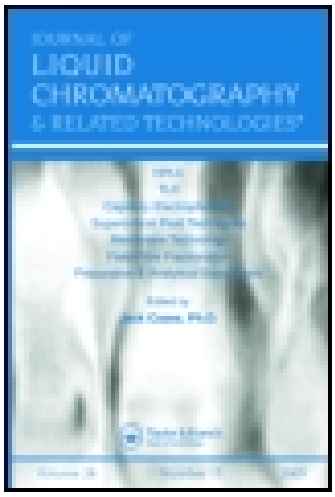

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Estimation of Lipophilicity of Some Polyoxygenated Steroids by the Means of Normal-Phase Thin-Layer Chromatography

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ESTIMATION OF LIPOPHILICITY OF SOME POLYOXYGENATED STEROIDS BY THE MEANS OF NORMAL-PHASE THIN-LAYER CHROMATOGRAPHY

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Abstract

Unmodified silica gel in combination with two mobile phases, acetone – n-hexane and acetonitrile – dichloromethane, was used in order to evaluate the capability of normal-phase (NP) chromatography on bare silica gel in estimation of lipophilicity of some polyoxygenated steroids. Soczewinski equation coefficients were employed as a measure of lipophilicity. The R_M^0 values obtained in NP-systems were correlated with those derived by extrapolation from reversed-phase (RP) systems. In addition, retention data, i.e. lipophilicity parameters determined in NP systems were compared with logP values calculated by use of several commercial computer programs. The results showed that chromatographic parameters R_M^0, and m obtained in NP system consisted of silica gel as stationary phase and acetone – n-hexane as mobile phase, are acceptable as the measures of lipophilicity of polyoxygenated steroids. The mechanism of retention was discussed.

KEYWORDS: Polyoxygenated steroids; Lipophilicity; Normal-phase chromatography; Silica gel

INTRODUCTION

The physicochemical characteristics of a substance depend on its structure and, to a great extent, affect its interactions with molecules governing in that manner substance's behavior in the various environments, biological, chemical or chromatographic. It is considered that partition of given substance between aqueous and organic phases determines both its permeation through biological membranes and retention behavior in reversed-phase chromatographic systems. This process includes non-covalent interactions related to substance's lipophilicity, which governs numerous processes, such as transport, distribution, and metabolism of molecules in biological systems.[1,2] Lipophilicity as a crucial physicochemical parameter in quantitative structure-activity relationships (QSAR), in drug and pesticide design as well as in toxicology studies in both the pharmaceutical and environmental sciences, is commonly expressed as logarithm of 1-octanol−water partition coefficient. Various experimental and calculation methods may be used for either measurement or estimation of the octanol-water partition coefficients.[2] Since each of them has certain advantages/disadvantages or some practical limitations, the most reliable results can be obtained by combination of different methods.

Besides tedious and time-consuming traditional shake-flask method, the various chromatographic methods are widely used for the indirect determination of lipophilicity. Due to similarity between octanol-water partitioning and chromatographic retention,

reversed-phase liquid chromatography is generally accepted as reliable method for determination of lipophilicity.[3] The reversed-phase high-performance liquid chromatographic (RPHPLC) method, described in the Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals, Test No. 117, is based on the distribution of the analyte between an expressively non-polar stationary phase (C-8 or C-18 alkyl-modified silica gel) and a polar mobile phase (a binary system methanol – water with a relatively high water content).[2-4] Linear relationships between the retention factors, and the standard lipophilicity parameter, log*P*, can be expected. Taking into account the same retention mechanism in HPLC and thin-layer chromatography (TLC), in numerous instances the fast, simple and reliable RPTLC method was used for determination of lipophilicity.

Although the mechanism of retention in NP chromatography is not based on partition, but on the adsorption process, governed mainly by hydrogen bond formation, in several published papers [5-9] the obtained results showed that NP retention data can also be used to express lipophilicity for the structurally closely related compounds.

