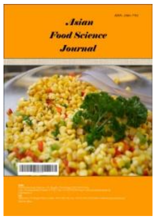

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Efficiency of Triple Bagging System and *Lippia multiflora* Leaves for the Vitamin Quality Preservation of Cowpea Seeds (*Vigna unguiculata* L. Walp)

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Authors' contributions

This study was carried out in collaboration among all authors. Author IF wrote the protocol, performed the laboratory analysis and wrote the first draft of the manuscript. Author NVA performed the statistical analysis of the result and achieved the manuscript revision. Author GHMB managed the literature, assisted the experiments implementation and the statistical analysis. Author KOC designed the study and supervised author IF in recovering the results. All authors read and approved the submitted manuscript.

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ABSTRACT

Aims: To assess the effectiveness of triple bagging systems with or without *Lippia multiflora* leaves on the vitamin-quality of cowpea seeds during storage.
Methodology: Cowpea seeds that have undergone any treatment were collected from producer in the southwest of Côte d'Ivoire. The fresh leaves of *Lippia multiflora* were collected and dried in sunlight for 7 days in center of Côte d'Ivoire. The storage bags used were obtained from the

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suppliers to Côte d'Ivoire in Abidjan. All this material was sent to the Laboratory of Biochemistry and Food Sciences, Felix Houphouet-Boigny University, Côte d'Ivoire to perform the experiment. Thus for the experiment realization, a central composite design (CCD) was used. The first factor of CCD consisted to six types of packaging, namely: one control with polypropylene bag (TST), one triple bagging batch (composed of 2 internal layers in independent high density polyethylene 80 mm thick and a woven bag polypropylene) without biopesticide (H0), and four batches (H1, H2, H3 and H4) containing respectively (0.7%; 2.5; 4.3%; and 5%) biopesticide. The second factor, storage time, it included six periods of observation (0; 1; 2; 4.5; 7 and 8 months). HPLC techniques were used for the separation and quantification of β-carotene, α-tocopherol (vitamin E) and the water-soluble vitamins (thiamine, riboflavin, niacin, pyridoxine and folic acid). The estimated daily intake of folic acid (vitamin B9) was also evaluated for Ivorian adult of 70 kg.

Results: The results of vitamin properties indicate a significant influence ($P < 0.05$) between the type of treatments and shelf life. Multivariate analysis results (PCA and AHC) indicate that the addition of at least 0.7% *Lippia multiflora* leaves in triple bagging systems makes preservation more efficient and preserves the vitamin quality of the cowpea seeds during 8 months. The contribution to meeting vitamin B9 requirements is palpable when cowpea seeds are stored for up to 8 months in the triple bagging systems associated with the biopesticide.

Conclusion: Cowpea seeds storage in triple bagging systems with *Lippia multiflora* leaves appears as a method of effective and inexpensive conservation to ensure the vitamin-quality of cowpea.

Keywords: Cowpea; conservation; triples bagging; biopesticide; vitamin characteristics.

1. INTRODUCTION

Cowpea seeds (*Vigna unguiculata*) constitute one of the bases of the food of Africa's dry lands populations [1]. Today, with an annual world production of 6.4 million tons [2], this legume food consumed in various form (doughnuts, boiled, mashed, paste and sauces) is really appreciated all over Africa because the seeds are valuable source of protein that can substitute meat, less expensive for majority of vulnerable people [3,4]. Cowpea seeds are also an important source of many vitamins particularly those of the B group containing folate (B9), riboflavin (B2) and thiamin (B1) [5,6]. These molecules are well known for their positive impact on the human organism [7].

In developing countries, vitamin deficiencies remain a serious public health problem. Conventional intervention programs in these areas depend on artificial vitamin supplementation [8]. Many of these programs have proved unsustainable due to their high operating costs, distribution problems or hard-to-reach areas [9-11].

The rational use of cowpea seeds in the diet of low-income populations in these areas where nutritional deficiencies are observed could represent a powerful and sustainable intervention tool. Unfortunately, cowpea is faced with storage and / or preservation problems mainly due to attacks caused by pests such as bruchids [12]. This situation is promoted by the lack of mastery of good post-harvest practices. Moreover, inadequate storage makes the seeds vulnerable to microorganisms (fungi and bacteria). These stock pests (bruchids and microorganisms) deteriorate the nutritive value and consequently the vitamin quality of seeds stored at the end of storage [13-15]. In order to address these stock destroyers, producers often resort to synthetic pesticides whose bad practices (misuse, lack of precaution in their handling and failure to meet the waiting periods for deficiency) can lead to the resistance of pests and diseases to environmental and health problems [16,17].

In view of the extensive damage caused by the use of these chemicals, the use of biopesticides as an alternative has been encouraged in recent decades [18,19]. Indeed, the use of plants and their derivatives to treat and protect foodstuffs is very old practice in rural areas. It is an effective means of control, guarantees biodiversity and is less expensive [20-22]. For the aromatic plants used, there is *Lippia multiflora*. It is a local and accessible plant in all regions of Ivory Coast whose insecticidal and / or insect repellent properties have been revealed by recent conservation work on cowpea [23-25].

Triple bagging systems that have been shown to be effective in extending the shelf life of cowpea seeds are also frequently used in the preservation of this pulse [26-28]. They consist of a double layer of high density independent polyethylene placed inside a polypropylene woven bag. Thus, the aim of this study is to evaluate the effects of triple bagging systems associated or not with *Lippia multiflora* leaves (biopesticide) on the vitamin profile of cowpea seeds during storage.

2. MATERIALS AND METHODS

2.1 Experimental Site

The experiment was carried out at the Laboratory of Biochemistry and Food Sciences, Biosciences Training and Research Unit at the University Felix HOUPHOUET-BOIGNY, Côte d'Ivoire. The different bags were stored in a laboratory storage room with average temperature and relative humidity of 28 ± 0.2ºC and 75.0 ± 1.0%, respectively. Wooden pallets have been placed on the floor as a support for the different types of packaging bags.

2.2 Biological Material

Cowpea seeds used belong to the local variety "Vya". They were collected from producers in the Loh-Djiboua region (5° 50′ North 5º 22′ West) just after harvest. After shelling, untreated seeds were sent to the laboratory for their packaging.

The leaves of *Lippia multiflora* (*Verbenaceae*) were collected in Gbeke region. They were dried out of the sun and then chopped in fine particles.

