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Determination of endocrine disrupting compounds using temperature-dependent inclusion chromatography I. Optimization of separation protocol

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ABSTRACT

In the present work we optimised the separation of battery of key UV non-transparent low-molecular-mass compounds having possible endocrine disrupting compounds (EDCs) activity or which may be used as the endocrine effect biomarkers. Simple optimization strategy was based on strong temperature effect that is driven by electrostatic interactions between macrocyclic mobile phase additives like cyclodextrins and eluted components of interest under C18 stationary phase and acetonitrile/water mobile phase conditions. Particularly, the effect of temperature involving native β-cyclodextrin and its hydroxypropyl derivative to improve separation of number of natural (*d*-equilenin, equilin, estetrol, estriol, estrone, 17β-estradiol, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, cortisol, cortisone, progesterone, testosterone, tetrahydrocortisol and tetrahydrocortisone) and artificial steroids (ethynylestradiol, norgestrel isomers, medroxyprogesterone, mestranol, methyltestosterone, norethindrone, 17α-estradiol) as well as non-steroidal compounds (diethylstilbesterol, bisphenol A, 4-*tert*-butylphenol, dimethyl phthalate, dibutyl phthalate and dioctyl phthalate) was investigated. It has been found that successful isocratic separation of 27 chemicals can be achieved using acetonitrile/water eluents modified with β-cyclodextrin or hydroxypropyl-β-cyclodextrin at concentration of 10 mM and temperature of 47 °C. Separation protocol is simple, reliable, direct and non-radioactive and may be easily adapted for rapid separation and quantification of wide range of given steroids and related EDCs in environmental samples, particularly those that are characterised by unstable biological matrix and components of interest load.

1. Introduction

Quantification of endocrine disrupting compounds (EDCs), particularly steroid hormones, in biological and environmental samples presents unique challenges for sample pre-purification, extraction, concentration and separation. In general, there are biological and physicochemical methodologies and the choice of method for determination of EDCs depends on the required outcome [1,2]. Biological methods are primarily used to determine the estrogenic potency and activity of compound, mixture or sample, whilst physicochemical techniques can qualify and quantify known compounds as well as identify unknown substances. In case of physicochemical methods, the complexity and wide range of components of interest levels in relation to interfering matrix of typical environmental samples often requires the use of advance separation techniques. From chromatographic point of view, steroids and related low-molecular-mass substances form a non-homogeneous group of solutes and therefore, many problems with extraction efficiency and good separation of multicomponent mixtures using common separation techniques were observed [3,4]. Therefore, the simultaneous measurement of the multiple forms of steroids like compounds in complex materials is still an unsolved analytical problem.

Most of the existing chromatographic protocols that are designed for screening of steroids like compounds in environmental samples are based on gas chromatography–mass spectrometry systems (GC–MS) [5–10]. However, direct quantification of such substances using GC–MS technique is strongly limited to volatility of components of interest. From that point of view chromatography based on liquid mobile phase including column chromatography (HPLC), planar chromatography (TLC), supercritical fluid chromatography (SFC) or capillary electrophoresis (CE) allows direct quantification of wide range of steroids despite of their volatility. Moreover, a LC chromatograph equipped with relatively simple and non-expensive diode-array UV–vis detector is capable for screening of wide range of steroids and pollutants that are present in environmental samples [2,3].

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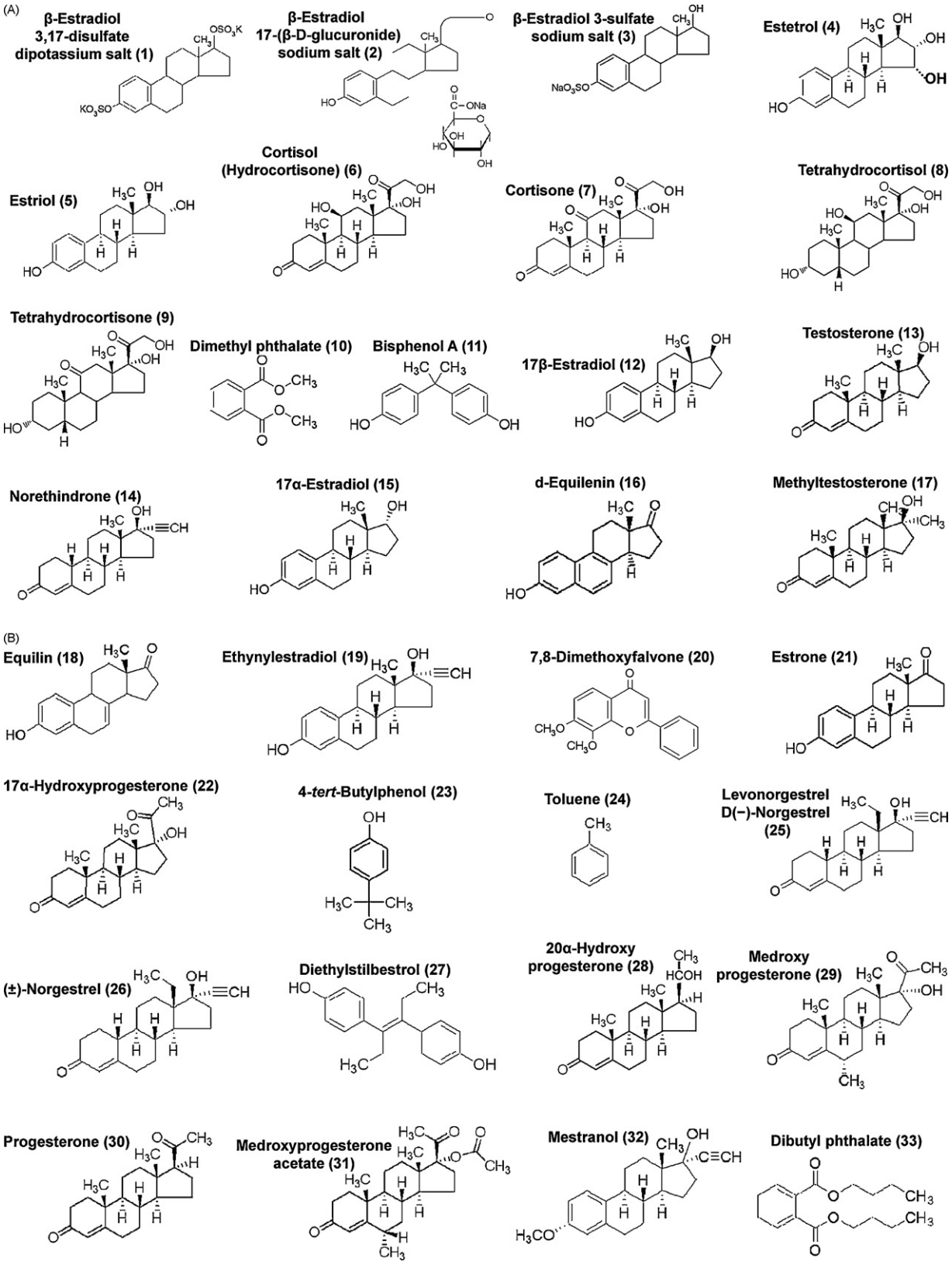

Fig. 1. Chemical structures of components of interest investigated.

In contrary to typical LC protocols that is based on gradient separation involving water (or buffer)–organic liquid mixtures, efficient separation of multicomponent samples consisted of low-molecular-mass analytes can be also achieved by a simple one-step isocratic (non-gradient) process. Isocratic method can be based on liquid chromatography driven by electrostatic interactions between macrocyclic additives like cyclodextrins (CDs) to the liquid mobile phase and eluted components of interest [11–13]. It has been

Table 1
Elution order and retention times of all components of interest chromatographed using mobile phase without macrocyclic additives (acetonitrile/water, 35:65, v/v) and 10 cm long C18 column (supelcosil LC-18, 5 μm). Remaining chromatographic conditions: separation temperature 20 °C; flow rate 1 mL/min; solutes numbers correspond to the elution order (Fig. 2) and chemical structures presented on Fig. 1A and B.

