# Evening Primrose Meal: A Source of Natural Antioxidants and Scavenger of Hydrogen Peroxide and Oxygen-Derived Free Radicals

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Evening primrose meal (EPM: 1% and 2%, w/w) reduced ($p \leq 0.05$) the formation of 2-thiobarbituric acid-reactive substances (TBARS), hexanal, and total volatiles in cooked comminuted pork by 43.6−72.6%. Phenolic compounds in the EPM were extracted under optimum conditions (with 56% acetone at 71 °C for 47 min) predicted by a multivariate analysis. The resulting evening primrose crude extract (EPCE) inhibited the bleaching of $\beta$-carotene in a model system. After 2 h of assay, the system containing 200 ppm EPCE [as (+)catechin equivalents] retained 86% of the initial $\beta$-carotene whereas the control retained only 11%. Inhibition of the formation of TBARS, hexanal, and total volatiles in the cooked comminuted pork containing 200 ppm EPCE [as (+)catechin equivalents] ranged from 67.3% to 97.5%. The EPCE inhibited the formation of conjugated dienes, hexanal, and total volatiles in stripped-bulk corn oil (18.5−63.6% inhibition) and stripped-corn oil-in-water emulsion systems (31.7−65.6% inhibition). Hydrogen peroxide ($H_2O_2$), hydroxyl radical ($^{\bullet}OH$), and superoxide radical ($O_2^{\bullet-}$) scavenging properties of the EPCE were comparable to those observed for authentic (+)catechin. At 200 ppm of the EPCE [as (+)catechin equivalents], a 100% quenching of $^{\bullet}OH$ and $O_2^{\bullet-}$ was evident. The EPCE scavenged 44−91% of $H_2O_2$ in the assay medium after 10 min as compared to 7% reduction in the control.

**Keywords:** *Antioxidants; free radicals; evening primrose; lipid oxidation; reactive-oxygen species, ROS; electron paramagnetic resonance, EPR*

## INTRODUCTION

Lipid oxidation is of great concern to the food industry because it leads to the development of undesirable off-flavors and potentially toxic reaction products (Maillard et al., 1996; Shahidi and Wanasundara, 1992). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tertiary*-butylhydroquinone (TBHQ), and propyl gallate (PG) may be added to food products to retard lipid oxidation (Winata and Lorenz, 1996). However, the use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (Hettiarachchy et al., 1996). Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Several sources of natural antioxidants are known, and some of them are currently used in a variety of food products (Metha et al., 1994; Inatani et al., 1982). Extracts of herbs such as rosemary (Bracco et al., 1981), thyme (Inatani et al., 1982), and sage (Pizzocaro et al., 1994), oilseeds such as sesame (Fukuda et al., 1985; Osawa et al., 1985), canola (Wanasundara et al., 1994), flax (Amarowicz et al., 1993; Oomah et al., 1995), soybean (Chen et al., 1995), and peanut (Duh and Yen, 1995), cereals such as rice (Asamari et al., 1996) and barley (Maillard et al., 1996), spices such as clove (Kramer, 1995), mustard (Shahidi et al., 1994), turmeric (Chipault et al., 1995), ginger rhizome (Lee et al., 1986), and fenugreek (Hettiarachchy et al., 1996), and beverages such as black tea (Ruch et al., 1989) and green tea (Amarowicz and Shahidi, 1996; Inoue et al., 1996; Ruch et al., 1989) have been reported to be antioxidative in various model systems. The antioxidant activity of these extracts has always been attributed to their phenolic constituents. For instance, the antioxidants in rosemary extracts have been identified as phenolics such as rosmarinic acid, rosemary diphenol, and rosmanol (Houlihan et al., 1984; Nakatani and Inatani, 1984). Several authors have reported the presence of flavonoids such as catechins in extracts of green and black teas. These phenolic compounds can retard lipid oxidation by donating a hydrogen atom or an electron to chain-initiating free radicals such as the hydroxyl and superoxide radicals (Cao et al., 1997; Silva et al., 1991). They can also neutralize the substrate-derived free radicals such as the fatty acid free radicals and alkoxy radicals (Cao et al., 1997; Packer and Glazer, 1990). Ruch et al. (1989) reported that tea extracts are capable of scavenging reactive-oxygen species (ROS), namely, hydrogen peroxide, superoxide, and hydroxyl radicals. This propertry of plant extracts has an important role in retarding lipid oxidation in food products and living tissues. Incorporation of such extracts in human foods not only preserves the wholesomeness of the food but also reduces the risk to humans of developing artherosclerosis and cancer (Ames, 1983; Namiki, 1990; Ramarathnam et al., 1995). Plant extracts, when added to lipid-containing foods, can also reduce the loss of α-tocopherol (Rice-Evans et al., 1996). Plant phenolics can regenerate α-tocopherol from tocopheryl free radical by donating an electron or a hydrogen atom. Some phenolic compounds present in plant extracts are reported to retard lipid oxidation through chelating transition metal ions such as those of iron, copper, and manganese (Rice-Evans et al., 1996).

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As mentioned earlier, one of the freely available sources of natural antioxidants is the meal of oilseeds. Evening primrose (*Oinothera biennis*), an oilseed crop grown mainly in North America, Europe, and Australia, has earned an important place in the pharmaceutical industry due to the presence of a high amount (9−10%, w/w) of $\gamma$-linolenic acid (GLA) in its seed oil (Gibson et al., 1992; Rahmatulla et al., 1994a,b; Redden et al., 1995). Evening primrose oil has been used for treating several skin disorders (Chapkin and Charmicheal, 1990; Engler et al., 1991, 1992; Huang et al., 1995). The meal after oil removal may retain a substantial amount of phenolic antioxidants (Lu and Foo, 1995) which may be concentrated by employing a proper extraction technique. It may be necessary to evaluate the extract in several model systems using different analytical techniques in order to draw a valid conclusion on its antioxidant efficacy. Several authors have reported that the antioxidant activity of plant phenolics differs greatly according to the physical and chemical properties of the model system in which they are evaluated. Frankel et al. (1996) reported that hydrophilic antioxidants such as Trolox (an α-tocopherol analogue) are more effective in bulk oil systems, whereas hydrophobic antioxidants, such as α-tocopherol, are more effective in oil-in-water emulsions. This phenomenon is due primarily to the differential affinities of the antioxidant compounds for oil−air and oil−water interfaces in bulk oil and oil-in-water emulsions, respectively. This property is greatly affected by the chemical nature of the antioxidant compound involved (Frankel et al., 1996).

One of the important aspects of the extraction of antioxidant compounds from plant materials is the selection of appropriate extraction conditions. It is not advisable to apply the conditions used for one kind of plant material to another because the diverse nature of natural antioxidants makes the generalized extraction conditions inefficient. However, this problem may be overcome by employing the response surface methodology (RSM), a tool used by many researchers to predict optimum experimental conditions to maximize various responses (Gao and Mazza, 1996; Wanasundara and Shahidi, 1996). The objectives of this study were to prepare an extract of evening primrose meal at optimum extraction conditions and to evaluate its antioxidant and ROS scavenging properties in various model systems.

