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ORIGINAL PAPER

Characterization of oxidized tannins: comparison of depolymerization methods, asymmetric flow field-flow fractionation and small-angle X-ray scattering

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Abstract Condensed tannins are a major class of plant polyphenols. They play an important part in the colour and taste of foods and beverages. Due to their chemical reactivity, tannins are not stable once extracted from plants. A number of chemical reactions can take place, leading to structural changes of the native structures to give so-called derived tannins and pigments. This paper compares results obtained on native and oxidized tannins with different techniques: depolymerization followed by high-performance liquid chromatography analysis, small-angle X-ray scattering (SAXS) and asymmetric flow field-flow fractionation (AF4). Upon oxidation, new macromolecules were formed. Thioglycolysis experiments showed no evidence of molecular weight increase, but thioglycolysis yields drastically decreased. When oxidation was performed at high concentration (e.g., 10 gL^{-1}), the weight average degree of polymerization determined from SAXS increased, whereas it remained stable when oxidation was done at low concentration (0.1 gL^{-1}), indicating that the reaction was intramolecular, yet the conformations were different. Differences in terms of solubility were observed; ethanol being a better solvent than water. We also separated soluble and non-water-soluble species of a much oxidized fraction. Thioglycolysis showed no big differences between the two fractions, whereas SAXS and AF4 showed that insoluble macromolecules have a weight average molecular weight ten times higher than the soluble ones.

Keywords Tannins · Oxidation · Molecular weight determination · Small-angle X-ray scattering · AF4-MALLS · Depolymerization

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Introduction

Two groups of flavonoids, anthocyanins and flavanols, play a major role in the development of the colour and taste (astringency and bitterness) of red wines and are particu-

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larly important to their quality. They can also be involved in colloidal instabilities (formation of hazes and precipitates) that are detrimental for this quality. Flavanols in grapes exist as monomers and as condensed tannins, i.e. oligomers and polymers of flavan-3-ol units primarily linked by C4–C8 bonds (Fig. 1). Native (*grape*) tannins differ by the nature of their constitutive units (catechin, epicatechin, epigallocatechin and epicatechin-gallate) as well as by their degree of polymerization. They constitute then a complex mixture of (macro)molecules with different structures. This complexity is increased during wine making and ageing: once extracted, flavonoids undergo several biochemical/chemical changes, leading to the formation of so-called derived pigments and tannins [1, 2]. These structural modifications are of importance in enology as these new compounds, which represent a large part of wine tannins, are expected to exhibit properties that are different from those of their precursors. Identification of the main reaction pathways and of the resulting structural changes is thus needed to establish the relationships between wine polyphenol composition and quality, but also to determine the impact of winemaking practices on this composition. A major difficulty is the identification of the chemical changes induced by tannin reactivity and their impact on their molecular weight distribution and conformation in solution. This information is necessary to link these changes with the technological and organoleptic properties of wine polyphenols. Problems encountered concern both the separation of these polymers for their structural analysis and the methods currently available to achieve this analysis.

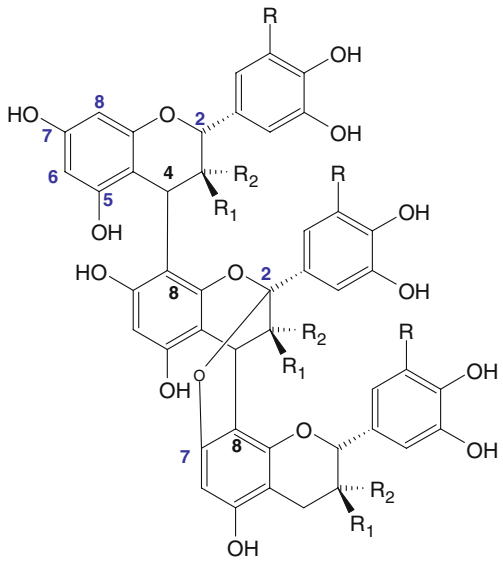

Fig. 1 Structure of condensed tannins. Flavanol monomer units can be linked by C4–C8 bonds (*upper unit*), C4–C6 bonds (not shown) or both a C4–C8 and an ether C2–O–C7 bond, also called A-type bond (*lower units*). A-type bonds are present for instance in native cranberry tannins

Methods used to characterize condensed tannins can be classified into several categories: colorimetric methods, chromatographic methods, depolymerization methods, etc. Various global quantification methods are based on the UV–Vis spectrophotometric quantification of coloured products formed after the reaction of polyphenols with e.g. Folin–Ciocalteu reagent [3] or with an aromatic aldehyde (e.g. vanillin [4] or dimethylaminocinnamaldehyde (DMAC) [5]). However, their results are considerably affected by the type of condensed tannins (also named proanthocyanidins) and the conditions used [6], or are even affected by other reducing substances such as some sugars or amino acids in the case of Folin–Ciocalteu test.

