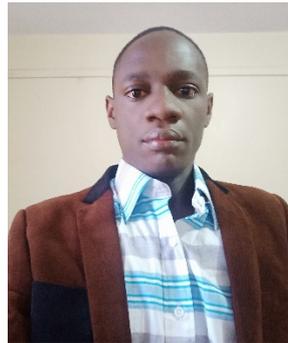


**GENETICALLY ENGINEERED EAST AFRICAN HIGHLAND BANANAS –  
PROXIMATE ANALYSIS AND EFFECT OF COOKING ON THE ENHANCED  
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## ABSTRACT

Micronutrient deficiency is a major challenge in the developing world. This is mainly attributed to over-reliance on starchy staples foods such as cassava, rice and banana among others, which are deficient in micronutrients such as vitamin A and iron. Strategies put in place to provide a solution to micronutrient deficiencies such as dietary supplementation of vitamin A and food fortification have not been successful in the developing world due to high costs and unreliable supply chains (food and medical). Biofortification of the easily accessible staple foods could help reduce this problem associated with micronutrient deficiency. On this account, the Biofortification project in Uganda under the National Banana Program developed transgenic East African Highland Bananas (EAHBs) (M9 and Nakitembe) with enhanced levels of provitamin A (PVA) using the Fe'i banana-derived phytoene synthase 2a (*MtPsy2a*) gene. To determine the nutritional quality of the transgenic bananas, an analysis of the proximate composition of the biofortified East African Highland Bananas was carried out. The effect of cooking on retention of provitamin A carotenoids (pVAC), was assessed using two cooking methods; boiling and steaming (most common methods of preparing cooking banana meals in Uganda). It was observed that there were no significant ( $P \leq 0.05$ ) differences in moisture content ( $P=0.4287$ ), carbohydrate ( $P=0.3966$ ), crude fat ( $P=0.4051$ ), crude fiber ( $P=0.3214$ ), protein ( $P=0.0858$ ) and ash content ( $P=0.1336$ ) between transgenic and non-transformed bananas. It was found that steaming, as a cooking method allowed for retention of more provitamin A carotenoids compared to boiling. Comparison of the cultivars on their retention of provitamin A carotenoids, results indicated that Nakitembe was superior to M9. Genetic engineering of bananas by biofortification has no effect on major food components in EAHBs (M9 and Nakitembe) and, therefore, genetically modified M9 and Nakitembe are substantially equivalent to the non-transgenic controls and the biofortified bananas can provide the necessary nutrients even after cooking. This data will inform subsequent steps for the commercialization of biofortified EAHBs.

**Key words:** M9, Nakitembe, Biofortification, Boiling, Steaming, Vitamin A, Proximate, HPLC



## INTRODUCTION

Banana is a perennial, unbranched herbaceous and monocotyledonous plant with underground rhizomes (corms), a fibrous root system and a pseudostem [1]. Taxonomically banana belongs to the order Zingiberales and family Musaceae. It grows up to 2 - 9 and 10 - 15 meters high in domesticated and wild types, respectively. Musaceae has two recognized genera namely, *Ensete* and *Musa* [ 2, 3].

Banana is an important cash crop but also a staple food crop providing starch and potassium in diets of many people living in tropical and sub-tropical regions [4]. More than 100 million people in Africa depend on banana and plantain for their food security [5]. It is a rich source of proteins, lipids, carbohydrates, starch and fiber [6], which are important components of the diet. It also provides a good source of major elements such as potassium, magnesium, phosphorous, calcium and vitamins B6 and C [7, 8, 9]. Bananas also have other important properties such as antidiarrheal and anti-ulcerogenic among other medicinal properties [10].

Most agronomically important cultivars, however, contain low levels of critical micronutrients such as iron and pro-vitamin A (pVA) [7, 11]. This may lead to serious malnutrition and diseases associated with malnutrition due to the high consumption such banana cultivars. Micronutrient deficiency is a major threat to the population, mostly affecting children and pregnant women especially in low income countries such as Uganda [12]. Despite micronutrient malnutrition reduction efforts, more than 40% of the world's population is suffering from at least one of the major micronutrient (iron, zinc, vitamin A or iodine) deficiencies [13] and their prevalence is rising, especially in undeveloped countries. In 2006, 20% of children (6-59 months) and 19% of childbearing age women were deficient in vitamin A in Uganda [14].

Overtime, different strategies have been used to solve the problem of micronutrient malnutrition. These include dietary supplementation of Vitamin A (VA) and food fortification. These have been successful especially in high risk populations in developed countries [15, 16]. Although these strategies have been successful in both developed and developing countries, they have been constrained by cost and unreliable food or medical supply chains, particularly in poor and rural communities [17].

Biofortification of staple foods could provide a solution for the developing countries [18]. Against this background, the East African Highland Bananas (EAHBs) with enhanced pVAC were developed through over expression of a Fe'i banana-derived phytoene synthase 2a (*MtPsy2a*) gene in two banana varieties namely M9 and Nakitembe [19] and [20]. Two promoters (*ubiquitin* and *ACO*), *Nopaline synthase* (NOS) terminator and *Neomycin Phosphotransferase* (NPTII) as a selectable marker were used in the gene construct. It is however unclear if the nutritional quality of the EAHBs was altered or remained the same following transformation. Proximate analysis was therefore carried out on the genetically engineered EAHBs [21] and the effect of cooking (heat) on the levels of pVAC was determined.

