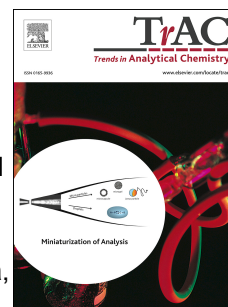


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State of the art and prospects of methods for determination of lipophilicity of chemical compounds

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# 1 State of the art and prospects of methods for determination of lipophilicity of 2 chemical compounds

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## 10 Abstract

11 Lipophilicity of the compounds is useful to (i) explain their distribution in biological systems, which is  
12 different in plant and in animal organisms, (ii) predict the possible pathways of pollutant transport in  
13 the environment, and (iii) support drug discovery process and select optimal composition in terms of  
14 bioactivity and bioavailability. The lipophilic properties can be determined by two main approaches,  
15 experimental, which apply instrumental techniques or computational, which is based on the complex  
16 algorithms. This review focuses primarily on various analytical methods that are used in the  
17 lipophilicity measurements. The classical methods and others based on chromatographic,  
18 electroanalytical and electroseparation approaches are compared and described in details. Modern  
19 solutions with chromatographic systems and their practical applications in the measurements of  
20 lipophilic and biomimetic properties of compounds have been included. However, there is an urgent  
21 need to standardize the high-throughput and reliable analytical procedure of the evaluation of  
22 lipophilic properties.

## 23 Abbreviations:

24 ACN, Acetonitrile; AGP,  $\alpha_1$ -Acid glycoprotein; CD, Conductivity detector; CE, Capillary  
25 electrophoresis; CHI, Chromatographic hydrophobicity index; CMC, Critical micelle concentration;  
26 CTAB, Cetyltrimethylammonium bromide; CV, Cyclic voltammetry; DDT,  
27 Dichlorodiphenyltrichloroethane; DMSO, Dimethyl sulfoxide; ECD, Electron capture detector; ED,  
28 Electrochemical detector; EDC, 1,2-Dichloroethane; EKC, Electrokinetic chromatography; ELSD,  
29 Evaporative light scattering detection; EOF, Electroosmotic flow; ESI-TOF-MS, Electrospray ionization  
30 time-of-flight mass spectrometry; FID, Flame ionization detector; FLD, Fluorescence detector; GC,

31 Gas chromatography; HSA, Human serum albumin; IAM, Immobilized artificial membrane; IPA,  
32 Isopropyl alcohol; IP-HPLC, Ion-pair high performance liquid chromatography; LI, Lipophilicity Index;  
33 IUPAC, International Union of Pure and Applied Chemistry; MeOH, Methanol; MEKC, Micellar  
34 electrokinetic chromatography; MEEKC, Microemulsion electrokinetic chromatography; MOPS, 4-  
35 Morpholinepropanesulfonic acid; MP, Mobile phase; MS, Mass spectrometry; NMR, Nuclear magnetic  
36 resonance; NP, Normal phase; OD-PVA, Octadecyl-poly(vinyl alcohol); ODS, Octadecyl-bonded  
37 silica; OECD, Organization for Economic Co-operation and Development; PBS, Phosphate buffer  
38 saline; PDMS, Polydimethylsiloxane; PGDP, Propylene glycol dipelargonate; PBS, phosphate buffer  
39 saline; PS, polystyrene; PS-DVB, Polystyrene-divinylbenzene; RID, Refractive index detector; RP-  
40 HPLC, Reversed-phase high performance liquid chromatography; RP-TLC, Reversed-phase thin-  
41 layer chromatography; RT, Retention time; RTILs, Room-temperature ionic liquids; SDS, Sodium  
42 dodecyl sulfate; SFM, Shake-flask method; SP, Stationary phase; SPME, Solid phase  
43 microextraction; SSM, slow stirring method TBAB, Tetrabutylammonium bromide; TEA, Triethylamine;  
44 THF, Tetrahydrofuran; TMAC, Tetramethylammonium chloride; UV-Vis, ultraviolet-visible  
45 spectrophotometry; VEKC, Vesicular electrokinetic chromatography.  
46 D, Distribution coefficient; k, Retention factor (liquid chromatography); k', Retention factor  
47 (electro separation methods); K, Distribution constant;  $K_{oa}$ , n-octanol-air partition coefficient;  $K_{oc}$ ,  
48 organic carbon-water partition coefficient; P ( $K_{ow}$ ), n-octanol/water partition coefficient; pKa, acid  
49 dissociation constant.

50

**51 Keywords:**

52 Lipophilicity; Shake-flask method; Potentiometric titration; High-performance liquid chromatography;  
53 Thin-layer chromatography; Capillary electrophoresis; Electrokinetic chromatography; Cyclic  
54 voltammetry

**55 Contents****56 1. Introduction****57 2. Classical methods for lipophilicity determination****58 3. Separation and electroanalytical methods for lipophilicity determination****59 3.1. Chromatographic methods****60 3.1.1. Thin-layer chromatography based methods**

**61 3.1.2. Liquid chromatography based methods****62 3.1.2.1. Chromatographic partition systems for measuring lipophilic properties****63 3.1.2.2. HPLC procedures for lipophilicity assessment****64 3.2. Electroseparation methods****65 3.3. Electroanalytical methods****66 4. Conclusion**

67

**68 1. Introduction**

69 According to International Union of Pure and Applied Chemistry (IUPAC) lipophilicity is the affinity of a  
70 molecule or a moiety for a lipophilic environment. It is commonly measured by the distribution  
71 behavior in a biphasic system [1], either liquid-liquid or solid-liquid system. This physicochemical  
72 property of a compound describes the balance between two major contributions: hydrophobicity and  
73 polarity. Hydrophobic effect relates to the tendency of non-polar compounds to prefer a non-aqueous  
74 environment to an aqueous one. Lipophilicity extends the hydrophobicity because of the polar term  
75 related to electrostatic interactions and hydrogen bonds. Therefore, lipophilicity comprises the  
76 favorable solute-solvent interactions that contribute to the distribution of a solute between two media:  
77 water and organic solvents. Other specific solubilizing media as biomembranes are also considered  
78 [2].

79 Different methods enable the measurement of solute-solvent interactions and based on them the  
80 lipophilicity of a compound can be determined. Many various lipophilicity descriptors have already  
81 been proposed, however the background point is the n-octanol-water partition coefficient P expressed  
82 as logP or logK<sub>ow</sub> [3]. It is defined as the ratio of the concentrations of a neutral compound or an  
83 ionizable compound in its neutral form in n-octanol (C<sub>O</sub>) and aqueous (water; C<sub>W</sub>) phases under  
84 equilibrium conditions (Eq. 1) [1,4–6].

$$85 \log P = \log(C_O/C_W) \quad (1)$$

86 Moreover, if a compound undergoes ionization in the aqueous phase another lipophilicity descriptor  
87 called distribution coefficient (logD) is used. It takes into account all forms of a compound, neutral and  
88 ionized, present at a given pH. For weak monoprotic acids or bases that are partially ionized in the



aqueous phase, distribution and partition coefficients are related through Eq. 2 and Eq. 3, respectively:

$$\log D_{\text{acids}} = \log P - \log(1 + 10^{\text{pH} - \text{pK}_a}) \quad (2)$$

$$\log D_{\text{bases}} = \log P - \log(1 + 10^{\text{pK}_a - \text{pH}}) \quad (3)$$

where  $\text{pK}_a$  is acid dissociation constant. According to these equations (Eq. 2, 3) the solubility of an ionizable compound increases exponentially with the difference between pH and  $\text{pK}_a$  [7].

The lipophilic properties of the compounds allow to characterize chemicals according to several aspects. First, they help to explain distribution of the compounds and to predict their transport in different biological systems considering the fact that the relations between lipophilic/hydrophilic properties are different in plant and animal organism. The second aspect considers the prognosis of the pollutant pathway in the environment. This knowledge allows to determine compound ability to be transported in and between the environment compartments or the possible place of its accumulation [3,8]. Partition coefficient  $K_{ow}$  is a useful index of the potential for bioaccumulation, bioconcentration and biomagnification of environmental pollutants. It has been assumed that bioaccumulation does not occur for substances with  $K_{ow}$  value lower than 2000 ( $\log K_{ow} < 3.3$ ), whereas easy bioaccumulated substances with tendency to biomagnification through food chain exhibit  $\log K_{ow}$  higher than 5 [9], which can be determined by some of the methods listed in Table 1. This coefficient is also related with n-octanol-air partition coefficient ( $K_{oa}$ ) that describes partitioning between atmosphere and terrestrial environment or the organic carbon-water partition coefficient ( $K_{oc}$ ) that is useful in the prediction of the mobility of organic soil contaminants [10–13]. The lipophilic properties are also useful for chemicals in respect to their optimal attribute for specific tasks in the industry [2,4].

The pathway of drugs and food components to reach their target site is complex since adsorption depends on solubility and permeability. Both properties are influenced by the lipophilicity of the compounds, however, in a different way. Moreover, the degree of ionization affects compound's lipophilicity and it influences its solubility and permeability thus absorption too [5,14–17]. The absorbed compounds undergo metabolic transformations, which can result in the changes of physicochemical properties such as the molecule size or mass, charge and lipophilicity [18,19]. Due to the complex nature of bioactive compounds in drugs and food as well as their different mechanism

117 of absorption and metabolism [7,20] the lipophilic characteristics performed on biomimetic stationary  
118 phases (SPs) are useful tools in the assessment of biological effects of these components.  
119 Consequently, the specific partition coefficients determined with the application of immobilized  
120 artificial membrane ( $K_{IAM}$ ), human serum albumin ( $K_{HSA}$ ) and  $\alpha_1$ -acid glycoprotein ( $K_{AGP}$ ), have been  
121 proposed [21].

122 Therefore, the lipophilic characteristics of environmental pollutants, food-derived bioactive compounds  
123 and drug ingredients can help to better understand their fate outside and inside the living organisms  
124 and to build models for biological absorption and partition processes trying to predict *in vivo*  
125 distribution of potential bioactive molecules (i.e. nutraceuticals and pharmaceuticals) [22,23].

126 Considering above, logP value of the solute allows to propose the fate of the chemical in the body as  
127 it describes compound ability to reach its intended target. Moreover, the lipophilicity of the compounds  
128 can be used as input parameters to design models for prediction of environmental transport of  
129 pollutants between water phase and natural organic matter in soil and sediments including the  
130 transport by biological membranes. In addition, the distribution between water and soil sediments  
131 relates also to chemicals of food chain and those undergoing the ingestion [3,13,16].

132 In recent years, the studies of lipophilic properties of bioactive compounds, including pharmaceuticals  
133 and natural products, as well as toxins, including environmental pollutants, have become a topic of  
134 increasing interest. In fact, a literature search performed on the Web of Science and Scopus  
135 databases revealed that the number of publications on lipophilicity and partition coefficient has  
136 quadrupled or even increased fivefold (Fig. 1) in the past two decades (28084 or 71601 until 1997,  
137 while between 1998-2018 around 153805 or 274422 scientific papers have been published according  
138 to Web of Science and Scopus search engine, respectively).

139 This tendency has provided development of different approaches in lipophilicity determination (see  
140 Table 1). We propose to divide them into classical methods and a set of methods based on different  
141 backgrounds: chromatographic, electroseparation, potentiostatic or voltamperometric (Fig.2). The well  
142 recognized classical methods are shake-flask technique or stir-flask technique, whereas a variety of  
143 chromatographic methods (reversed-phase thin-layer chromatography (RP-TLC) and reversed-phase  
144 high-performance liquid chromatography (RP-HPLC)) are today the most popular ones [24,25]. Main  
145 advantages and limitations of the lipophilicity determination techniques are shown in Table 1 and will

146 be discussed in details further. The logP values can be also calculated using various computer  
147 softwares or Internet available modules that applied different algorithms based on structural,  
148 atomistic, topological, electrotopological, or other considerations on a drawn chemical structure [26].

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149 **Table 1.** Comparison of methods for determination of lipophilicity based on their usefulness, main advantages and limitations.

