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ORIGINAL PAPER

Interactions between four common plant-derived phenolic acids and pectin, and its effect on antioxidant capacity

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Abstract
Phenolic acids (PAs) are molecules of vegetable origin with high antioxidant capacity (AOXC) attributed to their hydroxyl groups. Vegetable cells store PAs within vacuoles, and when disrupted through mastication or food processing, PAs can interact with other molecules like fibers, which alters their AOXC. Mango (*Mangifera indica* L.) cv Ataulfo contains PAs and fiber, particularly pectin. Previous reports indicate that PAs interact between themselves and affect each other's AOXC. The present work analyzed the interactions between the main PAs from mango cv Ataulfo and pectin, and how pectin affects their AOXC. We used UV–Vis spectrophotometry, fluorescence spectrophotometry and AOXC assays to study how pectin influences gallic, protocatechuic, chlorogenic and vanillic acid, individually, and in combinations of two, three and four PAs. Results showed that gallic acid was the most affected by the presence of pectin, which was related to its number and position of hydroxyl groups. The effect of pectin was not as obvious when three or four PAs were combined, suggesting that PAs preferentially interact among themselves, likely through hydrogen bonds. Pectin exerted mostly synergistic effects on AOXC values when added to one, two and four combined PAs; antagonistic effects were recorded when pectin was added to three combined PAs. The effect of pectin on AOXC was more noticeable when analyzed by the ORAC assay, and less when analyzed by the DPPH assay. Basic knowledge of the interactions between PAs and pectin is of great importance, since they are normally consumed alongside each other.

Keywords Mango · Phenolic acids · Spectrophotometry · Antioxidant capacity · Interactions

Introduction

Mango (*Mangifera indica* L.) cv Ataulfo is a tropical fruit grown in Southwestern Mexico, where it is commercially cultivated for local consumption and international export; it owes its popularity to the desirable organoleptic qualities of its edible portion. Mango pulp is a source of fiber, mainly pectin, and phenolic acids (PAs), whose consumption is related to beneficial health effects, attributed in part to their high antioxidant capacity (AOXC). Reports indicate that mango cv Ataulfo pulp has the highest AOXC values, as compared to other mango cultivars [1]. Previous experiments revealed that this high AOXC is due to the presence of four main PAs: gallic acid (highest contribution), chlorogenic acid, vanillic acid and protocatechuic acid, as well as their combined activities [2]. Experiments that focus on the interactions of these PAs are of great interest to the study of mango, and are equally relevant to other products, because they are also abundant in other fruits and vegetables.

When AOXC of the previously stated PAs was analyzed in different combinations, most showed synergistic effects, except for vanillic acid, which favored antagonistic effects [3]. PAs can also interact with other components of the food matrix when vegetable matter is disrupted by food processing, cooking, mastication, etc., or with host macromolecules

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after ingestion, resulting in changes to their AOXC, as compared to in vitro values where other molecules are not present. For example, pectin and other polysaccharides have been shown to bind chlorogenic acid, ferulic acid and caffeic acid in other foodstuffs [4, 5], suggesting that cell wall components have affinity for PAs and can form various interactions. Also, the AOXC of flavonoids can be masked when interacting with plasma proteins, which results in a lower than expected AOXC [6]. Others have shown that PAs found in mango peel (gallic acid, protocatechuic acid and syringic acid) interact strongly with cell wall polysaccharides, and are the major bound PAs in Raspuri and Badami mango cultivars [7]. Since PAs can interact with several compounds, their in vitro AOXC can be affected depending on the molecules present and if they interact through hydrogen bonds or hydrophobic interactions, which depends on the molecular structure of both. Additionally, different interactions will have particular impacts on the assay used to quantify AOXC, because the mechanism of action of some assays is based on hydrogen atom transfer, some on electron transfer and some on a combination of both [8]. Interactions between PAs and pectin can potentially modify the AOXC and bioactivity of PAs in living cells; PAs and pectin are both present in mango cv Ataulfo [9], and are also ubiquitous in other foods of vegetable origin. Because detailed information on the interactions between pectin and PAs is lacking, the main objective of the present work was to study the in vitro interactions that take place between them through spectroscopic methods, and the impact on AOXC as determined through different assays. We studied the effect of increasing pectin concentrations on commercial standards of gallic acid, protocatechuic acid, chlorogenic acid and vanillic acid, individually and in combinations of two, three and all four PAs. Comprehensive knowledge of these interactions will serve as basis for future in vitro or in vivo experiments that involve AOXC, bioavailability, bioaccessibility, bioactivity, pharmacokinetics, etc. of the PAs present in mango pulp and other plant-derived foods which contain them.

Materials and methods

Chemicals

Gallic acid (3,4,5-trihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), chlorogenic acid (3-*O*-caffeoylquinic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), pectin, ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)], AAPH [2,2′-azobis(2-methylpropionamidine) dihydrochloride], fluorescein, DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ [2,4,6-Tris(2-pyridyl)-s-triazine], sodium persulfate and Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

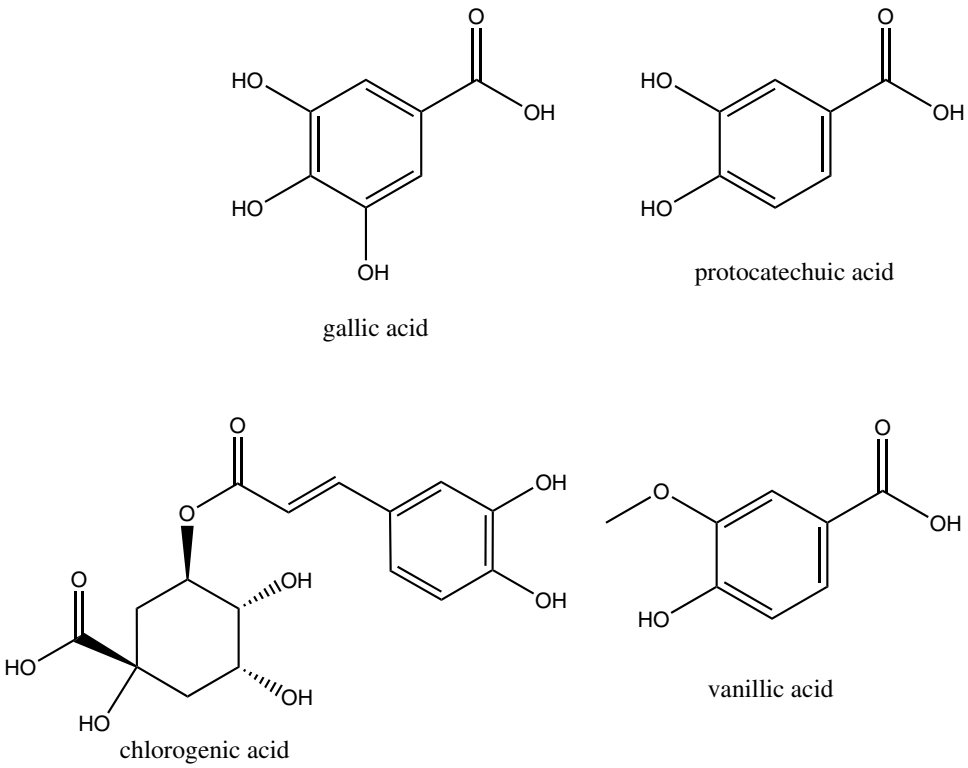


Fig. 1 Molecular structures of the analyzed PAs. Gallic acid has three free hydroxyl groups, protocatechuic acid has two, chlorogenic acid has five and vanillic acid has one. Intermolecular interactions and AOXC is related to the number and position of free hydroxyl groups

