Guidelines for evaluating the antioxidant activity of lignin via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Daryna Diment[a], *Oliver Musl*[b], *Mikhail Balakshin*[a,†] *and Davide Rigo*[a,*]

[a]Department of Bioproducts and Biosystems, School of Chemical Engineering, Aalto University, 02150, Espoo, Finland

[b]Department of Chemistry, Institute of Chemistry of Renewable Resources, University of Natural Resources and Life Sciences, Konrad-Lorenz-Strasse 24, A-3430 Tulln, Austria

†Deceased, 2022

*Corresponding author email: davide.rigo@unive.it

ABSTRACT

The most widespread procedure to measure the antioxidant activity of lignin is via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. So far, different experimental procedures (*i.e.,* different solvent, time, etc.) have been used to implement the DPPH methodology without estimating the effect of such modifications on the experimental procedure. To overcome this issue, the impact of the solvent, the time, and the type of substrate on the evaluation of the antioxidant activity (AoA) of lignin via the DPPH assay was investigated in this work. We found that multiple different parameters affect the evaluation of the AoA of lignin: i) the stability of the DPPH radical and the lignin solubility in a given solvent; ii) the importance of reaching steady state (the effect of time);

iii) the background noise associated with lignin absorbance at λ = 515 nm (used to monitor the DPPH radical scavenging); iv) lignin structure; v) providing a *normalized* radical scavenging index (nRSI); vi) comparing nRSI vs. inhibition percentage (IP) values. Overall, our investigation allowed us to provide guidelines on how to perform the DPPH assay for a more reliable evaluation of lignin AoA.

KEYWORDS: normalized radical scavenging index (nRSI), DPPH method, guidelines, modification, NMR

1. INTRODUCTION

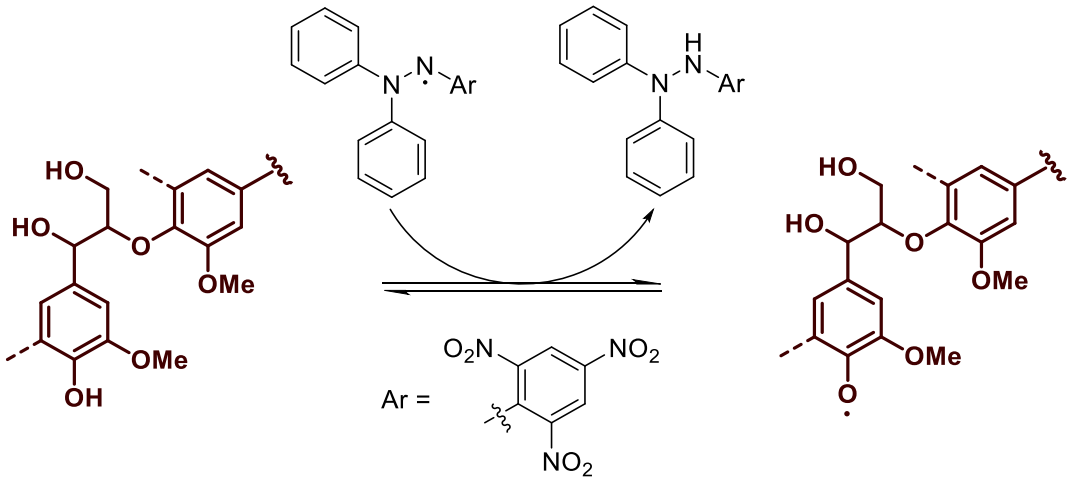
Lignin is an aromatic biopolymer which constitutes 5–30 wt% of lignocellulosic biomass.[1] The heterogenous lignin structure comprises mainly three types of phenylpropane units – *i.e.*, *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) – which are interlinked through ether linkages, such as β–O–aryl units, and C–C bonds like β–β, β–5, β–1 and 5–5, among others.[2–5] In addition, lignin boasts an abundance of different functionalities, such as phenolic and aliphatic hydroxyls (PhOH and AliphOH), carboxylic (–COOH), carbonyl (CO) and methoxy ($–OCH_3$) groups.[3]

The heterogeneity of lignin structure depends on the type of biomass and the processing conditions used to extract/separate it. For instance, upon severe pulping conditions (*i.e.*, Kraft pulping), the lignin structure changes drastically, especially by the cleavage of β–O–4 linkages, resulting in a decrease in molar mass and an increase in the amount of phenolic hydroxyl (PhOH) and carboxyl (–COOH) groups.[3,4,6] Other examples of biorefinery processes are organosolv and sulfite pulping. Organosolv pulping is a more recent method which allows for biomass fractionation using an organic solvent as the reaction media.[7] Sulfite pulping involves the chemical treatment of wood with SO_2 in water under acidic conditions and high temperature. This leads to the functionalization of lignin with sulfonic acid groups.[8,9] In addition, other emerging types of biorefineries showed flexibility and tunability for lignin structure and properties.[10–12] It is thus clear that the applied extraction process strongly affects the resulting lignin structure and its properties.

The abundance of lignin as a by-/co-product in established pulping processes or emerging biorefineries makes it an appealing substrate for various high value applications.[13] Currently, lignin is mainly used as a low value on-site biofuel.[14] Other more valuable strategies to valorize lignin are the production of biobased chemicals,[15,16] carbon fibers,[8] carbon foams,[17]

thermoplastics,[18] adhesives, and thermosetting resins,[19] among others. An interesting feature of lignins is the presence of phenolic –OH groups (PhOH) that endow them with antioxidant properties.[20] In detail, PhOH groups scavenge free radicals *via* an H atom donation and a subsequent phenoxy radical stabilization.[21]

Among the available assays to evaluate the antioxidant properties of compounds (*e.g.*, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid, ABTS; ferric ion reducing antioxidant potential, FRAP),[22] the "free radical" (DPPH) method is widely accepted and used by many researchers.[23] This relies on the calculation of the radical scavenging index (RSI; see experimental and ESI). For instance, Brand-Williams *et al.* used this method to investigate the antiradical activity of various phenolic compounds and different acids (ascorbic and iso-ascorbic) by measuring the reaction kinetics.[24] The concept is based on the interaction between a stable DPPH radical and a chosen scavenging substrate in solution. In other words, an anti-radical (antioxidant) molecule can scavenge the free DPPH radical and quench it. Scheme 1 reports the case of PhOH in the radical scavenge of DPPH radicals. In turn, the quenching capability of the compound is evaluated by measuring the related decrease in UV absorption of DPPH over time.



Scheme 1. Representation of the DPPH radical scavenging by lignin structural units

Hence, AoA is best described as a kinetic parameter, meaning how fast rather than how many (equilibrium) radicals are quenched over time.[25] This decay rate depends on the chemical structure, implying that AoA is a very specific property of a given compound. However, the evaluation of the AoA of lignin is a less trivial endeavor than it first appears as lignins and other plant extracts are more complex than (phenolic) model compounds. In the case of lignin, factors like heterogeneity, complexity, molar mass and molar mass distribution, and selective solubility complicate the evaluation of its AoA. For example, in the DPPH assay, the solvent choice has a big impact on the occurring reactions between lignin and DPPH. Different solvents interact in different ways with different lignin functionalities, which in turn influences the radical quenching kinetics. For all these reasons, the DPPH assay for lignin cannot determine the AoA (kinetic value) but rather the content of "reductive species" or "reductive potential" of a given substrate, which is generally defined as antioxidant capacity (AoC).[25,26] Nevertheless, since AoC is a more specific and less widespread term than AoA, AoA will be interchangeably used through the text even when referring to AoC. Overall, the AoA of lignin is often characterized by the radical scavenging index (RSI), which is related to the concentration at which a lignin substrate is able to scavenge 50 % of the initial DPPH radicals. This is also true for every other antioxidant assay used on lignins as well, and not specific to the DPPH assay.

