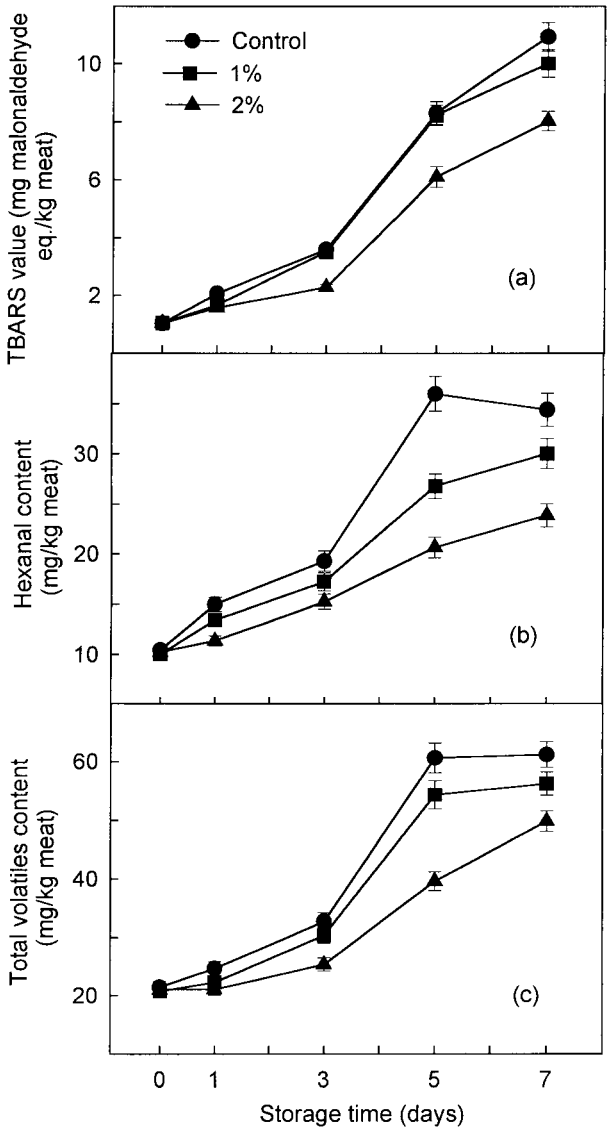

Fig. 1. Effect of borage meal (1 and 2%, w/w) on (a) TBARS, (b) hexanal and total (c) volatiles formation in a cooked lean pork model system. Results are mean values of three determinations ± standard deviation.

result in 200 ppm in the final assay medium (final volume was 800 μl). For 100 ppm concentration, 100 μl of the same stock solution was used, but the volume was adjusted to 200 μl by adding 100 ml of the buffer. Extract (200 μl) was mixed with 200 μl of 100 mM DMPO, 200 μl of 10 mM $FeSO_4$ and 200 μl of 10 mM hydrogen peroxide. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). After 3 min, 10 μl of the mixture was drawn into a syringe and transferred into a quartz capillary tube. The spectrum was recorded in an EPR spectrometer set at 2×10^5 Hz receiver gain, 1 G modulation amplitude, 200 s scan time, 3460 G centre field, 100 G sweep width and 0.5 s time constant (Shi, Dall & Jain, 1991).

2.12. Determination of superoxide-scavenging properties of the extract

A modified version of the method explained by Nishikimi, Rao and Yagi (1972) was employed. The

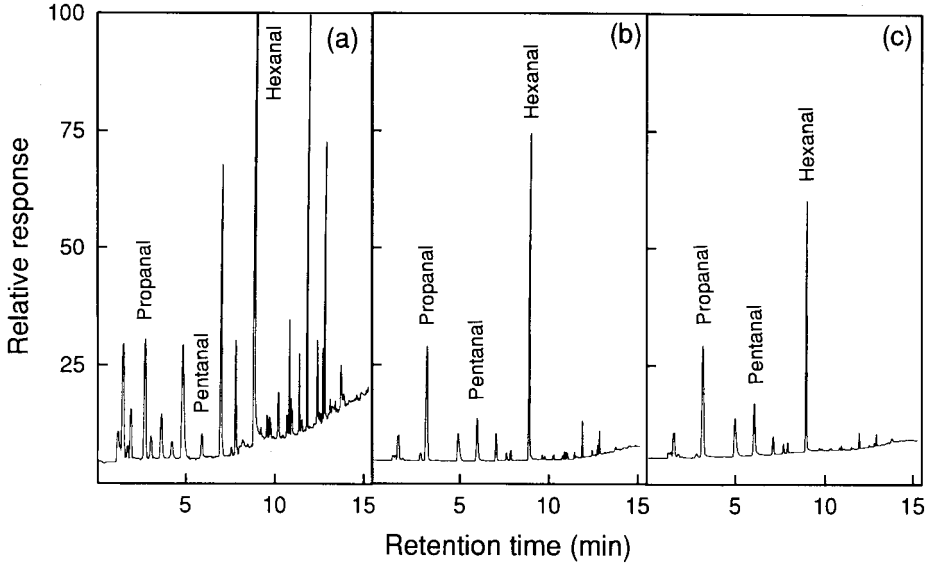

Fig. 2. GC chromatogram showing the dominance of hexanal among the volatiles formed in a cooked lean pork model system containing (a) no additives, (b) 1% borage meal and (c) 2% borage meal after three days of storage at 4°C.

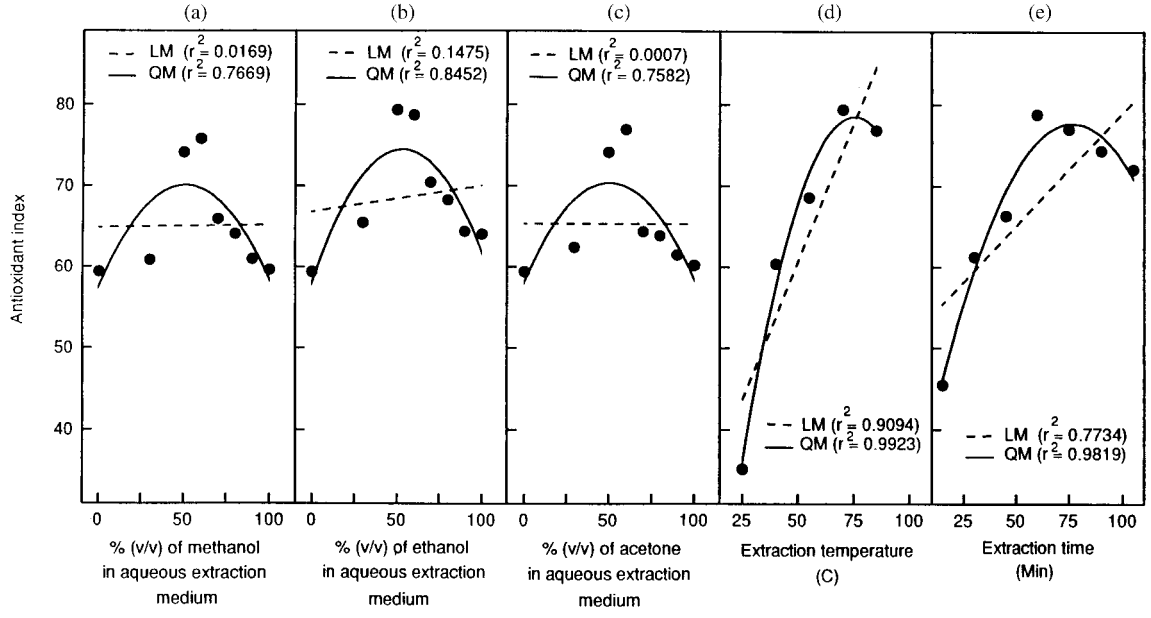

Fig. 3. Effect of different extraction media (a: methanol, b: ethanol, c: acetone), extraction temperature (d) and time (e) on antioxidant activity of borage extract. LM and QM denote linear model and quadratic models, respectively.

superoxide radical was generated with an enzymatic reaction. The reaction mixture contained 1 ml of 3 mM hypoxanthine, 1 ml of xanthine oxidase (100 mIU), 1 ml of 12 mM diethylenetriaamine-pentaacetic acid, 1ml of 178 μM nitro blue tetrazolium and 1 ml of the extracts (final concentration of the extract in the reaction mixture was 200 ppm). For 100 ppm concentration, 0.5 ml of stock extract solution was diluted with 0.5 ml of the buffer. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). The absorbances (at 560 nm) of systems were recorded at 0 min and then after every 10 min up to 60 min. For each system, the absorbance values were corrected by substracting 0 min readings from subsequent readings.

