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A method for obtaining plant unsaturated fatty acid ethyl esters with the use of transesterification process

Summary

For many years now a beneficial influence of polyunsaturated fatty acids (PUFA) on human body has been known. They are not only an essential structural component of cells but also an energy carrier and set a substrate for synthesis of biologically active compounds. Various supplements containing omega fatty acids are known: shark fish oil, cod livers. Nevertheless, due to their specific taste and smell, limited availability and oxidation of fatty acids during processing and storing, possibility of implementation of these nutraceuticals is quite narrow. In diet supplementation, ethyl esters of unsaturated fatty acids came out to be especially beneficial, having stronger anti-atherosclerotic and anti-arrhythmogenic properties. Conformation of isomers of these acids is also crucial - naturally occurring as cis form, or worsening vital cell traits and possibly showing side effects as trans form. Unfortunately, during production of unsaturated fatty acids ethyl esters, undesired isomerization occurs which results in the increased amount of trans isomers at a cost of cis isomers. Thus, we propose an innovative method for transesterification of plant unsaturated fatty acids in order to obtain ethyl esters with a high content of cis isomers and purity.

Key words: *transesterification, ethyl esters, omega acids, polyunsaturated fatty acids, cis isomers*

Metoda otrzymywania estrów etylowych nienasyconych roślinnych kwasów tłuszczowych z wykorzystaniem procesu transestryfikacji

Streszczenie

Od lat znany jest zbawienny wpływ wielonienasyconych niezbędnych kwasów tłuszczowych (WNKT) na organizm człowieka. Są nie tylko podstawowym składnikiem strukturalnym komórek, ale i nośnikiem energii oraz substratem do syntezy substancji biologicznie czynnych. Znane są suplementy zawierające kwasy tłuszczowe omega: tran rybi z rekina, wątróbek dorszy. Jednak ze względu na specyficzny smak i zapach tranu, limitowaną dostępność i utlenianie kwasów tłuszczowych podczas przetwarzania i przechowywania, możliwość stosowania tych nutraceutyków jest ograniczona. Szczególnie korzystne w suplementacji diety okazały się estry etylowe nienasyconych kwasów tłuszczowych o silniejszych właściwościach antymiadżycowych i antyarytmogennych. Istotna jest także konformacja izomerów tych kwasów - naturalnie występują w postaci cis, natomiast w formie trans skutkują pogorszeniem funkcji życiowych komórek i mogą wywołać działania niepożądane. Niestety podczas procesu wytwarzania estrów etylowych nienasyconych kwasów tłuszczowych dochodzi do niepożądanej izomeryzacji i wzrostu zawartości izomerów trans kosztem korzystnych izomerów cis. Zaproponowano więc nowatorską metodę transestryfikacji roślinnych nienasyconych kwasów tłuszczowych, pozwalającą na otrzymanie estrów etylowych o wysokiej zawartości izomerów cis i czystości.

Słowa kluczowe: *transestryfikacja, estry etylowe, kwasy omega, wielonienasycone kwasy tłuszczowe, izomery cis*

List of abbreviations

AA - arachidonic acid	*GC-HRMS - gas chromatography-high resolution mass spectrometry*
ALA - alpha-lipoic acid	*GLA - gamma-linoleic acid*
CVAAS - Cold Vapor Atomic Absorption Spectrometry	*ICP-MS - Inductively Coupled Plasma – Mass Spectrometry*
DHA - docosahexaenoic acid	*LA - linoleic acid*
EPA - eicosapentaenoic acid	*LDL-C - low-density lipoprotein-cholesterol*
GC - gas chromatography	*PUFA - polyunsaturated fatty acid*
GC/MS - gas chromatography-mass spectrometry	*SDA - stearidonic acid*
GC-MS/MS - gas chromatography-tandem mass spectrometry	

Introduction

Fats are an essential source of energy as well as polyunsaturated fatty acids (PUFA), which play a crucial role in human body (National Academy Press, Washington 1989). Fatty acids constitute a basic building material, from which an organism takes structural elements of the cell and tissues and substrates for synthesis of biologically active substances. Omega-3 and omega-6 acids are crucial for proper growth, development and functioning of many organs in the human body, especially within cardiovascular system, retina and brain (Gallai et al., 1995; Holm et al., 2001; Nowak, 2009).

