

## Ellagitannins with a Glucopyranose Core Have Higher Affinity to Proteins than Acyclic Ellagitannins by Isothermal Titration Calorimetry

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1 **ABSTRACT:**

2 The thermodynamics of the interactions of different ellagitannins with two proteins, namely bovine serum  
3 albumin (BSA) and gelatin, were studied by isothermal titration calorimetry. Twelve individual ellagitannins,  
4 including different monomers, dimers and a trimer, were used. The studies showed that several structural  
5 features affected the interaction between the ellagitannin and the protein. The interactions of ellagitannins with  
6 proteins were stronger with gelatin than with BSA. The ellagitannin-gelatin interactions contained both the  
7 primary stronger and the secondary weaker binding sites. The ellagitannin-BSA interactions showed very weak  
8 secondary interactions. The ellagitannins with a glucopyranose core had stronger interaction than C-glycosidic  
9 ellagitannins with both proteins. In addition, the observed enthalpy change increased as the degree of  
10 oligomerization increased. The stronger interactions were also observed with free galloyl groups in the  
11 ellagitannin structure and with higher molecular flexibility. Other smaller structural features did not show any  
12 overall trend.

13

14 **KEYWORDS:** *binding, bovine serum albumin, ellagitannin, gelatin, isothermal titration calorimetry,*  
15 *thermodynamics*

16

## 17 INTRODUCTION

18

19 Tannins are plant secondary metabolites, which could also be called plant specialized metabolites<sup>1,2</sup>. Plants  
20 produce them in their tissues to protect themselves against, for example pathogens and insect herbivores.  
21 Tannins are polyphenols that have the ability to bind and precipitate proteins and they can be divided into three  
22 groups: hydrolysable tannins, proanthocyanidins (syn. condensed tannins) and phlorotannins. Hydrolyzable  
23 tannins are further divided into simple gallic acid derivatives, gallotannins and ellagitannins. Ellagitannins  
24 (ETs) are a structurally complex group and individual structures vary from simple hexahydroxydiphenoyl  
25 (HHDP) esters to high oligomers with both varying degree of oligomerization and types of bonds between the  
26 monomers.<sup>3,4</sup> ETs have been stated as the most promising tannin class with potent biological activities, such  
27 as antimicrobial, antioxidant and antiparasitic activities.<sup>3,5-8</sup>

28           Some dietary tannins can have several beneficial effects in animal nutrition and health, for  
29 example, through enabling a better utilization of feed proteins, generating anthelmintic effects against  
30 gastrointestinal nematodes, and lowering nitrogenous and methane emissions.<sup>8-13</sup> The interactions between  
31 tannins and proteins plays an important role in these bioactivities observed. Tannins may bind dietary proteins  
32 and thus can reduce the degradation of these proteins in the rumen and they may also enhance the amount of  
33 protein available for digestion in the small intestine.<sup>12</sup> Tannins can also interact with digestive enzymes, such  
34 as  $\alpha$ -glycosidase,  $\alpha$ -amylase, lipase, pepsin, trypsin, and chymotrypsin, and thus inhibit their enzymatic  
35 activities.<sup>14,15</sup> These interactions are mostly regulated by non-covalent binding, i.e. van der Waals forces,  
36 hydrogen bonding, and other electrostatic forces.<sup>14</sup> Tannins can form soluble and/or insoluble complexes with  
37 proteins, and the tannin-protein interactions are both tannin- and protein-specific.<sup>16</sup> The studies on the effects  
38 of 27 individual ETs and 7 galloylglucoses and gallotannins on the egg hatching of pathogenic parasite  
39 *Haemonchus contortus* showed that several compounds have antiparasitic properties and clear structure-  
40 activity relationships were observed.<sup>8</sup> The mechanisms of action remained unclear but the main reason seemed  
41 to be that tannins bind to egg shell proteins and thereby disturb the egg hatching process.<sup>8</sup>

42           Isothermal titration calorimetry (ITC) is an ideal technique to measure biological binding  
43 interactions, such as the interactions between the tannin and the protein.<sup>17-24</sup> ITC can be used to measure the

44 thermodynamics of the interaction, i.e. the binding constant  $K$ , the enthalpy of binding ( $\Delta H_{\text{obs}}$ ) and the  
45 stoichiometry or number of binding sites ( $n$ ).<sup>24</sup> Most of the ITC studies on the interaction between tannins and  
46 proteins have focused on proanthocyanidins.<sup>19–21,23</sup> Oligomeric and polymeric proanthocyanidins cannot be  
47 purified as individual compounds and therefore mainly proanthocyanidin fractions have been used. This makes  
48 the interpretation of results and the determination of thermodynamic binding parameters more difficult.<sup>23</sup> The  
49 only exceptions are monomeric flavan-3-ols and cocoa proanthocyanidins consisting of epicatechin monomers  
50 which have been separated into different oligomers and studied in detail by ITC.<sup>20,23</sup>

51 In our previous study, we utilized a unique series of oligomeric tellimagrandin I –based ETs  
52 and studied their interaction with BSA by ITC.<sup>22</sup> The ET series from tetramers to octa–undecamers enabled  
53 the evaluation of the effect of the molecular size on the interaction and we could decouple the other structural  
54 features. The interactions of ETs with BSA revealed strong similarities: Enthalpy showed an increasing trend  
55 from the dimer to larger oligomers. Our studies highlighted the importance of molecular flexibility to maximize  
56 binding between the tannin and protein surface.<sup>22</sup> In this study, ET structures were selected so that they differed  
57 in the molecular flexibility and size and that they had different structural features (Fig. 1). These features  
58 included, for example, the tautomeric forms of the glucose core (glucopyranose versus acyclic core), different  
59 functional groups in their structures or the position of free hydroxyl group at C-1 ( $\alpha$  and  $\beta$  anomers). In  
60 addition, two proteins were used: BSA, a model for the globular proteins, and gelatin, a model for flexible  
61 proline-rich proteins.

62 Altogether, 12 ETs were selected and purified from different plant sources and their interactions  
63 were studied with BSA and gelatin by ITC. The aim was to broaden the knowledge on tannin-protein  
64 interactions and to study in detail how efficient different ETs are at binding with different proteins and to  
65 characterize the thermodynamics of these bindings.

66

## 67 MATERIALS AND METHODS

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69 **Isolation and Characterization of ETs.** ETs were extracted, isolated and purified from plant  
70 extracts and characterized by the methods previously described.<sup>4,22,25–28</sup> The plant material was collected and  
71 placed directly into 10 bottles of 1 L, which were then immediately filled with acetone, transferred to the  
72 laboratory, and stored in a cold room (4 °C) prior to the isolation of ellagitannins.<sup>22</sup> The preliminary  
73 fractionation was performed by Sephadex LH-20 chromatography and the final purifications of ellagitannins  
74 were made by preparative and semipreparative HPLC; all steps were followed by UPLC-ESI-MS.<sup>22</sup> The  
75 ellagitannins were identified based on their chromatographic elution order, UV spectra, molecular ions and  
76 characteristic fragment ions based on our previous work and literature as shown in Table 1. Pure ellagitannins  
77 were concentrated to the water phase and freeze-dried. The individual ellagitannins, their purities obtained by  
78 UPLC at 280 nm and the electrospray ionization mass spectrometric identification are presented in Table 1.  
79 .Monomeric ETs with a glycopyranose core, tellimagrandin I and tellimagrandin II (1 and 2 in Fig. 1), were  
80 isolated from the meadowsweet inflorescence (*Filipendula ulmaria*)<sup>29–31</sup> and geraniin (3 in Fig. 1) from the  
81 wood cranesbill leaves (*Geranium sylvaticum*)<sup>32</sup>. Acyclic ETs castalagin and vescalagin (4 and 5 in Fig. 1)  
82 were isolated from the purple loosestrife flowers and leaves (*Lythrum salicaria*)<sup>30,31,33</sup> and castavalonic and  
83 vescavalonic acids (6 and 7 in Fig. 1) from the English oak acorns (*Quercus robur*)<sup>34,35</sup>. The stereochemistry  
84 of castalagin and vescalagin were lately reinvestigated by computational methods and the  
85 nonahydroxytriphenoyl group (NHTP) was found to exist in (*S,R*) configuration.<sup>36,37</sup> Therefore, it is feasible  
86 that the NHTP group of vescavalonic and castavalonic acids is also in (*S,R*) configuration. Dimeric  
87 agrimoniin (8 in Fig. 1) was from the silverweed leaves (*Potentilla anserina*)<sup>25,31,38</sup>, gemin A (9 in Fig. 1) from  
88 the wood avens leaves (*Geum urbanum*)<sup>25,39</sup>, and sanguin H-6 and roshenin C (10 and 11 in Fig. 1) from the  
89 raspberry leaves (*Rubus idaeus*)<sup>25,38,40</sup>. In addition, trimeric lambertianin C (12 in Fig. 1) was isolated from the  
90 raspberry leaves.

