Absorption Coefficients of Phenolic Structures in Different Solvents Routinely Used for Experiments

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Abstract: Phenolic structures are of great interest due to their antioxidant properties and various postulated benefits on human health. However, the quantification of these structures in fruits and vegetables, as well as in vivo or in vitro experiments, is demanding, as relevant concentrations are often low, causing problems in exactly weighing the respective amounts. Nevertheless, the determination of used concentrations is often a prerequisite for accurate results. A possibility to quantify polyphenol is the use of UV/vis spectroscopy. Therefore, the absorption coefficients of selected phenolic structures were determined in three different solvents relevant for polyphenol research (water/methanol (50/50, v/v), water, and phosphate buffer at pH 7.5). To confirm the values based on weight and to avoid errors due to impurities, hygroscopic effects, and inadequate balance care, the mass concentrations were additionally determined by quantitative NMR (q-NMR). The coefficients presented in this article can help to quickly and easily determine accurate concentrations in a laboratory routine without wasting the often-precious standard compounds.

Keywords: polyphenols; anthocyanins; absorption coefficient; q-NMR

1. Introduction

For polyphenols and water-soluble secondary plant substances, many positive health effects have been proposed [1]. Besides identifying and quantifying phenolic structures in food [2–5], current research attempts to prove the postulated effects on human health have been performed by the use of in vivo and in vitro experiments [6–9]. Some previous investigations have focused on the holistic evaluation of the effects of polyphenolic extracts but not on the individual substances and their properties [10,11]. Our aim was to provide reliable data as a basis for further in-depth research into the quantification of individual phenolic structures and clarification of their interaction mechanisms. Quantification in biological samples and experiments into the effects and the biochemical mechanisms require stock solutions and dilutions with defined and precisely determined concentrations.

Particularly for physiologically relevant concentrations, the exact weighing is problematic. Isolated compounds might contain impurities of substances, which are not detectable by routinely applied methods such as HPLC-DAD-MS. In addition, commercially available phenolic standard compounds, with the exception of simple hydroxyl cinnamic acids, are cost-intensive and exhibit a limited shelf life in solution. Moreover, more hydrophobic phenolic structures can be dissolved in aqueous media (electrolyte solutions or buffers) only to a limited extent. Micro-balances fit to weigh sub-milligram amounts of substances are cost-intensive and require a strictly controlled environment. In addition, systematic errors can occur if they are not adequately maintained, serviced, and calibrated. Apart from general individual weighing errors, the lyophilized phenolic powders are often hygroscopic, which leads to corresponding weighing inaccuracies.
As polyphenols are aromatic substances, it is possible to determine their absorption at 280 nm by means of UV spectroscopy. According to the Bouguer–Lambert–Beer law, a substance’s light absorption is proportional to its concentration in a given solvent; however, this is limited to a substance- and solvent-specific maximum concentration. Particularly, phenolic compounds tend to form supramolecular structures at higher concentrations in aqueous solutions [12], which limit the linear proportionality [13]. With the expansion of the conjugated π-electron system, the maximum absorption shifts from 280 nm to higher wavelengths (bathochromic effect). Furthermore, the wavelength might shift when different solvents are used, due to pH-dependent equilibria. Therefore, we determined absorption coefficients for some phenolic structures (Figure 1) in three different solvents: water, aqueous methanol (50/50 v/v), and aqueous phosphate buffer at pH 7.5 at $\lambda_{\text{max}}$, the individual wavelength of maximum absorption, and at 280 nm for comparison.

Figure 1. Overview of phenolic compounds investigated.
As the determination of absorption coefficients requires a reliable and confirmed concentration determination, we compared the data based on weight with concentrations determined by quantitative NMR (q-NMR). In recent years, q-NMR has been proven as a fast, reliable, sample saving and nondestructible absolute method to determine concentrations [14–17]. The quantifications performed by q-NMR are based on specific proton signals of the different substances.

2. Results

The following tables combine the results we found. Table 1 lists the extinction coefficients determined at the substances’ individual wavelengths of maximum absorption ($\lambda_{\text{max}}$). In Table 2 the extinction coefficients measured at the common wavelength $\lambda = 280$ nm are given. Table 3 shows the extinction coefficients determined in strongly acidic aqueous solution, both at $\lambda_{\text{max}}$ and at $\lambda = 280$ nm.
Table 1. Absorption coefficients at $\lambda_{\text{max}}$ (individual) for different phenolic compounds in methanol/water, water, and phosphate buffer pH 7.5 using concentrations determined by balance and q-NMR.

