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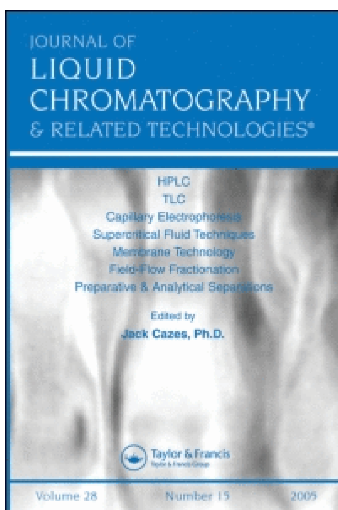

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Development and Validation of a Multidetector HPLC Method for the Determination of Antioxidant Flavonoids of some *Hypericum* L. Species

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Abstract: A multidetector approach for the HPLC analysis of methanolic extracts of three *Hypericum* species is performed under isocratic conditions through a C_{18} (ODS) analytical column. Variable wavelength UV-VIS detector (λ_{max}: 230 nm), fluorescence detector (ex: 250 nm em: 450 nm), and a programmable electrochemical detector (+1.30 V vs. Ag/AgCl) were employed simultaneously for detection. The mobile phase used was a combination of methanol and 0.01 M orthophosphoric acid (pH 7) (50:50, v/v). Retention time values were assigned relatively to those of the standards, and the flavonoid contents were quantified by the standard addition method. Rutin, quercetin-3′-glucoside (isoquercitrin), luteolin-4′-glucoside, quercetin-4′-glucoside, quercetin, naringenin, luteolin, and apigenin were chosen as the model compounds to undergo the validation studies with the three different detector systems. For the comparison of the detector performances, analytical method validation parameters were studied and displayed. Defined methods yielded pretty good results regarding linearity, precision (reproducibility), accuracy, limit of detection, and quantification.

Keywords: ECD, Flavonoids, FLD, HPLC analysis, *Hypericum*, UVD

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INTRODUCTION

Hypericum perforatum L., *Hypericum empetrifolium* Willd., *Hypericum triquetrifolium* Turra. are herbaceous perennial plants of the Hypericaceae family, which is distributed in Europe, Asia, Northern Africa, and America. This genus is represented by 400 species in the world and 77 species in Türkiye.[1] They have became very popular because of their reported beneficial controlled clinical trials, conducted during the past decade, and have confirmed the therapeutic use of alcoholic extracts of *H. perforatum* for the treatment of mild to moderate depressive disorders. Those pharmacological and therapeutic properties have been well documented and reviewed.[2–4]

HPLC analysis of different *Hypericum* species have been initially reported at various times.[5–16] Such approaches usually employed only one detector at a time such as; UV-Vis (UVD),[6–12,14–17] electrochemical (ECD),[5,14] fluorescence (FLD),[6,13,14] or mass detectors (LC/MS).[8,9,14,16,17] Thus, to our best knowledge, a HPLC method validation study, performing a comparison of three different detector systems simultaneously (HPLC-ECD, HPLC-UVD, and HPLC-FLD), for flavonoid analysis has not been reported yet. Flavonoids belong to a very rich group of molecules of various chemical structures; therefore, under isocratic conditions it would have been very hard to separate such a wide number of molecules simultaneously present in the extracts. Thus, only 8 flavonoids: rutin, isoquercitrin, luteolin-4′-glucoside, quercetin-4′-glucoside, quercetin, naringenin, luteolin, apigenin, were chosen as the model compounds to undergo the validation studies with the three different detector systems.

Briefly, the aim of our study was to make method validation studies and simultaneous detector performance comparisons for HPLC-ECD, HPLC-UVD, and HPLC-FLD systems to separate and quantify the model flavonoids in different three *Hypericum* species (*H. perforatum* L., *H. empetrifolium* Willd., *H. triquetrifolium* Turra.) present in Türkiye. Thus, we believe making an overview on the three detector systems, and the comparison of our obtained analytical method validation data for the *Hypericum* species will be a valuable contribution to the literature.

EXPERIMENTAL

Chemicals

Luteolin, naringenin, and quercetin-3′-glucoside (isoquercitrine) were obtained as standards from Biochemica. Quercetin dihydrate and apigenin were obtained from Aldrich. These commercially available flavonoids were donated by Assist. Professor Bintuğ Öztürk (Ege Univ.

