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FIRST AND SECOND DERIVATIVE SPECTROPHOTOMETRIC DETERMINATION OF FLAVONOIDS CHRYSIN AND QUERCETIN

Keywords: Flavonoids, Chrysin, Quercetin, simultaneous determination, derivative spectrophotometry

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ABSTRACT

The first and second derivative spectrophotometric methods have been developed for the determination of flavonoids chrysin and quercetin. The amplitudes in the first derivative spectrophotometric spectra at 256 nm and in the second derivative spectra at 268 nm were selected to determine chrysin, while the amplitudes at 233 and 269 nm in the first and at 241, 258, 387 and 403 nm in the second derivative spectra were chosen for the determination of quercetin. A linear relationship between derivative amplitudes and a concentration of compounds was found. Beer's law is obeyed over a concentration range of 2-20 $\mu g.mL^{-1}$ for both drug components. The method is rapid, simple, sensitive and does not require a separation step.

INTRODUCTION

Flavonoids constitute one of the largest groups of naturally occurring phenols. They occur in a variety of structural forms and include several groups as flavones, flavonols, anthocyanidins, isoflavonones, flavanones, dihydroflavonols, bioflavonoids, chalcones and aurones[1].

The interest in the flavonoids is due to their significant physiological activities suach as antibacterial, antiinflammatory, analgesic, local anesthetic, antiallergic, antiatherosclerotic, antimetastatic and antiviral[2]. Because of these diverse physiological activities and the increasing interest by the pharmaceutical industry, the qualitative and quantitative composition of the flavonoids are of great importance for their standardization.

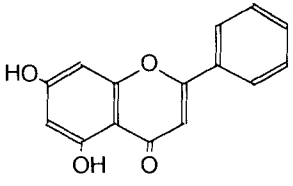
Flavonoids as a large heterogenous group of substances, imposes demands upon the techniques that are used for studying a single component, a class of compounds or the totality of them.

A quantitative analysis of the total content of flavonoids is based on UV spectrophotometry through the formation of coloured complex with some reagents as $AlCl_3$, $SbCl_3$, $ZrOCl$[3-5]. Paper chromatography (PC) was mainly used in the past for a separate determination of flavonoids[6], but PC lacks the resolution, speed and the quantitative accuracy. Thin-layer chromatography[7-8] offers greater resolution and speed than PC, but also suffers from quantitative accuracy. Gas chromatography (GC) is a fast, efficient and accurate technique for the analysis of complex mixtures and it has been applied to separate phenolic compounds[9,10]. However, GC requires a derivatization step, thermal degradation may occur and higher-molecular-weight compounds cannot be analyzed. In 1974, Ward and Pelter[11] published the first application of high-performance liquid chromatography (HPLC) to flavonoid analysis, and since then researchers have applied HPLC analysis to flavonoids in various plant samples[12-17]. Although HPLC provides a possibility for a sensitive and accurate procedure for the determination of flavonoids, the investigated sample required fractionation and purification.

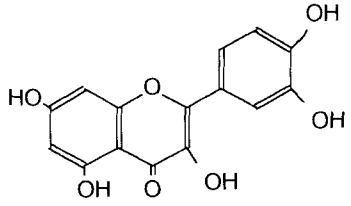
With tendency to eliminate preliminary clean-up procedure of extracts, it was decided to examine the possible application of UV derivative spectrophotometric method for a simultaneous determination of model mixtures of flavonoids. However, there is no data about a separate derivative spectrophtometric determination of flavonoids in the available literature.

Derivative spectrophotometry possesses different advantages including precise determination of λ_{max}, quantitative determination in presence of turbidity, enhancement of minor spectral features found on a broad background and quantitative separation of overlapping absorption peaks. It has been successfully used for the analysis of pharmaceutical dosage forms and for the determination of drugs alone or in mixtures[18-23].

Flavonoids, quercetin (3,5,7,3',4'- penthahydroxy flavone) and chrysin (5,7-dihydroxy flavone), which possess some physiological activities (spasmolytic, antiinflammatory, cytostatic, antiviral), commonly occur in medicinal plants extracts[24-26]. Quercetin is commonly prescribed in combination with ascorbic acid for the treatment of disease states characterised by capillary bleeding associated with increased capillary fragility[27].



Chrysin



Quercetin

Propolis is a resinous hive product, collected by bees, which contains a cosiderable number of flavonoid aglycones[28]. Interest in the propolis flavonoids was stimulated mainly by the suggestion that they are responsible for the physiological activity of propolis, especially the antibacterial, fungicidal and local anaesthetic activities[29].

