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Binding of an Oligomeric Ellagitannin Series to BSA: Analysis by Isothermal Titration Calorimetry

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1 **ABSTRACT:**

2 A unique series of oligomeric ellagitannins was used to study their interactions with bovine serum

3 albumin (BSA) by isothermal titration calorimetry. Oligomeric ellagitannins, ranging from

4 monomer up to heptamer and a mixture of octamer-undecamers, were isolated as individual pure

5 compounds. This series allowed studying the effects of oligomer size and other structural features.

6 The monomeric to trimeric ellagitannins deviated most from the overall trends. The interactions of

7 ellagitannin oligomers from tetramers to octa-undecamers with BSA revealed strong similarities. In

8 contrast to the equilibrium binding constant, enthalpy showed an increasing trend from the dimer to

9 larger oligomers. It is likely that first the macrocyclic part of the ellagitannin binds to the defined

10 binding sites on the protein surface and then the "flexible tail" of the ellagitannin coats the protein

11 surface. The results highlight the importance of molecular flexibility to maximize binding between

12 the ellagitannin and protein surfaces.

13

14 **KEYWORDS:** *Interactions, molecular size, polyphenol, protein, thermodynamics*

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18   **INTRODUCTION**

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20   Plants produce a variety of secondary metabolites including polyphenols in their tissues to protect

21   them for example against pathogens and insect herbivores. The most complicated polyphenol

22   structures are tannins which by definition have the ability to bind and precipitate proteins. Tannins

23   can be divided into condensed tannins, hydrolysable tannins (gallotannins and ellagitannins) in

24   addition to phlorotannins, which are found only in algae. For decades, ellagitannins were an

25   underestimated class of bioactive plant tannins.[1] However, ellagitannins are one of the most

26   promising tannin classes with potent biological activities, including antimicrobial and antioxidant

27   activities.[1,2] More than 1000 individual ellagitannins have been identified from plants and natural

28   ellagitannins larger than pentamers were recently reported.[3–5]

29   Dietary tannins can affect animal nutrition and health in several ways, for example

30   through enabling a better utilization of feed proteins, generating anthelmintic effects against

31   gastrointestinal nematodes and by lowering nitrogenous and methane emissions.[6–16] Tannins may

32   bind dietary proteins and thus reduce the degradation of these proteins in the rumen and may also

33   enhance the amount of protein available for digestion in the small intestine. Tannins can form

34   soluble and/or insoluble complexes with proteins and the tannin–protein interactions are both

35   tannin- and protein specific.[17] Bovine serum albumin (BSA) is a well-characterized model protein

36   and it has been widely used for the investigations of tannin-protein interactions.[18] Previous results

37   have shown that tannins have higher affinities to loosely structured globular proteins, such as BSA,

38   than to compact globular structures.[19]

39   Isothermal titration calorimetry (ITC) is a powerful technique to study the

40   thermodynamics of tannin–protein interactions. ITC has both a reference and a sample cell at a

41   constant temperature and the technique relies only on the detection of a heat effect upon binding; it

42   provides the accurate, rapid and label-free measurement of the thermodynamics of molecular

4

43 interactions.[20,21] In a single ITC experiment, the strength and stoichiometry of the interaction

44 between tannin and protein can be measured, i.e. the enthalpy (ΔH_{obs}) and stoichiometry of binding

45 (n) and the binding constant (K) can be determined. The binding of condensed tannins and

46 hydrolyzable tannins to proteins has been previously studied by ITC.[22–26] The binding parameters

47 have been related to the structural flexibility of tannins.[24,25] Most of the studies have been

48 conducted using condensed tannins. However, the use of condensed tannins as model compounds

49 poses particular problems as the determination of their molecular weight is not straight forward.[23]

50 Oligomeric and polymeric condensed tannins cannot be chromatographically separated and

51 therefore, they are usually isolated and purified as mixtures.[27–29] Therefore, their molecular weight

52 is an estimation based on the size distribution within a condensed tannin mixture, obtained for

53 example by acidic degradation in the presence of nucleophiles or by mass spectrometry.[30–35] It is

54 feasible, however, to isolate ellagitannins as pure compounds[4,36], and this offers a unique

55 opportunity to investigate the effects of different structural features, such as molecular size and

56 monomeric units.

57   In this study, we utilized ITC to study the interaction between ellagitannins and BSA.

58 We characterized the thermodynamics of the binding of tellimagrandin I-based oligomeric

59 ellagitannins[3,4] (Fig. 1) to bovine serum albumin BSA. The ellagitannin oligomers were a unique

60 series consisting of tellimagrandin I (monomer), oenothein B (dimer), oenothein A (trimer) plus a

61 tetramer, pentamer, hexamer and heptamer (Fig. 1) and a mixture of octamers to undecamers. These

62 ellagitannin oligomers are excellent model compounds for the ITC studies as they can be isolated as

63 individual pure compounds and are well-characterized with exact molecular weights.[3] This work is

64 the very first systematic investigation of ellagitannins using an oligomeric series so that we can

65 evaluate the effect of the molecular size and decouple this from other structural features, such as

66 functional groups, which has been a problem when interactions between condensed tannins and

67 proteins were studied. The oligomers consisted of similar monomeric units, which also enabled the

5

68   direct comparison of the interaction between the different oligomers based on the number of

69   monomeric tellimagrandin I units.

70

71   **MATERIALS AND METHODS**

72

73   **Materials.** Acetone (technical grade) used in the collection of plant materials was

74   from VWR International (Leuven, Belgium). Acetone and methanol (analytical grade) used in the

75   Sephadex LH-20 fractionations, methanol and acetonitrile (HPLC grade) used in the preparative

76   and semipreparative HPLC were obtained from VWR International (Fontenay-Sous-Bois, France).

77   LiChroSolv® acetonitrile (hypergade for LC-MS) was purchased from Merck KGaA (Darmstadt,

78   Germany) and formic acid (eluent additive for LC-MS) was from VWR International Ltd. (Poole,

79   England). The water was filtered through an Elgastat UHQ-PS purification system (Elga, Kaarst,

80   Germany) or with Synergy® UV water purification system (Millipore SAS, Molsheim, France).

81   BSA (purified by heat shock fractionation, pH 7, purity ≥98%, lyophilized powder, 66 kDa) was

82   purchased from Sigma-Aldrich (St. Louis, US).

83   **Isolation and Characterization of Ellagitannins.** A series of oligomeric

84   ellagitannins was purified: monomer, dimer, trimer, tetramer, pentamer, hexamer, heptamer and a

85   mixture of chromatographically non-separated octamers to undecamers.[3,4] The monomeric

86   ellagitannin, tellimagrandin I, was isolated from meadowsweet (*Filipendula ulmaria)* inflorescences

87   and the other oligomeric ellagitannins from fireweed (*Epilobium angustifolium*) inflorescences. The

88   inflorescences were collected during summer 2011 from southwest Finland. The plant material was

89   collected and placed directly into ten bottles of 1 L, which were then immediately filled with

90   acetone, transferred to the laboratory, and stored in a cold room (+4 ˚C) prior the isolation of

91   ellagitannins.

