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ORIGINAL PAPER

Concentrating Immunoprotective Phytoactive Compounds from Fruits and Vegetables into Shelf-stable Protein-rich Ingredients

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Abstract Co-delivery of edible proteins with health-protective fruit (muscadine grape) and vegetable (kale) phytoactive compounds was accomplished in a biofortified ingredient for use in convenient, portable food formulations. Polyphenolics were concentrated (10–42 mg/g range) in dry muscadine-protein matrices. Kale-fortified protein matrices also captured polyphenolics (8 mg/g), carotenoids (69 μg/g) and glucosinolates (7 μmol/g). Neither total phenolics nor glucosinolates were significantly diminished even after long term (6 months) storage at 4, 20, or 37 °C, whereas carotenoids degraded over time, particularly at higher temperatures. Dry biofortified phytoactive-protein ingredients allowed delivery of immunoprotective compounds from fruits and vegetables in a stable, lightweight matrix.

Keywords Muscadine · Kale · Polyphenolics · Carotenoids · Glucosinolates · Protein isolate

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Abbreviations

HP50	Hemp protein
SPI	Soy protein isolate
WPI	Whey protein isolate
TP	Total phenolics

Introduction

Dietary phytochemicals (phytoactives) from fruits and vegetables can reduce risks of chronic human diseases such as diabetes and cardiovascular disease [1–3], and provide adaptogenic, weight management, cognitive, and immunoprotective benefits [4–6]. However, most consumers fall far short of achieving the recommended daily consumption, due in part to the bulk and inconvenience of storing and preparing fresh produce, the high perishability, and the seasonal access. Hurdles to incorporating adequate fruits and vegetables into the diet are further exacerbated when meals are consumed in transit such as in school lunchboxes, or by motorists, cyclists, backpackers, or campers. A particularly heightened challenge is provision of health-protective phytoactives from produce into field combat rations for military personnel, in a form that will be lightweight, modular, portable, and have taste appeal, yet also maintain stability, shelf-life, and health-protective functionality [7]. Soldiers encounter demanding mental and physical challenges, and exposure to harsh environments combined with the physical requirements of combat lead to increased turnover and loss of nutrients, and impeded mental and physical performance [7, 8]. The relationship between strenuous physical activity, mental stress, and inflammation and immune system response is well established [8–10], and prompts a need to supplement rations with nutrients and nutraceuticals that

heighten alertness, improve immune function, and bolster metabolism.

Recently, we reported on a straightforward food grade technology for biofortification of edible proteins with fruit phytoactives [11, 12]. Both bioavailability and health-protective efficacy of the phytoactive constituents were enhanced by the co-delivery with edible proteins in a complexed aggregate matrix [13]. In this report, we used muscadine grape (a source of polyphenols) [14] and kale (a source of glucosinolates, carotenoids, and polyphenols) [15] as representative model candidates to evaluate the utility of this novel biofortification strategy to capture and concentrate the health benefits from both fruit and vegetables into shelf-stable, dry, lightweight ingredient matrices, amenable to formulation into a variety of foods.

Materials and Methods

Reagents and Ingredients All reagent-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). Sodium carbonate, gallic acid, β-carotene (synthetic, 93 %, powder), Folin-Ciocalteau reagent, butylated hydroxytoluene (BHT), sulfatase, and barium acetate were purchased from Sigma-Aldrich (St. Louis, MO), and β-cryptoxanthin from CaroteNature GmbH (Lupsingen, Switzerland). Benzyl glucosinolate was purchased from POS Pilot Plant Corp. (Saskatchewan, Canada). Fruits (Noble muscadine grape, The Muscadine Group, LLC, Pine Level, NC) and vegetables (curly kale, from a North Carolina grower, Kannapolis, NC) were harvested at grower-determined peak ripeness/stage of maturity for sale at local markets. Edible proteins included: soy protein isolate (SPI, 90 % protein, Archer Daniels Midland Company; Decatur, IL), hemp protein (HP50, Hemp Pro 50, 50 % protein, Manitoba Harvest Hemp Foods & Oils; Manitoba, Canada), and *Bi*Pro whey protein isolate (WPI, 95 % protein, Davisco Foods International, Inc.; Eden Prairie, MN).