The aim of the present study was to develop simple, sensitive and rapid UV-derivative spectrophotometric methods for the simultaneous determination of two flavonoids in the synthetic mixture (chrysin - quercetin), without a prior separation. The proposed methods were applied to determine both flavonoids in propolis ethanolic extract.

EXPERIMENTAL

Reagents and standard solutions

All solvents and reagents were of analytical reagent grade. Pharmaceutical grade standards, chrysin and quercetin, were purchased from Aldrich. Stock solutions of chrysin and quercetin (0,1 $mg.mL^{-1}$) were prepared in ethanol. Stock solutions were kept at 4°C and in these conditions they were stable for 5 days. Series of working standards of investigated flavonoids (2-20 $\mu g.mL^{-1}$) were obtained by dilution and mixing of the stock solutions.

Apparatus

Spectra were recorded on a Perkin-Elmer Lambda 16 dual-beam UV-visible spectrophotometer that was interfaced to a PC computer running UVWinLab software.

The spectrophotometric conditions used were as follows: maximum wavelength = 500 nm; minimum wavelength = 200 nm; scan speed = 240 nm/min; wavelength interval = 1 nm; slit = 1 nm; smooth = 0.

Analytical procedure

We assayed several series of solutions:

a) a set containing 2, 4, 8, 12, 16 and 20 $\mu g.mL^{-1}$ chrysin and quercetin, separately;

b) mixtures of the two flavonoids containing increasing quercetin concentrations between 4 - 12 $\mu g.mL^{-1}$ plus a constant chrysin concentration of 4, 8

and 12 $\mu g.mL^{-1}$, increasing chrysin concentrations between 4 and 12 $\mu g.mL^{-1}$ plus a constant quercetin concentration of 4, 8 and 12 $\mu g.mL^{-1}$, and increasing chrysin concentrations plus decreasing concentrations of quercetin over the ranges 2 - 16 $\mu g.mL^{-1}$.

Determination of chrysin and quercetin in ethanolic extract of propolis

Crude propolis sample was cut into small pieces and extracted with 96% ethanol for 24h. The obtained extract was filtered and the solvent was evaporated under reduced pressure. A suitable quantity of dry resinous residue was weighed, dissolved in 96% ethanol in order to obtain ethanolic extract of propolis with concentration of 80 $\mu g.mL^{-1}$. To determine both flavonoids, the absorption spectra of the sample were recorded as described above. The concentration of chrysin was proportional to the amplitude of the first derivative signal at 256 nm ($^1D_{256}$), while the concentration of quercetin was proportional to the amplitude at 403 nm in the second derivative spectra ($^2D_{403}$).

RESULT AND DISCUSSION

Flavonoids, chrysin and quercetin, possessing a number of unsubstituted hydroxyl groups, are polar compounds and as the old adage "like dissolves like" suggests, are generally moderately soluble in polar solvents such as ethanol, methanol, butanol, etc[20]. Extraction of most flavonoids from plant material, is usually carried out with ethanol or methanol, thus all UV spectra are recorded in ethanol solutions.

In Figure 1 are present the UV absorption (zero-order) spectra of chrysin, quercetin and their mixture (8 $\mu g.mL^{-1}$ in all instances). For a UV analysis, chrysin and quercetin can be assayed directly at their maximum at 270 and 375 nm respectively, but in combination, in which they are usually in propolis and also in plant extracts, there is no wavelength where only one flavonoid absorbs. This is due to the large overlap of the spectral bands of the two components. However,

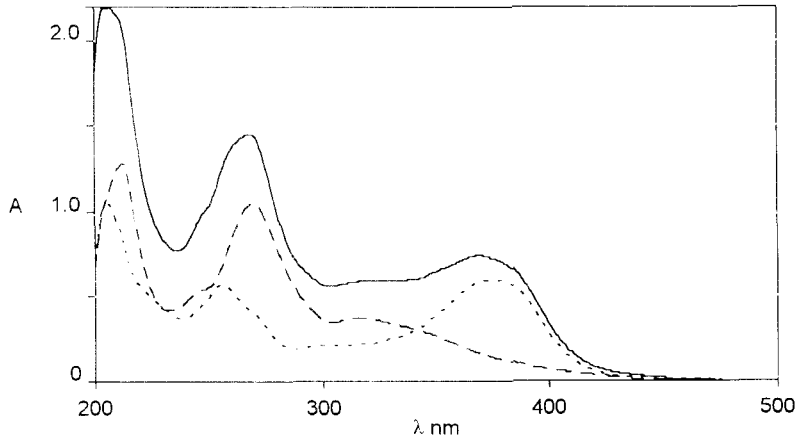


Figure 1. Absorption spectra of chrysin (– – –), quercetin (· · · · ·) and their mixture (———) (8 μg.mL^{-1} in all instances)

the first and second derivative spectra have spectral features that can be used for the simultaneous determination of these two components.

