Eur Food Res Technol (2011) 232:113–121
DOI 10.1007/s00217-010-1365-9

ORIGINAL PAPER

Precipitation of low molecular weight phenolic compounds of grape seeds cv. Carménère (*Vitis vinifera* L.) by whole saliva

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Received: 11 June 2010 / Revised: 10 September 2010 / Accepted: 17 September 2010 / Published online: 5 October 2010

Abstract Low molecular weight phenolic compounds (LMWP) contribute to astringency and bitterness, two important sensory attributes. This work aimed to study the interactions between human saliva and a group of LMWP from a grape seed extract, namely, monomers, dimers and trimers of flavan-3-ol plus gallic acid. LMWP in the sediment that was produced by mixing whole saliva with the grape seed extract were identified by high-performance liquid chromatography analysis (HPLC–DAD). Two assays to produce LMWP-salivary protein sediments were carried out. An indirect assay consisted of in vitro mixing aliquots of saliva with a constant volume of the grape seed extract followed by centrifugation and sediment analysis. In a direct assay, aliquots of the grape seed extract were mixed *in-mouth* with saliva, returned to a vessel and centrifuged. In each assay, polyphenol composition of the sediments varied according to the tannin/saliva vol/vol ratio although in both cases monomeric LMWP were the most abundant polyphenol components of the sediments. The study also suggested the need for a strict control of the in vitro experimental conditions used to mimic the in vivo conditions in which tannin–protein interactions do occur and produce astringency perception.

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Keywords Astringency · Salivary proteins · Tannin–protein interaction · Tannin–protein precipitation · Enological tannins

Abbreviations

HPLC–DAD	High-performance liquid chromatography–diode array detector
LMWP	Low molecular weight phenolic compounds
PRPs	Proline-rich proteins

Introduction

Polyphenols constitute one of the most common and widespread groups of substances in vegetables. Polyphenols include the tannin group, which comprises water-soluble compounds of molecular weights between 500 and 3,000 Da that confer a variety of special features to foods, such as, bitterness, color and astringency [1]. Astringency is a tactile sensation perceived as dryness and roughness throughout the oral cavity that is thought to be provoked by physicochemical interactions, followed by precipitation, between salivary proteins and tannins [2, 3]. Accordingly, interactions between tannins and proteins, particularly salivary proteins, have been extensively studied in order to understand the astringency [4–15].

Modifications in the profile of salivary proteins have been invoked as a mechanism affecting tannin–protein interactions and hence the intensity of perceived astringency [11, 15]. Salivary proline-rich proteins (PRPs), a highly diverse family of parotid gland-produced proteins among herbivorous and omnivorous mammals, including

man, have been shown to display a high affinity for tannins [14]. Currently, binding of tannins to salivary PRPs has been proposed as an initial step in the development of astringent sensations [12]. However, polyphenols also bind tightly to salivary histatins, α-amylase, lactoferrin, and mucins and a number of non-salivary proteins, such as, gelatin, bovine serum albumin and casein [2, 7, 14]. Bacon and Rhodes [6] had observed differential affinities between a single tannin and different proteins and suggested that epigallocatechin has a higher binding affinity for PRPs than for many other proteins. Binding studies with purified salivary proteins have shown that interactions depend on the nature of the protein and particularly on their level of glycosylation [15].

In open contrast with salivary proteins, properties of tannins participating in astringency-inducing mechanisms have been far less studied [3, 8, 10]. Two factors have been suggested to be critical in the affinity of tannins for salivary proteins: tannin size (e.g. degree of polymerization) and degree of galloylation (presence of gallic acid and their derivates in the tannin structure) [16]. For instance, tannic acid comprises a high proportion of gallotannins and shows a high affinity for salivary proteins whereas tannins with low degree of galloylation display a lower affinity for salivary proteins [6]. On the other hand, it has been shown that larger and more complex polyphenols interact more strongly with fragments of PRPs [17]. In this regard, it has been postulated that larger tannins form multiple bonds with adjacent proline residues and that also associate and stack with other tannin molecules after binding to proteins. In contrast, simple phenols would bind only to single proline residues [4]. In addition, some authors have observed that high molecular weight proanthocyanidins are selectively precipitated by the supernatant obtained from saliva spun at 10,000 g for 10 min while dimeric and trimeric proanthocyanidin oligomers remain in solution. On the other hand, in the presence of small amounts of proanthocyanidins, low molecular weight PRPs and probably histatins displayed a higher tendency to precipitate [18].

