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CAROTENOID QUANTIFICATION OF *CUCURBITA* SPP. BY SPECTROPHOTOMETRY, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PHOTOACOUSTICS

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ABSTRACT

Background. Photoacoustic spectroscopy (PAS) is a tool for the rapid and non-destructive identification of materials even without contact. In recent years, there have been several works concerning the applicability of PAS in food analytical measurements. The intention of this work is to identify whether there is a correlation between total carotenoid and the β-carotene content of pumpkin and squash measured by high-performance liquid chromatography (HPLC), spectrophotometry (SP) and PAS.
Material and methods. 'Crown prince F1', 'Veenas F1', 'Atlas F1' and 'Apollo F1' (SAKATA) were used as experimental materials. The samples were measured in a fresh state and in a lyophilised condition with HPLC, SP and PAS.
Results. The results of SP show that total carotene content varies according to the species and variety. Lyophilisation resulted in lower, although varying carotene content compared to the raw form. Typical PA spectra of pumpkins were determined (300–550 nm), normalized to the carbon black powder. At 17 Hz the amplitude and carotene content shows direct proportionality in the range investigated. Photoacoustic (PA) signal and carotenoid content of pumpkin samples gave a linear correlation ($R^2 = 0.9821$).
Conclusion. The measurement of PA spectra gives reliable information about the total carotene content of pumpkin and squash samples. These findings may allow the use of PAS as a fast tool for the carotenoid determination in squashes and give the possibility of instead for the results to be used for the evaluation of squash varieties currently used for industrial processing in functional food development.

Keywords: carotenoid, pumpkin, squash, photoacoustics

INTRODUCTION

Carotenoids are accessory pigments that take part in the light-harvesting processes of photosynthesis, and also in protecting the photosynthetic apparatus against photo-oxidative damage (Young, 1991)

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caused by reactive oxygen species (ROS). Moreover, β-carotene is the precursor of retinol (vitamin A) and is of great importance in sight (Chichili et al., 2005). These characteristics of carotenoids make them interesting to investigate from the perspectives of human health, especially when the level of ROS may exceed the constitutive amount of antioxidants in the human body that could lead to diseases (Aruoma, 1998). The genus Cucurbita has several species, such as butternut squash (*Cucurbita moschata* L.) and pumpkin (*Cucurbita maxima* L.) and these contain a considerable amount of carotenoids even in processed forms. The amount and form of carotenes may change due to heat or chemical treatment. The majority (>75%) of the most important forms of pro-vitamin A carotenes: α- and β-carotene cane be determined (Provesi et al., 2011) in *C. moschata* L. and in *C. maxima* L., as well after cooking, being pureed and having been stored for 180 days. For this reason, the genus Cucurbita is promising as a functional food ingredient in vegetable juices, syrups, jellies, jams, and purees (Provesi et al., 2011), snacks or chips (Konopacka et al., 2010). Determining the carotenoid content of processed food products is important, since the amount of the functional compound is among the major requirements of the consumers. There are several different methods for the determination of carotenoids from plant samples (Blanco-Díaz et al., 2014; 2015; Carvalho et al., 1995; Conti et al., 2015; Iacuzzo and Costa, 2009; Seo et al., 2005).

