



Original scientific paper

DOI: 10.17508/CJFST.2021.13.2.06

## Spectrophotometric Folin-Ciocalteu and Aluminium Chloride Method Validation for the Determination of Phenolic Acid, Flavan-3-ol, Flavonol, and Anthocyanin Content

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### ARTICLE INFO

#### Article history:

Received: November 6, 2020

Accepted: March 22, 2021

#### Keywords:

total polyphenols

total flavonoids

validation

### ABSTRACT

Spectrophotometric methods for the determination of total polyphenols and total flavonoids are often used because they are simple, sensitive, and precise. Before using these methods, they must be validated to ensure the quality of the obtained data and to prove that the method is suitable for its purpose. The aim of this study was to validate the spectrophotometric methods for total polyphenols and total flavonoids. The method for total polyphenols has been validated according to polyphenol standards, which belong to the groups of phenolic acids (*p*-hydroxybenzoic acid, sinapic acid, ferulic acid), flavan-3-ols ((-)-epicatechin), flavonols (myricetin, kaempferol), and anthocyanins (cyanidin-3-glucoside). For the validation of the method for total flavonoids, the same polyphenol standards belonging to flavan-3-ols, flavonols, and anthocyanins have been used. The validation parameters were linearity, sensitivity, limit of detection, limit of quantification, precision, and accuracy. The results of the validation for both methods were in acceptable ranges, except for kaempferol in the method for total flavonoids. In conclusion, these methods can be used for the determination of total polyphenols and total flavonoids by using calibration curves of studied polyphenol standards, except for the kaempferol standard in the method for total flavonoids, due to lower sensitivity.

### Introduction

Polyphenols are a large group of secondary plant metabolites (Manach et al., 2004). They are present in different plants and vegetables (Manach et al., 2004). Numerous polyphenol groups can be found in different fruit and vegetables, like phenolic acids (*p*-hydroxybenzoic acid, ferulic acid, sinapic acid), flavan-3-ols (epicatechin), flavonols (kaempferol, myricetin), anthocyanins (cyanidin), and others (Manach et al., 2004; Ramassamy, 2006). Many studies have shown their positive effects on human health (Camouse et al., 2005; Kampa et al., 2007; Pandey et al., 2009). Due to their positive properties, polyphenols have been widely investigated in various samples (Klimzcak et al., 2007; Turkmen et al., 2006; Paško et al., 2009). Various methods have been used to determine the polyphenol profile in samples, like chromatographic

methods or spectrophotometric methods (Tsao and Yang, 2003).

Methods that have been widely used are spectrophotometric methods, like the method for the determination of total polyphenols (Folin-Ciocalteu method) or the method for the determination of total flavonoids (Singleton et al., 1999; Zhishen et al., 1999). These methods are economical and simple, but they are not specific. Polyphenols which are determined with these methods are total polyphenols and total flavonoids. To assure the quality of the results, methods have to be validated (Araujo, 2009; González and Herrador, 2007).

Parameters for the validation include linearity, sensitivity, limit of detection, limit of quantification, precision, accuracy, and others. In order to evaluate the linearity and sensitivity of the method, it is necessary to construct a calibration line in order to obtain a linear equation of that line and

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the coefficient of determination (Araujo, 2009; González et al., 2007; Zwanziger et al., 1998). Calibrated methods can then be used for the determination of polyphenols in different samples (Klimczak et al., 2007; Paško et al., 2009; Turkmen et al., 2006). The limit of detection is the lowest concentration of the analyte that can be detected in the sample using an analytical method. The limit of quantification is the lowest concentration of the analyte that can be determined quantitatively by an analytical method (Taverniers et al., 2004). The limit of quantification is the level of analyte at which it can be accurately and precisely determined (Krull et al., 1999). In our earlier study (Matić et al., 2017), we validated the method for total polyphenols using the mentioned validation criteria for several polyphenols (gallic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, (+)-catechin, procyanidin B1 and B2, quercetin, quercetin-3-rutinsoide, phloretin, and phloretin-2-glucoside) and the method for total flavonoids for the same polyphenols, except for phenolic acids ((+)-catechin, procyanidin B1 and B2, quercetin, quercetin-3-rutinsoide, phloretin and phloretin-2-glucoside). This study is the continuation of that work.

