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African Journal of Biochemistry Research

Table of Contents: Volume 9 Number 10, November 2015

ARTICLES

Research Articles:

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Full Length Research Paper

Extraction, fractionation and assessment of antioxidant activities of active components of *Aframomum sceptrum* seeds

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A simple procedure of extraction, fractionation and assessment of organoleptic and antioxidant properties of *Aframomum sceptrum* seeds was carried out, and the results were compared with a currently used synthetic antioxidant, butylated hydroxy toluene (BHT). Extraction was carried out using Soxhlet extractor and fractionation of the ethanolic extract was accomplished using vacuum liquid chromatography (VLC). Various fractions of the ethanolic extract were obtained but similar fractions were pooled together to yield six fractions (F1-F6) as guided by thin layer chromatography (TLC) analysis. Fractions obtained were subjected to sensory, chemical, spectroscopic and storage assessment in order to evaluate their antioxidant potentials. Sensory characterization and antioxidant storage tests showed that fractions F4 and F5 had non-bitter taste and yellow colour in contrast to bitter taste and dark brown colour of the crude extract. Statistical analysis (P= 0.05) showed that the antioxidant effectiveness (AE) of the fractionated extracts F4 and F5 were 62.57 and 59.13% for groundnut oil and 64.50 and 62.91% for red palm oil, respectively, and compared favourably with the standard synthetic antioxidant used. The fractions can be used to extend the shelf life of food products.

Key words: Spices, fractionated extracts, antioxidant effectiveness, active components, sensory and spectroscopic characteristics.

INTRODUCTION

Free radicals can be generated by metabolic pathways in the body tissues or in food systems. Many synthetic chemicals such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) though very effective as antioxidants, have been known to have toxic and carcinogenic effects on humans (Yoshihara et al., 2010). Synthetic antioxidants may result in liver swelling and influence liver enzyme activities (Yoshihara et al., 2010). Therefore, considering these reports and the present trends towards naturally preserved food products, herbs and spices have been targeted as sources of natural antioxidants (Kimura et al., 2010; Brewer, 2011). The use of these plant materials as natural antioxidants for food, cosmetics and other application becomes necessary because of food safety issues: (1) safety, since they are part of food man has been eating for thousands of years

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(Bongoni et al., 2013); (2) good carry through effectiveness, since they survive processing operations; (3) their use is not guided by legislatory rules (Cohen and Ernst, 2010) and (4) their source is renewable. Seeds of *Aframomum sceptrum* have been shown to possess antioxidant activity superior to some currently used synthetic antioxidants (Auqustyniak et al., 2010). However, the seed and its crude extracts were bitter, a quality which could limit their application in food systems. The constraint serves as impetus to this study. Investigations carried out on fractionation and assessment of phytochemicals and antioxidant activities of fractionated active components of the *A. sceptrum* seeds were reported in this study.

MATERIALS AND METHODS

Seeds of *A. sceptrum* used in this study were purchased from a local market in Agbor, Delta State of Nigeria. They were identified and authenticated at the Botany Department of Delta State University, Abraka, Nigeria. The dried seeds were carefully removed from the pod manually. The removed seeds were cleaned - up of all extraneous materials and adhering particles by putting in a cotton sack, rubbed, then put in a tray and winnowed. The seeds were air-dried at 25°C for 2 days, to further reduce the moisture content. The air-dried seeds were pulverized into a fine powdery form using a Warren blender. Groundnut oil was purchased from local processor in Bida, Niger State, Nigeria. Palm oil was purchased from Nigerian Institute For Oil Palm Research (NIFOR) oil mill at Benin City, Edo State, Nigeria. Standard antioxidant used was BHT (Aldrich Chem. Co). All other chemicals used were of analytical grade.

Preparation of ethanolic extract of *A. sceptrum*

Solvent extraction of the seeds of *A. sceptrum* was done using the method stated in Mircea and Isabel (2015). An amount of 10 g of finely ground *A. sceptrum* powder was extracted with 100 ml of 95% ethanol in Soxhlet extractor for 8 h. The crude extract was evaporated to remove the solvent, and the percentage yield of the extract was determined.

Fractionation of *A. sceptrum* extracts using vacuum liquid chromatography (VLC)

Fifteen grams (15 g) of ethanolic crude extract was dissolved in 150 ml of ethanol (95%), followed by the addition of 120 g of pre-treated silica gel (Na ethanoate-treated gel). The mixture was mixed until thorough blend was obtained, then air-dried at 30°C in air-oven, and milled manually to obtain “pre-adsorbed sample”. Five grams (5 g) of the pre-adsorbed sample was subjected to vacuum liquid chromatography (VLC) on silica gel using a mobile phase (50ml each) gradient in toluene: ethyl ethanoate: methanol. Fractions were monitored by thin layer chromatography (TLC). Similar fractions were pooled together to produce six fractions (F1-F6), evaporated to dryness and kept in the dark for subsequent analysis (Perqola and Werz, 2010).

Characterization of crude and fractionated extract of *A. sceptrum*

Sensory characteristics of crude and fractionated extracts of *A. sceptrum* were determined following the procedure of Bongoni et al. (2013). For determination of chemical compounds, standard phytochemical screening procedure was carried out according to the method of Marcia et al. (2011). Total phenol was determined according to the method of Lu et al. (2011). The reducing power of crude and fractionated extract was determined using the method of Canabady-Rochelle et al. (2015). Other assays used to determine the antioxidant potentials of the extract include; DPPH spectrometric assay by the method of Chandrasekar et al. (2014), hydrogen peroxide scavenging activity by the method of Graf and John (2015), superoxide radical scavenging activity by the method of Shuhui et al. (2010), nitric oxide radical scavenging activity by the method of Anguo et al. (2003), hydroxyl radical scavenging activity according to the method of Isaksen and Dalsoren (2011), total flavonoid content by the method of Chen and Dingrong (2012), free fatty acid (AOCS, 2012) and peroxide value (AOCS, 2011). Ultra violet (UV) visible absorption spectra of crude and fractionated extracts were recorded on a UV- visible double beam recording spectrophotometer. Infrared (IR) spectral characteristic of crude and fractionated extracts were recorded on a Fourier- Transform- IR spectrophotometer.