## MATERIALS AND METHODS

**Materials.** Evening primrose seeds were obtained from Efamol, Inc., Kentville, NS, Canada. Seeds were stored in vacuum-packaged polyethylene pouches at −20 °C until used. Fresh pork shoulder meat (1 day after slaughter) was acquired from a local supermarket and most of its surface fat removed. The meat was ground twice in a meat grinder (Omega, Type12, Larry Sommers Ltd., Toronto, ON) using a 0.79 and then a 0.48 cm plate. Ground pork was vacuum-packaged in polyethylene pouches and stored in a freezer (Ultralow, 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, $\beta$-carotene, linoleic acid, Tween 40, butylated hydroxyanisole (BHA), sodium carbonate, mono- and di-basic sodium phosphate, 5,5-dimethyl-1-pyrroline *N*-oxide, nitro blue tetrazolium, hypoxanthine, xanthine oxidase, 2-heptanone, hexanal, (+)catechin, and Folin-Denis reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Hexane, methanol, ethanol, butanol, acetone, chloroform, hydrogen peroxide, ferrous sulfate, 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).

**Preparation of the Meal.** Evening primrose seeds were ground for 15 min and then defatted by blending with hexane (1:5 w/v, 5 min, three times) in a Waring Blendor (Model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. The resulting hexane mixture was then filtered through a Whatman No. 3 filter paper. Defatted seeds were air-dried for 12 h and stored in vacuum-packaged polyethylene pouches at −20 °C until used.

**Preparation of the Crude Extract.** Extraction of phenolic compounds present in evening primrose meal was carried out under reflux conditions in selected solvents in a thermostated water bath. The antioxidant compounds present in the evening primrose meal (6 g) were extracted into aqueous organic solvents (100 mL of 0−100% ethanol, methanol, or acetone) at different extraction temperatures (25−80 °C) and times (10−105 min). The slurry was centrifuged for 5 min at 4000*g* (ICE Centra M5, International Equipment Co., Needham Heights, MA) and the supernatant collected. This procedure was repeated two times, and the 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 $62 \times 10^{-3}$ mbar (Freezone 6, Model 77530, Labconco Co., Kansas City, MO). Extract yield (g/100 g of meal) and total phenolics in the EPCE (mg of catechin eq/g) were determined. The antioxidant index, calculated for EPCE [at 200 ppm total phenolics as (+)catechin equivalents] in a $\beta$-carotene-linoleate model system, was then determined.

The two concentrations (100 and 200 ppm) of the EPCE used in the experiments involving the determination of antioxidant efficacy and ROS scavenging activity were based on the weight of the EPCE required to obtain 100 and 200 ppm total phenolics [as (+)catechin equivalents] in the model system.

***β*-Carotene-Linoleate Model System.** A solution of $\beta$-carotene was prepared by dissolving 2.0 mg of $\beta$-carotene in 10 mL of chloroform. Two milliliters of this solution was pipetted into a 100 mL round-bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40 °C, 40 mg of 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 $\mu$L of the EPCE (in methanol) so that the final concentrations of the phenolics in the assay media were 100 and 200 ppm [as (+)-catechin equivalents]. The total volume of the systems was adjusted to 5.0 mL with methanol. BHA and authentic (+)-catechin 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, Hewlette 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 $\beta$-carotene were prepared for background subtraction (Miller, 1971). Antioxidant index (AI) was calculated using the following equation:

$$\text{AI} = 100(\beta\text{-carotene content after 2 h of assay/initial } \beta\text{-carotene content})$$

**Cooked Comminuted Pork Model System.** Ground pork was mixed with 20 wt % of deionized water in Mason jars (height 10 cm, internal diameter 6 cm). EPM (1% and 2%, w/w) or EPCE [100 and 200 ppm as (+)catechin equivalents] was added directly to meat, cooked in a water bath at 85 ± 2 °C for 40 min, and subsequently cooled to room temperature. 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 (Shahidi and Pegg, 1993). Inhibition of

conjugated dienes, hexanal, and total volatiles was calculated using the following equation:

$$\% \text{ inhibition} = 100 - 100(\text{value for the sample/value for the control})$$

**Bulk Stripped-Corn Oil Model System.** The extract [100 and 200 ppm as (+)catechin equivalents] and oil (5 g) were mixed well in 70 mL capped glass tubes (19 cm × 2 cm internal diameter). Systems were stored in a forced-air convection oven set at 60 °C for 7 days. Samples for conjugated dienes (0.2 g) and headspace (0.2 g) analyses were drawn on days 0, 1, 3, 5, and 7. Authentic (+)catechin was used for comparative purposes. Inhibition of conjugated dienes, hexanal, and total volatiles was calculated using the same equation as the one used for the meat model system.

**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 (19 cm × 2 cm internal diameter) containing 100 or 200 ppm extract [as (+)catechin equivalents] and stored in a forced-air convection oven at 60 °C. Authentic (+)catechin was used for comparative purposes. Aliquots for conjugated dienes (2 mL) and headspace (2 mL) analyses were drawn on days 0, 1, 3, 5, and 7. Oil was extracted with hexane and desolventized under nitrogen. The weight of the retrieved oil was recorded. Inhibition of the formation of conjugated dienes, hexanal, and total volatiles was then calculated.

**Determination of Hydrophilic, Hydrophobic, and Total Phenolic Contents of EPCE.** The EPCE was dissolved in methanol to obtain 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*g*. Absorbance of the supernatant was measured at 725 nm. Blank samples for each extract were used for background subtraction. The total phenolics content in the EPCE was determined using a standard curve prepared for (+)catechin (Swain and Hillis, 1959; Naczk et al., 1992). Total extracted phenolics were expressed as milligrams of (+)catechin equivalents per gram of EPCE. The EPCE (5 g) was fractionated into hydrophilic and hydrophobic components by mixing 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, and 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 determined.

**Determination of Proximate Composition of Meat.** The moisture, protein, and ash content of meat was determined according to the AOAC (1990) methods. The total lipid content was determined using the procedure of Bligh and Dyer (1959).

**Determination of 2-Thiobarbituric Acid-Reactive Substances (TBARS).** Samples, taken on days 0, 1, 3, 5, and 7, were used for TBARS determination according to the method of Siu and Draper (1978) as described by Shahidi and Hong (1991). TBARS values were calculated by multiplying absorbance readings by a factor of 3.4 which was determined from a standard curve prepared for1,1,3,3-tetramethoxypropane, a precursor of malonaldehyde (Wettasinghe and Shahidi, 1996). Inhibition of TBARS on day 7 was determined using the following equation:

$$\% \text{ inhibition} = 100 - 100(\text{TBARS value for the treated sample/TBARS value for the control sample})$$

**Headspace Volatile Analysis.** A Perkin-Elmer 8500 gas chromatograph equipped with an HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, PQ) was used for analysis of volatiles in cooked pork samples. A high-polarity Supelcowax 10 fused silica capillary column (30 m × 0.32 m 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 0.6 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 there for 5 min. The injector and flame ionization detector (FID) temperatures were adjusted to 280 °C and held throughout the analysis (Shahidi and Pegg, 1993; Wettasinghe and Shahidi, 1996).

