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# Effect of monovalent cations on the binding of amino acids to cholesterol

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The nature of the interaction between amino acids and cholesterol was shown by reversed-phase thin-layer chromatography (RP-TLC) in the presence of monovalent cations of different concentration. The degree of interaction between amino acids and cholesterol was affected by different salt solutions and by applying cholesterol to the chromatographic plates at higher concentration. The relative strength of interaction was determined by statistical evaluation of the results in each case. The objectives of the investigations were to study the retention behaviour of biologically active amino acids on alumina supports impregnated with cholesterol and to characterize the effects of monovalent cations on the interaction between amino acids and cholesterol.

## 1. Introduction

Cholesterol and its derivatives constitute an important class of membrane lipids, the steroids. Cholesterol is the major steroid constituent of animal tissues; other steroids play more important roles in plants. One important effect of sterol inclusion is an increase in membrane permeability to non-polar solutes. The presence of the steroids moves the polar heads further apart, giving greater access of non-polar substances to the lipid layers, while these materials also tend to be soluble in the steroids themselves [1–3].

A rapid and reliable LC-MS-MS method for the quantification of underivatized amino acids (such as glutamic acid, glutamine and other relevant amino acids) in exocellular media has been established. The separation of seven underivatized amino acids was achieved on a reversed-phase C-18 column with pentadecafluorooctanoic acid as a volatile ion-pair reagent. The technique seemed to be selective and sensitive and shows potential for the high-throughput quantification of amino acids from biological matrices [4].

Several papers have focused on enantio- and diastereoseparation of amino acids by HPLC techniques using either chiral stationary phases or chiral selectors [5–7] and by capillary electrophoresis [8–10].

Modern thin layer chromatography (TLC) for separation of amino acids and their various derivatives using the impregnation technique is also reported to be one of the most appropriate methods for the simultaneous determination of amino acids. Procedures for impregnating thin layer material with a variety of reagents for resolving compounds have been extensively discussed [11–13]. Reversed-phase thin-layer chromatography (RP-TLC) has become of significant importance in lipophilic and chromatographic characterization of analytes over the last few decades [14–18].

Large data matrices consisting of a great number of retention data determined under various chromatographic conditions cannot be evaluated by the traditional linear regression model nor can the information content of the matrix be assessed in detail. Numerous multivariate mathematical-statistical methods have been developed for the extraction of maximal information from complicated data matrices. These methods have also been successfully applied in chromatography for the evaluation of retention data and for quantitative-structure-retention relationship (QSSR) studies. Partial last square (PLS) modeling has been used in GC for the study of the retention behaviour of oxo compounds [19] and in HPLC for the QSSR of chalcones [20]. Canonical variate analysis has found application in pyrolysis GC-MS [21] and canonical correlation analysis for the QSSR study of nonionic surfactants in RP-TLC [22]. Cluster analysis has been employed for the classification of organic modifiers in RP-TLC [23]. Principal component analysis (PCA), a versatile and easy-to-use multivariate method, has been extensively applied in chromatography. Thus, it has been used for the evaluation of lipophilicity in RP-TLC [24], for QSSR studies in GC [25], for the characterization of hydrophobic interaction and hydrophobic interaction chromatographic media [26], etc. Each multivariate method listed above classifies the retention data according to both the strength and selectivity of the retention which is not possible when separation of the strength and selectivity of the effect is required. Spectral mapping technique (SPM), another multivariate mathematical-statistical method overcomes this difficulty [27]. The method divides the information into two matrices using the logarithm of the original data. The first one is a vector containing the potency values related to the overall effect. The second matrix (selectivity map) contains the information concerning the spectra of activity (the qualitative characteristics of the effect) [28]. SPM first calculates the logarithm of the members of the original data matrix facilitating the evaluation of the final plots in terms of log ratios. Then, SPM subtracts the corresponding column-mean and row-mean from each logarithmic element of the matrix calculating potency values. The source of variation remaining

in the centered data set can be evaluated graphically (selectivity map). SPM has been employed previously for elucidation of the relationship between the chemical structure and fungicidal activity of nonionic surfactants [29], for study of the inhibitory effect of surfactants on sunflower downy mildew [30], and for characterization of stationary phases in HPLC [31]. As the matrices of the selectivity matrices of SPM are generally multidimensional they cannot be evaluated by visual methods.