PUFA type n-3 and n-6 cannot be synthesized by human, thus must be delivered within diet. Basic PUFAs are α-linolenic acid (C 18:3) from n-3 family, a precursor of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) as well as linoleic acid (C 18:2), a precursor of arachidonic acid (AA). Supplementing with a proper proportion of these acids has a significant influence on the development and functioning of the human body (Kromhout, 1992; Connor, 1999).

ALA and LA should constitute 1/3 of a daily intake of fats, taking into account n-6 to n-3 ratio as 5:1 to 3:1 (Marciniak-Łukasiak and Krygier, 2004). It was shown that the excess of n-6 fatty acids in diet inhibits n-3 fatty acids metabolism, which can lead to disturbance in the balance of biologically active substances synthesized from these acids. In the human body, omega-3 and omega-6 acids are substrates of the same metabolic pathways. Their metabolites act antagonistically against each other. Metabolites of omega-6 acids show proinflammatory and prothrombotic effects, whereas omega-3 acids act anti-inflammatory and against platelet aggregation (Marciniak-Łukasiak and Krygier, 2004). That is why it is crucial to keep the right ratio between these two types of fatty acids in the diet. Numerous studies has shown that individuals consuming products rich with omega-3 acids have lower occurrence of cardiovascular diseases (Connor, 2000). Excessive consumption of n-6 acids inhibit metabolism of n-3 acids disturbing the balance between derivatives of these types of acids (Newton, 1996).

Human body possesses metabolic pathways, which enable synthesis of long-chain, unsaturated fatty acids not present in the diet. Here, LA and ALA act as precursors. Elongases elongate carbon chains and desaturases create additional double bonds, resulting in polyunsaturated fatty acid chains with no less than 20 C atoms (Behrouzian and Baist, 2003). The most important derivatives are: n-6 AA (from LA) as well as EPA and DHA (from ALA). For the metabolic conversion of n-6 LA and n-3 ALA compete the same enzymes, hence the excess of LA in a diet inhibits synthesis of EPA and DHA, and promotes synthesis of AA, which results in body homeostasis (Gurr, 1992; Hamilton et al., 1998). Long-chain polyunsaturated fatty acids are considered essential for human body. They are crucial for the proper development and functioning of the brain and retina (Newton, 1996).

Unsaturated fatty acids occur in different spatial configurations, depending on the location of hydrogen adjacent to a double carbon bond. Yet, it is noteworthy that number of C, its placement as well as a number of double bonds influence the properties and role of fatty acids in the human body. Depending on configuration of these fatty acids, isomers can be divided into two types: *cis* (*Z* configuration) and *trans* (*E* configuration). Naturally occurring fatty acids present *cis* configuration. Unsaturated *trans* fatty acids, when built into the cell membrane, result in deterioration of cell vital traits and may promote atherosclerotic lesions in blood vessels (Kozłowska-Wojciechowska, 2003).

Various plant-derived substances are widely known as healing agents. For many years it has been known that both plant oils and substances solved within, contain unsaturated fatty acids and vitamins, e.g. showing antioxidant effect (vitamin E), supporting immunity. Importantly, many studies confirmed health-supporting effect of plant-derived unsaturated fatty acids on human body. Of special interest are unsaturated fatty acid ethyl esters, both in terms of obtaining and implementation in health improvement and reduction of cardiovascular, skin, inflammatory and allergic diseases. PUFA esters show health-beneficial effect as well. Research has shown that plant sterol esters help reduce LDL-C level by 10-15% showing no side effects (Nguyen, 1999; Wen-Sen et al., 2018). For example, children with familial hypercholesterolemia had reduced LDL-C level when daily intake of plant sterol esters was at the level of 1.6 g (Amundsen et al., 2002). Esters of fatty acids are obtained in the transesterification process (Derosa et al., 2009; Adkins and Kelley, 2010; Cabo et al., 2012; D'Vaz et al., 2012; Black et al., 2016).

