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Reversed-phase high-performance liquid chromatography in quantitative structure–activity relationship studies of new fungicides

A. Niewiadomy[a,*], J. Matysiak[a], A. Żabińska[b], J. K. Różyło[b], B. Senczyna[a], K. Jóźwiak[c]

[a]*Department of Chemistry, University of Agriculture, Akademicka 15, 20-950 Lublin, Poland*
[b]*Faculty of Chemistry, M. Curie-Skłodowska University, 20-031 Lublin, Poland*
[c]*Department of Inorganic and Analytical Chemistry, Medical School, Staszica 6, Lublin, Poland*

Abstract

The retention behaviour of 2,4-dihydroxythiobenzanilides in a reversed-phase high-performance liquid chromatographic (HPLC) system has been examined. Using methanol–water or acetonitrile–water systems as the mobile phases, a linear relationship between the volume fraction of the organic modifier, φ, and the capacity factor, $\log k'$, was established for each solute over the whole examined range of methanol concentration and the limited range of acetonitrile content in the mobile phase. It was permitted to determine $\log k'_w$ values by extrapolation technique. For the system with acetonitrile the parabolic dependence of this relationship was also examined. The different correlation curves for each compound indicate selective effects upon retention due to solute–solvent and solute–stationary phase interaction. In the case of dihydroxythiobenzanilides buffering of the mobile phase at a proper pH is necessary. Studies on relationship between fungistatic activity of the compounds against the *Botrytis cinerea* and lipophility expressed by $\log k'_w$ have shown a great usefulness of HPLC method to evaluation of hydrophobic nature of biologically active substances in relation to biological systems.

Keywords: Quantitative structure–retention relationships; Mobile phase composition; Hydrophobicity; Organic modifiers; Pesticides; Dihydroxythiobenzanilides; Anilides

1. Introduction

The aim of studies on quantitative structure–activity relationships (QSARs) introduced by Hansch and co-workers [1,2], is the correlation of biological activity with chemical structure for congeneric series of compounds. Most of these studies, have shown that the inhibitory action of the substances is predominantly a function of their hydrophobic nature [3].

To the determination of hydrophobic nature of bioactive compounds, chromatographic techniques can be used, especially thin-layer chromatography (TLC) [4–6] and reversed-phase high-performance liquid chromatography (HPLC) [7–9].

The analytical tool to assess selective effects of specific molecular structures is the variation of the logarithm of capacity factor of the sample with the organic modifier content of the mobile phase [7,9,10]:

$$\log k' = -S\varphi + \log k'_w \quad (1)$$

where φ is the volume fraction of organic solvent in the water–organic solvent mixture; S is the slope of the regression curve, and should be related to the solvent strength of the pure organic solvent; $\log k'_w$ (lipophilicity index) is the logarithm of the capacity factor for 100% water as eluent. The obtained linear

*Corresponding author.

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dependences allow to extrapolate the data obtained for water–organic mixtures to water as mobile phase, even for compounds which do not migrate with water alone. It is shown that $\log k'_w$, a theoretical capacity factor obtained by extrapolation of retention data in binary solvent systems to pure aqueous eluent, is suitable for eliminating the selective effects and thereby for quantitatively describing the hydrophobic nature of solutes independently of the nature of the organic modifier and better show correlation with biological activity [9,11–13].

It was shown [14] that the effect of organic modifier is not strictly linear. They suggested that the relationship between solute retention ($\log k'$) and the concentration of the modifier φ can be expressed by the quadratic equation:

$$\log k' = \log k'_w + B\varphi + A\varphi^2 \quad (2)$$

Within the research of compounds with biological activity in the group of sulphur analogues of carboxyanilides it has been obtained 2,4-dihydroxythiobenzanilides modified in the *N*-aryl ring (Table 1), which have fungicidal properties [15]. This is a new group of the compounds which show the interactions typical for anilides, i.e., the ability for blocking of the functions of succinate-Q-reductase coenzyme [16,17] as well as the properties of sulphur inhibitors of respiration and oxidative phosphorylation processes [18–20]. The synthesized compounds were tested in relation to phytopathogenic fungi *Botrytis cinerea* to study the correlation between biological activity and lipophility parameters ($\log k'_w$) determining probably the ability of these compounds to diffuse through cell membranes.

