Carotenoid composition and vitamin A value of an Argentinian squash (*Cucurbita moschata*)

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SUMMARY. The carotenoid composition of butternut squash (*Cucurbita moschata*) cultivated in the province of Santiago del Estero, Argentina, was determined. The main carotenoids isolated were identified as β-carotene (β,β-carotene), α-carotene (β,ε-carotene), and lutein (β,ε–carotene-3,3'-diol) and the minor carotenoids, as phytofluene (7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene), ζ-carotene (7,8,7',8'-tetrahydro-ψ,ψ-carotene), neurosporene (7,8-dihydro-ψ,ψ-carotene), violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) and neoxanthin (5,6-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol). In some samples, 5,6,5',6'-β-carotene diepoxide, (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene) and flavoxanthin (5,8-epoxy-5,8-dihydro-β,ε–carotene-3,3'-diol) were detected. The presence of *cis*-isomers of β,β-carotene was also detected by HPLC. The vitamin A value obtained was 432 µg RE/ 100g fresh sample, which indicates that this vegetable is an important source of provitamin A.
Keywords: Carotenoids, vitamin A, butternut squash, *Cucurbita moschata*, squashes.

RESUMEN. Composicion de carotenoides y valor de vitamina A de una calabaza argentina *(Cucurbita moschata)*. Se determinó la composición de carotenoides de un tipo de calabaza (*Cucurbita moschata*), conocida como "anco coreano" cultivada en la provincia de Santiago del Estero, Argentina. Los principales carotenoides aislados fueron identificados como mayoritarios, β-caroteno (β,β-caroteno), α-caroteno (β,ε-caroteno), luteína (β,ε-caroteno-3,3'-diol) y como minoritarios, fitoflueno (7,8,11,12,7',8'-hexahidro-ψ,ψ-caroteno), ζ-caroteno (7,8-7',8'-tetrahidro-ψ,ψ-caroteno), neurosporeno (7,8-dihidro-ψ,ψ-caroteno), violaxantina (5,6,5',6'-diepoxi-5,6,5',6'-tetrahidro-β,β-caroteno-3,3'-diol) y neoxantina (5',6'-epoxi-6,7-didehidro-5,6,5',6'-tetrahidro-β,β-caroteno-3,5,3'-triol). En algunas muestras fueron detectados β-caroteno 5,6,5',6'-diepóxido (5,6,5',6'-diepoxi-5,6,5',6'-tetrahidro-β,β-caroteno) y flavoxantina (5,8-epoxi-5,8-dihidro-β,ε-caroteno-3,3'-diol). También fue detectada por HPLC la presencia de isómeros *cis* de β,β-caroteno. El valor de vitamina A obtenido fue 432 µg RE/ 100g de muestra fresca, lo cual indica que este vegetal es una fuente importante de provitamina A.
Palabras clave: Carotenoides, vitamina A, calabazas, anco coreano, *Cucurbita moschata*.

INTRODUCTION

Carotenoids are natural pigments, commonly found in vegetables, fruits, flowers and microorganisms as well as in the animal kingdom. These pigments play an important role in the human diet because of their provitamin A function (1).

Epidemiological evidence suggests that a diet rich in carotenoids is associated with decreased incidence of many serious diseases, such as cancer, atherosclerosis, cataracts, and age-related macular degeneration (2-4). It is suggested that this may occur via prevention of lipid peroxidation. The carotenoids protect cells and organisms against photooxidation and they can deactivate singlet oxygen, which is mutagenic, able to inactivate enzymes and damage DNA molecules and lipids (5-6).

The carotenoid composition varies with variety, culture, cultivation conditions, the state of maturity, the post-harvest and storage handling, the climate and the geographical localization, the type of sample and the part of plant (7-8). It must be also taken into account that the stability of the carotenoids is also variable in different foods even with the same processing and storage conditions (9-11).

Studies about carotenoids in the Republic of Argentina are scarce (12). Deficiency of vitamin A and its consequences such as xerophthalmia and blindness are endemic in many regions, particularly the Northwestern provinces of Jujuy, Salta, Santiago del Estero, Catamarca and Tucumán; other regions of the country do not have this problem (13).