6

92   The extraction and isolation of ellagitannins followed mainly the previously outlined

93   methods.[3,4] However, some modifications were made in order to enhance and speed up the large-

94   scale extractions and fractionations. The acetone extracts of fireweed inflorescences were combined

95   and concentrated to 300-500 mL of water phase. The concentrated water phases were fractionated

96   twice with Sephadex LH-20 chromatography. First, a rough fractionation was performed in a

97   beaker, and then careful fractionation was performed for a selected fraction by Sephadex LH-20

98   column chromatography as previously described.[37] The isolation of ellagitannins from the Sephadex

99   fractions was performed by preparative HPLC. The HPLC-DAD system consisted of a Waters

100   Delta 600 liquid chromatograph, a Waters 600 Controller, a Waters 2998 Photodiode Array

101   Detector and a Waters Fraction Collector III. The column (approximately 327 mm × 33 mm) was

102   manually filled with LiChroprep RP-18 (40-63 μm) material (Merck KGaA, Darmstadt, Germany).

103   The flow rate was 8 mL min^{-1} and the sparge rate for the helium flow 100 mL min^{-1}. Two eluents

104   were used: 1% formic acid (A) and methanol (B). The gradient was the following: 0–5 min, 100%

105   A; 5–180 min, 0-40% B in A; 180–220 min, 40–60% B in A; 220–240 min, 60-80% B in A. The

106   injection volume was 5 mL. The photodiode array detector was operating between 190−500 nm,

107   and ellagitannins were detected at 280 nm. The final purification of ellagitannins was performed by

108   semipreparative HPLC with the same HPLC-DAD system. The column was a Gemini C18 column

109   (150 × 21.2 mm, 10 μm, Phenomenex) and the eluents were 0.1 % formic acid (A) and acetonitrile

110   (B). Different gradients were used for different oligomers; for example, a typical gradient for larger

111   oligomers was as follows: 0–5 min, 17% B in A; 5–51 min, 17–47% B in A; 51–55 min, 47–70% B

112   in A. The flow rate was 8 mL min^{-1}and the sparge rate for the helium flow 100 mL min^{-1}. The

113   injection volume was 5 mL. The photodiode array detector was operating between 190−500 nm,

114   and ellagitannins were detected at 280 nm.

115   All steps in the extraction, isolation and preparative and semipreparative purifications

116   were followed by UPLC-DAD-MS (Acquity UPLC®, Waters Corporation, Milford, USA

7

117   combined with Xevo® TQ, Waters Corporation, Milford, USA). Samples were filtered with a

118   syringe filter (4 mm, 0.2 μm PTFE, Thermo Fisher Scientific Inc., Waltham, USA) prior to the

119   analysis. The Acquity UPLC® BEH Phenyl column (2.1 × 100 mm, 1.7 μm, Waters Corporation,

120   Wexford, Ireland) was used with two eluents: 0.1% formic acid (A) and acetonitrile (B). The

121   gradient was 0−0.5 min, 0.1% B in A; 0.5−5.0 min, 0.1−30.0 % B in A (linear gradient); 5.0−5.1

122   min, 30.0−90.0 % B in A (linear gradient); 5.1−8.5 min, column wash and stabilization. The flow

123   rate was 0.5 mL min^{-1} and the injection volume 5 μL. The photodiode array detector was operating

124   between 190−500 nm, and ellagitannins were detected at 280 nm. Mass spectrometer was operated

125   in a negative ionization mode and ions at *m/z* 160−1200 were scanned. Capillary voltage was set at

126   3.53 kV, cone voltage ramp was used between 20-70 V, desolvation temperature was set at 650 °C,

127   and source temperature at 150 °C. Desolvation and cone gas (N$_2$) flow rates were 1000 L h^{-1} and

128   100 L h$^-$, respectively. The ellagitannins were identified based on previous work.[3,36] Pure

129   ellagitannins were concentrated to the water-phase and freeze-dried.

130   **Isothermal Titration Calorimetry.** Titrations of ellagitannins into BSA (purity ≥

131   98%, lyophilized powder, 66 kDa; Sigma-Aldrich, St. Louis, US) were performed with a NanoITC

132   instrument (TA Instruments Ltd., Crawley, West Sussex, UK) as previously described[18,23,24] with

133   minor modifications. All solutions were prepared in 50 mM citrate buffer adjusted to pH 6. BSA

134   solution (10, 20, 30 or 40 μM) was placed in the 950 μL sample cell of the calorimeter and 3 mM

135   ellagitannin solution was loaded into the injection syringe. The ellagitannin studied was titrated into

136   the sample cell at 298 K as a sequence of 24 injections of 10 μL. The time delay between the

137   injections was 360 s. The content of the sample cell was stirred throughout the experiment to ensure

138   comprehensive mixing. All ellagitannins were studied with three replicate titrations; the pentameric

139   and heptameric ellagitannins were studied in duplicates because of limited amounts. Control

140   experiments included the titration of ellagitannin solution into buffer, the titration of buffer into

141   BSA solution and the titration of buffer into buffer. Control experiments of buffer titrated into BSA

8

142   solution and buffer into buffer resulted only in small or equal enthalpy changes for each successive

143   injection of buffer, and therefore, were not considered in the data analysis.[18,23,24] The control data of

144   ellagitannin titrated into buffer was always subtracted from the sample data as it was known that

145   ellagitannins tend to self-associate into aggregates due to hydrophobic groups; and therefore, when

146   injected from the syringe into buffer, they undergo an endothermic process of deaggregation. The

147   extent of deaggregation depends inversely on the concentration of ellagitannin already present in

148   the sample cell: therefore, successive injections of ellagitannins into buffer lead to observation of

149   progressively lower endothermic enthalpy changes as has been illustrated in earlier work.[22]

150   **Data Analysis.** Raw data from isothermal titration calorimetry were obtained as plots

151   of heat (μJ) against injection number and exhibited a series of peaks for each injection. The raw

152   data were transformed using the NanoAnalyze Data Analysis software (version 2.4.1., TA

153   Instruments) to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs}, kJ mol^{-1})

154   against molar ellagitannin:protein ratio. The estimated binding parameters were obtained from ITC

155   data using the same NanoAnalyze Data Analysis software. Data fits were obtained in two different

156   ways: using a single set of multiple binding sites and a model for two independent sets of multiple

157   binding sites. The quality of fits was determined by standard deviation.

