JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purple Carrot (*Daucus carota* L.) Polyacetylenes Decrease Lipopolysaccharide-Induced Expression of Inflammatory Proteins in Macrophage and Endothelial Cells

BRANDON T. METZGER,*,†,§ DAVID M. BARNES,§ AND JESS D. REED†

Department of Animal Science, University of Wisconsin—Madison, 1675 Observatory Drive, Madison, Wisconsin 53706, and Standard Process Inc., 1200 West Royal Lee Drive, Palmyra, Wisconsin 53156

Carrots (*Daucus carota* L.) contain phytochemicals including carotenoids, phenolics, polyacetylenes, isocoumarins, and sesquiterpenes. Purple carrots also contain anthocyanins. The anti-inflammatory activity of extracts and phytochemicals from purple carrots was investigated by determining attenuation of the response to lipopolysaccharide (LPS). A bioactive chromatographic fraction (Sephadex LH-20) reduced LPS inflammatory response. There was a dose-dependent reduction in nitric oxide production and mRNA of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) and iNOS in macrophage cells. Protein secretions of IL-6 and TNF-α were reduced 77 and 66% in porcine aortic endothelial cells treated with 6.6 and 13.3 μg/mL of the LH-20 fraction, respectively. Preparative liquid chromatography resulted in a bioactive subfraction enriched in the polyacetylene compounds falcarindiol, falcarindiol 3-acetate, and falcarinol. The polyacetylenes were isolated and reduced nitric oxide production in macrophage cells by as much as 65% without cytotoxicity. These results suggest that polyacetylenes, not anthocyanins, in purple carrots are responsible for anti-inflammatory bioactivity.

KEYWORDS: Polyacetylenes; falcarinol; falcarindiol; falcarindiol 3-acetate; anti-inflammatory; purple carrot; nitric oxide; hormetic.

INTRODUCTION

Carrots were studied almost 80 years ago (*1*) for their provitamin A activity. Carrots are associated with reduced incidence of lung and breast cancer (*2, 3*), improved vitamin A concentrations (*4–6*), modulation of immune function (*7*), and increased serum antioxidants (*8*). Carrots contain different combinations and quantities of macronutrients, fiber, vitamins, minerals, and phytochemicals including carotenoids, phenolics, polyacetylenes, isocoumarins, terpenes, and sesquiterpenes. Carrot varieties differ in color including white, orange, yellow, red, and purple. Carrot phytochemical diversity within and among varietals represents a major challenge to the functional food and dietary supplement industry interested in maximizing health benefits from carrots.

Purple carrots (*Daucus carota* L.) introduced into the U.S. carrot production (*9, 10*) are unique due to the presence of anthocyanins. In addition to conventional nutritional components, purple carrots contain more than 40 phenolic acids (*11*) and 5 predominant anthocyanins as acylated cyanidin glycosides (*12*).

Anthocyanins possess anti-inflammatory bioactivity because enriched fractions reduce chemokine, chemoattractants, and cell adhesion factors in endothelial cells (*13*). Isolated anthocyanins reduce nitric oxide release from macrophages (*14*). Cyanidin-3-glucoside reduces NF-κB activation in endothelial cells (*15*), whereas cyanidin decreases nitric oxide production in macrophages (*14*). Cyanidin-3-glucoside and cyanidin are the major anthocyanin and anthocyanidin, respectively, in purple carrot. In addition to the anthocyanins, purple carrots possess other phytochemicals with anti-inflammatory activity. 2,4,5-Trimethoxybenzaldehyde (gazarin), found in carrot (*16*), significantly inhibits cyclooxygenase II compared to four commercial nonsteroidal anti-inflammatory drugs (*17*). The polyacetylene compounds falcarinol and falcarindiol from *Angelica furcijuga*, also in carrot (*18–20*), inhibit nitric oxide production in mouse peritoneal macrophages. Falcarinol, also known as panaxynol in ginseng, inhibits lipoxygenase enzyme activity (IC_{50}) at micromolar concentrations (*21*).

We chose to study purple carrot as a food source with potential anti-inflammatory bioactivity due to the unique presence of anthocyanins and other reported phytonutrients. We measured nitric oxide production in addition to mRNA and inflammatory proteins in lipopolysaccharide (LPS)-induced macrophage and endothelial cells. We hypothesized that anthocyanins in purple carrot were primarily responsible for anti-inflammatory bioactivity.

* Author to whom correspondence should be addressed [e-mail bmetzger@standardprocess.com; telephone (262) 495-6442; fax (262) 495-6399].

† University of Wisconsin—Madison.

§ Standard Process Inc.

10.1021/jf073494t CCC: $40.75
Published on Web 04/24/2008

MATERIALS AND METHODS

Carrot Extraction. Purple carrot seed was provided by the University of Wisconsin—Madison, USDA-ARS Vegetable Crops Research Unit, Department of Horticulture. Carrots were grown in experimental raised beds (Standard Process Inc., Palmyra, WI), harvested in October 2006, ground using a Cuisinart (East Windsor, NJ), and freeze-dried (Virtis, Gardiner, NY). Freeze-dried carrot was ground to a fine powder with a Foss mill (Eden Prarie, MN). Purple carrot extracts containing anthocyanins were prepared with 7 g of dried carrot powder stirred for 2 h in the dark in 90 mL of acidified water (1% trifluoroacetic acid, TFA) or methanol (1% TFA). The slurry was centrifuged at 3480*g* for 10 min, and the supernatants were pooled after a further extraction with 45 mL of acidified water or methanol for 30 min. The aqueous (extract 1) and methanol extracts (extract 2) were applied to an activated 500 mg C18 solid phase extraction (SPE) cartridge (Phenomenex, Torrance, CA). The SPE cartridge was washed with 25 mL of 0.1% TFA and the sample eluted with 5 mL of acidified methanol (0.1% TFA) and volumetrically brought to 10 mL.