For adsorption from solution as a result of the competition between the solute and an electron donor solvent for the active sites on the adsorbent surface, Soczewinski equation coefficients were employed as a measure of lipophilicity:[5,10]

$$R_M = R_M^0 + m\log\varphi \qquad (1)$$

where φ is the volume fraction of polar component of the mobile phase, *m* is the slope of the linear correlation and the intercept of this equation is an extrapolated value

corresponding to φ = 0% polar modifier in mobile phase. The slope *m*, of the regression line is considered to be related to the specific hydrophobic surface area. If the retention mechanism is uniform within a series of compounds a good relationship between slope and intercept is anticipated.[11] Based on the obtained intercept and slope values, another lipophilic parameter, named C_0, which is computed by dividing the intercept by the slope, can also be used as the lipophilicity measure.[5] The advantage of this parameter is that it includes both the lipophilicity and specific hydrophobic surface area of the solute, and in some cases is more reliable in QSAR studies.

In addition, polar stationary phases can be used for measuring the lipophilicity of polar and charged compounds using a mobile phase containing water and a miscible aprotic solvent, usually acetonitrile. The retention mechanism in this type of normal-phase chromatography, so-called hydrophilic interaction liquid chromatography (HILIC) is very complex and include several different interactions which depend on the experimental conditions.[12,13]

In addition to an assessment of lipophilicity, the retention parameters obtained by NPTLC could be successfully used for prediction of biological activity and some *in-silico* ADME properties of compounds.[14]

Recently, estrogen derived polyoxygenated steroids, were synthesized.[15-18] These compounds possess steroidal backbone which is large and conformationally restricted skeleton. The presence of different functional groups, which can be quite easily

derivatized, made mentioned substances attractive building blocks for numerous applications. The chromatographic behavior of 31 polyoxygenated steroids on both silica gel and RP-18 silica thin layers using non-aqueous and aqueous-organic mobile phases, was the subject of our previous investigations.[19] It was concluded that the retention of the mentioned substances in NPC conditions depend on interactions of their polar functional groups and silanol groups of stationary phase. On the other hand, the influence of particular substance's moiety on the retention behavior of the investigated polyoxygenated steroids under reversed-phase condition was not be drawn due to the complex retention mechanism. Further investigation of chromatographic behavior of polyoxygenated steroids was focused on the detailed quantitative structure-retention relationship (QSRR) study.[20] The obtained results indicated the chromatographic system consisted of C-18 silica gel as stationary and methanol-water as mobile phase, as most suitable for lipophilicity determination of the studied compounds. It was assumed that the retention mechanism of these substances under RP conditions is governed by lipophilicity, steric, and hydrophilic interactions.

Based on the emphasized importance of hydrophilic interaction the aim of this study was to explore the possibility of application of the retention constants of 15 polyoxygenated steroids obtained in NPTLC systems in estimation of their lipophilicity. The obtained retention parameters were correlated with retention constants established under reversed phase conditions as well as with theoretically calculated log*P* values.

EXPERIMENTAL

The investigated compounds (Table 1) were synthesized according to published procedures. [15-18]

The TLC experiments were performed on 10 cm × 10 cm plates. An HPTLC developing chamber (CAMAG, Muttenz, Switzerland) in the tank configuration was used for this purpose. The plates were spotted with 2µl aliquots of freshly prepared solutions (approximately 2 mg/ml) in dichlorometane (DCM). Before development, the spotted plates were equilibrated for 15 min in a chromatographic chamber saturated with the vapor of the mobile phase being used.

The chromatographic systems were consisted of silica gel 60 F_{254} (Art. 5554, Merck, Darmstadt, Germany) with mobile phases: (1) 25-50% (v/v) acetone in *n*-hexane, in steps of 5% (v/v) and (2) 10-30% (v/v) acetonitrile (ACN) in dichloromethane, with increment of 5% (v/v).

