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Simultaneous Estimation of Pro-vitamin A (β-carotene), Vitamin A (Retinol), and Anthocyanins (Peonidin and Cyanidin) in Biofortified Sweet Potato Varieties Using UFLC-MS/MS

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KEYWORDS

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ABSTRACT:

Introduction: Sweet potatoes, a nutrient-dense crop are recognized as a potential functional food to mitigate an array of nutritional disorders. In India, sweet potato is considered a secondary staple crop due to its unique nutritional properties. Owing to its high consumption, food interventions like biofortification have been made to enrich the essential bioactive compounds (carotenoids and anthocyanins).

Objectives: The current study aims to develop and validate an optimized UFLC-MS/MS based analytical method for the simultaneous estimation of pro-vitamin A, vitamin A, and anthocyanins in two biofortified sweet potato varieties (*Bhu-Krishna* & *Bhu-Sona*).

Methods: A high throughput robust UFLC-MS/MS method was developed and validated for assaying enriched carotenoid content and anthocyanin content in two biofortified and one unfortified sweet potato varieties.

Results: The linearity range was 400 to 2000 ng/mL for cyanidin chloride, 850 to 3000 ng/mL for peonidin 3-glucoside, 900 to 3500 ng/mL for β-carotene and 60 to 500 ng/mL for retinol. Furthermore, the extraction process was optimized for beta carotene and anthocyanins from the sweet potato samples with an extraction efficiency of more than 85%.

Conclusion: The currently developed method was validated using ICH guidelines (Q2R1) and can be applicable in sweet potato quantification and *in vitro* digestion studies for determining the bioaccessibility, evaluate stability and access the nutrient interactions.

1. Introduction

Sweet potato (*Ipomoea batatas* L. Lam.), a member of the Convolvulaceae family, serves as a nutrient reservoir comprising carbohydrates, proteins, polyphenols, carotenoids, and other essential nutrients[1]. The major bioactive elements within them include carotenoids (such as β-carotene), phenolic acids (like caffeic acid), flavonoids (such as quercetin and myricetin), and anthocyanins (derivatives of cyanidin and peonidin) which play a crucial role in combating various serious illnesses[2]. In recent years, vitamin A deficiency (VAD) has been recognized as a major malnutrition in lower- and middle-income countries, affecting approx.30% of young children and 15% of pregnant women[3]. Numerous interventions including direct nutrient supplementation, fortification, and dietary diversification, have been implemented to address vitamin A malnutrition, but were a significant concern affecting the global population[4]. In contrast to the challenges associated with supplementation and the costs of fortification, the Government of India has adopted biofortification as an alternative strategy focusing on providing provitamin A

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through agricultural approaches. Given the widespread consumption of sweet potatoes, enhancing their carotenoids and anthocyanins content emerges as a valuable means to combat vitamin A malnutrition in affected groups.

Carotenoids are long conjugated isoprenoid molecules belonging to the class of hydrocarbons, synthesized mostly in all photosynthetic organisms[5]. Over 600 identified carotenoids, only 10%. play a significant role in promoting human health and reducing the risk of vitamin A deficiency and malnutrition[6]. Carotenoids are plant-synthesized precursors of vitamin A which are converted into vitamin A analogues i.e., retinoic acid, retinol, and retinyl esters[7]. Among these, β-carotene stands out as the most potent carotenoid with the highest conversion rates to retinol[8]. Anthocyanins are the other major group of water-soluble bioactive compounds mainly responsible for blue, red and purple colors. Chemically, anthocyanins comprise an aglycone (anthocyanidin), allied to one or more sugar moieties. Six primary anthocyanidins are cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin—comprise the majority and widely distributed. Notably, sweet potato cultivars are particularly abundant in derivatives of cyanidin and peonidin, which are widely found and characterized[9]. Anthocyanins have been shown in dietary studies to have high antioxidant and anti-mutagenic activity[10]. Recent investigations revealed that the simultaneous combination of bioactive compounds, when consumed together, can yield additive, synergistic, or antagonistic effects on biological activities. Remarkably, research has shown synergy in combinations such as cyanidin-3-glucoside with quercetin malvidin 3-glucoside, or peonidin-3-glucoside with catechin[11]. In a related study, the co-presence of all tested anthocyanins, excluding delphinidin-3-glucoside, demonstrated a significant increase in the cellular absorption of β-carotene in Caco-2 cells. This resulted in the augmentation of intracellular β-carotene content by the anthocyanins. Experiments were conducted using micelles enriched β-cryptoxanthin (5 mM) and β-carotene (5 mM) in the presence of hesperetin (250 mM), hesperidin (250 mM), naringenin (250 mM), ascorbic acid (50 mM), and iron exposed to Caco-2 cell monolayer. After 5 to 24 hours of incubation, hesperidin and hesperetin significantly increased the uptake of both β-cryptoxanthin and β-carotene by 1.7- and 1.6-folds, respectively, as determined by HPLC analysis[12].

