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Dedicated to Professor Emil Cordoș
on the occasion of his 80th anniversary

LC-MS/MS METHOD FOR DETERMINATION OF L-A-PHOSPHATIDYLCHOLINE FROM SOYBEAN

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ABSTRACT. The purpose of this study was to develop a quick procedure for the determination of α-phosphatidylcholine from soybean. The procedure is based on a simple solid-liquid extraction followed by a quick analytical method carried out with an LC-ESI-MS/MS system. A chromatographic column of 2×50 mm with a particle size of 2.5 µm was used. The LC-ESI-MS/MS method was developed in positive ionization mode. The analytical method was described in terms of: linearity, detection and quantification limits, accuracy and precision and matrix effect. The calibration curve was developed in the range of 5 to 200 ng/ml with a correlation coefficient r^2 of 0.995 and detection limit of 0.5 ng/ml. The extraction method was tested for the recovery degree. The recovery obtained was 97.2±1.2%. The method was used to determine the content of α-phosphatidylcholine from five soybean varieties from Romanian market. No significant differences were obtained between the five varieties regarding the α-phosphatidylcholine content.

Keywords: *lecithin, α-phosphatidylcholine, soybean, solid-liquid extraction, detection limits, accuracy.*

INTRODUCTION

The soybean (Glycine max (L.) Merrill) is economically the most important bean in the world [1]. In February 2016, the United States Department of Agriculture (USDA) estimated that the Global Soybean Production 2015/2016

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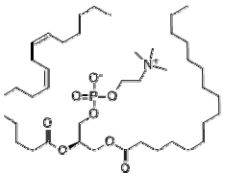
will be 320.51 million metric tons [2]. 10% of this production is directed to human consumption [3, 4]. When talking about human consumption, two important aspects are taken in consideration, the nutritional value and the impact on human health. The soybean contains all eight essential amino acids and it's a good source of fiber, iron, calcium, zinc and vitamins, has no cholesterol and is low in saturated fat [5]. It also contains bioactive components such as saponins, protease inhibitors, phytic acid and isoflavones [6].

One of the important products from soybeans is the commercial lecithin, a complex mixture of phospholipids (PL) containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA) and phosphatidylinositol (PI), sphingolipids, triglycerides, free fatty acids and glycolipids which are widely used as natural emulsifiers, wetting agents and baking improvers [7] as well as dietetics, cosmetics and pharmaceuticals [8]. From these phospholipids, PC is more important (Table 1).

Table 1. Basic information about L-α-phosphatidylcholine [9]

Chemical name	General data		Structural formula
L-α-phosphatidylcholine	Synonyms	L-α-Lecithin, 3-sn-Phosphatidylcholine, L-α-Phosphatidylcholine	
	Chemical class	Phospholipids	
	Chemical formula	$C_{42}H_{80}NO_8P$	
	Molecular weight ($g \cdot mol^{-1}$)	758.06	

L-α-phosphatidylcholine is in the largest concentration in the membrane; it supports all metabolism and is often used under the name of lecithin [10]. Due to the importance of PC, the detection method is very important. The use of analytical methods is critical in order to certify the composition of PC containing products. Analytical method such as: Fourier transform infrared (FTIR) spectrometry [8, 11], high performance liquid chromatography (HPLC) methods with UV detection [12, 13] and evaporative light scattering detector (ELSD) detector [12, 14], matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) [15] and thin-layer chromatography and 1H, 13C, 31P NMR [8] were used intensively in past years.

The purpose of this study is to obtain a quick LC-MS/MS analytical method for determining the L-α-phosphatidylcholine from soybeans. In order to obtain good analytical data, a reliable LC-MS/MS method was

developed. The method is described in terms of retention time, linear range, linear equation, correlation coefficient, detection and quantification limits.

RESULTS AND DISCUSSION

LC-MS/MS profile

Several experiments were performed in order to establish the ionization mode, the precursor and product ions (MRM transition) and the parameters specific to the MS/MS method. For L-α-phosphatidylcholine the protonated molecule $[M+H]^+$ was proved to be more abundant and, therefore selected for further investigation. The compound dependent parameters, specific to the investigated compound and with an important contribution to the sensitivity of the method, are presented in Table 2. The source dependent parameters, with an important contribution in optimizing the compound signal in HPLC conditions, and the HPLC parameters are presented in Table 2.

The MRM (multiple reaction monitoring) transition of the pair 760.5→184.1 for 100 ng/ml of L-α-phosphatidylcholine is presented in Figure 1.