The methanol extract as described above (extract 2) was fractionated by solid phase chromatography on Sephadex LH-20 (GE Healthcare, Piscataway, NJ). Briefly, the supernatant of the methanol extract was dried at <35 °C under vacuum by rotary evaporation (IKA, Wilmington, NC). The dried residue was resuspended in 20 mL of 0.1% TFA and applied to an open 2.5 × 20 cm glass chromatography column filled with 10 g of Sephadex LH-20 (GE Healthcare). The column was washed with 200 mL of 0.1% TFA and the sample eluted with 200 mL of acidified methanol (0.1% TFA). The collected eluant was dried by rotary evaporation to near dryness and brought to 25 mL with 0.1% TFA and applied to an activated 500 mg C18 solid phase extraction (SPE) cartridge (Phenomenex). The SPE cartridge was washed with 25 mL of 0.1% TFA and the sample eluted with 5 mL of acidified methanol (0.1% TFA) and volumetrically brought to 10 mL (fraction 1). Fraction 1 was 0.38% (w/w) of freeze-dried.

An anthocyanin-enriched fraction derived from purple carrot extract 1 was prepared by C18 SPE (fraction 2) according to the methods of Kammerer et al. (*11*).

Chromatographic Analysis. HPLC of purple carrot fraction 1 was performed on an Agilent 1100 series (Santa Clara, CA) system equipped with a quaternary pump, temperature-controlled autosampler, and column compartment, diode array detector, and Agilent Chemstation LC 3D software. An Eclipse XDB-C18 column (3 × 250 mm, 5μm, Agilent) was used to separate anthocyanins. The anthocyanins were separated by a step gradient consisting of 0.1% TFA (A) and methanol (B). The step gradient conditions were as follows: 0−5 min, hold at 20% B; 10 min, 40% B; 10−20 min, hold at 40% B; 25 min, 60% B; 25−35 min, hold at 60% B; 40 min, 80% B; 40−50 min, hold at 80% B; 52 min, 100% B; 52−57 min, hold at 100% B; 60 min, 20% B. The flow rate was 1 mL/min, and the injection volume was 60 μL. The detection wavelengths were 205, 280, and 520 nm.

HPLC analysis of polyacetylenes in purple carrot fraction 1 was performed by HPLC as previously described (*22*). The polyacetylenes falcarinol, falcarindiol, and falcarindiol 3-acetate were identified by characteristic UV spectral data and retention time. Peaks were isolated by preparative HPLC and verified by GC-MS according to the methods of Czepa and Hofmann (*16*).

Preparative HPLC (prep-LC) was performed on an Agilent 1200 system equipped with an automated fraction collector and controlled with Agilent ChemStation software. Samples were injected on an Agilent Eclipse XDB-C18 Prep HT (21.2 × 250 mm, 7 μm) column. The flow rate was 49 mL/min using the step gradient described above for analysis of purple carrot anthocyanins and 1.8 mL injection volume. The detection wavelengths were 205, 280, and 520 nm. Isolation of polyacetylene standards was performed by prep-LC, dried under vacuum, protected from light, and weighed on an analytical balance.

Quantitation of polyacetylenes in purple carrot fraction 1 and freeze-dried purple carrot was performed by GC-MS. Briefly, 1 g of freeze-dried material was extracted into 10 mL of dichloromethane with sonication for 10 min followed by a 10 and 5 mL subsequent extraction. After centrifugation at 4500*g*, the supernatants were pooled and dried under vacuum. The dried pellet was reconstituted in 0.75 mL of dichloromethane, and 1 μL was injected onto the GC (*16*). Quantitative determination of the polyacetylenes in freeze-dried carrot and fraction 1 with external standards indicated 199.0 ± 6.9, 1216.5 ± 24.7, and 200.0 ± 25.7 μg/g and 516.0, 2520.7, and 309.0 μM falcarinol, falcarindiol, and falcarindiol 3-acetate, respectively.

Folin−Ciocalteu Assay. Sample total phenolics were estimated by the Folin−Ciocalteu assay with gallic acid as a standard according to the method of Singleton and Rossi (*23*) and expressed as gallic acid equivalents (GAE).

RAW 264.7 Macrophages. The mouse macrophage cell line (RAW 264.7, ATCC TIB-71, Manassas, VA) was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Gaithersburg, MD). Cells were grown in a humidified incubator at 37 °C under 5% CO_2 and used after reaching confluence and prior to passage 25.

Nitric Oxide (NO) Production. Macrophages were cultured in 24-well plates (BD Falcon, Franklin Lakes, NJ) and incubated overnight (16 h) with or without purple carrot extracts or purple carrot fractions dried and reconstituted in the presence of 100 ng/mL LPS (Sigma, L6529) in high-glucose, phenol red free DMEM (Mediatech) containing 0.5% FBS, 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultured cells with or without LPS served as a positive or negative control, respectively. The production of NO was determined by measuring nitrite in culture medium by the Griess reaction (*24*). Briefly, 100 μL aliquots of medium were incubated with an equal volume of modified Griess reagent (Sigma Aldrich, St. Louis, MO). After 15 min, the absorbance was measured at 540 nm using a microplate spectrophotometer (BioTek, Winooski, VT). Appropriate blanks were run by combining equal volumes of treatment medium with water. Nitrite concentrations were determined on the basis of a standard curve generated with $NaNO_2$.

Cytotoxicity Assay. Cytotoxicity was assessed with the tetrazolium salt MTT (Promega, Madison, WI) as described. Cells were treated overnight with purple carrot extract or fractions followed by removal of the treatment medium and washed once with phenol red free DMEM followed by replacement with fresh medium containing the unreduced tetrazolium salt. After a 3 h incubation period, the cells were solubilized, and the absorbance of the reduced formazon dye was determined at 570 nm. Cytotoxicity was expressed relative to the positive control containing LPS.

Porcine Aortic Endothelial Cells (PAECs). PAECs were isolated from fresh swine aortas. Aortas were washed three times in 200 mL of sterile cold PBS containing 300 units/mL penicillin, 300 μg/mL streptomycin, 0.15 mg/mL gentamycin (Invitrogen), 2.25 ng/mL amphotercin B (Gibco), and 10 units/mL heparin (Elkins-Sinn, Cherry Hill, NJ) on ice. Cells were isolated in a laminar flow hood by scraping aortic arch sections with a scalpel blade. Cells were dispersed in 50 mL of M199 medium (Cambrex Bioscience, Walkersville, MD) containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL of streptomycin and plated onto eight 60 mm tissue culture dishes (BD Biosciences, San Jose, CA) coated with fibronectin (US Biological, Swampscott, MA) cultured in a 37 °C incubator under 5% CO_2. Plates were screened and selected for use after confluence and only when the morphological characteristics adhered to >95% of a cobblestone appearance (*25*).

