SOI: 1.1/TAS DOI: 10.15863/TAS

International Scientific Journal

Theoretical & Applied Science

p-ISSN: 2308-4944 (print) **e-ISSN:** 2409-0085 (online)

Year: 2017 **Issue:** 08 **Volume:** 52

Published: 21.08.2017 **http://T-Science.org**

SECTION 9: Chemistry and chemical technology

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VALIDATION OF THE METHODIC OF QUANTITATIVE DETERMINATION OF QUERCITIN IN THE MEDICINE "LIPOPHLAVONE, LIOPHILIZATE FOR EYE DROPS PREPARATION"

Abstract: *Validation of methodic of HPLC quantitative determination of quercitin in the medicine "Lipophlavone, liophilizate for eye drops preparation" was carried out. The methodic was developed at first and is a part of analytical normative documentation. The obtained data for parameters, demanded for validation of quantitative determination, show us, that the methodic can be used for analytical investigation of the medicine. To treat obtained data "Chemcalc" program was used.*

Key words: *methodic, validation, nanotechnological liposomal medicine, Chemcalc program.*

Language: *English*

Citation: Bida DS, Yurchenko OI, Chernozhuk TV, Kravchenko OA (2017) VALIDATION OF THE METHODIC OF QUANTITATIVE DETERMINATION OF QUERCITIN IN THE MEDICINE «LIPOPHLAVONE, LIOPHILIZATE FOR EYE DROPS PREPARATION». ISJ Theoretical & Applied Science, 08 (52): 43-51.

Soi: http://s-o-i.org/1.1/TAS-08-52-6 ***Doi***: crossref https://dx.doi.org/10.15863/TAS.2017.08.52.6

Introduction

At the modern stage of pharmacy development the new brunch of creation of the new medicines with antioxidant and inflammation activity is developed too. The most important natural antioxidants are bioflavonoids (rutine, quercitin, flacumine), which possess good antioxidant and anti inflammation activity due to catching of endorgenic radicals. On the base of such biflavonoid nanotechnological liposomal medicine "Lipophlavone, liophilizate for eye drops preparation" was created. The medicine consists of quercitin, phosphatidylcholine (active substances) and lactose like crioprotector. [1,p.5050;2,p. 20;3,p.15;4.p.20;5,p.17;6,p.7].

Development of methods of control of quality is part of pharmaceutical creation of medicines. To control the composition of the medicine the new methodic of qualitative determination of one of the active substance-quercitin, using high performance liquid chromatography (HPLC) method was developed. HPLC is universal and high effective method in analytical chemistry, because of it is widely used in modern analytical methodic. [7,p.75; 8,p.30].

HPLC method except of selectivity, is different from TLC and spectrophotometry in its sensibility. Because of we have small amount of quercitin in our medicine, we should use HPLC method. We can not use big samples of the medicine because of

nonhomogeneous distribution of quercitin in the samples. In this case we should use sample of the medicine from one vessel because at low concentrations an error of measurement makes contribution in the obtained results.

According to demands SPU 2.0 (State Pharmacopoeia of Ukraine) to carry out validation of methodic of quantitative determination in medicines forms we should estimate the next characteristics: accuracy, that included convergence and intralaboratory accuracy, selectivity (specify), linearity, diapason of use, robustness (stability of the methodic to changes) [10,p.20;12,p 5].

The purpose of the work is: to determine use of developed methodic to analytical investigation of the medicine, with use of "Chemcalc" program.

Experimental

Liquid chromatography HPLC Shimadzu LC 20AD, detector LT-ELSD SEDEX 85; dozen pipettes Vitlab from 100 to 1000 mkl; laboratory scale Kern 82 g\320 g, *d*=0.01mg/0.1mg; volumetric flasks Simax, of 10 and 100 ml volume; device to obtain high purified water Millipore/Millipore Direct-Q SUV were used for analysis.

Conditions of chromatography were: column Waters Xbridge Shield RP18 5 mkm × 250 mm × 4.6 mm; temperature of column was 55ºC; mobile phase B: metanole-acetonitrile, phase A 1% water solution of acetic acid; flows rate is 1 ml\min: volume of injected sample is 10 mkl; detection; wave lengths of detection is 371 nm. An algorithm of carrying out chromatography is in the Table 1.