Individual zones were detected by spraying the chromatograms with sulfuric acid reagent and heating until the spots became visible.

All experiments were performed at ambient temperature (22 ± 2 °C). All solvents used were of analytical grade purity.

RESULTS AND DISCUSSION

Normal-Phase Chromatography

The amphiphilic steroidal structure with substituents that are differing in their polarity allows good possibilities in separation of the polyoxygenated steroids. The difference in retention is based on the nature of the substituent's polarity attached to the steroidal structure.

Unmodified silica, as a polar adsorbent, was used as a stationary phase in combination with non-aqueous binary solvent systems as non-polar mobile phases containing acetone-*n*-hexane or acetonitrile-dichlormethane. The polarity (*P'*) of mobile phases used was in range of 0.12-0.25 and 0.34-0.38, respectively. Since the polarity of mobile phase acetonitrile-dichlormethane is higher, the investigated compounds showed the stronger retention with mobile phase acetone-*n*-hexane. Typical normal-phase chromatographic behavior was observed, *i.e.* the retention of the compounds increased with increasing amount of the less polar component of the mobile phase.

In all instances, the retention behavior of the studied substances was in accordance with their structural characteristics.

The conformation of benzyl substituent on position C17 of compound **1,** contributed to the stronger polar interactions of this compound with the polar stationary phase in comparison to the contribution of the polar interactions which exhibited butyl-group at the same position in compound **2**.

Introduction of chlorine and bromine at position C4 (compounds **7** and **8**) and keto–group at position C17 led to the stronger retention of these compounds. It could be expected that chlorine as a less voluminous substituent allows easier approach of molecule to the polar surface of stationary phase and make a stronger interactions with silica gel. However, opposite retention behavior was observed. The possible explanation can be based on the values of Hammet substituents constants, σ. Looking at the values of the Hammet constants for a chloro- and bromo-group at *para* position, it can be concluded that these substituents act as an electron acceptor, wherein the electronic parameter of the chloro-group at *para* position (0.227) is lower than for bromine (0.232).[21] Also, compound **9,** because of favorable conformation of chlorine at position 4 to the stationary phase, exhibited stronger retention than compound **7**.

By oxidation of quinol into epoxyquinol (compound **3**), specific interactions of epoxy group with polar stationary phase become stronger allowing the stronger retention of this compound. Within a group of epoxyquinols, compound **6,** which contain hydroxyl group on positions 3, 10 and 17, exhibited the strongest retention because of strong polar interactions with stationary phase. Reducing the polarity by introducing acetoxy-(compound **4**) or butoxy group (compound **5**) on position C17 the interactions with stationary phase become weaker and retention, therefore, decreased. This led to an increased solvation of these compounds by the non-polar molecules of the mobile phase, which weakened their specific interactions with the polar stationary phase.

In system with acetone-*n*-hexane mobile phase compound **14,** which contain acetoxy

groups at positions 1 and 3 and bromine at position 4, exhibited the stronger retention than compound **15** with acetoxy groups on positions 3 and 4 and bromine at position 2. However, with mobile phase containing acetonitrile-dichlormethane, the retention of these compounds was weaker and their retention order was opposite thus pointing to the specific effect of solvent system used.

Determination Of Lipophilicity Parameters

In study of lipophilicity of polyoxygenated steroids in NPC systems, the R_M values were plotted against logφ, where φ is the volume fraction of more polar organic component in the mobile phase. In both chromatographic systems used the linear dependences between retention constant and mobile phase composition were obtained with very high values of correlation coefficients (Tables 2 and 3).

Since the R_M values were plotted against the more polar component of mobile phase, more lipophilic compounds have lower values of lipophilicity parameters, R_M^0, in contrast to RP systems. The lower values of R_M^0 were obtained with acetonitrile-dichloromethane mobile phase than with solvent system acetone-*n*-hexane.