Spectrophotometry – SP is widely used in carotenoid quantification, mostly along with the determination of other photosynthetic pigments (Blanco-Díaz et al., 2014; 2015; Fu et al., 2018; Konopacka et al., 2010; Martínez-Valdivieso et al., 2015). Near infrared spectroscopy (NIRS) is also an effective tool for carotenoid determination (Martínez-Valdivieso et al., 2015). However, high-performance liquid chromatography with a diode array detector followed by mass spectrometry (HPLC-DAD/MS) is the most widely used analytical technique (Ahamad et al., 2007; Bushway and Wilson, 1982; Conti et al., 2015; Dietz et al., 1988; Provesi et al., 2011; Seo et al., 2005), because of its precision and selectivity. Photoacoustic spectroscopy – PAS enables the rapid and non-destructive identification of materials (Scotter, 1997) even without contact. The photoacoustic (PA) signals of cytochrome and haemoglobin suggested that PAS might be useful for obtaining information from biological samples (Rosencwaig, 1973; 1981) as well. In PAS the sample is irradiated by a modulated beam of radiation and the response of the sample is detected by a microphone (Tam, 1986). The fraction of the energy absorbed by the sample is converted to heat, as a result of which the temperature of the sample oscillates periodically at a frequency identical to that of the modulated radiation itself. The thermal waves that are generated eventually reach the surface of the sample and cause periodic heating and cooling of the contacting layer of the surrounding gas. Finally, the expansions and contractions of the gas give rise to acoustic waves; these are detected as a voltage by means of the microphone. It is termed a photoacoustic signal. The optical and thermal parameters of the sample and the contacting gas all play decisive roles in the generation of the PA signal. In order to eliminate the variation in the output power on the emission wavelength of the illuminating source, it is essential to normalise the PA signal of the sample to the one obtained (under identical experimental conditions) from the carbon black powder acting as a strongly absorbing reference sample. Since the normalised signal is a ratio, it is expressed in arbitrary units [a.u.] (Rosencwaig, 1973). The main advantages of the method are that the samples that are measured do not need special sample preparation; they can be applied without the use of chemicals and extraction methods. Therefore, there have recently been several studies regarding the applicability of PAS in food analytical measurements. The anthocyanin content was determined (Dóka et al., 2011) in sour cherry cultivars with PAS, but this technique was also used successfully to determine fat-free cocoa solids in dark chocolate Dóka et al., 2013); as well as the total carotenoid content (TCC) in seven different processed tomato products using laser-based photoacoustic spectroscopy (LPAS) (Bicanic et al., 2015).

The quantification of β-carotene and total carotenoids using LPAS compared to VIS spectrometry was also reported (Dóka et al., 2015). The correlation was rather high and proved the effectiveness of this non-destructive technique. Furthermore, the anthocyanin content of hard boiled candy showed linear correlation between LPAS and VIS measurements (Kovács et al., 2017).

Earlier results showed PAS to be an effective method for the quantification of anthocyanins and carotenoids, and the PAS signals were mostly compared to VIS spectrometry (Dóka et al., 2017). The intention of this work was therefore to identify if there was a correlation between total carotenoid and β-carotene content of pumpkin and squash measured by HPLC, SP and PAS in order to offer fast and reliable carotene quantification for the industrial use of squashes as functional food ingredients.

MATERIALS AND METHODS

Plant material and culture conditions

The experimental material was as follows: 'Crown prince F1' (SAKATA; *Cucurbita maxima* Duchesne), 'Veenas F1' (SAKATA; *Cucurbita moschata* Duchesne, Duchesne *ex* Poiret), 'Atlas F1' (SAKATA; *Cucurbita moschata* Duchesne, Duchesne *ex* Poiret), and 'Apollo F1' (SAKATA; *Cucurbita moschata* Duchesne, Duchesne *ex* Poiret). The features of the hybrids are detailed in Table 1.

All the hybrids were grown extensively with direct sowing and without irrigation in Csongrád county, Hungary. The sowing time was 10 April 2016. The plant density was 11,000 plant/ha, in 1.8 m-wide beds with a spacing of 0.5 m. The amount of nutrients applied as basic fertilisers were: 120 kg nitrogen, 50 kg phosphorus and 260 kg potassium. The harvest was at the end of October.

Lyophilisation

For freeze-drying pumpkin pulp, 3.0 grams were measured in 3 parallels. Prior to lyophilisation, the weighed samples were frozen and placed, as soon as possible, in the apparatus to avoid thawing. The type of device for lyophilisation was: (Christ Alpha 1–4 LSC – Martin Christ Gefriertrocknungsanlagen GmbH, An der UnterenSöse 50 37520 Osterode am Harz, Germany). The drying process took approximately 1 day with the following pressure and temperature parameters: 0.18 mbar at –10°C for 2 h and then raised to 0°C for 2–3 h. Finally, at +10°C, the samples were evaporated for about 18 h. The measurements were immediately conducted after lyophilisation.

