Article

Solubility of Luteolin and Other Polyphenolic Compounds in Water, Nonpolar, Polar Aprotic and Protic Solvents by Applying FTIR/HPLC

Saša Rajhard [1,2], Lucija Hladnik [1], Filipa A. Vicente [1,*], Stanko Srčič [2], Miha Grilc [1] and Blaž Likozar [1,*]

[1] Department of Catalysis and Chemical Reaction Engineering, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia; sasarajhard@gmail.com (S.R.); lucija.hladnik@ki.si (L.H.); miha.grilc@ki.si (M.G.)

[2] The Department of Pharmaceutical Technology, Faculty of Pharmacy, Aškerčeva Cesta 7, 1000 Ljubljana, Slovenia; stanko.srcic@ffa.uni-lj.si

* Correspondence: filipa.andre.vicente@ki.si (F.A.V.); blaz.likozar@ki.si (B.L.)

Abstract: In recent years, flavonoids have become a highly researched topic due to their health beneficial effects. Since flavonoids' solubility plays a significant role in their use in pharmaceutical, food, biological, and chemical areas, the determination of suitable solvents is crucial. Fourier transform infrared (FTIR) analysis was used to characterize functional groups of several flavonoids and phenolic compounds, namely luteolin, hesperidin, quercetin, naringenin, gallic acid and tannic acid. Concentration dependence on transmittance was evaluated for these compounds in ethanol. Afterwards, luteolin was chosen as a model flavonoid, with its concentration correlated with transmittance using 15 solvents with different polarities. Luteolin solubility was further corroborated with high-performance liquid chromatography (HPLC). These results shed light on using FTIR as a semi-quantitative method for the initial screening of solvents and the solubility of different compounds while saving time and solvents. Hence, HPLC would only be needed as a final step for the most promising solvents.

1. Introduction

Solubility data of flavonoid compounds are essential for the pharmaceutic industry and food, biological, chemical, and medicine branches [1]. It is one of the most important parameters being determined for purification processes, drug identification and for the preparation of dietary supplements and drug products [2]. Likewise, the determination of suitable solvents is also of the utmost importance to ensure an optimal process of extraction and separation of the compounds, especially considering the compounds stability, the system selectivity, and the solvent cost and safety [3,4]. In recent years, interest in flavonoids has been increased due to their numerous applications, including functional food and beverages, dietary supplements, and pharmaceuticals as a result of their multiple health-benefiting properties [5]. High potency and low systemic toxicity make these compounds applicable as alternatives to conventional therapeutic drugs [6]. Flavonoids can be divided into six subclasses: flavones, flavanols, flavanones, catechins, or flavanols, anthocyanidins and isoflavones [7]. Within the flavones, luteolin arises as one of the most prevalent ones, being commonly found in fruits, vegetables, seeds and beverages of plant origin, and presents numerous biological effects such as anti-oxidative, anti-inflammatory and anti-allergic. Hence, it has shown multiple cardio-protective results [7]. Additionally, luteolin has been widely studied for its anticancer properties, as it induces apoptotic cell death, inhibits cancer cell proliferation and suppresses tumor angiogenesis [8].

Flavonoids are commonly extracted using different extraction methodologies, for instance maceration, heat reflux extraction, Soxhlet extraction and hydrodistillation ex-

traction. However, these conventional techniques present some drawbacks, namely low extraction efficiency, long extraction times, high temperatures and the use of large amount of organic solvents [4]. Alternatively, solid-liquid extraction is a faster and simpler process with milder conditions that has also been proven efficient. In fact, Koleva et al. [9] showed that high amounts of flavonoid and phenolic compounds can be extracted with this technique. Furthermore, studies have shown that under the same extraction conditions, solvent selection is the most important parameter. Not only does it affect the extraction yield, but it also significantly affects the biological activity of the compound [10]. Hence, the solubility and stability screening of different flavonoids and phenolic compounds in different solvents is of crucial importance. The literature typically shows studies of a single flavonoid in a couple of solvents [11,12] or with an increase in the number of compounds under study by up to four but to a maximum of five solvents [13,14] evidencing a current lacuna in more complete studies. Polar solvents and mixtures of alcohol (such as methanol or ethanol) and water are used for extractions of polar compounds, whereas pure organic solvents such as chloroform, dichloromethane and diethyl ether are used for extractions of less polar and/or nonpolar compounds [15]. Selection of the extraction method has a great impact on the quantitative isolation of extracted phenols. Thus, the method applied should be determined by the chemical nature of the isolating compound, which varies accordingly to the solvent used and process conditions such as temperature, pH and extraction time [16,17].
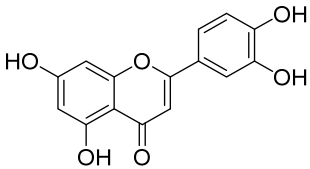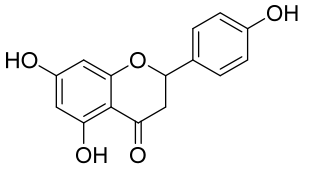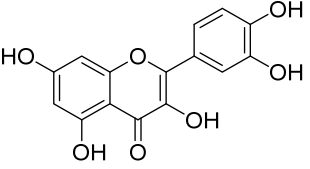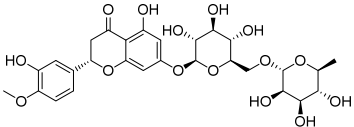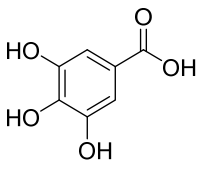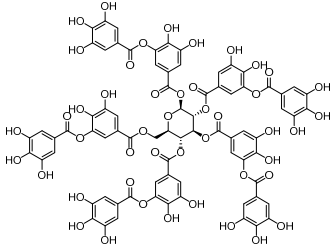
Regarding the quantification of flavonoids solubility, there are several methods reported, among which high-performance liquid chromatography (HPLC) is the most frequently used, as shown by Peng and Yan [17], Xiao and Yan [18] and Shakeel et al. [19], due to its accuracy and sensitivity. However, in spite of being a quick and efficient method, developing a new quantification method can be time consuming. This arises as the greatest downside of HPLC quantification. In this sense, the use of alternative approaches emerges as an attractive option, especially concerning initial solvents screening and optimization studies. Ferreira and Pinho [13] reported the use of a gravimetric method for the quantitative analysis of several flavonoids solubility whereas Zhang et al. [20] determined the solubility of naringenin and naringin through UV-VIS spectrophotometry. Another option to measure compound's solubility is Fourier-transform infrared spectroscopy (FTIR), as this is a quick, in-line, non-destructive method that can also be used as a quantification tool. Yet, it is rarely used to measure the solubility of flavonoids [21]. Therefore, the aim of this research was to shed some light on the current lacuna regarding more complete studies with a wider range of flavonoids and phenolic compounds, and solvents. Herein, different flavonoids, namely luteolin (flavone), naringenin (flavanone), hesperidin (glycosylated flavanone) and quercetin (flavanol), and two phenolic compounds, explicitly tannic acid and gallic acid, were firstly characterized through FTIR-ATR. This helps with gathering crucial knowledge about the functional groups of distinct flavonoids, which will consequently dictate the compounds solubility. Secondly, these compounds concentration was correlated with transmittance in a common studied solvent, namely ethanol. Afterwards, luteolin was chosen as a model flavonoid to further study its solubility in a total of 15 solvents with a wide range of polarities, using both FTIR-ATR and HPLC. This allowed the correlation between transmittance and concentration. Measuring solubility of flavonoids has already been done in several solvents, usually in water, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, ethyl acetate and acetonitrile [11,19,22]. However, measuring the solubility of luteolin in a wider range of solvents was rarely done. Thus, the results of this screening methodology can be applied in future experiments, regarding extractions of flavonoids with similar structure and for the determination and selection of suitable solvents.

