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ORIGINAL RESEARCH

Quantitative analysis of glucosinolates in organic cruciferous vegetables: Yield values between cold methanol and boiling water extraction methods

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Abstract

Introduction: Consumption of cruciferous vegetables has continued to gain more acceptance in Africa, for reasons including health benefits; reducing the risks of developing cancer, bone health, fighting heart disease and a good source of vitamins and minerals. The presence of glucosinolates, a class of organic anionic sulfur rich secondary metabolites confers the heath servicing capacities on crucifers.

Aims: This study is aimed at evaluating total and individual glucosinolates levels in cultivars widely consumed in Nigeria using high performance liquid chromatography (HPLC), through cold methanol and boiling water extraction treatments.

Materials and Methods: A reverse phase HPLC C18 column (Spherisorb 5µ ODS, 100 mm × 4.6 mm) was employed to evaluate the concentration of three intact glucosinolates: progoitrin (2R)-2-Hydroxybut-3-enyl), sinigrin (2-Propenyl) and sulforaphane in four (4) different types of cruciferous vegetables: Broccoli (Brassica oleracea L. italica), kale (Brassica oleracea L. acephala group), cauli flower (Brassica oleracea L. botrytis) and green cabbage (Brassica oleracea L. capitata). Glucosinolates as (desulfoglucosinolates) were quantified at 229 nm wavelength within the UV spectrum.

Results: The results show concentration of progoitrin, sinigrin and sulforaphane range 0.133-0.154, 0.590-0.640, and 0.820-0.980 respectively for boiling water method while the range 0.00-0.056, 0.108-0.302 and 0.364-0.398 for cold methanol treatment.

Conclusion: The level of glucosinolates investigated was observed higher in stems than the leaves of vegetables studied. ANOVA at $P < 0.05$ revealed varying degree of significant and non-significant differences between the two extraction methods used, boiling water extraction was observed to give a higher yield than cold methanol extraction treatment.

Keywords: Progoitrin, Crucifers, Sinigrin, Sulforaphane, Broccoli

All co-authors agreed to have their names listed as authors.

1. INTRODUCTION

Glucosinolates (GSLs) are a class of secondary plant metabolites found in crucifers: Brassicaceae, Koeberliniaceae, Moringaceae, Resedaceae and Tovariaceae. Crucifers are an important group of cultivated plants in the world [1- 3]. Over 100 different GSLs have been identified in a variety of vegetables such as broccoli, mustard seed, and brussels sprouts. So many GSLs have been characterized mainly by the R group (Figure 1.0) which can either be aromatic, indolic or aliphatic. GSLs may be enzymatically hydrolyzed by the enzyme myrosinase (thioglucoside glucohydrolase) to yield a variety of biologically-active products, including isothiocyanates, thiocyanates, nitriles, and oxazolidine-2-thiones [1].

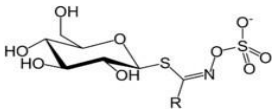

Figure 1.0: Glucosinolate structure; side group *R* varies [1].

The most commonly consumed cruciferous belong to the genus Brassicaeae and many are within the several varieties of *Brassica oleracea*, including cauliflower, cabbage and broccoli. GSLs belong to a group of thioglycosides, which occur naturally in cruciferous vegetables. GSLs are well known for their roles in plant resistance to insects and pathogens [4] and are largely found in the economically and nutritionally important Brassica crops [5]. Ciska et al., [6] reported that some products are formed from enzymatic or non-enzymatic hydrolysis of GSLs which are biologically of various diverse effects on human health [7]. These substances may also act as antioxidants that help in combating degenerative diseases [8 - 10]. The protective effect of cruciferous vegetables is attributed to isothiocyanates (ITC) and indoles. Both groups of compounds have been shown to reduce occurrence of cancer in experimental animals [11], and may have anti carcinogenic effects by several mechanisms [12]. Despite the great diversity of GSLs, only a limited number are commonly consumed within the human diet [1, 12]. Intake of cruciferous vegetables is encouraged as a part of a diet rich in a variety of fruits and vegetables, for cancer reduction and healthy body promotion [12, 13].

Crucifer vegetables play an important role in the American diet. In 1983, the United States produced 352,000 tons of fresh broccoli (*Brassica oleracea* L. italica group), and 259,000 tons of fresh cauliflower. The naturally occurring GSLs in the edible crucifers should be monitored because of their potential beneficial effects on health [14]. Total and individual GSLs content varies among species, cultivars, and plant parts. Environmental factors, such as solar

radiation, temperature variation and climatic conditions, within a geographical location are also other key factors that influence the determination of the GSL content in cruciferous vegetables [15]. Among the cultivated Brassicaceae, broccoli attracted attention after the discovery that it contains high levels of the isothiocyanate and sulforaphane [1-isothiocyanate-(4R)-(methylsulfinyl) butane], and of other GSLs derivatives thought to have anti carcinogenic properties [1].