Altogether, most of the referred studies on molecular mechanisms underlying astringency and focusing on the tannin component have been performed under experimental in vitro conditions or have used salivary fluids that may drastically differ from native salivary secretions both in structure, composition and properties [9, 13–15, 18] or have been based on the use of model proteins, polypeptides and tannins [11, 12, 19]. This work was aimed at identifying low molecular weight phenolic compounds (LMWP) from cv. Carménère berry grape seeds that may interact and precipitate while in contact either in vivo or in vitro with human saliva.

Materials and methods

Materials

Standards of gallic acid (G-7384), (+)-catechin (C-1251), (-)-epicatechin (E-1753) and (-)-epicatechin-3-*O*-gallate (E-3893) as well as 0.45-μm pore size membranes were acquired from Sigma Chemical Company, Saint Louis, Missouri, USA. HPLC grade acetonitrile and proanalysis solvents were purchased from Merck, Darmstadt, Germany.

Major instrumentation

The HPLC system (Agilent Technologies Santa Clara, CA., USA) used for the characterization of phenolic compounds consisted of a photodiode-array detector model G1315B, a quaternary pump model Quat G1311A, an autosampler model ALS G1329A and a reversed-phase Nova Pack C_{18} column (4 μm, 3.9 mm ID × 300 mm; Waters Corporation, Milford, MA., USA).

Grape samples

Plants of *Vitis vinifera* L. cv. Carménère, vintage 2008, grown in the Maule Valley at the VII Region of Chile were used. One thousand berries (25°Brix) selected from 200 plants were harvested on April 15th.

Extraction of phenolic compounds

All the seeds ($N = 1{,}000$, weighing 30.0 ± 0.5 g) were separated by hand from the berries, weighed and grounded. After adding two litters of distilled water, maceration was performed for 2 h at 20 °C under mechanical stirring. The suspension was centrifuged at 3,600 rpm for 15 min and filtered through a 0.45-μm pore size membrane.

HPLC–DAD analysis of individual phenolic compounds

The seed extract containing phenolic compounds was characterized as previously described [20]. Briefly, a 25-mL aliquot of the extract was re-extracted with ethyl ether (3 × 20 mL) and ethyl acetate (3 × 20 mL). The new extracts were combined and evaporated to dryness at 30 °C, re-dissolved in 1 mL of 50% (v/v) methanol/water and membrane-filtered (0.45 μm pore size). Fifty-microliter aliquots of the final solution were subjected to reversed-phase chromatographic separation at 20 °C using a Nova Pack C_{18} column. A photodiode-array detector was set at 280 nm. Two mobile phases were used: A, water/acetic acid (98:2 v/v) and B, water/acetonitrile/acetic acid

(78:20:2 v/v/v). A two-step gradient was carried out at a constant flow rate of 1.0 mL per min: 0–55 min, 100–20% A and 55–70 min, 20–10% A. Equilibration times of 15 min were allowed between injections. Each major peak in the HPLC chromatograms of the extracts was characterized by both retention time and UV absorption spectrum (from 210 to 360 nm). Identification of specific compounds was achieved by comparison of UV spectra and retention times against those of pure standards. The proanthocyanidins for which no standards were available, were identified by their retention time and spectral parameters reported by Santos-Buelga et al. [21]. Quantitative determinations were made by using both an external standard method with commercial standards and the Agilent ChemStation software. For gallic acid and procyanidin gallate quantification, a gallic acid linear standard curve was produced. For proanthocyanidin quantification, a (+)-catechin linear standard curve was produced. Results were expressed, respectively, as equivalents of gallic acid or catechin. All the qualitative and quantitative analyses of phenolic composition were performed in quintuplicate.

Saliva

A 31-year-old male volunteer, without history of smoking, alcoholism or medication consumption, with no evidence of disease and displaying both normal saliva flow (over 1 mL/min) and normal salivary protein profile [22] was included under the terms of a signed informed consent. Immediately before the experiments and following a mouth rinse with water, samples of whole saliva were always collected between 9:00 and 10.00 A.M. to minimize eventual diurnal variations in salivary composition [15]. Also, to minimize confounding variables, the volunteer consumed the same diet since 3 days before beginning the experiments. Saliva was conserved in an ice bath during the experiments. For reference, an aliquot of saliva was routinely saved for measuring protein concentration using a modification of the method of Bramhall and bovine serum albumin as reference [23].

