*Analusis*, **1998**, *26*, 400-406

# Application of multivariate mathematical-statistical methods to compare reversed-phase thin-layer and liquid chromatographic behaviour of tetrazolium salts in Quantitative Structure-Retention Relationships (QSRR) studies

G.A. Csiktusnádi-Kiss[1],*, E. Forgács[1], M. Markuszewski[2] and S. Balogh[3]

[1] *Institute of Chemistry, Chemical Research Centre, Hungarian Academy of Sciences, P.O. Box 17, 1525 Budapest, Hungary*
[2] *Medical University of Gdañsk, Dept. of Biopharmaceutics and Pharmacodynamics, Budapest, Hungary*
[3] *Reanal Fine Chemicals, Budapest, Hungary*

**Abstract.** The retention parameters of seven mono- and nine ditetrazolium salts were investigated by reversed-phase liquid chromatography (RP-LC) using polyethylene-coated alumina support with various concentration of eluent-mixtures and by reversed-phase thin-layer chromatography (RP-TLC) using the same support and eluent-mixtures. Principal component analysis (PCA) followed by non-linear mapping (NLM) and varimax rotation was used for the determination of molecular substructures and physicochemical parameters accounting for the separation in the different methods and circumstances. Calculations proved that the steric and electronic parameters have the highest influence on the retention of tetrazolium salts. This investigation uncovered that the thin-layer chromatography is strictly independent from liquid chromatography. It has been established that multivariate mathematical-statistical methods are the most appropriate ways for evaluation of large data matrices in various chromatographic departures.

**Key words**. Tetrazolium salts – thin layer chromatography – liquid chromatography – principal component analysis – cluster analysis – non-linear mapping – varimax rotation.

## Introduction

The widely used reversed-phase liquid chromatographic separation technique is especially useful to the separation of bioactive molecules [1].

Although the support is generally silica with covalently bonded hydrocarbons [2] or various polymers to the polar surface [3], the alumina is used more frequently with coated polymers on the surface as well [4]. Not only polyethylene [5-7] but also various types of other polymers such as polyamine [8], polystyrene [9] and polypyrrole chloride [10] have been coated to silica or alumina basis.

The polymer-coated alumina supports typically and successfully used the investigation or separation of basic solutes hardly eluted from silica-based supports such as various proteins [11], peptides [12], other bio-macromolecules [13], alkaline compounds [14] and a lot of drugs [15] and diagnostic materials [16] as well.

The tetrazolium-derivatives are wide-spreadingly used in the field of biology research [17], human medicine care [18] especially in the field of endocrinology, gastroenterology, cytology-histology, angiology, microbiology and virology, in microbiological laboratories [19] and in the field of bio-inorganic chemistry.

---

* Correspondence and reprints.

Received February 17, 1998; revised July 24, 1998; accepted September 1, 1998.

Thin-layer chromatography (TLC) is a relatively old technique among the various chromatographic separation methods, but it showed some marked advantages over the other chromatographic techniques. TLC and high-performance liquid chromatography (HPLC) are similar in many aspects. Both of them use a stationary and a mobile phase, and the solutes are separated according to differences in their affinities to the phases. It has often been indicated that TLC can be successfully used as a pilot method for HPLC because the retention behaviour of the solutes became predictable with its help [20,21]. However in certain cases, TLC is not suitable (or suitable only with restrictions) as a pilot method: the different surface pH of the TLC and HPLC supports may result in entirely different retention orders for acidic or basic compounds in adsorption systems [22].

The development of new calculation methods to interpret large data matrices obtained by the usage of highly automated chromatographic instruments is one of the major advances nowadays. The importance of using high performance mathematical methods to evaluate data matrices is especially increased in the field of QSRR research [23].

The modern multivariate mathematical-statistical methods e.g. principal component analysis (which is used to manifest the original variables with background-variables according to its correlation among themselves, that helps to recognise the possible connections in the observated matrices) [24], canonical correlation analysis [25], stepwise regression analysis [26], non-linear mapping and varimax rotation (which graphically transforms the principal-component loadings from the hypersphere into two-dimensional plane) [27], cluster analysis (which gives theoretically similar results like non-linear mapping but it transforms to one dimensional line) [28], etc. make the simultaneous assessment of a practically unlimited number of variables: therefore these methodologies are especially useful in various fields of chromatography such as thin-layer [29,30], gas [31,32], high-performance liquid chromatography [33,34], capillary zone electrophoresis [35] and micellar electrokinetic chromatography [36,37].

The objectives of the present investigation were to study the retention of the mono- and ditetrazolium salts on a polyethylene-coated alumina plate and column in ethanol-water mixtures at various concentrations of organic phase, to evaluate the retention data by multivariate mathematical-statistical methods and the comparison of the efficacy of the traditional and modified non-linear mapping technique and cluster analysis for this purpose, and to find the possible relationship between the retention characteristics and the physico-chemical parameters of the tetrazolium salts comparing the applicability of thin-layer (TLC) and high-performance liquid (HPLC) chromatography.

## Experimental

The IUPAC-names and chemical structures of the sixteen mono- and ditetrazolium salts are shown in tables I and II. The samples were separately dissolved in methanol to yield a concentration of 5 mg/mL for TLC and 0.05 mg/mL for HPLC.

The TLC plates were prepared as follows: 8 g of 20 – 80 μm diameter alumina particles coated with 2.5% polyethylene was mixed with water to reach a slurry and the slurry was spread on a glass plate of 20 × 20 cm. After an overnight drying at room temperature, 2 μL of sample solution were spotted onto the plate then it was developed with water-ethanol mixtures as eluents. Ethanol concentrations ranged from 0 – 45% (v/v) in steps 5% (v/v). The development was carried out in sandwich chambers (22 × 22 × 3 cm) at room temperature and the running distance was approx. 18 cm. The chambers were not presaturated. After development the plates were dried at 105 °C and the spots were visualised under UV light.