Due to their prevalence in cultivated vegetables, spices, oils and animal feed, GSLs and their hydrolysis products have been much studied in the context of their effects on human and animal nutrition [16]. GSLs and their metabolites have also been a focus of prevention studies of disorders linked to oxidative stress such as cancer and gastric ulcers [16] and more recently, potential undesirable dietary effects such as genotoxicity of GSLs [17].

The breakdown of GSLs has also been studied in respect of their potential uses as agricultural pesticides in a technique known as bio fumigation. In bio fumigation, a GSLs-rich crop is mulched into the field, releasing toxic secondary GSLs by-products, thereby reducing the incidence of pests, weeds and diseases in the arable and horticultural crops [18].

GSLs and their derived products prevent carcinogen molecules from reaching the target site or activating the important hepatic enzymes for the protection against several carcinogens [19, 20]. When consumed, they are transformed into bioactive compounds that possess anticancer properties, which trigger the body detoxification systems, slows down cancer cell growth and support deoxyribonucleic acid (DNA) repair. Plant GSLs concentration has been related to environmental conditions and cultivation methods (organically and conventionally farming methods) as it is particularly sensitive to the sulfur content in the soil [20 and 21]. Winkler et al., [22] reported that plants produced by organic cultivation have increased cytochrome P450 concentrations, which contributes to detoxificationof xenobiotics. Research suggests that GSLs can quicken the activity of the body natural antioxidant system.

As such, GSLs act as indirect antioxidants pulling the liver to produce detoxifying enzymes that block free-radical attack on deoxyribonucleic acid (DNA). GSLs and their metabolites have also been reported with roles in prevention studies of disorders linked to oxidative stress such as cancer and gastric ulcers [18, 23 and 24].

There is dearth of research reports on the quantification and other studies on GSL in variety of cruciferous vegetable cultivated or consumed in Nigeria that may help in formulating health policies.

The aim of this work was therefore to quantify total GSL concentrations in four cultivars commonly consumed in Nigeria, employing high performance liquid chromatography (HPLC), and to compare the yield from the two extraction treatments: cold methanol and boiling water methods. This will help in the preliminary information on the level of GSLs in cultivars, despite being grown on Nigeria soils as well as providing enlightenment on the importance of cruciferous vegetables. It helps the relevant agencies to also make required policies

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2. EXPERIMENTAL DETAILS

2.1 Reagents and Chemicals

All solvents and chemicals used were of HPLC grade and obtained from Sigma-Aldrich. They include HPLC grade water and HPLC grade methanol. Three standards; progoitrin (2R)-2-Hydroxybut-3-enylglucosinolates), sinigrin (2- Propenyl glucosinolates) and sulforaphane were also obtained.

2.2 Cruciferous vegetables Materials

Four (4) different types of cruciferous vegetables: broccoli (*Brassica oleracea L. italica*), kale (*Brassica, oleracea L. acephala*), cauli flower (*Brassica oleracea L. botrytis*) and green cabbage (*Brassica oleracea L. capitata*) used in this study were obtained from supermarkets and groceries in Ilupeju, Ikeja and Alimosho areas as well as open markets in Egbeda and Ojo in Lagos metropolis, Lagos State. This type of sampling is similar to a past study [25] where cruciferous samples were obtained from groceries and open markets. However, some studies made use of plants materials that are harvested directly from experimental fields [26-28]. Samples were wrapped looselyin aluminum foil and transported on dry ice box and thereafter stored in a freezer for three days in the laboratory.

2.3 Freeze drying and Tissue disruption

The vegetable samples were loaded into a freeze drier with maximum loading time of 30 seconds. Freeze dried plant tissues were homogenized to a roughly ground powder (approximately 0.1 cm particle size) using a grinder (Lloytron, E5601BK). Homogenized ground samples were milled (Retch, MM400) at a frequency of 20 Hz for 10 min. with 2 steel ball bearings to a fine powder (particle diameter <0.1 mm). Each vegetable was separated into the leaves and the stems with the exemption of Green Cabbage.). Samples were then sealed andstored at 20 °C. Freeze drying or lyophilisation was carried to remove water from GSLs containing tissues while preventing hydrolysis through thermal inhibition. This process allows subsequent tissue disruption without risking GSLs degradation.