	SFM	SSM	Potentiometric titration	TLC	HPLC	CE	EKC methods	CV
Measurement	Direct	direct	direct	indirect	indirect	indirect	indirect	indirect
LogP range	Classic: $-3 < \log P < 4$ Miniaturized: $-2 < \log P < 6$ SPME-based: $0 < \log P < 5$	$\log P > 5$	$-1 < \log P < 8$	$4 < \log P$	$-3 < \log P < 8$	$0 < \log P < 5$	$-1 < \log P < 7$	$-8 < \log P < 1$
Sample amount required	Classic: 10-50 mg Miniaturized: 1-10 mg SPME-based: <2 mg	10-50 mg	2-50 mg	2-10 mg	<1 mg	<1 mg	1-2 mg	1-10 mg
Partition solvent	n-octanol, aqueous buffer systems	water, n-octanol	n-octanol, KCl	water and organic solvent (e.g. MeOH, ACN, THF, dioxane)	aqueous buffer, ACN or MeOH	buffers	buffers, surfactants	1,2-dichloroethane or nitrobenzene, buffers
Consumption of organic solvent	Classic: high Miniaturized: low SPME-based: solventless	high	low	very low	Isocratic: low to medium Gradient: very low	low	low	medium
pH range	$0 < \text{pH} < 14$	$0 < \text{pH} < 14$	$1.8 < \text{pH} < 12.2$	$2 < \text{pH} < 11$	Isocratic: $1.5 < \text{pH} < 10.5$ (13*) Gradient: $1 < \text{pH} < 12$ (13*)	$2 < \text{pH} < 12$	$2 < \text{pH} < 12$	$0 < \text{pH} < 14$
Apparatus required	Classic: basic glassware and labware, UV-Vis spectrophotometer or LC system (LC-UV, LC-MS) or NMR detection Miniaturized: multi-well plates, liquid handling robot, shaker and centrifuge for plates, highly sensitive quantification system	basic glassware and labware, GC or LC system with appropriate detection (e.g. FID, ECD, MS, UV)	potentiometric titrator with a set of electrodes	manual or fully automated TLC system with detection unit (UV, FLD); fast, reproducible, high-throughput, but more expensive	LC system with various detection (UV, FLD, MS, MS/MS)	CE system with CD, ED, FLD, UV or MS detection	CE system with CD, ED, FLD, UV or MS detection	typical apparatus for electrochemical measurements





	(LC-UV/MS, LC-MS/MS, NMR); high-throughout, but more expensive approach			approach				
	SPME-based: SPME device, GC or LC system with appropriate detection (e.g. FID, ECD, MS, UV)							
Time consumption	Classic: ~1 day Miniaturized: 10-30 min; up to 200 compounds/day SPME-based: up to 2 h (depending on type of SPME fiber used)	2-4 days	up to 30 min; determination of lipophilicity profile of one compound	30-60 min; simultaneous analysis of several to several dozen compounds	Isocratic: 30-120 min (depends on the dimension of the column used); several compounds in single run Gradient: 5-20 min; up to tens of compounds per run	up to 60 min; simultaneous analysis of several to several dozen compounds	15-30 min; 20-30 compounds per run (2 h) in multi-well plate format	up to 30 min; single measurements
Lipophilicity index determined	Classic, miniaturized: logP SPME-based: logK	logP	logD, logP <sup>N</sup> , logP <sup>I</sup>	R <sub>M</sub> , R <sub>M0</sub>	Isocratic: logk, logk <sub>w</sub> , S, CHI, logP Gradient: CHI, k <sub>g</sub> , logP, logD	logP	logk', logP	logP <sup>0,i</sup>
Application	Neutral and ionizable compounds (only in their neutral form), limited to highly hydrophobic and sparingly soluble compounds	Highly hydrophobic compounds in neutral form	Ionizable compounds with acid-base properties	Neutral compounds, ionizable compounds only in their unionized form (appropriate mobile phase pH and composition is required)	Neutral compounds, ionizable compounds only in their unionized form (appropriate mobile phase pH and composition is required)	Cations and anions of inorganic and organic salts	Neutral compounds	Ionizable compounds

\* pH limit for polymer-based stationary phases of HPLC columns; CD – conductivity detector; ECD – electron capture detector; ED – electrochemical detector; FID – flame ionization detector; FLD – fluorescence detector; SPME – solid-phase microextraction; CHI – chromatographic hydrophobicity index; D – distribution coefficient; k – retention factor in LC methods; k' – retention factor in EKC methods; k<sub>g</sub> – apparent gradient capacity factor; k<sub>w</sub> – retention factor extrapolated to zero organic phase concentration; K – distribution constant; P – n-octanol/water partition coefficient; P<sup>I</sup> – partition coefficient of ionized forms; P<sup>N</sup> – partition coefficient of neutral forms; R<sub>M</sub> – retardation parameter; S – slope of the linear relationship between the organic solvent concentration (φ) and the logk



## 150 2. Classical methods for lipophilicity determination

151 The oldest and the most known method of lipophilicity measurement, shake-flask method (SFM), is  
152 based on liquid-liquid extraction using n-octanol/water system [24]. The analyzed substance is  
153 dissolved in two flasks, the first one filled with aqueous buffer solution and the second one containing  
154 an organic solvent (n-octanol). Both solutions should be saturated before the compound introduction,  
155 buffer with n-octanol, and n-octanol with water. These solutions are introduced to the laboratory  
156 separator and then shaken to achieve equilibration of the solute between the aqueous and organic  
157 phases as shown in Fig.3a [27]. After the equilibrium is reached, UV-Vis spectrophotometry or other  
158 appropriate analytical method is employed to determine the concentration of the compound dissolved  
159 in each phase [23].

160 Due to the simplicity and evident correlation with partitioning phenomenon, SFM is recommended as  
161 a benchmark procedure for the other methods. However, reliable measurement of lipophilicity by this  
162 method is only achievable in the logP range from -3 to 4 [27–29]. Furthermore, the procedure is  
163 tedious and time-consuming and requires relatively large amount of pure solutes. In addition, there is  
164 a possibility of formation of stable emulsions after the shaking step [28,30–33]. Emulsion in n-  
165 octanol/water system can be a serious problem, particularly in the case of the hydrophobic  
166 compounds. The  $\log K_{ow}$  depends on relative solubility of the compound in water and organic solvents  
167 and it has to be corrected for ionization. Moreover, due to amphiphilic properties, some compounds  
168 may behave as detergents [23,30,34]. Modifications of this classical method (Fig.3) enable to handle  
169 with some of limitations described above. In case of model of biological partition, different solutions  
170 are used to determine the partition coefficient. Aside from n-octanol/water set, which is characterized  
171 by the properties of hydroxyl group connected with function of hydrogen-bond donor and acceptor,  
172 some other solvents that allow to imitate different physiological cell barriers have been proposed.  
173 These systems include chloroform and water (a set with largely proton donors) or alkane (e.g.  
174 cyclohexane or dodecane) and water (a set without hydrogen acceptors and donors) or propylene  
175 glycol dipelargonate (PGDP) and water (a set with largely proton acceptors as in phospholipid  
176 membranes). PGDP, chloroform, n-octanol and cyclohexane are known as solvents that encoding  
177 important hydrogen bonding properties. Due to hydrogen bonding effects, the values of partition  
178 coefficient measured in these four solvents are different but their forces account for membrane  
179 partitioning. Lipophilicity parameters measured experimentally for these solvent systems are partially

180 dependent on the reference system and vary from classical n-octanol/water partition coefficient. The  
181 n-octanol/water model does not reflect the drug partitioning in biological structures anymore, thus  
182 these critical quartet (chloroform, cyclohexane, n-octanol and PGDP) has been proposed to be used  
183 instead of one organic solvent [35,36].

184 Miniaturization of SFM has led to the development of the high-throughput methods for screening of  
185 new targets with pharmacological effects. In traditional procedure even hundreds milliliters of each  
186 phase was used during the extraction. Nowadays, the volume of organic and aqueous solvents has  
187 trimmed down to less than 1 mL and the 96-well plate format of SFM has been presented [37]. The  
188 miniaturized SFM may be coupled with sensitive detection technique, such as mass spectrometry  
189 (MS). The LC-UV/MS systems have been successfully applied for the final determination step and the  
190 obtained results were in good correlation with literature values. The biggest advantages of the  
191 miniaturized method are rapidity, great flexibility, the use of small amount of solutes, the extended  
192 range of measured logP (from -2 to 6) and the potential to be fully automated. However, the  
193 miniaturization does not exclude the emulsion problem, especially for hydrophobic compounds  
194 [23,37–39].

195 In case of poorly water soluble substances, the lipophilicity may be determined by automated  
196 continuous sampling method, called filter probe method. This method is simple, partially automated  
197 and time-saving. Furthermore, computer program monitoring showed that filter probe method ensured  
198 greater accuracy and reproducibility [40]. However, during the analysis of the partition coefficient of  
199 highly hydrophobic compound, the concentration of this compound in organic phase is much higher  
200 than that in aqueous phase. In such case, contamination of aqueous phase by n-octanol layer is  
201 usually occurring during sampling. To solve this problem, the water-plug aspiration/injection method  
202 has been developed. In this method, the sample is taken by a small syringe with needle filled with a  
203 few microliters of water as a plug. This is expected to prevent contamination while the needle is  
204 passing through the n-octanol layer to reach sample in the aqueous layer, because the water plug  
205 stops the entrance of the n-octanol into the needle [38].

206 Another modification of classical methods applied for the determination of logP is the procedure  
207 based on nuclear magnetic resonance spectroscopy (NMR). The extraction step takes places in NMR  
208 tubes, content of which is vigorously mixed for 20 minutes. The concentration of analyte in water

209 phase is measured twice: before addition of the n-octanol and after the extraction. Then, P value is  
 210 calculated from the following equation (Eq. 4):

$$211 \quad P = (I_w - I_{w0})/I_w \quad (4)$$

212 where  $I_w$  and  $I_{w0}$  are the intensity of the signal of analyte in pure water and in water with n-octanol  
 213 added, respectively. This method was proposed by Cumming and Rücker and so far it has been used  
 214 to determine the partition coefficient of some common solvents, such as acetone and tetrahydrofuran  
 215 (THF) [41]. Moreover, it is well-suited for fast and easy measurement of n-octanol-water partition  
 216 coefficient of the compounds with sufficient water solubility and logP in the range from -1 to 1.

217 Another variation of the classical methods is the solid phase microextraction (SPME) developed by  
 218 Pawliszyn [42]. During the solventless extraction, the sample partitions between aqueous phase  
 219 (blood, urine or environmental water) and a fused silica fiber coated with a polymer. In SPME, the  
 220 equilibrium is established as the partition of analytes between the stationary and aqueous phase, and  
 221 it depends on distribution coefficients (D), the temperature of the sample, stirring rate, the ionic  
 222 strength of the solvent and the thickness of the fiber coating [43–45]. The amount of analyte adsorbed  
 223 by the fiber (N) at the equilibrium is defined by the following equation (Eq. 5):

$$224 \quad N = K \cdot V_s \cdot C_0 \quad (5)$$

225 where  $V_s$  is the volume of the SP,  $C_0$  is the initial concentration of analyte in aqueous phase, and K is  
 226 the distribution constant of analyte partitioning between the aqueous and stationary phase. However,  
 227 when the value of distribution constant is high and the volume of sample is small, there is a possibility  
 228 that analytes are mainly presented on the SP. In that case, the Eq. 6. should be used. According to  
 229 this equation, the distribution coefficient is defined as:

$$230 \quad K = (N \cdot V_{aq}) / (V_s \cdot (V_{aq} \cdot C_0 - N)) \quad (6)$$

231 where  $V_{aq}$  is the volume of aqueous phase [46,47].

232 In 1996 Dean et al. proposed the use of the fused-silica fiber coated with polyacrylate for  
 233 determination of the K of phenols. They confirmed that K values determined by SPME method can be  
 234 correlated with logP values. Their results indicated that SPME was an appropriate method for  
 235 estimating logP. Currently, the non-polar fiber coatings (i.e. polydimethylsiloxane (PDMS)) are  
 236 commonly used in this technique. Studies have shown that distribution coefficients obtained by SPME  
 237 with PDMS coating also correlate well with logK<sub>ow</sub> values [47,48]. SPME coupled with gas  
 238 chromatography, and less often with LC, and MS detection enables the determination of minimal

239 quantities of highly non-polar compounds in aqueous phase and minimizes the loss of volatile  
240 compounds [49].

241 Slow stirring method (SSM) follows the same principle as SFM. However, the emulsion formation is  
242 limited. Although this method allows to determine the logP of neutral compounds, it is time-  
243 consuming, rather expensive and requires a large amount of sample [50–52].

### 244 **3. Separation and electroanalytical methods for lipophilicity determination**

245 Currently, classical methods of lipophilicity determination are almost totally replaced by indirect  
246 methods that include chromatographic, electrochemical and electroseparation methods. In contrast to  
247 time-consuming equilibration, chromatography and other techniques provide measurement of  
248 extended range of lipophilicity during rapid analysis, in which the sample impurities usually do not  
249 affect the measurements [24,53].

