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Quantitative structure–chromatographic retention relationship study of six underivatized equine estrogens

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Abstract

Underivatized estrone (ES), equilin (EQ), equilenin (EQN) and their corresponding 17α-diols 17α-estradiol (ESD), 17α-dihydroequilin (DHEQ) and 17α-dihydroequilenin (DHEQN) were separated by TLC, RP-HPLC and capillary GC. Their dipole moments (μ) and Randić's connectivity indices ($^1\chi$) were determined as parameters of importance for the separation. The number of H atoms was taken as an additive structural parameter of importance for the quantitative structure–chromatographic retention relationship study (QSRR). Principal component analysis (PCA) was applied in order to find similarities and dissimilarities between 9 TLC and 10 RP-HPLC systems. PCA indicated that proton donor–proton acceptor interactions play the most important role for the TLC and RP-HPLC separation. The two-dimensional non-linear map of PC variables showed that the keto-estrogens (ES, EQ and EQN) and the corresponding diols (ESD, DHEQ and DHEQN) form two separate clusters. The relationship between GC retention of equine estrogens characterized by Kováts indices (KI), their $^1\chi$ and μ was expressed by the equation $KI/100 = a/^1\chi + b/\mu^2 + c$. The biological activity of the estrogens was related to $\log 1/\mu^2$.

Keywords: Quantitative structure–retention relationships; Estrogens; Estrone; Equilin; Equilenin; Estradiol; Dihydroequilin

1. Introduction

Estrone (ES), equilin (EQ), equilenin (EQN) and the corresponding 17α-diols 17α-estradiol (ESD), 17α-dihydroequilin (DHEQ) and 17α-dihydroequilenin (DHEQN), in the form of sodium sulfate salts are the most commonly prescribed estrogens for replacement therapy in estrogen-deficient women [1]. The structural and molecular formulae of equine estrogens are given in Fig. 1. They were analyzed in pharmaceuticals by HPTLC [2], capillary GC [3] and RP-HPLC [4]. To characterize their GC retention/physico-chemical properties relationship their Kováts indices (KI), steroid numbers (SN) and dipole moments (μ) were determined [5].

The aim of this study was to determine the relationship between the physico-chemical parameters of equine estrogens and their HPTLC, RP-HPLC and GC retention (QSRR study), as well as to correlate their physico-chemical properties to their biological activity. Principal component analysis (PCA) [6] was applied to find similarities and dissimilarities between the chromatographic systems and physico-chemical parameters of six equine estrogens, and the non-linear mapping technique [7]

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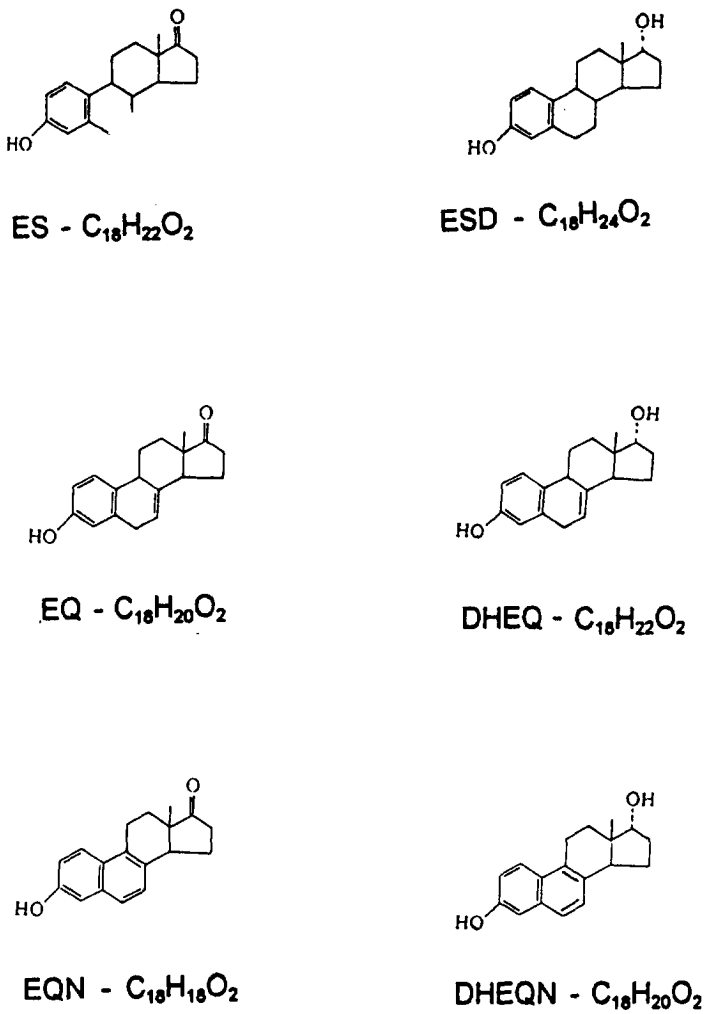