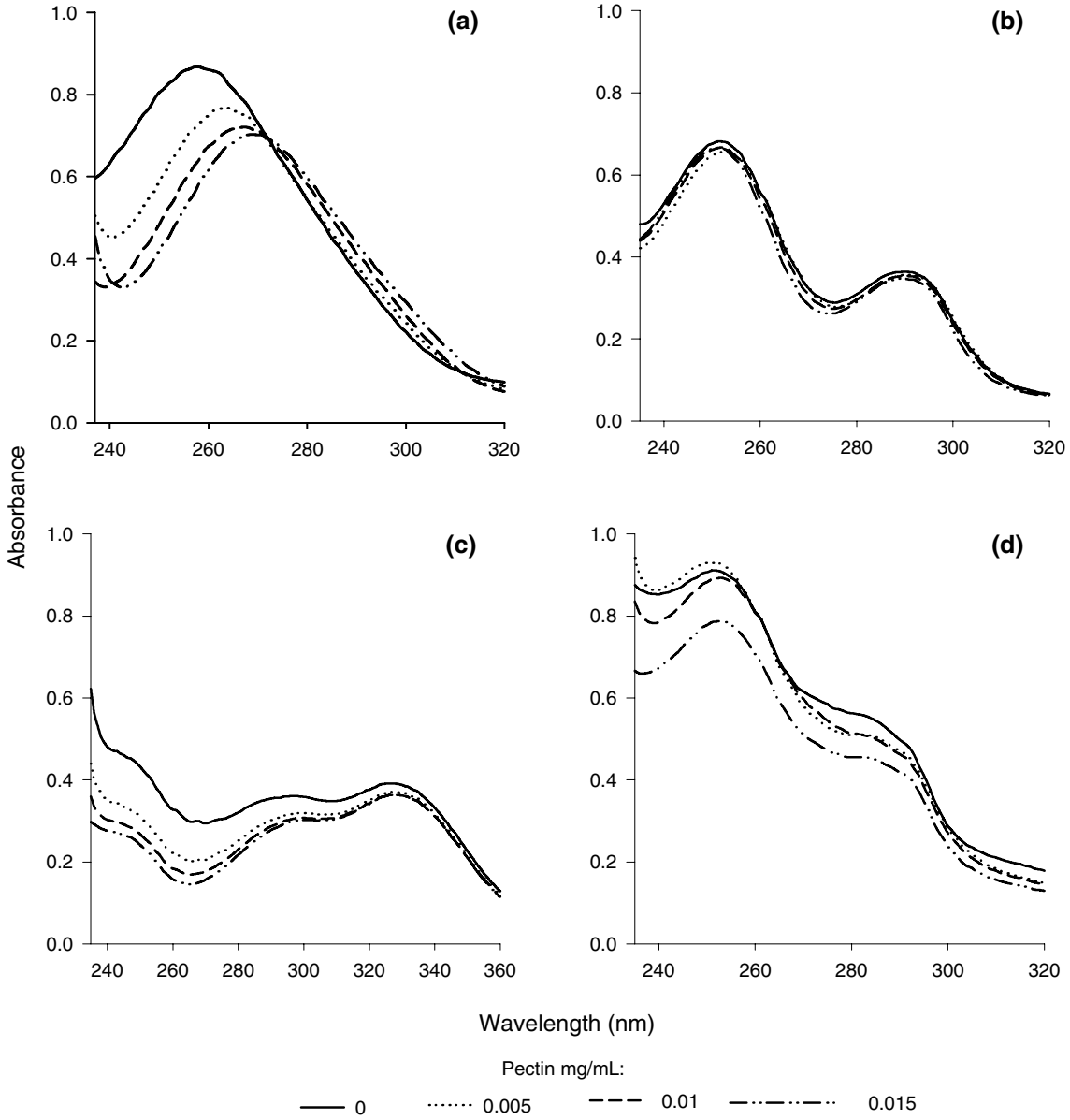


Fig. 2 UV–Vis spectra of individual PAs with increasing pectin concentrations. **a** Gallic acid, **b** protocatechuic acid, **c** chlorogenic acid, and **d** vanillic acid

UV–Vis and fluorescence spectrophotometry

Experiments were performed on individual PAs and their combinations in pairs, threes and all four together, with and without pectin. Spectrophotometric analyses were performed with a total combined PA concentration of 60 µM, while pectin concentration was increased from 0, 0.005, 0.010 to 0.015 mg/mL. For AOXC assays, pectin concentration was fixed at 0.015 mg/mL. The solvent used was methanol:water (8:2), pH was 7.0, and temperature was 26 ± 1 °C for all assays. Figure 1 shows the molecular structure of the PAs discussed herein.

PAs, pectin and their combinations were read from 200 to 800 nm in a UV–Vis spectrophotometer (Varian Cary 50 UV–Vis, Varian Australia, Mulgrave, Australia) in a 1 mL quartz cell.

Fluorescence emission was read up to 600 nm in a 1 mL quartz cell in a fluorometer (PicoMaster TCSPC Lifetime Spectrofluorometer, Photon Technology International, London, Ontario, Canada). Excitation wavelength (λ_{ex}) was 320 nm for chlorogenic acid, 296 nm for gallic acid, 280 nm for protocatechuic acid and 290 nm for vanillic acid. λ_{ex} of 290 nm was used when combining two or more PAs.