2. Materials and Methods

2.1. Chemicals

Table 1 presents all compounds studied in this work and their chemical structure, CAS number, purity, and supplier. The solvents used in the experiments are presented in Table 2 alongside their CAS number, purity, and supplier.

Table 1. Phenolic compounds studied in this work and their chemical structure, CAS number, purity and supplier.

	Chemical Structure	CAS Number	Purity (wt %)	Supplier
Luteolin		491-70-3	97.0%	Abcr GmbH
(±)-Naringenin		67604-48-2	≥95.0%	Aldrich
Quercetin		117-39-5	≥95.0%	Sigma
Hesperidin		520-26-3	≥80.0%	Sigma
Gallic acid		149-91-7	≥97.5%	Sigma Aldrich
Tannic acid		1401-55-4	≥87.5%	Sigma Aldrich

2.2. Sample Preparation

All experiments were carried out by dissolving the different compounds at 27 °C for 60 min while stirring at 150 rpm. The conditions were regulated using a IKAMAG magnetic stirrer with heating plate and were defined after preliminary experiments. The lack of sedimentation after centrifugation was evidence of a total dissolution. In the case of saturated solutions, samples presented a pellet after being centrifuged, hence the supernatant was collected and filtrated through 0.22 μm filters before being analyzed. The analysis of four luteolin concentrations (1, 2, 4 and 8 mg·mL^{-1}) was performed using FTIR-ATR spectroscopy (*cf.* FTIR analysis section).

Table 2. Solvents and their CAS number, purity and supplier.

Solvent	CAS Number	Purity (wt %)	Supplier
Methanol	67-56-1	≥99.8%	Honeywell
Ethanol	64-17-5	≥99.9%	Carlo Erba
n-Propanol	71-23-8	≥99.5%	Kemika
1-Butanol	71-36-3	≥99.5%	Merck
Ethyl acetate	141-78-6	≥99.9%	Honeywell
Acetonitrile	75-05-8	≥99.9%	Honeywell
Diethyl ether	60-29-7	≥99.7%	Merck
Acetone	67-64-1	≥99.5%	Honeywell
Toluene	108-88-3	≥99.5%	Merck
Dimethyl sulfoxide	67-68-5	≥99.9%	Merck
1.4-Dioxane	123-91-1	≥99.0%	Honeywell
Tetrahydrofuran	109-99-9	≥99.9%	Honeywell
Dichloromethane	75-09-2	≥99.5%	Merck
Benzene	71-43-2	≥99.0%	Sigma–Aldrich

Furthermore, three lower luteolin concentrations (0.003, 0.03 and 0.3 mg·mL^{-1}) were prepared as aforementioned, namely at 27 °C for 60 min with stirring at 150 rpm. Conditions were controlled with IKAMAG magnetic stirrer with heating plate. Supernatant was collected with a syringe and filtered through a 0.22 µm filter to be further analyzed using HPLC (*cf.* HPLC analysis section). Luteolin solubility was determined by calculating the amount of dissolved luteolin in these samples, using a pre-established calibration curve with DMSO as solvent (cf. Figure S1 in Supporting Information). Supplier's information of the purities was satisfactory; therefore, the experimental procedure did not include purification of the compounds.

To ensure a wide range of solubility data, 15 solvents with different polarity were selected: nonpolar (benzene, toluene, 1,4-dioxane, diethyl ether), polar aprotic (ethyl acetate, tetrahydrofuran, dichloromethane, acetone, acetonitrile, dimethyl sulfoxide) and polar protic (water, methanol, ethanol, 1-propanol, 1-butanol).

2.3. FTIR Spectroscopy

The FTIR analysis was performed using a FTIR-ATR spectrometer (Perkin Elmer, Spectrum Two). Each sample was analyzed with 32 scans in the region of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and an interval of 2 cm^{-1} at room temperature. The spectrometer has a high-performance DTGS (deuterated triglycine sulphate) MIR detector with a signal to noise ratio of 14,500:1.

2.4. HPLC Analysis

Luteolin solubility was determined with a UHPLC Fisher Thermo Scientific Ultimate 3000 using a Kinetex C18 column (5 µm, 100 mm × 4.6 mm). The mobile phase contained a mixture of water and trifluoroacetic acid in a volume ratio of 99.9:0.1 (mobile phase A) and acetonitrile and trifluoroacetic acid in a volume ratio of 99.9:0.1 (mobile phase B) in a gradient system as follows: 0–4 min, 100% mobile phase A; 4–6 min, mobile phase 40% A; 16–18 min 100% mobile phase B; 18–22 min, 100% mobile phase A. The elution flow rate was 1 mL.min^{-1} with an injection volume of 10 µL, the oven temperature was set to 30 °C and the detection wavelengths were 210 and 255 nm [22]. Luteolin purity (97%) was adequate and further purification was not needed.

Luteolin solubility was determined considering the average of three to six replicas, being the standard deviation also determined and presented alongside the average. Ex-

perimental values of luteolin solubility in mole fraction were calculated using Equation (1), where m_1 (g) is mass of luteolin, V_2 (mL) is volume of the solvent and ρ_2 (g mL^{-1}) is the solvents density. M_1 and M_2 are molar masses of luteolin (g mL^{-1}) and pure solvent (g mL^{-1}). Masses of luteolin in different solvents were acquired through HPLC results, while densities of solvents were obtained from the literature [23]. Molar solubility values are expressed *per* volume of a solvent since it concerns very diluted samples [19].

$$x = \frac{\frac{m_1}{M_1}}{\frac{m_1}{M_1} + \frac{V_2 \cdot \rho_2}{M_2}} \tag{1}$$

3. Results

As aforementioned, by studying different flavonoids through FTIR-ATR spectroscopy, it is possible to gather critical information about the functional groups that will play an important role in the compound dissolution. Therefore, several flavonoids, namely luteolin (flavone), naringenin (flavanone), hesperidin (glycosylated flavanone) and quercetin (flavanol), and two phenolic compounds, explicitly tannic acid and gallic acid, were selected to shed some light upon these compounds' solubility, in particular luteolin. FTIR-ATR analyses were performed for every compound in a concentration of 4 mg·mL^{-1} in ethanol and compared with the spectrum of pure solvent. Special attention was given to luteolin, as a representative of the flavonoids. Thus, this compound was studied in the presence of several solvents. Additionally, a correlation of luteolin solubility was attempted using both FTIR-ATR spectroscopy and HPLC. HPLC analysis was used to determine dissolution of luteolin at three initial concentrations: 0.003, 0.03 and 0.3 mg·mL^{-1}. Based on the amount of luteolin dissolved in these samples, the order of solvent suitability was determined.

3.1. FTIR Spectroscopy

The spectra of the different flavonoids and phenolic compounds, for instance luteolin, naringenin, quercetin, hesperidin, gallic and tannic acid dissolved in ethanol all show comparable IR spectra, as shown in Figure 1. Main differences between different spectra appear between 1760–1170 cm^{-1}, which is emphasized by a rounded rectangle, and can be directly observed in Figure S2 of SI. Here, the ethanol spectrum has been subtracted from the mixture solute-solvent. It is clear that all compounds present 6 main vibrations, with slight deviations among them: (i) a broad peak located at 3340 cm^{-1} corresponding to O-H stretching vibrations; (ii) peaks in the range of 2978 to 2896 cm^{-1} belong to stretching of the C-H bond; (iii) the peak at 1738 cm^{-1} is assigned to the C=O stretching; (iv) peaks in the range 1643 to 1568 cm^{-1} are a result of the C-C bond of the aromatic ring; (v) the peak at 1160 cm^{-1} corresponds to the C-O stretching and (vi) peaks at 1045 and 879 cm^{-1} are assigned to C-OH bond [24]. Luteolin, naringenin and quercetin are flavonoids with a very similar chemical structure, which can also be seen in the spectra, especially in the range 1696–1129 cm^{-1}. The structure of hesperidin differs from the rest of flavonoids, as it is a glycoside. This difference can be seen in the IR spectra as the peaks are not as intense. Tannic acid is a phenolic compound with the same functional groups as the flavonoids, only with a much more complex structure therefore, the peaks in the spectra are more intense in the range 1809 to 1144 cm^{-1}. The structure of gallic acid is comparable to tannic acid, only with less complexity, which is reflected in the peaks' lower intensity.

