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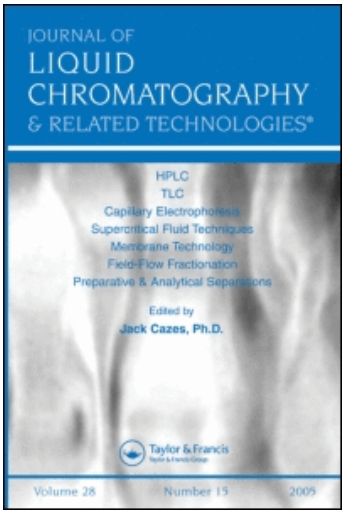

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Anđelija, Malenović , Darko, Ivanović , Biljana, Stojanović-Jančić and Mirjana, Medenica(2009) 'Robustness Testing of Microemulsion Liquid Chromatographic Separation of Simvastatin and its Impurities', Journal of Liquid Chromatography & Related Technologies, 32: 6, 874 — 889
To link to this Article: DOI: 10.1080/10826070902768161
URL: http://dx.doi.org/10.1080/10826070902768161

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Journal of Liquid Chromatography & Related Technologies®, 32: 874–889, 2009
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ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902768161

Robustness Testing of Microemulsion Liquid Chromatographic Separation of Simvastatin and its Impurities

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Abstract: Liquid chromatography employing the microemulsion as eluent was applied for the analysis of simvastatin and its six impurities. Previously, the optimized and validated method was tested to prove the method's capability to perform during the small changes of important parameters. In this paper, influence of parameters tipical for microemulsion liquid chromatography (MELC) on the method's robustness was presented.

For the results evaluation, and presentation, two statistical methods were applied. In order to designate the most convinient one, multiple regression (MR) and artificial neural networks (ANN) were applied and compared. A set of experiments was defined by the central composite design (CCD). Indepedent variables (SDS, *n*-buthanol and diisopropyl ether content) and dependent variables (retention factors of investigated substances) were the framework for both, MR and ANN. This study demonstrated that MR and radial basis function ANN are useful tools in understanding the effects of the investigated factors on the chromatographic system and definition of the robustness limits.

Keywords: Artificial neural networks, MELC, Multiple regression, Robustness testing, Simvastatin

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INTRODUCTION

Microemulsion liquid chromatography (MELC) is a relatively new chromatographic technique, which utilizes microemulsion as a mobile phase and has been shown to be suitable for the separation of a range of pharmaceutical compounds using both isocratic and gradient elution modes.[1,2] Validation and application of MELC for drug analysis can be found in some papers.[3–11] In this paper, testing of MELC method robustness, especially the influence of mobile phase constituents on method's robustness, was evaluated. As defined by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters.[12]

The robustness testing can be conducted using different kinds of experimenatal design, e.g., central composition design (CCD)[13] Plackett–Burman design,[14] full and fractional factorial design,[15] etc. Some papers gave detail illustration of experimental design application during the robustness/ruggidness testing.[16–18] On the other hand, in many papers, the combination of appropriate experimental design and neural networks was used in various phases of method development.[19–24] It is important to know that experimental design and artificial neural networks may be used separately or in a combination supporting one another. For that reason, this paper presents comparison of experimental data and predicted data derived from multiple regression (MR), e.g., second order polynomials, and predicted data from artificial neural networks (ANN) in an aim to examine robustness of the previously validated MELC method for separation and quantitation of simvastatin and its six impurities.[6]

For the analysis of simvastatin in various pharmaceutical dosage forms, the application of HPLC,[25–28] derivative spectrophotometry,[29,30] and MEKC[31] was reported. Also, a review of HPLC methods for the determination of simvastatin and atorvastatin in bioanalytical assays, pharmaceutical assays, and environmental applications was published.[32]

THEORY

Central Composite Design

The CCD consists of a full factorial 2^k design to which a star design is added. The CCD is completed by addition of a certain number of replications in the center point. For three factors to be considered, at least $8 + 6 + 1 = 15$ experiments are necessary.[33]

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Multiple Regression

The general purpose of multiple regression is to set the relationship between several independent or predictor variables and a dependent or criterion variable. Mathematical correlation could be presented as second order polynomial.

