# Evaluation and Improvement of Antioxidant Activity of Water-soluble Lignin Products from Steam Explosion Processing of Corn Stalk

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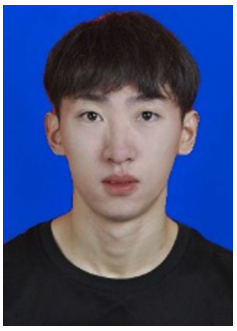

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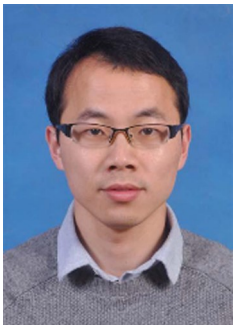

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**Abstract:** In this study, corn stalk was pretreated by steam explosion under various processing conditions, and the antioxidant activity of hydrolysates in the post-process washing liquor was analyzed using the 1, 1'-diphenyl-2-picrylhydrazyl (DPPH•) scavenging method. The yield and composition of the hydrolysates obtained under different treatment conditions were also determined; the results indicated that the steam explosion extent had a significant effect on both properties. Under optimized conditions (1.5 MPa, 20 min), the obtained hydrolysate had the highest phenolic compound yield (18.6 mg/g untreated corn stalk) and the highest radical scavenging capacity ($IC_{50}$=0.24 mg/mL). To confirm the positive effect of phenolic compounds on the antioxidant activity of the hydrolysate, one-step ethanol fractionation was carried out. Due to the enrichment of phenolic compounds, the ethanol-soluble fraction (ESF) exhibited significantly enhanced antioxidant activity with an $IC_{50}$ value of 0.06 mg/mL, which was close to that of butylated hydroxytoluene (BHT, $IC_{50}$ = 0.056 mg/mL). Consequently, this work indicated that phenolic compounds have a significant effect on the antioxidant activity of hydrolysate from steam-exploded corn stalk and that simple one-step ethanol fractionation is an effective way to enrich the phenolic compounds in the hydrolysate and improve the antioxidant activity.

**Keywords:** steam explosion; hydrolysate; antioxidant activity; phenolic compounds; ethanol fractionation
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## 1 Introduction

With the growing demand for energy and depletion of fossil fuels, the utilization of lignocellulosic biomass as an alternative feedstock for the production of fuels, materials, and chemicals is generating increased interest owing to the attractive properties of lignocellulosic biomass, including low cost, abundant availability, and low environmental impact [1−3]. Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin, which combine to form a complex matrix through covalent bonds, various intermolecular bridges, and van der Waals forces [1, 4]. Owing to its recalcitrant properties and the various constituents of each structural module in lignocellulose, pretreatment is a key step that can change or destroy the connections between molecules and improve the converted biomass into high value-added products [5−6]. Among the pretreatment approaches that have been developed, steam explosion is somewhat unique and remarkable, as it uses steam as a reaction and transfer medium without requiring additional chemicals [2, 7].

Steam explosion is considered an efficient pretreatment owing to its moderate energy consumption, small environmental impact, and low capital investment [8−9]. A steam explosion process typically involves exposing biomass to high pressure (0.6−4.0 MPa) saturated steam and then rapidly reducing the pressure, causing the material to undergo explosive decompression [10−11]. The autohydrolysis reactions, which are propagated by the organic acids generated from the acetyl groups of hemicelluloses, lead to the partial removal of hemicellulose and lignin. Moreover, the drop in pressure provides the advantage of effectively reducing the particle size, destroying the lignocellulosic substrate, making the biomass more fluffy, and thus significantly increasing the accessible surface area of cellulose [10, 12−13]. However, steam explosion pretreatment also leads to the formation of inhibitors from the partial degradation of hemicellulose-derived sugars and lignin. Inhibitors, such as furfural, phenolic compounds, and acetic acid, can significantly reduce the efficiency of enzymatic hydrolysis and glucose conversion during the fermentation process [5, 14−15]. Fortunately, the good water solubility of these inhibitors allows efficient removal from the pretreated substrate using a simple post-explosion washing process. After washing, a liquor fraction is obtained, which mainly contains the hydrolysates from the steam explosion processing, including hemicellulose sugars, their derivatives, and phenolic compounds. Due to the high content of hemicellulose sugars, the hydrolysates can be further converted into biofuels or biochemicals by fermentation using xylose as the main carbon source. However, as the inhibitors are also dissolved in the post-washing liquor, a detoxification process to remove the non-sugar hydrolysate components is necessary for efficient bioconversion [14, 16].