3.1.1. Luteolin Analysis in Presence of Different Solvents

It was previously mentioned that luteolin was chosen as the model flavonoid solute for the solubility systematic experiments. Therefore, this compound was dissolved in a total of 15 solvents, namely ethyl acetate, methanol, ethanol, propanol, butanol, acetonitrile, dichloromethane, diethyl ether, acetone, water, 1,4–dioxane, toluene, THF, benzene and DMSO, as shown in Figure 2. It should be stressed that solvents under study can disguise luteolin's peaks so, depending on the solvent, differences can only be seen in some regions. To facilitate the analysis and comparison, solvents with similar spectra were grouped and

discussed together. IR spectra of luteolin in methanol and ethanol are quite similar. There is a broad peak located at 3325 cm^{-1}, which is the result of O-H vibrations, and peaks in the range of 2832 to 2994 cm^{-1}, which are assigned to the C-H stretching vibrations. Peaks at 1656–1609 cm^{-1} are result of C=C vibrations of luteolin. C-O-C stretching vibrations are most likely seen at 1169 cm^{-1} and C–OH vibrations at 1045 cm^{-1} and 1022 cm^{-1} in the methanol and ethanol spectra, respectively [25,26].
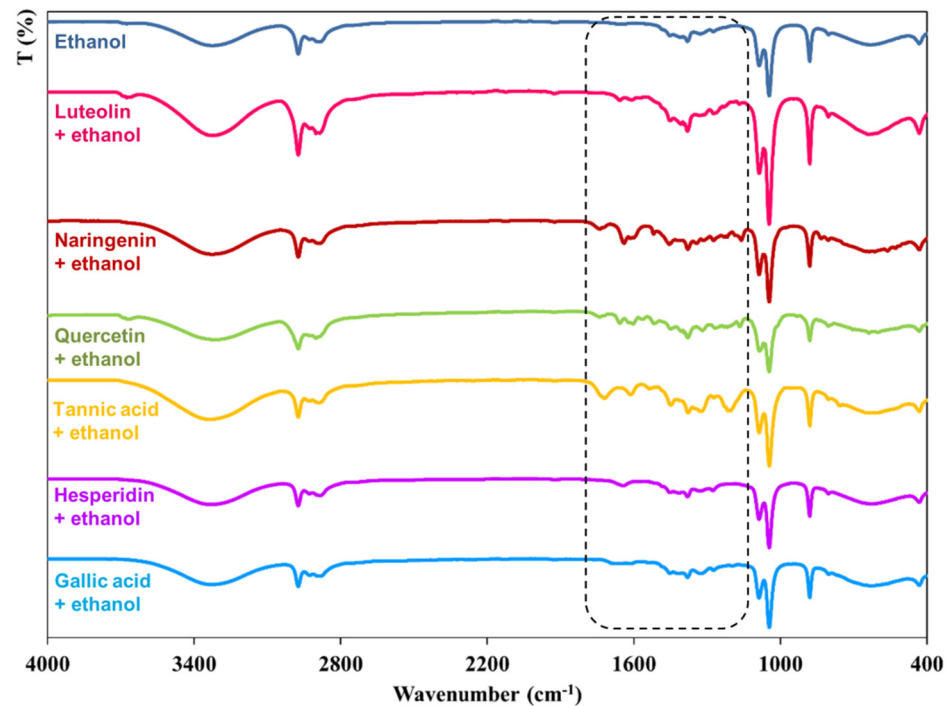

Figure 1. FTIR–ATR spectra of luteolin, naringenin, quercetin, tannic acid, hesperidin and gallic acid in ethanol.

Spectra of luteolin dissolved in propanol and butanol shows a broad peak at 3324 cm^{-1} which is a result of O-H vibrations and multiple peaks in the range of 2959 to 2873 cm^{-1}, which are assigned to asymmetric and symmetric C-H stretching. Peaks at 1658 and 1608 cm^{-1} belong to the C=C bond of luteolin, while the C-OH bond is located at 1071 cm^{-1} and at 1053 cm^{-1} in the butanol and propanol spectra, respectively [27].

Spectra of luteolin in acetone and ethyl acetate resemble each other. Peaks assigned to C-H vibrations are located at 3003 cm^{-1} in the acetone spectrum and at 2984 cm^{-1} in the ethyl acetate spectrum. In the acetone spectrum, the C=O bond is located at 1710 cm^{-1}, and peaks at 1358 cm^{-1} and 1219 cm^{-1} are associated with C-C stretching. Moreover, the C=O bond in ethyl acetate spectrum is present at 1737 cm^{-1}, C=C vibrations at 1656 cm^{-1}, and C-O stretches are located at 1233 cm^{-1} [28,29].

Luteolin dissolved in 1.4-dioxane shows a spectrum with multiple peaks in the range of 2959 to 2852 cm^{-1}, which are assigned to C-H stretching. There are peaks at 1655 and 1609 cm^{-1} which belong to C=C vibrations of luteolin. The peak located at 1116 cm^{-1} is a result of C-O-C bond. IR spectrum of luteolin in THF shows two peaks at 2973 and 2857 cm^{-1}, which correspond to the C-H stretching vibrations. Peaks at 1660 and 1604 cm^{-1} are assigned to C=C vibrations of luteolin, and peak at 1066 cm^{-1} is a result of C-O-C stretching vibrations [29].