Numerous methods have been extensively developed for studying fat-soluble vitamins using techniques like HPLC[13–15] and LCMS[16,17]. However, to the best of our knowledge, there is a dearth of concurrent analytical studies for anthocyanins and carotenoids using liquid chromatography-mass spectrometry emphasizing the need to develop a highly specific, robust, analytical method[18,19]. Therefore, the developed method will be applied to assess enriched content i.e., carotenoids and anthocyanins in biofortified sweet potato matrices. In the current study, a high throughput robust UFLC-MS/MS method was developed and validated for assaying enriched carotenoid content *i.e.,* β-carotene (Orange flesh sweet potato) and anthocyanins content *i.e.,* peonidin-3 glucoside & cyanidin -3-0- glucoside (Purple flesh sweet potato).

2. Objectives

The current study aims to develop and validate an optimized UFLC-MS/MS based analytical method for the simultaneous estimation of pro-vitamin A, vitamin A, and anthocyanins in two biofortified sweet potato varieties (*Bhu-Krishna* & *Bhu-Sona*).

3. Material & Methods

3.1 Chemicals and reagents

β-carotene (97.6%) was procured from Sigma Aldrich, Soldiers Spring Road, Laramine, USA. Retinol (95.02%) obtained from Sigma Aldrich, Soldiers Spring Road, Laramine, USA. Kuromanin chloride (cyanidin-3 (98%) was procured from Carbnio, Kukatpally, Hyderabad and Peonidin -3-O- glucoside (99.9%) was procured from Sigma Aldrich, St. Louis, USA. Solvents like Methanol (HPLC& LCMS grade) have been purchased from Biosolve Chimie, France. Ammonium formate (Analytical Grade) was obtained at Merck Life Science Limited, Mumbai, India and Ultra-pure water from the Elga water system (in-house) was used throughout the study.

3.1.1 Collection of sweet potato varieties and sample preparation

Fresh sweet potato tubers of two biofortified varieties i.e., orange flesh, purple flesh and unfortified variety (white flesh) were procured from the commercial

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markets of Bhubaneswar, Odisha, India. Approximately 2.2 kg of individual sweet potato varieties were washed, freeze-dried pulverized and sieved (60 mesh) to obtain a fine powder. The obtained fine powder was packed in airtight laminates and stored at 2-8°C until further analysis.

3.2 Preparation of master stock, working and quality control standard solutions

Master stock solutions of β- carotene, retinol, kuromanin chloride and peonidin -3-O- glucoside were separately prepared by using chloroform as a diluent for β- carotene, acidified methanol for anthocyanins, and acetonitrile for retinol at a concentration of 0.5 mg/mL. Working standard solutions of all standards were prepared using the mobile phase diluents for calibration standards, and quality control (QC) samples.

