IJFANS
International Journal of Food And Nutritional Sciences

Volume 4, Issue 1, Jan-Mar 2015, www.ijfans.com e-ISSN: 2320-7876

INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES

IMPACT FACTOR ~ 1.021

Official Journal of IIFANS

e-ISSN 2320 –7876 *www.ijfans.com*
Vol.4, Iss.1, Jan-Mar 2015

Research Paper *Open Access*

DETERMINATION OF INTERACTION BETWEEN SOME PLANET TANNINS AND MILK PROTEINS BY HPLC

Tamer Mohammed El-Messery[1*], Hala Mohamed Faker El-Din[1], Nayra Shaker Mehanna[1], Ali Abd-Elaziz Ali[2], Zakaria Mohamed Rezk Hassan[2], Ryszard Amarowicz[3]

[1]Dairy Science &Technology Department, National Research Center, Egypt, [2]Food Science Department; Faculty of Agriculture; Ain Shams University; Cairo; Egypt, [3]Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Division of Food Science, Olsztyn, Poland.

*****Corresponding author: tmelmessery@yahoo.com

Received on: 28th January, 2015 **Accepted on: 30h March, 2015**

ABSTRACT

Interaction between tannins fraction from different plants sources with milk proteins (β-casein, κ-casein, β-lactoglobulin (β-Lg) and α-lactoalbumin (α-La)) were determined by HPLC method with a diode array UV detector. The tannin fraction content was isolated from walnuts and green tea and authentic tannic acid. Tannic acid was the highest interaction with β casein and κ casein more than other tannins, in the same time tannins (green tea) was interaction with β casein and κ casein more than tannins (walnut). On the other hand tannic acid was the highest interaction with (β-Lg) and (α-La) more than other tannins, in the same time interaction tannins (walnut) with β-Lg was more than tannins (green tea), but interaction tannins (green tea) with α-La was more than tannins (walnut) but not significant. HPLC technique suitable to allows knowing which protein fraction interact with tannins.

Keywords: Tannins, Interaction, Milk proteins and HPLC method.

INTRODUCTION

Tannins, which are polymers of phenolic compounds, have been investigated for their interactions with proteins (Serafini *et al* 1997). Phenolic phytochemicals are abundant micronutrients in fruits and vegetables, and there is a growing body of evidence regarding their health benefits (Manach *et al* 2005). The role of food proteins in nutrition and health has long been well established. More recently, the roles of phenolic compounds in human nutrition and health have become known (Singh 2011). Consequently, investigations on protein-phenolic interactions have become the study of interest in many areas of food, nutrition and health. Polyphenols have been known to confer beneficial effects by acting as antioxidants; however they can also be harmful (Haslam 2000). Tannins consist of a polyhydric alcohol, such as glucose, to which is linked gallic acid or its dimer hexahydrodiphenic acid in ester linkages. Considerable structural variation is introduced by additional molecules of gallic acid linked depsidically to other gallic acid moieties. As the name implies, these compounds are easily hydrolyzed in alkali, giving rise to a polyhydric alcohol and gallic acid, in the case of gallotannins, or ellagic acid, the condensation product of hexahydrodiphenic acid, in the case of ellagitannins. An example of hydrolyzable tannin is the tannic acid pentagalloyl glucose (Paul *et al* 2002). Tannins are polyphenols that occur widespread in plant-based food. They are considered to be part of the plant defence system against environmental stressors. Tannins have a number of effects on animals, including growth-rate depression and inhibition of digestive enzymes. Tannins also have an effect on humans: They are, for example, the cause of byssinosis, a condition that is due to exposure to airborne tannin. Their biological effect is related to the great efficiency by which tannins precipitate proteins (Bennick 2002). Interactions between proteins and polyphenols can be reversible associations (via hydrogenbonding, hydrophobic interactions and van der Waals forces) that alter the solution properties of proteins, or can be permanent modifications of proteins. Reversible associations may or may not result in protein precipitation, dependent on factors such as ionic composition of solution and pH. Protein precipitation that occurs at low ratios of protein to polyphenolic may be reversed as the ratio increases (Luck *et al* 1994). Formation of tannin-protein complexes are based on the strong hydrophobic and hydrogen bond, tannin contain phenolic group that act as an excellence hydrogen donor, when tannin interacts with protein, the phenolic group will form a strong hydrogen bond with the carboxyl group in protein, the bonding formed is specific and depend on the structure of the protein and tannin (Antonello 2009). HPLC is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying or purifying the individual components of the mixture. By far, HPLC is the most popular technique used currently. Commonly used HPLC methods are based on reversed phase (Andrew *et al* 2003). HPLC method with a diode array UV detector can

apply for detection of phenolic compounds bound to protein (Karamać *et al* 2007). Tannins can bind to proteins through a variety of mechanisms such as a hydrogen bonding, hydrophobic interactions, and ionic bonding (Rubino *et al* 1996).

