

Carotenoid composition and content in products of sea buckthorn and peach as determined by high-performance liquid chromatography

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Summary: A study was conducted to analyse the carotenoids by high-performance liquid chromatography (HPLC) using reversed-phase cross linked end-capping and to study the pigment content and composition in sea buckthorn products as well as in freshly harvested fruits from peach trees grown under organic and integrated farming conditions. It was found that carotenoids in fruits of both crops occur mainly esterified with fatty acids in form of mono- and di-esters. The major carotenoids were esters of zeaxanthin, lutein, β -cryptoxanthin and β -carotene. The major carotenoids showed different response to processing of sea buckthorn being highly sensitive to thermal treatments such as blanching, cooking and drying. Significant differences were found between peach varieties in their carotenoid content, whereas the impact of organic farming on carotenoids formation was found to be variety-dependent. Two of the three varieties examined in this work, when cultivated under organic farming conditions contained lower carotenoid level as compared to that found in the fruits of the same varieties but produced in integrated farms.

Keywords: sea buckthorn, peach, carotenoids, liquid chromatography, processing, organic farming

Introduction

Fruits of sea buckthorn and peach are among those having special importance from nutritional therapeutic points of view. Such fruits have considerably high content of bioactive compounds beside carbohydrates, proteins, lipids, amino acids and vitamins (Geetha et al., 2002; Guliyev et al., 2004). Carotenoids, as natural colorants and bioactive nutrients, have received an increasing interest during the last decades. The reactive oxygen species-scavenging activity of carotenoids and other natural antioxidants has been found to be associated with reduced risk of serious diseases including cancer (Keleman et al., 2006; Larsson et al., 2010), cardiovascular disease (Arab and Steck, 2000) and age-related degeneration and disorder caused by the low concentration of macular pigment (Bone et al., 2000; Mozaffariech et al., 2003).

At post-harvest of plant crops, particularly during processing, carotenoids undergo some chemical alteration, such as isomerisation and oxidation on their molecules (Niizu and Rodriguez-Amaya, 2005). Such a chemical alteration can change, to a considerable extent, their chemical and biological properties (Granado et al., 1992; Molnár, 2009). Under certain biological conditions all-trans to cis geometrical isomerisation occur not only in processed foods but also in human serum (Pérez-Gálvez and Mínguez-Mosquera, 2005).

The diversity of carotenoid extract from some plant products is due to the formation of a large number of oxygen-containing xanthophylls, which occur esterified with fatty acids in form of mono- and di-esters (Biacs et al., 1989; Biacs and Daood 1994; Mínguez-Mosquera and Hornero-Méndez, 1994; Pérez Gálvez et al., 1999). Fatty acid esters of carotenoids differ substantially in their chemical and chromatographic properties from that of free ones. Depending on fatty acid moiety antioxidant activity of carotenoid esters may or may not be altered (Mustafuji et al., 1998; Pérez-Gálvez and Mínguez-Mosquera, 2002; Subagio and Morita, 2001 and 2003). To simplify the carotenoid extract, the pigment is often subjected to alkaline hydrolysis (saponification) to hydrolyse fatty acid esters and to dissociate their bounds with the macromolecules such as proteins (Ágos et al., 2007; Meléndez-Martínez et al., 2007; De Faria et al., 2009). However, conventional gradient elution is recommended to separate the individuals of carotenoid pigment even in saponified extracts.

The objective of the present work was to study the carotenoids composition of sea buckthorn, by applying recent liquid chromatographic (HPLC) method with the use of adsorbent having cross-linked C18 end-capping and gradient elution of water in acetone. Also, the comparison with the carotenoid composition of peach fruit and studying the changes in carotenoid content and composition of sea buckthorn as a function of processing was aimed.

Materials and methods

Materials

Fresh, frozen and processed sea buckthorn products were from Bio-Drug-Berta Ltd. (Fajsz, Hungary), while the fruits of peach were obtained from the experimental fields of the University of Debrecen (Debrecen, Hungary). Standard materials of all-trans β -carotene, lutein, Zeaxanthin, and lycopene were purchased from Sigma-Aldrich (Budapest, Hungary). β -cryptoxanthin and was obtained with the courtesy of the Institute of Bio- and Medical Chemistry of the University of Péch (Hungary). HPLC- and analytical grade organic solvents were from Merck (Darmstadt, Germany). A cross-linked Nucleodur ISIS C-18 UPLC column was from Machery-Nagel GmbH & Co. (Düren, Germany).

