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## HPLC analysis of carotenoids in particular carrot (*Daucus Carota* L.) cultivars

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Optimal conditions for analysis of carotenoids in carrot by HPLC, including sample preparation, were selected. Chromatograph Agilent 1200 with diode array detector was used. The sample components were separated on column ZORBAX Eclipse Plus C18 (3.0×100 mm; 1.8 μm) at temperature 22 deg C. The temperature in the autosampler was 10 deg C, injection volume — 2 μl. The isocratic solvent system consisting of acetonitrile — methanol — ethyl acetate (73:20:7, v/v/v) was chosen as the mobile phase. The flow rate of eluent was 0.4 ml/min. It is shown that 50 ml of acetone was needed for extraction of carotenoids from the 5 g of homogenized carrot samples (10-multiple excess). Separating capacity of the solvents (acetone, the mixture acetonitrile — methanol — ethyl acetate, dichloromethane) and their compatibility with the mobile phase was investigated. It was established experimentally that the acetone is the best solvent to recover dry residue of pigments. Developed method was used for the study of carotenoids in eleven carrot cultivars, zoned in Belarus. The main carotenoids of carrots were distributed as follows: α-carotene — 30–40%, β-carotene — 55–68%, lutein — 1–6% of carotenoid total amount. Cultivars Vitaminnaya-6 and Dordon were characterized by significant amount of β-carotene (90–100 μg/g) and α-carotene (53–61 μg/g). The largest quantity of lutein (4.3–6.5 μg/g) was contained in cultivars Dordon, Morelia, Nerac and Niland. The highest content of carotenoids (~155 μg/g) was observed in cultivars Dordon and Vitaminnaya-6. Carrot cultivars grown in Belarus were characterized by average contents of β-carotene, but the carotenoid total amount was high. Described HPLC method can be applied for the determination of the main carotenoids of carrot.

**Keywords:** carotenoids, analysis, HPLC method, carrot, cultivar.

## Анализ каротиноидов методом ВЭЖХ в отдельных сортах моркови

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Установлены оптимальные параметры анализа каротиноидов моркови методом высокоэффективной жидкостной хроматографии. Применен хроматограф Agilent 1200 с диодно-матричным детектором. Разделение компонентов произведено на колонке ZORBAX Eclipse Plus C18 (3.0×100 мм; 1.8 мкм) при температуре 22 град. С. Температура автосамплера — 10 град. С, объем инъекции — 2 мкл. В качестве подвижной фазы использована смесь растворителей: ацетонитрил: метанол: этилацетат (73:20:7, об/об/об) со скоростью потока 0.4 мл/мин. Показано, что полная экстракция пигментов из 5 г моркови достигается при 10-кратном избытке ацетона. Исследована разделяющая способность и совместимость с подвижной фазой растворителей (дихлорметана, ацетона, смеси ацетонитрил-метанол-этилацетат) для восстановления сухого остатка каротиноидов. Экспериментальным путем установлено, что наилучшим растворителем является ацетон. Разработанный метод применен для исследования качественного и количественного состава каротиноидов в 11 сортах моркови, районированной на территории Республики Беларусь. Установлено соотношение основных каротиноидов моркови, которое составляет: α-каротин — 30–40%, β-каротин — 55–68%, лютеин — 1–6% от общего количества. Исследования показали, что высоким содержанием β-каротина (90–100 мкг/г) и α-каротина (53–61 мкг/г) характеризуются сорта Витаминная-6 и Дордонь. Наибольшее количество лютеина (4.3–6.5 мкг/г) содержится в сортах Дордонь, Морелия, Нерак и Ниланд. Общее содержание каротиноидов является наибольшим (~155 мкг/г) в сортах Дордонь и Витаминная-6. Наименьшее количество каротиноидов наблюдается в сортах Монанта и Балтимор. Сорта моркови, произрастающей в Беларуси, характеризуются довольно высоким общим содержанием каротиноидов, при относительно небольшом соотношении β-каротина (58.9% от общего количества). Применение метода целесообразно при определении каротиноидного статуса различных сортов моркови.

