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CHROMATOGRAPHIC PARAMETERS AS TOOLS FOR PREDICTING THE BIOLOGICAL ACTIVITY OF AZO DERIVATIVES

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Preliminary assessment of the bioactive profile of azo derivatives was performed by applying drug-likeness rules. As the presumed criteria of lipophilicity, chromatographic parameters (R_M^0, m and C_0) were calculated in mixtures of water/methanol and water/acetonitrile by using reversed phase thin-layer chromatography (RPTLC C18/UV254s). The relationships between chromatographic parameters and relevant software parameters for the biological activity of azo derivatives were examined by linear regression and by two multivariate methods. Good linear relationships were obtained for each applied system. The multivariate methods show the similarity of chromatographic parameters (R_M^0, C_0) with standard measures of the lipophilicity and pharmacokinetic predictors. The chromatographic parameter m obtained in the same conditions exhibits better agreement with the drug-likeness and toxicity parameters. The polarity of the substituent was found to have a higher impact on the values of azo derivatives' bioactivity parameters than its electronic effects.

Keywords: chromatographic parameters; drug-likeness rules; biological activity parameters; multivariate methods; azo derivatives

ХРОМАТОГРАФСКИ ПАРАМЕТРИ КАКО АЛАТКИ ЗА ПРЕДВИДУВАЊЕ НА БИОЛОШКАТА АКТИВНОСТ НА АЗОДЕРИВАТИ

Извршена е прелиминарна процена на биоактивниот профил на азодеривати со примена на правила за „сличност со лек“. Како претпоставените критериуми за липофилност се пресметани, хроматографските параметри (R_M^0, m и C_0) во смеси вода/метанол и вода/ацетонитрил со примена на реверзнофазна тенкослојна хроматографија (RPTLC C18/UV254s). Зависноста помеѓу хроматографските параметри и релевантните софтверски параметри на биолошката активност на азодериватите е проверена со линеарна регресија и со две мултиваријабилни методи. Добиени се добри линеарни зависности за секој применет систем. Мултиваријабилните методи ја покажуваат сличноста на хроматографските параметри (R_M^0, C_0) со стандардните мерила на липофилност и на фармакокинетички параметри. Хроматографскиот параметар m добиен при исти услови покажува подобро совпаѓање со параметрите на сличност со лекот и за токсичност. Утврдено е дека поларитетот на супституентот има поголемо влијание врз вредноста на биоактивните параметри на азодериватите одошто врз нивните електронски ефекти.

Клучни зборови: хроматографски параметри; правила на „сличност со лек“; биолошки параметри на активност; мултиваријабилни методи; азодеривати

1. INTRODUCTION

The need for stable, resistant and intense colors has led to an increase in the production and consumption of the synthetic dyes. This has resulted in approximately 100,000 different types of dyes being used in the textile industry today [1]. Azo dyes are the most versatile and the most wide-

spread group used for coloring fabrics. In addition to the desired stability, they also show an extremely difficult degradability, which negatively affects aquatic ecosystems [2, 3]. The cleavage products of azo dyes show toxic, mutagenic, carcinogenic and teratogenic effects [4–7]. Also, it is known that azo derivatives and their metal complexes exhibit potent anti-inflammatory, antitumor and antimicrobial activity [8–10].

Conducting the preliminary conclusions regarding the activity, physico-chemical properties or retention behavior of azo derivatives based on their structures is possible by developing various mathematical models [11–13]. Selection of appropriate molecular descriptors for forming a meaningful mathematical model is a precondition to rationalizing the production of new azo dyes which would be in line with market, environmental and legal requirements [14]. *In silico* studies of the new potentially bioactive compounds usually include checking drug-likeness rules (the Lipinski's rule of five (Ro5), the Ghose's rule (GR) and the Veber's rule (VR)) as well as getting information about the compound's lipophilicity, a crucial molecular descriptor associated with its bioavailability, pharmacokinetics and toxicity [15–19]. Lipophilicity is usually defined by the partition coefficient, log*P*, but it is increasingly expressed by the parameters obtained by reversed phase thin-layer chromatography [20–22].

The existence, intensity and rate of biological activity of a compound are conditioned by a number of its pharmacokinetic properties. Among them, permeability primarily affects a compound's bioavailability, intestinal absorption, passage through the blood-brain barrier, efficacy or toxicity, and elimination through the kidneys and liver [23–25]. Also, information about the percent of plasma protein binding, *PPB*, is necessary since only the free concentration of the bioactive compound in tissues guarantees its biological effect [26].

Given that azo derivatives are invasive compounds, predicting the health risks for consumers exposed to them should include assessing a dye's ability to penetrate the skin barrier through the stratum corneum and through the ocular tissues [27, 28]. Predicting the harmful effects of the synthetic dyes should cover various aspects of toxicity, among which particular attention is paid to cardiac toxicity testing [29]. Since the aquatic ecosystems are often exposed to azo dyes, early research also includes predicting their ecotoxicity [30].

The aim of this study was an *in silico* estimation of the biological activity of selected azo derivatives. In the first phase, their compliance with the drug-likeness rules was checked. Alternative measures of lipophilicity for selected azo compounds were determined by using reversed phase thin-layer chromatography, RPTLC C18/UV254s, in the presence of two organic modifiers (methanol and acetonitrile). Also, their pharmacokinetic properties and toxicological profiles were evaluated. Correlation of the bioactivity parameters of the tested azo derivatives was examined by applying linear regression and two multivariate methods (Cluster analysis and Principal component analysis). High-quality mathematical models were obtained as confirmed by statistical parameters.

