

Statistical evaluation of DPPH, ABTS, FRAP, and Folin-Ciocalteu assays to assess the antioxidant capacity of lignins

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ARTICLE INFO

Keywords:

Antioxidant capacity

Lignin

Assay reproducibility

ABSTRACT

This research studies in detail four different assays, namely DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric ion reducing antioxidant potential) and FC (Folin-Ciocalteu), to determine the antioxidant capacity of standard substances as well as 50 organosolv lignins, and two kraft lignins. The coefficient of variation was determined for each method and was lowest for ABTS and highest for DPPH. The best correlation was found for FRAP and FC, which both rely on a single electron transfer mechanism. A good correlation between ABTS, FRAP and FC, respectively, could be observed, even though ABTS relies on a more complex reaction mechanism. The DPPH assay merely correlates with the others, implying that it reflects different antioxidative attributes due to a different reaction mechanism. Lignins obtained from paulownia and silphium have been investigated for the first time regarding their antioxidant capacity. Paulownia lignin is in the same range as beech wood lignin, while silphium lignin resembles wheat straw lignin. Miscanthus lignin is an exception from the grass lignins and possesses a significantly higher antioxidant capacity. All lignins possess a good antioxidant capacity and thus are promising candidates for various applications, e. g. as additives in food packaging or for biomedical purposes.

1. Introduction

Antioxidants play a crucial role in human health, as they are able to inhibit or delay undesired oxidation reactions, and thus prevent oxidative stress related to diseases like high blood pressure, neurodegenerative disorders or cancer. [1] However, the investigation of the antioxidant capacity is very complex, as no single method is capable to entirely depict the natural reactions occurring in vivo. [2] There are several factors influencing the efficacy of an antioxidant, the most important of which are the 3D structure, concentration, and intrinsic reactivity of the antioxidant to free radicals and other reactive oxygen species. Additionally, the temperature and kinetics of the redox reactions involved also exert influence on the potency of an antioxidant. All of these factors have to be considered when choosing a suitable antioxidant for a particular use. [3] Depending on the evaluation method of the respective assay, one can distinguish between the antioxidant activity as the rate of the chemical oxidation (reaction kinetics), and the antioxidant capacity as the oxidative conversion efficiency (stoichiometry of the reaction). [1,4]

There are different ways to classify antioxidant assays: one of them is

based on the reaction mechanism, which can be a hydrogen atom transfer (HAT) or a single electron transfer (SET). In most cases, though, more complex reactions like mixed HAT/SET, stepwise electron transfer-proton transfer, concerted electron-proton transfer, or sequential proton loss electron transfer occur. [5] Another classification differentiates between the reaction methodology used to evaluate antioxidant capacity, e.g. assays based on chemical reagents versus those evaluating at cellular levels. [6] The chemical-based assays can be further subdivided into different methods for assessing antioxidant activity: those based on the scavenging activity toward a stable free radical (2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)), the reduction of metal ions (ferric ion reducing antioxidant potential (FRAP), cupric ion reducing antioxidant capacity (CUPRAC), Folin-Ciocalteu (FC) assay) or competitive methods (oxygen radical absorbance capacity (ORAC), total reactive antioxidant potential (TRAP)). In this study, we used DPPH, ABTS, FRAP and FC, which are further specified in Table 1. These assays are the most frequently used antioxidant assays for lignin analysis from the methods mentioned above. All four are facile methods with a high throughput, do not require any special equipment, and can be

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implemented as “fixed-time” assays with reaction times between 12 and 30 min. However, their reactivity strongly depends on the sample type, which may lead to varying reaction times.

Nowadays, natural antioxidants are attracting increasing attention due to the need to substitute fossil-derived resources. [9] In addition, some synthetic antioxidants are cytotoxic and carcinogenic as shown in in vivo studies. [10] One potential candidate and a key topic in bio-refinery research is lignin, a polyphenolic biopolymer that can be extracted from various biomasses. [11] Its many phenolic hydroxyl groups is one reason for its antioxidant capacity, as they are able to terminate oxidative chain reactions and quench free radicals. [12] In addition, structural features like oxygen-bearing groups, e.g. methoxy groups [13], the molecular weight and polydispersity [14], or remaining oligosaccharides [15] influence the antioxidant properties. Currently, the number of studies investigating the antioxidant capacity of lignin is quickly growing, as nearly half of all publications regarding the four assays investigated in this article have been published in the past five years only (Fig. S1). The most frequently used antioxidant assays for lignin analysis are ABTS and DPPH, while FRAP and FC only play a minor role. Due to specific strengths and limitations, each assay can only investigate limited portions of the entire antioxidative capacity, particularly for complex samples such as 3D polymers. There are reviews summarizing the influence of isolation methods, pretreatments or postprocessing methods on the antioxidant properties [9,10], and studies using up to three different assays to measure the antioxidant capacity of lignins [16,17], but a comparison of these assays with appropriate statistical methods is missing. As the methodologies of different assays vary, a statistical evaluation of the results is crucial, so that data obtained from different assays can synergistically be put into a reasonable context.

This study addresses the complexity of the antioxidant capacity of any active compound, in particular natural polymers such as lignin. Therefore, the Trolox equivalent antioxidant capacity (TEAC) was determined using the DPPH, ABTS, and FRAP assay, and the total phenol content (TPC) was measured by the FC assay. For the first time, these four different assays were used to compare lignin's antioxidant capacity. The first section of this paper features a comparison of the assays using standard substances (Trolox / gallic acid) to investigate the reproducibility and sensitivity of each assay. This provides an assessment of the assay performance independently on the sample type. In the second section the correlations among the assays are examined. Finally, in the third section, the antioxidant capacities of lignins extracted identically from different biomasses are discussed.

2. Materials and methods

2.1. Materials

Miscanthus x giganteus, *Paulownia tomentosa* and *Silphium perfoliatum* were supplied by the Agricultural Faculty of the University of Bonn (field labs at Campus Klein-Altendorf, Rheinbach, Germany). All samples were milled and sieved to a particle size of 0.5–1 mm. Beech wood and wheat straw were obtained from the LXP Group (Teltow, Germany) with a particle size of 0.5–2 mm. Black liquor (spruce/pine mixture) was

received from Zellstoff- und Papierfabrik Rosenthal GmbH (Blankenstein, Germany, MERCER group).