Assays for interaction between seed tannins and saliva

1. *In vitro (or indirect) assay* Aliquots of saliva (1, 2 and 8 mL) from a single donor volunteer were mixed with 40 mL of the grape seed extract in centrifuge tubes. After a 5-min incubation at 37 °C, each mixture was centrifuged at 3,600 rpm during 5 min. The supernatants were eliminated whereas the sediments (1S/40T, 2S/40T and 8S/40T) were individually suspended in 50 mL of distilled water with the aid of mechanical stirring during 10 min. Each resulting suspension was divided into two halves and a half was used for the analysis of LMWP by using HPLC–DAD and the other half was placed on a glass plate and dried at 95 °C for 1 h to obtain the dry weight of the sediment (Fig. 1). Specific contents of LMWP were expressed in mg/Kg of dry sediment.
2. *In vivo (or direct) assay* A second assay was carried out by placing 20 and 40 mL of the grape seed extract

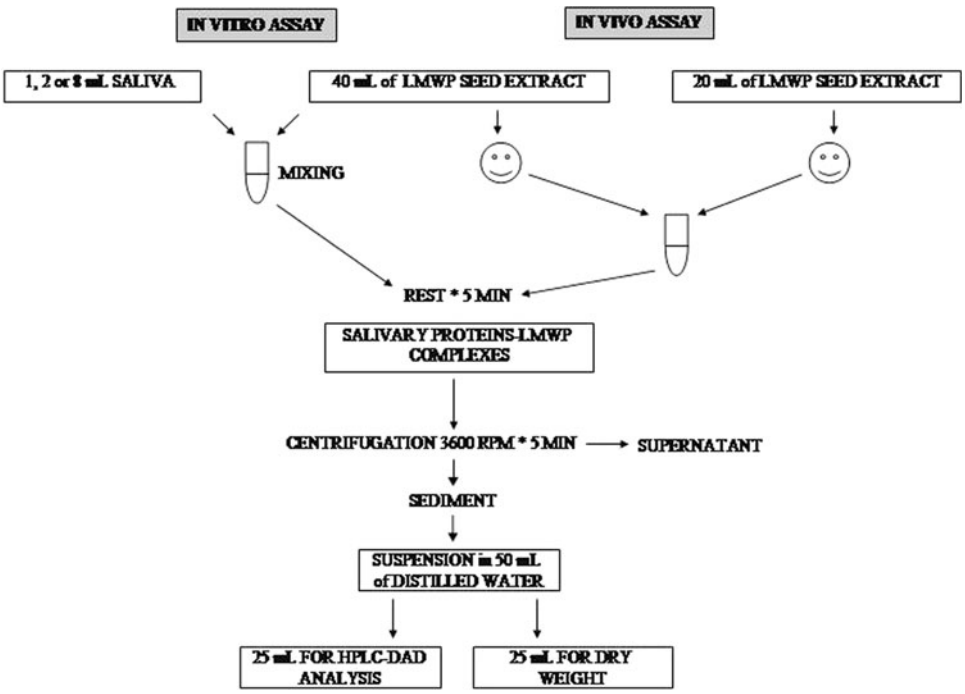

Fig. 1 In vitro and in vivo assays for tannin–salivary protein precipitation

Table 1 Low molecular weight phenolic compounds in aqueous extracts from grape seeds cv. Carménère

LMWP	mg/Kg of seeds*
Gallic acid	51.4 ± 5.2
Proanthocyanidin B3	37.0 ± 2.6
Proanthocyanidin B1	25.7 ± 0.5
(+)-catechin	585.8 ± 86.0
Trimer 1	12.1 ± 0.8
Proanthocyanidin B4	32.2 ± 3.1
Proanthocyanidin B2	96.4 ± 1.5
(-)-Epicatechin	343.2 ± 8.1
Proanthocyanidin 1	6.5 ± 2.0
Trimer 2	23.1 ± 3.0
Epicatechin-3-*O*-gallate	47.1 ± 3.3
Proanthocyanidin 2	15.7 ± 3.4
Total of proanthocyanidin gallates	228.5 ± 22.9

* Means ± standard deviations from quintuplicates

in the mouth of the single volunteer donor of saliva. After mixing thoroughly the extract with the saliva present in the mouth (15 s), the volunteer returned the mixture into centrifuge tubes. After a 5-min rest, the tubes were centrifuged and the resulting sediments (R/20T and R/40T) were analyzed as described above (Fig. 1). Both the direct and the indirect assays were carried out daily for 5 days. Each day was a replicate.

