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Fatty oil accumulation in vegetable soybean seeds and its thin-layer chromatography

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Abstract. This paper studies the accumulation of crude oil (triacylglycerides, monoacylglycerides, diacylglycerides, free fatty acids, phospholipids, tocopherols, pigments, sterols, waxes) in soybean vegetable samples. Samples were taken from two groups: grown in an experimental field and in protected ground of the Federal Scientific Center for Vegetable Growing in the Moscow Region. Both groups were observed in the phase of technical ripeness and in the phase of complete biological ripeness (finally ripe seeds). Soxhlet method as arbitration in analysis was used as suitable for the extraction of lipophilic substances. It was determined that the fat content in the technical ripeness phase in most soybean samples averaged 10.5%. In the phase of biological ripeness, the highest accumulation of fatty oil was observed in Hidaka and Nordic (17.6%). The oil content in vegetable forms of soybeans was consistently lower than that of grain varieties: in the phases of technical and biological ripeness by 55.6% and 22.0% (in relative values) respectively. Thus, he accumulation of oil in seeds is determined mainly genetically. The refractive index of vegetable and oil soybean was established equal on average 1.4755. According to this finding the soybean oil can be classified as semi-drying.
Thin layer chromatography (TLC) was used to study the lipophilic components of soybean fatty oil. It was found experimentally that the best separation of the components is achieved using an eluent system: carbon tetrachloride: chloroform in a 2: 3 ratio. It was found that the main fat-soluble compounds are the following (in order of increasing Rf in the chromatogram): phospholipids, monoacylglycerides, triacylglycerides, tocopherols, fatty acid esters. As a finding of the research vegetable soybean cultivated at 55 °N in both technical and biological ripeness phases significantly accumulate crude oil in the seeds. This crude oil contained ω-6, ω-3, phospholipids, and vitamin E.

Key words: vegetable soybean, *Glycine max* (L.) Merr., oil content, crude oil, fat, thin layer chromatography.

INTRODUCTION

The aim of the research is to study the oil content in vegetable-type soybean seeds in the R6 and R8 phases under conditions of 55 °N, and the selection of eluents in thin layer chromatography.

Soybean is a crop of dual industrial use: a source of protein and fat content varies in the range of 16–27% (Hymowitz et al., 1972; Degola et al., 2019; Novytska et al., 2020). Soybean oil is one of the most biologically valuable among all vegetable oils: a high content of polyunsaturated fatty acids (PUFA) - about 55–63%, a low proportion of saturated fatty acids - no more than 15% (Dornbos et al., 1992; Carson et al., 2011). The composition of fatty acids significantly affects the organoleptic qualities of vegetable soybeans and the nutritional value of oil (Haun et al., 2014; Li Qing-Tian et al., 2017).

Soybean oil contents 50–60 % linoleic oil. Its content has a close correlation with the amount of α-linolenic acid (up to 8%), which gives the oil a peculiar taste, aroma and contributes to its rapid oxidation (Kostik et al., 2013; Miao Long et al., 2020). The fat content in vegetable soybeans in the technical ripeness phase varies within 8.0–14.6% (on dry matter) (Rao et al., 2002; Shafigullin et al., 2020a) the active synthesis occurs almost until the onset of full biological ripeness (Rubel et al., 1972; Nadtochii et al., 2015).

Moisture evaporation under high air temperature causes water deficiency and decrease in fat synthesis. At the same time, the accumulation of protein increases (Tretyakov et al., 2005; Wijewardana et al., 2019) and respiratory processes intensify. A large amount of oxygen spends on the synthesis of PUFA. That’s why the iodine number decreases.

Fat content in soybean seeds from south (45–50° N) to north (55–59° N) regions increases in more than 2% The fraction of oleic acid decreases by 15.4% in absolute values. The proportion of linoleic acid increases by 5.5% in absolute values. The proportion of linolenic acid increases almost 3 times. The result of these biochemical processes is a general decrease in the ratio of ω-6 and ω-3 (3–4: 1) (Kucherenko et al., 2008). For this reason, soybean seed oil grown in northern conditions is less stable during storage, but more suitable for medicinal purposes and functional nutrition.