The most common derivative approaches for the construction of analytical calibration graphs are "peak to peak" and "peak to baseline" measurements (generally called graphical measurements) and " zero-crossing" measurements[31]. The suitability of different graphical and zero-crossing measurements were investigated in two derivative modes for both compounds. The measurements selected were those which exhibit the best linear response, give a zero or near zero intercept on the ordinate of the calibration graph, and are less affected by the concentration of other component.

The first and second-derivative spectra of chrysin and quercetin (8 μg.mL^{-1}) are shown in Figure 2. In the first-derivative mode, the zero-crossings of quercetin occur at 256 nm, while those of chrysin occur at 233 and 269 nm. In the second-derivative spectra, 268 nm is the zero-crossing wavelength of quercetin and 241 and 258 nm are those of chrysin.

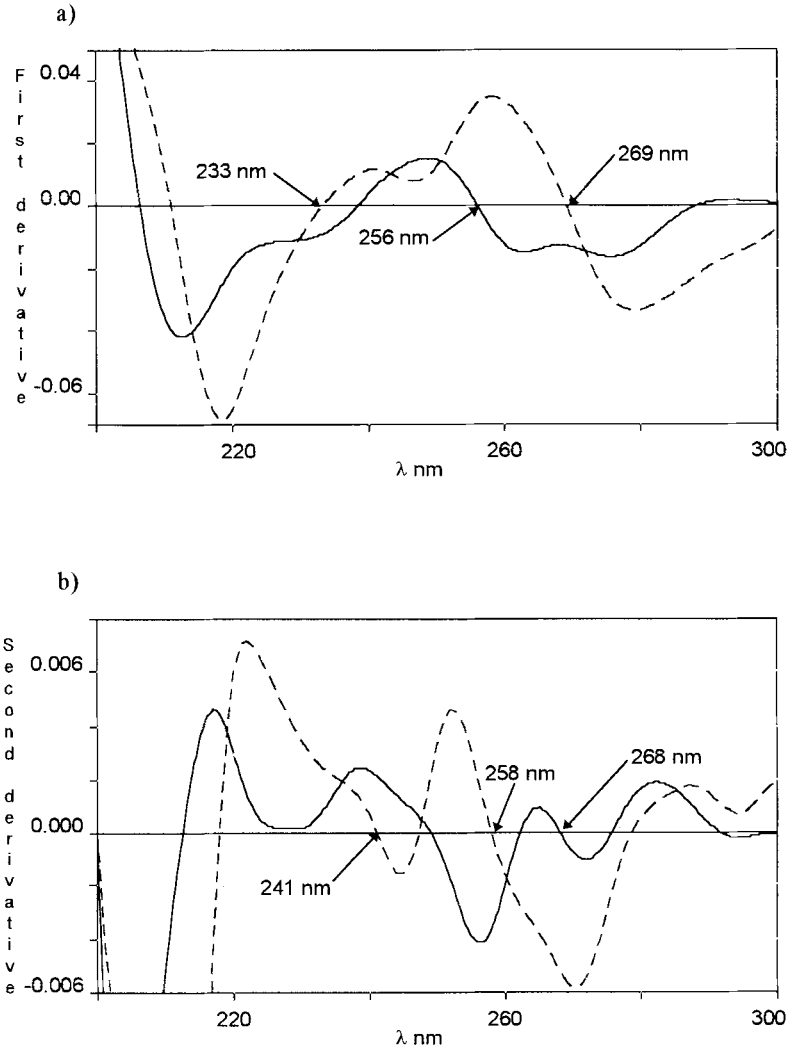


Figure 2. (a) First and (b) second derivative spectra of chrysin (– – –) and quercetin (——) (flavonoids concentracion 8 μg.mL^{-1})

First-derivative mode

Figure 3(a) presents a series of first-derivative spectra of mixtures of quercetin (12 $\mu g.mL^{-1}$) plus increasing amounts of chrysin (4, 8 and 12 $\mu g.mL^{-1}$), while Figure 3(b) shows a series of first-derivative spectra of mixtures of chrysin ($8\mu g.mL^{-1}$) plus increasing quantities of quercetin (4, 8 and 12 $\mu g.mL^{-1}$).