Preparation of Phytoactive-protein Matrices SPI or HP50 were combined with diluted muscadine juice concentrate (1:1, *v*/*v*) at a 100 g/L ratio at room temperature to allow sorption of medium-polarity polyphenolic constituents to the edible proteins [12]. After centrifugation, the pelleted polyphenol-protein complex was freeze-dried to create muscadine-SPI and muscadine-HP50 matrices. Matrices were ground into fine powders (flours) and stored at −20 °C. The supernatant (containing sugars, pectin, and water from the juice) was discarded.

The above procedure could not be used to prepare muscadine-WPI due to the relative solubility of whey and consequent loss of protein in the discarded supernatant that would occur during the centrifugation step. Instead, muscadine grape pomace (the ground waste material after juice processing, consisting of skins, seeds, and debris) was extracted in 50 % food-grade ethanol (1:5, *w*/*v*) by refluxing at 80 °C for 2 h, centrifuged at 4,000 rpm, and supernatant was filtered to afford the muscadine pomace extract. WPI was added to the hydro-alcoholic extract (100 g/L), ethanol removed by rotary evaporation, and remaining aqueous mixture lyophilized to yield dry powdered muscadine-WPI matrix.

Because raw kale contains myrosinase enzyme, which after cell disruption will cause autolytic breakdown of the health-protective glucosinolates [16], it was necessary to deactivate this enzyme prior to juicing. Fresh kale was placed into Ziploc® microwaveable steam bags and microwaved for 2 min at 90 % power (1.21 KW). This timing was empirically determined to be ideal for deactivation of the myrosinase without compromising the bioactive phytochemical constituents inherent to kale. Approx. 1.0 kg kale was juiced to produce 0.75 L of homogenous kale juice. Protein matrices (SPI, HP50 or WPI) were complexed with kale juice (100 g/L), lyophilized, ground to a fine powder and stored at −20 °C. This co-drying process captured and stabilized both the medium polarity polyphenolic and glucosinolate phytoactives and the low polarity carotenoids from the juice into the protein-rich matrix.

Measurement of Total Phenolics (TP) in the Matrices TP were extracted from each matrix fortified with muscadine grape or kale phytochemicals by treating samples (0.5 g) with 8 mL of 1 % acetic acid in 80 % aqueous methanol by sonication for 5 min at 55 °C. Samples were centrifuged (10 min) and the supernatant was collected in 25-mL volumetric flasks. The process was repeated on the pellet two more times, and the eluents were pooled together and brought to 25 mL with the extraction solvent. TP were determined with Folin-Ciocalteu reagent [17] and expressed as mg/g gallic acid equivalents (based on a gallic acid external standard curve, with a range of 25–500 μg/ml in 10 % ethanol/water solution).

Estimation of Carotenoid Content in Kale-SPI Matrix Kale-SPI samples were extracted for carotenoids using procedures described previously [18]. Briefly, 0.2 g samples were incubated with 9 mL of ethanol with 0.01 % butylated hydroxytoluene (BHT), at 40 °C for 10 min, then placed on ice where 3 mL of cold water was added followed by 3 mL hexane (0.01 % BHT), and then centrifuged at 3,000 rpm for 10 min at 10 °C. Supernatant hexane extraction was repeated 3x to collect approx. 9 mL extract, hexane was evaporated, dry residue was re-suspended in 1 mL hexane (0.01 % BHT), filtered using 0.2 μm PTFE syringe filters (Fisher Scientific, Pittsburg, PA) into 2-mL HPLC amber vials, and stored at −80 °C until HPLC analysis. Samples were injected (5 μL) into an Agilent 1260 Infinity system (Agilent Technology Inc., Santa Clara, CA) equipped with diode array detector

and autosampler (4 °C). Carotenoids were separated on a RP C_{30} column 250×4.6 mm and 5 μm (YMC America, Inc., Allentown, PA) using 0.05 % ammonium acetate in water (A), and acetonitrile: methanol: dichloromethane: triethylamine (75:20:5: 0.1 *v*/*v*, 0.01 % BHT (B). The solvent gradient system was performed as 95, 95, 100, 100, 95, and 95 % of B at 0, 10, 20, 50, 55, and 60 min, respectively, with a constant flow rate (1.8 mL/min) and column temperature (35 °C). Carotenoids were monitored at 450 nm, and DAD spectral data from 250 to 550 nm were stored to examine peak spectrum [19]. Carotenoid concentrations were calculated using the β-carotene standard curve with concentrations at 125, 63, 31, 16, 8, 4 and 2 μg/mL and data were presented as μg/g dry matrix (β-carotene equivalents).