Analyte	Peak numbers	Retention time (min)	Analyte	Peak numbers	Retention time (min)	Analyte	Peak numbers	Retention time (min)
β-Estradiol 3,17-disulfate	1	0.70	17β-Estradiol	12	7.94	4-*tert*-Butyl-phenol	23	14.78
β-Estradiol 17-(β-D-glucuronide)	2	0.74	Testosterone	13	8.44	Toluene	24	16.40
17β-Estradiol 3-sulfate	3	0.76	Norethindrone	14	9.51	Levo-norgestrel	25	17.64
Estetrol	4	1.53	17α-Estradiol	15	9.73	Norgestrel	26	17.64
Estriol	5	1.96	D-Equilenin	16	10.79	Diethyl-stilbesterol	27	22.63
Cortisol	6	2.39	Methyl-testosterone	17	11.07	20α-Hydroxy-progesterone	28	23.03
Cortisone	7	2.60	Equilin	18	11.90	Medroxy-progesterone	29	23.69
Tetrahydro-cortisol	8	2.88	Ethynyl-estradiol	19	12.00	Progesterone	30	44.11
Tetrahydro-cortisone	9	3.45	7,8-Dimethoxy-flavone	20	12.42	Medroxy-progesterone acetate	31	56.48
Dimethyl phthalate	10	4.83	Estrone	21	13.08	Mestranol	32	94.53
Bisphenol A	11	7.78	17α-Hydroxy-progesterone	22	13.56	Dibutyl phthalate	33	185.20

proved that such interaction is extremely temperature sensitive and therefore, liquid chromatography separation can be simply and very efficiently optimised using narrow range of temperature within sub-ambient and elevated areas [14–23]. It is noteworthy, that under particular conditions the association constant of host–guest complexes involving native cyclodextrins can change more than 200% per 1 °C [24]. The experimental data indicate that retention of inclusion complexes can be varied between two lines formed by the Van't Hoff plot of the cyclodextrin and the Van't Hoff plot of uncomplexed solute [25]. If the retention of macrocyclic additive is considerably lower than the retention of solutes chromatographed then the temperature can produce a massive change in separation power of the chromatographic system [25,26]. In low temperatures, the inclusion modifier action is more efficient due to high values of the binding constant of the complexes created and large differences in retention of free macrocycle and uncomplexed solutes. Therefore, in contrary to classical chromatographic systems without inclusion modifiers, the analysis time is shorter at low temperatures. Small change in the temperature of the mobile phase produce substantial differences in the separation power of a chromatographic system modified with inclusion agents. Particularly, the total analysis time of multicomponent mixture of low-molecular-mass compounds with a wide range of polarities can be reduced to a few minutes instead of hours in case of classical chromatographic systems [3,26]. This makes the procedure attractive for processing of number of low-molecular-mass compounds isolated from the complex environmental samples.

In our present work a simple optimization strategy for isocratic HPLC separation of multicomponent mixtures composed of UV non-transparent low-molecular-mass compounds having possible EDC activity is presented. Particularly, this work was focused on simultaneous separation of number of natural (*d*-equilenin, equilin, estetrol, estriol, estrone, 17β-estradiol, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, cortisol, cortisone, progesterone, testosterone, tetrahydrocortisol and tetrahydrocortisone) and artificial steroids (ethynylestradiol, norgestrel isomers, medroxyprogesterone, mestranol, methyltestosterone, norethindrone, 17α-estradiol) as well as non-steroidal compounds (diethylstilbesterol, bisphenol A, 4-*tert*-butylphenol, dimethyl phthalate, dibutyl phthalate and dioctyl phthalate) that may be considered as the environmental biomarkers for the endocrine disrupting phenomenon. Especially, the effect of temperature on separation efficiency and capability of temperature driven HPLC system for quantification of complex mixtures is discussed from practical point of view.

2. Experimental

2.1. Chemicals and reagents

Analytical standards of steroids and low-molecular-mass compounds (Fig. 1A and B) were obtained from Steraloids (London, UK) including estetrol, 20α-hydroxyprogesterone, cortisol, cortisone, ethynylestradiol, norgestrel isomers, medroxyprogesterone, mestranol, norethindrone and diethylstilbesterol. Equilin, *d*-equilenin, 17β-estradiol, 17α-hydroxyprogesterone, bisphenol A, 4-*tert*-butylphenol, dimethyl phthalate, dibutyl phthalate, dioctyl phthalate, 17β-estradiol 3-sulfate sodium salt, β-estradiol 3,17-disulfate dipotassium salt, β-estradiol 17-(β-D-glucuronide) sodium salt and 7,8-dimethoxyflavone (internal standard) were product of Sigma (St. Louis, MO, USA) whilst estriol, estrone, 17α-estradiol were obtained of Aldrich (Milwaukee, WI, USA). Testosterones (testosterone, methyltestosterone) were purchased from Polfa (Jelenia Góra, Poland), tetrahydrocortisol and tetrahydrocortisone were product of Koch-Light Labs. (Colnbrook,

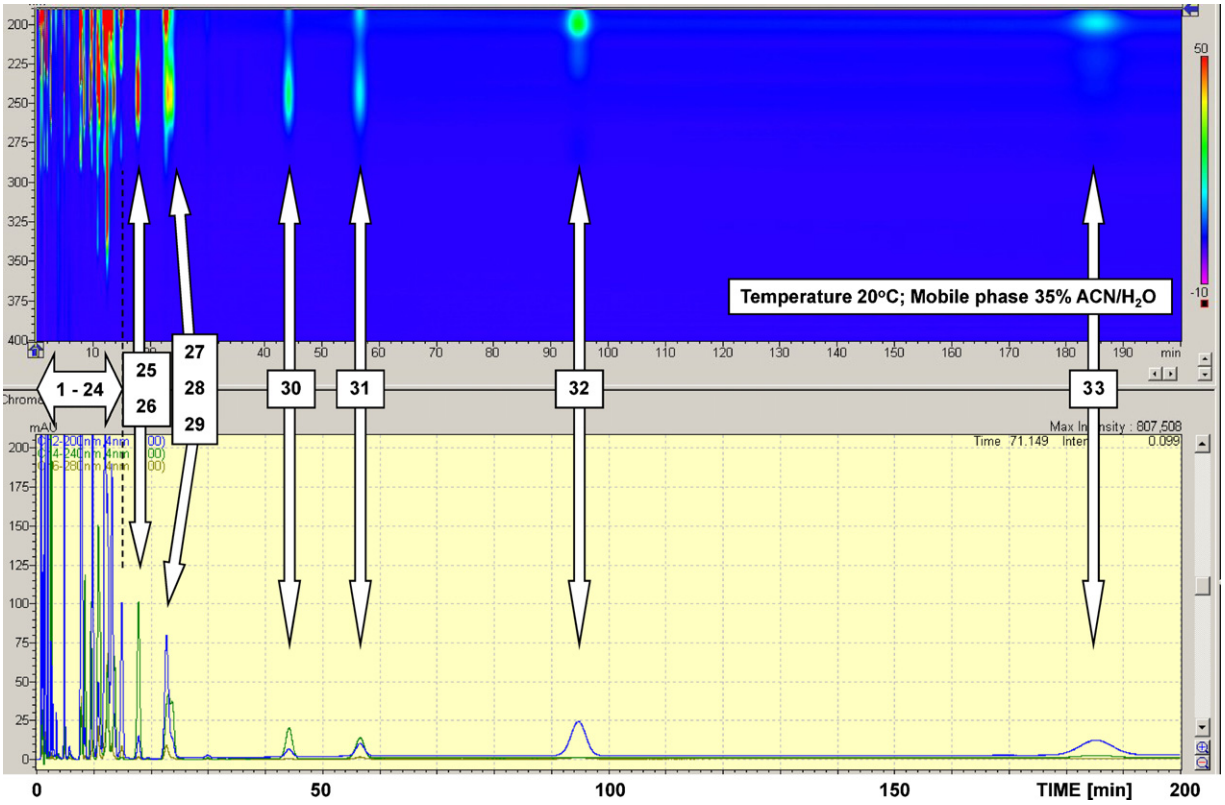

Fig. 2. Initial isocratic separation of all substances studied using mobile phase without macrocyclic additives. Peak numbers correspond to substances presented in Fig. 1A and B and Table 1. Chromatographic conditions: temperature 20 °C; 10 cm long C18 column (Supelcosil LC-18, 5 μm); mobile phase acetonitrile:water (35:65, v/v) without cyclodextrin; flow rate 1 mL/min.

