Interactions between polyphenol oxidation products and salivary proteins: Specific affinity of CQA dehydrodimers with cystatins and P-B peptide

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

ABSTRACT

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

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

1 Introduction

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

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

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

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

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

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

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

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

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

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

2 Material and Methods

2.1 Chemicals

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

2.2 Synthesis and CPC fractionation and purification of CQA oxidation products

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

2.3 Human saliva collection and acidic saliva preparation

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

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

2.4 Salivary proteins purification and characterization using ESI-MS

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

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

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

2.5 Interactions between CQA oxidation products and acidic saliva

2.5.1 Preparation of salivary protein controls

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

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

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

a) Interaction of a fixed SP concentration (603 µg/mL) with increasing concentrations of OP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL).

b) Interaction of a fixed concentration of OP (0.5 or 2.5 mg/mL) with increasing SP concentrations (72, 150, 302, 603, 965 and 1930 µg/mL).

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

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

2.6 Reversed phase HPLC-UV methods

- For protein analyses

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

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

- For polyphenol analysis

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

2.7 Fluorescence quenching analysis

Purified proteins (P-B peptide and cystatins) stock solutions were prepared at 30 μM in distilled water. Polyphenols were titrated at increasing concentrations (0 to 100 μM). Tryptophan residues of P-B peptide and cystatins were used as intrinsic fluorophore [setting the excitation wavelength (λ_{ex}) at 280 nm]. After mixing the proteins and the polyphenols solutions, the samples were pipetted to the fluorimeter cell. The emission spectra (from 290 to 450 nm) were recorded in a Horiba FluoroMax-4 Spectrometer. The cell was rinsed with water after each measurement. Fluorescence intensity for the different titrations was registered at 310 nm for P-B peptide and 347 nm for cystatins, the wavelengths of maximum fluorescence. Polyphenols intrinsic fluorescence (blank measurement) was also assessed for the different concentrations and they were subtracted to the respective sample of the complexes SP-polyphenol. Additionally, the optical density of each SP-polyphenol mixture was measured in order to use polyphenol concentrations with the lowest optical 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 kq – 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 Ka – 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 μg/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 μg/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;

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

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

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

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

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

3.1.2 Fixed concentrations of oxidation products

In order to understand the previous suggested saturation effect, further experiments were made with different concentrations of total SP. In these experiments, low (0.5 mg/mL) or high (2.5 mg/mL) fixed OP concentrations were mixed with increasing concentrations of SP (72 to 1930 µg/mL).

3.1.2.1 At low concentration of CQA dehydrodimers

The results of the interactions of a fixed OP concentration (0.5 mg/mL) with increasing concentrations of SP (72 to 1930 µg/mL) are shown in Figure 3.

The highest interaction was observed with cystatins and it was particularly visible in the assay at the lowest protein concentrations (Figure 3A). Indeed, more than 80% of cystatins were removed at lowest SP concentrations (72 and 150 µg/mL). As SP concentration increased, the percentage of remaining proteins (in supernatant) was higher probably due to the high ratio of protein/OP and the 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 µg/mL). This was followed by the highest precipitation of
313 CQA dehydrodimers (with around 30% of precipitation) at the SP concentration of 603 µg/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 µg/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 µg/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 µg/mL (Figure 4A).
323 Interestingly, at low SP concentrations (150, 302 µg/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 µg/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.

3.2 Interactions between individual CQA dehydrodimers and individual salivary proteins

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

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

Regarding the influence of the protein families, the determined K_{SV} indicate that both CQA dehydrodimers have a higher interaction with cystatins than with P-B peptide (Table 1). Indeed, the K_{SV} for the interactions between 705-3 and 705-4 with cystatins were 22766 M^{-1} and 54054 M^{-1}, respectively, while the K_{SV} values for the interaction with P-B peptide were 12760 M^{-1} and 11855 M^{-1}, respectively. Additionally, the interaction with P-B peptide seems not to be selective because the K_{SV} values for the interaction with both compounds (705-3 and 705-4) were similar (12760 $^{-1}$ and 11855 M^{-1}). This is not true for cystatins which seems to have a higher interaction with 705-4 (54054 M^{-1}), suggesting some selectivity. Remarkably, this selectivity was highlighted only for 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 M^{-1}, B6 = 6500 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.