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Faculty of Pharmacy, Türkiye). Luteolin-4-glucoside was isolated from the *Helichrysium* L. species by Professor Ali Hikmet Meriçli (İstanbul Univ. Faculty of Pharmacy, Türkiye). It was received from him as a gift and used without further purification. Rutin was obtained as a standard from Sigma. Solvents used for chromatography were methanol and phosphoric acid (HPLC ultragradient grade) supplied by J.T. Baker and Riedel-de Haen AG, respectively. Membranes (0.45 µm pore size) used for filtration of the samples were obtained from Sartorius AG (16555 Minisart©).

Apparatus

The liquid chromatographic system (Agilent 1100 series) supplied by SEM Company (Izmir, Türkiye) was equipped with an electrochemical detector (HP 1049-A, programmable electrochemical detector), a fluorescence detector (Agilent G1321A), a variable wavelength UV-VIS detector (Agilent G1314A), a pump (Agilent G1310A isocratic Pump), a manual injector (Agilent G1328A Rheodyne 7725İ) with 20 µL loop, and a chromatographic data processing software (HP ChemStation for LC Rev. A. 06. 03 [509]). The separation was performed using an octadecyl (C_{18}) column (Hichrom 5 C_{18}, 7.75×300 mm, 5 µm particle size).

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Plant Material

Fresh plants of *H. perforatum*, *H. triquetrifolium*, and *H. empetrifolium* from West Anatolia were used. Mainly, the aerial parts of the plants were selected. Voucher specimens of the plants were kept for the record in IZEF (Herbarium of Ege University, Faculty of Pharmacy, Department of Pharmaceutical Botany, http://www.izef.ege.edu.tr).

Procedure

Extraction of *Hypericum* Species

The crude drug was dried in the shade and finely powdered by a mill (Brabender OHG, Duisburg). A modified method of Wagner and Bladt was used for the extraction of the powdered plant.[18] Methanol at 80°C was used for soxhlation, using 750 mL methanol for 100 g crude drug and the extracts were dried in vacuo (yields are 25.94, 36.2, and 26.36%, respectively). After lyophilization (Labconco lyophilizateur, −50°C) of the extracts an approciate portion was weighed from each

and was dissolved in 4 mL of HPLC grade methanol. Dissolved extracts were then filtered through the membrane filters of 0.45 μm pore size (Sartorius AG - 16555 Minisart©) before injecting into the HPLC system.

Method Validation Process

There are no official guidelines on the correct sequence of validation experiments and the optimal sequence may depend on the method itself. Based on the experience cited in the literature,[19–24] for a liquid chromatographic method, the sequence in Table 3 has proven to be useful, according to which we proceeded with the full method validation for HPLC-ECD, HPLC-UVD, and HPLC-FLD systems. Therefore, the order of appearance of the studied parameters was as follows; specificity, linearity, precision (reproducibility), accuracy, limit of detection, and limit of quantification. The experimental methodology dealing with each individual validation parameter was as displayed in the table.

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Chromatographic Conditions

Chromatographic conditions were based on our previously submitted analytical method validation data of a similar group of compounds.[25] Briefly, separation of the model flavonoids was performed with a flow rate of 1 mL/min for 80 min, using an octadecyl (C_{18}) column (Hichrom 5 C_{18}, 7.75×300 mm, 5 μm particle size) at 20 ± 0.5°C. The absorbance of the flavonoids at 230 nm was monitored via a variable wavelength UV-Vis detector (UVD). For some of the standards (quercetin-4-glucoside, quercetin, naringenin, and luteolin), the fluorescence detector (FLD) was also applicable (ex: 250 nm em: 450 nm). Using the electrochemical detector (ECD), chronoamperometric detection was carried out at +1.30 V (vs. Ag/AgCl, 0.5 μA full scale) in the electrochemical flow cell. The solvents used and their proportions were as follows: methanol/0.01 M phosphoric acid (50/50 v/v). Both solvents were sonicated for degassing (ELMA LC 30/H ultrasonic bath) for 30 minutes before use. Each compound was tentatively identified by its unique retention time under the same conditions. Quantitative determinations were carried out by the external standard method based on peak heigths.