Fig. 1. The structural and molecular formulae of equine estrogens.

was used to facilitate the evaluation of the multi-dimensional data matrices of principal component (PC) loadings and PC variables.

2. Experimental

2.1. Materials

ES, EQ and EQN were obtained from Sigma (St. Louis, MO, USA), and ESD, DHEQ and DHEQN were gifts from Diosynth (Oss, Netherlands).

2.2. Apparatus and operating conditions

The TLC separation was carried out on silica gel 60 F_{254} HPTLC plates (Merck, Darmstadt, Germany) in a twin-trough developing chamber (Camag, Muttenz, Switzerland) after 30 min chamber saturation with the mobile phase. Sample preparation and chromatographic procedure were done as described in Ref. [2]. The mobile phases used for TLC separation of equine estrogens are listed in Table 1.

HPLC separation followed by UV absorbance detection at 280 nm was performed on a stainless-steel Nucleosil C_{18} column (250×4.6 mm I.D., 3

Table 1
Composition of HPTLC mobile phases

No.	Mobile phase	Composition
I	C–EA–TA	42:52.5:5.5 (v/v)
II	C–EA–TA	40:48:12 (v/v)
III	C–EA–TA	36:55:9 (v/v)
IV	Cl–D–TA	89:6.5:4.5 (v/v)
V	C–Cl–M–TA	41:47:5.2:6.8 (v/v)
VI	C–Cl–EA–TA	51:33:8:8 (v/v)
VII	C–EA–M–TA	60:26:9:5 (v/v)
VIII	C–Cl–D–TA	39.5:44.5:10:6 (v/v)
IX	C–Cl–D–TA	45:41:10:4 (v/v)
X	Dual separation in the mobile phases V and VI	

C, cyclohexane; Cl, chloroform; D, dioxane; EA, ethyl acetate; M, methanol; TA, triethyl amine.

μm particle size) in the mobile phases containing methanol (M), water (W), 2-propanol (P) and dichloromethane (DM) in different ratios. The instrumentation used was described in our previous report [4]. The composition of HPLC mobile phases, flow-rates and polarities (P') according to Snyder and Kirkland [8] are given in Table 2.

Capillary GC separation on serially coupled non-polar CP-Sil 5 CB and moderately polar CP-Sil 19 CB columns (Chrompack, Middelburg, Netherlands) and determination of Kováts indices (KI), steroid numbers and dipole moments (μ) of equine estrogens were reported previously [3,5].

Software for both PCA and non-linear mapping calculations were provided by Dr. Barna Bordás (Plant Protection Institute of Hungarian Academy of Sciences, Budapest, Hungary). Calculations were carried out on a IBM AT computer. Nine TLC systems (variables 1–9), 10 HPLC systems (variables 10–19), the number of dissociable hydrogen atoms and double bonds in the estrogen molecules (variables 20 and 21, respectively) were taken as the variables for the observations. The limit of variance explained was set to 99%. The non-linear mapping technique was used to facilitate the evaluation of the multi-dimensional data matrices of PC loadings and PC variables. The iteration was carried out to the point when the difference between the two last iterations was lower than 10^{-8}.