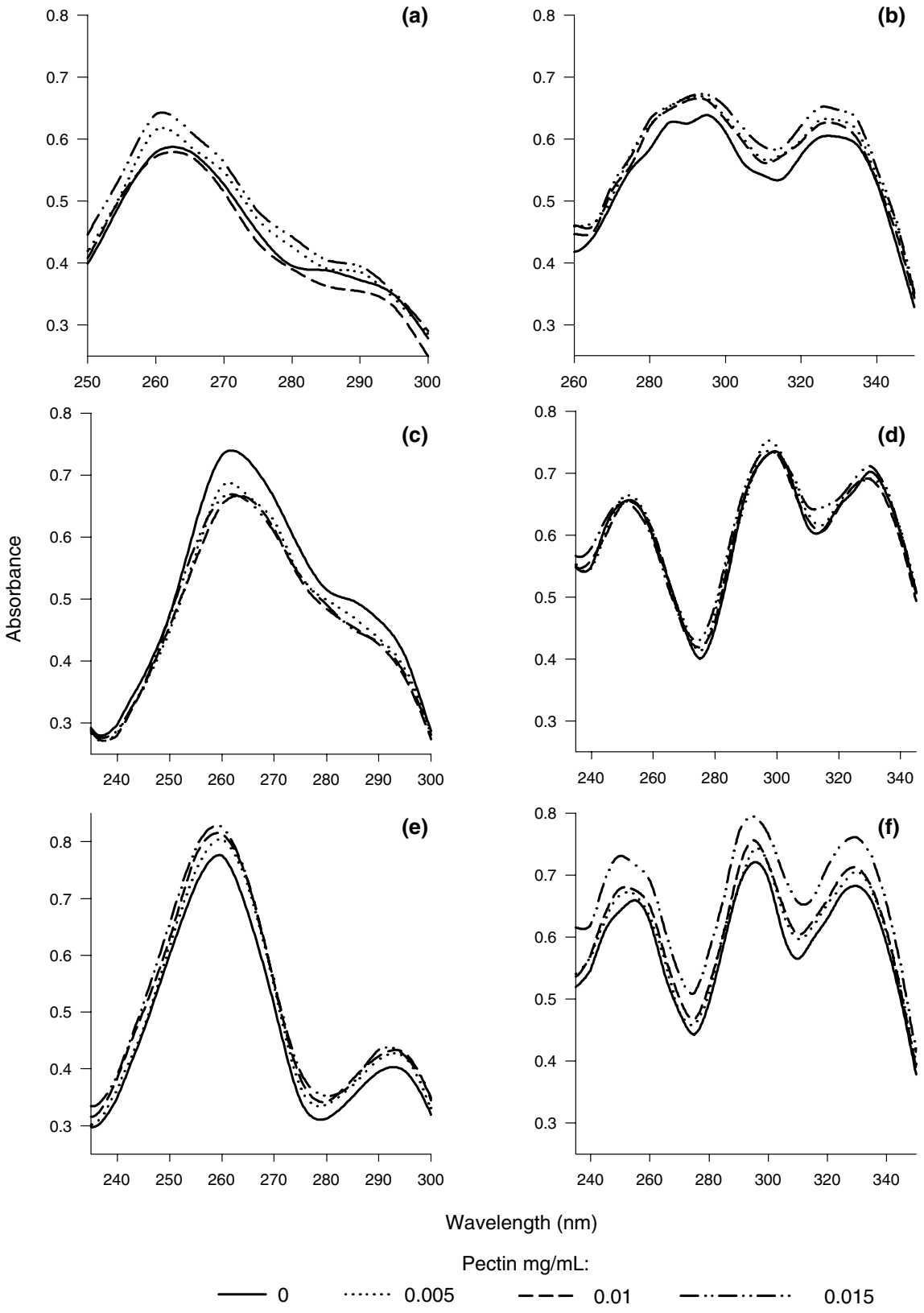


Fig. 3 UV–Vis spectra of two combined PAs with increasing pectin concentrations. **a** Gallic–protocatechuic, **b** gallic–chlorogenic, **c** gallic–vanillic, **d** protocatechuic–chlorogenic, **e** protocatechuic–vanillic and **f** chlorogenic–vanillic

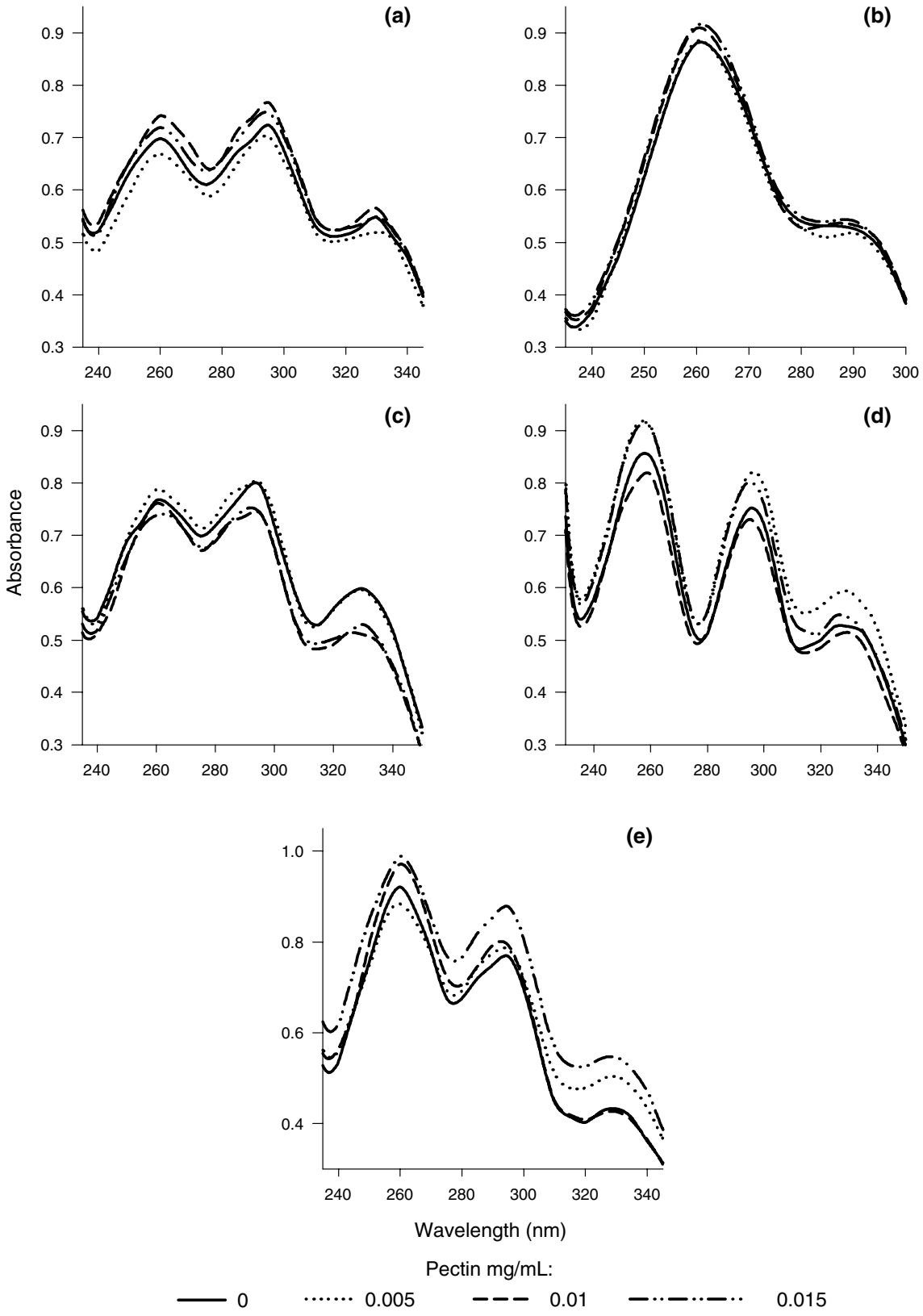


Fig. 4 UV–Vis spectra of three and four combined PAs with increasing pectin concentrations. **a** Gallic–protocatechuic–chlorogenic, **b** gallic–protocatechuic–vanillic, **c** gallic–chlorogenic–vanillic, **d** protocatechuic–chlorogenic–vanillic, **e** gallic–protocatechuic–chlorogenic–vanillic