In order to examine whether the R_M^0 values obtained in NP systems can be used for determination of substances' lipophilicity, these parameters were correlated with R_M^0 values from corresponding RP systems described in our previous paper.[19] The obtained correlations were presented in Figures 1 and 2. A very high correlation between mentioned parameters was obtained in systems containing acetone which justifies the

confidence of using R_M^0 values from NP system with acetone, as lipophilic parameters. Taking into account this correlation, it can be stated that some kind of partition is also possible in NP systems, since, during the chromatographic procedure, the substance is distributed between the two phases significantly differing from each other in polarity. However, the regression analysis performed on R_M^0 values obtained in NP and RP systems with acetonitrile was of lower statistical quality and, therefore, is practically unsuitable for determination of lipophilicity of polyoxygenated steroids. Similar unsuitability of acetonitrile as a solvent in the analysis of organic bases using RPHPLC was reported in the literature.[22] The influence of acetonitrile in producing of asymmetrical peaks was explained by its inability to form hydrogen bonds with residual silanols on RP stationary phase. Therefore, during the equilibration, acetonitrile molecules do not associate with the stationary phase forming a monolayer, which provides a hydrogen bonding capability in better agreement with *n*-octanol.

Considering the correlation between different retention factors (Table 4), it can be concluded that a high correlation coefficient was observed only for the relationship between R_M^0 and *m*, in system containing acetone – *n*-hexane as mobile phase. Because of that, the values of slopes could be considered as an alternative lipophilic parameter. Contrary, C_0 values obtained in normal-phase systems are not preferable for measuring of lipophilicity of polyoxygenated steroids , because of their low correlation with R_M^0 values.

The validation of chromatographic parameters as lipophilicity descriptors usually

requires their comparison with the partition coefficients in system *n*-octanol-water. The theoretical values of the partition coefficients were calculated from the molecular structures of compounds by the Internet module ALOGPS 2.1-vcclab.[23][Correlation between experimentally obtained lipophilic parameters, logP_{exp}, and theoretical values, log*P*, can be expressed by Collander-type equations:

$$\log P_{exp} = a_0 + a_1 \log P \qquad (2)$$

where a_0 and a_1 are constants.

The correlation matrix of linear regression analysis between different computational log*P* values and normal-phase retention constants, R_M^0, *m* and C^0, is presented in Table 5. It was found a very poor correlation between retention parameters and theoretically calculated log*P* values when acetonitrile was used as a component of mobile phase. This is in agreement with previously stated fact that solvent system containing acetonitrile is not a suitable solvent for determination of lipophilicity of polyoxygenated steroids based on very low correlation between R_M^0 values in NP and RP systems. In NP system containing acetone a very high coefficients of correlations between *KOWWIN* and values of R_M^0 and *m* were obtained and slightly lower with *C*log*P*, *IA*log*P* and *X*log*P*. Besides these, retention parameter, C^0 exhibited very high correlation with calculated *IA*log*P* values. The mentioned calculated lipophilicity parameters are differing in calculation procedure. The calculation of *KOWWIN* is based on atom/fragment contributions, *C*log*P*

is based on fragmental contributions, *IA*log*P* is based atom-type electrotopological state indices and neural network modeling and *X*log*P* is based on atom contributions [23] Since the chromatographic parameters R_M^0, and *m* obtained in NP chromatographic system containing acetone showed a very high correlation with theoretically calculated log*P* values, it can be concluded that these NP chromatographic parameters are acceptable as measures of lipophilicity of polyoxygenated steroids.

