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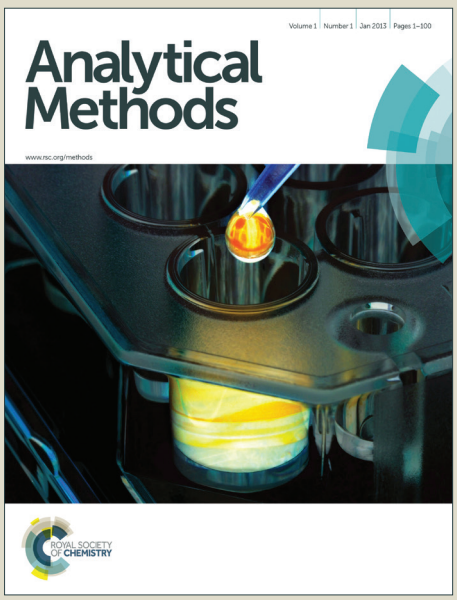

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A Rapid Validated HPLC Method for Determination of Sulforaphane and Glucoraphanin in Broccoli and Red Cabbage prepared by various cooking techniques.

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ABSTRACT

In this work, the effects of common cooking practices such as boiling, microwaving, steaming, and oven cooking and their influence on the amount and release of glucoraphanin (GCP) and sulforaphane (SFP) in broccoli and red cabbage were investigated using HPLC. These vegetables are approved for their beneficial effects and have preventing effects particularly against colon, lung, breast, and prostate cancers due to their glucosinolate content, therefore, development of an analytical method for determination of their glucosinolate profile is an important step for clinical studies. The HPLC method that is introduced in this study is fully validated and proved to be fast and effective. On the other hand, the importance of the methods of cooking these vegetables have been investigated and compared with each other that resulted in detecting SFN in all samples studied except in samples where whole broccoli was directly added into the boiled water.

Keywords: HPLC, glucoraphanin, sulforaphane, cooking methods, validation

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1. Introduction

Vegetables are good sources of natural antioxidants and biologically active components and play an important role in human nutrition in supplying certain constituents that are deficient in other foods [1]. Brassicaceous vegetables (e.g. broccoli and red cabbage) have been widely approved for their beneficial effects on human health and contain high concentration of vitamins, minerals and, in particularly, a special group of phytochemicals named glucosinolates [2]. While sulforaphane (the primary isothiocyanate from broccoli and red cabbage) and glucosinolate/ isothiocyanate that are present in these vegetables have potential importance in providing health benefits; moreover, the high flavonoids and carotenoids content of them increase their nutritious effects when compared to many other vegetables [3]. Myrosinase is an enzyme present in fresh broccoli and its sprouts, and is necessary to hydrolyze glucoraphanin (GCP), the inert glucosinolate precursor of sulforaphane (SFP), into the biologically active isothiocyanate. The process starts as soon as the fresh vegetable is chewed or otherwise any causing process could damage the cells. Consequently, there is partial conversion even before the compound reaches the stomach. Myrosinase is also present in the microbial flora of the lower intestine of animals and humans; hence, a significant fraction of GCP is expected to be hydrolyzed and become bioavailable as SFP by the time it fully passes the gastrointestinal system [4].

Even though broccoli is in general consumed raw because of its higher healing effects, its consumption after cooking is also very common. Traditional cooking methods such as conventional boiling in water, microwaving, steaming, or oven cooking may affect both the texture and the nutritional values of the vegetables. The nutrient losses occur due to plant tissue damage and subsequent loss of glucosinolates that may differ depending on the cooking treatments [5-8]. The disruption of the vegetable tissue brings glucosinolates into contact with myrosinase from within the intra- and inter-cellular vacuoles to initiate their

hydrolysis. However, cooking methods may partially or completely denature myrosinase [9]. Therefore, the acidity and temperature [10] of the medium, activity and cofactors of myrosinase and concentrations of residual glucosinolates may affect the nature and proportion of metabolites of glucosinolates produced during cooking and ingestion of cooked vegetables.

