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Comparative study of extraction of soy molasses isoflavones and *in vivo* bioconversion of daidzein into S-equol in rats models

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Abstract. We compared different extraction methods for isolation of isoflavones from soybean molasses. Since conventional extraction methods are time-consuming, expensive and unsustainable, we have compared them with the NADES extraction method, which does not have these disadvantages. Fermentation-assisted technique and extraction with Natural Deep Eutectic Solvents (NADES) were compared to the conventional extraction methods. Based on the results obtained, we selected the optimal technique for isoflavones isolation. Isoflavones were identified by gas chromatography equipped with mass spectrometer (GC-MS), whereas their quantities were determined using high performance liquid chromatography (HPLC). *In vivo* metabolism of daidzein to S-equol was performed in rats with quantification of a yield of S-equol as a result of daidzein *in vivo* conversion in rats' intestines.

Key words: soy molasses, S-equol, NADES, extraction, daidzein, genistein, fermentation, GC-MS, HPLC.

INTRODUCTION

Natural preparations and phytochemicals for food and dietary purposes or nutritional supplementation have attracted an increased attention during the last years. Especially, products rich in soy-derived polyphenols have been used for centuries in traditional medicine as well as in supplementary nutrition and food diets due to their nutritional and physiological properties, above all in regard to their health effects on the human organism (Kroyer & Hegedus, 2001).

Phytochemicals can be referred as plant-produced substances. The term 'plant-produced substances' is generally used to describe chemicals from plants that are not essential for nutrition but may affect health. It is well known that diets rich in vegetables, fruits, whole grains, legumes and nuts have health benefits. And there is no doubt that these effects are due to specific phytochemicals and nutrients. Since foods based on plants are complex mixtures of bioactive and biochemical compounds, the health effects of foods with special phytochemicals are related to the possible health effects of such phytochemicals.

Polyphenols are secondary metabolites and very common in the plant kingdom. At present, over 8000 different structures of flavonoids are known (Boeing, 1995). They are divided into numerous classes, i.e. phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavonols, flavanols, flavones, isoflavones, flavanones, proanthocyanidins), lignans and stilbenes, which are widely spread in plants and food of plant origin (Manach et al., 2004). These compounds are important constituents of fruit quality because of their contribution to the taste, colour and nutritional properties of fruits (Cheynier, 2005).

Polyphenols are exceedingly reactive compounds and good substrates for several enzymes, including glycosidases, esterases, polyphenoloxidases and peroxidases. They undergo several chemical and enzymatic reactions during food storage, after harvest and during processing. Although the manifestation of such reactions and their roles in the development or degradation of food quality are well documented, the structures of the resulting products are still not fully clarified.

Soy and soy foods are rich sources of isoflavones, which are shown to possess various biological activities. Isoflavones are naturally found in soybean in glycosylated state such as daidzin, glycitein and genistin with only a small percentage in the aglycone form (approximately 2–3%) (Hur & Rafii, 2000). Soy molasses (SM) is a by-product of soy protein concentrate processing containing 2–3% of isoflavones (Waggle, 2004). The principal soy isoflavones daidzein, genistein and glycitein are undergone to metabolic conversion in human subjects (Heinonen et al., 2002). About one-third of the human population are able to naturally metabolize the major soy isoflavone daidzein to equol. This isoflavone is converted into S-equol by the action of specific intestinal bacteria, mostly gram positive rods (Decroos et al., 2005; Yu et al., 2008; (Zhang et al., 2012b). S-equol has shown higher estrogenic effect and health benefits as compared to the isoflavones algycones.

Generally, the extraction of polyphenols from biological matrices has been carried out using conventional extraction techniques such as liquid-liquid extraction (LLE) and solid-liquid extraction (SLE). However, at present, these methods are being replaced with microwave-assisted extraction, ultrasound-assisted extraction or supercritical fluid extraction that are more advanced and used rather often for the extraction of polyphenols from plant materials. One of the crucial issues in chemical sciences is the development of green technology that enables to diminish negative impact of human activities and preserve the environment. The green technology comes up with new environmentally tolerable solubilization techniques by controlling physical properties of media such as pressure and temperature, the use of non-hazardous media and developing new green solvents (Paiva et al., 2014). The development of novel green solvents is one of the most prospective and innovative areas. In this context natural deep eutectic solvents has been investigated to replace current harsh organic solvents (Zhang et al., 2012a).