Ethanol (absolute), dimethyl sulfoxide (DMSO), glacial acetic acid and sodium acetate trihydrate were purchased from VWR chemicals (Darmstadt, Germany); hydrochloric acid, sodium carbonate (anhydrous) and 1,4-dioxane from Carl Roth (Karlsruhe, Germany); sodium hydroxide, iron(III) chloride hexahydrate, potassium persulfate, Folin-Ciocalteu reagent and gallic acid from Merck (Darmstadt, Germany); ABTS diammonium salt and DPPH from Alfa Aesar; Trolox® from Acros Organics; 2,4,6-Tri-(2-pyridyl)-s-triazin (TPTZ) from Thermo Scientific; and Indulin AT (IAT) from S3-chemical (Bad Oeynhausen, Germany). All chemicals were used without further purification.

2.2. Lignin isolation

Organosolv lignins (OSL) and kraft lignin (KL) were isolated as described in Rumpf et al. [18]. Briefly, the biomass and an aqueous ethanol solution were mixed with a solid-to-liquid-ratio of 1:8 and heated to 170 °C in a Parr reactor with a 4848 Reactor controller. Some miscanthus samples were subjected to an autohydrolysis step before pulping, to generate lignins with differing properties. Afterwards, the biomass was filtered, washed with aqueous ethanol solution, and three volumes of acidified water were added to the filtrate to precipitate the OSL. After washing the lignin with acidified water, samples were freeze-dried for 72 h. To confirm the reproducibility of the process, pulping was carried out at least in triplicate for each sample. In this way, a total of 50 OSL samples were generated (25 from miscanthus (ML), 16 from paulownia (PL), 3 from silphium (SL), 3 from beech wood (BL), and 3 from wheat straw (WL)).

KL was precipitated from black liquor using dilute HCl at pH = 2. The solid residue was obtained by centrifugation, washed with acidified water, and freeze-dried for 72 h. In addition, IAT as commercially available KL was investigated as external reference.

A detailed structural characterization of lignins from different biomasses was performed by Rumpf et al. [18], and an overview of mon-olignol ratios and molecular weight of the lignins compared in Section 3.3 is given in Table S1.

2.3. Determination of antioxidant capacity and TPC

2.3.1. DPPH assay

The DPPH assay was conducted as described in Alzagameem et al. [19] Briefly, 0.1 mL sample solution (1 mg mL⁻¹ lignin in dioxane+water, 90 + 10 (v/v)) were mixed with 3.9 mL DPPH radical solution (6 × 10⁻⁵ mol L⁻¹ in dioxane+water, 90 + 10 (v/v)) and the absorption was measured on a Hach Lange DR6000 after 30 min at 518 nm. A calibration was performed with six Trolox standards in the range of 30–230 mg L⁻¹.

The radical scavenging activity (RSA) is calculated using the following formula:

$$\text{RSA} [\%] = \left(1 - \left(\frac{A_{\text{sample}}}{A_{\text{blank}}} \right) \right) * 100\% \quad (1)$$

Table 1
Characteristics of the antioxidant assays used in this study. [3,4,7,8].

	DPPH	ABTS	FRAP	FC
Principle of method	Reaction with organic radical	Reaction with organic radical cation	Reaction with Fe(III) complex	Reaction with Mo(VI) complex
Reaction mechanism	Mixed mode (HAT & SET)	Mixed mode (HAT & SET)	SET	SET
End-product determination	Colorimetry (discoloration) at 518 nm	Colorimetry (discoloration) at 734 nm	Colorimetry (color formation) at 593 nm	Colorimetry (color formation) at 750 nm
Working pH	5–9	3–9	3.6	≈ 10
Polarity of antioxidants	Hydrophobic (only organic solvents)	Hydrophilic & lipophilic	Hydrophilic (only in aqueous solution)	Hydrophilic

where A_{sample} is the absorbance of the tested sample, and A_{blank} is the absorbance of the blank (solvent mixture instead of sample solution was mixed with the DPPH radical solution). From the Trolox calibration curve, the concentration of Trolox equivalent (TE) c_{TE} is calculated in a first step as follows:

$$c_{TE} \left[\frac{\mu\text{g}}{\text{mL}} \right] = \frac{RSA_{lignin} - b_{cal}}{m_{cal}} \quad (2)$$

where b_{cal} is the intercept of the calibration curve and m_{cal} is the slope of the calibration curve.

In a second step, the TEAC value is determined with the molar mass of Trolox M_{Trolox} and the concentration of the lignin sample solution c_{lignin} :

$$\text{TEAC} \left[\frac{\mu\text{mol}}{\text{mg}} \right] = \frac{c_{TE} \cdot \frac{1}{M_{Trolox}}}{c_{lignin}} \quad (3)$$

2.3.2. ABTS assay

The ABTS assay was conducted as described in García et al. [20] For this purpose, an ABTS radical cation solution (7 mM ABTS and 2.45 mM potassium persulfate in ethanol+water, 50 + 50 v/v) was prepared and incubated in the dark at 25 °C overnight. Then, the solution was diluted with 50 % (v/v) ethanol to achieve an absorption at 734 nm of 0.700 ± 0.005 . For the measurement of the RSA, 2.5 mL of diluted ABTS radical cation solution were mixed with 0.25 mL sample solution (0.15 mg mL^{-1} lignin in DMSO) and the absorption was measured on a Hach Lange DR6000 after 12 min at 734 nm. A calibration curve with six Trolox standards was measured in the range of $15\text{--}40 \text{ mg L}^{-1}$. RSA, c_{TE} , and TEAC were calculated as described for the DPPH assay in Section 2.3.1.