Table 2
Variable (factor) levels used in the response surface methodology[a]

Variable	Symbol	Coded variable levels[a]		
		−1	0	+1
Ethanol content in the extraction medium (% v/v)	X_1	30	50	70
Extraction temperature (C)	X_2	50	60	80
Extractin time (min)	X_3	30	60	90

[a] Coded variable (X_i) levels can be uncoded (x_i) by using equations: $x_1 = 20X_1 + 50$;$x_2 = 20X_2 + 60$; and $x_3 = 30X_3 + 60$.

Table 3
Face-centred cube design and observed responses[a]

Design point	Independant variable[b]			Response (Y)[c]
	X_1[d]	X_2[e]	X_3[f]	
1	−1	−1	−1	60.4
2	−1	−1	+1	62.3
3	−1	+1	−1	65.6
4	−1	+1	+1	68.7
5	+1	−1	−1	66.4
6	+1	−1	+1	65.6
7	+1	+1	−1	70.0
8	+1	+1	+1	71.4
9	−1	0	0	68.3
10	+1	0	0	70.8
11	0	−1	0	79.3
12	0	+1	0	77.2
13	0	0	−1	79.7
14	0	0	+1	74.8
15	0	0	0	80.3
16	0	0	0	81.2
17	0	0	0	79.8

[a] Nonrandomized.
[b] See Table 1 for uncoded variable levels.
[c] Average value of duplicate determinations except for design points 15–17.
[d] X_1: Ethanol content inthe extraction medium (%, v/v).
[e] X_2: Extraction temperature (C).
[f] X_3: Extraction time (min).

2.13. Determination of proximate composition of meat

Moisture, protein and ash contents of meat were determined according to the AOAC (1990) methods. Total lipid content was determined using the Bligh and Dyer (1959) procedure.

2.14. Tukey's studentized range test

One way analysis of variance (ANOVA) and Tukey's studentized range test (Snedecor & Cochran, 1980), based on data collected from various analytical techniques were carried out. Significance was established at $p \leqslant 0.05$.

3. Results and discussion

3.1. Antioxidant activity of borage meal

Fig. 1 depicts the effect of borage meal on lipid oxidation in a meat (moisture, protein, lipid and ash contents were 71.8, 20.2, 6.9 and 1.1%, respectively) model system. TBARS test measures the secondary oxidation products, mainly carbonyl compounds, such as the malonaldehyde (Tarladgis, Pearson & Dugan, 1964; Shahidi & Hong, 1991). Hexanal has also been used as an indicator of lipid oxidation in terrestrial animal and plant lipids as it is the dominant aldehyde formed during oxidation (Frankel, Hu & Tappel, 1989) (Fig. 2). Borage meal, at both levels tested, reduced ($p \leqslant 0.05$) the

Table 4
Estimated regression coefficients of the quadratic polynomial model

Parameter[a]	Estimated coefficient	Standard error
Linear		
β_0	−17.1765	14.3677
β_1	2.7029****[b]	0.4031
β_2	0.4910	0.4667
β_3	0.3188	0.2285
Quadratic		
β_{11}	−0.0251****[b]	0.0037
β_{22}	−0.0034	0.0037
β_{33}	−0.0026	0.0016
Interaction		
β_{12}	−0.0007	0.0021
β_{13}	−0.0009	0.0014
β_{23}	−0.0007	0.0014
β_{123}	–	
R^2	0.9439	
F ratio	13.091	
p value	0.00013	
CV, %	3.3737	

[a] Coefficients refer to the general model.
[b] ****Significant at 0.001 level.

formation of TBARS, hexanal and total volatiles formation in a concentration dependent manner. At 2% addition level, it reduced the TBARS, hexanal and total volatile formation by 26.5, 30.5 and 18.6%, respectively (Table 1). These results showed the ability of borage meal to retard lipid oxidation and suggested the presence of antioxidant compounds in the meal. Therefore, the concentration of these compounds using a solvent extraction procedure was intended.

3.2. Selection of an appropriate organic solvent and its factor levels (X_1)

Fig. 3 (a)–(c) shows the effect of aqueous methanol, ethanol and acetone at 0% (organic solvent:water ratio was 0:100, v/v) to 100% (organic solvent:water ratio was 100:0, v/v), as extraction media, on antioxidant activity of the resulting extract. The response behaved as a second order function of the independent variable (for all three solvents) as the second order models had higher correlation coefficients than those for linear models. For extraction media containing methanol and acetone, the antioxidant activity increased, reached a maximum, and then started to decline with the increasing solvent content. Maximum antioxidant activity was evident when the solvent content in the extraction medium was 60 and 50% for methanol and ethanol, respectively. For acetone, the maximum antioxidant activity occurred at 60%. In general, antioxidant activity of the extract prepared with acetone and methanol was low, while that of the extract prepared with 50% ethanol was the greatest. Therefore, aqueous ethanol was selected as

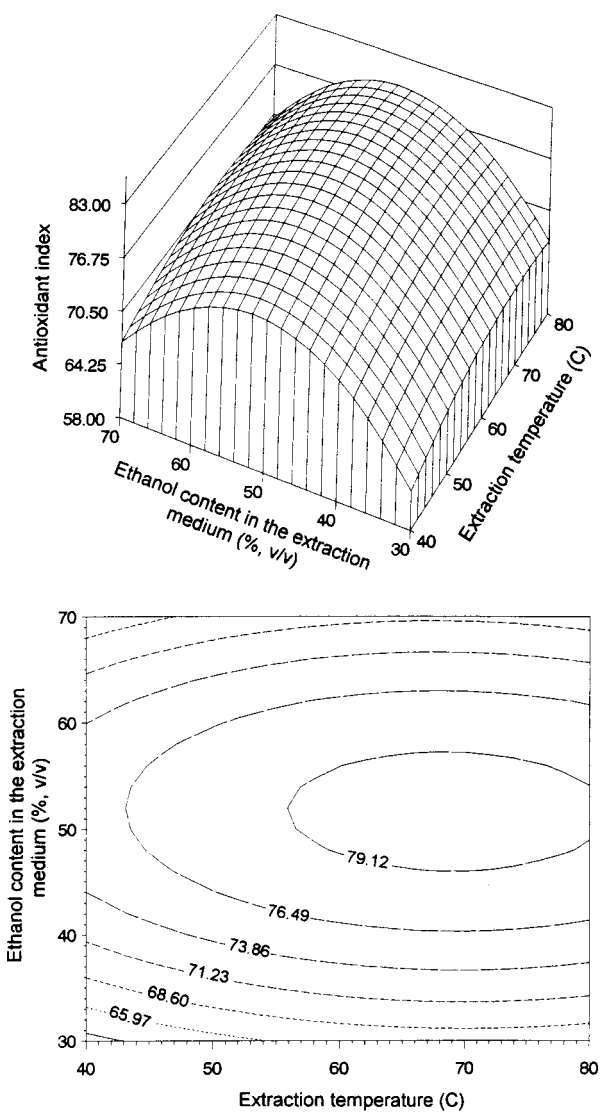

Fig. 4. Dependence of the antioxidant activity of borage extract on ethanol content in the aqueous extraction medium and extraction temperature.