This study was aimed to compare extraction yield of isoflavones obtained by ethyl acetate extraction, acetic acid extraction, fermentation-assisted extraction and extraction isoflavones with Natural Deep Eutectic Solvents (NADES), to identify and to determine *in vivo* conversion of daidzein into S-equol in rats' intestines.

MATERIALS AND METHODS

Materials

Daidzein, genistein, puerarin, vitexin, and 4'-hydroxyflavanone standards (HPLC grade) were purchased from Sigma Aldrich (Missouri, USA). Choline chloride and citric acid were supplied by Alfa Aesar Inc., USA. Soy molasses was procured from Agroproduckt CJSC, Kalingrad, Russia. Ethyl acetate and ethanol were of analytical grade. *Saccharomyces cerevisiae* species were purchased from open joint stock yeast factory, mink, Belarus.

Acetic acid extraction

To extract isoflavones, 10 g liquid soy molasses was suspended in 250 mL distilled water adjusted with acetic acid up to pH 4.5, and heated under reflux for 6 hours (Waggle, 2004). Hereafter, the isoflavone-contai,ning mass was suspended in 150 mL water/acetic acid and cooled up to 7 °C. After centrifugation at 8 rpm and 7 °C the supernatant was decanted, and the pellet was taken up in methanol, thoroughly vortexed and centrifuged at 10 rpm at room temperature. The supernatant was gathered and concentrated using rotating evaporator. The extract was then dissolved in 10 mL of methanol and stored at -20 °C until further usage.

Ethyl acetate extraction

Exactly 2.5 g of soy molasses was added 20 mL of ethyl acetate water solution (5 mL of ethyl acetate and 15 mL of distilled water) and mixed thoroughly using magnetic stirrer. The solution was allowed in a separatory funnel at different extraction time (20, 40, 60, 80 min, respectively). At the end of the extraction time, the mixture was centrifuged at 6,000 rpm for 15 min and the supernatant was collected and evaporated using a rotary evaporator under vacuum (60 rpm, 65 °C). The extract was dissolved in ethanol prior to GC-MS analysis (Duru et al., 2017).

Fermentation-assisted extraction

Activation of *Saccharomyces cerevisiae*

All activation procedures were performed under the laminar flow cabinet. 2 g of *Saccharomyces cerevisiae* was weighed and dissolved in 50 mL of distilled water at 30–35 °C. Then, 0.8 g of granulated sugar was added to the dissolved yeast and held for 30 min prior to inoculation (Duru et al., 2017).

Fermentation of soy molasses

Soy molasses was fermented according to the method described in (Siqueira et al., 2008) with slight modification. 100 g soy molasses was dissolved in 500 mL distilled water and thoroughly mixed. After that, the dissolved mixture was sterilized at a temperature of 121 °C for 15 min and cooled to room temperature. The activated yeast was inoculated in resulted mixture and kept in a batch fermenter in anaerobic conditions at room temperature. The end of fermentation was visually observed after 3 days as a termination of carbon dioxide evolution (Duru et al., 2017).

Fermented residue extraction

The fermented soy molasses was centrifuged at 6,000 rpm for 20 min, the yeast and supernatant were collected. The supernatant was evaporated using a rotary evaporator under vacuum (65 rpm, 60 °C), and extraction was performed using 70% ethanol and ethyl acetate water solution as earlier stated (Duru et al., 2017).

NADES extraction

Preparation of NADES

For extraction of soy molasses, NADES were prepared by mixing 1 : 1 equimolar quantities of choline chloride and citric acid (Table 1).

Table 1. NADES conditions used to perform isoflavone extraction

Materials	Components		Molar ratio	Solid/solvent ratio	Appearance
	HBA	HDB			
Soy molasses (viscous liquid)	Choline chloride	Citric acid	1 : 1	1 : 3	Transparent colourless liquid

HBA denotes hydrogen bond acceptors; HBD denotes Hydrogen bond donors.

Briefly, distilled water (30%) was added to the two-component mixture, the final mixture was heated at 60–80 °C under constant stirring until a transparent solution was observed. The prepared NADESs were stored in the dark until subsequent utilization.