Analysis of condensed tannins by high-performance liquid chromatography (HPLC) is difficult because proanthocyanidins (PAs) are complex mixtures. In reversed-phase HPLC or UPLC, the separation of large polymers (degree of polymerization, DP>4) is not possible [7]. In normal/diol phase HPLC, PAs (for instance cocoa PAs) can be separated according to their degree of polymerization up to DP 14 [8]. For higher molecular weight species, proanthocyanidins appear as a broad unresolved hump. Electrospray ionization (ESI) mass spectrometry has proven to be very efficient for structural analysis of polyphenols, but it has shown limitations in the evaluation of the molecular weight distributions of tannin mixtures. That is, ESI mass spectra of tannin mixtures are always dominated by the lowest molecular weight (MW) components with peak intensities diminishing as polymer chain length increases, in a pattern resembling exponential decay [9, 10]. This is also observed with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, although the latter has demonstrated a better ability to detect larger polymers than the ESI systems. The fractionation of crude tannin extracts prior to mass analysis greatly improves the detection of larger molecular weight species [9]. Size distribution in native tannin fractions can be obtained by size exclusion chromatography (SEC) [11]. However, there is a current lack in available polyphenolic standards for calibration (polystyrene standards are commonly used, but their use may lead to discrepancies); it is possible to perform a calibration with tannins with different molecular weight, but the obtention of highly pure and monodisperse fraction is delicate, and the monomeric composition of tannins plays an important part: for instance, grape seed tannins have a SEC behaviour different from grape skin tannins [11]. Last, but not least, calibration is done on hydrodynamic bases, without knowing if tannins are linear or branched. Due to difficulties in analysing polymeric species, condensed tannins are, therefore, depolymerised before HPLC analysis. The analysis of native condensed tannins is usually achieved by acid-catalyzed cleavage of the interflavan bond in the presence of a

nucleophilic reagent (e.g. benzylthioether or thioglycolic acid), followed by HPLC analysis: flavan-3-ol extender units are converted into the corresponding adducts, whereas the terminal units are released as monomeric flavan-3-ols [12–14]. This gives access to the number average degree of polymerization (DP) of the fraction and to its composition in flavanol units, but does not provide information on its polydispersity. Besides, these chromatographic techniques do not give information concerning tannin conformation in solution and the changes induced by their reactivity. Yet, this conformation is of importance as some of their properties are related to physico-chemical interactions, between themselves or with other biopolymers [1].

In addition to the limitations previously listed when dealing with native tannins, chemical changes occurring during wine ageing increase the initial polydispersity (changes in molecular weight, branching, etc.) and result in the formation of new covalent bonds between units, some of them being resistant to acid-catalyzed cleavage. Indeed, flavanol autoxidation, studied with monomers and dimers, can lead to both intermolecular and intramolecular reactions [15–17]. When dealing with polymers, competition between intra- and intermolecular reactions, as well as between internal and terminal units, is expected (Fig. 2). These competitions determine changes in tannin DP, in their flexibility and the type of polymers produced (linear versus branched polymers). As a consequence, the estimation of tannin DP becomes inaccurate during wine ageing: yields of the depolymerization reaction decrease. In addition, interactions with chromatographic supports are enhanced, leading to an irreversible adsorption. In order to improve the analysis of oxidized tannins, it is necessary to improve both tannin separation and detection. In this paper, we focused on two techniques: asymmetric flow field-flow fractionation (AF4) and small-angle X-ray scattering (SAXS), the first one being used to fractionate tannins, the second one being used to determine the conformation of native and oxidized tannins in solution.

AF4 is a separative technique which does not use any support. It is based on a liquid flow field in a semi-permeable channel. Because of the parabolic main flow profile, the macromolecules are size-separated (from the smallest to the largest ones) under a field of carrier solvent (named crossflow) [18]. A multi-angle laser light scattering detector (MALLS) associated with a concentration detector (UV or DRI) permits to determine size and absolute mass of the analyzed macromolecules without need for calibration (limitations due to the possible existence of ramified species are also swept aside). One of the main advantages of AF4 compared to SEC is the lack of interactions (in optimized conditions) between analytes and the membrane covering the channel. Purawatt et al. [19] studied the complexation of tannic and phytic acids with iron by means of AF4–ICP–OES (flow field–flow fractionation–inductively coupled plasma optical emission spectrometry), but to our knowledge, nobody has used AF4 to determine the size distribution of condensed tannins in ethanol. AF4 was used in this study in organic mode to evaluate the distribution and the mass of condensed oxidized tannins, using ethanol as carrier in the channel, with a UV detector and a multi-angle laser light scattering (MALLS) detector in order to obtain directly the molecular weight and the radius of gyration of separated tannins.

SAXS gives access to the weight average molecular weight and conformation of macromolecules in solutions [20]. In a previous work, we have used SAXS to study tannin conformation in ethanol. We also have evidenced that SAXS could be used, after calibration with native tannins, to determine changes in their weight average molecular weight and conformation after oxidation [21]. Attention in this work was focused on autoxidation reactions. In quite concentrated acidic solutions (5 gL^{-1},

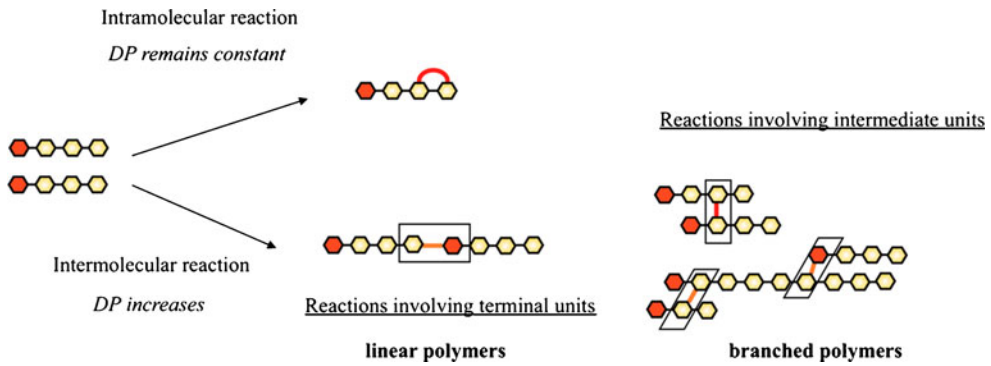

Fig. 2 Schematic representation of oxidized tannins. According to current knowledge on monomers and dimers, the creation of new bonds may occur between two macromolecules (intermolecular bonding) or on the same macromolecule (intramolecular bonding) leading to the formation of an A-type tannin. In the case of intermolecular reactions, the new bond is formed between A and B aromatic rings. Further oxidation may lead to additional cyclisation between rings A and B. In the case of polymers or oligomers, if bonding is purely intramolecular, the average DP should stagnate, whereas it should increase if intermolecular reactions take place

