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Monodisperse lignin fractions as standards in size-exclusion analysis Comparison with polystyrene standards

Vagner Roberto Botaroa,*, Antonio Aprígio da Silva Curvelob

a *Universidade Federal de São Carlos – Campus de Sorocaba, Rod. João Leme dos Santos, km 110, CEP 18052-780, CP 3031- Sorocaba, SP, Brazil*
b *Universidade de São Paulo – Instituto de Química de São Carlos, Av. Trab. São-carlense 400, CP 780 - CEP 13560-970, São Carlos, SP, Brazil*

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a b s t r a c t
The difficulty of preparing monodisperse lignin fractions on a large scale is a limiting factor in many applications. The present paper addresses this problem by examining the properties and size-exclusion behavior of lignin isolated by the acetosolv pulping process from post-extraction crushed sugarcane bagasse. The isolated lignin was subjected to a solvent pretreatment, followed by preparative gel permeation chromatography fractionation. The fractionswere analyzed by high-performance size-exclusion chromatography (HPSEC) and these samples showed a great decrease in polydispersity, compared to the original acetosolv lignin. Several fractions of very low polydispersity, close to unity, were employed as calibration curve standards in HPSEC analysis. This original analytical approach allowed calibration with these lignin fractions to be compared with the polystyrene standards that are universally employed for lignin molecular mass determination. This led to a noteworthy result, namely that the lignin fractions and polystyrene standards showed very similar behavior over a large range of molecular masses in a typical HPSEC analysis of acetosolv lignin.

1. Introduction

In a generalway, macromolecules and polymers are highly variable in their average molecular masses, distribution of molecular masses and consequently their polydispersities [1]. This heterogeneity is a result of the varied reactions that can occur during

the polymer synthesis. On the other hand, the properties of macromolecules and polymers are strongly affected by their mean molecular masses and polydispersities. In this context, preparativescale fractionation methods are important in that they enable more homogeneous fractions or even fractions of specific molecular mass to be obtained. Suchfractions are useful for the study of the relationships between molecular masses and structures of macromolecules and their properties [2].

An example of a very complex polymer is lignin isolated by various different pulping processes [3–6]. Lignin is a natural polymer occurring in plant cellwalls and represents, after cellulose, the second most abundant polymer in nature. The basic units are linked by at least 10 different linkages and form branchedchains of interpenetrating network in the cellwall. Owing to its verycomplex structure, lignin is an amorphous polymer with rather limited industrial use [7].

* Corresponding author. Tel.: +55 15 3202 2438; fax: +55 15 3202 2438.
E-mail address: vagner@ufscar.br (V.R. Botaro).

The extraction of the lignin from plant tissues necessarily involves the rupture of covalent bonds in the three-dimensional protolignin network, with a concomitant fall in molecular mass [8,9]. The molecular mass of lignin fragments depends not only on the isolation process but also on the raw material. Generally speaking, these fragments are quite heterogeneous in terms of size and consequently exhibit high polydispersities, which varywith the pulping process [3–7].

The chemical structure, relative molecular mass and relative molecular mass distribution of lignin depend on the age and also the type of plant (hardwood, softwood, grasses, etc.). As for synthetic polymers, the properties of lignin are closely related to its molecular mass [11]. Knowledge of this and other parameters can lead to a complete determination of the structural features of lignin [12,13]. A simple example is the relationship between the presence of condensed structures and the content of phenolic hydroxyl groups as a function of the molecular mass of lignin [14].

There are several methods of lignin fractionation that can be used to obtain fractions of reduced polydispersity and thus greater homogeneity [2]. Preparative gel permeation chromatography (GPC) is the technique used most commonly for this purpose. However, the fractions obtained by preparative GPC alone are not always sufficiently homogeneous, by virtue of the degree of heterogeneity of the original lignin. A sequence of solvent fractionation followed by GPC is described in this paper. This two-step fractionation process was employed to obtain lignin fractions with

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polydispersities close to unity. The paper focuses first on acetosolvextracted sugarcane bagasse lignin. In the hope of improving our understanding of the fragmentation in the acetosolv pulping

process, the molecular mass of fractions was determined after solvent fractionation combined with preparative GPC. The use of polystyrene (PS) standards for the determination of molecular mass of branched macromolecules such as lignin has always been an object of criticism in the literature. For this reason, a second objective of our work was to construct two different calibration curves, employing the monodisperse lignin fractions and PS standards, in order to examine this question. An important result was established, that lignin fractions and polystyrene standards exhibit very similar behavior over a large range ofmolecular masses in analytical high-performance size-exclusion chromatography (HPSEC).

