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QUANTITATIVE DETERMINATION OF PHYTOSTERINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

High performance liquid chromatography (HPLC) is one of the universal and highly effective modern methods applied to the pharmaceutical analysis and separation of various substances. Phytosterins are biologically active compounds that are of great interest because of their anti-cholesterol, anti-sclerosis and anti-triglyceride activity as well as because of their inhibition efficiency in the peroxide oxidation of lipids. This work reports results of elaboration of the HPLC method for quantitative determination of phytosterins in the lipophilic extract from the pumpkin pulp. Analysis of the total content of phytosterins in terms of cholesterol proves that the pulp extract can be used as a valuable source of phytosterins.

Keywords: *chromatogram, high performance liquid chromatography, phytosterins, quantitative determination.*

Introduction

A variety of the glycerine/aliphatic acids esters and some other accompanying compounds can be found in proteins and oils. The high molecular cyclic alcohols with the steroid basis known as sterins are among such accompanying compounds. They represent a part of proteins that does not undergo saponification. β-citosterin, cholesterol, stigmasterin, cycloartenol and ergosterin can be mentioned as main representatives of the vegetable sterins. There are some publications discussing an influence of phytosterins on the lipids metabolism and total status of the immune system and reporting their antitumor, anti-inflammatory and antivirus activity. Besides, the vegetable phytosterins can be used as a raw material for production of the steroid hormones [12, 13, 14, 15].

Various waste materials of the food processing industry can be used for the cost effective production of phytosterines. For example, it can be pumpkin millcake collected at juice or puree production. This substance is traditionally sent for utilization or can be used as the cattle feed. It should be mentioned that pumpkin is a traditional and very popular fruit in Ukraine that is used widely as a regular, dietary and health food. Pumpkin delivers a valuable set of bioactive compounds: vitamins, proteins, hydrocarbons, sterols, organic acids, peptides, polysaccharides and others [16, 17, 18].

Some industrial producers in Ukraine use pumpkin seed to press out the oil to be processed further as a raw material in pharmaceutical industry. The pulp is used in food industry for production of the juice and puree. As a result, a significant amount of waste materials is collected at such productions (like LLC "Kharkiv Natural Food") and can be recycled through extraction of various bioactive components remained in that substance.

The lipophilic extract was obtained preliminary from the wasted pulp of pumpkin (*Cucurbita pepo* (L.), plant family *Cucurbitaceae*) by the circulating extraction with n-hexane (1 weight part of solvent per 6 weight parts of the pulp) repeated until complete exhaustion of the material. Then the qualitative composition of the lipophilic fraction has been determined by chromato-mass-spectrometry and the following compounds were identified: hydrocarbons, phytosterines, aldehydes, ketones, terpenic compounds, aliphatic acids and vitamins [1, 2].

Since phytosterins are the main representatives of bioactive compounds of the lipophilic pumpkin extract, this work was aimed onto elaboration of a method of quantification of the phytosterins content in the extract.

There is an official method of determination of a content of phytosterins in the stearin fraction of vegetable oils. According to the first edition of the State Pharmacopeia of Ukraine (SPU) and fourth edition of the European Pharmacopeia, this investigation should be realized using the gas chromatography (GC) method. The method is based on obtaining of the unsaponifiable residue, which should be analyzed by the thin layer chromatography in order to separate the stearin fraction from the oil material. Then this fraction should be additionally analyzed by GC after processing with a required reactant [4-8, 9-11, 19, 21].

Methods

A method of HPLC has been proposed to perform a total content of phytosterins (see SPU, Appendix 1, 2.2.29).

A method of quantification of the total phytosterins content in terms of cholesterol:

Test solution: approx. 0.50 g of the pumpkin pulp extract should be placed in a 50 ml volumetric flask then add 25 ml of acetone and shake during 20 min, add more acetone to adjust the volume to the mark and stir again. Take 2.5 ml of the source solution and vacuum filtrate it through the glass filter POR-16.

Preparation of the working standard solution of cholesterol. A 0.50 g sample of the RS cholesterol should be placed in the 50 ml volumetric flask then add the P-graded acetone up to the mark and mix. Take 1 ml of that solution and put it into another volumetric flask of 10 ml, add the P-graded acetone up to the mark and mix. Use this solution only as freshly prepared.

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Testing of GS system. The system is considered suitable if the following conditions are observed: the cholesterol peak symmetry coefficient for the reference solution should not exceed 2.0 (SPU, edition 1, 2.2.29).

An Agiant 6420 Triple Quad mass-spectrometer was used to perform the quantitative determination of the total phytosterins content.