The aim of the study was to know characterize interactions between tannins fraction from different plants sources and milk proteins by HPLC.

MATERIALS AND METHODS

REAGENTS

All solvents were of HPLC grade and Trifluoroacetic acid, acetonitrile and acetic acid, were obtained from P.O.Ch. Company (Gliwice, Poland) or Merck (Darmstadt, Germany). Sephadex LH-20, tannic acid, vanillin, ferric chloride Sodium mono basic and Sodium di basic was purchased from Sigma–Aldrich Co. Ltd. (Poznan´, Poland). All other reagents were of at least ACS grade and were acquired from P.O.Ch. Company (Gliwice, Poland) Tannins (Elgin, IL, USA).

MATERIAL

Green tea and walnut were purchased in Olzans-CN LLC (Olsztyn, Poland), where as casein was precipitated from buffalo skim milk (Giza, Egypt) by acid. β-casein and κ-casein was purchased from Sigma–Aldrich Co. Whey protein isolate (WPI) was obtained from Davisco Foods International Inc., (Minnesota, USA).

EXTRACTION AND FRACTIONATION

The ingredient were ground in a coffee mill (BSH Bosch & Siemens Hausgera¨te GmbH, Munich, Germany) into fine powder (particle size<0.8 mm). A 60-g portion of green tea and walnut powder was extracted using 80% (v/v) aqueous acetone at a solid-to-solvent ratio of 1:8 in a thermostatic shaking water bath (357 Elpan, Lubawa, Poland), at 60°C for 15 min. Then, the supernate was filtered through filter paper and the extraction step was repeated twice more. The supernatants evaporated using Büchi Rotavapor R-200 (Büchi Labortechnik, Flawil, Switzerland) at 40°C, and aqueous residue was lyophilized (Lyph Lock 6 freeze dry system, Labconco, Kansas City, MO, USA). One gram of crude extract of green tea, lentil and walnut phenolic compounds was suspended in 10 mL of 96% (v/v) ethanol and applied onto a chromatographic column (30 mm *i.d.* x 230 mm) packed with lipophilic Sephadex LH-20 gel (Sigma– Aldrich). Firstly, low molecular weight phenolics were eluted gravimetrically using 96% (v/v) ethanol, and then, solvent was changed over to 50% (v/v) acetone in order to elute tannins. Acetone from tannin fraction was evaporated, and remaining water was lyophilized (**Kosińska *et al* 2011)**.

DETERMINATION INTERACTION B AND K CASEIN WITH TANNINS BY HPLC

SAMPLE PREPARATION

β and κ casein solution were prepared within (5 mg β and κ casein powder/ 1 ml 0.1 M Sodium phosphate buffer pH 5), Tannins solutions (tannic acid, green tea and walnut) were prepared (5 mg/ 1 ml water), and mixed two solution with ration 1:1 according to Ali 2002.

HPLC ANALYSIS

Interaction between tannins and casein fractions were determined using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a LC-10AD pump, SCTL 10A system controller and SPD-M 10A photo-diode array detector, software Class VP. Samples were performed by a TOSOHAAS, Exclusion chromatography column gel filtration, size of party of gel (7.8 mm x 30 cm, 5 µm) , size of column (7.8 mm x 30 cm, 6 µm). The mobile phase was: 0.1 M Sodium phosphate buffer pH 7, change from 50 min. Flow rates were: 1 ml/ min. The detection was monitored at 220 nm, and injection volume 20 µl, samples were filtration with whatman 0.45 µm.

DETERMINATION INTERACTION WHEY PROTEIN WITH TANNINS BY HPLC

SAMPLE PREPARATION

Whey proteins solution were prepared (1 mg whey protein powder/ 1 ml 0.1 M Sodium phosphate buffer pH 6), Tannins solution (tannic acid, green tea and walnut) were prepared (1 mg/ 1 ml water), and mixed two solution with ration 1:1.

HPLC ANALYSIS

Interaction between taninns and whey protein determined by a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of two pump (LC-10AD), SCTL 10A system controller and SPD-M 10A photo-diode array detector. Samples were performed by a Hypersil Bc C18 column (4.6 x 250 mm, 5 µm; Germany). The mobile phases were: A (5% acetonitrile-acetic acid + 0.1% trifluoroacetic acid) and B 0.06% trifluoroacetic acid, changes from 50 min. Flow rates were: 1 ml / min. The detection was monitored at 200 nm, and injection volume 20 µl, samples were filtration with whatman 0.54 µm PTFEW/GMF.

TOTAL PHENOLICS CONTENT (TPC)

The TPC of tannin fractions from plants material extract was determined using colorimetric assay with Folin-Ciocalteu phenol reagent according to Naczk and Shahidi 1989. Briefly, 0.25 ml of methanolic solution of tannin fractions was mixed with 0.25 ml of Folin-Ciocalteu reagent (diluted 1:1 with distilled water), and then, 0.5 ml of sodium carbonate saturated solution and 4 ml of water was added, and mixture was vortexed thoroughly (Genie2, Scientific Industries, Bohemia, NY, USA). Absorbance at 725 nm after 30 min color development was measured with Beckman DU-7500 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with prior centrifugation of samples. TPC was expressed as mg tannic acid equivalents per gram of extract or fraction from triplicate measurements.

