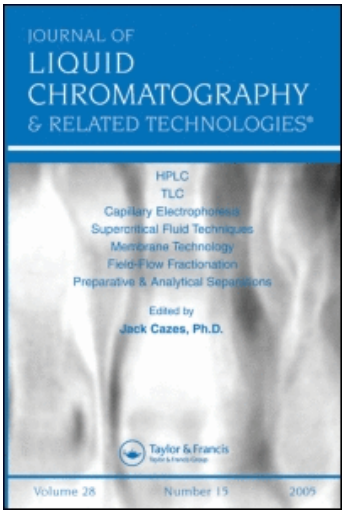

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Densitometry Application for Evaluation of the Visualizing Agents for Dehydroepiandrosterone

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Densitometry Application for Evaluation of the Visualizing Agents for Dehydroepiandrosterone

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Abstract: In our research the dehydroepiandrosterone (DHEA) was detected. The main aim of the work was to determine the possibility of application for the new visualizing agents for DHEA. Methylene violet, gentian violet, janus blue, methylene blue, and malachite green were used as visualizing agents. Chloramine T and rodamine B were used as comparative reagents described in literature.

DHEA solution was spotted on chromatographic plates and then dipped in solutions of visualizing reagents, dried and scanned using a densitometer. The plates without using visualizing agents, as well as after using procedure described in literature (using chloramine T) were also scanned. The bands with maximum absorption (λ_{max}) for DHEA without and with using visualizing agents were settled on the base of spectrodensitometric analysis. Consequently, the densitometric analysis of DHEA by (λ_{max}) determined was carried out. On the base of concentration values of investigated solution of DHEA and height and areas of densitometric bands, the relationships between heights of densitomteric bands and concentrations of DHEA, as well as between areas of densitometric bands and concentrations of DHEA were settled. The values of detectability and range of quantification, as well as the angle between tangents at the inflection points to the curves of the densitometric peaks (β) were determined.

Keywords: Dehydroepiandrosterone, Densitometry, TLC, Visualizing reagents

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INTRODUCTION

Thin layer chromatography (TLC) as well as other chromatographic methods is the most popular methods applied in scientific disciplines.[1] Thin layer chromatography is very often connected with other analytical methods, which improve and confirm detection results. TLC is very often used with mass spectrometry (MS), for example, for analysis of oligosaccharides,[2] nucleotides,[3] anti–inflammatory drugs,[4] alkaloids,[5] and polyether mixtures.[6] Another example is using TLC with infrared spectroscopy (IR) for identification of metal-dithizonates complexes[7] and cholesterol,[8] or coupling chromatography with NMR for determination of anthocyanins.[9]

The substance researched in this work was the dehydroepiandrosterone (DHEA). There are many different ways for determination of DHEA. Some of the methods can be: reversed phase ion-pair high performance liquid chromatography,[10] liquid chromatography/mass spectrometry,[11] high performance liquid chromatography[12] or high-performance thin-layer chromatography,[13] and liquid chromatography–tandem mass spectrometry.[14]

The method used for DHEA investigation in this work was thin layer chromatography. The chromatogram obtained during chromatographic separation can be used both for qualitative and quantitative characteristics of substances analyzed. However, in many cases, the visualization of the chromatogram is necessary. One of the ways of visualization is dipping the chromatograms in a solution of visualizing agent.

The main aim of the work was the determination possibility of the application of new agents visualizing one of the hormones that is dehydroepiandrosterone (DHEA). The agents taken into consideration were as follows: methylene violet, gentian violet, janus blue, methylene blue, and malachite green. Another purpose was the application of densitometry for estimation of the selected agents visualizing DHEA.

EXPERIMENTAL

Substances Investigated

The solutions obtained by dissolving the dehydroepiandrosterone in chloroform were used. The dehydroepiandrosterone (pure substance) was supplied by Merck, Germany, whilst chloroform was supplied by POCh, Poland. The concentration of the initial solution was 25 μg/5 μL. Then the next solutions were obtained by dilution of the initial solution. The concentrations of these solutions were: 25.00; 20.00; 16.00; 12.80; 10.24; 8.19; 6.55; 5.24; 4.19; 3.35; 2.68; 2.14; 1.72; 1.37; 1.10; 0.88; 0.70; 0.56; 0.45; 0.36; 0.29 μg/5 μL.