158

159   **RESULTS AND DISCUSSION**

160

161   **ITC Binding Isotherms and Data Fitting**. In this study, the interaction of seven

162   individual tellimagrandin I-based oligomeric ellagitannins (from monomer to heptamer) and a

163   mixture of larger oligomeric ellagitannins with BSA was investigated by ITC. For each

164   ellagitannin:BSA system studied, an exothermic interaction was observed. Fig. 2 shows ITC

165   binding isotherms for two of the ellagitannins studied, the trimer (oenothein A) and hexamer

166   binding to BSA. Experiments were carried out using two different concentrations of BSA, 20 μM or

9

167　30 μM, and this showed the good repeatability of the ellagitannin:BSA interaction. The data

168　showed that the interaction was not affected by BSA concentration suggesting no evidence of co-

169　operative binding. Upon the addition of ellagitannin, the interaction became less exothermic as the

170　binding sites of BSA became saturated. The ITC binding isotherms showed that an

171　ellagitannin:BSA molar ratio of approximately 10:1 corresponded to the inflection point for each

172　ellagitannin interaction and that there were multiple binding sites for the ellagitannin on the BSA

173　molecule.[18]

174　　　　　Two different binding models were used to fit the data, one assuming a single set of

175　multiple binding sites (a single-site model) and a second model assuming two independent sets of

176　multiple binding sites (a two-site model), as previously discussed in detail by Deaville et al.[25] In

177　Fig. 3, it can be seen that both models fit the data equally well for the ellagitannin trimer binding to

178　BSA, and thus the simpler single-site model seemed better justified as a binding model. However,

179　for many of the ellagitannins studied, both models provided an acceptable fit, but the two-site

180　model clearly exhibited a closer fit for the data. This was seen particularly at higher

181　ellagitannin:BSA molar ratios where a longer tail in the data was seen as the tannin:BSA interaction

182　was saturated. Therefore, the fit parameters for both binding models are shown in Tables 1 and 2 for

183　all ellagitannins studied. The second site showed very weak interaction with small binding

184　constants varying from 17 to 1.8×10^3 M^{-1} (Table 2) for all the ellagitannin:BSA interactions

185　studied. This weaker interaction might indicate a non-specific surface adsorption mechanism

186　following the more selective stronger binding of the ellagitannin, as indeed we had observed in

187　previous tannin:protein binding studies.[22–24] This was the first study where a purified oligomeric

188　series was investigated to look at the effect of oligomerization on ellagitannin:protein binding

189　behavior. In previous studies with hydrolysable tannins, a two-binding site model had been used. In

190　those examples, the binding constants had shown to be higher than values seen here, and the two-

191　site model had been more pronounced. Interestingly, those measurements had been carried out

10

192   using lower tannin concentrations (3 mg mL^{-1}, for example approximately 2 mM for oenothein B)

193   and over a longer timescale using more injections and a higher level of protein dilution than the

194   current experiments. From our experience it was clear that we could not directly compare data from

195   previous studies, which has used different experimental conditions that could impact on the

196   interaction, due to complicating factors such as the degree of self-association of tannin molecules.

197   The two-site model indicated two independent types of binding on the protein surface.

198   The second binding site highlighted the presence of a weaker interaction in addition to a more

199   selective stronger first binding site. As seen in Table 2, the weakness of the second binding site

200   meant that it was difficult to confidently identify trends across the oligomeric series for this

201   interaction. Therefore, we have focused on the single-binding site model for our discussion of the

202   ellagitannin:BSA interaction across the oligomeric series since this interaction was likely to relate

203   to a specific molecular interaction between the tannin and protein.

204   **Monomeric, Dimeric and Trimeric Ellagitannins.** Tables 1 and 2 show trends

205   linking the oligomer size to ellagitannin:BSA interaction, that suggested that the smaller

206   ellagitannins deviated from the overall trends seen. The interaction of monomeric tellimagrandin I

207   with BSA was stronger, in terms of K_a than expected in relation to the oligomer series. The binding

208   constant (K_a = 1.8 × 10^4 M^{-1}) was higher than for the dimeric oenothein B (5.7 × 10^3 M^{-1}) and

209   trimer (7.6 × 10^3 M^{-1}) and similar to hexameric and heptameric ellagitannins (1.7 × 10^4 M^{-1}). This

210   could be due to the additional free galloyl group as the monomer contained two adjacent free

211   galloyl groups (Fig. 1A) whereas oligomeric ellagitannins contained only one free galloyl group per

212   monomeric unit. Previously, Dobreva *et al.* had reported that the strong binding site was dependent

213   on tannin flexibility and possibly also free galloyl groups.[24]

214   The binding of the dimer, oenothein B, to BSA was also different in comparison to the

215   others. The equilibrium binding constant was the lowest (5.7 × 10^3 M^{-1}) as well as the enthalpy

216   change observed (-14 kJ mol^{-1}) indicating a relatively low affinity of oenothein B towards BSA.

11

217   Oenothein B was a macrocyclic, relatively rigid structure with less conformational flexibility

218   compared with all the other ellagitannins studied in this series (Fig. 1B). A previous study had

219   shown that the binding constant for the interaction with BSA was dependent on the structural

220   flexibility of the tannin molecule; and a loss of conformational freedom in the ellagitannin structure

221   impacted on its ability to bind to BSA.[25] The thermodynamic parameters for oenothein B (Table 1

222   and 2) were different to Dobreva et al.[24] although the overall conclusion linking BSA binding to

223   tannin flexibility was the same. As mentioned previously, differences in the experimental

224   procedures were likely to explain the quantitative difference in the binding interaction.[24] Our

225   findings suggested that the interaction between ellagitannin and protein might be dependent on the

226   ellagitannin concentration.

227   The trimeric oligomer, oenothein A, showed stronger binding to BSA than the dimer.

228   The binding constant was 7.5×10^3 M^{-1} and the enthalpy change was -45 kJ mol^{-1}. The trimer was

229   more flexible than the dimer as the additional monomeric unit was attached only via one bond (Fig.

230   1C). The presence of this conformationally free chain (flexible tail) allowed for a stronger

231   interaction in terms of K_a of the trimer versus the dimer and this was also seen for the larger

232   oligomers in terms of ΔH_{obs} (Fig. 4). These observations could be linked to the three-dimensional

233   structures of the oligomer series, where larger oligomers had a longer flexible chain attached to the

234   macrocycle.

235   **Larger Oligomeric Ellagitannins.** The interactions of ellagitannin oligomers from

236   trimers to octamer-undecamers with BSA revealed strong similarities. The stoichiometric number,

237   i.e. tannin to protein ratio, varied from just 9 to 12 (Table 1) and the equilibrium binding constant

238   was 10^3-10^4 M^{-1} for all ellagitannin-BSA interactions. The observed change in enthalpy was

239   exothermic and increased with the oligomeric size up to the heptamer. Exothermic interactions were

240   associated with hydrogen bonding or Van der Waals interactions. As the oligomeric size increased

241   the number of footholds, the points of interaction with the protein surface, increased.