The target amount of pVA in the fruit pulp was set at 20µg/g dry weight Beta Carotene Equivalent (BCE) to ensure alleviation of VAD. The goal was to ensure delivery of 50%



of the estimated activity requirement in vulnerable populations. This limit was reached with due consideration of the bioconversion of beta carotene to vitamin A (6:1) and retention of the carotenoid after cooking [22].

### Sample collection and processing

Full green fruit samples were collected from a confined field trial (CFT) at the National Agricultural Research Laboratories (NARL) for PVA extraction and analysis. Samples were collected from the top cluster, middle cluster and bottom cluster of the same bunch, processed and later combined to form a composite sample from which analysis was done. The samples included 5 transgenic lines of M9 (FT12041, FT12053, FT12095, FT12280 & FT12310) and 4 transgenic lines of Nakitembe (FT12390, FT12392, FT12412 & FT12468). Samples from non-transformed M9 and Nakitembe comprised the controls. Leaf tissue was also collected for the different lines and freeze dried for molecular characterization.

### Presence of transgene

Genomic DNA was extracted from 50 mg of freeze-dried leaf tissue using the modified CTAB protocol [23]. Polymerase chain reaction (PCR) (BIO RAD C1000 Touch Thermal cycler) was done to confirm the presence of the gene using specific primers for the different genes. Two primers; *Actin* (Actin forward: 5<sup>1</sup> - CTG GTG ATG GTG TGA GCC AC – 3<sup>1</sup>, Actin reverse: 5<sup>1</sup> – CAT GAA ATA GCT GAG AAA CG – 3<sup>1</sup>)(housekeeping gene) to check for presence of banana DNA and *NPTII* (NPTII Forward: 5<sup>1</sup> – TGA TTG AAC AAG ATG GAT TGC ACG C – 3<sup>1</sup>, NPTII Reverse: 5<sup>1</sup> – GAT GAA TCC AGA AAA GCG GCC AT – 3<sup>1</sup>) (selectable marker) to confirm the presence of the transgene in the transgenic lines.

### Proximate Analysis

Full green samples were harvested and transported to the laboratory. They were then processed by peeling and cutting the pulp into smaller cubes using a stainless knife, placed in a petri dish and kept at -80<sup>0</sup>C. The samples were then freeze dried at 100 μBar for 72 h in a BenchTop™ 4K Freeze Dryer (VirTis® SP Industries) and finally ground into a fine powder using a Mini-BeadBeater-8 (Biospec Products) from which the analysis was done. The analysis was done according to the Association of Official Analytical Chemists (AOAC) guidelines [24].

### Determination of moisture content

An empty petri dish was weighed (W<sub>1</sub>) using an analytical balance (Shimadzu TX423L, Uni Bloc). A full green banana fruit sample was processed by peeling and cutting the pulp into smaller cubes using a stainless knife and then placed in the petri dish. The combined weight of the sample and petri dish was taken (W<sub>2</sub>). The sample was then freeze dried for 72 hours in a BenchTop™ 4K Freeze Dryer (VirTis®, SP Industries). The weight of the sample after freeze drying (W<sub>3</sub>) was then taken. The moisture content was calculated as shown in equation (i).

$$\frac{W_2 - W_3}{W_2 - W_1} \times 100 \dots\dots\dots (i)$$



**Determination of ash content**

A silica crucible was first heated in a muffle furnace (CARBOLITE CWF 1200) at 550 °C for one hour, cooled in a desiccator and the initial weight taken. The process was repeated until the weight difference became less than 1 mg. After, 2 g of the sample was heated in a muffle furnace at 550° C for 6 h, cooled in desiccator and the weight of the ash was taken. The ash content was calculated as shown in equation (ii) [24].

$$\frac{\text{Weight of ash}}{\text{Weight of the sample}} \times 100 \dots\dots\dots(ii)$$

**Determination of crude fat**

The initial weight of the flask was taken by heating in a hot air oven overnight at 105 °C followed by cooling in a desiccator. The weight of the flask was then taken. The process was repeated until the weight difference became less than 1.0 mg (30 minutes of heating). Five g of the sample was then extracted with ethyl ether using Soxhlet apparatus for about 6 h. The extracted fat was then dried in a rotary evaporator and the weight determined. The percent crude fat was determined as shown in equation (iii) [24].

$$\frac{\text{Weight of fat}}{\text{Weight of the sample}} \times 100 \dots\dots\dots(iii)$$

**Determination of crude protein**

Crude protein was determined by Kjeldahl method following the AOAC method [24]. Accurately weighed 0.5 g of the sample was digested with 2 mL concentrated H<sub>2</sub>SO<sub>4</sub> and Kjeldahl catalyst (9 parts of K<sub>2</sub>SO<sub>4</sub> and one part of CuSO<sub>4</sub>) in a digestion chamber until it became clear. The blank test was also performed without the sample. After digestion, distillation in Kjeldahl distillation chamber (Buchi Kjelflex K-360) was done. Upon evaporation, the ammonia was condensed and titrated against 0.1 N HCl. Nitrogen concentration was calculated by equation (iv).

$$\text{Nitrogen \%} = (A - B) \times N \times f \times 0.014 \times 100 / S \dots\dots\dots(iv)$$

Where; A = volume (ml) of 0.1N HCL used in sample titration

B = volume (ml) of 0.1N HCL used in blank titration

f = Factor of standard HCL solution

S = weight of sample taken (g)

The protein content was then determined by multiplying the nitrogen concentration with the protein factor (6.25).

