

TANNIN-PROTEIN INTERACTION IS MORE CLOSELY ASSOCIATED TO ASTRINGENCY THAN TANNIN-PROTEIN COMPLEX PRECIPITATION

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5 **COMPLEX PRECIPITATION**
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ABSTRACT

The relative abilities of two enological tannins to interact with a single gelatin were compared with their relative abilities to elicit astringency. A trained sensory panel assessed astringency whereas the interaction between tannins and gelatin was estimated by observing the ability of tannins both to interfere with gelatin diffusion on a cellulose membrane and to form tannin-gelatin precipitates. HPLC chromatography and spectroscopic analysis showed that one of the tannins was hydrolysable tannin while the other one was a proanthocyanidin. The majority of the sensory panelists recognized the hydrolysable tannin as far more astringent than the proanthocyanidin. Finally we showed that the more astringent tannin interfered markedly with gelatin diffusion on the cellulose membrane but it failed to produce tannin-gelatin precipitation whereas the proanthocyanidin tannin both interfered with gelatin diffusion and was a powerful gelatin-precipitant. Thus, this study supports the hypothesis that astringency correlates better with gelatin-tannin interaction than gelatin-tannin precipitation.

Keywords: Astringency, tannin-protein interaction, tannin-protein binding, enological tannin, procyanidin, gallotannin, gelatin

INTRODUCTION

Astringency, a drying, roughing and sometimes puckering sensation in the mouth, is among the most relevant sensory features of some foods, in addition to color, aroma and bitterness (1). Red wine astringency has been mostly associated with condensed tannins or proanthocyanidins, particularly flavan-3-ol polymers, which come mainly from grape seeds and skins (2, 3). Other tannins that also contribute to wine astringency and that differ chemically from grape tannins are the hydrolysable tannins. These tannins come mostly from oak barrels or from other commercial products that are used during wine aging (4, 5, 6, 7, 8). Previous reports have shown that different tannins vary in the intensity of the astringency response they elicit (1, 9, 10). Astringency assessment is performed by a panel of trained tasters whose reports use to involve some degree of subjectiveness (11). Accordingly, the use of complementary chemical analytical methods for estimating astringency has become a common practice. The Gelatin Index, the most popular of these methods, gives an estimate of tannin reactivity towards proteins by measuring the concentration of procyanidins both before and after their precipitation by the addition of an excess of gelatin (12). However, a main drawback of this index is that gelatin, which is produced by partial hydrolysis of collagen from various sources, is a highly heterogeneous protein material whose activity as tannin precipitant may vary markedly among commercial products. For that reason, Llaudy et al. (13) have recently proposed a new method to assess astringency by using ovalbumin, a pure protein, as a tannin precipitant agent. This method, which estimates precipitation of tannin-protein complexes by measuring the 280 nm absorbance of the corresponding supernatant, is more reproducible than the Gelatin Index and correlates well with sensory assessment. However, considering that both tannins and ovalbumin absorb intensely at 280 nm, differential contribution of either tannin or protein to absorbance cannot be defined.

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3 Nevertheless, whatever the nature of the tannin-precipitant protein, both methods are taken as a
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5 measure of the magnitude of the interactions between tannins and protein and as a direct
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7 reflection of the ability of tannins to elicit an astringent response. Tannin-protein interaction and
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9 precipitation have been a main subject of study in the past few years (1, 2, 10, 13, 14, 15, 16, 17).
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11 According to Versari et al. (18), at wine pH the positively charged gelatins interact with the
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13 negatively charged tannins and only if a critical mass of tannin-protein complexes is reached they
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15 may be precipitated. Thus, interaction between tannins and proteins would be previous to
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17 precipitation of tannin-protein complexes. To test that hypothesis the experimental design must
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19 be able to distinguish between interaction and precipitation. Noncovalent immobilization of
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21 proteins on solid supports is a technique fundamental to a number of modern analytical methods
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23 in biochemistry and molecular biology (19, 20). For instance, protein detection and quantification
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25 methods using solid matrices involve the fixation of the protein sample by denaturation on a
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27 nonreactive cellulose surface and its staining with a dye that binds selectively, stoichiometrically
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29 and with high affinity to proteins (21). Tannins do not display some of those features what may
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31 be used advantageously to gain insight into protein-tannin interactions. The purpose of this study
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33 was to evaluate the relative ability of two enological tannins to interact with a single gelatin and
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35 to correlate this with the relative intensity of the astringency elicited by both tannins. Tannin-
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37 gelatin interaction was estimated by measuring the relative ability of tannins both to interfere
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39 with gelatin diffusion on a cellulose membrane as well as to induce precipitation of the protein. A
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41 trained sensory panel assessed astringency.
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MATERIALS AND METHODS