pH 3.5), SAXS patterns evidenced an increase of tannin weight average molecular weight and structure upon autoxidation, attributed to intermolecular reactions. Comparison between SAXS and thiolysis data indicated that intramolecular reactions also took place. Also, autoxidation induced the formation of water-insoluble species. In the present paper and to complete these initial findings, we have used SAXS to study the impact of the concentration on bond formation during autoxidation in dilute solutions.

Materials and methods

Materials

Deionized water was obtained with a Milli-Q system (Millipore, Billerica, MA, USA). Chemicals (solvents, organic acids and reagents) were of analytical grade and purchased from VWR and Merck (Hohenbrunn, Germany). The commercial epicatechin dimer B2 called thereafter A-2 was purchased from Extrasynthèse (France). Three apple (A-6n, A-14n and A-40n) and one grape (G-9n) tannins fractions were purified from apple parenchyma and grape seeds, as described before [21, 22]. These fractions are referred to as A-Xn or G-Xn, where 'A' stands for apple and 'G' for grape seeds, 'X' being the number average molecular weight determined by thioglycolysis. 'n' stands for native (i.e. non-oxidized tannins) and is replaced with 'ox' when oxidized tannins are studied. These fractions were used to get the relationship between the average DP and the scattering intensity at null Q obtained from SAXS experiments. In order to study the effect of concentration on oxidation mechanisms (intra- vs intermolecular reactions), the A-14 fraction was oxidized at pH 3.5 (which is considered as a relevant pH in enology) according to the procedure described in reference [21], either in dilute (0.1 g L^{-1}) or concentrated (10 gL^{-1}) solutions. Oxidations were stopped after 7, 14 and 25 days. Samples will be referred to as A-14ox dil or conc (for dilute or concentrated oxidation) yd, 'y' being the duration of oxidation in days.

Finally, the last grape seed tannin fraction used in this study was prepared in 2004 (*Vitis vinifera*, var. Shiraz) as described before [22]. Just after purification, its number average degree of polymerization was 15.6 determined by thiolysis (reaction yield, 78%). This native fraction, which is no longer available, will be referred to as G-15. Even though it was kept away from light and stored under vacuum, the fraction evolved (oxidation during storage) after 5 years (changes in colour and in thiolysis yield after analysis). We decided to use this highly oxidized fraction to determine if there were any size differences between native and oxidized tannins, and among these, if water-soluble ones were different from insoluble ones. Oxidized tannins were thus dispersed in an aqueous solution, and water-soluble (named G-15 ox-sol thereafter) and insoluble (G-15 ox-insol) species were separated by centrifugation. They were then freeze-dried and re-dissolved in ethanol for AF4 and SAXS. It is important to note that water-insoluble species were not fully dissolved in ethanol. Aggregates were eliminated by filtration before experiments.

Methods

Tannin analysis

Thioglycolysis was carried out with 100 μL of tannin solution (at a concentration of 1 gL^{-1} in methanol) added to 100 μL of thioglycolic acid solution (0.8% *v*/*v* in 0.2 M HCl in methanol), in a sealed glassware. The mixture was heated at 90 °C during 6 min and then cooled in ice. Ten microlitres of the end mixture was injected in the LC system, an Alliance Waters system (Milford, MA, USA) equipped with a photodiode array detector, and a Millenium32 data manager software. The column was a reversed-phase Atlantis C18 (250×2.1 mm, 5-μm packing) protected with a guard column of the same material (20×2.1 mm, 5-μm packing; Waters, Milford, MA, USA). Oven temperature was set at 38 °C. The solvent system was a gradient of solvent A (water/formic acid, 95:5, *v*/*v*) and solvent B (acetonitrile/water/formic acid, 80:15:5, *v*/*v*/*v*): initial 0% B, from 0 to 40% linear in 18 min, isocratic with 40% for 12 min, from 40% to 44% linear in 5 min, from 44% to 100% linear in 4 min, isocratic with 100% for 5 min followed by washing and re-equilibrating the column. Flow rate was 0.2 mL min^{-1}. All analyses were performed in triplicate, and calibration curves were established at 280 nm using external standards, either commercial ((+)-catechin and (−)-epicatechin) or isolated and purified in our laboratory (thioglycolic acid derivatives). The number average degree of polymerization (DPn) was calculated as the ratio between the summation of the molar concentrations of all released monomer constitutive units and the summation of the molar concentrations of terminal constitutive units.

SAXS

SAXS experiments were performed on the beamline SWING, at Synchrotron Soleil (Saint-Aubin, France). The incident beam energy was 12 keV (λ=1.03 Å); the distance from the sample to the Aviex CCD detector was 1,843 mm. The corresponding scattering vector $Q=4\pi \sin\theta/\lambda$ ranged from 0.005 to 0.529 $Å^{-1}$, where 2θ is the scattering angle and λ the incident wavelength. Experiments were performed at 25 °C. Several successive frames (typically 10) of 4 s each were recorded for both the sample and the

solvent (EtOH). We checked that X-rays did not cause any damage to the polyphenol molecules by comparing successive frames. The average intensity and experimental error of each set of frames were subsequently computed. Scattering from the solvent was measured and subtracted from the corresponding intensity of tannin solution. Concentrations ranged from 5 to 10 gL^{-1}.