2. Experimental

2.1. Acetosolv process

Approximately 100 g of dry fibers from crushed sugarcane was added to acetosolv pulping mixture in a glass reactor of 2.0 L capacity. The pulping mixture was composed of 1200 g glacial acetic acid, 5 g concentrated hydrochloric acid and 90 g water. The reactor was immersed in a thermostatic bath (115 ◦C) for a period of 3 h. The residual pulp was filtered and washed with a solution of acetic acid (93.0%, w/w). The filtrate (black liquor) was evaporated under reduced pressure, downto a volume of 75mL, and then added drop by drop to 750mL water. The precipitated lignin was filtered, washed with water until the washings reached a neutral pH and then dried in a desiccator.

2.2. GPC

Initially, about 9 g acetosolv lignin was dissolved in 20.0mL of a 9:1 (v/v) dioxane:water solution. The resulting solutionwas loaded on a Pharmacia preparative column (Bromma, Sweden): XK 50/100 with 80cm long×5.0cm I.D., packed with about 500 g Sephadex of dry LH-20 gel and eluted with the same 90% dioxane solvent, flowing at 1.0mL/min. During the experiment, 19 fractions of 60mL were collected with an automatic collector. The temperature of the column was maintained at 20 ◦C by an external water jacket. The fractions containing lignin were evaporated to dryness under reduced pressure at 40 ◦C and subsequently stored in a desiccator.

2.3. Preliminary treatment with methanol and fractionation by preparative GPC

About 1 0 g acetosolv lignin was mixed with 100mL methanol and shaken vigorously for 1 h at 25 ◦C to dissolve the lignin. The undissolved solid was filtered off and extracted again in the same conditions. The methanol solutions were evaporated to dryness under reduced pressure at 40 ◦C, in order to obtain the soluble lignin fraction.

The preparative systems and conditions described above were used in the fractionation of methanol-soluble lignin. In this case, 4.5 g lignin was dissolved in 15.0mL dioxane:water solution and the solution injected on to the preparative column. Eight fractions

were collected at this stage, with volumes varying from 75mL to 180mL. The solvent mixture was evaporated from each fraction under reduced pressure and the fractions dried in a desiccator.

2.4. Vapor pressure osmometry (VPO)

VPO is based on colligative properties of solutions and is therefore a useful technique to determine the absolute number-average molecular mass (¯Mn) of the lignin fractions. The system was calibrated with dibenzoyl (¯Mn 210.23) as standard. The solvent used was analytical-reagent grade tetrahydrofuran (THF) at 45 ◦C.

2.5. FTIR spectroscopy

The spectra were measured from pellets of KBr carrying 1% (w/w) sample.

2.6. HPSEC

The HPSEC experimental conditionswere: flow-rate 1.0 mL/min, solvent THF, sample concentration of 1.25mg/mL of THF, volume injected variable from10_L to20_L, UV–vis at 254 nmwavelength, and Pl-Gel columns in series: 500A; 103 A; 104 A. Calibration curves were constructed with polystyrene standards with the molecular mass given in Table 3. The elution profiles of polystyrene standards

Fig. 1. HPSEC profiles of PS standards: peaks 1–14 correspond to ps1 to ps 14, respectively (see Table 4). Peaks 15, 16 and 17 correspond to benzyl, ethylbenzene and toluene respectively.

Fig. 2. HPSEC profiles of lignin standards. Peaks 1–7 correspond to lignin fractions 1–7 m, respectively, a = acetosyringone and v = vanillin.

and very homogenous lignin fractions are shown in Figs. 1 and 2, respectively

3. Results and discussions

The structural heterogeneity of the lignin macromolecule has been described by many authors as one reason for its limited industrial use [1,2,10,15–18]. Others are its complex structure and molecular mass distribution, which have also hindered the characterization and identification of the polymer properties of the specific lignins isolated by various processes.

The great heterogeneity of lignin is related to variability in the biosynthetic process among the plant species used and to its location in various plant tissues. Other factors, especially the method of isolation, contribute to its structural heterogeneity. The isolation process can produce fragments in several ways and sizes, starting from the degradation of the three-dimensional network of protolignin [14].