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

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

4 Conclusion

CQA is the main hydroxycinamic acid ester in apple and the preferential substrate for PPO. Oxidation of CQA results in the formation of CQA dehydrodimers as one of the main oxidation products in apple juice and cider. As far as we know, this study highlighted for the first time, the specific interaction between several families of SP and CQA dehydrodimers (oxidation products). Indeed, protein/polyphenol precipitation revealed that OP presented a stronger interaction with 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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582

583

Figures Captions

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

Figure 2: Remaining SP or OP (peak area %) after interaction of a fixed SP concentration (603 µg/mL) with increasing concentrations of OP (0.5 mg/mL to 3.0 mg/mL). A) Different families of SP (p = 0.05, dol = 14); B: the individual CQA dehydrodimers (p = 0.05, dol = 11).

Figure 3. Remaining SP or OP (peak area %) after interaction of a fixed OP concentration (0.5 mg/mL) with increasing concentrations of SP (72 to 1930 µg/mL). A) different families of SP (p = 0.05, dol = 10), B) individual CQA dehydrodimers (p = 0.05, dol = 13).

Figure 4. Remaining SP or OP (peak area %) after interaction of a fixed OP concentration (2.5 mg/mL) with increasing concentrations of SP (72 to 1930 µg/mL). A: different families of salivary proteins (p = 0.05, dol = 10) B: individual CQA dehydrodimers (p = 0.05, dol = 13).

