

CHARACTERIZATION OF *ORTHOSIPHON STAMINEUS* BENTH EXTRACTS BY REVERSED-PHASE THIN LAYER CHROMATOGRAPHIC METHODS

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ABSTRACT. TLC is a powerful method used for separation of complex mixtures such as plant extracts. Employing different TLC techniques the separations can be improved. This paper presents a study of the *Orthosiphon stamineus* Benth extracts using isocratic reversed-phase thin layer chromatography (RP-TLC) and reversed-phase automated multiple development technique (RP-AMD). Methanol (SI) and a mixture of methanol–water–methyl acetate (SII) were used as extraction agents. *Orthosiphon stamineus* Benth. belongs to the Lamiaceae family. Its leaves contain rosmarinic acid, sinesetine and eupatorine as main compounds. After TLC separation the bioactive compounds from plant extracts were identified by comparison of the R_f values and *in situ* UV-Vis spectra with those of the standards and quantified using the calibration method. The rosmarinic acid was better extracted in the solvent mixture methanol–water–methyl acetate (10:10:80, v/v), while the sinesetine and eupatorine, which are more lipophilic, were better extracted in methanol. The study revealed the AMD technique superiority in comparison with the isocratic one.

Keywords: RP-TLC; RP-AMD; *Orthosiphon stamineus* Benth. extracts; rosmarinic acid; sinesetine; eupatorine.

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INTRODUCTION

Orthosiphon stamineus Benth. belongs to the Lamiaceae family and it is originary from Southeast Asia. *Orthosiphonis folium* was used from long time in different kidney diseases. The vegetal product contains caffeic acid derivatives (rosmarinic acid, cichoric acid, etc) and polymethoxylated flavonoids (sinesetine, eupatorine) [1,2]. Their structure is presented in figure 1.

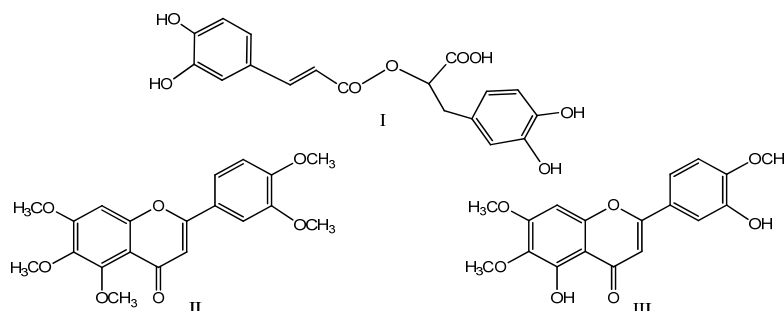


Figure 1. Structure of rosmarinic acid (I), sinesetin (II) and eupatorin (III)

Different techniques are used for identification and determination of these compounds. Usually separation methods using both, column [3-5] and planar [6-9] chromatography are preferred. Generally thin layer analyses are carried out on silica plates. Spectrophotometric methods for determination of caffeic acid derivatives are also used [3].

TLC is a versatile method for separating multicomponent mixtures. The separating power of isocratic TLC can be enhanced by two-dimensional development, multiple or automated multiple development (ADM). The AMD technique uses a solvent gradient and several development steps to separate compounds of widely different polarity. Most reported AMD applications have used gradients on normal phase plates [10-19].

This paper presents the qualitative and quantitative determination of rosmarinic acid, sinesetine and eupatorine from two extracts of *Orthosiphon stamineus* Benth. using isocratic RP-TLC and RP-AMD techniques. The identification of the separated compounds was performed based on chromatographic (R_f values) and non-chromatographic (*in situ* UV-Vis spectra) parameters. The efficiency of RP-TLC and RP-AMD separation techniques was achieved by comparison of R_f values. On the other hand, based on quantitative analyses, the efficiency of the extraction systems was correlated with analytes polarity.

RESULTS AND DISCUSSION

The chromatograms of developed plates using both chromatographic development methods RP-TLC and RP-AMD are presented in figure 2 and figure 3 respectively.