For headspace (HS) analysis, 4.0 g portions of homogenized pork samples (or 0.2 g of oil from bulk oil/emulsion) 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, we tempered frozen vials at room temperature for 30 min and then preheated them in the HS-6 magazine assembly at 90 °C for a 45 min equilibration. The pressurization time of the vial was 6 s, and the volume of the vapor 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 was accomplished using 2-heptanone as an internal standard (Shahidi and Pegg, 1993; Wettasinghe and Shahidi, 1996). Inhibition of hexanal and total volatiles was calculated using the following equation:

$$\% \text{ inhibition} = 100 - 100(\text{value for the treated sample/value for the control})$$

**Determination of Conjugated Dienes (CD).** Oil (0.02−0.04 g) was weighed into a 25 mL volumetric flask, dissolved in isooctane, and made up to the mark with the same solvent. The solution was thoroughly mixed, and the absorbance was read at 234 nm using a Hewlett-Packard 8452 A diode array spectrophotometer. Pure isooctane was used as the reagent blank. The CD value was calculated using the following equation:

$$\text{CD value} = \text{absorbance at 234 nm}/(\text{concentration of oil in g/100 mL} * \text{length of the cell in cm})$$

**Hydrogen Peroxide Scavenging Assay.** The EPCE [100 and 200 ppm as (+)catechin equivalents] was dissolved in 3.4 mL of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 μL of a 43 mM solution of hydrogen peroxide (prepared in the same buffer). Authentic (+)catechin was used as the reference antioxidant. 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 the assay medium was determined using a standard curve.

**Hydroxyl Radical Scavenging Assay.** 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 (Bruker ESP 300, Bruker Instruments, Inc., Billeria, MA). The EPCE was dissolved in 0.1 M phosphate buffer (pH 7.4) so that a 200 μL aliquot will result in 200 ppm of phenolics in the final assay medium (final volume was 800 μL). For 100 ppm concentration, 100 μL of the same EPCE stock solution was used, but the volume was adjusted to 200 μL by adding 100 μL 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

**Table 1. Variable (Factor) Levels Used in the Response Surface Methodology[a]**

| variable | symbol | coded variable levels −1 | 0 | +1 |
|---|---|---|---|---|
| acetone content in the extraction medium (%, v/v) | $X_1$ | 30 | 50 | 70 |
| extraction temperature (°C) | $X_2$ | 40 | 60 | 80 |
| extraction time (min) | $X_3$ | 30 | 60 | 90 |

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

and transferred into a quartz capillary tube. The spectrum was recorded in the EPR spectrometer set at $2 \times 10^5$ receiver gain, 1.0 G modulation amplitude, 200 s scan time, 3460 G center field, 100 G sweep width, and 0.5 s time constant (Shi et al., 1991). Authentic (+)catechin was used as the reference antioxidant.

**Superoxide Radical Scavenging Assay.** A modified version of the method explained by Nishikimi et al. (1972) was employed. The 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 diethylenetriamine-pentaacetic acid, 1 mL of 178 $\mu$M nitro blue tetrazolium, and 1 mL of the EPCE (final concentration of the phenolics in the reaction mixture was 200 ppm). For 100 ppm concentration, 0.5 mL of the stock EPCE solution was diluted with 0.5 mL of the buffer. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). Authentic (+)catechin was used as the reference antioxidant. The absorbance values (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 subtracting 0 min readings from subsequent readings.

**Response Surface Methodology (RSM).** The experimental design adopted for RSM was a three-factor three-level face-centered cube design with 15 different design points (Snedecor and Cochran, 1980; Mason et al., 1989; Gao and Mazza, 1996). 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). The response (Y, antioxidant index) at each design point was recorded. Duplicate extractions were carried out at all design points except for the center point (0, 0, 0) where triplicate extractions were carried out.

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

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

where $\beta_0$, $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and $x_i$ and $x_j$ are independent variables. Data were analyzed using the general linear model (GLM) and response surface regression (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. Verification experiments were carried out using combinations of variables at different levels (within the experimental range) to determine the validity of the model.

**Tukey's Studentized Range Test.** All other experiments used completely randomized block designs (CRD), and analyses were carried out in triplicate. The significance of differences among mean values was determined at $p \leq 0.05$ using analysis of variance (ANOVA) followed by Tukey's multiple range test (Snedecor and Cochran, 1980).

## RESULTS AND DISCUSSION

**Antioxidant Activity of the Evening Primrose Meal (EPM).** Figure 1 shows the effect of the EPM on lipid oxidation in a cooked comminuted pork (moisture, protein, lipid, and ash contents were 71.8%, 20.2%, 6.9%, and 1.1%, respectively) model system. The oxidative stability of cooked comminuted pork, stored at 4 °C, was assessed using TBARS and hexanal analyses. TBARS test measures the secondary oxidation products, mainly carbonyl compounds such as the malonaldehyde (Tarladgis et al., 1964; Shahidi and Hong, 1991). Hexanal, an oxidation product of $\omega$-6 fatty acids, has been used as an indicator of oxidation in terrestrial animal and plant lipids as it is the dominant aldehyde formed

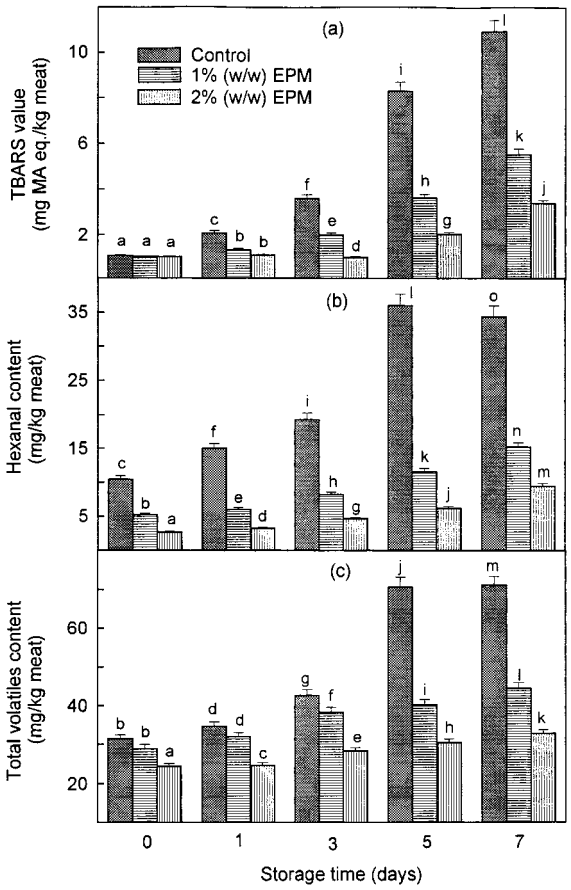


**Figure 1.** Effect of evening primrose meal (EPM, 1 and 2%, w/w) on the formation of TBARS, hexanal, and total volatiles in a cooked lean pork model system. Results are mean values of three determinations ± standard deviation. Bars sharing the same letter in a group of bars are not significantly ($p > 0.05$) different from one another.

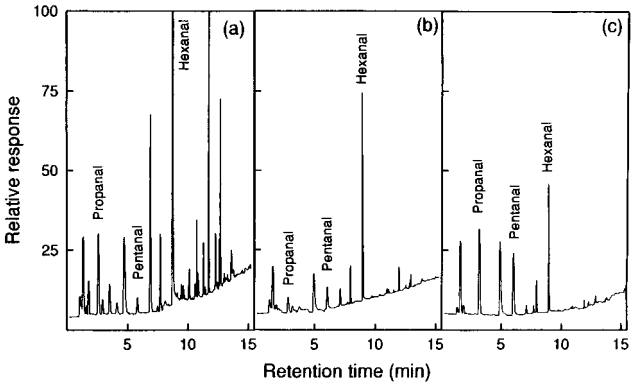


**Figure 2.** GC chromatogram showing the dominance of hexanal among the volatiles formed in a cooked lean pork model system containing no additives (a), 1% meal (b), and 2% meal (c) after 3 days of storage at 4 °C.