Denoting the height of the first derivative at 256 nm, the zero-crossing wavelength of quercetin, by h1 and the heights at 233 and 269 nm, the zero-crossing wavelengths of chrysin, by h2 and h3, we found that h1, h2 and h3 were proportional to the concentration of chrysin and quercetin, respectively. Moreover, the values of h1, h2 and h3 were not affected by the presence of quercetin and chrysin, respectively. It is important to point out distinct isosbestic points at 256 nm (Figure 3b) and at 233 and 269 nm (Figure 3a), which are corresponding to the zero-crossing wavelengths of the two flavonoids. At these wavelengths any change of the concentration of quercetin and chrysin has no effect on the separate determination of each flavonoid.

Second-derivative mode

In Figure 4(a) are reported a series of second-derivative spectra of mixtures of 12 $\mu g.mL^{-1}$ of chrysin and increasing concentrations of quercetin (4, 8 and 12 $\mu g.mL^{-1}$), while in Figure 4(b) are the second-derivative spectra of mixtures of 12 $\mu g.mL^{-1}$ of quercetin and increasing amounts of chrysin (4, 8 and 12 $\mu g.mL^{-1}$). Measurements at the "zero-crossing" wavelengths were the most appropriate for resolving mixtures of these compounds, thus signal at 268 nm (h4 - zero-crossing point for quercetin) was proportional to the chrysin content while the second derivative signal at 241 and 258 nm (h5 and h6 zero-crossing wavelengths for the chrysin signal) was proportional to the quercetin concentration.

By analyzing the second derivative spectra (figure 5), it was evident that the derivative signals $^2D_{387}$ (h7), $^2D_{403}$ (h8) and $^2D_{387,403}$ (h9) were specific for quercetin and gave possibility for graphical measures (base-line and peak-to-peak measurements).

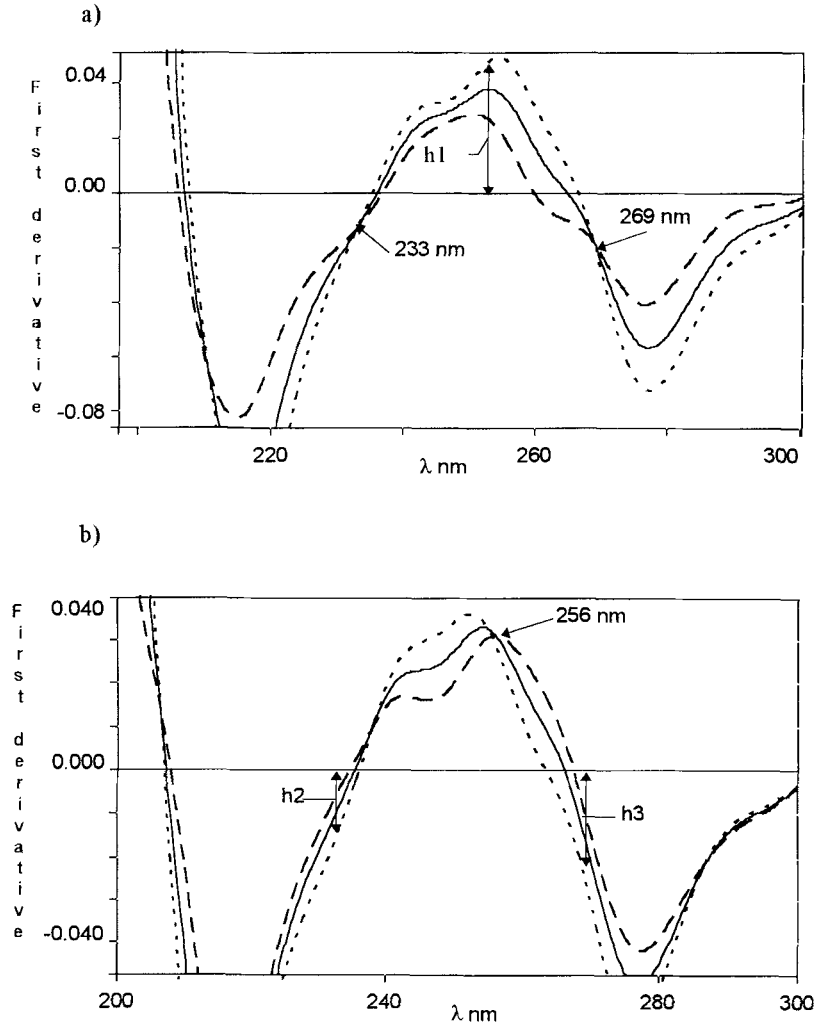


Figure 3. First derivative spectra of mixtures of chrysin and quercetin
a) Quercetin concentration 12 μg.mL^{-1} plus increasing amounts of chrysin: 4 (– – –); 8 (———) and 12 μg.mL^{-1} (· · · · ·)
b) Chrysin concentration: 8 μg.mL^{-1} plus increasing amounts of quercetin:4 (– – –); 8 (———) and 12 μg.mL^{-1} (· · · · ·)