***Investigated solution*:** to the vessel with the medicine, that consists of lipophilizated powder with contain of quercitin 0,75 mg, phosphatidylcholine 27,5 mg and lactose 40 мг, was added 2 ml of phase B, mixed up to total dissolution. The contain of the vessel is placed into volumetric flack of 10 ml volume, brought up to the mark by B phase and mixed. The solution is filtered through PTFE membrane with porous diameter of 0.45 mkm.

***Compensation solution*:** the sample of 0.1126 g of standard quercitin dyhydrate (Quercetin dihydrate CRS batch2, 90.5% $C_{15}H_{10}O_7$, EC no 204-187-1, RTECS No LK8950000) was placed into volumetric flack of 100 ml volume, dissolved in 70 ml of B phase and brought up to the mark by B phase and mixed. 0.75 ml of quercitin dyhydrate solution is placed into volumetric flack of 10 ml volume and dissolved in B phase. [9,p.30;11,p.10]

Results and discussion

At the first stage of our work an availability of chromatography system was estimated.

The results of availability of chromatography system are in the Table 2 (data by Shimadzu LCsolution Analysis Report).

According to demands of SPU 2.0 the results from table 2 approve an availability of chromatography system.

At the second stage of our work selectivity of methodic was estimated. There are only peak of quercetin is observed in this system. Peaks of phosphatidylcholine and lactose are not observed. It was proved by absence of phosphatidylcholine and lactose peaks on placebo chromatogram (and phosphatidylcholine and lactose solution in phase B). Spectral purification of the quercitin peak, obtained with use of diode-matrix detector for investigated and comparison solutions was checked up. An example of placebo chromatogram is on 1(*a*) Figure. Examples of chromatograms and peaks spectra for compensation and investigated solutions are on Fig. 1(*b*). And Fig. 1(*c*) respectively. Peak cleanness of quercitin in comparison solutions is observed at wave lengths 370.57 nm (Fig. 1(*b*)). Peak cleanness of quercitin in investigated solutions is observed at wave lengths 370.50 nm (Fig. 1(*c*)).

At the third stage of our work an accuracy of the methodic was estimated. An accuracy of the methodic was checked by establishing of comparison between well known true value (external international standard) and obtained value (an average value), using the methodic in a number of parallel measurements. An analysis of a number of samples, prepared according to external international standard Quercitin dihydrate CRS with well known concentrations of quercitin in three parallels of every sample. Five standard samples with well known concentration of quercitin were prepared from solution of standard quercitin sample. Also an analysis of samples, prepared from quercitin dihydrate with the same concentrations was carried out. Five standard samples with well known quercitin concentration were prepared from compensation solution. Five investigated samples were prepared from quercitin solution with concentration 1 mg\ml.

The results of an accuracy of the methodic are in Table 3.

Result: The obtained result 98.2±0.1 is not exceed an error of the methodic, described in analytical documentation in the diapason of use "Lipoflavone-nano" medicine, like 100±10% (from 0.67 up to 0.83 mg in the vessel).

At the fourth stage of our work linearity of the methodic was estimated.

To prove linear dependence of peak square from concentration in the solution were prepared 5 solutions with quercitin concentrations 0,6 mg/ml, 0,7 mg/ml, 0,8 mg/ml, 0,9 mg/ml, 1 mg/ml. To do it, 5 precise samples of Quercitin standard (Quercitin dihydrate CRS batch 2, 90.5% $C_{15}H_{10}O_7$, EC no 204-187-1, RTECS No LK8950000) in the diapason from 75% to 125% from contain in the medicine were taken and solutions in the B phase were prepared.

Every solution was analyzed trice and coefficients of linear regression were determined.

The results of linearity of the methodic are on pic. 2 and table 4.

At the fifth stage of our work the diapason of use of the methodic was estimated. Diapason of use of the methodic at quantitative determination in medicines is from 80 up to 120% from nominal content of active substance. Content of quercitin, according to analytical normative documentation of the medicine, is in diapason from 0,67 up to 0,83 mg in the vessel or 0,75 mg ±10% (100%±10%), what is conformed with demands according to medicines.

