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# Neural network and experimental design to investigate the effect of five factors in ion-interaction high-performance liquid chromatography

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**Abstract**

The effect of five experimental parameters on the ion-interaction chromatographic retention of pesticides characterized by different polarity was investigated by means of experimental design and artificial neural network treatments. The factors considered were: (1) the mobile phase pH; (2) *N*, the alkyl-chain length of the IIR (ion-interaction reagent); (3) *CM*, the organic modifier concentration in the mobile phase (4) *CR*, the concentration of IIR and (5) *F*, the flow-rate. The use of fractional design and Hoke design allowed useful information to be drawn about the retention mechanism involved and to build, through artificial neural network treatment (ANN), a model characterised by both descriptive and predictive ability. Four neurons and a bias unit were employed.

The ANN proved to be a useful instrument in the optimisation of the chromatographic separation, as regards resolution and total analysis time: the experimental retention obtained in the optimal conditions always differed within 14% from the predicted ones. © 1998 Elsevier Science B.V.

*Keywords:* Neural networks, artificial; Experimental design; Factorial design; Hoke design; Chemometrics; Optimization; Pesticides

## 1. Introduction

Ion-interaction chromatography is based on the use of a reversed stationary phase that is dynamically modified by a suitable ion-interaction reagent (IIR) added to the mobile phase. The IIR, when flowing in isocratic conditions, induces a dynamic modification of the surface of the stationary phase with the formation of a double electrical layer adsorbed onto it [1,2]. Anionic and cationic species can thus be retained. Since, likely, not all the original reversed-phase sites are modified, ion-interaction and conventional reversed-phase mechanisms can coexist and alternatively predominate, as a function of the experimental conditions.

Many variables are involved, such as the chemical properties and the concentration of the ion-interaction reagent, the pH and the ionic strength of the mobile phase. The effects of the different factors on retention are often non linear and/or interdependent on each other [3–6].

It must be considered that variations of the experimental conditions affect both the retention of

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the analytes and the extent and the properties of the moiety adsorbed onto the stationary phase.

Due to the dependence on so many factors, the technique is very versatile and can be made suitable to solving different separation problems. But, on the other hand, the optimisation of the experimental conditions can be very complex. This is true when using univariate methods but it represents a challenge also when using multivariate treatments, due to the large number of the variables which must be simultaneously treated.

By using alkylammonium salts as the ion-interaction reagent, a chemometric study has been already performed in this laboratory to optimize five independent variables [7], namely the ion-interaction reagent concentration *CR*, the alkyl chain length *N*, the organic modifier concentration *CM*, the mobile phase pH and the flow-rate *F*. For the cationic analytes atrazine and simazine the use of experimental design methods and multivariate analysis permitted to build mathematical models capable of correlating the chromatographic response (retention time) to the factors considered or to their combination. The models showed good descriptive ability, which contributed well to a better knowledge of the retention processes involved, together with a good predictive ability.

In this paper an optimisation treatment was performed by the use of artificial neural network in the separation of 5 widely diffused pesticides, characterised by different chemical properties.

In particular three quite hydrophilic species (the phenoxyacids: 2,4,5-trichlorophenoxyacetic acid or 2,4,5-T, 2,4-dichlorophenoxyacetic acid or 2,4-D and (±)2-(2,4-dichlorophenoxy) propionic acid or DCP), a less hydrophilic compound (2-methyl-4,6-dinitrophenol or DNOC), and a species characterised by predominantly lipophilic characteristics (5-bromo-6-methyl-5-butyluracil or bromacil) were considered. Structures are shown in Fig. 1.

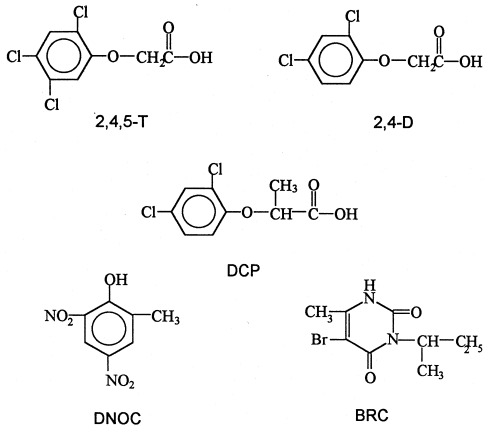


Fig. 1. Molecular structures of the five pesticides considered. 2,4,5-T=2,4,5-trichlorophenoxyacetic acid; 2,4-D=2,4-dichlorophenoxyacetic acid; DCP=(±)2-(2,4-dichlorophenoxy) propionic acid; DNOC=2-methyl-4,6-dinitrophenol; BRC=5-bromo-6-methyl-5-butyluracil.