The SAXS data were analyzed according to classical formulas for scattering from dispersions of particles or macromolecules in a homogeneous solvent [20]. For such dispersions, the intensity can be decomposed as a product of the intensity scattered by a single particle and a structure factor that describes interferences arising from different particles:

$$I(Q) = N_p\left(\rho_p - \rho_s\right)^2 V_p{}^2 P(Q) S(Q) \quad (1)$$

where ρ_p is the electron density of the particles, ρ_s that of the solvent, V_p the volume of a particle, N_p the number of particles per unit volume, $P(Q)$ the form factor of particle and $S(Q)$ the structure factor that describes the pair correlations between the positions of all particles. If the sample is a dilute solution, where the relative positions of the particles are not correlated, then $S(Q)=1$ at all Q values. If the sample is a dilute solution of polydisperse macromolecules, the intensity scattered in the $Q\rightarrow 0$ limit is proportional to a weight average molecular weight of macromolecules M_w, or degree of polymerization DP, and of the polymer concentration C:

$$I(Q \rightarrow 0) = I_0 \propto M_w C\left(\rho_p - \rho_s\right)^2 \quad (2)$$

I_0 is extrapolated from the experimental data after fitting them with different models for the form factor of the macromolecule [20, 23–25]. Spectra were finally fitted using the Fisher–Burford approximation [25] and taking the cross section of the macromolecular chain into account:

$$I_{FB,RCS}(Q) = I_0\left(1 + \frac{2}{3d_f}Q^2R_g^2\right)^{-\frac{d_f}{2}} \times e^{-\frac{Q^2R_{CS}^2}{2}} \quad (3)$$

where R_g the radius of gyration, d_f the self-similarity exponent, also called the fractal dimension, and R_{cs} is the radius of the chain cross section. Fits were done with standard software.

dn/dc determination

The differential index of refraction dn/dc at λ=620 nm was measured on an Optilab differential refractometer (Wyatt Technology Europe, Germany). Solutions of tannins in ethanol (with concentrations ranging from 0.2 to 2 mg L^{-1}) were injected, and data were treated using the software Astra 5.3.4.16 (Wyatt Technology Europe, Germany).

AF4-MALLS

Asymmetrical flow field-flow fractionation system used was a Postnova AF2000 (Postnova Analytics GmbH, Landsberg, Germany). Ethanol was used as mobile phase. The channel was equipped with a 350-μm spacer, and membrane (cut-off of 5 kDa) was made in cellulose material treated for organic solvents. Detector flow was set at 0.5 mL min^{-1}. A 4-min injection time and a 1-min transition time came before the elution step. The crossflow was set to 2 mL min^{-1}. The concentration detector was a Waters 486 UV spectrophotometer (Waters Corporation, Milford, MA, USA) tuned at 280 nm, and the MALLS was a seven-angle multi-angle light scattering detector (PN 3070) from Postnova (Postnova Analytics GmbH, Landsberg, Germany). Samples were prepared at a concentration of 0.5 mg mL^{-1} and filtered at 0.22 μm. An autosampler was used to inject 100-μL samples in the AF4 system. Data were treated with the AF2000 software from Postnova. For mass calculations, Zimm formalism was used with an extinction coefficient of 14.7 mL mg^{-1} cm^{-1}. The dn/dc was estimated about 0.248 mL g^{-1} for water-soluble tannins and 0.266 mL g^{-1} for water-insoluble tannins.

Recoveries of AF4 experiments were calculated comparing the area of the tannins UV peak (at 280 nm) after fractionation with the area of peaks obtained for the same injection but without any crossflow (only elution through the channel without fractionation):

$$R(\%) = \frac{S}{S_0} \quad (4)$$

with R the recovery, S the area obtained after fractionation and S_0 the area obtained for the same injection without any crossflow.

Results and discussion

Information about native tannins obtained from SAXS and thioglycolysis will be discussed first. These results will then be used to study oxidized tannins.

Native tannins

DPn: comparison of thioglycolysis and SAXS

Number average degree of polymerization, monomer composition and yields of the depolymerization reaction obtained by thioglycolysis on native tannins are compared in Table 1. In this calculation, only monomers and their thioglycolic acid derivatives were accounted for. For native tannins, reaction yields were in the range 70–85%.

Table 1 Number average degrees of polymerization DPn according to thioglycolysis, percentage of galloylation, yields of thiolysis before and after oxidation

	Tannin fraction	DPn	Yield of reaction (%)
Native fractions	A-6n	6±0.5	72±0.5
	G-9n	9±0.8	81±1.0
	A-14n	14±1.0	76±0.8
	A-40n	37.7±1.0	75±0.8
A-14 oxidized in diluted conditions	7 days	12.1±0.2	61.8±0.5
	14 days	11.6±0.5	46.5±0.5
	25 days	11.2±0.3	44.0±0.3
A-14 oxidized in concentrated conditions	7 days	12.7±0.3	67±3.0
	14 days	12.4±0.5	51±0.6
	25 days	12.1±0.4	41.6±0.5
Tannins oxidized in dry conditions	G-15n	15.6±0.6	78±0.8
	G-15ox-soluble	12.3±1.2	47±3.0
	G-15ox-insoluble	13.4±2.1	44±5.0

Experiments were done in triplicate

Small-angle X-ray spectra were recorded in ethanol, which is a better solvent than water, for native and oxidized fractions. The scattering curves of native tannins are compared in Fig. 3. They were obtained at concentrations C between 5 and 10 mg mL^{-1}, where the dilution is such that the SAXS intensities reflect the scattering from isolated macromolecules. They are typical of macromolecules in solution. Fitting parameters obtained with the Fisher–Burford equation are listed in Table 2.