CONDENSED TANNINS CONTENT

The method of Price *et al* 1978 was employed to determine condensed tannin fractions. Methanolic solutions of samples (1ml) at a concentration of 0.25 mg/ml were mixed with 5mL of vanillin reagent (obtained by dissolving of 0.5g vanillin in 100ml 4% (v/v)

concentrated hydrochloric acid). The absorbance of the mixture was measured after a 20-min period of reaction development at 500 nm using Beckman DU-7500 spectrophotometer. Due to the lack of appropriate standard for this assay, the results were expressed as absorbance units per g of sample (A500/g).

RESULTS AND DISCUSSION

THE TOTAL PHENOLIC CONTENT FOR TANNINS FRACTION

Phenolics are broadly distributed in the plant

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer (Dai and Mumper 2010, Nazni.P and Dharmalingam.R, 2013). Table 1 show that extracts tannin from walnut was the higher in phenolic content than that extract tannin from green tea.

THE CONDENSED TANNINS CONTENT

Condensed green tea tannins showed higher value of 0.360 than that of walnut of 0.076 (A500/g).

Table 1: **Total phenolic content and condensed tannin content of tannin fractions.**

	Total phenolic content (mg tanninc acid equivalents/g)	Condensed tannins content (A500/g)
Tannin from green tea	393.0109 ± 12	0.360
Tannin from walnut	877.4204 ± 22	0.076

INTERACTIONS BETWEEN TANNINS AND MILK PROTEINS USING HPLC

INTERACTIONS BETWEEN B AND K CASEIN WITH TANNINS BY HPLC

Fig. (1A) showed sharp peak for HPLC chromatogram of β-casein standard, and appear anther sharp peak of complex for β-casein-tannic acid at retention time 5.18 min. in the same figure tannic acid standard and tannic acid residual from interaction between β-casein and tannic acid appear as sharp peak at retention time 12.51 min.

Retention time of β-casein standard peak was 5.18 min. When the detector was set at 220 nm, and in the same time appear complex for β-casein-tannins (walnut) but tannins (walnut) standard and tannins (walnut) residual from interaction between β-casein and tannins (walnut) appear at time 12.3 min. Fig. (1B).

The chromatogram of complex β-casein-tannins (green tea) was characterised by the main peak with retention time of 5.24 min. and tannins (green tea) residual was observed on chromatogram at longer retention time of 13.4 min. Fig. (1C).

Fig. (2A) depicts a chromatogram of pure κ-casein. Retention time of this protein was 5 min. at same time appear κ-casein residual from interaction between κ-casein and tannic acid , in the same figure also appear tannic acid standard and tannic acid residual from this interaction at retention time at 12.3 min.

κ-casein standard and κ-casein residual from interaction κ-casein with tannins (green tea) were resolved under the chromatographic conditions at retention time 5 min. and observed tannins (green tea) residual from this interaction at time 14 min. Fig. (2B).

Fig. (2C) showed representative chromatograms obtained of κ-casein standard and κ-casein residual from interaction κ-casein with tannins (walnut) at retention time 5 min. Their retention times longer than that of tannins (walnut) standard and tannins (walnut) residual from this interaction at 12.3 min.

Interaction between β-casein and tannins was determined by a HPLC method, Figs. 1 A, 1B and 1C showed peaks for β-casein standard and comparison this peaks with complex β-casein-tannins and determined interaction within increasing percentage sample after interaction, while interaction between κ-casein and tannins was determined within percent of protein in the sample after incubation with tannins, Fig. (2A, 2B and 2C).

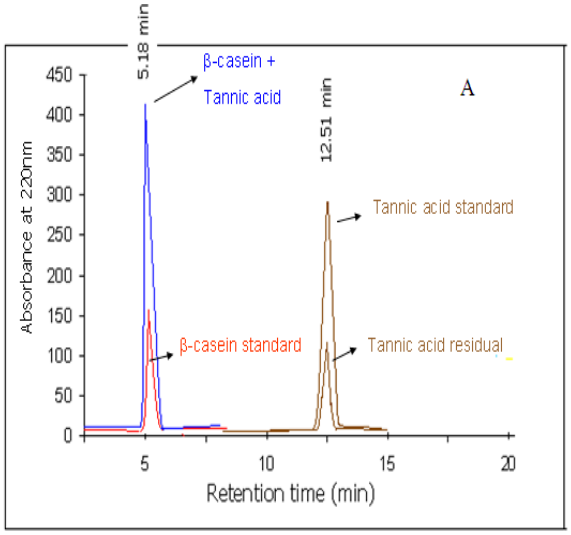
Tannic acid was highest interaction with β-casein showed higher interaction with tannic acid of 51.8% followed by green tea tannins of 11.2% and the lowest interaction was showed with walnut tannins of 9.8% as shown in table 2. Percent of interactions between κ-casein-green tea tannins was showed highest interaction of 71.4% followed by tannic acid was 44% and of 1.1% with walnut-tannins (Table 2).