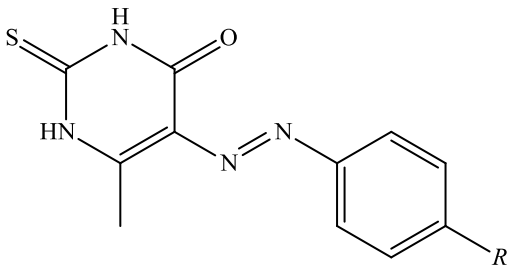
2. EXPERIMENTAL

2.1. *Chromatographic measurements*

Thin-layer chromatography was performed on 5 cm × 10 cm commercial plates (RP-18W/UV_{254} Macherey-Nagel GmBH and Co., Duren, Germany). As a mobile phase, mixtures of LC grade organic modifiers (methanol and acetonitrile, J. T. Baker, Deventer, the Netherlands) with filtered bi-distilled water were used. The content of the organic modifier, φ, in the mobile phase ranged from 36 % to 52 % (v/v) in 5 % increments. Solutions of the azo dyes (Table 1) were prepared with ethanol, p.a., (J. T. Baker, Deventer, the Netherlands) in the concentration of 2 mg ml^{-1}. Their synthesis and characterization are described in the literature [31].

T a b l e 1

Structures of the investigated azo derivatives

R	
H	
Cl	
Br	
F	
NO_2	
OH	
COOH	
$COCH_3$	
CH_3	
OCH_3	

The freshly prepared solutions of each derivative (0.2 μl) were spotted on the plates and developed by the ascending technique in saturated chambers at a temperature of 25 °C with previously prepared aqueous solutions of each organic

modifier. The front of solvent was approximately 5 cm, the chromatograms were developed for about 20 minutes, and after that the plates were dried in a stream of air. The examined compounds were detected under UV light at λ = 254 nm. For each solute-organic modifier combination, three chromatograms were developed, and the average R_f values were calculated.

The R_M values, characterizing the retention in TLC, were calculated according to the Bate-Smith and Westall equation [32]:

$$R_M = \log\left(\frac{1}{R_f} - 1\right) \quad (1)$$

Linear correlations between the R_M values of the compounds and the concentration of organic modifier in the mobile phases (φ) were calculated with the Soczewiński-Wachtmeister's equation [33]:

$$R_M = R_M^0 + m\varphi \quad (2)$$

where the intercept R_M^0 (chromatographic retention constant) represents an R_M value extrapolated to 0% v/v of organic modifier/water mobile phase system. The slope of the regression plot, *m*, is related to the specific hydrophobic surface area of the compound [34]. In addition to R_M^0, parameter *m* can be regarded as an alternative measure of lipophilicity.

Based on the slope and the intercept of the Soczewiński-Wachtmeister's equation, another parameter of lipophilicity, C_0, can be calculated [35]:

$$C_0 = -\frac{R_M^0}{m} \quad (3)$$

Parameter C_0 corresponds to the concentration of the organic component of the mobile phase for which the distribution of the analyzed substance between the mobile and stationary phase is equal (1:1). This parameter is considered more reliable in QSAR analysis because it also includes both chromatographic lipophilicity, R_M^0, and the specific hydrophobic surface area of the solute.

2.2. *In silico properties*

The values of the partition coefficient, log*P*, were calculated using virtual Computational Chemistry Laboratory, VCCLAB [36]. Molecular descriptors, values of pharmacokinetic predictors, and toxicity parameters were obtained using Molinspiration, SimulationPlus, and PreADMETonline programs [37–39].

2.3. *Chemometric analysis*

The experimental data were processed by Origin 6.1 software. Multivariate methods were performed by Statistica software v.13.5.017 (StatSoft Inc., Tulsa, OK, USA).

Cluster analysis (CA) and Principal component analysis (PCA) are performed on a matrix in which different measures of lipophilicity (R_M^0, *m*, C_0, log*P*), the pharmacokinetic predictors, and the toxicity parameters of examined compounds represented variables (columns) and the azo derivatives were rows. The matrix data were standardized to ensure equal impact of all analyzed parameters. In CA, the Euclidean distance was applied as a measure of diversity, and the clusters were formed by the Ward method. In PCA, the original data matrix was decomposed to a loading vector (including experimentally and software obtained parameters of lipophilicity, drug-likeness descriptors, pharmacokinetic parameters and toxicity parameters), and score vectors (including the examined azo derivatives).

3. RESULTS AND DISCUSSION

3.1. *Compatibility of the studied azo derivatives and the drug-likeness rules*

In compliance with Lipinski's rule of five, a potentially biologically active compound should possess the following: molecular weight ≤ 500; number of hydrogen bond acceptors ≤ 10 (2·5); number of hydrogen bond donors ≤ 5, and value of the partition coefficient log*P* ≤ 5.

On the other hand, according to the Ghose's rule, the molecular weight should be within 160–480, values of log*P* between –0.4 and 5.6, the total number of atoms in molecule within 20–70, and molar refractivity in the range 40–130.

According to Veber's rule, the number of rotatable bonds should be less than 10, the sum of hydrogen bond donors and hydrogen bond acceptors not over 12, and total polar surface area less than 140 Å^2. The relevant molecular descriptors of the examined azo derivatives are shown in Table 2 and Table 3.

It is obvious from the data shown in Table 2 and Table 3 that all of the examined azo derivatives fulfill all of the mentioned drug-likeness rules and thereby theoretically could be biologically active.