The freshly harvested fruits of both crops were cut into small pieces by a stainless steel knife and stored at -20°C in a vacuumed nylon sacs when not immediately analysed

Methods

Extraction of carotenoids

Five grams of fresh or processed products were crushed in a crucible mortar in the presence of 1 g of quartz sand and 0,5g ascorbic acid. The extraction procedure started with macerating with methanol to bind the water. The mixture was left to stand for few minutes and the supernatant was carefully decanted into a 100 ml Erlenmeyer flask. The residues in the mortar were further crushed with gradual addition of 50 ml of 1,2-dichloroethane-methanol (6:1) and the mixture was transferred to the flask containing methanol fraction. The liquid-liquid phase separation of carotenoids was initiated by adding few drops of water and mechanical shaking for 15 min at room temperature according to a previously described procedure (Biacs and Daood, 1994). The lower phase containing carotenoids was separated in a separating funnel, dehydrated on anhydrous Na_2SO_4 and evaporated under vacuum at maximum 40°C . The residues were re-dissolved in pure acetone and passed through a 0,45 μm PTFE syringe filter aid before injection onto HPLC column.

To saponify the carotenoid pigment, the extracted pigment was re-dissolved in 20 ml petroleum ether and subjected to alkaline hydrolysis with 20 ml of 30% KOH in methanol for 1 h at dark place. Carotenoids were then extracted with ethyl acetate, which was pooled and washed with double distilled water. The organic layer was then dried on anhydrous Na_2SO_4 and evaporated to dryness under vacuum at 40°C . The pigment was re-dissolved in HPLC grade acetone before injection.

HPLC conditions and instrument

A Waters Alliance liquid chromatographic instrument

consisting of a Model 2695 Separation Module (Gradient pump, auto-sampler and column heater) and a Model 2975 Photodiode-Array Detector was used. Operation and data processing were performed by Empower software.

Separation of carotenoids was performed on Nucleodur ISIS, 3 μm , 15 cm \times 4,6 mm, column with gradient elution of (A) water and (B) acetone. The elution started with 20% A in B, which changed to 10% A in B in 7 min then to 5% A in B in 5 min and stayed isocratic for 5 min and turned to 20% A in B in 3 min. The flow rate was 0.7 ml min^{-1} . Detection of carotenoids was performed between 200 and 700 nm. For quantification, each compound was detected at the maximum wavelength of its light absorbance.

Stock solutions for lycopene, zeaxanthin and β -carotene were prepared by dissolving 1 mg of standard materials (Sigma, St. Lo. USA) in 10 ml of petroleum ether using brown-coloured volumetric flasks. Working solutions, between 0 and 100 $\mu\text{g/ml}$, were prepared by dilution with HPLC acetone and injected onto the HPLC system. The recorded peak areas of standard solutions was Plotted versus concentrations to get the calibration curve using Microsoft xls 2007 program

Identification: The peaks were identified by comparing their spectral characteristics and retention times with those of standard materials and with literature data when available (Bauernfeind, 1981). The identification of any individual of carotenoids basing on only retention and spectral characteristics is considered tentative unless confirmed by standard or structural elucidation means such as mass spectroscopy (MS) or magnetic nuclear resonance (NMR).

Statistical analysis

Standard deviation and One-way analysis of variance (ANOVA) (Minitab statistical program) was performed to statistically analyse the differences between the samples.

Results and discussion

Carotenoid composition

HPLC profiles carotenoids from hydrolysed and unhydrolysed extract of sea buckthorn are shown in Figure 1 and 2. In such a crop, carotenoids occur mainly esterified with fatty acids in form of mono- and di-esters. Zeaxanthin was found dominant carotenoid followed by β -cryptoxanthin, β -carotene and mutatoxanthin. The fatty acid esters of the main xanthophylls were dominant with a ratio of esterified/unesterified was over 9/1. In general these results agree with the results of HPLC analysis of carotenoids from sea buckthorn fruits, which has been reported by Guliyev et al., (2004). The authors reviewed that fresh fruits distribute mainly carotenes and palmitat esters of zeaxanthin and β -cryptoxanthin.