## 2. Theory

### 2.1. Fractional factorial design and Hoke design

Factorial designs were first introduced by Fisher in 1926 [8]. Full and fractional designs, widely described elsewhere [9,10], are here shortly summarised.

In the factorial designs each factor is investigated at fixed levels: the most common is the two level factorial design, characterised by the orthogonality of the factors. Full factorial design contains all the possible combinations of the selected settings of the experimental factors, so that a 2-level full factorial design requires, $n$ being the number of the factors, $2^n$ experiments. Representing the two levels of each factors with $+1$ and $-1$, the $j$ columns of the experimental matrix is obtained writing $2^{j-1}$ times $-1$ and $2^{j-1}$ times $+1$ as many times to get $2^n$ values.

Full factorial design allows, directly from the experimental matrix, calculation of the effects of the factors and of all the possible multi-factor interactions: to calculate the effect of an original factor it is sufficient to sum up the responses obtained for each experiment with the sign of the correspondent element in the experimental matrix. The sum is then divided for half the number of the experiments. Analogously, the interactions effect can be calculated determining the columns of signs by multiplying the columns of signs corresponding to the factors taking part in the interaction.

As a disadvantage, besides the high number of

experiments, the 2-level full factorial design does not consider possible curvatures.

A way to perform a lower number of experiments, when treating the same number of factors, is the use of fractional factorial design. This is possible by associating new original factors to the higher order interaction columns of signs. This operation leads to a partial loss of information, since it results in it being impossible to discriminate between the effects or the interactions of the effects.

To determine the eventual presence of second order effects it is necessary to sample at least three levels of experiments. A diffused plan of this kind is the star design, in which the experiments are performed along the factor axes and at their intersection and which leads to the calculation of regression models containing first order and squared factors, but no interaction terms.

To obtain a model containing the main factors plus the interactions and the squared terms the use of a central composite design is required, which results from the addition of a factorial design and of a star design.

A particular kind of central composite design is the Hoke design [11]. This plan is useful whenever the results of the experiments lie on the limits of the dominion of the variables and these limits cannot be experimentally enlarged due to physico–chemical constrictions. This design consists in the superimposition of two star-designs indicated as external and internal star designs (see the scheme of an Hoke design for two factors and three levels in Fig. 2). For three-levels and $f$ factors, the Hoke design consists of $2(2f \times 2)+1)$ experiments.

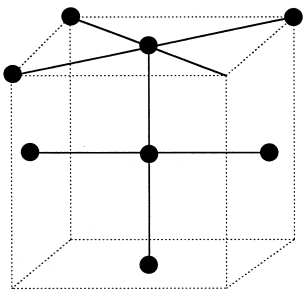

Fig. 2. Hoke design for two factors and three levels (9 experiments).

### 2.2. Artificial neural networks

The use of ANNs (artificial neural networks) is recently gaining a lot of interest and many kinds of ANN, simulating the activities of the human brain, have been developed. Applications in classification, modelling, mapping and association have been reported in literature [12,13]. The examples of chemical application concern kinetic studies [14], spectroscopy [15–17] and electrochemistry [18]. In HPLC optimisation ANNs have been used for peak-tracking [19], in response surface modelling and mobile phase optimisation [20].

This paper deals with the development, by means of ANN, of a surface response modelling for an ion-interaction RP–HPLC system.

A neural network treatment, with respect to MLR, can be very useful when non linear dependencies are supposed to be present, as those induced by pH variations [21,22] or unpredictable interactions between the variables.

Neural networks do not require the explicitation of a mathematical model. The final model is built through a continuous and iterative adjustment of the weights which are assigned to each variable $x_i$ considered, through the implicit building of the transfer function.