Oil content of vegetable forms increased, in comparison with oilseeds by an average of 2% in soybean cultivars (Glycine max (L.) Merr.), that grown in the Krasnodar region of Russia. The content of ω-6 and ω-3 decreased by 4% and 50% respectively because of the rejection of samples with an increased accumulation of linolenic acid, which imparts a bitter taste to vegetable oil and soybeans in the phase of technical ripeness (Petibskaya et al., 2006).

Gas chromatography–mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR) methods are most often used to analyze the chemical composition of vegetable oils (Tariq et al., 2011; Goryainov et al., 2012). Nevertheless, thin layer chromatography (TLC) is the simplest and most inexpensive for identification of fatty oil (Lobaeva et al., 2013). TLC is an analytical and operational method characterized by high efficiency and versatility, widely used in scientific research (Nazi et al., 1998). For the analysis of lipophilic components, it is important to select the optimal eluents for most qualitative separation the lipophilic components on a chromatogram, where a thin layer of adsorbent is used as a stationary phase (Fuchs et al., 2011).

METHODOLOGY

The soils of the experimental field are sod-podzolic medium loamy. The humus content is low, 1.3–4.6%. The reaction of the soil solution varies from acidic to close to neutral: pH_{KCl} = 4.5–6.9. The content of mobile phosphorus (P_2O_5) is sufficient, and averages 320–840 mg kg^{-1} of soil, and potassium (K_2O) is much less - 100–250 mg kg^{-1} of soil (Knyazkov et al., 2014). Soil cultivation included plowing in the fall, disking, early spring harrowing and pre-sowing cultivation. The predecessor was complete fallow.

The study of *Glycine max* (L.) Merr., samples was carried out in the laboratory of physiology and biochemistry of the Federal Scientific Vegetable Center during 2016–2018. Ten lines of soybeans were studies: two oilseeds, two universal and six vegetable ones. Most of the collection material was provided by the Federal Research Center 'Vavilov All-Russian Institute of Plant Genetic Resources'; breeding material from the Federal Scientific Vegetable Center collection was also used (Table 1).

There are morphological and economic characteristics, biological characteristics, biochemical parameters use to determine vegetable form (Shafigullin et al., 2020b). Universal cultivars have features of vegetable and oil varieties.

Soybean cultivar 'Okskaya' was a control as registered in the Russian State Register. The soybean cultivars were sown manually in open ground in the third decade of May in 2016 and 2018, in 2017 - in the first decade of July in protected ground (greenhouse) in three rows 1.5 m long, 45 cm interrow, standing density 40 pcs m^{-2}, harvesting area 15 m^3.

Table 1. Origin of soybean samples and directions of use

No.	Varieties	Origin	Type
1	Okskaya (standard)	Russia	grain (oilseed)
2	Soer-5	Russia	grain (oilseed)
3	Gokuwase Hayabusa Edamame	Japan	vegetable
4	Japaneese sampleA	Japan	vegetable
5	Nordik	Russia	universal
6	Hidaka	Japan	vegetable
7	740-1	Sweden	vegetable
8	Fiskeby III	Sweden	vegetable
9	Tundra	Canada	universal
10	Cha Kura Kake	Japan	vegetable

The crude oil content was determined according to Redfern et al. (2014). N-hexane (C6H14) was used as solvent due to its low toxicity, good dissolving properties and low boiling point (68.7 °C).

Analysis of variance was used for statistics using Microsoft Office Excel (2010) and then validated in Origin 9.1.

Thin layer chromatography (TLC) was performed on Merck plates (TLC Silica gel 60 F254) on silica gel (7×15) cm with a total area of 105 cm^2. The system was chosen as the most optimal eluent: carbon tetrachloride-chloroform in the ratio (8:12) mL. Chromatograms were developed in a desiccator with iodine vapor; the saturation time of the chromatogram in the chamber was about 30 min. The temperature of separation of lipid fractions by TLC is about 22 °C. The witnesses (taps) were: 10% solution of vitamin E (LLC 'Tula pharmaceutical factory') and 97% solution of lecithin (LLC 'Vitaprom'). The Rf index was calculated as the ratio of the distance (l) traveled by the substance to the distance (L) traveled by the solvent: l:L.

The refractive index (refractive index) was determined at a temperature of 25 °C using a laboratory refractometer RL 3 (Poland) in four replicates.

