

SZENT ISTVAN UNIVERSITY

**Adsorption characteristics and polarity relations of the separation by thin layer chromatography of bioactive chemical compounds having effects in the cultivated soil**

Summary of the Ph.D. thesis

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## 1. Introduction

It is inevitable to have prompt and efficient examination methods to solve the problems which arise during the modern production of medicine, food-products, paints, insecticides and artificial fertilizers. This demand resulted in the development of different methods of separation and their practical application. One branch of the quickly developing separation methods is chromatography, which is suitable for the separation of materials even in small quantities and with close structure. The different methods of chromatography: thin-layer chromatography, liquid chromatography and gas chromatography – are equally applicable to solve any practical problems. However, none of the methods of chromatography are suitable for separating and specifying every type of compound. Thin-layer chromatography is a simple, quick and efficient method, but its reproducibility and automatization do not comply in every case with the requirements raised against modern methods. High capacity methods of gas chromatography can make the separation and specification of volatile and non-volatile, organic and inorganic compounds possible. It sets a limit to the applicableness of gas chromatography that primarily it is suitable for separating volatile compounds. Although by various procedures of derivative production the analysis of many compounds can be accomplished through gas chromatography, creating by-products involve additional expenses and serves as a source of error. Present day, the development of liquid chromatography is the most conspicuous due to the new stationary phases, the more accurate methods of detecting and technologies of miniaturisation.

Usually, mathematical statistical methods are applied to the exploration of relations between retention features and physico-chemical parameters and the comparison of the retention qualities of different carriers.

The most frequently used method is probably the stepwise regression analysis. The stepwise regression analysis (SRA) is different from the conventional more than one variable regression analysis, because in the descriptive equations of the connections of dependent and independent variables it considers only those of the independent variables which exert significant influence on the dependent variables. The omission of the insignificant independent variables increases the authenticity of the calculations considerably.

### **The two applications of the method:**

- a. We can give the programme instructions to select  $n$  number of the independent variable which influences the dependent variable the most, or
- b. Give instructions to select all those independent variables which have the probability of influence on the dependent variable over a level of significance stated by us.

The calculations show us the chosen diagrams significance level attributed to the measurement data (F value), it shows us the percentage of the effect that the independent variables had on dependent variables ( $r^2$  or determination coefficient value) and the rate at which the independent variables influenced the dependent variables (path/coefficient or normalized slope value) However it is also possible to calculate the connection of structure-chromatographic retention without the determination of the physico-chemical characteristics. Principle component analysis (PCA) and factor analysis have been developed to reduce the number of variables.

These calculations examine the relationship of data's lines and columns, without attributing significance to any of the lines or columns, so that there can be no dependent variable and so that instead of the measured variables there may not be artificial background variables or phantom variables which would explain the majority of changes in the examined phenomenon, these are the principle components or factors.

The percentage value belonging to the principle components (factors) self value, shows us what percentage of the general changes can be explained with the help of the principle component or factor. Because none of the methods have statistical significance test we usually viewed principle components (factors) with a self value greater than 1.0 as information carriers. The measured or calculated variables that are in relation with one another are present in identical principle components or factors. The absolute value of the principle component or factor weight equals the force of the effect, while the positive or negative sign indicates its direction. Taking into account all the measured variables the chemicals that behave similarly are those that are found in great numbers in the same principle component or factor. If the principle components or factors number is  $n$ , then according to the variables significance they situate themselves in an  $n$  dimensional space, closest are the most similar, furthest the most differing ones. Because it is very hard to imagine this, more methods have been developed to portray this  $n$  dimensional space in two dimensions: varimax rotation, cluster analysis, two dimensional nonlinear mapping.

The principle component weight and principle component variables matrix primarily calculated by PCA place their elements strictly in a  $90^\circ$  (orthogonal) coordinate system, which with the rotation of the axle (giving up the orthogonality) can be set so that the values of the principle components weight may situate themselves maximally along the axle (varimax rotation)

The two dimensional nonlinear portrayal (nl map) technique projects an  $n$  dimensional matrix (principle component weight and values) in such a way onto a single plane, that their distance best represents their distance in an  $n$  dimensional space. Because of the method of mapping the points describing or influencing similar characteristics will be located close to one another, forming a group.

