Biologically important thiols in various organically and conventionally grown vegetables

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Summary

Biothiols are well-known beneficial antioxidants that protect cells from oxidative damage that potentially leads to cancer and Alzheimer's disease. This study investigated and compared the levels of important biological thiols including glutathione (L-glutamyl-L-cysteinyl glycine, GSH), *N*-acetylcysteine (NAC), captopril (CAP), cysteine (CYS), and γ-glutamyl cysteine (GGC) in a variety of organic versus conventional (industrially-produced) asparagus, spinach, green beans and red peppers, using a sensitive high-performance liquid chromatography (HPLC) technique. Only the contents of CYS, or CYS with NAC, were detected as significantly higher in organic green beans or organic asparagus, respectively. However, conventional spinach contained significantly higher amounts of all investigated thiols than contained the organic spinach. Results also showed that conventional or organic production methods did not significantly affect the contents of CAP, GSH or CYS in asparagus, green beans or red peppers. In conclusion, production methods do not appear to be the only factor affecting the thiol contents in vegetables.

Keywords

biothiols; conventional vegetables; organic vegetables

In recent years, food safety issues, have been given more attention worldwide by governments, politicians, health institutions and food industries as the primary factor in determining the food preferences of consumers. This has promoted the introduction of safer and healthier foods in the market places. Increasing consumer consciousness about food science could also influence successful marketing prospects for international business and the agricultural industry, as well as impacting politics and the purchasing behaviour of people.

Organic farming, considered a safe food production method, has become the fastest growing industry in the United States and in the European Union since 1990, resulting in an increasing demand for organic foods [1]. The perception of health risks posed by artificial fertilizers and chemical pesticides used in agricultural production has caused people to shift their food purchases towards more organic products, in spite of their sometimes higher direct costs [1–3]. It is generally thought that when agricultural products are grown with artificial fertilizers or pesticides, their natural defense mechanisms are impaired. On the other hand, it is also thought that organic foods tend to have more distinguishable sensory properties, higher nutritional value and contain more protective phytochemicals [4, 5]. In the literature, most of the studies which were designed to compare nutrient quality of produce grown by using organic or conventional methods indicated that organic products contained more vitamin C and less nitrates. Conversely, another study conducted in France on some food contaminants (lead, mercury, cadmium, arsenic, nitrites, nitrates, mycotoxins) claimed that organic foods could be categorized as unsafe [6]. In addition, too many differences in growing conditions of products or methods of analysis applied in these studies often make it difficult to compare these results [7].

Vegetables contain varied concentrations of important biologic thiols possessing antioxidant properties [8]. Thiols (or biological thiols) are compounds characterized as a type of mercaptans carrying a sulfhydryl functional group [9–11]. They protect cells from oxidative damage which leads to diseases such as cancer or Alzheimer's disease [8]. DEMIRKOL et al. [12] claimed that thiols could act

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as antimutation or anticarcinogenic agents. Due to these significant properties of thiols, interest in this topic has recently been rising [8, 12–15].

Thiols encompass compounds such as glutathione (GSH, L-γ-glutamyl cysteinyl-glycine), cysteine (CYS), *N*-acetylcysteine (NAC), γ-glutamyl cysteine (GGC) and captopril (CAP) [8]. The tripeptid form of GSH, which is predominantly intracellular and has a low molecular weight, is extensively found in the cells of animals and plants [14, 15]. In addition to GSH protecting cells from the toxic effect of reactive oxygen substances, it plays important roles in the synthesis of proteins and DNA, the synthesis of leucotrians used in the treatment of inflammations, and preservation of reduced forms of sulfhydryl groups in proteins [15]. Studies indicated that GSH protected plants from heavy metal poisoning and certain amounts of GSH induced accumulation of anthocyanins [16]. Its other physiological roles include storage and transport of CYS, plus a co-enzymatic role in several reactions with foreign compounds [9]. CYS and GSH delivery compounds have been used to protect normal cells from antitumor agents and radiation [17]. Another thiol compound, NAC, is a precursor of GSH and is an antioxidant as well. NAC has long been used as a mucolytic agent for treating chronical bronchitis as well as being an effective antidote for acetaminophen poisoning and the drug of choice at paracetamol intoxication [17–19]. Furthermore, according to results of one study, the addition of GSH, NAC or Vitamin C at the beginning of the reaction between nitrite and the drug, it had a similar effect on inhibiting mutations in vitro [20]. Nonetheless, after completion of the nitrosation reaction, only GSH or NAC provided inhibition. CAP, a synthetic thiol and inhibitor of angiotensin converting enzyme (ACE), has also been postulated as a free radical scavenger because of its terminal sulfhydryl group [21].