Adsorption Thin-layer Chromatography

Adsorption thin-layer chromatography was performed on aluminium plates precoated with 0.2 mm layer of a silica gel $60F_{254}$ (E.Merck, #1.05554). The plates were activated at 120°C for 30 min. The mixture of chloroform and acetone (POCh, Poland) in volume ratio 85:15 (v/v) was used as mobile phase.

The chromatographic plates were developed to height of 7.5 cm in the room temperature in a classical chamber (Camag, Switzerland), after their saturation with mobile phase (50 mL) during 30 minutes.

The solutions of DHEA were visualized by dipping them in 0.05% water solutions of visualizing agents (methylene violet, gentian violet, janus blue, methylene blue, malachite blue, rodamine B). The methylene violet and janus blue were supplied by Michrom Brand, England; gentian violet by Fluka AG, Switzerland; and methylene blue, malachite green, and rodamine B were supplied by POCh, Poland.

In chromatographic research, the reagent described in literature (solution of chloramine T[15]) was used. For the purpose of determination, the dehydroepiandrosterone using chloramine T, two solutions were prepared:

I – by dissolving 2.5 g of chloramine T (POCh, Poland) in 20 mL of distilled water and then diluted with 30 mL of methanol (Merck, Germany)
II – by mixing 47.5 mL of methanol and 2.5 mL of concentrated sulfuric (VI) acid.

After development, the chromatographic plates with solutions of DHEA spotted were dried in cold air during 5 minutes. Then, the plates were dipped in solution I and dried in warm air during 1 min. Next step was dipping in solution II and heating in temperature of 110°C during 10 min., again dipping into ammonia (25%) and again heating in temperature of 110°C during 5 minutes, and then cooling the plates and dipping in solution of *n*-hexane (POCh, Poland).[15]

Spectrodensitometric Analysis

The spectrum was performed using Camag Scanner TLC 3. The radiation sources were deuterium and wolfram lamps. Start wavelength was 200 nm and end wavelength was 800 nm. The slit dimensions were 8.00×0.40 nm, the scanning speed was $100\,\text{nm}\,\text{s}^{-1}$. The measurement mode was absorption (DHEA detection without the visualizing agent and in the case of methylene violet and chloramines T) and fluorescence (in the case of gentian violet, janus blue, malachite green, and rodamine B).

Spots Visualization Using Densitometer

Densitometric scanning was then performed at 254 nm with a Camag Scanner TLC 3 controlled by winCATS 1.4.1 software. The densitometric scanning was performed at various wavelengths depending on the visualizing agent used.

In the case of methylene violet the scanning was performed at wavelength of 200 nm, gentian violet –657 nm, janus blue –487 nm, malachite green –488 nm, and rodamine B –580 nm. Scanning plates without a visualizing agent was also performed at wavelength of 200 nm, and using chloramine T as visualizing agent at 361 nm. The radiation sources were deuterium and wolfram lamps.

Regression Analysis

The regression equations describing relationships between concentrations of DHEA and heights and between concentrations of DHEA and area of densitometric bands were achieved using computer program STATISTICA 7.1.

RESULTS AND DISCUSSION

Chromatograms Visualization using Solutions of Visualizing Agents

The chromatographic plates after development and drying were dipped in solutions of visualizing agents during 10 seconds. The new visualizing agents as follows: gentian violet, methylene violet, janus blue, methylene blue, malachite green, as well as described in literature: chloramine T and rodamine B,[15] were used for detection of DHEA. Chloramine T and rodamine B were used as comparative agents in relation to new proposed agents visualizing DHEA.

All obtained spots, except those obtained by dipping in solution of methylene blue were compact, and after dipping in solutions of visualizing agents and drying were scanned using a densitometer. In the case of methylene blue, spots were broadened and densitometric measurement was not possible.

