Characterization of Asparagus Lignin by HPLC

Jaramillo-Carmona S, Fuentes-Alventosa JM, Rodríguez-Gutiérrez G, Guillén-Bejarano R, Fernández-Bolaños J, Jiménez-Araujo A, Rodríguez-Arcos R.

Instituto de la Grasa (CSIC), Food Biotechnology Department, Avenida Padre García Tejero 4, 41012 Sevilla, Spain

Corresponding author:

Dra. Rocío Rodríguez-Arcos

Instituto de la Grasa (CSIC), Food Biotechnology Department. Avenida Padre García Tejero 4, 41012 Sevilla, Spain

Phone (+34) 954692516

Fax (+34) 954691262

rrodri@cica.es

Running head : Asparagus lignin ...

Journal section : Food Chemistry

Abstract

Lignin is the cell wall component most frequently associated with hardening. Its characterisation and quantification is very important to understand the biochemical modifications related to the changes in texture of vegetables such as asparagus (*Asparagus officinalis*), in which this organoleptic attribute is a very important quality factor. In this study, asparagus lignin from the basal sections of fresh and stored spears was analysed by using both methods, the traditional (Klason lignin) and the recently developed derivatization followed by reductive cleavage (DFRC) method. The latter is a simple and reproducible technique for lignin characterization based on a degradation procedure that produces analysable monomers and dimers by cleaving α - and β -aryl ethers in lignins. The primary monomers derived from DFRC degradation of lignins are essentially p-coumaryl peracetate, coniferyl peracetate and sinapyl peracetate.

In order to evaluate the efficiency of the DFRC method, our investigations have been carried on distinct sample types, including wood (data not shown), straw, and asparagus samples. The results have confirmed that lignin composition is affected by plant nature. It has been found that whereas wood samples mostly contain coniferyl units, plant foods such as straw and asparagus contain both coniferyl and guaiacyl units.

Key words: lignin composition, asparagus toughening, DFRC method, HPLC, Klason lignin

INTRODUCTION

The chemical basis of hardening is related with modifications of the cell wall composition and has traditionally been associated with lignification (Salunkhe and Desai

1984; Smith and Stanley 1987; Everson and others 1992). The presence of lignin is confined to vascular plants, where it is mainly deposited in cell walls of tissues involved in mechanical support or in water conduction such as xylem, sclerenchyma, phloem fibers and periderm. The effect of lignin on cell wall properties is thought to be mediated by the hydrophobicity of lignin and its incrustation and attachment to structural polysaccharides and proteins (Grabber and others 1996). Lignin is derived from the dehydrogenative polymerization of three different hydroxy-cinnamyl alcohols (or monolignols): *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. They give rise to the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the lignin polymer, respectively. The monolignols are C6-C3 phenylpropanoids and differ from each other only by their degree of methoxylation. In lignin, the C6-C3 units are interconnected by several types of ether and carbon-carbon linkages, but their relative number is not clearly established because different analytical methods give distinct results. Only a fraction of the lignin polymer is solubilized by most of the chemical degradation methods and isolation of parts of lignin always causes chemical changes in the structure (Monties 1998). An artificial lignin polymer, called dehydrogenation polymer (DHP) can be formed by dehydropolymerization of the lignin precursors, and it has been used to investigate *in vitro* lignin biosynthesis and structure (Freudenberg and Neish 1968). However, those synthetic lignins generated by oxidative polymerisation of *p*-hydroxycinnamyl alcohols differ structurally from natural lignins and they do not adequately model the three dimensional structure of lignified walls. The difficulty in obtaining knowledge about lignin structures *in vivo* is accompanied by the fact that lignin content and composition vary among plants. In general, the lignin of dicotyledonous gymnosperms results from the polymerization of coniferyl alcohol, whereas angiosperm

lignin is composed of both coniferyl and sinapyl alcohol. Monocotyledonous plants usually contain the three types of monomers (Baucher and others 1998). Different compositions can also be found within the same plant, among cell types of a single tissue. In asparagus, the lignin content is approximately threefold higher at the bottom of the spear than that at the top (Hennion and others 1992).

