# Identification, Separation and Quantification of Rosmarinic Acid from Extract of Orthosiphon by HPTLC

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*This paper presents a fast and accurate method Thin Layer Chromatography (TLC) for high performance quantification of rosmarinic acid present in the extract of Orthosiphon. Rosmarinic acid from studied samples was extracted with methanol and separate on High Performance Thin Layer Chromatography (HPTLC) silica gel 60 F254 plates with toluene, ethyl acetate, formic acid and water 3/3/1/0.2 (v / v / v / v) as the mobile phase for 17 min without derivatization. Evaluation of plates was made in UV wavelength of 366 nm. The correlation coefficient of the calibration curve was 0.99999 for rosmarinic acid and analysis repeatability was calculated on three applications of the same sample analyzed (≤ 0.93%). Based on the results, this method can be used in routine analysis for the determination of rosmarinic acid extract from Orthosiphon. Antioxidant properties of rosmarinic acid were highlighted by the detection of DPPH.*

*Keywords: chromatography, TLC, rosmarinic acid, extract from Orthosiphon, HPTLC*

Medicinal plants have played an essential role in the development of human culture. These plants are used in human or veterinary practice for therapeutic or prophylactic purposes due to their antioxidant qualities. Herbs are available in many forms including fresh, dried, capsules, tablets or bottled in liquid form [1-3].

Orthosiphon is, for example, a good source of antioxidant phenolic acids (caffeic acid derivatives), flavonoids (quercetin and myricetin), anthocyanidins (cyanidin and delphinidin) and flavan-3-ols (catechin) [2-5].

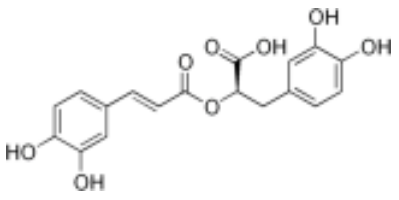
The antioxidants can be defined as substances able to inhibit or delay the oxidative damage of protein, nucleic acid and lipid caused by a dramatic increase of reactive oxygen species (ROS) through inhibiting the initiation or propagation of oxidizing chain reactions [2, 3, 6-10].

During the last decade, TLC and HPTLC have become important analytical techniques [11-13].

High Performance Thin Layer Chromatography (HPTLC) methods present many advantages including: simple sample preparation, low operating cost, short analysis time and simultaneous analysis of several samples [14-18].

Rosmarinic acid is an ester of caffeic acid and 3,4-dihidroxifenillactic acid. It was first isolated and characterized in 1958 by two Italian chemists M.L. Scarpatti and G. Oriente rosemary [1-2]. The chemical structure of rosmarinic acid is shown in the scheme 1.

It is usually found in species of the Lamiaceae family. However, it is also present in some species of fern, and hornwort [19].




Scheme 1. Chemical structure of rosmarinic acid

Rosmarinic acid has a number of interesting biological activities, such as antiviral, antibacterial, anti-inflammatory and antioxidant [1-3, 20-24].

There are few articles in the literature regarding the usage of these techniques for identification and quantification of phenolic compounds [21-28].

This paper presents a fast and accurate method -thin layer chromatography for high performance quantification of rosmarinic acid present in the extract of Orthosiphon. Rosmarinic acid from studied sample was extracted with methanol and separate on HPTLC plates, precoated with silica gel 60 $F_{254}$, with toluene, ethyl acetate, formic acid and distilled water as the mobile phase without derivatization.

## Experimental part

*Reagents and chemicals*

All reagents used in this study were of high purity. Rosmarinic acid was purchased from the Phytolab (Vestenbergsreuth). For preparation of the mobile phase toluene, ethyl acetate (Merck), formic acid (Fluka) and double distilled water were used. Double distilled water was produced from distiller Heraeus Destamat Bi-18. Methanol (Sigma-Aldrich) and 2,2-diphenyl-1-picrilhidrazil radical (DPPH*) were analytical - reagent grade. HPTLC plates (20 × 10 cm) silica gel 60 $F_{254}$ and magnesium chloride used for humidity control during plate development were purchased from Merck.