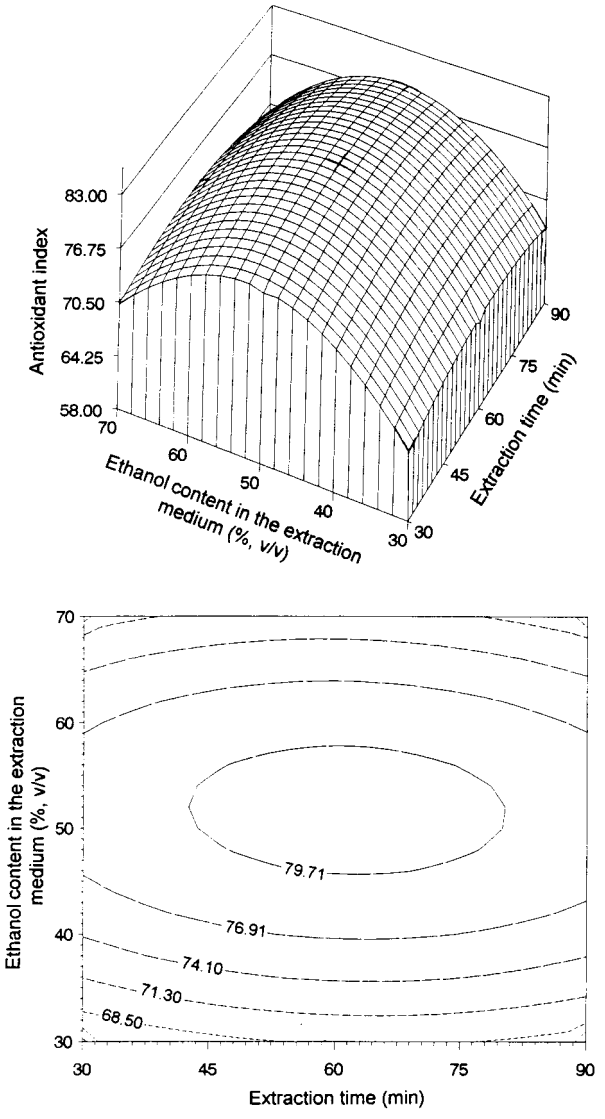

Fig. 5. Dependence of the antioxidant activity of borage extract on ethanol content in the aqueous extraction medium and extraction time.

the preferred extraction medium and 30, 50 and 70% ethanol were selected as the lower, middle, and upper design points, respectively.

3.3. Factor levels of extraction temperature (X_2) and time (X_3)

The effect of extraction temperature and time on antioxidant activity of the extract was a second order function [Fig. 3(d) and (e)]. The antioxidant activity of the borage extract prepared at 25°C was low, but gradually increased with increasing temperature (up to 70°C) and started to decrease afterwards. Therefore, variable levels of 40, 60 and 80°C were chosen as the lower, middle, and upper points, respectively. Results for extraction time studies showed that the shorter (15 min) or prolonged (105 min) extraction times are not suitable for the optimum antioxidant activity of the extracts. The three design points selected for time variable were 30, 60 and 90 min.

3.4. Response surface analysis

After the lower, middle, and upper design points for each variable were determined, they were assigned a code (Table 2) and an experimental design was devised using different factor combinations (Table 3). Table 3 also shows the experimental data observed for response variable. Regression coefficients of intercept, linear, quadratic, and interaction terms of the model were calculated using least square techniques and their significance was determined using *t*-test (Table 4). The linear and quadratic effects of ethanol content in the extraction medium (X_1) were significant. All other terms were insignificant. The polynomial model fitted to experimental data were highly significant ($p \leqslant 0.05$). Coefficient of determination (R^2) was 0.9439 and indicated that most of the variation observed for design points has been explained by the predicted polynomial model. Furthermore, results of the error analysis indicated that the lack of fit was insignificant ($p > 0.05$). Coefficient of variation (CV) of less than 5% indicated that the model is reproducible (Mason et al., 1989). The predicted second-order polynomial model was:

$$Y = -17.1765 + 2.7029X_1 + 0.4910X_2 + 0.3188X_3 - 0.0251X_1^2 - 0.0034X_2^2 - 0.0026X_3^2 - 0.0007X_1X_2 - 0.0009X_1X_3 - 0.0007X_2X_3$$

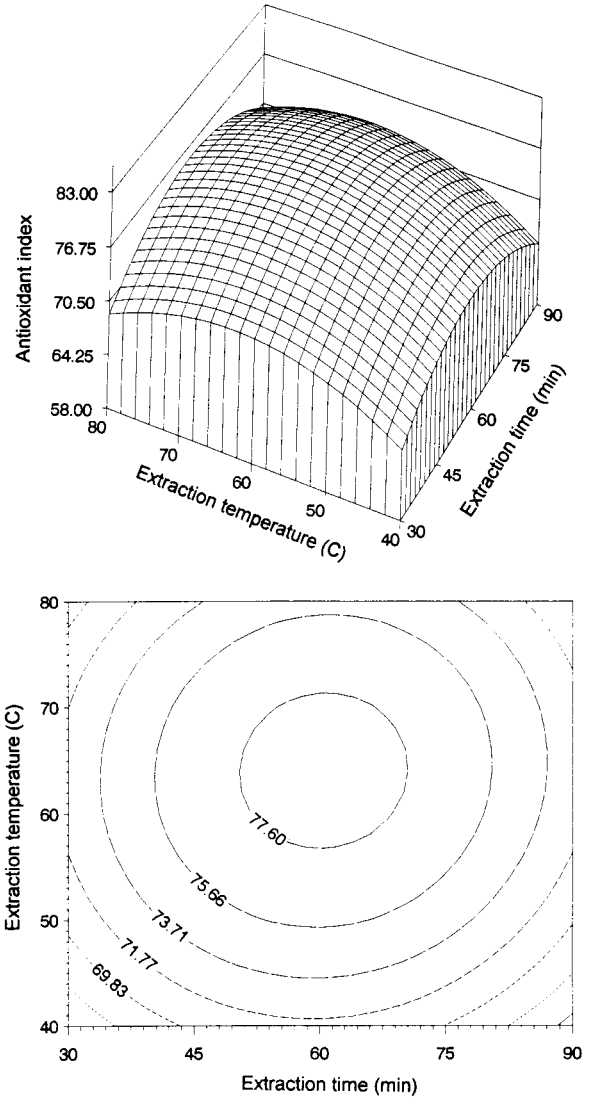

Fig. 6. Dependence of the antioxidant activity of borage extract on extraction temperature and time.

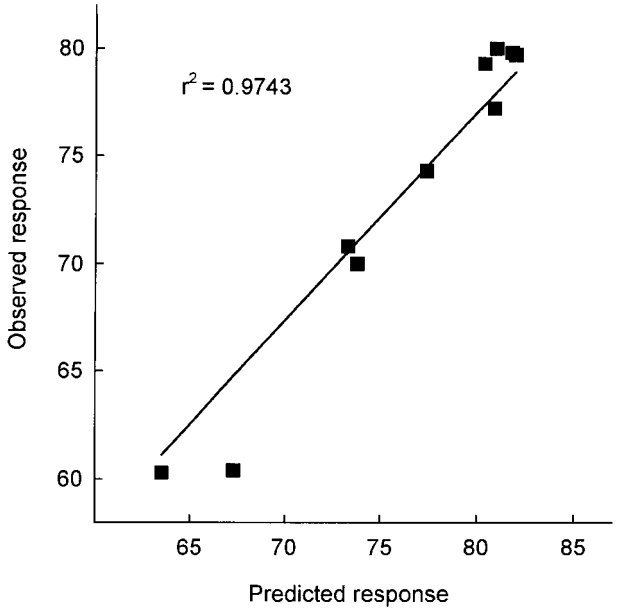

Fig. 7. Relationship between the predicted and observed response.

The model indicated that the linear effect of the solvent content in the extraction medium (X_1) had the greatest effect on antioxidant activity of the extract as it had the largest positive linear coefficient. The linear effect of X_1, X_2 and X_3 variables decreased in the following order: $X_1 > X_2 > X_3$.