Materials. “Tanin Gallique a l’alcool”, an alcoholic extract of tannins from the oak gall nut, and “Protanin R-instantáneo”, a 100% instantaneously soluble proanthocyanidic tannin, were purchased from Vinicas Industry Co., Santiago, Chile. “Gélatine Extra N°1”, a polyvalent gelatin, was purchased from Laffort Oenologie, Bordeaux, France. Tartaric acid, bovine serum albumin (BSA), tannic acid and standards of gallic acid (G-7384), protocatechuic acid (P-5630), protocatechuic aldehyde (E-24859), quercetin (Q-0125), miricetin (M-6760), kaempferol (K-420325), (+)-catechin (C-1251), (-)-epicatechin (E-1753) and (-)-epicatechin-galato (E-3893), were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA. Cellulose membranes (Whatman # 1) were purchased from Whatman Ltd., Maidstone, England. Acetic acid HPLC degree and acetonitrile HPLC degree were purchased from Merck, Darmstadt, Germany. Other pro-analysis solvents were obtained from Oxiquim-Chile. The HPLC system consisted of a photodiode-array detector Model G1315B, a pump Model Quat G1311A, an autosampler Model ALS G1329A (Agilent Technologies 1200) and a reversed phase Nova Pack C₁₈ column (4µm, 3.9 mm di x 300 mm) (Waters Corporation, Milford, M.A. USA). Absorbances were measured using an UNICAM UV-VIS spectrophotometer Model Helios-Gamma 2000.

Sensory Evaluation. Both enological tannin extracts were evaluated sensorially by a panel of 13 judges trained to rate intensity of astringency. A paired comparison test was used. Results from duplicate assessments were expressed in percentage and tested by chi-square analysis.

Chemical characterization of tannins. Stock solutions (3 g/L) of both enological tannins were prepared in hydroalcoholic solution (10% v/v ethanol and 0.5% w/v tartaric acid). Total phenol

content was determined by UV-absorptiometry at 280 nm (12) using gallic acid as standard. Total tannin content was measured by the method of Ribereau-Gayon and Stonestreet (22). The gelatin index of tannins was measured according to the method of Glories (11) using 50 ml of tannin stock solutions and 5 ml of 70 g/L gelatin. For HPLC–DAD analysis, tannins were extracted with ethyl ether (3 x 20 mL) and ethyl acetate (3 x 20 mL). The total extracts were evaporated to dryness at 30 °C, re-dissolved in 2 mL of 50% (v/v) methanol/water and membrane-filtered (0.45 µm pore size). Aliquots of 100 µl were subjected to reversed-phase fractionations at 20°C using a Nova Pack C₁₈ 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 tannin extracts was characterized both by its retention time and absorption spectrum (from 210 to 360 nm). Identification of specific compounds was achieved by comparison against pure standards. Quantification of specific components of the tannin extracts was performed by using external standards. Calibration curves were obtained by using gallic acid (gallotannins) and (+)-catechin (proanthocyanidins). All the qualitative and quantitative analyses of phenolic composition of tannins were performed in triplicate.