2.3 Storage Equipment

Storage bags used were constituted of polypropylene bags and triple bagging systems. The triple bagging systems obtained from the suppliers were composed of two internal layers of polyethylene liners (composed of 80 mm high density) and a third layer made from woven polypropylene. The two layers, one adapted inside the other, were enclosed in the polypropylene woven bag.

2.4 Protocol of Cowpea Seeds Preserving

The experiment lasted 8 months. It was implemented using the cowpea bagging modified method proposed by Konan et al. [29]. The cowpea bagging method was based on mixing a proportion of crushed dried leaves with a defined amount of cowpea seed. The packaging was packaged in triple-bottom bags and a polypropylene woven bag. In total one control and five experimental batches were constituted as follows:

- ✓ Control batch: 50 kg of cowpea seeds packed in polypropylene woven bag without biopesticide (TST);
- ✓ 1st experimental batch: 50 kg of cowpea seeds packed in triple bagging system without biopesticide (H0);
- ✓ 2nd experimental batch: 50 kg of cowpea seeds packed in triple bagging system containing 0.35 kg of *Lippia multiflora* leaf, that is 0.7% of biopesticide (H1);
- ✓ 3rd experimental batch: 50 kg of cowpea seeds packed in triple bagging system containing 1.25 kg of *Lippia multiflora* leaf, that is 2.5% of biopesticide (H2);
- ✓ 4th experimental batch: 50 kg of cowpea seeds packed in triple bagging system containing 2.15 kg of *Lippia multiflora* leaf, that is 4.3% of biopesticide (H3) and
- ✓ 5th experimental batch: 50 kg of cowpea seeds packed in triple bagging system containing 2.5 kg of *Lippia multiflora* leaf, that is 5% of biopesticide (H4).

The filling of the bags was made by alternating cowpea seeds and leaves as stratum.

2.5 Sampling

Sampling for analysis was carried out at different storage periods using a central composite design (CCD) [29]. The first analysis was done just before the conditioning for conservation (0 months). The aim was to determine base values (references) and then compare them to values obtained during preservation. Cowpea samples (2.5 kg) were then taken in triplicate at 1 month; 2 months; 4.5 months; 7 months and 8 months. Bag sampling was done randomly.

2.6 Vitamin Analysis

The concentrations in water-soluble vitamins and fat-soluble vitamins were determined using a high performance liquid chromatographic system (HPLC, mark Water Alliance). This system included a Waters pump, an automatic injector, a UV / PDA detector and a Servotrace recorder. The operating conditions were adapted to the type of required vitamins.

2.6.1 Samples preparation for HPLC separation

Two grams of finely ground samples were extracted vigorously with five excess of n-hexane solvent followed by centrifugation in the cold for 5 min at 3000 rpm. The organic solvent was aspirated and saved. The residue was reextracted with the same solvent and the same steps were repeated until the extract was almost colorless. The total volume of the extract was recorded and an aliquot was injected in the HPLC system.

Fat soluble vitamins were separated on a column Kromasil C18 of 30 X 4 mm (CIL CLUZEAV) in stainless steel. The mobile phase was a mixture acetonitrile / methanol (80/20, v / v), of HPLC grade and well furnished by MERCK (Germany). The column temperature was 30ºC, the elution length was 35 min and the flow rate was 1.2 mL / min.

Water soluble vitamins were separated on a Zorbax column to silica support post grafted in C18 (150 mm X 4.6 mm) with particles of 3 mm. The mobile phase was a mixture of ammonium acetate and methanol, of grade HPLC and furnished by MERCK (Germany). The flow rate was programmed to 2 mL / min on a length of 20 min.

Standard β-carotene and α-tocopherol were purchased from Fluka Chemie (Switzerland), while water soluble vitamins were purchased from Sigma-Aldrich (UK). Table 1 present the concentrations of the standard vitamins and different wavelengths used for injection in the HPLC system.

Table 1. Concentration of vitamins for injection and wavelengths used

Vitamins	Concentration range (µg/ml)	Wavelengths (nm)
Thiamine	0.1 to 3.5	270
Riboflavin	0.1 to 7	265
Niacin	0.2 to 4.5	256
Pyridoxine	0.5 to 12	257
Folic acid	0.5 to 5	280
α-Tocopherol	0.2 to 5.5	295
ß-Carotene	0.2 to 4.5	445

2.6.2 Validation of the vitamin analysis method

The validation of vitamins dosage by HPLC was been achieved by the application the NFV03-110 norm [30]. This process consists in the study of the linearity of the standardization range, the determination of the limits of detection and quantification, the calculation of the variation coefficient on tests of repeatability and reproducibility, as well as the calculation of the percentage of addition recovery measured.

2.6.2.1 Test of linearity

The linearity was tested between 0 and 125 µg / mL using 5 points of calibration: 0, 25, 50, 75 and 125 µg / mL, five distinct tests were carried out.

2.6.2.2 Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were calculated with the standards of the various required vitamins.

Ten (10) distinct tests were carried out and the values were obtained with the following formulas:

LOD = Average (MX) + 3 standard deviation
LOQ = Average (MX) + 10 standard deviation

2.6.2.3 Test of repeatability and reproducibility

To test the repeatability, 10 trials of extract from a reference sample were analyzed by HPLC. For reproducibility, 5 separate tests sample from a reference sample were analyzed by HPLC at intervals of several days. The standard concentrations are 1 mg / mL [31].

2.6.2.4 Test of accuracy

Ten separate trials from reference samples were analyzed to assess the recovery rate by the method used for determination of vitamins. The standard concentrations are 5 mg / mL.

2.7 Estimate of Folic Acid (vitamin B9) from Cowpea Seeds after Storage

Vitamin B9 was estimated according to the method of the Codex Alimentarius [32]. This method takes into account the concentrations in vitamins recovered in the food (cowpea seeds stored for 8 months) and the daily consumption of an adult individual of 70 kg in Ivory Coast according to the following formula:

Estimated Daily Intake (EDI) = C x Q

With: **C**, Vitamin B9 concentration measured; **Q**, cowpea seeds daily consumption with is 4.93 g/day of cowpea seed in Ivory Coast [33].

The contribution of cowpea seeds in daily requirement has been calculated also from the values of daily recommended intakes [34].

Contribution (%) = (EDI x 100) / DRI

With: DRI, daily recommended intake.