Table 1. Main features of the hybrids used in the experiment

Feature	'Crown prince F1'	'Veenas F1'	'Atlas F1'	'Apollo F1'
Maturity time, days	100	100	90	85–100
Skin colour	metallic grey blue	shiny orange	orange	shiny, deep orange
Fruit shape	round	pear-shaped	pear-shaped	elongated
Fruit size	medium	small–medium	large	medium–large
Fruit weight, kg	3–5	0.6–1	2–3	1.5–2
Flesh colour	deep orange	yellowish orange	orange	orange
Flavour	sweet, rich, nutty	sweet	sweet, high sugar content	sweet, high sugar content
Storage ability	excellent, up to 6 months	good, up to 4 months	good, up to 4 months	good, up to 4 months
Usage	fresh market	fresh market	fresh market and processing	fresh market and processing
Rind	thin	smooth	smooth	very smooth
Resistance	moderate: powdery mildew	moderate: powdery mildew	overall	moderate: ZYMV, PRSV
Harvest time	September–October	July	July–October	October

Chemicals

All chemicals and solvents used were analytical grade (>99%). Tetrahydrofuran (THF), methanol (MeOH) and acetonitrile (ACN) were obtained from VWR International (Radnor, Pennsylvania, United States). N-hexane was purchased from Molar Chemicals (Halásztelek, Hungary), L-ascorbic acid (99.7%, Reanal Laboratory, Hungary) and β-carotene synthetic standard from Alfa Aesar (99%, Haverhill, Massachusetts, United States).

Sample preparation for HPLC and SP measurement

3.0 grams of crushed, mixed and homogeneous pulps of raw and lyophilised pumpkin samples were measured on an analytical scale, in 3 parallel portions, in a 50--ml conical centrifuge tube, coated with aluminium foil in order to protect the sample from light degradation. 1.0 g ascorbic acid was added to the raw pumpkin. After adding 30 ml of 90:10 (v/v) MeOH:THF extraction solution, the samples were put on a mechanical shaker (KS-15 Control, Edmund Bühler GmbH) for 20 min and centrifuged (Micro 22 R, Hettich) at 4°C at 6,000 RPM for 3 min. The supernatants were transferred to screw-capped glass tubes covered with aluminium foil. The extraction process was repeated twice. The resultingsupernatants were filtered as follows: a single-use syringe (Omnifix 3 ml, B. Braun) was used with a 0.45 µm disposable syringe filter unit (MILLEX®-HN Syringe Driven Filter Unit (SLHN 013 NL, 0.45 µm, Millipore Ltd., 290 Concord Road, Billerica, MA 01921, USA).

Preparation of the standard reagent

Due to the light, oxygen and temperature sensitivity of β-carotene, preparation was carried out quickly in a dark and cool place. A 30-µg/ml stock solution was prepared, which was first dissolved in THF and after ultrasonic homogenization for 30 s in n-hexane was added in 20:80 (v/v) THF:n-Hexane ratio. Subsequently, the exact concentration of the freshly prepared stock solution was determined by a spectrophotometer at 453 nm. Calibration solutions were then prepared in a mixture of 90:10 (v/v) MeOH:hexane, where the calibration points were the following: 0.6, 1.5, 3, 6, 12 µg/ml. Hamilton syringes and two-sided glass pipettes were used to prepare the solutions.

Chromatographic conditions

The HPLC system used for the measurement was an LC-20 AV pump, a DGU 20 A3 degasser, and an SPD M20 A DAD (Shimadzu Scientific Instruments, Kyoto, Japan). The analytical column Phenomenex Kinetex C18 type (International 411 Madrid Avenue, Torrance, CA 90501-1430, USA) was a reversed phase C18 (100 mm × 2.1 mm × 2.6 µm) silica gel-coated column containing a KurdKatcher Ultra In-Linepre-column filter thermostated at 40°C. The volume injected was 10 µl. Elution was carried out in an isocratic manner. The mobile phase was 50:45:5 (v/v) ACN:MeOH:THF at a flow rate of 1 mL/min. The detector was set to 450 nm, which is the absorption maximum of β-carotene (Ahamad et al., 2007; Bushway and Wilson, 1982).

Spectrophotometric measurement

The prepared and filtered samples, as described earlier, were measured by a UV/VIS spectrophotometer (UV-1800 spectrophotometer, Shimadzu Scientific Instruments, Kyoto, Japan) on 450 nm. The measurements were done in triplicates, and the results were given as the averages and were expressed in ß-carotene equivalents (Dóka et al., 2011), a standard that is easy to acquire. The previously described calibration curve was used for quantification.