In this work, lignin was quantified from the residue left after saponification of cell wall material. The recently developed derivatization followed by reductive cleavage (DFRC) method was used for lignin analysis. This procedure has been optimised for the characterisation of the degradation products from both wood and grass cell walls. The DFRC method is a degradation procedure that produces analysable monomers and dimers by cleaving α - and β -aryl ethers in lignins (Ralph and Lu 1998). Lignin components were identified by HPLC, after being released from asparagus tissues. As it has previously been reported, it is difficult to achieve the measurement of lignin in herbaceous plants, including asparagus. Interferences of other cell wall components such as proteins and simple phenolics can overestimate the quantity of lignin; and on the other hand, incomplete hydrolysis of lignin may give erroneous lower values. This seemed to be the situation when asparagus lignin contents were analysed by using the DFRC method. In order to improve those results different factors have been investigated: derivatization temperature, lengthening AcBr treatment, particle size, reagents volume, and nature of sample. The objectives of the present work are to improve measurement of lignin by optimizing the DFRC method and to determine changes in lignin components after asparagus storage.

MATERIALS AND METHODS

Plant Material

Asparagus officinalis, var. Franklin, was obtained from a local commercial asparagus producer. Spears were cut to a distance of 18 cm from the tip, and then stored for 16 days at 4°C in airtight containers with an air current of saturated humidity. Fresh and stored samples were divided in three cylindrical portions of 5 cm length (apical, middle and basal) and lignin was determined in the cell wall material prepared from each of the three sections.

Straw samples from wheat (Straw-W) and barley (Straw-B) were extracted for alcohol insoluble residue (AIR) as described by Martín-Cabrejas and others (1994).

Lignin quantification by the Derivatization Followed by Reductive Cleavage method

Preparation of Cell Wall Material

Fresh and stored green asparagus (100 g) was blended in 15 g L⁻¹ aqueous lauryl sulphate (SDS) containing 5 mM Na₂S₂O₅ with an Omni-mixer homogeniser for 5 min. A few drops of octanol were added to reduce foaming. The homogenate was filtered through a 100 µm nylon mesh and the residue was ball-milled (Pascall, 0.5-L pot) at 1°C in 5 g L⁻¹ SDS containing 3 mM Na₂S₂O₅ for 12 h at 60 rpm. After filtering the homogenate through a 75-µm nylon mesh, the residue was suspended in cold water containing 3 mM Na₂S₂O₅, homogenized for 5 min and re-filtered. The latter procedure was repeated two times. Finally, the cell wall material (CWM) was further extracted with hot ethanol, for 10 min, to remove any alcohol soluble phenolics, washed with acetone and then air dried (Martín-Cabrejas and others 1994).

DFRC Lignin Quantification and Characterisation by the derivatisation followed by reductive cleavage

Wall-bound phenolics were released by sequential alkaline hydrolysis of isolated CWM as described by Hartley and Morrison (1991). The residue remaining after saponification of cell wall material was used for lignin analysis. Lignin composition was determined by modifying the derivatization followed by reductive cleavage (DFRC) procedure (Lu and Ralph 1997). To a round bottom flask containing lignin sample (the residue remaining after saponification of cell wall material), acetyl bromide (AcBr) stock solution (AcBr:acetic acid 20:80 by volume) was added. The mixture was heated at 50°C, for 3 h, and then the solvent was removed by rotary evaporation at 40°C. Prior to the establishment of these conditions, different variables have been investigated for optimisation: derivatization temperature (30-60°C), lengthening AcBr treatment (1-5 h), particle size (normally ground and freezer-milled samples), reagents volume (6-96 mL) and nature of sample (asparagus, wood and straw).

The residue was dissolved in the acidic reduction solvent (dioxane:acetic acid:water 5:4:1). Zinc powder (100 mg) was added to the well-stirred solution. Stirring was continued for 30 min. The mixture was filtered through glass fibre paper (GF-A Whatman, Maidstone, U.K.) and the filtrate was evaporated under vacuum, at 40°C, to dryness. The residue was used for HPLC quantification. The degraded products were dissolved in 1 ml of methanol and trans-cinnamic acid (16 µg) was added as internal standard. 100 µl of this solution was used for HPLC analysis.

Monoacetylated and diacetylated monolignols released from asparagus lignin were identified by HPLC and their retention times and spectrums were compared with those of authentic compounds. Monolignols were separated by using a SPHERISORB "S Chiral" column (25 cm x 4.6 mm i.d., 5 µm, Capital HPLC Ltd., Broxburn, West Lothian, U.K.). The elution was performed with progressive increasing acetonitrile levels

in 1 mM trifluoroacetic acid (TFA). The most suitable gradient profile for separation of coniferyl and sinapyl acetates was: using solvent A (water plus TFA to 1 mM) and solvent B (acetonitrile neutral plus TFA to 1 mM) in the following programme: initially A 100%, linear gradient over 50 min to A 65%, B 35%, exponential gradient over 15 min to B 100%, held isocratically at B 100% for 10 min, exponential gradient over 10 min to A 100%, held isocratically to A 100% for 5 min. The flow rate was maintained at 1 ml/min and the solvents were sparged with helium prior to use. Detection was with a Perkin Elmer Model 235C DAD. Quantitation was by integration of peak-areas at 265 nm, with reference to calibration s made using known amounts of pure compounds.