2.8 Statistical Analysis

The statistical analyses of data were carried out thanks to software SPSS (version 22.0) and STATISTICA (version 7.1). All tests related to the vitamins characteristics analyze were performed in triplicate and the results are expressed as mean ± standard deviation. An analysis of variance (repeated measures ANOVA) was first performed on all the results during the first four and a half months of conservation. It consisted in Analysis of Variance according to two factors: duration and type of treatments and then completed by a one-way Analysis of Variance (ANOVA 1) for the rest of storage period (7 and 8 months). The significant differences were highlighted by the Tukey post-hoc test at 5% threshold. Finally, Multivariate Statistical Analysis (MSA) including Principal Component Analysis (PCA) and Hierarchical Ascending Clustering Analysis (HAC) were performed to classify samples with similar behavior across all vitamins over storage time.

3. RESULTS

3.1 Results of Validation

The results of the validation are presented in Table 2. The coefficients of determination obtained for the linearity studies are between 0.996 and 0.999 for the different vitamins studied. The limits of detection are included between 25 µg/L and 135 µg/L, when the limits of quantification vary from 83 µg/L to 449 µg/L. The coefficients of variation determined for the repeatability oscillate between 1.0±0.05% and 1.7±0.04% and those of the tests of reproducibility are between 2.5±0.47% and 4.4±0.60%. For the extraction yields determined from the added additions, the rates are between 96.8±0.14% and 100.5±0.07%.

3.2 Evolution of the Contents of Cowpea Seeds Vitamins According to the Method and Time of Storage

Statistical test data used to evaluate all vitamins during storage are shown in Tables 3 and 4. The tests carried out reveal significant variations ($P < 0.05$) in the vitamins contents according to the duration and type of treatments (triple bagging and / or biopesticide). In addition, the interaction effect between these two variables has is significant (Table 3).

3.2.1 Water-soluble vitamins contents of cowpea seeds

The evolution of water-soluble vitamins contents are consigned in Tables 5 and 6. The results show that the levels of thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6) and folic acid (B9) decrease significantly during storage depending on the type of treatments.

The thiamine contents recorded at the beginning of storage (1.40 ± 0.11 µg/100 g) in cowpea seeds decreased significantly ($P < 0.05$) after 4.5 months of storage to 0.36 ± 0.06 µg/100 g in polypropylene control bag. The decrease is slight during the first five months of storage in triple bagging system without biopesticide with a value of 1.10 ± 0.05 µg/100 g. After five months of storage, the decline is very significant ($P < 0.001$) and reaches an average value of 0.94 ± 0.06 µg/100g in eight months. On the other hand, the vitamin B1 levels determined in the cowpea seeds stored in the triple bagging systems associated with biopesticide remain statistically constant ($P > 0.05$) during the 8 months of storage with an average value of 1.30 ± 0.06 µg/100 g (Table 6).

The riboflavin contents dropped significantly ($P < 0.05$) in polypropylene bag from 2.50 ± 0.12 µg/100 g to 0.36 ± 0.14 µg/100 g after 4.5 months storage. In the triple bagging system without biopesticide (H0), the vitamin B2 content remained virtually stable during the first four and a half months of storage. After 4.5 months, the riboflavin content rapidly decreased to reach after 8 months of storage, the value of 1.07 ± 0.07 µg/100g. The riboflavin content of batch H1 in 8 months of cowpea storage is lower than the other batches values in the triple bagging system with biopesticide. It is 2.06 ± 0.09 µg/100 g whereas for H2, H3 and H4 lots, the vitamin B2 contents are respectively 2.28 ± 0.13 µg/100 g, 2.32 ± 0.12 µg/100g and 2.48 ± 0.08 µg/100 g (Table 6).

With regard to niacin (vitamin B3) content, a significant decrease is also observed with the storage period in the polypropylene control bag. Vitamin B3 contained in cowpea seeds at the start of storage (1.63 ± 0.11 µg/100 g) drops to 0.13 ± 0.05 µg/100 g after 4.5 months of storage. The niacin content after 8 months of cowpea seeds storage is 1.00 ± 0.12 µg/100 g, 1.35 ± 0.22 µg/100 g, 1.45 ± 0.29 µg/100 g, 1.50 ± 0.24 µg/100 g and 1.58 ± 0.17 µg/100 g respectively for H0, H1, H2, H3 and H4 (Table 6).

As regards the pyridoxine (vitamin B6) content, with an average value of 0.99 ± 0,14 µg/100 g at the beginning of storage, the lowest levels were recorded after 4.5 months of storage in the polypropylene control bag (0.15 ± 0.05 µg/100 g) and after 8 months of storage in the triple bagging system without biopesticide (0.39 ± 0.08 µg/100 g). The vitamin B6 content after 8 months of cowpea storage in the triple bagging system with biopesticide remains above the values of the control group (TST) and triple bagging system without biopesticide (H0) with average grades of 0.58 ± 0.03 µg/100 g, 0.93 ± 0.11 µg/100 g, 0.94 ± 0.05 µg/100 g et 0.96 ± 0.06 µg/100 g respectively for H1, H2, H3 et H4 (Table 6).

Storage of cowpea also revealed a significant decrease in folic acid levels over time depending on the type of treatment. With an initial value of 8.65 ± 1.29 µg/100 g, the vitamin B9 content drops significantly to 2.67 ± 0.71 µg/100 g in the polypropylene batch after 4.5 months of storage. In the triple bagging system without biopesticide this content fell gradually during the first 7 months of storage from to 5.99 ± 0.98 µg/100 g. After 7 months of storage, this content dropped significantly to reach a value of 4.13 ± 0.85 µg/100 g after 8 months of storage. In triple bagging system lots supplemented with biopesticide, vitamin B9 levels did not decrease significantly ($P > 0.05$) over time. Mean values remained in the order of 8.00 ± 0.10 µg/100 g.