Sample preparation for photoacoustic measurement

The squash slices were crushed, mixed and homogenized by a blender. With each loading, 256 successive readings of lock-in amplifier were taken and the average value and standard deviation was calculated. The sample was then removed and the PA cell cleaned using a paper towel. The loadings were repeated three times and the average of the values measured was calculated. To record the whole spectrum of the pumpkin homogenate by the first setup, the samples were kept at room temperature until the sample was dried. Dried samples were crushed in a mortar and measured in a powdered form. The drying procedure was necessary in this case, because the intensity of the light behind the monochromator is too low (a few mW only) to give evaluable signal. This procedure of drying may lead to the loss of carotenoids (no microbiological spoilage was observed), although the spectra measured were not used

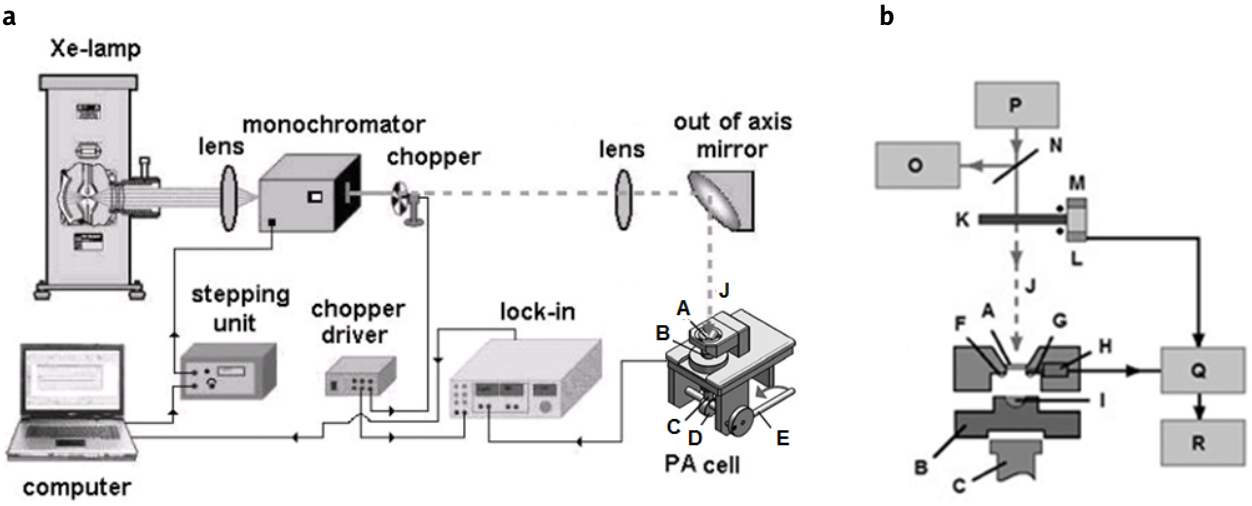

Fig. 1. The schematic diagrams of the homemade photoacoustic spectrometers with the Xe-lamp (**a**) and with the blue laser pointer (**b**): A – quartz window, B – sample holder, C – metal shaft, D – eccentric wheel, E – lever of sample holder, F – O-ring, G – capillary tube, H – microphone, I – sample, J – modulated laser beam, K – mechanical chopper, L – photodiode, M – LED, N – beam splitter, O – power meter, P – laser pointer, Q – lock-in amplifier, R – computer

for quantitative analysis. Via recording the PA spectra of the dried samples it was shown qualitatively that the analytical wavelength used by the laser is correct.

Photoacoustic spectroscopy

In PAS the sample to be investigated is irradiated by a modulated beam of radiation. A fraction of the energy absorbed by the sample is converted to heat, as a result of which the temperature of the sample oscillates periodically at a frequency identical to that of the modulated radiation itself. The thermal waves that are generated give rise to acoustic waves; these are detected by a microphone as voltages (termed photoacoustic (PA) signal).