#### 250 **3.1. Chromatographic methods**

251 Chromatographic methods have been applied for the lipophilicity determination since 1970s [54,55].  
252 Due to their many advantages and relatively few limitations, nowadays they are the most popular  
253 experimental indirect methods for estimating logP values.

##### 254 **3.1.1. Thin-layer chromatography based methods**

255 TLC is a chromatographic technique used for separation of non-volatile mixtures. The first application  
256 of TLC was the determination of impurities in pharmaceutical preparations and since 1938 this  
257 technique has been applied in diverse fields of chemistry. The adaptability of TLC may offer lots of  
258 new possibilities to evaluate lipophilic character [24]. In this case, different reversed-phase modes  
259 (see Table 2) are commonly used where the stationary phase is a foil coated with thin layer of silica or  
260 aluminum derivatives, both modified with hydrophobic ligand bounded covalently or by absorption,  
261 and the mixture of water and water-soluble organic solvent works as the mobile phase (MP) [36]. The  
262 retention of analytes can vary during changing the content of organic solvent in the MP and the  
263 activity of SP is based on the contribution of many specific parameters influencing the  
264 chromatographic behavior. The most important ones are the chemical structures of the sorbents, the  
265 surface area, the density of the free active centers per unit of sorbent surface area and the energy of  
266 intermolecular interactions between a molecule and a type of sorbent active centers. These

267 parameters affect the data obtained for measured lipophilicity of molecules, so the standardization of  
268 the TLC procedure in relation with biological impact is needed [24,56].

269 The most popular chromatographic lipophilicity descriptor is the retardation parameter ( $R_M$ ) defined by  
270 Bate-Smith and Westall through the following formula (Eq. 7) [57]:

$$271 \quad R_M = \log((1/R_f) - 1) \quad (7)$$

272 where the  $R_f$  is the retention factor, which is calculated as the ratio of the migration distances of solute  
273 and the solvent front. The  $R_M$  value depends linearly on the concentration of organic modifier in MP  
274 and this relationship is described by a TLC adapted Soczewiński-Wachtmeister equation [58,59] (Eq.  
275 8):

$$276 \quad R_M = -S \cdot \varphi + R_{Mw} \quad (8)$$

277 The value of  $R_{Mw}$  is extrapolated to pure water as a MP. The regression slope ( $S$ ) is directly linked to  
278 specific surface area of SP and is considered also as an alternative lipophilicity descriptor. The last  
279 factor,  $\varphi$ , represents the volume of organic solvent in the MP [24,58,59].

280 The RP-TLC is easy to perform and rapid. It requires small amount of the samples and allows the  
281 analysis of several compounds simultaneously. Moreover, there is no need to test pure compounds or  
282 to perform the problematic quantitative analysis. This technique is especially suitable for the  
283 investigation of compounds with low water solubility ( $\log P > 4$ ). Furthermore, the use of TLC plates  
284 coated with RP material allows to overcome some of the disadvantages associated with SFM,  
285 because the reversed-phase mode simulates the process of n-octanol-water partitioning [23,25].

286 Recently, new approaches in the lipophilicity determination using TLC have been reported [60–62].  
287 Janicka et al. have used micellar TLC and over-pressured-layer chromatography (OPLC) [60,63],  
288 where surfactants (anionic sodium dodecyl sulfate (SDS), cationic cetyltrimethylammonium bromide  
289 (CTAB) and non-ionic Brij-35) are used as the modifiers of MP. Due to amphiphilic character of  
290 micelles formed, both non-polar and polar interactions between them and solutes take place during  
291 the analysis. As a consequence, these systems are supposed to be more similar to biomembranes  
292 than in classical TLC [64]. Another method applied for the lipophilicity evaluation is normal-phase TLC  
293 (NP-TLC), where MP contains two organic solvents, polar (e.g. acetone, ethanol, ethyl acetate) and  
294 non-polar (e.g. benzene, cyclohexane, carbon tetrachloride, toluene). An excellent review of these  
295 TLC approaches for lipophilicity studies has been published lately [59].

296 **3.1.2. Liquid chromatography based methods**

297 The use of RP-HPLC as indirect method for the determination of lipophilic properties has been the  
298 subject of several reviews [6,23,65–68]. Nowadays it has become one of the most commonly used  
299 procedures in the lipophilicity studies recommended by Organization for Economic Co-operation and  
300 Development (OECD). In general, this method is based on dynamic partitioning of a compound  
301 between two immiscible phases, solid and liquid (SP of the column and MP) (Fig. 4), which is  
302 consistent with the IUPAC definition of partition coefficient [69]. Based on the solvophobic theory the  
303 interaction between the solute and the SP is considered as a reversible association of the solute  
304 molecules with the SP moiety. The distribution of the compound between the SP and MP is directly  
305 related to the chromatographic retention time (RT,  $t_R$ ). The solute retention factor ( $k$ ) is proportional to  
306 the ratio of the average number of analyte molecules in the SP ( $n_s$ ) to the average number of  
307 molecules in the MP ( $n_m$ ) during the chromatographic elution (Fig. 4). This lipophilicity index (LI) is  
308 usually expressed using a logarithmic scale and can be related to the distribution constant ( $K$ ) of the  
309 compound between the MP and SP, as shown in Figure 4. Therefore, the retention of dissolved  
310 compound is governed by this equilibrium constant [70].

311 Both equations shown in Figure 3 provide the theoretical basis for the partition data obtained from  
312 retention of the compound in the selected chromatographic system. In contrast to the determination of  
313 compound concentration required within the classical methods, only RT measurements are necessary  
314 to determine the LIs by RP-HPLC procedures and thus it is the main indicator of a real partition  
315 process. This chromatographic separation-based approach also provides other practical advantages:  
316 speed up the experimental work, good reproducibility, process automation, broad dynamic range, on-  
317 line detection (mainly UV-Vis or refractive index detector (RID)), small amount of sample required,  
318 independence of measurements from low compound solubility as well as impurities or degradation  
319 products. However, some limitations of the RP-HPLC method have also been noted, including (i)  
320 insufficient modeling of the n-octanol-water system for structurally diverse compounds, (ii) pore size  
321 effects for sorbents filling chromatographic column have no counterpart in the n-octanol-water  
322 partition system, (iii) possible interactions with the surface of the SP that not occur in the n-octanol-  
323 water system, (iv) time-consuming isocratic measurements in some cases, (v) limited pH working  
324 range for most of the SPs (2.0-7.5). In order to overcome these drawbacks, some solutions have  
325 been recently introduced, just to mention novel types of columns [21,71,72] that are designed to



326 mimic the n-octanol-water system or biological membranes and let to operate over a wide pH range  
327 (1.0-12.0). Moreover, application of short columns with smaller inner diameter results in acceleration  
328 of experiments and reduction of costs, especially reduced consumption of organic solvents.  
329 Separations using micellar MPs, and gradient elution procedures have been proposed lately  
330 [60,70,73,74].

### 331 3.1.2.1. Chromatographic partition systems for measuring lipophilic properties

332 One of the most important aspects in the lipophilicity assessment by HPLC is to develop a  
333 chromatographic system that mimics the standard n-octanol-water partition system as closely as  
334 possible. Therefore, various SPs as well as MP modifying agents have been introduced and tested for  
335 this purpose. Improved or newly-developed SPs for the lipophilicity studies were summarized by  
336 Kaliszan [75] and Giaginis et al. [76] and they include mainly silica-based, polymer-based and  
337 biomimetic phases as shown in Table 2. Octadecyl-bonded silica (ODS) and other alkyl groups  
338 bonded to silica core are one of the most commonly used SPs for HPLC-based lipophilicity  
339 measurements. However, the possibility of polar moieties interactions, including hydrogen bonding or  
340 electrostatic attraction, with the remaining free-silanol groups on the silica surface may affect the  
341 partitioning mechanism of RP-HPLC and thus results in increases in RTs and peaks asymmetry  
342 [67,75]. Therefore, the studies of lipophilic character of ionizable basic compounds on silica-based  
343 columns can be difficult and give overestimated results [77,78].

344 Some improvements of silica-based SPs have been recently proposed. End-capping of the free-  
345 silanol sites by short alkyl groups (i.e. trimethylsilyl group (TMS)) is usually performed in order to  
346 provide higher degree of silanization [79] and thus make the column packaging material more suitable  
347 for analysis of strong hydrogen-bonding and ionized compounds [80]. Another solution for reducing  
348 effect of residual silanols is embedding or end-capping polar groups (i.e. amide, carbamate, ether,  
349 sulfonamide or ammonium) in the alkyl chains [72]. Due to accurate mimicking of biopartitioning and  
350 good correlation with  $K_{ow}$ , alkylamide-silica HPLC columns are one of the most frequently applied  
351 phases of this type [71,81]. The possibility of use of MP with a high water content or even pure water  
352 without the risk of hydrophobicity collapse of these type of SPs is an additional advantage. However,  
353 the polar moieties incorporated into the silica backbone may interact with some analytes, i.e.  
354 polyphenols and thus addition of masking agents is also required [67,82]. Octanol-coated SPs were



355 successfully applied for the estimation of logP of neutral or basic compounds [83,84]. On the other  
356 hand, the problems associated with long-term stability of these columns may occur [6].

357 In order to overcome pH limitation of ODS columns and to make the lipophilicity assessment of basic  
358 analytes in their neutral form possible, new generation of SPs have been developed. They include:  
359 grafted polymer-silica hybrid columns [74,85,86], columns with hybrid organic-inorganic silica in which  
360 hydroxyl groups are replaced by methyl ones, and columns based on the bidentate technology that  
361 include a propylene bridge [71,72]. These types of column are protected from silanol interactions and  
362 have an extended pH range capability up to 12 [82,86].

363 More recently, the polymer-based SPs, including octadecyl-poly(vinyl alcohol) (OD-PVA), and  
364 polystyrene-divinylbenzene (PS-DVB) based columns, have also been successfully applied for the  
365 lipophilicity measurements [87–90]. In contrast to silica-based SPs, the polymer-based resins are  
366 rigid, macroporous, cross-linked polymers completely free of silanol groups and other polar sites, and  
367 hence irreversible binding of polar compounds is eliminated [77]. Furthermore, these columns are  
368 chemically inert in most organic solvents and stable over a wide pH range (1-13). However, their  
369 retention mechanism is governed by a different balance of structural properties (mainly  
370 dipolarity/polarizability parameter) as determined by linear solvation/free-energy relationships and  
371 thus obtained results may correlate better to alkane-water than to n-octanol-water partitions, which  
372 mimics the blood-brain partitioning more reliably [88,90]. Further evaluation of retention behavior on  
373 polymer-based SPs is needed for better use in the lipophilicity studies.

374 As an alternative choice for a more accurate description of compound distribution between various  
375 compartments *in vivo*, the SPs that could directly mimic biologically important elements and provide  
376 biomimetic characteristic are increasingly used in recent years. These biomimetic SPs include IAMs,  
377 liposomes and plasma proteins (i.e. HSA, AGP). The theoretical and practical aspects of using  
378 biomimetic columns and their detailed characterization have been the subject of several reviews  
379 [21,68,76,91–94]. The retention factors of compounds obtained using protein-based SPs can be  
380 easily converted to binding parameters such as  $\%HSA = 100 \times k / (k + 1)$ . These columns provide  
381 potential to simulate plasma protein binding, as retention mechanism incorporates other interactions  
382 than in n-octanol-water partitioning, especially those of electrostatic nature. It should be noted that  
383 since protein binding occurs naturally only at physiological conditions, there is no need to use MPs

384 with different pHs. Verification of protein SPs stability is also essential and can be easily ensured by  
385 HPLC analysis of racemic mixture of warfarin that should revealed the separation of enantiomers  
386 [21,68].

