2014

MEJORA GENÉTICA DEL CULTIVO DE CALABACÍN: INCREMENTO DEL VALOR AÑADIDO MEDIANTE LA OBTENCIÓN DE VARIEDADES CON MAYOR CALIDAD SENSORIAL Y NUTRICIONAL

Tesis Doctoral

DAMIÁN MARTÍNEZ VALDIVIESO

* Universidad de Córdoba

* IFAPA-Centro la Mojonera (Almería)

[Noviembre 2014]

TITULO: *Mejora genética del cultivo de calabacín: incremento del valor añadido mediante la obtención de variedades con mayor calidad sensorial y nutricional*

AUTOR: *Damián Martínez Valdivieso*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

www.uco.es/publicaciones
publicaciones@uco.es

Instituto de Investigación y Formación Agraria y Pesquera
CONSEJERÍA DE AGRICULTURA, PESCA Y DESARROLLO RURAL

UNIVERSIDAD D CÓRDOBA

TESIS DOCTORAL

MEJORA GENÉTICA DEL CULTIVO DE CALABACÍN: INCREMENTO DEL VALOR AÑADIDO MEDIANTE LA OBTENCIÓN DE VARIEDADES CON MAYOR CALIDAD SENSORIAL Y NUTRICIONAL

GENETIC IMPROVEMENT OF THE SUMMER SQUASH CROP: INCREASING ADDED VALUE BY OBTAINING VARIETIES WITH HIGHER SENSORY AND NUTRITIONAL QUALITY

Trabajo realizado en el Instituto de Investigación y Formación Agraria, Pesquera y Alimentaria (IFAPA) - Centro La Mojonera (Almería) y en el Departamento de Genética de la Universidad de Córdoba para optar al grado de Doctor por el Licenciado en Biología:

DAMIÁN MARTÍNEZ VALDIVIESO

Dirigido por:

Dra. Mercedes Del Río Celestino

IFAPA- Centro la Mojonera, Almería

Dra. Ángeles Alonso Moraga

Departamento Genética
Universidad de Córdoba

TÍTULO DE LA TESIS:

MEJORA GENÉTICA DEL CULTIVO DE CALABACÍN: INCREMENTO DEL VALOR AÑADIDO MEDIANTE LA OBTENCIÓN DE VARIEDADES CON MAYOR CALIDAD SENSORIAL Y NUTRICIONAL

DOCTORANDO: DAMIÁN MARTÍNEZ VALDIVIESO

INFORME RAZONADO DE LAS DIRECTORAS DE LA TESIS

La Tesis Doctoral de D. Damián Martínez Valdivieso se ha llevado a cabo en IFAPA-Centro La Mojonera de Almería, en el Área de Mejora y Biotecnología de Cultivos y en el Departamento de Genética de la Universidad de Córdoba. Durante el desarrollo de la Tesis Doctoral, el Doctorando no sólo ha superado ampliamente los objetivos planteados al comienzo de la misma, sino que ha desarrollado y validado técnicas experimentales de una gran utilidad para el grupo de investigación, que le han permitido obtener resultados muy relevantes, que han quedado patentes en 5 publicaciones con factor de impacto, y se están preparando otras 3 que en un futuro cercano serán publicadas. El Doctorando ha presentado sus resultados en diferentes congresos de ámbito nacional e internacional, y que han quedado reflejados en forma de 8 capítulos de libros, 7 pósters y una comunicación oral. Concretamente, como fruto del trabajo del Doctorando se han publicado tres trabajos relacionados con su Tesis Doctoral en varias revistas de referencia en nuestra área de investigación. Las publicaciones son las siguientes:

Martínez-Valdivieso D., Gómez P., Font R., Del Río-Celestino M. (2014). Mineral composition and potential nutritional contribution of 34 genotypes from different summer squash morphotypes. European Food Research and Technology

Martínez-Valdivieso D., Font R., Blanco-Díaz M.T., Moreno-Rojas J.M., Gómez P., Alonso-Moraga A., Del Río-Celestino M. (2014). Application of near-infrared reflectance spectroscopy for predicting carotenoid content in summer squash fruit. Computers and Electronics in Agriculture.

Martínez-Valdivieso D., Font R., Gómez P., Blanco-Díaz M.T., Del Río-Celestino M. (2014). Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy. Journal of the Science of Food and Agriculture.

Por todo ello, autorizamos la presentación de la Tesis Doctoral.

Córdoba, __10__ de ____noviembre_____ de ___2014____

Firma de las directoras

Fdo.: Mercedes Del Río Celestino Fdo.: Ángeles Alonso Moraga

Dra. Mercedes Del Río Celestino, Investigadora del Área de Mejora y Biotecnología de Cultivos del IFAPA- Centro la Mojonera, Almería y

Dra. Ángeles Alonso Moraga, Catedrática del Departamento de Genética de la Universidad de Córdoba, como directoras de esta Tesis,

INFORMAN:

Que el trabajo titulado **"MEJORA GENÉTICA DEL CULTIVO DE CALABACÍN: INCREMENTO DEL VALOR AÑADIDO MEDIANTE LA OBTENCIÓN DE VARIEDADES CON MAYOR CALIDAD SENSORIAL Y NUTRICIONAL"**, realizado por Damián Martínez Valdivieso, bajo la dirección de la Dra. Mercedes Del Río Celestino y la Dra. Ángeles Alonso Moraga, puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

Considerando que se encuentra concluida, dan el VºBº para su presentación y lectura.

Fdo.: Mercedes Del Río Celestino

Fdo.: Ángeles Alonso Moraga

Córdoba,10 de noviembre de 2014.

Córdoba, 10 de noviembre de 2014.

Esta investigación ha sido financiada por el Proyecto INIA-RTA00039-000, titulado “Mejora genética del cultivo de calabacín: incremento del valor añadido mediante la obtención de variedades con mayor calidad sensorial y nutricional” del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) y fondos FEDER .

Además el doctorando agradece al Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) por la concesión de la beca predoctoral para la realización de esta tesis.

TESIS POR COMPENDIO DE ARTÍCULOS

INFORME DEL FACTOR DE IMPACTO Y CUARTIL DEL JOURNAL CITATION REPORT DEL ÁREA EN LA QUE SE ENCUENTRAN LAS PUBLICACIONES PRESENTADAS

1. Martínez-Valdivieso D., Gómez P., Font R., Del Río-Celestino M. (2014). Mineral composition and potential nutritional contribution of 34 genotypes from different summer squash morphotypes. European Food Research and Technology DOI 10.1007/s00217-014-2308-7

Índice de impacto: 1.387, Cuartil: Q2

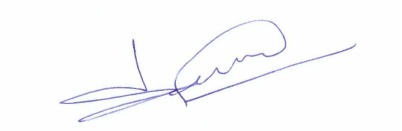
2. Martínez-Valdivieso D., Font R., Blanco-Díaz M.T., Moreno-Rojas J.M., Gómez P., Alonso-Moraga A., Del Río-Celestino M. (2014). Application of near-infrared reflectance spectroscopy for predicting carotenoid content in summer squash fruit. Computers and Electronics in Agriculture. http://dx.doi.org/10.1002/jsfa.6667

Índice de impacto: 1.486, Cuartil: Q1

3. Martínez-Valdivieso D., Font R., Gómez P., Blanco-Díaz M.T., Del Río-Celestino M. (2014). Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy. Journal of the Science of Food and Agriculture. http://dx.doi.org/10.1016/j.compag.2014.07.003

Índice de impacto: 1.879, Cuartil: Q1

En Córdoba, a 10 de noviembre de 2014



Fdo. Damián Martínez Valdivieso

Nota: esta Tesis Doctoral se presenta en parte en Inglés, ya que los capítulos que la conforman han sido publicados o enviados a diferentes revistas de investigación y se han editado los trabajos originales. Por esta misma razón las referencias de cada capítulo aparecen al final del mismo tal y como establecen las normas de cada una de las revistas.

ÍNDICE

Contents

AGRADECIMIENTOS

Acknowledgments

Es difícil agradecer en pocas palabras a todas las personas que, de una u otra manera, han formado parte de este período tan importante.

Todo esto no lo habría conseguido sin la ayuda de mi familia. Mi madre Concha y mi padre José siempre han estado a mi lado apoyándome y aconsejándome, gracias por haber hecho de mi lo que soy. Gracias a mi hermana Inma por todo, pero en especial por su apoyo durante estos últimos meses. Gracias a mi hermano José y a su mujer Inma por su ánimo, y por supuesto a mi sobrino Adrian y mi sobrina Alma por llenarme de alegría.

Gracias a mi directora la Dra Mercedes del Río Celestino por darme la oportunidad de realizar la tesis con ella y por todo el apoyo recibido durante estos cuatro años y por estar siempre dispuesta a ayudar con una sonrisa, porque sin ella nada de ésto hubiera sido posible: Muchas gracias Mercedes!!!!

Quisiera agradecer al Centro IFAPA la Mojonera de Almería y a todo su personal la colaboración prestada durante los años de elaboración de esta tesis. También me gustaría agradecer a los compañeros y compañeras del Departamento de Genética de la Universidad de Córdoba por la ayuda recibida para llevar a cabo parte de los experimentos que aquí se presentan, y en especial a mi directora la Prof. Ángeles Alonso Moraga por su amistad y hospitalidad. También agradecer al Centro IFAPA Alameda del Obispo por su colaboración con el equipo NIRS.

Gracias también al Dr Jim McCreight del Centro del USDA en Salinas (California), al Dr Hernán Ceballos del Centro Internacional de Agricultura Tropical en Cali-Palmira (Colombia), y al Dr John Lunn del Instituto Max Planck de Potsdam-Golm (Alemania) por recibirme en cada uno de estos países y por toda la ayuda recibida durante los meses de mi estancia. También dar las gracias a todos los amigos y amigas que he conocido en de cada uno de estos países por hacer que haya sido una experiencia única.

A todos mis amigos y amigas, en especial a los de mi pueblo (Cúllar). Por desgracia no puedo mencionar a todos pero gracias por darme momentos inolvidables, cariño y ser pacientes conmigo. Especial mención para Mari Carmen García por estar a mi lado y apoyarme durante tanto tiempo.

Por último hacer mención a la importante labor que efectúan los organismos INIA-IFAPA (Subprograma FPI-INIA) que me han permitido llevar a cabo esta investigación. Y gracias a los miembros del tribunal por su buena disposición y su colaboración.

RESUMEN

Actualmente, en una sociedad donde el consumidor exigente busca productos de calidad, la oportunidad de crecimiento del sector hortofrutícola pasa por la obtención de variedades que respondan a estas nuevas necesidades del mercado.

El calabacín (*Curcurbita pepo* L.) es un cultivo de gran importancia económica nacional e internacional que ha incrementado su superficie, su producción (169%) y su valor económico (111%) de forma significativa durante los últimos veinte años. Sin embargo, este crecimiento no se ha visto reflejado en un incremento en la obtención de nuevas variedades como ha ocurrido en otros cultivos.

En este trabajo se han llevado a cabo dos estrategias con el fin de obtener nuevas variedades de calabacín con alto valor añadido: 1) la caracterización físico-química, nutricional y biológica de cultivares de *C. pepo* (morfotipos de las subsp. *pepo y* subsp. *ovifera*) comparándolos con variedades testigo comerciales representativas de las áreas de producción españolas más importantes, y 2) programa de mejora con el desarrollo de dos ciclos de selección recurrente fenotípica a partir de una variedad híbrida comercial.

Asimismo con el objeto de que la mejora se realice de una forma eficiente, un requisito indispensable para la evaluación de las fuentes de variabilidad es el uso de métodos rápidos, fiables y no destructivos, por lo que en esta tesis se ha aplicado la espectroscopía de reflectancia en el infrarrojo cercano (NIRS) para la predicción del contenido de carotenoides y minerales en el fruto de calabacín.

Los resultados más relevantes de esta tesis han sido:

- La caracterización permitió la selección de un genotipo perteneciente al morfotipo marrow (cultivar tradicional) con un alto contenido en β-caroteno (45 mg kg^{-1} peso seco) en pulpa, dos veces mayor que la mayoría de los genotipos ensayados.

- A partir del análisis de componentes principales aplicado a los caracteres en estudio de los distintos cultivares se concluyó que los parámetros cromáticos a*, b*, Chroma* y hue (en piel y pulpa), peso seco, sólidos solubles, acidez total, luteina, β-caroteno y contenido total de carotenoides de la piel fueron los que contribuyeron en mayor medida a explicar la variación total de los datos.

- Asimismo, se ha demostrado que la técnica NIRS puede ser utilizada con precisión para la predicción del contenido total de carotenoides, luteína y β-caroteno en muestras liofilizadas de piel y pulpa de calabacín. El análisis de estos componentes,

se basa principalmente en la región espectral del visible (500 a 700 nm) y del infrarrojo (1432 a 2348 nm), asociadas a los grupos C-H de lípidos y almidón, grupo O-H del agua, así como proteínas y clorofilas.

- Desde el punto de vista de la calidad nutricional, se llevó a cabo el estudio del contenido en minerales en frutos de calabacín, encontrándose una amplia diversidad varietal, especialmente en P, K, Ca y Mg.

- De acuerdo con los coeficientes de determinación en la validación cruzada, las ecuaciones NIRS desarrolladas para la predicción del contenido de Ca (piel), P, K y Ca (pulpa) en muestras liofilizadas de *Cucurbita pepo* pueden ser utilizadas para propósitos de cribado, mientras que los resultados obtenidos para el contenido total de macrominerales (piel y pulpa), P, K y Mg (piel) pueden ser usadas en el control de calidad.

- Respecto al análisis de la actividad biológica *in vivo* e *in vitro* de la piel y la pulpa del calabacín y de otros componentes bioactivos (luteína, β-caroteno, zeaxantina y ácido dehidroascórbico) del fruto, los resultados indicaron que la actividad citotóxica que presentan ambos tejidos fue debida a la acción conjunta de varios de estos compuestos junto con otros no analizados en este trabajo. Asimismo, todas las concentraciones de las sutancias analizadas fueron antigenotóxicas en el Test SMART de *D. melanogaster*, a excepción de la concentración más alta de luteína, aunque aunque no se pudo observar efecto de dosis.

- A partir del programa de mejora, se desarrollaron dos ciclos de selección recurrente fenotípica sobre una población derivada de una variedad comercial, lo que ha permitido obtener cuatro subpoblaciones avanzadas de calabacín, en las que se intensificaron el carácter alto contenido en carotenoides en piel y pulpa, a través de la selección.

Esta tesis ha puesto en evidencia el gran potencial existente entre las accesiones de *C. pepo* caracterizadas, procedentes principalmente de España pero también de otros países, para futuros programas de mejora genética de dicha especie, sugiriendo, por tanto, una alta probabilidad de encontrar nuevos y valiosos recursos fitogenéticos, que den lugar a la obtención de variedades capaces de ofrecer al consumidor más exigente la calidad que está demandando en la actualidad.

SUMMARY

Currently, in a society where consumers are demanding high quality products, the opportunity for growth of the horticultural sector is through the development of varieties that respond to these new needs of the market

Summer squash (*Curcurbita pepo* L.) is a crop of great national and international economic importance, it has increased its production (169%) and economic value (111%) significantly during the past twenty years. However, this growth has not been reflected in an increase in the development of new varieties, as has happened in other crops.

In this study, we have carried out two strategies to obtain new varieties of summer squash with high added value: 1) the physicochemical, nutritional and biological characterization of *C. pepo* cultivars (morphotypes of subsp *pepo* and subsp*ovifera*) compared with Spanish commercial varieties representative of the most important production areas, and 2) a breedingprogrammewith the development of two cycles of phenotypic recurrent selection from a commercial hybrid variety.

Likewise, with the aim that the improvement is carried out in an efficient way, an essential requirement for the evaluation of the sources of variability is the use of fast, reliable and nondestructive methods. For this reason, in this thesis the near-infraredspectroscopy (NIRS) has been applied for the prediction of carotenoid and mineral contentin the summer squash fruit.

The main results of this Thesis were:

- The characterization allowed the selection of a genotype belonging to marrow morphotype (traditional cultivar) with a high β-carotenecontent (45 mg kg^{-1} dry weight) in pulp, two times higher than most genotypes tested.

- From the principal component analysis applied to the characters, in a study with different cultivars, it was found that the colourparameters a *, b *, Chroma * and hue (skin and pulp), dry weight, soluble solids, total acidity, lutein, β-carotene and total carotenoids in the skin, were the most important characters to explain the total variation in the data.

- It has also been demonstrated that the NIRS technology can be used accurately to predict total carotenoid, lutein and β-carotene content in skin and pulp lyophilized samples. Analysis of these components was primarily based on the visible (500 to 700

nm) and infrared (from 1432 to 2348 nm) spectral region, associated with the C-H groups of lipids and starch, water O-H group as well as protein and chlorophylls.

- From the point of view of nutritional quality, the study of the mineral content in summer squash fruits was conducted, finding a wide varietal diversity, especially in P, K, Ca and Mg.

- According to the coefficient of determination in the cross-validation, the NIRS equations developed to predict the Ca content (skin), P, K and Ca (pulp) in summer squash lyophilized samples, can be used for screening purposes, whereas the results for the total content of macro-elements (skin and pulp), P, K and Mg (skin) can be used in quality control.

- Regarding the analysis of the biological activity *in vivo* and *in vitro* of the summer squash skin and pulp and other bioactive compounds (lutein, β-carotene, zeaxanthin and dehydroascorbic acid) of the fruit T the results indicated that the cytotoxic activity observed in both tissues was due to the combined action of several of these compounds along with others not analyzed in this work. In addition, all the concentrations of the substances analyzed were antigenotoxic in SMART Test of *D. melanogaster*, although it did not observe dose effect.

- From the breeding programme, two cycles of phenotypic recurrent selection on a population derived from a commercial variety were developed, which has allowed us to obtain four advanced zucchini subpopulations in which the carotenoid content in the skin and pulp was increased.

This thesis has demonstrated the great potential among the *C. pepo* accessions characterized, mainly from Spain, but also from other countries, for future breeding programmes of this species, suggesting, therefore, a high probability of finding new and valuable plant genetic resources, which lead to the development of varieties able to offer the most demanding consumers the quality that is currently demanded.

INTRODUCCIÓN

Introduction

1. Situación actual de la agricultura y del cultivo del calabacín

El sector de Frutas y Hortalizas, en el que están incluidas las Hortícolas intensivas, sigue siendo el grupo de cultivos que ocupa el primer lugar en la Producción Final Agraria de Andalucía, el sector que genera más empleo y mayor renta general en la agricultura andaluza.

Para obtener cultivos hortícolas competitivos que den un buen rendimiento, que se comporten bien en condiciones adversas, que viajen bien a los mercados de exportación, que tengan un alto porcentaje de frutos de Categoría I, buen sabor, y buena apariencia y uniformidad, hay que lograr una mayor homogeneidad y calidad. Los alimentos frescos de alta calidad son altamente atractivos para todos los sectores que conforman el gremio alimentario.

Las inquietudes de los consumidores europeos sobre la seguridad de los alimentos, la necesidad de estabilizar los mercados alimentarios a niveles de calidad controlables, las exigencias de los grandes grupos europeos de la distribución demandantes de productos frescos, y el respeto por el medio ambiente, hacen necesario que los productos que lleguen al mercado cumplan unos estrictos sistemas de control (Sistemas Aenor-Fepex, Eurep-Gap, etc.) que garanticen el seguimiento de todo el proceso de producción. El consumidor hoy día busca seguridad, calidad (alimentos más frescos), comodidad (alimentos más acondicionados) y salud (alimentos más diversificados y funcionales).

Desde el punto de vista de la salud, la Unión Europea, a través del Decálogo Europeo contra el Cáncer, impulsa el consumo diario de una dieta rica en hortalizas y frutas. Cada vez hay más evidencias sobre los beneficios que para la salud tienen las dietas basadas en hortalizas por su reducción de la incidencia de tumores, reconocidas por el Consejo Nacional de Investigación de los Estados Unidos de Norteamérica desde 1982, que sigue haciendo una recomendación específica del consumo de hortalizas, especialmente aquéllas ricas en carotenoides (especialmente β-caroteno), vitamina C, y otros compuestos, los cuales aunque no se consideren factores esenciales de la dieta, por sus cualidades antioxidantes, llevan a cabo una serie de actividades que sugieren fuertemente que pueden tener propiedades anticarcinogénicas y antitumorales significativas.

Dentro de las cucurbitáceas, el calabacín (*Cucurbita pepo* L.) ha ido ganando importancia hasta convertirse en una de las hortícolas más importantes

económicamente en España. La producción española ha experimentado un incremento, paralelo al incremento en los rendimientos medios, debido fundamentalmente a la mayor importancia relativa del cultivo bajo plástico. Un gran porcentaje de la producción procede de Andalucía (con 4038 ha de cultivo protegido en Almería), siguiéndole en importancia Canarias y Cataluña. El resto de la producción se reparte entre distintas provincias tanto en cultivo al aire libre como en invernadero. Actualmente en Almería se concentra el 70% de la producción nacional, con 371.29 millones de kilos, con una superficie de 6358 hectáreas y con un valor total de la producción de calabacín de 197.11 millones de euros. A la exportación se destinó un volumen de 230.35 millones de kilos y un valor total de 206.54 millones de euros. (Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía, 2014).

Actualmente la apuesta por la calidad ha adquirido mayor importancia, y se constituye como uno de los grandes retos del sector hortofrutícola nacional. Obtener una producción con un alto porcentaje de fruto de primera es un problema principal de los cultivares, sobre todo aquellos que se usan en cultivos de otoño-invierno, en los que el efecto de las bajas temperaturas disminuye los beneficios del agricultor. Como el principal uso de calabacín es el consumo en fresco, entre la oferta de híbridos F_1 no hay muchos adaptados a las condiciones de temperatura de esos meses, y entre estas variedades la producción de calidad se ve muy reducida. El mercado demanda variedades que no pierdan sus cualidades de turgencia, color y sabor, que sean más productivas y de mayor calidad nutricional y que responda a las necesidades del consumidor que demanda más calidad y seguridad en los alimentos que consume.

2. Taxonomía de la especie *Cucurbita pepo*

El calabacín (*Cucurbita pepo* L.), pertenece al género *Cucurbita*, uno de los géneros cultivados más importantes económicamente. Está compuesto por veintidós especies silvestres y cinco especies cultivadas (Decker, 1988). Estas especies son *C. pepo*, *C. moschata*, *C. maxima*, *C. ficifolia* y *C. argirosperma*, siendo las tres primeras (*C. pepo, C. moschata* y *C. maxima*), las más importantes económicamente y las más ampliamente distribuidas.

Cucurbita pepo (2n=2x=40) es la especie más polimórfica dentro de la familia *Cucurbitaceae* (Duchesne, 1786; Naudin, 1856). Este polimorfismo se manifiesta especialmente en sus frutos (tamaño, forma, color, patrón de coloración, textura, etc), pero también en sus características vegetativas (hábito de crecimiento, longitud y grosor de los entrenudos, tamaño de las hojas, etc). La especie incluye 10 grupos

horticulturales (o morfotipos) y tipos silvestres, todos agrupados en 3 subespecies (Paris, 1986; Paris, 2000): a) subsp. *pepo* (Cocozelle, Vegetable Marrow, Zucchini, Pumpkin y 2 morfotipos ornamentales), b) subsp. *ovifera* (L.) Decker (syn. subsp. *texana* (Scheele) Filov) (Crookneck, Straightneck, Scallop, Acorn y un tipo ornamental), y c) subsp. *fraterna* (Bailey) Lira, Andres & Nee (incluye solo tipos silvestres) (Tabla 1) (Andres, 1987; Paris et al., 2003; Sanjur et al., 2002). Esta clasificación se ajusta a los tipos morfológicos, pero también corresponde con los resultados moleculares obtenidos con isoenzimas y marcadores de DNA (Paris et al., 2003).

El calabacín que se puede encontrar de manera habitual en los mercados se corresponde con el morfotipo Zucchini en estado inmaduro. La palabra Zucchini proviene del diminutivo en plural de la voz italiana “zucca” que significa calabaza de verano. Es el morfotipo de origen más reciente de *C. pepo*, ya que se diversificó en Italia más tarde que las otras variedades y de forma más restringida (París, 2001). Posteriormente se introdujo en Estados Unidos desde Italia durante los años veinte y en tan sólo diez años, se constituyó como un grupo bien definido (París, 1989). Las variedades de Zucchini que se cultivan actualmente son híbridos mejorados en América en los últimos 50 años, obtenidos a partir de variedades italianas, en su mayoría de frutos verde oscuro o amarillo, habiéndose convertido en el morfotipo más importante económicamente. El calabacín tipo Zucchini se caracteriza por sus frutos cilíndricos, nada o un poco afilados, con una relación longitud-anchura de 3.5 a 4.5 (Tabla 1). En la mayoría de los casos el fruto es verde oscuro imponiéndose sobre los tipos amarillentos iniciales. Es una planta rastrera y monoica, con flores unisexuales axilares.

Tabla 1. Descripción de los morfotipos de *Cucurbita pepo* (adaptado de Paris, 2001, 2008).

Morfotipo	Distribución	Forma fruto	Color fruto	Corteza (Piel)	Consumo	
subsp. *Pepo*						
Pumpkin	EEUU- Canadá, Europa-Asia Menor, México-Guatemala	Esféricos y ovalados con/sin costillas	Naranja o amarillo-verde	Lignificada/ tierna	Inmaduro-maduro y semillas	
Vegetable marrow	Este Medio, norte de África	Corto, cónico y cilíndrico	Variable	Lignificada	Inmaduro	
Cocozelle	Europa (Italia), Lejano Oeste, Turquía, Yugoslavia	Largo y bulboso acostillado o no	Rayado, verde claro sin rayas	Lisa	Inmaduro y flores femeninas	
Zucchini	Mundial	Uniformemente cilíndrico	amarillo y verde	Tierna	Inmaduro	
subsp. *ovifera*						
Scallop	Australia, EEUU, Europa	Plano, festoneado	Amarillo, blanco-verde	Lignificada	Inmaduro	
Acorn	EEUU-Canadá	Asurcado, turbinado	Verde	No lignificada	Maduro	
Crookneck	Sudoeste EEUU	Cuello estrecho, usualmente curvado y con verrugas	Amarillo	Gruesa, lignificada	Inmaduro	
Straightneck	EEUU, Europa	Cuello corto y constreñido	Amarillo	Lignificada	Inmaduro	

3. Mejora genética de calabacín

El morfotipo Zucchini es el más importante a nivel económico y el que copa los programas de mejora de las compañías de semillas. Se han conseguido algunos logros de interés, fundamentalmente orientados a mejorar la arquitectura de la planta, a optimizar su floración, diversificar el tipo de fruto y a mejorar la resistencia a algunas enfermedades. Los avances en la mejora de calabazas y calabacines se resumen en dos revisiones recientes (Ferriol y Picó, 2008; Paris, 2008). Por ejemplo, la mayoría de los materiales comerciales presentan hábito de crecimiento arbustivo (característica controlada por el gen *Bu*) (París, 2000; Paris y Brown, 2005), reducida ramificación y porte erecto, características que facilitan enormemente la recolección. Además, se han seleccionado cultivares precoces con tendencia al desarrollo de un mayor porcentaje de flores femeninas. Se han desarrollado también algunos cultivares con variación en el color y forma del fruto, y en el valor nutritivo (sabor y contenido en provitamina A) (Paris, 2008). En cuanto a las resistencias a enfermedades, la mejora se ha orientado fundamentalmente a las principales enfermedades causadas por virus y hongos. Entre los virus que afectan a Cucurbitáceas hay un grupo bastante amplio, consiguiendo resistencias a virus como: *Zucchini Yellow Mosaic Virus* (ZYMV), o *Cucumber Mosaic Virus* (CMV), pertenecientes los dos a distintas familias. Más recientemente se ha producido una fuerte incidencia del virus del rizado de tomate Nueva Delhi (ToLCNDV) cuya transmisión, de unas plantas a otras, se produce por mediación de la mosca blanca *Bemisia tabaci*, su único vector conocido. Entre las enfermedades causadas por hongos, destaca el oídio causado por dos especies, *Podosphaera xanthii* (Castagne) Braun & Shishkoff y *Golovinomyces cichoracearum* (DC) V.P. Heluta (Jahn et al., 2002).

La mayor parte de la mejora se ha realizado utilizando como fuentes de variabilidad cultivares de otros morfotipos de la subsp. *pepo* y de los morfotipos de la subsp. *ovifera*, para características como arquitectura de la planta, floración, tamaño, forma, calidad del fruto, maduración, postcosecha, aunque también se han utilizado otras especies cultivadas del género como *Cucurbita moschata* o *Cucurbita maxima*, con una menor eficiencia en el cruce (Diez et al., 2002; Lira-Saade y Montes-Hernandez, 1994).

En los bancos de germoplasma europeos existían, en 2002, 6937 entradas de *Cucurbita*, de los cuales 3541 eran de *C. pepo* (Diez et al., 2002). N.I. Vavilov Research Institute of Plant Industry (Federación Rusa) tiene la mayor colección con

2064 entradas, 1004 de ellos de *C. pepo* (Piskunova, 2002). Otra colección europea importante está en la Universidad Politécnica de Valencia (España), donde existen 925 entradas de *Cucurbita*, de los cuales 291 son de *C. pepo* (Picó, 2002).

En la mejora de las distintas especies de *Cucurbita* se han utilizado distintos métodos como la selección masal, la selección genealógica y la selección fenotípica recurrente, para aumentar caracteres tales como la producción, calidad y la resistencia a plagas y enfermedades (Whitaker y Robinson, 1986).

En la actualidad, las líneas de investigación que complementan los programas de mejora de calabacín se dirigen hacia: a) la secuenciación del genoma (Gong et al, 2012), y construcción del mapa genético basado en más de 300 SNPs (*Single Nucleotide Polymorphisms*) relacionados con la precocidad de la floración, la tendencia femenina de la planta, la forma, tamaño y coloración del fruto, además de otros caracteres que resultan de gran interés para la mejora (Esteras et al., 2012); b) el estudio del transcriptoma (Obrero, 2013) y c) la obtención de colecciones de mutantes mediante el uso de agentes químicos (Martínez-Valdivieso et al., 2012; Vicente-Dólera et al, 2014).

4**. Composición nutricional del fruto y semilla de calabacín**

El fruto inmaduro de *Cucurbita pepo* es comestible y tiene numerosos componentes nutricionales incluyendo polisacáridos, proteínas activas, aminoácidos esenciales, vitaminas, carotenoides y minerales. En los últimos años ha recibido una atención considerable por su valor nutricional y su efectos beneficiosos para la salud (Shokrzadeh et al., 2010). Es bajo en calorías, su componente principal es el agua (>90 % en estado inmaduro), por lo que resulta recomendable en dietas de adelgazamiento. Debido a su alto contenido en fibra tiene un suave efecto laxante y favorece el funcionamiento del aparato digestivo. Al ser bajo en sodio es muy apropiado en los casos de afecciones cardiovasculares (hipertensión, etc.), en el control del colesterol en sangre y de los niveles de azúcar, lo que le hace muy indicado en caso de diabetes por sus propiedades diuréticas. Además se caracteriza por su contenido en carotenoides, cuyas propiedades son muy amplias (Hidaka et al., 1987, Khachik y Beecher, 1988, Erdman, 1999).

Respecto a las semillas de *C. pepo*, el aceite extraído de ellas es rico en ácidos grasos insaturados, destacándose el linoléico (43-56 %) y el oleico (24-38 %). Además contiene tocoferoles beta y gamma (vitamina E) y carotenoides: luteolina y β-

carotenos. Otros componentes lipídicos son escualeno y esteroides (1%), entre los que destacan Δ7-esteroles como α-espinasterol, Δ7, (27)-estigmastatrien-3beta-ol, Δ7-estigmastenol, delta 7,25(27)-estigmastadien-3beta-ol y Δ7-avenasterol, β-D-glucopiranósidos y pequeñas cantidades de Δ5- y Δ8-esteroles. También triterpenos de núcleo multiflorano esterificados con ácido para-aminobenzoico, proteínas (31-51 %) y aminoácidos poco frecuentes como cucurbitina ó 3-amino-3-carboxipirrolidina (0,5-2%). La cucurbitita poseen propiedades antioxidantes, capaces de neutralizar los radicales libres. Además tiene acciones como antihelmíntico, inhibidor de la 5 α-reductasa y antiinflamatorio (Bombardelli et al., 1997; Younis et al., 2000).

5**. Caracterización de los recursos genéticos**

Los recursos genéticos, además de ser una necesidad para evitar la vulnerabilidad genética, son una oportunidad para encontrar en ellos aquellas características de calidad que el consumidor está demandando. Por tanto, la información asociada a estos recursos resulta de vital importancia de cara a su utilidad y aprovechamiento (González-Andrés y Pita-Villamil, 2001). Además, este conocimiento es esencial para explicar la actividad biológica de las entradas y para planear estrategias para el diseño de variedades que incrementen la salud del consumidor.

La caracterización es el establecimiento de todos los caracteres posibles de un cultivo. Entre estos enfoques podemos citar:

Caracterización físico-química: estudia las propiedades físicas y químicas relevantes para el material vegetal, como nivel de pH, color, firmeza, etc.

Forma del fruto

La forma del fruto también es un carácter muy importante desde el punto de vista del consumidor, ya que busca frutos con formas características: alargados o redondeados. Por otra parte, las formas redondeadas facilitan el transporte y almacenamiento y son menos susceptibles a recibir golpes durante su manejo (Llácer et al., 2006).

Firmeza de la pulpa

La dureza es quizá de los parámetros más importantes en los actuales planes de mejora debido a su influencia en la aceptación de una variedad por parte del consumidor (Monforte y Álvarez, 2006). Su determinación en la caracterización

de una variedad, presenta importantes implicaciones económicas. El ensayo más ampliamente utilizado para determinar la firmeza de la pulpa es el de cizallamiento con sonda (Pardo et al., 2000).

Una de las máquinas más modernas para la medición de la textura de los alimentos es el Analizador de Textura TA-XT2, comúnmente llamado texturómetro. Este aparato, específicamente diseñado para alimentos, tiene controles electrónicos muy precisos, una elevada sensibilidad y gran versatilidad. Utiliza un programa informático asociado (con mejoras constantes) que permite recoger los datos y las gráficas automáticamente. Tras el ensayo se genera una curva fuerza-distancia, en la que el punto más alto determina el valor de fuerza máxima, utilizado como medida de la dureza del alimento.

Porcentaje de materia seca

El porcentaje de materia seca (o porcentaje de humedad) no ha sido profundamente estudiado hasta el momento en variedades de calabacín, y podría jugar un papel importante en la futura mejora de esta especie. La liofilización es un proceso que permite su análisis, ya que conociendo lo que pesa la muestra en fresco y una vez seca, se puede conocer este parámetro. El proceso consiste en congelar el alimento y una vez congelado se introduce en una cámara de vacío para que se separe el agua por sublimación. De esta manera se elimina el agua desde el estado sólido del alimento al gaseoso del ambiente sin pasar por el estado líquido. Para acelerar el proceso se utilizan ciclos de congelación-sublimación con los que se consigue eliminar prácticamente la totalidad del agua libre contenida en el producto original. Esta técnica ha sido utilizada principalmente en el análisis de nuevas líneas mejoradas de cucurbitáceas (Lester, 2008).

Peso del fruto

El peso del fruto ha sido de interés por parte de varios investigadores, dada su relación directa con la producción. Es importante para el consumidor, porque es uno de los criterios principales para la selección del fruto, así como para la conservación y el transporte.

Color del fruto

Las tonalidades de la piel también han sido muy estudiadas, aunque no esclarecidas. Estudios destacados en especies de Cucurbita son los de Itle y Kabelka (2009), Francis et al. (1962) y Seroczynska et al. (2006).

Nivel de sólidos solubles (ºBrix)

El contenido de sólidos solubles es un criterio importante en la calidad sensorial de los frutos de cucurbitáceas (Yamaguchi et al., 1977; Monforte y Alvarez, 2006). El índice refractométrico o grados Brix se determina mediante el uso de un refractómetro digital. Con ello se obtiene el porcentaje de sólidos solubles presente en el zumo extraído de las muestras, dato que se asume en muchos casos como sinónimo del nivel de dulzor.

Nivel de pH.

Existen diversas maneras de medir la acidez en calabacín, pero por su sencillez el pH ha sido frecuentemente utilizado. Este valor se mide con un pHmetro que registra la diferencia de potencial eléctrico entre un electrodo de medición y otro de referencia.

Acidez titulable

La mayoría de las frutos son ricos en ácidos orgánicos que están normalmente disueltos en la vacuola de la célula, ya sea en forma libre o combinada como sales, ésteres, glucósidos, etc. La acidez libre (acidez titulable) representa a los ácidos orgánicos presentes que se encuentran libres y se mide neutralizando los jugos o extractos de frutas con una base fuerte, el pH aumenta durante la neutralización; y la acidez titulable se calcula a partir de la cantidad de base necesaria para alcanzar el pH del punto final de la prueba. En el caso de calabacín, la acidez titulable (TA) se expresa como % ácido cítrico (g/100 g de peso fresco) ya que es el ácido mayoritario (Jacobo-Valenzuela et al., 2011), se realiza a partir de una valoración con NaOH 0.1 mol/l (AOAC, 1984).

Caracterización sensorial

Con base en las normas internacionales (ISO, 13299, 2003) relativas al análisis sensorial, recientemente se ha desarrollado un vocabulario específico que contribuirá a la caracterización cualitativa de calabacín (Galán-Soldevilla et al., 2012; Ruíz Pérez-Cacho et al., 2012). El panel sensorial generó 19 atributos sensoriales: 4 para la apariencia (tono, luminosidad, brillo y textura visual; 7 para el olor/aroma (intensidad

global, frutado, vegetal, hierba, tierra, humedad y otros); 2 sabores básicos (dulce y ácido); 3 sensaciones trigeminales (refrescante, astringente y picante) y 3 para la textura (firmeza, carácter crocante y humedad). Además se generaron 5 atributos negativos de olor/aroma: plano, agrio, plástico/goma, pescado fresco y cámara. Con este lenguaje desarrollado se elabora la hoja de perfil sensorial para la caracterización sensorial de líneas en estudio.

Caracterización nutricional: la calidad nutritiva de un alimento está relacionada con su capacidad de proporcionar todos los nutrientes que favorezcan una buena salud y eviten la aparición de enfermedades. El aumento de la calidad nutricional de un fruto, aunque ha sido objeto de atención desde hace muchos años, no ha obtenido grandes avances hasta ahora, posiblemente porque implica el control de caracteres muy complejos que requieren programas de mejora de muy larga duración (Schuch et al., 1991; Harlander, 1993; Cubero, 2000; García-Olmedo et al., 2001). Los componentes nutricionales más estudiados en el material son: el contenido en carotenos, minerales, vitaminas, etc.

Contenido en carotenoides

Respecto a los carotenoides, estos compuestos han mostrado actividad como antioxidantes biológicos, protegiendo las células y los tejidos de los efectos perjudiciales de radicales libres y del oxígeno singlete. Su comportamiento antioxidante depende de la concentración y localización en las células diana, así como de otros factores (Van den Berg et al., 2000). La luteína y la zeaxantina actúan como protectores en la región macular de la retina humana (Snodderly, 1995). Además se ha observado un incremento de la función inmune (Bendich, 1989), protección contra quemaduras solares (Mathews-Roth, 1990) e inhibición del desarrollo de ciertos tipos de cáncer (Nishino, 1998). Es de destacar que a pesar de que la luteína no es una provitamina A, tiene una mayor eficacia antioxidante que otros carotenoides (*in vitro* inhibe la oxidación lipídica de una forma más eficiente que el β-caroteno, α-caroteno o el licopeno) (Castenmiller y West, 1998). Mencionar también que la biodisponibilidad de la luteína desde fuentes vegetales es mucho más alta en comparación a la del β-caroteno (Van Het Hof, 1999; Erdman, 1999). Por otra parte, pero no menos importante, resultan los β-carotenos en la salud ocular (Scalch, 1992). Una ingestión superior de este componente ayudará a prevenir enfermedades oculares como el

desarrollo de las cataratas o la pérdida de visión por degeneración de la retina (Varma et al., 1995, Seddon et al., 1994).

Trabajos anteriores han puesto de manifiesto la variabilidad que existe en el contenido de carotenoides (carotenos totales, luteína, β-caroteno, zexantina, etc.) en *C. pepo* (Ben-Amotz y Fishler, 1998; Tadmor et al., 2005; Muntean et al., 2006; Azevedo-Meleiro y Rodríguez-Amaya, 2007; Rodríguez-Amaya et al., 2008; El-Qudah, 2009).

Contenido en minerales

Los minerales son una parte integral de la dieta humana y su ingesta es un factor importante para mantener un óptimo estado de salud, ya que éstos están implicados en multitud de reacciones metabólicas, en la transmisión del impulso nervioso, la formación de hueso, la regulación del balance de agua y sal (Kalač y Svoboda, 2000), o el transporte de oxígeno en sangre (Harichan y Verma, 2013), además son de gran importancia en determinadas etapas como en la de crecimiento y desarrollo, aunque suponen una pequeña fracción respecto al peso total de una persona. El consumo de vegetales puede suponer una fuente importante de minerales; frutas y hortalizas contribuyen a la ingesta diaria del 35, 24 y 11 % de K, Mg y P, respectivamente (Levander, 1990).

Acido ascórbico

El total de vitamina C consiste en la suma de ácido ascórbico y su forma oxidada, ácido dehidroascórbico. Ambas formas, con actividad antiescorbútica, son interconvertibles entre sí, siendo la forma reducida la que se presenta principalmente en los tejidos de las plantas. Las propiedades antioxidantes se deben sólo a su forma reducida que puede ser destruida durante el procesamiento (Khaw et al., 2001). Ocupa un lugar destacado como compuesto bioactivo con actividad antioxidante en los alimentos. Su ingesta ayuda al desarrollo de dientes y encías, huesos, cartílagos, a la absorción del hierro, al crecimiento y reparación del tejido conectivo normal, a la producción de colágeno, metabolización de grasas, la cicatrización de heridas, enfermedades cardiovasculares, cáncer y enfermedades neurodegenerativas (Halliwell, 2001). A pesar de la importancia de esta vitamina, existen pocos trabajos destacados enfocados sobre el contenido de ácido ascórbico en distintas variedades de

melón. Sí es importante nombrar los estudios realizados por nuestro grupo (Blanco-Díaz et al., 2014).

6. Desarrollo de variabilidad para componentes de calidad: Importancia de métodos rápidos de cribado para la evaluación de estos componentes

La mejora genética de la calidad de las hortícolas requiere la evaluación de la variación existente en colecciones de germoplasma de las distintas especies y de la obtenidas por mutagénesis, así como de la evaluación de poblaciones segregantes en los programas de mejora. Esto precisa del análisis de un número elevado de muestras con objeto de identificar genotipos con la composición alterada deseada (Font et al., 2006).

Las metodologías analíticas convencionales para la determinación de carotenoides (cromatografía líquida de alta resolución) y minerales (espectrometría de absorción atómica) en matrices biológicas, muestran un alto grado de precisión en la medida, pero al mismo tiempo presentan grandes inconvenientes, como son el alto coste del análisis, lentitud de la operación, necesidad de personal especializado, destrucción de la matriz analizada, y polución del medio ambiente, debido al uso de reactivos químicos, entre otros.

Estos antecedentes han llevado a la búsqueda de tecnologías analíticas alternativas, que aunque perdiendo precisión en la cuantificación del analito, permitan un muestreo rápido y a bajo coste económico, redundando en una importante descarga analítica para el laboratorio. En este sentido, la Espectroscopía en el Infrarrojo Cercano (*Near Infrared Spectroscopy*-NIRS), ha mostrado un alto potencial para la predicción de carotenoides y minerales, tanto en matrices orgánicas como en inorgánicas. En relación a los objetivos de esta tesis, el NIRS viene siendo usado desde hace años en la predicción de carotenoides en frutos de banana (Davey et al., 2009), patata (Bonierbale et al., 2009), raíz de yuca (Sánchez et al., 2014) o en granos como el maíz (Brenna y Berardo, 2004), trigo duro (Edwards et al., 1996) o tritordeo (Atienza et al., 2005). Asimismo, también ha sido demostrado su potencial en el muestreo de minerales en muestras biológicas (Nilsson et al., 1996, Font et al., 2001, 2004a, 2004b, 2005) y en hortícolas de hoja (Villatoro-Pulido et al., 2011). Es por ello una técnica con posibilidades reales de ser empleada como método de muestreo rápido, sencillo y no contaminante en el control de la Seguridad Alimentaria. Las muestras de interés seleccionadas durante el proceso de mejora en base a la predicción NIRS, pueden ser posteriormente analizadas por los métodos de referencia para confirmar

los contenidos predichos, suponiendo una reducción muy importante en el número de análisis efectuados por los métodos de referencia en laboratorio.

La tecnología NIRS se basa en la emisión de un haz de luz sobre la muestra a analizar, la cual en función de su composición, o mejor aún, de la naturaleza de los enlaces presentes en sus moléculas, fundamentalmente de aquellos de tipo –CH, –NH y –OH, interaccionará con ellos absorbiendo una determinada cantidad de radiación electromagnética en el rango del infrarrojo cercano, de 780 a 2500 nm (Shenk et al., 2001).

Figura 1. NIRS modelo 6500 (Foss-NIRSystems, Inc., Silver Spring, MD, EE.UU.)

Figura 2. Muestras de calabacín liofilizadas y molidas en la cápsula del NIRS.

En nuestro estudio utilizamos un sistema espectrofotométrico NIR modelo 6500 (Foss-NIRSystems, Inc., Silver Spring, MD, EE.UU.) (Figura 1). Se trata de un equipo dispersivo, que registra los espectros mediante un barrido de longitudes de onda (cada espectro es el promedio de 32 barridos). El sistema permite un registro rápido del espectro (menos de 1 segundo), en el rango de 400 a 2500 nm con un intervalo espectral de 2 nm. Las muestras liofilizadas molidas (Figura 2) se analizaron por triplicado como log 1/R, (R=reflectancia).

El espectro NIRS está basado en los valores de absorbancia a las diferentes longitudes de onda en el rango espectral considerado, siendo los cambios en la respuesta del espectro NIR proporcionales a los cambios en la concentración de componentes químicos, o en las características físicas de la muestra a analizar (Workman y Shenk, 2004). Cada espectro NIRS contiene una importante cantidad de información, en muchos casos redundante, con superposición de bandas de absorción a lo largo de todo el espectro, lo que dificulta en muchos casos la extracción de información relevante (Shenk et al., 2001). Para extraer la información química relevante de cada muestra es necesario recurrir al uso de pretratamientos de la señal espectral, que permiten separar la información meramente química de las variaciones de origen físico (textura, tamaño, geometría de las partículas, etc.). Los pretratamientos más utilizados son la derivación, y los tratamientos de corrección del efecto *scatter* o radiación dispersa denominados corrección multiplicativa del efecto del *scatter*, *Standard Normal Variate* (SNV) y *Detrend* (DT) (Shenk y Westerhaus, 1995a; Naes et al., 2002; Heise y Winzen, 2004).

Para ello se desarrolla un modelo quimiométrico que relaciona la información espectral de las muestras que constituyen el colectivo de aprendizaje con sus valores para el parámetro en estudio, proporcionados por un método de referencia. Una vez desarrollado el modelo, éste permite predecir el contenido de otras muestras de características similares a las incluidas en el grupo de entrenamiento o calibración.

Disponer de un colectivo de calibración que aporte variabilidad, es tal vez uno de los factores más importantes en el desarrollo de ecuaciones NIRS. La variabilidad, en lo posible debe ser tanto espectral como física y química, similar a la que se espera posteriormente encontrar en el análisis de rutina diario de nuevas muestras (Williams y Norris, 2001). Shenk et al., (2001) y Williams (2001) señalan que las características que definen al colectivo de calibración empleado, determinan tanto la estabilidad como la exactitud de las ecuaciones desarrolladas.

La selección de muestras para constituir el colectivo de calibración puede ser realizada a través del uso de herramientas matemáticas diseñadas para la estructuración de la población y la elección de muestras representativas del colectivo. Así, por ejemplo el software WinISI (Infrasoft Internacional, Port Matilda, PA, EE.UU.) cuenta con los algoritmos CENTER y SELECT, que se basan en la realización de un análisis de componentes principales (ACP), seguido del cálculo de distancias entre los espectros de las distintas muestras en un espacio n-dimensional, a través generalmente de la distancia de Mahalanobis (Shenk y Westerhaus, 1995b, 1996). Al mismo tiempo, estos procedimientos permiten detectar aquellas muestras con comportamiento diferente, denominadas "anómalas" (outliers), cuya anomalía puede ser causada tanto por la información espectroscópica o química.

Para el desarrollo de una calibración existen diferentes métodos de regresión (Martens y Naes, 1989; Burns y Ciurczak, 1992 y 2001), siendo los más utilizados en las aplicaciones cuantitativas NIRS la Regresión Lineal Múltiple (RLM ó MLR), la Regresión por Componentes Principales (RCP o PCR), la Regresión mediante Mínimos Cuadrados Parciales (RMCP o PLS) y la Regresión mediante Mínimos Cuadrados Parciales Modificada (RMCPM o MPLS), siendo éstas dos últimas las más empleadas en aplicaciones agroalimentarias (Shenk y Westerhaus, 1995b, Pérez-Marín et al., 2007). El método de regresión MPLS (*Modified Partial Least Squares*) es una variante del PLS (transforma una regresión lineal en un nuevo sistema de ordenadas, con una dimensión menor que el espacio original de las variables independientes) y aporta la ventaja de la estandarización, es decir, que los residuos NIRS a cada longitud de onda, son divididos por la desviación estándar de los residuales a esa longitud de onda, antes de calcular el siguiente factor. Se dice que este método es a menudo más estable y preciso que el algoritmo PLS, siendo el número de factores de la regresión seleccionado por validación cruzada (Shenk y Westerhaus, 1995a).

Para determinar la exactitud y precisión de las ecuaciones obtenidas en la calibración de cada componente se llevó a cabo un procedimiento de validación cruzada. Es un algoritmo que selecciona diferentes colectivos de calibración y validación dentro de una población específica. El procedimiento consiste en dividir el colectivo de calibración en grupos (dependiendo del número de muestras). Cada grupo de validación es predicho una vez con la ecuación desarrollada a partir del resto de grupos. El procedimiento se repite hasta que todas las muestras hayan sido predichas

una vez. Este procedimiento además previene el sobreajuste del modelo (Shenk y Westerhaus, 1995a; Williams, 2001).

Los principales estadísticos que nos informan sobre la calidad de las ecuaciones obtenidas son el coeficiente de determinación R^2 (Williams y Norris, 1987), RPD (*Ratio of standard error of performance to standard desviation*), que mide la relación entre la desviación típica de los datos de referencia de un determinado constituyente y el error típico de validación cruzada (ETVC) para el colectivo de validación cruzada, y RER (*Ratio of standard error of performance to range of standard data*), que establece la relación entre el rango de los datos de referencia de un determinado constituyente y el ETVC para el colectivo de validación cruzada, son estadísticos muy útiles usados en la evaluación de aplicaciones NIRS (Williams, 2001).

7. Herramientas de laboratorio para medir el efecto antigenotóxico y anticarcinogénico de poblaciones de calabacín: Test SMART y línea celular HL60

Actualmente se dispone de numerosos protocolos de laboratorio que permiten distinguir entre productos medicinales y los simplemente saludables, entre los que destacan aquellos que analizan la actividad mutagénica o antimutagénica de las sustancias vegetales. Esto es posible debido a la relación existente entre mutagénesis y otros fenómenos toxicológicos, como la carcinogenicidad, la teratogenicidad, o el envejecimiento, y también a la relación entre efectos antimutagénicos y supresiones de promociones de cánceres (bio-antimutágenos) (De Flora y Ramel, 1988).

La elección de un sistema *in vivo* adecuado para la detección de agentes geno y antigenotóxicos es de gran importancia, ya que las biotransformaciones pueden estar sesgadas según el sistema de activación exógeno que se utilice en ensayos *in vitro*. La capacidad de metabolizar promutágenos a compuestos activos o inactivos no es exclusiva de mamíferos sino que aparece en todos los taxa biológicos (inclusive en plantas), por ello se propone el uso de *Drosophila melanogaster* como sistema *in vivo* de detección de anti y xenobióticos desde muy antiguo (Muller, 1927).

El ensayo de mutaciones y recombinaciones somáticas en alas de *Drosophila* (*Somatic Mutation And Recombination Test*-SMART) se basa en las alteraciones genéticas producidas en las células de discos imaginales alares de la larva, que pueden evidenciarse fenotípicamente en el tejido adulto después de la expansión clonal y la metamorfosis y fue desarrollado por Graf y colaboradores en 1984 (Graf et al., 1984). Este ensayo ha mostrado ser capaz de detectar actividad genotóxica en

compuestos de estructura química variada, tanto mutágenos directos como promutágenos, con diferentes métodos de acción genotóxica, como agentes alquilantes, intercalantes o formadores de aductos, tanto sólidos, como líquidos, gaseosos, simples o mezclas complejas (Graf et al., 1984; Alonso-Moraga y Graf, 1989; Graf at al., 1994; Osaba et al., 1999). Además, ante esta evidencia indirecta, los estudios bioquímicos dan cuenta de la presencia en *Drosophila* de enzimas implicados en el metabolismo de xenobióticos (Baars, 1980). Hoy día se considera un test de detección de primera línea; si además se tiene en cuenta su bajo coste, eficiencia, versatilidad y rapidez podemos considerar al test SMART como muy apropiado para usar en Toxicología Genética utilizando como modelo un eucariota *in vivo* que no necesita de activación metabólica exógena para detectar actividad de promutágenos y de antimutágenos.

Para estudios de antigenotoxicidad, el test SMART ofrece una gran variedad y flexibilidad en los protocolos pudiéndose aplicar a los más diversos compuestos. Se pueden realizar tanto co-tratamientos simultáneos (mutágeno y antimutágeno) como pre- y post- tratamientos de duraciones variables, combinando dos o más compuestos o utilizando mezclas complejas. Se ha comprobado la capacidad de detección de antimutágenos del test SMART en una gran variedad de compuestos tales como clorofilina, flavonoides diversos, vitamina C, y mezclas complejas como el café instantáneo o la cúrcuma (Hamss et al., 1999). Por todo ello hoy día se considera un test de detección de primera línea; si además se tiene en cuenta su bajo coste, eficiencia, versatilidad y rapidez podemos considerar al test SMART como muy apropiado para usar en Toxicología Genética utilizando como modelo un eucariota *in vivo* que no necesita de activación metabólica exógena para detectar actividad de promutágenos y de antimutágenos.

La evaluación de la quimiosensibilidad de líneas celulares cancerosas humanas es importante en los primeros pasos de *screening* necesarios para el desarrollo de sustancias anticancerosas, ya que es una metodología muy barata, rápida y evita el sacrificio de animales de sangre caliente. Estas células se caracterizan por proliferar continuamente en suspensión, aunque tras un periodo de cultivo, pierden su capacidad de división y se diferencian. Durante muchos años, diferentes líneas celulares han sido extensamente estudiadas para esclarecer los mecanismos de citotoxicidad y que inducen diferenciación y apoptosis y así poder controlar su proliferación en los organismos vivos (Collins et al., 1978; Conte-Anazetti et al., 2003).

Para ello se utiliza la línea celular HL60 (leucemia promielocítica humana), analizándose con diferentes ensayos la viabilidad celular, la proliferación/citotoxicidad y apoptosis celular: los ensayos Trypan blue de exclusión para la determinación de viabilidad, el MTT colorimétrico de formación de formazán para determinación de citotoxicidad/proliferación, y finalmente el método Giemsa May-Grünwald para evaluar la muerte celular por apoptosis (Carpentier et al., 1998).

La muerte celular o apoptosis juega un papel crucial en el desarrollo y el mantenimiento de la homeostasis y eliminación de células dañadas o que no son necesarias en un futuro. La correlación entre la inducción de la apoptosis y la citotoxicidad es una estrategia quimiopreventora interesante. El ensayo de inducción de apoptosis estudia el grado de fragmentación del ADN que ocurre durante la apoptosis entre otros mecanismos, pudiendo haberse observado inducción de apoptosis en algunas líneas celulares por el tratamiento con muchos tipos de compuestos diferentes.

JUSTIFICACIÓN DEL TRABAJO

Desde instituciones públicas y privadas en España, y en Andalucía en particular, se viene realizando en los últimos años una apuesta clara y decidida en investigación y transferencia dirigida a incrementar el valor añadido de los productos hortofrutícolas producidos en nuestro país. Esta apuesta viene de la mano de la necesidad de ganar competitividad y rentabilidad en el sector, como una respuesta al aumento de la competencia procedente de terceros países del arco mediterráneo, con los que no es posible competir en precio, así como a la exigencia de un incremento de la calidad en los productos tradicionales por parte de los mercados internos y externos.

Con el fin de dar respuesta a la problemática planteada, la cual arrastra una evidente pérdida de competitividad real sufrida por el sector hortofrutícola español y andaluz, el Ministerio de Ciencia y Tecnología, a través del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) y la Junta de Andalucía a través de la Consejería de Innovación, Ciencia y Empresa (CICE), vienen apoyando líneas específicas de investigación para incrementar el valor añadido de la producción agraria española y andaluza. Entre estas líneas se encuentran: 1) necesidad de aportar mayor valor añadido en la cadena comercial; 2) apuesta por la innovación y programas I+D+i y 3) la diversificación de la oferta hacia nuevas necesidades de los mercados.

En base a los criterios anteriormente mencionados, esta tesis aborda la caracterización de un cultivo que ha ido ganando peso en los últimos años como es el calabacín. La amplia variabilidad existente en una colección constituida por líneas pertenecientes a diferentes morfotipos de la especie *C. pepo*, y procedentes de diversos orígenes geográficos representa una fuente de germoplasma con múltiples posibilidades en el campo de la Mejora Genética Vegetal. Por tanto, se ha propuesto como principal objetivo de esta tesis el incremento del valor añadido del fruto de calabacín a través de un programa de mejora genética y la caracterización de una colección de germoplasma para la selección de líneas de *C. pepo* con mejores características físico-químicas, nutricionales, mayor capacidad antimutagénica y tumoricida (atribuible a los carotenoides y otros componentes antioxidantes presentes en su fruto) y alto contenido en minerales para su futuro uso en programas de mejora.

OBJETIVOS DE LA TESIS

Thesis objetives

Objetivo global 1. Caracterización física y química de la colección de germoplasma de *Cucurbita pepo* mantenida en el Centro IFAPA La Mojonera.

Objetivo específico 1.1. Caracterización morfológica y de otros importantes parámetros de calidad de las accesiones de *Cucurbita pepo* de la colección.

Objetivo específico 1.2. Caracterización del contenido en carotenoides y minerales en frutos de las accesiones de la colección de calabacín.

Objetivo específico 1.3. Aplicación de la técnica NIRS para el muestreo de carotenoides y minerales en frutos de calabacín.

Objetivo global 2. Estudio de genotoxicidad y antigenotoxicidad del fruto de dos accesiones de calabacín y de algunos de sus compuestos bioactivos.

Objetivo específico 2.1. Ensayo de genotoxicidad y antigenotoxicidad de la piel y la pulpa de las accesiones de calabacín y de algunos de sus compuestos bioactivos como son los carotenoides mayoritarios presentes en el fruto (luteína, β-caroteno y zeaxantina) y el ácido dehidroáscórbico, mediante el test SMART de *Drosophila melanogaster.*

Objetivo global 3. Estudio del potencial quimiopreventivo del fruto de dos accesiones de calabacín y de algunos de sus compuestos bioactivos en la citotoxicidad de células tumorales.

Objetivo específico 3.1. Ensayo de citotoxicidad y apoptosis con la línea celular tumoral de leucemia HL60 de la actividad de la piel y la pulpa de las dos accesiones y de algunos de sus compuestos bioactivos (luteína, β-caroteno, zeaxantina y ácido dehidroáscórbico).

Objetivo global 4. Programa de mejora genética en *Cucurbita pepo.*

Objetivo específico 4.1. Obtención de variedades de calabacín con alto contenido de carotenoides (alta calidad nutricional) mediante selección recurrente fenotípica.

CAPÍTULO I: Physical and chemical characterization in fruit from 22 summer squash (*Cucurbita pepo* L.) genotypes

Chapter I

Artículo enviado a:

Journal of the Science of Food and Agriculture

Damián Martínez-Valdivieso[a], Pedro Gómez[a], Rafael Font[b], Angeles Alonso-Moraga[c], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[b]Department of Postharvest Technology and the Agrifood Industry, IFAPA Center La Mojonera Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[c]Department of Genetics, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

ABSTRACT

Physical and chemical characters of 22 summer squash genotypes (traditional and commercial cultivars) were evaluated in fruit using correlation and principal component analysis. External and internal fruit quality was assessed by standard parameters (fruit weight and dimensions, firmness, color, dry matter, soluble solids, pH, total acidity) and nutritional quality (individual and total carotenoid content) in both, epicarp and mesocarp. A remarkable variation was observed in the total carotenoid content of epicarp (4452.93 mg kg^{-1} dry matter) and mesocarp (371 mg kg^{-1} dry matter), with the Cu-17 (traditional cultivar) and Cu-29 (commercial cultivar) genotypes exhibiting the greatest values, respectively. The Cu-11.genotype (traditional cultivar) had the highest mean content of β-carotene (45 mg kg^{-1} dry matter) in mesocarp, which was up to two times greater when compared with the majority of studied genotypes. Strong and significant positive relation was found between epicarp and mesocarp color parameters (a* and b* and Chroma*) with lutein and total carotenoid contents in fruit mesocarp (*r* >0.48**) suggesting that indirect selection for high carotenoid content within summer squash breeding material will be successful, easy to implement, and inexpensive. Multivariable analysis allowed the grouping of variables, with more important variables being the a*, b*, Chroma* and hue parameters (epicarp and mesocarp), dry matter, soluble solid, total acidity, lutein, β-carotene and total carotenoid contents of the epicarp. This material provides a way of allowing breeders to select and breed genotypes with higher levels of nutritional compounds and also increasing the dietary intake of the consumers.

Key words: *Cucurbita*, carotenoids, color, epicarp, mesocarp

INTRODUCTION

The genus *Cucurbita* L. (pumpkins and squash) is native to the Americas where there is evidence of their culture more than 10000 years ago (Smith, 1997), according to archaeological recordings, where *Cucurbita pepo* L. appears to be one of the first domesticated species (Hernándo-Bermejo and León, 1994; Aliu et al., 2011). They were dispersed to other continents by transoceanic voyagers at the turn of the 16th century and have become a familiar and important vegetable crop in many countries (Hernándo-Bermejo and León, 1994, Paris et al., 2006). The three widely grown species, *C. pepo* ('summer squash'), *C. maxima* Duch. ('winter squash') and *C. moschata* Duch. ('butternut squash') are extremely polymorphic in fruit characteristics (Loy, 2004; Paris et al., 2006). *C. pepo* is one of the most variable species in the plant kingdom (Naudin 1856). Taxonomically. *C. pepo* comprises eight morphotypes grouped into two sub-species, subsp. *pepo* L.: pumpkin, vegetable marrow, cocozelle and zucchini; and subsp. *ovifera* (L.) Decker (syn subsp. texana (Scheele) Filov): scallop, acorn, crookneck and straightneck) (Ferriol et al., 2003). The groups differ in geographical distribution and economic importance (Paris, 2010). In general, cocozelle and vegetable marrow groups with considerably long histories, were more variable than the zucchini group, of a more recent origin (Formisano et al., 2012), the nineteenth century (Hernándo-Bermejo and León, 1994), and has undergone intensive breeding in the United States and Europe (Paris, 2010). The zucchini group is the most commercially important of morphotypes and is widely grown throughout the Mediterranean basin, especially in Southeastern Spain (Almeria), where the production reaches more than 350000 tonnes each year (Consejeria de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucia, 2013). Its great economic value is based mainly on the culinary use of the immature fruits as vegetables. The great diversity of uses makes breeding objectives quite variable.

Most breeding programs aim to produce new cultivars with better traits such as plant architecture, flower optimization, diversification fruit type, attractive fruit color and resistance to diseases (Paris, 2008; Ferriol and Pico, 2008; Manzano et al., 2009, 2010). Lately, there is a considerable interest in determining the variation that may exist in the content of antioxidant compounds and other nutritional properties of fruit from different genotypes within *C. pepo* species (Blanco-Díaz et al., 2014; Martinez-Valdivieso et al., 2014a, 2014b). This would provide a way of allowing breeders to select and breed accessions with higher levels of nutritional compounds and also increasing the health of consumers.

The consumer preference for summer squash is greatly influenced by its external appearance, constituting the main physical property. Moreover, information on the content of soluble solid content and individual carotenoids in fruits could increase the dietary intake by the consumers. Thus, recently, because of high antioxidant activity and nutritional properties of squash, the ready-to-eat winter squash (*C. maxima* Duch.) snack was developed with high carotenoid content and good sensory prosperties (Konopacka et al, 2010).

Multivariate analysis is a useful tool for germplasm description and characterization and has been used to determine the relationships among cultivars and to study correlations among variables in other species (Iezzoni and Pritts, 1991). In winter squash, *Cucurbita maxima* Duch, (Whang et al., 1999; Seroczynska et al., 2006; Itle and Kabelka, 2009; Konopacka et al., 2010; Kim et al., 2012) a high positive correlation was found between the carotenoid content and its epicarp and mesocarp color. Information on the associations that may exist among physical and chemical characters may be beneficial for consumers interested in a healthier product.

The purposes of this study were to i) determine the variability of physical and chemical traits in the fruit of a germplasm collection of *C. pepo*. and ii) find possible relationships that may exist among physical and chemical characters in the summer squash fruit

MATERIAL AND METHODS

Plant material and greenhouse experiment

Twenty two genotypes were evaluated in this work (Table 1), currently kept in the Germoplasm Bank at the “IFAPA La Mojonera”. They were representatives of traditional and improved cultivars and were classified as follows: thirteen traditional genotypes of *C. pepo* ssp. *pepo* from Spain (belonging to the `Vegetable marrow´ group, and one of them to the `Pumpkin´ group) which are cultivated in small orchards used for self-consumption; three genotypes from Israel (`Vegetable marrow´); a genotype from Spain belonging to ssp. ovifera (L.) D.S.Decker corresponding to the `Scallop´ group; eight commercial hybrids belonging to the `Zucchini´ group representatives of the main commercial varieties currently offered in the market.

Table 1. List of 22 *Cucurbita pepo* genotypes included in this study, botanical classification, morphotype, country of origin and source of germoplasm.

Accession	Subspecies	Morphotype	Country of origin (Estate/Region)		Source of Germoplasm
Cu-1	ssp. pepo	vegetable marrow	Spain	(Aragón)	COMAV
Cu-4	ssp. pepo	vegetable marrow	Spain	(Andalucía)	COMAV
Cu-5	ssp. pepo	vegetable marrow	Spain	(Andalucía)	COMAV
Cu-7	ssp. pepo	vegetable marrow	Spain	(Cataluña)	COMAV
Cu-8	ssp. pepo	vegetable marrow	Spain	(Cataluña)	COMAV
Cu-9	ssp. pepo	vegetable marrow	Spain	(Cataluña)	COMAV
Cu-10	ssp. pepo	vegetable marrow	Spain	(Cataluña)	COMAV
Cu-11	ssp. pepo	pumpkin	Spain	(Cataluña)	COMAV
Cu-13	ssp. pepo	vegetable marrow	Spain	(Unknown)	COMAV
Cu-14	ssp. ovifera	scallop	Unknown	(Unknown)	COMAV
Cu-15	ssp. pepo	vegetable marrow	Israel	(Unknown)	Israel
Cu-17	ssp. pepo	vegetable marrow	Israel	(Unknown)	Israel
Cu-25	ssp. pepo	zucchini	Spain		Commercial hybrid
Cu-27	ssp. pepo	zucchini	Spain		Commercial hybrid
Cu-28	ssp. pepo	zucchini	Spain		Commercial hybrid
Cu-29	ssp. pepo	zucchini	Spain		Commercial hybrid
Cu-31	ssp. pepo	zucchini	Spain		Commercial hybrid
Cu-32	ssp. pepo	zucchini	Spain		Commercial hybrid
Cu-33	ssp. pepo	zucchini	Spain		Commercial hybrid
Cu-35	ssp. pepo	vegetable marrow	Spain	(Canarias)	COMAV
Cu-36	ssp. pepo	vegetable marrow	Israel	(Unknown)	Israel
Cu-37	ssp. pepo	zucchini	Spain		Commercial hybrid

The crop was grown during the spring-summer season of 2012. Seeds of these genotypes were germinated in rock-wool cubes (Grodan BV, 6040KD Roermond, NL) in a seedbed. When plants had developed three to four leaves they were transferred to 1 m large rock-wool slabs at a density of two plants/slab. Plants were grown in a greenhouse in the IFAPA Center in La Mojonera, Almería, Spain (36º47'19''N, 02º42'11''W; 142 m a.s.l.), following standard local cultural practices for both plant nutrition and insect pest and disease control.

Fruits were harvested at an immature stage because they are marketed this way. In order to obtain a sufficient sample volume for standard analytical methods, each sample was a combination of two fruits of the same plant. Upon harvest, they were processed preserving the epicarp and mesocarp of each fruit separately. Then, the physical and chemical traits were measured. For determining the carotenoid content, samples were packaged in polypropylene plastic containers and stored at -80℃, immediately. These samples were lyophilised using freeze drying equipment (Telstar LyoQuest, Germany), then were ground in a mill (Janke & Kunkel, mod. A10, IKA-Labortechnik) for about 20 seconds to pass a 0.5 mm screen, and stored at -80 ℃ until analysis.

Physical traits

The length of the fruit was measured from end to end with a flexible tape and expressed in centimeters. The diameter was measured using a digital caliper, expressed in millimeters. The weight of each fruit were obtained with a balance, expressed in grams.

Epicarp and mesocarp fruit color was determined using a Minolta spectrophotometer (Croma Meter CM-700D, Japan). Three measurements were performed on the surface (epicarp and mesocarp), and the color parameters L*, a*, and b* were registered. The hue angle [H^0 = arctangent (b^*/a^*)] and Chroma* [$C^* = (a^{*2} + b^{*2})^{1/2}$ parameters were then calculated.

Fruit texture with and without skin was determined on a Texture Analyzer TA.XT plus (Stable Micro Systems Ltd., Surrey, UK) with a cylindrical probe (diameter = 4 mm)with a penetration of 7 mm depth at a speed of 0.83 mm s^{-1}.

Freeze-drying was used for the determination of dry matter.

Chemical traits

Soluble solid content (SSC), pH and total acidity (TA) were determined in juice extracted using a food processor in two replicates of five samples. SSC was determined using a digital refractometer (model PR-1, Atago, SMART-1, Japan) and expressed as %, and TA was analyzed in juices by titration (Metrohm, Herisau, Suiza) with 0.1 N NaOH and expressed as % of citric acid content (g 100 mL^{-1}).

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 Tadmor et al. (2005). 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 of ethanol containing 1 mg mL^{-1} butylated hydroxytoluene (BHT) using a Polytron homogenizer. 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 85ºC. 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 □L of an acetonitrile/methanol/dichloromethane (45:20:35 v/v/v) solution, filtered through a 0.22 □m PTFE syringe filter (Millipore) directly to sample vials, and 10 □L were injected into the chromatograph. The initial mobile phase consisted of acetonitrile/methanol (97:3, v/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 30º C. The analyses were carried out on a HPLC apparatus equipped with binary pump, in-line vacuum degasser, autosampler injector, a *Waters C18 column* a 4.6 x 250 mm C18 60 Å 4 □m Nova-Pak column, and a Nova-Pak Sentry guard cartridge (Waters Co.) and a 996 diode array detector (Waters, Milford, MA) supported by the Empower chromatography manager computing system (Waters) which was used to detect colored carotenoids at 450 nm. The detector was set to monitor spectra from 265 to 500 nm, at a sampling rate of 1 spectrum/s and utilizing an

optical resolution of 2.4 nm. Compounds were identified by comparison of retention times, co-injection with known standards, and comparison of their UV-visible spectra with authentic standards. Quantification was carried out by external standardization. Full standard curves were constructed with five different concentrations for each carotenoid in triplicate. The curves which passed through or were very near the origin, were linear and bracketed the concentrations expected in the samples. Results were expressed on a dry weight (dw) basis.

Statistical Analyses

Data were subject to one-way analysis of variance (ANOVA), and Duncan's multiple range test was used in cases where significance at $P \leq 0.05$ variance was found among genotypes. Correlation analysis was assessed by the Pearson test. Principal component analysis (PCA) was applied to mean values of the measured traits for the 22 summer squash genotypes for which data from all measured traits were available. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

The mean values of physical and chemical characters in epicarp and mesocarp of the 22 summer squash genotypes are listed in Tables 2, 3, 4 and 5. ANOVA indicated significant ($P<0.05$) genotypic variation in individual parameters. This variability is essential for breeding programs focused on the selection of the most adequate lines.

The overall mean weight, length and diameter in the 22 genotypes were 828.2 g, 18.5 cm and 89.4 mm, varying from 223.8 (Cu-27) to 1687 g (Cu-35), from 5.9 (Cu-14) to 28 cm (Cu-29), and from 40.4 (Cu-33) to 131.6 mm (Cu-35), respectively (Table 2). Significant variation was found in weight, length and diameter among the genotypes, with Cu-35 and Cu-15 (vegetable marrow genotypes) having the highest mean weight and diameter. Cu-29 (zucchini) had the highest mean length, and Cu-14 (scallop) showed the lowest mean length.

Table 2. Weight (grams), length (centimeters) and diameter (millimeters) of 22 *Cucurbita pepo* genotypes.

Accession	Weight		Lenght			Diameter	
Cu-1	571,5	cdef	19,6	cdef	3,1	73,3	efg
Cu-4	1021,4	abcde	24,6	ab	7,0	84,3	def
Cu-5	1058,1	abcde	18,8	cdefg	1,9	106,7	abcd
Cu-7	688,4	bcdef	22,4	bcde	4,1	74,0	efg
Cu-8	1166,8	abcd	20,2	bcdef	5,4	96,6	cdef
Cu-9	1345,1	ab	20,6	bcdef	4,1	113,0	abcd
Cu-10	1201,2	abc	19,6	cdef	5,2	107,3	abcd
Cu-11	1242,1	abc	14,3	g	3,5	130,0	ab
Cu-13	991,4	abcdef	17,2	fg	5,0	92,3	cdef
Cu-14	326,9	ef	5,9	h	0,7	115,5	abc
Cu-15	1515,8	a	18,4	defg	1,3	129,7	ab
Cu-17	1083,7	abcde	17,6	efg	3,1	101,9	bcde
Cu-25	373,2	ef	9,6	h	1,2	90,6	cdef
Cu-27	223,8	f	18,4	defg	0,8	47,7	gh
Cu-28	419,5	def	23,1	bcd	1,6	55,6	gh
Cu-29	533,3	cdef	28,0	a	2,0	54,3	gh
Cu-31	372,6	ef	9,7	h	0,7	94,4	cdef
Cu-32	399,7	def	23,5	bc	2,1	51,7	gh
Cu-33	227,4	f	17,3	fg	2,3	40,4	h
Cu-35	1687,0	a	19,0	cdef	3,7	131,6	a
Cu-36	1254,9	abc	19,8	cdef	1,7	108,7	abcd
Cu-37	517,0	cdef	19,4	cdef	1,0	68,3	fgh

Means for weight, length and diameter were compared among accessions using Duncan's multiple comparison test. Means followed by a common letter are not significantly different from each other at $P < 0.05$.

This variability in our result is largely due to the fruit morphology. Each morphotype has a characteristic fruit shape. In general, pumpkin has oval or oblate fruit that is rounded or flat at the ends; scallop has almost discoidal fruit, with undulations or equatorial margins; vegetable marrow has short cylindrical fruit that is slightly broader at the apex, with a smooth rind which hardens and thickens on ripening; and zucchini has cylindrical fruit (Hernándo-Bermejo and León, 1994), except the commercial zucchini (Cu-31 and Cu-25) which have an oval or rounded fruit. Summer squash can be harvested over a wide range of sizes, from <50 g to >400 g, the acceptable size range functions according to market demand. Their differing fruit shapes result in their differential adaptations to various methods of culinary preparation (Paris, 2010).

The color is an indicator of ripeness in fruit and vegetables (Jacobo-Valenzuela et al., 2011). There was a high variability in color, especially for the fruit epicarp which varied from white, light to dark green, plain to minutely speckled with cream or green contrasting with yellow, orange or two colored. In this work, the flesh color types of summer squash genotypes were grouped into fluorescent yellow, yellow, whitish yellow and greenish yellow based on observations at the immature stage. Generally, the flesh color of squash includes a wide range of white, yellow, and orange (Gross, 1991). These colors are based on the specific carotenoid type and concentrations (Paris and Brown, 2005; Tadmor et al., 2005).

Based on the results of this study, the lightness (L*) of epicarp varied from 29.35 to 82.35 (Cu-28 and Cu-15). The redness (a*) and yellowness (b*) values of the epicarp ranged from -7.78 to 21.99 (Cu-8/Cu-11 and Cu-29) and from 6.19 to 61.91 (Cu-28 and Cu-29), respectively (Table 3). L* of mesocarp varied from 63.44 to 86.04 (Cu-27 and Cu-5), and a* and b* of the mesocarp ranged from -3.20 to 1.17 (Cu-32 and Cu-25), and from 13.92 to 40.17 (Cu-14 and Cu-25), respectively. The redness (a*) value of the greenish yellow type of mesocarp color was mostly calculated as negative values, tending to be more green. On the other hand, a* value of the yellow type of mesocarp color was calculated as positive values, more towards red. L* values were lower in epicap than in the mesocarp. *C** ranged in epicarp from 7.44 (Cu-28) to 66.18 (Cu-29), and in mesocarp from 14.00 (Cu-14) to 40.22 (Cu-25). H^{o} varied in epicarp from 70.16 (Cu-29) to 124.36 (Cu-28), and in mesocarp from 88.41 (Cu-25) to 102.87 (Cu-27). Accession with yellow epicarp (Cu-29) showed the highest values of a*, b* and *C**.