After the planning stage, when the set of experiments are done according to a statistical design, each experiment exhibited a result, i.e., value of the response variable, these data are analysed by means of multiple regression. This gives a model relating the factors to the results, showing which factors are important, and how they combine in influencing the results. As the model will be used to make predictions, e.g., how to set the factors to achieve desired (optimal) results, it was reviewed by analysis of variance (ANOVA).

ANOVA breaks up sums of squares in components and compares their size with a F-test. The desired result is that the model selected does not show significant lack of fit (i.e., the F test will be insignificant, $F_{tab} > F$). Lack of fit compares pure error (from replicated experiments) with residual error (model error). If residual error significantly exceeds pure error, the model will show significant lack of fit, and another model may be more appropriate.

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Artificial Neural Networks

An artificial neural network (ANN) is a data processing system consisting of a large number of simple, highly interconnected precessing elements in an architecture inspired by the structure of the brain. Radial basis function neural network (RBFNN) is a type of neural network used to solve several problems, such as modeling and classification. The RBFNN consists of three layers: input layer, hidden layer, and output layer. The input layer does not process the information. It only distributes the input vectors to the hidden layer. The hidden layer of RBFNN consists of a number of RBF units (n_h). Each neuron in the hidden layer employs a radial basis function as non-linear transfer function to operate on the input data. In general, there are several radial basis functions: linear, cubic, thin plate spline (TPS), Gaussian, multi-quadratic, and inverse multi-quadratic. The overall performance of RBFNN is evaluated in terms of root mean squared (RMS) error according to the equation:

$$rms = \sqrt{\frac{\sum_{i-1}^{n_s} (y_k - \hat{y}_k)^2}{n_s}}$$

where y_k is the desired output and $\hat{y}_k$ the actual output of the network, n_s the number of compounds in analyzed set.[34]

EXPERIMENTAL

Chemicals

All reagents used were of an analytical grade. Sodium dodecyl sulphate (SDS), was obtained from Sigma (St. Louis, MO, USA). Diisoprophyl ether and *n*-butanol – HPLC grade were manufactured by Riedel-deHäen (Sleeze, Germany). Water – HPLC grade, *di*-sodium hydrogen phosphate, J. T. Baker (Deventer, Netherlands) and orthophosphoric acid, Carlo Erba (Milan, Italy) were used to prepare a water phase. The substances used, simvastatin and its impurities, were of Ph. Eur. quality.

Chromatographic Conditions

The chromatographic system Waters Breeze consisted of Waters 1525 Binary HPLC Pump, Waters 2487 UV/VIS detector, and Breeze

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Table 1. Plan of experiments for CCD

		Factors		
	No	x_1	x_2	x_3
Full factorial design	1	1.0	8.0	3.0
	2	0.8	8.0	3.0
	3	1.0	6.0	3.0
	4	0.8	6.0	3.0
	5	1.0	8.0	1.5
	6	0.8	8.0	1.5
	7	1.0	6.0	1.5
	8	0.8	6.0	1.5
Star design	9	1.0	7.0	2.2
	10	0.8	7.0	2.2
	11	0.9	8.0	2.2
	12	0.9	6.0	2.2
	13	0.9	7.0	3.0
	14	0.9	7.0	1.5
Replications	15	0.9	7.0	2.2
	16	0.9	7.0	2.2
	17	0.9	7.0	2.2
	18	0.9	7.0	2.2

x_1 – content of diizopropylether (% w/v); x_2 – content of *n*-butanol (% w/v); x_3 – content of SDS (% w/v).

Software, Windows XP, for data collection. Separations were performed on the X-Terra™ 4.6 mm × 50 mm, 3.5 μm particle size column with UV detection at 238 nm. The flow rate was 0.3 mL min^{-1} and column temperature was set at 40°C. The Laboratory mixture was prepared of 0.5 mg mL^{-1} of simvastatin, 5 μg mL^{-1} of lovastatin, 2.5 μg mL^{-1} of hydroxy acid, 2.5 μg mL^{-1} of methyl–simvastatin, 2.5 μg mL^{-1} of acetate ester, 2.5 μg mL^{-1} of anhydro-simvastatin, and 2.5 μg mL^{-1} of simvastatin dimer in mobile phase. The samples were introduced through a Rheodyne injector valve with a 20 μL sample loop.