RESULTS AND DISCUSSION

Chromatograms of the Standards

Chromatograms of the flavonoid standards obtained with three different detectors; electrochemical (ECD), fluorescence (FLD), and UV-visible

(UVD) are displayed in Figure 1. As the standard solution, a mixture of flavonoids were prepared: 25 ppm rutin (A), 25 ppm isoquercitrin (B), 25 ppm luteolin-4′-glucoside (C), 25 ppm quercetin-4′-glucoside (D), 25 ppm quercetin (E), 25 ppm naringenin (F), 45 ppm luteolin (G), and 45 ppm apigenin (H). With ECD and UVD all standards could be detected, whereas with FLD only four of them (quercetin-4′-glucoside, quercetin, naringenin, and luteolin) were detectable. With FLD to detect all entire standards additional derivatization processes were necessary, as most of the flavonoids do not own native fluorescence properties. Rutin, isoquercitrin, and luteolin-4′-glucoside is best viewed with UVD. Best detector responses for quercetin, luteolin, and apigenin are obtained with ECD. Even though, quercetin-4′-glucoside, quercetin, naringenin, and luteolin were detectable with FLD, when compared to the other two detectors, due to the very low responses obtained, FLD isn't applicable for the detection of all entire flavonoids studied. With the other two applicable detectors (ECD and UVD) all flavonoids except quercetin-4′-glucoside show pretty much acceptable responses. The detector response characteristics of these compounds are summarized in the analytical method validation data.

The retention time (t_R) values assigned to the model flavonoids are as in Table 1. In accordance with the experimental HPLC conditions,

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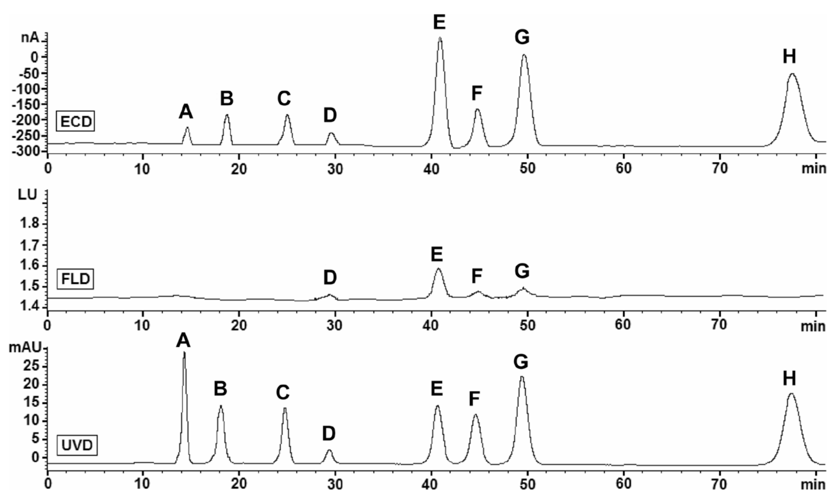

Figure 1. Typical chromatograms of the model flavonoid standards; rutin (A), isoquercitrin (B), luteolin-4′-glucoside (C), quercetin-4′-glucoside (D), quercetin (E), naringenin (F), luteolin (G), and apigenin (H) with electrochemical (ECD), fluorescence (FLD), and UV-Vis (UVD) detectors. Experimental conditions as in *Materials and Methods* section.

Table 1. Retention times obtained with different detectors for flavonoid standards. (ND* = Not Detectable) Experimental conditions as in *Matericals and Methods* section

Flavonoid Standard	t_R (UVD) (min)	t_R (FLD) (min)	t_R (ECD) (min)
Rutin	15.34	ND*	15.62
Isoquercitrin	18.03	ND*	18.34
Luteolin-4′-Glucoside	24.71	ND*	24.98
Quercetin-4′-Glucoside	29.30	29.42	29.59
Quercetin	40.59	40.70	40.87
Naringenin	44.55	44.74	44.82
Luteolin	49.41	49.57	49.62
Apigenin	77.49	ND*	77.59

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these model flavonoids leave the analytical column in an order of, rutin < isoquercitrin < luteolin-4′-glucoside < quercetin-4′-glucoside < quercetin < naringenin < luteolin < apigenin. Thus, the corresponding retention time values (t_R) are as of 15.34, 18.03, 24.71, 29.30, 40.59, 44.55, 49.41, and finally 77.49 minutes, respectively, with UVD. As the detectors are connected in series, there is a slight difference of t_R values for each compound, therefore, the t_R values for the remaining two detectors (FLD and ECD) are also displayed in Table 1. Because of detector response characteristics obtained and our previously submitted analytical method validation data of a similar group of compounds,[25] UVD is chosen as the optimum detector and the data obtained with it is used for the quantification of the model bioflavonoids.

