Liquid-liquid partition – HPLC assessment of provitamins A from fruits of *Cucurbita maxima Duch.*

Eduard MUNTEAN

University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, 3-5 Manastur Street, 400372-Cluj Napoca, Romania; Phone: 0264 596 384/ 213, E-mail: edimuntean@yahoo.com

Abstract
The provitamins A from fruits of *Cucurbita maxima Duch* were quantified using liquid – liquid partition followed by high performance liquid chromatography. The concentration of carotenoids in the plant matrix is 85.13 μg/ g (921.44 μg/ g dry weight), while the concentration of provitamins A, expressed in retinol – equivalents, is 31.45 RE/ g dry weight. Ten carotenoids were identified: two major ones (lutein and β-carotene) and eight minor carotenoids (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene and 15Z-β-carotene). From these, only five carotenoids are provitamins A: α–cryptoxanthin, β–cryptoxanthin, α-carotene, β-carotene and 9Z-β-carotene, but more than 95% of the provitamin A activity is due to β-carotene.

Key words: Carotenoids, provitamins A, HPLC, chromatography, food analysis, quality control

Resumé
Les provitamines A des fruits de *Cucurbita maxima Duch* ont été déterminées en utilisant la répartition liquide-liquide suivie par la chromatographie de liquides de haute performance. La concentration des caroténoïdes totales dans la matrice végétale étudiée est de 85.13 μg/ g (921.44 μg/ g matière sèche), tandis que la concentration des provitamines A est de 31.45 RE/ g matière sèche. On a identifié dix caroténoïdes: deus principales (la lutéine et le β-carotène) et huit minoritaires (la neoxanthine, la violaxanthine, la antheraxanthine, la zeaxanthine, la α-cryptoxanthine, la β-cryptoxanthine, le α-carotène et le 15Z-β- carotène). De toutes ces caroténoïdes seulement cinq sont provitamines A: la α-cryptoxanthine, la β-cryptoxanthine, le α-carotène, le β-carotène et le 15Z-β- carotène, mais plus de 95% de l'activité provitaminique A est dû au β-carotène.

Mots cles: caroténoïdes, provitamines A, HPLC, chromatographie, analyse des aliments,

Rezumat
Provitaminele A din fructe de *Cucurbita maxima Duch.* au fost determinate utilizând repartiția lichid-lichid urmată de cromatografia de lichide de înaltă performanță. Concentrația carotenoidelor totale în matricea vegetală investigată este de 85.13 μg/ g (921.44 μg/ g substanță uscată), iar concentrația provitaminelor A este de 31.45 RE/ g substanță uscată. Zece carotenoide au fost identificate: două majore (luteina şi β-carotenul) şi opt minore (neoxantina, violaxantina, anteraxantina, zeaxantina, α-criptoxantina, β-criptoxantina, α-carotenul şi 15Z-β-carotenul). Dintre acestea, doar cinci carotenoide sunt provitamine A: α–criptoxantina, β–criptoxantina, α-carotenul, β-carotenul şi 9Z-β-carotenul, însă peste 95% din activitatea provitaminică A se datorează β-carotenului.

Cuvinte cheie: Carotenoide, provitamine A, HPLC, cromatografie, analiza alimentelor, controlul calității

1. Introduction

Nowadays, fruits and vegetables have come into the people's attention due to their nutritional value and

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their health effects. Plants belonging to the genus *Cucurbita*, besides their considerable dietary value, host notable amounts of carotenoids in their fruits (Arima, 1988; Gross, 1991; Muntean, 2001).Consumed raw (in salads or juices) or cooked (in pies, baked etc.), they are sources of valuable nutrients in human diet.

Among micronutrients, provitamins A are especially considered by nutritionists due to their role in human health; knowing the provitamin A levels in the fruits and vegetables is an important task for using them rationally as food.

Thus, the aim of this work is to investigate the levels of provitamin A carotenoids from the fruits of *Cucurbita maxima Duch*. ("winter squash") using a new approach: liquid-liquid partition, followed by high performance liquid chromatography (HPLC). HPLC is the method of choice for carotenoids' analysis available to date (Cortes, 2004; Muntean, 2001) and usually it follows after an extraction step using organic solvents (Hidaka, 1987; Khachik, 1988; Muntean, 2003).

