Determination of total flavonoid content by aluminum chloride assay: A critical evaluation

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ABSTRACT

Flavonoids are important natural bioactive compounds. Quantitation of total flavonoid content (TFC) is widely performed using the aluminum chloride colorimetric assay against a flavonoid standard assuming equal responses from all flavonoids. The aim of this work was to critically evaluate the assay employing spike recovery in plant extracts and three authentic flavonoid standards (catechin, quercetin, and rutin). Due to the inherent variations in absorbance values at quantitation wavelengths between investigated flavonoids, the assay produced huge unacceptable differences in TFC. For trials involving $AlCl_3$ alone in standard solutions, false-positive results were obtained (63–124%) when quercetin content was expressed as rutin equivalents. Conversely, false-negative results were found (26–42%) when rutin concentration was expressed as quercetin equivalents. Similarly, unacceptable spike recoveries were recorded (8–106%) when involving $AlCl_3$ alone in standard solutions at all investigated wavelengths. For plant extracts, unacceptable differences (58–152%) in TFC were also obtained when either quercetin or rutin was used as a quantitation standard. When $AlCl_3$ is used in conjunction with sodium nitrite, unacceptable high or low recoveries were noted depending on the quantitation standard used. The findings of this work provide conclusive evidence highlighting conceptual and methodological flaws in the $AlCl_3$ colorimetric assay for the determination of TFC.

1. Introduction

Plants are a crucial source of diet and nutrition for humans and animals. They produce primary and secondary metabolites, which are chemical substances that differ in role and function. While primary metabolites are vital for the proper growth of plants, secondary metabolites do not play any obvious role in the internal economy of the plant (Delgoda & Murray, 2017; Stone & Williams, 1992). Despite the absence of any internal role, the unique chemical properties of secondary metabolites made them of high nutritional and medicinal values. These secondary metabolites possess antibiotic, antifungal, antioxidant, antiinflammatory, and antiviral properties and hence have applications in pharmaceutical, food, cosmetics, and fine chemical industries (Bor, Aljaloud, Gyawali, & Ibrahim, 2016; Dhama et al., 2018; Kallscheuer, Classen, Drepper, & Marienhagen, 2019; Pan, Xu, Shi, Tsang, & Zhang, 2018; Prasad et al., 2015; Shields, 2017; Widelski & Kukula-Koch, 2017).

There are three major classes of plant secondary metabolites, namely alkaloids, phenols, and terpenoids (Das & Gezici, 2018). Alkaloids originate from aromatic amino acids or from aspartate, glutamate, or glycine in the plant cell, whereas phenols, including polyphenols, are derived from plant aromatic amino acids. On the other hand, terpenoids are produced from intermediates of glycolysis. In this work, we focus on the determination of flavonoids, which are a sub-group of phenols. There are more than 10,000 phenolic compounds that have been isolated from plants so far. In general, phenolic metabolites are divided into seven groups: coumarins, flavonoids, phenolic acids, lignans, lignin, stilbenes, and tannins (Luna-Guevara, Luna-Guevara, Hernández-Carranza, Ruíz-Espinosa, & Ochoa-Velasco, 2018). Notably, more than half of the identified phenolics are flavonoids.

The total flavonoid content (TFC) in plants is usually determined colorimetrically after solvent extraction. One of the widely followed methods for the determination of TFC in plant extracts is the aluminum chloride colorimetric assay, where Al(III) is utilized as a complexing

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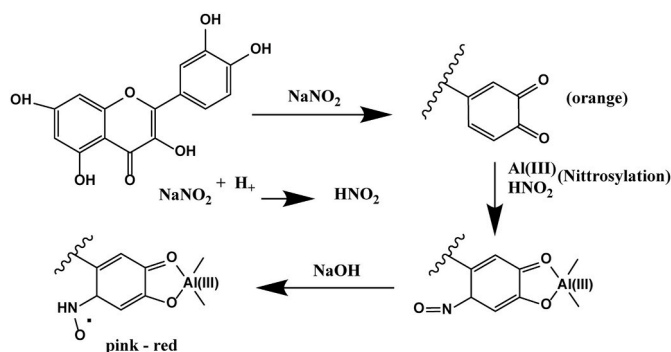
agent. The assay was first proposed by Christ and Muller in 1960 for the determination of flavonol derivatives in drugs (Christ & Müller, 1960). The method is based on the formation of chelates of Al(III)-flavonoids. Due to their many oxo and hydroxyl groups (Scheme 1), flavonoids have a great affinity to bind metal ions such as Al(III), mostly at a 1:1 ratio, depending on experimental conditions including pH value (Kasprzak, Erxleben, & Ochocki, 2015; Pyrzynska & Pękal, 2011). With time, the original method went through several amendments such as the introduction of sodium nitrite (NaNO$_2$) before the addition of AlCl$_3$. Sodium nitrite serves as a nitrating agent that is selective for aromatic vicinal diols (Barnum, 1977) to produce a flavoniod-nitroxyl derivative (Scheme 2) that are characterized by the appearance of a new absorption band at about 500 nm. As another amendment, the Al(III)-flavonoid complexation has been performed in the presence of acetate salt. The original assay and its amendments are experimentally evaluated in the present work.

According to its proposers, the determination of TFC using the AlCl$_3$ is only possible if the absorption of the metal chelates of the individual flavonoids present in a sample quantitatively have similar absorbance at a certain wavelength (Christ & Müller, 1960). However, despite this fact, the method was blindly and widely applied for the determination of TFC incorrectly assuming that different flavonoids have same absorption spectra at the region of interest.

Experimentally, upon the addition of AlCl$_3$ in the absence of NaNO$_2$, yellow-colored Al(III)-flavonoid complexes are formed, and their absorbance is subsequently measured at a specific wavelength in the

(a)

Basic chemical structure of flavonoids

Flavonols
= H, R = OH)
Quercetin: R$_1$ = R$_2$ = OH)
Myricetin: R$_1$ $_2$

Flavanols
= H, R = OH)
Catechin: R$_1$ = R = OH)
Gallocatechin: R$_1$ $_2$

Flavones
= R = H, R = OH)
Apigenin: R$_1$ = R = R $_2$ = H)
Chrysin: R$_1$ $_2$ $_3$

Flavanones
= R
ERiodictyol: R$_1$, R$_2$ = OH)
Hesperetin: R$_1$ = OH, R$_2$ = OCH$_3$)

(b)

Scheme 1. Flavonoids: general chemical structure and examples (**a**), and illustration of Al(III)-quercetin chelate (**b**).
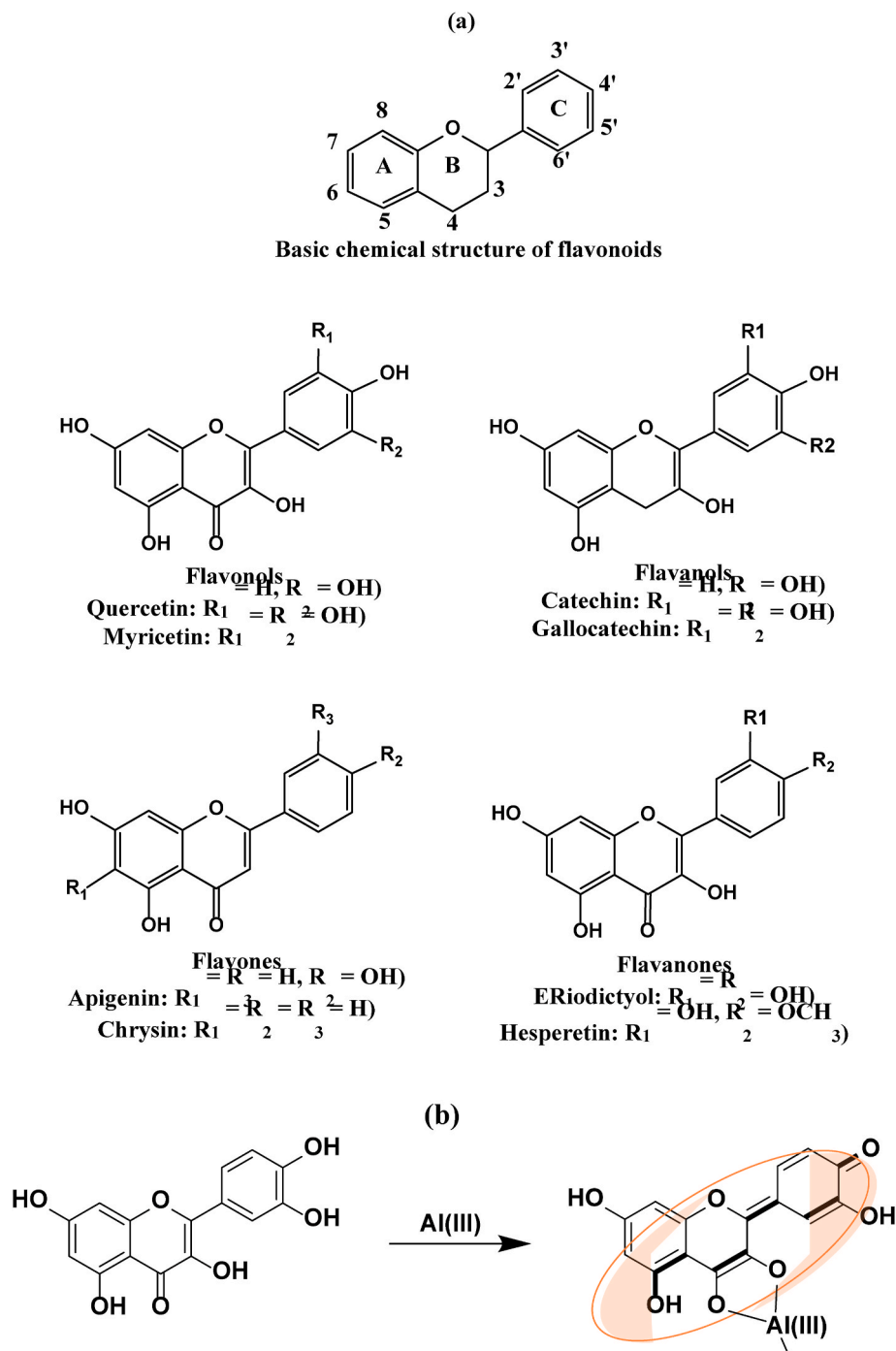
Scheme 2. Al(III)-flavonoids (quercetin is used as example) complexation in the presence of $NaNO_2$ (Mekonnen & Desta, 2021).

