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Estimation of folate content of cultivated and uncultivated traditional green leafy vegetables in Nigeria

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The aim of the study was to quantify the folate content of eight samples (seven raw and boiled traditional leafy vegetables and one fruit vegetable). The analysis of folate in the raw and cooked vegetable samples followed the process of extraction, deconjugation using chicken pancreas deconjugase, and derivatization. Through this process, all folate forms in the deconjugated sample extracts were converted to 5-methyltetrahydrofolic acid (THF-5CH$_3$) monosodium glutamate and/or diglutamate. Purification was done by affinity chromatography with Folate Binding Protein and Reversed-phase high performance liquid chromatography (RP-HPLC) equipped with fluorimetric detection was used to quantify folate. Folate content in the raw samples ranged from 21.5 µg/100 g (FW) in *Solanum macrocarpon* leaves to 183.4 µg/100 g (FW) in *Corchorus olitorius* and in the boiled samples values ranged from 8.5 µg/100 g (FW) in *S. macrocarpon* to 48.6 µg/100 g (FW) in *Launaea taraxacifolia* leaves. Loss of folate in the boiled vegetables varied from 46.6% in *L. taraxacifolia* to 88.4% in *Adansonia digitata*. The difference in folate content of the raw and boiled vegetables were found to be significantly different (p<0.05). Traditional green leafy vegetables studied are good sources of folate in their raw form. However, cooking of the vegetables resulted in considerable decrease of the folate content of the vegetables. Preparation methods of traditional leafy vegetables that will allow for optimal retention of folate are therefore necessary.

Key words: Folic acid, 5-methyltetrahydrofolic acid (THF-5CH$_3$) monosodium glutamate, indigenous vegetables, processing.

INTRODUCTION

Folate (Vit. B9) is a water-soluble B-vitamin, which is found in foods mainly as polyglutamate (Scott, 1999). It is involved in one-carbon transfer in DNA synthesis and amino acid metabolism in the methylation cycle, a deficiency of which results in megaloblastic anaemia (Scott, 1999). Folate is also involved in the conversion of

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Table 1. Selected Traditional green leafy vegetables (TGLVs) studied.

Scientific name	Family	English name	Local/ common name	Status
Amaranthus hybridus L.	Amaranthaceae	Pig weed	*Efo tete/ green*	Cultivated
Abelmoscus manihot (L.) Medikus (leaves and tender shoots)	Malvaceae	Okro leaves	*Ilasa*	Cultivated
Adansonia digitata	Malvaceae	Baobab leaves	*Luru / kuka*	Uncultivated
Corchorus olitorius L.	Malvaceae	Jute mallow	*Ewedu*	Cultivated
Crassocephalum crepidioides (Benth.) S. Moore	Asteraceae		*Ebolo*	Uncultivated
Launaea taraxacifolia (Willd.) Amin, ex C. Jeffrey	Asteraceae	African lettuce/ wild lettuce	*Yanrin*	Uncultivated
Solanum macrocarpon L.	Solanaceae		*Efo igbagba*	Cultivated/ wild
Abelmoscus esculentus (fruit- green pod)	Malvaceae	Okro fruit	*ila*	Cultivated

homocysteine to methionine, an amino acid (Scott et al., 2000). High levels of plasma homocysteine (Hyperhomocysteinaemia), has been identified as an independent risk factor for cardiovascular diseases and stroke (Scott and Weir, 1998), hence its importance in the prevention of cardiovascular diseases. Furthermore, folate is important for normal neural tube development; low maternal folate status could result in neural tubes defects (NTDs) in newborns (Wald and Sneddon, 1991). Birth prevalence of NTDs has been reported to be between 2.2 - 7 per 1000 births in Nigeria (Nnadi, 2016, Zaganjor et al., 2016, Anyanwu et al., 2015).

Vegetables and fruits are among the richest plant sources of folates in the diet, with 5-CH_3-H_4folate, being the most predominant form (Delchier et al., 2016; Hefni et al., 2010; Holasova et al., 2008). Foods fortified with synthetic folic acid are also rich sources in developed countries (Shohag et al., 2012; Hefni et al., 2010). In a review by Scott et al. (2000), vegetables were reported to be the largest single contributor to adult intake of folates in The Netherlands (39%), UK (35%) and Ireland (38%). A 4-week controlled dietary trial by Brouwer et al. (1999) showed that increasing consumption of folate rich vegetables and citrus fruits, significantly improved folate status and decreased plasma homocysteine levels in healthy volunteers.