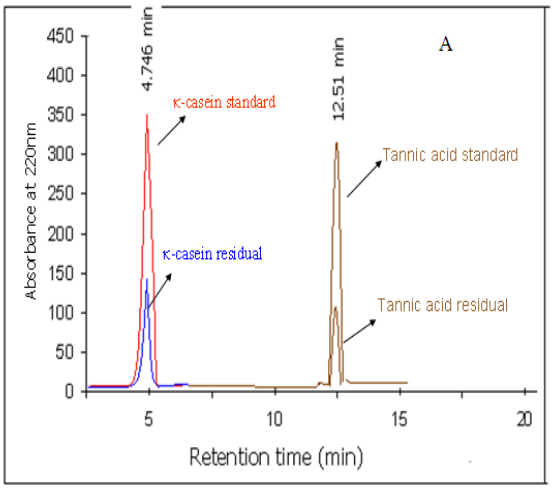
Table 2: Percent of interaction between β- and κ-casein with tannins

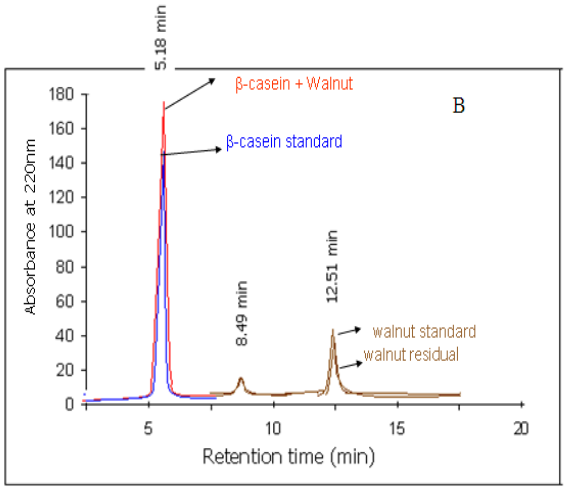
Tannins	β-casein	κ-casein
Tannic acid	51.8%	44%
Walnut	9.8%	1.1%
Green tea	11.2%	71.4%

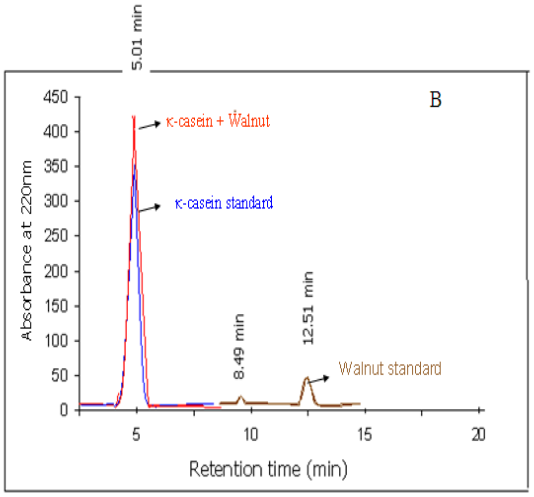
A majority of UV spectra of β-casein standard recorded as peaks on the SE-HPLC chromatograms was characterized by a maximum at 278 nm. Two maximum peaks, at 284 and 326 nm were noted for complex β-casein and tannic acid, while maximum peak for complex β-casein and tannins (green tea) was 280 nm. Whereas without maximum peaks for β-casein and tannins (walnut), Fig. (3).

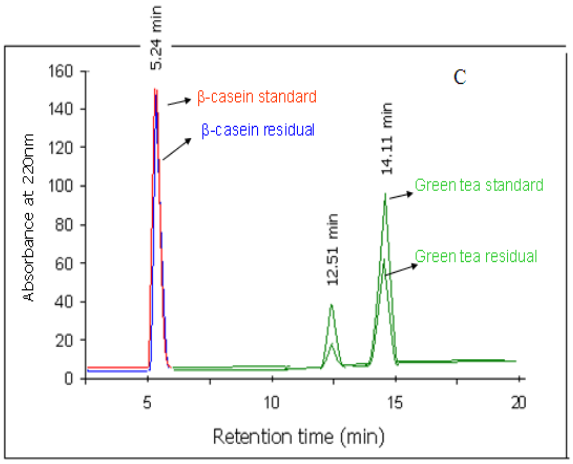
The maximum peak of UV spectra of κ-casein standard was 280 nm. Spectra of κ-casein after interaction with tannic acid was two maximum peaks at 280 and 310 nm., while without maximum peak for κ-casein after interaction with tannins (walnut). Two maximum peaks for κ-casein after incubation with tannins (green tea) was 278 and 324 nm. Fig. (4).











