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Determination of hypericin in St. John's wort by derivative spectrophotometry

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Abstract
Sum of hypericine content in St. John's Wort was determined by second order derivative spectrophotometry without using any separation or background correction technique and reagent. Calibration curves were constructed for the 5–25 μg ml^{-1} concentration range. As a reference method, reversed phase high performance liquid chromatographic procedure was developed. Commercially available samples of St. John's Wort were analyzed by the two methods and the results were statistically compared.

Keywords: Derivative spectrophotometry, herbal substances, St. John's Wort, hypericin

1. Introduction
Derivative spectrometry within the limits of the linear coordinate transformations of the spectrum is a method of processing of spectroscopic data simply, in which instead of the traditional spectrum its derivatives to the x-axis values are used. The using of derivatives reduces random error, for example, in practice it is often necessary to determine one component in the presence of impurities with absorption (background) in the analytical points. As a rule, previously the quality composition of the impurity and its spectrum is not exactly known, therefore, it remains to speculate on the background nature, based on the available information about the nature of the studied object [1].

The main advantages of derivative spectrophotometry are the more clearly determination of the wavelength for the absorption maximum of the detected substances; increasing of the method selectivity due to the narrowing of the absorption bands; reducing of the background signal. Derivative spectrophotometry allows to determine the substances absorbing at similar wavelengths and under mutual overlay of absorption spectra, increases the selectivity of the signals and the sensitivity of detection for disguised or weak absorption bands. For example, derivative spectrophotometric methods have been developed for the assay of caffeine in some pharmaceutical preparations [2].

The expensive equipment and more operator attention to the chromatographic methods prevent their application in small industrial laboratories where few analyses are performed for a day. Derivative spectrophotometry is a useful technique for taking of qualitative and quantitative information from spectra composed of unresolved bands, and for elimination of the effect of baseline shifts and baseline tilts. Now derivative spectrophotometry is a low-cost standard method of modern micro computerized UV/Vis spectrophotometry.

We consider the application of derivative spectrophotometry for determination of analytical markers in herbal substances. The St. John's Wort has a broad spectrum of biological activity. It exhibits the anti-inflammatory, antiseptic, astringent, photosensitizing, choleretic, diuretic, tonic effects, has antimicrobial and antiviral activity, is used for depressive mental disorders treatment in some cases. One of the major groups of active substances of the St. John's Wort, collected from two species of plants (*Hypericum perforatum* L. or *Hypericum maculatum* Crantz), are condensed anthracenediones, particularly hypericin and pseudohypericin [3, 4].

The extracts from St. John's Wort have intensive red colour due to the conjugation of simple chromophores in the molecule of a natural compound and the shift of the absorption maxima to the visible region (400–780 nm) of the spectrum. For the assay of biologically active substances in the St. John's Wort the photometric method is widely used, because the active ingredients of medicinal plants are colored compounds [4, 5]. However, this method has some significant drawbacks which can be removed using the derivative spectrophotometry in the visible region of the spectrum [6].

The purpose of this work is the designing of a direct and simple UV-spectrophotometric method for the determination of hypericin derivatives in St. John's Wort without separation or

background correction procedures. Several samples of St. John's Wort were analyzed to their hypericin derivatives. The results were compared with those ones obtained by the HPLC method.

2. Materials and Methods

For derivative spectrophotometric measurement a Specord 250 UV/Vis (Analytic Jena) spectrophotometer and 10 mm quartz cells were used. All spectra were recorded from 500 nm to 700 nm with 0,5 nm slit width, 50 nm min^{-1} scan speed and very high smoothing.

Chromatographic analyses were performed using the Agilent 1260 HPLC system consisting of a model including four-channels pump, autosampler and diode-array matrices (at 588 nm). A reverse-phase analytical column C-18 (250×4,6 mm, 5 µm) and an gradient mobile phase, containing 0,3% phosphoric acid / Acetonitrile / Methanol (table 1) were used [7]. The flow rate was 1,0 ml min^{-1}, column temperature 30 °C, injection volume 20 µl. Hypericine (Sigma, Lot BCBB0997) was used as standard.

Table 1: Ratio of solvents in mobile phase

Time (min)	0,3 phosphoric acid (%)	Acetonitrile (%)	Methanol (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

2.1 Preparation of sample solution

Test solution. Pulverize 10,0 g of St. John's Wort. Carefully weigh about 1,0 g and move it into a flask equipped with a condenser and protected from light, add 50 ml of 70% ethanol, and heat at 60 °C for 2 h with stirring. Cool to room temperature, centrifuge (2 min at 700 *g*), pass the solution through a PTFE membrane filter with 0,45 µm or finer pore size, and use the filtrate.