Mobile phases were prepared according to the plan of experiments presented in Table 1, by mixing all the microemulsion components and treating them with an ultrasonic bath for 30 min. The resulting transparent microemulsion was filtered through a 0.45 μm membrane filter, Alltech (Loceren, Belgium).

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RESULTS AND DISCUSSION

Robustness testing identifies the factors in the method, which have a significant effect on its results and anticipate the problems which may arise during its application. It allows limits to be set for all method parameters and, where they are very narrow, to underline in the protocol the limits permitted. The following steps can be identified in a robustness test: (a) identification of factors to be tested; (b) definition of different levels for the factors; (c) selection of the experimental design; (d) definition of experimental protocol (complete experimental set up); (e) definition of response to be determined; (f) execution of the experiments and determination of responses of the method; (g) calculation of effects; (h) statistical and/or graphical analysis of effects, and (i) drawing chemically relevant conclusions from statistical analysis and, if necessary, taking measures to improve the performance of the method.[16]

During the method development,[4] factors important for separation with microemulsion eluent were identified. Furthermore, the method was validated and applied for in the analysis of tablets.[5] Validation was done and the chromatographic conditions were as follows: column X-Terra™ 4.6 mm × 50 mm, 3.5 μm particle size, UV detection at 238 nm, the flow rate was 0.3 mL min^{-1}, column temperature was 40°C, and mobile phase consited of 0.9% w/v of diisopropyl ether, 1.7% w/v of SDS, 7.0% w/v of *n*-buthanol, and 90.4% w/v of aqueous 25 mM *di*-sodium hydrogenphosphate pH 7.0, adjusted with *ortho*-phosphoric acid. Hence, MELC presents the recently indroduced analytical method; the objective of our investigation was primary to evaluate the influence of mobile phase constituents on method robustness. Meaning that the other factors (column type and manufacturer, flow rate, temperature, wavelength,

pH of the mobile phase, buffer concentration) were kept on optimal level, while diisopropyl ether, SDS, and *n*-buthanol content were tested.

Then, definition of different levels for the factors were done. According to adequate planes of experiments (Table 1), the retention factor (k) values of analyzed substances were gathered and presented in Table 2.

Mathematical modeling using MR enabled derivation of second order polynomials. Results for the coefficients of second order polynomials are presented in Table 3.

On the basis of obtained second order polynomials, predicted values of outputs (retention factors) are calculated and presented in Table 4.

The ANOVA for the test of adequacy of the model is shown in Table 5.

Calculated values of SS_{lof} and statistical parameter F (lower then tabelar value – F_{tab}) showed that the obtained mathematical model is suitable for explaining the experimental results.

Experimentaly obtained k values were also tested using artificial neural networks. Radial basis function (RBFNN) gave the best performance of

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Table 2. Experimentaly obtained response factor values

	Responses						
No	k_1	k_2	k_3	k_4	k_5	k_6	k_7
1	0.668	2.519	2.796	3.625	4.098	4.944	9.300
2	0.950	2.975	3.168	3.950	4.794	5.719	11.300
3	1.180	3.768	4.315	5.344	6.314	7.597	22.438
4	1.396	4.525	5.256	6.469	7.766	8.731	28.725
5	0.981	3.288	3.600	4.250	5.106	6.350	11.894
6	1.188	4.181	4.656	5.075	5.956	6.463	15.825
7	1.544	5.869	6.694	8.150	8.781	10.113	26.118
8	1.819	6.513	7.480	9.356	11.275	13.625	23.980
9	1.075	3.631	4.100	5.313	6.081	7.450	18.750
10	1.050	3.550	4.000	5.130	6.210	7.230	18.830
11	1.025	3.413	3.825	4.850	5.475	6.569	12.740
12	1.280	4.820	5.570	6.890	8.530	10.270	25.560
13	1.000	3.430	3.930	4.600	5.880	6.970	9.310
14	1.360	5.140	5.810	7.090	8.790	10.500	13.730
15	1.140	4.500	5.170	6.330	7.880	9.200	12.860
16	1.140	4.520	5.210	6.370	7.940	9.250	12.960
17	1.150	4.570	5.260	6.360	8.020	9.310	13.000
18	1.160	4.620	5.310	6.430	8.120	9.380	13.240

