

Study of qualitative and quantitative content of amino acids in pumpkin seeds for further standardization of the herbal drug

Original Paper

Kotova E.E.¹, Kotov S.A.¹, Gontova T.M.², Kotov A.G.^{1✉}

¹Department of the State Pharmacopoeia of Ukraine,
State enterprise "Ukrainian scientific Pharmacopoeial
center for quality of medicines", 33, Astronomichna, Str.,
Kharkiv, 61085, Ukraine

²Department of Botany, National University of
Pharmacy, 4, Valentynivska Str.,
Kharkiv, 61168, Ukraine.

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Abstract **Aim:** Literary sources on qualitative composition, quantitative content of biologically active substances, and standardization methods of pumpkin seeds have been studied. It has been established that, along with Δ^7 -sterols, whose action is associated with treatment of the prostate gland, pumpkin seeds contain amino acids, cucurbitin in particular, which are responsible for their antihelminthic effect. The aim is to study the qualitative and quantitative content of amino acids in domestic samples of pumpkin seeds in order to clarify the possibility of pumpkin seed standardization for this class of biologically active substances.
Methods: The investigation of the possibility of amino acids' identification in the herbal drug by the thin-layer chromatography (TLC) method was carried out on silica gel 60 F₂₅₄ plates, Merck and Silica gel 60, Merck, on aluminum and glass supports in a mixture of the following solvents: butanol–acetone–acetic acid–water (35:35:10:20). The conclusions have been made based on the presence of characteristic zones of amino acids in the chromatograms after the treatment with ninhydrin solution. Quantitative determination of amino acids in the herbal drug was carried out by absorption spectrometry using HP spectrophotometer HP-8453 UV-VIS, Hewlett Packard (USA).
Results: The chromatographic profile of the amino acid fraction of domestic samples of pumpkin seeds was studied using a TLC technique. In the chromatograms of test solutions from all samples, the zones were detected at the level of histidine, aspartic acid, glutamic acid, glycine, and leucine zones, as well as a zone with color (olive) that differs from the other zones of amino acids and which is possibly a zone of cucurbitin.
On the basis of the results of qualitative research, the quantitative content of the sum of amino acids according to a UV method developed has been determined, which including purification from the fatty oil, further extraction with an aqueous alcohol solution, selection of aliquots for the reaction with ninhydrin solution, and subsequent determination of the absorbance of the test solution and the solution of glutamic acid at a wavelength of 400 nm.
It has been established that the content of the sum of amino acids expressed as glutamic acid in domestic samples is about 2%.
Conclusions: The research of the possibility of pumpkin seed standardization by the content of amino acids was carried out. The chromatographic profile of the amino acid fraction of domestic samples of pumpkin seeds was studied using a TLC method developed. The quantitative content of the amino acids sum by the absorption spectrophotometry method was investigated. The techniques developed will be recommended for inclusion in the draft of national monograph "Pumpkin seeds."

Keywords pumpkin seeds – amino acids – TLC method – UV-vis spectrophotometry method – standardization – SPhU

INTRODUCTION

Pumpkin seeds (*Cucurbita pepo* L.) are a well-known traditional herbal medicine that has been widely used for centuries. Modern use of herbal drugs (HDs) of pumpkin seeds is primarily associated with functional disorders of the bladder, including enlarged prostate gland [1-3]. This pharmacological activity of the HD is associated primarily with the content of fatty oil components, in particular with the content of characteristic Δ^7 -sterols, and so on [4,5]. In addition, pumpkin seeds have been used for a long period

of time, and especially in countries of the former Soviet Union, as an antihelminthic. This pharmacological action is associated with a sufficient amount of amino acids in the raw material, among which special attention is given to cucurbitin (3-amino-3-carboxypiperidine) because of its antihelminthic properties. This amino acid is capable of destroying parasitic worms; it is an inhibitor of histidine decarboxylase by biological activity [6].

