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1 **Interactions between polyphenol oxidation products and salivary proteins: specific affinity of**
2 **CQA dehydrodimers with cystatins and P-B peptide.**

3 Claudia Mariana Castillo-Fraire ^{a,c}, Elsa Brandão ^d, Pascal Poupard ^{b,c}, Jean-Michel Le Quéré ^{a,c},
4 Erika Salas ^e, Victor de Freitas ^d, Sylvain Guyot ^{a,c,*}, Susana Soares ^d

5 ^a INRAE UR BIA – Polyphenols, Reactivity, Processes, F-35653, Le Rheu, France

6 ^b IFPC (French Institute for Cider Production), F-35653, Le Rheu, France

7 ^c UMT ACTIA Nova²Cidre, F-35653, Le Rheu, France

8 ^d REQUIMTE/LAQV, Departamento de Química e Bioquímica, Faculdade de Ciências da
9 Universidade do Porto

10 ^e Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Circuito Universitario s/n,
11 Campus Universitario No. 2, CP 31125, Chihuahua, Mexico

12 * Corresponding author at INRA UR1268 BIA – Polyphenols, Reactivity, Processes, F-35653, Le
13 Rheu, France. E-mail address: sylvain.guyot@inra.fr (S. Guyot)

14 **Keywords:** polyphenols, chlorogenic acid, apple juice, human saliva, astringency

15 **Abbreviations:** CQA: 5'-O-Caffeoylquinic acid; EGCG: epigallocatechin gallate; SP: salivary
16 protein; PRPs: proline-rich proteins; bPRPs: basic bPRPs; gPRPs: glycosylated PRPs; aPRPs:
17 acidic PRPs; AS: acidic saliva; OP: oxidation products; PPO: polyphenoloxidase; CPC: centrifugal
18 partition chromatography; EEECC: elution-extrusion countercurrent chromatography; K_{SV}: Stern-
19 Volmer quenching constant; k_q: bimolecular quenching constant; TFA: trifluoroacetic acid; ACN:
20 acetonitrile; FRET: fluorescence resonance energy transfer; dof: degrees of freedom; SEM:
21 standard error of the mean; ANOVA: one-way analysis variance.

22

23 **ABSTRACT**

24 Throughout the apple juice and cider making process, polyphenols undergo enzymatic
25 oxidation which generate a great variety of polyphenol oxidation products. Since 5'-O-
26 Caffeoylquinic acid (CQA) is one of the major phenolic compounds and the preferential
27 substrate for polyphenoloxidase in apple juice, its oxidation leads to the formation of newly
28 formed molecules by which dehydrodimers (MW 706 Da) are included. Interactions of salivary
29 proteins (SP) with native polyphenols is a well-known phenomenon, but their interactions with
30 polyphenol oxidation products has not been studied yet. In this work, we decided to decipher
31 the interactions between CQA dehydrodimers and SP (gPRPs, aPRPs, statherins/P-B peptide,
32 and cystatins) using HPLC-UV and fluorescence. These results showed that contrary to what

33 was expected, CQA dehydromers presented a low interaction with PRPs, but revealed a
34 specific interaction with statherins/P-B peptide and cystatins. This work settles for the first time
35 the interactions between SP and polyphenol oxidation products.

37 **1 Introduction**

38 Polyphenols have an important impact in organoleptic properties of food, including colour,
39 bitterness and astringency (Li & Duan, 2018). Once food is in the mouth, polyphenols interact with
40 saliva which is rich in several proteins. The interaction of polyphenols with salivary proteins (SP),
41 mostly with proline-rich proteins (PRPs), results in a polyphenol-induced interaction and/or
42 precipitation of PRPs in the oral cavity, which has been described to be the main mechanism
43 responsible for astringency sensation (Canon, Ployon, & Mazauric, 2015; Charlton et al., 2002; de
44 Freitas & Mateus, 2012; Kallithraka, Barker, & Clifford, 1998; Ployon et al., 2018; Soares, Sousa,
45 Mateus, & De Freitas, 2012; Soares et al., 2011). Astringency is described as a complex tactile
46 sensation that takes a significant time to develop. This phenomenon was described as a loss of
47 lubrication, which involves dryness, tightening, roughness or puckering (Haslam, Williamson,
48 Baxter, & Charlton, 1999; Kallithraka et al., 1998; Soares, Brandão, Mateus, & de Freitas, 2017;
49 Soares et al., 2011; Soares, Sousa, et al., 2012). This sensation is strongly related to red wine, for
50 being a tannin-rich beverage, but it is also present in other beverages as tea, fruit juices, beer and
51 cider (Ferrer-Gallego et al., 2016; Lea & Arnold, 1978; Symoneaux, Baron, Marnet, Bauduin, &
52 Chollet, 2014).

53 In general, more than 85% w/w of the salivary proteome belongs to the major families as α -
54 amylase, carbonic anhydrase, histatins, mucins, statherin, P-B peptide, cystatins and PRPs
55 (Ekström, Khosravani, Castagnola, & Messana, 2012).

56 Among them salivary PRPs are classically divided into basic PRPs (bPRPs), glycosylated PRPs
57 (gPRPs) and acidic PRPs (aPRPs). Differences between aPRPs are based on their origin and
58 conformation, giving them diverse functions. Indeed aPRPs are called acidic because their first 30

59 amino acid residues of the N-terminal region present an acidic character; the remaining part is basic
60 and similar to bPRPs containing repeated sequences rich in proline and glutamine (Ekström et al.,
61 2012). aPRPs have an important role on the modulation of the salivary calcium ion concentration.
62 In addition, they are involved in the formation of acquired enamel pellicle and oral mucosal pellicle
63 (Ekström et al., 2012). bPRPs and gPRPs are the most complex group of salivary peptides, their
64 biological properties are not well-known, despite a component of the bPRP family has shown
65 antiviral activity (Robinovitch et al., 2001).

66 All through food process, oxidation is one of the most common reaction leading to food quality
67 modification (Fellow, 2000). In the particular case of cider and apple juice production, especially
68 during crushing and pressing, plant cells are disrupted, and vacuolar polyphenols are put in contact
69 with plastidial polyphénoloxydase (PPO). In presence of dioxygen, the reaction between PPO and
70 polyphenols leads to the formation of oxidation products (OP) (Guyot, Marnet, Sanoner, &
71 Drilleau, 2003; Poupard, Sanoner, Baron, Renard, & Guyot, 2011). In apple and apple-derived
72 products, the main ester of hydroxycinnamic acid is 5'-*O*-caffeoylquinic acid (CQA). In apple
73 juices the CQA concentration ranged from 0.2 to 1.0 g/L depending on the apple variety (Guyot,
74 Bernillon, Poupard, & Renard, 2008). The preferential substrate for PPO in apple juice is CQA, and
75 its oxidation lead to the formation of its corresponding *o*-quinone (Nicolas, Richard-Forget, Goupy,
76 Amiot, & Aubert, 1994). This highly reactive specie is immediately involved in different reaction
77 pathways that may implicate other polyphenols ((-)-epicatechin, procyanidins) or other CQA
78 molecules (Bernillon, Guyot, & Renard, 2004).