k_1 – retention factor of hydroxy acid; k_2 – retention factor of lovastatin; k_3 – retention factor of simvastatin; k_4 – retention factor of methyl simvastatin; k_5 – retention factor of acetete ester; k_6 – retention factor of anhydro simvastatin; k_7 – retention factor of simvastaitin dimer.

Table 3. Calculated coefficients of second order polynomials

	Simvastatin	Hydroxy acid	Lovastatin	Methyl–simvastatin	Acetate ester	Anhydro simvastatin	Simvastatin dimer
b_0	4.932	1.126	4.301	6.070	7.560	8.901	13.916
b_1	−0.306	−0.093	−0.264	−0.33	−0.562	−0.531	−1.016
b_2	−1.127	−0.238	−0.915	−1.446	−1.724	−2.029	−6.576
b_3	−0.878	−0.173	−0.780	−0.993	−1.106	−1.309	−1.047
b_{12}	0.037	−0.003	0.003	0.148	0.300	0.470	−0.223
b_{13}	0.066	0.002	0.037	0.073	0.150	0.215	−0.812
b_{23}	0.289	0.033	0.268	0.493	0.476	0.658	−1.023
b_{123}	0.105	−0.020	0.072	0.052	−0.111	−0.380	1.295
b_{11}	−0.577	−0.042	−0.459	−0.547	−0.984	−1.176	3.972
b_{22}	0.071	0.048	0.067	0.102	−0.984	−0.097	4.332
b_{33}	0.243	0.075	0.236	0.077	0.205	0.219	−3.298

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the system. For the network training K–Means, K–Nearest Neighbour and Pseudo–Invert (Linear Squares Optimization) were employed. Obtained values for root mean square (RMS) of the errors were 0.1876 for training set, 0.1744 for the verification set and 0.4125 for the testing set. Graphical presentation of neural network is given in Figure 1.

Table 4. Response factor values predicted using second order polynomials

	Responses						
No	k_1	k_2	k_3	k_4	k_5	k_6	k_7
1	0.715	2.566	2.856	3.698	4.076	4.938	9.520
2	0.944	2.870	3.051	3.812	4.523	5.393	11.032
3	1.172	3.710	4.248	5.204	6.194	7.502	22.575
4	1.308	4.314	5.012	6.118	7.397	8.315	28.374
5	1.032	3.373	3.692	4.449	5.259	6.572	12.695
6	1.186	4.113	4.570	5.063	5.860	6.365	16.138
7	1.539	5.875	6.659	8.136	8.836	10.246	26.836
8	1.762	6.340	7.267	9.131	11.08	13.437	24.210
9	0.991	3.578	4.050	5.193	6.013	7.193	16.872
10	1.177	4.106	4.661	5.853	7.137	8.255	18.904
11	0.936	3.454	3.876	4.726	5.708	6.774	11.672
12	1.412	5.283	6.130	7.618	9.156	10.830	24.824
13	1.029	3.757	4.298	5.153	6.659	7.810	9.571
14	1.374	5.317	6.053	7.140	8.870	10.424	11.666
15	1.126	4.301	4.932	6.070	7.559	8.900	13.916
16	1.126	4.301	4.932	6.070	7.559	8.900	13.916
17	1.126	4.301	4.932	6.070	7.559	8.900	13.916
18	1.126	4.301	4.932	6.070	7.559	8.900	13.916

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Table 5. ANOVA table for the test of adequacy of the model