At the sixth stage of our work an accuracy of the methodic was estimated. To determine accuracy of the method of quantitative determination, the investigated solution was chromatographed 5 times in one day according to the methodic. The criteria of accuracy is relative standard deviation. The results of accuracy estimation of the methodic are in the Table 4.

Relative standard deviation is Sr,% = 0,145. At the step of validation, laboratory accuracy according to the given conditions (equipment, climate, reagents, etc.) was determined.

At the seventh stage convergence of the methodic was estimated. To determine convergence of the results of analysis (as index of stability of the used HPLC method) 3 solutions with 2 parallel samples with concentrations of quercitin in diapason from 80 up to 120% were chromatographed. The results of estimation of convergence of the methodic are in the Table 5.

At the eights stage of our work robustness (stability of the methodic to changes) was estimated. To check robustness of chromatography we should determine stability of solutions with time, and an influence of subjective factors should be determined. An influence of subjective factors was not proved by our investigation of laboratory accuracy.

Using developed by us software «ChemCalck», calculations of quantitative determination of quercitin in the medicine “Lipophlavone, liophilizate for eye drops preparation” were done. The software was developed on C# programming **language with use** Windows Forms и Entity Framework Code First technologies.

At first were created three templates to carry out calculations:

- Parametrical, contained all of the parameters of chromatography.
- Template, contained all of information about sample preparation.
- General template, contained all information about sample and connect all of templates into one.

After we put our samples in the general database and with created template did treatment of the obtained experimental data.

As a result we obtained quantitative content of quercitin in the samples and statistic information about it. Use these results we can make an account, included all information about carried out analysis.

Conclusions

Validation of developed by us methodic of quantitative determination of quercitin in the medicine “Lipophlavone, liophilizate for eye drops preparation” was carried out. The methodic was done with use of HTML on chromatograph of Shimadzu company and diode-matrix detector Waters Xbridge Shield RP18 5 × 250 mm × 4.6 mkm. It was shown that the methodic can be used for analytical investigations of “Lipophlavone, liophilizate for eye drops preparation. It was proved by using developed by us a “Chemcalc” software.

Table 1

An algorithm of carrying out chromatography

Time (min)	Flow (ml/min)	Mobile phase A (% vol.)	Mobile phase B (% vol.)
0	1.0	40	60
19.0	1.0	40	60
20.0	1.0	10	90
27.0	1.0	10	90
28.0	1.0	40	60

Table 2

The results of availability of chromatography system

Compensation solution №1	Retention time, min	Peak square	Peak height	Tailing factor	Number of theoretical plates
1	9.030	2788725	107146	6.848	11.445
2	9.034	2776390	106326	6.731	11.232
3	9.034	2775399	106380	6.624	11.360
Mean values:		2780171			
Investigated solution № 1,	Retention time, min	Peak square	Peak height	Tailing factor	Number of theoretical plates
1	9.060	2693526	103435	6.773	11.008
2	9.056	2677276	101939	6.816	11.072
3	9.038	2681352	102094	6.735	11.012
Mean values:		2684051			
B(%)	**2** (USP38/NF33 Dietary Suplements: Quercitin) [13].				

Table 3

The results of an accuracy of the methodic (n = 9, P = 0.95, S_r = 0.36%).

№ of sample	Quercitin content in standard solution mg/ml «injected»	Quercitin substansion content in investigated solution mg/ml «found out»	Quercitin content «found out» from «injected»,%
1	0.068	0.066	98.18
2	0.079	0.077	98.13
3	0.090	0.088	98.15
4	0.101	0.099	98.09
5	0.113	0.111	98.37
Mean value:			98.184

Table 4

The results of estimation of linearity and accuracy.

Linearity of the methodic		Accuracy of the methodic (n = 5, P = 0.95, S_r = 0.145%)	
C (of standard solution), mg/ml	An average peak square	№	Content of quercitin, mg in the vessel
0.61	2049968.67	1	0.75
0.71	2441709.67	2	0.76
0.82	2800191.33	3	0.76
0.92	3158978.33	4	0.77
1.02	3560322	5	0.75

