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Analytical Methods

Determination of neurotoxic agents as markers of common vetch adulteration in lentil by LC-MS/MS

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ABSTRACT

Vicia sativa (common vetch), which contains β-cyanoalanine (BCA) and γ-glutamyl β-cyanoalanine (γGBCA) is used for adulteration of lentil and have neuro toxic effects on people and animals. Therefore the determination of BCA and γGBCA analytes in accurate and reliable manner has high importance for human and animal health. LC-IDMS/MS method has been developed and validated for quantitative analyses of β-cyanoalanine and ^{13}C labeled BCA is used as an internal standard in γGBCA analysis. In order to validate the method, linearity, recovery, precision (repeatability), intermediate precision, limit of detection, and limit of quantification parameters were investigated. The correlation coefficient was found to be greater than 0.99 for both analytes. The recoveriess were determined as 95.8% for BCA and 97.4% for γGBCA. The relative expanded measurement uncertainties of β-cyanoalanine and γ-glutamyl-β-cyanoalanine were obtained as 4.6% and 5.8%, respectively with the coverage factor, k, is 2 and at 95% confidence level.

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1. Introduction

Due to the rising requirement for food for ever-growing population, narrowing field of agriculture and excessive profit motivations, food adulteration is becoming increasingly common, which can cause irreparable adverse effects on human and animal health. Studies on identification of adulterations, especially in basic foods such as honey, meat products, milk, oil, and lentil, which is an important vegetarian food, have already been initiated. Lentil is adulterated by mixing it with *Vicia sativa* (common vetch; "fiğ" in Turkish), which is one of the species of the family Fabaceae. As it is very similar to lentil, it can only be identified by a botanical expert and/or experienced people by eye examination of shape of seed (Tate & Enneking, 1992). *Vicia sativa*, which has a very close nutritional value to that of lentil, contains β-cyanoalanine (BCA, up to 0.97%) and γ-glutamyl β-cyanoalanine (gGBCA, up to 2.6%). These substances well known as cause neurotoxic effects in people and animals (Enneking, 1995; Fushiya, Sato, Kusano, & Nozoe, 1993; Ressler, Nigam, Giza, & Nelson, 1963; Thavarajah, Thavarajah, Premakumara, & Vandenberg, 2012). The toxicity occurs by cyanide moiety of the amino acids, which is surprisingly widely present in nature, and is assimilated through different biological processes, among which β-cyano-l-alanine pathway is the best known one. As it is commonly known, ethylene biosynthesis stoichiometrically produces cyanide ions, which are deactivated by 3-cyanoalanine synthase enzyme. In this process, L-cysteine (**1**) serves as a precursor to assimilate the cyanide ions to β-cyano-L-alanine (**2**) (Scheme 1) (Yip & Yang, 1998). In the next biological process, β-cyano-L-alanine (**2**) is converted to asparagine (**3**) by enzymatic hydrolysis catalyzed by cyanoalanine hydratase (Scheme 1) (Castric, Farnden, & Conn, 1972).

To obtain more accurate and repeatable results and to reach lower quantitation limits for BCA measurement in any food product, such as those of common vetch and lentil, we developed an isotope dilution mass spectrometry (IDMS)-based method, which is the primary-ratio technique to determine the concentration of compounds in any matrice. In isotope dilution mass spectrometry (IDMS), at the start of the analysis, an isotopically labeled analogue (e.g., ^{13}C, ^{2}H for organic analytes) is added to the sample and allowed to reach equilibrium without any loss or isotopic fractionation. It compensates for any error at all stages of the analysis, i.e. from sample preparation to the final instrumental measurement (Binici, Bilsel, Karakas, Koyuncu, & Goren, 2013). The labeled β-[^{13}C]cyano-L-alanine was used to identify BCA by IDMS technique. The internal standard quantitation was applied for determination of γ-glutamyl β-cyanoalanine and labeled β-[^{13}C]cyano-L-alanine was as an internal standard.