3.3 Calibration standards, and Quality control (QC) sample preparations

Calibration standards for β carotene concentrations were prepared at 950, 1200, 1750, 2500, 3150 and 3500 ng/mL, whereas for kuromanin chloride the concentrations were 400, 600 850, 1000, 1800, 2000 ng/mL, for peonidin-3-0-glucoside the concentrations were 850, 1000, 1300, 1500, 2500 and 3000 ng/mL and 60, 75, 250, 320, 450 and 500 ng/mL for retinol. Spiked QC samples for β carotene were prepared by adding the working standard solutions to a blank matrix of sweet potato extract at concentrations of 950, 1750 and 3150 ng/mL. Further, kuromanin and peonidin QCs were prepared at concentrations of 400, 1000, 1800 and 900, 1500 & 2700 ng/mL, representing QC at LQC, MQC and HQC, respectively. For retinol, QC samples were prepared at concentrations of 75, 250 & 450 ng/mL, representing QC at LQC, MQC and HQC, respectively.

3.4. Preparation of extraction samples of β carotene and anthocyanin from sweet potato cultivars.

3.4.1. Beta carotene extraction

The β-carotene content of sweet potato samples was determined using organic solvents (acetone-petroleum ether) for the extraction as described in the literature [20,21]. Briefly, 20 mL of distilled acetone was mixed with 1 g of sweet potato powder for approximately 3 minutes. The resulting mixture was then transferred to a sintered glass filter, wrapped in aluminium foil, and subjected to vacuum filtration. 250 mL separating funnel was rinsed and filled with 20 mL of distilled petroleum ether. The acetone extract was carefully added to the separating funnel containing petroleum ether, allowing it to flow gently down the funnel wall. Subsequently, 150 mL of distilled water was added to the separating funnel, allowed to flow down the funnel wall, and left undisturbed for 5–10 minutes to facilitate separation into organic and aqueous layers. The aqueous layer, containing acetone, was discarded. This process was repeated 3 to 4 times, with 100 mL of distilled water added each time to remove residual acetone. The petroleum ether extract in the separating funnel was filtered and collected through a funnel containing a small amount of anhydrous sodium sulfate into a 25 mL volumetric flask. Further, the separating funnel was rinsed with 2 mL of petroleum ether using a pipette. 1 mL of eluent was dried under a nitrogen stream and reconstituted with a mobile phase ratio (2mL) for the determination of carotenoids (β-carotene). The complete process was carried out in the dark as described.

3.4.2. Anthocyanins extraction

The extraction protocol of anthocyanins was followed with some modifications described in the literature [22]. Briefly, 2 g of pulverized samples were weighed, and 5 mL of acidified methanol was added to them. The mixture was then placed in a rotary shaker, operating at 280 RPM, for 2 hours. Subsequently, it was centrifuged at 2000 RPM for 20 minutes to separate the supernatant, and this extraction process was repeated until the solution became colourless.

Further, 10 mL of the aforementioned extract was collected in a centrifugation tube and diluted with water. The sample was then evaporated down to 1.5 mL under a nitrogen flush. The resulting sample was subjected to solid phase extraction (SPE) using a Sep-Pac C_{18} column, which was activated with 2 mL of methanol followed by 2 mL of water. The sample was loaded onto the column, washed with 4 mL of water, and then with 1 mL of 0.2% acidified methanol. The collected sample was dried using nitrogen gas, and finally, reconstituted with a 1% solution of water and methanol in a 1:1 ratio (2mL) for analysis.

3.5. Liquid chromatography and Mass spectrometric conditions

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3.5.1 Chromatographic conditions

Reverse phase- ultra-performance liquid chromatographic study (RP-UFLC) was performed using Zorbax SB C_8 column (7.5 cm × 4.6 mm, 3µ) with a mobile phase (A&B) i.e., A consisting of formic acid (0.1% v/v) and B as acetonitrile (5: 92, v/v) at a flow rate of 0.5 mL min^{-1} and injection volume of 5 µL.

3.5.2 LC-MS/MS conditions

The eluted samples from LC were further determined using Shimadzu 8030 UFLC coupled with a triple quadrupole mass spectrometer. Electrospray ionization in positive ion mode and negative ion mode for (β carotene) was used as a mass interface and multiple reaction monitoring (MRM) as the acquisition mode. The MRM transitions are carried out with a block temperature of 350 ℃ and desolvation temperature of 250 ℃ was maintained with a detector voltage and CID gas set at 1.3 kV and 230 kPa, respectively. For nebulization and collision, nitrogen (99.95%) and argon gases (99.99%) were used and collision energy of 16 for β-carotene, -14 for retinol, -24 for peonidin-3-0-glucoside & -22 for kuromanin chloride were selected for better ionization. **(Table 1a & b)**.