Antioxidant incorporation and evaluation of antioxidant activities of *A. sceptrum*

The extracts and standard antioxidant were added into the oils at recommended concentration of 20 mg/100 g (0.02%) by direct addition at 50°C and stirred using BHT (standard antioxidant) as positive control and oils without additive as negative control. Antioxidant activities of the fractionated extracts were evaluated in treated oils by Schaal oven test. Treated samples were stored in an oven and maintained at a temperature of 63°C. Negative control was also placed under the same condition of storage as the treated sample. Oxidative stability was monitored by measuring peroxide values at regular intervals of seven days during the storage period (Akinmoladun et al., 2010). Decrease in the rate of formation of peroxides was used as a measurement of antioxidant activities (Akinmoladun et al., 2010).

Statistical analysis

Data obtained from evaluation of antioxidant activities at different concentrations using changes in peroxide values with time were subjected to statistical analysis and results obtained were tested for significant difference at 5% level using analysis of variance (ANOVA). Means were separated using Turkey’s test (Lowry, 2013).

RESULTS AND DISCUSSION

Phytochemical components of seeds, crude and fractionated extracts of *A. sceptrum*

Phytochemical screening of the seeds, crude extracts and fractions of *A. sceptrum* showed the presence of alkaloids, flavonoids, phenols, anthraquinones, tannins, saponins and glycosides (Table 1). This result is in agreement with earlier report of Marcia et al. (2011) on the class of secondary chemical compounds present in the seeds of *A. sceptrum* and plants belonging to the botanical family Zingiberaceae. The determination of the dominant secondary metabolites in the fractions is

Table 1. Phytochemical components in the seeds, crude and fractionated extracts of *A. sceptrum.*

Phytochemical	Seed	ETH	Fractions					
			1	2	3	4	5	6
Tannin	+	+	+	+	+	+	+	+
Saponin	++	++	+	+	+	-	-	+
Alkaloids	+++	++	++	++	++	-	+	++
Flavonoid	++	+++	+	+	+	+++	+++	+
Flavonol	+	+++	-	-	+	+++	++	-
Anthraquinone	+	+	-	-	-	-	+	-
Glycoside	+	+	+	+	+	+	+	+
Phenol	+	++	+	+	+	++	++	+

+++ Highly positive; ++ moderately positive; + slightly positive; - negative (not present).

necessary for structural elucidation of active compounds responsible for antioxidative activities. Flavonoid and its derivative flavonol were dominantly present in F4 as well as F5 with trace amount of anthraquinones. Flavonoids and their derivatives have been known to be the most active polyphenolic antioxidant (Marcia et al., 2011; Hussein et al., 2013). The effectiveness of flavonoid in retarding lipid oxidation in fat containing foods is related to their ability to act as free radical acceptors (Dai and Mumper, 2010) or as chelators of metal ions. The chelation by flavonoids is due to *ortho*-dihydroxy (3, 4-dihydroxyl) group on the β- ring in their chemical structure (Dai and Mumper, 2010). Flavonoid is also known to exhibit antimicrobial properties, while anthraquinone is known for its laxative properties. In line with Osbourn et al. (2011) and Erukainure et al. (2011), saponin and glycosides were detected in the seeds and fractions of *A. sceptrum.*

Sensory characteristics of crude and fractionated extracts

The active components of the seeds of *A. sceptrum* were separated into six fractions using vacuum liquid chromatography (VLC) and their sensory characteristics are shown in Table 2. The crude ethanolic extract was a brown viscous liquid with pungent and bitter taste. The dominating bitter taste is as a result of alkaloids present in *A. sceptrum* (Marcia et al., 2011). The various fractions obtained from VLC techniques were of varying degree of yellow colour with different kinds of taste, which could have been as a result of the dominant secondary metabolites. Fractions F4 and F5 were light-yellow and sunset yellow respectively in colour with non-bitter taste in contrast to dark colour and bitter taste associated with crude extracts. Such fractionated extracts will be good for preventing lipid peroxidation in food systems. Objectionable taste and unappealing colour are some of the characteristics that limit the application of antioxidants from natural sources (Karlen et al., 2011).

Table 2. Sensory characteristics of crude and fractionated extracts of *A. sceptrum.*

Antioxidant	Sensory characteristics	
	Colour	Taste
ETH	Dark brown	Bitter
STEAM	Brown	Bitter
F1	Golden yellow	Tasteless
F2	Reddish yellow	Slightly bitter
F3	Brown	Bitter
F4	Light yellow	Tasty (sweet)
F5	Sun-flower yellow	Slightly bitter
F6	Light yellow	Bitter

Spectroscopic analysis of crude and fractionated extracts of *A. sceptrum* - ultraviolet (UV) spectral characteristics of crude and fractionated extracts

Crude ethanolic extracts were separated into six fractions as guided by thin layer chromatography. Their UV spectral as extrapolated from UV curves is shown in Table 3. The crude extract (ETH) had strong absorption at 235 nm. The most potent fractions (F4 and F5) had strong absorptions at 240 and 210 nm respectively. Strong absorptions (primary and secondary) in the ultra violet region by the samples are diagnostic feature of unsaturation or non-bonded electron in the absorbing molecules (Akinmoladun et al., 2010). Free electron is a pre-requisite for antioxidant activity (Auqustyniak et al., 2010). In addition to this high wavelength of maximum absorption (λmax), the samples were characterized with large magnitude of molar extinction coefficient (ε). The magnititude of a molar coefficient for a particular absorption is directly proportional to the probability of the particular electronic transition, the more the given transition, the larger the extinction coefficient (Mariani et al., 2012). While both primary and secondary λmax of fraction F4 were higher than that of the standard antioxidant, BHT, the spectroscopic characteristics of the standard antioxidant is comparable to that of fraction F5.