UK) and progesterone was obtained from Merck (Darmstadt, Germany).

Mobile phase macrocyclic modifiers: β-cyclodextrin and its hydroxypropyl derivative were product of Fluka (Buchs, Switzerland). Organic liquids including methanol, ethanol and acetonitrile were obtained from Merck and used as received without further purification (LiChrosolv; HPLC grade). Double distilled water was used for binary mobile phase preparation.

2.2. High-performance liquid chromatography

Chromatographic experiments were performed using two columns: 10 cm and 25 cm length (Supelcosil LC-18, I.D. = 4.6 mm, 5 μm). Mobile phases were composed of acetonitrile/water (35:65, v/v) without and with addition of β-cyclodextrin and hydroxypropyl-β-cyclodextrin at level of 10 mM. Mobile phase flow rate was set at 1 mL/min. Column temperature ranging from 0 °C to 60 °C was controlled by foam insulated water jacket connected to circulating thermostat (Nestlab RTE7; product of Thermo Electron Corporation, Newington, NH, USA). HPLC system consisting of isocratic pump (LC-10ADvp), injector (Rheodyne 7725i, Rohner Park, CA, USA) with 20 μL loop, a SPD-M20A photodiode array detection (DAD) system and a computer system for data acquisition with software LC Solution (version 1.21 SP1; 2002–2005) was product of Shimadzu (Suzhou New District, Jiangsu, China). Analytical columns dead times were measured using $NaNO_3$ marker (POCh, Gliwice, Poland). Stock solutions of chromatographic standards were prepared in methanol at concentration of 1 mg/mL. Appropriate injecting solutions at concentration of 50 μg/mL were prepared in mobile phase (acetonitrile/water, 35:65, v/v) without macrocyclic additives.

3. Results and discussion

Present work was focused on separation of number of key natural and artificial steroids as well as low-molecular-mass non-steroidal compounds that may be considered as the environmental biomarkers of the endocrine disrupting phenomenon. Chemical structures of components of interest selected on that basis were presented on Fig. 1A and B. Initial measurement of the retention properties of all analytes was made using short C18 column (10 cm) and binary mobile phase acetonitrile/water (35:65, v/v). Chromatographic experiment was performed at temperature of 20 °C. Data in Table 1 clearly indicate that under such conditions complete elution of all solutes needs more than three hour of analysis. Moreover, a simultaneous quantitative determination can be strongly limited due to number of non separated peaks and highly unsymmetrical spread of the analytes peaks along the time axis (Fig. 2). Further selection of 27 target substances was based on the retention data observed. Particularly, fast eluting solutes (estradiol conjugates) with retention time close to dead time marker as well as strongly retarded compounds, which were eluted after progesterone (medroxyprogesterone acetate, mestranol and dibutyl phthalate) were omitted for further investigations. Moreover, preliminary separation experiment revealed that retention of medroxyprogesterone acetate, mestranol and dibutyl phthalate cannot be shortened using β-cyclodextrin mobile phase additive under acetonitrile/water and C18 stationary phase conditions. Final analytes selection was also based on the compounds polarity affecting fractionation using solid-phase extraction, which was described previously [16].

Results of temperature studies on the analytes retention using mobile phases unmodified and modified with 10 mM of β-

Table 2
Retention factor (k) data of 27 selected analytes measured on a C18 column (10 cm) at different temperatures using an acetonitrile/water (35:65, v/v) mobile phase without cyclodextrin (A) as well as modified with native β-cyclodextrin (B) and its hydroxypropyl derivative (C) at concentration of 10 mM; solutes number correspond to the elution order (Fig. 2) and chemical structures presented on Fig. 1A and B.