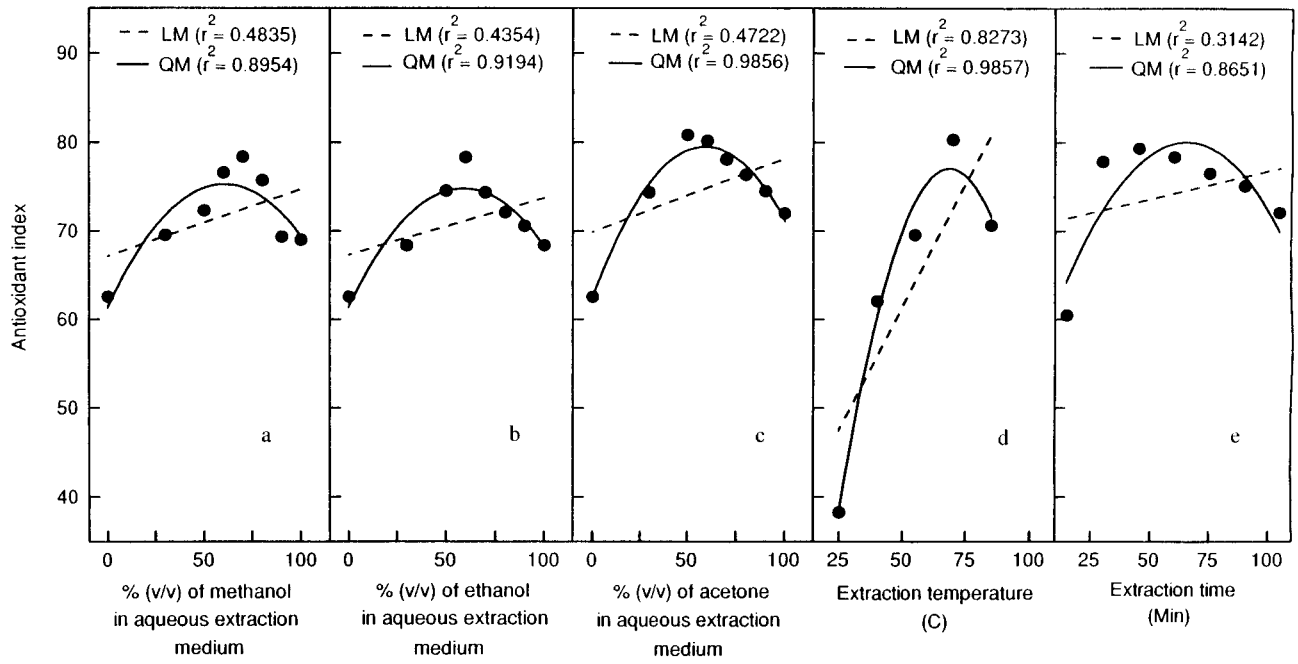


**Figure 3.** Effect of different extraction media (a, methanol; b, ethanol; c, acetone), extraction temperature (d), and time (e) on antioxidant activity of the evening primrose crude extract (EPCE). LM and QM denote linear and quadratic models, respectively.

during oxidation of linoleic acid (Frankel et al., 1989). Figure 2 depicts the dominance of hexanal among volatiles formed in cooked comminuted pork containing EPM. Evening primrose meal, at both levels tested, reduced ($p \leq 0.05$) the formation of TBARS, hexanal, and total volatiles in a concentration-dependent manner (Figure 1). On day 7, EPM at 2% (w/w) reduced ($p > 0.05$) the formation of TBARS, hexanal, and total volatiles by 68.8%, 72.6%, and 62.7%, respectively. These results showed the ability of EPM 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 carried out.

**Selection of an Appropriate Extraction Medium ($X_1$).** Figure 3a−c shows the effect of aqueous methanol, ethanol, and acetone at 0% (solvent−water ratio was 0:100, v/v) to 100% (solvent−water ratio was 100:0, v/v) as extraction media on antioxidant activity of the resulting extracts. 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 (significant at $p \leq 0.01$) than those for the linear models. For all three types of organic solvents used, 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 was acetone and its concentration was 50%. In general, antioxidant activity of the extract prepared with methanol and ethanol was low. Therefore, aqueous acetone was selected as the preferred extraction medium, and 30%, 50%, and 70% acetone were selected as the lower, middle, and upper design points, respectively.

**Selection of 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 (Figure 3d,e). The antioxidant activity of the EPCE prepared at 25 °C was low, but gradually increased with increasing temperature (up to 70 °C) and then started to decline. 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 course were 30, 60, and 90 min.

**Table 2. Face-Centered Cube Design and Observed Responses[a]**

| design point | independent variable[b] $X_1$ | $X_2$ | $X_3$ | response ($Y$)[c] |
|---|---|---|---|---|
| 1 | −1 | −1 | −1 | 65.8 |
| 2 | −1 | −1 | +1 | 60.6 |
| 3 | −1 | +1 | −1 | 77.8 |
| 4 | −1 | +1 | +1 | 68.4 |
| 5 | +1 | −1 | −1 | 55.1 |
| 6 | +1 | −1 | +1 | 58.6 |
| 7 | +1 | +1 | −1 | 65.2 |
| 8 | +1 | +1 | +1 | 64.7 |
| 9 | −1 | 0 | 0 | 80.8 |
| 10 | +1 | 0 | 0 | 62.8 |
| 11 | 0 | −1 | 0 | 72.3 |
| 12 | 0 | +1 | 0 | 74.0 |
| 13 | 0 | 0 | −1 | 68.8 |
| 14 | 0 | 0 | +1 | 76.3 |
| 15 | 0 | 0 | 0 | 79.5 |
| 16 | 0 | 0 | 0 | 78.1 |
| 17 | 0 | 0 | 0 | 76.2 |

[a] Nonrandomized. [b] See Table 1 for uncoded variable levels. $X_1$, acetone content in the extraction medium (%, v/v); $X_2$, Extraction temperature (°C); $X_3$, extraction time (min). [c] Average value of duplicate determinations except for design points 15−17.

**Response Surface Analysis.** After the lower, middle, and upper design points for each variable were determined, they were assigned a code (Table 1) and an experimental design was devised using different factor combinations (Table 2). Table 2 also shows the experimental data observed for response variable $Y$. Regression coefficients of intercept, linear, quadratic, and interaction terms of the model were calculated using least-squares procedures, and their significance was determined using the t-test (Table 3). Among the linear

**Table 3. Estimated Regression Coefficients of the Quadratic Polynomial Model**

| parameter[a] | estimated coefficient | standard error |
|---|---|---|
| intercept | | |
| $\beta_0$ | −24.4791 | 22.9281 |
| linear | | |
| $\beta_1$ | 1.2096 | 0.6300 |
| $\beta_2$ | 1.6981*** | 0.5556 |
| $\beta_3$ | 0.4600 | 0.2774 |
| quadratic | | |
| $\beta_{11}$ | −0.0110** | 0.0043 |
| $\beta_{22}$ | −0.0106** | 0.0043 |
| $\beta_{33}$ | −0.0054** | 0.0019 |
| interaction | | |
| $\beta_{12}$ | −0.0019 | 0.0025 |
| $\beta_{13}$ | −0.0034 | 0.0017 |
| $\beta_{23}$ | −0.0020 | 0.0017 |
| $\beta_{123}$ | | |
| $R^2$ | 0.9943 | |
| $F$ ratio | 13.177 | |
| $p$ value | 0.0013 | |
| CV, % | 4.0405 | |

[a] Coefficients refer to the general model: **, significant at 0.05 level; ***, significant at 0.01 level.

effects, the linear effect of the extraction temperature ($X_2$) was significant. All quadratic effects were also significant, but interaction effects were insignificant. The polynomial model fit to experimental data highly significanty ($p \leq 0.05$), and the coefficient of determination ($R^2$) was 0.9443 and indicated that the most variation observed for design points was explained by the predicted polynomial model. Furthermore, results of the error analysis indicated that the lack of fit was insignificant ($p \geq 0.05$). The coefficient of variation (CV) of less than 5% indicated that the model is reproducible (Mason et al., 1989). The fitted second-order polynomial model was the following:

$$Y = -24.4791 + 1.2096X_1 + 1.6981X_2 + 0.4600X_3 - 0.0019X_1^2 - 0.0106X_2^2 - 0.0054X_3^2 - 0.0019X_1X_2 - 0.0034X_1X_3 - 0.0020X_2X_3$$

The model indicated that the linear effect of the extraction temperature ($X_2$) was greatest 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 order $X_2 > X_1 > X_3$.

