Journal of Chromatographic Science, 2021, Vol. 59, No. 1, 40–46
doi: 10.1093/chromsci/bmaa074
Advance Access Publication Date: 27 October 2020
Article

Article

HPLC Gradient Retention of Tryptophan and its Metabolites on Three Stationary Phases in Context of Lipophilicity Assessment

Katarzyna Wicha-Komsta[1], Robert Skibiński[2], Tomasz Kocki[1], Waldemar A. Turski[1], and Łukasz Komsta[2,*]

[1]Department of Experimental and Clinical Pharmacology, Faculty of Medicine, Medical University of Lublin, Jaczewskiego 8b, 20-090 Lublin, Poland, and [2]Department of Medicinal Chemistry, Faculty of Pharmacy, Medical University of Lublin, Jaczewskiego 4, 20-090 Lublin, Poland

*Author to whom correspondence should be addressed. Email: lukasz.komsta@umlub.pl

Received 30 April 2020; Editorial Decision 4 September 2020

Abstract

This paper is a continuation of lipophilicity research on 14 compounds (tryptophan, kynurenine pathway products, auxin pathway products, serotonin pathway products, tryptamine, as well as two synthetic auxin analogs): indole-2-acetic acid sodium salt (IAA), serotonin, 5-hydroxy-L-tryptophan, tryptamine, L-tryptophan, L-kynurenine (KYN), kynurenic acid (KYA), 3-hydroxy-DL-kynurenine, naphtyl-1-acetamide, indole-3-propionic acid (IPA), naphthalene-1-acetic acid (NAA), indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPV), as well as melatonin. They were chromatographed in high performance liquid chromatography gradient conditions on tree stationary phases (C18, CN, DIOL) using three modifiers on each phase (methanol, acetonitrile and acetone). The resulting retention data was correlated with computational lipophilicity indices. Six compounds were proven to be ionized in neutral pH physiological conditions (IAA, KYA, IPA, NAA, IBA and IPV) and they were rechromatographed with acidic mobile phase to enhance the resulting dataset. It can be concluded that the retention times are highly correlated with lipophilicity regardless of used modifier and column and the main differentiating trend can be only connected to presence of naphthalene or indole ring. The principal component analysis, additive linear modeling, as well as multiplicative trilinear parallel factor analysis (PARAFAC) modeling helped to understand the internal structure of the obtained results.

Introduction

A recently increased interest on the kynurenine (KYN) pathway, which is a significant part of tryptophan (TPF) metabolism, is caused by several new scientific ideas about the compounds occurring in this process (1). The first interesting substance is kynurenine, created from tryptophan by breaking its five-membered ring, through formylkynurenine as an intermediate product. The next step of this metabolism, and equally interesting compound, is kynurenic acid (KYA), eliminated from human body with urine. Kynurenine can also undergo further decomposition to 3-hydroxykynurenine, 3-hydroxyantranilate then conversion to simple non-aromatic compounds (1).

In general, kynurenine can be perceived as a compound with a negative impact onto human organism, as it is an aryl hydrocarbon receptor agonist (2) and its content is increased during aging. Due to increased overproduction of this compound in response to infection or inflammatory state, mutated cells can escape from the immune system, leading to cancer development. Therefore, new analytical methods appear in recent literature, as an increase of the L-KYN/L-TRP ratio is the marker of the above process (3).