NADES-based ultrasound-assisted extraction of soy molasses

NADES-based ultrasound-assisted extraction (UAE) procedure was performed for the isolation of isoflavones from SM (Shang et al., 2019). NADES solution was immediately added to soy molasses in proportions which indicated in Table 1, and the mixture was processed by ultrasonic extraction at a frequency of 80 kHz and a power of 580 W at 55 °C for 3 hours. Isoflavones were gradually extracted into the NADES phase, and a viscous suspension was obtained. The suspension was centrifuged at 10,000 rpm for 10 min to separate the solid and liquid phases. The liquid extract was fractionated in a separatory funnel containing ethyl acetate, and the resulting organic layer was concentrated using rotatory evaporator until constant weight. The final extract was then stored at –20 °C until further usage.

Identification and quantification of isoflavones

Chromatographic conditions for acetic acid extraction

The standards of daidzein, puerarin and genistein as substances of interest were used, as well as 4'-hydroxyflavanone and vitexin, since they were considered suitable as internal or surrogate standards. The chromatographic setup is shown in Table 2. The compounds were separated on a reversed stationary phase and were detected with UV (254 nm). One run lasted 20 min and including 5 min equilibration time. In the beginning of each analysing day the column was washed with acetonitrile / water-formic acid (95 / 5) for 30 min. Samples were injected after short centrifugation (30 sec) using a table-top centrifuge to prevent solid components from blocking the column (Table 2).

Table 2. Chromatographic conditions used to perform isoflavones separation

Parameter	Chromatographic system / requirements
Stationary phase	Agilent Zorbax SB-C18, 3.5 µm, 2.1×150 mm
Mobile phase A	Water + 2 mL L^{-1} formic acid
Mobile phase B	Acetonitrile
Gradient program (A:B)	$85{:}15 \xrightarrow{4\ min} 60{:}40 \xrightarrow{3\ min} 50{:}50 \xrightarrow{5\ min} 20{:}80 \xrightarrow{1\ min} 5{:}95 \xrightarrow{2\ min} 15{:}85$ (5 min)
Flow rate	0.3 mL min^{-1}
Column temperature	35 °C
Detection	254 nm
Time per run	20 min
Injection volume	250 µL

Gas chromatography–mass spectrometry identification of isoflavones in extracts obtained by ethyl acetate and fermentation-assisted extractions

Analysis of the extracts was performed using a gas chromatograph (GCMS-QP2010 Ultra, Shimadzu) coupled to a quadrupole mass spectrometer with an electron ionization (EI) source (70 eV) and 5% diphenyl-95% dimethyl polysiloxane covered capillary column DB-5 MS (30 m × 0.25 mm, 0.25 µm). Helium was used as the carrier gas at a constant flow rate of 1.5 Ml min^{-1}. 1 µL of untreated extract was injected in the splitless mode (vent time, 60 sec), and compounds identification was carried out when comparing mass spectra obtained by Mass Spectral Library of the National Institute of Standards and Technology (NIST) (Duru et al., 2017). The temperature program began with an initial temperature of 50 °C kept for 1 min and increasing 25 °C min^{-1} until 200 °C, then increasing 3 °C min^{-1} until 240 °C when this temperature was maintained for 35 min. Total separation time was 55.3 min. A 5-minute delay was used to protect the MS ion multiplier from saturation. Full scan mode was used in the range of m/z 35–660. Aliquots of 1 µL of the samples (1 mg mL^{-1}) were injected in the splitless mode. The analytical conditions: source temperature 200 °C and injector temperature 240 °C. Capture times for isoflavone derivatives were the following: 23.256 min for daidzein; 25.698 min for genistein, 30.072 min for glycitein.

HPLC conditions for NADES extraction

Quantification of isoflavones content in the extract was performed using HPLC Agilent 1260 Infinity II system consisting of quaternary pump (Model G7111B), diode array UV detector (Model G7117C) coupled with DAD analysis software, autosampler (Model G7129A) with integrated column compartment and vacuum degasser module. A Poroshell 120 EC-C18 (3.0 mm × 100 mm × 2.7 µm, Agilent p/n 695975-302) reversed phase column was used for the separation of active compounds. The mobile phase consisted of solvent A, containing water and 0.1% acetic acid and solvent B, containing methanol and 0.1% acetic acid. A linear gradient elution of solvent B was applied from 5% up to 100% over 20 min, at a flow rate of 0.7 mL min^{-1}. The temperature of the column was kept at 30 °C, and injection volume was 5 µL. A quantitative analysis of the two peaks of isoflavone content in soy molasses was performed. The isoflavones were detected at 254 nm.