Itle and Kabelka (2009) obtained similar results for L*, a*, b*, *C** and H^{o} values in different genotypes of *C. pepo,* 76.5-83.8, -4.9-9.4, 9.8-64.5, 10.0-65.3 and 81.9-

102.9º, respectively, and in *C. moschata*, 70.5-83.2, -4.9-14.8, 31.5-71.4, 31.9-72.2 and 77.5-99.0º. L*, a*, and b* values in *C. moschata* obtained for Jacobo-Valenzuela et al. (2011) were less variable because they only use one genotype, and were 75.66, 9.69 and 18.88 in epicarp, and 73.19, 5.57 and 43.86 in mesocarp.

The force applied (firmness) to penetrate the epicarp was higher than those required in the mesocarp for all accessions (approximately 20% more). The firmness varied from 18.40 N (Cu-9) to 34.31 N (Cu-15) in epicarp, and from 14.36 N (Cu-9) to 26.26 N (Cu-15) in mesocarp (Table 4). The firmness is an interesting parameter because accessions with a resistant epicarp could be better for transport, but they must be tender enough to eat raw.

The overall mean dry matter (DM) in epicarp and mesocarp were 6.68 and 5.21 %, varying from 5.14 (Cu-9) to 10.15 % (Cu-15), and from 3.85 (Cu-7) to 6.56 % (Cu-36), respectively (Table 4). The DM content in the epicarp was greater than in the mesocarp for all accessions, except for Cu-11 genotype where epicarp and mesocarp had a similar content. The dry matter content at the immature stage was low and not highly variable, in mesocarp this parameter increases nearly linearly between 10 to 30 days after pollination (Loy, 2004). Consumer preferences, linked to the different end-uses and processing approaches, influence the target for DM in the fruit. Consumers generally prefer varieties with a high dry matter content (Murphy et al., 1966). Although fruit with a dry matter (DM) content of <20 % is watery and lacks flavor, fruit with over 28 % DM may be unacceptably dry except for processing purposes (Harvey et al., 1997).

Table 3. Color parameters (L*, a*, b*, *C and *H°*) in epicarp and mesocarp of 22 *Cucurbita pepo* genotypes.**

	Epicarp										Mesocarp									
	L*(D65)		a*(D65)		b*(D65)		*C**(D65)		*H°*(D65)		L*(D65)		a*(D65)		b*(D65)		*C**(D65)		*H°*(D65)	
Cu-1	38.84	hij	-5.88	efghij	12.84	f	14.17	ij	116.59	b	83.63	abc	-2.22	fghi	22.06	efghi	22.20	efghi	95.50	bcd
Cu-4	73.03	b	-6.01	efghij	29.51	c	30.13	de	101.58	hi	82.78	abc	-1.11	def	21.23	ghi	21.33	ghi	92.54	def
Cu-5	41.92	hi	-6.73	ghij	15.38	ef	16.87	hi	115.85	bc	86.04	a	-1.24	def	16.73	ij	16.77	ij	94.27	cde
Cu-7	53.17	fg	-7.38	hij	24.21	cd	25.37	defg	108.70	de	80.95	bcd	-2.09	fghi	22.91	efgh	23.02	defgh	94.85	bcde
Cu-8	58.03	def	-7.78	j	25.51	cd	26.70	def	107.26	defg	82.44	abcd	-2.21	fghi	21.62	fghi	21.75	fghi	95.40	bcd
Cu-9	62.09	cdef	-7.22	hij	24.81	cd	25.92	defg	106.56	defgh	82.38	abcd	-1.10	def	19.12	hi	19.17	hij	93.10	cdef
Cu-10	55.82	efg	-7.34	hij	25.09	cd	26.35	defg	108.16	def	83.98	abc	-1.57	defgh	19.16	hi	19.24	hij	94.68	bcde
Cu-11	64.44	bcde	-7.78	j	29.41	c	30.46	d	104.97	efgh	80.80	cd	-1.66	defgh	25.20	cdefg	25.29	cdefg	93.57	cdef
Cu-13	70.69	bc	-7.71	j	35.24	b	36.11	c	102.31	ghi	83.77	abc	-2.61	ghi	23.77	defgh	23.94	defgh	95.91	bc
Cu-14	82.01	a	-4.79	defg	20.18	de	20.74	gh	103.34	fgh	84.63	abc	-1.47	defg	13.92	j	14.00	j	95.98	bc
Cu-15	82.35	a	-3.57	d	24.25	cd	24.53	efg	98.32	i	80.86	cd	-1.99	efghi	22.86	efgh	22.95	efgh	94.90	bcde
Cu-17	66.24	bcd	-6.41	fghij	21.99	d	22.91	fg	106.34	efgh	85.21	ab	-2.22	fghi	28.40	cd	28.50	cd	94.50	cde
Cu-25	69.39	bc	17.82	b	57.78	a	60.98	b	74.73	j	82.25	abcd	1.17	a	40.17	a	40.22	a	88.41	g
Cu-27	32.39	jk	-5.15	defgh	9.75	fg	11.03	jk	119.87	b	63.44	e	-1.45	defg	18.72	hij	19.09	hij	102.87	a
Cu-28	29.35	k	-4.10	de	6.19	g	7.44	k	124.36	a	84.08	abc	-2.15	fghi	20.24	ghi	20.36	ghi	95.71	bc
Cu-29	65.83	bcd	21.99	a	61.91	a	66.18	a	70.16	k	81.76	abcd	0.07	bc	34.19	b	34.28	b	90.82	fg
Cu-31	39.32	hij	-3.44	d	10.96	fg	11.57	ijk	107.86	def	83.52	abc	-2.16	fghi	21.05	ghi	21.18	ghi	95.43	bcd
Cu-32	40.16	hij	-4.92	defg	10.98	fg	12.30	ijk	119.40	b	83.27	abc	-3.20	i	23.00	defgh	23.23	defgh	97.74	b
Cu-33	37.84	ijk	-5.31	defghi	11.71	fg	12.87	ijk	115.04	bc	81.44	bcd	-0.85	cde	27.00	cdef	27.02	cdef	91.85	ef
Cu-35	67.05	bcd	-7.57	ij	28.98	c	29.99	de	104.73	efgh	82.90	abc	-0.45	cd	23.67	defgh	23.70	defgh	90.84	fg
Cu-36	47.81	gh	-4.23	def	13.03	f	13.89	ij	111.42	cd	83.44	abc	-2.79	hi	29.81	bc	29.96	bc	95.25	bcd
Cu-37	71.47	bc	7.82	c	35.79	b	36.67	c	77.78	j	78.39	d	0.96	ab	27.39	cde	27.41	cde	88.03	g

Means for L*, a*, b*, *C** and H^o in epicarp and mesocarp were compared among accessions using Duncan's multiple comparison test. Means followed by a common letter are not significantly different from each other at $P < 0.05$.

Table 4. SSC (ºBrix), pH, TA (% Citric), dry matter (% DM) and firmness (N) in epicarp and mesocarp of 22 *Cucurbita pepo* genotypes.

	Epicarp										Mesocarp									
	SSC (ºBrix)		pH		TA		% DM		Firmness (N)		SSC (ºBrix)		pH		TA		% DM		Firmness (N)	
Cu-1	2,48	ef	6,92	abc	0,11	bc	6,92	bcde	20,49	bc	4,03	abc	6,58	abc	0,11	c	5,16	abc	16,64	cdef
Cu-4	3,15	bcdef	6,68	bcd	0,14	abc	6,97	bcde	22,41	bc	4,09	abc	6,65	abc	0,10	c	6,08	ab	17,51	cdef
Cu-5	2,45	ef	6,65	bcd	0,13	abc	6,12	cde	20,98	bc	4,08	abc	6,52	bc	0,10	c	4,51	bc	16,83	cdef
Cu-7	2,32	e	6,85	abcd	0,12	bc	5,69	de	20,48	bc	4,73	ab	6,78	ab	0,13	bc	3,85	c	16,76	cdef
Cu-8	3,08	bcdef	6,89	abc	0,13	abc	5,65	e	19,12	bc	4,74	a	6,64	abc	0,11	c	5,42	abc	15,93	def
Cu-9	3,99	abcd	6,32	d	0,13	abc	5,14	e	18,40	c	4,55	ab	6,68	abc	0,12	bc	4,91	abc	14,36	f
Cu-10	3,15	bcdef	7,00	abc	0,10	c	6,16	cde	22,48	bc	4,36	abc	6,57	bc	0,11	c	5,66	ab	17,78	bcdef
Cu-11	2,99	cdef	6,61	bcd	0,13	abc	5,46	e	21,05	bc	4,14	abc	6,60	abc	0,10	c	5,50	abc	17,56	cdef
Cu-13	3,40	abcdef	6,78	bcd	0,13	abc	5,95	de	21,12	bc	3,95	abc	6,51	bc	0,13	bc	5,11	abc	17,09	cdef
Cu-14	3,20	bcdef	6,61	bcd	0,10	c	7,03	bcde	26,18	b	3,39	c	6,82	ab	0,10	c	5,08	abc	20,07	bc
Cu-15	3,69	abcde	6,46	cd	0,12	abc	10,15	a	34,31	a	3,71	bc	6,36	c	0,15	ab	5,76	ab	26,26	a
Cu-17	3,74	abcde	6,59	bcd	0,16	a	8,28	abc	20,98	bc	4,12	abc	6,58	abc	0,12	bc	5,43	abc	16,99	cdef
Cu-25	4,40	ab	7,07	ab	0,11	bc	7,02	bcde	22,14	bc	4,07	abc	6,92	a	0,12	bc	6,09	ab	16,05	cdef
Cu-27	2,50	ef	6,56	bcd	0,13	abc	6,55	cde	22,14	bc	4,10	abc	6,81	ab	0,11	c	4,75	bc	19,29	bcde
Cu-28	3,74	abcde	6,69	bcd	0,13	abc	5,94	de	18,96	bc	4,01	abc	6,68	abc	0,11	c	4,70	bc	17,80	bcdef
Cu-29	4,23	abc	6,79	bcd	0,11	bc	6,82	bcde	24,18	bc	4,02	abc	6,85	ab	0,10	c	4,83	bc	21,57	b
Cu-31	3,48	abcdef	6,72	bcd	0,13	abc	6,39	cde	18,64	c	3,38	c	6,80	ab	0,12	bc	5,03	abc	15,59	fe
Cu-32	3,21	bcdef	6,61	bcd	0,13	abc	6,00	de	18,99	bc	3,91	abc	6,75	ab	0,11	c	4,61	bc	17,75	bcdef
Cu-33	2,46	ef	6,53	bcd	0,12	bc	6,48	cde	20,12	bc	3,88	abc	6,73	ab	0,11	c	5,76	ab	18,06	bcdef
Cu-35	2,74	def	7,34	a	0,10	c	7,91	bcd	19,04	bc	4,23	abc	6,67	abc	0,10	c	5,39	abc	16,32	cdef
Cu-36	4,55	a	6,60	bcd	0,14	ab	8,77	ab	21,06	bc	3,87	abc	6,61	abc	0,12	bc	6,56	a	16,91	cdef
Cu-37	3,28	abcdef	6,57	bcd	0,14	abc	5,54	e	24,38	bc	4,62	ab	6,51	bc	0,17	a	4,54	bc	19,92	bcd

Means for SSC, pH, TA, dry matter and firmness in epicarp and mesocarp were compared among accessions using Duncan's multiple comparison test. Means followed by a common letter are not significantly different from each other at $P < 0.05$.

Significant differences were found in soluble solid content (SSC) in epicarp and mesocarp (Table 4). The mesocarp SSC was higher than in the epicarp, except for Cu-36 and Cu-29 genotypes. The lowest and highest contents in epicarp were found in fruits of Cu-7 (2.32 ºBrix) and Cu-36 (4.55 ºBrix), respectively, while in the mesocarp were found among Cu-14 (3.39 ºBrix) and Cu-8 (4.74 ºBrix) genotypes, respectively. The SSC determined in the present work were lower than the results reported by Jacobo-Valenzuela et al., (2011) in the mature fruit of *C. moschata* (6.42). High SSC corresponds with high sugar content and is an important quality factor for sensory impression (Gajc-Wolska et al., 2005).

Total acidity (TA) and pH values were less variable, but significant differences were found among the accessions in both, epicarp and mesocarp (Table 4). The pH values ranged in epicarp from 6.32 (Cu-9) to 7.34 (Cu-35), and in mesocarp from 6.36 (Cu-15) to 6.92 (Cu-25). TA ranged in epicarp from 0.10 (Cu-10, Cu-35 and Cu-14) to 0.16 (Cu-17), and in mesocarp from 0.10 (Cu-4, Cu-5, Cu-11, Cu-35, Cu-14 and Cu-29) to 0.17 (Cu-25). Jacobo-Valenzuela et al. (2011) obtained a similar pH (6.77) in mesocarp of *C. moschata*, but their % citric acid was lower than our result (0.04%).

Considerable variation was found in the content of carotenoid compounds in epicarp and mesocarp from different summer squash genotypes (Table 5).The interest in the uptake of carotenoids has increased enormously during the last years since these lipophilic substances as non-nutritive nutrients have antioxidative properties (Jorgensen and Skibsted, 1993) and produce specific colouration of the food, which is one of the assessed visual quality attributes. In addition, carotenoids are compounds associated with a decrease in the risk of developing certain types of cancer (Giovannucci et al., 1995) and other degenerative and chronic diseases (Klipstein-Grobuschet al., 2000). In particular, lutein and zeaxanthin have been implicated in preventing age-related macular degeneration (Seddon et al., 1994), and β-carotene has provitamin A activity. The major carotenoid present in both fruit tissues was the xanthophyll lutein; zeaxanthin and the hydrocarbons β-carotene appeared in smaller amounts. Lower levels of neoxanthin, violaxanthin, α- and β-cryptoxanthin and α-carotene were also present (not shown). The epicarp showed higher carotenoid content than those in mesocarp, which agrees with results reported in other vegetables, reinforcing the hypothesis of an independent regulation of carotenoid biosynthesis in these tissues (Gross, 1987; Kato et al., 2004; Xu et al., 2006; Alquezar et al., 2008).

The range of the mean total and individual carotenoid contents in epicarp were for total carotenoid content from 68.33 (Cu-14) to 4452.93 mg kg^{-1} DW (Cu-17), lutein from 40.90 (Cu-14) to 4003.65 mg kg^{-1} DW (Cu-17), zeaxanthin from not identified to 125.08 mg kg-1 DW (Cu-17) and for β-carotene from not identified. to 267.64 mg kg^{-1} DW (Cu-27). For the mesocarp, mean values of total carotenoid content ranged from 34.93 (Cu-14) to 371.50 mg kg^{-1} DW (Cu-29), lutein from 31.49 (Cu-14) to 333.35 mg kg^{-1} DW (Cu-29), zeaxanthin from not identified to 5.83 mg kg^{-1} DW (Cu-7) and β-carotene from 3.44 (Cu-14) to 45.07 mg kg^{-1} DW (Cu-11).

Taking into account that high sugar and carotenoid contents are regarded as the better quality products in fruit *Cucurbita* (Gajewski et al., 2008) in our case Cu-36 could be the best accession.

Some authors have also reported data for carotenoid content in *Cucurbita*, lower, similar or higher than those shown in this work. Thus, comparable values were reported in zucchini by El-Quad et al. (2009), who found values in mesocarp for lutein, zeaxanthin and β-carotene of 23.4, 0.41 and 1.46 mg kg^{-1}, respectively. Tadmor et al. (2005) evaluated the carotenoid content in the mesocarp of five pairs of near-isogenic lines of *C. pepo*, and they obtained the following range for total carotenoids, lutein and β-carotene contents: 10.4-187.2, 6.4-143.2 and 4-44.8 mg kg^{-1} dw, respectively (assuming 92% of moisture).

Table 5. Lutein, zeaxanthin (Zea), β-carotene (Beta) and total carotenoids (Total) content in epicarp and mesocarp of 22 *Cucurbita pepo* genotypes.

	Epicarp								Mesocarp							
	Lutein		Zea		Beta		Total		Lutein		Zea		Beta		Total	
Cu-1	467,30	cd	6,35	c	15,96	de	489,61	cde	119,13	cde	1,20	b	7,26	cd	127,59	cd
Cu-4	1587,06	b	26,14	c	204,24	ab	1864,72	b	86,86	cde	2,35	ab	17,46	bcd	106,66	cd
Cu-5	872,28	bcd	0,00	c	21,37	de	893,80	bcde	109,81	cde	0,27	b	21,52	abcd	131,58	cd
Cu-7	1407,54	bc	0,00	c	43,19	de	1450,74	bc	141,80	cde	5,83	a	24,61	abcd	172,23	bcd
Cu-8	524,81	cd	0,00	c	8,45	e	533,26	cde	73,51	cde	0,35	b	23,87	abcd	97,73	cd
Cu-9	801,39	bcd	36,51	c	53,43	de	895,85	bcde	98,55	cde	0,33	b	15,75	bcd	114,64	cd
Cu-10	469,01	cd	15,66	c	29,90	de	519,43	cde	149,47	cde	2,03	ab	21,63	abcd	173,13	bcd
Cu-11	461,45	cd	15,98	c	39,98	de	530,48	cde	163,29	cd	1,95	ab	45,07	a	210,31	bc
Cu-13	563,13	cd	28,17	c	26,96	de	618,25	cde	104,67	cde	1,74	b	28,88	abcd	135,28	Cd
Cu-14	40,90	d	0,00	c	27,43	de	68,33	e	31,49	e	0,00	b	3,44	d	34,93	D
Cu-15	659,08	bcd	18,17	c	103,61	cd	814,67	bcde	86,56	cde	0,54	b	13,84	bcd	100,93	Cd
Cu-17	4003,65	a	125,08	a	224,10	a	4452,93	a	120,62	cde	0,00	b	9,76	bcd	130,37	Cd
Cu-25	615,61	bcd	0,00	c	63,91	cde	679,52	cde	284,71	ab	0,00	b	19,04	abcd	303,75	Ab
Cu-27	396,89	d	7,42	c	267,64	a	673,50	cde	63,74	de	0,79	b	13,45	bcd	77,97	Cd
Cu-28	1426,05	bc	0,00	c	39,80	de	1465,85	bc	142,00	cde	0,00	b	15,89	bcd	157,89	Cd
Cu-29	1035,88	bcd	19,42	c	83,38	cde	1241,17	bcd	333,35	a	3,13	ab	35,02	ab	371,50	A
Cu-31	796,66	bcd	0,00	c	42,13	de	838,78	bcde	194,94	bc	0,00	b	16,48	bcd	211,42	Bc
Cu-32	987,85	bcd	0,00	c	16,86	de	1004,71	bcde	137,69	cde	0,00	b	13,34	bcd	151,02	Cd
Cu-33	793,32	bcd	10,76	c	6,76	e	810,84	bcde	71,32	cde	1,41	b	8,82	bcd	81,55	Cd
Cu-35	209,08	d	6,33	c	25,43	de	243,86	de	154,71	cde	0,39	b	35,64	ab	190,73	Bc
Cu-36	3932,00	a	79,15	b	138,03	bc	4280,59	a	121,94	cde	0,00	b	32,62	abc	154,56	Cd
Cu-37	72,84	d	0,56	c	0,00	e	73,40	e	78,99	cde	1,44	b	4,58	d	85,01	Cd

Means for Lutein, zeaxanthin, β-carotene and total carotenoids in epicarp and mesocarp were compared among accessions using Duncan's multiple comparison test. Means followed by a common letter are not significantly different from each other at $P < 0.05$

Correlations among quality parameters in summer squash

Correlations between physical and chemical characters of summer squash fruits were analyzed and are shown in Table 6. The color parameters (L*, a*, b*, *C**) were positively correlated (*r* >0.53***), while negative correlations were found between these parameters and hue (*r* <-0.82) in both epicarp and mesocarp tissues. Strong and significant positive relation was found between epicarp and mesocarp color parameters (a*, b* and *C**) with lutein and total carotenoid contents in fruit mesocarp (*r* >0.48**). These results indicate that epicarp and mesocarp pigments are related with yellow and orange types of mesocarp color. Therefore, this will assure that indirect selection for high carotenoid content within summer squash breeding material will be successful, easy to implement, and inexpensive. Previous studies have also correlated the color measurement system with carotenoid content in winter squash (Whang et al., 1999; Seroczynska et al., 2006; Itle and Kabelka, 2009; Konopacka et al., 2010; Kim et al., 2012) and other vegetable crops such as tomato (D'Souza et al., 1992; Arias et al., 2000), sweet potato (Simonne et al., 1993; Ameny and Wilson, 1997), pepper (Reeves, 1987; Lee and Lee, 1992), carrot (Park et al., 1995) or cassava roots (Sánchez et al. 2014). A weak positive correlation was found between epicarp and mesocarp SSC with color parameter (L*, a*, b*, *C**), and negative with hue (*r*> 0.3*) (Table 6).

.

Table 6. Correlation coefficients of fruit physical and chemical characters of summer squash.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	1																														
2	n.s.	1																													
3	0,76	-0,43	1																												
4	n.s.	n.s.	n.s.	1																											
5	n.s.	n.s.	n.s.	n.s.	1																										
6	n.s.	n.s.	n.s.	0,31	-0,48	1																									
7	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1																								
8	-0,36	-0,29	n.s.	n.s.	n.s.	n.s.	n.s.	1																							
9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,44	n.s.	1																						
10	n.s.	n.s.	0,38	n.s.	n.s.	-0,33	n.s.	n.s.	n.s.	1																					
11	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,70	1																				
12	n.s.	-0,32	0,41	0,38	n.s.	n.s.	n.s.	n.s.	0,39	0,59	0,33	1																			
13	n.s.	n.s.	-0,28	0,45	n.s.	n.s.	0,36	0,39	0,31	n.s.	n.s.	0,53	1																		
14	n.s.	n.s.	n.s.	0,44	0,35	n.s.	0,29	0,36	0,32	0,28	n.s.	0,77	0,85	1																	
15	n.s.	n.s.	n.s.	0,44	0,36	n.s.	0,29	0,36	0,31	n.s.	n.s.	0,76	0,85	1,00	1																
16	n.s.	n.s.	n.s.	-0,39	n.s.	n.s.	-0,29	-0,28	-0,50	-0,43	n.s.	-0,86	-0,82	-0,93	-0,93	1															
17	n.s.	n.s.	n.s.	0,37	0,28	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1														
18	n.s.	n.s.	n.s.	n.s.	0,30	n.s.	0,29	0,30	0,34	n.s.	n.s.	0,58	0,72	0,79	0,79	-0,79	n.s.	1													
19	n.s.	n.s.	n.s.	0,51	0,34	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,42	0,77	0,77	0,78	-0,69	0,36	0,60	1												
20	n.s.	n.s.	n.s.	0,51	0,33	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,41	0,78	0,77	0,77	-0,69	0,35	0,59	1,00	1											
21	n.s.	n.s.	n.s.	n.s.	-0,30	n.s.	n.s.	n.s.	-0,31	n.s.	n.s.	-0,45	-0,42	-0,55	-0,55	0,58	-0,77	-0,68	-0,63	-0,62	1										
22	n.s.	n.s.	n.s.	0,33	0,30	n.s.	n.s.	n.s.	n.s.	0,37	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1									
23	n.s.	n.s.	n.s.	0,28	0,29	n.s.	n.s.	n.s.	n.s.	0,42	n.s.	0,33	n.s.	0,31	0,31	0,31	n.s.	n.s.	n.s.	n.s.	n.s.	0,37	1								
24	n.s.	n.s.	n.s.	0,38	n.s.	0,46	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,40	n.s.	1							
25	0,28	n.s.	n.s.	0,29	n.s.	0,42	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,32	n.s.	0,75	1						
26	n.s.	n.s.	n.s.	n.s.	n.s.	0,31	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0,49	n.s.	n.s.	n.s.	0,47	0,40	n.s.	0,50	0,56	1					
27	n.s.	n.s.	n.s.	0,38	n.s.	0,46	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,41	n.s.	1,00	0,78	0,56	1				
28	n.s.	n.s.	n.s.	0,38	0,34	n.s.	n.s.	0,28	n.s.	n.s.	-0,28	n.s.	0,54	0,55	0,55	-0,38	n.s.	0,35	0,61	0,61	-0,30	n.s.	0,28	n.s.	n.s.	n.s.	n.s.	1			
29	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1		
30	0,39	n.s.	0,31	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,59	n.s.	1	
31	n.s.	n.s.	n.s.	0,34	0,34	n.s.	n.s.	n.s.	n.s.	n.s.	-0,28	n.s.	0,48	0,52	0,53	-0,34	n.s.	0,32	0,58	0,59	-0,29	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0,99	0,29	0,69	1

1=Weight, 2=Length, 3=Diameter, Epicarp (4=SSC, 5=pH, 6=TA), Mesocarp (7=SSC, 8=pH, 9=TA), 10= Epicarp firmness, 11=mesocarp firmness, Epicarp (12=L*, 13=a*, 14=b*, 15=C*, 16=h), Mesocarp (17=L*, 18=a*, 19=b*, 20=C*, 21=h), 22=Epicarp DM, 23=Mesocarp DM, Epicarp (24=lutein, 25=zeaxanthine, 26= β-carotene, 27= Total carotenoids), Mesocarp (28=lutein, 29=zeaxanthine, 30= β-carotene, 31= Total carotenoids).

Principal component analysis (PCA)

PCA was applied to mean values of physical-chemical traits to study which parameters contributed most to the total data variation. The PCA carried out produced six components accounting for 35.6, 18.4, 10.2, 8.5, 7.2, 5.7% of variance, respectively. The first component was positively correlated with the a*, b* and *C** parameters (epicarp and mesocarp), while negative correlations had the hue parameters (epicarp and mesocarp). The most important variables integrated in the second component were the DM, SSC and TA, lutein, β-carotene, and total carotenoid contents of the epicarp. The third component was positively correlated with the citric acid, lutein, β-carotene and total carotenoid contents (mesocarp), and L* parameter (epicarp). The similarity among genotypes was examined when each sample was plotted using the first and second PC components, which retained 43.2% of the total variance (Figure 1). The highest PC1 values correspond to genotypes with yellow colored epicarp and mesocarp (low *H°* and high a*, b* and *C** (Cu-29 and Cu-25), whereas the negative values indicate genotypes with dark green colored epicarp (high *H°* and low a*, b* and *C**) (Cu-27, Cu-32, Cu-28, Cu-1). Positive values for PC2 indicate genotypes with high DM, SSC and citric acid, lutein, β-carotene and total carotenoid contents of the epicarp (Cu-17 and Cu-36) while the group of genotypes with negative PC2 values indicate genotypes with low DM, SSC and TA, lutein, β-carotene and total carotenoid contents of the epicarp (Cu-14, Cu-37 and Cu-35).

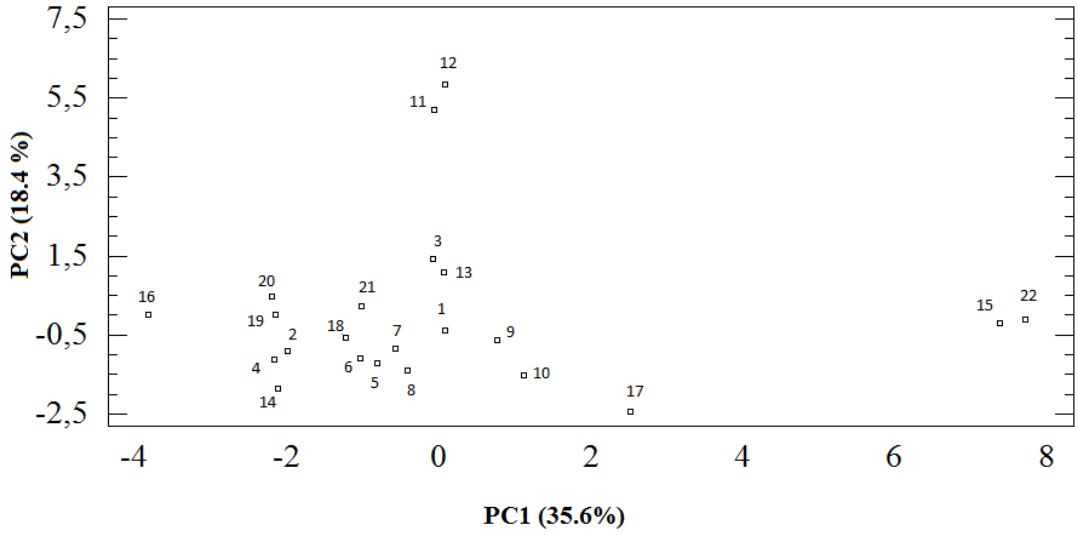


Figure 1. Plot of the first two PCA loading vectors. The labels correspond to sample notation given in the Table 1.

CONCLUSIONS

This study has demonstrated that significant genetic variability exists for the quality parameters analyzed in fruits of different summer squash genotypes. Significant variation was found in weight, length and diameter among the morphotypes, vegetable marrow genotypes having the highest mean weight and diameter, and Cu-14 (scallop) showed the lowest mean length by special shape. This variability in our result is mainly due morphotype. There was a high variability in color, especially for the fruit epicarp. The highest redness (a*) yellowness (b*) and Chroma* values showed Cu-29 because this accession have yellow epicarp. There was variability in the force applied to penetrate both tissues. Epicarp firmness was higher than mesocarp firmness for all accessions (approximately 20% more), being Cu-15 the accession with the highest firmness values in epicarp and mesocarp. The dry matter content at the immature stage was low and not highly variable, and DM content in the epicarp was greater than in the mesocarp for all accessions (except for Cu-11). Soluble solid content (SSC) is an important quality factor for sensory impression, but showed a low variability in both vegetal matrix, being Cu-36 and Cu-8 in epicarp and mesocarp the accessions with the highest values, respectively. Total acidity (TA) and pH values, others parameters related with the fruit quality, were less variable, but significant differences were found among the accessions, Cu-17 and Cu-35 in epicarp and Cu-25 for both parameters in mesocarp, were the accessions with the highest values. Respect to nutritional composition, carotenoids are compounds associated with a decrease of developing certain pathologies, such a some cancers. The carotenoid composition of *C. pepo* fruit tissues was highly variable, and significant differences were found among genotypes. Epicarp showed higher carotenoid content than mesocarp. Lutein was the major carotenoid present in both fruit tissues, and zeaxanthin and β-carotene showed the lowest amounts. Lutein and zeaxanthin are related with ocular health and β-carotene has provitamin A activity. Cu-17 (lutein and zeaxanthin) and Cu-27 (β-carotene) in epicarp, and Cu-29 (lutein), Cu-7 (zeaxanthin) and Cu-11 (β-carotene) showed the highest carotenoid contents. Taking into account both, high sugar and carotenoid contents (two important quality factors), Cu-36 could be a interesting accession to be used in a breeding program. The correlation results indicated that epicarp and mesocarp pigments (carotenoids) are related with yellow and orange types of mesocarp color; this information could be useful to select summer squash breeding material to improved carotenoids content.

REFERENCES

1. Alquezar B., Rodrigo M.J. and Zacarías L. 2008. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. Phytochemistry, 69: 1997–2007.

2. Aliu S., Haziri A., Fetahu S., Aliaga N., Rusinovci I., Haziri I., Arapi V. 2011. Morphological and nutritive variation in a collection of *Cucurbita pepo* L. growing in Kosova. Not Sci Biol, 3(2):119-122

3. Ameny M.A. and Wilson P.W. 1997. Relationship between Hunter color and β-carotene content in white-fleshed African sweetpotato. J Sci Food Agric, 73: 301-306.

4. Arias R., Lee T., Logendra L., Janes H. 2000. Correlation of lycopene measured by HPLC with the L*, a*, b* color readings of a hydroponic tomato and the relationship of maturity with color and lycopene content. Journal of Agricultural Food Chemistry, 48: 1697-1702.

5. Blanco-Díaz M.T., Del Río-Celestino M., Martínez-Valdivieso D., Font R. 2014. Use of visible and near-infrared spectroscopy for predicting antioxidant compounds in summer squash (*Cucurbita pepo* ssp *pepo*). Food Chem. 164:301-8.

6. Consejeria de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucia (2013). http://www.faeca.es/index.php/archivo/documentacion/126-frutas-y-hortalizas/1632-avance-de-lacampana-horticola-de-almeria-2012-2013-fuente-capma.html

7. D'Souza M.C., Singha S. and Ingle M. 1992. Lycopene concentration of tomato fruit can be estimated from chromaticity values. HortScience, 27(5): 465-466.

8. El-Qudah J.M. 2009. Identification and quantification of major carotenoids in some vegetables. Am J Appl Sci, 6(3): 492–497.

9. Ferriol M. and Picó B. 2008. Pumpkin and Winter Squash. In Handbook of Vegetable Breeding. Prohens-Tomas, J., F. Nuez and M.J. Carena (eds.), Springer. Berlin, Germany. pp 317-349.

10. Ferriol M., Picó B. and Nuez F. 2003. Genetic diversity of a germplasm collection of Cucurbita pepo using SRAP and AFLP markers. Theor Appl Genet, 107: 271–282.

11. Formisano G., Roig, C. Esteras C., Ercolano M.R., Nuez F., Monforte A.J., Picó M.B. 2012. Genetic diversity of Spanish *Cucurbita pepo* landraces: an unexploited resource for summer squash breeding. Genetic Resources and Crop Evolution, 59(6): 1169-1184.

12. Gajc-Wolska J., Gajewski M., Radzanowska J., Niemirowicz-Szczytt K., Korzeniewska A. 2005. The fruit quality of chosen hybrids and cultivars of pumpkin (*Cucurbita maxima* Duch.). Veg Crops Re. Bull, 63: 34-43.

13. Gajewski M., Radzanowska J., Danilčenko H., Jariene E., Cerniauskiene J. 2008. Quality of pumpkin cultivars in relation to sensory characteristics. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 36(1): 73-79.

14. Giovannucci E., Ascherio A., Rimm E. B., Stampfer M.J., Colditz G. A., Willet, W.C. 1995. Intake of carotenoids and retinol in relation to risk of prostate cancer. Journal of the National Cancer Institute, 87: 1767-1776.

15. Gross J. 1987. Pigments in Fruits. Academic Press, Londres.

16. Gross J. 1991. Pigments in vegetables: Chlorophylls and carotenoids. Van Nostrand Reinhold, New York, NY.

17. Harvey W.J., Grant D.G. and Lammerink J.P. 1997. Physical and sensory changes during the development and storage of buttercup squash. New Zealand Journal of Crop and Horticultural Science, 25: 341-351.

18. Hernándo-Bermejo J.E. and León J. 1994. Neglected Crops: 1492 from a Different Perspective. Plant Production and Protection Series No. 26, FAO, Rome, pp. 63-77.

19. Iezzoni A.F. and Pritts M.P. 1991. Applications of principal component analysis to horticultural research. HortScience, 26(4): 334–338.

20. Itle R.A. and Kabelka E.A. 2009. Correlation Between L*a*b* Color Space Values and Carotenoid Content in Pumpkins and Squash (Cucurbita spp.). Hortscience, 44(3): 633–637.

21. Jacobo-Valenzuela N., Zazueta-Morales J.J., Gallegos-Infante J.A., Aguilar-Gutierrez F., Camacho-Hernandez I.L., Rocha-Guzman N.E., Gonzalez-Laredo R.F.

2011. Chemical and physicochemical characterization of winter squash (*Cucurbita moschata* D.). Not Bot Horti Agrobot Cluj-Napoca, 39: 34–40.

22. Jorgensen K. and Skibsted, L.H. 1993. Carotenoid scavenging of radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. Z Lebensm Unters Forsch, 196: 423-429.

23. Kato M., Ikoma Y., Matsumoto H., Sugiura M., Hyodo H., Yano M. 2004. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiol, 134: 824–837.

24. Khachik F. and Beecher G.R. 1988. Separation of carotenol fatty acid esters by high performance liquid chromatography. J Chromatogr A, 449: 119–133.

25. Kim, M.J., Shim, C.K., Park, J.H., Hong, S.J., Lee, M.H., Han, E.J., Kim, Y.K., Jee, H.J. 2012. Introduction to Colorimetric Analysis for Assessment of Carotenoid Pigments in Squash Germplasm. Korean Journal of Breeding Science, 44: 433–443.

26. Klipstein-Grobusch K., Launer L.J., Geleijnse J.M., Boeing H., Hofman A., Wittemen J.C.M. 2000. Serum carotenoids and atherosclerosis: The Rotterdam Study. Atherosclerosis, 148: 49-56.

27. Konopacka D., Seroczyńska A., Korzeniewska A., Jesionkowska K., Niemirowicz-Szczytt K., Płocharski W. 2010. Studies on the usefulness of *Cucurbita maxima* for the production of ready-to-eat dried vegetable snacks with a high carotenoid content. LWT - Food Science and Technology, 43(2): 302-309.

28. Lee H.D. and Lee C.H. 1992. Studies on the quality evaluation of Korean red pepper by color measurement. Kor. J. Dietary Culture, 7: 105-112.

29. Lichtenthaler H.K. and Buschmann C. 2001. Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. Current protocols in food analytical chemistry.

30. Loy J.B. 2004. Morpho-physiological aspects of productivity and quality in squash and pumpkins (Cucurbita spp.). Critical reviews in plant sciences, 23(4): 337-363.

31. Manzano S., Martínez C., Domínguez V., Avalos E., Garrido D., Gómez P., Jamilena M. 2009. A major Gene Conferring Reduced Ethylene Sensitivity and Maleness in *Cucurbita pepo*. J Plant Growth Regul, 29: 73–80.

32. Manzano S., Martínez C., Gómez P., Garrido D., Jamilena M. 2010. Cloning and characterization of two CTR1-like genes in Cucurbita pepo: regulation of their expression during male and female flower development. Sex Plant Reprod, 23: 301–313.

33. Martínez-Valdivieso D., Font R., Blanco-Díaz M.T., Moreno-Rojas J.M., Gómez P., Alonso-Moraga A., and Del Río-Celestino M. 2014a. Application of near-infrared reflectance spectroscopy for predicting carotenoid content in summer squash fruit. Comput Electron Agric, 108: 71-79.

34. Martínez-Valdivieso D., Font R., Gómez P., Blanco-Díaz T., Del Río-Celestino M. 2014b. Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy. J Sci Food Agric. 2014 Mar 20. doi: 10.1002/jsfa.6667

35. Murphy E.F., Hepler P.R. and True R.H. 1966. An evaluation of the sensory qualities of inbred lines of squash *(Cucurbita maxima).* American Society for Horticultural Science Proceedings, 89: 483-490.

36. Naudin C. 1856. Nouvelles recherches sur les caracteres specifiques et les varietes des plantes du genre Cucurbita. Ann Sci Nat Bot, Ser. 4,6:5-73, 3 pl.

37. Obrero Á., González-Verdejo C.I., Die J.V., Gómez P., Del Río-Celestino M., Román B. 2013. Carotenogenic gene expression and carotenoid accumulation in three varieties of *Cucurbita pepo* during fruit development. Journal of Agriculture and Food Chemistry, 61: 6393-6403.

38. Paris H.S. 1996. Summer Squash: History, Diversity, and Distribution. Hort Technology Jan./Mar. 1996 6(1).

39. Paris H.S. 2008. Summer Squash. In: J. Prohens-Tomás, F. Nuez, M. J. Carena (eds.), Handbook of Plant Breeding. Vol I. Vegetables I. Springer New York, pp. 351-379.

40. Paris H.S. 2010. Characterization of the *Cucurbita pepo* collection at the Newe Ya ar research Center, Israel. PGR Newslett. 126: 41-45.

41. Paris H.S. and Brown R.N. 2005. The Genes of Pumpkin and Squash. HortScience, 40: 1620-1630.

42. Paris H.S., Daunay M.C., Pitrat M., Janick J. 2006. First Known Image of Cucurbita in Europe, 1503–1508. Annals of Botany, 98: 41–47.

43. Park S.W., Park Y. and Joung, H. 1995. Quality evaluation by color determination in Carrot (*Daucus carota* L.) Estimation of total carotenoid content by Colorimeter. J Kor Soc Hort Sci, 36: 481-485.

44. Reeves M.J. 1987. Re-evaluation of Capsicum color data. J Food Sci, 52: 1047-1049.

45. Sánchez T., Ceballos H., Dufour D., Ortiz D., Morante N., Calle F., Zum Felde T., Domínguez M., Davrieux F. 2014. Prediction of carotenoids, cyanide and dry matter contents in fresh cassava root using NIRS and Hunter color techniques. Food Chem, 151: 444–451.

46. Seddon J. M., Ajani U.A., Sperduto R. D., Hiller R., Blair N., Burton T.C., Farber M.D., Gragoudas E.S., Haller J., Miller D.T., Yannuzzi L.A., Willet W. 1994. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Journal of the American Medical Association, 272: 1413 1420.

47. Seroczyńska A., Korzeniewska A., Sztangret-Wiśniewska J., Niemirowicz-Szczytt K., Gajewski M. 2006. Relationship between carotenoids content and flower or fruit flesh colour of winter squash (*Cucurbita maxima* Duch.). Folia Hortic, 18(2): 51-61.

48. Simonne A.H, Kays S.J, Koehler P.E., Eilenmiller R.R. 1993. Assessment of β-carotene content in sweetpotato breeding lines in relation to dietary requirements. J Food Compos Anal. 6: 336-345.

49. Smith B.D. 1997. The Initial Domestication of *Cucurbita pepo* in the Americas 10,000 years ago. Science Washington D C, Vol. 276, No. 5314

50. Tadmor Y., Paris H.S., Meir A., Schaffer A.A., Lewinsohn E. 2005. Dual role of the pigmentation gene B in affecting carotenoid and vitamin E content in squash (*Cucurbita pepo*) mesocarp. J Agric Food Chem. 53: 9759–9763.

51. Xu C.J., Fraser P.D., Wang W.J., Bramley P. 2006. Differences in the carotenoid content of ordinary citrus and lycopene-accumulating mutants. J Agric Food Chem. 54: 5474–5481.

52. Whang H.J., Park Y.K. and Seog H.M. 1999. Carotenoid pigment of Pumpkin cultivated in Korea. Korean J Food & Nutr. 12: 508-512.

CAPÍTULO II: Mineral composition and potential nutritional contribution of 34 genotypes from different summer squash morphotypes

Chapter II

Artículo publicado en:

European Food Research and Technology

DOI 10.1007/s00217-014-2308-7

Damián Martínez-Valdivieso[a], Pedro Gómez[a], Rafael Font[b], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[b]Department of Postharvest Technology and the Agrifood Industry, IFAPA Center La Mojonera Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

ABSTRACT

Mineral concentrations were determined in fruit of 34 traditional and improved genotypes of *Cucurbita pepo*. Genotypes belong to two subspecies, the subsp. *pepo* (classified into zucchini, vegetable marrow and pumpkin morphotypes) and subsp. *ovifera* (with three varieties: texana, ozarkana and clypeata). Phosphorus, potassium, calcium, magnesium, iron, copper, manganese, zinc and sodium were analyzed, and two distinct patterns of mineral accumulation were found to be evident by cluster analysis. Genotypes in group 1 (zucchini and pumpkin) showed the highest concentrations of total minerals (24,338-62,136 mg kg^{-1} dry weight) as compared to the genotypes in group 2 (vegetable marrow, var. clypeata, var. texana and var. ozarkana). Some genotypes with significant concentrations for different minerals were identified, with the genotype Cu-2 (traditional zucchini) showing the highest concentrations for K, Ca, Mg, Fe, Mn, Zn and Na (4,615, 315, 300, 4.8, 3.03, 3.83 and 9.4 mg kg−1 dry weight, respectively). The zucchini morphotype was superior to other morphotypes studied in terms of contribution to the recommended dietary allowance of mineral content for both men and women. The mineral content of *C. pepo* fruit reported provides a valuable material for breeding programs to generate lines with a significant long-term beneficial impact on human health.

Keywords: *Cucurbita,* Minerals, Epicarp, Mesocarp, Potassium, Zucchini

INTRODUCTION

Cucurbitaceae is a large family including many economically important vegetable crops such as summer and winter squash and melon. *Cucurbita pepo* L., the species with the greatest monetary value of the genus, is consumed virtually worldwide and cultivated intensively in Southeastern Spain, concentrating most of the production in Almeria, reaching more than 350.000 tonnes during the season 2012/2013 [1, 2].

Cucurbita pepo has long been cultivated not only for food but also for their medicinal properties, which have been attributed to both fruit and plant parts [3]. These medicinal properties are due to bioactive compounds such as β-carotene, phenolics, flavonoids, vitamins (including vitamin A, B2, C and E), amino acids, carbohydrates and minerals (especially potassium) [3-6].

In relation to minerals, these compounds are an integral part of human and plant nutrition, and support biological processes during different stages of growth and development [7]. Humans obtain all essential elements mostly from higher plants [8], which is interesting from a nutritional point of view because fruits and vegetables usually contribute to 35, 24 and 11% respectively of the total K, Mg and P to the dietary intake of humans [9]. The proper vegetable consumption can improve the mineral and trace metal regulation and reduce cardiovascular diseases and certain cancer risks [10].

Mineral malnutrition can be addressed through dietary diversification, mineral supplementation, food fortification and/or increasing mineral concentrations in food products. Biofortification, which aims to increase mineral concentrations in edible crops, either agronomically or genetically using both conventional breeding and modern biotechnology, has been adopted by plant scientists to address this problem, and is considered to be the most promising and cost-effective approach to alleviate mineral malnutrition [8, 11].

Previous studies have determined a decrease in the mineral content (especially K, Ca, Mg, Fe, Cu and Na) of fruits and vegetables in recent decades [12]. Taking into account both the problem of malnutrition [8, 11] and the decline in the mineral content of fruits, efforts are necessary to improve the mineral content in vegetables widely distributed throughout the world such as summer squash.

The traditional landraces are an important genetic resource for plant breeders because of their considerable genotypic variations. Evaluation of the primitive cultivars and their

germplasm is used to develop new cultivars with additional nutritional value. A wider genetic base of a species such as *C. pepo*, thus, assumes priority in plant breeding research, developing new varieties for increased traits such as productivity and nutritive value [3].

There is limited information available concerning the genetic variability of the mineral composition of *C. pepo* or the breeding potential for mineral content improvement. Breeding programs have gotten some achievements of interest, mainly aimed at improving plant architecture, to optimize flowering, diversify the type of fruit and improve resistance to some diseases. Progress in improving pumpkins and squash is summarized in two recent reviews [2, 13].

Our study was designed to determine 1) the mineral content in the epicarp and mesocarp fruit; 2) the relationships among mineral contents; and 3) the potential contribution to human nutrition. Minerals selected for analysis were phosphorus, potassium, calcium, magnesium, iron, copper, manganese, zinc and sodium.

MATERIAL AND METHODS

Plant material and greenhouse experiment

A total of thirty-four genotypes, currently kept in the Germoplasm Bank at the "IFAPA La Mojonera" were evaluated in this work (Table 1). They were representatives of traditional and improved cultivars and were classified as follows: thirteen traditional genotypes of *C. pepo*. ssp. *pepo* from Spain (four belonging to the `zucchini´ group, eight to the `vegetable marrow´ group, and one to the `pumpkin´ group) which are cultivated in small orchards used for self-consumption; three genotypes from Israel (`vegetable marrow´); two genotypes from the USA belonging to *C. pepo* ssp. ovifera (L.) D.S.Decker: var. texana (Scheele) and var. ozarkana D.S.Decker-Walters; a genotype from Spain belonging to ssp. ovifera (L.) D.S.Decker var. clypeata corresponding to the `scallop´ group; fifteen commercial hybrids belonging to the `zucchini´ group representatives of the main commercial varieties currently offered in the market.

Table 1. List of 34 *Cucurbita* genotypes included in this study, botanical classification, morphotype, country of origin and source of germoplasm.

Accession	Subspecies	Morphotype	Country of origin (Estate/Region)	Source of Germoplasm
Cu-1	ssp. pepo	vegetable marrow	Spain (Aragón)	COMAV
Cu-2	ssp. pepo	zucchini	Spain (Aragón)	COMAV
Cu-3	ssp. pepo	zucchini	Spain (Andalucía)	COMAV
Cu-4	ssp. pepo	vegetable marrow	Spain (Andalucía)	COMAV
Cu-5	ssp. pepo	vegetable marrow	Spain (Andalucía)	COMAV
Cu-6	ssp. pepo	vegetable marrow	Spain (Asturias)	COMAV
Cu-7	ssp. pepo	zucchini	Spain (Cataluña)	COMAV
Cu-8	ssp. pepo	vegetable marrow	Spain (Cataluña)	COMAV
Cu-9	ssp. pepo	vegetable marrow	Spain (Cataluña)	COMAV
Cu-10	ssp. pepo	vegetable marrow	Spain (Cataluña)	COMAV
Cu-11	ssp. pepo	pumpkin	Spain (Cataluña)	COMAV
Cu-12	ssp. pepo	zucchini	Spain (Murcia)	COMAV
Cu-13	ssp. pepo	vegetable marrow	Unknown (Unknown)	COMAV
Cu-14	ssp. ovifera	scallop	Unknown (Unknown)	COMAV
Cu-15	ssp. pepo	vegetable marrow	USA (Arizona	Israel
Cu-16	ssp. pepo	vegetable marrow	Israel (Unknown)	Israel
Cu-17	ssp. pepo	vegetable marrow	Israel (Unknown)	Israel
Cu-18	ssp. ovifera	var. ozarkana	USA (Arkansas)	USDA
Cu-19	ssp. ovifera	var. texana	USA (Texas)	USDA
Cu-20	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-21	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-22	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-23	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-24	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-25	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-26	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-27	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-28	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-29	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-30	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-31	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-32	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-33	ssp. pepo	zucchini	Spain	Commercial hybrid
Cu-34	ssp. pepo	zucchini	Spain	Commercial hybrid

Seeds of these genotypes were germinated on wet filter paper in Petri dishes at room temperature for 2-4 days in the dark, after which they were transplanted into rock-wool cubes (Grodan BV, 6040KD Roermond, NL) in a greenhouse. When plants had developed three to four leaves, they were transferred to 1-m-large rock-wool slabs at a density of two plants/slab. Plants were grown in a greenhouse in the IFAPA Center in La Mojonera, Almería, Spain (36º47'19''N, 02º42'11''W; 142 m a.s.l.) from March to June 2011 following standard local cultural practices for both plant nutrition and insect pest and disease control.

Fruits (6-10) of each genotype were harvested at an immature stage because they are marketed this way (14-20 cm). Then, they were processed preserving epicarp and mesocarp of each fruit separately, packaged in polypropylene plastic containers and stored at -80ºC.

Dry matter content

Freeze-drying was used for the determination of dry matter. Sample lyophilization was performed using freeze drier equipment (Telstar LyoQuest, Germany) at -55ºC under vacuum (133 x 10^{-3} mBar) for 96 h per sample. Then, the samples were ground and frozen at -80ºC for further analysis.

Analysis of mineral composition of *Cucurbita pepo* fruit

The mineral content of the *C. pepo* fruit was determined using the dry mineralization method. The lyophilized samples were weighed into porcelain crucibles and were mineralized in a muffle furnace (Carbolite CWF 1200, UK) by incineration at 460ºC for 15 h. The ash was bleached after cooling by adding hydrochloric acid, then drying it on thermostatic hotplates and finally maintaining it in a muffle furnace at 460 ºC for 1 h. Subsequently, the elements contained in the ashes were dissolved in an aqueous solution of HCl and were filtered. Finally they were carried to a determined volume of water MiliQ (Millipore Corporation, Bedford, USA). The determination of the different minerals was performed using flame atomic absorption spectrophotometry for Ca, Mg, Fe, Cu, Mn and Zn, except for Na and K, which were analysed by flame atomic emission, and for P which was measured by UV/VIS spectrophotometry. Elemental analysis was performed with a Varian model 240 FS atomic absorption spectrophotometer equipped with an online pump system SIPS-20 and SP-3 auto-sampler, standard air-acetylene flame and single-element hollow cathode lamps and background correction with a deuterium lamp for Mn. Phosphorus was measured using

a spectrophotometer (Helios Alpha UV–Vis spectrophotometer model, Thermo Electron Corporation). All analysis were expressed as mg per kg of dry weight (dw).

Statistical Analysis

To assess the variability of individual mineral concentrations between genotypes, analysis of variance (ANOVA) were performed considering a randomized complete design with six to ten replications. LSD (least significant differences test) was used to compare means, and significance was accepted at $P = 0.05$ level.

The P, K, Ca, Mg, Fe, Cu, Mn, Cu, Zn and Na concentrations in mesocarp of the 34 genotypes were subject to cluster analysis on the basis of distances computed from quantitative variables using FASTCLUS procedure or k-means model.

Correlations between the mineral concentrations were assessed by the Pearson test. Statistical analysis was performed using SPSS.

RESULTS AND DISCUSSION

Dry matter in summer squash fruit

The dry matter content in mesocarp ranged from 3.8 to 12.5 % with the genotypes Cu-7 (zucchini) and Cu-18 (var. ozarkana) showing the lowest and highest values, respectively (Table 3). Because ssp. *pepo* fruits are harvested immature (about 5-7 days after anthesis), dry matter is only 4 to 9.6% [14-16], although in our study the highest contents were achieved by ssp. *ovifera*. Consumer preferences, linked to the different end-users and processing approaches, influence the target for dry matter of the fruit. Thus, the dry matter content of varieties is a critical parameter in squash (*Cucurbita maxima* Duchesne) breeding programs, as consumers generally prefer varieties with high dry matter content [17]. Although fruit with a dry matter (DM) content of <20% is watery and lacks flavour, fruit with over 28% DM may be unacceptably dry except for processing purposes [18]. In our case, it should be possible to produce biofortified summer squash combining high levels of dry matter (<12%) and high mineral content in fruit.

Mineral content in summer squash fruit

The distribution pattern of mineral concentrations in epicarp and mesocarp of 34 summer squash genotypes is shown in Fig. 1. Among the mineral contents, K concentration was positively skewed followed by Na, Cu, Zn and Ca in both fruit tissues

(Fig. 1). The genotypes studied varied considerably in elemental composition as shown by the range and coefficient of variation (CV). High CV was observed for Cu and Na in epicarp, and for Zn, Cu, Fe, Na and Ca in mesocarp (>40%) due to the different genotypes used in this work.

The mineral content of the fruit epicarp was higher than that found in the mesocarp for all the minerals, except Na (Fig. 1). From a nutritional point of view, it should take into account to avoid the removal of the epicarp during minimally processed fresh fruit considering the major portion of elements found in this fruit tissue.

Individual mineral contents determined in epicarp and mesocarp of the *C. pepo* accessions are shown in Tables 2 and 3, respectively. The analysis of variance (ANOVA) indicated significant (P<0.05) genotypic variation among accessions for elemental concentrations. This variability is essential for breeding programs focused on the selection of the most adequate lines.

Previous studies have reported that mineral content is influenced by several factors: degree of maturation, soil type, climatic and storage conditions, geographic location and especially genotype [19]. In the present work, the most important factor was the genotype since all the other factors were constant; thus the genotypes were grown under the same environmental conditions, analyzed at the same stage of maturation and stored under the same conditions.

Phosphorus is among the most abundant of mineral elements in the human body, and deficiencies in P nutrition have historically been seen in rickets and osteomalacia [20]. Significant differences were found in the P content between the genotypes, with Cu-20 (19100 mg kg^{-1}) and Cu-34 (8350 mg kg^{-1}) (both commercial zucchini) having the highest P mean contents in epicarp and mesocarp, respectively.

Potassium is an essential mineral that works to maintain the body's water and acid balance [21] and it plays a role in transmitting nerve impulses to muscles, in muscle contractions and in the maintenance of normal blood pressure [22]. This element was the major element found in *C. pepo* fruit. K content differed significantly among genotypes, with Cu-24 (49795 mg kg^{-1}) (commercial zucchini) and Cu-2 (46150 mg kg^{-1}) (traditional zucchini) genotypes having the highest mean content in epicarp and mesocarp, respectively (Tables 2, 3).

Calcium is an essential nutrient that plays a vital role in neuromuscular function, many enzyme-mediated processes, blood clotting, and providing rigidity to the skeleton by

virtue of its phosphate salts [7]. Significant differences were found in Ca content between the genotypes, with Cu-9 (9550 mg kg^{-1}) and Cu-5 (4750 mg kg^{-1}) (traditional vegetable marrow) having the highest mean calcium content in epicarp and mesocarp, respectively.

Magnesium is a constituent of bone and teeth and is closely associated with calcium and phosphorus. It has many functions in muscles and soft tissues, such as a cofactor of many enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis, and maintenance of the electrical potential of nerve tissues and cell membranes [23]. Magnesium content varied among the genotypes (Tables 2, 3), Cu-33 (4714 mg kg^{-1}) and Cu-31 (3050 mg kg^{-1}) (commercial zucchini) showed the highest mean Mg content in epicarp and mesocarp, respectively.

Iron performs several functions in the body; it helps in the formation of blood, it also helps in the transfer of oxygen and carbon dioxide from one tissue to another [24, 25]. Significant differences were found in Fe content among the genotypes, with Cu-33 (102 mg kg^{-1}) (commercial zucchini) in epicarp, and Cu-23 (55 mg kg^{-1}) (commercial zucchini) in mesocarp having the highest mean Fe contents (Tables 2, 3).

Copper is an essential trace element for humans and is a vital component of several enzymes [26]. Copper was the analyzed mineral present in lower proportion in *C. pepo*. Cu content changed depending on the genotypes, Cu-24 (14.5 mg kg^{-1}) and Cu-29 (4.9 mg kg^{-1}) (commercial zucchini) genotypes, had the highest mean Cu content in epicarp and mesocarp, respectively (Tables 2, 3).

Manganese plays an important role in all mental functions and aids in the transfer of oxygen from lungs to cells and it is important as an activator for enzyme reactions concerned with carbohydrate, fat and protein metabolism [27]. Significant variation was found in the Mn content of genotypes, with Cu-20 (55 mg kg^{-1}) (commercial zucchini) and Cu-5 (38 mg kg^{-1})(traditional vegetable marrow) genotypes having higher mean Mn content than all others in epicarp and mesocarp, respectively (Tables 2, 3).

Zinc plays a very important role in protein and carbohydrate metabolism and also helps in mobilizing vitamin A from its storage site in the liver and facilitates the synthesis of DNA and RNA necessary for cell production [24]. Zinc content varied among the genotypes, Cu-25 (77 mg kg^{-1}) and Cu-30 (274 mg kg^{-1}) (commercial zucchini) having the highest mean Zn contents in epicarp and mesocarp, respectively (Tables 2, 3).

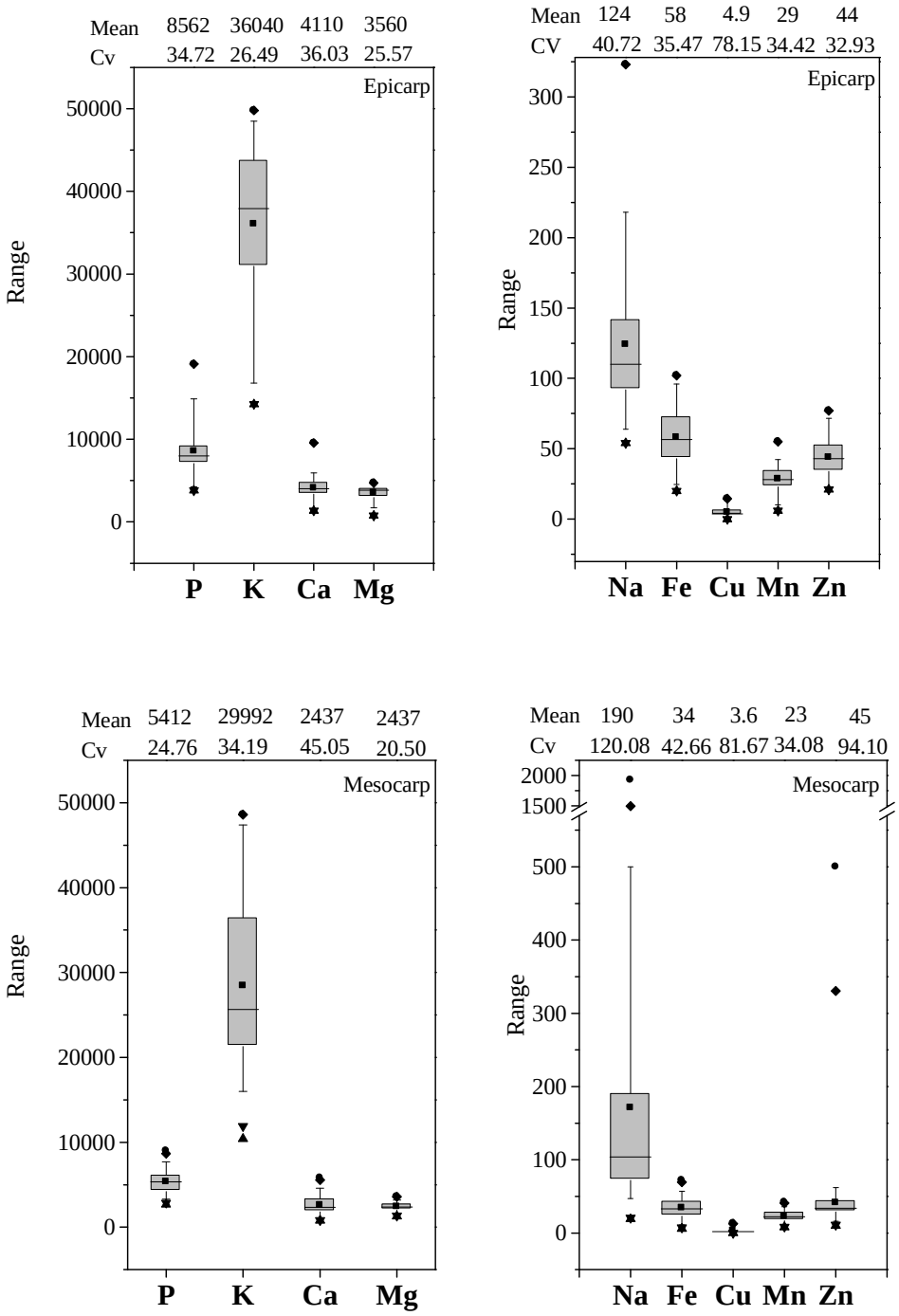


Fig. 1 *Box* and *whisker plots* for the mineral content of the 34 summer squash accessions. *Whiskers* denote minimum and maximum values, and *box* signifies 25th percentile, median, and 75th percentile with mean represented by a *filled square*. *Outliers* are represented by *asterisk*. Coefficient of variation (Cv) was included for indicating the variability among accessions.

Table 2. Epicarp mean dry matter (expressed in %) and mineral content (expressed in mg kg^{-1}) of the 34 genotypes of summer squash.

Ac	Dry matter	P	K	Ca	Mg	Fe	Cu	Mn	Zn	Na
Cu-1	6.9d-i	7163g-i	27771i-l	3833b-g	4027a-f	52h-k	0.8i	28e-j	38g-l	103bc
Cu-2	6.1e-i	9252c-g	41623a-f	4245b-e	4087a-e	73c-g	6.0e-h	33b-h	57c-e	111bc
Cu-3	6.7e-i	7753d-i	32473e-k	3782b-g	3856a-f	54g-k	2.7g-i	28f-j	51c-g	109bc
Cu-4	6.1e-j	7763d-i	40619a-g	4022b-f	3157f-i	49i-k	2.8g-i	28e-j	34h-o	154bc
Cu-5	4.8g-i	15100b	43800a-c	5200b-d	3625d-h	43j-n	2.8g-i	35b-g	28j-o	78c
Cu-6	7.0d-i	6450g-k	24850j-m	4250b-e	3900a-f	46j-m	1.0i	23i-k	37h-m	85c
Cu-7	5.7e-i	7403f-i	33261d-k	4044b-f	3867a-f	57g-k	2.8g-i	27g-j	47d-h	99bc
Cu-8	5.7e-i	8061c-h	38206b-h	3755b-g	3959a-f	57f-k	3.4g-i	28g-j	43e-i	122bc
Cu-9	5.1e-i	6749g-j	31785e-k	9550a	4091a-e	47i-l	0.8i	23h-k	43f-i	98bc
Cu-10	5.0f-i	7068g-i	38145b-h	4694b-e	4102a-e	60e-j	3.1g-i	23h-k	34h-o	140bc
Cu-11	5.1e-i	7950d-h	34250c-j	3350d-h	3550e-h	45j-m	3.5g-i	17k-n	41f-j	131 bc
Cu-12	6.7e-i	8993c-g	41955a-e	5925b	4523a-c	66d-i	3.3g-i	35b-g	47d-h	323a
Cu-13	3.8hi	7578e-i	37577b-i	3179d-h	4026a-f	38k-o	2.2hi	24h-k	40f-k	123bc
Cu-14	7.0d-h	6794g-j	24839j-m	4171b-e	3772b-g	43j-n	4.2f-i	35b-g	46d-h	61c
Cu-15	10.2b-d	5432h-k	23729k-n	2517e-h	1981 lk	20o	0.0i	14k-n	21o	99bc
Cu-16	6.7e-i	7192g-i	30927g-k	3509c-h	2801h-k	40j-o	1.4hi	23h-k	26k-o	92c
Cu-17	8.3b-f	6985g-i	28455h-k	3503c-h	2956g-j	29l-o	0.2l	20j-l	24l-o	105bc
Cu-18	22.0a	4100jk	16450mn	1700f-h	2250j-l	27m-o	4.5f-i	11l-n	21no	174bc
Cu-19	19.4a	3800k	14250m	1550g-h	1650l	24no	1.0i	6n	24m-o	159bc
Cu-20	7.8c-g	19100a	45950ab	3050d-h	2950g-j	88a-c	4.4f-i	55a	59b-d	85c
Cu-21	3.7i	8483c-g	43322a-d	4878b-e	3787b-g	70c-h	4.6f-i	36b-g	45d-h	109bc
Cu-22	7.7c-g	8473c-g	37656b-i	4694b-e	4660ab	73c-g	6.9d-g	31c-i	62bc	89c
Cu-23	11.4b	10484cd	44267a-c	4823b-e	2947g-j	72c-h	9.3b-e	38b-e	44e-i	158bc
Cu-24	4.2hi	10158c-f	49795a	5855bc	4473a-d	98ab	14.5a	39b-d	64a-c	154bc
Cu-25	10.6bc	8900c-g	34350c-j	2550e-h	3800b-g	71c-h	13.0ab	28f-j	77a	76c
Cu-26	8.4b-e	4900i-k	18200l-n	1350h	750m	39k-o	4.0f-i	10mn	31i-o	54c
Cu-27	6.5e-i	7934d-h	31527f-k	3346d-h	2403i-l	81b-d	8.6b-f	31c-i	40f-j	142bc
Cu-28	5.9e-i	8191c-h	47093ab	4825b-e	3683c-h	60e-j	4.0f-i	29d-j	35h-n	159bc
Cu-29	6.8d-i	14000b	44350a-c	3900b-g	4550a-c	78c-e	11.5-d	33b-h	73ab	87c
Cu-30	6.3e-i	10294c-e	47603ab	5079b-d	3959a-f	84a-d	9.7b-e	40bc	53c-f	137bc
Cu-31	6.4e-i	8582c-g	39984a-g	3670b-h	3657c-h	56g-k	6.0e-h	28e-j	42f-j	93c
Cu-32	6.0e-i	8952c-g	47144ab	4658b-e	4384a-e	53h-k	3.5g-i	30c-j	44e-i	141bc
Cu-33	6.5e-i	10146c-f	40404a-g	5415b-d	4714a	102a	11.7-c	38b-f	66a-c	229ab
Cu-34	5.8e-i	10928c	48751a	4869b-e	4158a-e	77c-f	8.3c-f	43b	59b-d	140bc
Mean	7.4	8562	36040	4110	3560	58	4.9	29	44	124

Means for dry matter and each individual and total minerals were compared among accessions using Tukey's multiple comparison test . Means followed by a common letter are not significantly different from each other at $P<0.05$.

Table 3. Mesocarp mean dry matter (expressed in %) and mineral content (expressed in mg kg-1) of the 34 genotypes of summer squash.

Ac	Dry matter	P	K	Ca	Mg	Fe	Cu	Mn	Zn	Na
Cu-1	5.2c-i	6167c-g	28733d-k	2233d-h	3033a	42a-g	1.3g-j	30b-f	38b	94bc
Cu-2	6.7c	5750e-k	46150a	3150b-f	3000ab	48a-d	1.5g-j	32a-d	46b	116bc
Cu-3	5.8c-h	5300e-m	32650c-i	2700c-g	2450a-f	21h-l	1.0h-j	31a-e	32b	206bc
Cu-4	6.3c-f	4650h-n	20000j-l	3350a-d	2400a-f	28g-k	0.5ij	22g-l	27b	151bc
Cu-5	4.5f-i	5400e-m	28450d-k	4750[a]	2500a-e	29g-k	1.5g-j	38a	25b	50c
Cu-6	4.8e-i	4100l-o	15600l	2850c-g	2250a-g	23h-l	0.0j	19j-n	23b	157bc
Cu-7	3.8i	4400j-o	22550i-l	2700c-g	2200b-g	24h-l	1.5g-j	19i-n	35b	119bc
Cu-8	5.4c-i	3150o	17700lk	1450gh	1500g	14kl	2.0f-j	16l-o	25b	225bc
Cu-9	5.2c-i	4300k-o	26100f-l	3100b-f	2500a-e	15j-l	2.0f-j	23g-l	30b	104bc
Cu-10	5.4c-i	3800no	21150j-l	3400a-d	2100d-g	9l	1.5g-j	23g-l	20b	136bc
Cu-11	5.5c-i	4900f-n	24000h-l	1750f-h	2400a-f	22h-l	4.5d-g	17k-o	38b	162bc
Cu-12	5.0c-i	7400a-d	30600d-j	4500ab	2950a-c	48a-d	2.5e-j	34a-c	42b	139bc
Cu-13	5.1c-i	6150c-g	22500i-l	2750c-g	2500a-e	36b-h	1.5g-j	27c-i	39b	117bc
Cu-14	5.1c-i	4200l-o	25400f-l	1200h	2000e-g	32d-j	3.5e-i	19j-n	31b	99bc
Cu-15	5.8c-h	4060l-o	26289e-l	1534gh	2089d-g	16j-l	3.4e-i	12no	33b	124bc
Cu-16	6.0c-g	4100l-o	16000 l	2033d-h	1667gf	17i-l	1.3g-j	10o	19b	193bc
Cu-17	5.4c-i	3918m-o	26088f-l	1554gh	1968e-g	20h-l	3.5e-i	13no	20b	101bc
Cu-18	12.5a	4550i-o	23800h-l	1800f-h	2700a-e	30f-k	3.0e-j	17k-o	33b	325bc
Cu-19	9.8b	4450i-o	26100f-l	1750f-h	2200b-g	18i-l	2.5e-j	12no	31b	250bc
Cu-20	6.4c-e	6100c-h	18050lk	1450gh	2050e-g	43a-g	2.5e-j	33a-d	37b	73c
Cu-21	3.8i	5400e-m	44500ab	3250b-e	2250a-g	51ab	8.0abc	26d-j	48b	117bc
Cu-22	5.3c-i	6300c-f	24750g-l	1450gh	2900a-d	43a-g	5.0c-f	23g-l	58b	60c
Cu-23	6.7cd	5800e-j	35500a-g	2450d-h	3000ab	55a	5.5b-e	26d-j	52b	62c
Cu-24	4.6e-i	5933d-i	36500a-f	3367a-d	3000ab	52ab	7.7a-d	27c-h	50b	399b
Cu-25	6.1c-g	6400b-e	34633b-h	3033c-f	2467a-f	55a	8.3ab	13m-o	62b	66c
Cu-26	4.8e-i	4700g-n	26400e-l	1900e-h	2414a-f	31e-k	4.0e-h	17k-o	33b	120bc
Cu-27	5.3c-i	6147c-h	43975a-c	2101d-h	2503a-e	50a-c	9.9a	20h-m	51b	256bc
Cu-28	4.7e-i	7500a-c	38050a-d	4000a-c	2400a-f	45a-g	1.5g-j	27c-h	36b	350bc
Cu-29	4.8e-i	6700b-e	44900ab	2000d-h	2500a-e	46a-f	10.5a	24e-k	67b	60c
Cu-30	4.1hi	5550e-l	43650a-c	2100d-h	2400a-f	42a-g	9.0a	29b-g	274a	1388a
Cu-31	5.0c-i	4400j-o	34450b-h	3050c-f	3050a	29f-k	2.0f-j	24e-k	30b	89bc
Cu-32	4.6e-i	7850ab	37550a-e	3250b-e	2150c-g	34c-i	0.5ij	24e-k	33b	225bc
Cu-33	5.8c-h	6144c-h	38818a-d	2290d-h	2665a-e	48a-d	7.6a-d	23f-k	56b	194bc
Cu-34	4.4g-i	8350a	38150a-d	3050c-f	2700a-e	47a-e	3.0e-j	36ab	52b	134bc
Mean	5.6	5412	29992	2437	2437	34	3.6	23	45	190

Means for dry matter and each individual and total minerals were compared among accessions using Tukey's multiple comparison test. Means followed by a common letter are not significantly different from each other at $P<0.05$.

Sodium is required by the body to regulate blood pressure and blood volume. It helps regulate the fluid balance in the body; it also helps in the proper functioning of the muscles and nerves [27]. The Na content in epicarp varied among genotypes. On average, Cu-12 (323 mg kg^{-1}) (traditional zucchini) and Cu-30 (1388 mg kg^{-1}) (commercial zucchini) had the highest Na mean content in epicarp and mesocarp, respectively (Tables 2, 3).

There are few studies concerning the mineral and trace elements in summer squash fruits. Ekholm et al. [28] reported that the entire fruit of *C. pepo* for P, K, Ca, Mg, Fe, Cu, Mn and Zn contains 6000, 46600, 3590, 3190, 73, 9, 27 and 40 mg kg^{-1}, respectively. These values are similar for K, Mg and Cu, lower for P, Ca, Mn and Zn and higher for Fe than those found in our study (considering mesocarp values, thus could have been superior taking into account epicarp contents).

Contrasting the results obtained by Harichan and Verma [9], who reported higher Ca, Fe and Mn contents in mesocarp than those found in epicarp of fruit of *C. pepo*. The values found in our study for K, Cu, Mg and Mn contents are higher than those obtained by Harichan and Verma [9] in epicarp (38364, <0.01, 662 and <0.01, mg kg^{-1}, respectively) and mesocarp (20337, <0.01, 438 and 4, respectively); and were lower for Ca, Fe and Na contents in mesocarp (7015, 92, 14947 mg kg^{-1}, respectively).

Our mean contents for P, K, Mg, Mn and Zn exceeded those described in fruit epicarp of *Cucurbita moschata* cv Cehualca (7999, 22550, 3442, 7 and 31 mg kg^{-1}, respectively) and those found in mesocarp for P, Mg, Fe, Mn and Zn (3040, 1590, 32, 3 and 24 mg kg^{-1}, respectively). In addition, *C. moschata* had a higher mean Fe content in epicarp (64 mg kg^{-1}) and K in mesocarp (42194 mg kg^{-1}) and in both epicarp and mesocarp for Ca (5857 and 6685 mg kg^{-1}, respectively), Cu (5.4 and 8.4 mg kg^{-1}, respectively) and Na (707 and 700 mg kg^{-1}, respectively) than *C. pepo* genotypes studied in this work [29].

Therefore, in the present study high mean levels of mineral contents such as P, Zn and Mn (8350, 274 and 38 mg kg^{-1}) were found in mesocarp as compared to previous studies. The high mean levels were found by the fact that a range of summer squash was included in this investigation.

Cluster analysis

The results corresponding to the mineral concentrations in mesocarp of the 34 genotypes grouped within morphotypes are shown in Figs. 2 and 3. The accessions

were clustered into two groups, and there was clear separation between accessions with high (zucchini and pumpkin) and low mineral contents (vegetable marrow, ozarkana, texana and scallop morphotypes). Genotypes in group 1 showed the highest mineral concentrations with mean values of 5155, 29685, 2250, 2495, 32.5, 4.7, 20.8 and 47.4 mg kg^{-1} for P, K, Mg, Fe, Cu, Mn and Zn, respectively (Figs. 2, 3).

This new understanding of patterns of diverse minerals accumulated in summer squash germplasm will be useful in setting objectives and selecting parents for breeding programs aimed at enhancing nutritional traits.

Correlations among mineral contents in summer squash

The relationships among minerals were analyzed by the Pearson correlation analysis (Table 4).

Significantly positive correlations were found among all individual minerals. The highest positive correlations ($r > 0.70^{***}$) in fruit epicarp were observed between P-Mn, Fe-Zn, K-Mn, Fe-Cu, and Fe-Mn; in the case of fruit mesocarp the highest positive correlations ($r>0.60^{***}$) were observed between P-Fe, K-Fe, Mg-Fe, and K-Cu. These results suggested that high Fe content might be accompanied by high P, K, Zn, Mg, Mn and Cu or viceversa. Therefore, selection of a mineral should respond favorably to the selection of other minerals.