In particular, a BNN (backpropagation neural network) has been used, that is typically characterized by three layers: the input, the hidden and the output: the general processing unit is reported in Fig. 3.

The back-propagation function is a development of neural networks and can use continuous values of the variables, scaled to range between $-1$ and $+1$. Both the input and the output values are expressed as real numbers. The activation of a neuron is defined as the sum of weighted input signals to that neuron:

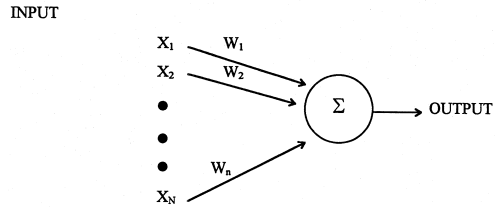


Fig. 3. Scheme of an artificial neuron.

$$A_k = \sum_i w_{ik} x_i + B_k$$

with $B_k$ being the bias value, $x_i$ the input variables and $w_{ik}$ the weights that must be adjusted.

$A_k$ is transferred to the neuron output by means of a transferability function that assumes the expression of a sigmoid transfer function *f(x)* with an activation value ranging between $-1$ and $+1$:

$$f(x) = \frac{1}{(1 + e^{-A_k})}$$

The function is centred on the zero value and this means that every net unit is switched on when activation is greater than zero and is switched out when activation is lower than zero. In order not to force the level 0 to assume the absolute value 0, an external input is added, called bias, which represents the value of the threshold, i.e. the value to which the unit naturally trends in the absence of any other external stimulation.

The learning mechanism of the back-propagation network works in a cyclic manner. Given $x_i$ as the input data and $D$ as the desired output value, the network computes successive values of the output $Y_i$ which are compared with $D_i$ and the error evaluated. This error is back-propagated and the weights successively adjusted up to reach convergence, i.e. when the error obtained becomes lower than that admitted.

## 3. Experimental

### 3.1. Apparatus

The analyses were carried out with a Merck–Hitachi Model L-6200 Lichrograph chromatograph (Tokyo, Japan) equipped with a two channel Merck–Hitachi Model D-2500 Chromato-integrator and interfaced with a Model L-4200 UV–Vis detector.

A Metrohm 654 pH meter (Herisau, Switzerland), equipped with a combined glass-calomel electrode was employed for pH measurements.

### 3.2. Reagents

Ultrapure water from a Millipore Milli-Q System (Milford, MA, USA) was used for the preparation of all the solutions. Octylamine and orthophosphoric acid were Fluka (Buchs, Switzerland) analytical-grade chemicals. Analytes (2,4,5-T, 2,4-D, DCP, DNOC and bromacil) were all purchased by Labservice Analytica (Anzola Emilia, Italy) and acetonitrile was Merck (Darmstadt, Germany) analytical grade chemicals.

### 3.3. Chromatographic conditions

An end-capped Superspher 100 RP-18 column (250.0×4.6 mm, 4 μm) (Merck) and a (15.0×4.6 mm) LiChrospher RP-18 (5 μm) guard precolumn were used.

The experiments planned by the experimental design required a number of eluents prepared with different combinations of the values of the 5 variables considered, ($N$ = alkyl chain length, mobile phase pH, $CM$ = organic modifier concentration, $CR$ = IIR concentration, $F$ = flow-rate). The table of experiments (Table 1) guarantees for the randomisation of the experiments.

The chromatographic system was conditioned by passing, in isocratic conditions, the eluent through the column until a stable baseline signal was reached and when reproducible capacity factors were obtained for three subsequent injections, (a minimum of 1 h, at flow-rate 1 ml/min, was usually necessary).

Spectrophotometric detection at 230 nm was employed.

## 4. Results

### 4.1. Fractional factorial design

Preliminary experiments permitted the identification of the variable space within which all the analytes present retention times greater than the dead time and lower than about 60 min.

A two-level fractional experimental design was first performed, that for 5 variables, consists of 16 experiments. The results (retention times, min) are reported in Table 1 where the signs + and − respectively indicate the maximum and the minimum value of the variables (see Table 2).