2.3.3. FRAP assay

The FRAP assay was conducted as described in Yang et al. [21] The FRAP reagent was freshly prepared before each measurement by mixing acetate buffer (300 mM), TPTZ (10 mM in HCl (40 mM)), and FeCl_3 (20 mM in dist. water) in a ratio of 10:1:1 (v/v/v) and incubated at 37 °C for 10 min before use. For the analysis, 3 mL FRAP reagent was mixed with 0.1 mL sample solution (0.5 mg mL^{-1} lignin in dioxane+water, 90 + 10) and incubated at 37 °C for 10 min before measuring the absorbance at 593 nm on a Hach Lange DR6000. A calibration curve with six Trolox standards was measured in the range of $40\text{--}190 \text{ mg L}^{-1}$.

For the evaluation of the FRAP assay, c_{TE} can be directly calculated from the absorbance of the lignin sample solution A_{lignin} from the calibration curve:

$$c_{TE} \text{ (FRAP)} \left[\frac{\mu\text{g}}{\text{mL}} \right] = \frac{A_{lignin} - b_{cal}}{m_{cal}} \quad (4)$$

Afterwards, the TEAC value can be calculated as described in Section 2.3.1.

2.3.4. Folin-Ciocalteu assay

The determination of the TPC was performed by the FC assay as described in Alzagameem et al. [19] For the analysis, 0.1 mL sample solution (2 mg mL^{-1} lignin in DMSO) were pipetted into a 10 mL volumetric flask, then 0.5 mL FC reagent and approx. 7 mL deionized water were added. One minute after adding the FC reagent, 1 mL saturated sodium carbonate solution was added, and the flask was filled up with deionized water. This measuring solution was incubated for 30 min at 40 °C before measuring the absorbance at 750 nm on a Hach Lange DR6000.

For evaluation, a calibration curve with gallic acid ($100\text{--}1000 \text{ mg L}^{-1}$) was measured, so results can be expressed as gallic acid equivalents (GAE) with the following equation:

$$\text{GAE} \left[\frac{\text{mg}}{\text{L}} \right] = \frac{A_{lignin} - b_{cal}}{m_{cal}} \quad (5)$$

where A_{lignin} is the absorbance of the lignin sample, b_{cal} is the intercept of the calibration curve, and m_{cal} is the slope of the calibration curve. The TPC is calculated from the GAE and the concentration of the lignin sample solution, c_{lignin} :

$$\text{TPC} [\%] = \frac{\text{GAE} \cdot 100\%}{c_{lignin}} \quad (6)$$

2.3.5. Statistical analysis

Statistical analysis was conducted using OriginPro 2016 (OriginLab, Northampton, MA, USA).

For each assay, standard solutions with six different concentrations were measured and expressed as mean \pm standard deviation (SD). Grubb's test was used to detect outliers ($p < 0.05$), and linear curve fitting (no weighting, confidence bands with 95 % confidence level) was employed resulting in slope (m), intercept (b), coefficient of determination (R^2), residual sum of squares (RSS), and root-mean-square error (RMSE). For a better comparison of the different assays, the method standard deviation (MSD) and the coefficient of variation (CV) of each method were calculated according to:

$$\text{MSD} = \frac{\text{RMSE}}{m} \quad (7)$$

$$\text{CV} = \frac{\text{MSD}}{\text{overall mean}} \cdot 100\% \quad (8)$$

Furthermore, standard scores were calculated in order to get a dimensionless value. [22] This standardization is performed by subtracting the mean and dividing by the SD. The resulting standard scores have a mean of 0 and a SD of 1 by definition. For the correlation of assays in Section 3.2, mean and SD of all 52 lignins were used.

For the comparison of lignins from different biomasses, OSL were isolated in triplicate from each biomass and a double determination per sample was conducted for each assay. The data were analyzed by one-way ANOVA and expressed as mean \pm SD ($n = 3$). The post hoc Tukey test was used for the comparison of means between groups. All tests were performed with 95 % significance ($p < 0.05$). Standard scores were calculated from mean and SD of the 16 lignins (3 from miscanthus, silphium, wheat straw, paulownia, and beech wood, respectively, and IAT). In addition, a relative antioxidant capacity index (RACI) was calculated from the standard scores according to Sun et al. [22] by averaging the standard scores from the four assays. In this way, a RACI is obtained for each lignin from threefold pulping per biomass and mean \pm SD were calculated per biomass.

3. Results and discussion

3.1. Comparison of assays with standard substances

For each spectrophotometric assay, a calibration curve with six standard concentrations was measured over several days. As influencing factors like temperature or light conditions may change from day to day, the calibration was performed at the same conditions to overcome this issue. Trolox was used as the standard for the DPPH, ABTS, and FRAP assays, respectively, and gallic acid was used for the FC assay. In Fig. 1, the calibration curve for each assay with mean \pm SD, linear fit, and 95 % confidence band are shown; the corresponding slope, intercept, R^2 and CV for each method are given in Table 2. Further results from linear curve fitting can be found in the Supplementary Information (Table S2). So far, no literature data are available in this regard. Even if the results are expressed as TEAC values (especially for the ABTS assay), neither calibration curves nor details on frequency or reproducibility of calibrations have been reported. In the following section, different calibration and validation approaches are discussed and compared between the different assays.