**Ключевые слова:** каротиноиды, исследование, ВЭЖХ метод, морковь, сорт.

## Introduction

It is known that food carotenoids are natural biologically active substances that protect the human body from infections, free radicals, prevent the growth of cancer cells [1]. Some of them, such as  $\beta$ -carotene, have provitamin A activity and contribute to the automatic regulation of the eye's sensitivity to light [2].

It is known that natural pigments in the range between yellow and red colors belong to carotenoids [3]. Carotenoids are divided into two classes – carotenes (nonsaturated hydrocarbons) and xanthophylls (oxygen-containing carotenoids).  $\beta$ -carotene is the most well-known representative of the first class. Carotenoids are synthesized by higher plants, algae, photosynthetic and non-photosynthetic bacteria, actinomycetes, filamentous fungi, yeasts, and are not synthesized by animals [4].

More than six hundred carotenoids were extracted and studied from plants, algae, bacteria and fungi [3]. Qualitative and quantitative composition of carotenoids found in food can be rather complicated and considerably differs depending on the plant species. For example, sea buckthorn contains 19 carotenoids (85% xanthophylls) with a total weight up to 32.3 mg/100 g; a pumpkin — up to 10.0 mg/100 g (30–40% xanthophylls) [5, 6]. The bright color of tomato fruits (pulp and peel) is mainly due to the presence of lycopene (62%); the rest of carotenoids are presented by xanthophylls [7].

The carotenoid content depends on the genetic characteristics of plants and can vary from year to year. Accumulation of carotenoids in cultivars can change in different agro-climatic and geographical growth conditions [8, 9]. Therefore, it is necessary to analyze the amount of carotenoids in plant varieties within several years [10].

Carrot (*Daucus carota* L.) is a classic source of carotenoids for people in many countries. The content of these compounds in different cultivars varies from 8.4 to 19.2 mg/100 g fresh weight.  $\alpha$ - and  $\beta$ -carotene are the major isomers of carrot carotenoids. Also carrot contains small amount of lutein [3]. According to [5] the average  $\beta$ -carotene content is 85–90% of the total carotenoids. In carrot grown in Europe part of  $\beta$ -carotene was found to be 72.3–78.5% of total carotenoids. In carrot cultivated in Asia it was on average 74% [6].

Carrot is important cultivated vegetable in many countries. Belarus is one of the world's largest producers of carrot (annually about 350000 MT). Production of carrots in Belarus in 2010 was 358102 MT [11]. Carrot and products based on it are the main source of carotene for the population. Therefore the data on the content of carotenoids in raw materials are relevant for food industry.

Analysis of international, interstate and state standards showed that spectrophotometric (EN 12136:1997<sup>1</sup>, ISO 6558–2:1992<sup>2</sup>, GOST 8756.22–80<sup>3</sup>) and chromatographic (EN

12823-2-2012<sup>4</sup>) methods are used for the determination of carotenoids in foods.

In the CIS countries the carotene in processed fruits and vegetables is determined according to standardized method GOST 8756.22–80. It is based on the photometric determination of carotene concentration in solution, obtained after extraction with an organic solvent.

Total amount of carotenoids and their composition in fruit and vegetable juices with carotenoid contents from 0.1 to 6 mg/100 g is measured by the method specified in STB GOST R 51443–2006<sup>5</sup>. The method is based on the precipitation of carotenoids with zinc hexacyanoferrate, extraction from the residue with reextraction and subsequent photometric determination of the content of total carotenoids. For analysis of the individual fractions extract is separated by column chromatography with aluminum oxide. Obtained fractions are the following: hydrocarbons, xanthophyll and cryptoxanthinethers.