As expected, the scattered intensities and radii of gyration increased with the degree of polymerization determined by thioglycolysis (Figure S1 in Electronic Supplementary Material). For native tannins, a linear relation between intensities from SAXS and molecular weights from thiolysis was obtained:

$$I_0/C = 3 \times 10^{-5} \times M_n \quad (5)$$

The correlation coefficient was 0.954 and took into account discrepancies between number and weight average molecular weights. This equation was exactly the same as

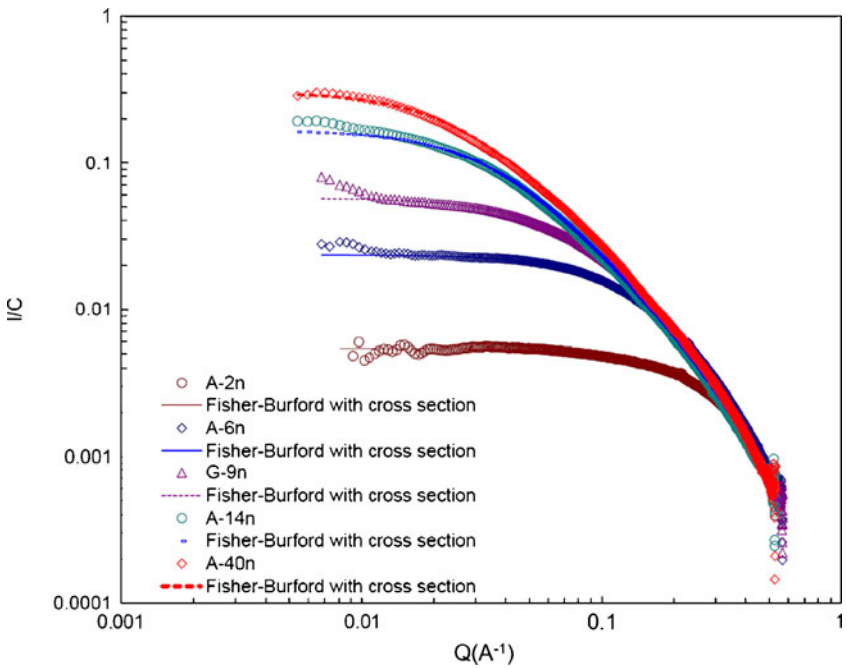

Fig. 3 SAXS intensity (normalized by concentration) of native tannins in the log I–log Q representation and fits obtained with the Fisher–Burford model

Table 2 Geometrical parameters derived from the Fisher–Burford model, taking the cross section of polymers into account

Tannin fraction	A-2n	A-6n	G-9n	A-14n	A-40n	A-14 ox-dil	A-14ox-conc	G-15ox-sol	G-15ox-insol
R_g (Å)	4.1	11.6	22.0	47.0	61.0	46.9	94.0	35.5	149.0
d_f	1.67	1.98	1.67	1.75	1.75	1.95	2.05	2.05	1.90
R_{cs} (Å)	3.00	2.75	3.20	2.70	2.80	2.10	2.00	2.50	/

already observed before, with other apple tannin fractions [21], which comforted us in the use of this calibration for oxidized tannins. In the section dealing with oxidized tannins, the SAXS intensities from oxidized tannins will be compared with those of native tannins, and this relation will be used to evaluate their average molecular weights.

Macromolecular parameters

As already observed [21], scattering curves were typical of macromolecules in solution, with cross section radii in the range 2.75–3 Å for epicatechin polymers. These cross section radii were slightly higher for polymers containing 20% of epicatechin gallate [21]: the presence of an additional galloyl group likely increases the chain thickness. Fractal dimensions d_f were all in the range 5/3–2, which corresponds to values classically observed with linear polymers in solution [26]: polymers in good solvent have a d_f of 1.5, linear swollen polymers a d_f of 5/3 and polymers in theta solvent have a d_f equals to 2.

Oxidized tannins

Comparison of oxidation in dilute and concentrated solutions

Autoxidation reactions were done in solution at two concentrations, differing from a factor of 100 (0.1 and 10 gL^{-1}). We expected to favour intermolecular reactions at high concentrations because a macromolecular chain has more probability to react with another neighbouring chain than at low concentration. Conversely, at very low concentrations, a macromolecule has more chances to react with itself.