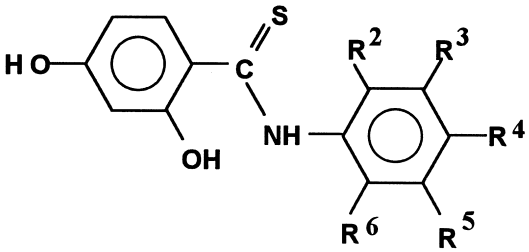
The aspects studied include the influence of the nature of organic modifier (methanol, acetonitrile), the buffering of the mobile phase and the structure of the substituents on the chromatographic parameters, $\log k'_w$, as well as the possibilities of utilisation of these parameters to evaluation of lipophilic nature of the compounds in relation to biological systems.

2. Experimental

2.1. Materials

The dihydroxythiobenzanilides used (Table 1) were synthesized in the laboratory at the Department of Chemistry in University of Agriculture, Lublin, Poland (new synthesis method, patent pending). All reagents were analytical reagent grade (LiChrosolv, gradient grade, Merck, Darmstadt, Germany). A 5-

Table 1



Chemical structure of 2,4-dihydroxythiobenzanilides

No.	Substituents	No.	Substituents
I	R^2–R^6=H	**X**	R^2=–Cl, R^4=–CH_3
II	R^3=–CH_3	**XI**	R^3=–CF_3
III	R^2, R^4=–CH_3	**XII**	R^3=–OCH_3
IV	R^3=–F	**XIII**	R^4=–OC_2H_5
V	R^2=–Cl	**XIV**	R^3=–OH
VI	R^3, R^4=–Cl	**XV**	R^4=–OH
VII	R^2, R^5=–Cl	**XVI**	R^2=–OH, R^4=–CH_3
VIII	R^2=–Br	**XVII**	R^4=–$CONHCH_2CO_2H$
IX	R^4=–I		

μm Eurosil Bioselect 300 RP-18 column (30 cm×4 mm, Vertex) was used.

2.2. UV–Vis

UV–Vis spectra were registered by means of UV-160A Shimadzu spectrophotometer equipped with quartz cuvette using ethanol solutions at pH 4 and 8. Interpretation of the spectra was limited to evaluation of the nature of transitions occurring in thiocarbamoyl system and to definition of mutually correlated changes in electron density with probability of equilibrium conformational transitions.

2.3. Chromatography

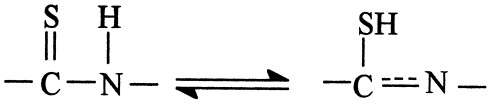
HPLC was carried out using a liquid chromatograph (Knauer) with a dual pump, a 20-μl sample injection valve and a UV–visible detector (320 nm). The mobile phase consisted of different volume fractions of methanol and acetonitrile in 10 m*M* acetate buffer (pH 4) as aqueous phase. The flow-rate was 0.5 ml/min at room temperature. The column dead time was determined by the injection of small amount of acetone dissolved in water.

2.4. Biological investigations

Sixteen compounds were tested for antifungal activity against *Botrytis cinerea*. A medium containing 20 g of malt extract and 20 g of agar in 1000 ml of distilled water was used. The medium was sterilized under a pressure of 1013 hPa at a temperature of 120°C for 15 min and then cooled to 60–70°C. Then, 5 mg of appropriate compound dissolved in 5 ml of acetone was added to the medium in such amount that the final volume was 250 ml. After mixing the medium was splashed onto the pans until a layer of 2–3 mm in thickness was obtained. Then the central inoculum of a 7-day spawn of fungi of 3 mm diameter (spawn of fungi to background) was sprayed on the background and subjected to incubation at 20°C. The measurements of spawn growth consisted in 'crosswise diameter' measurements, and presented values are the arithmetical means from two measurements. After 6 days, the degree of inhibition of spawn growth in preparation-containing medium in relation to control medium was calculated.