Analyte	Separation temperature (°C)				
	0	10	20	40	60
A: Mobile phase without cyclodextrin					
Estetrol (4)	1.06	1.02	1.00	0.96	0.92
Estriol (5)	1.71	1.64	1.59	1.48	1.36
Cortisol (6)	1.89	2.01	2.11	2.17	2.07
Cortisone (7)	2.44	2.42	2.40	2.28	2.10
Tetrahydrocortisol (8)	2.10	2.45	2.72	2.98	2.93
Tetrahydrocortisone (9)	2.92	3.25	3.46	3.54	3.32
Dimethyl phthalate (10)	6.96	6.07	5.41	4.38	3.58
Bisphenol A (11)	12.71	10.72	9.12	6.62	4.83
17β-Estradiol (12)	11.21	10.22	9.38	7.76	6.25
Testosterone (13)	10.53	10.31	10.04	9.11	7.89
Norethindrone (14)	13.16	12.25	11.42	9.66	7.95
17α-Estradiol (15)	13.67	12.70	11.74	9.67	7.70
d-Equilenin (16)	18.92	15.56	13.12	9.44	6.85
Metyltestosterone (17)	13.59	13.65	13.46	12.50	10.89
Equilin (18)	20.37	17.08	14.57	10.64	7.81
Ethynylestradiol (19)	19.28	16.79	14.72	11.21	8.41
7,8-Dimethoxyflavone (20)	18.58	16.68	15.20	12.41	9.86
Estrone (21)	22.00	18.74	16.16	11.96	8.77
17α-Hydroxyprogesterone (22)	19.12	17.85	16.66	14.05	11.33
4-*tert*-Butylphenol (23)	23.77	21.02	18.37	13.66	10.02
Toluene (24)	26.67	23.46	20.38	15.31	11.44
Levonorgestrel (25)	25.84	24.08	22.20	18.24	14.41
Norgestrel (26)	25.92	24.13	22.24	18.24	14.42
Diethylstilbesterol (27)	42.72	34.99	28.65	18.95	12.56
20α-Hydroxyprogesterone (28)	33.16	31.17	29.30	24.71	19.89
Medroxyprogesterone (29)	34.37	32.13	29.68	24.41	18.97
Progesterone (30)	76.45	65.35	56.62	42.00	30.51
B: Mobile phase with β-cyclodextrin additive					
Estetrol (4)	0.63	0.73	0.74	0.79	0.82
Estriol (5)	0.67	0.83	0.87	1.06	1.14
Cortisol (6)	1.07	1.21	1.41	1.71	1.81
Cortisone (7)	1.45	1.63	1.78	1.94	1.92
Tetrahydrocortisol (8)	0.57	0.86	1.18	1.83	2.25
Tetrahydrocortisone (9)	1.06	1.36	1.76	2.43	2.72
Dimethyl phthalate (10)	7.12	6.21	5.53	4.47	3.66
Bisphenol A (11)	2.85	2.98	3.12	3.27	3.13
17β-Estradiol (12)	1.03	1.48	2.10	3.58	4.38
Testosterone (13)	0.61	0.89	1.39	3.03	4.66
Norethindrone (14)	8.74	8.89	8.92	8.43	7.42
17α-Estradiol (15)	3.80	4.50	5.18	6.06	6.01
d-Equilenin (16)	9.94	9.68	9.26	7.85	6.23
Metyltestosterone (17)	3.72	4.98	6.27	8.19	8.70
Equilin (18)	5.32	6.05	6.67	6.96	6.22
Ethynylestradiol (19)	7.90	8.22	8.40	8.15	7.13
7,8-Dimethoxyflavone (20)	19.52	17.51	15.85	12.84	10.11
Estrone (21)	3.59	4.72	5.94	7.52	7.16
17α-Hydroxyprogesterone (22)	7.33	8.48	9.46	10.21	9.54
4-*tert*-Butylphenol (23)	4.62	5.03	5.37	5.80	5.74
Toluene (24)	22.66	20.67	18.39	14.31	11.00
Levonorgestrel (25)	18.28	18.55	18.25	16.43	13.75
Norgestrel (26)	18.05	18.40	18.15	16.41	13.74
Diethylstilbesterol (27)	1.85	2.77	4.21	8.30	9.78
20α-Hydroxyprogesterone (28)	2.45	3.94	6.27	11.72	14.12
Medroxyprogesterone (29)	22.40	23.76	24.06	22.16	18.32
Progesterone (30)	17.12	20.98	24.71	27.56	24.67
C: Mobile phase with hydroxypropyl-β-cyclodextrin additive					
Estetrol (4)	0.67	0.78	0.76	0.79	0.80
Estriol (5)	0.91	0.92	1.00	1.10	1.12
Cortisol (6)	1.19	1.37	1.54	1.75	1.80
Cortisone (7)	1.58	1.71	1.82	1.91	1.86
Tetrahydrocortisol (8)	0.97	1.15	1.49	2.06	2.34
Tetrahydrocortisone (9)	1.30	1.70	2.09	2.64	2.79
Dimethyl phthalate (10)	6.94	6.04	5.36	4.35	3.55
Bisphenol A (11)	2.60	2.71	2.85	3.03	2.96
17β-Estradiol (12)	3.92	4.13	4.35	4.58	4.42
Testosterone (13)	4.00	4.44	4.87	5.48	5.58
Norethindrone (14)	8.95	8.76	8.59	7.94	6.93
17α-Estradiol (15)	5.18	5.52	5.83	6.04	5.67
d-Equilenin (16)	10.85	9.67	8.78	7.22	5.74
Metyltestosterone (17)	5.38	6.11	6.77	7.70	7.87
Equilin (18)	8.77	8.14	7.73	6.83	5.80
Ethynylestradiol (19)	7.92	7.88	7.80	7.41	6.49
7,8-Dimethoxyflavone (20)	18.41	16.48	14.97	12.19	9.67
Estrone (21)	11.69	10.76	10.02	8.55	7.01
17α-Hydroxyprogesterone (22)	8.90	9.33	9.67	9.70	8.87
4-*tert*-Butylphenol (23)	4.34	4.69	5.00	5.43	5.41
Toluene (24)	22.46	20.12	17.85	13.79	10.55
Levonorgestrel (25)	19.09	18.50	17.64	15.46	12.82
Norgestrel (26)	19.09	18.47	17.62	15.47	12.81
Diethylstilbesterol (27)	15.13	14.19	13.41	11.58	9.31
20α-Hydroxyprogesterone (28)	8.40	9.26	10.16	11.69	12.11
Medroxyprogesterone (29)	20.41	21.21	21.38	19.90	16.71
Progesterone (30)	31.08	29.37	28.14	25.38	21.63

cyclodextrin and its hydroxypropyl derivative are presented within Table 2 and typical retention profiles of selected analytes (Van't Hoff plots) are presented in Fig. 3. To compare the intensity of host–guest interaction for both cyclodextrins investigated, the retention factor ratios ($k_{0\,mM\,CD}/k_{10\,mM\,CD}$) values were calculated (Table 3). This ratio is directly related to the number of moles of the solute that is present in the mobile phase, and can be used as the parameter simply reflecting the intensity of the host–guest interaction, occurring in chromatographic systems involving liquid mobile phases. In general, the values of the retention factor ratios presented in Table 3 indicate that native β-cyclodextrin interact more efficiently at low temperature region with the analytes investigated than do its hydroxypropyl counterpart. Weak supramolecular interaction observed for small (toluene) and less compact structures (dimethyl phthalate or 7,8-dimethoxyflavone) can be explained by the macrocycles cavity size and relatively strong competitive interaction between cyclodextrin and acetonitrile molecules that can exist in organic-solvent based systems [26,27]. Therefore, such compounds cannot enter the internal cavity of the macrocycles and form stable host–guest complex, which subsequently may affect analytes retention. Significant complex formation in the sub-ambient temperature region was observed for 17β-estradiol, testosterone, diethylstilbestrol, 20α-hydroxyprogesterone, bisphenol A, estrone, 4-*tert*-butylphenol, and progesterone. In such a case the observed retention factor ratios exceed 4. It should be noted that wide range of k ratios values within relatively narrow temperature range allows efficient optimization of multicomponent mixture separation. Moreover, high k ratio value for strong retarded steroid such progesterone allows significant reduction of total analysis time. Interestingly, it has been found that at high temperature region significant host–guest interaction may still exist, particularly for 4-*tert*-butylphenol, testosterone, methyltestosterone, diethylstilbestrol, bisphenol A, 17β-estradiol, and 20α-hydroxyprogesterone, regardless on the macrocycle type applied. It should be noted that advantage of mobile phase modified with β-cyclodextrin is enantioselectivity for norgestrel enantiomers. On the other hand hydroxypropyl-β-cyclodextrin and host–guest complexes involving this macrocycle are more soluble in acetonitrile/water mobile phase. Therefore, possible analytes precipitation on the column or within detector cell in case of low temperature chromatographic run and/or sample consisting of high matrix load (e.g. untreated waste water fraction) can be avoided.

Our data clearly demonstrate that temperature can be considered as the critical parameter for HPLC selectivity of investigated compounds. From practical point of view strong deviations from regular Van't Hoff plots of analytes investigated, which were

