Studies on the extraction of cyclic peptides from flax waste materials

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Introduction

Linseed is one of the oldest cultivated plants, with invaluable health properties. Nature has equipped linseed in nutritious plant elements sought after by human beings. People figured out long ago that frequent consumption of linseed (minimum 10 g a day) protects against such diseases as: atherosclerosis, hypertension, heart infarction, cerebral stroke, cataract. It also prevents certain types of cancer: breast, mucosal, uterine, prostate and colonic carcinoma. It shields the digestive tract (especially stomach) and acts as a laxative. It is used in inflammations of the digestive tract, in constipations or respiratory tract infections. It decreases the level of cholesterol and provides regeneration of the mucous membranes. It supports digestion thanks to a large content of cellulose. Externally broken-up seeds may be used in the form of compresses for abscesses and ulcers which are cured faster.

Linseed contains approx: 8% of mucus, 30÷40% of oil, 25% of proteins, glycosides, enzymes, mineral salts (iron, magnesium, copper, zinc, cobalt) [1].

Plant mucus is known as a natural coating substance, diluted in water into mucilage of a high viscosity and ductility. It naturally attenuates the impact of factors which irritate mucus membranes of the digestive and respiratory tract. Linseed is recommended in therapies of the digestive and respiratory tract or dermatological affections; in them linseed mucus is used for shielding, diarrhea, softening and alleviating. Based on genetically modified linseed, the team from the Institute of Biochemistry and Molecular Biology at the University of Warsaw led by prof. Jan Szopa-Skórkowski developed a dressing which proved to be salutary for patients dealing with chronic venous ulcerations or bedsores. The inventive dressing comprises three elements: fabric, emulsion and flax extract [1].

Linseed contains also approx: 40% of precious oil. Linseed oil is a unique vegetable oil which contains over 90% of unsaturated fatty acids. It contains approx. 60% of linoleic acid glyceride, 20% of linolenic acid glyceride, 5% oleic acid glyceride, 8% of saturated acid glycerides, 1% of free acids, as well as vitamin E and other compounds.

It is worth noting that this oil contains over 50% of fatty acids from the omega 3 family, which contain a very important link in proper cholesterol conversions in the human body. Fatty acids from the omega 3 family, belonging to so-called necessary unsaturated fatty acids (NNKT), are very rarely met in other vegetable oils. The majority of commonly consumed vegetable oils contain almost entirely polyunsaturated fatty acids from the omega 6 family. As civilization progresses, the consumption of omega 6 acids advances at the cost of omega 3 acids, which is the main cause for currently increasing occurrence of heart and circulatory system diseases.

The third important component of linseed is a very valuable and easily digestible protein which occurs in the seed in the amount of 25%. The digestibility indexes of linseed protein under the activity of pepsin and tripsin belong to one of the highest ones. They are higher even than digestibility indexes of milk protein, and the supplement of linseed protein with milk protein allows for obtaining an ideal composition of amino acids.

Flax protein is rich in essential amino acids, i.e. components which have to be provided from the outside and whose lack may cause numerous disorders in the human body. Flax protein, compared to other plants' proteins, contains relatively considerable amounts of tryptophan, an essential amino acid whose lack unfavorably influences the sight and favors the occurrence of skin diseases. In recent years cyclic peptides were separated from flax [1], so-called cyclopeptides composed of 8 or 9 cyclically connected amino acid molecules. They have a molecule structure similar to that of cyclosporine which is composed of 11 amino acids and is used as an immunosuppressant necessary during transplants.

Bioactive flax components include also recently discovered chemical compounds from the group of so-called phytohormones – plant hormones referred to as plant lignans [2, 3]. Lignans may occur in many plant species; linseed contains on average 700 times more of these substances than other, often consumed plant products. The hormone dependency of certain types of neoplastic diseases has already been pointed out, which led to further studies aimed at testing the protective effect of a diet rich in plant hormones with regard to certain types of neoplasms.

The problem is very interesting; thus, for some time we have been observing a true explosion of research studies on the relation between a diet containing linseed and prophylaxis and treatment of certain types of neoplasms in animals and people.

As of now, very promising results were obtained regarding a protective effect in certain types and stages of neoplastic, autoimmune diseases, as well as both types of diabetes. Moreover, scientific reports have appeared on the favorable impact of this diet on symptoms related to menopause and osteoporosis. A promising direction of studies is the application of cyclopeptides from flax as autoimmune drugs.