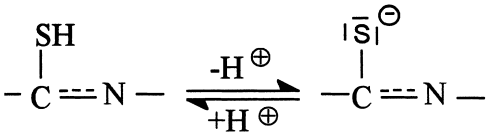
3. Results and discussion

Registration of electron spectra of substance **I** (non-substituted thiobenzanilide) at pH 4 and 8 confirmed earlier assumptions relating to structural changes dependent on pH of medium, which make chromatographic measurements for QSAR purposes impossible at any pH. This is due to tautomeric rearrangements of the type of:



(3)

as well as to the deprotonization (at pH>6):



(4)

promoted by increase of pH. Dissociation of the thiocarbamoyl system changes the nature of chromophores, which causes in turn shifts of energies of electron transitions of $n\rightarrow\pi^*$ and of $\pi\rightarrow\pi^*$ type. Thus all chromatographic measurements were carried out at pH 4.

The HPLC results are shown as plots of $\log k'$ versus volume fraction of organic modifier (Figs. 1 and 2), as intercepts of HPLC equations represented by the extrapolated $\log k'_w$ values and slopes of these equations, *S* (Figs. 3 and 4), and also as plots of $\log k'_{wM}$ (methanol–water) versus $\log k'_{wA}$ and $\log k'_{wA1}$ (acetonitrile–water), obtained from the linear (for $\varphi\leq 0.5$) and parabolic (for whole examined range of φ) extrapolation, respectively (Figs. 5 and 6).

The results in Fig. 1 and Table 2 indicate that linear relationships were obtained over all of the investigated concentration ranges for all studied substances in the methanol–water mobile phase. The different regression curves for each compound indicate selective effects upon retention due to solute–solvent and solute–stationary phase interactions. In a reversed-phase system, the retention depends on the

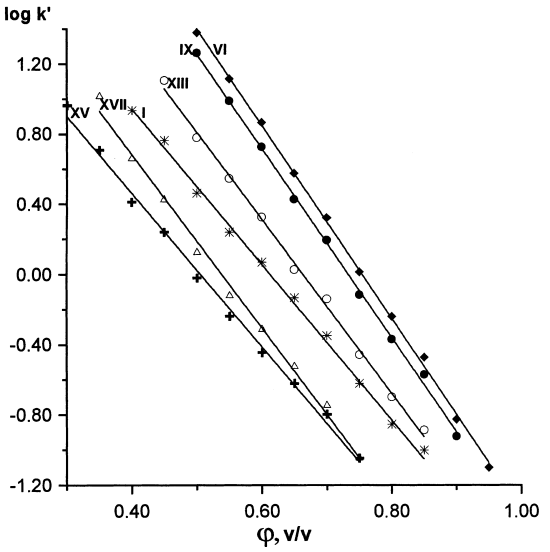


Fig. 1. Relationship between $\log k'$ values and the methanol concentration in the mobile phase. Notation of solutes as in Table 1.

molecular structure of the solute. The introduction of polar group (–OH) into unsubstituted compound **I** caused significant increase of affinity of compounds for water and the presence of nonpolar substituents caused the opposite effect; in this connection the

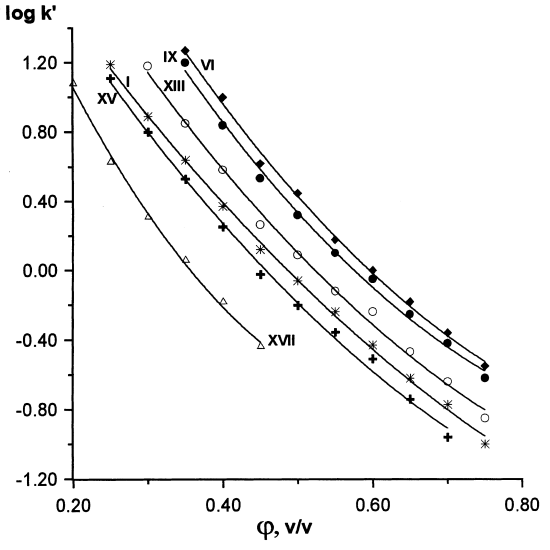


Fig. 2. Relationship between $\log k'$ values and the acetonitrile concentration in the mobile phase. Notation of solutes as in Table 1.