*Preparation of the sample to be analyzed*

For sample preparing, 0.2 g of the extract of Orthosiphon was weighed into a 10 mL volumetric flask on an analytical balance to four decimal places. The flask was brought to volume with methanol and stirred an ultrasonic bath for 30 min at 60°C. The solution was filtered through a 0.45 μm pore size cellulose filter and transferred to a 1.8 mL vial. Storage and preservation of the solution was done in a freezer at -20° C. The concentration of the solution to be analyzed was 0.025 g / mL.

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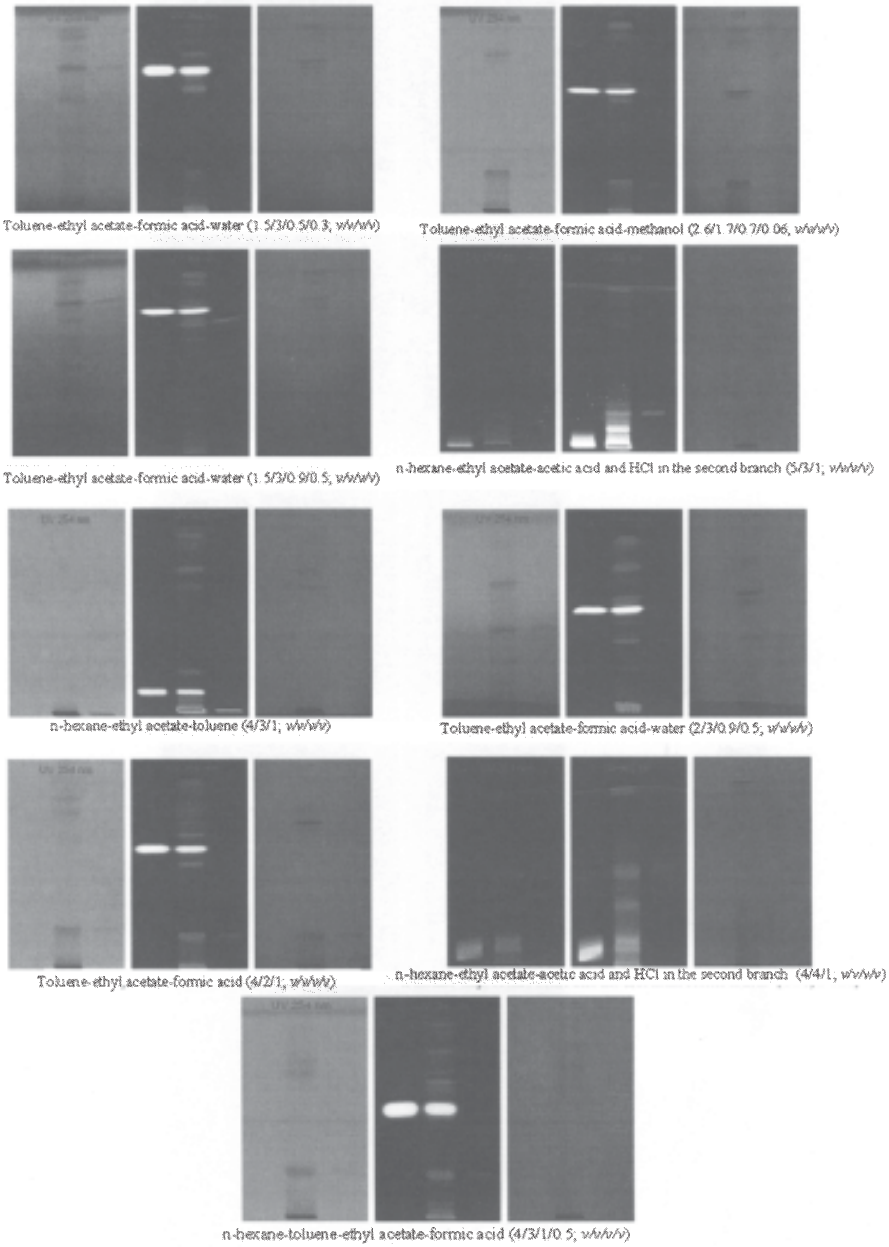



Fig. 1. The mobile phase preliminary selection

*Preparation of the reference solution*

As a reference solution (1 mg/10 mL), rosmarinic acid was dissolved in methanol. Storage was done in freezer at -20° C. The concentration of the obtained standard solution is 0.1 mg / mL.