2.4 Glucosinolates extraction

Extractions were carried out in two ways. In each case 50 µl of 20 mM sinigrin was added as internal standard.

2.5 Cold methanol extraction

A 5.0 ml of 80:20 of methanol: water at 20 °C was added to 0.1 g plant tissue and the internal standard was added. The sample was shaken and left to stand for 30 min at room temperature. The sample was then mixed at 70 rpm with a platform rocker (Bibby, STR6) for a further 30 min., before centrifugation at 4000 rpm (Jouan, model) for 10 min. Supernatant was then filtered through a 0.22 µm syringe filter (Millex GP) for direct injection on HPLC [29]

2.6 Boiling water extraction

A 25.0 ml of boiling water was added to 0.1 g of freeze dried and milled plant tissues in a 150 ml Erlenmeyer flask and the internal standard was added. Sample was heated at 100 °C and stirredwith a magnetic stirrer hot plate for 10 min. Sample was heated for a further 4 hrs at 70 °C before centrifugation at 4000 rpm for 10

mins. Sample was topped up to 20 ml level with deionized water [30].

2.7 HPLC analysis of desulfoglucosinolates

A Water 600E system controller attached to a Waters 717 auto sampler, Water 996 photodiodearray detector and Sphere Clone 5µ ODS(2) column (Phenomonex) were used for separation and detection of desulfo and intact GSLs. A reverse phase C18 column (Phenomonex, Spherisorb 5µ ODS (2), 100 mm × 4.6 mm) was equilibrated for 30 min with a mobile phase which consist of 100% distilled H_2O. Flow rate was set to 1 ml/min and samples separated according to the programme for desulfoglucosinolates. Mobile phase solutions were degassed for 30 min in a Sonicator (Decon, Sussex England). Solution A: 100% distilled H_2O Solution B: 70:30, distilled H_2O: acetonitrile. Desulfoglucosinolates were quantified using 229 nm wavelength within the UV spectrum. The HPLC PDA detector allowed a full spectrum analysis from 180 to 800 nm, allowing comparative UV-visible spectra analysis, which aided in identifying unknown GSLs. Through standard injections and HPLC-MS identification, the id's of the reported GSLs were confirmed. ISO 9167-1 [30]

2.6 Statistical analysis

The statistical analysis was entirely randomized in groups consisting of 2 treatments: Cold methanol and boiling water conditions. Descriptive analysis (Mean and standard deviation) of the data obtained was carried out. Analysis of variance (ANOVA) at $P < 0.05$ was also used for the significant differences in the GSLs concentration in the extraction methods and significant difference between leaves and stems of the plant samples.

3. RESULTS AND DISCUSSION

The descriptive statistics of GSLs levels in both leaves and stems of cruciferous vegetables analysed through both cold methanol and boiling water extraction methods are shown in Table 1. Three markers were made use of namely progoitrin (2R)-2-Hydroxybut-3-enyl), sinigrin (2-Propenyl) and sulforaphane. Calibration curve was generated for each of the standard using peak response against concentration (mg/L) sinigrin, progoitrin and sulforaphane respectively with the detail profile presented in Table 2.0. The pictorial (Bar Chart) of the GSLs concentration in cruciferous vegetables investigated is shown in Figure 2. Progoitrin, Sinigrin and Sulforaphane used as markers for the samples were found present in almost all the vegetables (broccoli, kale, cauliflower and cabbage) as shown in Table 1. The level of progoitrin for the hot extractions in all the samples were higher than that of the cold extraction as shown in Table 1.0, probably as a result of heat condition which helps in rate of reaction.

Table 1.0: Descriptive statistics of concentration of total glucosinolate in cruciferous for cold methanol and boiling water extraction methods