Varimax rotation, nonlinear mapping technique and cluster analysis were developed for reduction of the dimension of such matrices [32]. These techniques are theoretically similar as each method calculates and visualizes the relative distances between the members of data matrices. Unfortunately, significance tests cannot be applied to the results of SPM, making the reliability of the conclusions drawn from the results questionable. To our knowledge only one graphical approximation has been made to include standard deviation on the SPM of chromatographic retention data [33].

## 2. Investigations, results and discussion

During the experiments we investigated cholesterol migration on silica (Si) and alumina (Alu) supports using different solvents.

Table 1 shows $R_f$ values of cholesterol for different solvents and stationary phases.

The effect of the applied salt is to increase the polarity of the solvent mixture thereby promoting the migration of the hydrophil compounds on the plates, while at the same time their adhesion to the cholesterol covered aluminium-oxide plate is weakened. We have concluded that the hydrophilic character of the amino acids influenced the type of the TLC running and rapid migration could be achieved by using saline solution.

With an acetone/chloroform 1 : 1 system we were able to develop 5% cholesterol solution covered Alu plates and 15 amino acids to be analysed could be dropped onto the Alu plates. We had to ignore two amino acids (Pro, Oxy-Pro) from the series as they could hardly be detected using the ninhydrin reaction. Our results are summarized in Table 2. The mobile phases used were KCl, LiCl, CsCl, NaCl, RbCl of different concentration and distilled water. The statistical evaluation of our measurements shows that a linear relationship exists between results obtained by using 1.5% cholesterol and 5% cholesterol impregnated Alu plates, respectively. In Fig. 1 the mobile phase was water and in Fig. 2 0.16 M sodium chloride solution was applied as eluent.

In the case of 5% cholesterol solution covered Alu plates the aqueous mobile phase was hardly migrating on the impregnated Alu surface but the running was complete within 8 hours except for 5 M LiCl solution. Using salt solution (5 M LiCl) of the highest concentration the position of spots can hardly be estimated (running time is too long) due to the extremely large lateral diffusion but as a result of a qualitative evaluation we can conclude that

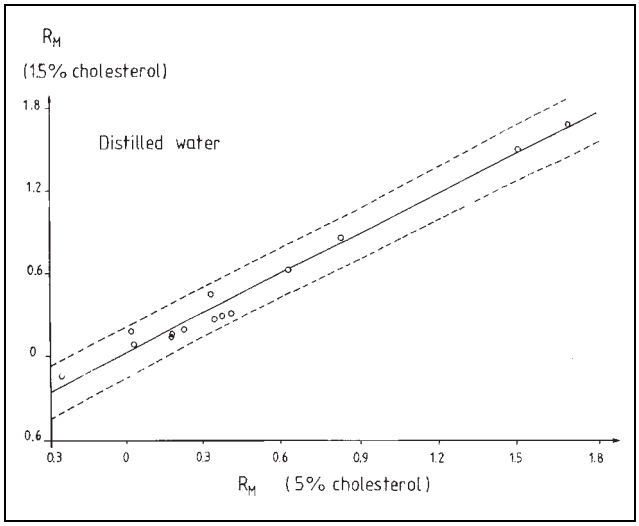


Fig. 1: Statistical estimation of the effect of cholesterol coverage on the interaction between cholesterol and amino acids using water as mobile phase

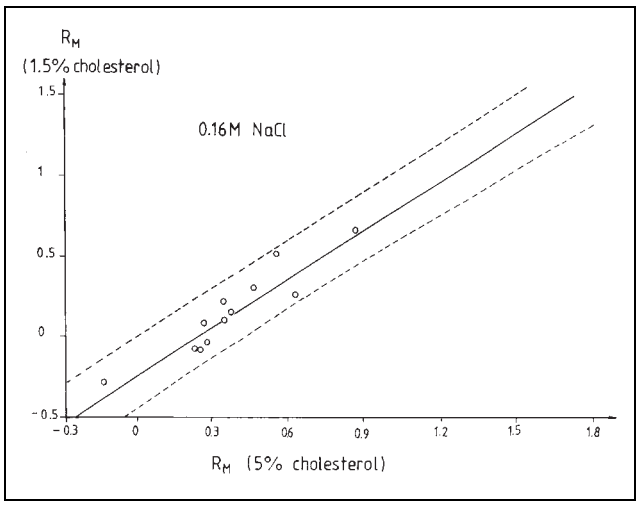


Fig. 2: Statistical estimation of the effect of cholesterol coverage on the interaction between cholesterol and amino acids using 0.16 M NaCl as mobile phase

higher binding strength can be observed at high salt concentration.