Figs. 4–6 depict the nature of the response surface. Canonical analysis of the response surface was performed in order to examine the nature of the stationary point and to obtain the critical values of the independent variables at the stationary point. Contour plots (Figs. 4–6) were also generated using the data obtained by canonical analysis. Since all eigen values were negative, the nature of the response at the stationary point was a maximum (antioxidant index, 80.70). Critical values of the three variables laid within the experimental region (X_1: 52%; X_2: 74°C and X_3: 62 min). Verification experiments showed that the experimental values were well in agreement with the predicted values (Fig. 7). The correlation coefficient between predicted and observed responses was 0.9743.

This study showed the necessity of using an appropriate solvent with the right polarity to maximize the antioxidant activity of phenolic extracts of plant materials. It also demonstrated the importance of selecting an appropriate extraction temperature and an extraction time when extracting plant phenolics.

3.5. Evaluation of antioxidant activity of the extract

Although a wide range of model systems is available for evaluation of antioxidant substances, the choice depends mainly upon the nature of the substances under investigation. There is evidence for discrepancies in antioxidant activities of substances when tested in different model systems. For example some hydrophilic antioxidants, i.e. Trolox (an analogue of α-tocopherol), were reported to be more effective than their lipophilic counterparts, i.e. α-tocopherol, in bulk oil, but less active in oil-in-water emulsions (Huang, Hopia, Schwarze, Frankel & German, 1996). Furthermore, a particular antioxidant can promote the formation of hydroperoxides at the early stages of oxidation and the same antioxidant may inhibit the formation of secondary oxidation products (aldehydes, ketones, alcohols and hydrocarbons) at later stages (Frankel, 1996; Frankel, Huang, Aeschbach & Prior, 1996). Therefore, the extract was evaluated for its antioxidant properties in different model systems using a variety of analytical techniques. Standard *trans*-sinapic acid and BHA were used as reference antioxidants.

3.6. Antioxidant activity of the extracts in the β-carotene-linoleate system

The extract prepared at the critical variable combination contained 413 mg/g phenolics as *trans*-sinapic acid equivalents. Its phenolics were composed of 89% (w/w) hydrophilic compounds and 11% (w/w) hydrophobic compounds. Fig. 8 shows the effects of the extract at 100 and 200 ppm concentration on β-carotene bleaching in a β-carotene-linoleate system. The extract exerted antioxidant activities at both concentrations tested, but the effect was more pronounced at 200 ppm. The system containing 200 ppm extract retained 81% of the initial β-carotene after 2 h of the assay whereas the control retained only 11%. Antioxidant efficacy of the extract in this system was comparable to that of 100 ppm standard *trans*-sinapic acid, but somewhat lower than that for 100 ppm BHA [Fig. 8(b)]. β-carotene in the model system undergoes rapid discoloration in the absence of an

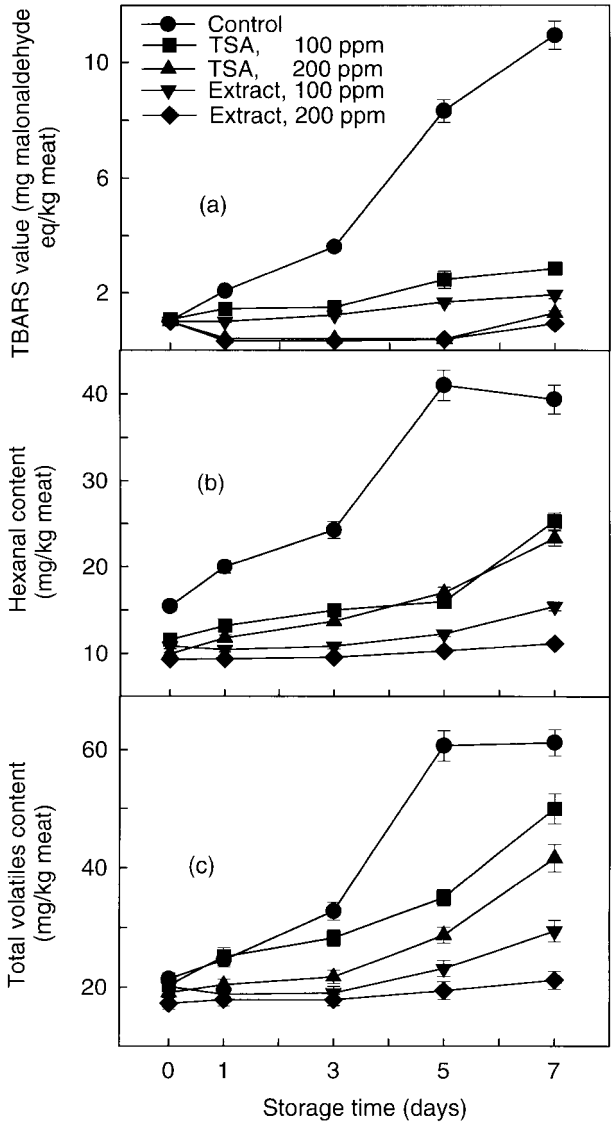

Fig. 8. Effect of borage extract on (a) TBARS, (b) hexanal and (c) total volatile formation in cooked lean pork stored at 4°C. Two concentrations of the extract used in the experiments, were based upon the total phenolics. TSA denotes *trans*-sinapic acid. Results are mean values of three determinations ± standard deviation.

antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene molecules loose their double bonds, the system loses its characteristic orange colour which can be monitored using spectrophotometry. Presence of a phenolic antioxidant can hinder the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system.

3.7. Antioxidant activity of the extract in the meat model system

In meat model system, borage extract exerted a concentration-dependent antioxidant effect. Both concentrations of the extract resulted in lower TBARS values through-out the entire storage period, but the effect at 200 ppm was almost comparable to that of the standard *trans*-sinapic acid at same concentration [Fig. 9(a), Table 5]. Standard *trans*-sinapic acid at 200 ppm exerted the greatest inhibition on TBARS formation. The inhibition of hexanal formation was similar for both concentrations [Fig. 9(b), Table 5]. Total volatile content of the systems containing extract was lower for both concentrations, but the effect was more pronounced at 200 ppm. The effect of standard trans-sinapic acid at both concentrations on volatile formation was greater ($p \leq 0.05$) than that exerted by the extracts [Fig. 9(c), Table 5].

Table 5
Inhabition[a] of TBARS, hexanal and total volatiles formation of cooked lean pork containing borage extract on day 7 of storage at 4°C[b]

Additive (ppm)		% Inhibition		
		TBARS	Hexanal	Total volatiles
Extract[c]	100	74.6 ± 4.2a	41.8 ± 2.7a	18.9 ± 1.0a
	200	88.3 ± 5.3b	47.3 ± 2.8a	32.5 ± 2.8b
TSA	100	82. ± 4.6ab	69.0 ± 4.9b	52.1 ± 2.8c
	200	92.1 ± 5.7	82.4 ± 41.c	65.4 ± 3.4d

[a] % Inhibition = 100 (value for sample/value for control) × 100.
[b] Results are mean value of three determinations ± standard deviation. Means sharing the same letter in a column are not significantly ($p < 0.05$) different from one another.
[c] As *trans*-sinapic acid (TSA) equivalents.