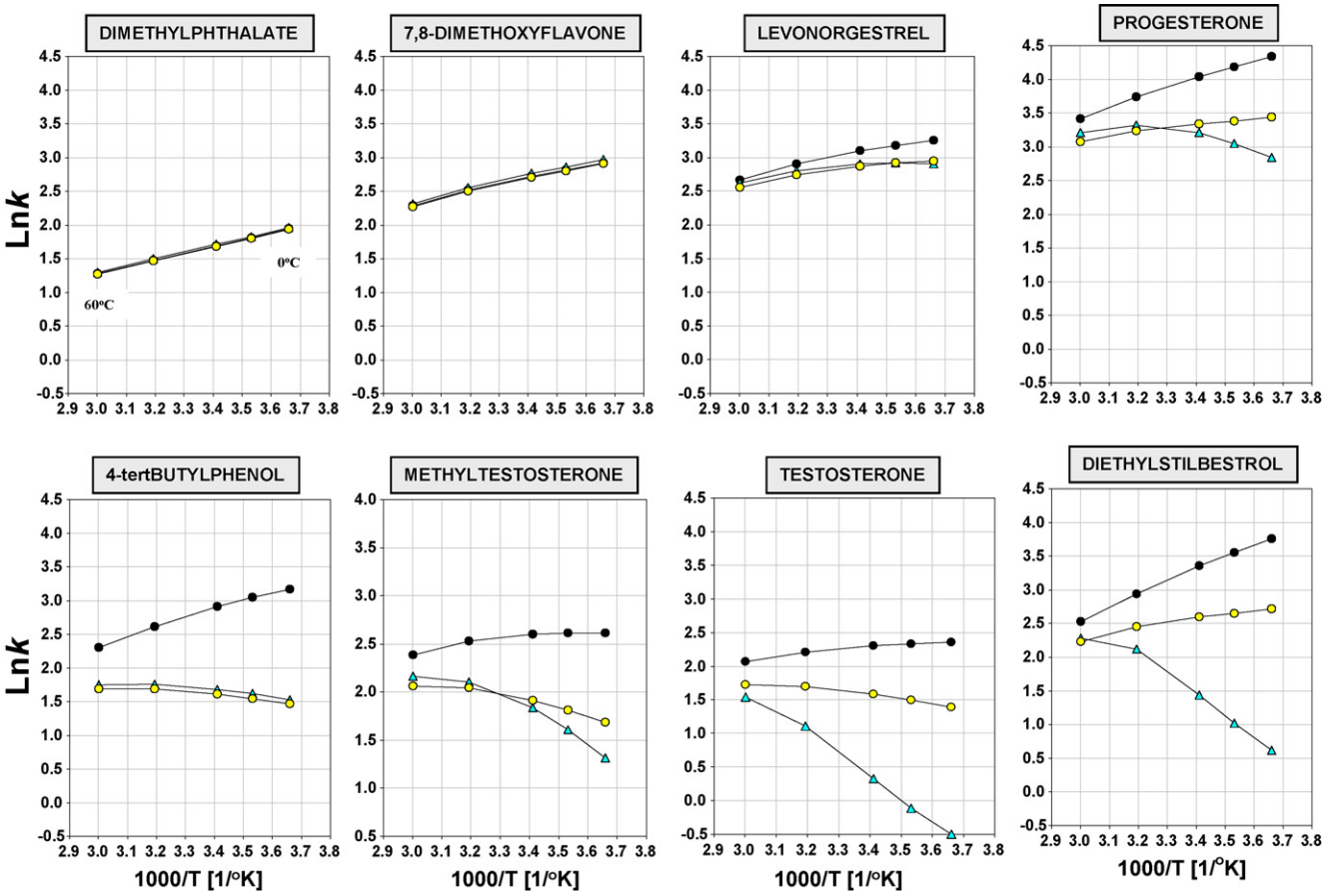

Fig. 3. Van't Hoff plots for selected analytes observed using mobile phases without cyclodextrin (black dots) as well with β-cyclodextrin (triangles) and hydroxypropyl-β-cyclodextrin (circles) at temperatures ranging from 0 °C to 60 °C.

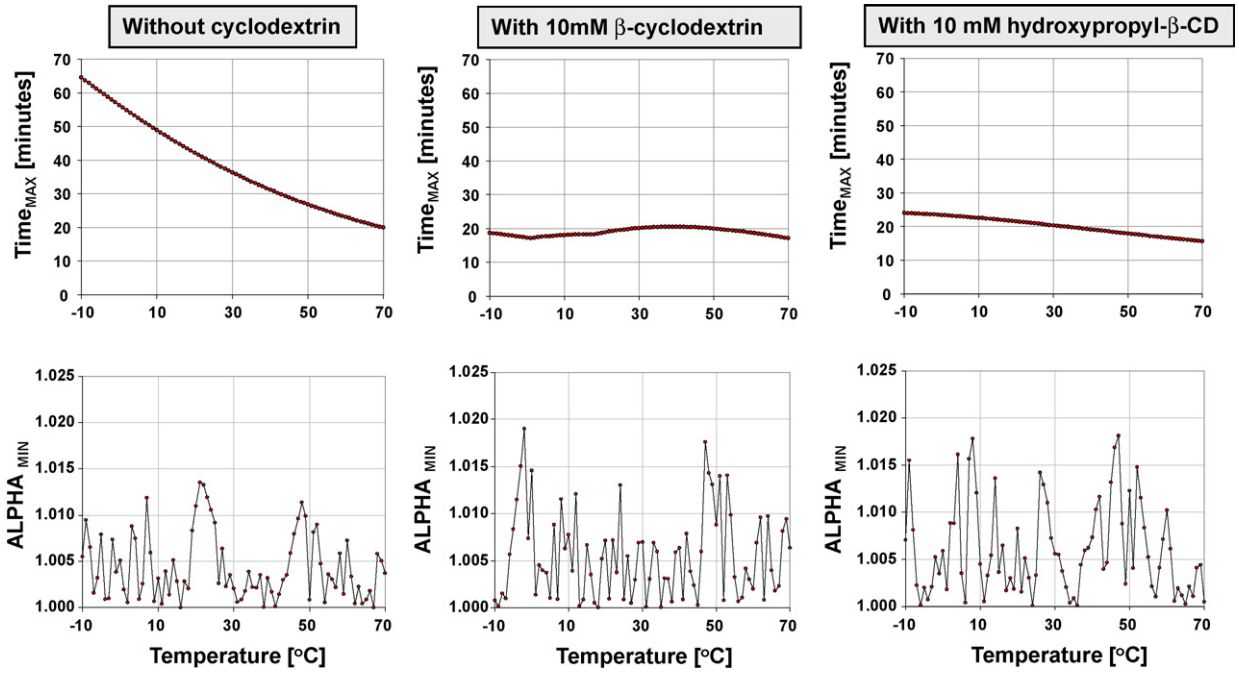

Fig. 4. Optimization plots concerning maximum analysis time (top) and maximum values of α_{MIN} parameter (bottom) for separation of 27 compounds (numbers 4–30) and temperatures ranging from −10 °C to +70 °C. Optimization parameter values were calculated for plain acetonitrile/water (35:65, v/v) (left), β-CD (middle) and HP-β-CD (right) modified mobile phases. Retention data calculations in step of 1 °C were based on the quadratric curve fit ($\ln k = a + bx + cx^2$) of the raw experimental data points obtained from 10 cm LC-18 column.

Table 3
Retention factor ratios ($k_{0\,mM\,CD}/k_{10\,mM\,CD}$) reflecting host–guest interaction intensity, calculated from the data presented in Table 2 for β-cyclodextrin (A) and hydroxypropyl-β-cyclodextrin (B); the numbers highlighted with bold font style correspond to minimum (*) and maximum (**) ratio values respectively.

