TADPOLE TOXICITY PREDICTION USING CHROMATOGRAPHIC SYSTEMS

Alejandro Fernández-Pumarega[a], Susana Amézqueta[a], Elisabet Fuguet[a,b], Martí Rosés[a]

[a] Departament de Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, 08028, Barcelona, Spain; [b] Serra Húnter Programme, Generalitat de Catalunya.

* Correspondence: Elisabet Fuguet, PhD.
e-mail: elifuguetj@ub.edu
Departament de Química Analítica, Universitat de Barcelona
c/ Martí i Franquès 1-11, 08028, Barcelona, Spain
Phone: (+34) 93 403 37 06
Fax: (+34) 93 402 12 33

ABSTRACT

Toxicity has been emulated in tadpole species through chromatographic systems. The parameter studied to evaluate the non-specific toxicity of a compound is the narcosis concentration (C_{nar}), which is defined as the concentration needed for the immobilization of the organism. Because experimental investigation with animals is lengthy, costly, technically difficult, and ethically questionable, there is a great interest in developing surrogate physicochemical systems able to emulate biological systems to obtain the same information in a faster, more economic, and easier manner.

In order to see which chromatographic systems would be able to emulate tadpole narcosis, both, tadpole narcosis data and data in several chromatographic and electrophoretic systems, were fitted to a linear solvation energy relationship (LSER) model. Thus, by comparison of the models it was possible to see which of the chromatographic systems were more similar to the biological one. The physicochemical systems that best emulate tadpole narcosis were an HPLC system based on an immobilized artificial membrane (IAM) column, and two micellar electrokinetic chromatography (MEKC) systems based on sodium taurocholate (STC) and a mixture of sodium dodecylsulphate (SDS) and Brij 35 as surfactants. A system based on a RP18 HPLC column also was selected for comparison because it is a common column in most analytical laboratories.

To establish the models, a set of compounds with known C_{nar} values were analyzed in the chromatographic, and electrophoretic selected systems and, then, the retention factor (k) was correlated to the concentration of narcosis. Statistics showed that the system based on STC micelles was the best to emulate toxicity in tadpoles. The robustness and predictive ability of the developed models were validated.

Keywords. Aquatic toxicity, tadpole, property estimation, micellar electrokinetic chromatography, high-performance liquid chromatography, surrogate systems.

HIGHLIGHTS

- Surrogate systems allow C_{nar} determination in a fast, cheap, and easy way
- Tadpole toxicity can be estimated through several chromatographic systems
- A sodium taurocholate MEKC system provides the better estimation results

1. Introduction

Aquatic wildlife is exposed to different chemical pollutants coming from industrial or municipal wastes or from urban or agricultural runoff. Tadpoles, amphibian anuran larvae, are useful models for studying environmental hazards due to their trophic importance, environmental sensitivity, research tractability and impeding extinction [1]. However, the experimental investigation through animal tests is usually a lengthy, costly, and technically difficult process, even ethically questionable. For these reasons, surrogate physicochemical systems capable of estimating toxicity in a faster, more economic and easier manner have become of potential interest. Liquid chromatography and capillary electrophoresis systems offer clear advantages over other physicochemical systems (based on liquid-liquid partition, for example) due to the high level of automation of the techniques, low cost, and low consumption of reagents, among others. Undoubtedly, implementation of physicochemical testing in common practices of toxicity estimation will contribute to more sustainable development of chemicals.

Several authors have proposed linear solvation energy relationship (LSER) equations that can be used to predict the narcotic or the lethal effect of chemical compounds [2–8]. These equations take into account several molecular descriptors related to structural, physicochemical, topological, geometrical, fragment, electrostatic, quantum chemical and thermodynamic parameters of the compounds.

Abraham et al. [2] proposed an equation (Eq. 1) to estimate the narcotic concentration of compounds to tadpoles through the solvation parameter model (SPM). This model assumes that narcotic and lethal effects are related to the partition of a solute between two phases, and this partition depends on five molecular descriptors:

$$\log (1/C_{nar}) = 0.582 + 0.770\,E - 0.696\,S + 0.243\,A - 2.592\,B + 3.343\,V \quad \text{(Eq. 1)};$$

n = 114, SD = 0.337, F = 217

E represents the excess molar refraction, *S* is the solute dipolarity/polarizability, *A* and *B* are the solute's effective hydrogen-bond acidity and hydrogen-bond basicity, respectively, and *V* is McGowan's solute volume. The coefficients of the equation are characteristic of the system and reflect its complementary properties to the corresponding solute property. In this case, the most important descriptors are the solute hydrogen bond basicity, that contributes negatively;

and the solute volume, which contributes positively. For any partition system, the coefficients of this correlation equation can be obtained by multiple linear regression analysis between the property to be correlated (which must be an equilibrium constant or some other free energy related property) and molecular descriptors for the set of compounds.