91 **Isothermal Titration Calorimetry.** A NanoITC instrument (TA Instruments Ltd., Crawley,  
92 West Sussex, UK) was used to measure the thermodynamics of titrations of ET into BSA (purity  $\geq$  98%,  
93 lyophilized powder, 66 kDa; Sigma-Aldrich, St. Louis, US, CAS 9048-46-8) or into gelatin (Gelatin, type B

94 derived from lime-cured tissue, purity approx. 225 Bloom which is proportional to the average molecular mass  
95 of 50 kDa, Sigma-Aldrich, CAS 9000-70-8). The measurements were performed as earlier described.<sup>22</sup> All  
96 solutions were prepared in 50 mM citrate buffer adjusted to pH 6. In a typical measurement for the interaction  
97 between the ET and BSA, 20 or 30  $\mu$ M BSA solution was placed in the 950  $\mu$ L sample cell of the calorimeter  
98 and 3 mM ET solution was loaded into the injection syringe. The ET studied was titrated into the sample cell  
99 at 298 K as a sequence of 24 injections of 10  $\mu$ L aliquots. The time delay between the injections was 360 s.  
100 Each ET-BSA interaction was measured at least with three replicates.

101 In a typical measurement for the interaction between ET and gelatin, different gelatin  
102 concentrations were used depending on the strength of the interactions. The different gelatin contents were  
103 0.3, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/mL. To get the molarities of the solution, the content of the solution was  
104 divided with the estimated molecular mass of gelatin (50 000 g/mol). The molarities of the gelatin solutions  
105 were thus approximately 6, 10, 15, 20, 30 and 40  $\mu$ M, respectively. Each ET-gelatin interaction was measured  
106 at least with three replicates.

107 Raw data were obtained as plots of heat ( $\mu$ J) against injection number and exhibited a series of  
108 peaks for each injection. Examples of thermograms are available in figures S1-S6 in Supporting Information.  
109 The plots of raw heat data were transformed using the NanoAnalyze Data Analysis software (version 2.4.1.,  
110 TA instruments) to obtain a plot of observed enthalpy change per mole of injectant ( $\Delta H_{\text{obs}}$ , kJ/mol) against  
111 molar ET:BSA ratio. The control data of ellagitannin titrated into buffer were always subtracted from the  
112 sample data as it was known that ellagitannins tend to selfassociate into aggregates and then undergo an  
113 endothermic process of deaggregation when titrated from the syringe into buffer.<sup>19,22</sup> Data fits and estimated  
114 binding parameters were obtained in two different ways: using a single set of multiple binding sites and a  
115 model for two independent sets of multiple binding sites. The quality of fits was determined by standard  
116 deviation.

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## 120 RESULTS AND DISCUSSION

121

122 **ITC Binding Isotherms and Data Fitting for the ET/BSA Interaction.** The interactions of twelve individual  
123 ETs (Figure 1) with BSA were studied by ITC. These ETs included three cyclic ET monomers, namely  
124 tellimagrandin I and tellimagrandin II and geraniin (ITC isotherms shown in Figure 2), four ET dimers, i.e.  
125 sanguiin H-6, roshenin C, agrimoniin and gemin A, and one ET trimer, i.e. lambertianin C (Fig. 3), and four  
126 acyclic ET monomers, namely castalagin, vescalagin, castavalonic acid and vescavalonic acid (Fig. 4).

127 Figures 2 and 3 show the ITC binding isotherms for the cyclic ET monomers, dimers and trimer  
128 binding to BSA. For these ET-BSA systems, an exothermic interaction was observed, and the interaction  
129 became less exothermic with the increasing injection number (increase in the tannin to protein molar ratio) as  
130 the binding sites of BSA became saturated. All experiments were performed in triplicate and using different  
131 concentrations of BSA, varying from 10  $\mu\text{M}$  to 40  $\mu\text{M}$  depending on the ET and the observed changes of  
132 enthalpy were detected. The interaction was not affected by BSA concentration, which suggested no evidence  
133 of co-operative binding as previously reported for oligomeric ETs.<sup>22</sup>

134 The data fittings were performed using two different binding models. One assumed a single set  
135 of multiple binding sites (later called as single-site model) and the other one two independent sets of multiple  
136 binding sites (later called as two-site model).<sup>21</sup> For sanguiin H-6 and lambertianin C, the first data points of  
137 the titration isotherm were not used in order to fit the data both in two-site and one-site models (Fig. 3C and  
138 D). We have observed similar shapes in tannin-protein binding isotherms before and have suggested that this  
139 could indicate synergism in protein binding, such that the presence of ellagitannin already bound to gelatin has  
140 an effect on the binding of subsequent tannin molecules.<sup>19</sup> There are also other possibilities that could explain  
141 this trend since there could be competing endothermic and exothermic interactions at play. In general, it can  
142 be seen that both binding models visually fit the data well for all of the ET/BSA systems with little difference  
143 in the agreement of the data fit curves and the data points for both binding models. Where a two-site model  
144 was used the second site showed usually a weaker binding with  $K_{a2}$  in the region of  $10^1$ - $10^3$   $\text{M}^{-1}$  (Table 2 and  
145 3) and a single-site model was able to equally well represent the data. In some cases, see for example  
146 lambertianin C and BSA (Fig. 3D), the two-site model clearly exhibited a slightly better fit to the data than the  
147 single-site binding model. Similar observation were made in our previous study<sup>22</sup>, and therefore, the fit

148 parameters for both binding models are shown in Tables 2 and 3. In addition, the estimated entropies for the  
149 ellagitannin-BSA interactions are presented as Supporting Information in Table S1. However, as all the  
150 interactions between ETs and BSA fit to the one-site model there is no clear justification for increasing the  
151 complexity of the model used to fit this data and therefore we will discuss the interactions between BSA and  
152 ETs based on the fittings obtained by the single-site model.

153 The ETs could be classified into three different groups based on the strength of their interactions  
154 with BSA; i.e. stronger, moderate and weaker interaction: i) Six ETs with a glupyranose core, i.e.  
155 tellimagrandin I, tellimagrandin II, agrimoniin, gemin A, sanguin H-6, and lambertianin C, had the strongest  
156 interactions with BSA and the thermodynamic binding parameters could be estimated for these interactions  
157 (Tables 2 and 3). ii) Two ETs with a glucopyranose core, i.e. geraniin and roshenin C, had moderate  
158 interactions with BSA (Figs. 2 and 3), and for these it was difficult to produce a clear fit to the data and  
159 therefore we have less confidence in our estimated thermodynamic binding parameters for these ET/BSA  
160 interactions. iii) All acyclic ETs, i.e. castalagin, vescalagin, castavalonic acid, and vescavalonic acid, had  
161 no interaction or very weak interactions with BSA (Fig. 4) and no fits or thermodynamic binding parameters  
162 were obtained. Therefore, it was immediately evident that binding of the ETs to BSA were stronger for ETs  
163 with a cyclic core than for those with an acyclic glucose core.

164 Both sanguin H-6 and lambertianin C binding to BSA exhibited an increase in  $\Delta H$  during early  
165 injection numbers (low molar ratio) which indicates that the presence of ET in the BSA/ET solution results in  
166 an initial increase in binding. This may indicate that previously bound ET on the protein is able to facilitate  
167 the binding of subsequent ET molecules injected into the solution. Such features in ITC binding isotherms  
168 have been seen in other studies as an indication of this kind of co-operative binding. We also noted above that  
169 gemin A exhibited different behavior in cases where the experimental procedure was different, and this may  
170 be explained if the method was able to provide enough time between injections to maximize the effect of this  
171 co-operative binding.

