

1 **Development of a direct ESI-MS method for measuring the tannin precipitation effect of**
2 **proline rich peptides and *in silico* studies on the proline role in tannin-protein**
3 **interactions**

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15 **Abbreviations:** TA, tannic acid; PGG, penta-O-galloyl- β -D-glucose; PRPs, proline rich
16 proteins; BK, bradykinin; BSA, bovine serum albumin.

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23 have influenced its outcome.

24 **Abstract**

25 Tannins are a heterogeneous class of polyphenols that are present in several plants and foods.
26 Their ability to interact and precipitate proline-rich proteins leads to different effects such as
27 astringency or antidiarrheal activity. Thus, evaluation of the tannin content in plant extracts
28 plays a key role in understanding their potential use as pharmaceuticals and nutraceuticals.
29 Several methods have been proposed to study tannin-protein interactions but few of them are
30 focused on quantification. The purpose of the present work is to set up a suitable and time
31 efficient method able to quantify the extent of tannin protein precipitation. Bradykinin, chosen
32 as a model, was incubated with increasing concentrations of 1,2,3,4,6-penta-O-galloyl- β -D-
33 glucose and tannic acid selected as reference of tannic compounds. Bradykinin not precipitated
34 was determined by a mass spectrometer TSQ Quantum Ultra Triple Quadrupole (direct infusion
35 analysis). The results were expressed as PC₅₀, which is the concentration able to precipitate
36 50% of the protein. The type of tannin-protein interaction was evaluated also after precipitate
37 solubilisation. The involvement of proline residues in tannin-protein interactions was confirmed
38 by repeating the experiment using a synthesized peptide (RR-9) characterized by the same
39 bradykinin sequence, but having proline residues replaced by glycine residues: no interaction
40 occurred between the peptide and the tannins. Moreover, modeling studies on PGG-BK and
41 PGG-RR-9 were performed to deeply investigate the involvement of prolines: a balance of
42 hydrophobic and H-bond contacts stabilizes the PGG-BK cluster and the proline residues exert
43 a crucial role thus allowing the PGG molecules to elicit a sticking effect.

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45 **Keywords:** tannins, proline rich proteins, mass spectrometry.

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1. Introduction

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Tannins are a heterogeneous class of polyphenols that are present in several plants and foods, such as fruits, cereals, wine, tea, cocoa and vegetables [1]. According to Bate-Smith and Swain, tannins are water-soluble phenolic compounds with a molecular weight between 500 and 3000 Da, characterized by many hydroxyl groups and capable of forming cross-linkages with proteins. These compounds are classified as hydrolysable and condensed tannins. Hydrolysable tannins are made of a monosaccharide core, such as glucose, partially or completely esterified with an organic acid, such as gallic acid (gallotannins) or ellagic acid (ellagitannins) [2]. Condensed tannins are more complex than hydrolysable tannins: they are polymers of flavan-3-ols linked through acid-labile carbon-carbon bonds [3].

As secondary metabolites of plants, tannins have a role in plant defense, discouraging herbivores from feeding on the plant. These compounds are also used in the production of leather and their levels need to be controlled in wine production to reduce astringency. All these actions are related to the effect of tannins in inducing protein precipitation. In particular, the interaction is known to occur between tannins and proteins rich in proline residues (PRPs) [4]. More recently, the protein precipitation effects induced by some plant components have also been considered as a possible explanation for some bioactive actions. Such effects occur when the precipitating protein (protein target) has a damaging effect or it is contained in infectious organisms. Based on such a mechanism, tannins and plant extracts rich in tannins have been proposed as antidiarrheal [5], antiviral [6,7], antibacterial agents [8–10] and to neutralize the toxic activities of snake venoms [11]. Moreover, a fully detailed study on the molecular interaction of tannins and in particular of penta-O-galloyl-d-glucopyranose with bradykinin has been carried out by NMR [12]. Bradykinin is a proline-rich peptide (Pro residues accounting for 30% of the residues) which acts as an inflammatory mediator and its complexing by tannins can partially explain the well-known anti-inflammatory properties of this class of compounds. More recently, the molecular interaction between tannins and different wheat-derived peptidic fractions, which contain a high content of proline residues and which are responsible for the onset of celiac disease, has been studied [13]. This study indicates that the aggregation between tannins and immunoreactive peptides could represent an important field in the potential protective effect of tannins on the cytotoxicity and/or the immunogenicity of gluten peptides.