Transesterification process is used due to the fact that non-modified chains of plant sterols have many traits limiting their use in the food industry (high melting point - at least 135°C; low solubility in oils). PUFA esters show better bioavailability and solubility in fats, not influencing taste and consistency of the final food products. Esterified plant long-chain unsaturated fatty acids are implemented in the production of: margarines, yoghurts, cheese spreads and cereal bars. Esterification increases PUFA solubility in oils from 2 to 20% (Jandacek et al., 1977; He et al., 2018).

To date, many types of reactions allowing conversion of plant fatty acids into ester form have been characterized. They can be divided into three main categories: catalyzed by acid or base compounds or enzymes (mainly lipases). Also many methods supporting esterification are known. For example, in the production of plant and algae sterol esters, ultrasounds and microwaves are helpful (Shang et al., 2015; Zheng et al., 2012). Ultrasounds reduce particles size, increasing the surface of the interaction with an enzyme and reaction efficiency. Furthermore, applying ultrasounds for 10h can accelerate the reaction 2x compared to stirring the reaction for 24h (Zheng et al., 2012). Microwaves increase activity and thermal stability of lipases. This helped to reduce esterification time 6x (Shang et al., 2015).

Transesterification in acidic environment can be divided into homogenous and heterogenous catalysis. The first one features low cost and high efficiency but also low quality of separation of final products. Usually H_2SO_4 is used as a catalyst, which enables conversion of plant sterols into fatty acid esters with efficiency of 98% (Meng et al., 2011). However implementation of this catalyst results in forming by-

products such as dehydrated sterols (at the level of 19%) (Meng et al., 2011; Yang et al., 2012). Heterogenous catalysis with the use of 0.2% tungstosilicic acid enabled conversion into plant sterol esters with efficiency at the level of 90% (Meng et al., 2006). Recently more popularity gained ionic liquids, such as choline chloride/tin (II) chloride ($ChCl{\cdot}2SnCl_2$). Esterification rate can reach 92%, nevertheless high temperature is needed for efficient reaction (150°C for 4h) (Yang et al., 2012).

Plant fatty acid esters can also be synthesized via transesterification with the use of base catalysts, such as metallic hydroxides, alkoxides and other oxides (Pouilloux et al., 2003; He et al., 2014; Valange et al., 2007). This type of transesterification allows for efficient reaction, high ester-formation rate and cost effectiveness (Deshpande et al., 2017). On the other hand there are some disadvantages, such as low selectivity and high by-products concentration (due to high temperature of reaction required by metallic hydroxides). Temperature required by metallic oxides is at least 170°C thus by-products are inevitably formed. For example, when this type of transesterification of plant sterols is performed at 230-250°C, the content by-products: diene and oxide sterols can reach 6.3% and 9%, respectively (Meng et al., 2011). This occurs during formation of soap, polymerization and oxidation, specific for this type of transesterification. It is also difficult to separate catalyst from the final product, increasing the amount of waste products during purification (Valange et al., 2007).

Another type of plant sterols conversion into esters is esterification carried out by enzymes. It has some advantages: mild conditions of reaction, relatively high selectivity, lower amount of by-products. Of special interest are lipases - biocatalysts with varying activity depending on their structure. During the last 20 years researchers described various examples of transesterification with the use of lipases from different sources in more than 40 papers (He et al., 2018).

Depending on substrate, different esterification efficiency with the use of lipases from *Candida rugosa* was reached. Example given, conversion of dodecanoic acid reached 75% (Miao et al., 2014), rapeseed sterols - 75% (Villeneuve et al., 2005), oleanolic acid - 97% (Kim and Akoh, 2007). Despite high conversion rate, lipases also possess a number of drawbacks. First, it is difficult to reuse these enzymes for another round of esterification, which makes this method complicated and expensive. Anyway, recently this obstacle was overcome by immobilizing the enzyme on different beads: synthetic polymers, biopolymers or hydrogels, which increases enzyme stability and catalytic efficiency (Zheng et al., 2013; Shuai et al., 2017). Nevertheless, the range of products from a specified lipase is closely related to the source of enzyme. Moreover, lipases are temperature sensitive: it influences enzyme activity and stability. On the one hand, temperature increase results in a better solubility of substrate, on the other - limits stability and activity of the enzyme. Consequently, for example, optimum temperature for immobilized lipase from *C. rugosa* needs a strict control of environmental conditions for the best transesterification ratio (He et al., 2018). Most importantly, a significant limitation for the use of lipases for transesterification is a low conversion rate of unsaturated *cis*-4, *cis*-6, *cis*-8 fatty acids (Jachmanián et al., 1996). Described transesterification procedures have a number of assets. Nevertheless, each of them has disadvantages limiting their use in the food industry.