* E-mail: fitex16@ukr.net

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According to [7], raw pumpkin seeds (*C. pepo*) contain high concentrations of most essential amino acids, among which the highest concentrations are observed in arginine, glutamine, and aspartic acid. According to [8], the content of cucurbitin in the seeds of *C. pepo* ranges from 0.18% to 0.66% and in *Cucurbita moschata* Duch, from 0.4% to 0.84%.

Pumpkin seeds are described in several pharmacopoeias of the world, namely in the article of the State Pharmacopoeia of the USSR XI (GF XI), V. 2, art. 78 "Pumpkin seeds" [9], monograph of the State Pharmacopoeia of the Republic of Belarus "Pumpkin Seeds" (GF RB) [10], monograph of the German Pharmacopoeia (DAB 10) "Kurbissamen" [11], monograph of the British Herbal Pharmacopoeia (BHP) "Pumpkin seed" [12], and article of the State Pharmacopoeia of the Russian Federation, 14 edition (GF RF 14) "Pumpkin Seeds" [13].

At present, there is no monograph on this type of HD in the State Pharmacopoeia of Ukraine (SPHU) that conduct a research related to its development is relevant.

The analysis of normative documents, namely, articles/monographs in the pharmacopoeias listed above has revealed the following.

Articles GF XI and GF RB include the following HD quality indicators: identification (macroscopy, microscopy) and quantitative indicators (humidity, ash, and foreign matter). In monographs DAB 10 and BHP, a method of identification of steroid compounds by thin-layer chromatography (TLC) is presented additionally. In GF RF 14, the TLC method for identification of substances detected by the solution of ninhydrin is also presented.

The purpose of this work is to study the qualitative and quantitative content of amino acids in domestic samples of pumpkin seeds to determine the possibility of HD standardization for this class of biologically active substances.

MATERIALS AND METHODS

For this experiment, we used seven batches of pumpkin seeds (*C. pepo* L.) (Fig. 1) collected in 2017–2018 and registered in the State Enterprise "Pharmacopoeia Center": No. 1: collected near Izum, Kharkiv region (RS 799); No. 2: collected near Chuguev, Kharkiv region (RS 800); No. 3: collected near Dergachi, Kharkiv region (RS 810); No. 4: collected near Valki, Kharkiv region (RS 802); No. 5: collected near Zmiev, Kharkiv region (RS 803); No. 6: collected near city Kharkiv (RS 804); and No. 7: collected near Merefa, Kharkiv region (RS 805). The identification and authentication of the plant material was carried out by Associate Prof. A.G. Vovk, Department of Experimental Support the Elaboration of Monographs on Herbal Drugs of the SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines" (Kharkov, Ukraine).

In the chromatographic study, TLC plates with a silica gel layer that meet the requirements of SPhU 2.0 "Chromatographic separating capacity" were used. Before use, the chromatographic plates were activated in a drying oven at 110°C for 1 h. The following equipment was used: Ultrasonic

bath SUPER RK100H "Bandelin" (Germany), glass vertical chamber, automatic spraying device ChromaJet DS20.

As standard substances, alanine, arginine, histidine, valine, glycine, leucine, glutamic and aspartic acids (>95%), pharmacopoeial reference standards of the State Pharmacopoeia of Ukraine were used.

Solvents (petroleum ether, alcohol [50%, v/v], methanol, butanol, acetone, water) and chemicals (acetic acid, ninhydrin) used in the experiments were of analytical grade.

The quantitative determination of amino acids in HD was carried out by absorption spectrometry using HP Spectrophotometer HP-8453 UV-VIS, Hewlett Packard (USA).

Method for determination of the quantitative contents of amino acids.

The basis for the determination of the amino acids' content was the technique described in [14] with changes that meet the requirements of SPhU 2.0.