Measurement of Secreted IL6 and TNF-α Protein Concentrations. Porcine IL-6 and TNF-α enzyme-linked immunosorbent assays (ELISA) from R&D Systems, Inc. (Minneapolis, MN) were performed according to the manufacturer's recommendations. Briefly, PAECs were plated in 24-well tissue culture plates and grown to confluence. Cells were incubated with purple carrot fraction 1 dried and reconstituted in phenol red free DMEM overnight (16 h) concurrently with 100 ng/mL LPS before passage 3. At the end of incubation, cell culture supernatants were immediately frozen at −80 °C and assayed for IL-6 and TNF-α.

Real-Time RT-PCR. Macrophages were plated in six-well plates. Total RNA was collected using the RNAqueous-4PCR kit (Ambion, Austin, TX). RNA concentrations were determined via a spectrophotometer (NanoDrop, Wilmington, DE) and used to generate cDNA using 2 μg of total RNA per the manufacturer's recom-

mendations (High Capacity cDNA kit, Applied Biosystems, Foster City, CA). TaqMan primer/probe sequences for iNOS (Mm0044-0485_m1), IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1), TNF-α (Mm00443258_m1), and eukaryotic18s rRNA (Hs9999-9901_s1) for real-time PCR were from Applied Biosystems. PCR was performed on an ABI 7300 Sequence Detection System. Each 25 μL reaction contained 5 μL of cDNA (diluted 1:20), 1× Taqman Universal PCR Mastermix (Applied Biosystems), and 1.25 μL of the primer/probe mixture. All 18s rRNA reactions were performed with 1.25 μL of the primer/probe diluted 1:6. Reaction parameters were as follows: 2 min at 50 °C then 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fold changes between treatment groups were determined using reaction efficiency (*26*): ratio = $(E_{target})^{\Delta CP}$target-(control−sample)/$(E_{ref})^{\Delta CP}$ref(control−sample). Each treatment was represented by two experiments done in triplicate, and each PCR reaction was carried out in duplicate.

Statistical Analysis. Data are expressed as means ± SEM. The statistical difference between treatments was determined by analysis in GraphPad Prism (San Diego, CA) by one-way analysis of variance (ANOVA) and Dunnett's multiple-comparison test with LPS serving as the control and $P < 0.05$ considered to be significant.

RESULTS

Purple Carrot Fraction 1 Reduces Nitric Oxide Dose Responsively in Macrophages. Macrophage cells were treated with purple carrot extracts 1 and 2 and fractions 1 and 2 standardized to total phenolics (μg of GAE). Purple carrot fraction 1 (22.7 ± 0.5 μmol/L, $P < 0.05$) reduced nitric oxide compared to LPS (29.8 ± 2.0), and purple carrot extracts 1 and 2 (29.7 ± 2.5 and 29.8 ± 1.7 μmol/L, respectively) with 2.5 μg/mL GAE (data not shown). A fraction enriched in anthocyanins (fraction 2) failed to reduce nitric oxide production in macrophages treated with 144 μg/mL GAE.

Nitric oxide production was reduced in macrophages treated dose dependently (**Figure 1A**) with ≥2.6 μg/mL purple carrot fraction 1, equivalent to ≥0.64 μg/mL GAE, or ≥0.6 mg/mL of freeze-dried carrot (**Table 1**). The concentration of nitrite was reduced 94 ± 0.8% with 41.0 μg/mL of fraction 1, whereas viability was not compromised up to 204.8 μg/mL fraction 1 (**Figure 1B**).

Purple Carrot Fraction 1 Reduces mRNA Expression of Inflammatory Proteins (iNOS, IL-6, IL-1β, TNF-α) in Macrophage Cells. LPS increased iNOS, IL-6, IL-1β, and TNF-α mRNA expression in macrophages 100-, 2000-, 400-, and 9-fold over no LPS treatment, respectively, as expected. Treatment with 6.6 or 13.3 μg/mL purple carrot fraction 1 suppressed inducible nitric oxide synthase mRNA levels 77.6 ± 7.9 and 84.6 ± 5.5% as compared to LPS alone (**Figure 2**). IL-6 expression was significantly reduced only with the 13.3 μg/mL treatment, whereas IL-1β expression was suppressed by both levels of carrot 46.9 ± 9.8 and 61.4 ± 11.0% compared to LPS alone. Purple carrot treatment of macrophages did not significantly modulate TNF-α mRNA expression.

Purple Carrot Fraction 1 Suppresses IL-6 and TNF-α Cytokine Secretion in PAECs. TNF-α and IL-6 protein concentrations in media were increased 3.5- and 5-fold, respectively, in endothelial cells treated with LPS. Concentrations of TNF-α in media of cells treated with 6.6 or 13.3 μg/mL purple carrot fraction 1 were decreased 34.2 ± 9.0 and 50.0 ± 10.4%, respectively, compared to LPS alone. At the same concentrations, purple carrot fraction 1 reduced IL-6 secretion 59.7 ± 3.1 and 22.9 ± 6.0% as compared to LPS alone (**Figure 3**). The MTT assay results indicated no difference in cell viability.

A Bioactive Purple Carrot Subfraction Is Devoid of Anthocyanins. Four subfractions derived from purple carrot fraction 1 were collected by prep-LC (**Figure 4A**). Bioactivity of the collected subfractions was determined in macrophages treated on an equal volume basis. Nitric oxide production was reduced only in cells treated with subfraction 3 from 56.2 ± 1.3 to 42.7 ± 1.9 μmol/L as compared to LPS alone (**Figure**