Principle component analysis and factor analysis cannot separate retention strength and retention selectivity from one another in the elements of a matrix. This problem can be solved using the Spectral map technique. Spectral mapping (Sp map) separates the matrix's elements retention strength (potency vector) and retention selectivity (selectivity matrix) from one another and gives their value as relation numbers. This method was developed originally for the design tests of medical active ingredients; however it quickly became clear how useful it is in chemical structure chromatographic retention tests.

The data from the Sp map method can be portrayed using nl map and CA (cluster analysis). It may be important in the examination of chemical structure chromatographic retention to know the measured (more than one chromatographic parameters) dependence upon the examined chemicals measured or calculated physico-chemical parameters.

## **2. The aims of my scientific work:**

Throughout my work my primary aim was to spread the use of thin-layer chromatography and liquid chromatography in separating the derivatives of pesticides, tenside materials and toxic, environmentally polluting pesticides and also to examine the parameters influencing separation

### **My scientific work extended to the examination of the following problems:**

- The stationary phase's materials and coatings effect on retention
- The mobile phase's pH and salt concentration effect on the examined chemicals retention
- The examination of molecular relations in thin-layer chromatographic systems (with the use of  $\beta$ -cyclodextrin and zein)
- I examined the differing mathematical-statistical methods expansion of applicability in analytical practice

### **My particular scientific objectives were:**

- a) To elaborate and carry out the separation of frequently used pesticides of wide-spread agricultural application, the decomposition products of pesticides and tenside materials on different chromatographical stationary phases and in different systems of chromatography.
- b) To examine the significance of the quality of the stationary phase (silicon-dioxyde or alumina) in different systems of chromatography (adsorption and reversed phase chromatography) and the possible changes of their qualities which may occur by physical (pyrolysis) or chemical impregnating (chemical agents of different molecular weight and vinyl content) effects. It was also my purpose to examine how changes in the characteristics of the stationary phase can effect the separation and retention of different pesticides.
- c) To specify the molecular parameters of fungicides by using adsorption and reversed phase thin-layer chromatography systems to obtain survey data which are used in the determination of those molecular parameters.
- d) To examine the interaction of different pesticides and  $\beta$ -cyclodextrin polymers dissolvable in water as giant molecules. I wanted to know how the retention of the pesticide-cyclodextrin complex changes.
- e) To examine the effect of the structure of benzonitrile ester molecule, what he has on the separation in the reversed phase thin-layer chromatography system. I wanted to ascertain whether there is a connection between the second ring structure of the chemical molecules and their biological activity, and to reveal the significance of sterical parameters.

- f) To examine the effect of benzonitril ester herbicides on soil bacteria. I used principle component-analysis to appraise the results.
- g) To study the binding strength and features of the organic solvents on the zein-covered (protein of corn) carbon HPLC column. In addition, I wished to study the binding of 18 non-ionic tenside materials with apolar ethylene-oxyde chain of different length on zein-covered alumina carrier layers by altering the content of methanol- and cation concentration and the pH of the mobile phase.
- h) To study, how the changing of the pH of the silica qualified as stationary influences the retention of the dansyl-amino-acid used as test material during the examination of adsorption and reversed phase thin-layer chromatography. I wanted to study how the pH change influences the retention characteristics of the stationary phase. I wanted to examine the impact of different salts (alkaline metal-chlorides) and acids (acetic acid, formic acid, etc) on the retention of dansyl-amino-acids in the reversed phase thin-layer chromatography system (salting out-, salting in effect).
- i) To study the modifying impact of the surface pH of silicon-dioxyde stationary phase on the reversed phase thin-layer chromatography retention of several amino-acids.
- j) To study the possible formation of peptyde-cyclodextrin complexes by imitating the retention circumstances in soil systems and studying the changes of the retention of the emerging complexes and analysing the effects of the salts on retention as well.
- k) To find the most appropriate stationary phases and conditions of reversed phase chromatography for separating non-ionic tenside materials. I wanted to accomplish the impregnation of the stationary phase with oils of different molecular weight and examine the effect of the impregnation on the non-ionic tenside materials.
- l) For the sake of the more proper applicability of the information contents of chromatographic results I set myself the aim of examining whether the application of covariance matrix or correlation matrix is more practical during the principle component analysis calculations.
- m) To accomplish the separation of aniline derivatives. With the aid of the obtained data I was looking for the connection between the characteristics of the column like reversed phase stationary phase and the retention behaviour of aniline derivatives. I applied more than one variant methods of calculation and tried to determine whether the non linear mapping or the cluster analysis is proper to perform the analyses and represent the results.