T a b l e 2

Selected molecular descriptors of the examined azo derivatives

R	MW	nON	nOHNH	natoms	MR cm^3mol^{-1}	nrotb	TPSA
H	246.295	5	2	27.0	70.15	2	73.381
Cl	280.740	5	2	28.0	74.75	2	73.381
Br	325.191	5	2	28.0	77.84	2	73.381
F	264.285	5	2	28.0	70.55	2	73.381
NO_2	291.292	8	2	30.0	77.72	3	119.205
OH	262.294	6	3	29.0	71.96	2	93.609
COOH	290.304	7	3	31.0	76.96	3	110.680
$COCH_3$	288.332	6	2	33.0	81.39	3	90.452
CH_3	260.322	5	2	31.0	76.04	2	73.381
OCH_3	276.321	6	2	32.0	77.40	2	82.615

MW – molecular weight; nON – number of hydrogen bond acceptors; nOHNH – number of hydrogen bond donors; natoms – the total number of atoms in molecule;
MR – molar refractivity; nrotb – the number of rotatable bonds; TPSA – total polar surface area

T a b l e 3

Software obtained logP values of the tested azo derivatives

R	AClog*P*	Alog*P*	Alog*Ps*	Mlog*P*	milog*P*	*kowwin*	XlogP_2	XlogP_3
H	2.19	2.47	2.53	1.49	2.00	2.11	2.06	1.99
Cl	2.81	3.14	3.23	1.77	2.68	2.76	2.68	2.61
Br	2.89	3.22	3.30	1.91	2.82	3.00	2.85	2.68
F	2.25	2.68	2.68	1.63	2.17	2.31	2.22	2.09
NO_2	2.20	2.37	2.58	1.31	1.96	2.51	1.95	1.82
OH	1.89	2.21	2.30	0.72	1.53	1.63	1.65	1.63
COOH	1.71	2.08	2.02	0.97	1.92	1.99	1.67	1.51
$COCH_3$	2.12	2.21	2.42	1.19	1.90	1.79	1.90	1.67
CH_3	2.51	2.96	2.89	1.77	2.45	2.66	2.49	2.35
OCH_3	2.09	2.46	2.62	1.00	2.06	2.19	1.97	1.96

Also, it is noticeable from Table 3 that different values of the partition coefficient, log*P*, were obtained for the same compound. This can be explained by the use of different mathematical methods in the software package for calculating log*P*. Regardless of the calculating method, the highest value of the partition coefficient was obtained for the compound with Br as a substituent, and the lowest value was obtained for the derivative with the most polar COOH group.

3.2. *Determination of the alternative measure of lipophilicity of the studied azo derivatives*

The values of the chromatographic parameters R_M^0, *m* and C_0 of the tested azo compounds are presented in Table 4.

The high values of the regression coefficients, *r*, indicate the validity of the linear R_M-φ dependencies in the chosen field of experimental work.

Bearing in mind that the chromatographic parameter R_M^0 depends solely on the nature of the compound and not on the solvent used, it was expected that the values would be similar. Despite this, it is noticeable that slightly higher values of the chromatographic parameter R_M^0 were obtained in methanol. This phenomenon can be explained by the fact that methanol, as a protic and very polar solvent, forms an association with water. In this way, the effect of the organic modifier is reduced and the elution power of the mobile phase is reduced, which results in a stronger retention of the tested compounds [40].

T a b l e 4

Chromatographic parameters obtained for the azo derivatives in applied modifiers

R	Modifier							
	Methanol				Acetonitrile			
	R_M^0	*m*	*r*	C_0	R_M^0	*m*	*r*	C_0
H	0.883	–1.952	0.999	0.452	0.679	–1.819	0.996	0.373
Cl	1.138	–2.296	0.998	0.496	1.109	–2.375	0.993	0.467
Br	1.217	–2.398	0.996	0.508	1.175	–2.452	0.995	0.479
F	0.956	–2.155	0.997	0.444	0.809	–2.055	0.997	0.394
NO_2	0.522	–1.510	0.995	0.346	0.356	–1.415	0.998	0.252
OH	0.701	–1.795	0.993	0.391	0.495	–1.589	0.996	0.312
COOH	0.306	–1.103	0.997	0.277	0.160	–1.156	0.992	0.138
$COCH_3$	0.414	–1.318	0.996	0.314	0.287	–1.295	0.995	0.222
CH_3	1.071	–2.285	0.999	0.469	1.030	–2.306	0.997	0.447
OCH_3	0.798	–1.893	0.998	0.422	0.635	–1.769	0.998	0.359

The retention of the compound is affected to a greater extent by the influence of the substituent`s nature. Nonpolar methyl as well as halogen substituents lead to a stronger retention compared to the basic molecule. Conversely, the presence of a polar substituent results in a lower retention in comparison to an unsubstituted molecule. This retention behavior was shown by derivatives with NO_2, COOH and $COCH_3$ groups, while a much stronger retention was obtained for derivatives with OH and OCH_3 groups. This was not unexpected because this deviation has been noticed in an earlier study [41]. Namely, these two groups are electron donors (they possess a negative value of the Hammett substituent constant, σ), and this causes the formation of an azotautomer that is more strongly bound to the stationary phase [42]. The Hammett substituent constant describes the electronic effects of a substituent bound to the basic molecule. The values of the Hammett substituent constants, σ, are given in Table S1.

Also, it is noticeable that the highest retention was observed for the derivative with Br as a substituent, and the lowest was for the compound with an COOH group in both applied modifiers.

From observing Table 4, it can be noticed that the *m* values change in accordance with changes in the R_M^0 values. Correlation of the mentioned parameters resulted in a linear dependence, which confirmed the assumption that they depend on the same physico-chemical parameters (Table 5).

T a b l e 5

Equations of R_M^0-m relationships of the azo derivatives in the modifiers used

Modifier	Equation	*r*	sd	*p*
Methanol	$R_M^0 = -0.512 - 0.702m$	0.993	0.038	$< 1 \cdot 10^{-4}$
Acetonitrile	$R_M^0 = -0.720 - 0.764m$	0.999	0.019	$< 1 \cdot 10^{-4}$

Additionally, the existence of a linear R_M^0-*m* dependence indicates that the selected azo derivatives can be seen as congeneric [43].