79 These reactions lead to a great diversity of newly formed molecules, of which structures and
80 properties are still not well known (Guyot et al., 2008; Poupard, Guyot, Bernillon, & Renard, 2008).
81 Previous studies in apple juice and cider have highlighted the presence of a family of OP
82 corresponding to CQA dehydrodimers (MW, 706 Da) (Bernillon et al., 2004; Guyot et al., 2008).
83 The antioxidant properties of these CQA dehydrodimers (MW, 706 Da) have been already studied,
84 revealing differences comparing to the native compound (CQA) (Wong-Paz, Muñoz-Márquez,

85 Aguilar, Sotin, & Guyot, 2015). Based on this, we state the hypothesis that these newly formed
86 molecules have quite different properties in comparison to their native ones.

87 In beverages such as tea, cider and red wine, the main responsible for astringency sensation are
88 flavonoids, including catechins, flavanols oligomers and polymers, because of their tanning
89 properties (Lesschaeve & Noble, 2005). Several studies dealing with the interaction of polyphenol
90 oxidation products and different proteins (albumins, globulins, lysozyme or gelatin) have shown,
91 that oxidation products (OP) have better tanning properties than native polyphenols (Bongartz et al.,
92 2016; Poupard, 2008; Prigent, Voragen, Visser, van Koningsveld, & Gruppen, 2007; Wildermuth,
93 Young, & Were, 2016; Yabuta, Koizumi, Namiki, Hida, & Namiki, 2001).

94 CQA dehydrodimers are the main products resulting from CQA oxidation (Castillo-Fraire, Poupard,
95 Guilois-Dubois, Salas, & Guyot, 2019; Wong-Paz et al., 2015), their interactions with SP (salivary
96 protein) and their impact on astringency sensation are expected to be different from that of CQA
97 native molecule.

98 As far as we know, the interactions between polyphenol oxidation products and SP and their
99 impacts on astringency sensation were only scarcely studied until now. This study aimed to reveal
100 for the first time, the specific interaction between several families of SP and CQA dehydrodimers
101 (oxidation products), which are present in apple juice and cider.

102 **2 Material and Methods**

103 **2.1 Chemicals**

104 All reagents used were of analytical grade. ACN (acetonitrile) was purchased from ChemLab, TFA
105 (trifluoroacetic acid) from Sigma-Aldrich and formic acid from VWR.

106 **2.2 Synthesis and CPC fractionation and purification of CQA oxidation products**

107 CQA dehydrodimers were synthesized by enzymatic oxidation. In order to remove the remaining
108 CQA, these OP were then fractionated using centrifugal partition chromatography (CPC) in
109 Elution-Extrusion Countercurrent Chromatography (EECCC) mode, as described in our previous
110 work (Castillo-Fraire et al., 2019). In the present work, this CPC fraction containing CQA
111 dehydrodimers was used for the interaction with salivary proteins (SP), but only six among them
112 were monitored (705-3, 705-4, 705-5, 705-6, 705-7 and 705-8) (Figure 1) using HPLC-UV.

113 Individual CQA dehydrodimers (705-3 and 705-4) used in fluorescence experiments were purified
114 (from CPC fraction) using semi-preparative HPLC as described in a previous work (Castillo-Fraire
115 et al., 2019).

116 **2.3 Human saliva collection and acidic saliva preparation**

117 Human saliva was collected and treated as reported previously (Soares et al., 2011). Briefly, human
118 saliva was recovered from seventeen healthy volunteers at 2 p.m. after privation of food or
119 beverages intake at least for 1h. Saliva was stabilized with trifluoroacetic acid (TFA, final
120 concentration 0.1%) to inactivate intrinsic proteases and precipitate the high molecular weight
121 proteins like mucin and amylase, and was then centrifuged (11500 rpm, 5 min) to remove the
122 insoluble particles. The supernatant, hereafter referred as acidic saliva (AS), was recovered and
123 used for the following experiments. The total protein concentration was measured by the Bradford
124 assay using bovine serum albumin as a standard and was determined to be 603 $\mu\text{g/mL}$. This AS
125 supernatant was then used for the interactions with the CQA dehydrodimers fraction. Five families

126 of SP were monitored using HPLC-UV: glycosylated PRPs (gPRPs), acidic PRPs (aPRPs),
127 cystatins, statherin and P-B peptide. As statherin and P-B peptide were co-eluted, they were
128 analyzed together.

129 **2.4 Salivary proteins purification and characterization using ESI-MS**

130 Additionally, part of the supernatant of the acidic saliva (AS) was placed in a cellulose dialysis
131 membrane (molecular-weight cut-off 3.5 kDa) for 24h at 4 °C with stirring against deionized water.
132 Then, the dialyzed saliva was lyophilized, and the resulting powder was re-solubilized in the
133 minimum volume of water to concentrate the total protein content.

134 This solution was purified using preparative HPLC to separate the different families of SP by their
135 retention time. The preparative fractions were lyophilized and the peptides present in each fraction
136 were identified by ESI-MS as described in the literature (Soares et al., 2018, 2011). Fractions
137 containing either cystatins or P-B peptide were used for Fluorescence assays.

138 The mass spectra of the cystatin fraction analysis and the corresponding deconvoluted spectra of the
139 identified cystatins is presented as Supplementary Information (Figure S1).

140 **2.5 Interactions between CQA oxidation products and acidic saliva**

141 **2.5.1 Preparation of salivary protein controls**

142 Salivary proteins (SP) control at fixed concentration was prepared by mixing AS (acidic saliva)
143 (603 µg/mL) (150µL) and water (50µL). SP controls for minor concentrations (72, 150 and 302
144 µg/mL) were prepared by dilution of the AS (603 µg/mL). The higher concentrations were prepared
145 by freeze-drying the control AS (603 µg/mL) until the expected concentration was reached.

146 **2.5.2 *In vitro* interactions between CQA dehydrodimers and acidic saliva**

147 The solution of OP containing CQA dehydrodimers (706 Da) was mixed with AS previously
148 isolated. Controls of SP and OP were separately prepared in water for each concentration and were
149 analysed using HPLC-UV. Two experiments were carried out:

150 a) Interaction of a fixed SP concentration (603 $\mu\text{g/mL}$) with increasing concentrations of OP (0.5,
151 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL).

152 b) Interaction of a fixed concentration of OP (0.5 or 2.5 mg/mL) with increasing SP concentrations
153 (72, 150, 302, 603, 965 and 1930 $\mu\text{g/mL}$).

154 For all the experiments, AS, water (if necessary), and the required volume of CQA dehydrodimers
155 (12 mg/mL), were mixed in that order to obtain the desired final concentration of SP and OP, in a
156 final volume of 200 μL .