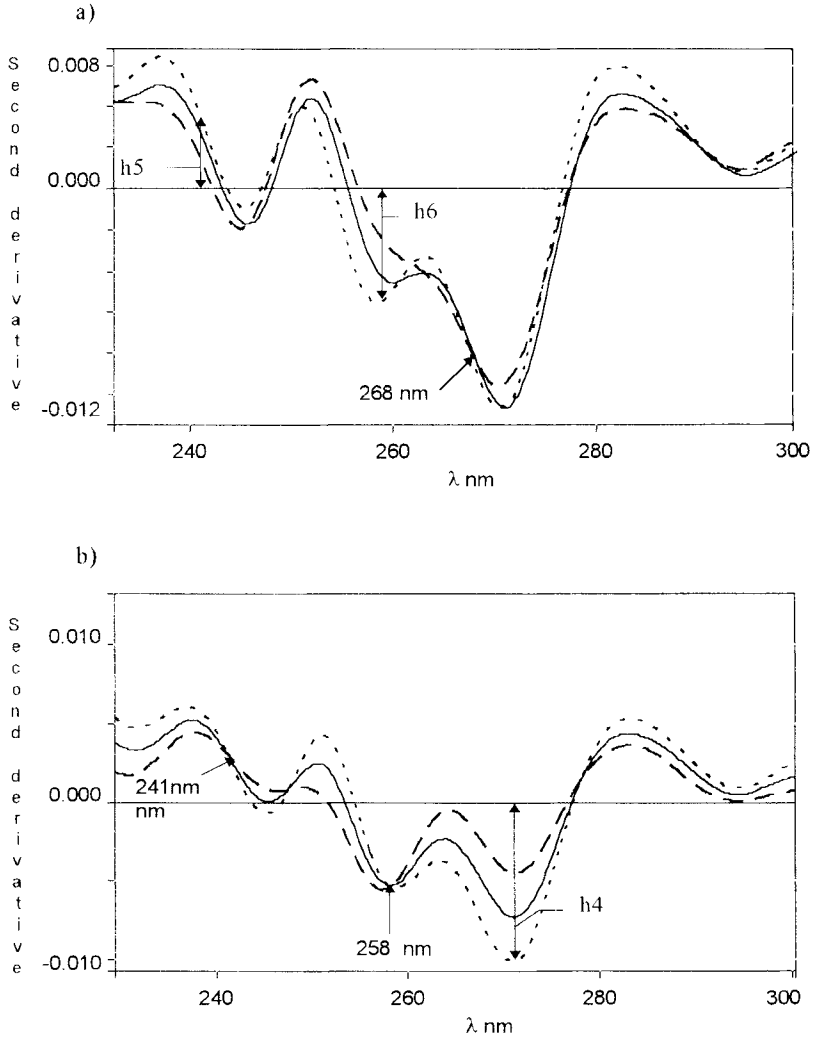


Figure 4. Second derivative spectra of mixtures of chrysin and quercetin
a) Chrysin concentration: 8 μg.mL^{-1} plus increasing amounts of quercetin:4 (– – –); 8 (———) and 12 μg.mL^{-1} (· · · · ·)
b) Quercetin concentration 12 μg.mL^{-1} plus increasing amounts of chrysin: 4 (– – –); 8 (———) and 12 μg.mL^{-1} (· · · · ·)