Immunosuppressants are drugs which inhibit or weaken immunological responses. An indication for their use is kidney and tissue transplants, as well as immunological diseases. Immunosuppressive activity is shown in: adrenal cortex hormones, antimetabolites which inhibit biosynthesis processes, alkylating drugs which inhibit the production of antibodies, antilymphocyte globulin containing antilymphocyte antibodies, cyclosporine. Immunosuppressants inhibit the process of producing antibodies and immunological cells in the organism, i.e. immunogenesis. In transplantology they are used to weaken the reaction of the recipient's organism to a transplanted tissue which for him is an antigen. The application of immunosuppressants directly after transplantation decreases the risk of rejecting the transplant.

A well-known and used immunosuppressant is cyclosporine. Cyclosporine A (Fig.1), the active substance of the drug, is a nonribosomal cyclic peptide consisting of 11 amino acids, produced by the *Tolypocladium inflatum* fungus, originally isolated from a sample of Norwegian soil.

The mechanism of cyclosporine involves bonding with cyclophilin, a cytosolic protein, in immunocompetent lymphocytes, mainly type T. A complex of these two proteins inhibits calcineurin, which in

normal circumstances is responsible for activating the transcription of interleukin-2. Moreover, it inhibits the production of lymphokines and the release of interleukins, which leads to reducing the function of T cell effectors.

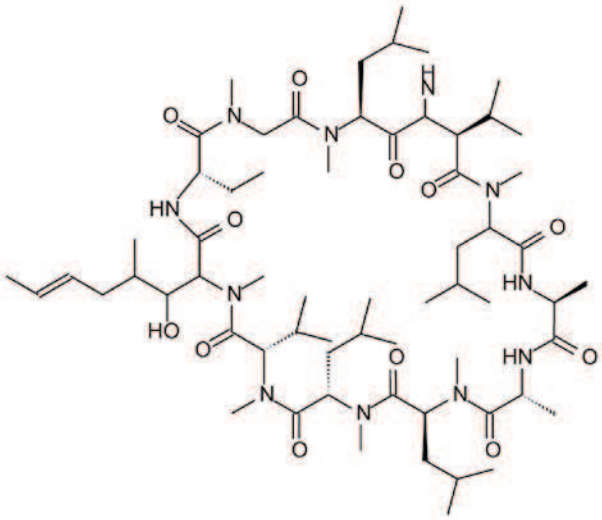

Fig. 1. The structure of cyclosporine A

Unfortunately, numerous side effects are observed in people who take cyclosporine – especially in patients treated for transplantological reasons. The most often include: kidney dysfunctions, arterial hypertension, tremors, headaches and hyperlipidemia. For this reason new, better immunosuppressants are constantly searched for.

Flax seeds were found to contain cyclic peptides which are an object of interest because of a similar structure to that of cyclosporine, which could suggest potential immunosuppressive activity.

Cyclopeptides are cyclic nona- or octapeptides with hydrophobic properties and a potential immunosuppressive activity.