Table 3. UV-spectral characteristics of crude and fractionated extracts of *A. sceptrum.*

Sample	λ_1 (nm)	A_1 (nm)	ε_1 (Lmole^{-1}cm^{-1})	λ_2 (nm)	A_2(nm)	ε_2(Lmole^{-1}cm^{-1})
ETH	235	2.821	19,219	263	2.506	17,835
F4	240	3.261	19,644	298	2.815	17,600
BHT	230	2.685	18,126	286	1.965	17,143
F1	217	2.203	5,620	222	0.581	2,264
F5	210	2.82	16,663	228	1.768	10,638
F3	226	2.143	3,642	272	1.398	2,533
F6	214	1.553	3,120	232	1.42	2,412
F2	218	2.166	3,200	262	1.02	2,760

Table 4. IR- spectral characteristics of the crude and fractionated extracts of *A. sceptrum.*

Sample	Vmax (cm^{-1})				
F1	3323 (b)	1288 - 1050 (b)	706 (w)	721 (w)	
F2	3466 (b)		780 (w)		
F3	3387 (b)	1208 (b)	816 (m)	780 (w)	640 (w)
F4	3364 (b)	1830 (s)	1452 (s)	965 (sh)	
F5	3449 (b)	1568 (sh)	1334 (s)	901 (v)	
F6	3359 (b)				
ETH	3386 (b)	1620 (sh)	1152 (s)	924 (w)	826 (w)

S = Strong; m = medium; w = weak; v = variable; b = broad; sh = sharp.

Infrared (IR) spectral characteristics of crude and fractionated extracts

Functional groups were assigned to IR – absorption peaks using functional group –IR frequency chart adapted from Neil (2005). Peaks of diagnostic value as extrapolated from IR curves (not shown) were identified and selected based on IR- functional group- antioxidant potential. The functional groups are phenolic hydroxyl group (≈3390 c m^{-1}), unsaturated/free electron potential (1600-1670cm^{-1}), carboxylate ions (1762 c m^{-1}), aromatic nuclei (800-900 c m^{-1}). The presence of peaks in the region and their intensities are indices of antioxidant potentials of crude and fractionated extracts. These properties are more pronounced in ETH, F4 and F5. The functional groups are the building blocks of antioxidant compounds (Table 4).

Antioxidant activities of *A. sceptrum* fractions in groundnut and red palm oils

Antioxidant activities of fractions of *A. sceptrum* and standard antioxidant (BHT) in the oils were evaluated. The peroxide values of the oil samples were determined when the oils were fresh and periodically at 7 days interval for 35 days as shown in Figures 1 and 2. Fractions F4 and F5 had the highest antioxidant effectiveness (AE) of 62.57 and 59.13% in groundnut oil, while 64.50 and 62.91% was observed in red palm oil respectively (Figures 3 and 4). The dominant secondary metabolites in the fractions are flavonoids, its derivative (flavonol) and anthraquinones. Flavonoid is a class of polyphenols with effective antioxidant activities (Hussein et al., 2013). Fraction F2 in which alkaloids are dominant had lower AE (32.62%) in groundnut oil and AE (42.42%) in red palm oil respectively when compared to activities of F4 and F5, showing that alkaloids are not the major contributors to the antioxidant properties of *A. sceptrum.* The antioxidant effectiveness (AE) of the fractions F4 and F5 were similar to that of BHT. The results indicated a comparable (p=0.05) effectiveness of fractions F4 and F5 to the reference antioxidant.

Relationship between phenolic content, reducing power and absorbance of extracts of *A. sceptrum*

Phenolic content, reducing power and absorbance of the fractions of *A. sceptrum* are shown in Table 5. Correlation (r=0.880) exists between phenolic content and reducing power. This suggested that the mode of antioxidant action of phenols in *A. sceptrum* is more of electron-transfer or abstraction of hydrogen from hydroxylic group of phenolic compounds than of resonance stability by phenyl moiety. There was also a slight relationship (r=

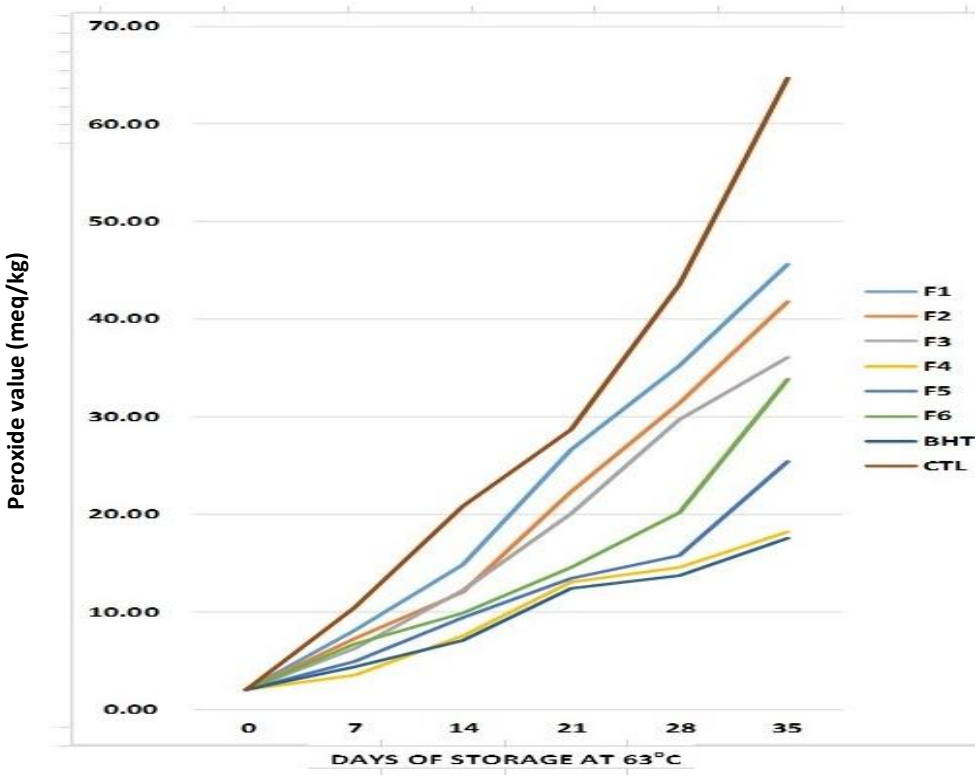

Figure 1. Antioxidant activity of fractions of *A. sceptrum* and BHT in groundnut oil at 0.02% concentrations.