3.8. Antioxidant activity of the extract in the bulk corn oil and corn oil-in-water emulsion systems

Corn oil stripped of its endogenous antioxidants was used in order to eliminate their effect from both bulk and emulsion systems. This facilitated the evaluation of antioxidant substances without the interference by endogenous antioxidants such as tocopherols. As

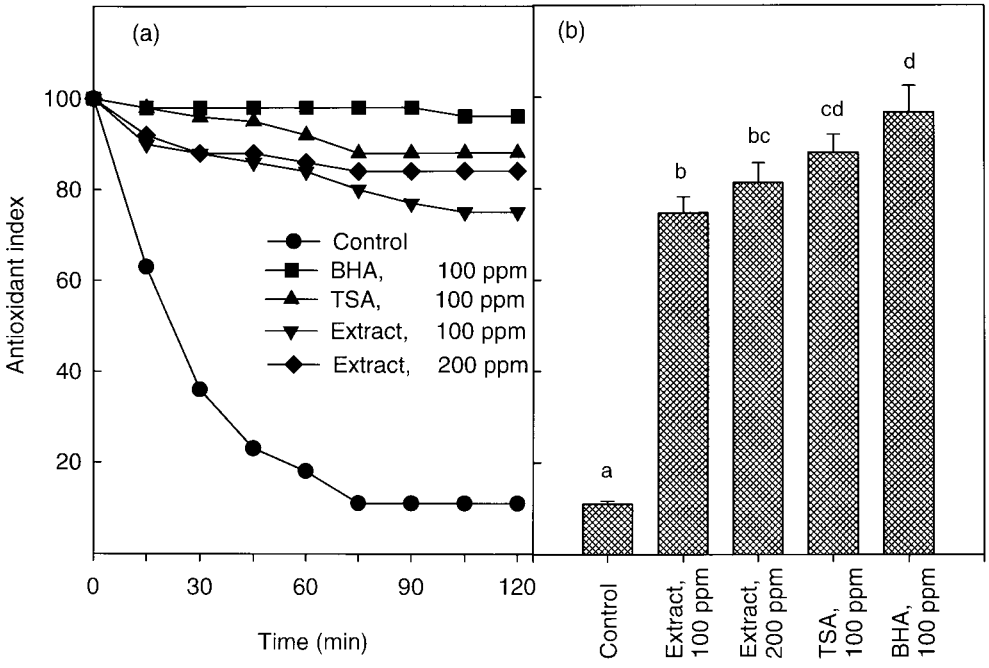

Fig. 9. Effect of borage extract on β-carotene bleaching over time in a β-carotene-linoleate model system (a). Statistical significance of the differences (at 120 min) among treatments (b) results are mean values of three determinations ± standard deviation; bars sharing the same letter are not significantly ($p > 0.05$) different from one another. Two concentrations of the extract used in the experiments were based upon the total phenolics. TSA denotes *trans*-sinapic acid.

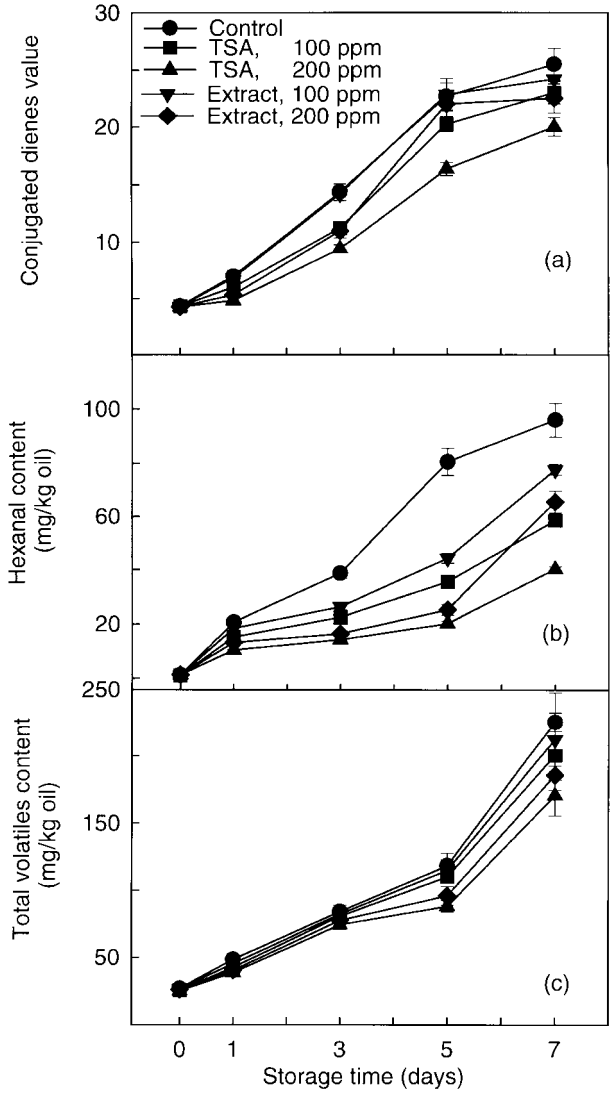

Fig. 10. Effect of borage extract on (a) conjugated diene (b) hexanal and (c) total volatile formation in a bulk stripped corn oil system. Two concentrations of the extract used in the experiments were based upon the total phenolics. TSA denotes *trans*-sinapic acid. Results are mean values of three determinations ± standard deviation.

depicted in Fig. 10, the extract reduced the formation of conjugated diene, hexanal and total volatiles in bulk corn oil. The statistical significance of the inhibition by the extract and *trans*-sinapic acid is given in Table 6. The extract was less effective on inhibition of conjugated diene formation (8–10%) as compared to its 27–49% inhibition of the formation of hexanal and total volatiles. The effect of the extract and *trans*-sinapic acid on lipid oxidation in oil-in-water emulsion system is shown in Fig. 11. The extract and the *trans*-sinapic acid were less effective in the emulsion system when compared to their high inhibition of conjugated diene and volatile formation in bulk corn oil (Table 6). This may be due to the fact that the extracts contained more hydrophilic phenolics (89%, w/w) than hydrophobic

Table 6
Inhibition[a] of conjugated dienes (CD), hexanal and total volatiles fromation in bulk corn oil and corn oil-in-water emulsion systems on day 7 of storage at 60°C[b]

Additive (ppm)		% Inhibition		
		CD	Hexanal	Total volatiles
Bulk stripped-corn oil model system				
Extract[c]	100	8.3 ± 0.5b	47.2 ± 2.8d	27.5 ± 1.1d
	200	10.6 ± 0.5bc	49.6 ± 2.0d	48.2 ± 2.3e
TSA	100	27.2 ± 1.3e	61.4 ± 3.4e	58.3 ± 2.2f
	200	44.8 ± 2.6f	68.0 ± 4.0f	68.8 ± 2.9g
Stripped-corn oil-in-water emulsion system				
Extract[c]	100	5.2 ± 0.3a	19.7 ± 0.9a	6.4 ± 0.2a
	200	12.4 ± 0.5c	32.2 ± 2.2b	18.8 ± 1.1c
TSA	100	10.00.5bc	39.4 ± 2.3c	11.40 ± 0.6b
	200	22.3 ± 1.0d	52.6 ± 3.1a	24.1 ± 1.0d

[a] % Inhibition = 100 (value for the sample/value for the control) × 100.
[b] Results are mean values of three determinations ± standard deviation. Means sharing the same letter in a column are not significantly ($p > 0.05$) different from one another.
[c] As *trans*-sinapic acid (TSA) equivalents.

phenolics (11%, w/w). According to Frankel, Huang, Kunner and German (1994), the natural antioxidants exert interfacial affinities between air–oil and oil–water interfaces that affect their activity in bulk oil and emulsion systems. In bulk corn oil, the hydrophilic antioxidants are oriented in the air–oil interface, protecting the oil phase, whereas the hydrophobic antioxidants remain dissolved in the oil phase. In emulsion systems, however, the hydrophilic antioxidants become diluted and less effective due to dissolving in the aqueous phase, but the hydrophobic antioxidants are oriented in the oil-water interfaces protecting the oil phase from oxidation. Therefore the borage extracts with very low hydrophobic phenolics were less effective in retarding lipid oxidation in the oil-in-water emulsion. In contrast, the extract had a very high content of hydrophilic phenolics which could efficiently orient at the air-oil interface, protecting bulk oil from oxidation. Being a hydrophilic compound, *trans*-sinapic acid more efficiently protected bulk oil from oxidation than it did in the emulsion systems and its effects were always superior to those of the extract at the same concentration.