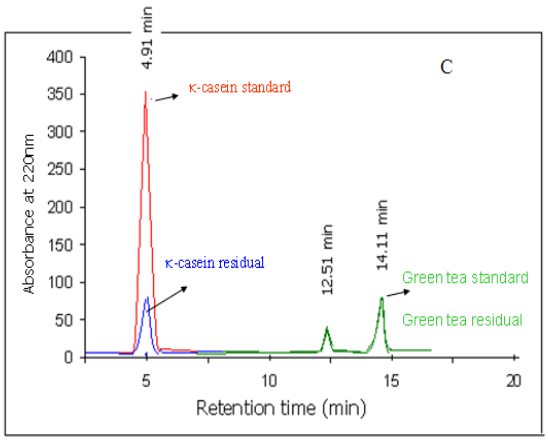


Fig. 1: HPLC chromatogram of β-casein standard and after interaction with tannins.

Fig. 2: HPLC chromatogram of κ-casein standard and after interaction with tannins

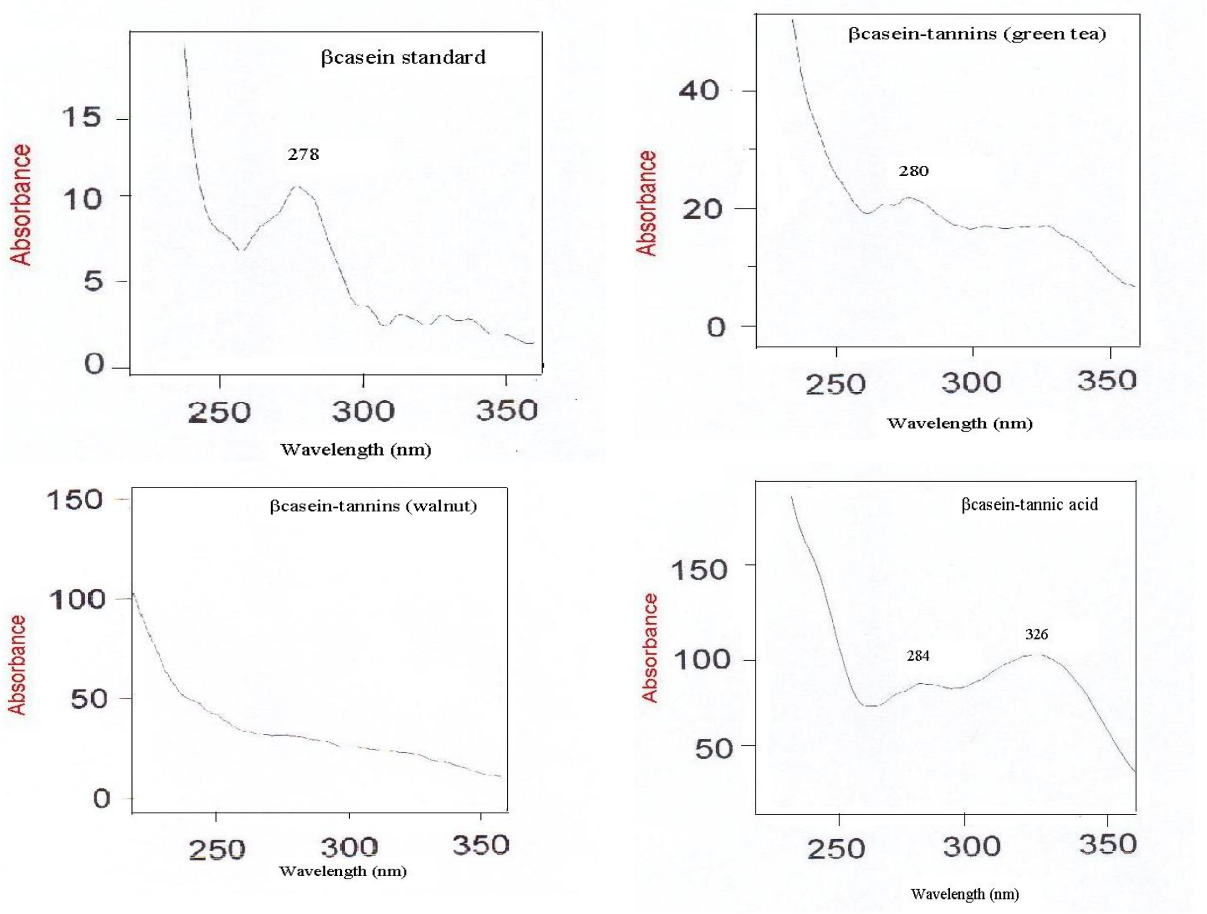


Fig. 3: UV spectra of complex between β-casein and tannins using HPLC method

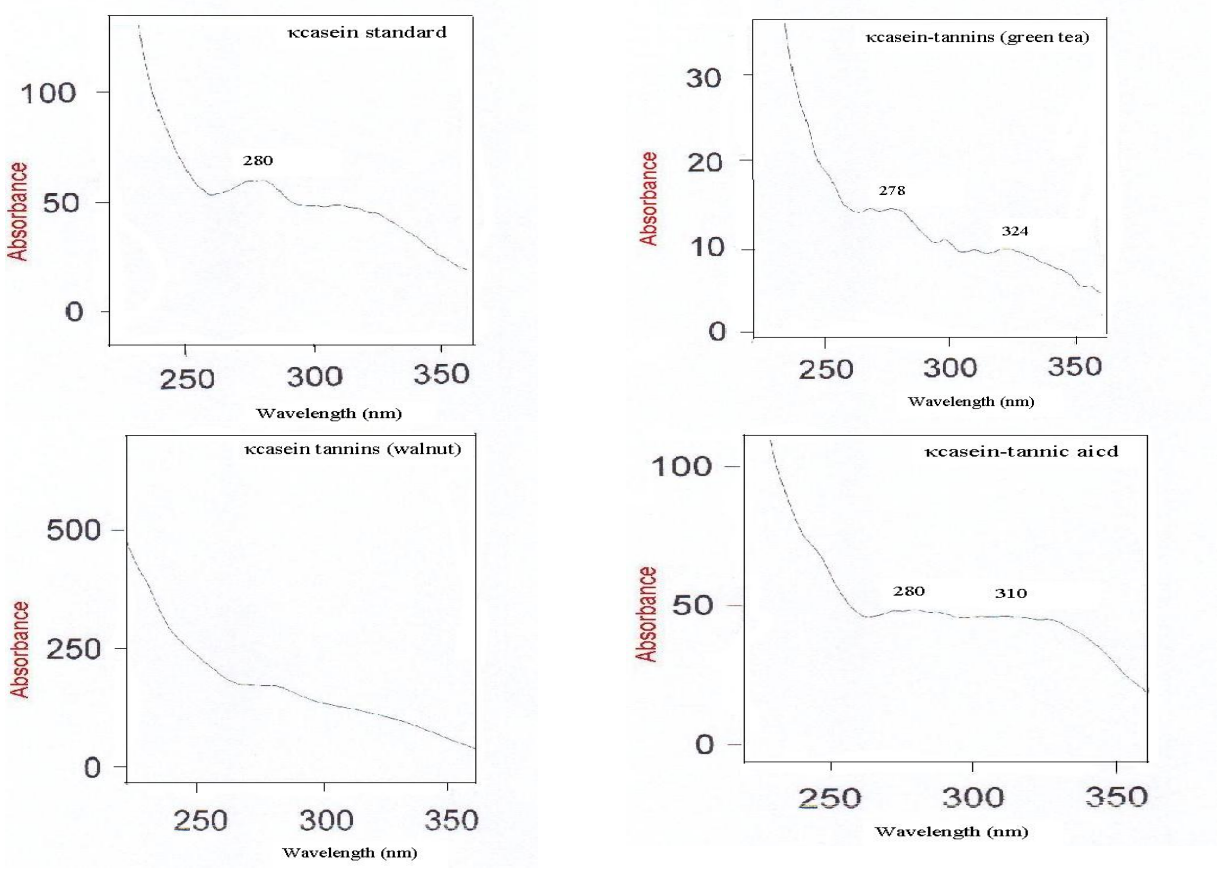


Fig. 4: UV spectra of complex between κ-casein and tannins using HPLC method

Table 3: Percent of protein in the sample after incubation with tannins

	Whey proteins		
Tannins	**β-Lg (a)**	**β-Lg (b)**	**α-La**
Tannic acid	94.4	86.6	60.2
Walnut	41.2	51	15.7