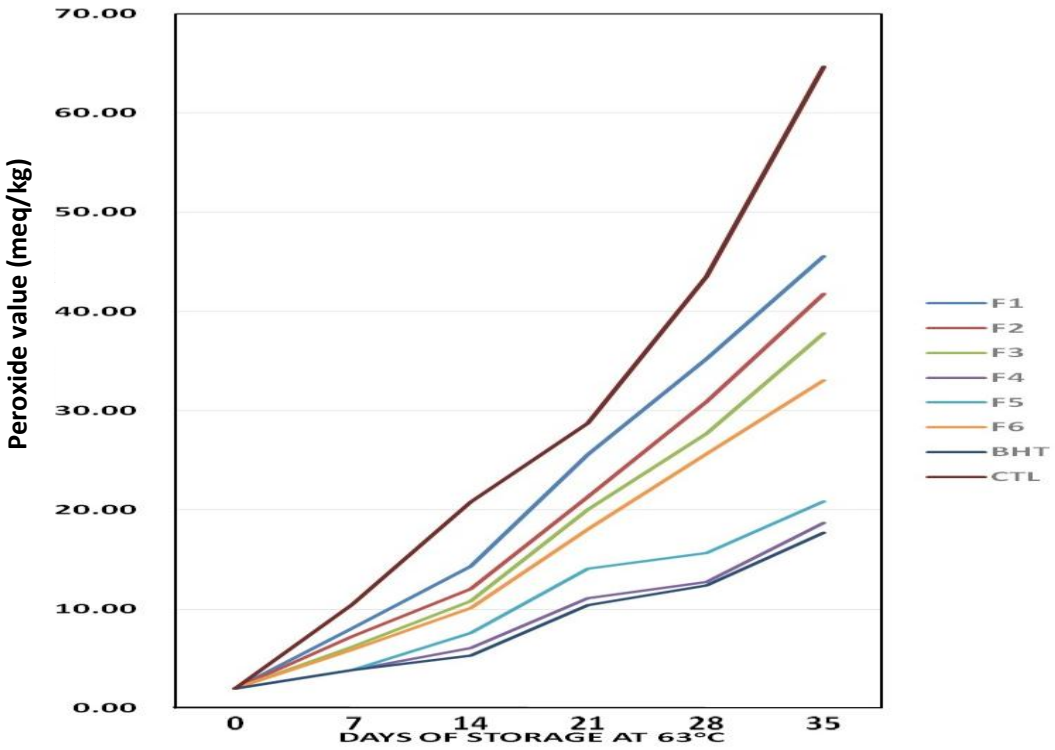

Figure 2. Antioxidant activity of fractions of *A. sceptrum* and BHT in red palm oil at 0.02% concentration.

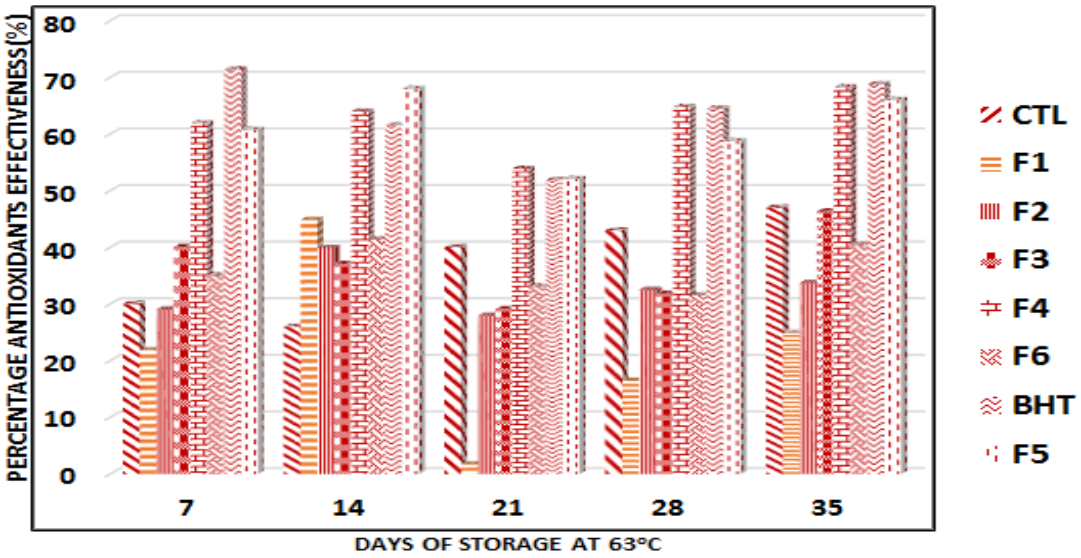

Figure 3. Percentage antioxidant effectiveness of fractions of *A. sceptrum* and BHT at 0.02% concentrations in groundnut oil.