157 The mixture was stirred and incubated at room temperature for 10 min, then it was centrifuged
158 (8000 g, 5 min). Supernatant was recovered for both analysis of proteins and polyphenols using two
159 separated HPLC-UV methods.

160 **2.6 Reversed phase HPLC-UV methods**

- 161 • For protein analyses

162 Ninety microliters (90 μL) of each solution (filtered and no-filtered) were analysed on a HPLC
163 Lachrom system (Merck Hitachi, L-7100) equipped with Kinesis C8 column (150 \times 2.1 mm, 5 μm
164 particle diameter). The eluents were (A) 0.2% aqueous TFA and (B) 0.2% TFA in acetonitrile/water
165 80/20 (v/v). The gradient applied was linear 10 to 45% of B in 40 min, at a flow rate of 0.50
166 mL/min . The column was then washed for 10 min with 100% eluent B to elute late-eluting proteins
167 and then stabilized with 10% B. The detection was carried out using a UV-vis detector (L-7420) at

168 214 nm. The results are expressed as area percentage of the chromatographic peak of protein
169 relatively to its control (SP without OP).

170 • For polyphenol analysis

171 The polyphenol profiles were analyzed by HPLC on a Hitachi Chromaster HPLC system [Poroshell
172 120 Agilent C18 column (250 × 4.6 mm, 2.7 μm particle diameter, 120 Å). Detection was carried
173 out at 280 nm using a diode array photo detector (5430)]. The volume injected was 40 μL, with a
174 flow rate of 0.4 mL.min⁻¹. The solvents were 0.1% (v/v) aqueous formic acid (A) and 0.1% (v/v)
175 formic acid in acetonitrile (B). The elution gradient was linear: 0 min, 7% B; 3 min, 13% B; 20 min,
176 15% B; 21– 50 min, 20% B; 60 min, 45% B. After the elution, the column was washed for 8 min
177 with 100% eluent B and equilibrated to 7% B for the next analysis. The results are expressed as area
178 percentage of the chromatographic peak of each compound relatively to its control (OP without SP).

179 **2.7 Fluorescence quenching analysis**

180 Purified proteins (P-B peptide and cystatins) stock solutions were prepared at 30 μM in distilled
181 water. Polyphenols were titrated at increasing concentrations (0 to 100 μM). Tryptophan residues of
182 P-B peptide and cystatins were used as intrinsic fluorophore [setting the excitation wavelength (λ_{ex})
183 at 280 nm]. After mixing the proteins and the polyphenols solutions, the samples were pipetted to
184 the fluorimeter cell. The emission spectra (from 290 to 450 nm) were recorded in a Horiba
185 FluoroMax-4 Spectrometer. The cell was rinsed with water after each measurement. Fluorescence
186 intensity for the different titrations was registered at 310 nm for P-B peptide and 347 nm for
187 cystatins, the wavelengths of maximum fluorescence. Polyphenols intrinsic fluorescence (blank
188 measurement) was also assessed for the different concentrations and they were subtracted to the
189 respective sample of the complexes SP-polyphenol. Additionally, the optical density of each SP-
190 polyphenol mixture was measured in order to use polyphenol concentrations with the lowest optical
191 densities to correct the inner filter effect. The probability of fluorescence resonance energy transfer

192 (FRET) between proteins and polyphenols was discarded after analysis of both absorption and
193 emission spectra.

194 The Stern-Volmer equation (equation 1) was used to address the fluorescence quenching data:

195
$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (\text{Equation 1})$$

196 F_0 – Intensity of fluorescence of the fluorophore before to the addition of quencher (polyphenol);

197 F – Intensity of fluorescence after the addition of quencher (polyphenol);

198 k_q – Bimolecular quenching constant;

199 τ_0 – Fluorophore lifetime (SP) in the lack of the quencher;

200 $[Q]$ – Quencher (polyphenol) concentration;

201 K_{SV} – Stern-Volmer quenching constant.

202 Using this equation, the results from the fluorescence quenching were presented in Stern-Volmer
203 plots (plots of F_0/F versus $[Q]$) to calculate the K_{SV} .

204 A linear Stern-Volmer plot usually point out that one type of mechanism occurs: static, that
205 suggests the formation of a stable fluorophore-quencher complex, or dynamic, usually linked to
206 collisional encounters of the fluorophore and quencher. Alternatively, a negative deviation
207 downward the x -axis occurs once different tryptophan residues of the protein occur in distinct
208 environments (environment a and environment b) and can be accessible in a different way to the
209 quencher (polyphenol). The typical Stern-Volmer plot of this type of interaction is a curve
210 downward to the x -axis, which is a distinctive feature of two fluorophore populations, one of which
211 is not accessible to the quencher. For these cases, the Stern-Volmer equation is modified into
212 equation 2:

213
$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a} \quad (\text{Equation 2})$$

214
$$f_a = \frac{F_{0a}}{F_{0b} + F_{0a}} \quad (\text{Equation 3})$$

215 F_0 – Fluorescence intensity before the addition of quencher (polyphenol);
216 ΔF – Difference between the fluorescence intensities before and after the addition of quencher
217 (polyphenol);
218 f_a – Fraction of the initial fluorescence accessible to quencher;
219 $[Q]$ – Quencher (polyphenol) concentration;
220 K_a – Stern-Volmer quenching constant;
221 F_{0a} – Intensity of the fluorescence without the quencher in environment a
222 F_{0b} – Intensity of the fluorescence without the quencher in environment b

223 **2.8 Data and statistical analysis**

224 In HPLC-UV graphics, bars represent the Student confidence intervals ($p = 0.05$) were calculated
225 according to the method proposed by Box, Hunter, & Hunter, (2005). In this method, degrees of
226 freedom (dof) were calculated by the sum of dof of each triplicate analysis, between 10 and 14
227 depending on the test.

228 Fluorescence assays were performed in triplicates and the presented values are the arithmetic means
229 and Standard Error of the Mean (SEM). The statistical significance of the difference between the
230 different experiments was assessed by one-way analysis variance (ANOVA) and the Tuckey test.
231 Differences were considered significant when $p < 0.05$. The statistical data were assessed by
232 GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA).

233 **3 Results and discussion**

234 **3.1 Interactions of CQA oxidation products with salivary proteins**

235 In order to assess the interactions between OP with SP, different concentrations of the CQA
236 dehydrodimer fraction (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL) were mixed with acidic saliva (AS) at
237 different concentrations of total salivary proteins (SP): 72, 150, 302, 603, 965 and 1930 $\mu\text{g/mL}$.

238 After 10 min incubation, a centrifugation was carried out to remove insoluble complexes. SP and
239 OP that remaining soluble in the supernatant were analysed using HPLC-UV.