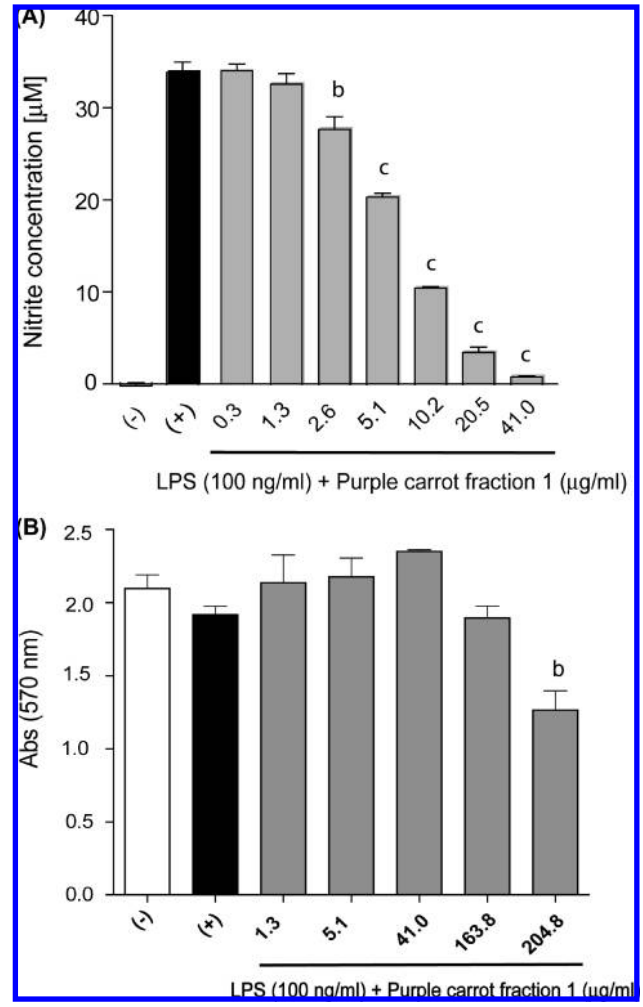

Figure 1. Effect of increasing concentration of purple carrot fraction 1 on LPS-induced nitric oxide production in macrophages. (**A**) Cells were treated overnight (16 h) concurrently with LPS and purple carrot. Nitric oxide production was determined by the Griess reagent, $n = 4$. (**B**) MTT assay results of cell viability ($n = 6$). b, $P < 0.01$, and c, $P < 0.001$, indicate difference from LPS positive control.

Table 1. Purple Carrot Fraction 1 Equivalents

purple carrot fraction 1[a]		
μg of gallic acid equiv/mL[b]	μg/mL	freeze-dried carrot[c] (mg/mL)
0.08	0.3	0.1
0.32	1.3	0.3
0.64	2.6	0.6
1.25	5.1	1.3
2.5	10.2	2.5
5	20.5	5.0
10	41.0	10.1

[a] Contains 2520.7 μM falcarindiol, 309.0 μM falcarindiol 3-acetate, and 516.0 μM falcarinol as determined by GC-MS. [b] Total phenolics determined by Folin−Ciocalteu assay. [c] Contains 1.22 mg/g falcarindiol, 0.20 mg/g falcarindiol 3-acetate, and 0.20 mg/g falcarinol as determined by GC-MS.

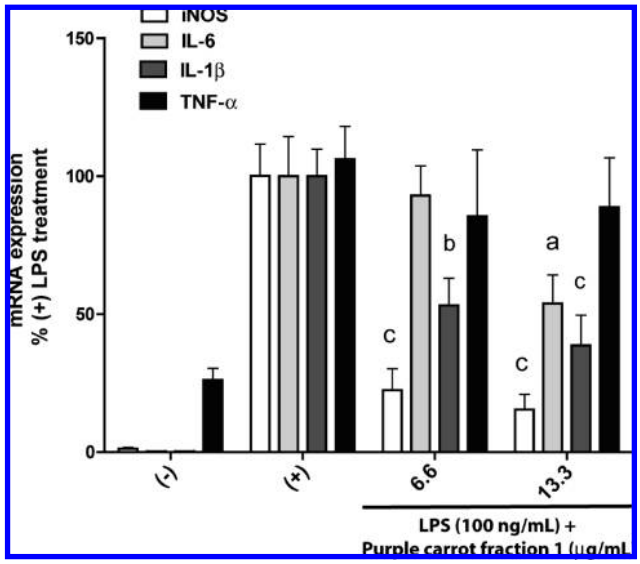

Figure 2. Effect of purple carrot fraction 1 on mRNA expression of inflammatory cytokines and iNOS in macrophages. Cells were incubated concurrently overnight (16 h) with LPS and 6.6 and 13.3 μg/mL purple carrot from LH-20. Real-time RT-PCR was performed ($n = 6$). a, $P < 0.05$; b, $P < 0.01$; and c, $P < 0.001$, indicate difference from LPS positive control.

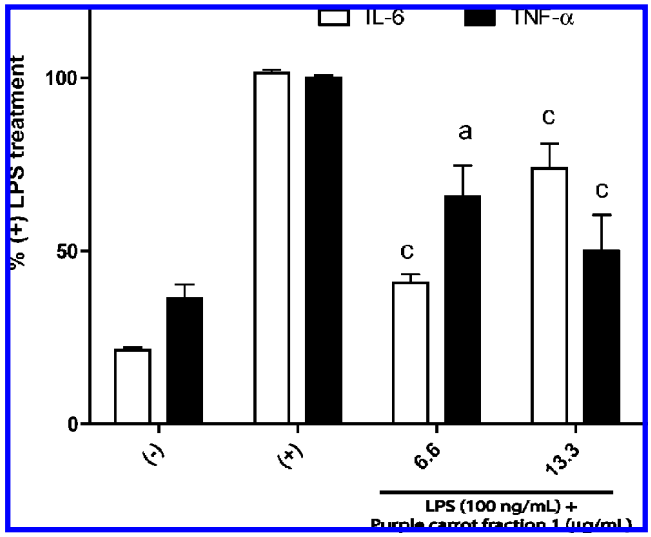

Figure 3. Effect of purple carrot from LH-20 on inflammatory cytokine production in PAECs. PAECs were treated with purple carrot fraction 1 overnight (16 h) concurrently with LPS and an aliquot of medium assayed by ELISA, TNF-α ($n = 4$), IL-6 ($n = 6$). a, $P < 0.05$, and c, $P < 0.001$, indicate difference from LPS positive control.

4B). Peaks with 205 nm absorbance (35−45 min), within subfraction 3, were isolated and reduced LPS-induced nitrite production (data not shown).

