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Polyphenol-Protein Complexes and Their Consequences for the Redox Activity, Structure and Function of Honey. A Current View and New Hypothesis – a Review

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There is increasing evidence that protein complexation by honey polyphenols is changing honey structure and function. This relatively less investigated filed of honey research is presented in a context of known mechanism of formation of the stable polyphenol-protein complexes in other foods. At a core of these interactions lies the ability of polyphenols to form non-covalent and covalent bonds with proteins leading to transient and/or irreversible complexes, respectively. Honey storage and thermal processing induces non-enzymatic oxidation of polyphenols to reactive quinones and enables them to form covalent bonds with proteins. In this short review, we present data from our laboratory on previously unrecognized types of protein-polyphenol complexes that differed in size, stoichiometry, and antioxidant capacities, and the implications they have to honey antioxidant and antibacterial activities. Our intent is to provide a current understanding of protein-polyphenol complexation in honey and also some new thoughts /hypotheses that can be useful in directing future research.

INTRODUCTION

Polyphenols and proteins are minor components of honeys but they can significantly influence honey antioxidant and antibacterial properties. The reason behind these effects relates to polyphenols' ability to bind proteins *via* non-covalent or covalent bonds and sequestering proteins into soluble or insoluble complexes. Binding affects function of both proteins and polyphenols. Proteins modified by polyphenols undergo conformation transitions that are changing their biological activity, as in a case of honey enzymes. Binding of polyphenols to proteins on the other hand, affects antioxidant activity of polyphenols because the binding engages the same functional groups that are involved in redox cycling; electron donation, or metal chelation. These functional alterations following protein and polyphenols complexations can be transient or irreversible, depending on whether non-covalent or covalent bonds are formed between these molecules. Environmental factors such as pH, temperature, ionic strength often modulate transient interactions toward formation of stable protein-polyphenols complexes with a long half-life. Here, we review these aspects of protein-polyphenol interactions and describe functional consequences they have on honey functions.

NON-COVALENT BINDING

Non-covalent binding between protein and polyphenols involves hydrogen bonds that are formed between electronegative atoms of nitrogen or oxygen, especially of amino ($-NH_2$) and hydroxyl (–OH) groups, and a positively charged hydrogen atom from neighboring hydroxyl or amino group of another polyphenol or protein molecules. Depending on polyphenol structure and degree of hydroxylation, the interaction may produce single or multiple hydrogen bonds that influence strength of the formed complexes [Haslam, 1974]. Hydrogen bonds between neighboring protein chains can create bridges that crosslink proteins into aggregates.

In addition to hydrogen bonds that involve polar groups, protein and polyphenols may interact *via* hydrophobic, non-polar aromatic rings of polyphenols and aromatic amino acids, (proline, phenylalanine, tyrosine, tryptophan, histidine) [Charlton *et al.*, 2002; Siebert, 1999]. Hydrophobic bonds influence the structure of the complex by stacking of aromatic rings of polyphenols against those of aromatic amino acids such as in the case of proline pyrrolidone rings in proline-rich proteins and galloyl rings of tannins [Baxter *et al.*, 1997; Siebert *et al.,* 1996]. Formation of protein-polyphenol complexes usually results from multiple cooperative hydrophobic and hydrogen binding and may lead to colloidal size aggregates. The non-covalent protein-polyphenol interactions are responsible for the haze formation in beers, wines and fruit

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juices [Siebert *et al*., 1996; Baxter *et al*., 1997; Siebert, 1999] and colloids in honey [Paine *et al*., 1934; Lothrop & Paine, 1931].

HONEY COLLOIDS - MELANOIDINS CONNECTION

Honey colloids are mixture of non-crystalline particles, vastly differing in sizes that are evenly dispersed and suspended in supersaturated sugar solution. They cannot be removed from honey by traditional membrane filtration or centrifugation because of their size heterogeneity, differences in solubility, and the particle association-dissociation dynamics. The presence of colloids affects physical properties of honey such as color, flavor, clarity, crystallization and thixotropy [Paine *et al*., 1934; Witczak *et al*., 2011]. The colloidal content in honey is low and varies between 0.1 to 1.0% for light and dark honey, respectively [Lothrop & Paine, 1931; Mitchell *et al*., 1955]. The chemical composition of honey colloids and the mechanism of their formation remained enigmatic for a long time. According to old literature, honey colloids consisted of proteins (enzymes), pigments (polyphenols) and some waxes [White, 1957]. More than 50% of its mass was found to be comprised of proteins [Mitchell *et al.,* 1955]. The fact that honey colloids could be precipitated upon dilution with water [Lothrop & Paine, 1931] suggested that they are hydrophobic in nature. Their isoelectric point was found to be 4.3 [White, 1957] therefore in honey, with the average pH of 3.9, colloids are positively charged.

High concentration of sugars in honey may increase colloidal stability. Usually, the stability and size of colloidal particles is strongly controlled by environmental conditions such as pH, temperature, or ionic strength. Since the interactions between colloid particles are governed by Coulombic electrostatic interactions and van der Waals interactions [Israelachvili, 1992], changes in pH and salt concentration increase the tendency of particles to coalesce to large aggregates and to promote their flocculation.