3.9. Hydrogen peroxide and hydroxyl radical scavenging activity of the extract

As depicted in Fig. 12, the extract exerted a concentration-dependent scavenging of hydrogen peroxide (H_2O_2). The concentration of H_2O_2 in the systems containing extract dropped very sharply during the initial 10 min period of the assay. During this period,

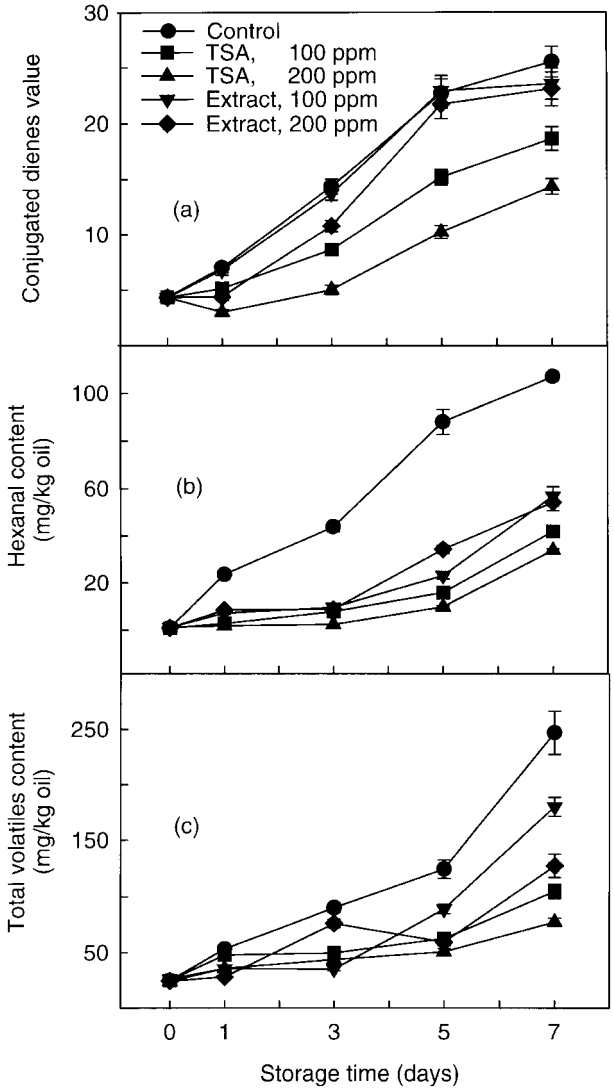

Fig. 11. Effect of borage extract on (a) conjugated diene, (b) hexanal and (c) total volatile formation in a stripped corn oil-in-water emulsion system. Two concentrations of the extract used in the experiments, were based upon the total phenolics. TSA denotes *trans*-sinapic acid.

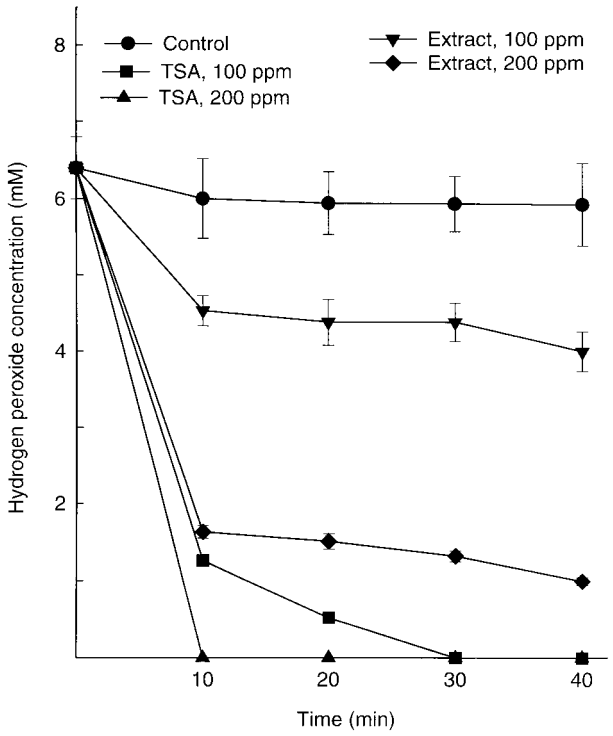

Fig. 12. Hydrogen peroxide-scavenging activity of borage extract. Two concentrations of the extract used in the experiments were based upon the total phenolics. TSA denotes *trans*-sinapic acid. Results are mean values of three determinations ± standard deviation.

the reduction ($p \leqslant 0.05$) in hydrogen peroxide concentration in the systems containing 0, 100 and 200 ppm extract was 3, 29 and 75% of the initial concentration, respectively. The decomposition of hydrogen peroxide into water may occur according to the following reaction:

$$H_2O_2 + 2H^+ + 2e \rightarrow 2H_2O$$

Since phenolic compounds present in the extract are good electron doners, they may accelerate the conversion of H_2O_2 into H_2O. However, H_2O_2 in the presence of iron ions can generate extremely reactive $^{\cdot}OH$ (Ruch, Chug & Klaunig, 1984). This reaction is sometimes called an iron-catalyzed Haber–Weiss reaction which can be written as:

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \qquad \text{(metal reduction)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

(Fenton reaction)

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^-$$

(Haber–Weiss reaction)

In this study $^{\cdot}OH$ generated via these reactions was spin-trapped with DMPO which resulted in a DMPO-OH adduct, a relatively stable free radical (Ruch et al., 1984; Halliwell & Gutteridge, 1985). This was done because the detection of $^{\cdot}OH$ as such is extremely difficult, due to its very short life time (Ruch et al., 1984). As depicted in Fig. 13, the DMPO-OH adduct generated a 1:2:2:1 quartet with hyperfine coupling parameters of 14.9 G (Yen & Chen, 1995). The intensity of this signal was reduced, but did not completely disappear, when 100 ppm extract was present in the assay medium. At 200 ppm, a 100% quenching of the signal was evident. There is well supported evidence that the phenolic compounds found in various plant materials possess free radical-scavenging properties. Shi et al.

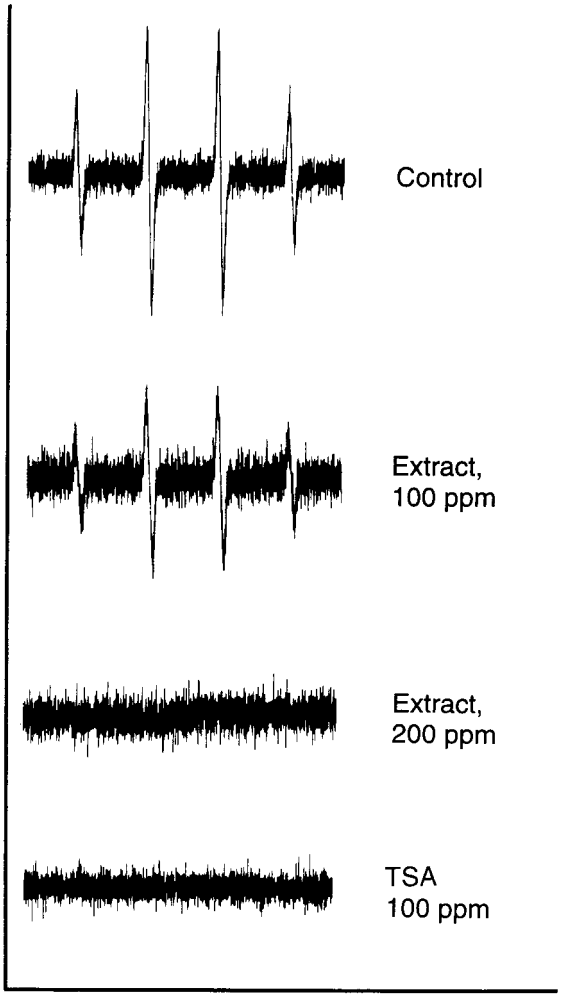

Fig. 13. EPR spectra showing the effect of borage extract on the scavenging of hydroxyl radical. Two concentrations of the extract used in the experiments were based upon the total phenolics. TSA denotes *trans*-sinapic acid.

(1991) demonstrated the ability of caffeine, a phenolic compound, to effectively scavenge ·OH. Husain et al. (1987) and van Acker et al. (1996) reported that flavonoids such as myrcetin, quercetin and rhamnetin were ·OH scavengers. They also noted that the effectiveness of such compounds increases with increasing number of hydroxyl groups attached to the aromatic B-ring. As is the case for many other free radicals, ·OH can be neutralized if it is provided with a hydrogen atom. The phenolic compounds present in the crude extract had the ability to donate a hydrogen atom to ·OH, resulting in the quenching of the EPR signal.