*Optimization of rosmarinic acid mobile phase separation corresponding to extract Orthosiphon*

For the separation of the rosmarinic acid from the extract of Orthosiphon a number of nine mobile phases have been tested (fig.1). A plate, precoated with silica gel $F_{254}$, of size 20 x 10 cm was cut into pieces of 5 cm, and developed with various solvent mixtures. The first application is standard sinensetin (2 µL), the second is the actual sample (10 µL) and the third one is standard rosmarinic acid (5 µL). Each plate has been assessed under UV light (at 254 and 366 nm) and white transmitted light after developing (fig. 1).

Optimal mobile phase was established from toluene-ethyl acetate-formic acid-water (fig. 2. A-H).

The optimal mobile phase for this study was: toluene-ethyl acetate-formic acid-water (3:3:1:0.2).

*Preparation of solutions for derivatization*

Preparation of the solution of DPPH *, 40 mg of 2,2-diphenyl-1-picrilhidrazil residue was dissolved in 200 mL of methanol. The solution was stable for at least a month.

## Results and discussions

*The working procedure*

After preparation of the samples and reference solution, the sample circuit and the syringe, were washed with methanol (solvent dilution). For the sample application was used a 25 µL syringe.

This operation is performed automatically to remove any existing impurities which can contaminate samples. After the circuit washing, the vials in which are the test samples was placed in the auto sampler and the plate in its specific compartment. Vials position in the auto sampler must be identical to the software. Thus, it was created the method and sequence in win CATS software of the device and starts the applying of the samples and standards.

Samples application was automatic using ATS 4 (CAMAG) at a distance of 8 mm from the bottom edge of the plate. This distance is called the starting line. The distance left-right (side) implementation plate with 16 applications it was 15 mm. The distance between the

