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Gastro-pancreatic release of phenolic compounds incorporated in a polyphenols-enriched cheese-curd

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ABSTRACT

As functional food, enriched cheese has recently been developed. The main objectives of this study were to investigate the role of casein in the retention of polyphenol during curd formation and the release of polyphenols during *in vitro* gastro-pancreatic digestion of polyphenols-enriched cheese and their contribution to the antioxidant activity of digested curd. Polyphenols showed high retention coefficient in curd. The retention coefficient of polyphenol was related to the binding affinity to casein and to their hydrophilicity. The polyphenols should be added before milk coagulation since the binding decreases as casein molecules aggregate. During *in vitro* gastro-pancreatic digestion steps, polyphenols were released from curd due to the dilution in gastric fluid and to casein proteolysis. The addition of polyphenols to curd determined a relevant increase of antioxidant activity respect to the curd control even a part of polyphenols is degraded by alkaline pH of pancreatic fluid. Our results suggested the possibility of producing highly nutritive value cheese with high release of the polyphenols during digestion. In addition, the whey, which contains polyphenols, can be involved in different products to maximize its utilization.

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1. Introduction

Over the last years, much more attention has been paid to polyphenolic compounds. They are the major source of antioxidants in human diet, and show a wide range of activities such as anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, angiogenesis and cell proliferation inhibitory activities (Conte, Pellegrini, & Tagliazucchi, 2003; Crozier, Jaganath, & Clifford, 2009; Verzelloni, Tagliazucchi, Del Rio, Calani, & Conte, 2011). Epidemiological studies and human intervention trials have associated a high intake of fruit and vegetables rich in phenolic compounds with a lower incidence of chronic diseases including diabetes, cardiovascular diseases and cancer (Del Rio et al., 2013).

The incorporation of bioactive compounds during the manufacturing of innovative functional foods became of important interest to improve the nutritional and healthy properties of certain types of food. Recent examples on this topic involved the incorporation of bioactive phenolic compounds in yogurt (Chouchouli et al., 2013), ice cream (Çam, Içyer, & Erdoğan, 2014) and cheese (Han et al., 2011a).

Cheese possesses a unique composition and structure, which actuate the researchers to try to apply different bioactive compounds to cheese with expectation to improve its nutritional and healthy qualities (Bandyopadhyay, Chakraborty, & Raychaudhuri, 2008; Joseph & Akinyosoye, 1997; Prudêncio, Prudêncio, Gris, Tomazi, & Bordignon-Luiz, 2008; Rinaldoni, Palatnik, Zaritzky, & Campderros, 2014). Recently, Han et al. (2011b) developed a functional cheese product containing polyphenolic compounds.

To exert their biological activity, phenolic compounds must be released from the curd during digestion. While polyphenols contained in the liquid matrices are promptly available for the absorption, this is not true for polyphenols contained in solid matrices such as polyphenols incorporated in cheese. In these foods, polyphenols must first be released to be bioaccessible, potentially bioavailable and able to exert their beneficial effects (Chiang, Kadouh, & Zhou, 2012; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010; Tagliazucchi, Verzelloni, & Conte, 2012).

The main objectives of this study were (i) to evaluate the retention coefficients of different types of polyphenol compounds revealing the mechanism by which these compounds are retained in and released from the curd; (ii) to measure the release of incorporated polyphenols during *in vitro* digestion; (iii) to evaluate the antioxidant activity released during gastro-pancreatic digestion steps.

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2. Materials and methods

2.1. Materials

Pasteurized whole bovine milk (3.1 g/100 mL protein and 3.6 g/100 mL fat) was purchased in a local market (Reggio Emilia, Italy). Liquid calf rennet was obtained from Educational Dairy Plant (Damanhour University, Damanhour, Egypt). Phenolic compounds, catechin, chlorogenic acid, ferulic acid, vanillic acid, gallic acid, *p*-coumaric acid, 3,4-dihydroxyphenylacetic acid and tannic acid were purchased from Sigma (Milan, Italy). Casein, calcium chloride, bile salts (mixture of sodium cholate and sodium deoxycholate), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP specifications) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were supplied by Sigma (Milan, Italy). Ethanol was supplied by Carlo Erba (Milan, Italy).