Antioxidants are substances that inhibit or prevent oxidation reactions by trapping and neutralizing free radicals and have been widely used in various fields, such as cosmetics, pharmaceuticals, food, and polymer processing [17]. Compared with synthetic antioxidants, there is increasing interest in antioxidants from natural sources owing to their high security and biocompatibility, strong antioxidant capacity, and sustainability [18]. In particular, antioxidants from low-cost and abundant lignocellulosic biomass sources have attracted considerable attention as a consequence of their economic efficiency and prevalence in feedstock [19]. Water-soluble hydrolysates from the hydrolysis of lignocellulosic materials contain hemicellulose-degraded oligosaccharides and phenolic compounds, which exhibit promising antioxidant properties. Cruz et al [20] studied the antioxidant activity of ethyl acetate-soluble hydrolysates obtained from the acid hydrolysis of several lignocellulosic materials using the 1, 1'-diphenyl-2-picrylhydrazyl (DPPH•) scavenging assay. They found that all hydrolysates showed antioxidant activity and that the hydrolysate from Eucalyptus wood possessed the strongest antioxidant property, which was comparable to the aqueous extracts from oolong and green teas. Conde

et al [21] proposed a route to produce antioxidants using hydrolysates obtained from the autohydrolysis of lignocellulosic wastes. Although previous studies have demonstrated that lignocellulosic biomass hydrolysates exhibit satisfactory antioxidant activity, there are still few efforts dedicated to exploring the antioxidant properties of hydrolysates in post-process washing liquor following steam explosion treatment.

In this study, the antioxidant activity of hydrolysates from post-process washing liquor of steam-exploded corn stalk was evaluated using the DPPH• scavenging method. The total yield of hydrolysate was measured gravimetrically, and the components (e. g., phenolic compounds and sugars) were determined using high performance liquid chromatography (HPLC) measurement. In order to confirm the components in the hydrolysate that predominantly contributed to the antioxidant activity, a simple one-step fractionation of the obtained hydrolysate was conducted by dissolving it in ethanol. Then, the hydrolysate was subdivided into two fractions (an ethanol-soluble fraction (ESF) rich in phenolic compounds and an ethanol-insoluble fraction (EIF) rich in sugar), and their antioxidant activities were further compared. The purpose of the current work was not only to evaluate the antioxidant activities of corn stalk hydrolysates under different steam explosion conditions, but also to identify the relationship between the antioxidant capacity and the components of the hydrolysate (e. g., sugars and phenolic compounds).

## 2 Experimental

### 2.1 Materials

Corn stalks obtained from Gongyi, He'nan province, China, were air-dried at room temperature before further processing. The chemical composition of raw corn stalk was as follows: lignin, (24.3 ± 0.9)% ; glucan, (33.9 ± 1.0)% ; xylan, (18.1 ± 0.1)% ; and moisture content, (10.9 ± 0.3)% . The monosaccharide model substances (glucose, xylose, and arabinose), DPPH• , and ethanol were purchased from Sigma-Aldrich, China. Phenolic compound model substances (*p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, vanillin, vanillic acid, syringaldehyde, syringic acid, ferulic acid, and *p*-coumaric acid) were obtained from Aladdin Industrial Corporation, China. All chemicals and solvents used in this study were analytical grade.

### 2.2 Steam explosion

Steam explosion treatment was conducted in a batch apparatus (QBS-200B, Zhengdao Bioenergy, He'nan province, China), which included a high-pressure reactor (5 L), a steam generator, and a receiver. Air-dried corn stalk (moisture content 8%) was cut into pieces of 3−4 cm, and then the moisture content was adjusted to 12% using distilled water. Corn stalk (approximately 200 g) was fed into the reactor and treated under six different conditions (Fig. 1). After reaching the holding time, the valve at the bottom of the reactor was opened rapidly to release the pressure, and the steam-exploded corn stalk was recovered in the receiver. The steam explosion severity index was calculated using the following formula [22].

$$S_o = t\exp\left(\frac{T - 100}{14.75}\right) \quad (1)$$

where $S_o$ is the steam explosion severity index, $t$ is the reaction time (min), and $T$ is the reaction temperature (℃).