Whether the chromatographically obtained parameters, R_M^0, *m* and C_0, can be used as alternative lipophilicity measures of the azo compounds was examined by their correlation with the software partition coefficient, log*P*, obtained by applying linear regression (Table 6).

Values of the basic statistical parameters shown in Table 6 (approximately $r > 0.940$, sd < 0.095 and $p < 0.05$) confirm the validity of the established linear relationships. Chromatographic parameters R_M^0, *m*, and C_0 of the azo derivatives obtained in both modifiers used are in good correlation with all of the calculated log*P* values (atomic, fragmental and property-contributed). This indicates their reliable application as lipophilicity measures in the given conditions of experimental work.

Table 6

Statistical parameters of the linear models R_M^0 - logP, m - logP and C_0 - logP for the azo derivatives

Methanol*		AClog*P*	Alog*P*	AlogP_s	Mlog*P*	milog*P*	kowwin	XlogP_2	XlogP_3
	r	0.982	0.989	0.959	0.941	0.974	0.973	0.996	0.984
R_M^0	sd	0.038	0.030	0.058	0.069	0.046	0.047	0.018	0.037
	p	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$6.32{\cdot}10^{-4}$	0.002	$2.06{\cdot}10^{-4}$	$2.21{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$
	r	0.946	0.979	0.927	0.936	0.950	0.958	0.975	0.953
m	sd	0.082	0.052	0.095	0.089	0.080	0.073	0.056	0.077
	p	0.001	$1.27{\cdot}10^{-4}$	0.003	0.002	0.001	$6.88{\cdot}10^{-4}$	$1.78{\cdot}10^{-4}$	$8.96{\cdot}10^{-4}$
	r	0.976	0.958	0.948	0.933	0.966	0.955	0.978	0.977
C_0	sd	0.010	0.013	0.014	0.016	0.011	0.013	0.009	0.009
	p	$1.69{\cdot}10^{-4}$	$6.99{\cdot}10^{-4}$	0.001	0.002	$4.07{\cdot}10^{-4}$	$8.00{\cdot}10^{-4}$	$1.27{\cdot}10^{-4}$	$1.48{\cdot}10^{-4}$
Acetonitrile*									
	r	0.982	0.998	0.968	0.909	0.975	0.976	0.992	0.985
R_M^0	sd	0.054	0.019	0.072	0.119	0.063	0.062	0.036	0.049
	p	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$3.56{\cdot}10^{-4}$	0.004	$1.79{\cdot}10^{-4}$	$1.70{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$
	r	0.966	0.993	0.952	0.922	0.968	0.971	0.986	0.974
m	sd	0.094	0.043	0.112	0.143	0.093	0.088	0.061	0.083
	p	$3.87{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$9.16{\cdot}10^{-4}$	0.003	$3.50{\cdot}10^{-4}$	$2.71{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$2.04{\cdot}10^{-4}$
	r	0.981	0.994	0.967	0.926	0.985	0.984	0.995	0.991
C_0	sd	0.013	0.007	0.017	0.026	0.012	0.012	0.007	0.009
	p	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$3.74{\cdot}10^{-4}$	0.003	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$	$< 1{\cdot}10^{-4}$

*Derivatives with NO_2, COOH and $COCH_3$ are excluded (polar substituents with positive values of Hammet constant)

3.3. *Correlation between the alternative measure of lipophilicity and selected pharmacokinetic predictors of the azo derivatives by applying linear regression analysis*

Table 7 shows the calculated values of selected pharmacokinetic predictors for the studied compounds.

Since the passage of the compounds through various biological membranes is closely related to its lipophilicity, it was assumed that more lipophilic derivatives would have better permeability. In line with expectations, the compound that most easily passed through the phospholipid bilayer of enterocytes was the most lipophilic derivative (Br as substituent). On the contrary, the derivative with an OH group substituent had the lowest value of P_{eff}.

Table 7

Pharmacokinetic predictors of the studied azo compounds

R	P_{eff} (cms^{-1})	*PPB* (%)	*BBB* (%)	pCornea (nms^{-1})	MDCK (nms^{-1})	$logK_{sp}$ (cmh^{-1})	*Tox herg* (pIC_{50})
H	2.020	81.126	0.075	117.415	70.065	–3.458	4.697
Cl	2.703	83.948	0.317	139.785	83.196	–3.515	5.047
Br	2.877	83.908	0.347	138.917	67.442	–3.402	5.023
F	2.558	81.934	0.189	109.121	86.521	–3.735	4.908
NO_2	2.394	85.040	0.135	22.291	45.788	–3.557	4.763
OH	1.204	78.538	0.032	60.032	33.571	–4.254	4.645
COOH	1.410	78.493	0.149	41.604	26.806	–3.819	4.413
$COCH_3$	2.163	80.728	0.099	82.798	65.565	–3.722	4.788
CH_3	2.380	83.323	0.527	124.085	68.063	–3.397	4.715
OCH_3	1.924	82.112	0.019	83.479	67.519	–3.703	4.762

Simulation plus: P_{eff} – human effective permeability in jejunum; MDCK – Madin-Darby canine kidney cell permeability line; pCornea – cornea permeability; ToxhERG – cardiac potassium channel encoded by the humanether-a-go-go gene; PreADMET: *PPB* – plasma protein binding; *BBB* – blood-brain barrier permeability; logK_{sp} – skin permeability

The values of the distribution parameter through the blood-brain barrier, *BBB*, indicate that the derivative with a CH_3 group had the greatest potential as a neuroactive substance (*BBB* > 0.4) [44].

Based on the value of the permeability coefficient, log K_{sp}, the best transdermal passage was exhibited by the derivative with a CH_3 substituent, and the weakest was the derivative with an OH group.

In addition, it was noticed that derivatives with a nonpolar group or a halogen substituent could exhibit the highest corneal permeability. This is consistent with studies showing that compounds with a log*P* between 2.5–2.9 have optimum corneal permeability [45].