We cannot resist the impression that the brown, colloidal material in honey described in old literature fits the description of melanoidins. Melanoidins are high molecular weight colloidal complexes that are formed in thermally processed foods as the result of the Maillard reaction [for review see, Wang *et al.,* 2011]. A structure of intact melanoidins has proven to be challenging to characterize at the molecular level and it remains mostly unknown. However, all food melanoidins contain proteins, polyphenols, sugars and the Maillard reaction products as the main components. Such structures have been also recently found in honey [Brudzynski & Miotto, 2011b, c].

Protein-polyphenol complexes are an integral part of both colloids and melanoidins. The colloid-melanoidin connection is supported by the following facts. Firstly, proteins, as surface active molecules are “colloidally” active and can readily assemble to high molecular weight aggregates *via* protein--protein interaction and/or interactions with other ligands including polyphenols [Haslam, 1996]. Secondly, the driving force in the protein-polyphenol complexation is a hydrophobic force/effect. This property is responsible for colloid formation, such as haze formation in beer as well as melanoidin formation, such as that of coffee. By making a connection between honey colloids and melanoidins, one could easily envision the location and milieu in which the protein-polyphenol interaction take place in honey. Despite uncertainty of the colloids-melanoidin connection, it is undoubtedly true that the protein-polyphenol complexes are highly relevant to physical, structural and functional properties of honey. There is a multitude of proteins of different functions in honey that can be target by polyphenols.

HONEY PROTEINS

Protein modification by polyphenols alters the biological activity of honey proteins in the way that it reduces their original function and nutritional benefits. Proteins are minor components of honey, amounting to 0.2 to 0.7% of honey mass [Bogdanov, 2008]. Together with nectar-carbohydrates they serve as the primary source of honeybee’s diet. Proteins of pollen origin are the main source of nitrogen for bees [Baroni *et al*., 2002; Iglesias *et al*., 2006]. Besides the nutritional role, proteins of pollen origin comprise of stress-inducible, pathogenesis-related proteins (PRP) [Midoro-Horiuti *et al.,* 2001; Breiteneder, 2004] that may enhance bee’s immunity and the tolerance to pathogens. To the group of pollen disease-resistant proteins belongs also the 31 kDa dirigent-like protein recently discovered in honey [Brudzynski *et al*., 2013].

However, majority of honey proteins are of bee origin and consist of enzymes involved in sugar metabolism: glucose oxidase [Schepartz & Subers, 1964], alpha-glucosidase (invertase) [White & Kushnir, 1966; Bonvehi *et al.,* 2000], beta--glucosidase [Pontoh & Low, 2002], alpha-amylase (diastase) [Oddo *et al*., 1995; Babacan & Rand, 2007], transglucosylase and phosphorylases. The proper functioning of these enzymes ensures an adequate supply of simple carbohydrates that are for bees a source of energy for flight and colony maintenance.

The most abundant non-enzymatic honey protein originating from bees is a 55–57 kDa glycoprotein, Major Royal Jelly Protein1 (MRJP1) or apalbumin-1 [Šimúth, 2001; Šimúth *et al*., 2004; Won *et al*., 2008]. The size and a high concentration of MRJP1 in honey suggested its nutritional role [Šimúth, 2001]. This view was initially supported by a finding that the MRJP1 protein can be specifically degraded by serine proteases, generating a substantial number of MRJP1-related peptides [Rossano *et al*., 2012]. However, the degradation products of MRJP1 were also shown to have immune-stimulatory activities [Majtan *et al*., 2006; Tonks *et al.*, 2003] and cell growth stimulatory activity [Watanabe *et al.,* 1998; Kamakura *et al.,* 2001; Majtan *et al.,* 2009].

The expanding number of functions of the MRJP1 includes its significant influence on honey structure. The MRJP1 has propensity for oligomerization, and in honey, it often forms a large, 350 kDa hetero-hexamer, known as apisin [Kimura *et al*., 1995, 1996] with a peptide named apisimin [Bilikova *et al*., 2002] that serves as a linker between the MRJP1 oligomers [Tamura *et al.,* 2009]. The MRJP1 molecule is highly glycosylated [Kimura *et al.,* 1995, 1996]. The MRJP1 size and presence of several reactive groups can influence the attraction and repulsion forces with other ligands. Thus, these features make the MRJP1 the most suitable candidate to be involved in colloid-melanoidin formation. Honey also con-

tains other types of MRJPs (MRPJ2 to 5) [Di Girolamo *et al.,* 2012] thereby creating a substantial pool of non-enzymatic proteins of honeybee origin.

In view of these multiple biological roles of honey proteins, it becomes instantly apparent that their interaction and complexation with polyphenols will have a significant impact on honey function and nutritional availability.