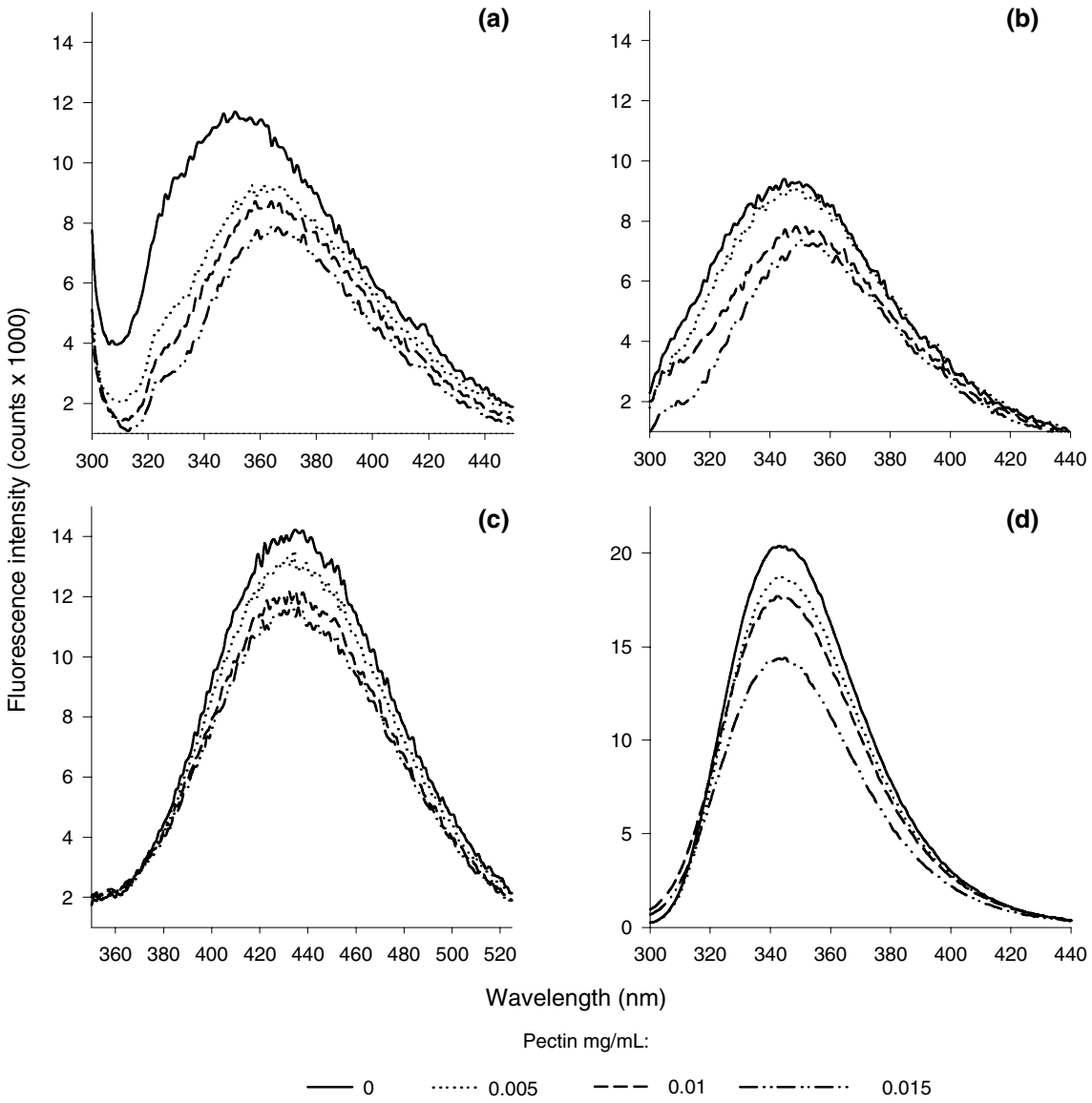


Fig. 5 Fluorescence emission spectra of individual PAs with increasing pectin concentrations. **a** Gallic acid, **b** protocatechuic acid, **c** chlorogenic acid, and **d** vanillic acid

Methanol:water (8:2) was used as instrument blank in both spectrophotometric assays.

Stern–Volmer fluorescence quenching constant (K_{SV})

Stern–Volmer constants (K_{SV}) were calculated from the fluorescence emission data by plotting F_0/F_Q against [Q], where F_0 and F_Q are the fluorescence emission in the absence and presence of a fluorescence quencher (pectin), respectively, and [Q] is the concentration of pectin (mg/mL). The slope of said plots is equal to K_{SV}, according to the Stern–Volmer equation (Eq. 1) [10]. K_{SV} values were not calculated when increasing pectin concentrations did not result in fluorescence quenching.

$$F_0/F_Q = 1 + K_{SV}[Q] \quad (1)$$

Antioxidant capacity

Oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC) and DPPH assays were used to determine AOXC, using Trolox as a standard. All results are expressed as μM of Trolox equivalents (μM TE). These particular AOXC assays were used because of their different mechanisms of action: hydrogen atom transfer (HAT) for ORAC, single electron transfer (SET) for FRAP and a mixed HAT/SET mechanism for TEAC and DPPH [8].

Table 1 Calculated Stern–Volmer quenching constants (K_{SV}) for phenolic acids and their combinations with pectin

PA	K_{SV} (mL/mg)	Correlation coefficient
G	45.93	0.9875
P	27.04	0.9796
C	16.71	0.9985
V	26.38	0.9462
GP	19.88	0.9857
GC	–	–
GV	–	–
PC	23.93	0.9399
PV	9.58	0.9622
CV	2.30	0.3533
GPC	15.69	0.9700
GPV	16.79	0.9465
GCV	24.33	0.9650
PCV	–	–
GPCV	–	–

A dash (–) indicates the K_{SV} was not calculated because the Stern–Volmer plot was not linear

G gallic acid, *P* protocatechuic acid, *C* chlorogenic acid, *V* vanillic acid

The ORAC assay measures the ability of antioxidants to inhibit the loss of fluorescence of fluorescein, caused by the AAPH radical. 150 µL of fluorescein (10 mM), 25 µL of sample and 25 µL of AAPH radical (240 mM) were added to a microplate well in the order mentioned. Readings were taken for 90 min at 37 °C with $\lambda_{ex} = 485$ nm and $\lambda_{em} = 520$ nm in a microplate reader (FLUOstar Omega, BMG Labtech, Cary, North Carolina, USA) [11].

