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# Structural characterization of red wine rhamnogalacturonan II

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**Abstract**

The pectic polysaccharide rhamnogalacturonan II (RG-II), which accounts for ~ 20% of the ethanol-precipitable polysaccharides in red wine, has been isolated from wine polysaccharides by anion-exchange chromatography. Four fractions enriched with RG-II were obtained and the RG-II then purified to homogeneity by Concanavalin A affinity and size-exclusion chromatographies. The glycosyl-residue compositions of the four RG-IIs are similar; all the RG-IIs contain the monosaccharides (apiose, 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, Kdo, Dha, and aceric acid) that are diagnostic of RG-II. The glycosyl-linkages of the neutral and acidic sugars, including aceric acid, were determined simultaneously by GC–EIMS analysis of the methylated alditol acetates generated from per-*O*-methylated and carboxyl-reduced RG-II. Two of the RG-IIs contain boron, most likely as a borate di-ester that cross-links two molecules of RG-II together to form a dimer. The dimer contains 3′- and 2,3,3′-linked apiosyl residues whereas the monomer contains only 3′-linked apiosyl residues which suggests that the borate di-ester is located on at least one of the apiosyl residues of RG-II. Although the wine RG-IIs all have similar structures they are not identical since they differ in the length and degree of methyl-esterification of the RG-II backbone and in the presence or absence of borate di-esters. Nevertheless, these studies show that the major

Abbreviations: RG-II, Rhamnogalacturonan II; Kdo, 3-Deoxy-D-*manno*-octulosonic acid; Dha, 3-Deoxy-D-*lyxo*-heptulosaric acid; Aceric acid, 3-*C*-Carboxy-5-deoxy-L-xylose; MALDI–TOF MS, Matrix-assisted laser desorption/ionization time of flight mass spectrometry; TMS, Per-*O*-trimethylsilylated methyl glycosides; CI–MS, Chemical ionization mass spectrometry; GC–EIMS, gas chromatography–electron impact mass spectrometry; ICP–AES, Inductively-coupled-plasma atomic-emission spectroscopy; HPAEC, High performance anion-exchange chromatography

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structural features of wine and primary cell wall RG-II are conserved. 

*Keywords:* Rhamnogalacturonan II; Wine; Grape; Pectin; Uronic acid; Aceric acid; Homogalacturonan; Dimer; Borate di-ester

## 1. Introduction

Rhamnogalacturonan II (RG-II) is a low molecular, structurally complex pectic polysaccharide that is released from the walls of plant cells by treatment with fungal endopolygalacturonase [1]. It has been isolated from the walls of suspension-cultured sycamore (*Acer pseudoplatanus*), Douglas fir (*Pseudotsuga menziesii*), and rice (*Oryza sativa*) cells [2–4]. RG-II is present in the cell walls of onion (*Allium cepa*) [5], kiwi fruit (*Actinidia deliciosa*) [6], *Bupleurum falcatum* roots [7], *Arabidopsis thaliana* [8] and *Panax ginseng* [9] leaves, and in a commercial enzyme preparation Pectinol AC [10]. RG-II has also been isolated from red wine by the authors and partially characterized [11] and is present in must obtained by pressing grape berries [12]. Since RG-II is a major component of wine polysaccharides it may have considerable importance in enology. For example, RG-II may be involved in electrostatic and ionic interactions with other wine constituents that lead to the formation of precipitates and haze since it has the highest negative charge density of the wine macromolecules [13].

RG-II contains twelve different glycosyl residues, including several rare and diagnostic monosaccharides such as apiose [2], 2-*O*-methyl-L-fucose [2], 2-*O*-methyl-D-xylose [2], aceric acid (3-*C*-carboxy-5-deoxy-L-xylose) [8], Kdo (3-deoxy-D-*manno*-octulosonic acid) [14], and Dha (3-deoxy-D-*lyxo*-heptulosaric acid) [15], and common monosaccharides involved in unusual glycosidic linkages, e.g. 3,4-linked fucose or fully substituted rhamnose. RG-II has four structurally well-defined oligosaccharide side chains [16] that are attached to a backbone composed of at least seven 1,4-linked α-D-galactosyluronic acid residues (Fig. 1) although the distribution of the side chains on the backbone has not been determined yet [17]. The results of recent studies [18,19] have shown that RG-II is present in plant cell walls predominantly as a dimer that is cross-linked by borate di-esters, probably located on one apiosyl residue. The chemical properties of this cross-link have led to the suggestion that RG-II plays an important role in determining the structure and functions of pectins in the walls of growing plants [19].

Although the main structural features of RG-II are conserved in all plants analyzed to date, there are discernible differences in the glycosyl-residue compositions of RG-II from different sources [3,10,11]. These variations result from differences in the length of the homogalacturonan backbone or from the loss of terminal non-reducing residues attached to the hepta- and octasaccharide side chains (A and B, Fig. 1). For example, the terminal α-L-rhamnose (residue B6) is absent in Pectinol AC RG-II [20] whereas the terminal α-D-galactose (residue A5) is present in wine RG-II in non-stoichiometric amounts [11]. Furthermore, the presence in sycamore and wine RG-II [21,11] of non-stoichiometric amounts of T-Ara*f* and T-Rha*p* residues may also lead to variations in the fine structure of RG-II. However, it has not been established if such differences