The main features of thioglycolysis results are the decrease of the reaction yield accompanied with the stagnation of the number average degree of polymerization. This has already been observed with thiolysis instead of thioglycolysis [21], but hypothesis and conclusions are the same (Fig. 4):

1. As stated before, flavanol autoxidation reactions create new bonds [15–17], and new structures are formed

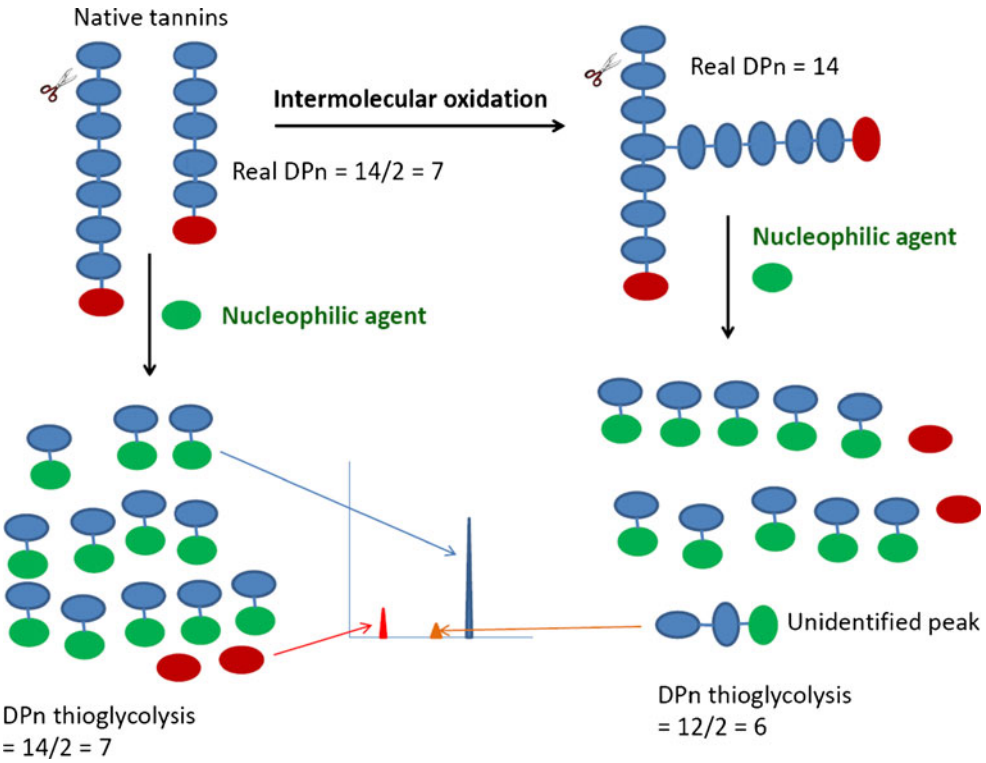

Fig. 4 Illustration of how thiolysis yields change with the formation of non-cleavable bonds such as those created by oxidation and how branching affects the determination of DPn

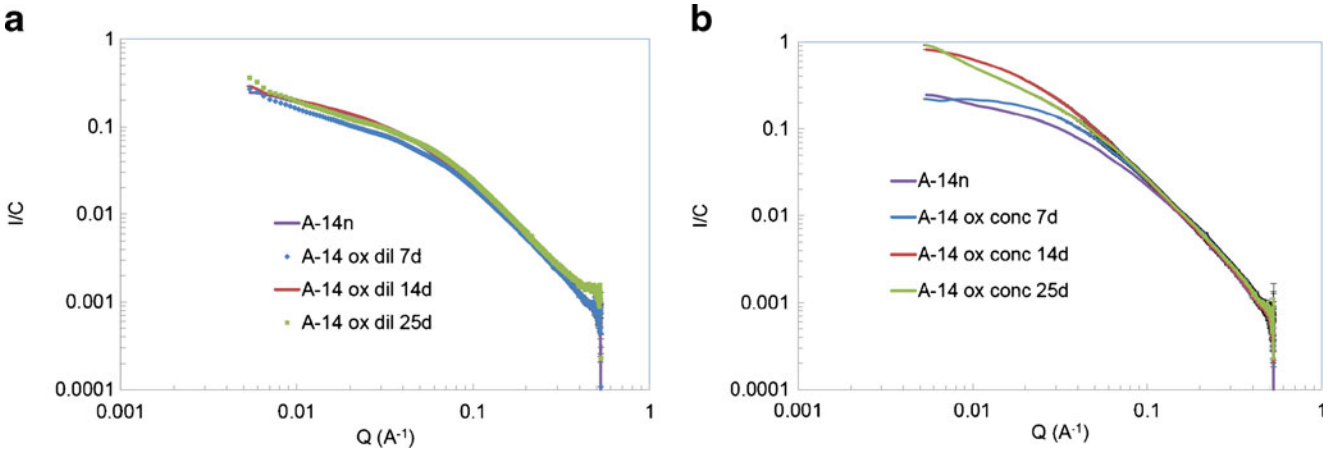

Fig. 5 Kinetics of oxidation followed by SAXS: SAXS intensity (normalized by concentration) of oxidized tannins in dilute (**a**) and concentrated (**b**) solutions, in the log *I*–log *Q* representation

(Fig. 2). These structures are partly resistant to acid-catalyzed cleavage. Thioglycolysis (or thiolysis) becomes thus inadequate to determine the number average degree of polymerization if only monomer units and their thioether derivatives are accounted for. The cleavage of oxidized tannins by thioglycolysis is likely incomplete: HPLC chromatograms taken after depolymerization show the apparition of new peaks when oxidized tannins are studied (results not shown). Some of these peaks may be thioglycolic acid A-type dimer and/or mixed A-type B-type trimers [27]. Taking these peaks into account would allow a better characterization of oxidized tannins; however, a full assignment (NMR study) and chain calibration would require isolation of a few milligrammes of these compounds.

2. The formation of branched polymers, having two, three or more so-called terminal units, is expected when intermolecular reactions take place. This results in an overestimation of the chain number because thiolysis is an end group titration, and thus in the underestimation of the DP.

The fact that both the formation of bonds resistant to thiolysis and the formation of intermolecular bonds play an important part in DP determination is illustrated by a scheme in Fig. 4. In these examples, the number average molecular weights determined by thiolysis slightly decrease, whereas the yields of thiolysis decrease, and the actual number average degree of polymerization either remains the same or increases [21].