STOICHIOMETRY OF PROTEIN-POLYPHENOL COMPLEXES

Association and dissociation between proteins and polyphenols is initially a surface phenomenon. The stability of complexes depends on the molecular size of interacting partners, their concentrations and external conditions in which the interaction takes place [Spencer *et al.*, 1988; Haslam, 1996]. Polyphenols of higher molecular size showed greater tendency to form stable complexes with proteins [De Freitas & Mateus, 2001]. In this respect, it has to be realized that proteins and polyphenols vastly differ in sizes. The average size of polyphenols is ~500 daltons while a typical size of protein is 30,000 daltons (in terms of their sequence size as determined by the number of amino acids). To produce stable complexes, the ratio of polyphenol to protein must be high to form multiple non-covalent bonds. Secondly, the strength of complexes is influenced by polyphenol hydrophobicity. At low polyphenol concentrations, their attachment to proteins does not change hydrophilic character of complexes and the complexes remain soluble. However, with the increased concentration of polyphenols, the protein-polyphenol complexes become more hydrophobic by encouraging interactions between non-polar residues of polyphenols and proteins. The intramolecular hydrophobic interactions between different protein-polyphenol complexes are the driving force toward aggregation and precipitation as large, insoluble aggregates [Spencer *et al.,* 1988; Naczk *et al.,* 2011]. Taken together, the polyphenols' hydrophobic aromatic rings and hydrophilic hydroxyl groups contribute to multiple, non-covalent bonds with proteins and act as multidenate ligands [Spencer *et al.,* 1988; Haslam, 1996].

The size and solubility of protein-polyphenol complexes are temperature-sensitive; elevated temperatures facilitate tendency to hydrophobic interactions by unfolding the proteins chains and exposing hydrophobic amino acids (hydrophobic effect). Thus, the size and number of protein-polyphenol complexation will increase with the raise of temperature. There are, however, other factors influencing the complexation.

POLYPHENOLS AUTO-OXIDATION AND FORMATION OF QUINONE-PROTEIN COVALENT BONDS

In addition to temperature, the environmental factors such as exposure to oxygen, presence of oxidizing agents (hydrogen peroxide), and the presence of transition metal ions significantly facilitates formation of covalent bonds that irreversibly link polyphenols with proteins. Such binding occurs upon polyphenol auto-oxidation in the presence of O_2. The auto-oxidation involves one- or two-step electron transfer (as it is shown for quercetin in Figure 1). Firstly, the transfer of an electron from polyphenol to O_2 leads to formation of *ortho*-semiquinone anion radical ($Q^{\bullet -}$). *o*-Semiquinone radicals, as unstable compounds, could undergo radical-radical reaction giving *o*-quinone and a reconstituted parent molecule. $Q^{\bullet -}$ radical can also react with molecular oxygen and give rise to a superoxide anion radical ($O_2^{\bullet -}$), initiating the redox cycle. Subsequently, the superoxide radical can be scavenged by the parent polyphenol and produce again *o*-semiquinone radicals and hydrogen peroxide (Figure 1). This redox cycle may continue until the system becomes depleted of oxygen.

In contrast to one electron transfer, the two-step electron transfer from the parent molecule gives more thermodynamically stable *o*-quinones [Methodieva *et al.,* 1999]. The formation of *o*-quinone is followed by a rapid isomerization to *para*-quinone methide intermediates. As potent electrophiles, quinones and quinone methides bind specifically and irreversibly to nucleophilic groups of amino acids such as sulfhydryl, amine, amide, indole or imidazole groups of proteins (Figure 1) [Methodieva *et al.,* 1999; Cilliers & Singleton, 1991, Hotta *et al.,* 2001].

The structure of polyphenols and their ability of redox cycling plays important role in formation of covalent bonds. Ability to auto-oxidize and hence to have pro-oxidant effect is directly related to the presence of (a) a catechol group on the B-ring (b) the 2, 3-double bond in conjugation with a carbonyl group at 4-position in the C-ring, and (c) the presence of hydroxyl groups at the 3 and 5 position (Figure 2) [Rice-Evans *et al.,* 1998; Bors *et al.,* 1990].

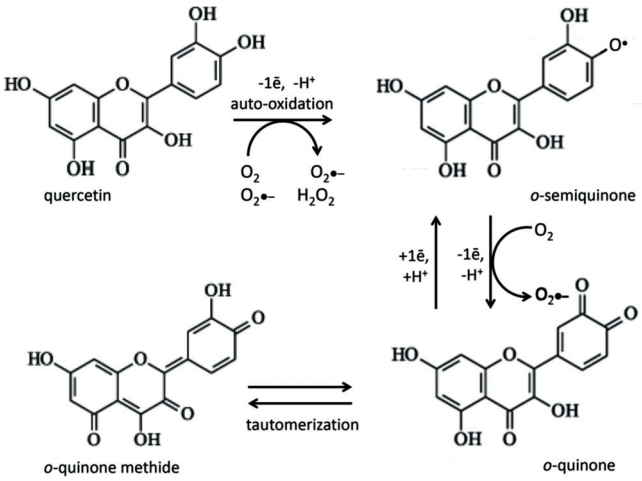

FIGURE 1. A simplified outline of auto-oxidation of quercetin to *o*-semiquinone radical and quinone by two subsequent electron transfers. *O*-semiquinone radical can react with molecular oxygen and give rise to a superoxide anion radical ($O_2^{\bullet -}$), producing pro-oxidant effect. Quinones and subsequent quinone methides react with proteins.

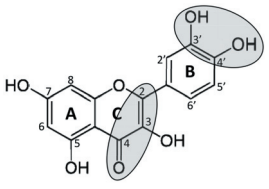

FIGURE 2. Structural determinants of flavonoids contributing to antioxidant and proxidant activities as shown in the molecule of quercetin: essential OH groups, 2, 3 –double bond and carbonyl-group at 4 position of C ring are highlighted.