Results

LMWP in grape seeds

As shown in Table 1 and Fig. 2, eleven flavonoid and non-flavonoid compounds were identified and quantified by HPLC–DAD analysis in the extract of Carménère seeds. Among those LMWP, we identified three monomers [(+)-catechin (C), (-)-epicatechin (EC) and (-)-epicatechin-3-*O*-gallate (ECG)], four dimeric proanthocyanidins [catechin-(4$\alpha \rightarrow 8$)-epicatechin (B4), epicatechin-(4$\beta \rightarrow 8$)-epicatechin (B2), catechin-(4$\alpha \rightarrow 8$)-catechin (B3) and epicatechin-(4$\beta \rightarrow 8$)-catechin (B1)], seven dimers esterified with gallic acid (GPs), two trimeric proanthocyanidins (T1 and T2), other two unidentified proanthocyanidins (P1 and P2) and only one non-flavonoid compound [gallic acid (GA)].

LMWP in the sediment produced by mixing saliva with a seed extract (in vitro *assay*)

Forty-milliliter aliquots of the extract were mixed in vitro with various volumes of whole saliva (0.86 mg protein/mL) in the range from 1 to 8 mL. After incubation for 5 min, the mixtures were centrifuged and the sediments were analyzed by HPLC–DAD. Figure 3 shows a representative chromatogram including identities of most of the peaks observed in the fractionation. Figure 4 shows that the composition of LMWP in the sediments produced by mixing different volumes of saliva with a single volume of seed extract is dependent upon the saliva to seed extract v/v ratio. Thus, the LMWP observed in the sediments of either 1 mL (Fig. 4a) or 2 mL (Fig. 4b) of saliva with 40 mL of seed extract (1S/40T and 2S/40T, respectively) were GA, C, EC, ECG and GPs. In addition, compared with the 1S/40T mixture, the sediment produced from the 2S/40T mixture showed a higher content of all the LMWP identified.

Likewise, the LMWP compounds present in the sediment corresponding to the 8S/40T mixture were the same as those present in the seed extract with the exception of trimer 1 (Fig. 4c). Concentrations of all the LMWP present in the 8S/40T sediment were higher than those observed in the 1S/40T and 2S/40T sediments, with the exception of GA, a LMWP whose highest concentration occurred consistently in the sediment of the 2S/40T mixture (Fig. 4b). Finally, in relation to the corresponding concentrations in

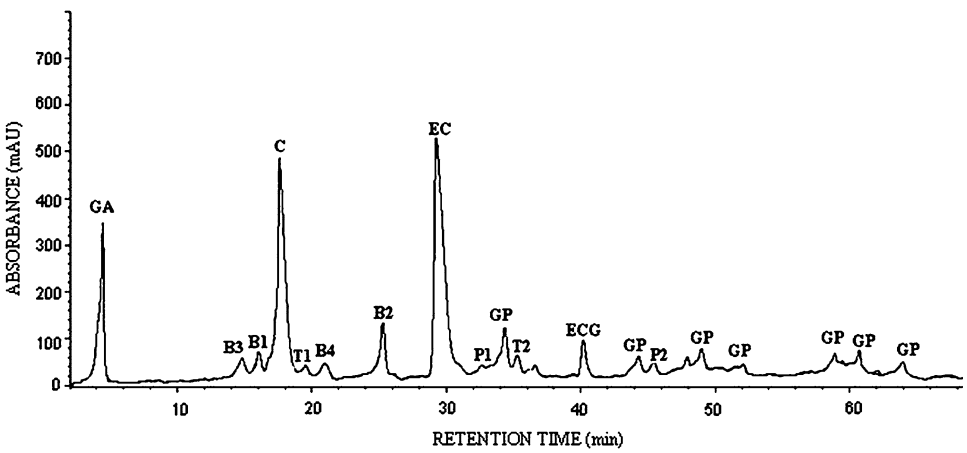

Fig. 2 Representative HPLC–DAD chromatogram of the aqueous grape seed extract from *Vitis vinifera* cv. Carménère. Identification of LMWP was mostly achieved by comparison of UV spectra and retention times against those of pure standards. Abbreviations of the LMWP names in this and the next figures are explained in the text (Results)