In Nigeria there is no mandatory fortification of foods with folic acid. This means that the population depends mostly on dietary sources for their folate intake. However, supplements are mostly recommended for women of reproductive age to meet up with their requirements for reproduction and prevent NTDs. It is important therefore to harness the potential contribution of readily available local dietary sources to folate intake like vegetables. There is great diversity of local cultivated and uncultivated traditional green leafy vegetables (TGLVs) in Nigeria that form a major component of several local dishes. Most of the TGLVs are traditionally washed and/ or boiled before consumption. Folate is reported to be vulnerable when subjected to such processing methods as boiling (Delchier et al., 2016). But information on the folate content of TGLVs in Nigeria is scant. Hence, the main purpose of this study was to quantify the folate content of selected TGLVs in fresh and boiled forms using an advanced method of quantification by high performance liquid chromatography (HPLC) with flourimeric detection.

The data provided in this study will be useful for educating the public about folate rich foods in order to encourage their consumption, recommend appropriate preparation methods and prevent consequences of the lack thereof. It will also help to fill some gaps in the recently published Nigerian food composition table (2017). It will facilitate better estimate of dietary intake of the vitamin from TGLVs among different age groups.

MATERIALS AND METHODS

Table 1 shows the names (scientific and local) and the status of eight vegetables studied, seven of which are green leafy vegetables and one fruit vegetable.

Sample collection and preparation

Fresh samples (500 g^{-1} kg) of TGLV (Table 1) vegetables were harvested from field locations in Nigeria except for *Adansonia digitata* that was purchased as dried ground samples (the available form in the market), *Corchorus olitorius*, and *Abelmoscus esculentus* (fruit- green pod) were also purchased from the market.

Edible portions (leaves and stalk) were sorted, and transported in cold chain to *Sécurité et Qualité des Produits d'Origine Végétale* (SQPOV) laboratory in Avignon, France, where they were further processed prior to folate analysis. At the laboratory, the edible portion was further sorted to remove damaged leaves (Ejoh et al. – In press).

Fifty grams of fresh TGLV samples were weighed in triplicate and each were ground in liquid nitrogen with A11 analytical mill (IKA, Staufen, Germany) and kept at -80°C. To obtain the boiled samples, 150 g of each vegetable sample (in triplicate) were boiled by adding the vegetables directly into boiling demineralized water and cooked for 5 min. The vegetable/liquid ratio was approximately 1 g/4 ml. The water from boiled TGLVs were drained (except for the resulting mucilaginous water from *C. olitorious* leaves and *A. esculentus* fruit liquids, that were not drained as is traditionally prepared). Samples were rapidly frozen at -30 ˚C, ground in liquid nitrogen and stored at -80 °C till analysis (Ejoh et al. In press).

Determination of folate

Folate extraction and measurement were done following the method of Delchier et al. (2012) with slight modifications.

(i) Principle of measurement: Folate is found in foods mainly as polyglutamate. The analysis of the fresh and cooked vegetable samples followed the process of extraction, deconjugation (using chicken pancreas conjugase), transformation (by a series of chemical reactions to convert all folate present in the deconjugated sample extracts, to 5-methyltetrahydrofolic acid (THF-5CH$_3$) monosodium glutamate and / or diglutamate), purification by affinity chromatography with Folate Binding Protein and quantification by HPLC equipped with fluorimetric detection.

(ii) Extraction: 10 g of sample was extracted using 30 ml 0.1 M phosphate buffer containing 1% ascorbic acid and boiled in a water bath at 100°C for 10 min. After cooling for 15 min, the volume was adjusted to 50 ml with phosphate buffer (0.1 M containing 1% ascorbic acid) and centrifuged for 10 min at 5000 g.

(iii) Deconjugation: 1 ml of chicken pancreas was added to 10 ml of extract and incubated at 37°C for 2 h.

(iv) Chemical transformation of folates: A series of chemical reactions to convert all folate present in the deconjugated sample extracts, to 5-methyltetrahydrofolic acid (THF-5CH$_3$) monosodium glutamate and / or diglutamate. 5 ml of 40% ascorbic acid pH buffer, 15 ml of Tris buffer, 1 ml of 2-octanol, 10 ml of NaBH4 (added very carefully and slowly because it is effervescent and toxic) were added into the beaker containing the sample extract. The content of the beaker was mixed thoroughly using magnetic stirrer and left to stand for 10 min. The pH of the sample solution in the beaker was adjusted to 7.4 by adding acetic acid 5 M. 80ul of 37% formaldehyde, and 10 ml of NaBH4 were added and stirred continuously. The pH was reduced to a value less than 1 by adding 37% HCl gradually. The mixture was left to stand for another 10 min to stop the formation of bubbles. The pH was adjusted to 5 by gradually adding 5 M NaOH and 10ml of NaBH4. The solution was left to stand for 20 min and later transferred into the 100 ml volumetric flasks. Tris buffer was added to the volumetric flask to make it to the mark. The flasks were covered with parafilm and mixed thoroughly by shaking. The solution was filtered with a 10 ml syringe attached to the 80 micron filters (with cellulose acetate membrane) into 15 ml falcon tubes and labeled.