The aim of work

Taking into account drawbacks of methods mentioned above, a method for plant fatty acid transesterification in order to obtain pure unsaturated fatty acid ethyl esters was not yet developed. Due to the beneficial effect of unsaturated fatty acids omega 3, omega 6 and omega 9 on human body, we established an innovative method for transesterification of plant oils to obtain a mix of unsaturated fatty acid ethyl esters with a high content of *cis* isomers. To overcome limitations of methods mentioned earlier, we propose an innovative technique of obtaining omega 3, 6, 9 fatty acid ethyl esters maintaining 95-100% *cis* isomers of unsaturated fatty acids contained in a plant material, limiting isomerization effect and undesired *trans* isomers content in the final product at the same time.

Material and methodology

One aim of our method was to provide a combination of ethyl esters of different unsaturated fatty acids contained in various plant oils. The oils used in tests are presented in Table 1.

For experiments, an example of the following plant oil mixture was as follows:
-primrose oil (1% to 30%),
-borage oil (1% to 30%),
-blackcurrant oil (1% to 10%),
-linseed oil (up to 100%),
and different quantitative combinations within specified limits were used. Importantly, due to a mono- and polyunsaturated fatty acids content in the source material, it was produced and stored in the temperature- and light-controlled environment and manufactured at nitrogen atmosphere. This also applies for conditions used for the following process for transesterification of fatty acids and its components (ethanol, KOH, evaporators, batch method). During manufacturing, raw oil slimes were clarified with sedimentation hence the presence of plant particles in the source material for transesterification process could be noted (microparticles of plant tissues, organic material, etc.). At all potential points of contact of substrates vulnerable to humidity (e.g. ethanol 99.9%) and carbon dioxide e.g. KOH, or products vulnerable to atmospheric oxygen (oils and mixtures of, esters), dry nitrogen atmosphere was applied.

Tab. 1. A summary of physical and chemical properties of plant oils used in transesterification process
Tab. 1. Zestawienie właściwości fizycznych i chemicznych olejów roślinnych użytych w procesie transestryfikacji

Oil Skurce; Źródło oleju	Main compounds; Główne związki	Specific gravity; Ciężar właściwy	Iodine index; Wskaźnik jodowy
Primrose seed oil; Olej z nasion wiesiołka	*Cis*-linoleic acid (LA) - 73.5 - 81.9%, gamma-linoleic (GLA) - 6.8 - 9.4% and oleic acid - 4.7 - 10.7%. Saturated fatty acids: palmitic (5.0 - 6.7%), stearic (1.1 - 2.9%). Contains saponins, polyphenos, glycolipids. Kwas cis-linolowy (LA) - 73.5 - 81.9%, gamma-linolenowy (GLA) - 6.8 - 9.4% i oleinowy- 4.7 - 10.7%. Nasycone kwasy tłuszczowe: palmitynowy (5.0 - 6.7%), stearynowy (1.1 - 2.9%). Zawiera saponiny, polifenole, glikolipidy.	0.915 - 0.935 g/ml	130 - 200% I
Borage oil; Olej z ogórecznika lekarskiego	Drying oil. Contains 18 - 25% gamma-linoleic acid (GLA) rich in flavonoids (quercetin, isorhamnetin, kaempferol), tannins, slime, acids: linolenic (38%), eicosenoic (4%), docosenoic (3%), mineral salts, saponins. Olej schnący. Zawiera 18 - 25% kwasu gamma-linolenowego (GLA) bogatego we flawonoidy (kwercetynę, izoramnetynę, kemferol), garbniki, śluz, kwasy: linolowy(38%), eikosenowy (4%), dokosenowy (3%), sole mineralne, saponiny.	0.900 - 0.940 g/ml	135 - 175% I
Blackcurrant oil; Olej z czarnej porzeczki	Contains over 80% polyunsaturated fatty acids, e.g.: linoleic (LA), *alpha*-linolenic (ALA), stearidonic (SDA). Phytosterols and tocopherols at the level of 1.5-2.5%. Zawiera ponad 80% wielonienasyconych kwasów tłuszczowych, np. kwas linolowy (LA), *alfa*-linolenowy (ALA), stearydynowy (SDA). Fitosterole i tokoferole na poziomie 1.5-2.5%.	0.910 g/ml	145 - 185% I
Linseed oil; Olej lniany	Drying oil. Contains over 50% of linolenic acid (omega-3), 15% linoleic acid (omega-6), 15% oleic acid (omega-9) and small quantities of saturated fatty acids. Olej schnący. Zawiera powyżej 50% kwasu linolenowego (omega-3), 15% kwasu linolowego (omega-6), 15% kwasu oleinowego (omega-9) oraz niewielkie ilości nasyconych kwasów tłuszczowych.	~ 0.931 g/ml	160 - 200% I