Such extractions are not selective and leads to a sample contamination with other liposoluble compounds, the consequences being difficult chromatographic separations. More than that, in provitamin A analysis it is not important to have the whole carotenoid profile of the sample, as only a small number of carotenoids are provitamins A. For avoiding such situation, liquid-liquid partition can be a convenient solution, as it removes unwanted ballast compounds (in the hypophase); the remaining epyphase contains finally the desired substances.

2. Materials and methods

Carotenoid standards were provided by F. Hoffman-La Roche, Switzerland. The solvents for chromatography were HPLC grade purity (ROMIL Chemicals); they were filtered through Whatman glass microfibre filters, then degassed in an ultrasonic bath, under vacuum, before use. Solvents for extraction were p.a. quality, freshly distilled.

Plant material. Ripe fruits of *Cucurbita maxima Duch*. were bought from the local marketplace of Cluj Napoca; the seeds and the placental tissue were removed, then the epicarp was peeled. The mesocarp was cut in small pieces, which were mixed and packed in sealed polyethylene bags, which were weighed and stored at -25°C until analysis.

Extraction and saponification. Carotenoids from fruits (samples between 5-10 g) were extracted in a blender using 50 mL methanol; 0.1 g butylated hydroxytoluene and 1 g $CaCO_3$ were added for avoiding oxidation and acidic isomerization during the extraction procedure. The resulting mixture was filtered under vacuum with a sintered-glass funnel and the solid material was re-extracted three times with acetone (50 mL). The resulting extract was washed ten times with distilled water, concentrated under reduced pressure in a Buchi rotary evaporator at 40°C and dissolved in 25 mL diethyl ether. It was saponified using 25 mL solution 30% KOH in methanol at room temperature for 16 hours. The unsaponifiable fraction was next extracted with diethyl ether and washed repeatedly with distilled water until free of alkali; the aqueous layers were re-extracted with small volumes of diethyl ether until colorless, then the organic layers were combined, washed several times with distilled water and evaporated to dryness under reduced pressure. The residue was dissolved in petroleum ether in an ultrasonic bath and subjected to liquid-liquid partition between hexane and a mixture of water : methanol (85 : 15, vol.); the epi-layer was partitioned repeatedly in this way until the hypo-layer was colorless.

Despite it is not necessary for provitamin A analysis, for checking purposes, in this study the resulted hypo-phases were combined and then carotenoids were transferred into diethyl ether. The final epyphase and hypophase solutions which resulted after partition were evaporated to dryness, then the residues were dissolved in 5 mL ethyl acetate and 20 μL aliquots from each were injected in HPLC system.

HPLC analysis was performed on a system consisting of: a Kontron Instruments pumping system 322, a Rheodyne 7125 injection valve with 20 μL loop, a Waters 990 photodiode array detector and a computer running Water 990 software for data analysis. Separations were carried out on by using a Nucleosil 120-5C_{18} column (250 x 4.6 mm, 5 μm particle size). Carotenoids were separated at room temperature, at a flow rate of 1 mL/min, under gradient conditions: initial conditions were 90% A, 10%B, then from 0 – 20 min. 30%A, 70% B, from 16 to 40 min. 90%A, 10%B. A is a mixture of acetonitrile : water (90:10, v/v) and B is ethyl acetate.

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The separations were monitored at 450 nm; peak identities were established by comparing their HPLC retention times and their VIS spectra with those of known reference carotenoids.

Quantification of the provitamins A was achieved by the external standard method; due to the lack of 15Z-β, β-carotene standard, the quantification of this carotenoid was based on the calibration curve for β-carotene (it has very similar spectra and chromatographic properties to that of β,β-carotene).

Visible absorption spectra were recorded on-line using the photodiode array detector of the HPLC system, being then compared with those obtained using reference carotenoids. For confirmation of the identity of individual carotenoids whose spectra were not in the library, the published maximum absorbance values (Britton, 1996) were used.