The most common assays to investigate lignins are the DPPH and

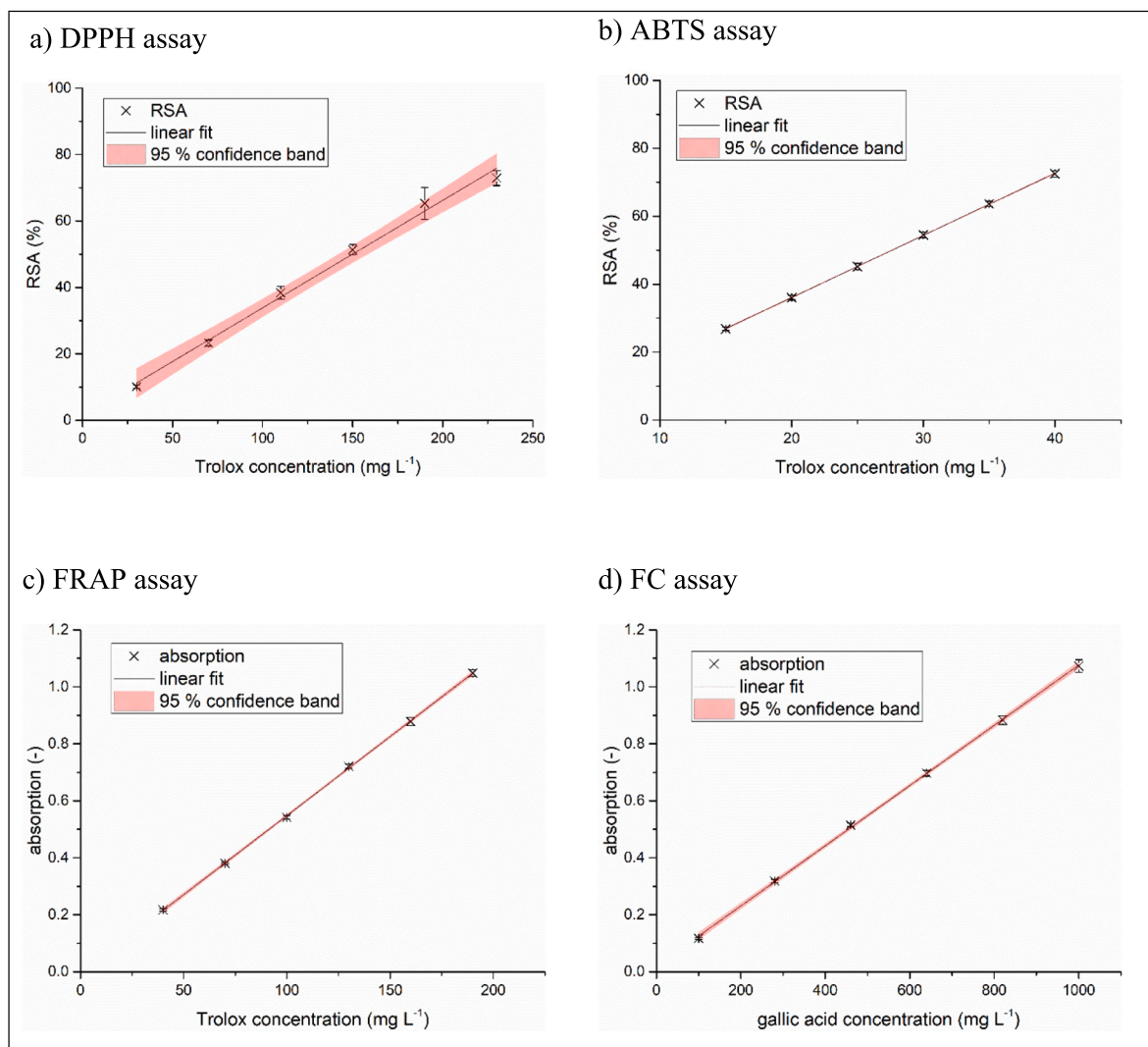


Fig. 1. Comparison of the calibration of the different assays given as mean \pm SD. No significant outliers were detected (0.05). RSA: radical scavenging activity.

Table 2

Results from linear curve fitting of the different assays. For slope and intercept, mean \pm SD are given.

	DPPH	ABTS	FRAP	FC
Slope m	0.323 \pm 0.013	1.831 \pm 0.006	0.006 \pm 0.000	0.001 \pm 0.000
Intercept b	1.508 \pm 1.957	-0.576 \pm 0.174	-0.007 \pm 0.005	0.633 \pm 0.019
R ²	0.99326	0.99996	0.99982	0.99972
CV	5.30 %	0.25 %	0.74 %	1.14 %

ABTS assay, both based on and accordingly named after an electron-deficient radical. [23] As a free stable radical, DPPH does not need to be prepared freshly like the ABTS radical cation. However, the ABTS assay is less prone to interference from colored samples due to the higher wavelength used. [24] Both are artificial and nonphysiologically relevant radicals, and thus only direct reactions of the radical with the antioxidant compound under investigation are measured. [25] The underlying reaction mechanisms cannot be assigned to either only HAT or SET, but are more complex as both occur in varying ratios depending on concentration and structure of the test compound, solvent and pH. In general, the electron transfer is very rapid, while hydrogen transfer is comparatively slow. This initial electron transfer is much faster in the ABTS assay due to the sterically hindered DPPH radical site, which is

difficult to access for phenols. [26] This becomes apparent when comparing the different reaction times: absorbance was measured after 12 min for ABTS, and after 30 min for DPPH. In addition, the absorbance was also measured after half of the respective reaction times, showing that in the ABTS assay, Trolox reacted completely causing no further change in absorbance after 6 min, while in the DPPH assay the absorbance decreased over the full reaction time (see Fig. S2). Again, this confirms the higher reactivity of the ABTS radical cation. [24] Next to the faster reaction, the ABTS assay is also more sensitive: the calibration curve of ABTS has a slope more than five times higher compared to the DPPH assay (Table 2). Thus, small changes in the concentration lead to larger differences in the RSA for ABTS than DPPH. Comparably, Samaniego Sánchez et al. used both assays to study the free-radical scavenging activity of olive oils, and found a Trolox standard curve slope nearly four times higher for the ABTS assay compared to the DPPH assay. [27] Moreover, the obtained R² for the ABTS assay (0.99154) is higher than the R² for the DPPH assay (0.98492). Analogously, we found lowest R² values for the Trolox calibration curve using the DPPH method (0.99326) and the highest with the ABTS assay (0.99996). To compare the reproducibility of different methods, the CV is a more meaningful parameter than R². The highest CV was obtained for the DPPH assay (5.30 %), while the ABTS assay showed the lowest one (0.25 %). Erel et al. developed an ABTS method to measure blood serum and determined a CV of 1.3–2.9 %, depending on the concentration level. [28]