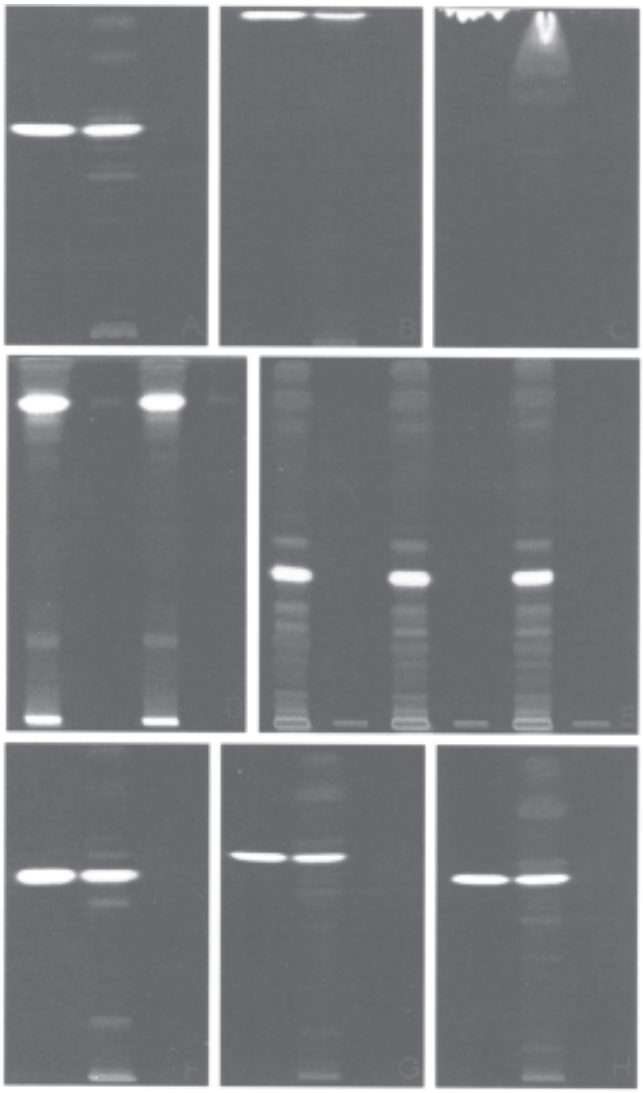


Fig. 2. Optimization of the mobile phase: A) Toluene-ethyl acetate-formic acid-water (2.5/3/0.9/0.5, v / v / v / v) B) Formic acid-acetone-dichloromethane (0.85/2.5/8.5, v / v / v / v), C) Water-methanol-acetic acid-dichloromethane (3/3/8/15, v / v / v / v), D) Ethyl acetate-formic acid-water (9/0.6/0.6, v / v / v / v), E) Methanol / ethyl acetate / toluene (0.5/4/5.5, v / v / v / v), F) Formic acid / ethyl acetate / toluene (1/4/5, v / v / v / v), G) Toluene-ethyl acetate-formic acid-water (2.5/3/1/0.2), H) Toluene-ethyl acetate-formic acid-water (3/3/1/0.2)

applications was 11.3 mm. The samples were applied in strips of 8 mm. Application rate was 150 nL/s.

After samples application, the plate was automatically submerged into automatic developing chamber (ADC2 CAMAG) being washed with methanol. The plate was dried for 4 min in a stream of cold air, while the humidity of the developing room reaches the optimum of 33 ± 2%. The humidity adjusting was done with magnesium chloride. Humidity at the analysis starting was 63.4 and 34.8% for analysis.

After drying, the plate was immersed into 10 mL of mobile phase. The migration distance was 60 mm, and the migration time was 17 minutes.

After development, the plate was dried for 3 minutes in a stream of cold air after being assessed under UV light at 366 nm with TLC Visualizer device (CAMAG) (fig. 3). UV light exposure time was 30 ms. Spectra recording was done using TLC Scanner 4 (CAMAG) at a wavelength of 327 nm with D (deuterium) lamp. The data were processed using win CATS software, version 1.4.7.2018 of CAMAG.

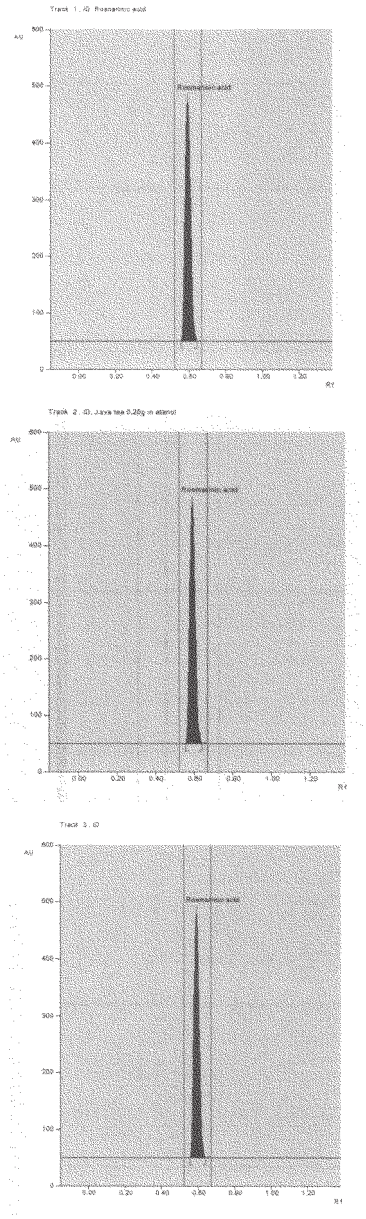


Fig. 3. HPTLC chromatograms rosmarinic acid extract of Orthosiphon

Finally, the plates were covered with a piece of glass of the same size as the board and wrap in aluminum foil.