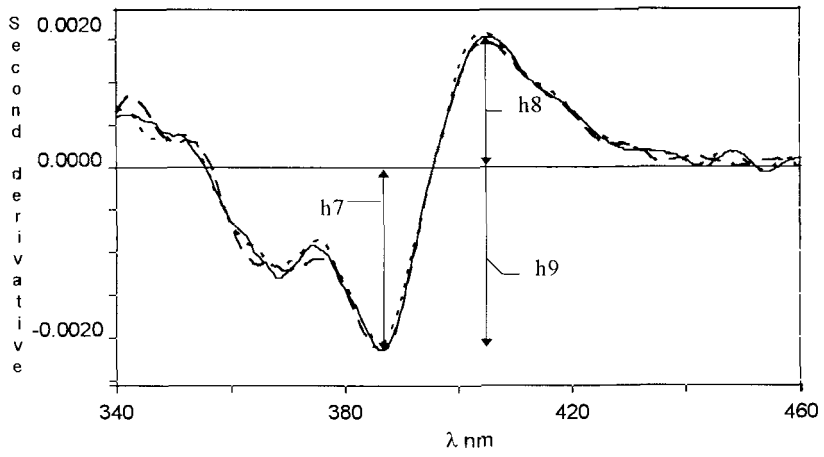


Figure 5. Set of second derivative spectra of 12 μg.mL^{-1} of quercetin plus increasing amounts of chrysin: 4 (– – –); 8 (———) and 12 (- - - - -) μg.mL^{-1}

Statistical analysis of results

Calibration graphs for chrysin were made by measuring the first derivative signal ($^1D_{256}$) and the second derivative signal ($^2D_{268}$) at the zero-crossing point for quercetin. On the other hand, calibration graphs for quercetin were made by measuring the first derivative signal ($^1D_{233}$ and $^1D_{269}$) and the second derivative signal ($^2D_{241}$ and $^2D_{258}$) at the zero-crossing point for chrysin, and also by measuring the amplitude from the minimum at 387 nm ($^2D_{387}$), from the maximum at 403 nm ($^2D_{403}$) and from peak-to-peak ($^2D_{387,403}$) of the second derivative spectra. The statistical data obtained from the different calibration graphs in the range 2-20 μg.mL^{-1} for both flavonoids, are given in Table 1.

The correlation coefficients of the regression equation are very close to unity and the intercepts on y-axis are very small. These obtained values indicate the good linearity of the calibration graphs and the adherence of the systems to Beer's law.

TABLE I

Linearity of the determination of chrysin and quercetin by the first and second derivative spectrophotometry

Compound	Signal measured		Intercept	Slope	Correlation coefficient
Chrysin	$^1D_{256}$	h1	$4.13x10^{-4}$ ($1.56x10^{-4}$)*	$4.00x10^{-3}$ ($2.38x10^{-5}$)**	0.9999
Quercetin	$^1D_{233}$	h2	$9.38x10^{-4}$ ($3.57x10^{-4}$)*	$1.06x10^{-3}$ ($3.61x10^{-5}$)**	0.9997
Quercetin	$^1D_{269}$	h3	$1.03x10^{-4}$ ($2.47x10^{-4}$)*	$1.49x10^{-3}$ ($3.95x10^{-5}$)**	0.9999
Chrysin	$^2D_{268}$	h4	$-8.66x10^{-5}$ ($4.71x10^{-5}$)*	$6.84x10^{-4}$ ($6.73x10^{-6}$)**	0.9998
Quercetin	$^2D_{241}$	h5	$6.41x10^{-5}$ ($3.39x10^{-5}$)*	$2.58x10^{-4}$ ($1.98x10^{-6}$)**	0.9998
Quercetin	$^2D_{258}$	h6	$3.98x10^{-5}$ ($4.59x10^{-5}$)*	$4.42x10^{-4}$ ($1.40x10^{-5}$)**	0.9999
Quercetin	$^2D_{387}$	h7	$6.35x10^{-5}$ ($2.72x10^{-5}$)*	$1.75x10^{-4}$ ($4.34x10^{-6}$)**	0.9998
Quercetin	$^2D_{403}$	h8	$1.33x10^{-5}$ ($2.78x10^{-6}$)*	$1.07x10^{-4}$ ($1.94x10^{-6}$)**	0.9997
Quercetin	$^2D_{387,403}$	h9	$8.47x10^{-5}$ ($4.78x10^{-5}$)*	$2.88x10^{-4}$ ($1.16x10^{-5}$)**	0.9998

n = 5; *Standard deviation of intercept; **Standard deviation of slope

To test accuracy and precision of all the proposed methods, a determination of the synthetic mixtures of chrysin and quercetin was carried out. The results are presented in Tables 2-4. Satisfactory results were obtained for the percentage of recovery (between 99.0 and 106.5%) and relative standard deviations (0.2 - 3.6%) for the determination of chrysin, by the first and second derivative measurements. On the other hand, the determination of quercetin gave the best results only by second derivative mode, measuring at 258 nm ($^2D_{258}$), (recoveries between 99.55 - 101.5% and RSD from 0.6 to 2.5%). All other proposed methods which were applied for the determination of quercetin showed

TABLE 2

Results obtained in the determination of chrysin in synthetic mixtures by using the proposed methods