No studies in the literature compared the thiol contents of vegetables grown by organic methods to vegetables produced by conventional methods. For that reason, the present study was designed to determine the contents of GSH, NAC, CYS, GGC and CAP in organic and in conventional vegetables.

MATERIALS AND METHODS

Reagents and chemicals

HPLC-grade acetonitrile, acetic acid, and *o*-phosphoric acid were purchased from Fisher Scientific (Fairlawn, New Jersey, USA). Glutathione (GSH, L-γ-glutamyl cysteinyl-glycine), γ-glutamyl cysteine (GGC), *N*-acetylcysteine (NAC), captopril (CAP), cysteine (CYS), oxidized glutathione (GSSG), *N*-(1-pyrenylmaleimide) (NPM), L-serine, trizma hydrochloride, and diethylenetriaminepentaacetic acid (DETAPAC) were purchased from either Sigma (St. Louis, Missouri, USA) or Aldrich (Milwaukee, Wisconsin, USA). Milli-Q (MQ) water with a resistivity of > 18.2 MΩ was produced by a Simplicity 185 water purification system (Millipore, Bedford, Massachusetts, USA) to prepare both HPLC mobile phases and biothiol standard solutions.

Vegetables

A suite of conventional vegetables, including spinach (*Spinacia oleracea*), green beans (*Phaseolus vulgaris*), green asparagus (*Asparagus officinalis*), and red bell peppers (*Capsicum annuum*) were obtained from a local grocery store in Rolla, Missouri, USA in April 2005. Organic vegetables were obtained from Diamond Organics Company, California, USA in April 2005. All of the studied vegetables from this supplier were rinsed three times with tap water for cleaning purposes.

HPLC System

A Finnigan high-performance liquid chromatography (HPLC) system (Thermo Electron, San Jose, California, USA), consisting of a vacuum membrane degasser, an injection valve with a 5 μl injection filling loop, two gradient pumps, an autosampler and a fluorescence detector, was used to determine the concentrations of biothiols and GSSG. The fluorescence detector was operated at an excitation wavelength of 330 nm and an emission wavelength of 376 nm. A reversed-phase Reliasil ODS-1 C18 column (5 μm, 250 mm × 4.6 mm; Column Engineering, Ontario, California, USA) was utilized for biothiol separation. The mobile phase consisted of 70% acetonitrile and 30% MQ water, and was adjusted to approximately pH 2.5 by addition of 1 ml of acetic acid and 1 ml of *o*-phosphoric acid per liter of the mobile phase. Prior to use, the mobile phase was vacuumed under sonication for 30 min to drive out dissolved gas bubbles.