The treated corn stalk was then washed with distilled water (1 L/200 g corn stalk) to dissolve the water-soluble hydrolysates. The mixture was filtered to separate it into a solid residue and the washing liquid [23]. The post-treatment washing process was repeated three times, and all washing liquids were mixed together as the post-washing liquor (PWL).

### 2.3 Component determination of hydrolysates in PWL

The concentrations of monosaccharides, oligosaccharides, acetic acid, and furfural in the PWL were analyzed using HPLC (Agilent 1200 HPLC, Agilent Technologies, US) equipped with an Aminex®HPX-87H ion exclusion column according to the National Renewable Energy Laboratory (NREL) analytical standard [24]. The acid-soluble lignin (ASL)

was measured using ultraviolet-visible (UV-vis) spectroscopy (UV-2550, Shimadzu, Japan) [24]. Phenolic compounds (including *p*-hydroxybenzoic acid, vanillic acid, syringic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde) were quantified by HPLC using a ZORBAX SB-C18 column (250 × 4.6 mm, Agilent Technologies, US) and an ultraviolet detector. The detection wavelengths of different phenolic compounds were chosen as follows: *p*-hydroxybenzoic acid and syringaldehyde were detected at 250 nm; syringic acid, vanillin, vanillic acid, and *p*-hydroxybenzaldehyde were detected at 280 nm; ferulic acid and *p*-coumaric acid were detected at 320 nm [25]. The mobile phase consisted of 60% acetic acid solution (1%) with 40% methanol at a flow rate of 0.6 mL/min. The temperature of the column was 30℃, and the temperature of the detector was 35℃.

### 2.4 Ethanol fractionation of hydrolysate in PWL

The solid hydrolysate in the PWL was separated by rotary evaporation and then subdivided into the ESF and EIF by ultrasound-assisted dissolution in ethanol, with a solid to liquid ratio of 1 ∶ 20 (*w*/*V*) at room temperature for 1 h. After fractionation, the ethanol-soluble hydrolysate was recovered by rotary evaporation. Both hydrolysate fractions (ESF and EIF) were stored at room temperature for further analysis.

### 2.5 DPPH• scavenging assay

The antioxidant activity of the hydrolysate was evaluated using the DPPH• scavenging assay, based on a previously described method with minor modifications [26]. The PWL with six different volumes ranging from 0.05 mL to 0.3 mL was diluted with methanol to 3 mL. The obtained solution was then mixed with 3 mL of 0.15 mmol/L DPPH-methanol solution at room temperature and kept away from light for 30 min. The absorbance of the mixture was determined through UV-vis spectrophotometry at 517 nm. For the antioxidant activity analysis of the ethanol-soluble hydrolysate and ethanol-insoluble hydrolysate, the solid samples were first dissolved in water and then diluted using methanol to test the DPPH• scavenging activity (RSA) value. Each test was performed in triplicate. The formula for RSA value of the samples was as follows [26].

$$\mathrm{RSA}(\%) = \left(1 - \frac{(A_1 - A_0)}{A_2}\right) \times 100\% \quad (2)$$

where $A_1$ is the absorbance of the DPPH-methanol solution with the sample, $A_2$ is the absorbance of the DPPH-methanol solution without sample, and $A_0$ is the absorbance of methanol with the sample.

## 3 Results and discussion

### 3.1 Yield and composition of hydrolysates

In the present study, the raw material (corn stalk) was pretreated by steam explosion processing under pressure ranging from 1.2 to 1.8 MPa with retention time varying from 5 to 30 min based on previous research [27]. Fig. 1(a) presents the corresponding yields of hydrolysates and residues in the PWL under the different steam explosion severities. The hydrolysate yields ranged between 12.02% and 20.82% (based on the raw material), which was in good agreement with a earlier publication [28]. This hydrolysate yield was higher than that reported for other hydrothermal pretreatment methods [8], such as hot-water prehydrolysis [29]. This was mainly attributed to the explosive effect during steam explosion processing, which enhanced the porosity and surface area of the pretreated biomass and facilitated the extraction of hydrolysates [8]. It can also be seen from Fig. 1(a) that the hydrolysate yield increased with the increase in steam explosion severity, which was attributed to the intensified hydrolysis of hemicellulose [30].