Trans-hydroxycinnamyl acetates **H_t**, **G_t** and **S_t** were prepared from parent hydroxycinnamyl alcohols by acetylation, and these compounds were used as standards to calculate lignin composition of plant cell walls.

Klason Lignin Quantification

Klason lignin was calculated as described by Theander and Westerlund (1986) with some modification. Both asparagus CWM and straw AIR, prepared as explained above, were analysed. A ground sample (150-250 mg) was weighed into a glass tube (100 ml), and 3 ml of 72% H₂SO₄ was added. The mixture was incubated at room temperature for 3 hours, shaking frequently. After adding 36 ml distilled water, the suspension was mixed and incubated at 100°C, for 2 ½ hours. The hydrolysis mixture was filtered through a Pyrex No. 2 glass filter, and the residue was washed with hot water, until free of acid. The glass filter, containing the filtered material, was dried, until constant weight, and Klason lignin was determined gravimetrically.

Statistical Analysis

The mean of three replicates was calculated. All data were analyzed using multivariate analysis of variance (ANOVA) followed by Fisher-LSD multicomparison test. The level of significance was $P < 0.05$.

RESULTS

Analysis of Plant Lignins by using a modified DFRC method

Lignin was characterized after degradation to analyzable monomers from native lignin. The primary monomers derived from DFRC degradation of lignin samples are essentially *p*-coumaryl peracetate, coniferyl peracetate and sinapyl peracetate (Fig 1). HPLC chromatograms showed that major peaks arise from guaiacyl and syringyl units involved in ether linkages. These units can be found as alcohol, monoacetylated monolignol or diacetylated monolignol. Coniferyl, sinapyl alcohol (commercially available), and *p*-coumaryl alcohol (provided by John Ralph) were acetylated, applied to the HPLC column and eluted with an acetonitrile gradient which enabled separation of alcohol, monoacetylated monolignols (A and B) and diacetylated monolignols (Fig. 2). Typical retention times and response factors of these compounds are shown in Table 1.

The presence of these compounds can be assessed through analysis of the spectral properties. The individual absorption spectrum of each compound was recorded, and the spectrums from *p*-coumaryl, coniferyl and sinapyl derivatives shown to be significantly different. In the spectra of *p*-coumaryl acetate, two peaks of absorbance maximum ($\lambda_{\max}$) were found, at 201 nm and 254 nm, and the absorbance minimum was at 225 nm. All coniferyl derivatives exhibited similar profiles: two absorbance maxima, at 214 nm and 260 nm, and a small shoulder at 300 nm. The absorbance minimum was at 240 nm (Fig. 1 and Table 1).

The spectrums of sinapyl derivatives show the following differences with respect to coniferyl compounds. Although also two absorbance maxima are present, they elute slightly later, at 220 and 265 nm, and there is no shoulder (Fig. 1).

The response factors (RF) have been based on gravimetric quantitation of small quantities of pure standards. The RF values, relative to the internal standard (trans-cinnamic acid) range from 0.1-0.4, reflecting the different absorption maxima of the *p*-coumaryl, coniferyl and sinapyl alcohol and acetates (Table 1).

Quantification and Characterization of Asparagus Lignin

As it has been reported previously (Rodríguez and others 1999, Rodríguez-Arcos and others 2002), the deposition of lignin that occurs during asparagus post-harvest storage is located in the middle and basal sections of the spear. In the present work, the values of lignin content for the middle and basal sections were not significantly different ($P < 0.05$), using either the Klason or the DFRC method (Table 2). Klason lignin values from the middle and basal section of fresh asparagus were 87.3 and 89.6 mg/g cell wall. After storage, those values increased to 110.6 and 107.0 mg/g cell wall. The values obtained by using the DFRC method were 2.73 and 3.06 mg/g cell wall for the middle and basal sections from fresh asparagus, and 4.13 and 4.21 mg/g cell wall for the respective stored samples. So, for investigating asparagus lignin composition, the cell wall material isolated from the basal section was used. In addition to asparagus, two different straw samples from wheat and barley have been investigated, in order to prove the efficiency of the DFRC method on both herbaceous and *woody* (straw) samples.