Biothiol Analysis

Because our preliminary work showed that the biothiol contents of a batch of a vegetable may vary significantly from one sample to another, 15 vegetables were analysed in replicate for both conventional and organic vegetable samples. Specifically, samples were replicates five times, with each replicate containing a mix of three cutlets (about 0.5 g per piece) cut from each individual vegetable. A cutlet represented a piece of vegetable from the central part of the vegetable body, cut across the whole central portion. As a result, the analysed biothiol level in spinach, green beans, asparagus and red peppers should represent the level occurring over the entire vegetable. The three-cutlet mix was placed in a serine borate buffer (SBB) to prevent potential oxidation of biothiols by atmospheric oxygen. The SBB buffer comprised 100 mM Tris-HCl, 10 mM borate, 5 mM serine and 1 mM diethylenetriaminepentaacetic acid (DETAPAC) with the final pH adjusted to 7.0 with a concentrated NaOH solution. The samples were homogenized in SBB buffer with a Tissue-Tearor (Biospec Products, Bartlesville, Oklahoma, USA) on ice for 2 min and centrifuged at 10 000 *g* for 15 min at a controlled temperature of 4 °C to extract biothiols from the vegetables. Thereafter, 40 μl of the supernatant were withdrawn and derivatized with *N*-(1-pyrenylmaleimide; NPM), which reacted with free sulfhydryl groups of biothiols to form fluorescent derivatives. Each sample was diluted with 210 μl of MQ water and derivatized with 750 μl of NPM (1 mM in acetonitrile). The resulting solution was vigorously mixed and allowed to react at an ambient temperature for 5 min. An HCl solution (10 μl, 2 M) was then added inside to stop the reaction. After being filtered through a 0.20 μm nylon filter (Advantec MFS, Dublin, California, USA), using a 3 ml syringe, the derivatized samples were injected into the HPLC system. The biothiol-NPM derivatives were separately eluted from the HPLC column with the mobile phase flowing isocratically at a rate of 1 ml.min^{-1}. GSH, GGC, NAC, CAP and CYS could be determined concurrently since all these biothiols form fluorescent derivatives with NPM [14]. The biothiol-NPM peaks were quantified with Thermo LC software (Thermo Electron, San Jose, California, USA). The linearity of standard calibration curves was confirmed over a concentration range of 0–10 μM for each biothiol in a mixture. The biothiol-NPM derivatives were eluted from the HPLC column in the following sequence: NAC (3.47 min), CAP (3.99 min), GSH (8.24 min), GGC (9.42 min), CYS (10.74 min).

GSSG Analysis

GSSG, the primary oxidation product of GSH, was determined by reacting 84 μl of vegetable supernatant with 16 μl of 2-vinylpyridine (Aldrich; 6.25% in ethanol) for 1 h to block any pre-existing GSH. After the reaction, 95 μl of an NADPH solution (Sigma; 2 mg.ml^{-1}) and 5 μl of a glutathione reductase solution (Sigma; 2 U.ml^{-1}) were added sequentially. An aliquot of 100 μl of the resulting solution was quickly withdrawn and mixed with 150 μl of MQ water and 750 μl of NPM (1 mM in acetonitrile) for derivatization. After 5 min, the reaction was stopped by adding 5 μl of HCl solution (2 M). The samples were then filtered through a 0.20 μm nylon filter and injected into the HPLC system [22]. The GSSG-NPM derivative was eluted from the HPLC column at 8.63 min.

Statistical analysis

The values represent mean ± *SD* (standard deviation) of five replicated samples. The one-way analysis of variance (ANOVA) test was used to analyse the data from the experimental and control groups, with p values of < 0.05 considered to be significant.

RESULTS

Asparagus

Five biothiols were detected in asparagus including GSH, CYS, CAP and NAC, only GGC was not found. As with spinach, the results demonstrated that conventionally produced asparagus contained significantly higher concentrations of GSH than the organic asparagus did (Fig. 1A; $p < 0.05$). On the other hand, organic or conventional asparagus contained approximately the same amount of CAP – (68.4 ± 11) nmol.g^{-1} or (92 ± 29) nmol.g^{-1}, respectively ($p < 0.05$). The results also indicated that the detected quantities of CYS and NAC were significantly higher in the organic asparagus, with baseline concentrations of (183 ± 32) nmol.g^{-1} and (177 ± 11) nmol.g^{-1}, respectively, than in conventional ones – (40 ± 13) nmol.g^{-1} and (106 ± 13) nmol.g^{-1}, respectively. In the organic asparagus, the amount of GSH detected was 71% less, but the amounts of CYS and NAC detected were 78% and 40% higher, respectively. Error bars in the figures represent the standard deviation of five replicates, with each replicate containing a mixture of three cutlets from each individual vegetable. The ratio of GSH to GSSG (reduced glutathione to oxidized glutathione) is a parameter that indicates significance of the oxidative stress. The mean GSH/GSSG ratio for the conventional asparagus (575 ± 61) was significantly higher than that for the organic asparagus (156 ± 24; $p < 0.05$, Tab. 1).