Table 4: Percent of tannins in the sample after incubation with whey proteins

Tannins	**α-La**	**β-Lg (b)**	**β-Lg (a)**
Tannic acid	39.8±6.2	13.4 ±5.6	5.6 ±3.7
Walnut	84.3 ±10.2	49.0 ±7.4	58.8 ±13.4
Green tea	79.0 ±6.7	49.3 ±10.4	80.6 ±12.3

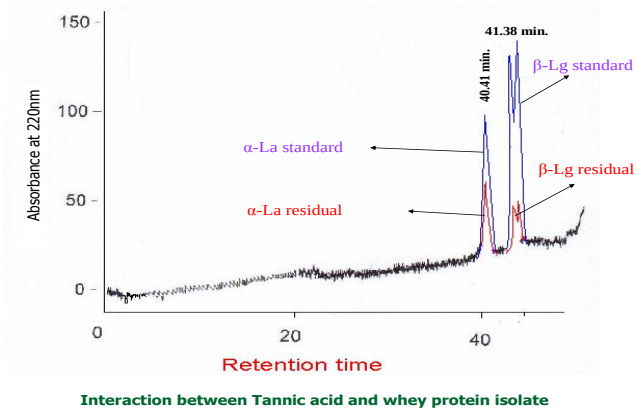


Fig. 5: HPLC chromatograms of α-lactalbumin and β-lactoglobulin standard and after interaction with tannic acid

INTERACTIONS BETWEEN WHEY PROTEIN ISOLATE AND TANNINS BY HPLC

Fig. (5) illustrated a HPLC chromatograms of α-lactalbumin and β-lactoglobulin as a standard and after interaction with tannic acid at retention time 40 and 42 min respectively.