Bowen et al. [7] described a more general correlation that should be applicable to different species of tadpoles (Eq. 2). To this end, they introduced a flag descriptor for each tadpole species different from *Rana Temporaria* (I_{pip} for *Rana Pipiens*, I_{jap} for *Rana Japonica*, I_{xen} for *Rana Xenopus laevis*, I_{brev} for *Rana Brevipoda porosa* and I_{not} for non-identified tadpole species).

$$\log (1/C_{nar}) = 0.543 + 0.736\,E - 0.367\,S - 0.049\,A - 2.852\,B + 3.225\,V + 0.043\,I_{pip} + 0.150\,I_{jap} + 0.524\,I_{xen} + 0.146\,I_{brev} + 0.473\,I_{not} \quad \text{(Eq. 2)};$$

n = 240, SD = 0.322, F = 331

Similarity of two different systems (i. e. a biological and a physicochemical one) can be measured when they both are characterized through the same mathematical model. In the same way that the tadpole narcosis, several physicochemical systems ruled also by the passive transport of solutes between two phases have been successfully described by means of the Abraham solvation parameter model [9]. When two systems are similar in terms of model coefficients, a good correlation is established between their solvation properties. Then, the biological property of a new chemical compound can be predicted just by determining the surrogate physicochemical property. The main advantage of this procedure over QSAR studies is that it is not necessary to know the molecular descriptor values of the new compound to estimate the biological property.

Several tools are available to compare the coefficients of different systems characterized by the SPM, in order to choose the physicochemical systems that can best emulate tadpole toxicity. Some comparison approaches, like the measurement of the *d* distance [10] are based on evaluating the difference between the coefficients of the respective equations. Other approaches are based on the estimation of the precision of the correlation between the biological property and the physicochemical one [11]. Finally, some common procedures such as principal component analysis (PCA) of the coefficients of the compared systems [12], or dendrogram plots [13] can also be applied. These methodologies provide different information about the similarity of the compared systems. The parameter *d* is the euclidian

distance between the SPM normalized coefficients, and provides information about how similar are the compared correlations. Instead, the prediction of the variance of the correlation between chromatographic and biological data is a measure of the precision with which a chromatographic system can emulate the biological one. This precision is a combination of the precision of biological data, the precision of chromatographic data, and finally the dissimilarity between the compared systems (which is related to the parameter *d*). The PCA reduces the chemical space dimensions by combination of the most significant variables, so the closest the compared systems are in the new dimensions, the more similar they are. Finally, the dendrogram clusters the different compared systems according to their similarities. Whereas *d* and the variance prediction provide direct comparison between pairs of systems (the biological and a physicochemical one), PCA and dendrogram compare the biological system to a set of selected physicochemical ones, so the final results can be affected by the nature of the physicochemical systems selected for comparison.

The aim of this work is to emulate nonspecific toxicity of neutral organic compounds to tadpoles (mainly *R. Temporaria* and *R. Japonica* tadpoles) through direct measurements in specific chromatographic and electrophoretic systems, basically high-performance liquid chromatography (HPLC) and micellar electrokinetic chromatography (MEKC) systems. Thus, the toxicity of test compounds could be directly estimated from the chromatographic retention factor of a simple linear correlation.

2. Materials and Methods

2.1. Equipment

HPLC measurements were done using a 10 A series chromatograph from Shimadzu (Kyoto, Japan) equipped with a quaternary pump and a diode array detector and fitted with either an IAM.DD 2 immobilized artificial membrane column (10 cm × 4.6 mm i.d., 12 μm particle size) (Regis Technologies, Morton Grove, IL, US) or a XBridge C18 column (15 cm × 4.6 mm i.d., 5 μm particle size) (Waters, Milford, MA, US).

MEKC measurements were done using the CE capillary electrophoresis system from Agilent Technologies (Santa Clara, CA, US) equipped with a diode array detector. The fused-silica separation capillary (40 cm effective length, 50 μm i.d.) was obtained from Composite Metal Services Ltd (Shipley, UK).

155 2.2. Reagents

156 Methanol (HPLC-grade), hydrochloric acid (25 % in water), sodium hydroxide (>99%),
157 sodium dihydrogenphosphate monohydrate (>99%), disodium hydrogenphosphate (>99%)
158 and sodium dodecyl sulfate (>99%) were from Merck. Acetonitrile (HPLC grade) was from
159 VWR International (West Chester, Pennsylvania, US). Taurocholic acid sodium salt
160 monohydrate (98%) was from Acros Organics (Geel, Belgium) and Brij 35 was from Scharlab
161 (Barcelona, Spain). Dodecanophenone (98%) was from Sigma-Aldrich. Water was purified
162 by a Milli-Q plus system from Millipore (Bedford, MA, US), with a resistivity of 18.2 MΩ
163 cm.
164 Tested substances were reagent grade or better and obtained from several manufacturers
165 (Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO, US), Carlo Erba (Milano,
166 Italy), Baker (Center Valley, PA, US), Panreac (Castellar del Vallès, Spain), Thermo Fisher
167 Scientific (Waltham, MA, US), Scharlab (Sentmenat, Spain).
168
169 2.3. Analysis by HPLC
170 In both cases (IAM and C18 columns) target compounds were analyzed with a binary system
171 consisting of aqueous buffer (pH 7.0, 60 %) and acetonitrile (40 %) at 1 mL min^{-1}. The
172 aqueous buffer was prepared dissolving 5 mM NaH_2PO_4 – 5 mM Na_2HPO_4 in HPLC-grade
173 water and neutralizing with hydrochloric acid or sodium hydroxide up to the desired pH. The
174 injection volume was 10 μL and the column temperature 25ºC. After a preliminary scan,
175 detection wavelengths were set at 200, 214, 240 and 254 nm depending on the compound
176 absorption profile. All measurements were taken in triplicate.
177 Solutes and the non-retained compound (potassium bromide) were dissolved in
178 acetonitrile:water (2:3) at 100 mg L^{-1}. In the case of alcohols, the final concentration was 40
179 % (v/v) to improve the compound detection. All solutions were passed through a 0.45 μm
180 nylon syringe filter obtained from Albet (Dassel, Germany) and placed into an HPLC vial.
181 The HPLC retention factor (*k*), was calculated according to Eq. 3.
182
183 $$k = \frac{t_R - t_0}{t_0} \qquad \text{(Eq. 3)}$$