240

241 **3.1.1 Fixed salivary protein concentration**

242 In this experiment (Figure 2), a fixed SP concentration of 603 $\mu\text{g/mL}$ was mixed with increasing
243 concentrations of OP from 0.5 to 3.0 mg/mL . The OP range of concentrations covered the
244 concentrations already reported for the CQA in apple juices (Guyot et al., 2008). However, the
245 range was extended in order to explore interactions at higher OP concentrations.

246 HPLC-UV analysis results after interaction of OP with the different families of SP are shown in
247 Figure 2. The results are expressed as area percentage of the chromatographic peak of each
248 compound relatively to its control (OP without SP). Figure 2A shows the area percentages of SP
249 that remain soluble in the supernatant after the interaction with OP.

250 Interestingly, a specific protein precipitation for statherin/P-B peptide and cystatins was observed,
251 even at the lowest OP concentration (0.5 mg/mL). Protein precipitation started being more obvious
252 at OP 1.0 mg/mL , observing a decrease of near 50% of these proteins (statherin/P-B peptide and
253 cystatins). This specificity toward SP was observed for all the tested concentrations and became
254 stronger as OP concentrations increased (Figure 2A). Under the tested conditions, gPRPs and
255 aPRPs reached their maximum of precipitation (near 50%) at 2.5 mg/mL of OP, whereas at the
256 same concentration, statherin/P-B peptide and cystatins were almost completely precipitated.

257 Our results are particularly different from previous studies that assessed polyphenol-SP interactions.
258 Most of previous works studied the interactions between SP and wine or grape tannins such as:
259 procyanidins, pyranoanthocyanins, pentagalloylglucose, trigalloylglucose, propyl gallate,
260 epigallocatechin gallate (Baxter, Lilley, Haslam, & Williamson, 1997; Brandão, Soares, Mateus, &
261 De Freitas, 2014; Canon, Giuliani, Paté, & Sarni-manchado, 2010; Canon et al., 2013; García-
262 Estévez et al., 2017; Luck et al., 1994; Pascal et al., 2007; Soares, Mateus, & de Freitas, 2012;

263 Soares, Sousa, et al., 2012; Soares et al., 2011). In fact, most of these works showed a strong
264 interaction of these polyphenols with specific families of PRPs (bPRPs, gPRPs, aPRPs) (Baxter et
265 al., 1997; Brandão et al., 2014; Canon et al., 2010, 2013, 2015; García-Estévez et al., 2017; Ployon
266 et al., 2018; Soares, Sousa, et al., 2012). As referred previously, the main similarity between the
267 different families of PRPs is the high content in proline residues. Thereafter, the different families
268 have a high content in other particular aminoacids, giving them diverse functions and physical-
269 chemical properties which could explain the different affinities of these proteins toward
270 polyphenols. In most cases, aPRPs is one of the main families presenting a strong interaction with
271 tannins (pentagalloylglucose, galloylated procyanidins, procyanidins), even if bPRPs, gPRPs and
272 statherin were also precipitated (Brandão et al., 2014; Canon et al., 2015; Ployon et al., 2018;
273 Soares, Mateus, et al., 2012; Soares, Sousa, et al., 2012; Soares et al., 2011). PRPs and statherin
274 have also been shown to be precipitated by tea polyphenols (Nayak & Carpenter, 2008). Recently, a
275 study focused on the individual interactions of the statherin, P-B peptide and cystatins toward
276 procyanidins dimers B3 and B6 highlighted that statherin and P-B peptide have a strong interaction
277 with these procyanidins, unlike to cystatins (Silva et al., 2017). Herein, it was observed that besides
278 statherin and P-B peptide, cystatins can also be highly precipitated in presence of CQA
279 dehydrodimers.

280 Furthermore, some of these studies revealed the importance of the polyphenol/protein ratio on the
281 type and (in)solubilisation of the aggregates. Previous works about the interaction of
282 epigallocatechin gallate (EGCG) with a PRP protein (IB5) observed that, at low polyphenol/protein
283 ratios, EGCG progressively coats the IB5 protein but no aggregation occurs, in contrast to higher
284 ratios, where IB5 proteins connect to each other through bridges between the EGCG molecules and
285 then aggregates are built (Canon et al., 2013; Pascal et al., 2007).

286

287 Regarding oxidation products, the interactions of six CQA dehydrodimers (from 705-3 to 705-8)
288 with SP were monitored on the basis of the decrease of their UV 280 nm peak on HPLC
289 chromatograms of the supernatants.

290 Figure 2B presents the area percentage of OP that remain in the supernatant after centrifugation.
291 Interestingly, five compounds (705-3 to 705-7) behave similarly for each OP concentration tested,
292 in contrary to compound 705-8 that is always more strongly precipitated.

293 The highest OP interaction with SP was observed at 0.5 mg/mL with around 30% reduction of the
294 CQA dehydrodimers (705-3 to 705-7). For higher concentrations (2.0 mg/mL to 3.0 mg/mL), less
295 than 10% of CQA dehydrodimers (705-3 to 705-7) were removed after precipitation and
296 centrifugation (Figure 2B), suggesting like a saturation effect. In fact, OP concentration is so high
297 that it has largely exceeded the maximum quantity of OP that can be complexed by that quantity of
298 the SP (603 $\mu\text{g/mL}$).

299 **3.1.2 Fixed concentrations of oxidation products**

300 In order to understand the previous suggested saturation effect, further experiments were made with
301 different concentrations of total SP. In these experiments, low (0.5 mg/mL) or high (2.5 mg/mL)
302 fixed OP concentrations were mixed with increasing concentrations of SP (72 to 1930 $\mu\text{g/mL}$).

303 **3.1.2.1 At low concentration of CQA dehydrodimers**

304 The results of the interactions of a fixed OP concentration (0.5 mg/mL) with increasing
305 concentrations of SP (72 to 1930 $\mu\text{g/mL}$) are shown in Figure 3.

306 The highest interaction was observed with cystatins and it was particularly visible in the assay at the
307 lowest protein concentrations (Figure 3A). Indeed, more than 80% of cystatins were removed at
308 lowest SP concentrations (72 and 150 $\mu\text{g/mL}$). As SP concentration increased, the percentage of
309 remaining proteins (in supernatant) was higher probably due to the high ratio of protein/OP and the
310 lack of OP molecules to complex with protein.

311 Regarding CQA dehydrodimers (Figure 3B), their precipitations were quite similar (around 15%) at
312 low SP concentrations (72, 150 and 302 $\mu\text{g/mL}$). This was followed by the highest precipitation of
313 CQA dehydrodimers (with around 30% of precipitation) at the SP concentration of 603 $\mu\text{g/mL}$.

314

315 3.1.2.2 At high concentration of CQA dehydrodimers

316 The results of the interactions of a fixed OP concentration (2.5 mg/mL) with increasing
317 concentrations of SP (72 to 1930 $\mu\text{g/mL}$) are shown in Figure 4.