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

Figure 1

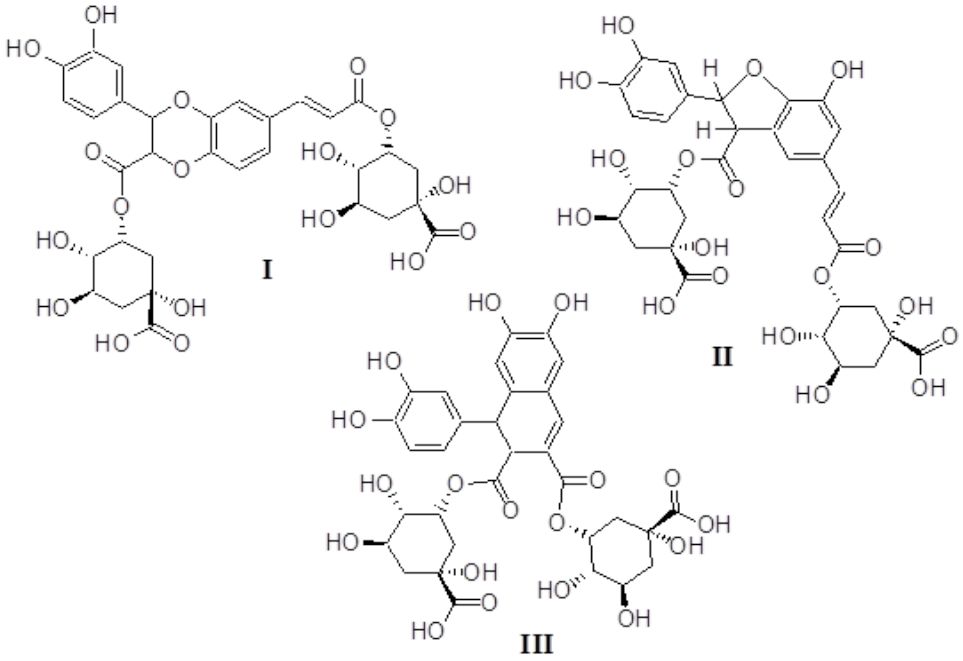

Figure 2

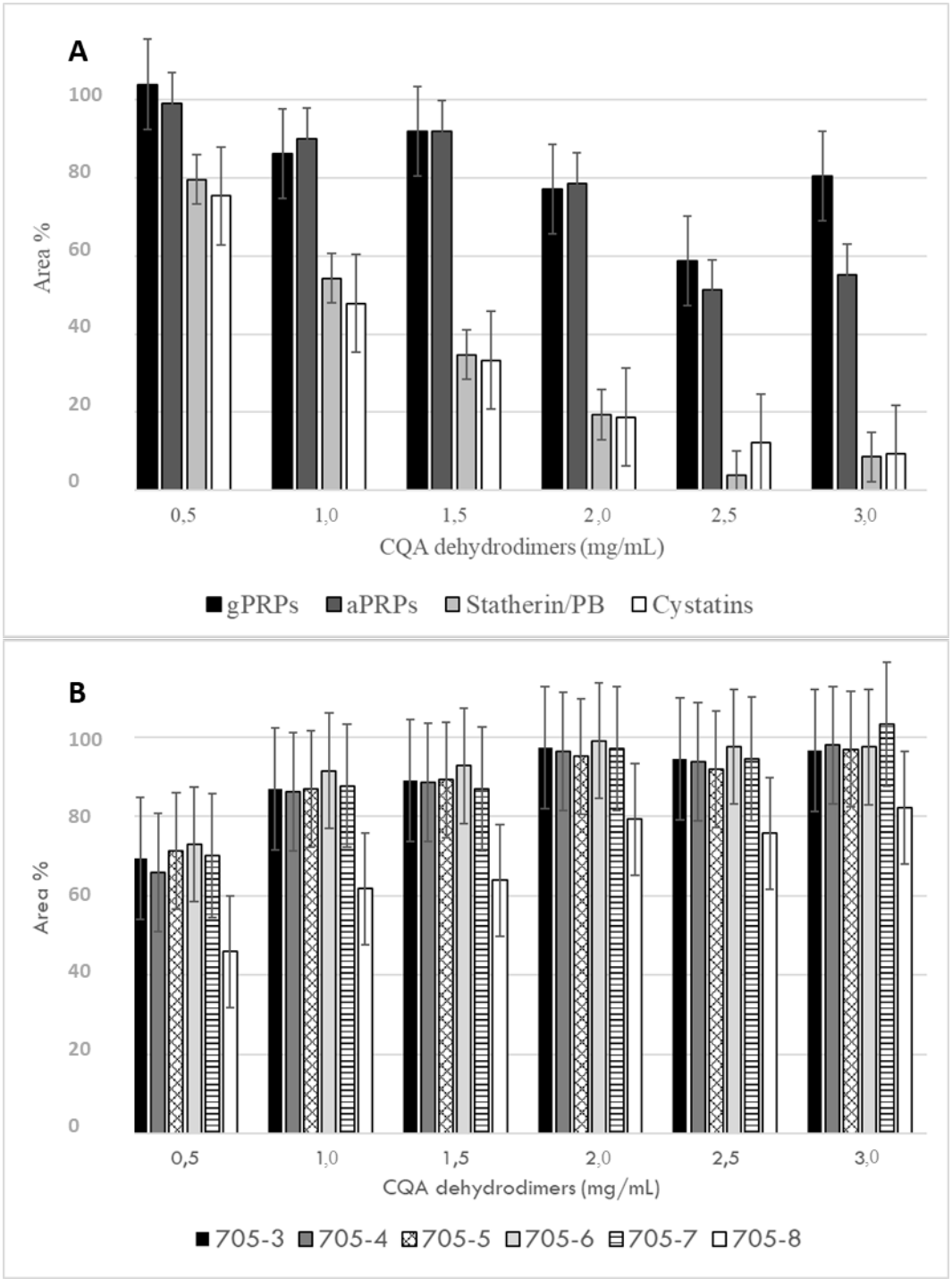
A
100
80
60
40
20
0
Area %
0,5
1,0
1,5
2,0
2,5
3,0
CQA dehydrodimers (mg/mL)
gPRPs
aPRPs
Statherin/PB
Cystatins
B
100
80
60
40
20
0
Area %
0,5
1,0
1,5
2,0
2,5
3,0
CQA dehydrodimers (mg/mL)
705-3
705-4
705-5
705-6
705-7
705-8