As minor individuals, γ - γ carotene, lycopene, rubinaxanthin and some of their epoxides and cis isomers could also be detected and tentatively identified. The

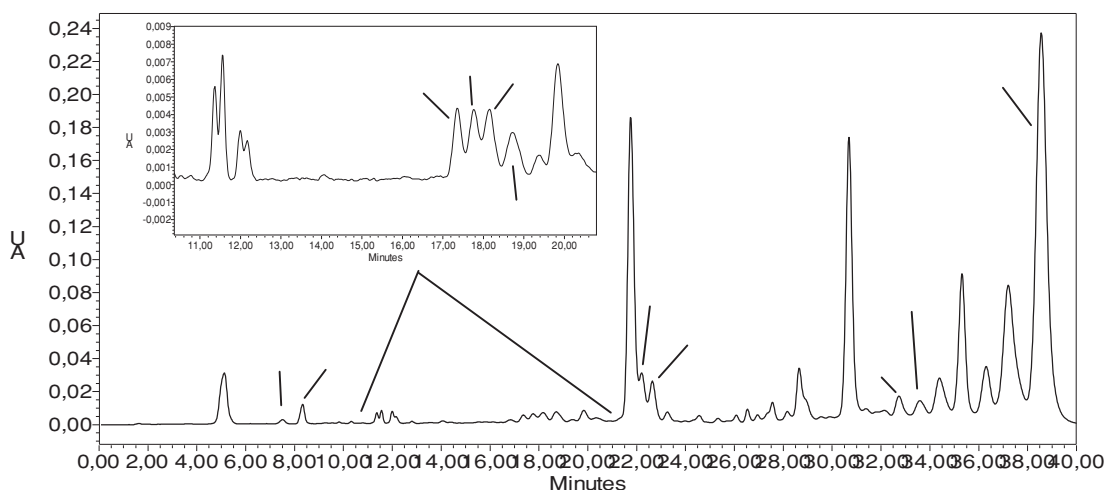


Fig.1: HPLC profile of carotenoids from un-hydrolysed extract of sea buckthorn. 1: zeaxanthin, 2,3: cis-zeaxanthin, 6: β -cryptoxanthin, 7: lycopene epoxide, 8: γ - γ -carotene, 9: lycopene, 10: cis-lycopene, 11: rubixanthin, 12: β -carotene, 13: 9Z- β -carotene, 14: 13Z- β -carotene, 15, 16: β -cryptox. ME, 17, 18, 19, 21: mutatoxanthin. DE, 20,23: zeaxanthinDE, 22: lutein DE

performance of the applied HPLC method was high enough to make it capable to separate not only the different esters of the different xanthophylls, but also the 9- and 13- cis isomers of the major carotenes, xanthophylls and their fatty acid esters. Since cis isomers were detected in both hydrolysed and un-hydrolysed sea buckthorn extracts, it can be concluded that biochemical pathway of cis-trans isomerisation is activated, to some extent, during ripening and post-harvest handling of such a product.

In case of peach (*Fig. 3*), the pigment consists of mainly β -carotene and fatty acid esters or free forms of violaxanthin, lutein, zeaxanthin and cryptoxanthin. The ratio of esterified/unesterified carotenoids was found to be around 2.5/1, which is well below the value found in sea buckthorn as mentioned before. The difference in degree of esterification of xanthophylls with fatty acids may affect colour intensity and stability of the pigment in different fresh or processed products of such crops since the fatty acid esters of xanthophylls have been found to be more stable than the free

forms towards oxidative degradation (*Biacs et al., 1992; Subagio and Morita, 2003*).

It is worthy to mention that most of carotenoids of sea buckthorn and peach are biologically active and may attribute to the therapeutic and immune-modulating properties of such fruit, which has been confirmed by some studies (*Guliyev et al., 2004*). Also, it is to be mentioned that occurrence of lutein, zeaxanthin and β -cryptoxanthin as fatty acid esters in fruits or vegetables does not reduce the biological activity of such carotenoids because mono- and di-esters can be hydrolysed by active de-esterase enzymes that function in the human intestine (*Pérez-Gálvez and Mínguez-Mosquera, 2005*), and moreover, antioxidant activity of carotenoid esters containing saturated fatty acids is almost the same of the correspondent free forms (*Matsufuji et al., 1998; Subagio and Morita, 2001*).