The aim of this study was to validate the spectrophotometric methods for total polyphenols and total flavonoids according to polyphenol standards, which belong to the groups of phenolic acids (*p*-hydroxybenzoic acid, sinapic acid, ferulic acid), flavan-3-ols ((-)-epicatechin), flavonols (myricetin, kaempferol), and anthocyanins (cyanidin-3-glucoside). The validation was carried out using validation parameters of linearity, sensitivity, limit of detection, limit of quantification, precision, and accuracy.

## Materials and methods

### *Chemicals and reagents*

Chemicals were purchased from J.T. Baker (Deventer, Netherlands) (methanol HPLC grade), then from Gram-mol (Zagreb, Croatia) (sodium carbonate, sodium nitrite), Kemika (Zagreb, Croatia) (Folin–Ciocalteu reagents), Carlo Erba (Chaussée du Vexin, France) (sodium hydroxide pellets), and Sigma-Aldrich (St. Louis, USA) (aluminium chloride anhydrous). Polyphenol standards were purchased from Sigma-Aldrich (St. Louis, USA) (*p*-hydroxybenzoic acid, (-)-epicatechin, myricetin, kaempferol, ferulic acid), Extrasynthese (Lyon, France) (cyanidin-3-

glucoside), and Acros Organics (New Jersey, USA) (sinapic acid).

### *The preparation of polyphenol standards*

Stock solutions of *p*-hydroxybenzoic acid, sinapic acid, ferulic acid, (-)-epicatechin, myricetin, and kaempferol were prepared in methanol. The concentration of the stock solution of cyanidin-3-glucoside was prepared in 0.1% HCL in methanol. The stock solutions were diluted for the preparation of calibration curves and the determination of linearity as follows: 1, 10, 50, 100, 200, and 500 mg/L for most standards, except for *p*-hydroxybenzoic acid (1, 10, 50, 100, 200, 500, and 1000 mg/L), ferulic acid (1, 10, 50, 100, 200, and 900 mg/L), cyanidin-3-glucoside (1, 10, 50, 100, 200, and 400 mg/L). For the precision and accuracy estimation, the concentrations of standards were (40, 70, and 150 mg/L).

### *The determination of total polyphenols*

The method for the determination of total polyphenols was described in our earlier paper (Matić et al., 2017). In short, the reaction mixture for the determination of total polyphenols was prepared in a glass tube by adding distilled water (1580 µL), the polyphenol standard (20 µL), the Folin-Ciocalteu reagent (100 µL), and Na<sub>2</sub>CO<sub>3</sub> (200 g/L) (300 µL). After mixing in a vortex (Grant Bio, Cambridgeshire, England), the tubes were incubated at 40 °C in a water bath. After 30 min, the absorbance was measured at 765 nm against a blank solution (which contained 20 µL of distilled water instead of a prepared solution) with a UV-Vis spectrophotometer (Selecta, UV 2005, Barcelona, Spain) (Singleton et al., 1999).

### *The determination of total flavonoids*

For the determination of total flavonoids (Zhishen et al., 1999), the reaction mixture was prepared in a glass tube by adding the following: 800 µL of distilled water, 200 µL of polyphenol standard, and 60 µL of NaNO<sub>2</sub> (5%). After 5 min, 60 µL of AlCl<sub>3</sub> (10%) was added. After additional 6 min, 400 µL of NaOH (1 mol/L) and 480 µL of distilled water were added into the reaction mixture. The solution was mixed in the vortex and absorbance was measured on the UV-Vis spectrophotometer (Selecta, UV 2005, Barcelona, Spain) at 510 nm against a blank solution (which contained 200 µL of distilled water instead of the polyphenol standard).

### *Validation parameters*

The validation parameters for the Folin-Ciocalteu and aluminium chloride method were linearity, limit

of detection (LOD), limit of quantification (LOQ), precision, accuracy, and sensitivity.

The linearity of both methods was determined for each polyphenol standard by determining the linear equation of the calibration line and the determination coefficient  $R^2$ .