CONCLUSION

In this study the possibility of using normal-phase TLC systems in the evaluation of lipophilicity of 15 polyoxygenated steroids was investigated. A very good linear correlations between R_M values and logarithm of volume percent of more polar component in mobile phase were found. The lipophilicity parameters obtained by NP chromatography were validated in relation to corresponding RP chromatographic data. Very high correlation between R_M^0 values obtained in NP and RP systems containing acetone indicated the confidence of using these NP retention data as lipophilic parameters. Also, the retention parameters R_M^0 and *m*, obtained in NP system containing acetone, showed a high correlations with computer calculated lipophilicity values *KOWWIN*, *C*log*P*, *IAlogP* and *X*log*P*. On the basis of the above mentioned findings it can be concluded that normal phase retention parameters, R_M^0 and *m*, obtained in chromatographic systems containing acetone, can be successfully applied to express lipophilicity of some polyoxygenated steroids.

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Table 1. Structures of the investigated compounds.

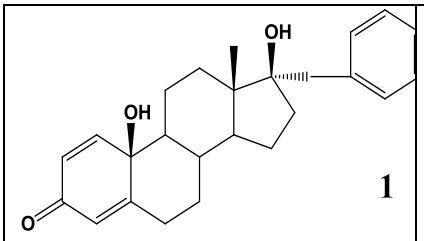
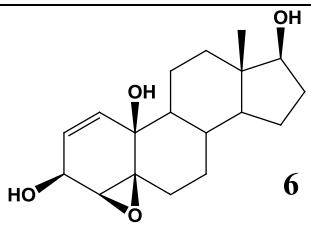
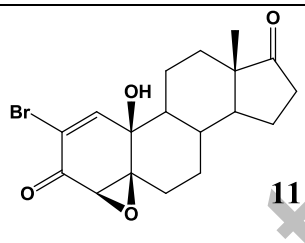
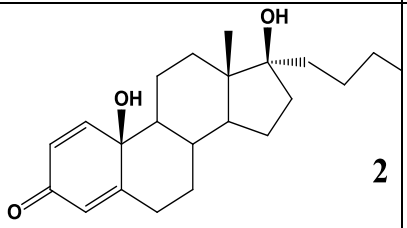
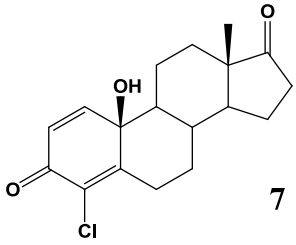
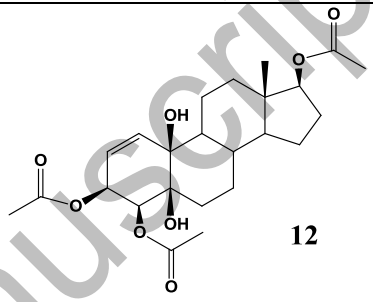
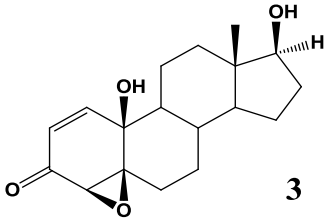
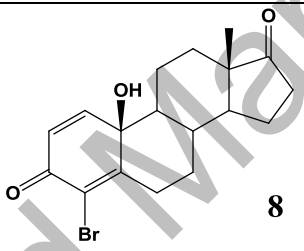
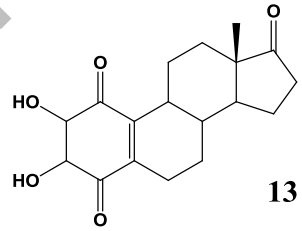
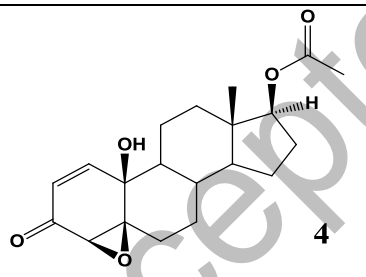
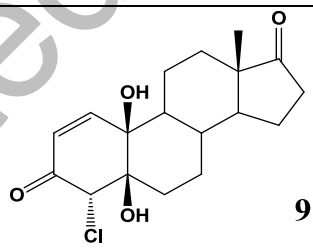
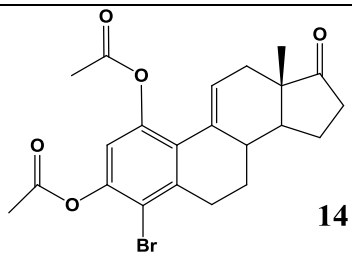
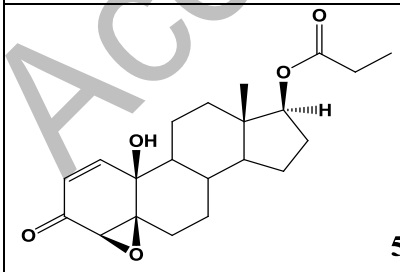
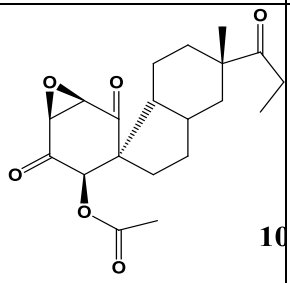
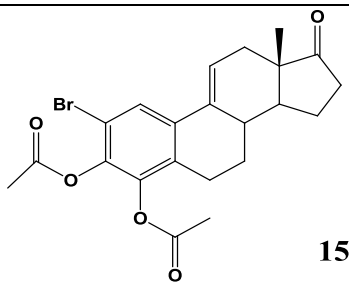
 1	 6	 11
 2	 7	 12
 3	 8	 13
 4	 9	 14
 5	 10	 15