Analyte	Dwell time Ms^{-1}	Q1 pre bias V	Q3 pre bias V	Collision energy eV	Retention time min
β-carotene	100	28	26	16	1.76
Retinol	100	-21	-17	-14	3.4
Peonidin-3-glucoside	100	-20	-14	-24	1.79
Kuromanin Chloride	100	-20	-29	-22	1.73

Table 1a: The optimized LC-MS/MS detection conditions for selected analytes

Analyte	Molecular weight g mol^{-1}	Ion mode	Precursor ion m/z	Product Ion
β-carotene	536.4	-ve	535.1	193
Retinol	286.4	+ve	269.2	95.1
Peonidin-3-glucoside	463.4	+ve	463.3	301.1
Kuromanin Chloride	484.8	+ve	448.9	286.9

Table 1b: The optimized LC-MS/MS detection conditions for selected analytes

The precursor ions(m/z) for β-carotene, retinol, peonidin-3-0-glucoside and kuromanin chloride were 535.1 , 269.2, 463.3 and 448.9 respectively and the product ions (m/z) were 193.0, 95.1, 301.1 and 286.9 **(Figure 1)**. The data acquisition was carried out using Lab solution software (Shimadzu Ltd., Mumbai, India).

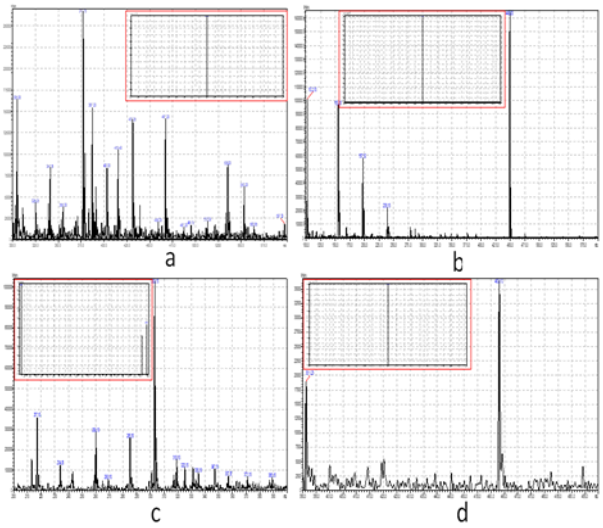

Figure 1. Mass and MRM spectra (image inside the box)f (a) β carotene in negative detection mode, (b) omanin chloride, (c) Retinol and (d) Peonidin 3-glucoside in positive detection mode.

3.6 Method performance and validation

Thr current method was calibrated and validated ing the ICH Q2 R1 guidelines[23]. The standard curves were detailed as follows: β-carotene (900-3500 ng/mL), Retinol (60-500 ng/mL), kuromanin chloride (400-2000 ng/mL) and Peonidin 3-glucoside (850-3000 ng/mL).

3.6.1. LOD and LOQ

The Limit of Detection (LOD) represents the minimum detectable amount of analyte in a sample, calculated as LOD = 3.3σ/S, and the Limit of Quantification (LOQ) signifies the minimum amount of analyte that can be quantitatively determined in a sample with adequate precision and accuracy. It is computed as 10σ/S. The σ

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denotes the standard deviation and S represents the slope of the calibration curve

3.6.2. Calibration and Linearity

Six calibration standards were prepared freshly for the calibration curve using a linear regression model with weighted $1/conc^2$.

3.6.3. Accuracy & Precision

Six replicates of QC samples of three concentrations levels of accuracy and precision batch were injected. 3 replicates were carried out within the same day, representing the intra-day precision and repeated three consecutive days representing the inter-day precision. Using the regression equation from the proposed methods, the expected concentration was obtained.