2.2. Cheese curd preparation

Polyphenols-enriched cheese was manufactured as described by Han et al. (2011b) with some modifications. Calcium chloride was added to the milk obtaining a final concentration of 6 mmol/L to compensate the effect on milk ingredient properties of pasteurization which decreases the concentration of free calcium and homogenization which decreases the dimension of fat micelles and increases the adsorption of κ-casein micelles on the fat globules. Different phenolic compounds were added to the milk as a solid compound to have a final concentration of 0.5 mg/mL, followed by stirring to obtain a homogenized solution. Rennet was firstly submitted to a clotting activity test as described by Berridge (1952), and then 1 mL was added to 20 mL of milk to be completely coagulated within 2 h at 35 °C. To separate the whey from the curd, the coagulated samples were centrifuged at 1300 *g* at 21 °C for 15 min. The curd and whey were weighed and measured volumetrically.

Casein curd was also prepared using casein solution at the same concentration as in milk (2.48 g/100 mL), in the presence and absence of catechin.

The samples containing polyphenols were treated as described above.

Curd moisture content (CMC) was calculated according to Pandey, Ramaswamy, and St-Gelais (2000).

2.3. Polyphenols determination and polyphenols retention coefficient (PRC)

To estimate the amount of polyphenols incorporated in curd, retention coefficient was determined for all the samples. Phenolic content was determined using high performance liquid chromatography (Jasco HPLC, Tokyo, Japan) equipped with a C18 column (HxSil C18 Reversed phase, 250 × 4.6 mm, 5 μm particle size, Hamilton Company, Reno, Nevada, USA). A volumetric injector Rheodyne (Cotati, CA, USA), and a temperature-controlled oven were utilized. An amount of 20 μL of each sample was used for injection with a gradient system of solvent A (1 mL/100 mL formic acid in water v/v) and solvent B (acetonitrile) as the mobile phase at a flow rate of 1 mL/min and the temperature was adjusted to 32 °C. The gradient system was linear, solvent B started from 4 mL/100 mL at 0 min and reached 25 mL/100 mL at 60 min; while in the case of measuring tannic acid, solvent B reached linearly 60 mL/100 mL after 60 min. Peaks for samples and standards were monitored at 280 nm. The calibration curves of standards polyphenols were used to quantify the polyphenols. PRC is the percentage of the amount of the polyphenols added to milk which remains in curd.

2.4. In vitro gastro-pancreatic digestion

The two-stage *in vitro* digestive model was adapted from Tagliazucchi et al. (2010) with some modifications. Curds were diluted 10 times with simulated gastric fluid containing 2 g/L of NaCl and 60 mmol/L HCl, pH 2.0, and homogenized for 2 min in a laboratory blender. The homogenates were adjusted to pH 2.0 with concentrated HCl and pepsin (315 U mL^{-1}) was added. The samples were incubated at 37 °C in a shaking bath for 2 h to simulate the gastric phase of digestion. At the end of the gastric digestion, the pH was brought to 7.5 with $NaHCO_3$ before adding 0.8 mg/mL pancreatin and 5 mg/mL of bile salts. The solution was then incubated again at 37 °C in a shaking bath to simulate the intestinal phase of digestion. After 2 h incubation, an aliquot of each sample was withdrawn and the pH was lowered to 2.0 to inactivate the enzymes and stabilize the polyphenols.

Aliquots of the samples were also withdrawn after the homogenization and after the gastric phase of digestion. A centrifugation was carried out on all aliquots at 1300 *g* at 21 °C for 15 min, the pellet and the supernatant were weighed, measured volumetrically and used for further analysis. Polyphenols were determined using high performance liquid chromatography (HPLC), following the same protocol as described in Section 2.3.

2.5. Fluorescence spectroscopy

The interaction between casein and the different polyphenols used in cheese was investigated by using fluorescence spectroscopy as reported by Tagliazucchi, Helal, Verzelloni, and Conte (2012) with some modification.

Fluorescence spectra were recorded at 35 °C in the range of 290–500 nm at an excitation wavelength of 280 nm using Jasco, FP-6200 spectrofluorometer (Tokyo, Japan). The intensity at 340 nm (tryptophan emission wavelength) was used to calculate the binding constant according to Dufour and Dangles (2005).

