Proanthocyanidins with a Low Degree of Polymerization are Good Inhibitors of Digestive Enzymes Because of their Ability to form Specific Interactions: A Hypothesis

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Abstract: Inhibition of target digestive enzymes is an accepted strategy to prevent diseases such as obesity and diabetes. Proanthocyanidins (PACs) are known for their ability to bind, inhibit, and precipitate enzymes, which makes them potential bioDrugs with an impact on the digestive process. PAC degree of polymerization (DP) is one of the structural features responsible for their differential inhibitory potency but the explanation for this phenomenon is still unclear. Pecan nut (*Carya illinoinensis* L.) kernels and nutshells are rich in oligomeric and polymeric PACs. We have used thiolysis and HPLC analyses to propose four theoretical model structures of PACs representative of four semipurified fractions obtained from pecan kernel and shell, which showed different inhibitory activity against intestinal lipases, amylases, and proteases. The noncovalent interactions between PACs and digestive enzymes were predicted by *in silico* methods through computational software. These observations are discussed in view of current literature on the biological effects of PACs with different DPs and allowed us to propose the hypothesis that "small oligomeric PACs could be digestive enzyme inhibitors due to their capacity to enter and bind the enzymes' specific cavities better than polymers and oligomers of medium and high molecular weight."

Keywords: *Carya illinoinensis*, enzyme inhibition, *in silico* analysis, proanthocyanidin structure

Introduction

The past three decades have witnessed a worldwide increase in the prevalence of obesity and related illnesses such as cardiovascular diseases and diabetes (Hruby & Hu, 2016). Several strategies can be aimed at maintaining a healthy body weight; some of the most useful include modification of dietary habits and the use of drugs that prevent the absorption of nutrients (Johansson, Neovius, & Hemmingsson, 2014). Currently Orlistat and acarbose are two of the most used synthetic drugs for the inhibition of digestive enzymes responsible of degradation and absorption of fats and carbohydrates, respectively (Al-Omar, Al-Suwailem, Al-Tamimi, & Al-Suhibani, 2006). The main advantage of these drugs is that they act only at the small intestine level and do not interfere with the central control of the digestive process (Yun, 2010). Although they are helpful, synthetic drugs have shown some undesirable side effects, such as gastrointestinal discomforts, flatulence, abdominal pain, and urgency or incontinence; also, they possess high costs, a disadvantage for low-income people who suffer from these disorders (Martinez-Gonzalez, Alvarez-Parrilla, et al., 2017; Yun, 2010).

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Several phytochemicals naturally found in plant foods have shown enzyme inhibitory activity and, therefore, have attracted scientific interest as potential antiobesity nutraceuticals (Martínez-Gonzalez, Díaz-Sánchez, et al., 2017; Martínez Gonzalez, Alvarez-Parrilla, et al., 2017). PACs are polyphenolic compounds recognized for their multiple pharmaco–biological effects, including antioxidant, hypoglycemic, and hypolipidemic properties, which make them potential for obesity management treatments (Atanasov et al., 2018). Some biological effects of PACs have been associated to their ability to complex and alter the activity of digestive enzymes, retarding degradation and absorption of nutrients from the diet (da Silva et al., 2014). PACs belong to the class of oligomeric and polymeric flavan-3-ols, they are highly complex chemical structures with two or more units of monomers joined by one (B-type PACs) or two (A-type PACs) interflavan bonds (Chai et al., 2018). The most common PACs are procyanidins, which contain only catechin/epicatechin units, but prodelphinidins, which contain gallocatechin/epigallocatechin units, are also abundant in nature. PACs can also contain afzelechin/epiafzelechin units, in this case they are known as propelargonidins (Chai et al., 2018), and 3-*O*-gallate derivatives of any of the previously mentioned monomers. PACs are present in plants as mixture of oligomers with degrees of polymerization (DP) between 2 and 10 and polymers with DP higher than 10. There is some evidence that demonstrates the influence of PAC structure, including monomer distribution, DP, and interflavanol bonds over their multiple bioactivities, including their capacity to inhibit digestive enzymes (Cui, Yang, & Li, 2015; Feng, Zhang, Li, Cui, and Chen, 2016; Zhou et al., 2014). The biological relevance of PACs with DP ≥4 is controversial since their bioavailability is limited, however, their inability to pass through the intestinal epithelium

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can be beneficial since they can be accumulated in the small intestine and colon where they can exert favorable effects (Neilson, O'Keefe, & Bolling, 2016), including inhibition of enzymes of the digestive tract and modulation of colonic microbiome.

