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Novel Coulometric Approach to Evaluation of Total Free Polyphenols in Tea and Coffee Beverages in Presence of Milk Proteins

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Abstract Electrogenerated hexacyanoferrate(III) ions have been used as coulometric titrant for the determination of total free polyphenols in beverages. Stoichiometric coefficients of natural flavonoids (rutin, quercetin, and taxifolin) in their reaction with $[Fe(CN)_6]^{3-}$ ions were established. The number of electrons involved in the oxidation corresponds to number of phenolic OH groups in the molecule of polyphenols. Proteins (casein and bovine serum albumin) bind polyphenols in the complexes that leads to significant decrease of free polyphenols portion (in the range of 5–76%). Ferric reducing power (FRP) of eight tea and 17 coffee samples was determined. The absolute FRP values ranged from 224 to 405 and from 241 to 488 C cup^{-1} for tea and coffee, respectively. Effect of milk proteins on total free polyphenols of tea and coffee and appropriate alteration of beverages FRP were evaluated. Milk addition decreases FRP of drinks in the range of 10–70% reflecting high content of inactive polyphenols.

Keywords Polyphenolic antioxidants · Antioxidant properties of beverages · Milk proteins · Constant-current coulometry · Electrogenerated hexacyanoferrate(III) ions · Food analysis

Introduction

Polyphenols are important natural antioxidants widely present in plant origins. They are classified by various groups depending on the number of phenol rings and structural elements bridging aromatic groups in the molecules. Thus, phenolic acids, flavonoids, stilbenes, and some other aromatic antioxidants are specified (Barbosa 2007). The flavonoids have common structure consisting of two aromatic rings (A and B) that are bonded together by three carbon atoms forming an oxygenated heterocycle (ring C) by intramolecular Michael reaction.

Polyphenols are abundant micronutrients in a human diet. The evidences for their role in the prevention of the degenerative diseases, e.g., cancer, cardiovascular, Parkinson, and Alzheimer diseases, are emerging. The health effects of polyphenols depend both on their amounts consumed and their bioavailability (Manach et al. 2004). Despite of the growing interest to the health benefits of polyphenols, only few investigations are concerned on the role of dietary components affecting polyphenols function in digestion and changing their antioxidant capacity. This is particularly true for flavonols and flavonones and proteins (Zhu et al. 1997), especially proline rich (Murray et al. 1994), which easily bind polyphenols in the complexes.

Tea refers to one of the most common drinks in a human regular diet. The traditions of tea consumption vary among various regions. Thus, in the UK, Ireland, and Canada, tea is consumed with a substantial amount of milk added (Weisburger 1997). The latter one contains various proteins, and interactions between polyphenols and proteins have been reported (Arts et al. 2001, 2002). The evaluation of the effect of such interaction on the antioxidant properties presents a subject of interest. Modest experimental evidences suggest the milk can diminish the antioxidant capacity of tea (Arts et al. 2002), as well as other drinks rich of polyphenols like dark chocolate (Serafini et al. 2003). The evaluation of free polyphenols content plays an important role.

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Several methods have been proposed for the determination of polyphenols. Most of them are based on determination of individual polyphenols in food samples using different types of chromatography with mass spectrometric (Cavaliere et al. 2008; Bolling et al. 2009; Buendía et al. 2010) and fluorescent detection (Bonerz et al. 2008; Vitrac et al. 2002), as well as spectrophotometry with UV detection (Khanchia et al. 2007). As known, these methods are very labor consuming and expensive. Furthermore, total content of polyphenols is necessary to evaluate in some cases.

Electrochemical methods are usually used for the determination of polyphenols. Different types of voltammetry on stationary electrodes (Ziyatdinova and Budnikov 2005) including chemically modified (Xiao et al. 2006; Guo et al. 2009) have been developed for polyphenols detection. Amperometric laccase-based biosensors for the determination of polyphenols in wines (Gomes and Rebelo 2003; Fernandes and Rebelo 2009) and plant extracts are described (Diaconu et al. 2010). Biosensor containing enzyme tyrosinase immobilized by the cross-linking method has been used for detection of polyphenols in tea (Abhijith et al. 2007). Biosensor based on the bi-immobilization of laccase and tyrosinase phenoloxidase enzymes and the Sonogel–Carbon as electrochemical transducer has been developed for estimation of the total polyphenol index in beer samples (ElKaoutit et al. 2007).