The FRAP assay measures the ability of antioxidants to reduce the ferric:TPTZ (Fe^{3+}:TPTZ) complex to the ferrous form (Fe^{2+}:TPTZ), which produces a change in coloration that can be measured at 593 nm. 20 µL of the sample were mixed with 280 µL of the FRAP reagent (25 mL of acetate buffer 300 mM pH 3.6, mixed with 2.5 mL of 20 mM ferric chloride and 2.5 mL of 10 mM TPTZ in 40 mM HCl), kept in the dark for 30 min, and read at 593 nm in a microplate reader (FLUOstar Omega) [12].

The TEAC assay is based on the ability of antioxidants to scavenge the ABTS radical ($ABTS^{\cdot+}$). The $ABTS^{\cdot+}$ radical was prepared by dissolving it in a sodium persulfate solution, incubated for 16 h at room temperature in total darkness, and its absorbance adjusted to 0.70 ± 0.02 at 754 nm. 5 µL of the sample were mixed with 245 µL of the previously prepared $ABTS^{\cdot+}$ radical for 5 min, and its absorbance was measured at 754 nm in a microplate reader (FLUOstar Omega) [13].

The DPPH assay is based on the reduction of the stable DPPH radical (·DPPH) exerted by antioxidant compounds, which also changes its color, this can be measured by a decrease in absorbance at 518 nm. 20 µL of the sample were added to 280 µL of a 1 mM DPPH solution. The mixture was incubated for 30 min under darkness, and the absorbance was measured at 518 nm in a microplate reader (FLUOstar Omega) [14].

Calculated LogP

Theoretical logarithm of partition coefficient (LogP) of all PAs was calculated using the Molinspiration Software [15]. LogP describes a compound's solubility: increasing LogP values indicate hydrophobicity, whereas decreasing LogP values indicate hydrophilicity. LogP values are related to bioavailability, metabolism, interactions, etc. of a given molecule, and is derived from molecular structure.

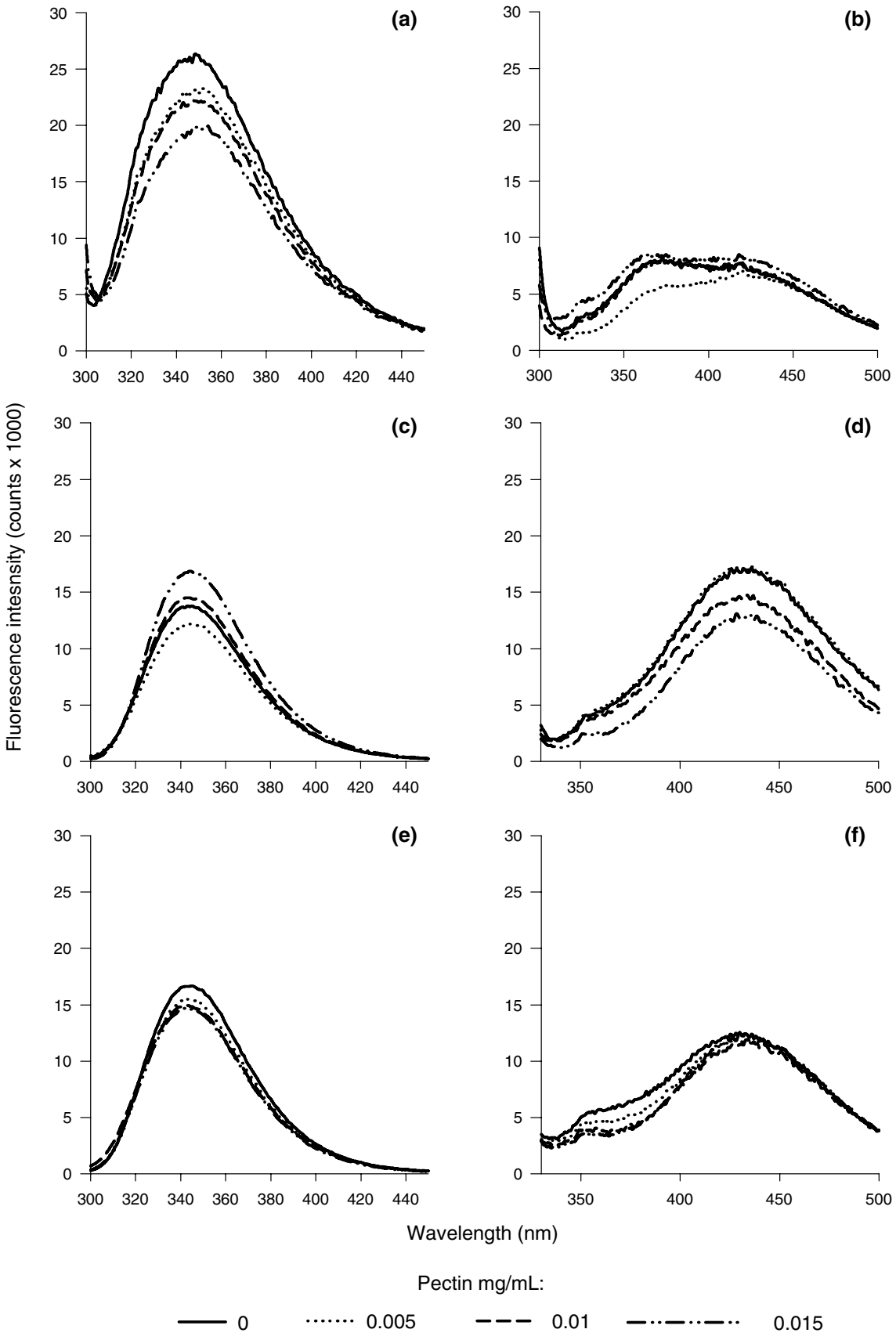
Statistical analyses

AOXC assays were performed in triplicate and presented as mean ± standard deviation. Results were analyzed by one-way ANOVAs when comparing one, two, three or four PAs with or without pectin, or by Student's t-test when analyzing the effect of pectin on individual PAs or their combinations. Results were considered significant when $p < 0.05$. All statistical analyses were performed in the Minitab (version 17) statistical software (Minitab, State College, Pennsylvania, USA).