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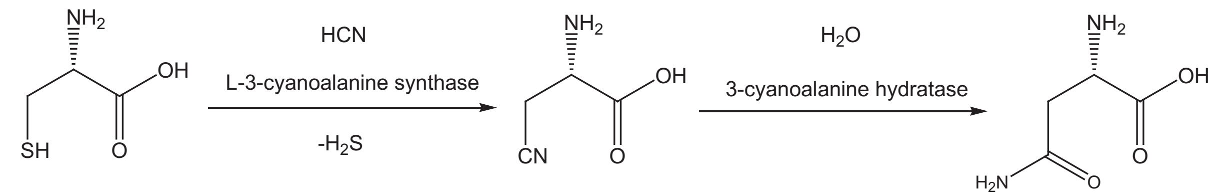

Scheme 1. Assimilation of cyanide ion via enzymatic pathway.

Some species of *Lathyrus* and *Vicia* produce neurotoxic factors causing neurolathyrism, which is characterized by "reflex irritability, weakness, spasticity, and rigidity of the leg muscles, could be followed by death" (Ressler & Malodeczky, 1962). β-Cyano-L-alanine itself and γ-L-glutamyl-β-cyano-L-alanine are two neurotoxic factors found in *Lathyrus sativus* and *Vicia sativa* (common vetch), videly used as animal feed (Friedmen & Shibko, 1972). Moreover, lentil, a daily staple of human diets, can easily be contaminated by the seeds of *Vicia sativa* during agricultural processing. Therefore, determination of the contamination level is very important from the perspective of human health.

Although DNA-based studies are available in the literature to identify adulteration or contamination with common vetch, they are not that reliable, as the measurements with biological techniques have high uncertainties (Bosmali, Ganopoulos, Madesis, & Tsaftaris, 2012; Chung, Kim, Suresh, Lee, & Cho, 2013). Therefore, development of better techniques for determination of the toxic compounds BCA and γGBCA is particularly important. Some studies already appeared in the literature for their isolation and qualitative analyses (Fushiya et al., 1993; Ressler & Malodeczky, 1962; Ressler, Tatake, Kaizer, & Putnam, 1997; Ressler et al., 1963), including an analytical method for detection of common vetch (*Vicia sativa* L.) in lentil, based on simple sample extraction and rapid high-performance liquid chromatography (HPLC-DAD) analysis of β-cyano-L-alanine together with γ-glutamyl-β-cyanoalanine. However, the detection limit of the proposed method for identifying adulteration was reported as 5% (Thavarajah et al., 2012).

Herein, we present the use of β-[^{13}C]cyano-L-alanine as an internal standard for determination of β-cyano-L-alanine and γ-gluta myl-β-cyanoalanine in lentil by propyl chloroformate derivatization liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2. Experimental

2.1. Chemicals and instrumentation

For the analytical experiments, β-cyanoalanine and labeled β-cyanoalanine were synthesized in our laboratories (Ghasemi & Secen, 2017). γ-Glutamyl β-cyanoalanine was provided by Dr. Max E. Tate from Department of Plant Science, University of Adelaide, Waite Campus, Glen Osmond SA 5064, South Australia. HPLC-grade acetonitrile was purchased from Fluka Company, Germany. HPLC-grade water was obtained using a Millipore Milli-Q water purification system. For the filtration of the samples, a Millex-HV 0.45-μm filter (Millipore) was used. The comparability of results over space and time is very important to assume the measurement as reliable, so then it becomes essential to link all the individual measurement results to some common, stable reference or measurement standard. Basen on the relationship between results and that reference, results can become comparable. This strategy of linking results to a reference is termed "traceability." In order to establish traceability, the purity assessments of β-cyanoalanine and γ-glutamyl β-cyanoalanine were performed by qNMR using NIST 350b (benzoic acid) and UME CRM 1301 (chloramphenicol), respectively. Purity of standards was determined as (85.1 ± 2.1)% and (95.0 ± 0.8)%, respectively, by qNMR. The expanded measurement uncertainty of compounds were obtained at 95% confidence level and the coverage factor, k, is 2. Labeled β-cyanoalanine was used as the internal standard (IS) for LC-MS/MS measurements.

2.2. LC-MS/MS measurements

LC-MS/MS analyses were conducted using a Zivak® HPLC and Zivak® Tandem Gold Triple Quadrupole (Istanbul, Turkey) mass spectrometer, equipped with a Phenomenex Luna 3 μm Phenyl-Hexyl column (150 × 2 mm i.d., 3 μm particle size). The mobile phase was composed of methanol (A) and water (B, 10 mM ammonium formate), the iscoratic program of which was 0–5.00 min 20% A and 80% B. The flow rate of the mobile phase was 0.25 mL/min, and the column temperature was set to 30 °C. The injection volume was 5 μL.