Ratio Ch/Q	Chrysin $\mu g.mL^{-1}$	Quercetin $\mu g.mL^{-1}$	Chrysin recovery % (RSD %) $^1D_{256}$	$^2D_{268}$
0.125	2	16	102,2 (0.2)	106.5 (1.6)
0.333	4	12	102.5 (2.1)	99.0 (3.2)
1.00	8	8	100.2 (1.2)	99.1 (3.6)
3.00	12	4	100.9 (1.0)	102.1 (2.8)
8.00	16	2	100.1 (1.5)	100.7 (2.7)

n = 5

TABLE 3

Results obtained in the determination of quercetin in synthetic mixtures by using the first derivative - mode

Ratio Q/Ch	Chrysin $\mu g.mL^{-1}$	Quercetin $\mu g.mL^{-1}$	Quercetin recovery % (RSD%) $^1D_{233}$	$^1D_{269}$
8.00	2	16	97.1 (0.1)	103.0 (1.0)
3.00	4	12	97.5 (0.9)	101.0 (1.8)
1.00	8	8	97.7 (1.1)	107.5 (3.3)
0.333	12	4	116.6 (5.3)	118.8 (2.2)
0.125	16	2	155 (17.6)	138.6 (19.3)

n = 5

TABLE 4

Results obtained in the determination of quercetin in synthetic mixtures by using the second derivative - mode

Chrysin	Quercetin	Quercetin recovery % (RSD %)				
$\mu g.mL^{-1}$	$\mu g.mL^{-1}$	$^2D_{241}$	$^2D_{258}$	$^2D_{387}$	$^2D_{403}$	$^2D_{387,403}$
2	16	100.3	101.5	102.1	105.3	103.5
		(0.5)	(0.6)	(1.1)	(4.4)	(1.2)
4	12	100.2	101.1	97.2	102.2	99.2
		(2,2)	(0.8)	(1.5)	(3.2)	(2.2)
8	8	103.3	100.8	95.8	110.6	101.7
		(3.0)	(0.9)	(2.2)	(3.5)	(3.6)
12	4	123.3	100.9	80.6	123.6	97.5
		(14.7)	(2.5)	(5.4)	(14.8)	(3.2)
16	2	167.6	99.55	40.3	140.7	79.6
		(16.3)	(2.4)	(28.6)	(15.5)	(8.79)

n = 5

good results for mixtures where the ratio of the two components was 8.00, 3.00 and 1.00 (recoveries between 95.8 and 110.6%) (Tables 3 and 4). It is important to point out that graphical measurement "peak to peak" at 387 and 403 nm ($^2D_{387,403}$) gave satisfactory recovery (97,5%) also for mixture with ratio 0.333 of two components (Table 4).

Therefore, it can be deduced that the zero-crossing method was the most appropriate for resolving mixtures of these compounds and gave satisfactory results in all investigated mixtures where the ratio of the two components was from 0.125 to 8.00. For all above mentioned reasons we propose to determine chrysin by the first derivative spectrophotometry at 256 nm ($^1D_{256}$) and by the second derivative spectrophotometry at 268 nm ($^2D_{268}$), while quercetin by measuring the second derivative signal at 258 nm ($^2D_{258}$).

Analytical application of derivative methods to propolis sample

The proposed methods were applied for the determination of flavonoids, chrysin and quercetin, in ethanolic extract of propolis (EEP).