Densitometric Determination of DHEA

On the basis of spectrodensitometric analysis the bands with maximum absorption (λ_{max}) for DHEA without and with using particular visualiz-

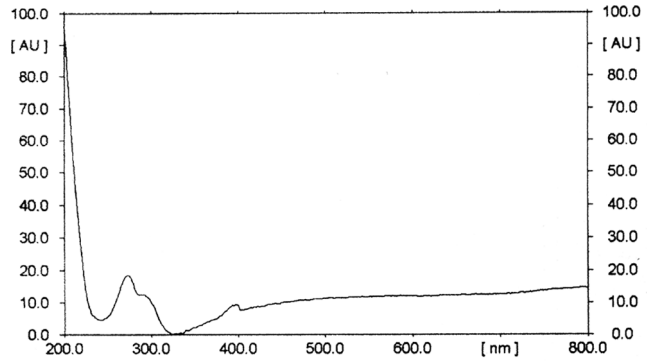

Figure 1. Spectrodensitogram of dehydroepiandrosterone without using the visualizing agent, $\lambda_{max} = 200$ nm.

ing agents (Figures 1–7) were settled. Then the densitometric analysis of DHEA was carried out at settled λ_{max}.

On the basis of values of DHEA concentrations, as well as heights (h) and areas (a) of densitometric bands obtained (Table 1), the relationships between height and DHEA concentration, as well as between area and DHEA concentration, were settled.

On the basis of these relationships was settled the range of concentrations, for which relationships are described by statistically significant mathematical functions. The relationships obtained are presented in Table 2.

In the case of determination of DHEA without using a visualizing agent and using chloramine T, the linear relationship was performed

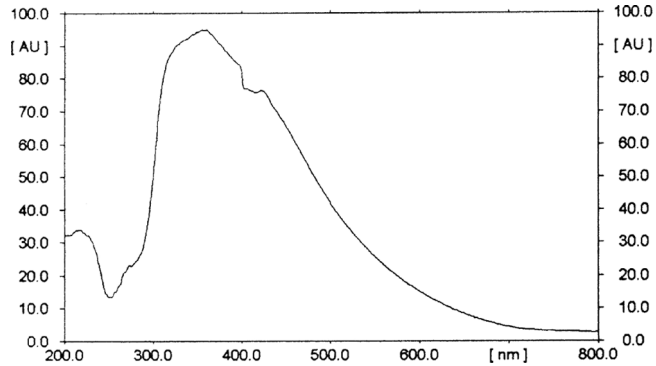

Figure 2. Spectrodensitogram of dehydroepiandrosterone after using solution of chloramine T, $\lambda_{max} = 361$ nm.

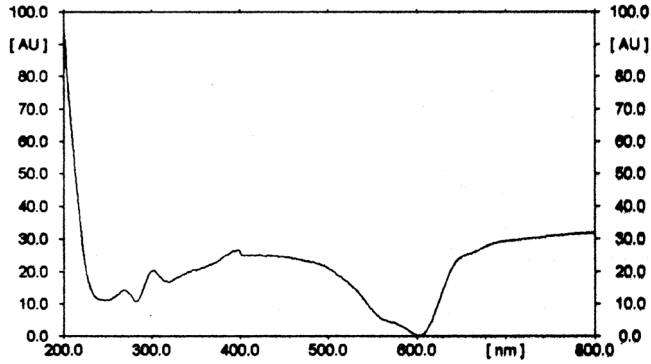

Figure 3. Spectrodensitogram of dehydroepiandrosterone after using solution of methylene violet, $\lambda_{max} = 200$ nm.

for lower values of concentrations. In the first case, relationships included concentrations from 0.45 to 3.35 μg/5 μL. Whereas, in the case of detection with using the solution of chloramine T, the relationships included concentrations from 0.45 to 4.19 μg/5 μL and from 0.45 to 3.35 μg/5 μL, for relationships with height and area of the densitometric band, respectively. In all four cases, the concentration value 0.88 μg/5 μL was omitted because of the gross error of the value.

Similarly, in the case of determination of DHEA using solution of rodamine B, equations describing the relationships between height or area of the densitometric band and DHEA concentration have large determination coefficients in the case of lower values of concentration (0.56 ÷ 5.24 μg/5 μL), and relationship with height of the densitometric band is described by polynomials of the second degree and relationship with area of densitometric band is described by linear function.

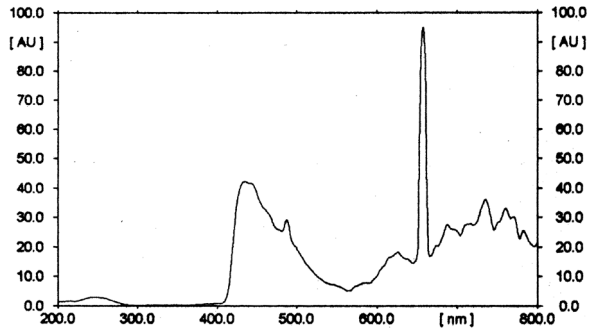

Figure 4. Spectrodensitogram of dehydroepiandrosterone after using solution of gentian violet, $\lambda_{max} = 657$ nm.