Stock solution. Powdered herbal drug (500) (2.9.12) is pretreated with *petroleum ether* (bp: 50–70°C) and the residual raw material is dried at a temperature not higher than 50°C for complete removal of the residual solvent.

Fat-free material (500) (2.9.12) is extracted with a certain amount of *alcohol* (50%, v/v).

Test solution. The stock solution is concentrated and 2-g/L *ninhydrin* solution is added and heated at 120°C for 20 minutes. The mixture is cooled and quantitatively transferred to a 100-ml volumetric flask.

Reference solution. It is prepared from accurately weighted mass of glutamic acid using the solution of 2 g/L *ninhydrin* and water.

The absorbance (2.2.25) of the test solution and the reference solution is measured in 40 min after the preparation at a wavelength of 400 nm using *water* as compensation liquid.

The content of amino acids, in percent, expressed as glutamic acid and fat-free material, is calculated using the following expression:

$$\frac{A_1 \times m_0 \times 40}{A_0 \times m},$$

where A_1 is the absorbance of the test solution at 400 nm; A_0 is the absorbance of the reference solution at 400 nm; m is the mass of the fat-free herbal drug to be examined, in grams.

RESULTS AND DISCUSSION

Taking into account the literature data on the presence of a significant amount of amino acids in pumpkin seeds, which are responsible for the pharmacological activity of HD, studies on the possibility of identifying using TLC method according to the presence of amino acids' characteristic zones in the chromatograms have been conducted.

Conditions described in article GF RF 14 "Pumpkin Seeds"

that proposed to identify HD by TLC method were studied previously. In this normative document, the raw material is treated with heated water to extract water-soluble substances and then filtered, and the aliquot of the filtrate is treated with alcohol at a temperature of 5°C. After filtration, the obtained solution is applied on the chromatographic plate with a layer of silica gel and developed in the mobile phase: propanol–anhydrous formic acid–anhydrous acetic acid (16:5:5); after the development, the plate is treated with 0.4% solution of ninhydrin in acetone, and the presence of at least four zones of different colors in the chromatogram is observed. It should be noted that the position of these zones in the chromatogram is not described in any way.

A study of the hydrophilic fraction of pumpkin seeds is described in several scientific papers [15, 16], which also describe the method of isolating the amino acid complex (called cucurbin) and which almost correspond to the method presented in GF RF 14. In these works, TLC method using the plates of Sylufol (taken out of production in the 90s of the last century) in the mobile phase: methanol–water–pyridine–10 M HCl (80:11.5:10:2.5) with the detection of 0.4% solution of ninhydrin was used to identify ninhydrin-positive substances; the characteristic zone that got olive color was different from the other zones and probably corresponded to the amino acid cucurbitin was described, but it was determined without the use of the reference standard.

As a result of the analysis of the techniques described, as well as previous studies, the following conditions for identification of amino acids of pumpkin seeds by TLC method were selected.

1) Preparation of the test solution.

Taking into account that seeds contain enough fatty oil (almost 50%), preliminary defatting of the raw material is obligatory because it allows separating the lipophilic part, which prevents chromatography. Conditions of the extraction of the amino acid complex (namely, the ratio of raw material to water is 1 g/10 ml and the heating conditions is 45 min on a water bath) are similar to those described in the abovementioned works, because they allow the quantitative extraction of the necessary substances [15]. After that, to remove high-molecular-weight substances from the water-soluble fraction, the aqueous extract is treated with methanol, filtered, and evaporated to remove alcohol to a volume of about 5 ml.

2) Reference solutions.

Taking into account the literature data on the amino acid content of pumpkin seeds, the following solutions of reference samples (RS) of amino acids were prepared: 5 mg of glycine, alanine, arginine, histidine, valine, aspartic acid, glutamic acid, and leucine were dissolved in 10 ml of methanol.

3) Mobile phase.

As a mobile phase, a mixture of solvents, butanol–acetone–acetic acid–water (35: 35: 10:20), was used, which is used in the SPhU monographs to identify amino acids in HDs [17].