Chromatograms of the Samples

Figure 2, displays a typical chromatogram of methanolic extracts of *H. triquetrifolium* studied with the three detector (ECD, FLD, and UVD) systems. When all samples are considered, the flavonoid content of this typical chromatogram is replicated for the two remaining species. Thus in qualitative means, quercetin-4′-glucoside (D), quercetin (E), and naringenin (F) are the common flavonoids present, where rutin (A) and apigenin (H) are rare compounds only present in *H. perforatum* and *H. empetrifolium*, respectively. Regarding the quantitative means, the amount of each flavonoid varies from sample to sample as expected and is displayed in Table 2.

The differences in the flavonoid profiles of the entire samples are summarized in Table 2. As can be seen, the amounts determined by HPLC analysis differs in each sample, rutin is only present in *H. perforatum.*

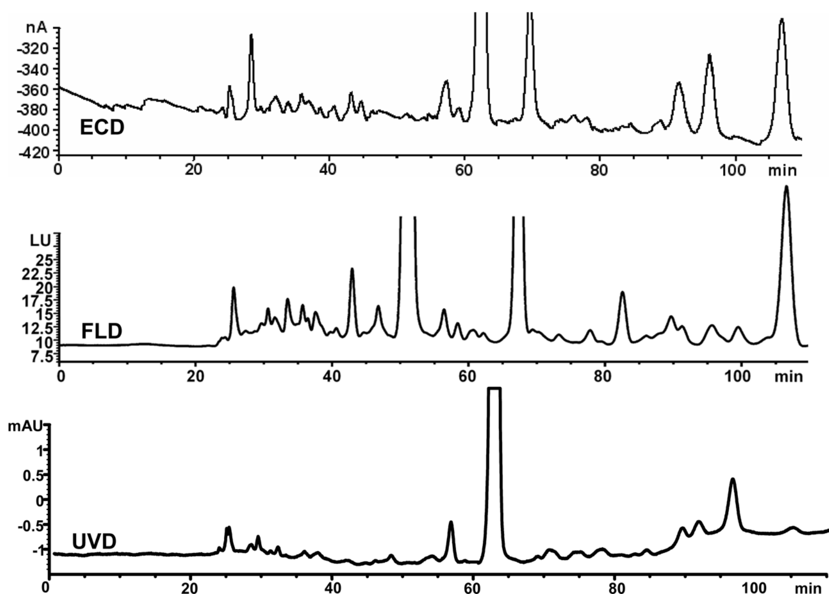

Figure 2. Typical chromatograms of the methanolic extracts of *H. Triquetrifolium*. Other conditions as in Figure 1.

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Isoquercitrin, luteolin-4′-glucoside, quercetin-4′-glucoside, quercetin, naringenin, and apigenin are determined in different samples with the highest/lowest amounts to be 4.56/3.12, 8.61/6.10, 83.97/46.53, 20.19/12.62, 24.56/14.56, and 16.11/11.04, respectively. Regarding luteolin, *H. empetrifolium* shows a similar trend of content at around ≅ 0.99 ppm levels while the compound is not detected in the remaining samples. The total flavonoid content is the highest in *H. perforatum* (150.10 ppm) and lowest in *H. empetrifolium* (99.71 ppm).

Analytical Method Validation

Analytical method validation data is discussed in Table 4a and 4b, which were obtained in accordance to the measurement methods summarized in Table 3. The validation parameters studied were specificity (with standards), linearity, precision, accuracy (Table 4a), limit of detection (LOD), and limit of quantification (LOQ) (Table 4b).