| PP      | Methanol/Water (50/50, v/v) | Water $[^a]$ | Phosphate Buffer pH 7.5 | Difference of $\varepsilon$ between Calculation Based on q-NMR and Balance $[^b]$ (%) |
|---------|-----------------------------|-------------|-------------------------|----------------------------------------------------------------------------------|
|         | Balance | NMR | Balance | NMR | Balance | NMR | Balance | NMR | Balance | NMR | Balance | NMR | Balance | NMR |
| GA      | 273     | 9507 | ± 436  | 9000  | ± 413  | 266  | 8021  | ± 166  | 7593  | ± 157  | 261  | 7406  | ± 288  | 7011  | ± 273  | 5.34 |
| COU     | 309     | 18,279 | ± 1237 | 18,131 | ± 1227 | 290  | 17,867 | ± 301  | 17,722 | ± 298  | 287  | 16,216 | ± 187  | 16,084 | ± 186  | 0.81 |
| CAF     | 322     | 15,458 | ± 590  | 14,792 | ± 565  | 315  | 14,606 | ± 601  | 13,976 | ± 575  | 312  | 12,073 | ± 266  | 11,553 | ± 255  | 4.31 |
| FER     | 320     | 15,573 | ± 555  | 16,203 | ± 580  | 314  | 14,365 | ± 391  | 14,948 | ± 407  | 310  | 13,738 | ± 544  | 13,662 | ± 541  | 4.05 |
| SIN     | 320     | 16,013 | ± 926  | 16,703 | ± 966  | 313  | 16,169 | ± 386  | 16,866 | ± 402  | 307  | 19,743 | ± 510  | 19,163 | ± 532  | 4.31 |
| CA      | 329     | 18,295 | ± 1435 | 18,091 | ± 1419 | 325  | 18,822 | ± 453  | 18,575 | ± 447  | 326  | 17,758 | ± 577  | 17,560 | ± 571  | 1.12 |
| CCA     | 329     | 18,106 | ± 391  | 17,842 | ± 386  | 326  | 18,177 | ± 275  | 17,912 | ± 271  | 327  | 16,145 | ± 220  | 15,910 | ± 217  | 1.46 |
| NCA     | 329     | 18,655 | ± 1084 | 18,323 | ± 1064 | 325  | 17,682 | ± 68   | 17,367 | ± 67   | 327  | 20,309 | ± 534  | 19,947 | ± 525  | 1.78 |
| DCQ     | 330     | 34,027 | ± 1672 | 34,515 | ± 1686 | 325  | 30,331 | ± 612  | 30,587 | ± 617  | 328  | 29,988 | ± 1422 | 30,234 | ± 1431 | 0.85 |
| CAT     | 280     | 41,75 | ± 160  | 40,47 | ± 155  | 280  | 37,70 | ± 71   | 36,555 | ± 69   | 280  | 34,422 | ± 191  | 3337  | ± 185  | 3.06 |
| EC      | 280     | 39,81 | ± 73   | 37,20 | ± 68   | 279  | 37,71 | ± 83   | 35,242 | ± 77   | 279  | 37,145 | ± 157  | 3470  | ± 147  | 6.56 |
| PC B1   | 281     | 73,64 | ± 78   | 75,34 | ± 80   | 280  | 70,66 | ± 60   | 72,229 | ± 62   | 280  | 61,616 | ± 699  | 699   | ± 715  | 2.30 |
| PC B2   | 281     | 74,96 | ± 223  | 79,99 | ± 237  | 280  | 68,10 | ± 83   | 72,311 | ± 88   | 280  | 66,986 | ± 189  | 7112  | ± 201  | 6.19 |
| PC C1   | 281     | 11,542 | ± 802  | 15,397 | ± 1070 | 280  | 10,432 | ± 392  | 13,917 | ± 524  | 280  | 9,783  | ± 533  | 13,051 | ± 711  | 33.40 |
| ECGG    | 277     | 10,735 | ± 819  | 11,958 | ± 912  | 275  | 10,438 | ± 190  | 11,628 | ± 211  | 277  | 9,525  | ± 255  | 10,610 | ± 284  | 11.39 |
| IRH-3rut| 257     | 22,001 | ± 744  | 22,381 | ± 756  | 256  | 18,925 | ± 499  | 19,252 | ± 508  | 270  | 19,760 | ± 844  | 20,101 | ± 859  | 1.73 |
| Q-3     | 258     | 19,568 | ± 938  | 25,053 | ± 1201 | 257  | 17,629 | ± 955  | 22,570 | ± 1223 | 269  | 17,545 | ± 368  | 22,464 | ± 471  | 28.03 |
| glec    | 358     | 16,317 | ± 731  | 21,515 | ± 964  | 352  | 13,915 | ± 782  | 18,349 | ± 1031 | 365  | 12,009 | ± 215  | 15,836 | ± 283  | 31.86 |
| RES     | 307     | 28,195 | ± 77   | 28,348 | ± 77   | 307  | 26,351 | ± 477  | 26,494 | ± 480  | 307  | 28,150 | ± 488  | 28,303 | ± 491  | 0.54 |
| PHL     | 287     | 15,585 | ± 267  | 15,139 | ± 260  | 286  | 14,986 | ± 100  | 14,557 | ± 97   | 325  | 18,164 | ± 179  | 17,643 | ± 176  | 2.86 |

$[^a]$ The respective pH values are provided in Table A1; $[^b]$ (ε<sub>q-NMR/ε<sub>balance</sub>)/ε<sub>balance</sub> × 100%; $[^c]$ the concentration of the solution was determined by UV spectroscopy with the absorption coefficient obtained in water; $[^d]$ the value is calculated with a second sample based on weight. $[^e]$ based on the values determined for PC B2, the values seem to be underestimated. $[^f]$ guaranteed purity is less than 90% (HPLC); therefore, the absorption coefficient might be underestimated. Due to unknown exact purity, the calculation is based on an estimated purity of 100%.
Table 2. Absorption coefficients at 280 nm for different phenolic compounds in methanol/water, water, and phosphate buffer pH 7.5 using concentrations determined by balance and q-NMR.