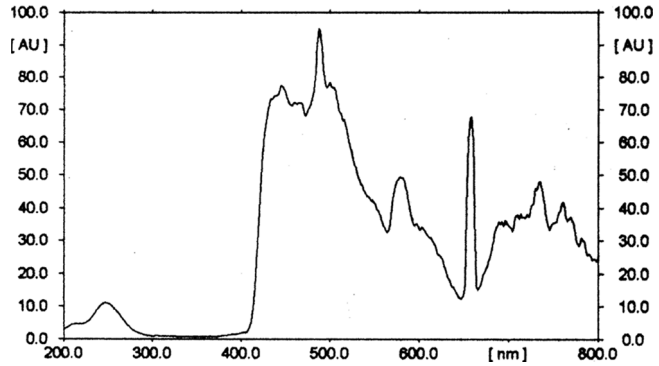

Figure 5. Spectrodensitogram of dehydroepiandrosterone after using solution of janus blue, $\lambda_{max} = 487$ nm.

In the case of detection using methylene violet, malachite green, and janus blue the relationships were described by polynomials of the second degree with determination coefficient being between 94.57 to 99.57%. These relationships were performed for higher range of concentrations from 5.24 to 25.00 μg/5 μL for methylene violet and malachite green, as well as from 2.68 to 25.00 μg/5 μL for janus blue.

In the case of detection with gentian violet, the functions statistically significant described the relationships for range of concentration of DHEA from 1.72 to 6.55 μg/5 μL and were characterized by linear function for relationship with height and area of densitometric band.

Equations presented in Table 2 can be used for calculation of DHEA concentration on the basis of known values of heights or areas of densitometric peaks.

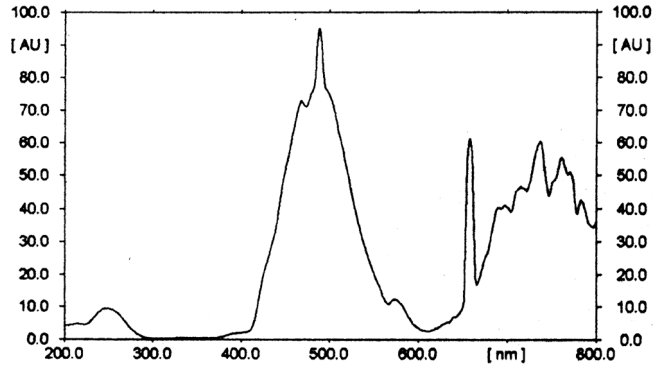

Figure 6. Spectrodensitogram of dehydroepiandrosterone after using solution of malachite green, $\lambda_{max} = 488$ nm.

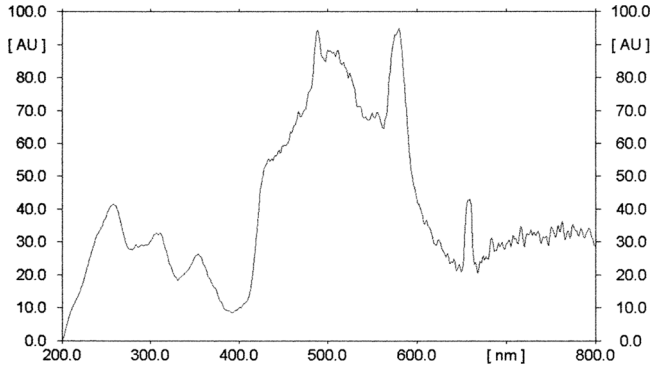

Figure 7. Spectrodensitogram of dehydroepiandrosterone after using solution of rodamine B, $\lambda_{max} = 580$ nm.

Parameters Describing the Results of DHEA Detection Without and With Visualizing Agents

The values of detectability and range of quantification of DHEA, as well as the values of angle between tangents at the inflection points to the curves of the densitometric peaks (β) are presented in Table 3.

All values of angle β were calculated for bands coming always from the same DHEA concentration (25 μg/5 μL).

