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Bioactivity of Antioxidants in Extruded Products Prepared from Purple Potato and Dry Pea Flours

Balunkeswar Nayak,[†,§] Rui Hai Liu,[§] Jose De J Berrios,[#] Juming Tang,[*,†] and Christopher Derito[§]

[†]Department of Biological Systems Engineering, Washington State University, Pullman, Washington 99163, United States

[§]Department of Food Science, Stocking Hall, Cornell University, Ithaca, New York 14853, United States

[#]Processed Foods Research Unit, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710, United States

ABSTRACT: Measuring antioxidant activity using a biologically relevant assay adds important evidence to aid in understanding the role of phytochemicals based on data from in vivo and chemical assays of extrusion processed purple potato and pea flours. A cellular antioxidant activity assay could provide biologically relevant information on bioactive compounds in raw as well as processed food products. The objective of this study was to investigate the complete phytochemical profiles, antioxidant activity, cellular antioxidant activity, and their contribution to bioactivity in purple potato flour, dry pea flour, raw formulations, and extrusion cooked products prepared with the above ingredients. The free fraction of extracts contributed 68, 64, and 88% to total phenolics, total antioxidant activity (ORAC value), and total flavonoids, respectively, in purple potato flour (PPF). Similarly, extracts in the free fraction contributed 87, 86, and 64% to total phenolics, total antioxidant activity (ORAC value), and total flavonoids, respectively, in dry pea flour (DPF). The amount of total phenolics and total flavonoids in purple potato flour and the antioxidant activity of PPF and DPF were comparable to published data. However, a higher amount in the total flavonoids and lower in the total phenolics of DPF were observed. Caffeic, *p*-coumaric, and ferulic acids were mostly observed in the bound extracts of raw formulations as in the extrudates, whereas chlorogenic acid was predominant in the free extracts. The extruded products had significantly higher ($p < 0.05$) content of total phenolics, ORAC antioxidant activity, and flavonoids, compared to the raw formulations. Extrusion processing increased the cellular antioxidant activity of the extrudates prepared from 35:65 and 50:50 PPF/DPF (w/w) of ingredients compared with control raw formulations in a dose-dependent manner. Increase of PPF significantly increased ($p < 0.05$) the cellular antioxidant activity of 35−50% PPF formulations.

KEYWORDS: purple potato flour, dry pea flour, extrusion, phenolics, antioxidant activity, flavonoids, cellular antioxidant activity, bioactivity

INTRODUCTION

Processed fruits and vegetables are generally believed to have less naturally occurring antioxidants than fresh products, resulting in reduced health benefits. One of the major reasons for this belief has been the measurement of antioxidant activity focused solely on vitamin C levels in foods as an indicator for health benefits of processed products.[1−4] However, Eberhardt et al.[5] observed that vitamin C contributed <0.4% to the total antioxidant activity in apples, indicating the importance of other phytochemicals toward total antioxidant activity in foods. Antioxidant compounds derived from fruits and vegetables act to prevent the formation of reactive oxygen, nitrogen, hydroxyl, and lipid species either by scavenging free radicals or by repairing or removing damaged molecules.[6] Bioactive phytochemicals are proposed to prevent chronic diseases such as cardiovascular disease, diabetes, and certain forms of cancers.[7−10] Bioactive phytochemicals exist in free, soluble-conjugated, and bound forms.[11] Bound phytochemicals, mostly in cell wall materials, are difficult to digest in the upper gastrointestine and may be digested by bacteria in the colon to provide health benefits and reduce the risk of colon cancer.[12] Only a small portion of flavonoids absorbed across the intestinal membrane are partly transformed to glucuronides and sulfates; however, the majority of the flavonoids are degraded by intestinal microflora. Several phenolic acids are produced by bacterial enzymes utilizing hydrolysis, dehydroxylation, cleavage of the heterocyclic oxygen-containing ring, and decarboxylation of flavonoids. Reabsorption of these phenolic acids increases the antioxidant protection with their radical scavenging ability.[13]

Many researchers have reviewed and documented the contributions of phenolic compounds including anthocyanins, individual phenolic acids, and carotenoids present in white and colored potato skin and flesh to antioxidant activity.[14−16] Wounding, boiling, baking, drum-drying, freeze-drying, and microwave cooking of white and colored potatoes had different effects on the total phenolic content.[17−19] Potato protein hydrolysate inhibited lipid oxidation of beef patties by scavenging free radicals,[20] and potato peel extracts retarded oxidation in radiation-processed lamb meat.[21]

Legumes such as dry peas are rich sources of protein and dietary fiber. Consumption of legumes helps prevent osteoporosis[22] and certain cancers[23] and reduces body lipid accumulation.[24] Various effects of thermally processed legumes on antioxidant activity, phenolic content, and potential health benefits were reported.[25,26]

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The combination of purple potato and dry pea may produce healthy snack foods incorporating natural color along with positive effects on human health. Limited studies have been done on the effect of processing on formulations of potatoes and legumes or to characterize the phytochemical profiles of their mixes, and their contributions to antioxidant activities. Metabolism, absorption, and bioactivity of health-beneficial phytochemicals could be improved by combining protein and individual phytochemicals. Liu[9] reported that the additive and synergistic effects of biologically active compounds from fruits, vegetables, and whole grains are responsible for health benefits.

The objective of this study was to investigate the complete phytochemical profiles that exist in free and bound forms, and their contribution to the total antioxidant activity in the raw ingredients (purple potato flour and dry pea flour), raw formulations, and extrusion cooked products prepared with the above ingredients. The bioactivity of phytochemicals in the raw and processed samples was also determined using the cellular antioxidant activity assay.[37] The cellular antioxidant activity assay has been used to assess antioxidants, foods, and dietary supplements due to its capability to consider cell uptake, distribution, and efficiency of protection against peroxyl radicals under physiological conditions.

MATERIALS AND METHODS

Chemicals and Reagents. Folin−Ciocalteu reagents, sodium nitrite, catechin, sodium borohydride, chloranil, vanillin, and gallic acid were purchased from Sigma (St. Louis, MO). Sodium hydroxide, hexane, aluminum chloride, and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA), whereas ethyl acetate, trifluoroacetic acid, methanol, hydrochloric acid, acetic acid, acetone, and ethanol were of analytical grade and were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Tetrahydrofuran and aluminum chloride were purchased from Fisher Scientific (Fair Lawn, NJ).

Preparation of Samples. 'Purple Majesty' potatoes (purchased from SLV Research Center, Colorado State University, Fort Collins, CO) were peeled, sliced, blanched, and drum-dried in the Food Processing Pilot Plant of Washington State University. Split dry peas (purchased from Giusto's Specialty Food, San Francisco, CA) and drum-dried potato flakes were pin-milled to produce flours at the Processed Foods Research Unit, Western Regional Research Center, USDA, Albany, CA. Raw formulations were prepared from purple potato flour (PPF) and dry pea flour (DPF) at selected concentrations (35:65, 50:50, and 65:35 PPF/DPF, w/w). Extruded products were prepared from PPF, DPF, and their formulations using a corotating twin-screw extruder (Micro 18, American Leistritz Extruder Corp., Branchburg, NJ) at 45 g/min feed rate, 300 rpm screw speed, 17% (wet basis) feed moisture, and barrel zones set at 80/100/110/120/130 °C (die temperature at 130 °C). The extrudates were milled to flour using a coffee grinder and stored at −80 °C until further use. The flours for routine analysis were stored at −20 °C.