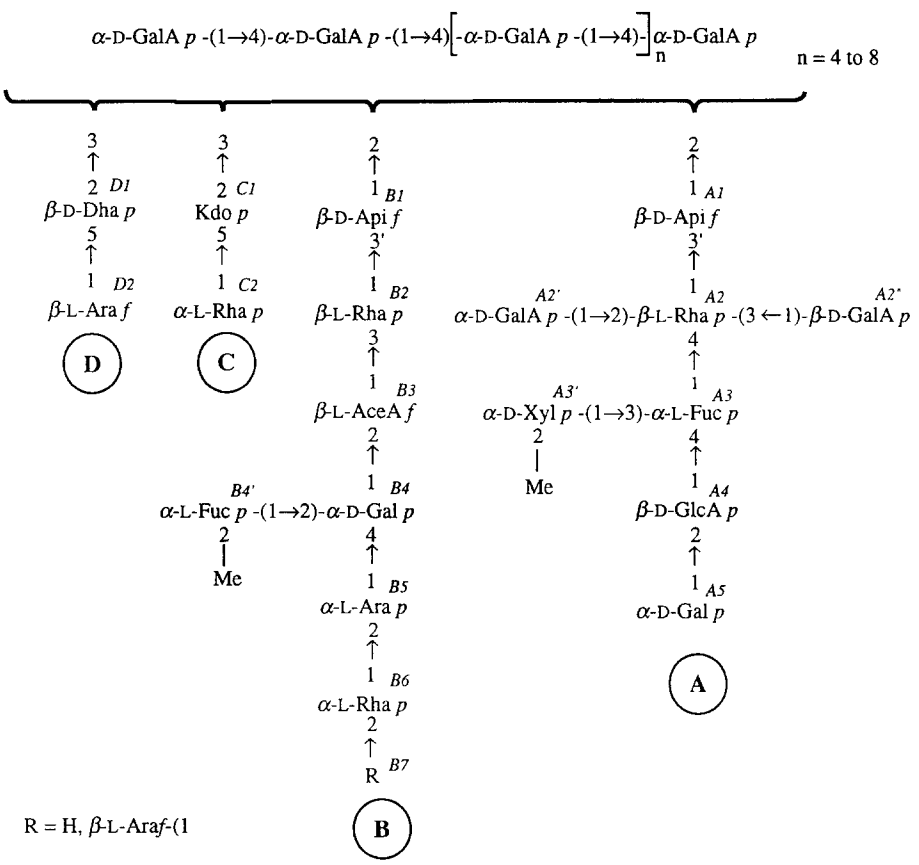


Fig. 1. Hypothetical structure of RG-II [1,17]. The figure shows the four oligoglycosidic side-chains A–D whose residues are numbered according to the model sequence.

result from partial enzymic fragmentation of RG-II during its isolation or if they reflect minor variations in the structures of RG-II in primary cell walls.

We now report that four distinct fractions of RG-II can be isolated from red wine by anion-exchange chromatography. The four RG-IIs have similar glycosyl-residue compositions even though there are discernible differences in the length and degree of methyl-esterification of the homogalacturonan backbone and in the ratios of borate di-ester cross-linked dimeric and monomeric RG-II.

## 2. Experimental

*Wine sample and recovery of colloids.*—Red wine (600 L) obtained after fermentation of mature Carignan noir grapes harvested in 1991 at the INRA-Pech Rouge/Narbonne Experimental Station was concentrated to 25 L by ultrafiltration (20

kDa cut off). Total colloids were precipitated by the addition of five volumes of cold ethanol (4 °C) to the concentrated wine [22]. The precipitate was then dissolved in water and dialysed. The salt-free colloid solution was adjusted to pH 3 with citric acid buffer (final concentration 20 mM) and loaded on a S-Sepharose Fast Flow cation-exchange column (Pharmacia, Sweden) [23]. The almost fully depigmented unbound fraction represented 97% of total wine polysaccharides (phenol–sulfuric acid assay) and was used as source of RG-II.

*Fractionation of total wine polysaccharides.*—The solution of wine polysaccharides (176.5 g by weight) in water was adjusted to pH 4.6 by the addition of sodium citrate (final concentration 40 mM) and fractionated in 9 portions on a DEAE-Macroprep column (5 × 80 cm; Bio-Rad, USA) equilibrated at 20 mL/min in 40 mM sodium citrate buffer pH 4.6. Bound polysaccharides were eluted by stepwise addition of NaCl (1 L/step; 10, 50, 150, 250 mM in starting buffer) to give four RG-II-containing fractions (**RG-II1**–RG-II**4**).

*Purification of wine rhamnogalacturonan II fractions.*—The four RG-II-containing fractions were separately dialysed against 50 mM sodium acetate, pH 5.6, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$, and loaded on a Concanavalin A-Ultrogel column (5 × 70 cm; Sepracor-IBF, France) equilibrated at 5 mL/min in the same buffer to remove the contaminating yeast mannoproteins. The RG-II from each fraction was then separated from high molecular weight polysaccharides by size-exclusion chromatography on a Sephacryl S-400 HR column (5 × 80 cm; Pharmacia) equilibrated at 7 mL/min in 50 mM sodium acetate buffer (pH 5) containing 50 mM NaCl or on a Sephacryl S-200 HR column (1.6 × 95 cm; Pharmacia) equilibrated in the same buffer. The elution of the polysaccharides was followed by using the phenol/sulfuric acid assay [24].

*Homogeneity and molecular weight determination.*—The homogeneity of the four RG-II fractions was determined by high-resolution size-exclusion chromatography by injecting portions (500 μg) of each purified RG-II on a Superdex-75 HR column (1 × 30 cm; Pharmacia) equilibrated at 0.6 mL/min in 25 mM ammonium formate pH 5.2. The eluant was monitored with a HP1057A refractometer. The column void volume was at ~ 14 min and the included volume was at ~ 34 min.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI–TOF MS) was performed on a Hewlett–Packard LDI 1700XP mass spectrometer operated in the positive ion mode at 30 kV accelerating voltage and a pressure of $6 \times 10^{-7}$ Torr. The mass spectrometer was calibrated with a mixture of malto-oligosaccharides. An aqueous solution of RG-II (10 mg/mL) was diluted 1:5 in aqueous 50% acetonitrile containing 100 mM 2,5-dihydroxybenzoic acid and 30 mM 1-hydroxyisoquinoline. Samples were desorbed/ionized from the probe tip with a nitrogen laser ($\lambda = 337$ nm) having a pulse width of 3 ns and delivering approximately 17 μJ of energy/laser pulse.