### 3. Materials and methods

#### 3.1 Materials

I included the following materials in my investigation:

- I. Pesticides, their derivatives and additives:  
Triphenyl-derivatives, aniline-derivatives,  $\beta$ -cyclodextrin polymers, 2-nitro-4-cyanophenyl esters, triazine derivatives, benzonitrile esters, triphenylmethane derivatives.
- II. Amino acids:  
L/amino acid, arginin, lysine, ornitin, histidin, isoleucin, leucin, norleucin, metionin, tiroyin, dansyl-amino acids
- III. Tenside materials:  
Nonylphenyl etoxylat oligomer, nonylphenyl ethylene oxide polymer
- IV. Solvents:  
Acetone, tetrahydrofuran, acetonitrile, 1,4-dioxane, 2-methoxyethanol, 2-ethoxyethanol, 2-propoxyethanol, methyl-ethyl-keton, ethylene glycol, propylene glycol, , glycerine, 1-proponol
- V. Fungies:  
Pleurotus ostreatus, Letinus edodes

#### 3.2 General liquid chromatographic methods

Because during the measurements the laboratory temperature changed immeasurably ( $\pm 1^\circ\text{C}$ ) I disregarded from thermostizing the chromatographic systems. Every retention time was calculated from the average of at least three parallel measurements. I used the logarithm of the retention capacity ( $\log k'$ ) to describe the retention. To write down the correlation between the eluents composition and the retention, I used the following equation:

$$\log k' = \log k'_0 + b.C$$

in which  $\log k'$  is the chemical compound's retention capacity in a given eluent composition,  $\log k'_0$  the chemical compound's retention capacity extrapolated on the mobile phases smaller elution force component (in the case of a reversed phase chromatography this is water), C the concentration of the component which has greater elution force in mobile phase. (bearing in mind the possibilities the extrapolation area in a group of compound was always identical)

The value of  $\log k'_0$  is the number used to describe the strength of interaction between the chemical compound and the stationary phase. 1. the equations intercept (b) I viewed as the value proportional to the chemical compound surface which makes contact with the carrier (in the case of phase a reversed chromatography this is the value of the chemical compound specific hydrophobic surface)

Because of the linear correlation value of  $\log k'_0$  and b in 1. equation is viewed as a justification for the homogeneity of a group of compound in the case of a given chemical group.

I calculated the characteristic correlation for every chemical group using the following equation:

$$\text{Log } k'_0 = A + B.b$$

### 3.3. General calculation methods

In the following I have compiled the advantages and disadvantages of the mathematical-statistical methods that I introduced.

Stepwise regression and modified Free-Wilson analysis

- Automatically singles out the significant independent variables

Principle component analysis

- Examines the system of relations-without significant dependent or independent variables
- Has no significance proof (only graphical estimate)

Spectral map

- Effect selectivity and force is separated
- Has no significance proof (only graphical estimate)

Two dimensional non-linear mapping

- The projection of points placed in distance proportion in two dimensional space
- Only graphical significance test

I used the following multiple variable mathematical-statistical methods to calculate the quantity relationship between retention characteristics and physicochemical parameters, as well as to compare retention characteristics of varying carries stationary phases:

#### 1). Step-by-step (stepwise) regression analysis (SRA)

During the calculations I did not limit the number of accepted independent variables, I set there significany level at 95%. I used the following mathematical-statistical parameters to evaluate the significant relationship between retention characteristics and physicochemical parameters:  $r$ = correlation coefficient (the strength of the relationship is indicated by a chosen independent in the case of a variable);  $s$ = the estimates standard error;  $F$ = value of the Fisher-test (the strength of the relationship is indicated by the presence of more than one independent variable in the equation);  $b$ = the regression coefficient of the independent variables, taking into account the original unit of measurement;  $b'$ = normalized slope value (path coefficient), the variation quotient given by the independent variables, independently from the variables original unit of measurement;  $r^2$ = the dependent variables variation quotient given by the independent variables.