range 410–440 nm. TFC is quantified from calibration curves based on a reference flavonoid standard measured at the same experimental conditions and at the wavelength. Among the most used flavonoid standards are quercetin, catechin, and rutin. The structure of these compounds is illustrated in Scheme 3. As noted above, the flaws in this approach arise from the assumption that all flavonoids available in a sample have the same extension coefficient and absorption maxima (λ_{max}) as the standard used in calibration. Indeed, various flavonoids have different absorbance values at λ_{max}, different λ_{max}, and many of them may not complex with Al(III) and hence will not be accounted for in the assay. Therefore, it is highly anticipated that the application of this assay for the determination of TFC in plants suffers from fundamental flaws that limit its validity and hence disqualify the assay to be used as a standard test method for the determination of TFC. Some of these concerns have been raised by some researchers (Chang, Yang, Wen, & Chern, 2002; Mammen & Daniel, 2012; Pękal & Pyrzynska, 2014) but despite that, the assay is still blindly and incorrectly applied for the determination of TFC till today. Experimental evidence highlighting the conceptual and methodical limitations of this assay are provided in the discussion part (section 3).

The goal of this investigation was to critically evaluate the aluminum chloride colorimetric assay for quantification of total flavonoid content. To achieve this goal, the following objectives were set: (i) evaluating the performance of the aluminum chloride colorimetric assay using authentic flavonoid standard solutions and real plant extracts, and (ii) assessing the validity and accuracy of the assay through spike recovery approach by calculating the recoveries of authentic flavonoid standards spiked both in experimental solutions and real plant extracts.
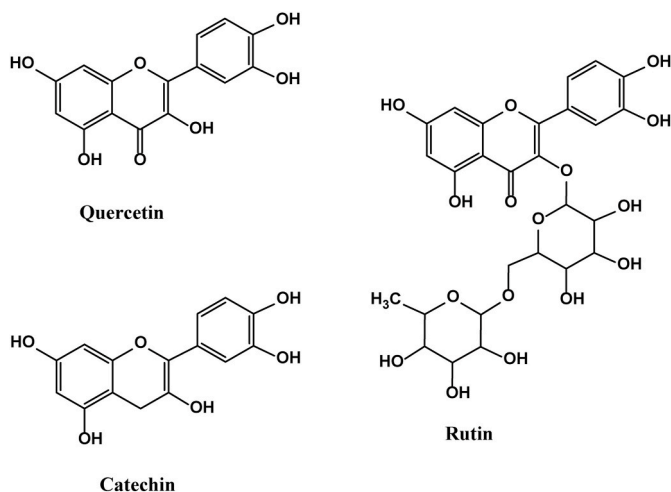
2. Materials and methods

2.1. Chemicals

All water used was of Type I obtained from Ultra-pure water purification system (0.055 μS/cm, Stakpure GmbH, Niederahr, Germany). Methanol (liquid chromatography grade, ≥99.8%) from Merck (Darmstadt, Germany). Ethanol (Analar, 96%) from VWR Chemicals (BDH, Fontenay-sous-Bois- France). Aluminum chloride (anhydrous sublimed, ≥99.8%) and sodium nitrate (ACS reagent, ≥99%) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (GPR, >99%) from Poole (Dorset, England). Sodium hydroxide (Analar 99%) from VWR Chemicals (BDH, USA). Sodium nitrite (>98%) from Fluka Chemika (Buchs, Switzerland). Quercetin (dihydrate, reference standard) from Thomas Scientific European Pharmacopeia (Strasbourg Cedex, France). Catechin from Phytolab GmbH & Co. (Vestenbergsgreuth, Germany). Rutin from United States Pharmacopeia (Rockville, MD, USA). Filter paper (90 mm medium size) from Ederol Binzer and Munktell (Battenberg, Germany).

2.2. Plants

The plants used in this work were desert medicinal plants collected from different Rawda sites in Qatar. The plant samples were dried at 50 °C inside a hot air oven, ground to powder using a commercial coffee grinder, passed through a 40-mesh sieve, and stored overnight at room temperature in clean airtight plastic bottles before being analyzed within 24 h after grinding.

2.3. Plants extraction and analysis

About 3.000 g of the plant powder (weighed accurately) was placed in an extraction glass flask followed by the addition of 30.0 mL of aqueous ethanolic solution (75% ethanol). The flask and its contents were placed in an ultrasonic bath and irradiated for 60 min. The temperature inside the bath was kept between 30 and 40 °C. The mixture was then filtered through a suction filtration system fitted with a borosilicate glass sintered funnel of a porous plate and an additional normal filter paper. The filtration funnel, filter paper, and flask were all washed with the solvent before filtering the plant mixture. The plant residue was washed three times with 5 mL solvent and the final volume was made to l00 mL with the solvent. For the colorimetric analysis of TFC, the following sequence of steps was followed using a 15 mL glass tube: 2.0 mL methanol followed by a known volume of the flavonoid standard (or 0.50 mL sample's clear extract, unless otherwise stated), 0.20 mL $AlCl_3$ (10% w/v), vortex mixing and 3.0 min equilibration time, 0.20 mL CH_3COONa (1.80 g/mL, when used), and the final volume was made to 5.0 mL using methanol. When $NaNO_2$ was used, the following steps in order were followed: 2.0 mL methanol followed by a known volume of the flavonoid standard (or 0.50 mL sample's clear extract, unless otherwise stated), 0.15 mL $NaNO_2$ (1.0 mol/L), vortex mixing, and 3.0 min equilibration time, 0.15 mL $AlCl_3$ (10% w/v), vortex mixing and 3.0 min equilibration time, 1.0 mL NaOH (1.0 mol/L), and the final volume was made to 5.0 mL using methanol. All solutions were vortex mixed after the last step and the tubes were stored in the dark for 40 min before UV–Vis analyses (UV-2600i, Shimadzu).

2.4. TFC quantification

The flavonoid content in the samples extracts and experimental solutions was quantified using calibration curves of individual flavonoid standards of catechin, quercetin, and rutin at six levels each (1.0–70.0 μg/mL) at various wavelengths. Spike recovery was calculated using the following formula: Spike recovery (%) = (obtained concentration × 100/spiked concentration).

Scheme 3. Chemical structures of quercetin, catechin, and rutin.