*Quantification of rosmarinic acid by HPTLC*

The presence of rosmarinic acid in the samples Orthosiphon was confirmed by 12 applications from the same sample. Determining the relative standard deviation was calculated for only three applications (fig. 4).

For the determination of the maximum absorbance from rosmarinic acid spectrum, this spectrum was recorded between 200 and 450 nm. Maximum absorbance was determined at a wavelength of 327 nm (fig. 5 and 6).

To determine this spectrum have been applied so far 10 µL of the sample and the reference solution.

After determining the maximum absorbance, plate was scanned at a wavelength of 327 nm using Scanner 4 (CAMAG). Recording of spectra was done using deuterium lamp. Other attempts have been made using other lamps, but the best results were obtained using deuterium lamp.

*Recording spectra using a mercury lamp (Hg)*

Identical volumes were applied both to the sample and the standard. The wavelength at which the recording was made spectra was 313 nm in fluorescence (fig. 7).

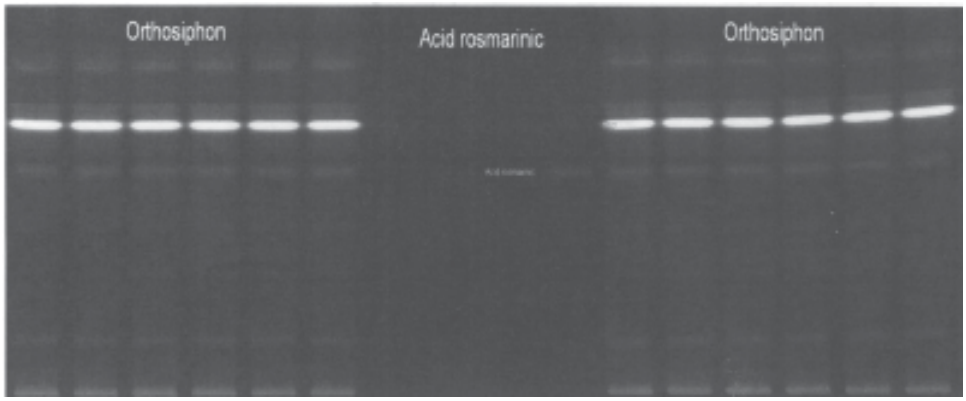



Fig. 4. Chromatogram separation of rosmarinic acid in the extract of Orthosiphon, after development in UV light at 366 nm

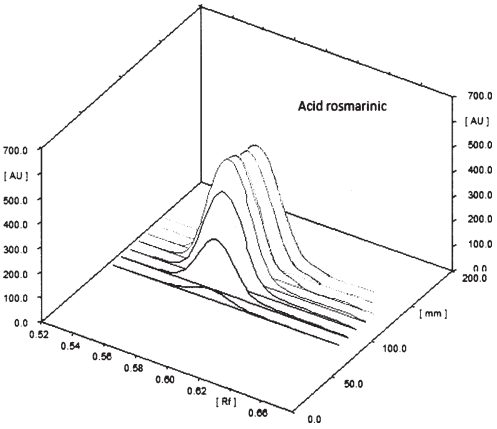



Fig. 5. 3D chromatogram rosmarinic acid

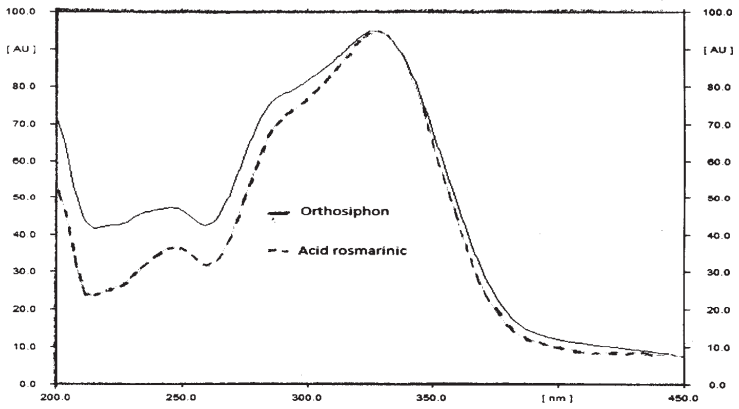



Fig. 6. The UV spectrum of rosmarinic acid identified in the extract of Orthosiphon

