REVERSE-PHASE HPLC IN THE ANALYSIS OF PLANT OILS. MONITORING OF AUTHENTICITY AND DETECTION OF ADULTERATION OF SEA BUCKTHORNE OIL

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Reverse-phase HPLC allowed detection of triglycerides (containing palmitoleic acid radicals) in sea buckthorne oil extracted from the parenchyma of the fruit on the background of the conventional triglycerides used as extractants. This allows rapid verification of the authenticity of the oil and detection of adulteration. Simple replacement of refractometric detection with a spectrophotometric procedure allows the carotenoid composition of the oil to be monitored at the same time.

Key words: Sea buckthorne oil, HPLC, adulteration, authenticity.

Sea buckthorne oil is one of the most important products of processing the fruits of the sea buckthorne *Hippophaë rhamnoides* L. The fruits of this plant are unusual in that the oil is present not only in the seeds, but also in the parenchyma of the fruit. Furthermore, the seeds are not currently of any great technological importance. Most of the oil accumulates in the parenchyma, i.e., up to 80% or more of the total quantity of oil (about 30% by weight in air-dried parenchyma) [1, 2]; it is from the parenchyma that this valuable product is extracted [1, 3, 4]. The oil of the parenchyma in pure form, which has an orange-brown coloration, contains concentrations of important biologically active substances which confer medicinal properties - carotenoids, group E and K vitamins, etc. [1]. And although difficulties in obtaining the oil in Russia led to the development of a substitute developed two decades ago, AEKOL [5], many pharmacies now include several brands of "Sea buckthorne oil" from various manufacturers. In this regard, the problems of standardizing the product and detecting adulteration of the oil have become relevant [6].

The aim of the present work was to develop a rapid and reliable method for verifying the authenticity of sea buckthorne oil (and to detect adulteration) using reverse-phase HPLC.

EXPERIMENTAL SECTION

Reverse-phase HPLC was performed using a chromatography system consisting of an Altex 110A pump, a Rheodyne 7100 doser valve with a loop of volume 20 μl, an RI 401 Waters detector, and an LC/9563 Spectromonitor (λ = 445 nm). Chromatograms were recorded and processed using a PP Multichrom 1.5 (Ampersand Ltd., 2005). The chromatography conditions were as follows: columns – 250 × 4 mm, Diasfer-110-C18, 5 μm, Chromasil-100-C18, 5 μm, Separon SGX C18, 5 μm; mobile phase – acetonitrile:acetone (10:90 by volume or 20:80 by volume); flow rate – 1 ml/min.

Chromatography was performed using oil solutions containing 5 – 20 mg per 1 ml of mobile phase (or acetone).

Sea buckthorne fruits of the varieties Perchik, Maslichnaya, Yubileinaya, Bochonok, Lyubitel'skaya, and Brat Krasnogo were collected at the end of August 2006 in the Belgorod State University botanical garden. Samples of "Sea buckthorn oil" were purchased in pharmacies in Belgorod. Studies used "Oleina" sunflower oil and "Maslo Makadamii" [translator's note: macadamia oil] from OOO "Natural'naya Kosmetika" [translator's note: "Natural Cosmetics"], "Maslo avokado" [translator's note: avocado oil] from OOO TPK "Aromaty Zhizni" [translator's note: "Life Aromas"].

Oil was extracted from samples of 5 – 10 g of fresh fruits (without seeds) by adding sequential portions of 5 – 10 ml of acetone; fruits were mashed beneath the solvent using a glass rod; each portion of extract was separated from the residue by filtration. The process was repeated until the solid residue was decolorized. All filtrate portions were combined and *n*-hexane was added, along with 10% aqueous sodium chloride. The hexane layer was washed with three portions of five volumes of 10% sodium chloride solution. Moisture was removed from the extract by holding it above calcined sodium sulfate. The oil from the parenchyma of the sea buckthorne fruit was extracted by vacuum distillation of *n*-hexane from the purified extract.