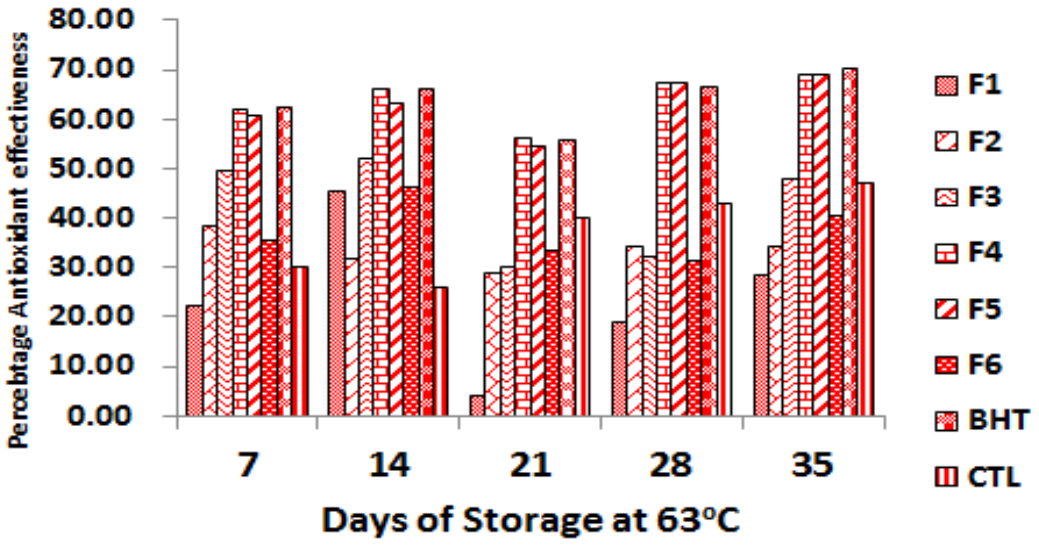

Figure 4. Percentage antioxidant effectiveness of fractions of *A. sceptrum* and BHT at 0.02% concentrations in red palm oil.

Table 5. Phenolic content, reducing power and absorbance at λmax of extracts of *A. sceptrum*.

Sample	Phenolic content (g/g ascorbic acid equivalent)	Reducing power	Absorbance at λmax
F1	0.700	0.010	2.300
F2	1.370	0.150	2.820
F3	1.430	0.121	2.810
F4	1.850	0.183	3.350
F5	1.420	0.140	2.600
F6	0.720	0.015	2.320
ETH	1.830	0.161	3.200

0.650) between total phenol content and absorbance at primary λmax. This implied that the phenolic compounds in the crude extracts and fractionated extracts are of variable qualities (Mouming et al., 2014).

Conclusion

The presence of bioactive compounds in *A. sceptrum* is an affirmation of its use in the management of various ailments, and consumption of this spice in combination of other foodstuff can help meet the nutritional needs of the individual. The fractionation of viscous, dark brown and bitter extracts of *A. sceptrum* yielded a non-viscous, yellow and tasty extract which compared favourably with the one of the currently used synthetic antioxidants. This fraction is recommended for use in food preservation.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Nitrate contents in some vegetable leaves in Sokoto Metropolis, Nigeria

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Popular Nigerian vegetables namely, cabbage, spinach, bitter leaf, water leaf, ewedu, roselle and lettuce obtained from Sokoto metropolis, Nigeria were analysed for nitrate contents by ultra-violet (UV)-spectrophotometric method. The fresh leaves of the samples were chopped and ground using mortar and pestle for the analysis. Absorbance of each sample was obtained in three replicate and the calibration graph of standards nitrate were used in determining the concentration of each sample. Cabbage, spinach, ugwu and lettuce contain the lowest amount of nitrate in this study (0.109 ± 0.035 µgml^{-1}, 1.530 ± 0.130 µgml^{-1}, 1.730 ± 0.328 µgml^{-1} and 2.185 ± 0.157 µgml^{-1}) in comparison with the nitrate contents of samples like roselle, ewedu, water leaf and bitter leaf which contain the highest amount (2.938 ± 0.060 µgml^{-1}, 3.682 ± 0.140 µgml^{-1}, 3.924 ± 0.160 µgml^{-1} and 4.351 ± 0.190 µgml^{-1}). These values fall within those recommended for nutritional purpose.

Key words: Nitrate contents, vegetables, Sokoto, absorbance.

INTRODUCTION

A number of ailments have their origin in our diet, either directly or indirectly. Many modern diseases are as a result of nutritional deficiencies. Fortunately, in many cases, by simply increasing the vegetables intake can solve these problems as long as they have not been ignored for too long (Mason, 2010; Sasathorn et al., 2015). Green vegetables are a major source of dietary nitrate intake. Nitrate may have several beneficial health effects mediated through reactive N intermediates, including antibacterial effects and effects on gastric mucosal integrity (Andra's et al., 2014; Keszei et al., 2013).

Nitrate is a naturally occurring compound that is part of nitrogen cycle, as well as approved food in which they play an important role in the nutrition and function of plants. Nitrates are important components of vegetables, they occur widely in our drinks and food (Okafor and Ogbonna, 2003; EFSA, 2008). High levels of nitrate tend to occur in the leaves whereas lower level occurs in the seeds and tubers. Nitrates are nitrogen-oxygen chemical units which combine with various organic and inorganic compounds (Croitoru, 2012). Once taken into the body, nitrates may be converted in to nitrites. Crop containing high level of nitrates can be identified by laboratory test. Nitrates in vegetables and fruits have no taste or smell (FFTC, 2007). Nitrates occur naturally in fruits and vegetables, but only in small quantities, they can rise to high levels in intensively grown crops (Croitoru, 2012).

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Nitrate concentrations in vegetables depend on the biological properties of the plant culture, light intensity, type of soil, temperature, humidity, frequency of plants in the field, plant maturity, vegetation period, harvesting time, storage time and source of nitrogen (Shohreh et al., 2015; Tamme et al., 2006).

The high concentration of nitrogen in fertilized soil may lead to the high nitrate level in edible vegetables and toxic level of nitrate may be produced by microbial activity in the gastrointestinal tract of the consumer of such vegetables (Tanaka et al., 1982; Thomson et al., 2007). Nitrates and their precursor's nitrites are both naturally occurring substances and are produced by living cells (Shohreh et al., 2015). They are involved in many important chemical reactions in the body. Vegetables and fruits sources of nitrates are considered healthy whereas preserved meat sources are not. Indeed 70 - 80% of our consumption of nitrates is thought to be from plant sources as well as from water (NYR, 2013; Contam, 2008). Nitrates are soluble salts of nitric acid. The solubility of nitrates is important, as they are absorbed in solution by plants through their root system (Tamme et al., 2006). Nitrates occur in the soil through the effect of lightning or atmospheric nitrogen and oxygen, and through the decay of dead plants and animals, as well as by use of fertilizers (Harwood, 2008).

High nitrate content is a potential human threat especially to infants, causing the problem known as methemoglobinemia, also called "blue baby syndrome" (Andra's et al, 2014). When nitrate is taken in by eating food and drinking water, it is converted in the gut to nitrite, which then combines with haemoglobin to form methemoglobin, thus, decreasing the ability of the blood to carry oxygen in the human body (FFTC, 2007).