PACs are found in many plant-derived foods, including wine, chocolate, nuts, and some fruits; they are also abundant in nonedible parts of the plants (leaves, seeds, peels, and roots), which are usually discarded in the food industry. Pecan (*Carya illinoinensis*) is a native nut from North America, where they are widely produced and consumed, although they are also exported worldwide (Alvarez-Parrilla, Urrea-López, & de la Rosa, 2018). Pecan and its nutshells are one of the best sources of food polyphenols, and at least one third of these compounds are PACs (Gu et al., 2002; Zhou et al., 2014). Although the structures of PACs present in pecan and especially in pecan nutshells have not been fully characterized, previous studies have reported that *C. illinoinensis* kernels are rich in oligomeric PACs (mostly procyanidins but also some prodelphinidins and gallates) within DP of 2 to 7 (Gong, & Pegg, 2017; Lerma-Herrera et al., 2017; Robbins, Ma, Wells, Greenspan, & Pegg, 2014); while the nutshells may contain oligomers and polymers (also a mixture of procyanidins, prodelphinidins, and gallates) with DP up to 12 or higher (Alvarez-Parrilla et al., 2018; Lerma-Herrera et al., 2017).

C. illinoinensis PACs can inhibit digestive enzymes (pancreatic lipase, α-amylase, and trypsin) with different inhibitory potency depending on their structure. Interestingly, the small to medium oligomers (DP < 7) were better inhibitors than polymers and large oligomers, which was somewhat unexpected (Vazquez-Flores et al., 2017). This prompted us to propose a hypothesis that would explain this behavior "PACs with a low DP may be good inhibitors of digestive enzymes because of their ability to form specific interactions with the enzymes' cavities." In order to demonstrate this asseveration, we will further analyze the structure of *C. illinoinensis* PACs and explore their probable binding sites in digestive enzymes (pancreatic lipase, α-amylase, and trypsin), by *in silico* modeling.

Premise I. PACs´ biological activity depends on structural features such as degree of polymerization and type of monomeric units

Determination of the structural features of PACs is not only an analytical challenge, but also necessary for a true understanding of their pharmaco–biological mechanisms and effects. It has been showed that bioactivity of PACs is deeply influenced by some structural features, for example, antioxidant activity of PACs is higher in polymeric molecules than in monomeric flavan-3-ols. This tendency is explained because, as the number of monomeric units linked in a polymeric PAC increases, the number of hydroxyl groups that can donate electrons to neutralize free radicals also increases (Sergent, Vanderstraeten, Winand, Beguin, & Schneider, 2012). DP seems to affect PAC's ability to inhibit enzymes in the same manner. Studies with angiotensin (enzyme related to hypertension and cardiovascular health) indicated that PAC´s inhibitory potency was directly dependent on the number of units present in the PAC molecule (Fernández, & Labra, 2013). Based on these studies, authors suggested that PACs with higher DP could exhibit better pharmaco–biological activities. This affirmation would be supported by the fact that the interaction between PACs and enzymes depends on the intrinsic capacity of PACs to form multiple hydrophilic and hydrophobic noncovalent linkages with the enzymatic proteins; these interactions are followed by the formation of large soluble or insoluble PAC–enzyme aggregates that alter the protein´s three dimensional conformation, reduce the catalytic activity, and finally, may induce the enzyme´s precipitation (Bandyopadhyay, & Ghosh, & Ghosh, 2012). However, the structure and concentration of PACs play an important role in the determination of the aggregation behavior between PACs and proteins (Bandyopadhyay et al., 2012). There may be a different phenomenon, also dependent of PACs structure, in which the enzyme inhibitory mechanism of PACs is similar to that exerted by well-known specific inhibitors (orlistat and acarbose), whose mechanisms involve covalent and noncovalent specific interactions with essential amino acid residues in the catalytic site of enzymes (Al-Omar et al., 2006; Guerciolini, 1997), but not protein aggregation and precipitation.

The relationship between PAC's structure and function is still a controversial issue yet, although there is evidence that sustains that DP should be high to increase their bioactivities, other authors suggest that PACs must have an optimal DP range in which their bioactivity is highest. For example, PACs extracted from persimmon peel with DP > 4, were less active in α-glucosidase inhibition than small oligomeric PACs with DP < 3 (Lee, Cho, Tanaka, & Yokozawa, 2007). PAC oligomers from apple with a DP of 5 were good inhibitors of pancreatic lipase activity but their inhibitory activity decreased as their DP increased to decamers (Sugiyama et al., 2007). The oligomeric PAC fraction (DP = 2 to10) isolated from cocoa bean (*Theobroma cacao*) had the highest inhibitory activity (compared with polymeric fractions) over pancreatic amylase, lipase, and phospholipase A2 (Gu, Hurst, Stuart, & Lambert, 2013). Similar effects were found for PACs from rowanberry (*Sorbus aucuparia*); the authors of this study found that a PAC crude fraction (containing oligomeric and polymeric PACs) was better inhibitor of pancreatic α-amylase than fractions enriched in polymeric PACs, although both were effective as enzyme inhibitors. This clearly suggest that oligomeric PACs mixed in the crude extracts may have enhanced α-amylase inhibition, but more detailed structure characterization of rowanberry PACs would be useful to explain this effect (Grussu, Stewart, & McDougall, 2011). These results have been obtained by comparing constant weights of PAC extracts, and some authors have pointed out that the bioactivity of PACs can be affected by the molar concentration of each fraction with different mean DP (mDP) (Jakobek, 2015), so this issue should be clarified in further studies.