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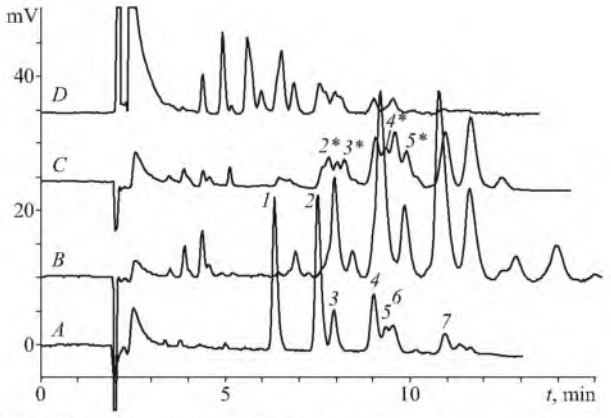

Fig. 1. Chromatograms of plant oils used as reference oils: *A*) sunflower oil; *B*) macadamia oil; *C*) avocado oil; *D*) sea buckthorne seed oil. Triglycerides: *1*) L_3; *2*) L_2O; *3*) L_2P; *4*) LO_2; *5*) L_2S; *6*) LOP; *7*) O_3; *2**) LPoO; *3**) LPoP; *4**) PoO_2; *5**) PoOP; the mobile phase was acetonitrile:acetone (10:90 by volume) at a flow rate of 1 ml/min.

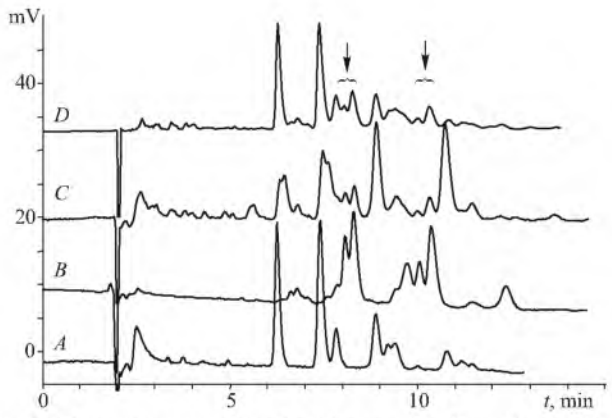

Fig. 2. Chromatograms of sea buckthorne oils: *A*) sunflower oil; *B*) sea buckthorne fruit parenchyma oil from the "Maslichnaya" variety; *C*) "Sea buckthorne oil from fruits and leaves," Katun' Oleum, Russian Federation; *D*) "Sea buckthorne oil," ZAO "Altaivitaminy," Russian Federation. The mobile phase was acetonitrile:acetone (10:90 by volume) at a flow rate of 1 ml/min; the arrows and brackets show the diagnostic peaks on chromatograms of the mixture of sea buckthorne oil with extractant oil.

Oil was extracted from sea buckthorne seeds with acetone after grinding the seeds in a porcelain mortar.

Oil triglycerides were named with letters corresponding to the fatty acid radicals: S = stearic (C18:0), P = palmitic (C16:0), O = oleic (9-*cis*-octadecenoic, $C18:1^{\Delta 9}$), and L = linoleic (9,12-*cis*-octadecadienoic, $C18:2^{\Delta 9,12}$). LOP identifies triglycerides containing linoleic, oleic, and palmitic acid residues (without identification of their positions in the molecule). Increments, for example $\Delta(L \rightarrow O)$, identify differences in the log retention factors of two triglycerides whose compositions differ in terms of only one acid radical: $\Delta(L \rightarrow O) = \lg k(L_2O) - \lg k(L_3) = \lg k(LOP) - \lg k(L_2P)$, and so on.

RESULTS AND DISCUSSION

Fatty acid composition is currently one of the properties used for monitoring oils [6]. Transfer from analysis of fatty acid composition by GLC of methyl esters prepared by chemical modification of study samples to monitoring of the triglyceride complex of oils by reverse-phase HPLC (without any sample treatment other than dissolution) requires determination of whether the triglycerides of sea buckthorne oil can be identified on the background of the plant oils conventionally used in Russia for extraction of sea buckthorne oil.