The purpose of the work was to study the accumulation of crude oil in vegetable soybean seeds at the technical and biological stages of development in the central region of Russia, as well as to select the optimal eluents for separating obtained oils by TLC and to study the lipophilic fractions of these compositions.

RESULTS AND DISCUSSION

Studying the oil content of soybean seeds

Increase in the accumulation of fat in seeds was observed in all. Thus, there was a twofold increase in the oil content, while it was slightly lower and amounted to 36.2% relative values. Oil content of seeds in grain varieties was higher in technical and biological ripeness. Technical ripeness (R6), the difference in fat content between the breeding forms decreased by almost 3 times in the phase of biological ripeness compare to technical ripeness (R6). This may indicate a more intensive process of oil synthesis in the initial stage of generative development in oilseeds, as well as a later onset of accumulation of triacylglycerides in vegetable forms. An increase in seed oil content, similar to protein content, goes to the terminal stage in ontogeny, i.e. shedding of leaves and a decrease in moisture content in seeds below 10%.

Accumulation of oil in the R6 phase in most of the vegetable forms of soybeans was almost at the same level and averaged 10.5% (in absolute values). Thus, in the phase of technical ripeness, vegetable soybean contains, in addition to protein, also a large proportion of polyunsaturated fatty acids in seeds (Fig. 1).

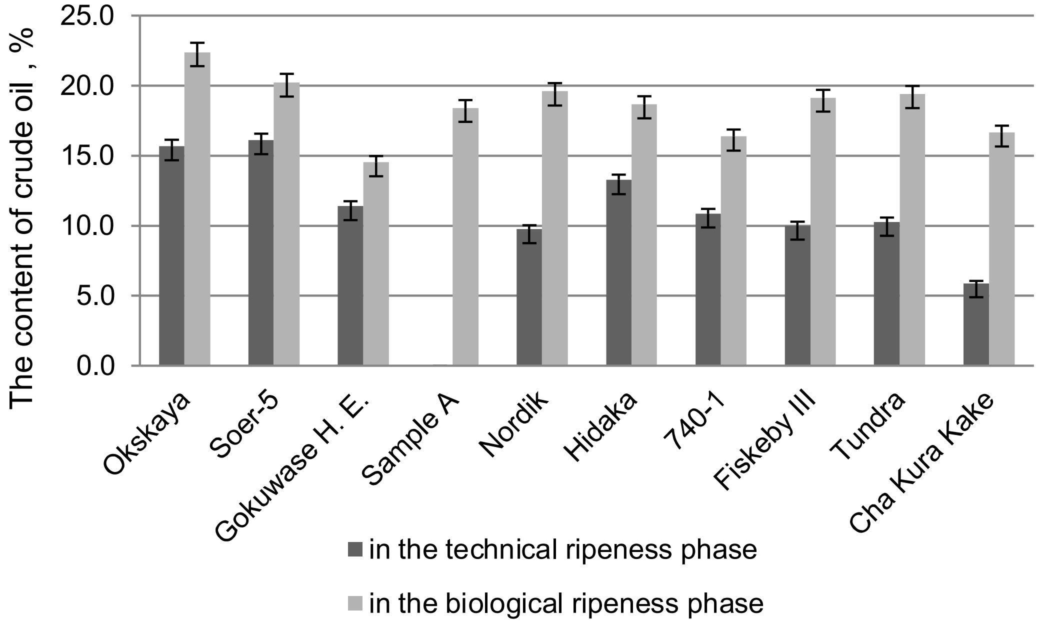

Figure 1. The content of crude oil in soybean seeds in the phase of technical and biological ripeness of samples, average 2016–2018 (dry weight).

Importantly, in 2017 the influence of non-standard growing conditions in greenhouses on biochemical processes was observed, which led to a decrease in the oil content of seeds in all samples by 30.4% (in relative values) compared to 2016 and 2018.