387 IAM columns introduced and patented by Pidgeon et al. [95] to model the lipid bilayers of the cells are  
388 prepared by covalent binding of phospholipids monolayers (i.e. phosphatidylcholine) to amine-  
389 modified silica support. IAM columns are highly stable with little phospholipid loss during analyses or  
390 storage [96] and commercially available, including the single chain and double chain SPs, which differ  
391 in the end-capping of free propylamine residues [97]. It is reported that double chain IAM phases  
392 better simulates the structure of natural phospholipids and hence the resulting lipophilicity indices  
393 correlate better with permeability data [98,99]. The amphiphilic character of phospholipid functional  
394 groups play an important role in IAM retention especially if charged molecules are analyzed. Thus,  
395 electrostatic interactions also affect the retention mechanism, which is mainly governed by  
396 hydrophobic/solvophobic interactions [81,97]. It should be emphasized that pure water can be used  
397 as a MP in IAM chromatography, which allows fast and direct determination of  $\log k$  value extrapolated  
398 to zero organic phase concentration referred as  $\log k_w$ . The addition of acetonitrile as organic modifier  
399 is recommended, when compounds with high affinity for the IAM SP are analyzed and then  $\log k_w$   
400 values require extrapolation. A novel SPs that may simulate cell membrane partitioning in the similar  
401 way as the IAM phases were introduced. They are *N,O*-dialkylphosphoramidate-based materials  
402 having in their structure amine groups, phosphate groups and hydrophobic long alkyl chains [100].  
403 Summarizing, the future trends in column development for the lipophilicity studies could be  
404 immobilization of other important proteins, enzymes or membrane lipids on the SP or introduction of  
405 similar functional groups to the silica surface and measure compound's interactions with them.

406 The mixtures of water and organic modifiers with some additives are commonly applied as MPs of  
407 chromatographic partitioning systems for the lipophilicity measurements. To speed up RP-HPLC  
408 analyses, especially of highly lipophilic compounds, methanol and acetonitrile are the most widely  
409 used modifiers. Methanol appears to be the most suitable organic solvent for the lipophilicity studies  
410 because it does not disturb the hydrogen-bonding network of water. On the other hand, acetonitrile,  
411 which generates the most asymmetrical peaks for basic analytes, proves to better simulate the  
412 'organic phase' [67,76]. The correlations between chromatographic hydrophobicity index (CHI,  $\phi_0$ ) and  
413  $\log P$  values reported by Valko et al. using fast gradient RP-HPLC procedures showed that acetonitrile

414 serves as a better organic modifier than methanol for both ODS and IAM columns [70,101]. According  
415 to the solvation equation, this results from the significant difference between the  $CHI_{MeOH}$  lipophilicity  
416 scale and the logP scale in terms of H-bond acidity, H-bond basicity, size and dipolarity/polarizability.  
417 In order to match the  $CHI_{ACN}$  scale with logP scale only H-bond acidity term should be considered  
418 thus acetonitrile was suggested as the preferred organic modifier by Valko et al. [70]. THF and  
419 isopropyl alcohol (IPA) are also used in some lipophilicity assays [73,102]. Due to the dissociation of  
420 most analytes in aqueous MPs, some chemical additives are required. These MP additives have been  
421 extensively discussed in many reviews [21,67,76,81,82] and therefore, only some important will be  
422 highlighted here. They can be divided into two main groups: ion suppressors and masking agents. In  
423 order to suppress dissociation of ionizable analytes and keep them in a neutral form different buffers  
424 have been applied, including morpholinepropanesulfonic acid, phosphate buffer and phosphate-  
425 buffered saline [82,84]. Ammonium acetate buffers are also used because they exhibit good  
426 compatibility with mass spectrometry. Acetic or perchloric acid and ammonia or triethylamine (TEA)  
427 were employed as ion suppressors in the lipophilicity assessment of weak acidic and weakly basic  
428 compounds, respectively [103,104]. Masking agents including hydrophobic amines (i.e. TEA, *n*-  
429 decylamine, *N,N*-dimethyloctylamine) and room-temperature ionic liquids (RTILs) are often used as  
430 MP additives to reduce or even to suppress silanophilic interactions. A small addition of amines (0.15-  
431 0.20%) is considered as the most suitable masking agents in combination with methanol as organic  
432 modifier [82,84]. Unlike amines, RTILs have no effect on the pH of MP. However, the use of RTILs  
433 complicates MS detection and may add noise or a background signal to UV detection [105,106]. In  
434 recent years, the addition of small amount of *n*-octanol to the methanolic MP has improved the HPLC  
435 determination of LIs. Almost 1:1 correlation between  $\log k_w$  and logP or logD was obtained with *n*-  
436 octanol-modified MP [86,107].



437 **Table 2. Chromatographic partition systems used in lipophilicity studies and their applications.**

Type of stationary phase	Mobile phase composition	Other important conditions	Type of analytes/samples	Measured LI	Ref.
<b>THIN-LAYER CHROMATOGRAPHY SYSTEMS</b>					
Silica gel TLC plate modified with: (1) cyanopropyl groups (CN), (2) octadecyl carbon chain (C <sub>18</sub> ), both with F <sub>254</sub> fluorescence indicator	(1) (a) Aqueous phase: CTAB Organic phase: ACN (b) Aqueous phase: SDS Organic phase: ACN (2) Aqueous phase: H <sub>2</sub> O Organic phase: MeOH	Saturation: 20 min Visualization: mixture of MeOH and sulfuric acid Detection: UV	25 Aromatic compounds (e.g. ethylbenzene, eugenol, fenitroton, nabumeton, phenol, vanillin)	R <sub>M</sub> , R <sub>Mw</sub>	[62]
Silica gel TLC plate modified with: (1) cyanopropyl groups (CN), (2) octadecyl carbon chain (C <sub>18</sub> ), (3) diol groups (DIOL), (4) octyl carbon chain (C <sub>8</sub> ), (5) dimethyl groups (C <sub>2</sub> ), all with F <sub>254</sub> fluorescence indicator	(a) Aqueous phase: H <sub>2</sub> O Organic phase: MeOH (b) Aqueous phase: H <sub>2</sub> O Organic phase: dioxane	Saturation: 20 min Detection: densitometric scanning	Naproxen	R <sub>Mw</sub>	[108]
Silica gel TLC plate modified with: (1) cyanopropyl groups (CN), (2) octadecyl carbon chain (C <sub>18</sub> ), (3) diol groups (DIOL), (4) octyl carbon chain (C <sub>8</sub> ), (5) dimethyl groups (C <sub>2</sub> ), all with F <sub>254</sub> fluorescence indicator	Aqueous phase: H <sub>2</sub> O Organic phase: ACN or MeOH	Saturation: 20 min Detection: UV	8 Cephalosporins	R <sub>Mw</sub> , R <sub>M</sub> , PC1/R <sub>M</sub>	[109]
Silica gel TLC plate modified with octadecyl carbon chain (C <sub>18</sub> ) / F <sub>254</sub> fluorescence indicator	Aqueous phase: H <sub>2</sub> O Organic phase: ACN or MeOH or acetone	Saturation: 20 min Detection: UV	6 Statin drugs	R <sub>Mw</sub> , C <sub>0</sub>	[110]
Silica gel TLC plate modified with: (1) cyanopropyl groups (CN), (2) octadecyl carbon chain (C <sub>18</sub> ), (3) octyl carbon chain (C <sub>8</sub> ), (4) amino groups (NH <sub>2</sub> ), all with F <sub>254</sub> fluorescence indicator	Aqueous phase: H <sub>2</sub> O Organic phase: CAN	Saturation: 10 min Visualization: ethanolic solution of bromocresol green treated with NaOH Detection: UV	4 Artificial and 13 natural sweeteners	R <sub>Mw</sub> , R <sub>M</sub>	[24]



Silica gel TLC plate modified with: (1) cyanopropyl groups (CN), (2) octadecyl carbon chain (C <sub>18</sub> ), (3) diol groups (DIOL), (4) octyl carbon chain (C <sub>8</sub> ), (5) dimethyl groups (C <sub>2</sub> ), all with F <sub>254</sub> fluorescence indicator	Aqueous phase: phosphate buffer Organic phase: MeOH	Visualization: fluorescamine/ 2,2-diphenyl-1-picrylhydrazyl Detection: UV	3 Amine neurotransmitters and 18 derivatives	R <sub>M</sub> , R <sub>MW</sub> , PC1/R <sub>M</sub>	[111]
Silica gel TLC plate modified with: (1) octadecyl carbon chain (C <sub>18</sub> ), (2) cyanopropyl groups (CN), both with F <sub>254</sub> fluorescence indicator	(1) Aqueous phase: H <sub>2</sub> O Organic phase: acetone, dioxane or MeOH (2) Aqueous phase: SDS Organic phase: THF	Saturation: 15 min Detection: UV	1,2,4-Triazoles (21 compounds)	R <sub>MW</sub>	[60]
Silica gel TLC plate modified with octadecyl carbon chain (C <sub>18</sub> ) / F <sub>254</sub> fluorescence indicator	Aqueous phase: H <sub>2</sub> O Organic phase: acetone or ACN or MeOH or THF or IPA	Detection: UV	15 Fluoroquinolones	R <sub>MW</sub>	[112]
Silica gel TLC plate modified with: (1) octadecyl carbon chain (C <sub>18</sub> ), (2) cyanopropyl groups (CN), both with F <sub>254</sub> fluorescence indicator	Aqueous phase: H <sub>2</sub> O Organic phase: MeOH	Saturation: 15 min Visualization: manganese chloride in sulfuric acid Detection: UV	Bile acids and their derivatives (27 compounds)	R <sub>MW</sub> , PC1/R <sub>M</sub>	[113]
Silica gel TLC plate modified with cyanopropyl groups (CN) / F <sub>254</sub> fluorescence indicator	(1) Aqueous phase: Brij (35) Organic phase: ACN (2) Aqueous phase: CTAB Organic phase: ACN (3) Aqueous phase: SDS Organic phase: acetone or dioxane or THF	Drying of plates: iodine vapor	13 Fatty acids and 4 polyphenols	P <sub>SW</sub> , K <sub>ma</sub>	[61]
Silica gel TLC plate modified with octadecyl carbon chain (C <sub>18</sub> ) / F <sub>254</sub> fluorescence indicator	Aqueous phase: H <sub>2</sub> O Organic phase: acetone or DMSO	Saturation: 15 min Detection: UV	4-Amino-7-chloroquinoline based compounds (18 compounds)	R <sub>MW</sub> , R <sub>M</sub>	[114]
<b>HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEMS</b>					
<b>SILICA-BASED COLUMNS</b>					
Kromasil C18 (250 mm × 4.6 mm, 5 μm particle size); Akzo Nobel / Eka Chemicals Inc.	Aqueous phase: H <sub>2</sub> O (10%) Organic phase: MeOH (90%)	HPLC mode: isocratic Detection: RID	Non-ionic surfactants (alcohol ethoxylates) including highly hydrophobic substances (logP > 6)	logk, logP	[115]
Spherex C18 (250 mm × 4.6 mm, 5 μm particle size); Phenomenex Inc.	Aqueous phase: H <sub>2</sub> O (35-90%) Organic phase: MeOH (10-65%) Masking agent: 10 mM TMAC	HPLC mode: isocratic Detection: UV (220 nm) Dead time marker: KBr	22 Penicilin drugs	logk, logk <sub>w</sub>	[116]
C18 column (250 mm × 4 mm, 5 μm)	Aqueous phase: H <sub>2</sub> O (15%)	HPLC mode: isocratic	16 Polycyclic aromatic hydrocarbons	logk, logP	[117]