Effects of technology

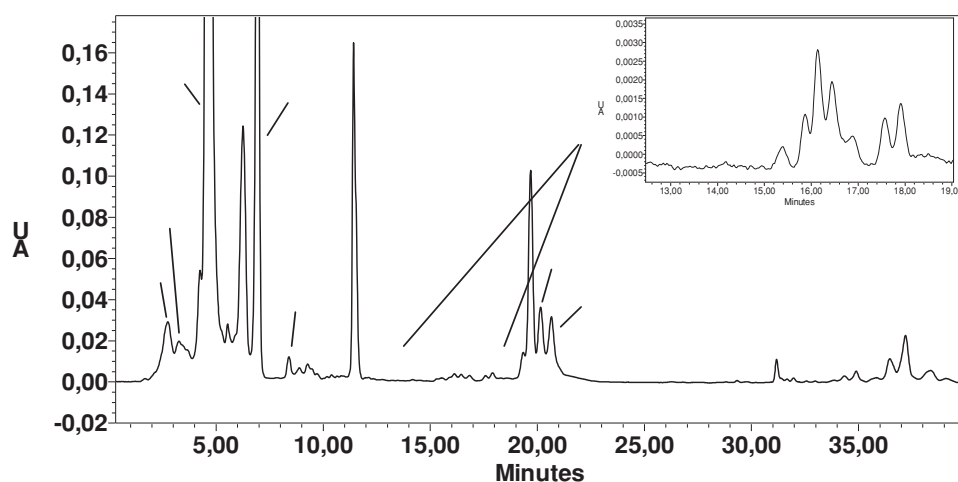


Fig 2: HPLC profile of carotenoids from hydrolysed extract of sea buckthorn. 1: mutatoxanthin, 2: lutein, 3: zeaxanthin, 4, 5: cis-zeaxanthin, 6: β -cryptoxanthin, 7: β -cryptoxanthin, 8: lycopene-epoxide, 9: γ - γ -carotene, 10: lycopene, cis-lycopene, 12, 13: rubixanthin, 14: β -carotene, 15: 9Z- β -carotene, 16: 13Z- β -carotene

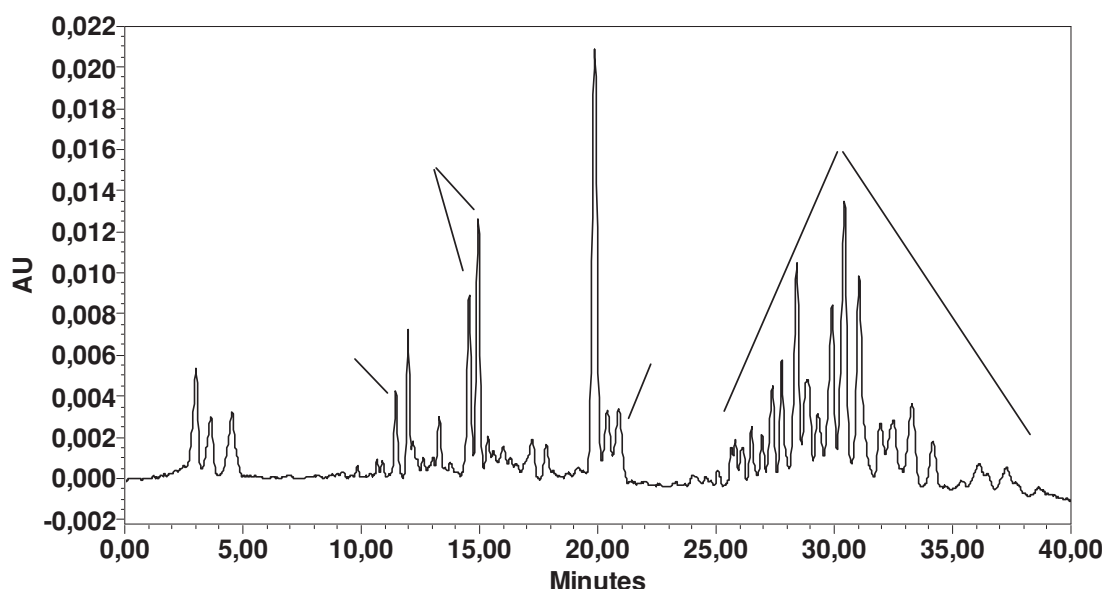


Fig 3: HPLC profile of carotenoids from ripe peach fruit. 1: lutein epoxide 2: zeaxanthin, 3: β -cryptoxanthin, 4: Zeaxanthin monoester, 5: Lutein monoesters, β -carotene, 7: 9Z- β -carotene + ζ -carotene, 8: 13Z- β -carotene, 9: xanthophyll diesters