Estimation of Glucosinolate Content in Kale-SPI Matrix Dry kale-SPI matrix samples were extracted in 50 % aqueous MeOH for glucosinolates, as previously described [20]. The supernatant was poured off into a new glass tube and saved on ice to prevent degradation during extraction procedure. The pellet was extracted twice and 1 mL from supernatant was combined with 150 μL of 0.5 M lead and barium acetate solution, vortexed, and centrifuged for 3 min at 2,000 rpm to allow proteins to precipitate. The supernatant from each 2-mL Eppendorf tube was poured off onto a drained polyprep chromatography column (Bio-Rad, Hercules, CA) containing precharged DEAE Sephadex A-25 (Sigma). Once the solution had passed through the column, 3 mL of 0.02 M pyridine acetate was added, followed by 3 mL water. To each polyprep column, 10 units of sulfatase suspended in 500 μL water were added and the columns were capped for 18 h to desulfate glucosinolates. Desulfanated glucosinolates were eluted from the polyprep columns with 3 mL water. Individual compounds were separated using a 1200 HPLC system attached to a 6510 Q-TOF (Agilent Technologies, Santa Clara, CA). The separation of glucosinolates was achieved using LiChrospher 100 RP C_{18} column, 250×4.6 mm×5 μm (Grace Davison Discovery Science, Deerfield, IL). The mobile phase was composed of solvent A (0.1 % ammonium acetate in H_2O with 0.1 % acetonitrile) and solvent B (100 % acetonitrile). The gradient system was 0, 25, 0, and 0 % of solvent B at 0, 32, 34, 36, and 40 min, respectively with a constant flow rate of 1 mL/min. Triplicate samples were analyzed for individual glucosinolate concentrations (μmol/g dry matrix) which were calculated in comparison to certified glucosinolate levels in a standard rapeseed reference material (BCR 367, Commission of the European Community Bureau of References, Brussels, Belgium).

Stability of the Phytochemicals in the Fortified Matrices Phytochemicals captured in the matrices were gauged over time up to 6 months storage at 4, 20, or 37 °C. Polyphenol stability was measured in muscadine-SPI, muscadine-HP50, kale-SPI, and kale-HP50 matrices, whereas glucosinolate and carotenoid stability was monitored in the kale-SPI matrix. Three random samples of each matrix were prepared and analyzed at each of the following time points: 0, 2, 4, 8, 12, 16, 20, and 24 weeks of storage in the dark at each of the three temperature treatments. Detailed analyses for each phytochemical class were performed as described above.

Statistical Analysis Statistical analyses for TP, carotenoid, and glucosinolate stability data were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, 2009). Data were analyzed by two-way ANOVA using PROC GLM procedures to compare phytochemical concentrations among treatments (4, 20, and 37 °C each at 0 to 24 weeks of storage). The statistical model was $y_{ijk} = \mu + T_i + S_j + R_k + TxS_{ij} + \varepsilon_{(ijk)}$; where y=response from the experimental unit, μ=overall mean, T=temperature, S=storage time, R=replication, TxS=temperature x storage time interaction, ε=experimental random error. Treatment mean separations were performed using the least significant difference (LSD) test at $P \leq 0.05$ to evaluate temperature and storage effects on concentration of sorbed phytoactives in matrices.

Results and Discussion

Concentration of Phytoactives in Matrices TP concentrations from muscadine captured in SPI, HP50 and WPI were 13.4, 10.5, and 42.3 mg/g DW, respectively (Table 1). TP levels were comparable for the two matrices prepared by complexing the edible proteins with diluted muscadine juice concentrate (muscadine-SPI and muscadine-HP50), however TP levels were nearly 4-fold higher for muscadine-WPI, which was prepared using the alternative method of co-drying muscadine pomace extract with WPI. The TP concentration in the diluted muscadine juice (6,265 mg/L) used to prepare muscadine-SPI and muscadine-HP50 was only slightly lower than the polyphenolic concentration in the pomace extract (~7,000 mg/L) used to prepare muscadine-WPI, however, in the latter method, the extract can be loaded at higher volumes and there are no losses of TP in discarded supernatant. The pelleted method efficiently concentrates and separates phytoactives from the large volume of water and sugars in fruit juices, but the co-drying technique can be used successfully for extracts that are low in or devoid of extraneous sugars, such as the muscadine pomace extract. The co-drying technique is also preferable when the protein matrix has a high tendency to partially dissolve in water, as was true for the WPI.