The homemade PA spectrometer used in this study comprised a 300 W Xe lamp (Oriel Technology), a monochromator (Jobin-Yvon, H-10, spectral resolution of 16 nm), a modulator and a homemade PA cell (Fig. 1a). After passing through the monochromator, the collimated beam of mechanically chopped (17 Hz) radiation was collected by a quartz lens and focused into the PA cell. Radiation enters the PA cell through a quartz window (diameter: 12.7 mm). A 3 mm-long capillary (inner diameter 300 µm) connects the miniature electret microphone (Sennheiser KE 4-211-2) with the part of the cell that accommodates the sample. The sample volume was about 0.13 cm^3 and the surface where the modulated light was absorbed was 1.6 cm^2. The sensitivity of the microphone is 10 mV/Pa at 1000 Hz. The PA signal was processed by a dual phase lock-in amplifier (Stanford SR530) with a 3-second time constant coupled to the computer.

In another PA set-up (Fig. 1b) in this study, a blue laser pointer (MBL-III-50, power 50 mW) was used. The continuous wave power emitted by this laser at 473 nm (fixed, monochromatic radiation) is substantially higher than that expected from the continuously tunable Xe lamp used as a source in the monochromator mentioned above. Because of the direct proportionality between the magnitude of the PA signal and the incident power of the excitation source, the availability of a strong radiation source results in a better signal.

RESULTS AND DISCUSSION

HPLC and SP measurements on raw and lyophilised samples

Since HPLC is a precise and widely used measurement for carotenoid quantification, as a first step, HPLC measurements were performed on raw samples, providing a basis for verifying the results of SP and

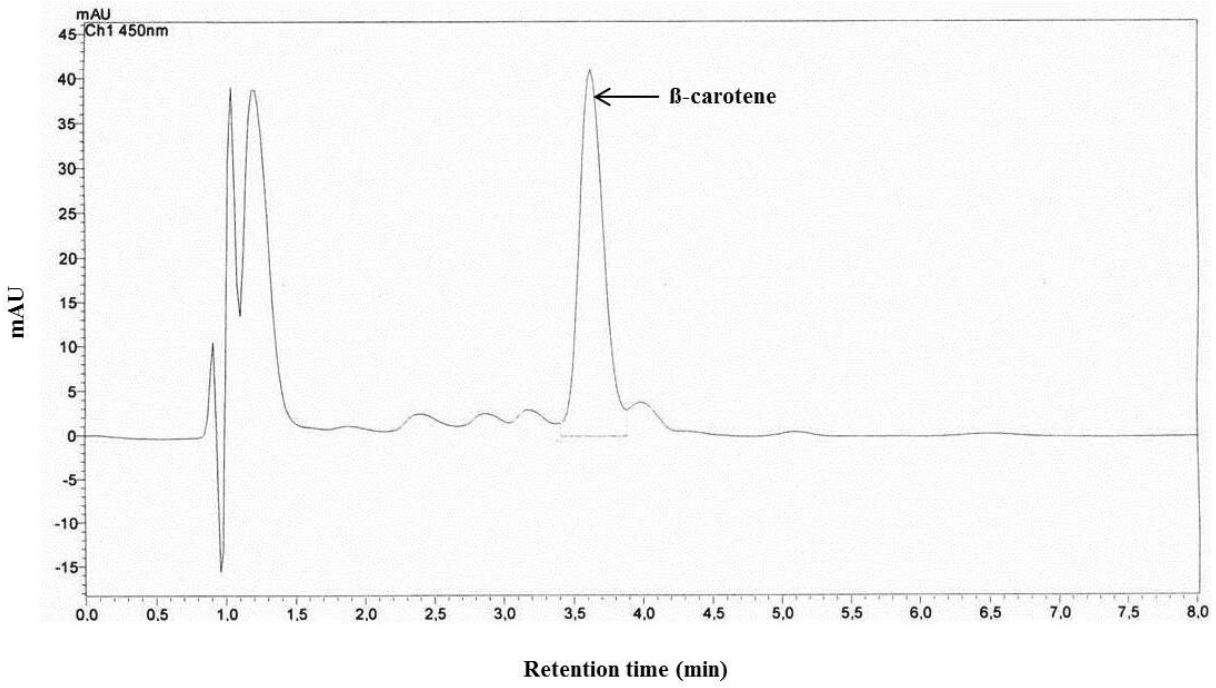

Fig. 2. HPLC chromatogram of β-carotene determination

PAS measurements. Figure 2 shows a typical chromatogram of β-carotene determination by HPLC.

