Asian Journal of Chemical Sciences

Volume 15, Issue 5, Page 156-165, 2025; Article no.AJOCS.145191
ISSN: 2456-7795

Evaluation of Antioxidant Contents and Structural Identification by HPTLC and HPLC-MS/MS of Carotenoids in the BF59XCIP1 Variety of *Ipomoea batatas* L.

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ajocs/2025/v15i5397

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://pr.sdiarticle5.com/review-history/145191

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Cite as: *Dominique Saga Kaboré, Benjamin Bazié, Mahamadi Ouedraogo, Remy K Bationo, Moumouni Koala, Constantin M Dabiré, Koussao Somé, Eloi Palé, and Pierre Duez. 2025. "Evaluation of Antioxidant Contents and Structural Identification by HPTLC and HPLC-MS/MS of Carotenoids in the BF59XCIP1 Variety of Ipomoea Batatas L". Asian Journal of Chemical Sciences 15 (5):156–165. https://doi.org/10.9734/ajocs/2025/v15i5397.*

Short Research Articles

Received: 30/06/2025
Published: 30/09/2025

ABSTRACT

Objective: Orange-fleshed sweet potatoes are an important source of nutrients for consumers thanks to the carotenoids they contain. Carotenoids are bioactive compounds which, thanks to their anti-radical properties, can protect the human body against oxidative stress. Oxidative stress is the cause of many degenerative diseases, such as cancer, blindness, and aging. This study aims to evaluate the antioxidant content of the orange-fleshed variety BF59XCIP1, obtained by crossing varieties adapted to local agroecological conditions, and to identify the main carotenoids in this variety.
Study Design: The work combined cutting, tuber harvesting, and various laboratory analyses. This work was carried out as part of my doctoral thesis.
Methodology: Two types of extracts were prepared. Extracts using an acetone-water-acetic acid solvent system (70:29.5:0.5 v/v/v) for the evaluation of antioxidant content and hexane extracts for structural identification. Antioxidant content was evaluated using the DPPH and FRAP methods. HPTLC-MS and HPLC-MS/MS analyses were used for carotenoid identification.
Results: antioxidant content assessed using the DPPH and FRAP methods was estimated at 0.082 mg ET/g and 0.183 mg ET/g of fresh plant material, respectively. For the structural identification of carotenoids, analysis of the two major spots by HPTLC-MS identified β-cryptoxanthin, zeaxanthin, and β-carotene. HPLC-MS/MS analysis identified capsanthin, echinenone, α-tocopherol, and β-carotene.
Conclusion: The BF59XCIP1 variety of orange-fleshed sweet potato is a source of vitamin A. Therefore, its consumption can compensate for vitamin A deficiency in malnourished children.

Keywords: Carotenoids; antioxidant; HPLC-MS/MS; HPTLC-MS.

1. INTRODUCTION

« Carotenoids are secondary metabolites, i.e., compounds synthesized by plants. They belong to the terpenoid family and comprise two major classes of compounds: carotenes and xanthophylls. Carotenoids are found in many fruits and vegetables (oranges, tomatoes, carrots, spinach, sweet potatoes, pumpkins) and are often responsible for the bright colors of these fruits and vegetables » (Sol et al., 2013). « Due to their coloring properties, carotenoids play an important role in plant physiology. Some carotenoids are precursors to vitamin A, which is one of their most important physiological functions » (Vílchez et al., 2011). « A correlation has been established between the consumption of vegetables and fruits rich in carotenoids and the prevention of several chronic degenerative diseases, such as cancer, inflammatory diseases, cardiovascular diseases, cataracts, and degeneration, among others » (Guerin et al., 2003), thanks to their antioxidant properties. Given the importance of these carotenoids, their qualitative and quantitative analysis in PDCO is necessary in order to promote their consumption as part of the fight against vitamin A deficiency. The objective of this study is to evaluate antioxidant content using spectrophotometry and to identify the structures of the major carotenoids in extracts of the BF59XCIP1 variety of *Ipomoea batatas* L. by combining chromatographic and spectral methods (HPLC-ESI-MS/MS; HPTLC-SM).