Solutions of the following ligands, catechin, tannic acid, chlorogenic acid, coumaric acid, ferulic acid, dihydroxyphenylacetic acid, vanillic acid and gallic acid were prepared in methanol. For each data point, 2 mL of 5 μmol/L casein (a mixture of α-and β-caseins dissolved in 10 mmol/L sodium phosphate buffer, pH 6.5) were transferred into a cuvette. After 5 min of equilibration at 35 °C, 0.01 mL of each of the above reported polyphenol methanol solution was added to cuvette. The added solutions of ligands were properly diluted in methanol to have a final ligand concentration between 1 and 100 μmol/L. The change in fluorescence emission intensity was measured after 10 min of the mixing with casein. The effect observed on the casein fluorescence emission spectrum with addition of methanol alone, was calculated and subtracted of the value of casein alone. Catechin, vanillic acid and 3,4-dihydroxyphenylacetic acid possess intrinsic fluorescence at the used excitation wavelength. Therefore, the emission spectrum of these phenolics was determined and subtracted from the emission spectra obtained for casein quenching.

The type of binding was assessed using the Stern-Volmer equation (Lacowitz, 2006).

For the kinetic analysis of ligand binding, non-linear regression analysis was performed using Graph Pad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). K_D (dissociation constant) and n (number of binding site) were calculated according to Rawel, Frey, Meidtner, Kroll, and Schweigert (2006) using the following equation:

$$F_0 - F = F_0 * L_0^n / (K_D + L_0^n)$$

and by plotting the graph of F_0–F versus L_0. F and F_0 are the measured fluorescence emission intensity of the casein solution in

Table 1
Curd yield, curd moisture content (CMC) and pH values of cheese curds and polyphenol retention coefficient (PRC) in cheese curd.

Treatment	Curd yield (g/100 g)	CMC (g/100 g)	pH	PRC (g/100 g)
Control	61.9 ± 1.5	68.9 ± 1.0^{a}	6.79	/
Catechin	63.8 ± 1.8	67.5 ± 1.4^{a,b}	6.78	84.8 ± 0.6^{a}
Tannic acid	65.7 ± 2.1	66.0 ± 1.8^{b}	6.70	86.8 ± 0.2^{a}
Chlorogenic acid	65.1 ± 1.2	66.5 ± 0.9^{a,b}	6.72	73.0 ± 1.4^{b}
p-Coumaric acid	62.0 ± 1.6	68.8 ± 1.2^{a}	6.75	70.3 ± 2.6^{b,c}
Ferulic acid	63.4 ± 1.5	67.8 ± 1.1^{a,b}	6.75	69.3 ± 0.3^{c,d}
3,4-Dihydroxyphenylacetic acid	63.7 ± 1.7	67.6 ± 1.3^{a,b}	6.77	63.7 ± 2.3^{e,f}
Vanillic acid	62.1 ± 1.4	68.8 ± 0.9^{a}	6.74	66.5 ± 1.1^{d,e}
Gallic acid	63.6 ± 0.5	67.7 ± 0.4^{a,b}	6.72	63.0 ± 1.1^{f}

Values in the same columns with different lowercase letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$).

the presence and absence of the ligand, respectively, and L_0 the total concentration of the ligand.

2.6. Radical scavenging activity determination

The radical scavenging activity of samples taken during the *in vitro* simulated digestion procedures was evaluated using the ABTS method as described in Re et al. (1999). Briefly, samples withdrawn after the homogenization and after the gastric and intestinal phase of digestion were centrifuged as described in Section 2.4 and 40 μL of supernatant were mixed with 1960 μL of ethanolic $ABTS^{\bullet+}$ solution. The mixture, protected from the light, was incubated in the spectrophotometer at 37 °C for 10 min; the decrease in absorbance at 734 nm was measured at the endpoint of 10 min. ABTS units of the samples were measured and calculated as Trolox equivalent antioxidant capacity (TEAC) using a standard curve of Trolox. The results were expressed as μmol/L of TEAC.

2.7. Statistics

Data are presented as mean ± SD for three replicates for each prepared sample. Linear regression analysis was performed using Graph Pad Instat (GraphPad Software, San Diego, CA, USA). Univariate analysis of variance (ANOVA) with Tukey post-test was applied using PASWStatistics 18.0 (SPSS Inc. Chicago, IL, USA) when multiple comparisons were performed. The differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Curd yield, curd moisture content (CMC) and polyphenols retention coefficient (PRC)

Table 1 reports the percent of curd formed from 20.68 g of milk (20 mL with a specific weight of 1.034). All the curds formed in the presence of polyphenols showed a non-significant increase in the yields respect to the control.