Constant-current coulometry is an attractive method from this point of view. It is characterized by simplicity, accuracy, and reliability and could be useful in polyphenols analysis. The aim of present work is the development of new method for the determination of total free polyphenols in beverages based on their reaction with electrogenerated $[Fe(CN)_6]^{3-}$ ions. Also, the effect of milk proteins on antioxidant properties of tea and coffee will be considered.

Materials and Methods

Reagents

Rutin and quercetin were purchased from Aldrich (Germany), taxifolin from Fluka (Germany). Their stock solutions (75 $\mu g mL^{-1}$) were prepared by dissolving a definite amount of the substance in ethanol and the stock solutions of β-casein from bovine milk (BioUltra, Germany) and bovine serum albumin (Reanal, Hungary) of the same concentration in water. Model solutions of polyphenols with proteins were prepared by mixing their stock solutions in appropriate *v*/*v* ratio. Other chemicals were of analytical reagent grade and used as received. Double-distilled water was used for the measurements.

Apparatus

Coulometric measurements were carried out using P-5827M potentiostat (Russia) with four-electrode two-compartment electrochemical cell. A bare platinum foil with the surface area of 1 cm^2 was used as the working electrode and a platinum wire as the auxiliary electrode. Anode and cathode compartments were separated by semipermeable diaphragm from porous glass. A pair of polarized platinum electrodes was used for detection of the titration end-point. Surface of the platinum electrodes was cleaned by HNO_3 and then rinsed thoroughly with double-distilled water prior to measurements.

Procedures

Electrochemical generation of $[Fe(CN)_6]^{3-}$ ions was carried out from 0.1 M $K_4Fe(CN)_6$ in 2 M KOH at the current density of 5 $mA cm^{-2}$ that provides a 100% current yield (Ziyatdinova et al. 2009). The titration end-point was detected biamperometrically (ΔE=200 mV).

Coulometric titration was carried out in a 50.0-mL cell containing 20.0 mL of the supporting electrolyte. The generating circuit was switched on, and a certain value of the indicator current was attained. Then, an aliquot of 50–1,000 μL of the solution tested was added and timer started. The titration end-point was indicated by reaching the initial value of the current. The timer was stopped, and the generating circuit was switched off.

Ferric reducing power (FRP) of samples was expressed by the quantity of electricity, Coulombs (C), spent for the titration, and recalculated per a cup of beverage.

All the experiments were carried out at temperature 23±2 °C.

Sample Preparation

Commercial trade names of teas and coffee available on local market were investigated. Tea extracts were prepared

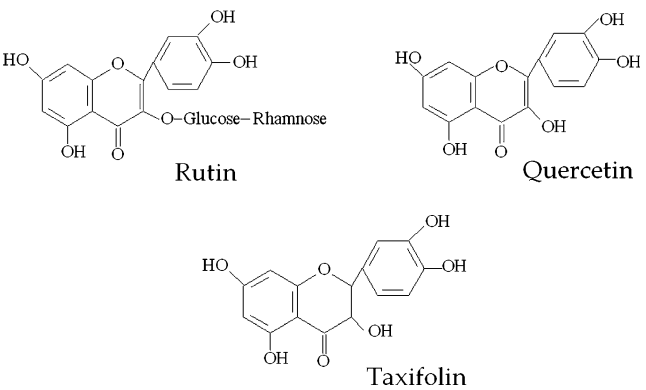

Fig. 1 Structures of natural polyphenols investigated

Table 1 Stoichiometric coefficients of the reactions between polyphenols and electrogenerated $[Fe(CN)_6]^{3-}$ ions

Compound	ν(compound):ν($[Fe(CN)_6]^{3-}$)
Rutin	1:4
Quercetin	1:5
Taxifolin	1:5

by soaking one tea bag or a definite amount of tea leaves (2 g) in 200 mL of boiling water for 3 min. Then, the tea extracts were cooled to room temperature, filtered through the filter paper, and used for titration without any other treatment.