Table 2. Statistical parameters for chromatographic system 1.

Compound	R_M^0	***m***	***r***	C_0	***SD***	***P***	***N***
1	5.040	3.151	0.999	1.599	0.01	<0.0001	6
2	5.068	3.170	0.998	1.599	0.024	<0.0001	6
3	4.884	3.023	0.996	1.616	0.033	<0.0001	6
4	4.325	2.852	0.995	1.516	0.037	<0.0001	6
5	4.278	2.867	0.988	1.492	0.055	<0.0001	6
6	5.082	2.839	0.985	1.790	0.062	<0.0001	6
7	7.063	4.395	0.996	1.607	0.048	<0.0001	6
8	6.901	4.270	0.997	1.616	0.036	<0.0001	6
9	7.943	4.781	0.998	1.661	0.031	<0.0001	6
10	6.405	4.031	0.995	1.589	0.046	<0.0001	6
11	6.595	4.124	0.994	1.599	0.055	<0.0001	6
12	5.870	3.569	0.999	1.645	0.018	<0.0001	6
13	6.605	3.994	0.994	1.654	0.055	<0.0001	6
14	4.510	3.000	0.994	1.503	0.042	<0.0001	6
15	4.676	3.136	0.991	1.491	0.054	<0.0001	6

Table 3. Statistical parameters for chromatographic system 2.

Compound	R_M^0	***m***	***r***	C_0	***SD***	***P***	***N***
1	1.709	1.646	0.996	1.038	0.029	0.0002	5
2	1.825	1.687	0.993	1.082	0.043	0.0007	5
3	2.199	1.479	0.994	1.487	0.034	0.0005	5
4	1.755	1.849	0.997	0.949	0.028	0.0001	5
5	1.469	1.723	0.996	0.853	0.033	0.0003	5
6	1.907	0.847	0.93	2.251	0.072	0.0221	5
7	2.552	2.035	0.992	1.254	0.056	0.0008	5
8	2.388	1.961	0.992	1.218	0.054	0.0008	5
9	2.662	1.877	0.997	1.418	0.031	0.0002	5
10	1.638	1.735	0.993	0.944	0.043	0.0006	5
11	1.539	1.588	0.995	0.969	0.034	0.0004	5
12	3.155	2.938	0.987	1.074	0.101	0.0017	5
13	2.265	1.949	0.993	1.162	0.049	0.0006	5
14	1.182	1.904	0.979	0.621	0.085	0.0034	5
15	1.328	1.867	0.979	0.711	0.085	0.0036	5

Table 4. Statistical parameters for correlation between different retention factors.