| PP    | Methanol/Water (50/50, v/v) | Water[a] | Phosphate Buffer pH 7.5 | Difference of ε between Calculation Based on q-NMR and Balance[b] |
|-------|-----------------------------|----------|-------------------------|---------------------------------------------------------------|
|       | Balance | NMR     | Balance | NMR     | Balance | NMR     |                                             |
|       | ε/(L·mol⁻¹·cm⁻¹) | ε/(L·mol⁻¹·cm⁻¹) | ε/(L·mol⁻¹·cm⁻¹) | ε/(L·mol⁻¹·cm⁻¹) | ε/(L·mol⁻¹·cm⁻¹) | ε/(L·mol⁻¹·cm⁻¹) |                                             |
| GA    | 8635 ± 521  | 8174 ± 493  | 5901 ± 595  | 5586 ± 563  | 3703 ± 98   | 3505 ± 92   | 5.34                                              |
| COU   | 14,035 ± 471 | 13,921 ± 467 | 15,982 ± 548 | 15,852 ± 544 | 15,470 ± 162 | 15,344 ± 160 | 0.81                                              |
| CAF   | 10,491 ± 564 | 10,039 ± 540 | 12,376 ± 550 | 11,843 ± 526 | 11,722 ± 247 | 11,217 ± 237 | 4.31                                              |
| FER   | 10,310 ± 408 | 10,728 ± 425 | 11,786 ± 363 | 12,264 ± 378 | 13,041 ± 511 | 13,570 ± 532 | 4.06                                              |
| SIN   | 5894 ± 365  | 6148 ± 381  | 7985 ± 718  | 8329 ± 749  | 6042 ± 310  | 6303 ± 323  | 4.31                                              |
| CA    | 8002 ± 500  | 7913 ± 494  | 10,119 ± 264 | 9987 ± 260  | 9231 ± 262  | 9128 ± 259  | 1.12                                              |
| CCA   | 7893 ± 189  | 7778 ± 186  | 9176 ± 138  | 9042 ± 136  | 7942 ± 127  | 7826 ± 125  | 1.46                                              |
| NCA   | 8544 ± 477  | 8392 ± 468  | 9189 ± 69   | 9026 ± 68   | 10,292 ± 239 | 10,108 ± 235 | 1.78                                              |
| DCQ   | 14,961 ± 669 | 15,087 ± 675 | 15,644 ± 341 | 15,776 ± 343 | 15,188 ± 562 | 15,317 ± 567 | 0.85                                              |
| CAT   | 4175 ± 160  | 4047 ± 155  | 3770 ± 71   | 3655 ± 69   | 3442 ± 191  | 3337 ± 185  | 3.06                                              |
| EC    | 3981 ± 73   | 3720 ± 88   | 3754 ± 83   | 3508 ± 78   | 3702 ± 159  | 3459 ± 149  | 6.56                                              |
| PC B1 | 7346 ± 79   | 7515 ± 81   | 7066 ± 60   | 7229 ± 62   | 6161 ± 699  | 6302 ± 715  | 2.30                                              |
| PC B2 | 7482 ± 227  | 7945 ± 241  | 6810 ± 83   | 7231 ± 88   | 6698 ± 189[c] | 7112 ± 201[c] | 6.19                                              |
| PC C1 | 11,518 ± 802 | 15,366 ± 1070 | 10,432 ± 392 | 13,917 ± 524 | 9783 ± 533[c] | 13,051 ± 711[c] | 33.40                                             |
| ECGC  | 10,544 ± 806 | 11,745 ± 898 | 9970 ± 197  | 11,106 ± 219 | 9319 ± 205  | 10,381 ± 229 | 11.39                                             |
| IRH-3- | 8958 ± 313  | 9112 ± 319  | 8190 ± 155  | 8331 ± 158  | 13,588 ± 587 | 13,823 ± 597 | 1.73                                              |
| Q-3-  | 7898 ± 296[f] | 10,112 ± 379 | 7474 ± 452[f] | 9569 ± 579  | 11,166 ± 201[f] | 14,296 ± 257 | 28.03                                             |
| RES   | 13,731 ± 150 | 13,805 ± 151 | 13,483 ± 217 | 13,556 ± 218 | 13,889 ± 184[c] | 13,964 ± 185[c] | 0.54                                              |
| PHL   | 14,187 ± 229 | 13,781 ± 223 | 13,940 ± 83  | 13,541 ± 81  | 8318 ± 185  | 8080 ± 180  | 2.86                                              |

[a] The respective pH value is provided in Table A1; [b] (ε_q-NMR-balance)/ε_balance × 100%; [c] the concentration of the solution was determined by UV spectroscopy with the absorption coefficient obtained in water; [d] the value is calculated with a second sample based on weight. [e] based on the values determined for PC B2, the values seem to be underestimated. [f] guaranteed purity is less than 90% (HPLC); therefore, the absorption coefficient might be underestimated. Due to unknown exact purity, the calculation is based on an estimated purity of 100%.
Table 3. Absorption coefficients for different anthocyanins in potassium chloride buffer at pH 1 at 520 nm and $\lambda_{\text{max}}$ using concentrations determined by balance.