318 Regarding the highest OP concentration (2.5 mg/mL), the selectivity effect toward the different
319 families of SP was only visible for higher protein concentrations (Figure 4A). Indeed, the totality of
320 the proteins, even gPRPs and aPRPs, were precipitated at the lowest SP concentrations (72 and 150
321 $\mu\text{g/mL}$). Besides, around 90% of cystatins and statherin/P-B peptide were removed for almost all
322 SP concentrations tested, except for the highest one at 1930 $\mu\text{g/mL}$ (Figure 4A).

323 Interestingly, at low SP concentrations (150, 302 $\mu\text{g/mL}$) and high OP concentration (2.5 g/L),
324 although proteins were completely precipitated (Figure 4A), the individual concentrations of CQA
325 dehydrodimers that remained in the supernatant were not noticeably impacted. This could be
326 justified by the fact that the necessary amount of OP to precipitate all the SP (at these low SP
327 concentrations) was too small to be detected by the HPLC-UV monitoring since it corresponded to
328 a very low part of the initial amount of OP (Figure 4B). For higher SP concentrations (965 and 1930
329 $\mu\text{g/mL}$), the precipitation of CQA dehydrodimers was higher and therefore clearly observable by
330 the HPLC-UV analysis leading to around 20% decrease of the initial concentration. These higher
331 SP concentrations also allowed to observe the greater affinity of 705-8 in comparison to the other
332 CQA dehydrodimers.

333 Based on all OP-SP interactions studied, we hypothesize that a low number of molecules (OP) is
334 necessary to significantly precipitate the SP.

335 3.2 Interactions between individual CQA dehydrodimers and individual salivary proteins

336 Based on HPLC-UV previous results, CQA dehydrodimers presented higher interactions with
337 statherins/P-B peptide and cystatins. In order to characterize the interactions between individual
338 compounds (SP and OP), the most abundant CQA dehydrodimers in the CPC fraction (705-3 and
339 705-4) were purified as well as P-B peptide and cystatins from AS. Then, interactions between
340 these individual compounds were studied by fluorescence quenching.

341

342 For interactions between 705-3 and both purified proteins (P-B peptide and cystatins), it was
343 observed a linear Stern-Volmer plot (Figure 5a), as for interaction of 705-4 with P-B peptide
344 (Figure 5b). According to equation 1, the Stern-Volmer quenching constant (K_{SV}) is directly
345 determined for these interactions (Table 1). For the interaction between 705-4 and cystatins, a
346 Stern-Volmer plot with a downward-curve toward x-axis was observed. To determine the
347 corresponding Stern-Volmer quenching constant (K_{SV}) (Table 1), a modified form of this plot is
348 represented (Figure 5c) based on equation 2. Both constants correspond to the ability to interact
349 despite the different forms of determination which is related to the quenching (interaction)
350 mechanism.

351 Regarding the influence of the protein families, the determined K_{SV} indicate that both CQA
352 dehydrodimers have a higher interaction with cystatins than with P-B peptide (Table 1). Indeed, the
353 K_{SV} for the interactions between 705-3 and 705-4 with cystatins were 22766 M^{-1} and 54054 M^{-1} ,
354 respectively, while the K_{SV} values for the interaction with P-B peptide were 12760 M^{-1} and 11855
355 M^{-1} , respectively. Additionally, the interaction with P-B peptide seems not to be selective because
356 the K_{SV} values for the interaction with both compounds (705-3 and 705-4) were similar (12760^{-1}
357 and 11855 M^{-1}). This is not true for cystatins which seems to have a higher interaction with 705-4
358 (54054 M^{-1}), suggesting some selectivity. Remarkably, this selectivity was highlighted only for
359 705-4, even if both compounds (705-3 and 705-4) have the same dihydrobenzofuran-type structures

360 (Figure 1) (Castillo-Fraire et al., 2019). However, this selectivity was not observed in HPLC-UV
361 assays, since no differences were observed on the remaining areas after interaction of CQA
362 dehydrodimers with AS.

363 In a similar work, interactions of procyanidins B3 and B6 with cystatins were explored. The results
364 showed low K_{SV} for both procyanidins ($B3 = 5400 \text{ M}^{-1}$, $B6 = 6500 \text{ M}^{-1}$) indicating a low ability to
365 interact with cystatins (Silva et al., 2017).

366

367 P-B peptide and cystatins tryptophan residues were used as intrinsic fluorophores. P-B peptide has
368 two tryptophan residues (Uniprot database, accession number P02814) while cystatins have two or
369 three tryptophan residues (Uniprot database, accession numbers P01036, P01037, P09228). The
370 tryptophan residues can occur in distinct environments of the protein structure and therefore, can be
371 differently accessible to the quencher (polyphenol) (Lakowicz, 2006). A linear Stern-Volmer plot is
372 generally indicative of a single class of tryptophan, all equally accessible to quencher (Lakowicz,
373 2006). This has been observed for the interaction of both CQA dehydrodimers with the P-B peptide
374 and for the interaction of the 705-3 with cystatins. On the other hand, for the interaction of the
375 polyphenol compound 705-4 with cystatins, it was observed a curve downward towards x-axis,
376 which is a typical Stern-Volmer plot for a situation with two fluorophore populations, one of which
377 is not accessible to the quencher. Additionally, this type of interaction could even lead to spectral
378 shifts due to the selective quenching of exposed versus buried tryptophan residues. In fact, a shift of
379 the cystatins emission spectra to higher wavelengths upon interaction with increasing
380 concentrations of the polyphenol compounds 705-4 was also readily observed (Figure 5d).

381 This has been justified as those tryptophan residues emitting at lower wavelengths are quenched
382 more readily than the higher wavelengths of tryptophan. In fact, the quenched residues display an
383 emission maximum at 363 nm while the protected residues display an emission maximum at 347
384 nm.

385 To differentiate the type of mechanisms involved (dynamic or static), it is necessary to calculate the
386 bimolecular quenching constant (k_q). When the k_q values are higher than the diffusion-controlled
387 limited value ($1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), it suggests higher affinities and stronger interactions, and a static
388 mechanism is more likely to occur with the formation of a stable complex between protein and
389 quencher (Lakowicz, 2006; Silva et al., 2017; Soares, Mateus, & De Freitas, 2007). This is the case
390 of all interactions between both OP (705-3 and 705-4) with peptide P-B and cystatins as they
391 present similar values (5.0, 5.9, $4.6 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$), except for interaction between cystatins and
392 compound 705-4 where k_q value is higher ($12.8 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$) suggesting a selective interaction
393 according its Stern-Volmer quenching constants (K_{SV}).