Epidemiological studies indicate that brassicaceous vegetables might have preventing effects on cancers, particularly against colon, lung, breast, and prostate cancers [11-15]. The chemoprotective effects of brassicaceous vegetables have been found to be correlated to their glucosinolate content [2]. Therefore, the determination of the glucosinolate profile (especially GCP and SFP contents) of a given brassicaceous vegetable happens to be a necessary step in the study of its chemopreventive activity. Numerous analytical methods including high-performance liquid chromatography (HPLC) [16-18], gas chromatography (GC) [19,20], GC with mass detector [21], micellar electrokinetic capillary chromatography (MECC) [22-24], and hydrophilic interaction liquid chromatography (HILIC) [25] have been previously used for determination of glucosinolate profile of fresh broccoli [26], broccoli seeds [27], broccoli sprouts [28], as well as many other HPLC based analytical methods [29-31]. These published methods have some limitations such as the occurrence of thermal degradation of SFP in the injection ports of GC/MS equipment [32] in GC method, the role of composition of mobile phase and percentage of organic solvent in it and the long analysis time needed for all chromatographic methods. Moreover, method validation for GCP and SFP has been performed only in a few published methods [17].

Because of its selectivity, sensitivity, and overall versatility, the development of a reliable and validated HPLC method has been received considerable attention in the quality control and quantitative determination of organic compounds. Owing to widespread use of the technique in routine and its benefits such as rapid set-up of the instrumentation, versatility

and low cost, it is important that a specific HPLC method is developed and thoroughly validated [33].

The different sources of variability throughout the food production chain, differences between similar methods of food procedures and various analytical techniques reported in the literature make direct comparison and interpretation of the present data difficult. Therefore, in this work, the effects of common cooking practices such as boiling, microwaving, steaming, and oven cooking and their influence on the amount and release of GCP and SFP in broccoli and red cabbage were investigated. Based on the general consumption habits, they were prepared in different ways and their contents of SFN and GCP were analyzed. Because red cabbage is generally consumed raw, the contents were determined in an uncooked form. The results of red cabbage were compared with broccoli prepared according to the preferred consumption habits. The second goal of this study was to develop and validate a rapid high-performance liquid chromatography (HPLC-DAD) method for the determination of GCP and SFP in these vegetables. The method was optimized and evaluated for fresh and lyophilized cooked broccoli and fresh red cabbage, and the applicability of the method was demonstrated. Emphasis was placed on broccoli due to the preference of the consumers and its economic importance.

2. Materials and methods

2.1. Materials

Fresh broccoli (*Brassica olearacea* L. var.*italica* Plenck) was purchased from a local market. Glucoraphanin (1-S-[(1E)-5-(methylsulfinyl)-N-(sulfonatooxy)pentanimidoyl]-1-thio-β-D-glucopyranose) was supplied from Phytolab GmbH & Co. KG (Bavaria, Germany) and sulforaphane (1-Isothiocyanato-4-methylsulfinylbutane) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical/HPLC grade and purchased from either Pancreac Quimica (Germany) or Riedel-de Haen (Germany).

2.2. Apparatus

Two certificated HPLC systems were used. HPLC1 was HP Agilent chromatographic system consisting of a Model Agilent 1100 series with a Model Agilent series G-1365B DAD detector, Model Agilent 1100 series G-1329A auto sampler. On the other hand, HPLC2 was HP Agilent chromatographic system consisting of a Model Agilent 1100 series with a Model Agilent series G-1315B DAD detector, Model Agilent 1100 series G-1367A auto sampler.

2.3. Separation Conditions

Separations were carried out with a Zorbax Extend-C_{18} column (250×4.6mm i.d., 5 μm) for SFN and with a Zorbax Eclipse SB-aq column (150×4.6mm i.d., 5 μm) for GCP. For SFN, isocratic elution was performed with a mixture of acetonitrile:water (30:70 v/v) at the flow rate of 0.60 mL/min. and the run time of the assay was 10 minutes. For GCP, isocratic elution was also performed with a mixture of acetonitrile:water:formic acid (1:99:0.1 v/v/v) at the flow rate of 1 mL/min and the run time was 6 minutes. The sample injection volume for both was 50 μL. Analyses were carried out at ambient temperature (20^{o}C).