Extraction of Free Phenolic Compounds. Free phenolic compounds in the ingredients, raw formulations, or extruded flours were extracted using the method reported by our research group in previous manuscript.[27,28] Briefly, 1−2 g of flour sample was homogenized with 50 mL of 80% chilled acetone for 10 min using a high-speed homogenizer (Brinkmann model PT 10/35). After centrifugation of homogenized flour at 2500*g* for 5 min, the supernatant was removed and extraction was repeated once more. Supernatants were pooled, evaporated at 45 °C to dryness, and reconstituted with water to a final volume of 10 mL. The extracts were stored at −75 °C until use.

Extraction of Bound Phenolic Compounds. Bound phenolics in the ingredients, raw formulations, or extruded flours were extracted using the method reported by our research group in previous manuscripts.[11,28,29] Briefly, 1−2 g flour samples were extracted twice with 80% chilled acetone with centrifugation at 2500*g* for 5 min, and the supernatant was discarded after each extraction. The residues were digested with 20 mL of 2 M sodium hydroxide at room temperature for 1 h with shaking under nitrogen gas. The mixture was neutralized with an appropriate amount of hydrochloric acid and extracted with hexane to remove lipids. The final solution was extracted five times with ethyl acetate. The ethyl acetate fraction was evaporated to dryness. The resulting residues were reconstituted in 10 mL of water and stored at −75 °C until use.

Determination of Total Phenolics. The total phenolics of each extract were determined using methods previously described by Singleton et al.[30] and modified in our laboratory.[31,32] Briefly, 400 μL of deionized water and 100 μL of a known dilution of the extract or standard solution were added to a test tube. Folin−Ciocalteu reagents (100 μL) was added to the solution and allowed to react for 6 min. Then, 1 mL of 7% sodium carbonate solution and 800 μL of deionized water were added to the test tubes, and the mixture was mixed well. The color developed for 90 min at room temperature, and absorbance was read at 760 nm using a MRX II DYNEX spectrophotometer (Dynex Technologies, Inc., Chantilly, VA). The measurements for free, bound, and total phenolics were compared with a standard curve of prepared gallic acid solutions and expressed as micrograms of gallic acid equivalents (GAE) per gram dry weight (DW) sample ± SD for triplicate extracts.

Determination of Individual Phenolic Acids. Chlorogenic, caffeic, *p*-coumaric, and ferulic acids in sample extracts were quantified using a reverse phase HPLC procedure employing a Supelcosil LC-18-DB, 150 mm × 4.6 mm, 3 mm, column as reported previously.[33] Briefly, isocratic elution was conducted with 20% acetonitrile in water adjusted to pH 2 with trifluoroacetic acid, at a flow rate of 1.0 mL/min, delivered using a Waters 515 HPLC pump (Waters Corp., Milford, MA). A Waters 2487 dual-wavelength absorbance detector was used for UV detection of analytes at 280 nm. Data signals were acquired and processed on a PC running Waters Millennium software, version 3.2 (1999). The retention times of the standards, chlorogenic, caffeic, *p*-coumaric, and ferulic acids, were 3.3, 4.7, 7.4, and 8.4 min, respectively. The phenolic acid concentrations of sample extracts were extrapolated from the pure phenolic acid standard curves (chlorogenic acid, $r^2 = 0.99$; caffeic acid, $r^2 = 0.99$; *p*-coumaric acid, $r^2 = 0.99$; and ferulic acid, $r^2 = 0.99$). Twenty microliter injections were made in each run, and areas were used for all calculations. The selected individual peaks were identified by the retention times and co-injection of the pure standards. The method was validated by the recovery of chlorogenic acid. The percentage recovery for chlorogenic acid was 96.5 ± 6.22 ($n = 3$).

Determination of Total Flavonoids. The total flavonoid contents of the samples were determined using the method of sodium borohydride/chloronil (SBC) total flavonoid assay with modifications.[34] The stored sample extracts for total phenolic analysis were thawed and added to test tubes (15 × 150 mm), then dried at 45 °C under nitrogen gas, and reconstituted in 1 mL of THF/EtOH (1:1, v/v). Catechin standards (0.3−10.0 mM) were prepared fresh each day before use in 1 mL of THF/EtOH (1:1, v/v). Each test tube with 1 mL of sample solution or 1 mL of catechin standard solution had 0.5 mL of 50 mM $NaBH_4$ solution and 0.5 mL of 74.56 mM $AlCl_3$ solution added and was then shaken in an orbital shaker (Laboratory-Line Instruments, Inc., Melrose Park, IL) for 30 min at room temperature. Then an additional 0.5 mL of $NaBH_4$ solution was added to each test tube with continual shaking for another 30 min at room temperature. Cold acetic acid solution (2.0 mL of 0.8 M, 4 °C) was added to each test tube, and the solutions were shaken in an orbital shaker in the dark for 15 min after thorough mixing. Then, 1 mL of 20 mM chloranil was added to each tube, which was heated at 95 °C under shaking for 60 min in a reciprocal shaking bath (Precision

Scientific Inc., Chicago, IL). The temperature in the reciprocal shaking bath was maintained using glycerin. The reaction solutions were cooled using tap water, and the final volume was brought to 4 mL using methanol. Then, 1 mL of 1052 mM vanillin was added to each tube, followed by mixing. Concentrated HCl (2 mL of 12 M) was added to each tube, and the reaction solutions were kept in the dark for 15 min after thorough mixing. Aliquots of the final reaction solutions (200 μL) were added to each well of a 96-well plate after centrifuging for 3 min at 2500*g*, and absorbances were measured at 490 nm using a MRX Microplate Reader with Revelation workstation (Dynex Technologies). Total flavonoids were expressed as micrograms of catechin equivalents per gram of dry weight sample. Data were reported as the mean ± SD for three replicates.