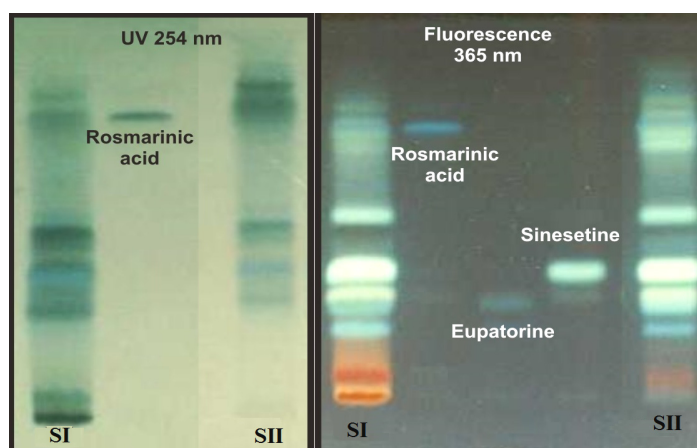


Figure 2. The chromatograms obtained using the isocratic technique

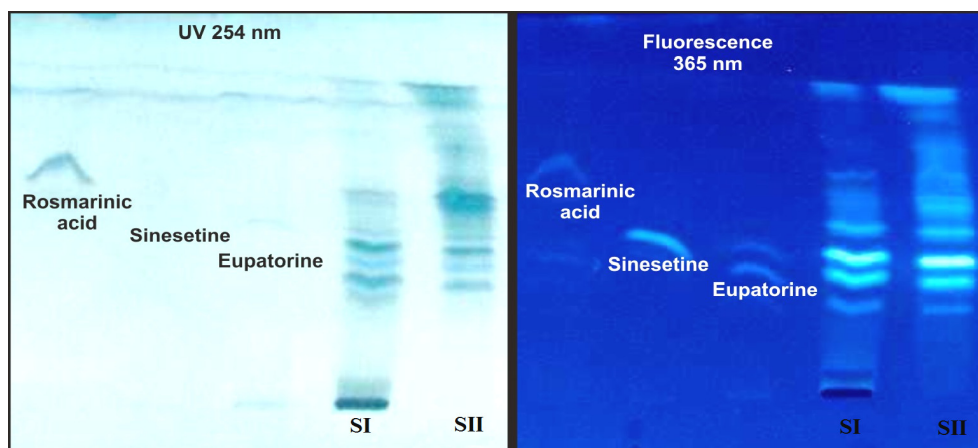


Figure 3. The chromatograms obtained using the AMD technique

The photodesitograms obtained by scanning the plates at 254 nm in reflectance mode and at 400 nm in fluorescence mode (excitation wavelength at 365 nm) are presented in Figures 4 – 6.

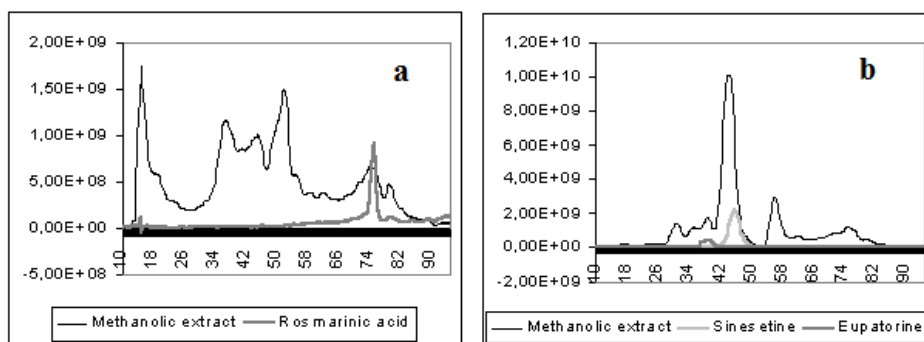


Figure 4. The densitogram of the methanolic extract (SI) separated by RP-TLC development. Visualization mode: (a) UV at 254 nm; (b) fluorescence at 400 nm.