2.4. Cooking treatments of broccoli and red cabbage

2.4.1. Preparation of broccoli samples

2.4.1.1. Boiling

400 g of whole broccoli was immersed into boiling water and were drained off after being boiled for 6 minutes. After 30 minutes of a waiting period, the solid part (broccoli) was removed and aqueous extract was directly lyophilized and coded as ***1***. The solid part was cut into small pieces by using a blender in the presence of water and waited for 30 minutes. Then, it was filtered and lyophilized and coded as ***2***.

2.4.1.2. Cooking in oven

400 g of broccoli was cut into small pieces by using a blender. After waiting for 30 minutes, broccoli was cooked for 45 minutes in an oven adjusted to 135 ^{o}C. Firstly, cooked broccoli was extracted with 600 mL dichloromethane (CH_2Cl_2) at 35 ^{o}C by using a magnetic stirrer (750-800 rpm Heidolph MR 30004) and then, filtered. The filtrate was evaporated under reduced pressure to dryness and coded as ***3***. Secondly, the remaining solid part after extraction was extracted with 500 mL distilled water. The filtrate was lyophilized and coded as ***4***.

2.4.1.3. Cooking in microwave

400 g of broccoli was cut into small pieces by using a blender in the presence of 200 mL distilled water. After waiting for 1 hour, broccoli was put in a pyrex plate and cooked in a microwave oven for 45 minutes adjusted to second power (home type microwave). After cooling, cooked broccoli was extracted by using the same procedure as described in part 2.4.1.2 and CH_2Cl_2 extract was coded as ***5*** and lyophilized H_2O extract was coded as ***6***.

2.4.1.4. Squeezing in juice extractor

400 g of broccoli was cut into small pieces by using a blender and aqueous part was directly lyophilized and encoded as ***9.*** Then, the smashed broccoli was also extracted by

using the same procedure as described in part 2.4.1.2. Extraction with CH_2Cl_2 was coded as ***7*** and lyophilized H_2O extract was coded as ***8***.

2.4.1.5. Steaming

400 g of broccoli was cooked in a steam cooker for 30 minutes. Then, broccoli was extracted by using the same procedure as before (section 2.4.1.2) and CH_2Cl_2 extract was coded as ***10*** and lyophilized H_2O extract was coded as ***11.***

2.4.2. Preparation of red cabbage

400 g of red cabbage was cut and extracted with 800 mL CH_2Cl_2 as described above (section 2.4.1.2) and coded as ***12*** and lyophilized H_2O extract was coded as ***13***.

2.5. Preparation of standard stock solutions

5.3 mg sulforaphane (SFP) was transferred into a 5 mL volumetric flask and dissolved with acetonitrile (named SFP-MS). The working solution of sulforaphane was prepared as follows: 0.943 mL of SFP-MS was taken and transferred into a 10 mL volumetric flask and dissolved in an acetonitrile:water (30:70) mixture (see the mobile phase composition). This solution was named SFP-DS1. Then, 1.0 mL of SFP-DS1 was transferred into a 10 mL volumetric flask and dissolved with the same solvent mixture. This solution was named SFP-DS2 and was used to prepare the standard solutions for the calibration curve. 2.0 mg glucoraphanin (GCP) was transferred into a 10 mL volumetric flask and dissolved in distilled water (named GCP-MS). This stock solution was used for construction of the calibration curve.

2.6. Preparation of mobile phases of HPLC Column

2.6.1. Preparation of the mobile phase for sulforaphane determination (Acetonitrile:water, 30:70)

300 mL of acetonitrile and 700 mL of distilled water were measured by using a measuring cylinder and were transferred into the reagent bottle. After complete mixing,

degassed mobile phase was filtered with 0.45 μm filter paper, and the reagent bottle was named and dated.

2.6.2. Preparation of the mobile phase for glucoraphanin determination (Acetonitrile: water: formic acid, 1:99:0.1 v/v/v)

10 mL of acetonitrile and 990 mL of distilled water were mixed in a reagent bottle by addition of 1 mL formic acid. After complete mixing, degassed mobile phase was filtered with 0.45 μm filter paper, and the reagent bottle was named and dated.