3. Results and discussion

The TFC content is usually determined using the AlCl₃ colorimetric assay without or with NaNO₂ (a nitrating agent) at an absorption wavelength between 400 and 550 nm depending on the reagents used. The use of sodium or potassium acetate in conjunction with AlCl₃ has also been reported in many investigations. When AlCl₃ (with or without acetate salt) is used in the absence of NaNO₂, TFC is determined at a wavelength between 410 and 440 nm, depending on the flavonoid standard employed (Aryal et al., 2019; Chandra et al., 2014; Cimpoiu, Cristea, Hosu, Sandru, & Seserman, 2011; Dahech et al., 2013; Zhang et al., 2013), whereas the 510 nm band is usually utilized when using AlCl₃ in the presence of NaNO₂ (Al-Rimawi et al., 2017; Perez-Perez et al., 2014; Phuyal, Jha, Raturi, & Rajbhandary, 2020). Some of the important experimental conditions reported in the literature for the TFC determination using the AlCl₃ colorimetric assay are summarized in Table 1. As evident from this table, TFC was determined under different experimental parameters. When using quercetin as a standard, TFC was determined at 415, 420, 430, 440, and 520 nm. Surprisingly, no absorption spectra were presented in any of these references to justify the wavelength selection.

In the present work, we pursued the following approach to assess the validity of the AlCl₃ colorimetric assay for the determination of TFC: (i)

employing the assay for analyzing three flavonoid standard solutions (i. e., quercetin, catechin, and rutin) individually or in mixtures, (ii) employing the assay in real plant extracts, and (iii) calculating the recoveries of flavonoid standards spiked in experimental solutions and plant extracts. These tests were carried out using AlCl₃ alone, with acetate salt, and with AlCl₃ in conjunction with NaNO₂. Spike recoveries were calculated at different wavelengths and expressed as quercetin, catechin, or rutin equivalents. Although the present work focuses on the absorption range between 400 and 550 nm, a wider absorption range between 250 and 600 nm is displayed in many cases to provide the reader with a holistic view.

3.1. The AlCl₃ colorimetric assay in the absence of NaNO₂

The discussion will be limited to quercetin, catechin, and rutin, the three flavonoid standards that are frequently employed in the AlCl₃ colorimetric quantification of TFC in plant extracts.

3.1.1. Interaction of AlCl₃ with flavonoids standard solutions in the absence of NaNO₂

The UV–Vis spectra for quercetin, catechin, and rutin standards, alone and upon complexing with AlCl₃ in the absence of NaNO₂ are shown in Fig. 1. The addition of AlCl₃ to quercetin resulted in a bathochromic (red) shift in λ_{max} from 367 nm to 428 nm. Rutin, on the other hand, showed similar interaction as that of quercetin but the shift was from 351 nm to 415 nm. Interestingly, catechin did not seem to interact with Al(III), where no appreciable change in λ_{max} was observed. Furthermore, catechin, alone or complexed, did not show any appreciable absorbance beyond 320 nm.

The flavonoids-metal binding is expected to occur at three sites: the 3-hydroxy – 4-carbonyl groups ("3–4 site" in B-ring); the 5-hydroxy group (A-ring) – 4-carbonyl group (C–ring) ("4–5 site"); and 3′,4′-dihydroxy groups ("3′–4′ site" in C-ring) (Scheme 1) (Chang et al., 2002; Cornard & Merlin, 2002; Tuli, 2019). Al(III) complexation to 3,4-site will result in the formation of a new chromophore (highlighted in Scheme 1b). The resulted red-shifted absorption band for quercetin and rutin appeared at 428 nm and 415 nm, respectively (Fig. 1). Such interaction will not occur in the case of catechin due to the lack of a carbonyl group at the binding site and hence the chromophore will not form. Consequently, catechin is not suitable as a standard in the AlCl₃ colorimetric assay for the determination of TFC. However, catechin can be employed as a calibration standard in the assay when NaNO₂ is used as discussed in section 3.2.

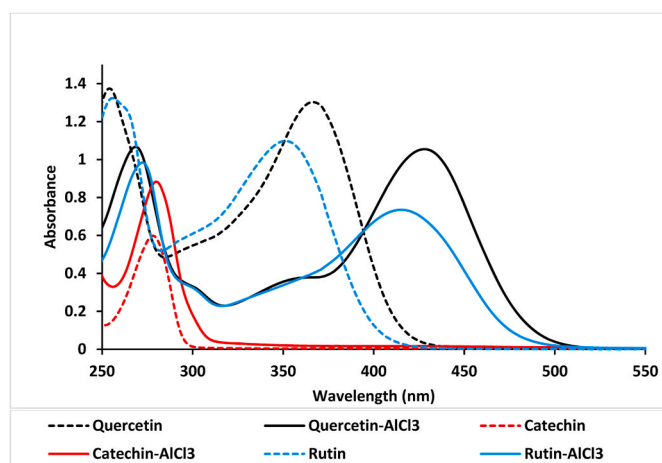As displayed in Fig. 1, the absorbance of the quercetin-Al(III)

Table 1
Use of flavonoid standards in the AlCl₃ colorimetric determination of TFC.

Flavonoid type	λ, nm	Use of other reagents	Solvent	References
Quercetin	415	CH₃COOK	MeOH–H₂O	Aryal et al. (2019)
Quercetin	415	CH₃COONa	MeOH– H₂O	Ondua, Njoya, Abdalla, and McGaw (2019)
Quercetin	415	CH₃COOK	MeOH– H₂O	Shah et al. (2019)
Quercetin	415	CH₃COOK	H₂O	Ab Rahman et al. (2018)
Quercetin	415	CH₃COONa	EtOH– H₂O	Sembiring, Elya, and Sauriasari (2017)
Quercetin	415	CH₃COOK	EtOH– H₂O	Chang et al. (2002)
Quercetin	440	CH₃COONa	EtOH– H₂O	Dahech et al. (2013)
Quercetin	520	CH₃COOK	MeOH–EtOH– H₂O	Engida et al. (2013)
Quercetin	415	–	MeOH– H₂O	(W. Wang et al., 2019)
Quercetin	420	–	MeOH–H₂O	Chandra et al. (2014)
Quercetin	420	–	EtOH–H₂O	(Ordoñez, Gomez, Vattuone, & lsla, 2006)
Rutin	415	–	MeOH	Lazarova et al. (2020)
Rutin	415	CH₃COOK	MeOH–H₂O	Jaradat, Hussen, and Al Ali (2015)
Rutin	415	CH₃COOK	MeOH–H₂O	Madaan, Bansal, Kumar, and Sharma (2011)
Rutin	430	CH₃COONa	EtOH–H₂O	Cimpoiu et al. (2011)
Rutin	410	CH₃COOK	EtOH–H₂O	Zhang et al. (2013)
Catechin	415	–	MeOH	Ramamoorthy and Bono (2007)
Catechin	510	NaNO₂–NaOH	H₂O	Munekata et al. (2020)
Quercetin	510	NaNO₂–NaOH	MeOH–H₂O	Phuyal et al. (2020)
Quercetin	425	NaNO₃–NaOH	MeOH–H₂O	Gomes et al. (2017)
Catechin	510	NaNO₂–NaOH	H₂O	Al-Rimawi et al. (2017)
Catechin	510	NaNO₂–NaOH	MeOH–H₂O	Perez-Perez et al. (2014)
Catechin	510	NaNO₃–NaOH	MeOH–H₂O	(M. Jain Kassim et al., 2011)
Rutin	510	NaNO₂–NaOH	MeOH–H₂O	Yan et al. (2021)
Rutin	510	NaNO₂–NaOH	MeOH–H₂O	(L. Wang, Luo, Wu, Liu, & Wu, 2018)
Rutin	450	NaNO₂–NaOH	MeOH–H₂O	Fu et al. (2010)
Rutin	510	NaNO₃–NaOH	EtOH–H₂O	Chen et al. (2019)

Fig. 1. Absorbance spectra of the interaction Al(III) with quercetin (19.8 µg/mL), rutin (30.9 µg/mL), and catechin (38.6 µg/mL alone and 77.1 µg/mL complexed) in the absence and presence of AlCl₃.

complex maximizes at 428 nm. Similar findings have been reported in the literature when using MeOH, EtOH, or H$_2$O in any mixture, where λ_{max} falls in the 425–430 nm range (Cornard & Merlin, 2002; Mammen & Daniel, 2012; Pękal & Pyrzynska, 2014). Therefore, the use of any wavelength outside the 428–430 nm will unnecessarily reduce the method's sensitivity and hence compromises the quality of the results when used for quantitative purposes. Most of the cited references (Table 1) applied correctly the 415 nm (λ_{max}) when using rutin as standard. Surprisingly, in some of the references cited in Table 1, the wavelength selection was inappropriate, and no evidence provided to support this selection. For example, the use of 520 nm to quantify TFC as quercetin equivalents (Engida et al., 2013) is impractical as the Al (III)-quercetin complex does not exhibit any appreciable absorbance at that wavelength (see Fig. 1). Furthermore, catechin at 415 nm has been used for TFC determination (Ramamoorthy & Bono, 2007), though it does not show any absorption beyond 320 nm (see Fig. 1 and section *3.1.4* for additional evidence). These observations highlight the importance of validating analytical methods following known quality control protocols before obtaining defensible results.