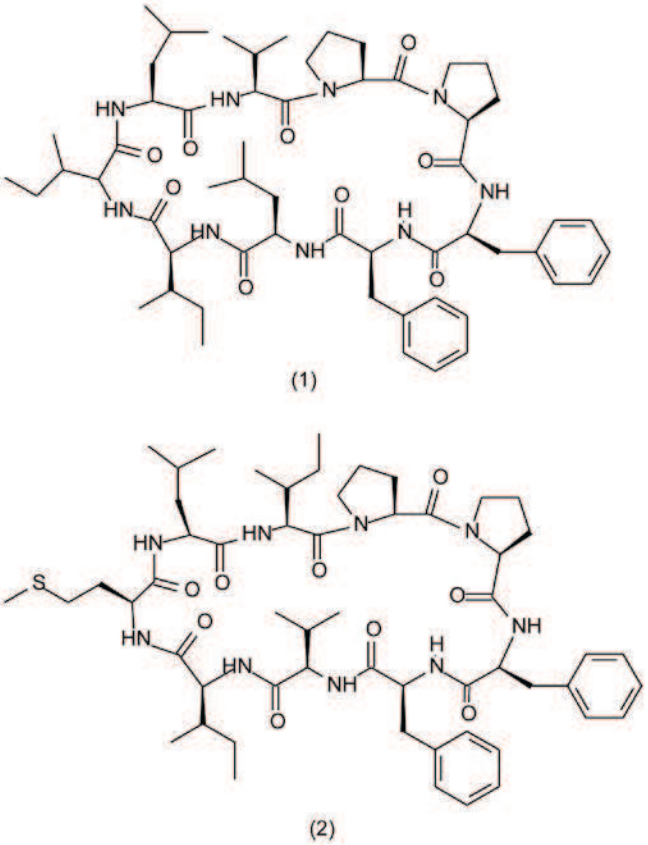

Fig. 2. Structures of cyclopeptides CLA (1) and CLB (2)

Cyclopeptide A (CLA) was first isolated in 1959 from raw linseed oil by Kaufmann and Tobschirbel [1]. 10 years later the primary structure of CLA (1) (Fig. 2) as a cyclic nonapeptide was specified. The CLA cyclo(PPFFLIILV)nonapeptide molecule contains two amino acid radicals of proline, two phenylalanines, leucine, two isoleucines, leucine and one valine radical. The CLB cyclo(PPFFVIMLI) particle (2) (Fig. 2) [4] is composed of two amino acid radicals of proline, two phenylalanines, valine, isoleucine, methionine, leucine, isoleucine.

For these cyclononapeptides, the mutual bonding of amino acids and configurations around chirality centers were determined using mass spectrometry, spectroscopy ^{1}H-NMR and ^{13}C-NMR, as well as 2D spectroscopy NMR (HMBC, NOESY).

Up to now, structures were separated and specified for 13 cyclic peptides from linseed. They are cyclic:

- nonapeptides (CLA, CLB, CLC) [4]
- octapeptides (CLD, CLE, CLF, CLG, CLH, CLI, CLP, CLW, CLX) [4, 5]
- bicyclic decapeptide (BCD).

The similarity of an amino acid sequence between cyclosporine and CLA suggested an immunosuppressive activity of the cyclopeptide which has been confirmed by numerous medical tests [6, 7]. It was stated that the activity of CLA on the body's immune system is analogous to the function mechanism of cyclosporine.

Based on initial medical studies it has also been stated that cyclopeptides, compared to cyclosporine, show a similar immunosuppressive activity in lower doses, they have a considerably lower toxicity and do not cause side effects. One has to wait for a confirmation of these studies because of time-consuming and costly medical tests which occur according to strictly defined procedures.

Moreover, cyclopeptides also show other favorable effects, e.g. antimalarial [8].

Our studies are aimed at separating cyclopeptide fractions from oils, linseed cake or waste chaff and their division into individual constituents using a chromatographic method and identification through spectroscopic methods [9]. The obtained cyclopeptide fractions will be subjected to a test of pharmacological activity in selected directions. The following methods were used for separating cyclopeptides from flax:

1. extraction of CO_2 in a liquid or supercritical state.
2. solvent extraction.

Studies on the extraction of cyclic peptides from linseed cake and chaff using CO_2 in a liquid or supercritical state

Supercritical extraction is a process of exchanging mass in which compressed gases in supercritical conditions are used instead of classic solvents. This form of extraction uses a positive (from the point of view of mass exchanging processes) property of gases and liquids, i.e. low viscosity at a relatively high density. As a result, the extractant has a high solubility capacity and good penetration properties. The solubility capacity for the same solvent changes in a broad range and depends on the temperature and pressure of the process. It is possible to conduct extraction with liquid gas, under high pressure and a temperature lower than its critical temperature.

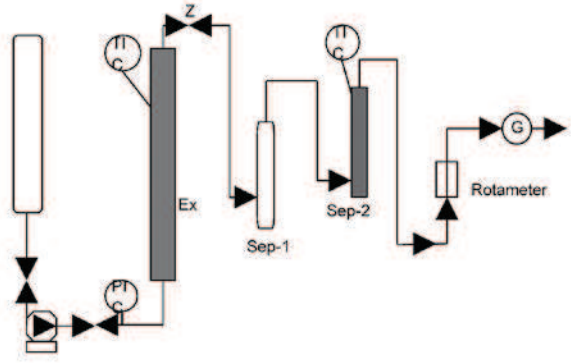

Fig. 3. Scheme of laboratory apparatus for supercritical extraction

A simplified diagram of a laboratory installation for testing supercritical extraction was presented in Figure 3.