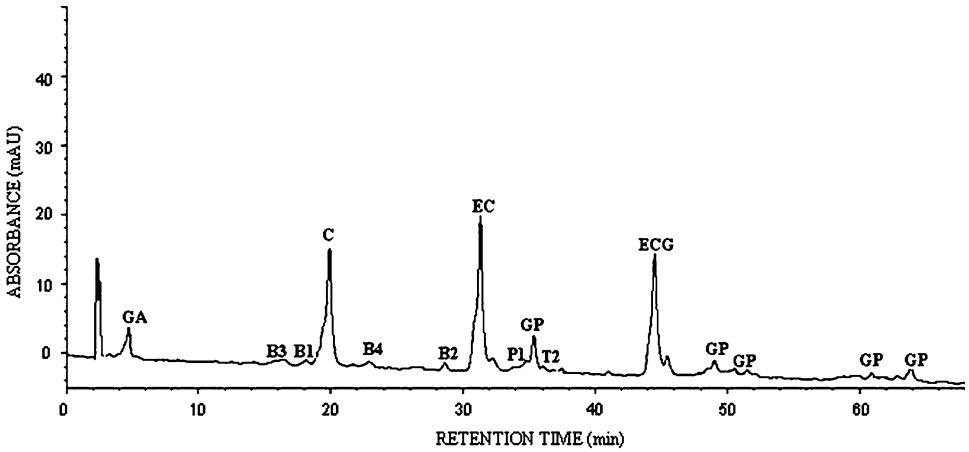

Fig. 3 Representative HPLC–DAD chromatogram of the sediment produced by in vitro mixing of 8 mL of whole saliva with 40 mL of grape seed extract (8S/40T)

the seed extract, ECG was the LMWP displaying the highest precipitated fraction in all the three sediments, followed by either GA in the 1S/40T and 2S/40T sediments or the proanthocyanidin B1 in the 8S/40T sediment.

LMWP in the sediment obtained after mouthrinsing with a seed extract (in vivo *assay*)

After mouthrinsing during 15 s with either 20 or 40 mL of the seed extract, the volunteer returned the mouth content corresponding to each experimental condition into a centrifuge tube. After centrifugation at 3,600 rpm for 5 min, the sediments were analyzed using HPLC–DAD as described previously. Figure 5 is a representative HPLC–DAD chromatogram of those sediments. Sediments obtained after mouthrinsing with 20 mL of the seed extract (R/20T) contained GA, C, EC, ECG, proanthocyanidin B1 and B2, proanthocyanidin P1 and GPs (Fig. 6a). In the sediments obtained after mouthrinsing with 40 mL of the seed extract (R/40T), besides those LMWP, dimeric proanthocyanidins B3 and B4 were also observed (Fig. 6b). Finally, this latter sediment showed the highest contents of the identified LMWP in these assays.

Discussion

Astringency has been described as a sensation of roughness and/or ruggedness in the mouth surface and has been attributed to interactions of phenolic compounds from a wide variety of vegetables, food and drinks with salivary proteins [11, 12]. Both salivary proline-rich proteins and histidine-rich proteins (histatins) have been shown to exhibit a high affinity for tannins and so they have been usually involved in molecular models for astringency perception [13, 14]. Since comprehensive reviews on salivary proteins interacting with polyphenols are available, our study was focused on the analysis of the phenolic component of tannin–salivary protein complexes. To do so, we investigated their presence in the tannin–salivary protein precipitates, which are the ultimate expression of the occurrence of tannin–salivary protein interactions. The main phenolic compounds thought to be responsible for wine astringency are flavan-3-ol polymers, commonly designated as proanthocyanidins or condensed tannins [1, 4, 7]. In the case of wine, these compounds are extracted from grape skins and seeds during the stage of vinification. Both the nature, level of galloylation and average degree of polymerization of tannins would strongly influence their interaction with salivary proteins [4, 6, 18].

In order to assess the effect of LMWP on the formation of insoluble tannin–salivary protein complexes, they were differentially extracted from grape seeds by using distilled water as solvent. By HPLC–DAD analysis, we observed a qualitative and quantitative composition of flavonoid and non-flavonoid compounds that was similar to the one reported by other authors [24]. The most abundant compounds in the extract were C and EC monomers, followed by GA and ECG. Various dimeric and trimeric proanthocyanidins were identified, such as seven proanthocyanidin gallates. These low molecular weight compounds have been found to influence both astringency and bitterness sensations [9, 25]. Thus, Pelleg et al. [25] evaluated the intensities of both astringency and bitterness of seven flavonoid compounds by using a time-intensity procedure. Those seven stimuli included two flavan-3-ol monomers [(+)-catechin and (-)-epicatechin], three dimers and two trimers. As the degree of polymerization increased among the three proanthocyanidin classes, both the maximum intensity and the total duration of bitterness decreased whereas astringency maximum intensity increased.

