Computers and Electronics in Agriculture 108 (2014) 71–79

Contents lists available at ScienceDirect

Computers and Electronics in Agriculture

journal homepage: www.elsevier.com/locate/compag

Application of near-infrared reflectance spectroscopy for predicting carotenoid content in summer squash fruit

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ARTICLE INFO

Article history:
Received 31 March 2014
Received in revised form 21 June 2014
Accepted 7 July 2014

Keywords:
Least squares regression
Cucurbita pepo
Carotenoid
Lutein
β-Carotene

ABSTRACT

The potential of near-infrared reflectance spectroscopy (NIRS) for predicting total carotenoid, lutein and β-carotene contents in skin and flesh of *Cucurbita pepo* fruits was assessed. The carotenoid contents were performed by HPLC, and were regressed against different spectral transformations by modified partial least square (PLSm) regression. Coefficients of determination in the external validation varied from 0.81 to 0.96, which characterize those equations as having from good to excellent quantitative information. The standard deviation (SD) to standard error of prediction ratio (RPD) and range to standard error of prediction ratio (RER) were variable for the different fruit part and compounds, and showed values that were characteristic of equations suitable for screening purposes. PLSm loading plots corresponding to the first terms of the equations showed that effects of the C–H group of starch and lipids, O–H group of water, as well as protein and chlorophyll, were most important in modeling prediction equations. The use of NIRS represents an important breakthrough in breeding for improved nutritional quality of summer squash fruit.

1. Introduction

The botanical family *Cucurbitaceae*, commonly known as cucurbits, includes several economically and nutritionally important vegetable crops cultivated worldwide, such as cucumber, melon, watermelon and pumpkins, gourds and squashes (Schaefer et al., 2009).

Cucurbita genus ($2n = 2 \times = 40$), that include gourds, squashes and pumpkins, has been less studied. It contains some of the earliest domesticated plant species (Smith, 2005). Today, three of them, *C. pepo* L., *C. moschata* Duchesne, and *C. maxima* Duchesne, have considerable impact on human nutrition, being appreciated for their medical and nutritional properties (Ferriol and Picó, 2008; Paris, 2008; Shokrzadeh et al., 2010). *C. pepo* is the most economically important species and has a great range of variation for shape, size, and color (Paris, 2002). The *C. pepo* fruits can be picked either when immature or fully mature, and this type of use determines the cultural techniques and breeding objectives.

Cultivated *C. pepo* is considered to comprise two subspecies each one including several cultivar-groups, ssp. *pepo* (pumpkin, vegetable marrow, cocozelle, and zucchini) and ssp. *ovifera* (acorn, scallop, crookneck, and straightneck) (Paris, 1986; Ferriol et al., 2003). Shape and size in fruits are under polygenic control (Emerson, 1910; Sinnott, 1936), whereas over a dozen major genes have been identified that affect fruit color, and differences in the genetic control of carotenoid content between skin and flesh of the fruit have been detected (Paris, 2000; Tadmor et al., 2000). These differences in the pattern of carotenoid accumulation between skin and flesh have also been observed in fruits of other species, reinforcing the hypothesis of an independent regulation of carotenoid biosynthesis in these tissues (Kato et al., 2004; Xu et al., 2006; Alquezar et al., 2008).

Abbreviations: NIRS, near-infrared spectroscopy; PCA, principal component analysis; PLSm, modified partial least-squares; R^2, coefficient of determination in the external validation; RER, ratio of the range to standard error of prediction (performance); RPD, ratio of the standard deviation to standard error of prediction (performance); SD, standard deviation; SEL, standard error of laboratory; SEP, standard error of performance; SNV-DT, standard normal variate-detrending; VIS, visible.

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http://dx.doi.org/10.1016/j.compag.2014.07.003
0168-1699/

Fruit skin of *Cucurbita* has been found to be a higher source of phytochemicals, such as carotenoid (Obrero et al., 2013) and it also exhibits antioxidant properties (Anter et al., 2011). Information about the carotenoid composition of the fruit tissues could be used in breeding programs to increase their value as health-promoting food by means of the combination of genotypes carrying genes with high carotenoid content in both, skin and flesh.

Animals cannot synthesize carotenoids (*in vivo*, α-carotene, β-carotene, and β-cryptoxanthin are transformed in vitamin A), so that they must eat vegetables. The consumption of carotenoid-rich foods has been associated with a decrease in the risk of developing certain types of cancer (Giovannucci et al., 1995) and other degenerative and chronic diseases (Klipstein-Grobusch et al., 2000). In particular, lutein and zeaxanthin are xanthophylls without provitamin A activity, but have been implicated in preventing age-related macular degeneration (Seddon et al., 1994). The dietary intake of these xanthophylls is generally low (0.6–3 mg day^{-1}) (Leth et al., 2000; Johnson, 2002) and apparently, daily intakes of 4–20 mg are required in order to achieve positive effects in human visual functions (Granado et al., 2003).

HPLC methods useful to determine carotenoids in different foods required the previous extraction of the analyte from the matrix, so many difficulties may arise regarding their stability over the whole procedure (Mínguez-Mosquera and Hornero-Méndez, 1993). In fact, carotenoids and xanthophylls are very sensitive to heat and acids, which may cause trans–cis isomerization and structural changes, these problems being strengthened by light and/or oxygen.