2. MATERIALS AND METHODS

2.1 Plant Material

The plant material consisted of the BF59XCIP1 variety of orange-fleshed sweet potato. This variety, whose cuttings were provided by INERA, was grown in the LCOPA experimental garden at Joseph KI-ZERBO University.

2.2 Methods

2.2.1 Extraction

For qualitative analysis, 30 g of fresh crushed plant material was extracted successively with

100 mL, 50 mL, and 50 mL of hexane by maceration. The filtrates obtained were collected for saponification. In fact, 30% KOH in methanol was added to the crude extract. The mixture was left to stand at room temperature for 3 hours away from light. After liquid-liquid partitioning in a separating funnel using hexane, the organic phase obtained was concentrated to dryness and used for the various analyses.

To evaluate the antioxidant content, extracts were prepared using a solvent system of acetone-water-acetic acid (70:29.5:0.5 v/v/v). Five grams of crushed tubers were macerated using a sonicator for 1 hour in 10 mL of the solvent system. After filtration, two further extractions were carried out with 5 mL of the same solvent system for 30 min. The filtrates were combined for analysis.

2.2.2 Evaluation of Antioxidant Content

The total antioxidant content was determined by spectrophotometry using two of the three commonly used methods: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and the Ferric Reducing Antioxidant Power (FRAP) method. These methods are based on different chemical mechanisms. The DPPH method measures the ability of an antioxidant to trap the radical (Tapiero et al., 2002) by transferring electrons from the sample to the antioxidant. The FRAP method evaluates the reducing power of a sample.

DPPH method: This method measures the absorbance of the discolored DPPH° radical solution by the activity of a quantity of the sample being studied. Fifty µL of each extract is added to 200 µL of the DPPH reagent. After 10 minutes of incubation, the absorbances are read at 515 nm using a microplate reader (MP96 spectrophotometer, SAFAS). The absorbances of the samples studied are plotted on a standard curve ($y = -14.194x + 0.6017$; $R^2 = 0.9997$) previously established using Trolox as a reference antioxidant. The contents are thus expressed in mg of Trolox equivalents per gram of fresh plant material. All measurements are repeated three times.

FRAP method: in this method, a ferric salt, Fe(III)(TPTZ)2Cl3 (TPTZ = 2,4,6-tripyridyl-s-triazine) is used as an oxidant (Tapiero et al., 2002 ; Strain and IF, 1996). The absorbance of the intense blue discoloration at 595 nm is measured with a microplate reader (MP96 spectrophotometer, SAFAS). To a mixture of 30 µL of distilled water and 20 µL of each of the appropriately diluted samples, 200 µL of the FRAP reagent is added. A standard curve is established using Trolox as the reference antioxidant. The results, determined from the calibration curve equation ($y = 18.957x + 0.2917$; R2= 0.9985), were expressed in mg of Trolox Equivalents (TE) per gram of plant material. All measurements were repeated three times.

2.2.3 HPTLC-MS Analysis

Chromatography was performed on a 20 cm x 10 cm silica gel 60 F254 HPTLC plate (Merck, Germany). The mobile phase was a petroleum ether-acetone-ethyl acetate system (85:15:5 v/v/v). Five µL and 6 µL of saponified extracts were applied in 5 mm bands using a CAMAG Vision CAT automatic thin-layer chromatography (TLC) sampler. The mass spectrometer used to identify carotenoids in PDCO extracts was a Quadrupole Time of Flight (QTOF, 6520 series) equipped with an Electro-Spray ionization source in positive mode (ESI+). Mass spectra were obtained in a mass range of m/z 400-600. The MS parameters were as follows: collision gas was helium; capillary temperature was 250°C.