	Simvastatin	Hydroxy acid	Lovastatin	Methyl–simvastatin	Acetate ester	Anhydro simvastatin	Simvastatin dimer
SS_{pe}	0.000086	0.00012	0.00056	0.00021	0.00011	0.00094	0.0780
SS_t	436.90	25.75	337.11	656.33	951.24	1321.20	566.64
SS_{mean}	438.24	26.03	337.37	656.86	951.69	1322.30	566.99
SS_{corr}	0.0106	0.0101	0.0002	0.0003	0.0002	0.0009	0.0042
SS_{fact}	1.359	0.069	1.038	2.004	2.994	3.214	14.372
SS_r	1.478	0.063	1.031	2.019	2.996	3.198	14.439
SS_{lof}	1.475	0.0619	1.035	2.004	2.989	3.192	14.357
F	0.846	1.201	1.014	0.985	0.999	1.010	0.991

SS_{pe} – Sum of squares due to purely experimental uncertainty.
SS_t – Total sum of squares.
SS_{mean} – Sum of squares due to the mean.
SS_{corr} – Sum of squares corrected to the mean (corrected sum of squares).
SS_{fact} – Sum of squares due to the factors (due to regression).
SS_r – Sum of squares of residuals.
SS_{lof} – Sum of squares due to lack of fit.
F_{tab} – 4.494 ($p = 0.05$).

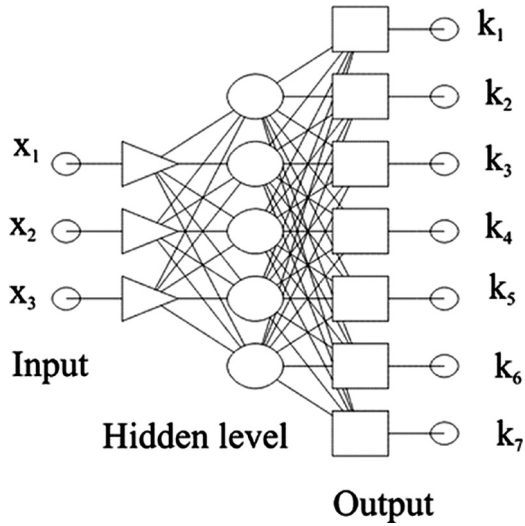

Figure 1. Graphical presentation of neural network (x_1 – content of diizopropylether (% w/v), x_2 – content of *n*-butanol (% w/v), x_3 – content of SDS (% w/v); k_1 – retention factor of hydroxy acid; k_2 – retention factor of lovastatin; k_3 – retention factor of simvastatin; k_4 – retention factor of methyl simvastatin; k_5 – retention factor of acetete ester; k_6 – retention factor of anhydro simvastatin; k_7 – retention factor of simvastaitin dimer).

Table 6. Responses factor values predicted using ANN

	Responses						
No	k_1	k_2	k_3	k_4	k_5	k_6	k_7
1	0.684	2.477	2.754	3.530	4.089	4.978	8.337
2	0.926	2.803	3.028	3.497	4.232	4.722	11.797
3	1.196	4.130	4.727	5.894	6.624	7.853	23.314
4	1.431	4.648	5.354	6.676	8.151	9.603	26.095
5	1.096	4.182	4.643	5.547	6.156	7.179	12.681
6	1.296	4.665	5.166	6.019	7.028	8.014	14.211
7	1.484	5.394	6.137	7.423	8.243	9.618	25.720
8	1.695	6.052	6.973	8.600	10.330	12.191	27.169
9	1.037	4.094	4.671	5.717	6.755	8.0233	13.616
10	1.297	4.694	5.360	6.461	8.219	9.599	15.902
11	0.899	3.494	3.890	4.598	5.732	6.674	6.378
12	1.449	5.359	6.232	7.726	9.467	11.245	23.547
13	0.931	3.245	3.695	4.541	5.779	6.816	12.753
14	1.340	5.246	5.969	7.142	8.638	10.103	15.268
15	1.121	4.405	5.054	6.115	7.780	9.174	12.015
16	1.121	4.405	5.054	6.115	7.780	9.174	12.015
17	1.121	4.405	5.055	6.115	7.780	9.174	12.015
18	1.121	4.405	5.055	6.115	7.780	9.174	12.015

