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A high-throughput, simultaneous analysis of carotenoids, chlorophylls and tocopherol using sub two micron core shell technology columns

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ABSTRACT

A high-throughput, robust and reliable method for simultaneous analysis of five carotenoids, four chlorophylls and one tocopherol was developed for rapid screening large sample populations to facilitate molecular biology and plant breeding. Separation was achieved for 10 known analytes and four unknown carotenoids in a significantly reduced run time of 10 min. Identity of the 10 analytes was confirmed by their UV–Vis absorption spectras. Quantification of tocopherol, carotenoids and chlorophylls was performed at 290 nm, 460 nm and 650 nm respectively. In this report, two sub two micron particle core-shell columns, Kinetex from Phenomenex (1.7 μm particle size, 12% carbon load) and Cortecs from Waters (1.6 μm particle size, 6.6% carbon load) were investigated and their separation efficiencies were evaluated. The peak resolutions were >1.5 for all analytes except for chlorophyll-a′ with Cortecs column. The ruggedness of this method was evaluated in two identical but separate instruments that produced CV < 2 in peak retentions for nine out of 10 analytes separated.

1. Introduction

Epidemiological studies suggest consuming cruciferous vegetables enhance human health benefits due to their bioactive compounds. Cruciferous vegetables belonging to the family *Brassicaceae* and are particularly rich in hydrophilic glucosinolates, organic acids, phenolics and hydrophobic (fat soluble) bioactive compounds [1,2]. The hydrophobic bioactives include colored pigments such as carotenoids, chlorophylls and tocopherol (Fig. 1). Carotenoids impart a wide array of colors ranging from pale yellow (lutein) to bright red (lycopene), and serve to protect photosynthetic apparatus from oxidation in plants. They benefit humans by acting as powerful free-radical scavengers and providing pro vitamin A activity. Tragically, over half a million deaths of children underage the age five group are reported annually due to β-carotene (provitamin A) deficiency, a major carotenoid found in plants [3,4]. Carotenoids are also implicated in decreasing the incidence of various eye diseases [5]. Consumption of *Brassica* vegetables can compensate for deficiencies of these compounds by providing β-carotene, lutein and tocopherols, which are related to reduced incidences of these degenerative diseases. The required DRI (dietary reference intakes) for adults and children above four years age is 1700 μg for β-carotene [6]. The consumption of 100 g/day of broccoli can adequately meet this daily requirement of β-carotene [7].

In the US alone, the annual cruciferous vegetable market accounts for over a billion dollars [8]. Carotenoids and chlorophylls greatly influence the produce quality and market value with the color intensity they impart. The concentrations of these compounds in the plant tissue are highly variable and influenced by the genetics, agro-ecosystems, climate and duration of light [9–11]. Sample degradation during prolonged analysis is a major concern in carotenoid analysis. Fruits such as mango, peaches and mandarins contain esterified carotene and xanthophylls requires sample saponification prior to quantification [12–14]. But the majority of carotenes, xanthophylls and chlorophylls in cruciferous vegetables are non-esterified and do not require saponification [7]. Bypassing this 16 h saponification step would prevent degradation of these compounds during extraction. Additionally, a wide range of absorption maxima and polarities make it extremely difficult to simultaneously quantify these compounds at a single wavelength. Although a plethora of quantitation methods exists for analysis of these compounds, only a handful of them have the ability to simultaneously quantify these compounds [7,15,16]. Hence, developing

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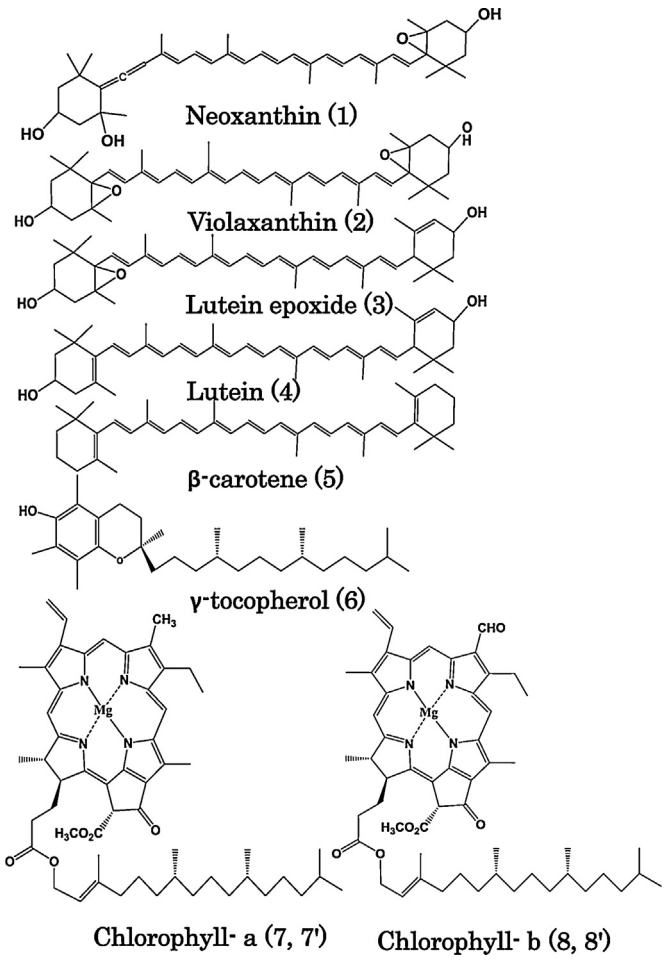