All the mentioned above techniques are applied only to products of fruit and vegetable processing. Therefore, to determine the carotene in raw carrots it is necessary to adapt these methods or develop new ones. HPLC method is widely used to determine the pigments in plants, so the aim of this research was to choose optimal conditions of sample preparation and HPLC separation for carotenoids of carrot.

## Materials and Methods

Carrot roots of the following cultivars (harvest 2013), grown in the Republic of Belarus, were used for the method development (Table 1).

Samples of varietal carrot were collected in the farms of Minsk district. The farms are located in the central agro-climatic zone of Belarus with the length of the growing period — 185–195 days, annual rainfall — 550–650 mm. This region is characterized by moderately humid climate with mild winters and short, moderately warm, long summer and is favorable for growing carrots [12].

The scheme of carrot carotenoids analysis shown in Fig. 1.

Accuracy of measurement of carotenoids contents in cultivar depends on a representativeness of the roots taken for the analysis [3]. To fulfill this requirement, 8–12 roots were selected from each carrot cultivar. Averaged sample of carrot was washed, dried, peeled, milled by the vegetable shredder to a particle size of ~1 mm and mixed carefully. 5 g of shredded carrots was taken immediately and homogenized in a mortar with quartz sand. Extraction of carotenoids from the homogenized samples was carried out with acetone until discoloration. For this purpose, 50 ml of acetone was needed for each sample (10-multiple excess). Acetone, being the water-miscible organic solvent, was selected as extracting agent since the extraction of carotenoids was carried out from fresh carrots containing ~85% moisture. Furthermore, acetone allows to extract all of the carotenoids, presented in the carrot matrix.

<sup>1</sup> EN 12136:1997, Fruit and vegetable juices. Determination of total carotenoid content and individual carotenoid fractions, 1997.

<sup>2</sup> ISO 6558–2:1992, Fruits, vegetables and derived products from them. Determination of carotene. Part 2: Practical Methods, 1992.












<sup>3</sup> GOST 8756.22–80, Products of fruits and vegetables. Method for determination of carotene, 1981.

<sup>4</sup> EN 12823-2-2012, Food products. Determination of vitamin A by HPLC. Part 2: Determination of  $\beta$ -carotene, 2013.

<sup>5</sup> STB GOST R 51443–2006, Fruit and vegetable juices. Method for determination of total carotenoids and their fractional composition, 2006.

Table 1

Characteristic of carrot cultivars

Cultivar	Characteristic	Cultivar	Characteristic
<p><b>Baltimor F1</b></p> 	<p>Root — from medium to long, cylindrical, with a blunt tip. Core and peel — orange. Root mass 115–230 g. Taste good and excellent.</p>	<p><b>Nantskaya F1</b></p> 	<p>Root — from 15 to 20 cm, root mass 100–200 g, bright orange color. Core and peel — orange. Form of root: cylindrical, flat head, slightly pointed tip. Taste good.</p>
<p><b>Bangor F1</b></p> 	<p>Root — narrow, elongated, orange, smooth, head slightly dented. Core and peel — orange. Root mass 125–210 g. Taste good.</p>	<p><b>Morelia F1</b></p> 	<p>Root long, fusiform with an obtuse base. Core and peel — orange. Root mass 110–190 g. Taste good and excellent.</p>
<p><b>Dordon F1</b></p> 	<p>Root — 18–20 cm, cylindrical, with a blunt tip, smooth, aligned at shape and size. Core and peel — orange. Root mass 80–130 g. Taste good and excellent.</p>	<p><b>Niland F1</b></p> 	<p>Root — medium, cylindrical, with a blunt tip. Core and peel — orange. Root mass 90–100 g. Taste good and excellent.</p>
<p><b>Monanta</b></p> 	<p>Root — from medium to long, with a rounded tip, smooth, orange, flat-rounded head, the core is small, orange. Root mass 75–115 g. Taste good and excellent.</p>	<p><b>Riga F1</b></p> 	<p>Root — from medium to long, cylindrical, with a blunt tip, elongated head. Core and peel — orange. Root mass 80–165 g. Taste good.</p>
<p><b>NeracF1</b></p> 	<p>Root — from medium to long, cylindrical, with elongated tip, elongated head. Core and peel — red. Root mass 130–160 g. Taste good.</p>	<p><b>Sircana F1</b></p> 	<p>Root — 18–20 cm, cylindrical, with a well-executed tip, aligned at shape and weight, intense orange. Very small core, the color is indistinguishable from the rest of the pulp. Root mass 50–160 g. Taste good.</p>
<p><b>Vitaminnaya-6</b></p> 	<p>Root — 13–15 cm, cylindrical, with a blunt tip. Root mass 80–120 g. Core and peel — red-orange, the core is small, round, color differs little from the pulp. Taste good and excellent.</p>		