Analyte	Separation temperature (°C)				
	0	10	20	40	60
A: $k_{0\,mM\,CD}/k_{10\,mM\,CD}$ ratio values for β-cyclodextrin					
Estetrol (4)	1.69	1.39	1.35	1.21	1.12
Estriol (5)	2.54	1.96	1.82	1.40	1.19
Cortisol (6)	1.76	1.66	1.49	1.27	1.14
Cortisone (7)	1.68	1.48	1.35	1.17	1.09
Tetrahydrocortisol (8)	3.69	2.86	2.31	1.63	1.30
Tetrahydrocortisone (9)	2.76	2.38	1.96	1.46	1.22
Dimethyl phthalate (10)	0.98	0.98	0.98	0.98	0.98
Bisphenol A (11)	4.46	3.59	2.92	2.03	1.54
17β-Estradiol (12)	10.91	6.90	4.47	2.17	1.43
Testosterone (13)	17.38	11.53	7.22(**)	3.00(**)	1.69
Norethindrone (14)	1.51	1.38	1.28	1.15	1.07
17α-Estradiol (15)	3.60	2.82	2.27	1.60	1.28
d-Equilenin (16)	1.90	1.61	1.42	1.20	1.10
Metyltestosterone (17)	3.66	2.74	2.15	1.53	1.25
Equilin (18)	3.83	2.82	2.18	1.53	1.26
Ethynylestradiol (19)	2.44	2.04	1.75	1.38	1.18
7,8-Dimethoxyflavone (20)	0.95(*)	0.95(*)	0.96(*)	0.97(*)	0.98(*)
Estrone (21)	6.13	3.97	2.72	1.59	1.23
17α-Hydroxyprogesterone (22)	2.61	2.10	1.76	1.38	1.19
4-*tert*-Butylphenol (23)	5.15	4.18	3.42	2.35	1.75(**)
Toluene (24)	1.18	1.13	1.11	1.07	1.04
Levonorgestrel (25)	1.41	1.30	1.22	1.11	1.05
Norgestrel (26)	1.44	1.31	1.23	1.11	1.05
Diethylstilbesterol (27)	23.10(**)	12.63(**)	6.80	2.28	1.28
20α-Hydroxyprogesterone (28)	13.56	7.92	4.67	2.11	1.41
Medroxyprogesterone (29)	1.53	1.35	1.23	1.10	1.04
Progesterone (30)	4.47	3.12	2.29	1.52	1.24
B: $k_{0\,mM\,CD}/k_{10\,mM\,CD}$ ratio values for hydroxypropyl-β-cyclodextrin					
Estetrol (4)	1.58	1.30	1.32	1.21	1.14
Estriol (5)	1.88	1.78	1.60	1.35	1.21
Cortisol (6)	1.59	1.47	1.37	1.24	1.15
Cortisone (7)	1.54	1.42	1.32	1.19	1.13
Tetrahydrocortisol (8)	2.16	2.13	1.83	1.45	1.25
Tetrahydrocortisone (9)	2.24	1.90	1.66	1.34	1.19
Dimethyl phthalate (10)	1.00(*)	1.01(*)	1.01(*)	1.01(*)	1.01(*)
Bisphenol A (11)	4.89	3.96	3.20	2.18	1.63
17β-Estradiol (12)	2.86	2.47	2.15	1.70	1.41
Testosterone (13)	2.63	2.32	2.06	1.66	1.41
Norethindrone (14)	1.47	1.40	1.33	1.22	1.15
17α-Estradiol (15)	2.64	2.30	2.01	1.60	1.36
d-Equilenin (16)	1.74	1.61	1.49	1.31	1.19
Methyltestosterone (17)	2.52	2.23	1.99	1.62	1.38
Equilin (18)	2.32	2.10	1.88	1.56	1.35
Ethynylestradiol (19)	2.43	2.13	1.89	1.51	1.30
7,8-Dimethoxyflavone (20)	1.01	1.01	1.02	1.02	1.02
Estrone (21)	1.88	1.74	1.61	1.40	1.25
17α-Hydroxyprogesterone (22)	2.15	1.91	1.72	1.45	1.28
4-*tert*-Butylphenol (23)	5.47(**)	4.49(**)	3.67(**)	2.52(**)	1.85(**)
Toluene (24)	1.19	1.17	1.14	1.11	1.09
Levonorgestrel (25)	1.35	1.30	1.26	1.18	1.12
Norgestrel (26)	1.36	1.31	1.26	1.18	1.13
Diethylstilbesterol (27)	2.82	2.47	2.14	1.64	1.35
20α-Hydroxyprogesterone (28)	3.95	3.37	2.88	2.11	1.64
Medroxyprogesterone (29)	1.68	1.51	1.39	1.23	1.14
Progesterone (30)	2.46	2.23	2.01	1.66	1.41

observed within relatively small temperature range (Table 4), should allow us to improve overall separation and reduce total analysis time of mixture studied. Based on data presented in Table 5 the effect of temperature on separation efficiency was computed for temperatures ranging from −10 °C to +70 °C with 1 °C step and assessed with help of simple optimization criteria. Particularly, total analysis time and maximum values of the α_{MIN} parameter (Fig. 4), which can be simply derived from the plots reflecting α values between adjacent peaks (not shown) were considered. Based on such criteria the best separation temperatures for mixture studied and eluents unmodified and modified with cyclodextrins were selected. Especially, temperature of 48 °C for unmodified mobile phase and 47 °C for eluents consisting of both macrocyclic additives were selected. This particular selection was tested using 25 cm analytical column and appropriate chromatograms were presented in Fig. 5. As can be seen, the addition of both macrocyclic modifiers allows baseline separation of key steroids including cortisol, cortisone, norethindrone and 17α-estradiol. It is noteworthy that in both cases total retention time (less than 55 min) and distribution symmetry along chromatogram time axis can be significantly improved in comparison to separation performed using mobile phase without cyclodextrins. Reported total analysis time is also very comparable to the gradient protocols described in the literature [2,3]. It should be also noted that the total analysis time described in this arti-

Table 4
Regression coefficients (intercept, slope) and determination coefficient (r^2) of the regression equation $\ln k$ = intercept + slope(1000/T) for the investigated compounds chromatographed on C18 stationary phase and acetonitrile/water (35:65, v/v) eluent as well as mobile phases modified with β-cyclodextrin and hydroxypropyl-β-cyclodextrin.[a].

Analyte	Mobile phase: without cyclodextrin			With β-cyclodextrin			With hydroxypropyl-β-CD		
	Slope	Intercept	r^2	Slope	Intercept	r^2	Slope	Intercept	r^2
Estetrol (4)	0.21 (0.01)	−0.71 (0.03)	0.994	−0.36 (0.09)	0.9 (0.3)	0.835	−0.2 (0.1)	0.5 (0.3)	0.616
Estriol (5)	0.34 (0.02)	−0.70 (0.05)	0.993	−0.8 (0.1)	2.5 (0.3)	0.949	−0.36 (0.05)	1.2 (0.2)	0.947
Cortisol (6)	−0.14 (0.08)	1.2 (0.3)	0.479	−0.8 (0.1)	3.1 (0.3)	0.962	−0.6 (0.1)	2.5 (0.4)	0.919
Cortisone (7)	0.23 (0.05)	0.1 (0.2)	0.886	−0.4 (0.1)	2.0 (0.4)	0.844	−0.25 (0.08)	1.4 (0.3)	0.752
Tetrahydrocortisol (8)	−0.5 (0.1)	2.6 (0.5)	0.808	−1.5 (0.2)	5.4 (0.8)	0.928	−1.4 (0.1)	5.1 (0.4)	0.974
Tetrahydrocortisone (9)	−0.2 (0.1)	1.8 (0.4)	0.424	−1.5 (0.2)	5.5 (0.6)	0.960	−1.1 (0.2)	4.6 (0.7)	0.960
Dimethyl phthalate (10)	1.00 (0.01)	−1.73 (0.03)	0.999	1.003 (0.008)	−1.71 (0.03)	0.999	1.01 (0.01)	−1.75 (0.04)	0.999
Bisphenol A (11)	1.47 (0.04)	−2.8 (0.1)	0.998	−0.17 (0.07)	1.6 (0.2)	0.625	−0.22 (0.06)	1.8 (0.2)	0.821
17β-Estradiol (12)	0.88 (0.05)	−0.8 (0.2)	0.991	−2.3 (0.2)	8.3 (0.7)	0.974	−0.19 (0.07)	2.1 (0.2)	0.713
Testosterone (13)	0.44 (0.07)	0.8 (0.2)	0.934	−3.2 (0.1)	11.2 (0.5)	0.993	−0.52 (0.08)	3.3 (0.3)	0.929
Norethindrone (14)	0.76 (0.05)	−0.2 (0.2)	0.986	0.25 (0.09)	1.3 (0.3)	0.716	0.38 (0.06)	0.8 (0.2)	0.923
17α-Estradiol (15)	0.87 (0.07)	−0.5 (0.2)	0.981	−0.7 (0.2)	4.0 (0.5)	0.878	−0.15 (0.09)	2.2 (0.3)	0.449
d-Equilenin (16)	1.53 (0.03)	−2.66 (0.08)	0.999	0.7 (0.1)	−0.3 (0.4)	0.932	0.95 (0.04)	−1.1 (0.1)	0.994
Methyltestosterone (17)	0.34 (0.08)	1.4 (0.3)	0.848	−1.3 (0.2)	6.1 (0.7)	0.924	−0.58 (0.09)	3.9 (0.3)	0.925
Equilin (18)	1.45 (0.03)	−2.3 (0.1)	0.999	−0.2 (0.2)	2.6 (0.6)	0.377	0.61 (0.04)	−0.05 (0.1)	0.986
Ethynylestradiol (19)	1.25 (0.05)	−1.6 (0.2)	0.995	0.1 (0.1)	1.6 (0.4)	0.374	0.29 (0.07)	1.0 (0.3)	0.835
7,8-Dimethoxyflavone (20)	0.95 (0.05)	−0.5 (0.2)	0.993	0.99 (0.05)	−0.6 (0.2)	0.993	0.97 (0.04)	−0.6 (0.2)	0.994
Estrone (21)	1.39 (0.04)	−2.0 (0.2)	0.997	−1.1 (0.3)	5.3 (0.9)	0.840	0.76 (0.05)	−0.3 (0.2)	0.988
17α-Hydroxyprogesterone (22)	0.79 (0.07)	0.1 (0.2)	0.978	−0.4 (0.2)	3.5 (0.6)	0.660	−0.002 (0.09)	2.2 (0.3)	0.000
4-*tert*-Butylphenol (23)	1.32 (0.07)	−1.6 (0.2)	0.991	−0.33 (0.08)	2.8 (0.3)	0.857	−0.34 (0.07)	2.8 (0.2)	0.891
Toluene (24)	1.29 (0.05)	−1.4 (0.2)	0.995	1.11 (0.07)	−0.9 (0.2)	0.989	1.15 (0.06)	−1.1 (0.2)	0.992
Levonorgestrel (25)	0.89 (0.07)	0.04 (0.3)	0.979	0.4 (0.1)	1.4 (0.4)	0.836	0.60 (0.08)	0.8 (0.3)	0.951
Norgestrel (26)	0.89 (0.07)	0.03 (0.2)	0.980	0.4 (0.1)	1.4 (0.4)	0.815	0.60 (0.08)	0.8 (0.3)	0.951
Diethylstilbesterol (27)	1.86 (0.07)	−3.0 (0.2)	0.996	−2.6 (0.3)	10 (1)	0.964	0.72 (0.08)	0.1 (0.3)	0.967
20α-Hydroxyprogesterone (28)	0.77 (0.08)	0.7 (0.3)	0.972	−2.7 (0.3)	11 (1)	0.958	−0.57 (0.07)	4.2 (0.2)	0.959
Medroxyprogesterone (29)	0.90 (0.09)	0.3 (0.3)	0.973	0.3 (0.2)	2.0 (0.5)	0.576	0.3 (0.1)	2.0 (0.4)	0.640
Progesterone (30)	1.39 (0.05)	−0.7 (0.2)	0.995	−0.6 (0.2)	5.0 (0.8)	0.636	0.5 (0.05)	1.5 (0.2)	0.972