I used the stepwise or step-by-step regression analysis, when I examined the relationship of a chromatographic characteristic and more than one structural or physicochemical variable.

#### 2). Principle component analysis (PCA)

I used principle component analysis in the cases when there where no significant dependent or independent variables and I wished to examine the relation of chromatographic and structural characteristics simultaneously.

I set the variation quotient given by the principle component at 99.9% in all cases. I solved the portrayal of our PCA results using varimax rotation, CA and nl map techniques. All three methods are suitable for the reduction of the principle components weight and variable dimensionality. I continued the nl maps iteration until the difference between the two lastly made iteration following was less than  $10^{-8}$ .

### 3). Spectral mapping (Sp map)

I used the Sp map techniques when I wished to examine the regression strength and selectivity separately. I portrayed the results with the help of the nl map technique. In this cases the difference of two lastly made iteration was less than  $10^{-8}$ .

The calculations were made using software developed by COMPUDRUG Ltd, Budapest (SRA) and Dr. Bordás Barna, MTA Plant Protection Research Institute, Budapest (PCA, NL map, CA, SP map)

## 3.4. Physicochemical parameters applied in the calculations

Retention- I used the following calculated parameters to reveal the relationship between the molecular parameters:

$\pi$  = the substituents constants characterizing hydrophobicity; H-Ac and H-Do= indicator variables for proton acceptor and proton donor properties. M-Re= molar refractivity. F and R are the Swain and Luton's electronic parameters characterizing the inductive and resonance effects.

$\sigma$  = Hammetts constant characterizing the electron withdrawing power of the substituents;  $E_s$ = Taft-constant characterizing the steric effects of substituents;  $B_1$  and  $B_4$ = the Sterimol parameters which characterizes the width and breadth of the molecule

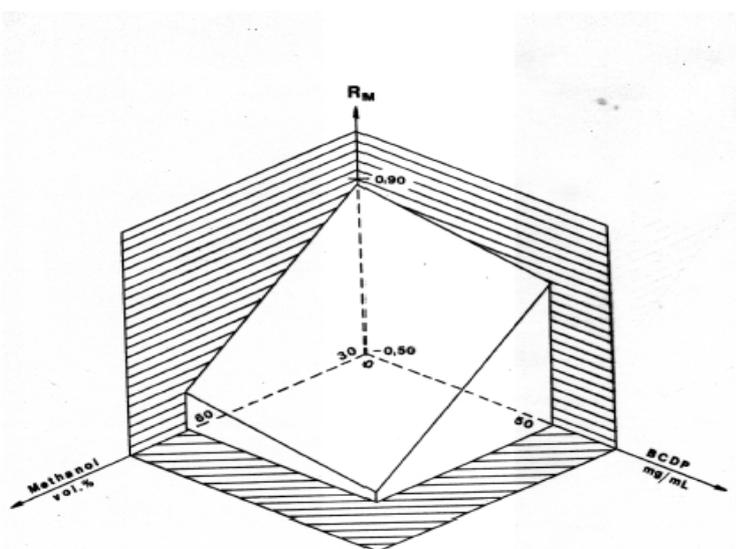
## 4. Summary of the results

1. I carried out the quick, effective, and easily repeatable tests (analyses and separation) on varying chromatographic stationary phases and in different chromatographic systems.of the pesticides, pesticide derivatives and tenside materials which has(2-nitro-4-cyanophenyl esters, triphenylmethane derivatives, nonylphenyl ethylene oxide polymers etc.) effects on the soil and influence the soils life

2. Throughout my experimental work I established and with later calculations proved, that both the stationary phase's original characteristics and the impregnating agents' characteristics had a role in the thin-layer chromatographic separation. 2-nitro-4-cyanophenyl esters and triphenyl methane derivatives, as pesticides separation the carriers maintained there retention characteristics even after impregnation, and so their original characteristics influence the above mentioned pesticides separation.