78 Based on these recent findings it seems that, beside salivary rich peptides such histatins,
79 interaction of tannins with bioactive proline rich peptides deserves some interest; in particular
80 a valuable method able to measure the precipitating effect of tannins towards damaging
81 peptides could be useful in order to identify potential bioactive compounds and/or the plant
82 extracts containing them.

83 The aim of the present work is 1) to set-up an analytical method able to measure the ability of
84 tannins and/or plant extracts to interact with and precipitate peptides rich in proline and 2) to
85 evaluate the importance of proline in tannin-protein interaction. Bradykinin (BK) was chosen as
86 a target for tannin binding, not only because it is a peptide rich in proline residues, but also due
87 to its involvement in inflammatory disorders such allergies and the common cold, hence
88 representing a potential target peptide of the tannin precipitation effect. Tannic acid (TA) and
89 penta-O-galloyl- β -D-glucose (PGG) were used as reference compounds of tannins.

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92 **2. Materials and Methods**

93 2.1. Chemicals and reagents

94 HPLC-grade water was prepared with a Milli-Q water purification system (Millipore, Milan, Italy).
95 Ammonium acetate was from Riedel-de Haën (Seelze, Germany). Bradykinin acetate, pure
96 tannic acid (TA, CAS number 1401-55-44), 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG),
97 formic acid and LC-grade and analytical-grade organic solvents were from Sigma-Aldrich
98 (Milan, Italy). The internal standard peptide LVNEVTEF was custom synthesized by Sigma-
99 Aldrich (Milan, Italy). The peptide RR-9 RGGGFSGFR was synthesized by PRIMM (Milan,
100 Italy).

101 2.2. Bradykinin-tannin co-precipitation assay

102 100 μ M bradykinin was dissolved in 50 mM acetate buffer pH 7.4 and 25 μ L samples were
103 spiked with of PGG or TA at the following concentrations: 10 (only for PGG), 50, 100, 150, 200,
104 250, 500, 1000 (only for TA) μ M performed in 3 replicates. The mixtures were incubated for 10
105 minutes at 37°C under gentle shaking (1400 rpm on a Thermomixer) and then centrifuged for
106 10 minutes at 14000 rpm. The supernatant (25 μ L) was diluted 1:10 with H₂O/CH₃CN/HCOOH
107 (70/30/0.1, % v/v), spiked with the peptide LVNEVTEF as internal standard (10 μ M final

108 concentration) and analyzed by direct infusion MS as below detailed in order to quantify the
109 amount of bradykinin not precipitated by tannins. The precipitates were then dried, dissolved in
110 H₂O/CH₃CN/HCOOH (70/30/0.1, % v/v) and analyzed by MS in order to verify the presence of
111 bradykinin.

112 2.3. Supernatant and precipitate analyses by mass spectrometry

113 30 µL of the supernatant was injected by an automated sample injection into a TSQ Quantum
114 Ultra Triple Quadrupole (Thermo Finnigan, Milan, Italy) equipped with an ESI-source. An HPLC
115 Surveyor MS Pump (Thermo Finnigan, Milan, Italy) pumped the sample at 25 µL/min with an
116 isocratic phase H₂O/CH₃CN/HCOOH (70/30/0.1, % v/v). The analyses were performed in
117 positive ion mode and with the following ion source parameters: capillary temperature 270° C;
118 spray voltage 4.5 kV; capillary voltage 35 V; tube lens voltage 114 V. The flow rate of the
119 nebulizer gas (nitrogen) was 15 a.u. The mass spectrometer operated in full mass scan and
120 the Q3 was used as detector with a scan range 450-1300 *m/z*.