The $R_M$ value characterising the retention in TLC was calculated separately for each compound and for each ethanol concentration of the eluent:

$$R_M = \log(1/R_F - 1). \qquad (1)$$

The $R_M$ values were separately extrapolated to zero ethanol concentration:

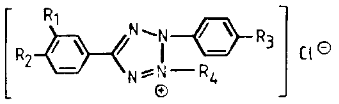
$$R_M = R_{M0} + b_{TLC} \cdot C \qquad (2)$$

where $C$ is the concentration of ethanol in the eluent (%, v/v) and $R_M$ is the actual $R_M$ value of the tetrazolium sample determined at this ethanol concentration. $R_{M0}$ and $b_{TLC}$ values from equation (2) were considered as the best means of estimating the lipophilicity ($R_{M0}$) [38] and the contact hydrophobic surface area ($b_{TLC}$) [39] of the tetrazolium salts.

The HPLC system contained a polyethylene-coated alumina (Al-PEE) column prepared in our laboratory (250 × 4 mm I. D. filled with 5 μm diameter alumina particles coated with 2.5% polyethylene) with a Shandon (Pittsburgh, PA, USA) analytical HPLC packing pump by the procedure proposed for the filling of reversed-phase columns. The flow rate was 0.7 mL/min and the detection wavelength was 254 nm. Although these molecules absorb better in higher wavelengths – because they are colour molecules – we carried out the measures at the mentioned lower wavelength because the circumstances of the measurement-sequence were designed for purity control too. At this wavelength the main components adsorb still enough good and the possible impurities also come into view.

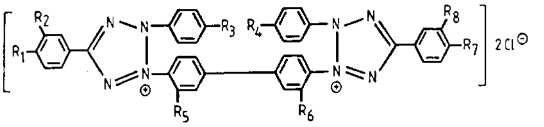
The HPLC system consisted of a Gilson Model 307 Piston Pump (Gilson, Villiers-le-Bel, France), a Gilson Model 116 variable-wavelength UV detector (Gilson), a Valco injector (Valco Instruments, Houston, TX, USA) with

**Table I.**



| *name* | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Triphenyltetrazolium chloride; TTC | -H | -H | -H | -Phe |
| Iodonitrotetrazolium chloride; INT | -H | -H | - I | -Phe-p-$NO_2$ |
| p-Nitrotetrazolium chloride; NTC | -H | -H | -$NO_2$ | -Phe |
| p-Nitrotetrazolium violet; NVT | -H | -H | -$NO_2$ | -Naph |
| Tetrazolium violet; VT | -H | -H | -H | -Naph |
| o-Tolyltetrazolium red; TRT | -H | -H | -H | -Phe-o-$CH_3$ |
| Iodopiperonyltetrazolium chloride; IPC | $R_1$-O-$CH_2$-O-$R_2$ | | -H | -Phe-p-I |

**Table II.**



| name | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | $R_8$ |
|---|---|---|---|---|---|---|---|---|
| Veratryltetrazolium chloride; VTC | $-OCH_3$ | $-OCH_3$ | -H | -H | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ |
| p-Anisylnitrotetrazolium blue; ANBT | $-OCH_3$ | -H | $-NO_2$ | $-NO_2$ | $-OCH_3$ | $-OCH_3$ | $-OCH_3$ | -H |
| m-Nitroneotetrazolium violet; NNVT | -H | $-NO_2$ | -H | -H | -H | -H | -H | $-NO_2$ |
| Tolidine-ditetrazolium violet; TVD | -H | -H | -H | -H | $-CH_3$ | $-CH_3$ | -H | -H |
| p-Nitrotetrazolium blue; pNBT | -H | -H | $-NO_2$ | $-NO_2$ | $-OCH_3$ | $-OCH_3$ | -H | -H |
| Neotetrazolium chloride. NTC | -H | -H | -H | -H | -H | -H | -H | -H |
| Tetrazolium blue; BT | -H | -H | -H | -H | $-OCH_3$ | $-OCH_3$ | -H | -H |
| Piperonyltetrazolium blue; PBT | $R_1$-O-$CH_2$-O-$R_2$ |  | -H | -H | $-OCH_3$ | $-OCH_3$ | $R_7$-O-$CH_2$-O-$R_8$ |  |
| m-Nitrotetrazolium blue; mNBT | -H | $-NO_2$ | -H | -H | $-OCH_3$ | $-OCH_3$ | -H | $-NO_2$ |

a 10 µL sample loop and a Waters 740 integrator (Waters-Millipore, Milford, MA, USA).

Mixtures of ethanol and water were used as eluents. The ethanol concentrations ranged from 90 – 97.5% (v/v) in steps 1.25% (v/v) because of the high sensitivity of the system for changing the concentration of the organic compound in the eluent. The retention time of each compound in each eluent was determined with three consecutive measures. The experiments were carried out at room temperature (22 ± 2 °C).

As the correlation between the logarithm of the capacity factor (*k'*) and the ethanol concentration in the eluent is generally linear in HPLC, the relationship was calculated:

$$\log k' = \log k'_0 + b_{\mathrm{HPLC}} \cdot C \qquad (3)$$

where $k'$ is the capacity factor; $k'_0$ value is the intercept and it is used to estimate the lipophilicity [40]; $b_{\mathrm{HPLC}}$ is the slope, related to the contact hydrophobic surface area of solutes in contact with support [39] and $C$ is the ethanol concentration in the eluent (%, v/v). Equation (3) was separately applied for each solute.