In light of these considerations, elucidation of lignin AoA needs to be performed via a thoughtful (standardized) procedure and accompanied by a comprehensive structural characterization of lignin to draw valid conclusions.[20] However, a standardized and universal procedure has not been properly developed yet.[27,28] The reported RSI values of lignin were measured using (slightly) different experimental procedures, which impairs the comparability of the obtained values. For instance, solvents like aqueous dioxane/methanol,[29],[30] methanol (MeOH), [31] dimethyl sulfoxide

(DMSO),[32] and ethanol (EtOH)[33] have been used. In addition, the RSI values were measured at different time frames, such as 15 min,[34] 30 min,[32], [35] and 24 h.[29] [36] In some cases, the authors did not mention the time frame for the analysis.[37] A wider scope of lignin AoA is provided in recent reviews.[38,39]

Overall, a standardized procedure that considers the influence of these parameters on the RSI values is still missing. The aim of the current work is to provide reliable and comprehensive insights into the effect of parameters, like the solvent, the time and the type of lignin substrate/structure. In addition, we here give guidelines on the best practices to evaluate the AoA of lignin via the DPPH assay. To the best of our knowledge, no similar works have been reported so far. This work represents a step towards a universal method to investigate the AoA of lignin.

2. EXPERIMENTAL

2.1 Materials and chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical was purchased from Thermo Fisher Scientific. Analytical grade methanol (≥99.8 %), dioxane (≥99.0 %), acetone (C_3H_6O, 95 vol%), and α-tocopherol (vitamin E, ≥95.5%) were purchased from Sigma-Aldrich. Softwood kraft lignin (Indulin AT) and Alcell are commercially available technical lignins. The other lignin substrates were produced according to our previously reported procedures.[10–12,20]

2.2 The preparation of lignin substrates

For the current work, lignin samples of different origin and structure were selected (Table 1). Lignin derivatization (S3–6, Table 1) was performed according to Diment et al.[20] Briefly, Indulin AT underwent structural modifications by selectively acetylating (masking) all –OH groups with acetic anhydride (S3, Table 1), methylating benzylic –OH and –COOH groups using acidic methanol (S4, Table 1), methylating phenolic –OH groups with dimethylsulfide at 80 °C (S5, Table

1), and sodium borohydride ($NaBH_4$) reduction of C=O groups of keto- and aldehyde-types (S6, Table 1). One partially ethoxylated lignin sample was prepared as described by Rigo et al.[12] (S7, Table 1). Two additional samples were prepared according to our recently reported biorefinery procedure[10,40] (S8 and S9, Table 1). Further details on the sample preparations are reported in the ESI.

2.3 AoA evaluation via the DPPH method

The antioxidant properties of the lignin samples were conducted according to Diment et al. with certain adjustments.[20] The effect of time, solvent and lignin substrate was investigated.

2.3.1 *Normalized radical scavenging method*.

Lignin samples were dissolved in 90 vol% dioxane (aq.), 90 vol% MeOH (aq.), or 90 vol% acetone (aq.) at different concentrations (0.12–0.6 mg mL^{-1}). DPPH was dissolved in 99.8 vol% MeOH, 90 vol% MeOH (aq.), 90 vol% acetone (aq.), or 90 vol% dioxane (aq.) at a concentration of 76 µmol L^{-1}. Then, 0.7 mL of the lignin solution was added to 27 mL of DPPH solution and vigorously mixed. The proportion between the DPPH and lignin solutions was kept at 39:1 (v/v) for all experiments. DPPH radical scavenging was determined by UV-VIS spectrometry as the change in DPPH concentration over time (0, 5, 15, 30 min, and 1, 2, 6, 24, 48 h) (*vide infra*). The rate of DPPH scavenging was determined by linearly fitting the first time points (5, 15, 30 min) and the steady state of the reaction was defined as the maximum of scavenging curve. Normalization accounted for the initial absorbance of the lignin solutions and the degradation of DPPH over time in a blank sample. Further details on the calculations to evaluate the normalized RSI (nRSI) of the lignin samples are reported in the ESI. The calculated nRSI values are based on two replicated measurements.

2.4 ^{31}P nuclear magnetic resonance spectroscopy (NMR)

Determination and subsequent quantification of various hydroxyl groups was performed by ^{31}P NMR according to our optimized method.[2] The spectra were recorded on a Bruker NMR Spectrometer AV III 400 with an acquisition time set to 1 s, relaxation delay of 5 s, and number of scans set to 128. Briefly, ca. 40 mg of each lignin sample was dissolved in a 0.4 mL of a freshly prepared mixture of pyridine and $CDCl_3$ (1.6:1, v/v). Following that, 100 µL of e-HNDI solution (0.3 µmol mg^{-1}) as an internal standard (IS) and 50 µL of chromium(III) acetylacetonate solution (11.4 mg mL^{-1}) were added. Lastly, 100 µL of derivatization agent (TMDP) was added and the vial was vortexed until homogeneous before being transferred into an NMR tube. The resulting spectra underwent phasing and calibration according to the signal of the 2,2′-oxybis(4,4,5,5-tetramethyl-1,3,2-dioxaphospholane) water-derivatized product at 132.2 ppm. A linear function was used for baseline correction.

2.5 UV-VIS spectroscopy

UV-VIS spectroscopy of the lignin:DPPH solutions was performed on an UV-2550 UV-VIS spectrophotometer (Shimadzu) using 10 mm way length quartz cuvettes. The scavenging of radicals leads to a shift in the UV-VIS spectra of DPPH, *i.e.*, a decrease in absorbance at λ = 515 nm, which was thus selected as measurement wavelength.

3. RESULTS AND DISCUSSION

The aim of the present study was to investigate the effect of solvent, time and substrate on the measurement of antioxidant properties of lignin. For this endeavor, a mixture of MeOH and 90 vol% dioxane (aq.) (39:1, v/v) and three different solvents (*i.e.*, 90 vol% MeOH (aq.), 90 vol% acetone (aq.), and 90 vol% dioxane (aq.)) were used, labeled solvent **A**, **B**, **C**, and **D**, respectively

(Table 2). The selected solvents are commonly used to solubilize lignin and DPPH. Several lignin substrates were tested, including industrially available Kraft (Indulin AT) and organosolv (Alcell) lignins, selectively modified Indulin AT, partially ethoxylated hardwood lignins, and lignin-carbohydrate complexes (see Table 1, experimental, and ESI).

Table 1. Lignin samples of various origin and structural peculiarities that were chosen for this study

Entry	Sample	Label	Origin	Structure	Ref.[a]
1	Indulin AT	S1	SW, Kraft	unmodified	n.a.
2	Alcell	S2	HW, organosolv	unmodified	n.a.
3	Ind-Ac	S3	SW, Kraft	Acetylated	[41]
4	Ind-Me	S4	SW, Kraft	Methylated benzylic –OH	[41]
5	Ind-Ph	S5	SW, Kraft	Methylated phenolic –OH	[42]
6	Ind-R	S6	SW, Kraft	Reduced (C=O to –OH)	[41]
7	EtO-lignin	S7	HW, AqSO	Partially ethoxylated	[12]
8	AqSO-LCC	S8	HW, AqSO	Lignin carbohydrate complexes (LCCs)	[10]
9	AqSO-lignin	S9	HW, AqSO	"Pure" lignin	[10]

n.a. = not applicable. [a]Reference for the experimental procedure for the sample preparations. HW = hardwood. SW = softwood. AqSO = AquaSolv Omni.[10,11]

3.1 Degradation of DPPH over time

The first issue to consider when evaluating the AoA via the DPPH method is the DPPH self-degradation over the time.[43] Thus, prior to the evaluation of RSI, we investigated the DPPH self-degradation in the different solvents of choice (Figure 1). Within the first 2 h, the degradation is low in all solvents, while, after this time point, DPPH experienced a rapid degradation in solvent **D** (dioxane-based) and a moderate-to-neglectable degradation in other solvents (**A**, **B**, and **C**).