From observing the MDCK values, it can be noted that all of the examined azo derivatives have permeability (25–500 $nm \cdot s^{-1}$). Again, higher permeability was obtained for derivatives with nonpolar and halogen substituents [46].

The azo derivative with the OH group in unbound form reaches the site of action in the highest amount, while the derivative with the highest binding affinity for plasma proteins was the derivative with the NO_2 group.

The hERG IC_{50} values indicate that the studied derivatives with Cl and Br can be considered as hERG channel blockers (hERG $IC_{50}<10 \mu mol \cdot l^{-1}$) [38].

In order to check the existence of a dependence between chromatographic parameters determined by TLC and selected pharmacokinetic predictors, linear regression analysis was applied. The results are presented in Table 8.

T a b l e 8

Basic statistical parameters of the linear models' chromatographic parameters – pharmacokinetic predictors for the azo derivatives

Methanol*		P_{eff}	*PPB*	*BBB*	MDCK	pCornea	$logK_{sp}$	*Tox herg*
	r	0.932	0.901	0.829	0.744	0.941	0.781	0.789
R_M^0	sd	0.074	0.088	0.114	0.123	0.069	0.128	0.126
	p	0.002	0.005	0.021	0.090	0.002	0.038	0.035
	r	0.929	0.885	0.881	–	0.898	0.730	0.764
m	sd	0.094	0.118	0.120	–	0.112	0.173	0.164
	p	0.002	0.008	0.009	–	0.005	0.062	0.046
	r	0.918	0.901	0.739	–	0.975	0.846	0.778
C_0	sd	0.018	0.019	0.030	–	0.010	0.024	0.028
	p	0.004	0.006	0.058	–	$1.93 \cdot 10^{-4}$	0.016	0.039
Acetonitrile*								
	r	0.908	0.912	0.865	–	0.911	0.752	0.774
R_M^0	sd	0.120	0.117	0.143	–	0.119	0.188	0.181
	p	0.005	0.004	0.012	–	0.004	0.051	0.041
	r	0.920	0.911	0.880	–	0.906	0.744	0.772
m	sd	0.144	0.152	0.175	–	0.156	0.246	0.234
	p	0.003	0.004	0.009	–	0.005	0.055	0.042
	r	0.921	0.988	0.833	–	0.931	0.821	0.763
C_0	sd	0.028	0.011	0.040	–	0.026	0.041	0.046
	p	0.009	$1.98 \cdot 10^{-4}$	0.040	–	0.007	0.045	0.077

*Derivatives with NO_2, COOH and $COCH_3$ are excluded.

Results presented in Table 8 confirm that the linear regression analysis gave, on average, satisfactory correlations between the chromatographic parameters and the selected pharmacokinetic predictors. Significant dependence between chromatographic parameters and MDCK values were not obtained. This could be explained by the fact that MDCK permeability not only depends on passive diffusion (which depends strongly on the compound's lipophilicity), but it is also regulated by P-glycoprotein cell influx and efflux [47].

3.4. *Correlation between the alternative measure of lipophilicity and the selected toxicity parameters of azo derivatives by applying linear regression analysis*

In Table 9 are presented the software values of the effective concentration, EC_{50}, $mg \cdot kg^{-1}$ as a measure of the acute toxicity of the tested compounds for the following test organisms: Algae, Daphnia, Medaka and Minnow (Table 9).

T a b l e 9

Computational EC_{50} values for the studied azo derivatives on the selected test organisms

R	*Algae* at	*Daphnia* at	*Medaka* at	*Minnow* at
H	0.0489	0.0615	0.0075	0.0068
Cl	0.0247	0.0308	0.0021	0.0020
Br	0.0217	0.0251	0.0015	0.0016
F	0.0384	0.0544	0.0059	0.0033
NO_2	0.0450	0.0533	0.0060	0.0045
OH	0.0389	0.0668	0.0091	0.0071
COOH	0.0352	0.0639	0.0087	0.0074
$COCH_3$	0.0382	0.0659	0.0092	0.0091
CH_3	0.0266	0.0371	0.0028	0.0024
OCH_3	0.0366	0.0611	0.0076	0.0071

It can be noted that the derivative with a Br substituent is the most toxic among all of the examined azo dyes, while all of the tested derivatives are most toxic to the *Minnow* species.

The relationship between the experimentally determined lipophilicity (chromatographic parameters, R_M^0, *m* and C_0) of the studied compounds and the values of their parameters of toxicity (EC_{50}) for the different test organisms was evaluated by applying linear regression. The correlation matrix of the established dependencies is presented in Table 10.

T a b l e 10

Basic statistical parameters of the linear models R_M^0-EC_{50}, m-EC_{50}, and C_0-EC_{50} for tested azo derivatives

Methanol*		*Algae*	*Daphnia*	*Medaka*	*Minnow*
	r	0.781	0.970	0.980	0.940
R_M^0	sd	0.127	0.050	0.040	0.070
	p	0.038	$2.93\cdot10^{-4}$	$1.04\cdot10^{-4}$	0.002
	r	0.804	0.958	0.978	0.979
m	sd	0.151	0.073	0.052	0.052
	p	0.029	$6.79\cdot10^{-4}$	$1.32\cdot10^{-4}$	$1.25\cdot10^{-4}$
	r	0.687	0.929	0.937	0.853
C_0	sd	0.032	0.016	0.016	0.023
	p	0.088	0.002	0.002	0.014
Acetonitrile*					
	r	0.842	0.988	0.997	0.952
R_M^0	sd	0.154	0.045	0.022	0.087
	p	0.018	$< 1\cdot10^{-4}$	$< 1\cdot10^{-4}$	$9.38\cdot10^{-4}$
	r	0.832	0.977	0.994	0.969
m	sd	0.204	0.079	0.042	0.091
	p	0.020	$1.57\cdot10^{-4}$	$< 1\cdot10^{-4}$	$3.25\cdot10^{-4}$
	r	0.805	0.975	0.990	0.930
C_0	sd	0.040	0.016	0.010	0.025
	p	0.029	$1.88\cdot10^{-4}$	$< 1\cdot10^{-4}$	0.002

*Derivatives with NO_2, COOH and $COCH_3$ are excluded.