Over the course of 3 years, oilseed samples consistently averaged 22% higher crude fat than vegetable samples. The maximum oil content among vegetable samples was observed in the Hidaka and Nordic lines (17.6%). The Gokuwase Hayabusa Edamame sample had a lesser oil content than other cultivars (33.3% lower than average mean in group of vegetable). It belongs to the ancient species cultivated more than 40 years ago. Fat content is mainly determined by hereditary factors; therefore this indicator can also be considered as one of the biochemical characteristics for selection. These findings correspond to earlier research (Chen Liang et al., 2018). The total crude oil yield was slightly lower than total crude oil yield of the vegetable soybean samples. The highest yield of crude oil was observed in Nordic breeding line and 740-1 vegetable form. In 2016, there was a higher fat collection in almost all samples, which is explained by increased seed productivity (Table 2).

Table 2. Content and yield of crude oil in seeds of soybeans in the phase of biological ripeness

No.	Varieties	Crude oil content, %					Crude oil yield, g plant^{-1}		
		2016	2017	2018	Average in 3 years	Vσ, %	2016	2018	Average in 2 years
1	Okskaya	19.7 ± 0.6	16.0 ± 0.5	22.4 ± 0.7	19.4 ± 1.8	16.4	3.6 ± 0.1	2.5 ± 0.1	3.0 ± 0.5
2	Soer-5	20.5 ± 0.6	17.5 ± 0.5	20.2 ± 0.6	19.4 ± 1.0	8.6	3.4 ± 0.1	4.0 ± 0.1	3.7 ± 0.3
3	Gokuwase Hayabusa Edamame	13.2 ± 0.4	9.2 ± 0.3	14.5 ± 0.4	12.3 ± 1.6	22.6	4.2 ± 0.1	1.4 ± 0.0	2.8 ± 1.4
4	Sample A	17.6 ± 0.5	12.8 ± 0.4	18.4 ± 0.6	16.3 ± 1.8	18.7	3.4 ± 0.1	2.6 ± 0.1	3.0 ± 0.4
5	Nordik	18.7 ± 0.6	13.2 ± 0.4	19.6 ± 0.6	17.2 ± 2.0	20.3	6.7 ± 0.2	3.3 ± 0.1	5.0 ± 1.7
6	Hidaka	17.9 ± 0.5	17.4 ± 0.5	18.7 ± 0.6	18.0 ± 0.4	3.5	2.5 ± 0.1	3.6 ± 0.1	3.1 ± 0.6
7	740-1	17.1 ± 0.5	13.8 ± 0.4	16.4 ± 0.5	15.8 ± 1.0	11.0	5.1 ± 0.2	2.8 ± 0.1	4.0 ± 1.1
8	Fiskeby III	15.5 ± 0.5	12.9 ± 0.4	19.1 ± 0.6	15.9 ± 1.8	19.7	4.7 ± 0.1	2.4 ± 0.1	3.5 ± 1.1
9	Tundra	17.6 ± 0.5	12.2 ± 0.4	19.4 ± 0.6	16.4 ± 2.2	22.8	3.2 ± 0.1	3.3 ± 0.1	3.2 ± 0.1
10	Cha Kura Kake	17.0 ± 0.5	13.0 ± 0.4	16.6 ± 0.5	15.5 ± 2.2	14.4	6.1 ± 0.2	1.8 ± 0.1	3.9 ± 2.2
	LSD_{05}	2.1	2.5	2.2	2.1				

In the course of studying the refractive index (refractive index) of the crude oil of the samples, it was found that in soybean seeds of both breeding directions, the fat has almost the same values in the ability to refract a ray of light. It can be concluded that soybean oil is semi-drying in terms of the content of unsaturated fatty acids, since the refractive index is proportional to the content of the latter (Table 3).

Table 3. Refractive index of crude oil of soybean samples, average for 2016–2018

Type of soybean varieties	Refractive index
oilseed	1.4765 ± 0.00001
vegetable	1.4744 ± 0.00001

Thin layer chromatography of oils.

The isolated crude oil from the seeds was studied by thin layer chromatography (TLC), in which vitamin E (tocopherol) and soybean lecithin standards were used as witnesses, as well as flaxseed and olive unrefined oils for comparison. Eluents of non-polar and polar nature were tested in various ratios for the selection of the mobile phase in order to optimally separate all the main lipophilic fractions.