In the present study, single volumes of the extract of grape seeds containing LMWP were mixed with varying volumes of whole saliva from a single volunteer; that is, saliva accumulated in mouth and expectorated passively. At variance of a number of studies, saliva was not

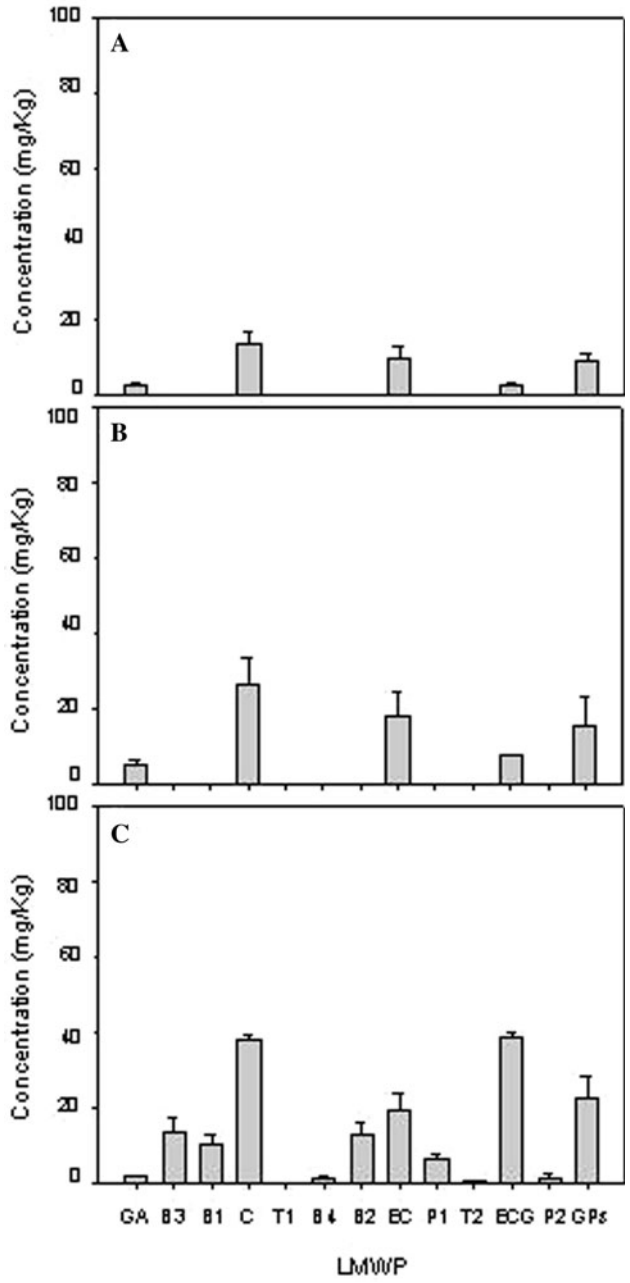

Fig. 4 Contents of LMWP in the sediments produced by in vitro mixing of various volumes of saliva (1, 2 or 8 mL) with a constant volume (40 mL) of grape seed extract. **a** 1S/40T, **b** 2S/40T, **c** 8S/40T. Contents are expressed in mg of LMWP/Kg of sediment dry weight

subjected to any kind of preparative fractionation prior to the experiments. After centrifugation, the sediments displayed a differential composition of LMWP, as assayed by HPLC-DAD. Sediments of the 1S/40T and 2S/40T mixtures comprised three flavan-3-ol monomers (C, EC and ECG), GA and some GPs, with a higher concentration of each of those LMWP in the 2S/40T sediment. Most of the seed extract LMWP were precipitated when the relative volume of saliva was risen up to 8S/40T. Studies on the stoichiometry of tannin–protein interactions have shown that less tannin is required to precipitate proteins from concentrated solutions than from dilute ones [26]. In addition, at high protein concentrations, direct bridging between proteins and epigallocatechin gallate does occur [27]. Recent studies from our laboratory have shown that an equivalence point, that is, the lowest amount of tannin needed to fully precipitate a protein, can be defined for a definite pair of a tannin and a protein [28]. Thus, the equivalence point for the LMWP extract and saliva could be estimated from the amount of total tannin present in a given volume of the extract that displays the ability to fully precipitate the amount of protein present in a given volume of saliva. However, because of the differential precipitation of LMWP with whole saliva, as shown in the present study, full precipitation of salivary protein at the equivalence point may occur together with the precipitation of just a subset of the LMWP present in the extract. Interestingly, ECG was the LMWP whose presence in the composition of all the sediments in relation to its presence in the seed extract was the highest among all the LMWP; that is, ECG seems to have been the most active precipitant of salivary proteins or, at least, a highly selected member of the LMWP-salivary protein complexes. This important observation might well be due to the relative high number of potentially reactive sites and substituents in ECG, such as the flavonoid skeleton allowing hydrophobic bonds and the three OH groups of the gallic acid residue each corresponding to a potential hydrogen-bond forming site between polyphenols and proteins [5, 8]. Although abundant data from literature suggest that a larger part of these interactions may be governed by non-covalent binding, more recent evidence have shown that under oxidizing conditions, oxidation intermediates of reaction products of polyphenols can also form covalents bonds with nucleophiles, such as aminoacids or thiol groups [29, 30].