Table 2
Composition of the mobile phases used for HPLC separation of equine estrogens on a Nucleosil C_{18} column

No.	Mobile phase	Composition	P'
I	M–W–P–DM	43:52:3:2 (v/v)	7.68
II	M–W–P–DM	48.1:47.6:2.8:1.5 (v/v)	7.46
III	M–W–P–DM	49.4:46.8:2.5:1.3 (v/v)	7.43
IV	M–W–P–DM	50.4:46:2.45:1.25 (v/v)	7.40
V	M–W–P–DM	50.65:45.35:2.75:1.25 (v/v)	7.35
VI	M–W–P–DM	51.7:44.45:2.7:1.15 (v/v)	7.31
VII	M–W–P–DM	52.8:44.2:2:1 (v/v)	7.31
VIII	M–W–P–DM	51.2:44.9:2.7:1.2 (v/v)	7.33
IX	M–W–P	60:38:2 (v/v)	7.01
X	M–W–P–DM	45:42.5:7.5:5 (v/v)	7.07

DM, dichloromethane; M, methanol; P, 2-propanol; W, water; P', polarity.

3. Results and discussion

For QSRR study three groups of parameters can be used [9]: additive structural parameters, physico-chemical properties and topological indices. The number of H-atoms of equine estrogens was considered as an additive structural parameter, while dipole moments and Randić connectivity indices were taken as physico-chemical properties and topological parameters of importance for the separation.

Equine estrogens are chemically closely related compounds (see Fig. 1) and that is why their HPTLC and HPLC separation was performed in mobile phases composed of three or four different solvents.

Table 3
Solvatochromic parameters of HPTLC and HPLC solvents

Solvent	$\log L^{16}$	R_2	π_2^H	$\sum\alpha_2^H$	$\sum\beta_w^H$
TA	3.040	0.101	0.15	0.00	0.79
C	2.964	0.305	0.10	0.00	0.00
EA	1.911	0.142	0.64	0.00	0.45
D	2.892	0.329	0.75	0.00	0.64
Cl	2.480	0.425	0.49	0.15	0.02
DM	2.019	0.387	0.57	0.10	0.05
M	0.970	0.278	0.44	0.43	0.47
P	1.764	0.212	0.36	0.33	0.56
W	0.260	0.000	0.45	0.82	0.35

C, cyclohexane; Cl, chloroform; D, dioxane; DM, dichloromethane; EA, ethyl acetate; M, methanol; P, 2-propanol; TA, triethyl amine; W, water; $\log L^{16}$, partition coefficient of a solvent between air and hexadecane at 290 K; R_2, solute excess molar refractivity; π_2^H, solute dipolarity–polarizability; $\sum\alpha_2^H$, solvent hydrogen bond acidity; $\sum\beta_2^H$, solvent hydrogen bond basicity.

Table 4
R_f and R_m values of equine estrogens separated by HPTLC on silica gel with mobile phases as listed in Table 1

Estrogen		Mobile phase									
		I	II	III	IV	V	VI	VII	VIII	IX	X
ES	R_f	0.64	0.626	0.733	0.72	0.845	0.36	0.57	0.556	0.55	0.7
	R_m	−0.226	−0.225	−0.533	−0.41	−0.736	−0.25	−0.127	−0.152	−0.087	−0.367
EQ	R_f	0.56	0.466	0.706	0.68	0.775	0.293	0.533	0.506	0.48	0.645
	R_m	−0.105	0.058	−0.382	−0.327	−0.536	0.382	−0.057	−0.011	0.034	−0.259
EQN	R_f	0.4	0.32	0.52	0.613	0.704	0.173	0.48	0.373	0.33	0.5
	R_m	0.176	0.327	−0.035	−0.2	−0.376	0.678	0.035	0.363	0.307	0
ESD	R_f	0.52	0.453	0.693	0.533	0.662	0.253	0.453	0.44	0.406	0.58
	R_m	−0.035	0.081	−0.354	−0.058	−0.292	0.469	0.081	0.105	0.165	−0.14
DHEQ	R_f	0.376	0.36	0.573	0.386	0.563	0.16	0.4	0.333	0.32	0.46
	R_m	0.22	0.249	−0.128	0.2	−50.11	0.72	0.176	0.301	0.327	0.069
DHEQN	R_f	0.293	0.253	0.453	0.306	0.465	0.106	0.36	0.173	0.23	0.41
	R_m	0.382	0.469	0.081	0.354	0.061	0.923	0.249	0.678	0.525	0.158

The solvatochromic parameters of solvents used, taken from the literature [10,11], are listed in Table 3.