Assays of tannin-gelatin interaction. In a final volume of 1000 µl, a constant amount of tannin (750 µg) was mixed thoroughly with one of a series of gelatin dilutions (range from 250 to 0 µg) and allowed to stand for 5 minutes at room temperature. After vortex mixing again an aliquot of 15 µl taken from each of the tubes was spotted punctually on a cellulose membrane. The

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3 membrane was then dried under a light-lamp, fixed for 5 minutes in 5% trichloroacetic acid,
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6 rinsed for 5 minutes in 80% ethanol, stained for protein during 20 minutes in 0.5% Coomassie
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8 blue R-250 dissolved in 45% isopropanol/10% acetic acid and washed in 7% acetic acid until
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10 clear background. Following a final wash in distilled water the membrane was dried again under
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12 a light-lamp (**Figure 1**). In this diffusion assay on cellulose membranes, a blue-stained circular
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14 spot represents the diffusion area of the protein present in the sample. Blue diffusion areas
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16 observed at varying gelatin/tannin ratios were visually compared with gelatin diffusion in the
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18 absence of tannin (control). In a variant assay, the Eppendorf tubes containing the tannin/gelatin
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20 mixtures were centrifuged at 5000 rpm for 5 minutes and, after visual inspection of the
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22 sediments, 30- μ l aliquots were taken from each supernatant and were orderly spotted on a
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24 cellulose membrane. The membrane was processed for protein detection as described above. In
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26 this assay (precipitation assay), a complete loss of protein staining represents the equivalence
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28 point of gelatin precipitation. Assays were performed in duplicate by each of two independent
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30 experienced operators.
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RESULTS

Sensory Analysis. The proanthocyanidin and hydrolysable tannins were assayed for astringency by means of a paired comparison test conducted by a trained sensory panel. In two successive assessments, 84% and 76% of the panelists reported that the hydrolysable tannin was more astringent than the proanthocyanidin tannin (Chi squared, $p < 0.05$).

Characterization of enological commercial tannins. Table 1 shows a physicochemical characterization of both enological tannins on a weight basis, the content of total phenols in the hydrolysable tannin was about four-fold the one in the proanthocyanidin tannin whereas the concentration of total tannins in the proanthocyanidin tannin was 2.6 times the one in the hydrolysable tannin. The hydrolysable tannin presented a significantly higher gelatin index as compared to the proanthocyanidin tannin. As shown in representative HPLC-DAD chromatograms, both tannins presented gallic and protocatechuic acids (**Figure 2**). In the proanthocyanidic tannin, we also observed protocatechuic aldehyde, flavonols, proanthocyanidins and procyanidin gallate. Gallotannins were identified only in the hydrolysable tannin. Table 1 summarizes the relative contributions of a number of compounds identified in each of both enological tannins. No significant differences (Tukey test, $p > 0.05$) were observed in the content of protocatechuic acid and protocatechuic aldehyde of both tannins. By contrast, both tannins showed significant differences ($p < 0.05$) in the content of most of the individual phenolic compounds identified by HPLC-DAD. As expected, the hydrolysable tannin showed a much higher content of gallotannins and gallic acid. As also expected, the proanthocyanidin tannin displayed a higher content of proanthocyanidins and procyanidin gallates.