3. I determined that stationary phase cellulose is best suited for the effective separation of nonionic tenside materials, because these materials have a small lipophilicity. The  $R_M$  (lipophilicity) difference in the value of nonionic tenside materials is greater on cellulose carriers than on other carrier surfaces. It was also proved in the reverse phase chromatographic system that silicone oils molecular weight barely effects the nonionic tenside materials retention, but with the increase of impregnation the retention of nonionic tenside materials also increased
  
4. I pointed out the role of sterical parameters in the separation process of benzonitrile esters in reverse phase thin-layer chromatography. During the study of benzonitrile ester herbicides effect on bacteria species living in the soil I recognized that the effect on the microorganism's growth depends evenly on the species of the microorganism and the herbicide's chemical structure.
  
5. I realized the separation of 37 tiophosphiril-glycin amid (fungi killing agent) using adsorption and reversed phase thin-layer chromatographic systems. I proved that these chemical compound's molecular parameters are definable in thin-layer chromatographic systems.
  
6. I proved using calculations in the case of 18 generally used pesticides that these chemicals react with  $\beta$ -cyclodextrin and that the examined pesticides molecules agrochemical characteristics is changing with this reaction (adsorption, leakage, decomposition etc.). The significance of this is in the formation of complexes in the soil and the formed complexes effect on the soil.

Effect of methanol and a water-soluble  $\beta$ -cyclodextrin (BCDP) concentrations on the lipophilicity ( $R_M$  value) of cyprofuram.

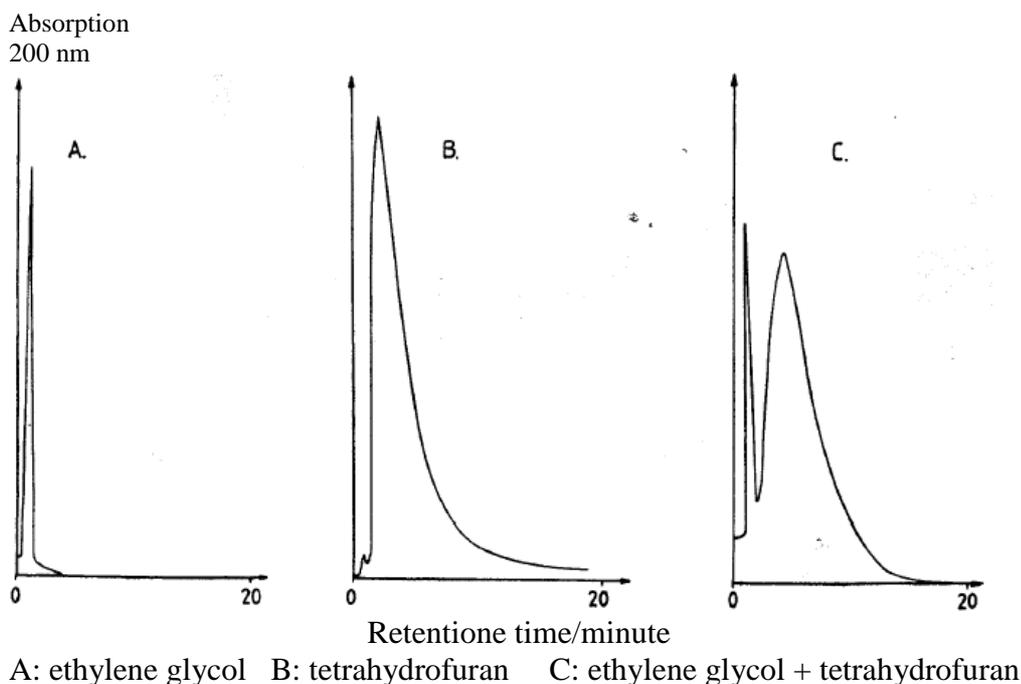


I proved that with the increase of  $\beta$ -cyclodextrin concentration and mobile phase methanol concentration the lipophilicity of pesticides decreased, there  $R_f$  value increases, so their retention will be smaller. The pesticide -  $\beta$ -cyclodextrin relationship mainly depends upon the apolar surfaces physicochemical parameters. The retention of the complex depends which part of the insecticide molecule, which ligand doesn't fit into the complex.