*Analytical methods.*—Glycosyl-residue compositions were determined by GC analysis of the per-*O*-trimethylsilylated methyl glycosides [25]. The TMS derivatives were separated on a fused-silica DB-1 capillary column (30 m × 0.32 mm i.d., 0.25 μm film) using temperature programming (120 °C to 200 °C at 1.5 °C/min) with $H_2$ as the carrier gas and identified by CI–MS on a HP-5989 MS-Engine (Hewlett–Packard, USA).

Protein contents were measured according to Lowry et al. [26]. Methyl groups were assayed after de-esterification of RG-II in 0.5 M KOH (25 °C, 30 min), by enzymic oxidation of methanol by alcohol oxidase followed by colorimetric determination of formaldehyde [27]. Acetic acid was determined [11] by the enzymic–UV method (Boehringer–Mannheim, Germany).

The B, Ca, K, and Na contents were determined by inductively-coupled-plasma atomic-emission spectroscopy (ICP–AES) performed with a Jarrell-Ash 965 Atomcomp Plasma Emission spectrometer. Samples of wine RG-II (3–5 mg/mL in water) were injected into the nebulizer of the spectrometer and analyzed according to the manufacturer's instructions.

*Glycosyl-linkage determination.*—The glycosyl-linkage compositions of wine RG-II were determined by GC–EIMS of the partially methylated alditol acetates. RG-II (4 mg) was methylated using sodium methyl sulfinyl carbanion and methyl iodide in dimethyl sulfoxide [28]. Half of the methylated sample was then carboxyl-reduced with lithium triethylborodeuteride (Superdeuteride®, Aldrich, USA) [23,25]. Both methylated or methylated and carboxyl-reduced samples were hydrolyzed with 2 M trifluoroacetic acid (75 min at 120 °C), reduced with $NaBH_4$ and acetylated as described [29]. Partially methylated alditol acetates were analysed [11] either on a fused-silica DB-225 capillary column (30 m × 0.32 mm i.d., 0.25 μm film) using temperature programming (170 °C for 15 min, then 5 °C/min to 210 °C) or on the same fused-silica DB-1 capillary column as for TMS derivatives analyses but using temperature programming (145 °C for 10 min, then 2 °C/min to 190 °C). The identity of each methyl ether was confirmed by EIMS and their areas were corrected by response factors [30].

*Generation and study of RG-II backbone fragments.*—RG-II backbone fragments [19] were generated by partial acid hydrolysis of RG-II (10 mg) in 0.1 M trifluoroacetic acid (16 h at 80 °C). The acid was removed by evaporation and the products analysed by high performance anion-exchange chromatography (HPAEC) with a Dionex DX-300 chromatography system equipped with a PAD detector using a CarboPac PA-1 column (0.4 × 25 cm; Dionex, USA) with a CarboPac PA-1 guard column (0.4 × 5 cm). The column was eluted at 1 mL/min with a gradient of sodium acetate in 100 mM NaOH: 100 mM sodium acetate (0–5 min); linear gradient up to 500 mM sodium acetate (5–25 min); linear gradient up to 700 mM sodium acetate (25–50 min) [21]. The column was reequilibrated in initial conditions for 10 min prior to sample injection.

The elution times of the backbone fragments were compared with the elution times of 1,4-linked α-D-oligogalacturonides generated by treating polygalacturonic acid (Sigma, USA), 0.2% solution in 0.1 M sodium acetate pH 4.8, with a purified endopolygalacturonase (Megazyme, Australia, 4 nkat/mL, 40 °C, 2 min) and with those of di- and tri-galacturonic acid (Sigma, USA). The RG-II backbone fragments were also treated by the purified endo-polygalacturonase (28 nkat/mL, 40 °C, 16 h).

## 3. Results and discussion

*Isolation and purification of four RG-IIs from red wine.*—RG-II was detected, by the presence of its diagnostic sugars, in four fractions (RG-II**1-4**) obtained by DEAE

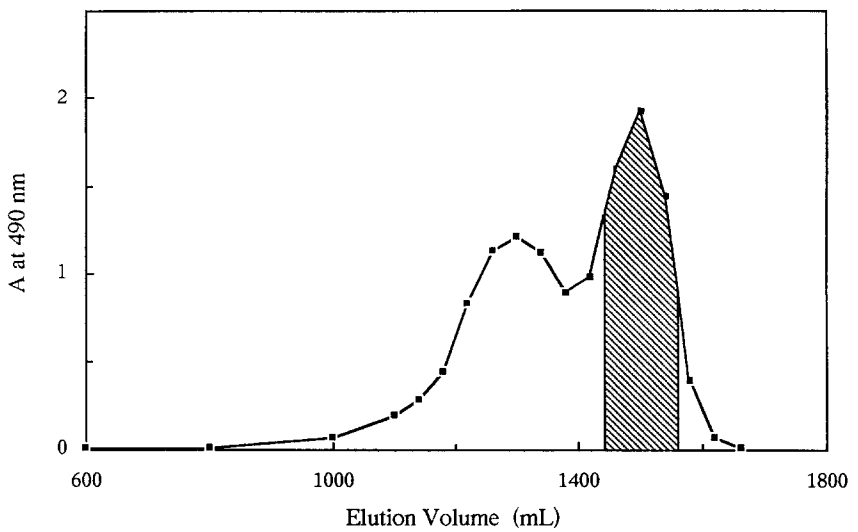


Fig. 2. Purification of a wine RG-II fraction by size-exclusion chromatography on Sephacryl S-400 HR. Fractions were assayed for total sugars by the phenol/sulfuric assay at 490 nm. The hatched area indicates the RG-II2 fraction that was collected.