In-vivo S-Equol production protocol

Animals and diet

Seven female rats (6 months aged) were obtained from the Institute of Natural Sciences and Mathematics of the Ural Federal University and were housed in pair polycarbonate cages at temperature approximately 24.9 ± 0.5 °C, then fed with NIH-31 rodent diet (not soy-enriched and isoflavones-containing) and water ad libitum according to the Guidelines for Animal Care and Experimentation of the National Food Research Institute. Fecal pellets were collected from all rats before any diet treatment for analysis. A pair of rats which served as control was fed with the regular NIH-31 rodent diet, while the other animals were on the same diet supplemented with daidzein 1,000 ppm. At the end of the first and the second weeks, fecal pellets were collected and immediately processed for analysis and *in vitro* faecal incubation.

Extraction of samples for analysis of daidzein metabolites

Extraction procedures were carried out as described in (Rafii et al., 2007), fecal pellets from rats that consumed diet supplemented with daidzein and control rats were collected and soaked in water to make a slurry. Ethyl acetate was used for extraction, and the fecal slurry was extracted 3 times. The solvent was removed by evaporation, then the sample was dissolved in ethanol.

Data statistical analysis

All experiments were conducted in triplicates. Experimental results are expressed as mean values. Data were analyzed by one-way *ANOVA* or *t-test* at a 95% confidence level. A *P-value* < 0.05 was considered statistically significant. Statistical analyses were done using graphPad Prism 8.

RESULTS AND DISCUSSION

Identification of isoflavones

Daidzein, genistein and glycitein have been found to be major isoflavones among all extracts analysed by GC-MS. The structures were identified by analysing MS spectral data and comparing them with those from the Mass Spectral Library of the NIST. The identities and retention time of each individual peaks are presented in the Fig. 1. The glycitein content in soy molasses is relatively lower as compared to other detected isoflavones that was shown by their relatively reduced peak in Fig. 1 (Duru et al., 2017). As shown in the Fig. 1, the ethyl acetate extracts contained other substances which both were originated from soy molasses such as Hydroxy-2-methyl-4-pyrone (sugar), Palmitic acid, cis, cis-Linoleic acid, 2-Hydroxy-1-(hydroxymethyl)ethyl ester (fatty acids and fatty acids esters), Clionasterol (phytosterol) and came from solvent used (n-Butyl acetate).

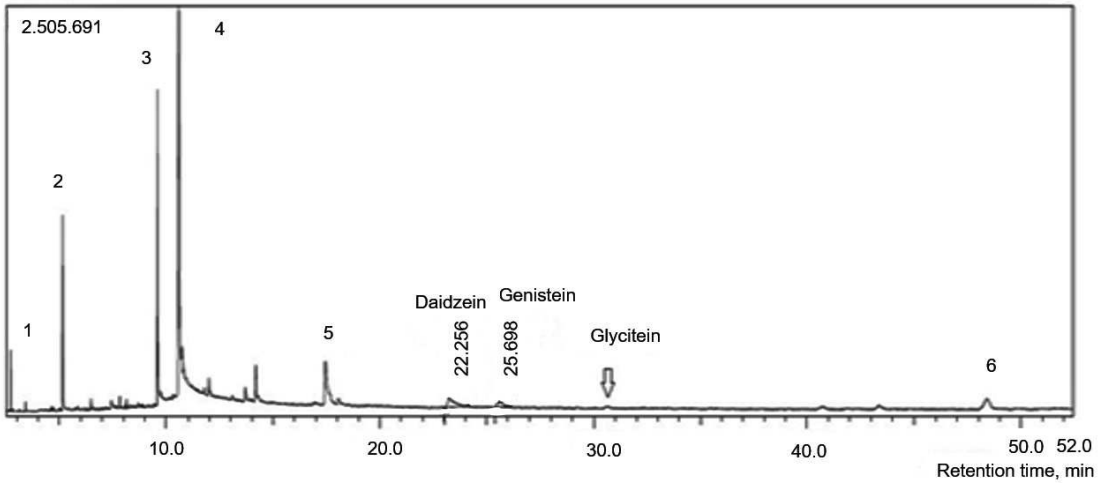

Figure 1. GC-MS chromatogram of ethyl acetate-water extract after fermentation. Other substances identified in the ethyl acetate - water extract are the following: 1 – n-Butyl acetate; 2 – 3-Hydroxy-2-methyl-4-pyrone; 3 – Palmitic acid; 4 – cis, cis-Linoleic acid; 5 – 2-Hydroxy-1-(hydroxymethyl)ethyl ester; 6 – Clionasterol.