Fig. 1(b) shows the composition (expressed in mg/g untreated corn stalk) of hydrolysates from corn stalk pretreated by steam explosion at different severities. The hydrolysate consisted of a mixture of water-soluble oligosaccharides (xylan and glycan), monosaccharides (glucose, xylose, and arabinose), sugar degradation products (acetic acid and furfural), and phenolic compounds. The results showed that the steam explosion severity had a significant effect on the composition of the hydrolysates. The content of total

monosaccharides in the hydrolysates increased from 62.96 to 132.78 mg/g untreated corn stalk with the increase in steam explosion severity from 3.29 (1.2 MPa, 5 min) to 4.20 (1.5 MPa, 20 min); this yield increase is ascribed to the intensified hydrolysis of hemicellulose during processing [31]. However, upon further increasing the processing time to 30 min, the total monosaccharides decreased slightly to 130.1 mg/g untreated corn stalk; this decrease is mainly attributed to the further dehydration of xylose into furfural (Fig. 1 (b)). The increase in steam explosion severity from 3.29 to 3.90 led to the decline of water-soluble oligosaccharide content. Moreover, water-soluble oligosaccharides were not detected when the steam explosion severity was further increased to 4.20 and 4.37 (1.5 MPa, 30 min). These results were presumably caused by further hydrolysis of the oligosaccharides into monosaccharides, including furfural and acetic acid, under higher severity steam explosion processing [31–32]. In addition, a small amount of ASL was also detected in the hydrolysate; the steam explosion severity had an insignificant effect on its content (Fig. 1 (b)).

As shown in Fig. 1(c), the phenolic compound content in the hydrolysates ranged from 6.8 to 18.6 mg/g untreated corn stalk, which was consistent with results previously published [25]. The highest yield of phenolic compounds was obtained under the steam explosion severity of 4.20 (1.5 MPa, 20 min); it decreased sharply to 11.2 mg/g untreated corn stalk when the steam retention time was prolonged to 30 min. The reason for the decrease in the content of phenolic compounds may be that, with the extension of the steam treatment time, the phenolic compounds and furfural underwent polycondensation reactions to form water-insoluble products [33].

Eight phenolic monomers were found in the hydrolysate, including two phenolic acids (*p*-coumaric acid and ferulic acid), three lignin monomeric aldehydes (syringaldehyde, *p*-hydroxybenzaldehyde, and vanillin), and three lignin monomeric acids (*p*-hydroxybenzoic acid, vanillic acid, and syringic acid).