As previously said, the results of the experiments of this design permit the estimation of the effects of

Table 1
Fractional Factorial Design. Experimental retention times (min) for the five pesticides as a function of mobile phase pH, alkyl-chain length *N*, organic modifier concentration *CM*, ion-interaction reagent concentration *CR* and mobile phase flow-rate *F*

| Exp. | pH | *N* | *CM* | *CR* | *F* | 2,4,5-T | 2,4-D | DCP | BRC | DNOC |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | − | − | − | − | + | 23.6 | 12.7 | 23.2 | 14.2 | 33.6 |
| 2 | + | − | − | − | − | 6.3 | 5.8 | 5.8 | 23.9 | 5.8 |
| 3 | − | + | − | − | − | 58.9 | 33.7 | 53.9 | 25.6 | 64.4 |
| 4 | + | + | − | − | + | 14.3 | 8.6 | 10.3 | 12.9 | 10.3 |
| 5 | − | − | + | − | − | 19.8 | 13.4 | 21.7 | 13.8 | 30.2 |
| 6 | + | − | + | − | + | 3.2 | 3.2 | 3.2 | 7.6 | 3.2 |
| 7 | − | + | + | − | + | 11.8 | 7.8 | 12.1 | 7.9 | 16.5 |
| 8 | + | + | + | − | − | 7.4 | 6.2 | 6.6 | 12.9 | 7.2 |
| 9 | − | − | − | + | − | 47.0 | 25.5 | 44.5 | 26.5 | 60.2 |
| 10 | + | − | − | + | + | 8.8 | 5.6 | 6.6 | 14.3 | 6.1 |
| 11 | − | + | − | + | + | 54.3 | 29.8 | 43.2 | 14.5 | 41.3 |
| 12 | + | + | − | + | − | 61.3 | 32.8 | 42.6 | 24.0 | 40.0 |
| 13 | − | − | + | + | + | 11.1 | 7.5 | 11.8 | 7.8 | 16.4 |
| 14 | + | − | + | + | − | 6.6 | 5.6 | 5.9 | 13.4 | 6.4 |
| 15 | − | + | + | + | − | 32.7 | 20.9 | 30.8 | 13.8 | 36.5 |
| 16 | + | + | + | + | + | 8.7 | 6.2 | 7.1 | 7.4 | 8.4 |

The factor experimental values range between − and +, (see Table 2).

Table 2
Factor experimental range

| | pH | *N* | *CM* | *CR* | *F* |
|---|---|---|---|---|---|
| + | 8 | 8 | 40 | 0.010 | 0.9 |
| − | 4 | 4 | 30 | 0.001 | 0.5 |

the principal factors and of their second order interactions, that are presented in Table 3. It is very easy to read the information contained in the data of the table, since the effect of the different factors is expressed by a numeric value and the sign gives the direction of the effect.

It can be so observed that the lowest factor effects are played on retention of bromacil: this result can be correlated to the predominantly lipophilic properties of this species with respect to the more hydrophilic phenoxyacids and DNOC. It has been already assumed [23] that under the same experimental conditions of stationary and mobile phase, analytes characterised by different chemical properties can be retained through different interaction mechanisms.

Table 3
The effects of the factors and of their interactions calculated for the five analytes from the fractional factorial design

| Factors | 2,4,5-T | 2,4-D | DCP | BRC | DNOC |
|---|---|---|---|---|---|
| *N* | 15.4 | 8.3 | 10.5 | −0.3 | 7.8 |
| *CR* | 10.7 | 5.3 | 7.0 | 0.4 | 5.5 |
| *CM* | −21.7 | −10.5 | −16.4 | −8.9 | −17.1 |
| pH | −17.8 | −9.7 | −19.1 | −1.0 | −26.5 |
| *F* | −13.0 | −7.8 | −11.8 | −8.4 | −14.3 |
| *N*×*CR* | 5.5 | 14.3 | 3.2 | −0.4 | 1.4 |
| *N*×*CM* | −103 | −5.5 | −6.9 | 5.6 | −4.7 |
| *N*×pH | 1.3 | 0.1 | 0.8 | −0.2 | 3.3 |
| *N*×*F* | −4.8 | −2.5 | −3.5 | 0.0 | −3.5 |
| *CR*×*CM* | −6.4 | −2.9 | −3.9 | −0.31 | −2.9 |
| *CR*×pH | 2.9 | 6.5 | 2.1 | 0.1 | 3.1 |
| *CR*×*F* | −3.2 | −1.1 | −1.9 | 0.0 | −3.4 |
| *CM*×pH | 5.5 | 2.6 | 5.7 | 0.5 | 7.9 |
| *CM*×*F* | 5.1 | 2.5 | 4.1 | 2.6 | 5.4 |
| pH×*F* | 1.4 | 1.1 | 3.4 | 0.4 | 6.5 |