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Table 7. Regression statistics for ANN

	Simvastin acid		Lovastatin		Simvastatin		Methyl simvastatin		Acetyl simvastatin		Anhidro simvastatin		Simvastatin dimer	
	Tr.	Vr.	Tr.	Vr.	Tr.	Vr.	Tr.	Vr.	Tr.	Vr.	Tr.	Vr.	Tr.	Vr.
Data Mean	1.22	1.11	4.40	4.18	5.01	4.77	6.11	5.87	7.35	7.16	8.62	8.38	17.69	12.95
Data S.D.	0.29	0.07	1.14	0.67	1.35	0.82	1.69	0.88	2.00	1.46	2.43	1.57	7.15	0.26
Error S.D.	0.08	0.06	0.33	0.15	0.38	0.16	0.55	0.05	0.59	0.30	0.83	0.16	1.95	3.08
Abs E. Mean	0.06	0.06	0.27	0.13	0.31	0.15	0.43	0.26	0.45	0.23	0.61	0.11	1.59	2.81
S.D. Ratio	0.27	0.77	0.29	0.22	0.28	0.19	0.33	0.06	0.29	0.21	0.34	0.10	0.27	11.81
Correlation	0.962	0.994	0.967	0.994	0.963	0.996	0.956	0.995	0.955	0.997	0.939	0.998	0.962	0.686

Tr: Training; Vr: Varification.

Values predicted by the ANN are given in Table 6.

Regression statistics for all variables are given in Table 7.

In order to compare two suggested methods, correlations among experimental data and predicted values for output obtained from second order polynomials and from ANN were calculated and presented in Table 8.

Calculated correlation coefficients indicate good correlation among experimental and obtained data. It can be concluded that both mathematical presentations of the results are satisfactory. Some advantages could be recognized in the ANN method, e.g., it can be used for linear and non linear systems; it can handle more input and output variables then MLR and it can work with theoretical and hystorical data for better prediction.

After the statistical evaluation of effects, graphical analysis was also done in order to facilitate the drawing relevant conclusions stage. As the easiest way for graphical presentation of the results, a three–D graph as the graphical method was chosen enabling vizualization of the analyzed system. Three–D graphs were constructed as k (investigated substance) = f (% SDS, % butanol), because diizopropylether was the factor with the lowest influence on the chromatographic behavior of the investigated substances. The resulting graphs are presented in Figure 2 for: (a) hydroxy acid, (b) lovastatin, (c) simvastatin, (d) methyl simvastatin, (e) acetete ester, (f) anhydro simvastatin, and (g) simvastatin dimer.

Thorough examination of the obtained three–D graphs showed that hydroxy acid and lovastatin are not as affected by the changes of mobile phase composition, so the final conclusion of the proposed method robustness should not be made according to those components chromatographic behavior.

Retention behavior of simvastatin, methyl simvastatin, acetete ester, and anhydro simvastatin proved to be similar because of the small differences in their structure. Those structural differences lead to polarity differences and enabled separation of those compounds with fine tuning of mobile phase composition. At the same time, similarity in structures facilitated precise setting of limits during robustness testing.

However, chromatographic behavior of the simvastatin dimer should be closely examined before making a final conclusion. As the most

Table 8. Correlations among experimental data and predicted values for output

		k_1	k_2	k_3	k_4	k_5	k_6	k_7
r	MR	0.9680	0.9698	0.9066	0.9724	0.8722	0.9788	0.9843
	ANN	0.9243	0.8989	0.9024	0.9017	0.9148	0.9068	0.9212

r – coefficient of correlation.