4) Stationary phase.



Figure 1: Pumpkin seeds: dried drug substance

Silica gel 60 F₂₅₄ and Silica gel 60, Merck on aluminum, and glass support were used.

5) The volume of application.

As a result of the selection of different aliquots of the application volume for test solutions, as well as preliminary studies on sensitivity of amino acid zones to the detective reagent, the following application volumes have been chosen: for test solutions, 50 µl; for solutions of RS alanine, arginine, histidine, valine, glycine, and leucine, 10 µl; for solutions of glutamic and aspartic acids, 25 µl.

6) Detection.

To detect amino acid zones, the plate is sprayed with ninhydrin solution (prepared by dissolving 1.0 g of ninhydrin in 50 mL of ethanol [96%] and adding 10 mL of glacial acetic acid), followed by heating at a temperature of (100–100°C) for 5 minutes; this detection is unified because it is used in the monographs of the SPhU to detect amino acid zones.

Figures 2 and 3 show the chromatograms obtained by identifying amino acids in pumpkin seed samples. As can be seen from the figures, the zones at the level of histidine, aspartic acid, glutamic acid, glycine and leucine zones are detected in chromatograms of the test solutions from all samples. In addition, in all chromatograms, the available zone is located close to the starting line, the color of which (olive) differs from other pink–purple zones of amino acids; we suppose this zone can be a zone of cucurbitin, taking into account similar descriptions given in works [15, 16].

Taking into account the results of the previous qualitative study, which showed the presence of amino acids in the studied samples of pumpkin seeds, the quantitative content of this group of biologically active compounds was determined.

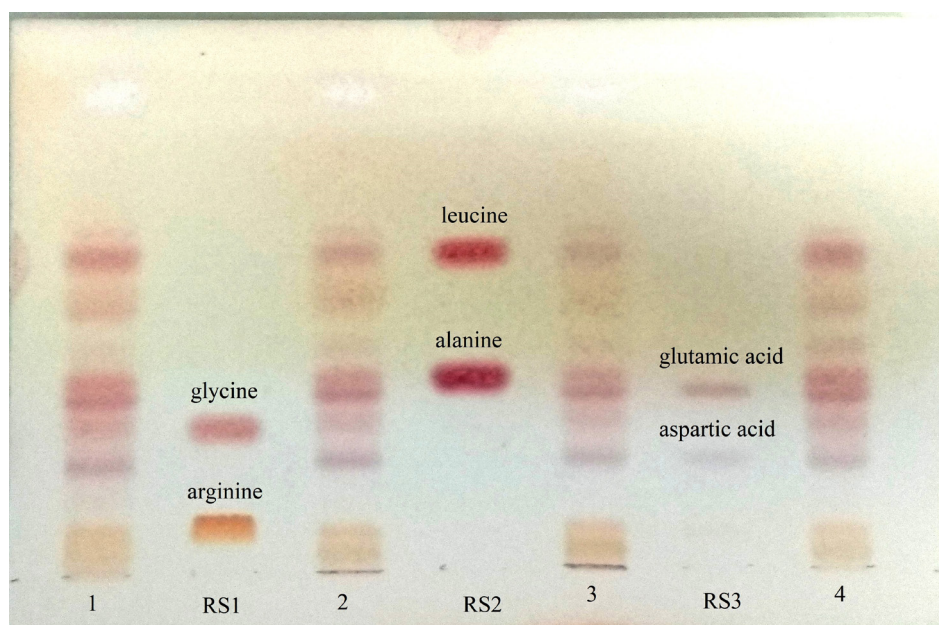


Figure 2: The chromatograms obtained for the identification of amino acids of various samples of pumpkin seeds under conditions of the procedure developed : 1,2,3,4, test solutions obtained from different series (RS 799, 800, 801, 802); RS 1, solution of arginine (zone with lower R_f) and glycine (zone with higher R_f); RS 2, solution of alanine (zone with lower R_f) and leucine (zone with higher R_f); RS 3, solution of aspartic acid (zone with lower R_f) and solution of glutamic acid (zone with higher R_f)