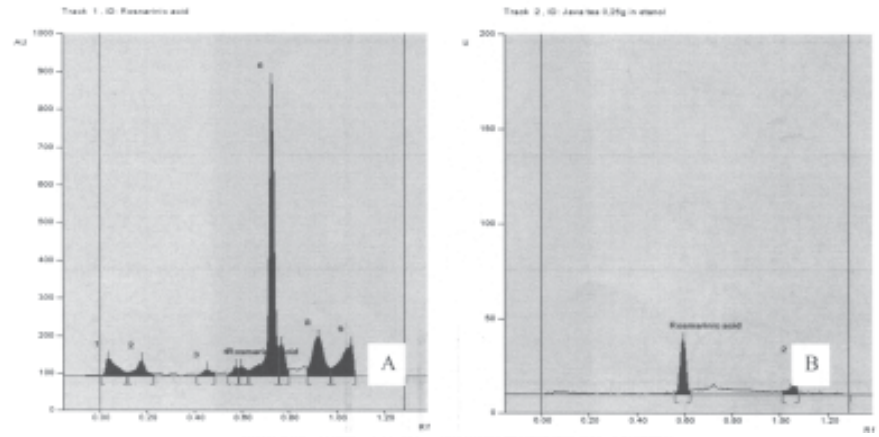



Fig. 7. HPTLC chromatograms of the sample to be analyzed (A) and of reference solution (B), at 313 nm

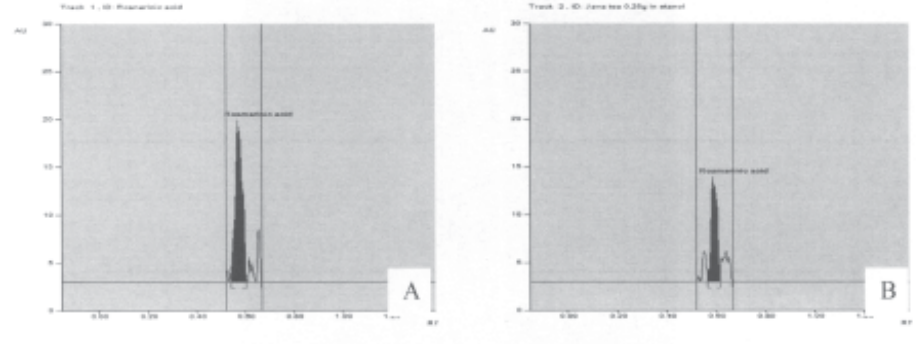



Fig. 8. HPTLC chromatograms of the sample to be analyzed (A) and of reference solution (B) at 327 nm, in fluorescence

*Recording spectra using a Deuterium (D) lamp*

Identical volumes were applied to both the sample and the standard. The wavelength at which the spectra recording were made was 327 nm in fluorescence (fig. 8).

*Recording spectra using a deuterium lamp (D)*

Identical volumes were applied to both the sample and the standard. The wavelength at which the recording was made spectra was 327 nm, the absorbance.

Record absorbance spectra seem much better than in fluorescence. Peaks seem perfectly separated and easily evaluated (fig. 9).

Quantitative determinations were made using calibration curve. In order to achieve the calibration curve four different sizes of the reference solutions 1, 3, 6, 9 μL were applied (points marked in fig. 10).