In addition to binding the nucleophilic amino acids groups, semiquinone radicals may undergo radical-radical reactions between themselves generating oligomers and polyphenol polymers [Cilliers & Singleton, 1991; Hotta *et al*., 2001; Bors *et al*., 2004]. Such radical-induced reactions underlie dimerization and oligomerization of caffeic acids [Bors *et al.,* 2004]. These oligomeric forms that possess multiple catechol rings have been shown to be much more effective radical scavengers than monomeric polyphenols [Bors *et al.,* 2004].

Thus, polyphenol auto-oxidation generates irreversible, covalent bonds with proteins and other polyphenols. The new structures acquire either increased antioxidant (scavenging free radicals) as in a case of caffeic acid oligomers or pro-oxidant capacities (generating free radicals) as in a case of quinone-protein complexes. Once the polyphenol auto-oxidation is initiated, it proceeds spontaneously with time.

"PROTEIN-TYPE" AND "POLYPHENOL-TYPE" COMPLEXES IN HONEY

In honey, the interaction between protein and polyphenols increases with time of storage and after heat treatment. The nature of protein-polyphenol complexation was studied using size-exclusion chromatography, polyacrylamide gel electrophoresis, ORAC and LC-ESI-MS [Brudzynski & Miotto, 2011a, b; Brudzynski *et al.,* 2013]. The implementation of this methodology allowed separation of honey complexes onto two groups that differed in size, stoichiom-

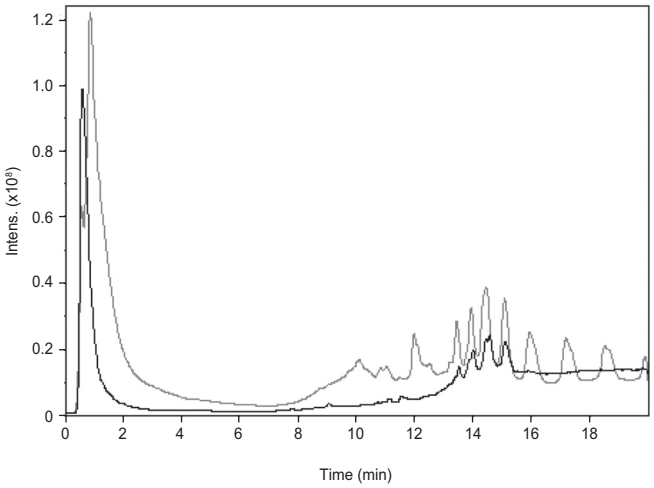

FIGURE 3. Total ion current chromatograms (TICs) in the negative ion mode for LC-ESI-MS analysis of "protein-type" (upper line) and "polyphenol-type" (bottom line) complexes isolated from honey.