184 where t_R corresponds to the solute retention time and t_0 is the column hold-up time
185 determined by the aqueous potassium bromide solution.
186
187 2.4. Analysis by MEKC

The target compounds were analyzed using two different pseudostationary phases: a 50 mM solution of taurocholic acid sodium salt monohydrate and 20 mM phosphate aqueous buffer adjusted to pH 7.0; and a mixture of surfactants that consists of 50 mM sodium dodecyl sulfate (SDS), 10 mM polyethylene glycol dodecyl ether (Brij 35) and also 20 mM phosphate aqueous buffer adjusted to pH 7.0.

Solutes and dodecanophenone (micellar marker) were dissolved to 500 mg L^{-1} in acetonitrile:aqueous buffer (1:1) in the case of the 50 mM sodium taurocholate (STC) system and to 200 mg L^{-1} and in methanol:micellar phase (1:3) in the case of 50 mM SDS – 10 mM Brij 35 system. In the case of alcohols, their final concentration was 40 % (v/v) to improve the compound detection. All solutions were passed through a 0.45 μm nylon syringe filter from Albet and placed into a CE vial.

Prior to analysis, the capillary was activated by the following washing sequence: water (5 min), 1 M NaOH (10 min), water (5 min), 0.1 M NaOH (10 min), water (5 min) and micellar solution (15 min). As daily conditioning, the capillary was flushed with water for 5 min, followed by 0.1 M NaOH (5 min), water (5 min) and micellar solution (15 min). Before each separation, the capillary was flushed with water (0.25 min), 0.1 M NaOH (1 min), water (0.25 min), and micellar solution (3 min).

The injection was done during 3 s at 50 mbar, the capillary temperature was 25ºC, and the voltage was +15 kV. After a preliminary scan, wavelengths were set at 200, 214, 240 and 254 nm depending on the compound absorption profile. All measurements were taken in triplicate.

The MEKC retention factor (*k*), was calculated according to Eq.4.

$$k = \frac{t_m - t_{eof}}{\left(1 - \frac{t_m}{t_{mc}}\right) t_{eof}} \qquad \text{(Eq. 4)}$$

where t_m is the solute migration time, t_{eof} corresponds to the migration time of methanol or acetonitrile (used to determine the electroosmotic flow), and t_{mc} is the migration time of dodecanophenone (used to determine the migration time of the micelles).

2.5. Biological and physicochemical systems comparison

Four tools have been used to predict the physicochemical systems that could better emulate the tadpole narcosis, and those with similar characteristics to the biological system will be selected for ongoing experimental tests.

2.5.1. d distance parameter

The similarity between two systems both characterized by means of the SPM can be measured through the *d* distance parameter [10], which is calculated according to the following expression:

$$d = \sqrt{(e_{ui} - e_{uj})^2 + (s_{ui} - s_{uj})^2 + (a_{ui} - a_{uj})^2 + (b_{ui} - b_{uj})^2 + (v_{ui} - v_{uj})^2} \qquad \text{(Eq. 5)}$$

where *e*, *s*, a, *b*, and *v* are the coefficients of the molecular descriptors in the SPM, the subscript *u* means that all coefficients have been previously normalized and the subscripts *i*, *j* represent the two compared systems. The coefficients are normalized by dividing each of the five coefficients by vector length (*l*), and *l* is calculated as follows:

$$l = \sqrt{e^2 + s^2 + a^2 + b^2 + v^2} \qquad \text{(Eq. 6)}$$

Considering the coefficients of any system as a vector in a five-dimensional space, the *d* parameter measures the distance between the normalized unitary vectors of a pair of systems. Thus, the *d* distance provides a measure of the similarity between the two considered systems: the smaller *d* is, the closer the two systems are. It is commonly assumed that distances below 0.25 indicate that the two compared systems are quite similar [10].