**Determination of crude fiber**

A dry sample (5g) was boiled with 0.25N H<sub>2</sub>SO<sub>4</sub> for 30 minutes under reflux condenser followed by filtration with pyrex glass filter, washed with hot water and boiled with 0.313N NaOH. It was again filtered, washed with boiling water followed by 0.5 N H<sub>2</sub>SO<sub>4</sub> and 50% ethanol. The residue was dried in an oven at 130 °C for 2 h. The dry weight of



the digested sample was then taken, incinerated in a muffle furnace at 600 °C for 30 min, cooled in a desiccator and weight of the ash was measured. The crude fiber content was calculated based on 100 g of the freeze-dried sample using equation (v) [24].

$$\frac{(\text{Dry weight of digested sample} - \text{Weight of ash})}{\text{Weight of sample}} \times 100 \dots\dots\dots (v)$$

**Determination of total carbohydrates**

The total carbohydrates was determined by the method of difference according to the formula (equation (vi))

$$100 - (\text{Moisture} (\%) + \text{Ash} (\%) + \text{Crude protein} (\%) + \text{Crude fat} (\%)) \dots\dots\dots (vi)$$

**Effect of cooking on pro-vitamin a content**

Full green fruits were collected from the field and peeled in a dimly lit laboratory because carotenoids are light sensitive. Peeled samples were then placed in a saucepan containing water (boiling) or wrapped in banana leaves and then placed into a saucepan containing banana fiber, banana leaf stalk and sufficient water (steaming). This was then tightly covered and placed on a heater (IGNIS gas stove) to start the heating process. Boiling was done for 30 minutes and steaming done for one and half hours. The cooked samples were mashed using a mingling stick, allowed to cool and then wrapped in clearly labelled foil. These were then stored at -80°C. After the samples were freeze dried, ground and provitamin A extraction and analysis done as described by Buah *et al.* [25]. Fresh samples were included in this test as controls. All the samples (boiled, steamed, fresh) were collected from the same bunch.

**Statistical Analysis**

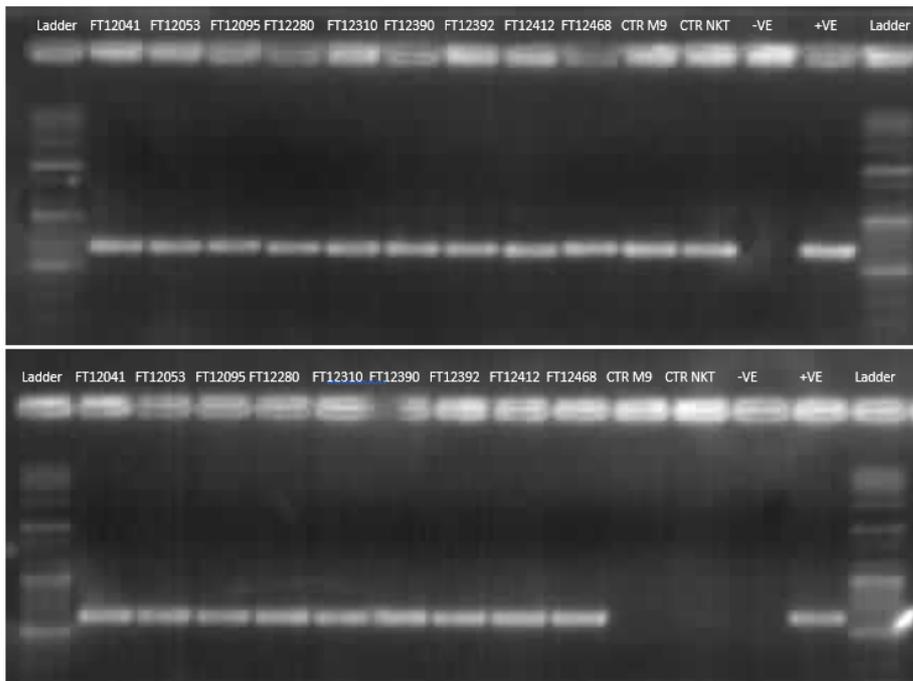
Graphpad prism 5 package was used for analyzing the data and for graphical representation of the data. Unpaired, one tailed T-test were used to compare non-transgenic controls and transgenics means (for proximate analysis) and one-way analysis of variance (Turkey’s multiple comparison test) used to compare Beta Carotene Equivalents (BCE) means; statistical differences were reported at 95% confidence level (p<0.05).