Linearity

Regarding the linearity of the obtained calibration plots, concentration ranges and the model flavonoids were as follows: 25–200 ppm,

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Table 2. Flavonoid profiles of *Hypericum* species

Methanolic extract	Flavonoids (ppm)								
	rutin	iso-quercitrin	luteolin-4′-glucoside	quercetin-4′-glucoside	quercetin	naringenin	luteolin	apigenin	Total
H. triquetrifolium	ND*	ND*	8.61	78.28	12.62	14.56	ND*	11.04	125.11
H. empetrifolium	ND*	3.12	6.10	46.53	19.78	23.19	0.99	ND*	99.71
H. perforatum	0.71	4.56	ND*	83.97	20.19	24.56	ND*	16.11	150.10

Table 3. Proposed sequence of analytial method validation experiments for a LC method

Validation Parameters	Measurement Methods
1. Specificity with standards	Sufficient separation of all compounds. Resolution factor >2.5
2. Linearity	Inject 5 standards containing the full working concentrations. Inject each standard 3 times. Average the peak area. Plot the averaged peak area vs. concentration. Calculate the linear regression.
3. Precision of the amounts	Inject a standard at three different concentrations 5 times. Calculate relative standard deviation of peak areas.
4. Accuracy	Spike a blank sample wit the analyte at three different concentrations. Calculate the deviation of the results obtained with the HPLC method to be validated with the true value.
5. Intermediate precision	Inject 3 standards at different concentrations over 15 working days. The analysis should be conducted by 3 different operators using columns from 3 different batches. Measure the precision of amounts.
6. Limit of detection (LOD)	Inject a standard with a concentration close to the detection limit 3 times. Average signal height and baseline noise. LOD = 3 × signal height × standard amount/baseline noise
7. Limit of quantitation (LOQ)	Specify a precision limit for the amount at the limit of quantitation. Prepare six standard solutions with the amounts in the range from the expected limit of quantitation to 20 times this amount. Inject all samples 6 times and calculate the standard deviations of the amounts. Plot the standard deviations versus the amounts. Take the specified standard deviation at the corresponding LOQ amount from the plot
8. Specificity with real samples	Use samples with analytes. Check peak purity with a diode-array detector and/or a mass selective detector. Run the sample under different chromatographic columns and/or with different columns.

(*Continued*)

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Table 3. Continued

Validation Parameters	Measurement Methods
9. Ruggedness	Check precision and accuracy in different laboratories
10. Robustness	Systematically change chromatographic conditions. Examples: column temperature, flow rate, gradient composition, pH of mobile phase, detector wavelength. Check influence of parameters on separation and/or peak areas.

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12.5–100 ppm, 12.5–100 ppm, 13–400 ppm, 12.5–100 ppm, 12.5–100 ppm, 11.48–200 ppm, 11.48–200 ppm for rutin (R), isoquercitrin (I), luteolin-4′-glucoside (L4), quercetin-4′-glucoside (Q4), quercetin (Q), and naringenin (N) and luteolin (L) and apigenin (A), respectively. When calibration plots and R^2 values were taken into consideration, mostly ECD displayed more linear responses such as; 0.9999, 0.9755, 0.9997, 0.9643, 0.9952, and 0.9998 for R, I, L4, Q4, Q, and N, respectively. For L and A, UVD performed more linearly with the R^2 values being 0.9942 and 0.9939. Even though the results of the FLD were summarized in the same table, these results were not taken into account, as obviously they had poorer linearity characteristics.

Precision

Precision was studied as the precision of the amounts. Multi-injections of 11.48 (L and A), 12.5 (I, L4, Q and N), 13 (Q4), 25 (R) ppm standards ($n = 10$) were performed for each detector, respectively. FLD was applicable to only Q4, Q, N, and L, while with UVD and ECD all entire standards were detectable. When SD values of the detector responses were taken into account, the precision of the detectors increased in the order FLD > UVD > ECD. Calculated "mean $\pm$ SD" values of the entire standards obtained by each detector are summarized in Table 4a. SD values varied in the range $\pm$ 0.01 to 0.03, 0.01 to 0.04, and 0.05 to 0.12 for FLD, UVD, and ECD, respectively.