For HPTLC separation of equine estrogens on silica gel stationary phase, cyclohexane was selected as a basic non-polar solvent, modified with ethyl acetate (strong dipolar interactions and proton–acceptor properties), triethyl amine (similar properties as ethyl acetate, but stronger proton acceptor), methanol and chloroform (strong proton donors) and dioxane (strong proton acceptor and induction interactions). R_F and R_m values of equine estrogens separated on silica gel are listed in Table 4. A graphical presentation of relationship between the number of H-atoms and R_m values in the mobile phases I, V and X is shown in Fig. 2. Similar results were obtained with other mobile phases used. The correlation coefficient of the dependence between the number of H-atoms of the keto-steroids and the corresponding diols, and the R_m values in the 10 TLC mobile phases varied between −0.97 and −0.99.

RP-HPLC separation of equine estrogens on C_{18} stationary phase was performed in the mobile phases

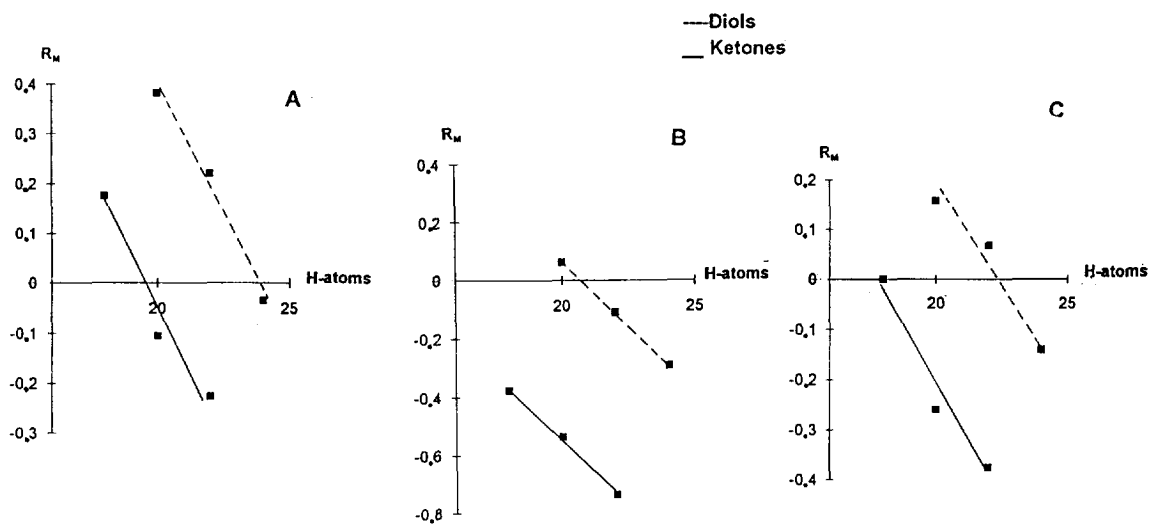

Fig. 2. Relationship between the number of H-atoms and R_m values of equine estrogens separated on silica gel 60 F_{254} HPTLC plates with I (A), V (B) and X (C) as the mobile phase.