As a preliminary step preceding esterification was a complete homogenization of an oil mixture. Progressive cavity pump with 3 - 8 cavities (e.g. DWO station on KAS 3, Unister Plus Mirosław Pytlik) was used. This enabled to obtain homogenous oil mixture, regardless of quantitative combination of different raw oils. The mixture was lucid, did not stratify even after a few days of storage. At this stage, concentration of *cis* isomers was determined with GC/MS and over 95% of *cis* isomers were still preserved. For the test purpose, comparison with a sonic cavitation was done. This method enabled us to obtain homogenous oil mixture, nevertheless significant amount of *cis* isomers spontaneously converted into *trans* isomers.

Total homogenization of an oil mixture was followed by the essential esterification process. Batch method was applied, meaning alternately running two reactors - while one carries the reaction, second is filled and prepared for transesterification process with oils mixture, ethanol and catalyst. Transesterification in the present of KOH was carried out at the temperature below 49°C (within the ranges of 40°C and 49°C), at atmospheric pressure and at least 16x alcohol molar excess against triglycerides (plant oils).

One of the most unwanted final product features is the presence of catalyzers in the final product. In order to eliminate unreacted alcohol, two cascading thin-wall vacuum evaporators were applied. First of them was used to evaporate majority of anhydrous ethanol, and second to evaporate remaining ethanol from the effluent. In order to accelerate the process of ethanol evaporation, oil mixture containing ethanol was sprayed (perpendicular to the collecting surface of the chamber). This enabled evaporation of the majority of ethanol at the temperature below 49°C in less than 20 minutes. Vapor diffuser was also applied which enabled limiting of transferring oil droplets to the outlet of vapor. Dual evaporation step enabled us to achieve <0.1% ethanol content in the final product. The yield of the transesterification process reached 90.42% (90.42 g of ethyl esters obtained from 100 g of raw oil).

Following ethanol evaporation, we also propose a robust method for removal of catalyzer, soap and foam, enabling us to obtain a high-purity final product. It was divided into 5 steps, as follows. First, short-term batch separators were used to roughly stratify ester and glycerine fractions. Later, ester fraction was subject to centrifugation, allowing removal of residual and emulsified glycerine fraction (purification step). Purified ester fraction was further purified by storing ester fraction in a whole-day separator. Remaining quantities of glycerine fraction were further removed by slow gravitational stratification and residual alcohol was removed via flushing ester fraction with nitrogen. At this step, pure ethyl ester fraction was already obtained. To even further purify the product, it was clarified with another centrifuge. Such thoroughly purified product was transferred to centrifuge storage compartment. Till investigation of ethyl ester fraction composition, it was subsequently collected at nitrogen-saturated atmosphere.

Kempiński i in., (2018). A method for obtaining plant unsaturated fatty acid ethyl esters with the use of transesterification process. *Inżynieria Przetwórstwa Spożywczego*, *2/4*(26), 22-29.