Interestingly, DPPH was found almost fully stable in solvent **C** (acetone-based) with ca. 3 % degradation over the 24 h period. The degradation in solvent **B** (MeOH-based) was 5 times higher than in solvent **C** (15 %) after 24 h. These results are consistent with the ability of protic solvents to transfer an H· radical when compared to aprotic solvents and, thus, promote DPPH quenching (*i.e.*, MeOH vs. acetone, respectively). The degradation in solvent **D** (dioxane-based) was found to be the highest at ca. 70 %. A plausible path for the instability of DPPH in dioxane or its mixture(s) may involve its reaction with peroxide species present in ethers.[44] The latter finding is consistent with the higher value (20 %) achieved after 24 h in solvent **A** (MeOH+dioxane-based) compared to **B** (MeOH-based).

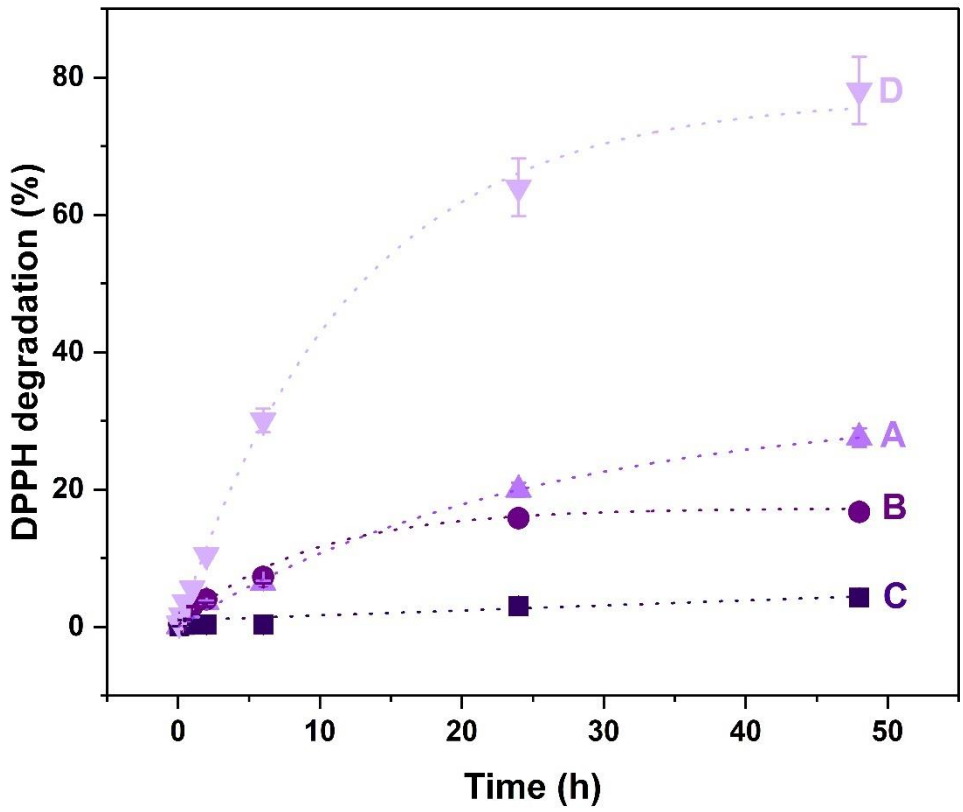


Figure 1. DPPH degradation over a 48-h period in different solvents. Where: -▲- MeOH:90 vol% dioxane (aq.) = 39:1 (v/v) (Solvent **A**); -●- 90 vol% MeOH (aq.) (Solvent **B**); -■- 90 vol% acetone (aq.) (Solvent **C**); -▼- 90 vol% dioxane (aq.) (Solvent **D**). Data are also reported in Table S2.

Additionally, we studied the rate of DPPH degradation (D_r) in the used solvents (Table 2). We report D_r as the slope of the fitting of the first three time points, where a linear correlation was found (5, 15 and 30 min; Figure S2). As expected, D_r values followed the same trend as the DPPH degradation after 24 h ranking the self-degradation in the order solvent **D**>**A**>**B**>**C**.

Table 2. Degradation rate of DPPH (D_r) for the solvents used in the study

Label	Solvent system	D_r (mmol mol^{-1} t^{-1})
Solvent **A**	MeOH:90 vol% dioxane (aq.)	2.0
Solvent **B**	90 vol% MeOH (aq.)	1.5
Solvent **C**	90 vol% acetone (aq.)	0.4
Solvent **D**	90 vol% dioxane (aq.)	7.4

Our results demonstrate the importance of selecting an appropriate solvent and moreover, correcting the radical scavenging index by the DPPH self-degradation over time (*i.e.*, normalized RSI, nRSI; see ESI for more details) is strongly recommended. We can, thus, express a corrected DPPH absorbance (cDPPH_{abs}) as:

$$\mathrm{cDPPH}_{abs} = \mathrm{DPPH}_0(\mathrm{absorbance}_{t=0}) - \mathrm{DPPH}_t(\mathrm{absorbance}_{t=x}) \quad (1)$$

Where DPPH_0 is the initial absorbance and DPPH_t is the absorbance at the time point of interest (t = x). The cDPPH_{abs} has been used to evaluate the lignin AoA in the present work.

3.2 The effect of solvent

An additional important aspect to consider when determining the AoA of lignin is the possible absorbance of certain lignin structures at the same wavelength of DPPH (λ = 515 nm). We therefore measured the absorbance of each lignin substrate (A_l) without the addition of DPPH in the selected solvents (Table 2) at λ = 515 nm. The initial absorbance in the DPPH experiment (*i.e.*, DPPH_0) was then corrected by A_l to eliminate bias due to the respective lignin's absorbance at the measurement wavelength (see ESI).

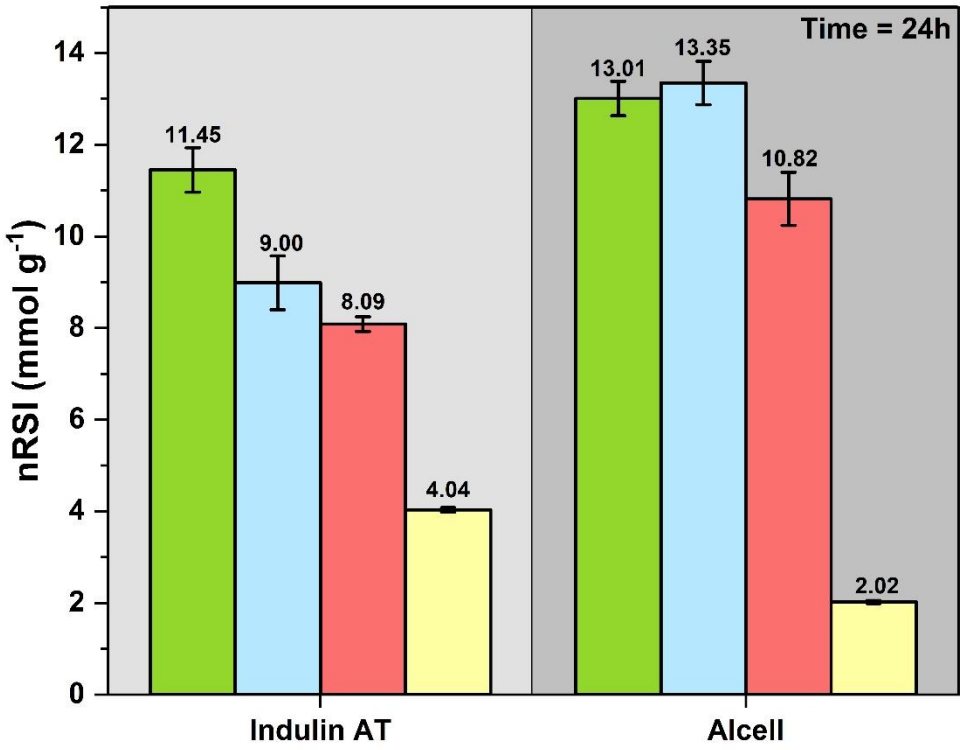


Figure 2. Average nRSI values in mmol g^{-1} for Indulin AT and Alcell in different solvent systems after 24 h, where ■ solvent **A**; ■ solvent **B**; ■ solvent **C**; ■ solvent **D**. Data are also reported in Table S3 and IP values in Figure S3.