Measurement of Antioxidant Activity (ORAC). The antioxidant activity of the samples (ingredients, raw formulations, and extruded products) were determined using the oxygen radical absorbance capacity (ORAC) assay described by Prior et al.[35] and modified in our laboratory.[36] Briefly, 20 μL of blank, Trolox standard, or sample extracts in 75 mM potassium phosphate buffer, pH 7.4 (working buffer), was added to triplicate wells in a black, clear-bottom, 96-well microplate. The triplicate samples were distributed throughout the microplate and were not placed side-by-side, to avoid any effect on readings due to location. In addition, no outside wells were used, as use of those wells results in greater variation. A volume of 200 μL of 0.96 μM fluorescein in working buffer was added to each well and incubated at 37 °C for 20 min, with intermittent shaking, before addition of 20 μL of freshly prepared 119 mM ABAP in working buffer using a 12-channel pipetter. The microplate was immediately inserted into a Fluoroskan Ascent FL plate reader (ThermoLabsystems) at 37 °C. The decay of fluorescence at 538 nm was measured with excitation at 485 nm every 4.5 min for 2.5 h. The area under the fluorescence versus time curve for the samples minus the area under the curve for the blank were calculated and compared to a standard curve of the area under the curve for 6.25, 12.5, 25, and 50 μM Trolox standards minus the area under the curve for blank. ORAC values were expressed as mean micromoles of Trolox equivalents (TE) per gram of dry weight sample ± SD for triplicate data.

Cellular Antioxidant Activity (CAA) Assay. The assay for CAA was performed following the procedures of Wolfe and Liu.[37] Briefly, HepG2 liver cancer cells from the passages of 5 and 7 were seeded at a density of 6×10^4 in 100 μL of complete growth medium per well on a 96-well microplate in a humidified 5% CO_2 incubator at 37 °C. The wells on the boundary of the microplate were filled with 200 μL of PBS. Twenty-four hours after seeding, the growth medium was removed and the wells were washed with PBS. The wells were treated in triplicate with 100 μL of solutions containing different concentrations of antioxidant extracts plus 25 μM DCFH-DA dissolved in antioxidant treatment media for 1 h at 37 °C. Then treatment media were removed, and wells were washed with 100 μL of PBS to remove extracellular residues. One hundred microliters of 600 μM ABAP in oxidant treatment medium (HBSS) was applied to all of the cells, and the microplate was immediately placed into a Fluoroskan Ascent FL plate reader (ThermoLabsystems, Franklin, MA) at 37 °C. Emission was measured every 5 min for 1 h at 538 nm after excitement at 485 nm. The blank wells contained cells treated with DCFH-DA, HBSS, and antioxidant extracts without ABAP, whereas the control wells contained cells treated with DCFH-DA, HBSS, and ABAP without antioxidant extracts.

The area under the curve for fluorescence (after subtraction of blank and initial fluorescence values) versus time was integrated to calculate the CAA value at each concentration of the sample extracts as

$$\text{CAA unit} = 1 - \left(\int \text{SS} / \int \text{CA}\right)$$

where $\int$SA is the integrated area under the sample fluorescence versus time curve and $\int$CA is the integrated area from the control curve. The median effective dose (EC_{50}), that is, the dose required to give a 50% inhibition for sample extract, was determined from the median effective plot of $\log(f_a/f_u)$ versus log(dose), where f_a is the fraction affected (CAA unit) and f_u is the fraction unaffected (1 − CAA unit) by the treatment. In the experiments, quercetin was used as a standard.

Statistical Analyses. All results were reported as the mean ± SD for at least three analyses for each type of extraction, and each extract was analyzed in triplicate. Results were subjected to ANOVA, and significance differences between means were determined using Tukey's multiple-comparison test run on SAS (version 9.1, SAS Institute Inc., Cary, NC). Correlations among various parameters were also evaluated using Pearson's correlation coefficient.

RESULTS

Phenolic Contents. The phenolic contents of the ingredients, raw formulations, and extruded products were determined using the Folin−Ciocalteu method and expressed as μg GAE/g DW sample (Figure 1). Free, bound, and total phenolics in PPF were 2008 ± 118, 932 ± 66, and 2940 ± 183 μg GAE/g DW sample, respectively. DPF had 366 ± 20, 55 ± 2, and 421 ± 19 μg GAE/g DW sample of free, bound, and total phenolics, respectively. The extruded products prepared from 35, 50, and 65% PPF (1947 ± 60, 2692 ± 42, and 3977 ± 36 μg GAE/g DW sample, respectively) had significantly higher ($p < 0.05$) free phenolic contents than their raw formulations (919 ± 28, 1552 ± 53, and 2290 ± 92 μg GAE/g DW sample, respectively). The bound phenolic contents of the extruded products prepared from 35, 50, and 65% PPF (175 ± 8, 378 ± 28, and 331 ± 35 μg GAE/g DW sample, respectively) were significantly lower ($p < 0.05$) than their raw formulations (415 ± 26, 478 ± 21, and 451 ± 31 μg GAE/g DW sample, respectively). The total phenolic contents of the extruded products prepared from 35, 50, and 65% PPF (2122 ± 53, 3070 ± 62, and 4308 ± 64 μg GAE/g DW sample, respectively) were significantly higher ($p < 0.05$) than their raw formulations (1334 ± 51, 2030 ± 39, and 2741 ± 120 μg GAE/g DW sample, respectively). Table 1 shows the percentage contributions of free and bound phenolics to the total phenolic content of raw ingredients and extruded products.

Flavonoid Contents. The flavonoid contents of the ingredients, raw formulations, and extruded products were determined using the SBC assay and were expressed as μg catechin eq/g DW sample (Figure 2). The free, bound, and total flavonoid contents of PPF were 5495 ± 42, 724 ± 23, and 6219 ± 417 μg catechin eq/g DW sample, respectively. The free, bound, and total flavonoid contents of DPF were 1305 ± 36, 731 ± 71, and 2036 ± 72 μg catechin eq/g DW sample, respectively. The free flavonoid contents of the extruded products prepared from 35, 50, and 65% PPF were 2606 ± 220, 2929 ± 153, and 3468 ± 64 μg catechin eq/g DW sample, respectively, and were significantly higher ($p < 0.05$) than their raw formulations (1944 ± 100, 2196 ± 68, and 2890 ± 103 μg catechin eq/g DW sample, respectively). The extruded products prepared from 35, 50, and 65% PPF had significantly higher ($p < 0.05$) bound flavonoid contents (592 ± 52, 470 ± 32, and 290 ± 27 μg catechin eq/g DW sample, respectively) than their raw samples (273 ± 24, 289 ± 26, and 230 ± 19 μg catechin eq/g DW sample, respectively). The total flavonoid content of the extruded products prepared from 35% PPF (3198 ± 220 μg catechin eq/g DW sample) was significantly higher ($p < 0.05$) than its raw formulation (2217 ± 124 μg catechin eq/g DW sample). Similarly, the extruded products prepared from 50 and 65% PPF (3400 ± 149 and 3758 ± 89 μg catechin eq/g DW sample, respectively) were significantly higher