Table 1

Number-average molecular mass (¯Mn) and weight-average molecular mass (¯Mw) of 19 acetosolv lignin fractions obtained by preparative GPC.

Fraction	¯Mw (HPSEC)	¯Mn (HPSEC)	¯Mw/ ¯Mn	Mass (%)
Acetosolv 14	320	1250	11.4	100
1d	50 000	1740	28.7	5.2
2d	28 970	1360	21,3	11.2

3d 26 300 1200 21.8 9.1
4d 19 900 1370 14.5 15.1
5d 8 000 1160 6.9 17.7
6d 2 170 860 14.0 11.4
7d 3 900 720 5.4 9.0
8d 920 490 1.9 7.8
9d 3 180 720 4.4 4.5
10d 740 460 1.6 3.3
11d 950 470 2.0 2.6
12d 810 410 2.0 0.7
13d 630 290 2.2 0.7
14d 570 330 1.7 0.5
15d 1 580 310 5.1 0.2
16d 700 330 2.1 0.2
17d 14 260 270 51.9 0.1
18d 1 190 370 3.2 0.1
19d 3 830 340 11.0 0.8

The fractionation of the acetosolv lignin from crushed sugarcane in a preparative GPC column supplied 19 fractions, which were characterized by HPSEC. As shown in Table 1, the fractions of lower-molecular-mass (8d, 10d, 11d, 12d, 13d, 14d, and 16d) had low polydispersities [average weight–average molecular mass (¯Mw/ ¯Mn)], from 1.6 to 2.2.

The molecular mass and polydispersities show a discontinuous and not homogeneous separation profile. This fact is related to the limitation of the Sephadex LH-20 column in the fractionation of very heterogeneous acetosolv lignin. The fractions of lignin beyond the upper exclusion limit of the column were eluted at the same retention volume and thus have high polydispersities. The exclusion limit of Sephadex LH-20 can vary in accordance with the solvent employed, but usually it is between 4000 and 6000. This limit is adequate for acetosolv lignin, which has more than 60% (w/w) of fragments with molecular mass below 5000.

If another stationary phase, such as Sephadex LH-60, was used, the efficiency of separation of the higher-molecular-mass fractions, above 6 kDa, could be improved. The fractionation profile is shown in Fig. 3 and exhibits a characteristic bimodal behavior, as described elsewhere for lignin [2,14].

In order to eliminate the higher-molecular-mass fractions, the acetosolv lignin was subjected to a preliminary treatment with

Fig. 3. Lignin elution profile produced by preparative GPC.

Table 2

¯M
w and (¯Mn) values of fractions obtained by preparative fractionation of methanolsoluble
lignin.

Fraction ¯Mw
(HPSEC)
¯M

n
(HPSEC)
(¯Mw/ ¯Mn) ¯Mn (VPO)
THF
Mass
(%)
Acetosolv 14 320 1250 11.4 – 100
Methanol (insoluble) 5120 1450 3.5 – 45.1
Methanol (soluble) 2 020 1000 2.02 – 54.9
1m 5320 2770 1.92 2270 11.9
2m 3010 2340 1.29 2490 22.3
3m 1860 1600 1.16 2040 14.4
4m 1500 1180 1.27 1160 17.4
5m 910 780 1.17 770 10.4
6m 650 530 1.23 630 11.4
7m 460 380 1.21 350 8.3
8m 440 340 1.29 – 3.6

methanol. The resulting fragmentation promoted a significant decrease in the polydispersity of the fractions, compared to the acetosolv lignin. Even the methanol-insoluble fraction exhibited a mean molecular mass (determined by HPSEC) much lower than the original value, due to its low solubility in THF.

The data in Table 2 show that the extraction with methanol, followed by preparative GPC fractionation of the solution, resulted in samples of extremely lowpolydispersity. Seven of the eight fractions showed values of ¯Mw/ ¯Mn from1.16 to 1.29. Both the molecular mass and the polydispersities show continuous and homogeneous profiles with respect to the separation time, owing to the capacity of the column to permeate selectively the methanol-soluble lignin, which is free of high-molecular-mass fragments. The seven fractions of lowest ¯Mn (by VPO) were employed as lignin standards in the calibration curve in a typical HPSEC analysis, in a comparative study with the polystyrene standards.