Figure 3

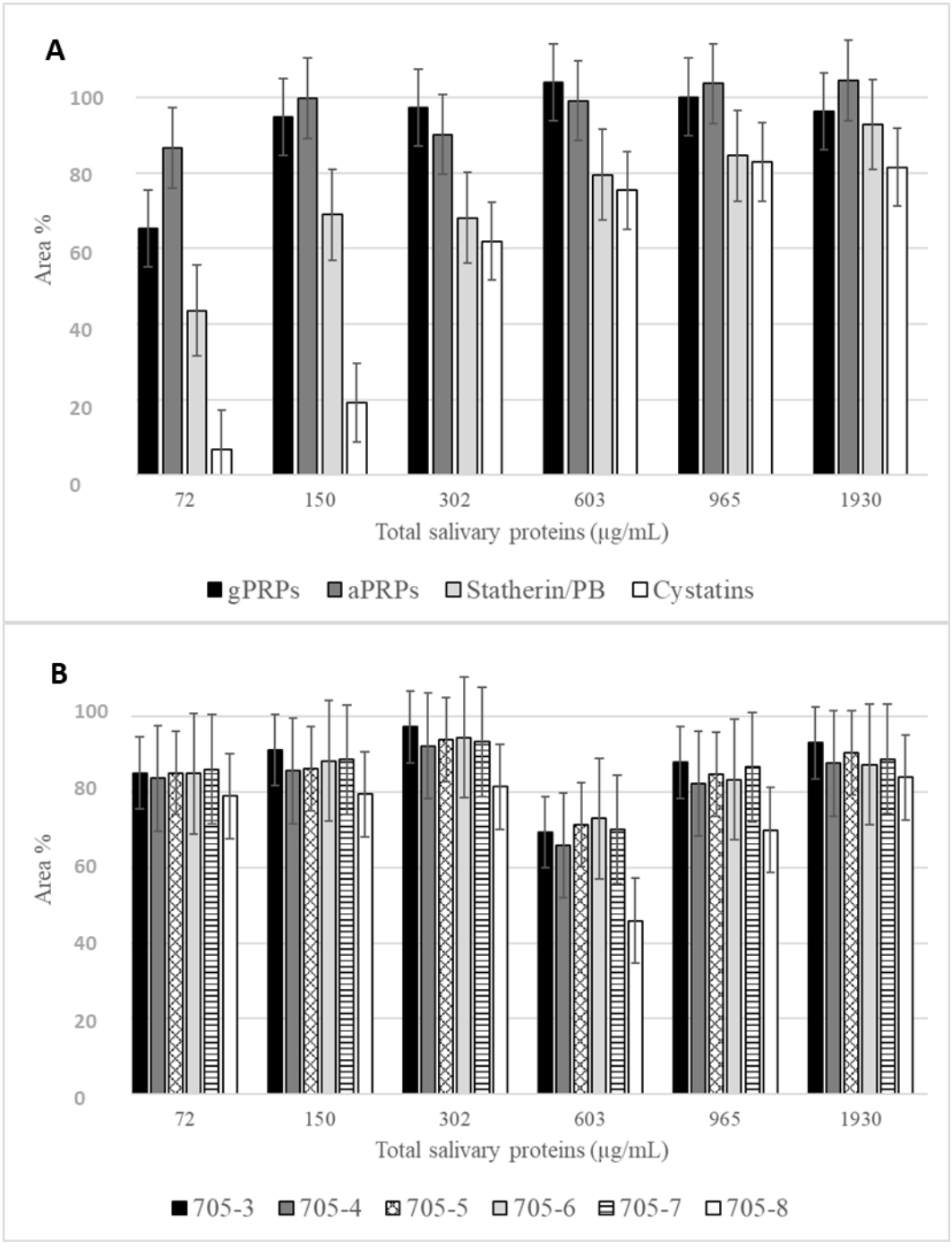
A
100
80
60
40
20
0
Area %
72
150
302
603
965
1930
Total salivary proteins (µg/mL)
gPRPs
aPRPs
Statherin/PB
Cystatins
B
100
80
60
40
20
0
Area %
72
150
302
603
965
1930
Total salivary proteins (µg/mL)
705-3
705-4
705-5
705-6
705-7
705-8