3.10. Superoxide radical-scavenging activity of the extract

The superoxide radical generated in the xanthine/xanthine oxidase system was readily scavenged by the extract. For the control, the characteristic ink blue colour of the

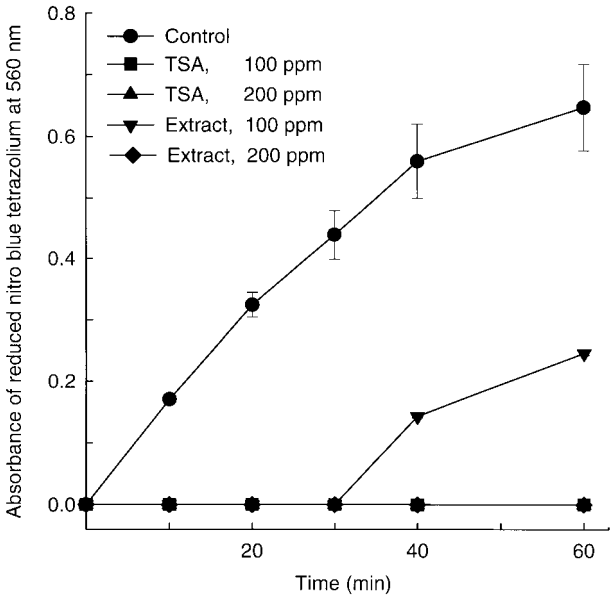

Fig. 14. Effect of borage extract on the superoxide radical as explained by the intensity of the reduced nitro blue tetrazolium indicator. Two concentrations of the extract used in the experiments were based upon the total phenolics. TSA denotes *trans*-sinapic acid. Results are mean values of three determinations ± standard deviation. Note: symbols for TSA and extract are overlapped.

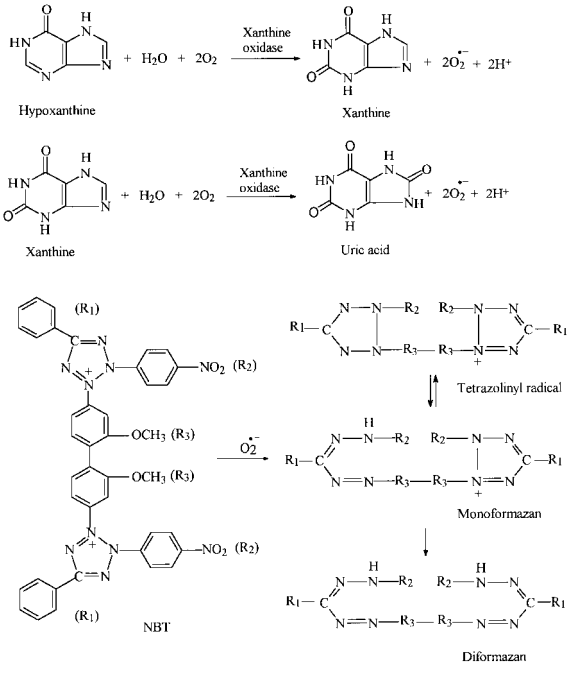

Fig. 15. Generation of the superoxide radical in hypoxanthine/xanthine oxidase system and its reaction with nitro blue tetrazolium indicator.

reduced nitro blue tetrazolium was observed after 10 min of the assay and its intensity increased throughout the entire assay period, indicating the generation of superoxide radical (Fig. 14). Nitro blue tetrazolium in the assay medium containing the crude extract at 200 ppm was not reduced even after 1 h of the assay, indi-

cating the superoxide-scavenging ability of the extract. Nitro blue tetrazolium in the medium containing 100 ppm extract started to reduce after 40 min into the assay. Fig. 15 shows the mechanism by which the superoxide radical is generated and its reaction with nitro blue tetrazolium.

4. Conclusions

Borage meal and its extract possess antioxidant properties which are concentration-dependent. Concentration of antioxidant compounds present in the borage meal should be carried out under optimized extraction conditions in order to acquire an extract with enhanced antioxidant activity. The antioxidant activity of the extract was a maximum when it was prepared by extracting with 52% ethanol at 74°C for 62 min. Antioxidant efficacy of botanical extracts should be evaluated in a variety of model systems using several different indices because the effectiveness of such antioxidant materials is largely dependent upon the chemical and physical properties of the system to which they were added and a single analytical protocol adopted to monitor the lipid oxidation may not be sufficient to make a valid judgment. The ability of borage extract to retard lipid oxidation is attributable to the ability of its phenolic constituents to quench reactive oxygen species. Borage extract may be added to bulk oils and meat products in place of synthetic antioxidants in order to retard lipid oxidation. However, the low content of hydrophobic phenolics in the extract might make it less antioxidative in oil-in-water emulsion systems.

Acknowledgements

We are grateful to Dr. Lawrence K. Thompson, Department of Chemistry, Memorial University of Newfoundland, St. John's, Canada, for assisting with EPR spectroscopy. We are also thankful to the Natural Sciences and Engineering Research Council of Canada for providing financial support.

References

Amarowicz, R., Wanasundara, U., Wanasundara, J., & Shahidi, F. (1993). Antioxidant activity of ethanolic extracts of flaxseed in a β-carotene-linoleate model system. *Journal of Food Lipids, 1*, 111–117.

AOAC (1990). *Official methods of analysis* (15th ed.). Association of Official Analytical Chemists: Washington, DC.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemical Physiology, 32*, 911–917.

Box, G. E. P., & Wilson, K. G. (1951). On the experimental attainment of optimum conditions. *Journal of the Royal Statistic Society, 13*, 1–45.

Chapkin, R. S., & Carmichael, S. L. (1990). Effects of dietary n-3 and n-6 polyunsaturated fatty acids on macrophage phospholipid classes and subclasses. *Lipids, 25*, 827–834.

Chiarpotto, E., Scavazza, A., Leonarduzzi, G., Camandola, S., Biasi, F., Teggia, P. M., Garavoglia, M., Robecchi, A., Roncari, A., & Poli, G. (1997). Oxidative damage and transforming growth factor b1 expression in pretumoral and tumoral lesions of human intestines. *Free Radical Biology and Medicine, 22*, 889–894.

Engler, M. M., Engler, M. B., & Paul, S. M. (1992). Effects of dietary borage oil rich in gamma-linolenic acid on blood pressure and vascular reactivity. *Nutrition Research, 12*, 519–528.

Engler, M. M., Karanian, J. W., & Salen. Jr., N (1991). Influence of dietary polyunsaturated fatty acids on aortic and platelet fatty acid composition in the rat. *Nutrition Research, 11*, 753–763.

Fukuda, Y., Osawa, T., Namiki, M., & Ozaki, T. (1985). Studies on antioxidative substances in sesame seeds. *Agricultural Biological Chemistry, 49*, 301–306.

Frankel, E. N., Hu, M.-L., & Tappel, A. L. (1989). Rapid headspace gas chromatography of hexanal as a measure of lipid peroxidation in biological samples. *Lipids, 24*, 976–981.

Frankel, E. N., Huang, S.-W., Kanner, J., & German, J. B. (1994). Interfacial phenomina in the evaluation of antioxidants: bulk oils vs emulsions. *Journal of Agricultural and Food Chemistry, 42*, 1054–1059.

Frankel, E. N. (1995). Antioxidants in lipid foods and their impact on food quality. *Food Chemistry, 57*, 51–55.

Frankel, E. N., Huang, S.-W., Aeschbach, R., & Prior, E. (1996). Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion. *Journal of Agricultural and Food Chemistry, 44*, 131–135.

Gao, L., & Mazza, G. (1996). Extraction of anthocyanin pigments from purple sunflower hulls. *Journal of Food Science, 61*, 600–603.

Gibson, R. A., Lines, D. R., & Neumann, M. A. (1992). Gamma linolinic acid (GLA) content of encapsulated evening primrose products. *Lipids, 27*, 82–84.

Halliwell, B., & Gutteridge, J. M. C. (1985). *Free radicals in biology and medicine*. Oxford Science Publications, Oxford University Press: Oxford.