Recent studies also point out that oligomeric PACs (DP < 5) exhibited better bioactivities as antioxidant and antiinflamatory in biological systems, because they can be transported more efficiently to cells than polymeric PACs whose bioavailability is very limited due to their high number of monomeric units (Zhang et al., 2016). Although it can be argued that absorption into cells is not a critical issue for the inhibitory activity of PACs against digestive enzymes, which are secreted into the lumen of the small intestine; these observations point out that PAC DP may have opposed effects on their bioactivity, depending on their mode of interaction with their molecular targets. Nevertheless, the poorly absorbed oligomeric and polymeric PACs may be accumulated in the small intestine and colon, promoting other type of benefits such as the balance of gut microbiota (Cires, Wong, Carrasco-Pozo, & Gotteland, 2017; Neilson et al., 2016).

The proportion of prodelphinidins in a PAC mixture has also been suggested as an important structural characteristic that regulates their ability to inhibit and inactivate enzymes. Authors explained this as a consequence of the higher number of hydroxyl groups in prodelphinidins in comparison with procyanidins, because protein binding and inactivation are associated with hydrogen bonding of carbonyl groups from peptide bonds and polar

groups in amino acid side chains with hydroxyl groups from PACs. Based on this, authors postulate that PACs with more hydroxyl groups, like prodelphinidins or PACs containing galloyl moieties, would be better enzyme inhibitors by forming multiple interactions inducing protein precipitation and inactivation (Saminathan et al., 2014). However, studies with pine coat (*Araucaria angustifolia*) suggest the opposite, since this species contains PACs rich in procyanidins with better inhibitory effect over saliva and pancreatic α-amylase than prodelphinidin-rich PACs found in *Acacia mearnsii* (da Silva et al., 2014). It is reasonable to point out that PACs are able to inhibit enzymes with different potencies, depending on various structural characteristics, but the simple presence of prodelphinidins rich in hydroxyl groups or a high DP does not ensure inhibition; authors propose that the PAC molecule requires an appropriate conformation with adequately positioned hydroxyl groups for optimizing PAC–enzyme interaction and, consequently, increasing the enzyme inhibition (da Silva et al., 2014).

In order to fully understand how PAC structure is related to their bioactivity, it is crucial to start from a well-elucidated PAC molecule and this must be done using different complementary analytical techniques that allow a broad description of the structure, necessary to achieve a good prediction of PAC bioactivity (Yokota, Kimura, Ogawa, & Akihiro, 2013). The following premise describes the elucidation of the structure of *C. illinoinensis* PACs, using an analytical technique that had not been previously used in these samples and integrating the new results with the previously published ones, in order to propose several model molecules representative of pecan nut kernel and shell PACs.

Premise II. PAC fractions isolated from the kernels and shells of *C. illinoinensis* possess characteristic structures with different DPs and inhibitory activity toward digestive enzymes

PACs are polymeric polyphenols that are present in nature as complex mixtures of different sizes, types of interflavan bonds, and monomer composition; for this reason, the application of diverse analytical techniques is required in order to characterize their structure (Lorrain, Ky, Pechamat, & Teissedre, 2013). Normal-phase chromatography is a good tool to analyze PACs, it can determine their DP with high resolution, although depolymerization reactions followed by reverse-phase chromatography with a mass detector provide more information about PAC features such as monomer distribution, mDP, and type of interflavan bond (Neilson et al., 2016). Normal-phase chromatography has been extensively used to separate PACs by molecular weight (DP) in diverse foods (Lorrain et al., 2013; Neilson et al., 2016). In this technique, low-molecular weight compounds (low DP) eluted first, followed by higher molecular weight (high DP) compounds (Amarowicz, & Pegg, 2006) and multiple compounds with the same DP generally coelute. PACs were extracted and purified from pecan kernels and shells as previously described (Vazquez-Flores et al., 2017) and analyzed by normal-phase HPLC and thiolysis depolimerization reaction.

Normal-phase HPLC was performed using a Luna silica column 250 × 4.6 mm, 5 μm, and 100 Å pore size (Phenomenex, Torrance, CA, USA) at 35 °C. Samples (1 mg/mL in methanol) were injected in a binary solvent system. Solvent A: dichlorometane, methanol, water, and acetic acid (82:14:2:2) and the same mixture, in a relation of 84:14:1:1 was used as solvent B. Ten microliters were injected and chromatograms registered at 280 and 320 nm. Monomers and dimers were identified by comparison of their retention times with those of commercial standards, and oligomers with a higher DP were tentatively identified according to previously published results on the identification of pecan kernel PACs by this same technique (Gong, & Pegg, 2017; Robbins et al., 2014). Figure 1A shows that the PACs from kernel were constituted by a mixture of monomers to hexamers, trimers and tetramers being the most abundant forms (>60%). mDP was calculated (Lerma-Herrera et al., 2017) as 4.1 ± 0.3. Shell PAC extracts could not be resolved by normal-phase HPLC, probably due to their higher mDP, however, complementary chromatographic techniques were used for their characterization.