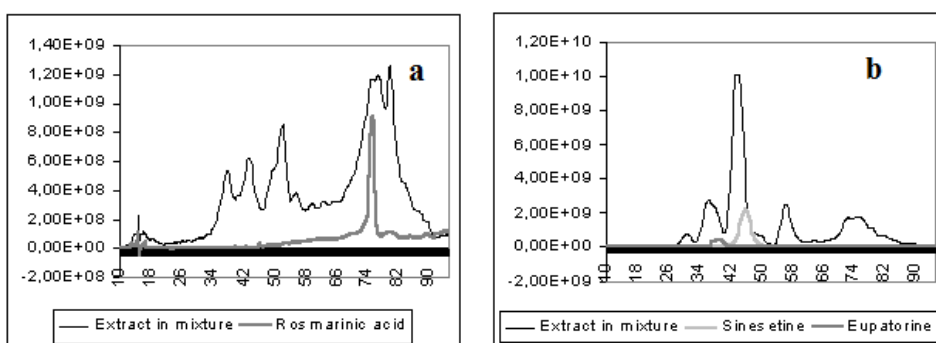


Figure 5. The densitogram of the solvent mixture extract (SII) separated by RP-TLC development. Visualization mode: (a) UV at 254 nm, (b) fluorescence at 400nm

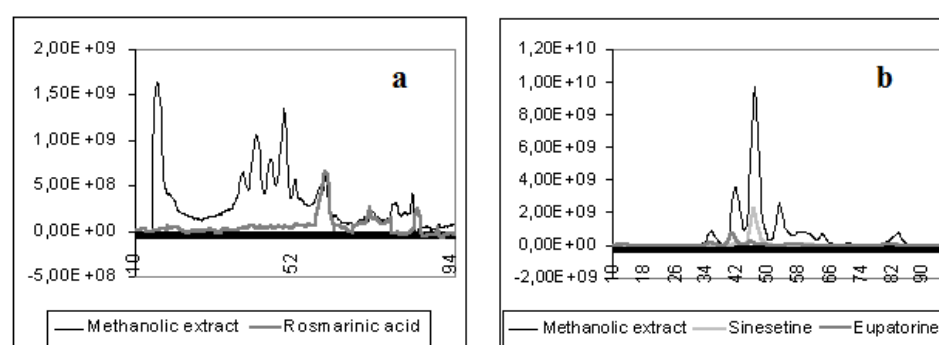


Figure 6. The densitogram of the methanolic extract (SI) separated by RP-AMD development. Visualization mode: (a) UV at 254 nm; (b) fluorescence at 400 nm.

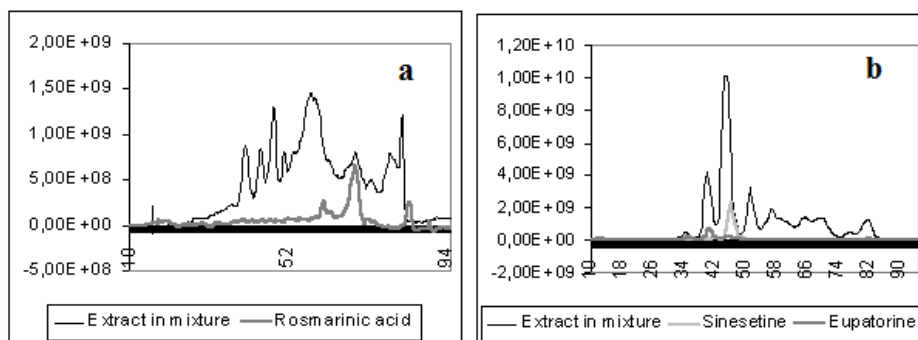


Figure 7. The densitogram of the solvent mixture extract (SII) separated by RP-AMD development. Visualization mode: (a) UV at 254 nm, (b) fluorescence at 400nm.

Comparing the photodensitograms from Figure 4a with Figure 5a and Figure 6a with Figure 7a, respectively, it can be observed a difference between the polarities of extracted compounds. In methanol (SI) were extracted most non-polar compounds than by using the solvent mixture methanol – water – methyl acetate (10:10:80, v/v), which extracted more polar compounds. This observation is confirmed by a pronounced peak at the start line and also by decreasing the concentration of the more polar compounds that are located in the top area of the plate. Furthermore, it can be observed a good separation of rosmarinic acid, a more polar compound having a higher R_f value, from sinesetine and eupatorine - less polar compounds that are situate in the middle zone of the plate.

On the other hand, comparing photodensitograms obtained by RP-AMD developing method with than obtained by RP-TLC, it can be observed a better separation efficiency achieved with the first mentioned technique.

Moreover, comparison of figures 4a with 6a and 5a with 7a, respectively, it can be observed an increased number of separated compounds obtained by RP-AMD vs RP-TLC method. The RP-AMD method gives a more specific fingerprint for plant extracts.