7. At the same time I proved, that small molecular weighted peptides most probably develop cluster complex formation with hydroxypropil-  $\beta$ -cyclodextrin, because the peptides lipophilicity, as well as retention gradually decreased during the increased concentration of hydroxypropil-  $\beta$ -cyclodextrin in the mobile phase. The binding and mobility of similar peptide complexes developed in the soil changes in the same way.

8. I described the physicochemical parameters influencing the strength of the bond with corn proteins (zein) in the case of 12 potentially environment polluting chemicals (organic solvents). With this I pointed out how the soils agrochemical state (for example salt concentration) influences the developing interaction. With my examinations I proved the binding of nonionic tenside materials to zein. I established that the number of hydrophilic ethylene oxide units in the tenside materials molecule affects the strength of the bond most greatly. The methanol and salt concentration of the chromatographic system only secondarily affect the bond. At the same time however the salts concentration, their chemical characteristics and pH significantly influence the tenside materials corn protein (zein) interaction selectivity.

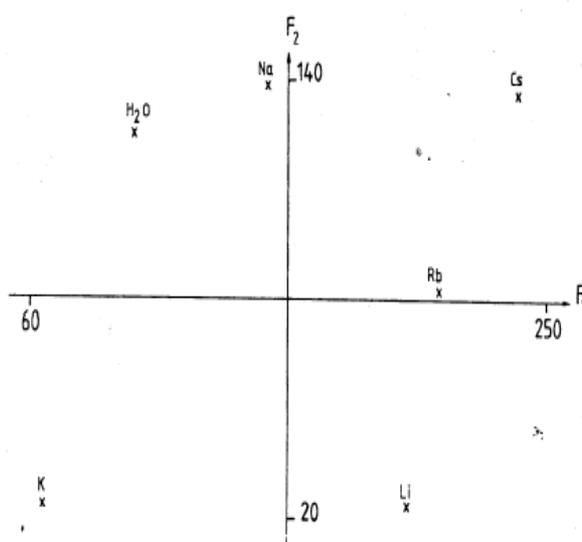
Typical chromatograms of organic solvents on zein-coated carbon stationary phase



9. In the case of Dansyl amino acids I justified, that the salt concentration of the mobile phase has a complex effect on the retention (salting in, salting out effect). In the mobile phase the presence of the acetic acid, formic acid, propionic acid etc. also induces the salting in and salting out effect. In the case of every Dansyl amino acid the minor concentration of the acids increased retention. I attributed this effect to the decreased dissociation of the polargroups of the eluent molecules which had the effect of increased lipophilicity (salting out effect). The acid solvents with greater concentrations reduced retention. The acid molecules that had not dissociated probably acted as an organic mobile phase (salting in effect)

10. For the effective separation of amino acids, as bioactive chemicals we must choose the suitable chromatographic circumstances. It is best in the case of this bioactive chemical group to experiment with varying the pH of the mobile phase in a reversed phase thin-layer chromatography. I found that in the case of double based amino acids (arginin, lysine, ornitin) lipophilicity and retention increased when the pH was increased. The cause of this is that impregnation, which assures the reversed phase, can never be perfect, so electrostatic interaction (hydrophil) develops between uncovered exposed silanol groups and amino acid groups. Even the pH changes in the places of active silanol groups influence the retention of amino acids. The increased or decreased adsorption capacity of the uncovered silanol groups takes place in effect of the present cations and anions (We are facing a dual retention mechanism)

11. I proved the usability of principle component analysis and Stepwise regression analysis in chromatographic data assessment with the new use of mathematical-statistical methods. This is shown by the diagram below and the description belonging to it:



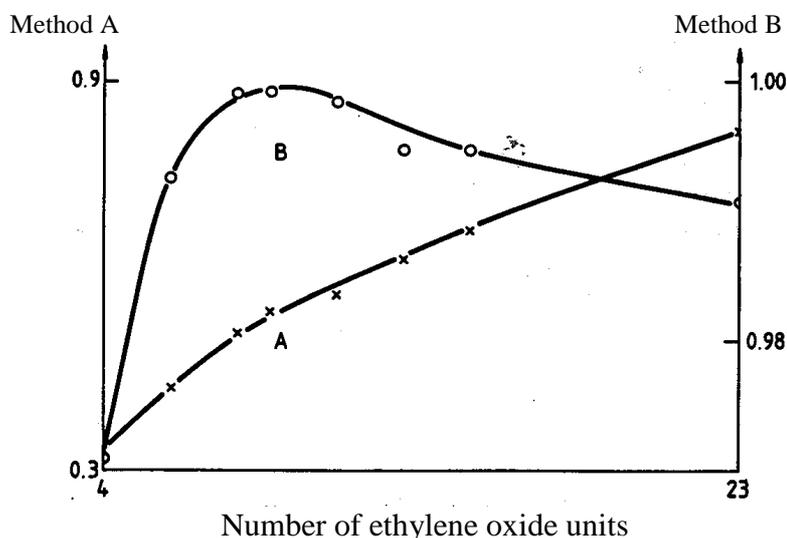
Similarity and difference between the relative effect on mobile phase HP- $\beta$ -CD-peptide interaction. The number of iterations is 59, the maximum error is  $1,12 \times 10^{-2}$ . The two dimensional nonlinear portrayal of PCA's weight.

Non dimensional numbers are found on the scale of the map which only show the points positioned in the two dimensional plane.

I examined the effect of differing concentrations of hydroxypropil- $\beta$ -cyclodextrin (HP- $\beta$ -CD) on the reversed phase thin-layer chromatographic behavior of a few small molecular weight peptides, both in distilled water and in solvents containing 0.05M LiCl, NaCl, KCl, RbCl, and CsCl, and I determined the relative strength of HP- $\beta$ -CD-peptide interaction in function of mobile phase HP- $\beta$ -CD concentration. The peptides lipophilicity steadily decreased with the increasing concentration of HP- $\beta$ -CD in the mobile phase, which proved the interaction between peptides and HP- $\beta$ -CD (probably cluster complex formation). I established using principle component analysis that every salt influences the interaction differently. (See the diagram above). On the two dimensional nonlinear portrayal the peptides organized themselves into groups, in accordance to the amino acids monomer characteristics, but not to the amino acid units number, this lets us believe, that in the HP- $\beta$ -CD cavity there is only one terminal amino acid.

12. With the assessment of chemical groups chromatographic separation data I proved that principle component analysis done from correlation and covariance matrixes may have differing results. My calculations proved the advantages of principle component analysis done from covariance matrix in chemical data assessment.

Relationship between the number of ethylene oxide groups per molecule and the principal component loadings.



Method A: covariance matrix

Method B: correlation matrix

I established that nonionic tenside materials' (nonyl-phenyl ethoxyeth oligomers) retention values did not follow the rules of additivity and changed nonlinearly with the growth of the ethylene-oxide chain. The silicon oils molecular weight does not really affect the retention of nonyl-phenyl ethoxyeth oligomers, however if the rate of impregnation is increased so is the retention. It became clear from the calculations that the use of the correlation matrix distorts the data; this is why the use of the covariance matrix is advised.

13. Regarding the reduction of the dimensionality of the data matrix I pointed out that two dimensional nonlinear portrayal and cluster analysis are effective tools for visualizing the results. Then again in the case environmental polluting aniline derivatives I established that the above mentioned calculation methods containing more than one variable used for the assessment of data and relations (stationary phases, examined materials) themselves influence the results. Due this, there is great need for the use of more calculation methods and the critical comparison of the results.

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