3.2.2 Fat-soluble vitamins contents of cowpea seeds

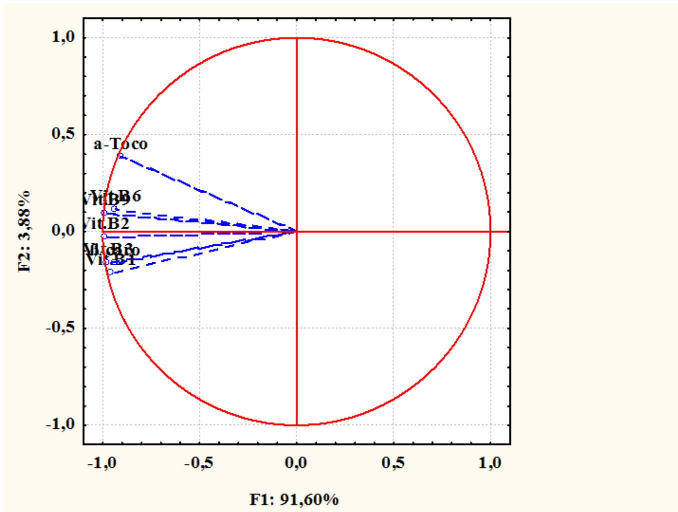
The α-tocopherol and ß-carotene are two fat-soluble vitamins that have been identified in the preserved cowpea seeds (Table 7). The results show that the content of these vitamins evolve differently in cowpea seeds during the 4.5 months storage period for the polypropylene control bag (TST); 8 months for triple bagging without biopesticide (H0) and four triples baggings systems with different proportions of biopesticide (H1: 0.7%; H2: 2.5%; H3: 4.3% and H4: 5%). With an average value of 0.10 ± 0.01 µg/100g prior to storage, the α-tocopherol content decreased significantly ($P < 0.05$) over time. The lowest levels were obtained after 4.5 months of storage in the polypropylene control bag (0.05 ± 0.01 µg/100 g) and 8 months of storage in triple bagging system without biopesticide (0.05 ± 0.01 µg/100 g). However, in triple bagging system in the presence of biopesticide, the α-tocopherol contents remained constant and no significant difference ($P > 0.05$) was observed during the 8 months of storage regardless of the biopesticide concentration, with an average value of 0,09 ± 0,01 µg/100 g (Table 8).

About the ß-carotene content, it remains statistically constant both in the triple bagging without biopesticide (H0) and in the 4 triple bagging system supplemented with biopesticide throughout the storage period (Table 8). Moreover in polypropylene control bag, it remains constant for up to 4.5 months when it is neither quantifiable (Table 7).

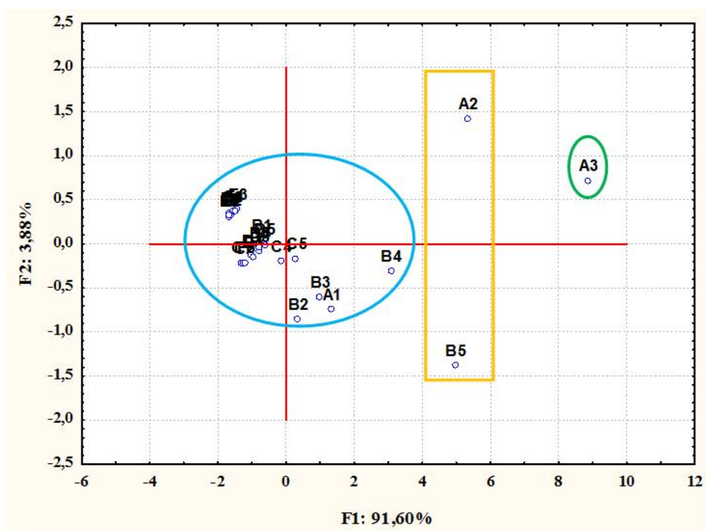
3.3 Multivariate Parameters

The Principal Components Analysis (PCA) of the different cowpea samples related to the seven (7) vitamin parameters permitted to show two (2) axes explaining the essential variability that were axis 1 and 2. These 2 axes expressed 95.48% of the total variability. The correlation circle indicated that the vitamins are strongly and negatively correlated to the F1 axis (Fig. 1a). This axis is good vitamin quality indicator of the seeds preserved.

The projection of the samples studied highlighted 3 groups of individuals (Fig. 1b). The group 1 consists mainly in individual from polypropylene control batch at 4.5 months of storage (A3). It is very clearly distinguished from over samples by very low levels of vitamins. The second group includes samples resulting from polypropylene bag at 2nd month of storage (A2) and the triple bagging without biopesticide at 8th month of storage (B5). These contain average levels of vitamins. The third group contains all the samples kept in the triple bagging system with biopesticide throughout the experiment, those from the triple bagging system without biopesticide from 1 to 7 months of storage (B1, B2, B3 and B4), the initial sample (Ei) and that of polypropylene bag at 1st month of storage (A1). These samples are characterized by high contents of vitamins.



(a)



(b)

Fig. 1. Correlation drawn between the F1-F2 factorial design of the principal component analysis and the vitamin characteristics (a) and Individuals (b) deriving from the cowpea samples studied

Vit. B1: vitamin B1, Vit. B2: vitamin B2, Vit. B3: vitamin B3, Vit. B6: vitamin B6, Vit. B9: vitamin B9, α-toco: α-tocopherol, ß-caro: ß-carotene, Ei: initial sample, A1: polypropylene bag at 1 month, B1: triple bagging without biopesticide at 1 month, C1, D1, E1, F1: triple bagging with 0.7%, 2.5%, 4.3% and 5% of biopesticide at 1 month A2: polypropylene bag at 2 months, B2: triple bagging without biopesticide at 2 months, C2, D2, E2, F2: triple bagging with respectively 0.7%, 2.5%, 4.3% and 5% biopesticide at 2 months of conservation. A3: polypropylene bag at 4.5 months, B3: triple bagging without biopesticide at 4.5 months, C3, D3, E3, and F3: triple bagging with respectively 0.7%, 2.5%, 4.3% and 5% biopesticide at 4.5 months of storage. B4: triple bagging without biopesticide at 7 months, C4, D4, E4, and F4: triple bagging with 0.7%, 2.5%, 4.3% and 5% biopesticide at 7 months of storage. B5: triple bagging without biopesticide at 8 months, C5, D5, E5, and F5: triple bagging with 0.7%, 2.5%, 4.3% and 5% biopesticide at 8 months of storage

Table 2. Tests results of analytic validation of the HPLC vitamins dosage

Vitamins	Linearity		CV Repeat. (%, n= 10)	CV Reprod. (%, n= 15)	EYAV (%, n= 10)	LOD (µg/L)	LOQ (µg/L)
	ESL	CD (R^2)					
α-Tocopherol	Y= 836.2x - 5800	0.997	1.7±0.04	3.1±0.51	100.5±0.07	98±0.23	326±0.41
ß-Carotene	Y= 326.6x + 152.9	0.999	1.5±0.12	4.4±0.60	98.7±0.88	125±0.69	416±0.25
Thiamine	Y= 723.4x + 1346	0.998	1.3±0.10	3.2±0.98	97.3±0.55	62±0.17	206±1.09
Riboflavin	Y= 4787x + 7107	0.998	1.6±0.94	3.6±0.22	96.8±0.14	54±0.29	179±0.76
Niacin	Y= 462.5x – 331.5	0.999	1.4±0.73	3.4±0.63	99.1±0.18	64±0.01	213±1.62
Pyridoxine	Y= 550.9x + 627.1	0.996	1.0±0.05	2.8±0.41	97.7±0.59	25±0.38	83±0.47
Folic acid	Y= 942.4x – 1615	0.999	1.2±0.21	2.5±0.47	98.6±0.44	33±0.75	109±0.15