Similarly, comparable TP concentrations were observed for kale-SPI, kale-HP50, and kale-WPI prepared by the co-drying method: 8.7, 8.0, and, 8.3 mg/g dry weight, respectively. Kale polyphenols included kaempferol, quercetin and

hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and sinapic) [21].

Primary carotenoids identified and measured in the kale-SPI matrix were lutein and β-carotene, in addition to neoxanthin, violaxanthin, lutein epoxide, and β-cryptoxanthin. A representative HPLC chromatogram is presented in Fig. 1. Carotenoid compound identification was in agreement with previous publications [22]. Lutein (36.4 μg/g) and β-carotene (25 μg/g) constituted 88 % of the total carotenoids in kale-SPI. As carotenoids are very low polarity compounds, they do not have the strong affinity to proteins as expected for medium polarity polyphenolic compounds or glucosinolates, therefore, the co-drying method was required to sorb these phytoactives to protein matrices. Carotenoids are recognized antioxidant compounds and are linked to prevention of age related diseases, particularly macular degeneration [23].

A total of eight glucosinolates were identified in kale-SPI matrix (Fig. 2), including glucoiberin (**1**), progoitrin (**2**), glucoraphanin (**3**), sinigrin (**4**), gluconapin (**5**), glucobrassicin (**6**), gluconasturtiin (**7**), and neoglucobrassicin (**8**). Glucososinolate compound identification was in agreement with previous publications [16, 20]. Based on the chemical structure of the side chain, glucosinolates are categorized into three classes, aliphatic (compounds **1**–**5**; 5.3 μmol/g matrix), indolyl (compounds **6** and **8**) and aromatics (compound **7**). The last two classes constituted a minor proportion in kale-SPI (2.2 μmol/g matrix). The major glucosinolates observed in kale-SPI were glucoiberin (**1**) and sinigrin (**4**). However, an appreciable amount of glucoraphanin (**3**) was also observed in kale-SPI matrix (0.23 μmol/g), a compound associated with prevention against several types of cancers [24, 25]. The bioactivity of glucosinolates is attributed to their hydrolysis products even at low concentrations [26], but this can also depend on their profile in plant tissue [27]. In a recent study, as little as 3–5 servings of broccoli per week were associated with a 30 % or 40 % decrease in risk for a number of cancers [28]. In our study, glucosinolates were maintained in their intact form during extraction by deactivating the glucosinolate hydrolysis enzyme (myrosinase), a required enzyme for the release of the health beneficial (but unstable) isothiocyanates. A recent study has shown that human colonic microbiota can perform the hydrolysis of glucosinolates [29].

Phytoactive Equivalencies To compare phytoactive equivalents in the matrices to fresh muscadine grapes and kale leaves, concentrations were calculated and converted on a per fresh plant tissue basis. In a recent survey for 10 muscadine varieties [14], TP averaged 21.8, 3.7, and 0.2 mg/g FW in seed, skin, and pulp, respectively. The muscadine-HP50 and muscadine-SPI, which used muscadine juice as the TP source,

Table 1 Total phenolics in muscadine and kale-fortified matrices [soy protein isolate (SPI) and hemp protein (HP50)] over a period of 6 month storage at three different temperatures (4, 20 and 37 °C)