12

242   All oligomers contained the same macrocyclic part in their structure, i.e. dimeric

243   oenothein B (Fig. 1B) formed by the dimerization of two tellimagrandin I monomers via two *m*-

244   DOG-type linkages. The *m*-DOG-type linkages are frequently found in ellagitannin oligomers: in

245   these linking units, the *O*-donating hydroxyl group is part of an hexahydroxydiphenoyl group and

246   the galloyl group is the acceptor.[2] In trimeric and larger ellagitannin oligomers, the additional

247   monomeric units were attached by one *m*-DOG-type linkage forming an elongated chain or tail to

248   this macrocyclic part (Fig. 1). It was likely that first the macrocyclic part of the ellagitannin bound

249   to the protein (to the defined binding sites on the protein surface resulting in little difference in the

250   value of n for each tannin) and then the "flexible tail" of the ellagitannin coated the protein surface.

251   Therefore, a trend in terms of binding affinity could be observed for this oligomeric ET series. This

252   observation differed from the previous studies of polyphenol binding to proteins where an increase

253   in the binding affinity with molecular size had been observed, but where there were also differences

254   in terms of flexibility, molecular structures and in some cases, the purity of the tannins that

255   influenced the interaction.[38]

256   **ITC Analysis Based on Monomeric Concentrations**. In an effort to remove the

257   effect of oligomerization and molecular weight, the data were also fitted by assuming a monomeric

258   concentration for the ellagitannins as shown in Table 3 using a single-site binding model. The

259   oligomers consisted of similar monomeric units, which enabled the direct comparison of the

260   different oligomers based on the number of monomer units. By doing this, the trend seen in Table 1

261   for ΔH_{obs} was largely removed. Focusing on the dimer and larger oligomers the variation seen for

262   ΔH was small at -13.9 ± 2.6 kJ per mole of monomer. However, the number of binding sites on the

263   ellagitannin increased with the oligomer size, since the binding of the flexible chain was now

264   considered as individual molecules. We saw that the K_a was smaller per monomer compared with

265   our molecular calculations, since the interaction was now split across multiple molecules.

13

266   The different analyses of the data seemed to fit the concept that there were two

267   different binding events; first the binding of the rigid ring of the dimer was followed by the binding

268   of the flexible units of the tail. It was for this reason why in previous studies two-site binding

269   models had been needed and why we fitted our current data also using the two-site binding model

270   (Table 2). However, often the second binding site was a lot weaker and less specific than the first

271   one. As mentioned previously, the fits obtained using a two-site model were good fits, and

272   marginally better than the single-site model for the larger ellagitannins. In particular, oligomers

273   from tetramer to heptamer all showed good two-site binding fits to the data with nine distinct strong

274   binding sites ($n = 9$, $K_a = 10^4 - 10^5$ M^{-1}) and a second weak binding site, a K_a of approximately 10^2

275   and a high n ranging from 35-90.

276   Previous studies had shown that the interaction of tannins with proteins could be a

277   surface phenomenon where tannins coated the surface of the protein.[18,25,39] Our data supported this

278   observation in two ways. Firstly, the flexible elongated chain in oligomeric ellagitannins appeared

279   to coat the protein surface. Secondly, when the data were fitted using monomeric concentrations,

280   the tannin:protein binding stoichiometries increased x-fold, where x was the number for the degree

281   of oligomerization, and the ΔH and K_a values converged. We observed that there were

282   approximately nine specific binding sites on the surface of the protein, but that further interactions,

283   akin to non-specific surface adsorption, occurred allowing the flexible chain of the tannin oligomers

284   to subsequently bind to the surface.

285   In conclusion, this unique series of oligomeric ellagitannins allowed us to study the

286   effect of molecular size on the interaction between ellagitannins and BSA. The novel results

287   showed that the interactions of ellagitannin oligomers from trimers to octamer-undecamers with

288   BSA revealed strong similarities. The monomeric and dimeric ellagitannins deviated from the

289   overall trends seen. Our studies highlighted the importance of molecular flexibility to maximize

290   binding between the tannin and protein surface. This systematic investigation of ellagitannins used

14

291 an oligomeric series and was able to decouple for the first time structural features, such as

292 functional groups present and purity, from molecular weight.

293

294 ABBREVIATIONS USED

295 BSA, bovine serum albumin; HPLC-DAD, high-performance liquid chromatography diode array

296 detection; ITC, isothermal titration calorimetry; UPLC-DAD-MS, ultra-performance liquid

297 chromatography diode array detection mass spectrometry

298

299 ACKNOWLEDGEMENTS

303

304 REFERENCES

305

306 (1) Okuda, T.; Yoshida, T.; Hatano, T.; Ito, H. Ellagitannins renewed the concept of tannins. In

307 *Chemistry and Biology Ellagitannins: An underestimated class of bioactive plant*

308 *polyphenols*; Quideau, S, Ed.; World Scientific Publishing, Singapore, 2009; pp. 1–54.

309 (2) Yoshida, T.; Hatano, T.; Ito, H.; Okuda, T. Structural diversity and antimicrobial activities

310 of ellagitannins. In *Chemistry and Biology Ellagitannins: An underestimated class of*

311 *bioactive plant polyphenols*; Quideau, S, Ed.; World Scientific Publishing, Singapore, 2009;

312 pp. 55–93.

313 (3) Karonen, M.; Parker, J.; Agrawal, A.; Salminen, J.-P. First evidence of hexameric and

314 heptameric ellagitannins in plants detected by liquid chromatography/electrospray ionisation

315 mass spectrometry. *Rapid Commun. Mass Spectrom*. **2010**, *24*, 3151–3156.

15

316   (4) Salminen, J.-P.; Karonen, M. Chemical ecology of tannins and other phenolics: we need a

317   change in approach. *Funct. Ecol.* **2011**, *25*, 325–338.

318   (5) Quideau, S.; Deffieux, D.; Douat-Casassus, C.; Pouységu, L. Plant polyphenols: chemical

319   properties, biological activities, and synthesis. *Angew. Chem. Int. Ed.* **2011**, *50*, 586–621.

320   (6) Lowry, J.B.; McSweeney, C.S.; Palmer, B. Changing perceptions of the effect of plant

321   phenolics on nutrient supply in the ruminant. *Aust. J. Agric. Res.* **1996**, *47*, 829–842.

322   (7) McNeill, D.M.; Osborne, N.J.; Komolong, M.; Nankervis, D. Condensed tannins in the

323   genus *Leucaena* and their nutritional significance for ruminants. *ACIAR Proc.* **1998**, *86*,

324   205–214.