Hypericine stock solution (1 mg ml^{-1}) was prepared by dissolving of 10,0 mg of hypericine in 10,0 ml of 70% ethanol. This solution was freshly prepared and was diluted to obtain standard solutions for the preparation of calibration curves.

3. Results and Discussion

When conducting analyses by HPLC at the 588 nm wavelength the peaks of the four substances, including pseudohypericin (Retention time (RT) 41, 3 min) and hypericin (RT=43, 2 min) were detected in the chromatogram of the extract from the St. John's Wort (Fig. 1).

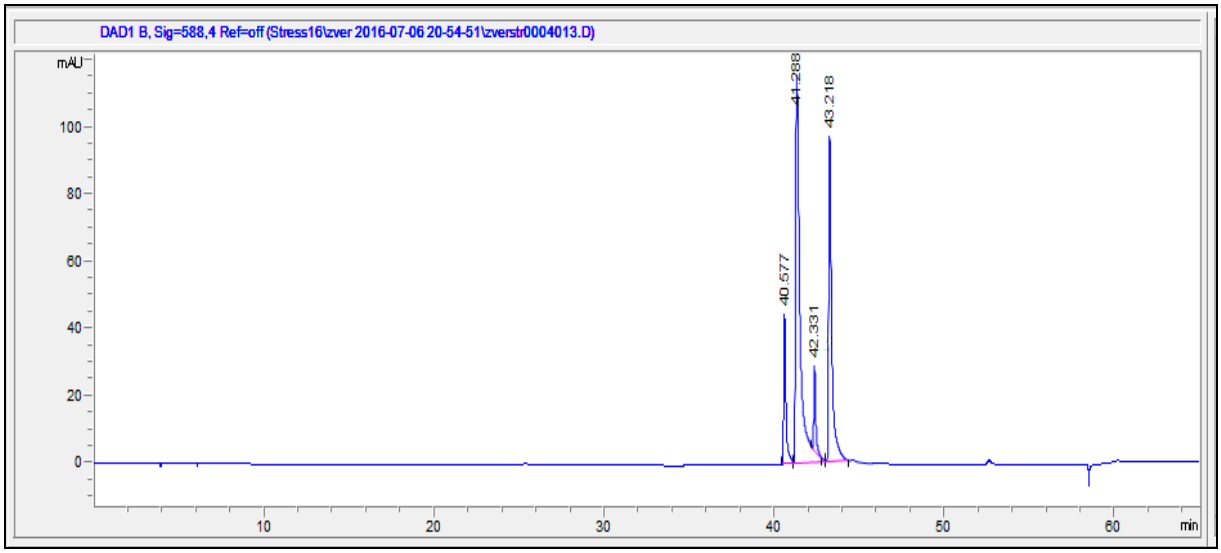

Fig 1: Chromatogram of extract St. John's Wort at wavelength 588 nm

Each of these substances contributes to the absorption at the wavelength of 588 nm. Absorption spectra of pseudohypericin and hypericin are almost identical, the spectra of impurities (Fig. 2 and Fig. 3) differ from the spectrum of hypericin and pseudohypericin (Fig. 4 and Fig. 5).