Quantitative determination of daidzein concentration

Daidzein contents of ethyl acetate extracts from fermented soy molasses obtained at 60 and 80 min of extraction time were quantified. The chromatographic peaks on the chromatograms before (a) and after adding daidzein as a standard (b) at retention time 5.9 min were recorded and used to calculate the daidzein concentration of extraction at different extraction times (Fig. 2) (Duru et al., 2017).

The daidzein concentration in the extract at different extraction time was determined using standard addition HPLC method (Cimetiere et al., 2013). The highest amount of daidzein was detected at 80 min' extraction compared to 60 and 40 min' ones (Table 3). It can be supposed that contact time between solvent and soy molasses affects the daidzein yield. Similarly, previous study (Wu et al., 2012) reported that prolonged extraction time increased the puerarin yield and total isoflavones content from the root of *Pueraria lobata radix*, when using ultrasound-assisted extraction method. Another study conducted on the extraction of isoflavones from soybean sprout cotyledon using different ethanol and extraction time supported that the yield of total isoflavones increases with extraction time (Cho et al., 2009).

Table 3. Extraction time versus daidzein concentration obtained in fermentation-assisted extraction

Time (min)	Daidzein content (mg per 100 g)
80	5.58 ± 0.005
60	5.05 ± 0.005
40	4.44 ± 0.005

However, there was contradictory report from earlier study conducted on the extraction of isoflavones from soybean flour using 70% aqueous ethanol containing 0.1% acetic acid, the authors reported that total and individual isoflavones content did not change at extraction time of 1, 4, 20 and 24 hours. (Carrão-Panizzi et al., 2002). It has been suggested that the constant agitation was the reason for the equal extraction yield providing better contact between the soybean particles and the solvent, regardless of different extraction duration (Duru et al., 2017).