2.2.4 HPLC-MS/MS Analysis

HPLC coupled with tandem mass spectrometry using positive mode electrospray ionization (HPLC-ESI-MS/MS) was used in this study to identify and characterize carotenoids. An Agilent Technology Infinitely Better 1290 HPLC system was used for chromatographic separation. It is equipped with a 100 mm long, 2.1 mm diameter, 5 µm particle size, water X-terra reverse phase C18 column. Elution was performed with mobile phase A (acetonitrile; methanol 85:15 v/v + 0.1% formic acid) and mobile phase B (water + 0.1% formic acid) as described by Jeevan Kumar Prasain (2005). The elution gradient over time, expressed as a percentage of the volume of mobile phase A and mobile phase B, was programmed as follows: 0 to 7 min, 95% A; 8 to 15 min, 100% A. The flow rate was maintained at 1 mL/min and the column temperature at 25°C.

3. RESULTS AND DISCUSSION

3.1 Antioxidant Content

The antioxidant content of the local PDCO variety BF59XCIP1 is expressed in mg ET/g of plant material. These contents are 0.082±0.002

and 0.183±0.001 respectively by the DPPH and FRAP methods. These values are significantly different. This difference could be explained by their reaction mechanisms. The DPPH method measures the ability of an antioxidant to trap the DPPH° radical (Tapiero et al., 2002) by transferring electrons from the antioxidant sample. However, the FRAP method evaluates the reducing power of the sample. Similarly, Hinneburg et al. found in 2006 that cumin hydrodistillate has antioxidant activity, which varies depending on the type of test used (Hongnan et al., 2014). The relatively low antioxidant content assessed by the DPPH method could also be explained by the structure of the DPPH° radical. This variety has higher antioxidant content compared to the BF59XCIP4 variety, which is 0.069 and 0.171 mg ET/g of fresh plant material, but lower compared to the TUSKEGEE ORANGE variety, which is 0.132 and 0.297 mg ET/g of fresh plant material, but lower than those of the TUSKEGEE ORANGE variety, which are 0.132 and 0.297 mg ET/g of fresh plant material respectively by the DPPH and FRAP methods (Saga, 2021).

3.2 Identification of the Main Carotenoids by HPTLC-MS

The identity of the bands in the extract chromatogram was further confirmed by mass spectrometry, i.e., using the Camag TLC-MS interface, which allows direct coupling of TLC to MS.

Two major spots were analyzed. Spots 1 and 2 with respective Rf values of 0.3 and 0.87 (Fig. 1).

MS analysis of spot 1 (Rf= 0.3) shows two peaks corresponding to two molecular ions at m/z 537.4 and 569.4, named A and B respectively (Fig. 2). The molecular ion at m/z 537.4 (≃537) corresponds to the mass calculated from the formula $C_{40}H_{56}$. Compounds such as β, α-carotene, and lycopene have the same molecular formula. Compound A can be either β or α-carotene, or lycopene. The molecular ion at m/z 569.4 (569) corresponds to the mass calculated from the formula $C_{40}H_{56}O_2$. This is either lutein or zeaxanthin. Lutein and zeaxanthin differ only in the position of a double bond on the rings (Fig. 3). Lutein and zeaxanthin are powerful fat soluble antioxidant nutrients. Lutein and zeaxanthin are two xanthophylls found in the macular pigment of the eyes.These molecules may play an important role in the prevention of age-related macular degeneration (AMD), cataracts, and other eye disorders. Indeed, studies have shown that lutein has a shorter retention time than zeaxanthin (Regnard et al., 2014). Compound B could therefore be lutein.