121 Xcalibur 2.0.7 version was used for the relative quantification of bradykinin. A processing
122 method was set up in order to obtain an automated integration of the areas of the *z* = 2 (*m/z*
123 530.9) peak of BK and *z* = 1 (950.5 *m/z*) peak of the internal standard. The ICIS peak integration
124 parameters set were: smoothing points 7; baseline window 40; area noise factor 5; peak noise
125 factor 10; minimum peak high (S/N) 3. This processing method was applied in the Quan
126 Browser window of Xcalibur for the analysis of all the samples. The results were verified and
127 manually corrected where the integrations were not appropriate.

128 20 µL of the precipitate dissolved was injected by an automated sample injection into a LTQ -
129 Orbitrap XL mass spectrometer (Thermo Scientific, Milan, Italy) equipped with an ESI-source.
130 An HPLC UltiMate 3000 (Thermo Scientific, Milan, Italy) pumped the sample at 25 µL/min with
131 an isocratic phase H₂O/CH₃CN/HCOOH (70/30/0.1, % v/v). The analyses were performed in
132 positive ion mode and with the following ion source parameters: capillary temperature 270° C;
133 spray voltage 4.5 kV; capillary voltage 35 V; tube lens voltage 114 V. The flow rate of the
134 nebulizer gas (nitrogen) was 15 a.u. After mass spectrometry analysis, the deconvolution was
135 carried out with MagTran 1.02 software.

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137 2.4. Modeling studies

138 The PGG conformational space was explored by a MonteCarlo procedure which produced
139 10000 optimized geometries by randomly rotating the peptide backbone torsions as implanted
140 in the VEGA program [14]. The resulting 10000 PGG conformations were clustered according
141 to their structural similarity; in detail, two geometries are considered non-redundant if they differ
142 by more than 60° in at least one rotor. The so derived best five PGG conformations underwent
143 docking simulations based on the recently resolved NMR structure of bradykinin (BK, PDB Id:
144 6F3V). All 10 frames included in the deposited NMR structure were considered in docking
145 simulations which were performed using PLANTS and considering the entire bradykinin
146 structure [15]. For each BK frame, 10 PGG poses were generated and ranked using the
147 ChemPLP score with the speed equal to 1. The BK frame affording the best docking results
148 (frame #6) was then utilized to generate the RR-9 peptide by manually replacing the proline by
149 glycine residues. The conformational profile of the obtained RR-9 peptide was explored by the
150 MonteCarlo procedure as described above by generating 100000 optimized conformations.
151 The best five obtained RR-9 geometries underwent docking calculations with the PGG ligand
152 as already described for BK. Next, and by applying the same docking procedures in an
153 incremental way, the best performing BK frame (#6) was also utilized to generate two clusters:
154 the first was composed of three BK molecules, while the second cluster comprised three BK
155 molecules in complex with three PGG ligands. The optimized clusters were then neutralized
156 and inserted in a 80 Å side cubic box including about 3750 water molecules. After an initial
157 minimization to optimize the relative position of the solvent molecules, the two systems
158 underwent 50 ns MD simulations using Namd [16] and adopting the same MD characteristics
159 described elsewhere [17]. The produced trajectories were finally wrapped using PBCTools [18]
160 and analyzed by performing MM-GBSA calculations.

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163 **3. Results**

164 3.1. Set-up of a ESI-MS method to measure the tannin precipitation effect

165 Several ESI-MS approaches to obtain a better understanding of the non-covalent interaction
166 between tannins and peptides and which in particular have been found suitable for the study of
167 the (1) molecular interactions, (2) the binding stoichiometry and (3) the evaluation of the
168 complex stability, have been reported.