Rubio et al. investigated three different versions of the ABTS assay to study the antioxidant capacity in canine serum, showing that the variation in the procedure could lead to different results in linearity and CV. [29] They found interassay CVs based on five replicate determinations in three samples across analytical runs on different days of 3.7–9.8 % for the method with the smallest error. These values are higher than the CV determined here, which might be due to the measured real samples instead of a standard substance as in this study. Generally speaking, CVs determined using the analyte sample are more meaningful for the specific application; yet a better comparison of the different assays independent of the application is achieved when standard substances like Trolox or gallic acid are investigated. Analogous tendencies were observed for the DPPH assay: when determining the repeatability over several days using the sample itself, Mikami et al. found CVs between 1.38 and 9.32 % for several crops [30], while Plank et al. obtained an intra-laboratory repeatability of 2.4–7.2 % for different foods and beverages. [31] In contrast, Cheng et al. reported a standard (ferulic acid) in duplicate for seven consecutive days and obtained a CV of only 3.46 %. [32] Lucas-Abellán et al. used both ABTS and DPPH to measure the antioxidant activity of resveratrol, leading to a better inter-day variation of the ABTS assay (13 %) than for the DPPH assay (15 %). [33]

The other two assays investigated are the FRAP and the FC assays; both are based on a SET reaction. In presence of an antioxidant, during the FRAP and FC assay, Fe^{3+} gets reduced to Fe^{2+} and Mo^{6+} to Mo^{5+} , respectively, resulting in a change of color that can be monitored photometrically. The FC assay is most commonly used to determine the phenol content, but as not only phenols but all oxidizable groups interact with the reagent, it is rather a measure of the reduction capacity, which directly correlates with phenolic content and antioxidant capacity. [25] Both, FC and FRAP are simple, rapid and inexpensive methods with a good reproducibility. [3] Furthermore, the FRAP assay possesses a high sensitivity and precision, with the disadvantage of non-physiological measurement conditions at low pH. [34] The obtained R^2 and CV data are in between the values determined for DPPH and ABTS assay, namely R^2 of 0.99982 and 0.99972 and CV of 0.74 % and 1.14 % for FRAP and FC assay, respectively. For the FRAP assay, FeSO_4 is normally used as standard, so a comparison with a Trolox calibration for this assay is rare. Stratil et al. compared various compounds for the calibration of the four assays used in this study, including Trolox, FeSO_4 and gallic acid, but as they used other units for their representation, the calibration equations are not comparable and R^2 data were not reported. [35] However, they concluded that Trolox seems to be the best standard not only for FRAP, but also for ABTS and DPPH assay. Other studies using FeSO_4 as the calibration standard for the FRAP assay obtained $R^2 > 0.99$ [36,37], supporting our findings with Trolox. Halvorsen et al. validated the FRAP assay for antioxidants in foods and found an inter-day repeatability in standard solutions $< 3\%$ [38], which also applies for the CV determined here. However, values concerning the optimization and validation of a method can be compared best for the FC assay. Bastola et al. evaluated different single and mixed phenolic standards for the FC assay and found that gallic acid was the best one among the single phenolic standards with a R^2 of 0.9946 and the least relative error when measuring three solutions with a known concentration of phenolics. [39] Other studies using the FC assay include the quantification of polyphenols in fruit juices and ciders with an intra-day repeatability of 0.66 % [40], the determination of the TPC in various teas with an inter-day precision $< 1.5\%$ for gallic acid standard solutions at different concentration levels [41], and the validation of the method for total phenolics quantification in açai with a CV of 2.58 % [42]. The CV determined in this study is 1.14 % and does not deviate in great extent from the values found in literature.

Only one study on TPC and antioxidant capacity used the same four assays and calibration standards as investigated here: Lu et al. obtained CVs of 1.15–3.18 % for ABTS, 0.96–5.08 % for DPPH, 0.68–2.23 % for FRAP, and 0.88–7.92 % for FC, respectively, for extracts of different onion and shallot varieties. [43] Similar to our study, ABTS and FRAP

showed the lowest CV, while DPPH and FC had greater variation.

In conclusion, the CVs determined for the DPPH, FRAP, and FC assay are comparable to literature data for the repeatability of the measurement of the standard solutions over several days, while for the ABTS assay a lower CV was identified. As the measuring procedures as well as the samples investigated differ from those used here, the findings are still within a reasonable magnitude. When comparing the radical scavenging assays DPPH and ABTS directly, ABTS has the higher R^2 and lower CV and thus seems favorable regarding reaction time, sensitivity, and reproducibility. One possible explanation might be the higher sensitivity of DPPH radical toward the reaction environment compared to ABTS radical cation, so small changes of e.g. the solvent, pH or temperature might have a more significant impact on the outcome of the measurement [26] and thus lead to a higher variability. Moreover, the absorbance of the DPPH radical decreases to a higher extent upon exposure to light. [25] To avoid this problem, it might be helpful to adjust the DPPH measurement solution to a certain absorption right before the assessment of the samples instead of a defined concentration, as it is also done in the ABTS assay. Nevertheless, a CV of 5.30 % for a wet-chemical method is still satisfactory and in the expected literature range, even when it is slightly higher than for the other assays.

3.2. Correlation of assays

The antioxidant capacities of the 52 lignins in total were correlated with each other to figure out which assays lead to comparable results, and which differ. As it is difficult to compare absolute values due to the varying units, standard scores were calculated and plotted against each other. The graphs as well as the corresponding slope and R^2 of the linear fits are given in Table 3.