325   (8) Min, B.R.; Barry, T.N.; Attwood, G.T.; McNabb, W.C. The effect of condensed tannins on

326   the nutrition and health of ruminants fed fresh temperate forages: a review. *Anim. Feed Sci.*

327   *Technol.* **2003**, *106*, 3–19.

328   (9) Waghorn, G.C.; McNabb, W.C. Consequences of plant phenolic compounds for

329   productivity and health of ruminants. *Proc. Nutr. Soc.* **2003**, *62*, 383–392.

330   (10)   Kebreab, E.; Mills, J.A.N.; Crompton, L.A.; Bannik, A.; Dijkstra, J.; Gerrits, W.J.J.

331   An integrated mathematical model to evaluate nutrient partition in dairy cattle between

332   animal and environment. *Anim. Feed Sci. Technol.* **2004**, *112*, 131–154.

333   (11)   Givens, D.I.; Rulquin, H. Utilisation by ruminants of nitrogen compounds in silage-

334   based diets. *Anim. Feed Sci. Technol.* **2004**, *114*, 1–18.

335   (12)   McWilliam, E.L.; Barry, T.N.; López-Villalobos, N.; Cameron, P.N.; Kemp, P.D. The

336   effect of different levels of poplar (*Populus*) supplementation on the reproductive

337   performance of ewes grazing low quality drought pasture during mating. *Anim. Feed Sci.*

338   *Technol.* **2004**, *115*, 1–18.

339   (13)   McWilliam, E.L.; Barry, T.N.; López-Villalobos, N.; Cameron, P.N.; Kemp, P.D.

340   Effects of willow (*Salix*) supplementation for 31 and 63 d on the reproductive performance

16

341   of ewes grazing low quality drought pasture during mating. *Anim. Feed Sci. Technol.* **2005**,

342   *119*, 87–106.

343   (14)   Mlambo, V.; Smith, T.; Owen, E.; Mould, F.L; Sikosana, J.L.N.; Mueller-Harvey, I.

344   Tanniniferous *Dichrostachys cinerea* fruits do not require detoxification for goat nutrition:

345   in sacco and *in vivo* evaluations. *Livestock Prod. Sci.* **2004**, *90*, 135–144.

346   (15)   Mueller-Harvey, I. Unravelling the conundrum of tannins in animal nutrition and

347   health. *J. Agric. Food Chem.* **2006**, *86*, 2010–2037.

348   (16)   Mueller-Harvey, I.; Mlambo, V.; Sikosana, J.L.N.; Smith, T.; Owen, E.; Brown, R.H.

349   Octanol-water partition coefficients for predicting the effects of tannins in ruminant

350   nutrition. *J. Agric. Food Chem.* **2007**, *55*, 5436–5444.

351   (17)   Asquith, T.N.; Butler, L.G. Interactions of condensed tannins with selected proteins.

352   *Phytochemistry* **1986**, *25*, 1591–1593.

353   (18)   Dobreva, M.A.; Frazier, R.A.; Mueller-Harvey, I.; Clifton, L.A.; Gea, A.; Green, R.J.

354   Binding of pentagalloyl glucose to two globular proteins occurs via multiple surface sites.

355   *Biomacromolecules* **2011**, *12*, 710–715.

356   (19)   Hagerman, A.E.; Butler, L.G. The specificity of proanthocyanidin-protein

357   interactions. *J. Biol. Chem.* **1981**, 4494–4497.

358   (20)   Lewis, E.A.; Murphy, K.P. Isothermal titration calorimetry. In *Methods in Molecular*

359   *Biology*: *Protein-ligand interactions: Methods and applications*, Nienhaus, G.U., Ed.;

360   Humana Press Inc., Totowa, 2005; 305, pp. 1–15.

361   (21)   Ghai, R.; Falconer, R.J.; Collins, B.M. Applications of isothermal titration calorimetry

362   in pure and applied research–survey of the literature from 2010. *J. Mol. Recognit.* **2012**, *25*,

363   32–52.

17

364   (22)   Frazier, R.A.; Papadopoulou, A.; Mueller-Harvey, I.; Kissoon, D.; Green, R.J.

365   Probing protein–tannin interactions by isothermal titration microcalorimetry. *J. Agric. Food*

366   *Chem.* **2003**, *51*, 5189–5195.

367   (23)   Frazier, R.A.; Deaville, E.R.; Green, R.J.; Stringano, E.; Willoughby, I.; Plant, J.;

368   Mueller-Harvey, I. Interaction of tea tannins and condensed tannins with proteins. *J. Pharm.*

369   *Biomed. Anal.* **2010**, *51*, 490–495.

370   (24)   Dobreva, M.A.; Green, R.J.; Mueller-Harvey, I.; Salminen, J.-P.; Howlin, B.J.;

371   Frazier, R.A. Size and molecular weight affect the binding of ellagitannins to bovine serum

372   albumin. *J. Agric. Food Chem.* **2014**, *62*, 9186–9194.

373   (25)   Deaville, E.R.; Green, R.J.; Mueller-Harvey, I.; Willoughby, I.; Frazier, R.A.

374   Hydrolyzable tannin structures influence relative globular and random coil protein binding

375   strengths. *J. Agric. Food Chem.* **2007**, *55*, 4554–4561.

376   (26)   Kilmister, R.L.; Faulkner, P.; Downey, M.O.; Darby, S.J.; Falconer, R.J. The

377   complexity of condensed tannin binding to bovine serum albumin – An isothermal titration

378   calorimetry study. Food Chem. **2016**, *190,* 173–178.

379   (27)   Guyot, S.; Doco, T.; Souquet, J.-M.; Moutounet, M.; Drilleau, J.-F. Characterization

380   of highly polymerized procyanidins in cider apple (*Malus sylvestris* var. *Kermerrien*) skin

381   and pulp. *Phytochemistry* **1997**, *44*, 351–357.

382   (28)   Guyot, S.; Matner, N.; Laraba, D.; Sanoner, P.; Drilleau, J.-F. Reversed-phase HPLC

383   following thiolysis for quantitative estimation and characterization of the four main classes

384   of phenolic compounds in different tissue zones of a French cider apple variety (*Malus*

385   *domestica* var. *Kermerrien*). *J. Agric. Food Chem.* **1998**, *46,* 1698–1705.

386   (29)   Karonen, M.; Loponen, J.; Ossipov, V.; Pihlaja, K. Analysis of procyanidins in pine

387   bark with reversed-phase and normal-phase high-performance liquid chromatography–

388   electrospray ionization mass spectrometry. *Anal. Chim. Acta* **2004**, *522*, 105–112.

18

389   (30)   Thompson, R.S.; Jacques, D.; Haslam, E.; Tanner, R.J.N. Plant proanthocyanidins.

390   Part I. Introduction; the isolation, structure and distribution in nature of plant procyanidins.

391   *J. Chem. Soc.*, *Perkin Trans. 1* **1972**, *11*, 1387–1399.