To find correlation between the physico-chemical parameters of solutes and their retention data, PCA and cluster analysis were applied [24]. To elucidate the influence of PCA on the data evaluation the cluster analysis was also applied to the original data matrix. The observations (independent variables) were the tetrazolium salts and the parameters of equation (2), equation (3) with the calculated physico-chemical values for each sample were the variables of following calculation. The physico-chemical parameters included in the calculation were: polarizability ($P'$) [41], refractivity ($\rho$) [42,43], 10 base logarithm of the lipophil-hydrophil character ($\log \pi$) [43,44], Van der Waals surface ($VdW_{\mathrm{surface}}$) [45-48], Van der Waals volume ($VdW_{\mathrm{volume}}$) [47], water accessible surface ($WA_{\mathrm{surface}}$) [45-48], water accessible volume ($WA_{\mathrm{volume}}$) [47], total energy ($E_{\mathrm{total}}$), binding energy ($E_{\mathrm{binding}}$), heat formation ($Q_f$), energy of the higher occupied molecular orbit ($eV_{\mathrm{HOMO}}$), energy of the lower unoccupied molecular orbit ($eV_{\mathrm{LUMO}}$), minimum charge of the atoms ($\delta_{\mathrm{min}}$), maximum charge of the atoms ($\delta_{\mathrm{max}}$) and dipole moment ($\xi$). These parameters were computed by HyperChem 5.01 with ChemPlus Extension (Hypercube, Waterloo, Canada). Structures were first optimised using molecular mechanic calculations [49]. The molecular modelling structural descriptors (energetic parameters) were computed using semi-empirical calculation method Austin Model 1 [50-52]. Altogether nineteen variables were enrolled in the investigation.

The PCA was calculated from the correlation matrix. Two-dimensional non-linear map and cluster dendogram of the PC loadings and variables were also calculated [26]. The inclusion of non-linear map technique (NLM) and cluster analysis in the evaluation was motivated by the consideration that they calculate and visualise the relative distances between the members of the matrix. The iteration of NLM was carried out to the point where the difference between the two last iterations was lower than $10^{-8}$.

To compare their information content, linear correlations were calculated between the corresponding co-ordinates of non-linear mapping and varimax rotation around two axes. These techniques are theoretically similar techniques: both methods calculate and visualise the relative distances between the members of the data matrix:

$$Y_{1,2} = a + b \cdot X_{1,2} \qquad (4)$$

where $Y_{1,2}$ are the co-ordinates of non-linear map; $X_{1,2}$ are the co-ordinates of varimax rotation.

## Results and discussion

The necessity of very different eluent systems used to elute samples with TLC and HPLC could explain only the great difference between selectivity of these chromatographic methods in respect of tetrazolium compounds. This phenomenon will try to more support later in present study.

The parameters of equation (2) are listed in table III. The relationship between these parameters ($R_M$, $b_{\mathrm{TLC}}$) was linear in all instances. In most cases the coefficient of correlation was greater than 0.99 confirming the applicability of the equation. The slope and intercept values differed considerably from each other and in addition they were divided into two different groups according to their chemical structure: the monotetrazolium salts are in the first group (Nos. 1-7), the ditetrazolium salts are in the second group (Nos. 8-16). This means that the two groups of tetrazolium salts can be easily separated from each other by TLC in ethanol-water eluent system. The parameters of equation (3) are compiled in table IV. In this case the relationship between these parameters ($\log k'$, $b_{\mathrm{HPLC}}$) was mostly approached by linear regression too. The intercept and slope values however, did not differ considerably from each other so it is not sure that the separation might be successfully improved by using an adequately chosen eluent system.

Why do the tetrazoles show this conspicuous different behaviour in thin-layer and liquid chromatographic systems whereas the support is the same? According to the microanalytical results the carbon contents of the support – related to the percentage of polyethylene covering – was statistically the same (8.023 ± 0.0004%) before and after the measurement of tetrazoles which means that the coating was not eluted from the column. The answer was found during the evaluation of the results of principal component analysis.

The original data matrix is shown in table V. PCA results are summarised in table VI. Five principal components explain more than 94% of the total variance. These results indicate that the 19 physicochemical and chromatographic parameters can be substituted by five background (imaginary) variables loosing only less than 6% of the total information. The principal component analysis does not prove but mathematically indicates the existence of these background-variables as measurable parameters.

The majority of the physicochemical parameters of molecules have high loadings in the first PC component indicating the marked influence of these parameters on the mode of retention of the polymer-coated alumina support. The lipophyl-hydrophyl character has high loading only in the second PC component with the minimum and maximum charge of atoms. This result indicates that mainly the shape and the polarity of molecules have great influence on their retention on polyethylene-coated support. The lipophyl-hydrophyl character of the molecules has only less influence on the retention characteristic.