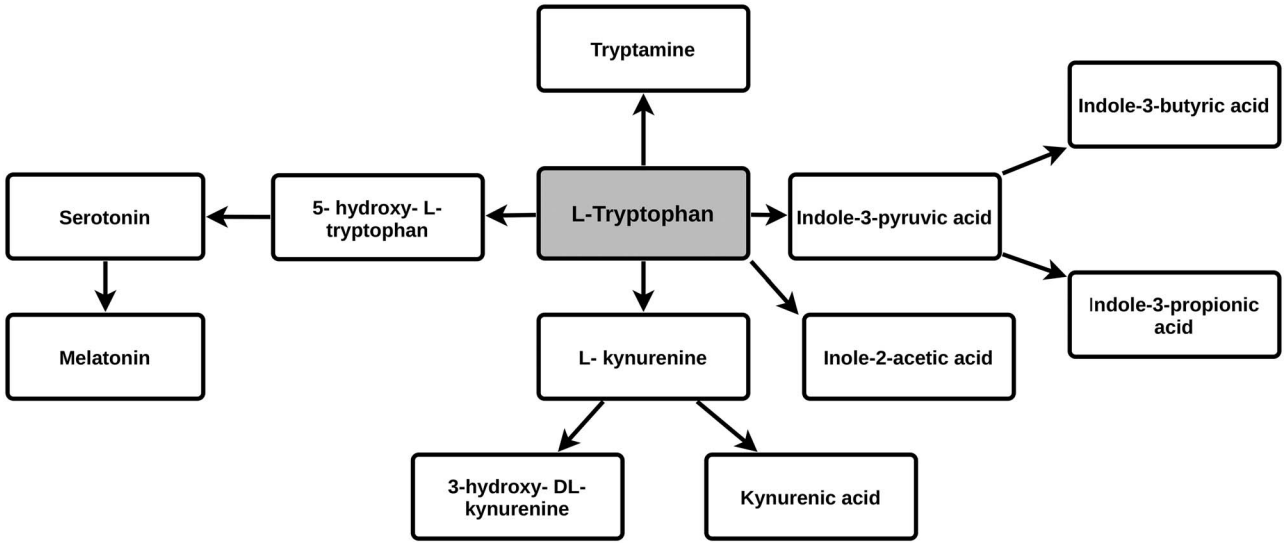

Figure 1. Metabolism of tryptophan.

On the contrary, kynurenic acid has neuroprotective properties, and it is involved in autoimmune, anti-inflammatory, antiepileptic and even anticancer processes. It is also the only known endogenous antagonist of N-methyl-D-aspartate receptor and has a weak affinity to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (4).

Besides the kynurenine pathway, tryptophan can be decarboxylated to tryptamine, or hydroxylated to 5-hydroxytryptophan, which is the substrate of serotonin and melatonin synthesis (Figure 1). Plants can oxidize tryptophan to indole-3-pyruvate, which is a main substrate of the synthesis of auxin hormones: indole-acetate, indole-propionate and indole-butyrate. Various secondary substrates (mainly alkaloids) containing indole ring in the molecule can be also formed (5).

Our previous research (6) focused on preliminary lipophilicity research of tryptophan metabolites and some analogs by means of thin-layer chromatography. The main motivation of undertaking this topic was the fact that there are some unclear details in tryptophan biochemistry, which can be directly connected with the lipophilicity. First of all, kynurenic acid almost does not pass the blood–brain barrier, whereas kynurenine is able to do it (4). The kynurenic acid is synthesized inside the brain and does not come from the blood. We showed that the kynurenine pathway represents subsequent lowering the lipophilicity during each stage, whereas the lipophilicity increases in the other pathways. Although a loss of one amino group and closing the ring to form quinolone moiety (conversion from kynurenine to kynurenic acid) should increase the lipophilicity, kynurenic acid is the less lipophilic compound under the investigated conditions due to its ionization.

These facts inspired us to undertake some additional high performance liquid chromatography (HPLC) study, using several modifiers and stationary phases.

Experimental

The investigated substances indole-2-acetic acid sodium salt (IAA), serotonin (SER), 5-hydroxy-L-tryptophan (HTR), tryptamine (TPA), L-tryptophan (TPF), L-kynurenine (KYN), kynurenic acid (KYA), 3-hydroxy-DL-kynurenine (HKY), naphtyl-1-acetamide (NAC), indole-3-propionic acid (IPA), naphthalene-1-acetic acid (NAA), indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPV), as well as melatonin (MEL) were purchased from Sigma-Aldrich (USA).

The HPLC system from Waters (USA) consisted of an Alliance e2695 separation module and 2998 PDA detector. This set was powered by Empower Pro v.2.0 software. Separation was carried out on a LiChrospher® 100 columns (125 mm × 4.0 mm i.d., with a particle size of 5 µm) from E. Merck, filled with C18, CN and DIOL. Each column was thermostated at 35°C.