The solvent is fed from the cylinder to the extractor using the P pump equipped with a manometer and working with the PIC pressure regulation system. The extractor was placed in a thermostatic water bath thanks to which it was possible to carry out the process in a constant, assigned temperature. A Z expansion valve is placed on the outlet piping from the extractor, which maintains pressure in the extractor. The pressure-free receiving node of the extract consisted of separators Sep-1, Sep-2 and unit for measuring the amount of the gaseous solvent. The momentary flow is measured using a rotameter, and the total amount – using a gas flowmeter, marked on the diagram with the letter G. The heavier extract is collected into glass, replaceable containers placed inside the Sep-1 separator, while the light extract is gathered into glass containers placed inside separator Sep-2 which is cooled to -30°C using a laboratory cryostat.

The studies of supercritical extraction pertained to:

- the influence of temperature and CO_2 flow on the rate of the CO_2 extraction process
- the impact of initial solvent extraction using selected organic solvents on the composition and amount of products obtained in the final CO_2 extraction
- fractioning CO_2 extraction products depending on the duration of the process.

The influence of temperature on the rate of extraction and the content of CLA from raw linseed cake through CO_2

When testing the influence of temperature on the extraction of raw linseed cake through CO_2, trials were performed at a temperature of 80°C, 60°C, 40°C and 27°C, under a pressure of 20 MPa. For temperatures 80°C, 60°C and 40°C the extractant was CO_2 in a supercritical state, while at 27°C the extractor was liquid CO_2. In the tests, waste material was used from "Modron" flax in the form of chaff and cake.

The supercritical extraction at 60°C was conducted until a practical disappearance of the extract stream and lasted 118 h. This experiment was adopted as a reference system for extraction at remaining temperatures. For the purpose of comparing the obtained data, experiment results were converted into 100 g of fed plant material, and the mass increment of the extract in time was related to the amount of used up CO_2. The results of conducted experiments were presented in graphic form in Figure 4.

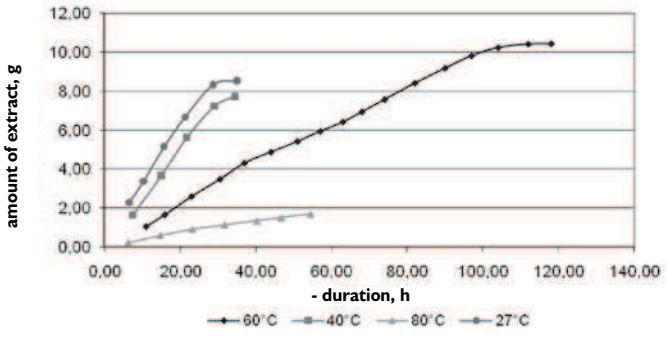

Fig. 4. The influence of temperature on the rate of extraction

The obtained experimental data show that extraction of linseed cake with liquid CO_2, conducted at a temperature of 27°C, is the fastest and most efficient. The increase of extraction temperature leads to a clear slowing down of the process. The influence of temperature and time of conducting the process on the change of CLA concentration in specific extraction samples was presented in Figure 5.

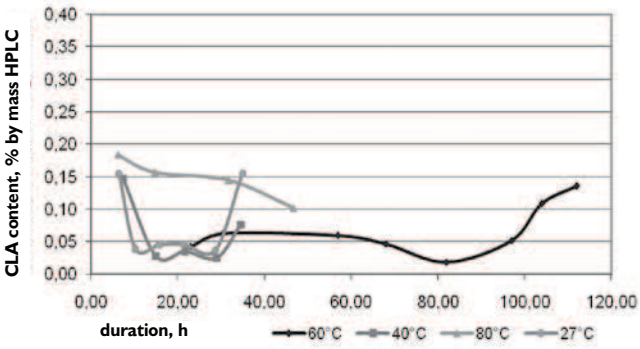

Fig. 5. Change of CLA concentration, depending on the time of extraction

The changing amounts of CLA collected in subsequent extract samples depending on the duration of the CO_2 extraction were calculated based on an analysis of HPLC and sample weight. The amount of collected CLA in the extract, depending on the time of extraction, was presented in Figure 6.