Sunflower oil is the most commonly used in Russia. This contains triglycerides formed mainly from the linoleic, oleic, palmitic, and stearic acid radicals; previous studies have demonstrated that differences between the chromatography profiles of sunflower oils from different producers on the Russian market are minimal [7]. In terms of chromatography profile, sunflower oil differs only slightly from many other oils, including maize and pumpkin oils and grapeseed oil; the appearance of small quantities of the α-linolenic acid radical ($C18:3^{\Delta 9,12,15}$) in the triglyceride composition does not produce any great increase in the complexity of the chromatogram of soy oil [8, 9].

A characteristic feature of oil from the parenchyma of sea buckthorne fruits is that the triglycerides contain significant quantities of palmitoleic (9-*cis*-hexadecenoic, $C16:1^{\Delta 9}$, Po) and vaccenic (11-*cis*-octadecaenoic, $C18:1^{\Delta 11}$) acid radicals. The proportion of palmitoleic acid in the oil from the parenchyma ranges, according to different sources, from 13% to 51% of the total quantity of acids [4, 6, 10 – 13], the most likely content being in the range 25 – 30%. It should be noted that the ranges were significantly smaller in each of the studies cited. These differences may be associated with differences between varieties, cultivation conditions, and the level of maturation of the fruits used. However, we found no studies giving metrologically grounded conclusions regarding the adequacy of the results obtained from analyses performed with a chemical modification stage (conversion of triglycerides into methyl esters). Evidence for the significance of these results cannot be obtained by "added-found" comparison in the case of real oils. There is therefore value in analyzing unmodified oils by NMR [14, 15].

Palmitoleic acid is quite rarely found in relatively large quantities in plant oils. Apart from sea buckthorne, the accumulation of this compound is also characteristic of, for example, avocado and macadamia oils [16, 17]. Using the increments approach and a number of previously identified changes in the retention times of triglycerides on substitution of some unsaturated acid radicals with others [8, 9, 18], the following relationship between increments can be proposed:

$$\Delta(Po \rightarrow O) < \Delta(L \rightarrow O).$$

Thus, triglycerides formed with palmitoleic acid must elute later than triglycerides in which palmitoleic acid is replaced by linoleic acid. The chromatograms of oils shown in Fig. 1 indicate that this suggestion was completely supported. It was typical that in the case of avocado oil, the re-

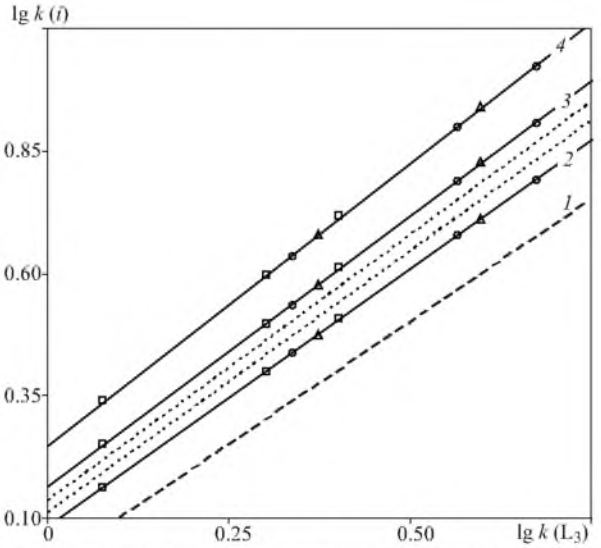

Fig. 3. Plots of relative retentions of triglycerides: *1*) lg$k$(L_3); *2*) lg$k$(L_2O); *3*) lg$k$(LO_2); *4*) lg$k$(O_3) relative to lg$k$(L_3); data obtained for stationary phases Diasfer-110-C18 (○), Separon SGX (□), and Chromasil-100-C18(○); dotted lines show the boundaries of the elution zone characteristic for sea buckthorne oil triglycerides.