Storage		Muscadine fortified[a]		Kale fortified[b]	
Time	Temperature	SPI	HP50	SPI	HP50
Week 0	–[c]	13.4(0.6)de	10.5(1.1)g	8.7(0.3)a	8(1.1)a
Week 2	4 °C	12.9(0.3)efg	10.6(0.4)fg	7.7(0.1)cde	6.4(0.1)d–g
	20 °C	13.1(0.1)d–g	11.2(0.5)efg	7.5(0.3)c–h	6.8(0.1)b–e
	37 °C	13.4(0.2)de	10.8(0.6)fg	7.5(0.1)c–h	6.4(0.3)def
Week 4	4 °C	13.0(0.3)efg	10.7(0.3)fg	7.9(0.5)bcd	6.6(0.6)b–e
	20 °C	12.6(0.3)g	11.0(0.3)fg	7.5(0.6)c–h	6.4(0.3)def
	37 °C	13.6(0.5)d	11.4(0.6)ef	7.0(0.1)g–j	6.3(0.2)e–h
Week 8	4 °C	11.8(0.3)h	9.2(0.8)h	5.5(0.5)k	5.8(0.4)gh
	20 °C	11.1(1.1)i	9.2(0.7)h	5.2(0.5)k	6.0(0.4)fgh
	37 °C	11.9(0.2)h	9.3(0.3)h	4.5(0.4)l	4.3(0.2)i
Week 12	4 °C	13.6(0.9)d	12.6(0.8)bcd	8.0(0.3)bc	6.8(0.4)bcd
	20 °C	12.8(0.5)fg	11.9(0.8)de	7.6(0.6)c–f	6.8(0.3)bcd
	37 °C	14.4(0.5)bc	11.8(0.8)de	6.4(0.3)j	5.8(0.1)h
Week 16	4 °C	13.5(0.3)de	12.3(0.4)cd	7.7(0.2)cde	6.5(0.2)c–f
	20 °C	13.3(0.4)def	12.5(0.1)bcd	7.2(0.2)e–h	6.9(0.1)bcd
	37 °C	14.8(0.5)bc	13.1(0.6)bc	7.1(0.3)f–i	6.8(0.2)bcd
Week 20	4 °C	13.1(0.4)d–g	12.2(0.4)d	6.6(1.4)ij	6.7(0.6)c–d
	20 °C	13.3(0.1)def	13.1(0.6)cb	6.9(0.1)hij	6.6(0.2)c–d
	37 °C	14.9(0.8)b	14.1(0.3)a	7.1(0.3)f–i	7.1(0.6)b
Week 24	4 °C	15.8(0.5)a	14.3(0.5)a	7.2(0.1)e–h	6.9(0.3)bcd
	20 °C	14.4(0.3)bc	12.6(1.9)bcd	7.4(0.3)d–h	7.0(0.3)bc
	37 °C	14.3(0.6)c	13.3(0.1)b	8.4(0.1)ab	7.8(0.3)a

[a],[b] total phenolics mg/g DW of matrix, as gallic acid equivalents. Values between parenthesis are standard deviations (n=3). Means with different letters within columns are significantly different at $P<0.05$. [c] SPI matrix at the start of the experiment (stored at −20 °C immediately after lyophilization)