A linear binary gradient program was used: the water-like phase (deionized water obtained with SolPure 7 deionization system, or 0.01 M phosphate buffer pH 2.0 made with Baker UltraPure salts) and organic modifier, with constant flow rate 1 mL/min. Three modifiers (methanol (MET), acetonitrile (ACN) and acetone (ACT)) was of gradient grade (Merck).

Each experiment was conducted with the same gradient program: column was equilibrated at 30% of organic modifier, then after sample injection increased immediately and linearly during 10 min to 100%. The concentration was then switched during the next minute back to 30% and the last 4 min of each run was treated as the time for reaching equilibrium before the next injection. Detection of all compounds was carried out at 220 nm; however, this was not a critical value due to qualitative nature of the experiments.

Six of analyzed compounds (IAA, KYA, IPA, NAA, IBA and IPV) were chromatographed with deionized water (abbreviated with "minus" anion sign) and in pH 2 phosphate buffer (without minus sign). Therefore, as the result, we obtained a matrix (20 compounds × 9 retention times).

The computations were performed inside open source R environment (version 3.6.2, www.r-project.org). Linear model fitting was performed with built-in function "lm", principal component analysis with built-in function "prcomp". Stepwise Akaike information criterion (AIC) selection was carried out with "stepAIC" function from built-in MASS package. PARAFAC model was fitted using "multiway" package. Computational lipophilicities were obtained using Virtual Computational Chemistry Laboratory (www.vcclab.org).