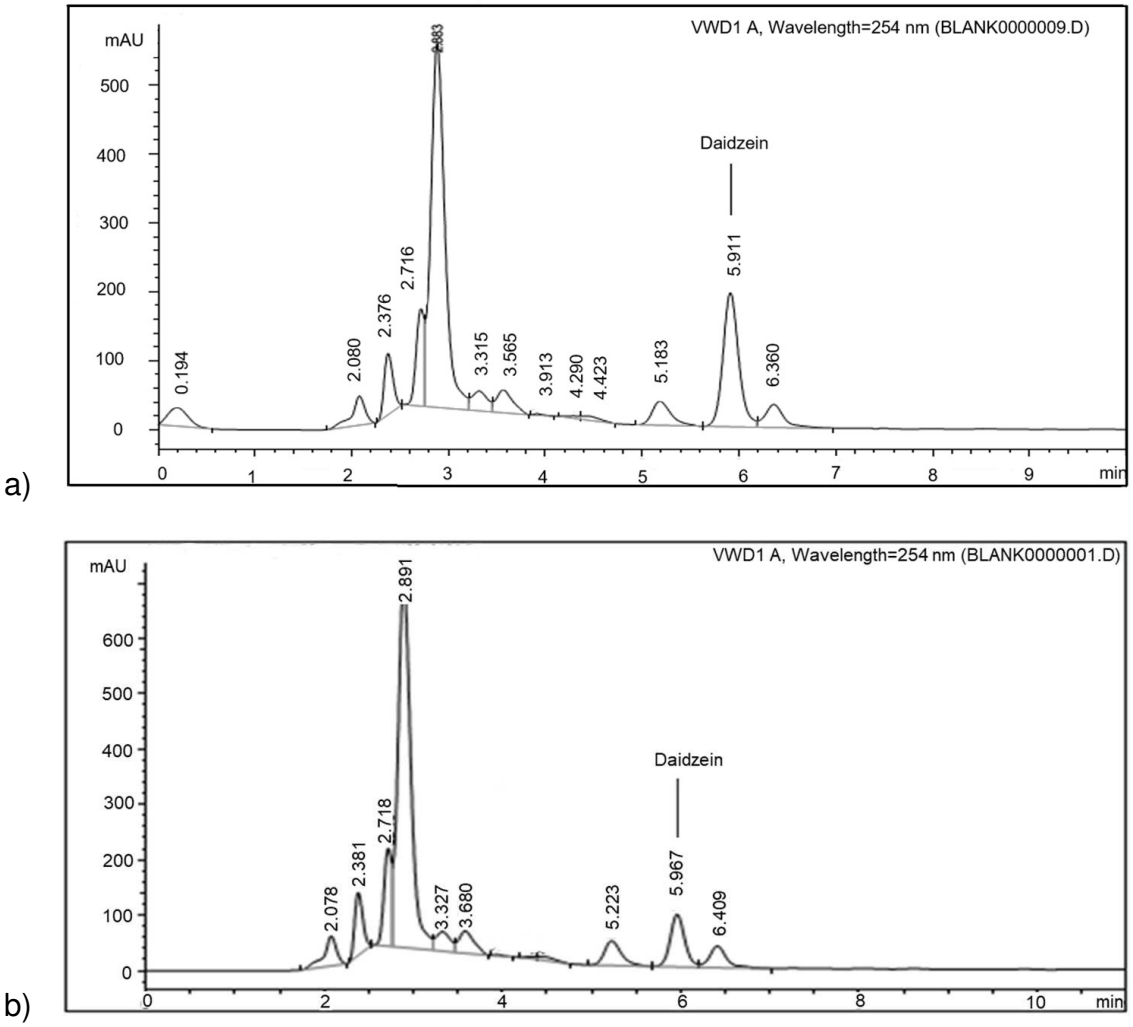

Figure 2. HPLC profile of the extract obtained from fermentation of soy molasses using ethyl acetate water solution at extraction time 60 min. Extract with addition of daidzein standard (a), without its addition (b).

Soy Molasses (maceration)

Table 4 shows the results for the measuring daidzein and genistein quantities in soy molasses using the principle of maceration.

According to the literature, the content of daidzein in soy molasses is 0.37 g per 100 g (Gu & Gu, 2001). In (Gu & Gu, 2001), there was found 0.98 g / 100 g of genistein.

Again, only a limited comparison with values in the literature can be drawn. The values presented in the literature were generated by NMR or HPLC-MS, which are more sensitive than selected ones for the present work using HPLC-UV analysis. Therefore, a deviation is possible.

In principle, the values determined were slightly less than those found in literature. This can be explained by the more extensive clean up after maceration. While Gu et al. dissolved the soy molasses in ethanol and purified

Table 4. Results of quantification of different isoflavones in maceration extract of soy molasses (SM)

Sample	Daidzein (mg per 100 g)	Genistein (mg per 100 g)
SM	5.61 ± 0.005	25.34 ± 0.007

it by a microfilter, the method used in this work included more workup steps (Gu & Gu, 2001). This can lead to a loss of analytes, but at the same time also cause a purer extract.

NADES extraction

Soy molasses extract was analysed by HPLC-DAD system. Daidzein and genistein were only identified. The results in Table 5 showed that the contents of the isoflavones determined are corresponding to those published in (Gu and Gu, 2001). The chromatogram below indicates the peaks of both daidzein and genistein as identified by HPLC in Fig. 3.

Table 5. Results of quantification of different isoflavones in NADES extract of soy molasses (SM)

Sample	Daidzein (mg per 100 g)	Genistein (mg per 100 g)
SM	5.21 ± 0.005	5.69 ± 0.007