2.7. Preparation of standard solutions for SFN and GCP

The concentrations of working solutions were varied in the range of 90-360 ng/mL (300 ng/mL was 100%) and 600-2400 ng/mL (2000 ng/mL was 100%) for SFN and GCP, respectively. All of the subsequent dilutions for working standards were prepared by using mobile phase of SFN and GCP, respectively. Separate standard calibration graphs were constructed by plotting the area underneath the chromatograms versus concentrations of the standards.

2.8. Preparation of spiked samples

SFN and GCP spiked samples were prepared in three different concentrations (80, 100, and 120 %) in three replicates. For SFN, 100% spiked solution was prepared as follows: 0.300 mL SFN-DS2 was taken and transferred into the 10 mL volumetric flask. 0.200 mg/mL sample was added into the same volumetric flask. The volume was brought to 10 mL by addition of the mobile phase of SFN. 80 and 120 % spiked solutions of SFN were prepared in the same way.

For GCP, 100% spiked solution was prepared as follows: 0.100 mL GCP-MS was taken and transferred into a 10 mL volumetric flask. 0.200 mg/mL sample was added into the same volumetric flask. The volume was brought to 10 mL by addition of the mobile phase of SFN. 80 and 120 % spiked solutions of GCP were prepared in the same way.

2.9. Method validation

Analytical method validation was performed in accordance to ICH guidelines [33]. Assay validation involved linearity, sensitivity, stability, precision and accuracy, recovery and specificity.

2.9.1 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of the analyte in the sample. Calibration curves were acquired by plotting the peak area against the concentration of calibration standards. The concentrations used for generating calibration curves for SFP were within the range 90-360 ng/mL and for GCP were within the range 600-2400 ng/mL. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.999 or greater.

2.9.2. Sensitivity

Limit of detection (LOD) and quantification (LOQ) were estimated from the signal-to-noise ratio. The detection limit is defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The quantization limit is defined as the lowest concentration level that provided a peak height with a signal-to-noise ratio higher than 10, with precision (RSD%) and accuracy (Bias%) within ±10%. LOD and LOQ values of HPLC method were determined to be 29.7 and 90 ng/mL for SFN and to be 198 and 600 ng/mL for GCP, respectively.

2.9.3. Stability

Stability of standard solutions at 4 °C refrigerator temperature for 2 weeks (short-term) and -20 °C for 1 month (long-term) were investigated. All the solutions were protected from light and the peak area of the solutions prepared for stability studies and kept for short

term and long term stability studies were compared with the solutions freshly prepared at equivalent concentrations.

2.9.4. Precision and Accuracy

The intra-day precision was estimated by analyzing four replicates containing either SFN or GCP at six samples. The inter-assay precision was determined by analyzing six samples on four different runs. Accuracy was calculated on the basis of quotient of the averaged measurements and the nominal value and expressed in percentage. The extraction recoveries of SFP and GCP were determined by comparing the responses of the spiked analytes extracted from replicate samples (n = 4) with the response of analytes from non-extracted standard solutions at equivalent concentrations. Analytic interferences were investigated by recovery percentage test.

2.9.5. Specificity

The specificity of an analytical method may be defined as the ability to unequivocally determine the analyte in the presence of additional components such as impurities, degradation products and matrix components. Specificity was evaluated by comparison of the chromatograms of standard solutions and sample solutions.

3. Results and Discussions

The effects of different preparation techniques on the contents of glucosinolates in broccoli and red cabbage were investigated in the present study. Glucosinolates are one of the most important bioactive compounds in broccoli and red cabbage. Several isothiocyanates derived from methylsulfinyl aliphatic glucosinolates, such as SFN (derived from GCP) in broccoli, are regarded to reduce the risk of cancers [34-36]. The HPLC method that is reported here provides a simple procedure to determine the concentrations of SFN and GCP in the samples prepared by different cooking or preparation techniques in some brassicaceous vegetables (i.e. broccoli and red cabbage) as described in the experimental part by DAD detection at 202 and 235 nm for SFN and GCP, respectively. Different chromatographic conditions for the quantification of SFN and GCP were optimized by performing preliminary trials with the corresponding reference standards.