Preliminary assays to compare different methods that have traditionally been used for lignin analysis were performed (results not shown). Based on the results of these

assays, in this study, acetyl bromide (AcBr) was used for hydrolysis of the cell wall material. Not only the content (Table 2) but also the composition of lignin within asparagus cell walls was determined. Typical composition of lignins from asparagus and straw samples are shown in table 3. Lignin was quantified as the sum of the different monolignols detected by HPLC analysis.

It was found that asparagus lignin consisted mainly of coniferyl (60%) and sinapyl (40%) units, and the majority of these were present as monoacetylated and diacetylated monolignols. The monoacetylated compounds comprised 67% of the total coniferyl and 77% of the total sinapyl complement.

As it can be observed in Table 3, the use of the DFRC method for the quantification of lignin, provides additional information about its composition.

Optimization and Evaluation of the DFRC Method

As it has previously been reported, it is difficult to reliably quantify lignin in herbaceous plants, including asparagus (Rodríguez and others 1999). Interferences by other cell wall components such as proteins and simple phenolics can result in overestimation of the quantity of lignin, and on the other hand, incomplete hydrolysis of lignin may result in underestimation (Südekum and others 1997). The second seems to be the situation when asparagus lignin contents were analysed by using the DFRC method. In order to improve the quantification method different factors were investigated: derivatization temperature, length of AcBr treatment, particle size, reagents volume, and nature of plant material. In relation to temperature and length of the treatment (Table 4), our results are consistent to those reported by Hatfield and others (1999). They suggested that treatment of samples at 50⁰C, during 3 h are the optimal conditions to derivatize

lignins from wood samples. Also, reducing the particle size and increasing the reagents volume results in some improvements (Table 4). Straw-wheat samples that had shown to be highly lignified (15% Klason lignin) have been used to test the influence of those two parameters in the recovery of lignin components: Scaling up the volume of reagents by a factor of two meant an important increase of monomer yields. Successive increases of solvents volume were also tested, but no improvements were observed (Table 4). Grinding of the samples in a freezer-mill for 10 min resulted in significant increases ($P<0.05$) in the yields of lignin monomers. Nevertheless, this factor did not appear as to be as important as the volume of reagents (Table 4).

It was observed that some residue remained after AcBr treatment of both normally-ground and freezer-milled samples. Scaling up the amount of solvents by a factor of 2 resulted in a complete solubilization of the samples, forming a clear solution.

It has been suggested that there is considerable heterogeneity in the amount and nature of the lignin deposited in walls of different cell types and in different parts of individual cell walls (Chesson and others 1997). This study has confirmed that not only the total content of lignin, but also its composition and structure can differ among different plant materials and tissue types.

Among the different factors that influence lignin monomers yields, our investigations revealed that particle size and reagents volume were the most determinant. The results from the comparative analysis of different plant samples (straw-wheat, straw-barley, fresh asparagus and stored asparagus) that were extracted with 6 and 12 mL of reagents, and using two different milling treatments are shown in Table 5. It can be concluded that the best conditions for characterizing plant lignins consisting on grinding

the samples (50 mg cell wall material) in a freezer-milling, for 10 min, and then extracting them with 12 mL of DFRC reagents.

As explained above, in asparagus, the lignin content increased from the top to the bottom of the spear. Besides, significant differences ($P<0.05$) were found in relation to both lignin content and composition by comparison of the middle and bottom sections from fresh and stored asparagus (Tables 2 and 5). It can be assumed that differences in cell wall lignin composition will affect the mechanical properties of asparagus tissues.

Our results are consistent with those reported by Terashima and Fukushima (1989), who suggested a mechanism that regulates the deposition of syringyl and guaicyl units in lignin. The compositional changes on lignin constituents may affect the extractability of the different lignins. Bernard-Vailhé and others (1998) found that in transgenic tobacco plants with reduced cinnamyl alcohol dehydrogenase (CAD) activity, which was supposed to decrease the formation of hydroxy-cinnamyl alcohols and, therefore, reduce the lignin content, the lignin contents did not change. However, there was an important reduction in the syringyl/guaicyl molar ratio, suggesting modifications in lignin composition and structure. For similar lignin content, the decrease in the proportion of syringyl units meant an increase in the accessibility of hydrolytic enzymes, such as glycanases, to the substrate and therefore an improvement of the lignin digestibility (Reeves 1987; Jung and Deetz 1993).