Processing of sea buckthorn

Sea buckthorn is often harvested at biological ripeness stage when the fruits turn yellow-orange in colour. Because of being climacteric fruit, biochemical processes including carotenoid biosynthesis are expected to continue in biological ripeness stage, which should proceed towards technological ripeness or over-ripeness. At the later stage flavour, colour and taste of the fruits should approach the desired level for both fresh consumption and processing.

Table 1 shows the carotenoid content of freshly harvested fruits and processed products of sea buckthorn produced in the previous seasons (2009) in addition to one product (nectar) stored for 5 years in dark and cool store. The HPLC analysis of carotenoid composition of sea buckthorn extracts showed that content, in dry matter, of free zeaxanthin is significantly lower and that of β -carotene and all of the di-esters is higher in fresh fruits than their content found in the dry matter of the frozen fruits and nectar produced at the same season. This is most likely due to probable variation in ripening stage of fresh fruits taken for analyses and fruits subjected to different processing. It is to be mentioned that due to the limited capacity of the laboratories it was not possible to immediately analyse the fresh fruits, which were kept at cool store at 5–10 °C for 3 days after harvest. It seemed that at such conditions fruits preceded in ripening towards full or

even over-ripeness, at which carotenoid biosynthesis and esterification with fatty acids can be accomplished. In pilot plants and factories such ripeness cannot be ensured with great batches of this crop to be processed.

Among products of 2009, the lowest level of total and some individuals of carotenoid pigment of sea buckthorn fruits was found in freeze-stored samples, in which the total carotenoid content was significantly lower ($p=0.01$) by approximately 34% as related to the content in fresh fruits or

Table 1: Carotenoid content of different products of sea buckthorn

Karotinoidok	Concentration ug/100g dry matter					
	Fresh	Frozen	Nectar	Nectar 2004	Jam	Ground product
Zeaxanthin	292±	638±	599±	651±	118±	607±
9-Z zeaxantin	10±	tr	135±	148±	6±	tr
13-Z zeaxantin	19±	tr	161±	67±	12±	35±
β -cryptoxanthin	17±	49±	71±	33±	3±	4±
Mutatoxantin ME	60±	134±	80±	71±	7±	ny
Rubixanthin	10±	39±	65±	48±	10±	5±
Lycopene	15±	86±	123±	18±	8±	tr
$\gamma\gamma$ - carotene	67±	407±	411±	106±	11±	tr
β -carotene	1315±	2561±	2071±	476±	206±	566±
9-Z β -carotene	55±	99±	134±	60±	20±	40±1
13-Z β -carotene	43±	206±	501±	118±	12±	25±
Mutatoxantin DE	517±	530±	591±	454±	19±	30±
β -cryptoxanthin ME	1265±	786±	955±	478±	69±	138±
Zeaxanthin DE	16125±	5485±	11028±	6279±	1184±	938±
Cisz-zeaxanthin DE	168±	112±	2199±	1873±	127±	tr
Lutein DE	1630±	422±	668±	548±	105±	108±
Total carotenoid	17070±2	11444±	17860±	13344±	1834±	5058±

nectar. The impact of processing treatments as well as ripening stage of fruits may stand behind the reason for the low level of carotenoids in freeze-stored product.

Cold-break extraction followed by passing through a sieve and heat treatment at 100 °C for 10 min to produce the nectar was found to have no significant effect on the total carotenoid content although substantial variation was noticed in the proportion of the individuals of carotenoid pigment as mentioned before. In case of nectar stored for 5 years at cool store, the content of most of carotenoids, except that of zeaxanthin, was significantly lower ($p=0.05$) than that determined in nectar samples produced in 2009. Since nectars were stored in a glass jars without removal of molecular oxygen, the loss of carotenoids was expected to be taken place as a function of oxidative deterioration.

