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Isolation and LC-MS (APCI)$^{+}$ analysis of carotenoids from red pepper: Influence of capsaicin as a major constituent of capsicum on physicochemical properties of carotenoids solubilized micelles and their bioaccessibility

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Abstract
HPLC separation of carotenoids eluted on C30 column as found in the following order; epoxy-carotenoids, hydroxy-carotenoids, and hydrocarbon carotenoids. Capsanthin and capsorubin were detected as characteristic pigments in the red pepper. The optimized HPLC condition separated and quantified carotenoids. The mass spectra of each carotenoid confirm by its characteristic ions**.** The mass spectra signals for epoxy-carotenoids are characterized by [M + H]+; *m/z* 601.3], [M + H]+; *m/z* 584.6] for capsanthin, loss of the water (lutein) or no loss of water (zeaxanthin) moiety from the respective quasimolecular ions representing the lutein backbone ([M + H - H2O]+; *m/z* 551) and β-carotene [M + H]+; *m/z* 536.8] The capsanthin rich fraction eluted on silica column yielded 92 ± 3% purity. The APCI^{+ve} detector parameters adjusted suitably to give ion signal intensity for identification of peaks using respective standards. Red pepper digested sample fractioned larger particle-sized micelles than only carotenoids and carotenoids+capsaicin solubilized micelles. The PDI of carotenoids+capsaicin indicated the narrow distribution of particle size and stable zeta potential than other groups. The viscosity of carotenoids+capsaicin was lower than red pepper, and only carotenoids digested derived micelles. Smaller particle size, stable zeta potential, uniformly dispersed, and less viscous micelles found suitable for improving bioaccessibility/bioavailability of carotenoids at the gastrointestinal region. Therefore, increased bioaccessibility of carotenoids observed in capsaicin treatments than the sample without capsaicin. These results suggested that co-consumption of phytoconstituents with carotenoid-rich diet affected bioavailability of carotenoids. Also, understanding its regulatory mechanism at the intestinal level is important for the enteral nutrition of carotenoids.

Keywords: capsicum, carotenoids, capsaicin, bioaccessibility, micelles

Introduction

Carotenoids are natural compounds that exhibit yellow, orange, and red colors found in photosynthesizing organisms such as green plants and their parts, algae, seaweeds, and marine bacteria (Britton *et al*. 2009) [6]. Epidemiological and clinical studies have demonstrated that dietary consumption of carotenoids rich fruits and vegetables reduces the risk of cardiovascular diseases, age-related degenerative diseases, and certain cancers in humans (Krinsk *et al*. 2003) [16]. In general, carotenoids classified as hydrocarbon carotenoids composed only of carbon and hydrogen, such as carotenes and lycopene, and oxygenated xanthophylls carotenoids consists of epoxy-, hydroxy-, methoxy-, and keto- functional groups (Britton *et al*. 2007) [8]. Carotenoids cannot be synthesized by animals and humans and need to obtain from plants source as a diet. Several findings suggested that carotenoids are involved in preventing oxidative stress and inflammatory responses, which are the leading cause of several chronic diseases. Carotenoids contain a conjugated backbone composed of isoprene units, usually inverted at the center of the molecule, imparting symmetry. Changes in the geometrical configuration of the double bonds result in the existence of many cis and trans-isomers. Among carotenoids, capsanthin is the most crucial carotenoid found in Capsicum annuum (red pepper) and showing antioxidant and immune-enhancing activities (Rhim *et al*. 2011; Topuz*et al*. 2011) [28]. Red pepper comprises various carotenoids, such as capsanthin, capsorubin, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, antheraxanthin, violaxanthin, phytofluene, and steroids, including capsicoside distinctively. One of the chief constituents is capsaicin, lipophilic antioxidants improving peripheral circulation and degenerative diseases (Mohd *et al*. 2019) [23]. Capsanthin and capsorubin are characteristic pigments found in the genus capsicum, besides other carotenoids such as β-cryptoxanthin, zeaxanthin, and β-carotene may also contribute to the red pepper fruit color. Capsaicin is the main pungency compound found in red pepper (Othman *et al*., 2011) [26]. As a spicy by-product, this ingredient is used to treat pain and inflammation-associated health problems (Shakhidoyatov *et al*. 2001; Kim *et al*. 2009) [32, 14]. Red pepper is widely used as a vegetable and food additive, as this spice fruit is considered a good source of capsanthin. Capsanthin is the primary carotenoid found in paprika and is present in an acylated form with fatty acids (Biacs *et al*. 1989) [3]. Oshima *et al*. (1997) [25] revealed that dietary capsanthin is absorbed into the body and distributed to HDL in more significant amounts than to LDL compared to hydrocarbon carotenoids. Xanthophylls can act as antioxidants against free radicals and ROS in lipoproteins (Lim *et al*. 1992) [20]. Further, they seem to be involved in the primary defense mechanism against oxidative damage/stress in the lipoproteins. Also, xanthophylls' are attributed to affect lipid metabolism and maintain favorable lipid profiles in blood. Though the possible biological function of capsanthin is known,

however, its bioaccessibility and bioavailability at the gastrointestinal level are not well detailed. Therefore, it is reasonable to explore red pepper or capsanthin's ingestion for the various health benefits. Against this background, the current study aimed to examine analytical methods for isolation and analysis of capsanthin as a nutraceutical compound. This study also evaluated influence of capsaicin or red pepper supplementation on micellization and carotenoid bioaccessibility by simulated digestion.