The sensitivity of methods was evaluated by comparing the slopes of the calibration lines.

The limit of detection (LOD) (Eq. (1)) and the limit of quantification (LOQ) (Eq. (2)) were determined by using the standard deviation of the intercept (SD) and the mean slope (S) of the calibration line. LOD and LOQ were expressed in mg/L.

$$\text{LOD} = 3.3 \frac{\text{SD}}{S} \text{ Eq. (1)}$$

$$\text{LOQ} = 10 \frac{\text{SD}}{S} \text{ Eq. (2)}$$

Intra-assay precision was determined by measuring three different concentrations of each standard and by calculating the coefficient of variation (CV) (%) according to Eq. (3) (SD is the standard deviation of the measured concentration and S is the mean concentration of the standard).

$$\text{CV} = \frac{\text{SD}}{S} \times 100 \text{ Eq. (3)}$$

Accuracy was expressed through the percentage of recovery by measuring known concentrations of standards at three concentration levels and by calculating the recovery according to Eq. (4) ( $\gamma_c$  is the measured concentration obtained from the calibration curve (mg/L),  $\gamma_r$  is the prepared concentration of the standard solution (mg/L), and R is recovery (%)).

$$R = \frac{\gamma_c}{\gamma_r} \times 100 \text{ Eq. (4)}$$

### Statistical analysis

MS Excel (Redmond, USA) was used for data analysis. Calibration curves were constructed with two replicate samples of each concentration of

polyphenol, each measured twice (n=4). Regression was used for obtaining the linear equation and the determination coefficient  $R^2$ . Precision and recovery were based on three different concentration levels of polyphenols, each made in two replicates and each measured three times (n=6).

### Results

Spectrophotometric methods for total polyphenols and total flavonoids are often used for the determination of polyphenols because they are simple, sensitive, and precise (Prior et al., 2005). Due to these good features, they can be methods of choice in some experiments. For example, they can be used for the determination of individual polyphenols in model solutions exposed to different experimental conditions. In these cases, it is necessary to validate the spectrophotometric methods according to each individual polyphenol. The methods for total polyphenols and for total flavonoids in this study were validated for various polyphenols through the determination of linearity, limit of detection, limit of quantification, sensitivity, precision, and recovery.

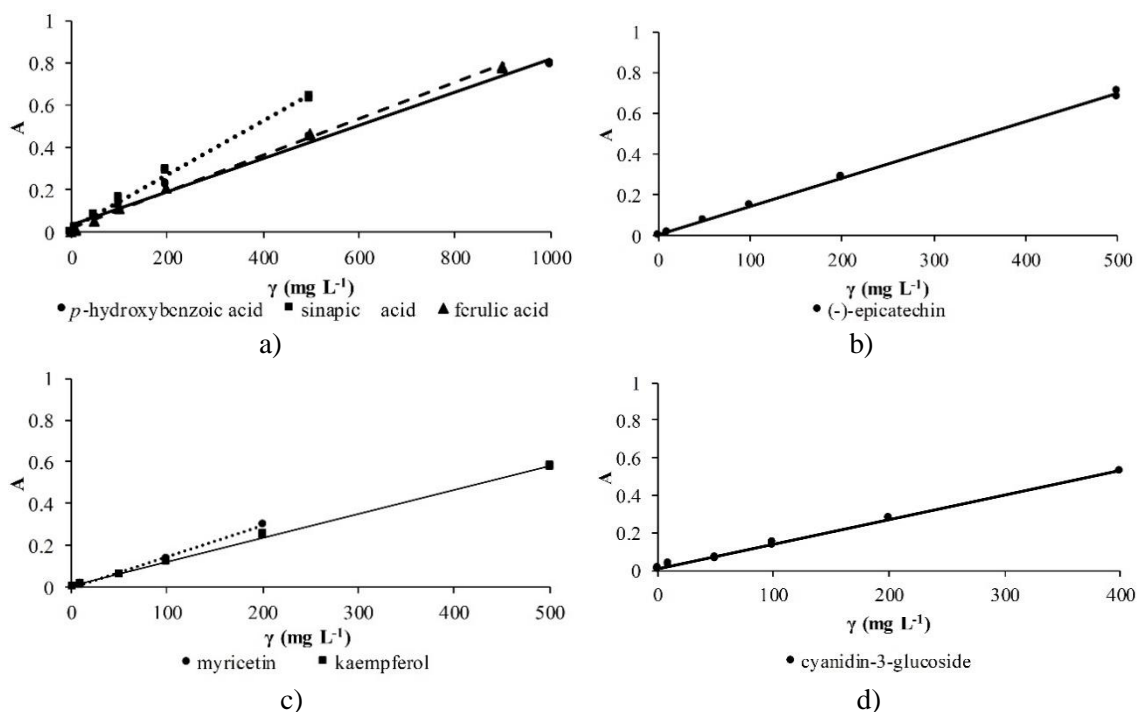
Table 1 presents the linearity, limit of detection, and limit of quantification of the spectrophotometric method for the determination of total polyphenols. The linearity of the method was estimated through the determination coefficient  $R^2$ . For all polyphenols,  $R^2$  was from 0.9916 to 0.9991, which allowed us to suggest that the method is linear for all studied polyphenols. The LOD was from 0.33 to 1.31 mg/L and the LOQ was from 1.01 to 3.96 mg/L, which is an acceptable range. Figure 1 presents the calibration curves of standards obtained with the method for total polyphenols. The sensitivity of the method can be determined according to the slopes of the calibration curves (Table 1). The slopes of all calibration curves were in the range from 0.0008 to 0.0015 L/mg. The lowest sensitivity was found for *p*-hydroxybenzoic acid (0.0008 L/mg) and the highest for myricetin (0.0015 L/mg).