This study on wood, straw and asparagus has confirmed the correlation between syringyl units and degradability. The application of the DFRC method, which is a simple and reproducible technique for lignin characterization, on *woody (straw)* lignins meant a complete solubilization of the samples, whereas an insoluble residue was left for asparagus samples, even when the samples were extracted with higher amounts of

solvent. Further characterization of lignin composition revealed that no syringyl units were present in wood samples and that the percent of syringyl units was lower in lignins from straw (32%), than in asparagus lignins (40%). These results strongly suggest that lignin structure is important for the extent, and possibly, the rate of cell wall degradability.

Regarding lignin quantification, the values of lignin calculated as recovery of its constituent monomers were much lower than the results obtained from the traditional methods such as Klason lignin. These discrepancies were found for both woody and asparagus samples, but were larger in the latter, in which the content of *DFRC* lignin was less than 10% of *Klason* lignin.

The hard fact remains that the measurement of lignins in plant material is difficult to achieve and there is no general method to quantify this complex cell wall polymer. However, the application of the *DFRC* modified procedure provides interesting information about structural characteristics of lignins isolated from both wood and herbaceous plants.

Covalent intermolecular bonds may also be implied in the different solubility of plant lignins, particularly those between lignin and carbohydrate. Further studies are needed to understand which structures in addition to syringyl units have an effect on cell wall degradability. Piquemal and others (1998) reported that the increase in the syringyl over guaiacyl (S/G) ratio was accompanied by increases of cell wall-bound phenolics, mostly ferulic acid, in stems of tobacco plants. The occurrence of cell wall-linked ferulic acid in substantial amounts is a specific feature of grasses, whereas it is usually detected as a minor or trace cell wall component in the xylem of dicotyledons. Recent investigations have demonstrated that cell walls of asparagus spears contain significant

quantities of phenolic esters, particularly FA and its dehydrodimers, which increase considerably during post-harvest storage. These findings are consistent with a role of phenolic esters in the maturation and storage-related toughening of asparagus (Rodríguez-Arcos and others 2002, 2004).

The attachment mode of ferulic acid to the cell wall is through ester bonds, cleaved by mild alkaline hydrolysis (Jacquet and others 1995; Ralph and others 1994). Nevertheless there may be other modes of incorporation of these acids, based on their enzymatic dehydrogenation to phenoxy radicals. These radicals are actually prone to participating in a variety of ether and carbon-carbon interunit linkages, resulting in the cross-linking of distinct cell wall polymers and thus in the strengthening of the lignocellulosic material. Further investigations are currently being developed in order to isolate and characterize the phenolic ethers that can participate in the formation of cross-links between polysaccharides and lignin within cell walls, which we have recently detected in asparagus tissues.

CONCLUSION

Our most recent investigations on asparagus toughening (Rodríguez-Arcos and others 2002, 2004) provide evidence that the deposition of lignin during asparagus storage is not the key point in the process of toughening that this vegetable suffers. So, gravimetric determinations of lignin by using the Klason method are not the most suitable system since there are many other components (polysaccharides, proteins, other phenolics) that can interfere with those measurements in asparagus tissues. Modifications in cell wall structure, mainly formation of ferulic cross-links between different polymers, seem to be much more significant than the increase of lignin in asparagus toughening. So,

future research on this subject should be focused in gaining further knowledge about the participation of lignin constituents in the formation of those cross-links, and for this purpose the structural information that the DFRC provides is of great interest.

REFERENCES

- Baucher M, Monties B, Van Montagu M, Boerjan W. 1998. Biosynthesis and genetic engineering of lignin. *Critical Rev Plant Sci* 17:125-97.
- Bernard-Vailhé MA, Besle JM, Maillot MP, Cornu A, Halpin C, Knight M. 1998. Effect of down-regulation of cinnamyl alcohol dehydrogenase on cell wall composition and on degradability of tobacco stems. *J Sci Food Agric* 76:505-14.
- Chesson A, Provan GJ, Russell W, Scobbie L. 1997. Characterisation of lignin from parenchyma and sclerenchyma cell walls of the maize internode. *J Sci Food Agric* 73:10-6.
- Everson HP, Waldron KW, Geeson JD, Browne KM. 1992. Effects of modified atmospheres on textural and cell wall changes of asparagus during shelf-life. *Intl J Food Sci Technol* 27:187-99.
- Freudenberg K, Neish AC. 1968. *Constitution and Biosynthesis of Lignin*. Berlin: Springer-Verlag. 122 p.
- Grabber JH, Ralph J, Hatfield RD, Quideau S, Kuster T, Pell AN. 1996. Dehydrogenation polymer-cell wall complexes as a model for lignified grass walls. *J Agric Food Chem* 44:1453-9.
- Hartley RD, Morrison WH. 1991. Monomeric and dimeric phenolic acids released from cell walls of grasses by sequential treatment with sodium hydroxide. *J Sci Food Agric* 55:365-75.