Two to six chromatograms were recorded for injection of 50 µl samples of the reference solution. Then the standard deviation (RSD) value was calculated. A series of n_0 chromatograms was considered completed after RSD value had satisfied requirements given in SFU 2.2.46, N, Table 2.2.46-2.

The total sterins content in the extract (X, %, in terms of cholesterol) was calculated by the formula:

$$X = \frac{\sum S_i \cdot 50 \cdot m_0 \cdot 1 \cdot 100}{S_0 \cdot m \cdot 1000 \cdot 50 \cdot 10},$$

where $\sum S_i$ – the mean value of all sterin peaks area in the reference solution's chromatogram;

$S_{0.}$ – the mean peak area for cholesterol obtained from the reference solution's chromatogram;

m_0 – the RS-grade cholesterol sample mass, g;

m – the extract sample mass, g.

Results

The *Agiant 6420 Triple Quad* mass-spectrometer (№ DE33220210, 06.05.15) was employed to detect phytosterins. First, the chromatograms were recorded for the reference solution with injected sample volume of 50 µl. From 2 to 6 chromatograms (n_0) were obtained and then the relative standard deviation (RSD) value was calculated for the cholesterol peak areas.

This stage was considered completed after RSD reached the value described in the SPU 2.2.46, N, Table. 2.2.46-2.

Then the series of chromatograms were recorded for the pure solvent, experimental solution and reference solution. A number of the parallel chromatograms (n) had to be equal or greater that for the chromatography column testing under the following conditions:

- The column size 250 x 4.0 mm, filling: the sorbent impregnated with octyl silica gel (L7) as the stationary phase; particles size 5 µ XTerra MS C8 (Waters) or similar, which meets the requirements of the chapter «Suitability of the chromatography system»;
- sample rate – 1 ml/min;
- column temperature – 30 °C;
- mobile phase A: 0.005 M deaerated aqueous (P) ammonium formate solution;
- mobile phase B: 0.005 M deaerated ammonium formate solution in the 90:10 mixture of acetonitrile P and water P$
- leaching mode – gradient, according to the given description;
- sampling volume – 50 µl;
- detector mass-spectrometer (Agilent 6420 Triple Quad);
- detector setting:
- ionization type: positive, electric spray (+ESI);
- detection mode: mass scan for the range between 100 to 1000 a.m.u;
- fragmenter voltage – 250 V;
- nitrogen temperature – 350 °C;
- nitrogen supply – 10 ml/min;
- pressure at nebulizer – 35 PSI;
- capillary voltage – 4 kV.

The complete ionic current chromatograms and the mass-spectra for the reference solution of cholesterol and the lipophilic extract from the pumpkin pulp are given in Figures 1-4 while the chromatograms processing data are given in Tables 1-2.

All quantitative results representing the total content of phitosterins in terms of cholesterol are given in Table 3.

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Discussion

It has been found that the total content of phytosterins in the lipophilic extract from the pumpkin pulp is 2.025 % in terms of cholesterol. Therefore, this extract can be considered as a promising source of phytosterins. The results of this work can further be used for elaboration of the raw material specifications and pumpkin quality input control methods.

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Table 1. Data developed from the chromatogram of the reference sample of cholesterol

Holding time	Peak area	Peak height
106,1	913,85	95,45

Figure 1. Chromatogram of the reference sample of cholesterol.