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3 **Diffusion of gelatin and tannins on cellulose membranes.** Stock solutions of both tannins (1.5
4 mg/mL) and gelatin (4.4 mg/mL) were serially diluted with either hydroalcoholic solution
5 (tannins) or distilled water (gelatin). Each one of the serial dilutions of gelatin and tannins were
6 punctually spotted on cellulose membranes and processed for detecting protein with the protein-
7 binding dye Coomassie blue R-250. As shown in **Figure 3A**, gelatin diffused radially and
8 homogeneously from the spotting site on the cellulose membrane. Distribution of gelatin was
9 clearly identified at the whole range of concentrations in the assay. At higher concentrations of
10 gelatin the protein was distributed all over the circle moistened by the sample. A lower
11 concentration, gelatin was partly retained by its interaction with the cellulose matrix (**Figure 3A**).
12 By contrast, most of the serial dilutions of both tannins remained undetected against the cellulose
13 background, excepting a faint staining of the sample of stock solution of the hydrolysable tannin
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34 **Differential interaction of tannins with a single gelatin.** Interaction of tannin with protein may
35 result in soluble and insoluble tannin-protein complexes. This might be evidenced either by a
36 decreased diffusion of the protein on the cellulose membrane, by the appearance of precipitates
37 following the centrifugation of the mix or by the disappearance of the protein from the
38 corresponding supernatant. Under the experimental conditions in this study, gelatin solutions in
39 the range 250 to 0.0 μ g were easily detected and found to diffuse homogeneously on the cellulose
40 membrane (**Figure 4A**). When fix amounts of the hydrolysable or proanthocyanidin tannins
41 (750 μ g) were combined with serially diluted solutions of gelatin over the range from 250 to
42 0.0 μ g followed by centrifugation and spotting of the supernatants, protein diffusion on the
43 cellulose membrane was found to be markedly reduced over the whole series of protein
44 concentrations (**Figures 4B and 4C**). At variance of the hydrolysable tannin, the
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3 proanthocyanidin tannin not only diminished the protein diffusion but it also provoked the whole
4 disappearance of the protein from the corresponding supernatant (“equivalence point”) (**Figure**
5 **4C**). That observation suggested that the proanthocyanidin tannin is also a precipitant of the
6 protein. Altogether, these studies indicate that under the present experimental conditions both
7 tannins interact with gelatin but that only the interaction of proanthocyanidin tannin with gelatin
8 results in precipitation of the complexes.
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20 **Time-dependency of the interaction between tannins and gelatin.** So far most of the assays
21 for interaction between tannins and gelatin had been performed at 10 minutes following their
22 mix. When this assay was repeated at 48 hours following the mix of tannins and gelatin we did
23 not observe any major difference. Likewise, precipitation of tannin-gelatin complexes as
24 observed either indirectly on the cellulose membranes (**Figures 4B and 5B**) or directly in the
25 tubes after centrifugation showed no major difference (**Figures 4C and 5C**) excepting slight
26 displacements in the equivalence point toward a lower amount of gelatin (**Figure 4C**).
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DISCUSSION

Enological tannins are used in wine industry to ensure sensory balance, sensory complexity, color stability and inhibition of lacase activity (23). However, the influence of tannins on the properties of wine will depend on their chemical nature (e.g. hydrolysable or proanthocyanidin). Tannins as a whole have been closely associated to the sensation of astringency. Wine astringency is usually evaluated using trained panels of tasters (11). On the other hand, the ability of tannins to produce astringency has been usually associated to their ability to precipitate proteins (17). Thus, the Gelatin Index, a method based on measuring the relative fraction of unprecipitated tannins from an aliquot of wine mixed with an excess of a commercial gelatin, has become a sort of Golden Standard for astringency. Among enologists, however, the utility of this index for assessing astringency has become highly controversial mostly because of its low reproducibility and frequent disagreement with trained sensory panel scores (13). A recent effort to improve reproducibility and the acceptance of this type of biochemical assays is based on the replacement of gelatin by ovalbumin as the precipitant protein (13). This method also analyses a correlation between precipitation of tannin-protein complexes and astringency. In the present study we have investigated the relative abilities of two enological tannins to precipitate a single enological gelatin. Both tannins were shown to differ markedly in their chemical nature (HPLC chromatography and spectroscopic analysis) as well as in the astringency they produce (sensory panel assessment). Surprisingly, we found that the tannin that was more astringent was also a far poorer protein precipitant than the less astringent tannin. However, we also found that the more astringent tannin interacts with the commercial gelatin without producing its precipitation. Thus, the results of the present study support the hypothesis that astringency correlates better with gelatin-tannin interaction than with gelatin-tannin precipitation. To perform this study we took