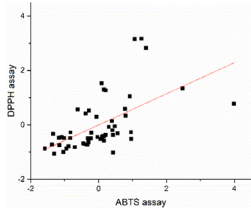
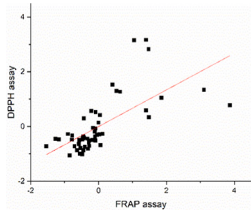
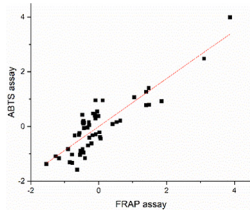
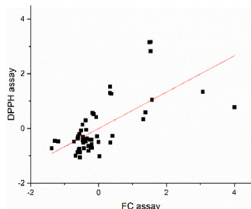
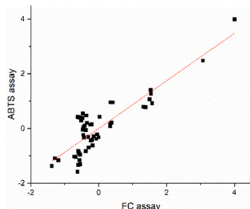
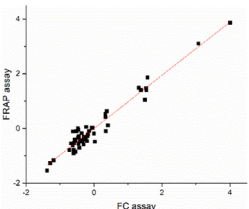
The highest correlation ($R^2 = 0.944$) can be found for the FRAP and FC assay, which is not surprising as both rely on the same mechanism, the SET. The ABTS assay also correlates well with FRAP ($R^2 = 0.763$) and FC ($R^2 = 0.757$), while for the DPPH assay only weak correlations exist with FRAP ($R^2 = 0.447$), FC ($R^2 = 0.440$) and ABTS ($R^2 = 0.326$).

Similar results were obtained by Wootton-Beard et al., who studied the antioxidant capacity and total polyphenol content of vegetable juices with strong positive correlations between FRAP and FC ($R^2 = 0.96$), ABTS and FRAP ($R^2 = 0.87$), and ABTS and FC ($R^2 = 0.89$), and relatively weak correlations between DPPH and FRAP ($R^2 = 0.53$), DPPH and FC ($R^2 = 0.50$), and DPPH and ABTS ($R^2 = 0.45$). [44] Zhang et al. analyzed the antioxidant capacities of flavonoids with the four assays and also found the highest correlation between FRAP and FC ($R^2 = 0.90$) and the lowest between DPPH and ABTS ($R^2 = 0.47$). [23] Moreover, they calculated bond dissociation enthalpies to characterize the hydrogen-atom-donating ability, and ionization potentials to evaluate the scavenging activity of the test compounds, leading to the conclusion that the DPPH assay is closer to the HAT mechanism than the other three assays. This is also supported by the fact that the FRAP assay, as a non-radical SET-based method, has a low relation with the HAT mechanism, and thus is suggested to be used together with other methods to distinguish the dominant mechanisms for different antioxidants. [3] All in all, this supports our findings that FRAP, FC and ABTS show strong positive correlations ($R^2 > 0.75$) with each other, while the DPPH assay merely correlates ($R^2 < 0.5$) with the others. However, such findings cannot be transferred in general but depend on the origin and structure of the investigated sample. Other studies in fact found good correlations ($R^2 > 0.8$) for all four assays, e.g. for aqueous acetone extracts from lentils [45], guava fruit extracts [46], and phenolic extracts from crabapples [47]. In contrast, some authors could not find any significant correlation at all between FRAP and the other three assays when studying citrus fruit extracts [48], while Vázquez-Gutiérrez et al. could not find significant differences for the antioxidant activities of onion extracts determined by the ABTS assay, but only with FRAP and DPPH, respectively. [49]

This emphasizes the need to thoroughly study each assay worth

Table 3

Correlation of the four assays (DPPH, ABTS, FRAP, FC) among each other using standard scores.

	DPPH	ABTS	FRAP	FC
DPPH	—	Slope = 0.571 ± 0.116 $R^2 = 0.326$	Slope = 0.668 ± 0.105 $R^2 = 0.447$	Slope = 0.663 ± 0.106 $R^2 = 0.440$
ABTS		—	Slope = 0.873 ± 0.069 $R^2 = 0.763$	Slope = 0.870 ± 0.070 $R^2 = 0.757$
FRAP			—	Slope = 0.972 ± 0.033 $R^2 = 0.944$
FC				—

considering for the respective sample type. Rácz et al. used seven different assays to study 13 berry genotypes and 12 sour cherry cultivars, respectively, and found differences in the performance of the assays using chemometric methods depending on the sample type. [2] Pellegrini et al. investigated various beverages, oils, fruits and vegetables with the ABTS and FRAP assay, resulting in different correlation coefficients between the assays for each food product. [50] Next to the assay itself, the pretreatment of the samples plays a crucial role: when investigating extracts from plants for example, the extraction solvent significantly influences the antioxidant capacity. [46,51] Moreover, the polarity/solubility can be a limiting factor when choosing an assay. As this is, to the best of our knowledge, the first study using all four assays for the characterization of lignins, no reference data for correlation coefficients between these assays were reported yet. Nevertheless, the results of our study seem to be plausible when examining related samples like (poly)phenols, and the range of correlation coefficients indicates that varying antioxidative attributes are reflected, which might arise due to a different reaction mechanism.

3.3. Comparison of lignins from different biomasses

In Fig. 2, the TEAC values from DPPH, ABTS, and FRAP assays, respectively, and the TPC from FC assay are shown as an example for one OSL from each biomass extracted under the same conditions. The corresponding raw data as well as RSA for DPPH and ABTS assay can be found in Table S3 and Table S4.

When comparing the four assays referring to the lignin samples, the DPPH assay cannot discriminate the OSL from each other, but only from the IAT, which makes it the least selective assay with the highest CV (see Section 3.1). Using the FRAP assay, only SL can be distinguished from the other OSL, so that in total with the IAT three groups can be discriminated. With the ABTS and FC assays, even four different groups can be observed. Similarities and differences between assays become more apparent using standard scores (Fig. 3): the FRAP and FC assays show the same tendencies, as expected from the results of the correlation in Section 3.2., while especially for BL and SL, there are large differences

between DPPH and the other assays.

For DPPH and ABTS assay, the RSA is always calculated in a first step. It can be useful to compare lignins among each other, but only as long as the employed concentrations and measuring conditions are the same. Thus, a wide range of RSA values for lignins of different biomass origin and/or isolation process can be found in literature ranging from 20 to 78 % for miscanthus lignins [52,53], 10–87 % for wheat straw lignin [54,55], and 5–64 % for beech lignin [19,56] for the DPPH assay. Another evaluation method is the calculation of the IC₅₀ value, which is, however, much more time-consuming as a standard curve for each sample is needed to determine the concentration which scavenges 50 % of the respective radical. [10] Hence, Trolox was used as standard substance in this study to obtain a calibration curve that can be applied for all lignins and is timesaving when many samples are measured repeatedly.