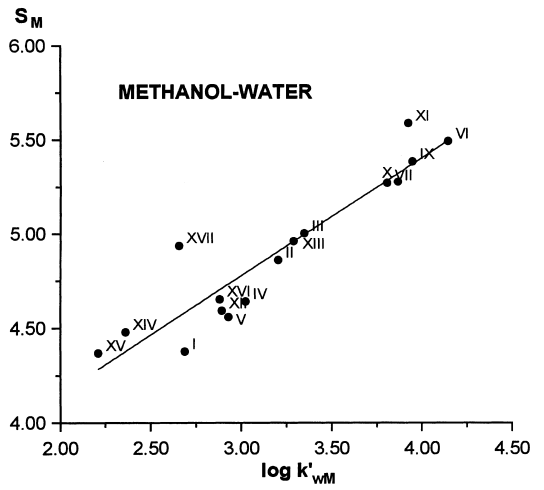


Fig. 3. Relationship between slopes S_M and intercepts $\log k'_{wM}$ of the HPLC equation as described by Eq. (5).

especially high lipophility shows the compounds containing –Cl, –I, and $-CF_3$ substituents. It can be observed that there is an increase in the selectivity at higher water contents in the eluent, caused by an increasing contribution of hydrophobic interaction.

The constant S value suggested by Biagi et al. [10] for given column and given organic modifier is not kept for all substances. It is dependent on the nature of N-amide substitution. The compounds containing lipophilic substituents are characterized by higher

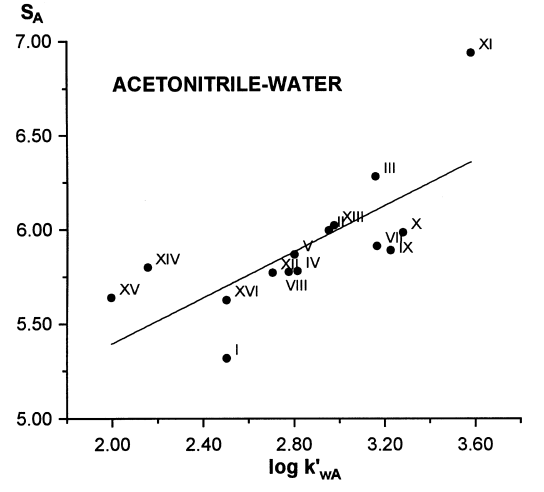


Fig. 4. Relationship between slopes S_A and intercepts $\log k'_{wA}$ of the HPLC equation as described by Eq. (6).

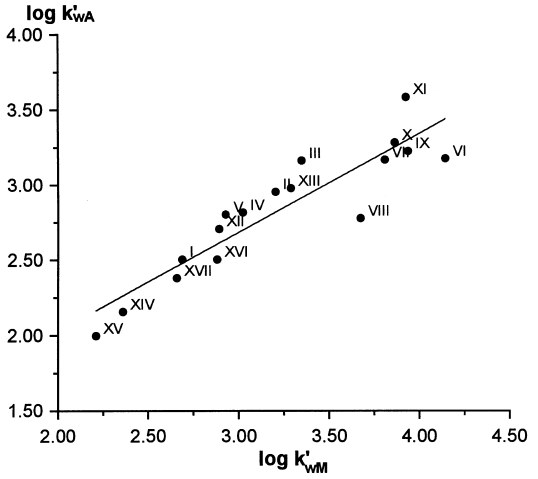


Fig. 5. Relationship between $\log k'_w$ values obtained from linear extrapolation from methanol ($\log k'_{wM}$) and the acetonitrile system ($\log k'_{wA}$).

slope values, even $S>5$, whereas the lowest values of S are shown by thiobenzanilides containing hydroxyl substituent (Table 2). Similar trends are observed for pyridazinone herbicides [7]. For the examined group of 2,4-dihydroxythiobenzanilides, S depends not only on the solvent strength of the mobile phase but also to a considerable extent on specific interaction between solutes, stationary phase and mobile phase.