2.5.2. Estimation of the correlation precision between systems

In order to estimate the precision of biological-chromatographic correlations (Eq. 7), the approach described elsewhere [14] has been used.

$$\log SP_{bio} = q + p \log SP_{chrom} \qquad \text{(Eq. 7)}$$

where SP_{bio} is the solute biological property, SP_{chrom} is the solute chromatographic property, and q and p are the ordinate and slope of the correlation, respectively.

In short, the correlation precision (SD_{corr}) can be considered as the sum of three different contributions: the biological data precision (SD_{bio}^2), the chromatographic data precision ($(p_{cal} \cdot SD_{chrom})^2$) (where p_{cal} is the calculated slope) and the error due to the dissimilarity between systems (SD_d^2). SD_{bio} and SD_{chrom} values are obtained from the respective SPM

characterizations. In order to know p_{cal} and also SD_d^2 the biological property and the chromatographic property are calculated through their SPM equations. The slope of the correlation between both calculated set values provides p_{cal}, and the SD of the correlation can be entirely attributed to the dissimilarity between both systems. For this purpose the set of solutes employed in the characterization of the toxicity to tadpoles (Eq. 2) have been considered (114 solutes) [2].

2.5.3. Principal component analysis

The principal component analysis (PCA) tool is used to transform the input data in a multivariate space (normalized Abraham equation coefficients) to a new multivariate space (principal components space) whose axes are uncorrelated and rotated with respect to the original space. The main reason to transform the data in principal component analysis is to explain data variability by eliminating redundancy. Therefore, a PCA is performed with the normalized coefficients of the tadpole narcosis system and the ones of a selection of physicochemical systems. The main PCs plot (scores plot) distributes the different systems in the new chemical space, so that systems with similar characteristics are close in the scores plot.

2.5.4. Dendrogram plot

The dendrogram tool uses a hierarchical clustering algorithm, and is a diagram that shows the distances between pairs of sequentially merged classes. First, the distances between each pair of classes (the biological and the different physicochemical systems) are calculated through the normalized coefficients of the SPM equations. Then, the closest pair of classes are merged. Successively, the next closest pair of classes are merged, and the succeeding closest too, until the classes are all merged. After each merging, the distances between all pairs of classes are updated. The distances at which the signatures of classes are merged are used to construct the dendrogram [15]. In this work, clustering has been performed using the euclidean distance (straight line distance) and the single-linkage method (the distance between two groups is defined as the distance between their two closest members).

Those physicochemical systems located nearer to the biological system in the dendrogram plot will have more similar chemical characteristics and will be good candidates to emulate the tadpole toxicity.

2.6. Validation

To prove the ability of the surrogate system to estimate $\log(1/C_{nar})$ values, the set of compounds is divided into two subsets: a training set and a test set [16]. The aim of the training set is to establish a group of compounds that can be used to calibrate the HPLC system. Then, the test set is used to validate the goodness of the calibration set to predict new $\log(1/C_{nar})$ values.

To construct the two subsets a PCA based on the Abraham's molecular descriptors of the compounds is performed. In this way, compounds are distributed in the scores plot according to their physicochemical properties, and a representative selection of compounds of different nature can be done for both, the training set and the test set. Next, a multiple linear regression is performed only with the compounds of the training set. General statistic rules are followed [16] to guarantee the robustness of the system: the coefficients of the regression (intercept and slope) must have a good significance level and have to be similar to those of the global regression equation (with all compounds), the variance (SD^2) has to be of the same order of that of the biological data; the determination coefficient (R^2) and the correlation cross-validation coefficient (Q_{LMO}^2) must be above 0.6; and the Fisher's F parameter must be high. To perform the external validation the experimental $\log(1/C_{nar})$ values are plotted against the values predicted through the correlation equation of the training set. To ensure the model predictive ability good concordance has to be observed between the experimental and the predicted values; the slope of the trend line has to be near unity and the intercept near zero; the variance (SD^2) has to be of the same order of that of the biological data, the determination coefficient (R^2) must be above 0.6; the correlation cross-validation coefficient (Q_{LMO}^2) must be above 0.5; and Fisher's F parameter must be significant.

2.7. Data analysis

Principal component analysis (PCA) and dendrogram plot were performed with Matlab package version R2007a from MathWorks (Natick, MA, USA). Excel 2010 from Microsoft (Redmond, WA, US) was used for data calculations and multiple linear regression analyses. Substances' pK_a values and Abraham descriptors were obtained from Percepta database version 2014 from ACD/Labs (Toronto, Canada).

3. Results and discussion

3.1. Selection of systems to emulate nonspecific toxicity of neutral organic compounds

A large number of physicochemical systems including solvent-water partition, electrokinetic chromatography (EKC), and HPLC systems have been characterized through the SPM. Therefore, the first step was to check through the *d* distance parameter the ones closest to the toxicity to tadpoles. From this first comparison 14 physicochemical systems, detailed in Table 1, were selected. The SPM equations of these systems are provided in the Supporting Information (SI-1) and the *d* distance parameter compared to tadpole toxicity in Table 2. This selection comprises the octanol-water partition system (1), two liquid chromatography systems (2-3), seven MEKC systems (4-10), two electrokinetic chromatography (EKC) systems based on polymeric surfactants (11-12), one microemulsion electrokinetic chromatography (MEEKC) system (13), and a liposome electrokinetic chromatography (LEKC) system (14). All of the systems included in Table 2 show *d* values under 0.25 and thus are candidates to good tadpole toxicity modelling. However, those with best perspectives are those based on SDCV (5), STC (6), SDS-Brij (7), pDHCHAt-2-Na (10) and AGESS (11) surfactants.