The potency values were calculated as described above for each amino acid and they are summarized in Table 3. Using stepwise regression analysis we concluded that potency values are correlated only with $pK_{\alpha COOH}$ of several physicochemical parameters examined, and the correlation coefficient is as high as $r = 0.6186$. Comparing this value to the $r_{Table,\,98\%} = 0.5923$ strong correlation between potency values and $pK_{\alpha COOH}$ values was found.

The following equation shows the relationship between potency values of amino acids (PV) and $pK_{\alpha COOH}$ values:

$$PV = 14.90 - 5.73(\pm 2.02)\, pK_{\alpha COOH} \qquad (1)$$

**Table 1: $R_f$ values of cholesterol with alumina (Alu) and silica (Si) supports**

| | $CHCl_3$ | Dichlormethane | Acetone | Benzene | Toluene | Hexane | I-Propanol | I-Propanol:hexane 1 : 1 | Acetone : chloroform | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 3 : 1 | 1 : 1 | 1 : 3 |
| Alu | 0.47 | 0.21 | front | 0.04 | 0.03 | not det | front | front | front | front | front |
| Si | 0.42 | 0.22 | front | not det | not det | not det | 0.83 | front | front | front | front |

**Table 2: $R_M$ values of amino acids using different mobile systems with 5% cholesterol impregnation**

| Sample | Mobile phase | | |
|---|---|---|---|
| | 0.05 M KCl | 0.1 M KCl | 0.16 M KCl |
| Ala | −0.31 | −0.18 | −0.12 |
| Asn | 0.63 | 0.83 | 0.69 |
| Asp | 1.51 | 1.38 | 1.51 |
| Cys | 1.51 | 1.51 | 1.51 |
| Phe | 0.60 | 1.00 | 0.75 |
| Gly | 0.18 | 0.39 | 0.25 |
| Gln | 0.16 | 0.33 | 0.27 |
| Glu | 0.95 | 1.00 | 0.87 |
| Leu | 0.25 | 0.79 | 0.37 |
| Met | 0.33 | 0.52 | 0.37 |
| Nleu | 0.50 | 0.52 | 0.48 |
| Orn | 0.23 | 0.37 | 0.23 |
| Ser | 0.31 | 0.60 | 0.31 |
| Thr | 0.55 | 0.52 | 0.43 |
| Val | −0.02 | −0.02 | −0.05 |

| Sample | Mobile phase | | | |
|---|---|---|---|---|
| | 0.05 M LiCl | 0.1 M LiCl | 0.16 M LiCl | 1 M LiCl |
| Ala | 0.12 | 0.03 | 0.12 | 0.00 |
| Asn | 0.95 | 0.87 | 0.91 | 0.58 |
| Asp | 1.69 | 1.51 | 1.69 | 1.51 |
| Cys | 1.51 | 1.51 | 1.69 | 1.51 |
| Phe | 0.91 | 0.63 | 0.69 | 0.60 |
| Gly | 0.72 | 0.33 | 0.45 | 0.09 |
| Gln | 0.66 | 0.25 | 0.43 | 0.09 |
| Glu | 0.95 | 0.95 | 0.87 | 0.75 |
| Leu | 0.60 | 0.41 | 0.29 | 0.35 |
| Met | 0.79 | 0.41 | 0.25 | 0.33 |
| NLeu | 0.69 | 0.48 | 0.37 | 0.48 |
| Orn | 0.52 | 0.35 | 0.27 | 0.10 |
| Ser | 0.48 | 0.43 | 0.23 | 0.29 |
| Thr | 0.35 | 0.41 | 0.29 | 0.45 |
| Val | −0.14 | −0.07 | −0.39 | 0.16 |

| | 0.05 M CsCl | 0.1 M CsCl | 0.16 M CsCl |
|---|---|---|---|
| Ala | −0.33 | −0.14 | 0.18 |
| Asn | 0.60 | 0.6 | 0.87 |
| Asp | 1.51 | 1.51 | 1.38 |
| Cys | 1.51 | 1.51 | 1.51 |
| Phe | 0.58 | 0.83 | 0.55 |
| Gly | 0.27 | 0.60 | 0.25 |
| Gln | 0.19 | 0.60 | 0.41 |
| Glu | 0.95 | 0.91 | 1.06 |
| Leu | 0.48 | 0.75 | 0.48 |
| Met | 0.35 | 0.66 | 0.33 |
| NLeu | 0.52 | 0.60 | 0.31 |
| Orn | 0.39 | 0.48 | 0.19 |
| Ser | 0.35 | 0.16 | 0.39 |
| Thr | 0.43 | 0.16 | 0.48 |
| Val | −0.03 | −0.25 | −0.05 |