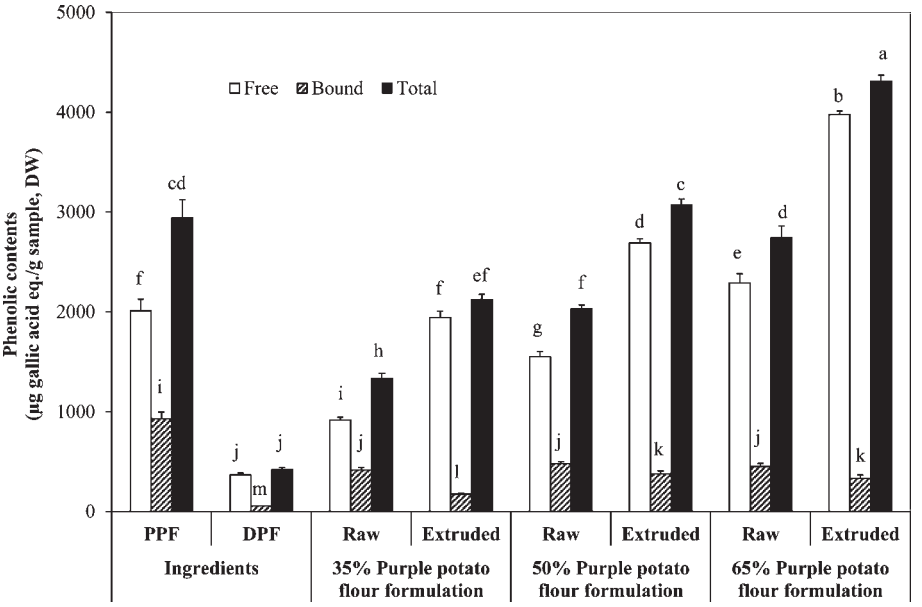

Figure 1. Phenolic contents of ingredients, raw formulations, and extruded products prepared from purple potato flour (PPF) and dry pea flour (DPF) formulations. The selected formulations for preparing the extrudates contained x% of PPF and $(100 - x)$% of DPF. Bars with different letters are significantly different ($p < 0.05$).

Table 1. Percentage Contributions of Phytochemicals in Free and Bound Extracts of Ingredients (Purple Potato Flour and Dry Pea Flour), Raw Formulations, and Extruded Products[a] to Total Phenolics, Total Antioxidant Activity, and Total Flavonoids ($n = 3$)

		total phenolics (%)		total antioxidant activity (ORAC value) (%)		total flavonoids (%)	
		free	bound	free	bound	free	bound
purple potato flour (PPF)		68	32	64	36	88	12
dry pea flour (DPF)		87	13	86	14	64	36
35% PPF[b]	raw	69	31	63	37	88	12
	extruded	92	8	88	12	82	18
50% PPF	raw	76	24	74	26	88	12
	extruded	88	12	88	12	86	14
65% PPF	raw	84	16	83	17	93	7
	extruded	92	8	93	7	92	8

[a] Extruded products prepared from PPF and DPF formulations. [b] The selected formulations for preparing the extrudates contained x% of PPF and $(100 - x)$% of DPF.

($p < 0.05$) flavonoid contents than their raw formulations (2486 ± 53 and 3120 ± 111 μg catechin eq/g DW sample, respectively).

Individual Phenolic Acids. Detected individual phenolic acids present in the raw formulations and extruded products are presented in Table 2, expressed as μg/g DW sample. Chlorogenic acid was the prominent phenolic acid in free phytochemicals, whereas caffeic and *p*-coumaric acids were prominent in bound phytochemicals of the samples. Quantities of caffeic, *p*-coumaric, and ferulic acids were either less or not detected in free phytochemicals of the raw formulations and extruded products. Chlorogenic acids in the free and bound phytochemicals of the extruded products were 984−2967 and 574−609 μg/g DW sample, respectively, and were significantly higher ($p < 0.05$) than in their raw formulations (298−1011 and 243−253 μg/g DW sample, respectively). Extrusion of raw formulations significantly lowered ($p < 0.05$) the caffeic acid content (from 306−339 to 77−168 μg/g DW sample), whereas processing increased the quantity of *p*-coumaric acid (from 334−463 to 362−970 μg/g DW sample) in the bound phytochemicals of all raw formulations except 35% PPF. Quantities of ferulic acids in the extruded products (159−188 μg/g DW sample) were also significantly higher ($p < 0.05$) than their raw formulations (57−59 μg/g DW sample). Individual phenolic acids, except caffeic acid, in the total phytochemicals were significantly higher ($p < 0.05$) in the extruded products compared to their raw formulations. However, no significant difference ($p > 0.05$) was observed in the content of total *p*-coumaric acid in the extruded product prepared from 35% PPF , compared to its raw formulation.

Total Antioxidant Activity. The total antioxidant activities of the ingredients, raw formulations, and extruded products were determined using the ORAC assay and expressed as μmol TE/g DW sample (Figure 3). The free, bound, and total ORAC values of PPF were 47.82 ± 1.66, 26.80 ± 0.89, and 74.62 ± 2.53 μmol

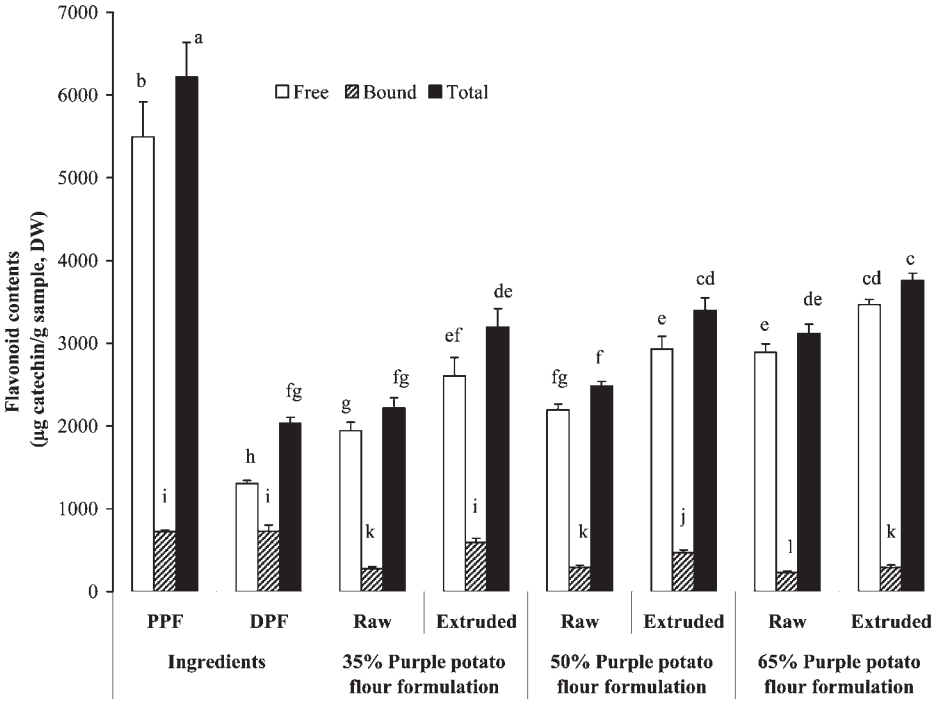

Figure 2. Flavonoid contents of ingredients, raw formulations, and extruded products prepared from purple potato flour (PPF) and dry pea flour (DPF) formulations. The selected formulations for preparing the extrudates contained x% of PPF and $(100 - x)$% of DPF. Bars with different letters are significantly different ($p < 0.05$).