Macroprep chromatography of wine polysaccharides. RG-II1 did not bind to the column at pH 4.6, RG-II2 was eluted with 10 mM NaCl, RG-II3 was eluted with 50 mM NaCl and RG-II4 was eluted with 150 mM NaCl. The four fractions all contained manno-proteins and arabinogalactans that were eliminated by a combination of Concanavalin A affinity and Sepharose S-400 HR size exclusion (Fig. 2) chromatographies. Fraction RG-II4 required an additional step on Sepharose S-200 HR. The recoveries of RG-II1 through 4 were 0.5, 13.2, 13.7, and 5.8 g, respectively from 176.5 g of total wine polysaccharides. Thus, RG-II, which accounts for ~ 20% of the ethanol-precipitable polysaccharides, is the second most abundant grape-deriving polysaccharides in red wine; arabinogalactans are the most abundant [23].

*The glycosyl-residue, O-acetyl, and methyl-ester contents of wine RG-IIs.*—The four purified fractions have similar glycosyl-residue compositions (Table 1) and contain those monosaccharides, 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, apiose, Kdo, Dha, and aceric acid, that are diagnostic of RG-II. The molar ratios of the glycosyl-residues, except aceric acid, are in agreement with the theoretical values expected from the proposed structure of RG-II (Fig. 1) with ~ 3.5 mol% for one residue in the molecule. The low recovery of aceric acid may be due to the acid-lability of this sugar [31].

All the RG-IIs contain ~ 2 *O*-acetyl groups per monomeric RG-II, a result that is consistent with the suggestion that side chain B (Fig. 1) contains two *O*-acetyl groups [21]. In contrast, the degree of methyl-esterification of the four fractions differed (Table 1). RG-II1 contains ~ 3 methyl-esterified uronic acid residues whereas RG-II4 contains only ~ 2 methyl-esterified uronic acid residues. Thus, our results provide evidence that some of the uronic acid residues of RG-II are methyl-esterified, a feature which has been suggested for sycamore RG-II [17], although the procedure used for its purification from

Table 1
Composition analysis of four RG-II fractions isolated from red wine

| | RG-II1 | RG-II2 | RG-II3 | RG-II4 |
|---|---|---|---|---|
| % of total wine polysaccharides | 0.3 | 7.5 | 7.8 | 3.3 |
| Rhamnose [a] | 14.7 | 16.8 | 16.9 | 18.3 |
| 2-*O*-$CH_3$-Fucose [a] | 4.0 | 3.7 | 4.2 | 4.4 |
| Fucose [a] | 3.7 | 3.0 | 3.6 | 3.5 |
| 2-*O*-$CH_3$-Xylose [a] | 3.5 | 2.8 | 3.1 | 3.1 |
| Apiose [a] | 5.8 | 6.2 | 7.2 | 7.4 |
| Arabinose [a] | 10.5 | 11.2 | 9.7 | 9.6 |
| Galactose [a] | 7.0 | 6.6 | 5.0 | 4.9 |
| Aceric acid [a] | 2.4 | 1.2 | 2.2 | 1.5 |
| Galacturonic acid [a] | 35.7 | 38.2 | 37.2 | 36.9 |
| Glucuronic acid [a] | 5.4 | 3.3 | 3.4 | 3.5 |
| Kdo [a] | 4.4 | 4.4 | 5.0 | 4.4 |
| Dha [a] | 2.9 | 2.6 | 2.5 | 2.5 |
| Methanol [b] | 1.5 | 1.4 | 1.3 | 1.1 |
| Acetic acid [b] | 1.5 | 1.6 | 1.8 | 1.7 |
| Protein [b] | 1.1 | 0.6 | 0.6 | 0.6 |
| B [b] | nd [c] | nd [c] | 0.09 | 0.06 |
| Ca [b] | 0.19 | 0.51 | 0.39 | 0.47 |
| K [b] | 0.13 | 0.09 | 0.13 | 0.21 |
| Na [b] | 2.49 | 3.40 | 2.11 | 1.83 |

[a] Mol%.
[b] Percent of dry matter.
[c] Not detected.

plant cell walls includes a de-esterification step prior to endo-polygalacturonase treatment [4,25]. Furthermore, the differences in the extent of methyl-esterification of wine RG-II may be one of the factors that contribute to their separation by anion-exchange chromatography.

*The homogeneity and boron content of wine RG-IIs.*—Previous studies have shown that RG-II exists as a monomer and a dimer that is cross-linked by borate di-esters [18,19]; these two forms are well separated by Superdex-75 HR size-exclusion chromatography [19]. We now provide evidence that wine RG-II also exists as a monomer and a dimer that contains borate di-esters.

The Superdex-75 HR profiles obtained with the four fractions shows that RG-II**1** and **2** are predominantly monomer (Fig. 3a and b), whereas RG-II**3** (Fig. 3c) is predominantly a dimer (87% of the total RI response). In contrast, RG-II**4** is 55% monomer and 45% dimer (Fig. 3d). ICP–AES analysis established that only RG-II**3** and RG-II**4** contain boron (Table 1) and provides additional evidence that at least some of the wine RG-II is cross-linked by borate di-esters. Boron is known as a wine inorganic constituent for more than a century [32] and more recent $^{11}B$ NMR studies have reported the presence of borate di-esters in red wine [33].