Results and discussion

As a result of transesterification process, an oleic, orange-yellowish substance was obtained. We decided to investigate the composition of this final product of transesterification process obtained from mixed plant oils listed in Tab. 1. To investigate fatty acids composition, analysis of five samples (Fig. 1., Tab. 2.) was done. The final product obtained with proposed novel method for transesterification of plant oils was analyzed with the use of GC/MS (gas chromatography coupled with mass spectrometry) (Agilent Technologies 122-2362 DB-23, 60 m x 0,250 mm, 0,25 Micron, 40-250/260 C; conditions were applied as follows: initial temp: 60°C; ramp 1: 175°C (25°C/min); ramp 2: 230°C (4°C/min); flow: 1,145 mL/min; pressure: 20 psi; standards used: "Fatty acid ethyl ester Standard Pack" (Item: 10008188), "γ-linolenic acid ethyl ester" (Item: 9000738) (Cayman Chemical Company)).

Surprisingly, significant amounts of high-purity unsaturated fatty acid ethyl esters were detected (Fig. 1.). Based on retention time, ethyl esters of the following fatty acids were detected (marked in red) as: palmitic acid (RT 15.926), stearic acid (RT 18.752), oleic acid (RT 19.168), linoleic acid (RT 19.979), gamma-linolenic acid (RT 20.469), alpha-linolenic acid (RT 21.098).

Most importantly, in terms of the negative influence of *trans* isomers on human health, no additional peaks were detected (Fig. 1.). The only detected peaks of fatty acid ethyl esters detected (marked in red), belonged to *cis* isomers. Hence, no *trans* isomers were present in the final product of the proposed transesterification process.

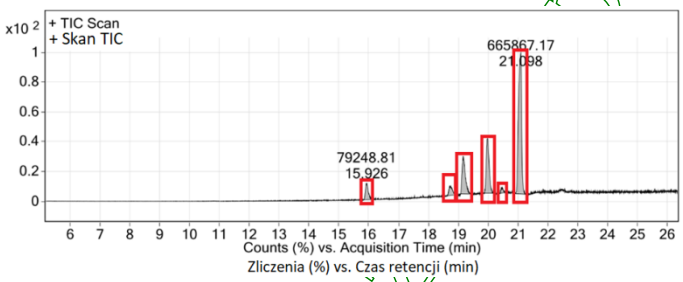

Fig. 1. GC/MS chromatogram of selected sample from the final product of transesterification. Peaks of ethyl esters of the following fatty acids were detected (from the left): palmitic acid, stearic acid, oleic acid, linoleic acid, gamma-linolenic acid, alpha-linolenic acid

Rys. 1. Chromatogram pochodzący z analizy GC/MS dla wybranej próby produktu finalnego procesu transestryfikacji. Wykryto piki estrów etylowych następujących kwasów (od lewej): kwasu palmitynowego, kwasu stearynowego, kwasu oleinowego, kwasu linolowego, kwasu gamma-linolenowego i kwasu alfa-linolenowego

To further analyze the ratio between ethyl esters of saturated and unsaturated fatty acids, we calculated participation of each ethyl ester fraction in the final product. Ethyl esters of saturated fatty acids: palmitic and stearic constituted only less than 13% of the total ethyl esters. Remaining ethyl esters of fatty acids: oleic, linoleic and linolenic represented about 87%.

Tab. 2. A fatty acid ethyl esters profile of five samples obtained with the novel procedure for transesterification of plant oils specified in Tab.1.

Tab. 2. Procentowy profil estrów etylowych kwasów tłuszczowych pięciu próbek otrzymanych metodą transestryfikacji roślinnych kwasów tłuszczowych wymienionych w Tabeli 1.