Alternative methods include spectrophotometry in the visible range to determine the total carotenoid content. For crops such as *Cucurbita* in which the carotenoid content of fruit tissue consists primarily of lutein and pro-Vit A carotenoids (pVACs) such as β-carotene (Rodríguez-Amaya, 1997; Ben-Amotz and Fishler, 1998), Vis-spectroscopy can provide an estimate of tissue vit A nutritional contents. However, both HPLC and spectrophotometric analyses involve lengthy and labor-intensive extraction protocols with large volumes of organic solvents, solvent partitioning, and/or saponification steps (Schulz et al., 2000; Zandomeneghi et al., 2000). Although these methodologies for carotenoid content determination offer a high level of precision they have some handicaps, such as the high cost of analysis, slowness of operation, and use of hazardous chemicals. In contrast, near-infrared spectroscopy is a valuable technique that offers speed, minimal sample preparation, low cost of analysis, and also the sample is analyzed without using chemicals, making it possible to conduct large numbers of analyses in a short time. Near-infrared reflectance spectroscopy (NIRS) has been widely used in breeding programs and within the food industry (Font et al., 2006; Blanco-Díaz et al., 2014; Martínez-Valdivieso et al., 2014), and it has been applied to the analysis of carotenoid contents in maize (Brenna and Berardo, 2004), tritordeum (Atienza et al., 2005), durum wheat (Edwards et al., 1996), banana (Davey et al., 2009), potato (Bonierbale et al., 2009) and fresh cassava roots (Sánchez et al., 2014).

In this work, we were interested in developing methodologies for the high-throughput analysis of fruit carotenoid contents as encountered in breeding and germplasm-screening programs. For this, the objective of this work was to evaluate the potential of NIRS for predicting carotenoid contents in skin and flesh of the fruit from a wide variety of summer squash genotypes using standardized HPLC protocols.

2. Material and methods

2.1. Summer squash cultivars selection

Summer squash (*C. pepo* subsp. *pepo*) cultivars representing a diverse collection of genetic material were selected for this study. The selected cultivars from 2 morphotypes (120 vegetable marrow and 110 zucchini) were grown following standard local cultural practices for both plant nutrition and insect pest and disease control in the Center IFAPA La Mojonera (36°47′19″N, 02°42′11″W; 142 m a.s.l.). Fruits were harvested at the immature stage (commercial size).

2.2. Sampling

The skin (epicarp) of the fruits was peeled and the remaining fruit tissue (flesh) was cut into small cubes after removal of seeds. For each sample (150 g fresh weight) skin and flesh tissues were pooled separately from two fruits, mixed, and immediately stored at −80 °C. The samples (250 skin and 250 flesh) were lyophilised using freeze drying equipment (Telstar LyoQuest, Germany), then were ground in a mill (Janke & Kunkel, mod. A10, IKA®-Labortechnik) for about 20 s to pass a 0.5 mm screen, and stored at −80 °C until analysis.

The samples were freeze-dried to eliminate the strong absorbance of water in the infrared spectral region which overlaps with important bands of compounds which are present in low concentration (Venyaminov and Prendergast, 1997).

2.3. Analysis of summer squash fruit carotenoids

Total carotenoid concentration was determined by spectrophotometry as described by Lichtenthaler and Buschmann (2001). Individual carotenoid concentration was determined by reverse phase HPLC after saponification as detailed in Obrero et al. (2013). Biological samples were prepared in triplicate and each biological sample was further analysed in triplicate. All manipulations were performed in ice and under subdued artificial light conditions with headspaces of containers flushed with oxygen free nitrogen to help prevent carotenoid degradation.

The carotenoids were extracted from the rehydrated sample with 5 ml ethanol containing 1 mg mL^{-1} butylated hydroxytoluene (BHT) using a Polytron homogenizer. All steps were carried out in darkness or under gold fluorescent light to prevent possible photodegradation of products.

Samples were saponified in order to hydrolyze esterified carotenoids that might complicate the chromatographic determinations (Khachik and Beecher, 1988). One millilitre of a 40% w/v KOH methanolic solution was added to each tube, and the samples were saponified for 10 min at 85 °C. The samples were cooled in an ice bath, and 2 mL of ice-cold water was added. The suspensions were extracted twice with 2 mL of hexane by vigorous vortexing followed by a 2000*g* centrifugation for 10 min at room temperature. The upper hexane layers were pooled and evaporated to dryness in a Savant SpeedVac apparatus and resuspended. Immediately before injection the carotenoids were dissolved in 800 μL of an acetonitrile/methanol/dichloromethane (45:20:35 *v*/*v*/*v*) solution, filtered through a 0.22 μm PTFE syringe filter (Millipore) directly to sample vials, and 10 μL were injected into the chromatograph. The initial mobile phase consisted of acetonitrile/methanol (97:3, *v*/*v*) containing 0.05% (*v*/*v*) triethylamine. We used a linear gradient of dichloromethane from 0% to 10% in 20 min at the expense of acetonitrile, and then the dichloromethane was kept constant at 10% until the completion of the runs. The flow rate was 1.0 mL/min while the column temperature was 30 °C.

The analyses were carried out on a HPLC apparatus equipped with binary pump, in-line vacuum degasser, autosampler injector, a Waters Symmetry C18 column (4.6 mm × 150 mm, 5 μm) and a 996 diode array detector (Waters, Milford, MA) supported by the Empower chromatography manager computing system (Waters) was used to detect colored carotenoids at 450 nm.