As also clear from Fig. 1, quercetin and rutin displayed clear differences in both the absorbance at λ_{max} and the λ_{max} itself. The quercetin relative absorbance at λ_{max} is more than double that of rutin at its λ_{max}, whereas the difference in λ_{max} was 13 nm. Such differences imply that quantification of TFC using the AlCl$_3$ spectrophotometric assay is dependent on the flavonoids standard used, where different standards result in different TFC values. For example, samples with no or low catechin content will show high false-positive results when quercetin or rutin is used as a quantitation standard. Similarly, high false-positive results will be obtained for rutin when using quercetin as a quantitation standard. Oppositely, low results for quercetin will be obtained when using catechin as standard.

To quantify such false contributions, quercetin and rutin calibration curves measured at various wavelengths were constructed. The calibration curves were made using six standard solutions of 1.0–70.0 µg/mL at six wavelengths (400, 410, 415, 420, 430, and 440 nm). The obtained coefficient of determination (R^2) values were better than 0.999 for quercetin and better than 0.998 for rutin at all six wavelengths. The concentrations of quercetin in each standard at each wavelength were then calculated considering rutin as a standard (i.e., quercetin content expressed as rutin equivalents). Similar calculations were performed for rutin standards where rutin content is expressed as quercetin equivalents. The results of this activity are summarized in Table 2. When quercetin concentrations were expressed as µg/mL rutin equivalents, false-positive values ranging between 63 and 124% were obtained depending on the selected wavelength. On the other hand, false-negative results of 42 to 26% were found when rutin concentrations are expressed as µg/mL quercetin equivalents. As demonstrated in sections 3.1.2 and 3.1.4, the flaws in this assay become very evident for samples containing catechin (or any other flavonoid of similar absorbance behavior), where large false-negative results were obtained as catechin-Al(III) complex does not show any appreciable absorbance after 320 nm (refer to Fig. 1).

3.1.2. Spike recoveries of flavonoids in solution in the absence of NaNO$_2$

The limitations of this assay have been further highlighted

Table 2a
Quercetin concentration in experimental standard solutions expressed as µg/mL rutin equivalents at various wavelengths.

Added concentration [a]	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm
0.0	0.6	0.6	0.6	0.6	0.6	0.6
0.8	1.5	1.7	1.8	1.8	1.8	1.7
7.9	11.5	14.0	15.0	15.8	16.2	15.0
19.8	32.7	40.5	43.6	45.9	47.4	43.4
39.6	61.7	76.7	82.7	87.0	89.4	81.6
69.4	109.7	136.2	146.6	152.2	154.9	141.2

Table 2b
Rutin concentration in experimental standard solutions expressed as µg/mL quercetin equivalents at various wavelengths.

Added concentration [a]	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm
0.0	0.3	0.3	0.3	0.3	0.3	0.3
1.2	1.2	1.3	1.3	1.3	1.2	1.0
6.2	4.1	4.5	4.5	4.5	4.1	3.5
15.4	8.3	9.0	9.1	9.0	8.3	7.0
30.9	19.2	20.8	21.0	20.8	19.1	16.1
74.1	47.3	50.9	51.3	50.6	46.2	38.6

[a] Concentration of flavonoids added to experimental solution.

employing spike recoveries of flavonoids standard solutions as demonstrated in Fig. 2 and Table 3. For this trial, three solution mixtures containing two or three of the investigated flavonoid standards were prepared and reacted with AlCl$_3$ as described in section 2.3. Low recoveries were obtained at all wavelengths in most of the trials, especially for solutions #2 and #4 (average recoveries 8–59%), which both contain catechin. Moderate to very good recoveries (67–106%) were obtained for solution #3, which does not contain any catechin.

3.1.3. Interaction of AlCl$_3$ with flavonoids in plant extracts in the absence of NaNO$_2$

The absorbance spectra of the plant extracts before and after treatment with AlCl$_3$ are shown in Fig. 3. The treatment of plant extracts with AlCl$_3$, resulted in the appearance of a new high-absorbance band that maximizes at 400 or 415 nm (Fig. 3b). The TFC in the samples' extracts was determined at different wavelengths using the calibration curves discussed in section 3.1.1. To provide numerical evidence for the dependence of TFC value on the flavonoid standard used in quantitation, the TFC in plant extracts was quantified based on both quercetin and rutin as listed in Table 4. Clearly, the use of quercetin as a quantitation standard resulted in different TFC values for each sample from those obtained when using rutin, and the difference is clearly wavelength-dependent (compare Tables 4a and 4b). Because rutin has lower absorbance signals than quercetin at all these wavelengths, use of rutin as a quantitation standard always resulted in higher TFC values. The calculated %differences between quercetin and rutin as quantitation standards are listed in Table 4c. It is obvious that %differences are unacceptably high ranging between 58 and 152%.

Another important point to consider related to the methodical limitations of the assay is that all plant extracts investigated in this work are colored (light-dark green) and therefore expected to have some absorption in the 400–450 nm wavelength range at which TFC
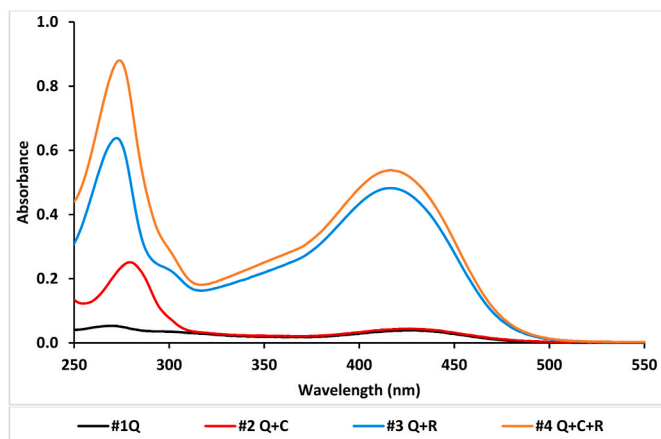
Fig. 2. Absorbance spectra for quercetin standard solutions spiked with rutin and catechin, either individually or as a whole. Q means quercetin alone at 0.8 µg/mL, C is catechin at 19.3 µg/mL, and R is rutin at 18.5 µg/mL.

Table 3a
Spike recoveries in experimental solutions expressed as µg/mL rutin equivalent at various wavelengths.

Solution # [a]	Added conc. [b]	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm	%Recovery ($x \pm s.d.$)
1	0.8	1.9	2.1	2.2	2.3	2.3	2.2	–
2	20.1	2.3	2.6	2.8	2.9	2.9	2.7	13.4 ± 1.1
3	19.3	19.8	21.7	22.0	21.9	20.4	17.4	106.4 ± 9.2
4	38.6	22.0	24.1	24.5	24.4	22.8	19.5	59.2 ± 5.0

Table 3b
Spike recoveries in experimental solutions expressed as µg/mL quercetin equivalent at various wavelengths.

Solution # [a]	Added conc. [b]	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm	%Recovery ($x \pm s.d.$)
1	0.8	1.1	1.2	1.3	1.3	1.4	1.2	–
2	20.1	1.3	1.6	1.6	1.7	1.7	1.6	7.9 ± 0.7
3	19.3	12.5	13.6	13.9	13.8	12.8	10.9	67.0 ± 5.8
4	38.6	13.9	15.2	15.4	15.4	14.4	12.3	37.3 ± 3.2

[a] Solution #1: quercetin alone at 0.8 µg/mL, #2: quercetin (0.8 µg/mL) + catechin (19.3 µg/mL), #3: quercetin (0.8 µg/mL) + rutin (18.5 µg/mL), #4: quercetin (0.8 µg/mL) + catechin (19.3 µg/mL) + rutin (18.5 µg/mL).
[b] Concentration of flavonoids added to experimental solution.

determination is made. Such original absorbance may not be, partially or fully, due to the presence of flavonoids. The possible contribution of the original color to the color formed after the Al(III) treatment forms the basis of another important point to consider when discussing the methodical limitations of the AlCl₃ assay. This limitation is indeed related to the use of appropriate blank, which was not considered in most of the published work. When flavonoids in samples' extracts are complexed with Al(III), a new high-absorbance band that maximizes at 400 or 415 nm is formed (Fig. 3b). As obvious from Fig. 3a, all untreated plant extracts have measurable absorbance values ranging between 0.1 and 0.6 absorption units. Similar behavior is observed in some flavonoid standards (Fig. 1). This original absorbance is expected to positively contribute to the overall absorbance values measured at λ_{max} at which the TFC determination is made. To explore this possibility, we spiked sample plant extracts with a mixture containing the three standards and measured the absorbances. As clear from Fig. 3c, an increase in absorbance was noticed at the absorbance range of interest for all solutions. For some of the analyzed samples (e.g., #14, Fig. 3b), the original absorbance represents ~50% of the absorbance value taken at 415 nm after the addition of AlCl₃. Such contribution can be eliminated by using a blank that contains the sample solution and all reagents except AlCl₃. In fact, this later approach was practiced by only a few researchers (W. Wang et al., 2019), while most of the other studies used only a water or solvent blank. The use of NaNO₂ in alkaline medium, one of the amendments made to the original assay, eliminates such interference as will be discussed in section 3.2.