Great destruction of carotenoids was also expected as a function of drastic heat treatments applied in the preparation of jam, which contained about 10% of the total carotenoids found in fresh fruits. In addition, dilution with added sugar and pectin should be considered as an additional reason for the high loss of carotenoids in jam samples from sea buckthorn.

The carotenoid composition and content of ground product of sea buckthorn is different from that of fresh fruits, nectars and jam because this product consists of branches, leaves and fruits of the crop. The purpose of preparation of such a mixture was to offer a product with increased medicinal effect exploiting the high biological and therapeutic properties of leave and branch extracts (Geetha et al., 2002; Gyliyev and Yildirim, 2004). The product is prepared by washing and thermal drying followed by course grinding of fruits, leaves and branches of sea buckthorn. Although addition of leaves and branches had a dilution effect on the carotenoids of the fruits it caused the concentration of Zeaxanthin to be at close level to that determined in frozen fruits or nectars and level of β -carotenes to be 20–25% of that recorded for the best nectar despite high degradation usually takes place on carotenoids as a result of thermal drying.

As a function of thermal treatments either in blanching before freezing or sterilization after cold break extraction in nectar processing, marked increase was recorded for cis-forms of both carotenes and xanthophyll esters. It has been repeatedly mentioned in literature that carotenoid degradation starts with all-trans to cis isomerisation, which is usually initiated by heat treatments in food processing (Shierle et al., 1997; Nguyen and Schwartz, 1998; Faulks and Southon, 2005; Fratianni et al., 2010). However, in case of nectar produced in 2004 and stored in a cool store for 5 years, the content of β -carotene and its cis- isomer was lower than that of the other products

revealing the sensitivity of these compounds to oxidative stress during un-controlled storage of such a product.

Cultivation technology and variety of peach

In a project investigating the effect of organic and integrated farming on the carotenoid content of different varieties, samples of ripe fruits were taken for analysis in two seasons (2008 and 2009). As shown in Table 2 the different varieties differ substantially in their carotenoid content, but not in the pigment composition. All the varieties listed in the table are of yellow-coloured pulp. Those varieties of white or faint green pulp were excluded from the analysis of carotenoids. The range estimated for the total carotenoids content was 0.42–0.98 mg per 100 g edible portion, which is close to the average found for peach varieties of British Columbia tree, and lower than the 1.2–5.4 mg/100 g recorded for Australian canning clingstone varieties (*Bauernfeind*, 1981).

In the two different seasons, the impact of organic farming on carotenoid content was found to be variety-dependent. With Early Redhaven and Panada varieties carotenoid content of peach from organic farming was significantly lower ($p=0.05$) than that of fruits from integrated farms, while opposite results were obtained with Cresthaven, which contained significantly higher carotenoid level ($p=0.01$) when

Table 2: Content of carotenoids in fruits of different peach varieties cultivated with organic and conventional farming conditions

Component	Concentration ug/100g						
	Early RH Integ.	Early RH Bio	Padana Integ.	Padana Bio	CrH Integ.	CrH Bio	Sweet Lady Integ.
Lutein-epoxide	11,4	9,1	34,4	20,1	10,4	82,2	200,6
Zeaxanthin	21,1	16,2	23,4	19,0	18,5	15,2	14,2
Zeaxanthin ME	3,1	2,4	1,8	0,6	0,4	0,6	2,1
β -cryptoxanthin	24,6	18,6	35,1	13,5	14,9	36,4	48,0
Lutein ME	25,3	22,0	72,1	19,7	15,4	18,7	57,6
β -carotene	77,2	67,7	190,1	104,6	130,5	174,7	216,3
β -cryptoxanthin ME	131,2	107,6	180,1	72,0	100,2	129,8	115,4
Zeaxanthin DE	15,7	15,1	30,2	12,8	12,7	10,6	11,6
Lutein DE	144,6	112,3	332,5	126,8	97,1	110,9	209,4
Violaxanthin DE	71,3	55,6	88,0	41,0	47,3	53,6	77,0
Total	525,5	426,5	987,6	430,2	447,5	632,6	952,2

cultivated under organic farming conditions. As regards the response of the individual compounds to the type of cultivation technology, lutein epoxide and β -cryptoxanthin exhibited the higher response than that noticed with the other carotenoids that were moderately responding to the change in the cultivation conditions.

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