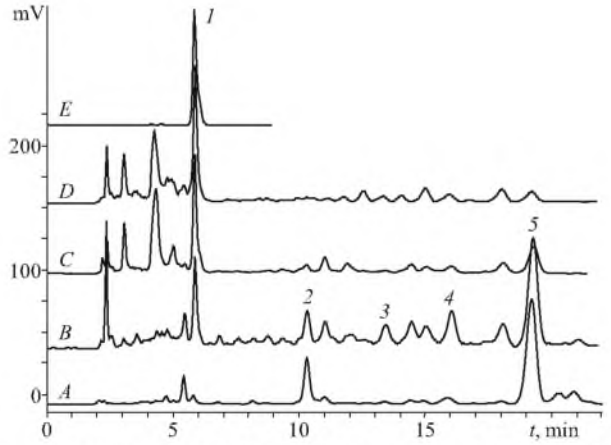

Fig. 4. Chromatograms of carotenoid complexes: *A*) physalis fruit extract; *B*) sea buckthorne oil of the Yubileinaya variety; *C*) sea buckthorne oil of the Perchik variety; *D*) "Sea buckthorne oil," ZAO "Altaivitaminy;" *D*) adulterated. *1*) β-Carotene; *2*) β-cryptoxanthine palmitate; *3*, *4*, *5*) zeaxanthine dimyristate, myristate-palmitate, and dipalmitate. The mobile phase was acetonitrile:acetone (20:80 by volume) at a flow rate of 1 ml/min.

tention range of triglycerides L_2O-L_2P and LO_2-LOP contained "duplicates" (LPoO and LPoP, and PoO_2 and PoOP). We note that oil from sea buckthorne seeds contained significantly more unsaturated acids than oil from the parenchyma of the fruits, and that these were difficult to identify on the background of, for example, flax oil.

Thus, it is not surprising that oil from the sea buckthorne fruit parenchyma was clearly distinct in terms of the retention times of several triglycerides from linoleic-oleic oils (Fig. 2) and could be detected on this background. The presence of sea buckthorne oil in the extract resulted in the appearance of a pair of peaks after the triglyceride of composition L_2P and a group of peaks I the range from LOP to O_3 of the latter triglyceride, which was virtually absent in oil from sea buckthorne fruit parenchyma.

Method for verifying the authenticity (or adulteration) of sea buckthorne oil.

1) A chromatography system with refractometric detection and a column of size 250 x 4 mm filled with a reverse-phase sorbent (such as Diasfer-110-C18, 5 μm, BioKhimMak, Moscow) was used, with a mobile phase consisting of acetonitrile and acetone (10:90 by volume) at a flow rate of 1 ml/min. The chromatography system must satisfy the following requirements: efficiency for L_3 at least 8000 theoretical plates, retention time of L_3 at least 6 min.

For stationary phases with significantly less hydrophobicity as compared with that of Diasfer-110-C18, the acetonitrile content in the mobile phase must be increased for these requirements to be met.

2) A "reference" oil solution is prepared: 100 ± 10 mg of food-quality sunflower oil (from "Oleina," "Sloboda," "Milora," or virtually any other producer – our investigations indicate that sunflower oil enriched with oleic acid is not available on the Russian market) is dissolved in 5 ml of mobile phase.

3) Chromatograms are recorded and must be analogous to those shown in Fig. 1*a*. The retention times (t_R) of the L_3, L_2O, LO_2, and O_3 peaks are used to determine t_0, the "thermodynamic dead retention time," as:

$$\frac{t_R(L_3)-t_0}{t_R(L_2O)-t_0}=\frac{t_R(L_2O)-t_0}{t_R(LO_2)-t_0}=\frac{t_R(LO_2)-t_0}{t_R(O_3)-t_0}.$$

An "analogous" chromatogram is one which has a similar number of main peaks with similar intensity ratios. Other oils can be used as the "reference" oil as long as their chromatography profiles are similar to that of sunflower oil, i.e., maize, pumpkin, etc.