3.1.4. Spike recoveries of flavonoids in plant extracts in the absence of NaNO₂

The absorbance spectra for two of the samples, unspiked and spiked with known amounts of each of the three flavonoid standards, alone or combined, are shown in Fig. 4. As evident from this figure and as expected, catechin spiked to samples did not show any increase in the samples' absorbance at the wavelength range of interest, whereas, both quercetin and rutin did. Recoveries of spiked flavonoids were also calculated and expressed as quercetin and catechin equivalents as listed in Table 5. Acceptable recoveries were only obtained for spiked quercetin in both samples at all wavelengths of interest when TFC was expressed as quercetin equivalent. The same findings were observed for spiked rutin when TFC was expressed as rutin equivalents. Conversely, very low to medium recoveries for spiked rutin as well as the spiked three flavonoids when results were expressed as quercetin equivalents. Same low findings were noted for spiked quercetin as well as the mixture of the three spiked flavonoids when results were expressed as rutin equivalents. This activity clearly demonstrates the conceptual flaws and

the limited capabilities and invalidity of the AlCl₃ colorimetric assay for TFC determination.

3.1.5. Effect of acetate salts on Al(III)-flavonoids complexation

The use of sodium or potassium acetate in the AlCl₃ colorimetric determination of TFC has been frequently reported in the literature (Table 1). Acetate salts are generally employed in the AlCl₃ assay in the absence of NaNO₂, however, their usefulness is questionable (Mammen & Daniel, 2012; Pękal & Pyrzynska, 2014). We explored the effect of CH₃COONa on the Al(III)-flavonoids interaction and found that adding CH₃COONa has no or minimal effect on both the λ_{max} and the absorbance at λ_{max} as shown in Fig. 5. Based on this finding, supported by the literature, acetate salts play no important role in the TFC determination using the AlCl₃ colorimetric assay.

3.2. The AlCl₃ colorimetric assay in the presence of NaNO₂

3.2.1. Interaction of AlCl₃ with flavonoids standard solutions in the presence of NaNO₂

The absorption spectra for the interaction of Al(III) with each of the three flavonoids in the absence and presence of NaNO₂ are presented in Fig. 6. The addition of NaNO₂ resulted in the appearance of several new high absorption bands in the catechin spectrum, which were not there in its absence. These new bands appeared after 320 nm including the one at 495 nm which is suitable for TFC determination. The appearance of these new red-shifted bands can be attributed to the reaction of nitrite with the phenolic hydroxyl groups to produce colored nitrophenols (Patnaik & Khoury, 2004). For rutin, the treatment with NaNO₂ resulted in a new band that maximizes between 488 and 497 nm. Rutin's major band undergone a redshift from 415 to 493 nm and a decrease in absorbance at λ_{max} upon the addition of NaNO₂. Similarly, quercetin went a redshift from 428 to 495 nm but with a broad shoulder and much lower absorbance values. One of the advantages of employing NaNO₂ in the AlCl₃ colorimetric assay is its interaction with most of the flavonoids, which in turn is expected to minimize the underestimation of TFC that is common when NaNO₂ is used (see sections 3.1.3 and 3.1.4).

Another important point to consider about the advantages of using NaNO₂ in conjunction with AlCl₃ is the large redshift in λ_{max} it makes. As explained in the last paragraph of section 3.1.3, there is a possible contribution from the original color of some flavonoid standards and plant extracts solutions. Such contribution is eliminated when using NaNO₂ as the quantitation is done at ~500 nm, where the original contribution is negligible. In this case, the TFC determination can be simply done against a solvent blank.
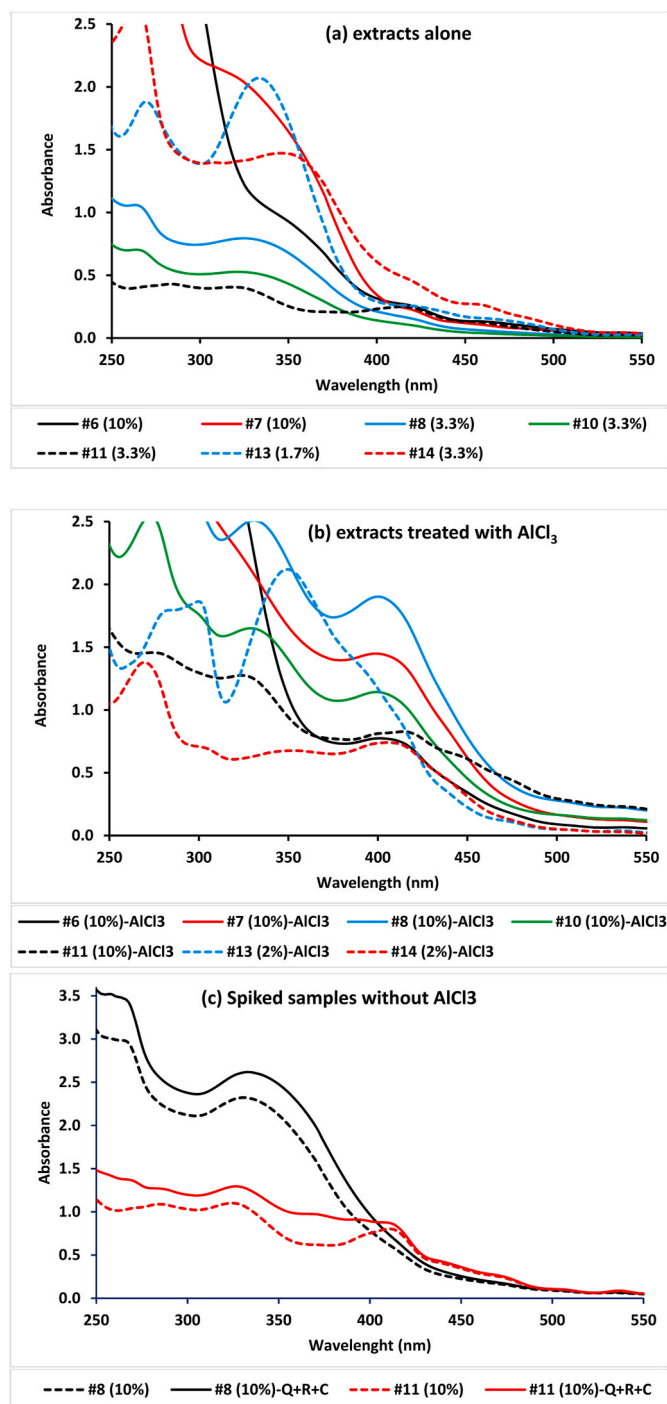
Fig. 3. Absorbance spectra of the plants extracts without AlCl₃ (**a**), with AlCl₃ (**b**), and standard-spiked in the absence of AlCl₃ (**c**). The numbers in brackets are the percent (v/v) of the crude extracts in the analyzed solutions, the numbers before the brackets are the samples numbers. Q: quercetin spiked at 4.0 μg/mL, R: rutin spiked at 6.2 μg/mL, C: catechin spiked at 3.9 μg/mL.

3.2.2. Spike recoveries of flavonoids in experimental solutions in the presence of NaNO₂

Spike analyses have been also performed in flavonoid standard solutions as shown in Fig. 7, where quercetin standard solution was spiked with rutin and catechin, either individually or mixed. The mixture of flavonoid standards produced a broad band that maximizes at ~485 nm. Although, any wavelength between 430 and 530 nm can be applied for the quantitation of TFC when using NaNO₂ in conjunction with AlCl₃ without much loss in sensitivity, it is recommended to use λ_{max} (i.e., 485

Table 4a

TFC in plant extracts expressed as μg/mL **quercetin** equivalents at various wavelengths.