ESL, equation of standard lines; CD, coefficient of determination; CV repeat, coefficient of variation from repeatability test; CV reprod, coefficient of variation from reproducibility test; EYAV, extraction yield from added vitamins; LOD, limit of detection; LOQ, limit of quantification

Table 3. Statistical data (Repeated measure ANOVA) of cowpea seeds vitamin levels under treatment during preservation

SOV	Stat Para.	B1	B2	B3	B6	B9	α-Toc	ß-Car
Durations	df	3	2.17	1.78	3	3	1.81	3
	SS	0.73	2.27	1.30	0.65	20.84	0.00	0.00
	F	17.62	14.69	9.44	12.91	4.89	3.18	1.06
	P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.05**	**<0.05**	**>0.05**
Error	df	36	26.08	21.31	36	36	21.71	36
	SS	0.50	1.85	1.65	0.60	51.11	0.01	0.00
Treatments	df	5	5	5	5	5	5	5
	SS	3.15	9.14	4.20	2.74	82.05	0.00	0.00
	F	62.25	29.19	40.64	28.68	23.21	9.66	0.76
	P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**>0.05**
Error	df	12	12	12	12	12	12	12
	SS	0.12	0.75	0.25	0.23	8.49	0.00	0.00
Durations x Treatments	df	15	10.87	8.88	15	15	9.05	15
	SS	1.90	6.03	3.11	1.18	41.50	0.00	0.00
	F	9.17	7.81	4.52	4.72	1.95	1.16	0.38
	P	**<0.001**	**<0.001**	**<0.05**	**<0.001**	**<0.05**	**<0.05**	**>0.05**

SOV, source of variation; Stat Para, statistical parameters; df, degree of freedom; SS, sum of squares; F, value of the statistical test; P, probability value of the statistical test, B1: vitamin B1 (Thiamine); B2: vitamin B2 (Riboflavin); B3: vitamin B3 (Niacin); B6: vitamin B6 (Pyridoxine); B9: vitamin B9 (Folic acid); α-Toc: (α-tocopherol); ß-Car: (ß-carotene)

Table 4. Statistical Data (ANOVA 1) of Cowpea seeds vitamin levels under treatment during preservation

Effect	Stat para	B1	B2	B3	B6	B9	α-Toc	ß-Car
Treatments	df	4	4	4	4	4	4	4
	SS	0.34	3.80	0.62	0.82	37.58	0.00	0.00
	F	21.10	94.47	3.26	37.02	6.48	11.79	0.44
	P	**<0.001**	**<0.001**	**<0.05**	**<0.001**	**<0.001**	**<0.001**	**>0.05**
Error	df	10	10	10	10	10	10	10
	SS	0.04	0.10	0.47	0.06	14.49	0.00	0.00
Total	df	14	14	14	14	14	14	14
	SS	0.38	3.90	1.09	0.87	52.07	0.00	0.00

Stat Para, statistical parameters; df, degree of freedom; SS, sum of squares; F, value of the statistical test; P, probability value of the statistical test, B1: vitamin B1 (Thiamine); B2: vitamin B2 (Riboflavin); B3: vitamin B3 (Niacin); B6: vitamin B6 (Pyridoxine); B9: vitamin B9 (Folic acid); α-Toc: (α-tocopherol); ß-Car: (ß-carotene)

Table 5. Evolution of the water-soluble vitamin contents of cowpea seeds preserved according to different treatments for 4.5 months

Vitamins	Storage time (month)	TST	H0	H1	H2	H3	H4
Thiamine (µg/100 g)	0	1.40 ± 0.11^{aA}	1.40 ± 0.11^{aA}	1.40 ± 0.11^{aA}	1.40 ± 0.11^{aA}	1.40 ± 0.11^{aA}	1.40 ± 0.11^{aA}
	1	1.10 ± 0.05^{bB}	1.29 ± 0.06^{bA}	1.39 ± 0.01^{aA}	1.40 ± 0.01^{aA}	1.40 ± 0.01^{aA}	1.40 ± 0.19^{aA}
	2	0.38 ± 0.02^{cB}	1.28 ± 0.06^{bA}	1.38 ± 0.19^{aA}	1.39 ± 0.01^{aA}	1.39 ± 0.10^{aA}	1.39 ± 0.09^{aA}
	4.5	0.36 ± 0.06^{cB}	1.10 ± 0.05^{cA}	1.30 ± 0.06^{aA}	1.35 ± 0.34^{aA}	1.38 ± 0.05^{aA}	1.38 ± 0.09^{aA}
Riboflavin (µg/100 g)	0	2.50 ± 0.12^{aA}	2.50 ± 0.12^{aA}	2.50 ± 0.12^{aA}	2.50 ± 0.12^{aA}	2.50 ± 0.12^{aA}	2.50 ± 0.12^{aA}
	1	2.00 ± 0.09^{bB}	2.38 ± 0.12^{bA}	2.48 ± 0.03^{aA}	2.49 ± 0.11^{aA}	2.50 ± 0.49^{aA}	2.50 ± 0.45^{aA}
	2	1.14 ± 0.13^{cB}	2.30 ± 0.14^{bA}	2.45 ± 0.13^{aA}	2.48 ± 0.07^{aA}	2.49 ± 0.12^{aA}	2.50 ± 0.32^{aA}
	4.5	0.36 ± 0.14^{dB}	2.15 ± 0.15^{cA}	2.36 ± 0.31^{aA}	2.38 ± 0.59^{aA}	2.42 ± 0.21^{aA}	2.49 ± 0.19^{aA}
Niacin (µg/100 g)	0	1.63 ± 0.11^{aA}	1.63 ± 0.11^{aA}	1.63 ± 0.11^{aA}	1.63 ± 0.11^{aA}	1.63 ± 0.11^{aA}	1.63 ± 0.11^{aA}
	1	1.36 ± 0.09^{bB}	1.41 ± 0.18^{bA}	1.60 ± 0.27^{aA}	1.61 ± 0.27^{aA}	1.63 ± 0.20^{aA}	1.63 ± 0.10^{aA}
	2	0.65 ± 0.18^{cB}	1.39 ± 0.26^{bA}	1.60 ± 0.36^{aA}	1.60 ± 0.27^{aA}	1.63 ± 0.06^{aA}	1.63 ± 0.12^{aA}
	4.5	0.13 ± 0.05^{dC}	1.33 ± 0.30^{bB}	1.51 ± 0.35^{aA}	1.53 ± 0.27^{aA}	1.55 ± 0.15^{aA}	1.59 ± 0.07^{aA}
Pyridoxine (µg/100 g)	0	0.99 ± 0.14^{aA}	0.99 ± 0.14^{aA}	0.99 ± 0.14^{aA}	0.99 ± 0.14^{aA}	0.99 ± 0.14^{aA}	0.99 ± 0.14^{aA}
	1	0.49 ± 0.03^{bC}	0.90 ± 0.12^{bB}	0.99 ± 0.17^{aA}	0.99 ± 0.02^{aA}	0.99 ± 0.17^{aA}	0.99 ± 0.02^{aA}
	2	0.20 ± 0.05^{cC}	0.57 ± 0.06^{cB}	0.97 ± 0.05^{aA}	0.98 ± 0.23^{aA}	0.99 ± 0.04^{aA}	0.99 ± 0.03^{aA}
	4.5	0.15 ± 0.05^{cC}	0.54 ± 0.03^{cB}	0.89 ± 0.06^{bA}	0.95 ± 0.04^{aA}	0.98 ± 0.04^{aA}	0.98 ± 0.08^{aA}
Folic acid	0	8.65 ± 1.29^{aA}	8.65 ± 1.29^{aA}	8.65 ± 1.29^{aA}	8.65 ± 1.29^{aA}	8.65 ± 1.29^{aA}	8.65 ± 1.29^{aA}