The values of all the statistical parameters indicate that the established mathematical models are satisfactory. Thus, the dependence between chromatographic parameters and software obtained

EC_{50} values suggests the possibility of using the parameters for predicting the toxicity of tested azo compounds.

3.5. *Study of the azo derivatives'biological activity parameters using multivariate analysis*

In modern research, multivariate analysis represents an indispensable tool because it enables the classification of a large amount of data from different origins as well as the identification and proper exclusionof those that are redundant [48, 49]. In order to achieve a more comprehensive *in silico* study of potentially biologically active azo derivatives, two multivariate methods were applied (CA and PCA).

3.5.1. *Results of cluster analysis*

A dendrogram of the studied parameters of biological activity is shown in Figure 1, while Figure 2 shows the dendrogram of the tested derivatives.

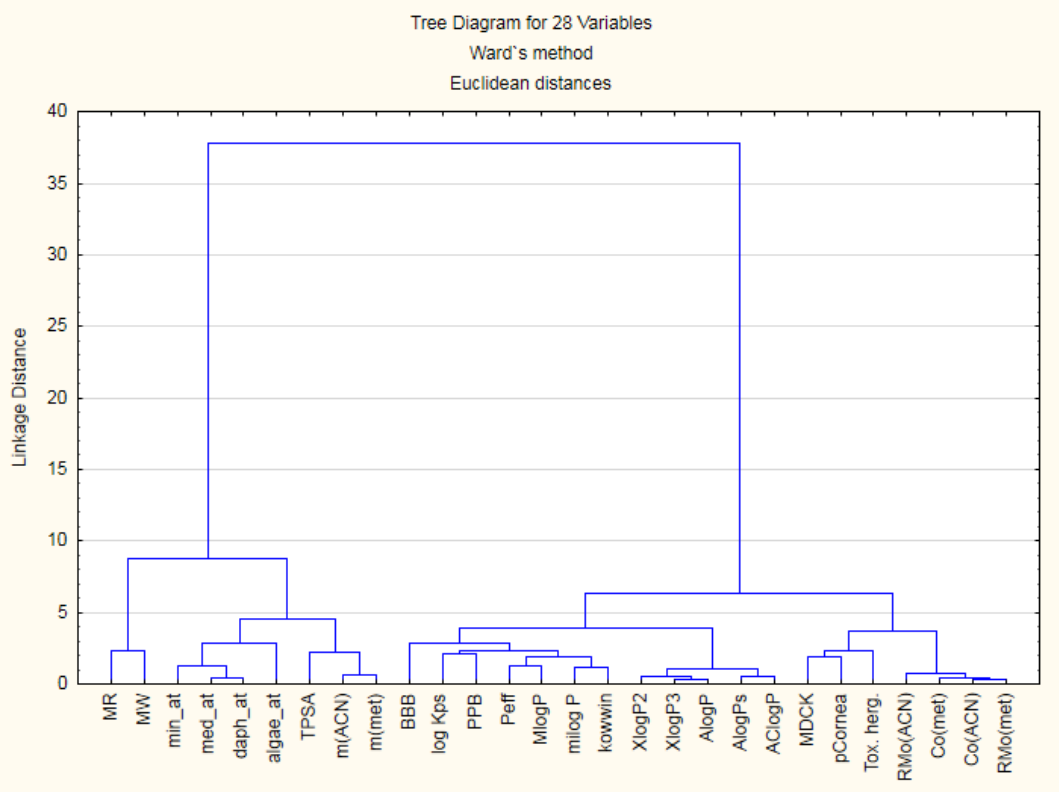


Fig. 1. Dendrogram of the examined biological activity parameters

Figure1 shows that the CA grouped the studied parameters of biological activity into two clearly defined clusters. The first cluster includes the chromatographic parameters, *m*, determined in both of the modifiers used, the toxicity parameters, and drug-likeness descriptors. The second cluster contains pharmacokinetic predictors, mathematically determined parameters of lipophilicity, and other chromatographic parameters obtained in both modifiers. This distribution of the examined parameters of biological activity indicates a greater similarity between the chromatographic parameter *m* and the toxicity parameters as well as a greater closeness of the chromatographic parameters (R_M^0 and C_0) with the software obtained values of the lipophilicity (log*P*) and pharmacokinetic predictors.

Within the cluster formed, the separation of parameters into sub-clusters can be observed. As expected, MR and MW form one cluster, and a second includes parameters of toxicity, while the chromatographic parameters *m* with TPSA (otherwise conditioned by this descriptor) form the third subgroup.

Three sub-clusters are also noticeable in the second cluster. The first sub-cluster predominantly includes pharmacokinetic predictors, the second log*P* values (atom-based extra separated), and the third chromatographic parameters (R_M^0 and C_0).