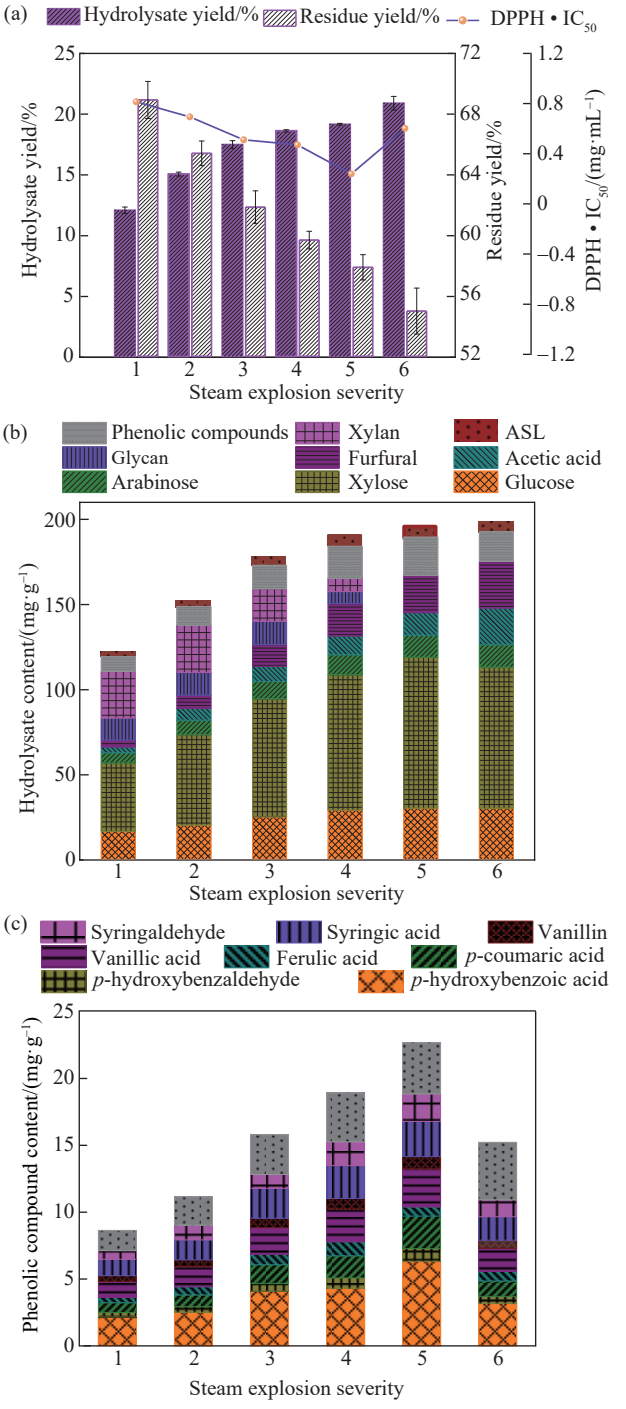


Fig. 1 (a) Hydrolysate yield and residue of corn stalk under different steam explosion severities, as well as the $IC_{50}$ values of hydrolysates; (b) effect of steam explosion severity on composition of hydrolysate in PWL (mg/g untreated corn stalk); (c) effect of steam explosion severity on composition of phenolic compounds in hydrolysates (mg/g untreated corn stalk). The numbers from 1 to 6 in the *x*-axis represent steam explosion at 1.2 MPa for 5 min, 1.5 MPa for 5 min, 1.8 MPa for 5 min, 1.5 MPa for 10 min, 1.5 MPa for 20 min, and 1.5 MPa for 30 min, respectively.

The phenolic acids were previously reported to be present in herbaceous plants, linking lignin and hemicelluloses through aromatic ether and ester bonds [34–35]. The three types of lignin monomers produced by the depolymerization of lignin confirmed the GHS-type (guaiacyl unit (G), syringyl unit (S), *p*-hydroxyphenyl unit (H)) of lignin in the corn stalk. It can also be observed from Fig. 1(c) that the proportion of lignin monomeric acids is relatively higher than that of the two phenolic acids and the corresponding monomeric aldehydes, which is consistent with the results reported previously [25].

### 3.2 Antioxidant and RSA

A frequently used DPPH• scavenging assay was applied to evaluate the antioxidant capacity of the hydrolysates of corn stalk produced by steam explosion pretreatment under different pretreatment severities. The RSA values of the hydrolysates under six different conditions are shown in Fig. 2(a), and their corresponding $IC_{50}$ values are presented in Fig. 1(a). All six hydrolysates exhibited DPPH• scavenging capacities in a dose-dependent manner (Fig. 2(a)). The RSA values of the hydrolysates increased from an $IC_{50}$ of 0.81 mg/mL to an $IC_{50}$ of 0.24 mg/mL with the enhancement of steam explosion severity from 3.29 (1.2 MPa, 5 min) to 4.20 (1.5 MPa, 20 min). However, an obvious decline of hydrolysate RSA value was observed when the severity of steam explosion was further increased to 4.37 (1.5 MPa, 30 min). Therefore, the hydrolysate in PWL of corn stalk after steam explosion treatment at 1.5 MPa and 20 min showed the highest RSA value with an $IC_{50}$ of 0.24 mg/mL. Conde et al [21] analyzed the RSA value of hydrolysates from autohydrolysis of four different lignocellulosic wastes and found that their $IC_{50}$ values ranged from 0.30 to 0.73 mg/mL. Similar findings ($IC_{50}$ of 0.23−0.39 mg/mL) have been reported for the ethyl acetate-soluble hydrolysates from acid hydrolysis of Eucalyptus wood [36].