172 Table 2 and Figure 2 compare our three monomeric cyclic ETs and show that the binding with  
173 BSA is strongest for tellimagrandin I ( $K_a = 1.8 \times 10^4 \text{ M}^{-1}$ ) compared to tellimagrandin II ( $K_a = 7.3 \times 10^{-3} \text{ M}^{-1}$ ).  
174 As could be expected, the weakest binding with BSA of our ET monomers studied was with geraniin, which  
175 also exhibits a more rigid constrained structure compared to tellimagrandin I and II. For all these three ETs,



176  $\Delta H$  is similar. It is not clear if there is any substantial trend or information that can be gained from the values  
177 of  $n$  (number of ET molecules binding to each BSA molecule). Our fit consistently gave tellimagrandin II a  
178 relatively high value for  $n$  compared to the other ETs (Table 2). Previously, there has been a suggestion that  
179 weak binding between tannins and BSA can result from unspecific adsorption that has a weak binding affinity  
180 to the BSA surface. The weak  $K_a$  seen for tellimagrandin II coincides with a higher fitted value for  $n$  and may  
181 well indicate such an adsorption event. To further support the link between high values for  $n$  and non-selective  
182 adsorption, tellimagrandin II has poorer water-solubility than other ETs (based on unpublished octanol-water  
183 coefficients), and thus a greater tendency towards surface adsorption. This type of adsorption might also be  
184 expected for ETs with greater flexibility in their structure allowing for less conformational restraints and  
185 increased ability to non-selectively binding to protein surfaces.<sup>17,18</sup> Our dimeric and trimeric ETs show  
186 variations in binding that are greater than that seen for tellimagrandin II and geraniin, with the trimeric  
187 lambertianin C exhibiting the strongest interaction ( $K_a = 1.1 \times 10^5 \text{ M}^{-1}$ ). Overall, we see a link between the  
188 oligomerization and the strength of interaction between ET and BSA. As seen here, our previous studies found  
189 that the interaction of monomeric tellimagrandin I with BSA was stronger, in terms of  $K_a$ , than expected in  
190 relation to the oligomeric series of ETs.<sup>22</sup> However, that oligomeric series contained similar monomeric units  
191 in all the oligomers, which enabled the direct comparison of the interaction between the different oligomers  
192 based on the number of monomeric units and without the effects of the other structural features, such as  
193 functional groups.<sup>22</sup> In general for polyphenols, the increase in the binding affinity with the molecular size  
194 have also been observed previously even if other differences in the molecular structures are present.<sup>41</sup>  
195 However, for quercetin and quercetin 3-*O*- $\beta$ -D-glucopyranoside binding with BSA, the opposite has been  
196 reported.<sup>42</sup>

197           The dimeric ETs that we investigated exhibited similar behavior in terms of their binding to  
198 BSA, agrimoniin, gemin A and sanguin H-6 had equilibrium binding constants varying between  $1.1$ - $1.7 \times 10^4$   
199  $\text{M}^{-1}$ , and similar values for  $\Delta H$  and  $n$ . Agrimoniin and gemin A are structurally closely similar; the main  
200 difference is that agrimoniin contains four HHDP groups whereas gemin A has three HHDP groups and two  
201 free galloyl groups and that the orientation at C-1 of the glucose is  $\alpha$  in agrimoniin and  $\beta$  in gemin A. Two of  
202 the binding parameters for gemin A,  $\Delta H_1 = -45 \text{ kJ mol}^{-1}$  and  $n = 14$ , are similar to the previously measured  
203 values,  $\Delta H_1 = -47 \text{ kJ mol}^{-1}$  and  $n_1 = 13$ , but the equilibrium binding constant  $1.1 \times 10^4 \text{ M}^{-1}$  is different to the

204 previous one  $1.8 \times 10^6 \text{ M}^{-1}$ .<sup>17</sup> In previous study, ETs were titrated into the sample cell in two titration events  
205 where the syringe was filled within the run, i.e. first the sequence of 24 injections, then the filling of the syringe  
206 and then the sequence of 24 injections.<sup>17</sup> The current measurements were performed as a single titration event  
207 without any additional distractions to the titration, and this means that the titration volume and time taken for  
208 the experiment to complete are different. Sanguiin H-6 and roshenin C only differ by the latter lacking one  
209 HHDP group, but still they showed very different behaviors in terms of BSA binding. Our fits for roshenin C  
210 are poor because of the observed weakness of the interaction with small variation in  $\Delta H$  and show significant  
211 variability in terms of the strong binding site between the two binding model fits. We are unable to identify  
212 any structural reason to explain why the roshenin/BSA interaction is weak and we are unable to provide  
213 confident fit parameters for this ET. Nevertheless, this finding shows that not only galloyls are important for  
214 increasing tannin-protein interaction, but also the presence vs. absence makes a difference.

215           Acyclic ETs castalagin, vescalagin, castavalonic and vescavalonic acids had very weak  
216 interactions with BSA based on the isotherms (Fig. 4). No reliable fits or thermodynamic binding parameters  
217 were obtained. The weak interaction cannot be due to low water-solubility as acyclic ETs are very water-  
218 soluble.<sup>28</sup> The other reason could be the relatively rigid conformation of acyclic ETs having NHTP groups in  
219 their structures (Fig. 1). The interaction between vescalagin and BSA has been previously studied and found  
220 to be weak in comparison to other ETs.<sup>17</sup> The interaction between acyclic ETs and BSA were so weak that we  
221 could not evaluate the effects of other structural features, such as the role of free COOH present in  
222 vescavalonic and castavalonic acids or the effects of the  $\alpha$  or  $\beta$  orientation at C-1 of the glucose.

223           **ITC Binding Isotherms and Data Fitting for the ET/Gelatin Interaction.** The interaction of  
224 the ETs with gelatin is shown in Figures 5-7 and Tables 4-5. The cyclic ET monomers in Fig. 5 and the cyclic  
225 ET dimers and trimer in Fig. 6 all show an exothermic interaction between the ET and gelatin showing a  
226 gradual decrease in exothermicity as the binding sites of gelatin become saturated. In general, the interaction  
227 was stronger between gelatin and ETs than between BSA and ETs. As for our ET-BSA data, the data fittings  
228 were performed using the single-site model and the two-site model. However, for the interaction with gelatin  
229 it can be seen that overall the two-site model fit the data better. For example, for the interaction between gemin  
230 A and gelatin, the two-site model clearly visually exhibited a closer fit (Fig. 6B) and the calculated SD for the  
231 fits and thermodynamic binding parameters (Table 5) also supported the presence of the secondary binding

232 site. Similar observations were made for all the other ETs (Figs. 5 and 6, Tables 4 and 5). For the trimer ET,  
233 lambertianin C (Fig. 6D), the data showed no evidence of a second-site binding and the ET-gelatin binding  
234 isotherm showed a strong primary binding site. As for our BSA data, the fit parameters for both binding models  
235 are shown in Tables 4 and 5. In addition, the estimated entropies for the ellagitannin-gelatin interactions are  
236 presented as Supporting Information in Table S1. Given that the interaction between gelatin and ETs are  
237 approximately ten-fold stronger (see  $K_{al}$  values), it may not be surprising that a second, perhaps less specific  
238 binding (or non-selective adsorption to the protein) could be observed.

239                 Similarly to the interaction between different ETs and BSA, it was evident that the interactions  
240 between ETs and gelatin were stronger for ETs with a glucopyranose core than for acyclic ETs as the observed  
241 changes of enthalpy were higher for these ETs than for acyclic ETs (Fig. 6 in comparison to Fig. 7). The ETs  
242 can be classified into two groups based on the strength of their interactions with gelatin. i) Seven ETs with a  
243 glucopyranose core, *i.e.* tellimagrandin I, tellimagrandin II, agrimoniin, gemin A, sanguin H-6, lambertianin  
244 C, and roshenin C had stronger interactions with gelatin (Figs. 5 and 6) and the thermodynamic binding  
245 parameters could be estimated for these interactions (Tables 4 and 5). ii) Four acyclic ETs, *i.e.* castalagin,  
246 vescalagin, castavalonic acid, and vescavalonic acid, had no interaction or very weak interactions with  
247 gelatin (Fig. 7) and no fits or thermodynamic binding parameters were obtained. For both BSA and gelatin,  
248 geraniin is an exception to this rule, where it behaved more like the acyclic ETs and for gelatin no binding  
249 parameters could be obtained.

250                 In general, the interactions between gelatin and ETs are independent of ET concentration as  
251 also seen to the interaction between ETs and BSA. However, there seems to be some exceptions, see for  
252 example geraniin in Fig. 5C which shows the ITC data for the titration of geraniin into gelatin solutions of  
253 varying concentrations (two replicates for 20  $\mu$ M gelatin, 30  $\mu$ M gelatin and 40  $\mu$ M gelatin). The shapes of  
254 the isotherms are completely different in comparison to those of other ETs with a glucopyranose core.  
255 Typically the interaction between the protein and ET gets smaller with increasing injections (molar ratio) as  
256 the saturation of the binding site on the protein occurs (Fig. 6 shows example of this usual behavior). However,  
257 initially at low molar ratio the interaction between gelatin and geraniin gets stronger when more geraniin is  
258 added (Fig. 5C) and the shape of the interaction changes with the increasing protein concentration. The  
259 biphasic shape of the isotherms and dependence on protein concentration are similar to the ITC isotherms of

260 SDS titration into lysozyme.<sup>43</sup> There is a gradual increase in the binding enthalpy that reaches a plateau region,  
261 with a maximum enthalpy change of the binding of approximately  $-20$  kJ/mol, followed by a decrease in the  
262 measured enthalpy change as the protein binding sites become saturated. This would suggest that initial binding  
263 is co-operative in that the presence of pre-bound ET initially promotes the exothermicity of the interaction.  
264 Also, the binding is stronger in a higher protein concentration solution which could indicate oligomerization  
265 of the protein and more complex protein-ET intermolecular structures. Others have also observed biphasic  
266 binding isotherms in cases where the ligand induces oligomerization of the protein.<sup>44</sup> It is possible given the  
267 structure of gelatin that geraniin is able to alter its secondary structure to promote oligomerization. Although  
268 less pronounced, the acyclic ETs (Fig. 7), particularly castalagin, appear to exhibit a similar behavior.