Thiolysis has proven to be one of the most efficient depolymerization reactions for PAC analysis (Chai et al., 2015). The technique permits to identify and differentiate terminal units from internal units, so monomer distribution can be deduced, it also allows the identification of the types and number of interflavan bonds, distinguishing between A- or B-type PAC (Shelembe, Cromarty, Bester, Minnaar, & Duodu, 2012), since A-type linkages are resistant to thiolysis depolymerization (Yokota et al., 2013). Thiolysis of PAC fractions isolated from pecan kernels and shells was carried out according to Guyot et al. (2001) with slight modifications, and the identification of its products was achieved by reversed-phase HPLC coupled to mass spectrometry (Mouls, & Fulcrand, 2012; Shelembe et al., 2012). Thiolysis products of PACs isolated from pecan kernel showed catechin and epicatechin as terminal units (Figure 1B, peaks 1 and 2) according to their molecular weight (m/z 289) and comparison of their retention time with that of commercial standards. While adducts formed with the nucleophile benzyl mercaptan (peaks 3 to 6) corresponded to internal units of epigallocatechin (m/z 427), catechin (m/z 411), epicatechin (m/z 411), and (epi)catechin-3-gallate (m/z 563). The mDP of the PAC extracts can be calculated according to Eq.1:

$$\mathrm{mDP} = \frac{(\mathrm{FTU} + \mathrm{Add})}{\mathrm{FTU}} \quad (1)$$

where FTU is the abundance of the free terminal units (sum of the abundance of each molecular ion) and Add is the abundance of the internal units (abundance of molecular ions of benzyl mercaptan adducts). Using this technique, mDP of kernel PAC was calculated as 5.5 ± 0.4, slightly higher than the mDP calculated by normal-phase HPLC.

The same methods were used to analyze the thiolytic products of the three PAC fractions isolated from pecan nutshell (PACS1, PACS2, and PACS3) as previously described (Vazquez-Flores et al., 2017). PACs from the three shell fractions showed signals of catechin and epicatechin as internal units (no epigallocatechins and 3-gallates were observed). PACS1 contained only catechin as terminal unit, while PACS2 had only epicatechin and PACS3 contained both types of terminal units (Figure 1C). The mDPs were the main difference among shelI PAC fractions: PACS1 had a mDP of 18 ± 0.8, PACS2 presented a mDP of 6.6 ± 0.5, while PACS3 had a mDP of 3.6 ± 0.5. These results were close to those calculated previously by phloroglusinolysis and butanolysis in the same fractions (Vazquez-Flores et al., 2017). No A-type PACs were detected in pecan shell or kernel; this is consistent with other authors who describe that the interflavan B-type bond is the most frequent linkage in PACs from almond, hazelnut, and walnut (Alasalvar, & Bolling, 2015).

Considering both the results presented in the present article, as well of those previously published, we are able to propose a theoretical model of PAC molecule for each extract or fraction