Table 2. Quantities of Free, Bound, and Total Individual Phenolic Acids Present in Extracts of Ingredients (Purple Potato Flour (PPF) and Dry Pea Flour (DPF)), Raw Formulations, and Extruded Products[a] (Mean ± SD, n = 3)

	free (μg/g sample, DW)				bound (μg/g sample, DW)				total (μg/g sample, DW)			
	chloro	caffeic	*p*-coum	ferul	chloro	caffeic	*p*-coum	ferul	chloro	caffeic	*p*-coum	ferul
PPF	714 ± 55d	14 ± 1b	52 ± 0.3b	nd	202 ± 8c	801 ± 55a	1019 ± 34a	109 ± 11b	916 ± 55e	815 ± 56a	1071 ± 35a	109 ± 11b
DPF	2.0 ± 1g	nd	nd	nd	236 ± 20b	15 ± 3 f	nd	22 ± 3d	260 ± 25g	15 ± 3f	nd	22 ± 3d
35% raw	298 ± 24f	10 ± 1c	6 ± 1d	nd	253 ± 27b	306 ± 45b	334 ± 10e	59 ± 1c	551 ± 46f	316 ± 46b	340 ± 10e	59 ± 1c
PPF[b] extruded	984 ± 10c	nd	nd	nd	581 ± 60a	77 ± 6 e	362 ± 11d	188 ± 12a	1565 ± 66c	77 ± 7e	362 ± 11e	188 ± 12a
50% raw	540 ± 77e	13 ± 1b	37 ± 8c	nd	253 ± 33b	339 ± 39b	387 ± 54cd	57 ± 7c	794 ± 107e	353 ± 39b	425 ± 47d	57 ± 7c
PPF extruded	2011 ± 211b	nd	nd	nd	609 ± 60a	168 ± 17c	970 ± 113ab	179 ± 27a	2620 ± 170b	168 ± 17c	970 ± 113ab	179 ± 27a
65% raw	1011 ± 42c	23 ± 4a	117 ± 18a	nd	243 ± 21b	309 ± 52b	463 ± 35c	58 ± 4c	1254 ± 63d	333 ± 55b	581 ± 51c	58 ± 4c
PPF extruded	2967 ± 267a	nd	nd	nd	574 ± 81a	93 ± 7d	857 ± 73b	159 ± 21a	3541 ± 348a	93 ± 7d	857 ± 73b	159 ± 21a

[a] Extruded products prepared from PPF and DPF formulations. [b] The formulation contains x% of PPF and $(100 - x)$% of DPF. Values in each column with no letters in common are significantly different ($p < 0.05$). Abbreviations: chloro, chlorogenic acid; p-coum, coumaric acid; ferul, ferulic acid; nd, not detected.

TE/g DW sample, respectively. The free, bound, and total ORAC values of DPF samples were 8.86 ± 0.88, 1.43 ± 0.07, and 10.29 ± 0.92 μmol TE/g DW sample, respectively. The free ORAC values of extruded products prepared from 35, 50, and 65% PPF (45.48 ± 2.34, 75.29 ± 5.32, and 113.83 ± 6.67 μmol TE/g DW sample, respectively) were significantly higher ($p < 0.05$) than those of their raw formulations (17.69 ± 1.56, 33.36 ± 4.27, and 57.80 ± 1.44 μmol TE/g DW sample, respectively). Conversely, the bound ORAC values of extruded products prepared from 35 and 65% PPF (6.08 ± 0.23 and 8.60 ± 1.42 μmol TE/g DW sample, respectively) were significantly lower ($p < 0.05$) than those of their raw formulations (10.28 ± 0.52 and 12.25 ± 0.64/g DW sample, respectively). However, there was no significant difference ($p > 0.05$) in the ORAC values of bound phytochemicals in the extruded products prepared from 50% PPF (9.93 ± 0.54 μmol TE/g DW sample) formulation compared to its raw formultion (11.58 ± 1.74 μmol TE/g DW sample). The extruded products prepared from 35, 50, and 65% PPF had significantly higher ($p < 0.05$) ORAC values (51.57 ± 2.57, 85.22 ± 5.81, and 122.43 ± 7.52 μmol TE/g DW sample, respectively) than those of their raw formulations (27.98 ± 1.87, 44.95 ± 5.92, and 70.05 ± 1.00 μmol TE/g DW sample, respectively).

Cellular Antioxidant Activity. The CAA of the raw formulations and extruded products was quantified using the protocol of the CAA method and expressed as EC_{50} in mg/mL and CAA

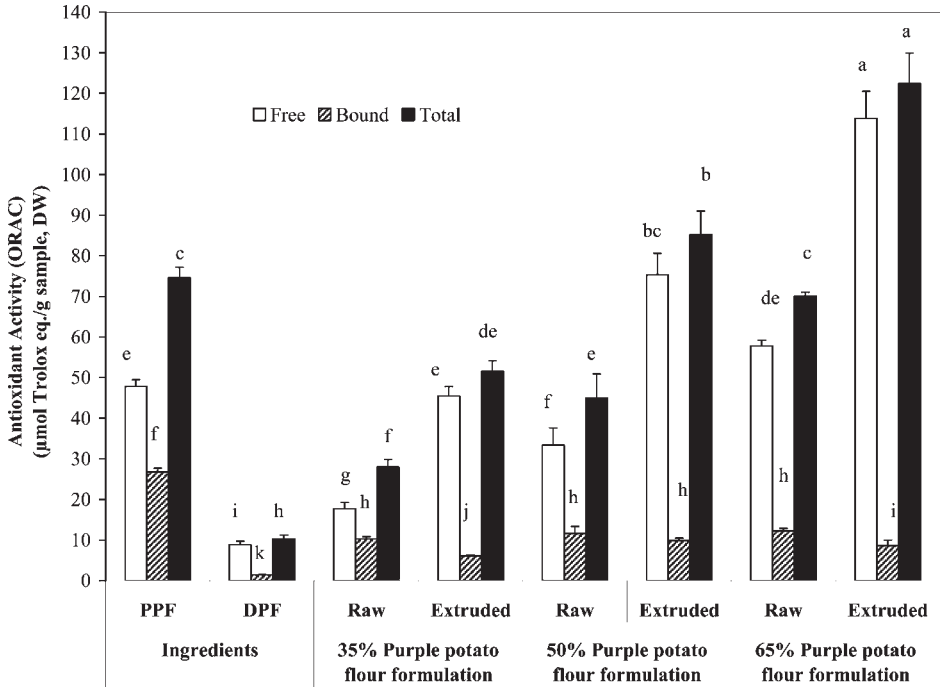

Figure 3. Antioxidant activity of ingredients, raw formulations, and extruded products prepared from purple potato flour (PPF) and dry pea flour (DPF) formulations. The selected formulations for preparing the extrudates contained x% of PPF and $(100 - x)$% of DPF. Bars with different letters are significantly different ($p < 0.05$).