269 For the interaction between the ETs with a glycopyranose core and gelatin, there is a clear link  
270 between strength of binding and oligomerization of the ETs. Generally weaker interactions observed for the  
271 monomers ( $K_{al}$  range  $0.8-1.8 \times 10^5 \text{ M}^{-1}$ ) compared to the dimers ( $K_{al} = 1.5-13 \times 10^5 \text{ M}^{-1}$ ), and a strongest  
272 interaction seen for the trimer ET ( $K_{al} = 19 \times 10^5 \text{ M}^{-1}$ , Table 5).

273 In summary, we had a selection of purified ETs and were able to determine their interactions  
274 with selected proteins, i.e. BSA and gelatin, in addition to the thermodynamic parameters related to this  
275 interaction. Given the importance of this interaction in defining the biological activities of these molecules and  
276 the current difficulties in studying such systems, the use of purified tannins provides structure-function  
277 information that has previously been difficult to derive from less purified tannin fractions. BSA and gelatin  
278 model different aspects of protein structure; BSA as a globular well-characterized protein and gelatin as a  
279 proline-rich random coil structure that are also exhibited in salivary proline-rich proteins. Our data clearly  
280 shows a difference in protein binding behavior of ETs with cyclic and acyclic glucose cores, showing very  
281 weak binding to the acyclic structures where the ET tends to have a less open structure and relatively rigid  
282 conformation. Our data show that ETs with a glucopyranose structure are able to bind more strongly to the  
283 protein. These observations were observed for binding to both proteins. As expected, the binding to gelatin  
284 was stronger than to BSA which indicates the importance of the more flexible protein structure on tannin  
285 binding behavior. The binding was also dependent on the oligomerization of the ET, with the larger ET binding  
286 more strongly. It is clear that the ETs bind to multiple sites on the surface of the protein and those able to wrap  
287 around the protein structure and increase foot-holds to the protein surface are able to bind more strongly. For

288 the ET-gelatin binding, a two-site binding model better described the interaction of the dimeric ETs and we  
289 observe for all ET-protein interactions relatively high values for  $n$ ; this shows that the ET-protein interaction  
290 is not dependent on a specific binding domain but is less selective with regions of higher and lower binding  
291 affinity that may be related to hydrophobicity or surface charged regions rather than specific tertiary binding  
292 regions. Thus, it may be easier to consider these interactions as a non-selective adsorption behavior particularly  
293 when considering the second-binding site.

294 In most cases, a tannins biological activity, for example as an anthelmintic compound, may be  
295 defined by how it interacts with proteins. Our data showed that acyclic ETs with NHTP groups had weaker  
296 interaction with proteins than the ETs with a glucopyranose core. Similarly, the presence of NHTP groups was  
297 shown to be an important factor in the anthelmintic effects of ETs, as detected by a decrease in the inhibition  
298 activity of ETs against egg hatching of *H. contortus*.<sup>8</sup> It is thus probable that these types of ITC experiments  
299 described in this paper are able to reveal the possible significant or non-significant role of tannins in many  
300 aspects related to tannin-ruminant interactions.

301

#### 302 ABBREVIATIONS USED

303 BSA, bovine serum albumin; DAD, diode array detection; ESI, electrospray ionization; ET, ellagitannin;  
304 HHDP, hexahydroxydiphenoyl; HPLC, high-performance liquid chromatography; ITC, isothermal titration  
305 calorimetry; MS, mass spectrometry; NHTP, nonahydroxytriphenoyl; UPLC-, ultra-performance liquid  
306 chromatography

307

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312

#### 313 ASSOCIATED CONTENT

314 **\*S Supporting Information**

315 The Supporting Information is available free of charge on the ACS Publications website at DOI: x.

316 Figures S1-S6. Examples of thermograms for the interaction of ellagitannins with BSA and gelatin. The  
317 thermograms include the raw heat data ( $\mu\text{J/s}$ ) from where the control experiment is not subtracted.

318 Table S1. Estimated entropies for the interaction of ellagitannins with BSA and gelatin fitted by two-site and  
319 one-site binding models.

320 (PDF)

321

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463 **Notes**

464 The authors declare no competing financial interest.

## FIGURE CAPTIONS

**Figure 1.** Individual ellagitannins studied for their interactions with BSA and gelatin. A refers to gallic acid, DHHDP to dehydrohexahydroxydiphenoyl group, G to galloyl group, GOD to linking between a galloyl and an HHDP group, GOG to linking between two galloyl groups, HHDP to hexahydroxydiphenoyl group and NHTP to nonahydroxytriphenoyl group.

**Figure 2.** Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of monomeric ellagitannins with a glucopyranose core: tellimagrandin I (A), tellimagrandin II (B), and the ITC binding isotherms for the interaction of monomeric ellagitannin geraniin (C) with 20  $\mu\text{M}$  BSA in two replicate measurements (■ and □) and 30  $\mu\text{M}$  BSA ( $\Delta$ ) and 40  $\mu\text{M}$  BSA in two replicates ( $\times$  and +).

**Figure 3.** Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of the ellagitannin dimers with a glucopyranose core: agrimoniin (A), gemin A (B), sanguin H-6 (C), and trimer lambertianin C (D) with 30  $\mu\text{M}$  BSA, and the ITC binding isotherms for the interaction of dimeric ellagitannin roshenin C (E) with 20  $\mu\text{M}$  BSA (■) and 30  $\mu\text{M}$  BSA in two replicate experiments ( $\Delta$  and  $\times$ ).

**Figure 4.** ITC binding isotherms for the interaction of acyclic ellagitannins castalagin (A) with 10  $\mu\text{M}$  BSA (■), 20  $\mu\text{M}$  BSA ( $\Delta$ ) and 30  $\mu\text{M}$  BSA ( $\times$ ), vescalagin (B) with 20  $\mu\text{M}$  BSA (■) and 30  $\mu\text{M}$  BSA in two replicate experiments ( $\Delta$  and  $\times$ ), castavalonic acid (C) with 20  $\mu\text{M}$  BSA (■) and 30  $\mu\text{M}$  BSA in two replicate experiments ( $\Delta$  and  $\times$ ) and vescavalonic acid (D) with 20  $\mu\text{M}$  BSA (■) and 30  $\mu\text{M}$  BSA in two replicate experiments ( $\Delta$  and  $\times$ ).

**Figure 5.** Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of ellagitannins with a glucopyranose core: tellimagrandin (A) I with 10  $\mu\text{M}$  gelatin and tellimagrandin II (B) with 20  $\mu\text{M}$  gelatin. In addition, ITC binding isotherms for the

interaction of monomeric ellagitannin geraniin (C) with 6  $\mu\text{M}$  gelatin in two replicate measurements (■ and □) and with 10  $\mu\text{M}$  gelatin ( $\Delta$ ) and 20  $\mu\text{M}$  gelatin ( $\times$ ).

**Figure 6.** Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the experimental data (■) for the interaction of ellagitannins with a glucopyranose core: agrimoniin (A), gemin A (B), sanguin H-6 (C), lambertianin C (D), and roshenin C (E) with 20  $\mu\text{M}$  gelatin.

**Figure 7.** ITC binding isotherms for the interaction of acyclic ellagitannins castalagin (A), vescalagin (B), castavalonic acid (C), and vescavalonic acid (D) with 6  $\mu\text{M}$  gelatin (■), 10  $\mu\text{M}$  gelatin ( $\Delta$ ) and 20  $\mu\text{M}$  gelatin ( $\times$ ).