Vitamins	Storage time (month)	TST	H0	H1	H2	H3	H4
(µg/100 g)	1	6.19 ± 1.31^{bB}	8.44 ± 0.53^{aA}	8.54 ± 1.82^{aA}	8.64 ± 0.16^{aA}	8.64 ± 0.39^{aA}	8.64 ± 0.85^{aA}
	2	5.03 ± 1.01^{cC}	7.56 ± 1.99^{bB}	8.44 ± 0.34^{aA}	8.58 ± 0.56^{aA}	8.60 ± 0.53^{aA}	8.64 ± 1.70^{aA}
	4.5	2.67 ± 0.71^{dD}	6.89 ± 0.14^{bB}	8.24 ± 0.87^{aA}	8.34 ± 1.28^{aA}	8.41 ± 0.41^{aA}	8.54 ± 1.35^{aA}

Means (±SD) with different upper-case/lower-case letters in the same line/column are different at 5% probability test. With TST: control polypropylene bag; H0: triple bagging without biopesticide; H1: triple bagging with 0.7% biopesticide (w / w); H2: triple bagging with 2.5% biopesticide (w / w); H3: triple bagging with 4.3% biopesticide (w / w); H4: triple bagging with 5% biopesticide (w / w)

Table 6. Evolution of the water-soluble vitamin contents of cowpea seeds preserved for 7 and 8 months according to different treatments

Vitamins	Storage time (month)	H0	H1	H2	H3	H4
Thiamine (µg/100g)	7	1.01 ± 0.05^{b}	1.27 ± 0.06^{a}	1.30 ± 0.08^{a}	1.37 ± 0.05^{a}	1.37 ± 0.07^{a}
	8	0.94 ± 0.06^{b}	1.25 ± 0.06^{a}	1.29 ± 0.09^{a}	1.30 ± 0.05^{a}	1.37 ± 0.05^{a}
Riboflavin (µg/100g)	7	1.55 ± 0. 07^{c}	2.11 ± 0.13^{b}	2.30 ± 0.14^{ab}	2.33 ± 0.01^{ab}	2.48 ± 0.11^{a}
	8	1.07 ± 0.08^{c}	2.06 ± 0.09^{b}	2.28 ± 0.13^{ab}	2.32 ± 0.12^{ab}	2.48 ± 0.08^{a}
Niacin (µg/100g)	7	1.21 ± 0.19^{a}	1.48 ± 0.17^{a}	1.50 ± 0.29^{a}	1.53 ± 0.26^{a}	1.59 ± 0.08^{a}
	8	1.00 ± 0.12^{a}	1.35 ± 0.22^{a}	1.45 ± 0.29^{a}	1.50 ± 0.24^{a}	1.58 ± 0.17^{a}
Pyridoxine	7	0.50 ± 0.08^{c}	0.71 ± 0.04^{b}	0.93 ± 0.06^{a}	0.96 ± 0.04^{a}	0.98 ± 0.09^{a}
(µg/100g)	8	0.39 ± 0.08^{b}	0.58 ± 0.03^{b}	0.93 ± 0.11^{a}	0.94 ± 0.05^{a}	0.96 ± 0.06^{a}
Folic acid (µg/100g)	7	5.99 ± 0.98^{a}	7.83 ± 0.17^{a}	8.22 ± 0.74^{a}	8.24 ± 1.96^{a}	8.44 ± 0.51^{a}
	8	4.13 ± 0.85^{b}	7.66 ± 2.25^{a}	8.00 ± 0. 10^{a}	8.20 ± 0.36^{a}	8.34 ± 1.16^{a}

Means (±SD) with different lower-case letters in the same line are different at 5% probability test. With H0: triple bagging without biopesticide; H1: triple bagging with 0.7% biopesticide (w / w); H2: triple bagging with 2.5% biopesticide (w / w); H3: triple bagging with 4.3% biopesticide (w / w); H4: triple bagging with 5% biopesticide (w / w)

Table 7. Evolution of the fat-soluble vitamin contents of cowpea seeds preserved according to different treatments for 4.5 months