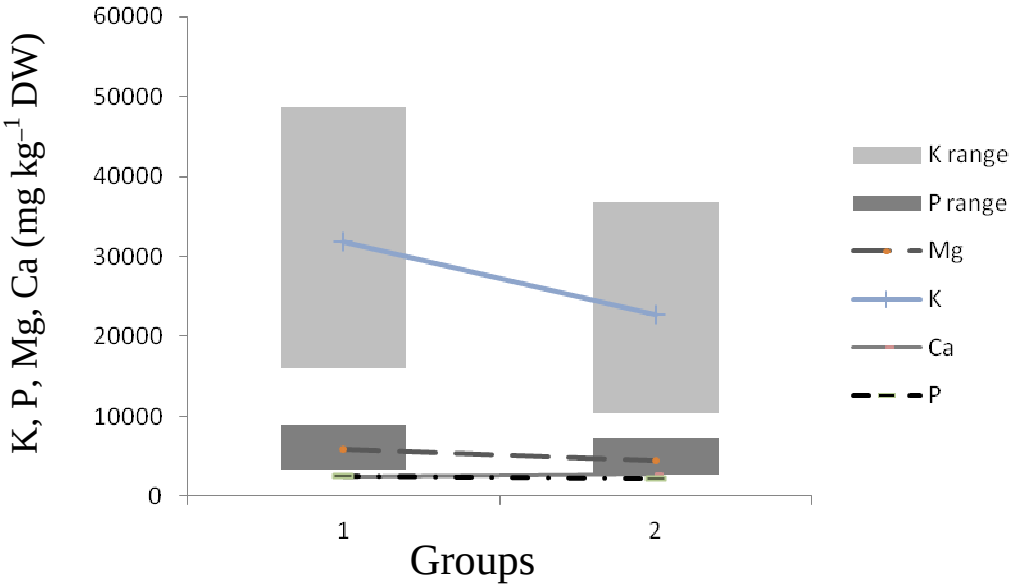


Fig. 2. Mean of mineral concentrations of the two groups obtained by cluster analysis of the 34 *C. pepo* accessions. *K* potassium, *P* phosphorus, *Mg* magnesium, *Ca* calcium. *Shaded bars* indicate range of K concentration in each group.

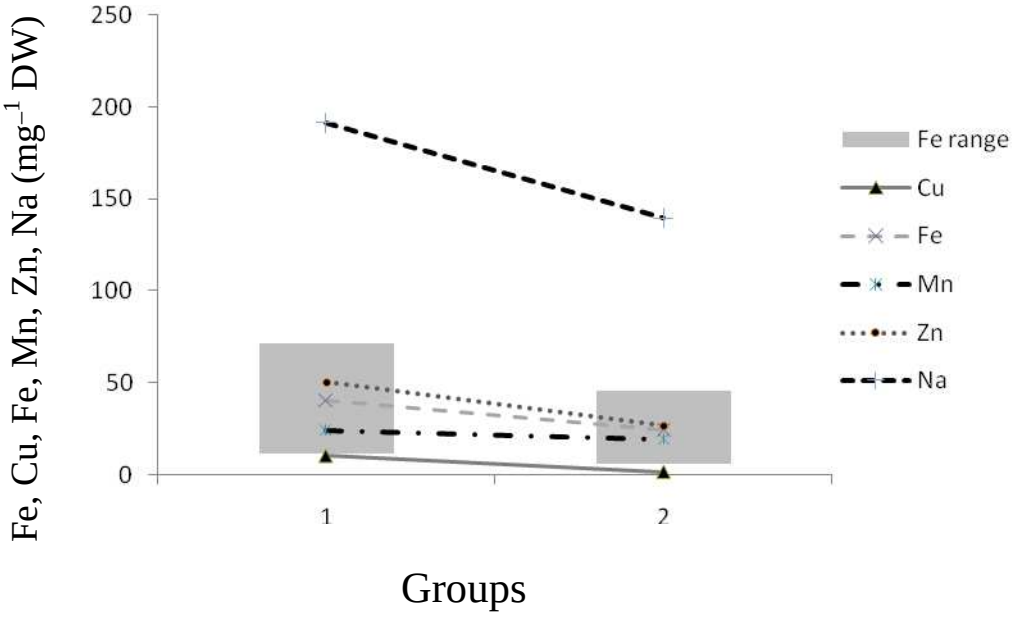


Fig. 3. Mean of mineral concentrations of the two groups obtained by cluster analysis of the 34 *C. pepo* accessions. *Fe* iron, *Cu* copper, *Mn* manganese, *Zn* zinc, *Na* sodium. *Shaded bars* indicate range of Fe concentration in each group.

Potential nutritional contribution of summer squash fruit

Table 5 shows the potential contribution of daily intake of 200 g of summer squash fresh fruits (mesocarp) toward the dietary requirements of minerals. The daily requirements of an adult person (men and women) are as follows (mg d^{-1}): 700 P, 3100 K, 900-1000 Ca, 300-350 Mg, 9-18 Fe, 1.1 Cu, 1.8-2.3 Mn, 7-9.5 Zn, 1300-1500 Na [30]. According to our data, the highest contribution was for potassium. Most of the genotypes belonging to group 1 (zucchini and pumpkin) contain up to 20-25% of the RDA (Recommended Dietary Allowance) for adults for potassium. Deficiencies of iron and zinc are, together with vitamin A and iodine deficiency, globally the leading causes of malnutrition [7, 10, 31]. A portion size (200 g fresh weight) of most of the genotypes will provide at least 5% of the RDA for zinc and 2% of the RDA of iron for adults. For phosphorus, and magnesium, an average portion size of zucchini will provide up to 20% of the RDA for adults. Genotypes of vegetable marrow contain up to 10% of calcium. For copper and manganese, zucchini morphotype showed the highest contents with up to 10 and 7.5%, respectively of the RDA for adults. In addition, the low concentration of sodium (<2%) and the presence of a high amount of potassium recommend the utilization of *C. pepo* in an antihypertensive diet.

Table 4. Correlation coefficients between mineral element contents for epicarp and mesocarp in summer squash fruits.

	P		K		Ca		Mg		Fe		Cu		Mn		Zn	
K	0.66[a]	***														
	0.59[b]	***														
Ca	0.24	*	0.48	***												
	0.40	***	0.34	**												
Mg	0.31	*	0.56	***	0.56	***										
	0.54	***	0.49	***	0.42	***										
Fe	0.54	***	0.63	***	0.35	**	0.53	***								
	0.73	***	0.64	***	0.29	*	0.64	***								
Cu	0.37	**	0.48	***	0.07	n.s.	0.24	n.s.	0.71	***						
	0.25	*	0.60	***	-0.05	n.s.	0.19	n.s.	0.56	***						
Mn	0.79	***	0.73	***	0.44	**	0.52	***	0.71	***	0.43	***				
	0.59	***	0.43	***	0.50	***	0.54	***	0.46	***	0.03	n.s.				
Zn	0.47	***	0.49	***	0.22	n.s.	0.59	***	0.78	***	0.67	***	0.57	***		
	0.15	n.s.	0.32	**	-0.07	n.s.	0.10	n.s.	0.26	*	0.33	**	0.09	n.s.		
Na	0.00	n.s.	0.19	n.s.	0.21	n.s.	0.14	n.s.	0.25	*	0.11	n.s.	0.08	n.s.	0.01	n.s.
	0.04	n.s.	0.24	*	-0.01	n.s.	-0.02	n.s.	0.05	n.s.	0.24	*	0.10	n.s.	0.31	**

[a]Correlation coefficient for Epicarp
[b] Correlation coefficient for Mesocarp
n.s.= no significantly different; *significantly different at P<0.05, ** significantly different at P<0.01, *** significantly different at P < 0.001.

Table 5. Daily nutrient requirements for both men and women an adult person (mg d−1) and potential contribution (%) by 200 g fresh fruits of different morphotypes to nutrient requirements of this life stage.

Element	Recommended (mgd^{-1})	Zucchini	Vegetable marrow	Pumpkin	Scallop	var. oxarkana	var. texana
P	700[a]	8.0 - 20.6	6.2 - 16.7	7.5 - 13.0	8.0 - 11.2	10.3 - 10.5	9.1 - 11.2
K	3100	8.3 - 25.1	5.4 - 19.0	11.0 - 21.2	13.0 - 13.3	12.2 - 12.3	13.5 - 13.5
Ca	900-1000	1.4 - 8.2	2 - 10.3	3 - 9.1	2 - 2.3	3 - 3.4	3 - 3.2
Mg	300-350	10.1 - 19.2	6.9 - 17.1	9.1 - 14.4	10.7 - 10.7	13.3 - 15.5	10.7 - 12.8
Fe	9-18	1.2 - 6.6	0.6 - 4.6	1.4 - 3	2.8 - 3.5	2.5 - 3.4	1.6 - 2.0
Cu	1.1	0.1 - 10.4	0.1 - 3.2	1.6 - 4.8	1.6 - 4.0	1.6 - 3.2	1.6 - 2.4
Mn	1.8-2.3	2.8 - 7.5	1.6 - 6.9	1.4 - 3.0	3.0 - 3.6	2.8 - 3.0	2 - 2.1
Zn	7-9.5	4.3 - 13.5	1.8 - 8.5	2.3 - 6.9	5.3 - 5.7	5.5 - 6	5.3 - 5.7
Na	1300-1500	0 - 2.2	0 - 0.3	0.1 - 0.3	0.1 - 0.2	0.1 - 0.6	0.3 - 0.4

[a] Cuervo,et al. [30].

CONCLUSIONS

This research demonstrates that significant genetic diversity exists for mineral concentration in summer squash germplasm, with potassium as the highest mineral analyzed.

Two different patterns of mineral accumulation were found from the mineral content of fruit mesocarp, for a representative set of 34 summer squash genotypes. The highest total mineral concentrations were found for zucchini and pumpkin morphotypes (group 1) where P, K, Mg, Fe, Cu, Mn and Zn were the major elements found. In this sense, we have also identified genotypes that can be used as sources of variation for the improvement of these nutrients in the fruit like Cu-2, Cu-23, Cu-25, Cu-29, Cu-30, Cu-31 and Cu-34 genotypes belonging to zucchini morphotype, but also Cu-5 among those belonging to vegetable marrow morphotype. In addition, it is possible to develop high dry matter and high mineral content lines, nevertheless, their usefulness for commercial exploitation will depend on the adequate integration of the genes controlling the high dry matter trait (present in the Cu-18 genotype) into inbred lines with a high potential to develop agronomically acceptable hybrids. However, further investigation is required before mineral contents are taken into account in breeding programs. Thus, the study of mineral content in summer squash genotypes grown in various environments would clarify the role of the genotype, environment and genotype by environment (G x E) interactions in mineral content.

To this date few studies have been carried out using diverse germplasm of summer squash to assess the mineral content in fruit. Thus, the values for P, Mn and Zn is to our knowledge higher than any value previously reported for *C. pepo.*

The zucchini morphotype was superior to other morphotypes in terms of contribution to the recommended dietary allowance of P, K, Ca, Mg, Fe, Cu, Mn, Zn and Na for adults with up to 21, 25, 8, 19, 7, 10, 7, 13 and 2%, respectively.

This new understanding of patterns of diverse minerals accumulated in summer squash germplasm will be useful in setting objectives and selecting parents for breeding programs aimed at enhancing nutritional traits.

ACKNOWLEDGEMENTS

Financial support was provided by the Spanish Project INIA-RTA2009-00039-000, FEDER and FSE funds. Damián Martínez-Valdivieso has a contract from INIA-IFAPA (Subprograma FPI-INIA), co-financed by FSE funds (Programa Operativo FSE de Andalucía 2007-2013_"Andalucía se mueve con Europa"). We would also like to thank Nicholas Davies for his help in grammatical revision of the manuscript.

References

1. Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía, http://www.faeca.es/index.php/archivo/documentacion/126-frutas-y-hortalizas/1632-avance-de-la-campana-horticola-de-almeria-2012-2013-fuente-capma.html (2013)

2. Paris HS (2008) Summer Squash. p. 351-379. In: J. Prohens-Tomás, F. Nuez, M. J. Carena (eds.), Handbook of Plant Breeding. Vol I. Vegetables I. Springer New York

3. Aliu S, Rusinovci I, Fetahu S, Zogaj R (2012) Nutritive and mineral composition in a collection of *Cucurbita pepo* L. grown in Kosova. Food and Nutrition Sciences 3:634-638

4. Obrero Á, González-Verdejo CI, Die JV, Gómez P, Del Río-Celestino M, Román B (2013) Carotenogenic gene expression and carotenoid accumulation in three varieties of *Cucurbita pepo* during fruit development. Journal of Agriculture and Food Chemistry 61:6393-6403

5. Rouphael Y, Colla G (2005) Growth, yield, fruit quality and nutrient uptake of hydroponically cultivated zucchini squash as affected by irrigation systems and growing seasons. *Scientia Horticulturae* 105:177-195

6. Tamer CE, İncedayi B, Parseker AS, Yonak S, Çopur ÖU (2010) Evaluation of several Quality Criteria of Low Calorie Pumpkin Dessert. *Notulae Botanicae Horti Agrobotanici* Cluj-*Napoca* 38:76-80

7. FAO/WHO (2001) Human Vitamin and Mineral Requirements. Report of a joint FAO/WHO Expert Consultation, Bangkok, Thailand

8. White PJ, Broadley MR (2009) Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytologist 182:49-84

9. Levander OA (1990) Fruit and vegetable contribution to dietary mineral intake in human health and disease. HortScience 25:1486-1488

10. Harichan S, Verma VN (2013) Investigation of nutrient Elements in *Cucurbita pepo* using Atomic Absorption Spectrometry. International Letters of Chemistry, Physics and Astronomy 2:11-17

11. White PJ, Broadley MR (2005) Biofortifying crops with essential mineral elements. Trends in plant science 10:586-593

12. Mayer, A.M., 1997. Historical changes in the mineral content of fruits and vegetables. British Food Journal 99, 207 – 211.

13. Ferriol M, Picó B (2008) Pumpkin and Winter Squash. In Handbook of Vegetable Breeding. Prohens-Tomas, J., F. Nuez and M.J. Carena (eds.), Springer. Berlin, Germany. pp 317-349

14. Aboul-Nasr MH, Damarany AM, Abdalla MMA (2002) Yield and its components of some summer squash (*Cucurbita pepo* L.) genotypes. In: DN Maynard (ed) *Cucurbitaceae* 2002, ASHS Press, Alexandria, VA, USA, pp 88–94

15. Audrey H, Beany AH, Stoffella PJ, Roe N, Picha DH (2002) Production, Fruit Quality, and Nutritional Value of Spaghetti Squash. In: *J. Janick*, A *Whipkey* (*eds*) *Trends* in *new crops* and *new uses*. *ASHS Press*, *Alexandria*, *VA*, USA, *pp 445–448*.

16. Kmiecik W, Lisiewska Z (1994) The influence of potassium sorbate addition on the quality of pickled zucchini fruit. Roczniki Państwowego Zakładu Higieny 45:301-309

17. Murphy EF, Hepler PR, True R.H (1966) An evaluation of the sensory qualities of inbred lines of squash *(Cucurbita maxima).* American Society for Horticultural Science Proceedings 89:483-490

18. Harvey WJ, Grant DG, Lammerink JP (1997) Physical and sensory changes during the development and storage of buttercup squash. New Zealand Journal of Crop and Horticultural Science 25:341-351

19. Šimič D, Sudar R, Ledencan T, Jamnrovic A, Zdunic Z, Brkic I, Kovacevic V (2009) Genetic variation of bioavailable iron and zinc in grain of a maize population. Journal of Cereal Science 50:392-397

20. Portale AA (1999) Blood calcium, phosphorus, and magnesium, in Favus MJ (ed): Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Philadelphia, PA, Lippincott, Williams & Wilkins, pp 115-118

21. Whitney E, Rolfes S (1999) *Understanding nutrition*. Belmont, Ca., USA, West/Wadsworth. Eighth ed. (ed. W. Rolfes).

22. Lambert IH, Hoffmann EK, Pedersen SF (2008) Cell volume regulation: physiology and pathophysiology. Acta Physiologica 194:255-282

23. Rude RK, Shils ME (2006) Magnesium. In: Shils ME, Shike M, Ross AC, Caballero B, Cousins RJ, eds. Modern Nutrition in Health and Disease.10th ed. Baltimore: Lippincott Williams&Wilkins; pp. 223-247

24. Guthrie HA (1989) Introductory Nutrition (7th ed.). Time mirror Mosby college publishers, Boston

25. McDonald A, Edwards, RA, Greenhulgh FD, Morgan CA (1995) Animal Nutrition. Prentices Hall, London pp.101-122

26. Harris ED (1983) Copper in human and animal health. p.44 in "Trace Elements in Health Ed. Rose, J. Butterworths. London

27. Payne WJA (1990) An Introduction to Animal Husbandry in the Trophics. Longman Publishers Singapore pp 92-110

28. Ekholm P, Reinivuo H, Mattila P, Pakkala H, Koponen J, Happonen A, Hellström J, Ovaskainen M (2007) Changes in the mineral and trace element contents of cereals, fruits and vegetables in Finland. Journal of Food Composition and Analysis 20:487-495

29. Jacobo-Valenzuela N, Zazueta-Morales JJ, Gallegos-Infante JA, Aguilar-Gutiérrez F, Camacho-Hernández IL, Rocha-Guzmán NE, González-Laredo RF (2011) Chemical and physicochemical characterization of winter squash (*Cucurbita moschata* D.). *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 39:34-40

30. Cuervo M, Abete I, Baladia E, Corbalán M, Manera M, Basulto J, ***et al***. (2010) Ingestas dietéticas de referencia (IDR) para la población española. Federación Española de Sociedades de Nutrición, Alimentación y Dietética (FESNAD), Ediciones Universidad de Navarra

31. Ezzatti M, Lopez AD, Rodgers Vander HS, Murray CJ (2002) Selected major risk factors and global regional burden of disease. Lancet 360: 1347–1360

CAPÍTULO III: Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy

Chapter III

Artículo publicado en:

Journal of the Science of Food and Agriculture

DOI 10.1002/jsfa.6667

Damián Martínez-Valdivieso[a], Rafael Font[b], Pedro Gómez[a], Teresa Blanco-Díaz[b], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[b]Department of Postharvest Technology and the Agrifood Industry, IFAPA Center La Mojonera Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

ABSTRACT

Background: Efforts through conventional breeding to improve the mineral content in horticultural crops have not always been successful mainly due to the fact that standard analytical methods are both costly and time-consuming. We investigated the feasibility of applying near infrared reflectance spectroscopy (NIRS) to the estimation of essential mineral composition in the skin and flesh of summer squash fruits (*Cucurbita pepo* subsp. *pepo*) using 200-samples set from diverse morphotypes.

Results: The coefficients of determination in the external validation (R^2 VAL) obtained for the skin and flesh of the fruit were: total mineral content, 0.84 and 0.70; P, 0.74 and 0.62; K, 0.83 and 0.67; Ca, 0.57 and 0.60; Mg, 0.78 and 0.45; Fe, 0.78 and 0.65; Cu, 0.67 and 0.66; Mn, 0.67 and 0.64; Zn, 0.80 and 0.79 and Na, 0.33 and 0.33, respectively.

Conclusions: NIRS combined with different spectral transformations by modified partial least-squares (MPLS) regression has shown to be useful in determining the mineral composition of summer squash fruit, being a fast and low-cost analytical technique. Components such as chlorophyll, starch and lipids were used by MPLS for modeling the predicting equations. The promotion of micronutrient-rich summer squash varieties could have a significant long-term beneficial impact on the health of mineral deficient human populations.

Keywords*:* summer squash; biofortification; NIRS; potassium; iron; calcium

INTRODUCTION

Mineral elements perform a variety of functions in plant cells, and are essential for growth and development in plants as well as in animals and humans. Humans obtain all essential elements mostly from higher plants.[1] Unfortunately, it is estimated that over three billion people are currently malnourished because of lack of minerals, especially iron and zinc, in their diet.[1,2] Biofortification, which aims to increase mineral concentrations in edible crops, either agronomically or genetically using both conventional breeding and modern biotechnology, has been adopted by plant scientists to address this problem, and is considered to be the most promising and cost-effective approach to alleviate mineral malnutrition.[1,3]

Cucurbita pepo L. (2 n = 40), the most economically important crop of the *Cucubita* genus[4], displays eight commercial morphotypes grouped into two sub-species (subsp. *pepo* L.: pumpkin, vegetable marrow, cocozelle and zucchini; subsp. *ovifera* (L.) Decker (syn subsp. *texana* (Scheele) Filov): scallop, acorn, crookneck and straightneck). The main economic value of the species resides in the consumption of its immature fruits as vegetables, commonly known as summer squashes.

Summer squash (*Cucurbita pepo* L.) is an important commercial crop for protected cultivation in the Mediterranean region, especially in Almería (Spain), where the production reaches more than 350000 tonnes each year.[5] Fruits are normally grown under greenhouse conditions using a drip-irrigation system during the spring-summer and the summer-autumn seasons in order to respond to the high demand for this fresh product in both national and international markets.[6]

Previous studies reported that summer squash fruits are a mineral source for human nutrition.[6,7] However, there is no knowledge of any study about the genetic variability of mineral composition of summer squash germplasm collections. Good characterization of this germplasm is needed in order to be useful for breeders and farmers throughout the world.

Chemical determinations of different minerals in vegetables are commonly performed by current techniques, which are time-consuming, expensive and labor-intensive[8]; therefore, they are not adequate for selecting superior lines from a number of summer squash germplasm lines. Thus, a rapid and cost-effective method is in high demand to evaluate mineral quality for *Cucurbita* breeding programs.

Near-infrared spectroscopy (NIRS) is known as a powerful tool for analysis of chemical and physical properties, and it has been applied for determining nutritive value in food and agricultural commodities.[9,10]

Although minerals theoretically do not absorb energy in the near-infrared spectrum, NIR reflectance spectroscopy has been successfully used for determining mineral concentration in plant tissues, due to the association between minerals and organic functional groups in the food matrix and the effect on O□H bonding. The estimation of mineral elements by NIR reflectance spectroscopy is therefore generally dependent on the presence of those elements in mixtures of organic or hydrated compounds and salts (cations and anions).[11] Both macro- and micro-minerals may influence the metabolism of NIR-absorbent components by their effects on plant physiology.[12]

To date, although NIR reflectance spectroscopy has been used to measure mineral content in legumes, forages, grapes, peanuts, woody materials, sediments, wine, cheese, and meat samples it has not been reported in major horticulture crops.[11,13-19]

Since Almería is an important exporter of summer squash, it is necessary to study the composition of the material, to ensure product quality, and to assess the potential use of secondary products such as the skin during the processing to produce puree, minimally processed, frozen and dried products. This study investigates the feasibility of using NIRS to estimate essential mineral composition in the flesh and skin of summer squash fruit, which would have potential application in quality control and in plant breeding programmes as a tool for rapid selection of plants.

MATERIALS AND METHODS

Samples

Plants of summer squash from different morphotypes (vegetable marrow, zucchini and pumpkin) were grown following standard local cultural practices for both plant nutrition and insect pest and disease control in the Center IFAPA La Mojonera (36º47'19''N, 02º42'11''W; 142m a.s.l.). Fruits were harvested from two consecutive seasons (spring-summer and summer-fall) in 2011-12 at the immature stage (commercial size). Skin (n=100) and flesh (n=100) samples (40 zucchini, 30 vegetable marrow and 30 pumpkin) were frozen in sealed polyethylene bags immediately after harvest, and subsequently were lyophilized and ground for analysis. In order to obtain a sufficient volume of meal for standard analytical methods and NIRS analysis, each sample was a combination of two fruits of the same plant.

Reference analysis

For mineral composition analysis of the summer squash the dry mineralization method was used. Washed and freeze-dried samples were weighed into porcelain crucibles and then incinerated in a muffle furnace at 460ºC for 15 h. The ash was bleached after cooling by adding hydrochloric acid, then drying it on thermostatic hotplates and finally maintaining it in a muffle furnace at 460 ºC for 1 h. After cooling, ash recovery was diluted in volumetric flasks with MilliQ water (Millipore Corporation, Bedford, USA).

The determinations (Ca, Mg, Fe, Cu, Mn and Zn) were carried out by flame atomic absorption spectrophotometry, except for Na and K, which were analysed by flame atomic emission and for P which was measured by UV-visible spectrophotometry.

Elemental analyses were performed with a Varian model 240 FS atomic absorption spectrophotometer (Varian, Palo Alto, CA, USA) equipped with a pump system online SIPS-20 and SP-3 auto-sampler, standard air–acetylene flame and single-element hollow cathode lamps and background correction with deuterium lamp for Mn. Phosphorus was measured on a spectrophotometer (Helios Alpha UV–visible spectrophotometer model; Thermo Electron Corporation, Cambridge, UK).

Calculations of total mineral content were based on the sum of individual minerals. All analysis was done in duplicate and expressed as mg per Kg of dry weight. The standard error of laboratory (SEL) was calculated based on duplicate analysis of each sample.[20]

NIRS analysis, calibration and validation development

Spectra of skin and flesh freeze-dried samples of summer squash were obtained in a near infrared spectrophotometer (NIRSystems mod. 6500; Foss-NIRSystems, Inc., Silver Spring, MD, USA) in the reflectance mode, acquiring their spectra over a wavelength range from 400 to 2500 nm (visible and near infrared regions). Samples were scanned in triplicate and the average spectrum was used to develop the multivariate models. Reflectance data was stored as log (1/*R*) (where *R* is the reflectance) at 2 nm intervals (1050 data points).

Samples recorded as an NIR file were checked for spectral outliers [spectra with a standardized distance from the mean (H)>3 (Mahalanobis distance)], using principal component analysis. The objective of this procedure was to detect and, if necessary, remove possible samples whose spectra differed from the other spectra in the set.[21]

Calibration equations for total mineral, P, K, Ca, Mg, Fe, Cu, Mn, Zn and Na were developed using the program GLOBAL v. 1.50 (WINISI II, Infrasoft International, LLC, Port Matilda, PA, USA). Calibration equations were computed using the raw optical data [log(1/*R*), or first or second derivatives of the log(1/*R*) data, with several combinations of derivative (gap) sizes and smoothing [i.e. (0, 0, 1, 1; derivative order, segment of the derivative, first smooth, second smooth); (1, 4, 4, 1); (1, 10, 10, 1); (2, 5, 5, 2); (2, 20, 20, 2)]. Wavelengths from 400 to 2500 nm every 8 nm, were used to perform the different calibration equations. The regression method employed to correlate spectral information and mineral content in the samples was modified partial least squares (MPLS). This regression method is a soft-modelling method[22,23] for constructing predictive models when the factors are many and highly collinear. The final objective of the mathematical procedure is to reduce the high number of spectral data points (absorbance values from 400 to 2500 nm every 2 nm, i.e. 1050 items of data) and to eliminate the correlation of absorbance values presented by neighbouring wavelengths.[24] Standard normal variate and detrend transformations (SNV-DT) were used to correct baseline offset due to scattering effects (differences in particle size among samples).[25] Cross-validation was performed on the calibration set for determining the best number of terms to use in the equation, as well as to determine the ability of each equation to predict an unknown.[26] For any trait analyzed in the external validation, accessions were divided into two groups so that approximately two-thirds could be used for calibration and one-third for the external validation. Calibration and validation accessions were randomly selected, but they were adjusted so that their content standard deviations were similar to ensure that the range and distribution of the two groups would be comparable.

The coefficient of determination (R^2) and standard error (SE) were calculated for both cross-validation and external validation. For all of the parameters analyzed, the mathematical pretreatment that yielded the minimum standard error of cross-validation (SECV) value was considered to be optimal. The predictive ability of the mathematical model was assessed from the coefficient of determination (R^2) and the dimensionless parameters RPD,[10] which is the ratio of the standard deviation for the validation samples to the standard error of prediction (SEP) and RER, which is defined as the ratio of the range in the reference data (validation set) to the SEP. The mathematical expressions of these statistics are as follow:

$$\text{RPD}= SD\left\langle\left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1}$$

where: y_i = lab reference value for the *i*-th sample; $\hat{y}$ = NIR measured value; *N* is the number of samples, *K* is the number of wavelengths used in an equation; and SD is the standard deviation.

$$\text{RER}= range\left\langle\left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1}$$

The definitions of the variables are as given for RPD.

RESULTS AND DISCUSSION

Reference values

One of the main objectives in developing NIRS calibrations is to ensure that a suitable range of the trait of interest is sampled and the level of precision in the reference method is acceptable.

Table 1 shows the descriptive statistics, mean, range, standard deviation (SD) and coefficient of variation (CV) for the elements measured in summer squash samples. The samples analysed varied considerably in elemental composition as shown by the range and CV. High CV was observed for Ca, Fe, Cu and Zn (>40%) possibly due to the different seasons and varieties used. The variability in elemental composition in calibration set was considered suitable for developing NIR calibrations for these elements.

Pearson's correlation coefficients among total and individual mineral contents are shown in Table 2 for the flesh and skin of the summer squash fruit. Significantly positive correlations were observed between them. Total mineral content was significantly positively correlated with all individual minerals for both, flesh and skin, with K (r=0.98) exhibiting the highest correlation. The low and high correlations shown between the total mineral content and many of the individual minerals in the fruit, and also between several pairs of elements, could explain the different prediction accuracy of the NIRS models. Thus, the low correlation exhibited for Na with other minerals, in both flesh and skin, could clarify partially the reduced prediction accuracy of this NIRS model compared to the others.

Table 1. Mean, range, standard deviation (SD), coefficient of variation (CV) and standard error of laboratory (SEL) of total and individual minerals for flesh and skin of summer squash fruit. Data expressed as mg kg^{-1} (dry weigth).

Mineral	Min	Max	Mean	S.D.	CV	SEL
Flesh						
Total	18930	62130	38700	11100	28.68	2984
P	2700	9000	5320	1290	24.25	380
K	10400	48600	28110	8490	30.20	2740
Ca	800	5100	2458	1050	42.72	330
Mg	1300	3500	2370	450	18.98	184
Fe	6	59	32	13.2	41.25	4.3
Cu	0.1	13	3.1	2.9	93.54	0.9
Mn	9	36	21.1	6.6	31.28	2.1
Zn	1	76	36.1	13.8	38.22	3.6
Na	23	200	108.76	46.29	42.56	17
Skin						
Total	20550	73820	45240	15870	35.08	3590
P	3700	10700	7040	2110	29.97	442
K	13300	49500	30850	10970	35.56	2375
Ca	100	5900	3165	1297	40.97	513
Mg	100	4700	3150	1160	36.82	235
Fe	14	95	58.5	19.8	33.85	5.4
Cu	0.1	13	4.4	3.2	72.72	1.3
Mn	5	42	24.1	10.3	42.74	3.2
Zn	17	81	41.8	16.2	38.76	3.6
Na	31.0	184.0	95.8	38.5	40.18	15

Data expressed as mg kg-1 (dry weigth).

Table 2. Pearson's correlation coefficient (*r*) among mineral contents for flesh and skin in summer squash fruits.

	P		K		Ca		Mg		Fe		Cu		Mn		Zn		Na	
K	0.48[a]	***																
	0.73[b]	***																
Ca	0.15	ns	0.36	***														
	0.34	*	0.54	***														
Mg	0.53	***	0.38	***	0.25	*												
	0.50	***	0.72	***	0.65	***												
Fe	0.69	***	0.52	***	0.10	ns	0.63	***										
	0.59	***	0.69	***	0.40	**	0.63	***										
Cu	0.25	**	0.59	***	-0.04	ns	0.20	ns	0.54	***								
	0.47	***	0.43	***	0.03	ns	0.27	ns	0.69	***								
Mn	0.59	***	0.35	***	0.24	*	0.54	***	0.43	***	0.00	ns						
	0.77	***	0.75	***	0.52	***	0.67	***	0.70	***	0.43	***						
Zn	0.18	ns	0.31	***	-0.08	ns	0.13	ns	0.27	*	0.36	***	0.11	ns				
	0.54	***	0.59	***	0.28	*	0.68	***	0.84	***	0.70	***	0.62	***				
Na	0.04	ns	0.27	***	0.00	ns	0.00	ns	0.05	ns	0.31	***	0.11	ns	0.32	***		
	0.13	ns	0.24	ns	0.26	ns	0.24	ns	0.29	*	0.03	ns	0.16	ns	0.07	ns		
Total	0.57	***	0.99	***	0.39	***	0.44	***	0.57	***	0.57	***	0.40	***	0.29	***	0.13	*
	0.79	***	0.98	***	0.63	***	0.78	***	0.71	***	0.42	***	0.80	***	0.62	***	0.25	***

[a]Correlation coefficient for flesh

[b] Correlation coefficient for skin

n.s.= no significantly different; *significantly different at $P<0.05$, ** significantly different at $P<0.01$, *** significantly different at $P < 0.001$.

Spectral features

Typical log(1/*R*) spectra for freeze-dried samples of summer squash, captured by the instrument (Foss Foss-NIRSystems-6500), together with the most relevant absorption bands, are shown in Fig. 1a (skin) and Fig. 1b (flesh).

In the visible region, spectra displayed two peaks characteristic of fruit and vegetable produce -at 440 nm and 670 nm- which correspond to electronic transitions in the blue and red, respectively. Thus, the band at 670 nm has been assigned to chlorophyll,[27] which near 680 nm has a strong inverse correlation with sugar content.[28]

The NIR region of the spectrum (Fig. 1) showed characteristic absorption bands in both tissues (skin and flesh) tested here and especially in skin, these peaks were at 1200 nm and 1448 nm characteristic of sugar absorption;[29] at 1916 nm related to first overtones of water; 1724 and 2348 nm related to C–H stretch first overtones and combination bands of lipids;[30] at 2280 nm related to O–H+C–O deformation, O–H stretch plus deformation, and O–H+C–C stretch of starch.[29]

For NIR spectra, principal component analysis (PCA) was performed on the second derivative (2, 5, 5, 2; SNV+DT) of the spectra as preliminary data examination in order to check the sample population (Fig. 2). For skin samples of summer squash, principal components 1 and 2 explained the 94,14% of the entire spectral variability in the data, and one H-outlier was found. For flesh samples of summer squash, PCs 1 and 2 explained the 94% of the spectral variation.

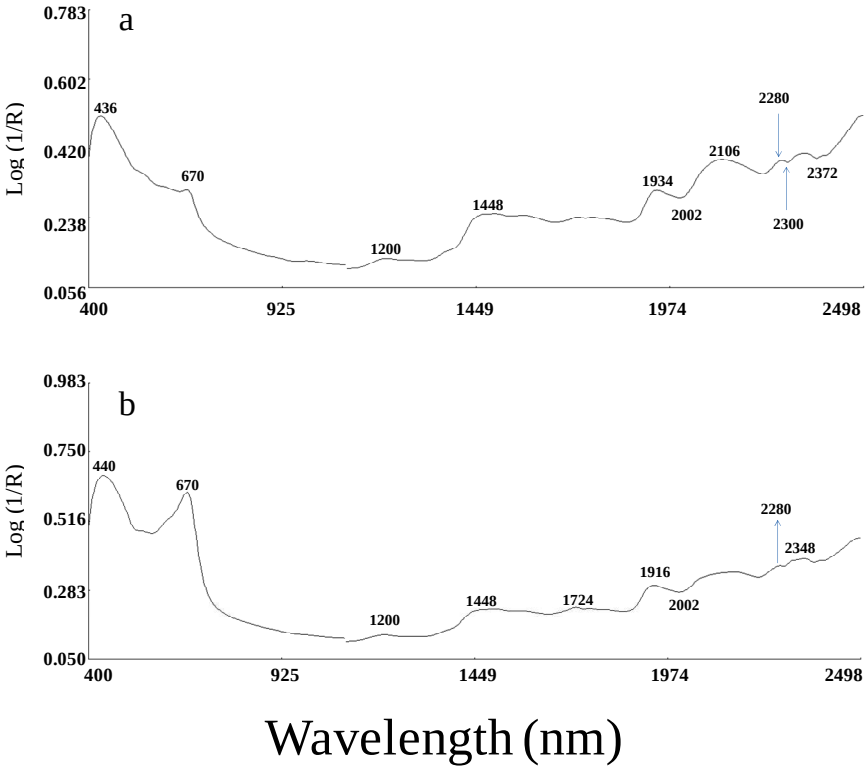


Figure 1. Typical log(1/*R*) spectra for flesh (a) and skin (b) of summer squash fruits.

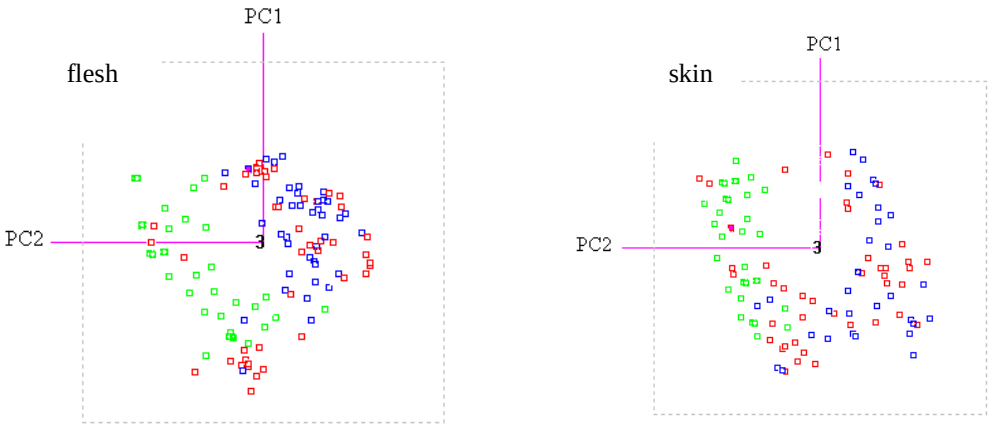


Figure 2. Score plots, obtained by applying principal component analysis to NIR spectra of flesh and skin, in the area defined by the first two principal components. Symbols indicate different samples used in this study. Solid, open and gray squares indicate samples from zucchini, vegetable marrow and pumpkin, respectively.

Calibration and validation

The descriptive statistics of calibration and external validation are shown in Table 3. For the external validation analysis, samples were divided into two groups: 74 and 75 for calibration of skin and flesh samples, respectively, and 25 for external validation.

Statistics obtained in the cross-validation and external-validation processes for minerals are shown in Table 4. For all the minerals studied in this work, the second derivative transformation of the raw optical data, with an interval of 5 nm, and 5 nm and 2 nm for the first and second smooth, respectively, yielded the equations with the highest accuracy in the external validation. Validation of the developed calibration models resulted in coefficients of determination, R^2, ranging from 0.33 for Na (flesh and skin) to 0.84 for total mineral (skin). On the basis of guidelines for interpretation of R^2 VAL,[31,32] the division of NIR calibration models was as follows: (1) R^2= 0.3 to 0.49, poor correlation models, Mg (flesh) and Na (flesh and skin); (2) R^2= 0.50 to 0.69, models usable for rough sample screening, Ca, Mn, Cu, (flesh and skin) and P, K, Fe (flesh); (3) R^2= 0.7 to 0.9, models usable for sample screening, total mineral, Zn (flesh and skin) and P, K, Fe and Mg (skin).

The coefficients of determination R^2 obtained in this work (Table 4) for the different trace minerals agreed with those reported by other authors in grapes[11], forages and legumes.[13,33-35] These authors reported similar results for Fe (R^2: 0.74), Zn (R^2: 0.72), and K (R^2: 0.82) and better results for Na (R^2: 0.83), Cu (R^2: 0.82), Mn (R^2: 0.74) and Ca (R^2: 0.75).

Figure 3 and Fig. 4 show the comparison of laboratory-predicted and NIRS-predicted content of validation set samples. In terms of RPD and RER coefficients, predictive ability of the equations in this work extended from 1.05-2.69, and 4.31-11.03, respectively.

For Na (in the flesh and skin) and Mg (flesh), the validation yielded RPD (1.05-1.44) and RER (4.31-5.93) values, which limit the application of NIRS for predicting these elements. However, for the rest of minerals, the external validation yielded RPD (1.56-2.69) and RER (4.04-11.03) values, indicative of models that could be used for screening purposes, which can be very useful as a selection tool in summer squash breeding programs.

Some examples of similar correlations between element concentration and apparent absorption have been reported in relation to the determination of total trace elements

and macronutrients in other matrices. Some authors have also reported RPD data for mineral analysis in plants, lower, similar or higher than those shown in this work. Thus, comparable RPD values were reported by Ruano-Ramos *et al*.[37] (1.69-3.67) to evaluate NIRS calibrations for ash content in grassland samples. Other authors, demonstrated the utility of NIRS calibrations to enable very rough screening for ashes and calcium in intact seeds of common beans (RPD 2.03 and 2.4), whereas the RPD values for Mg were lower (RPD 1.33-1.5)[38], while Andrés *et al*.[39] reported an RPD value of 1.05 for ashes by NIRS in natural meadows. On the other hand, Cozzolino *et al.*[11] reported a SD to standard error of prediction ratio in validation from 1.5 for the prediction of Fe and Ca to 2.2 for Mg in grapes. Sauvage *et al.*[40] obtained RPD ratios that ranged from 2.70 to 2.85 for PLS calibrations of Na, K, Mg, and Ca in white wines by using NIR transmission. Cozzolino and Morón[13] developed successful equations predicting macro-elements in legume crops, which presented RPD ratios of 1.61 to 2.38 for Zn and Mn, respectively. Similar ratios were reported for legume forage crops by Moron and Cozzolino[41], who found values that ranged from 1.69 to 2.32 for P, Mg and Phan-Thien *et al.*[17] reported remarkable results estimating peanut essential minerals by NIRS with RPD ratios that ranged from 1.85 (Ca) to 2.25 (K). However, prediction errors related to micro-nutrients and trace metals are sometimes higher than those previously mentioned, depending on the element being predicted. Vázquez de Aldana *et al.*[33] found standard error of prediction in an external validation to SD ratios of 1.51 to 1.88 for manganese and zinc, respectively, in grasslands. Font *et al.*[35] reported RPD ratios that ranged from 1.34 (Zn) to 1.72 (Pb) in *Brassica juncea* plants grown in polluted soils. Much more diverse was the prediction data reported by Clark *et al.*[42] for various macro- and micronutrients in three forage species. The RPD ratio found by these authors showed values that ranged from 0.71 (Fe) to 2.08 (K) in alfalfa.

To evaluate the predictive ability of equations in relation to the overall error of the reference method, the SEL was calculated and related to SEP. The SEP/SEL ratio shown by total and individual mineral equations (1.54-2.64), classified them as having good precisions.[20]

MPLS Loadings

To reduce the spectral information of the samples by creating a much smaller number of new orthogonal variables (factors) which are combinations of the original data, and which retain the essential information needed to predict the composition, MPLS regression was employed (Fig. 5). It has been stated that the success of estimation via

NIRS of specific mineral elements in some grasses and legumes is usually dependent on the occurrence of those elements in either organic or hydrated molecules.[33,42]

Figure 5a and b show loading plots corresponding to the best models obtained for predicting mineral contents in both flesh and skin. These plots show the areas across the spectral range where variance has influenced computing of the model to a greater or lesser degree, and the direction (positive or negative).

The areas of the spectrum exerting greatest weight on model fitting were between 500 and 704nm in the visible region related to absorptions by plant pigments; the NIR region showed that the wavelengths at 1140, 1400, 1490 and 1900 nm related to the absorption of glucides and water and 1900-2400 nm related with C-H combinations and overtones had also a large influence on the different calibration models developed[11,43,44] (Fig. 5). A marked positive influence was noted at 1930 nm, an absorption area characteristic of cellulose.[29]

Other authors who used NIRS for predicting minerals in forages, grapes, rocket, wine and legumes reported similar absorption regions, although some differences at specific wavelength absorptions were found. Because trace elements are found in different complexes and the complexes appear to be different both within and among forages and legumes, this will lead to differences in selected wavelengths.[11,13,18,23] Thus, some minerals (e.g. Ca and P) may be indirectly detected through their linkage with diverse organic complexes such as chelates and pigments.[11,43,44] This is the case with P-OH in phosphate associated with chelates or organic matter, Ca with malate and Mg with chlorophyll. For the other elements the loadings were spread along the NIR region, being difficult to assign to any particular bond or chemical structure.[42-44]

Table 3. Descriptive statistics of calibration and validation sets in summer squash fruits.

	Calibration set			Validation set		
Trait	range	mean	SD	range	mean	SD
Flesh						
Total	18930-62130	37567	11458	16633-61761	36920	10527
P	2700-9000	5231	1345	3300-8200	5500	1264
K	14100-48600	28298	9884	10400-47600	28148	8890
Ca	800-5100	2445	1062	800-4600	2572	1524
Mg	1300-3500	2416	408	1400-3400	2455	521
Fe	6-59	33	13	9-66	33	16
Cu	0.1-13	3.5	2.9	0.1-12	2.6	2.9
Mn	9-36	21	6.6	9-42	22	8.1
Zn	1-76	36	14	18-62	36	12.4
Na	23-200	103	44.4	24-200	112	49
Skin						
Total	20550-73820	45944	16258	23250-72144	41685	16405
P	3700-10700	7040	2108	3900-10497	7010	1995
K	13300-49500	30852	10974	15400-48900	30310	11475
Ca	100-5900	3308	1370	150-5800	2934	1516
Mg	100-4700	3151	1157	110-4624	2980	1173
Fe	14-95	51	19.7	15-90	48.2	18.3
Cu	0.1-13	4.5	3.5	0.1-12	4.1	3.12
Mn	5-42	24.1	10.3	6-41	25	9.81
Zn	17-81	42	16	18-79	41	15.1
Na	31-184	96	36.3	32-183	86	37

Table 4. Cross-validation and external validation statistics for summer squash fruit measured by near infrared spectroscopy.

	NT [a]	R^2CV [b]	SECV [c]	R^2VAL [d]	SEP [e]	RPD [f]	RER [g]
Flesh							
Total	6	0.79	4923	0.70	5263	2.00	8.57
P	6	0.85	724	0.62	795	1.60	6.16
K	5	0.73	5238	0.67	5558	1.60	6.69
Ca	7	0.63	638	0.60	862	1.76	4.40
Mg	2	0.47	335	0.45	337	1.44	5.93
Fe	6	0.63	8	0.65	9.32	1.71	6.11
Cu	5	0.67	1.6	0.66	1.7	1.70	7.00
Mn	5	0.74	3.4	0.64	4.7	1.72	7.02
Zn	6	0.83	4.4	0.79	5.68	2.18	7.74
Na	2	0.38	36.3	0.33	39.8	1.18	4.42
Skin							
Total	7	0.84	6761	0.84	6913	2.37	7.07
P	7	0.85	860	0.74	989	2.02	6.67
K	7	0.85	4225	0.83	4642	2.47	7.22
Ca	6	0.62	1027	0.57	969	1.56	5.83
Mg	7	0.84	464	0.78	525	2.23	8.80
Fe	6	0.80	9.4	0.78	9.0	2.69	11.03
Cu	6	0.70	1.7	0.67	2.0	1.56	5.95
Mn	5	0.75	4.6	0.67	5.0	1.96	5.1
Zn	7	0.83	6.5	0.80	6.56	2.30	9.3
Na	1	0.38	33	0.33	395	1.05	4.31

[a] NT: number of terms of the equation selected in the cross-validation
[b] R^2 CV: coefficient of determination in cross-validation.
[c] SECV: standard error of cross-validation.
[d] R^2VAL: coefficient of determination in the external validation
[e] SEP: standard error of performance
[f] RPD: ratio SD to SEP
[g] RER: ratio of the range to standard error of prediction (performance)

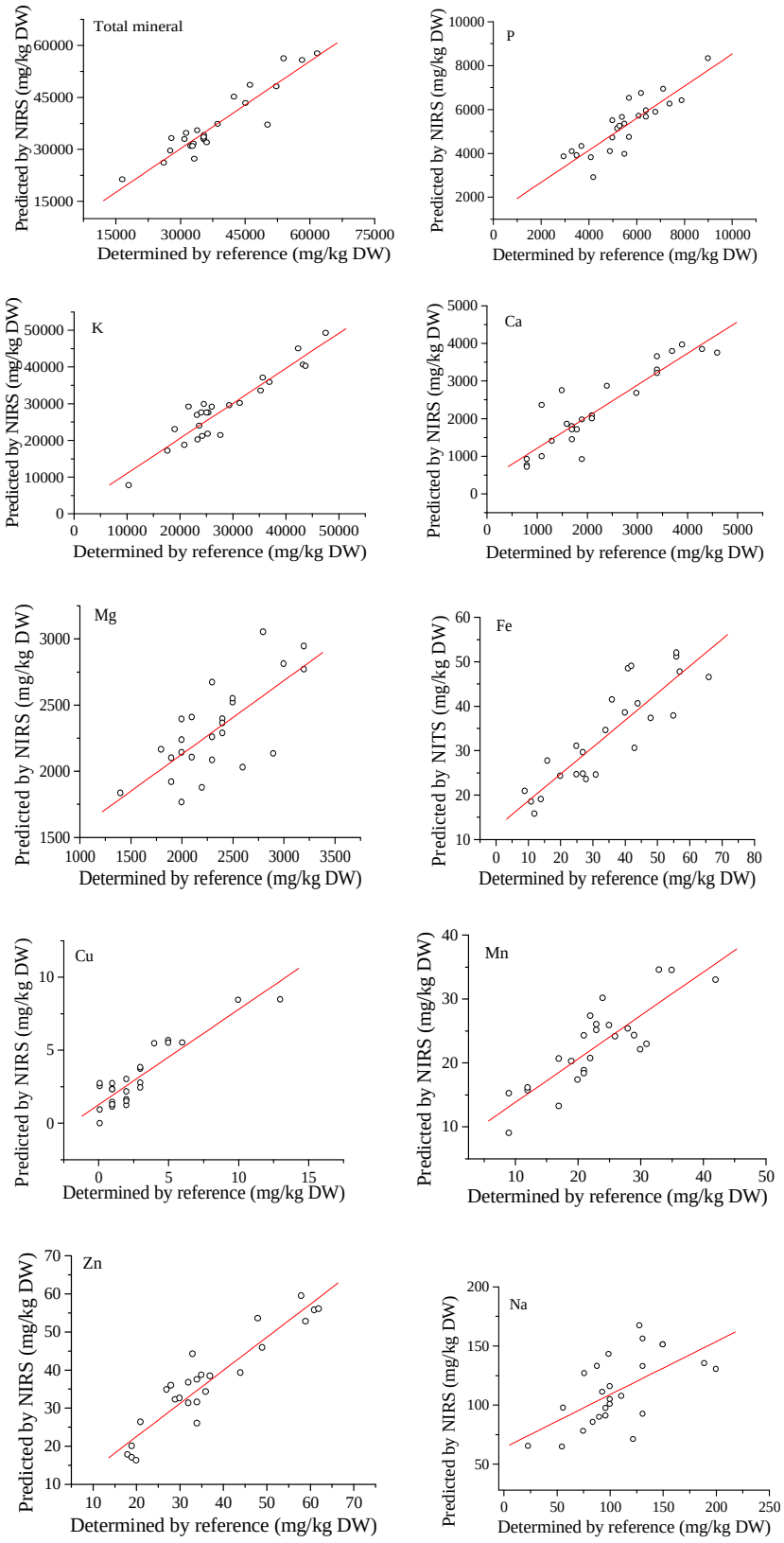


Figure 3. External validation scatter plot for near infrared predicted values versus reference values for total and individual mineral content in flesh of summer squash fruits.

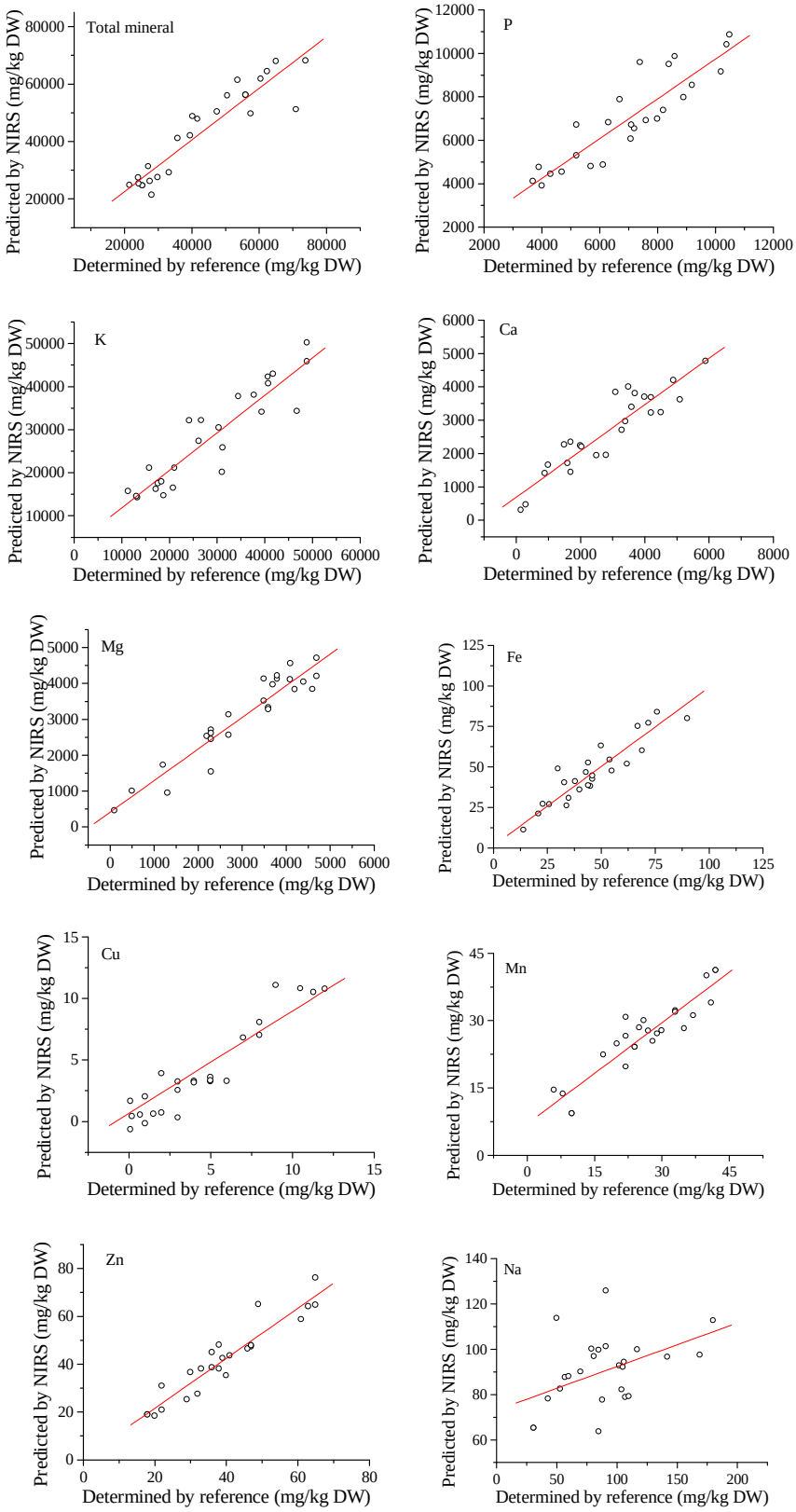


Figure 4. External validation scatter plot for near infrared predicted values versus reference values for total and individual mineral content in skin of summer squash fruits.

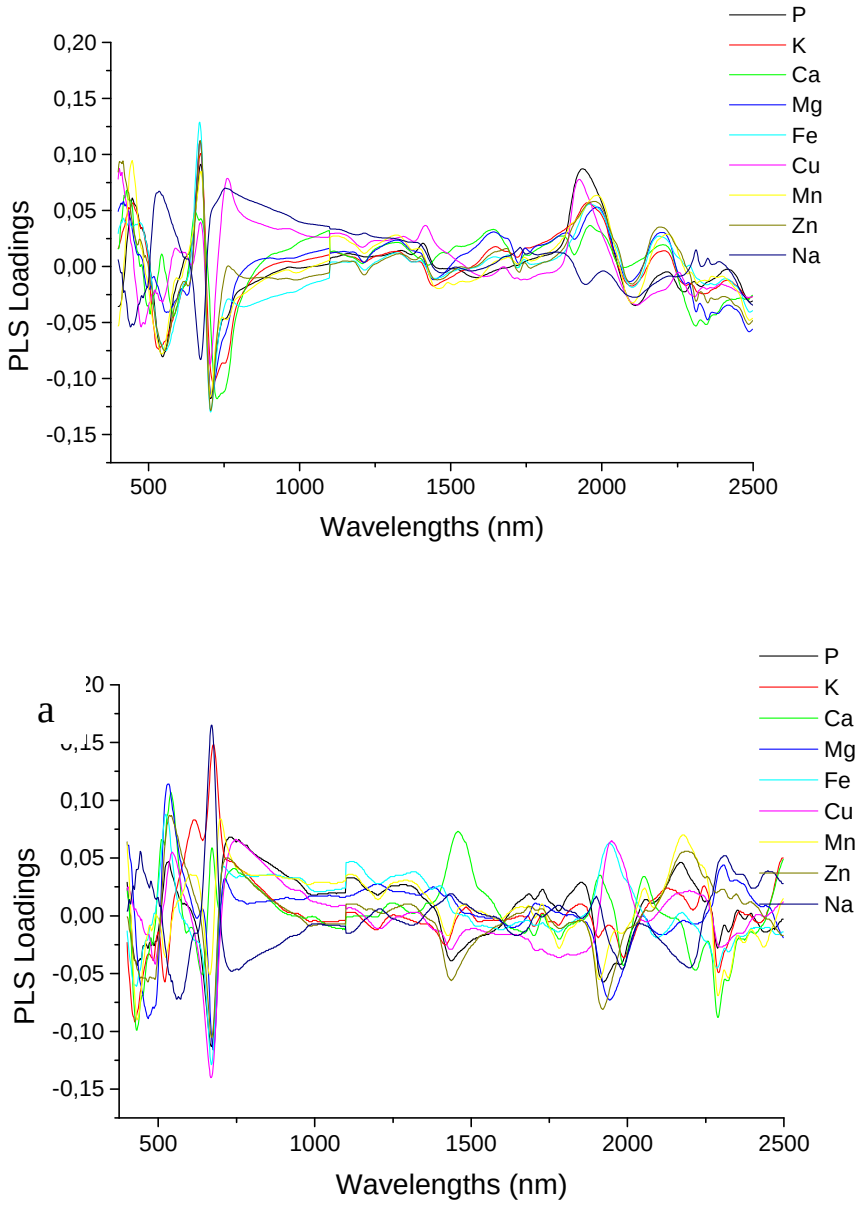


Figure 5. Modified partial least squares (MPLS) loadings for elements measured in flesh (a) and skin (b) of summer squash fruits using near infrared reflectance spectroscopy.

CONCLUSIONS

From the data reported in this work it is concluded that the NIRS technology can be used for screening purposes of total mineral, P, K, Ca, Fe, Cu, Mn, Zn (in the flesh and skin) and Mg (skin) contents in freeze-dried samples of summer squash. The use of this technique represents an important reduction of the analysis time, at a low cost and without using hazardous chemicals, and will be used for quality control and in future research aiming to select the best genotypes after the screening of thousands of plants in a breeding program of summer squash fruit.

Furthermore, deficiencies during the irrigation of the summer squash crop, which affect the quality parameters of the fruit (mineral composition), might be detected early in the greenhouse, which is interesting from a nutritional point of view because fruits and vegetables contribute highly to the dietary intake of humans.

ACKNOWLEDGEMENTS

Financial support was provided by the Spanish Project INIA-RTA00039-000, FEDER and FSE funds. Damián Martínez-Valdivieso has a contract from INIA-IFAPA (Subprograma FPI-INIA), co-financed by FSE funds (Programa Operativo FSE de Andalucia 2007-2013_"Andalucia se mueve con Europa"). We thank Nicholas Davies for his help in grammatical revision of the manuscript.

REFERENCES

1 White PJ and Broadley MR, Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol* **182**: 49-84 (2009).

2 Welch RM and Graham RD, Breeding for micronutrients in staple food crops from a human nutrition perspective. *J Exp Bot* **55**:353-364 (2004).

3 White PJ and Broadley MR, Biofortifying crops with essential mineral elements. Trends *Plant Sci* **10**:586-593 (2005).

4 Paris H, Summer Squash, in *Handbook of Plant Breeding, Vegetables I.* Part 4, ed. by Prohens J, Nuez, F. Volume 1. Springer, 351-381 (2008).

5 Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía, http://www.faeca.es/index.php/archivo/documentacion/126-frutas-y-hortalizas/1632-avance-de-la-campana-horticola-de-almeria-2012-2013-fuente-capma.html (2013).

6 Rouphael Y and Colla G, Growth, yield, fruit quality and nutrient uptake of hydroponically cultivated zucchini squash as affected by irrigation systems and growing seasons. *Sci Hortic* **105**:177-195 (2005).

7 Aliu S, Rusinovci I, Fetahu S and Zogaj R, Nutritive and Mineral Composition in a Collection of *Cucurbita pepo* L. Grown in Kosova. J *Food* Nutr Sci **3**:634-638 (2012).

8 Munter RC, Halverson TL, Anderson RD, Quality assurance for plant tissue analysis by ICP-AES. *Commun Soil Sci Plant Anal* **15**:1285-1322 (1984).

9 Batten GD, Plant analysis using near infrared reflectance spectroscopy: The potential and the limitation. *Aust J Exp Agr* **38**:697–706 (1998).

10 Williams P and Norris K, Near-Infrared Technology in the Agricultural and Food Industries, St. Paul, MN: AACC (2001).

11 Cozzolino D, Cynkar W, Shah N and Smith P, Quantitative analysis of minerals and electric conductivity of red grape homogenates by near infrared reflectance spectroscopy. *Comput Electron Agric* **77**:81-85 (2011).

12 Font R, Río-Celestino M and Haro-Bailón A, 2007 Near-Infrared Reflectance Spectroscopy, in *Phytoremediation*, ed. by Willey N. New Jersey: Humana Press, 205-217 (2007).

13 Cozzolino D and Morón A, Exploring the use of near infrared reflectance spectroscopy (NIRS) to predict trace minerals in legumes. *Anim Feed Sci Tech* **111**:161-173 (2004).

14 González-Martín I, González-Pérez C, Hernández-Méndez J and Alvarez-García N, Mineral analysis (Fe, Zn, Ca, Na, K) of fresh Iberian pork loin by near infrared reflectance spectrometry: Determination of Fe, Na and K with a remote fibre-optic reflectance probe. *Analytica Chimica Acta* **468**:293-301 (2002).

15 Moros J, De Vallejuelo, SF, Gredilla A, De Diego A, Madariaga JM, Garrigues S and De La Guardia M, Use of reflectance infrared spectroscopy for monitoring the metal

content of the estuarine sediments of the Nerbioi-Ibaizabal River (Metropolitan Bilbao, Bay of Biscay, Basque Country). *Environ Sci Technol* **43**:9324-9320 (2009).

16 Petisco C, García-Criado B, De Aldana BRV, Zabalgogeazcoa, I., Mediavilla, S. and García-Ciudad A, Use of near-infrared reflectance spectroscopy in predicting nitrogen, phosphorus and calcium contents in heterogeneous woody plant species. *Anal Bioanal* Chem **382**: 458-465. (2005).

17 Phan-Thien KY, Golic M, Wright GC and Lee NA, Feasibility of estimating peanut essential minerals by near infrared reflectance spectroscopy. *Sens Instrum Food Qual Saf* **5**:43-49 (2011).

18 Villatoro-Pulido M, Moreno Rojas R, Muñoz-Serrano A, Cardeñosa V, Amaro López MA, Font R and Del Río-Celestino M, Characterization and prediction by near-infrared reflectance of mineral composition of rocket (*Eruca vesicaria* subsp. *sativa* and *Eruca vesicaria* subsp. *vesicaria*). *J Sci Food Agr* **92**:1331-1340 (2012).

19 Wu D, He Y, Shi J and Feng S, Exploring Near and Midinfrared Spectroscopy to Predict Trace Iron and Zinc Contents in Powdered Milk. *J Agric Food Chem* **57**:1697-1704 (2009).

20 Font R, Del Río-Celestino M and De Haro-Bailon A, The use of near-infrared spectroscopy (NIRS) in the study of seed quality components in plant breeding programs. *Ind Crop Prod* **24**:307-313 (2006).

21 Shenk JS and Westerhaus MO, Population structuring of near infrared spectra and modified partial least squares regression. *Crop Sci* **31**:1548-1555 (1991).

22 Naes T, Isaksson T, Fearn T and Davies TA, User-friendly guide to multivariate calibration and classification. Chichester, UK: NIR Publications (2002).

23 Wold S, Sjöström M and Eriksson L, PLS-regression: a basic tool of chemometrics. *Chemometr Intell Lab.* **58:**109-130 (2001).

24 Shenk JS and Westerhaus MO, The application of near infrared reflectance spectroscopy (NIRS) to forage analysis, in *Forage Quality, Evaluation and Utilization, USA: American Society of Agronomy*, ed. by Fahey GC Jr, Collins M, Mertens DR, Moser LE and Madison WI. Crop Science Society of America, Soil Science Society of America, 406-450 (1994).

25 Barnes RJ, Dhanoa MS and Lister SJ, Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra. *Appl Spectrosc* **43**:772-777 (1989).

26 Shenk JS, Workman J and Westerhaus M, Application of NIR Spectroscopy to Agricultural Products. In *Handbook of Near Infrared Analysis*, ed. by Burns DA and Ciurczac EW. New York: Marcel Dekker, 419-474 (2001).

27 Tkachuk R and Kuzina FD, Chlorophyll analysis of whole rapeseed kernels by near infrared reflectance. *Can J Plant Sci* **62**:875-884 (1982).

28 Stchur P, Cleveland D, Zhou J and Michel RG, A review of recent applications of near infrared spectroscopy, and of the characteristics of a novel Pb SCCD array-based near-infrared spectrometer. *Appl Spectrosc* **37**: 383-428 (2002).

29 Osborne BG, Fearn T and Hindle PH, Practical NIR Spectroscopy with Applications in Food and Beverage Analysis. Harlow: Longman Scientific & Technical (1993).

30 Murray I, The NIR spectra of homologous series of organic compounds, in *Proceedings of the International NIR/NIT Conference*, ed. by Hollo J, Kaffka KJ, Gonczy JL. Budapest, Hungary: Akademiai Kiado, 13-28 (1986).

31 Shenk JS and Westerhaus MO, Calibration the ISI way. In *Near infrared spectroscopy: The future waves*, ed. by Davies AMC and Williams PC. Chichester: NIR Publications, 198–202 (1996).

32 Williams PC and Sobering DC, How do we do it: a brief summary of the methods we use in developing near infrared calibrations. In *Near infrared spectroscopy: The future waves*, ed. by Davies AMC and Williams PC. Chichester: NIR Publications, 185-188 (1996).

33 Vazquez De Aldana BR, García Criado B, García Ciudad A and Pérez Corona ME, Estimation of mineral content in natural grasslands by near infrared reflectance spectroscopy. *Commun Soil Sci Plant* **26**:1383-1396 (1995).

34 Clark DH, Cary EE and Mayland HF, Analysis of trace elements in forages by near infrared reflectance spectroscopy. *Agron. J* **81**:91-95 (1989).

35 Font R, Del Río M and De Haro A, Use of Near Infrared Spectroscopy to evaluate heavy metal content in *Brassica juncea* cultivated on the polluted soils of the Guadiamar River area. *Fresen Environ Bull* **11**:777-781 (2002).

36 Murray I, Forage analysis by near infrared reflectance spectroscopy, in Sward *Management Handbook,* ed. by Davies A, Baker RD, Grant SA and Laidlaw AS. UK: British Grassland Society, 285-312 (1993).

37 Ruano-Ramos A, García-Ciudad A and García-Criado B, Determination of nitrogen and ash contents in total herbage and botanical components of grassland systems with near infrared spectroscopy. *J Sci Food Agr* **79**:137–143 (1999).

38 Plans M, Simó J, Casañas F, Sabaté J, Near-infrared spectroscopy analysis of seed coats of common beans (*Phaseolus vulgaris* L.): a potential tool for breeding and quality evaluation. *J Agric Food Chem* **60**(3):706-12 (2005).

39 Andrés S, Giráldez F J, López S, Mantecón AR and Calleja A, Nutritive evaluation of herbage from permanent meadows by near infrared reflectance spectroscopy 1. Prediction of chemical composition and *in vitro* digestibility. *J Sci Food Agr* **85**:1564-1571 (2005).

40 Sauvage L, Frank D, Stearne J and Millikan MB, Trace metal studies of selected white wines: an alternative approach. *Anal Chim Acta* **458**:223-230 (2002).

41 Morón A and Cozzolino D, Determination of macro elements in alfalfa and white clover by near-infrared reflectance spectroscopy. *J Agric Sci* **139**, 413-423 (2002).

42 Clark DH, Mayland HF and Lamb RC, Mineral analysis of forages with near infrared reflectance spectroscopy. *Agron J* **79**:485-490 (1987).

43 Miller CE, Chemical principles of near infrared technology, in *Near-Infrared technology in the agricultural and food industries*, ed. by Williams P and Norris K. American Association of Cereal Chemists (AACC), St. Paul, Minnesota, USA, 19-37 (2001).

44 Workman J and Weyer L, Practical guide to interpretive near-infrared spectroscopy. London: Taylor and Francis (2008).

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

Chapter IV

Artículo publicado en:

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

http://dx.doi.org/10.1016/j.compag.2014.07.003

Damián Martínez-Valdivieso[a], Rafael Font[b], María Teresa Blanco-Díaz[b], José Manuel Moreno-Rojas[c], Pedro Gómez[a], Ángeles Alonso-Moraga[d], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, Center IFAPA La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain
[b]Department of Postharvest Technology and the Agrifood Industry, Center IFAPA La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain
[c]Department of Postharvest technology and the Agrifood Industry, Center IFAPA Alameda del Obispo, 14080 Córdoba, Spain
[d]Department of Genetics, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

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.

Keywords: least squares regression; *Cucurbita pepo*; carotenoid; lutein; β-carotene

Abbreviations used:

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.

1. Introducción

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 × = 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 et al., 2003). 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).

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*, β-carotene, α-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 (0.6–3mg 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

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 (Minguez-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 et al., 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 labour-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 (*Cucurbita 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 (36º47'19''N, 02º42'11''W; 142m 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℃. The samples (250 skin and 250 flesh) were lyo philised 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 ℃ 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 et al., 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 85ºC. 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 30º 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 x 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.

Compounds were identified by comparison of retention times, co-injection with known standards, and comparison of their UV-visible spectra with authentic standards.

Quantification was carried out by external standardization. Full standard curves were constructed with five different concentrations for each carotenoid in triplicate. The curves passed through or were very near the origin, were linear and bracketed the concentrations expected in the samples.

Results were expressed on a dry weight (DW) basis.

2.4. Scanning samples for NIRS analysis

A NIR Systems Model 6500 spectrophotometer (Foss-NIRSystems, Inc., Silver Spring, MD, USA) equipped with a transport module was used to perform the NIRS analyses. Samples were analyzed as ground flesh and skin. Spectra were recorded in reflectance mode. In this mode, a ceramic standard is placed in the radiant beam, and the diffusely reflected energy is measured at each wavelength, before and after reading the sample. Spectra of the samples were recorded once from each sample, and were obtained as an average of 32 scans. The ceramic and the sample spectra were used to generate the final log (1/*R*) spectrum. Samples were placed for the analysis in a diameter round cell holder. This cell is composed of quartz glass and anodized aluminium to avoid absorption.

Principal component analysis (PCA) of the whole set of spectra (skin and flesh samples) was performed using raw optical data in order to stablish population boundaries. To do this the second derivative transformation of the original spectra was performed prior to PCA analysis to enhance spectral differences between types of samples (Naes et al., 2002).

2.5. Developing NIRS equations

Spectra corresponding to the skin and flesh samples separately, were sorted on the basis of the reference values for each component, from the lowest to the highest, and then divided into calibration and validation groups in a rate 2:1 (154 calibration and 76 validation).

In a first step, the full wavelength range (from 400 to 2500 nm, at 2 nm intervals) was used for calibration. In most cases, the visible region of the spectrum (400-700 nm) provided efficient contribution to calibration of fruit components, as color is often correlated with flesh and skin characters (i.e., chlorophyll and pigments, protein, moisture and carbohydrates) (Williams and Sobering, 1996).

Calibrations were performed by using the GLOBAL v. 1.50 software (WINISI II, Infrasoft International, LLC, Port Matilda, PA, USA). Calibration equations were computed by using the raw optical data (log 1/*R*), or first or second derivatives of the log 1/*R* data, with several combinations of segment (smoothing) and derivative (gap) sizes [i.e., (0, 0, 1, 1; derivative order, segment of the derivative, first smooth, second smooth); (1, 4, 4, 1); (1, 10, 10, 1); (2, 5, 5, 2); (2, 10, 10, 2] (Shenk*,* 1992). The use of derivative spectra instead of the raw optical data to perform calibration is a way of solving problems associated with overlapping peaks and baseline correction (Hruschka, 1987). A first-

order derivative of log (1/*R*) results in a curve containing peaks and valleys corresponding to the point of inflection on either side of the log (1/*R*) peak. While the second-order derivative calculation results in a spectral pattern display of absorption peaks pointing down rather than up, with an apparent band resolution (Shenk, 1992). The gap size and amount of smoothing used to make the transformation will also affect the number of apparent absorption peaks.

Among the different methods based on selected wavelengths or in full-spectrum which are available in commercial chemometric softwares, the full-spectrum methods have yield calibration equations that perform better with the types of seed material, and components that are the object of this study. Specially, modified partial least squares (PLSm) regression has been revealed as noteworthy for assessing seed components. PLS performs a linear regression in a new coordinate system with a lower dimensionality than the original space of independent variables. The PLS factors are determined by the maximum variance of independent (spectral data) variables and by a maximum correlation with the dependent (chemical) variable. The model actually uses only the primary, most important factors, the 'noise' being encapsulated in the less important factors. Regression is performed in the space spanned by the new reduced coordinate system of the orthogonal factors.

In addition to derivatives, standard normal variate and de-trending (SNVD) algorithms (Barnes et al., 1989) were applied to the derived spectra to minimize baseline offset due to scattering effects caused by differences in particle size or path-length variation among samples.

In this study, cross-validation was computed based on the calibration set for determining the optimum number of terms to be used in building the calibration equations.

2.6. Validation of the equations

An external validation procedure was carried out to determine the accuracy and precision of the equations obtained in the calibration for each component in each species. To evaluate the accuracy of the equations, different statistics were used, namely, the coefficient of determination (r^2) (Williams and Norris, 1987); the RPD, which is the ratio of the standard deviation (SD) for the validation samples to the standard error of prediction (performance) (SEP), and the RER, which is the ratio of the range in the reference data (validation set) to the SEP (Williams and Sobering, 1996).

As far as possible, we also calculated the ratio SEP to standard error of laboratory (SEL), as this statistic allows the error of NIRS to be put in perspective to the error in the reference method. The mathematical expressions of these statistics are as follow:

$$r^2 = \left(\sum_{i=1}^{n}(\hat{y}-\bar{y})^2\right)\left(\sum_{i=1}^{n}(y_i-\bar{y})^2\right)^{-1} \qquad (1)$$

where: $\hat{y}$ = NIR measured value; $\bar{y}$ = mean "y" value for all samples; y_i = lab reference value for the *i*th sample.

$$\text{RPD} = SD\left\langle\left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1} \qquad (2)$$

where: y_i = lab reference value for the ith sample; $\hat{y}$ = NIR measured value; N= number of samples, *K*= number of wavelengths used in an equation; SD= standard deviation.

$$\text{RER} = range\left\langle\left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1} \qquad (3)$$

where: y_i = lab reference value for the ith sample; $\hat{y}$ = NIR measured value; N= number of samples, K= number of wavelengths used in an equation.ratio.

$$\text{SEP/SEL} = \left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\left\langle\left[\left(\sum_{i=1}^{n}(y_1-y_2)^2\right)(2N)^{-1}\right]^{1/2}\right\rangle^{-1} \quad (4)$$

where: y_i = laboratory reference value for the ith sample; $\hat{y}$ = NIR measured value; y1 and y2= laboratory reference values for 2 duplicates of the same sample; N= number of samples, *K*= number of wavelengths used in an equation.

2.7. Correlation plot of total carotenoid content versus wavelength in summer squash fruit

The correlations of the total carotenoid content versus wavelength for each sample were obtained by using the whole set of samples, to identify those spectral regions more highly correlated with the total carotenoid content in the tissues of summer squash flesh. Spectral data was standardized by using SNVqDT (Barnes et al., 1989)

to interpret in a simpler way the correlation plot of spectral data versus total carotenoid content in the whole set of samples. This mathematical pre-treatment of the spectral data eliminates the background of constant correlation due to any existing relationship between total carotenoid content and particle size. In theory, areas matching absorption bands in the spectra of the constituent being measured should have positive correlations in the correlation plot, while areas corresponding to absorption bands in the spectra of other constituents could have positive, negative or zero correlations depending on the inter-correlations between constituents (Osborne et al., 1993).

2.8. MPLS loading plots

The MPLS loading plots of the first three factors generated from the MPLS regression performed on the second derivative transformation of the raw optical data (2, 5, 5, 2; SNV +DT) were calculated for total carotenoid content. MPLS regression constructs its factors capturing as much of the variation in the spectral data as possible by using the reference values actively during the decomposition of the spectral data. By balancing the spectral and chemical information the method reduces the impact of large but irrelevant spectral variations in the calibration modelling (Martens and Naes, 1992).

The loading plots show the regression coefficients of each wavelength to the parameter being calibrated for each factor of the equation. Wavelengths represented in the loading plots as more highly participative in the development of each factor are those of more variation and better correlated to the parameter in the calibration set. In the second derivative, peaks pointing downwards indicate positive influence of absorbers on the development of the equations, while peaks pointing upwards indicate negative correlations. In this work we use band assignments from literature, to relate some major absorption bands in the spectrum of summer squash fruit with the main wavelengths used by MPLS to construct the first three MPLS terms for the total carotenoid equation.