**RESULTS AND DISCUSSION**

**Presence of transgene**

Amplification of Actin, a house keeping gene, was successful in both transgenic and non-transgenic. This confirmed successful isolation as well as the presence of good quality banana DNA. Control M9 and control NKT were confirmed as wild-types since there was no amplification with *NPTII* primers. In the other lines, *NPTII* amplified and were confirmed as transgenics as shown in Figure 1. Lines FT12041, FT12053, FT12095, FT12280 and FT12310 are M9 transgenics while lines FT12390, FT12392, FT12412 and FT12468 are Nakitembe transgenics.





**Figure 1: Stained agarose gel showing PCR products obtained using Actin and NPTII primers. Actin gene was amplified to test for presence of banana DNA in the different line (A). NPTII gene (selectable marker) was amplified to test for the presence of the transgene (B). -ve was the water control (negative control), +ve was the plasmid DNA (positive control). X-axis – molecular marker (ladder) sizes, X-axis – sample IDs**

### Proximate composition of the transgenic banana

In line with this study, proximate analysis was done to ascertain that genetically modified EAHB lines are substantially equivalent to the non-transgenic lines. This is a requirement to ensure food safety according to the Organization for Economic Cooperation and Development (OECD) food safety concept [26]. The proximate composition for all the samples tested is presented in Table 1.

In the EAHBs, moisture contributed the highest percentage in both transgenic and non-transgenic lines ( $77.23 \pm 1.49\%$ ), followed by carbohydrates ( $16.57 \pm 0.99\%$ ), ash ( $3.43 \pm 0.20\%$ ), fiber ( $1.43 \pm 0.58\%$ ), fat ( $0.56 \pm 0.01\%$ ) and protein ( $0.13 \pm 0.04\%$ ). The results showed that crude fiber ( $P=0.3214$ ), crude ash ( $P=0.1336$ ), moisture content ( $P=0.4287$ ), crude fat ( $P=0.4051$ ), protein ( $P=0.0858$ ) and carbohydrate content ( $P=0.3966$ ) in the transgenic plants are not significantly ( $P>0.05$ ) different from non-transgenic controls. Therefore, *MtPsy2a* gene inserted into the different EAHBs by genetic engineering did not affect the composition of carbohydrates, ash, fiber, protein, moisture and fat levels in the EAHBs. Generally, M9 samples contained more carbohydrate and crude ash compared to Nakitembe lines. Transgenic lines, both M9 and Nakitembe (except FT12392) contained more ash than the non-transgenic controls. Nakitembe lines generally contained higher levels of crude fiber and crude fat than M9

lines. All Nakitembe transgenics contained more crude fat than the Nakitembe control whereas in the M9 cultivar, the control line contained more crude fat than the transgenic lines.

From the analysis, both the transgenic and non-transformed lines contained all the nutrients analyzed for proximate (therefore no proximate parameter was completely lost by addition of *MtPsy2a* gene in the EAHBs). Moisture content for the analyzed lines were above 70% (between 75%-79%) which lie within the range (77.85% to 82.22%) obtained from other studies by Auta and Kumurya [27] and Odenigbo [28] of 71% to 75%. From the findings, moisture content in transgenic lines was substantially equivalent to the non-transformed controls.

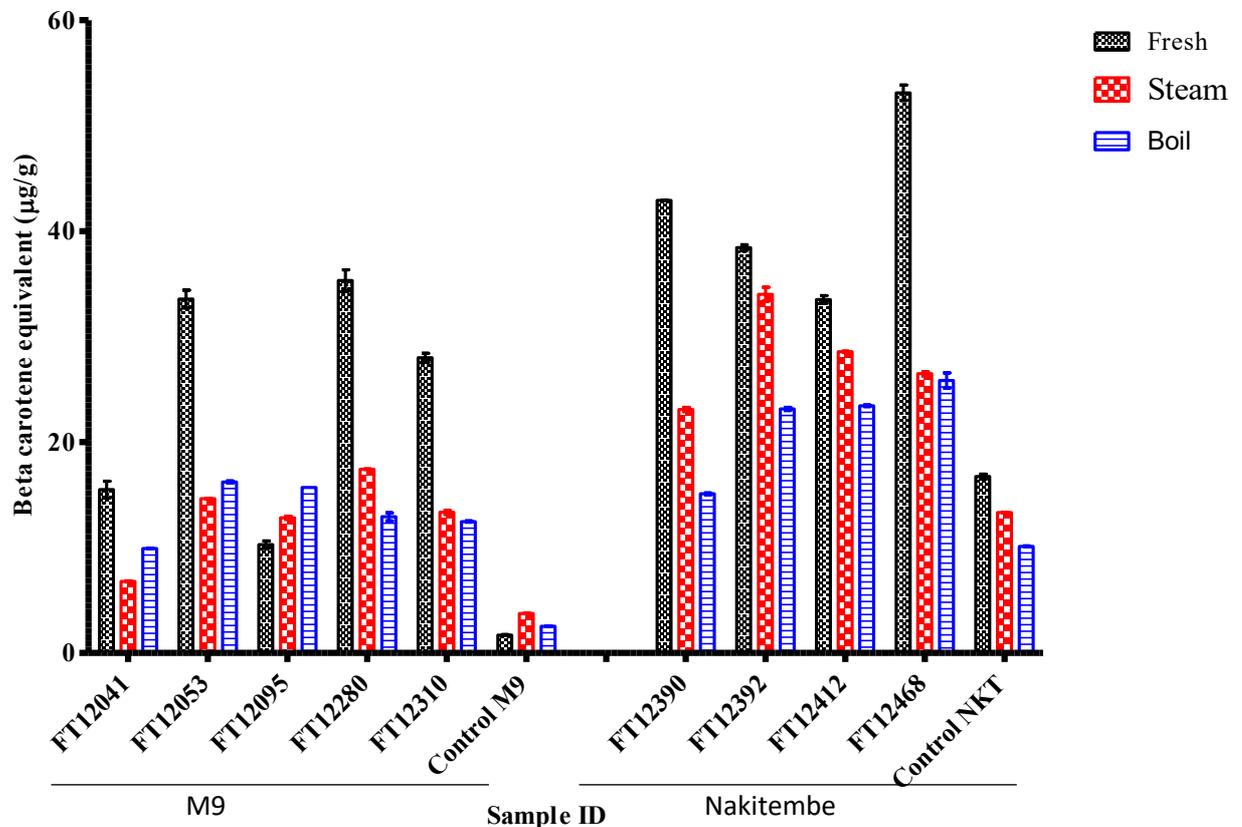
The carbohydrate content ranged from 14% to 19%, which was highest in comparison to other nutrients. Bananas being starchy staples are expected to have higher quantities of carbohydrates compared to other nutrients [29]. From our findings, there was no significant difference in the carbohydrate content between transgenic and non-transformed controls, implying that the *MtPsy2a* gene does not affect the carbohydrate content in the EAHBs.