In the same table, curd moisture content, pH, and polyphenol retention coefficient are reported. One of the most important characteristics of cheese curd is the moisture content, which affects many factors like yield, texture properties and calculation of the nutritional values based on dry weight. The addition of different polyphenols to milk had no significant effect on the moisture content in the majority of samples. However, a slight significantly ($P < 0.05$) decrease in CMC in case of the addition of tannic acid was noticed. According to Han et al. (2011a), this decrease can be attributed to hydrophobic interaction between milk proteins and polyphenols, which would reduce the quantity of entrapped water in protein polymeric networks during the formation of cheese curd.

The retention coefficient values of phenols investigated ranged between 63.0 ± 1.1 (gallic acid) and 86.8 ± 0.2 g/100 g (tannic acid).

Retention coefficient is an important parameter to evaluate the residual amount of additives such as polyphenols. A higher retention coefficient obtained in curd, a lower loss of functional ingredients in whey occurred. A high retention coefficient of the curd predicts a high retention during the cheese processing.

To explain the differences in the retention coefficient between the various polyphenols utilized, it is important to consider the media in which they are distributed (curd and whey). The coefficient of retention of polyphenols depends on various factors such as the interaction between specific or non-specific binding sites on the protein molecules, the solubility in water and in lipid micelles, the distribution between solid matrix and liquid phase of the curd. The decrease of pH or temperature decreases the solubility of phenols and a part of the phenols may come out of the liquid phase and be trapped in the pellet curd. An important factor, which can affect the retention coefficient, is the hydrophilicity of the polyphenols. The different types of polyphenols used in our study showed different degree of hydrophilicity. We separated and determined the phenolic compounds by chromatography on C-18 column eluted by gradients of formic acid in water as hydrophilic and acetonitrile as hydrophobic component of mobile phase. The elution of phenolic compounds from C-18 column gives us an evaluation of their hydrophilicity. From elution data, it resulted that the rank order of hydrophilicity at low pH, was gallic acid > 3,4-dihydroxyphenylacetic acid > vanillic acid ≈ catechin ≈ chlorogenic acid > *p*-coumaric acid > ferulic acid > tannic acid.

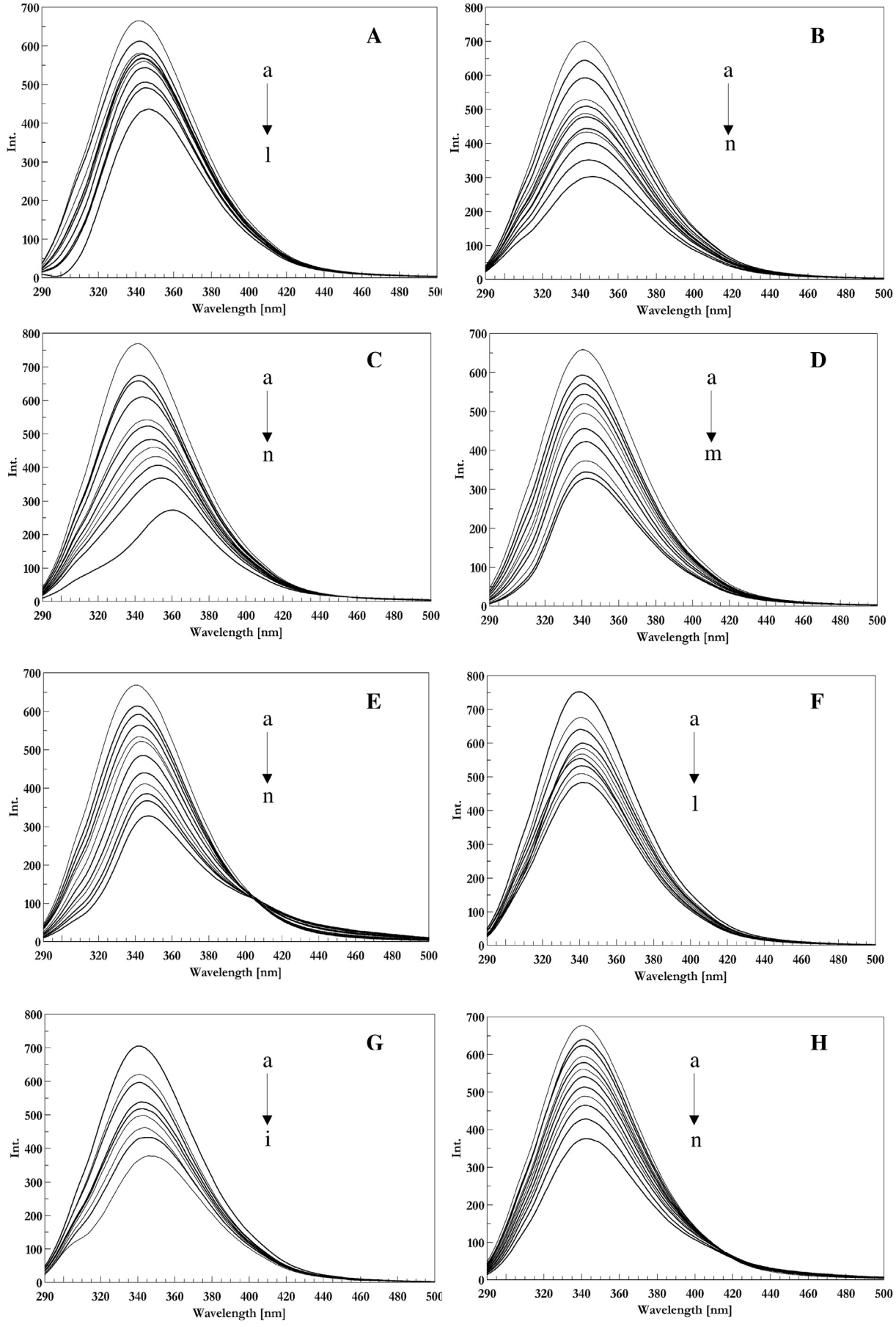
Correlation analysis showed a positive correlation between the retention time value on C-18 column and the polyphenol retention coefficient in curd (Pearson $r = 0.644$; $P = 0.0085$), which confirms the role of polyphenols hydrophilicity on the retention coefficient in cheese curd.