It was experimentally found that the mobile phase CCl_4:$CHCl_3$ in the ratio (10:10) cm^3, (15: 5) cm^3, (15:5) cm^3 n-hexane: ethanol (95%) in the ratio (20:0.5) cm^3, as well as pure carbon tetrachloride and chloroform (20 cm^3 each), did not separate fractions, or were separated, but not completely. For example, using pure carbon tetrachloride (20 cm^3), or the system: n-hexane: ethanol (95%) in the ratio (20:0.5) cm^3, the chromatogram almost did not 'stretch' the fat, but only chloroform 20 cm^3, or the system: carbon tetrachloride: chloroform in the ratio (05:15) cm^3- on the contrary, the length of the plate was not enough even to separate compounds at the level of triacylglycerides (TAG) (Fig. 2).

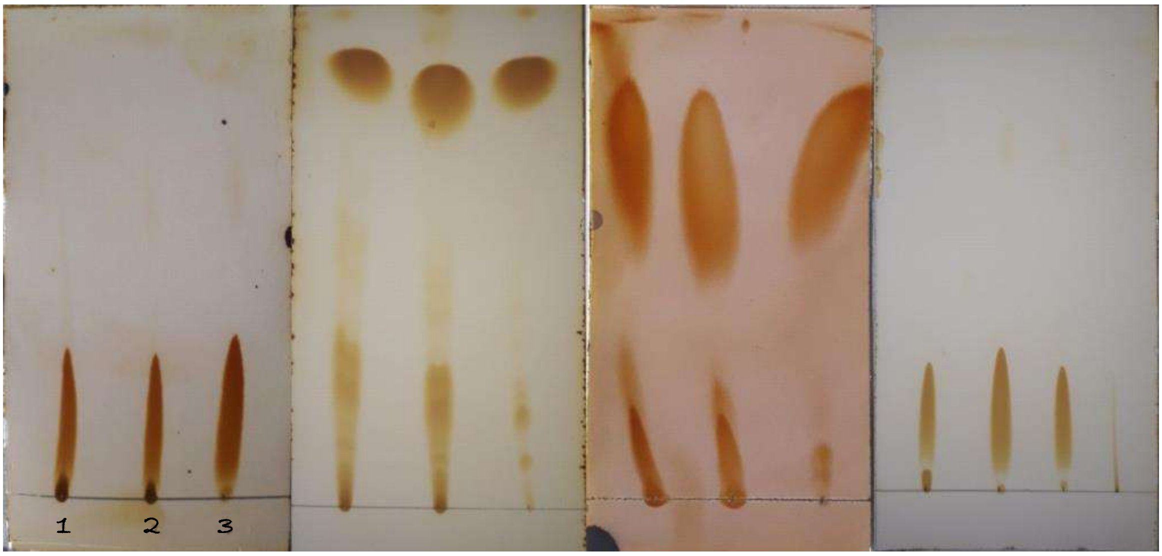

Figure 2. Thin layer chromatography of the obtained crude fat from soybean seeds using eluents in various ratios: 1 – n-hexane: ethanol (95%) in the ratio (20:0.5) cm^3; 2 – chloroform 20 cm^3; 3 – carbon tetrachloride: chloroform in the ratio (5:15) cm^3; 4 – carbon tetrachloride 20 cm^3 (left to right).

The transit time of the eluent from the start line to the finish line was 50 minutes using pure carbon tetrachloride, or the system: n-hexane: ethanol (95%) in the ratio (20:0.5) cm^3, about 30 min., using the system CCl_4:$CHCl_3$ in the ratio (8:12) cm^3, and about 20 min. using chloroform 20 cm^3, or the mobile phase CCl_4:$CHCl_3$ in the ratio (5:15) cm^3.

It has been proved that the eluents: carbon tetrachloride-chloroform in the ratio (8:12) cm^3 have the optimal separation of all the main fat-soluble fractions. The diagrams of these chromatograms are shown in Figs 3–4. The most significant spots in terms of area and size belong to TAG - the main components of vegetable oils, incl. soybean. Linseed and olive oils, taken for comparison, visually showed similar chromatographic results.