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3 advantage of a procedure based on protein immobilization on solid cellulose membranes
4 followed by protein staining with Coomassie blue under conditions in which tannins remain
5 mostly undetected. At variance with conventional assays, our study was designed to observe not
6 only precipitation of protein-tannin complexes but also to observe interaction between protein
7 and tannins resulting in soluble tannin-protein complexes. When serially decreasing amounts of
8 single gelatin were mixed with a constant amount of a tannin extract and after a centrifugation
9 step aliquots of the supernatants were orderly placed on a cellulose membrane we observed a
10 progressive disappearance of the protein because of precipitation of tannin-gelatin complexes. In
11 this precipitation assay, the equivalence point is the experimental condition resulting in full
12 disappearance of the protein from the supernatant and represents an objective parameter for
13 comparison between tannin samples. In addition, the outcome of this assay can be contrasted with
14 the direct observation of the sediments in the centrifuge tubes. When both enological tannins
15 under consideration in the present study were compared as to their relative abilities to produce
16 protein precipitation, either by the precipitation assay on cellulose membranes or by direct
17 inspection of the sediments in the centrifuge tubes, a striking difference became evident. In
18 effect, over the whole range of experimental concentrations of the gelatin, the hydrolysable
19 tannin was fully ineffective in forming insoluble complexes whereas the proanthocyanidin tannin
20 was able to produce the full precipitation of the protein. A first major conclusion of this
21 comparison was that the ability of the hydrolysable tannin to elicit astringency cannot be properly
22 represented by its ability to form insoluble tannin-gelatin complexes. In this study we also
23 observed the mode of diffusion of the gelatin in solution when spotted on a cellulose membrane.