2.2.1. Preparation of standard solutions

10 mg of β-Cyanoalanine and 10 mg of γ-glutamyl β-cyanoalanine were weighed into the 25-mL volumetric flask and dissolved in a portion of 0.1 N HCl and *n*-propanol (70:30; v/v)% mixture and so first native stock solution was prepared. Then, 5 mL of first native stock solution was measured and added into to 50 mL volumetric flask and completed to volume thus 40 mg/L second native stock solution was prepared. On the other hand, the labeled β-cyanoalanine first stock solution was prepared by dissolving 20 mg of solid material in 10 mL volumetric flask and adjusting to the volume. Then 250 μL of first labeled stock solution was measured and added into the 25 mL volumetric flask and completed to the volume and so 20 mg/L second labeled stock solution was obtained. The calibration solution were prepared in 5 mL volumetric flask by adjusting the concentration of native compounds at seven levels as 0.05, 0.1, 0.5, 1, 2, 5, and 20 mg/L and the labeled as 2 mg/L at each level.

2.2.2. Extraction of analytes from lentil

2.2.2.1. Extraction procedure. The lentil and common vetch samples were pulverized in a mill, and 1 g of sample was weighed into a round-bottomed flask. In order to apply method, 12.5 mL of 20 mg/L BCA labeled second stock solution was added over the sample. After adding 50 mL of water, the mixture was heated at 95–102 °C for 1 h, 20 mL of sample was centrifuged at 5000 rpm (G-Force:1844), and then 100 μL of the supernatant was used for the derivatization step. The same procedure repeated for acidic extraction by 0.1 N HCl solution.

2.2.2.2. Derivatization of compounds with propyl chloroformate. 100 μL of extract was transferred into a 1.5-mL vial and 200 μL of *n*-propanol:pyridine (7:1) solution was added and the vial was vortexed for 30 s. Then, 200 μL of isooctane propyl chloroformate mixture (4:1) was added and the vial was vortexed additional for 30 s. To finalize derivatization of BCA and γGBCA and to get simultaneous extraction of measurands 200 μL of

Table 1
LC-MS/MS parameters of developed method for BCA and γGBCA analysis in lentil.

Compound	Parent ion	Daughter ion	Capillary Voltage	Collision Energy
BCA	260.00	243.00	50.000	6.000
IS	261.00	244.00	50.000	6.000
γGBCA	414.40	354.00	50.000	9.000

chloroform, isooctane, and propyl chloroformate mixture (20:12:1) was added to vial and was vortexed for 30 s. Finally, 500 μL of HCl solution (5%) was added to vial and it was vortexed once more for 30 s. The final solution was centrifuged for 2 min at 14,000 rpm. Then 200 μL of supernatant was transferred to a 1.5-mL HPLC vial and the solvent was evaporated under nitrogen at room temperature. After a drying process for 200 μL of the mobile phase added to vial and vortexed for 30 s. 5 μL of this sample was injected to liquid chromatography for quantification.

3. Results and discussions

3.1. Analytical chemistry

3.1.1. Optimization of HPLC methods and LC–MS/MS procedure

After evaluation of several mobile phases to obtain a good separation for the targeted peaks on the HPLC column, the best gradient was decided as methanol and water with ammonium formate. We attempted to analyze the samples without any derivatization step but unfortunately we coudn't get any reasonable result because the molecular ion peak of an interference in matrice was detected as M^+ 414, which is the same ion value like the labeled standard has. Then, the analytes were derivatized with propyl chloroformate, which resulted in improved ionization efficiency and the method was validated according to the proposed protocol described in Section 2.2.2.

The ESI technique is better than APCI technique for good ionization of small and relatively polar compounds (Gulcin et al., 2011; Kalın, Gulcin, & Goren, 2015). The optimum ESI parameters in triple quadrupole mass spectrometry were observed as 2.40 mTorr (4.6×10^{-5} psi) CID gas pressure, 5000 V ESI needle voltage, 600 V ESI shield voltage, 300 °C drying gas temperature, 50 °C API housing temperature, 55 psi nebulizer gas pressure, and 34 psi drying gas pressure. The molecular ions used for quantification are given in Table 1 and separation of the analytes' peaks was presented in Fig. 1.