Sample #	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm
6	22.1	17.5	15.7	13.9	10.8	9.5
7	41.1	32.1	28.6	25.6	20.4	18.0
8	54.0	41.7	36.8	32.6	25.6	22.4
10	32.5	25.0	22.0	19.4	15.1	13.1
11	23.2	18.9	17.7	16.6	14.4	14.4
13	33.3	22.1	18.2	14.6	9.2	7.3
14	21.0	17.0	15.3	13.6	10.7	9.5

Table 4b

TFC in plant extracts expressed as μg/mL **rutin** equivalents at various wavelengths.

Sample #	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm
6	35.0	31.9	30.5	28.7	24.9	24.0
7	65.0	58.4	55.7	52.6	47.0	45.3
8	85.2	75.9	71.5	66.9	58.9	56.2
10	51.4	45.5	42.9	39.9	34.8	33.0
11	36.7	34.5	34.4	34.2	33.2	36.4
13	52.6	40.3	35.4	30.1	21.3	18.6
14	33.3	30.9	29.7	28.0	24.7	24.0

Table 4c

%Difference in TFC in plant extracts when using quercetin and rutin as quantitation standards.

	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm
Mean	58.1	82.0	94.4	106.0	130.9	152.4
s.d. ($n = 7$)	0.2	0.1	0.0	0.3	0.7	1.1

nm as demonstrated in the present work). The most frequent absorbance wavelength reported in the literature is 510 nm (Table 1).

Spike recoveries calculated at 500 and 510 nm are summarized in Table 6. No pronounced differences in recoveries were observed at any of the two wavelengths. Although good recoveries for quercetin were obtained when results are expressed as either quercetin or rutin equivalents, unacceptable high recoveries (146–217%) for quercetin-catechin and quercetin-catechin-rutin mixtures were found when results are expressed as either quercetin or rutin equivalents. Furthermore, unacceptable low recoveries (42–53%) for quercetin and quercetin-rutin mixture were obtained when results are expressed as catechin equivalents. However, medium to good recoveries (72–93%) for quercetin-catechin and quercetin-catechin-rutin mixtures were found when results are expressed as rutin equivalents. These findings along with those reported in the previous sections provide conclusive evidence about the dependency of the TFC on the flavonoid standard used in the quantitation, which in turn highlight the conceptual flaws of the AlCl₃ colorimetric assay for the determination of TFC.

3.2.3. Interaction of AlCl₃ with flavonoids in plant extracts in the presence of NaNO₂

In a similar way used for flavonoid standard solutions, treatment of plant extracts with NaNO₂ produced a new broad band at λ_{max} between 495 and 500 nm (Fig. 8). The use of NaNO₂ in conjunction with AlCl₃ resulted in a larger redshift when compared to the use of AlCl₃ alone. The shift, which is sample-dependent, was from 330 to 400 nm (Fig. 3a) to ~400 nm when using AlCl₃ alone (Fig. 3b) and to ~500 nm when NaNO₂ was used in conjunction with AlCl₃. This large redshift at the quantitation λ_{max} reduces much the contribution of the samples' original absorbance in the final absorbance after the treatment with NaNO₂ (Fig. 8, also consult last paragraph in section *3.1.3*).

The TFC in samples' extracts using NaNO₂–AlCl₃ was quantified against standard solutions of quercetin, catechin, and rutin treated
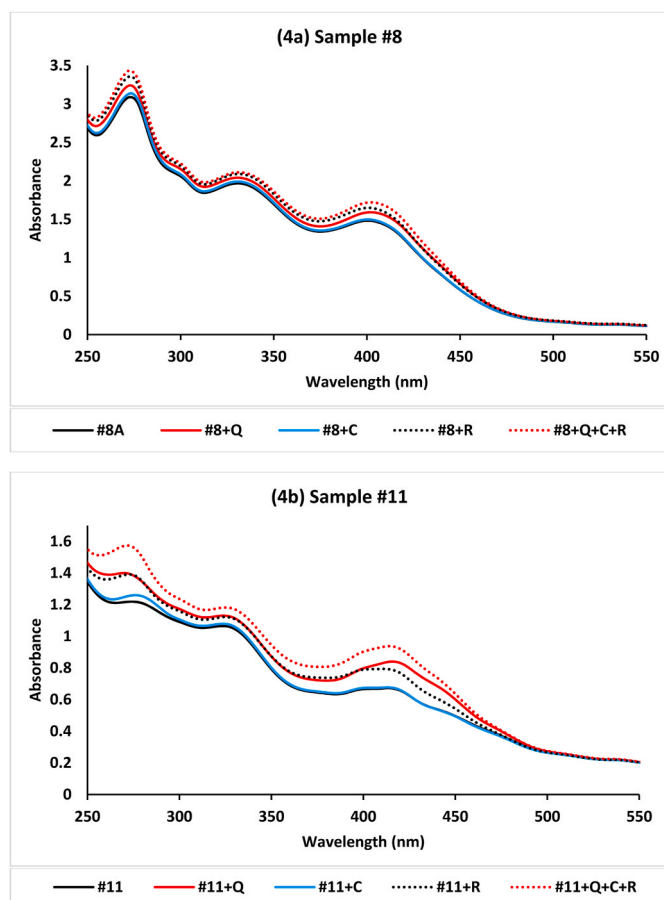
Fig. 4. Absorbance spectra for samples #8 (**4a**) and #11 (**4b**) unspiked and spiked with flavonoid standards. Q refers to quercetin spiked at 4.0 μg/mL, C: catechin at 3.9 μg/mL, and R: rutin at 6.3 μg/mL.

Table 5

Spike recoveries at various wavelengths: plant extracts spiked with known amounts of flavonoid standards.

ID [a]	Spiked amount	400 nm	410 nm	415 nm	420 nm	430 nm	440 nm
Recovered amount expressed as μg/mL quercetin equivalent (sample #8)							
8 + Q	4.0	3.2	3.1	3.0	2.9	2.6	2.4
8 + C	3.9	0.7	0.6	0.5	0.4	0.2	0.1
8 + R	6.2	4.9	4.0	3.6	3.2	2.5	2.2
8 + Q + C + R	14.0	6.9	5.9	5.5	5.0	4.2	3.6
Recovered amount expressed as μg/mL rutin equivalent (sample #8)							
8 + Q	4.0	5.3	5.7	5.9	6.0	6.3	6.4
8 + C	3.9	1.3	1.1	1.0	0.9	0.7	0.6
8 + R	6.2	8.0	7.4	7.0	6.7	6.1	5.8
8 + Q + C + R	14.0	11.0	9.1	10.7	10.4	9.9	9.4
Recovered amount expressed as μg/mL quercetin equivalent (sample #11)							
11 + Q	4.0	4.0	4.7	5.0	5.2	5.1	4.4
11 + C	3.9	0.5	0.4	0.4	0.3	0.3	0.2
11 + R	6.2	3.7	3.8	3.6	3.4	2.8	2.2
11 + Q + C + R	14.0	6.9	7.6	7.7	7.7	7.1	5.9
Recovered amount expressed as μg/mL rutin equivalent (sample #11)							
11 + Q	4.0	6.4	7.7	8.1	8.3	8.2	7.1
11 + C	3.9	0.9	0.9	0.8	0.7	0.6	0.5
11 + R	6.2	6.1	6.1	5.8	5.5	4.6	3.6
11 + Q + C + R	14.0	11.1	12.2	12.4	12.3	11.3	9.4

[a] Samples #8 and #11 spiked with known amounts of flavonoid standards. **Q** refers to quercetin spiked at 4.0 μg/mL, **C**: catechin at 3.9 μg/mL, and **R**: rutin at 6.3 μg/mL.

similarly as the samples at 500 and 510 nm. The calibration curves were constructed using five calibration standards containing 1.0–77.0 μg/mL. The obtained coefficient of determination (R^2) values was better than 0.991 for quercetin and better than 0.998 for catechin and rutin at the two wavelengths. When the TFC concentrations in plant extracts is expressed as quercetin equivalents at both 500 and 510 nm, the results were always almost double than those expressed as catechin equivalents. The mean difference between the two calculations was 53.3% (*range* 48–66%, *s.d.* 5.4%, n 8). On the other hand, calculated TFC values expressed as quercetin equivalents were slightly lower that those expressed as rutin equivalents for all samples with a mean difference of −12% (*range* -23 – 17%, *s.d.* 12%, n 8). This activity demonstrates again the dependency of TFC on the flavonoid standard employed in the quantitation process, which highlights further the conceptual and methodical flaws of the AlCl₃ colorimetric assay.