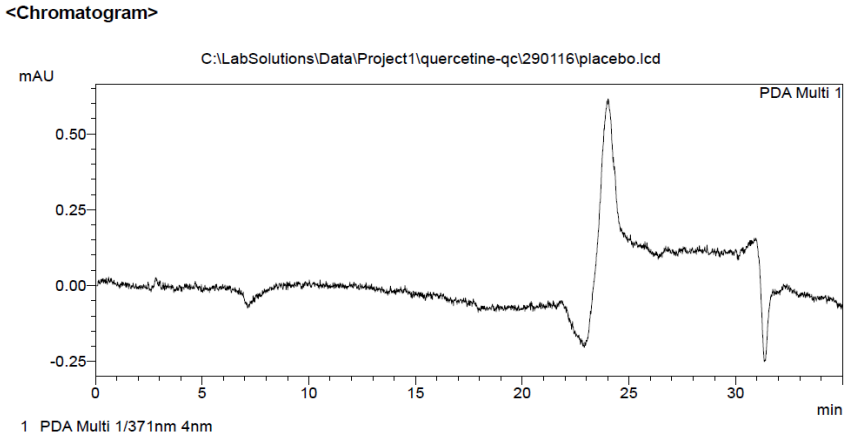
Table 5

The results of estimation of convergence of the methodic

№ of injection	Peak square	An average peak square	Qercetin content , mg	Qercetin content from nominal, %
Content of quercitin 80% from nominal one (S_r = 1.17%)				
1	2135727	2135989	0.61	81.3
2	2136251			
Content of quercitin 100% from nominal one (S_r = 1.77%)				
1	2693526	2685401	0.77	102.5

2	2677276			
Content of quercitin 120% from nominal one (S_r =0.79%)				
1	3176012	3111484.5	0.89	118.7
2	3046957			

An average relative standard deviation of peaks squares is 1,24%.



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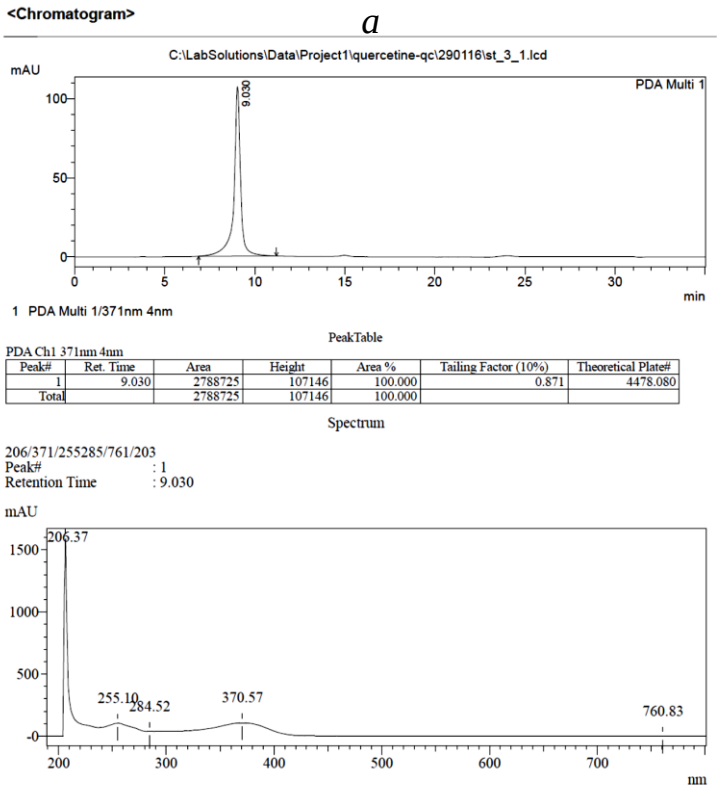