From data obtained, arises that the angle β for the band coming from DHEA on the plate dipped in the solution of chloramine T has the lowest numerical value. Whereas, the largest value of angle β was obtained for DHEA band on the plate dipped in solutions of malachite green and rodamine B. The values of angles β point out that the DHEA band after detection using chloramine T is more compact, whilst the most broadened band comes from DHEA visualized using solution of malachite green.

The most compact densitometric band of DHEA was obtained after detection using methylene violet. In the case of detection using methylene violet, janus blue, and malachite green the ranges of quantification included higher values of DHEA concentration. In remaining cases the ranges of quantification included lower values of DHEA concentration.

The detectability of DHEA was also determined with use of particular visualizing agents (Table 3). The lowest value of detectability was stated in the case of DHEA determination without using a visualizing agent and also in the case of detection using solutions of chloramine T and rodamine B.

The experiment showed the possibility of usefulness of the new visualizing agents that is methylene violet, gentian violet, janus blue, malachite

Table 1. Values of heights (h) and areas (a) of densitometric bands of DHEA obtained without and with using particular visualizing agents

Concentration (μg/μL)	Without visualizing agent		Chloramine T		Methylene violet		Gentian violet		Janus blue		Malachite green		Rodamine B	
	h (AU)	a (AU)	h (AU)	a (AU)	h (AU)	a (AU)	h (AU)	a (AU)	h (AU)	a (AU)	h (AU)	a (AU)	h (AU)	a (AU)
25	295.4	14103.5	521.9	41300.6	170.3	6178.0	46.7	3114.8	72.9	5994.9	34.1	1907.6	65.7	5349.6
20.00	302.7	14335.5	518.5	38922.3	148.5	4868.0	52.3	3422.5	80.0	6303.1	38.5	2303.4	69.3	4444.6
16.00	256.5	10840.2	491.4	37300.1	132.7	4150.2	36.8	2078.6	50.7	3542.6	34.7	1625.6	63.2	4351.4
12.80	237.4	9490.4	434.4	31807.6	107.5	3128.4	33.0	1867.7	78.8	4956.6	29.6	1655.0	65.0	4314.8
10.24	216.4	8287.9	424.3	28341.8	87.9	2524.4	33.0	1753.6	62.3	3813.8	30.6	1573.7	53.6	3196.9
8.19	186.4	6572.2	355.4	22669.8	64.4	1849.1	37.4	1951.1	62.7	3919.7	28.9	1428.0	60.7	3751.1
6.55	164.2	5882.9	341.7	21522.9	37.7	927.1	44.0	2052.6	60.6	3262.9	25.6	1255.1	56.3	3357.4
5.24	180.3	6620.9	360.9	23952.5	24.7	560.6	34.5	1722.7	55.4	3320.3	21.9	816.7	41.3	2272.7
4.19	173.1	6522.8	352.9	24098.5	16.0	282.3	35.2	1743.5	51.6	3116.6	17.3	836.6	35.5	2047.9
3.35	115.2	4041.7	306.7	16853.1			30.6	1437.9	39.5	1463.5			33.8	1767.2
2.68	91.2	3311.4	257.7	13615.0			28.4	1200.3	32.2	1320.8			28.1	1410.6
2.14	73.7	2546.8	216.9	9712.8			22.8	982.4	15.3	415.7			29.5	1435.5
1.72	62.0	1914.9	189.7	8357.9			26.5	1036.5					30.4	1432.9
1.37	48.2	1490.6	166.7	6947.4			22.6	786.2					27.8	1298.9
1.10	40.6	1323.0	138.4	5764.1			20.6	592.8					41.0	1819.8
0.88	44.4	1371.3	156.9	6889.8									28.4	1139.8
0.70	32.1	938.9	105.7	4419.1									31.1	1376.7
0.56	26.1	816.9	96.8	3971.7									29.6	1004.1
0.45	20.6	639.4	83.4	3405.6									40.9	2353.7
0.36	14.6	405.2	44.2	1741.5									35.5	1545.6
0.29	15.0	516.1	33.1	1173.7									44.0	2332.4

Table 2. Equations describing the relationships between values of height (h) or area (a) of densitometric band and concentrations of dehydroepiandrosterone (c)