Luteolin dissolved in DMSO shows a spectrum with two peaks at 2989 and 2911 cm^{-1}, which are assigned to C-H stretching. There are additional C-H bending peaks at 1407 and 1309 cm^{-1}. Peaks located at 1655 and 1603 cm^{-1} are result of C=C vibrations and peaks at 1251 and 1230 cm^{-1} are most likely the result of C-O-C bond [28].
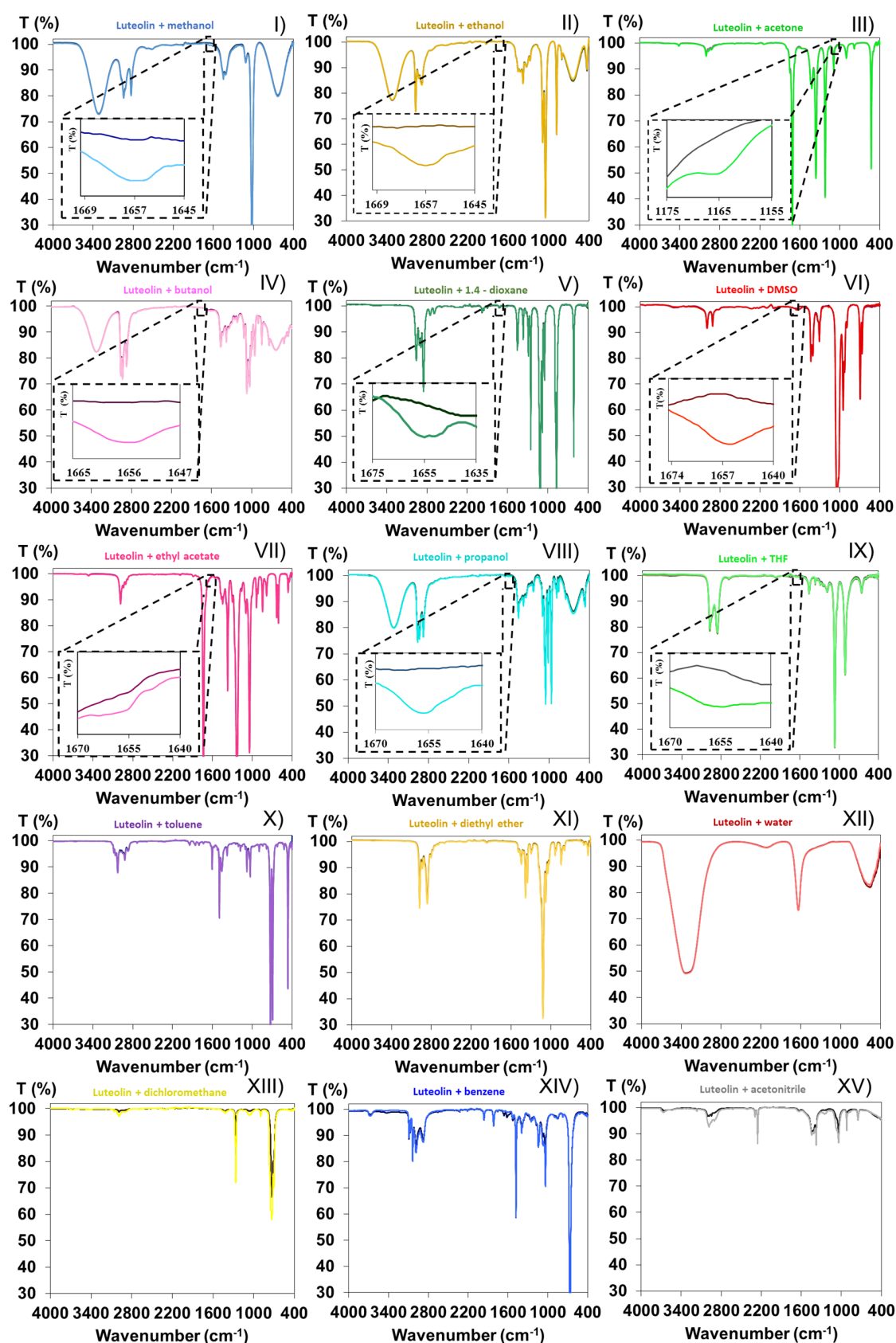
Figure 2. FTIR–ATR spectra of luteolin dissolved in various solvents. Spectrum of pure solvent is presented with darker color, while the spectrum of luteolin dissolved in the respective solvent has a lighter color: (**I**) methanol, (**II**) ethanol, (**III**) acetone, (**IV**) butanol, (**V**) 1,4-dioxane, (**VI**) DMSO, (**VII**) ethyl acetate, (**VIII**) propanol, (**IX**) THF, (**X**) toluene, (**XI**) diethyl ether, (**XII**) water, (**XIII**) dichloromethane, (**XIV**) benzene and (**XV**) acetonitrile.

Lastly, there is the attempt to dissolve luteolin in the remaining studied solvents, namely diethyl ether, acetonitrile, dichloromethane, water, benzene and toluene. Herein, the IR spectra only displayed peaks representing the functional groups of the solvents, i.e., there were no distinctive peaks of luteolin in these systems. These results evidence that there was either no solubilization of luteolin in these solvents or that the solvents' peaks are overlapping luteolin's peaks as they are much more intense. As such, diethyl ether, acetonitrile, dichloromethane, water, benzene and toluene were not used for more FTIR spectroscopy studies.

In summary, from the analyses that have been carried out, it is possible to gather a wavenumber range corresponding to the different functional groups' vibrations of the flavonoids and phenolic compounds, as simplified in Table 3. The region 3375–3324 cm^{-1} is associated with O-H stretching and the region of 3026–2832 cm^{-1} corresponds to C-H stretching. Stretching band of the C=O group can be found at 1737-1710 cm^{-1} and the C=C bond of the aromatic nucleus is located in the range 1660-1466 cm^{-1}. The region of 1261–1066 cm^{-1} represents the stretching of the C-O-C bond while the 1071–1022 cm^{-1} region represents C-OH vibration. Experimental data obtained from this research is consistent with several other studies describing IR spectra of flavonoids, such as the study performed by Silva et al. [30], where –OH bond was located at 3627–2971 cm^{-1}, C=O bond at 1712–1704 cm^{-1}, C=C at 1609–1608 and 1519–1516 cm^{-1}, C-OH at 1281–1278 cm^{-1} and C-O-C at 1207–1062 cm^{-1}.

Table 3. IR spectra data (cm^{-1}) of luteolin in presence of the different solvent studied in this work.

	IR Vibration Peaks at Specific Wavenumbers (cm^{-1})					
Solvent	**O-H**	**C-H**	**C=O**	**C-C**	**C-O-C**	**C-OH**
Ethanol	3325	2973–2901	/	1656–1609	1169	1045
Methanol	3325	2994–2832	/	1654–1611	1168	1022
1.4–Dioxane	/	2959–2852	/	1655–1609	1116	/
Ethyl acetate	/	2984	1737	1656	1233	/
Acetone	/	3003	1710	1358–1219	/	/
DMSO	/	2989-2911	/	1655–1603	1251–1230	/
THF	/	2973–2857	/	1660–1604	1066	/
Butanol	3324	2959–2873	/	1658–1608	/	1071
Propanol	3324	2952–2877	/	1658–1608	/	1053

Note: For the remaining solvents, namely diethyl ether, acetonitrile, dichloromethane, water, benzene and toluene, IR spectra showed no distinctive peaks for the compound apart from the solvent peaks.

As presented in Table 3, there are significant shifts of peaks that correspond to the same functional group between solvents. The most important factor that determines positions of peaks is the chemical structure of the solvent and its functional groups. However, the shifts between the positions of each group are most likely the result of solute-solvent interactions. As reported by Zhong et al. [31], hydrogen bonding significantly influences the position of the C=O bond of the flavonoid. The magnitude of a shift depends on the strength of the hydrogen bond. The other literature reports show that it is common to observe hydrogen bonding shifts in aqueous solutions of alcohols [32]. Therefore, it can be concluded that whenever a compound has groups involved in hydrogen bonding, significant changes can be observed in the spectra. Furthermore, luteolin has many –OH groups that can either be acceptors or donors for hydrogen bonding, hence explaining the multiple shifts of the functional group position between the solvents.