- 336 Hatfield RD, Grabber J, Ralph J, Brei K. 1999. Using the acetyl bromide assay to
337 determine lignin concentrations in herbaceous plants: some cautionary notes. *J Agric*
338 *Food Chem* 47:628-32.
- 339 Hennion S, Little CHA, Hartmann C. 1992. Activities of enzymes involved in
340 lignification during the postharvest storage of etiolated asparagus spears. *Physiol Plant*
341 86:474-8.
- 342 Jacquet G, Pollet B, Lapierre C, Mhamdi F, Rolando C. 1995. New ether-linked ferulic
343 acid-coniferyl alcohol dimers identified in grass straws. *J Agric Food Chem* 43:2746-51.
- 344 Jung HJG, Deetz DA. 1993. Cell wall lignification and degradability. In: Jung HG,
345 Buxton DR, Hatfield RD, Ralph J, editors. *Forage cell wall structure and digestibility*.
346 *Am Soc Agron.* p 315-46.
- 347 Lu F, Ralph J. 1997. Derivatization followed by reductive cleavage (DFRC method), a
348 new method for lignin analysis: protocol for analysis of DFRC monomers. *J Agric Food*
349 *Chem* 45:2590-2.
- 350 Martin-Cabrejas MA, Waldron KW, Selvendran RR. 1994. Cell wall changes in Spanish
351 pear during ripening. *J Plant Physiol* 144:541-8.
- 352 Monties B. 1988. Preparation of dioxane lignin fractions by acidolysis. *Meth Enzymol*
353 161:31-5.
- 354 Piquemal J, Lapierre C, Myton K, O'Connell A, Schuch W, Grima-Pettenati J, Boudet
355 AM. 1998. Down-regulation of cinnamoyl-CoA reductase induces significant changes of
356 lignin profiles in transgenic tobacco plants. *Plant J* 13:71-83.
- 357 Ralph J, Lu F. 1998. The DFRC method for lignin analysis. 6. A simple modification for
358 identifying natural acetates on lignins. *J Agric Food Chem* 46:4616-9.

- 359 Ralph J, Quideau S, Grabber JH, Hatfield RD. 1994. Identification and synthesis of new
360 ferulic acid dehydrodimers present in grass cell walls. J Chem Soc (Perkin trans) 1:3485-
361 98.
- 362 Reeves JB. 1987. Lignin and fiber compositional changes in forages over a growing
363 season and their effects on in vitro digestibility. J Dairy Sci 70:1583-94.
- 364 Rodríguez R, Jiménez A, Guillén R, Heredia A, Fernández-Bolaños J. 1999. Postharvest
365 changes in white asparagus cell wall during refrigerated storage. J Agric Food Chem
366 47:3551-7.
- 367 Rodríguez-Arcos R, Smith AC, Waldron KW. 2002. Effect of storage on wall-bound
368 phenolics in green asparagus. J Agric Food Chem 50:3197-203.
- 369 Rodríguez-Arcos R, Smith AC, Waldron KW. 2004. Ferulic acid cross-links in asparagus
370 cell walls in relation to texture. J Agric Food Chem 52:4740-50.
- 371 Salunkhe DK, Desai BB. 1984. Asparagus and artichoke. In: Postharvest biotechnology
372 of vegetables. Florida: CRC Press. p 117-127.
- 373 Smith JL, Stanley DW. 1987. Nonenzymatic lignification of asparagus?. J Texture
374 Studies 18:339-58.
- 375 Südekum K-H, Voigt K, Monties B, Stangassinger M. 1997. Spectrophotometric
376 investigations on lignin in wheat (*Triticum aestivum* L.): influence of cell wall
377 preparation, solvent and standard. J Agric Food Chem 45:1220-8.
- 378 Terashima N, Fukushima K. 1989. Biogenesis and structure of macromolecular lignin in
379 the cell wall of tree xylem as studied in microautoradiography. In: Lewis NG & Paice
380 MG, editors. Plant cell wall polymers, biogenesis and biodegradation. Washington:
381 American Chemical Society. p160-8.

382 Theander O, Westerlund EA. 1986. Studies on dietary fiber. 3. Improved procedures for
383 analysis of dietary fiber. J Agric Food Chem 34:330-6.