**Table III.**

| tetrazolium | $R_M = R_{M0} + b_{TLC}C$ | | | |
|---|---|---|---|---|
| salts samples | $R_{M0}$ | $b_{TLC} \times 10^2$ | $s_b \times 10^2$ | $r$ |
| 1 | 1.22 | 5.41 | 1.58 | 0.9974 |
| 2 | 1.32 | 4.75 | 1.62 | 0.9954 |
| 3 | 1.09 | 4.87 | 1.04 | 0.9986 |
| 4 | 1.51 | 5.33 | 2.83 | 0.9902 |
| 5 | 1.69 | 5.67 | 2.47 | 0.9934 |
| 6 | 1.25 | 5.30 | 2.11 | 0.9953 |
| 7 | 1.95 | 6.21 | 2.16 | 0.9958 |
| 8 | 3.34 | 8.91 | 6.99 | 0.9850 |
| 9 | 2.99 | 7.35 | 5.98 | 0.9839 |
| 10 | 3.70 | 10.21 | 3.00 | 0.9983 |
| 11 | 3.25 | 8.94 | 3.11 | 0.9970 |
| 12 | 2.67 | 7.21 | 2.77 | 0.9963 |
| 13 | 2.13 | 6.48 | 5.39 | 0.9766 |
| 14 | 3.61 | 9.61 | 1.45 | 0.9995 |
| 15 | 3.31 | 8.14 | 2.34 | 0.9984 |
| 16 | 3.02 | 7.53 | 3.36 | 0.9960 |

**Table IV.**

| tetrazolium | $\log k' = \log k'_0 + b_{HPLC}C$ | | | |
|---|---|---|---|---|
| salts samples | $\log k'_0$ | $b_{HPLC} \times 10^2$ | $s_b \times 10^3$ | $r$ |
| 1 | 4.89 | 4.67 | 5.18 | 0.9820 |
| 2 | 7.51 | 7.60 | 17.52 | 0.9507 |
| 3 | 6.82 | 6.92 | 5.86 | 0.9929 |
| 4 | 8.79 | 8.98 | 16.57 | 0.9676 |
| 5 | 9.24 | 9.45 | 19.81 | 0.9588 |
| 6 | 8.96 | 9.15 | 19.26 | 0.9584 |
| 7 | 7.41 | 7.53 | 13.99 | 0.9672 |
| 8 | 7.39 | 7.57 | 8.32 | 0.9881 |
| 9 | 8.41 | 8.46 | 20.19 | 0.9241 |
| 10 | 8.73 | 8.76 | 19.13 | 0.9353 |
| 11 | 10.74 | 10.98 | 6.02 | 0.9970 |
| 12 | 10.14 | 10.32 | 11.75 | 0.9873 |
| 13 | 6.32 | 6.48 | 4.98 | 0.9884 |
| 14 | 4.79 | 4.88 | 5.34 | 0.9825 |
| 15 | 7.56 | 7.69 | 4.86 | 0.9941 |
| 16 | 4.34 | 4.04 | 10.94 | 0.9054 |

**Table V.**

| Tetrazolium | Parameters | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| salts | $P'$ | $\rho$ | $\log \pi$ | $VdW_S$ | $VdW_V$ | $WA_S$ | $WA_V$ | $E_{total}$ | $E_{bind}$ | $Q_f$ | $eV_{HOMO}$ | $eV_{LUM}$ | $\delta_{min}$ | $\delta_{max}$ | $\xi$ | $\log k'$ | $b_{HPLC}$ | $R_{M0}$ | $b_{TLC}$ |
| 1 | 35.61 | 92.46 | 5.75 | 323.13 | 286.51 | 545.39 | 914.75 | –79600.00 | –4070.00 | 414.90 | –12.72 | –4.44 | –0.14 | 0.18 | 1.76 | 4.89 | 4.67 | 1.22 | 5.41 |
| 2 | 42.48 | 112.19 | 9.96 | 379.59 | 334.21 | 628.22 | 1060.00 | –106000.00 | –4210.00 | 427.40 | –12.73 | –5.73 | –0.32 | 0.57 | 9.76 | 7.51 | 7.60 | 1.32 | 4.75 |
| 3 | 37.45 | 99.78 | 5.71 | 349.16 | 305.37 | 587.59 | 980.86 | –98800.00 | –4250.00 | 407.90 | –12.68 | –5.55 | –0.32 | 0.57 | 6.99 | 6.82 | 6.92 | 1.09 | 4.87 |
| 4 | 44.72 | 116.23 | 6.71 | 397.71 | 351.62 | 632.88 | 1090.00 | –11200.00 | –5020.00 | 427.20 | –12.25 | –5.43 | –0.32 | 0.57 | 6.37 | 8.79 | 8.98 | 1.51 | 5.33 |
| 5 | 42.88 | 108.91 | 6.76 | 372.13 | 332.30 | 597.10 | 1030.00 | –92100.00 | –4830.00 | 413.60 | –12.02 | –4.68 | –0.15 | 0.17 | 2.79 | 9.24 | 9.45 | 1.69 | 5.67 |
| 6 | 37.44 | 97.50 | 6.22 | 343.23 | 302.58 | 564.04 | 959.04 | –83200.00 | –4370.00 | 386.40 | –12.46 | –5.15 | –0.20 | 0.17 | 3.62 | 8.96 | 9.15 | 1.25 | 5.30 |
| 7 | 42.97 | 110.63 | 6.70 | 378.67 | 337.56 | 632.44 | 1070.00 | –105000.00 | –4390.00 | 355.80 | –11.59 | –5.34 | –0.20 | 0.23 | 5.60 | 7.41 | 7.53 | 1.95 | 6.21 |
| 8 | 85.27 | 222.77 | 9.63 | 852.61 | 106.28 | 2770.00 | 635.54 | –224000.00 | –10300.00 | 601.80 | –12.20 | –6.41 | –0.21 | 0.18 | 9.58 | 7.39 | 7.57 | 3.34 | 8.91 |
| 9 | 84.01 | 224.50 | 10.04 | 794.85 | 693.47 | 1200.00 | 2130.00 | –241000.00 | –9860.00 | 697.40 | –12.88 | –6.72 | –0.22 | 0.57 | 10.42 | 8.41 | 8.46 | 2.99 | 7.35 |
| 10 | 74.12 | 196.64 | 3.32 | 666.81 | 594.24 | 1040.00 | 1820.00 | –197000.00 | –8370.00 | 849.20 | –14.29 | –7.12 | –0.35 | 0.57 | 11.61 | 8.73 | 8.76 | 3.70 | 10.21 |
| 11 | 74.11 | 194.08 | 12.08 | 657.88 | 589.05 | 995.81 | 1780.00 | –166000.00 | –8600.00 | 811.30 | –13.60 | –6.70 | –0.22 | 0.17 | 0.20 | 10.74 | 10.98 | 3.25 | 8.94 |
| 12 | 79.07 | 209.56 | 2.82 | 729.63 | 643.63 | 1100.00 | 1970.00 | –219000.00 | –9110.00 | 777.80 | –13.73 | –6.96 | –0.32 | 0.57 | 13.45 | 10.14 | 10.32 | 2.67 | 7.21 |
| 13 | 70.44 | 184.00 | 11.14 | 621.66 | 566.09 | 969.47 | 1700.00 | –159000.00 | –8030.00 | 826.50 | –13.62 | –6.77 | –0.13 | 0.18 | 2.32 | 6.32 | 6.48 | 2.13 | 6.48 |
| 14 | 80.05 | 208.46 | 10.01 | 733.35 | 650.90 | 1120.00 | 1990.00 | –181000.00 | –8780.00 | 748.40 | –13.50 | –6.60 | –0.21 | 0.17 | 4.10 | 4.79 | 4.88 | 3.61 | 9.61 |
| 15 | 75.38 | 196.92 | 10.64 | 679.12 | 605.47 | 1040.00 | 1850.00 | –220000.00 | –9860.00 | 634.00 | –12.55 | –6.50 | –0.21 | 0.18 | 3.64 | 7.56 | 7.69 | 3.31 | 8.14 |
| 16 | 79.07 | 209.56 | 2.82 | 735.41 | 644.38 | 1110.00 | 1980.00 | –219000.00 | –9120.00 | 770.02 | –14.18 | –6.78 | –0.34 | 0.57 | 10.18 | 4.34 | 4.04 | 3.02 | 7.53 |