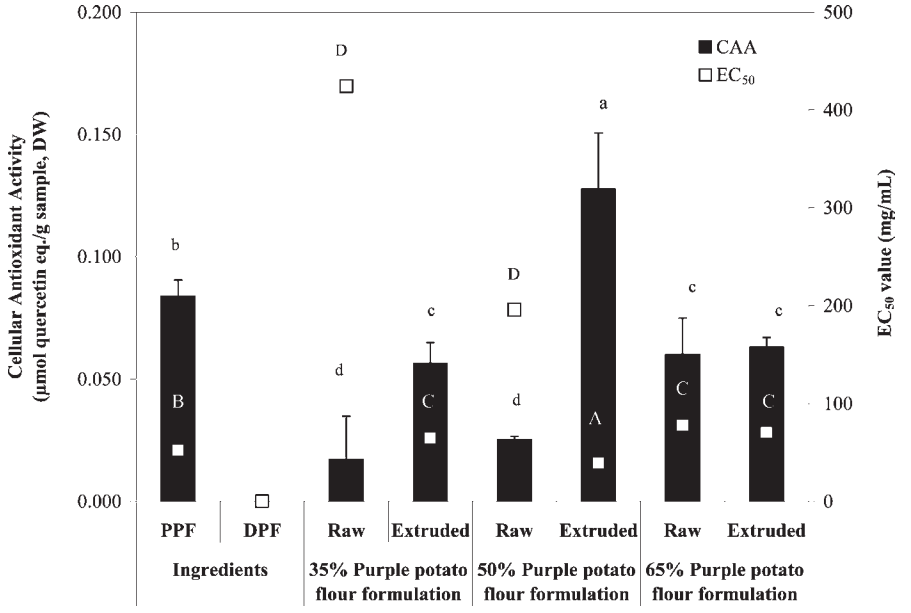

Figure 4. Cellular antioxidant activity (CAA) and EC_{50} value of ingredients, raw formulations, and extruded products prepared from purple potato flour (PPF) and dry pea flour (DPF) formulations. The selected formulations for preparing extrudates contained x% of PPF and $(100 - x)$% of DPF. Bars with different lower case letters are significantly different ($p < 0.05$) for CAA, whereas different upper case letters are significantly different ($p < 0.05$) for EC_{50}.

values in μmol quercetin equivalent (QE)/g DW sample (Figure 4). The EC_{50} value of PPF sample was 52.1 ± 4.0 mg/mL (CAA = 0.084 μmol QE/g DW sample), whereas no CAA activity was detected in DPF sample. The EC_{50} values of the extruded product prepared from 35% PPF (64.7 ± 9.0 mg/mL) and 50% PPF (38.9 ± 7.4 mg/mL) were significantly lower ($p < 0.05$) than those of their raw formulations (424.2 ± 344 and 195.8 ± 10 mg/mL, respectively). However, no significant difference in the EC_{50} values was observed between the raw (77.8 ± 19 mg/mL) and extruded products (70.3 ± 4.4 mg/mL) prepared from 65% PPF. Similarly, the CAA values of 35, 50, and 65% PPF raw formulations (0.017 ± 0.018, 0.025 ± 0.001, and

Table 3. Correlation Analysis of Phenolics, Antioxidant Activity (ORAC), and Cellular Antioxidant Activity (CAA) of Ingredients (Purple Potato Flour and Dry Pea Flour), Raw Formulations,[a] and Extruded Product[b]

	free extract			bound extract			total		
	total phenolics	total ORAC value	CAA	total phenolics	total ORAC value	CAA	total phenolics	total ORAC value	CAA
phenolics		0.9780 (<0.0001)	0.6565 (0.0005)		0.3295 (0.1159)	0.3936 (0.0570)		0.9847 (<0.0001)	0.6988 (0.0001)
flavonoids	0.6636 (0.0004)	0.6288 (0.0010)	0.6008 (0.0019)	−0.3210 (0.1262)	−0.2752 (0.1931)	0.0677 (0.7534)	0.5814 (0.0029)	0.5555 (0.0048)	0.5832 (0.0028)
chlorogenic acid	0.8843 (<0.0001)	0.9176 (<0.0001)	0.6047 (0.0017)	0.5117 (0.0106)	0.5583 (0.0046)	0.5605 (0.0044)	0.8529 (<0.0001)	0.8897 (<0.0001)	0.6181 (0.0013)
caffeic acid	0.0240 (0.9113)	−0.0655 (0.7610)	−0.0708 (0.7423)	0.1912 (0.3707)	0.1121 (0.6019)	0.2377 (0.2634)	0.1878 (0.3796)	0.1073 (0.6178)	0.2299 (0.2799)
p-coumaric acid	0.1575 (0.4622)	0.0926 (0.6668)	0.0880 (0.6827)	0.8402 (<0.0001)	0.8329 (<0.0001)	0.8551 (<0.0001)	0.8465 (<0.0001)	0.8318 (<0.0001)	0.8532 (<0.0001)
ferulic acid	nd	nd	nd	0.6419 (0.0007)	0.6583 (0.0005)	0.7197 (<0.0001)	0.6419 (0.0007)	0.6583 (0.0005)	0.7197 (<0.0001)
ORAC value	0.8762 (<0.0001)		0.3514 (0.0923)	0.3188 (0.1290)		0.2749 (0.1936)	0.9847 (<0.0001)		0.6984 (0.0001)

[a] The formulations contain x% of purple potato flour (PPF) and (100 − x)% of dry pea flour (DPF). [b] Extruded products prepared from PPF and DPF formulations. Correlation coefficients were calculated as r^2. Significant levels are given in the parentheses.

0.06 ± 0.015 μmol QE/g DW sample, respectively) and extruded products (0.56 ± 0.008, 0.128 ± 0.023, and 0.063 ± 0.004 μmol QE/g DW sample, respectively) followed the same trend as their EC_{50} values. The EC_{50} values of quercetin standard in all of the replicates were in the range of 3.35−4.5 mg/mL, which were similar to those reported by Wolfe and Liu.[37]

Correlation Analyses. Relationships among total phenolics, total ORAC values, and CAA with individual phenolic acids and flavonoids in free and bound forms were determined using Pearson's correlation coefficient (Table 3). The free phenolic content was significantly correlated ($p < 0.05$) to total ORAC values ($r^2 = 0.98$) and CAA values ($r^2 = 0.66$). Similar correlation patterns were observed between total phenolics and total ORAC values and CAA values. The free and total flavonoids were significantly correlated ($p < 0.05$) to total phenolics, ORAC, and CAA values. Among individual phenolic acids, free and total chlorogenic acid contents were significantly correlated ($p < 0.05$) to the total phenolics, ORAC, and CAA values. *p*-Coumaric and ferulic acids in the bound and total extracts significantly correlated ($p < 0.05$) to the total phenolics, ORAC, and CAA values. However, caffeic acid did not show any positive correlation with the total phenolics, ORAC, or CAA values. The total ORAC values were significantly correlated ($p < 0.05$) with CAA values ($r^2 = 0.69$).