Indulin AT (S1) and Alcell (S2) were selected to further investigate the effect of the solvent. After 24 h of measurement time, the highest nRSI values were found for S1 and S2 in solvent **B** (MeOH-based) and solvent **C** (acetone-based), respectively (Figure 2). It must be noted that S1 and S2 were not fully soluble in solvent **B**, thus the AoA was evaluated solely of the soluble part (82 and 90 % for S1 and S2, respectively; see ESI and Table S3). In contrast, S1 and S2 were fully soluble in all the other systems (Table S3), which further highlights the importance of selecting an appropriate solvent. Incomplete dissolution complicates the interpretation of AoA, since we only

characterize a certain lignin fraction and not the whole sample. Thus, to draw consistent conclusions, we compared the contents of –OH and –COOH groups present in the MeOH soluble fractions (S1-sol and S2-sol) with S1 and S2 (Table 3, entries 1-4). As we recently reported,[20] PhOH groups are the most important functional groups to determine the AoA of lignin. ^{31}P NMR analysis showed no significant difference in PhOH groups between S1 and S1-sol, thus the nRSI was assumed not to be much affected by the incomplete dissolution. In contrast, S2-sol showed a ca. 20 % decrease in PhOH content compared to S2 which suggests an underestimation of nRSI in solvent **B**.

Table 3. Quantification of –OH groups in lignin samples of various origin and structural peculiarities used in the current work by ^{31}P NMR. Spectra of the analyzed samples are reported in the Figure S4.

Entry	**Label**	**–OH/–COOH groups (mmol g^{-1})**							Ref.
		Aliph.	**5-Sub**	**Gnc**	**H**	**–COOH**	**PhOH**	**Total –OH**	
1	S1	2.49	1.63	1.67	0.23	0.33	3.53	6.02	[20]
2	S1-sol	1.97	1.53	1.59	0.25	0.79	3.38	5.35	Current paper
3	S2	1.69	2.73	1.02	0.25	0.37	4.00	5.69	
4	S2-sol	1.46	2.25	0.84	0.23	0.46	3.31	4.77	
5	S3	0.10	0.08	0.04	0.03	0.33	0.14	0.25	[20]
6	S4	1.92	1.66	1.60	0.30	0.18	3.56	5.48	
7	S5	2.12	0.23	0.09	0.02	0.33	0.38	2.46	
8	S6	2.87	1.62	1.81	0.38	0.34	3.81	6.68	
9	S7	2.11	1.97	0.57	0.11	0.08	2.65	4.76	Current paper
10	S8	3.19	2.73	0.65	0.10	0.21	3.48	6.67	
11	S9	2.06	2.84	0.63	0.16	0.33	3.63	5.70	

Aliph. = aliphatic –OH groups. 5-Sub. = 5-substituted –OH groups. Gnc = G-type non conjugated –OH groups.

Lower nRSI values (ca. 1.4 and 1.2 times for S1 and S2, respectively) were obtained using solvent **A** compared to solvent **B** for both substrates. Moreover, very low nRSI values of 4.04 and

2.02 mmol g^{-1} were observed using solvent **D** for S1 and S2, respectively (*i.e.*, a 65 and 84 % decrease in nRSI compared to solvent **B** for S1 and S2, respectively). Overall, the observed trend for nRSI values was **B**>**C**>**A**>**D**, so MeOH>Acetone>MeOH+dioxane>dioxane.

These results suggest that polar protic solvents such as MeOH provide higher nRSI compared to polar aprotic solvents like acetone and dioxane. To explain these finding, we should consider the two main scavenging mechanisms for plant antioxidants: proton-coupled electron transfer (PCET) and sequential proton loss electron transfer (SPLET).[34] In brief, PCET is solely governed by the homolytic dissociation of the O–H bond of active –OH groups. The SPLET mechanism consists of an initial proton loss followed by electron transfer. This mechanism is firmly favored in alkaline conditions.[34] In both cases, phenolic protons are involved. The environment (solvent) can influence the rate and type of reaction based on its polarity, ionization potential, pH, etc.[25,34] For instance, in polar protic solvents, SPLET mechanism has been proven to exist.[25] In addition, it is important to highlight that the solvent should ensure that the scavenging reaction proceeds effectively, that the radicals are able to interact with the substrate while favoring mass transfer. Consequently, the ability of polar protic solvents to facilitate the proton and electron transfer – mass transfer – via SPLET mechanism is consistent with the higher nRSI values observed in solvent **B**. Additionally, the possibility of MeOH to form H-bonds should be an additional aspect to consider when explaining the higher nRSI.[25]

Comparing now solvent **A** and **D** (both dioxane-based) with solvent **C** (acetone-based) as polar aprotic solvents, the differences in the nRSI can be associated with different polarity and the high degradation of DPPH in solvent **D**, as discussed in the previous section. We also attribute the lower nRSI values obtained in the dioxane-based solvents to the presence of peroxide reactive species in ethers which somehow influence the radical scavenging.

Taking into account several factors such as solubility of the substrates, solvation effects, DPPH degradation, IP development rate, solvent toxicity and the resulting nRSI values, we concluded that the most efficient solvent for the determination of antioxidant properties using the DPPH assay is solvent **C** (acetone-based).