[a] Calculations were based on the retention data set listed in Table 2; number of samples = 5; the values in parentheses indicate the standard errors of coefficients at a 95% significance level.

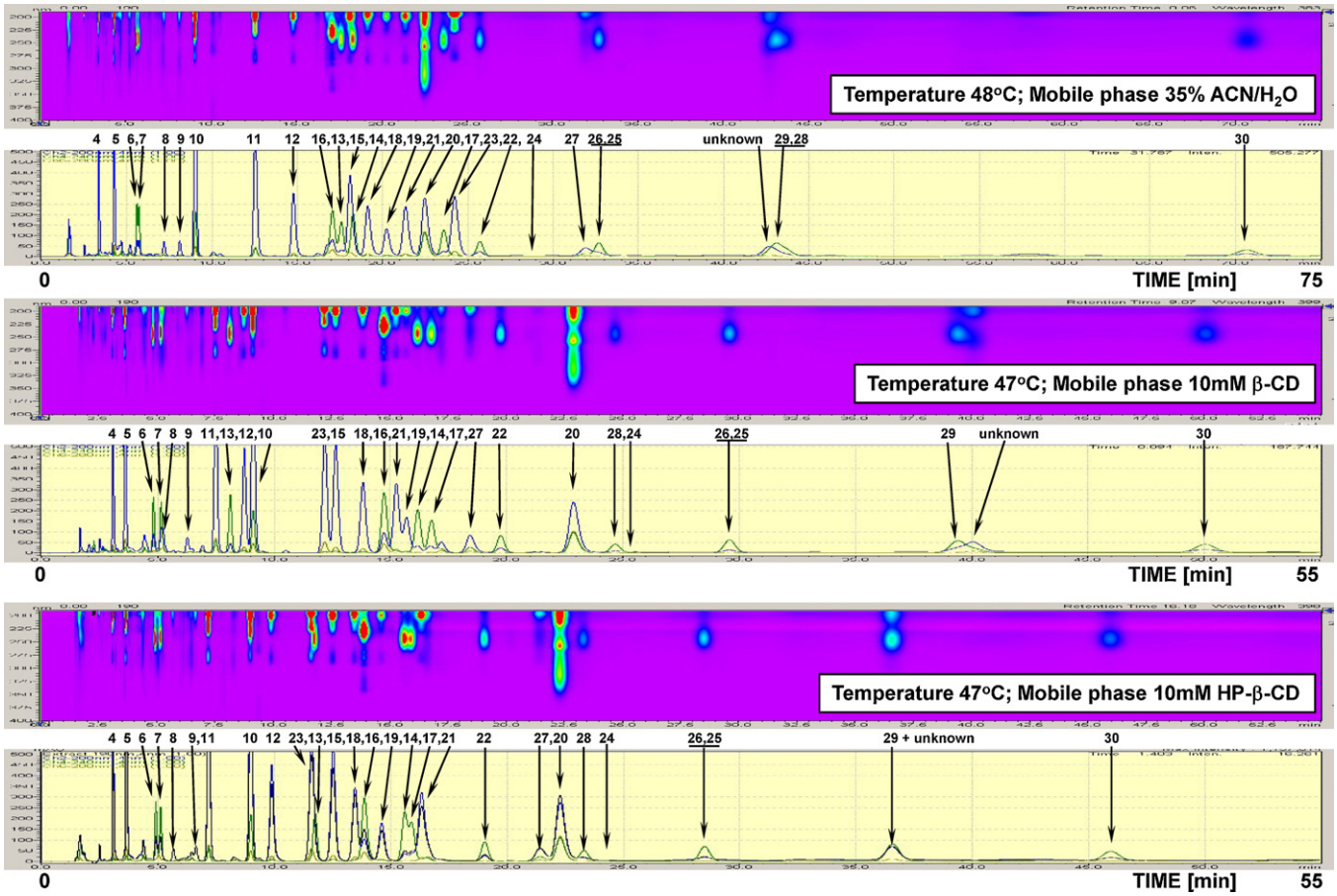

Fig. 5. Isocratic separation of 27 substances on 25 cm LC-18 column. Temperature selection was based on the maximum values of α_{MIN} and minimum analysis time parameters from the optimization plots presented in Fig. 4; Chromatograms and DAD scans: mobile phase acetonitrile/water (35:65, v/v) (top); 10 mM β-cyclodextrin (middle), 10 mM hydroxypropyl-β-cyclodextrin (bottom). Peak numbers correspond to substance numbers listed in Table 1.

Table 5
Regression coefficients (a, b, c) and determination coefficient (r^2) of the quadratic equation $\ln k = a + bx + cx^2$ (where $x = 1000/T$) for investigated compounds chromatographed on C18 stationary phase and acetonitrile/water (35:65, v/v) eluent (A) as well as mobile phases modified with β-cyclodextrin (B) and hydroxypropyl-β-cyclodextrin (**C**)[a].