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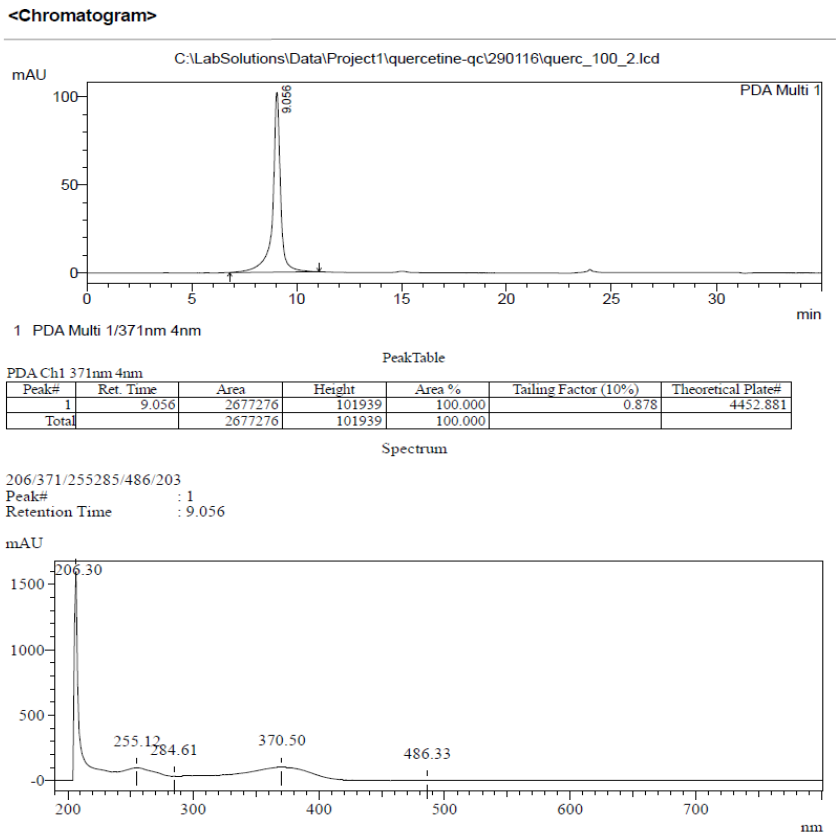
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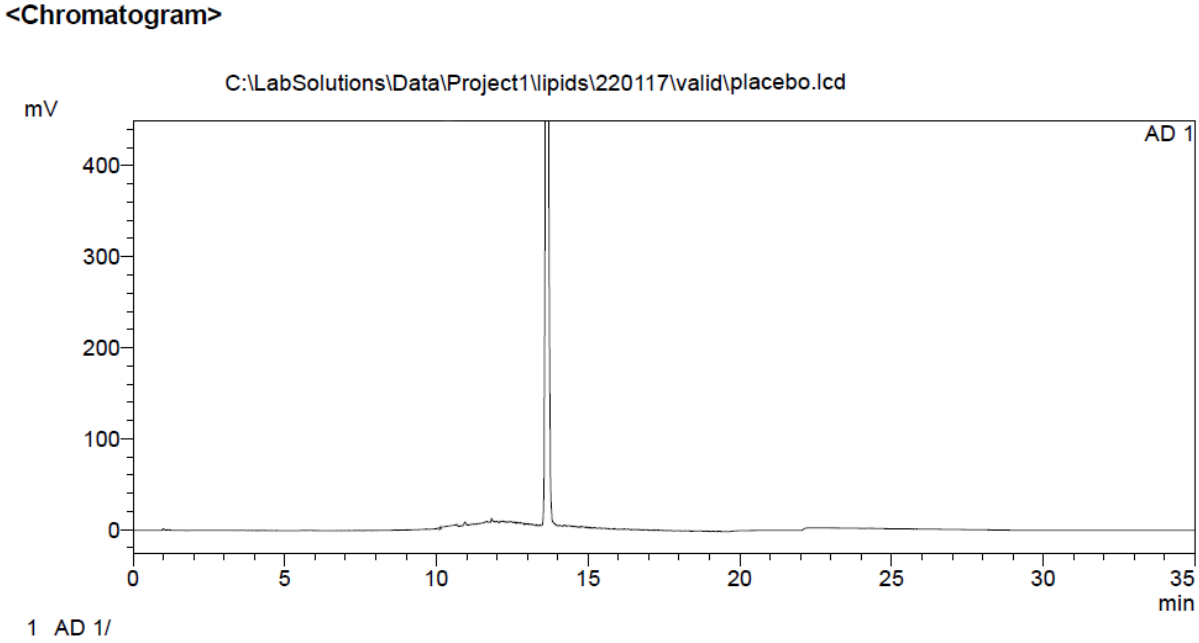
mAU
PDA Multi 1
9.056
min
1 PDA Multi 1/371nm 4nm
PeakTable
PDA Ch1 371nm 4nm
Peak# | Ret. Time | Area | Height | Area % | Tailing Factor (10%) | Theoretical Plate#
1 | 9.056 | 2677276 | 101939 | 100.000 | 0.878 | 4452.881
Total | | 2677276 | 101939 | 100.000 |
Spectrum
206/371/255285/486/203
Peak# : 1
Retention Time : 9.056
mAU
206.30
255.12
284.61
370.50
486.33
nm