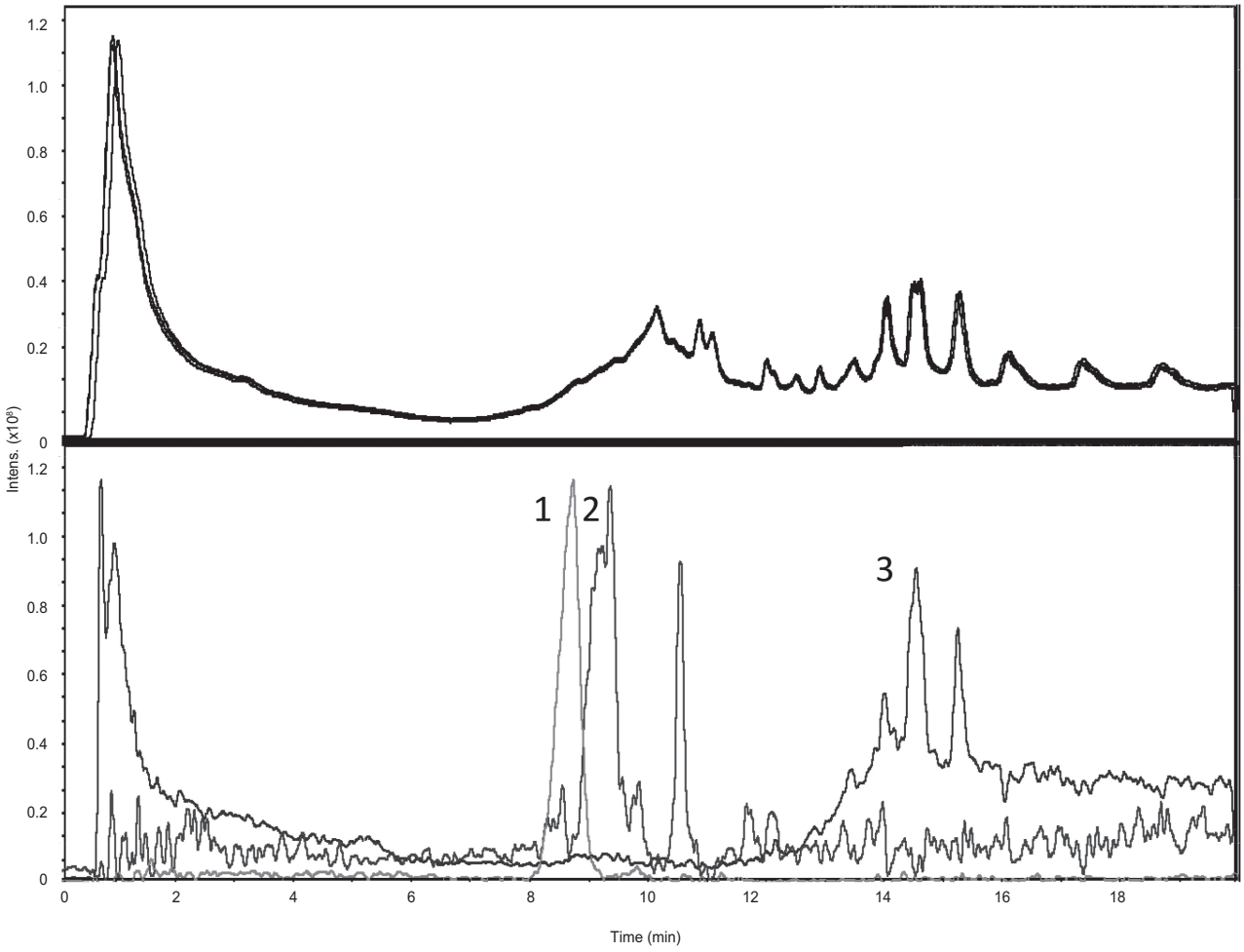

FIGURE 4. Extracted ion mass chromatogram of "protein-type" complexes against target mass ions of *p*-coumaric acid m/z 163 (peak 1), ferulic acid m/z 193 (peak 2), and caffeic acid m/z 179 (peak 3) (lower panel). The position of extracted mass ions is related to the total ion current chromatogram (upper panel).

etry and antioxidant/pro-oxidant capacity. Considering data on browning indexes, characteristic UV spectra (200 nm to 400 nm), color, molecular size, and ORAC values obtained before and after heat-treatment, the complexes were identified as melanoidins [Brudzynski & Miotto, 2011a, b].

Determination of the total protein and phenolic contents in these complexes provided a general assessment of the stoichiometry of the reactants expressed as percentage of the total mass. The stoichiometry results, together with SDS-PAGE, revealed that high molecular weight complexes (230–180 kDa) were enriched in proteins ("protein-type" complexes) while lower molecular size complexes (110–85 kDa) were enriched in polyphenols ("polyphenol-type" complexes) [Brudzynski *et al.,* 2013].

In addition to differences in size and protein to polyphenol ratio, the complexes presented different polyphenol profiles in LC-ESI-MS. The direct comparison of the total ion currents (TICs) during 20 min elution showed that "polyphenol-type" complexes were lacking hydrophilic compounds that were contributing to mass spectrum at retention times of 7 to 13 min (Figure 3) [Brudzynski & Miotto, 2011b]. Furthermore, screening of mass ion profiles against target mass ions of caffeic (m/z 179), ferulic (m/z 193) and *p*-coumaric (m/z 163) acids demonstrated that these simple, monophenolic acids were absent in "polyphenol-type" complexes but occurred in "protein-type" complexes (Figures 6 and 4, respectively). The phenolic acids, specifically caffeic acid, appeared as unknown conjugates that were eluted at longer retention time than RT=4.19 min expected for a standard caffeic acid monomer under the chromatographic conditions used (Table 1). We have noted however that caffeic acid mass ion of m/z 179 co-eluted together with m/z 253 (chrysin) at RT 13.9 min, m/z 255 (pinocembrin) at RT=14.4 and with m/z 283 (acetatin) at RT 15.2 (Figure 4). Although interesting, these very early results do not warrant any suggestion of possible association between above-mentioned flavonoids and caffeic acid in honey. The same screening method used against target mass ions of pinobanksin (m/z 271), pinocembrin (m/z 285), chrysin (m/z 253) and acacetin (m/z 283) supported the presence of these flavonoids in both types of complexes (Figures 5 and 6). These polyphenols have been chosen as typical representatives of phenolics in honey [Pyrzynska & Biesaga, 2009]. Their identification was based on a comparison with mass ions and retention times of standards under chromatographic conditions described previously (Table 1) [Brudzynski & Miotto, 2011]. The low polarity of "polyphenol-type" complexes (longer elution times) indicated their hydrophobic nature.