Figure 5 shows the SAXS curves of native and oxidized A-14 tannins. In the case of A-14 oxidized at high concentration, an increase of the intensity scattered at low Q was observed. This reflects an increase of the weight average molecular weight of the macromolecules. In the case of tannins oxidized at low concentration, we did not observe such an increase. Radii of gyration and I_0 values were again derived from a fit with Eq. 3, and the ratio $M_w$ox/$M_w$nat (Table 3) was calculated using Eq. 5.

According to I_0/C values, the DP of apple tannins increased by less than 10% after 25 days oxidation in dilute solutions, whereas it was multiplied by five in concentrated solutions. These results show that even if there was a slight decrease of the apparent number average DP determined by thioglycolysis, the weight average DP measured through SAXS either remained stable or increased: intramolecular reactions almost exclusively took place in dilute solutions, whereas intermolecular reactions occurred in concentrated ones. This does not exclude the possibility of the formation of intramolecular bonds in the latter case. In concentrated solutions, we found $\frac{I_{ox}}{I_n} \propto \left(\frac{R_{g\,ox}}{R_{gn}}\right)^{d_f}$ with d_f=2.27. The radius of gyration increased slightly less than expected in the case of the growth of Gaussian polymers (where d_f=2). This is consistent with the formation of denser, branched structures [28].

Comparison of thiolysis, SAXS and AF4 on water-soluble and water-insoluble oxidized fractions

In this section, we evaluated the use of AF4-MALLS coupling to obtain the mass distribution of oxidized tannins. The molecular weights of water-soluble and water-insoluble tannins determined by thioglycolysis, SAXS and AF4-MALLS were compared. To do so, we used a much

Table 3 Effect of oxidation on I_0/C values and thus on the weight average M_w

Tannin fraction	I_0/C	$M_w$ox/$M_w$nat
A-14n	$0.168 \pm 1 \times 10^{-3}$	
A-14ox-dil	$0.181 \pm 2 \times 10^{-3}$	1.07
A-14-ox-conc	$0.851 \pm 3 \times 10^{-3}$	5.07

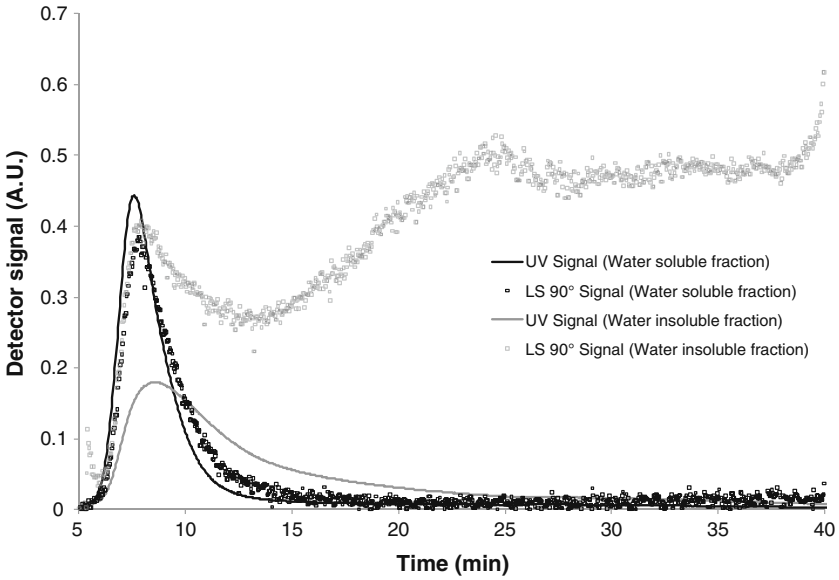

Fig. 6 Comparison of UV and MALLS fractograms obtained with water-soluble and -insoluble fractions of oxidized tannins in ethanol. Analytical conditions: detector flow rate, 0.5 mL min^{-1}; crossflow rate, constant at 2 mL min^{-1} during 25 min then decreasing linearly to 0 mL min^{-1} until 40 min; 5 kDa membrane; injected volume, 100 μL

oxidized tannin fraction, G-15ox, which has become partly insoluble in water. The aim of this experience was to evidence structural differences between water-soluble and water-insoluble tannins.

Number average degree of polymerization, monomer composition and yields of the depolymerization reaction obtained by thioglycolysis are compared in Table 1. It should be reminded that for native tannins, reaction yields were in the range 70–80%, whereas the yield decreased with oxidized tannins. The calculated DP hardly decreased from 15.6 to 12.3 and 13.4, but the thiolysis yield decreased by 40% upon oxidation. We compare in Fig. 6 the fractograms of the insoluble and soluble part of the oxidized grape seed tannin fraction. Insoluble tannins exhibit a broader size distribution, with an increase of the MALLS signal at higher elution times combined with an important UV peak tailing. Soluble oxidized tannins had a weight average molecular weight determined by MALLS of 3.7×10^4 g mol^{-1} and a recovery of 75%, whereas insoluble ones had a M_w of 3.43×10^5 g mol^{-1} and a recovery of 93%. This increase in recovery observed with water-insoluble tannins also suggests that there are fewer chains having a molecular weight smaller than the membrane cut-off, consistent with globally larger chains. It can also suggest a lower adsorption on the membrane surface. Due to the small dimensions of the studied macro-