It is therefore necessary to evaluate the composition of carotenoids present in foods of this region of the country to determine their nutritional values in terms of provitamin A activity.

Butternut squash is one of the most highly consumed vegetables in the region. It is economical and it is available throughout the year. For this reason, it is urgent to determine its carotenoid composition to evaluate it as a source of provitamin A carotenoids and of other carotenoids that may be beneficial to health.

MATERIAL

Butternut squashes with weights around 1kg were analyzed. These squashes were obtained in different markets in the city of Santiago del Estero as well as in the city of La Banda, province of Santiago del Estero, Argentina.

Each sample was peeled and cut into four transverse sections. Two opposite sections of each squash were taken and homogenized first in a mixer. From the homogenized part, 50 g were taken, to which 25 g of Hyflosupercel and 100 ml cold acetone were added and the extraction was carried out by mixing in a second blender.

Carotenoids determination

A. Analysis by chromatography and UV visible spectroscopy:

Extraction

The carotenoids were extracted from the vegetable tissue with cold acetone in a blender (14). The extract was transferred to petroleum ether, and then saponified with 10% KOH in methanol so as to hydrolyze carotenoid esters (14). After washing with water to remove alkali, the saponified extract was dried over anhydrous sodium sulfate for a few minutes and concentrated in a rotary evaporator (temperature under 40°C).

The separation of the carotenoids was performed by open column chromatography on MgO: Hyflosupercel (1:2) as the stationary phase, followed by rechromatography of mixtures on a neutral alumina column (15).

The identification of the carotenoids was based on a) their chromatographic behavior, i. e., order of elution from the open column, R_f values on silica gel thin layer chromatography (TLC) plates and retention times in high performance liquid chromatography (HPLC) compared with those of standard samples (16), b) the chemical reactions of functional groups (17) and c) their UV-visible spectra by considering the wavelength of maximum absorption and the shape of the spectrum (fine structure) which is characteristic of the chromophore (18-19).

Eight carotenoids were isolated from the squash samples. The first fraction obtained from the MgO: Hyflosupercel (1:2) column eluted with petroleum ether, was a colorless compound with the typical absorption spectrum and chromatographic behavior of phytofluene. The second fraction was eluted from the column with 2-6% ethyl ether in petroleum ether. Its R_f of 0.90 on silica gel plates developed with 5% methanol in toluene is typical of a carotene. The absence of epoxy groups was indicated by the lack of color change of the sample on a silica gel plate, when exposed to HCl fumes. This behavior and the absorption spectrum were characteristic of α- carotene. The third fraction, eluted with 6-10% ethyl ether in petroleum ether, also had R_f 0.90 on silica-gel plates developed with 5% methanol in toluene. The absence of epoxy groups was determined as for the previous fraction. The absorption spectrum and chromatographic behavior of this compound were typical of β-carotene.

The fourth fraction, eluted with 10-12% ethyl ether in petroleum ether, also has R_f 0.90 on silica-gel plates developed with 5% methanol in toluene. The absence of epoxy groups was verified as before. From its absorption spectrum and its chromatographic behavior, this compound was identified as ζ-carotene.

The fifth fraction was eluted with 2-10% of acetone petroleum ether. The absorption spectrum and its R_f 0.90 on silica-gel plates developed in 5% methanol in toluene were consistent with the identification of this compound as neurosporene.