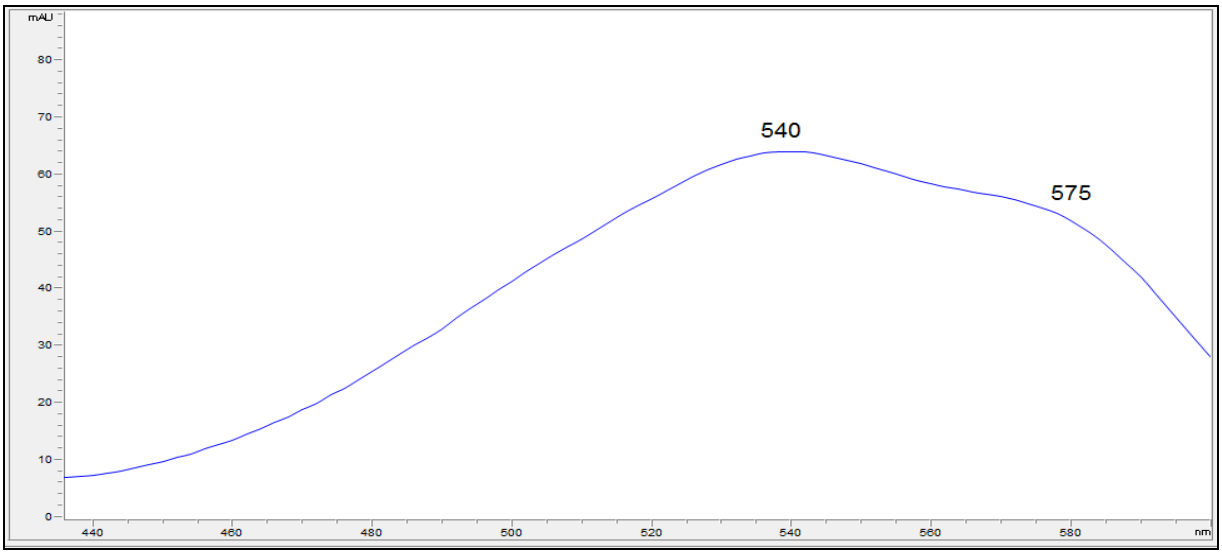

Fig 2: Absorption spectra of impurities with RT=40,6 min

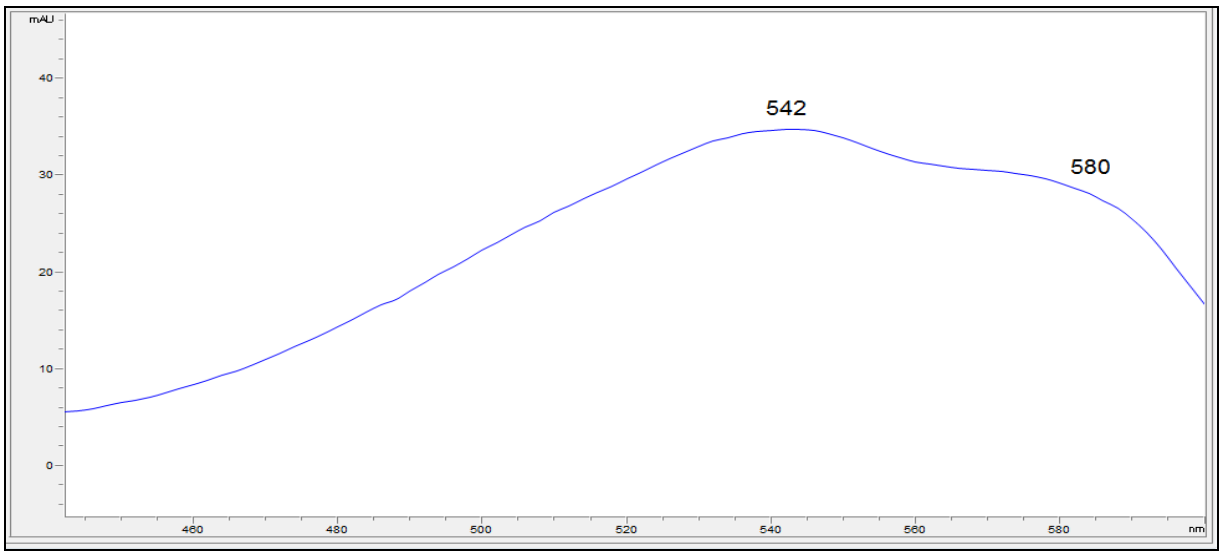

Fig 3: Absorption spectra of impurities with RT=42,3 min

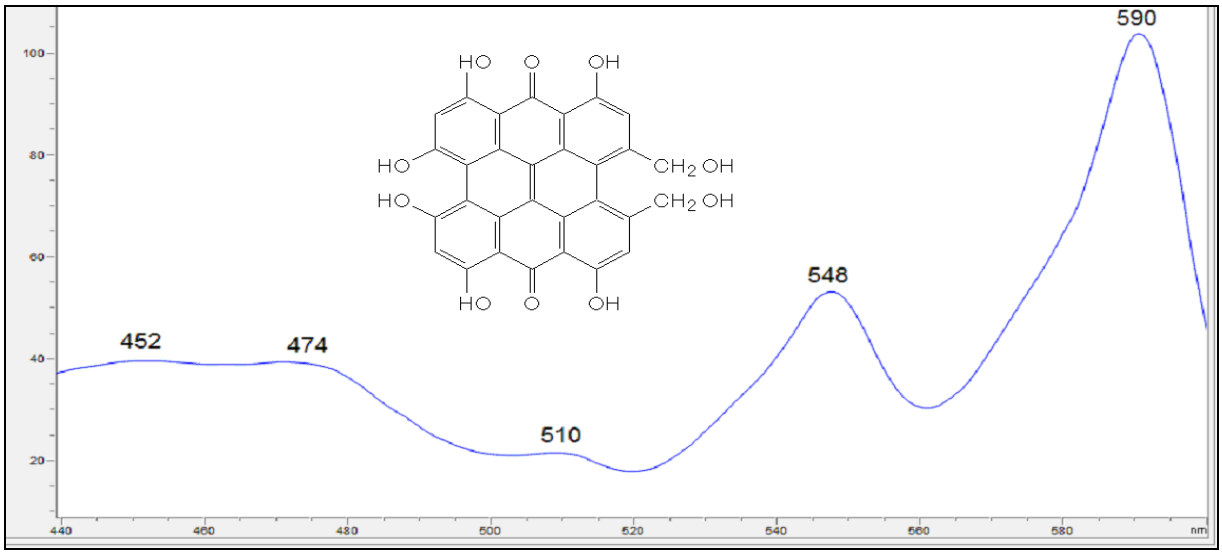

Fig 4: Absorption spectra of pseudohypericin with RT=41,3 min

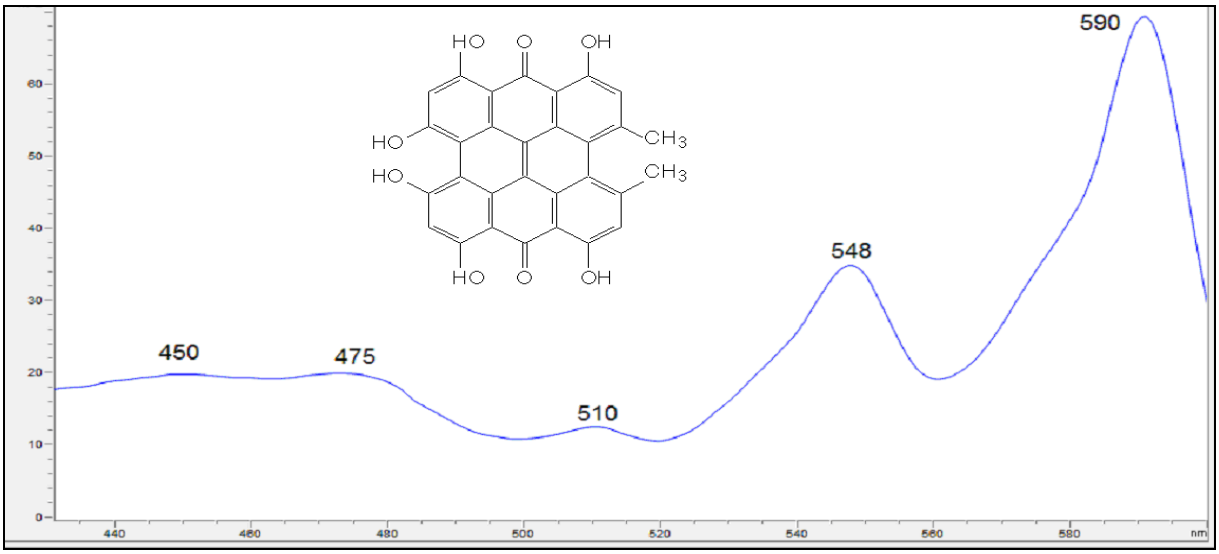

Fig 5: Absorption spectra of hypericin with RT=43,2 min

The spectra of all substances are summarized in the spectrophotometric method, therefore we obtain overstated results for the quantitative content of hypericins amount. The use of derivative spectrophotometry allows to eliminate the background influence of impurities (Fig. 6).