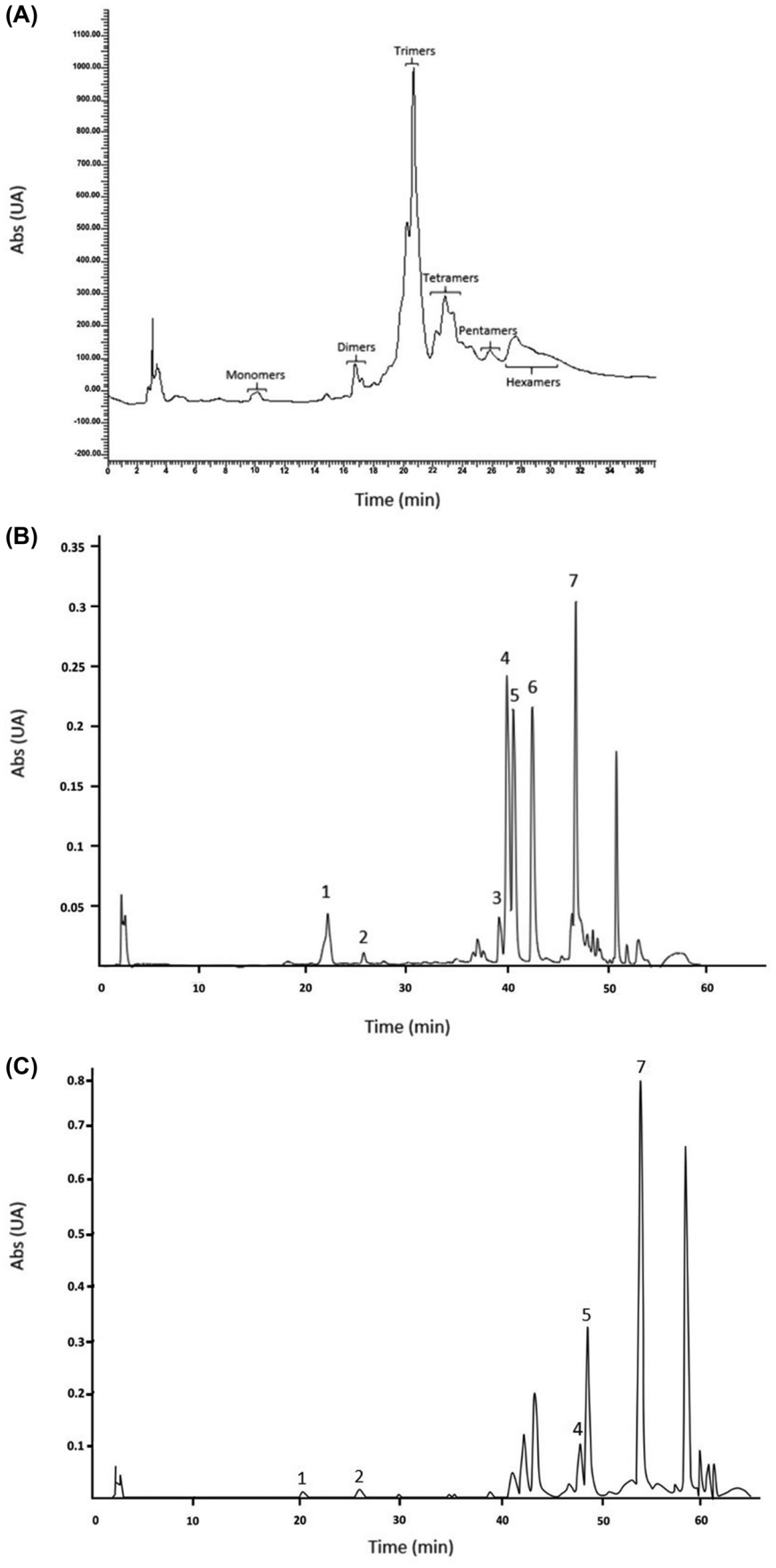

Figure 1—Elucidation of PAC structure by chromatographic techniques. (A) Normal-phase chromatogram of PAC from pecan kernel at 280 nm. Monomers come out first, followed by PAC with increasing degree of polymerization. (B) Reversed-phase chromatogram at 280 nm of *Carya illinoinensis* kernel thiolyisis products. (C) Reversed-phase chromatogram at 280 nm form PACS3 thiolyisis products. Peaks in B and C are: 1, free catechin (terminal unit); 2, free epicatechin (terminal unit); 3, epigallocatechin adduct; 4, catechin adduct; 5, epicatechin adduct; 6, (epi)catechin-3-gallate adduct; and 7, benzyl mercaptan.