Our observations also suggest that precipitation of the LMWP present in the seed extract depends positively on the concentration of saliva in the mix. According to Pascal et al. [27], at low EGCG–protein ratios, EGCG binds progressively a model protein in suspension without inducing aggregation. At higher ratios, EGCG bridges the partially bound protein molecules and provokes the appearance of EGCG–protein aggregates. Such in vitro observation would support the hypothesis that interindividual differences in salivary volume and salivary protein composition may be of major relevance in differential astringency perception toward the tannin content of a food [11, 14]. In this regard, total protein contents in whole stimulated saliva of a single group of patients have been shown to vary from 1.1 to 3.8 mg/mL [31]. Also, average volume of saliva in the mouth of healthy subjects has been

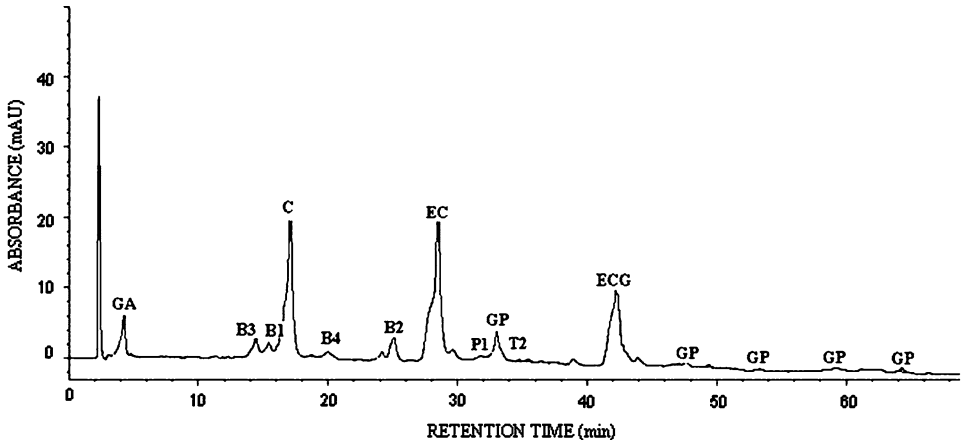

Fig. 5 Representative HPLC–DAD chromatogram of the sediment produced by mouthrinsing with a 40-mL aliquot of grape seed extract (R/40T). In vivo assay