3.1. Optimization of the Analytical Methods

To find optimum mobile phase composition, different kinds of mobile phase compositions were tested. Based on literature survey [17] and our experimental results, the mobile phase compositions were chosen for SFN and GCP as described in the experimental part. The developed method has several advantages over the previously published methods. First of all, the amount of organic solvent was reduced for determination of GCP and the analyzing time was shorten to about half of the published results [17]. For SFN, analysis time was less than 8 minutes as opposed to 15 minutes in the published method [17]. Moreover, the experimental temperature condition was decreased to room temperature from 36 ^{o}C without losing the resolution and the limit of quantification was lowered to 0.090 μg/mL from 4 μg/mL.

The retention times of standards of SFN and GCP were given in Table 1. Sharp, symmetrical and well-resolved peaks were obtained for both standards. The variation in

retention time for seven replicate injections of SFN and GCP reference solutions (300 ng/mL for SFN and 2000 ng/mL for GCP) gave RSDs of 0.030% for SFN and 0.042% for GCP.

3.2. System suitability test

The system suitability tests are an integral part of HPLC method development. These tests ensure that the method can generate results of acceptable accuracy and precision. The criteria will be based on the actual performance of the method which is determined during its validation. The USP suggests that the system suitability tests be performed prior to analysis [37]. System suitability for the proposed method was evaluated. The parameters tested for system suitability included selectivity factor, asymmetry factor, resolution, tailing, theoretical plates, retention time and RSD% of retention time and peak area.

The results from system suitability tests are given in Table 1 for each studied compound with %RSD values. Usually, at least two of these criteria are required to demonstrate system suitability for the proposed method. As shown in Table 1, the presented chromatographic conditions ensure sufficient retention of all compounds, since the capacity factor values satisfied the conditions. Furthermore, since values of resolution factors of adjacent peaks were greater than 1.0, the proposed method has enabled excellent resolution of both analytes. Therefore, the results obtained from system suitability tests are in good agreement with the USP requirements.

3.3. Standards Chromatograms and Calibration Curve

The chromatograms of SFN and GCP standards are given in Figure 1 and 2, respectively.

3.3. Analytical Method Validation

3.3.1. Linearity

The relationship between the standards and the responses were linear, y=mx+b, within the ranges of the analytical procedure where x was the concentration of the standard in ng/mL

and y was the response (peak area). The calibration curves were obtained by using the linear least square equations, and correlation coefficients and other related validation data are reported in Table 2.

3.3.2. Sensitivity

The limit of detection (LOD) and quantification (LOQ) were calculated in accordance with 3.3s/m and 10s/m criteria [34, 35], respectively, where s is the standard deviation of the peak area (for seven replicates) for the sample and m is the slope of the calibration curve, determined from the linearity investigation. These results also are reported in Table 2. Precision, accuracy and reproducibility of the method were assessed by performing replicate analysis of standard solutions. Repeatability and reproducibility were characterized by RSD% in Table 2.

3.3.3. Stability

The short term stability of the standard solutions was controlled by analyzing a standard solution aged at 4 ^{o}C for two weeks, in dark against a freshly prepared sample. The long term stability was performed by preparation of standard solutions and preventing from daylight at -20 ^{o}C for one month. The results showed that the working standard solutions were stable and the obtained peak area for the assay standard solutions did not change over the periods.

3.3.4. Precision and Accuracy

Precision of the assay method was determined for both intra-day and inter-day variations by analyzing the quality control samples for six replicates. The RSD values for intra-day studies were 2.22% and 1.22% for SFN and GCP, respectively. On the other hand, the inter-day values were 3.77% and 3.91% for SFN and GCP, respectively. The accuracy of the method was proved by recovery tests carried out by analyzing the sample and the known amount of standard added. The results are given in Table 3.