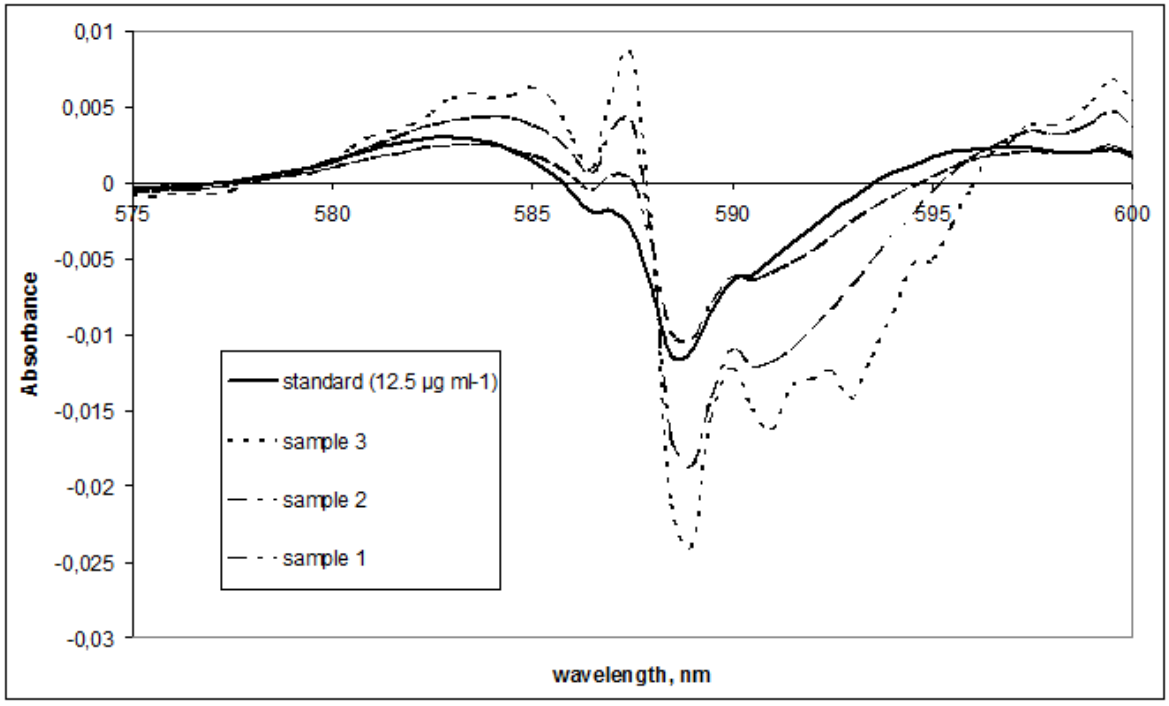

Fig 6: Second order of derivative spectrophotometry solution of hypericine (12,5 µg ml^{-1} and three samples of extracts St. John's Wort)

The use of higher orders derivatives and calculation to larger number of base points did not lead to better results. Therefore the processing of the spectra was performed using of second order with five base points. The minimum value was determined at a wavelength of 589 nm. Calibration curve was built on the results at five points (Fig. 7).

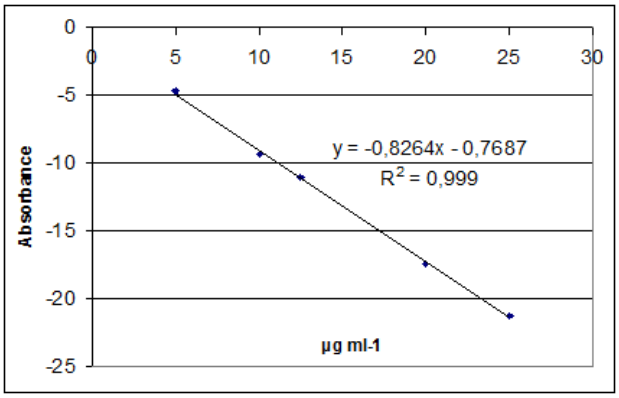

Fig 7: Calibration curve depending of second order absorbance from concentration of hypericin (range 5…25 µg ml^{-1})

Data for the comparative analysis of the three samples of the St. John's Wort are presented in table 2.

Table 2: Results of analysis sum of hypericins in St. John Wort by different methods, % (n=5, P=0, 95)

	Spectrophotometry	Derivative spectrophotometry	HPLC
1 sample	0,212±0,020	0,142±0,009	0,149±0,011
2 sample	0,123±0,014	0,062±0,006	0,066±0,005
3 sample	0,097±0,012	0,048±0,006	0,049±0,004

As seen from table 2, in all cases the results of spectrophotometric determination exceed the results of reference method (HPLC). The results obtained using the second derivative in the calculations do not differ from results obtained using HPLC.

Thus, the technique of determination of the hypericin derivatives (hypericin and pseudohypericin) in the St. John's Wort by the method of derivative spectrophotometry was developed. This technique is simpler and faster in compare to HPLC, and also gives more exact results compared to the spectrophotometric determination of European Pharmacopeia 8.0 [9].

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