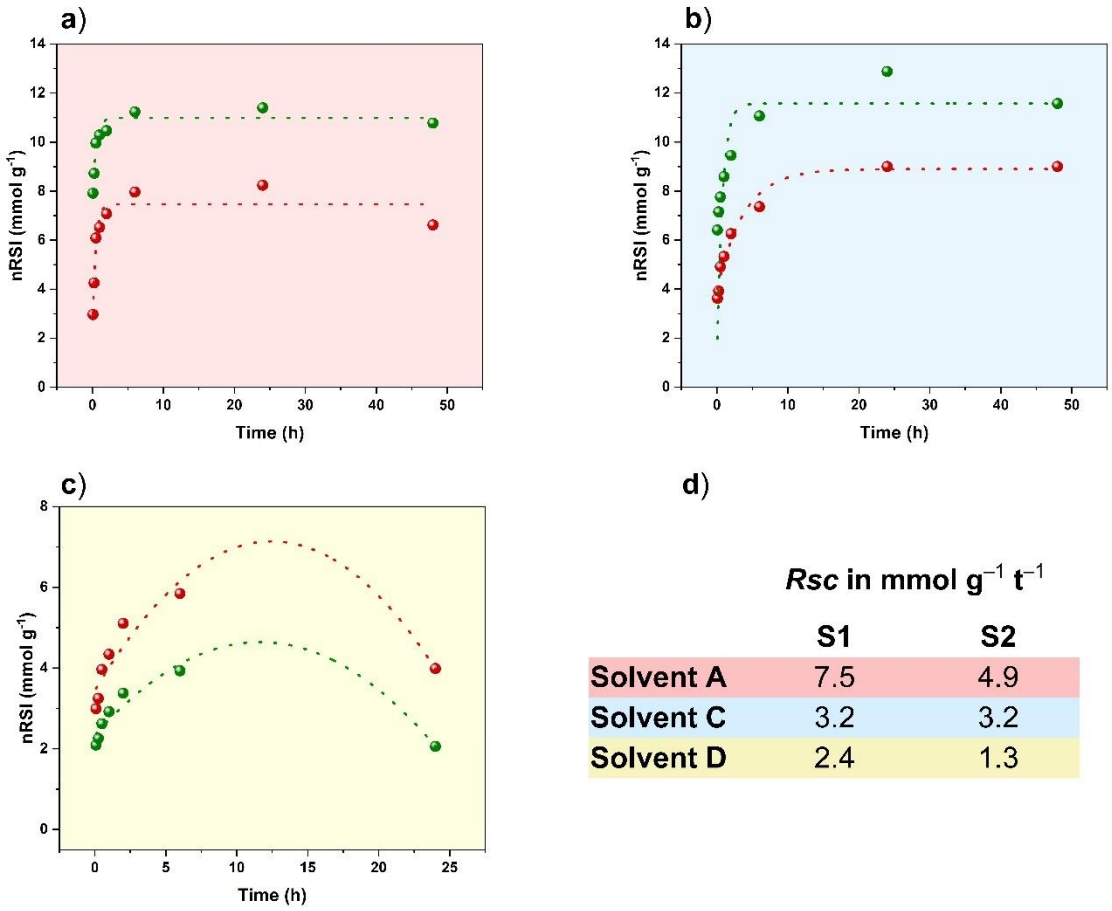
3.3 The effect of measurement time

As mentioned in the introduction, the number of scavenged radicals and the rate of radical scavenging depends on the type of substrate, the solvent, the temperature, the occurrence of side reactions, and the self-degradation rate of DPPH. It is therefore of key importance to ensure that steady state – the point when the amount of scavenged radicals does not change (thermodynamic equilibrium) – is reached before evaluating the antioxidant properties.[24,25] Thus, the scavenging reaction should be monitored over a sufficiently long time when determining the AoA of lignins.

In this part, S1 and S2 were used as substrates in solvent **A**, **C** and **D** (Figure 3 and 4). We decided to not include solvent **B** due to the solubility issues we encountered, as discussed in the previous section. In addition to nRSI values, the number of scavenged radicals over time (*i.e.*, inhibition percentage, IP) was determined after 5, 15, 30 min, and 1, 2, 6, 24, 48 h, respectively, since for complex samples such as lignins, IP might be considered an even better indicator of the scavenging reaction compared to nRSI.

As expected, clear differences were found for the used solvents. In all solvents, the scavenging activity within the first 5 min was very high (15–40 %) after which IP grew moderately for 6 h (20–67 %) until a steady state was reached (Figure 4). For both substrates, steady state was reached faster in solvent A (MeOH+dioxane-based) compared to solvent C (acetone-based) after 6 h and 10 h, respectively, while no clear steady state was observed in solvent **D** (dioxane-based) (Figure 3). As previously discussed, polar protic solvents such as MeOH facilitate radical scavenging, and thus steady state was reached faster compared to aprotic solvents. In contrast, the presence of

peroxide reactive species in dioxane had a detrimental effect on the reaction due to enhanced self-degradation of DPPH with time. This led to a decrease in the nRSI after reaching the steady state (solvent **A**), or to the obscuring of a potential steady state (solvent **D**).



Rsc in mmol g^{-1} t^{-1}

	S1	S2
Solvent A	7.5	4.9
Solvent C	3.2	3.2
Solvent D	2.4	1.3

Figure 3. nRSI values calculated for each time point of the experiment for Indulin AT and Alcell in a) solvent **A**; b) solvent **C**; c) solvent **D**, where: -●- Indulin AT, -●- Alcell. d) Rate of the scavenging reaction (R_{sc}) in the used solvents.

Linear fitting of the nRSI values between 5, 15 and 30 min – the timeframe where the trend is linear (Figure 3) – allowed to determine the rate of the scavenging reaction (R_{sc}) in different solvents (Figure 3d and Figure S5). R_{sc} represents how fast the radical scavenging of DPPH is happening and, thus, provides kinetic information on the scavenging reaction. S1 and S2 showed

higher reaction rates in solvent **A**, followed by solvent **C** and lastly solvent **D** (7.5 and 4.9, 3.5 and 3.5, and 2.4 and 1.3 mmol g^{-1} t^{-1}, respectively). Interestingly, no clear correlation was noticed for the rate of DPPH degradation and the lignin-promoted rate of radical scavenging (*vide infra*). Since both DPPH self-degradation and its scavenging by lignin are radical reactions, they were expected to correlate with each other.

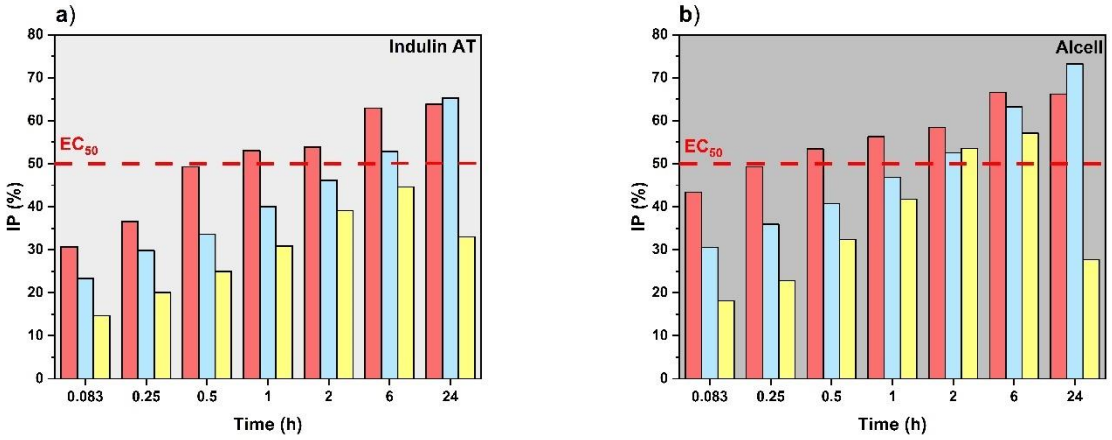


Figure 4. Change in inhibition percentage (IP, %) over 24 h for S1(a) and S2 (b) in different solvents, where ■ solvent **A**; ■ solvent **C**; ■ solvent **D**.

One should keep in mind that nRSI is a number which is calculated via a multi-step procedure. In brief, nRSI is calculated as the inverse value of the effective concentration (EC_{50}) of lignin, normalized by the DPPH concentration (Eq. 2).

$$\mathrm{nRSI} = \frac{1}{\mathrm{nEC_{50}}} \tag{2}$$

Where nEC_{50} is the extrapolated lignin concentration at which 50 % of DPPH radicals were scavenged, normalized by DPPH concentration. More details on all the calculations are reported in the ESI. Therefore, its value can dramatically change even for small differences in IP values. In other words, small differences (5 %) in the nRSI of different samples are generally within

experimental error. Therefore, we encourage to always compare both the nRSI and IP values to have a more comprehensive picture of lignin AoA.

3.4 The effect of substrate and lignin structure

The influence of the lignin substrate on the radical scavenging relies on its structural peculiarities affecting both the proton abstraction rate and the stability of the radical formed. Nine structurally different lignin substrates were tested using the normalized RSI approach (Table S4). As discussed in the previous section, solvent **B** was again not included in this part of the study, since the solubility of S1 and S2 in the latter was not complete (see ESI). We ensured that steady state was reached before providing the nRSI values (t = 24 h).