3.3.5. Specificity

The chromatograms obtained from standard solutions were identical with that obtained from sample solutions containing equivalent concentrations of SFN and GCP. The representative chromatograms (Fig. 1 and 2) show no other peaks on the retention time of SFN and GCP, in addition, the retention times did not change. There were no peaks on the injection of blank solutions and the chromatograms showed that there were no interferences from the matrix of the sample. Based on these results, the proposed methods can be considered specific.

Satisfactory sensitivity, accuracy and precision were noted. The methods that provide reproducible results are easy to perform and sensitive enough for the determination of SFN and GCP in the complex matrixes.

3.2. Effect of Cooking Ways on content of SFN and GCP in Broccoli and Red Cabbage

In general, vegetables are prepared at home on the basis of convenience and taste preference rather than retention of nutrient and health-promoting contents [1,2]. It is known that cooking methods may induce significant changes in chemical composition, affecting the bioavailability and content of effective ingredients in vegetables. For the brassicaceous vegetables (i.e. broccoli and red cabbage), the glucosinolates content (e.g. SFN and GCP) are particularly important. The preparation procedures such as boiling, microwaving, steaming, squeezing, and oven cooking were based on the common cultural dietary habits in the world. A more integrated analysis of nutritional change of broccoli and red cabbage were studied in this work. Hence, the amount of SFN and GCP in broccoli and red cabbage might be changed as well.

As described in the experimental part, twelve different samples of broccoli and red cabbage were prepared and analyzed by using HPLC-DAD method. It is well known that cooking processes could affect the concentration and eventually biological activities of

different plant ingredients [38] and degradation rate is dependent on the food matrix in which the glucosinolates are located. Several processing methods particularly using a thermal treatment lead to an inactivation of enzymes, e.g. myrosinase, generally resulting in low loss of glucosinolates. Therefore, the nutritional quality of the vegetables depends not only on the nutrient content when harvested but also on the changes occurring during postharvest handling, storage conditions, processing, and preparation prior to consumption. During these stages the glucosinolate–myrosinase system may be modified. It has been stated [10] that the concentration of glucosinolates in broccoli may vary by 5–10-fold at each step of the food production chain because of the differences attributed to cultivar, environmental and genetic factors, industrial processing, storage and domestic cooking. The cumulative nature of this variation at various steps of the food production chain leads to considerable uncertainty in assessing rates of exposure of glucosinolates and their metabolites at target tissues. Despite the extensive literature on the hydrolysis of glucosinolates, their distribution in fresh brassicaceous plants and their health benefits, information on the effects of processing of them on the glucosinolate–myrosinase system is relatively scarce and inconsistent. It has been argued[10] that the extent of leaching of glucosinolates during boiling of broccoli is negatively related to the amount of broccoli and cooking water used. The unaccountable losses as the result of thermal, enzymatic degradation or volatilization of glucosinolates during cooking have been pointed out earlier in another study [10]. The cooking time and cut sizes of broccoli have been shown to be an important parameter for the amount of glucosinolates remaining in the samples after the cooking process. However, common consumption method of red cabbage is raw. For that reason red cabbage was not cooked and analyzed as such. An increase of 78% in total glucosinolate concentration after microwaving 300 g coarsely-chopped red cabbage under the conditions of 900W for 4 min 48 s is reported but this outcome is attributed to a higher chemical extractability of glucosinolates from

cooked cabbage when compared to its raw counterpart and might have been an analytical artifact [8]. These results suggest that broccoli may be more prone to cellular disruption and loss of glucosinolates than red cabbage because of its loose structure. As indicated in Table 4, percentage amounts of SFN were considerably higher in red cabbage samples ***12*** and ***13*** as oppose to other samples of broccoli. The amounts of SFN and GCP in these samples are summarized in Table 4.