3. Results and discussion

3.1. Reference values for summer squash carotenoid contents

The summer squash varieties used in this work were chosen on the basis of the need to cover as wide a range of fruit carotenoid contents as possible.

The major carotenoids present in the flesh and skin of all of the samples were the xanthophyll lutein and the hydrocarbons β-carotene. Lower levels of neoxanthin,

violaxanthin, zeaxanthin,α- and β-cryptoxanthins and α- carotene were also present (not shown).

An overview of the mean, range, standard deviation (SD) and coefficient of variation (CV) for total, lutein and β-carotene contents measured in summer squash samples are given in **Table 1**.

The samples analysed varied considerably in elemental composition as shown by the range and CV. High CV was observed for total and individual contents (>70%) possibly due to the different varieties used. The variability in elemental composition in calibration set was considered suitable for developing NIR calibrations for these traits.

The qualitative and quantitative carotenoid content exhibited by the samples in this study covered most of the variability reported in the literature for *C. pepo* (Ben-Amotz and Fishler, 1998; Tadmor et al., 2005; Azevedo-Meleiro and Rodríguez-Amaya, 2007; Rodríguez-Amaya et al., 2008; El-Qudah, 2009).

The total-carotenoid and lutein contents varied from 68-428 µg/g dw and 53-421.7 µg/g dw, respectively, in flesh of *C. pepo* fruits. These results were superior to those found in a previous study evaluating the flesh of five pairs of near-isogenic lines of *C. pepo* (10.4 to 187.2 µg/g dw and 6.4-143.2 for total and lutein contents, respectively, assuming 92% of moisture) by Tadmor et al. (2005).

The β-carotene content varied from 1.3 to 23.9 µg/g dw in flesh of *C. pepo*. These values were lower than those found by Tadmor et al. (2005) which varied from 4 to 44.8 µg/g dw in flesh samples.

To this date few studies have been carried out in *C. pepo* to assess the carotenoid content in fruit skin. The carotenoid content was higher in the skin of fruit which agrees with results reported in other vegetables (Gross, 1987). As the major carotenoid of this matrix is lutein, with high concentration in skin, it is worth mentioning in this context some benefits mentioned in the literature which are associated with lutein. Lutein is a xanthophyll which can be found in retina, where its main function is to protect photoreceptive cells from oxygen radicals generated in photochemical processes; it thus plays a main role in the prevention of age-related macular degeneration (Scalch, 1992; Khachik et al., 1999). Many studies showed that by consuming vegetables and vegetable products with a high lutein and zeaxanthin content, the risk of cataracts is reduced, as well as the risk of age-related macular degeneration (Seddon et al., 1994;

Varma et al., 1995, Beatty et al., 1999; Segasothy and Phillips, 1999; Hammond and Caruso-Avery, 2000).

A previous study has suggested that 6 mg of lutein a day might decrease the risk of age related macular degeneration by 43 % (Seddon et al., 1994). This amount is the same as the daily consumption of: 2 salad bowls of spinach, ~7 kg tomatoes, ~1 kg corn, one salad bowl of kale or, as revealed by this study ~ 375 g *Cucurbita* flesh (considering 200 mg lutein kg^{-1} dw which is equal to 16 mg lutein kg^{-1} fresh weight) (Muntaun et al., 2006).

Despite lutein is not a provitamin A, it is a more effective antioxidant than many other carotenoids (it inhibits *in vitro* the lipid oxidation, in a more efficient manner than α-carotene, β-carotene or lycopene). One must mention also that the lutein biodisponibility from plant sources is much higher in comparison to that of β-carotene (Castenmiller and West, 1998; Van Het Hof et al., 1999, 2000; Erdman, 1999).

3.2. Summer squash reflectance spectrum

Fig. 1 showed typical NIR spectra obtained for the different summer squash samples analyzed. Clearly it can be seen that a considerable contribution is due to the visible wavelength range (400-700 nm), and this may be of relevance for highly colored summer squash samples. This range has indeed been used to measure carotenoids in durum wheat and maize by NIRS (Brenna and Berardo, 2004, Murray and Williams, 1987). Vis/NIR spectra also indicated that the absorption profiles yield information in the NIR region where carotenoids (lutein) absorb strongly as it has been reported previously (Davey et al., 2009).

Fig. 2 shows the second derivative (2, 5, 5, 2; SNV+DT) average NIR spectrum of freeze-dried samples from summer squash skin (2a) and flesh (2b) used to conduct this work (n=230). The average spectrum matched all the absorption bands, any shift of absorption maxima (lmax) being observed between them. In both Figures, the average spectrum showed bands in the visible region of the spectrum presenting lmax at 676 nm and 700nm, which correspond to electronic transitions in the red. Thus, the band at 676 nm has been assigned to chlorophyll (Tkachuk and Kuzina, 1982).

Table 1. Mean, range, standard deviation (SD), coefficient of variation (CV) and standard error of laboratory (SEL) of total and individual carotenoids for flesh and skin of summer squash fruit. Data expressed as mg kg^{-1} (dry weigth).

	Range	Mean	SD	CV	SEL
Flesh					
Total carotenoid	67.1-451.2	185.0	130.8	0.70	15.14
Lutein	50.3-434.3	172.0	120.4	0.70	11.48
β-carotene	0-24	7.02	5.31	0.75	1.25
Skin					
Total carotenoid	85.0-1822	836.1	499.4	0.94	52.31
Lutein	78.4-1529	720.3	413.1	0.88	47.76
β-carotene	0-194	38.12	38.36	1.39	5.26

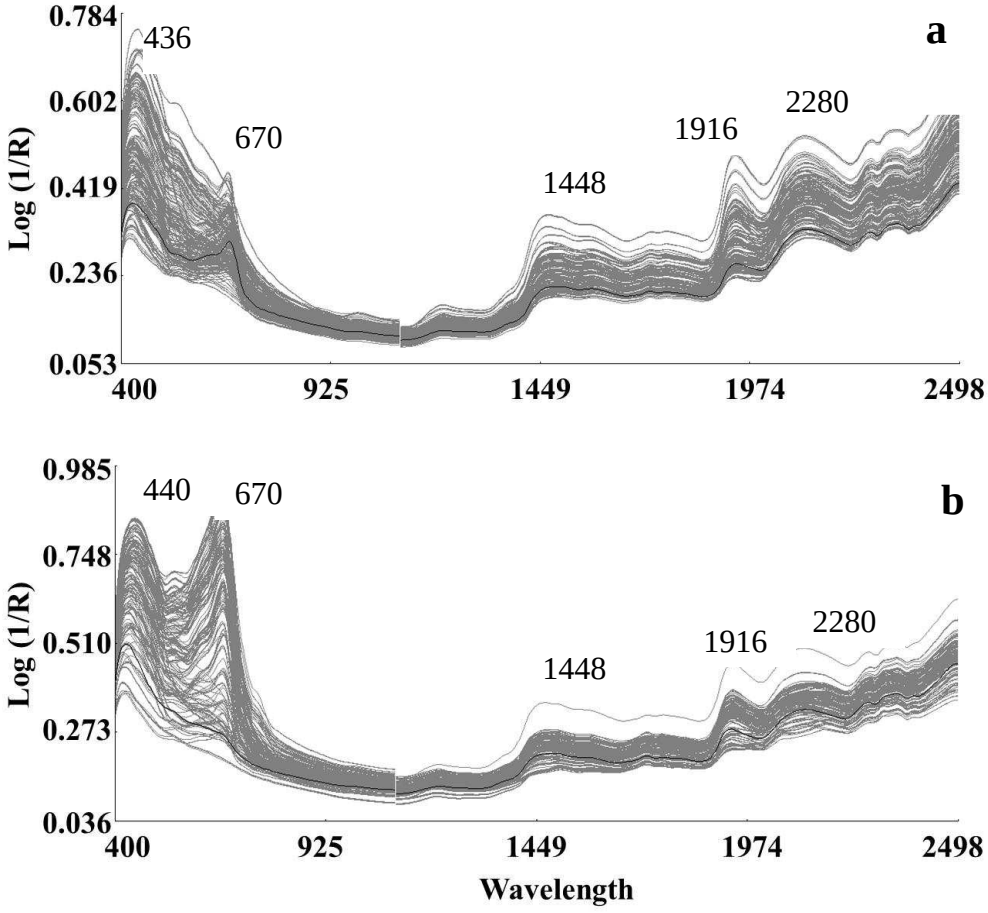


Fig. 1. Typical NIR spectra obtained for the different samples of skin (a) and flesh (b) fruits analysed.

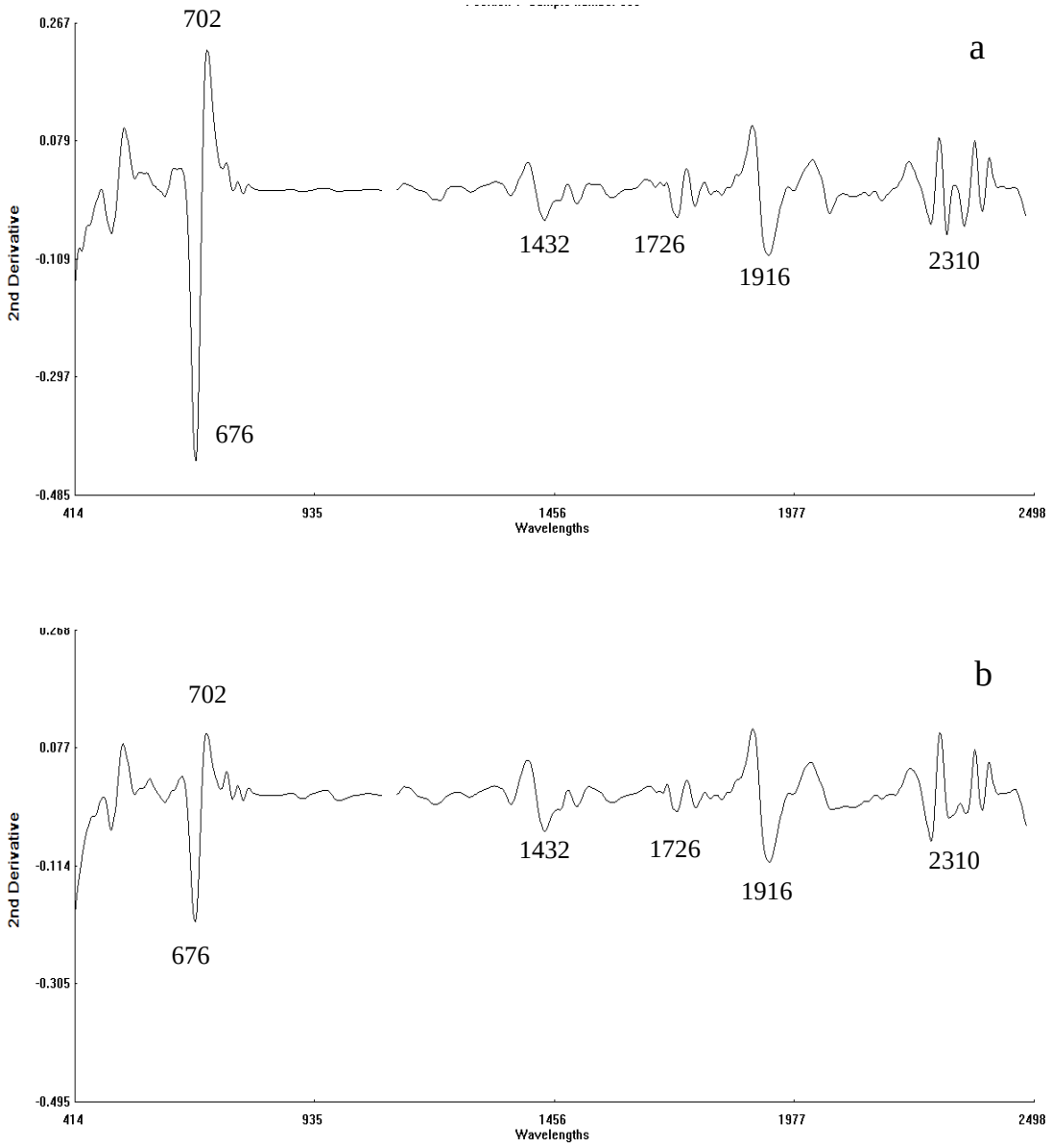


Fig. 2. Near infrared mean spectrum of Cucurbita samples of skin (a) and flesh (b) fruits using SNVD and second derivative as treatment.

The NIR region of the spectrum (**Fig. 2a**) showed characteristic absorption bands at 1432 and 1916 nm related to O-H stretch second and first overtone of water, respectively; 1726, 2310 and 2348 nm related to C-H stretch first overtone and combination bands of lipids (Murray, 1986). At 2274 nm related to O-H+C-O deformation, O-H stretch plus deformation, and O-H+C-C stretch of starch (Osborne et al., 1993),

Principal component analysis showed a clear separation between both groups of samples, i.e., skin and flesh. This spectral variation was almost entirely explained by second versus third principal components (PC2 and PC3) (**Fig. 3**).

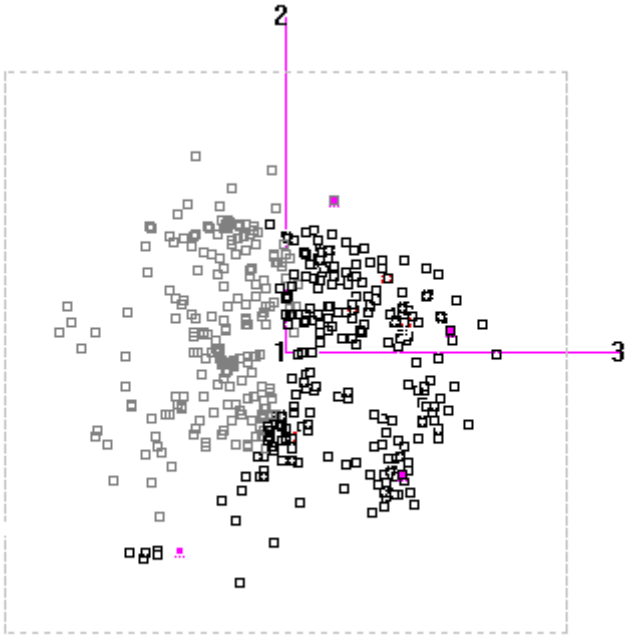


Fig. 3. Score plots, obtained by applying PCA to NIR spectra of skin and flesh, in the area defined by the second versus third principal components (PC2 and PC3). Gray and black squares indicate samples from skin and flesh, respectively.

3.3. Correlation plot for total carotenoid content versus wavelength

Fig. 4 showed plots of the weighted regression coefficients for the prediction of the total carotenoid concentrations in skin (4a) and flesh (4b) from the second derivatives

of the Vis/NIR spectral absorption data. The higher correlations between spectra and chemical composition for total carotenoids fall into the visible (range 500-700 nm) and NIR (range 1100-2378 nm), (defined as the relationship among the substance and wavelengths, absorptivity maxima as derived by second derivative). These results are similar to those obtained in other species as maize, potato and banana for carotenoid content analysis (Brenna and Berardo, 2004, Bonierbale et al., 2009; Davey et al., 2009). In particular, two clear negative peaks around 502 and 678 nm corresponding to the absorption peaks of around 532 and 676 nm in the original spectra (**Fig. 4a**) seem to be particularly important. Other absorption bands in the NIR region of the spectrum around 1158, 1168, 1230, 1950, 2270 and 2378 nm were also important which agrees with previous studies (Brenna and Berardo, 2004; Davey et al., 2009).

3.4. Modified partial least square loadings

MPLS loading plots allow an observation of wavelengths with high variation in the calibration set that may be associated with spectral regions of known chemical origin (Durkee, 1971).

Fig. 5 represents the MPLS loading spectra for factors 1, 2 and 3, respectively. It can be concluded that pigments existing in the tissues of the summer squash fruit greatly influenced the three MPLS loadings of the second derivative transformation (2, 5, 5, 2; SNV + DT). This is in agreement with the correlations existing between total carotenoid content and apparent absorption in our samples, in which high correlations were shown in the visible region of the spectrum (**Fig. 5**). Of the first three factors of the selected equation (2, 5, 5, 2; SNV + DT), the third MPLS loading was the most highly correlated with total carotenoid content. It is worth noting the influence of the band at 680 and 704 nm in modeling this third factor, which is related to the absorption in the red region by chlorophyll as has been mentioned above.

Other absorptions due to water (1412 nm), N–H combination tones of amide (1916 nm), O–H deformation plus C–O deformation of starch (2284 and 2324 nm) and also C-H combination tones by lipids (2308 and 2348 nm) (Osborne et al., 1993) highly influenced the two first factors of the equation.

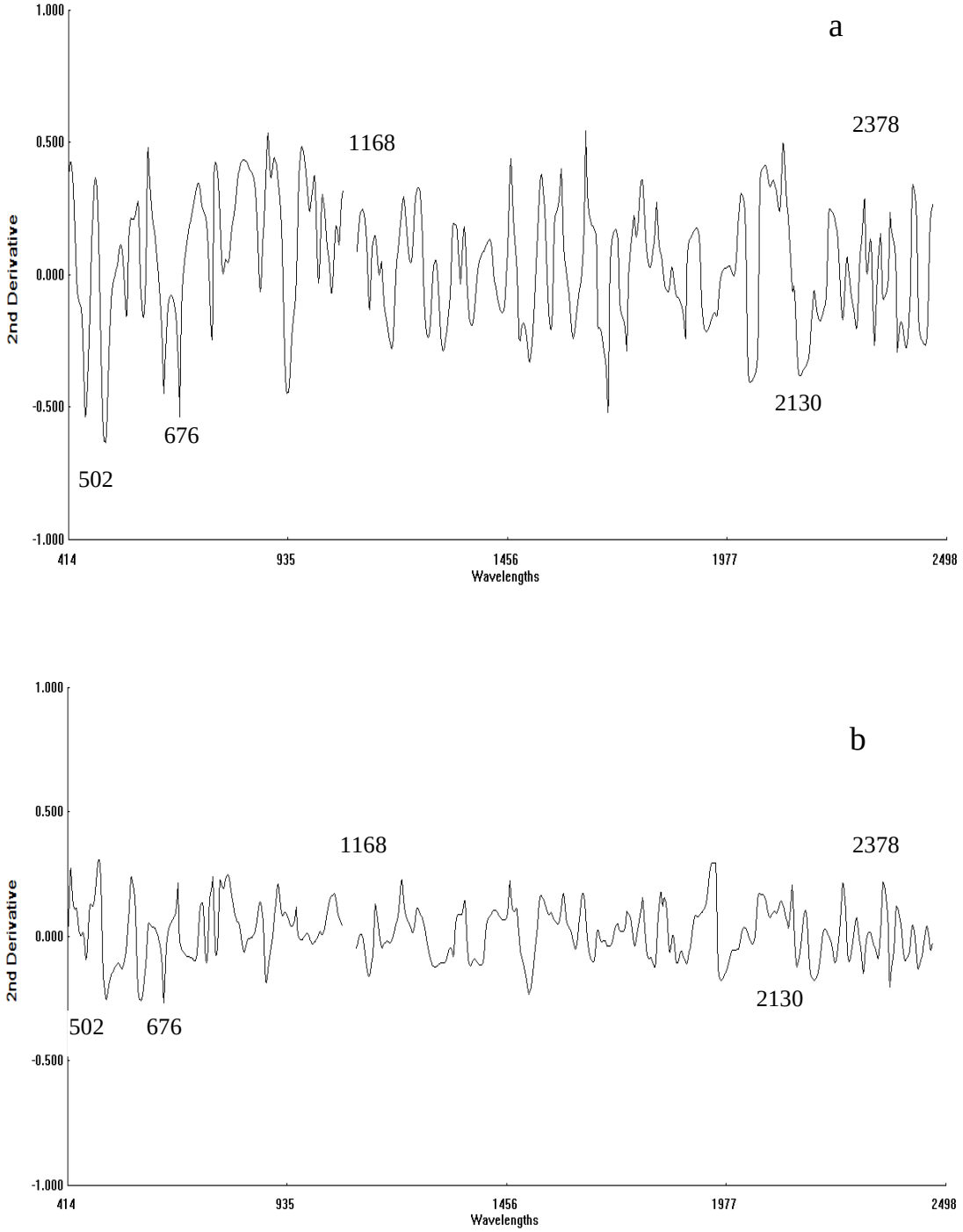


Fig. 4. Wavelength correlation of total carotenoid content in Cucurbita samples of skin (a) and flesh (b) fruits using SNVD and second derivative as treatment.

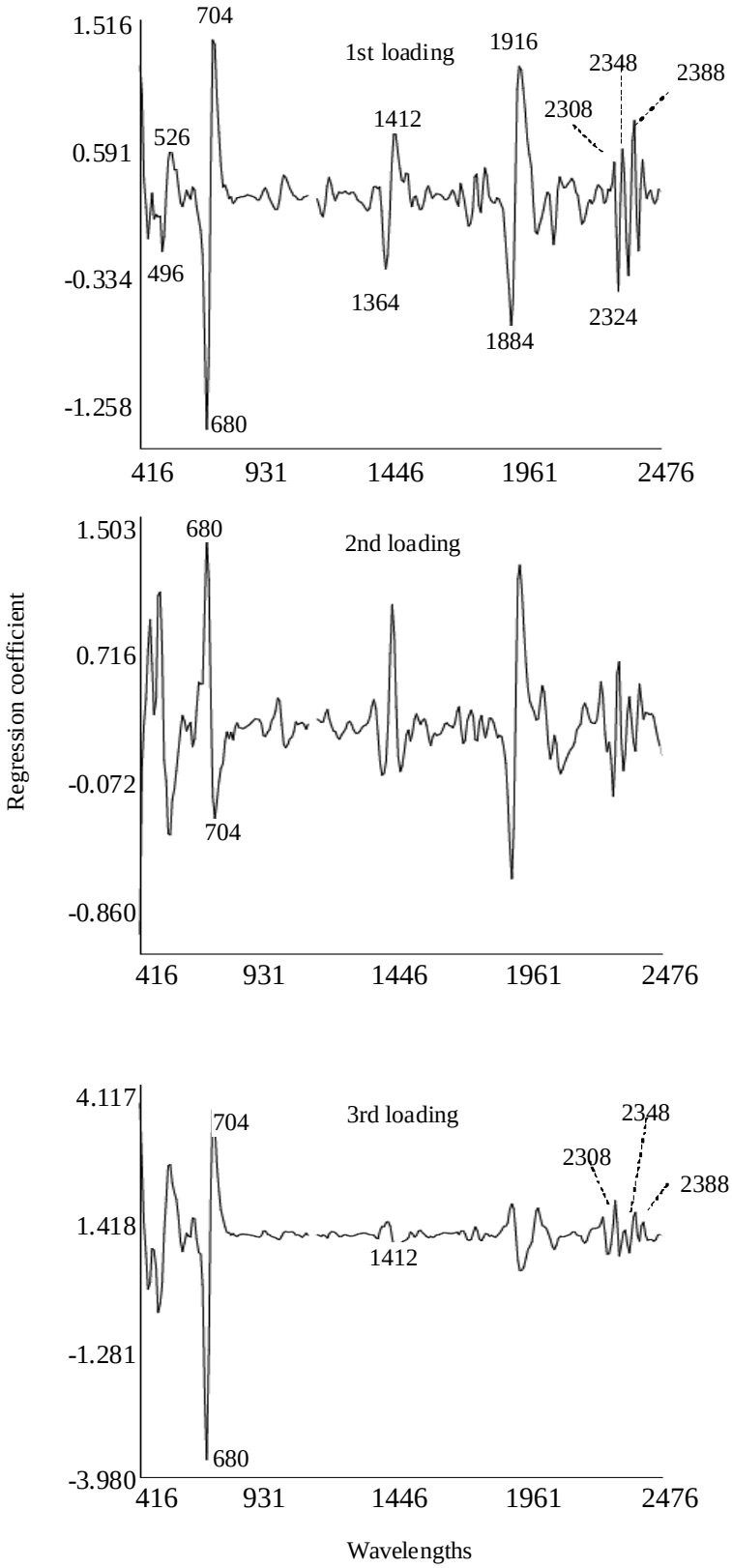


Fig. 5. Modified Partial least squares (MPLS) loadings for total carotenoid content measured in flesh of summer squash fruits using near infrared reflectance spectroscopy.

3.5. NIRS Analysis.

In **Table 2** are reported the statistics of the external-validation for the different carotenoids, including standard errors of performance (SEP) and R^2 values for the equations of best fit obtained for each of the traits.

The SEP obtained in the validation were lower than their respective S.D.s, indicating that NIRS is able to determine total carotenoids, lutein and β-carotene contents in fruits of *C. pepo*.

R^2 values for the validation ranged from 0.81 for β-carotene in flesh to 0.96 for lutein content in flesh.

The usefulness and accuracy of the developed models were evaluated on the basis of the R^2 and RPD values (Williams and Sobering, 1996). The R^2 values give an indication of the percentage variation in the *Y* variable that is accounted for by the *X* variable. Therefore R^2 values above 0.50 indicate that over 50% of the variation in *Y* is attributable to variation in *X*, and this allows discriminations between high and low concentrations to be made. Higher R^2 values improve discrimination, and models with a R^2 of 0.60-0.82 can be used for screening and approximate quantitative predictions, models with R^2 values between 0.83-0.90 can be used for many applications, while models with values of 0.92-0.96 are suitable for most applications including quality assurance, and those above 0.98 for all applications.

In our study, external-validation resulted in coefficients of determination (1-VR) of 0.95, 0.96 and 0.81 for total carotenoid, lutein and β-carotene contents, respectively in flesh (**Table 2**, **Fig. 6**), indicating that the 95%, 96% and 81% of the variability present in the data was explained by the respective calibration equations. In skin, external-validation resulted in coefficients of determination (1-VR) of 0.90, 0.88 and 0.84 for total carotenoid, lutein and β-carotene contents, respectively (**Table 2**, **Fig. 6**), indicating that the 90%, 88% and 85% of the variability present in the data was explained by the respective calibration equations.

The values for R^2 shown by the equations in this work, indicated good quantitative information (0.81 for β-carotene in flesh), useful for many applications (0.90, 0.88 and 0.84 for total carotenoid, lutein and β-carotene in skin, respectively) and excellent quantitative information (0.95 and 0.96 for lutein and total carotenoid content in flesh, respectively) (Shenk and Westerhaus, 1996) (**Table 2**, **Fig. 6**).

The R^2 statistics were still comparable with previously reported results for NIRS calibrations for β-carotene and total carotenoid contents in banana and cassava (R^2:0.89-0.91 and 0.84-0.88, respectively), and for lutein in maize grain (R^2:0.96); higher than those obtained for lutein in banana and potato (R^2:0.30 and 0.70, respectively) and for β-carotene in potato and maize grains (R^2:0.56 and 0.70, respectively) (Brenna and Berardo, 2004, Bonierbale et al., 2009; Davey et al., 2009; Sanchez et al., 2013).

The prediction ability of the NIR calibration equations is determined by many authors according to the relationship between the spread in composition of agricultural products and the error of the performance (SEP). Thus, if the error in estimation is large compared with the spread (as SD and range) in composition, then regression has increasing difficulty in finding stable calibrations (Murray, 1986, 1993; Williams and Sobering, 1996). On the basis of the statistics RPD and RER, most of the equations were higher than 2 and 9, respectively, thus being useful for screening purposes (Williams and Sobering, 1996). To evaluate the predictive ability of equations in relation to the overall error of the reference method, the SEL was calculated and related to SEP. The SEP/SEL ratio shown by total and individual carotenoid equations (1.6-2.33) classified them as having good precisions (Font et al., 2006).

Some authors have also reported SD/SEP data for carotenoid analysis in plants, higher, similar to or even lower than those shown in this work. Davey et al. (2009) reported RPD ratios of 1.16, 2,74 and 3.34, for the prediction of lutein, β-carotene and total carotenoid content, respectively in banana. Brenna and Bernardo (2004) obtained RPD ratios that ranged from 1.78 to 5.07 for calibrations of lutein, β-carotene and total carotenoids in maize grains. Bonierbale et al (2009) reported successful equations predicting carotenoids in potato, which presented RPD ratios of 2.1, 2 and 3.3 for lutein, β-carotene and total carotenoid content, respectively. Recently, Sánchez et al., (2014) have developed successful equations predicting total and individual carotenoids in fresh cassava roots, with RPD values of 3 and 3.2 for total carotenoid content determined by HPLC and spectrophotometer, respectively and 3.5 for the prediction of β-carotene content.

Table 2. Validation statistics for equations of different carotenoids developed over flesh and skin of fruit of *Cucurbita pepo* (n=154 for calibration and n=76 for validation).

	NT	range	mean	SD	SEP	r^2	RPD	RER
Flesh								
Total carotenoid	11	68-428	188.6	136.7	31.7	0.95	4.31	11.35
Lutein	11	53-421.7	172.6	128.8	26.8	0.96	4.81	13.77
β-carotene	9	1.3-23.9	7.52	5.27	2.27	0.81	2.32	9.95
Skin								
Total carotenoid	10	87-1819	892.4	476.4	104.6	0.90	4.55	16.55
Lutein	9	79-1685	783.9	419.2	87.37	0.88	4.79	18.38
β-carotene	9	0.3-192.6	42.93	41.44	8.46	0.84	4.89	22.73

[a]NT: number of terms of the equation selected in the cross-validation
SD: standard deviation of the data in the validation set
SEP: standard error of performance
r^2: coefficient of determination in the external validation
RPD: ratio of the standard deviation to standard error of prediction (performance)
RER: ratio of the range to standard error of prediction (performance)

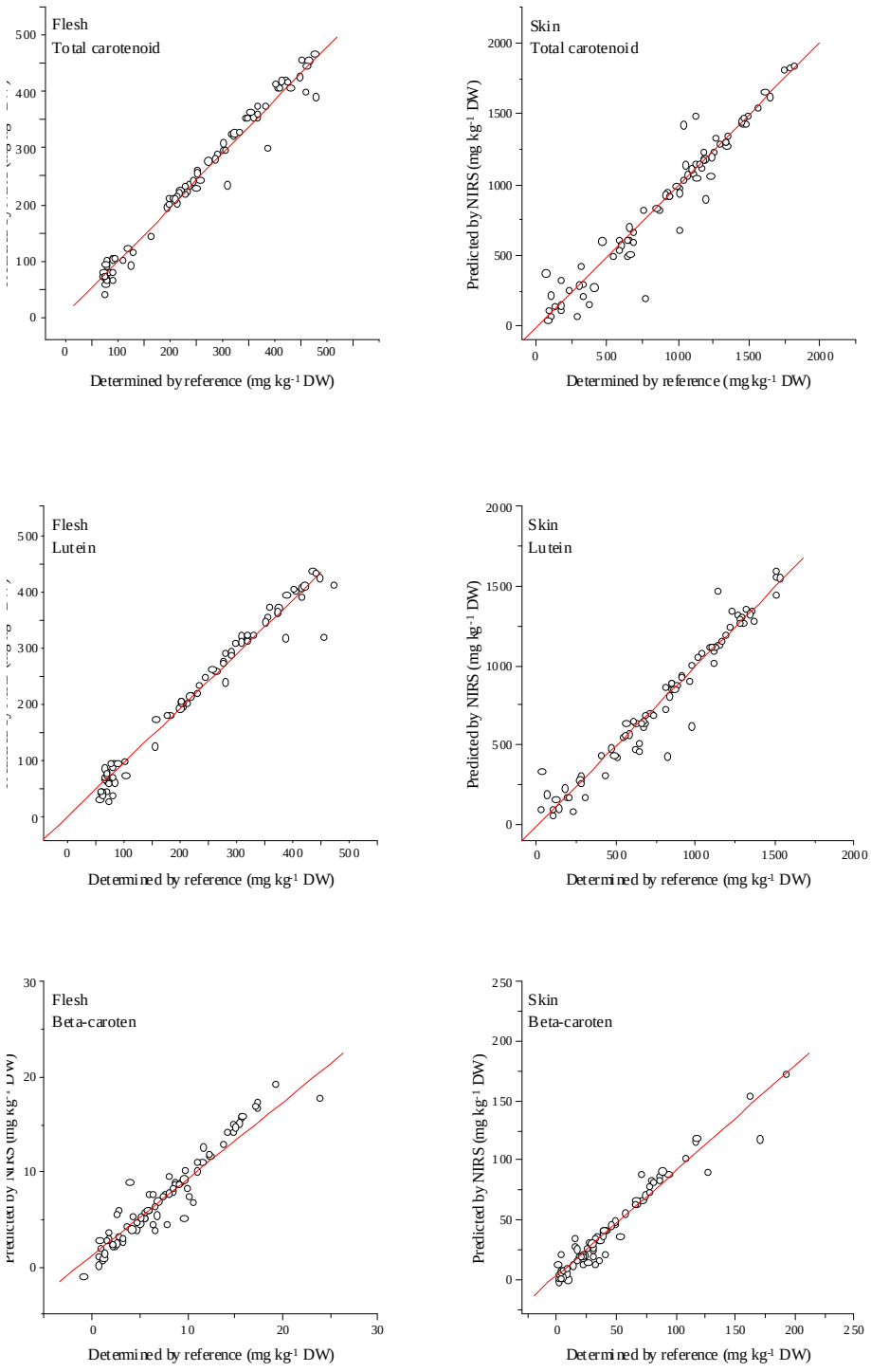


Fig. 6. Near infrared predicted values versus reference values for total and individual carotenoid content in skin and flesh of summer squash fruits.

4. Conclusions

The results from this study demonstrate that Vis/NIRS has good potential for the high-throughput screening of total carotenoid content and in particular for the individual carotenoid contents of lyophilized *Cucurbita* fruit samples. Despite the relatively small sample group used to develop the predictive models, the procedure shows good accuracy for total carotenoids, lutein and β-carotene contents, but it remains to be seen whether larger sample sets will improve models sufficiently to enable the reliable prediction of the concentrations of other carotenoid compounds present. Clearly, the fact that Vis/NIRS is a non-destructive analytical method and only requires minimal sample preparation will help prevent sample degradation during analysis. The disadvantages of Vis/NIRS are the low discriminatory power with respect to minor carotenoid species (as zeaxanthins) and the lower sensitivity compared to high-resolution chromatographic procedures.

When compared to conventional laboratory analyses, NIRS appears to be an attractive alternative technique because of its rapidity, simplicity, safety, and low operational costs.

This is of particular importance in nutritional quality evaluation, quality plant-breeding programs, species resource identification, and the healthy processing of summer squash foods in which a large number of samples must be analyzed.

Acknowledgements

The authors wish to express their thanks to the Project INIA RTA00039-000 and FEDER for the funding of this research. Damián Martínez-Valdivieso was supported by an INIA (Instituto de Investigación y Tecnología Agraria y Alimentaria) contract. We thank Nicholas Davies for his help in the grammatical revision of the manuscript.

5. References

Alquezar, B., Rodrigo, M.J., Zacarías, L., 2008. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. Phytochemistry 69, 1997-2007.

Anter, J., Fernández-Bédmar, Z., Villatoro-Pulido, M., Martínez-Valdivieso, D., Font, R., Muñoz-Serrano, A., Alonso-Moraga, A., Del Río-Celestino, M., 2011. Cytotoxicity and in vivo antigenotoxicity of some horticultural food cultivated in Southern Spain. Toxicology Letters, 205, S60-S179.

Atienza, S.G., Avila, C. M., Ramírez, M. C., Martí, A., 2005. Application of near infrared reflectance spectroscopy to the determination of carotenoid content in tritordeum for breeding purposes. Australian Journal of Agricultural Research 56 (1), 85-89.

Azevedo-Meleiro, C. H., Rodríguez-Amaya, D.B., 2007. Quantitative and qualitative differences in carotenoids composition among *Cucurbita moschata*, *Cucurbita maxima* and *Cucurbita pepo*. Journal of Agricultural and Food Chemistry 55, 4027–4033.

Barnes, R.J., Dhanoa, M.S., Lister, S.J., 1989. Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra. Applied Spectroscopy 43, 772–777.

Beatty, S., Boulton, M., Henson, D., Koh, H.H., Murray, I.J., 1999. Macular pigment and age related macular degeneration. The British Journal of Ophthalmology 83, 867-877.

Ben-Amotz, A., Fishler, R., 1998. Analysis of carotenoids with emphasis on 9-cis β-carotene in vegetables and fruits commonly consumed in Israel. Food Chemistry 62, 515-520.

Berardo, N., Brenna, O., Amato, A., Valoti, P., Pisacane, V., Motto, M., 2004. Carotenoids concentration among Maize genotypes measured by near infrared reflectance spectroscopy (NIRS). Innovative Food Science and Emerging Technologies 5, 393–398.

Blanco-Díaz, M.T., Del Río Celestino, M., Martínez-Valdivieso, D., Font, R. 2014. Use of visible and near-infrared spectroscopy for predicting antioxidant compounds in summer squash (*Cucurbita pepo* ssp *pepo*). Food Chemistry 164, 301-308.

Bonierbale, M., Grüneberg, W., Amoros, W., Burgos, G., Salas, E., Porras, E., zum Felde, T., 2009. Total and individual carotenoid profiles in *Solanum phureja* cultivated potatoes: II. Development and application of near-infrared reflectance spectroscopy (NIRS) calibrations for germplasm characterization. Journal of Food Composition and Analysis 22 (6), 509-516.

Brenna, O. V., Berardo, N., 2004. Application of Near-Infrared Reflectance Spectroscopy (NIRS) to the evaluation of carotenoids content in maize. Journal of Agricultural and Food Chemistry 52, 5577-5582.

Castenmiller, J.J.M., West, C.E., 1998. Bioavailability and bioconversion of carotenoids. The Annual Review of Nutrition 18, 19- 38.

Davey, M.W., Saeys, W., Hof, E., Ramon, H., Swennen, R. L., Keulemans, J., 2009. Application of Visible and Near-Infrared Reflectance Spectroscopy (Vis/NIRS) to determine carotenoid contents in banana (*Musa* spp.) fruit flesh. Journal of Agricultural and Food Chemistry 57, 1742–1751.

Durkee, A. B., 1971. The nature of tannin in rapeseed (*Brassica campestris*). Phytochemistry 10, 1583−1585.

Edwards, N.M., Dexter, J.E., Sobering, D.C, Williams, P.C., 1996. Whole grain prediction of durum wheat yellow pigment by visible-NIR reflectance spectroscopy. In: Near Infrared Spectroscopy: The Future Waves, Proceedings 7th International Conference on Near-Infrared Spectroscopy, Montreal, Canada, August, 1995, Davies, A.M.C., Williams, P.C. (eds). NIR Publications, Chichester, UK., pp. 462-465

El-Qudah, J.M., 2009. Identification and Quantification of Major Carotenoids in Some Vegetables. American Journal of Applied Sciences 6(3), 492-497.

Emerson, R.A., 1910. The inheritance of sizes and shapes in plants. The American Naturalist 44, 739–746.

Erdman, J.W. Jr., 1999. Variable bioavailability of carotenoids from vegetables. American Journal of Clinical Nutrition 70, 179-180.

Ferriol, M., Picó, B., 2008. Pumpkin and Winter Squash. In Handbook of Plant Breeding. Vegetables I. Part 4. Volume 1. Edited by: Prohens J, Nuez, F. Springer; 317-349.

Ferriol, M., Picó, B., Nuez, F., 2003. Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRAP and AFLP markers. Theoretical and Applied Genetics 107, 271-282.

Font, R., Del Río-Celestino, M., De Haro-Bailón, A., 2006. The use of near-infrared spectroscopy (NIRS) in the study of seed quality components in plant breeding programs. Industrial Crops and Products 24, 307-313

Giovannucci, E., Ascherio, A., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Willet, W. C., 1995. Intake of carotenoids and retinol in relation to risk of prostate cancer. Journal of the National Cancer Institute. 87, 1767-1776.

Granado, F., Olmedilla, B., Blanco, I., 2003. Nutritional and clinical relevance of lutein in human health. British Journal of Nutrition 90, 487–504.

Gross, J., 1987. *Pigments* in fruits. Academic Press. Londres

Hammond, B.R. Jr., Caruso-Avery, M., 2000. Macular pigment optical density in a Southwestern sample. Investigative Ophthalmology & Visual Science 41, 1492-1497.

Hruschka, W.R., 1987. Data analysis: Wavelength selection methods. In. Williams, P.C., Norris, K.H. (Eds.), Near-Infrared Technology in the Agricultural and Food Industries, St. Paul, Minn.: American Association Cereal Chemistry, pp. 35-55.

Johnson, E.J., 2002. The role of carotenoids in human health. Nutrition in Clinical Care 5, 56–65.

Lichtenthaler, H. K., Buschmann, C., 2001. Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. Current Protocols in Food Analytical Chemistry. http://dx.doi.org/10.1002/0471142913.faf0403s01.

Kato, M., Ikoma, Y, Matsumoto, H., Sugiura, M., Hyodo, H., Yano, M., 2004. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiology 134, 824-837.

Khachik, F., Beecher, G.R., 1988. Separation of carotenol fatty acid esters by high-performance liquid chromatography. Journal of Chromatography A, 449, 119-133.

Khachik, F., Bertram, J.S., Huang, M.T., Fahey, J.W., Talalay, P., 1999. Dietary carotenoids and their metabolites as potentially useful chemoprotective agents against cancer. In: L. Packer, M. Hiramatsu, and T. Yoshikawa (eds.). Antioxidant food supplements in human health. Academic Press, San Diego, CA, p. 203–229.

Klipstein-Grobusch, K., Launer, L. J., Geleijnse, J. M., Boeing, H., Hofman, A., Wittemen, J. C. M., 2000. Serum carotenoids and atherosclerosis: The Rotterdam Study. Atherosclerosis 148, 49-56.

Leth, T., Jakobsen, J., Andersen, N.L., 2000. The intake of carotenoids in Denmark. European Journal of Lipid Science and Technology 102, 128–132.

Martens, H., Naes, T., 1992. Multivariate Calibration. John Wiley & Sons.

Martínez-Valdivieso, D., Font, R., Gómez, P., Blanco-Díaz, T., Del Río-Celestino, M. 2014. Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy Journal of the Science of Food and Agriculture doi: 10.1002/jsfa.6667.

Mínguez-Mosquera, M.I., Hornero-Méndez, D., 1993. 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 41, 1616-1620.

Mínguez-Mosquera, M.I., Hornero-Mendez, D., 1997. Change in provitamin A during paprika processing. Journal of Food Protection 60, 853-857.

Muntean, E., Lazar, V., Muntean, N., 2006. HPLC-PDA analysis of carotenoids and chlorophylls from *Cucurbita pepo* L. convar. giromontina fruits. Buletinul Universitatii de Stiinte Agricole si Medicina Veterinara Cluj Napoca Seria Agricultura 62, 94-99.

Murray, I., 1986. The NIR spectra of homologous series of organic compounds. In: Hollo, J., Kaffka, K. J., Gonczy, J. L. (Eds.), Proceedings International NIR/NIT Conference. Budapest, Hungary: Akademiai Kiado. pp. 13-28.

Murray, I., 1993. Forage analysis by near reflectance spectroscopy. In: Davies, A., Baker, R. D., Grant, S. A., Laidlaw, A. S. (Eds.), Sward Management Handbook, British Grassland Society: UK, pp. 285-312.

Murray, I., Williams, P.C., 1987. Chemical principles of near infrared technology. In: Williams, P. C., Norris, K. (Eds.), Near Infrared Technology in the Agricultural and Food Industries AACC: St. Paul, MN, pp 17-34.

Naes, T., Isaksson, T., Fearn, T., Davies, T., 2002. A User-friendly guide to multivariate calibration and classification. NIR Publications, Chichester, UK. 420 pp.

Obrero, Á, González-Verdejo, C.I., Die, J.V., Gómez P., Del Río-Celestino M, Román B., 2013. Carotenogenic gene expression and carotenoid accumulation in three varieties of *Cucurbita pepo* during fruit development. Journal of Agricultural and Food Chemistry 61, 6393-6403.

Osborne, B.G., Fearn, T., Hindle, P., 1993. Practical NIR Spectroscopy with applications in food and beverage analysis. Longman Scientific and Tecnnical. London, UK.

Paris H.S., 2003. Genetic control of irregular striping, a new phenotype in *Cucurbita pepo*. Euphytica 129, 119-126

Paris H.S., 2008. Summer Squash. In. Prohens J, Nuez, F. (Eds.), Handbook of Plant Breeding. Vegetables I. Part 4. Volume 1. Springer. pp.351-381.

Paris, H.S., 1986. A proposed subspecific classification for *Cucurbita pepo*. Phytologia 61, 113-138.

Paris, H.S., 2000. *Quiescent intense* (*qi*), a gene that affects young but not mature fruit color intensity in *Cucurbita pepo*. Journal of Heredity 91, 333–339.

Rodríguez-Amaya, D.B., 1997. Carotenoids and food preparation: The retention of provitamin A carotenoids in prepared, processed, and stored foods. Opportunities for Micronutrient Intervention (OMNI), Arlington.

Rodríguez-Amaya, D.B., Kimura, M., Godoy, H.T., Amaya-Farfan, J., 2008. Update Brazilian database on food carotenoids: Factors affecting carotenoid composition. Journal of Food Composition and Analysis 21, 445-463.

Sánchez, T., Ceballos, H., Dufour, D., Ortiz, D., Morante, N., Calle, F., Zum Felde, T., Domínguez, M., Davrieux, F., 2014. Prediction of carotenoids, cyanide and dry matter contents in fresh cassava root using NIRS and Hunter color techniques. Food Chem. 151, 444–451.

Scalch, W., 1992. Carotenoids in the retina: a review of their possible role in preventing or limiting damage caused by light and oxygen. Experientia Supplementum 62, 80-298.

Schaefer, H., Heibl C., Renner S.S., 2009. Gourds afloat: a dated phylogeny reveals an Asian origin of the gourd family (*Cucurbitaceae*) and numerous oversea dispersal events. Proceedings of the Royal Society B: Biological Sciences 276, 843-851.

Schulz, H., Quilitzsch, H., Drews, H., Krüger, H., 2000. Estimation of minor components in caraway, fennel and carrots by NIRSs comparison of results from dispersive and Fourier transform instruments. International Agrophysics 14, 249-253.

Seddon, J. M., Ajani, U. A., Sperduto, R. D., Hiller, R., Blair, N., Burton, T. C., Farber, M. D., Gragoudas, E. S., Haller, J., Miller, D. T., Yannuzzi, L. A., Willet, W., 1994. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Journal of the American Medical Association 272, 1413 1420.

Segasothy, M., Phillips, P.A., 1999. Vegetarian diet: panacea for modern lifestyle diseases?. Quarterly Journal of Medicine 92, 531-544.

Shenk, J.S., 1992. Networking and calibration transfer. ln: Making Light Work: Advances in Near lnfrared Spectroscopy, VCH, London, pp. 223-228.

Shenk, J.S., Westerhaus, M.O., 1991. Population structuring of near infrared spectra and modified partial least squares regression. Crop Science 31, 1548-1555.

Shenk, J.S., Westerhaus, M.O., 1996. Calibration the ISI way. In: Murray, and Cowe (Eds), Near Infrared Journal of the American Medical Association**,** NIR Publications, Chichester, UK. pp.198-202.

Shokrzadeh, M., Azadbakht, M., Ahangar, N., Hashemi, A., Saeedi, Saravi, S.S., 2010. Cytotoxicity of hydro-alcoholic extracts of *Cucurbita pepo* and *Solanum nigrum* on HepG2 and CT26 cancer cell lines. Pharmacognosy Magazine 6, 176-179.

Smith, B.D., 2005. Reassessing Coxcatlan Cave and the early history of domesticated plants in Mesoamerica. In: Proceedings of the National Academy of Sciences, Volume 102, Issue 27, pp. 9438-9445.

Sinnott, E.W., 1936. A developmental analysis of inherited shape differences in cucurbit fruits. American Naturalist 70, 245-254.

Tadmor, Y., Larkov, O., Meir, A., Minkoff, M., Lastochkin, E., Edelstein, M., Levin, S., Wong, J., Rocheford, T., Lewinsohn, E., 2000. Reversed-phase high performance liquid chromatographic determination of vitamin E components in maize kernels. Phytochemical Analysis 11, 370-374.

Tadmor, Y., Paris, H.S., Meir, A., Schaffer, A.A., Lewinsohn, E., 2005. Dual role of the pigmentation gene B in affecting carotenoid and vitamin E content in squash (*Cucurbita pepo*) mesocarp. Journal of the Agriculture and Food Chemistry 53, 9759-9763.

Tkachuk, *R.,* Kuzina, F.D., 1982. Chlorophyll analysis of whole rape-seed kernels by near infrared reflectance. Canadian Journal of Plant Science 62, 875-884.

Van Het Hof, K.,H., West, C.E., Westrate, J.A., Hautvast, J.G., 2000. Dietary factors that affect the bioavailability of carotenoids. Journal of Nutrition 130, 503-506.

Van Het Hof, K.H., Brouwer, I.A., West, C.E., Haddeman, E., Steegers-Theunissen, R.P., Van Dusseldorp, M, Weststrate, J.A, Eskes, T.K., Hautvast, J.G., 1999. Bioavailability of lutein from vegetables is 5 times higher than that of beta-carotene. American Journal of Clinical Nutrition 70(2), 261-268.

Varma, S.D., Devamanoharan, P.S., Morris, S.M., 1995, Prevention of cataracts by nutritional and metabolic antioxidants. CRC Critical Reviews in Food Science and Nutrition 35 (1-2), 111- 129.

Venyaminov, S.Y., Prendergast, F.G., 1997. Water (H_2O and D_2O) molar absorptivity in the 1000–4000 cm^{-1} range and quantitative infrared spectroscopy of aqueous solutions. Analytical Biochemistry 248, 234–245.

Williams, P.C., Norris, K., 1987. Qualitative applications of near-infrared reflectance spectroscopy. In: Williams, P.C., Norris, K. (Eds.), Near-Infrared Technology in the Agricultural and Food Industries, the American Association of Cereal Chemists, St. Paul, Mn, pp.241-246.

Williams, P.C., Sobering, D.C., 1996. How do we do it: a brief summary of the methods we use in developing near infrared calibrations. In: Davies, A.M.C.,Williams, P.C. (Eds.), Near Infrared Spectroscopy: The Future Waves. NIR Publications, Chichester, pp. 185–188.

Xu, C.J., Fraser P.D., Wang, W.J., Bramley, P., 2006. Differences in the carotenoid content of ordinary citrus and lycopene-accumulating mutants. Journal of Agricultural and Food Chemistry 54*,* 5474–5481.

Zandomeneghi, M., Festa, C., Carbonaro, L., Galleschi, L., Lenzi, A., Calducci, L., 2000. Front-surface absorbance spectra of wheat flour: determination of carotenoids. Journal of Agricultural and Food Chemistry 48, 2216-2221.

CAPÍTULO V: Selection for high carotenoid content in zucchini (*Cucurbita pepo* subp. *pepo*)

Chapter V

Artículo en preparación:

Damián Martínez-Valdivieso[a], Pedro Gómez[a], Ángeles Alonso-Moraga[b], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[b]Department of Genetics, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

Abstract

Cucurbita pepo subsp. *pepo* is the most economically important species of the genus and is one of the most widely cultivated in the world. The increasing demand for developing new materials with high levels of essential micronutrients such as carotenoids has brought about the implementation of different breeding programmes. Recurrent selection methods have been effectively used by summer squash breeders to improve the performance of populations for quantitatively inherited traits. The main obstacle for screening the total carotenoid content (TCC) is the amount of labour and expense involved in measuring this compound by reference methods (high-performance liquid chromatography and UV-visible spectrophotometry) in comparison with color measurement systems. The objectives of this work were i) to determine if the carotenoid content of summer squash (*C. pepo subp. pepo*) can be correlated with colorimetric analysis using the CIE L*a*b* color value system for being used as method for rapid selection, and ii) to evaluate the efficiency of recurrent selection for two cycles in "Yellow zucchini" aimed at increasing TCC. Using the colour parameters (L*, *a**, *b**, Chroma and Hue angle) we found correlation correlation (R^2 = 0.84) beetween hue angle and carotenoid content in fruit pulp. Results indicated that summer squash responded well to the methodology employed with consistent and significant gains for TCC.

Key words: zucchini, carotenoids, color, recurrent selection

Introduction

Zucchini (*Cucurbita pepo* L. morphotype Zucchini) is an important greenhouse crop in South-Eastern Spain, mainly in the province of Almería. The zucchini fruit is harvested immature and almost the entire harvest is exported from Spain to other European countries. Increased production of this crop can be attributed to its versatility, it can be consumed directly in purees, juices, soups, creams, sweets, pastries, and preserves or used as raw material for the agro-industrial processing of flours and dehydrated products.

The main components of summer squash fruit are carbohydrates which represent the 85-90% of the total dry matter. Other minor compounds found in this vegetable with high nutritional value are carotenoids, chlorophyll pigments (*a* and *b*), total phenolic compounds (TPC), ascorbic acid (AA), minerals (calcium, iron, phosphorus) and amino acids such as thiamin and niacin (Martínez-Valdivieso et al., 2014a, b, c; Blanco-Díaz et al., 2014; Møller and Loft, 2004; Oloyede et al., 2012)

In relation to the TCC in fruit, these are natural pigments widely distributed in nature, and are responsible for the yellow-to-intense orange color in plants (Arima and Rodríguez-Amaya, 1990). These pigments are found in the chromoplasts of higher plants, but their color may be masked by chlorophyll (Zaccari et al., 2007). β-carotene, abundant in several varieties of squash, is a principal precursor of vitamin A, which plays a role in many bodily functions: it enhances the immune system and decreases the risk of degenerative diseases such as cellular aging, cancer, cardio vascular diseases, arteriosclerosis, age-related muscular degeneration and cataract formation (Astorg, 1997; Clevidence et al., 2000; Rodríguez-Amaya, 2001; Bramley, 2003; Southon and Faulks, 2003).

Recently, different programs have been used for improving morphological, physiological and nutritional characters, disease resistance and high yield. Among these initiatives is the mutation breeding (Vicente-Dólera et al., 2014) and the evaluation of the carotenoid content in summer squash germplasm where the maximum level detected was 451.2 mg g^{-1} of dry weight (Martínez-Valdivieso et al., 2014b). Other methodology used effectively in genus *Cucurbita* is the recurrent selection. Within the breeding species of the genus *Cucurbita* it´s possible to use “selfing” because although the plants are out-crossing plants, there is virtually no loss of vigour due to inbreeding (Allard, 1971; Abd El-Al et al., 1973; Whitaker and Robinson, 1986; Robinson, 1999). Recurrent selection methods have been used to

improve the performance of populations for quantitatively inherited traits (Ceballos et al., 2013; Nienhuis and Lower, 1989). Recurrent selection is a cyclical process, which, except for mass selection, includes three phases: (i) development of progenies, (ii) progeny evaluation, and (iii) recombination of selected families or progenies. Although most recurrent selection methods include these three phases, they vary in types of progenies evaluated (i.e., inbred, full-sib, half-sib, etc.); number of progenies evaluated; number of selected families (i.e., 5, 10, 20, 30, etc.); parental control; and the type of progenies intermated. It is this flexibility in the different types of methods and different parameters that has led to the utilization of a wide range of recurrent selection methods for population improvement with an equally wide range of responses. Even with the diversity of recurrent selection methods, two goals remain common throughout, increasing the mean performance of the population and maintaining the genetic variability in the population to facilitate long-term selection.

One reason recurrent selection for TCC has traditionally not been used extensively is the amount of labour and expense involved in measuring this compound by UV-Vis spectrophotometer or HPLC. If a fair prediction of carotenoid content could be obtained, this rapid and inexpensive method could be very useful in breeding summer squash for enhanced carotenoid levels. Previous studies have correlated color measurement systems with carotenoid content in vegetable crops such as tomato (Arias et al., 2000; D'Souza et al., 1992), sweet-potato (Ameny and Wilson, 1997; Simonne et al., 1993), cassava (Sánchez et al., 2014), carrot (Park et al., 1995), pepper (Reeves, 1987; Lee and Lee, 1992), winter-type squash (Francis, 1962; Seroczynska et al., 2006) and pumpkin and squash (Itle and Kabelka, 2009).

Based on these aspects, the objectives of this work were i) to determine if the carotenoid content of summer squash (*C. pepo* subp. *pepo*) can be correlated with colorimetric analysis using the CIE L*a*b* colour value system for being used as method for rapid selection, and ii) to evaluate the efficiency of recurrent selection for two cycles in "Yellow zucchini" aimed at increasing TCC.

Material and methods

The "Yellow zucchini" commercial hybrid cultivar was selected on the basis of their high carotenoid content (Martínez-Valdivieso et al., 2014b).

Inbreeding and recurrent selection procedure:

The Figure 1 illustrates the chronology of cycling recurrent selection in summer squash for increasing carotenoid content. Each population obtained was represented by 200 plants.

Original selection cycle: Commercial seeds of “Yellow zucchini”, were collected from local seed retailer, and mixed together to form the base population P_0

The P_0 plants were grown following standard local cultural practices for both plant nutrition and insect pest and disease control in the IFAPA Center La Mojonera (36º47'19"N, 02º42'11"W; 142 m. a.s.l.). Then, co ntrolled self-pollinations were attempted on each plant, for this, female flowers were protected before anthesis to prevent the transfer of pollen by insects, and each P_0 plant was self-pollinated by hand early in the morning. At 60–80 days after self-pollination, plants with desirable phenotype (fruit with the lowest Hue angles) were selected and P_1 seeds from fruits at the mature stage were extracted.

In the second year, separate progeny rows were grown from selfed seeds of the selected plants. Then, the progenies were intercrossed in all possible combinations by hand, and an equal amount of seed from each cross was composited to produce the next generation. This completes the original selection cycle.

First recurrent selection cycle: For recurrent selection, several desirable plants were selected from the composited population obtained from the original selection cycle; they were self-pollinated and then plants with desirable were selected on the basis of phenotype.

The next year, progeny rows were grown from the selfed seed and all possible intercrosses were made by hand. Equal seeds from all the intercrosses were composited to produce the next generation.

This constitutes the first recurrent selection cycle. The population could be subjected to one or more recurrent selection cycles.

Colorimetric analysis for estimating the TCC in fruit mesocarp

A total of 200 fruits were harvested for colorimetric and UV-visible spectrophotometer analysis from P_1 population. Fruits were harvested mature at the time they were harvested for seed extracting. Mesocarp fruit color was recorded using a Minolta CR-400 Colorimeter (Minolta Camera Co., Ltd., Ramsey, NJ) tristimulus color analyzer, equipped with an 8 mm diameter measuring area.

Each fruit was sliced transversely, and L*, a*, and b* color space measurements from the fruit mesocarp were recorded within five minutes to avoid discoloration. Therefore, avoiding the seed cavity and surrounding tissue, three random measurements were carried out on the surface of the mesocarp, and colour parameters L*, a*, and b* were registered. The L* coordinate indicates darkness or lightness of color and ranges from black (0) to white (100). Coordinates, a* and b*, indicate color directions: +a* is the red direction, –a* is the green direction, +b* is the yellow direction, and –b* is the blue direction. Chroma (C*) is the saturation or vividness of color. Hue angle (Hº) is the basic unit of color and can be interpreted, for example, as 0º= red and 90º = yellow. Both chroma and hue are derived from a* and b* using the following equations:

$C^* = (a^{*2} + b^{*2})^{1/2}$

H^0 = arctangent (b*/a*)]

Statistical analysis

Pearson correlation coefficients (r) and linear regression (R^2) between color space values and carotenoid content were calculated from the means of the fruits using the CORR and REG procedure of SAS (Statistical Analysis System Version 9.2; SAS Institute, Cary, NC).

Results and discussion

Figure 2 shows some fruits obtained from the P_1 population, which ranged from green to yellow-orange.

Mean color space values a* (+a* red direction; –a* green direction) ranged from -4.12 to 35.33 in epicarp and from 0.13 to 26.77 in mesocarp. The mean color space value, b* (+b* yellow direction; –b* blue direction) was from -0.23 to 65.1 in epicarp and from 14.05 to 68.33 in mesocarp. The fruit mesocarp showed hue angles of 90º or less (the lowest being 65.08) ranging from light yellow to dull yellow-orange, mean a* values varied from 0.13 to 26.77, and mean b* values ranged from 14.05 to 68.33. Fruit with hue angles of 90º or greater (the highest being 198.8º) for epicarp had yellow to green skin, mean a* values ranging from 00.02 to -4.12, and mean b* values varying from -0.23 to 26.75. Genotypes with hue angles of 90º or less (the lowest being 53.6º) had yellow to dull yellow-orange skin, mean a* values ranging from 0.09 to 35.33, and mean b* values between 10.61 and 65.1.

Correlations between colorimetric values and TCC in flesh

Pearson correlation coefficients (r) between color space values, L*, a*, b*, Chroma, and Hue, with TCC measured in this study, were calculated. The color value L* (lightness or darkness) correlated negatively with total carotenoids in mesocarp (r =-0.72**). The color value a* (color direction in red or green) was correlated with total carotenoids in mesocarp (r = 0.89**). The color value b* (color direction in yellow or blue) and Chroma (saturation or vividness of color) correlated with total carotenoids in mesocarp (r = 0.83*** and r = 0.84***, respectively). Hue (tint of color, an angular measure) was strongly correlated with total carotenoids (r = –0.92***). The negative correlation observed between Hue and the carotenoids measured in this study suggests that as Hue angles decrease, carotenoid concentrations would increase.

Studies relating colorimetric values with total carotenoids in winter type squash have previously been reported. Francis (1962) evaluated cultigens of *C. maxima* and *C. moschata* and identified moderate to strong correlations between L* (r = –0.78), a* (r = 0.83), b* (r = 0.79), chroma (r = 0.93), and hue (r = –0.96) with total carotenoids. In a study evaluating *C. maxima* germplasm, Seroczynska et al. (2006) reported poor to fair correlations between L* (r = –0.53***), a* (r = 0.77***), b* (r = 0.76***), and chroma (r = 0.77***) with total carotenoids. Itle and Kabelka (2009) obtained strong correlations between color value a* (r=0.91***), b* (r=0.75**), chroma (r=0.76**), hue (-0.83**) and TCC and weak correlations between L* (r=0.66) and TCC. After comparing our findings with those of Francis (1962), Serocyznska et al. (2006) and Itle and Kabelka (2009) similar correlations and strengths were found

Regression equation and R^2 value for the prediction of total carotenoid content based on hue, in our findings, are provided in Table 1. The R^2 value indicated that 84% of the TCC variation can be accounted for by the change in hue. Although regression equations based on these correlations may account for only 84% of the variation for TCC, they may still be useful for estimating this concentration.

Figure 3 is presented to illustrate trends related to selection cycles of both the skin and pulp color. There were consistent gains throughout the different cycles, in agreement with data presented in Table 2, showing the variability of the Hue angle in the populations for two recurrent selection cycles. It is important to emphasize that the comparison among the populations has been performed during different years , even though the first (P_0) and last (P_4) populations were grown in the same season.

Within the P_1 population, the genotype with the highest Hue value measured in seedling nurseries for both epicarp and mesocarp was discovered, as well as other

genotypes with lower levels (Table 2). This type of segregation suggests that this specific trait inheritance is controlled by more than one gene. However, the rapid gains attained demonstrate high heritabilities.

The average TCC level from the P_0 population was 315 and 1104.6 mg Kg^{-1} dry weight, in mesocarp and epicarp, respectively, which can be considered the baseline at the start of this work. This value was surpassed in both, mesocarp and epicarp during the second selection cycle , in fact, the P_4 population ranged from 385 to 508.04 and from 1415 to 1600 mg Kg^{-1} dry weight. This suggests that successive selection cycles would allow to further increase the TCC and reduce the coefficient of variation in the population.

The direct comparison of the results reported here with other studies is difficult because of the differences in traits and selection methods. Our study was not designed to answer questions regarding the genetic basis of the observed responses to selection; however, we believe that two main reasons may be responsible for this. First, the results are consistent with effects of a few major quantitative trait loci associated with the TCC. Previous inheritance studies indicated that carotenoid content (with color range from white to orange) are controlled by at least three genes (Paris and Nerson, 1986), *D* (*Dark*), *l-1* (*light coloration-1*), and *l-2* (*light coloration-2*) that have a major effect on fruit rind color intensity. A fourth fruit-color gene, designated *B* (*Bicolor*), does not noticeably affect the fruit´s exterior color intensity but rather its Hue angle (Shifriss, 1965). Fruit-flesh color is also affected by these genes. One additional gene that effects squash flesh color is the dominant Wf (white flesh), which confers a white flesh color by preventing yellow pigment accumulation (Paris and Nelson-Brown, 2005). Second, we only selected for TCC, and a direct response to selection for a single trait is expected to be larger than the response when selecting simultaneously for multiple traits (Falconer and Mackay, 1996).

Conclusions

Although data from two selection cycles suggested that gains have been made through time, future work will be necessary to compare high TCC material developed growing them together in two different locations. This is an important finding as it indicates that summer squash responded well to the methodology employed and therefore fruit in this species with highest added value can be obtained.

Acknowledgements

The authors wish to express their thanks to the Project INIA RTA00039-000 and FEDER for the funding of this research. Damián Martínez-Valdivieso was supported by an INIA (Instituto de Investigación y Tecnología Agraria y Alimentaria) contract. We thank Nicholas Davies for his help in the grammatical revision of the manuscript.

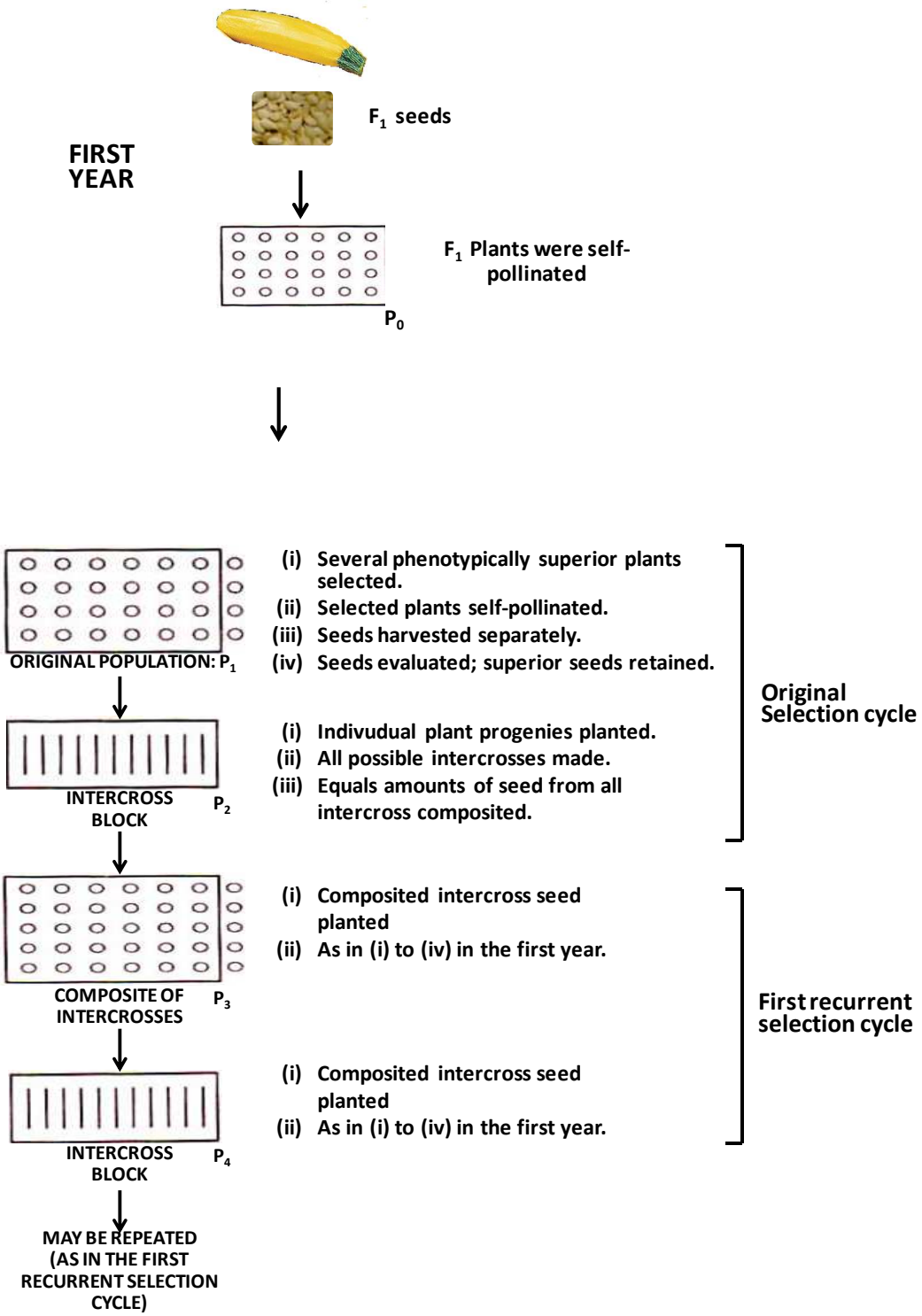


Figure 1. Illustration of the chronology of cycling recurrent selection in summer squash for increasing carotenoid content.

Figure 2. Some fruits obtained from the P_1 population.

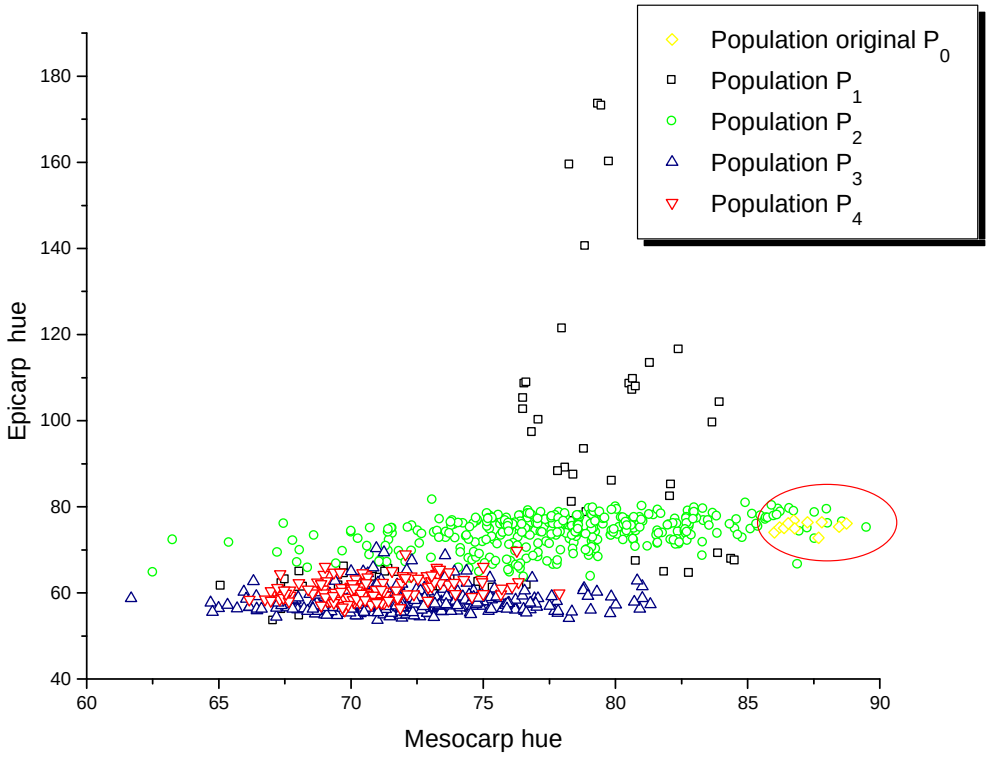


Figure 3. Scatter plot for mesocarp and epicarp Hue angles from populations obtained in two cycles of selection.

Table 1. Regression equation and R^2 coefficient between the best space value and carotenoid content in mesocarp summer squash.

Mesocarp	Hue	R^2 Coefficient
Regression equations	y= -10.262+1175.9	0.8464

Values shown are based on 200 fruits from three colorimetric measurements per fruit

Table 2. Estimation of variability of the Hue angle in the base population (P_0) and selected population for two recurrent selection cycles

	Population	Hue	Mean	Coefficient variation
Epicarp	P_0	72.81-77.11	75,54	1.62
	P_1	53.58-173.55	76.89	35.92
	P_2	61.1-81.97	74.26	4.7
	P_3	53.68-70.37	58.05	4.39
	P_4	55.98-69.8	60.78	4.24
Mesocarp	P_0	86.01-90	87.38	1.03
	P_1	61.08-84.52	74.57	6.79
	P_2	62.52-89.51	77.87	5.58
	P_3	61.68-81.97	74.26	4.35
	P_4	66.16-77.88	70.77	3.32

References

1. Allard R.W.1971. Principles of genetic improvement .Sào Paulo: Edgard Blūchner.381 P.

2. Abd El–Al Z.E., Khalf–Allah A.M. Shenoud G.S. 1973. Effect of visual selection and inbreeding on some quantitative characters of summer squash. Alex J Agric Res, 21: 277.

3. Ameny M.A. and Wilson P.W. 1997. Relationship between Hunter color values and b-carotene contents in white-fleshed African sweetpotatoes (*Ipomoea batatas* Lam). J Sci Food Agr, 73: 301–306.

4. Arias R., Lee T.C., Logendra L., Janes H. 2000. Correlation of lycopene measured by HPLC with the L*, a*, b* color readings of a hydroponic tomato and the relationship of maturity with color and lycopene content. J Agr Food Chem, 48: 1697–1702.

5. Arima H.K. and Rodríguez-Amaya D.B. 1990. Carotenoid composition and vitamin A value of squash and pumpkin from northeastern Brazil. Arch. Latinoam. Nutr. 40: 284–292.

6. Astorg P. 1997. Food carotenoids and cancer prevention: An overview of current research. Trends Food Sci Technol, 8: 406–413.

7. Blanco-Díaz M.T., Del Río-Celestino M., Martínez-Valdivieso D., Font R. 2014. Use of visible and near-infrared spectroscopy for predicting antioxidant compounds in summer squash (*Cucurbita pepo* ssp *pepo*). Food Chem. 164:301-8.

8. Bramley P. 2003. Chapter 13: The genetic enhancement of phytochemicals: The case of carotenoids. In: Johnson, I.T. (ed.). Phytochemical Functional Foods. Woodhead Publishing Limited, Cambridge, England. pp. 153–179.

9. Ceballos H., Morante N., Sánchez T., Ortiz D., Aragón I., Chávez A.L., Pizarro M., Calle F., Dufour D. 2013. Rapid Cycling Recurrent Selection for Increased Carotenoids Content in Cassava Roots. Crop Science, 53: 1-10.

10. Clevidence B., Inke P., Smith J.R., Cecil J. 2000. Bioavailability of carotenoids from vegetables. HortScience, 35(4): 585–588.

11. D'Souza M.C., Singha S., Ingle M. 1992. Lycopene concentration of tomato fruit can be estimated from chromaticity values. HortScience, 27: 465–466.

12. Falconer D.S. and Mackay T.F.C. 1996. Introduction to quantitative genetics. Longman, Essex, England.

13. Francis F.J. 1962. Relationship between flesh color and pigment content in squash. Amer. Soc. Hort. Sci. 81:408–414.

14. Itle R.A. and Kabelka E.A. 2009. Correlation Between L*a*b* Color Space Values and Carotenoid Content in Pumpkins and Squash (*Cucurbita* spp.). Hortscience, 44(3): 633–637.

15. Lee H.D. and Lee C.H. 1992. Studies on the quality evaluation of Korean red pepper by color measurement. Kor. J. Dietary Culture, 7: 105-112.

16. Martínez-Valdivieso D., Font R., Gómez P., Blanco-Díaz M.T., Del Río-Celestino M. 2014a. Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy. Journal of the Science of Food and Agriculture. http://dx.doi.org/10.1016/j.compag.2014.07.003

17. Martínez-Valdivieso D., Font R., Blanco-Díaz M.T., Moreno-Rojas J.M., Gómez P., Alonso-Moraga A., Del Río-Celestino M. 2014b. Application of near-infrared reflectance spectroscopy for predicting carotenoid content in summer squash fruit. Computers and Electronics in Agriculture. http://dx.doi.org/10.1002/jsfa.6667.

18. Martínez-Valdivieso D., Gómez P., Font R., Del Río-Celestino M. 2014c. Mineral composition and potential nutritional contribution of 34 genotypes from different summer squash morphotypes. European Food Research and Technology. DOI 10.1007/s00217-014-2308-7

19. Møller P.and Loft S. 2004. Interventions with antioxidants and nutrients in relation to oxidative DNA damage and repair. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 551: 79–89.

20. Nienhuis J. and Lower R.L. 1988. Comparison of two recurrent selection procedures for yield in two pickling cucumber population.J.Amer.Soc. Hort. sci.113: 272–276.

21. Oloyede F.M., Agbaje G.O., Obuotor E.M., Obisesan I.O. 2012. Nutritional and antioxidant profiles of pumpkin (*Cucurbita pepo* Linn.) immature and mature fruits as influenced by NPK fertilizer. Food Chemistry, 135: 460-463.

22. Paris H.S. and Nerson H. 1986. Genes for intense pigmentation of squash. J Hered, 77: 403-409.

23. Paris H.S. and Nelson-Brown R. 2005. The genes of pumpkin and squash. HortScience, 40: 1620–1630.

24. Park S.W., Park Y. and Joung H. 1995. Quality evaluation by color determination in Carrot (*Daucus carota* L.) Estimation of total carotenoid content by Colorimeter. J Kor Soc Hort Sci, 36: 481-485.