DISCUSSION

Phytochemicals in food samples are present in both free and bound forms.[11,12] Without accounting for bound phytochemicals, the total phytochemical content will be underestimated.[11] Therefore, studying free and bound phytochemicals provides a more accurate information on total phytochemicals and the contributions of free, soluble-conjugated, and bound to the total phenolics and their total antioxidant activity. The cellular antioxidant activity assay, used in this study, is a biologically relevant means to quantify the bioactivity of antioxidants in food products as it takes into consideration the cellular uptake, metabolism, and distribution of bioactive compounds in the cell.[37,38] The values of cellular antioxidant activities of the samples were also evaluated using ORAC. This study was designed to determine the phytochemicals, their relationships, and contributions to total antioxidant activity in potato and dry pea flours, raw formulations, and extrusion cooked snack foods made from the raw formulations.

Our study showed that majority of the phenolics in PPF and DPF were present in free rather than in bound form (Table 1). The quantity of total phenolics in whole potato cultivars grown in the Andes Mountains of South America was in the range of 1120 − 12370 μg GAE/g DW sample, which is within the range of our study determined on flesh potato flour.[39] Results from a Colorado potato breeding program have shown that there was no difference in the total phenolic content between whole 'Purple Majesty' potato processed thru microwave cooking, boiling or baking compared to the unprocessed potatoes. In our study (Figure 1) we determined that the extrudates obtained from flesh potato flour had higher concentration in total phenolics than raw formulations. This indicates that the type of cooking/processing have a significant effect on the phenolic content in the final product. Additionally, the type of potato cultivar may had also an effect on the reported result.[19] The percentage contribution of free phenolics to the total was 68%, whereas bound phenolics contributed 32% in potato flour (Table 1). This is similar to the results of Chu et al.,[40] who reported the contributions of 60 and 40% by free and bound phenolics, respectively, to the total phenolics in white potatoes with skin.[40] Therefore, the total phenolic contents reported in the previous literature may have been underestimated by not including the bound phenolics in the results of their study. Bound phenolics might be associated with the plant cell walls that survive upper gastrointestinal digestion and reach the colon, where most of them are released by intestinal microflora.[11,12] The total phenolic content in DPF in the present study (Figure 1) was 3−5-fold lower than in the

reported literature.[25,26] This could be due to (i) use of previously milled flours compared to whole raw legumes by other researchers or (ii) different solvents for extraction of phenolic compounds.[41] Free phenolics in DPF contributed more (87%) to the total phenolics than bound ones (13%). The raw formulations from potato and dry pea flours in selected concentrations had 69−84% free phenolics. Extrusion cooking of raw formulations increased the percentage contributions of free phenolics to the total and decreased the contributions from bound phenolics (Table 1). Increase in the total phenolics in the extruded food products (50−60%) rather than the raw formulations could be due to (i) breaking of conjugated phenolics into free phenolics and (ii) leaching of soluble fibers, proteins, and other nonphenolic soluble components such as mono-, di-, and oligosaccharides.[26] Han and Baik[26] reported a similar increase in the total phenolics of cooked and soaked chickpeas, yellow peas, green peas, and soybeans. However, in another paper, the total phenolics of cooked legumes were significantly reduced ($p < 0.05$) when compared to uncooked samples.[25]

Free and bound phytochemicals in potato flour contributed 64 and 36%, respectively, to the total ORAC antioxidant activity, similar to their contributions to the total phenolics. The total ORAC value in potato flour was in the range of hydrophilic ORAC values (28.25−250.67 μmol TE/g DW) of Andean potato cultivars.[39] The individual ORAC values of free and bound phytochemicals could not be compared due to limited information in the literature. The total ORAC value in DPF was comparable to that of reported in the literature[42] and had similar contributions from free (86%) and bound (14%) phytochemicals as was the case for total phenolics (Table 1). Han and Baik[26] reported that free and bound phytochemicals contributed 68 and 32%, respectively, to the total TEAC antioxidant activity of yellow peas. Processing of the all raw formulations using extrusion cooking significantly increased ($p < 0.05$) the total ORAC values of the extruded products. Increase in the total ORAC values followed the same pattern as in total phenolics of the extruded products. It was also observed that the ORAC values of bound phytochemicals significantly decreased ($p < 0.05$) and ORAC values of free phytochemicals increased in the extruded products. This phenomenon could be attributed to (i) breaking of conjugated phytochemicals to release free phytochemicals,[31] (ii) prevention of enzymatic oxidation, and (iii) darker colors of the extruded products indicating formation of Maillard reaction products having antioxidant properties.[43] The ORAC values of processed green peas, yellow peas, and chickpeas were significantly increased ($p < 0.05$) (27−114, 12−67, and 25−40%, respectively) after pressure boiling as compared to the raw legumes.[42] However, the FRAP values and DPPH antioxidant activity of legumes were decreased significantly ($p < 0.05$) by conventional and pressure boiling.[25,42] Increases in the antioxidant activities of sweet corn, teas, and tomatoes with thermal processing were also reported.[4,31,44]

Quantities of free flavonoids in potato and dry pea flours were significantly higher ($p < 0.05$) when compared to their bound flavonoids. The total flavonoids of both flours followed a similar pattern as free flavonoids, because of the larger contribution from free flavonoids (Table 1). As we previously stated, other authors have also indicated that there is limited literature available to compare free, bound, and total flavonoids in colored potatoes.[45] The total flavonoids of DPF in our study were higher when compared to the reported total flavonoid contents of whole[41,46] and dehulled yellow peas.[47] Quantities of free flavonoids increased after extrusion cooking of all the raw formulations. Interestingly, the contents of bound flavonoids were also significantly increased ($p < 0.05$) in the extruded products when compared to their raw formulations. Choi et al.[48] reported a significant increase of free flavonoids in shiitake mushrooms after heat treatment at 100 and 121 °C when compared to raw mushrooms. However, the investigators observed decreases in bound flavonoids after heat treatment. Increase in the flavonoid contents of the extrudates could be attributed to (i) disruption of plant cell walls providing better extractability, (ii) breaking of chemical bonds of higher molecular weight polyphenols and forming soluble low molecular weight polyphenol compounds, and (iii) interconversion of flavonoids in different forms.[49] Variyar et al.[50] reported an increase in antioxidant potential of soybeans with the dose of γ-irradiation due to increased levels of genistin (an isoflavone) and degradation products of diadzein.