As anticipated, the four fractions also contain significant amounts of Na, K, and Ca (Table 1).

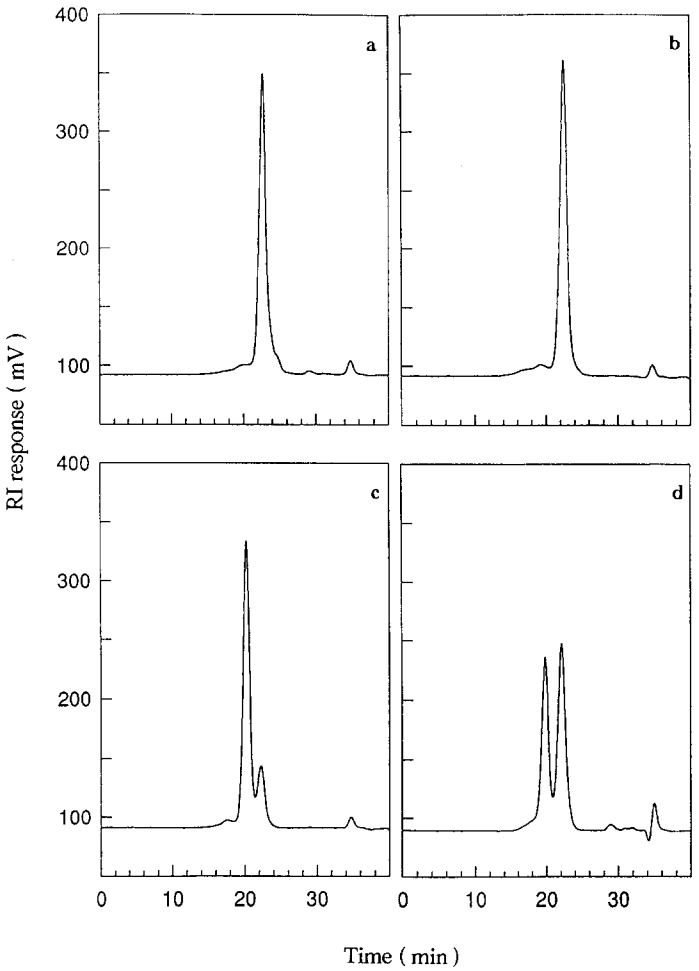


Fig. 3. Superdex-75 high-resolution size-exclusion chromatography of RG-II**1** (a), **2** (b), **3** (c) and **4** (d).

*The molecular weight of wine RG-II2.*—The molecular weight of wine RG-II has been estimated to be 5.3 kDa by light scattering and 9.8 kDa by universal calibration of size-exclusion chromatography [11]. However, the presence of monomer or dimer of RG-II in the preparation used in that study could not be established. We have now determined the molecular weight of RG-II**2** by MALDI–TOF MS analysis. The interpretation of the MS data is facilitated by the fact that RG-II**2** is almost exclusively in its monomeric form (Fig. 3b). The MALDI–TOF mass spectrum of RG-II**2** (Fig. 4) contains a broad signal at $m/z$ 4717 that we have assigned to the $[M + H]^+$ ion of monomeric RG-II. This value is in good agreement with the calculated molecular weight of the wine RG-II monomer (weight = 4684 Da) if it is assumed that the backbone contains eight galactosyluronic acid residues and that there are two *O*-acetyl groups and

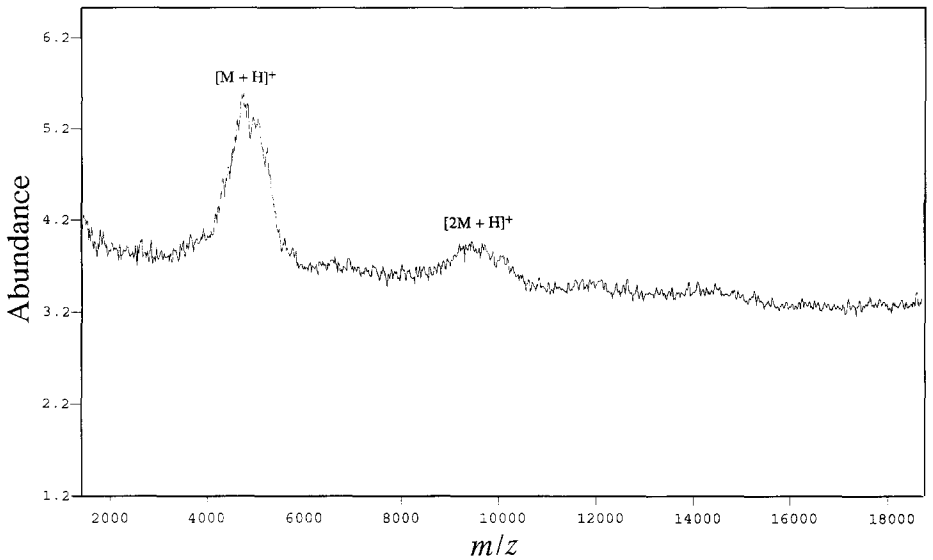


Fig. 4. MALDI–TOF mass spectrometry of RG-II2. The mass spectrum contains one main peak corresponding to the single charged $[M+H]^+$ ion at $m/z$ 4717. The signal at $m/z$ 9500 may correspond to the $[2M+H]^+$ ion since RG-II2 contains virtually no dimer (Fig. 3).

three methyl-ester groups present. The broad signal at $m/z$ 9500 may correspond to the $[2M + H]^+$ ion of monomeric RG-II since RG-II2 contains virtually no dimer.