| ACY   | $\varepsilon_{520\text{nm}}$ /$(\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1})$ | $\lambda_{\text{max}}$ /nm | $\varepsilon_{\lambda_{\text{max}}}$ /$(\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1})$ | $\varepsilon$ According to [10] |
|-------|-------------------------------------------------|-----------------|---------------------------------|---------------------|
| PEL-3-glc | 15,849 ± 2070 | 497 | 21,843 ± 2825 | 27,300 |
| CYD-3-glc | 25,526 ± 428 | 510 | 26,953 ± 464 | 26,900 |
| DPD-3-glc | 26,935 ± 680 | 516 | 27,087 ± 671 | 22,800 |
| PET-3-glc | 26,821 ± 1386 | 516 | 26,892 ± 1353 | 28,800 |
| PEO-3-glc | 23,926 ± 898 | 510 | 25,141 ± 931 | 28,800 |
| MLV-3-glc | 27,911 ± 437 | 518 | 27,923 ± 443 | 28,800 |

3. Discussion

The absorption coefficients in methanol/water for COU, CAF, FER, and SIN are comparable with the values found by Rubach with 18,800, 15,800, 13,300, and 16,700 L·mol$^{-1}$·cm$^{-1}$, at $\lambda_{\text{max}}$, respectively [18]. The structures of hydroxycinnamic acids are pH-dependent. In water, the pH values are concentration-dependent and range from 4.9 to 5.2 (Table A1). In buffer, the carboxylic group tends to dissociate, which explains the hypsochromic shifts in $\lambda_{\text{max}}$ and the decrease in absorption in phosphate buffer due to an increased formation of the negatively charged structures (Figure 2). The $pK_a$ values, calculated by ChemAxon and listed in the HMDB data bank [19], are in a similar range with 4.00, 3.64, 3.77, and 3.61 for COU, CAF, FER, and SIN, respectively, and explain the increased bathochromic shifts. The values for the absorption coefficient calculated with a concentration based on balance or q-NMR are in a good agreement.

![Figure 2](image-url) UV spectra of hydroxycinnamic acids in water/methanol (50/50, v/v, black), water (red), and phosphate buffer pH 7.5 (blue). (A), coumaric acid; (B), caffeic acid; (C), ferulic acid; (D), sinapinic acid. Concentrations are different for the four hydroxycinnamic acids but similar among the solvents.

The absorption coefficients for chlorogenic acid derivatives are independent of the ester position and the solvent (Tables 1 and 2, Figure 4A). Surprisingly, esterified with quinic acid, the absorption coefficient is roughly 25% higher compared to free CAF. The
significantly lower absorption at 280 nm underlines the importance to quantify these phenolic compounds separately at their individual absorption maxima or summarized at 320 nm. Our values determined in water and methanol/water are in good agreement with a former study by Rubach. Here, 19,500, 18,000, and 18,400 L·mol⁻¹·cm⁻¹ were found for chlorogenic (3'), neochlorogenic (4'), and cryptochlorogenic (5') acid [18]. The UV spectra of chlorogenic acids are not significantly influenced by the solution’s pH values (Figure 4A). In water, the pH values of the isomers are significantly different, with 5.0 (CA), 4.6 (CCA), and 5.6 (NCA) (Table A1). However, the carboxylic group of the quinic acid with a $pK_a$ of 3.3 [19] is widely distanced from the aromatic system, which is responsible for the absorption in the UV range. DCQ contains two independent CAF units and, therefore, the absorption should be doubled. However, the data are closer to the sum of the absorption of a chlorogenic acid and CAF.

Our values for CAT and EC are in agreement with the literature. A value of $\varepsilon = 3988$ L·mol⁻¹·cm⁻¹ has been reported for CAT and EC in methanol at 280 nm [20]. The absorption coefficients for the two dimers (PC B1 and B2) are in a similar range and are roughly doubled compared to the monomers. The trimer PC C1 follows the same trend comparing the data obtained by balance. In pure water and, in particular, in phosphate buffer, the absorption is reduced. In water, the pH value of all flavanols investigated is about pH 6 (Appendix A Table A1) and we interpret this more as an effect of the solvent’s dielectric constant, than an effect of the $pK_a$ ($pK_a$ CAT/EC = 9) [19]. The q-NMR data of the procyanidins are suspicious. Due to the formation of rotamers, quantification of the procyanidins by NMR is hampered. Fortunately, in methanol/water, the sum of the signals for the six protons of the B- and E-ring and the two diastereomeric protons at position F 4 are suitable to quantify the dimers, ignoring the different ratios of the two rotamers [21,22] (Supplementary Material). For the trimer PC C1, the number of rotamers is even higher (up to 4) [22,23], significantly influencing signal intensity and, therefore, integration.

The UV spectra of the flavonoids IRH-3-rut and Q-3-glc show two maxima around 260 nm (B-ring) and around 360 nm (A and C-ring) (Figure 3). Gitelson et al. reported an absorption coefficient for quercetin-rutinoside of 25,400 L·mol⁻¹·cm⁻¹ at 358 nm in 80% aqueous methanol [24]. This is higher than the value of 21,515 ± 964 L·mol⁻¹·cm⁻¹ found in this study for Q-3-glc (based on q-NMR, Table 1). The absorption coefficient calculated with the mass concentration $\gamma$ based on weight is markedly reduced. Due to the unknown purity of Q-3-glc and problems with precipitations, we rather trust the value based on NMR. The pH of the aqueous solution is 6.6 and 6.0 for IRH-3-rut and Q-3-glc, respectively. Both compounds have $pK_a$ values of 6.4 [19], and an increased formation of the deprotonated structure is obvious, comparing the spectra in water and buffer at pH 7.5. The most acidic position is the hydroxyl group at position 7 (A-ring). However, due to mesomeric effects, the negative charge is transferred to position 4' in the B-ring and a bathochromic shift of $\lambda_{max}$ is observed for both maxima.