Materials and methods

Chemicals: Red pepper was procured from local market. β-carotene, lutein, capsanthin, butylated hydroxytoluene, and capsaicin were procured form Sigma Chemicals (St. Louis, MO USA). Ammonium acetate, sodium bicarbonate, oleic acid, 2-mono-acyl-glycerol, phosphatidylcholine, bile salt, pancreatin, pepsin, and sodium taurocholate were purchased from Hi-Media Chemical Laboratories (Mumbai, India). All other chemicals and solvents of analytical and HPLC grades were purchased from Sisco Research Laboratories (Mumbai, India) unless otherwise mentioned.

Extraction of capsanthin from red pepper

The red pepper was purchased from a local supermarket. A known quantity of red pepper (*Capsicum annum* L.) was taken, grounded with an addition of 0.1% butylated hydroxytoluene (BHT) and 2 g of sodium sulfate. Followed by, a blended sample (20 g) was extracted for carotenoids in a clean conical flask using ice-cold acetone (100 mL) until the sample became colorless. This pooled extract was filtered using Whatman no. 1 filter paper and subjected for phase separation. In brief, the acetone crude extract was mixed and shaken well with n-hexane, then the upper hexane layer was collected in a separating funnel. This process was repeated by washing with water till the extract became colorless (Figure 1). The pooled hexane layers were collected and evaporated to dryness under nitrogen gas. The dried carotenoid residue re-dissolved in a known volume of mobile phase (acetonitrile/methanol/dichloromethane, 60:20:20 v/v/v) and analyzed by HPLC. Fractions rich in capsanthin were collected and evaporated to dry under N2 gas and subjected to silica gel column chromatography, and sequentially eluted with a solvent mixture of dichloromethane with methanol (1:1, v/v). The fractionated solvent extract was dried using N2 gas to obtain purified capsanthin (Aizawa & Inakuma, 2007). The characterization of all the carotenoids and purity of isolated capsanthin was determined by LC-MS (APCI)+ve techniques.

HPLC analysis

The concentration of carotenoids in a red pepper sample was determined by HPLC (DAD, Shimadzu, Japan). HPLC system consists of an LC-10AD pump with SPD10A UV–Visible absorbance detector (Shimadzu, Kyoto, Japan) and a personal computer equipped with Ezchrome chromatography data system software (Scientific Inc., Pleasanton, CA). Carotenoids were separated and quantified on the C30 column (5 µm; 250 × 4.6 mm; Princeton, Cranbury, USA) separately. Acetonitrile/methanol/ dichloromethane (60:20:20, v/v/v) containing 0.1 % ammonium acetate was used as a mobile phase. An isocratic analysis was performed at a flow rate of 1 mL min−1 at 450 nm. The carotenoids quantified from their peak area using respective standard curves. The peak identities and λ-max of each carotenoid were confirmed by their retention time and characteristic spectra of standard chromatograms recorded under similar conditions (Lakshminarayana *et al*. 2005) [18].

LC-MS analysis

LC-MS analysis was performed on a Waters 2996 modular HPLC system with autosampler, gradient pump, thermoregulator, and diode array detector (DAD) coupled to a Q-TOF Ultima (UK) mass spectrometer. The $APCI^{+ve}$ source heated at 130 °C, and a probe was kept at 500 °C. The corona voltage was optimized to 5 kV, HV lens to 0.5 kV, and cone voltage to 30 V. Nitrogen was used as a sheath, and the flow rate of drying gas was 100 28 and 300 L/h respectively. Mass spectra of carotenoids were acquired with an m/z 0–1,200 scan range, and the UV absorption was recorded at 450 nm using a DAD. The MS identities of each carotenoid in the sample were confirmed using respective reference standards. The delay time from PDA to the mass spectrometer was observed to be 0.10 min. Data were processed with Mass Lynx 3.2 software. For LC-MS analysis, the mobile phase and condition mentioned in the HPLC analysis section without 0.1 % ammonium acetate. All the isolation, extraction, and analysis of samples were done under dim yellow light to prevent photooxidation at 4 °C (Schweiggert *et al*. 2005) [30].

Bioaccessibility of carotenoids

Red pepper/capsicum sample or only carotenoids (β-carotene: lutein: capsanthin/100 µM each) or capsaicin plus carotenoids samples were added separately and homogenized in 25 mL of Hank's balanced salts solution (HBSS) containing 150 µmol/L BHT and 6% of fat (lecithin: olive oil, 1:1 ratio) by weight in the meal. This carotenoid meal mixed by stirring with a glass rod and homogenized for 30 s at 50 °C in micro-oven. This procedure was repeated three times at one-minute intervals. The microwaved carotenoid meal was lubricated by adding 0.5% α-amylase and allowed for 2-3 mins to start oral digestion. Further, the digestion process was carried out by acidifying the homogenized samples attained pH-2 using porcine pepsin (0.04 g/mL in 0.1 mmol/L HCl) followed by incubation (37 °C) in an orbital shaking water bath for 1 h at 100 rpm to complete stomach phase of exposure. Then, to mimic the intestinal phase, the pH increased to 5.3, followed by the addition of bile extract and pancreatin (9 mL containing 2 mg/mL pancreatin and 12 mg/mL bile extract in 100 mmol/L sodium bicarbonate solution). Final concentrations of pancreatic and bile extract in the reaction mixture were 0.4 and 2.4 mg/mL, respectively glycodeoxycholate (0.8 mmol/L), taurodeoxycholate (0.45 mmol/L), taurocholate (0.75 mmol/L), porcine pancreatin (0.08 g mL/L), and cholesterol esterase (1 U/ mL). Further, the pH of the sample increased to 7.4 using NaOH. After that, the obtain digesta (approximately 25 mL) was further incubated at 37 oC for 2 h in an orbital shaking water bath at 250 rpm to complete the intestinal phase digestion process. Followed by, digesta was ultra-centrifuged (194000 g for 60 min) and collected the aqueous phase of the micellar fraction. The resulting supernatant was filter-sterilized using a syringe and surfactant-free cellulose acetate filter (0.2 µm) to remove microcrystalline aggregates. This aqueous fraction considers as a source of carotenoids' rich micellar fraction. All the preparation and manipulation with the carotenoid samples were performed under the subdued

yellow light to minimize the carotenoid photo-decomposition. The carotenoid content in each treatment's micellar fractions was quantified using HPLC (Lakshminarayana *et al*. 2006). Likewise, the above procedure was done using two and three different spices treated at an equimolar ratio (not to exceed 1%) to understand the synergistic influence on carotenoids bioaccessibility and bioavailability. The content of capsaicin and carotenoids in red pepper was analyzed and quantified by HPLC as per the standard procedure (Othman, *et al*. 2011; Lakshminarayana *et al*. 2005) [26, 18].