*Glycosyl-linkage compositions of the four wine RG-IIs.*—The four RG-II fractions were per-*O*-methylated [28] and the methyl-esterified carboxyl groups then reduced with lithium triethylborodeuteride prior to hydrolysis, reduction, and acetylation. This procedure was similar to that reported in the study of Pectinol RG-II [15] but the second methylation step was omitted. This procedure allows the galactose derivatives originating from galactosyluronic acid residues to be identified unambiguously since they are *O*-acetylated at C-6 [23]. For example, a 4-linked galactosyluronic acid residue generates 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-6,6′-dideuterio-galactitol. In contrast, the galactosyl residues in RG-II all give derivatives that are methylated at C-6. The identification of all the derivatives and especially the presence of dideuteriohexitols arising from acid monosaccharides were confirmed by EI–MS analyses. Furthermore, this procedure can be used to determine the linkage of the aceryl residues since the methylated alditol acetate derivative corresponding to 2-linked aceric acid was identified by GC–EIMS (Fig. 5). This is, to our knowledge, the first time that the linkage of aceric acid could be successfully characterized by glycosyl-linkage composition analysis. This might be due to the lack in our procedure of a remethylation step after carboxyl-reduction with Li-triethylborodeuteride contrary to previous studies on RG-II [4,8,20].

The glycosyl-linkage compositions of the four RG-IIs are, with the exception of the apiosyl residues, similar (Table 2) and are in accordance with the relative sugar molar ratios obtained from compositional (TMS derivatives) analyses. Almost all the derivatives identified originate from the known glycosyl residues of RG-II (Fig. 1) and molar

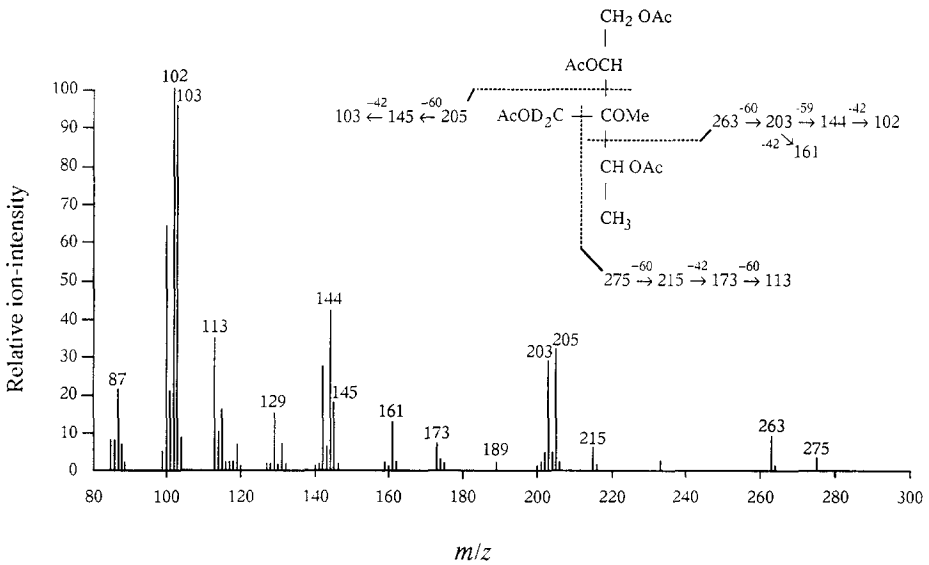


Fig. 5. EI–mass spectrum of 3-methyl ether of aceric acid (relative retention time to myo-inositol hexaacetate, 0.553 on DB-1), obtained after per-*O*-methylation of RG-II, followed by carboxyl-reduction of the uronosyl groups with Li-triethylborodeuteride, hydrolysis, reduction, and acetylation. Secondary fragment ions are indicated.

ratios are in agreement with the theoretical values expected (see for instance the 3- and 2,3,4-linked rhamnosyl, terminal non-reducing 2-*O*-methylfucosyl, terminal non-reducing 2-*O*-methylxylosyl, 3,4-linked fucosyl, 2,4-linked galactosyl residues). Thus, glycosyl-linkage analysis confirm that wine and cell wall RG-II [1] have similar and conserved structures. However, we have detected in wine RG-II two glycosyl-linkages (2,4-linked Rha*p* and 3,4-linked Gal*p*, see Table 2) that are not present in sycamore RG-II. The 3,4-linked Gal*p* residue is also present in Pectinol [20] and Douglas fir [3] RG-II which suggests that some of the terminal non-reducing galactosyl residues attached to side chain A (Fig. 1) are substituted with other sugars. However, the possibility cannot be discounted that the 3,4-linked Gal*p* results from the incomplete methylation of the terminal non-reducing galactosyl residue.

Total apiose represents ~ 9% of total methyl ethers in the four fractions. However, apiose is present only as 3′-linked residues in RG-II**1** and RG-II**2** whereas it is also present as 2,3,3′-linked residues in the dimer and boron-containing RG-II**3** and RG-II**4**. The amount of 2,3,3′-linked apiose is maximum in RG-II**3** (87% dimer) and provides additional evidence that the borate di-esters may be located on C-2 and C-3 of an apiosyl residue of the RG-II molecule [19].