392   (31)   Guyot, S.; Marnet, N.; Sanoner, P.; Drilleau, J.-F. Direct thiolysis on crude apple

393   materials for high-performance liquid chromatography characterization and quantification of

394   polyphenols in cider apple tissues and juices. *Methods Enzymol.* **2001**, *335*, 57–70.

395   (32)   Matthews, S.; Mila, I.; Scalbert, A.; Pollet, B.; Lapierre, C.; Hervé du Penhoat,

396   C.L.M.; Rolando, C.; Donnelly, D.M.X. Method for estimation of proanthocyanidins based

397   on their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem.* **1997**,

398   *45*, 1195–1201.

399   (33)   Ohnishi-Kameyama, M.; Yanagida, A.; Kanda, A.; Nagata, T. Identification of

400   catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser

401   desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass

402   spectrometry. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 31–36.

403   (34)   Vivas, N.; Nonier, M.-F.; Vivas de Gaulejac, N.; Absalon, C.; Bertrand, A.; Mirabel,

404   M. Differentiation of proanthocyanidin tannins from seeds, skins and stems of grapes (*Vitis*

405   *vinifera*) and heartwood of Quebracho (*Schinopsis balansae*) by matrix-assisted laser

406   desorption/ionization   time-of-flight   mass   spectrometry   and   thioacidolysis/liquid

407   chromatography/electrospray ionization mass spectrometry. *Anal. Chim. Acta* **2004**, *513*,

408   247–256.

409   (35)   Karonen, M.; Leikas, A.; Loponen, J.; Sinkkonen, J.; Ossipov, V.; Pihlaja, K.

410   Reversed-phase HPLC-ESI/MS analysis of birch leaf proanthocyanidins after their acidic

411   degradation in the presence of nucleophiles. *Phytochem. Anal.* **2007**, *18*, 378–386.

19

412   (36)   Moilanen, J.; Salminen, J.-P. Ecologically neglected tannins and their biologically

413   relevant activity: chemical structures of plant ellagitannins reveal their *in vitro* oxidative

414   activity at high pH. *Chemoecology* **2008**, *18*, 73–83.

415   (37)   Baert, N.; Karonen, M.; Salminen, J.-P. 2015. Isolation, characterisation and

416   quantification of the main oligomeric macrocyclic ellagitannins in *Epilobium angustifolium*

417   by ultra-high performance chromatography with diode array detection and electrospray

418   tandem mass spectrometry. *J. Chromatogr. A.* **2015**, *1419*, 26–36.

419   (38)   Ozdal, T.; Capanoglu E.; Altay, F. A review on protein-phenolic interactions and

420   associated changes. *Food Res. Int.* **2013**, *51*, 954–970.

421   (39)   Spencer, C.M.; Cai, Y.; Russell, M.; Gaffney, S.H.; Goulding, P.N.: Magnolato, D.;

422   Lilley, T.H.; Haslam, E. Polyphenol complexation–some thoughts and observations.

423   *Phytochemistry* **1988**, *27*, 2397–2409.

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434   **FIGURE CAPTIONS**

435

436   **Figure 1.** The structures of (A) monomeric ellagitannin tellimagrandin I, (B) dimeric ellagitannin

437   oenothein B, (C) trimeric ellagitannin oenothein A, and (D) tetrameric tellimagrandin I-based

438   ellagitannin.

439

440   **Figure 2.** Typical ITC binding isotherms for the interaction of ellagitannin (A) trimer (oenothein A)

441   and (B) hexamer with 20 µM BSA (■) and 30 µM BSA in two replicate experiments (Δ and ×).

442

443   **Figure 3**. Single-site (short dashed line) and two-site (long dashed line) binding models fitted to the

444   experimental data (■) for the interaction of ellagitannin (A) monomer (tellimagrandin I), (B) dimer

445   (oenothein B), (C) trimer, (D) tetramer, (E) pentamer, (F) hexamer, (G) heptamer and (H) a mixture

446   of octamer-undecamers with 30 µM BSA.

447

448   **Figure 4.** Plots of (A) ΔH and (B) K_a vs degree of oligomerization for the interaction of

449   ellagitannin oligomers with BSA fitted by single-site binding model.

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Table 1. Estimated Thermodynamic Binding Parameters for the Interaction of Ellagitannin Oligomers with BSA Fitted by a Single-site Binding Model[a]

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer-undecamers
K_{a1}[b]	18403 ± 5052	5739 ± 794	7552 ± 136	26810 ± 2708	23200	16925 ± 551	16925	35520
ΔH_1[c]	-24 ± 3	-14 ± 4	-45 ± 5	-69 ± 3	-80	-88 ± 3	-93	-93
n_1	6 ± 1	12 ± 2	11 ± 1	9 ± 1	9	10 ± 1	10	9
SD	13	11	11	71		94		

[a] SD = standard deviation around fit obtained by NanoAnalyze software; n = 3, except for ellagitannin pentamer, heptamer and octamer to undecamer n =2 due to the limited amount of individual oligomers. [b] K_{a1} (M^{-1}) is the equilibrium binding constant for the single set of multiple binding sites. [c] ΔH_1 (kJ mol^{-1}) is the corresponding enthalpy.

22

Table 2. Estimated Thermodynamic Binding Parameters for the Interaction of Ellagitannin Oligomers with BSA Fitted by a Two-site Binding Model[a]

	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer-undecamer
K_{a1}[b]	22188 ± 6280	9786 ± 1597	11863 ± 1090	72560 ± 10124	76620	55890 ± 5419	35280	42940
ΔH_1[c]	-20 ± 3	-14 ± 1	-33 ± 3	-52 ± 4	-56	-63 ± 5	-73	-88
n_1	6 ± 2	6 ± 1	11 ± 1	9 ± 1	9	9 ± 1	9	9
K_{a2}[b]	1828 ± 1815	1138 ± 998	350 ± 93	184 ± 19	262	115 ± 11	174	17
ΔH_2[c]	-10 ± 9	-7 ± 3	-9 ± 3	-19 ± 3	-44	-59 ± 2	-27	-24
n_2	4 ±1	36 ± 12	32 ± 14	90 ± 11	35	59 ± 1	84	83
SD	12	7	14	50		73		

[a] SD = standard deviation around fit obtained by NanoAnalyze software; n = 3, except for ellagitannin pentamer, heptamer and octamer-undecamer n =2 due to the limited amount of individual oligomers. [b] K_{a1} and K_{a2} (M^{-1}) are the equilibrium binding constants for the two set of multiple binding sites. [c] ΔH_1 and ΔH_2 (kJ mol^{-1}) are the corresponding enthalpies.