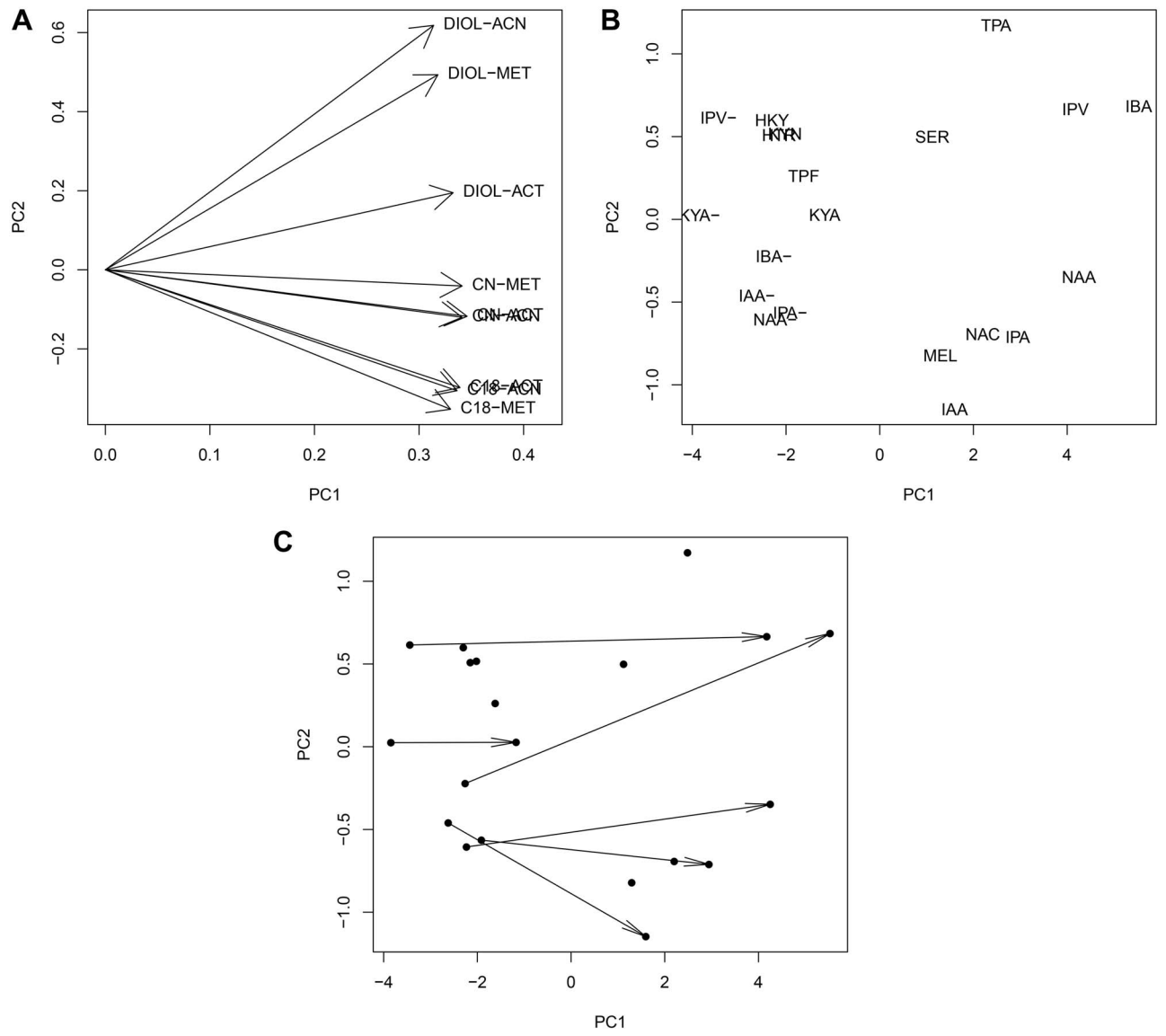

Figure 2. Principal Component Analysis of the retention time matrix: (A) loadings, (B) scores and (C) scores indicating the retention change from ionized to non-ionized form as arrows.

Results

The final retention time matrix had 20 rows (14 non-ionized +6 ionized compounds) and 9 columns (three modifiers × three columns). It was compared to the arithmetic mean of obtained computational indices (sorted according to value): HKY: -1.72, KYN: -1.43, HTR: -0.88, TPF: -0.54, SER: 0.83, KYA: 1.13, IPV: 1.21, MEL: 1.4, TPA: 1.41, IAA: 1.56, IPA: 1.88, NAC: 2.04, IBA: 2.29 and NAA: 2.68.

Principal Component Analysis, done on the retention time matrix, placed 91.5% of information in the first principal component and 4.6% of information in the second one (Figure 2). Next, nine linear models, fitted to the dependence: log P = at + b, where t is retention time, were fitted (Figures 3 and 4). Correlation coefficients were inside range 0.51–0.95 (mean 0.81).

To investigate the contribution of modifiers and stationary phases onto the retention time, a following multivariate model was then fitted to these dataset:

$$t = \beta_0 + \beta_{c0} + \beta_{m0} + (\beta + \beta_c + \beta_m) \log P, \quad (1)$$

... where t is the retention time and the intercept and slope terms are modeled additively as some initial value for C18 and MET, respectively (β_0, β), optionally modified for other columns (β_{c0}, β_c) and modifiers (β_{m0}, β_m). The model fitted well with R^2 = 0.864 (Table I).

The last attempt in the data analysis was to fit the one-component PARAFAC model. For this task, the retention times were arranged as a cube (tensor), having 20 rows (compounds), 3 columns (modifiers) and 3 slices (columns). The model assumes that the retention time is a product of three "contributions"—from compound, modifier and adsorbent:

$$t_{ijk} = a_i b_j c_k, \quad (2)$$

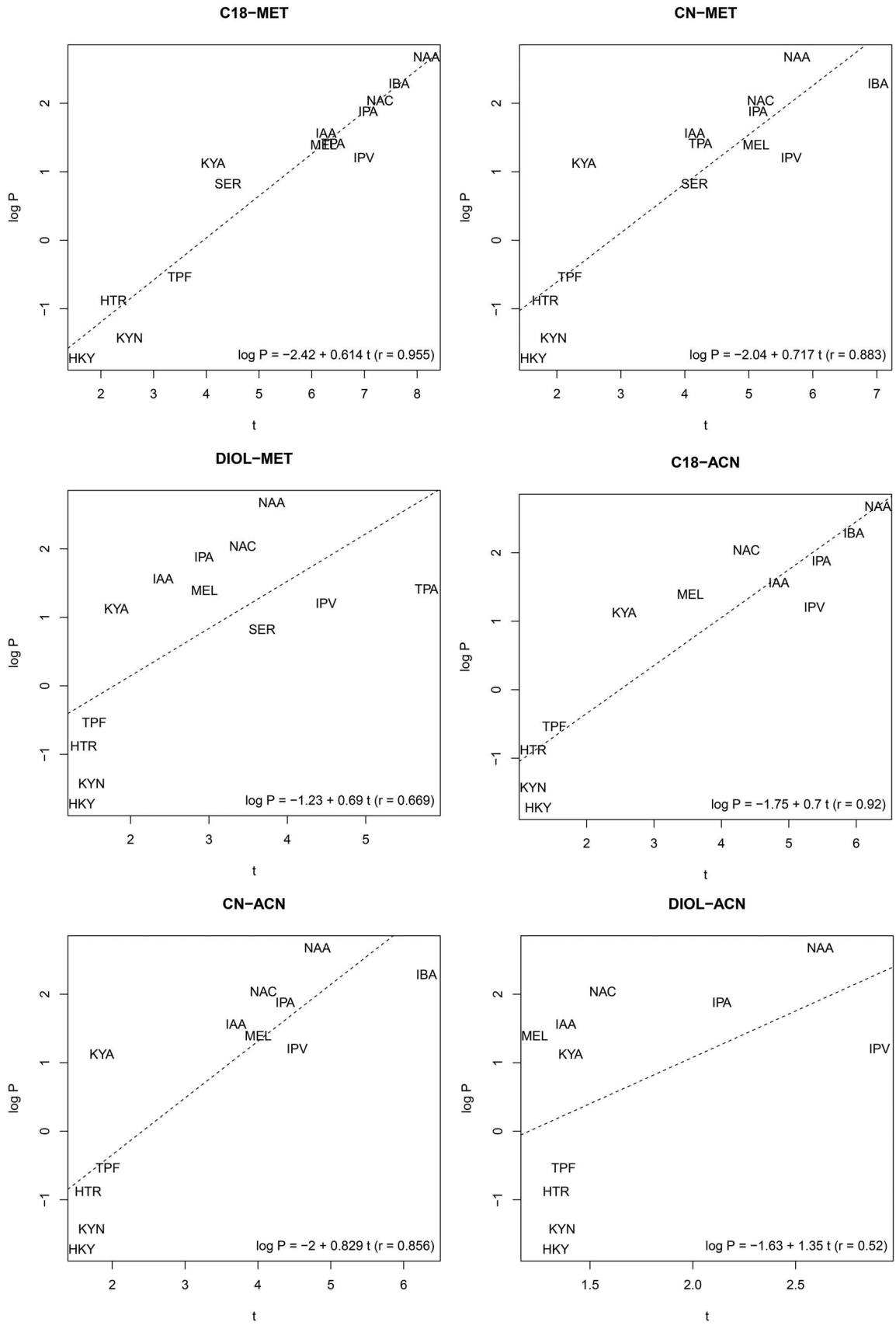

Figure 3. Dependence between the retention time and average computational lipophilicity for nine used chromatographic systems.