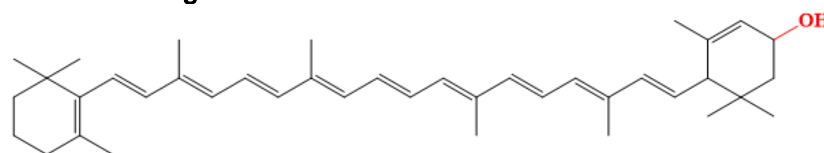
Mass spectrum analysis (HPTLC-MS) of spot 2 (Rf= 0.87) reveals three peaks corresponding to molecular ions at m/z 537.4, 553.7, and 568.5, labeled C, D, and E, respectively (Fig. 2). As in the case of spot 1, the molecular ion at m/z 537.4 (≃537) corresponds to the mass calculated from the formula C40H56. The masses of carotenoids such as lycopene, β, γ, and α-carotene correspond to this molecular formula. Compound C would be either lycopene or one of its isomers. However, several studies have shown that β-carotene (Fig. 5) is the major component in PDCOs (Kimura et al., 2007 ; Pott et al., 2003). Compound C could correspond to β-carotene. The molecular ion at m/z 553.7 could correspond to one of the isomers of cryptoxanthin (β-cryptoxanthin and α-cryptoxanthin) with the formula $C_{40}H_{56}O$ (Fig. 4). The structural difference between these two molecules is related to the position of the hydroxyl group and the double bond of the second nucleus of the molecule. In addition, the hydroxyl group of α-cryptoxanthin is allylic (Fig. 6) and therefore easy to eliminate compared to the hydroxyl group of β-cryptoxanthin, which is linked to a secondary carbon with saturated neighboring C-C bonds. According to the work reported by Koala, (2015) and Somé et al.(2004), orange-fleshed sweet potatoes contain β-cryptoxanthin. It should be noted that β-cryptoxanthin is a powerful antioxidant. It is one of the xanthophylls that also have slight provitamin A activity (Melendez-Martinez et al., 2004). It has about half the provitamin A activity of β-carotene (Delia et al., 2004). Dietary β-cryptoxanthin is thought to be a preventive agent against certain cancers (Binns et al., 2004) and rheumatoid arthritis (Pattison et al., 2004). Compound D could be identified as β-cryptoxanthin. The molecular ion at m/z 568.5 (≃569) corresponds to the mass calculated from the formula C40H56O2. Lutein and zeaxanthin (Fig. 3) are two carotenoids corresponding to this molecular formula. The structural difference between these two compounds is the presence of the allylic OH group in the structure of lutein. In addition, this hydroxyl group is easier to eliminate in the form of water to give a stable fragment ion. However, zeaxanthin does not have an allylic hydroxyl group, so its molecular ion is stable. Furthermore, previous studies have already shown the presence of these carotenoids in PDCO (Somé et al., 2004). "Compound E could therefore be identified as zeaxanthin. It is a

compound known as macular pigment. It is a powerful antioxidant that helps trap free radicals formed in photoreceptors, thus protecting the macula from light-induced damage and age-related macular degeneration. In addition, zeaxanthin is a colorant widely used in the cosmetics and food industries. It may also play an essential role in cancer prevention" (Nishino et al., 2002). Zeaxanthin is what gives the macular tissue in the eye its characteristic yellow colour.

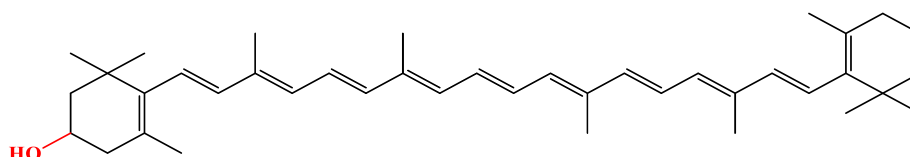
Fig. 1. HPTLC spectrum of the BF59XCIP1 variety