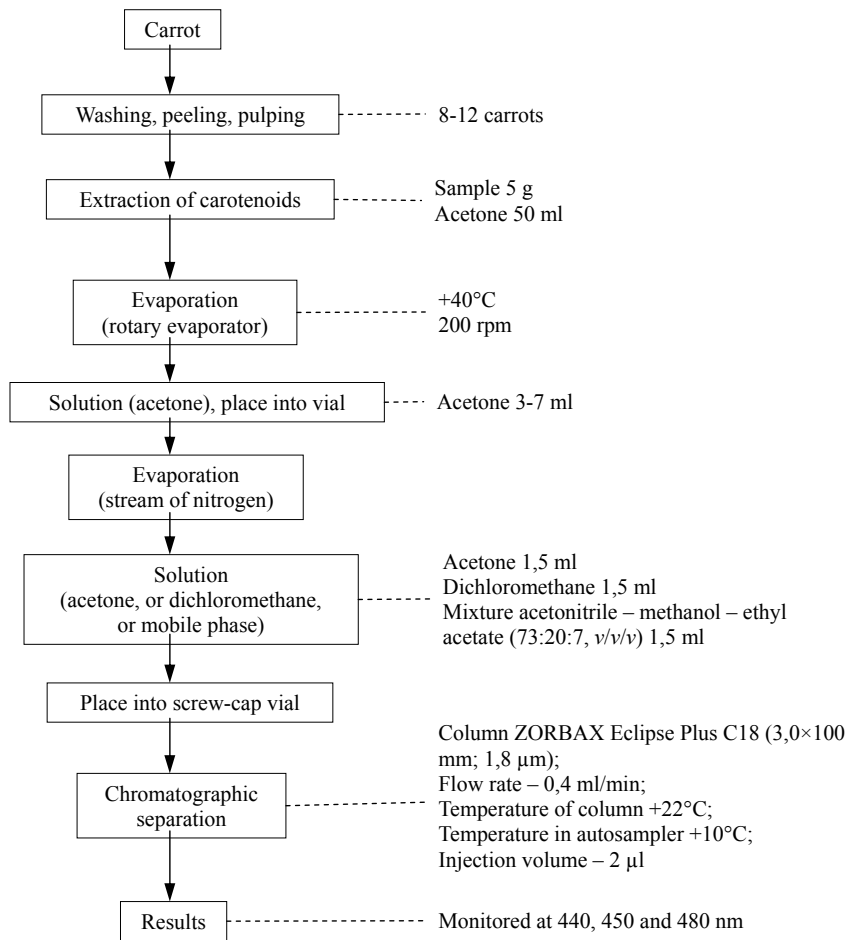


Fig. 1. The scheme of carotenoids analysis by HPLC method

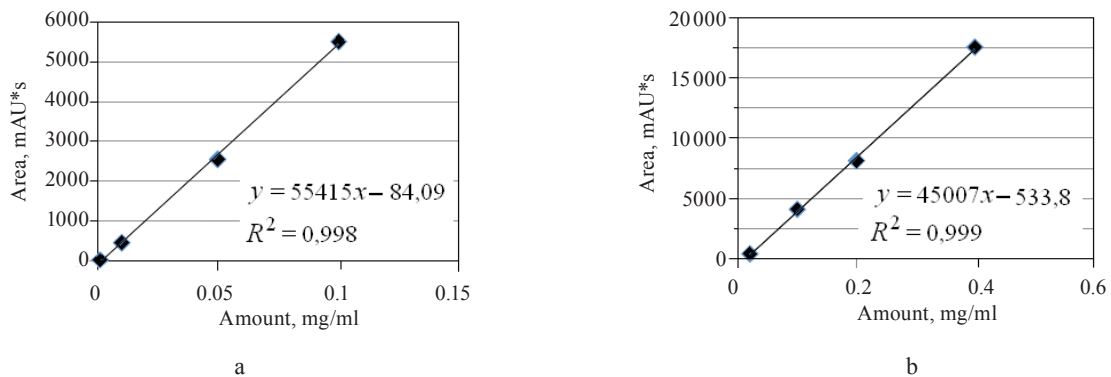


Fig. 2. Calibration curves: a — lutein; b —  $\beta$ -carotene

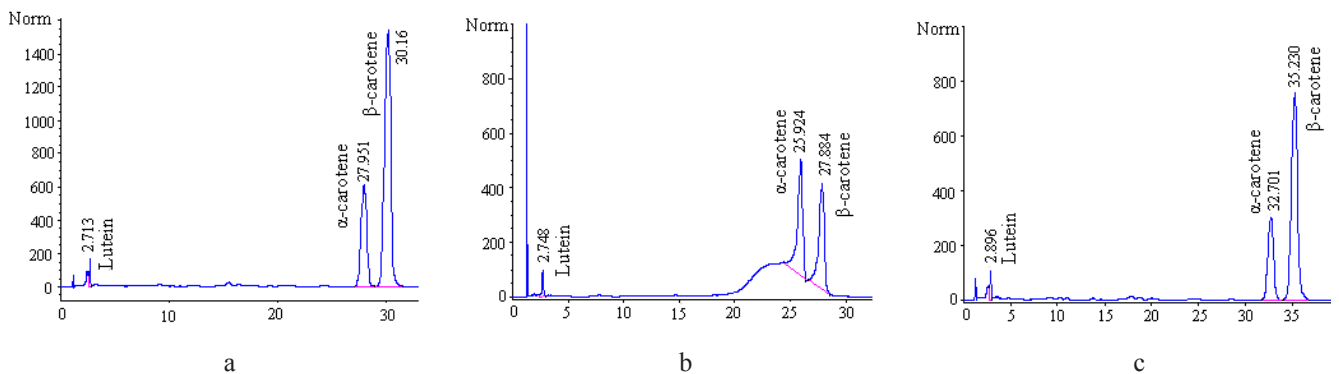


Fig. 3. Separation of carotenoid residue, which was reconstituted with 1.5 ml of various solvents:  
a — acetone; b — dichloromethane; c — mixture acetonitrile – methanol – ethyl acetate

Subsequently the solvent was evaporated on a rotary vacuum evaporator at a temperature not above 40 °C. To avoid destruction of carotenoids and strong adhesion to the glass walls of the flask evaporation was carried out not to complete dryness [3]. The pigments residue in a round bottom flask was dissolved in a small amount of acetone (3–7 ml), and quantitatively transferred into a vial, wherein the solvent was again evaporated to dryness under a stream of nitrogen. The resulting residue of carotenoids was reconstituted with 1,5 ml of various solvents (acetone, dichloromethane, acetonitrile — methanol — ethyl acetate 73:20:7, v/v/v) and placed in a screw-cap vial for HPLC analysis.

To avoid quantitative losses of carotenoids and changes in their structure during the analysis, caused by influence of oxygen, light and temperature, experiment was carried out as fast as possible (within no more than an hour) under subdued light. Samples were analyzed immediately after preparation. Sometimes they were stored as a dry residue no more than three days at a temperature –20 °C in a dark place.