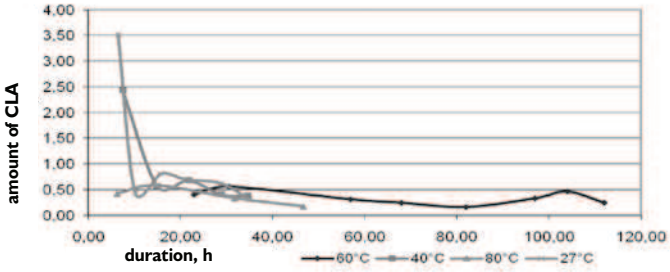

Fig. 6. Amount of CLA in subsequent extract fractions

A comparison of experimental results of CLA concentration through a CO_2 extraction of raw linseed cakes at different temperatures shows that lower process temperatures cause a clear increase in the amount of CLA received in the initial extract portions. At temperatures of 27°C and 40°C the initial, relatively high concentration of CLA in the extract rapidly decreases from the 10÷12th hour of the process. The weight amounts of the received extract are clearly higher at the beginning of the process.

Thus, it seems that in order to initially concentrate the CLA stream through a CO_2 extraction of raw linseed cake the process should be conducted at a low temperature, for no longer than 10÷12 h.

Impact of initial solvent extraction parameters on the content of CLA in extracts of CO_2

Subsequent studies used initial solvent extraction of linseed cake: with hexane, cyclohexane and toluene at their boiling points. Then, the cakes were subjected to extraction through CO_2. This led to an almost complete removal of all elements extracted with solvents. The amounts of extract obtained through supercritical CO_2 extraction in each of these samples were small.

The CO_2 extraction of linseed cake subjected to initial solvent extraction was conducted at a temperature of 60°C.

The results of marked changes of CLA concentration in samples of the extract received during the process of CO_2 extraction and the amounts of CLA calculated based on them in individual samples were presented in Figure 7.

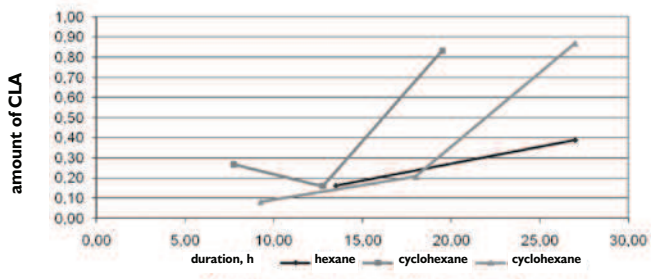

Fig. 7. Change of CLA concentration in the subsequent samples after the initial CO_2 extract solvent extraction

The amounts of CLA included in subsequent extract samples received during CO_2 extractions, calculated based on HPLC analyses and the weight of samples were presented in Figure 8.

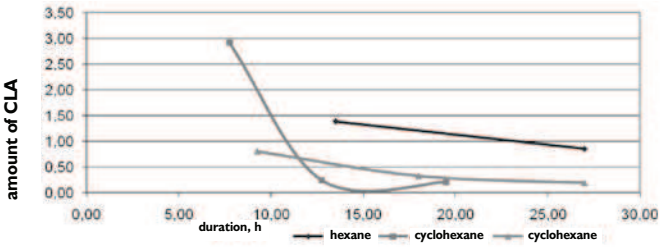

Fig. 8 Changing the amount of CLA contained in subsequent samples of the extract

Initial solvent extractions of linseed cake, conducted at the solvents' boiling points, caused the removal of elements searched by us. The amounts of extract obtained during subsequent extraction of these cakes using supercritical CO_2 were too small to be used for further CLA isolation.

A comparison of results of CO_2 supercritical extractions of raw linseed cakes and those subjected to initial solvent extraction at room temperature shows that for hexane and cyclohexane a slight increase was obtained of the average CLA concentration in the CO_2 extract.

After conducting a couple of experiments it was stated that solvents used in tests extract cyclopeptides together with oil.

The total times of conducting CO_2 extraction after an initial solvent extraction are shortened from 118 h and in the case of raw cakes – to 30 h. The amount of CLA included in subsequent portions of the extract is decreased in comparison to the CO_2 extraction of raw cakes.