Results and discussion

Effect of pectin on the UV–Vis spectra of phenolic acids

Figure 2 shows the UV–Vis spectra of individual PAs with increasing pectin concentrations. The presence of pectin caused a hypochromic effect on the spectrum of gallic acid, and a redshift of its λ_{max}. Similar results were observed on the spectrum of chlorogenic acid, particularly at $\lambda < 280$ nm, and vanillic acid with the highest pectin concentration. Pectin had a negligible effect on the spectrum of protocatechuic acid. Since three out of four compounds presented a hypochromic effect, it is likely that they are interacting with pectin. The noticeable redshift on the spectrum of gallic acid also suggests that its interactions with pectin were stronger than those of the other compounds. Interestingly, the molecular structure of gallic acid is similar to the structure of protocatechuic acid, differing only in the number of hydroxyl groups; protocatechuic acid has only two hydroxyl groups, gallic acid has three. Considering that protocatechuic acid was minimally affected by pectin, the presence of a hydroxyl


(a)
(b)
(c)
(d)
(e)
(f)
Fluorescence intesnsity (counts x 1000)
Wavelength (nm)
Pectin mg/mL:
0
0.005
0.01
0.015

◂**Fig. 6** Fluorescence emission spectra of two combined PAs with increasing pectin concentrations. **a** Gallic–protocatechuic, **b** gallic–chlorogenic, **c** gallic–vanillic, **d** protocatechuic–chlorogenic, **e** protocatechuic–vanillic and **f** chlorogenic–vanillic

group at position five in gallic acid strongly promotes its interaction with pectin.

Calculated LogP values for vanillic, protocatechuic, gallic and chlorogenic acid were 1.19, 0.88, 0.59 and − 0.45 respectively. Considering that low LogP values are characteristic of polar compounds, the molecular structures of the PAs and the recorded changes to their spectra, hydrogen bonds are the most likely interactions between pectin and PAs. Other authors have proposed that hydroxyl groups of various phenolic compounds from grapes may form hydrogen bonds with matrix polysaccharides, which is in accordance with our results [16]. Interactions of various phenolics with the food matrix have also been reported in cereals [4].

Figure 3 shows the UV–Vis spectra of two combined PAs with increasing pectin concentrations. Four out of six combinations presented a hyperchromic effect (gallic–protocatechuic acid, gallic–chlorogenic acid, protocatechuic–vanillic acid and chlorogenic–vanillic acid), one combination presented a hypochromic effect (gallic–vanillic acid) and one combination was unaffected by the addition of pectin (protocatechuic–chlorogenic acid). Because pectin caused mostly hyperchromic effects, PAs may be interacting among themselves, which decreases the surface area available to absorb photons, but when pectin is added, these interactions are disrupted and the absorbance increases. The likely interactions between PAs are hydrogen bonds. The gallic–vanillic acid combination had an opposite effect, suggesting pectin does not disrupt the previously-formed hydrogen bonds that can take place between both PAs. The null effect of pectin on the protocatechuic–chlorogenic combination may be mediated by protocatechuic acid, since it was the only PA that was not affected by pectin when individually analyzed.

Figure 4a–e show the UV–Vis spectra of the combination of three and four PAs, respectively, with increasing pectin concentrations. No obvious trend is noticeable when three PAs are combined, suggesting that the effect of pectin could not be reliably detected by UV–Vis. A hyperchromic effect is apparent when all four PAs are combined, particularly at 260, 290 and 330 nm. The hyperchromic effect seen when all four PAs were combined suggests that pectin disrupts the bonds that could be forming between PAs, similarly to the recorded effect of two PAs.

Effect of pectin on the fluorescence emission spectra of phenolic acids

Figure 5 shows the fluorescence emission spectra of individual PAs with increasing pectin concentrations. The effect on the spectra of all PAs is fluorescence quenching, which indicates that they are interacting with pectin, and similar to the UV–Vis spectra, the λ_{max} of gallic acid was redshifted.

Table 1 lists the K_{SV} values for all PAs and their combinations with increasing pectin concentrations. K_{SV} value of gallic acid was the highest, indicating that its interaction with pectin was the strongest, followed by protocatechuic acid, vanillic acid and chlorogenic acid. This is similar to the effect that various polyphenols exert on the fluorescence of bovine serum albumin, where higher K_{SV} values are indicative of higher affinities [17]. Considering the molecular structure of the PAs (Fig. 1), the hydroxyl group at position five of gallic acid is important when interacting with pectin, because in its absence (protocatechuic acid) their interaction is greatly reduced. This is similar to the trend recorded when UV–Vis was used, and once again shows that some changes may not be detected by one method and require the aid of a second one.

Figure 6 shows the fluorescence emission spectra of two combined PAs with increasing pectin concentrations. Four out of six combinations presented fluorescence quenching in the presence of pectin (gallic–protocatechuic acid, protocatechuic–chlorogenic acid, protocatechuic–vanillic acid and chlorogenic–vanillic acid), while the remaining combinations (gallic–chlorogenic acid and gallic–vanillic acid) showed minimal or mixed effects. These results suggest that PAs interact closely with pectin, similarly to when individually analyzed, which results in fluorescence quenching. K_{SV} values (Table 1) of protocatechuic–chlorogenic acid and gallic–protocatechuic acid combinations show that they were the most affected by pectin, while K_{SV} and coefficient of correlation of chlorogenic–vanillic acid combination are both low, suggesting that the effect of pectin was minimal. Because pectin did not exert fluorescence quenching on the gallic–chlorogenic acid and gallic–vanillic acid combinations, K_{SV} values were not calculated, which suggests stronger interactions between these PAs, as compared to their interaction with pectin, and are therefore less affected by it.

Figure 7a–e show the fluorescence emission spectra of three and four combined PAs, respectively, with increasing pectin concentrations. Three combinations showed a concentration-dependent fluorescence quenching (gallic–protocatechuic–chlorogenic acid, gallic–protocatechuic–vanillic acid and gallic–chlorogenic–vanillic acid), while a minimal increase in fluorescence occurs in the protocatechuic–chlorogenic–vanillic acid combination. Changes in fluorescence are negligible when all four PAs were combined. K_{SV} values (Table 1) show that the gallic–chlorogenic–vanillic acid combination was affected most by pectin. The null effect of pectin on the protocatechuic–chlorogenic–vanillic acid combination suggests that