Trolox calibration is usually the standard procedure of the ABTS assay [56], but also works well for the DPPH and FRAP assay. There is only one further study that investigated all three assays for lignin evaluation using Trolox as standard: Sun et al. reported a steam-explosion process followed by alkali and alkaline ethanol delignification to extract lignins from bamboo, and obtained TEAC values of 0.282–0.447 $\mu\text{mol TE mg}^{-1}$ lignin (DPPH), 1.485–2.273 $\mu\text{mol TE mg}^{-1}$ lignin (ABTS), and 0.579–0.767 $\mu\text{mol TE mg}^{-1}$ lignin (FRAP). [57] Gong et al. studied acetic acid lignin and milled wood lignin from bamboo and obtained TEAC values of 0.265–0.633 $\mu\text{mol TE mg}^{-1}$ lignin for DPPH and 0.819–1.441 $\mu\text{mol TE mg}^{-1}$ lignin for ABTS. [58] In general, these values are slightly higher than those obtained here, which might be caused by differences in lignin origin or pulping process, but also varying assay parameters including environmental factors. The absolute TEAC values are highest for ABTS and lowest for DPPH, which is not only common for lignins but also reported for fruits [59], vegetables [35], or olive oil [27], due to the higher sensitivity of the ABTS assay. The TPC values are also in accordance with literature data, where values from 15.6 to 32.2 % can be found for OSL. [20,60] For lignins from paulownia and silphium no reference data exists yet. It can be assumed that PL shows a comparable antioxidant capacity as BL, as both are

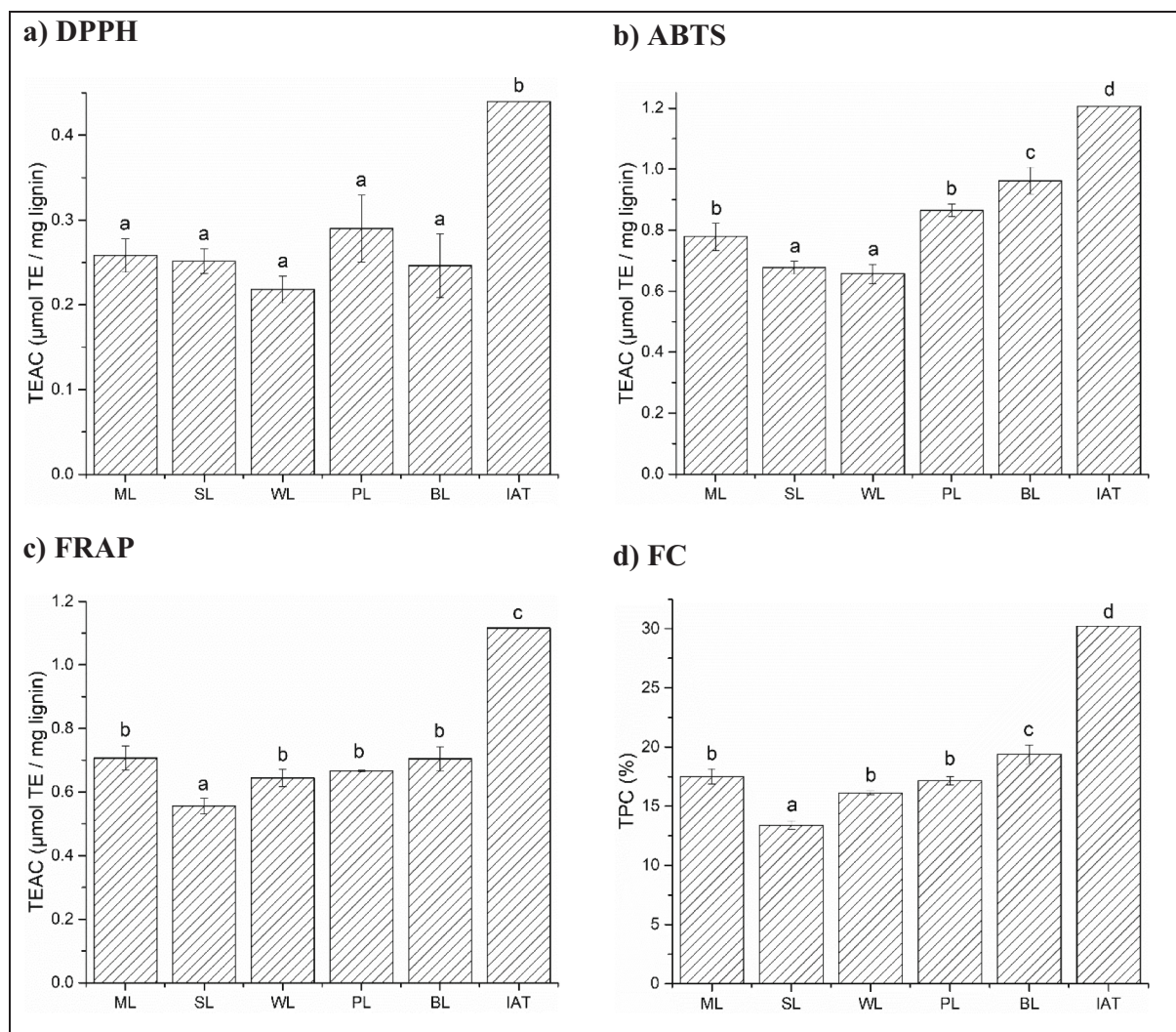


Fig. 2. Comparison of TEAC and TPC of lignins (ML: miscanthus lignin, SL: silphium lignin, WL: wheat straw lignin, PL: paulownia lignin, BL: beech wood lignin, IAT: Indulin AT), results expressed as mean \pm SD. Different letters indicate a significant difference between the values (One-way ANOVA; $p < 0.05$; $n = 3$).