Physicochemical properties of mixed micelles

Particle size, zeta potential, and PDI of micellar fraction obtain through simulated digestion of red pepper or purified carotenoids (100 µM β-carotene + 100 µM lutein + 100 µM capsanthin) or 1% capsaicin plus carotenoids (n=3) were measured using Zetasizer Nano ZS (Malvern, South borough MA) with measuring range from 0.3 nm to 5 µm. A refractive index for oil of 1.47 and water of 1.33 was used for the calculation of particle size. Also, the viscosity of the micellar samples was measured at 37 °C. 0.1 to 100 s^{-1} (up curve) and 100 to 0.1 s−1 (down curve) range was used to monitor shear rate using DHR-S rheometer (TA Instruments, USA) (Verrijssen *et al* 2014; Cervantes-Paz *et al* 2016).

Statistical analysis

Data tested for homogeneity of variances by the Bartlett test. When homogenous variances were confirmed, the data were further analyzed using ANOVA (Assistant software, v.7.7). Tukey's test analyzed the differences between experimental and control groups. The differences between the experimental samples were considered significant levels at $p < 0.05$.

Table 1: Physical properties of micelles obtain after simulated digestion of red pepper, and standard micelles.

Samples	Particle size (nm)	Viscosity (mPa.s)	PDI	Zeta potential (mV)
¶**Red pepper**	283.0 ± 12[a]	132.15± 10.20 [a]	0.42 ± 0.02[a]	-12.13 ± 0.7[a]
***Carotenoids**	170.5± 23[b]	82.3 ± 8.21[b]	0.62 ± 0.10[b]	-18.30 ± 2.11[b]
¥**Carotenoids +Capsaicin**	223.1± 18[c]	111.35 ± 5.02[a]	0.90 ± 0.72[c]	-42.2 ± 4.21[c]

¶Carotenoids solubilised micelles obtain through simulated digestion.
***Carotenoids (β-carotene +LUT+CAPS) solubilised micelles prepared using purified carotenoid.
¥Carotenoids solubilised micelles prepared with capsaicin standard.

Values are mean of ± SD of three samples. Values not sharing common superscript letters within a column are statistically significant ($p > 0.05$).

Table 2: Influence of capsaicin as a major spice active constituents on bioaccessability of carotenoids* under simulated digestion condition.

Experimental groups	Bioaccessability of carotenoids (µM)		
	β-Carotene	Lutein	Capsanthin
Red pepper	2.13 ± 0.5[a]	6.05 ±1.32[a]	9.10 ± 0.12[a]
¶Carotenoids	4.85 ± 0.78[b]	10.21 ± 1.24[b]	15.32 ± 0.45[b]
¶Carotenoids + ¥Capsaicin	7.35 ± 1.23[c]	13.02 ± 0.78[c]	19.10 ± 1.33[c]

*Values are mean ± SD (n=5), Values not sharing a common superscript within a column are statistically significant (p> 0.05). ¶Carotenoids: β-Carotene+Lutein+Capsanthin. Each carotenoid concentration has chosen 100 µM. ¥Capsaicin concentration: 1%.