VPO is a directmethod to determine thenumber-averagemolecular mass of a sample directly. A comparison of the data obtained by VPO and HPSEC shows a great number of coincident values, confirming the reliability of the HPSEC technique for molecular mass determination.

Elemental analysis data for the eight fractions obtained from methanol-soluble lignin are presented in Table 3. These data can be divided into two different groups. The higher-molecular-mass fractions (fraction 1m to fraction 4m) had a slightly higher carbon content and lower oxygen content than the fractions with lowermolecular-mass. These differences are due to the incorporation of oxygen atoms during the acetosolv pulping process, as a result of solvolytic or hydrolytic rupture of polymer linkages, promoted respectively by acetic acid or water.

The eight fractionswere also analyzed by infrared spectroscopy. The spectra of fractions 1m and 6m showed the same absorption bands, as can be seen in Fig. 4. On the other hand, for fractions 7m

and especially 8m, it is possible to observe significant differences in the spectra. The main differences were observed in the region of stretching bands for C O and O–H, C–H out of the plane and C–O at 1377 cm−1 and 1117 cm−1. The O–H absorption band was moved to lowerwave numbers and broadened, for both fractions 7m and 8m.

Table 3
Elemental analysis of the eight fractions obtained from methanol-soluble lignin.

Fraction	Carbon (%)	Hydrogen (%)	Oxygen (%)
1m	61.70	5.60	32.70
2m	61.37	5.47	33.16
3m	61.88	5.53	32.60
4m	61.78	5.79	32.43
5m	59.60	5.76	34.64
6m	60.51	6.15	33.34
7m	59.75	6.08	34.17
8m	59.86	5.34	34.80

Fig. 4. FTIR spectra of lignin fractions 1m, 6m, 7m and 8m.

In the same way, the carbonyl group stretching bands were moved to lower wave numbers and, particularly in fraction 8m, another small band is visible at 1630cm−1. These differences, including the peak at 1630cm−1, can be attributed to the presence of *p*-coumaric acid, which is typically found in sugarcanewaste and is also present in large amounts in the low-molecular-mass fractions.

The eight samples of low polydispersity (1–8m) were used as standards for the calibration of HPSEC columns in a comparative study with polystyrenes. These commercial polystyrene standards have been used in determinations of average values and distributions of molecular mass in several lignins. The standard lignin fractions were also used to extend the calibration curve to lowermolecular-mass. The respective retention times of the polystyrene and lignin standards are presented in Table 4. The molecular mass values for the lignin fractions presented in Table 4 refer to $\bar{}$Mn, obtained by VPO in THF, as described previously.

The calibration curves obtained are shown in Fig. 5, where good agreement can be observed between the points plotted with polystyrene and lignin standards. It should also be noted that the experimental points obtained with lignin standards complete the calibration curve traced from the polystyrene data perfectly.

Table 4
HPSEC retention times of calibration curve standards.

Standard Identification	Retention time in HPSEC (min)	Molecular mass (Da)
PS		
ps1	14.05	770 000
ps2	14.54	330 000
ps3	15.04	195 000
ps4	16.30	66 000
ps5	17.55	30 300

ps6 18.13 22 000
ps7 19.32 11 600
ps8 20.23 7000
ps9 20.88 5050
ps10 21.67 3250
ps11 22.20 2450
ps12 22.93 1700
ps13 23.74 1060
ps14 24.75 580
Dibenzoyl 27.22 210
Ethylbenzene 28.39 106
Toluene 28.93 92
Lignin
1m 22.94 2 490
2m 23.39 2 270
3m 24.09 2 040
4m 24.79 1 160
5m 25.33 770
6m 25.67 630
7m 26.74 350
Acetosyringone 28.15 196
Vanillin 28.58 152

Fig. 5. HPSEC profile of sugarcane bagasse lignin extracted by the acetosolv process.
Calibration curves constructed with PS standards and lignin fractions.
Table 5
Mw and *Mn* values of acetosolv lignin obtained by PS and lignin calibration curves.
Calibration curve $\bar{}$Mw (Da) $\bar{}$Mn (Da) ($\bar{}$Mw/ $\bar{}$Mn)
Lignin 2560 1450 1.76
PS standards 2280 1490 1.53
The results presented here might theoretically be extrapolated to fractions of higher-molecular-mass. However, the behavior of such fractions would be expected to deviate from such an extrapolation. The main reason for this is that the larger molecules are more branched than those of lowermass. Such a large extension of the branches in the molecule increases its hydrodynamic volume significantly, making its properties diverge fromthose of the linear chains of the polystyrene standards.
In this study of the molecular masses of the acetosolv fractions, the divergent behavior mentioned above was not observed, indicating that the fractions of lignin soluble in methanol are not as branched as has been proposed for the higher-molecular-mass fractions of milled-wood lignin (MWL). Thus, the acetosolv lignin fraction was eluted in the HPSEC columns in the same way as the flexible chains of the PS standards.
The calibration curve drawn in Fig. 5 was obtained from a cubic polynomial equation applied to the polystyrene data. It can be seen that the experimental points of the lignin standards covered a range