Total carotenoids were determined by VIS-spectrophotometry (Britton, 1996).

The provitamin A concentrations were expressed in retinol equivalents (RE), according to the requirements of FAO/WHO (FAO/ WHO, 1988):

1 RE = 6 μg β-carotene = 12 μg of other provitamins A

3. Results and discussion

The total carotenoid content of the *Cucurbita maxima Duch.*fruits is 85.13 μg/ g or 921.44 μg/ g dry weight, while the provitamins A level is 31.45 RE/ g dry weight.

The HPLC profile of the provitamins A is revealed in figure 1, where it is obvious that the major carotenoid is β, β-carotene; this is responsible for more than 95% of the provitamin A activity, as can be seen in table 1. In very small amounts are present other two carotenes (α-carotene and 9Z-β, β-carotene) and two xanthophylls (α–cryptoxanthin and β–crypto-xanthin). To verify the quality of liquid-liquid partition, the resulted hypophase was also checked; the corresponding HPLC chromatogram is presented in figure 2.

Table 1. Provitamins A in *Cucurbita maxima Duch*.

Peak no.	Carotenoids	Concentrations [mg/ g dry weight]
6	α - cryptoxanthin	2.21
7	β-cryptoxanthin	6.09
8	α-carotene	2.13
9	β, β-carotene	179.42
10	15Z-β, β -carotene	8.11
R.E. / g dry weight		31.45

A.U.

As the hypophase was analyzed under the same chromatographic conditions, a comparison between the chromatogram reported in figure 1 and the one from figure 2 reveals that there is no contamination of the hypophase with carotenoids from the epyphase. Five carotenoids were identified in the epyphase: a major one, lutein, and for minor ones (neoxanthin, violaxanthin, anteraxanthin and zeaxanthin), but no ones were quantified, as they have no provitamin A activity. In fact all provitamins A goes to the epyphase and for this reason the hypophase separation is useless in routine analysis of provitamin A carotenoids.

4. Conclusions

This research proved that the proposed analytical method is a good alternative to provitamin A analysis, despite it involves a supplementary step: liquid – liquid partition.

Besides the main purpose-the assessment of the provitamins A from *Cucurbita maxima Duch.*. fruits, this study allowed an overview of the chromatographic profile of the carotenoids from the above-mentioned plant matrix. Ten carotenoids were identified: two major ones (lutein and β-carotene) and eight minor carotenoids (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene and 15Z-β-carotene). From these, only five carotenoids are provitamins A: α–cryptoxanthin, β–cryptoxanthin, α-carotene, β-carotene and 9Z-β, β-carotene, but more than 95% of the provitamin A activity is due to β-carotene.

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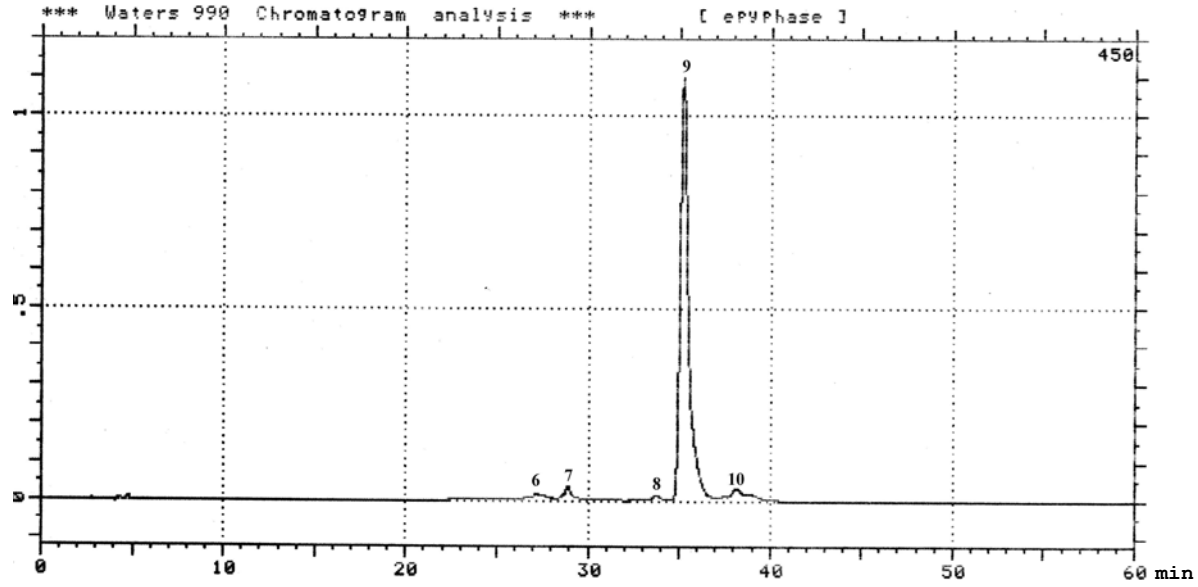