Vitamins	Storage time (month)	TST	H0	H1	H2	H3	H4
α-Tocopherol	0	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}
(µg/100g)	1	0.08 ± 0.03^{aAB}	0.09 ± 0.01^{aA}	0.09 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.03^{aA}	0.10 ± 0.00^{aA}
	2	0.08 ± 0.03^{aAB}	0.08 ± 0.01^{aAB}	0.09 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}
	4.5	0.05 ± 0.01^{bB}	0.08 ± 0.01^{aAB}	0.09 ± 0.01^{aA}	0.09 ± 0.01^{aA}	0.10 ± 0.02^{aA}	0.10 ± 0.01^{aA}
ß-Carotene	0	0.02 ± 0.00^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.00^{aA}
(RE/100g)	1	0.02 ± 0.01^{aA}	0.02 ± 0.01^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.01^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.01^{aA}
	2	0.01 ± 0.00^{aA}	0.02 ± 0.01^{aA}	0.02 ± 0.01^{aA}	0.02 ± 0.01^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.00^{aA}
	4.5	< LOQ	0.02 ± 0.00^{aA}	0.02 ± 0.00^{aA}	0.02 ± 0.01^{aA}	0.02 ± 0.01^{aA}	0.02 ± 0.00^{aA}

Means (±SD) with different upper-case/lower-case letters in the same line/column are different at 5% probability test. With TST: control polypropylene bag; H0: triple bagging without biopesticide; H1: triple bagging with 0.7% biopesticide (w / w); H2: triple bagging with 2.5% biopesticide (w / w); H3: triple bagging with 4.3% biopesticide (w / w); H4: triple bagging with 5% biopesticide (w / w); < LOQ= below the limit of quantification

Table 8. Evolution of the fat-soluble vitamin contents of cowpea seeds preserved for 7 and 8 months according to different treatments

Vitamins	Storage time (month)	H0	H1	H2	H3	H4
α-Tocopherol	**7**	0.07 ± 0.01^{b}	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}	0.10 ± 0.01^{a}
(µg/100g)	**8**	0.05 ± 0.02^{b}	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}	0.09 ± 0.00^{a}	0.09 ± 0.01^{a}
ß-Carotene	**7**	0.01 ± 0.00^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}
(RE/100g)	**8**	0.01 ± 0.00^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}

Means (±SD) with different lower-case letters in the same line are different at 5% probability test. With H0: triple bagging without biopesticide; H1: triple bagging with 0.7% biopesticide (w / w); H2: triple bagging with 2.5% biopesticide (w / w); H3: triple bagging with 4.3% biopesticide (w / w); H4: triple bagging with 5% biopesticide (w/ w)

The Ascending hierarchical classification (AHC) corroborates the variability observed in the PCA (Fig. 2). Indeed, at the gene distance of 60, the dendrogram shows three clusters of cowpea samples during storage. The first cluster is polypropylene control individual at 4.5 months of storage (A3) with lower contents of vitamins. The second cluster encloses individuals resulting from polypropylene bag at 2nd month of storage (A2) and the triple bagging system without biopesticide at 8th month of storage (B5). The samples in this class have vitamin content close to those of first class. The all samples of cowpea deriving from triple bagging system with different proportion of biopesticide, those from triple bagging system without biopesticide from 1 to 7 months of storage, the initial sample and that of polypropylene bag at 1st month of storage constitute the third cluster. These are distinguished by high levels of vitamins.

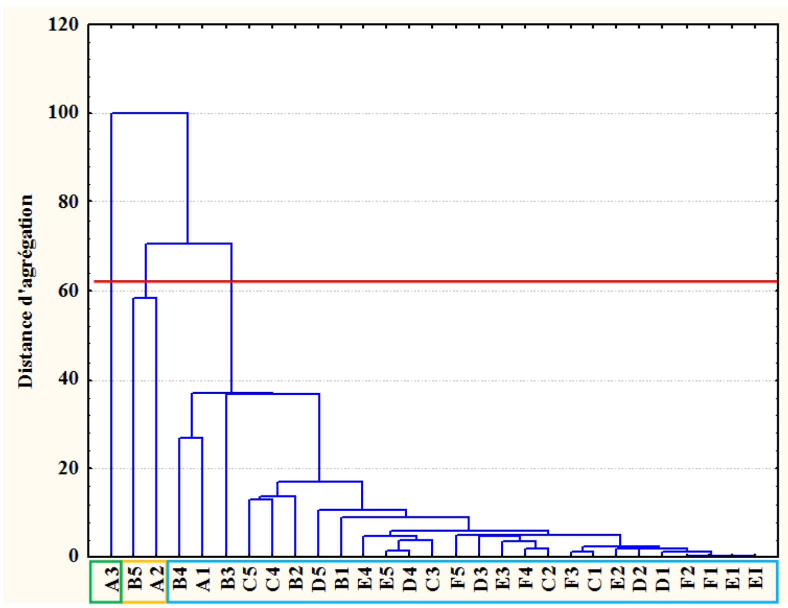


Fig. 2. Ascending hierarchical clustering (dendrogram) with the vitamin characteristics of cowpea seeds preserved according to different treatments

Ei: initial sample, A1: polypropylene bag at 1 month, B1: triple bagging without biopesticide at 1 month, C1, D1, E1, F1: triple bagging with 0.7%, 2.5%, 4.3% and 5% of biopesticide at 1 month A2: polypropylene bag at 2 months, B2: triple bagging without biopesticide at 2 months, C2, D2, E2, F2: triple bagging with respectively 0.7%, 2.5%, 4.3% and 5% biopesticide at 2 months of conservation. A3: polypropylene bag at 4.5 months, B3: triple bagging without biopesticide at 4.5 months, C3, D3, E3, and F3: triple bagging with respectively 0.7%, 2.5%, 4.3% and 5% biopesticide at 4.5 months of storage. B4: triple bagging without biopesticide at 7 months, C4, D4, E4, and F4: triple bagging with 0.7%, 2.5%, 4.3% and 5% biopesticide at 7 months of storage. B5: triple bagging without biopesticide at 8 months, C5, D5, E5, and F5: triple bagging with 0.7%, 2.5%, 4.3% and 5% biopesticide at 8 months of storage

Table 9. Estimated folic acid intake in cowpea seeds stored at 8 month according to the consumption level

Vitamin	RDI*	Current Ivorian consumption: 4.98 g		Projection for a consumption of 100 g of cowpea seeds	
Folic acid (vitamine B9)	200 µg/j	**EAI (µg/j)**	**EAI (%)**	**EI (µg/j)**	**EI (%)**
		0.41	0.21	8.34	4.17

RDI: recommended daily intake [33]; EAI: estimated mean intake level per day from the 4.98 g of cowpea seeds consumed; EI: estimated intake for 100 g of cowpea seeds consumed*

3.4 Dietary Intake of Folic Acid from Cowpea Seeds after Storage

Table 9 above shows the contribution of vitamin B9 (folic acid) deriving from the intake of cowpea seeds stored for 8 months.