Vegetables	Progoitrin				Sinigrin				Sulforaphane	
	Hot Extract	Average value	Cold Extract	Average value	Hot Extract	Average value	Cold Extract	Average value	Hot Extract	Average value
Broccoli Leaf	0.154		0.056		0.222		0.512		0.075	
	0.133	0.144± 0.02	0.000	0.028± 0.04	0.341	0.281± 0.24	0.548	0.530± 0.18	0.017	0.046 ±0.11
Broccoli Stem	0.315		0.166		2.624		1.329		-	
	0.290	0.303± 0.02	0.091	0.128± 0.05	2.464	2.544± 0.04	0.723	1.025± 0.27	-	-
Kale Leaf	0.591		0.302		5.167		2.506		-	
	0.648	0.620± 0.04	0.108	0.205± 0.13	5.877	5.522± 0.6	0.625	1.565± 1.2	-	-
Kale Stem	1.988		0.920		14.938		8.255		0.059	
	1.927	1.958± 0.04	0.770	0.845± 0.1	14.606	14.73± 0.07	6.545	7.40±1.1	0.114	0.086 ±0.1
Cauli Flower Leaf	0.822		0.364		7.032		2.875		-	
	0.978	0.900± 0.11	0.397	0.381± 0.02	8.525	7.779± 1.24	3.697	3.286± 0.73	-	-
Cauli Flower Stem	1.714		0.189		13.603		1.626		0.003	
	1.702	1.708± 0.01	0.413	0.301± 0.16	12.986	13.29± 0.27	3.150	2.38±1.2	0.001	0.002 ±0.15
Green Cabbage	0.356	0.356	0.028	0.028	3.117	3.117	0.723	0.723	0.017	0.017

- = Not detected

Table 2 .0: Calibration curves for the three marker compounds.

Standards	Calibration curve	R^2
Progoitrin	y = 8E+06x - 426.75	1.0000
Sinigrin	y = 1E+07x - 7686.8	1.0000
Sulforaphane	y = 1E+07x - 652.1	0.9998

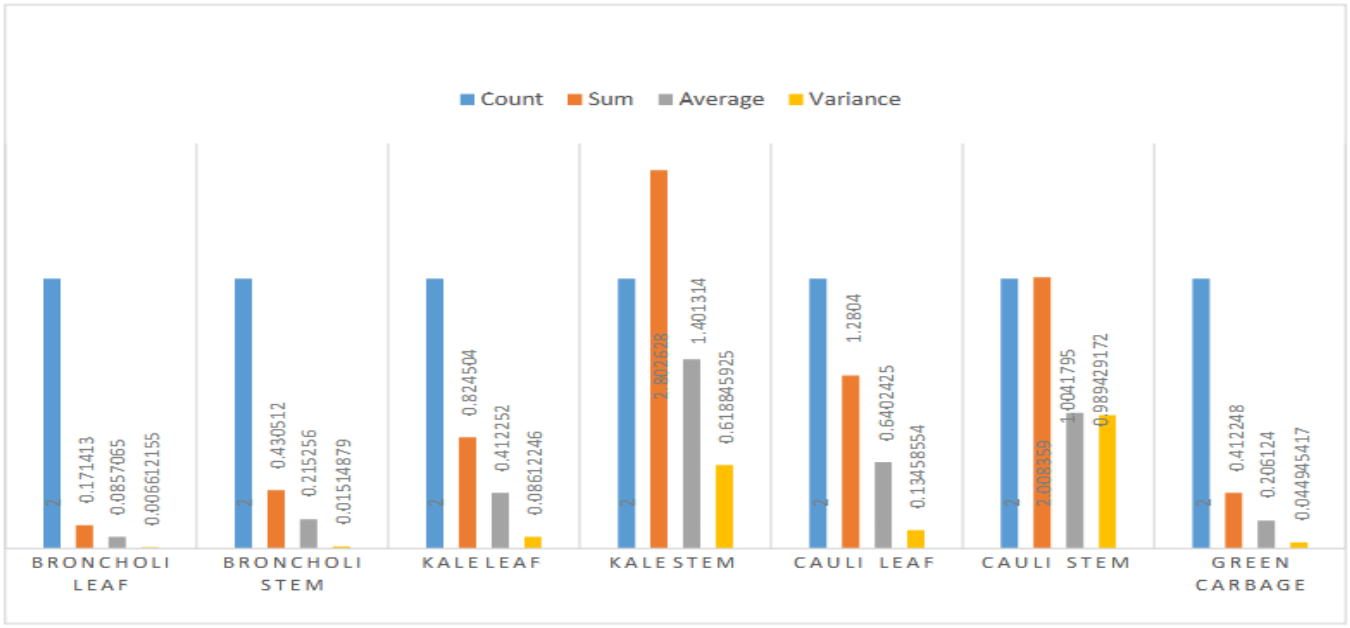

Figure 2. The bar chart of glucosinolates concentration in each of the cruciferous vegetables investigated

The same (higher level of progoitrin in the hot extractions) was also observed in the sinigrin level in the various samples. Sulforaphane was onlyfound in the stem of kale and cauliflower and in the leaf of broccoli. Progoitrin and sinigrin level in the stems of all the vegetables analysed were higher than the levels found in the leaves except in cauliflower where the level (mg/g) in the leaves and stems are 0.381 and 0.301 for progoitrin and 3.286 and 2.388 for sinigrin respectively. This observation is similar to the values reported by Aires et al, [31] where the highest GSL levels were found in the stems.