Back to the PCA results, while the thin-layer chromatographic parameters ($R_{M0}$, $b_{TLC}$) have high loadings in the first PC component, the high-performance liquid chromatographic parameters (log $k'_0$, $b_{HPLC}$) have high loadings only in the third PC component which explain only approx. the 11% of the total variance. This result shows that the TLC system is strictly different from the HPLC system for tetrazolium salts under circumstances mentioned above. Since the two methods show different behaviour in the reversed-phase environment, the TLC is not suitable as a pilot method in this case.

The two-dimensional non-linear map of PCA loadings is shown in figure 1. The TLC retention parameters form the first distinct cluster with polarizability ($P'$), refractivity ($\rho$), heat formation ($Q_f$) and Van der Waals surface ($VdW_{surface}$) values of the samples. An other cluster (second) appears with binding, total and the LUMO-energy ($E_{binding}$, $E_{total}$ and $eV_{LUMO}$) values. The third cluster including the HPLC retention parameters is quite separated from the first, second and the fourth cluster which contains water accessible and Van der Waals volume ($WA_{volume}$, $VdW_{volume}$) values. These results show that the retention of tetrazoles on a polymer coated alumina plate depends on their molar refractivity, steric effects and electronic forces. The far distance of the cluster of HPLC parameters indicates its difference from TLC. With removing the negative signs from the PC loadings matrix wanted to avoid this problem. The found values are shown in figure 2. which seems to be a same distribution, what was expected. Some of the PC loadings divided into two clusters in this figure: one of them contains the TLC parameters and almost all the physicochemical parameters, and the other one contains the HPLC parameters only. This spread supports our opinion mentioned above, and confirms the difference of HPLC from TLC again. It is very important that the parameters of lipophilicity, dipole moment, minimum and maximum charge of the atoms are not included in any cluster of the two-dimensional non-linear map calculated with two different ways. This is a very interesting result which means that these molecular features determine the retention behaviour by oneself both in the case of TLC and HPLC. These parameters cannot be replaced with background variables, as these parameters are not correlated with each other and with the rest.

The tetrazolium salts structurally belong to two great groups, divided into two clusters indeed in figure 3 which is the two-dimensional non-linear map of PCA variables. This figure reflects to the dendogram (Fig. 4) of cluster analysis calculated from the original data matrix. The difference appeared between two figures is explained by the fact that the PCA modifies the data matrix.

Good linear correlations were found between the first co-ordinates of two-dimensional non-linear map and varimax rotation ($n$ = 19):

$$n\text{lmap}_1 = 141.61 + (73.44 \pm 7.29) \times \text{varimax}_1$$

$$r_{\text{calc.}} = 0.9405; \qquad r_{99.9\%} = 0.6932.$$

The correlation between the second co-ordinates was not significant. These data indicate that both the varimax rotation and the non-linear mapping technique can be used for the two-dimensional visualisation of retention data matrices. The results obtained by the methods are similar but not identical.