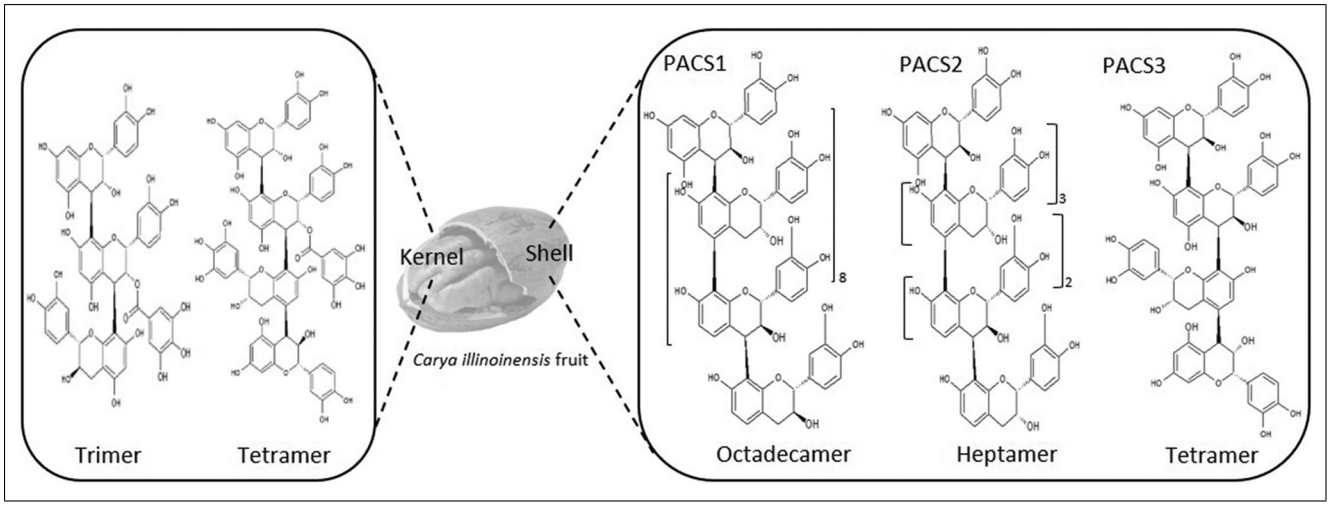

Figure 2–*Carya illinoinensis* fruit portions and model PACs elucidated by chromatographic techniques. Kernel model PAC trimer epicatechin/epicatechin–3-gallate/catechin(EC/ECG/C) and tetramer epicatechin–epicatechin–3-gallate–epigallocatechin–catechin (EC/EC3G/EGC/C). Model PACs from shell: fraction PACS1 octadecamer catechin–(epicatechin–catechin)$_8$–catechin (C/(EC–C)$_8$/C), fraction PACS2 heptamer catechin–(epicatechin)$_3$–(catechin)$_2$–epicatechin (C/(EC)$_3$/(C)$_2$/EC), and fraction PACS3 tetramer catechin/catechin/epicatechin/epicatechin (C/C/EC/EC).

(Figure 2). For the kernel extract, two model molecules are proposed: a trimer consisting of epicatechin/epicatechin–3-gallate/catechin and a tetramer (epicatechin–epicatechin–3-gallate–epigallocatechin–catechin). For shell PACs, three models were proposed, one for each shell fraction: an octadecamer for PACS1 integrated by catechin–(epicatechin–catechin)$_8$–catechin, an heptamer for PACS2 integrated by catechin–(epicatechin)$_3$–(catechin)$_2$–epicatechin, and a tetramer for PACS3 integrated by catechin/catechin/epicatechin/epicatechin. These model PAC molecules from *C. illinoinensis* fruit were classified as oligomeric, except for the one representing PACS1 that is classified as polymeric (>10 DP) (Feng et al., 2016).

The isolated PAC fractions had previously shown a good inhibitory activity against digestive enzymes: pancreatic α-amylase, pancreatic lipase, and trypsin (Vazquez-Flores et al., 2017). The best inhibitors were the fractions with the lowest mDP (except for trypsin for which the low-mDP shell fraction showed low activity). In order to further investigate the reason for the better activity of low-DP oligomers, we have carried out computational (*in silico*) analyses to predict the specific interactions and binding sites of the different *C. illinoinensis* PACs, in order to evaluate how the differences in PAC's size can influence the binding site and, consequently the enzyme inhibitory potency.

Premise III. Small oligomeric PACs from *C. illinoinensis* interact with small cavities of digestive enzymes while larger PACs form interactions with the proteins´ surfaces

Recently, *in silico* analyses (also known as molecular modeling or docking) have been used as a relevant and unique tool to explain or predict dynamics and mechanisms of enzymatic inhibition, providing information about specific interactions and conformation of protein–inhibitor complexes at atomic level (Escobedo-González et al., 2015; Liu et al., 2017; Zhou et al., 2014; Martínez Gonzalez, Alvarez-Parrilla, et al., 2017; Chai et al., 2018). In this study, we performed molecular dockings for the three digestive enzymes: pancreatic α-amylase, lipase, and trypsin and their binding with model PACs from *C. illinoinensis*. The PAC model structure predicted for fraction PACS1 could not be optimized in 3D (PyMol Molecular Graphics System 1.3v software) because of its polymeric dimension (DP = 18), so molecular predictions could not be estimated, therefore, only four PAC molecules were analyzed: a trimer and a tetramer representative of kernel PACs, a tetramer and an heptamer for shell PACs. Figure 3 shows the molecular docking simulations (UCSF Chimera 1.12v software) of the interaction between the four model PACs and pancreatic α-amylase, simulations with other enzymes showed similar behavior, although the interaction with α-amylase presented the clearest tendency. Pancreatic α-amylase is one of the α-glucosidases recently recognized as therapeutic target for the prevention and control of diabetes *mellitus* type 2 (Liu et al., 2017); it hydrolyzes α-1-4 glycosidic linkages in starch, and its active site contains a catalytic triad formed by Asp^{197}, Glu^{233}, and Asp^{300}. Figure 3 shows that trimeric and tetrameric PACs bound with amino acid residues from this triad: kernel PACs (trimer and tetramer) bound with Asp^{197} and Asp^{300} (Figure 3A and 3B), while the shell tetramer interacted with Asp^{300} and Glu^{233} (Figure 3D). A different behavior was observed for larger oligomers like the shell heptamer (Figure 3C), which showed interactions with the enzyme´s surface, in a region distant to the catalytic site cavity. Actually, it is apparent that almost one half of the PAC molecule remained without interacting with the enzyme. This suggests that even if heptamers or bigger PACs can establish numerous hydrophilic interactions with the proteins, they are mostly superficial interactions, since the size of the PAC molecule impairs its entrance to the enzyme´s catalytic site, and consequently, the inhibitory activity of large PACs is lower than that of medium or small oligomers. This observation also suggested that, since interactions are mostly with the protein´s surface, high-DP PAC could be better at precipitating proteins despite being worst specific inhibitors. It has been demonstrated that proteins can aggregate and precipitate with PACs in the small intestine lumen when proteins are near to their isolectrical point (Cires et al., 2017), which was not the case with the three digestive enzymes analyzed in the present study.