3.6.4. Recovery

The recovery was evaluated by analyzing six replicates of QC samples, including low, mid, and high concentrations, alongside extracted and screened blank matrix samples. Six replicates were processed for all the prepared quality control standards. The recovery of analytes was determined by comparing the results of the extracted samples with recovery standards.

4. Results & Discussion

4.1 Chromatographic separation and analysis

Chromatograms for β-carotene, retinol, kuromanin chloride and peonidin 3-glucoside were obtained and illustrated in **Figure 2**. The noninterference of analytes in the blank chromatogram signified the specificity of the optimized method (Figure 3). The calibration curve was found to be linear for all the analytes i.e., β-carotene (900-3500 ng/mL), retinol (60-500 ng/mL), kuromanin chloride (400-2000 ng/mL) and peonidin 3-glucoside (850-3000 ng/mL) indicating the method's adequate reliability and reproducibility. The retention periods of β-carotene, kuromanin chloride, peonidin-3-0-glucoside and retinol were found to be 1.76, 1.73, 1.79 and 3.40 min, and the analyte peaks obtained were of acceptable form and were well resolved. Analytes were further fragmented using MRM and ESI MS/MS. The mass transition ions for the respective analytes are as follows, β-carotene (535.1, 193.0), Retinol (269.2, 95.1), kuromanin chloride (463.3, 301.1) and peonidin 3-glucoside (448.9, 286.9) (precursor ion and product ion, *m/z*) and respectively in **Figure 1.**

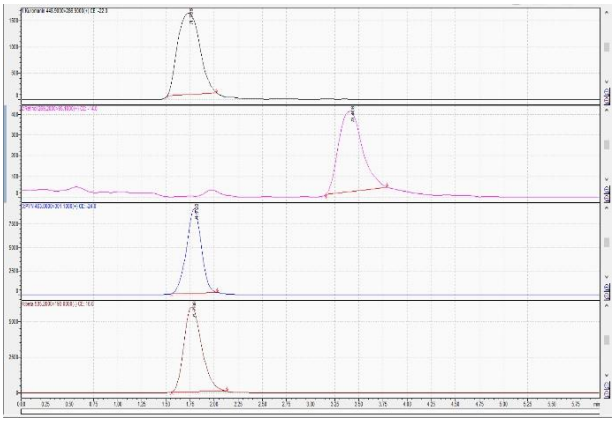

Figure 2. LCMS-MS chromatograms of the selected analytes i.e., Kuromanin chloride, Retinol, Peonidin-3glucoside and β-carotene

4.2 Method Validation

4.2.1 Selectivity & Specificity

The selectivity of the method was determined by analysing eight independent blank sources. The blank chromatogram (**Figure 3)** played a pivotal role as a crucial control, ensuring the reliability of the developed method and the specificity of the developed method.

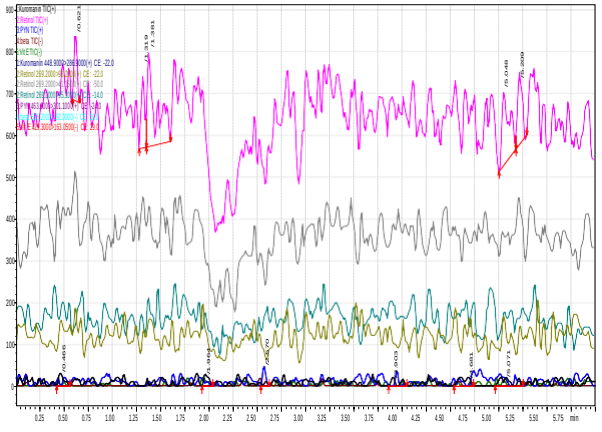

Figure 3. Total ion chromatogram of Blank matrix

4.2.2 Sensitivity

Sensitivity was determined based on signal-to-noise (S/N) ratio with six replicates (n=6) of mixed standard (LOQ level). There were no interferences with the blank, and neither the matrices were affected. The mean values

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were found to be within the S/N 3:1 acceptability requirement.