Alternatively, the variance of the final correlation ($SD_{corr\ cal}^2$) between tadpole narcosis data and the physicochemical property (either the partition coefficient or the retention factor) of the selected physicochemical systems was estimated. Results are shown also in Table 2, together with the three different contributions to the final variance: SD_{bio}^2, $(p_{cal}\ SD_{chrom})^2$, and (SD_d^2). The intercept (q_{cal}) and the slope (p_{cal}) of the calculated correlations are also included. Since $(p_{cal}\ SD_{chrom})^2$ is usually much lower than SD_{bio}^2, a low overall variance and therefore a good performance will be obtained if SD_d^2 is lower or similar to SD_{bio}^2. Taking this criterion into account, all systems except for the one based on a RPC18 column (2) could emulate well tadpole narcosis. However, the systems of STC (6), SDS-Brij (7), and pDHCHAt-2-Na (10), with the lowest $SD^2_{corr\ cal}$ (~ 0.14-0.15), should best emulate nonspecific toxicity of neutral organic compounds to tadpoles when looking at the global variance of the correlations. Other appropriate systems could be the ones based on SDS (4), and TTAB (12) micelles, and the LEKC system (14) ($SD^2_{corr\ cal}$ ~ 0.16-0.17).

Figure 1 shows the PC2 vs PC1 plot resulting from the PCA, considering the SPM normalized coefficients of the biological system and the physicochemical systems as the initial attributes. PC1 and PC2 represent 82 % of the data variability. STC system (6) is the closest physicochemical system to the tadpole narcosis and, according to this tool, is probably the best strategy to model the toxicological parameter. Other systems such as the ones based on SDCV (5) and pDHCHAt-2-Na (10) could also be good candidates. In the case of HPLC techniques, the IAM-40% Acetonitrile system (3) is the one nearer to the biological one.

Finally, Figure 2 shows the dendrogram plot considering the SPM normalized coefficients of the biological system and the physicochemical systems as variables. According to this tool, the closest cluster to the tadpole narcosis (15) is the one that includes octanol-water (1), SDS MEEKC (13), STC (6), pDHCHAt-2-Na (10) and SDCV(5) systems. As just shown in the PC plot analysis, the IAM-40% Acetonitrile (3) is the HPLC system nearer to the biological one. Chromatographic systems are generally preferable to water-partition systems because they usually are more economic, easier to automate and faster. According to the results obtained through the different comparison tools, two HPLC systems and two EKC systems have been selected to correlate their experimental values against the ones of the tadpoles systems. As regard HPLC systems, the one based on an IAM column (3) is the one that can better model the toxicity to tadpoles, and has been included in the experimental study. Although the C18-40% Acetonitrile systems may be a very weak model for tadpole toxicity estimation, a C18 column system (RP18-40% Acetonitrile, system 2) has been studied experimentally due to its large availability in routine analysis laboratories.

In general electrophoretic systems seem to model best the tadpole toxicity rather than HPLC systems. STC (6) and SDS-Brij (7), both micellar systems, will be experimentally considered as according to the different comparison tools they are expected to be two of the best systems to model tadpole toxicity. As regards the rest of the systems that could be good candidates, they have been discarded by different reasons: SDCV (5) has been discarded because it has a high UV-Vis absorbance and may interfere with the tested substances detection; pDHCHAt-2-Na (10) and AGESS (11) polymeric surfactants are not commercially available; SDS-MEKC and MEEKC systems (4 and 13, respectively) will not be analyzed due to the more promising SDS-Brij 35 MEKC system has been selected; the LEKC system could also be used to emulate toxicity, but it has not been considered because liposomes are expensive, unstable, and difficult to prepare. The other systems included in Table 1 are not expected to model tadpole toxicity as well as the ones selected.

3.2. Selection of the solutes to be tested

About 250 substances with known narcotic concentration or 12h/24h-LC50 (lethal concentration that causes 50 % of the population death 12h/24h after the administration of a toxic compound) values have been considered in this work [3,4,17–20]. It has been shown that these two concentrations are very close and can be added up [7]. Compiled data basically correspond to two tadpole species, *R. temporaria* and *R. japonica*. When toxic concentration

values are available for different species, all of them have been taken into consideration for the correlation study.