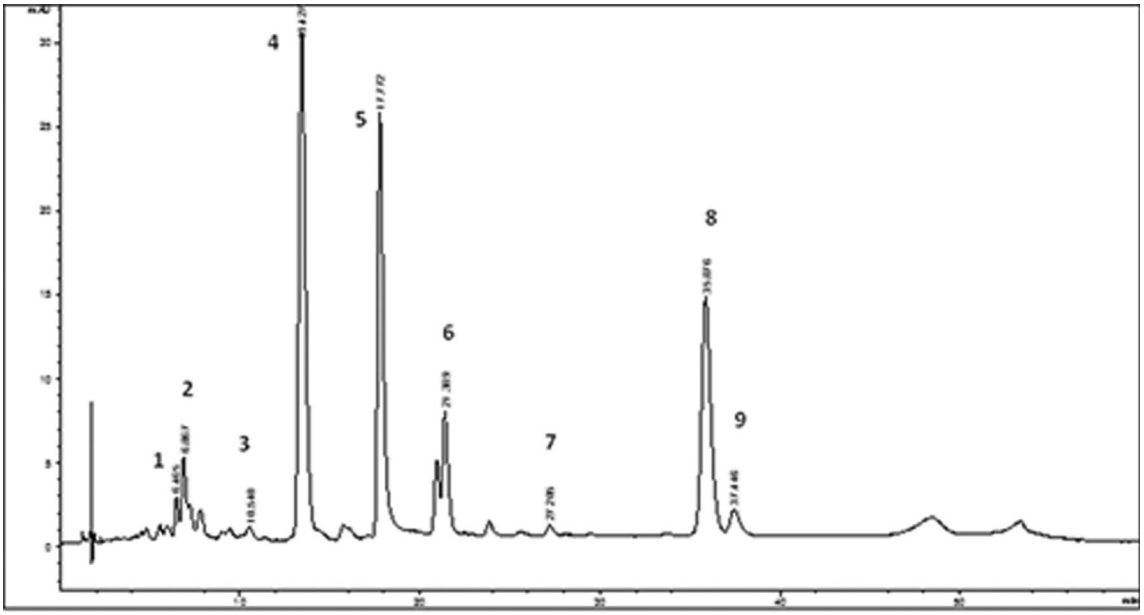

Fig. 1 HPLC chromatogram showing the carotenoid profile in the kale-fortified soy protein isolate matrix (SPI) at the start of the stability experimentation. Compound identification: ***1***=neoxanthin, ***2***=violaxanthin, ***3***=lutein expoxide, ***4***=lutein, ***5***=chlorophyll b, ***6***=chlorophyll a, ***7***=β-cryptoxanthin, ***8***=all *trans* β-carotene, ***9***=*cis* β-carotene

resulted in 3 times higher concentration of TP in the matrix, as compared to the fruit, whereas muscadine-WPI, which used pomace as the TP source and a co-drying technique, concentrated up to 11 times more TP on a per volume basis (that is, only 7 g of ingredient provided a TP equivalent of a full 75 g serving size of muscadine grape fruits). In a recent large survey of kale varieties, TP, glucosinolates, and carotenoids averaged 3.5, 0.7, and 0.2 mg/g fresh matter, respectively [15]. For the kale-SPI matrices, TP was 4 times concentrated, and glucosinolates were 12 times concentrated compared to the

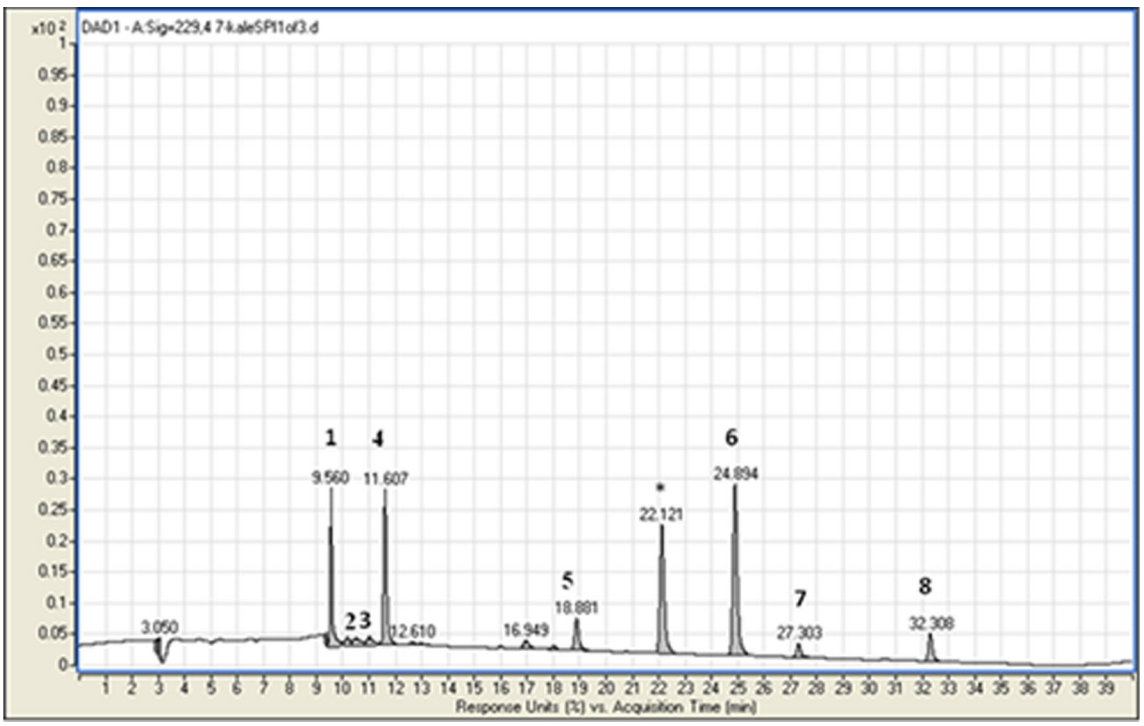

Fig. 2 HPLC chromatogram showing the glucosinolate profile in the kale- fortified soy protein isolate matrix (SPI) at the start of the stability experimentation. Compound identification: ***1***=glucoiberin, ***2***=progoitrin, ***3***=glucoraphanin, ***4***=sinigrin, ***5***=gluconapin, ***6***=glucobrassicin, ***7***=gluconasturtiin, ***8***=neoglucobrassicin. *=benzyl glucosinolate (internal standard)