In previous studies, it was suggested that the two main substances in the hydrolysates of lignocellulosic biomass that exhibit antioxidant activities are oligosaccharides and phenolic compounds [21, 36–38]. In this study, the hydrolysate obtained under the lowest steam explosion severity (1.2 MPa, 5 min) had the highest oligosaccharide content; however, it showed the lowest antioxidant activity. In addition, with decreasing oligosaccharide content in the hydrolysates, the opposite trend in RSA was observed. Though there were no oligosaccharides in the hydrolysate obtained from steam explosion treatment at 1.5 MPa and 20 min, the hydrolysate obtained from which exhibited the highest RSA among the six samples. Therefore, it was concluded that the oligosaccharides were not the substances that predominantly contribute to the antioxidant activity of the hydrolysates, which means that the phenolic compounds may be the main reason for the high antioxidant activity of the hydrolysates.

Interestingly, the profile of total phenolic compound content was consistent with the change in antioxidant

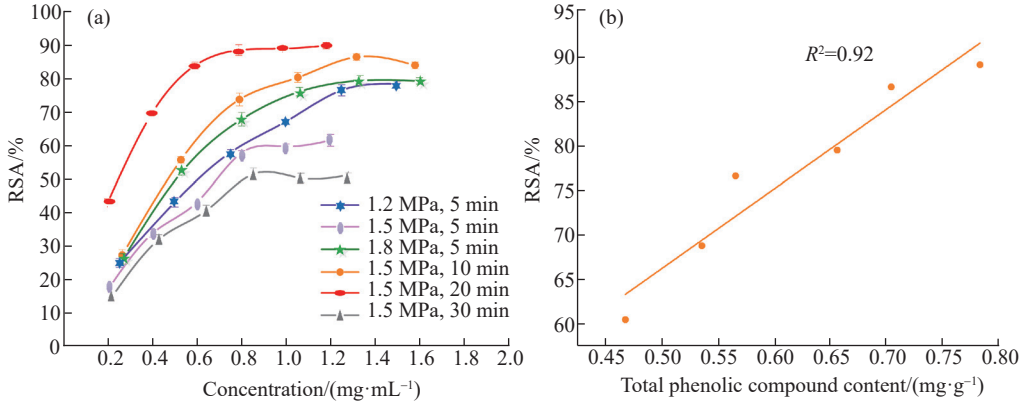


Fig. 2 (a) Effect of steam explosion severity on RSA values of hydrolysates in PWL of steam exploded corn stalk; (b) a linear fitting between the total phenolic compound content and the corresponding RSA values of hydrolysates

activity of the hydrolysates. As shown in Fig. 2(b), a visibly linear relationship ($R^2$=0.92) between the total phenolic compound content and RSA was observed. The results indicate that the antioxidant capacity of hydrolysates obtained from steam-exploded corn stalks is highly dependent on their phenolic compound content [39]. Previous studies have suggested that phenolic compounds are promising antioxidants, and their antioxidant activity is generally determined by the amount of phenolic hydrogen available for the hydrogen donation reaction and the stabilization of the resulting phenoxyl radicals formed by hydrogen donation [39−40].