25. Reeves, M.J. 1987. Re-evaluation of Capsicum color data. J Food Sci, 52:1047–1049.

26. Robinson R.W.1999. Rational methods for producing hybrid cucurbit seed.J of New seeds, 1: 1–47.

27. Rodríguez-Amaya D.B. 2001. A guide to carotenoid analysis in food. ILSI International Life Sciences Institute, Washington DC.

28. Sánchez T., Ceballos H., Dufour D., Ortiz D., Morante N., Calle F., Zum Felde T., Domínguez M., Davrieux F. 2014. Prediction of carotenoids, cyanide and dry matter contents in fresh cassava root using NIRS and Hunter color techniques. Food Chem, 151: 444–451.

29. Seroczynska A., Korzeniewska A., Sztangret-Wisniewska J., Niemirowicz-Szczytt K., Gajewski M. 2006. Relationship between carotenoid content and flower or fruit flesh colour of winter squash (*Cucurbita maxima* Duch.). Horticulturae, 18: 51–61.

30. Shifriss O. 1965. The unpredictable gourds. Am Hortic Mag, 44: 184-201.

31. Simonne A.H., Kays S.J., Koehler P.E., Eitenmiller R.R. 1993. Assessment of beta-carotene content in sweet potato (*Ipomoea batatas* Lam.) breeding lines in relation to dietary requirements. J Food Compost Anal, 6: 336–345.

32. Southon S. and Faulks R. 2003. Chapter 7: Carotenoids in food: Bioavailability and functional benefits. In: Johnson, I.T. (ed.). Phytochemical Functional Foods. Woodhead Publishing Limited, Cambridge, England. pp. 117–127.

33. Vicente-Dólera N, Pinillos V, Moya M, Del Río-Celestino M, Pomares-Viciana T, Román B, Gómez P. 2014. An improved method to obtain novel mutants in *Cucurbita pepo* by pollen viability. Scientia Horticulturae. 169: 14–19.

34. Whitaker T.W. and Robinson R.W.1986. Squash breeding .In.Basset M.I.(ed) Breeding vegetable crops.Westport : Avi.P.209 – 242.

35. Zaccari F., Galietta G., Durán A., Soto B., Gratadoux V. 2007. Cuantificación de β-caroteno en zapallos (*Cucurbita* sp.) cultivados en Uruguay. In: Memorias, V Congreso Iberoamericano de Tecnología Postcosecha y Agroexportaciones. Montevideo, Uruguay. pp. 544–549.

CAPÍTULO VI: Role of zucchini and its distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxicity, cytotoxicity and apoptotic effects

Chapter VI

Artículo en preparación:

Damián Martínez-Valdivieso[a], Rafael Font[b], Zahira Fernández-Bedmar[c], Tania Merinas-Amo[c], Marcos Mateo-Fernández[c], Ángeles Alonso-Moraga[c], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[b]Department of Postharvest Technology and the Agrifood Industry, IFAPA Center La Mojonera Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[c]Department of Genetics, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

ABSTRACT

Zucchini (*Cucurbita pepo* subsp. *pepo*) is one seasonal vegetable with high nutritional and medical value. Many useful properties of this fruit are attributed to its bioactive compounds. The purpose of this study was to demonstrate the safety and suitability of the use of Zucchini fruit ("Yellow" and "Light Green" varieties), along with some of its bioactive components, carotenoids (lutein, β-carotene and zeaxanthin) and dehydroascorbic acid. In order to measure these activities, three types of assays were carried out: (i) determination of the safety and DNA-damage protecting ability against free radicals by using the Somatic Mutations and Recombinations Test of *Drosophila melanogaster*; (ii) cytotoxic activity (using the promyelocytic leukaemia HL60 cell line) and (iii) apoptotic effect evaluated using a DNA fragmentation assay based on the formation of internucleosomal units. Results showed that: (i) all the substances were safe, non-genotoxic, at the assayed concentrations, (ii) all the substances were antigenotoxic (ranging from 11 to 100 % inhibition) in combined treatments with the genotoxicant hydrogen peroxide, except the highest concentration of lutein, thereby confirming its antioxidant capacity, (iii) "Yellow" Zucchini epicarp and mesocarp exhibited the highest cytotoxic activity (IC_{50} > 0.1 mg/ml and 0.2 mg/ml, respectively), iv) "Light Green" Zucchini skin and pulp and the mix of the active compounds (β-carotene, zeaxanthin and dehydroascorbic acid) also exhibited cytotoxic activity but IC_{50} was not reached. Taking into account the safety, antigenotoxicity, cytotoxicity and apoptotic effect data obtained in the different assays, Zucchini can be a candidate as a nutraceutical food because it is not genotoxic, is able to protect DNA against free radicals and inhibits the tumour cell growing.

Key words: *Cucurbita*, cancer, chemoprevention, antigenotoxicity, carotenoid

INTRODUCTION

It is currently accepted that diet can affect the overall process of carcinogenesis by different mechanisms: its constituents may contain cancer-causing substances as well as many cancer preventive agents (Kuno et al., 2012). Inappropriate dietetic habits are estimated to be the cause of more than one third of cancer deaths (Fernández-Bedmar et al., 2011).

There are more than 20000 species of plants used in traditional medicines, and these are all potential reservoirs for new drugs (Hamamouchi, 2002) and are a potential source of chemical constituents with antitumor and cytotoxic activities (Saha et al., 2011). Protective elements in a cancer prevention diet include selenium, folic acid, vitamin B-12, vitamin D, chlorophyll and antioxidants such as the carotenoids (α-carotene, β-carotene, lycopene, lutein, cryptoxanthin) (Donaldson, 2004).

Summer squash (*Cucurbita pepo* subsp*. pepo*) is a seasonal vegetable that contains a number of beneficial micronutrients such as minerals, carotenoids, vitamin C, phenolic compounds, etc. (Blanco-Díaz et al., 2014, Martínez-Valdivieso et al., 2014a, Martínez-Valdivieso et al., 2014b, Martínez-Valdivieso et al., 2014c). In the past it has been used in traditional folk medicine to treat colds and alleviate aches, due to its antioxidant/anti-radical, anti-carcinogenic, anti-inflammatory, antiviral, antimicrobial and analgesic activities (Menéndez et al. 2006; Møller and Loft, 2004; Oloyede et al., 2012; Shokrzadeh et al. 2010, Wang et al, 2007).

Studies of genotoxicity/antigenotoxicity and cytotoxicity are rapid methods to assess the innocuousness and possible beneficial effects of single compounds or complex mixtures and also foods (Anter et al. 2011a and b, Villatoro-Pulido et al. 2012, 2013; Tasset-Cuevas et al., 2013). The Somatic Mutation and Recombination Test (SMART) of *Drosophila melanogaster* is a suitable eukaryotic tool for genotoxicity and antigenotoxicity studies (Graf et al., 1984) and is a one-generation test based on the loss of heterozygosity of two suitable recessive markers (*mwh* and *flr*), due to different genotoxic events (*i.e.* mitotic recombination, mutation and chromosomal aberration). HL60 human leukaemia cells have been used in cytotoxicity assays in order to determine the tumoricide activity of some vegetable matrices (Villatoro-Pulido et al., 2012).

There are a few studies where the cytotoxic activity of *C. pepo* has been checked. In this way, Shokrzadeh et al. (2010) reported different cytotoxic activities of the *C. pepo* leaf extracts on normal and cancer cell lines. Wang et al., (2007) observed a significant dose-dependent inhibitory effect against HeLa and HepG cell growth of the extract of *C. pepo* fruits. Menéndez et al., (2006) also reported also a significant decrease of the growth of prostatic hyperplasia by extract of *C. pepo* seeds at the tested concentrations (400 and 200 mg/kg). However no studies have been carried out to demonstrate the presence or absence of *in vivo* activity of Zucchini fruit and about the mechanisms of action of their antioxidant compounds.

In the case of the genotoxic or antigenotoxic activities of antioxidant compounds such as the ascorbic acid (the reduced form of dehydroascorbic acid), most of the reported data (Collins,1999; Hartman and Shankel, 1990; Odin, 1997) indicates that it is anti-mutagenic. Although antioxidant protection is assumed for ascorbic acid against oxidative damage, the details of this protection are not yet completely understood. In fact, under different conditions, ascorbic acid seems to have co-genotoxic activity instead of the normal antigenotoxic action (Kaya et al. 2002).

Studies about carotenoids have also been performed in relation to decrease cancer risk. Thus, the chemopreventive action of β-carotene is effective mainly at the beginning of the carcinogenic process or in the initial stages of its promotion, inhibiting the formation of pre-neoplastic lesions in some *in vitro* and *in vivo* experimental models (Moreno et al., 1995; He et al., 1997; Gradelet et al., 1997). Lutein and zeaxanthin, naturally occurring carotenoids, have shown to reduce the risk of cataracts and age-related macular degeneration (Ravikrishnan et al., 2011). Zeaxanthin is related with the reduction of melanoma cells viability (Bi et al., 2013). Lutein caused significant DNA damage in human retinal pigment epithelial cells (Kalariya et al., 2009).

The aim of this work was to demonstrate the safety and suitability of the use of Zucchini fruit, along with some of its active components, carotenoids (lutein, β-carotene, and zeaxanthin) and other antioxidant compounds (dehydroascorbic acid, chlorophyll a and chlorophyll b), with respect to DNA integrity, and to establish the possible *in vivo* genotoxic and *in vitro* cytotoxic effects. In order to measure these properties, three types of assays were carried out: genotoxicity and antigenotoxicity (*in vivo* analysis using the *Drosophila melanogaster* model), and cytotoxicity (*in vitro* assays using the promyelocytic leukaemia HL60 cell line). In this way, the beneficial

effect of the consumption of Zucchini could be explained, and the investigation of its mechanism of action may lead to major advances in the prevention of human cancer.

MATERIAL AND METHODS

Plant material

Two different varieties of *C. pepo* belonging to Zucchini morphotype were evaluated in this work: “Light Green” (elongated in shape with light green skin) and “Yellow” (elongated in shape with yellow skin). They were representatives of Zucchini commercial cultivars currently offered in the market. Seeds of these varieties were germinated on wet filter paper in Petri dishes at room temperature for 2-4 days in the dark, after which they were transplanted into rock-wool cubes (Grodan BV, 6040KD Roermond, NL) in a greenhouse. When plants had developed three to four leaves they were transferred to 1 m large rock-wool slabs at a density of two plants/slab. Plants were grown in a greenhouse in the IFAPA Center in La Mojonera, Almería, Spain (36º47'19"N, 02º42'11"W; 142 m a.s.l.) from March to June 2011 following standard local cultural practices for both plant nutrition and insect pest and disease control. Six fruits of each variety were harvested at an immature stage, and they were processed preserving epicarp and mesocarp of each fruit separately, packaged in polypropylene plastic containers and stored at -80ºC. Sample was lyophilized using freeze drier equipment (Telstar LyoQuest, Germany) at -55 ºC under vacuum (133 x 10^{-3} mBar) for 96 h per sample. Then, the samples were ground and frozen at -80ºC for further extractions and biological analyses.

Antioxidant compounds

The compounds used in this study were purchased from: lutein and β-carotene (Cat. Number 07168 and 22040, respectively, Fluka Chemika-bioChemika, Buchs, Switzerland), zeaxanthin (Cat. Number 0307S, Extrasynthese, Lyon, France) and dehydroascorbic acid (Cat. Number 261556, Sigma-Aldrich, Missouri, USA). The carotenoids were dissolved in ethanol prior to addition to the corresponding culture media *i.e.* in water for flies treatment, or in RPMI 1640 medium for HL60 cell culture at the time of the experiment. The final concentration of ethanol was 1 % in the culture media.

Determination of the carotenoid content

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. Individual carotenoid concentration was determined by reverse phase HPLC (High-Performance Liquid Chromatography) after saponification as detailed in Martínez-Valdivieso et al. (2014c). The carotenoids were extracted from the rehydrated sample with 5 ml ethanol containing 1 mg mL-1 butylated hydroxytoluene (BHT) using a Polytron homogenizer. Samples were saponified in order to hydrolyze esterified carotenoids that might complicate the chromatographic determinations (Khachik et al., 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 85ºC. 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 □L of an acetonitrile/methanol/dichloromethane (45:20:35 v/v/v) solution, filtered through a 0.22 □m PTFE syringe filter (Millipore) directly to sample vials, and 10 □L were injected into the chromatograph. The initial mobile phase consisted of acetonitrile/methanol (97:3, v/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 30º 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 x 150 mm*, *5* □m) 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. Compounds were identified by comparison of retention times, co-injection with known standards, and comparison of their UV-visible spectra with authentic standards. Quantification was carried out by external standardization. Full standard curves were constructed with five different concentrations for each carotenoid in triplicate. The curves passed through or were very near the origin, were linear and bracketed the concentrations expected in the samples. Results were expressed on a dry weight (DW) basis.

Once the content of the selected antioxidant compounds was evaluated in the epicarp and the mesocarp of *C. pepo* fruit, it performed the genotoxicity, cytotoxicity and apoptosis assays.

Extraction and analysis of vitamin C

The vitamin C analysis was carried out with freeze dried lyophilized samples stored at −80 Cº. Five grams of samples were homogenized in 10mL of MeOH/H_2O (5:95) plus citric acid (21 g L^{-1}) with EDTA (0.5 g L^{-1}) and 4mM NaF. Homogenates were then filtered through cheesecloth and C18 Sep-Pak cartridges (Waters, Milford, MA). Ascorbic acid (AA) and dehydroascorbic acid (DHA) contents were determined as described by Zapata and Dufour (1992). HPLC analyses were performed after derivatization of DHA into the fluorophore 3-(1,2-dihydroxyethyl) furol [3,4-b]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride (OPDA). Samples of 20 µL were analyzed by using a Merck-Hitachi (Tokyo, Japan). The analyses were carried out on a HPLC apparatus equipped with binary pump, in-line vacuum degasser, autosampler injector, a Waters and a 996 diode array detector (Waters, Milford, MA) supported by the Empower chromatography manager computing system (Waters). Separations of DFQ and AA were achieved on a Kromasil 100 C18 column (250mm×4 mm; 5µm particle size; Tecnokroma, Barcelona, Spain). The mobile phase was MeOH/H_2O (5:95, v/v) containing 5mM cetrimide and 50mM potassium dihydrogen phosphate at pH 4.5. The flow rate was 0.9mLmin^{-1}. The detector wavelength was initially set at 348 nm and after elution of DFQ, the wavelength was manually shifted to 261 nm for AA detection. Standard solutions, column conditioning and derivatization procedures have been previously described by Gil et al. (1999).

Genotoxicity and antigenotoxicity tests

The principles and basic procedures for the *Drosophila* wing spot test have been described by Graf et al. (1984), and in previous works of our group (Anter et al., 2011; Fernandez-Bedmar et al., 2011). Two strains of flies carry wing genetic markers on the left arm of chromosome 3: *multiple wing hair* (*mwh*, 3-0.3) and *flare* (*flr*^{3}, 3-38). The transheterozygous larvae were obtained by crossing *mwh*/*mwh* males and *flr*^{3}/TM3, *Bd*^{S} virgin females. Hybrid eggs derived from crossing optimally fertile flies were collected over an 8 h period. Larvae emerged 72 ± 4 h later were cleaned from remaining feeding medium with distilled water, and subsequently transferred to treatment vials. These vials contained 0.85g of *Drosophila* Instant Medium (formula 4-24, Carolina Biological Supply, Burlington NC, USA) wetted with 4 ml of the epicarp

and mesocarp of *C. pepo* and their antioxidant compounds solutions at physiological concentrations for *Drosophila melanogaster*: 0.25 and 8 mg/ml of epicarp and mesocarp of each variety, 0.039 and 0.615 µM lutein, 0.0003 and 0.0689 µM β-carotene, 0.0001 and 0.105 µM zeaxanthin, and 0.003 and 0.107 mM dehydroascorbic acid. Concurrent negative controls with the solvent alone (water) and positive controls with hydrogen peroxide (120 mM) were also run. Antigenotoxicity tests were carried out by mixing the mutagen (hydrogen peroxide, 120 mM) with the compounds solution. After emergence, adult flies were collected from the treatment vials and stored in 70% ethanol. The wings of the flies were removed under a stereomicroscope using a pair of entomological tweezers, similar number of males and females-wings were mounted in Faure's solution on microscope slides and inspected, under 400× magnification, for the presence of clones of cells. The mutant clones were classified into three types: (1) small single spots, containing one or two cells; (2) large single spots, containing three or more cells; and (3) twin spots, containing adjacent *mwh* and *flr*^{3} cells (Graf et al., 1984). The appearance of twin spots indicated the recombinogenic activity of the chemotherapeutic agent.

Data evaluation and statistical analysis

Differences between quality compounds in epicarp and mesocarp from "Yellow" and "Light Green" zucchini were assessed by Student´s t-test. Data normality was tested prior to analysis. SPSS Version 10.0 software was used to perform all statistical analyses (SPSS, 2000).

The frequencies of spots per fly of treated series were compared with its corresponding negative control series (water) to analyze SMART data. For maximum power, statistical analyses were done exclusively for the total number of spots recovered. The non parametric *U* test of Mann, Whitney and Wilcoxon was used to avoid weak positive and inconclusive results on SMART and to achieve a well defined statistical diagnosis whether a given treatment should be regarded as genotoxic or not (Frei and Würgler, 1995). The inhibition percentages (IP) were calculated using the total spots per wing with the following formula (Abraham, 1994):

IP = [(Genotoxine alone - Genotoxine plus active compound)/ Genotoxine alone]*100

Cell culture and cytotoxicity assay

HL60 cells were cultured in suspension at 37 ℃ in RPMI 1640 medium (Biowhittaker, BE12-167F) supplemented with 10% fetal bovine serum (Biotewhittaker, DE14-801F), Glutamine (Sigma, G7513) and an antibiotic-antimycotic solution (Sigma, A5955) in a humidified atmosphere containing 5% CO_2. Cultured HL60 cell (2×10^5 cells/ml) was treated for 72 h with epicarp, mesocarp and different compounds at the concentrations in which they are found in the fruit: epicarp (0.015-0.25 mg/ml) and mesocarp (0.015-0.5 mg/ml) from "Yellow" *C. pepo*, and epicarp and mesocarp (0.031-0.5 mg/ml) from "Light Green" *C. pepo*, lutein (0.0039-0.1237 µM), β-carotene (0.023-0.466 µM), zeaxanthin (0.022-0.879 µM), dehydroascorbic acid (0.0341-0.681 mM). A mix of β-carotene, zeaxanthin and dehydroascorbic acid at the same concentrations above indicated was also prepared to check if the cytotoxic effects were in additive or synergic way.

The reactivity of trypan blue (Sigma, 93595), a vital dye, is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable. Non-viable cells stained purple-violet, whereas viable ones remained unstained. Trypan blue was added to cell cultures at a 1:1 ratio, and 20 µl of cell suspension was loaded into a Neubauer chamber. The cells were counted under an inverted microscope at 100 x magnification. After the incubation period, a growth curve was established and IC_{50} values (concentration of test compound causing 50% inhibition of cell growth) were estimated. Curves are expressed as survival percentage with respect to controls growing at 72 h. All data are the average of at least three independent experiments.

Assessment of proapoptosis DNA fragmentation

The HL60 tumoral cell line was used with the aim of analyse the apoptotic induction. HL60 cells ($1.5x10^6$/ml) were treated with the same concentrations of the different compounds for 5 h. A non-treated cells control was included for each experiment. Treated cells were collected, were centrifuged at 4000 rpm for 5 minutes and washed with PBS. The DNA was extracted using a commercial kit (Dominion mbl, MBL 243) and the resulting total DNA was treated with RNAse for 30 minutes at 37ºC. Samples of 1.5 µg of DNA were mixed with loading buffer and loaded onto a pre-solidified 2% agarose gel containing ethidium bromide. The agarose gels were run at 50 V for 90 minutes in TBE buffer and then observed and photographed under UV light.

RESULTS

Quantitation of antioxidant compounds

The mean content values of the different active compounds in epicarp and mesocarp of the two summer squash varieties are listed in Table 1. The two varieties differed in the content of all the compounds analyzed. The “Yellow” Zucchini carotenoid content was significantly higher than “Light Green” Zucchini in both tissues, except zeaxanthin in mesocarp. It should be noted that the lutein epicarp content of “Yellow” Zucchini is seven times higher than “Light Green” Zucchini. Further in the latter variety, β-carotene was not detected in epicarp. Dehydroascorbic acid content of “Light Green” Zucchini was similar in both tissues, but mesocarp of “Yellow” Zucchini was ten fold higher than epicarp. In addition, dehydroascorbic acid mesocarp content of “Yellow” Zucchini was significantly higher than “Light Green” Zucchini.

Genotoxicity and antigenotoxicity analysis of *C. pepo* and their components

The results of chronic treatment of *Drosophila melanogaster* transheterizygous larvae with Zucchini epicarp and mesocarp and their different antioxidant compounds, alone or combined with one fixed concentration (120 mM) of hydrogen peroxide in the *Drosophila* wing spot assay (SMART) are presented in Table 2 and Table 3. The positive control hydrogen peroxide (H_2O_2) behaved as a genotoxin inducing oxidative stress and DNA damage, giving the expected result. It exhibited a total mutation rate (0.45 mutant clones/wing), which triplicated the negative control (water) rate (0.13). This ratio ensures the accuracy of the genotoxicity and antigenotoxicity concurrent assays (Romero-Jiménez et al., 2005).

All the compounds were non-genotoxic in the SMART at the tested concentrations (Table 2). There was no significant increase in the numbers of small single spots and total spots in the case of Zucchini epicarp and mesocarp and single compounds at the studied doses. Mutation rates were even lower than the negative water control. The only case with high but non-significant mutation rate was the highest concentration of lutein with 0.45 total spots/wing for 0.615 µM. Zeaxanthin and β-carotene induced a non-significant increase of mutation frequencies (0.05 and 0.15 total spots/wing for 0.0001 and 0.105 µM, and 0.00 and 0.05 total spots/wing for 0.0003 and 0.0689 µM, respectively) and, similarly, dehydroascorbic acid did not induce significant increases in the frequency of mutant spots at any of the two tested concentrations (0.003 and 0.107 mM) after larval feeding. Thus, in the wing spot test *in vivo* model, carotenoids and dehydroascorbic acid did not appear to be genotoxic.

Table 3 shows the results of the antigenotoxicity assays. The results obtained in the combined treatment of larvae with Zucchini epicarp and mesocarp (0.25 and 8 mg/mL) and H_2O_2 showed non-significant differences when compared to the negative control Then the SMART test showed that epicarp and mesocarp of Zucchini and bioactive compounds were able to detoxify the genotoxic activity of hydrogen peroxide although no dose effect was observed. (Table 3). The mutation frequencies at β-carotene, zeaxanthin and dehydroascorbic acid were even smaller than that obtained with the negative control. Only the highest concentration of lutein showed significant differences with the negative control (0.47 for 0.615 µM).

The inhibition percentage (IP) of genotoxicity by epicarp and mesocarp of “Yellow” Zucchini was 72 and 61 % and 100, and 11 % at 0.25 and 8 mg/mL, respectively (Figure 1). The IP of genotoxicity by epicarp and mesocarp of “Light Green” Zucchini was 56 and 78 %, and 83 and 89 % at the same concentrations, respectively. The fruit antioxidant compounds would act as an antimutagens against hydrogen peroxide in SMART. The highest concentration of mesocarp of “Yellow” Zucchini (8 mg/mL) showed the highest IP (100 %). The active compounds selected of Zucchini exhibited a IP that ranged from 72 to 100 % (on average 83 %), with the exception of the highest concentration of lutein that was not able to detoxify any effect of hydrogen peroxide (Figure 1).

It should be noted that the SMART test is suitable to detect the anti/pro-mutagenic effects of some chemicals (Kaya et al. 2002; Villatoro-Pulido et al., 2009; Anter et al., 2011a and b; Fernández-Bedmar et al., 2011). The results reported here are of interest when investigating the different ways in which summer squash and their antioxidant compounds can interfere *in vivo* with the mechanisms of genotoxic agents.

Table 1. Epicarp and mesocarp mean contents (lutein, β-carotene, zeaxanthin, dehydroascorbic acid) from two zucchini varieties ("Yellow" and "Ligh Green"), expressed in mg Kg^{-1} dry weight.

	Yellow	Light Green
Epicarp		
Lutein	1036.9 a	135 b
β-carotene	99.5 a	n.d. b
Zeaxanthin	18.6 a	1.7 b
DHA	369.3 b	592 a
Mesocarp		
Lutein	362.7 a	63.2 b
β-carotene	31.6 a	4.0 b
Zeaxanthin	1.9 a	1.7 a
DHA	3200 a	683.3 b

Means within the same row followed by the same letter are not significantly different using Student´s t test at P<0.05. n.d.: no detected.

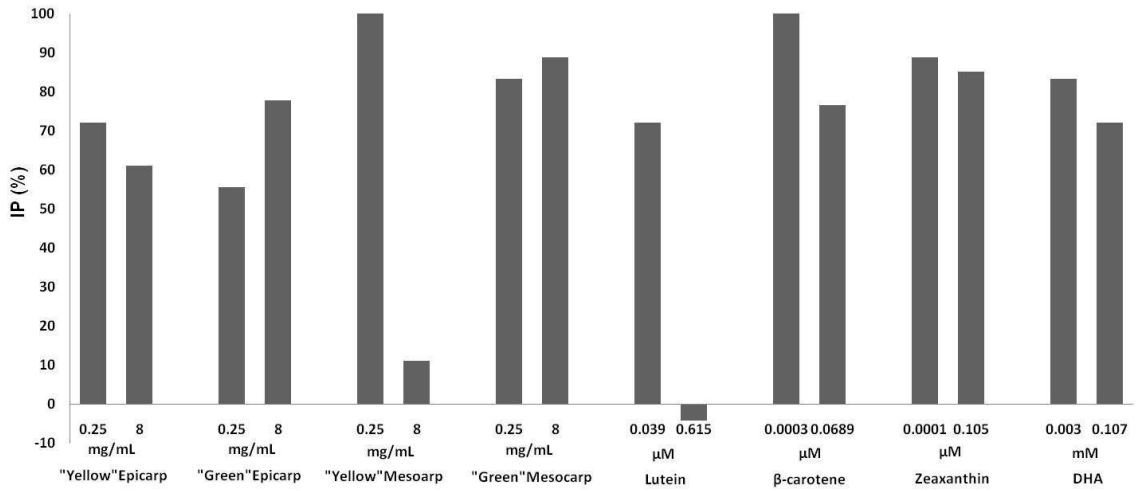


Figure 1. Inhibition percentages (IP) of Zucchini epicarp and mesocarp and its bioactive compounds against H_2O_2-induced damage in the *Drosophila* wing spot test.

Table 2. Genotoxicity (single treatment) of epicarp and mesocarp of two varieties of *C. pepo* and its active compounds (lutein, β-carotene, zeaxanthin and dehydroascorbic acid) in the *Drosophila* Wing Spot Test.

Compounds	Number of wings	Small spot (1-2 cells) (m=2)		Large spot (>2 cells) (m=5)		Twin spot (m=5)		Total spot (m=2)		
H_20 only	40	0.08	3	0.00	0	0.05	2	0.13	5	
Epicarp										
Yellow										
0.25 mg/ml	40	0.15	6	0.00	0	0.03	1	0.18	7	n.s.
8 mg/ml	40	0.00	0	0.00	0	0.03	1	0.03	1	n.s.
Green										
0.25 mg/ml	40	0.10	4	0.03	1	0.00	0	0.13	5	n.s.
8. mg/ml	40	0.05	2	0.00	0	0.00	0	0.05	2	n.s.
Mesocarp										
Yellow										
0.25 mg/ml	40	0.08	3	0.03	1	0.00	0	0.10	4	n.s.
8 mg/ml	40	0.05	2	0.00	0	0.00	0	0.05	2	n.s.
Green										
0.25 mg/ml	40	0.03	1	0.03	1	0.00	0	0.05	2	n.s.
8 mg/ml	40	0.03	1	0.03	1	0.00	0	0.05	2	n.s.
Single compounds										
Lutein										
0.039 µM	40	0.18	7	0.05	2	0.00	0	0.23	9	n.s.
0.615 µM	40	0.33	13	0.08	3	0.05	2	0.45	18	n.s.
β-carotene										
0.0003 µM	36	0.08	3	0.00	0	0.00	0	0.00	0	n.s.
0.0689 µM	40	0.03	1	0.03	1	0.00	0	0.05	2	n.s.
Zeaxanthin										
0.0001 µM	40	0.05	2	0.00	0	0.00	0	0.05	2	n.s.
0.105 µM	40	0.10	4	0.03	1	0.03	1	0.15	6	n.s.
DHA										
0.003 mM	40	0.10	4	0.03	1	0.00	1	0.13	5	n.s.
0.107 mM	40	0.15	6	0.03	1	0.00	0	0.18	7	n.s.

Data were evaluated by the non nonparametric U test of Mann, Whitney and Wilcoxon.
n.s., nonsignificant (P>0.05);*significant (P<0.05).
H_2O_2, hydrogen peroxide; m, multiplication factor for the assessment of significantly negative results.

Table 3. Antigenotoxicity (combined treatment) of epicarp and mesocarp of two varieties of *C. pepo* and its active compounds (lutein, β-carotene, zeaxanthin and dehydroascorbic acid) in the *Drosophila* Wing Spot Test.

Compounds	Number of wings	Small spot (1-2 cells) (m=2)		Large spot (>2 cells) (m=5)		Twin spot (m=5)		Total spot (m=2)		
H_20 only	40	0.08	3	0.00		0.05	2	0.13	5	
H_20_2 (120 mM)	40	0.38	15	0.13	5	0.03	1	0.45	18	*
Epicarp										
Yellow										
0.25 mg/ml	16	0.13	2	0.00	0	0.00	0	0.13	2	n.s.
8 mg/ml	40	0.15	6	0.03	1	0.00	0	0.18	7	n.s.
Green										
0.25 mg/ml	40	0.13	5	0.05	2	0.03	1	0.20	8	n.s.
8 mg/ml	40	0.05	2	0.03	1	0.03	1	0.10	4	n.s.
Mesocarp										
Yellow										
0.25 mg/ml	18	0.00	0	0.00	0	0.00	0	0.00	0	n.s.
8 mg/ml	40	0.38	15	0.00	0	0.03	1	0.40	16	n.s.
Green										
0.25 mg/ml	40	0.05	2	0.03	1	0.00	0	0.08	3	n.s.
8 mg/ml	40	0.05	2	0.00	0	0.00	0	0.05	2	n.s.
Single compounds										
Lutein										
0.039 µM	32	0.09	3	0.03	1	0.00	0	0.13	4	n.s.
0.615 µM	32	0.25	8	0.16	5	0.06	2	0.47	15	*
β-carotene										
0.0003 µM	12	0.00	0	0.00	0	0.00	0	0.00	0	n.s.
0.0689 µM	38	0.08	3	0.03	1	0.00	0	0.11	4	n.s.
Zeaxanthine										
0.0001 µM	40	0.03	1	0.00	0	0.03	1	0.05	2	n.s.
0.105 µM	30	0.07	2	0.00	0	0.00	0	0.07	2	n.s.
DHA										
0.003 mM	40	0.08	3	0.00	0	0.00	0	0.08	3	n.s.
0.107 mM	40	0.10	4	0.03	1	0.00	0	0.13	5	n.s.

Data were evaluated by the non nonparametric U test of Mann, Whitney and Wilcoxon.
n.s., nonsignificant (P>0.05);*significant (P<0.05).
H_2O_2, hydrogen peroxide; m, multiplication factor for the assessment of significantly negative results.

Effects on tumoral growth of HL60 cells and apoptosis

Cultured mammalian cells provide an important tool for evaluating the cytotoxicity of compounds with potential therapeutic activity (Paillard et al., 1999). Trypan blue assessment was used to check the cytotoxicity of epicarp and mesocarp of two summer squash varieties and the main antioxidant compounds of the fruit.

A wide range of concentrations was used for every compound. Figures 2 and 3 show the relative tumour growth inhibition for the substances assayed. The results are expressed as survival percentage with respect to the controls (% viability). The shapes of the curves were different for each case. Epicarp and mesocarp of “Yellow” Zucchini showed the most negative slope and the highest tumoricidal effect in the trypan blue exclusion assay with a IC_{50} > 0.1 mg/ml and 0.2 mg/ml, respectively. Epicarp and mesocarp of “Light Green” Zucchini also showed a negative slope, but not reaching $IC_{50.}$ The rest of compounds assayed did not reach IC_{50} at the tested concentrations, although some cytotoxic effect were observed in some of them (zeaxanthin, dehydroascorbic acid, β-carotene and the mix of the compounds) (Figure 3). We did not observe any cytotoxic effect of lutein at the chosen concentrations. Taking these observations together, it appears that the cytotoxic action of Zucchini epicarp and mesocarp cannot be related to the antioxidant effect of a single antioxidant compound, but rather to the total interactions between different compounds of such a complex mixture.

Once cytotoxicity assays of Zucchini epicarp and mesocarp and some of its antioxidant compounds were performed, a visible assay of DNA fragmentation was carried out in order to investigate whether the mechanism undergoing the cytotoxicity was mediated via apoptosis. The degradation of genomic DNA into oligonucleosomal fragments is a hallmark of apoptosis. This DNA fragmentation endpoint was analyzed by conventional agarose gel electrophoresis. The HL-60 cell line was treated for 5 h with the same concentrations as in the trypan blue cytotoxicity assay. As shown in Figure 4, the highest concentrations of "Light Green" Zucchini mesocarp and, in a lesser extent, epicarp showed an internucleosomal fragmentation ladder pattern of DNA for HL60 treated cells. This did not occur in the control and some lower concentrations. A dose-dependent effect is suggested."Yellow" Zucchini, β-carotene, zeaxanthin and DHA did not show any internucleosomal fragmentation ladder pattern (Figures 4 and 5).

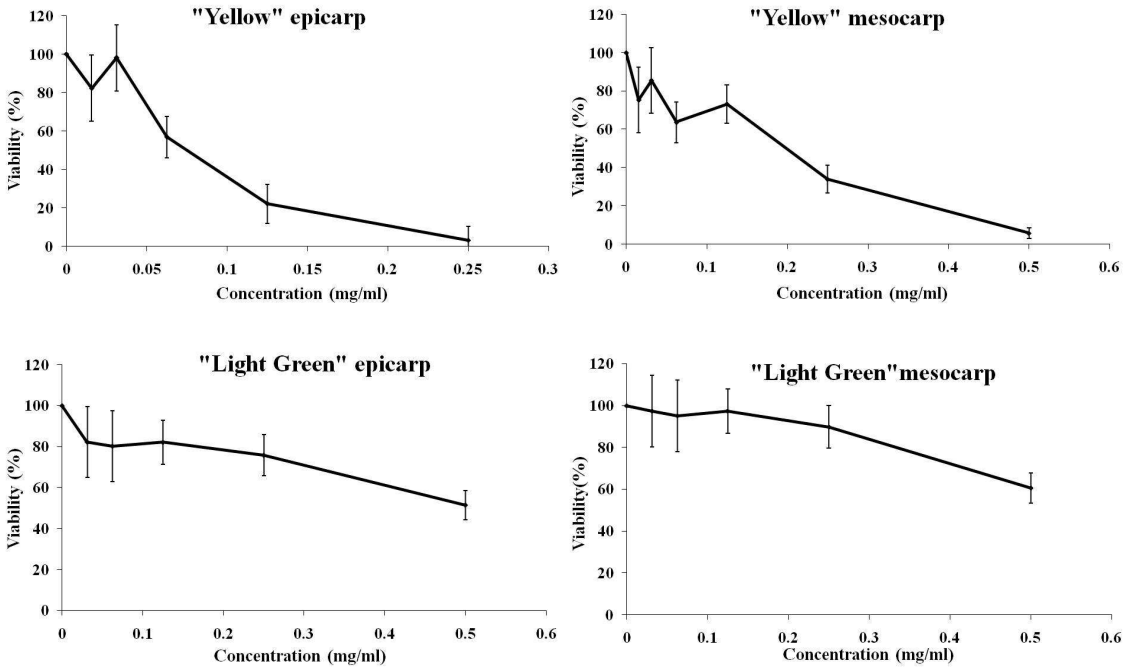


Figure 2. Effects of Zucchini epicarp and mesocarp on viability of HL-60 cells. Cell viability was assessed after 72 h by trypan blue exclusion test assay. The data are expressed as percentages of control (mean ±SD from three independent experiments).

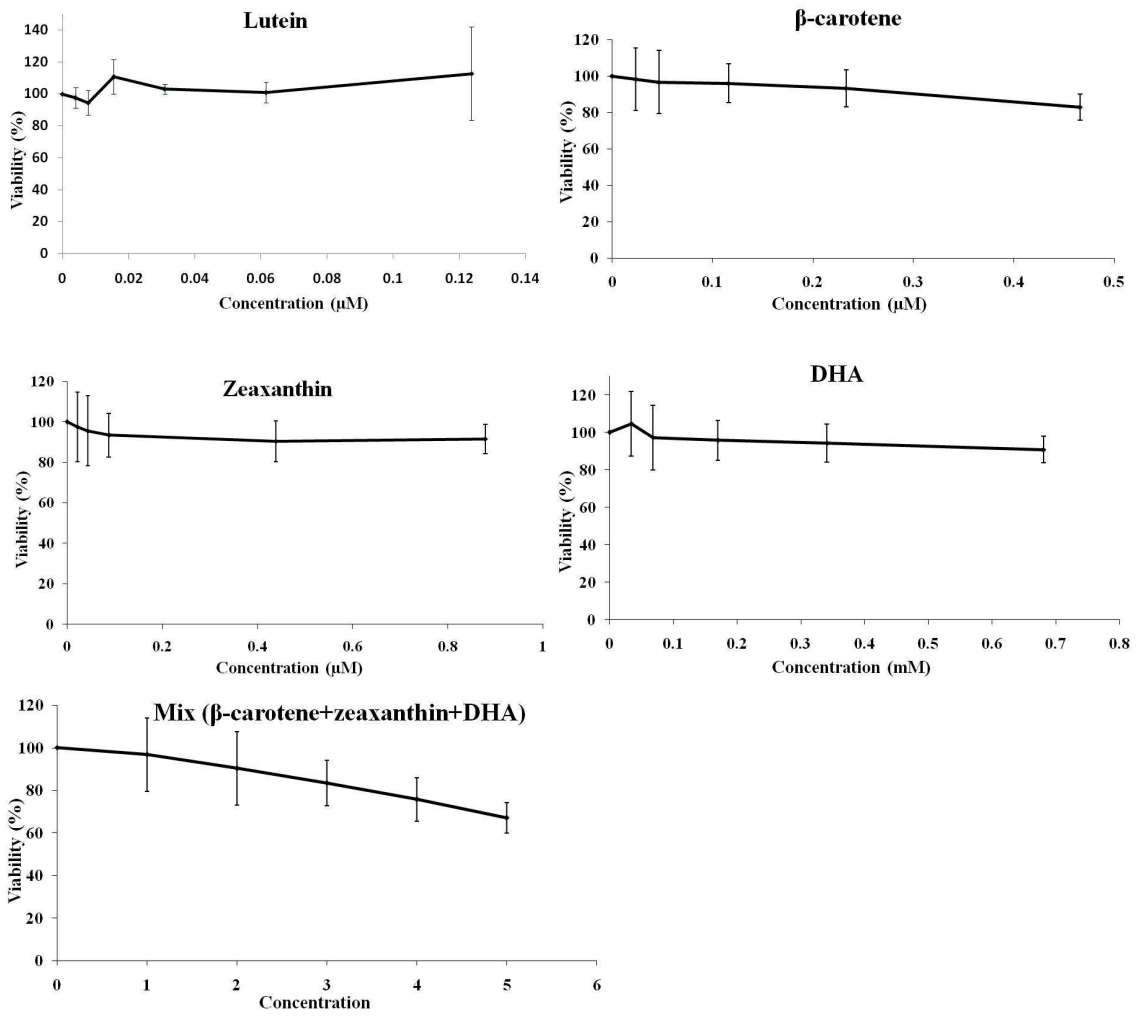


Figure 3. Effects of lutein, β-carotene, zeaxanthin, dehydroascorbic acid (DHA) and mix of compounds (β-carotene, zeaxanthin and DHA) on viability of HL-60 cells. Cell viability was assessed after 72 h by trypan blue exclusion test assay. The data are expressed as percentages of control (mean ±SD from three independent experiments).

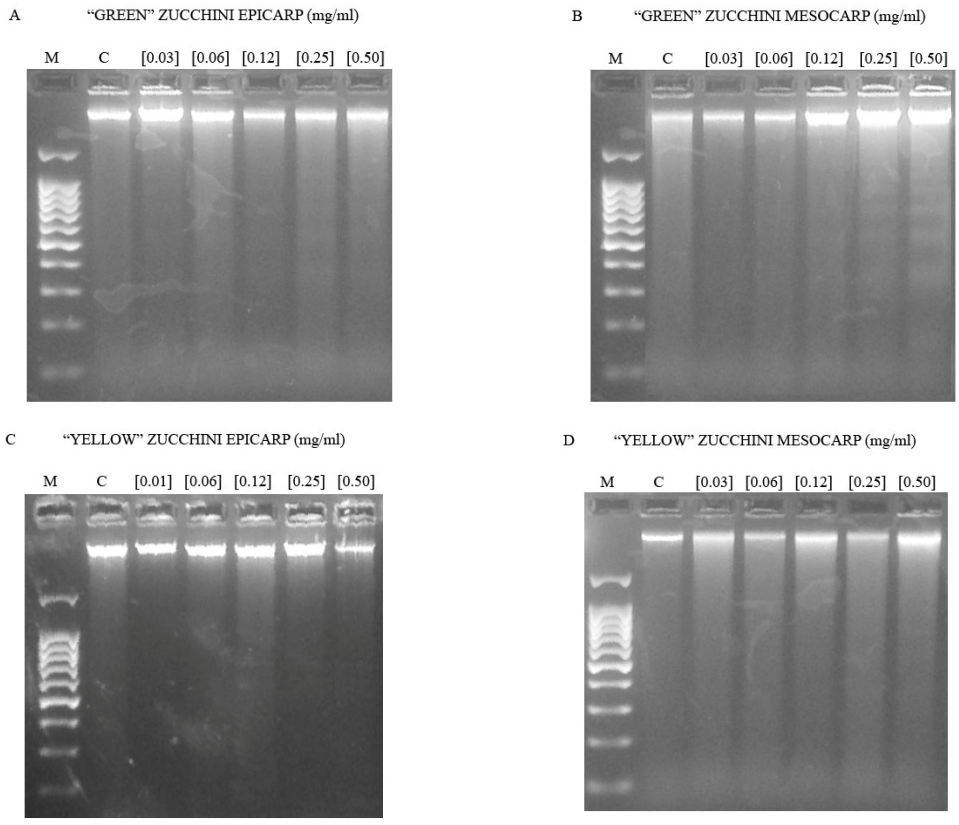


Figure 3. Nucleosomal DNA fragmentation. HL-60 cells were exposed to various concentrations of Zucchini epicarp and mesocarp for 5h. DNA was extracted from cells and was subject to 2% agarose gel electrophoresis at 50 V for 90 min. (A) "Green" Zucchini epicarp: Control (lane 1), 0.03 mg/ml (lane 2), 0.06 mg/ml (lane 3), 0.12 mg/ml (lane 4), 0.25 mg/ml (lane 5) and 0.50 mg/ml (lane 6). (B) "Green" Zucchini mesocarp: Control (lane 1), 0.03 mg/ml (lane 2), 0.06 mg/ml (lane 3), 0.12 mg/ml (lane 4), 0.25 mg/ml (lane 5) and 0.50 mg/ml (lane 6). (C) "Yellow" Zucchini epicarp: Control (lane 1), 0.01 mg/ml (lane 2), 0.06 mg/ml (lane 3), 0.12 mg/ml (lane 4), 0.25 mg/ml (lane 5) and 0.50 mg/ml (lane 6). (D) "Yellow" Zucchini Mesocarp: Control (lane 1), 0.03 mg/ml (lane 2), 0.06 mg/ml (lane 3), 0.12 mg/ml (lane 4), 0.25 mg/ml (lane 5) and 0.50 mg/ml (lane 6). M indicates DNA size marker.

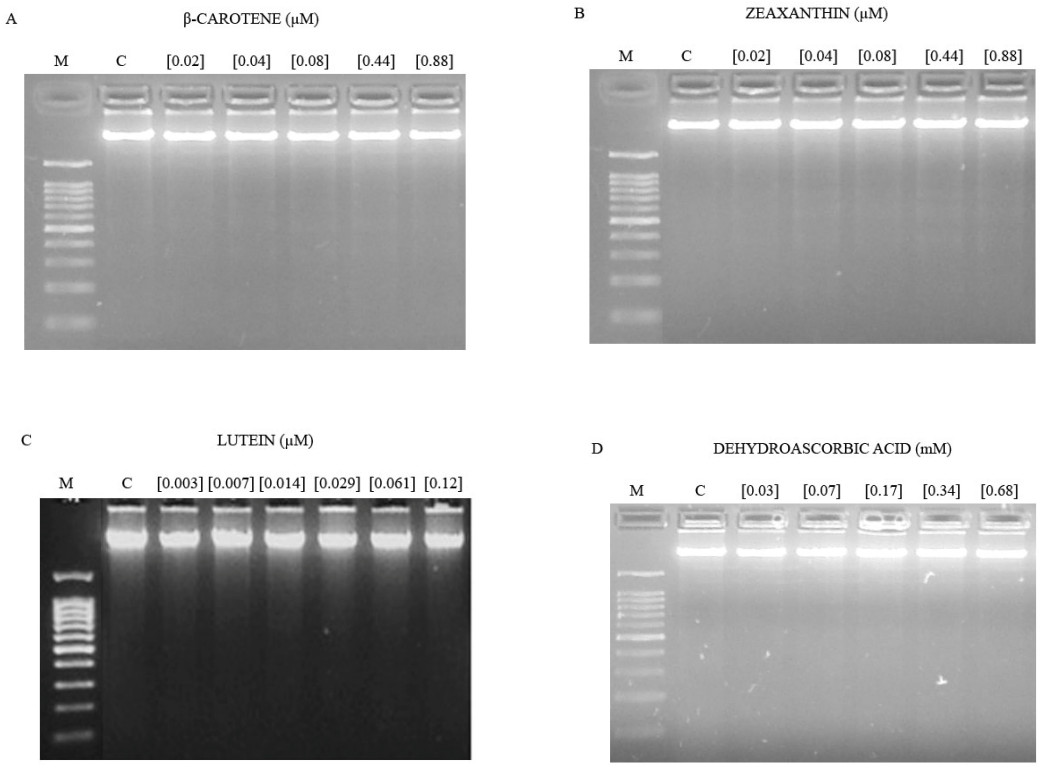


Figure 4. Nucleosomal DNA fragmentation. HL-60 cells were exposed to various concentrations of β-carotene, zeaxanthin, lutein and dehydroascorbic acid for 5h. DNA was extracted from cells and was subject to 2% agarose gel electrophoresis at 50 V for 90 min. (A) β-carotene: Control (lane 1), 0.02 μM (lane 2), 0.04 μM (lane 3), 0.08 μM (lane 4), 0.44 μM (lane 5) and 0.88 μM (lane 6). (B) zeaxanthin: Control (lane 1), 0.02 μM (lane 2), 0.04 μM (lane 3), 0.08 μM (lane 4), 0.44 μM (lane 5) and 0.88 μM (lane 6). (C) lutein: Control (lane 1), 0.003 μM (lane 2), 0.007 μM (lane 3), 0.014 μM (lane 4), 0.029 μM (lane 5), 0.06 μM (lane 6) and 0.12 μM (lane 7). (D) dehydroascorbic acid: Control (lane 1), 0.03 mM (lane 2), 0.07 mM (lane 3), 0.17 mM (lane 4), 0.34 mM (lane 5) and 0.68 mM (lane 6). M indicates DNA size marker.

DISCUSSION

Common prevention strategies include avoiding exposure to known cancer-causing agents, enhancement of host-defense mechanisms against cancer, life style modifications and chemoprevention (Sun et al., 2004). Chemoprevention is basic for the reversion, inhibition and prevention of cancer, and, optimally, requires the use of non-toxic agents that inhibit molecular steps during the carcinogenic pathway (Fimognari et al., 2007). Prevention by dietary phytochemicals is an important approach in cancer management (Surh, 2003).

The first step of the present work was to characterise the different active compounds in Zucchini epicarp and mesocarp. As the results show, both varieties differ greatly in content of bioactives compounds in both tissues. The antioxidant contents exhibited by the samples were included in the variation interval found in the literature for *C .pepo*. Lutein and β-carotene contents in epicarp and mesocarp fall within the range reported in previous studies (Martínez-Valdivieso et al., 2014c), but our lutein mesocarp content in "Yellow" Zucchini (362.7 mg Kg^{-1} dry weight) was higher than reported in mesocarp by Tadmor et al. (2005) (143.2 mg Kg^{-1} dry weight, assuming 92% of moisture); El-Qudah (2009) analyzed the lutein, β-carotene and zeaxanthin contents in fruit of Zucchini and their results were lower than ours for the three carotenoids (23.4, 1.46 and 0.41 mg Kg^{-1} dry weight, respectively). As reported in previous studies, ascorbic acid (vitamin C) is a very unstable compound and it easily transforms into its oxidized form, dehydroascorbic acid (Retsky et al., 1993). In our study, samples were lyophilized, so that virtually all the ascorbic acid is present as dehydroascorbic acid. Blanco et al. (2014) obtained ascorbic acid values in *C. pepo* epicarp ranged from 150 to 6530 mg Kg^{-1} dry weight, and in mesocarp from 280 to 4790 mg Kg^{-1} dry weight.

The SMART test has been showed to be a good predictor of safe/harmful substances due to the ability of *Drosophila* larvae to activate many procarcinogens and to the possibility for individually treating of thousands of somatic cells (Graf et al., 1998). The main objective of this assay was to evaluate the safety of Zucchini epicarp and mesocarp for the first time, as well as their major active components (lutein, β-carotene, zeaxanthin and dehydroascorbic acid) with respect to the lack of induction of genetic damage. The wing spot test for Zucchini tissues and their components resulted non-significant at the assayed concentrations when compared to the water control.

Antigenotoxic agents generally exhibit desirable chemotherapeutic effects that could be efficient in the strategy of cancer control (Anter et al., 2011b). H_2O_2 was used as a

positive control in SMART because it is reported that it generates DNA damage through oxygen-radical mechanisms, gene mutation, chromosomal aberration and DNA single-strand breaks (Rueff et al., 1993). Nevertheless, it is not known the epigenetic interactions that can occur between oxidative elements (H_2O_2) and others active compounds (lutein, β-carotene, zeaxanthin and DHA) acting mutually. The concentrations of the selected compounds showed to be antigenotoxic in the SMART test of *Drosophila melanogaster*. The strongest inhibition ability was detected with the lowest concentration of "Yellow" Zucchini mesocarp (0.25 mg/mL) and the lowest concentration of β-carotene (0.0003 µM) against the genotoxic effects of H_2O_2 in the imaginal discs of *Drosophila* (100 % for both).

The cytotoxicity assessment is an *in vitro* bioassays needed in the evaluation of the chemopreventive effects of a substance as a fast, not expensive and informative first step of screening. The human cell line HL60 provides a reliable model to study the cytotoxic effect of chemopreventive substances and the mechanisms underlying this potential activity (Villatoro-Pulido et al., 2009).

Some examples of similar results in relation to the cytotoxicity of *C. pepo* in the *in vitro* cytogenetic assay with different cell lines has been reported. Wang et al., (2007) observed a significant dose-dependent inhibitory effect against HeLa and HepG cell growth using ethanolic extracts of *C. pepo* fruits. Menéndez et al., (2006) remarked a significant decrease of the growth prostatic using a lipophilic extract of *C. pepo* seeds at doses of 400 and 200 mg/kg. Shokrzadeh et al. (2010) used hydro-alcoholic extracts of leaves of C. *pepo* on normal [Chinese hamster ovarian cells (CHO) and rat fibroblast] and cancer (HepG2 and CT26) cell lines with the following rank of inhibition ability: CHO < fibroblast < CT26 < HepG2 (being the lowest and the highest IC_{50} for HepG2: 132.6 µg/ml, and for fibroblast:293.2 µg/ml) cell lines. Other studies revealed that aerial parts of other *Cucurbita* species such a *C. maxima* also possesses significant anticancer activity in Ehrlich ascites carcinoma model in mice which may be due to its cytotoxicity and antioxidant properties (Saha et al., 2011).

Anti-proliferative properties and induction of apoptosis in tumor cells are suitable when the health-protecting effect of antioxidants is assessed (Anter et al., 2011b). Apoptosis plays critical roles in development and maintenance of homeostasis, in multicellular organism. During apoptosis, intracellular contents are not released and potentially harmful inflammatory responses are prevented. Apoptosis cell death is characterized by a fragmentation DNA into 180-200 pb nucleosomal units (Anter et al., 2011a). The

detection of DNA fragmentation in HL60 exposed to different concentrations of epicarp and mesocarp of "Light Green" Zucchini variety, indicated that the cells exposed to these concentrations suffer cell death via apoptosis and therefore their activity could be catalogued like an antiproliferative action. The compounds that promote apoptosis should become an important addition to the arsenal of target-specific drugs in the fight against cancer. The results of our study have shown fragmentation of DNA upon treatment of HL60 cells with epicarp and mesocarp of "Light Green" Zucchini, indicating the involvement of apoptosis.

In the present study, we demonstrated that a complex mixture such a Zucchini epicarp and mesocarp, inhibited proliferation in the human promyelocytic cell line HL60. In addition, our results provide a basic knowledge about interactions of phytochemicals with biological systems that may be useful for the design of functional foods. Sharoni et al. (2002) hypothesized that a single micronutrient cannot replace the power of the concerted action of multiple agents derived from a diet rich in fruits and vegetables.

Carotenoids have been studied widely to demonstrate if these colorful compounds can decrease the cancer risk (Donaldson, 2004). Dias et al., (2009) obtained similar results in the SMART test for β-carotene using higher concentrations than ours (1, 2 and 4 mg/mL): β-carotene was not genotoxic and was able to detoxify up to 95 % of the mutation induced by doxorubicin. Zhang and Omaye (2001) demonstrated that the antioxidant and pro-oxidant effects of β-carotene are dependent of both, the level of oxygen and the β-carotene concentration. As for most biological tissues, when the oxygen level is low, β-carotene, as other carotenoids, becomes important as an antioxidant (Burri, 1997). The chemopreventive action of β-carotene is effective mainly in the beginning of the carcinogenic process or in the initial stages of its promotion, inhibiting the formation of pre-neoplastic lesions in experimental models *in vitro* and *in vivo* (Moreno et al., 1995; He et al., 1997; Gradelet et al., 1997). The ability of β-carotene to inhibit cell growth has been established in several tumor cells including melanoma (Hazuka et al., 1990), prostate (Williams et al., 2000), colon (Palozza et al., 2002b), lung, breast and oral mucosa cancer (Schwartz et al., 1992). In our study β-carotene did not show cytotoxicity (0.5 µM) which agree with Sacha et al. (2005) who used β-carotene in the concentrations available *in vivo* (10 µM) and did not affect leukemic cell lines (HL60). Higher doses (ID_{50} = 27 µM) of β-carotene used in HL60 cells by Palozza et al. (2002a) modulated cell cycle progression and induced apoptosis in a dose-dependent manner; nevertheless the delay in cell growth by β-carotene was highly coincident with the increased intracellular ROS production and oxidized

glutathione content induced by the β-carotene. In the other hand, Sacha et al. (2005) also informed that β-carotene stimulated apoptosis in HL60 by modulating the expression of the regulatory genes at 10 µM concentration. β-carotene at 10 µM and 50 µM concentrations also stimulated apoptosis in B16F cells (melanoma) (Guruvayoorappan and Kuttan, 2007) and in the cell line MCF-7 (Cui et al., 2007), respectively. Although epidemiologic studies have demonstrated that a high intake of vegetables containing β-carotene decreases the risk of cancer, different studies have revealed that β-carotene supplementation to smokers resulted in a high incidence of lung cancer, it could alternatively behave as an antioxidant or as a prooxidant molecule, depending on its redox potential and on the cellular environment (Palozza et al., 2002a). Studies tend to agree that overall intake of carotenoids is more protective than a high intake of a single carotenoid (Donaldson, 2004) as β-carotene may be a marker for intake of fruits and vegetables, but it does not have a powerful protective effect in isolated pharmacological doses. So, a variety of fruits and vegetables is still a better anti-cancer strategy than just using a single vegetable high in a specific carotenoid.

Respect to the rest of carotenoid (lutein and zeaxanthine), previous work informed that lutein caused significant DNA damage in a dose- and time-dependent manner in human retinal pigment epithelial cells (ARPE-19) at a concentration ranging among 10 and 50 µM (Kalariya et al., 2009). The cytotoxic effects of zeaxanthin on two human uveal melanoma cell lines (SP6.5 and C918) were studied and compared to effects on normal ocular cells (uveal melanocytes, retinal pigment epithelial cells, and scleral fibroblasts); zeaxanthin reduced the cell viability of melanoma cells in a dose-dependent manner (10, 30, and 100 μM), with IC_{50} at 40.8 and 28.7 μM in SP6.5 and C918 cell lines, respectively. Zeaxanthin did not affect the viability of normal ocular cells even at the highest levels tested (300 μM), suggesting that zeaxanthin has a selectively cytotoxic effect on melanoma cells; in addition, zeaxanthin (30 μM) induced apoptosis in melanoma cells (Bi et al., 2013). Roberts et al. (2008) checked phototoxicity of fullerol induced by UVA *in vitro* with human lens epithelial cells (HLE B-3). When cells were pretreated with lutein (20 µM added 2 h prior to irradiation with UVA), the singlet oxygen quencher-lutein significantly protected against fullerol photodamage, phototoxic damage decreased by half. Brandão-Lencart and Sousa-Martins (2013) evaluated the safety profile of Lutein/Zeaxanthin (L/Z)-based natural dye solutions at different dilutions of the dyes (1/15, 1/30, 1/60, and 1/120) using *in vitro* cell culture models, retinal pigment epithelial cells (ARPE-19) and human corneal

epithelial cells (HCE), and they demonstrated that there was no dye-related cytotoxicity in all dyes tested. Other studies have been conducted with rats where the toxicity of zeaxanthin and lutein present in different commercial preparations have been evaluated, and no toxicologically relevant findings were noted in any study (Ravi et al., 2014; Ravikrishnan et al., 2011, Thurnham and Howard, 2013). Moreover in these studies, the results of zeaxanthin mutagenicity testing in *Salmonella typhimurium* model did not reveal any genotoxicity (Ravi et al., 2014; Ravikrishnan et al., 2011, Thurnham and Howard, 2013), and neither in *Escherichia coli* (Thurnham and Howard, 2013).

Low blood levels of ascorbic acid are detrimental to health and vitamin C is correlated with overall good health and cancer prevention (Donaldson, 2004). Dehydroascorbic acid did not show cytotoxicity in our studies at 0.7 mM, but Park et al. (2005) informed that L-ascorbic acid (the reduced form of dehydroascorbic acid) showed cytotoxicity and induces apoptosis of malignant cells (HL60, NB4 and NB4-R1) *in vitro*, when they used higher concentration than our (0.25-1 mM). Moreover, dehydroascorbic acid (0.5-1 mM) was tested for its protective effect against phototoxic damage induced by fullerol, there was found some protection against metabolic damage (Roberts et al., 2008).

The results obtained in genotoxicity and antigenotoxicity assays of Zucchini epicarp and mesocarp an its different bioactive compounds (lutein, β-carotene, zeaxanthin and dehydroascorbic acid) indicated that Zucchini was not genotoxic and is safe. "Light Green" and "Yellow" Zucchini showed antimutagenic capacity, but the capacity of "Light Green" variety was higher on average. This difference in the antigenotoxic activities could be due to the different content of bioactive molecules. With the current data, authors suggest that lutein could be the responsible molecule for the different antimutagenic activity of two varieties, as although lutein is not genotoxic, it is at the limit of significance. Moreover lutein was antigentoxic at the lowest concentration, but at highest concentration enhances the genotoxicity of hydrogen peroxide. With respect to the cytotoxicity results in the present study, "Light Green" Zucchini showed a lower activity than “Yellow” Zucchini. The differences in carotenoids content could partially explain the different cytotoxic activities, as can be observed in Figure 3, where the inhibition of the tumoral growth of the mix of the compounds is equivalent to the inhibitions of individual compounds separately (zeaxanthin, β-carotene and dehydroascorbic acid).

CONCLUSIONS

C. pepo is an important crop and source of human food around the world. Our results confirmed the safety, the antigenotoxicity and chemopreventive potential of Zucchini and some of its compounds using the SMART test as *in vivo* model and the cytotoxicity HL60 cells *in vitro* model. Antigenotoxicity assays indicated that all the concentrations were antigenotoxic showing different inhibition percentages (ranged from 11 to 100 % inhibition) in combined treatments with the genotoxicant hydrogen peroxide, with the exception of the highest concentration of lutein. The leukaemic cell line HL60 provides an important model for studying the mechanisms and relationships between cytotoxicity, apoptosis and antitumor efficacy of different substances and compounds. Epicarp and mesocarp of "Yellow" Zucchini exhibited the highest cytotoxic activity (IC_{50} > 0.1 mg/ml and 0.2 mg/ml, respectively); the rest of the bioactive compounds assayed did not reach IC_{50} at the tested concentrations, although some cytotoxic effect were observed in some of them (zeaxanthin, dehydroascorbic acid, β-carotene and the mix of the compounds).

The result of the present investigation is quite encouraging and it explores the potent Zucchini anticancer activity probably because of its direct cytotoxic effect which is further potentiated by its antioxidant properties. The Zucchini fruit showed promising results, and can play beneficial roles in human nutrition and health status. We conclude that fruit of *C. pepo* and their components were safe, able to inhibit significantly the H_2O_2-induced damage and exhibit antiproliferative and pro-apoptotic properties toward HL60 tumour cells. The information generated from *in vitro* and *in vivo* studies about the Zucchini phytochemical profile that contributes to improve the consumer's health is essential in order to select potential accessions for using in breeding programs.

REFERENCES

1. Abraham S.K. 1994. Antigenotoxicity of coffee in the *Drosophila* assay for somatic mutation and recombination, Mutagenesis 9: 383–386.

2. Anter J., Fernández-Bedmar Z., Villatoro-Pulido M., Demyda-Peyras S., Moreno-Millán M, Alonso-Moraga A., Muñoz-Serrano A., Luque de Castro M.D. 2011b. A pilot study on the DNA protective, cytotoxic and apoptotic-induced roles of olive leaf extracts. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. Article in Press. Doi:10.1016/j.mrgentox.2011.05.005.

3. Anter J., Romero-Jiménez M., Fernández-Bedmar Z., Villatoro-Pulido M., Analla M., Alonso-Moraga Á., Muñoz-Serrano A. 2011a. Antigenotoxicity, cytotoxicity, and apoptosis induction by apigenin, bisabolol, and protocatechuic acid. Journal of medicinal food, 14(3): 276-283.

4. Bez G.C., Jordão B.Q., Vicentini V.E.P., Mantovani, M.S. 2001. Investigation of genotoxic and antigenotoxic activities of chlorophylls and chlorophyllin in cultured V79 cells. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 497(1): 139-145.

5. Bi M.C., Rosen R., Zha R.Y., McCormick S.A., Song E., Hu D.N. 2013. Zeaxanthin induces apoptosis in human uveal melanoma cells through Bcl-2 family proteins and intrinsic apoptosis pathway. Evidence-Based Complementary and Alternative Medicine. Volume 2013, Article ID 205082, 12 pages.

6. Blanco-Díaz M.T., Del Río-Celestino M., Martínez-Valdivieso D., Font R. 2014. Use of visible and near-infrared spectroscopy for predicting antioxidant compounds in summer squash (*Cucurbita pepo* ssp *pepo*). Food Chem. 164:301-8.

7. Brandão-Lencart J. and Sousa-Martins D. 2013. Cytotoxicity study of lutein-based dyes in a cell-culture *in vitro* model. Technical Literature. Kemin Industries, Inc., U.S.A.

8. Burri B.J. 1997. β-carotene and human health: a review of current research. *Nutr. Res.* 17: 547-580.

9. Carr A.C. and B. Frei. 1999. Toward a new recommended dietary allowance for Vitamin C based on antioxidant and health effects in humans. Am J Clin Nutr 69: 1086–1107.

10. Collins A.R. 1999. Oxidative DNA damage, antioxidants, and cancer, BioEssays, 21: 238–246.

11. Collins S.J., Gallo P.C., Gallagher R.E. 1977. Continuous growth and differentiation of human promyelotic leukaemia cell line (HL60) in suspension culture. Nature, 270: 347–349.

12. Cui Y, Lu Z, Bai L, Shi Z, Zhao W.E., Zhao B. 2007. β-carotene induces apoptosis and up-regulates peroxisome proliferator-activated receptor gamma

expression and reactive oxygen species production in MCF-7 cancer cells. Eur J Cancer, 43: 2590-2601.

13. Dias C.D., Araújo B.C., Dutra E.S,, Nepomuceno J.C. 2009. Protective effects of β-carotene against the genotoxicity of doxorubicin in somatic cells of *Drosophila melanogaster*. Genetics and Molecular Research, 8 (4): 1367-1375

14. Donaldson M.S. 2004. Nutrition and cancer: A review of the evidence for an anti-cancer diet. Nutrition Journal, 3, 19. doi:10.1186/1475-2891-3-19

15. El-Qudah JM. 2009. Identification and quantification of major carotenoids in some vegetables. Am J Appl Sci, 6 (3): 492‑497.

16. Fernández-Bedmar Z., Anter J.; de La Cruz-Ares S., Muñoz-Serrano A., Alonso-Moraga, Á.; Pérez-Guisado J. 2011. Role of Citrus Juices and its Distinctive Components in the Modulation of Degenerative Processes: Genotoxicity, Antigenotoxicity, Cytotoxicity and Longevity Scopes. Journal of Toxicology and Environmental Health, 74: 15-16.

17. Fimognari C and Hrelia P. 2007. Sulforaphane as a promising molecule for fighting cancer. Mut Res; 635: 90-104.

18. Ghobrial I.M., Witzig T.E. and Adjei A.A. 2005. Targeting apoptosis pathways in cancer therapy. CA—A Cancer Journal for Clinicians, 55: 178-194.

19. Gil M.I., Ferreres F., Tomás-Barberán F.A. 1999. Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. J Agric Food Chem, 47: 2213–2217.

20. Gonzalez C.A. and Riboli E. 2006. Diet and cancer prevention: Where we are, where we are going. Nutrition and Cancer, 56: 225-231.

21. Gradelet S., Astorg P., Le Bon A.M., Berges R., et al. 1997. Modulation of aflatoxin B1 carcinogenicity, genotoxicity and metabolism in rat liver by dietary carotenoids: evidence for a protective effect of CYP1A inducers. Cancer Lett, 114: 221-223.

22. Graf U., Würgler F.E., Katz A.J., Frei H., Juon H., Hall C.B., Kale P.G. 1984. Somatic mutation and recombination test in *Drosophila melanogaster*. Environ. Mutagen. 6:153-188.

23. Graf U., Abraham S.K., Guzmán-Rincón J., Würgler F.E. 1998. Antigenotoxicity studies in *Drosophila melanogaster*, Mutat Res, 402:203–209.

24. Guruvayoorappan C and Kuttan G. 2007. β-carotene down-regulates inducible nitric oxide synthase gene expression and induces apoptosis by suppressing bcl-2 expression and activating caspase-3 and p53 genes in B16F-10 melanoma cells. Nutr Res, 27: 336-342.

25. Hail N.Jr., Cortes M., Drake E.N., Spallholz J.E. 2008. Cancer chemoprevention: A radical perspective. Free Radical Biology and Medicine, 45: 97-110.

26. Hamamouchi M. 2002. Medicinal plants in Morocco: Traditional use, marketing, and strategies for conservation and increasing value. Esperance Med, 9: 454–8.

27. Hartman P.E. and Shankel D.M. 1990. Antimutagens and anticarcinogens: a survey of putative interceptor molecules, Environ Mol Mutagen, 15: 145–182.

28. Hazuka M.B., Edwards-Prasad J., Newman F., Kinzie J.J., Prasad K.N. 1990. β-carotene induces morphological differentiation and decreases adenylate cyclase activity in melanoma cell culture. J Am Coll Nutr, 9: 143– 149.

29. He Y., Root M.M., Parker R.S., Campbell T.C. 1997. Effects of carotenoid-rich food extracts on the development of preneoplastic lesions in rat liver and on *in vivo* and *in vitro* antioxidant status. Nutr Cancer, 27: 238-244.

30. Kalariya N.M., Ramana K.V., Srivastava S.K., van Kuijk F.J. 2009. Genotoxic effects of carotenoid breakdown products in human retinal pigment epithelial cells. Curr Eye Res, 34(9): 737-47.

31. Karasavvas N., Cárcamo J.M., Stratis G., Golde D.W. 2005. Vitamin C protects HL60 and U266 cells from arsenic toxicity. Blood, 105(10): 4004-4012.

32. Kaya B., Creus A., Velázquez A., Yanikoğlu A., Marcos R. 2002. Genotoxicity is modulated by ascorbic acid: Studies using the wing spot test in Drosophila. Mutation Research/Genetic Toxicology and Environmental Mutagenesis,520(1): 93-101.

33. Khachik F. and Beecher G.R. 1988. Separation of carotenol fatty acid esters by high performance liquid chromatography. J Chromatogr A, 449: 119–133.

34. Kuno T., Tsukamoto T., Hara A., Tanaka T. 2012. Cancer chemoprevention through the induction of apoptosis by natural compounds. Journal of Biophysical Chemistry, 3(2): 156-173.

35. Martin K.R. 2006. Targeting apoptosis with dietary bioactive agents. Experimental Biolology and Medicine (Maywood), 231: 117-129.

36. Martínez-Valdivieso D., Gómez P., Font R., Del Río-Celestino M. 2014a. Mineral composition and potential nutritional contribution of 34 genotypes from different summer squash morphotypes. European Food Research and Technology. DOI 10.1007/s00217-014-2308-7

37. Martínez-Valdivieso D., Font R., Gómez P., Blanco-Díaz M.T., Del Río-Celestino M. 2014b. Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy. Journal of the Science of Food and Agriculture. http://dx.doi.org/10.1016/j.compag.2014.07.003

38. Martínez-Valdivieso D., Font R., Blanco-Díaz M.T., Moreno-Rojas J.M., Gómez P., Alonso-Moraga A., Del Río-Celestino M. 2014c. Application of near-infrared reflectance spectroscopy for predicting carotenoid content in summer squash fruit. Computers and Electronics in Agriculture. http://dx.doi.org/10.1002/jsfa.6667

39. Menéndez A.B., Capó J.T., Menéndez-Castillo R.A., González O.L., Domínguez C.C., Sanabria M.L.G. 2006. Evaluation of *Cucurbita pepo* L. lipophilic extract on androgen-induced prostatic hiperplasia. Rev Cubana Plant Med v.11 n.2 Ciudad de la Habana abr.-jun.

40. Møller P. and Loft S. 2004. Interventions with antioxidants and nutrients in relation to oxidative DNA damage and repair. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 551: 79–89.

41. Moreno F.S., Wu T.S., Penteado M.V., Rizzi M.B., et al. 1995. A comparison of β-carotene and vitamin A effects on a hepatocarcinogenesis model. Int J Vitam Nutr Res, 65: 87-94.

42. Odin A.P. 1997. Vitamins as antimutagens: advantages and some possible mechanisms of anti-mutagenic action, Mutat Res, 38: 39–67.

43. Paillard F., Finot F., Mouche I., Prenez A., Vericat J.A. 1999. Use of primary cultures of rat hepatocytes to predict toxicity in the early development of new chemical entities. Toxicol In Vitro, 13: 693–700.

44. Palozza P., Serini S., Torsello A., Boninsegna A., Covacci V., Maggiano N., Ranelletti F.O., Wolf F.I., Calviello, G. 2002a. Regulation of cell cycle progression and apoptosis by β-carotene in undifferentiated and differentiated HL-60 leukemia cells: Possible involvement of a redox mechanism. International Journal of Cancer, 97(5): 593-600.

45. Palozza P., Serini S., Torsello A., Di Nicuolo F., Piccioni E., Ubaldi V., Pioli C., Wolf F.I., Calviello G. 2002b. Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by beta-carotene through down-regulation of cyclin A and Bcl-2 family proteins. Carcinogenesis, 1: 11–18.

46. Park S., Park C.H., Hahm E.R., Kim K., Kimler B.F., Lee S.J., Kim W.S., Jung C.W., Park K., Riordan H.D., Lee J.H. 2005. Activation of Raf1 and the ERK pathway in response to l-ascorbic acid in acute myeloid leukemia cells. *C*ellular signalling, 17(1): 111-119.

47. Ravi K.B., Reddy K.R., Shankaranarayanan J., Deshpande J.V., Juturu V., Soni M.G. 2014. Safety evaluation of zeaxanthin concentrate (OmniXan™): Acute, subchronic toxicity and mutagenicity studies. Food and Chemical Toxicology, 72: 30-39.

48. Ravikrishnan R., Rusia S., Ilamurugan G., Salunkhe U., Deshpande J., Shankaranarayanan J., Shankaranarayanan M.L., Soni M.G. 2011. Safety assessment of lutein and zeaxanthin (Lutemax™ 2020): Subchronic toxicity and mutagenicity studies. Food and Chemical Toxicology, 49(11): 2841-2848.

49. Reed J.C. 1999. Dysregulation of apoptosis in cancer. Journal of Clinical Oncology, 17: 2941-2953.

50. Reddy L., Odhav B., Bhoola, K.D. 2003. Natural products for cancer prevention: a global perspective. Pharmacol. Therapeut. 99:1-13.

51. Retsky K.L., Freeman M.W., Frei B. 1993. Ascorbic acid oxidation product (s) protect human low density lipoprotein against atherogenic modification. Anti-rather than

prooxidant activity of vitamin C in the presence of transition metal ions. Journal of Biological Chemistry, 268(2): 1304-1309.

52. Riboli E. and Norat T. 2003. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. American Journal of Clinical Nutrition, 78: 559S-569S.

53. Roberts J.E., Wielgus A.R., Boyes W.K., Andley U., Chignell C.F. 2008. Phototoxicity and cytotoxicity of fullerol in human lens epithelial cells. Toxicology and applied pharmacology, 228(1): 49-58.

54. Romero-Jiménez M., Campos-Sánchez J., Analla M., Muñoz-Serrano A., Alonso-Moraga A. 2005. Genotoxicity and antigenotoxicity of some traditional medicinal herbs. Mutat Res, 585: 147-155.

55. Rueff J., Bras A., Cristovao L., Mexia J., Sa da Costa M., Pires V. 1993. DNA strand breaks and chromosomal aberrations induced by H_2O_2 and 60Co gamma-radiation. Mutat Res, 289: 197–204.

56. Sacha T., Zawada M., Hartwich J., Lach Z., Polus A., Szostek, M., Libura M., Bodzioch M., Dembinska-Kiec A., Skotnicki A.B., Goralczyk R., Wertz K., Riss G., Moele C., Langmann T., Schmitz G. 2005. The effect of β-carotene and its derivatives on cytotoxicity, differentiation, proliferative potential and apoptosis on the three human acute leukemia cell lines: U-937, HL-60 and TF-1. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1740(2): 206-214.

57. Saha P., Mazumder U.K., Haldar P.K., Naskar S., Kundu S., Bala A., Kar B. 2011. Anticancer activity of methanol extract of *Cucurbita maxima* against Ehrlich ascites carcinoma. International Journal of Research in Pharmaceutical Sciences, 2(1): 52-59.

58. Sarkar D, Sharma A, Talukder G. 1994. Chlorophyll and chlorophyllin as modifiers of genotoxic effects. Mutat Res, 318: 239-247.

59. Schwartz J. and Shklar G. 1992. The selective effects of carotenoids and α-tocopherol on human cancer cell lines in vitro. J Oral Maxillofac Surg, 50: 367–373.

60. Sharoni Y., Danilenko M., Walfisch S., Amir H., Nahum A., Ben-Dor A., Levy J. 2002. Role of gene regulation in the anticancer activity of carotenoids. Pure and applied chemistry, 74(8): 1469-1477.

61. Shokrzadeh M., Azadbakht M., Ahangar N., Hashemi A., Saeedi-Sarav S.S. 2010. Cytotoxicity of hydro-alcoholic extracts of *Cucurbita pepo* and *Solanum nigrum* on HepG2 and CT26 cancer cell lines. Pharmacognosy Magazine*,* 6: 176–179.

62. Stan S.D., Kar S., Stoner G.D., Singh S.V. 2008. Bioactive food components and cancer risk reduction. Journal of Cellular Biochemistry, 104: 339-356.

63. Sun S.Y., Hail N. Jr. and Lotan R. 2004. Apoptosis as a novel target for cancer chemoprevention. Journal of Na- tional Cancer Institute, 96: 662-672.

64. Surh YJ. 2003. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer, 3: 768-780.

65. Tadmor Y., Paris H.S., Meir A., Schaffer A.A., Lewinsohn E. 2005. Dual role of the pigmentation gene B in affecting carotenoid and vitamin E content in squash (*Cucurbita pepo*) mesocarp. J Agric Food Chem. 53: 9759–9763.