Figures 4, 5, and 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 (Box and Hunter, 1957; Mead, 1989). Contour plots (Figures 4, 5, and 6) were also generated using the data obtained by ridge analysis of the response surface. Since all eigen values were negative, the nature of the response at the stationary point was a maximum (antioxidant index, 80.60). Critical values of the three variables were located within the experimental region ($X_1$, 56%; $X_2$, 71 °C; and $X_3$, 47 min). Verification experiments showed that the experimental values were well in agreement with the predicted values (Figure 7). The correlation coefficient between predicted and observed values for the response was 0.9621.

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.

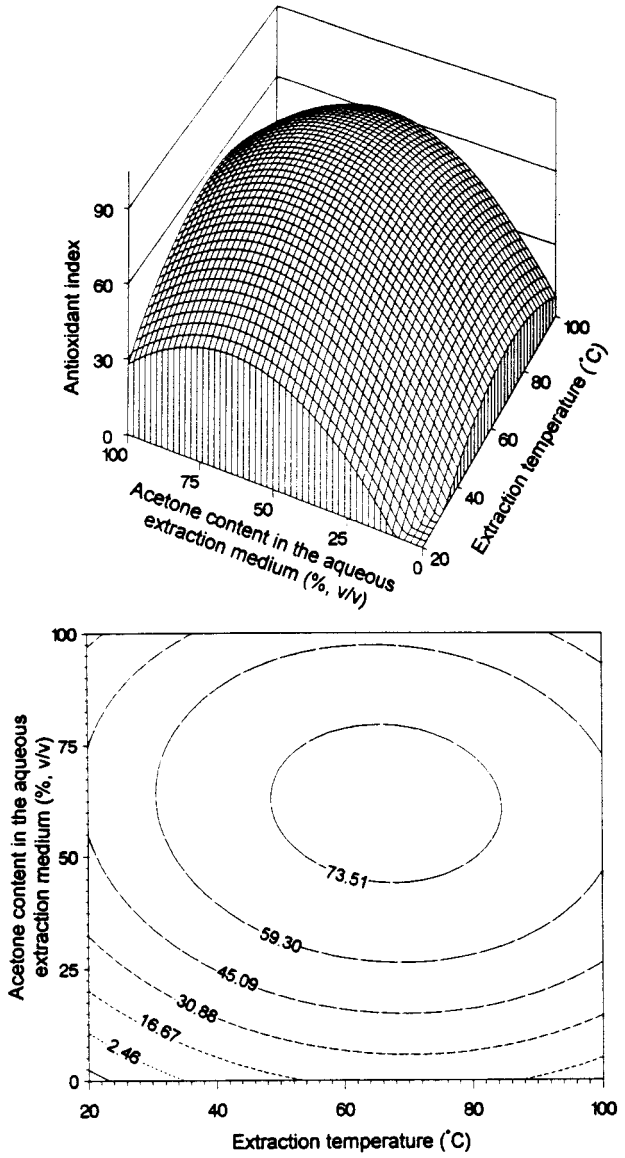


**Figure 4.** Dependence of the antioxidant activity of the evening primrose crude extract (EPCE) on acetone content in the aqueous medium and extraction temperature. Extraction time was maintained at a constant level.

**Evaluation of the Antioxidant Activity of EPCE.** Although a wide range of model systems are available for evaluation of the potency of antioxidant substances, the choice depends mainly upon the chemical nature of their constituents. There is evidence for discrepancies in antioxidant activities of substances when tested in different model systems. For example some hydrophilic antioxidants, that is, Trolox (an analogue of α-tocopherol), were reported to be more effective than their lipophilic counterparts, that is, α-tocopherol, in bulk oil, but less active in oil-in-water emulsions (Huang et al., 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 et al., 1996). Therefore, the extract was evaluated for its antioxidant properties in different model systems using a variety of analytical techniques.

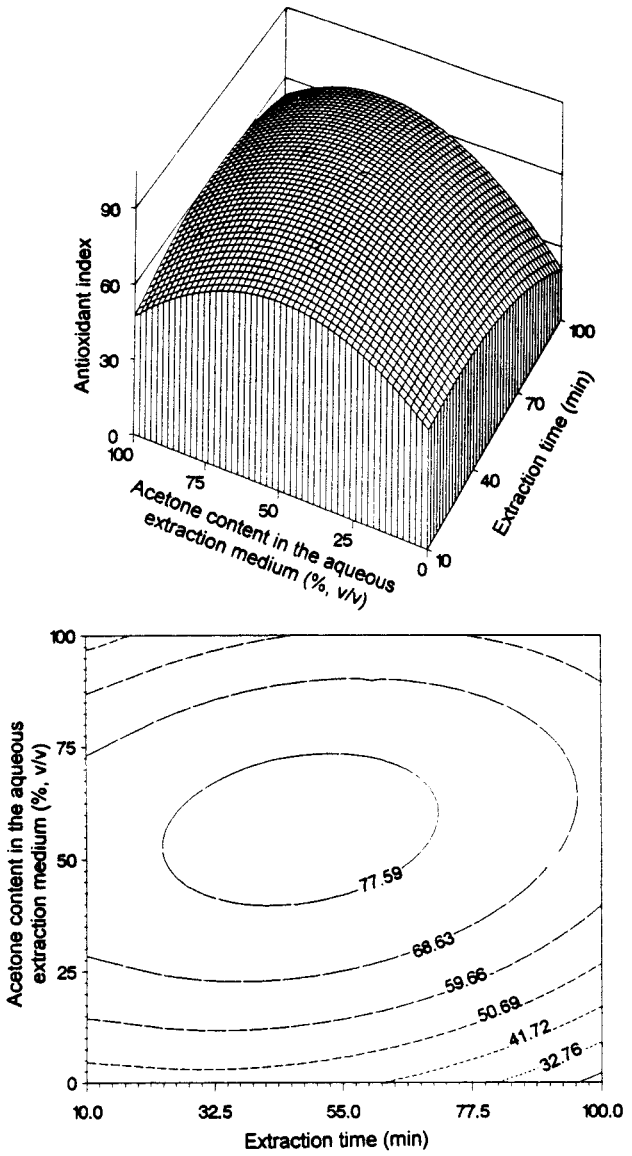


**Figure 5.** Dependence of the antioxidant activity of evening primrose crude extract (EPCE) on acetone content in the aqueous medium and extraction time. Extraction temperature was maintained at a constant level.