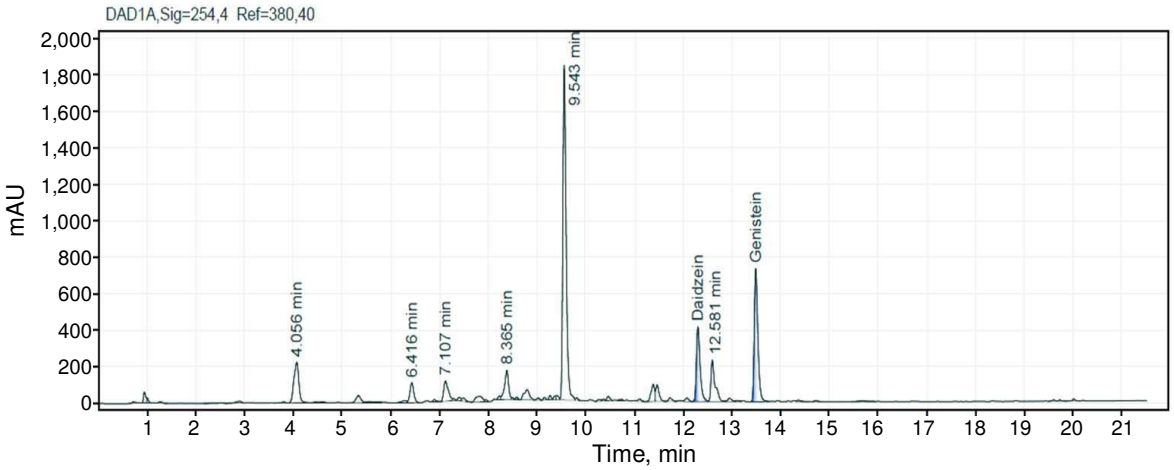

Figure 3. Representative HPLC-UV chromatographic profile for NADES extraction of soy molasses.

Comparison of different methods of extraction

As it is seen, the conventional extraction method with acetic acid-water resulted in the highest yield of isoflavones. However, it is more time-consuming method. We believe that it is necessary to modify the extraction method using NADES. Since this method uses non-toxic solvents, it is cheaper and takes less time. Ethyl acetate extracts from fermented soy molasses resulted in less isoflavones yields.

Characterization of equol

***In vivo* metabolism of daidzein**

Fecal extracts from control rats and those that were fed with the daidzein supplemented diet were analysed. Equol was not identified in fecal extract from control rats as shown in Fig. 4. From numerous research articles, it is known that microbiologically produced equol is S-equol as compared to that which is obtained by chemical transformations (Heemstra et al., 2006; Vázquez et al., 2020). Both daidzein and equol were identified in fecal extract obtained after one week of consumption of the diet supplemented with daidzein with 95% conversion as shown in Fig. 5. After two weeks of consumption there was complete metabolism of daidzein to equol as shown in Fig. 6 (compare with Fig. 4 for control rats' group).

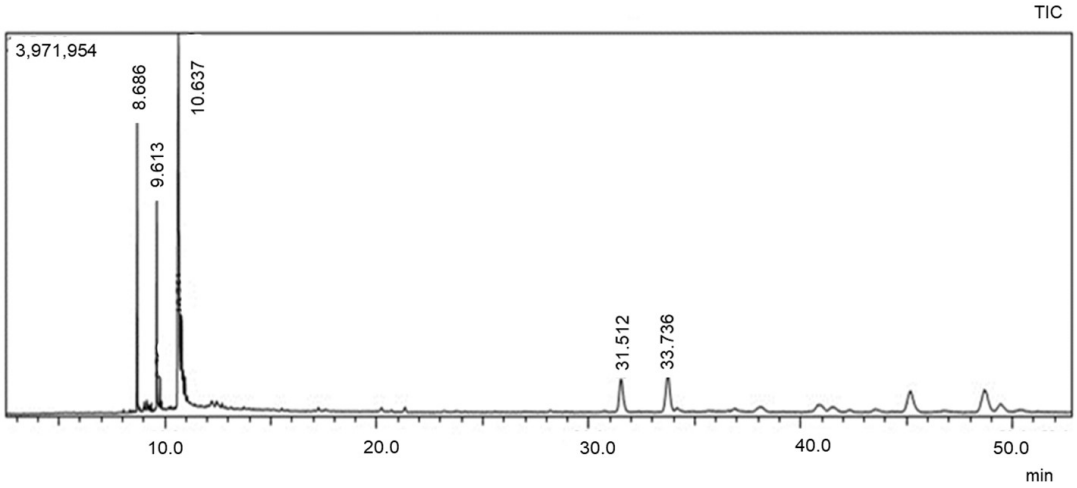

Figure 4. GC-MS of fecal extract from control rats.