Accuracy

Accuracy was checked with a spiked blank sample. The very same concentration values of standards used during Precision studies were

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Table 4a. Specifity, linearity, precision and accuracy of the validated method for different detectors. Experimental conditions as in *Experimental* section

Validation Parameters	Method	Experimental Results		
		ECD	UVD	FLD
Specifity (with standards)	Sufficient separation of all compounds, resolution factor > 2.5	$R > 2.5$	$R > 2.5$	$R > 2.5$
Linearity	Calibration plots were obtained for the range 12.5 to 200 ppm at different concentrations for each compound.			
rutin		$y = 1.7543x + 6.1419$ $R^2 = 0{,}9999$	$y = 31.878x - 111{,}438$ $R^2 = 0{,}9971$	ND*
isoquercitrin		$y = 4{,}6138x - 28{,}275$ $R^2 = 0{,}9755$	$y = 0{,}7992x - 1{,}68$ $R^2 = 0{,}9509$	ND*
luteolin-4′-glucoside		$y = 3{,}9674x - 18{,}54$ $R^2 = 0{,}9997$	$y = 0{,}9708x - 2{,}72$ $R^2 = 0{,}9993$	ND*
quercetin-4′-glucoside		$y = 1{,}0832x + 5{,}855$ $R^2 = 0{,}9643$	$y = 0{,}185x + 0{,}645$ $R^2 = 0{,}9507$	$y = 0{,}236x + 0{,}880$ $R^2 = 0{,}9706$
quercetin		$y = 16{,}221x - 31{,}145$ $R^2 = 0{,}9952$	$y = 1{,}005x - 3{,}145$ $R^2 = 0{,}9913$	$y = 0{,}2685x - 0{,}91$ $R^2 = 0{,}9921$
naringenin		$y = 2{,}6836x - 4{,}195$ $R^2 = 0{,}9998$	$y = 0{,}426x - 2{,}255$ $R^2 = 0{,}9953$	$y = 0.312x + 0.483$ $R^2 = 0.9936$

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luteolin		$y = 6,3441x + 12,505$ $R^2 = 0,9733$		$y = 0,7569x - 0,765$ $R^2 = 0,9942$		$y = 0,9674x - 1,540$ $R^2 = 0,9962$	
apigenin		$y = 4,1658x + 36,405$ $R^2 = 0,9254$		$y = 0,5658x - 0,24$ $R^2 = 0,9939$		ND*	
Precision	Multinjection (n = 10) of R (25 ppm), I (12.5 ppm), L4 (12.5 ppm), Q4 (13 ppm), Q (12.5 ppm), N (12.5 ppm), L (11.48 ppm), A (11.48 ppm), standards. SD of results calculated.	R (24.90±0.06 ppm)		R (24.91±0.01 ppm)		ND*	
		I (12.38±0.05 ppm)		I (12.32±0.02 ppm)		ND*	
		L4 (12.47±0.05 ppm)		L4 (12.40±0.03 ppm)		ND*	
		Q4 (12.90±0.06 ppm)		Q4 (12.83±0.02 ppm)		Q4 (12.03±0.01 ppm)	
		Q (12.20±0.07 ppm)		Q (12.10±0.03 ppm)		Q (12.21±0.02 ppm)	
		N (12.46±0.10 ppm)		N (12.36±0.03 ppm)		N (12.33±0.02 ppm)	
		L (11.36±0.11 ppm)		L (11.04±0.04 ppm)		L (10.71±0.03 ppm)	
		A (11.33±0.12 ppm)		A (11.29±0.02 ppm)		ND*	
		Detected	%Rel. Error	Detected	%Rel. Error	Detected	%Rel. Error
Accuracy	Blank sample spiked with the analyte at lowest concentration. Deviation of the results were calculated. R (25 ppm), I (12.5 ppm), L4 (12.5 ppm), Q4 (13 ppm), Q (12.5 ppm), N (12.5 ppm), L (11.48 ppm), A (11.48 ppm)	24.87±0.09 ppm	−0.52	24.71±0.02 ppm	−1.66	ND*	ND*
		12.08±0.11 ppm	−3.36	12.28±0.03 ppm	−1.76	ND*	ND*
		12.45±0.09 ppm	−0.40	12.11±0.03 ppm	−3.12	ND*	ND*
		12.89±0.09 ppm	−0.85	12.43±0.05 ppm	−4.39	11.94±0.02 ppm	−8.15
		11.67±0.21 ppm	−6.64	11.90±0.04 ppm	−4.80	12.18±0.04 ppm	−2.56
		11.96±0.19 ppm	−4.32	12.23±0.04 ppm	−2.16	12.26±0.03 ppm	−1.92
		10.86±0.22 ppm	−5.40	11.01±0.04 ppm	−4.09	10.56±0.04 ppm	−8.01
		11.23±0.18 ppm	−2.18	11.02±0.03 ppm	−4.00	ND*	ND*