The calibration curve of rosmarinic acid is described by a polynomial equation. Peaks analyzed samples were

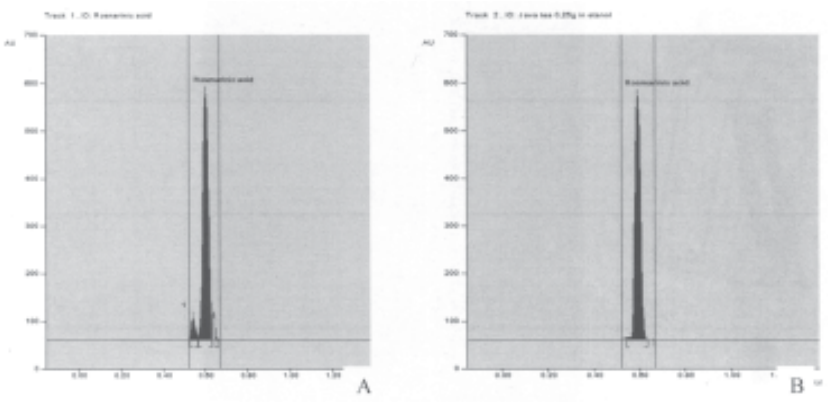


Fig. 9. HPTLC chromatograms of the sample to be analyzed (A) and of reference solution (B) at 327 nm
Rosmarinic acid has a Rf of 0.58

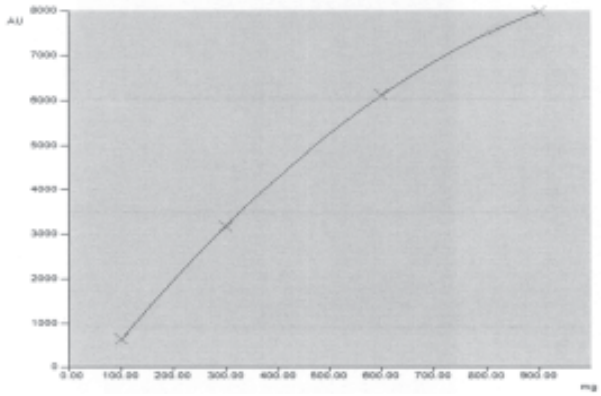


Fig. 10. Polynomial calibration curve of rosmarinic acid

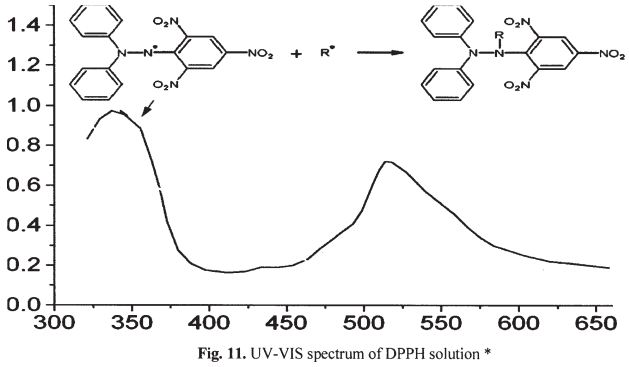


Fig. 11. UV-VIS spectrum of DPPH solution

| Compound | Polynomial Calibration (evaluated by area) | Domain of calibration (ng/band) | Coefficient of correlation (r) | Relative standard deviation (sdv, %) |
|---|---|---|---|---|
| Rosmarinic Acid | $Y_A = -0.006x^2 + 15.147x - 834.358$ | 100-900 | 0.99999 | 0.53 |

**Table 1**
THE CHARACTERISTICS OF THE CALIBRATION CURVE USED FOR THE QUANTIFICATION OF ROSMARINIC ACID

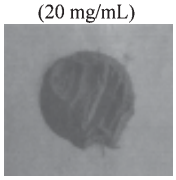
| Extract solution concentration | Volume applied (µL) | Content (ng/band) | Content (%) | Repetability (*%RSD*, n = 3) |
|---|---|---|---|---|
| Orthosiphon (20 mg/mL)  | 5 | $P_1$ = 809.56<br>$P_2$ = 803.40<br>$P_3$ = 818.47<br>Media = 810.48 | $P_1$ = 0.8096<br>$P_2$ = 0.8034<br>$P_3$ = 0.8185<br>Media = 0.8105 | 0.93 |