Table 2 presents the linearity, limit of detection, and limit of quantification of the spectrophotometric method for the determination of total flavonoids. For all polyphenols,  $R^2$  was in the range from 0.9744 to 0.9978, which suggests a good linearity of the method for all polyphenols. The LOD was from 0.097 to 2.33 mg/L and the LOQ was from 0.29 to 7.07 mg/L, which is an acceptable range.

**Table 1.** Linearity, limit of detection (LOD), and limit of quantification (LOQ) of the spectrophotometric method for the determination of total polyphenols<sup>1</sup>

Standard	Range mg/L	Calibration equation	R <sup>2</sup>	LOD mg/L	LOQ mg/L
<b>Phenolic acids</b>					
<i>p</i> -hydroxybenzoic acid	1-1000	$y=0.0008x+0.0369$	0.9916	1.17	3.53
sinapic acid	1-500	$y=0.0013x+0.0186$	0.9951	1.31	3.96
ferulic acid	1-900	$y=0.0009x+0.0175$	0.9973	0.78	2.36
<b>Flavan-3-ols</b>					
(-)-epicatechin	1-500	$y=0.0014x+0.0062$	0.9991	0.33	1.01
<b>Flavonols</b>					
myricetin	1-200	$y=0.0015x-0.0032$	0.9972	0.62	1.89
kaempferol	1-500	$y=0.0012x+0.0041$	0.9985	0.58	1.77
<b>Anthocyanins</b>					
cyanidin-3-glucoside	1-400	$y=0.0013x+0.0108$	0.9982	0.36	1.09

<sup>1</sup> The results are based on two replicate samples of each standard concentration, each measured twice (n=4)



**Fig. 1.** Calibration curves of standards obtained by the spectrophotometric methods for the determination of total polyphenols. a) phenolic acids; b) flavan-3-ols; c) flavonols; d) anthocyanins

**Table 2.** Linearity, limit of detection (LOD), and limit of quantification (LOQ) of the spectrophotometric method for the determination of total flavonoids<sup>1</sup>

Standard	Range mg/L	Calibration equation	R <sup>2</sup>	LOD mg/L	LOQ mg/L
<b>Flavan-3-ols</b>					
(-)-epicatechin	1-200	$y=0.0024x+0.0085$	0.9978	0.09	0.29
<b>Flavonols</b>					
myricetin	1-500	$y=0.0013x+0.0244$	0.9961	0.54	1.63
kaempferol	1-500	$y=0.0001x+0.0013$	0.9744	2.33	7.07
<b>Anthocyanins</b>					
cyanidin-3-glucoside	1-200	$y=0.0013x+0.0003$	0.9967	0.36	1.09