Kale stems have the highest level of GSL in the samples for hot and cold extraction in progoitrin (1.96 / 0.85) mg/g, (14.8 / 7.40) mg/g in sinigrin and hot (0.09) mg/g in sulforaphane. The content of progoitrin and sinigrin in either the leaves or the stems of broccoli, kale, cauliflower and cabbage were higher than the defined limit set by the Chinese pharmacopoeia of 0.2 mg/g. It is observed that the level of sinigrin in each of the sample were higher than that level found in progoitrin. This trend was also observed in the work of Lee et al., [32]. Variance tests were carried out through ANOVA. Tables 3.0 and 4.0 show test result of total GSLs comparism between cold methanol and boiling water extraction methods and between GSLs levels of the stem and leaves in various cruciferous vegetables. There are significant differences between cold methanol and boiling water extraction methods in broccoli stem, kale leaves and stem, cauliflower leaves and stem and green cabbage in both progoitrin and sinigrin standards.

Table 3: ANOVA of total glucosinolates levels of the stem and leaves in various cruciferous vegetables

Standard (Marker)	Broccoli leaf	Broccoli stem	Kale leaf	Kale Stem	Cauliflower Leaf	Cauliflower stem	Green Cabbage
Progoitrin	0.0862± 0.08	0.216 ±0.126	0.413±0.29	1.402±0.78	0.64±0.36	1.004±0.9	0.103
Sinigrin	0.406 ±0.18	1.785± 1.07	3.54± 2.79	11.07±5.18	5.49±3.22	7.83±7.7	0.100
Sulforaphane	0.023±0.011	-	-	0.043±0.061	-	0.001±0.001	0.0085±0.012

- = Not detected P ≤ 0.05 * = Non significant difference ** =significant difference

Table 4: ANOVA of total levels of the cold methanol and boiling water extraction methods.

	Method	Broccoli	Kale	Cauliflower	Green cabbage
Progoitrin	Boiling	0.268±0.112	1.29±0.96	1.34±0.63	0.178±0.25
	Cold	0.078±0.07	0.603±0.45	0.341±0.054	0.01±0.014
Sinigrin	Boiling	1.41±1.59	10.12±6.5	10.53±3.9	1.57±0.24
	Cold	0.775±0.35	4.48±4.1	2.85±0.61	0.36±0.51
Sulforaphane	Boiling	0.023±0.03	0.046±0.066	0.001±0.014	0.087±0.12
	Cold	-	-	-	

- = Not detected P ≤0.05 * = Non significant difference **=significant difference

A remarkable significant difference was also observed in bronccoli stem and leaves, kale stem and leaves and cauliflower 3 stems and leaves for sinigrin and progoitrin in the two extraction methods. However, a non-significant difference was noticed in cauliflower leaves and stem in cold methanol extraction method for progoitrin

4.0 CONCLUSION

High performance liquid chromatography (HPLC) was successfully used to quantify three GSLs namely: Progoitrin (2R)-2-Hydroxybut-3-enyl), sinigrin (2-Propenyl) and sulforaphane in four (4) different types of commonly consumed cruciferous vegetables; Broccoli (*Brassica oleracea L. italica*), Kale (*Brassica, leracea L. acephala group*), Cauli flower (*Brassica oleracea L. botrytis*) and Green cabbage (*Brassica oleracea L. capitata*) through both Cold methanol and boiling water extraction methods. Progoitrin, sinigrin and sulforaphane used as marker for the samples were present in almost all the vegetables (broccoli, kale, cauliflower and cabbage. The level of progoitrin and sinigrin extractions in all the samples were higher in the hot extraction than that of the cold extraction.

Sulforaphane was only found in the stem of kale and cauliflower and in the leaf of broccoli. Progoitrin and sinigrin level in the stems of all the vegetables analysed were higher than the levels found in the leaf except in cauliflower. The level of progoitrin and sinigrin either in the leaves or the stems of broccoli, kale, cauliflower and cabbage were higher than the defined limit set by the Chinese pharmacopoeia of 0.2 mg/g. Variance tests by ANOVA reveal varying degree of significant and non- significant difference when comparingthe two extraction methods and when comparing GSLs levels in stems and leaves of cruciferous vegetable investigated.

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COMPETING INTERESTS

The authors declare that there is no competing interest.

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