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Phytochemical phenolics in organically grown vegetables

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Fruit and vegetable intake is inversely correlated with risks for several chronic diseases in humans. Phytochemicals, and in particular, phenolic compounds, present in plant foods may be partly responsible for these health benefits through a variety of mechanisms. Since environmental factors play a role in a plant's production of secondary metabolites, it was hypothesized that an organic agricultural production system would increase phenolic levels. Cultivars of leaf lettuce, collards, and pac choi were grown either on organically certified plots or on adjacent conventional plots. Nine prominent phenolic agents were quantified by HPLC, including phenolic acids (*e.g.* caffeic acid and gallic acid) and aglycone or glycoside flavonoids (*e.g.* apigenin, kaempferol, luteolin, and quercetin). Statistically, we did not find significant higher levels of phenolic agents in lettuce and collard samples grown organically. The total phenolic content of organic pac choi samples as measured by the Folin-Ciocalteu assay, however, was significantly higher than conventional samples ($p < 0.01$), and seemed to be associated with a greater attack the plants in organic plots by flea beetles. These results indicated that although organic production method alone did not enhance biosynthesis of phytochemicals in lettuce and collards, the organic system provided an increased opportunity for insect attack, resulting in a higher level of total phenolic agents in pac choi.

Keywords: Phytochemicals / Organically / Vegetables / Phenolics / Conventional System

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1 Introduction

Epidemiological studies consistently link diets high in fruits and vegetables to decreased risks for several human chronic diseases [1–5]. Multiple components present in plant foods offer health benefits to consumers including vitamins, minerals, and fiber; however, recent studies have shown that phytochemicals present in fruits and vegetables offer health benefits beyond basic nutrition and that cancer prevention may be due, at least in part, to their bioactive phytochemicals [6–12].

Phytochemicals are present ubiquitously in plants. They appear to function in plant protection; for example, phenolics, one class of phytochemicals including phenolic acids and flavonoids, were allelopathic against neighboring plants [13–15]. These compounds have multiple additional roles including to attract insects for seed dispersion and pollination or to repel them in self-defense [16, 17]. The phenolics are produced in plants as secondary metabolites via the shikimic acid pathway [18]. Phenylalanine ammonia-lyase (PAL), the key enzyme catalyzing the biosynthesis of phenolics from the aromatic amino acid phenylalanine, is responsive to biotic and abiotic stresses such as high UV light, low temperatures, insect attack, pathogen infection, or nutrient deficiency; thus, environmental conditions can influence the amount and types of phenolic compounds present in plants [17–20].

Recent studies of others and us have shown that phytochemicals can play chemopreventive roles in regards to human cancer by modulation of the cancer cell cycle, proliferation inhibition, and induction of apoptosis [9, 11, 2]. The phenolics, in particular, have apparent antioxidant properties and prevent stimulation of cancer cells' oxidative stress-responsive genes [21–23]. Besides antioxidation, flavonoids and phenolic acids can upregulate tumor suppressor genes and

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inhibit oncogenes [24], induce apoptotic death in vitro and in animals [25–28], and cause cell cycle arrest [29, 30]. Flavonoids have been associated with reduced incidence of other chronic diseases such as heart disease, cerebrovascular disease, asthma, and type 2 diabetes [10].

Given the mounting data in support of the role of phenolics in the prevention of cancer and other chronic diseases, methods to improve the phenolic content of plant foods would be of extreme benefit to human health. Plant geneticists have attempted to improve phytochemical levels through traditional breeding programs or through bioengineering of PAL leading to secondary metabolite accumulation. However, accumulating evidence suggests that phytochemical content may be affected simply by a plant's environment [31–34].

Organic production systems that promote biodiversity and use few off-farm inputs such as chemical fertilizers and pesticides may tend to increase environmental stresses on plants [35]. Consequently, the use of organic agriculture may be a way to increase the phytochemical content of plant foods.

The growing popularity of organic agriculture makes understanding the relationship between phytochemical levels and farming particularly relevant. Statistics indicate that the attractiveness of both organic farming practices and organic products has increased in recent years. Certified organic cropland in the United States more than doubled between 1992 and 1997 [36], and greater than 24 million hectares of cropland is managed organically worldwide [37]. Organic food sales in the USA have grown 20–25% annually since 1990 [38], and a major consumer watchdog group listed organic foods as one of the top ten food trends of this decade, with sales of organic foods in the US alone expected to exceed $22 billion by 2010 [39].