| | 0.05 M RbCl | 0.1 M RbCl | 0.16 M RbCl | 1 M RbCl |
|---|---|---|---|---|
| Ala | −0.09 | −0.27 | −0.19 | −0.21 |
| Asn | 0.79 | 0.75 | 0.66 | 0.52 |
| Asp | 1.69 | 1.69 | 1.51 | 1.38 |
| Cys | 1.69 | 1.69 | 1.51 | 1.38 |
| Phe | 0.91 | 0.95 | 0.52 | 0.72 |
| Gly | 0.50 | 0.50 | 0.16 | 0.39 |
| Gln | 0.43 | 0.25 | 0.10 | 0.39 |
| Glu | 1.12 | 0.95 | 0.83 | 0.83 |
| Leu | 0.39 | 0.27 | 0.33 | 0.52 |
| Met | 0.48 | 0.33 | 0.48 | 0.52 |
| NLeu | 0.43 | 0.41 | 0.43 | 0.63 |
| Orn | 0.33 | 0.27 | 0.31 | 0.19 |
| Ser | 0.37 | 0.29 | 0.43 | 0.35 |
| Thr | 0.37 | 0.31 | 0.58 | 0.39 |
| Val | −0.10 | −0.12 | 0.14 | −0.21 |

| Sample | Distilled water | Mobile phase | | | |
|---|---|---|---|---|---|
| | | 0.05 M NaCl | 0.1 M NaCl | 0.16 M NaCl | 1 M NaCl |
| Ala | −0.25 | −0.02 | 0.19 | −0.14 | −0.37 |
| Asn | 0.63 | 0.72 | 0.79 | 0.55 | 0.60 |
| Asp | 1.69 | 1.51 | 1.38 | 1.51 | 1.38 |
| Cys | 1.51 | 1.51 | 1.51 | 1.51 | 1.38 |
| Phe | 0.33 | 0.58 | 0.75 | 0.63 | 0.60 |
| Gly | 0.18 | 0.25 | 0.27 | 0.23 | 0.09 |
| Gln | 0.02 | 0.23 | 0.21 | 0.25 | 0.07 |
| Glu | 0.83 | 0.83 | 0.79 | 0.87 | 0.72 |
| Leu | 0.03 | 0.39 | 0.33 | 0.87 | 0.39 |
| Met | 0.18 | 0.37 | 0.39 | 0.27 | 0.39 |
| NLeu | 0.23 | 0.43 | 0.43 | 0.35 | 0.52 |
| Orn | 0.37 | 0.29 | 0.18 | 0.37 | 0.23 |
| Ser | 0.35 | 0.41 | 0.33 | 0.43 | 0.19 |
| Thr | 0.41 | 0.41 | 0.39 | 0.41 | 0.31 |
| Val | −0.05 | −0.09 | −0.05 | −0.23 | −0.09 |

The key role of $pK_{\alpha COOH}$ can be attributed to the fact that there is significant difference between the properties of the protonated and deprotonated carboxyl group in binding processes. This phenomenon can be explained by the presence of the free hydroxyl group in cholesterol, which is likely to be the determinative subunit in the cholesterol molecule. Thus, the affinity of amino acids towards cholesterol is primarily influenced by their degree of dissociation.

In order to identify all subunits responsible for the retention behaviour of the molecules, cluster analysis was carried out. We established that amino acids with highly dissociable hydrogen ions bind strongly to cholesterol compared to neutral and basic compounds which are less susceptible to interact with cholesterol.