Hettiarachchy, N. S., Glenn, K. C., Gnanasambandam, R., & Johnson, M. G. (1996). Natural antioxidant extracts from fenugreek (*Trigonella foenumgraecum*) for ground beef patties. *Journal of Food Science, 61*, 516–519.

Huang, S.-W., Hopia, A., Schwarze, K., Frankel, E. N., & German, J. B. (1996). Antioxidant activity of α-tocopherol and trolox in different lipid substrates: bulk oils vs oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 44*, 444–452.

Husain, S. R., Terao, J., & Matsushita, S. (1987). Effect of browning reaction products of phospholipids on antioxidation of methyl linoleate. *Journal of the American Oil Chemistry Society, 63*, 1457–1460.

Huang, S.-Y., Lin, X., Redden, P. R., & Horrobin, D. F. (1995). *In vitro* hydrolysis of natural and synthetic γ-linolenic acid-containing triacylglycerols by pancreatic lipase. *Journal of the American Oil Chemists' Society, 72*, 625–631.

Iuliano, L., Colavita, A. R., Leo, R., Pratico, D., & Violi, F. (1997). Oxygen free radicals and platelet activation. *Free Radical Biology and Medicine, 22*, 999–1006.

Lu, F., & and Foo, Y. (1995). Phenolic antioxidant components of evening primrose. In A. S. H. Ong, E. Niki, & L. Parker, *Nutrition, ipids, health, and disease* (pp. 86–95). American Oil Chemists' Society Press: Champaign, IL.

Mason, R. L., Gunst, R. F., & Hess, J. L. (1989). *Statistical design and analysis of experiments: with applications to engineering and science*. Wiley Series in Probability and Mathematical Statistics. New York: John Wiley and Sons.

Mead, R. (1988). *The design of experiments: statistical principles for practical application*. Cambridge, UK: Cambridge University Press.

Miller, H. E. (1971). A simplified method for the evaluation of antioxidants. *Journal of the American Oil Chemists' Society, 18*, 439–452.

Naczk, M., Shahidi, F., & Sullivan, A. (1992). Recovery of rapeseed tannins by various solvent systems. *Food Chemistry, 45*, 51–54.

Nishikimi, M., Rao, N. A., & Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. *Biochemical Biophysical Research Communications, 46*, 849–854.

Oomah, B. D., Kenaschuk, E. O., & Mazza, G. (1995). Phenolic acids in flaxseed. *Journal of Agricultural and Food Chemistry, 43*, 2016–2019.

Packer, M., & Glazer, A. N. (1990). *Methods in enzymology (Vol. 186 Part B). Oxygen radicals and antioxidants*. San Diego, CA: Academic Press.

Pryor, W. A. (1966). *Free radicals*. McGraw–Hill Series in Advanced Chemistry. New York: McGraw-Hill Book Company.

Rahmatullah, M. S. K. S., Shukla, V. K. S., & Mukherjee, K. D. (1994a). γ-Linolenic acid concentrates from borage and evening primrose oil fatty acids via lipase-catalyzed esterification. *Journal of the American Oil Chemists' Society, 71*, 563–573.

Rahmatullah, M. S. K. S., Shukla, V. K. S., & Mukherjee, K. D. (1994b). Enrichment of γ-linolenic acid from evening primrose oil and borage oil via lipase-catalyzed hydrolysis. *Journal of the American Oil Chemists' Society, 71*, 569–573.

Ramarathnam, N., Osawa, T., Ochi, H., & Kawakishi, S. (1995). The contribution of plant food antioxidants to human health. *Trends in Food Science and Technology, 6*, 75–82.

Redden, R. P., Lin, X., Fahey, J., & Horrobin, F. (1995). Stereospecific analysis of the major triacylglycerol species containing γ-linolenic acid in evening primrose oil and borage oil. *Journal of Chromatography A, 704*, 99–111.

Ruch, R. J., Chug, S. U., & Klaunig, J. E. (1984). Spin trapping of superoxide and hydroxyl radicals. *Methods in Enzymology, 105*, 198–209.

Ruch, R. J., Cheng, S.-J., & Klaunig, E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chineese green tea. *Carcinogenesis, 10*, 1003–1008.

SAS Institute Inc. (1990). *Statistical analytical systems user's guide* (Vol. 2). Carg, NC. SAS: Institute Incorporated.

Shahidi, F., & Hong, C. (1991). Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. *Journal of Food Biochemistry, 15*, 97–105.

Shahidi, F., & Pegg, R. B. (1993). Hexanal as an indicator of meat flavour deterioration. *Journal of Food Lipids, 1*, 177–186.

Shahidi, F., & Wanasundara, P. K. J. P. D. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32*, 67–103.

Shahidi, F., Wanasundara, U. N., & Amarowicz, R. (1994). Natural antioxidants from low-pungency mustard flour. *Food Research International, 27*, 489–493.

Shi, X., Dalal, N. S., & Jain, A. C. (1991). Antioxidant behavior of caffeine: efficient scavenging of hydroxyl radicals. *Food and Chemical Toxicology, 29*, 1–6.

Silva, J. M. R. D., Darman, N., Fernandez, Y., & Mitjavila (1991). Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. *Journal of Agricultural and Food Chemistry, 39*, 1549–1552.

Siu, G. M., & Draper, H. H. (1978). A survey of the malonaldehyde content of retail meats and fish. *Journal of Food Science, 43*, 1147–1149.

Snedecor, G. W., & Cochran, W. G. (1980). *Statistical methods* (7th ed.). Ames, IA: The Iowa State University Press.

Swain, T., & Hillis, W. E. (1959). The phenolic constituents of *Prunes domesticus* (L.). The quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture, 10*, 63–68.

Tarladgis, B. G., Pearson, A. M., & Dugan Jr., L. R. (1964). Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods. II-formation of the TBA-malonaldehyde complex without acid-heat treatment. *Journal of the Science of Food and Agriculture, 15*, 602–607.

van Acker, S. A. B. E., van den Berg, D.-J., Tromp, M. N. J. L., Griffioen, D. H., van Bennekom, W. P., van der Vijgh, W. J. F., & de Bast, A. (1996). Structural aspects of antioxidant activity of flavonoids. *Free Radical Biology and Medicine, 20*, 331–342.

Wanasundara, U., Amarowicz, R., & Shahidi, F. (1994). Isolation and identification of an antioxidative component in canola meal. *Journal of Agricultural and Food Chemistry, 42*, 1285–1290.

Wanasundara, P. K. J. P. D., & Shahidi, F. (1996). Optimization of hexametaphosphate-assisted extraction of flaxseed proteins using response surface methodology. *Journal of Food Science, 61*, 604–607.

Webber, C., Podda, M., Rallis, M., Thiele, J. J., Traber, G. M., & Packer, L. (1997). Efficacy of topically applied tocopherols and tocotrienols in protection of murine skin from oxidative damage induced by UV-irradiation. *Free Radical Biology and Medicine, 22*, 761–769.

Wettasinghe, M., & Shahidi, F. (1996). Oxidative stability of cooked comminuted lean pork as affected by alkali and alkali-earth halides. *Journal of Food Science, 61*, 1160–1164.

Wolff, R. L., & Sébédio, J.-L. (1994). Characterization of γ-linolenic acid geometrical isomers in borage oil subjected to heat treatments (deodorization). *Journal of the American Oil Chemists' Society, 71*, 117–126.

Yanishlieva, N. V., & Marinova, E. M. (1995). Effects of antioxidants on the stability of triacylglycerols and methyl esters of fatty acids of sunflower oil. *Food Chemistry, 54*, 377–382.

Yen, G.-C., & Chen, H.-Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry, 43*, 27–32.

Yuting, C., Rongliang, Z., Zhongjian, J., & Yong, J. (1990). Flavonoids as superoxide scavengers and antioxidants. *Free Radical Biology and Medicine, 9*, 145–150.

Zhang, D., Yasuda, T., Yu, Y., Zheng, P., Kawabata, T., Ma, Y., & Okada, S. (1996). Ginseng extract scavenges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. *Free Radical Biology and Medicine, 20*, 145–150.