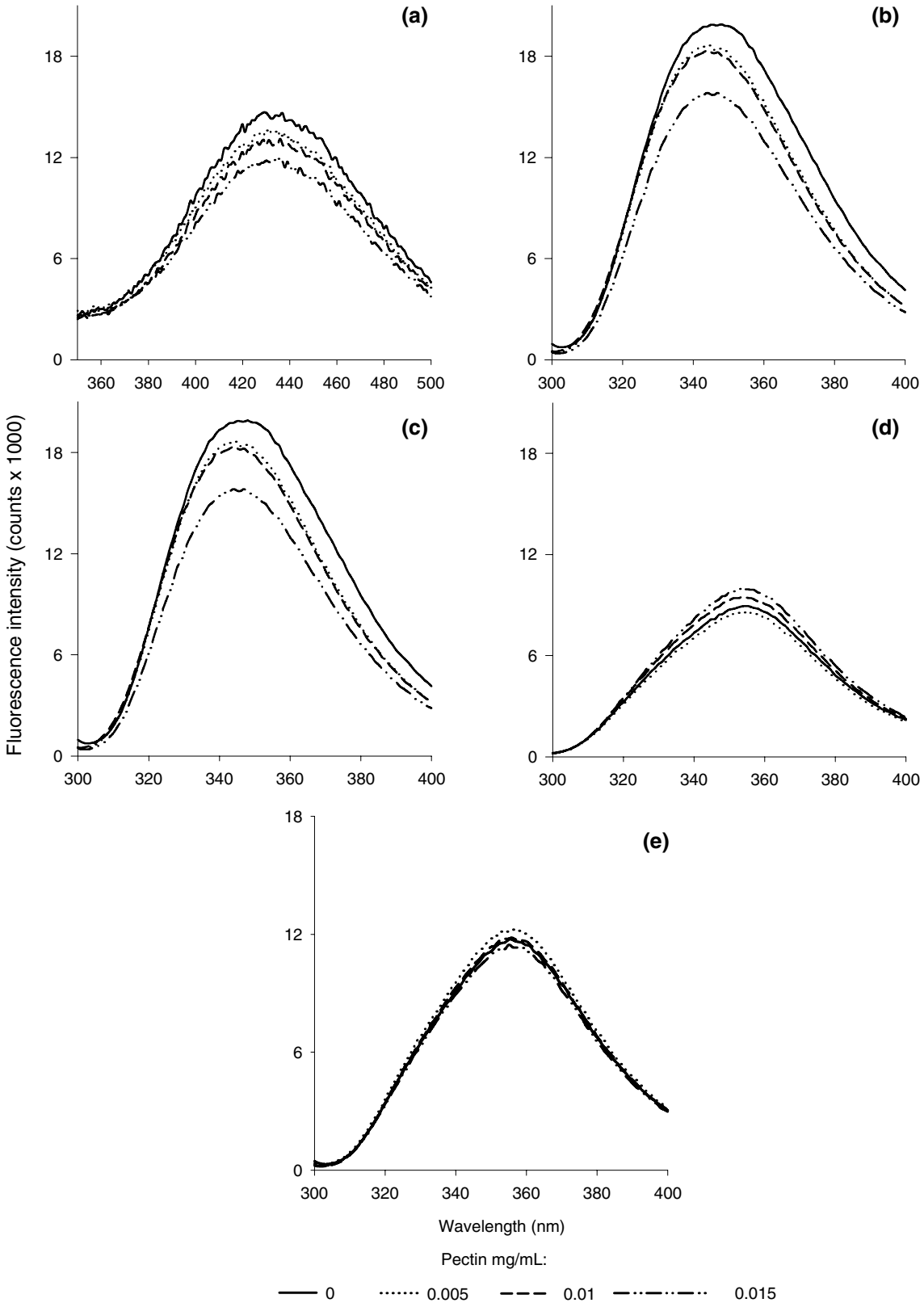


Fig. 7 Fluorescence emission spectra of three and four combined PAs with increasing pectin concentrations. **a** Gallic–protocatechuic–chlorogenic, **b** gallic–protocatechuic–vanillic, **c** gallic–chlorogenic–vanillic, **d** protocatechuic–chlorogenic–vanillic, **e** gallic–protocatechuic–chlorogenic–vanillic

Table 2 Antioxidant capacity of phenolic acids combined with pectin

PA	Pectin	ORAC[H]		FRAP[S]		TEAC[HS]		DPPH[HS]	
		−	+	−	+	−	+	−	+
E			0 ± 0		0 ± 0		8 ± 1		3 ± 1
G		(242 ± 5)^{a}	(165 ± 6)^{a,*}	(114 ± 7)^{a}	(98 ± 10)^{b}	(407 ± 12)^{a}	(252 ± 9)^{a,*}	(225 ± 9)^{a}	(219 ± 11)^{a}
P		(235 ± 4)^{a}	(323 ± 14)^{a,*}	(123 ± 6)^{a}	(129 ± 5)^{a}	(144 ± 7)^{c}	(172 ± 6)^{b,*}	(85 ± 11)^{b}	(91 ± 6)^{b}
C		(162 ± 9)^{b}	(191 ± 12)^{b,*}	(66 ± 4)^{b}	(69 ± 5)^{c}	(115 ± 6)^{d}	(108 ± 8)^{c}	(53 ± 4)^{c}	(60 ± 4)^{c}
V		(158 ± 6)^{b}	(239 ± 6)^{a,*}	(45 ± 7)^{c}	(45 ± 3)^{d}	(225 ± 8)^{b}	(66 ± 2)^{d,*}	(26 ± 3)^{d}	(32 ± 2)^{d}
GP		(322 ± 11)^{b}	(421 ± 12)^{a,*}	(353 ± 8)^{a}	(324 ± 11)^{a}	(306 ± 11)^{a}	(326 ± 10)^{a,*}	(263 ± 8)^{a}	(253 ± 4)^{a}
PC		(217 ± 12)^{d}	(248 ± 17)^{e,*}	(224 ± 6)^{b}	(74 ± 9)^{d,*}	(266 ± 9)^{b}	(310 ± 3)^{b,*}	(185 ± 12)^{b}	(221 ± 13)^{b}
GV		(428 ± 12)^{a}	(392 ± 11)^{b,*}	(95 ± 4)^{c}	(95 ± 6)^{c}	(312 ± 8)^{a}	(318 ± 11)^{ab}	(153 ± 11)^{b}	(188 ± 12)^{c}
GC		(313 ± 10)^{b}	(309 ± 11)^{c}	(23 ± 2)^{d}	(83 ± 8)^{cd,*}	(99 ± 8)^{e}	(94 ± 6)^{d}	(107 ± 8)^{c}	(102 ± 4)^{d}
PV		(267 ± 11)^{c}	(270 ± 10)^{d}	(98 ± 4)^{c}	(143 ± 7)^{b,*}	(192 ± 10)^{c}	(158 ± 8)^{c,*}	(59 ± 11)^{d}	(62 ± 3)^{e}
CV		(317 ± 12)^{b}	(315 ± 9)^{c}	(27 ± 4)^{d}	(73 ± 8)^{d,*}	(133 ± 8)^{d}	(149 ± 10)^{c}	(19 ± 10)^{e}	(18 ± 6)^{f}
GPC		(264 ± 6)^{b}	(336 ± 15)^{a,*}	(394 ± 10)^{a}	(400 ± 14)^{a}	(346 ± 10)^{b}	(309 ± 12)^{c,*}	(301 ± 13)^{a}	(267 ± 18)^{a,*}
GPV		(229 ± 9)^{c}	(328 ± 12)^{a,*}	(340 ± 11)^{b}	(210 ± 13)^{c,*}	(398 ± 11)^{a}	(351 ± 10)^{b,*}	(254 ± 12)^{b}	(196 ± 12)^{b,*}
GCV		(325 ± 8)^{a}	(223 ± 7)^{c,*}	(218 ± 7)^{c}	(186 ± 11)^{c,*}	(310 ± 11)^{c}	(196 ± 11)^{d,*}	(197 ± 10)^{c}	(92 ± 11)^{c,*}
PCV		(232 ± 10)^{c}	(298 ± 9)^{b,*}	(197 ± 9)^{c}	(343 ± 12)^{b,*}	(192 ± 15)^{d}	(403 ± 12)^{a,*}	(106 ± 9)^{d}	(256 ± 10)^{a,*}
GPCV		298 ± 6	291 ± 7	376 ± 6	(416 ± 7)*	467 ± 8	451 ± 9	299 ± 3	(338 ± 3)*