particle size)	Organic phase: MeOH (85%)	Detection: UV (254 nm)	(3.3 < logP < 6.3)		
Altima C18 (150 mm × 4.6 mm, 5 μm particle size); Hichrom Ltd.	Aqueous phase: 0.02 M MOPS buffer, pH 7.2 (25-70%) Organic phase: MeOH (30-75%)	HPLC mode: isocratic Detection: UV-Vis (215 and 500 nm) or RID	57 Terpenoids including monoterpene hydrocarbons and oxygenated terpenes: alcohols, aldehydes, ketones, acetates (1.81 < logP < 4.48)	log <sub>k<sub>w</sub></sub> , logP	[118]
Gemini C18 hybrid silica-based columns (150 mm × 4.6 mm and 50 mm × 4.6 mm, 5 μm particle size); Phenomenex Inc.	Aqueous phase: (1) 20 mM Na <sub>2</sub> HPO <sub>4</sub> , pH 3.0 (2) 20 mM Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0 (3) 20 mM Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> , pH 10.0 Organic phase: MeOH (40-55%)	HPLC mode: isocratic Detection: UV (254 nm)	28 Pharmaceuticals including: basic (local anesthetics, β-blockers), acidic (non-steroidal anti-inflammatory drugs) and neutral (steroid hormones) drugs.	log <sub>k<sub>w</sub></sub> , S	[86]
Kromasil C18 (250 mm × 4.6 mm, 5 μm particle size); Akzo Nobel / Eka Chemicals Inc.	Aqueous phase: H <sub>2</sub> O (20%) Organic phase: MeOH (80%)	HPLC mode: isocratic Detection: UV-Vis (λ <sub>max</sub> for each compound) Dead time marker: NaNO <sub>3</sub>	21 Persistent organic pollutants (POPs; 2.0 < logP < 7.0) including model compounds and synthetic organochlorine pesticides (DDT and DDT-related compounds)	log <sub>k<sub>w</sub></sub> , logP	[119]
XBridge-C18 column packed with bridged ethylene hybrid (BEH) particles (50 mm × 3 mm, 2.5 μm particle size); Waters Corporation	Aqueous phase: (1) 10 mM HCOONH <sub>4</sub> (pH: 2.5, 3.3, 4.1, 8.9, 9.7) (2) 10 mM CH <sub>3</sub> COONH <sub>4</sub> (pH: 4.9, 5.8) (3) 10 mM NH <sub>4</sub> HCO <sub>3</sub> (pH: 6.8, 10.5) Organic phase: MeOH	HPLC mode: two pH/organic modifier gradient sets Detection: ESI-TOF-MS (range 50-1200 m/z) Dead volume marker: citric acid	40 drugs including antibiotics, antidepressant, β-blockers, anti-arrhythmic agents, anticoagulants, antipsychotics, hypertensive drugs, anesthetic drugs, antispasmodic drugs, anti-inflammatory drugs, antifungal drugs, analgesic and antipyretic drugs	log <sub>k<sub>w</sub></sub> , logP, logD, pK <sub>a</sub> *	[120]
LiChroCART Purosphere RP-18e (125 mm × 3 mm, 5 μm particle size), Zorbax Eclipse XDBC8 (150 mm × 4.6 mm, 5 μm particle size), Discovery RP-Amide C16 (150 mm × 4.6 mm, 5 μm particle size), LiChrospher 100 CN (250 mm × 4 mm, 5 μm particle size), and Kinetex PFP (150 mm × 2.1 mm, 2.6 μm particle size)	Aqueous phase: 0.1% HCOOH Organic phase: MeOH (50-70%)	HPLC mode: isocratic Detection: UV (254 nm) Dead time marker: uracil	22 antioxidant compounds including phenolic acids, flavonoids, anthocyanins, xantonoids, proanthocyanidins	log <sub>k<sub>w</sub></sub> , log <sub>k<sub>w</sub></sub>	[121]
Luna C18(2) (150 mm × 4.6 mm, 5 μm particle size), Candeza CD-C18 (150 mm × 4.6 mm, 3 μm particle size), TSK-gel ODS-80TS (150 mm × 4.6 mm, 5 μm	Aqueous phase: 0.1% HCOOH Organic phase: ACN with 0.1% HCOOH	HPLC mode: gradient Detection: UV (254 nm)	21 antitumor acridinone (imidazoacridinone and triazoloacridinone) derivatives	log <sub>k<sub>w</sub></sub> , logP	[122]



particle size), Ascentis C18 (150 mm × 4.6 mm, 5 μm particle size), Unison UK-C18 (150 mm × 4.6 mm, 3 μm particle size), and Zorbax SB-C8 (75 mm × 4.6 mm, 3.5 μm particle size)					
LiChroCART LiChrospher RP-18e (250 mm × 4 mm, 5 μm particle size), LiChroCART Purosphere RP-18e (125 mm × 3 mm, 5 μm particle size), Zorbax Eclipse XDBC8 (150 mm × 4.6 mm, 5 μm particle size)	Aqueous phase: (1) H <sub>2</sub> O with NH <sub>3</sub> aq, pH 9.6 (2) H <sub>2</sub> O with HCOOH, pH 2.8 Organic phase: MeOH	HPLC mode: isocratic Detection: UV (230 nm), MS Dead time marker: acetone	10 compounds with increased toxicity (mycotoxins and alkaloids) and 12 amines with important biological activity	k, k <sub>0</sub> , S, PC1/k	[123]
<b>POLYMER-BASED COLUMNS</b>					
PRP-1 column (150 mm × 4.1 mm, 10 μm particle size); Hamilton company and ACT-1 (150 mm × 4.6 mm, 10 μm particle size); Interaction chemicals	Aqueous phase: 0.1 M CH <sub>3</sub> COONH <sub>4</sub> , pH 4.6 (35 or 30%) Organic phase: ACN (65 or 70%)	HPLC mode: isocratic Detection: UV (210, 230 or 254 nm) Dead time marker: NaNO <sub>3</sub>	40 Compounds from different classes: non-H bonders, single amphiprotics (with 1 hydroxyl or amide substituent), and double amphiprotics	logk	[124]
Asahipak ODP 50-4D (150 mm × 4.6 mm, 5 μm particle size); Shodex Group, Showa Denko K.K.	<u>HPLC experiments</u> Aqueous phase: (1) H <sub>2</sub> O (2) 20 mM NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> , pH 2.0 (3) 20 mM NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> , pH 10.0 Organic phase: MeOH and ACN for experiments with each of the aqueous phases <u>IP-HPLC experiments</u> Aqueous phase: (1) 20 mM NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> , pH 7.0 (2) 20 mM NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> , pH 7.0 + 10 mM TBAB Organic phase: MeOH	HPLC mode: isocratic and gradient IP-HPLC mode: isocratic Detection: UV-Vis (λ <sub>max</sub> for each compound)	72 Chemical compounds including 24 neutral compounds, 20 weak acid compounds, 14 strong acid compounds, 14 basic compounds	logk <sub>w</sub> , CHI, k <sub>g</sub>	[87]
C18-derivatized PS-DVB column (Act-I, 50 mm length); Interaction chemicals	Aqueous phase: H <sub>2</sub> O (40%) Organic phase: MeOH (60%)	HPLC mode: isocratic Detection: UV (230 nm) Dead time marker: NaNO <sub>3</sub>	50 Different chemical compounds including non-hydrogen bonding compounds, acids/alcohols, bases, and hydrogen bonding acceptors (-0.20 < logP < 3.88)	logk, logP	[125]
ODP-50 column (20 mm × 4 mm, 5 μm particle size); Supelco	Aqueous phase: (1) 26 mM CF <sub>3</sub> COOH, pH 2.0 (2) 10 mM Na <sub>3</sub> PO <sub>4</sub> , pH 7.0 (3) 10 mM Na <sub>3</sub> PO <sub>4</sub> , pH 10.0	HPLC mode: gradient Detection: UV (260, 285 nm)	16 Compounds with antimicrobial activity from group of 3(2 <i>H</i> )-isothiazolones	logP	[126]





	Organic phase: MeOH				
PRP-1 column (150 mm × 4.6 mm, 5 μm particle size); Hamilton company	Aqueous phase: 25 mM CH <sub>3</sub> COONH <sub>4</sub> buffer of pH (1) 4.5, (2) 7.2, (3) 9.8 Organic phase: ACN	HPLC mode: isocratic and gradient Detection: UV or ELSD	21 Commercially available drugs and 24 biologically active marine natural products	log <sub>k<sub>w</sub></sub> , CHI, logP	[77]
ODP-50 column (20 mm × 4 mm, 5 μm particle size); Supelco	Aqueous phase: 10 mM Na <sub>3</sub> PO <sub>4</sub> adjusted to pH (1) 2, (2) 7, (3) 10 Organic phase: MeOH	HPLC mode: gradient Detection: UV (260-285 nm)	120 Different chemical compounds including 26 pharmaceuticals, 24 fungicides, 25 herbicides, 25 insecticides and 20 miscellaneous	logP	[90]
Supelguard ODP-50 column (20 mm × 4 mm); Supelco	Aqueous phase: 15 mM potassium phosphate buffer adjusted to pH (1) 2.0 (H <sub>3</sub> PO <sub>4</sub> ), (2) 7.4, (3) 11.0 (KOH) Organic phase: MeOH	HPLC mode: gradient Detection: UV (230, 260 nm)	15 β-blockers and structurally related compounds	logD	[89]
Asahipak ODP-50-4B column (50 mm × 4.6 mm, 5 μm particle size); Asahi Chemicals	Aqueous phase: 20 mM phosphate buffer prepared using <i>n</i> -octanol - saturated H <sub>2</sub> O and adjusted to pH (1) 3.0, (2) 4.0, (3) 7.0 Organic phase: MeOH with addition of 0.25% <i>n</i> -octanol	HPLC mode: isocratic Detection: UV-Vis (λ <sub>max</sub> for each compound) Dead time marker: uracil	Set of 41 compounds including model solutes (13 acids, 8 neutrals, 5 bases) and 15 drugs (-0.69 < logP < 4.80)	logk, log <sub>k<sub>w</sub></sub> , S	[88]
PLRP-S column (50 mm × 4.6 mm, 5 μm particle size), Agilent	Aqueous phase: 20 mM CH <sub>3</sub> COONH <sub>4</sub> , pH 7.0 Organic phase: ACN (10, 15 and 20%)	HPLC mode: isocratic Detection: UV-Vis	Amyloid β-peptides: Aβ <sub>12-28</sub> and Aβ <sub>25-35</sub>	logk	[127]
PLRP-S column (50 mm × 4.6 mm, 5 μm particle size), Agilent	Aqueous phase (pH range 2-11): (1) 0.1% formate buffer (2) 0.1% CH <sub>3</sub> COONH <sub>4</sub> (3) 10 mM triethylamine Organic phase: ACN (80%)	HPLC mode: isocratic Detection: UV (215, 254, 280, 310 nm) and MS Dead time marker: astemizole (pH ~ 2) valsartan (pH ~ 10)	3 Sets of compounds: (1) 44 neutral compounds (simple organic molecules, commercial drugs), (2) 10 compounds (5 pairs) capable and incapable to form intramolecular hydrogen bonds, (3) 76 commercial drugs	logk	[128]
<b>BIOMIMETIC COLUMNS</b>					
IAM.PC.DD2 column (150 mm × 4.6 mm, 10 μm particle size); Regis Technologies Inc.	Aqueous phase: (1) 0.1% (v/v) HCOOH, pH 2.8 (2) 10 mM CH <sub>3</sub> COONH <sub>4</sub> , pH 7.0 Organic phase: MeOH	HPLC mode: gradient Detection: UV (230, 254 nm)	32 Analogs of 4-hydroxycoumarin (biologically active compounds)	CHI	[129]





IAM.PC.MG (150 mm × 4.6 mm, 10 μm particle size) and IAM.PC.DD2 (100 mm × 4.6 mm, 10 μm particle size); Regis Technologies Inc.	Aqueous phase: 0.1 M phosphate buffer of pH (1) 7.0, (2) 5.5 Organic phase: ACN (15-30% if required)	HPLC mode: isocratic Detection: UV ( $\lambda_{\max}$ for each compound)	10 Quinolone antibacterial agents, including both acidic and zwitterionic congeners	logk, logk <sub>w</sub> , logP	[130]
IAM.PC.MG (150 mm × 4.6 mm, 10 μm particle size) and IAM.PC.DD2 (30 mm × 4.6 mm, 12 μm particle size) columns; Regis Technologies Inc., ChromTech chiral-HSA and ChromTech chiral-AGP (50 mm × 4 mm, 5 μm particle size) columns; Supelco	<u>For IAM columns:</u> (1) High purity water (2) 20 mM MOPS with pH of 4.2 and 7.4 (3) PBS with pH of 4.2 and 7.4 (4) 50 mM CH <sub>3</sub> COONH <sub>4</sub> , pH 7.4 <u>For the HSA and AGP columns:</u> (1) High purity water (2) PBS with pH of 4.2 (only AGP), 5.0 (only HSA) and 7.0 (3) 50 mM CH <sub>3</sub> COONH <sub>4</sub> , pH 7.0	HPLC mode: isocratic Detection: UV-Vis (205 nm)	11 Selenium species including methylseleninic acid, methylselenocysteine, dimethylselenourea, selenites Se(IV), selenates Se(VI), seleno-DL-methionine, L-selenocystine, selenocystamine, selenourea, dimethyl selenide, dimethyl diselenide	logk, logk <sub>w</sub> , logD	[5,131]
IAM.PC.DD2 (100 mm × 3 mm), Chiralpack-HSA and Chiralpack -AGP (50 mm × 3 mm); Regis Technologies Inc.	Aqueous phase: 50 mM CH <sub>3</sub> COONH <sub>4</sub> , pH 7.4 Organic phase: ACN (IAM column), IPA (HSA and AGP columns)	HPLC mode: gradient Detection: UV (254 nm)	117 Marketed drugs	logk, logP	[102]
Chiral-HSA and Chiral-AGP (100 mm × 4 mm); Chrom Tech Ltd.	<u>For HSA column:</u> Aqueous phase: 10 mM phosphate buffer, pH 7.0 Organic phase: IPA (20% required only for nebivolol) <u>For AGP column:</u> Aqueous phase: 10 mM phosphate buffer, pH 7.0 (75% or 90%) Organic phase: MeOH	HPLC mode: isocratic Detection: UV ( $\lambda_{\max}$ for each compound)	13 β-blockers (enantiomers) including acebutolol, alprenolol, atenolol, betaxolol, labetalol, metoprolol, nadolol, nebivolol, oxprenolol, pindolol, propranolol, sotalol, and timolol	logk, logk <sub>w</sub> , logP, logD	[91]
Chiral-HSA (50 mm × 3 mm); Chrom Tech Ltd., and RexChrom IAM PC2 (CH <sub>2</sub> ) <sub>12</sub> (150 mm × 4.6 mm); Fisher Scientific	Aqueous phase: 50 mM CH <sub>3</sub> COONH <sub>4</sub> , pH 7.4 Organic phase: IPA (HSA column), ACN (IAM column)	HPLC mode: gradient Detection: UV (230, 254 nm)	68 Drug molecules	logK <sub>HSA</sub> , CHI <sub>IAM</sub>	[73,101]