A European study examining marketplace purchasing decisions determined that perceived health benefits, rather than concerns for the environment, most likely drive consumers to buy organic foods [40]. Consumer concerns could be related to expectations of reduced pesticide residues on organic compared to conventional product, or of higher nutritional value of organic produce. Data supporting these supposed benefits are few and controversial. No differences were found in macro or micronutrient composition between organic and conventional fruits, vegetables, and grains grown under controlled field or greenhouse production conditions or acquired at the market [41–44], or at least there was insufficient evidence to support or refute any claims [45–46]. Vitamin C was an exception and was increased by organic agriculture in some of the samples examined [42, 43, 45]. Differences in macronutrients, vitamins, and minerals between conventional and organic produce likely would not influence the nutritional status of consumers in developed countries since none of these nutrients are particularly deficient in these populations; however, an examination of concentrations of phytochemicals present in organic versus conventional plant foods may be necessary in order to properly assess any differences in potential health benefits [41, 45, 47]. Again, only a few studies have addressed these possible differences, and the results are contradictory and inconclusive. Organic marionberries and corn showed significantly higher levels of total phenolics than their conventional counterparts [48] did; likewise, organic peaches and pears had significantly higher levels of some individual phenolic acids and total polyphenols than those grown using conventional methods [49]. Conversely, organic production methods had no consistent effects on the levels of phenolics in strawberries [34].

In order to accurately draw any conclusions regarding the alleged nutritional superiority of organic food, it is necessary to continue investigating the effects, if any, that organic production methods have on the levels of phytochemicals in plant foods. This research is focused on the effects of organic farming methods on levels of secondary metabolites, specifically phenolic acids and flavonoids, present in leafy lettuce, collards, and pac choi vegetable crops. Furthermore, in contrast to previous studies, this study examined a large number of phenolic compounds included an assay for total phenolics, and accounted for environmental variability among samples.

2 Materials and methods

2.1 Samples

Leaf lettuce (*Lactuca sativa* L. cv. Kalura and Red Sails), collards (*Brassica oleracea* L. cv. Top Bunch), and pac choi (*Brassica rapa* L. cv. Mei Qing) were cultivated under organic and conventional production systems in adjacent replicated plots during the summer 2003 at the Kansas State University Research and Extension Center, Olathe, Kansas. The organic plots were certified under the USDA National Organic Program. Organic and conventional fertilizer regimes provided equivalent rates of nitrogen to the crops at standard recommended rates based on soil tests. The conventional system received a pre-plant application of 13-13-13 (N-P-K), followed by fertigation with calcium nitrate (15.5-0-0) during crop growth, while the organic system received a pre-plant application of Hu-More (1-1-1), a compost/alfalfa mix, followed by fertigation with fish emulsion (5-1-1). Leaf lettuce and collards did not receive any insecticide treatments; however, organic (pyrethrin) and conventional insecticides (permethrin) were applied to pac choi to control flea beetle damage. Fully expanded, but not outer leaves were taken from three plants per plot and freeze-dried immediately after harvest. Six freeze-dried samples per plot were randomly selected for analyses of individual and total phenolics as mentioned as follows.

2.2 Extraction of phenolic agents

One g of freeze-dried vegetable samples was refluxed in 50 mL of 80% aqueous ethanol containing 20 ppm of 98% 2-naphthoic acid (Aldrich Chemical Co., Milwaukee, WI) as an internal standard for 1 hour. The lost ethanol was replaced after the mixture cooled to room temperature, followed by centrifugation. Twenty mL of the supernatant was evaporated under reduced pressure until dry and then redissolved in 8 mL of water. Two mL of this water solution was subsequently purified by solid phase extraction (SPE) using reverse-phase Accubond ODS C-18 columns (Agilent Technologies, Stockport, Cheshire, U. K.) preconditioned with 2 mL of methanol and 2 mL of water. After washing the column with 2 mL of water, the phenolic agents were eluted with 2 mL of methanol.

2.3 Folin-Ciocalteu assay for total phenolics

A 50-µL aliquot of the clear supernatant obtained from SPE was neutralized with 50 µL of 2 M NaOH and diluted to exactly 5 mL with 5 mM, pH 7.4 phosphate buffer. Two microliters of the resulting supernatant was mixed with 200 µL of Folin and Ciocalteu's Phenol reagent (Sigma-Aldrich, St. Louis, MO) and 400 µL of saturated Na_2CO_3 for 3 min followed by mixing with 1.4 µL distilled water. After exactly 60 min incubation in the dark at room temperature, the solution was read spectrophotometrically at 725 nm. The 3,3′,4′,5,7-pentahydroxyflavone (quercetin, Sigma-Aldrich) was used as an external standard, and total phenolic content was reported in quercetin equivalents based on a calibration curve.