66. Tanaka T., Shnimizu M. and Moriwaki H. 2012. Cancer chemoprevention by carotenoids. Molecules, 17(3): 3202-3242.

67. Tasset-Cuevas I., Fernández-Bedmar Z., Lozano-Baena M.D., Campos-Sánchez J., de Haro-Bailón A., Muñoz-Serrano A., Alonso-Moraga Á. 2013. Protective Effect of Borage Seed Oil and Gamma Linolenic Acid on DNA: In Vivo and In Vitro Studies. PloS one, 8(2): e56986.

68. Thurnham D.I. and Howard A.N. 2013. Studies on meso-zeaxanthin for potential toxicity and mutagenicity. Food and Chemical Toxicology, 59: 455-463.

69. Villatoro-Pulido M, Font R, De Haro-Bravo MI, Romero-Jimenez M, Anter J, De Haro Bailon A, Alonso-Moraga A, Del Rio-Celestino M. 2009. Modulation of genotoxicity and cytotoxicity by radish grown in metal-contaminated soils. Mutagenesis; 24: 51–57.

70. Villatoro-Pulido M., Font R., Saha S., Obregón-Cano S., Anter J., Muñoz-Serrano A., De Haro-Bailón A. Alonso-Moraga A., Del Río- Celestino M. 2012. In vivo biological activity of rocket extracts (*Eruca vesicaria* subsp. *sativa* (Miller) Thell) and sulforaphane. Food Chem Tox, 50(5): 1384-1392.

71. Villatoro Pulido M., Priego Capote F., Álvarez Sánchez B., Saha S., Philo M., Obregón Cano S., De Haro-Bailón A, Font R, Del Río-Celestino M. 2013. An approach

to the phytochemical profiling of rocket [Eruca sativa (Mill.) Thell]. Journal of the Science of Food and Agriculture, 93(15): 3809-3819.

72. Wang D.C., Pan H.Y., Deng X.M., Xiang H., Gao H.Y., Cai H,. et al. 2007. Cucurbitane and hexanorcucurbitane glycosides from the fruits of *Cucurbita pepo* cv *dayangua*. J Asian Nat Pro Res, 9: 525–9.

73. Williams A.W., Boileau T.W.M., Zhou J.R., Clinton S.K., Erdmann Jr. J.V. 2000. β-carotene modulates human prostate cancer cell growth and may undergo intracellular metabolism to retinol. J Nutr, 130: 728–732.

74. Zapata S. and Dufour J.F. 1992. Ascorbic, dehydroascorbic and isoascorbic acid simultaneous determinations by reverse phase ion interaction HPLC, J Food Sci. 57: 506–511.

75. Zhang P. and Omaye S.T. 2001. Antioxidant and prooxidant roles for b-carotene, a-tocopherol and ascorbic acid in human lung cells. Toxicol In Vitro, 15: 13-24.

DISCUSIÓN GENERAL DE LA TESIS

Thesis general discussion

En el presente trabajo se ha abordado la caracterización físico-química, nutricional, así como el análisis de su actividad biológica, de las variedades que forman parte de la colección de germoplasma de la especie mantenida en el Centro IFAPA-La Mojonera, tanto variedades tradicionales con diferentes orígenes geográficos como variedades comerciales que pueden encontrarse en el mercado, para la selección de material vegetal con potencial para ser utilizado como parentales donantes en programas de mejora genética

Uno de los objetivos generales del presente trabajo ha sido contribuir al conocimiento de los recursos fitogenéticos del calabacín como herramienta para la mejora de la especie. Se ha caracterizado la variabilidad físico-química y nutricional del fruto en accesiones de calabacín mantenidas en el Banco de Germoplasma del Centro IFAPA-La Mojonera. La selección de material vegetal procedente de variedades tradicionales puede representar una importante fuente de recursos, ya que puede aportar valor añadido a variedades comerciales ya disponibles. El material vegetal ha consistido principalmente en accesiones pertenecientes a diferentes morfotipos de *Cucurbita pepo* subsp. *pepo*, incluidas tanto variedades tradicionales como comerciales, y una accesión de *Cucurbita pepo* subsp. *ovifera* (Cu-14). Los resultados han revelado una alta variabilidad en las 22 accesiones evaluadas (Tabla 1, Capítulo 1), tanto en la piel como en la pulpa del fruto, para la mayoría de caracteres evaluados: peso, longitud, diámetro, color, firmeza, materia seca, contenido en sólidos solubles, acidez total y pH (Tablas 2, 3 y 4 Capítulo 1). A partir del análisis de componentes principales aplicado a los caracteres en estudio de los distintos cultivares se concluyó que los parámetros cromáticos a*, b*, Chroma* (*C**) y Hue (*H°*) (en piel y pulpa), peso seco, sólidos solubles, acidez total, luteina, β-caroteno y contenido total de carotenoides del epicarpo fueron los que contribuyeron en mayor medida a explicar la variación total de los datos (Figura 1, Capítulo 1). La variabilidad observada ha sido similar a la encontrada en estudios previos de la especie y en especies cercanas (Itle y Kabelka. 2009; Jacobo-Valenzuela et al., 2011).

En la caracterización física de los frutos se observaron diferencias significativas para peso, longitud, diámetro, debidas principalmente a las diferencias morfológicas entre los morfotipos de la especie (Tabla 2, Capítulo 1); el morfotipo marrow presentó los pesos y diámetros más altos, y la accesión perteneciente al morfotipo scallop (Cu-14) mostró la menor longitud por la forma especial de su fruto. Las diferencias en estos parámetros entre morfotipos han sido descritas por otros autores (Hernándo-Bermejo y León, 1994) y coinciden con nuestros resultados. Las diferencias entre los frutos de los

morfotipos se deben esencialmente a la selección que se ha hecho durante años por parte de los agricultores y productores con el objetivo de conseguir una diferente adaptación de las variedades y un diferente uso culinario de sus frutos (Paris, 2010).

En cuanto al color, otro importante parámetro relacionado con la calidad, hubo una gran variabilidad entre las accesiones para los parámetros cromáticos, especialmente en la piel. Cabe destacar que los mayores valores de a*, b* y *C** los obtuvieron las accesiones de piel amarilla, especialmente la accesión Cu-29 (zucchini comercial) (Tabla 3, Capítulo 1). Itle y Kabelka (2009) obtuvieron valores similares para L*, a*, b*, *C** y el ángulo *Hº* en *C. pepo* y *C. moschata*, en cambio Jacobo-Valenzuela et al. (2011) obtuvieron una menor variabilidad quizás debido a que sólo evaluaron el color en la variedad *C. moschata* cv Cehualca.

Destacar que la fuerza o firmeza requerida para atravesar la piel fue mayor que la de la pulpa, aproximadamente un 20% mayor (Tabla 4, Capítulo 1). Conocer como varía este parámetro entre accesiones es interesante porque puede ser un factor a tener en cuenta en programas de mejora centrados en conseguir variedades con una mayor resistencia a los daños por golpes que pueden producirse durante la manipulación o el transporte de los frutos.

El porcentaje de materia seca del fruto en estado inmaduro fue inferior al 6 % para la mayoría de las accesiones (Tabla 4, Capítulo 1) (Tablas 2 y 3, Capítulo 2) (Aboul-Nasr et al., 2002; Audrey et al., 2002; Kmiecik y Lisiewska, 1994), siendo el porcentaje medio en la piel de 6.68 y en la pulpa de 5.21 (Tabla 4, Capítulo 1). Según Loy (2004) este parámetro aumenta en la pulpa entre los 10 y los 30 días tras la polinización. En general, la piel tiene un mayor contenido de materia seca que la pulpa. Las accesiones Cu-15 en la piel (10.15 %) y Cu-36 en la pulpa (6.56 %) (Tabla 4, Capítulo 1) serían candidatas para ser utilizadas en un programa de mejora centrado en aumentar este parámetro. Las preferencias de los consumidores en cuanto al contenido de materia seca van unidas al uso final que se le va a dar al producto. Murphy et al. (1966) indicaron que los consumidores prefieren un alto contenido de materia seca en frutos de variedades de *C. maxima*. Según Harvey et al. (1997), por lo que frutos con un porcentaje superior al 28 % no serían aptos para el consumo en fresco, pero sí para algunos tipos de procesado.

Respecto al contenido en sólidos solubles (SSC) se encontraron diferencias significativas entre las accesiones estudiadas para ambas, la piel y pulpa (Tabla 4, Capítulo 1). Cu-8 (4.74 ºBrix) y Cu-36 (4.55 ºBrix) fueron las accesiones con los

valores más altos de SSC. Este parámetro está relacionado con el contenido en azúcares y es un importante factor de calidad implicado en la percepción sensorial del producto (Gajc-Wolska et al., 2005). Nuestros contenidos resultaron inferiores a los obtenidos por Jacobo-Valenzuela et al. (2011) en fruto de *C. moschata* (Tabla 4, Capítulo 1).

Los valores obtenidos para pH y acidez total (TA) presentaron un estrecho rango de variación (Tabla 4, Capítulo 1), aunque se encontraron diferencias significativas entre las accesiones para piel y pulpa, siendo Cu-35 (tradicional vegetable marrow) y Cu-25 (zucchini comercial) las accesiones con mayor pH. Nuestros resultados para pH son similares a los obtenidos en otras especies de *Cucurbita* (Jacobo-Valenzuela et al., 2011). En cambio el valor de acidez total (0.04 %) fue más bajo que los de nuestro estudio (>0.10 % para ambos tejidos).

En cuanto a la composición nutricional del fruto, se encontró una considerable variación en el contenido de carotenoides de piel y pulpa del fruto de *C. pepo*. El contenido total de carotenoides, luteína, zeaxantina y β-caroteno variaron en la piel de la siguiente manera: 68.33-4452.93 (Cu-14 y Cu-17, respectivamente), 40.90-4003.65 (Cu-14 y Cu-17, respectivamente), no detectado-125.08 (Cu-17), no detectado-267.64 (Cu-27) mg kg^{-1} de peso seco; y en la pulpa: 34.93-371.50 (Cu-14 y Cu-29, respectivamente), 31.49-333.35 (Cu-14 y Cu-29, respectivamente), no detectado-5.83 (Cu-7), 3.44-45.07 (Cu-14 y Cu-11, respectivamente) mg kg^{-1} peso seco (Tabla 5, Capítulo 1). Como indican nuestros resultados, la luteína fue el carotenoide mayoritario en ambos tejidos, piel y pulpa. Además, el contenido de carotenoides en la piel fue superior que en la pulpa, resultado que coincide con el de otros autores, reforzando la hipótesis de que hay una regulación de la biosíntesis de carotenoides independiente en ambos tejidos (Gross, 1987; Kato et al., 2004; Xu et al., 2006; Alquezar et al., 2008). El-Quad et al. (2009) obtuvieron valores de luteína en *C. pepo* inferiores a los nuestros (23.4 mg kg^{-1}), en cambio los valores de zeaxantina y β-caroteno estuvieron dentro de nuestro rango (0.41 y 1.46 mg kg^{-1}, respectivamente). También se obtuvieron rangos más estrechos en los estudios realizados por Tadmor et al. (2005) para el contenido total, luteína y β-caroteno en pulpa de calabacín con: 10.4-187.2, 6.4-143.2 y 4-44.8 mg kg^{-1} de peso seco, respectivamente.

El interés por el contenido en este tipo de compuestos ha aumentado en los últimos años al atribuírseles propiedades antioxidantes (Jorgensen y Skibsted, 1993). La luteína se encuentra en la retina del ojo donde protege a las células fotorreceptoras de

las especies reactivas de oxígeno que se forman durante los procesos fotoquímicos, además juega un importante papel en la prevención de la degeneración macular que se produce con la edad (Scalch, 1992; Khachik et al., 1999). La ingesta de luteína y zeaxantina se ha relacionado con la reducción del riesgo de sufrir cataratas o degeneración macular (Seddon et al., 1994; Varma et al., 1995, Beatty et al., 1999; Segasothy y Phillips, 1999; Hammond y Caruso-Avery, 2000). La luteína a pesar de no ser una provitamina A como el β-caroteno, es un antioxidante más efectivo que otros carotenoides, propiedad a tener en cuenta ya que es el carotenoide que se presenta en mayor concentración en calabacín.

La ingesta diaria de luteína y zeaxantina es generalmente baja (0.6-3 mg día^{-1}) (Leth et al., 2000; Johnson, 2002), se recomienda una ingesta diaria de estas xantofilas de entre 4-20 mg para que tengan efectos beneficiosos sobre las funciones visuales (Granado et al., 2003). Según nuestros resultados, para alcanzar una ingesta diaria de 20 mg sería necesario el consumo de 1250 g de pulpa de *C. pepo* (considerando una concentración de 200 mg luteína kg^{-1} en peso seco de calabacín que corresponde a 16 mg luteína kg^{-1} peso fresco), presente en las accesiones Cu-29, Cu-25 y Cu-31 (Tabla 5, Capítulo 1).

Además, los carotenoides son pigmentos responsables de la coloración que presentan muchos tipos de frutos. La relación que existe entre el color y el contenido de carotenoides se puso de manifiesto en el análisis de correlación entre caracteres físico-químicos y nutricionales en *C. pepo* (Tabla 6, Capítulo 1). Existe una fuerte correlación positiva entre los parámetros del color (a*, b* y *C**) de la piel y de la pulpa, y el contenido total de carotenoides y luteína de la pulpa, por lo que se podrían seleccionar líneas con alto contenido de carotenoides teniendo en cuenta solamente el color del fruto. Estudios previos han correlacionado los parámetros de color y. el contenido de carotenoides en otras especies del género *Cucurbita* (Whang et al., 1999; Seroczynska et al., 2006; Itle y Kabelka, 2009; Konopacka et al., 2010) y otras especies vegetales como tomate (D'Souza et al., 1992; Arias et al., 2000), boniato (Simonne et al., 1993; Ameny y Wilson, 1997), pimiento (Reeves, 1987; Lee y Lee, 1992), zanahoria (Park et al., 1995), o más recientemente en yuca (Sánchez et al., 2014).

Otros importantes nutrientes evaluados en este trabajo fueron los minerales. En las 34 accesiones analizadas (Tabla 1, Capítulo 2), el contenido mineral total e individual de la piel fue más alto que el de la pulpa para todas las accesiones, excepto Na. Las

accesiones con los contenidos más altos (expresados como mg kg^{-1}) en la piel y la pulpa, respectivamente, fueron: P: Cu-20 (19100) y Cu-34 (8350) (zucchini); K: Cu-24 (49795) (zucchini comercial) y Cu-2 (46150) (zucchini tradicional); Ca: Cu-9 (9550) y Cu-5 (4750) (vegetable marrow); Mg: Cu-33 (4714) y Cu-31 (3050) (zucchini comerciales); Fe: Cu-33 (102) y Cu-23 (55) (zucchini comerciales); Cu: Cu-24 (14.5) y Cu-29 (4.9) (zucchini comerciales); Mn: Cu-20 (55) (zucchini comercial) y Cu-5 (38) (vegetable marrow); Cu-25 (77) y Cu-30 (274) (zucchini comerciales); Na: Cu-12 (323) (zucchini tradicional) y Cu-30 (1388) (zucchini comercial). Las accesiones Cu-2, Cu-23, Cu-25, Cu-29, Cu-30, Cu-31 y Cu-34 pertenecientes al morfotipo zucchini y Cu-5 perteneciente a vegetable marrow podrían ser utilizadas como fuente de variación en programas de mejora para incrementar estos nutrientes en el fruto

Hay pocos estudios en los que se hayan evaluado el contenido mineral en la piel y la pulpa del fruto de *C. pepo*. Ekholm et al. (2007) obtuvieron valores similares para K, Mg y Cu, más bajos para P, Ca, Mn y Zn, y un valor más alto para Fe, considerando los valores de la pulpa. Harichan y Verma (2013) obtuvieron contenidos más altos de Ca, Fe y Mn en pulpa que los nuestros en la piel, en cambio, nuestros resultados para K, Cu, Mg y Mn fueron más altos que los obtenidos por estos autores en la piel y en la pulpa, y fueron más bajos para Ca, Fe y Na en la pulpa. Jacobo-Valenzuela et al. (2011) estudiaron la composición mineral en la piel y la pulpa de *C. moschata* cv Cehualca, y encontraron contenidos inferiores en la piel para P, K, Mg, Mn y Zn, y en la pulpa para P, Mg, Fe, Mn y Zn; sin embargo, los valores fueron mayores para Fe en la piel y K en la pulpa y para Ca, Cu, y Na en ambos tejidos. Por tanto, los contenidos de P, Zn y Mn en la pulpa de *C. pepo* en esta tesis fueron más altos que los reportados en los trabajos anteriormente citados.

A partir de un análisis cluster dos patrones diferentes de acumulación de minerales fueron evidentes, donde los genotipos en el primer grupo pertenecientes a los morfotipos zucchini y pumpkin presentaron los contenidos más altos (24338 y 62136 mg kg^{-1} peso seco) en comparación con los genotipos del segundo grupo con los morfotipos de vegetable marrow, var. clypeata, var. texana y var. ozarkana (Figura 1, Capítulo 1).

Los requerimientos diarios de una persona adulta (hombres y mujeres) son (mg d^{-1}): 700 P, 3100 K, 900-1000 Ca, 300-350 Mg, 9-18 Fe, 1.1 Cu, 1.8-2.3 Mn, 7-9.5 Zn, 1300-1500 Na (Cuervo et al., 2010). La contribución potencial de la ingesta diaria de 200 gr de calabacín (pulpa) se muestra en la Tabla 5 (Capítulo 2), agrupando las

accesiones por morfotipos. El morfotipo zucchini fue superior a los demás morfotipos, aportando el porcentaje más alto a la ingesta diaria recomendada, que para P, K, Ca, Mg, Fe, Cu, Mn, Zn y Na sería del 21, 25, 8, 19, 7, 10, 7, 13 y 2%, respectivamente. Las deficiencias de Fe, Zn, junto con las de vitamina A e iodina, son las principales causas de malnutrición a nivel global (FAO/WHO, 2001; Harichan y Verma, 2013; Ezzatti et a al.,2002), por lo que la información que se aporta en estos estudios es importante para seleccionar parentales que puedan utilizarse en programas de mejora centrados en aumentar el contenido de alguno o varios de estos nutrientes.

Otro de los objetivos de la tesis fue desarrollar calibraciones NIRS para predecir tanto el contenido mineral (P, K, Ca, Mg, Fe, Cu, Mn, Zn y Na) como el de carotenoides (carotenoides totales, luteína, y β-caroteno).

Respecto los modelos de predicción desarrollados para determinar el contenido mineral en fruto de *C. pepo*, se encontró una clara contribución de la región visible y NIRS del espectro en las calibraciones (Figuras 1 y 5, Capitulo 3). Los estadísticos de la validación cruzada y externa de las ecuaciones desarrolladas para los diferentes minerales de la piel y de la pulpa del fruto de *C. pepo* pueden observarse en la Tabla 4 (Capítulo 3). Los valores de R^2 variaron entre 0.33-0.84, que corresponden al Na en piel y pulpa y al total mineral en piel, respectivamente. En función de R^2 los modelos desarrollados para Mg (pulpa) y Na (piel y pulpa) serían de poca utilidad ($0.3<R^2<0.49$), la fiabilidad de los modelos para predecir Ca, Mn, Cu (piel y pulpa) y P, K, Fe (pulpa) aumentaría un poco ($0.50<R^2<0.69$) y podrían utilizarse para seleccionar material vegetal, en cambio sí se consiguieron buenos resultados para los modelos desarrollados para los minerales totales y Zn (piel y pulpa) y para el P, K, Fe y Mg (piel). Estos resultados están de acuerdo con los reportados por otros autores en uva (Cozzolino et al., 2011), forrajes y legumbres (Cozzolino y Morón, 2004; Vazquez De Aldana et al., 1995; Font et al., 2002), ya que obtuvieron valores similares para Fe (R^2: 0.74), Zn (R^2: 0.72) y K (R^2: 0.82) aunque mejores resultados para Na (R^2: 0.83), Cu (R^2: 0.82), Mn (R^2: 0.74) y Ca (R^2: 0.75).

Hubo una clara contribución de las regiones visible y NIRS del espectro en las calibraciones desarrolladas para la predicción del contenido en carotenoides (Figuras 1, 2, 4 y 5, Capitulo 4). Estas regiones fueron también utilizadas por otros autores para predecir mediante NIRS el contenido en carotenoides en otros cultivos (Brenna y Berardo, 2004, Murray y Williams, 1987. Bonierbale et al., 2009; Davey et al., 2009).

Los coeficientes de determinación (R^2) de las ecuaciones NIRS obtenidos en esta tesis son comparables a los reportados por otros autores en banana y yuca para el β-caroteno y el total de carotenoides (R^2: 0.89-0.91 y 0.84-0.88, respectivamente) (Davey et al., 2009; Sánchez et al., 2014), o en el grano de maíz para la luteína (R^2:0.96), y más altos que los obtenidos para la luteína en banana y patata (R^2:0.30 y 0.70), y para β-caroteno en patata y en grano de maíz (R^2:0.56 y 0.70) (Brenna y Berardo, 2004; Bonierbale et al., 2009).

Otro de los objetivos de esta tesis fue incrementar el contenido total de carotenoides en fruto de calabacin a través de un programa de selección recurrente fenotípica, dado que esta metodología ha sido empleada con éxito en *Cucurbita* y en otras especies (Abd El-Al et al., 1973; Ceballos et al., 2013; Nienhuis and Lower, 1989, Whitaker and Robinson, 1986; Robinson, 1999). El principal obstáculo por lo que este método de mejora no ha sido extensamente utilizado es la enorme carga de laboratorio (HPLC ó espectrofotometría UV-VIS) que representa el análisis del contenido de carotenos en piel y pulpa del fruto, además del esfuerzo económico. Por este motivo, y dado que en trabajos previos se había demostrado con éxito la alta correlación entre los parámetros del color y el contenido en carotenoides, decidimos estudiar la posibilidad del uso del espectrocolorímetro como herramienta de selección. Como material vegetal de partida se utilizó la variedad híbrida comercial de calabacín "Yellow zucchini", la cuál mostró el más alto contenido total en carotenoides en mesocarpo (designada como Cu-29 en el Capítulo I).. Se obtuvieron cuatro poblaciones (dos por ciclo) a lo largo de cuatro años de selección (la figura 1 ilustra el esquema aplicado). La población P_o, fue la que presentó el coeficiente de variación más alto, tanto en mesocarpo como en epicarpo, producto de la segregación de plantas F_2. En esta generación se obtuvieron frutos con la piel de color verde, verde claro y distintas tonalidades de amarillos, y también se observó una amplia variabilidad para el color de la pulpa. Este tipo de segregación es consistente con una herencia para este carácter controlada por varios genes, lo que coincide con los resultados de estudios previos en *C. pepo* (Tadmor et al., 2005).

El mejor coeficiente de correlación entre los parámetros del color y el contenido en carotenos en pulpa (determinado mediante espectrofotometría) se obtuvo con el ángulo hue (r=0.92**). No fue posible obtener una ecuación de regresión aceptable para el contenido de carotenos en piel a partir de la población P_0 debido a la existencia de frutos de color verde (clorofilas). En la Figura 2 (Capítulo V) se presenta el diagrama de dispersión para los ángulos hue de piel y pulpa para las distintas poblaciones obtenidas (incluida la población original), que coincide con los datos

mostrados en la Tabla 2 (Capítulo V). Aunque en la Figura 2 se han representado las poblaciones recolectadas en diferentes años (por tanto las condiciones ambientales pudieron ser diferentes), la población original fue desarrollada junto a la población P_4, La Tabla 2 muestra una disminución de los coeficientes de variación y de los valores hue a lo largo de las diferentes poblaciones obtenidas. Los resultados fueron consistentes con un incremento en el contenido en carotenoides de la pulpa y la piel del calabacin en la población P_4 (385-508.04 y 1415-1600 mg kg^{-1} de peso seco) en comparación con la población original (315 y 1104.6 mg kg^{-1} de peso seco). Por tanto, aunque en el futuro será necesario avanzar más con la obtención de un tercer ciclo de selección, y en la comparación de todas la poblaciones en dos localidades para una mejor evaluación, este trabajo ha puesto de manifiesto que la herramienta de selección basado en el color y el método de mejora son adecuados para conseguir un mayor valor añadido del fruto.

Por otra parte, nuestros resultados confirman la seguridad, la antigenotoxicidad y el potencial quimiopreventivo del calabacín (Zucchini) y de algunos de los componentes bioactivos de su fruto (luteína, β-caroteno, zeaxantina y ácido dehidroascórbico) usando el modelo *in vivo* SMART y el modelo *in vitro* de citotoxicidad con la línea celular HL60. Ambas variedades difieren enormemente en casi todos los contenidos de los compuestos bioactivos de su fruto (Capítulo 6, Tabla 1).

Los agentes antigenotóxicos generalmente exhiben efectos quimioterapéuticos deseables y podrían ser utilizados como una estrategia eficiente para el control del cáncer (Anter et al., 2011). Las concentraciones utilizadas de la piel y la pulpa de las dos variedades de calabacín (“verde” y “amarillo”) y de los principales compuestos bioactivos de su fruto, los carotenoides (luteína, β-caroteno y zeaxantina) y ácido dehidroascórbico (forma oxidada del ácido ascórbico, es decir, la vitamina C) fueron antigenotóxicas en el test SMART de *Drosophila melanogaster*, mostrando distintos porcentajes de inhibición del daño genético (variando entre el 11 y el 100 %) en los tratamientos en los que se utilizó agua oxigenada como genotoxina, a excepción de la concentración más alta de luteína (0.615 µM) (Capítulo 6, Figura 1). Los porcentajes más altos de inhibición los mostraron la concentración más baja de pulpa del calabacín “amarillo” (0.25 mg/mL) y la concentración más baja de β-caroteno (0.0003 µM), siendo de un 100% para ambos compuestos.

En cuanto al análisis de la citotoxicidad, es conocido que el uso de la línea celular HL60 proporciona un modelo seguro para estudiar los efectos citotóxicos de

sustancias con potencial quimiopreventivo (Villatoro-Pulido et al., 2009). Se utilizó un amplio rango de concentraciones en las sustancias ensayadas. La piel y la pulpa del calabacín "amarillo" mostraron el mayor efecto tumoricida, mostrando una IC_{50} > 0.1 mg/ml y 0.2 mg/ml; la piel y la pulpa del calabacín "verde" también fueron citotóxicas, pero no se alcanzó la IC_{50} (Capítulo 6, Figura 2). Los compuestos bioactivos analizados tampoco alcanzaron la IC_{50} a las concentraciones ensayadas, pero se observaron efectos citotóxicos en distinto grado en algunos de ellos (zeaxantina, ácido dehidroascórbico, β-caroteno y la mezcla de compuestos), en cambio la luteína no tuvo ningún efecto citotóxico (Capítulo 6, Figura 3). En cuanto a los ensayos de apoptosis, únicamente la pulpa, y en menor medida la piel del calabacín "verde", mostraron fragmentación y por tanto una acción antiproliferativa, sugiriéndose un efecto que depende de la dosis a tenor de los resultados (Capítulo 6, Figuras 4 y 5).

En este respecto hay pocos estudios en el que se haya evaluado al calabacín en experimentos *in vivo* o *in vitro*. Wang et al., (2007) usaron extractos etanólicos del fruto y observaron un efecto inhibitorio dependiente de la dosis sobre el crecimiento de dos líneas celulares (HeLa y HepG). Menéndez et al., (2006) utilizaron el extracto lipofílico de las semillas de calabacín y observaron un descenso significativo del crecimiento prostático que se produce en la hiperplasia prostática inducida por andrógenos. Otros autores también utilizaron extractos etanólicos, pero en este caso de las hojas del calabacín, en distintas líneas celulares, normales y cancerosas, observando una mayor inhibición en este último tipo celular (Shokrzadeh et al., 2010).

Los carotenoides han sido ampliamente estudiados, y se ha demostrado que su consumo puede descender el riesgo de cáncer (Donaldson, 2004). Dias et al., (2009) obtuvieron resultados similares en el test SMART para el β-caroteno usando unas concentraciones mayores que las nuestras (1, 2 and 4 mg/mL), ya que no resultó genotóxico y fue capaz de disminuir en un 95 % la frecuencia de mutaciones inducida por la doxorubicina. Se ha demostrado que el β-caroteno, al igual que otros carotenoides y otros compuestos bioactivos, tienen efectos antioxidantes o pro-oxidantes, dependiendo de los niveles de oxígeno y de β-caroteno (Zhang and Omaye, 2001). En nuestro estudio el β-caroteno no mostró citotoxicidad (0.5 µM), resultado que coincide con el de otros autores que usaron la misma línea celular pero a unas concentraciones más altas (10 µM), en cambio estos mismos autores y también otros, informaron de que este compuesto producía fragmentación a mayores concentraciones que las nuestras (Sacha et al. 2005; Palozza et al., 2002).

En cuanto a los demás carotenoides estudiados en este trabajo, la luteína, a unas concentraciones más altas que las nuestras, causó daños en el ADN de manera significativa en la línea celular ARPE-19, dependiente del tiempo y la dosis utilizada (Kalariya et al., 2009). Bi et al. (2013) comprobaron la citotoxicidad de la zeaxantina en líneas celulares tumorales comparándolas con otras líneas celulares normales y observaron que no afectaba a la viabilidad de las células normales, pero en cambio si afectaba a la viabilidad de las líneas celulares tumorales dependiendo de la dosis, por lo que sugerían un efecto citotóxico selectivo. Además, otros estudios demostraron que la zeaxantina no era genotóxica utilizando como modelos a *Salmonella typhimurium* (Ravi et al., 2014; Ravikrishnan et al., 2011, Thurnham and Howard, 2013), y *Escherichia coli* (Thurnham and Howard, 2013).

Por otra parte, estudios realizados con el ácido ascórbico (forma reducida del ácido dehidroascórbico) mostraron que era citotóxico e inducía apoptosis en células malignas, utilizando unas concentraciones más altas que las utilizadas en este estudio (Park et al., 2005).

Según nuestros resultados, existe una gran variabilidad para la mayoria de los componentes implicados en la calidad del calabacín, en las accesiones de la colección de germoplasma del Centro IFAPA-La Mojonera. Además se han encontrado posibles candidatos para utilizar como parentales en programas de mejora cuyo objetivo sea incrementar el contenido de uno o de varios de estos parámetros, ya que algunos de ellos están correlacionados. Hay que destacar también la gran variabilidad encontrada en los componentes nutricionales del fruto, dada su importancia y relación con el mantenimiento de una buena salud, ya que los minerales están implicados en multitud de funciones en el organismo y los carotenoides son compuestos con propiedades antioxidantes cuyo consumo está relacionado con la disminución de padecer diferentes enfermedades, entre ellas, ciertos tipos de cáncer. Por esta razón es importante desarrollar métodos de muestreo rápidos, seguros y efectivos para localizar genotipos con un alto contenido en estos nutrientes, y el NIRS ha demostrado ser una herramienta con prometedores resultados para este propósito en programas de mejora. Además ha quedado demostrado que el calabacín responde bien a la selección recurrente, y se ha conseguido un aumento significativo del contenido total de carotenoides, logro importante debido a su implicación en el mantenimiento de la salud, y a que la mejora de su contenido nutricional puede significar dar un valor añadido como producto de mercado. En este sentido, nuestros resultados confirman

que el calabacín es un alimento seguro y con potencial quimiopreventivo, lo que le añade valor como alimento.

CONCLUSIONES

Las conclusiones que se derivan de los trabajos de investigación realizados en la presente Tesis son las que se exponen a continuación:

1. Existe una alta variabilidad morfológica (peso, longitud y diámetro) en las accesiones de calabacín (*Cucurbita pepo*) procedentes de distintos orígenes geográficos, variabilidad debida principalmente a las diferencias existentes entre los morfotipos de la especie estudiados.

2. También se encontró una amplia variabilidad, tanto en la piel como en la pulpa, para los parámetros relacionados con la calidad físico-química (color, firmeza, materia seca, contenido sólidos solubles, acidez total, pH) y calidad nutricional (carotenoides y minerales), sugiriendo, por tanto, una alta probabilidad de encontrar nuevos y valiosos recursos fitogenéticos en colecciones de germoplasma para su uso en programas de mejora genética. Los caracteres físico-químicos y nutricionales más relevantes para explicar la variabilidad total de los datos fueron los parámetros cromáticos a*, b*, Chroma* y Hue (en piel y pulpa), y peso seco, sólidos solubles, acidez total, luteina, β-caroteno y contenido total de carotenoides en la piel.

3. Las accesiones de *C. pepo* fueron una buena fuente de carotenoides, especialmente en luteína, carotenoide mayoritario en el fruto. Las accesiones Cu-17 (luteina y zeaxantina), y Cu-27 (β-caroteno) en piel pertenecientes a los morfotipos Vegetable Marrow y Zucchini respectivamente, y Cu-29 (luteina), Cu-7 (zeaxantina) y Cu-11 (β-caroteno) en pulpa, pertenecientes a los morfotipos Zucchini, Vegetable Marrow y Pumpkin respectivamente, presentaron los contenidos más altos para los distintos carotenoides analizados y podrían ser utilizadas para mejorar el contenido de carotenos en un programa de mejora de la especie centrado en aumentar uno o varios de .estos componentes.

4. La espectroscopía en el infrarrojo cercano (NIRS) puede ser utilizada como un método de muestreo rápido para la determinación del contenido de carotenoides (carotenoides totales, luteína y β-caroteno) en la piel y la pulpa del fruto del calabacín con un mínimo procesado (liofilización). El análisis de estos componentes, se basa principalmente en la región espectral del visible (500 a 700 nm) y del infrarrojo (1432 a 2348 nm), asociadas a los grupos C-H de lípidos y almidón, grupo O-H del agua, así como proteínas y clorofilas.

5. Las accesiones de *C. pepo* fueron también una buena fuente de minerales, particularmente el morfotipo zucchini, aportando un porcentaje adecuado a la ingesta

diaria recomendada de una persona adulta, que para P, K, Ca, Mg, Fe, Cu, Mn, Zn y Na sería del 21, 25, 8, 19, 7, 10, 7, 13 y 2%, respectivamente. Las accesiones Cu-2, Cu-23, Cu-25, Cu-29, Cu-30, Cu-31, Cu-34 pertenecientes al morfotipo Zucchini y Cu-5 perteneciente al morfotipo Vegetable Marrow, podrían ser utilizadas como fuente de variación en programas de mejora cuyo objetivo sea mejorar el contenido de estos minerales en fruto.

6. Las ecuaciones NIRS desarrolladas para la predicción del contenido de Ca (piel), P, K y Ca (pulpa) en muestras liofilizadas de *Cucurbita pepo* pueden ser utilizadas para propósitos de cribado, mientras que los resultados obtenidos para el contenido total de minerales (piel y pulpa), P, K y Mg (piel) pueden ser usadas en el control de calidad. representando una herramienta útil, rápida, de bajo coste y sin la utilización de productos químicos tóxicos.

7. Las correlaciones significativas entre algunos de los parámetros cromáticos y el contenido total de carotenoides hace posible su uso en programas de mejora en esta especie para la selección de genotipos con alto contenido en estos componentes.

8. Los resultados obtenidos a partir del programa de selección recurrente indicaron que el cultivo de calabacín respondió bien a dicha metodología con consistentes y significativas ganancias para el contenido total en carotenoides.

9. Nuestros resultados confirman la seguridad, la antigenotoxicidad y el potencial quimiopreventivo del calabacín (Zucchini) y de algunos de los componentes bioactivos de su fruto (luteína, β-caroteno, zeaxantina y ácido dehidroascórbico) usando el modelo *in vivo* SMART y el modelo *in vitro* de citotoxicidad con la línea celular HL60.

10. Los ensayos de antigenotoxicidad indicaron que las concentraciones usadas mostraron distintos porcentajes de inhibición (variando entre el 11 y el 100 %) en los tratamientos combinados con la genotoxina peróxido de hidrógeno, a excepción de la concentración más alta de la luteína.

11. La piel y la pulpa del calabacín “amarillo” mostraron la actividad citotóxica más elevada(IC_{50} > 0.1 mg/ml y 0.2 mg/ml, respectivamente); el resto de compuestos bioactivos analizados no alcanzaron la IC_{50} a las concentraciones ensayadas pero se observaron efectos citotóxicos en distinto grado en algunos de ellos (zeaxantina, ácido dehidroascórbico, β-caroteno y la mezcla de compuestos).

CONCLUSIONS

The conclusions derived from the research performed in this Thesis are those listed below:

1. There is great morphological variability (weight, length and diameter) among zucchini accessions (*Cucurbita pepo*) from different geographical origins. This variability is primarily due to differences among morphotypes of this species.

2. A great variation was found in both skin and pulp for physicochemical characters (colour, firmness, dry matter, soluble solid content, total acidity, pH) and nutritional quality (carotenoids and minerals). This suggests a high probability of finding new and valuable plant genetic resources in germplasm collections for using in breeding programmes. The most relevant physicochemical and nutritional characters to explain the total variability of the data were the colour parameters a *, b *, Chroma, Hue (in skin and pulp), and dry matter, soluble solids, total acidity, lutein, β carotene and total carotenoids (in the skin).

3. *C. pepo* accessions were a good source of carotenoids, especially lutein, which was the major carotenoid in the fruit. Cu-17 (lutein and zeaxanthin) and Cu-27 (β-carotene) accessions in skin, belonging to morphotypes Zucchini and Vegetable Marrow respectively, and Cu-29 (lutein), Cu-7 (zeaxanthin) and Cu-11 (β-carotene) in pulp, belonging to Zucchini, Vegetable Marrow and Pumpkin morphotypes, respectively, showed the highest levels for different carotenoids and could be used in breeding programmes focused on increasing one or more of these components.

4. The near-infrared spectroscopy (NIRS) can be used as a rapid method for the prediction of the carotenoid content (total carotenoids, lutein and β-carotene) in skin and pulp of summer squash samples with minimal processing (lyophilization). Analysis of these components, is mainly based on the spectral region of the visible (from 500 to 700 nm) and infrared (from 1432 to 2348 nm), associated with the CH groups of lipids and starch, water OH group as well as protein and chlorophylls.

5. *C. pepo* accessions were also a good source of minerals, particularly Zucchini morphotype, providing a good percentage of the recommended adult daily intake, for P, K, Ca, Mg, Fe, Cu, Mn, Zn and Na would be 21, 25, 8, 19, 7, 10, 7, 13 and 2% respectively. Cu-2, Cu-23, Cu-25, Cu-29, Cu-30, Cu-31, Cu-34 accessions belonging to morphotype Zucchini and Cu-5 belonging to Vegetable Marrow morphotype, could be used as a source of variation in breeding programmes aimed at improving the mineral content in fruit.

6. NIRS equations developed to predict Ca (in skin), P, K and Ca (in pulp) contents in lyophilized samples of *Cucurbita pepo* can be used for screening purposes. While the NIRS equations for the total content of minerals (in skin and pulp), P, K and Mg (in skin) may be used in quality control, representing a useful, quick, low cost tool without using toxic chemicals.

7. Significant correlations among some of the colour parameters and total carotenoid content make its use possible in *C. pepo* breeding programmes for the selection of genotypes with a high content of these components.

8. Results indicated that summer squash responded well to the recurrent selection method with consistent and significant gains for total carotenoid content.

9. Our results confirmed the safety, antigenotoxicity and chemopreventive potential of Zucchini and some of its fruit bioactive compounds (lutein, β-carotene, zeaxanthin and dehydroascorbic acid) using the SMART test as *in vivo* model and the cytotoxicity HL60 cells *in vitro* model.

10. Antigenotoxicity assays indicated that the used concentrations showed different inhibition percentages (ranging from 11 to 100 %) in combined treatments with the genotoxicant hydrogen peroxide, with the exception of the highest concentration of lutein.

11. “Yellow” Zucchini skin and pulp exhibited the highest cytotoxic activity (IC_{50} > 0.1 mg/ml and 0.2 mg/ml, respectively); the rest of the bioactive compounds assayed did not reach IC_{50} at the tested concentrations, although cytotoxic effects were observed in some of them (zeaxanthin, dehydroascorbic acid, β-carotene and the mix of the compounds).

REFERENCIAS DE LA INTRODUCCIÓN Y LA DISCUSIÓN GENERAL

Introduction and general discussion references

1. Aboul-Nasr MH, Damarany AM and Abdalla MMA. 2002. Yield and its components of some summer squash (*Cucurbita pepo* L.) genotypes. In: Maynard DN (ed) Cucurbitaceae. ASHS Press, Alexandria, pp 88–94.

2. Alonso-Moraga A and Graf U. 1989. Genotoxicity testing of antiparasitic nitrofurans in the Drosophila wing somatic mutation and recombination test. Mutagenesis. 4: 105-1.

3. Alquezar B, Rodrigo MJ and Zacarías L. 2008. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. Phytochemistry. 69: 1997–2007.

4. Anter J, Fernández-Bedmar Z, Villatoro-Pulido M, Demyda-Peyras S, Moreno-Millán M, Alonso-Moraga A, Muñoz-Serrano A, Luque de Castro MD. 2011b. A pilot study on the DNA protective, cytotoxic and apoptotic-induced roles of olive leaf extracts. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. Article in Press. Doi:10.1016/j.mrgentox.2011.05.005.

5. AOAC. 1984. Official methods of analysis. 14th Ed. Virginia, USA: Association of Official Analytical Chemists, pp 414–420.

6. Arias R, Lee T, Logendra L and Janes H. 2000. Correlation of lycopene measured by HPLC with the L*, a*, b* color readings of a hydroponic tomato and the relationship of maturity with color and lycopene content. Journal of Agricultural Food Chemistry. 48: 1697-1702.

7. Audrey H, Beany AH, Stoffella PJ, Roe N, Picha DH. 2002. Production, fruit quality, and nutritional value of spaghetti squash. In: Janick J, Whipkey A (eds) Trends in new crops and new uses. ASHS Press, Alexandria, pp 445–448.

8. Ameny MA and Wilson PW. 1997. Relationship between Hunter color and β-carotene content in white-fleshed African sweetpotato. J. Sci. Food Agric. 73: 301-306.

9. Andres TC. 1987. *Cucurbita* fraterna, the closest wild relative and progenitor of *C. pepo*. Cucurbit Genet Coop. Rep. 10: 69–71.

10. Atienza SG, Avila CM, Ramírez MC, Martí A. 2005. Application of near infrared reflectance spectroscopy to the determination of carotenoid content in tritordeum for breeding purposes. Australian Journal of Agricultural Research. 56(1): 85-89.

11. Azevedo-Meleiro CH and Rodríguez-Amaya DB. 2007. Quantitative and qualitative differences in carotenoids composition among *Cucurbita moschata*, *Cucurbita maxima* and *Cucurbita pepo*. Journal of Agricultural and Food Chemistry. 55: 4027–4033.

12. Baars AJ. 1980. Biotransformation of xenobiotics in *Drosophila melanogaster* and its relevance for mutagenicity testing. Drug Metabolism Review 11: 191-221.

13. Beatty S, Boulton M, Henson D, Koh HH, Murray IJ. 1999. Macular pigment and age related macular degeneration. The British Journal of Ophthalmology. 83: 867-877.

14. Ben-Amotz A and Fishler R. 1998. Analysis of carotenoids with emphasis on 9-cis β-carotene in vegetables and fruits commonly consumed in Israel. Food Chemistry. 62: 515-520.

15. Bendich A. 1989. Carotenoids and the immune response. Journal of Nutrition. 119: 112-115.

16. Bi MC, Rosen R, Zha RY, McCormick SA, Song E, Hu DN. 2013. Zeaxanthin induces apoptosis in human uveal melanoma cells through Bcl-2 family proteins and intrinsic apoptosis pathway. Evidence-Based Complementary and Alternative Medicine. Volume 2013, Article ID 205082, 12 pages.

17. Blanco-Díaz MT, Del Río Celestino M, Martínez-Valdivieso D, Font R. 2014. Use of visible and near-infrared spectroscopy for predicting antioxidant compounds in summer squash (*Cucurbita pepo* ssp *pepo*). Food Chemistry. 164: 301–308.

18. Bombardelli E and Morazzoni P. 1997. *Cucurbita pepo* L. Fitoterapia. 68(4): 291-301.

19. Bonierbale M, Grüneberg W, Amoros W, Burgos G, Salas E, Porras E, zum Felde T. 2009. Total and individual carotenoid profiles in *Solanum phureja* cultivated potatoes: II. Development and application of near-infrared reflectance spectroscopy (NIRS) calibrations for germplasm characterization. Journal of Food Composition and Analysis. 22 (6): 509-516.

20. Brenna OV and Berardo N. 2004. Application of Near-Infrared Reflectance Spectroscopy (NIRS) to the evaluation of carotenoids content in maize. Journal of Agricultural and Food Chemistry. 52: 5577-5582.

21. Burns DA and Ciurczak EW. 1992. Handbook of Near Infrared Analysis. Burns, D.A., Ciurczak, E.W. (Eds.). Practical spectroscopy series. 13. Marcel Dekker, Inc. New York, USA.

22. Burns DA and Ciurczak EW. 2001. Handbook of Near-Infrared Analysis. Marcel Dekker, Inc. New York, USA. Kader A (ed). University of California, Division of Agriculture and Natural Resources, Oakland, California, USA. pp 55-62.

23. Carpentier Y, Mayer P, Bobichon H, Desoize B. 1998. Cofactors *in vitro* induction of apoptosis in HL60 cells by all-trans retinoic acid (ATRA) Biochemical Pharmacology. 55: 177-184.

24. Castenmiller JJM and West CE. 1998. Bioavailability and bioconversion of carotenoids. Ann. Rev. Nutr. 18, 19–38.

25. Ceballos H, Morante N, Sánchez T, Ortiz D, Aragón I, Chávez AL, Pizarro M, Calle F, Dufour D. 2013. Rapid Cycling Recurrent Selection for Increased Carotenoids Content in Cassava Roots. Crop Science, 53: 1-10.

26. Collins SJ, Ruscetti FW, Gallagher RE, and Gallo RC. 1978. Terminal differentiation of human promyelocytic leukaemia cells induced by dimethyl sulfoxide and other polar compounds. Proceedings of the National Academy of Sciences USA. 75: 2458–2462.

27. Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía. 2014. Observatorio de precios y mercados. Análisis de rentabilidad de hortícolas protegidos : calabacín verde. Campaña 2012/2013.

28. Conte-Anazetti M, Silva-Melo P, Duran N, Haun M. 2003. Comparative cytotoxicity of dimethylamide-crotonin in the promyelocytic leukemia cell line (HL60) and human peripheral blood mononuclear cells. Toxicology. 88: 261–274.

29. Cozzolino D and Morón A. 2004. Exploring the use of near infrared reflectance spectroscopy (NIRS) to predict trace minerals in legumes. Anim Feed Sci Tech. 111: 161-173.

30. Cozzolino D, Cynkar W, Shah N and Smith P. 2011. Quantitative analysis of minerals and electric conductivity of red grape homogenates by near infrared reflectance spectroscopy. Comput Electron Agric. 77: 81-85.

31. Cubero JI. 2000. Mejora genética clásica en la producción de nuevas variedades. En: La biotecnología aplicada a la agricultura. Ed. Mundi-Prensa. S.A. Madrid.

32. Cuervo M, Abete I, Baladia E, Corbalán M, Manera M, Basulto J, et al. 2010. Ingestas dietéticas de referencia (IDR) para la población española. Federación Española de Sociedades de Nutrición, Alimentación y Dietética (FESNAD), Ediciones Universidad de Navarra.

33. Davey MW, Saeys W, Hof E, Ramon H, Swennen RL, Keulemans J. 2009. Application of Visible and Near-Infrared Reflectance Spectroscopy (Vis/NIRS) to determine carotenoid contents in banana (*Musa* spp.) fruit flesh. Journal of Agricultural and Food Chemistry. 57: 1742–1751.

34. Decker DS. 1988. Origin(s), evolution, and systematics of *Cucurbita pepo* (*Cucurbitaceae*). Econ. Bot. 42: 4-15.

35. De Flora S and Ramel C. 1988. Mechanisms of inhibitors of mutagenesis and carcinogenesis. Classification and overview. *Mutations Research* 202: 285-313.

36. Dias CD, Araújo BC, Dutra ES, Nepomuceno JC. 2009. Protective effects of β-carotene against the genotoxicity of doxorubicin in somatic cells of *Drosophila melanogaster*. Genetics and Molecular Research, 8 (4): 1367-1375.

37. Diez MJ, Pico B and Nuez F. 2002. Cucurbit Genetic Resources in Europe, International Plant Genetic Resources Institute, Ad hoc meeting, January 2000, Adana, Turkey. IPGRI, Rome, Italy. pp 58.

38. Donaldson MS. 2004. Nutrition and cancer: A review of the evidence for an anti-cancer diet. Nutrition Journal, 3, 19. doi:10.1186/1475-2891-3-19

39. Duchesne AN. 1786. Essai sur l'histoire naturelle des courges. Pankoucke CJ, Paris, p 46.

40. D'Souza MC, Singha S and Ingle M. 1992. Lycopene concentration of tomato fruit can be estimated from chromaticity values. HortScience. 27(5): 465-466

41. Edwards NM, Dexter JE, Sobering DC, Williams PC. 1996. Whole grain prediction of durum wheat yellow pigment by visible-NIR reflectance spectroscopy. In: Near Infrared Spectroscopy: The Future Waves, Proceedings 7th International

Conference on Near-Infrared Spectroscopy, Montreal, Canada, August, 1995, Davies, A.M.C., Williams, P.C. (eds). NIR Publications, Chichester, UK, pp 462-465.

42. Ekholm P, Reinivuo H, Mattila P, Pakkala H, Koponen J, Happonen A, Hellström J, Ovaskainen M. 2007. Changes in the mineral and trace element contents of cereals, fruits and vegetables in Finland. Journal of Food Composition and Analysis. 20: 487-495.

43. El-Qudah JM. 2009. Identification and Quantification of Major Carotenoids in Some Vegetables. American Journal of Applied Sciences. 6(3): 492-497.

44. Erdman JWJR. 1999. Variable bioavailability of carotenoids from vegetables. Am.J.Clin.Nutr. 70: 179-180.

45. Esteras C, Gómez P, Monforte AJ, Blanca J, Vicente-Dólera N, Roig C, Nuez F, Picó B. 2012. High-throughput SNP genotyping in *Cucurbita pepo* for map construction and quantitative trait *loci* mapping. BMC Genomics. 13:80.

46. Ezzatti M, Lopez AD, Rodgers Vander HS, Murray CJ. 2002. Selected major risk factors and global regional burden of disease. Lancet. 360: 1347–1360.

47. FAO/WHO. 2001. Human Vitamin and Mineral Requirements. Report of a joint FAO/WHO Expert Consultation, Bangkok, Thailand.

48. Ferriol M and Picó B. 2008. Pumpkin and Winter Squash. Invited chapter. Handbook of Plant Breeding. Vol I. Vegetables I. Springer New York (ISBN 978-0-387-72291-7), pp 317-349.

49. Font R, Del Río M and De Haro A. 2002. Use of Near Infrared Spectroscopy to evaluate heavy metal content in *Brassica juncea* cultivated on the polluted soils of the Guadiamar River area. Fresen Environ Bull. 11:777-781.

50. Font R; Del Rio-Celestino M and De Haro-Bailon A. 2006. The use of near-infrared spectroscopy (NIRS) in the study of seed quality components in plant breeding programs. Industrial Crops And Products. 24: 307-313

51. Font R, Del Río-Celestino M, Simón M, Aguilar J, De Haro A. 2001. Uso de la espectroscopía en el infrarrojo cercano para la determinación de metales pesados y arsénico en suelos contaminados. Conferencia Internacional de Estadística en Estudios Medioambientales Cádiz (España). Noviembre.

52. Font R, Del Río-Celestino M, Vélez D, De Haro A, Montoro R. 2004a. Visible and Near Infrared Spectroscopy as a technique for screening the inorganic arsenic content in the red swamp crayfish (*Procambarus clarkii* girard). Analytical Chemistry. 76(14): 3893-3898.

53. Font R, Del Río-Celestino M, Vélez D, Montoro R, De Haro A. 2004b. Use of Near-Infrared Spectroscopy for determining the total arsenic content in prostrate amaranth. The Science of the Total Environment. 327: 93-104.

54. Font R, Vélez D, Del Río-Celestino M, De Haro-Bailón A, Montoro R. 2005. Screening inorganic arsenic in rice by Visible and Near-Infrared Spectroscopy. Microchimica Acta. 151: 231-239.

55. Francis FJ. 1962. Relationship between flesh color and pigment content in squash. Amer Soc Hort Sci. 81:408–414.

56. Gajc-Wolska J, Gajewski M, Radzanowska J, Niemirowicz-Szczytt K, Korzeniewska A. 2005. The fruit quality of chosen hybrids and cultivars of pumpkin (*Cucurbita maxima* Duch.). Veg Crops Res Bull. 63: 34-43.

57. Galán-Soldevilla H., Ruiz-Pérez-Cacho P., Blanco-Díaz M.T., del Río-Celestino M., Font R. 2012. Estudio de la calidad sensorial de variedades de calabacín fresco cultivadas en Almería. XIII Congreso Nacional de Ciencias Hortícolas. Almería. 16-20 Abril 2012.

58. García-Olmedo F, Sanz-Magallón G and Marín-Palma E. 2001. La agricultura española ante los retos de la biotecnología. Instituto de Estudios Económicos. Ed. Impresos y Revistas. S.A. Madrid.

59. Gong L, Paris HS, Nee MH, Stift G, Pachner M, Vollmann J, Lelley T. 2012. Genetic relationships and evolution in Cucurbita pepo (pumpkin, squash, gourd) as revealed by simple sequence repeat polymorphisms. Theor Appl Genet. 124(5): 875-91.

60. González-Andrés F and Pita-Villamil J M. 2001. Conservación y Caracterización de Recursos Fitogenéticos. Publicaciones I.N.E.A.

61. Graf U, Würgler FE, Katz AJ, Frei H, Juon H, Hall CB, Kale PG. 1984. Somatic mutation and recombination test in *Drosophila melanogaster*. Environmental Mutagenesis. 6: 153–188.

62. Graf U, Alonso-Moraga A, Castro R, Diaz E. 1994. Genotoxicity testing of different types of beverages in the wing somatic mutation and recombination test. Food and Chemical Toxicology. 32: 423–430.

63. Granado F, Olmedilla B and Blanco I. 2003. Nutritional and clinical relevance of lutein in human health. British Journal of Nutrition. 90: 487–504.

64. Gross J. 1987*. Pigments* in fruits. Academic Press. Londres.

65. Halliwell B. 2001. Vitamin C and genomic satbility. Mutat Res. 18: 29-35.

66. Hammond BRJr and Caruso-Avery M. 2000. Macular pigment optical density in a Southwestern sample. Investigative Ophthalmology & Visual Science. 41: 1492-1497.

67. Hamss R, Idaomar M, Analla M, Muñoz-Serrano A, Sánchez-Campos J, Alonso-Moraga A. 1999. A dose dependent antigenotoxic effect of turmeric. Mutat Res. 446(1): 135-139.

68. Harichan S and Verma VN. 2013. Investigation of nutrient Elements in *Cucurbita pepo* using Atomic Absorption Spectrometry. International Letters of Chemistry, Physics and Astronomy. 2:11-17.

69. Harlander S. 1993. Genetic engineering of foods: a US perspective. Trends in Food Science and Technology. 4: 301-305.

70. Harvey WJ, Grant DG and Lammerink JP. 1997. Physical and sensory changes during the development and storage of buttercup squash. N Z J Crop Horti Sci. 25: 341–351.

71. Heise HM and Winzen R. 2004. Chemometrics in Near-Infrared Spectroscopy. In: Near-Infrared Spectroscopy. Principles, Instruments, Applications. Siesler HW, Ozaki Y, Kawata S, Heise HM (eds). WILEY-VCH, Weinheim, Germany, pp 125-162.

72. Hernándo-Bermejo JE and León J. 1994. Neglected Crops: 1492 from a Different Perspective. *Plant Production and Protection Series No*. 26, FAO, Rome, pp 63-77.

73. Hidaka T, Anno T and Nakatsu S. 1987, Composition and vitamin A value of the carotenoids of pumpkins of different colors. J.Food Biochem. 11: 59-68.

74. ISO, 13299. 2003. Sensory analysis-Methodology-General Guidance for establishing a sensory profile.

75. Itle RA and Kabelka EA. 2009. Correlation Between L*a*b* Color Space Values and Carotenoid Content in Pumpkins and Squash (Cucurbita spp.). Hortscience. 44(3): 633–637.

76. Jacobo-Valenzuela N, Zazueta-Morales JJ, Gallegos-Infante JA, Aguilar-Gutierrez F, Camacho-Hernandez IL, Rocha-Guzman NE, Gonzalez-Laredo RF. 2011. Chemical and physicochemical characterization of winter squash (*Cucurbita moschata* D.). Not Bot Horti Agrobot Cluj-Napoca. 39:34‑40

77. Jahn M, Munger HM and McCreight JD. 2002. Breeding cucurbit crops for powdery mildew resistance. In: Bélanger RR, Bushnell WR, Dik AJ and Carver TLW. The powdery mildews a comprehensive treatise. APS Press, St. Paul, Minnesota, pp 239–248.

78. Johnson EJ. 2002. The role of carotenoids in human health. Nutrition in Clinical Care. 5: 56–65.

79. Jorgensen K and Skibsted LH. 1993. Carotenoid scavenging of radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. Z Lebensm Unters Forsch. 196: 423-429.

80. Kalač P and Svoboda L. 2000. A review of trace element concentrations in edible mushrooms. Food Chemistry. 69: 273-281.

81. Kalariya NM, Ramana KV, Srivastava SK, van Kuijk FJ. 2009. Genotoxic effects of carotenoid breakdown products in human retinal pigment epithelial cells. Curr Eye Res, 34(9): 737-47.

82. Kato M, Ikoma Y, Matsumoto H, Sugiura M, Hyodo H, Yano M. 2004. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiology. 134: 824-837.

83. Khachik F and Beecher GR. 1988. Separation and identification of carotenoids and carotenol fatty acid esters in some squash products by liquid chromatography. I. Quantification of carotenoids and related esters by HPLC. J Agric Food Chem. 36: 929-937.

84. Khachik F, Cohen L and Zhao Z. 1999. Metabolism of dietary carotenoids and theirpossible role in prevention of cancer and macular degeneration. In: Functional Foods (American Chemical Society Symposium Series), Shibamoto T (ed.). Oxford University Press. 71-85.

85. Khaw KT, Bingham S, Welch A, Luben R, Wareham N. 2001. Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective population study. European Prospective Investigation into Cancer and Nutrition. Lancet. 357: 657–663.

86. Kmiecik W and Lisiewska Z. 1994. The influence of potassium sorbate addition on the quality of pickled zucchini fruit. Rocz Panstw Zakł Hig. 45: 301–309.

87. Konopacka D, Seroczyńska A, Korzeniewska A, Jesionkowska K, Niemirowicz-Szczytt K, Płocharski W. 2010. Studies on the usefulness of *Cucurbita maxima* for the production of ready-to-eat dried vegetable snacks with a high carotenoid content. LWT-Food Science and Technology. 43(2): 302-309.

88. Lester GE. 2008. Antioxidant, sugar, mineral, and Phytonutrient concentrations across edible fruit tissues of orange-fleshed Honeydew melon (*Cucumis melo* L.). J Agric Food Chem. 56: 3694-3698.

89. Lee HD and Lee CH. 1992. Studies on the quality evaluation of Korean red pepper by color measurement. Kor. J. Dietary Culture. 7: 105-112.

90. Leth T, Jakobsen J and Andersen NL. 2000. The intake of carotenoids in Denmark. European Journal of Lipid Science and Technology. 102: 128–132.

91. Levander OA. 1990. Fruit and vegetable contributions to dietary mineral intake in human health and disease. HortScience. 25: 1486-1488.

92. Lira-Saade R and Montes-Hernandez S. 1994. Cucurbits (*Cucurbita* spp.). In: Neglected crops: 1492 from a different perspective- Hernandez-Bermejo JE and Leon J (eds). FAO Plant Production and Protection Series No. 26. FAO, Rome.

93. Llácer G, Díez MJ, Carrillo JM, Badenes ML. 2006. Mejora genética de la calidad en plantas. Sociedad Española de Ciencias Hortícolas. Sociedad Española de Genética. Universidad Politéctica de Valencia. Valencia.

94. Loy JB. 2004. Morpho-physiological aspects of productivity and quality in squash and pumpkins (*Cucurbita* spp.). Critical reviews in plant sciences. 23(4): 337-363.

95. Martens H and Naes T. 1989. Multivariate Calibration. John Wiley & Sons. Chichester, UK.

96. Martínez-Valdivieso D, Gómez P, Vicente-Dólera N, Rubira F, Moya M, Del Río-Celestino M. 2012. Obtención y caracterización de mutantes morfológicos en calabacín (*Cucurbita pepo* spp. *pepo*). Actas de Horticultura. 60: 71-75.

97. Mathews-Roth MM. 1990. Plasma concentration of carotenoids after large doses of beta-carotene. American Journal of Clinical Nutrition. 52: 500-501.

98. Menéndez AB, Capó JT, Menéndez-Castillo RA, González OL, Domínguez CC, Sanabria MLG. 2006. Evaluation of *Cucurbita pepo* L. lipophilic extract on androgen-induced prostatic hiperplasia. Rev Cubana Plant Med v.11 n.2, Ciudad de la Habana abr.-jun.

99. Monforte AJ and Álvarez JM. 2006. Mejora de la calidad del melón. In: Llácer G, Díez MJ, Carrillo JM and Badenes ML (eds). Mejora genética de la calidad en plantas. Sociedad Española de Ciencias Hortícolas. Sociedad Española de Genética. Universidad Politéctica de Valencia. Valencia.

100. Muller HJ. 1927. Artificial transmutation of the gene. Science. 66: 84-87.

101. Muntean E, Lazăr V and Muntean N. 2006. HPLC-PDA analysis of carotenoids and chlorophylls from *Cucurbita pepo* convar.*giromontina* fruits. Bulletin of Agricultural Sciences and Veterinary Medicine. Agriculture. 62: 94-99.

102. Murphy EF, Hepler PR and True RH. 1966. An evaluation of the sensory qualities of inbred lines of squash (*Cucurbita maxima*). Am Soc Horti Sci Proc. 89: 483–490.

103. Murray I and Williams PC. 1987. Chemical principles of near infrared technology. In: Williams PC and Norris K (eds). Near Infrared Technology in the Agricultural and Food Industries AACC: St. Paul, MN, pp 17-34.

104. Naes T, Isaksson T, Fearn T, Davies A. 2002. A User-Friendly Guide to Multivariate Calibration and Classification. NIR Publications, Chichester, UK.

105. Naudin C. 1856. Nouvelles recherches sur les caracteres specifiques et les variete´s des plantes du genre Cucurbita. Ann Sci Nat Bot IV 6:5–73, pl. 1–3.

106. Nilsson MB., Dabakk E, Korsman T, Renberg I. 1996. Environ. Sci. Technol. 30: 2586-2590.

107. Nishino H. 1998. Cancer prevention by carotenoids. Mutation Research. 402: 159-163.

108. Obrero Á, González-Verdejo CI, Die JV, Gómez P, Del Río-Celestino M, Román B. 2013. Carotenogenic gene expression and carotenoid accumulation in three varieties of *Cucurbita pepo* during fruit development. Journal of Agriculture and Food Chemistry. 61: 6393-6403.

109. Osaba L, Aguirre A, Alonso A, Graf U. 1999. Genotoxicity testing of six insecticides in two crosses of the *Drosophila* wing spot test. Mutation Research. 439: 49-61.

110. Palozza P, Serini S, Torsello A, Boninsegna A, Covacci V, Maggiano N, Ranelletti FO, Wolf FI, Calviello G. 2002. Regulation of cell cycle progression and apoptosis by β-carotene in undifferentiated and differentiated HL-60 leukemia cells: Possible involvement of a redox mechanism. International Journal of Cancer, 97(5): 593-600.

111. Pardo JE, Alvarruiz A, Varón R, Gómez R. 2000. Quality evluation of melon cultivars. Correlation among physical-chemical and sensory parameters. Journal of food quality. 23: 161-170.

112. Park SW, Park Y and Joung H. 1995. Quality evaluation by color determination in Carrot (*Daucus carota* L.) Estimation of total carotenoid content by Colorimeter. J Kor Soc Hort Sci. 36: 481-485.

113. Paris HS. 1986. A proposed subspecific classification for *Cucurbita pepo*. Phytologia. 61: 133–138.

114. Paris HS. 1989. Historical records, origins, and development of the edible cultivar groups of *Cucurbita pepo* (*Cucurbitaceae*). Economic Botany. 43: 423–443.

115. Paris HS. 2000, History of the cultivar-groups of *Cucurbita pepo*. In: Horticultural Reviews, Janick J (ed). Wiley, New York. 25: 71–170.

116. Paris HS. 2001. History of the cultivar-groups of *Cucurbita pepo*. Horticultural Reviews. 25: 71–170.

117. Paris HS. 2008. Summer Squash. Invited chapter. Handbook of Plant Breeding. Vol I. Vegetables I. Springer New York (ISBN 978-0-387-72291-7). pp 317-349

118. Paris HS and Brown RN. 2005. The genes of pumpkin and squash. HortScience. 40: 1620–1630.

119. Paris HS, Yonash N, Portnoy V, Mozes-Daube N, Tzuri G and Katzir N. 2003. Assessment of genetic relationships in *Cucurbita pepo* (*Cucurbitaceae*) using AFLP, ISSR, and SSR markers. Theor Appl Genet. 106: 971–978.

120. Park S, Park CH, Hahm ER, Kim K, Kimler BF, Lee SJ, Kim WS, Jung CW, Park K, Riordan HD, Lee JH. 2005. Activation of Raf1 and the ERK pathway in response to l-ascorbic acid in acute myeloid leukemia cells. *C*ellular signalling, 17(1): 111-119.

121. Pérez-Marín D, Garrido-Varo A and Guerrero JE. 2007. Non-linear regression methods in NIRS quantitative analysis. *Talanta*, 72, 28-42.

122. Picó B, Díez MJ, Ferriol M, Fernández de Córdova P, Valcárcel, Nuez F. 2002. Status of the cucurbit collection at COMAV, Spain. In: Cucurbit Genetic Resources in Europe. Ad hoc meeting, January 2000, Adana, Turkey. IPGRI, Rome, Italy.

123. Piskunova T. 2002. Status of the cucurbit collections in Russia. In: Cucurbit Genetic Resources in Europe. Ad hoc meeting, January 2000, Adana, Turkey. IPGRI, Rome, Italy.

124. Ravi KB, Reddy KR, Shankaranarayanan J, Deshpande JV, Juturu V, Soni MG. 2014. Safety evaluation of zeaxanthin concentrate (OmniXan™): Acute, subchronic toxicity and mutagenicity studies. Food and Chemical Toxicology, 72: 30-39.

125. Ravikrishnan R, Rusia S, Ilamurugan G, Salunkhe U, Deshpande J, Shankaranarayanan J, Shankaranarayanan ML, Soni MG. 2011. Safety assessment of lutein and zeaxanthin (Lutemax™ 2020): Subchronic toxicity and mutagenicity studies. Food and Chemical Toxicology, 49(11): 2841-2848.

126. Reeves MJ. 1987. Re-evaluation of Capsicum color data. J Food Sci. 52: 1047-1049.

127. Robinson RW.1999. Rational methods for producing hybrid cucurbit seed.J of New seeds, 1: 1–47.

128. Rodríguez-Amaya DB, Kimura M, Godoy HT, Amaya-Farfan J. 2008. Update Brazilian database on food carotenoids: Factors affecting carotenoid composition. Journal of Food Composition and Analysis. 21: 445-463.

129. Ruiz Pérez-Cacho P., Blanco-Díaz M.T., Pérez-Vicente A., Fayos A., Font R., Galán-Soldevilla H. 2012. Effect of stored temperature and time on the sensory characteristics of minimally processed zucchini. 5th European Conference on Sensory and Consumer Research. Switzerland. September 2012.

130. Sacha T, Zawada M, Hartwich J, Lach Z, Polus A, Szostek M, Libura M, Bodzioch M, Dembinska-Kiec A, Skotnicki AB, Goralczyk R, Wertz K, Riss G, Moele C, Langmann T, Schmitz G. 2005. The effect of β-carotene and its derivatives on cytotoxicity, differentiation, proliferative potential and apoptosis on the three human acute leukemia cell lines: U-937, HL-60 and TF-1. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1740(2): 206-214.

131. Sánchez T, Ceballos H, Dufour D, Ortiz D, Morante N, Calle F, Zum Felde T, Domínguez M, Davrieux F. 2014. Prediction of carotenoids, cyanide and dry matter contents in fresh cassava root using NIRS and Hunter color techniques. Food Chem. 151: 444‑451.

132. Sanjur OI, Piperno DR, Andres TC and Wessel-Beaver L. 2002. Phylogenetic relationships among domesticated and wild species of *Cucurbita* (*Cucurbitaceae*) inferred from a mitochondrial gene: implications for crop plant evolution and areas of origin. Proc Natl Acad Sci USA. 99: 535–540.

133. Scalch W. 1992. Carotenoids in the retina: a review of their possible role in preventing or limiting damage caused by light and oxygen. EXS (Supplement to Experientia). 62: 280-298.

134. Schuch W, Kanczler J, Robertson D, Hobson G, Tucker G, Grierson D, Bright S, Bird C. 1991. Fruit quality characteristics of transgenic tomato fruit with altered polygalacturonase activity. HortScience 26: 1517-1520.

135. Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, Yannuzzi LA, Willett WC. 1994. Dietary carotenoids, vitamins A, C and E and advanced macular degeneration. J Am Med Assoc. 272: 18: 1413-1420.

136. Segasothy M. and Phillips PA. 1999. Vegetarian diet: panacea for modern lifestyle diseases?. Quarterly Journal of Medicine. 92: 531-544.

137. Seroczyńska A, Korzeniewska A, Sztangret-Wiśniewska J, Niemirowicz-Szczytt K, Gajewski M. 2006. Relationship between carotenoids content and flower or fruit flesh colour of winter squash (Cucurbita maxima Duch.). Folia Hortic. 18(2): 51-61.

138. Shenk JS and Westerhaus MO. 1995a. Analysis of Agriculture and Food Products by Near Infrared Reflectance Spectroscopy. Monograph, NIRSystems, Inc., 12101 Tech Road, Silver Spring. MD 20904. USA.

139. Shenk JS and Westerhaus MO. 1995b. Routine Operation, Calibration, Development and Network System Management Manual. NIRSystems, Inc., 12101 Tech Road, Silver Spring. MD 20904. USA.

140. Shenk JS and Westerhaus MO. 1996. Calibration the ISI way. In: Near Infrared Journal of the American Medical Association, Murray and Cowe (eds). NIR Publications, Chichester, UK, pp198-202.

141. Shenk JS, Workman J and Westerhaus M. 2001. Application of NIR spectroscopy to agricultural products. In Handbook of Near Infrared Analysis. 2nd Edition. Burns, D.A., Ciurczac, E.W. (Eds.). Marcel Dekker, Nueva York. (USA), pp 419-474.

142. Shokrzadeh M, Azadbakht M, Ahangar N, Hashemi A, Saeedi Saravi SS. 2010. Cytotoxicity of hydro-alcoholic extracts of *Cucurbita pepo* and *Solanum nigrum* on HepG2 and CT26 cancer cell lines. Phcog Mag. 6: 176-179.

143. Simonne AH, Kays SJ, Koehler PE, Eilenmiller RR. 1993. Assessment of β-carotene content in sweetpotato breeding lines in relation to dietary requirements. J Food Compos Anal. 6: 336-345.

144. Snodderly DM. 1995. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. American Journal of Clinical Nutrition. 62: 1448-1461.

145. Tadmor Y, Paris HS, Meir A, Schaffer AA, Lewinsohn E. 2005. Dual role of the pigmentation gene B in affecting carotenoid and vitamin E content in squash (*Cucurbita pepo*) mesocarp. J Agric Food Chem. 53: 9759–9763.

146. Thurnham DI and Howard AN. 2013. Studies on meso-zeaxanthin for potential toxicity and mutagenicity. Food and Chemical Toxicology, 59: 455-463.

147. Van den Berg H, Faulks R, Fernando-Granado H. Hirschberg J, Olmedilla B, Sandmann G, Southon S, Stahl W. 2000. The potential for the improvement of carotenoid levels in foods and the likely systemic effects. Journal of the Science of Food and Agriculture. 80: 880-912.

148. Van Het Hof KH, West CE, Westrate JA, Hautvast JG. 2000. Dietary factors that affect the bioavailability of carotenoids. J Nutr. 130: 503-506.

149. Varma SD, Devamanoharan PS and Morris SM. 1995. Prevention of cataracts by nutritional and metabolic antioxidants. CRC Critical Reviews in Food Science and Nutrition. 35 (1-2): 111-129.

150. Vazquez De Aldana BR, García Criado B, García Ciudad A and Pérez Corona ME. 1995. Estimation of mineral content in natural grasslands by near infrared reflectance spectroscopy. Commun Soil Sci Plant. 26:1383-1396.

151. Vicente-Dólera N, Pinillos V, Moya M, Del Río-Celestino M, Pomares-Viciana T, Román B, Gómez P. 2014. An improved method to obtain novel mutants in *Cucurbita pepo* by pollen viability. Scientia Horticulturae. 169: 14–19.

152. Villatoro-Pulido M, Font R, De Haro-Bravo MI, Romero-Jimenez M, Anter J, De Haro Bailon A, Alonso-Moraga A, Del Rio-Celestino M. 2009. Modulation of genotoxicity and cytotoxicity by radish grown in metal-contaminated soils. Mutagenesis; 24: 51–57.

153. Villatoro-Pulido M, Moreno-Rojas R, Muñoz-Serrano A, Cardeñosa V, Amaro-López MA, Font F, Del Río-Celestino M. 2011. Characterization and prediction by near-infrared reflectance of mineral composition of rocket (*Eruca vesicaria* subsp. *sativa* and *Eruca vesicaria* subsp. *vesicaria*). J Sci Food Agric. 92(7): 1331-40.

154. Wang DC, Pan HY, Deng XM, Xiang H, Gao HY, Cai H, et al. 2007. Cucurbitane and hexanorcucurbitane glycosides from the fruits of *Cucurbita pepo* cv *dayangua*. J Asian Nat Pro Res, 9: 525–9.

155. Whang HJ, Park YK and Seog HM. 1999. Carotenoid pigment of Pumpkin cultivated in Korea. Korean J Food & Nutr. 12: 508-512.

156. Whitaker TW and Robinson RW. 1986. Squash breeding .In.Basset M.I.(ed) Breeding vegetable crops. Westport: Avi. pp 209-242.

157. Williams PC. 2001. Implementation of Near-Infrared technology. In Near-Infrared Technology in the Agricultural and Food Industries, 2nd edition, Williams PC, Norris K (eds). American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA, pp145-169.

158. Williams PC and Norris K. 1987. Near-Infrared Technology in the Agricultural and Food Industries. AACC, Inc., USA.

159. Williams PC and Norris K. 2001. Near-Infrared Technology in the Agricultural and Food Industries, 2nd edition. American Association of Cereal Chemists, Inc. St. Paul, Minnesota, USA.

160. Workman JJr and Shenk J. 2004. Undestanding and using the near-infrared spectrum as an analytical method. In: Near-Infrared Spectroscopy in Agriculture. Roberts CA, Workman J, Reeves III JB (eds). ASA, CSSA y SSSA, Inc., Madison, Wisconsin, USA, pp 3-10.

161. Xu CJ, Fraser PD, Wang WJ, Bramley P. 2006. Differences in the carotenoid content of ordinary citrus and lycopene-accumulating mutants. Journal of Agricultural and Food Chemistry. 54: 5474–5481.

162. Yamaguchi M, Hughes DL, Yabumoto K, Jennings WG. 1977. Quality of cantaloupes: variability and attributes. Sci Hort. 6: 59-70.

163. Younis YM, Ghirmay S and Al-Shihry SS. 2000. African *Cucurbita pepo* L. properties of seed and variability in fatty acid composition of seed oil. Phytochem. 54(1):71-5.

164. Zhang P and Omaye ST. 2001. Antioxidant and prooxidant roles for b-carotene, a-tocopherol and ascorbic acid in human lung cells. Toxicol In Vitro, 15: 13-24.

ANEXOS: Publicaciones en revistas

Annexes

ANEXO I: Capítulo II

Mineral composition and potential nutritional contribution of 34 genotypes from different summer squash morphotypes

Artículo publicado en:

European Food Research and Technology

DOI 10.1007/s00217-014-2308-7

Damián Martínez-Valdivieso[a], Pedro Gómez[a], Rafael Font[b], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[b]Department of Postharvest Technology and the Agrifood Industry, IFAPA Center La Mojonera Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

B

Eur Food Res Technol
DOI 10.1007/s00217-014-2308-7

ORIGINAL PAPER

Mineral composition and potential nutritional contribution of 34 genotypes from different summer squash morphotypes

Damián Martínez-Valdivieso · Pedro Gómez · Rafael Font · Mercedes Del Río-Celestino

Received: 18 February 2014 / Revised: 25 July 2014 / Accepted: 9 August 2014

Abstract Mineral concentrations were determined in fruit of 34 traditional and improved genotypes of *Cucurbita pepo*. Genotypes belong to two subspecies, the subsp. *pepo* (classified into zucchini, vegetable marrow and pumpkin morphotypes) and subsp. *ovifera* (with three varieties: texana, ozarkana and clypeata). Phosphorus, potassium, calcium, magnesium, iron, copper, manganese, zinc and sodium were analyzed, and two distinct patterns of mineral accumulation were found to be evident by cluster analysis. Genotypes in group 1 (zucchini and pumpkin) showed the highest concentrations of total minerals (24,338–62,136 mg kg^{-1} dry weight) as compared to the genotypes in group 2 (vegetable marrow, var. clypeata, var. texana and var. ozarkana). Some genotypes with significant concentrations for different minerals were identified, with the genotype Cu-2 (traditional zucchini) showing the highest concentrations for K, Ca, Mg, Fe, Mn, Zn and Na (4,615, 315, 300, 4.8, 3.03, 3.83 and 9.4 mg kg^{-1} dry weight, respectively). The zucchini morphotype was superior to other morphotypes studied in terms of contribution to the recommended dietary allowance of mineral content for both men and women. The mineral content of *C. pepo* fruit reported provides a valuable material for breeding programs to generate lines with a significant long-term beneficial impact on human health.