Interaction of α-lactalbumin and β-lactoglobulin with other tannins (Walnut and green tea) were appear as two peaks at HPLC chromatograms in same retention time, Fig. (6 and 7) respectively.

Tannic acid interacted with α-lactalbumin and β-lactoglobulin more than other tannins, while β-lactoglobulin interacted with tannins more than α-lactalbumin. The determination of interaction between whey proteins fractions and tannins was depend on the percent of protein in the sample after incubation with tannins, (Table 3,4).

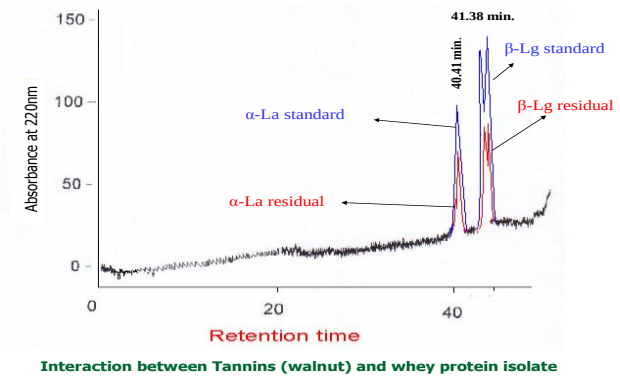


Fig. 6: HPLC chromatograms of α-lactalbumin and β-lactoglobulin standard after interaction with tannins (walnut).

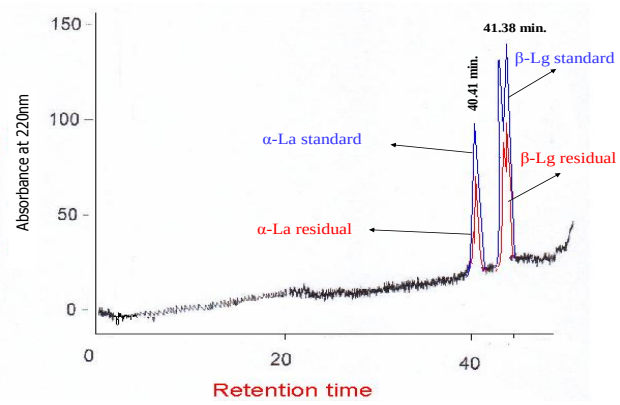


Fig. 7: HPLC chromatograms of α-lactalbumin and β-lactoglobulin standard after interaction with tannins (green tea).

Interaction between tannins and protein depends on structure of phenolic, where some complexes exhibit hydrogen bonding and electrostatic interaction plays a dominant role in the stabilization of the peptide by tannins. The π-OH type of interaction also observed in the peptide stabilization, tannins molecule have been placed appropriately near the side chain groups of the peptide. The functional groups para-OH (ρ-OH) meta-OH (m-OH) and COOH of tannins have been assumed to act as a hydrogen bond donor/acceptor for different side chain groups of amino acid (Madhan *et al* 2001).

The quinones on the rings react to form permanent covalent bonds with compounds such as proteins and sulphur-containing compounds (Singh 2011). These explain the higher interaction evaluation of tanninc acid than other tannins fraction.

Because of the greater electronegativity of oxygen, the carbonyl (C=O) is a stronger dipole than the N-C dipole. The presence of a C=O dipole and, to a lesser extent a N-C dipole, allows amides to act as H-bond acceptors. In primary and secondary amides, the presence of N-H dipoles allows amides to function as H-bond donors as well. Thus amides can participate in hydrogen bonding with water and other protic solvents; the oxygen atom can accept hydrogen bonds from water and the N-H hydrogen atoms can donate H-bonds. As a result of interactions such as these, the water solubility of amides is greater than that of corresponding hydrocarbons (Mehanna *et al* 2014).

Milk proteins have a considerable amount of proline, thehydrophobic attraction between proline and the phenolic group is stabilised by H-bond formation between phenolic ring groups and the bis-alkyl substituted amide

nitrogen of the proline amino group. The interaction is a complex one, with water also likely to play a part (Luck *et al* 1994) and this interaction was non-covalent (Yuksel *et al* 2010).

This may due to the presence of more sulphur containing amino acid (cysteine) in whey protein rather than casein which forming covalent bonds via a quinone mediated mechanism including thiol group (Hassan *et al* 2013).