Instant coffee (2 g) was soaked in 150 mL of hot water. Roasted coffee beans were grinded, and then 4 g were added to 150 mL of hot water and left for 4 min.

Statistics

All the measurements were performed in five replications. Mathematical and statistical evaluation was performed with significance level α=0.05 by SPSS for Windows software (SPSS Inc., USA). All data are expressed as the $X\pm\Delta X$, with X as average value and ΔX as confidence interval. The difference of parameters was performed by Student's t test. A $p<0.05$ was considered as statistically significant.

Results and Discussion

Reactions of Polyphenols with Electrogenerated Hexacyanoferrate(III) Ions

Constant-current coulometry with electrogenerated $[Fe(CN)_6]^{3-}$ ions as titrant opens new opportunities for the investigation of reaction stoichiometry between polyphenols and one-electron carriers, as well as for the evaluation of polyphenols content in the presence of proteins. The transfer of one electron from a substrate molecule is not complicated by additional chemical reactions involving the titrant and hence provides 100% current yield.

Flavonoids, i.e., rutin, quercetin, and taxifolin, were chosen as model compounds (Fig. 1). Titration of the standard solutions of natural polyphenols showed their reactions with titrant were fast and quantitative. The number of electrons involved in the oxidation stage corresponds to the number of OH groups present in the molecules of polyphenols. Oxidation leads to formation of appropriate carbonyl derivatives (Ziyatdinova et al. 2010). For rutin, only aromatic hydroxy groups are oxidized. Saccharide residue is not involved in the reaction with electrogenerated $[Fe(CN)_6]^{3-}$ ions. This was confirmed by titration of glucose and rhamnose performed in the same experimental conditions. The stoichiometric coefficients of the reactions calculated from coulometry experiment are presented in Table 1.

Coulometric Determination of Free Polyphenols in Presence of Proteins

It was shown that milk proteins, e.g., casein from bovine milk and BSA, did not react with electrogenerated $[Fe(CN)_6]^{3-}$ ions which are rather soft oxidants. It is well known that casein exists in quaternary structure and BSA as a B-form (Harrington et al. 1956). This leads to spatial hindrances of the reaction with rather large titrant ions.

Interaction of polyphenols with proteins was investigated in a model system consisting of a polyphenol and a protein in different ratios (v/v) and expressed as a content of free polyphenol in the mixture.

Casein and BSA bind polyphenols investigated (Fig. 2). The content of polyphenol available for oxidation decreased with an increase in protein quantities in the mixture. This corresponds well to data obtained earlier with catechin and gallic acid derivatives, as well as caffeine by electrokinetic chromatography (Kartsova and Alekseeva 2008).

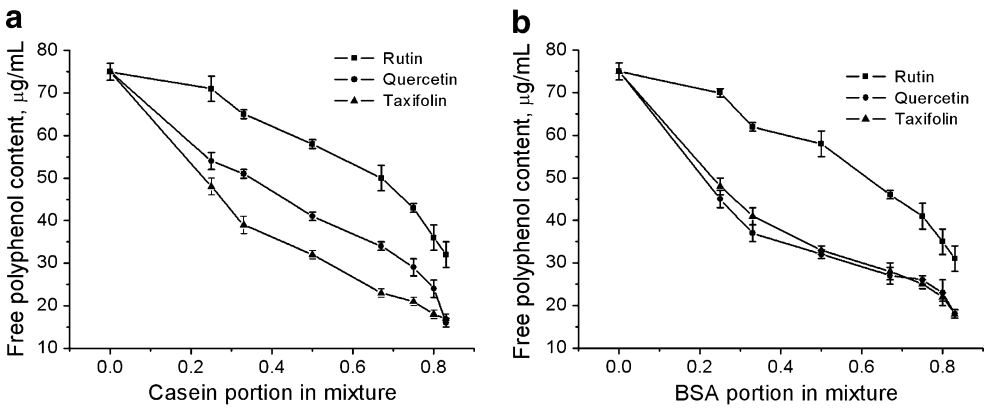

Fig. 2 Free polyphenol content depending on the protein content in the mixture: **a** casein and **b** BSA