Chiral-HSA (50 mm × 3 mm); Chrom Tech Ltd.	Aqueous phase: PBS, 0.157 M K <sup>+</sup> /Na <sup>+</sup> , pH 7.0 Organic phase: ACN and IPA (5-20%)	HPLC mode: isocratic Detection: UV (220, 254 nm)	63 Structurally diverse basic, acidic and neutral drugs	logk, S, logk <sub>w</sub>	[132]
IAM.PC.MG (150 mm × 4.6 mm) and IAM.PC.DD2 (100 mm × 4.6 mm); Regis Technologies Inc.	Aqueous phase: 0.1 M phosphate buffer, pH 7.0 Organic phase: ACN (10-30% if required)	HPLC mode: isocratic Detection: UV (λ <sub>max</sub> for each compound)	14 Basic drugs spanning a wide lipophilicity range	logk, logk <sub>w</sub>	[133]
IAM.PC.MG (150 mm × 4.6 mm); Regis Technologies Inc.	Aqueous phase: 16 mM phosphate buffer, pH 7.4 Organic phase: ACN (4-27%)	HPLC mode: isocratic Detection: UV (λ <sub>max</sub> for each compound; 225-290 nm)	11 Arylpropionic non-steroidal anti-inflammatory drugs	logk, logk <sub>w</sub>	[134]
IAM.PC.DD2 (100 mm × 4.6 mm); Regis Technologies Inc.	Aqueous phase: 10 mM phosphate buffer of pH (1) 3.0, (2) 5.0, (3) 7.4 Organic phase: ACN (≤ 30% if required)	HPLC mode: isocratic Detection: UV or MS/MS	86 Monoprotic positively charged amines including monopolar amines, dipolar amines, amines with polar N moieties, polycyclic amine structures, and complex multifunctional amine structures	logk, logk <sub>w</sub>	[135]

ACN – acetonitrile; AGP – α<sub>1</sub>-acid glycoprotein; Brij 35 – polyoxyethylene lauryl ether; CTAB – cetyltrimethylammonium bromide; DDT – dichlorodiphenyl-trichloroethane; DMSO – dimethyl sulfoxide; DVB – divinylbenzene; ELSD – evaporative light scattering detection; ESI-TOF-MS – electrospray ionization time-of-flight mass spectrometry; HSA – human serum albumin; IPA – isopropyl alcohol; IP-HPLC – ion-pair high performance liquid chromatography; LI – lipophilicity index; MeOH – methanol; MOPS – 4-morpholinepropanesulfonic acid; MS – mass spectrometry; PBS – phosphate buffer saline; PS – polystyrene; RID – refractive index detector; SDS – sodium dodecyl sulfate; TBAB – tetrabutylammonium bromide; THF – tetrahydrofuran; TMAC – tetramethylammonium chloride; \* possibility to determine acid dissociation constant (pK<sub>a</sub>) in the same HPLC run

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## 445 3.1.2.2. HPLC procedures for lipophilicity assessment

446 As summarized in details in recent reviews [21,23,67,68,76], the HPLC-based procedures in isocratic  
447 and gradient elution mode have been widely used for determination of various LIs, mainly  $\log k_w$ ,  $\phi_0$   
448 and S (slope of the linear relationship between the organic solvent concentration ( $\phi$ ) and the  $\log k$ ).  
449 Therefore, here only the main principles of most commonly applied HPLC approaches will be  
450 discussed.

451 Most of the reported procedures are based on isocratic measurements of compounds retention that is  
452 normalized by column dead time and expressed as retention factor (see Fig.4). In isocratic method,  
453 several HPLC analyses at different MP compositions (min. 4 various concentrations of organic  
454 modifier) must be carried out for each compound, which requires preliminary experiments and is more  
455 time-consuming. The obtained results should be extrapolated to the same MP composition, normally  
456 to pure aqueous phase. The Snyder-Soczewinski equation (Eq. 9) is the most commonly used for  
457 such extrapolation [136]:

$$458 \log k = -S \cdot \phi + \log k_w \quad (9)$$

459 Although this relationship is non-linear in the full range of organic modifier concentration, a good  
460 approximation within the working limits of  $-0.5 < \log k < 1.5$  is ensured [68]. If necessary, a quadratic  
461 model (Eq. 10) can be applied:

$$462 \log k = A \cdot \phi^2 + B \cdot \phi + \log k_w \quad (10)$$

463 where A and B are regression coefficients.  $\log k_w$  is regarded as the most representative LI, since its  
464 value is of the same order of magnitude as  $\log P$  or  $\log D$ . Both isocratic retention factors ( $\log k$  or  
465  $\log k_w$ ) are directly correlated to n-octanol-water  $\log P/\log D$  via Collander equation (Eq. 11):

$$466 \log P/\log D = a \cdot \log k_w + b \quad (11)$$

467 where a and b are linear regression coefficients determined by analyzing a set of standard  
468 compounds according to OECD guidelines [69].

469 Assessment of LIs of highly lipophilic compounds using standard polycratic approach is difficult or  
470 rather impossible due to RTs being too long and thus analysis becomes very time-consuming and  
471 labor-intensive. Therefore, a new approach to determine  $\log k_w$  through the correlation with  $\log k$  was  
472 introduced [137]. As the S and  $\log k_w$  parameters indicating mechanism of retention give a linear  
473 relationship for structurally related compounds (see Eq. 12) [28], the  $\log k_w$  value can be evaluated



474 based on  $\log k$  measured only for the corresponding organic modifier concentration according to the  
 475 following formula (Eq. 13):

$$476 \quad S = a \cdot \log k_w + b \quad (12)$$

$$477 \quad \log k_w = (b \cdot \varphi + \log k) / (1 - a \cdot \varphi) \quad (13)$$

478 where  $a$  and  $b$  correspond to the slope and the intercept, respectively.

479 In order to speed up the lipophilicity evaluation, various gradient HPLC methods have been proposed,  
 480 and as a result a new LI namely CHI was introduced by Valkó et al. [138,139]. It links the isocratic and  
 481 gradient retention together and represents the volume of organic modifier in MP for which the  
 482 amounts of solute distributed between two phases are equal ( $k = 1$ ,  $\log k = 0$ ). The CHI is computed  
 483 as follows:

$$484 \quad \varphi_0 = -\log k_w / S \quad (14)$$

485 It has been shown that gradient RTs ( $t_{Rg}$ ) correlate well with isocratically determined CHI values and  
 486 thus give a straight line when plotted against the CHIs obtained for given set of standards [70,138].  
 487 The slope ( $a$ ) and the intercept ( $b$ ) of the calibration equation (Eq. 15) are used to convert  $t_{Rg}$  of  
 488 analytes to CHI scale (range of 0-100) that is useful for inter-laboratory study and database creation:

$$489 \quad \varphi_0 (\text{CHI}) = a \cdot t_{Rg} + b \quad (15)$$

490 The CHI values can also be converted to  $\log P$  scale. However, the best correlation was observed for  
 491 neutral form of compounds. Considering the H-bond acidity or the H-bond count terms, the  
 492 relationship between  $\log P / \log D$  and CHI can be improved as reported by Valkó et al. [70].

493 As an alternative LI, an apparent capacity factor ( $k_g$ ) was introduced by Krass et al. [140] and defined  
 494 as follows:

$$495 \quad k_g = (V_g - V_d - V_m) / V_m \quad (16)$$

496 where  $V_g$  is gradient retention volume,  $V_d$  is system delay volume and  $V_m$  is column dead volume. A  
 497 good correlation between  $k_g$  and  $\log k_w$  obtained in a series of isocratic HPLC runs was reported.

498 Nowadays, the rapid-gradient RP-HPLC methods using high flow rate and short columns to reduce  
 499 the analysis time (up to ~5 min) with a negligible loss of resolution have been widely applied for the  
 500 lipophilicity assessment. The use of MS detection allows to determine CHIs for mixture of compounds,  
 501 which further accelerates the experimental work [141]. Another improvement has been proposed by  
 502 Wiczling et al. [120,142], who employed time-of-flight MS to enlarge the number of analytes in



503 individual HPLC run and to facilitate their identification in complex mixture. They have developed a  
504 double pH/methanol gradient procedure for the simultaneous determination of  $\log k_w$  and  $pK_a$  values  
505 based on two gradient RTs of a compound (one is an organic phase, the other is a pH gradient), with  
506 a screening rate of about 100 compounds/day.

507 Summarizing, using gradient RP-HPLC methods, reproducible LIs can be obtained in a high-  
508 throughput way. The current trend is also focused on the use of chemometric tools, such as principal  
509 component analysis, to extract the meaningful and interpretable features from the multivariate HPLC  
510 raw data on lipophilicity and then provide new highly descriptive LIs (i.e. principal components and  
511 their derivatives) [26,121].

### 512 3.2. Electroseparation methods

513 Electroseparation techniques used to determine the lipophilic properties are based generally on  
514 capillary electrophoresis (CE). In this method, analytes migrate through electrolyte solutions (buffers  
515 with pH adjusted to certain values) under the influence of electric field. They can be separated in two  
516 different ways: according to ionic mobility or/and partitioning into an alternate phase via non-covalent  
517 interactions [143,144]. Migration of the analytes is confined by the sum of two factors: electroosmotic  
518 mobility ( $\mu_{eo}$ ) and intrinsic electrophoretic mobility ( $\mu_{ep}$ ). This limitation is caused by the action of  
519 electroosmotic flow (EOF) of the buffer solution, which is defined as a bulk liquid motion that results  
520 when the external electric field interacts with the net surplus of charged ions in the diffuse part of the  
521 electrical double layer [145,146]. The velocity of the EOF ( $u_{eo}$ ) can be defined as:

$$522 \quad u_{eo} = \mu_{eo} \cdot E \quad (17)$$

523 where  $E$  is the intensity of the applied electric field (V/m). The electroosmotic mobility ( $\mu_{eo}$ ) may be  
524 experimentally determined by measuring the migration time of a neutral analyte.

525 The use of CE has few advantages such as small amount of compounds required, speed and  
526 relatively easy automation and low consumption of solvents. In many cases CE is used as an  
527 alternative or complementary technique to HPLC due to its high separation efficiency [6,53,147].  
528 Hence, CE remains to play an important role in separation of medium size molecules (e.g. peptides,  
529 oligosaccharides and oligonucleotides), as well as in analysis of large macromolecules, such as  
530 polysaccharides, nucleic acids and synthetic polyelectrolytes. [143,148,149].