3.1.2. Method validation

The linearity, recovery, precision (repeatability), intermediate precision, limit of detection (LOD) and limit of quantification (LOQ) parameters were evaluated during the method validation. EURACHEM and IUPAC (EURACHEM/CITAC, 2012) guidelines were

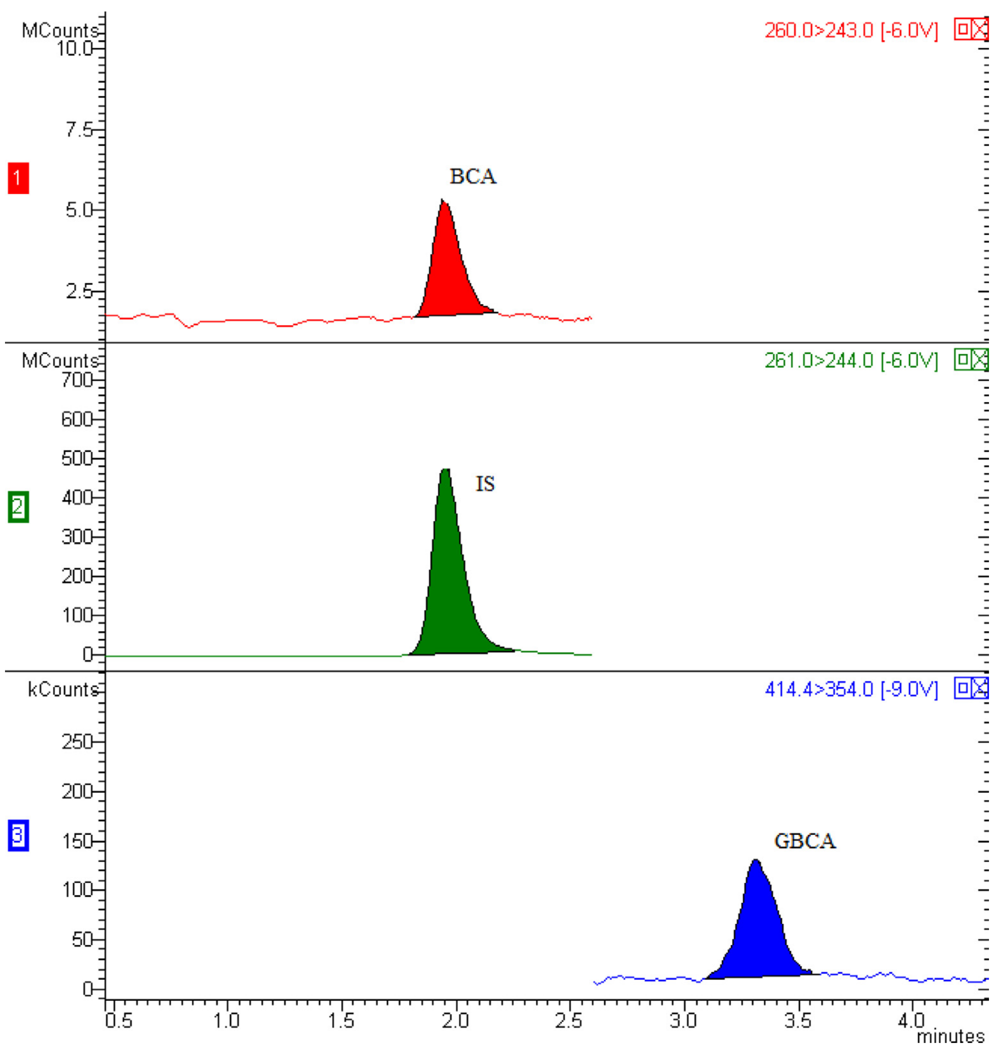

Fig. 1. Spiked sample chromatogram of BCA and γGBCA by LC-MS/MS (0.1 mg/kg).