According to Directive 2008/100/CE, the recommended daily intake of vitamin B9 for adults (70 kg) is 200 µg [33]. The mean folate (B9) daily intake from cowpea seeds stored for 8 months is 0.41 µg. This value represents 0.21% of the recommended intake for an equivalent daily consumption of cowpea stated at 4.93 g per capita in Côte d'Ivoire [32]. However, a consumption of 100 g of cowpea seeds would provide 8.34 µg of vitamin B9, which would cover 4.17% of the recommended daily intake for this vitamin.

4. DISCUSSION

The low coefficients of variation obtained from the reproducibility and repeatability tests (1.0 to 4.4%) reflect the stability and satisfactory accuracy details for HPLC technique used. Moreover, the extraction yields of the standard addition and the linearity effectively reflect the reliability of this technique. Also, the low values of the limits of detection and quantification are guarantees of sensitivity of the proportioning of the vitamins contained in the samples studied by HPLC system.

Biotic and abiotic factors generally affect the quantity and nutritional quality of stored cowpea seeds. In order to get an idea about the conservation of various vitamins of cowpea seeds during storage, analyzes were carried out during all sampling periods. Vitamins considered as essential organic matter, without intrinsic energy value which the man cannot synthesize or in sufficient quantity must be brought to him by the food. However, under uncontrolled conditions of storage / preservation of cowpea, these vitamins undergo some modifications [14,15]. The results of this study show that the preservation technique using *Lippia multiflora* leaves is effective in preserving the vitamin quality of cowpea seeds during storage. Indeed, for all the vitamins, the highest contents were recorded in the triple bagging systems added to the *Lippia multiflora* leaves compared to the triple bagging system without biopesticide and the control bag (polypropylene) which record the lowest values at the end of storage.

During storage, the decrease in the levels of fat-soluble vitamins observed in the control group is due to the hydrolysis and oxidation of lipids. However, a plausible explanation for the ß-carotene contents stability of cowpea seeds in triple bagging system without biopesticide could be the presence of α-tocopherol in the food matrix. In fact, α-tocopherol is the naturally predominant form of tocopherols in cowpea seeds [35]. It is a powerful antioxidant that is widely used in the food industry to reduce the ß-carotene degradation by its antioxidant capacity [36,37]. Our findings corroborate those of Che et al. [38]. These authors showed that vitamin E prevented the oxidative degradation of ß-carotene in sorghum grains and increased stability during grains storage. Also Taleon et al. [39] work in Zambia showed that tocopherols reduce the degradation of carotenoids in biofortified maize under different storage conditions. Thus, for these different authors, the degradation of α-tocopherol was correlated with the attenuated degradation of ß-carotene during grains storage, suggesting that α-tocopherol could protect ß-carotene against oxidative degradation. In addition to this protective function, this molecule is known to be effective in delaying the onset of a variety of degenerative diseases such as cardiovascular diseases, cancers, inflammatory diseases and in the maintenance of the immune system [40]. Regarding water-soluble vitamins, cowpea seeds are sources of many vitamins including those of B complex such as folic acid, riboflavin, niacin, thiamine and pyridoxine. These molecules play an important role in metabolism, particularly of carbohydrates (thiamine), proteins and lipids (riboflavin and pyridoxine) [7]. Generally, a significant decrease in the different levels of water-soluble vitamins in the seeds stored in the polypropylene bag can be attributed to the nature of damage caused by the parasitic insects. In fact, during the different phases of their development, stocks pests use large amounts of vitamins as food for the maintenance of life and the accomplishment of larval development and metamorphosis [41]. Similar comments were made by Jood and Kapoor [42] reporting a substantial loss of vitamins (riboflavin, thiamin and niacin) at different levels of infestation. Also, these losses can be explained by the distribution of vitamins in grain constitution [43]. Indeed, vitamins have been described as the best nutrients distributed and assessed with proteins in cowpea seeds [44]. Thus, insects that feed on the rich parts of these nutrients also alter the vitamins.

In triple bagging system without biopesticide, the changes observed both in α-tocopherol and vitamins B levels could also be explained by low levels of oxygen in these systems. The moisture and heat produced can then accelerate the breathing process and in turn the degradation of vitamins such as thiamine and pyridoxine in these systems. Moreover, in the over experimental batches, the low levels of oxygen and the presence of biopesticide create an environment not conducive to the development of the insects and associated microorganisms. This could translate the stability of the vitamins of the seeds preserved in these systems. Indeed, Baoua et al. [45] and Garcia-Lara et al. [46] reported that when the beans are naturally infested by insects before storage in hermetic silos, the available oxygen inside the silos decreases by 5% and is due to insect respiration. The resulting consequence is the maximum reduction of grain damage at the end of storage. If in such condition the grain integrity is well preserved, there’s a possibility that the rate of deterioration of vitamins during the storage of cowpea seeds remains low. In addition, the anti-appetent, insecticidal and repellent effects of *Lippia multiflora* on *Callosobruchus maculatus*, a cowpea insect pest during storage were highlighted by Ilboudo et al. [24] and Konan [25].

The recommended daily intake of folic acid (vitamin B9) for an adult according AJR [34] is 0.2 mg/day. Therefore, the estimated intake of vitamin B9 from cowpea seeds stored during the 8 months of storage are below the required reference values and would not meet the adult folate requirement. The low vitamin B9 contribution of cowpea seeds could be explained by the low level of consumption of cowpea (4.93 g). However, taking into account the vitamin potential of cowpea seeds, if the daily consumption of cowpea in Côte d’Ivoire increased to 100 g following a change in diet, the vitamin B9 content of the seeds after 8 months of storage in triple bagging system with biopesticide would cover a satisfactory level of folic acid requirements.

5. CONCLUSION

The study confirmed the importance of setting up adequate systems (triple bagging with or without biopesticide) to preserve vitamin quality of cowpea. The triple bagging technique without biopesticide has shown its advantages in extending the shelf life of cowpea seeds for 7 months. However, the use of *Lippia multiflora* leaves have been able to maintain the vitamin composition of cowpea during the 8 months of storage. In addition, the contribution to meeting the vitamin B9 requirements of cowpea seeds stored in triple bagging system with biopesticide may be more assured following a change in diet in Côte d’Ivoire. Therefore, the technology developed in this study could be an alternative to the use of synthetic pesticides in the protection of cereals and legumes. It is inexpensive, easy to perform and protects the environment and human health. However, this study must be deepened in order to preserve organoleptic characteristics of the cowpea at the end of storage.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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