**Figure 3.** UV-spectra of IRH-3-rut (A) and Q-3-glc (B) in water/methanol (50/50, v/v, black), water (red), and phosphate buffer pH 7 (blue). Concentrations are different for the two flavonoids but similar between the solvents.
For EGCG ($pK_a$ 7.99) [19], the UV absorption spectra in water ($pH$ value is 6.0) and phosphate buffer are different (Figure 4B). However, the impact on the absorption coefficient is marginal. For PHL, a strong bathochromic shift and an increased absorption are observed in phosphate buffer (Figure 4C). This is due to the increased formation of the deprotonated, anionic PHL species ($pK_a$ 7.87 [19], $pH$ in water is 6.0).

Figure 4. UV spectra of CA (A), EGCG (B), and PHL (C) in water/methanol (50/50, v/v, blue), water (orange), and phosphate buffer $pH$ 7.5 (gray).

For anthocyanins, a wide variety of absorption coefficients are available in the literature, and some of them have been summarized by Giusti and Wrolstad [25]. However, the data vary in the wavelength of absorption and the solvent used. In particular, the $pH$ value plays an important role for anthocyanins due to the $pH$-dependent equilibration between the red flavylum cation and the colorless hemicetal. Therefore, $pH$ values were checked for all anthocyanidin NMR dilutions to be $pH \leq 1.1$. Nevertheless, our values for the absorption coefficients differ significantly between the calculations based on the balance and q-NMR (16–40% higher in the calculation based on q-NMR, Table 3, Supplementary Material Table S1). Despite difficulties in weighing the hygroscopic anthocyanidins, we assumed a systematic underestimation by q-NMR. Data from the literature, in particular, the value of 26,900 L·mol$^{-1}$·cm$^{-1}$ for CYD-3-glc [25], support this. Therefore, we diluted two acidic (0.1% DCl) aqueous stock solutions of DPD-3-glc (1 g/mL, 1.6 g/mL) with potassium chloride buffer $pH$ 1 and methanol-$d_4$ and determined the solutions’ mass concentrations by q-NMR. A significant concentration difference (~20%) was observed between samples in buffer at $pH$ 1 and acidic methanol-$d_4$/D$_2$O (50/50, v/v) (Supplementary Material Table S2, Figure S1). Excluding the protons at position 6 and 8 (A-Ring), the mass concentration determined in methanol-$d_4$/D$_2$O was similar to the mass concentration calculated by weight. Reduced integrals for protons at these positions have also been reported for other flavonoids [21].

The partial NMR excitation due to insufficient relaxation delay was checked by comparing spectra recorded with shorter vs. longer recycle delays and was found to be
irrelevant. Due to a sample pH below 1.1, the formation of significant amounts of hemicetals can also be excluded. It is conspicuous that the NMR resonances are broader in spectra obtained from buffered samples than in spectra from aqueous methanolic samples, this could be caused by self-association of the anthocyanidins in aqueous media. Such supramolecular aggregates are known to lead to reduced quantification due to the aggregates’ slower tumbling rate (stochastic rotational and diffusion motion in the solution). The longer correlation times of such aggregates lead to faster T2 (spin-spin) relaxation and can induce signal broadening [26].

The focus of the investigation was aqueous solvents because in vitro experiments are usually performed in buffer. However, some polyphenols have limited solubility in water; therefore, HPLC-DAD standard stock solutions are often prepared in aqueous alcohol, and quantification with q-NMR also requires relatively high concentrations. Therefore, aqueous methanol was also included in the study. Despite limited solubility, stacking and hydratization in aqueous solvents might be problematic for quantification. If the molecules form more than simple van der Waals interactions with the solvent, as with hydrogen bonds or (de-)protonation equilibria, NMR signal intensities may be influenced due to the carry-over of water presaturation into the molecule (NOE).

Supramolecular stacking has an impact on the absorption spectra and the absorption coefficient and on the NMR resonances, too. However, for the UV/vis spectra, this effect is negligible due to high dilutions (1:50–1:400, 1:10,000 for CA to measure absorptions in the range of 0.1–1.4); for NMR, we observed (as expected) signal broadening and lowered intensities, and these effects were inversely proportional to the sample temperature during measurement. However, due to the limited amounts of substances and due to their tendency to degrade, we did not systematically acquire spectra at T(sample) > RT.

4. Materials and Methods
4.1. Materials, Solvents, and Reagents

(-)-Epicatechin (EC) (95.1% purity HPLC), 3-O-cafeeoquinic acid (chlorogenic acid, CA) (99% titration with NaOH), 5-O-cafeeoquinic acid (neochlorogenic acid, NCA) (99.5% HPLC), phlorizin dihydrate (PHL) (99% purity), trans-sinapinic acid (SIN) (99.1% HPLC, 100.1% titration), and trans-ferulic acid (FER) (99.8% purity HPLC; 99.8% titration) were stored at room temperature. 4-O-cafeoylquinic acid (cryptochlorogenic acid, CCA) (99.6% HPLC) and epigallocatechin gallate (EGGC) (99% HPLC) were stored at 4 °C and quercitin-3-O-glucoside (Q-3-glc) (91.4% HPLC), as well as resveratrol (RES) (100% HPLC) at −20 °C. These phenolic structures were obtained from Sigma Aldrich (Darmstadt, Germany).