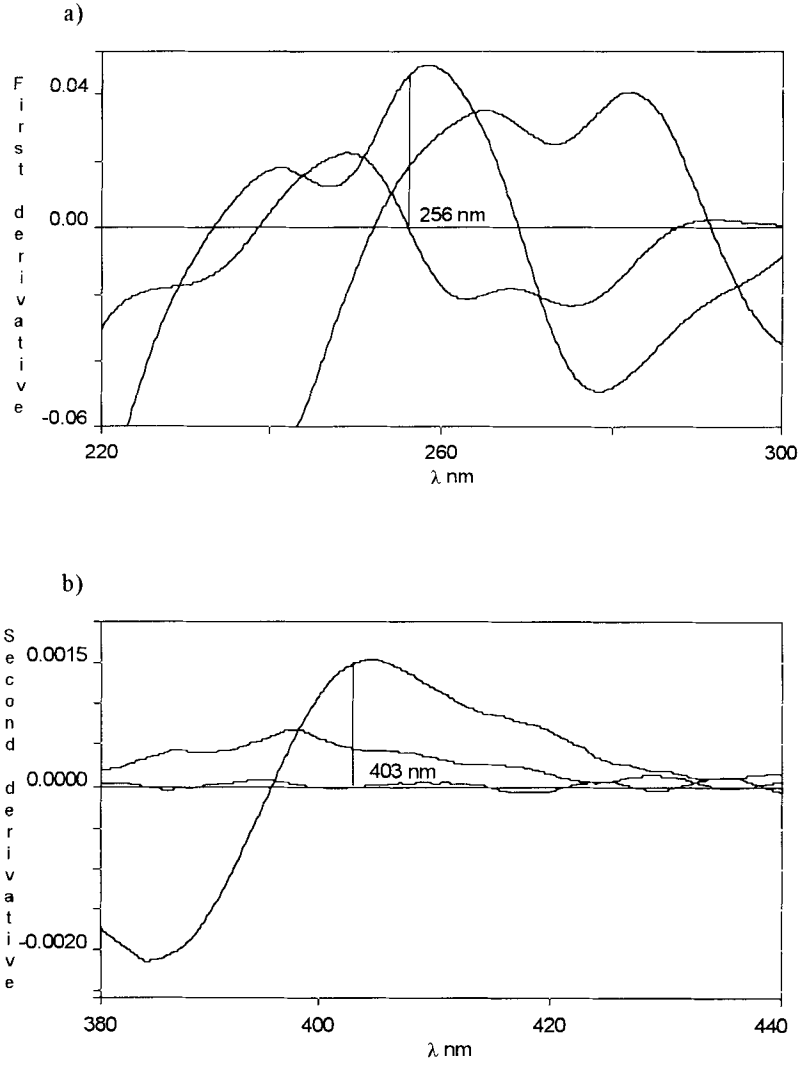


Figure 6. (a) First and (b) second derivative spectra of chrysin 12 $\mu g.mL^{-1}$ (------), quercetin 12 $\mu g.mL^{-1}$ (·····) and ethanolic extract of propolis 80 $\mu g.mL^{-1}$ (——)

TABLE 5

Results obtained in the determination of chrysin and quercetin in ethanolic extract of propolis (EEP) by using the proposed methods

Chrysin ($\mu g.mL^{-1}$) $^1D_{256}$				Quercetin ($\mu g.mL^{-1}$) $^2D_{403}$			
Added	Found	Recovery (%)	RSD (%)	Added	Found	Recovery (%)	RSD (%)
0	4.3907	/	/	0	3.3403	/	/
2	6.3984	100.12	1.59	2	5.4743	102.51	1.98
8	12.4836	100.75	0.35	8	12.0003	105.82	2.46
12	16.5956	101.25	0.20	12	15.7729	102.82	1.37

n = 3

Figure 6 shows the first (a) and second (b) derivative spectra of flavonoids (12 $\mu g.mL^{-1}$) and EEP (80 $\mu g.mL^{-1}$).

Considering the above investigated amplitudes, amplitude at 256 nm of the first derivative spectra was chosen for the quantitative determination of chrysin in ethanolic extract of propolis. This derivative signal gave very good results in the determination of chrysin in the investigated synthetic mixtures. On the other side, although the amplitude of the signal at 258 nm in the second derivative spectra gave the best results for the determination of quercetin in synthetic mixtures, we couldn't use it, because propolis extract contains other constituents which significantly interfere at this wavelength. For these reasons, the derivative signal at 403 nm in the second derivative spectra was chosen for the quantitative determination of quercetin in EEP, because of the absence of interferences from other propolis constituents at this wavelength (Figure 6 b).

It is important to point out that we investigated all possible amplitudes in the first and second derivative spectra for the quantitative determination of these two flavonoids in synthetic mixtures. On the other side, propolis extracts, as well

as plant extracts differ among each other in regards to their sources, amounts of flavonoids and ways of extracts production. Therefore, it is recommended that the validity of these proposed amplitudes for the quantitative determination of chrysin and quercetin in synthetic mixtures, have to be verified for each real sample, before applying the derivative methods.

In order to verify the accuracy of the described method, recovery experiments by the standard addition method were carried out. The obtained results (Table 5), recoveries between 100.12 - 101.25 % (RSD from 0.20 to 1.59%) for chrysin and 102.51 - 105.82 % (RSD from 1.37 to 2.46%) for quercetin, were satisfactory and confirmed the accuracy of the method.

CONCLUSION

The results demonstrated that the first and second derivative spectrophotometric methods can be applied to the simultaneous determination of flavonoids, chrysin and quercetin without significant mutual interference. These methods are simple, sensitive, rapid and do not require any preliminary purification or treatment of the samples containing these two flavonoids.

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