384

385 **Acknowledgements**

386 This work was supported by the Spanish CICYT (Comisión interministerial de Ciencia y
387 Tecnología), Project AGL2007-63703/ALI; and MMA (Ministerio de Medio Ambiente),
388 Project B008/2007/1-02.5. J.M.F-A. is the holder of a grant FPI from IFAPA (Instituto de
389 Investigación y Formación Agraria y Pesquera), Junta de Andalucía (Spain)

Table 1. Diagnostic absorption wavelengths of peaks ($\lambda_{\max}$) and of troughs ($\lambda_{\min}$), response factors (RF) in relation to trans-cinnamic acid and retention times (R_t) of coniferyl and sinapyl derivatives that constitutes plant lignins, analysed by HPLC.

Compound	$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)	RF	R_t (min)
Coniferyl alcohol	213.9, 265.2, 298.3	240.9	0.45	11.6
Sinapyl alcohol	222.0, 274.0	245.7	0.20	15.0
trans-Cinnamic acid	276.5	232.0	1.00	18.4
Coniferyl monoacetylated A	212.6, 253.8, 294.9	236.2	0.11	19.9
Sinapyl monoacetylated A	219.3, 263.2	239.6	0.19	23.8
Coniferylmonoacetylated B	214.6, 267.3, 301.7	243.0	0.11	30.0
Sinapyl monoacetylated B	219.3, 263.2	239.0	0.20	32.7
<i>p</i> -Coumaryl diacetylated	201.0, 254.0	225.0	0.05	38.6
Coniferyl diacetylated	214.6, 256.8, 297.0	237.6	0.11	41.7
Sinapyl diacetylated	219.3, 265.2	239.0	0.19	44.3

Table 2. Lignin content from different plant materials (%cell wall)

	Fresh asparagus		Stored asparagus	
	Klason	DFRC	Klason	DFRC
TOP	6.59±0.29	0.18±0.02	6.51±0.21	0.26±0.04
MIDDLE	8.73±0.40	0.27±0.04	11.06±0.46	0.41±0.05
BASAL	8.96±0.24	0.31±0.03	10.70±0.27	0.42±0.06
Straw-W	15.51±0.99	2.36±0.22		
Straw-B	13.98±0.69	1.76±0.19		

W: wheat, B: barley

Data are the mean of three replicates

Table 3. Proportions of lignin degradation products from several plant materials

	Lignin composition (%Relative)			
	Straw-W	Straw-B	Fresh asparagus	Stored asparagus
Coniferyl alcohol	n.d	n.d.	n.d.	n.d.
Coniferyl monoacetylated	35	33	41	40
Coniferyl diacetylated	31	30	20	19
Sinapyl alcohol	14	13	n.d.	n.d.
Sinapyl monoacetylated	7	7	30	33
Sinapyl diacetylated	10	8	9	8
<i>p</i> -Coumaryl diacetylated	3	9	n.d.	n.d.

W: wheat, B: barley

n.d.: no detected

Data are the mean of three replicates

Table 4. Effects of temperature, time, volume of acetyl bromide reagents and particle size on lignin analysis by the DFRC method on straw-wheat sample

Temperature	Lignin%	Time (h)	Lignin%	volume (ml)	Lignin%	Particle size	Lignin%
40°C	1.42±0.13 ^a	1	1.86±0.16 ^a	6	0.54±0.04 ^a	N.G., 5 min	1.62±0.10 ^a
50°C	2.36±0.17 ^b	2	1.96±0.13 ^a	12	2.36±0.17 ^b	F.M., 5 min	2.28±0.14 ^b
60°C	2.38±0.08 ^b	3	2.36±0.17 ^b	24	2.32±0.21 ^b	N.G., 10 min	1.93±0.05 ^c
70°C	2.35±0.11 ^b	4	2.40±0.24 ^b	48	2.36±0.14 ^b	F.M., 10 min	2.36±0.17 ^d
80°C	2.28±0.05 ^b	5	2.37±0.05 ^b	96	2.34±0.20 ^b	N.G., 15 min	2.09±0.13 ^c

N.G.: normally ground (in a coffee mill) samples; F.M.: freezer-milled samples

Data are the mean of three replicates

Data with the same letter within the same column were not significantly different ($P<0.05$)

Table 5. Quantification of lignin after DFRC degradation (mg / g CWM)