Binding of *C. illinoinensis* model PACs with pancreatic lipase and trypsin showed similarities with α-amylase, in that the higher DP molecule preferably bound the protein´s surface, although differences in the binding sites could explain the different inhibitory potency toward each enzyme. For pancreatic lipase, responsible

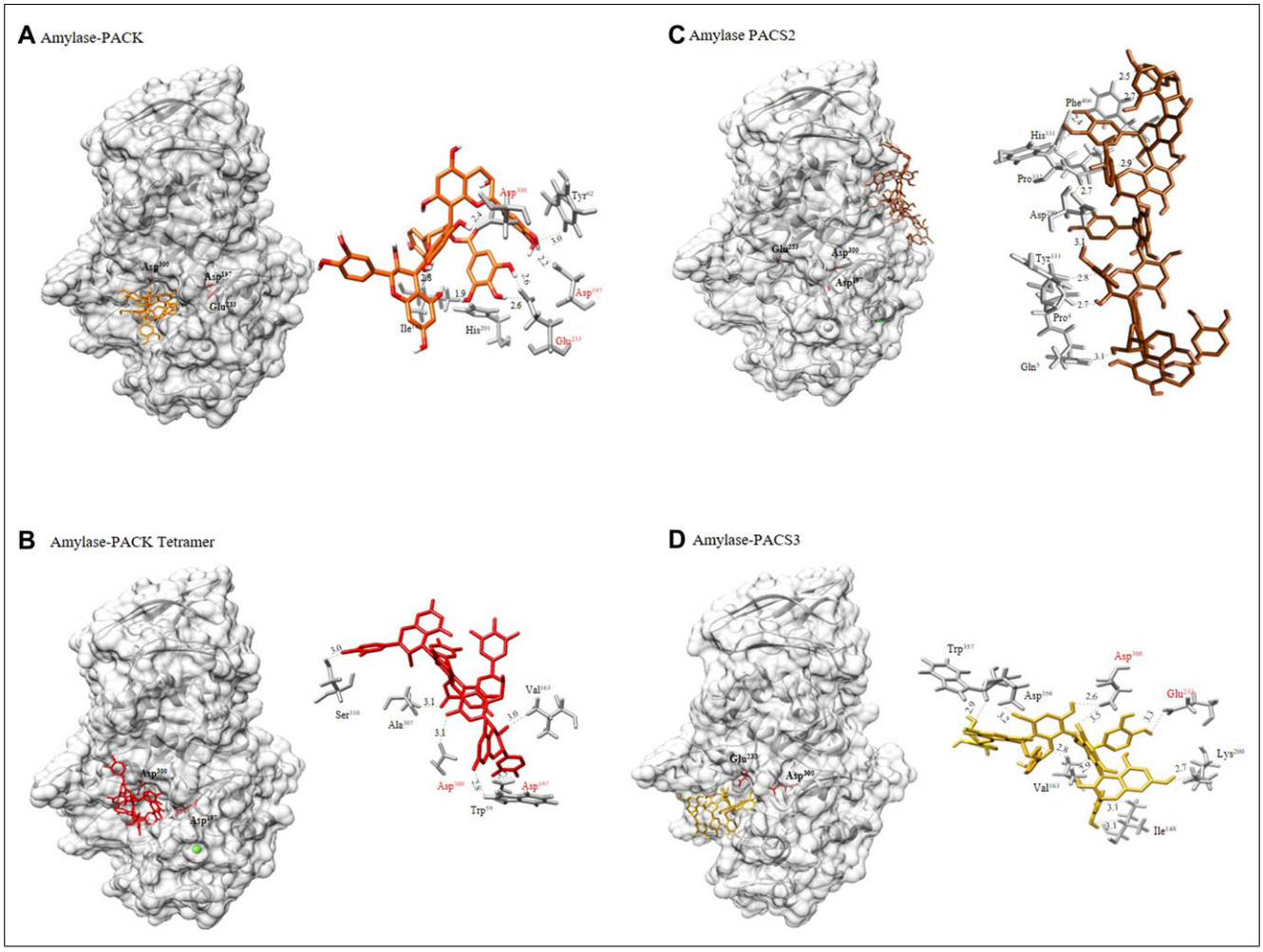

Figure 3–Molecular docking simulations of the interaction of model PACs from *C. illinoinensis* and pancreatic α-amylase. (A) Trimer of kernel; (B) tetramer of kernel; (C) heptamer of shell; and (D) tetramer for shell. Distances between amino acid residues and PAC are given in Å.