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Table 4b. LOD and LOQ of the validated method for different detectors. Experimental conditions as in *Experimental* section

Validation Parameters	Method	Experimental Results					
		ECD		UVD		FLD	
Limit of Detection (LOD) (ppm)	LOD = [(B_{av} + 3 SD) − b] a^{-1}	rutin	0.49	rutin	0.40	rutin	ND*
		isoquercitrin	0.87	isoquercitrin	0.17	isoquercitrin	ND*
	B_{av} = Baseline (average)	luteolin-4′-glucoside	0.76	luteolin-4′-glucoside	0.16	luteolin-4′-glucoside	ND*
	SD = Standard deviation of baseline (n = 3)	quercetin-4′-glucoside	0.22	quercetin-4′-glucoside	1.03	quercetin-4′-glucoside	0.14
	b = intercept	quercetin	0.27	quercetin	0.16	quercetin	0.38
	a = slope of calibration plot	naringenin	0.59	naringenin	0.35	naringenin	0.05
		luteolin	0.16	luteolin	0.27	luteolin	0.09
		apigenin	0.19	apigenin	0.27	apigenin	ND*
Limit of Quantification (LOQ) (ppm)	LOQ = [(B_{av} + 10 SD) − b] a^{-1}	rutin	1.49	rutin	1.39	rutin	ND*
		isoquercitrin	1.25	isoquercitrin	0.59	isoquercitrin	ND*
		luteolin-4′-glucoside	1.21	luteolin-4′-glucoside	0.50	luteolin-4′-glucoside	ND*
		quercetin-4′-glucoside	2.35	quercetin-4′-glucoside	2.03	quercetin-4′-glucoside	0.56
		quercetin	0.37	quercetin	0.41	quercetin	0.97
		naringenin	1.25	naringenin	0.44	naringenin	0.21
		luteolin	0.44	luteolin	0.51	luteolin	0.32
		apigenin	0.62	apigenin	0.50	apigenin	ND*

spiked and the found concentration values with the %relative errors were displayed in Table 4a. Regarding the Relative error values, a similar increasing trend in the accuracy was observed; FLD > UVD > ECD.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ values were calculated according to the equations in Table 4B. With ECD, the lowest/highest LOD values are 0.16/0.87 ppm for L and I, respectively. Similarly, with UVD, 0.16/1.03 ppm values are calculated for L4, Q, and Q4, respectively. Even though FLD is not applicable to four of the standards, 0.05/0.38 ppm was the lowest/highest LOD values calculated for N and Q, respectively.

Regarding the LOQ values, the lowest/highest values with ECD 0.37/2.35 ppm for Q and Q4, with UVD 0.41/2.03 ppm were calculated for Q and Q4, respectively. Although four of the standards were not detectable with FLD, the lowest/highest values were found to be 0.21/0.97 ppm for N and Q, respectively.

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CONCLUSIONS

Briefly, the current paper is focused on the HPLC analysis of methanolic extracts of three *Hypericum* species (*H. perforatum*, *H. empetrifolium*, *H. triquetrifolium*), performed under isocratic conditions. Even though methanolic extracts contain a wide spectrum of components, a group of compounds, the flavonoids, with a strong antioxidant activity were mainly studied. On the other hand, flavonoids belong to a very rich group of molecules of various chemical structures; therefore, under isocratic conditions it would have been very hard to separate such a wide number of molecules simultaneously present in the extracts. Thus, only eight flavonoids: rutin, isoquercitrin, luteolin-4′-glucoside, quercetin-4′-glucoside, quercetin, naringenin, luteolin, and apigenin were chosen as the model compounds to undergo the full validation studies with the three different detector systems. The defined methods yielded good results regarding linearity, precision (reproducibility), accuracy, limit of detection, and quantification.

To the best of our knowledge, a HPLC method validation study to perform a simultaneous comparison of three different detector systems (ECD, UVD, and FLD) for flavonoid analysis has not yet been reported. Therefore, the analytical method validation results of the paper may be a contribution to the literature.

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