24 In effect, this protein in solution diffuses freely, radially and homogeneously after being placed
25 punctually on a cellulose membrane. That mode of diffusion was markedly affected when the
26 protein was mixed with tannins. In this assay, even the lowest hydrolysable tannin/gelatin and
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3 proanthocyanidin tannin/gelatin ratios were found to be associated to a marked restriction of
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5 gelatin diffusion on the cellulose membrane. Such decreased gelatin diffusions are indicative of
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7 interactions between tannins and gelatin. Thus, the more astringent hydrolysable tannin affected
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9 gelatin diffusion without provoking its precipitation at all the experimental conditions in the
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11 study. On the other hand, for the series of gelatin concentrations mixed with a given amount of
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13 the proanthocyanidin tannin, we could compare the equivalence point with the relative amount of
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15 tannin affecting gelatin diffusion. In this case, the equivalence point was obtained at a
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17 proanthocyanidin tannin/gelatin ratio much lower than those found to affect gelatin diffusion on
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19 the cellulose membrane, that is, we observed a range of proanthocyanidin tannin/gelatin ratios in
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21 which the gelatin-tannin complexes are soluble in nature whereas higher proanthocyanidin
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23 tannin/gelatin ratios induce the formation of insoluble tannin-gelatin precipitates. It remains to be
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25 seen whether those insoluble proanthocyanidin-gelatin complexes are simple aggregates of