169 To our knowledge, no studies based on MS have been reported for the measurement of the
170 precipitating effects of tannins towards proline rich peptides which is the aim of this paper.
171 Bradykinin was selected as target peptide and tannic acid (TA) and penta-O-galloyl- β -D-
172 glucose (PGG) as reference compounds of tannins.

173 **Figure 1** summarizes the assay. After incubating the target peptide with tannin at different final
174 concentrations, the sample is centrifuged and the relative content of the target peptide in the
175 supernatant determined by ESI-MS using LVNEVTEF as internal standard. The precipitating
176 effect is determined by calculating the residual amount of the target peptide in the supernatant
177 in relation to a sample containing the target peptide and prepared in the absence of tannins
178 (100% of the peptide).

179 **Figure 2** shows the ESI-MS spectra of BK spiked with the internal standard. The ions at m/z
180 530.9 and m/z 1060.6 refer to the $[M+2H]^{2+}$ and $[M+H]^+$ of BK, respectively, while the ion at m/z
181 950.5 is attributed to the $[M+H]^+$ of the internal standard.

182 In preliminary experiments carried out by LC-ESI-MS/UV, neither TA nor PGG up to a
183 concentration of 1 mM significantly precipitated the IS. As shown in **Figure 3A**, the intensity of
184 BK peaks (but not that of the internal standard) decreases as the concentration of TA increases.

185 **Figure 3B** shows the dose-dependent precipitating effect of TA which started at 50 μ M (% of
186 residual bradykinin = 87.88 ± 12.52) and reached a plateau at 500 μ M ($13.00 \pm 12.36\%$). **Figure**
187 **3C** reports the % of residual BK values for each concentration tested. A similar precipitating
188 effect was observed for PGG (**Figure 4A, B and C**). The protein precipitation potency for each
189 tannin was then quantified as the concentration able to precipitate the 50% of BK 100 μ M (PC₅₀).
190 The calculated values are 112.3 μ M and 84.6 μ M for TA and PGG, respectively.

191 To confirm the presence of BK in the precipitates, the pellet obtained by precipitation was
192 dissolved in H₂O/CH₃CN/HCOOH (70/30/0.1, % v/v) and analyzed by direct infusion in a LTQ-
193 Orbitrap XL mass spectrometer. **Figures 5 and 6** show the presence of $[M+3H]^{3+}$ and $[M+2H]^{2+}$
194 ions of BK without any covalent adduct with TA or PGG, as can be appreciated by the
195 deconvoluted spectra.

196 3.2. Study of the involvement of proline residues in bradykinin precipitation

197 In order to understand the involvement of Pro residues in BK precipitation an analogue peptide
198 (RR-9), where the Pro residues are replaced by Gly, was tested. **Figure 7** shows the MS
199 spectrum of RR-9 characterized by the ions at m/z 470.7 and 940.2 which represent the $[M+H]^+$

200 and $[M+2H]^{2+}$ ions, respectively. PGG was then selected as precipitating tannin. As shown in
201 **Figure 8**, PGG did not induce any significant precipitating effect up to 250 μM while only a weak
202 effect was observed at 500 μM .