394 Specific interactions of OP with cystatins or statherin/P-B could be explained by structural
395 differences. Contrary to what was observed with tannins (Silva et al., 2017), OP presented a low
396 affinity for unstructured proteins (PRPs), but a high interaction was observed toward structured
397 proteins (cystatins). In the particular case of statherin, despite it has been previously referred as
398 unstructured protein (only partly folded) (Ramasubbu, Thomas, Bhandary, & Levine, 1993), a high
399 interaction with OP was observed. However, statherin could present a "conformational change" that
400 would privilege interaction with OP. Moreover, P-B peptide is frequently included in the basic PRP
401 family, but it presents several characteristics suggesting a functional relationship with statherin,
402 which could explain the high interaction with OP (Ekström et al., 2012)

403 **4 Conclusion**

404 CQA is the main hydroxycinnamic acid ester in apple and the preferential substrate for PPO.
405 Oxidation of CQA results in the formation of CQA dehydrodimers as one of the main oxidation
406 products in apple juice and cider. As far as we know, this study highlighted for the first time, the
407 specific interaction between several families of SP and CQA dehydrodimers (oxidation products).
408 Indeed, protein/polyphenol precipitation revealed that OP presented a stronger interaction with
409 statherin/P-B peptide and cystatins than with PRPs (gPRPs and aPRPs). These results were not

410 expected since based on previous studies, polyphenols (tannins) interaction with PRPs are clearly
411 stronger (Baxter et al., 1997; Brandão et al., 2014; García-Estévez et al., 2017; Luck et al., 1994;
412 Soares, Mateus, et al., 2012; Soares, Sousa, et al., 2012; Soares et al., 2011).

413 Interactions between SP and OP are highly impacted by the ratio SP/OP. In the tested conditions,
414 we hypothesize that a low number of OP is necessary to significantly precipitate SP.

415 Regarding, fluorescence quenching assays, the specific interaction with cystatins seemed to be a
416 little bit stronger than P-B peptide interaction, based on K_{SV} . This result is quite interesting since in
417 previous works cystatins presented the lowest interactions with polyphenols belonging to the
418 procyanidins class compared to other SP (Silva et al., 2017).

419 The impact that these specific interactions with SP could have on mouthfeel/sensation is still
420 unknown. Sensorial analyses are needed to fully understand the contribution of these newly formed
421 compounds on the taste of polyphenol-rich beverages.

422

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429

430

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Figures Captions

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586

587 **Figure 1.** CQA dehydrodimers structures: compounds 705_3 and 705_4 = I, 2,3-
588 dihydro-1,4-benzo-dioxan type; 705_5 and 705_6 = II, 2,3-dihydrobenzofuran-
589 type; 705_7 and 705_8 = III, 1,2-dihydro-naphthalene-type (Castillo-Fraire et al.,
590 2019).

591

592 **Figure 2:** Remaining SP or OP (peak area %) after interaction of a fixed SP concentration (603
593 $\mu\text{g/mL}$) with increasing concentrations of OP (0.5 mg/mL to 3.0 mg/mL). A) Different families of
594 SP ($p = 0.05$, dol = 14); B: the individual CQA dehydrodimers ($p = 0.05$, dol = 11).

595

596 **Figure 3.** Remaining SP or OP (peak area %) after interaction of a fixed OP concentration (0.5
597 mg/mL) with increasing concentrations of SP (72 to 1930 $\mu\text{g/mL}$). A) different families of SP ($p =$
598 0.05, dol = 10), B) individual CQA dehydrodimers ($p = 0.05$, dol = 13).

599

600 **Figure 4.** Remaining SP or OP (peak area %) after interaction of a fixed OP concentration (2.5
601 mg/mL) with increasing concentrations of SP (72 to 1930 $\mu\text{g/mL}$). A: different families of salivary
602 proteins ($p = 0.05$, dol = 10) B: individual CQA dehydrodimers ($p = 0.05$, dol = 13).

603

604 **Figure 5.** Stern-Volmer plots for the fluorescence quenching of (●) P-B peptide and (o) cystatins
605 upon titration with increasing concentrations of CQA dehydrodimers 705-3 (a) and 705-4 (b). The
606 presented data are the means and SEM of a triplicate assay. c: Modified Stern-Volmer plot based on
607 equation 2; d: fluorescence spectra of cystatins recorded at λ_{ex} 280 nm for the interaction of
608 cystatins (30 μM) with increasing concentrations of CQA dehydrodimers 705-4 (0 to 100 μM).