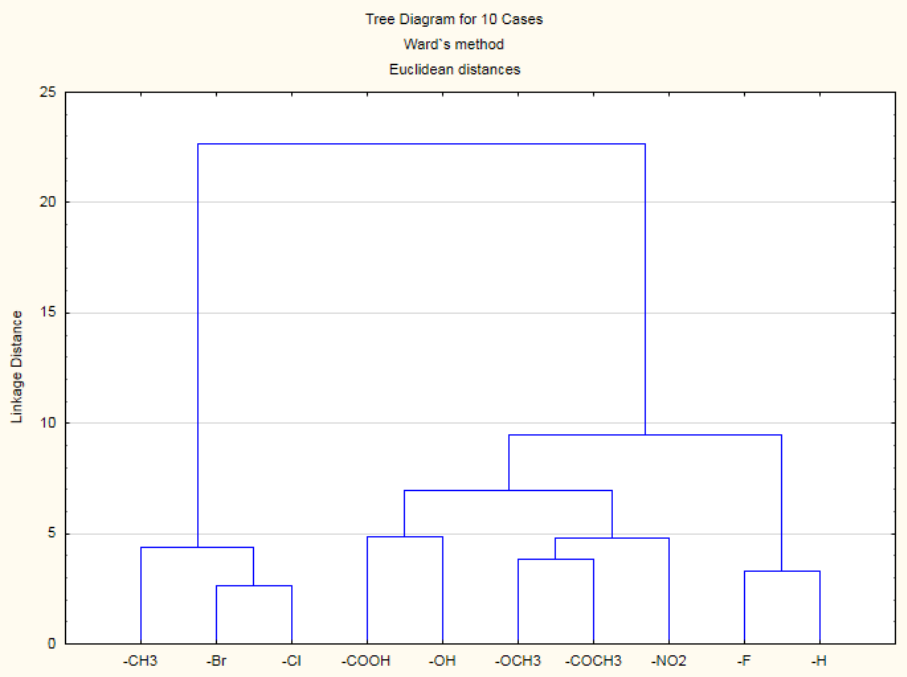


Fig. 2. Dendrogram of the studied azo derivatives

The CA placed the examined azo derivatives into two groups (Fig. 2). The first cluster includes azo derivatives with nonpolar and halogen substituents (CH_3, Br and Cl), except the one with F as a substituent. Compounds with a polar substituent and the unsubstituted molecule form the other cluster. It is assumed that the difference in size and polarizability of fluorine could be the reason for the separation of this derivative from other halogenated derivatives.

This grouping of the studied azo compounds indicates that their potential biological activity is mostly conditioned by the chemical nature of the presented substituent.

3.5.2. *Results of principal component analysis*

By decomposing the original data matrix to loading vectors and score vectors, PCA enables the elimination of excessive information as well as a significant reduction in the volume of analyzed data. The newly obtained principal components, PC, represent the linear dependence of the original variables. Only those newly formed components whose sum exceeds 80 % are considered to be valid for further analysis.

Figure S1 shows that the three principal components describe about 89 % of the total variables.

The partition of the examined parameters of the biological activity of the studied derivativesis shown in Figure 3 (loading plot).

It can be noticed that the first principal component (PC1) classifies the studied parameters in two clearly defined groups. The first group (negative PC1) includes the chromatographic parameter *m*, toxicity parameters and drug-likeness descriptors, while the second group (positive PC1) consists of the chromatographic parameters (R_M^0 and C_0), pharmacokinetic parameters and mathematically determined parameters of lipophilicity (log*P*). Based on this division, as well as in the case of CA, the similarity among the studied parameters of biological activity is confirmed.

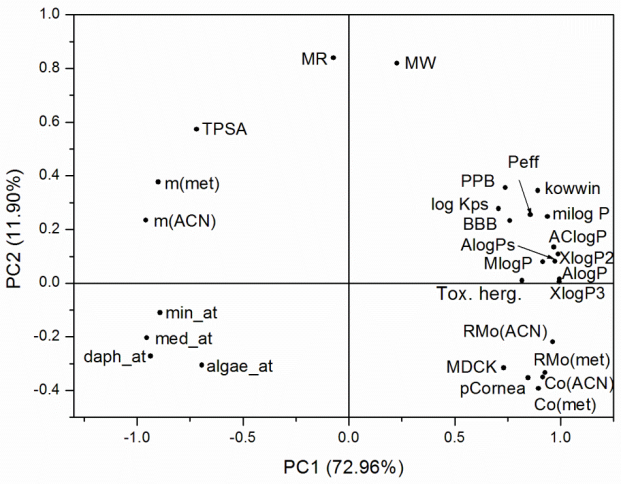


Fig. 3. Eigenvalues of correlation matrix for the studied compounds

No less significant, the second principal component further organizes the studied parameters. Namely, based on the PC2 value, within the negative PC1, the chromatographic parameter, *m*, is separated from the parameters of toxicity. Also, within the negative PC1, the software parameters of lipophilicity (log*P*) and pharmacokinetic parameters are separated from the chromatographic parameters, R_M^0 and C_0.

This partition not only shows PCA's ability to detect similarities among the analyzed bioactivity parameters but also the fine dissimilarities incurred as result of different ways of determination.

Figure 4 shows a grouping of the studied derivatives performed by applying PCA to variables, (score plot). It is evident that the first two principal components achieve a good classification of the tested compounds based on the substituent properties.