**Table VI.**

| *Background variables* | *Eigenvalues* | *Cumulative explained variance values (%)* |
|---|---|---|
| 1 | 10.22 | 53.77 |
| 2 | 3.15 | 70.33 |
| 3 | 2.07 | 81.22 |
| 4 | 1.78 | 90.57 |
| 5 | 0.66 | 94.03 |

| *Parameters* | *No. of principal components* | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| $P'$ | 0.98 | 0.14 | 0.04 | −0.10 | 0.05 |
| $\rho$ | 0.98 | 0.11 | 0.03 | −0.10 | 0.06 |
| log $\pi$ | 0.11 | 0.72 | 0.19 | 0.14 | 0.55 |
| $VdW_{surface}$ | 0.96 | 0.14 | 0.04 | −0.20 | 0.06 |
| $VdW_{volume}$ | 0.73 | −0.18 | −0.15 | 0.60 | 0.12 |
| $WA_{surface}$ | 0.60 | 0.32 | 0.16 | −0.70 | −0.02 |
| $WA_{volume}$ | 0.80 | −0.16 | −0.14 | 0.52 | 0.12 |
| $E_{total}$ | −0.91 | −0.08 | 0.03 | 0.16 | −0.06 |
| $E_{binding}$ | −0.96 | −0.18 | −0.06 | 0.11 | −0.05 |
| $Q_f$ | 0.93 | 0.00 | −0.06 | 0.25 | −0.10 |
| $eV_{HOMO}$ | −0.70 | 0.33 | 0.29 | −0.34 | 0.22 |
| $eV_{LUMO}$ | −0.95 | 0.13 | −0.05 | −0.06 | −0.11 |
| $\delta_{minimum}$ | −0.23 | 0.88 | −0.07 | 0.17 | −0.02 |
| $\delta_{maximum}$ | 0.17 | −0.92 | 0.00 | −0.10 | 0.28 |
| $\xi$ | 0.41 | −0.72 | 0.08 | −0.48 | 0.11 |
| log $k'_0$ | 0.01 | −0.14 | 0.96 | 0.22 | −0.08 |
| $b_{HPLC}$ | 0.01 | −0.12 | 0.97 | 0.22 | −0.06 |
| $R_{M0}$ | 0.93 | 0.18 | 0.05 | −0.05 | −0.16 |
| $b_{TLC}$ | 0.85 | 0.22 | 0.06 | −0.04 | −0.33 |

| *Tetrazolium salts* | *No. of principal components* | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 1 | −4.29 | 1.14 | −2.30 | 0.04 | −0.76 |
| 2 | −2.70 | −1.62 | 0.07 | −0.42 | 1.46 |
| 3 | −3.31 | −1.87 | −0.62 | −0.50 | 0.40 |
| 4 | −3.10 | −1.64 | 0.96 | −0.02 | 0.41 |
| 5 | −3.58 | 1.03 | 1.15 | 0.52 | −0.63 |
| 6 | −3.84 | 0.29 | 0.86 | 0.44 | −0.66 |
| 7 | −3.20 | 0.50 | 0.07 | −0.36 | −0.13 |
| 8 | 2.23 | 2.40 | 1.12 | −4.30 | −0.23 |
| 9 | 3.33 | −0.25 | 0.63 | 0.19 | 1.63 |
| 10 | 3.61 | −2.41 | 0.41 | 0.19 | −1.50 |
| 11 | 2.23 | 1.70 | 2.14 | 2.08 | −0.33 |
| 12 | 3.14 | −2.67 | 1.45 | 0.17 | −0.39 |
| 13 | 1.18 | 1.93 | −1.14 | 1.35 | 0.60 |
| 14 | 2.96 | 1.76 | −2.06 | 0.57 | −0.11 |
| 15 | 2.09 | 1.75 | 0.15 | 0.55 | 0.52 |
| 16 | 3.26 | −2.05 | −2.88 | −0.49 | −0.28 |

Summarising our results it can be concluded that the polymer coated alumina support is suitable for the mapping of the chromatographic character of different tetrazolium salts and for TLC separation as well. Although TLC is usually suitable as a pilot method for HPLC, in present case, with present conditions, the thin-layer chromatographic method proved to be very different from the HPLC method. Furthermore it may become more clear that during separation of these compounds probably the mixture of steric and electronic interactions have higher possible roles than lipophilicity. Nevertheless, it is a great deal to evaluate further investigations for possible replacement of background variables found by us by measurable physico-chemical parameters. We must declare that the conclusions discussed above are valid only for this special data set.

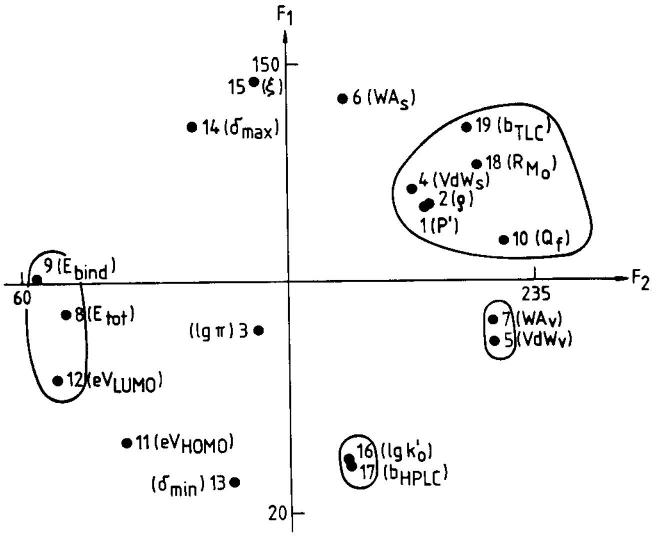


**Fig. 1.** Similarities and differences between the retention characteristics and physico-chemical parameters of mono- and ditetrazolium salts. Two dimensional non-linear map of PC loadings. Number of iterations: 182, maximum error: $4.7 \times 10^{-2}$. For symbols see experimental.