While ion-interaction mechanisms govern the retention of the more hydrophilic analytes, conventional reversed-phase mechanisms likely intervene in determining the retention of the more lipophilic bromacil: the unmodified sites still present on the reversed-phase surface are used. In agreement with this hypothesis bromacil retention does not practically depend on the alkyl-chain length $N$ (value of the coefficient $= -0.30$), on the concentration of the IIR (0.36) and very little also on the mobile phase pH ($-0.96$). On the contrary, the major effects on bromacil retention are played by the flow-rate and the organic solvent concentration, as is usual for a reversed-phase mechanism.

For all the other analytes, a dependence of retention on the other factors considered was verified. In particular it can be observed that the retention decrease induced by organic solvent increase is generally higher than that observed in a reversed-phase mode. This result can be explained by considering, besides the increased eluotropic strength of the eluent, the effect that the organic solvent exerts on the modification of the stationary phase, when the organic solvent competes for the surface of the stationary phase with the moiety already adsorbed. The increased solvent eluotropic strength and the lower extent of surface modification play concomitant roles, in decreasing retention of the more hydrophilic analytes [24,25].

Also, as regards the effect of the interaction between the factors, it is possible (Table 3) to distinguish a different behaviour of bromacil with respect to the other analytes.

### 4.2. Hoke design

A new plan of experiments was set, again considering the chemical restrictions of the system (as for example the pH range compatible with the silica-based stationary phase) and imposing a reasonable total analysis time ($\leq 60$). To evaluate the eventual presence of second order dependencies, a three-level design is required.

In our conditions, where the space of the variables cannot be further enlarged, and the maximum of the response surface obtained from the factorial design resulted in being shifted towards the limits of the variable dominion, the Hoke design seemed to be the most suitable. As said, this consists in two star designs (internal and external) and it requires for 5 factors ($f$): $(2f \times 2) + 1 = 21$ experiments. The levels investigated ($-$, 0, $+$) are reported in Table 4a,b. In Table 5a–c are shown the results obtained for the Hoke design, where 0 indicates the central point.

### 4.3. Artificial neural networks

To build a model able to correlate the retention of the analytes to the different experimental conditions, taking into account possible nonlinear dependence between retention and the experimental parameters, a back-propagation neural network function was used.

The algorithm used, developed by one of the authors, consists of four neurons and a bias unit (Fig. 4).

The treatment started with three neurons and the number of calculation cycles was increased up to convergence (response error between two successive cycles lower than $1 \cdot 10^{-5}$) . The correlation coefficient resulting was too poor, so that it was necessary to introduce another neuron. With four neurons, 150 cycles were required to achieve convergence. Correlation coefficients were always greater than 0.9911. In Fig. 4, the final weights obtained after the completion of the iterative processes are presented, together with the $R^2$ correlation coefficients.

In order to verify the predictive ability of the model in the chromatographic optimization process, two sets of conditions able to guarantee the resolution of all the analytes in a reasonable total analysis time were identified. For this purpose, a grid-search algorithm and the obtained ANN model were used. The neural model was used to calculate the retention for the different analytes under given

Table 4
Factor experimental values ranges for the Hoke Design

| | pH | *N* | *CM* | *CR* | *F* |
|---|---|---|---|---|---|
| a) *Internal star* | | | | | |
| + | 8 | 8 | 40 | $10.0 \cdot 10^{-3}$ | 0.9 |
| − | 4 | 4 | 30 | $1.0 \cdot 10^{-3}$ | 0.5 |
| 0 | 6 | 6 | 35 | $5.5 \cdot 10^{-3}$ | 0.7 |
| b) *External star* | | | | | |
| + | 7 | 7 | 38 | $8.0 \cdot 10^{-3}$ | 0.8 |
| − | 5 | 5 | 32 | $1.0 \cdot 10^{-3}$ | 0.6 |
| 0 | 6 | 6 | 35 | $5.5 \cdot 10^{-3}$ | 0.7 |