Repeatability of the extraction process of linseed cake using CO_2

In order to perform an evaluation of the repeatability of the CO_2 extraction process a portion of linseed cake was subjected to initial solvent extraction using cyclohexane at room temperature and divided into two portions. Each of them was separately subjected to CO_2 extraction in supercritical conditions, at a temperature of 60°C and pressure of 20.0 MPa. In the first case the extract was collected into a container together, noting down an increment of extract mass over time. In the second experiment extract portions were collected and weighed separately. The concentration of CLA was marked in extract samples.

Based on the results of markings and the weight of the obtained extract, a weight balance was compiled of the total amount of CLA obtained in each sample. In the second experiment the final concentration was calculated, based on the total amount of CLA in each of the three samples and the total weight of the extract. The obtained result of the final concentration calculation differed by 0.002 from the value of the concentration marked using the HPLC method in the extract collected in total (in one portion) from the first experiment. As a result of both conducted experiments, similar weight amounts of the extract were obtained in similar time.

Coincident extraction results of an identical material obtained in both samples prove the repeatability of the studies processes.

Studies on the extraction of cyclic peptides from linseed cakes and chaff using traditional solvents

Within the work, an attempt was made to isolate cyclic peptides using traditional organic solvents. For the purpose of the studies the following solvents were selected: hexane, cyclohexane, methanol and toluene. The extraction process was conducted periodically using solvents at room temperature and their boiling points. Chaff and linseed cake were used as materials.

The studies commenced with the extraction of cyclic peptides from linseed cakes, using hexane at boiling point in the Soxhlet apparatus. The extraction was carried out for 14 h; its result was the obtaining (after evaporating the solvent) of 15.06% of extract expressed in means of the used material. The extract contained 0.085% of CLA. Next, the effectiveness was checked of the extraction of cyclic peptides from cakes using hexane at room temperature. Extraction using hexane lasted 6 h; its result was obtaining 13.97% of extract with a 0.016% content of CLA. It was observed that hexane at room temperature extracts a similar amount of extract as compared to extraction at boiling point in which it contains five times less CLA cyclopeptide. The increase of the extraction temperature to the solvent's boiling point (69°C) results in a slight increase of the extract amount by 1.1% and an increase of CLA content by 0.069%.

The same cake initially extracted using hexane at room temperature was extracted using toluene or methanol at boiling point. As a result of hexane extraction, after evaporating the solvent, 2.55% of extract was obtained which contained 0.5% of CLA. On the other hand, as a result of applying hot methanol an extract was obtained which constituted 9.03% of cake mass and contained 0.001% of CLA.

It has been observed that methanol at boiling point allows for obtaining a large amount of extract with a very low content of CLA as compared to toluene which gives a small amount of extract with a large content of CLA. Toluene at boiling point extracts CLA a lot better than methanol.

A comparison was made of the content of CLA in extracts obtained using cyclohexane at room temperature and boiling point. As a result of extraction using cyclohexane at room temperature, 8.35% of extract was obtained with a 0.037% CLA content. On the other hand, extraction using boiling cyclohexane allowed for obtaining a material constituting 10.06% of the initial cake mass, containing 0.083% of CLA.

Cyclohexane at boiling temperature extracts twice as much cyclopeptide CLA compared to one at room temperature. At the same time, at room temperature it contains twice as much CLA as compared to hexane.

The content of cyclopeptide in plant material is very small and does not exceed 0.02%; moreover, cakes contain approx. 10% of oil which changes the solubility of cyclopeptides and influences their extraction.

In order to extract the maximum amount of CLA, it has been decided to perform a multiple extraction of the same plant material. At the first stage, linseed cake is subjected to extraction using toluene at a temp. of 20°C in order to extract linseed oil. The obtained extract constituted 7.26% of the initial mass of the used plant material and contained 0.17% of CLA. Then, cakes were subjected to a triple extraction using toluene at a temp. of 90°C. The obtained extracts contained 2.33%, 0.55% and 0.23% of linseed cake mass.

In total, 22.30% mg of CLA was extracted from 100 g of cake.

Toluene at room temperature extracted 0.170% of CLA, while increasing temperature up to 90°C influenced the increase of CLA to 0.293% of the extract mass.

Extraction using toluene at room temperature led to the extraction of a considerable amount of cyclic peptides (0.32%) apart from linseed oil.