Isolated Purple Carrot Polyacetylenes Decrease Nitric Oxide Production in Macrophage Cells. UV spectral maxima of the peaks in prep-LC subfraction 3 (35−45 min, **Figure 4A**) as described previously (*22*) were confirmed to be polyacetylene compounds falcarindiol, falcarindiol 3-acetate, and falcarinol by GC-MS (*16*). Polyacetylenes from purple carrot fraction 1 were collected after elution from prep-LC to yield 1.4, 1.1, and 1.0 mg of falcarindiol, falcarindiol 3-acetate, and falcarinol, respectively. The purity of the collected polyacetylenes was >99.5% for each of the three compounds by HPLC and GC-MS. Macrophages treated with 0.5−20 μmol/L falcarindiol, falcarindiol 3-acetate, and falcarinol reduced nitric oxide production at concentrations of ≥5 μmol/L (**Figure 5**). Falcarindiol was the most bioactive polyacetylene and reduced nitric oxide by 65.3 ± 2.0% at the 10 μmol/L concentration. No cell cytotoxicity was observed with the exception of 20 μmol of falcarindiol/L. Treatment of macrophage cells with equal molar combinations of falcarinol, falcarindiol 3-acetate, and falcarindiol offered no greater reduction in nitric oxide production compared to isolated polyacetylenes at the same concentration (data not shown). The composition of falcarindiol, falcarindiol 3-acetate, and falcarinol in freeze-dried purple carrot was 0.02, 0.01, and 0.06% (w/w), respectively.

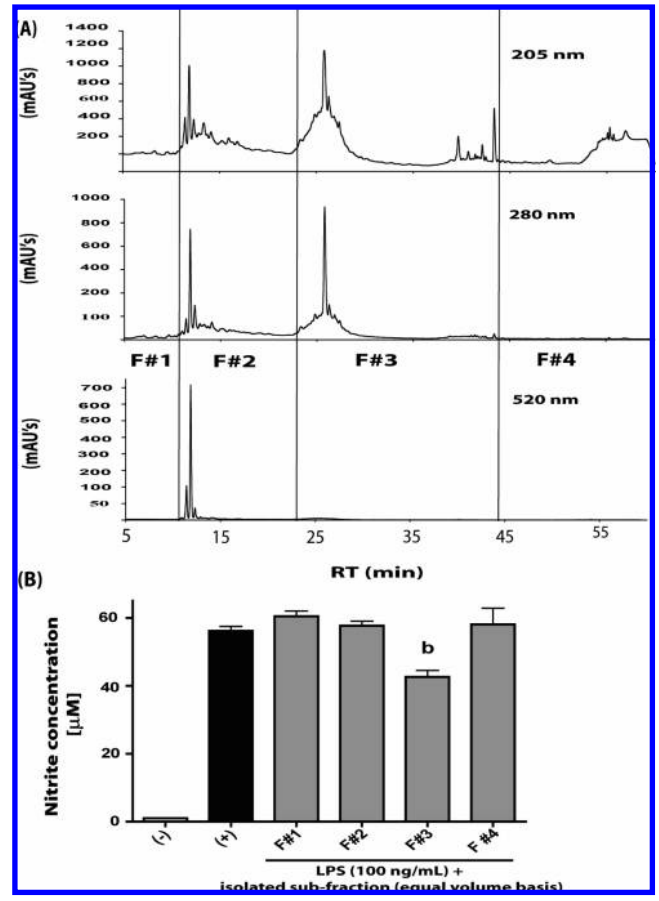

Figure 4. Subfractionation and bioactivity determination of purple carrot fraction 1. (**A**) HPLC chromatogram of purple carrot fraction 1 (205, 280, and 520 nm). Four subfractions were obtained by prep-LC (fractions 1−4). Fraction 1 contained no peaks at the wavelengths monitored. Fraction 2 contained anthocyanins as indicated by 520 and 280 nm absorbance. Fraction 3 contained phenolic acid compounds (280 and 205 nm) and polyacetylenes (205 nm). Fraction 4 contained later eluting peaks with no strong absorbance at the wavelengths monitored. (**B**) Bioactivity of prep-LC subfractions (fractions 1−4) from purple carrot fraction 1 on LPS-induced nitrite production. Volumes collected from each subfraction (fractions 1−4) were reduced to dryness and volumetrically brought to 10 mL with methanol. Macrophages were treated with an equal volume of each subfraction ($n = 4$). b indicates difference from LPS positive control ($P < 0.01$).

DISCUSSION

This study demonstrates that a purple carrot fraction from LH-20 chromatography (fraction 1) reduces LPS inflammatory response in macrophage and endothelial cells. Characterization

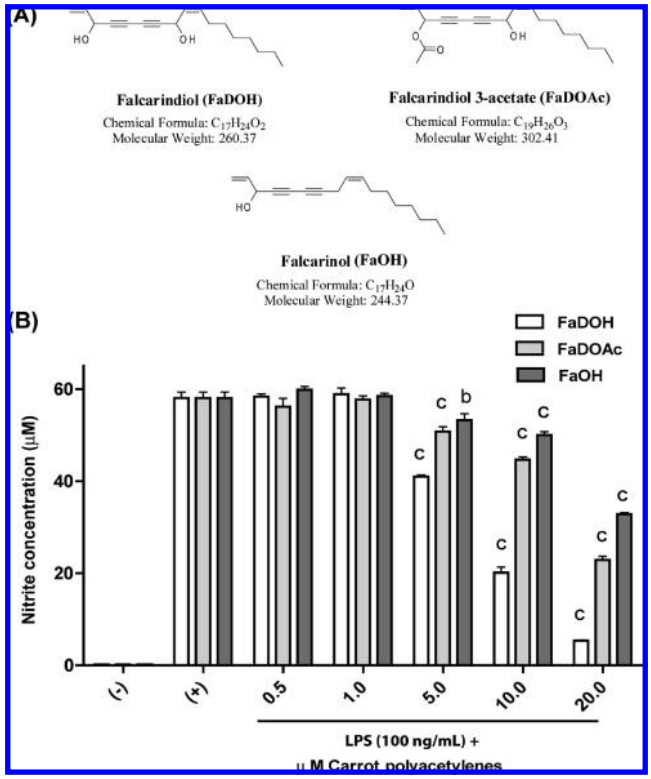

Figure 5. Bioactivity of isolated polyacetylenes on LPS-induced nitric oxide production in macrophages. (**A**) Chemical structures of the polyacetylenes. (**B**) Falcarinol, falcarindiol 3-acetate, and falcarindiol prepared by preparative LC chromatography were >99.5% pure by HPLC and GC-MS prior to treating cells with 0.5−20 μmol of polyacetylene/L ($n = 4$). b, $P < 0.01$, and c, $P < 0.001$, indicate difference from LPS positive control.

of fraction 1 by prep-LC led to the discovery of a subfraction containing polyacetylenes, not anthocyanins, which is responsible for the reduced nitric oxide production in macrophage cells. Furthermore, isolation of crude anthocyanins by SPE (fraction 2) confirmed the lack of bioactivity of anthocyanins up to 144 μg of GAE when compared to fraction 1, which reduced nitric oxide at 2.5 μg of GAE.