(v) Purification of folates: Purification by affinity chromatography with Folate Binding Protein followed the process of column conditioning using 0.1 M phosphate buffer. Passing of 10 ml filtered sample through the column gel and elution of folates with 8 ml eluent solution consisting of 0.02 M DL-dithiothreitol (DTT) and 0.02 M trifluoroacetic acid (TFA) into a beaker containing 40 µl of 60% NaOH and 200 ul of 25% amino acid. The volume was adjusted to 10 ml with eluent solution.

(vi) Quantification of folates: HPLC equipped with fluorimetric detection (RF-10AXL, Shimadzu Inc., Kyoto, Japan). Column Licrospher ® RP-18 Column 250 × 4.6 mm, 5 µm. Precolumn Guard 7.5 × 4.6 mm Licrospher ® RP-18 5 microns; column oven temperature: 30°C; Autosampler Temperature: 4°C; flow rate is 0.8 ml per min; 25 µl of the eluted solution was injected; Mobile phases: ultrapure Water + 0.1% formic acid (v/v) and acetonitrile.

Analysis was carried out in three technical replicates. Data was summarized using means and standard deviations in MS Excel software (2010) and paired sample t-test was used to compare folate content of fresh and boiled samples at p=0.05.

RESULTS AND DISCUSSION

Folate content of raw TGLVs

The results of moisture and folate analysis are presented in Table 2. The predominant form of folate in all the TGLVs analysed was 5 methyl tetrahydrofolate (5-CH$_3$-H$_4$folate). The other forms where quantified, but represent less than 1% of the total content of folate, explaining why all other forms was grouped as one single "folate" class and results expressed in (µg/100 g) FW.

Four of the TGLVs in their fresh form (*C. olitorius, Launaea taraxacifolia, Crassocephalum crepididiodes and Amaranthus hybridus)* contained between 62 and 183 µg/100 g of folate, *Corchorus olitorius* having the highest. *A. esculentus* fruit (okra) had 106 µg/ 100 g of folate and dried *Adansonia digitata* powder had 149.64 µg/ 100 g. Green leafy vegetables are considered good sources of dietary folate, especially when they have concentrations of >50 µg/100 g edible portion of fresh vegetable or fruit (Ogle et al., 2001). In this form most of the vegetables fall into the category of good sources of folate except *Abelmoscus manihot* and *Solanum macrocarpon.* In fresh form, the TGLVs have the potential to contribute as much as 46% of 400 µg RDA for women of reproductive age in Nigeria.

The paucity of previous data on the folate content of TGLVs in Nigeria, makes it difficult to make comparisons. In the recently published Nigerian Food composition table (Sanusi et al., 2017), folate values for *Amaranthus* sp, *A. digitata* dried leaves and *Abelmoscus* leaves were borrowed from other food composition tables. The folate values documented were higher than obtained for similar vegetables in our study. There was no value for folate content of *C. olitorius*, one of the most popular TGLV in Nigeria, and for uncultivated TGLVs like *S. macrocarpon*, *L. taraxacifolia,* and *C. crepidioides* in the database.

Comparing data from literature outside Nigeria for similar vegetables, van der Walt et al. (2009) reported a variation in the folate content of *Amaranthus* sp from three locations in South Africa; the values ranged from 72 to 130 µg/100 g of fresh sample, 75 µg/100 g and 56 µg/100 g for *Amaranthus* sp and *Solanum* sp, respectively reported by van Jaarsveld et al. (2014). These values were higher than the values obtained for raw *A. hybridus* sp and *Solanum* sp (FW) in the present study (Table 2). The only exception was *C. olitorius* for which we obtained a considerably higher value than what was reported by van Jaarsveld et al. (2014), but it was within the range reported for folate content of spinach by Delchier et al. (2012) and Shohag et al. (2012).

The varaiation in folate values could be as a result of post-harvest conditions of the raw materials and or variety of vegetable used or differences in analytical methods. The studies mentioned above used microbiological methods for determination of folates, while HPLC with flourimetric detection was used in our

Table 2. Moisture and Folate content of raw and boiled TGLVs (µg/100 g) FW.