As a second step, the SP measurements were carried out at 453 nm on the very same raw samples used in the HPLC measurements. To evaluate these results, total carotene content determination values were used, since currently PAS is not a compound-selective technique and correlates with total carotene content. The total carotene content of the samples showed a similar pattern as for the HPLC measurement, although the measured values were higher (Fig. 3).

The lowest total carotene content is expressed as mg/100 g fresh weight (FW) basis. The results present two samples from the same hybrids consecutively (e.g. Crown prince F1-1, Crown prince F1-2). In 'Crown prince F1' the total carotene content was (5.90; 4.78 mg/100 g FW), the highest values were obtained in 'Veenas F1' (13.29; 10.27 mg/100 g FW), whereas in 'Atlas F1' (7.75; 5.67 mg/100 g FW) and 'Apollo F1' (7.47; 5.49 mg/100 g FW) the values obtained were lower (Fig. 3).

Afterwards, the lyophilised samples were measured by HPLC. The results showed a different pattern of the preservation of total carotene content. Lyophilisation resulted in lower concentrations of carotene in all samples compared to the raw condition of Cucurbita, except for Atlas II, which may be due to the degradation of the carotene content of pumpkins and squashes as the result of the drying process itself. Song et al. (2017) found that microwave freeze-drying of pumpkin pulp results in the degradation of lutein, α-, and ß-carotenes.

The lowest lyophilised carotene content (Fig. 2) was found in 'Crown prince F1' (4.93; 4.80 mg/100 g FW), the highest was the 'Veenas F1' (10.26; 14.08 mg/100 g FW), and 'Atlas F1' (7.35; 5.76 mg/100 g FW) and 'Apollo F1' (6.43; 5.16 mg/100 g FW). 'Veenas F1' has of 79% β-carotene compared to that of the raw one (Fig. 2), whereas 'Crown prince F1' has 92% and 'Atlas F1' 98.3% and 'Apollo F1' 90%.

The carotene content determination revealed distinct differences among the carotene content of varieties for raw and for lyophilised samples as well. 'Veenas F1', 'Apollo F1' and 'Atlas F1' are squash (*Cucurbita moshata* L.) types that normally have a more intensive orange colour compared to *C.*

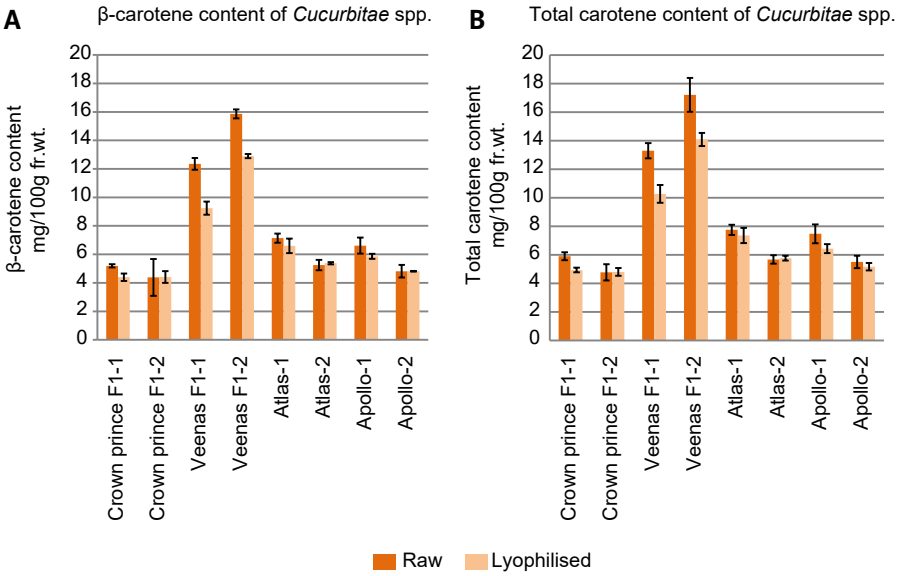

Fig. 3. Changes in β-carotene content of three raw and lyophilised *Cucurbitae* varieties determined by HPLC (**A**). Changes in total carotene content of three raw and lyophilised *Cucurbitae* varieties determined by SP (**B**). Data represent the averages and the standard deviations of triplicate measurements