<sup>1</sup> The results are based on two replicate samples of each standard concentration, each measured twice (n=4)

Figure 2 presents the calibration curves of standards obtained by the method for total flavonoids. The slopes of all calibration curves were in the range from 0.0001 to 0.0024 L/mg. The lowest sensitivity was found for kaempferol (0.0001 L/mg) and the highest for (-)-epicatechin (0.0024 L/mg) (Table 2). Due to the very low sensitivity for kaempferol, it should be used carefully as a polyphenol standard, since it can give results with lower precision. It was found that the slope of the calibration curve for gallic acid was 0.08 and for catechin it was 0.0778 (in linear range of 1 to 8 mg/L) (Medina-Remón et al., 2009). Furthermore, the higher the slope of a calibration curve, the better the sensitivity, and a more sensitive method can provide more precise results. Moreover, a method is sensitive if a small change in concentration causes a large change in the measured signal (Taverniers et al., 2004).

Table 3 presents the obtained results for the precision and accuracy of the method for total polyphenols. For the precision, the results were in the range from 1.19% to 11.70%, while accuracy was in the range from 98.23% to 109.24%. Table 4 presents the results for precision and accuracy of the method for total flavonoids. The precision was from 1.05% to 6.31% for all polyphenols except for kaempferol (from 7.98 to 97.99%). Accuracy was from 98.51% to 113.65%

except for kaempferol (from 67.50 to 144.67%). The results of precision and accuracy for all standards were in the acceptable range, except for kaempferol. The slope of the calibration curve for kaempferol (0.0001 L/mg) was very low, which reflects to very low sensitivity, precision, and accuracy of the method for kaempferol.

The method for total polyphenols is based on the use of the Folin-Ciocalteu reagent and sodium carbonate, which provide the basic conditions in which the dissociation of phenolic protons occurs. The phenolate anions yielded from this reaction can reduce the Folin-Ciocalteu reagent, and the colour of the solution changes into blue (Huang et al., 2005; Singleton et al., 1999). This allows the measurement of absorbances in the UV/Vis spectrum which are proportional to the concentration. In this study, the method for the determination of total polyphenols showed good validation parameters for all the individual studied polyphenols. This suggests that the method can be used in experiments where those individual polyphenols are monitored under different experimental conditions. This method was also used for the determination of the total polyphenols in plums (Kim et al., 2003), mushrooms (Palacios et al., 2011), amaranth and quinoa seed (Paško et al., 2009), and others.