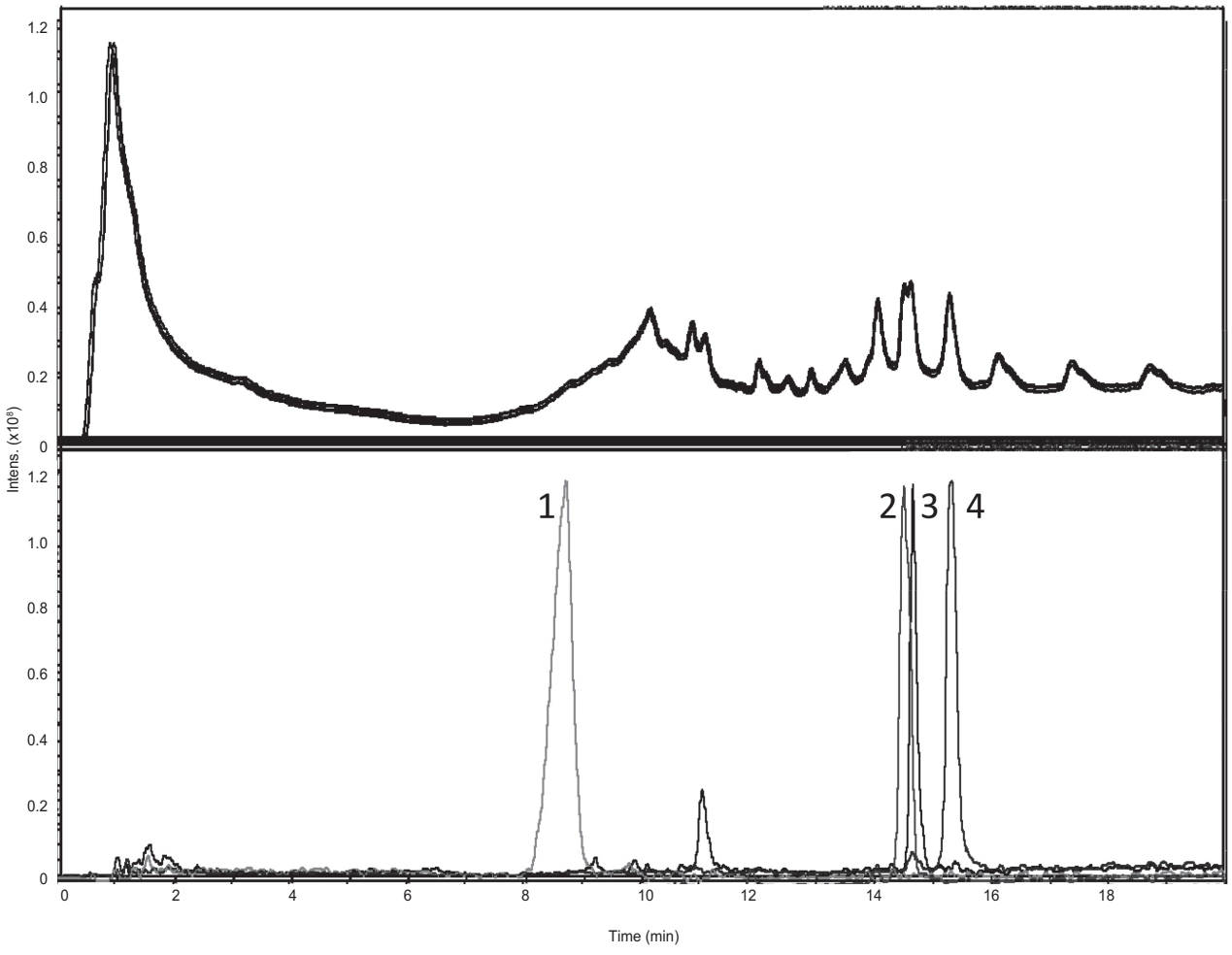

FIGURE 5. Extracted ion chromatogram of "protein-type" complexes against target mass ions of *p*-coumaric acid m/z 163 (peak 1), chrysin m/z 253 (peak 2), pinocembrin m/z 255 (peak 3) and acecatin m/z 283 (peak 4) (lower panel). The upper panel presents the TIC.