23

**Table 3. Estimated Thermodynamic Binding Parameters for the Interaction of Ellagitannin Oligomers with BSA Fitted by a Single-site Binding Model. The Concentrations of Oligomers Have Been Set to the Corresponding Monomeric Concentrations in Order to Remove the Impact of Molecular Weight[a]**

|	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer-undecamer
K_{a1}[b]	18403 ± 8750	2391 ± 1364	2517 ± 79	6703 ± 1182	4645 ± 368	3082 ± 372	2420 ± 135	5119 ± 2760
ΔH_1[c]	-24 ± 6	-12 ± 11	-15 ± 3	-17 ± 1	-16 ± 1	-15 ± 1	-13 ± 1	-9 ± 1
n_1	6 ± 1	21 ± 13	30 ± 7	37 ± 4	46	57 ± 3	67	85 ± 8
SD	13	9	11	71		94		39

[a] SD = standard deviation around fit obtained by NanoAnalyze software; n = 3, except for ellagitannin pentamer, heptamer and octamer to undecamer n =2 due to the limited amount of individual oligomers. [b] K_{a1} (M^{-1}) is the equilibrium binding constant for the single set of multiple binding sites. [c] ΔH_1 (kJ mol^{-1}) is the corresponding enthalpy.
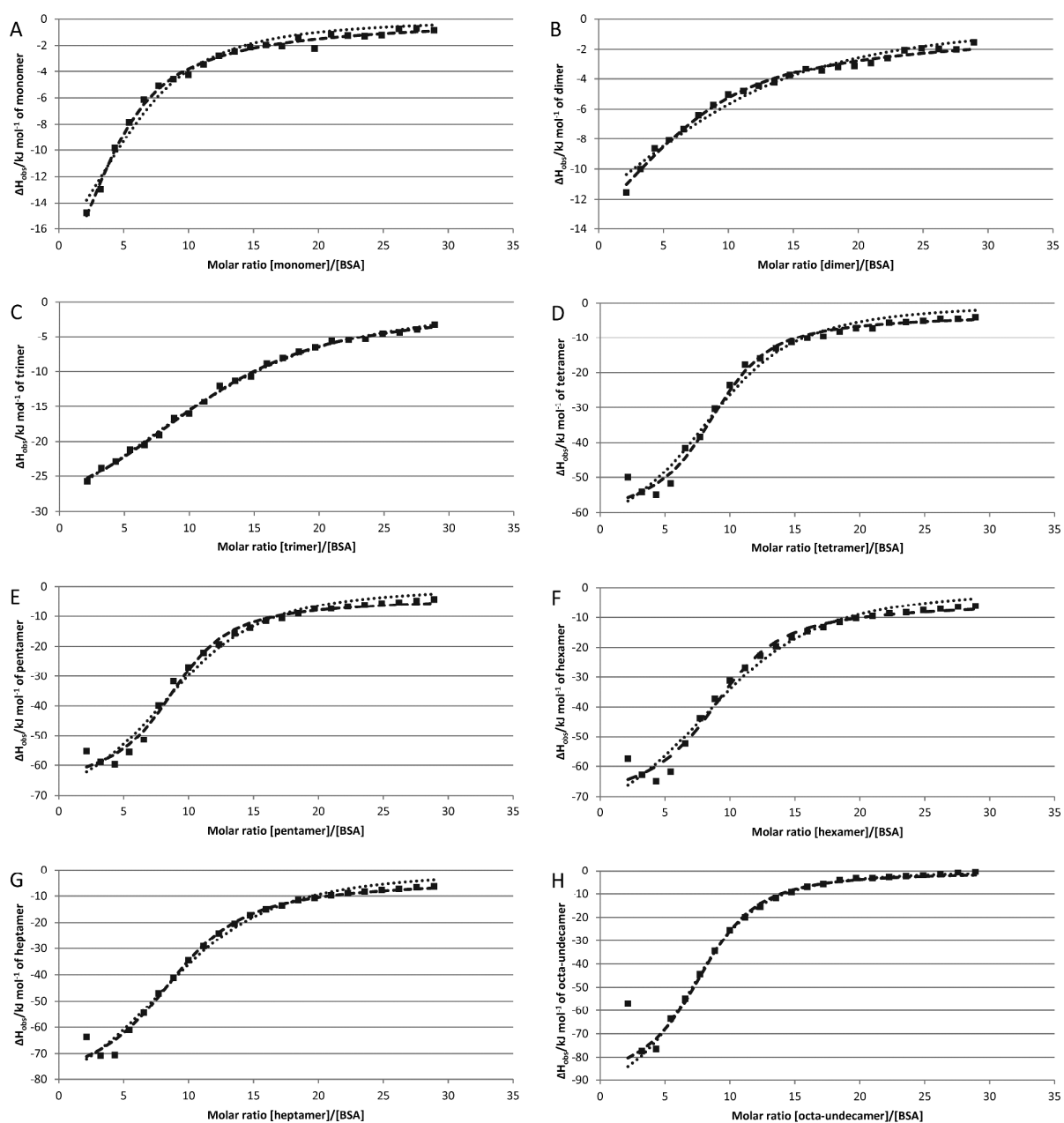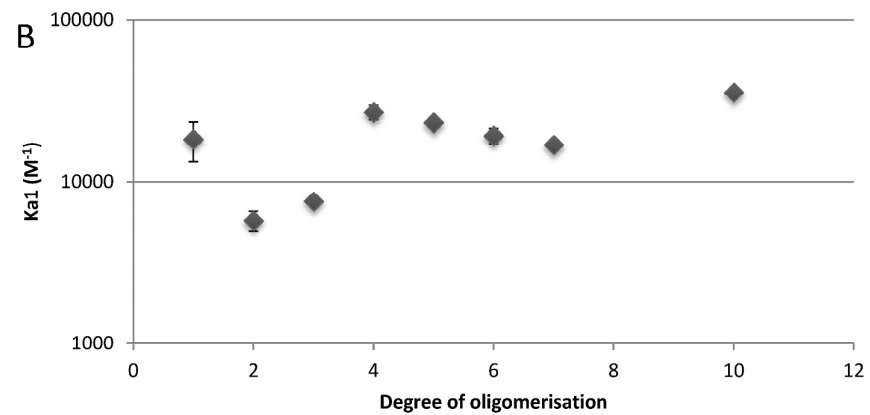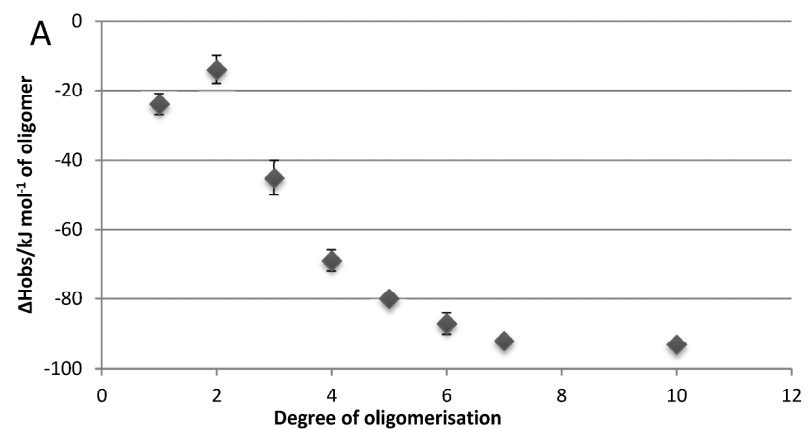
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Figure 1.