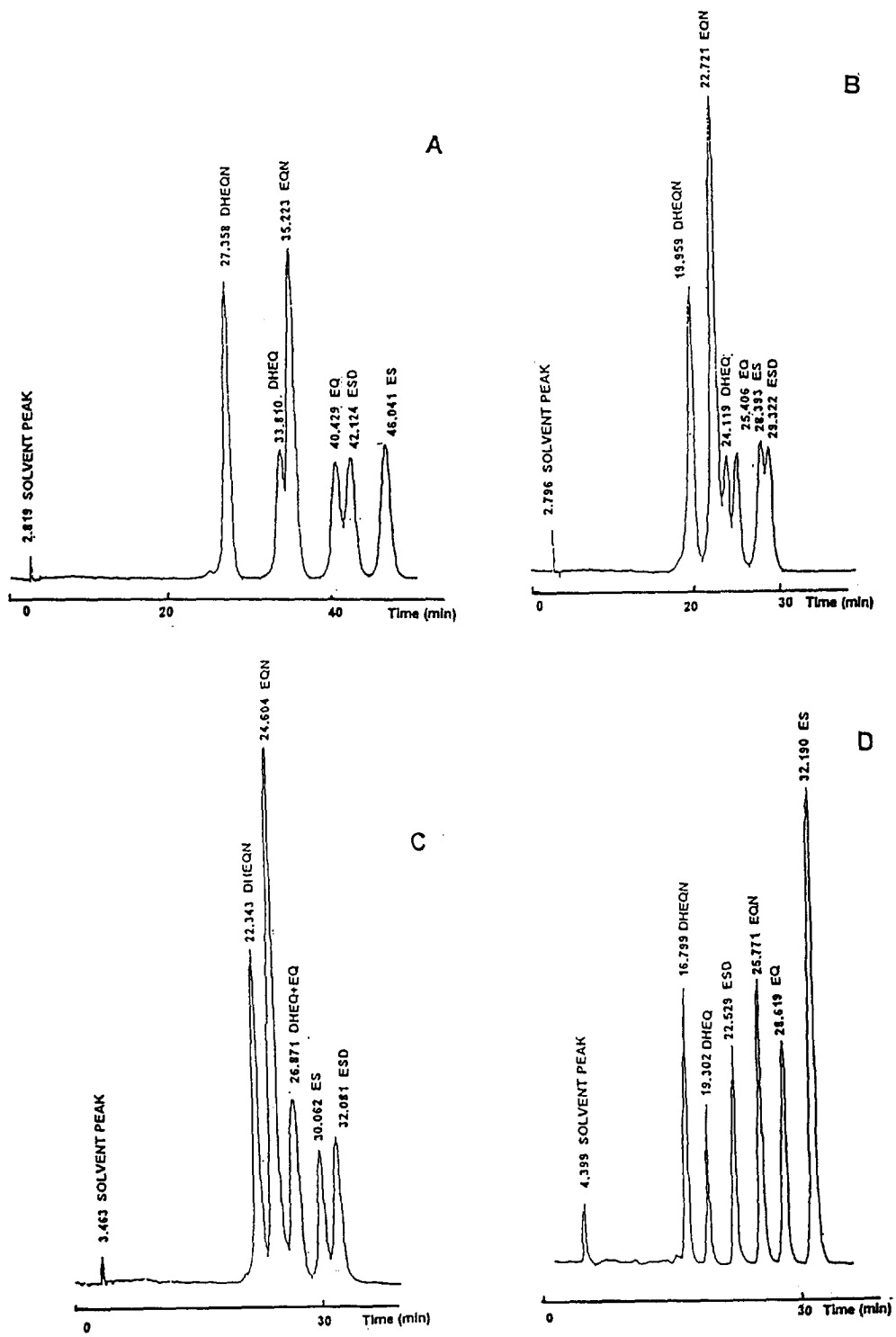

Fig. 3. RP-HPLC chromatograms of equine estrogens separated on a Nucleosil C_{18} column with the mobile phase I (A), II (B), VII (C) and X (D). UV absorbance detection at 280 nm; loop injection (20 μl).