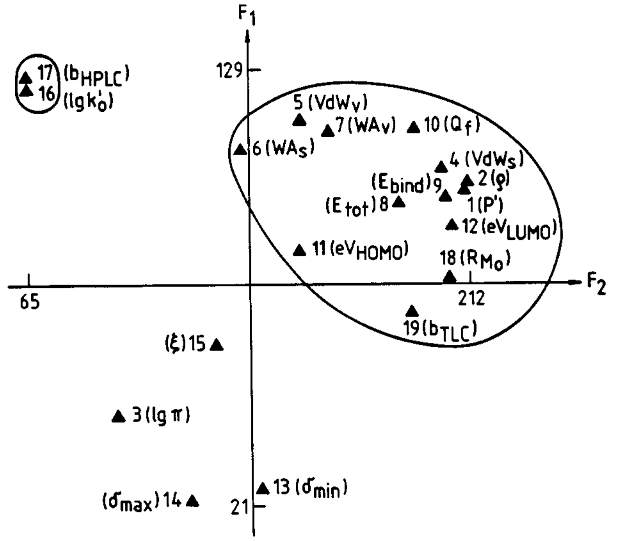


**Fig. 2.** Similarities and differences between the retention characteristics and physico-chemical parameters of mono- and ditetrazolium salts. Two dimensional non-linear map of PC loadings after elimination of negative signs. Number of iterations: 168, maximum error: $1.8 \times 10^{-2}$. For symbols see experimental.

According to the results it could be said that principal component analysis combined with two-dimensional nonlinear mapping and/or varimax rotation and cluster analysis is an adequate way to evaluate large retention data matrices for the aims of QSRR-investigations.

## Acknowledgements

This work has financially supported by the Hungarian-Polish and Hungarian-Spanish Scientific Co-operation.

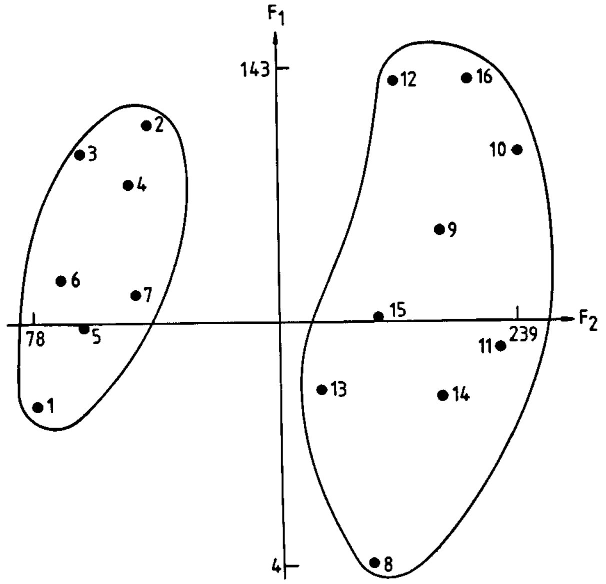


**Fig. 3.** Similarities and differences between the retention characteristics and physico-chemical parameters of mono- and ditetrazolium salts. Two dimensional non-linear map of PC variables. Number of iterations: 191, maximum error: $3.9 \times 10^{-2}$. For symbols see experimental.

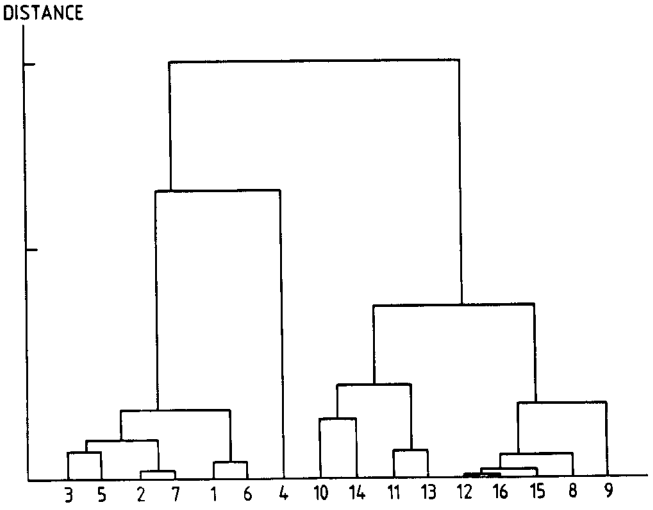


**Fig. 4.** Similarities and differences between the retention characteristics and physico-chemical parameters of mono- and ditetrazolium salts. Cluster dendogram calculated from the original data matrix. For symbols see experimental.

## References

1. Gami-Yilinkou, R.; Kaliszan, R. *J. Chromatogr.* **1991**, *550*, 573.
2. Kirkland, J. J.; Dilks, C. H.; Henderson, J. E. *LC·GC* **1993**, *6*, 436.
3. Claessens, H. A.; Vermeer, E. A.; Cramers, C. A. *LC·GC* **1993**, *6*, 692.
4. Hanson, M.; Unger, K. K.; Mant, C. T.; Hodges, R. S. *J. Chromatogr.* **1992**, *599*, 77.
5. Forgács, E. *J. Liquid Chromatogr.* **1993**, *16*, 3757.