Another important observation is that fluorescence densitometry at 400 nm has a higher specificity than UV at 254 nm. Having natural fluorescence, sinesetine and eupatorine can be selectively identified by fluorescence photodensitometry.

The interest compounds from extracts were identified based on chromatographic parameter - R_f , which are similar with the standards ones, in both visualization modes – fluorescence and fluorescence quenching (Table 1).

Table 1. The R_f value of the separated compounds

Compound	Extract type	RP-TLC	RP-AMD
Rosmarinic acid	Standard	0.94	0.73
	Methanolic extract	0.93	0.73
	Extract in solvent mixture	0.94	0.73
Sinesetine	Standard	0.54	0.43
	Methanolic extract	0.53	0.44
	Extract in solvent mixture	0.54	0.43
Eupatorine	Standard	0.45	0.35
	Methanolic extract	0.46	0.36
	Extract in solvent mixture	0.45	0.34

Moreover, *in situ* UV-Vis spectra between 200 - 500 nm were register for spots having the same R_f . The spectra of the standards (rosmarinic acid, sinesetine and eupatorine) and their corresponding spots from both extracts are showing similar shapes (Figures 8-10).

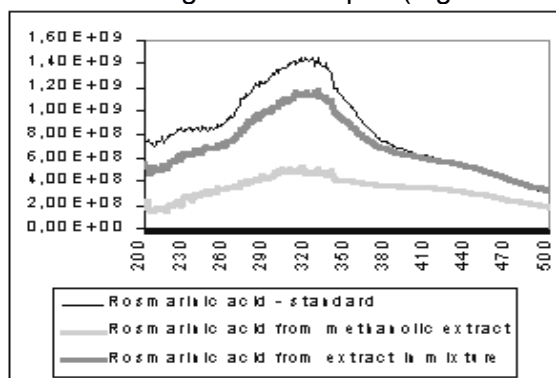


Figure 8. *In situ* UV-Vis spectra for rosmarinic acid (RP-AMD separation)

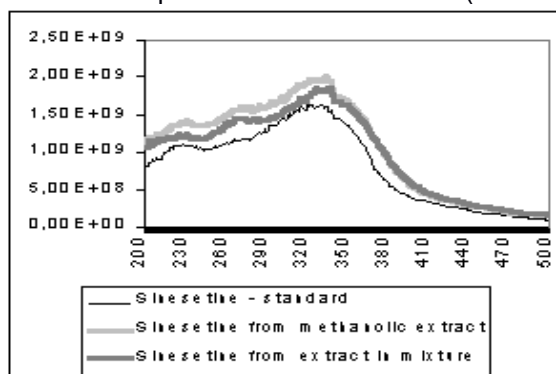


Figure 9. *In situ* UV-Vis spectra for sinesetine (RP-AMD separation)

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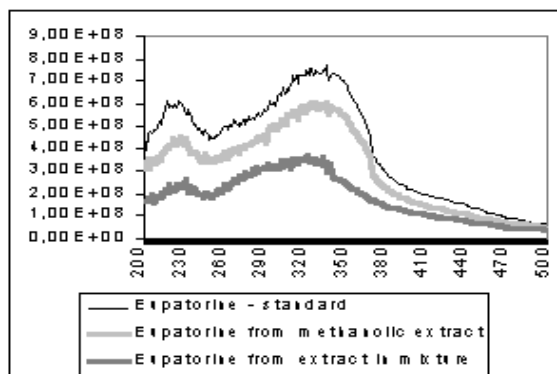


Figure 10. *In situ* UV-Vis spectra for eupatorine (RP-AMD separation)

Quantitative determination of the analytes was carried out by scanning the RP-TLC plates at 254 nm. The calibration functions were obtained by linear regression. Linear relationships between area (Y) and analyte quantity/spot (X) were obtained (Table 2).

Table 2. Calibration curves parameters

Compound	Rosmarinic acid	Sinesetin	Eupatorin
Working range - concentration (mg/ml)	0.108-0.540	1.500-6.000	1.050-4.200
Working range (µg/spot)	1.1 – 5.5	15.0 - 60.0	10.5 – 42.0
Slope(±t*s/n ^{1/2})	3914.80(±947.62)	2690.90(±109.75)	576.84(±100.91)
Intercept(±t*s/n ^{1/2})	714.32(±339.44)	3134.50(±416.25)	207.72(±267.85)
r	0.989	0.999	0.994

The percent content (% mg/g) of rosmarinic acid, sinesetin, and eupatorin in the studied plant, computed based on measured spot area and the equation of calibration curve, taking in account the extract volume and the analyzed quantity of the dry plant are presented in Table 3.