(+) -Catechin (CAT) (99.5% HPLC-PDA) and 4,5-O-dicafeoylquinic acid (DQA) (99.2% HPLC-PDA) were purchased from Phytolab GmbH & Co. KG (Germany) and stored at 4 °C. The procyanidins (PC) B1 (97.39%), B2 (96.72%), and C1 (97.41%), as well as trans-caffeic acid (CAF) (99.90% HPLC UV), trans-p-cumaric acid (COU) (99.76% HPLC-UV), and isorhamnetin-3-O-rutinoside (IRH-3-rut) (99.06% HPLC-UV), were also purchased from Phytolab and stored at −80 °C (PCs) and room temperature, respectively.

The anthocyanin-3-O-glucosides cyanidin-3-O-glucoside (CYD-3-glc) (99.66% HPLC), delphinidin-3-O-glucoside (DPD-3-glc) (98.11% HPLC), malvidin-3-O-glucoside (MLV-3-glc) (99.10% HPLC), pelargonidin-3-O-glucoside (PLG-3-glc) (98.95% HPLC), peonidin-3-O-glucoside (PEO-3-glc) (98.27% HPLC) were obtained as chlorides from Phytolab GmbH & Co. KG (Germany) and stored at −80 °C.

Na₂HPO₄ and NaH₂PO₄·H₂O were obtained from Roth (Karlsruhe, Germany) to prepare 100 mM of phosphate buffer at pH 7.5. Sodium hydroxide and hydrochloric acid (Grüßing, Germany) were used to adjust the pH value. For NMR experiments, D₂O and methanol-d₄ were purchased from Eurisotop (Saarbrücken, Germany), and the methanol used to dilute the samples for UV spectroscopy was acquired from Fisher Scientific (Loughborough, UK). All reagents and solvents were of analytical grade and ultrapure water (ELGA PurLab flex, Veolia Waters, Celle, Germany) was used throughout.
4.2. Preparation of the Stock Solutions

Polyphenols were weighed using an AT 20 (Mettler Toledo; Gießen, Germany) balance. Anthocyanin stock solutions were prepared in ultrapure water containing 0.1% HCl, and all other phenolic structures were dissolved in 0.5 mL of methanol-d$_4$ and subsequently mixed with 0.5 mL of D$_2$O. All solvents were degassed and samples were stored at −20 °C. The compounds and mass concentrations (γ) determined by the balance and NMR are listed in Table 4.

Table 4. Mass concentration γ of the phenolic solutions based on the weights and determined with q-NMR at two different solutions.

| PP  | γ Balance by mg/L | Protons Used for Quantification[a] | γD by q-NMR spectroscopy (mg/L) | Average difference between Balance/NMR (in %) | Literature for Signal Assignment |
|-----|--------------------|------------------------------------|---------------------------------|---------------------------------------------|-------------------------------|
| GA  | 2368               | H 2,6                             | 2573                            | ± 36                                        | [27]                          |
| COU | 1158               | H$_6$; H 2,6; H 3,5; H$_8$         | 1219                            | ± 26                                        | [28]                          |
| CAF | 1088               | H$_6$; H 2; H 6; H 5; H$_8$        | 1164                            | ± 14                                        | [29]                          |
| FER | 1244               | H$_6$; H 2,6; H 5; H$_8$; H 7      | 1251                            | ± 28                                        | [30]                          |
| SIN | 2094               | H$_6$; H 2,6; H 7,8; H$_8$         | 2041                            | ± 17                                        | [31]                          |
| CA  | 26,990             | H$_6$; H 2, 6; H 6; H 5; H$_8$     | 27,451                          | ± 78                                        | [32]                          |
| CCA | 2976               | H$_6$; H 2, 6; H 5; H$_8$          | 3016                            | ± 2                                         | [33]                          |
| NCA | 6096               | H$_6$; H 2, 6; H 5; H$_8$          | 6197                            | ± 5                                         | [34]                          |
| DCQ | 3100               | H$_{v, 6}$; H 2, 6; H 6, 6; H 5, 5; H$_8$ | 3088                            | ± 7                                         | [35]                          |
| CAT | 1502               | H 2,5, H$_{6, 6}$; H 4, eq/ax      | 1550                            | ± 1                                         | [36]                          |
| EC  | 9362 [a]           | H 2, 5, 6; H 4, eq/ax              | 10,025                          | ± 3                                         | [37]                          |
| PC B1 | 3954              | H B 2, 5, 6; H 4, eq/ax           | 3865                            | ± 1                                         | [38]                          |
| PC B2 | 5114              | H B 2, 5, 6; H 4, eq/ax           | 4816                            | ± 1.5                                       | [39]                          |
| PC C1 | 2492              | H B 2, 5, 6; H 4, eq/ax           | 1868                            | ± 7                                         | [40]                          |
| EC CG | 1144              | H 2, 6; H 2, 6; H 4, eq/ax        | 1036                            | ± 5                                         | [41]                          |
| IRH-3-rut | 1002             | H 2, 6; H 6; H 5; H 13            | 998                             | ± 7                                         | [42]                          |
| Q-3-glc | 2576            | H 2, 6; H 6; H 5; H 6; H 8        | 1954                            | ± 29                                        | [43]                          |
| RES | 1112 [c]           | H 2, 6; H 2, 6; H 2, 6; H 4       | 1144                            | ± 19                                        | [44]                          |
| PHL | 4679               | H 2, 6; H 3, 5; H 3, Hb           | 4817                            | ± 7                                         | [45]                          |

[a] for further information, see spectra provided in the Supplementary Material Figure S2, [b] dilution factor 6, [c] dilution factor 2, [d] pre-dissolved in 0.5 µL of DMSO-d$_6$, [e] purity declaration was >90% (HPLC). The difference between balance and qNMR is reduced to 15% assuming a purity of 90% for the Q-3-glc standard compound.