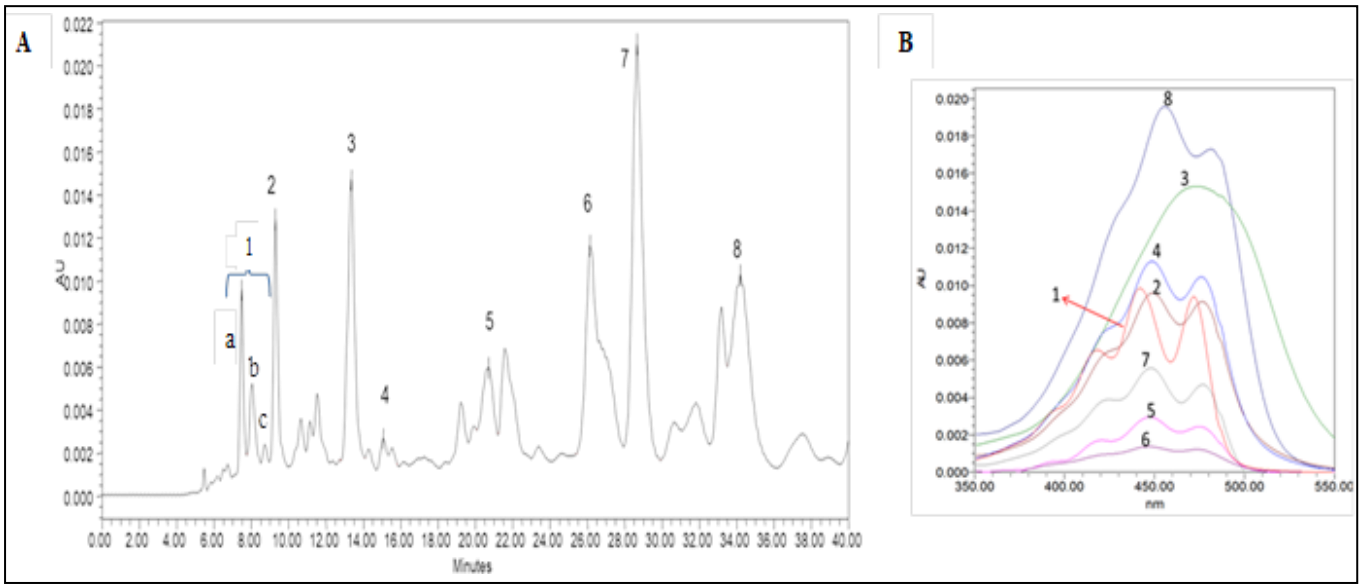

Fig 1: Typical HPLC profile (A) and Uv-vis spectra of carotenoids isolated from red pepper sample. The order of major carotenoids eluted as follows: 1. Epoxy-carotenoids (a. violaxanthin, b. neoxanthin c. capsorubin), 2. Lutein, 3. Capsanthin, 4. Zeaxanthin, 5. Phytofluene, 6. β-Cryptoxanthin, 7. α- Carotene, and 8. β-Carotene.

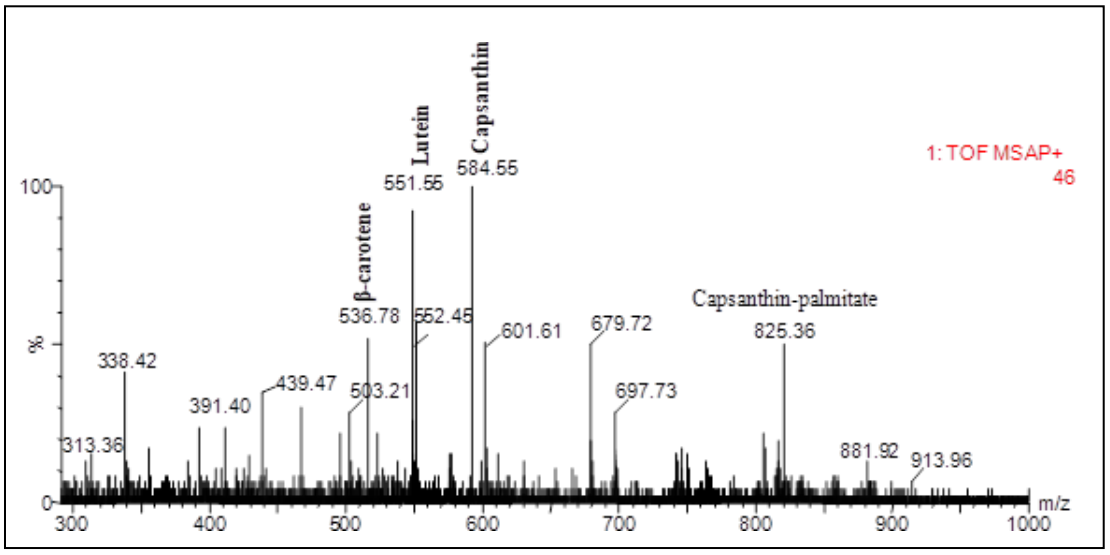

Fig 2: Typical MS $(APCI)^{+}$ profile of red pepper analysed by direct inclusion of crude extract of carotenoids.