6. McNeff, C.; Carr, P. W. *Anal. Chem*. **1995**, *67*, 2350.
7. Jilge, G.; Unger, K. K.; Esser, U. ; Schafer, H. J.; Rathergeber, G.; Müller, W. J. *J. Chromatogr*. **1989**, *476,* 37.
8. Kennedy, L. A.; Kopaciewicz, W., Regnier, F. E. *J. Chromatogr*. **1986**, *359,* 73.
9. Kurganov, A.; Kuzmenko, O.; Davankov, V. A.; Eray, B.; Unger, K. K.; Trübinger, U. *J. Chromatogr*. **1990**, *506*, 391.
10. Hailin, G.; Wallace, G. G. *J. Chromatogr*. **1991**, *588,* 25.
11. Hansen, M.; Unger, K. K.; Mant, C. T.; Hodges, R. S. *J. Chromatogr*. **1992**, *599,* 65.
12. Hansen, M.; Unger, K. K.; Mant, C. T.; Hodges, R. S. *J. Chromatogr*. **1992**, *599,* 77.
13. Van Landuyt, K. G.; Thienpont, L. M.; De Leenheer, A. P.; Stöckl, D. *J. Chrom. Sci*. **1994**, 32, 294.
14. Buszewski, B.; Schmid, J.; Albert, K.; Bayer, E. *J. Chromatogr*. **1991**, *552,* 415.
15. Gawdzik, B. *J. Chromatogr.* **1992**, *600,* 115.
16. Huskey, S. -E. W.; Miller, R. R.; Chiu, S. -H. L. *Drug Metab. Disp*. **1993**, *21*, 792.
17. Kambur, M. *Anal. Lett*. **1994**, *27,* 1999.
18. Yoshida, M.; Eguchi, T.; Ikekawa, N.; Saijo, N. *Interfer. Cytokin. Res*. **1995**, *15*, 265.
19. Gribbon, L. T.; Barer, M. R. *Appl. Environ. Microbiol*. *61*, **1995**, 3379.
20. Rozylo, J. K.; Janicka, M. *J. Liquid Chromatogr*. **1991**, 14, 3197-3212.
21. Rozylo, J. K.; Janicka, M. *J. Planar Chromatogr*. **1996**, *9,* 418-424.
22. Cserháti, T.; Forgách, E. *J. Chrom. Sci*. **1997**, *35,* 383-391.
23. Kaliszan, R. Quantitative Structure-Chromatographic Retention Relationships, Wiley-Interscience, New York, 1987.
24. Mardia, K. V.; Kent, J. T.; Bibbi, J. M. Multivariate Analysis, Academic Press, London, 1979; pp 213-254.
25. Orloci, L.; Rao, C. R.; Stitler, W. M. Multivariate Methods in Ecological Work, International Cooperative Publishing House, Fairland, MD, 1979.
26. Mager, H. Moderne Regressionsanalyse, Salle Sauerlanlander, Frankfurt am Main, 1982; pp 135-157.
27. Sammon Jr, J. W. *IEEE Trans. Comput*. **1969**, *C18,* 401-407.
28. Dillon, W. R. Multivariate Analysis, Wiley, New York, 1984; pp 157-208.
29. Forgách, E. *Fresenius J. Anal. Chem*. **1944**, *349,* 743-745.
30. García-March, F. J.; Antón-Fos, G. M.; Pérez-Giménez, F.; Salabert-Salvador, M. T.; Cercós-del-Pozo, R. A.; de Julián-Ortiz, J. V. *J. Chromatogr. A* **1996**, *719,* 45.
31. Szymoniak, K.; Chretien, J. R. *J. Chromatogr*. **1987**, *404,* 11-22.
32. Poole, S. K.; Kollie, T. O.; Poole, C. F. *J. Chromatogr. A* **1994**, *664*, 229.
33. Chretien, J. R.; Righezza, N.; Hassani, A.; Meklati, W. Y. *J. Chromatogr*. **1992**, *609,* 261-267.
34. Petrusewicz, J.; Gami-Yilinkou, R.; Kaliszan, R.; Pilarski, B.; Foks, H. *Gen. Pharmakol*. **1993**, *24*, 17.
35. Liang, H.-R.; Vuorela, H.; Vuorela, P.; Riekkola, M. -L.; Hiltunen, R. *J. Chromatogr. A* **1998**, *798*, 233.
36. Chen, Y. -C.; Lin, C. -E. *J. Chromatogr. A* **1998**, *802*, 95.
37. Wu, H. -L.; Huang, C. -H.; Chen, S. -H.; Wu, S. -M. *J. Chromatogr. A* **1998**, *802,* 107.
38. Gasparic, J. *Adv. Chromatogr.* **1992**, *31,* 153.
39. Horváth, Cs.; Melander, W.; Molnár, I. *J. Chromatogr*. **1976**, *125*, 129.
40. Valkó, K.; Olajos, S.; Cserháti, T. *J. Chromatogr*. **1990**, *499,* 361.
41. Miller, K. J. *J. Am. Chem. Soc*. **1990**, *112*, 8533.
42. Ghose, A. K.; Crippen, G. M. *J. Chem. Inf. Comput. Sci*. **1987**, *27*, 21.
43. Viswanadhan, V. N.; Ghose, A. K.; Revankar, G. N.; Robins, R. K. *J. Chem. Inf. Comput. Sci.* **1989**, *29*, 163.
44. Ghose, A. K.; Pritchett, N. N.; Crippen, G. M. *J. Comp. Chem.* **1988**, *9*, 80.
45. Hasel, W.; Hendrickson, T. F.; Still, W. C. *Tet. Comput. Meth.* **1988**, *1*, 103.
46. Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127-6129.
47. Bodor, N.; Gabanyi, Z.; Wong, C. *J. Am. Chem. Soc*. **1989**, *111*, 3783.
48. Gavezotti, A. *J. Am. Chem. Soc.* **1983**, *10*, 5220.
49. Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127.
50. Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. *J. Am. Chem. Soc.* **1985**, *107*, 3902.
51. Dewar, M. J. S.; Dieter, K. M. *J. Am. Chem. Soc.* **1986**, *108*, 8075.
52. Stewart, J. J. P. *J. Comp. Aided Mol. Design* **1990**, *4*, 1.