**Table 1.** The Individual Ellagitannins Tested, Their Purities Obtained by UPLC at 280 nm and Electrospray Ionization Mass Spectrometric (ESI-MS) Identification

| No. | Ellagitannin      | Purity (%) | ESI-MS identification ( <i>m/z</i> )   | Literature |
|-----|-------------------|------------|--|------------|
| 1   | Tellimagrandin I  | 97.5       | 785 [M-H] <sup>-</sup>   | 27,31      |
| 2   | Tellimagrandin II | 97.9       | 937 [M-H] <sup>-</sup> , 301 [ellagic acid-H] <sup>-</sup>   | 27,29      |
| 3   | Geraniin          | 98.3       | 951 [M-H] <sup>-</sup> , 933 [M-H <sub>2</sub> O-H] <sup>-</sup> ,   | 32         |
| 4   | Castalagin        | 99.6       | 933 [M-H] <sup>-</sup> , 466 [M-2H] <sup>2-</sup> ,<br>301 [ellagic acid-H] <sup>-</sup>   | 30,31,33   |
| 5   | Vescalagin        | 94.1       | 933 [M-H] <sup>-</sup> , 915 [M-H <sub>2</sub> O-H] <sup>-</sup> , 466 [M-2H] <sup>2-</sup> ,<br>457 [M-H <sub>2</sub> O-2H] <sup>2-</sup> , 301 [ellagic acid-H] <sup>-</sup>                   | 30,31,33   |
| 6   | Castavalonic acid | 99.6       | 1101 [M-H] <sup>-</sup> , 528 [M-COOH-H] <sup>2-</sup>   | 34,35      |
| 7   | Vescavalonic acid | 95.6       | 1101 [M-H] <sup>-</sup> , 1083 [M-H <sub>2</sub> O-H] <sup>-</sup> ,<br>528 [M-COOH-H] <sup>2-</sup> ,<br>519 [ [M-H <sub>2</sub> O-COOH-H] <sup>2-</sup> ,<br>301 [ellagic acid-H] <sup>-</sup> | 34,35      |
| 8   | Agrimoniin        | 97.7       | 934 [M-2H] <sup>2-</sup> , 301 [ellagic acid-H] <sup>-</sup>   | 25,29,31   |
| 9   | Gemin A           | 98.2       | 935 [M-2H] <sup>2-</sup> , 301 [ellagic acid-H] <sup>-</sup>   | 25,39      |
| 10  | Sanguiin H-6      | 97.6       | 934 [M-2H] <sup>2-</sup> , 301 [ellagic acid-H] <sup>-</sup>   | 25,40      |
| 11  | Roshenin C        | 93.8       | 783 [M-2H] <sup>2-</sup> , 301 [ellagic acid-H] <sup>-</sup>   | 38         |
| 12  | Lambertianin C    | 95.6       | 934 [M-3H] <sup>3-</sup> , 301 [ellagic acid-H] <sup>-</sup>   | 25,40      |

**Table 2.** Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Monomeric Ellagitannins with BSA Fitted by Two-Site and One-Site Binding Models. The Values for Tellimagrandin I Have Been Previously Published.<sup>22</sup> SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

| <b>Two-Site</b>                 | <b>Tellimagrandin I</b> | <b>Tellimagrandin II</b> |
|---------------------------------|-------------------------|--------------------------|
| $K_{a1}$ ( $M^{-1}$ )           | 22188 ± 6280            | 8308 ± 6248              |
| $\Delta H_1$ ( $kJ\ mol^{-1}$ ) | -20 ± 3                 | -33 ± 5                  |
| $n_1$                           | 6 ± 2                   | 29 ± 1                   |
| $K_{a2}$ ( $M^{-1}$ )           | 1828 ± 1815             | 36 ± 13                  |
| $\Delta H_2$ ( $kJ\ mol^{-1}$ ) | -10 ± 9                 | -28 ± 6                  |
| $n_2$                           | 4 ± 1                   | 110 ± 27                 |
| SD                              | 12 ± 2                  | 28 ± 6                   |

| <b>One-Site</b>                 | <b>Tellimagrandin I</b> | <b>Tellimagrandin II</b> |
|---------------------------------|-------------------------|--------------------------|
| $K_{a1}$ ( $M^{-1}$ )           | 18403 ± 5052            | 7284 ± 5408              |
| $\Delta H_1$ ( $kJ\ mol^{-1}$ ) | -24 ± 3                 | -37 ± 6                  |
| $n_1$                           | 6 ± 1                   | 30 ± 1                   |
| SD                              | 13 ± 4                  | 25 ± 6                   |

**Table 3.** Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Dimeric and Trimeric Ellagitannins with BSA Fitted by Two-Site and One-Site Binding Models. SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

| <b>Two-Site</b>                 | <b>Agrimoniin</b> | <b>Gemin A</b>   | <b>Sanguiin H-6</b> | <b>Lambertianin C</b> |
|---------------------------------|-------------------|------------------|---------------------|-----------------------|
| $K_{a1}$ ( $M^{-1}$ )           | $35687 \pm 20734$ | $16413 \pm 3912$ | $35360 \pm 8262$    | $156900 \pm 16108$    |
| $\Delta H_1$ ( $kJ\ mol^{-1}$ ) | $-18 \pm 4$       | $-37 \pm 1$      | $-18 \pm 1$         | $-25 \pm 2$           |
| $n_1$                           | $16 \pm 1$        | $14 \pm 1$       | $17 \pm 1$          | $11 \pm 1$            |
| $K_{a2}$ ( $M^{-1}$ )           | $686 \pm 245$     | $784 \pm 176$    | $1134 \pm 273$      | $3117 \pm 4760$       |
| $\Delta H_2$ ( $kJ\ mol^{-1}$ ) | $-8 \pm 1$        | $-8 \pm 2$       | $-6 \pm 1$          | $-3 \pm 1$            |
| $n_2$                           | $46 \pm 1$        | $30 \pm 1$       | $94 \pm 12$         | $37 \pm 13$           |
| SD                              | $19 \pm 3$        | $31 \pm 3$       | $20 \pm 2$          | $20 \pm 8$            |

| <b>One-Site</b>                 | <b>Agrimoniin</b> | <b>Gemin A</b>   | <b>Sanguiin H-6</b> | <b>Lambertianin C</b> |
|---------------------------------|-------------------|------------------|---------------------|-----------------------|
| $K_{a1}$ ( $M^{-1}$ )           | $17140 \pm 5892$  | $11470 \pm 1223$ | $13337 \pm 4242$    | $107180 \pm 37045$    |
| $\Delta H_1$ ( $kJ\ mol^{-1}$ ) | $-24 \pm 4$       | $-45 \pm 4$      | $-26 \pm 2$         | $-28 \pm 1$           |
| $n_1$                           | $17 \pm 1$        | $14 \pm 1$       | $19 \pm 1$          | $11 \pm 1$            |
| SD                              | $20 \pm 4$        | $29 \pm 3$       | $21 \pm 3$          | $26 \pm 1$            |



**Table 4.** Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Monomeric Ellagitannins with Gelatin Fitted by Two-Site and One-Site Binding Models. SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

| <b>Two-Site</b>                 | <b>Tellimagrandin I</b> | <b>Tellimagrandin II</b> |
|---------------------------------|-------------------------|--------------------------|
| $K_{a1}$ ( $M^{-1}$ )           | $175600 \pm 28614$      | $84207 \pm 16299$        |
| $\Delta H_1$ ( $kJ\ mol^{-1}$ ) | $-14 \pm 3$             | $-56 \pm 1$              |
| $n_1$                           | $52 \pm 19$             | $31 \pm 1$               |
| $K_{a2}$ ( $M^{-1}$ )           | $88693 \pm 53165$       | $7092 \pm 3005$          |
| $\Delta H_2$ ( $kJ\ mol^{-1}$ ) | $-11 \pm 2$             | $-3 \pm 2$               |
| $n_2$                           | $120 \pm 55$            | $59 \pm 6$               |
| SD                              | $15 \pm 2$              | $55 \pm 12$              |

| <b>One-Site</b>                 | <b>Tellimagrandin I</b> | <b>Tellimagrandin II</b> |
|---------------------------------|-------------------------|--------------------------|
| $K_{a1}$ ( $M^{-1}$ )           | $7592 \pm 1492$         | $76143 \pm 5888$         |
| $\Delta H_1$ ( $kJ\ mol^{-1}$ ) | $-39 \pm 9$             | $-59 \pm 2$              |
| $n_1$                           | $67 \pm 31$             | $31 \pm 1$               |
| SD                              | $36 \pm 2$              | $48 \pm 11$              |

**Table 5.** Estimated Thermodynamic Binding Parameters for the Interaction of Cyclic Dimeric and Trimeric Ellagitannins with Gelatin Fitted by Two-Site and One-Site Binding Models. SD = Standard Deviation Around Fit Obtained by NanoAnalyze Software; n = 3

| <b>Two-Site</b>                      | <b>Agrimoniin</b> | <b>Gemin A</b>   | <b>Sanguiin H-6</b> | <b>Roshenin C</b> | <b>Lambertianin C</b> |
|--------------------------------------|-------------------|------------------|---------------------|-------------------|-----------------------|
| $K_{a1}$ (M <sup>-1</sup> )          | 169667 ± 43966    | 1275667 ± 114001 | 220500 ± 18163      | 150600 ± 35982    | 1866000 ± 268836      |
| $\Delta H_1$ (kJ mol <sup>-1</sup> ) | -65 ± 5           | -64 ± 3          | -63 ± 4             | -30 ± 4           | -91 ± 7               |
| $n_1$                                | 18 ± 2            | 15 ± 1           | 16 ± 1              | 24 ± 2            | 11 ± 1                |
| $K_{a2}$ (M <sup>-1</sup> )          | 11267 ± 6161      | 42425 ± 11770    | 42197 ± 8074        | 42063 ± 22578     | 250 ± 65              |
| $\Delta H_2$ (kJ mol <sup>-1</sup> ) | -10 ± 1           | -17 ± 1          | -12 ± 1             | -57 ± 8           | -22 ± 10              |
| $n_2$                                | 52 ± 3            | 21 ± 2           | 38 ± 2              | 30 ± 2            | 15 ± 2                |
| SD                                   | 78 ± 22           | 45 ± 18          | 66 ± 20             | 57 ± 13           | 55 ± 14               |

| <b>One-Site</b>                      | <b>Agrimoniin</b> | <b>Gemin A</b>  | <b>Sanguiin H-6</b> | <b>Roshenin C</b> | <b>Lambertianin C</b> |
|--------------------------------------|-------------------|-----------------|---------------------|-------------------|-----------------------|
| $K_{a1}$ (M <sup>-1</sup> )          | 74353 ± 10169     | 417533 ± 104665 | 72583 ± 14192       | 17007 ± 5878      | 1534000 ± 129526      |
| $\Delta H_1$ (kJ mol <sup>-1</sup> ) | -76 ± 5           | -81 ± 2         | -78 ± 3             | -57 ± 8           | -93 ± 5               |
| $n_1$                                | 20 ± 2            | 16 ± 1          | 17 ± 1              | 30 ± 3            | 11 ± 1                |
| SD                                   | 117 ± 20          | 88 ± 9          | 123 ± 12            | 96 ± 16           | 60 ± 7                |

Fig. 1.