These results suggest that the individual glucosinolates may behave differently according to the cooking method. Information on the effect of the extent of cooking on the glucosinolate-related characteristics of brassicaceous plants and on the relationship between the residual glucosinolate concentrations and plant myrosinase activity and the formation of metabolites of glucosinolates produced, is limited. However, other methods like cutting, shredding or chewing of the raw broccoli do not inactivate myrosinase and epithiospecifier protein, resulting in a hydrolysis of glucosinolates in broccoli to isothiocyanates, nitriles, oxazolidinethiones and various indole decomposition products. For raw, crushed or shredded broccoli sulforaphane nitrile has been identified as the major hydrolysis product derived from glucoraphanin [39]. These findings are supported by the results of samples ***8*** and ***9*** where the samples were squeezed. Another study on glucoraphanin degradation in freshly homogenized broccoli led to sulforaphane as the main product instead of sulforaphane nitrile, but the homogenate in this study had been treated for 5 minutes at 50 ^{o}C [21]. However, our study suggests that the thermal degradation of broccoli does not contain SFN in the liquid part of the samples ***1*** and ***2***, whereas SFN was found in samples ***3*** *and* ***4*** where the whole broccoli was extracted first with dichloromethane then with water at a temperature above 110 ^{o}C. Another study on glucoraphanin degradation rate suggests that when the temperature was increased from 80 to 123 ^{o}C, the amount of GCP was increased for broccoli and red cabbage [40]. This finding was supported by our observations. Due to the fact that at higher

temperatures (above 100 ^{o}C), myrosinase enzyme was being inactivated, it could not convert GCP into SFN (see Table 4). Different methods for processing of broccoli and red cabbage are listed in Table 4 and is pointed out that different processing conditions affect the formation and the possible bioavailability of GCP and SFN. As shown in Table 4, SFN were found in all samples prepared by different techniques except in samples ***1*** and ***2***. GCP were found to be present only in samples ***1***, ***2***, ***3***, ***5***, ***7***, ***8***, and ***12***. These findings might be attributed to the fact that either all the GCP were converted into SFN or, in contrast, not detecting any SFN might be due to the thermal decomposition of myrosinase that results in high concentration of GCP. In the preparation methods, boiling of broccoli is especially important due to the common practice of cooking. It is publicly believed that consumption of broccoli as in the sample ***2*** would help to reduce the risk of the cancers. However, our finding revealed that GCP was not converted into SFN by this preparation method (see Table 4). This result may imply that myrosinase enzyme was degraded at high temperature (above 100^{o}C). Therefore, if one would like to get the expected health benefits by this cooking procedure, microbiota of individuals should contain myrosinase enzyme in their gut.

4. Conclusions

The proposed analytical methods provide a very short analysis time (10 and 6 minutes for SFN and GCP, respectively), and LODs and LOQs values are comparatively reduced. This study reveals that the preparation methods of broccoli and red cabbage directly affect the amounts of SFN and GCP. Of all the twelve samples studied, SFN was absent only in samples ***1*** and ***2*** in which whole broccoli was directly added into the boiled water. SFN was found neither in solid nor liquid parts of the cooked broccoli. As opposed to this, GCP were determined in the same samples. These results strongly suggest that the way of preparation of broccoli and red cabbage before the consumption is very important in terms of their SFN contents. This work indicates that benefits of the vegetables largely depend on their methods of cooking. Therefore, a statement should not be declared on the healthiest way of preparation of broccoli or red cabbage without having any scientific confirmation on the amount of SFN and GCP left in the vegetable after the treatment.

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Table 1: System suitability parameters

	Observed Value				Recommended value
	SFN		GCP		
	Value	%RSD	Value	%RSD	
Retention time (t_R, min)	7.20	0.03	3.15	0.04	
Tailing factor (T)	1.23	3.67	1.04	2.97	≤ 2
Asymmetry factor (A)	1.36	2.81	1.03	3.21	0.95–1.20
Capacity factor	13.39	0.03	5.31	0.05	> 1
Resolution (R_s)	12.11	1.95	3.79	2.86	> 2
Theoretical plates (N)	14817	1.22	4100	1.87	> 2000
Selectivity factor (α)	2.06	4.86	1.39	0.09	> 1

Table 2: Validation Paramaters of SFN and GCP

	SFN	GCP
Linearity Range (ng/mL)	90-360	600-2400
Slope	0.34126	0.01226
Intercept	-2.46351	0.02793
Correlation coefficient (r)	0.99985	0.99935
Limit of Detection (LOD, ng/mL)	29.7	198
Limit of Quantification (LOQ, ng/mL)	90	600