2.4 HPLC characterization and quantitation of individual phenolics

According to our previous publication [50], 100 µL of the clear supernatant obtained from SPE as mentioned above was injected into a Beckman "Gold Nouveau" HPLC system (Beckman Instruments, Fullerton, CA) equipped with a guard column (Alltech, Deerfield, IL) coupled to an Alltima C-18 (250 × 4.6 mm; id 4 mm) RP column (Alltech). Elution was carried out at a flow rate of 0.8 mL/min with the following solvent system: A = methanol, B = 10% acetic acid in water v/v. The following gradient elution scheme was utilized: 100% B in A v/v, decreasing linearly to 90% B in A in 5 min, holding at 90% B in A for 5 min, decreasing linearly to 60% B in A in 20 min, and linearly down again to 30% B in A in 10 min. After holding at 30% B in A for 6 min, B was increased linearly back to 100% in A in 5 min, thus equilibrating the system for subsequent injections. The phenolics were monitored with a dual channel diode array detector at 280 and 355 nm simultaneously, and peaks were scanned between 190 and 450 nm for identification purposes. Retention times and UV/VIS scans of the analytes were compared with those of authentic material of the phenolic acids 3,4-dihydroxycinnamic acid (caffeic acid, Sigma-Aldrich) and 3,4,5-trihydroxybenzoic acid (gallic acid, Sigma-Aldrich) and the flavonoids 4′,5,7-trihydroxyflavone (apigenin, Aldrich Chemical Co., Milwaukee, WI), 3,5,7-trihydroxy-2-[4-hydroxyphenyl]-4H-1-benzopyran-4-one (kaempferol, Sigma-Aldrich), 3′,4′,5,7-tetrahydroxyflavone (luteolin, Sigma-Aldrich), quercetin, kaempferol-3-O-glucoside (Indofine Chemical, Hillsborough, NJ), luteolin-7-O-glucoside (Indofine Chemical), and quercetin-3-rutinoside (rutin, Acros Organics, Geel, Belgium) (Fig. 1) and by co-chromatography with the authentic material. Individual phenolics present in the samples were quantified using their peak areas and calibration curves obtained from authentic material. Additionally, the internal standard was used to adjust values for analyte loss during the extraction procedure.

2.5 Statistical analysis

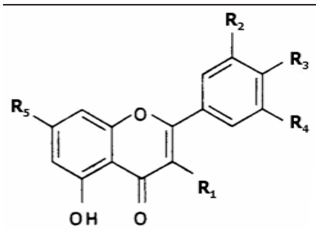
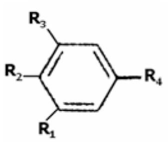
The SAS statistical system 8.0 (SAS Institute, Cary, NC) was used for statistical analysis. For phenolic levels, the significance of differences between organic and conventional systems was determined by one-way ANOVA and comparisons were analyzed by Tukey's post-hoc test. Data are presented as means ± SEM of six samples per group. Differences were considered significant at $p = 0.05$.

3 Results

3.1 Individual phytochemicals

The phenolic acids (caffeic acid and gallic acid) and the aglycone and glycoside forms of the flavonoids (apigenin, kaempferol, luteolin, and quercetin) (Table 1) were detectable by HPLC. A representative HPLC chromatogram of standard phenolics and sample extracts has been published separately [50]. Table 2 shows concentrations of individual flavonoids and phenolic acids in three cultivars of leafy green vegetables and pac choi. The predominant form of the flavonoids in leafy greens was their glucose conjugate or glycoside form, with levels considerably higher than those found for the aglycone forms. All the three glycoside flavonoids were not detectable in pac choi samples.

Table 1. Chemical structures of phenolic compounds tested in this study

		Functional group				
		R_1	R_2	R_3	R_4	R_5
	*Flavonoid*					
	Apigenin	H	H	OH	H	OH
	Luteolin	H	OH	OH	H	OH
	Kaempferol	OH	H	OH	H	OH
	Quercetin	OH	OH	OH	H	OH
	Luteolin-7-O-glucoside	H	OH	OH	H	O-Glu
	Kaempferol-3-O-glucoside	OH	H	OH	H	O-Glu
	Quercetin-3-rutinoside	OH	OH	OH	H	O-Glu
	*Phenolic acid*					
	Caffeic acid	H	OH	OH	CH_2CH_2COOH	
	Gallic acid	OH	OH	OH	COOH	

Although organic samples often exhibited moderately higher levels of individual phytochemicals than did their conventional counterparts, the differences were predominantly insignificant except for kaempferol-3-O-glucoside in the top brunch collard green that was significantly higher in organic than conventional samples.