Carrots contain the polyacetylene compounds falcarindiol, falcarindiol 3-acetate, and falcarinol (*16, 22, 27*). Bioactivity of polyacetylenes from various food sources indicates anti-inflammatory, antifungal, antibacterial, and reduced platelet aggregation (*18*). Polyacetylenes decrease the development of induced preneoplastic lesions in rat colon (*28*) and reduce LPS-stimulated nitric oxide production in mouse peritoneal macrophages (*29*). Polyacetylenes are active at low concentrations (1 ng/mL) and stimulate cell proliferation, whereas high concentrations inhibit proliferation (*19*), indicative of a hormetic effect with potent bioactive properties.

To our knowledge there are no reports of anti-inflammatory bioactivity of carrot polyacetylenes and no comparative reports of the three polyacetylenes in carrot. This is the first reported bioactivity investigation of purple carrot. Although falcarinol is reported to be the most bioactive cytotoxic polyacetylene (*19, 30*), this study found falcarindiol to be the most active in reducing nitrite production in macrophage cells followed by falcarindiol 3-acetate and falcarinol. Falcarinol stimulates primary epithelial cell proliferation at concentrations between 0.004 and 0.4 μM and is cytotoxic in the same cell type at concentrations of <4 μM (*19*). However, this study found no cytotoxic effect up to 20 μM falcarindiol in macrophages. The cytotoxic threshold of falcarinol is dependent on cell type with ED_{50} values 20 times higher in noncancerous human fibroblast cells than cancerous gastric adenocarcinoma cells (*31*). The concentration of polyacetylenes in carrot range from 20−100 mg/kg of fresh weight (*16, 32*). In this study the concentration of purple carrot polyacetylenes from freeze-dried carrot equaled 0.09% (w/v), equivalent to previously reported levels. However, it should be noted that our extraction methodology was not optimal for a thorough extraction of polyacetylenes. The bioactivity in RAW macrophage cells in this study is comparable to that of a methanolic extract from *Angelica furcijuga* root, which reduces nitric oxide 50% in mouse peritoneal macrophages at concentrations of 4.8 and 4.4 μM falcarinol and falcarindiol, respectively (*33*).

Macrophage TNF-α mRNA levels were reduced, although not significantly, by purple carrot fraction 1, in contrast to reduced TNF-α protein secretion in PAEC. Different effects of purple carrot fraction 1 on TNF-α protein and mRNA may be reflective of different cell types, or a post-transcriptional mechanism that suppresses TNF-α secretion. Similarly, quercetin, a flavonoid with anti-inflammatory bioactivity (*34*), decreases nitric oxide production and TNF-α protein secretion in macrophages while ineffectively modulating TNF-α mRNA levels (*35*). TNF-α secreted from activated macrophages is tightly governed (*36*), and elevated TNF-α levels play a role in cytostatic and cytotoxic activities in malignant cells (*37*); hence, concentration differences may be reflective of the polyacetylenes' antiproliferative properties (*38*).

Time course studies of mRNA expression in LPS-stimulated macrophages indicate differential regulation of cytokines and iNOS. TNF-α, IL-6, and IL-1β mRNA levels peak by 8 h (*39, 40*), whereas iNOS mRNA remains elevated up to 24 h (*41*). Our 16 h experiments may have missed the peak mRNA expression level for some genes; however, we still saw large increases in LPS-stimulated mRNA compared to cells not stimulated with LPS. Macrophage TNF-α and IL-1β protein levels in the media corresponded with mRNA profiles after 16 h of incubation (unpublished data).

Isolated polyacetylenes from purple carrot indicate bioactivity, but a cruder LH-20 preparation (fraction 1) also exhibits bioactivity and contains polyacetylenes, anthocyanins, phenolic acids, and carotenoids. Nitrite production was reduced maximally by 65% with 10 μmol/L falcarindiol with subsequent cytotoxicity at 20 μmol/L; in contrast, macrophage cells treated with purple carrot from LH-20 reduced nitrite by as much as 94% with no cytotoxicity. Polyacetylenes decrease the majority of nitric oxide production in macrophage cells, but other phytochemicals in purple carrot fraction 1 increase bioactivity and decrease cytotoxic effects.

Purple carrot remains unique compared to other carrot varieties due to the presence of anthocyanins, previously characterized (*12, 42–45*), with reported health benefits. The prominent anthocyanin pigment in purple carrot, cyanidin-3-glucoside, increases endothelial nitric oxide synthase (eNOS) expression in bovine aortic endothelial cells, an important factor in modulating blood pressure and endothelial dysfunction (*46*). Purple carrot anthocyanins may also function to reduce oxidative stress because similar acylated anthocyanins from purple cabbage suppress paraquat-induced stress in rats (*47*). In addition, anthocyanins quench peroxyl radicals (*48*), increase serum antioxidant status in rats (*49*), and reduce intracellular reactive oxygen species and lipid peroxidation (*50*). Thus, purple carrot consumption offers several health benefits due to the diverse mixture of phytochemicals present in this unique food.

Experiments from this study indicate the anti-inflammatory bioactivity of polyacetylenes in a concentrated methanol extract from purple carrot derived from LH-20 chromatography. Polyacetylenes in carrot represent an underappreciated class of phytochemicals with demonstrated anti-inflammatory bioactivity important to the field of functional foods and nutritional supplements. Further work is needed to investigate potential synergistic or additive effects of anthocyanins and other phytochemicals in purple carrot fraction 1 with polyacetylenes at concentrations not studied here, as well as a thorough comparison of other carrot varietals that also possess polyacetylenes. Polyacetylenes, such as those in purple carrot, should be considered when potential bioactive phytochemicals are investigated in other foods/herbs, which may often be overlooked due to their low UV absorbance.

ACKNOWLEDGMENT

We thank the Standard Process farm staff including Christine Mason and Darren Poulson for their assistance in cultivating purple carrots in addition to Phil Simon from the University of Wisconsin, ARS, for providing carrot seed.