Galacturonic acid is present as terminal non-reducing, 4-, 3,4-, 2,4-, and 2,3,4-linked residues (Table 2). These residues are all known to be components of sycamore RG-II even though the 2,3,4-linked Gal*p*A residue has not been included in any structural model of RG-II. We suggest that one of the 4-linked Gal*p*A residues in the backbone may be substituted with two oligosaccharide side chains since the results of previous

Table 2
Glycosyl-linkages of four carboxyl-reduced RG-II fractions isolated from red wine

| Methyl ether | Linkage | Residue | RG-II1 | RG-II2 | RG-II3 | RG-II4 |
|---|---|---|---|---|---|---|
| 2,3,4-Rha [a] | terminal | B6, C2 | 6.0 | 5.9 | 7.2 | 7.4 |
| 3,4-Rha | 2-linked | B6 | 8.2 | 5.8 | 6.3 | 6.2 |
| 2,4-Rha | 3-linked | B2 | 4.5 | 4.6 | 4.9 | 4.6 |
| 3-Rha | 2,4-linked | unknown | 1.7 | 1.7 | 0.8 | 0.8 |
| Rha | 2,3,4-linked | A2 | 4.4 | 4.6 | 5.0 | 4.5 |
| Total rhamnose [b] | | | (24.8) | (22.6) | (24.2) | (24.3) |
| 2,3,4-Fuc | terminal | B4′ | 3.2 | 3.1 | 3.3 | 3.4 |
| Total 2-*O*-$CH_3$-fucose | | | (3.2) | (3.1) | (3.3) | (3.4) |
| 2-Fuc | 3,4-linked | A3 | 3.1 | 2.9 | 3.7 | 3.6 |
| Total fucose | | | (3.1) | (2.9) | (3.7) | (3.6) |
| 2,3,4-Xyl | terminal | A3′ | 3.1 | 3.0 | 2.9 | 2.6 |
| Total 2-*O*-$CH_3$-xylose | | | (3.1) | (3.0) | (2.9) | (2.6) |
| 2,3-Api | 3′-linked | A1 B1 | 9.0 | 9.3 | 6.5 | 8.1 |
| Api | 2,3,3′-linked | A1 or B1 | 0.0 | 0.0 | 2.1 | 0.7 |
| Total apiose | | | (9.0) | (9.3) | (8.6) | (8.8) |
| 2,3,5-Ara | terminal | B7, D2 | 7.2 | 6.4 | 6.8 | 7.1 |
| 3,4-Ara | 2-linked | B5 | 1.9 | 2.1 | 0.8 | 0.9 |
| 4-Ara | 2,3-linked | B5 | 2.0 | 2.2 | 3.6 | 3,4 |
| Total arabinose | | | (11.1) | (10.7) | (11.2) | (11.4) |
| 2,3,4,6-Gal | terminal | A5 | 1.6 | 1.6 | 1.3 | 1.3 |
| 2,6-Gal | 3,4-linked | unknown | 2.3 | 2.3 | 1.3 | 1.4 |
| 3,6-Gal | 2,4-linked | B4 | 3.9 | 4.2 | 4.1 | 4.0 |
| Total galactose | | | (7.8) | (8.1) | (6.7) | (6.7) |
| 3-Acer | 2-linked | B3 | 2.3 | 2.1 | 1.9 | 1.7 |
| Total aceric acid | | | (2.3) | (2.1) | (1.9) | (1.7) |
| 2,3,4-GalA [c] | terminal | A2′, A2″ | 10.3 | 11.1 | 11.2 | 10.7 |
| 2,3-GalA [c] | 4-linked | | 11.5 | 9.0 | 9.7 | 10.9 |
| 2-GalA [c] | 3,4-linked | | 4.6 | 7.0 | 6.5 | 5.0 |
| 3-GalA [c] | 2,4-linked | | 4.1 | 4.7 | 5.9 | 5.5 |
| GalA [c] | 2,3,4-linked | | 2.4 | 3.9 | 3.3 | 2.5 |
| Total GalA | | | (32.9) | (35.7) | (36.6) | (35.7) |
| 2,3,4-GlcA [c] | terminal | A4 | 0.3 | 0.2 | 0.4 | 0.6 |
| 3,4-GlcA [c] | 2-linked | A4 | 2.4 | 2.3 | 2.3 | 3.1 |
| Total GlcA | | | (2.7) | (2.5) | (2.7) | (3.7) |

[a] 2,3,4-Rha denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol, etc.
[b] Relative mole percent of each parent sugar family (sum of ethers from one sugar type) within total sugars.
[c] 6,6′-dideuterated ether.

studies do not exclude this possibility [20]. However, additional evidence is required to substantiate the suggestion.

Dha and Kdo are both degraded under the acidic conditions used to cleave glycosidic linkages [15] and their methyl ethers were not determined in this study.

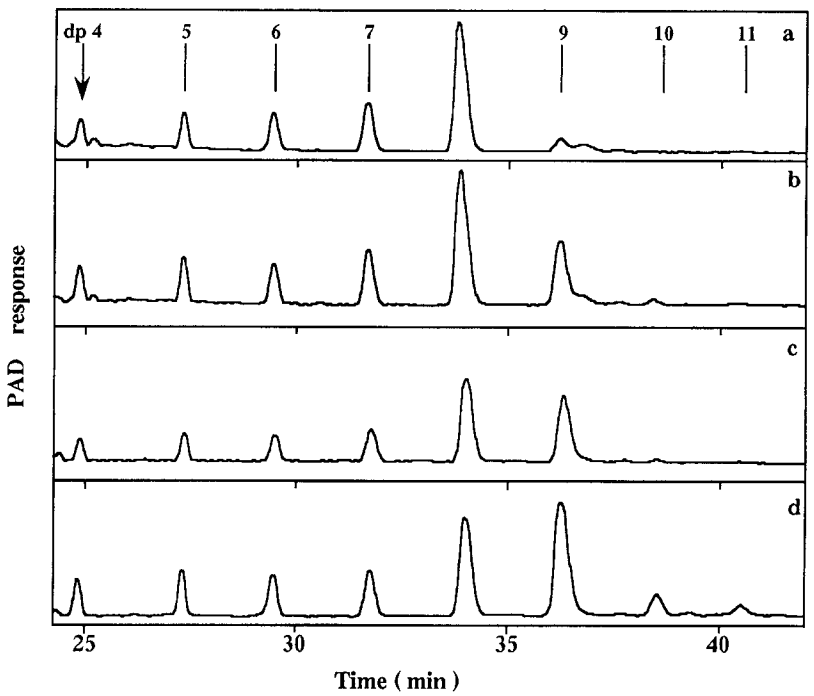


Fig. 6. HPAEC analysis of the backbone fragments released by partial acid hydrolysis of RG-II1 (a), RG-II2 (b), RG-II3 (c) and RG-II4 (d). Elution times of a series of 1,4-linked α-D-galacturonides with their respective dp are shown. Peaks eluting during the initial 20 min correspond to side-chain fragments released by partial acid hydrolysis [21] and are not shown.