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204 3.3. Modelling studies

205 **Figure 9** reports (on the left) the best putative complex between BK and PGG as computed by
206 initial docking calculations and reveals that it is stabilized by a fine balance of both hydrophobic
207 and polar contacts. The former involve two galloyl moieties plus the central glucose carbon
208 skeleton which approach Pro2 and Pro3 and are reinforced by π - π stacking with Phe8. Polar
209 interactions involve almost all galloyl groups which stabilize a rich network of reinforced H-
210 bonds with the charged termini plus Arg1 and Arg9. The reported complex suggests that the
211 proline residues are both involved in direct apolar contacts and, due to their rigidifying effect,
212 are responsible for retaining the charged residues exposed and far enough to optimize their H-
213 bonds with PGG. This supposition finds encouraging confirmation when analyzing the
214 computed complexes between PGG and RR-9 (complexes not shown). Here, the replacement
215 of prolines with glycines markedly increases the peptide flexibility and thus the charged groups
216 are able to stabilize intramolecular salt bridges which shield and render them unavailable for
217 contacting PGG. Indeed, the best obtained PGG-RR-9 complex appears to be almost
218 exclusively stabilized by π - π stacking which involves nearly all galloyl rings with Phe5 and
219 Phe8, reinforced by weak H-bonds between the PGG ester functions and the BK backbone
220 atoms. These clear differences are reflected in the primary ChemPLP scores as seen in the
221 best (-100.31 kcal/mol vs. -90.59 kcal/mol. for BK and RR-9, respectively) and average (-92.35
222 \pm 4.21 kcal/mol vs -82.12 \pm 7.01 kcal/mol. for BK and RR-9, respectively) score values.
223 Remarkably, 35 out of the 50 best PGG-BK complexes show scores better than the global
224 minimum as computed by PGG-RR-9 simulations. These notable score differences suggest
225 that even simple docking simulations might be successful in predicting the precipitation effect
226 of tannins.

227 With a view to gaining deeper insights into the molecular mechanisms by which PGG exerts its
228 precipitation effect, the dynamic behaviours of the two clusters composed of a BK trimer with
229 and without PGG molecules were compared by MD simulations. **Figure 9** shows the profile of
230 the interaction energies experienced by the BK peptide in the two MD runs as computed by the

231 MM-GBSA approach. These energies can be seen as a measure of the complex stability and
232 reveal that the inclusion of the PGG molecules markedly increases such a stability (the energy
233 averages are equal to -69.05 ± 7.60 kcal/mol and -38.67 ± 8.44 kcal/mol with and without PGG,
234 respectively). In detail, the stability increase is almost completely ascribable to a strengthening
235 of the van der Waals interactions (the van der Waals energy averages are equal to $-66.01 \pm$
236 8.11 kcal/mol and -36.72 ± 7.12 kcal/mol with and without PGG, respectively) while the
237 electrostatic terms are similarly marginal in both simulations (the electrostatic energy averages
238 are equal to -3.04 ± 5.31 kcal/mol and -1.95 ± 4.70 kcal/mol with and without PGG,
239 respectively). The key role of hydrophobic contacts is confirmed by the right complex in **Figure**
240 **9** which shows the BK-PGG cluster as obtained at the end of the MD run. One may observe
241 that PGG remains precisely inserted between two bradykinin peptides and even at the end of
242 the simulation the contacts between PGG and the proline residues maintain a pivotal role thus
243 emphasizing the relevance of the hydrophobic interactions in determining the PGG precipitation
244 effects.

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247 **4. Discussion**

248 Several methods have been set-up to measure the protein precipitation effect of tannins and
249 the most popular are those based on BSA as protein target and colorimetric assays to measure
250 the unbound protein fraction in the supernatant or tannins in the precipitated protein-tannin
251 fraction. Makkar H.P. [19] determined the precipitated BSA by ninhydrin assay and Haruo
252 Kawamoto [20] quantified the precipitated BSA by HPLC-UV. Two methods were based on the
253 use of filter disk and membrane after tannin fractions or plant extract immobilization on these
254 kinds of surface: the extent of the interaction was determined as reduction of the BSA diffusion
255 [21] or by gamma counting of BSA ^{125}I -labelled adsorbed [22]. Scott H. reported a miniaturized
256 method [23] which aimed to reduce the amount of volume in the assay by using a microplate to
257 measure the unprecipitated BSA by the Bradford assay. On the other hand, some methods were
258 based on the analysis of a tannin co-precipitated fraction after solubilisation of the precipitate
259 by reaction with ferric chloride followed by quantification of the total absorbance (510 nm)
260 [24,25].