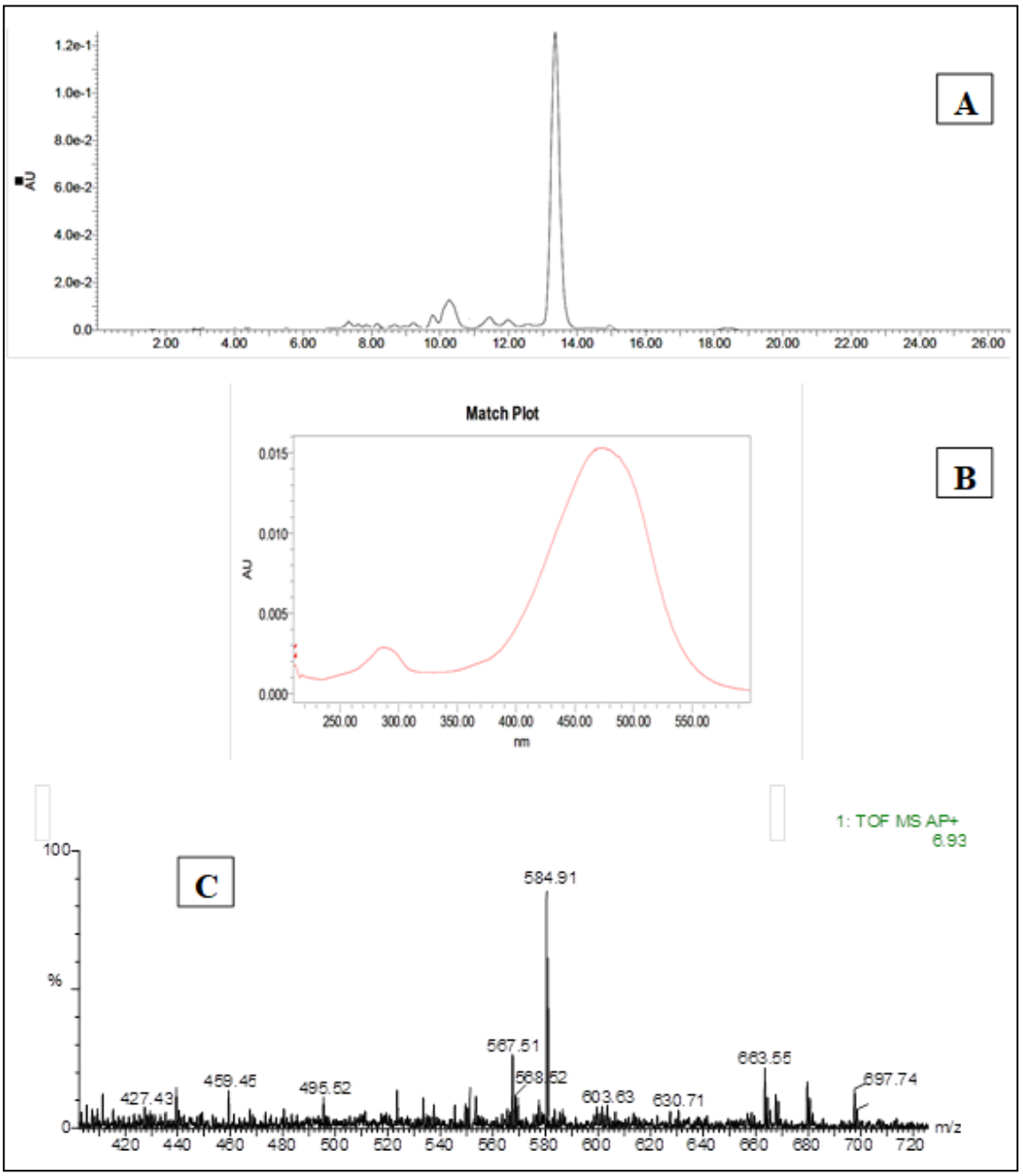

Fig 3: Typical LC-MS $(APCI)^{+ve}$ profile of purified capsanthin isolated from red pepper. (A) LC profile, (B) Characteristic Uv-vis spectra, (C) Mass spectra.

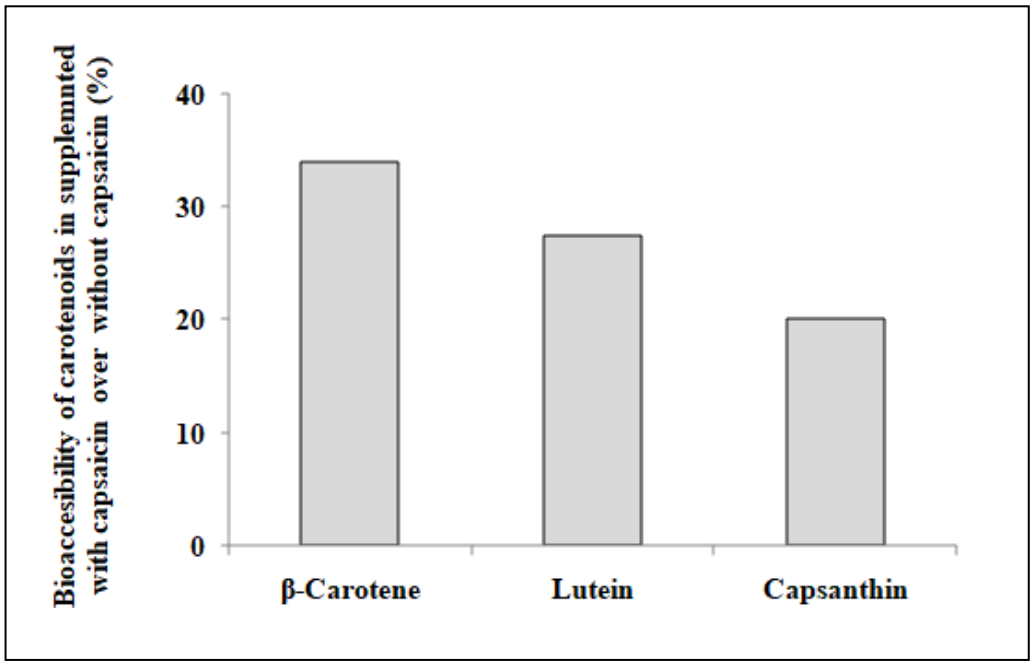

Fig 4: Percent difference of carotenoids bioaccessibility after simulated digestion of carotenoids sample with capsaicin over without capsaicin