A PCA analysis of the 250 solutes has been done according to their SPM molecular descriptors. In this way they are distributed in the scores plot according to their physicochemical properties. PC1 and PC2 explain 95 % of the data variability. Descriptors with higher influence on PC1 are V and S, whereas E descriptor has a higher influence on PC2. Three criterions have been followed to select the compounds that will further be analyzed in the chosen chromatographic systems: firstly, substances must cover all the PCA chemical space to assure a set of compounds chemically diverse; secondly, they must be neutral at the working pH, since all the SPM equations compared stand only for neutral compounds; finally, selected compounds must have chromophore groups due to detection requirements. According to these statements, a selection of 65 compounds of known toxicity data was done. From these 65 substances, 37 belong to *R. temporaria* and 30 solutes to *R. japonica*. Figure SI-1 shows the final distribution of the 65 compounds, and also the distributions according the two species of tadpoles. It can be noted that not many compounds with negative PC2 have been selected; this is due to the lack of chromophore groups on these aliphatic substances. The rest of the graphic areas are covered with the selected solutes.

3.3. Evaluation of the performance of chromatographic systems to estimate nonspecific toxicity to tadpoles

After selecting the four more interesting physicochemical systems, the retention factors of the selected compounds were determined in these systems. Then, a regression analysis of experimental log ($1/C_{nar}$) values vs log *k* value was done, according to (Eq. 7).

The results of the experimental correlations are shown in Table 3 (global) and Figure 3. According to the statistics, both MEKC systems (6 and 7) show the best correlation parameters (smallest slope and ordinate SD, and highest R^2). They also have associated the lowest experimental correlation variances ($SD_{corr\ exp}^2$), which agrees and indeed are better than the corresponding initial predictions ($SD_{corr\ cal}^2$, Table 2). HPLC system 3 (IAM column, 40 % acetonitrile) could also provide good estimations with only slightly higher errors. The HPLC system 2 (C18 column, 40 % acetonitrile) shows a little worse performance to estimate nonspecific toxicity to tadpoles as predicted by the variances study (SD_d^2 value, Table 2).

Most of the data available in the literature correspond to two tadpole species, *R. temporaria* and *R. japonica*. Hence, an independent correlation for these two species and a both combined correlation using a flag descriptor for *R. Japonica* (I_{jap}) have been performed. The indicator

variable I_{jap} is set to 1 when the toxicological property has been evaluated on *R. japonica* species and to 0 otherwise. Solutes considered outliers were excluded from the correlations. Substances that have been found as outliers in some of this correlations are o-dinitrobenzene, ethan-1,2-diol, 2,4-dinitrotoluene, 2-methylpropan-2-ol, coumarine, resorcinol and 1-chloro-4-nitrobenzene. The maximum number of outliers in a correlation is 5 (IAM with a flag descriptor correlation). Results are shown in Table 3 (*R. temporaria* and *R. Japonica*), and Figures 4 and 5. STC MEKC system (6) shows the best correlation parameters to emulate the nonspecific toxicity to *R. temporaria* tadpoles. However, SDS-Brij 35 MEKC system (7) could be an alternative method. In the case of *R. japonica*, poor correlations have been obtained with the MEKC systems. Instead, the HPLC system based on an IAM column (3) shows a very good correlation to toxicity of this tadpole species.

The correlation introducing a flag descriptor has been done according to Eq. 8 (Figure 6).

$$\log (1/C_{nar}) = q + p \log k + i\, I_{jap} \qquad \text{(Eq. 8)}$$

Although in some instances the value of the *i* coefficient is very close to zero (Table 3), statistics of the multiple linear correlation are slightly better than the ones of the global correlation. Therefore, the system that best can emulate tadpole toxicity is the one based on STC micelles, when a mixture of tadpole species (model with flag descriptor) or only *R. Temporaria* tadpoles are considered, whereas the system based on an HPLC column is the best to emulate specific toxicity for *R. Japonica* tadpoles.

3.4. Validation of the best chromatographic models

Next, the selected models have been internally and externally validated in order to check their robustness and predictive ability, respectively. However, in addition to the three models already mentioned, the IAM model with flag descriptor has also been validated since statistics are also within the acceptable range, and HPLC equipment is much more common in analysis laboratories rather than capillary electrophoresis one.

To perform the model's validation, the set of solutes (65 for the general models and 37 and 30 for *R. Temporaria* and *R. Japonica*, respectively) were divided into a training set (around 2/3 of the compounds) and a test set (around 1/3 of the compounds). To ensure all type of compounds were included both in the training and test sets, this selection was done on the basis of a PCA on the solute SPM descriptors (Table SI-2). For the internal validation, the four selected models were calculated again, but only with the solutes of the training set. Table

4 shows the correlation parameters obtained. Equations' coefficients are similar to those of the models with all solutes (Table 3), which is indicative of the robustness of the models. Adequate determination coefficients, standard deviations and F values were obtained. Furthermore, an additional parameter, the leave-several-out cross-validated squared correlated coefficient was calculated. This coefficient was higher than 0.6 in all cases, which also points out the robustness of the selected systems [16].

Finally, the external validation was performed. A regression was done between the experimental toxicity and the one predicted through the training set equations toxicity data for the compounds of the test set. Table 5 shows the correlation parameters and statistics, including the leave-several-out cross-validated squared correlated coefficient. According to statistics, all models show good prediction ability. However, when the slope and the intercept of the correlations are examined, the model of STC with a flag descriptor is the one that has a slope value closer to 1 and an intercept value closer to 0, which indicates a better correlation between the experimental predicted values.