Table 2 Carotenoids and glucosinolates in kale-fortified soy protein isolate matrix (SPI) over a period of 6-month storage at three different temperatures (4, 20, and 37 °C)

Storage		Carotenoids[a]				Glucosinolates[b]		
Time	Temperature	Lutein	β-carotene	Other carotenoids	Total	Aliphatic	Indolyl/ aromatic	Total
Week 0	–[c]	36.4(1.9)a	25.0(0.8)a	7.9(1)a	69.4(4.9)a	5.3(0.2)cde	2.2(0.1)a	7.5(0.2)a–d
Week 2	4 °C	31.5(3.2)bc	22.2(2.5)ab	4.9(1.1)bc	58.7(10)bc	6.6(0.2)ab	1.6(0.1)d–i	8.3(0.3)ab
	20 °C	30.1(1.2)cd	20.6(0.8)bc	5.0(0.7)b	55.7(3)bcd	7.1(0.4)a	1.4(0.1)hij	8.5(0.5)a
	37 °C	25.1(1.2)e–h	16.0(0.4)fgh	2.9(0.3)de	44.1(3.1)fg	6.9(0.3)a	1.6(0.0)d–i	8.5(0.3)a
Week 4	4 °C	34.2(0.8)ab	22.7(0.6)ab	5.8(0.6)b	62.7(6.9)ab	5.8(1.2)bcd	1.9(0.1)a–f	7.6(1.2)abc
	20 °C	30.2(1.6)bcd	19.1(0.9)cde	4.5(0.5)bc	53.7(4.0)cd	6.2(1.5)abc	2.0(0.1)a–d	8.2(1.3)ab
	37 °C	19.5(0.4)ij	10.7(0.6)jk	2.0(0.0)ef	32.1(1.2)hi	4.4(0.0)e–i	1.8(0.1)b–g	6.3(0.2)d–h
Week 8	4 °C	29.1(3.5)cde	18.4(2.3)c–f	5.4(2.1)b	52.9(11.2)cde	4.5(0.2)e–h	2.0(0.1)abc	6.5(0.2)d–g
	20 °C	22.4(2.6)ghi	13.6(1.9)hi	2.8(0.3)de	38.7(7.6)gh	6.3(0.3)abc	1.9(0.2)a–d	8.2(0.1)ab
	37 °C	19.5(1.0)ij	9.1(0.1)k	2.2(0.2)de	30.3(2.2)i	4.2(0.3)f–i	1.7(0.0)d–i	5.8(0.3)e–h
Week 12	4 °C	26.1(0.2)d–f	16.5(0.2)efg	3.6(0.4)cd	46.1(2.2)ef	4.9(0.4)d–g	2.2(0.2)ab	7.1(0.6)b–e
	20 °C	21.2(0.6)hi	12.3(0.4)ij	2.4(0.5)de	36.0(2.0)hi	4.1(1.2)f–i	1.7(0.5)c–g	5.9(1.7)e–h
	37 °C	8.9(0.4)k	3.3(0.2)l	0.7(0.2)fg	12.9(0.9)j	4.1(0.1)f–i	1.5(0.1)f–g	5.7(0.2)fgh
Week 16	4 °C	32.0(1.1)bc	19.3(0.5)cd	5.0(0.5)b	56.3(1.7)bcd	5.1(0.2)def	1.9(0.1)a–e	7.0(0.3)b–e
	20 °C	22.9(1.5)ghi	12.7(0.8)ij	2.8(0.3)de	38.4(2.3)gh	4.9(0.5)d–g	1.7(0.0)c–h	6.6(0.5)c–f
	37 °C	9.4(0.3)k	4.1(0.4)l	0.5(0.1)g	13.9(1.0)j	4.0(0.2)ghi	1.3(0.1)ij	5.3(0.2)gh
Week 20	4 °C	28.7(0.5)c-f	17.7(0.3)def	4.7(1.0)bc	51.1(1.0)def	4.7(0.8)e–h	1.8(0.1)c–g	6.5(0.9)c–g
	20 °C	20.0(0.7)ij	10.4(0.1)jk	2.3(0.5)de	32.7(1.4)hi	3.7(0.7)hi	1.4(0.2)hij	5.1(0.9)h
	37 °C	8.1(0.2)k	3.3(0.3)l	0.7(0.3)fg	12.2(0.3)j	4.0(0.2)ghi	1.3(0.1)j	5.3(0.2)gh
Week 24	4 °C	24.7(0.4)fgh	14.5(0.8)ghi	4.6(1.6)bc	43.8(1.6)fg	4.6(0.3)e–i	2.0(0.1)a–d	6.6(0.4)c–g
	20 °C	16.4(7.6)j	10.7(0.4)jk	2.7(0.2)de	29.8(0.9)i	3.5(1.1)i	1.5(0.4)g–j	5.0(1.5)h
	37 °C	6.1(0.0)k	2.2(0.4)l	0.5(0.2)g	8.8(0.6)j	4.0(0.1)ghi	1.5(0.1)hij	5.4(0.1)fgh