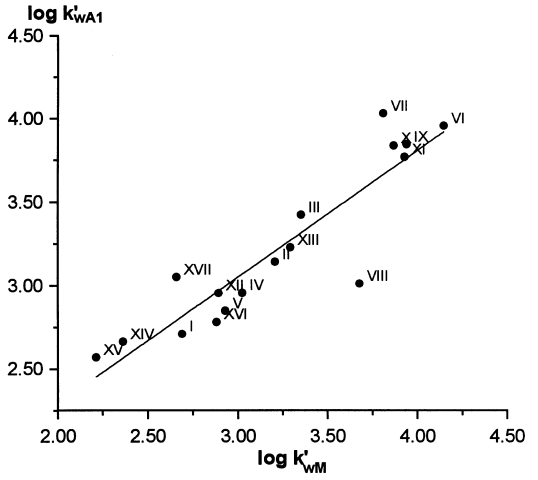


Fig. 6. Relationship between $\log k'_w$ values obtained from linear extrapolation from the methanol system ($\log k'_{wM}$) and parabolic extrapolation from the acetonitrile system ($\log k'_{wA1}$).

The correlation between $\log k'$ and volume fraction of acetonitrile in organic phase may be described by a parabolic equation or by two linear equations (Table 2, Fig. 2), and one of these equations, including the range of lower content of organic modifier in eluent, may be utilised to linear extrapolation of $\log k'_{wA}$. In this system it can be observed that there is an analogous effect of substitution on the changes of retention in relation to the non-substituted compound (*I*), but this effect is characterized by lower selectivity.

The intercepts ($\log k'_w$) in Eq. (1) represent the extrapolated $\log k'_w$ values, i.e., the theoretical $\log k'$ values at 0% organic solvent. The intercepts $\log k'_w$ and slopes for different organic solvent in the mobile phase are reported in Table 2 and Figs. 3 and 4. The correlation between intercepts $\log k'_w$ and slope S are described by the following equation:

$$S_M = 0.625 \log k'_{wM} + 2.904 \quad (n = 16, r = 0.931, s = 0.152) \tag{5}$$

$$S_A = 0.608 \log k'_{wA} + 4.179 \quad (n = 15, r = 0.730, s = 0.253) \tag{6}$$

In the case of methanol, a good correlation coefficient was obtained for the relationship between slope and $\log k'_{wM}$. A significantly worse correlation was obtained for acetonitrile–water mobile phase.

The data in Table 2 show that the extrapolated $\log k'_{wM}$ values from the HPLC system with methanol–water as mobile phase are much higher then those obtained for acetonitrile–water phases from linear extrapolation. These values are similar to the $\log k'_{wM1}$ parameters obtained from parabolic correlation. The correlations between the capacity factor values are described by the following equation:

$$\log k'_{wA} = 0.658 \log k'_{wM} + 0.709 \quad (n = 17, r = 0.913, s = 0.179) \tag{7}$$

$$\log k'_{wA1} = 0.758 \log k'_{wM} + 0.777 \quad (n = 17, r = 0.912, s = 0.208) \tag{8}$$

For both techniques, identical correlation coefficients were obtained, which creates the possibility of

Table 2
Parameters $\log k'_w$ and S obtained from the linear Eq. 1 for the methanol and acetonitrile system and $\log k'_{wA1}$ values from the quadratic equation (Eq. 2) for the acetonitrile–water phase