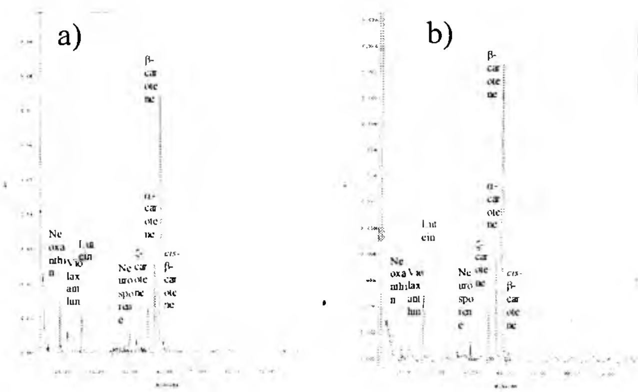
The sixth fraction eluted with 10-20% acetone in petroleum ether, was shown by TLC on silica gel plates to consist of a mixture of 2 carotenoids with R_f values 0.44 and 0.20 (25% ethyl acetate in benzene), and 0.82 and 0.55 (5% methanol in toluene). The mixture was separated by rechromatography on a neutral alumina column. The presence of lutein (major component in the mixture) as well as violaxanthin could be shown by the absorption spectra and the R_f values in TLC silica gel plates. The presence of epoxy groups in the more polar pigment was suggested by the change of color to blue in TLC plates when they were exposed to the HCl fumes and by the hypsochromic shift of 40 nm in the absorption spectrum.

The seventh fraction was eluted with 20% acetone into petroleum ether. The presence of epoxy groups was suggested by the change of color from yellow to green on the silica gel plates. The R_f value of 0.09 TLC silica gel plate eluted with 25% ethyl acetate in benzene and 0.22 in ether, the order of elution from the open column and the presence of epoxy group suggest that this compound may be neoxanthin.

B. Analysis by high performance liquid chromatography (HPLC)

The saponified and unsaponified extracts were analyzed by HPLC with a photodiode array detector (Figure 1). A high efficiency liquid chromatograph (Waters 2690) with automatic injection was used equipped with a photodiode-array detector (Waters 996) and a C_{18} Spherisorb ODS-2 end capped (5 μm, 4.6 x 250 mm) column. As mobile phase a mixture of methanol- acetonitrile- ethyl acetate was used, with the following gradient: 95% acetonitrile-5% methanol to 100% ethyl acetate. The flow rate was 0.5 ml/min and the injection volume was 10 μl.

FIGURE 1
HPLC chromatograms of an Argentinian squash (*Cucurbita moschata*) unsaponified (a) and saponified (b) extracts



Conditions: C_{18} Spherisorb ODS-2, end-capped (5μm, 4.6 x 250 mm) column. Photodiode array detector. Mobile phase: a mixture of methanol-acetonitrile-ethyl acetate with the following gradient: 95% acetonitrile, 5% methanol, to 100% ethyl acetate. Flow rate: 0.5 ml/min. Injection volume: 10 μl.

The identification was based upon the UV- visible absorption spectrum, obtained by the photodiode array detector and the retention times in the chromatograms compared with standards obtained from natural sources. The results obtained from the comparison with standard samples agree with the previous tentative identification.

The following carotenoids in decreasing order of concentration were identified by their retention times in the chromatogram obtained from the unsaponified extract (Figure.1.a): all-*trans*-β-carotene (39.0 minutes), α-carotene (37.3 minutes), ζ-carotene (35.3 minutes), neurosporene (32.0 minutes), lutein (15.7 minutes), violaxanthin (11.4 minutes), *cis*-β-carotene (39.8 minutes) and neoxanthin (9.1 minutes). The same carotenoids with a small increase in the peak corresponding to lutein were observed in the chromatogram obtained from the saponified extract which suggests the presence of lutein esters in the original sample (Figure. 1.b).

The analysis by HPLC allowed the detection of a *cis* isomer of β-carotene by means of its visible spectrum although it is not possible to speculate on the position of this *cis* double bond with just this information. This compound was not isolated from its all-*trans*-isomer in the open column chromatography.

In some samples, 5,6,5',6'-β-carotene diepoxide (5,6,5',6'-diepoxy-5,6,5',6'- tetrahydro-β,β-carotene) and flavoxanthin (5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3'-diol) were detected, which were identified as artifacts formed during the analysis.