Table 2 Ferric reducing power of tea (n=5; p=0.95)

Tea	Package type	FRP, C cup^{-1} (250 mL)	RSD, %
"Greenfield," Delicate Keemun	Tea bags	224±3	1.2
"Ahmad," English Tea No. 1		405±14	2.8
"Princess Noori," Earl Grey		273±3	0.85
"Ahmad," Earl Grey		339±3	1.3
"Princess Java," Earl Grey	Leaves	277±5	1.3
"Greenfield," Green Mellissa	Tea bags	250±5	1.8
"Hyleys," English Green Tea		299±2	0.64
"Ahmad," Jasmine Green Tea	Leaves	374±9	1.8

It should be noted that quercetin and taxifolin are bonded stronger than rutin. This is probably caused by glycoside residue in rutin structure that complicates the formation of a complex.

In general, binding between polyphenols and proteins is mainly resulted from intermolecular interactions. Different mechanisms of such interaction can be realized depending on the protein structure. Under non-oxidizing conditions, non-covalent interactions including hydrogen bonds and hydrophobic forces stabilize polyphenol–protein complexes. To some extent, electrostatic interactions involving phenylalanine residues (π-electrons of aromatic ring) can take place (Viljanen 2005).

In polyphenol–protein interaction, a strong hydrogen bond is formed between the carbonyl functions of the amino acids or peptide backbone and the isolated phenolic hydroxyl groups of polyphenols (Charlton et al. 2002). Structure and flexibility of a protein play an important role in this case (Frazier et al. 2003). The size and stoichiometry of aggregates formed depend on the components ratio (Siebert et al. 1996).

In hydrophobic binding, the aliphatic and aromatic side chains of a protein molecule, preferably proline residues, and the aromatic ring of a polyphenol stabilize the complex formed (Viljanen 2005). The protein structure affects the interaction, too. Linear proteins are involved in face-to-face stacking, whereas the interaction with globular proteins, e.g., α-lactalbumin and BSA, involves only the residuals located on their surface (Carvalho et al. 2004).

In general, the interaction between polyphenols and proteins may occur via multisite (several phenolic compounds bind to a single protein molecule) or multidentate interaction (one phenolic compound binds to several protein sites or molecules). The type of interaction depends on the nature and molar ratio of both phenolic compound and protein (Viljanen 2005). As known, quercetin is bonded by BSA through the hydrophobic interaction mainly (Wang et al. 2006).

Table 3 Ferric reducing power of coffee (n=5; p=0.95)

Coffee	Type of processing	FRP, C cup^{-1} (125 mL)	RSD, %
Arabica "Tradition"	Coffee beans	241±3	0.92
"Vending Espresso Paulig"		283±7	1.9
"Dallmayr Promodo"	Ground coffee	259±4	1.3
"Jockey Traditional"		331±5	1.3
"Grandos Exclusive"	Freeze-dried instant coffee	441±10	1.9
"Grandos Egoiste Noir"		488±16	2.6
"Nescafe Cap Colombie"		335±7	1.7
"Carte Noire"		337±6	1.3
"Vipcafe Black Label"		344±8	1.8
" Jockey Triumph"		344±8	1.8
"Jacobs Monarch"		455±7	1.1
"Tchibo Exclusive"		291±4	1.1
"Ambassador Adora"		336±3	0.83
"Moccona Excellent"	Spray-dried instant coffee	331±4	1.1
"Maxwell House"		294±3	0.75
"Nescafe Classic"		311±2	0.55
"Dancafe"		334±10	2.5

Thus, proteins interacting with flavonols (5–76% of bonding) significantly decrease the portion of free polyphenols.