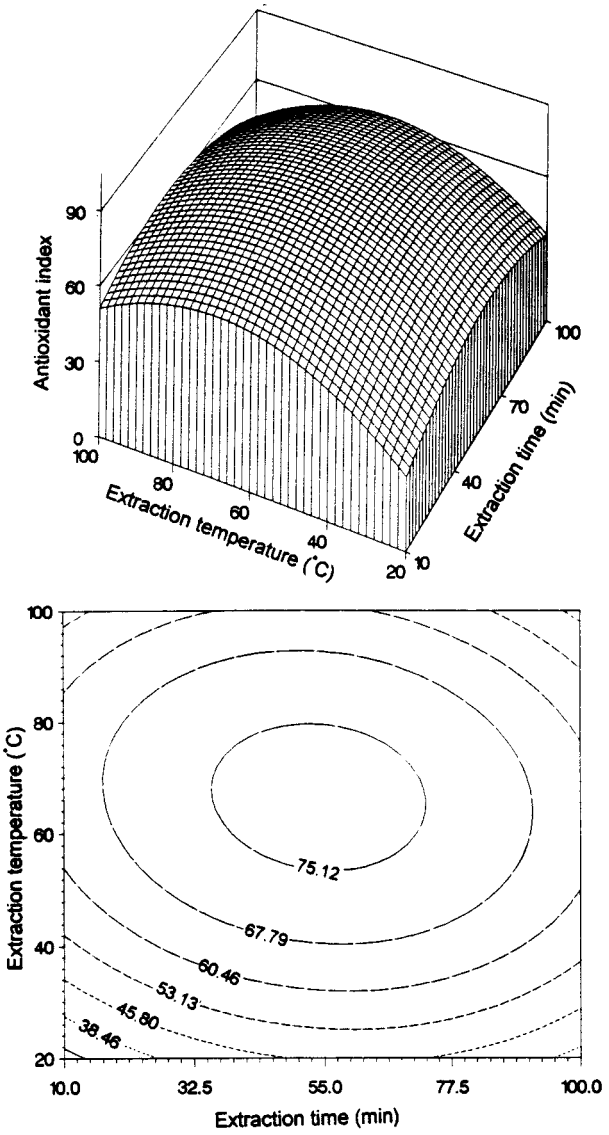


**Figure 6.** Dependence of the antioxidant activity of the evening primrose crude extract (EPCE) on extraction temperature and time. Acetone content in the aqueous extraction medium was maintained at a constant level.

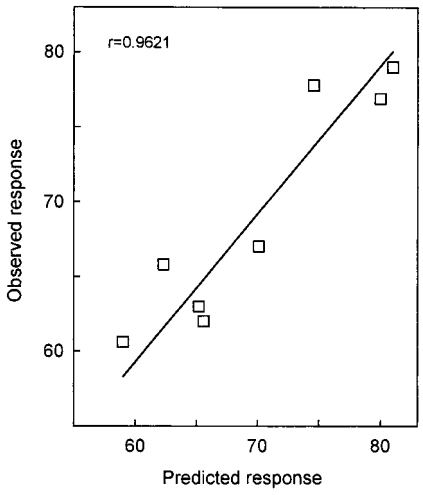


**Figure 7.** Relationship between the predicted and observed response (antioxidant index) for the verification experiments.

**Antioxidant Activity of the EPCE in a β-Carotene-Linoleate System.** The extract prepared at the critical variable combination ($X_1$, 56%; $X_2$, 71°C; and $X_3$, 47 min) contained 304 mg/g of phenolics as (+)catechin equivalents. Its phenolics were composed of 61% (w/w) hydrophilic and 39% (w/w) hydrophobic compounds. Figure 8 shows the effects of the extract at 100 and 200 ppm [as (+)catechin equivalents] on the bleaching of β-carotene in a model system. The EPCE exerted a strong antioxidant activity at both levels tested. The systems containing EPCE retained more than 86% of the initial β-carotene after 2 h, whereas the control retained only 11%. Antioxidant efficacy of the extract in this system was comparable to that of BHA and authentic (+)catechin (Figure 8). β-Carotene in this model system undergoes rapid discoloration in the absence of an 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 diallylic methylene groups attacks the highly unsaturated β-carotene molecule. As β-carotene molecules loose their double bonds, the system loses its characteristic orange color which can be monitored spectrophotometri-

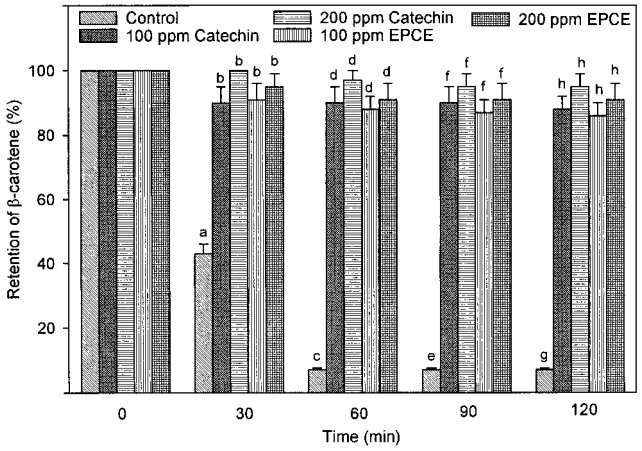


**Figure 8.** Effect of the evening primrose crude extract [EPCE as (+)catechin eq] on $\beta$-carotene bleaching in a $\beta$-carotene-linoleate model system. Results are mean values of three determinations ± standard deviation. Bars sharing the same letter in a group of bars are not significantly ($p > 0.05$) different from one another. After 2 h, the assay medium containing 100 ppm BHA retained >98% of the initial $\beta$-carotene (results not shown).

cally. The presence of a phenolic antioxidant can hinder the extent of $\beta$-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

**Antioxidant Activity of the EPCE in a Cooked Comminuted Pork Model System.** In this model system, EPCE exerted a strong antioxidant effect. Both concentrations of the EPCE resulted in lower TBARS values throughout the entire storage period, and the effect at 200 ppm [as (+)catechin equivalents] was comparable to that of the authentic (+)catechin at the same concentration (Figure 9a); the magnitude of this effect on the inhibition of TBARS formation was the highest. TBARS values of cooked comminuted pork containing 100 ppm EPCE were always higher than those of samples containing catechin at the same concentration. The inhibition of hexanal formation was also dependent upon the concentration of the EPCE in the system (Figure 9b). Total volatiles content of the systems containing EPCE was lower for both concentrations, but the effect was more pronounced at 200 ppm [as (+)catechin equivalents]. On day 7, the effect of authentic (+)catechin at both concentrations on volatile formation was lower ($p \leq 0.05$) than that exerted by the EPCE (Figure 9c).