Table 2. LC-MS/MS parameters for determining L-α-phosphatidylcholine

Compound	**L-α-phosphatidylcholine**
MS/MS parameters	
Ionization mode	Pozitive
Compound dependent parameters	DP (V):86.00; EP (V):4.50; CEP (V): 30.00; CE (V):39.00; CXP (V):4.00
Source dependent parameters	CUR:15.00 psi; CAD:Medium; IS:4500.00V; TEM:600°C; GS1:25.00 psi; GS2:35.00 psi
MRM transition	760.5→184.1
HPLC parameters	
Chromatographic column	Phenomenex Synergi Fusion 2.5µm, 2×50 mm
Flow rate	0.5 ml/min
Column temperature	40°C;
Injection volume	40 µl
Mobile phase:	H_2O:CH_3CN (10:90 v/v)

Method evaluation

The LC/ESI(+)-MS/MS was evaluated in terms of retention time, linear range, linear equation, correlation coefficient, detection and quantification limits and accuracy. The obtained values are presented in Table 3. The matrix effect was -8% and was not taken in consideration for further investigations. The recovery of the extraction method was evaluated and obtained result was 97.2±1.2%.

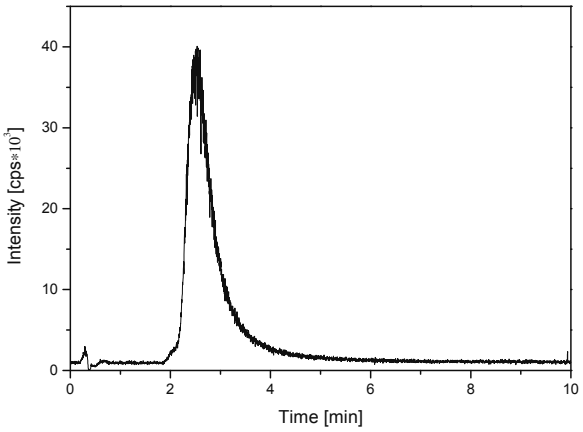


Figure 1. MRM chromatogram for 100 ng/ml of L-α-phosphatidylcholine

Table 3. Experimental and statistical parameters of LC-ESI-MS/MS method for L-α-phosphatidylcholine determination

Experimental parameters			
Retention time t_R (min)	2.54		
Linear range (ng/ml)	5-200		
Linear equation	$1.85x*10^4+976$		
Correlation coefficient r^2	0.995		
Detection limit LOD (ng/ml)	0.5		
Quantification limit LOQ (ng/ml)	1.5		
Statistical parameters			
	Concentration (ng/ml)		
	20	40	60
Intra-day			
Mean ±SD (ng/ml)	19.64±0.21	39.82±0.22	59.84±0.42
RSD (%)	1.07	0.55	0.70
Inter-day			
Mean ±SD (ng/ml)	19.51±0.25	39.77±0.31	59.79±0.45
RSD (%)	1.28	0.78	0.75

Real sample experiments

Five varieties of soybean were acquired from a Romanian market. The soybean varieties were tested with the developed LC-MS/MS method in order to evaluate the content of L-α-phosphatidylcholine. Three samples from every variety of soybeans were investigated. The results are presented in Table 4.

Table 4. Concentrations of L-α-phosphatidylcholine in the five investigated soybean varieties

Soybean variety	**L-α-phosphatidylcholine (mg/kg)**				
	1	**2**	**3**	**4**	**5**
Experimental values	2804	3351	2978	3002	3411
	2815	3354	2971	3015	3415
	2809	3348	2988	3019	3418
MEAN	**2809**	**3351**	**2978**	**3015**	**3415**

A rough analysis of the results shows that L-α-phosphatidylcholine represents ~0.3% of the soybean content. In this situation, it can be stated that the differences between the soybean varieties when spoken about L-α-phosphatidylcholine content are insignificant.

CONCLUSIONS

A LC-MS/MS method was developed for identification and quantification of L-α-phosphatidylcholine from soybean. An HPLC Agilent 1200 series coupled with an ABI Sciex 3200 QTRAP mass spectrometer with a TurboV ionization source was used in ESI positive ion mode. The chromatographic column Phenomenex Synergi Fusion 2.5 μm, 2×50 mm showed a good chromatographic peak. The specific parameters for mass spectrometer and also HPLC were identified and selected to assure the most sensitive response of the equipment. The calibration curve was developed in the range of 5 to 200 ng/ml with a correlation coefficient r^2 of 0.995 and detection limit of 0.5 ng/ml. An extraction procedure was tested and the recovery was 97.2±1.2%.

The developed method was used to test the content of L-α-phosphatidylcholine from five varieties of soybean acquired from a Romanian market. There were no significant differences between the content of the investigated soybean varieties, the content of L-α-phosphatidylcholine being ~0.3% of the soybean content in all soybean varieties.