3.1.2. Correlation of Flavonoids and Phenolic Compounds Concentration and Transmittance

FTIR spectroscopy has been used to analyze the correlation between transmittance and concentration of the solution. According to Beer–Lambert's law and the relation of absorbance to transmittance, there is a direct correlation between transmittance and concentration. This implies that transmittance decreases with higher concentration, therefore the intensity of peaks is increased. As shown in Figure 3, the correlation between transmittance and the compounds concentration was evaluated for luteolin, quercetin, naringenin, hesperidin, tannic acid and gallic acid while being dissolved in ethanol. This gave crucial insights about the possibility of applying FTIR spectroscopy as a (semi-)quantitative method to infer about the solubility of flavonoids and phenolic compounds. Whenever possible, calibration curves were generated from the IR spectra to demonstrate the linear relationship between the absorbance and concentration, which are shown in Figures S3 and S4 of SI, alongside the respective equations. The linear relationship could not be determined in samples in which hesperidin and gallic acid were dissolved in ethanol since the intensity of the peaks did not increase consecutively with higher concentration.
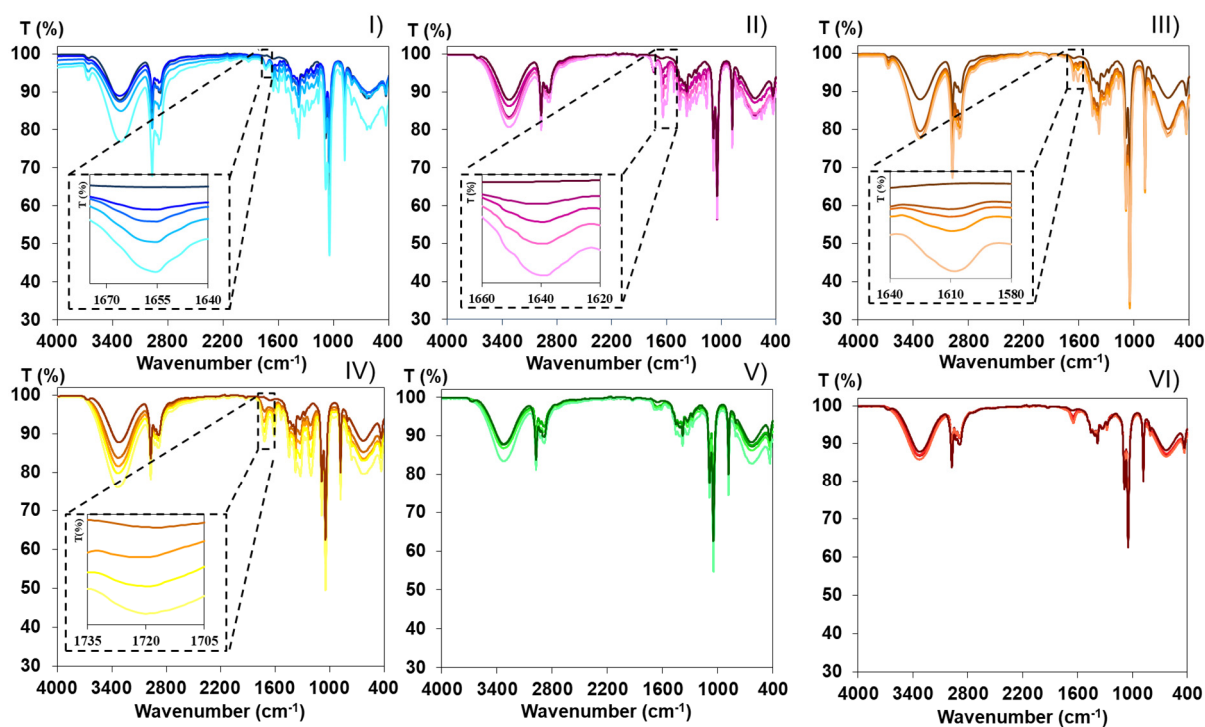

Figure 3. FTIR–ATR spectra of 1, 2, 4 and 8 mg·mL^{-1} of (**I**) quercetin, (**II**) naringenin, (**III**) luteolin, (**IV**) tannic acid, (**V**) gallic acid and (**VI**) hesperidin dissolved in ethanol. Spectrum of pure solvent is presented with darker color, while the spectrum of the dissolved compound in solvent has a lighter intensity with the color intensity increasing with compound concentration.

As previously mentioned, luteolin was selected as the model flavonoid, therefore it was used to make a correlation between transmittance and concentration for a broader range of solvents. These results are presented in Figure 4. Whenever the intensity of peaks increased with the concentration, calibration curves were determined as shown in Figure S5 of SI. A linear relationship was determined for samples where DMSO, THF, butanol, 1.4-dioxane, ethanol, acetone and propanol were used as solvents, which indicates that solubility is higher than 8 mg·mL^{-1}. This is also evident from Figure S5 where samples are presented before and after centrifugation. In samples where the linear relationship was determined, there was no sedimentation after centrifugation. Non-linear relationship was determined for samples where methanol and ethyl acetate were used, which indicates that solubility in these solvents is lower than 8 mg·mL^{-1}. Evidence for saturated solution is

also sedimentation after centrifugation, which can be seen in samples with methanol and ethyl acetate in Figure S6.
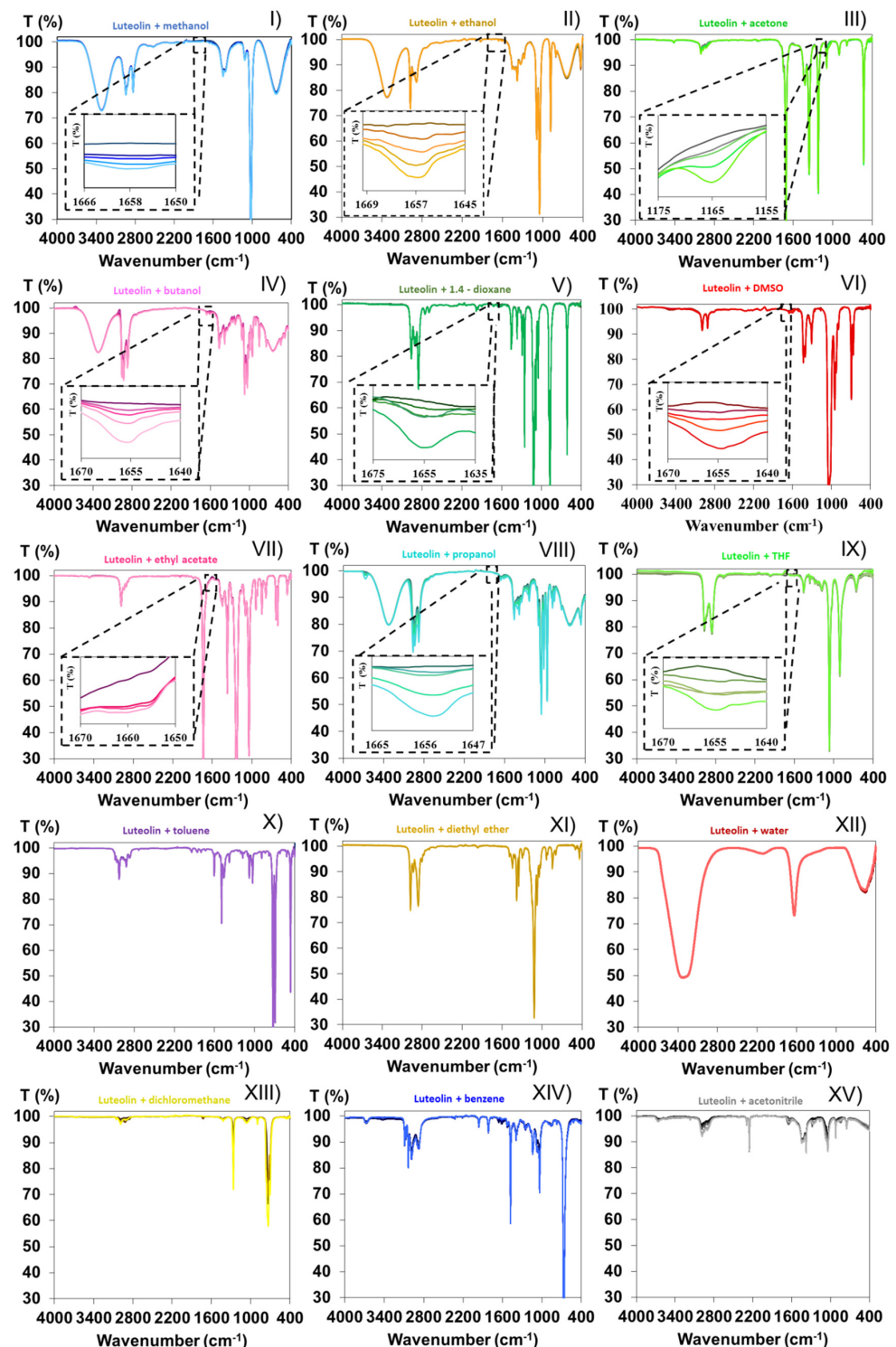

Figure 4. FTIR–ATR spectra of 1, 2, 4 and 8 mg·ml^{-1} of luteolin dissolved in various solvents. Spectrum of pure solvent is presented with darker intensity, while the spectrum of luteolin dissolved in solvent has a lighter intensity with the color intensity increasing with luteolin concentration: (**I**) methanol, (**II**) ethanol, (**III**) acetone, (**IV**) butanol, (**V**) 1,4-dioxane, (**VI**) DMSO, (**VII**) ethyl acetate, (**VIII**) propanol, (**IX**) THF, (**X**) toluene, (**XI**) diethyl ether, (**XII**) water, (**XIII**) dichloromethane, (**XIV**) benzene and (**XV**) acetonitrile.