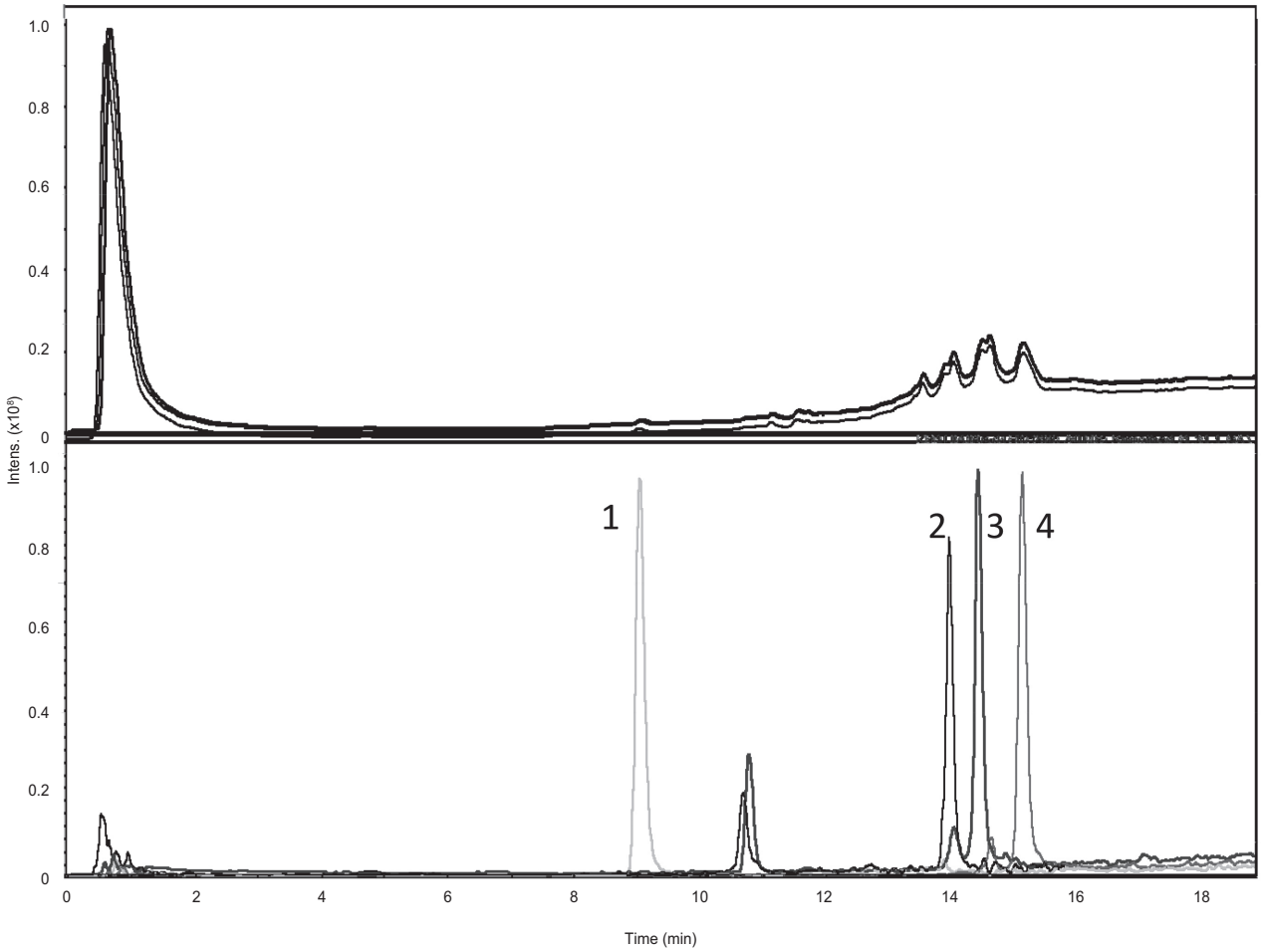

FIGURE 6. Extracted ion chromatogram of "polyphenol-type" complexes against target mass ions of pinobanksin m/z 271 (peak 1), chrysin m/z 253 (peak 2), pinocembrin m/z 255 (peak 3) and acecatin m/z 283 (peak 4). The upper panel present the TIC of "polyphenol-type" complexes.

Another feature differentiating "protein-type" from "polyphenol-type" complexes was the level of their antioxidant/pro-oxidant capacity. The radical scavenging activity of "protein-type" complexes was up to 2.5 fold higher than that of "polyphenol-type" complexes in unheated honeys and increased up to 29.5 fold after heat-treatment of honey, as indicated by ORAC values [Brudzynski & Miotto, 2011b]. Polyphenols present in "protein-type" and "polyphenol-type" complexes also showed a distinct susceptibility to auto-oxidation. This was determined colorimetrically using a dye, NitroBlue Tetrazolium (NBT). In the presence of redox-active quinones, the NBT was reduced producing the purple formazan [Paz *et al.*, 1991]. The reaction proceeds under alkaline conditions in the presence of glycine as a reducing agent that reduces quinones to hydroquinones. Hydroquinones in turn, reduce the NBT to formazan. Thus, the intensity of formazan staining directly correlated with the levels of quinone-protein complexes that underwent reduction to hydroquinones. By employing this method, it occurred that "protein-type" complexes possessed a limited reducing activity toward NBT and therefore had less quinones/ hydroquinones, while "polyphenol-type" complexes strongly reduced the dye [Brudzynski *et al.,* 2013].

The importance of these differences is implicit in the fact that in "protein-type complexes" the reduced, non-oxidized polyphenols may carry antioxidant activity, while the oxidized polyphenols of "polyphenol-type" complexes may contribute to pro-oxidant effects. These interesting preliminary observations need to be further thoroughly investigated.

FUNCTIONAL CONSEQUENCE OF PROTEIN--POLYPHENOL COMPLEXATION: LOSS OR GAIN OF FUNCTION

Enzyme inactivation

The interactions of proteins and polyphenols influence the structural and functional properties of both molecules. The covalent interactions of proteins with polyphenols may be responsible for the inactivation of honey enzymes; alpha-amylase (diastase), alpha-glucosidase, and D-fructofuranoside-fructohydrolase, invertase [White *et al.,* 1964; Huidobro *et al.,* 1995; Oddo *et al*., 1999; Semkiw *et al*., 2010]. Although such relationship in the case of honey enzymes has not yet been established, other studies showed a clear connection between polyphenol bindings and inhibition of variety of enzymes [Haslam, 1996; Harborne & Williams, 2000; Narayana *et al.,* 2001; Charlton *et al*., 2002]. Honey has also been shown to inhibit polyphenol oxidase activity and enzymatic oxidation of polyphenols [Oszmianski & Lee, 1990].

TABLE 1. Summary of retention times of polyphenolic standards and polyphenols in "protein-type" and polyphenol-type" complexes.

Polyphenol	[M-H] -m/z	Chemical structure	Standard Retention Time (min)	Protein-type compounds Retention Time (min)	Polyphenol-type compounds Retention Time (min)
Caffeic acid	179	3,4-Dihydroxy-cinnamic acid	4.19	13.9; 14.5; 15.1	
p-coumaric acid	163	4-hydroxycinnamic acid	8.7	8.7	
Ferulic acid	193	3-methoxy-4-hydroxycinnamic acid	9.5	9.5	
Pinobanksin	271	3,5,7-trihydroxyflavanone	9.1	9.1; 11.0	9.1
Pinocembrin	255	5,7-dihydroxyflavanone	14.4	14.4;13.9	14.4;10.7
Chrysin	253	5,7-dihydroxyflavone	14.0	14.0	14.0
Acacetin	283	5,7-Dihydroxy-4'-methoxyflavone Apigenin-4'-methylether	15.1	15.2	15.1; 14.6

Changes in antioxidant capacities

With respect to polyphenols, their binding to proteins usually causes a decrease of their antioxidant activities [Rohn & Rawel, 2004]. Often, the antioxidant capacities of protein--polyphenol complexes were found to be lower than the sum of the antioxidant capacities of individual components [Arts *et al*., 2001]. However, in contrast, antioxidant and metal chelating activities were shown to be retained by melanoidins of beer [Morales & Jimenes-Perez, 2004], bread crust [Michalska *et al.,* 2008], vinegar [Tagliazucchi *et al.,* 2010] and wines [Lopez de Lerma *et al*., 2010].