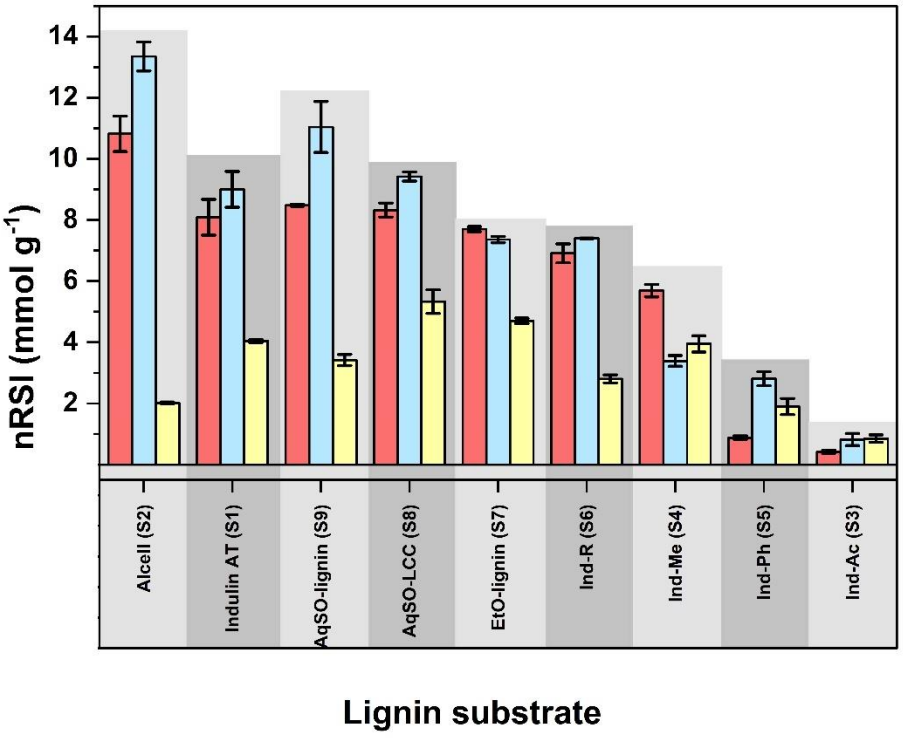


Figure 5. Average nRSI values obtained for different lignins in ■ solvent **A**; ■ solvent **C**; ■ solvent **D**. Data and associated errors are also reported in Table S4.

In a previous study, we emphasized that PhOH play a preeminent role in the AoA of lignin, while aliphatic −OH groups and −COOHs are detrimental to nRSI.[20] In addition, we found that masking benzylic −OH groups (*e.g.*, in β−O−4 structures) is detrimental for lignin AoA.[20] The observed impact of the lignin structure on the AoA will be discussed in this context (compare Tables 1 and 3, and Figure 4). Alcell (S2) showed the highest values followed by AqSO-lignin (S9) and Indulin (S1), which is in line with their high PhOH groups content (4.00, 3.63, and 3.53 mmol g^{-1}, respectively). Differences in AoA between S2, S9, and S1 can be further explained by the number of aliphatic −OH groups which follow the trend S1>S9>S2 (2.49, 2.06, and 1.69 mmol g^{-1}, respectively). This supports our recent findings on the detrimental effect of aliphatic −OH groups on lignin AoA.[20] Modified Indulin lignin samples (S3–S6) with (partially) masked benzylic –OH groups showed the expected decrease in AoA compared to unmodified Indulin, regardless of the used solvent. We can thus corroborate our previous findings via an updated methodology for nRSI evaluation.[20] The AquaSolv Omni (AqSO) biorefinery[10,11] lignin samples (S7–9) exhibited remarkable differences, especially in solvent **C**. The ethoxylated sample (S7) – mainly ethoxylated at the benzylic position[12] – showed the lowest AoA among them (S7–9), once again in line with our previous findings on the detrimental effect of methylation of benzylic –OHs.[20] The AqSO-lignin sample (S9) showed higher AoA compared to AqSO-LCC (S8) which relies on the higher PhOH (beneficial) and lower aliphatic OH (detrimental) groups content in S9 compared to S8.

An additional aim of this part of the work was to determine whether the nRSI of a lignin obtained in different solvents are correlated, with the calculation of a conversion factor as the ultimate goal. A conversion factor would help to roughly estimate the nRSI in different solvents for comparison with literature values. However, we want to stress that measurement parameters are often not

ideally defined in literature and thus comparisons should be met with caution. In this work, conversion factors have been calculated as the ratio of the nRSI values obtained in different solvents:

$$Conversion\ factor = \frac{nRSI_{Solvent\ X}}{nRSI_{Solvent\ Y}} \quad (2)$$

An uncertainty associated with the conversion factors was calculated as well (see ESI).

Though the number of tested samples does not represent a statistical set, the calculated conversion factors still allow for a rough comparison of nRSI obtained in different solvents with uncertainties below 20 % of the conversion factors.

Table 4. nRSI conversion factors for lignin samples in the tested solvents and associated errors

Conversion factor		
Solvent C → A (Acetone → MeOH+dioxane)	**Solvent C → D** (Acetone → dioxane)	**Solvent A → D** (MeOH+dioxane → dioxane)
0.87 ± 0.11	0.60 ± 0.13	0.85 ± 0.11

3.5 Vitamin E as a Standard Antioxidant

Vitamin E is a lipophilic antioxidant that plays imperative role in protecting cells from oxidative stress caused by free radicals.[45] Vitamin E is widely used as an antioxidant in various industries due to its ability to prevent oxidation, thus extending the shelf life and maintaining the high quality of products.[45,46] Its antioxidant properties are of prime importance in food, cosmetics, and pharmaceuticals sectors.[45] For all these reasons, we selected vitamin E as a standard antioxidant to compare with our lignin samples. Solvent **D** was not considered due to the very high DPPH degradation (see discussion in the previous sections). We included solvent **B** to make the

discussion more comprehensive, even though we previously encountered solubility issues with the lignin samples.

Vitamin E showed an extremely high IP rate in all solvents (Figure 7a). Steady state was reached within the first 15 min of the reaction (ca. 24 times faster – on average – compared to lignin samples), by inhibiting ca. 80 % of the DPPH radicals in solvent **C** and more than 95 % of the DPPH radicals in solvents **A** and **B** (Figure 7a). This exemplifies why AoA is commonly defined as a kinetic parameter for small molecules.[25] Especially the outstanding IP values in solvent **B** are in good agreement with literature,[47–49] since MeOH as a polar protic solvent (solvent **B**) facilitates the SPLET mechanism which results in a higher IP rate as well as in higher nRSI values (Figure 7B).

As expected, the change in IP showed that the rate of radical scavenging for vitamin E is much faster compared to lignin regardless of the used solvents (Figure 7a). For instance, vitamin E can scavenge DPPH ca. 2.6 times faster than S1 when comparing their change in IP in solvent **C** (compare Figures 7a and 4a). However, comparing the nRSI values of vitamin E and Alcell when steady state was reached (15 min and 24 h for vitamin E and Indulin AT (S1) in solvent **C**, respectively), a 15% higher nRSI value was found for the lignin sample (7.7 and 9.0 mmol g^{-1} for vitamin E and Indulin AT, respectively; Figures 2 and 7b, and Table S4). As discussed in the introduction and throughout the text, we suggest considering both the nRSI values and the IP trends over the time to draw more reliable and comprehensive conclusions on the AoA of lignin. Nevertheless, we also demonstrated that certain lignin samples (*e.g.*, S1 and S2) and vitamin E work almost equally in the long run, eventually resulting in both high IP rates and nRSI values. In addition, testing vitamin E proved the effectiveness of the developed procedure for nRSI evaluation.