In the other samples, the peak intensity did not increase alongside the concentration, namely for diethyl ether, acetonitrile, dichloromethane, water, benzene and toluene. This indicates that all samples contained saturated solutions and that solubility in these solvents is lower than 1 mg·mL^{-1}. These results are also in good agreement with other studies which report that luteolin solubility in DMSO, propanol, ethanol and butanol is greater than 8 mg·mL^{-1}, while its solubility in methanol and ethyl acetate is above 1 mg·mL^{-1} and below 1 mg·mL^{-1} in water [19,33].

Overall, these results show the possibility of using FTIR-ATR spectroscopy as a semi-quantitative method, however a more comprehensive range of increasing concentrations should be included for more accurate and precise calibration curves.

FTIR spectroscopy has been used in several studies as a quantification tool, for instance to determine the amount of polymer recovered during the final product polishing, allowing the recovery of the system phase formers for further reuse [34]. It has also been applied for the quantitative determination of biogenic silica in marine sediments due to its fast, efficient and economical analysis. However, it was not always possible to achieve accurate and reliable results in addition to the fact that the target compound had to be present in high amounts in order to be detected [35]. Another example is the quantitative analysis of ciprofloxacin in pharmaceutical tablets. Herein, all validation parameters were found to be highly satisfactory, indicating linearity, accuracy and adequate detection and quantification limit, though it had lower precision than other methods evaluated [36]. In this sense, we decided to use HPLC to further infer about luteolin solubility in all the 15 solvents studied in this work, since it is not only a more precise technique, but it is also commonly used to determine luteolin solubility.

3.2. HPLC Analysis of Luteolin Solubility

For HPLC analysis, luteolin solubility was evaluated at three lower concentrations, using the integration of a previously determined calibration curve with DMSO as solvent, as the lowest concentration studied in FTIR already resulted in peak saturation.

The real amount of luteolin dissolved in various solvents is shown in Figure 5, considering the attempt to prepare solutions of 0.3 mg·mL^{-1}. Moreover, it is listed in Table S1 of SI the real amounts of luteolin that were possible to be dissolved considering the attempt to prepare solutions of approximately 0.003, 0.03 and 0.3 mg·mL^{-1}. It should be stressed that for all the higher concentrations studied using FTIR, most samples seemed to be visually dissolved, as shown in Figure S7 of SI. However, HPLC results showed this was not always the reality. HPLC technique has many advantages, such as accurate and precise quantitative analysis and high-sensitive detection [37]. Therefore, this analysis provided more in-depth information about the real solubility of luteolin in solvents.

Dissolution of luteolin at approximately 0.3 mg·mL^{-1} in solvents is decreasing in the following order: DMSO > THF > butanol > 1.4-dioxane > methanol > diethyl ether > ethanol > ethyl acetate > acetone > propanol > acetonitrile > dichloromethane > water > toluene and benzene. The amount of luteolin dissolved in DMSO was 0.31 ± 0.08 mg·mL^{-1}, followed by 0.30 ± 0.03 mg·mL^{-1} in THF and 0.29 ± 0.08 mg·mL^{-1} in butanol. Dissolved amount in 1.4–dioxane, methanol, diethyl ether, ethanol, ethyl acetate, acetone, propanol and acetonitrile was 0.28 ± 0.03 mg·mL^{-1}, 0.28 ± 0.04 mg·mL^{-1}, 0.27 ± 0.06 mg·mL^{-1}, 0.25 ± 0.05 mg·mL^{-1}, 0.25 ± 0.04 mg·mL^{-1}, 0.21 ± 0.04 mg·mL^{-1}, 0.21 ± 0.03 mg·mL^{-1} and 0.20 ± 0.02 mg·mL^{-1}, respectively. Luteolin showed very low solubility in dichloromethane and water. For toluene and benzene, luteolin was not detected. Thus, luteolin may be considered insoluble in these solvents or negligible solubility.

The results obtained indicate that the most suitable solvent for luteolin dissolution is DMSO, since the entire amount of luteolin that was weighted was dissolved. These results are in good agreement with other studies, in which it was shown that the solubility of luteolin was the highest in DMSO compared to any other solvent evaluated [19,33]. Comparing the best and worst solvents for luteolin dissolution, it is evident that as a highly polar organic solvent, DMSO is a great hydrogen bond acceptor, while flavonoids are great

hydrogen bond donors. This is most likely the reason that DMSO was the most promising solvent for luteolin dissolution. In contrast, toluene and benzene are highly non-polar organic solvents that cannot establish hydrogen bonding, hence not being able to dissolve luteolin.
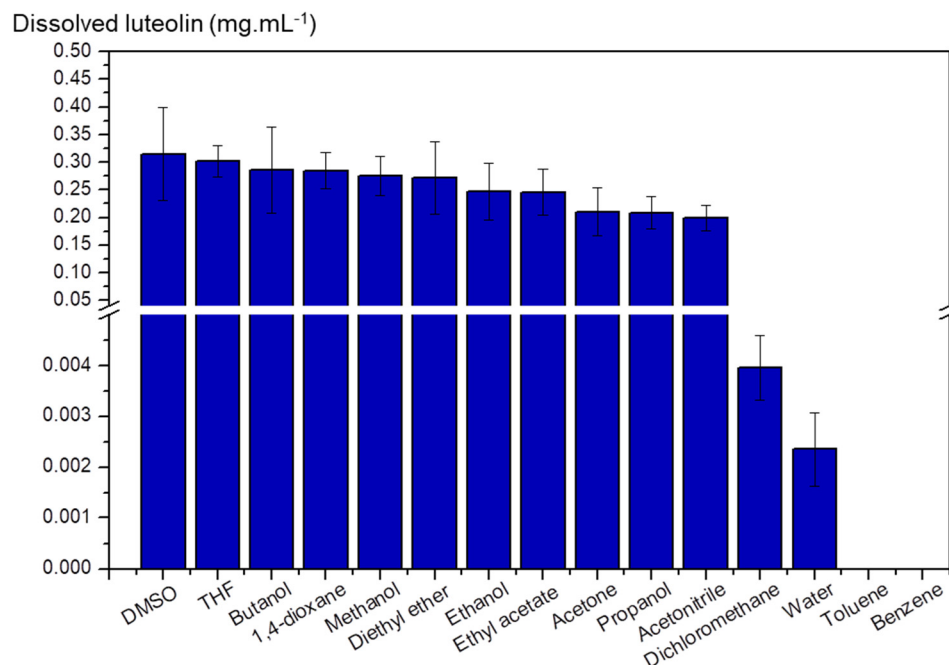

Figure 5. Values of luteolin solubility in a systematic series of solvents, at 27 °C.

When comparing the HPLC with the previous data obtained using FTIR-ATR spectroscopy, these results are in good agreement, since it is possible to conclude with both methods that luteolin is poorly soluble in dichloromethane and water, and completely insoluble in toluene, benzene. On the other hand, DMSO, THF, butanol, 1.4-dioxane, methanol, ethanol, ethyl acetate, acetone and propanol all showed good potential for luteolin dissolution while using both analytical methods. Additionally, these results also showed that for a faster screening, FTIR will definitely be the best option as: (i) it allows the analysis of higher concentrations of flavonoids without requiring sample dilution, and (ii) it leads to more accurate results at concentrations higher than 1 mg·mL^{-1} due to the more intense peaks. In contrast, HPLC requires prior knowledge of the concentrations under study owing to signal saturation at lower concentrations, namely between 0.5 and 1 mg·mL^{-1}. The only exception was diethyl ether, since FTIR results showed that luteolin was either not soluble in this solvent or the solvent's peaks were hindering luteolin's. Nevertheless, when lower concentrations were analyzed in HPLC, it was clear that almost all the amount of the flavonoid weighted was dissolved in this solvent. As such, it is possible to conclude that luteolin's solubility in diethyl ether is higher than 0.3 mg·mL^{-1} and lower than 1 mg·mL^{-1}, which can be further confirmed macroscopically through Figure S5.