EXPERIMENTAL SECTION

Standards and reagents

Lyophilized powder of L-α-phosphatidylcholine from egg yolk (≥99%) used to prepare the standard solutions was purchased from Sigma-Aldrich (Steinheim, Germany). Methanol LC-MS Optigrade (≥99.8%) used to prepare the stock solution and Acetonitrile LC-MS Optigrade (≥99.8%) used for mobile phase were purchased from LGC Standards. Chloroform anhydrous (≥99%) used for extraction was purchased from Sigma-Aldrich (Steinheim, Germany). Ultra pure water was obtained by using a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA).

Standard solution preparation

The stock solution (1mg/ml) was obtained by dissolving 1 mg of l-α-phosphatidylcholine in 1 ml of CH_3OH. Two solutions, one of 100 ng/ml l-α-phosphatidylcholine in CH_3OH and the other of 100 ng/ml l-α-phosphatidylcholine in CH_3CN were prepared from the stock solution and used for method development and optimization. Six concentration levels of 5, 25, 50, 75, 100 and 200 ng/ml were prepared by diluting the stock solution with a mixture of H_2O/CH_3CN (10:90 v/v). These standard solutions were used for obtaining the calibration curve. Three concentration levels of 20, 40 and 60 ng/ml were prepared by diluting the stock solution with a mixture of H_2O/CH_3CN (10:90 v/v) and were used for accuracy and precision studies.

Sample extraction

The soybeans were chopped using a special grinding mill. 1ml of chloroform was added on 0.5 mg of soy. The mix was centrifuge at 10°C for 20 min using a speed rotation of 15000 RPM. The supernatant was recovered and then dried using a rotary evaporator. The solution was reconstituted with 1ml of H_2O/CH_3CN (10:90 v/v).

Analytical equipment

A high performance liquid chromatograph HPLC Agilent 1200 Series coupled with an ABI Sciex 3200 QTRAP mass spectrometer was used for this study.

LC-MS/MS profile development

The MS/MS profile is developed in three steps. First step, a solution of 100 ng/ml l-α-phosphatidylcholine in CH_3OH was injected directly in MS

in order to establish the ionization mode. Also, the parent and the product ions were selected at this step. Second step, a solution of 100 ng/ml l-α-phosphatidylcholine in CH_3OH was injected directly in MS in order to establish the compound dependent parameters (DP (declustering potential), EP (entrance potential), CE (collision energy) and CXP (collision cell exit potential)). Third step, after connecting the HPLC, a solution of 100 ng/ml l-α-phosphatidylcholine in CH_3CN was injected into the LC-MS/MS system and the source dependent parameters (CUR (curtain gas), CAD (collision gas), IS (ionspray voltage), TEM (temperature), GS1 (gas 1) and GS2 (gas 2)) were established.

The HPLC parameters: chromatographic column, flow rate, column temperature, injection volume and mobile phase were established after several experiments and finalize the LC-MS/MS method.

Method evaluation

The LC/ESI(+)-MS/MS was evaluated in terms of retention time, linear range, linear equation, correlation coefficient, detection and quantification limits, accuracy and precision.

Linearity. Six levels of concentration ranging from 5 to 200ng/ml were prepared by successive dilution with mobile phase from the stock solution. The calibration curve was obtained by plotting the peak area to corresponding concentrations. Useful information such as: linear equation and correlation coefficient were obtained.

Detection and quantification limits. Detection (LOD) and quantification (LOQ) limits were estimated by analyzing standard solutions at levels producing signals at signal-to-noise ratios of 3 and 10 respectively.

Accuracy. The intra- and inter- day accuracies were estimated by preparing three concentration levels which were used in both experiments. For intra-day study three replicas of three concentration levels were analyzed. For inter-day study three concentration levels were analyzed once per day for three consecutive days.

Matrix effect. The matrix effect was evaluated as follows. The value obtained for the analyte of interest after extraction was compared with pure solutions prepared in mobile phase containing equivalent amounts of the analyte of interest. The difference in response between the extracted sample and the pure solution multiplied by 100 and divided by the pure solution response determines the degree of matrix effect occurring to the analyte in question under chromatographic conditions [16]. The target for the matrix effect value was chose to be between -20% and +20%.

Recovery. The recovery (R) of the extraction method was determined by using the standard addition method. A sample of androsterone was

extracted and then measured (*initial amount*). Then, other sample of the same celery was spiked with a known concentration of analyte (*spiked amount*), extracted and then measured (*final amount*).

The recovery was calculated using the following equation:

R (%)=100×(final amount - initial amount)/spiked amount.

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