LITERATURE CITED

(1) Moore, T. Vitamin A and carotene: the association of vitamin A activity with carotene in the carrot root. *Biochem. J.* **1929**, *23*, 803–811.

(2) Pisani, P.; Berrino, F.; Macaluso, M.; Pastorino, U.; Crosignani, P.; Baldasseroni, A. Carrots, green vegetables and lung cancer: a case-control study. *Int. J. Epidemiol.* **1986**, *15*, 463–468.

(3) Longnecker, M. P.; Newcomb, P. A.; Mittendorf, R.; Greenberg, E. R.; Willett, W. C. Intake of carrots, spinach, and supplements containing vitamin A in relation to risk of breast cancer. *Cancer Epidemiol. Biomarkers Prev.* **1997**, *6*, 887–892.

(4) Ncube, T. N.; Greiner, T.; Malaba, L. C.; Gebre-Medhin, M. Supplementing lactating women with pureed papaya and grated carrots improved vitamin A status in a placebo-controlled trial. *J. Nutr.* **2001**, *131*, 1497–1502.

(5) Tang, G.; Qin, J.; Dolnikowski, G. G.; Russell, R. M.; Grusak, M. A. Spinach or carrots can supply significant amounts of vitamin A as assessed by feeding with intrinsically deuterated vegetables. *Am. J. Clin. Nutr.* **2005**, *82*, 821–828.

(6) Dosti, M. P.; Mills, J. P.; Simon, P. W.; Tanumihardjo, S. A. Bioavailability of β-carotene (βC) from purple carrots is the same as typical orange carrots while high-βC carrots increase βC stores in Mongolian gerbils (*Meriones unguiculatus*). *Br. J. Nutr.* **2006**, *96*, 258–267.

(7) Watzl, B.; Bub, A.; Briviba, K.; Rechkemmer, G. Supplementation of a low-carotenoid diet with tomato or carrot juice modulates immune functions in healthy men. *Ann. Nutr. Metab.* **2003**, *47*, 255–261.

(8) Garcia-Closas, R.; Berenguer, A.; Jose Tormo, M.; Jose Sanchez, M.; Quiros, J. R.; Navarro, C.; Arnaud, R.; Dorronsoro, M.; Dolores Chirlaque, M.; Barricarte, A.; Ardanaz, E.; Amiano, P.; Martinez, C.; Agudo, A.; Gonzalez, C. A. Dietary sources of vitamin C, vitamin E and specific carotenoids in Spain. *Br. J. Nutr.* **2004**, *91*, 1005–1011.

(9) Simon, P. W. B7262, purple carrot inbred. *HortScience* **1997**, *32*, 146–147.

(10) Simon, P. W. Plant pigments for color and nutrition. *HortScience* **1997**, *32*, 12–13.

(11) Kammerer, D.; Carle, R.; Schieber, A. Characterization of phenolic acids in black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1331–1340.

(12) Kurilich, A. C.; Clevidence, B. A.; Britz, S. J.; Simon, P. W.; Novotny, J. A. Plasma and urine responses are lower for acylated vs nonacylated anthocyanins from raw and cooked purple carrots. *J. Agric. Food Chem.* **2005**, *53*, 6537–6542.

(13) Youdim, K. A.; McDonald, J.; Kalt, W.; Joseph, J. A. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. *J. Nutr. Biochem.* **2002**, *13*, 282–288.

(14) Wang, J.; Mazza, G. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-γ-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 4183–4189.

(15) Xia, M.; Ling, W.; Zhu, H.; Wang, Q.; Ma, J.; Hou, M.; Tang, Z.; Li, L.; Ye, Q. Anthocyanin prevents CD40-activated proinflammatory signaling in endothelial cells by regulating cholesterol distribution. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 519–524.

(16) Czepa, A.; Hofmann, T. Structural and sensory characterization of compounds contributing to the bitter off-taste of carrots (*Daucus carota* L.) and carrot puree. *J. Agric. Food Chem.* **2003**, *51*, 3865–3873.

(17) Momin, R. A.; De Witt, D. L.; Nair, M. G. Inhibition of cyclooxygenase (COX) enzymes by compounds from *Daucus carota* L. seeds. *Phytother. Res.* **2003**, *17*, 976–979.

(18) Christensen, L. P.; Brandt, K. Bioactive polyacetylenes in food plants of the Apiaceae family: occurrence, bioactivity and analysis. *J. Pharm. Biomed. Anal.* **2006**, *41*, 683–693.

(19) Hansen, S. L.; Purup, S.; Christensen, L. P. Bioactivity of falcarinol and the influence of processing and storage on its content in carrots (*Daucus carota* L.). *J. Sci. Food Agric.* **2003**, *83*, 1010–1017.

(20) Baranska, M.; Schulz, H.; Baranski, R.; Nothnagel, T.; Christensen, L. P. In situ simultaneous analysis of polyacetylenes, carotenoids and polysaccharides in carrot roots. *J. Agric. Food Chem.* **2005**, *53*, 6565–6571.

(21) Alanko, J.; Kurahashi, Y.; Yoshimoto, T.; Yamamoto, S.; Baba, K. Panaxynol, a polyacetylene compound isolated from oriental medicines inhibits mammalian lipoxygenases. *Biochem. Pharmacol.* **1994**, *48*, 1979–1981.

(22) Christensen, L. P.; Kreutzmann, S. Determination of polyacetylenes in carrot roots (*Daucus carota* L.) by high-performance liquid chromatography coupled with diode array detection. *J. Sep. Sci.* **2007**, *30*, 483–490.

(23) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

(24) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.* **1982**, *126*, 131–138.

(25) Carrillo, A.; Chamorro, S.; Rodriguez-Gago, M.; Alvarez, B.; Molina, M. J.; Rodriguez-Barbosa, J. I.; Sanchez, A.; Ramirez, P.; Munoz, A.; Dominguez, J.; Parrilla, P.; Yelamos, J. Isolation and characterization of immortalized porcine aortic endothelial cell lines. *Vet. Immunol. Immunopathol.* **2002**, *89*, 91–98.

(26) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45.

(27) Kidmose, U.; Hansen, S. L.; Christensen, L. P.; Edelenbos, M.; Larsen, E.; Norbaek, R. Effects of genotype, root size, storage, and processing on bioactive compounds in organically grown carrots (*Daucus carota* L.). *J. Food Sci.* **2004**, *69*, S388−S394.