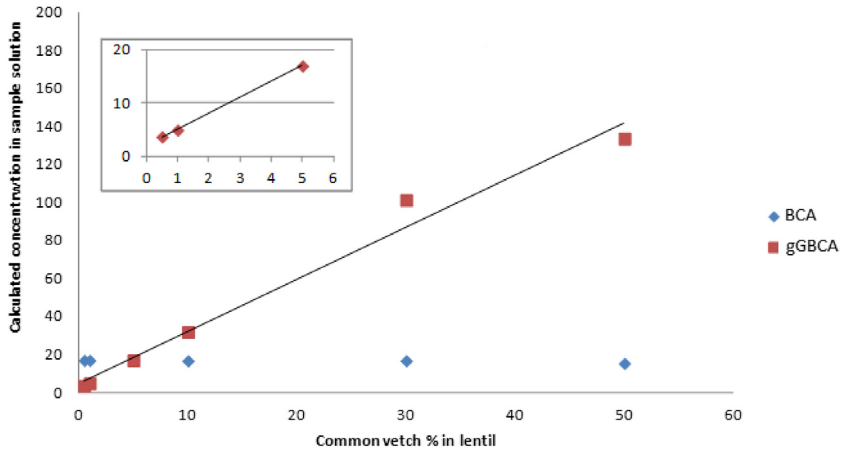

Fig. 2. Linearity graph for determination of adulteration of lentil by common vetch.

followed as references. In-house synthesized labeled β-cyanoalanine (Ghasemi & Secen, 2017) was used as an internal standard in all experiments.

3.1.2.1. Linearity and working range. The linearity of the method for β-cyanoalanine and γ-glutamyl β-cyanoalanine was assessed by analyzing standard solutions in the range of 0.05–20 mg/L. Correlation coefficients of the method for both analytes in the working range was obtained as ⩾0.999. The linear regression equation was $y = 0.054x + 0.0073$ for β-cyanoalanine and $y = 0.0070x - 0.018$ for γ-glutamyl β-cyanoalanine, where y is the peak area and x is the concentration in mg/L.

Linearity study for the determination of adulteration by common vetch was also performed, and a good linearity was observed between 0.5% and 50% for common vetch (Fig. 2). The developed method allows the determination of even lower adulteration or contamination levels in lentil samples (Fig. S1).

3.1.2.2. LOD and LOQ. The LOD and LOQ values of β-cyanoalanine and γ-glutamyl β-cyanoalanine analytes were calculated via signal to noise (S/N) ratio. The LOD value of β-cyanoalanine and γ-glutamyl β-cyanoalanine was obtained as 0.2 µg/L and 0.02 µg/L, respectively by multiplying the S/N ratio with 3. Besides LOQ values were determined by multiplying the S/N ratio with 10 and obtained as 0.67 µg/kg and 0.07 µg/kg for β-cyanoalanine and γ-glutamyl β-cyanoalanine, respectively.

3.1.2.3. Recovery, precision (repeatability) and intermediate precision. The recovery study of the method was performed with spiked lentil samples at 1, 5 and 20 mg/L concentration levels for each analytes. Replicate recovery tests were conducted on the same and different days with different concentrations. The mean of recovery at each level was determined as 95.8% for β-cyanoalanine and 97.4% for γ-glutamyl β-cyanoalanine. The representative chromatograms and data are given in Figs. S1 and S2 (Supporting information) and Table 3.

For the precision (repeatability) study, to apply the method in two replicate, 6 lentil samples spiked at 3 different concentration levels (0.5, 2, and 5 mg/L) so that at each contraction level two lentil samples will be spiked. The method was applied on samples during the one day. The precision (repeatability) was identified by standard deviation and 0.1 and 0.6 values were obtained for β-cyanoalanine and γ-glutamyl β-cyanoalanine, respectively for each concentration level.

For the intermediate precision study, again 6 lentil samples spiked at 3 different concentration levels (0.5, 2 and 5 mg/L) were prepared and tested over a period of 2 days in 3 weeks, and the relative standard deviations were calculated as 4.2% and 4.6% for both analytes. The results of validation parameters and uncertainty data are given in Tables 2 and 3 and 3.

Table 2
Calibration parameters of method.

Compound	Linear regression equation	R^2	Reported linear range (mg/kg)
BCA	$y = 0054x + 0{,}0073$	0,9996	0.05–20
γGBCA	$y = 00070x - 0{,}018$	0,9992	0.05–20

Table 3
Results of validation parameters and relative expanded measurement uncertainty of method.