The extraction yield of rosmarinic acid - a hydrophilic compound, was higher when SII extraction system was used. On the other hand, when methanol was used, more lipophilic compounds like sinesetin and eupatorin are favored.

Table 3. The content of rosmarinic acid, sinesetine, and eupatorine in *Orthosiphon stamineus* Benth.

Rosmarinic acid % mg/g(±t*s/n ^{1/2})	Sinesetine % mg/g(±t*s/n ^{1/2})	Eupatorine % mg/g(±t*s/n ^{1/2})
Methanolic extract (SI)		
0,021±(0,0004)	0,852±(0,0154)	0,424±(0,0071)
Extract in solvent mixture (SII) methanol – water – methyl acetate (1:1:8, v/v)		
0,031±(0,0006)	0,837±(0,0154)	0,405±(0,0071)

CONCLUSIONS

The two studied *Orthosiphon stamineus* Benth. extracts can be characterized using reversed phase – thin layer chromatography and automated multiple development on reversed phase, because the RP-TLC and RP-AMD techniques show good separation of the main compounds from *Orthosiphon stamineus* Benth. leaves. The separations with AMD technique were better than those with isocratic RP-TLC. The characterization of the studied extracts was performed by separation and identification of two main compound classes: the caffeic acid derivatives (rosmarinic acid) respectively the polymethoxylated flavonoids (sinesetine and eupatorine).

EXPERIMENTAL SECTION

Materials, reagents and apparatus

The experiment was performed using acetonitrile and methyl acetate obtained from Roth, (Germany), methanol supplied from Euromedica (Romania) and acetic acid from Chimopar (Romania). Rosmarinic acid (I) was supplied from Roth (Germany), sinesetine (II) and eupatorine (III) were obtained from Extrasynthese (France). *Orthosiphon stamineus* Benth. leaves were obtained from Caesar & Loretz (Germany).

TLC RP18-Kieselgel F₂₅₄ (20x20 cm) plate used for chromatographic separation were purchased from Merck (Germany).

There were used a Desaga AS-30 automated applicator (Germany), a Camag AMD instrument (Muttenz Switzerland) and a Desaga CD 60 photodensitometer (Germany).

Sample Preparation

Bioactive compounds from 20 g crushed leaves of *Orthosiphon stamineus* Benth were extracted by cold extraction (10 days) with 100 mL extraction solvent. Two different systems were used for extraction: methanol (SI) and methanol-water-methyl acetate (10:10:80, v / v) mixture (SII). The second extraction system has already been optimized for a high yield of polyphenols extraction [20,21]. Both extracts were concentrated to 10 ml.

Experimental Conditions for Isocratic RP-TLC and RP-AMD Separation

Both separations were carried out on RP-silicagel plates. Methanolic solutions of rosmarinic acid (1.08 mg/mL), sinesetine (6.00 mg/mL) and eupatorine (4.20 mg/mL) were used as standards. 20 µL of each sample and 10 µL from each standard solution were applied as bands (1 cm) using an

automated applicator. Isocratic elution was performed in normal chamber using the mixture acetonitrile – water – acetic acid (55:44:1, v/v) as mobile phase. The developing distance was 8 cm. Gradient elution for RP-AMD was performed in 12 steps, on 8 cm (final developing distance), with acetonitrile – water – acetic mixtures of different composition, starting with the most polar one (Table 4).

Table 4. Mobile phase for AMD

Bottle no.	1	2	3	4	5
Steps no.	1-2	3-4	5-6	7-9	10-12
Water	79	69	59	49	39
Acetonitrile	20	30	40	50	60
Acetic acid	1	1	1	1	1

The scanning densitometry of the plates was performed at 254 nm and at 400 nm in fluorescence mode (excitation wavelength - 365 nm). The *in situ* UV-Vis spectra of sinasetin, eupatorin and rosmarinic acid spots from standards and extracts were obtained in the range of 200 - 500 nm.

The quantitative analyses were performed using calibration curves of three standards determined in the same chromatographic conditions as the isocratic analyses were performed. All determinations were made in triplicate and the result is the average of the individual values.

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