	Range of quantification (μg/5μL)	Equation	n	R^2 (%)	s	F	Eq. no.
Without visualizing agent	0.45–3.35 (except 0.88)	$h = 31.7169\ (\pm 0.6416)c + 7.0603\ (\pm 1.1721)$	9	99.71	1.82	2443	1
	0.45–3.35 (except 0.88)	$a = 1200.5000\ (\pm 20.0353)c$	9	99.14	109.80	3590	2
Chloramine T	0.45–4.19 (except 0.88)	$h = 72.7787\ (\pm 1.7411)c + 58.6062\ (\pm 3.7983)$	10	99.54	6.57	1747	3
	0.45–3.35 (except 0.88)	$a = 5021.1500\ (\pm 139.9880)c$	9	97.22	767.20	1287	4
Rodamine B	0.56–5.24 (except 0.70; 1.10 and 2.68)	$h = 0.4630\ (\pm 0.0368)c^2 + 28.1935\ (\pm 0.4549)$	8	96.35	0.95	4640	5
	0.56–5.24 (except 0.70; 1.10 and 2.68)	$a = 264.5610\ (\pm 10.710)c + 906.6870\ (\pm 50.143)$	8	99.12	44.86	677	6
Gentian violet*	1.72–6.55	$h = 3.7828\ (\pm 0.5630)c + 17.7416\ (\pm 2.2707)$	7	90.03	2.40	45	7
	1.72–6.55	$a = 224.0120\ (\pm 25.6072)c + 625.8230\ (\pm 103.2750)$	7	93.87	109.40	76	8
Methylene violet	5.24–25.00	$h = -0.3025\ (\pm 0.0394)c^2 + 16.3589\ (\pm 1.1894)c - 52.2039\ (\pm 7.6068)$	8	99.57	4.06	581	9
	5.24–25.00	$a = -5.2696\ (\pm 1.6257)c^2 + 436.6460\ (\pm 49.1042)c - 1542.9500\ (\pm 314.0550)$	8	99.47	167.82	472	10
Malachite green	5.24–25.00 (except 12.8 and 20.00)	$h = -0.0666\ (\pm 0.0106)c^2 + 2.5744\ (\pm 0.3281)c + 11.2155\ (\pm 2.0001)$	6	97.80	0.95	67	11

	5.24–25.00 (except 12.8 and 20.00)	$a = -5.4308\ (\pm 0.4888)c^2 + 212.1310\ (\pm 10.6123)c$	6	94.64	107.76	446	12
Janus blue	2.68–25.00 (except 8.19; 10.24 and 16.00)	$h = -0.2213\ (\pm 0.0345)c^2 + 7.6364\ (\pm 0.9407)c + 18.5204\ (\pm 4.2795)$	8	96.38	3.98	67	13
	2.68–25.00 (except 8.19; 10.24 and 16.00)	$a = -15.1149\ (\pm 2.1822)c^2 + 615.2380\ (46.2016 \pm)c$	8	94.57	475.14	297	14

*Densitometric measurement was carried out using only wolfram lamp for all equations $p \leq 0.01$.

Table 3. Detectability values, range od quantification of DHEA and values of the angle between tangents at the inflection points to the curves of the densitometric peaks (β)

Visualizing agent	Detectability (μg/5μL)	Range of quantification (μg/5μL)*	$\beta(^\circ)$
Without visualizing agent	0.29	0.45 ÷ 3.35	14
Chloramine T	0.29	0.45 ÷ 4.19	10.5
Methylene violet	4.19	5.24 ÷ 25.00	18
Gentian fiolet	1.10	1.72 ÷ 6.55	32
Janus blue	2.14	2.68 ÷ 25.00	40
Malachite green	4.19	5.24 ÷ 25.00	98
Rodamine B	0.29	0.56 ÷ 5.24	74.5

*Range of quantification is described for relationships between height of densitometric band and concentration DHEA.

green, and as described in the literature, rodamine B and chloramine T for detection of dehydroepiandrosterone (DHEA).

The usefulness of densitometry for detection of DHEA without and with using particular visualizing agents was also confirmed. The detectability and range of quantification of DHEA without and with using visualizing agents could be determined with application densitometry. The lowest detectability of DHEA was 0.29 μg/5 μL.

Induction of spectrum as a result of DHEA visualization using visualizing agents widen the analytical significance of results obtained.

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