During the next experiment, cyclohexane was used for initial oil extraction. Extraction was conducted at room temperature for 13 h; as a result of this process, 8.35% of extract was obtained, with a 0.091% content of CLA. Then, the same cakes were extracted three times using toluene at a temp. of 90°C. The extracts contained 1.57%, 0.30% and 0.18% mass of linseed cake. All the obtained extracts were subjected to analysis using the HPLC method for marking the amount of cyclopeptides, arriving at the following results: toluene extract I – 0.69%, II – 0.58% and III – 0.58% CLA.

In total, 20.02% mg of CLA was obtained from 100 g of cake.

In both experiments a very similar general amount of the CLA cyclopeptide was obtained. The use of cyclohexane for initial oil extraction meant that toluene extracts contained 7.4 mg CLA more than in the experiment in which toluene was used for initial extraction. The results of the discussed experiments were illustrated in Figure 9.

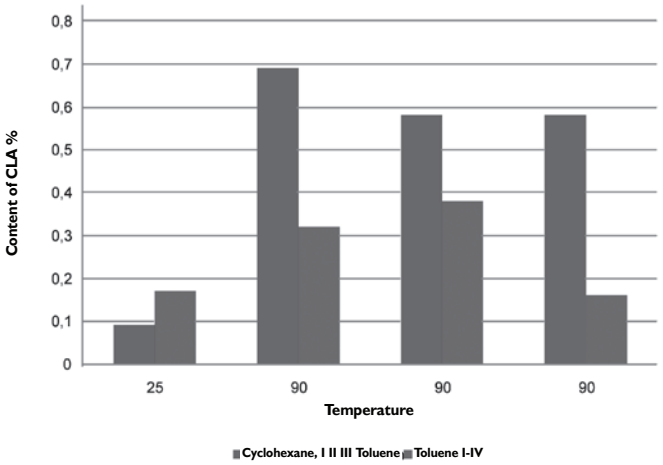

Fig. 9. The results of solvent extraction of linseed oil cake

The second waste material used for studies was linseed chaff. In order to determine the content of CLA in chaff and to compare it with the content of these peptides in linseed chaff, an experiment was conducted in conditions similar to those for linseed chaff.

Extraction from linseed chaff was carried out with an initial extraction of plant material with hexane for 7 h at room temperature, and then it was extracted three times with toluene for 7-8 h at boiling point. Hexane extract constituted 0.91% of the used chaff's mass, with a content of CLA equal to 0.001%. Extractions conducted using toluene constituted 1.68%, 0.57% and 0.49% recalculated to the mass of the used material, and the content of CLA in extracts equaled 0.144%, 0.044% and 0.005%. In total, 3.64 g of extract was obtained with a 3.64% efficiency recalculated into initial chaff mass.

In total, chaff extracts contained 2.67 mg of CLA.

On the other hand, in similar conditions a total amount of 15.23 mg of CLA was obtained from linseed cake (2.23 mg of CLA in hexane extract, and approx. 13 mg of CLA in toluene extract).

The content of CLA in linseed chaff turned out to be very small. In order to confirm this result, an additional extraction was performed in identical conditions, using a new portion of material.

Extraction from chaff was conducted with an initial oil separation of the material with cyclohexane at room temperature, and then with toluene at boiling point. Cyclohexane extract constituted 0.03% of the used chaff's mass, with a content of CLA equal to 0.010%. On the other hand, the content of CLA in toluene extract equaled 0.053% of the mass of the extract obtained with an efficiency of 1.39%. In total, 1.42 g of extract was obtained (which constitutes 1.42% of the initial chaff mass). *In total, extracts contain 0.743 mg of CLA.*

A graphic comparison of obtained data for linseed cakes and chaff is presented in Figure 10.

From the obtained data it stands that the content of CLA in chaff is a lot lower than the content of CLA in linseed cake. The most favorable result of extracting CLA from linseed cake was obtained using an initial cleaning with cyclohexane at room temperature, and then a triple extraction using toluene at boiling point.

Data collected from the above experiments indicate that the use of hexane, cyclohexane and toluene at room temperature in the initial solvent extraction with the aim of removing oil causes a partial washing out of oil in individual extractions. Toluene extract contains the highest amount of CLA (0.17%), while its amount is twice smaller in cyclohexane extract (0.09%), and in hexane extract – ten times smaller (0.016%).