Fig. 2. HPTLC spectrum of spots 1 and 2 of variety BF59XCIP1

Fig. 3. Structures of zeaxanthin and lutein



α-cryptoxanthin



β-cryptoxanthin

Fig. 4. Structures of β-cryptoxanthin and α-cryptoxanthin

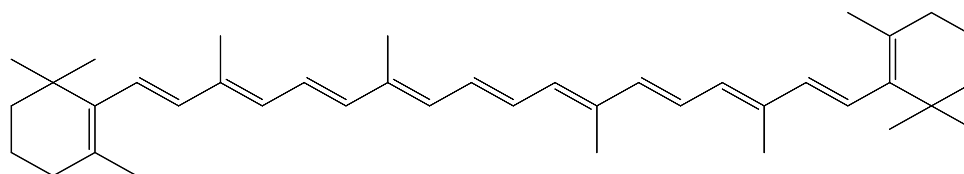


Fig. 5. Structures of β-carotene

3.3 Identification of Compounds in the BF59XCIP1 Variety by HPLC-MS/MS

"The LC-MS/MS method can be used to characterize carotenoids present as minor components in several fruits, as demonstrated by the analysis of extracts from pineapple (Physalis peruviana), kiwano (Cucumis metuliferus), mango (Mangifera indica), and pumpkin (Cucurbita pepo)" (Kimura et al., 2007). The identification of the different carotenoid isomers is based on the characteristic fragmentation pattern. Chromatographic analysis identified four peaks labeled 1, 2, 3, and 4 with retention times of 0.788, 0.94, 3.33, and 9.652 min, respectively, corresponding to four compounds (Fig. 6). These peaks correspond in LC-MS to molecular ions at m/z: 585, 551, 431, and 537, respectively. The observed sequence of chromatographic peaks reflects the decreasing polarity of the eluted compounds, with the more polar xanthophylls being eluted first. This is related to the polarity of the mobile phase used in our case. The intensities of the peaks corresponding to the molecular ions are greater than those of the fragment ions. This could be explained by the collision energy used during their fragmentation. It could even explain the absence of certain fragments of these molecular ions.

The molecular ion at m/z 585 corresponds to the mass calculated from the formula $C_{40}H_{56}O_3$. It corresponds to the most polar compound. The presence of three oxygen atoms could explain this polarity. LC-MS/MS analysis of this molecular ion gives a fragment ion at m/z 249 corresponding to $[M+H-C_{24}H_{32}O]^+$ (Fig. 7). This compound 1 is thought to be capsanthin (Fig. 8).

The molecular ion at m/z 551.200 corresponds to the mass calculated from the formula $C_{40}H_{54}O$. Carotenoids (xanthophylls) have the same chromophore. The difference in retention times between the molecular ions at m/z 551 and 585 can be explained by the difference in the number of oxygen atoms. Thus, the molecular ion at m/z 551 would have fewer oxygen atoms than the one at 585. In addition, the MS/MS spectrum of this molecular ion gives a fragment ion at m/z 459. This fragment ion corresponds to the loss of toluene C_7H_8 $[M+H-C_7H_8]^+$ (Fig. 9). This compound 2 can be identified as echinenone (Fig. 10).

From the analysis of the general and individual chromatograms, the peaks corresponding to masses 413 and 431 have the same retention

times (Fig. 11). The peak corresponding to m/z 413 could therefore correspond to the fragment ion of the molecular ion at m/z 431. This fragment ion corresponds to the loss of a water molecule [M+H-H2O]+. In addition, the fragment ion at m/z 301 corresponds to the loss of a water molecule and a C8H17 molecule [M+H-H2O-C8H17]+ (Fig. 11). This compound 3 can be identified as α-tocopherol (5,7,8-trimethyltocol), also known as vitamin E (Fig. 11).

The retention time tR=9.65 min corresponding to peak 4 of the chromatogram shows that the corresponding molecular ion is less polar than the other three ions. This molecular ion could belong to the carotenes subgroup. In mass spectrometry, this molecular ion gives a mass at m/z 537 corresponding to the mass calculated from the formula C40H56 and two fragment ions at m/z 415 [M+H-C9H15]+ and 282 [M+H-C19H27]+ (Fig. 12). The masses of lycopene, β, γ, and α-carotene correspond to this molecular formula. Compound 4 is either lycopene or one of its isomers. The presence of these compounds has already been reported in PDCOs in the literature. However, these studies show that β-carotene is the major compound (Somé et al., 2004). Compound 4 can be identified as β-carotene (Fig. 13).