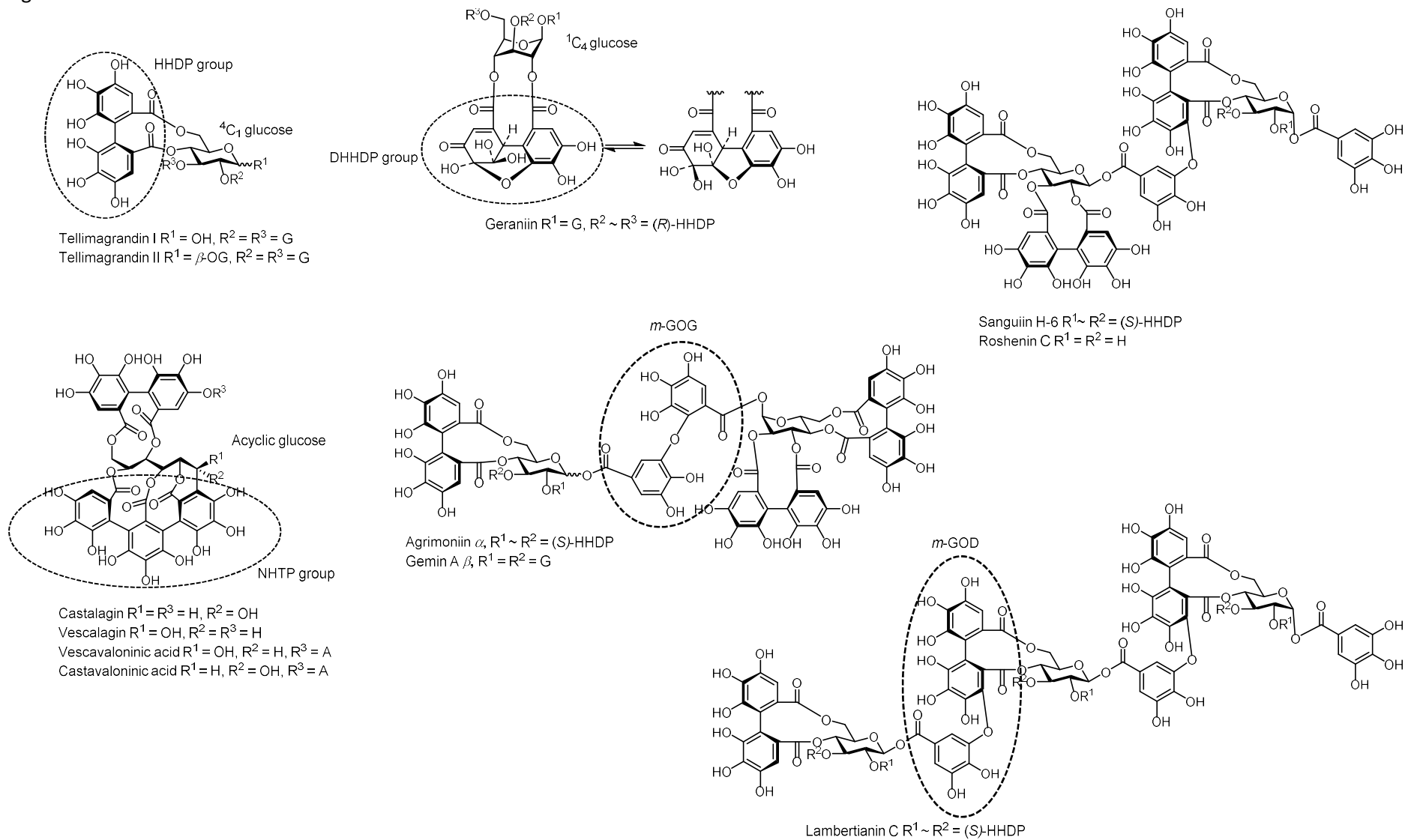


Fig. 2

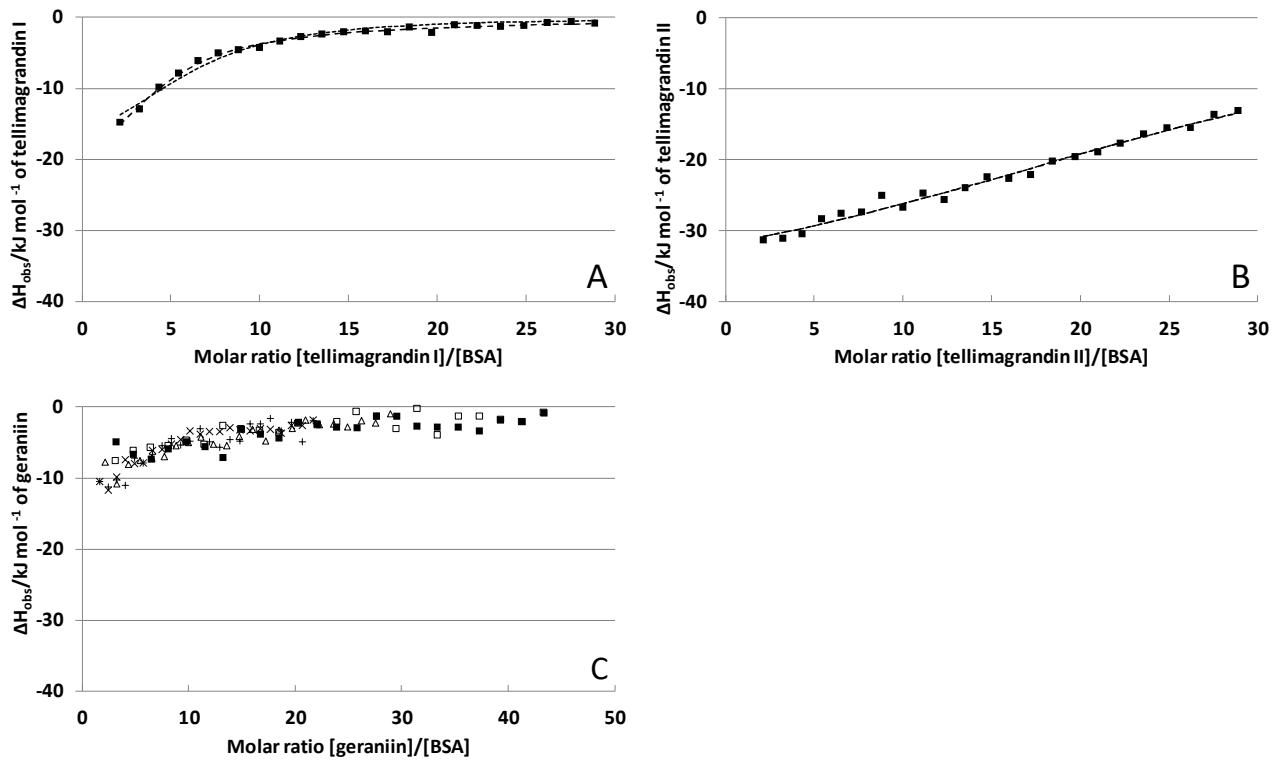


Fig. 3

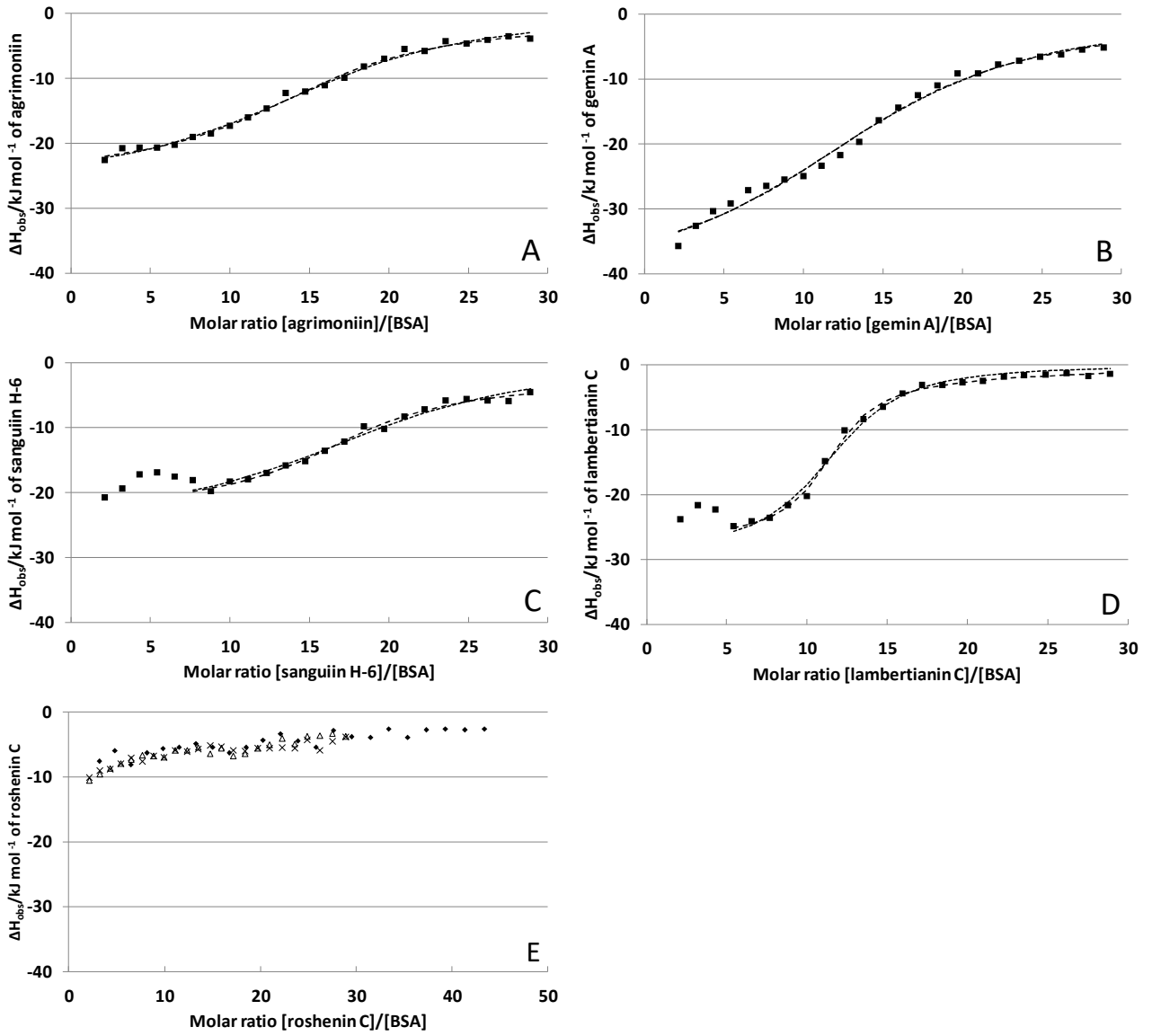