**Antioxidant Activity of the EPCE in 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 in both bulk and emulsion systems. This facilitated the evaluation of antioxidant substances without the interference by endogenous antioxidants such as tocopherols. As depicted in Figure 10, the EPCE reduced the formation of conjugated dienes, hexanal, and total volatiles in the bulk corn oil. The EPCE was less effective in inhibiting the formation of conjugated dienes as compared to its relatively high inhibition of the formation of hexanal and total volatiles. The effect of the EPCE and authentic (+)catechin on lipid oxidation in the oil-in-water emulsion system is shown in Figure 11. On day 7, the EPCE and authentic (+)catechin inhibited the formation of conjugated dienes and hexanal more effectively in the emulsion system than in the bulk corn oil. The effect of EPCE on total

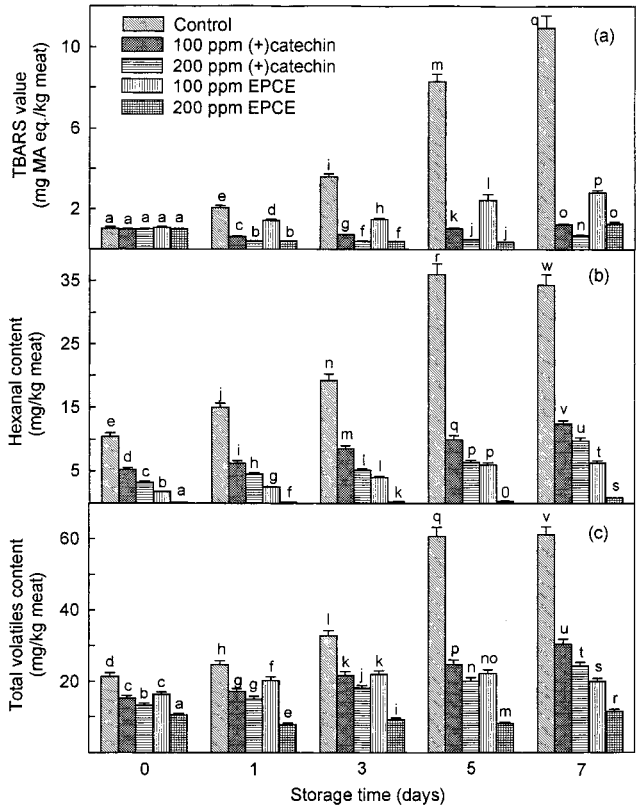


**Figure 9.** Effect of the evening primrose crude extract [EPCE as (+)catechin eq] on the formation of TBARS (a), hexanal (b), and total volatiles (c) in cooked lean pork stored at 4 °C. Results are mean values of three determinations ± standard deviation. Bars sharing the same letter in a group of bars are not significantly ($p > 0.05$) different from one another.

volatiles formation was similar for both systems. These trends may be attributed to the fact that EPCE contained more hydrophilic (61%, w/w) than hydrophobic phenolics (39%, w/w). According to Frankel et al. (1994), the natural antioxidants exert 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, thus 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 their solubility in the aqueous phase, but the hydrophobic antioxidants are oriented in the oil−water interfaces, thus protecting the oil phase from oxidation. Therefore, the EPCE with nearly 40% (w/w) hydrophobic phenolics may effectively retard the oxidation of oil-in-water emulsions. The EPCE also contained 61% (w/w) hydrophilic phenolics which could efficiently orient themselves at the air−oil interface, thus protecting the bulk oil from oxidation. Being a hydrophilic compound, (+)catechin more efficiently protected the bulk oil from oxidation than the emulsion systems and its effects in bulk oil were always superior to those of the EPCE at the same concentration. In emulsion system, authentic (+)catechin exhibited a remarkably high ($p \leq 0.05$) inhibition on the formation of hexanal.

**Hydrogen Peroxide and Hydroxyl Radical Scavenging Activity of the EPCE.** As shown in Figure 12, EPCE exerted a concentration-dependent scavenging of hydrogen peroxide ($H_2O_2$). The concentration of $H_2O_2$ in the systems containing EPCE dropped very sharply

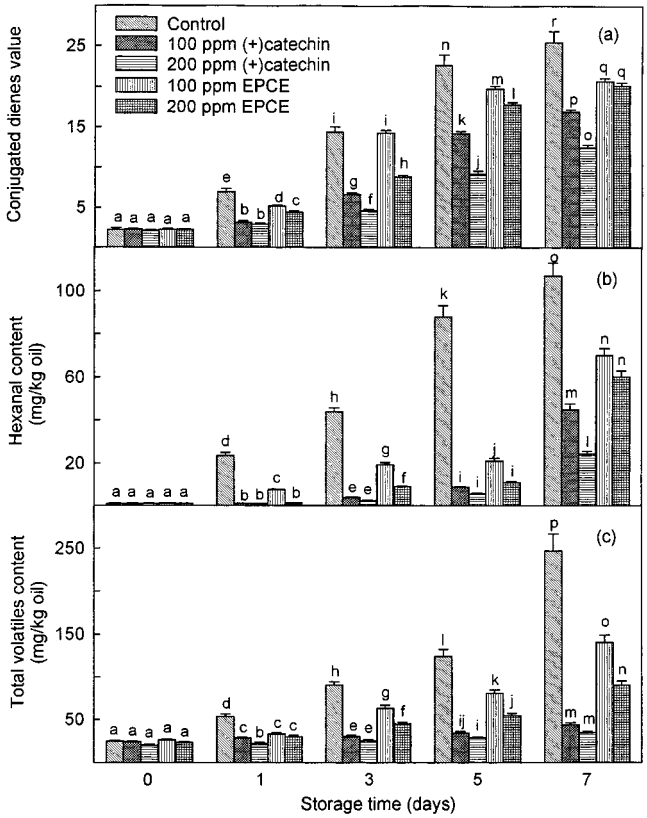


**Figure 10.** Effect of the evening primrose crude extract [EPCE as (+)catechin eq] on the formation of conjugated dienes (a), hexanal (b), and total volatiles (c) in a bulk stripped corn oil system. Results are mean values of three determinations ± standard deviation. Bars sharing the same letter in a group of bars are not significantly ($p > 0.05$) different from one another.

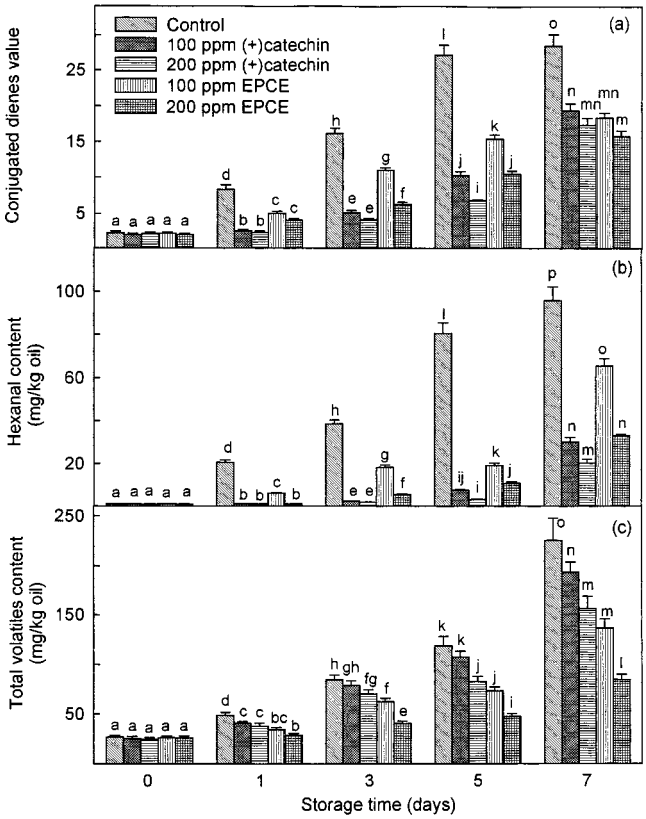


**Figure 11.** Effect of evening primrose crude extract [EPCE as (+)catechin eq] on the formation of conjugated dienes (a), hexanal (b), and total volatiles (c) in a stripped corn oil-in-water emulsion system. Results are mean values of three determinations ± standard deviation. Bars sharing the same letter in a group of bars are not significantly ($p > 0.05$) different from one another.