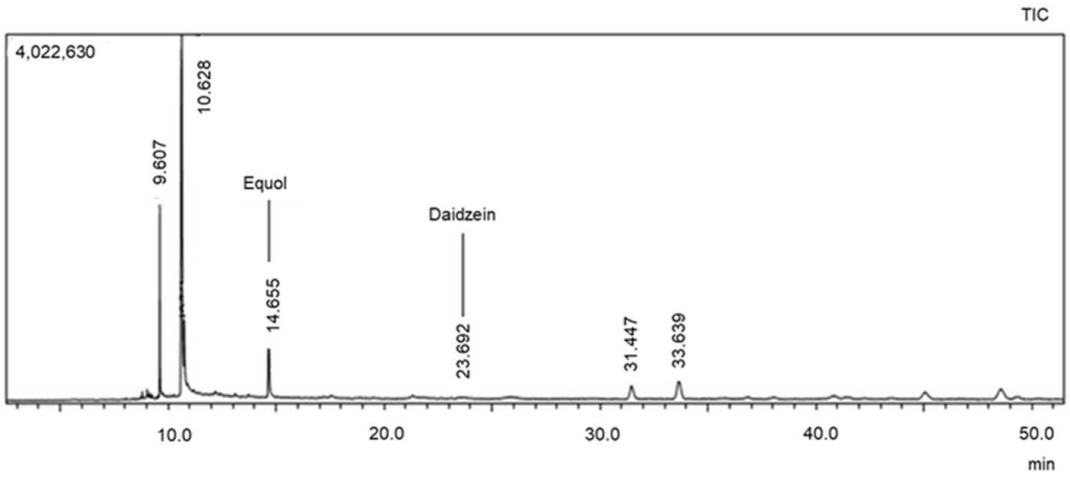

Figure 5. GC-MS of fecal extract from test rats after 1 weeks of daidzein enriched diet consumption.

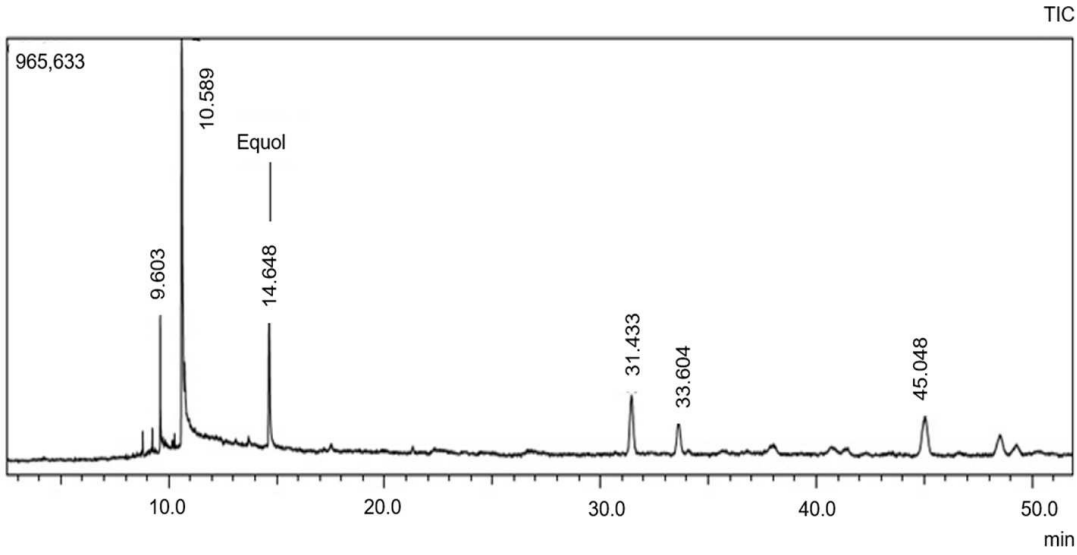

Figure 6. GC-MS of fecal extract from test rats after 2 weeks of daidzein enriched diet consumption.

CONCLUSIONS

Several methods for extraction of isoflavones from soy molasses have been compared. The conventional acetic acid-water extraction method provided the highest yield of isoflavones as compared to fermentation-assisted and NADES extraction, although is more time-consuming. NADES extraction explores non-toxic solvents, is cheaper and takes less time, therefore is very promising as compared to other studied methods.

Further research on optimization of extraction conditions would allow to achieve the highest yield of the isoflavones extracted from soy molasses using these solvents. Daidzein, one of the major isoflavones, can be metabolized completely to S-equol using *in vivo* rat model after two weeks of consumption of the diet supplemented with daidzein by laboratory animals.

The conditions of working with the animals were consistent with the regulations of the European Convention ET/S 129 (1986) and Directives 2010/63/EU.

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