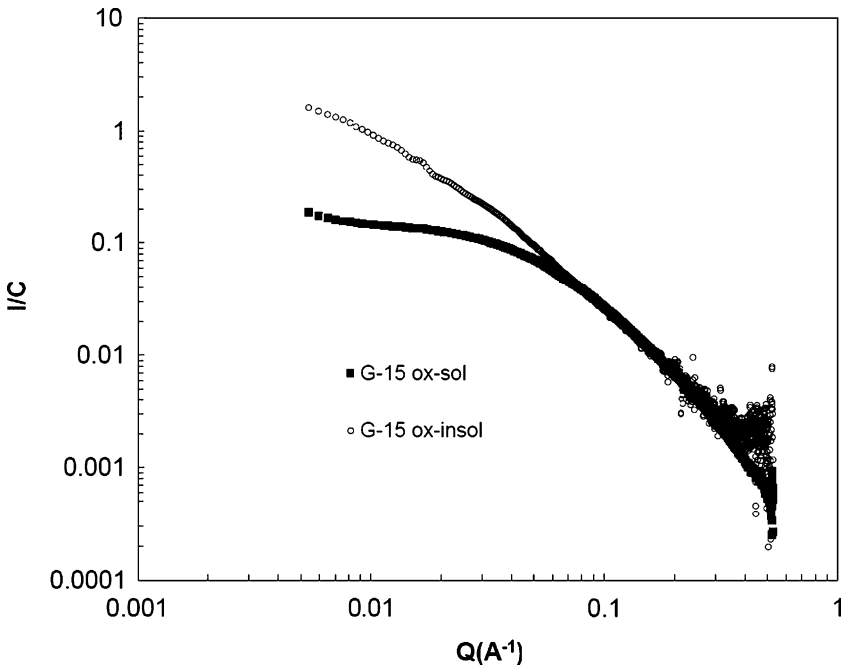

Fig. 7 SAXS intensity (normalized by concentration) in the log *I*–log *Q* representation of oxidized grape seed tannins. *Black squares*: water-soluble fraction; *black circles*: insoluble fraction. At low *Q* values, the insoluble fraction scatters more than ten times, indicating that there is a factor of 10 between the two weight average molecular weight

molecules (R_g<10 nm), the MALLS detection could not give reliable radii of gyration values.

The ratio of the molecular weights of soluble and insoluble fractions was almost 10. This value is strikingly high compared to thiolysis results that suggested that both fractions had roughly the same DP. This is in accordance with the fact that depolymerization on oxidized tannins is not total and induces products that are not accounted for in DP calculation.

Figure 7 shows the SAXS intensities of water-soluble and -insoluble tannins dissolved in ethanol. Radii of gyration and I_0 values (Tables 1 and 2) were derived from a fit with Eq. 3. I_0/C values of the two species were found to differ by a factor of 11, meaning that insoluble tannins in water have a weight average molecular weight about 11 times larger than the soluble ones. Their radius of gyration differed by a factor of 4.

Results obtained with AF4-UV-MALLS and SAXS were quite consistent (10% error is commonly admitted in SAXS M_w determination) and showed the limitations of the thioglycolysis on oxidized tannins: we actually observed an increase of weight average molecular weight with oxidation (the M_w of the native fraction, estimated from its DP and its percentage of galloylation is 5.10^3 g mol^{-1}). Moreover, the water-insoluble fraction has a larger weight average DP than the soluble one. However, if relative values were consistent (i.e. there is a factor of 10 between soluble and insoluble fractions), absolute values differed. Further investigations will be needed to understand the origin of these differences. This could be due for example to the fact that we did our SAXS calibration with native species and considered that ρ_p in Eq. 1 was the same for native and oxidized tannins.

On the whole, these results indicated that AF4-MALLS, a lab-bench system, is a promising tool to investigate the size distribution of tannins in different solvents, but it is not sufficient to determine their radius of gyration and obtain structural information (form factor). SAXS is necessary due to the small dimensions of tannins, even when they are oxidized. However, this tool can be used to determine aggregate sizes, but also to determine the size distribution of complexes formed between tannins and other biomacromolecules.

Conclusion

In accordance with previous results, we evidenced that usual classical analytical methods are inadequate for the analysis of oxidized tannins. Two techniques were presented in order to palliate this: SAXS, which can be used to determine changes in tannins weight average molecular weight and conformation after oxidation, and AF4, which allows separation without chromatographic support. We confirmed that SAXS is a powerful technique to determine the conformations of native and oxidized tannins. It allows following the evolution of the degree of polymerization of tannins during oxidation, which is not possible yet with standard depolymerization techniques. However, SAXS is not easily accessible, which makes thus AF4-MALLS, a lab-bench system, a promising tool to investigate the size distribution of tannins in different solvents. Its main advantage is to minimize interactions due to the very small membrane area in contact with tannins. However, the light scattering detection has limitations: if the molecular weight determination is reliable, size measurements are not accurate for molecules having a radius of gyration less than 10 nm (e.g. native tannins). This limitation vanishes when the aim is to study aggregation, or to determine the size distribution of complexes formed between tannins and other biomacromolecules.

Back to enological considerations, we evidenced the impact of concentration on oxidation mechanisms: oxidation in dilute solutions (≤0.1 gL^{-1}, 'white wine' conditions) led to the formation of macromolecules with roughly the same weight average molecular weight, consistent with intramolecular reactions, whereas oxidation in concentrated solutions ('red wine' conditions) led to the formation of higher molecular weight species, consistent with both mechanisms. Finally, SAXS also evidenced molecular weight differences between water-soluble and non-water-soluble tannins, confirmed by AF4. The tools presented here will be helpful to identify the structural changes which occur during wine ageing.

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