4) Sea buckthorne test oil solution is prepared: 100 ± 10 mg of sea buckthorne oil is dissolved in 5 ml of mobile phase and a chromatogram is recorded. The group of "problem" (not separated in these conditions) triglycerides with retention times between $t_R(L_2P)$ and $t_R(LO_2)$, i.e., in the range between the two dotted lines in Fig. 3 is considered:

$$1.068\ \log k(L_3) + 0.110 < \log k(i) < 1.090\ \log k(L_3) + 0.136.$$

The presence of these peaks is evidence for the presence of sea buckthorne oil in the sample. These peaks are absent from adulterated samples.

It is known that substitution of the stationary phase (even between different batches of the same brand) can cause significant changes in the elution order of mixture components. However, analysis of the relative retentions [19] of weakly polar sorbates (including triglycerides) makes the relative retention equation insensitive to changes in C18 stationary phases (including changes in brand) over a wide range of

mobile phase compositions. This is supported by the data presented in Fig. 3, showing the retention parameters of triglycerides obtained using three different stationary phases (Diasfer-110-C18, Chromasil-100 C18, and Separon SGX C18). The coordinates of each point are defined by the log of the retention factor of the corresponding triglyceride and the log of the retention factor of L_3, which is selected as the reference substance in identical conditions. The plots were constructed using mobile phases containing 0%, 10%, 20%, and 34% (by volume) acetonitrile in acetone.

Rapid monitoring of sea buckthorne oil is performed using conditions in which complete separation of triglyceride peaks is not obtained. If required, the degree of separation can be increased by using eluants with higher volume proportions of acetonitrile (more than 25% by volume) or running two columns connected in sequence; however, this compromises the rapidity of the method.

This method was used for analysis of a series of "Sea buckthorne oils" acquired in retail shops and fruit parenchyma oil from several varieties of sea buckthorne from the collection of the botanic garden of Belgorod State University. Chromatograms of sea buckthorne fruit parenchyma oils of all six varieties tested were essentially identical, while the coloring of the fruits varied from yellow to orange-red. There were significant differences in the chromatograms of commercial "sea buckthorne oils." Samples from ZAO "Altaivitaminy" (on the background of a sunflower-type oil) and "Katun' Oleum" (on the background of oleic-linoleic oil) contained 30 – 50% sea buckthorne oil; a sample from OOO NPF "Altaiskii buket" and three samples of "Sea buckthorne oil" (as stated on the labels) yielded only good-quality sunflower oil.

The second measure regarded as important for monitoring is the carotenoid composition of the oil. While the spectrophotometric method for analyzing total carotenoids is clearly simple and accessible, its informativeness in general is sometimes dubious, especially considering the importance of the biologically active non-saponifying oil residues (see [20] and the references cited therein). The method we have proposed above can also be used to monitor the carotenoid composition of the oil in analogous conditions using the same samples of oil, but using a spectrophotometric detector instead of a refractometric detector.

The accumulation of carotenoids in sea buckthorne fruits depends on the variety; fruit coloration is one of the characteristic parameters of varieties. Differences in carotenoid complexes are very large, even for the varieties studied here, which were grown in identical conditions (Fig. 4). In fruits with yellow coloration, the main components of the complex were zeaxanthine diesters, while red fruits contained mainly a component with a retention time in the elution range of xanthophyll monoesters. We note that for all the sea buckthorne varieties studied here, β-carotene accounted for no more than 20% of the total chromatogram peak area. One therefore has to wonder whether there is any meaning in expressing carotenoid contents in sea buckthorne oil per unit β-carotene, given that zeaxanthine (the main component) lacks provitamin activity. Those samples of "Sea buckthorne oils" which were the best in terms of parenchyma oil content were found to contain zeaxanthine diesters, though the proportion of β-carotene was notably greater than in fruits grown in Belgorod. Finally, samples identified as adulterated in terms of their triglyceride compositions had chromatograms with a single (β-carotene) peak.

Thus, the reverse-phase HPLC method can be used for rapid verification of authenticity (or detection of adulteration) of sea buckthorne oils in terms of their triglyceride compositions using refractometric detection and/or in terms of carotenoid composition (using the same oil samples as used for refractometric detection of triglycerides) after replacement of the detector with a spectrophotometric detector.

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