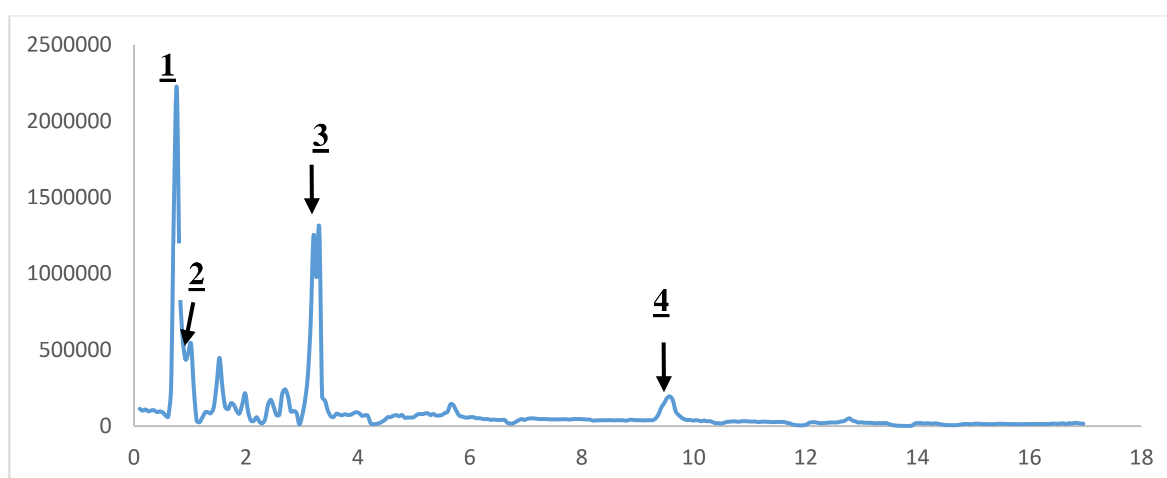


Fig. 6. Chromatogram of the extract from variety BF59XCIP1

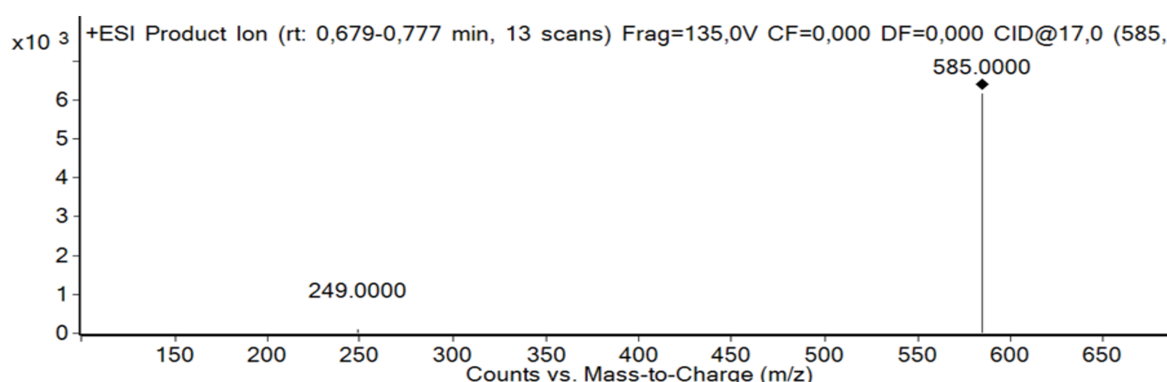


Fig. 7. SM/SM spectrum of capsanthin

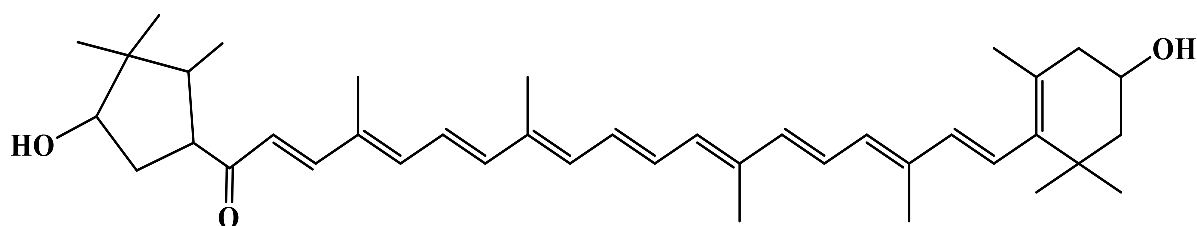


Fig. 8. Structure of capsanthin

Fig. 9. SM/SM spectrum of echinenone

Fig. 10. Structure of echinenone

Fig. 11. Mass spectrum of compound 3

Fig. 12. Structure of α-tocopherol

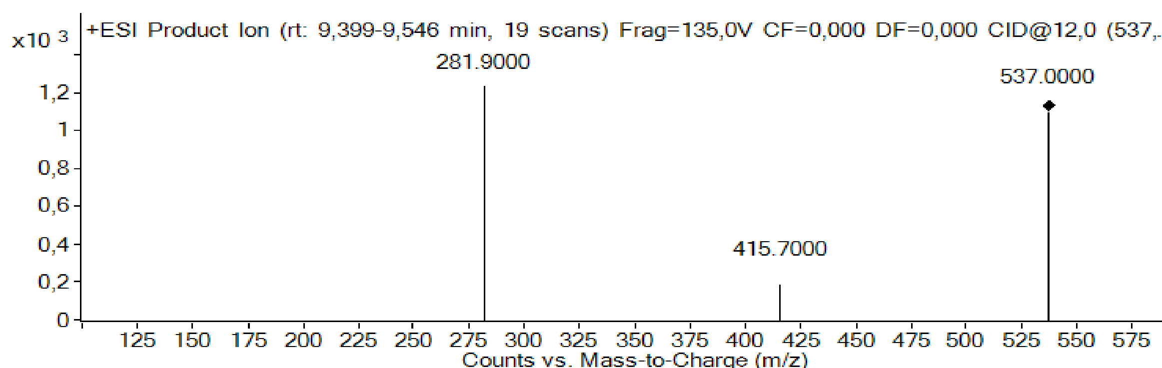


Fig. 13. SM/SM spectrum of β-carotene

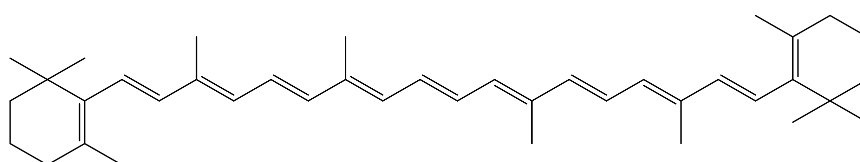


Fig. 14. Structure of β-carotene

4. CONCLUSION

Quantitative analysis of antioxidant content was performed using two of the three commonly used methods: DPPH and FRAP. Antioxidant content was found to be 0.082 and 0.183 mg ET/g of plant material using the DPPH and FRAP methods, respectively. From the qualitative analysis, four carotenoid molecules were identified: zeaxanthin, α-cryptoxanthin, α-tocopherol, and β-carotene. It appears that this variety contains vitamin E, a powerful antioxidant, and β-carotene, a molecule with provitamin A activity.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to Joseph KI-ZERBO University and the National Center for Scientific and Technological Research (CNRST) through the Institute for the Environment and Agricultural Research (INERA) for allowing me to conduct this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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