The hydrophobicity of the surface sites of milk proteins was decreased in the presence of green teaflavanoids. The decrease in protein surface hydrophobicity was explained by the hydrophobic binding between milk proteins and green tea flavanoids (Yuksel *et al* 2010).

CONCLUSIONS

The interaction of tannins fraction isolated from walnut and green tea with milk proteins resulted an insoluble and soluble complexes formation. Similarly, the most extensive HPLC analysis was observed in the interaction between milk protein and tannic acid rather than tannins isolated from walnut and green tea. This must be taken into consideration for the biologicalactivities of tannins from walnut and green tea.

REFERENCES

- Ali H. Protein-phenolic interactions in food. M.Sc. Thesis, Department of Food Science and Agricultural Chemistry, Macdonald campus, McGillb University Monteral, Québec, Canada. 2002.
- Andrew AO, Amarowicz R, Peiquiang Y, John JM, David DMA. Rapid HPLC method for determination of major phenolic acid in plant material. Pol. J. Food Nutr. Sci., 2003; 12/53(1):53-57.
- Antonello C. Tannins: fascinating but sometimes dangerous molecules. Cornell University Department of Animal Science. 2009; [online] from: http://www.ansci.cornell.edu/plants/toxicagents/tannin.html.
- Bennick A. Interaction of Plant Polyphenols with Salivary Proteins. Crit Rev Oral Biol Med. 2002; 13(2):184-196.
- Dai J, Mumper RJ. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules 2010; 15:(7):313-7352.
- Hagerman AE, Butler LG. Protein precipitation method for the quantitative determination of tannins. J Agric Food Chem. 1978; (26):809-812.
- Haslam E, Williamson M, Carlton A. Protein-polyphenol interactions. International Congress and Symposium Series - Royal Society of Medicine 2000; (226):25-33.
- Hassan ZMR, El Din HMF, Ali AA, Mehanna NSH, El-Messery TM. Interaction of Some Low Molecular Weight Phenolics with Milk Proteins. World Applied Sciences Journal. 2013; (23) 182-187.
- Karamac´ M, Kosińska A, Rybarczyk A, Amarowicz R. Extraction and chromatographic separation of tannin fractions from tannin-rich plant material. Pol J Food Nutr Sci. 2007; (57):471–474.
- Kosińska A, Karamać M, Penkacik K, Urbalewicz A, Amarowicz R. Interactions between tannins and proteins isolated from broad bean seeds (*Vicia faba* Major) yield soluble and non-soluble complexes. Eur Food Res Technol. 2011; (233):213-222.
- Luck G, Liao H, Murray NJ, Grimmem HR, Warminski EE, Williamson MP, Lilley TH, Hasam E. Polyphenols, astringency and proline-rich proteins. Phytochemistry. 1994; 37(2):357-371.
- Madhan B, Thanikaivelan P, Subramanian V, Raghava RJ, Balachandran UN, Ramasami T. Molecular mechanics and dynamics studies on the interaction of gallic acid with collagen-like peptides, Chem. Phys. Latter.2001; (346): 334-340.
- Manach C, Williamson G, Mirand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. American Journal of Clinical Nutrition. 2005; 81(1):230S-242.
- Mehanna NS, Hassan ZMR, El-Din HMF, Ali AA, Amarowicz R, El-Messery TM. Effect of Interaction Phenolic Compounds with Milk Proteins on Cell Line. Food and Nutrition Sciences. 2014; (5): 2130-2146.
- Naczk M, Shahidi F. The effect of methanoi ammonia water treatment on the content of phenolic acids of canola. Food Chem. 1989; (31):159–164
- Nazni.P and Dharmaligam.R, Isolation and Separation of Phenolic Compound from Coriander Flowers, International Journal of Agricultural and Food Science, 2013; Vol.4, Iss.1, pp. 13-21.
- Paul WH, Forkner R, Hunter M, Hagerman A. Determination of hydrolyzable tannins (gallotannins and ellagitannins) after reaction with potassium iodate. J. Agric. Food Chem. 2002; (50):1785−1790.
- Price ML, Van Scoyoc S, Butler LGA. Critical evaluation of the vanillic reaction as an assay for tannin in sorghum grain. J Agric Food Chem. 1978; (26):1214–1218.
- Rubino MI, Arntfield SD, Nadon CA, Bernatsky A. Phenolic protein interaction in relation to the gelation properties of canola protein. Food Res. Int. 1996; (29):653–659.
- Serafini M, Maiani G, Ferro-Luzzi, A. Effect of ethanol on red wine tannin-protein (BSA) interaction. J Agri Food Chem. 1997; 45 (8):3148–3151.
- Singh R. Interaction of phytochemicals with dairy ingredients- Ph. D. Thesis. University of Auckland, New Zealand. 2011.
- Yuksel Z, Avci E, Erdem YK. Characterization of binding interactions between green tea flavanoids and milk proteins Food Chemistry. 2010; (121): 450–456.