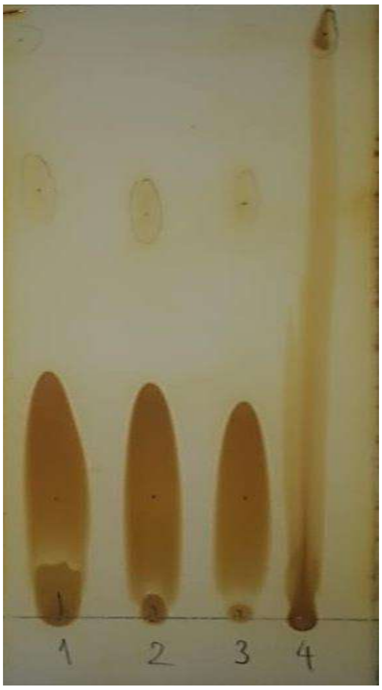

Figure 3. Thin layer chromatography of the obtained crude fat from soybean seeds: 1 – crude oil from oil seeds of the Soer-5 variety, 2016; 2 – unrefined linseed oil; 3 – vitamin E solution; 4 – lecithin solution.

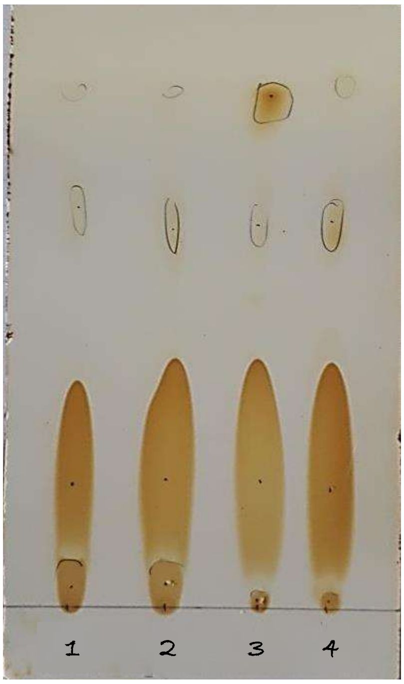

Figure 4. Thin-layer chromatography of the obtained crude fat from soybean seeds: 1 – crude oil from the seeds of the oil variety Soer-5, 2016; 2 – crude oil from the seeds of the vegetable sample Cha Kura Kake, 2016; 3 – unrefined olive oil; 4 – vitamin E solution.

In accordance with the standards and literature data, the separated lipid fractions were analyzed and the Rf index was calculated (Sherma & Rabel, 2018). It was found that the standards (tocopherol and lecithin) appear as spots with Rf 0.66 and 0.01, respectively. Based on the obtained chromatograms, a conclusion was made about the presence of tocopherol and phospholipids in crude soybean oil, based on similar Rf values, which is also confirmed by literature (Touchstone et al., 1980). Also, other adsorption spots were found on the chromatogram, which are supposed to correspond to monoacylglycerides and fatty acid esters (Table 4).

Table 4. Rf index of separated lipid fractions from crude oil of soybean samples, average for 2016–2018

No.	Separated lipid fractions	Value Rf
1	Phospholipids	0.01 ± 0.00
2	Monoacylglycerides	0.04 ± 0.01
3	Triacylglycerides	0.20 ± 0.03
4	Tocopherols	0.66 ± 0.10
5	Fatty acid esters	0.95 ± 0.14

CONCLUSSION

The oil content of seeds in vegetable forms of soybeans, in comparison with cereals, in the phase of technical and biological ripeness is less by 55.6 and 22.0%, respectively (in relative values). It does not drop below 16% under standard growing conditions. The accumulation of fatty oil is an important biochemical characteristic of vegetable-type soybeans. The most valuable lines (Sample A, Hidaka) in the phase of complete biological maturity showed a fat content of 17.1%. Oil accumulation is mainly dependent on genetic factors and is one of the main biochemical characteristics suitable for selection.

It can be concluded that thin layer chromatography is used in a qualitative analysis for the content of the main fat-soluble components (phospholipids, monoacylglycerides, triacylglycerides, tocopherols, fatty acid esters); their different content is shown in comparison with olive and linseed oils.

Optimal separation of fat-soluble fractions by thin layer chromatography was achieved by using eluents carbon tetrachloride: chloroform in a ratio of 2: 3. The use of this mobile phase made it possible to separate the main components of a lipophilic nature that are part of the fatty soybean oil: phospholipids, monoacylglycerides, triacylglycerides, tocopherols, fatty acid esters.

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