Quantification by HPLC

The carotenoid content in unsaponified and saponified extracts were determined by HPLC. The HPLC (KONIK 500A) was equipped with a KONIK UV-Visible 200 detector to 450 nm, a C_{18} VYDAC 218TP54 (5μm, 4.6 x 250 mm) column. Methanol was used as mobile phase at 1 ml/min and the injection volume was 20μl. The samples were dried under N_2 and re-dissolved in 3ml ethyl acetate-methanol (1:1) at the time of the injection in the chromatograph.

The quantification was carried out by means of calibration curves, constructed with a minimum of five concentrations for each standard, determined by UV-Visible spectroscopy, applying the Beer Law and using the absorption coefficients presented by Davies (19).

The standards of β-carotene, α-carotene and lutein were obtained from natural sources and purified by open column and thin layer chromatography. The purity of the standards was verified by HPLC.

The carotenoid composition determined by HPLC is shown in Table 2.

TABLE 1
UV-visible Absorbance Maxima of the carotenoids extracted from butternut squash

Carotenoids	λ_{max} UV-Vis Spectra	λ_{max} HPLC-PAD Spectra[c]
all-*trans*-β-carotene	447-474[a]	454.9-481.5
α-carotene	419-442-471[a]	426.2-448.9-476.6
lutein	425-445-472[b]	424.3-447.6-474.4
ζ-carotene	376-397-422[a]	380.1-400.7-424.7
neurosporene	417-439-468[a]	416.5-441.6-470.6
flavoxanthin	404-424-451[b]	d
5,6,5',6'-diepoxy-β-carotene	421-440-468[b]	d
violaxanthin	419-441-470[b]	417.0-441.6-470.6
phytofluene	345-364[a]	d

a. Determined in petroleum ether. b. Determined in ethanol. c. Determined in methanol-ethyl acetate-acetonitrile (mobile phase). d. Non detected.

TABLE 2
Carotenoid content of a butternut squash from Argentina

Carotenoids	β-carotene	α-carotene	Lutein
Concentration[a] ($\mu g\ g^{-1}$)	23 ± 4	6 ± 1	3 ± 1

[a]Determined in a fresh squash by HPLC. Means and standard deviation for 5 samples.

Quantification by UV-visible spectroscopy

Quantification was carried out for the isolated major compounds by UV-visible spectroscopy as recommended by Davies (19). The value found for β-carotene concentration in row butternut squash was 20 ± 4 μg g^{-1}. This result is similar, within the experimental errors, with that obtained by HPLC. Besides, according to the chromatogram (Fig. 1), the *cis*-isomer concentration is much lower than that of the all *trans* β-carotene and therefore not important enough to be worth considering the different provitamin A activities (20)

The concentration determined for α-carotene was 8 ± 2 μg g^{-1} and for lutein (in the mixture with violaxanthin) 5 ± 1 μg g^{-1}.

Vitamin A values

On basis of β-carotene and α-carotene as provitamin A, a value of 437,14 μg RE/100g, was obtained for butternut squash from Santiago del Estero, Argentina, calculated according to Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (21).

CONCLUSIONS

In the present work the composition of carotenoids present in butternut squashes *(Cucurbita moschata)* was determined. The analysis of the samples was carried out, applying the method of Rodriguez-Amaya (14-15).

Eight carotenoids were isolated and by their features (chromatographic behavior, absorption spectrum, and chemical reactions) were identified as phytofluene, β-carotene, α-carotene, ζ- carotene, neurosporene, lutein, violaxanthin and neoxanthin. The presence of *cis*-β-carotene was detected by HPLC.

The obtained results coincide with the literature about the carotenoid compositions in squashes and pumpkins (22), although it must be taken into account that the carotenoid composition varies with factors as climate, geographical position, state of maturity, among others.

From the realized analysis, it can be concluded that this type of squashes constitutes an important source of vitamin A. The carotenoids found in these squashes, as mentioned previously may also bring other health benefits because of their antioxidant activity.

Due to these properties, butternut squashes consumption is recommended. Moreover, they are easy to cultivate in our region, taste good, are available all year round and are economical. In this way, the nutritional deficiency in vitamin A, which causes many diseases, may be reduced.

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