Total Free Polyphenols in Beverages Containing Milk Proteins

Tea and coffee are one of the main sources of polyphenols in the common human diet. Their total free polyphenols were determined in the presence of milk proteins. The polyphenols are main antioxidants present in these beverages, so that alteration of drinks' antioxidant properties expressed via FRP will reflect the changes in the content of free polyphenols.

For this purpose, the FRP of drinks was first determined using electrogenerated $[Fe(CN)_6]^{3-}$ ions. As stated above, this value corresponds to total amount of low-molecular antioxidants, preferably polyphenols.

The FRP of tea extracts is presented in Table 2. Green and black teas showed similar FRP values (305±35 C cup^{-1}) caused by presence of polyphenol bonding proteins (16–25%). Glutenines and albumins are main soluble proteins presented in tea (Harbowy and Balentine 1997; Graham 1992). Albumins give bigger contribution. Green tea is rich of albumins that partially inactivate polyphenols. Black tea extract contains mainly glutenines because albumins are decomposed during tea fermentation. Glutenines bind polyphenols to a less extent. Therefore, the content of free polyphenols in green and black tea extracts appears the same.

As regards absolute values, the highest FRP value was observed for "Ahmad" trademark, most expensive in the trademarks considered.

The FRP data for beverages under investigation correlate with total antioxidant capacity based on the reaction with electrogenerated bromine. The correlation coefficients are 0.98 and 0.90 for tea and coffee, respectively.

FRP value of instant coffee samples changes insignificantly for all the trademarks investigated (Table 3). However, freeze-dried instant coffee showed higher FRP than

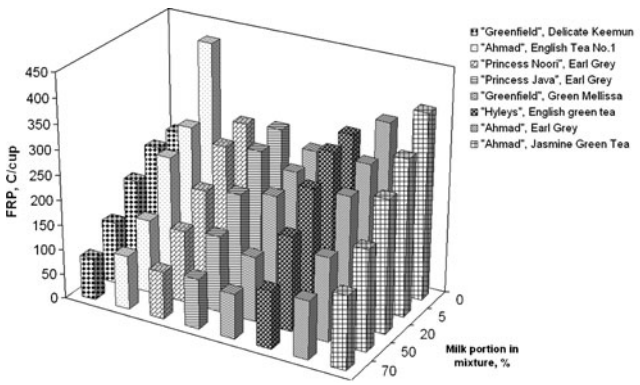

Fig. 3 Masking effect of milk proteins on tea antioxidant properties

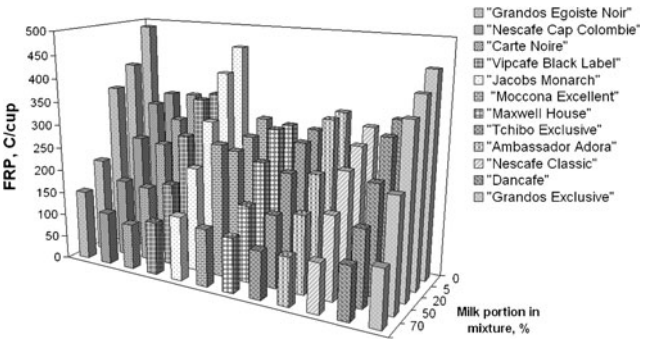

Fig. 4 Alteration in FRP of instant coffee in presence of milk

that spray-dried (375±22 vs. 318±9 C cup^{-1}, $p>0.05$). It can be explained by the method of beans treatment. Spray-dried coffee is produced by dehydration of coffee extract at high pressure in hot air flow that leads to oxidation of polyphenols. Freeze-dried coffee is prepared by freezing extract and dehydration in vacuum. This procedure promotes conservation of the polyphenols in the final product (Viani and Petracco 1986; Pintauro 1975).

Coffee beans possess significantly lower FRP than instant coffee (289±18 vs. 359±19 C cup^{-1}, $p<0.05$). Probably, dehydration stage promotes collection of components of instant coffee. Furthermore, extra addition of caffeine and other compounds with antioxidant properties can be used for instant coffee.