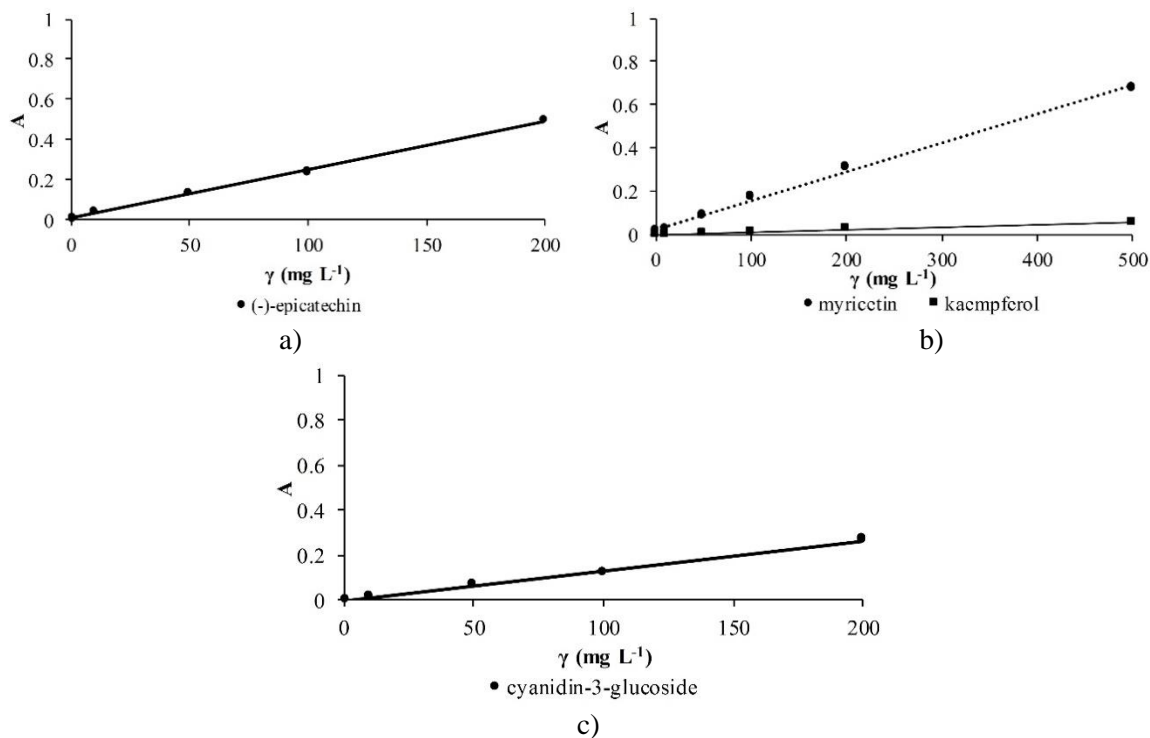


Fig. 2. Calibration curves of standards obtained by the spectrophotometric methods for the determination of total flavonoids. a) flavan-3-ols; b) flavonols; c) anthocyanins

**Table 3.** Precision<sup>1</sup> and accuracy<sup>2</sup> of the spectrophotometric methods for the determination of total polyphenols

Standard	Concentration mg/L	Intra-assay precision %	Recovery %
<b>Phenolic acids</b>			
<i>p</i> -hydroxybenzoic acid	40	1.84	98.23
	70	8.76	102.56
	150	3.84	108.42
sinapic acid	40	2.72	107.82
	70	8.28	100.44
	150	1.19	108.07
ferulic acid	40	2.97	107.87
	70	3.49	105.03
	150	4.57	106.30
<b>Flavan-3-ols</b>			
(-)-epicatechin	40	6.30	107.38
	70	2.02	105.24
	150	2.48	107.05
<b>Flavonols</b>			
myricetin	40	10.04	101.44
	70	1.85	109.24
	150	2.10	105.87
kaempferol	40	11.70	108.82
	70	2.91	108.21
	150	3.55	104.20
<b>Anthocyanins</b>			
cyanidin-3-glucoside	40	8.56	107.44
	70	1.52	102.42
	150	7.88	108.31

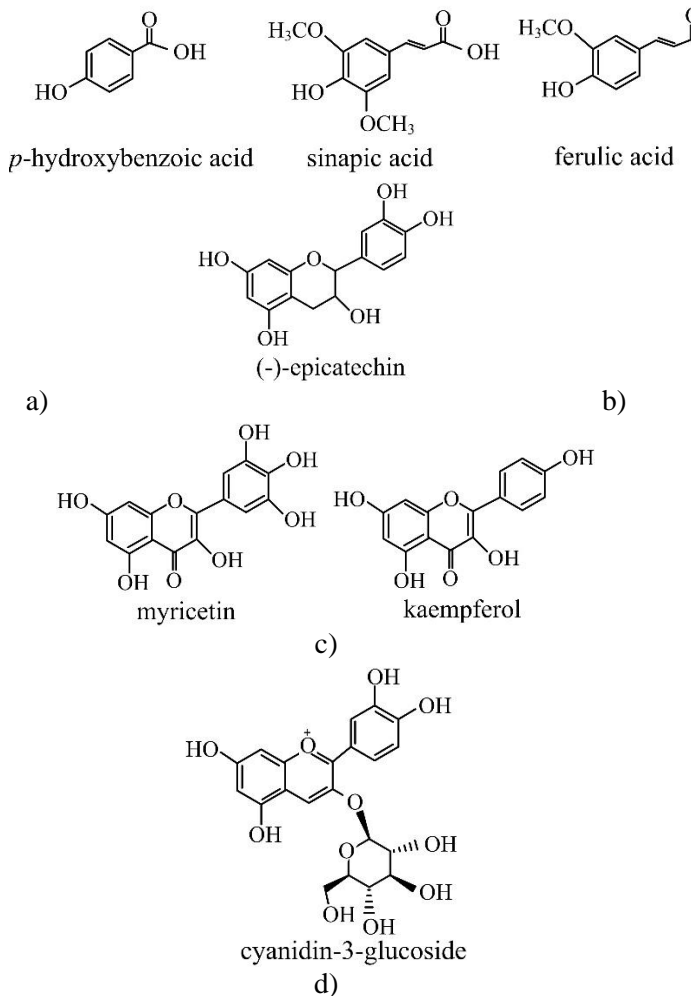
<sup>1</sup> Expressed through intra-assay precision (one analyst, the same laboratory, each concentration made in two replicates and each measured three times (n=6))<sup>2</sup> Expressed through recovery**Table 4.** Precision<sup>1</sup> and accuracy<sup>2</sup> of the spectrophotometric methods for the determination of total flavonoids