4.3. Quantification Based on $^3$H-NMR

Absolute quantification of the polyphenols was performed in solution by quantitative nuclear magnetic resonance spectroscopy (qNMR) at the Chemical and Veterinary Investigation Office Karlsruhe (Chemisches Veterinär- und Untersuchungsamt, Karlsruhe, Germany). The measurement was carried out in methanol-d$_4$/D$_2$O (50/50, v/v) for the initial concentration and an appropriate dilution to check for concentration-dependent impacts. Initially, anthocyanins were quantified at two different concentrations (1.2–2.3 mM, pH 3.70) for the next quantification by $^3$H-NMR. The compounds and mass concentrations (γ) determined by the balance and NMR are listed in Table 4.

In general, the volume of 600 µL of the stock solutions was transferred into a 5 mm NMR tube and NMR spectra were recorded on a 400 MHz Bruker Avance (Bruker Biospin, Germany) equipped with a BBI 400S1 H-BB-D-05 Z probe and an automatic sample changer (Sample Xpress). Proton spectra were acquired using the pulse program nosygppr1d_d7.
(1D NMR spectra) with presaturation of the water signal and an additional (fully passive) d7 delay limiting the presaturation irradiation to the d1 delay immediately before the excitation pulse. See Figure 5 as an example, for more spectra, see the Supplemental Material, Figure S2. To obtain an optimal and comparable excitation for all samples, the 90° pulse was calibrated for each sample using Bruker’s PULSECAL routine. With a time domain (TD) of 128 k, 128 scans with 4 dummy scans were acquired, using a spectral width (SW) of 20.56 ppm (8223 Hz), an acquisition time (AQ) of 7.97 s, and a receiver gain (RG) of 32. Delay 1 (D1) and delay 7 (D7) were set to 4.00 and 60.0 s, respectively. The sample temperature was set at 300 K (±0.1 K). All spectra were automatically phased and baseline-corrected. NMR spectra were analyzed using TopSpin version 4.06 (Bruker Biospin, Germany) and compound concentrations were determined using the PULCON principle (pulse length-based concentration determination) according to [14,39,40]. 1H-NMR spectra of Quantification Reference solutions (QuantRef, = external standards), containing known, purity-corrected concentrations of the certified reference substances lactic acid and citric acid (aqueous QR for anthocyanins) or diethyl phthalate and 1,2,4,5-tetrachloro-3-nitrobenzene (organic QR for nonanthocyanin phenolic structures) were used to calculate the ERETIC factor according to Equation (1).

$$f_{ERETIC} = \frac{I_{Ref} \times SW_{Ref} \times M_{Ref}}{SI_{Ref} \times \gamma_{Ref, \text{corr}} \times N_{H, \text{Ref}} \times 1000} \left( \text{in \( \frac{\text{a.u.} \times \text{ppm} \times L}{\text{mmol}} \)} \right)$$  

where:

- $I_{Ref}$ = absolute integral of the reference signal;
- $SW_{Ref}$ = spectral width;
- $M_{Ref}$ = molar mass;
- $SI_{Ref}$ = number of data points of the processed reference spectrum;
- $\gamma_{Ref, \text{corr}}$ = mass concentration of reference substance, adjusted for purity;
- $N_{H,\text{Ref}}$ = number of protons per reference molecule giving this resonance.

The following factor was used to quantify the anthocyanins according to Equation (2).

$$\gamma_{An} = \frac{I_{An} \times SW_{An} \times M_{An}}{SI_{An} \times f_{ERETIC} \times N_{H,\text{An}} \times f_{\text{dil}}} \times \frac{P_{An}}{P_{Ref}} \times \frac{NS_{Ref}}{NS_{An}} \left( \text{in \( \frac{mg}{L} \)} \right)$$

where:

- $\gamma_{An}$ = analyte mass concentration;
- $I_{An}$ = absolute integral of analyte in sample;
- $SW_{An}$ = spectral width;
- $M_{An}$ = molar weight of analyte;
- $SI_{An}$ = no. of data points of the processed analyte spectrum;
- $f_{ERETIC}$ = mean value ERETIC factor from QuantRef;
- $N_{H,An}$ = number of protons per analyte molecule giving this resonance;
- $f_{\text{dil}}$ = dilution factor from analyte stock solution to measurement sample;
- $P_{An}$ = excitation pulse length used for the analyte sample (in µs);
- $P_{Ref}$ = excitation pulse length used for the QuantRef solution (in µs);
- $NS_{Ref}$ = number of recorded scans for the reference spectrum;
- $NS_{An}$ = number of recorded scans for the analyte spectrum.