531 For analysis of both charged and neutral compounds electrokinetic chromatography (EKC) methods,  
532 including micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic  
533 chromatography (MEEKC) and vesicular electrokinetic chromatography (VEKC) are applied [150].  
534 MEKC has gained in popularity for the indirect estimation of logP values of small molecules. The main  
535 difference between MEKC and CE is that the solution contains a surfactant, which concentration is  
536 greater than critical micelle concentration (CMC), so the concentration of monomers reaches a critical  
537 value when only micelles are present. These micelles are the pseudo-SP and the analytes are  
538 separated by differential partitioning between them and surrounding buffer solution (MP) [151].  
539 Usually, MEKC is performed in open capillaries under the alkaline conditions to generate a strong  
540 EOF. Anionic micelles of SDS is the most common surfactant for MEKC systems and it was the first  
541 surfactant used for logP determination [152]. In comparison with n-octanol/water partition system, the  
542 advantage of MEKC results from the large difference in hydrogen-bond basicity, and to a lesser  
543 extent, dipolarity/polarizability, for partition into SDS micelles. Although, a better partition model is  
544 provided by sodium N-dodecyl-N-methyltaurine (SDMT), this surfactant has not been used for  
545 estimating logP [6]. However, Ibrahim et al.[153] investigated the use of bile salts as a pseudo-SP in  
546 MEKC. The results showed that sodium deoxycholate (SDC) was the most appropriate surfactant  
547 used to estimate lipophilic properties of fungicides. Due to the fact that bile salts can be found in  
548 biological systems, the SDC-MEKC method is considered to be environmentally friendly. More  
549 detailed information on the MEKC principle and the reagents used are summarized by Terabe [151]  
550 and Silva[152]. As in the case of LC, determination of logP is based on the relationship between this  
551 coefficient and the retention factor [154]. This factor in MEKC technique may be defined as (Eq. 18):

$$552 \quad k' = (t_R - t_0)/(t_0 \cdot (1 - (t_R/t_{mc}))) \quad (18)$$

553 where  $t_R$ ,  $t_0$  and  $t_{mc}$  are the migration times of the solute, the EOF marker (e.g. methanol), and the  
554 micelle marker (e.g. Sudan III or Sudan IV), respectively [151]. Compared to SFM, MEKC has the  
555 advantages of speed, wide dynamic range, high sample throughput and small sample size [150,151].

556 For indirect determination of logP values MEEKC can also be applied. This technique can be seen as  
557 extension of MEKC, however in this case the microemulsion is used as migrating pseudo-SP.  
558 Microemulsions are immiscible oils droplets in water (usually heptane or octane droplets) that are  
559 stabilized by co-surfactants and surfactants situated at the surface of the droplet [147,155,156]. The  
560 most commonly used surfactant and co-surfactant in MEEKC are SDS and 1-butanol, respectively



561 [6,156]. The mechanism of separation in MEEKC is based on differential partitioning of analyte into  
562 migrating microemulsion, which is related to analyte hydrophobicity. Therefore, the migration time of  
563 analyte is proportional to its hydrophobicity, so its retention factor ( $k'$ ) is related to values of  $\log P$ . To  
564 determine the  $k'$  value it is necessary to precisely measure the migration time of EOF marker ( $t_0$ ) and  
565 microemulsion marker ( $t_{me}$ ), such as octanophenone, along with analyte ( $t_R$ ) and then to calculate the  
566 retention factor from the following formula [150] (Eq. 19):

$$k' = (t_R - t_0) / (t_0 \cdot (1 - (t_R/t_{me}))) \quad (19)$$

567  
568 In order to determine  $\log P$  values, Xia et al. [157] developed novel MEEKC approach based on peak-  
569 shift assay. In comparison to conventional approach, this method provides the  $\log P$  values of the  
570 compounds without the use of reference substances with known  $\log P$  values reported in the literature.  
571 Fernández- Pumarega et al. [158] presented the applicability of MEEKC in estimation of  $\log D$  of acidic  
572 drugs at several ionization degrees. They reported that the overestimation of  $\log D$  was observed only  
573 at degree of ionization higher than 99.5%. However, further research is needed to check if this  
574 behavior can occur with other compounds. This paper showed that MEEKC can be an alternative to  
575 other approaches used in lipophilicity assessment. Compared to MEKC, in MEEKC better  
576 solubilization properties for water insoluble compounds has been observed [156,159]. Furthermore,  
577 the hydrogen bond effects that affect the partitioning behavior of some solutes during lipophilicity  
578 determination are minimal in MEEKC compared to RP-HPLC or MEKC. This may be due to the  
579 addition of co-surfactant (1-butanol) that minimizes electrostatic interactions as reported by Ishihama  
580 et al. [160]. They also showed that MEEKC provides better correlation of retention factor with  $\log P$   
581 determined by SFM than MEKC and RP-HPLC methods for which the hydrogen acceptor and  
582 hydrogen donor effects must be considered. In addition, some limitations of SFM (e.g. large amount  
583 of high purity sample, lack of automation) and chromatographic methods (e.g. limited pH range,  
584 lifetime of SP) are overcome in the MEEKC [155].

585 Another EKC technique applied for  $\log P$  determination is VEKC. Vesicles are self-assembling,  
586 organized structures that contain continuous bilayer of monomers and enclose an aqueous core  
587 region used as potential models of synthetic membranes. In nature, this formation occurs through the  
588 aggregation of phospholipids to form liposomes [6]. Hence, vesicles synthesized from phospholipid  
589 aggregates may be used as pseudo-SP in VEKC. These vesicles are inherently physiological, but  
590 they are stable only in multilamellar form. Due to the fact that in EKC analysis the monolamellar forms





are preferred, the use of liposomes requires some additional laborious preparation procedures allowing the production of monolayer vesicles [161]. However, the vesicles can also be synthesized using oppositely charged surfactants and double ionic surfactants, such as bis(2-ethylhexyl)sodium sulfosuccinate in a phosphate buffer containing 10% (v/v) methanol (an anionic, double chain surfactant known as Aerosol OT or AOT) or hexadecyltrimethylammonium bromide-sodium octyl sulfate (double ionic surfactant, known as CTAMB-SOS) [6,150]. Jiang et al. [162] investigated the influence of the molar ratio of cationic to anionic surfactants on the vesicle properties and the performance of VEKC. It is well known that due to the electrostatic attractions among oppositely charged head groups of ionic surfactants and the hydrophobic interaction of their hydrocarbon tails the strong synergistic effects may occur. Some of physical properties of vesicles can be modified by changing the ratio of ionic surfactants. These changes affect the performance of VEKC. It has been reported that the molar ratio of 3:7 and 5:5 systems used as pseudo-SP had similar selectivity. However, the second system showed rapid and efficient separation for nonpolar substances [162]. Because of the similarity of surfactant vesicles and liposomes, both VEKC systems may be used not only as separation models for estimation of logP of various neutral and charged compounds, but also as a model for environmental and biological partitioning (e.g. permeability, diffusion or membrane transport) [6,163–165]. To obtain logP values, the retention factor ( $k'$ ) determined by VEKC technique should be calculated according to following formula (Eq. 20):

$$k' = (t_R - t_0)/(t_0 \cdot (1 - (t_R/t_v))) \quad (20)$$

where  $t_v$ ,  $t_R$  and  $t_0$  are the migration times of vesicle marker (e.g. dodecanophenone or octylbenzene), the solute and the EOF marker, respectively [163]. The use of VEKC for determination of n-octanol-water partition coefficients has similar advantages to MEKC. However, it is difficult to obtain reproducible results using this technique [150].

### 3.3. Electroanalytical methods

A variety of electrochemical methods have been developed, but the most frequently chosen methods for lipophilicity assessment are potentiometric and voltammetric ones [166]. Potentiometric methods were described for the first time in 1952 and since that time, they have been applied in different branches of science and industry. The determination of lipophilic profile of compound directly from a single acid-base titration in dual-phase partition solvent system is fast, however, it is necessary to know the acid dissociation constant ( $pK_a$ ) of tested compounds in case of using some of these



621 methods. Potentiometric methods create a good alternative to SFM especially in the case of acidic or  
622 basic compounds. Furthermore, the use of these methods enable to determine the logP in range of -1  
623 to 8. Unfortunately, they require a relatively large amount of samples [27,32].

624 Classical potentiometric titration can be used for the evaluation of the partition of ionizable  
625 compounds or ion-paired substances into the organic phase. Simple titration procedure, together with  
626 calculation of  $pK_a$  values involved the ionization of water in n-octanol, has been proposed by Scherrer  
627 and Donovan [167]. During the measurement, the  $pK_a$  of analytes is determined by adding high  
628 precision titrators. A potentiometric titration procedure in KCl/water-saturated n-octanol provides a link  
629 to logP through the thermodynamic cycle of ionization and partitioning (Fig. 5). The use of this titration  
630 method in order to apply it for the determination of substituent ion pair stabilization values (IPS) may  
631 give more accurate data concerning logD calculations.

632 The greatest advantage of potentiometric titration over other methods is the independence of  
633 measurements from the magnitude of logP value. In the same time, a reproducibility of a few  
634 hundredths of a logP in the calculated difference between  $\log P^N$  (neutral forms) and  $\log P^I$  (ionized  
635 forms) is maintained.

636 Titrations in KCl/water-saturated n-octanol provide essential data to advance our understanding of ion  
637 pair partitioning in n-octanol, but the ultimate goal of the lipophilicity studies is the application of this  
638 knowledge to the anisotropic world of cells, membranes, proteins, and nucleic acids [23,167].

639 Cyclic voltammetry (CV) has been used for the indirect determination of the lipophilicity of organic  
640 salts. Generally, typical CV system consists of four-electrode potentiostat, the aqueous phase and  
641 nitrobenzene or 1,2-dichloroethane (EDC) as the organic phase [50,168]. Bouchard et. al. proposed  
642 to use CV for examination of the lipophilicity of quaternary ammonium drugs and proved that the  
643 partition of some ions is influenced by Galvani potential difference, not ion-pairing phenomenon. The  
644 obtained values of standard water-EDC partition coefficient of ions correlated well ( $R^2 = 0.94$ ) with  
645 logP values of neutral molecular structures closest to these ions that were calculated by computer  
646 algorithms [168]. In contrast to traditional SFM, the partition coefficient determined by CV does not  
647 depend on experimental conditions. Furthermore, the use of this technique enables to estimate very  
648 low logP values ranging from - 8 to 1. The main disadvantages of CV are the limited number of



649 solvent systems that can be applied for lipophilicity determination and the large amount of sample  
650 required (1-10 mg) [50,53,168,169].

#### 651 **4. Conclusion**

652 Lipophilicity is an essential physicochemical property, which affects biological activities, assimilation  
653 and environmental fate of compounds. Despite the importance of lipophilicity, its evaluation is still  
654 problematic and constantly attracts researchers' attention. Nowadays, classical methods are almost  
655 completely replaced by instrumental ones, in particular separation techniques. Compared with the  
656 classical approach, the use of chromatographic or EKC methods does not require a long analysis time  
657 and a large amount of high purity samples. The purity of the sample may be problematic for unstable  
658 analytes and may lead to false results. Since only retention factors must be determined using  
659 separation techniques, the analytical quantification is not required. This drawback of classical liquid-  
660 liquid system (i.e. SFM) is really serious in the case of highly lipophilic or sparingly soluble  
661 compounds due to the need for a highly sensitive quantification system. In turn, potentiometric  
662 titration as fully automated and time-saving instrumental technique is only applicable to acidic and  
663 basic compounds of high purity and may not be suitable for highly hydrophobic molecules due to  
664 solubility problems. Chromatographic methods, especially TLC and fast HPLC, considered as  
665 economical and environmentally friendly solutions, can be used to determine lipophilicity of both  
666 neutral and dissociable compounds. However, the pH and composition of the mobile phase should be  
667 carefully selected in order to ensure that the compound is in its neutral form. Furthermore, new  
668 solutions have been introduced to reduce the limitations of indirect separation-based methods,  
669 including employment of new HPLC columns, addition of masking agents to mobile phase,  
670 modifications of CE, application of surfactants in TLC experiments, and development of new  
671 lipophilicity indices. The gradient HPLC methods proposed as alternative to isocratic ones give other  
672 additional advantages, particularly speed up experimental process and reduce costs. However, there  
673 is still an urgent need for valid, standardized and high-throughput procedures to determine the  
674 lipophilicity of chemical compounds.

675 Future trends may also include (i) design of new biomimetic SPs through immobilization of important  
676 biomolecules or binding similar functional groups to the silica surface in order to mimic partitioning in  
677 different biological and environmental systems thus help to better understand these phenomena, (ii)

678 development of new biological partition/distribution models using well designed HPLC retention  
679 properties, (iii) application of chemometric approaches to provide new highly descriptive LIs and build  
680 large data bases for comparison purposes, (iv) estimation of potential kinetic aspects of partitioning  
681 based on changes in the symmetry and width of chromatographic peak as a function of the MP flow  
682 rate.