Values were presented in mole fraction for easier comparison with results from other authors such as Shakeel et al. [19] and Peng et al. [33]. Study from Peng et al. [33] presented a list of 6 solvents that are also included in our research in the following order: DMSO, butanol, ethyl acetate, ethanol, methanol and water. Herein, DMSO is the best solvent whereas water is the worst. The same order of solvents was achieved in our research with the exception of DMSO, as it is shown in Table S2. Moreover, these studies showed higher solubility data for luteolin. However, both works used different methods to determine the flavonoid solubility, hence resulting in some differences with our data as we used a lower dissolution time. Shakeel et al. [19] used shake flask method and stirred the saturated solution for 72 h, while Peng et al. [33] waited for the solution to settle for 24 to 36 h. Nonetheless, both works performed saturation studies while we intended to demonstrate

that FTIR spectroscopy could be used as a fast quantification tool hence, not promoting directly the sample saturation. In this sense, none of these studies for luteolin solubility can be directly compared with our data, being more a guideline for the trend observe.

4. Conclusions

In this study, the FTIR-ATR analyses were carried out for luteolin, naringenin, hesperidin, quercetin, gallic acid and tannic acid in ethanol in order to characterize different flavonoids and gather essential knowledge about their functional groups. Additionally, solubility of luteolin was further studied in several solvents, using both FTIR-ATR spectroscopy and HPLC. The correlation between transmittance and concentration was evaluated for each solvent and characteristic peaks were described. From this research, it was concluded that, for some solvents, luteolin solubility could not be determined solely by FTIR spectra. Thus, luteolin solubility was also evaluated using HPLC as the analyzing method. Based on the experimental data obtained, order of suitable solvents for luteolin dissolution was determined. The solubility of luteolin was the highest in DMSO followed by THF, butanol, 1,4–dioxane, methanol, diethyl ether, ethanol, ethyl acetate, acetone, propanol, acetonitrile, dichloromethane, water, toluene and benzene, respectively. These results are of great importance, as they can be used in further studies for determining the appropriate solvents for extraction and separation of luteolin, as well as other flavonoids. Moreover, it was here evidenced that FTIR spectroscopy can be used as a fast quantification tool for solubility determination upon the proper analysis of several concentrations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9111952/s1, Figure S1. Luteolin dissolution with time, Figure S2. FTIR-ATR spectra of luteolin, naringenin, quercetin, tannic acid, hesperidin and gallic acid in ethanol after the solvent spectrum has been subtracted, Figure S3. Calibration curve of luteolin in DMSO for HPLC analysis, Figure S4. Calibration curves from the IR spectra of (I) quercetin at 1654 cm^{-1}, (II) naringenin at 1637 cm^{-1}, (III) luteolin at 1612 cm^{-1} and (IV) tannic acid at 1716 cm^{-1} dissolved in ethanol, Figure S5. Luteolin calibration curves from the IR spectra in: I) methanol at 1658 cm^{-1}, (II) ethanol at 1656 cm^{-1}, (III) acetone at 1164 cm^{-1}, (IV) ethyl acetate at 1655 $cm^{-1}cm^{-1}$, (V) 1.4-dioxane at 1656 cm^{-1}, (VI) DMSO at 1655 cm^{-1}, (VII) propanol at 1656 cm^{-1}, (VIII) THF at 1658 cm^{-1}, (IX) butanol at 1656 cm^{-1}, Figure S6. Dissolution of luteolin at concentrations 1, 2, 4, 6, 8, 10 mg·mL^{-1} before (left) and after centrifugation (right) in I) methanol, II) ethanol, (III) acetone, (IV) ethyl acetate, (V) 1.4-dioxane, (VI) DMSO, (VII) propanol, (VIII) THF, (IX) butanol, (X) diethyl ether, (XI) acetonitrile, (XII) dichloromethane and XIII) water. Samples (I), (IV), (V), (X), (XI), (XII) and (XIII) present sedimentation, Figure S7. Luteolin dissolved in distinct solvents, Table S1. Real amount of dissolved luteolin (mg·mL^{-1}) in different solvents mixed in proportions of 0.3, 0.03 and 0.003 mg·mL^{-1} at 27 °C, Table S2. Experimental solubilities of luteolin in several solvents calculated in mole fractions at 27 °C.

Author Contributions: S.R.: Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing—original draft. L.H.: Conceptualization, Supervision. F.A.V.: Conceptualization, Supervision, Validation, Writing—review and editing. S.S.: Writing–review and editing. M.G.: Writing—review and editing. B.L.: Conceptualization, Funding acquisition, Project administration, Supervision, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interes.