Fig. 1. Structures of neoxanthin (1), violaxanthin (2), lutein epoxide (3), lutein (4), β-carotene (5), tocopherol (6), chlorophyll a, isomer (7, 7′) and chlorophyll b, isomer (8, 8′) chromatographically separated in UPLC system.

a fast and simultaneous high-throughput method for quantification of carotenoids, chlorophylls and tocopherol is warranted for rapid screening of a vast number of samples required by plant breeders to select recombinant lines with enhanced or modified levels of these health enhancing compounds.

The recently introduced Ultra High Performance Liquid chromatography (UPLC) technology has transformed the quantification methods of natural products and pharmaceuticals, which led to significantly low analysis time [17]. The UPLC technology primarily utilizes sub-two-micron particles in their columns for routine separations and analysis, which enhances the sensitivity of these instruments. Although some studies used sub-two micron particle technology, they have not focused on developing high-throughput analytical methods [7,18]. However, a recent study conducted by Maurer et al., has achieved the rapid method, but the identity of chlorophylls in their simultaneous analysis remained unclear [19]. Previous techniques have found that core shell particles produce greater applicability toward height equivalent theoretical plate (HETP) and reduced dispersion over traditional porous particles [20]. Although recently reported carotenoid methods have used UPLC/UHPLC technologies, the utility of the core shell column technology was not explored in the area of plant pigment compounds [7,19]. The objective of the current study is to develop a high-throughput, sensitive and simultaneous UPLC method, and using core shell particle technology to quantify carotenoids, chlorophylls, and tocopherol and to evaluate the applicability of the method.

2. Materials and methods

2.1. Chemicals, standards and stock solutions

The mobile phase chemicals, ammonium acetate and HPLC-grade chloroform, were purchased from Sigma–Aldrich (St. Louis, MO, USA), while HPLC-grade acetonitrile (ACN), methanol (MeOH) and tetra hydrofuron (THF) were purchased from Fischer Scientific (Pittsburg, PA, USA). The β-carotene standard was purchased from ChromaDex (Irvine, CA, USA), while chlorophyll-a, and γ-tocopherol were obtained from Sigma–Aldrich (St. Louis, MO, USA). The Millipore water is obtained from Milli Q system, EMD Millipore Co. (Billerica, MA, USA).

2.2. Plant material and applicability

The field grown commercial broccoli cultivars including Marathon, Imperial, Gypsy, Peto-7, Majestic, were evaluated for levels of five carotenoids, four chlorophylls and one tocopherol. Broccoli plants (10) were transplanted in the piedmont research station, Salisbury, NC, on September 11, 2009, when the transplants are approximately five weeks old. Standard practices were applied for pest control and fertilization. At the commercial maturity stage, broccoli heads were harvested, freeze dried, and extracted for carotenoids, tocopherols, and chlorophylls.

2.3. Calibration curves, accuracy, quantification and precision

The calibration curve and its linearity were determined by injecting standards within the concentration range of the samples. Ten serial dilutions of standard concentrations ranging from 0.0005 μg to 0.25 μg were prepared by dissolving β-carotene standard in THF. Aliquot of 5 μL was injected into UPLC column. The calibration curves were prepared by plotting peak area against corresponding standard concentrations and the linearity was checked according to reported standard practices [21]. The quantification of other carotenoids was done by multiplying the respective response factor with reference to β-carotene standard. The standard response factors for neoxanthin, violaxanthin, lutein epoxide and lutein were obtained from previous reports [20]. The concentrations of carotenoids are calculated as:

$$CC = \left\{ \frac{\text{Area} + \beta_i}{\beta_s} \right\} \times R_f \quad (1)$$

where CC is the carotenoid concentration, β_i is intercept and β_s is slope of β-carotene regression curve while R_f is the response factor for specific carotenoid with respect to β-carotene. Standard stock solutions of chlorophyll-a and tocopherol were dissolved in ethanol to prepare 50 μg/mL concentrations and stored at −80 °C until the calibrations were prepared. Later, chlorophyll a and tocopherol stock solutions were serially diluted with concentrations ranging from 0.0005 to 0.25 μg/5 μL injections. The linearity of the regression curves provided the accuracy of the method. Precision of the chromatographic method is obtained by studying the inter-day and intra-day variations of five different broccoli cultivars as described by Piller et al., [22]. Each sample was extracted twice and injected into the UPLC system. The sample extraction followed the same procedure as detailed in Section 2.4. The broccoli samples were injected into the column at time intervals of 0 h, 12 h and 24 h time difference.

2.4. Extractions and sample preparation

Immediately after harvest, the selected broccoli cultivars were flash frozen in liquid nitrogen and then stored at −80 °C until