Figure 4

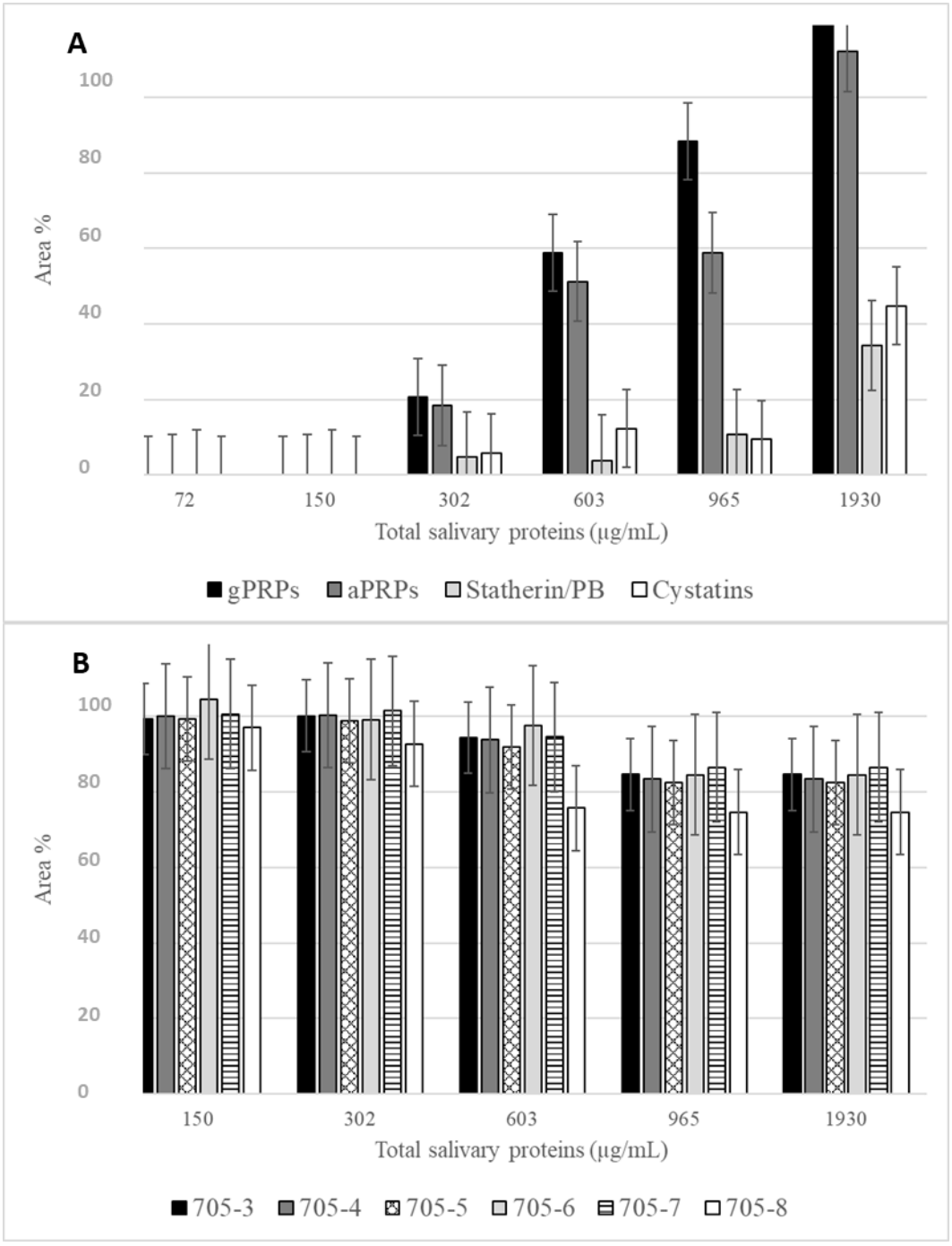
A
100
80
60
40
20
0
Area %
72
150
302
603
965
1930
Total salivary proteins (µg/mL)
gPRPs
aPRPs
Statherin/PB
Cystatins
B
100
80
60
40
20
0
Area %
150
302
603
965
1930
Total salivary proteins (µg/mL)
705-3
705-4
705-5
705-6
705-7
705-8

Figure 5

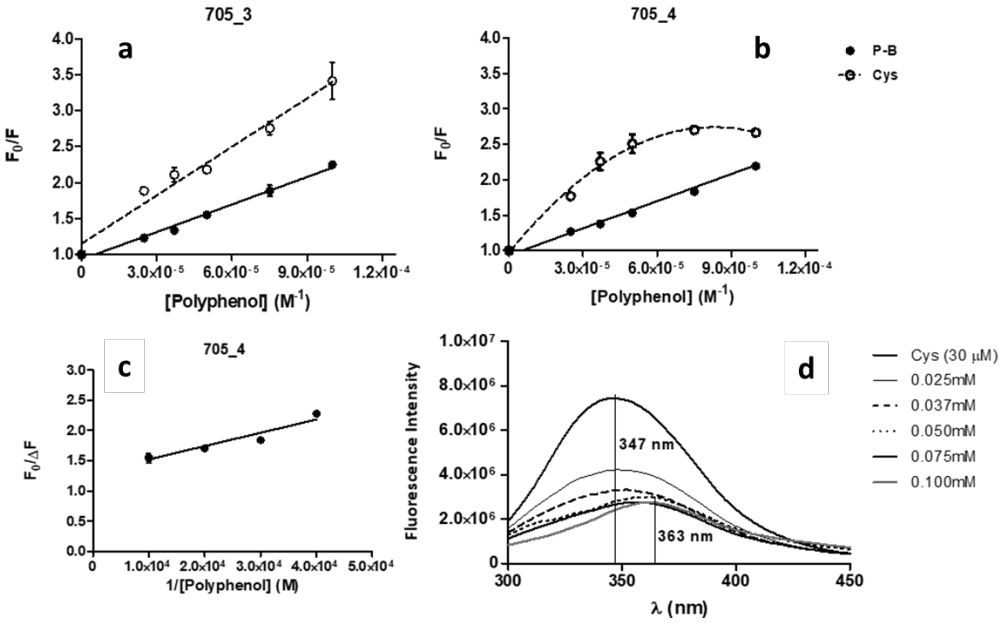
705_3
a
F0/F
[Polyphenol] (M-1)
705_4
b
P-B
Cys
F0/F
[Polyphenol] (M-1)
c
705_4
F0/ΔF
1/[Polyphenol] (M)
d
Fluorescence Intensity
347 nm
363 nm
λ (nm)
Cys (30 μM)
0.025mM
0.037mM
0.050mM
0.075mM
0.100mM

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