Determination of the mass concentration $\gamma$ was performed in duplicate and calculated as an average for the protons specified in Table 4. Signals for integration were selected having a low multiplicity and showing complete relaxation during the delay between the scans. The proton spectra are provided in the Supplementary Material.
4.3. Quantification Based on 1H-NMR

Absolute quantification of the polyphenol s was performed in solution by quantitative nuclear magnetic resonance spectroscopy (qNMR) at the Chemical and Veterinary Investigation Office Karlsruhe (Chemische Veterinär- und Untersuchungsamt, Karlsruhe, Germany). The measurement was carried out in methanol-d4/D2O (50/50, v/v) for the initial concentration and an appropriate dilution to check for concentration-dependent impacts. Initially, anthocyanins were quantified at two different concentrations (1.2–2.3 mM, diluted 1:4 and 1:6) in 0.2 M potassium chloride buffer adjusted to pH = 1 with 0.2 M of HCl and D2O. The pH value of the samples ranged between 1.05 and 1.10 after 1 h of equilibration. To investigate the systematic difference between the balance and qNMR, stock solutions of delphinidin-3-glucoside (D2O, 0.1% DCl) were diluted in potassium chloride buffer and acidic methanol-d4/D2O (50/50, v/v, pH 1).

Figure 5. 1H-NMR example spectrum of procyanidin-B2 (1868 mg/L) in methanol-d4/D2O (50/50, v/v). The signals in the range of 6.5–7.15 ppm (the six protons of Ring B and E) and 2.6–3.0 ppm (the two diastereomeric protons F4) were used for summary quantification (Figure 1, 1H-NMR spectra with signal assignments for all PP are provided in the Supplemental Material Figure S4, including references).

4.4. Determination of the Absorption Coefficient

The absorptions were determined in duplicate by UV/Vis spectroscopy (Spectrostar Nano, BMG, Labtech, Ortenberg, Germany, UV-Cuvette semi micro-cuvette d = 1 cm, Helma Analytics, Muehlheim, Germany) after equilibration for, at minimum, three different dilutions. The absorption coefficients $\varepsilon$ (in $\text{L mol}^{-1} \text{cm}^{-1}$) were calculated according to Equation (3) for each concentration and then expressed as mean ± standard deviation.

$$\varepsilon = \frac{\text{Abs} \times f_{\text{dil}} \times M_{\text{an}}}{\gamma_{\text{an}} \times l \times 1000} \text{ (in L cm} \times \text{mol})$$ (3)

$\text{Abs} =$ absorption at $\lambda_{\text{max}}$ or 280 nm;
$M_{\text{an}} =$ molar weight of the anthocyanin;
$\gamma_{\text{an}} =$ average mass concentration of the anthocyanin determined by q-NMR;
$l =$ path length (1 cm);
$f_{\text{dil}} =$ dilution factor;
$1000 =$ conversion factor.

5. Conclusions

This article provides absorption coefficients for some phenolic structures in solvents generally used in experiments. The data also help to work with precise concentrations at low amounts during experiments and to save time and money. Commonly, it is recommended to use the absorption coefficients at $\lambda_{\text{max}}$; however, due to equipment limitations, it might sometimes be required to use the coefficient obtained at 280 nm.
**Supplementary Materials:** Table S1: Absorption coefficients of anthocyanidin-3-glucosides calculated by mass concentration $\gamma$ determined by balance and q-NMR in aqueous buffer at pH 1. Figure S1: Proton spectra recorded with a 400 MHz spectrometer and used for quantification, including signal assignment based on the literature of delphinidin-3-O-glucoside in buffer. Table S2: Mass concentration $\gamma$ determined by q-NMR in acidic methanol/water (50/50, v/v) and potassium chloride buffer pH 1. Figure S2: Proton spectra recorded with a 400 MHz spectrometer and used for quantification, including signal assignment based on the literature and own 2D NMR spectra.

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**Conflicts of Interest:** All authors declare that there is no conflict of interest.

**Sample Availability:** Not available.

**Appendix A**

**Table A1.** pH values for aqueous solution of the phenolic compounds in the specified concentration range.

| PP         | c/µM | pH   |
|------------|------|------|
| GA         | 35   | 4.62 ± 0.21 |
| COU        | 18   | 4.92 ± 0.24 |
| CAF        | 15   | 4.96 ± 0.18 |
| FER        | 16   | 4.92 ± 0.22 |
| SIN        | 23   | 5.16 ± 0.20 |
| CA         | 8    | 5.00 ± 0.36 |
| CCA        | 21   | 4.62 ± 0.30 |
| NCA        | 17   | 5.64 ± 0.08 |
| DCQ        | 6    | 5.26 ± 0.16 |
| CAT        | 26   | 5.94 ± 0.17 |
| ECAT       | 81   | 6.10 ± 0.11 |
| PC B1      | 27   | 6.07 ± 0.34 |
| PC B2      | 25   | 6.13 ± 0.08 |
| PC C2      | 26   | 5.97 ± 0.26 |
| ECGG       | 6    | 6.02 ± 0.01 |
| IRH-3-rut  | 16   | 6.62 ± 0.07 |
| Q-3-glc    | 14   | 6.02 ± 0.22 |
| RES        | 9    | 6.33 ± 0.44 |
| PHL        | 27   | 6.01 ± 0.03 |
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