Fig. 4

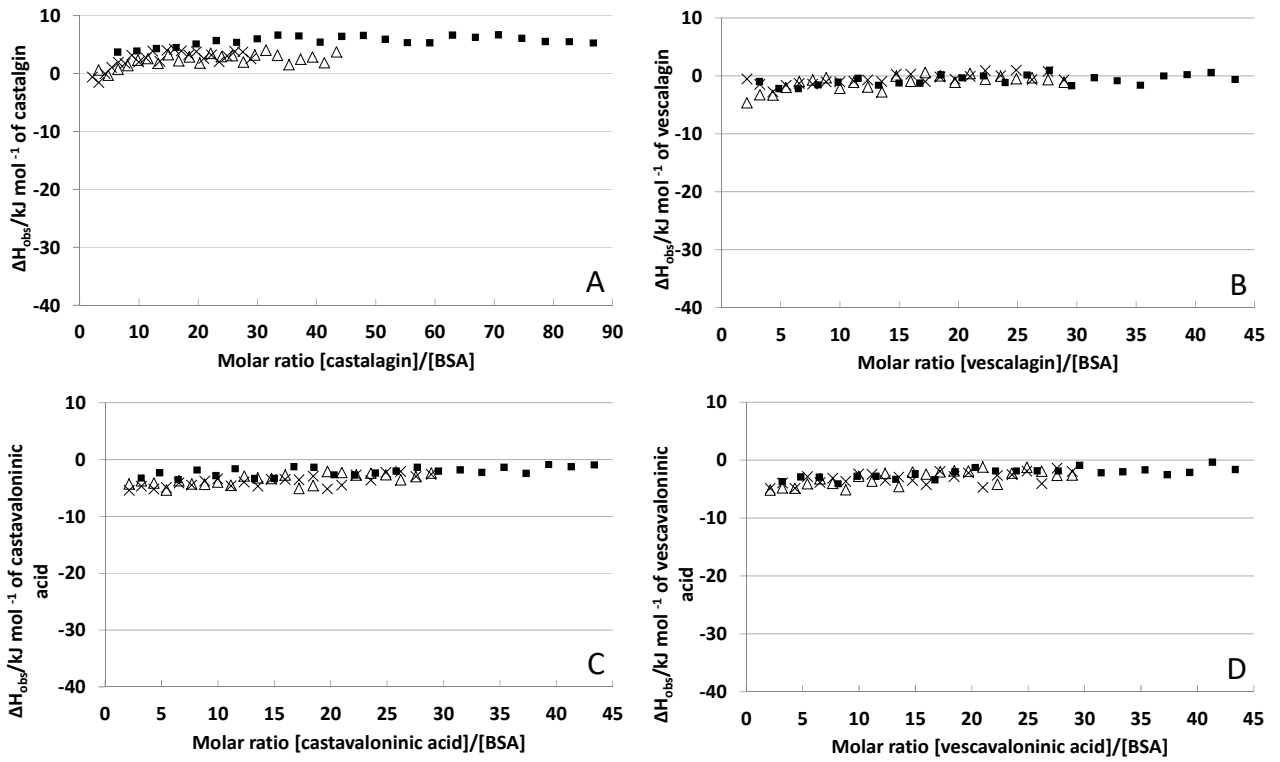


Fig. 5

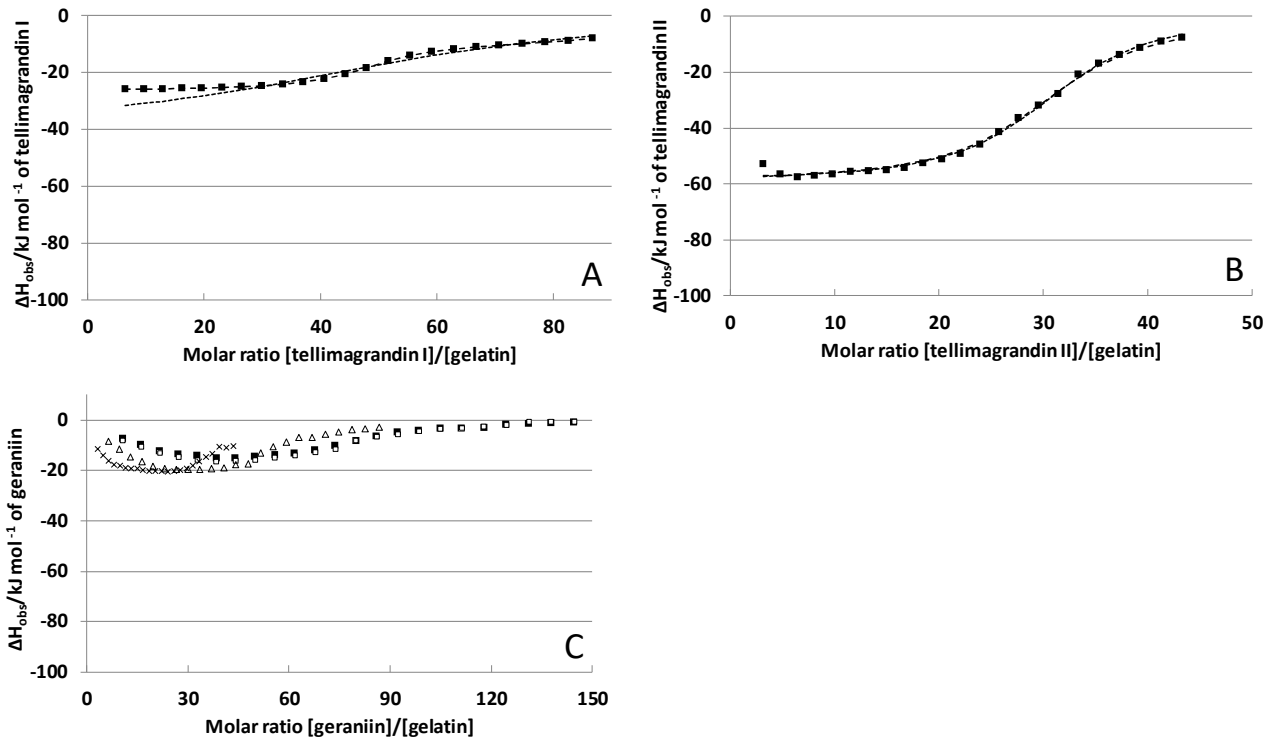


Fig. 6

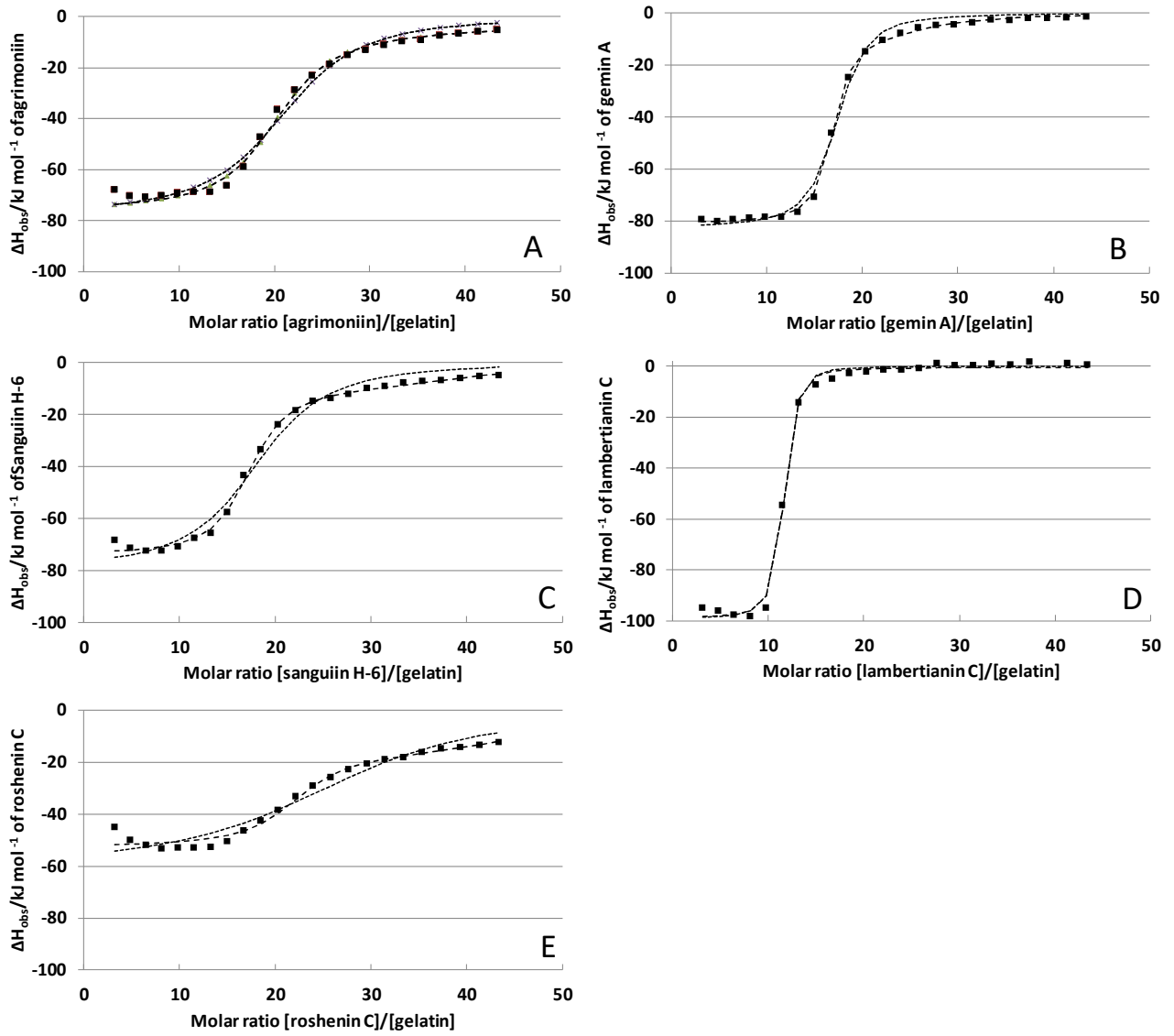