a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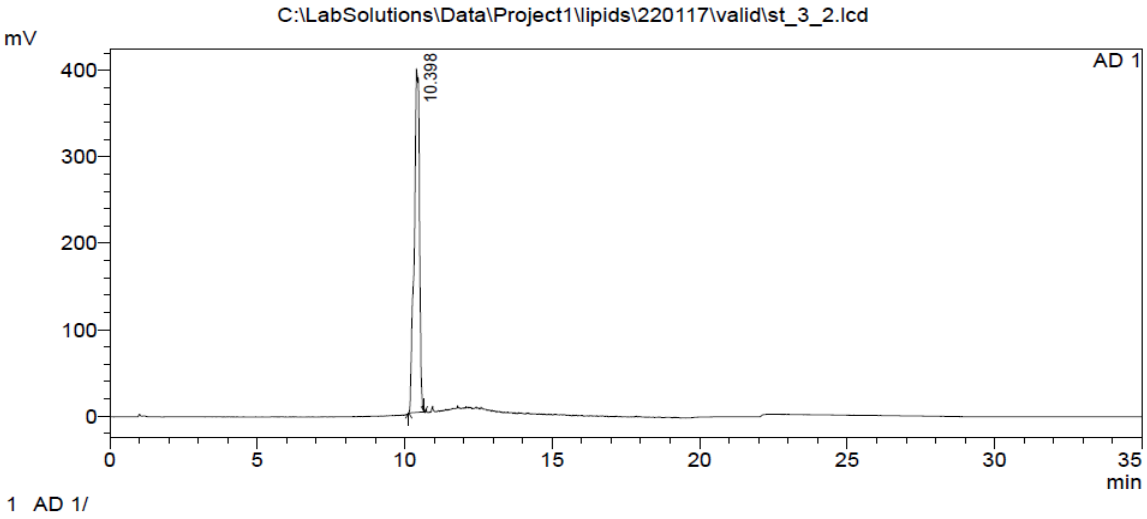

PeakTable

ELSD Ch1

Peak#	Ret. Time	Area	Height	Area %	Theoretical Plate#	Tailing Factor (10%)
1	10.398	4943115	397638	100.000	21366.110	0.932
Total		4943115	397638	100.000		

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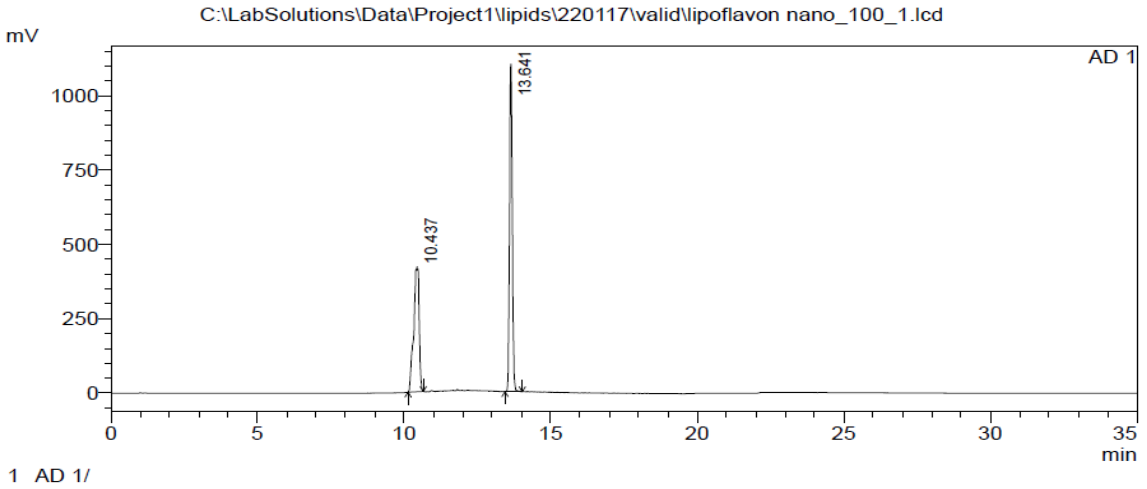
C