It can also be observed from Fig. 3 that, among the six lignin-derived monomers, the H-unit monomers (*p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid) showed insignificant antioxidant activities when compared with the G-unit monomers (vanillic acid and vanillin) and the S-unit monomers (syringic acid and syringaldehyde), which confirmed that the methoxylation of the aromatic ring may have a positive effect on antioxidant activity [41−42]. Moreover, syringic acid (methoxylated at C3 and C5) and ferulic acid (methoxylated at C3) displayed superior antioxidant capacities with RSA values of 96.40% and 95.21% at 0.3 mg/mL, respectively, whereas the lowest RSA value was found for the methoxyl-free *p*-coumaric acid with an RSA value of 15.02% at the same concentration. The results suggest that an increase in methoxylation is responsible for the improved antioxidant activities of lignin monomeric acids, which is consistent with the DPPH• scavenging activities mechanism proposed in previous studies [26, 41]. It was also notable that, for all four kinds of phenolic compounds (H-unit, G-unit, S-unit monomers, and phenolic acids), the acids exhibited visibly higher antioxidant activities than the corresponding aldehydes. This is possibly attributed to the position of the carbonyl groups in the side chains, because the electron-acceptor properties of carbonyl groups have a prominent positive influence on the antioxidant activity of phenolic compounds in the DPPH• test [41]. However, compared with phenolic compounds, the sugars (glucose, xylose, and arabinose) hardly exhibited DPPH• scavenging activities, as their RSA values were all less than 1.5% at the tested concentrations. Therefore, these results confirm that phenolic compounds are the main substances responsible for the antioxidant activity of hydrolysates, and the hydrolysates obtained from steam-exploded corn stalk can be applied as an effective source of antioxidant.

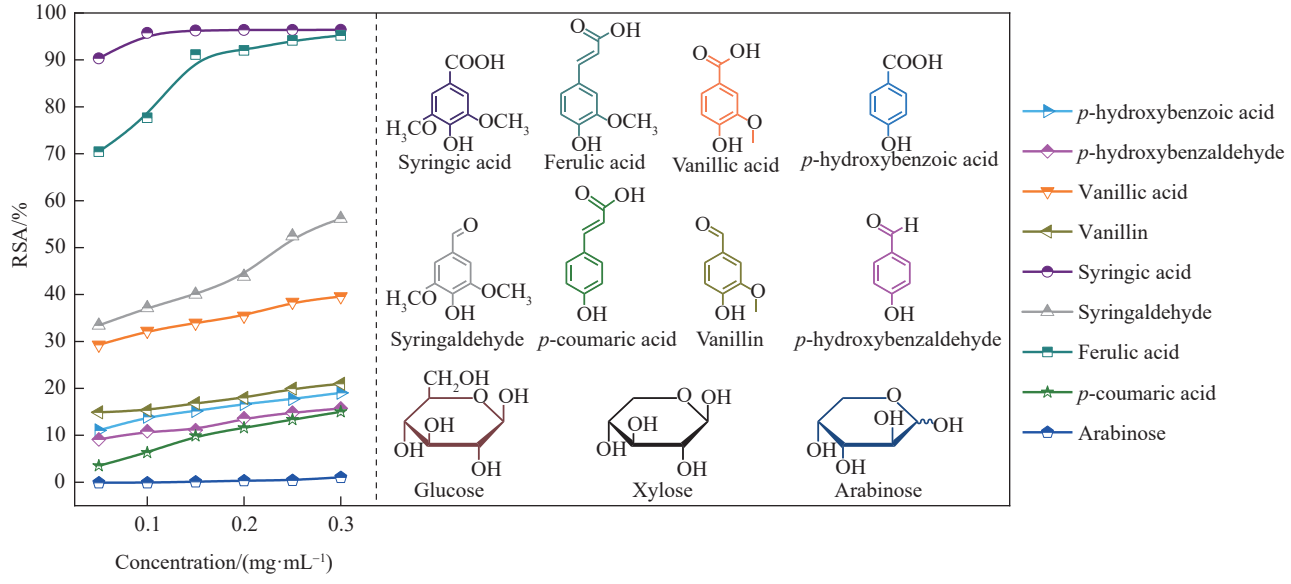


Fig. 3 RSA values of main substances in hydrolysates at different concentrations, including monosaccharides and phenolic compound model substances