Local/common name	Scientific name	Condition	Moisture (%)	Folate content µg/100 g edible portion	Folate loss (%)	p-value*
Ewedu	*Corchorus olitorius*	Fresh	82.59	183.36± 12.92	74.2	0.002
		Boiled	95.13	47.31 ± 3.73		
*Luru / kuka	*Adansonia digitata*	Dried	*nd*	149.64 ± 13.89	88.4	0.002
		Boiled	90.11	17.36 ± 2.10		
Okro/ ila	*Abelmoscus esculentus fruit*	Fresh	87.09	105.95± 15.63	66.3	0.07
		Boiled	95.77	40.15± 4.88		
Ilasa	*Abelmoscus manihot*	Fresh	77.05	46.75 ± 9.49	*nd*	
Efo tete/ green	*Amaranthus hybridus*	Fresh	84.34	62.15 ± 13.72	48.2	0.06
		Boiled	91.58	32.19 ± 4.85		
Yanrin	*Launaea taraxacifolia*	Fresh	88.12	91.13 ± 11.23	46.6	0.02
		Boiled	92.16	48.63 ± 8.41		
Efo igbagba	*Solanum macrocarpon*	Fresh	84.82	21.54 ± 3.08	60.6	0.03
		Boiled	89.55	8.48 ± 1.19		
Ebolo	*Crassocephalum crepidioides*	Fresh	89.07	70.98 ± 4.95	75.8	0.003
		Boiled	94.10	17.18 ± 1.72		

Total folates in sample (µg/100 g) FW, is equivalent of folic acid. *Significance $p < 0.05$.
nd – Not determined (only raw sample was analysed).

study. It has been noted in literature that folate content of foods quantified using microbiological assay result in higher average values (Delchier et al., 2016) and HPLC methods are usually lower by 20 to 50% (Shohag et al., 2012). However, the HPLC method used in our study has several advantages over the widely used microbiological assay which uses *Lactobacillus casei* as test organism, but does not respond to polymer derivatives (Scott et al., 2000). The advantages include the following; it allows for the analysis of total folates and it is also sensitive to the presence of the different mono - and polyglutamate forms of the folates; a satisfactory day-to-day repeatability (never more than 10%) and a very low detection limit (0.02 pmol per injection); the recovery rates vary from 78% depending on the form of folate being studied; use of chicken pancreas conjugase which more effectively converts the different folate polyglutamates into folate diglutamates (Delchier et al., 2016; Ndaw et al., 2001).

Folate content of boiled TGLVs

Boiling caused a considerable decrease in the folate content of the TGLVs: it ranged from 46% in *L. taraxacifolia* to 88% in *A. digitata*, the values were all less than 50 µg/ 100 g of boiled TGLVs (Table 2). The

difference in folate content of fresh and boiled samples were found to be statistically significant for all TGLVs ($p < 0.05$). Studies have shown that folates are prone to losses by leaching when in direct contact with water (Bureau et al., 2015; Delchier et al., 2013, 2012). Delchier et al. (2012) reported that when spinach and green beans were boiled in water, there was a > 50% loss of folate from the vegetables. Our findings are within the range for folate loss in boiled vegetables in previous literature (up to 94%) (Bureau et al., 2015, Delchier et al., 2012, Holasova et al., 2008).

Given that most leafy vegetables are consumed in their cooked form in Nigeria, preparation methods of TGLVs that would allow for optimal retention of folate are necessary. Since home-cooking methods like boiling of vegetables lead to folate loss by leaching into surrounding liquids (Delchier et al., 2012), adding TGLVs directly to soups/ sauces, may be more beneficial in retaining folate in such diets. Consequently the practice of boiling TGLVs in water which is discarded thereafter should be discouraged to avoid folate loss.

Conclusion

The folate content of seven TGLVs and one fruit

vegetable consumed in Nigeria revealed an average content of 91.4 µg/100 g which was within the range reported in literature for raw green leafy vegetables. Thus, TGLVs represent a non-negligible source of folate in the diets of populations who consume them in environments where these vegetables are readily available and affordable. Their production should also be encouraged where they are underutilised and dissappearing. However, major losses (65.7% in average) were also observed due to the traditional cooking method of boiling TGLVs. Hence minimal processing, or direct inclusion to soups and sauces should be encouraged.

The database for folate content of TGLVs in Nigeria is scant. Our study therefore provides original data to fill some of these gaps in subsequent updates of the Nigerian food composition table, thus enriching the content of the Nigerian food/ nutrient database. It also provides data for three underutilised and uncultivated TGLVs (*L. taraxacifolia, S. macrocarpon* and *C. crepidioides*). The availability of data on folate content of TGLVs will also make it possible to estimate the contribution to folate intake from these vegetables in the population and for promoting increased consumption of TGLVs rich in folate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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