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27 soluble proanthocyanidin tannin-gelatin complexes or rather the insoluble complexes are formed
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29 with participation of particular components of the tannin extract once they reach a critical
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31 concentration. Finally, our study strongly contradicts the wide use of the conventional Gelatin
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33 Index. To further substantiate this view and considering that the Gelatin Index comprises a 3-day
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35 incubation period of the “reactants”, we additionally controlled our precipitation assay on
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37 cellulose membranes by incubating the mixtures of gelatin with each of the experimental tannins
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39 for either 10 minutes or 2 days. In these studies, the observed patterns of gelatin-tannin
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41 interactions, with or without precipitation of the corresponding complexes, were mostly
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43 unaffected by the time of incubation. Thus, an expedite and objective evaluation of interactions
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45 of gelatin and tannins or tannin-containing foods, whether or not gelatin-tannin complexes
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47 precipitate, may be more informative about the subjective sensation of astringency that foods
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49 may elicit.
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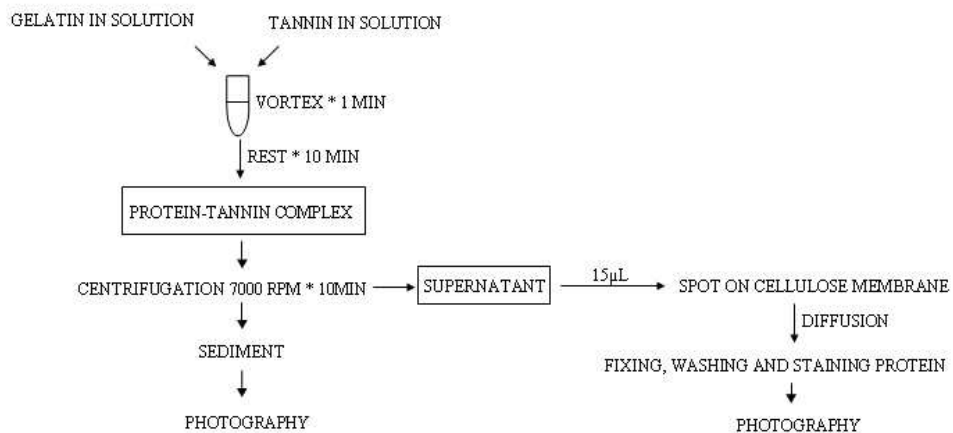
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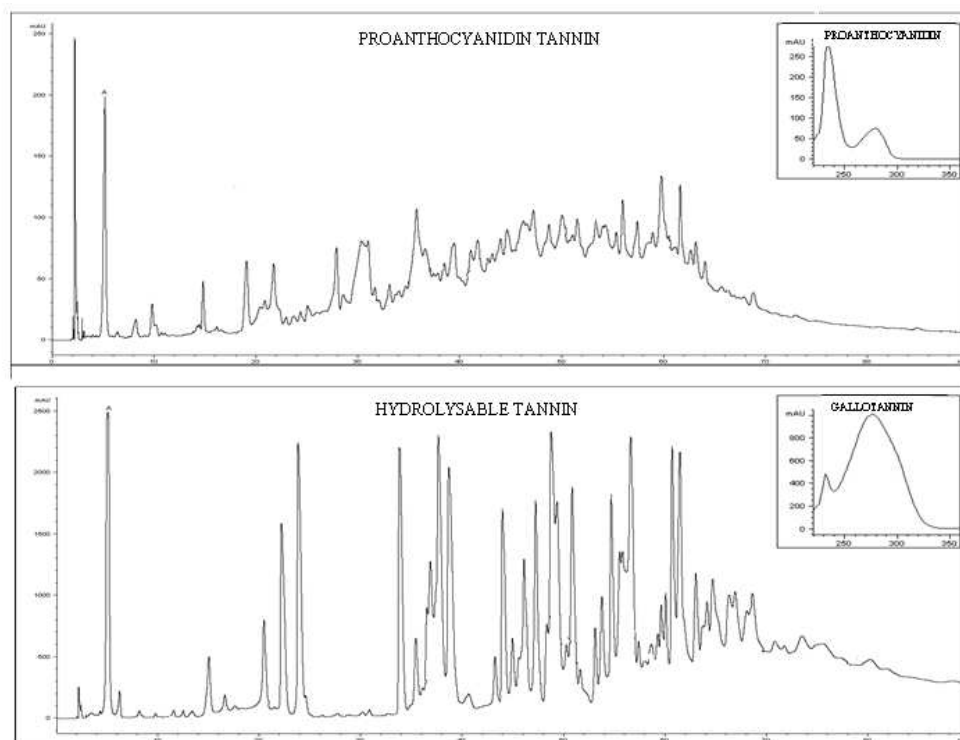
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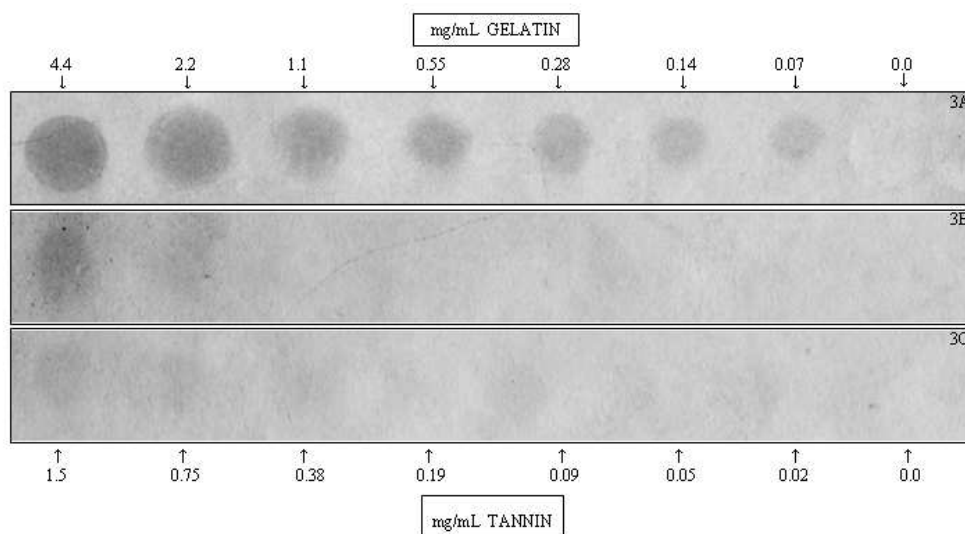
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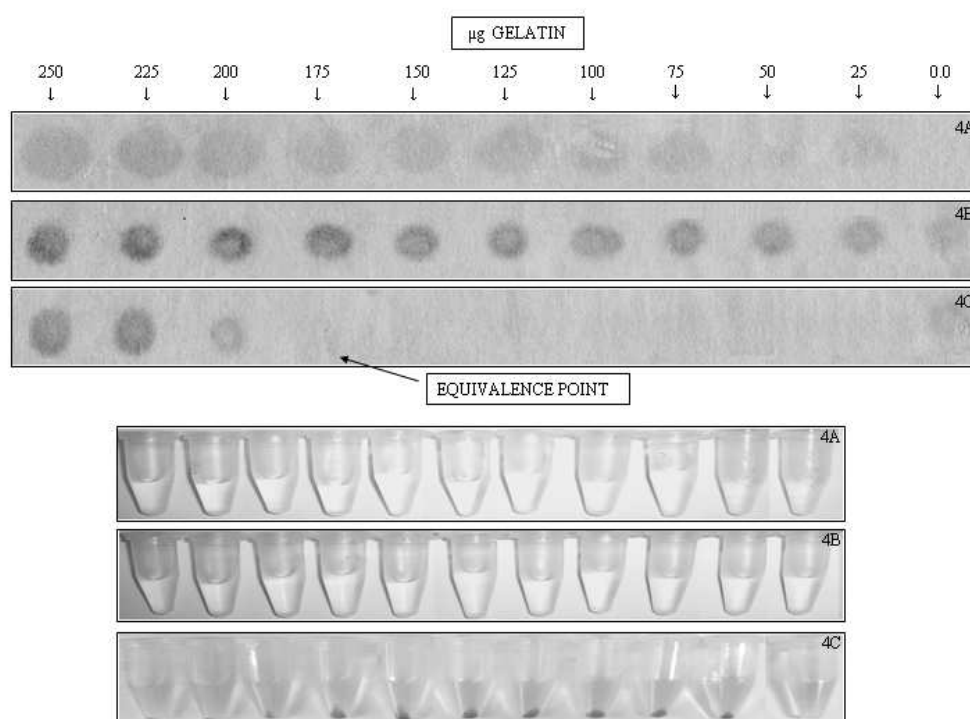
Assay for detection of tannin-gelatin interactions
254x190mm (72 x 72 DPI)