The total concentration of PAs was 60 µM, the concentration of pectin was fixed at 0.015 mg/mL. Results are expressed as mean ± standard deviation (n = 3). All units are µM Trolox equivalents. Different superscript letters indicate significant differences ($p < 0.05$) in AOXC per assay

G gallic acid, *P* protocatechuic acid, *C* chlorogenic acid, *V* vanillic acid

An asterisk (*) indicates significant difference ($p < 0.05$) for that value, due to the presence of pectin, per assay

[H]Indicates the mechanism of action of the assay is hydrogen atom transfer (HAT); [S]Indicates the mechanism of action of the assay is single electron transfer (SET); [HS]Indicates the mechanism of action of the assay is a combination of hydrogen atom transfer and single electron transfer (HAT/SET)

they preferentially interact among themselves, similarly to when all four PAs were present.

Fluorescence spectrophotometry detected the effects of pectin when three PAs were present, but not when four PAs were present, while the opposite occurred when using UV–Vis.

Effect of pectin on the antioxidant capacity of phenolic acids

Table 2 shows the AOXC of PAs and their combinations with and without pectin. When the AOXC of pectin and individual PAs was evaluated, pectin had negligible (TEAC and DPPH) or null (ORAC and FRAP) AOXC values. Gallic acid consistently showed high AOXC in the absence of pectin. These results are similar to those previously reported [3], where a high AOXC for gallic acid is attributed to its three optimally-located hydroxyl groups that can readily reduce other compounds. Pectin caused a decrease in ORAC values of gallic acid and increased them for the remaining compounds. The addition of pectin significantly ($p < 0.05$) decreased TEAC values of gallic and vanillic acid, the latter being the most affected, while the TEAC value of protocatechuic acid increased significantly ($p < 0.05$). Pectin had a null effect on DPPH and FRAP results of all four PAs.

Considering the mechanism of ORAC and TEAC assays (HAT and HAT/SET, respectively), it is apparent that pectin had a stronger impact on HAT-based assays. In contrast, pectin has minimal effect when the assay's mechanism is SET, suggesting that hydroxyl groups may be involved in hydrogen bond formation and were less available to transfer hydrogen atoms. Previous results show that hydroxyl groups of all four PAs discussed here (in addition to caffeic acid, ferulic acid and *p*-coumaric acid), do in fact form hydrogen bonds in the DPPH assay, which was corroborated by proton nuclear magnetic resonance (^{1}H NMR) [18]. Others have shown that AOXC assays are strongly influenced not only by the antioxidant molecules being assayed, but also by the presence of other molecules like amino acids, proteins, solvents and others [19]. Pectin by itself had no apparent effect on the TEAC assay, but can nevertheless have an impact when combined with PAs, as corroborated by our results.

According to Table 2, when two PAs were combined with pectin, it had no significant effect on the ORAC values of three combinations, it significantly ($p < 0.05$) increased them in two combinations and it significantly ($p < 0.05$) decreased the values of one combination. Pectin significantly ($p < 0.05$) increased FRAP values of three combinations, it significantly ($p < 0.05$) decreased the values of one combination and had no effect on two combinations. Pectin had no significant effect on the TEAC values of three combinations, it significantly ($p < 0.05$) increased them in two combinations and it significantly ($p < 0.05$) decreased the values of one combination. Pectin had no significant effect on the DPPH values of any combination. Overall results indicate that AOXC values were mostly increased when pectin was added, also, FRAP assay had the most significant changes. Because FRAP is based on a SET mechanism, it appears that pectin favored electron transfer over hydrogen atom transfer. Considering the previously proposed interactions among PAs, pectin may disrupt them and favor electron transfer, which reduces the radicals used to quantify AOXC.

According to Table 2, AOXC values of three combined PAs shows that only one combination was not affected by the addition of pectin in all assays. The most noticeable change was in the protocatechuic–chlorogenic–vanillic acid combination, where the addition of pectin significantly ($p < 0.05$) increased its AOXC in all assays. The overall tendency was a decrease in AOXC values, in contrast to the combination of two PAs, where an overall increase was found. These results suggest that pectin favors transfer of hydrogen atoms and electrons when three PAs are combined, and that these changes were evident on all assays used to quantify AOXC.

According to Table 2, AOXC values of four combined PAs were significantly ($p < 0.05$) increased when analyzed by FRAP and DPPH, and no effect is seen in ORAC and TEAC assays. This suggests that pectin favored electron transfer and hindered hydrogen atom transfer, similar to the combinations of two PAs.

Conclusions

Spectrophotometric analyses showed that phenolic acids interact with pectin with varying affinity, gallic acid showed the strongest affinity and was the most affected by pectin, which was related to the number and position of its hydroxyl groups. Increasing the number of phenolic acids may allow interactions among them that were harder to discern by a single method, the combined use of UV–Vis and fluorescence spectrophotometry assisted in interpreting the results.

The addition of pectin to phenolic acids or their combinations did not show a uniform or predictable response in antioxidant capacity, either towards an increase or towards a decrease. However, the effects of pectin were more noticeable when analyzed with the ORAC assay, and in contrast, the DPPH assay was the least sensitive to the presence of pectin. Additional, more specific studies may be useful to determine why the presence of pectin is more evident on ORAC than on DPPH.

Bioactivity of phenolic acids may be related to their antioxidant capacity and their interactions with macromolecules. In the case of pectin, our data showed that these interactions

are not straightforward, and that experimental data is needed to firmly confirm an effect. Further analyses may be useful to determine the precise mechanism of action of how a phenolic acid interacts with pectin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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