Spinach

Only three biothiols were detected in spinach, including GSH, GGC and CYS. The concentration of GHS (377 ± 38) nmol.g^{-1} in conventional samples was found to be significantly higher than the

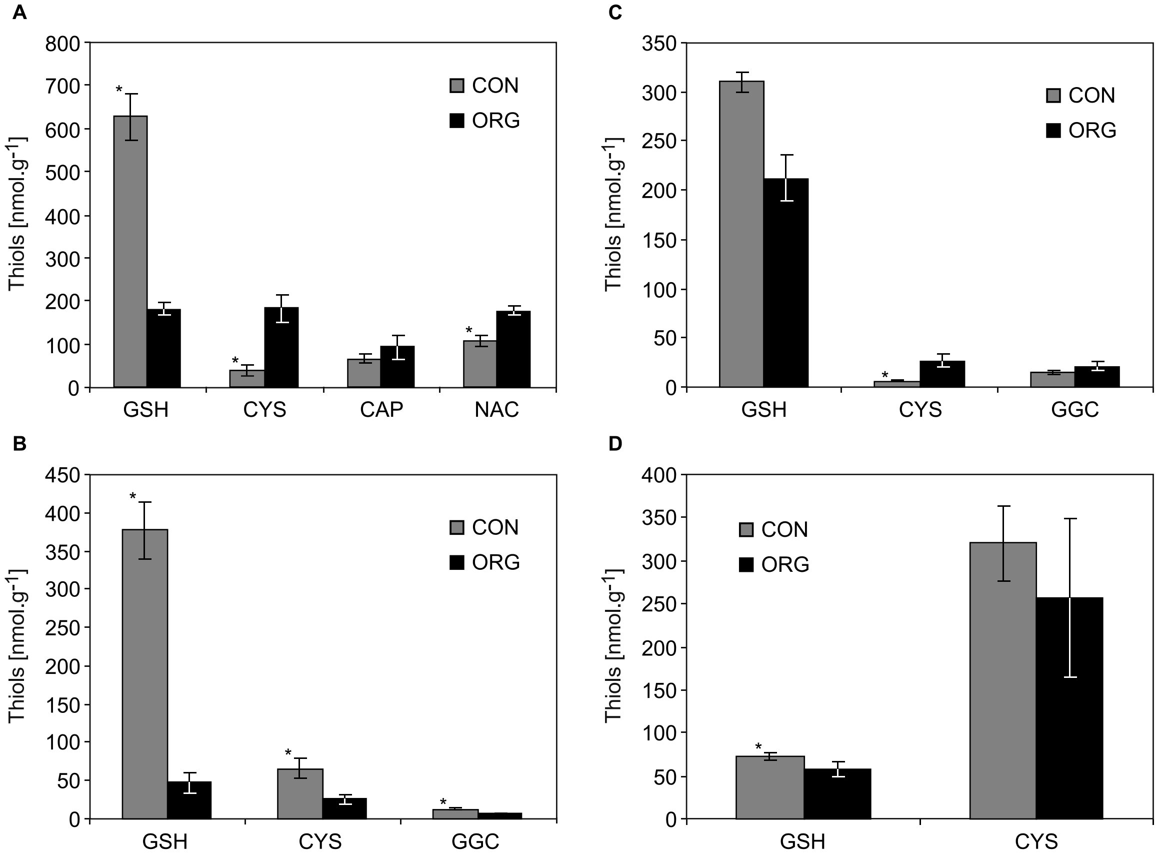

Fig. 1. Thiol compositions of conventionally and organically grown asparagus (A), spinach (B), green beans (C) and red pepper (D).

Values are mean ± SD. CON – conventionally grown, ORG – organically grown. * – Significantly different when compared with organic red peppers ($p < 0.05$, $n = 5$).

concentration of GHS (47 ± 14) $nmol.g^{-1}$ in the organic samples ($p < 0.05$). Moreover, the concentrations of GGC and CYS in conventional spinach samples were also significantly higher than those in organic spinach samples ($p < 0.05$, Fig. 1-B). In fact, the levels of GSH, GGC and CYS in the organic samples were 87.5%, 60% and 46% less than in conventional samples, respectively. Results further showed that the mean of the GSH/GSSG ratio for conventional spinach (4.3 ± 1.2) was significantly different from that for organic spinach (0.78 ± 0.19; $p < 0.05$, Tab. 1).