(28) Kobaek-Larsen, M.; Christensen, L. P.; Vach, W.; Ritskes-Hoitinga, J.; Brandt, K. Inhibitory effects of feeding with carrots or (−)-falcarinol on development of azoxymethane-induced preneoplastic lesions in the rat colon. *J. Agric. Food Chem.* **2005**, *53*, 1823–1827.

(29) Yoshikawa, M.; Nishida, N.; Ninomiya, K.; Ohgushi, T.; Kubo, M.; Morikawa, T.; Matsuda, H. Inhibitory effects of coumarin and acetylene constituents from the roots of *Angelica furcijuga* on D-galactosamine/lipopolysaccharide-induced liver injury in mice and on nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages. *Bioorg. Med. Chem.* **2006**, *14*, 456–463

(30) Bernart, M. W.; Cardellina, J. H.; Balaschak, M. S.; Alexander, M. R.; Shoemaker, R. H.; Boyd, M. R. Cytotoxic falcarinol

oxylipins from *Dendropanax arboreus*. *J. Nat. Prod.* **1996**, *59*, 748–753.
(31) Matsunaga, H.; Katano, M.; Yamamoto, H.; Fujito, H.; Mori, M.; Takata, K. Cytotoxic activity of polyacetylene compounds in *Panax ginseng* C. A. Meyer. *Chem. Pharm. Bull. (Tokyo)* **1990**, *38*, 3480–3482.
(32) Zidorn, C.; Johrer, K.; Ganzera, M.; Schubert, B.; Sigmund, E. M.; Mader, J.; Greil, R.; Ellmerer, E. P.; Stuppner, H. Polyacetylenes from the Apiaceae vegetables carrot, celery, fennel, parsley, and parsnip and their cytotoxic activities. *J. Agric. Food Chem.* **2005**, *53*, 2518–2523.
(33) Matsuda, H.; Murakami, T.; Kageura, T.; Ninomiya, K.; Toguchida, I.; Nishida, N.; Yoshikawa, M. Hepatoprotective and nitric oxide production inhibitory activities of coumarin and polyacetylene constituents from the roots of *Angelica furcijuga*. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2191–2196.
(34) Ruiz, P. A.; Braune, A.; Holzlwimmer, G.; Quintanilla-Fend, L.; Haller, D. Quercetin inhibits TNF-induced NF-κB transcription factor recruitment to proinflammatory gene promoters in murine intestinal epithelial cells. *J. Nutr.* **2007**, *137*, 1208–1215.
(35) Wadsworth, T. L.; Koop, D. R. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem. Pharmacol.* **1999**, *57*, 941–949.
(36) Beutler, B.; Cerami, A. The biology of cachectin/TNF—a primary mediator of the host response. *Annu. Rev. Immunol.* **1989**, *7*, 625–655.
(37) Camussi, G.; Albano, E.; Tetta, C.; Bussolino, F. The molecular action of tumor necrosis factor-α. *Eur. J. Biochem.* **1991**, *202*, 3–14.
(38) Young, J. F.; Duthie, S. J.; Milne, L.; Christensen, L. P.; Duthie, G. G.; Bestwick, C. S. Biphasic effect of falcarinol on caco-2 cell proliferation, DNA damage, and apoptosis. *J. Agric. Food Chem.* **2007**, *55*, 618–623.
(39) Shin, N. R.; Lee, D. Y.; Shin, S. J.; Kim, K. S.; Yoo, H. S. Regulation of proinflammatory mediator production in RAW264.7 macrophage by *Vibrio vulnificus* luxS and smcR. *FEMS Immunol. Med. Microbiol.* **2004**, *41*, 169–176.
(40) Westra, J.; Doornbos-van der Meer, B.; de Boer, P.; van Leeuwen, M. A.; van Rijswijk, M. H.; Limburg, P. C. Strong inhibition of TNF-α production and inhibition of IL-8 and COX-2 mRNA expression in monocyte-derived macrophages by RWJ 67657, a p38 mitogen-activated protein kinase (MAPK) inhibitor. *Arthritis Res. Ther.* **2004**, *6*, R384–R392.
(41) Srisook, K.; Cha, Y. N. Biphasic induction of heme oxygenase-1 expression in macrophages stimulated with lipopolysaccharide. *Biochem. Pharmacol.* **2004**, *68*, 1709–1720.
(42) Alasalvar, C.; Grigor, J. M.; Zhang, D.; Quantick, P. C.; Shahidi, F. Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. *J. Agric. Food Chem.* **2001**, *49*, 1410–1416.
(43) Glassgen, W. E.; Wray, V.; Strack, D.; Metzger, J. W.; Seitz, H. U. Anthocyanins from cell suspension cultures of *Daucus carota*. *Phytochemistry* **1992**, *31*, 1593–1601.
(44) Kammerer, D.; Carle, R.; Schieber, A. Detection of peonidin and pelargonidin glycosides in black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2407–2412.
(45) Schwarz, M.; Wray, V.; Winterhalter, P. Isolation and identification of novel pyranoanthocyanins from black carrot (*Daucus carota* L.) juice. *J. Agric. Food Chem.* **2004**, *52*, 5095–5101.
(46) Xu, J. W.; Ikeda, K.; Yamori, Y. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. *Hypertension* **2004**, *44*, 217–222.
(47) Igarashi, K.; Kimura, Y.; Takenaka, A. Preventive effects of dietary cabbage acylated anthocyanins on paraquat-induced oxidative stress in rats. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 1600–1607.
(48) Rossetto, M.; Vanzani, P.; Lunelli, M.; Scarpa, M.; Mattivi, F.; Rigo, A. Peroxyl radical trapping activity of anthocyanins and generation of free radical intermediates. *Free Radical Res.* **2007**, *41*, 854–859.
(49) Han, K. H.; Matsumoto, A.; Shimada, K. I.; Sekikawa, M.; Fukushima, M. Effects of anthocyanin-rich purple potato flakes on antioxidant status in F344 rats fed a cholesterol-rich diet. *Br. J. Nutr.* **2007**, 1–8.
(50) Choi, E. H.; Chang, H. J.; Cho, J. Y.; Chun, H. S. Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities. *Food Chem. Toxicol.* **2007**, *45*, 1873–1881.

Received for review November 29, 2007. Revised manuscript received February 7, 2008. Accepted March 5, 2008. Supported by Standard Process, Inc., Palmyra, WI.

JF073494T