**Table 2**
QUANTIFICATION OF ROSMARINIC ACID IN THE EXTRACT OF ORTHOSIPHON

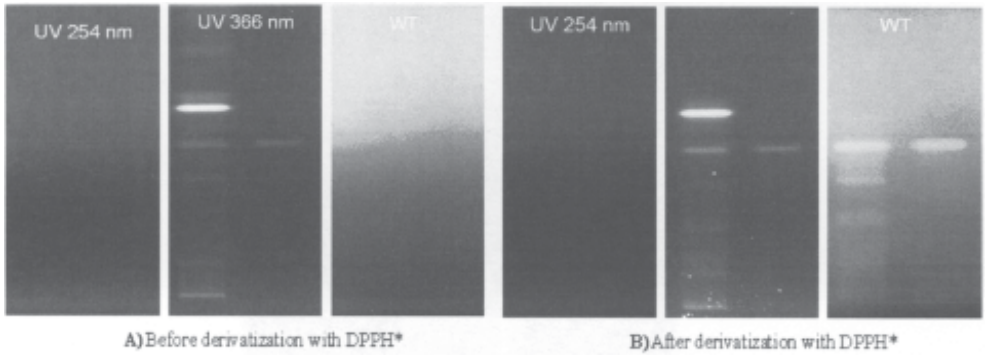



Fig. 12. Chromatographic results before (A) and after (B) derivatization samples *

evaluated by area determination. The characteristics of the calibration curve are presented in table 1.

*The calculation of existing rosmarinic acid content in the samples analyzed*

The calculations were made using win CATS software. We obtained a good repeatability of the three applications which will determine a high relative standard deviation. Repeatability was ≤ 0.93%.

Volume of 1 µL of reference solution applied with 100 ng /µL of rosmarinic acid (3 µL - 300 ng / µL, 6 µL - 600 ng / µL, 9 µL - 900 ng / µL). The software calculates the amount of rosmarinic acid expressed in ng / band. To express results in percentages, the quantity expressed in ng / band divides the volume of each sample and then applied to each solution concentration applied. After the calculus the amount of rosmarinic acid is expressed in ppm.

With the help of the relation 1 ppm = 0.0001% is calculated as a percentage. The amount of the rosmarinic acid is shown in table 2.

*Detection of DPPH **

DPPH * is one of the most stable organic radicals with one nitrogen, is commercially available and has a maximum absorbance in visible light at 517 nm (fig. 11).

For the detection of anti-oxidant activity of a 0.5 mM solution of 2,2-diphenyl-1-picrilhidrazil radical in methanol was prepared. After developing, the plate was immersed in this solution using TLC Chromatogram Immersion Device (fig. 12 A and B). Before derivatization with DPPH * was making: Application 1 - extract Orthosiphon and Application 2 - rosmarinic acid standard.

The immersion rate and time were set at 3 cm /s respectively 2 s. After plate's immersion, they were first dried for 90 s at room temperature, but in the dark. This first drying was followed by a second one at 60 °C for 30 s.

Plates were assessed with transmitted white light. Areas with antioxidant compounds have been identified immediately as yellow on a purple background, for each compound exhibiting.

If the outdoor exposure of the plates is prolonged, there are identified yellowish spots. The plate was monitored for 24 h.

## Conclusions

Rosmarinic acid from studied Orthosiphon sample was extracted with methanol and separate on High Performance Thin Layer Chromatography (HPTLC) silica gel 60 F254 plates with toluene, ethyl acetate, formic acid and water and evaluation plates made in UV wavelength of 366 nm.

Rosmarinic acid was separated from the extract of Orthosiphon using optimal mobile phase: toluene-ethyl acetate-formic acid-water (3:3:1:0.2) in 17 min, without derivatization.

Rosmarinic acid is the compound majority of analyzed compounds (rosmarinic acid, sinensetin, β-sitosterol, caffeic acid and chlorogenic acid) in the extract of Orthosiphon.

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