Phenolic acids are sources of dietary phenols, which are linked to cellulose, lignin, and proteins through ester bonds. For example, chlorogenic acid has strong antioxidant activity and is already demonstrated to have several desirable effects on biochemical processes involved in carcinogenesis.[51] *p*-Coumaric acid was the major phenolic acid observed in potato flour, followed by chlorogenic, caffeic, and ferulic acids (Table 2). Most of the *p*-coumaric and caffeic acids were observed in bound phenolic fraction, whereas chlorogenic acid was higher in free phenolics. Ferulic acid was observed only in bound phenolics. Adom and Liu reported that >93% of ferulic acids were present in the bound form in corn, wheat, oats, and rice.[11] The content of free chlorogenic acid we reported in this study was similar to the total chlorogenic content in 'Purple Majesty' potatoes found in a Colorado potato breeding program.[19] Interestingly, our results demonstrated that bound phenolics contributed additional ∼20% of total chlorogenic acid, which was commonly underestimated in published literature. Lewis et al.[52] and Lachman et al.[53] reported in detail the presence of chlorogenic, caffeic, *p*-coumaric, ferulic, and other phenolic acids in colored potatoes. Chlorogenic acid, followed by ferulic and caffeic acids, was detected mostly in bound phenolics of DPF, whereas *p*-coumaric acid was not detected. Dry pea flour contained only chlorogenic acid in free phenolic fraction. The content of total chlorogenic acid in this study was higher when compared to the reported content of whole yellow peas.[25] The investigators, however, reported having *p*-coumaric acid along with chlorogenic and gallic acids in yellow peas. A multifold increase in chlorogenic acid in free and bound phenolic fractions of the formulations rather than raw ingredients could be due to the binding of an *o*-hydroxy phenolic group in chlorogenic acid to protein via the bidentate hydrogen bond.[54] The mechanism for binding of chlorogenic acid to sunflower proteins involved both hydrogen bonding and covalent linkages between oxidized phenolics and nucleophilic amino acid side chains, such as lysine and cysteine.[55] The behavior of caffeic acid in the raw formulations could also be due to the same mechanism of protein-bound phenolic interactions as chlorogenic acid. Quantities of chlorogenic acids in free and bound phenolics in all the extruded products were significantly higher ($p < 0.05$) when compared to their raw formulations (Table 2). A similar pattern was observed in total chlorogenic acid contents of the extruded products. Xu and Chang[25] reported significant increases in gallic, chlorogenic, and total phenolic acids in yellow peas after pressure boiling. Significant decreases in caffeic acid in bound fraction and total phenolics were observed in the extruded products when compared

to their raw formulations, whereas those decreases were not detected in free phenolics. A decrease in caffeic acid contents with exposure of potato strips to home processing conditions was reported.[56] Total *p*-coumaric and ferulic acid contents were increased in the extruded products compared to their raw formulations, mostly due to increases in their bound phenolics. However, caffeic, *p*-coumaric, and ferulic acids were not detected in free phenolic extractions of the extruded products. The undetected phenolic acids in the extrudates may be due to alkaline hydrolysis that partly or completely broke down original phenolic acids. Increases in the total chlorogenic, *p*-coumaric, and ferulic contents in the extruded products could be attributed to the alkaline hydrolysis of caffeic acid present in conjugated phenolics. The reasons for change in the individual phenolic acids during extrusion cooking could be explained according to Fleuriet and Macheix[57] as (i) oxidative degradation of phenolic acids, (ii) breakdown of conjugated phenolics and release of free phenolic acids, and (iii) formation of complex phenolic acids.

Increases in the contents of chlorogenic, *p*-coumaric, and ferulic acids and low molecular phenolic acids from the breakdown of caffeic acid could have increased the total phenolics in the extruded products. Thermal decomposition of caffeic acid generated compounds such as tetraoxygenated phenylinadan isomers with higher antioxidant activity than caffeic acid.[58] Analysis of individual phenolic acids suggested that the breakdown of higher polyphenols and formation of novel compounds after processing could have increased the total phenolics as well as antioxidant activity. Significant positive correlations among individual phenolic acids except caffeic acid to the total phenolics and total ORAC antioxidant activity justify these assumptions. Increases in the total phenolics after processing and a strong correlation ($r^2 = 0.9847$) with ORAC antioxidant activity showed that phenolics were mostly responsible for the chemical antioxidant activity in the extruded products.

The bioactivity of phytochemicals in free phytochemical extracts in the ingredients, raw formulations, and extruded products was studied using the CAA assay. Potato flour showed potent CAA with an EC_{50} of 52 mg/mL. Lower EC_{50} values indicate higher CAA. Dry pea flour had no measurable CAA. CAA were observed in all of the raw formulations and their extruded products (Figure 4). The extruded products prepared from 50% PPF had higher CAA and lower EC_{50} value among the extrudates ($p < 0.05$) and also had much higher CAA ($p < 0.05$) and lower EC_{50} value ($p < 0.05$) than its raw formulation. The effect of extrusion on the products prepared from 65% PPF was not significantly different ($p > 0.05$) in EC_{50} or CAA values. Similar results were observed in the 35% PPF raw formulation; CAA values were increased in the extrudates when compared to its raw formulation (Figure 4). There was no specific pattern observed in EC_{50} values of the extruded products. It could be possible that products extruded from a 50% PPF raw formulation had a better composition of potato and dry pea flour for possible additive and synergistic effects of phytochemicals responsible for the higher CAA as suggested by Liu.[9] The CAA value of the extruded product from 50% PPF formulation was comparable with those of plums and red grapes and higher than those of cherries, kiwifruit, mangoes, peaches, and pears.[36] The extruded products had higher CAA when compared to the raw formulations, which may be due to extrusion processing increasing the amount of bioaccessible phytochemicals as well as increased cellular uptake of those phytochemicals.

In summary, a comprehensive study on the contributions of free and bound phytochemicals to the total phenolics, flavonoids, ORAC antioxidant activity, and CAA in 'Purple Majesty' potato and dry pea flours is presented in this study. Raw formulations of PPF and DPF and their extrudates were analyzed in detail with regard to changes in individual phenolic acids, bound and free phytochemicals, and their contributions to ORAC antioxidant activities after extrusion processing. In most of the extruded products, the total phenolics, flavonoids, and ORAC values increased after processing. The CAA were observed in potato flour, raw formulations, and extruded products. Extrusion processing improved CAA of 50% PPF raw formulation. Measuring the antioxidant activity using the CAA assay in the extruded food products is important for assessing the bioactivity of processed foods, because it is more biologically relevant than chemical antioxidant assays.[37] Further studies on the antioxidant activities of individual phytochemicals and changes during processing are needed to understand the detailed mechanism of the breakage and formation of total phytochemicals and their contribution to bioactivity.

AUTHOR INFORMATION

Corresponding Author

*Postal address: 256, LJ Smith Hall, Department of Biological Systems Engineering, Washington State University, Pullman, WA 99163. Phone: (509) 335-2140. Fax: (509) 335-2722. E-mail: jtang@wsu.edu.

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ABBREVIATIONS USED

PPF, purple potato flour; DPF, dry pea flour; PBS, phosphate-buffer saline; THF, tetrahydrofuran; EtOH, ethyl alcohol; ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride; CAA, cellular antioxidant activity; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; HBBS, Hank's balanced salt solution; FRAP, ferric reducing/antioxidant power; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalent; QE, quercetin equivalents; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; TRAP, total radical-scavenging antioxidant parameter.

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