Some studies have raised a concern about cancer causing-potential of nitrates and nitrites which are used as preservative and colour enhancing agents in meat. Nitrates react with amino acids to form nitrosamine which has been reported to cause cancer in humans (ATDSR, 2007; Pham et al., 2008).

Tanaka et al. (1982) reported a sensitive and direct spectrophotometric method for the determination of nitrates in vegetables using 2-sec-butylphenol. The basis for this method is that 2-sec-butylphenol reacts quantitatively with nitrate in acidic solution. Gaya and Alimis (2006) also reported a spectrophotometric determination of nitrate in vegetables using phenol. The method is based on the measurement of the absorbance of yellow sodium nitrophenoxide formed via the reaction of phenol with the vegetable-based nitrate in the presence of sulphuric acid.

This current work demonstrated the effectiveness of a standard calibration plot for the determination of the concentration of nitrate in an unknown sample. The investigation also aimed at determining the contents of nitrate in the vegetable leafs consumed in Sokoto, Nigeria using spectrophotometric method and also to see if the level of nitrate found in the leafs is in line with the approved daily in take.

MATERIALS AND METHODS

Materials

Sodium hydroxide (MF: NaOH, MW: 40.00 g/mol, CAS: 1310-73-2, Assay: 97%), silver sulphate (MF: Ag_2SO_4, MW: 311.80 g/mol, CAS: 10294-26-5, Assay: 99.99%), sodium carbonate (MF: Na_2CO_3, MW: 105.99 g/mol, CAS: 497-19-8 Assay: 99.99%), sulphuric acid (MF: H_2SO_4, MW: 98.08 g/mol, CAS: 7664-93-9, Assay: 99.99%), toluene (MF: $C_5H_5CH_3$, MW: 92.14 g/mol, CAS: 108-88-3, Assay: 99.80%) and phenol (MF: C_6H_5OH, MW: 94.11 g/mol, CAS: 108-95-2, Assay: 99.50%), were purchased from Sigma-Aldrich (Dorset, UK). Vegetable samples, namely: spinach, lettuce, water leaf, bitter leaf, ugwu, ewedu, roselle and cabbage were purchased from Sokoto fish, meat and vegetable market. The materials were used as received.

Methods

Wavelength determination (λ_{max})

A stock solution of 100 μgml^{-1} of nitrate was prepared in deionised water to determine the lambda max. Subsequently, a standard solution of 25 μgml^{-1} was prepared in a 25 ml volumetric flask, and a Cary 60 UV/Vis spectrophotometer was used to determine the wavelength of maximum absorption in the range 200 - 800 nm. Figure 1 shows the spectrum of nitrate.

Calibration graph

A Cary 60 UV/Vis spectrophotometer (Agilent technologies) was used to determine the concentration of nitrate. A stock solution of 100 μgml^{-1} of nitrate was prepared in deionised water. Absorbance of 8 standards solutions (0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, 25.0 μgml^{-1}) each with three replicates were determined at 282 nm. Beer's Law calibration plots of absorbance versus concentration of nitrate showed no deviation from linearity with regression coefficients ≥ 0.9999 and an intercept of 0.0039.

Sample preparation

Fresh vegetable samples were chopped and ground using mortar and pestle till homogenous slurry was formed, 10 g of the slurry was taken in to a 250 ml beaker. 70 ml of deionised water and 2.5 ml of 4% NaOH solution were added. The content of the beaker was warmed at 80°C for 25 min with occasional shaking. The resulting solution was filtered through a fluted filter paper into a 100 ml volumetric flask and made up to the mark with deionised water. An aliquot of 4 ml of the diluted solution was taken into a test tube cooled in an ice. 1 ml of 5% Ag_2SO_4 solution was added followed by subsequent addition of 7 ml of concentrated H_2SO_4 solution and 0.1 ml of 5% phenol solution. The solution was allowed to stand for 20 min with occasional shaking and the resulting mixture was extracted with toluene after shaking for another 10 min in a 50 ml separating funnel. The lower aqueous layer was discarded, the organic phase was washed twice with 10 ml of deionised water by shaking for 2 min and each time discarding the aqueous phase. The organic phase was extracted again by shaking for 1 min with 10 ml of 10% Na_2CO_3 solution and then the resultant product was collected in a test tube. The procedure was carried out for all the vegetable samples as described above.

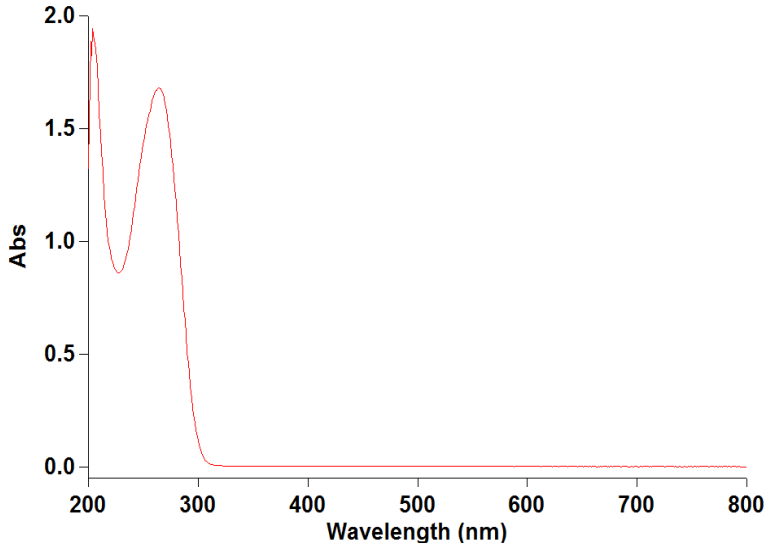

Figure 1. UV spectrum of nitrate.

Table 1. Absorbance (A.U) and concentration (µgml^{-1}) of nitrate + STDEV.