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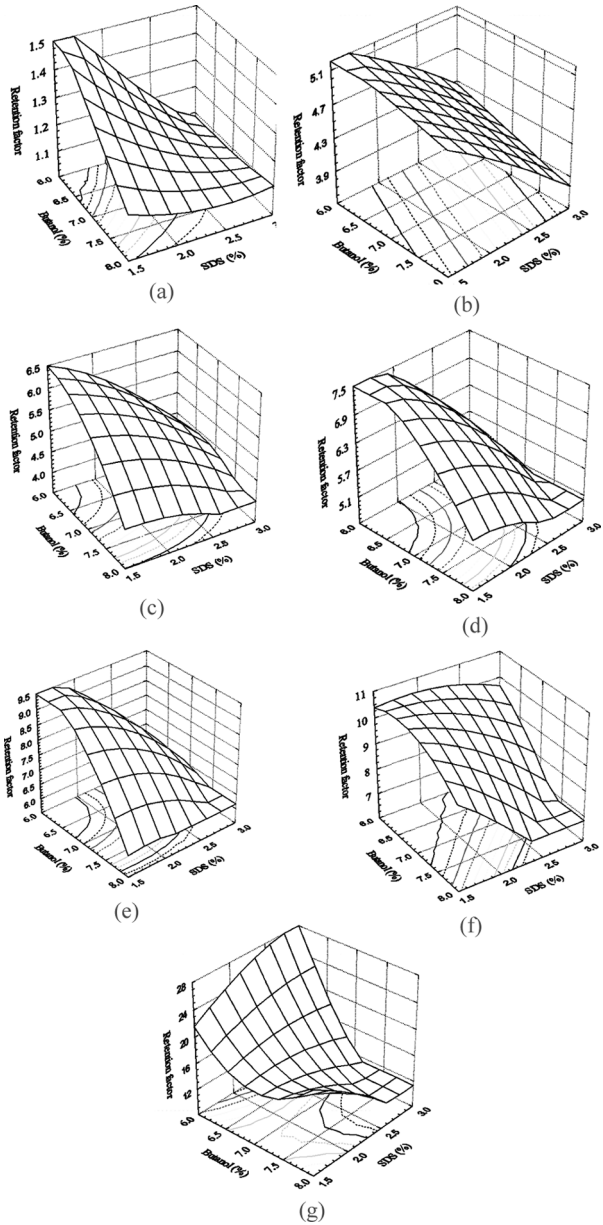

Figure 2. Three–D graph: (a) k (hydroxy acid) = f (SDS, butanol); (b) k (lovastatin) = f (SDS, butanol); (c) k (simvastatin) = f (SDS, butanol); (d) k (methyl simvastatin) = f (SDS, butanol); (e) k (acetete ester) = f (SDS, butanol); (f) k (anhydro simvastatin) = f (SDS, butanol); (g) k (simvastatin dimer) = f (SDS, butanol).

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hydrophobic compound, simvastatin dimer was the most affected by the changes in mobile phase polarity. The shape of the three–D graph presented in Figure 2g. showed that this compound is the most affected by the small changes in mobile phase composition.

As can be seen from the graphical representation of performed robustness testing, if the content of *n*-butanol is from 7.0% w/v to 7.5% w/v and content of SDS from 1.5% w/v to 2.5% w/v the proposed method will be robust. In microemulsion, the LC content of surfactant and cosurfactant, as selectivity modifiers, should be strictly controled in order to obtain valid results. The complexity of the presented mixture of seven components is the best example, because for the simpler mixtures predictions will be easier to make.

CONCLUSIONS

In this paper for statistical analysis of effects in robustness testing of MELC, proposed for simvastatin and its six impurities separation, MR and RBFNN were employed. Input data for both methods were results for retention factors obtained from the set of experiments defined by the central composite design. Whereas both methods have the same starting point and the final graphical representation as three–D graphs, method adequacy was established by statistical evaluation. One method is complimentary to the other with each having its own advantages. This approach gave thorough information about the investigated system, so the proper analysis enabled exact prediction of every change. In that way, behavior of the investigated system with the small changes in method conditions was defined. In microemulsion liquid chromatographic analysis of simvastatin and its six impurities, the content of surfactant and cosurfactant should be strictly controled, so the content of *n*–butanol should be from 7.0% w/v to 7.5% w/v and content of SDS from 1.5% w/v to 2.5% w/v in order to maintain method performance.

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ACKNOWLEDGMENT

The authors thank the Ministry of Science of Republic of Serbia for supporting these investigations in Project 142077 G.

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Received September 28, 2008
Accepted October 27, 2008
Manuscript 6416

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