As shown in Fig. 3, amino acids of similar retention properties form separate groups according to their acid-base characters (designated numbers see in Table 3). The position of the clusters determines the liability of amino acids to bind to cholesterol so the closer two clusters of amino acids are to each other the higher is the possibility for behaving similarly during binding processes. The binding differences are based on the characteristics of the subunits present in the molecules which allows us to predict bind-

**Table 3: Calculated potency values of amino acids**

| Sample number | Amino acid | Potency values |
|---|---|---|
| 1 | Ala | −0.45 |
| 2 | Asn | 3.11 |
| 3 | Asp | 6.64 |
| 4 | Cys | 6.65 |
| 5 | Phe | 3.01 |
| 6 | Gly | 1.40 |
| 7 | Gln | 1.23 |
| 8 | Glu | 3.91 |
| 9 | Leu | 1.77 |
| 10 | Met | 1.78 |
| 11 | NLeu | 2.02 |
| 12 | Orn | 1.30 |
| 13 | Ser | 1.52 |
| 14 | Thr | 1.77 |
| 15 | Val | −0.38 |

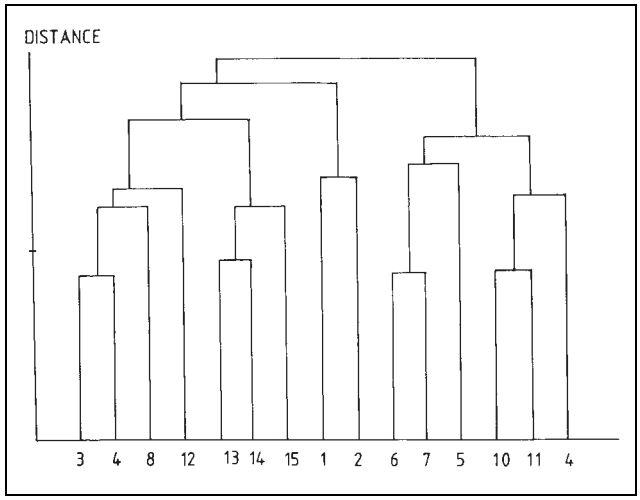


Fig. 3: Statistical evaluation of the strength of interaction between amino acids and cholesterol on the basis of the results of cluster analysis

ing affinities of various amino acids with regard to the active molecular groups.

PCA was carried out to estimate the effect of physicochemical parameters of monovalent cations on the interaction between cholesterol and amino acids. As a result of PCA we established that two principal components explain the overwhelming majority (94%) of total variance indicating that the 18 original variables can be substituted by two background variables with only negligible loss of information. The first eigenvalue was 14.98 with 83.21% as the relevant variance %, the second eigenvalue was 1.92 with a further 10.64% as the relevant variance %. PCA shows that the potency values of amino acids are significantly influenced by the following physicochemical parameters: ion radii relevant to coordination numbers, hydration enthalpy, ion radii in the case of hydrated cations, enthalpy, $T^*S$, bond distances in $Cl^-$ lattice.

The amino acids examined were divided into 3 groups according to their retention behaviours. The first group was Ala, Val, Orn, Gly, Gln, the second one was Leu, Ser, Met, Thr, NLeu, and finally Asp, Asn, Phe, Glu, Cys belong to the third group. The first five amino acids bind only marginally to cholesterol, where as the highest binding affinities were observed in the third group. In Fig. 4