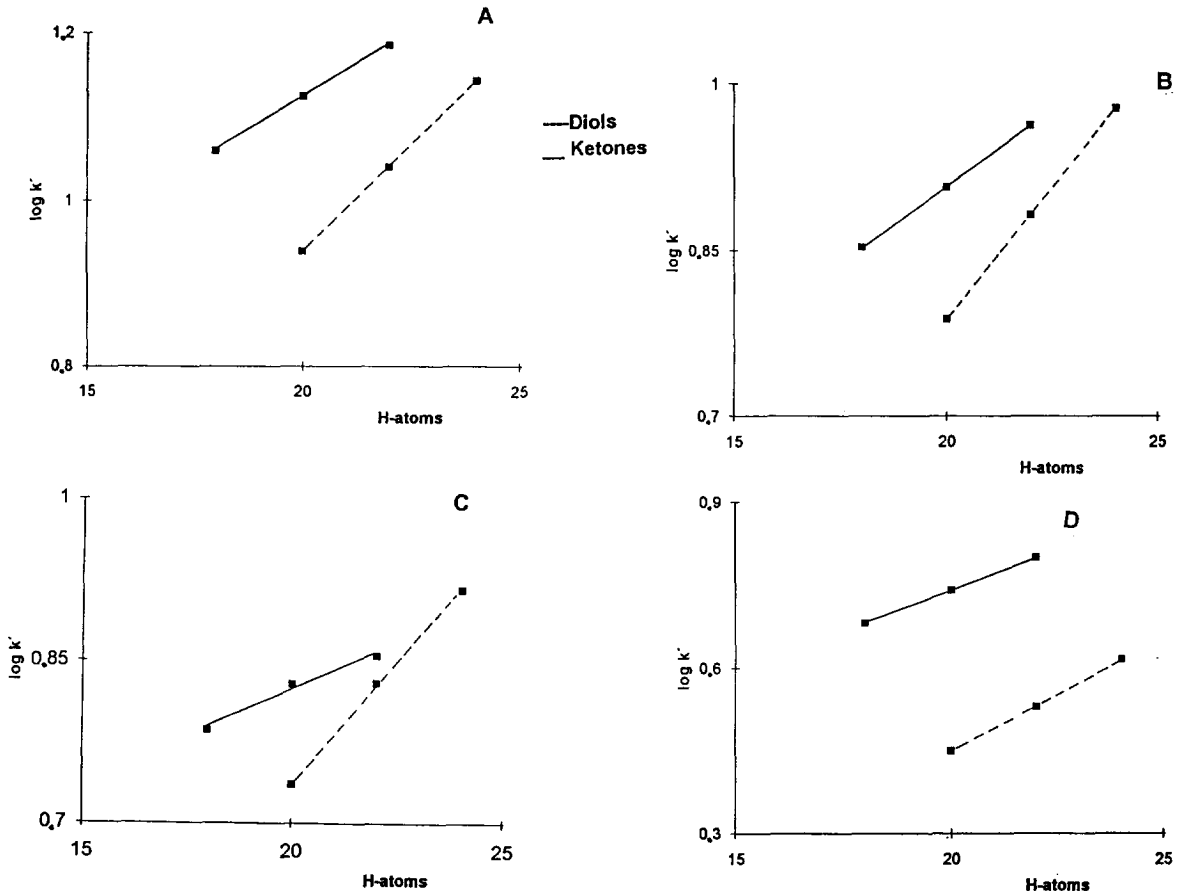

Fig. 4. Relationship between the number of H-atoms and log k' of equine estrogens separated by HPLC with the mobile phase I (A), II (B), VII (C) and X (D).

composed of water as a basic component, modified by methanol (strong proton donor with important dipolar interactions), 2-propanol (proton–acceptor interactions) and dichloromethane (strong proton donor).

The log k' values of equine estrogens separated in 10 different HPLC mobile phases are given in Table 5. Solvent (M–W, 1:1, v/v) retention was taken as column hold-up time. Chromatograms obtained in the mobile phases I, II, VII and X are presented in

Table 5
log k' of equine estrogens obtained by HPLC separation with mobile phases I–X

Estrogen	log k'									
	I	II	III	IV	V	VI	VII	VIII	IX	X
EQN	1.06	0.853	0.841	0.806	0.831	0.763	0.786	0.745	0.483	0.681
EQ	1.125	0.907	0.894	0.868	0.874	0.814	0.829	0.806	0.527	0.741
ES	1.186	0.962	0.942	0.909	0.927	0.863	0.885	0.856	0.57	0.8
DHEQN	0.939	0.788	0.784	0.751	0.783	0.706	0.736	0.707	0.443	0.449
DHEQ	1.041	0.882	0.877	0.843	0.874	0.797	0.83	0.788	0.527	0.53
ESD	1.144	0.977	0.971	0.936	0.957	0.887	0.917	0.879	0.607	0.615

Table 6
GC retention indices, dipole moments, Randić connectivity indices and relative biological activities of equine estrogens