Concentration (µgml^{-1})	Absorbance (A.U)			Mean (A.U)	STDEV
	1st	2nd	3rd		
0	0	0	0	0	0
2.5	0.0857	0.0897	0.0911	0.0889	0.0028
5.0	0.1832	0.1756	0.1727	0.1772	0.0054
7.5	0.2732	0.2820	0.2676	0.2743	0.0073
10.0	0.3627	0.3672	0.3575	0.3625	0.0049
12.5	0.4559	0.4586	0.4522	0.4556	0.0032
15.0	0.5439	0.5425	0.5399	0.5421	0.0020
20.0	0.7349	0.7335	0.7283	0.7322	0.0035
25.0	0.9262	0.9198	0.9165	0.9208	0.0049

UV/visible spectroscopy

Absorbance eight of the standards and the samples were measured with Cary 60 UV/Visible spectrophotometer at 282 nm. The samples containing total nitrate content were placed into a cell equipped with a quartz window. Measurements were made in triplicates for both the standards and the samples. Concentrations in the liquid samples were analysed using the equation of the graph and the mean of the absorbance of nitrate and their corresponding standard deviations were calculated.

RESULTS AND DISCUSSION

Calibration graph

Table 1 shows the absorbance of standard concentration of nitrate in three replicate with their mean and standard deviation. These results were utilised in building a calibration graph of the known standards. The data presented in Table 1 can also be seen in Figure 2.

The data point represents the mean of 3 results with SD error bars. The graph shows the line equation and R^2 value is included to aid further calculations. The error bars on the graph are very small which shows the significance of the observed data to the linear relationship.

From Table 1, the mean of three replicates of the absorbances were plotted against the concentration of the standards and standard deviation where each replicate was used to calculate error bars on each data point. Looking at the graph (Figure 2), the plot generated exhibited an excellent linearity, the equation of the graph $y = 0.0368 x – 0.0039$ shows the gradient of 0.0368, intercept – 0.0039 and $R^2 = 0.9999$ which is very close to +1. The observed results are due to careful handling during solution preparation, making the calibration plot less susceptible to random errors which can affect the

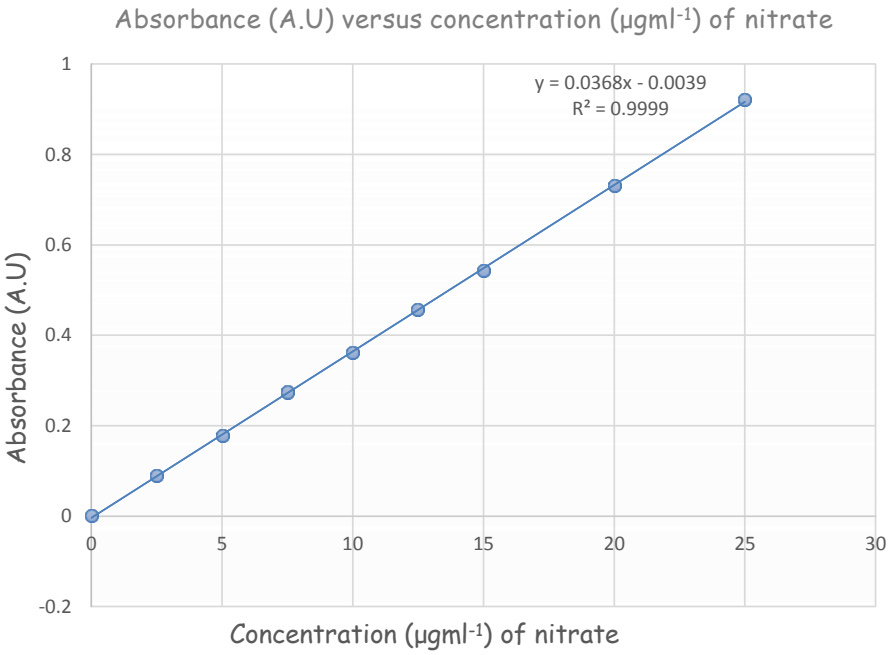

Figure 2. Graph of absorbance (A.U) versus concentration (µgml^{-1}) of nitrate.

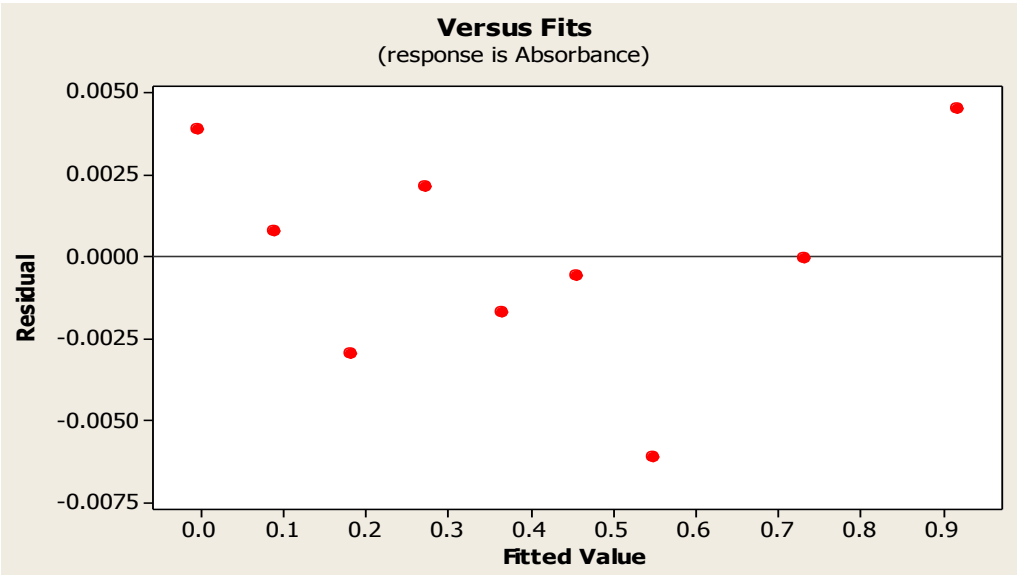

Figure 3. Residual versus fitted value of the calibration graph.

results.