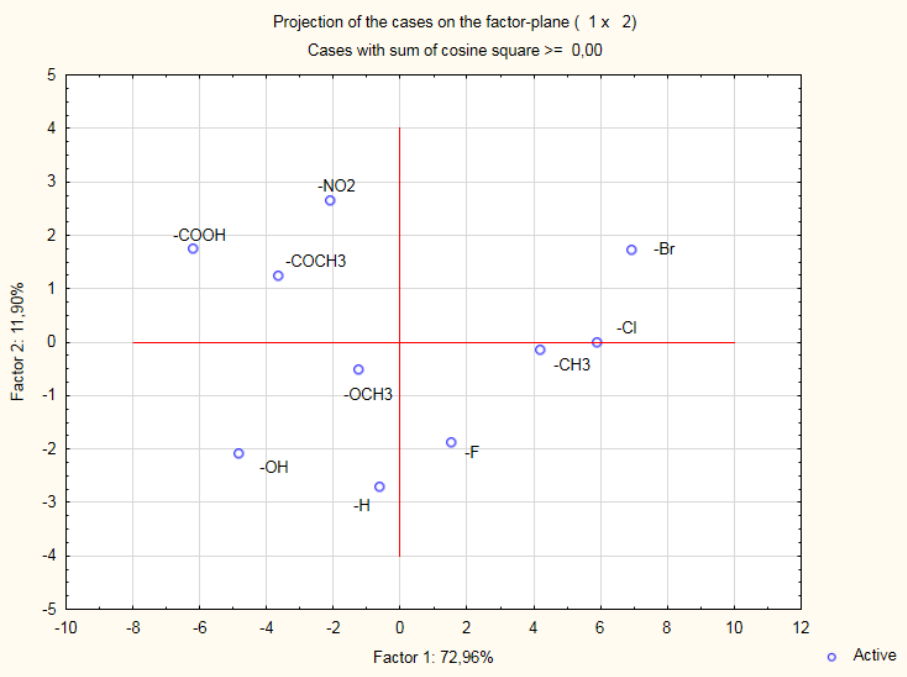


Fig. 4. Loading plot as a result of PC1 versus PC2

The first principal component (PC1) divides the derivatives based on the polarity of the substituent presented in the molecule. The results obtained are similar to the results obtained by the Cluster analysis.

The first group whose PC1 is negative consists of derivatives with polar substituents (NO_2, OH, COOH, $COCH_3$ and OCH_3), while the second group with positive PC1 includes derivatives with non-polar and halogen substituents (F, Cl, Br and CH_3).

It was assumed that PCA as well as CA clearly separated the investigated compounds based on polarity of the substituent, so the values obtained for PC1 were compared with the Hansch parameter (π) using the linear regression method. The Hansch parameter represents the contribution of a particular substituent to the lipophilicity of the molecule. Table S2 gives the values of the Hansch parameter for substituents presented in the analyzed compounds.

The relationship between the values of PC1 and the Hansch parameter is shown in Figure 5.

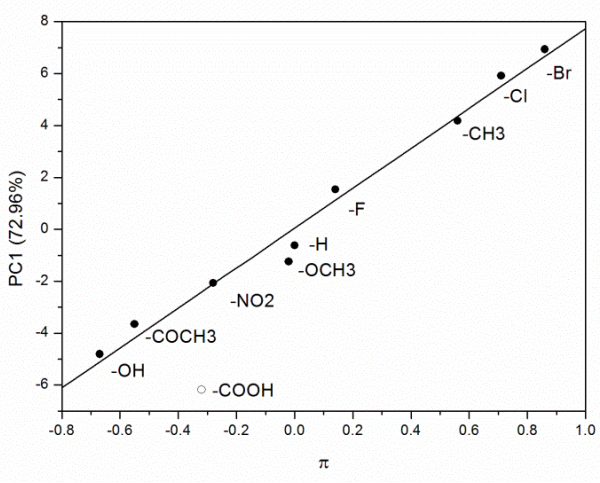


Fig. 5. Relationship between the values of PC1 and the Hansch parameter

The obtained PC1-π linear dependence is described by the following equation:

$$\begin{aligned} PC1 &= 0.0454 + 7.695\pi, \qquad r = 0.991 \\ sd &= 0.604 \qquad\qquad p < 1\cdot10^{-4} \end{aligned} \quad (4)$$

The high value of the correlation coefficient indicates that the polarity of the substituent presented in the molecule has a high impact on the studied properties of the azo derivatives.

Also, the distribution based on PC2 values is noticeable. It is considered that this partitioning is based on the electronic effects of the substituent attached to the molecule. Compounds with negative PC2 values contain substituents that have the ability to donate electrons and further increase reactivity, except for F. These substituents (OH, OCH_3, F and CH_3) favor further substitution in the ortho and para position. Compounds with positive PC2 have deactivating substituents, and they favor further substitution in meta (NO_2, COOH, $COCH_3$) or ortho- and para- positions (Cl and Br).

The quantitative dependence of the substituent electronic effects on the properties of the azo derivatives was checked by the change of the PC2 value in the function of the Hammett substituent constant, σ. Unfortunately, no significant correlation was obtained.

4. CONCLUSION

In order to obtain information about the bioactive profile of the studied azo derivatives, it was found that they fulfill Lipinski, Ghose and Veber drug-likeness rules. As an alternative measure of lipophilicity, the chromatographic parameters (R_M^0, m and C_0) of the tested azo derivatives were determined by thin layer chromatography on reverse phases (RPTLC C18/ UV254s). It was found that the chromatographic behavior of the tested derivatives is caused by the nature of the presented substituent and to a lesser extent by the nature of the applied modifier. Based on the software-derived lipophilicity parameters (log$P$), pharmacokinetic predictors, and ecotoxicity parameters, it was concluded that derivatives with nonpolar or halogen substituents show the best permeation through different biological membranes. Also, none of the derivatives exhibited cardiotoxicity, and for the selected test organisms, the most toxic was the most lipophilic derivative (Br as substituent).

The relationship between chromatographic parameters, standard measures of lipophilicity, pharmacokinetic predictors and the toxicity parameters of potentially bioactive azo derivatives was examined by linear regression and two multivariate methods (CA and PCA). Thereby, good linear relationships were obtained for each applied system. The results of multivariate methods show the great resemblance of the chromatographic parameters, R_M^0 and C_0 obtained in both modifiers used with standard measures of lipophilicity and pharmacokinetic predictors. On the other hand, the chromatographic parameter m, obtained in the same conditions, exhibits better agreement with the drug-likeness and toxicity parameters.

In addition, it was concluded that the polarity of a substituent present in the azo molecule influences the values of the bioactivity parameters more than its electronic effect.

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