Overall, the rate of radical scavenging (kinetics) was the main difference between the best performing lignin samples and vitamin E, while the differences in the total number of scavenged radicals (thermodynamics) were less pronounced. One should consider that the importance of how fast or how many radicals are quenched depends on the application the antioxidant is intended for. For instance, in certain applications an active ingredient that prevents aging in the long term is needed while in others quenching must be fast. Therefore, the discussed lignin behavior might be exploited in long-lasting antioxidant formulations (*i.e.*, coatings, paints, among others).

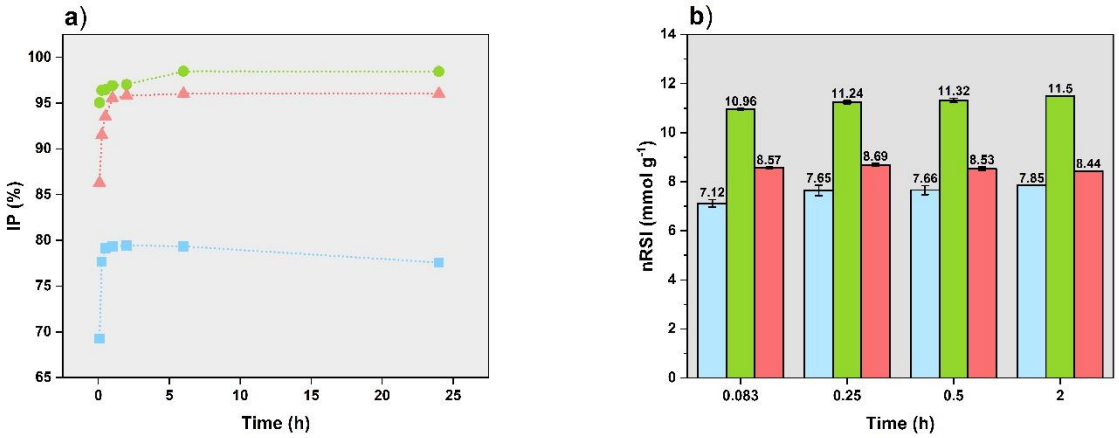


Figure 7. Quantitative representation of the antioxidant properties of vitamin E. a) change in IP (%) over time of vitamin E in -▲- solvent **A**; -●- solvent **B** and -■- solvent **C**. b) nRSI over time in ■ solvent A; ■ solvent B and ■ solvent C

3.6 Guidelines for the evaluation of the AoA of lignin

After investigating the effect of multiple parameters on the calculation/evaluation of nRSI values – and thus on the AoA of lignin – we here provide guidelines for performing the DPPH assay on lignin samples.

- **DPPH degradation**: DPPH stability in the chosen solvent significantly affects the nRSI determination. Therefore, we suggest measuring the absorbance of the DPPH solution at the

starting time and at various time intervals to track any changes due to self-degradation. If the degradation is higher than 20 %, we suggest using a different solvent or solvent system.

- **Lignin absorbance**: lignin samples may show absorbance at the measurement wavelength of DPPH (λ = 515 nm). For this reason, we suggest to always eliminate the lignin "background noise" as discussed in section 3.2 and in the ESI.
- **Steady state:** steady state is reached at different times in different solvents, which depend on the rate of the scavenging reaction. We suggest to always monitor the reaction over a longer timeframe to ensure steady state is considered when determining the nRSI. In addition, our results suggest that the time needed to establish steady state is more dependent on the solvent than on the substrate type.
- **nRSi vs. IP:** nRSI is related to the amount of lignin needed to scavenge 50 % of the DPPH radicals and is extrapolated following a multi-step procedure (see ESI). In contrast, IP directly represents the number of scavenged radicals, which might be more representative for complex samples such as lignins. In light of this, we suggest discussing and comparing the IP and nRSI development over time to provide comprehensive information on the AoA of a lignin sample.
- **Solvent:** the used solvent plays crucial role in nRSI evaluation as it predetermines the DPPH scavenging mechanism as well as the above-discussed rate of steady state establishment and DPPH stability. An appropriate solvent for the experiment should provide excellent solubility for both the lignin substrate and DPPH, and good stability of the radical in the chosen solvent, while favoring the radical transfer. Among the used solvents, solvent **C** (acetone-based) showed the most promising suitability for the evaluation of antioxidant properties. Solvent **C** was particularly effective in maintaining DPPH stability, with minimal

degradation observed over a 24 h period. However, it cannot be ruled out that other solvent systems exist that show an equal or even superior performance in fast and effective antioxidant properties screening of lignin. Future studies could elaborate on this aspect to further optimize the solvent composition.

- **Lignin structure:** the structure of the substrate affects the scavenging rate by its ability to form stable radicals and the presence/absence of electron-donating or electron-withdrawing groups, as well as other intra- and inter-molecular interactions. In this study, all solvent systems showed only a low to moderate impact of the substrate used compared to the effect of other parameters (*i.e.*, time, solvent, etc.). For all the tested lignin samples, AoA was therefore better expressed as a thermodynamic value – amount of scavenged radicals at the equilibrium (steady state) – rather than a kinetic one. On the other hand, as expected, small molecules such as vitamin E show a different behavior and their AoA should be indicated as a kinetic value.
- ***In-vitro* vs. *in-vivo*:** the obtained nRSI values are only an indication and should not be taken as an absolute value. The *in-vitro* – our study – DPPH assay is a good method to compare and screen the AoA of several lignin samples. Nevertheless, we suggest using a combination of both *in-vitro* and *in-vivo* tests to confirm the effectiveness of the lignin AoA. This aspect will be investigated in a follow-up work.

4. CONCLUSIONS

Based on the classic DPPH assay, we provided a comprehensive evaluation of the dependance of the calculated normalized radical scavenging index (nRSI) values – as an indication of the antioxidant activity (AoA) of lignin – on different parameters, including the solvent, the measurement time and the type of lignin substrate. Our results allowed us to provide guidelines

for evaluating the AoA of lignin via the DPPH assay and more reliably compare the properties of different lignin samples. 90 vol% acetone (aq.) was found to be the most appropriate solvent based on several important parameters, including complete solubility of lignin and DPPH, DPPH stability, solvation effects, inhibition percentage (IP) development rate, solvent toxicity and the obtained nRSI values. Additionally, it was highlighted that steady state establishment, and a low DPPH degradation are important to accurately and consistently evaluate and compare lignin AoA. Conversion factors to compare the nRSI values obtained for the same lignin samples in different solvents have been proposed. Overall, for a more reliable evaluation and comparison of the antioxidant properties of lignin samples, we suggest considering both the nRSI values and the IP development over the time.

ACKNOWLEDGEMENTS

D.D., and D.R. gratefully acknowledge the support from the Academy of Finland (Grant 341596) and the FinnCERES BioEconomy flagship.

References

[1] M. Y. Balakshin, E. A. Capanema, *RSC Adv* **2015**, *5*, 87187–87199.

[2] M. Balakshin, E. Capanema, *Journal of Wood Chemistry and Technology* **2015**, *35*, 220–237.

[3] A. Berlin, M. Balakshin, in *Bioenergy Research: Advances and Applications*, Elsevier Inc., **2014**, 315–336.

[4] M. Y. Balakshin, E. A. Capanema, C. L. Chen, H. S. Gracz, *J Agric Food Chem* **2003**, *51*, 6116–6127.

[5] M. Y. Balakshin, E. A. Capanema, R. B. Santos, H. M. Chang, H. Jameel, *Holzforschung* **2016**, *70*, 95–108.

[6] G. Zinovyev, I. Sulaeva, S. Podzimek, D. Rössner, I. Kilpeläinen, I. Sumerskii, T. Rosenau, A. Potthast, *ChemSusChem* **2018**, *11*, 3259–3268.