The protein content ranged from 0.1% to 0.3%. This was less than the ranges from other studies [30] and can be due to the differences in environmental conditions [32]. For a sample to be considered a rich source of proteins, it should provide 12% of its calorific value from proteins [30]. This suggests that M9 and Nakitembe cultivars are not adequate sources of protein.

From the findings, proximate composition in the transgenic EAHBs was comparable to that in the non-transgenic controls. Therefore, provitamin A enhanced transgenic EAHBs are substantially equivalent to the non-transformed lines proximate in terms of proximate composition. This was similar to findings from Ramakanth *et al.* [33] who studied nutritional composition of genetically modified peanut varieties and concluded that genetic modification did not cause any substantial change in transgenic peanut when compared to traditional varieties. A similar study on transgenic rice also revealed that apart from the enhanced iron and zinc in the transgenic seeds, the nutritional quality in transgenic lines were not significantly different from non-transgenic lines [34]. Venneria and Monastra [35] also concluded that genetic modification events are similar nutritionally to the conventionally developed varieties of wheat, tomato and corn. This is because genetic engineering techniques are precise and specific and, therefore, proximate composition of the EAHBs was not affected by the *MtPsy2a* gene.

**Effect of processing on Provitamin A levels in EAHBs**

The results for BCE levels after steaming and boiling are shown in Figure 3.



**Figure 3: Provitamin A levels in EAHBs. Comparison between the fresh samples and the cooked samples (mean±SD, n=2)**

**Figure 3: Provitamin A levels in EAHBs. Comparison between the fresh samples and the cooked samples (mean±SD, n=2)**

All fresh transgenic Nakitembe lines crossed the  $20\mu\text{g/g}$  Dry weight (DW) limit. Three out of the five fresh transgenic M9 lines also crossed the limit. Both controls were below the limit. The highest levels of BCE were observed in Nakitembe line FT12468 ( $53.11\pm 1.03\mu\text{g/g}$  DW). Boiling reduced the provitamin A levels in the biofortified bananas. The reduction was more in M9 than Nakitembe. Among the Nakitembe transgenics, 80% remained above the  $20\mu\text{g/g}$  DW limit after boiling (apparent retention was used as described by [36]). This suggests that the Nakitembe cultivar had a higher retention than M9. Average reduction in BCE levels in M9 transgenics was 51.7% after boiling, while average reduction in Nakitembe transgenics after boiling was 45.1%. Retention in Nakitembe control after boiling was 66.44%. Steaming reduced the provitamin A levels in the two cultivars. The reduction in pVA levels was more in M9 than in Nakitembe. The average reduction/loss after steaming in M9 transgenics was 53.91% while the average reduction in Nakitembe transgenics was only 28.59%. Therefore, Nakitembe had a better retention after cooking.

The BCE levels of the banana observed after cooking (boiling and steaming) were lower compared to the fresh values in all transgenic and non-transformed lines. This is because carotenoids are subject to degradation by physical and chemical means such as high temperature [37,38]. Boiling and steaming effect on the levels of provitamin A carotenoids was not uniform. Some lines had higher retention after boiling while others had higher retention after steaming. This is consistent with findings by Murador *et al.*, [38] that also observed both reduction and increase in the carotenoids levels among transgenic lines after cooking. Generally, there were lower levels of BCE after boiling compared to steaming suggesting that boiling had a lower retention for provitamin A carotenoids than steaming. All M9 transgenic lines did not exceed the 20 $\mu$ g/g DW after processing (both cooking and steaming). This suggests that M9 cultivar had lower retention compared to Nakitembe cultivar. This does not agree with findings from Mbabazi [22] where M9 had a higher retention after processing than Nakitembe. The effect of cooking on the amount of non-pVA lutein and pVA carotenoids is shown in Figure 4. There was an increase in the amount of non pVA lutein and a reduction in the amount of pVA carotenoids ( $\alpha$ -carotene and *trans*  $\beta$ -carotene). This accounts for the observed reduction in BCE levels.