4. Conclusions

Two different chromatographic systems, one based on MEKC with STC micelles and another one based on HPLC with an IAM column (40 % acetonitrile), have been selected as the best systems to estimate toxicity to tadpoles. Different models have been established with solutes of different nature. The model with STC is the best choice to estimate the toxicity to *R. Temporaria* tadpoles, and also it is the system that provides the model with the best prediction ability when all species of tadpoles are considered. For the latter case, models with the flag descriptor (that discriminate between different tadpole species) provide slightly better statistics than global models where the different tadpole species are not differentiated. Whereas, the HPLC system with an IAM column (40 % acetonitrile) is the one that better predicts the toxicity to *R. Japonica* tadpoles.

The four comparison tools used in this work have been very valuable to identify those physicochemical systems (previously characterized through the SPM) that can best emulate a given biological one. In fact, the four mathematical approaches pointed out STC as one of the closest systems to tadpole narcosis, and in all cases the IAM-40% Acetonitrile system was the closest system based on HPLC. SDS-Brij 35 was discarded for validation in front of STC, although the established models were only slightly worse than the ones of STC, being a good alternative to the STC system.

In conclusion, this work provides two different methodologies to estimate tadpole toxicity through direct correlation to chromatographic measurements in an easy, fast economic and sustainable way.

ACKNOWLEDGEMENTS

Financial support from the Ministerio de Economía y Competitividad from the Spanish Government (CTQ2014-56253-P), and the Catalan Government (2014SGR277) is acknowledged.

REFERENCES

[1] W. a Hopkins, Amphibians as models for studying environmental change., ILAR J. 48 (2007) 270–277.

[2] M.H. Abraham, C. Rafols, Factors that influence tadpole narcosis. An LFER analysis, J. Chem. Soc. Perkin Trans. 2. (1995) 1843.

[3] H. Huang, X. Wang, W. Ou, J. Zhao, Y. Shao, L. Wang, Acute toxicity of benzene derivatives to the tadpoles (Rana japonica) and QSAR analyses, Chemosphere. 53 (2003) 963–970.

[4] X. Wang, Y. Dong, S. Xu, L. Wang, S. Han, Quantitative Structure-Activity Relationships for the Toxicity to the Tadpole Rana japonica of Selected Phenols, Bull. Environ. Contam. Toxicol. 64 (2000) 859–865.

[5] X. Wang, Y. Dong, L. Wang, S. Han, Acute toxicity of substituted phenols to Rana japonica tadpoles and mechanism-based quantitative structure-activity relationship (QSAR) study, Chemosphere. 44 (2001) 447–455.

[6] a Leo, C. Hansch, C. Church, Comparison of parameters currently used in the study of structure-activity relationships., J. Med. Chem. 12 (1969) 766–771.

[7] K.R. Bowen, K.B. Flanagan, W.E. Acree, M.H. Abraham, C. Rafols, Correlation of the toxicity of organic compounds to tadpoles using the Abraham model, Sci. Total Environ. 371 (2006) 99–109.

[8] D.M. Cimpean, C.F. Poole, Systematic search for surrogate chromatographic models of biopartitioning processes., Analyst. 127 (2002) 724–729.

[9] C.F. Poole, T.C. Ariyasena, N. Lenca, Estimation of the environmental properties of compounds from chromatographic measurements and the solvation parameter model, J. Chromatogr. A. 1317 (2013) 85–104.

[10] E. Lázaro, C. Ràfols, M.H. Abraham, M. Rosés, Chromatographic estimation of drug disposition properties by means of immobilized artificial membranes (IAM) and C18 columns, J. Med. Chem. 49 (2006) 4861–4870.

[11] M. Hidalgo-Rodríguez, E. Fuguet, C. Ràfols, M. Rosés, Estimation of biological properties by means of chromatographic systems: Evaluation of the factors that contribute to the variance of biological-chromatographic correlations, Anal. Chem. 82 (2010) 10236–10245.

[12] E. Fuguet, C. Ràfols, E. Bosch, M.H. Abraham, M. Rosés, Selectivity of single, mixed, and modified pseudostationary phases in electrokinetic chromatography, Electrophoresis. 27 (2006) 1900–1914.

[13] J. a. Castillo-Garit, Y. Marrero-Ponce, J. Escobar, F. Torrens, R. Rotondo, A novel approach to predict aquatic toxicity from molecular structure, Chemosphere. 73 (2008) 415–427.

[14] M. Hidalgo-Rodríguez, E. Fuguet, C. Ràfols, M. Rosés, Modeling nonspecific toxicity of organic compounds to the fathead minnow fish by means of chromatographic systems, Anal. Chem. 84 (2012) 3446–3452.

[15] ArcGis Resources, Understanding multivariate classification, (2012). http://resources.arcgis.com/en/help/main/10.1/index.html#/Understanding_multivariate_classification/009z000000pr000000/ (accessed June 6, 2015).