261 However, such methods are not suitable for the measurement of the precipitation of target
262 peptides when used at micromolar level and contained in small sample volumes (not higher
263 than one hundred microliters). Such conditions are required when using bioactive peptides,
264 which are expensive or difficult to synthesize and/or isolate. The method here reported fulfils
265 these requirements since the volume of the samples is in the order of hundreds of microliters
266 and the concentration of the target peptides is in a micromolar range, depending on the MS
267 analyzer used. In the present work, by using triple quadrupole, which is quite a common
268 instrument, the peptide was used at a final concentration of 100 micromolar but the
269 concentration could be further reduced when more sensitive analyzers, such as qTOF or
270 orbitraps, are available. Moreover, the sample volumes can be further reduced if micro-wells
271 are used and, in this case, a high throughput method can also be adapted. Despite the fact that
272 the triple quadrupole mass spectrometer is not the best instrument in terms of sensitivity when
273 used in full MS acquisition mode, good reliability and precision can be reached and better
274 results can be obtained if compared to colorimetric assays.

275 The second part of the paper was aimed at better characterizing the involvement of Pro
276 residues in the tannin precipitation effects of BK. By using the developed method we found that
277 the presence of Pro residues is required for tannins to induce BK precipitation, since the peptide
278 analogue, with Pro residues substituted with Gly (RR-9) was only slightly precipitated at the
279 highest concentration (500 μM), while no effect was observed at 84.6 μM , which is the
280 concentration required to PGG for precipitating BK.

281 The involvement of Pro was then further investigated by molecular modelling studies. The
282 obtained results confirm that the stabilizing interactions in the BK-PGG cluster comprise a
283 precise balance of hydrophobic and H-bond contacts in which the proline residues exert a
284 crucial role thus allowing the PGG molecules to elicit a sticking effect by shielding the polar
285 groups of the peptide. The analysis of the PGG effects on the bradykinin folding reveals that on
286 average the peptide assumes more extended conformations when interacting with PGG as
287 described by the end-to-end distance averages (here the distance between the charged termini)
288 which are equal to $15.33 \pm 0.66 \text{ \AA}$ and $12.32 \pm 0.89 \text{ \AA}$ for the simulations with and without PGG,
289 respectively. This effect is clearly understandable by considering that the bradykinin peptides
290 tend to assume the extended conformations which maximize their interactions with PGG. As
291 discussed above, the capacity to retain extended conformations is primarily due to the
292 rigidifying effect of the proline residues and is further increased by the PGG itself, which

293 however does not induce dramatic conformational changes of the peptide as assessed by the
294 rmsd analysis of the superimposed peptide conformations [26]. The so computed rmsd values
295 show indeed almost identical resulting averages ($5.21 \pm 1.76 \text{ \AA}$ and $5.13 \pm 2.36 \text{ \AA}$ for the
296 simulations with and without PGG, respectively) thus suggesting that the structural differences
297 observed with PGG are ascribable to fine conformational shifts by which BK optimizes its
298 interactions with PGG without exerting unfolding effects.

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301 **5. Conclusions**

302 In conclusion, an *in vitro* method was set-up for the quantitative determination of the tannin
303 precipitation effect of proteins and proline rich peptides. The method could be useful to test the
304 ability of tannins to precipitate and hence inactivate damaging peptides and also to evaluate
305 the selectivity of the precipitation effect. Modelling studies confirmed the crucial role of Pro
306 residues for the interaction between tannins and the peptide. Some proline rich peptides can
307 be considered as potential targets of tannins such as bradykinin, an inflammatory mediator
308 which also exerts its pro-inflammatory activity in the g.i. tract, and wheat-derived peptidic
309 fractions which contain a high content of proline residues and which are responsible for the
310 onset of celiac disease. The method could be applied to isolated compounds as well as to
311 complex matrices such as plant derivatives and fractions. The precision of the method was
312 found satisfactory since the CV% was always lower than 20% for all the concentrations tested.
313 Finally, the method is time efficient thus permitting the rapid screening of several plant extracts
314 and compounds able to complex and precipitate damaging target peptides.