[7] A. Johansson, O. Aaltonen, P. Ylinen, *Biomass* **1987**, *13*, 45–65.

[8] J. F. Kadla, S. Kubo, R. A. Venditti, R. D. Gilbert, A. L. Compere, W. Griffith, *Carbon N Y* **2002**, *40*, 2913–2920.

[9] Z. Guo, L. Olsson, *Process Biochemistry* **2014**, *49*, 1231–1237.

[10] D. Tarasov, P. Schlee, A. Pranovich, A. Moreno, L. Wang, D. Rigo, M. H. Sipponen, C. Xu, M. Balakshin, *Green Chemistry* **2022**, *24*, 6639–6656.

[11] P. Schlee, D. Tarasov, D. Rigo, M. Balakshin, *ChemSusChem* **2023**, *16*, e202300549.

[12] D. Rigo, N. Kohlhuber, L. Fliri, D. Diment, M. Cho, I. Sumerskii, M. Hummel, A. Potthast, M. Balakshin, *Green Chemistry* **2024**, *26*, 2623–2637.

[13] M. Y. Balakshin, E. A. Capanema, I. Sulaeva, P. Schlee, Z. Huang, M. Feng, M. Borghei, O. J. Rojas, A. Potthast, T. Rosenau, *ChemSusChem* **2021**, *14*, 1016–1036.

[14] D. D. S. Argyropoulos, C. Crestini, C. Dahlstrand, E. Furusjö, C. Gioia, K. Jedvert, G. Henriksson, C. Hulteberg, M. Lawoko, C. Pierrou, J. S. M. Samec, E. Subbotina, H. Wallmo, M. Wimby, *ChemSusChem* **2023**, *16*, e202300492.

[15] J. E. Holladay, J. F. White, J. J. Bozell, D. Johnson, **2007**, DOI: 10.2172/921839.

[16] C. Amen-Chen, H. Pakdel, C. Roy, *Bioresour Technol* **2001**, *79*, 277–299.

[17] J. S. Rodrigues, A. D. S. M. de Freitas, C. C. Maciel, C. Guizani, D. Rigo, M. Ferreira, M. Hummel, M. Balakshin, V. R. Botaro, *Int J Biol Macromol* **2023**, *240*, 124460.

[18] R. T. Mathers, *J Polym Sci A Polym Chem* **2012**, *50*, 1–15.

[19] D. Di Francesco, D. Rigo, K. Reddy Baddigam, A. P. Mathew, N. Hedin, M. Selva, J. S. M. Samec, *ChemSusChem* **2022**, *15*, e202200326.

[20] D. Diment, O. Tkachenko, P. Schlee, N. Kohlhuber, A. Potthast, T. M. Budnyak, D. Rigo, M. Balakshin, *Biomacromolecules* **2024**, *25*, 200–212.

[21] J. L. Wen, B. L. Xue, F. Xu, R. C. Sun, A. Pinkert, *Ind Crops Prod* **2013**, *42*, 332–343.

[22] J. Rumpf, R. Burger, M. Schulze, *Int J Biol Macromol* **2023**, *233*, 123470.

[23] T. Kurechi, K. Kikugawa, T. Kato, *Chem Pharm Bull (Tokyo)* **1980**, *28*, 2089–2093.

[24] W. Brand-Williams, M. E. Cuvelier, C. Berset, *LWT - Food Science and Technology* **1995**, *28*, 25–30.

[25] M. C. Foti, *J Agric Food Chem* **2015**, *63*, 8765–8776.

[26] M. Olszowy-Tomczyk, *Chemical Papers* **2021**, *75*, 6157–6167.

[27] J. Tao, T. Morikawa, I. Toguchida, S. Ando, H. Matsuda, M. Yoshikawa, *Bioorg Med Chem* **2002**, *10*, 4005–4012.

[28] S. Domenek, A. Louaifi, A. Guinault, S. Baumberger, *J Polym Environ* **2013**, *21*, 692–701.

[29] T. Dizhbite, G. Telysheva, V. Jurkjane, U. Viesturs, *Bioresour Technol* **2004**, *95*, 309–317.

[30] X. Pan, J. F. Kadla, K. Ehara, N. Gilkes, J. N. Saddler, *J Agric Food Chem* **2006**, *54*, 5806–5813.

[31] L. An, G. Wang, H. Jia, C. Liu, W. Sui, C. Si, *Int J Biol Macromol* **2017**, *99*, 674–681.

[32] S. You, Y. Xie, X. Zhuang, H. Chen, Y. Qin, J. Cao, T. Lan, *Biochem Eng J* **2022**, *180*, 108335.

[33] Z. Guo, D. Li, T. You, X. Zhang, F. Xu, X. Zhang, Y. Yang, *ACS Sustain Chem Eng* **2020**, *8*, 12110–12119.

[34] J. Ponomarenko, M. Lauberts, T. Dizhbite, L. Lauberte, V. Jurkjane, G. Telysheva, *Holzforschung* **2015**, *69*, 795–805.

[35] L. de Sousa Nascimento, F. I. D. da Mata Vieira, V. Horácio, F. P. Marques, M. F. Rosa, S. A. Souza, R. M. de Freitas, D. E. A. Uchoa, S. E. Mazzeto, D. Lomonaco, F. Avelino, *Int J Biol Macromol* **2021**, *181*, 241–252.

[36] T. Zhang, H. Zhou, Y. Fu, Y. Zhao, Z. Yuan, Z. Shao, Z. Wang, M. Qin, *ChemSusChem* **2020**, *13*, 4478–4486.

[37] M. Matos, F. C. Claro, T. A. M. Lima, F. Avelino, F. A. Hansel, G. M. Maciel, D. Lomonaco, W. L. E. Magalhães, *J Anal Appl Pyrolysis* **2021**, *156*, 105175.

[38] K. Li, W. Zhong, P. Li, J. Ren, K. Jiang, W. Wu, *Int J Biol Macromol* **2023**, *251*, 125992.

[39] X. Lu, X. Gu, Y. Shi, *Int J Biol Macromol* **2022**, *210*, 716–741.

[40] D. Diment, J. Löfgren, M. Alopaeus, M. Stosiek, M. Cho, C. Xu, M. Hummel, D. Rigo, P. Rinke, M. Balakshin, **2024**, arXiv preprint DOI: 10.26434/CHEMRXIV-2024-F0R5L.

[41] G. F. Zakis, Functional analysis of lignins and their derivatives, TAPPI Press, **1994**.

[42] H. Sadeghifar, C. Cui, D. S. Argyropoulos, *Ind Eng Chem Res* **2012**, *51*, 16713–16720.

[43] P. Thirunavukkarasu, T. Ramanathan, L. Ramkumar, R. Shanmugapriya, G. Renugadevi, *Journal of Medicinal Plants Research* **2011**, *5*, 4754–4758.

[44] D. E. Clark, *Chem Health Saf* **2001**, *8*, 12–22.

[45] İ. Gulcin, *Archives of Toxicology 2020 94:3* **2020**, *94*, 651–715.

[46] A. M. Ribeiro, B. N. Estevinho, F. Rocha, *Food Hydrocoll* **2021**, *121*, 106998.

[47] H. H. Hamdo, H.-H. Housam, K. Warid, A.-A. Zaid, *Article in International Journal of Pharmacy and Pharmaceutical Sciences* **2014**.

[48] H.-Y. Zhang, H.-F. Ji, *New J. Chem* **2006**, *30*, 503–504.

[49] R. Kumar, D. Padmalaxmi, A.L. Udupa, N. Ojeh, V. Patil, P. Kodancha, D. Benegal, A. Benegal, S.R. Rao, S. Nisarga, H.V. Shubha, *Pharmacologyonline* **2011**, 833-841.