A

B

C

Macrocyclic part

D

Flexible tail
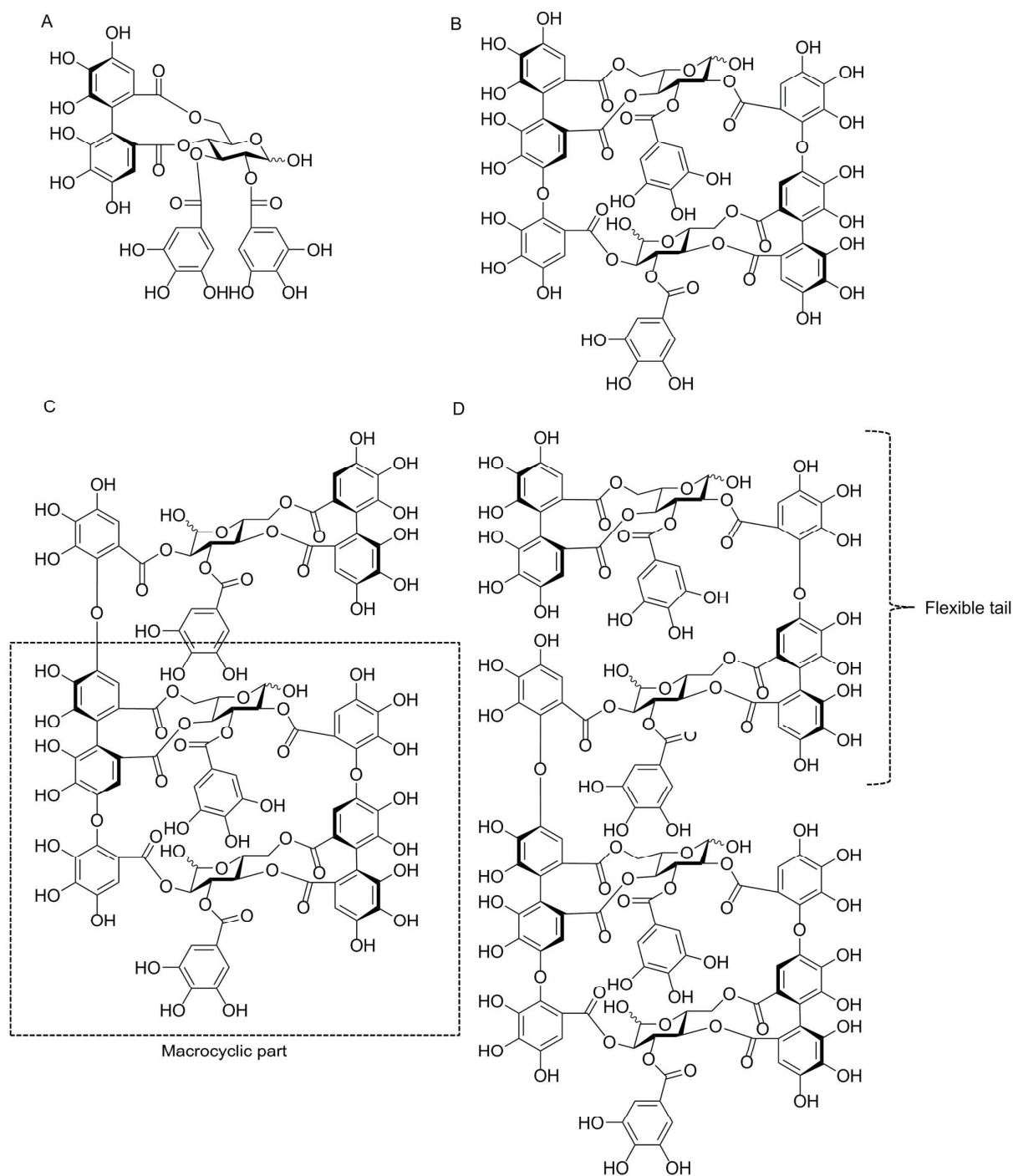
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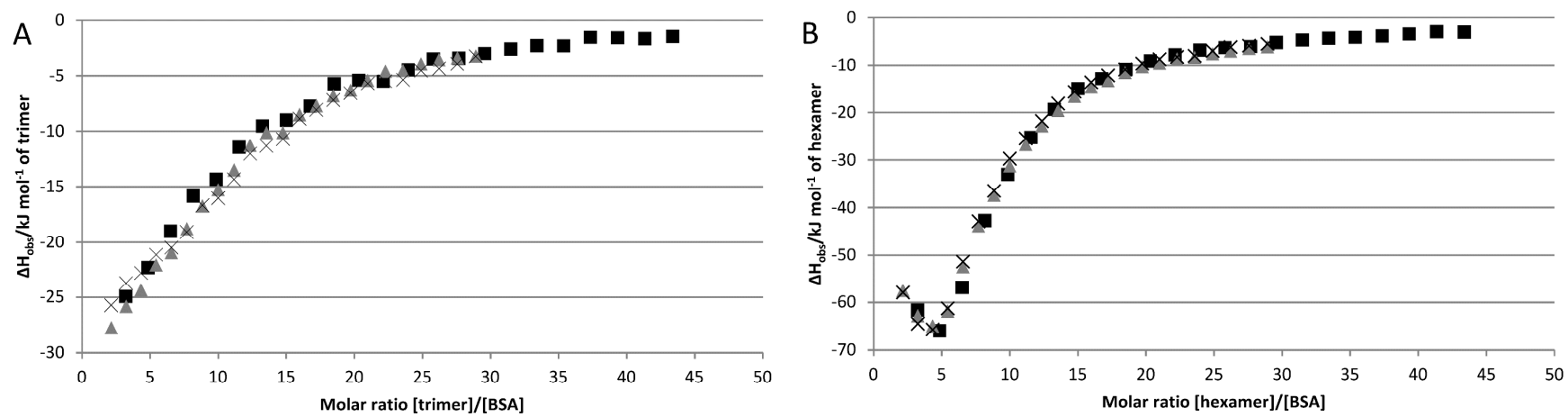
Figure 2.

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Figure 3.

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Figure 4.

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Graphic for table of contents