315

316 **Figure legends**

317 **Figure 1** – Graphic representation of the bradykinin precipitation assay.

318 **Figure 2** – ESI-MS spectrum of a solution of BK (10 μM) spiked with the IS. Ions at 530.9 m/z
319 and 1060.6 m/z are the $[\text{M}+2\text{H}]^{2+}$ and $[\text{M}+\text{H}]^+$ of BK, respectively. The ion at 950.5 m/z refers
320 to the $[\text{M}+\text{H}]^+$ of the internal standard.

321 **Figure 3** - Dose-dependent precipitating effect of BK induced by TA. A) Tannic Acid dose-
322 dependently reduces the relative abundance of the ion at m/z 530.9 refers to BK $[\text{M}+2\text{H}]^{2+}$ in

323 respect to the abundance of the ion at m/z 950.5 attributed to the internal standard
324 (LVNEVTEF). TA concentrations: 0 μM (a), 50 μM (b), 100 μM (c), 150 μM (d), 200 μM (e), 250
325 μM (f), 500 μM (g), 1000 μM (h). B) Plot showing the residual BK in respect to TA concentration.
326 Values are mean \pm SD of three replicates. C) Mean % of residual BK for each concentration
327 tested.

328 **Figure 4** - Dose-dependent precipitating effect of BK induced by PGG. A) PGG dose-
329 dependently reduces the relative abundance of the ion at m/z 530.9 refers to BK $[\text{M}+2\text{H}]^{2+}$ in
330 respect to the abundance of the ion at m/z 950.5 attributed to the internal standard
331 (LVNEVTEF). TA concentrations: 0 μM (a), 10 μM (b), 50 μM (c), 100 μM (d), 150 μM (e), 200
332 μM (f), 250 μM (g), 500 μM (h). B) Plot showing the residual BK in respect to TA concentration.
333 Values are mean \pm SD of three replicates. C) Mean % of residual BK for each concentration
334 tested.

335 **Figure 5** – Analysis of the precipitate occurred between TA and BK: in the upper panel the
336 spectrum of $[\text{M}+3\text{H}]^{3+}$ and $[\text{M}+2\text{H}]^{2+}$ BK ions; in the lower panel the deconvoluted spectrum
337 obtained with MagTran confirms the identity of BK.

338 **Figure 6** - Analysis of the precipitate occurred between PGG and BK: in the upper panel the
339 spectrum of $[\text{M}+3\text{H}]^{3+}$ and $[\text{M}+2\text{H}]^{2+}$ BK; in the lower panel the deconvoluted spectrum obtained
340 with MagTran confirms the identity of BK.

341 **Figure 7** - ESI-MS spectrum of a solution of RR-9 (10 μM) spiked with the IS. Ions at 470.7 m/z
342 and 940.2 m/z are the $[\text{M}+2\text{H}]^{2+}$ and $[\text{M}+\text{H}]^+$ of RR-9, respectively. The ion at 950.5 m/z refers
343 to the $[\text{M}+\text{H}]^+$ of the internal standard.

344 **Figure 8** - Profile of RR-9 reduction in the supernatant by PGG increasing concentration.

345 **Figure 9** - Dynamic profile of the complex stability with (grey line) and without (dashed grey
346 line) PGG molecules as assessed by MM-GBSA calculations based on the two performed MD
347 runs. At the bottom, two representative BK-PGG complexes are shown. On the left the best
348 BK-PGG complex as obtained by docking simulations. On the right, a portion of the last frame
349 of the MD simulations with PGG (in both complexes prolines are coloured in orange for easy
350 identification).

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