Compound Name	LOD/LOQ (µg/kg)	Recovery (%)	Precision (SD)	Intermediate Precision (%)	U (%)
BCA	0.2/0.67	95.8	0.1	4.2	4.6
γGBCA	0.02/0.07	97.4	0.6	4.6	5.8

3.2. Estimation of uncertainty

3.2.1. Identification of measurement uncertainty

The uncertainty sources as calibration curve (C), purity of reference standards (P), stock solutions (Css), weighing of samples (w), repeatability (Rp), and recovery (rc) for the method applied here were identified based on the 2004 EURACHEM Guide (Kalın et al., 2015; GUM (Guide to the expression of uncertainty in measurement), 2014; EURACHEM/CITAC, 2012). In order to obtain expanded measurement uncertainty, the standard measurement uncertainty values of each parameter were combined according to Eq. (1) and it was multiplied with 2, which is the coverage factor at 95% confidence level.

Eq. (1) was used to estimate combined standard measurement uncertainty.

$$\frac{u_c(A)}{C_A} = \sqrt{\left(\frac{u(C)}{C}\right)^2 + \left(\frac{u(P)}{P}\right)^2 + \left(\frac{u(C_{SS})}{C_{SS}}\right)^2 + \left(\frac{u(w)}{w}\right)^2 + \left(\frac{u(Rp)}{Rp}\right)^2 + \left(\frac{u(rc)}{rc}\right)^2} \quad (1)$$

The performance of the method was shown by presenting the relative expanded measurement uncertainty as 4.6% for β-cyanoalanine and 5.8% for γ-glutamyl-β-cyanoalanine in Table 3. The principle of estimation of uncertainties was discussed extensively in our previous studies. Thus, we do not repeat that information here (Binici et al., 2013; Goren et al., 2015; Gulcin, Bursal, Sehitoglu, Bilsel, & Goren, 2010).

The main uncertainty comes from the weighing of the analytes, repeatability and the purity of standards.

3.2.2. Application of the method for the determination of adulteration of lentil

The LC-MS/MS method was applied successfully for routine analyses of β-cyanoalanine and γ-glutamyl-β-cyanoalanine in lentil and any species from the family Fabaceae. In this study, we showed that the lentil samples, adulterated with common vetch within the range of $0.5_{(w/w)}$% to $50_{(w/w)}$%, could successfully be determined. In general, because of the consistent presence of the BCA synthase enzyme in Fabaceae species, most BCA analyses do not give different results from species to species. However, regarding the γGBCA amount in different species, differences can clearly be observed. Therefore, we can use that compound as a marker for the determination of adulteration or contamination of lentil by *Vicia sativa* or any kind of vetch. To our best knowledge, determination of adulteration of lentil by common vetch has been performed by thin-layer chromatography (TLC) and botanical assessment of seeds in the laboratories of the Ministry of Food, Agriculture, and Livestock in Turkey and in many other countries. Only a single HPLC method has been reported recently (Thavarajah et al., 2012). On the other hand, this method cannot detect adulteration rates lower than 5%, and the reliability of the method is not as good as that of the LC-MS/MS method reported herein. Thus, the method developed here could be a very good tool for routine test laboratories worldwide.

4. Conclusion

On the basis of LC-MS/MS measurements we did not obtain a significant difference in the BCA contents of lentil and common vetch. BCA content was determined in the range of 0.06–0.08% in many lentil species obtained from a local market in Istanbul, and there was no meaningful difference between vetch and lentil regarding the BCA content (Fig. 1). However, considering the γGBCA content, it is easy to observe that gGBCA is a very good vetch marker in lentil and lentil products. According to our study, LC-MS/MS measurements with ^{13}C-labeled BCA could be a very useful tool for determining the adulteration or contamination of lentils by common vetch. This determination can be up to <0.05% (Figs. S1 and S2), while the determination limit of adulteration of lentil with common vetch was reported as 5% by HPLC UV in the literature (Thavarajah et al., 2012). However, as the lentil species also have trace amount of γGBCA, it should not be assumed lower than 0.1%.

When the vetch samples were boiled in water for 1 h, There is no difference observed between BCA and γGBCA contents during the extraction procedure. However, when the vetch samples were boiled in 0.1 N HCl, decomposition of 99.2% of the γGBCA was determined by LC-MS/MS. Therefore, even decomposition is not so strong at room temperature in acidic media, we do not recommend the use of acidified water for any kind of extraction for γGBCA analyses from any matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.11.079.

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