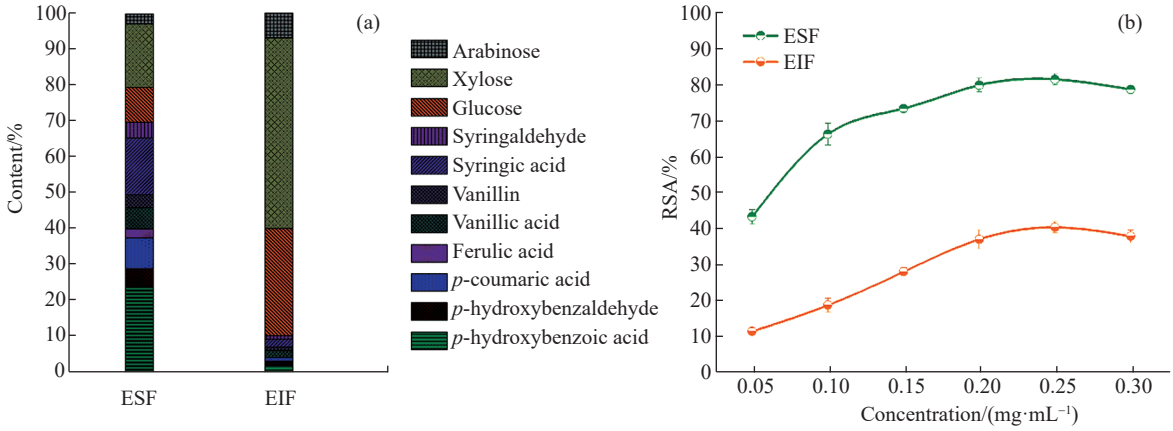


Fig. 4 (a) Contents of phenolic compounds and sugars in ESF and EIF; (b) RSA values of the ESF and EIF

### 3.3 Ethanol fractionation of hydrolysate and antioxidant activities of fractions

To further confirm the positive effect of phenolic compounds on the antioxidant activity of hydrolysate, a simple one-step fractionation method using ethanol was applied to subdivide the hydrolysate (obtained at 1.5 MPa and 20 min) into two fractions: the ESF (10.8 wt% of total hydrolysate) and the EIF. As shown in Fig. 4(a), the content of phenolic compounds in the ESF was 71 wt%, whereas the corresponding value for the EIF was only 12 wt%. However, the sugar content in the EIF was approximately three times higher than that in the ESF. The results showed that the signals originating from sugars were dominant in the spectra of EIF, whereas ESF exhibited visibly higher intensity of signals corresponding to the phenolic compounds. These results were consistent with the composition analyses of EIF and ESF.

The RSA values of the two fractions are shown in Fig. 4(b). The ESF had an $IC_{50}$ value of 0.06 mg/mL, which was considerably lower than that of the unfractionated hydrolysate. It is important to note that the $IC_{50}$ value of the EIF was close to that of butylated hydroxytoluene (BHT) ($IC_{50}$ value of 0.056 mg/mL) [26, 43], indicating its potential application as a commercial antioxidant. However, the EIF exhibited much inferior antioxidant activity compared with the ESF, as the percentage of scavenged free radicals was only 40% at a concentration of 0.30 mg/mL. These results verified that the phenolic compound content in the hydrolysate was critical to its antioxidant activity. Consequently, simple one-step fractionation by ethanol dissolution is an efficient way to enhance the antioxidant activity of the hydrolysate.

## 4 Conclusions

Corn stalk was subjected to steam explosion pretreatment under various operating conditions, and the hydrolysates obtained from the post-washing liquor were used for analysis of the 1, 1' -diphenyl-2-picrylhydrazyl (DPPH•) scavenging activity. The free radical scavenging activity of the hydrolysate was highly dependent on the phenolic compound content rather than the oligosaccharide content. The steam explosion severity had a remarkable effect on the phenolic compound content in the hydrolysate and, accordingly, its free radical scavenging activity. Among the six steam explosion severities tested in this work, the hydrolysate obtained from 1.5 MPa and 20 min presented the highest phenolic compound content and the highest radical scavenging activity. A simple one-step fractionation by dissolving the hydrolysate in ethanol resulted in the subdivision of the hydrolysate into two fractions: an ethanol-soluble fraction rich in phenolic compounds and an ethanol-insoluble fraction rich in sugars. The ethanol-soluble fraction exhibited significantly enhanced DPPH• scavenging capacity compared to the original hydrolysate owing to the high content of phenolic compounds. In conclusion, the study demonstrated that the phenolic compound

content in hydrolysate is a key factor in its antioxidant activity, and the one-step ethanol fractionation can prominently enhance the antioxidant activity of hydrolysate through the enrichment of phenolic compounds.

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