Green beans

Similar to spinach, three biothiols, GSH, GGC and CYS, were detected in green beans with baseline concentrations of (310 ± 10) $nmol.g^{-1}$, (15 ± 2) $nmol.g^{-1}$and(6.5 ± 0.4) $nmol.g^{-1}$,respectively

Tab. 1. The ratio GSH/GSSG in conventionally and organically grown vegetables.

Vegetables	Conventional	Organic
Asparagus	575 ± 61*	156 ± 24
Spinach	4.3 ± 1.2*	0.78 ± 0.19
Green Bean	34 ± 10*	47 ± 19
Red Pepper	53 ± 14*	31 ± 6

All values are averages of five samples ± SD. * – $p < 0.05$ compared the ratio of GSH/GSSG in conventional vegetables to that in organic ones.

(Fig. 1-C). The levels of GSH (212 ± 23) $nmol.g^{-1}$ or GGC (21 ± 5) $nmol.g^{-1}$ in organically grown green beans were not significantly different from those produced conventionally ($p < 0.05$). However, the amount of CYS in organic green beans was 330.7% higher than that in conventional green beans. The mean GSH/GSSG ratio for conventional green beans (34 ± 10) was significantly lower than that for organic green beans (47 ± 19; $p < 0.05$; Tab. 1).

Red peppers

Two biothiols, GSH and CYS, were detected in red peppers with baseline concentrations of (73 ± 4) $nmol.g^{-1}$ and (320 ± 44) $nmol.g^{-1}$, respectively (Fig. 1-D). According to results, organic red peppers contained significantly smaller amounts of GSH, (58 ± 9) $nmol.g^{-1}$, $p < 0.05$. In addition, the amounts of CYS were not significantly different in organic and conventional red peppers ($p < 0.05$). Red peppers, compared to other vegetables investigated, contained much more CYS. Results also indicated that the mean GSH/GSSG ratio (53 ± 14) for conventional red peppers was significantly different from that for organic red peppers (31 ± 6; $p < 0.05$; Tab. 1).

DISCUSSION

The growing popularity of organic foods has increased, not only because of the general assumption that these foods are free of insecticides, herbicides and synthetic fertilizers, but also because of the belief that these foods are nutritionally superior to conventional foods. Studies focussing on this issue have been unable to confirm consistent differences in the levels of certain macro and micronutrients in organic or in conventional foods, including fruits and vegetables [5, 23–26]. Most of these studies determined that the amounts of trace elements, minerals, Vitamin A, Vitamin B and beta carotene were essentially the same in organic and in conventional vegetables. The present study also comparatively demonstrated that the use of conventional or organic methods of production did not significantly change the concentrations of CAP, GSH or CYS in asparagus, green beans or red peppers.

Some of the thiols investigated in this study were determined to be higher in organic vegetables than in conventional ones. For instance, the level of CYS in organic green beans and the levels of CYS and NAC in the organic asparagus were detected to be higher. Some studies of phytochemicals in various organic fruits and vegetables explained this phenomenon especially being the result of an increased environmental stress (insects, diseases, fertilizers, weather) placed on the plant due to organic farming [27, 28]. Although genetics are the primary determinant of the composition of secondary metabolites, environmental and phytopathogenic stress also play roles in the production of plant defense compounds. In fact, under stressful conditions, some catalysing enzymes might be activated and could cause an increase in the levels of plant secondary metabolites [3, 29]. For instance, some studies demonstrated that organic tomatoes contained more lycopene and organic potatoes and peaches contained more polyphenols than conventional ones [1, 3]. However, we also reported that the conventional spinach contained higher amounts of thiols than the organic spinach. The reason for this result can be explained if the environmental pressure in the place where the organic spinach grew was not sufficient to induce significant differences between the thiol contents. In accordance with our results, Young et al. [29] also did not find consistent differences between the amounts of phytochemicals in vegetables that were grown organically or those that were conventionally produced because of an inadequate stress.