4.2.3 Calibration and linearity

The correlation coefficients (R^2) of standard β-carotene, Retinol, Peonidin-3 glucoside and Kuromanin chloride were not less than 0.99, showing a good linear correlation (R^2 >0.99). The calibration curves were linear in the range of β-carotene (900-3500 ng/mL), retinol (60-500 ng/mL), kuromanin chloride (400-2000 ng/mL) and peonidin 3-glucoside (850-3000 ng/mL). The details are illustrated in **Table 2.**

Table 2: Linear ranges, regression equation, coefficient of determination (R^2), LOD, and LOQ of calibration curves for selected analytes**.**

Parameters	B-carotene	Retinol	Kuromanin Chloride	Peonidin-3 glucoside
Range(ng/mL)	950-3500	60-500	400-2000	850-3000
Regression(y=mx+c)	y = 370.38x + 26128	y = 242.58x + 629.28	y = 108.33x - 4757.3	y = 337.79x - 20087
R^2	0.9961	0.9997	0.9989	0.9969
LOD (ng/mL)	300	18	130	270
LOQ (ng/mL)	900	60	1400	810

4.2.4 Accuracy and precision

The intra-day and inter-day accuracy and precision were determined at all QC levels including a lower limit of quantification (LOQ) in a blank sweet potato matrix (n=18). The extraction efficiency and reproducibility are established by the recovery studies. which was determined at low, medium and high QC levels of six replicates for the recovery of β-carotene, Retinol, Peonidin-3 glucoside and Kuromanin chloride from selected sweet potato matrix resulting in the overall recovery of 75.40 to 86.60% are presented in **Table 3 a & b.**

Table 3a: Percentage recovery, intra, and inter-day accuracy and precision analysis of analytes in spiked matrix

Analyte	QC samples	Analyte conc (ng/mL)	Mean conc (ng/mL) (n=3)
	LQC	900	710±6.5
Kuromanin	MQC	1500	1210±6
	HQC	2700	2200±7.8
Kuromanin	LQC	400	320±3.1
	MQC	1000	810±5.2
β-carotene	HQC	1800	1530±5.9
	LQC	950	720±6.5
β-carotene	MQC	1750	1370±8.5
	HQC	3150	2500±7.9
Retinol	LQC	75	59±0.8
	MQC	250	205±1.9
Retinol	HQC	450	390±2.5

Table 3b: Percentage recovery, intra, and inter-day accuracy and precision analysis of analytes in spiked matrix

	Intraday		Interday	
Analyte	**Accuracy (%) ±sd**	**Precision**	**Accuracy (%) ±sd**	**Precision**
Peonidin				
	78.8±**0.37**	0.91	78.1±**0.41**	0.95
	80.6±**0.20**	0.49	79.9±**0.24**	0.53
	81.4±**0.2**	0.35	81.1±**0.39**	0.39

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Kuromanin	80±**0.21**	0.96	79.8±**1.2**	0.99
	81±**0.2**	0.64	80.9±**0.81**	0.68
	85±**0.04**	0.38	84.3±**0.41**	0.41
β-carotene	75.7±**0.43**	0.9	75.1±**1.3**	0.94
	78.2±**0.38**	0.62	77.9±**0.68**	0.68
	79.3±**0.31**	0.31	78.9±**0.35**	0.34
	78.6±**0.32**	1.3	78.1±**0.98**	1.39
Retinol	82±**0.24**	0.92	79.8±**0.67**	0.98
	86.6±**0.05**	0.64	86.2±**0.31**	0.67

4.3 Evaluation of selected bioactive compounds in White flesh(unfortified), orange flesh (Bhu-sona) and purple flesh (Bhu Krishna) sweet potato varieties of India