Fatty acid ethyl esters profile; Profil estrów etylowych kwasów tłuszczowych	**Sample 1; Próbka 1**	**Sample 2; Próbka 2**	**Sample 3; Próbka 3**	**Sample 4; Próbka 4**	**Sample 5; Próbka 5**	**Mean; Średnia**
Palmitic acid ethyl ester C16:0 [%]; Ester etylowy kwasu palmitynowego C16:0 [%]	5,66	9,03	7,73	6,68	10,36	**7,89**
Stearic acid ethyl ester C18:0 [%]; Ester etylowy kwasu stearynowego C18:0 [%]	3,97	6,23	4,42	4,23	6,06	**4,98**
Oleic acid ethyl ester C18:1 [%]; Ester etylowy kwasu oleinowego C18:1 [%]	14,94	14,2	15,62	15,05	16,22	**15,21**
Linoleic acid ethyl ester C18:2 [%]; Ester etylowy kwasu linolowego C18:2 [%]	19,51	18,49	20,42	18,96	19,24	**19,32**
Gamma-linolenic acid ethyl ester C18:3 [%]; Ester etylowy kwasu gamma-linolenowego C18:3 [%]	1,57	2,28	1,9	2,03	2,24	**2,00**
Alpha-linolenic acid ethyl ester C18:3 [%]; Ester etylowy kwasu alfa- linolenowego C18:3 [%]	54,35	49,77	49,91	53,05	45,88	**50,60**

Besides analysis with GC, for safety reasons, the final product was further analyzed for the presence of heavy metals applying analysis with ICP-MS (Inductively Coupled Plasma – Mass Spectrometry) and CVAAS (Cold Vapor Atomic Absorption Spectrometry). Beneficially, as expected, significant amounts of vitamins A, D_3, E and K were detected (0.59 mg, 0.078 mg, 353 mg and 0.209 mg on average per kg of final product, respectively). Noticeably, high levels of sterol compounds were present (per 100 g of fat on average): brassicasterol (<1.0 mg), kaempferol (57.7 mg), stigmasterol (10.8 mg), β-sitosterol (211.4 mg), Δ5-avenasterol (45.3 mg). Microbiological analysis was also done. The product was considered

microbiologically safe, since number of colony-forming units was defined as below 10/g of the final product. For the investigation of purity in terms of the presence unreacted catalyst in the final product, acidic value was calculated and determined as 0.05 g KOH/g of the final product. Glycerine was measured as below detection level (below 10 mg/kg of the final product). We also decided to investigate the presence of di- and monoglycerides - intermediates of the transesterification process (Tab. 3.). All of investigated intermediates were also considered at low level.

Tab. 3. Amounts of intermediates: di- and monoglycerides in the final product of transesterification of plant oils specified in Tab.1.

Tab. 3. Ilość produktów pośrednich: di- i monoglicerydów w produkcie finalnym procesu transestryfikacji olejów roślinnych wymienionych w Tabeli 1.

Intermediates of transesterification of plant oils [g/100g]
Produkty pośrednie transestryfikacji olejów roślinnych [g/100g]

Compound Związek	Amount Ilość
Monolaurine (monolauryna)	<0,10
Monolinolein (monolinoleina)	0,234 [±0,072]
Monomyristine (monomirystyna)	<0,10
Monoolein (monooleina)	0,111 [±0,068]
Monopalmitine (monopalmityna)	<0,10
Monopalmitolein (monopalmitooleinian)	<0,10
Monostearin (monostearyna)	<0,10
1,2-Distearin (1,2-distearyna)	<0,20
1,2-Diolein (1,2-dioleina)	<0,20
1,2-Dipalmitin (1,2-dipalmityna)	<0,20

Iodine index was also measured for the final product and set as 196.8% I. Taking into account iodine index values of different plant oils used in the transesterification process, the amount of unsaturated fatty acids did not change significantly during the process and remained relatively high.

Discussion

Unsaturated fatty acids are not only an essential structural component of cells but also an energy carrier and set a substrate for synthesis of biologically active compounds (Nowak, 2009). Besides number of carbon double bonds, configuration of fatty acids is important as well. Only *cis* isomers show beneficial effects on the human body, whereas *trans* configuration causes deterioration of cell vital traits and can promote atherosclerotic lesions in blood vessels (Kozłowska-Wojciechowska, 2003). It is also known that ethyl esters of plant fatty acids can reduce cardiovascular, skin, inflammatory and allergic disorders even more efficiently compared to non-modified carbon chains. Recently, an increasing interest is observed in techniques that allow for obtaining such esters. Fatty acid esters are produced as a result of transesterification reaction.