Analysis of the chromatograms before and after cooking (both transgenic and non-transgenic control) revealed new peaks after cooking. In the transgenic line (Figure 5) peaks (not identified) were observed at a retention time between 10 and 12 minutes (as shown by the red pointers) after boiling and steaming (not seen in the fresh sample). Similarly, in the control line (Figure 6) peaks (not identified) were observed at a retention time between 10 and 15 minutes (as shown by the red pointers) after boiling and steaming (not seen in the fresh sample). These may be due to a change from one carotenoid to another leading to a reduction in the overall BCE levels after cooking. This is probably a result of isomerization after cooking of the banana samples. Acids, light and heat promote isomerization of *trans*-carotenoids to *cis*-form [40].

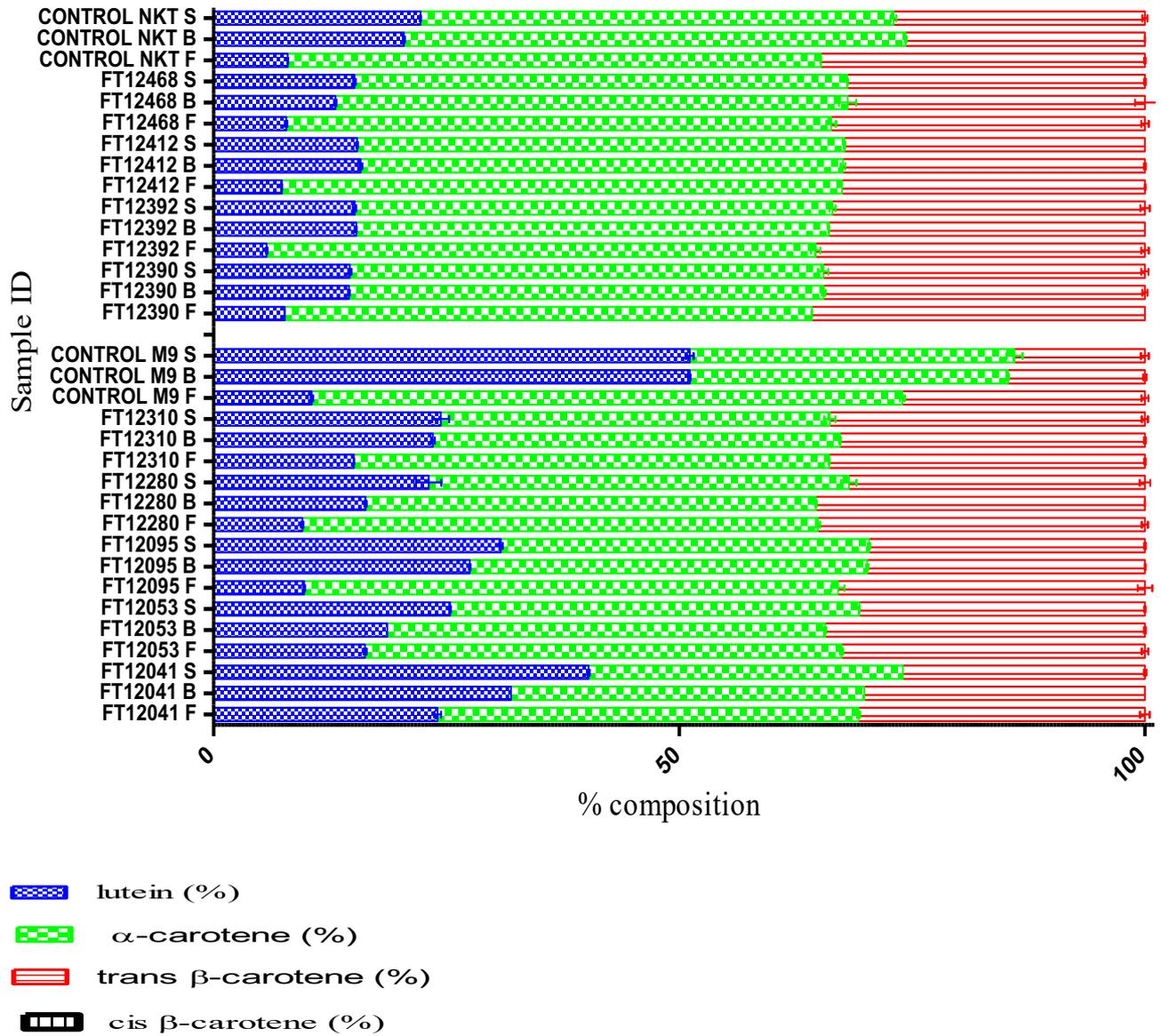
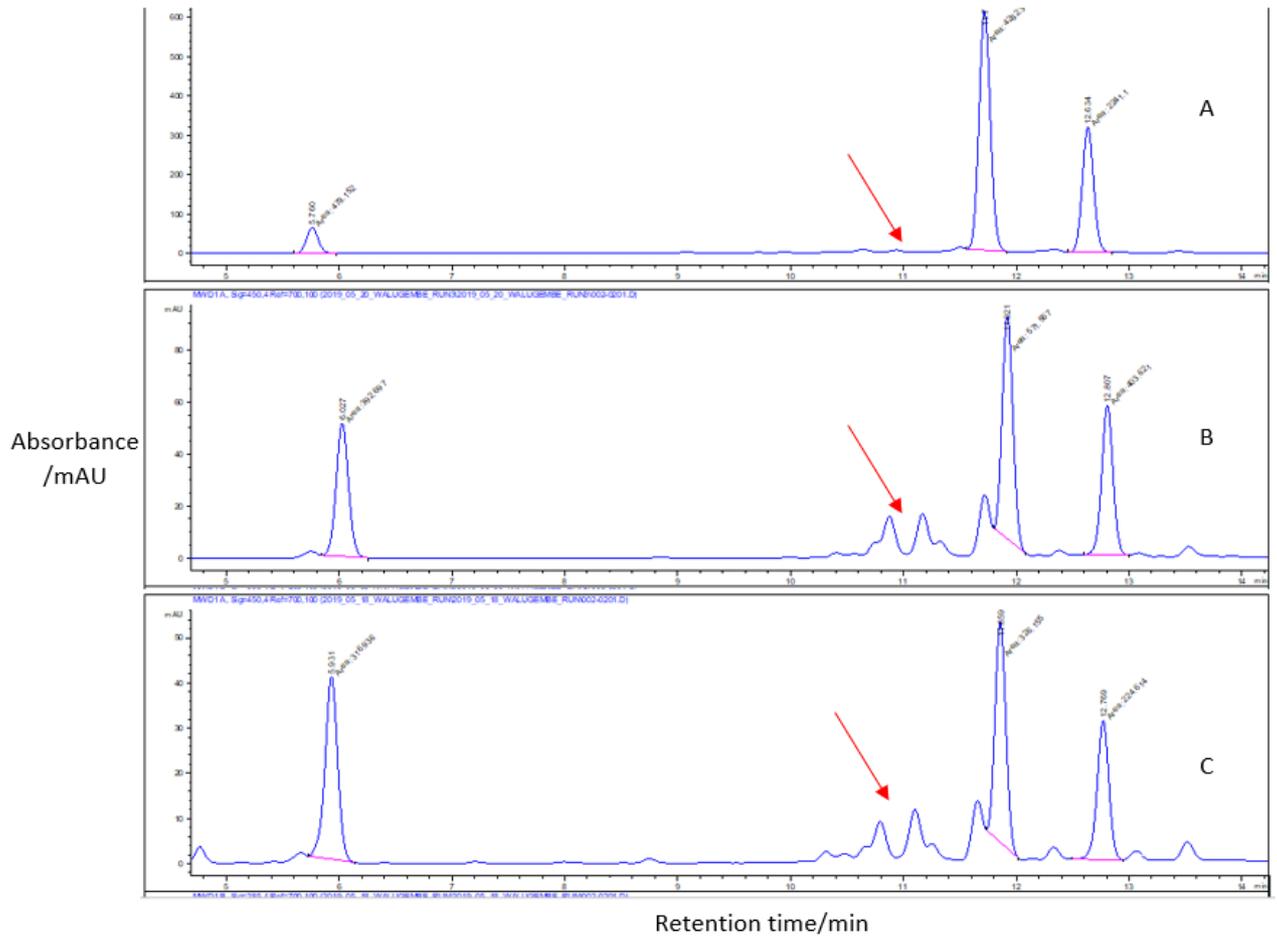
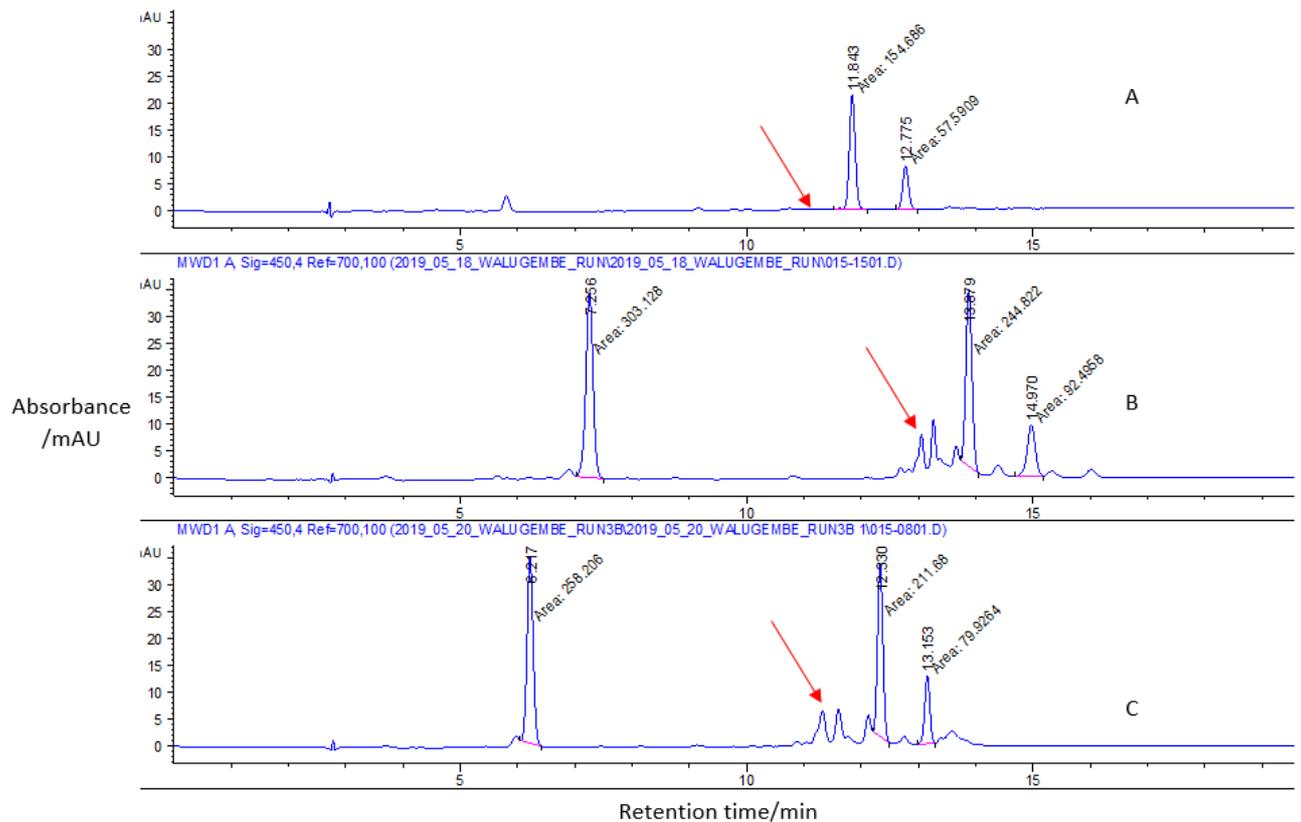