[a] *Each value is obtained from seven experiments*

[b] Between-day reproducibility is determined from seven different runs over a week period

Tablo 3: Recovery studies for SFN and GCP

Concentration in %	Recovery % SFN	Recovery % GCP
80%	99.87	89.68
	96.28	92.27
	99.09	90.48
100 %	99.05	90.67
	93.48	90.28
	93.27	90.34
120 %	91.91	81.19
	92.51	85.62
	90.16	86.67
Average	95.07	88.58
SD	3.59	3.46
% RSD	3.77	3.91

Table 4: Amount of SFN and GCP based on different preparation techniques in broccoli and red cabbage.

Sample	*SFN (ng/mL)*	*GCP (ng/mL)*	*% SFN*	*% GCP*
1	-	*8932.1*	-	*4.47*
2	-	*2121.6*	-	*1.06*
3	*36.2*	*2634*	*0.018*	*1.31*
4	*80.1*	-	*0.040*	-
5	*32.2*	*5449.7*	*0.016*	*2.72*
6	*73.5*	-	*0.037*	-
7	*55.7*	*10502.2*	*0.028*	*5.25*
8	*90.3*	*1474.5*	*0.045*	*0.74*
9	*88.1*	-	*0.044*	-
10	*65.1*	-	*0.033*	-
11	*25.2*	-	*0.013*	-
12	*107.6*	*3841.1*	*0.054*	*1.92*
13	*125.8*	-	*0.063*	-

Figure 1: Representative chromatogram of SFN: (a) mobile phase, (b) blank and (c) SFN standard (300 ng/mL)

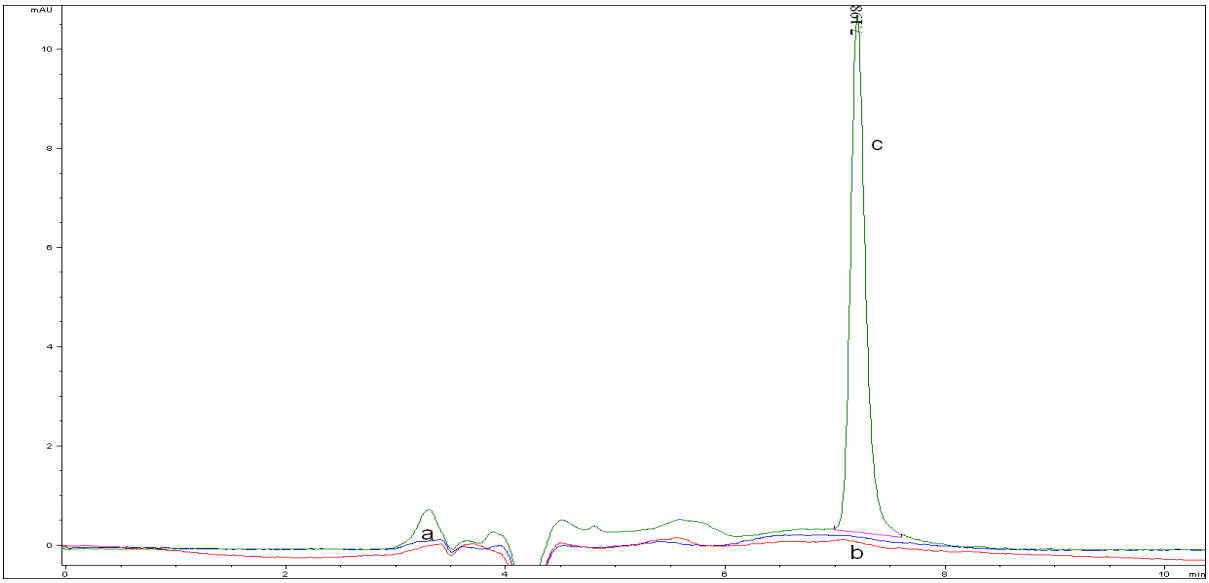

Figure 2: Representative chromatogram of GCP: (a) mobile phase, (b) blank and (c) GCP standard (2000 ng/mL)