HPLC-DAD chromatograms of two enological tannins and representative UV spectra of most of the corresponding identified components (procyanidins or gallotannins).
254x190mm (72 x 72 DPI)

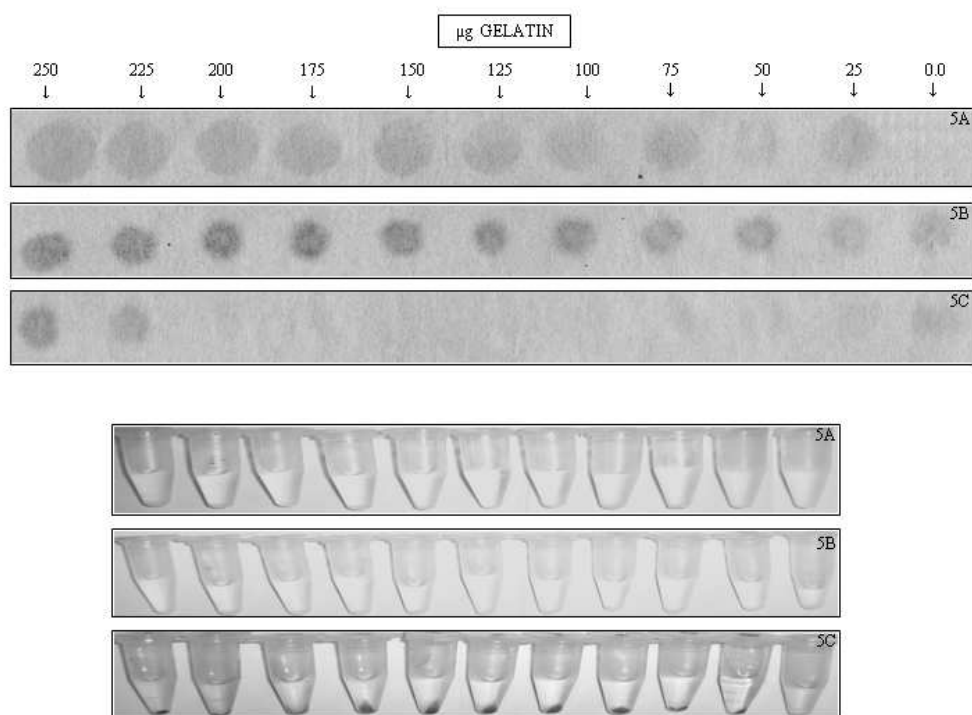


Differential detection of gelatin and enological tannins by a protein-binding dye on cellulose membranes. Fifteen- μ l aliquots of either gelatine in solution (Figure 3A), hydrolysable tannin (Figure 3B) or proanthocyanidin tannin (Figure 3C) were spotted and processed. Concentrations of gelatin (top) and tannins (bottom) are indicated.
254x190mm (72 x 72 DPI)



Interaction between gelatin and tannins. Gelatin in solution (range 0-250 μg from right to left) (Figure 4A) was mixed with 750 μg of either hydrolysable tannin (Figure 4B) or proanthocyanidin tannin (Figure 4C) in final 1-mL and incubated for 10 minutes. After centrifugation only proanthocyanidin tannin-gelatin precipitates were observed (Eppendorf tubes in Figure 4C). Fifteen- μg aliquots of the supernatants from the whole series of tubes were spotted on cellulose membranes and allowed to diffuse. Note that the hydrolysable tannin restricted markedly the free diffusion of gelatine (Figure 4B) whereas the proanthocyanidin tannin both affected protein diffusion (low tannin/protein ratio) and provoked then its full disappearance from the supernatant (precipitation) (Figure 4C).

254x190mm (72 x 72 DPI)



32 Interaction between gelatin and tannins at later times. Both precipitations of tannin-gelatin
33 complexes as well as gelatin diffusion on cellulose membranes shown in the experiment of Figure 4
34 were re-evaluated after a 48-hours period of incubation. Note that at this time the results are
35 mostly undistinguishable from those observed at short times of incubation.

36 254x190mm (72 x 72 DPI)

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