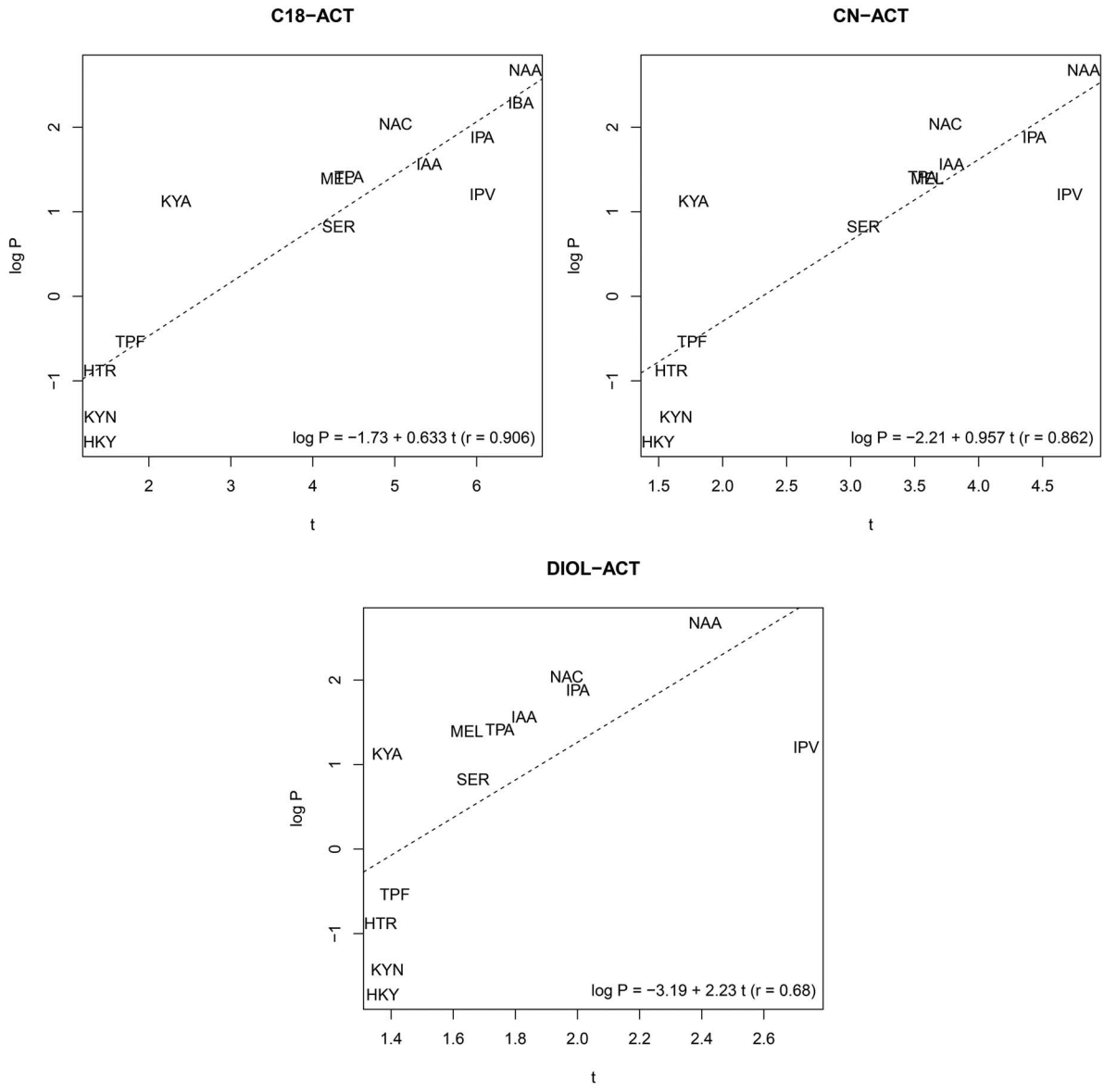

Figure 3. Continued.

Table I. The coefficients of linear additive model for computing retention time from the lipophilicity

		Estimate	±SE	t	$p(t)$
β_0		3.8	0.18	21	1.5e-39
β		1.5	0.11	14	8.2e-25
β_{c0}	CN	-0.5	0.2	−2.5	0.014
	DIOL	-1.4	0.2	−7	2.9e-10
β_{m0}	ACN	-0.88	0.2	−4.3	3.3e-05
	ACT	-0.82	0.2	−4.1	7.4e-05
β_c	CN	-0.42	0.12	−3.3	0.0011
	DIOL	-0.98	0.13	−7.7	6.6e-12
β_m	ACN	-0.3	0.13	−2.4	0.017
	ACT	-0.31	0.13	−2.4	0.017