In honey, the incorporation of polyphenol-protein complexes into melanoidin was responsible for both gain and loss of melanoidins' antioxidant activity [Brudzynski & Miotto, 2011a]. In unheated honeys, the radical scavenging activity of melanoidins depended on the initial content and antioxidant activity of polyphenols. In light- and medium-colored honeys possessing low ORAC values, heat treatment caused a strong increase in antioxidant activity of melanoidins with the concomitant accelerated formation of high molecular weight melanoidins. Melanoidins of light- and medium honeys gained the antioxidant capacity. In contrast, heat treatment of dark, buckwheat honeys, that possessed a high polyphenol content and high initial antioxidant activity, resulted in an overall reduction of ORAC values and a decrease of polyphenol content in melanoidin complexes. Melanoidins of dark honey lost the antioxidant capacity. As a result, much stronger correlation was observed between phenolic content and antioxidant capacity of melanoidins in unheated honeys than in heated honeys ($R=0.89$, $p<0.0002$ and $R=0.72$, $p<0.007$, respectively) [Brudzynski & Miotto, 2011].

Evidence that thermal treatment of honey did not produce uniform effects on antioxidant capacity of honeys was also reported by other researchers [Turkmen *et al*., 2006; Šarić *et al*., 2013]. It has to be appreciated that heat-treatment of honey accelerated the global reduction-oxidation pathways, and several products of the Maillard reaction could contribute to the antioxidant capacities, in addition to polyphenols. We have also noted that heat-treatment of buckwheat honeys caused accelerated formation of very large, insoluble, brown aggregates that precipitate out of solution and these events coincided with the reduction of antioxidant capacities of soluble melanoidins [Brudzynski & Miotto, 2011b].

Our preliminary observations of two types of protein--polyphenol complexes, the "protein-type" and "polyphenol-type", carrying antioxidant and pro-oxidant capacities in honeys requires further, thorough investigation. However, the existence of these complexes may provide a previously unrecognized link through which the polyphenol interaction with proteins could directly impact either antioxidant or pro-oxidant capacities of food products.

Inhibition of antibacterial activity

An important functional consequence of protein-polyphenol interaction is the loss of honey's antibacterial activity during storage [Brudzynski & Kim, 2011]. Polyphenols alone have been shown to contribute to antibacterial activity by binding and inactivating proteins of crucial importance for bacterial survival [Haslam, 1996; Cushnie & Lamb, 2005]. In honey, such role has been assigned to pinocembrin [Bogdanov, 2011].

However, the highest relevance to antibacterial activity of honey might be related to polyphenol oxidation and the propensity of quinones to react with proteins. Probable targets for quinone bindings on the bacterial cell are proteins of cell envelope and cell wall, surface-exposed adhesins and membrane-bound enzymes [Cowan, 1999]. The decrease of honey's antibacterial activity during storage could be explained by increasing complexation of quinones with honey proteins that in turn, reduced quinone availability to react with bacterial proteins. This explanation found a partial support in the observation that the decline in antibacterial activity during storage coincided with increasing formation of melanoidins as indicated by a significant browning ($p<0.0025$), increased concentration of UV-absorbing compounds ($p<0.0001$), and the appearance of polymeric structures [Brudzynski & Kim, 2011].

CONCLUDING REMARKS

From these results, a picture emerges that redox properties of polyphenols are at the center of polyphenol-protein interactions in honey. The polyphenol auto-oxidation leads to formation of irreversible, covalent bonds between quinones and proteins. The increased complexation of proteins by polyphenols during honey storage correlated with a loss

of enzyme activities, a decrease of antibacterial activity and a change of the balance between antioxidant-pro-oxidant capacities. In relation to the latter, we presented data on previously unrecognized types of protein-polyphenol complexes that differed in size, stoichiometry, and antioxidant capacities. The possible implication of this finding is that "polyphenol--type" complexes could carry mostly pro-oxidant activity due to presence of oxidized polyphenols while "protein-type "complexes could be responsible for antioxidant activity. The differences in redox cycling between these two types of complexes could partially explain a loss or gain of antioxidant capacities observed in different honeys after heating. Another new hypothesis presented here is a potential association between honey flavonoids and polyphenols such as association of caffeic acid with flavones-, chrysin and acecatin, and flavanone-, pinocembrin. A phenomenon of co-pigmentation between anthocyanins with caffeic, coumaric, chlorogenic, sinapic, and ferulic acids is well recognized in wines [Boulton, 2001]. The importance of co-pigmentation in wines is in the protection of anthocyanins from oxidation and that it could also merit study in honey. Finally, the environmental factors are significantly implicated in honey structure and function by modulating oxidation-reduction reactions in honey.

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