References

1. Daneshfar, A.; Baghlani, M.; Sarabi, R.S.; Sahraei, R.; Abassi, S.; Kaviyan, H.; Khezeli, T. Solubility of citric, malonic, and malic acids in different solvents from 303.2 to 333.2 K. *Fluid Phase Equilib.* **2012**, *313*, 11–15. [CrossRef]
2. Martínez, F.; Jouyban, A.; Acree, W.E. Pharmaceuticals solubility is still nowadays widely studied everywhere. *Pharm. Sci.* **2017**, *23*, 1–2. [CrossRef]
3. Noubigh, A.; Aydi, A.; Mgaidi, A.; Abderrabba, M. Measurement and correlation of the solubility of gallic acid in methanol plus water systems from (293.15 to 318.15) K. *J. Mol. Liq.* **2013**, *187*, 226–229. [CrossRef]
4. Manzoor, M.F.; Ahmad, N.; Ahmed, Z.; Siddique, R.; Zeng, X.A.; Rahaman, A.; Muhammad Aadil, R.; Wahab, A. Novel extraction techniques and pharmaceutical activities of luteolin and its derivatives. *J. Food Biochem.* **2019**, *43*, e12974. [CrossRef]
5. Gómez, E.; Requejo, P.F.; Tojo, E.; Macedo, E.A. Recovery of flavonoids using novel biodegradable choline amino acids ionic liquids based ATPS. *Fluid Phase Equilib.* **2019**, *493*, 1–9. [CrossRef]
6. Feizi, S.; Jabbari, M.; Farajtabar, A. A systematic study on solubility and solvation of bioactive compound chrysin in some water + cosolvent mixtures. *J. Mol. Liq.* **2016**, *220*, 478–483. [CrossRef]
7. Luo, Y.; Shang, P.; Li, D. Luteolin: A Flavonoid that has multiple cardio-protective effects and its molecular mechanisms. *Front. Pharmacol.* **2017**, *8*, 692. [CrossRef] [PubMed]
8. Lin, Y.; Shi, R.; Wang, X.; Shen, H.-M. Luteolin, a Flavonoid with Potential for Cancer Prevention and Therapy. *Curr. Cancer Drug Targets* **2008**, *8*, 634–646. [CrossRef]
9. Koleva, V.; Simeonov, E. Solid liquid extraction of phenolic and flavonoid compounds from *Cotinus coggygria* and concentration by nanofiltration. *Chem. Biochem. Eng. Q.* **2015**, *28*, 545–551. [CrossRef]
10. Truong, D.H.; Nguyen, D.H.; Ta, N.T.A.; Bui, A.V.; Do, T.H.; Nguyen, H.C. Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of severinia buxifolia. *J. Food Qual.* **2019**, *2019*, 1–9. [CrossRef]
11. Shakeel, F.; Alshehri, S.; Ibrahim, M.A.; Elzayat, E.M.; Altamimi, M.A.; Mohsin, K.; Alanazi, F.K.; Alsarra, I.A. Solubility and thermodynamic parameters of apigenin in different neat solvents at different temperatures. *J. Mol. Liq.* **2017**, *234*, 73–80. [CrossRef]
12. Zi, J.; Peng, B.; Yan, W. Solubilities of rutin in eight solvents at T = 283.15, 298.15, 313.15, 323.15, and 333.15 K. *Fluid Phase Equilib.* **2007**, *261*, 111–114. [CrossRef]
13. Ferreira, O.; Pinho, S.P. Solubility of flavonoids in pure solvents. *Ind. Eng. Chem. Res.* **2012**, *51*, 6586–6590. [CrossRef]
14. Zhang, H.; Wang, M.; Chen, L.; Liu, Y.; Liu, H.; Huo, H.; Sun, L.; Ren, X.; Deng, Y.; Qi, A. Structure-solubility relationships and thermodynamic aspects of solubility of some flavonoids in the solvents modeling biological media. *J. Mol. Liq.* **2017**, *225*, 439–445. [CrossRef]
15. Stalikas, C.D. Extraction, separation, and detection methods for phenolic acids and flavonoids. *J. Sep. Sci.* **2007**, *30*, 3268–3295. [CrossRef] [PubMed]
16. Ben Ahmed, Z.; Yousfi, M.; Viaene, J.; Dejaegher, B.; Demeyer, K.; Mangelings, D.; Heyden, Y. Vander Determination of optimal extraction conditions for phenolic compounds from: Pistacia atlantica leaves using the response surface methodology. *Anal. Methods* **2016**, *8*, 6107–6114. [CrossRef]
17. Peng, B.; Yan, W. Solubility of luteolin in ethanol + water mixed solvents at different temperatures. *J. Chem. Eng. Data* **2010**, *55*, 583–585. [CrossRef]
18. Xiao, M.; Yan, W.; Zhang, Z. Solubilities of apigenin in ethanol + water at different temperatures. *J. Chem. Eng. Data* **2010**, *55*, 3346–3348. [CrossRef]
19. Shakeel, F.; Haq, N.; Alshehri, S.; Ibrahim, M.A.; Elzayat, E.M.; Altamimi, M.A.; Mohsin, K.; Alanazi, F.K.; Alsarra, I.A. Solubility, thermodynamic properties and solute-solvent molecular interactions of luteolin in various pure solvents. *J. Mol. Liq.* **2018**, *255*, 43–50. [CrossRef]
20. Zhang, L.; Song, L.; Zhang, P.; Liu, T.; Zhou, L.; Yang, G.; Lin, R.; Zhang, J. Solubilities of naringin and naringenin in different solvents and dissociation constants of naringenin. *J. Chem. Eng. Data* **2015**, *60*, 932–940. [CrossRef]
21. Großhans, S.; Rüdt, M.; Sanden, A.; Brestrich, N.; Morgenstern, J.; Heissler, S.; Hubbuch, J. In-line Fourier-transform infrared spectroscopy as a versatile process analytical technology for preparative protein chromatography. *J. Chromatogr. A* **2018**, *1547*, 37–44. [CrossRef] [PubMed]
22. Bertrand, A.; Morel, S.; Lefoulon, F.; Rolland, Y.; Monsan, P.; Remaud-Simeon, M. Leuconostoc mesenteroides glucansucrase synthesis of flavonoid glucosides by acceptor reactions in aqueous-organic solvents. *Carbohydr. Res.* **2006**, *341*, 855–863. [CrossRef] [PubMed]
23. Common Solvents Used in Organic Chemistry: Table of Properties. Available online: https://organicchemistrydata.org/solvents/ (accessed on 20 October 2021).
24. Unsalan, O.; Erdogdu, Y.; Gulluoglu, M.T. FT-Raman and FT-IR spectral and quantum chemical studies on some flavonoid derivatives: Baicalein and Naringenin. *J. Raman Spectrosc.* **2009**, *40*, 562–570. [CrossRef]
25. Michniewicz, N.; Muszyński, A.S.; Wrzeszcz, W.; Czarnecki, M.A.; Golec, B.; Hawranek, J.P.; Mielke, Z. Vibrational spectra of liquid 1-propanol. *J. Mol. Struct.* **2008**, *887*, 180–186. [CrossRef]
26. Xu, W.F.; Zhang, Z.Y.; Wang, Q.; Zhu, H.J.; Zheng, C.L. Research of the dyeing thermodynamics and supramolecular structure of luteolin on wool fabric. *Wool Text. J.* **2015**, *43*, 6–9.

27.   Smith, B.C. The C-O Bond, Part I: Introduction and the Infrared Spectroscopy of Alcohols. *Spectroscopy* **2017**, *32*, 14–21.

28.   Roy, S.; Mallick, S.; Chakraborty, T.; Ghosh, N.; Singh, A.K.; Manna, S.; Majumdar, S. Synthesis, characterisation and antioxidant activity of luteolin-vanadium (II) complex. *Food Chem.* **2015**, *173*, 1172–1178. [CrossRef]

29.   Baranović, G.; Šegota, S. Infrared spectroscopy of flavones and flavonols. Reexamination of the hydroxyl and carbonyl vibrations in relation to the interactions of flavonoids with membrane lipids. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2018**, *192*, 473–486. [CrossRef]

30.   Silva, S.D.; Feliciano, R.P.; Boas, L.V.; Bronze, M.R. Application of FTIR-ATR to Moscatel dessert wines for prediction of total phenolic and flavonoid contents and antioxidant capacity. *Food Chem.* **2014**, *150*, 489–493. [CrossRef]

31.   Zhong, Y.; Chen, Y.; Feng, X.; Sun, Y.; Cui, S.; Li, X.; Jin, X.; Zhao, G. Hydrogen-bond facilitated intramolecular proton transfer in excited state and fluorescence quenching mechanism of flavonoid compounds in aqueous solution. *J. Mol. Liq.* **2020**, *302*, 112562. [CrossRef]

32.   Zhang, J.; Zhang, P.; Ma, K.; Han, F.; Chen, G.; Wei, X. Hydrogen bonding interactions between ethylene glycol and water: Density, excess molar volume, and spectral study. *Sci. China Ser. B Chem.* **2008**, *51*, 420–426. [CrossRef]

33.   Peng, B.; Zi, J.; Yan, W. Measurement and correlation of solubilities of luteolin in organic solvents at different temperatures. *J. Chem. Eng. Data* **2006**, *51*, 2038–2040. [CrossRef]

34.   Vicente, F.A.; Santos, J.H.P.M.; Pereira, I.M.M.; Gonçalves, C.V.M.; Dias, A.C.R.V.; Coutinho, J.A.P.; Ventura, S.P.M. Integration of aqueous (micellar) two-phase systems on the proteins separation. *BMC Chem. Eng.* **2019**, *1*, 4. [CrossRef]

35.   Melucci, D.; Zappi, A.; Poggioli, F.; Morozzi, P.; Giglio, F.; Tositti, L. ATR-FTIR spectroscopy, a new non-destructive approach for the quantitative determination of biogenic silica in marine sediments. *Molecules* **2019**, *24*, 3927. [CrossRef]

36.   Pandey, S.; Pandey, P.; Tiwari, G.; Tiwari, R.; Rai, A.K. FTIR spectroscopy: A tool for quantitative analysis of ciprofloxacin in tablets. *Indian J. Pharm. Sci.* **2012**, *74*, 86–90. [CrossRef]

37.   Sylvestre N'cho, J.; Fofana, I.; Hadjadj, Y.; Beroual, A. Review of physicochemical-based diagnostic techniques for assessing insulation condition in aged transformers. *Energies* **2016**, *9*, 367. [CrossRef]