Result and discussion

Major pigments in samples used in the present study consist of three classes of carotenoids. In the order of chromatographic elution on a C30 column, epoxy-carotenoids eluted initially, followed by hydroxy-carotenoids and hydrocarbon carotenoids. In red pepper, capsanthin and capsorubin are characteristic of the genus capsicum, besides other carotenoids such as β-cryptoxanthin, zeaxanthin, and β-carotene may also contribute to the pepper fruit color. During pepper fruit ripening, selective xanthophyll pigment esterified with fatty acids and increases versus a gradual decrease of free pigments and is directly linked to the transformation of chromoplasts. In the fruit's ripened stage, a balance between free and esterified carotenoids exists. Besides the variations in their levels, it seems largely dependent on the variety and indices of the fruit's physiological maturity: Capsicum annum and Capsicum frutescence primarily used as coloring and flavoring food products. The capsicum/spice is believed to be a rich source of keto-carotenoids; however, it may exist some time in esters form (the content of carotenoid esters varies depending on the ripening stage). The detectable xanthophylls and hydrocarbon carotenoids peaks were confirmed and quantified based on their retention time, characteristic UV-vis and mass spectra, and peak areas of reference standards eluted under isocratic conditions. The concentration of selected carotenoids, such as β-carotene, lutein, and capsanthin, were shown 7990, 7150, 9352 area/p moles under the standardized condition, respectively. Under the standardized condition, we separated and quantified (µg/100g of the edible portion) carotenoids is as follows: violaxanthin (650), lutein (1310), zeaxanthin (458), capsanthin (11025), β-cryptoxanthin (235), α-carotene (178), β-carotene (1987), phytoene (448) and phytofluene (254), respectively (Kimura and Rodriguez-Amaya. 2002; Rodríguez-Rodríguez *et al.* 2020) separated these carotenoids by HPLC under gradient condition, which required more than 50 min to separate them. In contrast, this study reports that all the major carotenoids separated within 40 min under isocratic conditions (Figure 1). Further, carotenoids' content may vary due to species variation, part of the plant, degree of maturity, stage of harvest, cultivation, and pre-and postharvest handling practices (Arathi *et al.*, 2017). Also, the variation of epoxy- and hydroxy-carotenoids within the same parts may differ due to transformation in the end group of one of these carotenoids may be responsible for forming the allenic end group. The LC-MS spectra of carotenoids, separated from the respective samples, further confirmed their identity (Figure 2). The mass spectra signals are characterized by [M + H]+; *m/z* 601.3] for neoxanthin/violaxanthin, [M + H]+; *m/z* 584.6] for capsanthin, loss of the water (lutein or no loss of water (zeaxanthin) moiety from the respective quasi molecular ions, which is found in each MS, representing the lutein backbone ([M + H - H2O]+; *m/z* 551) and carotene showed the mass peak at 536.8, respectively. Breithaupt *et al.* (2003) [5] and Wingerath *et al.* (2006) [37] reported a similar mass spectra pattern for lutein and its isomers using matrix-assisted laser desorption/ionization mass spectrometry and APCI-MS. Furthermore, each carotenoid's mass spectra eluted from the samples were compared with the respective standards' mass spectra. The purity of column eluted individual carotenoids on silica column was ranged between 92 ± 3 % for capsanthin (Figure 3). The absorption maxima (λ_{max}) for carotenoids isolated from red pepper was found to be comparable with the reported values in the literature (Britton, 1995) [7] (Figure 3). This study aimed to optimize the analytical conditions for enumeration of carotenoids using HPLC and LC-MS $(APCI)^{+ve}$. The APCI detector parameters were adjusted suitably to give ion signal intensity for the identification of peaks. The solvents in the mobile phase, such as acetonitrile (60 %), methanol (20 %), and DCM (20 %), appeared not to affect the formation of positive ions. PDA measured an integrated chromatographic peak area at 450 nm. HPLC-MS was very sensitive, permitting detection limits at 10 p moles with signal-to-noise criteria of 10. Under the standardized condition, carotenoids'

characteristics detected, as shown in Figures 2 & 3. The purified capsanthin was comparable with the reference standard, which can further use for nutritional biochemistry and toxicological studies. Dietary consumption of capsanthin-rich sources provides majorly mono- and di-ester forms of capsanthin. Though consumption of capsanthin esters, while absorption esters are hydrolyzed by esterase and lipases. Therefore, carotenoids esters are not detected in blood and tissues (Chitchumroonchokchai & Failla. 2006) [12].

Further, this study was aimed to evaluate the influence of spice (red pepper) or spice compound supplementation (capsaicin) on carotenoids solubilization or micellization (percent bioaccessibility) and physicochemical properties of micelles. The spice selected is used as major dietary additives and rich in unique carotenoids such as capsanthin and capsorubin. Therefore we have chosen red pepper for the evaluation of carotenoids bioaccessibility/ bioavailability. Further, spice-active compound capsaicin, a highly lipophilic compound, stimulates the digestive enzymes and juices for enhanced absorption of lipids and fats. The bioaccessibility revealed that carotenoids were more accessible from red pepper or co-supplementation of capsaicin to the carotenoids meal than carotenoids sample digested without capsaicin. Before and after *in vitro* digestion, carotenoids were isolated and analyzed by LC-MS and confirmed its isomerization or degradation ((Figure 2 & 3). Under the simulated condition, except carotenoids isomerization (<3%), we are not found any deterioration of carotenoids during the process of sample and digestion. Previously studies have shown that phytoconstituents like fibers, lipids, flavonoids, and spices thought to be affected carotenoids absorption (Castenmiller, 1998; Lakshminarayana *et al*. 2009; Mamatha *et al*. 2011; Yonekura *et al*. 2007; Veda *et al*. 2011; Nagao *et al*. 2000; Borel *et al*. 2003; Sha 2016) [10, 17, 21, 38, 35, 24, 4]. Solubilization of carotenoids and construction of desired micelles favours intestinal absorption of carotenoids. Therefore, carotenoids' bioaccessibility/bioavailability variation depends on the source composition vs. composition of micelles (Reboul *et al*. 2006) [27]. Carotenoids are absorbed mainly through passive diffusion and partially actively mediated through transporter protein. Therefore, carotenoid permeability through the intestinal water layer presumed not only physical parameters of micelles but also the chemical properties of phytoconstituents present in the fruits, vegetables, grains/pulses, or spices (Sy., 2012). In general, vegetable oils/ dietary fats and bile salt appear to be necessary for the efficient solubilization of lipophilic capsanthin and other carotenoids. Further, specific lipids soluble flavonoids, alkaloids including spices like capsaicin, may influence micellization and their absorption process. The bioaccessibility of carotenoids depends on hydrophobic interaction found in capsaicin and carotenoids. The present study opines that carotenoids' interaction with other dietary phytoconstituent affects micellization and intestinal absorption processes. Therefore, we presume that permeability through the intestinal water layer and subsequent uptake process of carotenoid from the micelles is influenced by their physicochemical properties, including specific spice active compounds as detailed. The physicochemical properties- size, charge, PDI, and viscosity of micellar carotenoids, micelles obtained simulated digestion of red pepper are shown in Table 1. Particle size is a vital characteristic for cellular uptake of micelles, indicating efficient delivery of nutrients. Particle size results showed a significant difference in red pepper digested sample (283.0 ± 12 nm) than only carotenoids solubilized micelles (170.5± 23 nm) and carotenoids+capsaicin based micelles (223.1 ± 18 nm), respectively. The red pepper digested micellar sample's particle size was more significant than the other two groups (Table 1). Whereas only carotenoids solubilized micelles yielded smaller particle sizes than carotenoids+capsaicin. These differences are attributed due to the composition of micelles derived from the red pepper and its phytoconstituents. The PDI was better in carotenoids+capsaicin (0.90 ± 0.72), indicating the narrow distribution of particle size than carotenoids (0.62 ± 0.10) and red pepper (0.42 ± 0.02). Zeta potential is one more important characteristic indicating the stability of micelles. Zeta potential was found highly stable in carotenoids+capsaicin (-42.2 ± 4.21) than carotenoids (-18.30 ± 2.11) and red pepper (-12.13 ± 0.7) (Table 1). The viscosity of carotenoids+capsaicin (111.35 ± 0.02) was the lowest of red pepper (132.15 ± 10.20) and carotenoids (82.3 ± 8.21). The desired physicochemical properties like smaller particle size, stable zeta potential, uniformly dispersed, and less viscous properties suggested appropriate. These properties may support improve the bioavailability of nutrients in the gastrointestinal region. Therefore, we observed increased bioaccessibility of carotenoids in capsaicin treatment was higher by 15 in β-carotene, 21 in lutein, and 31 % in capsanthin than simulated digestion of only carotenoids without capsaicin. Furthermore, the red pepper digested micellar sample demonstrates the possible interaction of other dietary matrix or phytoconstituents on carotenoids' solubilization compared to other groups. In this context, the bioaccessibility results of three carotenoids are positively correlated with the physicochemical properties of micellar fraction obtained by simulated digestion (Table. 2).