Mobile phase	**Equation**	**Statistical parameters**
Acetone - *n*-hexane	R_M^0 = -0.454(±0.343) + 1.730(±0.095)m	r = 0.981, SE = 0.233, P<0.0001
	R_M^0 = -5.188(±5.846) + 6.800(±3.653)C_0	r = 0.458, SE = 1.066, P = 0.0854
ACN - DCM	R_M^0 = 0.749(±0.569) + 0.677(±0.307)m	r = 0.521, SE = 0.488, P = 0.0462
	R_M^0 = 1.287(±0.426) + 0.603(±0.356)C_0	r = 0.424, SE = 0.518, P = 0.1148

Table 5. The correlation matrix between R_M^0, m and C_0 and computer calculated log*P* values.

	Acetone – *n*-hexane			**ACN - DCM**		
	R_M^0	m	C_0	R_M^0	m	C_0
AlogP*	-0.529	-0.489	-0.345	-0.613	-0.313	-0.210
IAlogP*	**-0.818**	**-0.727**	**-0.718**	-0.657	-0.175	-0.422
AB/logP*	-0.598	-0.538	-0.476	-0.531	-0.028	-0.398
QlogP*	-0.408	-0.316	-0.574	-0.367	0.266	-0.584
Cosmofrag*	-0.466	-0.414	-0.389	-0.343	0.177	-0.443
milogP1	-0.717	-0.730	-0.203	-0.473	-0.384	0.069
KOWWIN*	**-0.961**	**-0.946**	-0.406	-0.659	-0.410	-0.123
XlogP*	**-0.743**	**-0.767**	-0.150	-0.393	-0.344	0.093
ClogP**	**-0.851**	**-0.838**	-0.370	-0.457	-0.156	-0.210
logP**	-0.317	-0.214	-0.574	-0.535	0.040	-0.555

Figure 1. Correlation between R_M^0 values obtained in NP and RP chromatographic systems containing acetone.

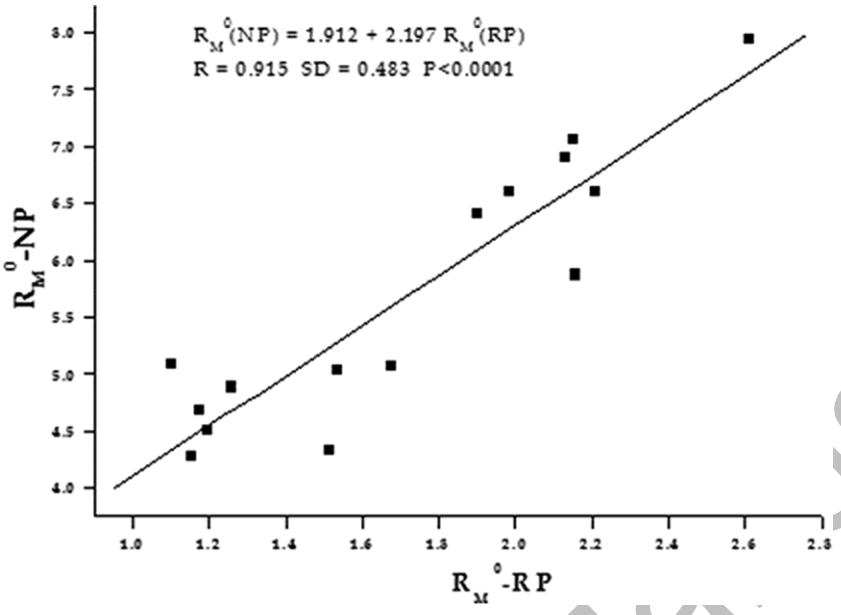


Figure 2. Correlation between R_M^0 values obtained in NP and RP chromatographic systems containing acetonitrile.