Standard	Concentration mg/L	Intra-assay precision %	Recovery %
<b>Flavan-3-ols</b>			
(-)-epicatechin	40	6.31	108.33
	70	1.05	98.51
	150	3.23	99.31
<b>Flavonols</b>			
myricetin	40	5.98	113.65
	70	2.71	102.13
	150	4.01	108.86
kaempferol	40	97.99	67.50
	70	23.04	129.05
	150	7.98	144.67
<b>Anthocyanins</b>			
cyanidin-3-glucoside	40	2.94	100.06
	70	5.02	101.14
	150	2.78	101.73

<sup>1</sup> Expressed through intra-assay precision (one analyst, the same laboratory, each concentration made in two replicates and each measured three times (n=6))<sup>2</sup> Expressed through recovery

The method for the determination of total flavonoids is based on the reaction of aluminium ions with flavonoid molecules under basic conditions. The sensitivity of the method depends on the number of hydroxyl groups on the flavonoid molecule and on the properties of the aluminium ion (Malešev and Kuntić,

2007; Zhu et al., 2010). In the flavonol group, the number of OH groups on the kaempferol molecule is lower than on the myricetin molecule (Figure 3). It is possible that the number of OH groups on the kaempferol molecule lowered the absorbance and the sensitivity of the method.



**Fig 3.** Chemical structures of polyphenols. a) phenolic acids; b) flavan-3-ol; c) flavonols; d) anthocyanin

Also, it was shown that standards with no catechol group showed very low absorbances at 510 nm (Pękal and Pyrzynska, 2014), like kaempferol in this study. It was found in the literature that the method for total flavonoid determination was also inadequate for the kaempferol standard (Denni and Mammen, 2012), like in our study. The use of the kaempferol standard does not look like a good solution, since the results would probably not be sensitive enough at low concentrations. The method showed good validation parameters for other polyphenols. This method was also used for the determination of total flavonoids in persimmon leaves (Sun et al., 2011).

## Conclusion

In this study, the spectrophotometric method for the determination of total polyphenols was validated according to polyphenol standards which belong to the groups of phenolic acids (*p*-hydroxybenzoic acid, sinapic

acid, ferulic acid), flavan-3-ols ((-)-epicatechin), flavonols (myricetin, kaempferol), and anthocyanins (cyanidin-3-glucoside). The method for total flavonoids was validated for polyphenol standards belonging to flavan-3-ols ((-)-epicatechin), flavonols (myricetin, kaempferol), and anthocyanins (cyanidin-3-glucoside). Validation parameters like linearity, sensitivity, limit of detection, limit of quantification, precision, and accuracy were determined. The results of validation for both methods were in acceptable ranges, except for kaempferol in the method for total flavonoids. Namely, the slope of the calibration curve of kaempferol was very low and the results for precision and accuracy were not in the acceptable range. The kaempferol standard in the method for total flavonoids should be used carefully.

**Funding:** This work has been fully supported by the Croatian Science Foundation under project number HRZZ-IP-2016-06-6777.

**Conflicts of Interest:** The authors declare no conflict of interest.

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