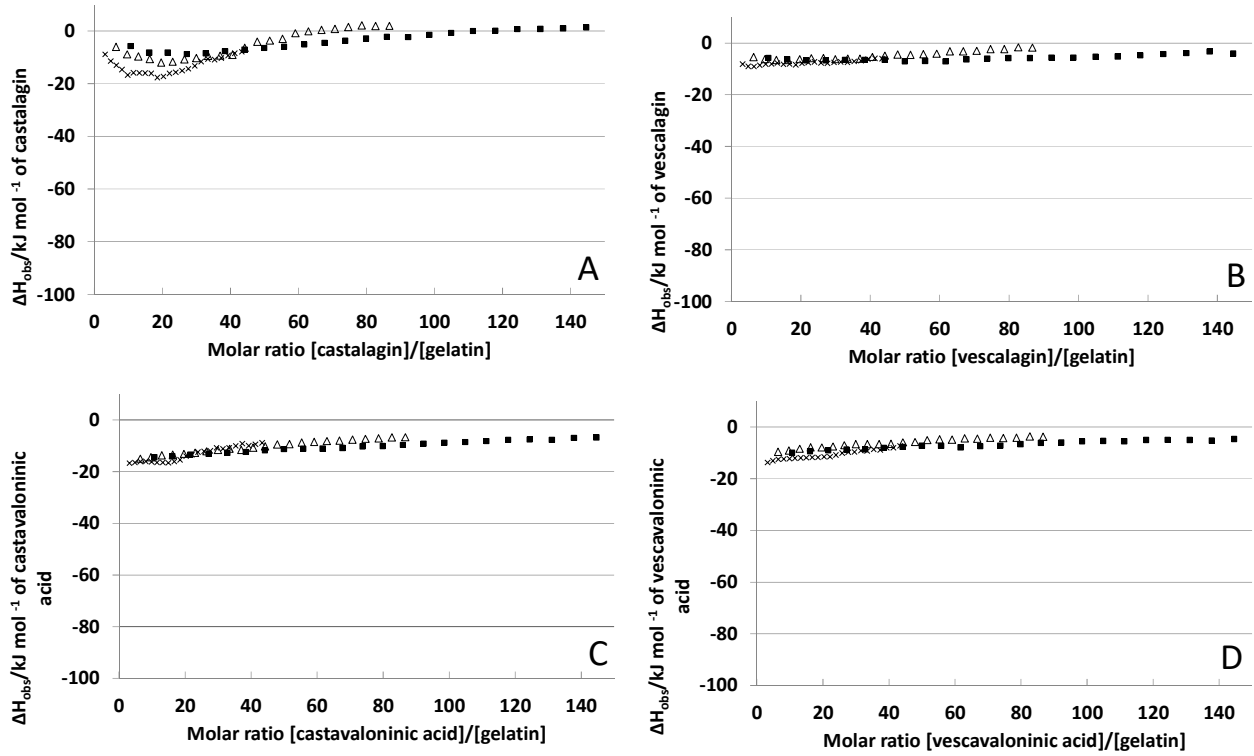
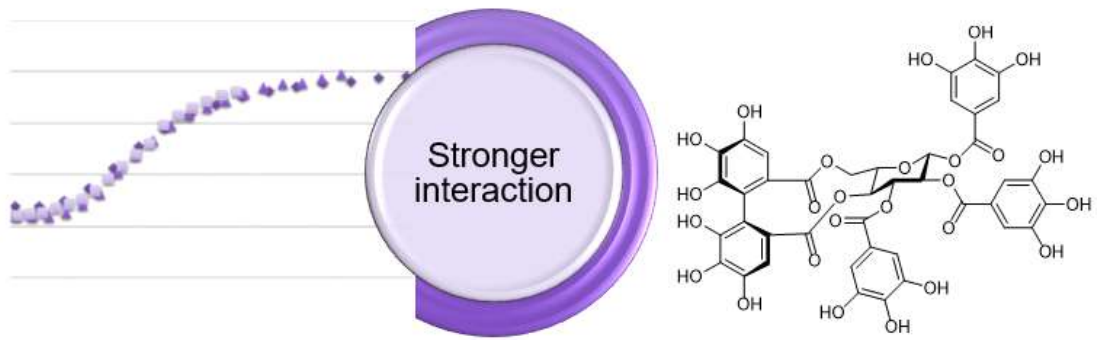




Fig. 7



Graphic for table of contents



Gelatin > BSA