The orange color of these sweet potatoes is indicative of high carotenoid content, particularly β-carotene, which is crucial for human health. In this current analysis, significant variations in β-carotene content were observed among the different sweet potato varieties. The orange flesh sweet potato (Bhu-sona) exhibited the highest concentration of β-carotene, with a remarkable 8 mg/100g dry basis **(Table 4).** Moreover, very few studies have assessed the beta-carotene levels (alone) in sweet potatoes. For instance, Van Jaarsveld *et.al.,* estimated the beta-carotene concentration of medium-sized Orange Fleshed Sweet Potatoes (OFSP) which ranged between 132 and 194 µg/g by HPLC analysis[24]. In another study, Sheikh Nazrul *et al*., estimated the beta carotene levels in three orange flesh types grown in Bangladesh. The authors demonstrated that Kamala Sundari (BARI SP-2) showed the highest carotenoid concentration, measuring 4500 µg/100 g via HPLC analysis[20]. In our study, the carotenoid concentration from orange-flesh sweet potato was 8mg/100g which is comparatively high than the previously reported. Further, the purple flesh sweet potato (Bhu Krishna) displayed a substantial but lower β-carotene content of 2.98 mg/100g, while the white flesh sweet potato registered the lowest level at 0.2 mg/100g. In one of the studies, Suhair *et.al*., estimated carotenoid content in orange, yellow and purple sweet potato varieties of Malaysian origin. Among these three varieties, the purple flesh exhibited 113.86 µg/g of β-carotene compared with orange flesh (376.03 µg/g). These outcomes align with previous research indicating that orange-fleshed sweet potatoes are rich sources of β-carotene, a precursor to vitamin A[25].

Contrastingly, anthocyanins were undetected in both orange and white flesh sweet potatoes **(Table 4).** The absence of anthocyanins in the orange and white flesh of sweet potatoes is consistent with their characteristic colors, as anthocyanins are responsible for the purple, red, and blue hues in many fruits and vegetables. The presence of anthocyanins in purple-fleshed sweet potatoes not only contributes to their vibrant color but also enhances their nutritional profile due to the antioxidant properties associated with these compounds[26]. The analysis of anthocyanins, specifically peonidin 3-glucoside and kuromanin chloride, revealed an intriguing pattern. Purple flesh sweet potatoes (Bhu Krishna) displayed the highest anthocyanin content with 37.8 mg/100g dry basis, underscoring their potential as a valuable source of these antioxidant compounds. For instance, in a study, Yeong *et.al.,* assessed the anthocyanin content using (UHPLC-(ESI)-q-tof-MS) in five purple flesh sweet potato varieties, (Sinjami, Jami, Danjami, Yeonjami, and Borami) grown and harvested in Korea. Their study displayed 943 to 3962 mg/kg dry weight of cyanidin-3-glucoside[27]. In another study, Vishnu *et.al.,* identified total anthocyanin content with 43.4 mg/100g peonidin 3-o-glucoside using HR-ESI-MS in purple flesh sweet potato (Bhu Krishna) variety[28].

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Table 4: Anthocyanin and carotenoid (β- carotene) content in selected sweet potato genotypes

	Orange Flesh Sweet Potato (Bhu Sona)	
Class of compounds	**Compound**	**mg/100g dw**
Anthocyanins	Cyanidin-3 0 glucoside Kuromanin chloride	-ND- -ND-
Total Anthocyanins		-ND-
Provitamin A	β- carotene	8.23
	Purple Flesh Sweet Potato (Bhu Krishna)	
Class of compounds	**Compound**	**mg/100g dw**
Anthocyanins	Cyanidin-3 0 glucoside Kuromanin chloride	26 11.8
Total Anthocyanins		37.8*
Provitamin A	β- carotene	2.98
	Normal Flesh Sweet Potato	
Class of compounds	**Compound**	**mg/100g dw**
Anthocyanins	Cyanidin-3 0 glucoside Kuromanin chloride	-ND- -ND-
Total Anthocyanins		-ND-
Provitamin A	β- carotene	0.2

*****Expressed as mg cyanidin-3-glucoside/100 g Dry weight (dw); ND- Not detected (Less than LOD values)

5. Conclusion

The developed method is robust and specific for estimating both β-carotene and anthocyanin simultaneously. To date, very limited concurrent methods are been developed in estimating both beta carotene and anthocyanins in food matrices The currently developed method provides insights to access the enriched content (β-carotene and anthocyanins) in biofortified sweet potato matrices and could be useful in bioaccessibility studies in different food matrices.

Conflict of interest -

The authors declare no conflict of interest.

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