Estrogen	KI/100	$^1\chi$	$1/^1\chi$	μ^2	$1/\mu^2$	BA[a]
ES	27.36	7.5833	0.1386	5.43	0.184	0.4
EQ	27.45	7.2825	0.1373	7.29	0.137	2.0
EQN	28.37	7.0108	0.1426	4.12	0.243	[b]
ESD	27.25	7.735	0.12928	2.56	0.391	0.06
DHEQ	25.51	7.435	0.13449	4.08	0.245	0.12
DHEQN	28.25	7.1641	0.13958	1.96	0.510	0.0

[a] Biological activity of conjugated equine estrogens according to Ref. [14].
[b] Data not given.

Fig. 3. Figs. 4A–D show the linear relationship between the number of H-atoms of keto-estrogens and the corresponding diols, and log k' in the mobile phases I, II, VII and X, respectively. The correlation coefficients in 10 different mobile phases are between 0.999 and 1.000, both for the ketones and diols.

The retention behavior of equine estrogens in 19 chromatographic systems (9 TLC and 10 HPLC mobile phases) forms a multi-dimensional space determined by an unknown number of independent variables. A PCA method of the multi-dimensional space reduction was used to analyze the data. Along a new, mathematically defined axis (principle components), not identical with original independent variables, are significantly oriented retention data. Thus this new axis, a principle component, represents a new hypothetical independent variable (eigen vector). If the original results are described by means of two principle components (the sum of the eigen vector values is close to 100%, the 1st eigen value is 70.96%, the 2nd eigen value is 26.87%), which means that two theoretical chromatographic systems are sufficient to describe the retention behavior of the 19 chromatographic systems. TLC and HPLC retention data form two distinct clusters within this space (Fig. 5). Point 20, representing the number of dissociable H-atoms in the steroid molecules, is close to the retention data which indicates that this molecular parameter exerts a considerable influence on the separation (see also Figs. 2 and 4). This point is nearer to the cluster of TLC systems, suggesting that proton donor–proton acceptor properties of solutes have a higher effect on the retention in adsorption TLC than in HPLC. Point 21, representing the number of π electrons in the ring A and B of the estrogen structure, is further from the clusters, indicating that the $\pi-\pi$ interactions do not play a predominant role in the separation, but certainly have an influence on the proton donor activity of the OH group in position 3.

The two-dimensional non-linear map of PC variables entirely supports our previous conclusion (Fig.

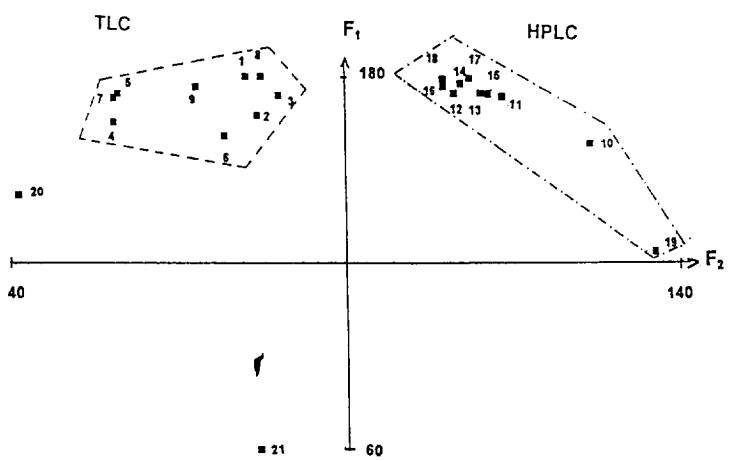

Fig. 5. Similarities and dissimilarities between the physico-chemical parameters and retention characteristics of equine estrogens. Shown is a two-dimensional non-linear map of principal component loadings. Number of iterations, 238; maximum error, 3.23×10^{-3}. Symbol identification: 1–9, nine TLC systems; 10–19, 10 HPLC systems; 20, number of dissociable H-atoms; 21, number of double bonds in rings A and B of the estrogens.