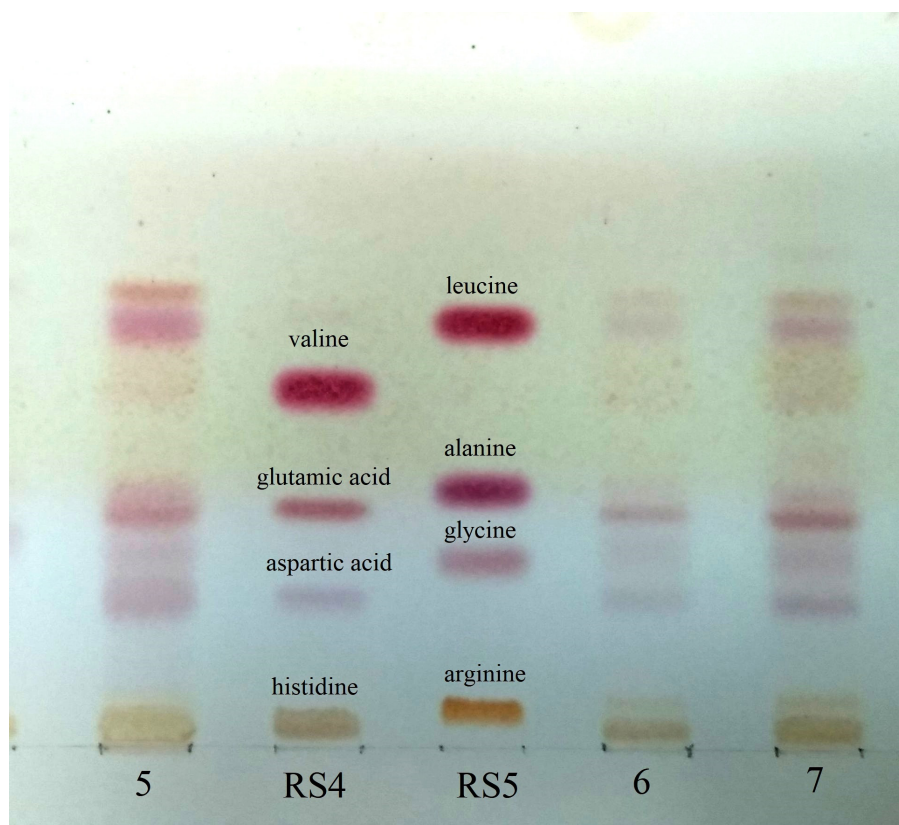


Figure 3: The chromatograms obtained for the identification of amino acids of various samples of pumpkin seeds under conditions of the procedure developed : 5,6,7, test solutions obtained from different series (RS 803, 804, 805); RS 4, solution of histidine, aspartic acid, glutamic acid, valine (in order of increasing R_f); RS 5, solution of arginine, glycine, alanine, and leucine (in order of increasing R_f)