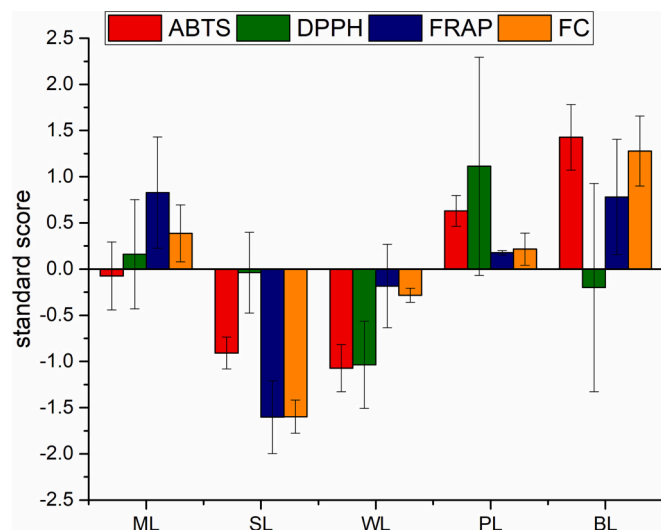


Fig. 3. Comparison of standard scores of lignins (ML: miscanthus lignin, SL: silphium lignin, WL: wheat straw lignin, PL: paulownia lignin, BL: beech wood lignin) using four different assays, results expressed as mean \pm SD.

hardwood lignins. In the DPPH and FRAP assay, they show no significant differences, while in the ABTS and FC assay they do. SL most likely resembles WL: both are indistinguishable in the DPPH and ABTS assay but show differences in the FRAP and FC assay. ML, despite being a grass lignin like SL and WL, it rather resembles the wood lignins and is indifferent from PL for all assays.

Next to the comparison of absolute values, standard scores were calculated for a better comparison of the individual biomasses (Fig. 3). SL and WL show negative standard scores without exception, while PL is the only lignin with only positive scores. By averaging all assays with the help of the RACI (Fig. 4), two groups could be identified: PL, BL, and ML show a higher antioxidant capacity than SL and WL. Despite ML being a grass lignin like SL and WL, it rather resembles the wood lignins PL and BL regarding its antioxidant capacity. As the RACI is an averaged index, it does not represent a specific antioxidant property but only a reasonably accurate ranking. [22] When examining the various processes that play a role in the antioxidant behavior of lignins, it might not be sufficient to condense the results to only a single value for each lignin. However, if a simple benchmark is needed to choose the one lignin that is most suitable for a particular application, e.g. as antioxidant, it might be helpful to use the RACI as decision guidance. Nevertheless, it is inevitable to use different assays to study the various aspects of antioxidant capacity and get a more meaningful rank of the antioxidant effectiveness.

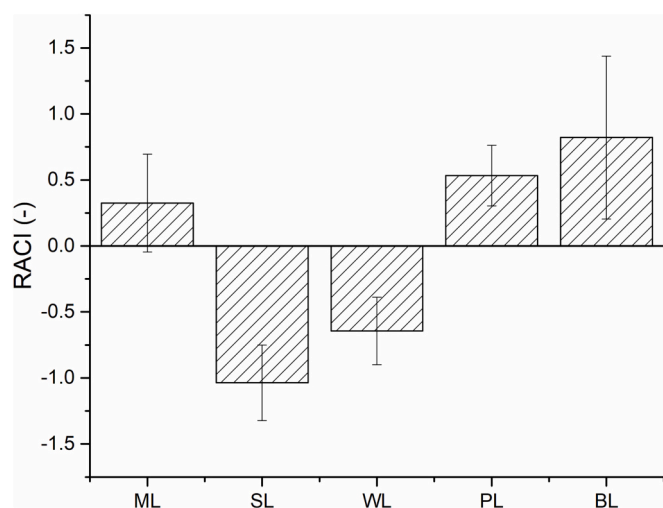


Fig. 4. Relative antioxidant capacity index (RACI) of lignins (ML: miscanthus lignin, SL: silphium lignin, WL: wheat straw lignin, PL: paulownia lignin, BL: beech wood lignin) expressed as mean \pm SD.

4. Conclusion

All four assays are appropriate methods to determine the antioxidant capacity of complex polymers like lignins. The ABTS assay possesses the lowest CV when measuring Trolox over several days and is favorable regarding reaction time, sensitivity, and reproducibility. Strongly positive correlations ($R^2 > 0.75$) were found between FRAP, FC and ABTS assays, while the DPPH assay shows only weak correlations with the other three ($R^2 < 0.5$). But solely because the DPPH assay merely correlates with the other assays, this does not mean that it is not suitable for the measurement of lignins. In general, it is always better to not only select one assay to measure the antioxidant capacity, as it is such a complex property, but choose different ones not closely correlated with each other to better understand which mechanisms play a crucial role for the specific antioxidant under investigation. Lignins from paulownia and silphium were studied for the first time regarding their antioxidant capacity, showing that wood lignins possess higher TEAC and TPC values than grass lignins, except for miscanthus. All four assays combined show that all investigated lignins possess good antioxidant capacities and thus are candidates for further investigations.

CRediT authorship contribution statement

Jessica Rumpf: Conceptualization, Formal Analysis, Investigation, Funding, Visualization, Writing – original draft preparation.

René Burger: Conceptualization, Writing – review & editing.

Margit Schulze: Conceptualization, Funding Acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This study was supported by the BMBF (grant no. 13FH102PX8) and EFRE/NRW (grant no. EFRE 0500035). J.R. gratefully acknowledges a scholarship given by the Graduate Institute of the Bonn-Rhein-Sieg University of Applied Sciences. The authors thank Ralf Pude, Georg Völkerling and Jan Niklas Frase (INRES, University of Bonn, Germany) for providing the biomasses, and Laura Berger (Bonn-Rhein-Sieg

University of Applied Sciences, Germany) for assistance in lignin recovery from wheat straw and beech wood. In addition, we gratefully acknowledge the final English revision by Michael Larkins at the East Carolina University Brody School of Medicine.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2023.123470>.

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