Conclusion

Isolation and supplementation of high pure capsanthin in its native form is tough due to its vulnerable chemical structure, which leads to isomerization or oxidation. The extraction and purification methods under the subdued yellow light at low temperature (4 ºC) yielded high pure capsanthin as confirmed by LC-MS $(APCI)^{+ve}$ techniques. The smaller particle size, stable zeta potential, homogeneously dispersed, and desired viscous micelles improving bioaccessibility/bioavailability in the gastrointestinal region. Therefore, increased bioaccessibility of carotenoids observed in capsaicin treatments than the sample without capsaicin. These results suggested that co-consumed phytoconstituents along with carotenoid-rich diet might affect the bioaccessibility /bioavailability of carotenoids. Furthermore, this study provides greater insight into how spices like red pepper modulate carotenoids' absorption in human beings despite the abundant consumption of its sources, mainly green leafy vegetables and fruits. The agonist and antagonist influence of spice active compounds at intestinal cells on uptake, distribution, and disposition of carotenoids, including capsanthin, need to be detailed using *in vivo* models. Perception of carotenoid absorption regulation is vital for enteral nutrition to combat vitamin-A deficiency and age-related macular degeneration health burden.

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References

1. Arathi BP, Sowmya PR, Vijay K, Baskaran V, Lakshminarayana R. Progress in Enrichment and Metabolic Profiling of Diverse Carotenoids in Tropical Fruits: Importance of Hyphenated Techniques. Phytonutritional Improvement of Crops,2017:271.
2. Aizawa K, Inakuma T. Quantitation of carotenoids in commonly consumed vegetables in Japan. Food Sci Technol Res,2007:13:247–252.
3. Biacs PA, Daood HG, Pavisa A, Hajdu F. Studies on the carotenoid pigments of paprika (Capsicum annuum L. var Sz-20). Journal of Agricultural and Food Chemistry,1989:37(2):350-353.
4. Borel P. Factors affecting intestinal absorption of highly lipophilic food microconstituents (fat-soluble vitamins, carotenoids and phytosterols. Clinical Chemistry and Laboratory Medicine,2003:41(8):979-994.
5. Breithaupt DE, Weller P, Grashorn MA. Quantification of carotenoids in chicken plasma after feeding free or esterified lutein and capsanthin using high-performance liquid chromatography and liquid chromatography-mass spectrometry analysis. Poultry Science, 2003:82(3):395-401.
6. Britton G, Khachik F. Nutrition and Health. Carotenoids in Food. Birkhauser Verlag, 5, 2009.
7. Britton G. Structure and properties of carotenoids in relation to function. FASEB,1995:9:1551–1558.
8. Britton G. Relationship between the colour and the chemical structure of carotenoid pigments. J Food Chem,2007:101:1145–1150.
9. Britton G. Structure and properties of carotenoids in relation to function. The FASEB Journal, 1995:9(15):1551-1558.
10. Castenmiller JJ, West CE. Bioavailability and bioconversion of carotenoids. Annual review of nutrition,1998:18(1):19-38.
11. Cervantes-Paz B, de Jesús Ornelas-Paz J, Pérez-Martínez JD, Reyes-Hernández J, Zamudio-Flores PB, Rios-Velasco C, *et al*. Effect of pectin concentration and properties on digestive events involved on micellarization of free and esterified carotenoids. Food Hydrocolloids,2016:60:580-588.
12. Chitchumroonchokchai C, Failla ML. Hydrolysis of zeaxanthin esters by carboxyl ester lipase during digestion facilitates micellarization and uptake of the xanthophyll by Caco-2 human intestinal cells. The Journal of nutrition,2006:136(3):588-594.
13. During A, Dawson HD, Harrison EH. Carotenoid transport is decreased and expression of the lipid transporters SR-BI, NPC1L1, and ABCA1 is down-regulated in Caco-2 cells treated with ezetimibe. The Journal of Nutrition,2005:135(10):2305-2312.
14. Kim JS, Park M, Lee DJ, Kim BD. Characterization of putative capsaicin synthase promoter activity. Molecules and Cells,2009:28(4):331-339.
15. Kimura M, Rodriguez-Amaya DB. A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. Food chemistry,2002:78(3):389-398.
16. Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Annual Review of Nutrition, 2003:23(1):171-201.
17. Lakshminarayana R, Raju M, Keshava Prakash MN, Baskaran V. Phospholipid, oleic acid micelles and dietary olive oil influence the lutein absorption and activity of antioxidant enzymes in rats. Lipids, 2009:44(9):799-806.