[a] μg/g DW matrix (β-carotene equivalents), other carotenoids included neoxanthin, violaxanthin, lutein epoxide, and β-cryptoxanthin. [b] μmol/g DW of matrix. Values between parenthesis are standard deviations (n=3). Means with different letters within columns are significantly different at $P<0.05$. [c] SPI matrix at the start of the experiment (stored at −20 °C immediately after lyophilization)

fresh kale leaves. In contrast, carotenoids measured in kale-SPI were available in the matrices at approximately the same levels as in the fresh kale leaf. TP, carotenoids, and glucosinolates available in the recommended serving size of kale leaves (67 g) could be delivered in 16, 67, and 5.6 g of kale-SPI matrices, respectively. These values can depend on many factors including the plant species used, the type of phytoactives, and the type of matrices selected as observed in this study and previous reports [11, 12]. In this study, polyphenolic and glucosinolate components were concentrated in matrices, but carotenoids were not concentrated beyond the levels inherently present in kale leaves.

Stability of Phytoactives Captured in Matrices Long term stability of the bound phytoactives was evaluated in muscadine-SPI and muscadine-HP50 (TP), and kale-SPI (TP, carotenoids, and glucosinolates). Phytoactive constituents were extracted from the fortified matrices stored at 4, 20 and 37 °C, at time intervals of 0, 2, 4, 8, 12, 16, 20 and 24 weeks.

The stability data (shelf-life) for phytoactives sorbed into matrices of muscadine-SPI and muscadine-HP50 revealed efficient TP stability for up to 6 months of storage at 4, 20, or 37 °C (Table 1). The muscadine TP was comprised of colored pigments (anthocyanins) and colorless flavonoids. While anthocyanins are prone to degradation with time [12], they were protected in the protein matrices and storage did not appear to affect the values for TP in matrices. Similar trends were observed in kale-SPI matrix samples, where comparable concentrations were observed after 24 weeks of storage (Table 1). Carotenoids were significantly less stable over time and subject to degradation especially at higher temperatures (20 and 37 °C) (Table 2). Over 6 months of storage, about 63 % of the total carotenoids remained stable (were not degraded) in the kale-SPI matrix samples stored at 4 °C. At 37 °C, over 88 % of total carotenoids were degraded or could not be detected in the samples. Lutein appeared to be the most stable carotenoid, as its concentration did not decline significantly over 4 months of storage at 4 °C. Carotenoids are known to be susceptible to oxidation and degradation over time particularly after extraction [30] and our results indicate that an antioxidant additive is

warranted to preserve carotenoids during storage in the matrix. For glucosinolates, stability data for kale-SPI showed that the aliphatic and indol glucosinolate classes as well as total glucosinolates showed reasonable stability over 6 months of storage at 4 °C (Table 2). A non-significant decline in the total glucosinolates was observed with temperature storage of 20 or 37 °C. After 6 months, glucosinolates were 90, 70, and 70 % of the initial content at 4, 20, and 37 °C, respectively.

Conclusion

Fortified matrices efficiently sorbed target phytoactives from kale and muscadine. While both SPI and HP50 matrices showed comparable phytochemical sorption efficiency, muscadine was a better source of TP, while kale was a resource for carotenoids and glucosinolates. With long term storage, TP and glucosinolates showed no significant degradation, while carotenoids degraded faster, particularly at high storage temperatures. To the best of our knowledge, this is the first report to address the stability of three significant phytoactive classes complexed with edible protein isolates when stored over 6 months at three different temperature regimes.

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Conflict of interest The authors declare no conflicts of interest.

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