D. Martínez-Valdivieso · P. Gómez · M. D. Río-Celestino (✉)
Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain
e-mail: mercedes.rio.celestino@juntadeandalucia.es

R. Font
Postharvest Technology and the Agrifood Industry, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

Keywords *Cucurbita* · Minerals · Epicarp · Mesocarp · Potassium · Zucchini

Introduction

Cucurbitaceae is a large family including many economically important vegetable crops such as summer and winter squash and melon. *Cucurbita pepo* L., the species with the greatest monetary value of the genus, is consumed virtually worldwide and cultivated intensively in southeastern Spain, concentrating most of the production in Almeria, reaching more than 350,000 tonnes during the season 2012/2013 [1, 2].

Cucurbita pepo has long been cultivated not only for food but also for their medicinal properties, which have been attributed to both fruit and plant parts [3]. These medicinal properties are due to bioactive compounds such as β-carotene, phenolics, flavonoids, vitamins (including vitamin A, B2, C and E), amino acids, carbohydrates and minerals (especially potassium) [3–6].

In relation to minerals, these compounds are an integral part of human and plant nutrition, and support biological processes during different stages of growth and development [7]. Humans obtain all essential elements mostly from higher plants [8], which is interesting from a nutritional point of view because fruits and vegetables usually contribute to 35, 24 and 11 %, respectively, of the total K, Mg and P to the dietary intake of humans [9]. The proper vegetable consumption can improve the mineral and trace metal regulation and reduce cardiovascular diseases and certain cancer risks [10].

Mineral malnutrition can be addressed through dietary diversification, mineral supplementation, food fortification and/or increasing mineral concentrations in food products.

Published online: 24 August 2014

Biofortification, which aims to increase mineral concentrations in edible crops, either agronomically or genetically using both conventional breeding and modern biotechnology, has been adopted by plant scientists to address this problem and is considered to be the most promising and cost-effective approach to alleviate mineral malnutrition [8, 11].

Previous studies have determined a decrease in the mineral content (especially K, Ca, Mg, Fe, Cu and Na) of fruits and vegetables in recent decades [12]. Taking into account both the problem of malnutrition [8, 11] and the decline in the mineral content of fruits, efforts are necessary to improve the mineral content in vegetables widely distributed throughout the world such as summer squash.

The traditional landraces are an important genetic resource for plant breeders because of their considerable genotypic variations. Evaluation of the primitive cultivars and their germplasm is used to develop new cultivars with additional nutritional value. A wider genetic base of a species such as *C. pepo*, thus, assumes priority in plant breeding research, developing new varieties for increased traits such as productivity and nutritive value [3].

There is limited information available concerning the genetic variability of the mineral composition of *C. pepo* or the breeding potential for mineral content improvement. Breeding programs have gotten some achievements of interest, mainly aimed at improving plant architecture, to optimize flowering, diversify the type of fruit and improve resistance to some diseases. Progress in improving pumpkins and squash is summarized in two recent reviews [2, 13].

Our study was designed to determine (1) the mineral content in the epicarp and mesocarp fruit; (2) the relationships among mineral contents; and (3) the potential contribution to human nutrition. Minerals selected for analysis were phosphorus, potassium, calcium, magnesium, iron, copper, manganese, zinc and sodium.

Materials and methods

Plant material and greenhouse experiment

A total of thirty-four genotypes, currently kept in the Germplasm Bank at the 'IFAPA La Mojonera' were evaluated in this work (Table 1). They were representatives of traditional and improved cultivars and were classified as follows: thirteen traditional genotypes of *C. pepo*. ssp. *pepo* from Spain (four belonging to the 'zucchini' group, eight to the 'vegetable marrow' group, and one to the 'pumpkin' group) which are cultivated in small orchards used for self-consumption; three genotypes from Israel ('vegetable marrow'); two genotypes from the USA belonging to *C. pepo* ssp. ovifera (L.) D.S.Decker: var. texana (Scheele) and var. ozarkana D.S.Decker-Walters; a genotype from Spain belonging to ssp. ovifera (L.) D.S.Decker var. clypeata corresponding to the 'scallop' group; fifteen commercial hybrids belonging to the 'zucchini' group representatives of the main commercial varieties currently offered in the market.

Seeds of these genotypes were germinated on wet filter paper in Petri dishes at room temperature for 2–4 days in the dark, after which they were transplanted into rock-wool cubes (Grodan BV, 6040KD Roermond, NL) in a greenhouse. When plants had developed three to four leaves, they were transferred to 1-m-large rock-wool slabs at a density of two plants/slab. Plants were grown in a greenhouse in the IFAPA Center in La Mojonera, Almería, Spain (36°47′19″N, 02°42′11″W; 142 m a.s.l.), from March to June 2011 following standard local cultural practices for both plant nutrition and insect pest and disease control.

Fruits (6–10) of each genotype were harvested at an immature stage because they are marketed this way (14–20 cm). Then, they were processed preserving epicarp and mesocarp of each fruit separately, packaged in polypropylene plastic containers and stored at −80 °C.

Dry matter content

Freeze-drying was used for the determination of dry matter. Sample lyophilization was performed using freeze drier equipment (Telstar LyoQuest, Germany) at −55 °C under vacuum (133×10^{-3} mBar) for 96 h per sample. Then, the samples were ground and frozen at −80 °C for further analysis.

Analysis of mineral composition of *Cucurbita pepo* fruit

The mineral content of the *C. pepo* fruit was determined using the dry mineralization method. The lyophilized samples were weighed into porcelain crucibles and were mineralized in a muffle furnace (Carbolite CWF 1200, UK) by incineration at 460 °C for 15 h. The ash was bleached after cooling by adding hydrochloric acid, then drying it on thermostatic hotplates and finally maintaining it in a muffle furnace at 460 °C for 1 h. Subsequently, the elements contained in the ashes were dissolved in an aqueous solution of HCl and were filtered. Finally, they were carried to a determined volume of water MiliQ (Millipore Corporation, Bedford, USA). The determination of the different minerals was performed using flame atomic absorption spectrophotometry for Ca, Mg, Fe, Cu, Mn and Zn, except for Na and K, which were analyzed by flame atomic emission, and for P which was measured by UV–Vis spectrophotometry. Elemental analysis was performed with a Varian model 240 FS atomic absorption spectrophotometer equipped with an online pump system SIPS-20 and SP-3 auto-sampler, standard air-acetylene flame and single-element hollow cathode lamps and background correction with a deuterium lamp for Mn. Phosphorus was measured using

Table 1 List of 34 *Cucurbita* genotypes included in this study, botanical classification, morphotype, country of origin and source of germplasm

Accession	Subspecies	Morphotype	Country of origin (Estate/Region)	Source of germplasm
Cu-1	ssp. pepo	Vegetable marrow	Spain (Aragón)	COMAV
Cu-2	ssp. pepo	Zucchini	Spain (Aragón)	COMAV
Cu-3	ssp. pepo	Zucchini	Spain (Andalucía)	COMAV
Cu-4	ssp. pepo	Vegetable marrow	Spain (Andalucía)	COMAV
Cu-5	ssp. pepo	Vegetable marrow	Spain (Andalucía)	COMAV
Cu-6	ssp. pepo	Vegetable marrow	Spain (Asturias)	COMAV
Cu-7	ssp. pepo	Zucchini	Spain (Cataluña)	COMAV
Cu-8	ssp. pepo	Vegetable marrow	Spain (Cataluña)	COMAV
Cu-9	ssp. pepo	Vegetable marrow	Spain (Cataluña)	COMAV
Cu-10	ssp. pepo	Vegetable marrow	Spain (Cataluña)	COMAV
Cu-11	ssp. pepo	Pumpkin	Spain (Cataluña)	COMAV
Cu-12	ssp. pepo	Zucchini	Spain (Murcia)	COMAV
Cu-13	ssp. pepo	Vegetable marrow	Unknown (Unknown)	COMAV
Cu-14	ssp. ovifera	Scallop	Unknown (Unknown)	COMAV
Cu-15	ssp. pepo	Vegetable marrow	USA (Arizona)	Israel
Cu-16	ssp. pepo	Vegetable marrow	Israel (Unknown)	Israel
Cu-17	ssp. pepo	Vegetable marrow	Israel (Unknown)	Israel
Cu-18	ssp. ovifera	Var. Ozarkana	USA (Arkansas)	USDA
Cu-19	ssp. ovifera	Var. Texana	USA (Texas)	USDA
Cu-20	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-21	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-22	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-23	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-24	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-25	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-26	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-27	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-28	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-29	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-30	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-31	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-32	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-33	ssp. pepo	Zucchini	Spain	Commercial hybrid
Cu-34	ssp. pepo	Zucchini	Spain	Commercial hybrid

P phosphorus, *K* potassium, *Ca* calcium, *Mg* magnesium, *Fe* iron, *Cu* copper, *Mn* manganese, *Zn* zinc, *Na* sodium

a spectrophotometer (Helios Alpha UV–Vis spectrophotometer model, Thermo Electron Corporation). All analyses were expressed as mg per kg of dry weight (dw).

Statistical analysis

To assess the variability of individual mineral concentrations between genotypes, analysis of variance (ANOVA) was performed considering a randomized complete design with six to ten replications. LSD (least significant differences test) was used to compare means, and significance was accepted at $P = 0.05$ level.

The P, K, Ca, Mg, Fe, Cu, Mn, Cu, Zn and Na concentrations in mesocarp of the 34 genotypes were subject to cluster analysis on the basis of distances computed from quantitative variables using FASTCLUS procedure or k-means model.

Correlations between the mineral concentrations were assessed by the Pearson test. Statistical analysis was performed using SPPS.

Results and discussion

Dry matter in summer squash fruit

The dry matter content in mesocarp ranged from 3.8 to 12.5 % with the genotypes Cu-7 (zucchini) and Cu-18 (var.

ozarkana) showing the lowest and highest values, respectively (Table 3). Because ssp. *pepo* fruits are harvested immature (about 5–7 days after anthesis), dry matter is only 4–9.6 % [14–16], although in our study the highest contents were achieved by ssp. *ovifera*. Consumer preferences, linked to the different end users and processing approaches, influence the target for dry matter of the fruit. Thus, the dry matter content of varieties is a critical parameter in squash (*Cucurbita maxima* Duchesne) breeding programs, as consumers generally prefer varieties with high dry matter content [17]. Although fruit with a dry matter (DM) content of <20 % is watery and lacks flavor, fruit with over 28 % DM may be unacceptably dry except for processing purposes [18]. In our case, it should be possible to produce biofortified summer squash combining high levels of dry matter (<12 %) and high mineral content in fruit.

Mineral content in summer squash fruit

The distribution pattern of mineral concentrations in epicarp and mesocarp of 34 summer squash genotypes is shown in Fig. 1. Among the mineral contents, K concentration was positively skewed followed by Na, Cu, Zn and Ca in both fruit tissues (Fig. 1). The genotypes studied varied considerably in elemental composition as shown by the range and coefficient of variation (CV). High CV was observed for Cu and Na in epicarp, and for Zn, Cu, Fe, Na and Ca in mesocarp (>40 %) due to the different genotypes used in this work.

The mineral content of the fruit epicarp was higher than that found in the mesocarp for all the minerals, except Na (Fig. 1). From a nutritional point of view, it should take into account to avoid the removal of the epicarp during minimally processed fresh fruit considering the major portion of elements found in this fruit tissue.

Individual mineral contents determined in epicarp and mesocarp of the *C. pepo* accessions are shown in Tables 2 and 3, respectively. The analysis of variance (ANOVA) indicated significant ($P < 0.05$) genotypic variation among accessions for elemental concentrations. This variability is essential for breeding programs focused on the selection of the most adequate lines.

Previous studies have reported that mineral content is influenced by several factors: degree of maturation, soil type, climatic and storage conditions, geographic location and especially genotype [19]. In the present work, the most important factor was the genotype since all the other factors were constant; thus, the genotypes were grown under the same environmental conditions, analyzed at the same stage of maturation and stored under the same conditions.

Phosphorus is among the most abundant of mineral elements in the human body, and deficiencies in P nutrition have historically been seen in rickets and osteomalacia [20]. Significant differences were found in the P content between the genotypes, with Cu-20 (19,100 mg kg^{-1}) and Cu-34 (8,350 mg kg^{-1}) (both commercial zucchini) having the highest P mean contents in epicarp and mesocarp, respectively.

Potassium is an essential mineral that works to maintain the body's water and acid balance [21], and it plays a role in transmitting nerve impulses to muscles, in muscle contractions and in the maintenance of normal blood pressure [22]. This element was the major element found in *C. pepo* fruit. K content differed significantly among genotypes, with Cu-24 (49,795 mg kg^{-1}) (commercial zucchini) and Cu-2 (46,150 mg kg^{-1}) (traditional zucchini) genotypes having the highest mean content in epicarp and mesocarp, respectively (Tables 2, 3).

Calcium is an essential nutrient that plays a vital role in neuromuscular function, many enzyme-mediated processes, blood clotting, and providing rigidity to the skeleton by virtue of its phosphate salts [7]. Significant differences were found in Ca content between the genotypes, with Cu-9 (9,550 mg kg^{-1}) and Cu-5 (4,750 mg kg^{-1}) (traditional vegetable marrow) having the highest mean calcium content in epicarp and mesocarp, respectively.

Magnesium is a constituent of bone and teeth and is closely associated with calcium and phosphorus. It has many functions in muscles and soft tissues, such as a cofactor of many enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis, and maintenance of the electrical potential of nerve tissues and cell membranes [23]. Magnesium content varied among the genotypes (Table 2, 3), and Cu-33 (4,714 mg kg^{-1}) and Cu-31 (3,050 mg kg^{-1}) (commercial zucchini) showed the highest mean Mg content in epicarp and mesocarp, respectively.

Iron performs several functions in the body; it helps in the formation of blood, and it also helps in the transfer of oxygen and carbon dioxide from one tissue to another [24, 25]. Significant differences were found in Fe content among the genotypes, with Cu-33 (102 mg kg^{-1}) (commercial zucchini) in epicarp and Cu-23 (55 mg kg^{-1}) (commercial zucchini) in mesocarp having the highest mean Fe contents (Tables 2, 3).

Copper is an essential trace element for humans and is a vital component of several enzymes [26]. Copper was the analyzed mineral present in lower proportion in *C. pepo*. Cu content changed depending on the genotypes, Cu-24 (14.5 mg kg^{-1}) and Cu-29 (4.9 mg kg^{-1}) (commercial zucchini) genotypes, and had the highest mean Cu content in epicarp and mesocarp, respectively (Tables 2, 3).

Manganese plays an important role in all mental functions and aids in the transfer of oxygen from lungs to cells, and it is important as an activator for enzyme reactions concerned with carbohydrate, fat and protein metabolism [27]. Significant variation was found in the Mn content

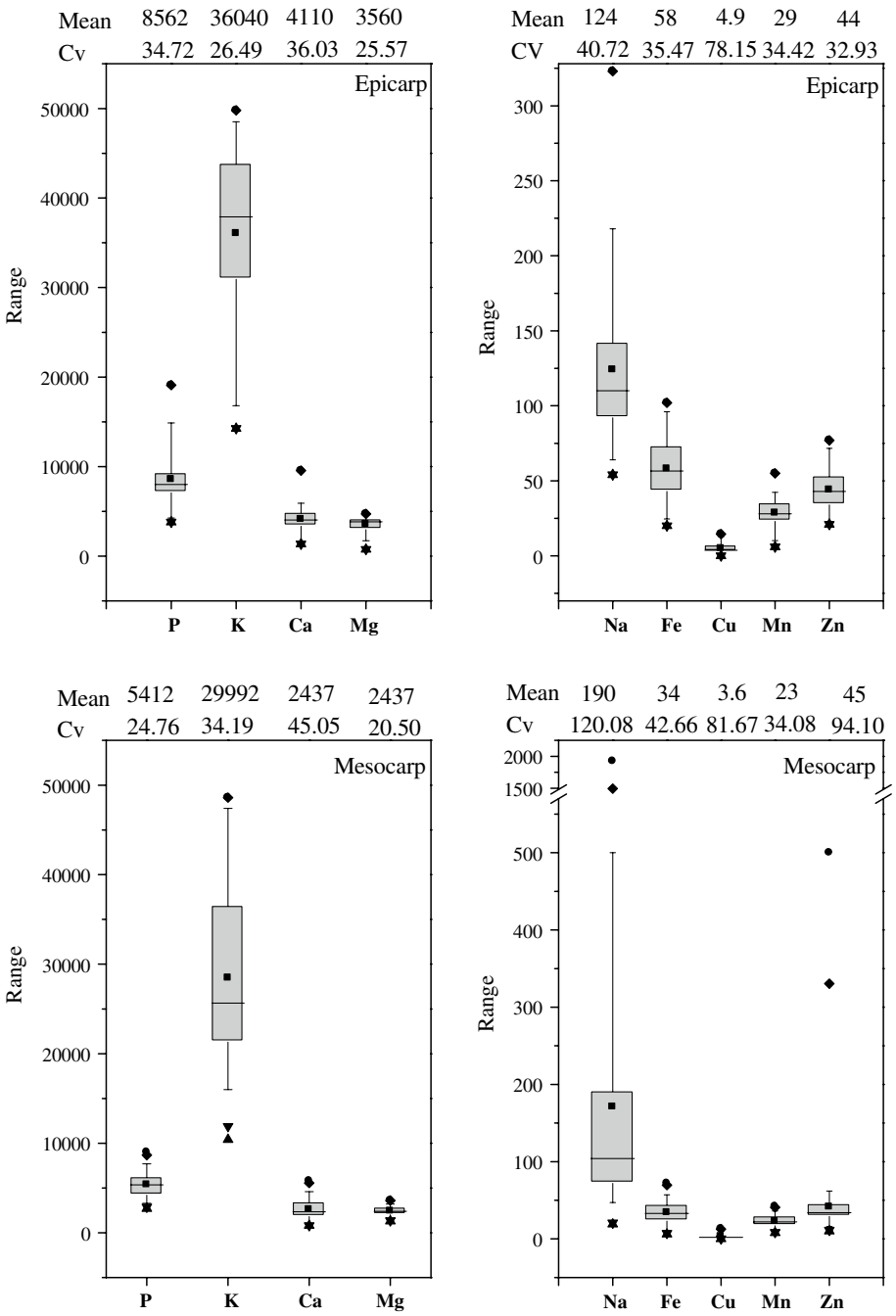


Fig. 1 *Box* and *whisker plots* for the mineral content of the 34 summer squash accessions. *Whiskers* denote minimum and maximum values, and *box* signifies 25th percentile, median, and 75th percentile with mean represented by a *filled square*. *Outliers* are represented by *asterisk*. Coefficient of variation (Cv) was included for indicating the variability among accessions

of genotypes, with Cu-20 (55 mg kg^{-1}) (commercial zucchini) and Cu-5 (38 mg kg^{-1}) (traditional vegetable marrow) genotypes having higher mean Mn content than all others in epicarp and mesocarp, respectively (Tables 2, 3).

Zinc plays a very important role in protein and carbohydrate metabolism and also helps in mobilizing vitamin A from its storage site in the liver and facilitates the synthesis of DNA and RNA necessary for cell production [24]. Zinc content varied among the genotypes, with Cu-25 (77 mg kg^{-1}) and Cu-30 (274 mg kg^{-1}), (commercial zucchini) having the highest mean Zn contents in epicarp and mesocarp, respectively (Tables 2, 3).

Sodium is required by the body to regulate blood pressure and blood volume. It helps regulate the fluid balance in the body; it also helps in the proper functioning of the muscles and nerves [27]. The Na content in epicarp varied among genotypes. On average, Cu-12 (323 mg kg^{-1}) (traditional zucchini) and Cu-30 (1,388 mg kg^{-1}) (commercial zucchini) had the highest Na mean content in epicarp and mesocarp, respectively (Tables 2, 3).

Table 2 Epicarp mean dry matter (expressed in %) and mineral content (expressed in mg kg^{-1}) of the 34 genotypes of summer squash

A	Dry matter	P	K	Ca	Mg	Fe	Cu	Mn	Zn	Na
Cu-1	6.9d–i	7,163g–i	27,771i–l	3,833b–g	4,027a–f	52h–k	0.8i	28e–j	38g–l	103bc
Cu-2	6.1e–i	9,252c–g	41,623a–f	4,245b–e	4,087a–e	73c–g	6.0e–h	33b–h	57c–e	111bc
Cu-3	6.7e–i	7,753d–i	32,473e–k	3,782b–g	3,856a–f	54g–k	2.7g–i	28f–j	51c–g	109bc
Cu-4	6.1e–j	7,763d–i	40,619a–g	4,022b–f	3,157f–i	49i–k	2.8g–i	28e–j	34h–o	154bc
Cu-5	4.8g–i	15,100b	43,800a–c	5,200b–d	3,625d–h	43j–n	2.8g–i	35b–g	28j–o	78c
Cu-6	7.0d–i	6,450g–k	24,850j–m	4,250b–e	3,900a–f	46j–m	1.0i	23i–k	37h–m	85c
Cu-7	5.7e–i	7,403f–i	33,261d–k	4,044b–f	3,867a–f	57g–k	2.8g–i	27g–j	47d–h	99bc
Cu-8	5.7e–i	8,061c–h	38,206b–h	3,755b–g	3,959a–f	57f–k	3.4g–i	28g–j	43e–i	122bc
Cu-9	5.1e–i	6,749g–j	31,785e–k	9,550a	4,091a–e	47i–l	0.8i	23h–k	43f–i	98bc
Cu-10	5.0f–i	7,068g–i	38,145b–h	4,694b–e	4,102a–e	60e–j	3.1g–i	23h–k	34h–o	140bc
Cu-11	5.1e–i	7,950d–h	34,250c–j	3,350d–h	3,550e–h	45j–m	3.5g–i	17k–n	41f–j	131bc
Cu-12	6.7e–i	8,993c–g	41,955a–e	5,925b	4,523a–c	66d–i	3.3g–i	35b–g	47d–h	323a
Cu-13	3.8hi	7,578e–i	37,577b–i	3,179d–h	4,026a–f	38k–o	2.2hi	24h–k	40f–k	123bc
Cu-14	7.0d–h	6,794g–j	24,839j–m	4,171b–e	3,772b–g	43j–n	4.2f–i	35b–g	46d–h	61c
Cu-15	10.2b–d	5,432h–k	23,729k–n	2,517e–h	1,981lk	20o	0.0i	14k–n	21o	99bc
Cu-16	6.7e–i	7,192g–i	30,927g–k	3,509c–h	2,801h–k	40j–o	1.4hi	23h–k	26k–o	92c
Cu-17	8.3b–f	6,985g–i	28,455h–k	3,503c–h	2,956g–j	29l–o	0.2I	20j–l	24l–o	105bc
Cu-18	22.0a	4,100jk	16,450mn	1,700f–h	2,250j–l	27m–o	4.5f–i	11l–n	21no	174bc
Cu-19	19.4a	3,800k	14,250m	1,550g–h	1,650l	24no	1.0i	6n	24m–o	159bc
Cu-20	7.8c–g	19,100a	45,950ab	3,050d–h	2,950g–j	88a–c	4.4f–i	55a	59b–d	85c
Cu-21	3.7i	8,483c–g	43,322a–d	4,878b–e	3,787b–g	70c–h	4.6f–i	36b–g	45d–h	109bc
Cu-22	7.7c–g	8,473c–g	37,656b–i	4,694b–e	4,660ab	73c–g	6.9d–g	31c–i	62bc	89c
Cu-23	11.4b	10,484cd	44,267a–c	4,823b–e	2,947g–j	72c–h	9.3b–e	38b–e	44e–i	158bc
Cu-24	4.2hi	10,158c–f	49,795a	5,855bc	4,473a–d	98ab	14.5a	39b–d	64a–c	154bc
Cu-25	10.6bc	8,900c–g	34,350c–j	2,550e–h	3,800b–g	71c–h	13.0ab	28f–j	77a	76c
Cu-26	8.4b–e	4,900i–k	18,200l–n	1,350h	750m	39k–o	4.0f–i	10mn	31i–o	54c
Cu-27	6.5e–i	7,934d–h	31,527f–k	3,346d–h	2,403i–l	81b–d	8.6b–f	31c–i	40f–j	142bc
Cu-28	5.9e–i	8,191c–h	47,093ab	4,825b–e	3,683c–h	60e–j	4.0f–i	29d–j	35h–n	159bc
Cu-29	6.8d–i	14,000b	44,350a–c	3,900b–g	4,550a–c	78c–e	11.5–d	33b–h	73ab	87c
Cu-30	6.3e–i	10,294c–e	47,603ab	5,079b–d	3,959a–f	84a–d	9.7b–e	40bc	53c–f	137bc
Cu-31	6.4e–i	8,582c–g	39,984a–g	3,670b–h	3,657c–h	56g–k	6.0e–h	28e–j	42f–j	93c
Cu-32	6.0e–i	8,952c–g	47,144ab	4,658b–e	4,384a–e	53h–k	3.5g–i	30c–j	44e–i	141bc
Cu-33	6.5e–i	10,146c–f	40,404a–g	5,415b–d	4,714a	102a	11.7–c	38b–f	66a–c	229ab
Cu-34	5.8e–i	10,928c	48,751a	4,869b–e	4,158a–e	77c–f	8.3c–f	43b	59b–d	140bc
Mean	7.4	8,562	36,040	4,110	3,560	58	4.9	29	44	124

Means for dry matter and each individual and total minerals were compared among accessions using Tukey's multiple comparison test. Means followed by a common letter are not significantly different from each other at $P < 0.05$

P phosphorus, *K* potassium, *Ca* calcium, *Mg* magnesium, *Fe* iron, *Cu* copper, *Mn* manganese, *Zn* zinc, *Na* sodium

Table 3 Mesocarp mean dry matter (expressed in %) and mineral content (expressed in mg kg^{-1}) of the 34 genotypes of summer squash

Ac	Dry matter	P	K	Ca	Mg	Fe	Cu	Mn	Zn	Na
Cu-1	5.2c–i	6,167c–g	28,733d–k	2,233d–h	3,033a	42a–g	1.3g–j	30b–f	38b	94bc
Cu-2	6.7c	5,750e–k	46,150a	3,150b–f	3,000ab	48a–d	1.5g–j	32a–d	46b	116bc
Cu-3	5.8c–h	5,300e–m	32,650c–i	2,700c–g	2,450a–f	21h–l	1.0h–j	31a–e	32b	206bc
Cu-4	6.3c–f	4,650h–n	20,000j–l	3,350a–d	2,400a–f	28g–k	0.5ij	22g–l	27b	151bc
Cu-5	4.5f–i	5,400e–m	28,450d–k	4,750[a]	2,500a–e	29g–k	1.5g–j	38a	25b	50c
Cu-6	4.8e–i	4,100l–o	15,600l	2,850c–g	2,250a–g	23h–l	0.0j	19j–n	23b	157bc
Cu-7	3.8i	4,400j–o	22,550i–l	2,700c–g	2,200b–g	24h–l	1.5g–j	19i–n	35b	119bc
Cu-8	5.4c–i	3,150o	17,700lk	1,450gh	1,500g	14kl	2.0f–j	16l–o	25b	225bc
Cu-9	5.2c–i	4,300k–o	26,100f–l	3,100b–f	2,500a–e	15j–l	2.0f–j	23g–l	30b	104bc
Cu-10	5.4c–i	3,800no	21,150j–l	3,400a–d	2,100d–g	9l	1.5g–j	23g–l	20b	136bc
Cu-11	5.5c–i	4,900f–n	24,000h–l	1,750f–h	2,400a–f	22h–l	4.5d–g	17k–o	38b	162bc
Cu-12	5.0c–i	7,400a–d	30,600d–j	4,500ab	2,950a–c	48a–d	2.5e–j	34a–c	42b	139bc
Cu-13	5.1c–i	6,150c–g	22,500i–l	2,750c–g	2,500a–e	36b–h	1.5g–j	27c–i	39b	117bc
Cu-14	5.1c–i	4,200l–o	25,400f–l	1,200h	2,000e–g	32d–j	3.5e–i	19j–n	31b	99bc
Cu-15	5.8c–h	4,060l–o	26,289e–l	1,534gh	2,089d–g	16j–l	3.4e–i	12no	33b	124bc
Cu-16	6.0c–g	4,100l–o	16,000l	2,033d–h	1,667gf	17i–l	1.3g–j	10o	19b	193bc
Cu-17	5.4c–i	3,918m–o	26,088f–l	1,554gh	1,968e–g	20h–l	3.5e–i	13no	20b	101bc
Cu-18	12.5a	4,550i–o	23,800h–l	1,800f–h	2,700a–e	30f–k	3.0e–j	17k–o	33b	325bc
Cu-19	9.8b	4,450i–o	26,100f–l	1,750f–h	2,200b–g	18i–l	2.5e–j	12no	31b	250bc
Cu-20	6.4c–e	6,100c–h	18,050lk	1,450gh	2,050e–g	43a–g	2.5e–j	33a–d	37b	73c
Cu-21	3.8i	5,400e–m	44,500ab	3,250b–e	2,250a–g	51ab	8.0abc	26d–j	48b	117bc
Cu-22	5.3c–i	6,300c–f	24,750g–l	1,450gh	2,900a–d	43a–g	5.0c–f	23g–l	58b	60c
Cu-23	6.7cd	5,800e–j	35,500a–g	2,450d–h	3,000ab	55a	5.5b–e	26d–j	52b	62c
Cu-24	4.6e–i	5,933d–i	36,500a–f	3,367a–d	3,000ab	52ab	7.7a–d	27c–h	50b	399b
Cu-25	6.1c–g	6,400b–e	34,633b–h	3,033c–f	2,467a–f	55a	8.3ab	13m–o	62b	66c
Cu-26	4.8e–i	4,700g–n	26,400e–l	1,900e–h	2,414a–f	31e–k	4.0e–h	17k–o	33b	120bc
Cu-27	5.3c–i	6,147c–h	43,975a–c	2,101d–h	2,503a–e	50a–c	9.9a	20h–m	51b	256bc
Cu-28	4.7e–i	7,500a–c	38,050a–d	4,000a–c	2,400a–f	45a–g	1.5g–j	27c–h	36b	350bc
Cu-29	4.8e–i	6,700b–e	44,900ab	2,000d–h	2,500a–e	46a–f	10.5a	24e–k	67b	60c
Cu-30	4.1hi	5,550e–l	43,650a–c	2,100d–h	2,400a–f	42a–g	9.0a	29b–g	274a	1,388a
Cu-31	5.0c–i	4,400j–o	34,450b–h	3,050c–f	3,050a	29f–k	2.0f–j	24e–k	30b	89bc
Cu-32	4.6e–i	7,850ab	37,550a–e	3,250b–e	2,150c–g	34c–i	0.5ij	24e–k	33b	225bc
Cu-33	5.8c–h	6,144c–h	38,818a–d	2,290d–h	2,665a–e	48a–d	7.6a–d	23f–k	56b	194bc
Cu-34	4.4g–i	8,350a	38,150a–d	3,050c–f	2,700a–e	47a–e	3.0e–j	36ab	52b	134bc
Mean	5.6	5,412	29,992	2,437	2,437	34	3.6	23	45	190

Means for dry matter and each individual and total minerals were compared among accessions using Tukey's multiple comparison test. Means followed by a common letter are not significantly different from each other at $P < 0.05$

P phosphorus, *K* potassium, *Ca* calcium, *Mg* magnesium, *Fe* iron, *Cu* copper, *Mn* manganese, *Zn* zinc, *Na* sodium

There are few studies concerning the mineral and trace elements in summer squash fruits. Ekholm et al. [28] reported that the entire fruit of *C. pepo* for P, K, Ca, Mg, Fe, Cu, Mn and Zn contains 6,000, 46,600, 3,590, 3,190, 73, 9, 27 and 40 mg kg^{-1}, respectively. These values are similar for K, Mg and Cu, lower for P, Ca, Mn and Zn and higher for Fe than those found in our study (considering mesocarp values, thus could have been superior taking into account epicarp contents).

Contrasting the results obtained by Harichan and Verma [9], who reported higher Ca, Fe and Mn contents in mesocarp than those found in epicarp of fruit of *C. pepo*. The values found in our study for K, Cu, Mg and Mn contents are higher than those obtained by Harichan and Verma [9] in epicarp (38,364, <0.01, 662 and <0.01, mg kg^{-1}, respectively) and mesocarp (20,337, <0.01, 438 and 4, respectively), and were lower for Ca, Fe and Na contents in mesocarp (7,015, 92, 14,947 mg kg^{-1}, respectively).

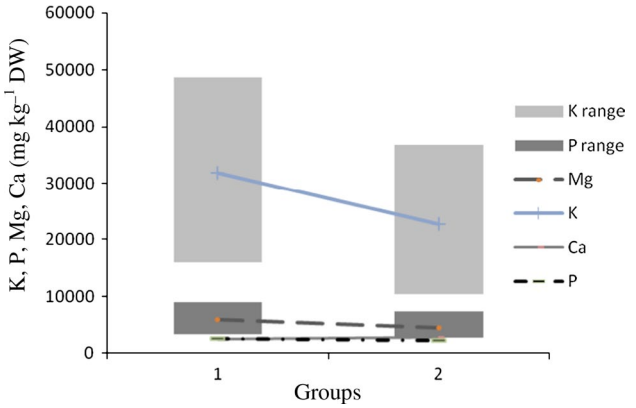


Fig. 2 Mean of mineral concentrations of the two groups obtained by cluster analysis of the 34 *C. pepo* accessions. *K* potassium, *P* phosphorus, *Mg* magnesium, *Ca* calcium. *Shaded bars* indicate range of K concentration in each group

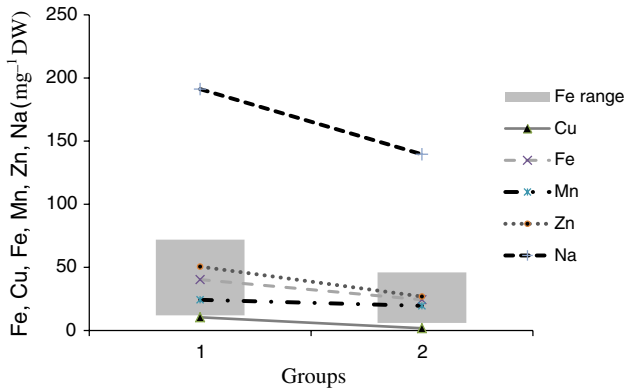


Fig. 3 Mean of mineral concentrations of the two groups obtained by cluster analysis of the 34 *C. pepo* accessions. *Fe* iron, *Cu* copper, *Mn* manganese, *Zn* zinc, *Na* sodium. *Shaded bars* indicate range of Fe concentration in each group

Our mean contents for P, K, Mg, Mn and Zn exceeded those described in fruit epicarp of *Cucurbita moschata* cv Cehualca (7,999, 22,550, 3,442, 7 and 31 mg kg^{-1}, respectively) and those found in mesocarp for P, Mg, Fe, Mn and Zn (3,040, 1,590, 32, 3 and 24 mg kg^{-1}, respectively). In addition, *C. moschata* had a higher mean Fe content in epicarp (64 mg kg^{-1}) and K in mesocarp (42,194 mg kg^{-1}) and in both epicarp and mesocarp for Ca (5,857 and 6,685 mg kg^{-1}, respectively), Cu (5.4 and 8.4 mg kg^{-1}, respectively) and Na (707 and 700 mg kg^{-1}, respectively) than *C. pepo* genotypes studied in this work [29].

Therefore, in the present study high mean levels of mineral contents such as P, Zn and Mn (8,350, 274 and 38 mg kg^{-1}) were found in mesocarp as compared to previous studies. The high mean levels were found by the fact that a range of summer squash was included in this investigation.

Cluster analysis

The results corresponding to the mineral concentrations in mesocarp of the 34 genotypes grouped within morphotypes are shown in Figs. 2 and 3. The accessions were clustered into two groups, and there was clear separation between accessions with high (zucchini and pumpkin) and low mineral contents (vegetable marrow, ozarkana, texana and scallop morphotypes). Genotypes in group 1 showed the highest mineral concentrations with mean values of 5,155, 29,685, 2,250, 2,495, 32.5, 4.7, 20.8 and 47.4 mg kg^{-1} for P, K, Mg, Fe, Cu, Mn and Zn, respectively (Figs. 2, 3).

This new understanding of patterns of diverse minerals accumulated in summer squash germplasm will be useful in setting objectives and selecting parents for breeding programs aimed at enhancing nutritional traits.

Correlations among mineral contents in summer squash

The relationships among minerals were analyzed by the Pearson correlation analysis (Table 4).

Significantly positive correlations were found among all individual minerals. The highest positive correlations ($r > 0.70$***) in fruit epicarp were observed between P–Mn, Fe–Zn, K–Mn, Fe–Cu and Fe–Mn; in the case of fruit mesocarp, the highest positive correlations ($r > 0.60$***) were observed between P–Fe, K–Fe, Mg–Fe and K–Cu. These results suggested that high Fe content might be accompanied by high P, K, Zn, Mg, Mn and Cu or vice versa. Therefore, selection of a mineral should respond favorably to the selection of other minerals.

Potential nutritional contribution of summer squash fruit

Table 5 shows the potential contribution of daily intake of 200 g of summer squash fresh fruits (mesocarp) toward the dietary requirements of minerals. The daily requirements of an adult person (men and women) are as follows (mg day^{-1}): 700 P, 3,100 K, 900–1,000 Ca, 300–350 Mg, 9–18 Fe, 1.1 Cu, 1.8–2.3 Mn, 7–9.5 Zn, 1,300–1,500 Na [30]. According to our data, the highest contribution was for potassium. Most of the genotypes belonging to group 1 (zucchini and pumpkin) contain up to 20–25 % of the RDA (recommended dietary allowance) for adults for potassium. Deficiencies of iron and zinc are, together with vitamin A and iodine deficiency, globally the leading causes of malnutrition [7, 10, 31]. A portion size (200 g fresh weight) of most of the genotypes will provide at least 5 % of the RDA for zinc and 2 % of the RDA of iron for adults. For phosphorus and magnesium, an average portion size of zucchini will provide up to 20 % of the RDA for adults. Genotypes of vegetable marrow contain up to 10 % of calcium. For copper and manganese, zucchini morphotype showed the

Table 4 Correlation coefficients between mineral element contents for epicarp and mesocarp in summer squash fruits

	P	K	Ca	Mg	Fe	Cu	Mn	Zn
K	0.66[a]***							
	0.59[b]***							
Ca	0.24*	0.48***						
	0.40***	0.34**						
Mg	0.31*	0.56***	0.56***					
	0.54***	0.49***	0.42***					
Fe	0.54***	0.63***	0.35**	0.53***				
	0.73***	0.64***	0.29*	0.64***				
Cu	0.37**	0.48***	0.07n.s.	0.24n.s.	0.71***			
	0.25*	0.60***	−0.05n.s.	0.19n.s.	0.56***			
Mn	0.79***	0.73***	0.44**	0.52***	0.71***	0.43***		
	0.59***	0.43***	0.50***	0.54***	0.46***	0.03n.s.		
Zn	0.47***	0.49***	0.22n.s.	0.59***	0.78***	0.67***	0.57***	
	0.15n.s.	0.32**	−0.07n.s.	0.10n.s.	0.26*	0.33**	0.09n.s.	
Na	0.00n.s.	0.19n.s.	0.21n.s.	0.14n.s.	0.25*	0.11n.s.	0.08n.s.	0.01n.s.
	0.04n.s.	0.24*	−0.01n.s.	−0.02n.s.	0.05n.s.	0.24*	0.10n.s.	0.31**

P phosphorus, *K* potassium, *Ca* calcium, *Mg* magnesium, *Fe* iron, *Cu* copper, *Mn* manganese, *Zn* zinc, *Na* sodium

n.s. No significantly different; * significantly different at $P < 0.05$, ** significantly different at $P < 0.01$, *** significantly different at $P < 0.001$

[a] Correlation coefficient for epicarp

[b] Correlation coefficient for mesocarp

Table 5 Daily nutrient requirements for both men and women an adult person (mg day^{-1}) and potential contribution (%) by 200 g fresh fruits of different morphotypes to nutrient requirements of this life stage

Element	Recommended (mg day^{-1})	Zucchini	marrow	Pumpkin	Scallop	var. ozarkana	var. texana
P	700[a]	8.0–20.6	6.2–16.7	7.5–13.0	8.0–11.2	10.3–10.5	9.1–11.2
K	3,100	8.3–25.1	5.4–19.0	11.0–21.2	13.0–13.3	12.2–12.3	13.5–13.5
Ca	900–1,000	1.4–8.2	2–10.3	3–9.1	2–2.3	3–3.4	3–3.2
Mg	300–350	10.1–19.2	6.9–17.1	9.1–14.4	10.7–10.7	13.3–15.5	10.7–12.8
Fe	9–18	1.2–6.6	0.6–4.6	1.4–3	2.8–3.5	2.5–3.4	1.6–2.0
Cu	1.1	0.1–10.4	0.1–3.2	1.6–4.8	1.6–4.0	1.6–3.2	1.6–2.4
Mn	1.8–2.3	2.8–7.5	1.6–6.9	1.4–3.0	3.0–3.6	2.8–3.0	2–2.1
Zn	7–9.5	4.3–13.5	1.8–8.5	2.3–6.9	5.3–5.7	5.5–6	5.3–5.7
Na	1,300–1,500	0–2.2	0–0.3	0.1–0.3	0.1–0.2	0.1–0.6	0.3–0.4

P phosphorus, *K* potassium, *Ca* calcium, *Mg* magnesium, *Fe* iron, *Cu* copper, *Mn* manganese, *Zn* zinc, *Na* sodium

[a] Cuervo et al. [30]

highest contents with up to 10 and 7.5 %, respectively, of the RDA for adults. In addition, the low concentration of sodium (<2 %) and the presence of a high amount of potassium recommend the utilization of *C. pepo* in an antihypertensive diet.

Conclusions

This research demonstrates that significant genetic diversity exists for mineral concentration in summer squash germplasm, with potassium as the highest mineral analyzed.

Two different patterns of mineral accumulation were found from the mineral content of fruit mesocarp, for a representative set of 34 summer squash genotypes. The highest total mineral concentrations were found for zucchini and pumpkin morphotypes (group 1) where P, K, Mg, Fe, Cu, Mn and Zn were the major elements found. In this sense, we have also identified genotypes that can be used as sources of variation for the improvement of these nutrients in the fruit like Cu-2, Cu-23, Cu-25, Cu-29, Cu-30, Cu-31 and Cu-34 genotypes belonging to zucchini morphotype, but also Cu-5 among those belonging to vegetable marrow morphotype. In addition, it is possible to develop high dry matter and high mineral content lines; nevertheless, their usefulness for commercial exploitation will depend on the adequate integration of the genes controlling the high dry matter trait (present in the Cu-18 genotype) into inbred lines with a high potential to develop agronomically acceptable hybrids. However, further investigation is required before mineral contents are taken into account in breeding programs. Thus, the study of mineral content in summer squash genotypes

grown in various environments would clarify the role of the genotype, environment and genotype by environment (G × E) interactions in mineral content.

To this date, few studies have been carried out using diverse germplasm of summer squash to assess the mineral content in fruit. Thus, the values for P, Mn and Zn are to our knowledge higher than any value previously reported for *C. pepo*.

The zucchini morphotype was superior to other morphotypes in terms of contribution to the recommended dietary allowance of P, K, Ca, Mg, Fe, Cu, Mn, Zn and Na for adults with up to 21, 25, 8, 19, 7, 10, 7, 13 and 2 %, respectively.

This new understanding of patterns of diverse minerals accumulated in summer squash germplasm will be useful in setting objectives and selecting parents for breeding programs aimed at enhancing nutritional traits.

Acknowledgments Financial support was provided by the Spanish Project INIA-RTA2009-00039-000, FEDER and FSE funds. Damián Martínez-Valdivieso has a contract from INIA-IFAPA (Subprograma FPI-INIA), cofinanced by FSE funds (Programa Operativo FSE de Andalucía 2007-2013_" Andalucía se mueve con Europa"). We would also like to thank Nicholas Davies for his help in grammatical revision of the manuscript.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

References

1. Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía (2013). http://www.faeca.es/index.php/archivo/documentacion/126-frutas-y-hortalizas/1632-avance-de-la-campana-horticola-de-almeria-2012-2013-fuente-capma.html
2. Paris HS (2008) Summer squash. In: Prohens-Tomás J, Nuez F, Carena MJ (eds) Handbook of plant breeding, vol I. Vegetables I. Springer, New York, pp 351–379
3. Aliu S, Rusinovci I, Fetahu S, Zogaj R (2012) Nutritive and mineral composition in a collection of *Cucurbita pepo* L. grown in Kosova. Food Nutr Sci 3:634–638
4. Obrero Á, González-Verdejo CI, Die JV, Gómez P, Del Río-Celestino M, Román B (2013) Carotenogenic gene expression and carotenoid accumulation in three varieties of *Cucurbita pepo* during fruit development. J Agric Food Chem 61: 6393–6403
5. Rouphael Y, Colla G (2005) Growth, yield, fruit quality and nutrient uptake of hydroponically cultivated zucchini squash as affected by irrigation systems and growing seasons. Sci Hortic 105:177–195
6. Tamer CE, İncedayi B, Parseker AS, Yonak S, Çopur ÖU (2010) Evaluation of several quality criteria of low calorie pumpkin dessert. Not Bot Horti Agrobot Cluj-Napoca 38:76–80
7. FAO/WHO (2001) Human vitamin and mineral requirements. Report of a joint FAO/WHO Expert Consultation, Bangkok, Thailand
8. White PJ, Broadley MR (2009) Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol 182:49–84
9. Levander OA (1990) Fruit and vegetable contribution to dietary mineral intake in human health and disease. HortScience 25:1486–1488
10. Harichan S, Verma VN (2013) Investigation of nutrient elements in *Cucurbita pepo* using atomic absorption spectrometry. Int Lett Chem Phys Astron 2:11–17
11. White PJ, Broadley MR (2005) Biofortifying crops with essential mineral elements. Trends Plant Sci 10:586–593
12. Mayer AM (1997) Historical changes in the mineral content of fruits and vegetables. Br Food J 99:207–211
13. Ferriol M, Picó B (2008) Pumpkin and winter squash. In: Prohens-Tomas J, Nuez F, Carena MJ (eds) Handbook of vegetable breeding. Springer, Berlin, pp 317–349
14. Aboul-Nasr MH, Damarany AM, Abdalla MMA (2002) Yield and its components of some summer squash (*Cucurbita pepo* L.) genotypes. In: Maynard DN (ed) *Cucurbitaceae* 2002. ASHS Press, Alexandria, pp 88–94
15. Audrey H, Beany AH, Stoffella PJ, Roe N, Picha DH (2002) Production, fruit quality, and nutritional value of spaghetti squash. In: Janick J, Whipkey A (eds) Trends in new crops and new uses. ASHS Press, Alexandria, pp 445–448
16. Kmiecik W, Lisiewska Z (1994) The influence of potassium sorbate addition on the quality of pickled zucchini fruit. Rocz Państw Zakł Hig 45:301–309
17. Murphy EF, Hepler PR, True RH (1966) An evaluation of the sensory qualities of inbred lines of squash (*Cucurbita maxima*). Am Soc Horti Sci Proc 89:483–490
18. Harvey WJ, Grant DG, Lammerink JP (1997) Physical and sensory changes during the development and storage of buttercup squash. N Z J Crop Horti Sci 25:341–351
19. Šimič D, Sudar R, Ledencan T, Jamnrovic A, Zdunic Z, Brkic I, Kovacevic V (2009) Genetic variation of bioavailable iron and zinc in grain of a maize population. J Cereal Sci 50:392–397
20. Portale AA (1999) Blood calcium, phosphorus, and magnesium. In: Favus MJ (ed) Primer on the metabolic bone diseases and disorders of mineral metabolism. Lippincott, Williams & Wilkins, Philadelphia, pp 115–118
21. Whitney E, Rolfes SR (1999) Water and the major minerals. In: Whitney E, Rolfes SR (eds) Understanding nutrition, 8th edn. West/Wadsworth, Belmont, pp 366–401
22. Lambert IH, Hoffmann EK, Pedersen SF (2008) Cell volume regulation: physiology and pathophysiology. Acta Physiol 194:255–282
23. Rude RK, Shils ME (2006) Magnesium. In: Shils ME, Shike M, Ross AC, Caballero B, Cousins RJ (eds) Modern nutrition in health and disease, 10th edn. Lippincott Williams & Wilkins, Baltimore, pp 223–247
24. Guthrie HA (1989) Introductory nutrition, 7th edn. Time Mirror Mosby College Publishers, Boston
25. McDonald A, Edwards RA, Greenhulgh FD, Morgan CA (1995) Animal nutrition. Prentices Hall, London, pp 101–122
26. Harris ED (1983) Copper in human and animal health. In: Rose J (ed) Trace elements in health. Butterworths, London, p 44
27. Payne WJA (1990) An introduction to animal husbandry in the trophics. Longman, Singapore, pp 92–110
28. Ekholm P, Reinivuo H, Mattila P, Pakkala H, Koponen J, Happonen A, Hellström J, Ovaskainen M (2007) Changes in the mineral and trace element contents of cereals, fruits and vegetables in Finland. J Food Compos Anal 20:487–495
29. Jacobo-Valenzuela N, Zazueta-Morales JJ, Gallegos-Infante JA, Aguilar-Gutiérrez F, Camacho-Hernández IL, Rocha-Guzmán NE, González-Laredo RF (2011) Chemical and physicochemical characterization of winter squash (*Cucurbita moschata* D.). Not Bot Horti Agrobot Cluj-Napoca 39:34–40

30. Cuervo M, Abete I, Baladia E, Corbalán M, Manera M, Basulto J et al (2010) Ingestas dietéticas de referencia (IDR) para la población española. Federación Española de Sociedades de Nutrición, Alimentación y Dietética (FESNAD), Ediciones Universidad de Navarra

31. Ezzatti M, Lopez AD, Rodgers Vander HS, Murray CJ (2002) Selected major risk factors and global regional burden of disease. Lancet 360:1347–1360

ANEXO II: Capítulo III

Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy

Artículo enviado y publicado en:

Journal of the Science of Food and Agriculture

DOI 10.1002/jsfa.6667

Damián Martínez-Valdivieso[a], Rafael Font[b], Pedro Gómez[a], Teresa Blanco-Díaz[b], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, IFAPA Center La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

[b]Department of Postharvest Technology and the Agrifood Industry, IFAPA Center La Mojonera Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain

D

Research Article

Received: 4 October 2013 Revised: 11 March 2014 Accepted article published: 20 March 2014 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.6667

Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy

Damián Martínez-Valdivieso,[a] Rafael Font,[b] Pedro Gómez,[a] Teresa Blanco-Díaz[b] and Mercedes Del Río-Celestino[a*]

Abstract

BACKGROUND: Efforts through conventional breeding to improve the mineral content in horticultural crops have not always been successful mainly due to the fact that standard analytical methods are both costly and time-consuming. We investigated the feasibility of applying near infrared reflectance spectroscopy (NIRS) to the estimation of essential mineral composition in the skin and flesh of summer squash fruits (*Cucurbita pepo* subsp. *pepo*) using a 200-sample set from diverse morphotypes.

RESULTS: The coefficients of determination in the external validation (R^2 VAL) obtained for the skin and flesh of the fruit were: total mineral content, 0.84 and 0.70; P, 0.74 and 0.62; K, 0.83 and 0.67; Ca, 0.57 and 0.60; Mg, 0.78 and 0.45; Fe, 0.78 and 0.65; Cu, 0.67 and 0.66; Mn, 0.67 and 0.64; Zn, 0.80 and 0.79 and Na, 0.33 and 0.33; respectively.

CONCLUSIONS: NIRS combined with different spectral transformations by modified partial least-squares (MPLS) regression has shown to be useful in determining the mineral composition of summer squash fruit, being a fast and low-cost analytical technique. Components such as chlorophyll, starch and lipids were used by MPLS for modelling the predicting equations. The promotion of micronutrient-rich summer squash varieties could have a significant long-term beneficial impact on the health of mineral deficient human populations.

Keywords: summer squash; biofortification; NIRS; potassium; iron; calcium

INTRODUCTION

Mineral elements perform a variety of functions in plant cells, and are essential for growth and development in plants as well as in animals and humans. Humans obtain all essential elements mostly from higher plants.[1] Unfortunately, it is estimated that over three billion people are currently malnourished because of lack of minerals, especially iron and zinc, in their diet.[1,2] Biofortification, which aims to increase mineral concentrations in edible crops, either agronomically or genetically using both conventional breeding and modern biotechnology, has been adopted by plant scientists to address this problem, and is considered to be the most promising and cost-effective approach to alleviate mineral malnutrition.[1,3]

Cucurbita pepo L. ($2n = 40$), the most economically important crop of the *Cucubita* genus,[4] displays eight commercial morphotypes grouped into two sub-species (subsp. *pepo* L.: pumpkin, vegetable marrow, cocozelle and zucchini; and subsp. *ovifera* (L.) Decker (syn subsp. *texana* (Scheele) Filov): scallop, acorn, crookneck and straightneck). The main economic value of the species resides in the consumption of its immature fruits as vegetables, commonly known as summer squashes.

Summer squash (*Cucurbita pepo* L.) is an important commercial crop for protected cultivation in the Mediterranean region, especially in Almería (Spain), where the production reaches more than 350 000 tonnes each year.[5] Fruits are normally grown under greenhouse conditions using a drip-irrigation system during the spring–summer and the summer–autumn seasons in order to respond to the high demand for this fresh product in both national and international markets.[6]

Previous studies reported that summer squash fruits are a mineral source for human nutrition[6,7] However, there is no knowledge of any study about the genetic variability of mineral composition of summer squash germplasm collections. Good characterisation of this germplasm is needed in order to be useful for breeders and farmers throughout the world.

Chemical determinations of different minerals in vegetables are commonly performed by current techniques, which are time-consuming, expensive and labour-intensive;[8] therefore, they are not adequate for selecting superior lines from a number of summer squash germplasm lines. Thus, a rapid and cost-effective method is in high demand to evaluate mineral quality for *Cucurbita* breeding programmes.

* *Correspondence to: Mercedes Del Río-Celestino, IFAPA-Centro La Mojonera, Plant Breeding and Biotechnology, Almería, Spain. E-mail: mercedes.rio.celestino@juntadeandalucia.es*

a *IFAPA-Centro La Mojonera, Plant Breeding and Biotechnology, Almería, Spain*

b *IFAPA-Centro La Mojonera, Postharvest Technology and Food Industry, Almería, Spain*

Near infrared spectroscopy (NIRS) is known as a powerful tool for analysis of chemical and physical properties, and it has been applied for determining nutritive value in food and agricultural commodities.[9,10]

Although minerals theoretically do not absorb energy in the near infrared spectrum, NIR reflectance spectroscopy has been successfully used for determining mineral concentration in plant tissues, due to the association between minerals and organic functional groups in the food matrix and the effect on O—H bonding. The estimation of mineral elements by NIR reflectance spectroscopy is therefore generally dependent on the presence of those elements in mixtures of organic or hydrated compounds and salts (cations and anions).[11] Both macro- and micro-minerals may influence the metabolism of NIR-absorbent components by their effects on plant physiology.[12]

To date, although NIR reflectance spectroscopy has been used to measure mineral content in legumes, forages, grapes, peanuts, woody materials, sediments, wine, cheese, and meat samples it has not been reported in major horticulture crops.[11,13–19]

Since Almería is an important exporter of summer squash, it is necessary to study the composition of the material, to ensure product quality, and to assess the potential use of secondary products such as the skin during the processing to produce puree, minimally processed, frozen and dried products. This study investigates the feasibility of using NIRS to estimate essential mineral composition in the flesh and skin of summer squash fruit, which would have potential application in quality control and in plant breeding programmes as a tool for rapid selection of plants.

Table 1. Mean, range, standard deviation (SD), coefficient of variation (CV) and standard error of laboratory (SEL) of total and individual minerals for flesh and skin of summer squash fruit

Mineral	Min	Max	Mean	SD	CV	SEL
Flesh						
Total	18 930	62 130	38 700	11 100	28.68	2 984
P	2 700	9 000	5 320	1 290	24.25	380
K	10 400	48 600	28 110	8 490	30.20	2 740
Ca	800	5 100	2 458	1 050	42.72	330
Mg	1 300	3 500	2 370	450	18.98	184
Fe	6	59	32	13.2	41.25	4.3
Cu	0.1	13	3.1	2.9	93.54	0.9
Mn	9	36	21.1	6.6	31.28	2.1
Zn	1	76	36.1	13.8	38.22	3.6
Na	23	200	108.76	46.29	42.56	17
Skin						
Total	20 550	73 820	45 240	15 870	35.08	3 590
P	3 700	10 700	7 040	2 110	29.97	442
K	13 300	49 500	30 850	10 970	35.56	2 375
Ca	100	5 900	3 165	1 297	40.97	513
Mg	100	4 700	3 150	1 160	36.82	235
Fe	14	95	58.5	19.8	33.85	5.4
Cu	0.1	13	4.4	3.2	72.72	1.3
Mn	5	42	24.1	10.3	42.74	3.2
Zn	17	81	41.8	16.2	38.76	3.6
Na	31.0	184.0	95.8	38.5	40.18	15

Data expressed as mg kg^{-1} (dry weight).

MATERIALS AND METHODS

Samples

Plants of summer squash from different morphotypes (vegetable marrow, zucchini and pumpkin) 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 from two consecutive seasons (spring–summer and summer–autumn) in 2011–2012 at the immature stage (commercial size). Skin ($n = 100$) and flesh ($n = 100$) samples (40 zucchini, 30 vegetable marrow and 30 pumpkin) were frozen in sealed polyethylene bags immediately after harvest, and subsequently were lyophilised and ground for analysis. In order to obtain a sufficient volume of meal for standard analytical methods and NIRS analysis, each sample was a combination of two fruits of the same plant.

Reference analysis

For mineral composition analysis of the summer squash the dry mineralisation method was used. Washed and freeze-dried samples were weighed into porcelain crucibles and then incinerated in a muffle furnace at 460°C for 15 h. The ash was bleached after cooling by adding hydrochloric acid, then drying it on thermostatic hotplates and finally maintaining it in a muffle furnace at 460°C for 1 h. After cooling, ash recovery was diluted in volumetric flasks with MilliQ water (Millipore Corporation, Bedford, MA, USA).

The determinations (Ca, Mg, Fe, Cu, Mn and Zn) were carried out by flame atomic absorption spectrophotometry, except for Na and K, which were analysed by flame atomic emission and for P which was measured by UV–visible spectrophotometry.

Elemental analyses were performed with a Varian model 240 FS atomic absorption spectrophotometer (Varian, Palo Alto, CA, USA) equipped with a pump system online SIPS-20 and SP-3 auto-sampler, standard air–acetylene flame and single-element hollow cathode lamps and background correction with deuterium lamp for Mn. Phosphorus was measured on a spectrophotometer (Helios Alpha UV–visible spectrophotometer model; Thermo Electron Corporation, Cambridge, UK).

Calculations of total mineral content were based on the sum of individual minerals. All analysis was done in duplicate and expressed as mg per kg of dry weight. The standard error of laboratory (SEL) was calculated based on duplicate analysis of each sample.[20]

NIRS analysis, calibration and validation development

Spectra of skin and flesh freeze-dried samples of summer squash were obtained in a near infrared spectrophotometer (NIRSystems mod. 6500; Foss-NIRSystems, Inc., Silver Spring, MD, USA) in the reflectance mode, acquiring their spectra over a wavelength range from 400 to 2500 nm (visible and near infrared regions). Samples were scanned in triplicate and the average spectrum was used to develop the multivariate models. Reflectance data was stored as $\log(1/R)$ (where R is the reflectance) at 2 nm intervals (1050 data points).

Samples recorded as an NIR file were checked for spectral outliers [spectra with a standardised distance from the mean (H) > 3 (Mahalanobis distance)], using principal component analysis. The objective of this procedure was to detect and, if necessary, remove possible samples whose spectra differed from the other spectra in the set.[21]

Calibration equations for total mineral, P, K, Ca, Mg, Fe, Cu, Mn, Zn and Na were developed using the program GLOBAL v. 1.50 (WINISI II; Infrasoft International, LLC, Port Matilda, PA, USA). Calibration

equations were computed using the raw optical data [log(1/*R*), or first or second derivatives of the log(1/*R*) data, with several combinations of derivative (gap) sizes and smoothing [i.e. (0, 0, 1, 1; derivative order, segment of the derivative, first smooth, second smooth); (1, 4, 4, 1); (1, 10, 10, 1); (2, 5, 5, 2); (2, 20, 20, 2)]. Wavelengths from 400 to 2500 nm every 8 nm, were used to perform the different calibration equations. The regression method employed to correlate spectral information and mineral content in the samples was modified partial least squares (MPLS). This regression method is a soft-modelling method[22,23] for constructing predictive models when the factors are many and highly collinear. The final objective of the mathematical procedure is to reduce the high number of spectral data points (absorbance values from 400 to 2500 nm every 2 nm, i.e. 1050 items of data) and to eliminate the correlation of absorbance values presented by neighbouring wavelengths.[24] Standard normal variate and detrend transformations (SNV-DT) were used to correct baseline offset due to scattering effects (differences in particle size among samples).[25] Cross-validation was performed on the calibration set for determining the best number of terms to use in the equation, as well as to determine the ability of each equation to predict an unknown.[26] For any trait analysed in the external validation, accessions were divided into two groups so that approximately two-thirds could be used for calibration and one-third for the external validation. Calibration and validation accessions were randomly selected, but they were adjusted so that their content standard deviations were similar to ensure that the range and distribution of the two groups would be comparable.

The coefficient of determination (R^2) and standard error (SE) were calculated for both cross-validation and external validation. For all of the parameters analysed, the mathematical pretreatment that yielded the minimum standard error of cross-validation (SECV) value was considered to be optimal. The predictive ability of the mathematical model was assessed from the coefficient of determination (R^2) and the dimensionless parameters RPD,[10] which is the ratio of the standard deviation for the validation samples to the standard error of prediction (SEP) and RER, which is defined as the ratio of the range in the reference data (validation set) to the SEP. The mathematical expressions of these statistics are as follows:

$$\mathrm{RPD} = \mathrm{SD}\left\langle\left[\left(\sum_{i=1}^{n}\left(y_i-\hat{y}_i\right)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1}$$

where y_i is the laboratory reference value for the i-th sample, $\hat{y}$ is the NIR measured value, N is the number of samples, K is the number of wavelengths used in an equation, and SD is the standard deviation.

$$\mathrm{RER} = \mathrm{range}\left\langle\left[\left(\sum_{i=1}^{n}\left(y_i-\hat{y}_i\right)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1}$$

The definitions of the variables are as given for RPD.

RESULTS AND DISCUSSION

Reference values

One of the main objectives in developing NIRS calibrations is to ensure that a suitable range of the trait of interest is sampled and the level of precision in the reference method is acceptable.

Table 1 shows the descriptive statistics, mean, range, standard deviation (SD) and coefficient of variation (CV) for the elements

Table 2. Pearson's correlation coefficient (*r*) among mineral contents for flesh and skin in summer squash fruits

	Mineral								
Mineral	P	K	Ca	Mg	Fe	Cu	Mn	Zn	Na
K	0.48[a]***	–	–	–	–	–	–	–	–
	0.73[b]***	–	–	–	–	–	–	–	–
Ca	0.15 NS	0.36***	–	–	–	–	–	–	–
	0.34*	0.54***	–	–	–	–	–	–	–
Mg	0.53***	0.38***	0.25*	–	–	–	–	–	–
	0.50***	0.72***	0.65***	–	–	–	–	–	–
Fe	0.69***	0.52***	0.10 NS	0.63***	–	–	–	–	–
	0.59***	0.69***	0.40**	0.63***	–	–	–	–	–
Cu	0.25**	0.59***	−0.04 NS	0.20 NS	0.54***	–	–	–	–
	0.47***	0.43***	0.03 NS	0.27 NS	0.69***	–	–	–	–
Mn	0.59***	0.35***	0.24*	0.54***	0.43***	0.00 NS	–	–	–
	0.77***	0.75***	0.52***	0.67***	0.70***	0.43***	–	–	–
Zn	0.18 NS	0.31***	−0.08 NS	0.13 NS	0.27*	0.36***	0.11 NS	–	–
	0.54***	0.59***	0.28*	0.68***	0.84***	0.70***	0.62***	–	–
Na	0.04 NS	0.27***	0.00 NS	0.00 NS	0.05 NS	0.31***	0.11 NS	0.32***	–
	0.13 NS	0.24 NS	0.26 NS	0.24 NS	0.29*	0.03 NS	0.16 NS	0.07 NS	–
Total	0.57***	0.99***	0.39***	0.44***	0.57***	0.57***	0.40***	0.29***	0.13*
	0.79***	0.98***	0.63***	0.78***	0.71***	0.42***	0.80***	0.62***	0.25 ***

[a] Correlation coefficient for flesh.
[b] Correlation coefficient for skin.
NS, no significant difference;
* significantly different at $P < 0.05$,
** significantly different at $P < 0.01$,
*** significantly different at $P < 0.001$.

measured in summer squash samples. The samples analysed varied considerably in elemental composition as shown by the range and CV. High CV was observed for Ca, Fe, Cu and Zn (>40%) possibly due to the different seasons and varieties used. The variability in elemental composition in calibration set was considered suitable for developing NIR calibrations for these elements.

Pearson's correlation coefficients among total and individual mineral contents are shown in Table 2 for the flesh and skin of the summer squash fruit. Significantly positive correlations were observed between them. Total mineral content was significantly positively correlated with all individual minerals for both, flesh and skin, with K ($r = 0.98$) exhibiting the highest correlation. The low and high correlations shown between the total mineral content and many of the individual minerals in the fruit, and also between several pairs of elements, could explain the different prediction accuracy of the NIRS models. Thus, the low correlation exhibited for Na with other minerals, in both flesh and skin, could clarify partially the reduced prediction accuracy of this NIRS model compared to the others.

Spectral features

Typical log(1/*R*) spectra for freeze-dried samples of summer squash, captured by the instrument (Foss-NIRSystems-6500), together with the most relevant absorption bands, are shown in Fig. 1a (skin) and Fig. 1b (flesh).

In the visible region, spectra displayed two peaks characteristic of fruit and vegetable produce – at 440 nm and 670 nm – which correspond to electronic transitions in the blue and red, respectively. Thus, the band at 670 nm has been assigned to chlorophyll,[27] which near 680 nm has a strong inverse correlation with sugar content.[28]

The NIR region of the spectrum (Fig. 1) showed characteristic absorption bands in both tissues (skin and flesh) tested here and especially in skin, these peaks were at 1200 nm and 1448 nm characteristic of sugar absorption;[29] at 1916 nm related to first overtones of water; 1724 and 2348 nm related to C—H stretch first overtones and combination bands of lipids;[30] at 2280 nm related to O—H + C—O deformation, O—H stretch plus deformation, and O—H + C—C stretch of starch.[29]

For NIR spectra, principal component analysis was performed on the second derivative (2, 5, 5, 2; SNV + DT) of the spectra as preliminary data examination in order to check the sample population (Fig. 2). For skin samples of summer squash, principal components 1 and 2 (PCs 1 and 2) explained 94.14% of the entire spectral variability in the data, and one H-outlier was found. For flesh samples of summer squash, PCs 1 and 2 explained 94% of the spectral variation.

Calibration and validation

The descriptive statistics of calibration and external validation are shown in Table 3. For the external validation analysis, samples were divided into two groups: 74 and 75 for calibration of skin and flesh samples, respectively, and 25 for external validation.

Statistics obtained in the cross-validation and external-validation processes for minerals are shown in Table 4. For all the minerals studied in this work, the second derivative transformation of the raw optical data, with an interval of 5 nm, and 5 nm and 2 nm for the first and second smooth, respectively, yielded

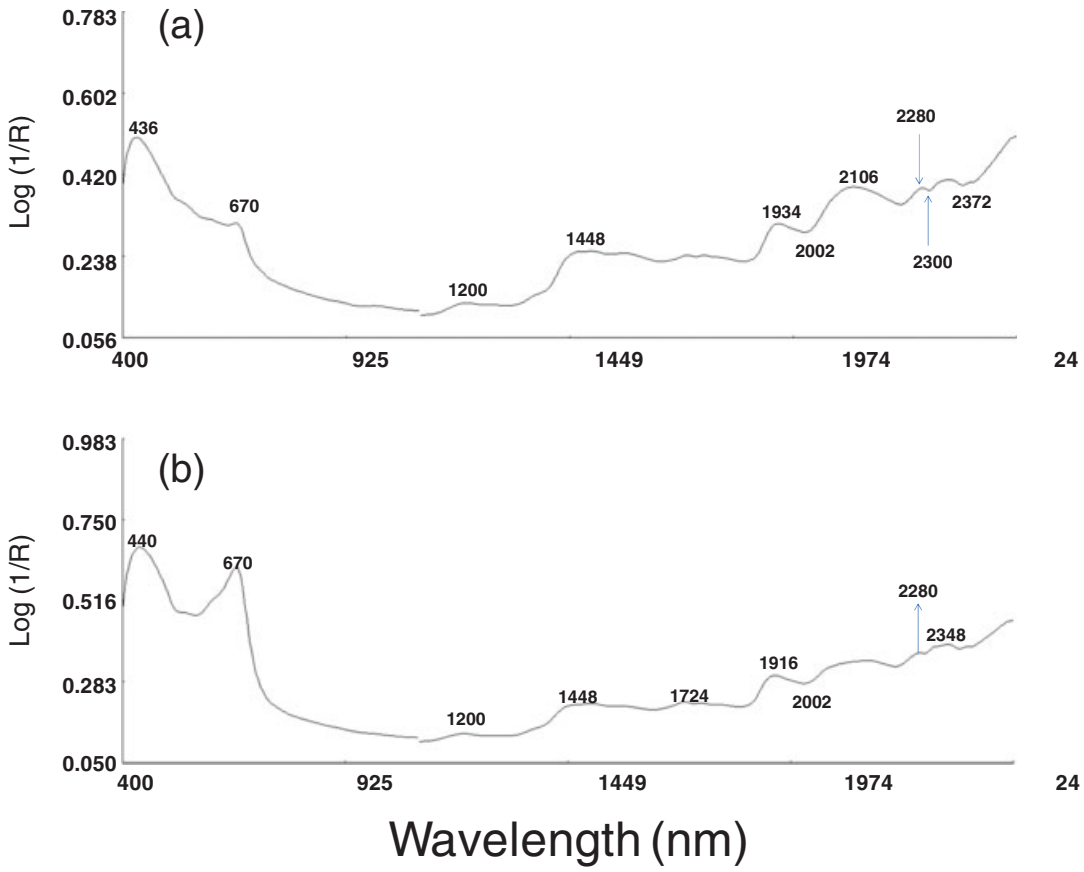


Figure 1. Typical log(1/*R*) spectra for flesh (a) and skin (b) of summer squash fruits.

Table 3. Descriptive statistics of calibration and validation sets in summer squash fruits

	Calibration set			Validation set		
Trait	Range	Mean	SD	Range	Mean	SD
Flesh						
Total	18 930–62 130	37 567	11 458	16 633–61 761	36 920	10 527
P	2 700–9 000	5 231	1 345	3 300–8 200	5 500	1 264
K	14 100–48 600	28 298	9 884	10 400–47 600	28 148	8 890
Ca	800–5 100	2 445	1 062	800–4 600	2 572	1 524
Mg	1 300–3 500	2 416	408	1 400–3 400	2 455	521
Fe	6–59	33	13	9–66	33	16
Cu	0.1–13	3.5	2.9	0.1–12	2.6	2.9
Mn	9–36	21	6.6	9–42	22	8.1
Zn	1–76	36	14	18–62	36	12.4
Na	23–200	103	44.4	24–200	112	49
Skin						
Total	20 550–73 820	45 944	16 258	23 250–72 144	41 685	16 405
P	3 700–10 700	7 040	2 108	3 900–10 497	7 010	1 995
K	13 300–49 500	30 852	10 974	15 400–48 900	30 310	11 475
Ca	100–5 900	3 308	1 370	150–5 800	2 934	1 516
Mg	100–4 700	3 151	1 157	110–4 624	2 980	1 173
Fe	14–95	51	19.7	15–90	48.2	18.3
Cu	0.1–13	4.5	3.5	0.1–12	4.1	3.12
Mn	5–42	24.1	10.3	6–41	25	9.81
Zn	17–81	42	16	18–79	41	15.1
Na	31–184	96	36.3	32–183	86	37

the equations with the highest accuracy in the external validation. Validation of the developed calibration models resulted in coefficients of determination, R^2, ranging from 0.33 for Na (flesh and skin) to 0.84 for total mineral (skin). On the basis of guidelines for interpretation of R^2 VAL,[31,32] the division of NIR calibration models was as follows: (1) $R^2 = 0.3$ to 0.49, poor correlation models, Mg (flesh) and Na (flesh and skin); (2) $R^2 = 0.50$ to 0.69, models usable for rough sample screening, Ca, Mn, Cu, (flesh and skin) and P, K, Fe (flesh); (3) $R^2 = 0.7$ to 0.9, models usable for sample screening, total mineral, Zn (flesh and skin) and P, K, Fe and Mg (skin).

The coefficients of determination R^2 obtained in this work (Table 4) for the different trace minerals agreed with those reported by other authors in grapes[11], forages and legumes.[13,33–35] These authors reported similar results for Fe ($R^2 = 0.74$), Zn ($R^2 = 0.72$), and K ($R^2 = 0.82$) and better results for Na ($R^2 = 0.83$), Cu ($R^2 = 0.82$), Mn ($R^2 = 0.74$) and Ca ($R^2 = 0.75$).

Figure 3 and Fig. 4 show the comparison of laboratory-predicted and NIRS-predicted content of validation set samples. In terms of RPD and RER coefficients, predictive ability of the equations in this work extended from 1.05 to 2.69, and from 4.31 to 11.03, respectively.

For Na (in the flesh and skin) and Mg (flesh), the validation yielded RPD (1.05–1.44) and RER (4.31–5.93) values, which limit the application of NIRS for predicting these elements. However, for the rest of minerals, the external validation yielded RPD (1.56–2.69) and RER (4.04–11.03) values, indicative of models that could be used for screening purposes, which can be very useful as a selection tool in summer squash breeding programmes.

Some examples of similar correlations between element concentration and apparent absorption have been reported in relation to the determination of total trace elements and macronutrients in other matrices. Some authors have also reported RPD data for mineral analysis in plants, lower, similar or higher than those shown in this work. Thus, comparable RPD values were reported by Ruano-Ramos *et al.*[37] (1.69–3.67) to evaluate NIRS calibrations for ash content in grassland samples. Other authors, demonstrated the utility of NIRS calibrations to enable very rough screening for ashes and calcium in intact seeds of common beans (RPD 2.03 and 2.4), whereas the RPD values for Mg were lower (RPD 1.33–1.5),[38] while Andrés *et al.*[39] reported an RPD value of 1.05 for ashes by NIRS in natural meadows. On the other hand, Cozzolino *et al.*[11] reported a SD to standard error of prediction ratio in validation from 1.5 for the prediction of Fe and Ca to 2.2 for Mg in grapes. Sauvage *et al.*[40] obtained RPD ratios that ranged from 2.70 to 2.85 for PLS calibrations of Na, K, Mg and Ca in white wines by using NIR transmission. Cozzolino and Morón[13] developed successful equations predicting macro-elements in legume crops, which presented RPD ratios of 1.61 to 2.38 for Zn and Mn, respectively. Similar ratios were reported for legume forage crops by Moron and Cozzolino,[41] who found values that ranged from 1.69 to 2.32 for P, Mg and Phan-Thien *et al.*[17] reported remarkable results estimating peanut essential minerals by NIRS with RPD ratios that ranged from 1.85 (Ca) to 2.25 (K). However, prediction errors related to micro-nutrients and trace metals are sometimes higher than those previously mentioned, depending on the element being predicted. Vázquez de Aldana *et al.*[33] found standard error of prediction in an external validation to SD ratios of 1.51 to 1.88 for manganese and zinc, respectively, in grasslands. Font *et al.*[35] reported RPD ratios that ranged from 1.34 (Zn) to 1.72 (Pb) in *Brassica juncea* plants grown in polluted soils. Much more diverse was the prediction data reported by Clark *et al.*[42] for various macro- and micronutrients in three forage species. The RPD ratio found by these authors showed values that ranged from 0.71 (Fe) to 2.08 (K) in alfalfa.