Figure 1. HPLC chromatogram of the epyphase.
Peak identities are: 6 – α-χryptoxanthin; 7 – β-cryptoxanthin; 8 – α-carotene, 9 – β,β-carotene; 10 – 9Z-β,β-carotene

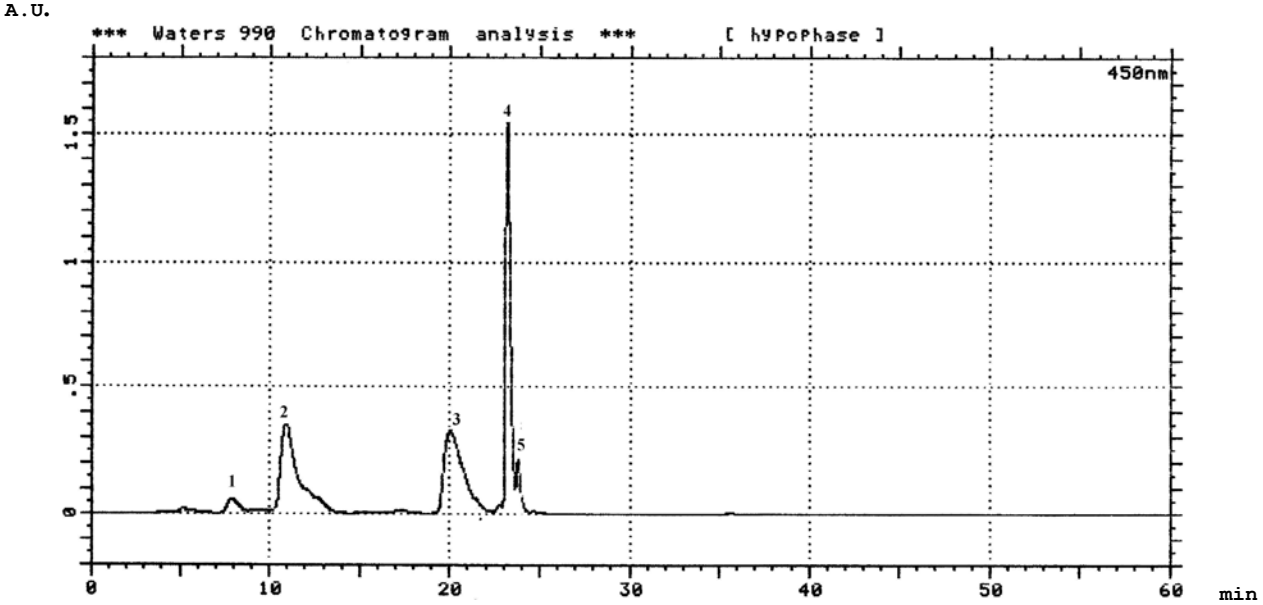

Figure 2. HPLC chromatogram of the hypophase.
Peak identities are: 1 – neoxanthin; 2 – violaxanthin; 3 – anteraxanthin; 4 – lutein; 5 – zeaxanthin.

This work also proved that *Cucurbita maxima Duch.* fruits are rich sources of provitamins A, so that they can fulfill the average daily requirement for an adult person (500 – 600 R.E./ day) which can be found in ~250 g raw fruit.

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