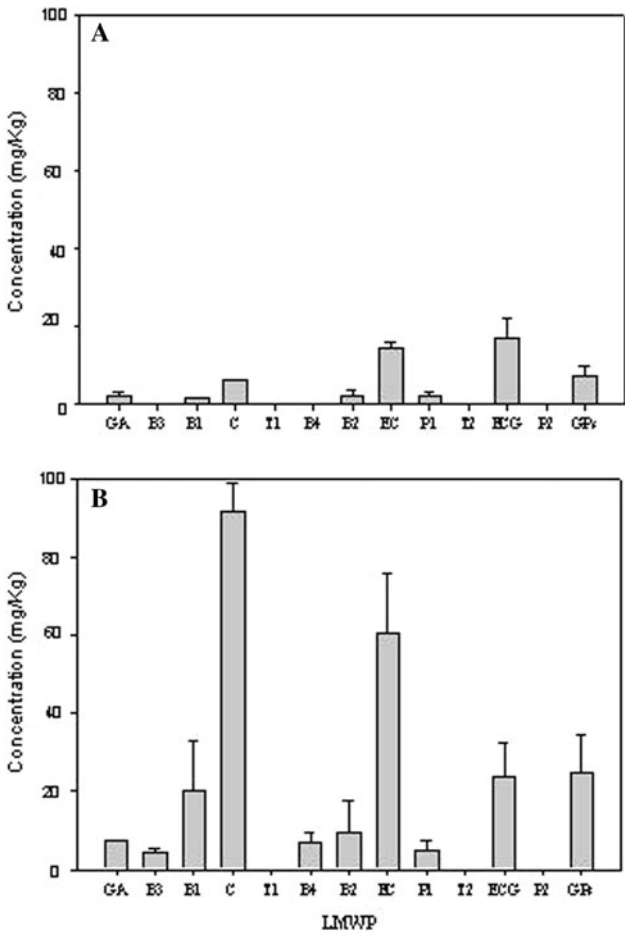

Fig. 6 Contents of LMWP in the sediments produced by mouthrinsing with either, **a** 20 mL of grape seed extract (R/20T), or **b** 40 mL of grape seed extract (R/40T). Contents are expressed in mg of LMWP/Kg of sediment dry weight

reported to be around 1 mL per mouth although this parameter seems to vary markedly in a sex- and age-dependent manner. Besides, the rate of saliva production is around 1 mL/min [32, 33]. Considering that set of parameters and the fact that in sensory evaluation tasters use to place around 15–20 mL of the tastant solution in the mouth [34], the experiment in this study consisting of the assessment of the sediment composition obtained from an in vitro 2S/40T mixture would represent, at least with regard to the volume to volume ratio, an experimental condition mimicking a real one. Under those conditions, precipitation of LMWP was only partial.

In the present study, we also conducted comparative in vivo assays. In these very uncommon assays in the literature, the same saliva donor volunteer placed either 20 or 40 mL of the same seed extract used in the in vitro assays, mixed it with saliva in the mouth and returned it into centrifuge tubes to obtain then the corresponding R/20T and R/40T sediments. While the R/20T sediment showed the presence of two-thirds of the LMWP present in the seed extract, the R/40T sediment displayed almost all the LMWP of the extract excepting the trimeric proanthocyanidins. Once again, relative to its presence in the seed extract, in these in vivo experiments ECG was the LMWP with the highest precipitation ratio.

Comparatively, the R/40T sediment showed the highest contents of all the LMWP, being the ECG, EC and C monomers (decreasing order) the ones displaying the highest levels in the sediment. Thus, this assay would demonstrate that the amount of sample used in tasting is of high relevance because the higher the volume of sample the higher the precipitation of LMWP compounds from the seed extract. As shown in this study, several LMWP compounds observed in the R/40T sediment were not present in the R/20T sediment. Those differences may well result in differences in the perceived astringency by the taster. On the other hand, our study has also shown that significant differences may occur between the observations made on the composition of insoluble complexes obtained by similar mixtures of saliva and seed extract through the use of in vivo and in vitro assays. Factors accounting for those differences are a matter of ongoing experiments in our laboratory.

A number of reports have described the formation of insoluble complexes between high molecular weight phenolics and salivary proteins and its correspondence with astringency perception [1–4, 19]. In this study, we have shown that LMWP that have been extracted from grape seeds, particularly flavanol monomers, display a marked tendency to interact with and precipitate salivary proteins. This main finding from our study would suggest that the low astringency, and bitterness, that is perceived in seeds from more mature grapes, may well be associated not only with the presence of flavan-3-ol polymers but with the decrease in the content of flavan-3-ol monomers. The latter one has been observed in studies with seeds during grape ripening [35]. In various experiments we have now observed that ECG was the LMWP compound from the seed extract with the highest precipitated fraction with respect to its content in the seed extract. By contrast, trimer 1 was not present in any sediment in the various assays of the study. These observations would suggest that the seed extract comprises LMWP displaying either a high or a low affinity for salivary proteins, such as ECG and trimer I, respectively.

Altogether, this study has shown that LMWP from grape seeds may interact differentially with the salivary protein fraction to become part of insoluble complexes. These interactions may well be part of the molecular mechanisms underlying astringency perception and should be taken into account in the interpretation of changing sensory attributes during grape maturation. Standardization of experimental protocols for the assessment of those interactions is strongly needed.

Acknowledgments This study was partially supported by grants Fondecyt-Chile 1080559, Corfo-Innova Tecnovid-05CTE02-04 and DI- Universidad de Chile Mult-05/35-2.

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