maxima. The carotene content of all three squash types was higher than 'Crown prince F1'. 'Veenas F1', however, turned out to have the highest β-, and total carotene content, even in lyophilised form. From an industrial point of view, the results of the lyophilised β-carotene content of the samples show that 'Veenas F1' is probably the best variety for processing, because its β-carotene and the TCC content of this genotype is relatively high in its raw form, but also remains high after lyophilisation. These findings may be important for the utilisation of this hybrid, since previously it was mostly used as a fresh market product (Table 1). However, these results indicate its good processing perspectives as well.

Photoacoustic measurement on raw and lyophilised samples

As the next step, the PA spectra of the samples were recorded. Figure 3 shows the typical PA spectra of three dried pumpkin samples between 300 nm and 550 nm, normalized to the carbon black powder. All eight pumpkins produced PA signals but the spectra of the samples practically overlapped each other; therefore one sample with low-, one with moderate- and one sample with high ß-carotene content was selected as representative for this group. The normalized PA spectra of the three samples mentioned were clearly distinguished. There was only one characteristic wavelength band in the spectral range investigated. However, the PA signal decreased with an increasing wavelength around 475 nm, and a characteristic peak representing the carotenoids of the pumpkin samples occurred.

In the PA spectroscopy, the signal increased linearly along with the absorbed light intensity. Therefore, a diode laser which emits ten times higher light intensity than the Xe lamp at 473 nm was used. The use of a monochromator was not necessary in that case. Despite the fact that this wavelength does not coincide with the maximum of the PA signal (478 nm), the PA signal increases significantly with the carotenoid content; this resulted in a better signal-to-noise ratio. Figure 4 presents the normalized amplitude of the PA signal to the power of the laser at 473 nm plotted against the TCC (determined by SP) in raw pumpkin. Each point is the average of three consecutive measurements. The experimental geometry and the modulation frequency were the same in all PA experiments.

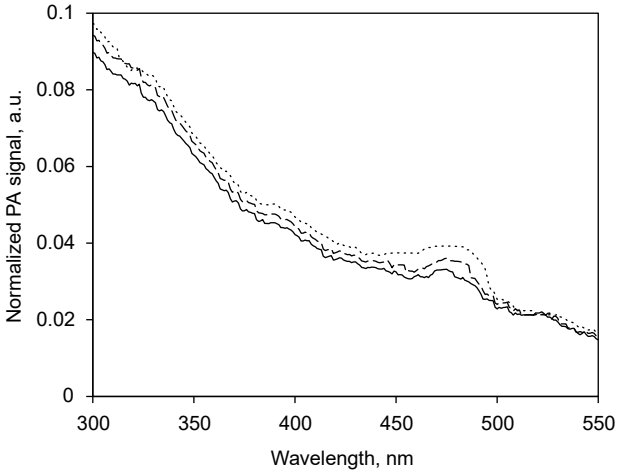

Fig. 4. Typical normalized PA spectra of lyophilised pumpkin samples between 300 and 550 nm: 'Crown prince F1' – continuous line (——), 'Veenas F1 2' – dashed line (- - - -), 'Atlas F1' – dotted line (·····)

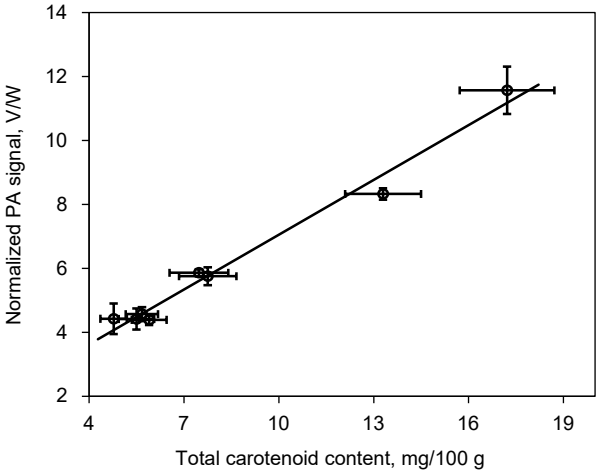

Fig. 5. The PA signal (obtained at 473 nm and 17 Hz) plotted versus the total carotenoid content (determined by spectrophotometry) of raw pumpkins. Data represent the averages and the standard deviations of triplicate measurements

As for the power of the laser changed during the measurements, the values are expressed in VW^{-1}.