Mixtures of tea and coffee with pasteurized milk (2.5% of fat) containing 5%, 20%, 50%, and 70% (*v*/*v*) of milk were prepared for the further evaluation of milk proteins effect of antioxidant properties of beverages.

Masking effect of milk on FRP of tea was observed (Fig. 3). This effect did not depend on the tea sort so that the shift of FRP observed for various teas after milk addition was about the same. Moderate amounts of milk (less than 50%) did not affect FRP significantly. In the only exception "Ahmad" English Tea No. 1, the FRP decreased dramatically by 30% (5% of milk in the mixture). Tea with high content of milk, very popular in UK, showed the smallest FRP caused by high degree of polyphenols inactivation.

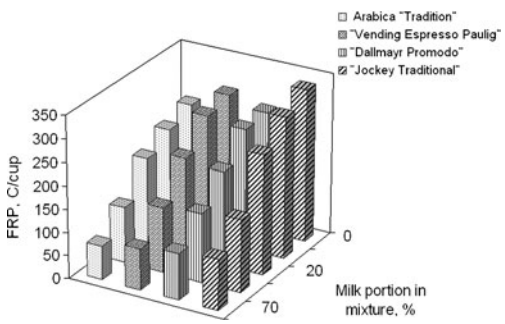

Fig. 5 Effect of milk proteins on FRP of coffee beans

Epigallocatechin gallate and epicatechin gallate are main polyphenols contained in tea and bonding to proteins. Their content decreases by 70–80% at 1:1 tea/milk ratio (Catterall et al. 2003). Epicatechin, epigallocatechin, and caffeine (Ferruzzi and Green 2006) also interact with proteins but give smaller contribution. Bonding of tea catechins with milk proteins is caused by strong hydrophobic interactions between galloil rings of polyphenols and proline-containing fragments of peptide molecule followed by stabilization due to formation of hydrogen bonds (Murray et al. 1994; Baxter et al. 1997).

Similar masking effect of milk was found for the instant coffee (Fig. 4). FRP of it decreased gradually until 1:1 coffee/milk ratio with following change on 50% at higher content of milk.

FRP value of coffee beans changes insignificantly until 20% of milk is added. Then, it decreases twofold (Fig. 5). This coincides with literary data obtained for espresso (Sánchez-González et al. 2005) and instant coffee (Dupas et al. 2006). Maillard reaction products, phenolic acids (chlorogenic, caffeic, ferulic, and *p*-coumaric), and their ethers interact with milk proteins and thus effect on FRP of coffee.

Summarizing the results obtained, it should be noted that milk proteins bind polyphenols present in beverages under investigation and therefore affect on the antioxidant properties of tea and coffee. These results are in good agreement with the data reported earlier (Arts et al. 2002; Alexandropoulou et al. 2006). Casein that averages 80% of the total protein content is a main milk protein binding polyphenols. α-Lactoalbumin, BSA, and β-lactoglobulin provide less contribution (Brown and Wright 1963).

Conclusions

New approach for the evaluation of total free polyphenols content in presence of proteins using electrogenerated $[Fe(CN)_6]^{3-}$ ions as coulometric titrant has been developed. The method characterized by simplicity, accuracy, speed, and reliability, as well as cost-efficiency, could be successfully used in food analysis.

Proteins (casein and BSA) bind rutin, quercetin, and taxifolin and decrease significantly their activity. Masking effect of milk proteins on FRP of tea and coffee reflecting content of free polyphenols in system was observed. Milk proteins bind polyphenols of drinks via intermolecular interactions and thus decrease their antioxidant effect.

It could be noted that better knowledge of dietary polyphenols properties is essential for further study of their behavior in organism. After the consumption of polyphenols, presence of other dietary antioxidants and matrix components like proteins, lipids, and polysaccharides has to be taken into account for correct evaluation of polyphenols biological activity. Investigations of polyphenols sources and human diet permit to give some dietary recommendations for population as well as for design of new foodstuffs.

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