The current study, the first to investigate the concentrations of GSH detected in various vegetables that were organically or conventionally grown, determined that the GSH contents in organic spinach, asparagus and red peppers was lower than that in conventional ones. Fundamental differences between organic and conventional production systems, particularly soil fertility management, have the potential to affect the nutritive composition of plants and, in particular, secondary plant metabolites [4]. Most of the synthetic fertilizers used in conventional farming are composed of more nitrogen than those that are used in organic farming. Generally, the amino acids or the protein contents of plants increased parallel with increasing amounts of nitrogen in their growing conditions. The results of one study supported the issue by reporting detection of higher amounts of proteins or amino acids in conventionally grown spinach, beets, carrots, tomatoes and potatoes than in those grown organically [27]. To obtain the nitrogen needed for synthesis of GSH, consisting of three amino acids [30], the GSH levels in plants using conventional production methods must be increased by using the high nitrogen contents of synthetic fertilizers. Also, the antocyanin level, which is induced by the GSH level in a plant [16], was detected as being distinctively higher in conventional grapes than in organic ones [31]. Accordingly, the GHS levels in conventional grapes, must also be higher, which further supports our findings.

According to some studies, the occurrence of contaminants (heavy metals or mycotoxins) in a plant tissue can reduce the amount of thiols [32, 33]. Evidently thiols, especially CYS and GSH, may protect the cells in a plant tissue from damage caused by such contaminants. Detoxication of mycotoxins and heavy metals is a pivotal capacity of organisms, in which GSH plays an important role. In plants, mycotoxins are conjugated to the thiol group of GSH, and heavy metal ions form complexes as thiolates with GSH-derived phytochelatins (PCs). In fact, one study determined excessive amounts of lead, cadmium or mycotoxins (patulin) in the organic vegetables [6]. The other possible explanation is that the organic vegetables tested in this study could have possibly been contaminated with mycotoxins or heavy metals, which would have reduced the amounts of GSH detected in spinach, green beans, asparagus and red peppers.

Conventional food production is also characterized by the use of synthetic herbicides, pesticides and insecticides. Several lines of evidence indicate a qualitative involvement of herbicides safeners in the protection of GSH, CYS and GGC in plant tissues [34, 35]. Herbicide safeners are a group of different synthetic compounds that are able to protect some plants against injury from certain herbicides. The mechanism of safener action has been found to involve the action of glutathione *S*-transferases, a family of enzymes promoting the conjugation of GSH [36–38]. These findings could explain the present detection of high amounts of GSH in vegetables grown by conventional production techniques, including the possible use of a herbicide containing herbicide safeners.

In this study, the ratio of GSH/GSSG was also analysed. In plant cells, GSH is synthesized by using glutamic acid and cysteine. However, under the oxidative stress, GSH in cells is oxidized, which leads to an increase in the concentration of GSSG. The ratio of GSH/GSSG is generally used as an indicator to provide information about the cellular redox state and oxidative capacity of cells. This ratio is usually found to be higher than 10 in normal physiological conditions [39]. However, in our study, the ratio of GSH/GSSG in organic and conventional spinach was detected as being less than 10, which indicates that spinach is exposed to too much oxidative stress because of its delicate leafy structure. The present study also demonstrated that the ratio was higher in conventional vegetables (except for green beans) than in the organic ones. This means that conventional spinach, asparagus and red peppers with a higher ratio of GSH/GSSG than organic vegetables, must have been exposed to less oxidative stress (e. g. insects, fungi, malnutrition,) than the organically produced vegetables.

CONCLUSION

Based on the results of this study, it cannot be concluded that organic vegetables contain higher amounts of biothiols than conventional samples. Some thiols were even detected in smaller amounts in organic vegetables, possibly due to the environmental stress. If the defense-related thiols of vegetables and fruits are of an important nutritional value in the diet, then organic vegetables cannot be expected to promote better health than conventional ones. Further investigation will be needed to determine the thiols contents of fruits produced organically and conventionally.

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