18. Lakshminarayana R, Raju M, Krishnakantha TP, Baskaran V. Determination of major carotenoids in a few Indian leafy vegetables by high-performance liquid chromatography. Journal of Agricultural and Food Chemistry,2005:53(8):2838-2842.
19. Lakshminarayana R, Raju M, Krishnakantha TP, Baskaran V. Enhanced lutein bioavailability by lyso-phosphatidylcholine in rats. Molecular and Cellular Biochemistry,2006:281(1):103-110.
20. Lim BP, Nagao A, Terao J, Tanaka K, Suzuki T, Takama K. Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism,1992:1126(2):178-184.
21. Mamatha BS, Baskaran V. Effect of micellar lipids, dietary fiber and β-carotene on lutein bioavailability in aged rats with lutein deficiency. Nutrition, 2011:27(9):960-966.
22. Minguez-Mosquera MI, Hornero-Mendez D. Separation and quantification of the carotenoid pigments in red peppers (*Capsicum annuum* L.), paprika, and oleoresin by reversed-phase HPLC. Journal of Agricultural and Food Chemistry,1993:41(10):1616-1620.
23. Mohd Hassan N, Yusof NA, Yahaya AF, Mohd Rozali NN, Othman R. Carotenoids of capsicum fruits: Pigment profile and health-promoting functional attributes. Antioxidants,2019:8(10):469.
24. Nagao A, Maeda M, Lim BP, Kobayashi H, Terao J. Inhibition of β-carotene-15, 15′-dioxygenase activity by dietary flavonoids. The Journal of Nutritional Biochemistry,2000:11(6):348-355.
25. Oshima S, Sakamoto H, Ishiguro Y, Terao J. Accumulation and clearance of capsanthin in blood plasma after the ingestion of paprika juice in men. The Journal of Nutrition,1997:127(8):1475-1479.
26. Othman ZAA, Ahmed YBH, Habila MA, Ghafar AA. Determination of capsaicin and dihydrocapsaicin in capsicum fruit samples using high performance liquid chromatography. Molecules,2011:16(10):8919-8929.
27. Reboul E, Richelle M, Perrot E, Desmoulins-Malezet C, Pirisi V, Borel P. Bioaccessibility of carotenoids and vitamin E from their main dietary sources. Journal of Agricultural and Food Chemistry,2006:54(23):8749-8755.
28. Rhim JW, Hong SI. Effect of water activity and temperature on the color change of red pepper (*Capsicum annuum* L.) powder. Food Science and Biotechnology,2011:20(1):215-222.

29. Rodríguez-Rodríguez E, Sánchez-Prieto M, Olmedilla-Alonso B. Assessment of carotenoid concentrations in red peppers (Capsicum annuum) under domestic refrigeration for three weeks as determined by HPLC-DAD. Food Chemistry,2020:6:100092.
30. Schweiggert U, Kammerer DR, CarleR, Schieber A. Characterization of carotenoids and carotenoid esters in red pepper pods (Capsicum annuum L.) by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Rapid Communication in Mass Spectrometry,2005:19(18): 2617-2628.
31. Sha Zhang, Xiaoyong Wang. Effect of vesicle-to-micelle transition on the interactions of phospholipid/sodium cholate mixed systems with curcumin in aqueous solution. J Phys Chem B.2016:120:7392-7400.
32. Shakhidoyatov RK, Sagdullaev BT. Capsaicine in Capsicum annuum condensed extract determined by HPLC. Chemistry of Natural Compounds, 2001:37(6):575-576.
33. Sy C, Gleize B, Dangles O, Landrier JF, Veyrat CC, Borel P. Effects of physicochemical properties of carotenoids on their bioaccessibility, intestinal cell uptake, and blood and tissue concentrations. Molecular Nutrition & Food Research,2012 :56(9):1385-1397.
34. Topuz A, Dincer C, Özdemir KS, Feng H, Kushad M. Influence of different drying methods on carotenoids and capsaicinoids of paprika (Cv., Jalapeno). Food Chemistry,2011:129(3):860-865.
35. Veda S, Srinivasan K. Influence of dietary spices on the *in vivo* absorption of ingested β-carotene in experimental rats. British Journal of Nutrition, 2011:105:1429-1438.
36. Verrijssen TA, Balduyck LG, Christiaens S, Van Loey AM, Van Buggenhout S, Hendrickx ME. The effect of pectin concentration and degree of methyl-esterification on the *in vitro* bioaccessibility of β-carotene-enriched emulsions. Food Research International,2014:57(8):71-78.
37. Wingerath T, Stahl W, Kirsch D, Kaufmann R, Sies H. Fruit juice carotenol fatty acid esters and carotenoids as identified by matrix-assisted laser adsorption ionization (MALDI) mass spectrometry. Journal of Agricultural and Food Chemistry,2006:449(8):2006-2013.
38. Yonekura L, Nagao A. Intestinal absorption of dietary carotenoids. Molecular Nutrition & Food Research, 2007:51(1):107-115.
39. Zinhoum RA, Negm AA, Abd-El Hameed SM, Afify A. Efficiency of some safe methods in controlling Stegobium paniceum on capsicum minimum plant. International Journal of Entomology Research. 2019;4:07-14.