The HPLC method was used for qualitative and quantitative analysis of major carotenoids in the samples. Chromatograph Agilent 1200 with diode array detector was used. The sample components were separated on column ZORBAX Eclipse Plus C18 (3.0×100 mm; 1.8 μm) at temperature 22 °C. The temperature in the autosampler was +10 °C, injection volume — 2 μl. The isocratic solvent system consisting of acetonitrile — methanol — ethyl acetate (73:20:7, v/v/v) was chosen as the mobile phase. The flow rate of eluent was 0.4 ml/min.

To select mobile phase we used the following criteria: it should be characterized by low viscosity, inertness to carotenoids and low toxicity. Also, the mobile phase in combination with C18 column must provide good separation both of nonpolar carotenes (α- and β-carotene) and polar xanthophylls (lutein) of carrots. These requirements are satisfied by mobile phases based on acetonitrile and methanol, which are often used in the analysis of carotenoids [13–18]. Other solvents also may be added (hexane, dichloromethane, ethyl acetate, etc.) to optimize the conditions of chromatographic separation. Based on the described criteria and analysis of literature the mixture acetonitrile — methanol — ethyl acetate (73:20:7, v/v/v) was chosen as mobile phase.

Results were monitored at wavelengths of 440, 450 and 480 nm. Identification was carried out by the retention times of appropriate carotenoids standards (β-carotene (Fluka, № 22040), lutein (ROTH, No 5671) and the absorption spectra.

The quantity of carotenoids was determined by external calibration method. Stock solutions of standards were prepared in dichloromethane in concentration 1 mg/ml. They were stored in sealed amber vials at the temperature –70 °C for use over an extended period. The working solutions were prepared in the day of analysis by step-by-step dilution in acetone. The following final standard concentrations were obtained: 0.02, 0.1, 0.2 and 0.4 mg/ml for β-carotene and 0.001, 0.05, 0.01, 0.1 mg/ml for lutein respectively.

Final carotenoid content in μg/g wet weight was determined by the formula:

$$m = \frac{\text{Amount} [\mu\text{g/ml}] \cdot 1.5 [\text{ml}]}{m_{\text{sample}} [\text{g}]}$$

## Results and Discussion

Linear calibration curves were obtained throughout the concentration range: 0.001–0.1 mg/ml for lutein and 0.02–0.4 mg/ml for β-carotene (Fig. 2). Calibration curves showed good linearity with coefficients of correlation greater than 0.99.

Samples of the carrot «Sircana» were used for developing of the method. Different solvents were used to recover the carotenoid dry residue. Obtained chromatograms are shown in Fig. 3. Good separation of components was obtained for samples recovered in acetone and the mobile phase. The samples dissolved in dichloromethane had unsatisfactory chromatographic separation.

As acetone provides less retention times, is compatible with the mobile phase and carotenes and xanthophylls are equally well dissolved in it, acetone was used to recover dry residue of pigments in further experiments.

Fig. 4 shows the standard chromatogram of the sample of carrots, obtained by the above scheme of analysis.

Developed method was used for the study of carotenoids in eleven carrot cultivars, zoned in Belarus. The contents of individual carotenoids for studied samples are shown in Fig. 5. The main carotenoids of carrots are distributed as follows: α-carotene — 30–40%, β-carotene — 55–68%, lutein — 1–6% of carotenoid total amount. These data are well consistent with literature [3, 19, 20]. Cultivars Vitaminnaya-6 and Dordon are characterized by significant amount of β-carotene (90–100 μg/g) and α-carotene (53–61 μg/g). The largest quantity of lutein (4.3–6.5 μg/g) is contained in cultivars Dordon, Morelia, Nerac and Niland. The total amount of carotenoids in all cultivars, found as the sum of α-, β-carotene and lutein, is presented in Table 2.