The graph shows the residual on the vertical axis and fitted value (independent variable) on the horizontal axis. The linear regression model for the data is appropriate because the points in the graph are randomly distributed on the horizontal axis indicating a good fit of the graph in Figure 2.

In order to examine whether the set of data in Figure 2 is a good fit, residuals of the line of the graph above was investigated, this graph revealed a fairly random distribution relationship between the residual and concentration, thus signifying that a decent fits to the data (Figure 2) is provided by a straight line model.

Linearity and the best-fit line were obtained by linear regression (Figure 3) which operates by obtaining the line that gives a minimum value for the sum of the squares of the distances of all the points from that line. The best-fit line occurs at the standard concentration of 20 µgml^{-1} and

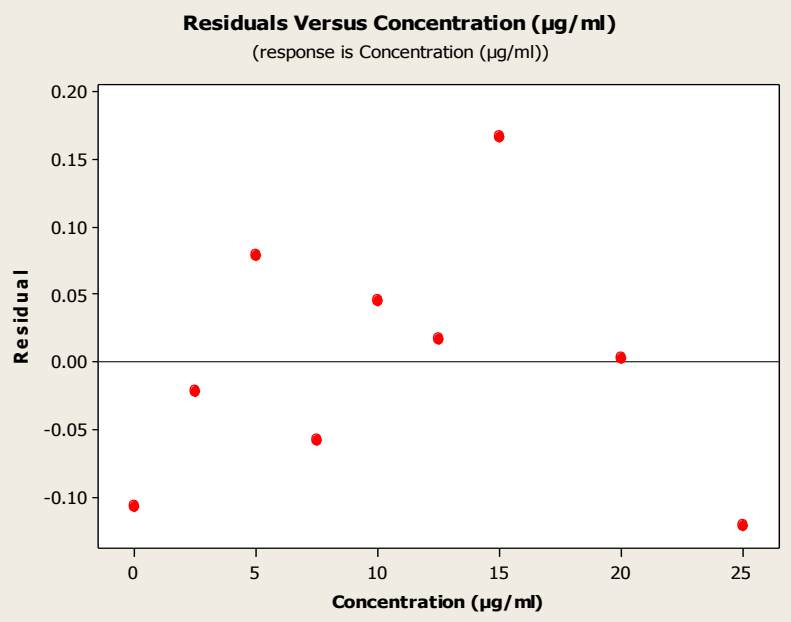

Figure 4. Residual versus concentration (µgml^{-1}) of the calibration graph.

Table 2. Total nitrate content in vegetables.

Sample	Botanical name	Amount of nitrate (µgml^{-1})
Cabbage	*Brassica Sativa*	0.109 ± 0.035
Ugwu	*Theifricia Occidentalis*	1.730 ± 0.328
Ewedu	*Chochorus Sativa*	3.682 ± 0.140
Water leaf	*Talinun Triangulare*	3.924 ± 0.160
Bitter leaf	*Vernonia Amygdalinan*	4.351 ± 0.190
Spinach	*Spinacia Oleracea*	1.530 ± 0.130
Lettuce	*Lactiva Sativa*	2.185 ± 0.157
Roselle	*Herbiscus Sabdariffa*	2.938 ± 0.060

the least fit-line occur at concentration of 25 µgml^{-1}. The accuracy of the plot was checked by plotting a graph of residual versus fitted values and also a plot of residual as a function of concentration (Figures 3 and 4). Data generated from the two plots is randomly distributed which signifies the certainty of the graph. As discussed earlier, looking at the trend pattern in Figures 3 and 4, data is randomly distributed and the instrument response increases as the concentration of the standards increased. Problems of non-linear range and matrix effect that normally occur due to an instrument problem were not witnessed which also signifies that the results obtained relied on interpolation (certainty of the results).

In summary, the resultant calibration graph proved suitable for use in the nitrate analysis.

LOD and LOQ

Detection limits and quantification limits. The limits of detection (LOD) of the proposed method were determined at a signal-to-signal ratio of 3, whereas the limits of quantification were obtained at a signal-to-signal ratio of 10. The results showed LOD of 0.522 µgml^{-1} and LOQ of 17.391 µgml^{-1} for the graph analysed.

Nitrate contents in the vegetable samples

Eight leafy vegetables samples were collected from local market during the period of June 2010. Three replicates of each sample were analysed and nitrate contents were evaluated as the mean of three measurements. The contents obtained are detailed in Table 2.

Spectrophotometric analysis was carried out in order to determine the amount of nitrate in the vegetable samples. The amount of nitrate from within all the vegetable was studied using Cary 60 spectrophotometer. From the Table 2, it shows that nitrate content was found in detectable amount in all the vegetables investigated. The

table illustrates the amount of nitrate in the vegetable samples. Vegetables such as cabbage, spinach, ugwu and lettuce contain the lowest amount of nitrate in this study (0.109 ± 0.035 μgml^{-1}, 1.530 ± 0.130 μgml^{-1}, 1.730 ± 0.328 μgml^{-1} and 2.185 ± 0.157 μgml^{-1}) in comparison with the nitrate contents of samples like Roselle, ewedu, water leaf and bitter leaf which contain the highest amount (2.938 ± 0.060 μgg^{-1}, 3.682 ± 0.140 μgg^{-1}, 3.924 ± 0.160 μgml^{-1} and 4.351 ± 0.190 μgml^{-1}). These results are in comparison with the study carried out by Ann et al. (2014) on how high-nitrate vegetable diet increases plasma nitrate and nitrite concentrations and also reduces blood pressure in healthy women.

Conclusion

The locally available vegetables are valuable and natural sources of nitrate. The results show that these vegetable leaves are a very good source of nitrate. This can be testified from the fact that nutritive recommendation for nitrate is 3.70 $mgkg^{-1}$ body weight. This paper presents a spectrophotometric method usable for simultaneously determining nitrate in vegetables with high sensitivity, accuracy and precision. Such method is extremely important in the biomedical research regarding the formation of nitric oxide and in the toxicological research regarding the presence of nitrate as toxins in vegetables or biological material. Other techniques such as HPLC and GC-MS could also be adopted in improving this research.

Conflict of interests

The authors did not declare any conflict of interest.

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