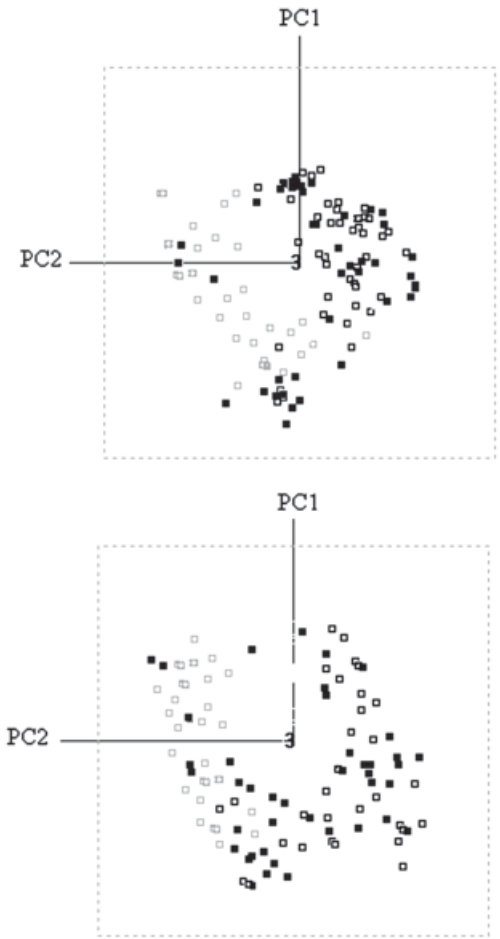


Figure 2. Score plots, obtained by applying principal component analysis to NIR spectra of flesh and skin, in the area defined by the first two principal components. Symbols indicate different samples used in this study. Solid, open and gray squares indicate samples from zucchini, vegetable marrow and pumpkin, respectively.

Table 4. Cross-validation and external validation statistics for summer squash fruit measured by near infrared spectroscopy

Mineral	NT[a]	R^2 CV[b]	SECV[c]	R^2 VAL[d]	SEP[e]	RPD[f]	RER[g]
Flesh							
Total	6	0.79	4923	0.70	5263	2.00	8.57
P	6	0.85	724	0.62	795	1.60	6.16
K	5	0.73	5238	0.67	5558	1.60	6.69
Ca	7	0.63	638	0.60	862	1.76	4.40
Mg	2	0.47	335	0.45	337	1.44	5.93
Fe	6	0.63	8	0.65	9.32	1.71	6.11
Cu	5	0.67	1.6	0.66	1.7	1.70	7.00
Mn	5	0.74	3.4	0.64	4.7	1.72	7.02
Zn	6	0.83	4.4	0.79	5.68	2.18	7.74
Na	2	0.38	36.3	0.33	39.8	1.18	4.42
Skin							
Total	7	0.84	6761	0.84	6913	2.37	7.07
P	7	0.85	860	0.74	989	2.02	6.67
K	7	0.85	4225	0.83	4642	2.47	7.22
Ca	6	0.62	1027	0.57	969	1.56	5.83
Mg	7	0.84	464	0.78	525	2.23	8.80
Fe	6	0.80	9.4	0.78	9.0	2.69	11.03
Cu	6	0.70	1.7	0.67	2.0	1.56	5.95
Mn	5	0.75	4.6	0.67	5.0	1.96	5.1
Zn	7	0.83	6.5	0.80	6.56	2.30	9.3
Na	1	0.38	33	0.33	395	1.05	4.31

[a] NT, number of terms of the equation selected in the cross-validation.
[b] R^2 CV, coefficient of determination in cross-validation.
[c] SECV, standard error of cross-validation.
[d] R^2 VAL, coefficient of determination in the external validation.
[e] SEP, standard error of performance.
[f] RPD, ratio SD to SEP.
[g] RER, ratio of the range to standard error of prediction (performance).

To evaluate the predictive ability of equations in relation to the overall error of the reference method, the SEL was calculated and related to SEP. The SEP/SEL ratio shown by total and individual mineral equations (1.54–2.64), classified them as having good precisions.[20]

MPLS loadings

To reduce the spectral information of the samples by creating a much smaller number of new orthogonal variables (factors) which are combinations of the original data, and which retain the essential information needed to predict the composition, MPLS regression was employed (Fig. 5). It has been stated that the success of estimation via NIRS of specific mineral elements in some grasses and legumes is usually dependent on the occurrence of those elements in either organic or hydrated molecules.[33,42]

Figure 5a and b shows loading plots corresponding to the best models obtained for predicting mineral contents in both flesh and skin. These plots show the areas across the spectral range where variance has influenced computing of the model to a greater or lesser degree, and the direction (positive or negative).

The areas of the spectrum exerting greatest weight on model fitting were between 500 and 704 nm in the visible region related to absorptions by plant pigments; the NIR region showed that the wavelengths at 1140, 1400, 1490 and 1900 nm related to the absorption of glucides and water and 1900–2400 nm related with C—H combinations and overtones had also a large influence on the different calibration models developed[11,43,44] (Fig. 5). A marked positive influence was noted at 1930 nm, an absorption area characteristic of cellulose.[29]

Other authors who used NIRS for predicting minerals in forages, grapes, rocket, wine and legumes reported similar absorption regions, although some differences at specific wavelength absorptions were found. Because trace elements are found in different complexes and the complexes appear to be different both within and among forages and legumes, this will lead to differences in selected wavelengths.[11,13,18,23] Thus, some minerals (e.g. Ca and P) may be indirectly detected through their linkage with diverse organic complexes such as chelates and pigments.[11,43,44] This is the case with P—OH in phosphate associated with chelates or organic matter, Ca with malate and Mg with chlorophyll. For the other elements the loadings were spread along the NIR region, being difficult to assign to any particular bond or chemical structure.[42–44]

CONCLUSIONS

From the data reported in this work it is concluded that NIRS technology can be used for screening purposes of total mineral, P, K, Ca, Fe, Cu, Mn, Zn (in the flesh and skin) and Mg (skin) contents in freeze-dried samples of summer squash. The use of this technique represents an important reduction of the analysis time, at a low

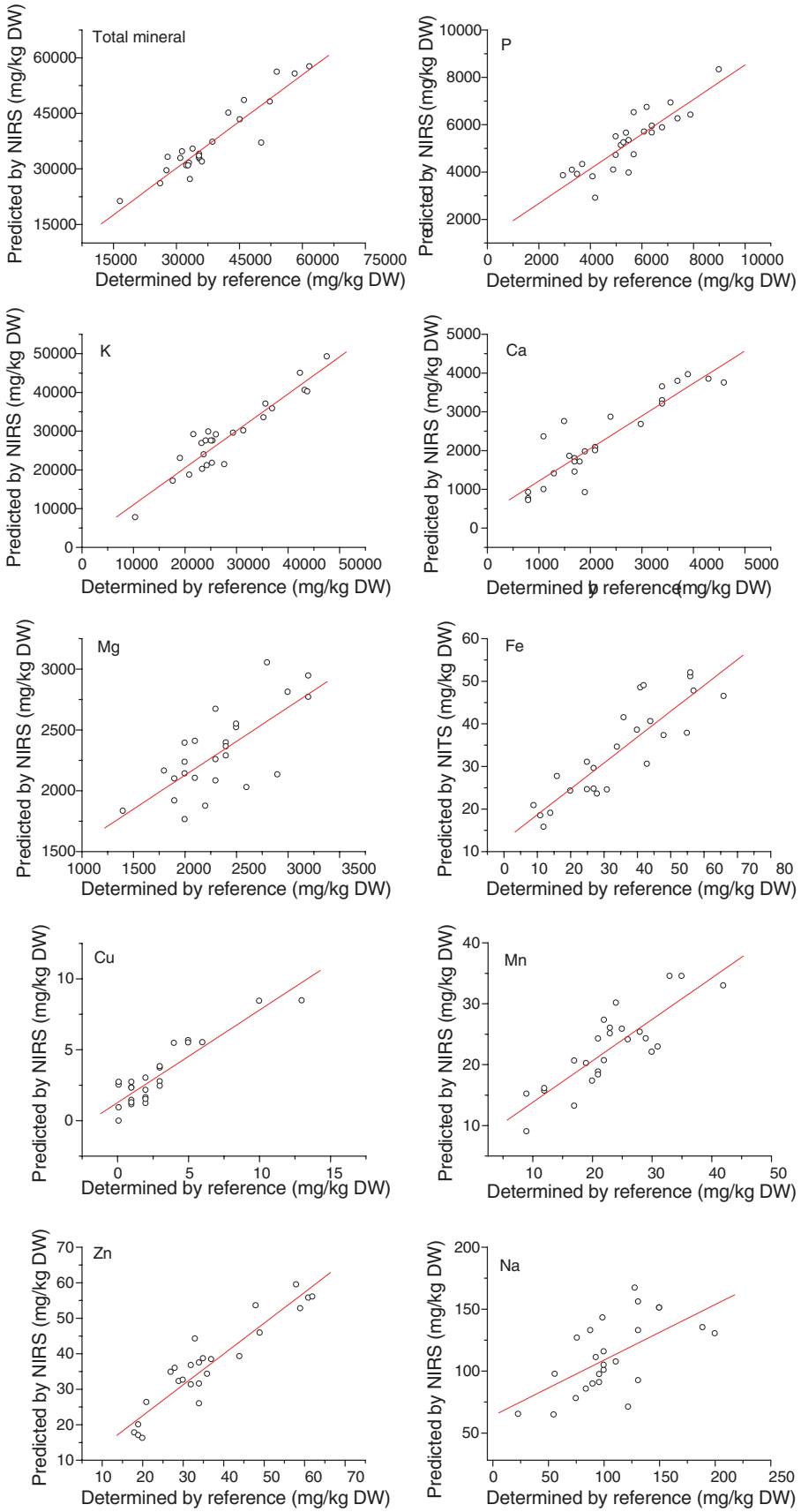


Figure 3. External validation scatter plot for near infrared predicted values versus reference values for total and individual mineral content in flesh of summer squash fruits.

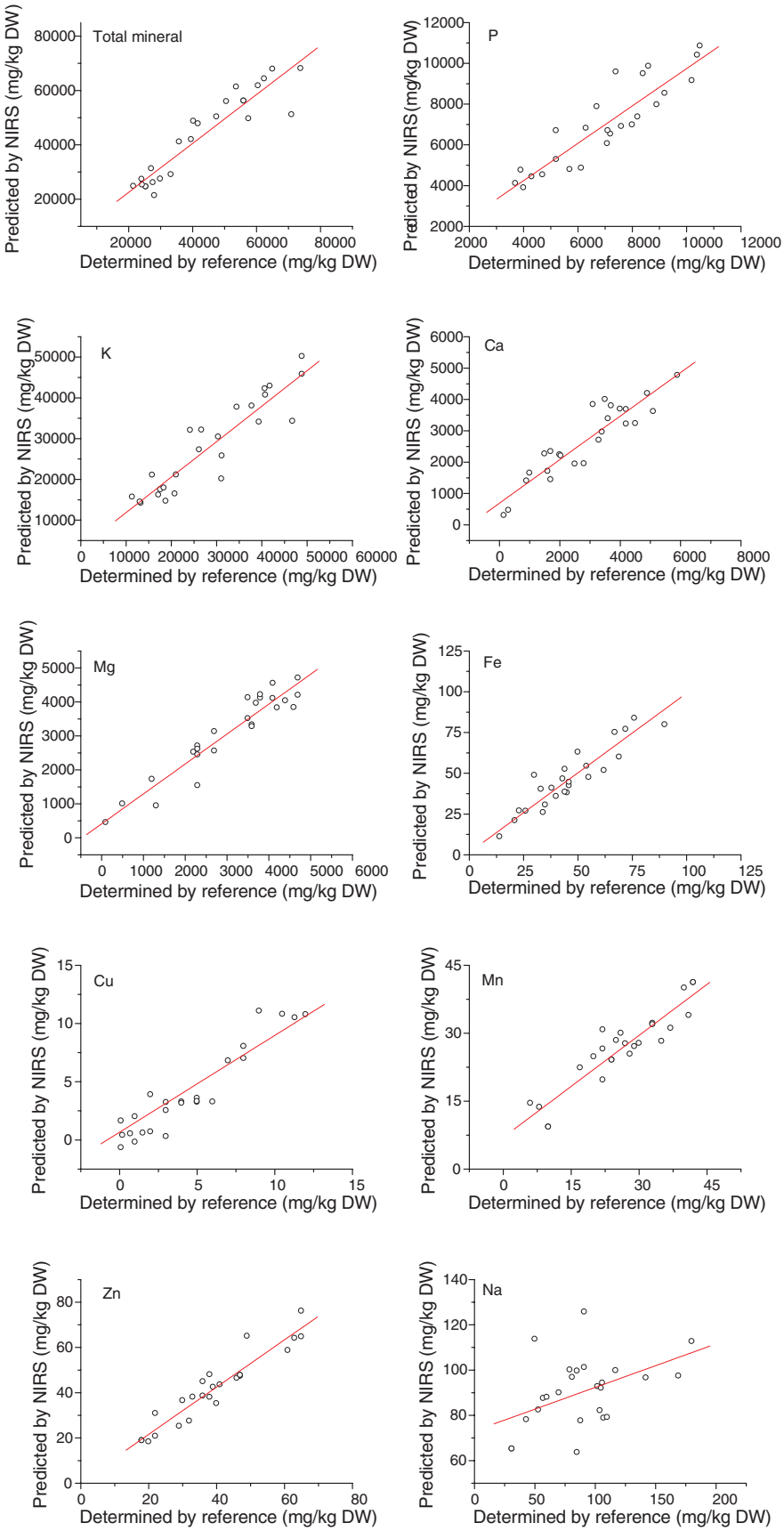


Figure 4. External validation scatter plot for near infrared predicted values versus reference values for total and individual mineral content in skin of summer squash fruits.

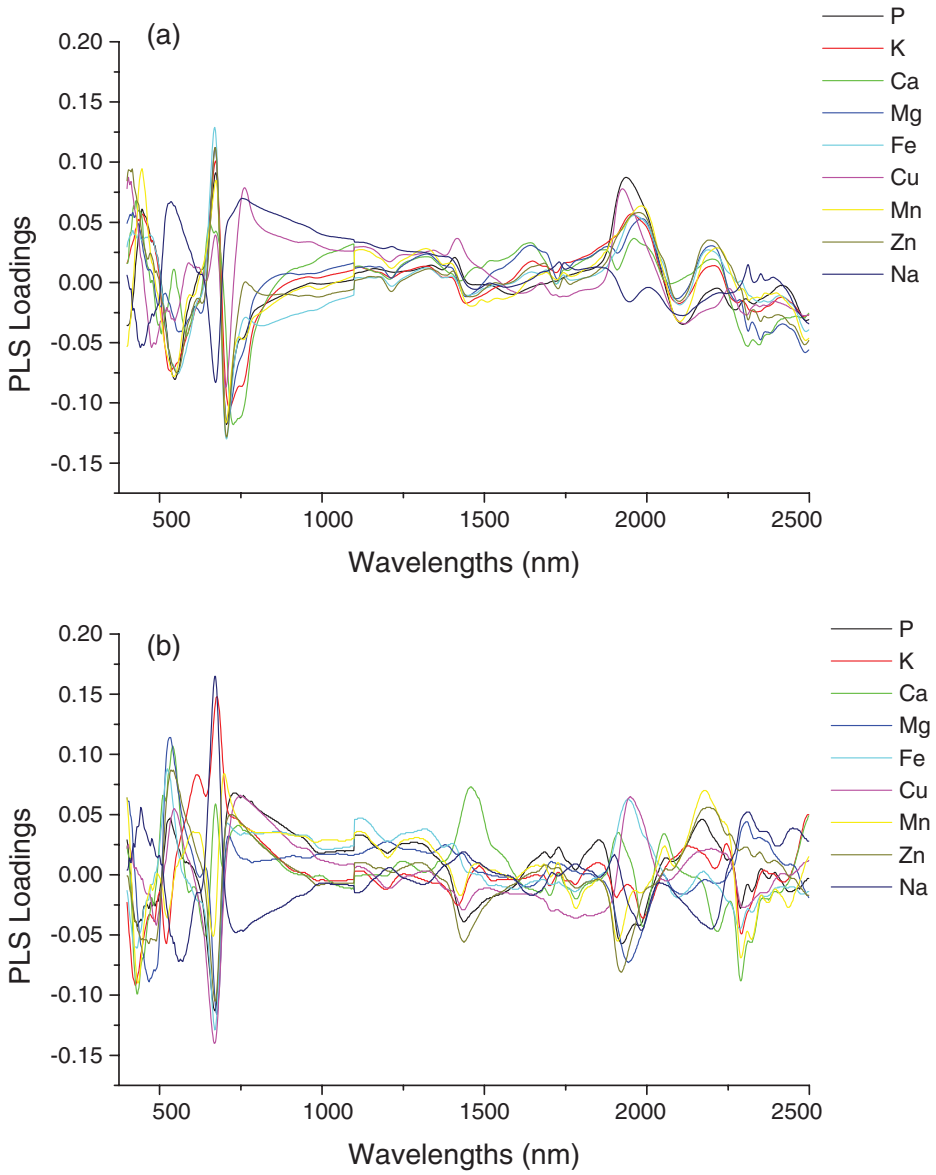


Figure 5. Modified partial least squares (MPLS) loadings for elements measured in flesh (a) and skin (b) of summer squash fruits using near infrared reflectance spectroscopy.

cost and without using hazardous chemicals, and will be used for quality control and in future research aiming to select the best genotypes after the screening of thousands of plants in a breeding programme of summer squash fruit.

Furthermore, deficiencies during the irrigation of the summer squash crop, which affect the quality parameters of the fruit (mineral composition), might be detected early in the greenhouse, which is interesting from a nutritional point of view because fruits and vegetables contribute highly to the dietary intake of humans.

ACKNOWLEDGEMENTS

Financial support was provided by the Spanish Project INIA-RTA00039-000, FEDER and FSE funds. Damián Martínez-Valdivieso has a contract from INIA-IFAPA (Subprograma FPI-INIA), co-financed by FSE funds (Programa Operativo FSE de Andalucia 2007-2013, 'Andalucia se mueve con Europa'). We thank Nicholas Davies for his help in grammatical revision of the manuscript.

REFERENCES

1 White PJ and Broadley MR, Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol* **182**:49–84 (2009).
2 Welch RM and Graham RD, Breeding for micronutrients in staple food crops from a human nutrition perspective. *J Exp Bot* **55**:353–364 (2004).
3 White PJ and Broadley MR, Biofortifying crops with essential mineral elements. *Trends Plant Sci* **10**:586–593 (2005).
4 Paris H, Summer squash, in *Handbook of Plant Breeding, Vegetables I*, Part 4, ed. by Prohens J and Nuez F. Springer, New York, USA, pp. 351–381 (2008).
5 Consejería de Agricultura, Pesca y Medio Ambiente de la Junta de Andalucía, [Online] http://www.faeca.es/index.php/archivo/documentacion/126-frutas-y-hortalizas/1632-avance-de-la-campana-horticola-de-almeria-2012-2013-fuente-capma.html (2013).
6 Rouphael Y and Colla G, Growth, yield, fruit quality and nutrient uptake of hydroponically cultivated zucchini squash as affected by irrigation systems and growing seasons. *Sci Hort* **105**:177–195 (2005).

7 Aliu S, Rusinovci I, Fetahu S and Zogaj R, Nutritive and mineral composition in a collection of *Cucurbita pepo* L. grown in Kosova. *J Food Nutr Sci* **3**:634–638 (2012).
8 Munter RC, Halverson TL and Anderson RD, Quality assurance for plant tissue analysis by ICP-AES. *Commun Soil Sci Plant Anal* **15**:1285–1322 (1984).
9 Batten GD, Plant analysis using near infrared reflectance spectroscopy: The potential and the limitation. *Aust J Exp Agric* **38**:697–706 (1998).
10 Williams P and Norris K, *Near-Infrared Technology in the Agricultural and Food Industries*. American Association of Cereal Chemists (AACC), St. Paul, MN (2001).
11 Cozzolino D, Cynkar W, Shah N and Smith P, Quantitative analysis of minerals and electric conductivity of red grape homogenates by near infrared reflectance spectroscopy. *Comput Electron Agric* **77**:81–85 (2011).
12 Font R, Río-Celestino M and Haro-Bailón A, Near-infrared reflectance spectroscopy, in *Phytoremediation*, ed. by Willey N. Humana Press, New Jersey, pp. 205–217 (2007).
13 Cozzolino D and Morón A, Exploring the use of near infrared reflectance spectroscopy (NIRS) to predict trace minerals in legumes. *Anim Feed Sci Technol* **111**:161–173 (2004).
14 González-Martín I, González-Pérez C, Hernández-Méndez J and Alvarez-García N, Mineral analysis (Fe, Zn, Ca, Na, K) of fresh Iberian pork loin by near infrared reflectance spectrometry: Determination of Fe, Na and K with a remote fibre-optic reflectance probe. *Anal Chim Acta* **468**:293–301 (2002).
15 Moros J, De Vallejuelo SF, Gredilla A, De Diego A, Madariaga JM, Garrigues S, *et al.*, Use of reflectance infrared spectroscopy for monitoring the metal content of the estuarine sediments of the Nerbioi-Ibaizabal River (Metropolitan Bilbao, Bay of Biscay, Basque Country). *Environ Sci Technol* **43**:9324–9320 (2009).
16 Petisco C, García-Criado B, De Aldana BRV, Zabalgogeazcoa I, Mediavilla S and García-Ciudad A, Use of near-infrared reflectance spectroscopy in predicting nitrogen, phosphorus and calcium contents in heterogeneous woody plant species. *Anal Bioanal Chem* **382**:458-465 (2005).
17 Phan-Thien KY, Golic M, Wright GC and Lee NA, Feasibility of estimating peanut essential minerals by near infrared reflectance spectroscopy. *Sens Instrum Food Qual Saf* **5**:43–49 (2011).
18 Villatoro-Pulido M, Moreno Rojas R, Muñoz-Serrano A, Cardeñosa V, Amaro López MA, Font R, *et al.*, Characterization and prediction by near-infrared reflectance of mineral composition of rocket (*Eruca vesicaria* subsp. *sativa* and *Eruca vesicaria* subsp. *vesicaria*). *J Sci Food Agric* **92**:1331–1340 (2012).
19 Wu D, He Y, Shi J and Feng S, Exploring near and mid-infrared spectroscopy to predict trace iron and zinc contents in powdered milk. *J Agric Food Chem* **57**:1697–1704 (2009).
20 Font R, Del Río-Celestino M and De Haro-Bailon A, The use of near-infrared spectroscopy (NIRS) in the study of seed quality components in plant breeding programs. *Ind Crop Prod* **24**:307–313 (2006).
21 Shenk JS and Westerhaus MO, Population structuring of near infrared spectra and modified partial least squares regression. *Crop Sci* **31**:1548–1555 (1991).
22 Naes T, Isaksson T, Fearn T and Davies TA, *User-friendly Guide to Multivariate Calibration and Classification*. NIR Publications, Chichester (2002).
23 Wold S, Sjöström M and Eriksson L, PLS-regression: A basic tool of chemometrics. *Chemom Intell Lab Syst* **58**:109–130 (2001).
24 Shenk JS and Westerhaus MO, The application of near infrared reflectance spectroscopy (NIRS) to forage analysis, in *Forage Quality, Evaluation and Utilization, USA: American Society of Agronomy*, ed. by Fahey GC Jr, Collins M, Mertens DR, Moser LE and Madison WI. Crop Science Society of America, with the Soil Science Society of America, Madison, WI, USA, pp. 406–450 (1994).
25 Barnes RJ, Dhanoa MS and Lister SJ, Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra. *Appl Spectrosc* **43**:772–777 (1989).
26 Shenk JS, Workman J and Westerhaus M, Application of NIR spectroscopy to agricultural products, in *Handbook of Near Infrared Analysis*, ed. by Burns DA and Ciurczac EW. Marcel Dekker, New York, pp. 419–474 (2001).
27 Tkachuk R and Kuzina FD, Chlorophyll analysis of whole rapeseed kernels by near infrared reflectance. *Can J Plant Sci* **62**:875–884 (1982).
28 Stchur P, Cleveland D, Zhou J and Michel RG, A review of recent applications of near infrared spectroscopy, and of the characteristics of a novel Pb SCCD array-based near-infrared spectrometer. *Appl Spectrosc* **37**:383–428 (2002).
29 Osborne BG, Fearn T and Hindle PH, *Practical NIR Spectroscopy with Applications in Food and Beverage Analysis*. Longman Scientific & Technical, Harlow (1993).
30 Murray I, The NIR spectra of homologous series of organic compounds, in *Proceedings of the International NIR/NIT Conference*, ed. by Hollo J, Kaffka KJ and Gonczy JL. Akademiai Kiado, Budapest, pp. 13–28 (1986).
31 Shenk JS and Westerhaus MO, Calibration the ISI way, in *Near Infrared Spectroscopy: The Future Waves*, ed. by Davies AMC and Williams PC. NIR Publications, Chichester, pp. 198–202 (1996).
32 Williams PC and Sobering DC, How do we do it: A brief summary of the methods we use in developing near infrared calibrations, in *Near Infrared Spectroscopy: The Future Waves*, ed. by Davies AMC and Williams PC. NIR Publications, Chichester, pp. 185–188 (1996).
33 Vazquez De Aldana BR, García Criado B, García Ciudad A and Pérez Corona ME, Estimation of mineral content in natural grasslands by near infrared reflectance spectroscopy. *Commun Soil Sci Plant* **26**:1383–1396 (1995).
34 Clark DH, Cary EE and Mayland HF, Analysis of trace elements in forages by near infrared reflectance spectroscopy. *Agron J* **81**:91–95 (1989).
35 Font R, Del Río M and De Haro A, Use of near infrared spectroscopy to evaluate heavy metal content in *Brassica juncea* cultivated on the polluted soils of the Guadiamar River area. *Fresenius Environ Bull* **11**:777–781 (2002).
36 Murray I, Forage analysis by near infrared reflectance spectroscopy, in *Sward Management Handbook,* ed. by Davies A, Baker RD, Grant SA and Laidlaw AS. British Grassland Society, Reading, USA, pp. 285–312 (1993).
37 Ruano-Ramos A, García-Ciudad A and García-Criado B, Determination of nitrogen and ash contents in total herbage and botanical components of grassland systems with near infrared spectroscopy. *J Sci Food Agric* **79**:137–143 (1999).
38 Plans M, Simó J, Casañas F and Sabaté J, Near-infrared spectroscopy analysis of seed coats of common beans (*Phaseolus vulgaris* L.): a potential tool for breeding and quality evaluation. *J Agric Food Chem* **60**:706–712 (2005).
39 Andrés S, Giráldez FJ, López S, Mantecón AR and Calleja A, Nutritive evaluation of herbage from permanent meadows by near infrared reflectance spectroscopy 1. Prediction of chemical composition and *in vitro* digestibility. *J Sci Food Agric* **85**:1564–1571 (2005).
40 Sauvage L, Frank D, Stearne J and Millikan MB, Trace metal studies of selected white wines: An alternative approach. *Anal Chim Acta* **458**:223–230 (2002).
41 Morón A and Cozzolino D, Determination of macro elements in alfalfa and white clover by near-infrared reflectance spectroscopy. *J Agric Sci* **139**:413–423 (2002).
42 Clark DH, Mayland HF and Lamb RC, Mineral analysis of forages with near infrared reflectance spectroscopy. *Agron J* **79**:485–490 (1987).
43 Miller CE, Chemical principles of near infrared technology, in *Near-Infrared Technology in the Agricultural and Food Industries*, ed. by Williams P and Norris K. American Association of Cereal Chemists (AACC), St. Paul, MN, pp. 19–37 (2001).
44 Workman J and Weyer L, *Practical Guide to Interpretive Near-Infrared Spectroscopy*. Taylor and Francis, London (2008).

ANEXO III: Capítulo IV

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

Artículo enviado y publicado en:

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

http://dx.doi.org/10.1016/j.compag.2014.07.003

Damián Martínez-Valdivieso[a], Rafael Font[b], María Teresa Blanco-Díaz[b], José Manuel Moreno-Rojas[c], Pedro Gómez[a], Ángeles Alonso-Moraga[d], Mercedes Del Río-Celestino[a]

[a]Department of Plant Breeding and Crop Biotechnology, Center IFAPA La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain
[b]Department of Postharvest Technology and the Agrifood Industry, Center IFAPA La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain
[c]Department of Postharvest technology and the Agrifood Industry, Center IFAPA Alameda del Obispo, 14080 Córdoba, Spain
[d]Department of Genetics, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

F

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

Damián Martínez-Valdivieso [a], Rafael Font [b], María Teresa Blanco-Díaz [b], José Manuel Moreno-Rojas [c], Pedro Gómez [a], Ángeles Alonso-Moraga [d], Mercedes Del Río-Celestino [a,*]

[a] Department of Plant Breeding and Crop Biotechnology, Center IFAPA La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain
[b] Department of Postharvest Technology and the Agrifood Industry, Center IFAPA La Mojonera, Camino San Nicolás, 1, 04745 La Mojonera, Almería, Spain
[c] Department of Postharvest technology and the Agrifood Industry, Center IFAPA Alameda del Obispo, 14080 Córdoba, Spain
[d] Department of Genetics, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

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.

* Corresponding author. Tel.: +34 950156453; fax: +34 950153444.
E-mail address: mercedes.rio.celestino@juntadeandalucia.es (M. Del Río-Celestino).

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.

Compounds were identified by comparison of retention times, co-injection with known standards, and comparison of their UV–visible spectra with authentic standards.

Quantification was carried out by external standardization. Full standard curves were constructed with five different concentrations for each carotenoid in triplicate. The curves passed through or were very near the origin, were linear and bracketed the concentrations expected in the samples.

Results were expressed on a dry weight (DW) basis.

2.4. Scanning samples for NIRS analysis

A NIR Systems Model 6500 spectrophotometer (Foss-NIRSystems, Inc., Silver Spring, MD, USA) equipped with a transport module was used to perform the NIRS analyses. Samples were analyzed as ground flesh and skin. Spectra were recorded in reflectance mode. In this mode, a ceramic standard is placed in the radiant beam, and the diffusely reflected energy is measured at each wavelength, before and after reading the sample. Spectra of the samples were recorded once from each sample, and were obtained as an average of 32 scans. The ceramic and the sample spectra were used to generate the final log (1/*R*) spectrum. Samples were placed for the analysis in a diameter round cell holder. This cell is composed of quartz glass and anodized aluminium to avoid absorption.

Principal component analysis (PCA) of the whole set of spectra (skin and flesh samples) was performed using raw optical data in order to stablish population boundaries. To do this the second derivative transformation of the original spectra was performed prior to PCA analysis to enhance spectral differences between types of samples (Naes et al., 2002).

2.5. Developing NIRS equations

Spectra corresponding to the skin and flesh samples separately, were sorted on the basis of the reference values for each component, from the lowest to the highest, and then divided into calibration and validation groups in a rate 2:1 (154 calibration and 76 validation).

In a first step, the full wavelength range (from 400 to 2500 nm, at 2 nm intervals) was used for calibration. In most cases, the visible region of the spectrum (400–700 nm) provided efficient contribution to calibration of fruit components, as color is often correlated with flesh and skin characters (i.e., chlorophyll and pigments, protein, moisture and carbohydrates) (Williams and Sobering, 1996).

Calibrations were performed by using the GLOBAL v. 1.50 software (WINISI II, Infrasoft International, LLC, Port Matilda, PA, USA). Calibration equations were computed by using the raw optical data (log 1/*R*), or first or second derivatives of the log 1/*R* data, with several combinations of segment (smoothing) and derivative (gap) sizes [i.e., (0, 0, 1, 1; derivative order, segment of the derivative, first smooth, second smooth); (1, 4, 4, 1); (1, 10, 10, 1); (2, 5, 5, 2); (2, 10, 10, 2)] (Shenk, 1992). The use of derivative spectra instead of the raw optical data to perform calibration is a way of solving problems associated with overlapping peaks and baseline correction (Hruschka, 1987). A first-order derivative of log (1/*R*) results in a curve containing peaks and valleys corresponding to the point of inflection on either side of the log (1/*R*) peak. While the second-order derivative calculation results in a spectral pattern display of absorption peaks pointing down rather than up, with an apparent band resolution (Shenk, 1992). The gap size and amount of smoothing used to make the transformation will also affect the number of apparent absorption peaks.

Among the different methods based on selected wavelengths or in full-spectrum which are available in commercial chemometric softwares, the full-spectrum methods have yield calibration equations that perform better with the types of seed material, and components that are the object of this study. Specially, modified partial least squares (PLSm) regression has been revealed as noteworthy for assessing seed components. PLS performs a linear regression in a new coordinate system with a lower dimensionality than the original space of independent variables. The PLS factors are determined by the maximum variance of independent (spectral data) variables and by a maximum correlation with the dependent (chemical) variable. The model actually uses only the primary, most important factors, the 'noise' being encapsulated in the less important factors. Regression is performed in the space spanned by the new reduced coordinate system of the orthogonal factors.

In addition to derivatives, standard normal variate and detrending (SNVD) algorithms (Barnes et al., 1989) were applied to the derived spectra to minimize baseline offset due to scattering effects caused by differences in particle size or path-length variation among samples.

In this study, cross-validation was computed based on the calibration set for determining the optimum number of terms to be used in building the calibration equations.

2.6. Validation of the equations

An external validation procedure was carried out to determine the accuracy and precision of the equations obtained in the calibration for each component in each species. To evaluate the accuracy of the equations, different statistics were used, namely, the coefficient of determination (r^2) (Williams and Norris, 1987); the RPD, which is the ratio of the standard deviation (SD) for the validation samples to the standard error of prediction (performance) (SEP), and the RER, which is the ratio of the range in the reference data (validation set) to the SEP (Williams and Sobering, 1996). As far as possible, we also calculated the ratio SEP to standard error of laboratory (SEL), as this statistic allows the error of NIRS to be put in perspective to the error in the reference method. The mathematical expressions of these statistics are as follow:

$$r^2 = \left(\sum_{i=1}^{n}(\hat{y}-\bar{y})^2\right)\left(\sum_{i=1}^{n}(y_i-\bar{y})^2\right)^{-1} \quad (1)$$

where $\hat{y}$ = NIR measured value; $\bar{y}$ = mean "*y*" value for all samples; y_i = lab reference value for the *i*th sample.

$$\mathrm{RPD} = SD\left\langle\left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1} \quad (2)$$

where y_i = lab reference value for the *i*th sample; $\hat{y}$ = NIR measured value; N = number of samples, K = number of wavelengths used in an equation; SD = standard deviation.

$$\mathrm{RER} = \mathrm{range}\left\langle\left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\right\rangle^{-1} \quad (3)$$

where y_i = lab reference value for the *i*th sample; $\hat{y}$ = NIR measured value; N = number of samples, K = number of wavelengths used in an equation ratio

$$\mathrm{SEP/SEL} = \left[\left(\sum_{i=1}^{n}(y_i-\hat{y}_i)^2\right)(N-K-1)^{-1}\right]^{1/2}\left\langle\left[\left(\sum_{i=1}^{n}(y_1-y_2)^2\right)(2N)^{-1}\right]^{1/2}\right\rangle^{-1} \quad (4)$$

where y_i = laboratory reference value for the *i*th sample; $\hat{y}$ = NIR measured value; y_1 and y_2 = laboratory reference values for 2 duplicates of the same sample; N = number of samples, K = number of wavelengths used in an equation.

2.7. Correlation plot of total carotenoid content versus wavelength in summer squash fruit

The correlations of the total carotenoid content versus wavelength for each sample were obtained by using the whole set of samples, to identify those spectral regions more highly correlated with the total carotenoid content in the tissues of summer squash flesh. Spectral data was standardized by using SNVqDT (Barnes et al., 1989) to interpret in a simpler way the correlation plot of spectral data versus total carotenoid content in the whole set of samples. This mathematical pre-treatment of the spectral data eliminates the background of constant correlation due to any existing relationship between total carotenoid content and particle size. In theory, areas matching absorption bands in the spectra of the constituent being measured should have positive correlations in the correlation plot, while areas corresponding to absorption bands in the spectra of other constituents could have positive, negative or zero correlations depending on the inter-correlations between constituents (Osborne et al., 1993).

2.8. MPLS loading plots

The MPLS loading plots of the first three factors generated from the MPLS regression performed on the second derivative transformation of the raw optical data (2, 5, 5, 2; SNV + DT) were calculated for total carotenoid content. MPLS regression constructs its factors capturing as much of the variation in the spectral data as possible by using the reference values actively during the decomposition of the spectral data. By balancing the spectral and chemical information the method reduces the impact of large but irrelevant spectral variations in the calibration modeling (Martens and Naes, 1992).

The loading plots show the regression coefficients of each wavelength to the parameter being calibrated for each factor of the equation. Wavelengths represented in the loading plots as more highly participative in the development of each factor are those of more variation and better correlated to the parameter in the calibration set. In the second derivative, peaks pointing downwards indicate positive influence of absorbers on the development of the equations, while peaks pointing upwards indicate negative correlations. In this work we use band assignments from literature, to relate some major absorption bands in the spectrum of summer squash fruit with the main wavelengths used by MPLS to construct the first three MPLS terms for the total carotenoid equation.

3. Results and discussion

3.1. Reference values for summer squash carotenoid contents

The summer squash varieties used in this work were chosen on the basis of the need to cover as wide a range of fruit carotenoid contents as possible.

The major carotenoids present in the flesh and skin of all of the samples were the xanthophyll lutein and the hydrocarbons β-carotene. Lower levels of neoxanthin, violaxanthin, zeaxanthin, α- and β-cryptoxanthins and α- carotene were also present (not shown).

An overview of the mean, range, standard deviation (SD) and coefficient of variation (CV) for total, lutein and β-carotene contents measured in summer squash samples are given in Table 1.

The samples analysed varied considerably in elemental composition as shown by the range and CV. High CV was observed for total and individual contents (>70%) possibly due to the different varieties used. The variability in elemental composition in calibration set was considered suitable for developing NIR calibrations for these traits.

The qualitative and quantitative carotenoid content exhibited by the samples in this study covered most of the variability reported in the literature for *C. pepo* (Ben-Amotz and Fishler, 1998; Tadmor et al., 2005; Azevedo-Meleiro and Rodríguez-Amaya, 2007; Rodríguez-Amaya et al., 2008; El-Qudah, 2009).

The total-carotenoid and lutein contents varied from 68 to 428 μg/g dw and 53–421.7 μg/g dw, respectively, in flesh of *C. pepo* fruits. These results were superior to those found in a previous study evaluating the flesh of five pairs of near-isogenic lines of *C. pepo* (10.4–187.2 μg/g dw and 6.4–143.2 for total and lutein contents, respectively, assuming 92% of moisture) by Tadmor et al. (2005).

The β-carotene content varied from 1.3 to 23.9 μg/g dw in flesh of *C. pepo*. These values were lower than those found by Tadmor et al. (2005) which varied from 4 to 44.8 μg/g dw in flesh samples.

To this date few studies have been carried out in *C. pepo* to assess the carotenoid content in fruit skin. The carotenoid content was higher in the skin of fruit which agrees with results reported in other vegetables (Gross, 1987). As the major carotenoid of this matrix is lutein, with high concentration in skin, it is worth mentioning in this context some benefits mentioned in the literature which are associated with lutein. Lutein is a xanthophyll which can be found in retina, where its main function is to protect photoreceptive cells from oxygen radicals generated in photochemical processes; it thus plays a main role in the prevention of age-related macular degeneration (Scalch, 1992; Khachik et al., 1999). Many studies showed that by consuming vegetables and vegetable products with a high lutein and zeaxanthin content, the risk of cataracts is reduced, as well as the risk of age-related macular degeneration (Seddon et al., 1994; Varma et al., 1995; Beatty et al., 1999; Segasothy and Phillips, 1999; Hammond and Caruso-Avery, 2000).

A previous study has suggested that 6 mg of lutein a day might decrease the risk of age related macular degeneration by 43% (Seddon et al., 1994). This amount is the same as the daily consumption of:, 2 salad bowls of spinach, ~7 kg tomatoes, ~1 kg corn, one salad bowl of kale or, as revealed by this study ~375 g *Cucurbita* flesh (considering 200 mg lutein kg^{-1} dw which is equal to 16 mg lutein kg^{-1} fresh weight) (Muntean et al., 2006).

Despite lutein is not a provitamin A, it is a more effective antioxidant than many other carotenoids (it inhibits *in vitro* the lipid oxidation, in a more efficient manner than α-carotene, β- carotene or lycopene). One must mention also that the lutein biodisponibility from plant sources is much higher in comparison to that of β-carotene (Castenmiller and West, 1998; Van Het Hof et al., 1999, 2000; Erdman, 1999).

3.2. Summer squash reflectance spectrum

Fig. 1 showed typical NIR spectra obtained for the different summer squash samples analyzed. Clearly it can be seen that a considerable contribution is due to the visible wavelength range (400–700 nm), and this may be of relevance for highly colored

Table 1
Mean, range, standard deviation (SD), coefficient of variation (CV) and standard error of laboratory (SEL) of total and individual carotenoids for flesh and skin of summer squash fruit. Data expressed as mg kg^{-1} (dry weight).

	Range	Mean	SD	CV	SEL
Flesh					
Total carotenoid	67.1–451.2	185.0	130.8	0.70	15.14
Lutein	50.3–434.3	172.0	120.4	0.70	11.48
β-Carotene	0–24	7.02	5.31	0.75	1.25
Skin					
Total carotenoid	85.0–1822	836.1	499.4	0.94	52.31
Lutein	78.4–1529	720.3	413.1	0.88	47.76
β-Carotene	0–194	38.12	38.36	1.39	5.26

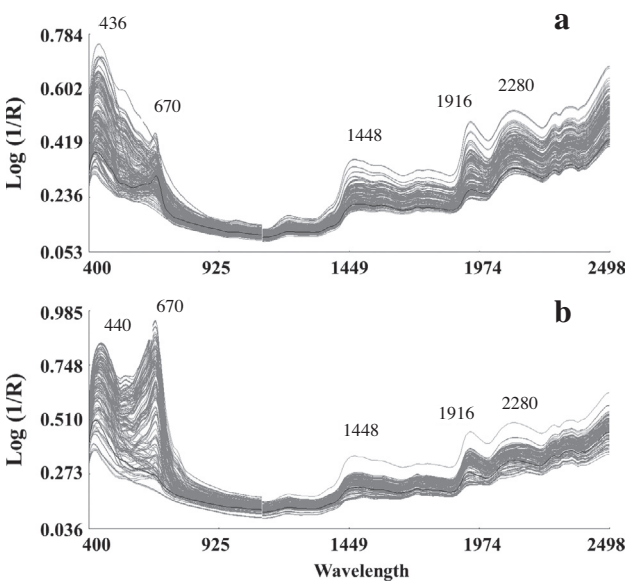


Fig. 1. Typical NIR spectra obtained for the different samples of skin (a) and flesh (b) fruits analysed.

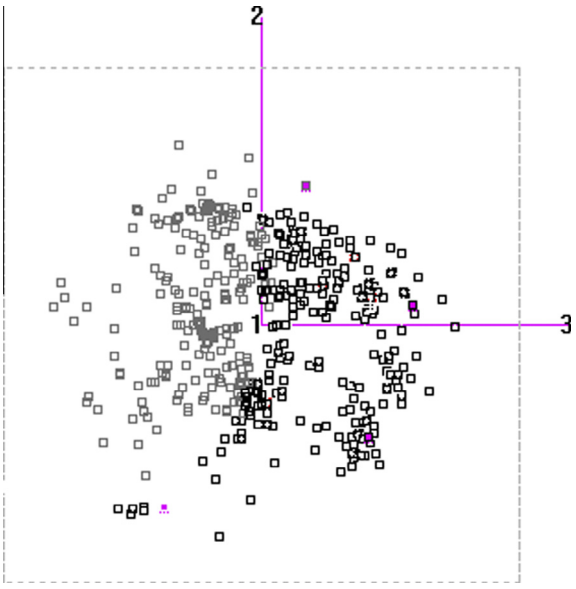


Fig. 3. Score plots, obtained by applying PCA to NIR spectra of skin and flesh, in the area defined by the second versus third principal components (PC2 and PC3). Gray and black squares indicate samples from skin and flesh, respectively.

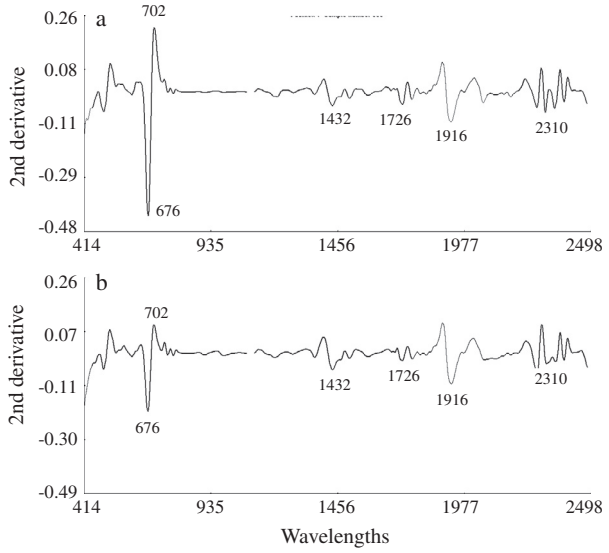


Fig. 2. Near infrared mean spectrum of *Cucurbita* samples of skin (a) and flesh (b) fruits using SNVD and second derivative as treatment.

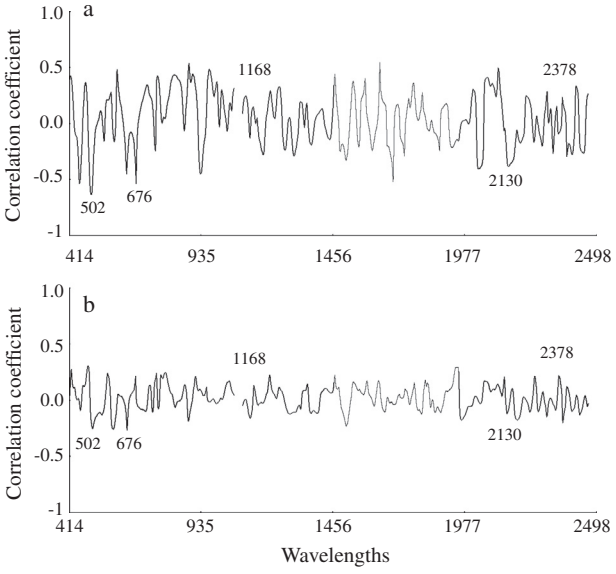


Fig. 4. Wavelength correlation of total carotenoid content in *Cucurbita* samples of skin (a) and flesh (b) fruits using SNVD and second derivative as treatment.

summer squash samples. This range has indeed been used to measure carotenoids in durum wheat and maize by NIRS (Brenna and Berardo, 2004; Murray and Williams, 1987). Vis/NIR spectra also indicated that the absorption profiles yield information in the NIR region where carotenoids (lutein) absorb strongly as it has been reported previously (Davey et al., 2009).

Fig. 2 shows the second derivative (2, 5, 5, 2; SNV + DT) average NIR spectrum of freeze-dried samples from summer squash skin (2a) and flesh (2b) used to conduct this work (n = 230). The average spectrum matched all the absorption bands, any shift of absorption maxima (lmax) being observed between them. In both Figures, the average spectrum showed bands in the visible region of the spectrum presenting lmax at 676 nm and 700 nm, which correspond to electronic transitions in the red. Thus, the band at 676 nm has been assigned to chlorophyll (Tkachuk and Kuzina, 1982).

The NIR region of the spectrum (Fig. 2a) showed characteristic absorption bands at 1432 and 1916 nm related to O–H stretch second and first overtone of water, respectively; 1726, 2310 and 2348 nm related to C–H stretch first overtone and combination bands of lipids (Murray, 1986). At 2274 nm related to O–H + C–O deformation, O–H stretch plus deformation, and O–H + C–C stretch of starch (Osborne et al., 1993).

Principal component analysis showed a clear separation between both groups of samples, i.e., skin and flesh. This spectral variation was almost entirely explained by second versus third principal components (PC2 and PC3) (Fig. 3).

3.3. Correlation plot for total carotenoid content versus wavelength

Fig. 4 showed plots of the weighted regression coefficients for the prediction of the total carotenoid concentrations in skin (4a) and flesh (4b) from the second derivatives of the Vis/ NIR spectral

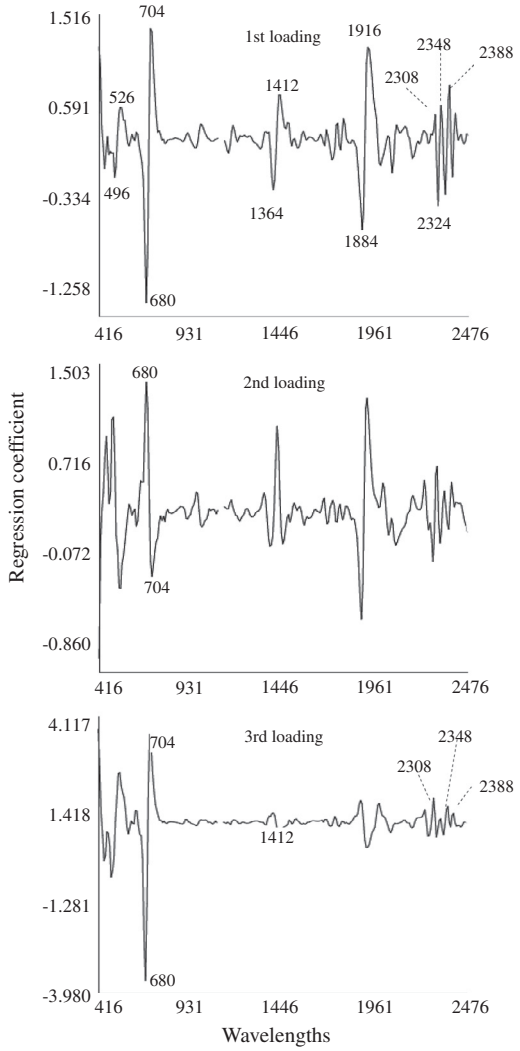


Fig. 5. Modified Partial least squares (MPLS) loadings for total carotenoid content measured in flesh of summer squash fruits using near infrared reflectance spectroscopy.

absorption data. The higher correlations between spectra and chemical composition for total carotenoids fall into the visible (range 500–700 nm) and NIR (range 1100–2378 nm), (defined as the relationship among the substance and wavelengths, absorptivity maxima as derived by second derivative). These results are similar to those obtained in other species as maize, potato and banana for carotenoid content analysis (Brenna and Berardo, 2004; Bonierbale et al., 2009; Davey et al., 2009). In particular, two clear negative peaks around 502 and 678 nm corresponding to the absorption peaks of around 532 and 676 nm in the original spectra (Fig. 4a) seem to be particularly important. Other absorption bands in the NIR region of the spectrum around 1158, 1168, 1230, 1950, 2270 and 2378 nm were also important which agrees with previous studies (Brenna and Berardo, 2004; Davey et al., 2009).

3.4. Modified partial least square loadings

MPLS loading plots allow an observation of wavelengths with high variation in the calibration set that may be associated with spectral regions of known chemical origin (Durkee, 1971).

Fig. 5 represents the MPLS loading spectra for factors 1, 2 and 3, respectively. It can be concluded that pigments existing in the tissues of the summer squash fruit greatly influenced the three MPLS loadings of the second derivative transformation (2, 5, 5, 2; SNV + DT). This is in agreement with the correlations existing between total carotenoid content and apparent absorption in our samples, in which high correlations were shown in the visible region of the spectrum (Fig. 5). Of the first three factors of the selected equation (2, 5, 5, 2; SNV + DT), the third MPLS loading was the most highly correlated with total carotenoid content. It is worth noting the influence of the band at 680 and 704 nm in modeling this third factor, which is related to the absorption in the red region by chlorophyll as has been mentioned above.

Other absorptions due to water (1412 nm), N–H combination tones of amide (1916 nm), O–H deformation plus C–O deformation of starch (2284 and 2324 nm) and also C–H combination tones by lipids (2308 and 2348 nm) (Osborne et al., 1993) highly influenced the two first factors of the equation.

3.5. NIRS analysis

In Table 2 are reported the statistics of the external-validation for the different carotenoids, including standard errors of performance (SEP) and R^2 values for the equations of best fit obtained for each of the traits.

The SEP obtained in the validation were lower than their respective S.D.s, indicating that NIRS is able to determine total carotenoids, lutein and β-carotene contents in fruits of *C. pepo*.

R^2 values for the validation ranged from 0.81 for β-carotene in flesh to 0.96 for lutein content in flesh.

Table 2
Validation statistics for equations of different carotenoids developed over flesh and skin of fruit of *Cucurbita pepo* (n = 154 for calibration and n = 76 for validation).

	NT[a]	Range	Mean	SD[b]	SEP[c]	r^{2d}	RPD[e]	RER[f]
Flesh								
Total carotenoid	11	68–428	188.6	136.7	31.7	0.95	4.31	11.35
Lutein	11	53–421.7	172.6	128.8	26.8	0.96	4.81	13.77
β-Carotene	9	1.3–23.9	7.52	5.27	2.27	0.81	2.32	9.95
Skin								
Total carotenoid	10	87–1819	892.4	476.4	104.6	0.90	4.55	16.55
Lutein	9	79–1685	783.9	419.2	87.37	0.88	4.79	18.38
β-Carotene	9	0.3–192.6	42.93	41.44	8.46	0.84	4.89	22.73

[a] NT: number of terms of the equation selected in the cross-validation.
[b] SD: standard deviation of the data in the validation set.
[c] SEP: standard error of performance.
[d] r^2: coefficient of determination in the external validation.
[e] RPD: ratio of the standard deviation to standard error of prediction (performance).
[f] RER: ratio of the range to standard error of prediction (performance).

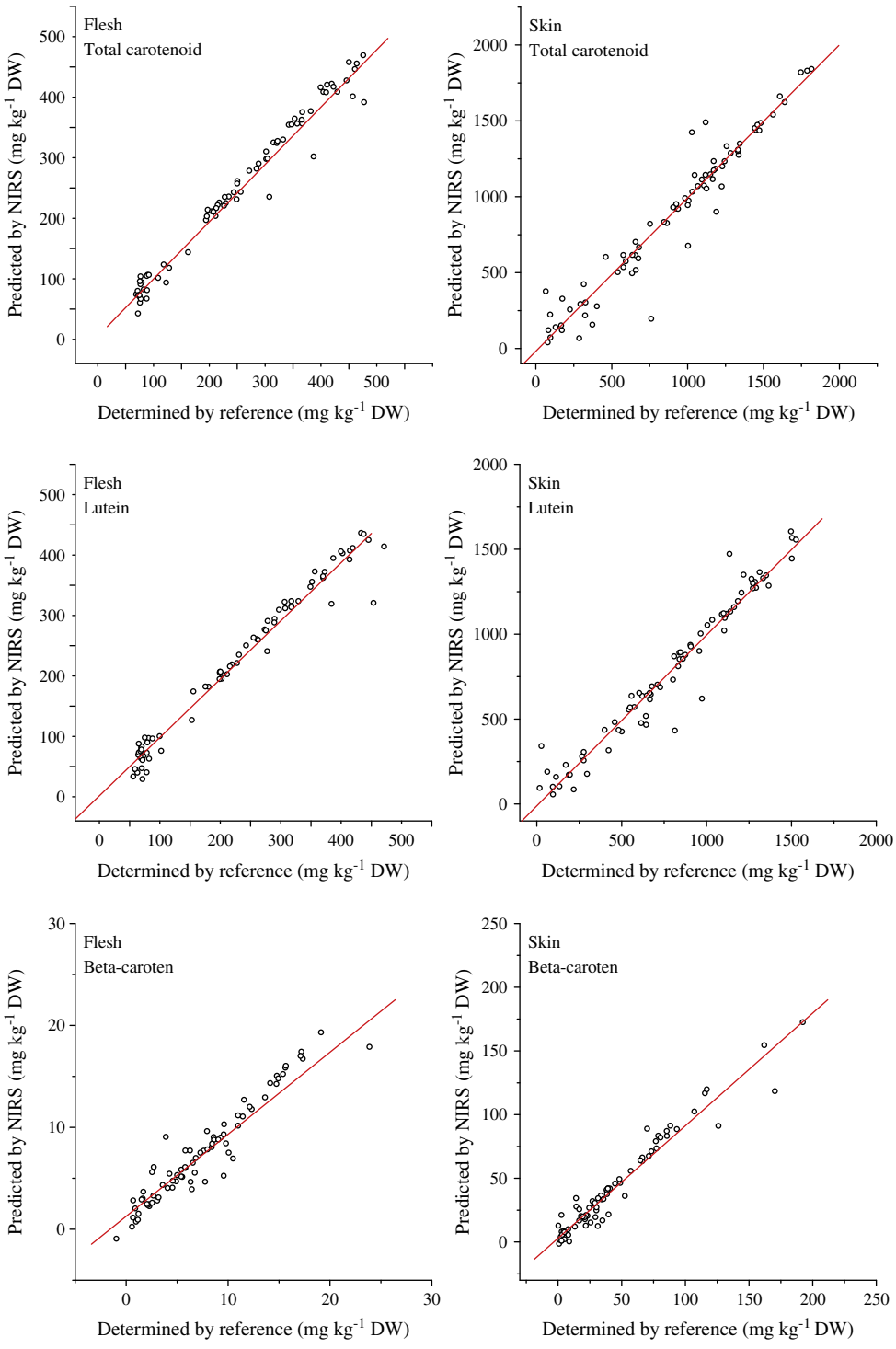


Fig. 6. Near infrared predicted values versus reference values for total and individual carotenoid content in skin and flesh of summer squash fruits.

The usefulness and accuracy of the developed models were evaluated on the basis of the R^2 and RPD values (Williams and Sobering, 1996). The R^2 values give an indication of the percentage variation in the *Y* variable that is accounted for by the *X* variable. Therefore R^2 values above 0.50 indicate that over 50% of the variation in *Y* is attributable to variation in *X*, and this allows discriminations between high and low concentrations to be made. Higher R^2 values improve discrimination, and models with a R^2 of 0.60–0.82 can be used for screening and approximate quantitative predictions, models with R^2 values between 0.83 and 0.90 can be used for many applications, while models with values of 0.92–0.96 are suitable for most applications including quality assurance, and those above 0.98 for all applications.

In our study, external-validation resulted in coefficients of determination (1-VR) of 0.95, 0.96 and 0.81 for total carotenoid, lutein and β-carotene contents, respectively in flesh (Table 2, Fig. 6), indicating that the 95%, 96% and 81% of the variability present in the data was explained by the respective calibration equations. In skin, external-validation resulted in coefficients of determination (1-VR) of 0.90, 0.88 and 0.84 for total carotenoid,

lutein and β-carotene contents, respectively (Table 2, Fig. 6), indicating that the 90%, 88% and 85% of the variability present in the data was explained by the respective calibration equations.

The values for R^2 shown by the equations in this work, indicated good quantitative information (0.81 for β-carotene in flesh), useful for many applications (0.90, 0.88 and 0.84 for total carotenoid, lutein and β-carotene in skin, respectively) and excellent quantitative information (0.95 and 0.96 for lutein and total carotenoid content in flesh, respectively) (Shenk and Westerhaus, 1996) (Table 2, Fig. 6).

The R^2 statistics were still comparable with previously reported results for NIRS calibrations for β-carotene and total carotenoid contents in banana and cassava (R^2: 0.89–0.91 and 0.84–0.88, respectively), and for lutein in maize grain (R^2: 0.96); higher than those obtained for lutein in banana and potato (R^2: 0.30 and 0.70, respectively) and for β-carotene in potato and maize grains (R^2: 0.56 and 0.70, respectively) (Brenna and Berardo, 2004; Bonierbale et al., 2009; Davey et al., 2009).

The prediction ability of the NIR calibration equations is determined by many authors according to the relationship between the spread in composition of agricultural products and the error of the performance (SEP). Thus, if the error in estimation is large compared with the spread (as SD and range) in composition, then regression has increasing difficulty in finding stable calibrations (Murray, 1986, 1993; Williams and Sobering, 1996). On the basis of the statistics RPD and RER, most of the equations were higher than 2 and 9, respectively, thus being useful for screening purposes (Williams and Sobering, 1996). To evaluate the predictive ability of equations in relation to the overall error of the reference method, the SEL was calculated and related to SEP. The SEP/SEL ratio shown by total and individual carotenoid equations (1.6–2.33) classified them as having good precisions (Font et al., 2006).

Some authors have also reported SD/SEP data for carotenoid analysis in plants, higher, similar to or even lower than those shown in this work. Davey et al. (2009) reported RPD ratios of 1.16, 2.74 and 3.34, for the prediction of lutein, β-carotene and total carotenoid content, respectively in banana. Brenna and Berardo (2004) obtained RPD ratios that ranged from 1.78 to 5.07 for calibrations of lutein, β-carotene and total carotenoids in maize grains. Bonierbale et al. (2009) reported successful equations predicting carotenoids in potato, which presented RPD ratios of 2.1, 2 and 3.3 for lutein, β-carotene and total carotenoid content, respectively. Recently, Sánchez et al. (2014) have developed successful equations predicting total and individual carotenoids in fresh cassava roots, with RPD values of 3 and 3.2 for total carotenoid content determined by HPLC and spectrophotometer, respectively and 3.5 for the prediction of β-carotene content.

4. Conclusions

The results from this study demonstrate that Vis/NIRS has good potential for the high-throughput screening of total carotenoid content and in particular for the individual carotenoid contents of lyophilized *Cucurbita* fruit samples. Despite the relatively small sample group used to develop the predictive models, the procedure shows good accuracy for total carotenoids, lutein and β-carotene contents, but it remains to be seen whether larger sample sets will improve models sufficiently to enable the reliable prediction of the concentrations of other carotenoid compounds present. Clearly, the fact that Vis/NIRS is a non-destructive analytical method and only requires minimal sample preparation will help prevent sample degradation during analysis. The disadvantages of Vis/NIRS are the low discriminatory power with respect to minor carotenoid species (as zeaxanthins) and the lower sensitivity compared to high-resolution chromatographic procedures.

When compared to conventional laboratory analyses, NIRS appears to be an attractive alternative technique because of its rapidity, simplicity, safety, and low operational costs.

This is of particular importance in nutritional quality evaluation, quality plant-breeding programs, species resource identification, and the healthy processing of summer squash foods in which a large number of samples must be analyzed.

Acknowledgements

The authors wish to express their thanks to the Project INIA RTA00039-000 and FEDER for the funding of this research. Damián Martínez-Valdivieso was supported by an INIA (Instituto de Investigación y Tecnología Agraria y Alimentaria) contract. We thank Nicholas Davies for his help in the grammatical revision of the manuscript.

References

Alquezar, B., Rodrigo, M.J., Zacarías, L., 2008. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. Phytochemistry 69, 1997–2007.

Anter, J., Fernández-Bédmar, Z., Villatoro-Pulido, M., Martínez-Valdivieso, D., Font, R., Muñoz-Serrano, A., Alonso-Moraga, A., Del Río-Celestino, M., 2011. Cytotoxicity and in vivo antigenotoxicity of some horticultural food cultivated in Southern Spain. Toxicol. Lett. 205, S60–S179.

Atienza, S.G., Avila, C.M., Ramírez, M.C., Martí, A., 2005. Application of near infrared reflectance spectroscopy to the determination of carotenoid content in tritordeum for breeding purposes. Aust. J. Agric. Res. 56 (1), 85–89.

Azevedo-Meleiro, C.H., Rodríguez-Amaya, D.B., 2007. Quantitative and qualitative differences in carotenoids composition among *Cucurbita moschata*, *Cucurbita maxima* and *Cucurbita pepo*. J. Agric. Food Chem. 55, 4027–4033.

Barnes, R.J., Dhanoa, M.S., Lister, S.J., 1989. Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra. Appl. Spectrosc. 43, 772–777.

Beatty, S., Boulton, M., Henson, D., Koh, H.H., Murray, I.J., 1999. Macular pigment and age related macular degeneration. Br. J. Ophthalmol. 83, 867–877.

Ben-Amotz, A., Fishler, R., 1998. Analysis of carotenoids with emphasis on 9-cis β-carotene in vegetables and fruits commonly consumed in Israel. Food Chem. 62, 515–520.

Blanco-Díaz, M.T., Del Río Celestino, M., Martínez-Valdivieso, D., Font, R., 2014. Use of visible and near-infrared spectroscopy for predicting antioxidant compounds in summer squash (*Cucurbita pepo* ssp *pepo*). Food Chem. 164, 301–308.

Bonierbale, M., Grüneberg, W., Amoros, W., Burgos, G., Salas, E., Porras, E., Zum Felde, T., 2009. Total and individual carotenoid profiles in Solanum phureja cultivated potatoes: II. Development and application of near-infrared reflectance spectroscopy (NIRS) calibrations for germplasm characterization. J. Food Compos. Anal. 22 (6), 509–516.

Brenna, O.V., Berardo, N., 2004. Application of Near-Infrared Reflectance Spectroscopy (NIRS) to the evaluation of carotenoids content in maize. J. Agric. Food Chem. 52, 5577–5582.

Castenmiller, J.J.M., West, C.E., 1998. Bioavailability and bioconversion of carotenoids. Ann. Rev. Nutr. 18, 19–38.

Davey, M.W., Saeys, W., Hof, E., Ramon, H., Swennen, R.L., Keulemans, J., 2009. Application of visible and near-infrared reflectance spectroscopy (Vis/NIRS) to determine carotenoid contents in banana (*Musa* spp.) fruit flesh. J. Agric. Food Chem. 57, 1742–1751.

Durkee, A.B., 1971. The nature of tannin in rapeseed (*Brassica campestris*). Phytochemistry 10, 1583–1585.

Edwards, N.M., Dexter, J.E., Sobering, D.C., Williams, P.C., 1996. Whole grain prediction of durum wheat yellow pigment by visible-NIR reflectance spectroscopy. In: Davies, A.M.C., Williams, P.C. (Eds.), Near Infrared Spectroscopy: The Future Waves, Proceedings 7th International Conference on Near-Infrared Spectroscopy, Montreal, Canada, August, 1995. NIR Publications, Chichester, UK, pp. 462–465.

El-Qudah, J.M., 2009. Identification and quantification of major carotenoids in some vegetables. Am. J. Appl. Sci. 6 (3), 492–497.

Emerson, R.A., 1910. The inheritance of sizes and shapes in plants. Am. Nat. 44, 739–746.

Erdman Jr., J.W., 1999. Variable bioavailability of carotenoids from vegetables. Am. J. Clin. Nutr. 70, 179–180.

Ferriol, M., Picó, B., 2008. Pumpkin and winter squash. In: Prohens, J., Nuez, F. (Eds.), Handbook of Plant Breeding. Vegetables I. Part 4. Springer, vol. 1, pp. 317–349.

Ferriol, M., Picó, B., Nuez, F., 2003. Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRAP and AFLP markers. Theor. Appl. Genet. 107, 271–282.

Font, R., Del Río-Celestino, M., De Haro-Bailón, A., 2006. The use of near-infrared spectroscopy (NIRS) in the study of seed quality components in plant breeding programs. Ind. Crops Prod. 24, 307–313.

Giovannucci, E., Ascherio, A., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Willet, W.C., 1995. Intake of carotenoids and retinol in relation to risk of prostate cancer. J. Natl Cancer Inst. 87, 1767–1776.

Granado, F., Olmedilla, B., Blanco, I., 2003. Nutritional and clinical relevance of lutein in human health. Br. J. Nutr. 90, 487–504.

Gross, J., 1987. *Pigments* in Fruits. Academic Press, Londres.

Hammond Jr., B.R., Caruso-Avery, M., 2000. Macular pigment optical density in a Southwestern sample. Invest. Ophthalmol. Vis. Sci. 41, 1492–1497.

Hruschka, W.R., 1987. Data analysis: wavelength selection methods. In: Williams, P.C., Norris, K.H. (Eds.), Near-Infrared Technology in the Agricultural and Food Industries. St. Paul, Minn., American Association Cereal Chemistry, pp. 35–55.

Johnson, E.J., 2002. The role of carotenoids in human health. Nutr. Clin. Care 5, 56–65.

Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and Carotenoids: Measurement and Characterization by UV–VIS spectroscopy. Current Protocols in Food Analytical Chemistry. http://dx.doi.org/10.1002/0471142913.faf0403s01.

Kato, M., Ikoma, Y., Matsumoto, H., Sugiura, M., Hyodo, H., Yano, M., 2004. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiol. 134, 824–837.

Khachik, F., Beecher, G.R., 1988. Separation of carotenol fatty acid esters by high-performance liquid chromatography. J. Chromatogr. A 449, 119–133.

Khachik, F., Bertram, J.S., Huang, M.T., Fahey, J.W., Talalay, P., 1999. Dietary carotenoids and their metabolites as potentially useful chemoprotective agents against cancer. In: Packer, L., Hiramatsu, M., Yoshikawa, T. (Eds.), Antioxidant Food Supplements in Human Health. Academic Press, San Diego, CA, pp. 203–229.

Klipstein-Grobusch, K., Launer, L.J., Geleijnse, J.M., Boeing, H., Hofman, A., Wittemen, J.C.M., 2000. Serum carotenoids and atherosclerosis: the Rotterdam Study. Atherosclerosis 148, 49–56.

Leth, T., Jakobsen, J., Andersen, N.L., 2000. The intake of carotenoids in Denmark. Eur. J. Lipid Sci. Technol. 102, 128–132.

Martens, H., Naes, T., 1992. Multivariate Calibration. John Wiley & Sons.

Martínez-Valdivieso, D., Font, R., Gómez, P., Blanco-Díaz, T., Del Río-Celestino, M., 2014. Determining the mineral composition in *Cucurbita pepo* fruit using near infrared reflectance spectroscopy. J. Sci. Food Agric. http://dx.doi.org/10.1002/jsfa.6667.

Mínguez-Mosquera, M.I., Hornero-Méndez, D., 1993. Separation and quantification of the carotenoid pigments in red peppers (*Capsicum annuum* L.), paprika, and oleoresin by reversed-phase HPLC. J. Agric. Food Chem. 41, 1616–1620.

Muntean, E., Lazar, V., Muntean, N., 2006. HPLC-PDA analysis of carotenoids and chlorophylls from *Cucurbita pepo* L. convar. giromontina fruits. Buletinul Universitatii de Stiinte Agricole si Medicina Veterinara Cluj Napoca Seria Agricultura 62, 94–99.

Murray, I., 1986. The NIR spectra of homologous series of organic compounds. In: Hollo, J., Kaffka, K.J., Gonczy, J.L. (Eds.), Proceedings International NIR/NIT Conference. Akademiai Kiado, Budapest, Hungary, pp. 13–28.

Murray, I., 1993. Forage analysis by near reflectance spectroscopy. In: Davies, A., Baker, R.D., Grant, S.A., Laidlaw, A.S. (Eds.), Sward Management Handbook. British Grassland Society, UK, pp. 285–312.

Murray, I., Williams, P.C., 1987. Chemical principles of near infrared technology. In: Williams, P.C., Norris, K. (Eds.), Near Infrared Technology in the Agricultural and Food Industries AACC: St. Paul, MN, pp. 17–34.

Naes, T., Isaksson, T., Fearn, T., Davies, T., 2002. A User-friendly Guide to Multivariate Calibration and Classification. NIR Publications, Chichester, UK, 420 pp.

Obrero, Á., González-Verdejo, C.I., Die, J.V., Gómez, P., Del Río-Celestino, M., Román, B., 2013. Carotenogenic gene expression and carotenoid accumulation in three varieties of *Cucurbita pepo* during fruit development. J. Agric. Food Chem. 61, 6393–6403.

Osborne, B.G., Fearn, T., Hindle, P., 1993. Practical NIR Spectroscopy with Applications in Food and Beverage Analysis. Longman Scientific and Technical, London, UK.

Paris, H.S., 2002. Genetic control of irregular striping, a new phenotype in *Cucurbita pepo*. Euphytica 129, 119–126.

Paris, H.S., 2008. Summer Squash. In. Prohens, J., Nuez, F. (Eds.), Handbook of Plant Breeding. Vegetables I. Part 4. Springer, vol. 1, pp. 351–381.

Paris, H.S., 1986. A proposed subspecific classification for *Cucurbita pepo*. Phytologia 61, 113–138.

Paris, H.S., 2000. *Quiescent intense* (*qi*), a gene that affects young but not mature fruit color intensity in *Cucurbita pepo*. J. Hered. 91, 333–339.

Rodríguez-Amaya, D.B., 1997. Carotenoids and Food Preparation: The Retention of Provitamin A Carotenoids in Prepared, Processed, and Stored Foods. Opportunities for Micronutrient Intervention (OMNI), Arlington.

Rodríguez-Amaya, D.B., Kimura, M., Godoy, H.T., Amaya-Farfan, J., 2008. Update Brazilian database on food carotenoids: Factors affecting carotenoid composition. J. Food Compos. Anal. 21, 445–463.

Sánchez, T., Ceballos, H., Dufour, D., Ortiz, D., Morante, N., Calle, F., Zum Felde, T., Domínguez, M., Davrieux, F., 2014. Prediction of carotenoids, cyanide and dry matter contents in fresh cassava root using NIRS and Hunter color techniques. Food Chem. 151, 444–451.

Scalch, W., 1992. Carotenoids in the retina: a review of their possible role in preventing or limiting damage caused by light and oxygen. Exper. Supplement. 62, 80–298.

Schaefer, H., Heibl, C., Renner, S.S., 2009. Gourds afloat: a dated phylogeny reveals an Asian origin of the gourd family (*Cucurbitaceae*) and numerous oversea dispersal events. Proceed. Roy. Soc. B: Biol. Sci. 276, 843–851.

Schulz, H., Quilitzsch, H., Drews, H., Krüger, H., 2000. Estimation of minor components in caraway, fennel and carrots by NIRSs comparison of results from dispersive and Fourier transform instruments. Int. Agrophys. 14, 249–253.

Seddon, J.M., Ajani, U.A., Sperduto, R.D., Hiller, R., Blair, N., Burton, T.C., Farber, M.D., Gragoudas, E.S., Haller, J., Miller, D.T., Yannuzzi, L.A., Willet, W., 1994. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. J. Am. Med. Assoc. 272, 1413–1420.

Segasothy, M., Phillips, P.A., 1999. Vegetarian diet: panacea for modern lifestyle diseases? Quart. J. Med. 92, 531–544.

Shenk, J.S., 1992. Networking and calibration transfer. In: Making Light Work: Advances in Near Infrared Spectroscopy, VCH, London, pp. 223–228.

Shenk, J.S., Westerhaus, M.O., 1996. Calibration the ISI way. In: Murray, Cowe (Eds.), Near Infrared Journal of the American Medical Association, NIR Publications, Chichester, UK, pp. 198–202.

Shokrzadeh, M., Azadbakht, M., Ahangar, N., Hashemi, A., Saeedi, Saravi S.S., 2010. Cytotoxicity of hydro-alcoholic extracts of *Cucurbita pepo* and *Solanum nigrum* on HepG2 and CT26 cancer cell lines. Pharmacogn. Mag. 6, 176–179.

Smith, B.D., 2005. Reassessing Coxcatlan Cave and the early history of domesticated plants in Mesoamerica. Proc. Natl. Acad. Sci. 102 (27), 9438–9445.

Sinnott, E.W., 1936. A developmental analysis of inherited shape differences in cucurbit fruits. Am. Natur. 70, 245–254.

Tadmor, Y., Larkov, O., Meir, A., Minkoff, M., Lastochkin, E., Edelstein, M., Levin, S., Wong, J., Rocheford, T., Lewinsohn, E., 2000. Reversed-phase high performance liquid chromatographic determination of vitamin E components in maize kernels. Phytochem. Anal. 11, 370–374.

Tadmor, Y., Paris, H.S., Meir, A., Schaffer, A.A., Lewinsohn, E., 2005. Dual role of the pigmentation gene B in affecting carotenoid and vitamin E content in squash (*Cucurbita pepo*) mesocarp. J. Agric. Food Chem. 53, 9759–9763.

Tkachuk, R., Kuzina, F.D., 1982. Chlorophyll analysis of whole rape-seed kernels by near infrared reflectance. Can. J. Plant Sci. 62, 875–884.

Van Het Hof, K.H., West, C.E., Westrate, J.A., Hautvast, J.G., 2000. Dietary factors that affect the bioavailability of carotenoids. J. Nutr. 130, 503–506.

Van Het Hof, K.H., Brouwer, I.A., West, C.E., Haddeman, E., Steegers-Theunissen, R.P., Van Dusseldorp, M., Weststrate, J.A., Eskes, T.K., Hautvast, J.G., 1999. Bioavailability of lutein from vegetables is 5 times higher than that of beta-carotene. Am. J. Clin. Nutr. 70 (2), 261–268.

Varma, S.D., Devamanoharan, P.S., Morris, S.M., 1995. Prevention of cataracts by nutritional and metabolic antioxidants. CRC Critical Rev. Food Sci. Nutr. 35 (1–2), 111–129.

Venyaminov, S.Y., Prendergast, F.G., 1997. Water (H_2O and D_2O) molar absorptivity in the 1000–4000 cm^{-1} range and quantitative infrared spectroscopy of aqueous solutions. Anal. Biochem. 248, 234–245.

Williams, P.C., Norris, K., 1987. Qualitative applications of near-infrared reflectance spectroscopy. In: Williams, P.C., Norris, K. (Eds.), Near-Infrared Technology in the Agricultural and Food Industries, the American Association of Cereal Chemists, St. Paul, Mn, pp. 241–246.

Williams, P.C., Sobering, D.C., 1996. How do we do it: a brief summary of the methods we use in developing near infrared calibrations. In: Davies, A.M.C., Williams, P.C. (Eds.), Near Infrared Spectroscopy: The Future Waves. NIR Publications, Chichester, pp. 185–188.

Xu, C.J., Fraser, P.D., Wang, W.J., Bramley, P., 2006. Differences in the carotenoid content of ordinary citrus and lycopene-accumulating mutants. J. Agric. Food Chem. 54, 5474–5481.

Zandomeneghi, M., Festa, C., Carbonaro, L., Galleschi, L., Lenzi, A., Calducci, L., 2000. Front-surface absorbance spectra of wheat flour: determination of carotenoids. J. Agric. Food Chem. 48, 2216–2221.