[16] K. Roy, On some aspects of validation of predictive quantitative structure–activity relationship models, Expert Opin. Drug Discov. 2 (2007) 1567–1577.

[17] E. Overton, Studien über die Narkose, zurgleich eing Bertrag zur allgemeinen Pharmakologie., Jena, Gustav Fischer, 1901.

[18] E. Overton, Studies on Narcosis, Chapman and Hall, London, 1991.

[19] K. Meyer, H. Hemmi, Beitrage zur theorie der Narkose. III, Biochem. Z. 277 (1935) 39–71.

[20] Y. Kita, L.J. Bennett, K.W. Miller, The partial molar volumes of anesthetics in lipid bilayers, Biochim. Biophys. Acta - Biomembr. 647 (1981) 130–139.

[21] M.H. Abraham, H.S. Chadha, G.S. Whiting, R.C. Mitchell, Hydrogen bonding. 32. An analysis of water-octanol and water-alkane partitioning and the Δlogp parameter of seiler, J. Pharm. Sci. 83 (1994) 1085–1100.

[22] E. Fuguet, C. Ràfols, E. Bosch, M.H. Abraham, M. Rosés, Solute-solvent interactions in micellar electrokinetic chromatography: III. Characterization of the selectivity of micellar electrokinetic chromatography systems, J. Chromatogr. A. 942 (2002) 237–248.

[23] E. Fuguet, C. Ràfols, E. Bosch, M.H. Abraham, M. Rosés, Erratum. Solute-solvent interactions in micellar electrokinetic chromatography: III. Characterization of the selectivity of micellar electrokinetic chromatography systems, J. Chromatogr. A. 1216 (2009) 6877–6879.

[24] M.D. Trone, M.G. Khaledi, Characterization of Chemical Selectivity in Micellar Electrokinetic Chromatography. 4. Effect of Surfactant Headgroup, Anal. Chem. 71 (1999) 1270–1277.

[25] S.K. Poole, C.F. Poole, Characterization of Surfactant Selectivity in Micellar Electrokinetic Chromatography, Analyst. 122 (1997) 267–274.

[26] M.M. Rosés, C. Ràfols, E. Bosch, A.M. Martínez, M.H. Abraham, A.M. Martınez, Solute – solvent interactions in micellar electrokinetic chromatography Characterization of sodium dodecyl sulfate – Brij 35 micellar systems for quantitative structure – activity relationship modelling, J. Chromatogr. A. 845 (1999) 217–226.

[27] M. Hidalgo-Rodríguez, E. Fuguet, C. Ràfols, M. Rosés, Solute–solvent interactions in micellar electrokinetic chromatography: VII. Characterization of sodium cholate–

sodium deoxycholate mixed-micellar systems, J. Chromatogr. A. 1217 (2010) 1701–1708.

[28] W. Shi, C.P. Palmer, Effect of pendent group structures on the chemical selectivity and performance of sulfonated copolymers as novel pseudophases in electrokinetic chromatography., Electrophoresis. 23 (2002) 1285–95.

[29] S. Schulte, C.P. Palmer, Alkyl-modified siloxanes as pseudostationary phases for electrokinetic chromatography., Electrophoresis. 24 (2003) 978–83.

[30] M.H. Abraham, C. Treiner, M. Roses, C. Rafols, Y. Ishihama, Linear free energy relationship analysis of microemulsion electrokinetic chromatographic determination of lipophilicity, J. Chromatogr. A. 752 (1996) 243–249.

[31] S.T. Burns, A.A. Agbodjan, M.G. Khaledi, Characterization of solvation properties of lipid bilayer membranes in liposome electrokinetic chromatography, J. Chromatogr. A. 973 (2002) 167–176.

FIGURE CAPTIONS

Figure 1: PCA scores plot of the biological (Δ) and the selected physicochemical (O) systems.

Figure 2: Dendrogram plot of the biological (15) and the selected physicochemical (1-14) systems.

Figure 3: Plots of $\log(1/C_{nar})$ vs. experimental log k of the studied physicochemical systems (IAM - 40 % acetonitrile; RP18 - 40 % acetonitrile; STC; SDS-Brij). Global model for all tadpole species. Solid lines are the plot of the regression equation.

Figure 4: Plots of $\log(1/C_{nar})$ vs. experimental log k of the studied physicochemical systems (IAM - 40 % acetonitrile; RP18 - 40 % acetonitrile; STC; SDS-Brij). Model for *R. Temporaria* tadpoles. Solid lines are the plot of the regression equation

Figure 5: Plots of $\log(1/C_{nar})$ vs. experimental log k of the studied physicochemical systems (IAM - 40 % acetonitrile; RP18 - 40 % acetonitrile; STC; SDS-Brij). Model for *R. Japonica* tadpoles. Solid lines are the plot of the regression equation.

Figure 6: Plots of $\log(1/C_{nar})$ vs. experimental log k of the studied physicochemical systems (IAM - 40 % acetonitrile; RP18 - 40 % acetonitrile; STC; SDS-Brij). Model for all tadpole species considering a flag descriptor. Solid lines are the plot of the regression equation.