3.2 Total phenolic content

The Folin-Ciocalteu assay was used to determine spectrophotometrically total phenolic content in quercetin equivalents. As shown in Table 2, no significant differences were found between organic and conventional vegetables except for pac choi. Organic samples of pac choi suffered greater insect attack than did the conventional samples. Severity of damage to the leaves was visibly noticeable as shown in Fig. 1. Although no significant differences were found in individual phenolic levels between organic and conventional pac choi samples, we found significantly higher levels of total phenolics ($p < 0.01$) in the organic samples compared to conventional ones (Fig. 1).

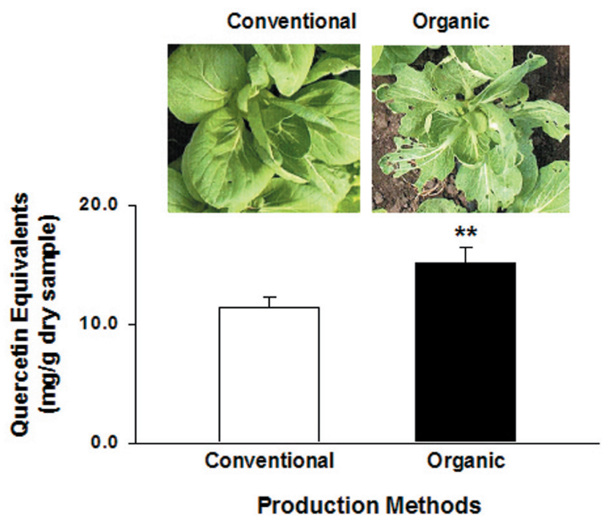


Figure 1. Total phenolic contents as measured by Folin-Ciocalteu assay in the pac shoi samples grown conventionally and organically, respectively. The top shows the differential insect attack in the representative samples. Total phenolic contents in quercetin equivalents at mg/g dry sample are shown as mean ± SEM of six samples per group. ***p* < 0.01 versus the conventional samples.

4 Discussion

The growing popularity of organic foods has been fueled not only by a general consumer perception that these foods are free of insecticide, herbicide and non-organic fertilizers but also by an attitude that these foods are nutritionally superior to conventional foods. Studies focusing on this issue have been unable to find consistent differences in the levels of certain macro and micronutrients of organic and conventional foods, including fruits and vegetables [41–46]. It has been hypothesized that because of a tendency to increase environmental stresses on the plant and thus the activity of PAL, organic production techniques may cause elevated levels of plant secondary metabolites. The increase of the biologically active phytochemicals in plant foods, therefore, may offer health benefits against several chronic diseases. A few studies have attempted to examine levels of phytochemicals in plant foods grown using organic and conventional methods; however, the published results have been contradictory and, for the most part, inconclusive.

In this controlled crop production study, organically grown vegetable samples were cultivated side-by-side to a conventional counterpart on a certified organic facility. Individual phenolics were detected and quantified by HPLC, and the

Table 2. Comparison of mean individual phytochemical and mean total phenolic levels in vegetables grown organically and conventionally