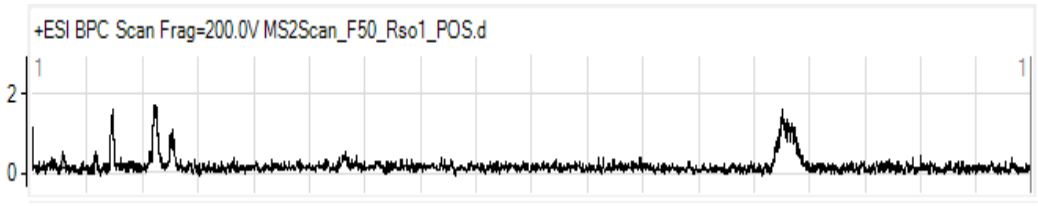

Figure 2. Mass-spectrum of the solution of the reference sample of cholesterol

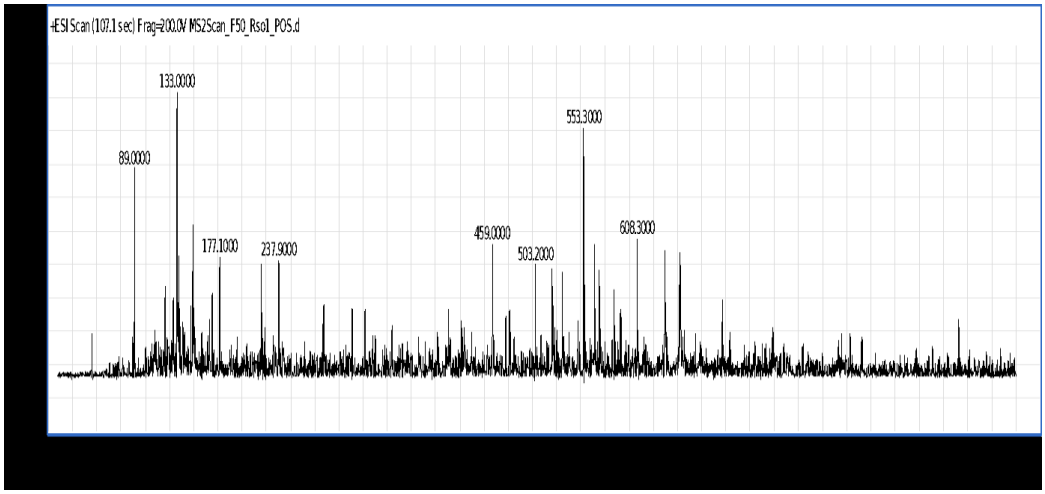

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Figure 3. Chromatogram of the lipophilic extract from the pumpkin pulp.

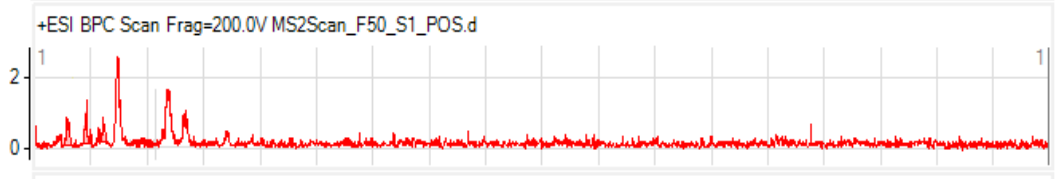

Table 2. Data developed from the chromatogram of the lipophilic extract from the pumpkin pulp

Holding time	Peak area	Peak height
124,3	41963,2	5455,47
180,8	3511,09	625,53
208,8	51279,88	3720,81
224,1	9732,6	945,98
266,8	41773,46	3711,14
293,9	19529,84	1616,92
322,4	30956,6	2138,04
397,1	18371,42	2108,38
430,5	32549,08	2774,35

Remark. Total peaks area = $190086610 \cdot 10^{-4}$.

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Figure 4. Mass-spectrum of a component with retention time 430.5 min taken from the lipophilic extract from pumpkin pulp.

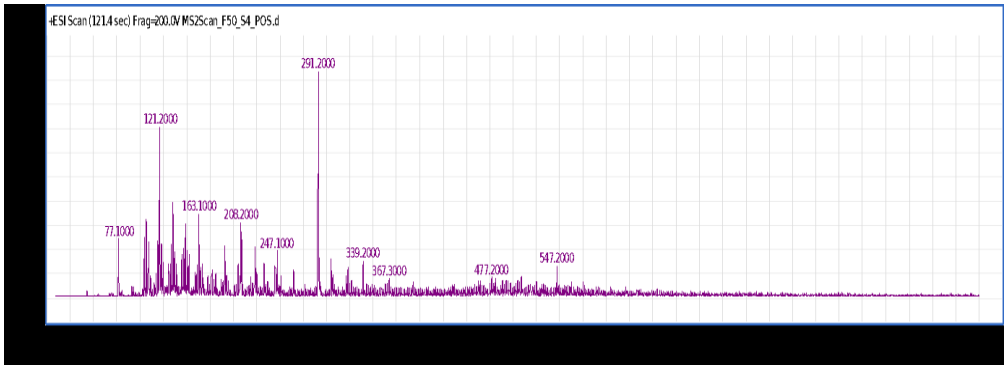

Table 3. Quantitative contents of total phytosterins in terms of cholesterol (%)

Parameter	Sample	
	Reference solution of *cholesterol P*	Lipophilic extract from the pumpkin pulp
Sample mass (m), g	0.50	516.1
Peak area, (S_0)	913.85	–
Total peak area calculated by the test solution chromatograms, (S_i)	–	1900866
Total sterins content in the extract X (in terms of cholesterol), %	–	2.025

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