Analyte	a	b	c	r^2
A: Mobile phase without cyclodextrin				
Estetrol (4)	−0.5553	0.1146	0.0140	0.994
Estriol (5)	−2.0945	1.1825	−0.1269	0.998
Cortisol (6)	−7.3972	5.0448	−0.7790	0.996
Cortisone (7)	−4.6668	3.0961	−0.4313	0.998
Tetrahydrocortisol (8)	−11.598	8.0943	−1.2902	0.999
Tetrahydrocortisone (9)	−10.9900	7.5495	−1.1622	0.999
Dimethyl phthalate (10)	−1.9414	1.1313	−0.0196	0.999
Bisphenol A (11)	−6.7316	3.8368	−0.3563	0.999
17β-Estradiol (12)	−5.6730	3.8338	−0.4439	0.999
Testosterone (13)	−6.0497	4.5676	−0.6209	0.999
Norethindrone (14)	−5.5811	4.0215	−0.4900	0.999
17α-Estradiol (15)	−7.6565	5.1717	−0.6465	0.999
d-Equilenin (16)	−4.9121	2.8920	−0.2044	0.999
Methyltestosterone (17)	−6.9619	5.3994	−0.7609	0.999
Equilin (18)	−5.4429	3.3624	−0.2877	0.999
Ethynylestradiol (19)	−6.6548	4.3018	−0.4580	0.999
7,8-Dimethoxyflavone (20)	−5.0802	3.6926	−0.4120	0.999
Estrone (21)	−6.4751	4.1080	−0.4086	0.999
17α-Hydroxyprogesterone (22)	−6.7443	4.9211	−0.6212	0.999
4-*tert*-Butylphenol (23)	−9.0138	5.7864	−0.6716	0.999
Toluene (24)	−7.0117	4.6752	−0.5088	0.999
Levonorgestrel (25)	−7.5742	5.4886	−0.6916	0.999
Norgestrel (26)	−7.5239	5.4545	−0.6858	0.999
Diethylstilbesterol (27)	−9.8709	5.9974	−0.6216	0.999
20α-Hydroxyprogesterone (28)	−6.9926	5.4260	−0.6994	0.999
Medroxyprogesterone (29)	−8.5847	6.2586	−0.8053	0.999
Progesterone (30)	−6.1787	4.6879	−0.4963	0.999
B: Mobile phase modified with 10 mM β-cyclodextrin				
Estetrol (4)	−5.1797	3.3505	−0.5636	0.997
Estriol (5)	−6.0060	4.3583	−0.7705	0.982
Cortisol (6)	−5.8441	4.5909	−0.8139	0.994
Cortisone (7)	−8.8512	6.1192	−0.9835	0.999
Tetrahydrocortisol (8)	−15.7330	11.7420	−2.0757	0.999
Tetrahydrocortisone (9)	−11.7050	8.9188	−1.5600	0.998
Dimethyl phthalate (10)	−1.7982	1.0561	−0.0080	0.999
Bisphenol A (11)	−5.2395	4.0036	−0.6251	0.968
17β-Estradiol (12)	−12.1720	10.1450	−1.8627	0.997
Testosterone (13)	0.7621	3.1085	−0.9461	0.997
Norethindrone (14)	−7.8648	5.7879	−0.8327	0.997
17α-Estradiol (15)	−11.4920	8.6486	−1.4060	0.997
d-Equilenin (16)	−11.6830	7.6154	−1.0372	0.999
Methyltestosterone (17)	−15.7800	11.9480	−1.9887	0.999
Equilin (18)	−15.6410	10.8020	−1.6593	0.995
Ethynylestradiol (19)	−9.8002	7.0190	−1.0322	0.987
7,8-Dimethoxyflavone (20)	−5.4205	3.8820	−0.4346	0.999
Estrone (21)	−22.0290	15.4450	−2.4810	0.996
17α-Hydroxyprogesterone (22)	−13.4180	9.8409	−1.5387	0.998
4-*tert*-Butylphenol (23)	−5.2162	4.5024	−0.7268	0.997
Toluene (24)	−7.8350	5.2995	−0.6300	0.999
Levonorgestrel (25)	−10.1800	7.4087	−1.0474	0.999
Norgestrel (26)	−10.4510	7.5877	−1.0770	0.999
Diethylstilbesterol (27)	−16.2420	13.4290	−2.4137	0.992
20α-Hydroxyprogesterone (28)	−21.5470	16.9310	−2.9527	0.997
Medroxyprogesterone (29)	−13.9660	9.9825	−1.4528	0.999
Progesterone (30)	−19.9730	14.5300	−2.2676	0.996
C: Mobile phase modified with 10 mM hydroxypropyl-β-cyclodextrin				
Estetrol (4)	−4.6792	2.9287	−0.4807	0.998
Estriol (5)	−0.2931	0.5469	−0.1356	0.952
Cortisol (6)	−8.5281	6.0485	−1.0033	0.999
Cortisone (7)	−6.9768	4.8154	−0.7607	0.997
Tetrahydrocortisol (8)	−5.8993	5.2556	−0.9998	0.991
Tetrahydrocortisone (9)	−15.3270	10.8670	−1.8051	0.999
Dimethyl phthalate (10)	−1.5973	0.9145	0.0137	0.999
Bisphenol A (11)	−3.7357	3.1104	−0.5003	0.969
17β-Estradiol (12)	−4.8751	4.0218	−0.6333	0.978
Testosterone (13)	−4.9932	4.4992	−0.7535	0.998
Norethindrone (14)	−5.5364	4.2256	−0.5780	0.996
17α-Estradiol (15)	−7.3202	5.6196	−0.8666	0.983
d-Equilenin (16)	−4.8435	3.2217	−0.3412	0.999
Methyltestosterone (17)	−5.8495	5.2827	−0.8813	0.999
Equilin (18)	−3.8908	2.9287	−0.3482	0.997
Ethynylestradiol (19)	−6.3442	4.7495	−0.6701	0.989
7,8-Dimethoxyflavone (20)	−4.9584	3.5941	−0.3948	0.999
Estrone (21)	−5.0701	3.6343	−0.4314	0.999
17α-Hydroxyprogesterone (22)	−7.3631	5.7971	−0.8714	0.970
4-*tert*-Butylphenol (23)	−4.1744	3.8415	−0.6287	0.995
Toluene (24)	−7.1245	4.8069	−0.5493	0.999
Levonorgestrel (25)	−7.3405	5.5081	−0.7370	0.999
Norgestrel (26)	−7.2728	5.4674	−0.7310	0.999
Diethylstilbesterol (27)	−7.4414	5.2809	−0.6851	0.997
20α-Hydroxyprogesterone (28)	−2.3434	3.4102	−0.5985	0.995
Medroxyprogesterone (29)	−11.558	8.4745	−1.2276	0.998
Progesterone (30)	−3.3209	3.4466	−0.4378	0.995

[a] Calculations were based on the retention data set listed in Table 2; number of samples = 5.

cle can be considerably reduced. It is expected to complete the separation of investigated mixture within 10–15 min. This can be perform by increasing mobile phase flow rate, which was set in our experiment at 1 mL/min, using instrumentation that operates at higher pressures than those used in classical HPLC like extreme-high-pressure liquid chromatography (X-LC) or very-high-pressure liquid chromatography (VHPLC) systems [28–31].

4. Conclusions

Taking into account the separation capability, total analysis time as well as separation protocol low cost and simplicity, the chromatographic method described in this paper is comparable with gas chromatography, gradient liquid chromatography or high performance planar chromatography, methods commonly used for steroid profiling in biological or environmental samples. The results of this research extend our knowledge concerning optimization of multiple isocratic separation of low-molecular-mass compounds, characterized by wide range of polarities, via temperature-driven supramolecular interactions involving macrocyclic mobile phase additives. Particularly, successful simultaneous separation of 27 components of interest that may be present in biological or environmental samples was described using eluents consisting of β-cyclodextrin or its hydroxypropyl derivative. The presented separation protocol, can be easily implemented for biomedical and environmental analysis of low-molecular-mass bioactive compounds that are present in the multicomponent samples from untreated and treated sewage water containing high level of interfering substances.

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