for the hydrolysis of 50% to70% of dietary fats (Ivanov, Nomura, Malfanov, Sklyar, & Ptitsyn, 2011), kernel trimer and tetramer and shell tetramer bound through hydrogen bonds in the region where lipase and colipase (lipase´s coenzyme) are joined. Co-lipase plays a very important role for triacylglycerol degradation, promoting a proper environment for the water–lipid interface (Lowe, 2002), therefore, disruption of this region by *C. illinoinensis* trimer and tetramers blocks the possibility of an appropriate medium for lipid degradation. The shell heptamer had a different behavior, it interacted with colipase but in a region that was not critical for the lipase–colipase union, explaining its lower lipase inhibitory activity. Also, the fact that PACs did not interact with lipase active site, but did so with the active site of α-amylase, could help to explain the observation that α-amylase is usually more sensitive than lipase to inhibition by PACs (He, Lv, & Yao, 2006; Vazquez-Flores et al., 2017). Finally, for trypsin, kernel PACs (trimer and tetramer) interacted with its active site, both with some residues of the catalytic triad (formed by His^{57}, Asp^{102}, and Ser^{195}) and with residues in the substrate binding pocket. In contrast, the shell tetramer was predicted to bind to a site different than the active site, showing that in this specific enzyme–PAC interaction the DP was not the decisive factor. The shell heptamer bounds to only one active site residue (His^{57}), but a large proportion of the molecule interacted with residues from the enzyme´s surface. It is important to mention that in the previously published paper, trypsin activity was very little affected by the smallest shell fraction (represented by the shell tetramer, which did not bind the active site) while the shell fractions with larger mDP were better inhibitors, although not as good as the kernel fraction (represented by the kernel trimer and tetramer that were capable of inserting themselves in the substrate binding site).

In summary, all small oligomers (DP = 3 to 4) were predicted to be capable of entering the enzymes´ cavities, although the binding sites were different in each enzyme and could also be affected by the monomer composition of the oligomers. The larger oligomer (DP = 7) was predicted to be able to form more enzyme–PAC interactions, although most of them with the enzyme's surface, distant from the active sites or sites critical for enzyme activity, moreover some parts of the PAC molecule were predicted to remain unbound. Despite being unable to perform the docking analysis with the polymer (DP = 18), we may speculate that it could show a behavior similar to the heptamer.

Final Considerations

PACs possess multiple biological activities, including their ability to inhibit digestive enzymes. Digestive enzyme inhibition can be viewed as a promising strategy to control obesity and diabetes, although inhibition of digestive proteases can have undesirable

consequences. In this scenario, it is important to understand which factors regulate the inhibitory potency of PACs with different structures toward different digestive enzymes. PAC size, expressed as its DP or mDP for PAC mixtures, is one of the structural features that strongly modulates PACs´ activities, however, there is no scientific consensus about how and why is PAC DP related to enzyme inhibition or other biological activities. In the present hypothesis of the article, we have first predicted, by reviewing literature and providing new analytical evidence, the most probable structures of different PACs isolated from *C. illinoinensis* kernels and shells. Next, using these model structures and *in silico* analysis tools, we have predicted that small oligomeric PACs (DP = 3 to 4) are able to interact with essential cavities involved in the enzymatic activity of pancreatic α-amylase, lipase and trypsin, while larger oligomers (DP = 7) may form more enzyme–PAC interactions but most of them with the enzyme's surface. These observations, in addition to previously published work about the differential enzyme inhibitory activity of PAC fractions with different mDP, allowed us to propose the hypothesis that "PACs with a low DP may be good inhibitors of digestive enzymes because of their ability to form specific interactions with the enzymes' cavities." Moreover, we can suggest that PACs with a high DP, by interacting mostly with the enzymes surface may be more active at inducing protein precipitation. Therefore, two different enzyme inhibition mechanisms could be predicted: for small oligomers, a specific inhibitory mechanism derived from PAC–protein interactions in specific binding cavities and for large oligomers and polymers a mechanism dependent of protein aggregation and precipitation. We believe that experimental and analytical testing of this hypothesis may have a major impact on the understanding of the health-related biological actions of PACs and other food phenolic compounds.

Conflicts Of Interest

All authors declare no conflict of interest in this article.

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Author Contributions

Alma A. Vazquez-Flores performed all the analysis and wrote the first draft of the manuscript. Alejandra I. Martinez-Gonzalez and Angel G. Díaz-Sánchez participated in the implementation of the computational simulations (docking) and its interpretation. Emilio Alvarez-Parrilla and Gustavo A. González-Aguilar participated in the design of the model PACs from the available experimental data and critically revised the hypothesis and manuscript. Cristóbal Aguilar-González participated in the HPLC analysis of thiolysis products and critically revised the hypothesis and manuscript. Laura A. de la Rosa was in charge of the study design, hypothesis formulation, and manuscript revision. All authors discussed the results and contributed to the final version of the manuscript.

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