of the chromatogram in which practically 85% of the analyte was collected.

As expected fromthe concordance of the two curves, the ¯Mw and ¯M n values obtained for acetosolv lignin are very close, as shown in Table 5.

4. Conclusions

The fractionation process employed in this study led to lignin fractions of low polydispersity with evenly graded molecular masses. The lignin fractions were not homogeneous in their chemical structure, as significant differences could be observed in the elemental analyses of the C, H and O contents of the different fractions. The FTIR analyses also indicated that the fractions of different molecular masses have different chemical compositions and functional groups. Finally, the results obtained in the comparative study between lignin and polystyrene standards are very important for the establishment of a standard approach to estimate molecular masses of lignins by HPSEC. Severe criticisms have often been made about the use of polystyrene standards in these analyses. This result shows clearly and conclusively that, depending on the molecular mass in question, it is perfectly possible to use polystyrene standards in the calibration of HPSEC columns for lignin molecular mass analyses.

References

[1] D.L. Kaplan (Ed.), Biopolymers fromrenewable resources, Springer, Berlin, 1998, p. 292.

[2] V.R. Botaro, Deslignificac¸ ão organossolve de bagac¸ o de cana-de-ac¸ úcar: fracionamento e benzilacão das ligninas obtidas, Ph.D. Thesis, Chemistry Institute of São Carlos – USP, Brazil, 1996, p. 65.

[3] E. Adler,Wood Sci. Technol. 11 (1977) 169.

[4] H.S. Abreu, A.M. do Nascimento, M.A. Maria, Wood Fiber Sci. 31 (1999) 426.

[5] V.R. Botaro, C.G. Santos, A.R. Costa, G. Arantes Jr., Appl. Surf. Sci. 183 (2001) 120.

[6] J. Tsujino, H. Kawamoto, S. Saka,Wood Sci. Technol. 37 (2003) 299.

[7] B. Cathala, T. Saake, O. Faix, B. Monties, J. Chromatogr. A 1020 (2) (2003) 229.

[8] R. Singh, S. Singh, K.D. Trimukhe, K.V. Pandare, K.B. Bastawade, D.V. Gokhale, A.J. Varma, Carbohydr. Polym. 62 (2005) 57.

[9] W.G. Glasser, C.A. Barnett, Y. Sano, J. Appl. Polym. Sci., Appl. Polym., Symposia 37 (1983) 441.

[10] S.Y. Lin, C.W. Dence (Eds.),Methods in Lignin Chemistry, Springer-Verlag, Berlin, 1992, p. 55.

[11] A. Tejado, C. Pen˜a, J. Labidi, J.M. Echeverria, I. Mondragon, Bioresour. Technol.
(2007) 1655.
[12] O. Ringena, S. Lebioda, R. Lehnen, B. Saake, J. Chromatogr. A 1102 (1–2) (2006)
154.
[13] A. Barakat, B. Chabbert, B. Cathala, Phytochemistry 68 (15) (2007) 2118.
[14] K.V. Sarkanen, C.H. Ludwig, Lignins-Occurrence Formation and Structure and
Reactions, Wiley-Interscience, New York, 1971, 345.
[15] R. Van Dy Nguyen, U. Schurr, D. Meier, J. Anal. Appl. Pyrol. 77 (2) (2006)
95.
[16] C. Pouteau, B. Cathala, P. Dole, B. Kurek, B. Monties, Ind. Crops Prod. 21 (1) (2005)
101.
[17] S. Hirose, T. Hatakeyama, H. Hatakeyama, Thermochim. Acta 431 (1–2) (2005)
76.
[18] X.F. Sun, R.C. Sun, P. Fowler, M.S. Baird, Carbohydr. Polym. 55 (4) (2004)
379.