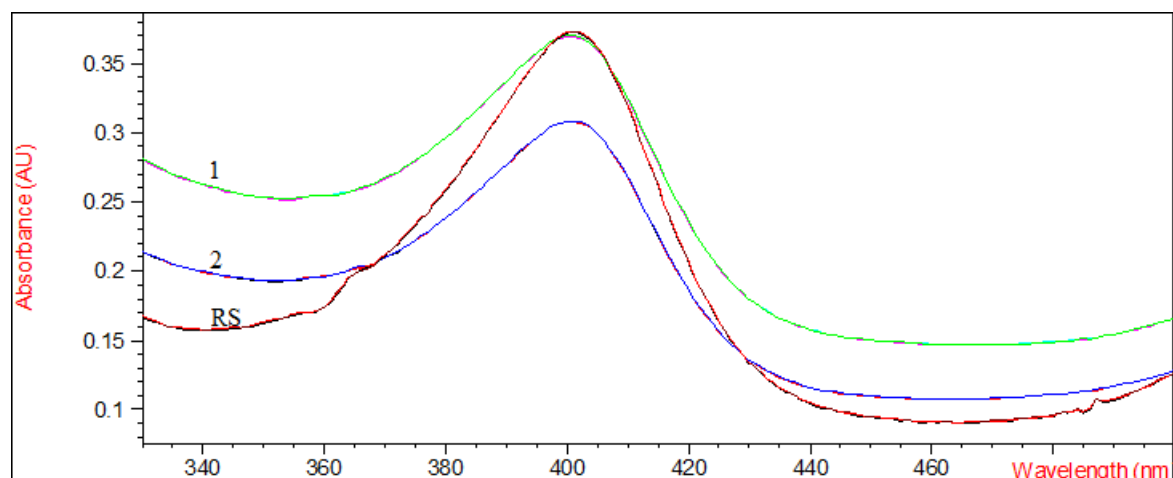


Figure 4: Typical absorption spectra obtained by assay amino acids content: 1,2, spectra of the test solutions obtained from the series of pumpkin seeds; RS, spectrum of solution of glutamic acid

Table 1: Results of amino acids assay expressed as glutamic acid and fat-free raw material in the different samples of pumpkin seeds

RS (batches of pumpkin seeds)	The content of the amino acids expressed as glutamic acid
799	2.0 ± 0.05
800	2.1 ± 0.03
801	1.9 ± 0.04
802	1.8 ± 0.05
803	2.5 ± 0.07
804	1.7 ± 0.03
805	1.8 ± 0.04

The data (g/100 g fat-free raw material) are presented as mean $\pm$ SEM ($n = 3$)

For the analysis of amino acids, methods based on the reaction with ninhydrin are widely used. In [14], the conditions for the ninhydrin reaction were optimized, and it was shown that the products of the reaction of α -amino acids with aqueous ninhydrin solution are characterized by the highest stability in time and have a single maximum absorption at a wavelength of 400 ± 2 nm. On this basis, the authors developed methods for the quantitative determination of α -amino acids in medicines and plant materials, which are based on the reaction with 0.2% aqueous solution of ninhydrin. The developed technique differs with sufficient accuracy (relative error of the separate definition result does not exceed $\pm 3\%$) and accessibility. Taking into account the results of these studies, the method described has been tested on seven samples of pumpkin seeds and the conditions for quantitative determination of the amino acids sum (with the changes indicated above) were selected. The research was carried out with the weights of defatted raw material, which was processed in a Soxhlet apparatus with petroleum ether for 5 h for the complete separation of fatty acid. As a standard, glutamic acid was used, the content of which is predominant in pumpkin seeds in accordance with

all literary sources. Figure 4 shows the absorption spectra of test solutions prepared from pumpkin seeds and a solution of glutamic acid obtained under conditions of the method developed. Table 1 shows the results of the quantitative determination of amino acids in the studied series.

CONCLUSION

The research on the possibility of pumpkin seeds standardization by the content of amino acids was carried out.

The chromatographic profile of the amino acid fraction of domestic samples of pumpkin seeds was studied using a TLC method developed. In the chromatograms of the test solutions from all samples of raw materials, zones at the level of histidine, aspartic acid, glutamic acid, glycine, and leucine zones were detected, as well as a zone whose color was different from the other zones of amino acids, which is possibly a zone of cucurbitin, was also detected.

The quantitative content of the amino acids sum was investigated using the absorption spectrophotometry method. It has been established that the content of the amino

acids sum expressed as glutamic acid in domestic samples is about 2%. The techniques developed will be recommended for inclusion in the draft of national monograph "Pumpkin seeds."

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