Table 5
Hoke Design. Experimental retention times (min) for the five pesticides as a function of the five factors considered

| pH | *CM* | *CR* | *F* | *N* | 2,4,5-T | 2,4-D | BRC | DCP | DNOC |
|---|---|---|---|---|---|---|---|---|---|
| a) *Internal star* | | | | | | | | | |
| + | 0 | 0 | 0 | 0 | 7.04 | 5.33 | 11.30 | 5.92 | 6.34 |
| − | 0 | 0 | 0 | 0 | 8.32 | 6.08 | 11.04 | 7.07 | 8.45 |
| 0 | + | 0 | 0 | 0 | 6.56 | 5.15 | 10.02 | 5.70 | 6.40 |
| 0 | − | 0 | 0 | 0 | 14.02 | 8.80 | 15.94 | 10.61 | 10.85 |
| 0 | 0 | + | 0 | 0 | 9.41 | 6.66 | 11.71 | 7.68 | 8.29 |
| 0 | 0 | − | 0 | 0 | 6.56 | 5.01 | 11.78 | 5.54 | 6.08 |
| 0 | 0 | 0 | + | 0 | 7.07 | 5.17 | 10.16 | 5.86 | 6.32 |
| 0 | 0 | 0 | − | 0 | 10.16 | 7.30 | 13.86 | 8.37 | 8.93 |
| 0 | 0 | 0 | 0 | + | 11.73 | 8.05 | 9.38 | 11.84 | 10.16 |
| 0 | 0 | 0 | 0 | − | 6.75 | 5.06 | 12.37 | 5.70 | 6.11 |
| b) *External star* | | | | | | | | | |
| + | 0 | 0 | 0 | 0 | 7.20 | 5.41 | 11.49 | 6.02 | 6.27 |
| − | 0 | 0 | 0 | 0 | 17.39 | 11.07 | 11.89 | 17.36 | 23.20 |
| 0 | + | 0 | 0 | 0 | 5.86 | 4.80 | 9.28 | 5.22 | 5.76 |
| 0 | − | 0 | 0 | 0 | 17.33 | 10.37 | 17.57 | 12.80 | 12.05 |
| 0 | 0 | + | 0 | 0 | 9.92 | 6.98 | 11.73 | 8.10 | 8.58 |
| 0 | 0 | − | 0 | 0 | 4.67 | 4.05 | 11.84 | 4.10 | 4.40 |
| 0 | 0 | 0 | + | 0 | 6.40 | 4.69 | 9.01 | 5.36 | 5.63 |
| 0 | 0 | 0 | − | 0 | 11.89 | 8.58 | 16.80 | 9.79 | 10.29 |
| 0 | 0 | 0 | 0 | + | 18.80 | 12.05 | 11.92 | 14.72 | 14.91 |
| 0 | 0 | 0 | 0 | − | 5.65 | 4.45 | 11.87 | 4.90 | 5.33 |
| c) *Central point* | | | | | | | | | |
| 0 | 0 | 0 | 0 | 0 | 9.2 | 6.5 | 11.8 | 7.8 | 8.0 |

conditions and the grid-search algorithm was used to select the best conditions of resolution. Total analysis times of 20 and 40 min respectively were imposed. The two sets of variables so evaluated are reported in Table 6.