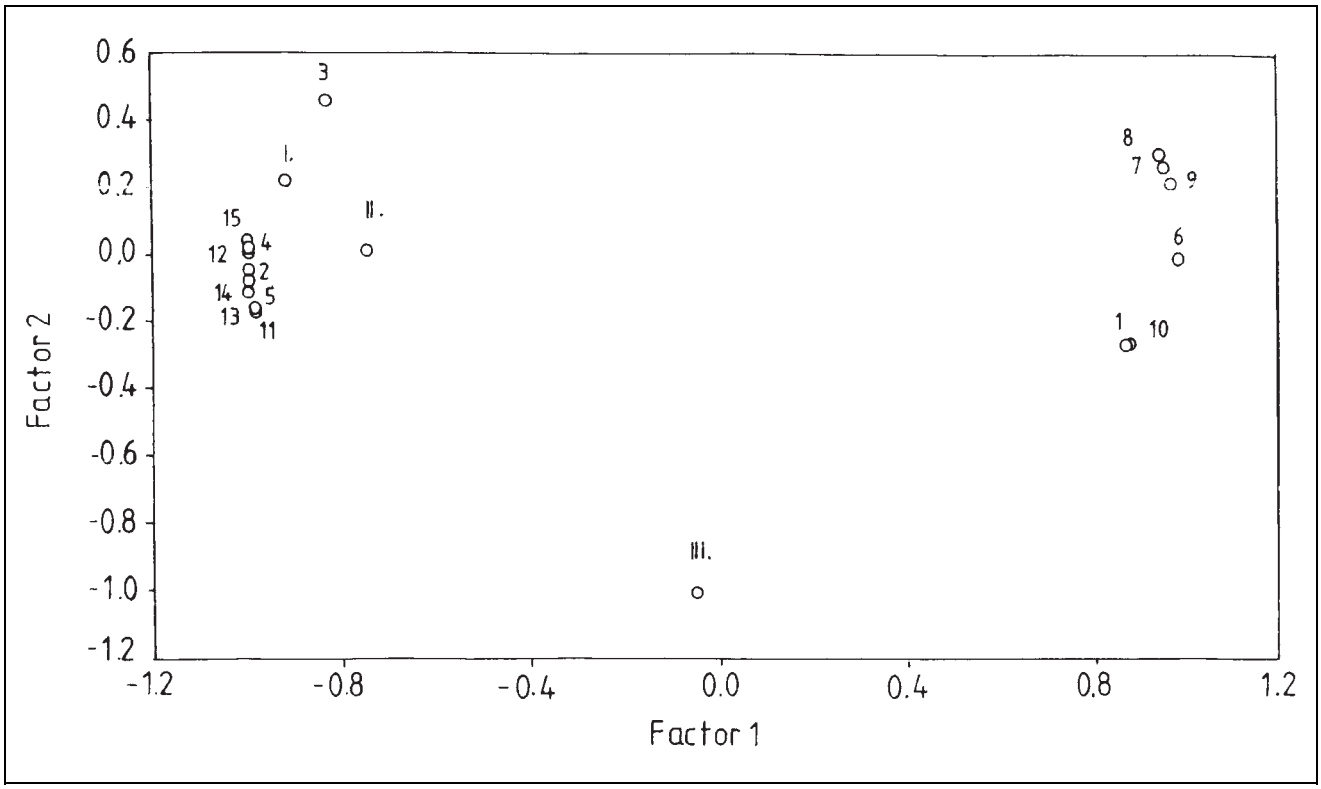


Fig. 4: Factor loadings of principal component analysis
I. first amino acid group: Ala, Val, Orn, Gly, Gln, II. second amino acid group: Leu, Ser, Met, Thr, Nleu, III. third amino acid group: Asp, Asn, Phe, Glu, Cys
1. first coordination number, 2. ion radii relevant to first coordination number (Å), 3. second coordination number, 4 ion radii relevant to second coordination number (Å), 5. hydration enthalpy (kCal/mol), 6.–8. ionization energies (kJ/mol), 9. electron affinity (kJ/mol), 10. theoretical lattice energy (in terms of $Cl^-$ ions ) (kJ/mol), 11. calculated value of lattice energies (kJ/mol), 12. ion radii for hydrated cation (Å), 13. enthalpy (kJ/mol), 14. $T \cdot S$ (kJ/mol), 15. bond distance in $Cl^-$ lattice

the results of Varimax rotation can be seen. It can be concluded from the results that multivariate statistical methods can be successfully applied to the evaluation of chromatographic retention data.

It can be concluded from the results that monovalent cations present in the mobile phase have significant impact on the interaction between amino acids and cholesterol using reversed-phase thin-layer chromatography (RP-TLC). The strength of the interaction depends on both the acid-base properties of amino acids and the physicochemical characteristics of the solutes. As we found, the degree of carboxyl group dissociation is one of the most important factors determining the binding affinities of amino acids to cholesterol. The relative contribution of several physicochemical parameters of monovalent cations to the effect of salts on the interaction was also elucidated. The statistical evaluation of our measurements gives sufficient information about the effect of salts on binding processes on cholesterol impregnated TLC plates.

## 3. Experimental

### *3.1. Materials and apparatus*

The silica plates used were DC-Alufolien, Kieselgel 60 $F_{254}$ $20 \times 20$, and the aluminium-oxide plates were DC-Aluminiumoxide $F_{254}$ plates $20 \times 20$ (Merck, Darmstadt, Germany). Cholesterol was a commercial product.

Solubility and running tests were carried out to decide what solvent or mixture of solvents should be used to reach optimal separation conditions. 5 mg cholesterol was dissolved in 1 ml chloroform and it was used as the sample material for TLC running.

By using an acetone system a maximum 1.5% cholesterol solution could be prepared with regard to solubilization parameters. Impregnation of Alu plates was therefore carried out with this solution and 17 amino acids were spotted onto 1.5% cholesterol solution covered plates. The first Alu plate was immersed in distilled water, the second one in 0.16 M NaCl solution.