*Determination of the degree of polymerization of the backbone of the wine RG-IIs.*—Evidence was reported in two previous studies that the backbone of sycamore RG-II is composed of at least seven and up to eleven 1,4-linked Gal*p*A residues [17,21]. We now provide evidence showing that the backbone of wine RG-II contains at least eight and up to fifteen residues. Backbone fragments were generated by treating the wine RG-IIs with 0.1 M trifluoroacetic acid for 16 h at 80 °C. The hydrolysates were then analyzed by HPAEC and each one shown to contain a series of components that co-chromatographed with standard 1,4-linked α-D-galacturonides (Fig. 6). These components are 1,4-linked α-D-galacturonides since they are hydrolyzed by treatment with endo-polygalacturonase (Fig. 7). However, the average dp's of the homogalacturonan backbone of the four wine RG-IIs are not identical. For example, the backbones of RG-II1 and RG-II4 have an average dp of 8 and 9, respectively (Fig. 6). Furthermore, fragments up to a dp of 15 were detected in RG-II4 (Fig. 7). These results confirm that the dp of the backbone varies and provide additional evidence that RG-II is linked to homogalacturonan. We conclude that the variation in length of the backbone may result from the incomplete hydrolysis of the homogalacturonan to which RG-II is linked. This

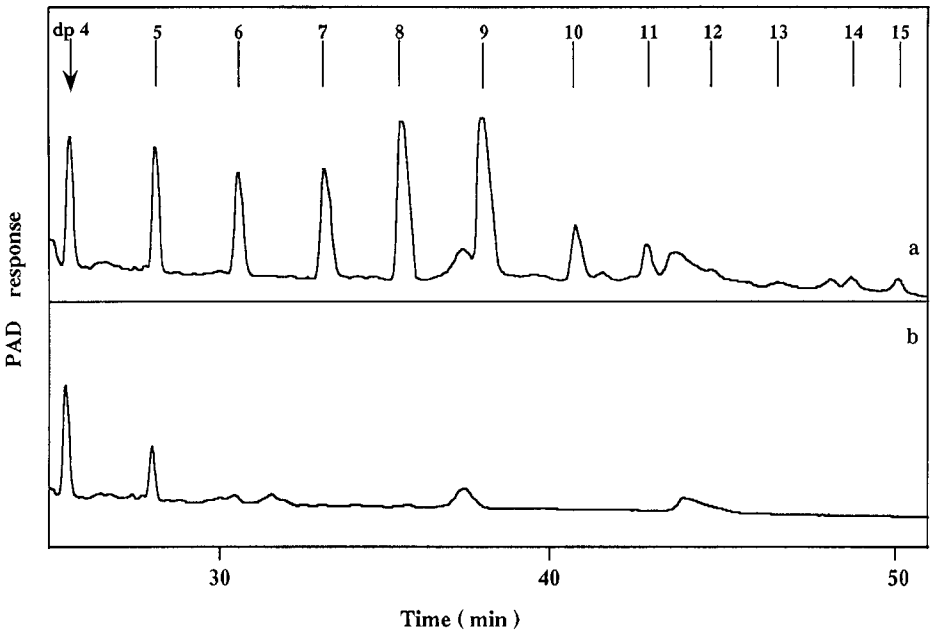


Fig. 7. HPAEC analysis of the backbone fragments released by partial acid hydrolysis of RG-II**4**, before (a) and after (b) treatment by a purified endo-polygalacturonase. Elution times of a series of 1,4-linked α-D-galacturonides with their respective dp are shown.

variation may be another factor which contributes to the separation of wine RG-II by anion-exchange chromatography.

## 4. Conclusions

The results of this study have confirmed that red wine is an abundant source of RG-II since the wine sample used in the present study contained at least 50 mg/L of RG-II and that wine and primary cell wall RG-II have similar, if not identical, structures. RG-II is present in wine as a monomer and a dimer that is cross-linked by a borate di-ester located on a 3′-linked apiosyl residue [19]. The fact that the RG-II dimer is present in red wine, which typically has a pH between 3.5 and 3.8, provides additional evidence that the di-ester cross-link is somewhat stable between pH 3 and 4 [19]. Nevertheless, the dimer may have been partially hydrolyzed in the red wine or during the sample preparation since the monomer accounts for 55% of the wine RG-II studied. However, the possibility cannot be discounted that similar amounts of monomeric and dimeric RG-II are present in grape berries. Furthermore, the ratios of monomeric and dimeric RG-II may be determined by the boron status of the grape plants. Boron is an essential microelement for the growth of the vine [34].

There are discernible differences in the structures of the four RG-IIs isolated from red wine polysaccharides by anion-exchange chromatography. These differences include

variations in the length and degree of methyl-esterification of the homogalacturonan backbone and the presence or absence of borate di-esters. Such differences will be reflected in the negative charge density of RG-II and thus determine the chromatographic properties of RG-II.

The role of RG-II in determining the properties of red wine remains to be determined. Nevertheless, we have shown that RG-II can be readily isolated from red wine in quantities that will facilitate the structural characterization of this complex polysaccharide which remains a challenging task.

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