At 17 Hz the relationship between the amplitude and carotene content was directly proportional in the range being investigated.

There was a linear correlation (R^2 = 0.9821) between the PA response and the carotenoid content of the pumpkin samples (Fig. 4). Finally, the lyophilised samples were measured photoacoustically using a diode laser. Figure 5 shows the normalized PA signal at 473 nm versus the TCC. The experimental conditions were the same as previously.

The correlation between the PA signal and the TCC was linear again with a rather good coefficient of determination (R^2 = 0.983). The obtained signal was conspicuously higher than on the fresh pumpkin samples. The main reason is the internal reflection of the samples that increases in the lyophilized samples, while in fresh samples such a reflection is absent. The slope of the fitted line was four and a half times higher than of the fresh samples. The use of a monochromator was not necessary in that case. Despite the fact that this wavelength does not coincide with the maximum of the PA signal (478 nm), the PA signal increases significantly with the carotenoid content; this resulted in a better signal to noise ratio.

Finally, the lyophilised samples were measured photoacoustically using a diode laser. Figure 5 shows the normalized PA signal at 473 nm versus the TCC. The experimental conditions were the same as previously. The correlation between the PA signal and the TCC was

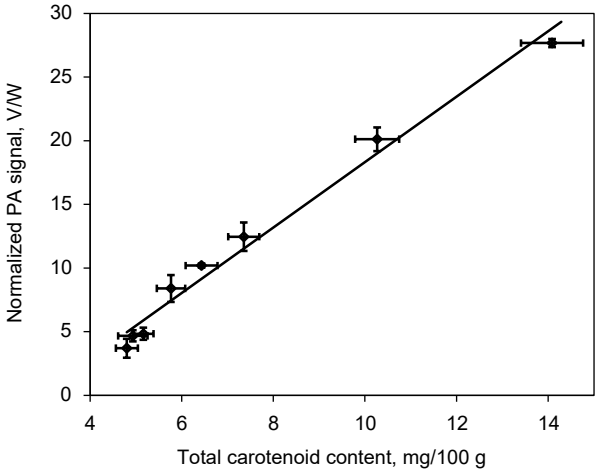

Fig. 6. The PA signal (obtained at 473 nm and 17 Hz) plotted versus the total carotenoid content (determined by spectrophotometry) of lyophilised pumpkins. Data represent the averages and the standard deviations of triplicate measurements

linear again with a rather good coefficient of determination (R^2 = 0.983). The signal obtained was conspicuously higher than with the fresh pumpkin samples.

The main reason is the internal reflection of the samples, which increases in the lyophilised samples, while in fresh samples such a reflection is absent. The slope of the fitted line was four and a half times higher than for the fresh samples.

Earlier works have been conducted in order to elucidate whether the LPAS technique is applicable for rapid and simple quantification of the total carotene content of apricot jams (Dóka et al., 2013) and these have found a linear correlation between the changes in total carotene content and the PA signal.

The PAS correlated linearly with the TCC R^2 = 0.9821 (Fig. 4). This result is in accordance with the findings of other authors: the changes of a well-known factor were in connection with the changes in PAS signals: for carotenoids in tomato (Bicanic et al., 2015) and in apricot jam (Dóka et al., 2015), for the anthocyanin content of hard boiled candy (Kovács et al., 2017) and for buckwheat grain (Dóka et al., 2017).

CONCLUSIONS

Measurement of PA spectra gives reliable information about the total carotenoid content of pumpkin and squash samples since measurements of PA signal from 300 nm to 500 nm showed an absorption peak at 473 nm of three samples with different carotenoid content (Fig. 3). Photoacoustic – PA also has the advantage of measuring without chemical extraction compared to the most used VIS spectrometric and HPLC measurements. Nevertheless, the results indicate the possibility of distinguising TCC among varieties by PAS in further studies. These findings may allow the use of PAS as a fast tool for carotenoid determination in squashes and provides a possibility for the results can be used to evaluate currently used squash varieties for industrial processing in functional food development.

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