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Figure 1

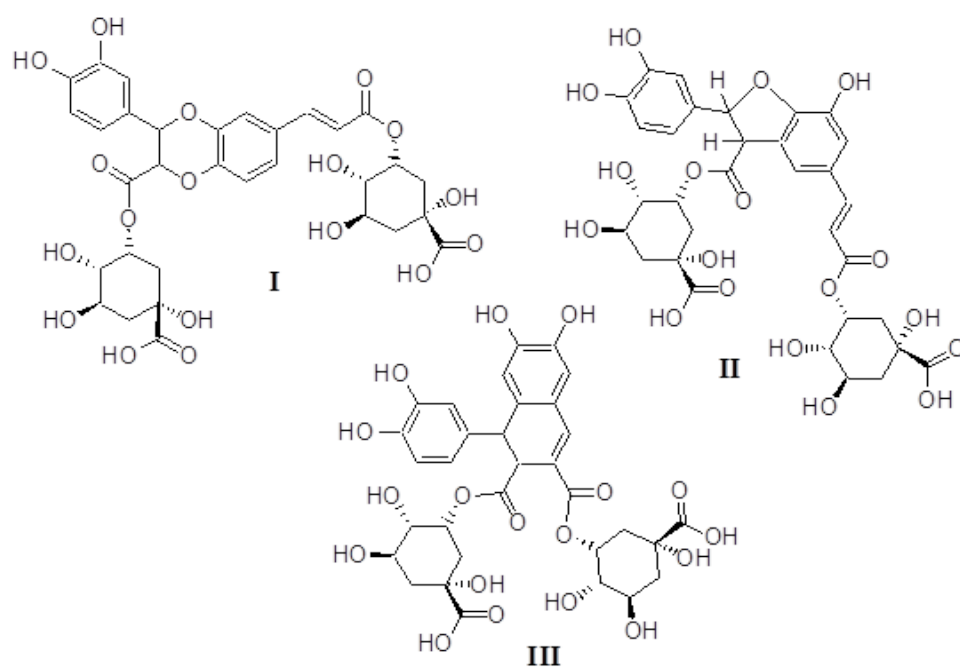


Figure 2

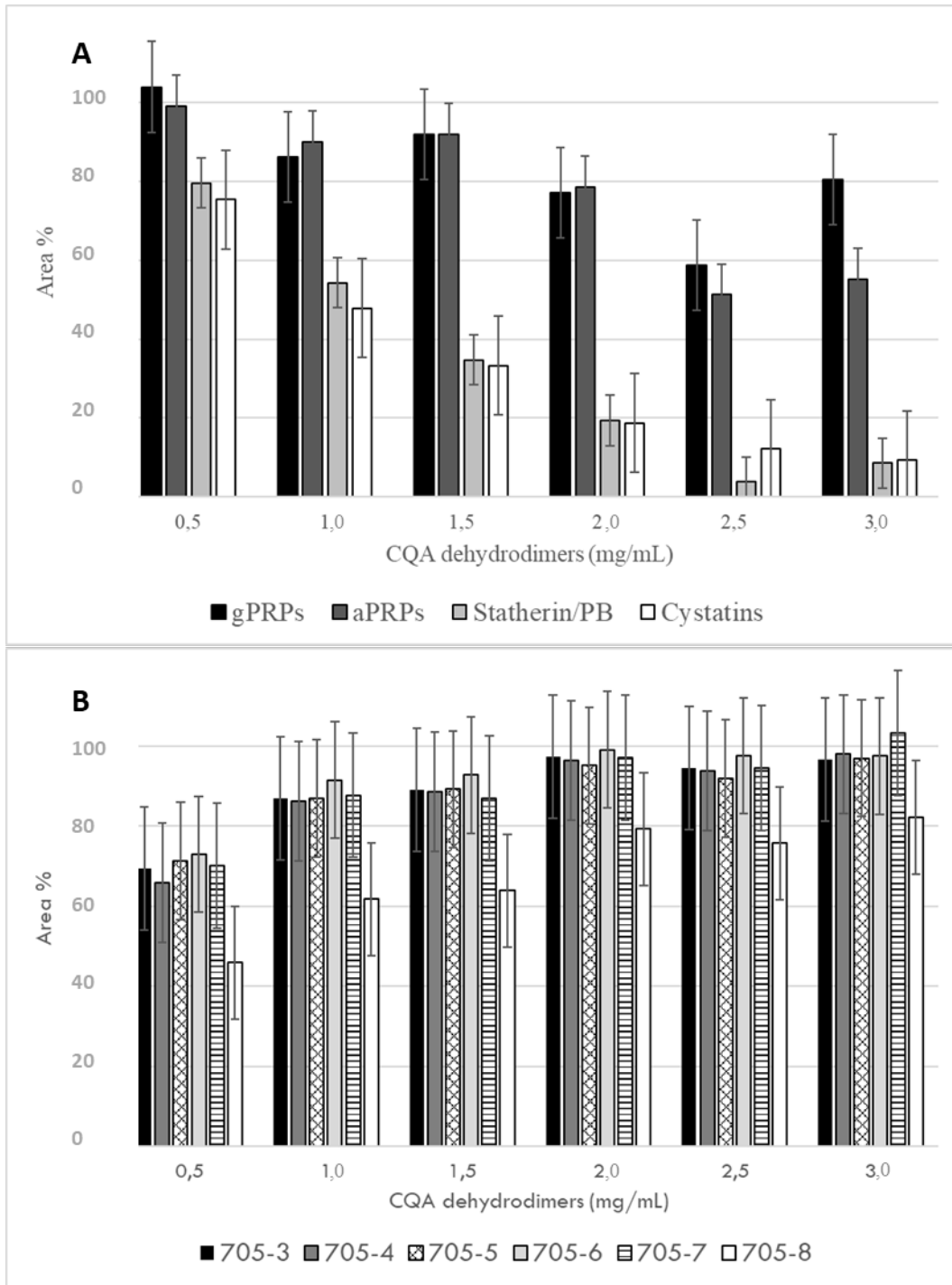


Figure 3

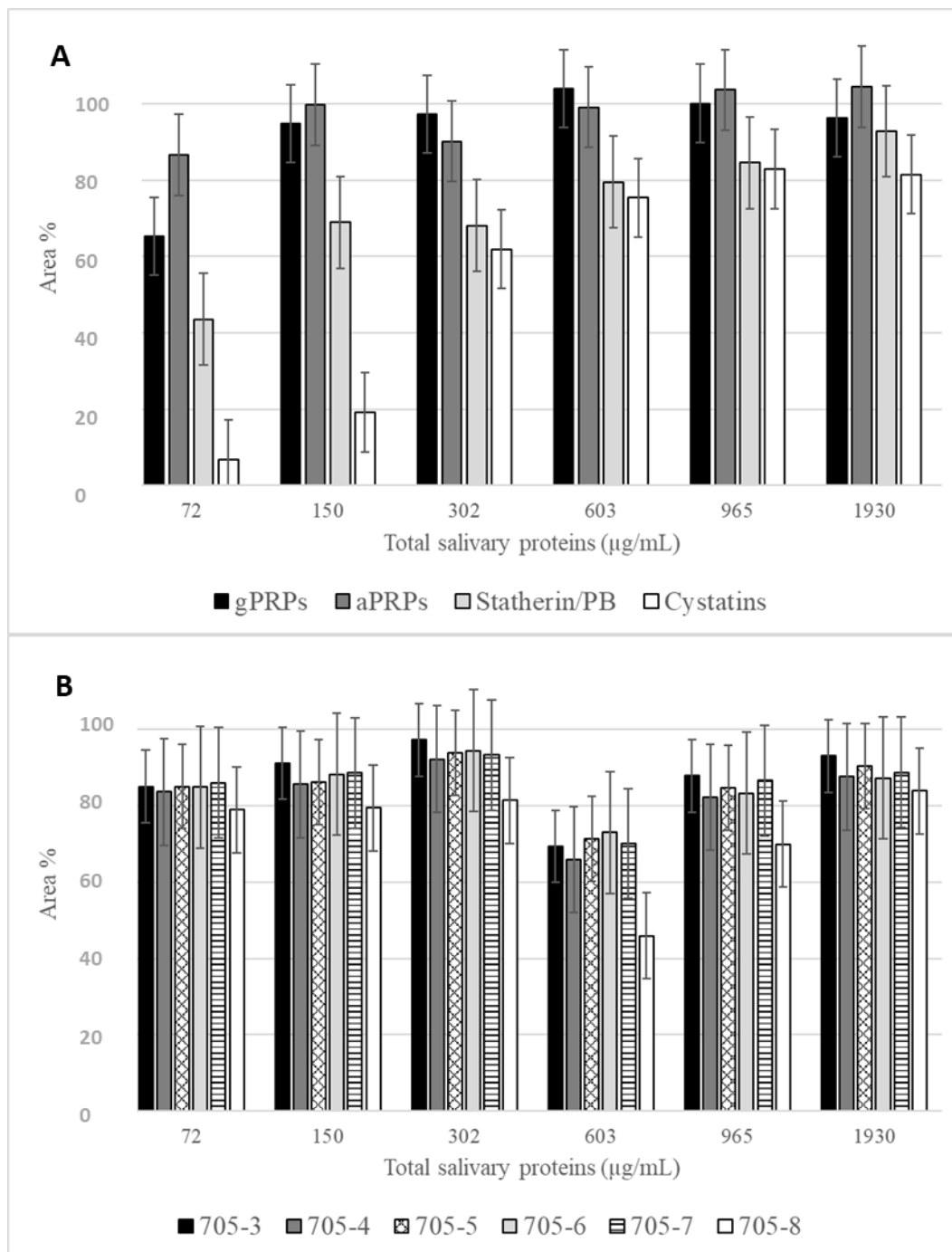


Figure 4

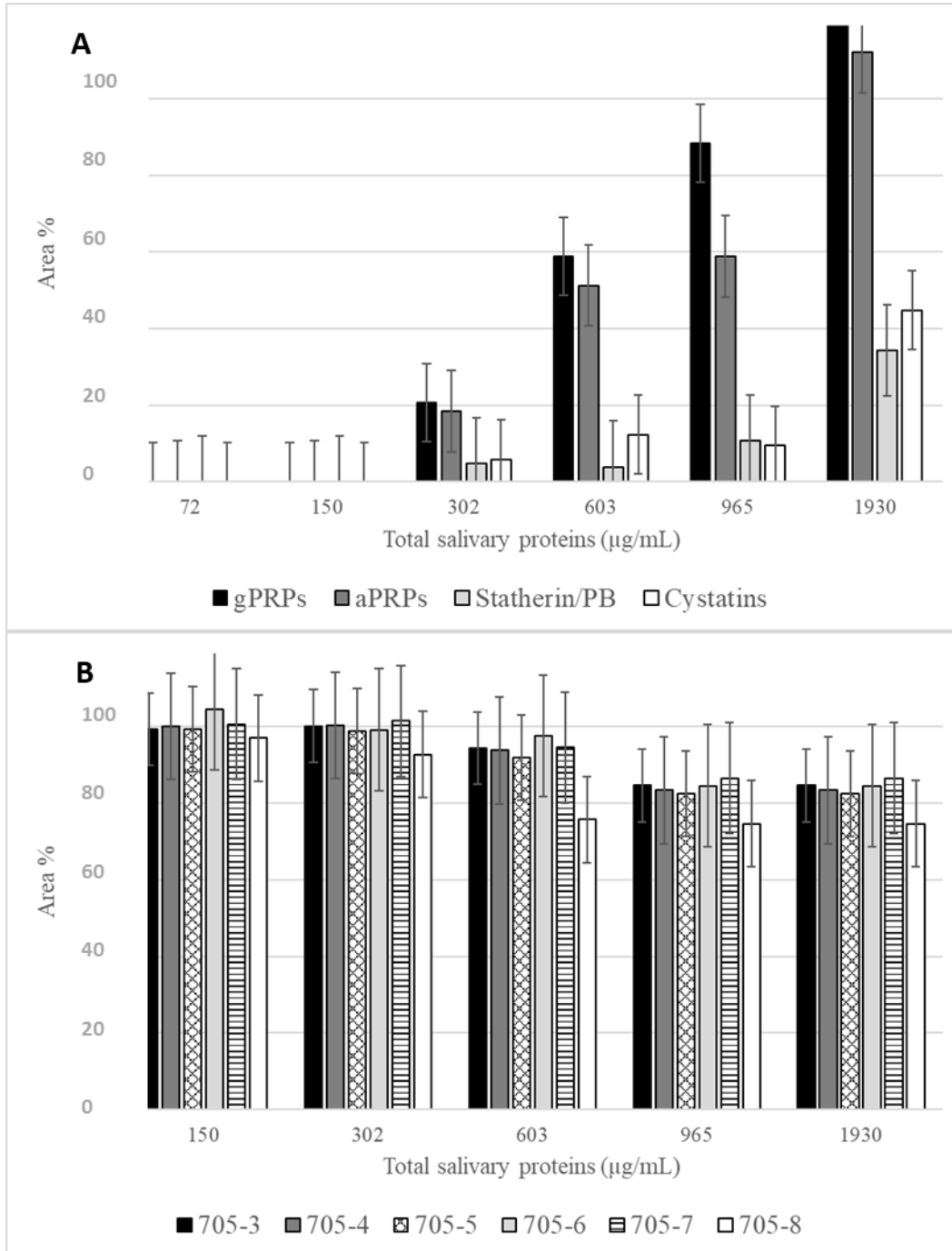


Figure 5

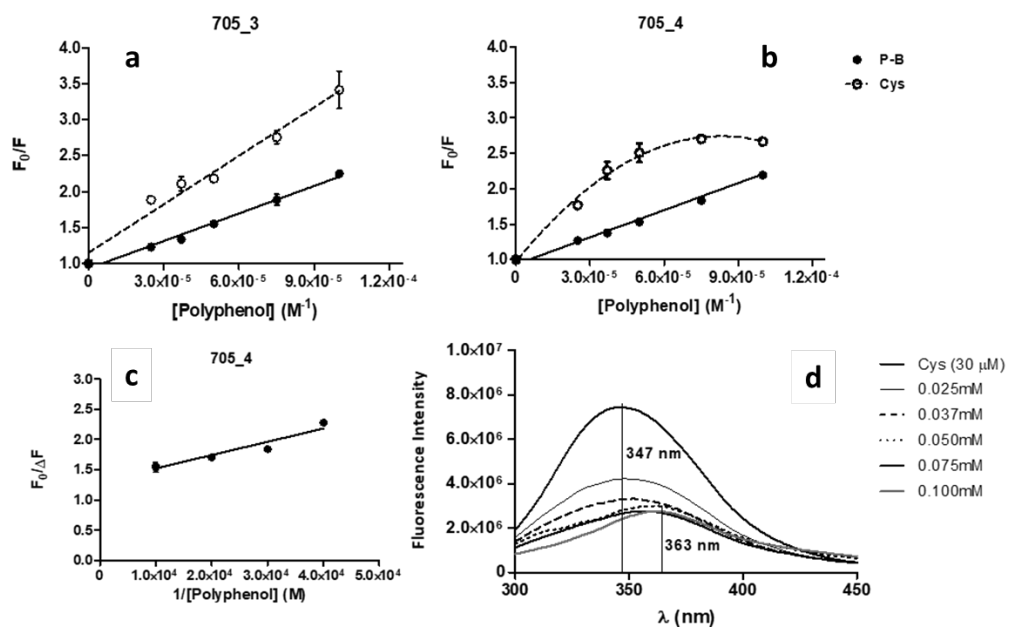


Table 1. Stern-Volmer quenching constants (K_{SV}) and biomolecular quenching constants (kq) for the interaction of P-B peptide and cystatins with the individual CQA dehydrodimers.

Polyphenol	705-3		705-4		
	Protein	P-B peptide	Cystatins	P-B peptide	Cystatins
$K_{SV} (M^{-1})$		12760 ± 723	22766 ± 1735	11855 ± 325	54054 ± 14236
$kq (10^{+12} M^{-1}s^{-1})$		5.0 ± 0.3	5.9 ± 0.4	4.6 ± 0.1	12.8 ± 0.4