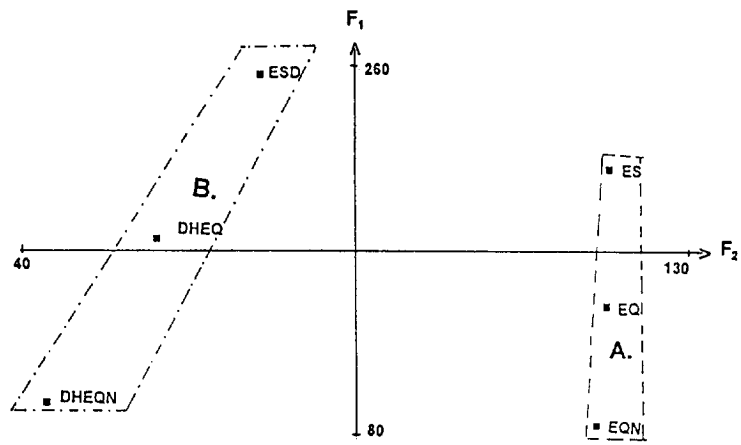

Fig. 6. Similarities and dissimilarities between keto-estrogens and the corresponding diols. Shown is a two-dimensional non-linear map of principal component variables. Number of iterations, 17; maximum error, 6.87×10^{-5}.

6). ESD, DHEQ and DHEQN have a dissociable H-atom in position 17 and form a separate cluster, providing again the marked role of the proton donor–proton acceptor interactions in the retention.

A multiparametral approach to QSRR study given by Gassiot-Matas and Firpo-Pamies [12] was expressed by the equation $I=a^{1}\chi+b\mu^{2}+c$, where I is a GC retention index, a, b and c are constants, $^{1}\chi$ is the Randić connectivity index [13] and μ is the dipole moment. The values of interest for this study are summarized in Table 6.

The relationship between GC retention, $^{1}\chi$ and μ^{2} of equine estrogens obtained by multiparametral regression analysis is expressed by the equation:

$$\mathrm{KI}/100 = a/^{1}\chi + b/\mu^{2} + c$$

where a is 100.8 (standard error 20.55), b is 2.57 (standard error 0.68), c is 13.2 (standard error 2.85) and the multiple regression coefficient is 0.9571. The relative biological activity of equine estrogens is related to $\log 1/\mu^{2}$, as shown in Fig. 7.

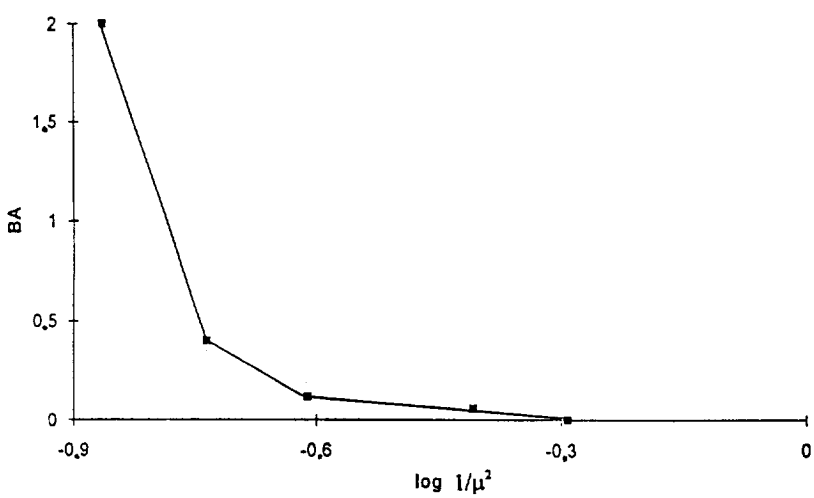

Fig. 7. Relationship between $\log(1/\mu^{2})$ and the relative biological activity (BA) of equine estrogens according to Ref. [14].

4. Conclusions

The experimental results of TLC and HPLC of equine estrogens indicate the important role of H-atoms for the separation. This observation is supported by PCA and the non-linear mapping technique, and the results confirm that proton donor–proton acceptor properties of the solutes play the most important role in the TLC and HPLC separation. GC retention of the estrogens is related to their connectivity indices and dipole moments by the equation: $KI/100=a^{1}\chi+b/\mu^{2}+c$. The relative biological activity is related to $\log 1/\mu^{2}$.

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