3.2.4. Spike recoveries of flavonoids in plant extracts in the presence of $NaNO_2$

Samples of plant extracts were spiked with individual flavonoid standards (at ∼ 8 μg/mL each) or with a mixture of the three flavonoids (at 24 μg/mL total). The absorption spectra before and after spiking are shown in Fig. 9. The percentage recoveries were calculated and expressed as quercetin equivalents, rutin equivalents, and catechin equivalents at 500 and 510 nm. The results obtained at the two wavelengths were close to each other. When recoveries were expressed as quercetin equivalents, good recoveries were obtained for the mixed standards (mean 120 ± 7.8%, n 4). However, unacceptable mean recoveries were obtained for both rutin (172%) and catechin (281%). Similarly, unacceptable mean recoveries were obtained for both

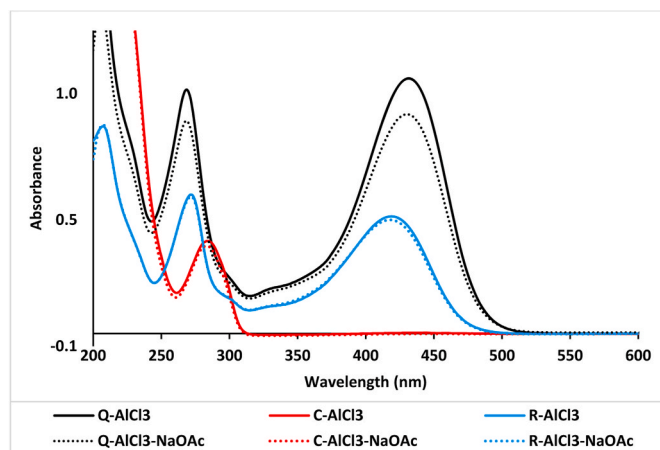**Fig. 5.** Absorbance spectra of Al(III)-flavonoids interaction in the absence and presence of CH₃COONa. Q means quercetin at 13.2 μg/mL, C: catechin at 25.2 μg/mL, and R; rutin at 13.0 μg/mL.

quercetin (44%) and catechin (149%) when results erre expressed as catechin equivalents. Likewise, unacceptable high mean recoveries were obtained for rutin (221%), catechin (343%), and the mixed standards (147%) when results are expressed as rutin equivalents. Details of the recoveries are listed in Table 7. This trial demonstrates once more the dependency of TFC on the flavonoid standard employed in the quantitation process, which highlights further the conceptual and methodical flaws in this assay.
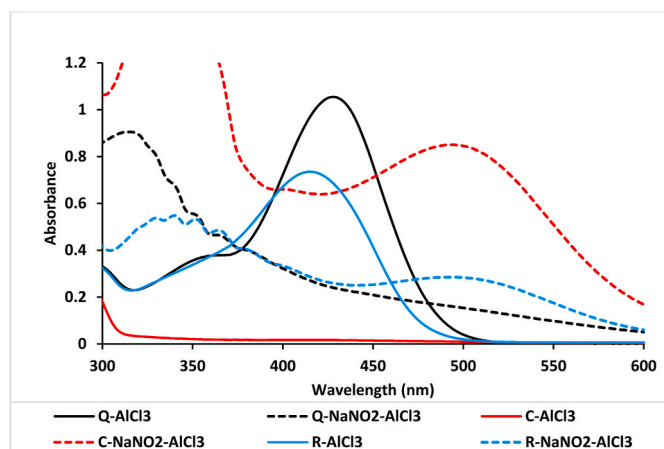
Fig. 6. Absorbance spectra of Al(III)-flavonoids interaction in the absence and presence of NaNO$_2$.
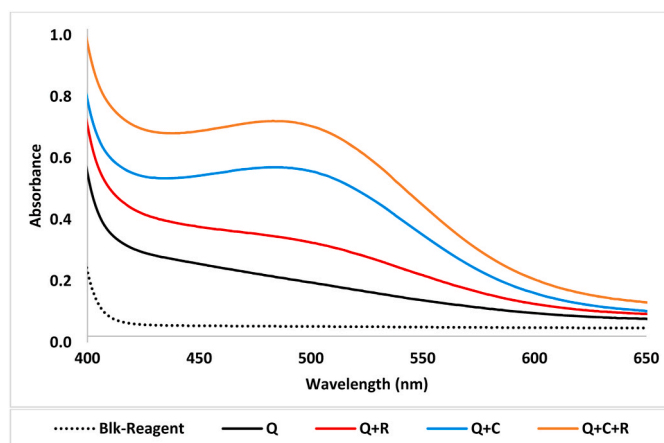

Fig. 7. Absorbance spectra for quercetin standard solutions spiked with rutin and catechin, either individually or as a whole. Q: quercetin alone at 15.06 μg/mL, C: Catechin (14.66 μg/mL), R: Rutin (14.82 μg/mL).

3.3. Use of NaNO$_3$ in the AlCl$_3$ colorimetric assay

The use of sodium nitrate (NaNO$_3$) in the AlCl$_3$ colorimetric assay for the determination of TFC has been reported by some authors (Table 1) (Chen et al., 2019; Gomes et al., 2017; M. Jain; Kassim, 2011; Patnaik & Khoury, 2004). No spectra or explanation about the effect of NaNO$_3$ were provided in any of these references. To assess the effect of NaNO$_3$ on the AlCl$_3$ assay, we performed additional tests on the three flavonoid standards and plant extracts using the same procedure described in section 2.3 in the absence and presence of NaOH. When compared to the treatment with AlCl$_3$ alone (Fig. 1), the addition of NaNO$_3$ in the

absence of NaOH resulted in a slight redshift in λ_{max} from 428 to 440 nm for quercetin and from 415 to 428 nm for rutin. No appreciable effect on the absorbance at λ_{max} was observed for both standards (compare Fig. 10 with Fig. 1). Catechin did not show any changes. On the other hand, the addition of NaNO$_3$ in basic medium made large changes in the absorption spectra of all standards. It resulted in a blueshift for both quercetin and rutin and the appearance of new bands for catechin at 350 nm, 425 nm, and 500 nm (broad weak band). Similar observations have been found for plant extracts as shown in Fig. 11. These trials show that the use of NaNO$_3$ does not improve the suitability of the assay for the TFC quantification.

4. Conclusions

The widely employed aluminum chloride colorimetric assay for the determination of the total flavonoid content (TFC) in plant extracts was experimentally evaluated in present work. The accuracy of the assay was assessed at several wavelengths in the absence and presence of sodium nitrite using spike recoveries in real plant extracts and three authentic flavonoid standards (quercetin, catechin, and rutin). As various flavonoids have different chemical structures, noticeable differences in their absorption spectra are indisputable. Accordingly, determination of TFC according to aluminum chloride colorimetric assay becomes dependent on the flavonoid standard employed in the quantitation process as demonstrated in the current work, where unacceptable high or low false
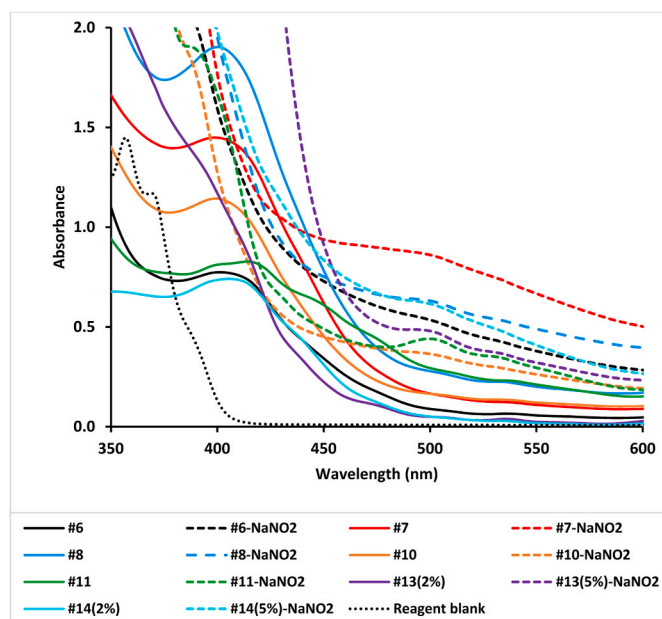

Fig. 8. Absorbance spectra of the interaction of Al(III) with plant extracts in the absence and presence of NaNO$_2$. %Samples in test solutions was 10% for all samples except where otherwise specified.