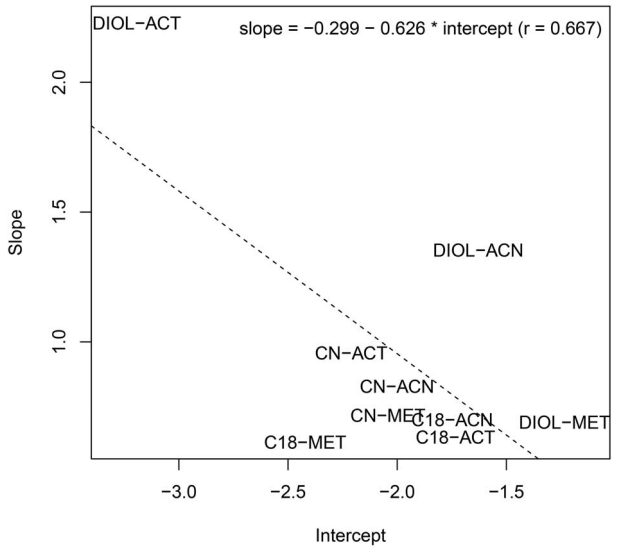

Figure 4. Slopes and intercepts of equations modeling lipophilicity from retention time (for each equation see Figure 3).

... where t_{ijk} is the retention time of *i*-th compound, obtained with *j*-th modifier on *k*-th adsorbent and *a*, *b* and *c* are some independent "contributions" of each of these three factors. The results are shown in Figure 5.

Discussion

The preliminary experiments with the analyzed compounds showed that during the chromatographic process in neutral pH (carried out with deionized water), six compounds (IAA, KYA, IPA, NAA, IBA and IPV) are present in ionized form and therefore have much shorter retention time than one could expect, taking into the account their lipophilicity. Therefore, all experiments with these compounds are annotated with "minus" sign (IAA^-, KYA^- and so on). Further experiments using pH 2 phosphate buffer allowed to obtain the retention times for non-ionized forms, abbreviated in further results without minus sign.

It can be easily seen (Figure 2A) that the first component represent the main intercorrelated trend in the retention, which is strictly dependent on lipophilicity of the compounds. High values of PC1 indicate high lipophilicity (IBA, IPV and NAA, see Figure 2B), whereas low value is characteristic for low lipophilicity compounds (TPF, KYA and HKY) and all ionized forms of lipophilic compounds. The second component represent differences between retention on different adsorbents—high value is obtained with DIOL, low with C18, whereas CN column gives some intermediate value. The main interpretation of this trend is connected with indole and naphthalene as a main skeleton of the substance. In general (Figure 2B) indole compounds have high PC2 and naphthalene compounds low values. This trend is slightly disturbed for ionized compounds (like IBA); however, it can be easily seen (Figure 2C) that the ionization process shifts the retention parameters almost horizontally, thus PC2 value almost does not change during ionization process.

Analyzing computational lipophilicity indices obtained with six algorithms (ALOGPs, AClogP, ALOGP, MLOGP, XLOGP2 and XLOGP3) one can conclude that all these indices are strictly intercorrelated. Unscaled PCA places 96.6% variance in the first PC, whereas scaled PCA stores there 94.8%. That is why it was sufficient to use the arithmetic mean as one index.

The fitting quality of the linear models (Figure 3) is visibly sorted according to the stationary phase: from the best to the worst: C18 (MET, ACN and ACT), CN (MET, ACT and ACN) and DIOL (ACT, MET and ACN). The worst correlations on DIOL adsorbent is caused by very low retention times regardless on the lipophilicity. However, these retention times do not suggest ionization of any compound, rather exceptional shortening of the retention time, especially with acetone.

Investigating the dependence between the slope and intercept (Figure 4), it can be seen that quite large correlation is caused mainly by DIOL-ACT as outlying observation. The rest of chromatographic systems are far away from lying on some straight line. Nevertheless, DIOL column gives equations above the main trend (higher slope), whereas CN and C18 are below the line. C18 column shows the lowest slope values and larger range of intercepts, whereas all CN systems are located together close to each other, with a middle value of intercept and low value of the slope.

Looking at the results of the linear additive modeling, the regression diagnostics showed no significant interactions between columns and modifiers. The optimality of this modeling was confirmed by stepwise optimization. When initial model was fitted with all possible interactions, then optimized stepwise using "stepAIC" function, the resulted optimal model was the additive model identical with the formula above.