Presented conventional methods for transesterification of fatty acids described so far possess a number of both advantages and disadvantages. To date, no ultimate method for transesterification of plant fatty acids was described in the literature, enabling to obtain pure fraction of fatty acid esters with a high content of desired *cis* isomers. For example, transesterification in acidic environment is cost-efficient and has high effectiveness. Nevertheless, high temperature is necessary for the reaction to occur and by-products are not easy to separate from the final product (Meng et al., 2011; Yang et al., 2012). Alternatively, fatty acid esters can be obtained through transesterification with the use of base catalysts. Similarly to transesterification in acidic environment, the method is cost-efficient and results in high ester-formation rate as well. On the other hand, low selectivity of desired final products, high reaction temperature, relatively high amounts of by-products and difficulties with the separation of catalyst from the final product is inevitable drawbacks of this method (Pouilloux et al., 2003; Meng et al., 2011; He et al., 2014; Valange et al., 2007). In this respect, another method was proposed as a reasonable alternative. Of special interest is transesterification with the use of enzymes, namely biocatalysts. Until now, more than 40 papers describe the use of lipases from different sources (He et al., 2018). Generally they possess high conversion rate, have high selectivity towards desired final products, enable implementation of mild reaction conditions but are hard to separate from the final product when not immobilized (Zheng et al., 2013; Shuai et al., 2017). Because of temperature sensitivity, they require strict control of reaction conditions. What is more, they present low conversion rate of unsaturated *cis* isomers, which is a serious limitation of this method in terms of obtaining nutraceutical of high quality (Jachmanian et al., 1996).

Taking into account a beneficial effect of unsaturated fatty acid esters in *cis* configuration on the human body and limitations of methods described, we propose a novel method for transesterification of plant fatty acids. This comprises the following stages. Initially, processing mixture plant oils with a progressive cavity pump enabled us to obtain homogenous and lucid plant slime, subsequently subjected to transesterification process. Transesterification in the presence of KOH is carried out at the temperature below 49°C, at atmospheric pressure and at least 16x alcohol molar excess against triglycerides (plant oils). One of the most important steps allowing for obtaining pure final product is a unique two-stage ethanol evaporation system allowing for less than 0.1% ethanol content in the final product. Following ethanol removal, we also applied a method allowing for removal of catalyzer, soap and foam from the final product. This consisted of a specific combination of short- and long-term separators, centrifugation and flushing ester fraction with nitrogen. To preserve a high content of *cis* isomers in the ester fraction, samples were bottled at the nitrogen atmosphere and subjected to GC/MS, GC-MS/MS, GC-HRMS and ICP-MS analysis. Elimination of the presence of oxygen starting from the storing of oils, via the whole process to packaging allowed for elimination of unwanted conversion of *cis* isomers into *trans* isomers. Obtained fatty acid ethyl ester fraction had a high content of converted unsaturated fatty acids - 87% of the total fraction of ethyl esters. What is truly advantageous against other methods listed in this paper, preservation of a high content of *cis* isomers in the final product is noticeable, as no unidentified peaks were present in the chromatograms of all samples subjected to analysis. Stand-

ards applied for GC/MS analysis allowed for identification of solely *cis* isomers

Conclusions

As awareness of the role of nutrition in human health increases, higher demand on nutraceuticals is observed. Variety of methods for obtaining such nutraceuticals was described, but no ultimate method lacking disadvantages limiting their use in the food industry was developed so far. Ideally, transesterification process resulting in formation of nutraceuticals would provide fatty acid ethyl esters of high purity (lacking catalyst) and *cis* isomer content, low amounts of by-products as well as recognized as safe for use in the food industry. In this paper we demonstrate a method allowing for obtaining a final product meeting all those requirements. Such product of transesterification was obtained from a mixture of oils: primrose seed, borage, blackcurrant and linseed. Based on the results of GC/MS, GC-MS/MS, GC-HRMS, ICP-MS and microbiological analyses of the final product of transesterification, such technique can be successfully applied in the food industry. Based on the previous findings on the impact of specific components of the final product on human health, especially plant unsaturated fatty acid ethyl esters, the nutraceutical obtained by applying this novel method can be implemented in human diet and positively influence health status.

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