PeakTable

ELSD Ch1

Peak#	Ret. Time	Area	Height	Area %	Theoretical Plate#	Tailing Factor (10%)
1	10.437	5458291	421200	42.667	20182.173	0.804
2	13.641	7334544	1101607	57.333	89475.595	1.103
Total		12792835	1522807	100.000		

Width at 50% Height
0.182
0.102

Figure 1 - Chromatogram of placebo (*a*), chromatogramme and spectra of quercitin peak of compensation solution (*b*), of investigated solution (*c*).

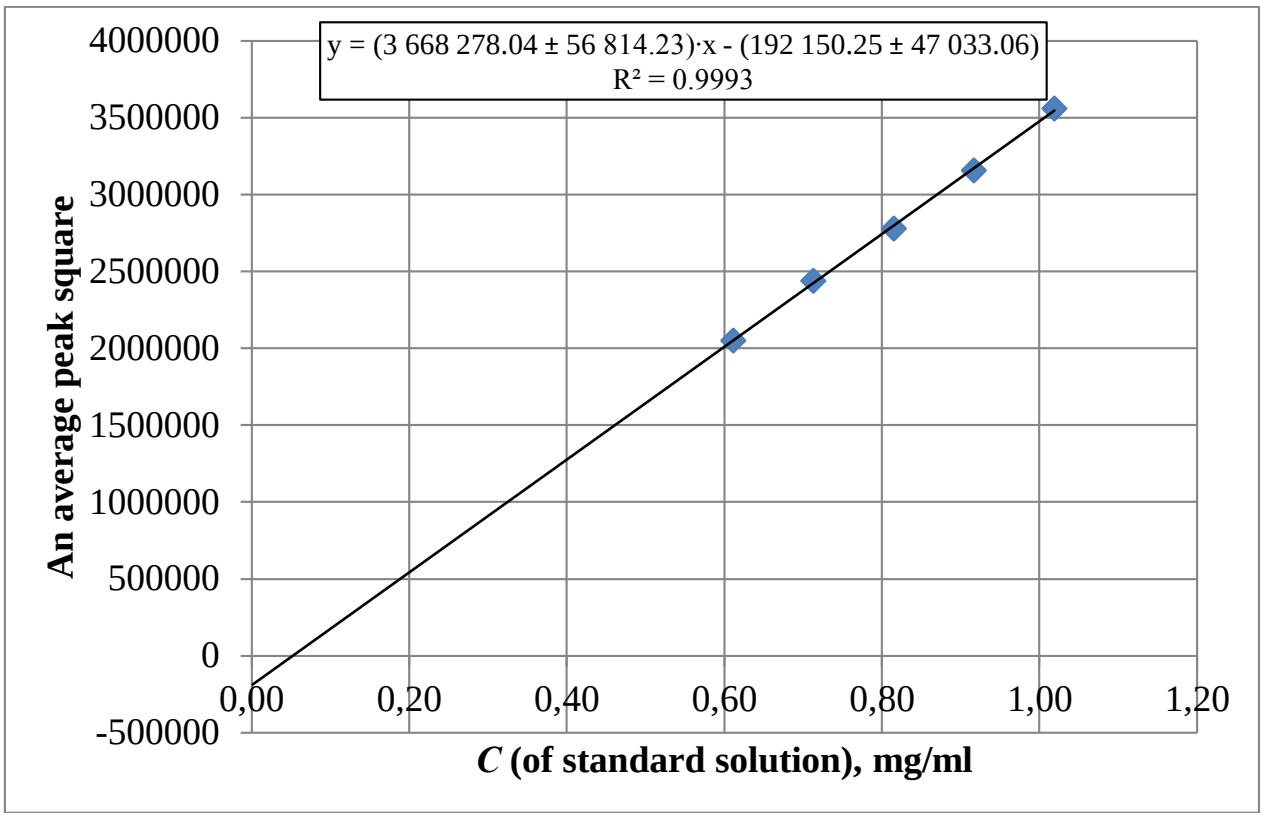


Figure 2 - Dependence of the average peak square from standard solution concentration to estimate linearity of the methodic.

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