From the regression diagnostics (Table I) it can be deduced, that the average retention shift (change of intercept term) between C18 and CN equals to 30 s, whereas DIOL gives 1.4-min shorter retention times than C18. Use of acetonitrile or acetone instead of methanol results in retention time shorter by ~45 s. Nevertheless, the slope of the dependence is also lowered by other modifiers and columns than C18-MET—by 0.3 for modifier change, 0.4 when changed to CN and 0.98 when changed to DIOL. All these terms are significantly different than zero.

PARAFAC model fitted to these data had surprisingly high R2 equal to 0.979, which indicates that the multiplicative modeling of the retention works very well in this case. The contribution of compound (Figure 5) is very well correlated with lipophilicity (for non-ionized compounds $r = 0.916$). Contributions of modifiers equal to 2.92, 2.98 and 3.89 for ACN, ACT and MET, respectively. The stationary phase contribution is the lowest for DIOL (0.615), intermediate for CN (0.999) and the largest for C18 (1.270).

Conclusions

The preliminary previous research, done on one stationary thin layer chromatography (TLC) phase with one modifier (RP8 and methanol, respectively), was significantly widened in the current paper to three modifiers and three stationary phases. It can be assumed that six investigated compounds (IAA, KYA, IPA, NAA, IBA and IPV) exist in ionized form under physiological conditions. The retention times were strictly correlated with lipophilicity regardless of used modifier and stationary phase and the main trend differentiating the retention among adsorbents is the difference between indole and naphtalene ring interaction with stationary phase. Our findings also further prove the assumption that kynurenic acid is ionized under physiological condition, which can be a significant suggestion in studies on its pharmacodynamical behavior.

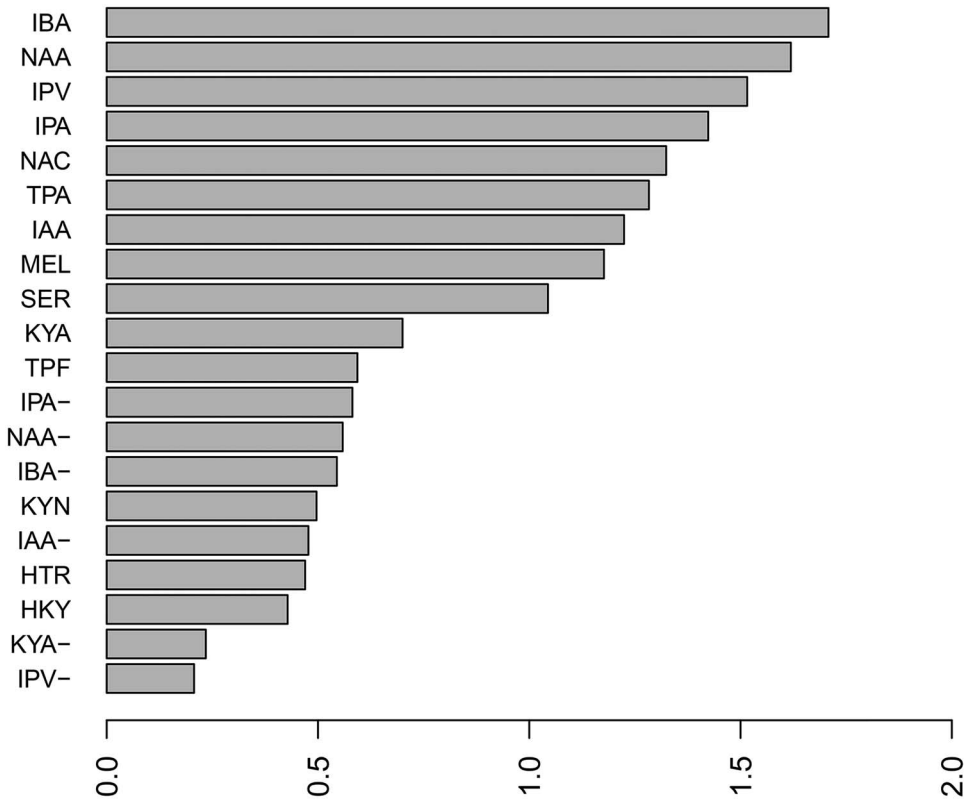

Figure 5. First score of PARAFAC, correlated with lipophilicity, representing the contribution of a compound to the retention.

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