In our study an acetone/chloroform 1 : 1 system proved to be ideal for solubilization of cholesterol (preparation of 5% cholesterol solution) as well as helping cholesterol to migrate.

Alumina supports were impregnated by overnight predevelopment in a 5% cholesterol solution prepared with an acetone : chloroform 1 : 1 mixture.

Amino acids (Ala, Asn, Asp, Cys, Phe, Gly, Gln, Glu, Leu, Met, NLeu (norleucine), Orn, Ser, Thr, Val, OxyPro (oxyproline), Pro) were purchased from Sigma Chemical and were used as received. Analytes were dissolved in a water-isopropanol mixture (3 : 1, v/v) at a concentration of 5 mg/mL and 5 µl of solutions were separately spotted onto the plates. The Mobile phase consisted of distilled water or an aqueous solution of LiCl, NaCl, KCl, RbCl, or CsCl at concentrations of 0.05 M, 0.1 M, 0.16 M, or 1 M.

Development was carried out in sandwich chambers ($22 \times 22 \times 3$ cm) at ambient temperature, the distance of development being about 16 cm. After development the plates were dried at 100 °C and the spots of analytes were revealed by the ninhydrin reagent. In order to increase the sensitivity of detection the plates were sprayed with 2 M aqueous acetic acid prior to the ninhydrin reaction.

### *3.2. Theoretical background and statistical calculations*

The $R_M$ value characterizing the molecular hydrophobicity in reversed-phase thin-layer chromatography was calculated for each solute in each eluent:

$$R_M = \log (1/R_f - 1) \quad (2)$$

where $R_f$ is the retention factor value of the analyte.

When the coefficient of variation of the parallel determination was higher than 6% the $R_M$ value was omitted from the following calculations. This procedure was motivated by the fact that the standard error of traditional TLC measurements is generally lower than 6%. A higher standard error indicates inadequate experimental conditions and biased data.

For data evaluation SPM was carried out as follows: 19 eluent systems formed the rows and the $R_M$ values of 15 different amino acids formed the columns ($19 \times 15$ matrix).

SPM calculates the potency values of amino acids and their selectivity map.

The dimension of the SPM selectivity maps was reduced to two by cluster analysis, iteration being continued to the point when the difference between the last two iterations was smaller than $10^{-8}$.

To identify the physicochemical parameters and molecular substructures of solutes that significantly influence their capacity of binding to cholesterol, stepwise regression analysis (SRA) [34] was used. In traditional multilinear regression analysis the presence of independent variables that exert no significant influence on the dependent variable lessens the significance level of the independent variables that significantly influence the dependent variable. To overcome this difficulty, stepwise regression analysis automatically eliminates from the selected equation the insignificant independent variables which have no significant impact on the strength and selectivity, thus increasing the information power of the calculation.

SRA was carried out to evaluate the retention behaviour of amino acids with respect to their molecular properties. The potency values of amino acids were the dependent variables, and $pK_{\alpha COOH}$ (where $K_{\alpha COOH}$ means the first dissociation constant), $pK_{\alpha NH2}$ (where $K_{\alpha NH2}$ means the protonation constant of $NH_2$ group), pI (where I stands for isoelectric point), and the hydrophobic ($z_1$), steric ($z_2$) and electronic ($z_3$) parameters of amino acids were the independent variables.

The hydrophobic ($z_1$), steric ($z_2$) and electronic ($z_3$) parameters of amino acids were calculated as described by Jonsson et al. [35]. The number of independent variables accepted was not limited and the acceptance limit was set at the 98% significance level. Software for stepwise regression analysis was written by CompuDrug Ltd, Budapest, Hungary.

PCA was used to determine the effect of salts on the interaction between amino acids and cholesterol. The potency values of amino acids and the various physicochemical parameters of cations such as coordination numbers, ion radii relevant to coordination numbers (Å), hydration enthalpy (kcal/mol), ionization energies (kJ/mol), electron affinities (kJ/mol), lattice energies (in terms of $Cl^-$ ions) (kJ/mol), the calculated values of lattice energies (kJ/mol), ion radii in the case of hydrated cations (Å), enthalpy (kJ/mol), $T \cdot S$ (kJ/mol), and bond distances in the $Cl^-$ lattice (15 in total) were the principal component variables. Calculations were carried out by Statistica 5.5 software (StatSoft Inc., Tulsa, OK, USA).

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