No.	Methanol–water					Acetonitrile–water						
	$\log k' = -S\varphi + \log k'_{wM}$					$\log k' = -S\varphi + \log k'_{wA}$					Quadratic relationships	
	S_M	$\log k'_{wM}$	n	r	s	S_A	$\log k'_{wA}$	n	r	s	$\log k'_{wA1}$	n
I	4.379	2.689	10	0.999	0.035	5.318	2.503	5	0.999	0.015	2.712	11
II	4.861	3.206	10	0.996	0.068	5.995	2.954	4	0.999	0.003	3.143	10
III	5.003	3.351	10	0.996	0.074	6.281	3.162	4	0.999	0.010	3.425	10
IV	4.641	3.024	9	0.998	0.035	5.780	2.816	4	0.999	0.009	2.958	10
V	4.560	2.929	9	0.998	0.036	5.867	2.802	4	0.999	0.017	2.849	10
VI	5.493	4.148	10	0.999	0.026	5.525	3.177	3	0.977	0.085	3.958	9
VII	5.272	3.811	9	0.999	0.037	5.911	3.167	4	0.994	0.051	4.031	8
VIII	4.518	3.676	11	0.999	0.029	5.775	2.777	4	0.999	0.021	3.013	9
IX	5.385	3.951	9	0.999	0.028	5.891	3.227	4	0.993	0.053	3.845	9
X	5.278	3.870	9	0.999	0.032	5.984	3.282	4	0.995	0.044	3.837	9
XI	5.588	3.929	9	0.997	0.063	6.937	3.584	3	0.997	0.084	3.770	9
XII	4.593	2.893	10	0.999	0.029	5.771	2.706	5	0.999	0.013	3.272	11
XIII	4.961	3.292	9	0.998	0.037	6.022	2.978	4	0.999	0.019	3.230	10
XIV	4.480	2.362	10	0.998	0.043	5.800	2.156	4	0.992	0.057	2.666	8
XV	4.370	2.211	10	0.998	0.044	5.640	1.996	4	0.992	0.057	2.571	7
XVI	4.654	2.881	10	0.999	0.029	5.627	2.503	5	0.999	0.012	2.784	10
XVII	4.937	2.659	9	0.997	0.053	6.760	2.379	4	0.991	0.071	3.051	6

selection of procedure. The slopes and intercept of Eqs. (7) and (8) differ from 1 and 0, respectively, which shows that the nature of the organic modifier significantly affects $\log k'_w$. Other HPLC data also suggest a different influence of methanol and acetonitrile on the retention of organic compounds [21,22]. Most probably, these differences arise from the chemical nature of the two organic modifiers. Methanol exhibits both hydrogen donor and acceptor abilities and will therefore easily be incorporated into the network of water molecules, whereas acetonitrile can serve only as a hydrogen acceptor and will charge the structure of the mobile phase. The moderate correlation coefficient indicates that $\log k'_w$ indeed reflects basically the same molecular properties of the solute in both solvents, but these properties contribute differently to retention.

Investigating the relation between lipophility and activity has revealed theoretical capacity coefficients determined for aqueous mobile phase with fungistatic activity (FA) of dihydroxythiobenzanilides against *Botrytis cinerea* (Figs. 7 and 8). For both organic modifiers the parabolic dependences were obtained, described by equations:

$$\mathrm{FA} = -45.158(\log k'_{wM})^2 + 293.746 \log k'_{wM} - 405.554 \quad (n = 16) \tag{9}$$

$$\mathrm{FA} = -50.336(\log k'_{wA})^2 + 288.321 \log k'_{wA} - 346.908 \quad (n = 16) \tag{10}$$

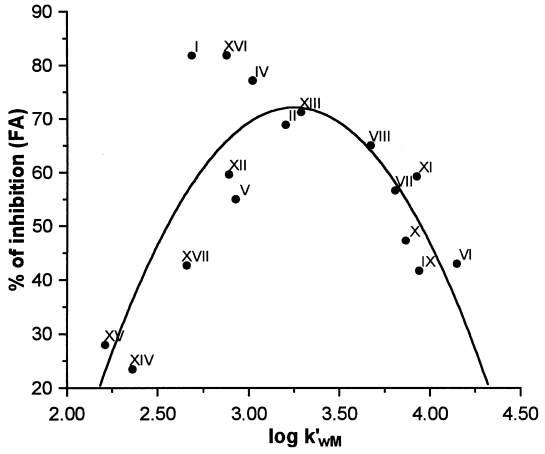


Fig. 7. The relationship between the antifungal activity of the 2,4-dihydroxythiobenzanilides at concentration of 20 μg ml^{-1} and lipophilicity parameter, $\log k'_{wM}$.