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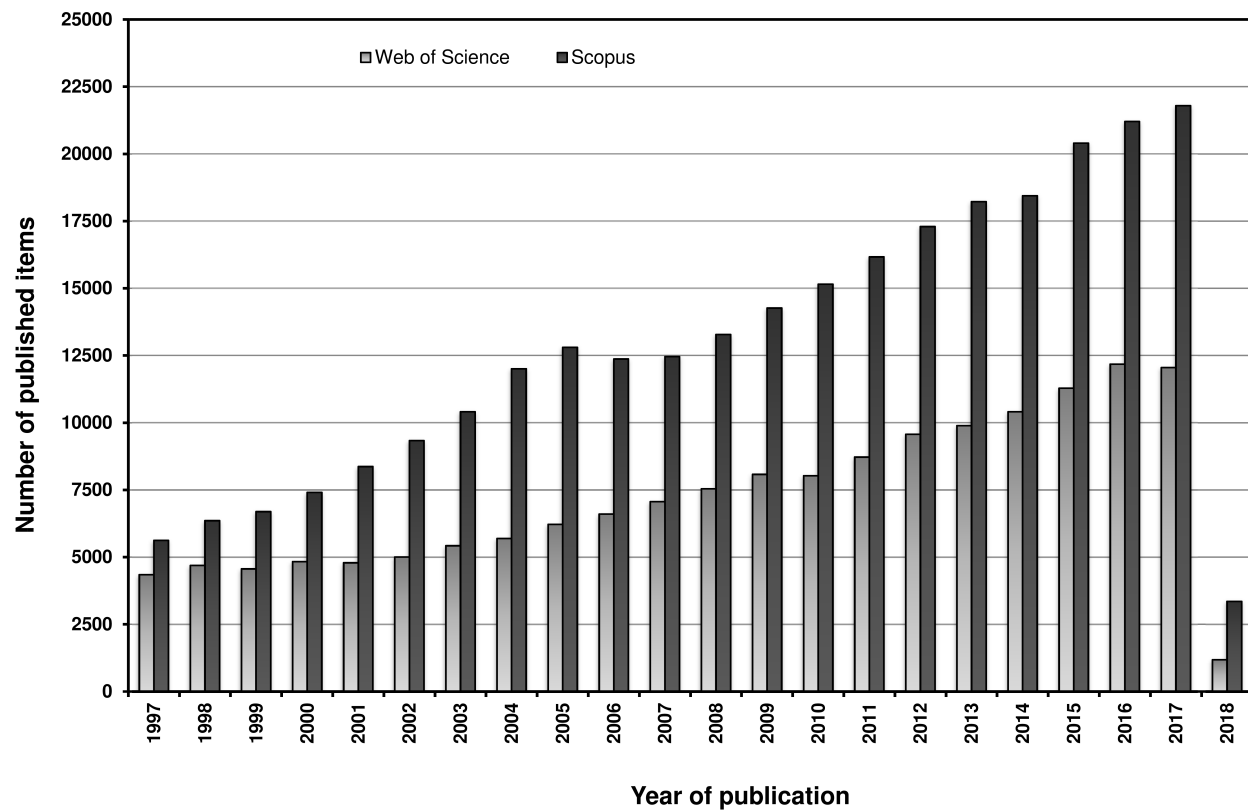
**Fig. 1.** Charts that shows the total number of publications by year that include the phrases “lipophilicity” or “lipophilic properties” or “partition coefficient” or “distribution coefficient” or “hydrophobicity” in the title, abstract, keywords or text, as searched through the Web of Science and Scopus databases. The difference in search results may be due to the type of published items that are considered by these search engines (i.e. original papers, reviews, book chapters, conference papers, short survey, etc.).

**Fig. 2.** Proposed classification of methods of lipophilicity assessment.

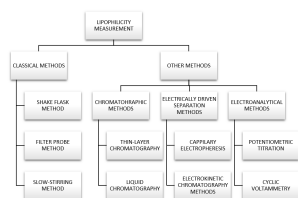
**Fig. 3.** Schematic presentation of direct experimental approaches of lipophilicity determination: a) procedure of the shake-flask method (SFM), b) the water-plug aspiration/injection method, c) modification of SFM based on NMR measurement, d) modification of SFM based on solid phase microextraction [26, 27, 38, 41]

**Fig. 4.** Partition process of less (white particles) and higher lipophilic (grey particles) compounds between stationary and mobile phases during chromatographic run. The retention factor ( $k$ ) of compound obtained on the HPLC stationary phase is proportional to its affinity (partition or distribution coefficient;  $K$ ) to the bonded groups presented on the surface of the column packing material, as depicted in the equations, where  $n_s$  and  $n_m$  stands for average numbers of molecules in the stationary and mobile phase, respectively,  $C_s$  and  $C_m$  are the molar concentrations of analyte in the stationary and mobile phase, respectively,  $V_s$  and  $V_m$  are the volumes of stationary and mobile phase, respectively  $t_R$  and  $t_0$  are the retention times of the analyte and the unretained compound, i.e. uracil, thiourea (known as column dead time).

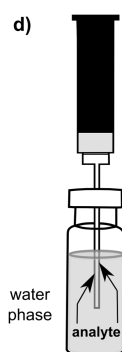
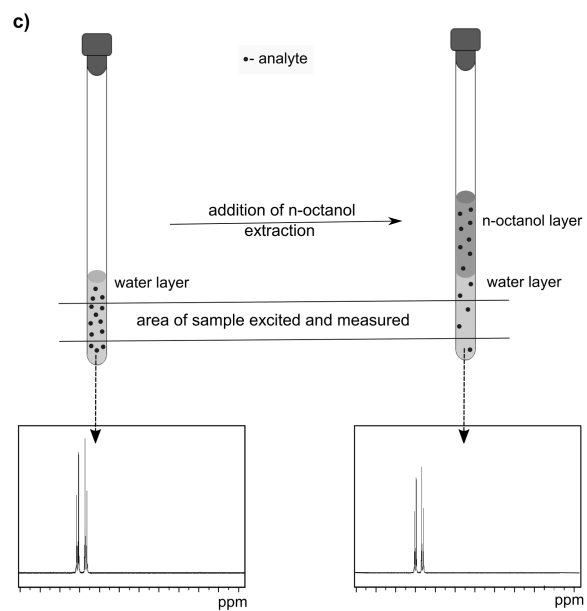
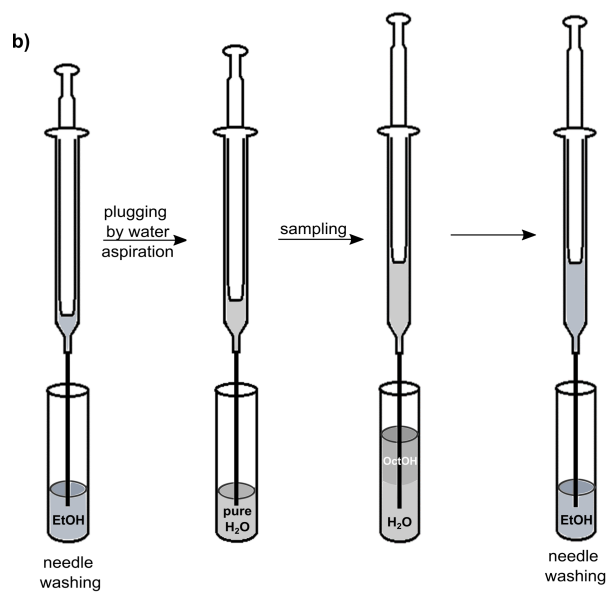
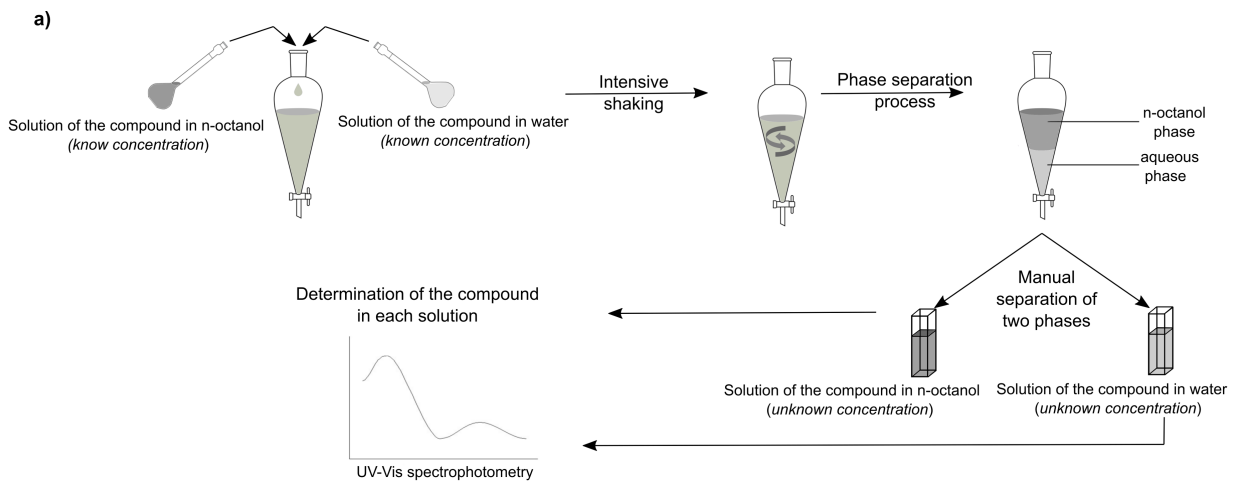
**Fig. 5.** The thermodynamic cycle of the ionization and partitioning of acid (HA) between n-octanol and aqueous phases based on Scherer and Donovan [167]. The  $pK_a$  in n-octanol ( $pK_{a_0}$ ) is defined to be equal to the pH of the aqueous phase (0.15M KCl) which is in equilibrium with the partition system when the concentration of acid ( $HA_0$ ) and anions ( $A_0^-$ ) in n-octanol phase are equal. Hence, the knowledge of  $pK_{a_0}$ , partition coefficient of neutral forms of a compound ( $\log P^N$ ) and  $pK_a$  in aqueous phase allows to calculate the partition coefficient of the ionized forms ( $\log P^I$ ). When the circle of equilibria is present, the difference between  $\log P^N$  and  $\log P^I$  is the same as the difference between  $pK_a$  values in aqueous and n-octanol phases.

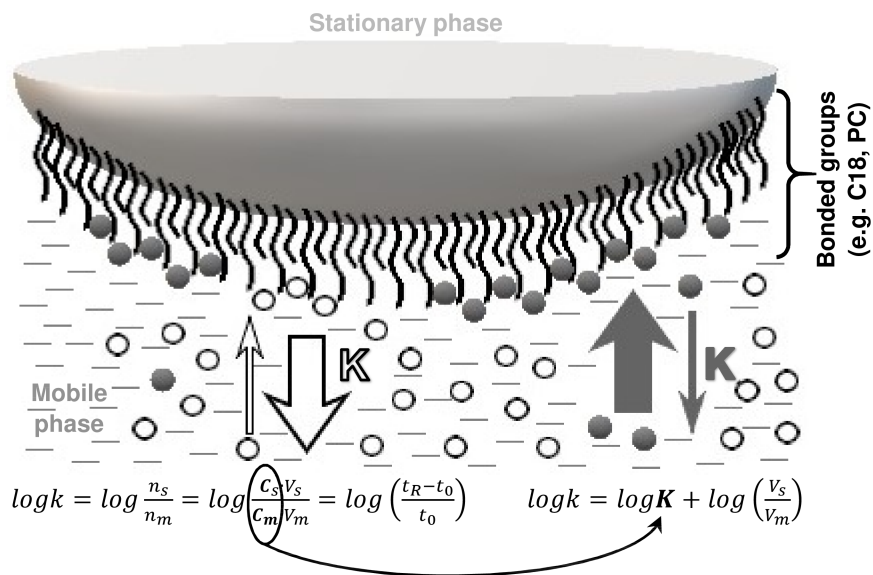






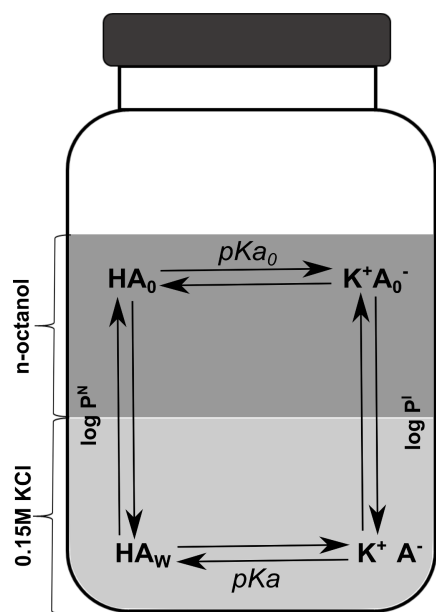
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- The role of lipophilicity in biological, environmental and technical science.
- Lipophilicity assessment using classical and modern approaches.
- Advantages and limitations of methods of lipophilicity determination.
- Novel solutions in chromatographic methods used for lipophilicity determination.

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