Table 2

**Total content of carotenoids from carrots grown on the territory of Belarus**

Cultivar	Carotenoids concentration, μg/g
Baltimor	51,4
Bangor	101,8
Dordon	156,7
Monanta	39,4
Morelia	69,3
Nantskaya	77,7
Nerac	106,1
Niland	94,4
Riga	89,1
Sircana	98,4
Vitaminnaya-6	156,2

The highest content of carotenoids (~155 μg/g) was observed in cultivars Dordon and Vitaminnaya-6. Cultivars Bangor, Nerac, Sircana contained less carotenoids (98.4–106.1 μg/g). Cultivars Monanta and Baltimor had low carotenoid contents.

Studied cultivars are characterized by very similar ratio of individual carotenoids — β-carotene:α-carotene:lutein = 58.9:32.4:3.3% of total amount. In accordance with [20] ratio of carotenoids in five studied samples of carrots grown in Moravia was the following: β-carotene:α-carotene:lutein:lycopene = 75.77:23.82:2.08:0.24%. Usually the part of β-carotene in

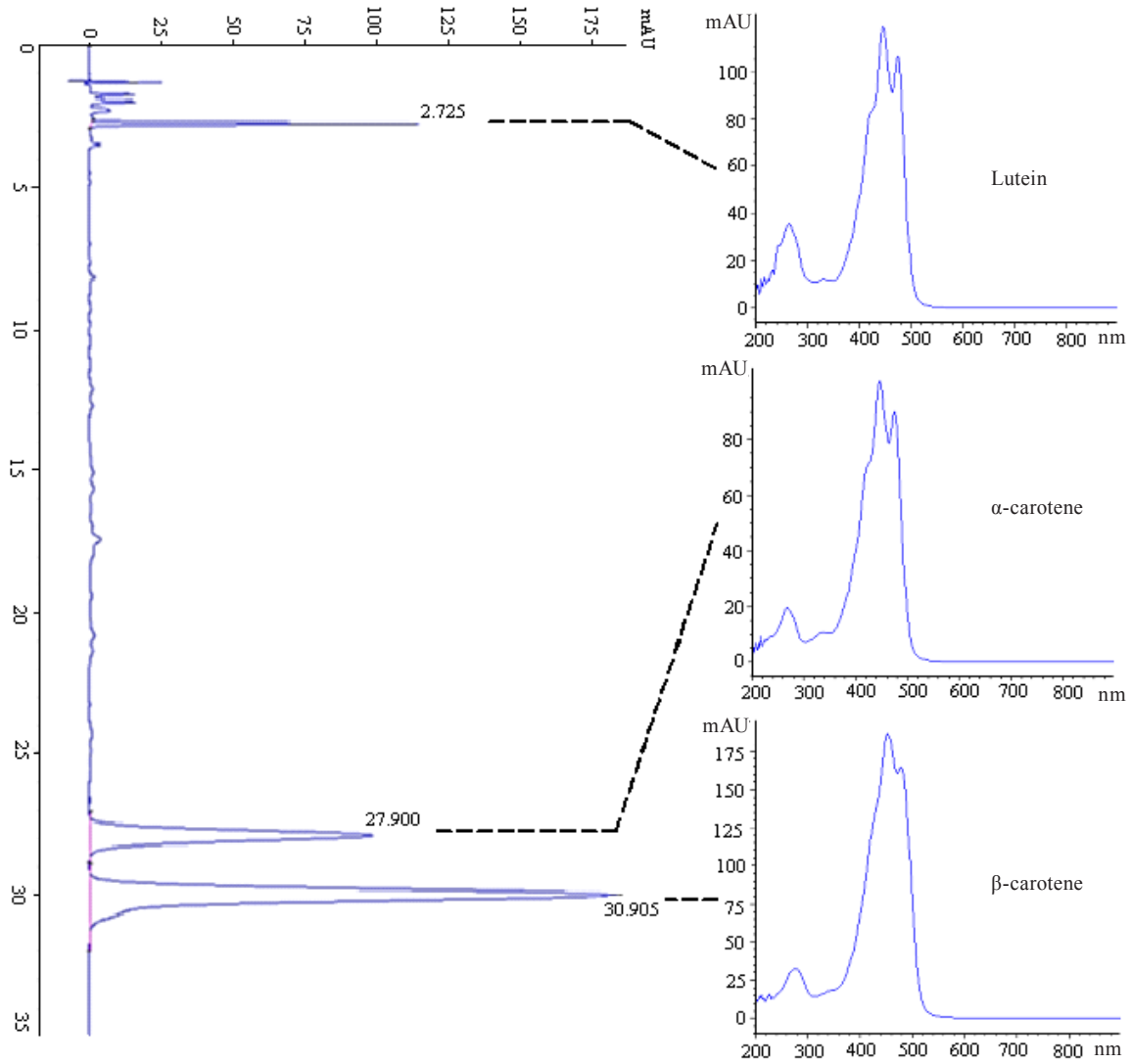


Fig. 4. HPLC chromatogram of the extract from carrot

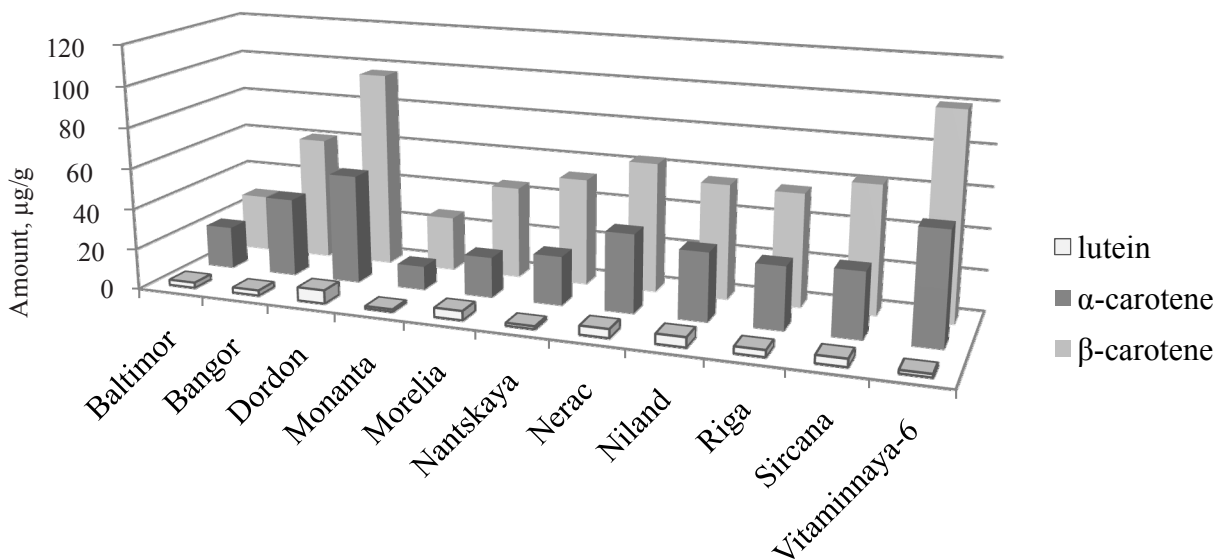


Fig. 5. Content and proportion of lutein, α- and β-carotene in 11 cultivars of carrots grown on the territory of Belarus

carrots grown in Eurasia is about 74% [5, 6]. Socarrot cultivars grown in Belarus are characterized by lower contents of  $\beta$ -carotene, but the total amount of carotenoids was high and varied depending on cultivar.

### Conclusions

Described HPLC method is sensitive, reliable, efficient, rather simple and can be applied for the determination of the main carotenoids of carrot:  $\alpha$ -,  $\beta$ -carotene, lutein. This method was used for qualitative and quantitative carotenoid analysis in 11 carrot cultivars grown in Belarus.

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