Table 6
Spike recoveries measured at 500 and 510 nm for quercetin (Q) standard solution spiked with known amounts of catechin (C) and rutin (R) standards (15.06, 14.82, 14.66 μg/mL for quercetin, rutin, and catechin, respectively). The mixture of the three contains 44.54 μg/mL as total flavonoid).

| Wavelength nm	Q1	Q1+R1	Q1+C1	Q1+C1+R1	Q	Q + R	Q + C	Q + C + R
%recoveries expressed as **quercetin equivalents**								
500 nm	131.5	105.1	198.4	163.4	124.6	102.2	173.5	145.6
510 nm	92.1	88.7	190.9	161.2	106.2	96.6	174.5	149.0
%recoveries expressed as **catechin equivalents**								
500 nm	44.9	43.4	92.9	78.5	53.0	47.6	85.3	72.8
510 nm	43.4	42.4	92.2	78.0	50.9	46.6	84.5	72.3
%recoveries expressed as **rutin equivalents**								
500 nm	108.7	102.6	216.9	182.7	124.9	111.2	198.2	168.8
510 nm	105.9	100.8	215.6	181.8	120.5	109.1	196.6	167.8

(a)

(b)
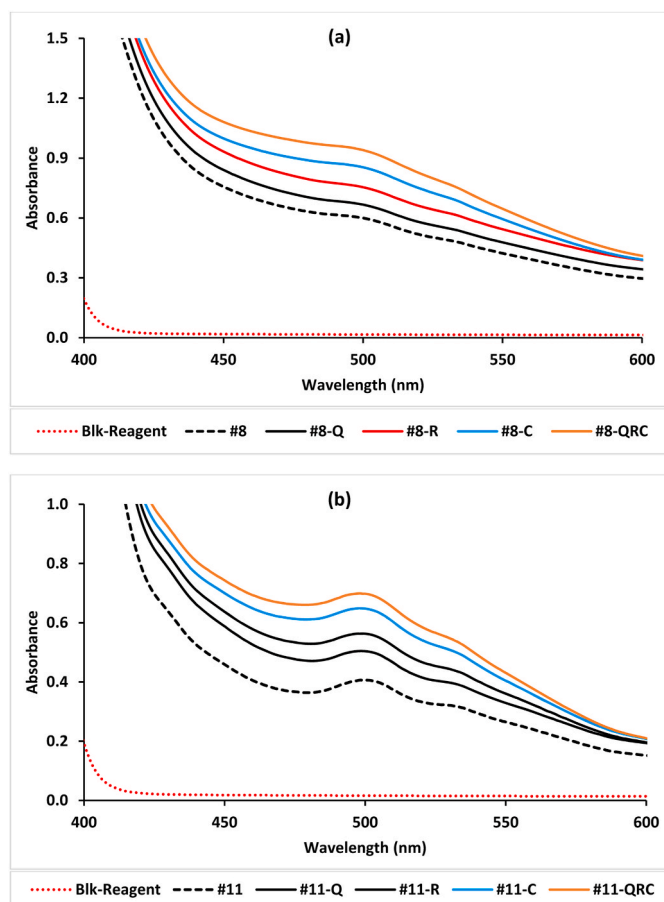
Fig. 9. Absorbance spectra for samples #8 (**a**) and #11(**b**) unspiked and spiked with flavonoid standards. Q refers to quercetin spiked at 7.93 μg/mL, C: catechin at 8.10 μg/mL, and R: rutin at 8.03 μg/mL %Samples in test solutions was 10% for all samples.

Fig. 10. Absorbance spectra of Al(III)-flavonoids interaction when using $NaNO_3$ in the absence and presence of NaOH. Quercetin (Q), rutin (R), and catechin (C) concentrations were 7.9, 15.4, and 19.3 μg/mL, respectively.

11 (a)

11 (b)

Fig. 11. Absorbance spectra of Al(III)-plant extracts interaction when using $NaNO_3$ in the absence (**a**) and presence (**b**) of NaOH.

Table 7
%Spike recoveries measured at 500 and 510 nm for plant extracts spiked with known amounts of flavonoid standards (7.93, 8.03, 8.10, and 24.06 μg/mL for quercetin, rutin, catechin, and mixture of the three, respectively).

Quercetin	Rutin	Catechin	Mixture	
%recoveries expressed as **quercetin equivalents**				
Min.	62.8	161.3	270.6	109.9
Max.	111.9	177.1	296.4	128.0
Mean	82.5	172.3	280.7	120.4
s.d.	20.8	7.5	12.1	7.8
%recoveries expressed as **catechin equivalents**				
Min.	33.3	85.5	143.4	58.3
Max.	59.3	93.9	157.1	67.8
Mean	43.7	91.3	148.8	63.8
s.d.	11.0	4.0	6.4	4.1
%recoveries expressed as **rutin equivalents**				
Min.	76.8	197.3	331.0	134.4
Max.	136.9	216.6	362.4	156.5
Mean	100.9	210.7	343.3	147.2
s.d.	25.5	9.2	14.8	9.6

recoveries (*range* 33–343%) were obtained depending on the flavonoid standard used in the TFC quantitation process. Another remarkable point to consider is, the use of acetate salts has no noticeable effect on the TFC determination when using the $AlCl_3$ colorimetric assay. The extensive experimental data presented in this work highlights the major conceptual and methodical flaws the aluminum chloride colorimetric assay inherently suffers from that limit its validity for the quantitation of TFC.
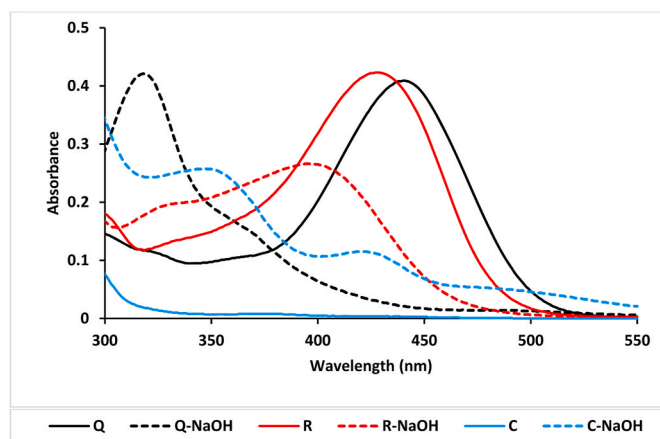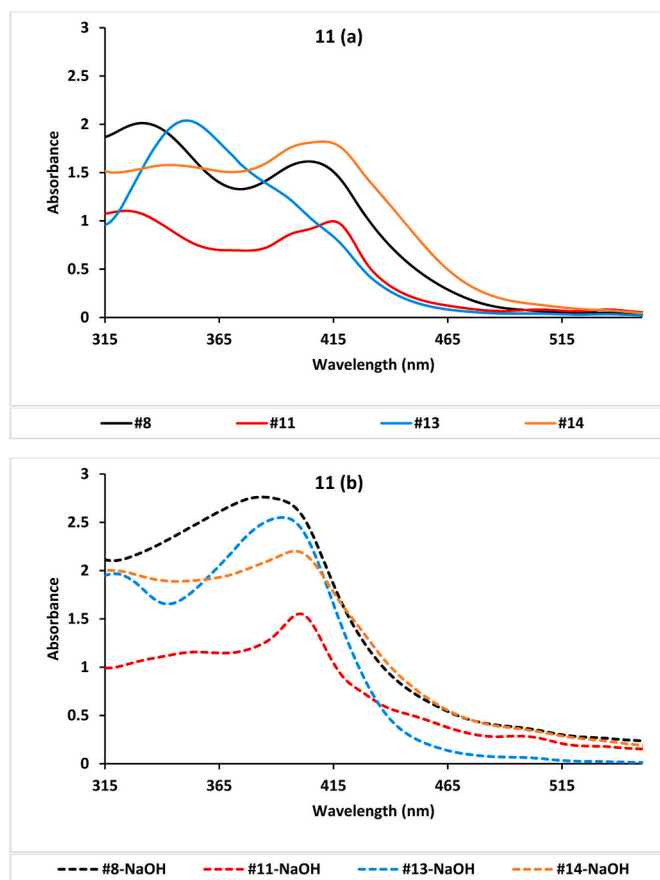
CRediT authorship contribution statement

Amjad M. Shraim: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing, Project administration. **Talaat A. Ahmed:** Plant collection, Writing – review & editing, Project administration. **Md Mizanur Rahman:** Funding acquisition, Writing – review & editing. **Yousef M. Hijji:** Discussion, Writing – review & editing.

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Declaration of competing interest

None.

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