Normally ground, 6 mL	Straw-W	Straw-B	Fresh asparagus	Stored asparagus
Coniferyl alcohol	n.d	n.d.	n.d.	n.d.
Coniferyl monoacetylated	2.31±0.16	1.15±0.06	0.08±0.00	0.12±0.00
Coniferyl diacetylated	2.05±0.13	1.05±0.04	0.04±0.00	0.06±0.00
Sinapyl alcohol	0.93±0.08	0.46±0.04	n.d.	n.d.
Sinapyl monoacetylated	0.46±0.05	0.24±0.03	0.06±0.01	0.10±0.00
Sinapyl diacetylated	0.66±0.03	0.28±0.01	0.02±0.00	0.02±0.00
<i>p</i> -Coumaryl diacetylated	0.20±0.01	0.31±0.02	n.d.	n.d.
Total monomers yield	6.61±0.47	3.49±0.21	0.20±0.02	0.30±0.02
Freezer milled, 6 mL	Straw-W	Straw-B	Fresh asparagus	Stored asparagus
Coniferyl alcohol	n.d	n.d.	n.d.	n.d.
Coniferyl monoacetylated	2.52±0.16	2.34±0.06	0.11±0.01	1.08±0.07
Coniferyl diacetylated	2.23±0.07	2.13±0.21	0.03±0.00	0.51±0.02
Sinapyl alcohol	1.00±0.05	0.92±0.03	n.d.	n.d.
Sinapyl monoacetylated	0.50±0.02	0.50±0.04	0.08±0.00	0.89±0.03
Sinapyl diacetylated	0.72±0.01	0.57±0.04	0.03±0.00	0.22±0.02
<i>p</i> -Coumaryl diacetylated	0.22±0.02	0.64±0.02	n.d.	n.d.
Total monomers yield	7.19±0.33	7.10±0.41	0.25±0.02	2.70±0.14
Normally ground, 12 mL	Straw-W	Straw-B	Fresh asparagus	Stored asparagus
Coniferyl alcohol	n.d	n.d.	n.d.	n.d.
Coniferyl monoacetylated	6.75±0.46	5.21±0.44	0.21±0.01	1.60±0.14
Coniferyl diacetylated	5.98±0.19	4.74±0.12	0.10±0.00	0.76±0.04
Sinapyl alcohol	2.70±0.14	2.05±0.16	n.d.	n.d.
Sinapyl monoacetylated	1.35±0.11	1.11±0.04	0.15±0.01	1.32±0.08
Sinapyl diacetylated	1.93±0.06	1.26±0.05	0.05±0.00	0.32±0.00
<i>p</i> -Coumaryl diacetylated	0.58±0.02	1.42±0.03	n.d.	n.d.
Total monomers yield	19.29±0.98	15.79±0.84	0.51±0.03	4.00±0.27
Freezer milled, 12 mL	Straw-W	Straw-B	Fresh asparagus	Stored asparagus
Coniferyl alcohol	n.d	n.d.	n.d.	n.d.
Coniferyl monoacetylated	8.26±0.25	5.81±0.41	1.27±0.11	1.68±0.15
Coniferyl diacetylated	7.32±0.44	5.28±0.16	0.62±0.02	0.80±0.04
Sinapyl alcohol	3.30±0.37	2.29±0.09	n.d.	n.d.
Sinapyl monoacetylated	1.65±0.13	1.23±0.08	0.93±0.03	1.39±0.07
Sinapyl diacetylated	2.36±0.26	1.41±0.08	0.28±0.02	0.34±0.00
<i>p</i> -Coumaryl diacetylated	0.71±0.02	1.58±0.17	n.d.	n.d.
Total monomers yield	23.60±1.47	17.60±0.99	3.10±0.18	4.21±0.27

W: wheat, B: barley. Data are the mean of three replicates

Fig. 1. Three-dimensional elution profile of monomers derived from DFRC degradation of lignin samples, detected by DAD scanning between 200 and 360 nm. 1, p-coumaryl peracetate, 2, coniferyl peracetate, 3, sinapyl peracetate.

Fig.2. HPLC elution profile of: A) coniferyl derivatives. 1, coniferyl alcohol, 2, coniferyl monoacetylated A, 3, coniferyl monoacetylated B, 4, coniferyl diacetylated; B) sinapyl derivatives. 1, sinapyl alcohol, 2, sinapyl monoacetylated A, 3, sinapyl monoacetylated B, 4, sinapyl diacetylated

Fig. 3. HPLC elution profile of lignin components of asparagus cell wall. 1, coniferyl monoacetate B, 2, sinapyl monoacetate B, 3, coniferyl diacetate, 4, sinapyl diacetate.