during the initial 10 min period of the assay. During this period, the reduction ($p \leq 0.05$) in the concentration of hydrogen peroxide in the systems containing 100 and 200 ppm EPCE [as (+)catechin equivalents] was 44% and 91% of the initial amount, 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 EPCE are good electron donors, they may accelerate the conversion of $H_2O_2$ to $H_2O$. However, $H_2O_2$ in the presence of iron ions can generate extremely reactive hydroxyl radicals ($^{\bullet}OH$) (Ruch et al., 1984). This reaction, an iron-catalyzed Haber-Weiss reaction (Pryor, 1966), can be written as

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

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \text{ (Fenton reaction)}$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^- \text{ (Haber-Weiss reaction)}$$

In this study, $^{\bullet}OH$ generated via these reactions was spin-trapped with DMPO, thus forming a DMPO−OH adduct, a relatively stable free radical (Ruch et al., 1984; Halliwell and Gutteridge, 1985). This was done because the detection of $^{\bullet}OH$, as such, is extremely difficult due to its very short lifetime (Ruch et al., 1984). As depicted

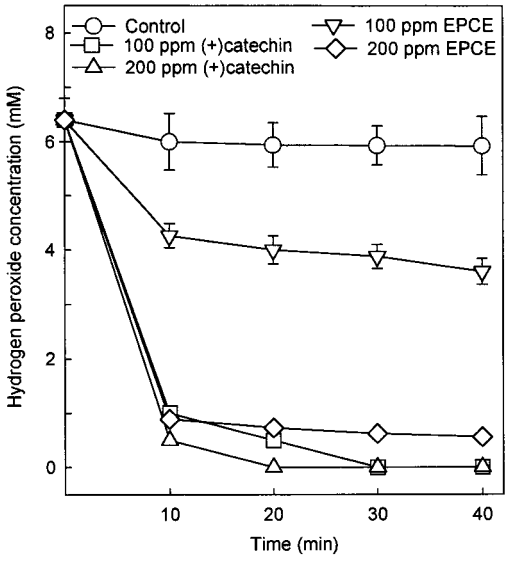


**Figure 12.** Hydrogen peroxide scavenging activity of the evening primrose crude extract [EPCE as (+)catechin eq].

in Figure 13, the DMPO−OH adduct generated a 1:2:2:1 quartet with a hyperfine coupling constant of 14.9 G (Yen and Chen, 1995). The intensity of this signal was reduced, but did not completely disappear, when 100 ppm of the extract [as (+)catechin eq] was present in the assay medium. At a 200 ppm level of the extract [as (+)catechin equivalents], a 100% quenching of the signal was evident. Free radical scavenging properties

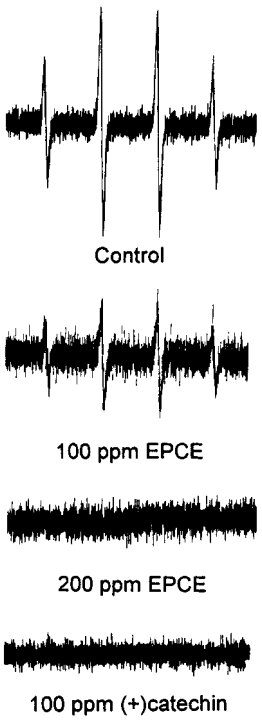


**Figure 13.** EPR spectra showing the effect of the evening primrose crude extract [EPCE as (+)catechin eq] on the scavenging of the hydroxyl radical.

of phenolic compounds found in various plant materials are well-recognized. Shi et al. (1991) demonstrated the ability of caffeine, a phenolic compound, to effectively scavenge the hydroxyl radical (•OH). Yuting et al. (1990) and van Acker et al. (1996) reported that flavonoids such as myricetin, quercetin, and rhamnetin were effective •OH scavengers. They also noted that the effectiveness of such compounds increases with increasing the number of hydroxyl groups attached to the aromatic B-ring of the molecules. 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.

**Superoxide Radical Scavenging Activity of the EPCE.** The superoxide radical, generated in a hypoxanthine/xanthine oxidase system, was readily scavenged by the EPCE. For the control, the characteristic ink blue color of the reduced nitro blue tetrazolium was visible after 10 min and its intensity increased throughout the entire assay period indicating the generation of the superoxide radical (Figure 14). The assay medium containing 200 ppm EPCE [as (+)catechin equivalents] did not develop ink blue color during the entire assay period. This is indicative of the complete quenching of the $O_2^{\bullet -}$ by EPCE. The ink blue color was developed in the medium containing 100 ppm EPCE [as (+)catechin equivalents] after 10 min into the assay, but its intensity was much lower than that of the control.

## CONCLUSIONS

Evening primrose meal and its extract possess antioxidant properties which are concentration-dependent. Thus, antioxidant compounds present in evening primrose meal were isolated, and their content was determined under optimized extraction conditions. The antioxidant activity of the extract was at a maximum when

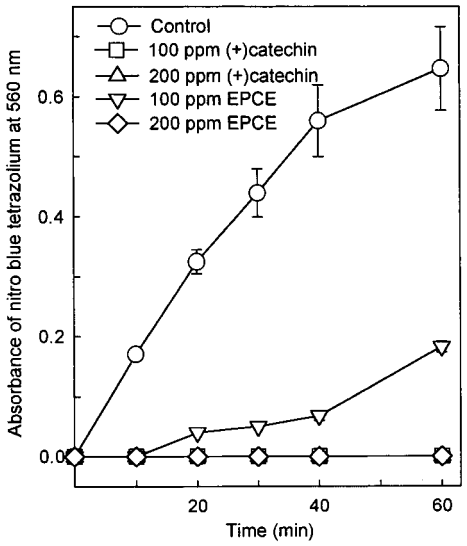


**Figure 14.** Effect of the evening primrose extract [EPCE as (+)catechin eq] on the superoxide radical as exhibited by the intensity of the reduced nitro blue tetrazolium indicator. Note: symbols for (+)catechin and extract are overlapped.

56% acetone at 71 °C for 47 min was used for the extraction. The antioxidant efficacy of plant extracts should be evaluated in a variety of model systems using several indicators because the effectiveness of such antioxidant materials is largely dependent upon the chemical and physical properties of the system to which they are added. Furthermore, a single analytical protocol to monitor oxidation may not be sufficient enough to make a valid judgment, and hence several indicators must be employed. The ability of the crude extract of evening primrose meal to retard lipid oxidation is attributable to the ability of its phenolic constituents to quench reactive oxygen species. The presence of substantial amounts of both hydrophilic and hydrophobic phenolics in the crude extract may make it suitable to be used as an antioxidant mixture in the bulk oil and oil-in-water emulsion systems.

## ABBREVIATIONS USED

TSA, *trans*-sinapic acid; eq, equivalents; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, tertiary butyl hydroquinone; PG, propyl gallate; ROS, reactive oxygen species; DNA, deoxyribonucleic acid; GLA, $\gamma$-linolenic acid; PUFA, polyunsaturated fatty acids; RSM, response surface methodology; TCA, trichloroacetic acid; AI, antioxidant index; TBA, 2-thiobarbituric acid; TBARS, 2-thiobarbituric acid-reactive substances; FID, flame-ionization detector; HS, headspace; GC, gas chromatography; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; CV, coefficient of variance; ANOVA, analysis of variance.

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