The results reported in Table 7 show a very

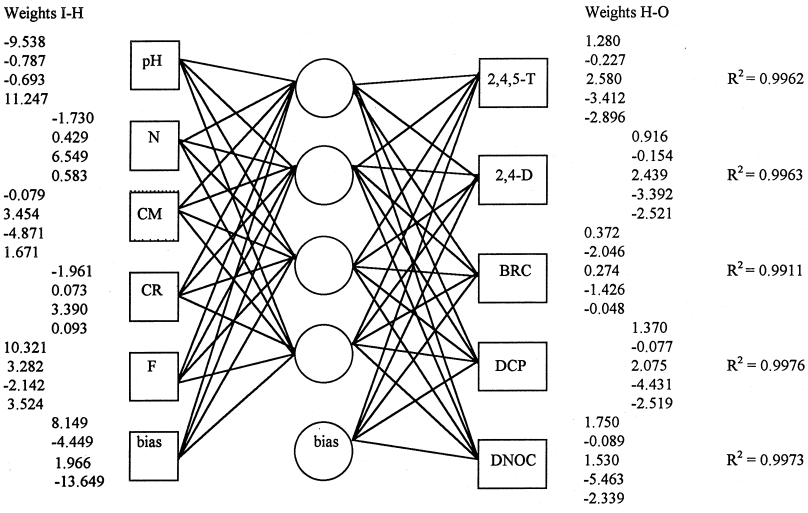


Fig. 4. Scheme of the ANN used, consisting of four neurons and a bias unit; final weights and correlation coefficients $R^2$.

Table 6
The two sets of conditions given by the ANN model, imposing 20 (experiment 1) and 40 min (experiment 2) as total analysis time and the resolution of all the analytes

| Exp. | pH | *N* | *CM* | *CR* | *F* |
|---|---|---|---|---|---|
| 1 | 5.3 | 6 | 30.0 | $3.0\times10^{-3}$ | 0.6 |
| 2 | 4.6 | 5 | 30.0 | $8.0\times10^{-3}$ | 0.5 |

satisfactory agreement, (ranging between 2.5–14.2%) between the predicted and the experimental retention times. As an example, Fig. 5 reports the separation obtained in about 20 min.

It is also interesting to note that in the two sets of conditions, the experimental elution sequence of the analytes is different. This is likely due to the chemical properties of the analytes and in particular to the already discussed lower hydrophilicity of bromacil and partially of DNOC. Their retention is therefore less affected, with respect to the other analytes, by most of the involved factors which characterize ion-interaction mechanisms. It is worthwhile to underline that the ANN model was able to predict this elution sequence inversion, that is an example of how complex the dependence of retention is on the different variables involved.

In conclusion, it can be said that the Neural Network model seems to have 'learnt' well, the mechanisms that govern the retention of the analytes in the ion-interaction chromatographic conditions here used.

Table 7
Predicted and experimental retention times. Total analysis time imposed: a) 20 min and b) 40 min

| | Predicted retention time | Experimental retention time |
|---|---|---|
| a) *Exp. 1* | | |
| 2,4,5-T | 18.1 | 17.1 |
| 2,4-D | 11.2 | 10.5 |
| DCP | 14.5 | 13.0 |
| BRC | 19.9 | 20.4 |
| DNOC | 16.2 | 14.1 |
| b) *Exp. 2* | | |
| 2,4,5-T | 34.3 | 30.3 |
| 2,4-D | 19.8 | 18.3 |
| DCP | 30.5 | 27.8 |
| BRC | 24.1 | 25.5 |
| DNOC | 37.9 | 32.5 |

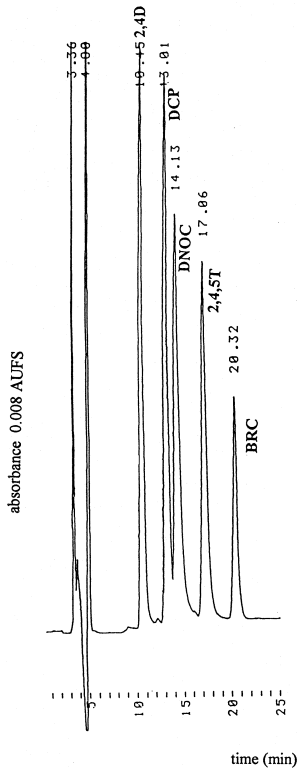


Fig. 5. Chromatogram obtained in the experimental conditions predicted by the ANN model and reported in Table 6 (experiment 1). Separation of 2,4-D, DCP, DNOC, 2,4,5-T and BRC (1.0 mg/l each). Stationary phase: Merck 4-μm Superspher 100 RP-18. Mobile phase: 3.0 m*M* hexylamine orthophosphate in water–acetonitrile (70:30), at pH 5.30. Flow-rate, 0.6 ml/min. Spectrophotometric detection at 230 nm.

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