Figure 4: Carotenoid composition for fresh and cooked lines. F- Fresh, B-Boil and S-Steam. Mean $\pm$ SD, n=2



**Figure 5: Chromatograms for transgenic sample before and after cooking. Fresh sample (A), Boiled sample (B) and Steamed sample (C). This was to compare the changes that occur in the transgenic banana with cooking (boiling and steaming)**



**Figure 6: Chromatograms for non-transgenic line before and after cooking. Fresh sample (A), Boiled sample (B) and steamed sample (C)**

The reduced levels for BCE after cooking were probably due to isomerization. Cooking (heat) leads to change from trans-carotenes to cis-carotenes [39] and [40]. This reduces the trans- $\beta$ -carotenes and increases the cis- $\beta$ -carotenes. From the calculation for BCE, cis- $\beta$ -carotene contributes a small percentage to the BCE. Therefore, a reduction in the trans- $\beta$ -carotene could have led to an overall reduction in the BCE amounts.

## CONCLUSION

In conclusion, provitamin A enhanced EAHBs studied were substantially equivalent to the non-transgenic controls. Therefore, genetic engineering does not appear to significantly affect the proximate composition of the EAHBs. Steaming, as a method of processing EAHBs resulted in better retention of provitamin A and is therefore recommended as a better method for preparation of cooking banana.

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**Table 1: Proximate analysis result for the different samples. Mean±SD, n=3**

Sample ID	Moisture	Ash	Fat	Fiber	Protein	Carbohydrates
FT12041	75.8761±0.214	3.8±0.071	0.44±0.028	0.65±0.042	0.7±0.031	18.53±0.183
FT12053	78.1319±0.082	3.7±0.141	0.29±0.014	1.06±0.057	0.6±0.031	16.21±0.183
FT12095	75.9637±0.469	3.5±0.035	0.05±0.042	1.53±0.071	0.6±0.031	18.36±0.564
FT12280	79.2148±0.931	3.5±0.177	0.35±0.042	1.25±0.071	0.8±0.031	14.83±1.105
FT12310	75.3218±2.043	3.5±0.141	0.26±0.057	1.34±0.057	1.0±0.495	18.64±2.688
FT12390	77.4202±1.330	3.3±0.000	0.57±0.042	2.92±0.028	0.7±0.062	15.11±1.282
FT12392	77.9206±0.042	3.1±0.106	0.8±0.028	1.87±0.071	0.9±0.031	15.41±0.136
FT12412	79.2073±0.601	3.4±0.000	1.59±0.042	1.62±0.057	0.7±0.031	13.48±0.471
FT12468	76.3161±1.571	3.4±0.035	0.86±0.085	0.97±0.042	0.6±0.031	17.91±1.524
CONTROL M9	77.2984±0.198	3.4±0.000	0.56±0.057	1.13±0.042	0.8±0.031	16.87±0.266
CONTROL Nakitembe	76.8293±0.199	3.2±0.035	0.43±0.014	1.34±0.057	1.3±0.000	16.89±0.164

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