Phytochemical[a]		Cultivar[b]	Summer 2003 trial	
			Organic	Conventional
Aglycone flavonoids	Apigenin	KA	0.5 ± 0.1	0.3 ± 0.1
		RS	0.6 ± 0.1	0.4 ± 0.1
		TB	0.6 ± 0.2	0.9 ± 0.2
		PC	1.0 ± 0.2	1.2 ± 0.2
	Kaempferol	KA	2.0 ± 0.2	1.6 ± 0.2
		RS	1.9 ± 0.2	1.6 ± 0.2
		TB	2.1 ± 0.2	2.0 ± 0.2
		PC	1.8 ± 0.3	2.4 ± 0.3
	Luteolin	KA	1.8 ± 1.6	1.1 ± 1.8
		RS	2.2 ± 1.6	1.6 ± 2.2
		TB	6.0 ± 1.5	1.9 ± 1.6
		PC	16.1 ± 1.9	11.4 ± 1.9
	Quercetin	KA	0.7 ± 0.4	0.2 ± 0.4
		RS	1.0 ± 0.4	0.7 ± 0.4
		TB	1.1 ± 0.4	0.7 ± 0.4
		PC	3.4 ± 1.0	3.4 ± 1.0
Phenolic acids	Caffeic acid	KA	8.9 ± 1.0	8.2 ± 1.0
		RS	9.5 ± 1.0	10.6 ± 1.0
		TB	6.8 ± 1.0	5.4 ± 1.1
		PC	7.1 ± 1.3	7.2 ± 1.3
	Gallic acid	KA	8.1 ± 2.6	9.4 ± 2.6
		RS	14.5 ± 2.6	9.2 ± 2.6
		TB	16.2 ± 2.6	17.0 ± 2.6
		PC	25.5 ± 0.9	19.2 ± 3.4
Glycoside flavonoids	Kaempferol-3-O-glucoside	KA	9.8 ± 3.0	1.7 ± 3.3
		RS	10.5 ± 3.3	9.1 ± 3.3
		TB	30.2 ± 3.0	12.8 ± 3.0*
		PC	ND[c]	ND
	Luteolin-7-O-glucoside	KA	18.5 ± 13.7	24.1 ± 15.3
		RS	56.1 ± 13.7	85.4 ± 13.7
		TB	15.5 ± 13.7	18.3 ± 13.7
		PC	ND	ND
	Quercetin-3-rutinoside (Rutin)	KA	40.1 ± 16.2	16.7 ± 17.4
		RS	169.9 ± 16.2	184.5 ± 16.2
		TB	24.8 ± 16.2	14.8 ± 16.2
		PC	ND	ND
Total phenolics		KA	5.2 ± 1.9	5.2 ± 1.9
		RS	13.5 ± 1.9	12.5 ± 1.9
		TB	9.5 ± 1.9	9.4 ± 1.9
		PC	15.2 ± 0.9	11.5 ± 0.9**

a) Unit = µmol/g dry sample for aglycone flavonoids and phenolic acids; Unit = mg quercetin equivalent/g dry sample for total phenolics.
b) Abbreviations: KA, Kalura leaf lettuce; RS, Red Sails leaf lettuce; TB, Top Bunch collard green; PC, Pac Choi.
c) ND = not detectable. *$p < 0.05$, **$p < 0.01$ (conventional *vs*. organic, mean ± SEM, $n = 6$ samplers per group).

total phenolic content was quantified by the Folin-Ciocalteu assay.

Consistent with findings from Crozier et al [51], the predominant forms of flavonoids present in the leafy green samples were glycosides rather than aglycones. As summarized in Table 2, organic production statistically did not significantly increase individual or total phenolic levels when compared to conventional. Variability in the levels of individual phenolics appeared to be a major factor in the failure to find statistical significance. While this experimental design had controlled well for abiotic environmental stresses (*e.g.*, fertilizer, weather, sunlight, temperature, *etc*.), the biotic environmental stresses (*e.g.*, microorganism infection, insect damage, *etc.*) might not have been enough to induce significant differences in phenolic levels between organic and conventional samples. The organic and conventional pac choi samples received an application of either organic or conventional insecticide during cultivation to control attack by flea beetles. This resulted in differential insect control with the organic samples suffering greater damage. Interestingly, these organic samples showed significantly higher levels of total phenolics than did their conventional counterparts (Fig. 2). In this case, biotic stress seemed to be great enough to overcome the variability among samples.

A few studies by others have compared the contents of certain phytochemicals between organic and conventional fruits and vegetables. Asami *et al.* [48] and Carbonaro *et al.* [49] used agricultural production techniques to examine phenolic levels in organic and conventional plant foods and found a higher level of certain phenolics in organic samples than in those grown conventionally. A Swiss group [52] assayed apples and found significantly higher levels of phenolics in the organic fruit. However, the Finnish researchers Häakkinen and Törrönen [34] analyzed strawberries for differences in phenolic content between organic and conventional systems and found no differences. Interestingly, the Swiss study retrieved samples from non-controlled, neighboring production facilities rather than from organic or conventional plots within a facility, and the Finnish study compared strawberries grown on 17 different production facilities, with some designated as organic and some as conventional. Therefore, the experimental design of those previous studies was different in regards to controlling environmental factors, which could lead to inconclusive results.

Taken together, this study differed from previous studies in several aspects. Organic and conventional vegetables were grown side-by-side on a controlled and certified organic production facility, eliminating the bias induced by uncontrolled environmental factors that may have influenced results from previous studies. Furthermore, nine individual phenolics were examined, combined with an assay for total phenolics. Under these controlled growing conditions, no differences were found in levels of individual and total phenolics between leaf lettuce and collards grown organically and conventionally. In response to insect attack, organic pac choi samples produced higher levels of total phenolics than conventional samples, suggesting that insect attack might be a biotic stress factor contributing to higher levels of total phenolic agents in some vegetables from organic production systems.

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