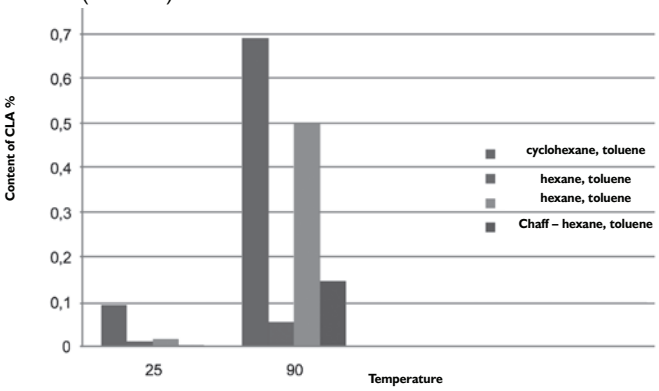

Fig. 10. Results of solvent extraction of linseed chaff and cake

In all the cases in which initial oil extraction was used from raw plant material, cyclopeptides were always present, next to oil fatty acids and their glycerides. Vegetable oils probably increase the solubility of CLA in non-polar solvents, even at room temperature. The results of these experiments were presented in Figure 11.

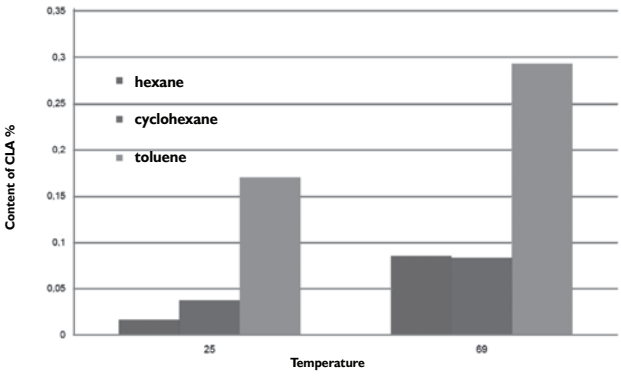

Fig. 11. CLA concentration dependence on the type of solvent and extraction temperature

Extract obtained at room temperature using hexane contained 0.016% of CLA. On the other hand, at boiling point the content of CLA in the extract equaled 0.085%

In the case of using cyclohexane, the contents of CLA in the extract obtained at room temperature equaled 0.037%, while at boiling temperature – 0.083%.

Summary

Cyclopeptides were separated from linseed cake and chaff (*Linum usitatissimum* L.). The separation was carried out using two methods:

1) extraction through supercritical CO_2.

2) traditional solvent extraction.

Studies were carried out of the dependence of the cyclopeptide extraction using CO_2 on temperature. It was stated that the highest efficiency of the CLA cyclopeptide (0.006%) is obtained at a temperature of 25÷40°C at a pressure of 20 MPa from raw cakes. The extract contains 0.08% of CLA cyclopeptide.

The efficiency of the CLA cyclopeptide extraction of cakes using CO_2 (after an initial cyclohexane extraction) equals 0.001%, while the concentration in the extract is higher and equals 0.11%.

For the purpose of studies on developing the most effective method of obtaining the CLA cyclopeptide in solvent extraction, three solvents were used: toluene, hexane, cyclohexane at room temperature and boiling point. Studies have shown that high temperature of solvents increases the content of the CLA cyclopeptide in the obtained extracts.

A method has been developed which allows for obtaining the highest amount of CLA cyclopeptide from linseed cakes. It involves an initial extraction using cyclohexane at room temperature, and then a triple extraction using toluene at boiling point. The purpose of the initial extraction is to remove oil remains from cakes. This has allowed for separating 0.02% CLA cyclopeptide from plant material. The product contained 0.66% of CLA cyclopeptide.

In the case of linseed chaff, both of the extraction methods – through CO_2 and solvent extraction – provided a similar amount of extract, i.e. 1.4÷1.7% of the material's mass. However, the concentration of the CLA cyclopeptide in extracts is different and equals 0.01% in the CO_2 extract, and 0.05% in the solvent extract.

Based on the obtained data it was stated that extraction using CO_2, as well as solvent extraction may be used as a basis for isolating cyclopeptides from flax.

Based on the identification it was stated that the extract obtained using CO_2 contains mainly cyclopeptide CLA (approx. 80%), while solvent extract contains, next to cyclopeptide CLA, also comparable amounts of cyclopeptide CLE.

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