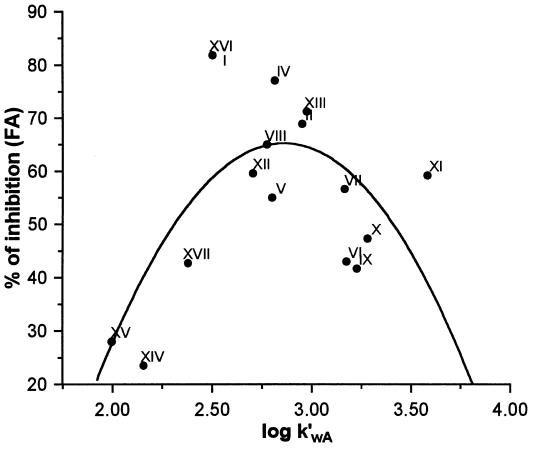


Fig. 8. The relationship between the antifungal activity of the 2,4-dihydroxythiobenzanilides at concentration of 20 μg ml^{-1} and lipophilicity parameter, $\log k'_{wA}$.

From the above, it results that some optimal value of $\log k'_w$ exists for which appropriately substituted compounds from 2,4-dihydroxythiobenzanilide group will show maximal fungistatic activity against *Botrytis cinerea*. For methanol used as organic modifier, $\log k'_w$ values should be contained in the range of 2.75–3.25, and for acetonitrile in the range of 2.50–3.00. Some deviations observed for the tested groups of the compounds may be explained by the fact that the lipophility is not the only factor determining the activity of the compound. Probably this factor determines mainly the transport, but does not define unequivocally the inhibition properties which are difficult to define and even more difficult to evaluate, although the obtained results show that capacity coefficients determined by the RP-HPLC method may be very helpful to evaluate the hydrophobicity of the compounds from the thiobenzanilide group for the purposes of QSAR analysis.

4. Conclusions

(1) Because of the tautomeric rearrangement in thiocarbamoyl system and the possibility of promotion of dissociation by the pH of medium, the most optimal pH at which HPLC measurements should be performed for QSAR purposes appears to be pH 4.

(2) The linear relationship between $\log k'$ and volume fraction of methanol in mobile phase has been obtained in whole examined concentration range, permitting determination of k'_{wM} values. Relation between $\log k'_w$ and volume percent of acetonitrile may be described by two linear equations or by a quadratic equation, making possible the extrapolation of the data to water as a mobile phase.

(3) The obtained $\log k'_w$ values are dependent on the type of organic modifier and mode of extrapolation, although these quantities do not show mutual correlation.

(4) The linear relationship between $\log k'_w$ and S values of Eq. (1) was found for both methanol–water and acetonitrile–water systems, which is one of the basic features of the chromatographic determination of hydrophobicity of closely related compounds.

(5) The quadratic equations (QSAR) obtained (between percent of inhibition and $\log k'_w$ parameters) show that $\log k'_w$ can be used for the prediction of the antifungal activity of newly synthesized agents for plant protection.

(6) It has stated that methanol used as organic modifier appears to be significantly more useful (owing to the wide range of linearity and higher selectivity) for evaluation of lipophility of the compounds in the 2,4-dihydroxythiobenzanilides group utilised in QSAR analysis. Obtained $\log k'_w$ values describe successfully the affinity of the compounds for biological systems and can be utilised to programming of synthesis towards the potentially most active compounds.

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