It should be pointed out that, besides hydrophobicity, other characteristics of the molecules such as the molecular weight and the shape may affect the elution time on C-18 chromatographic column. Four investigated molecules have very similar molecular

Table 2
Binding constants, quenching type and number of binding sites (n) for polyphenols-casein complexes as determined by the quenching of the tryptophan fluorescence.

Polyphenol	Ksv ($\times 10^3$ mol^{-1})	Kq ($\times 10^{10}$ mol^{-1} s^{-1})	Quenching type	K_D (μmol/L)	n
Catechin	50.3 ± 3.8	838.3 ± 63.3	Static	21.9 ± 1.6	0.95 ± 0.09
Tannic acid	509.9 ± 39.3	8498.3 ± 655.0	Static	1.8 ± 0.1	0.91 ± 0.08
Chlorogenic acid	27.9 ± 0.6	465.0 ± 10.0	Static	37.9 ± 2.3	0.88 ± 0.06
p-Coumaric acid	18.6 ± 0.8	310.5 ± 13.8	Static	75.2 ± 4.4	1.14 ± 0.09
Ferulic acid	16.9 ± 0.7	281.7 ± 11.7	Static	73.2 ± 5.1	1.20 ± 0.11
3,4-Dihydroxyphenylacetic acid	11.4 ± 0.7	189.0 ± 11.7	Static	92.2 ± 5.5	1.27 ± 0.11
Vanillic acid	17.4 ± 0.8	290.0 ± 13.3	Static	90.1 ± 8.3	1.16 ± 0.15
Gallic acid	4.1 ± 0.1	68.3 ± 1.7	Static	240.4 ± 11.2	0.99 ± 0.06

Kq was calculated by dividing the experimentally measured *Ksv* for 6×10^{-9} s that is the maximum lifetime (τ_0) of the fluorophore (tryptophan) in the absence of quencher as reported by Dufour and Dangles (2005). Data are means ± SD ($n = 3$).


A
a
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B
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n
C
a
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D
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m
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F
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Int.
Wavelength [nm]

weights which are 164.16, 168.15, 170.12, 168.15 and 194.18 Da for coumaric, 3,4-dihydroxyphenylacetic, gallic, vanillic, ferulic acids, respectively. The highest molecular weight is that of tannic acid (1701.20 Da). The molecular weight of catechin and chlorogenic acid are 290.27 and 354.31 Da, respectively. Considering the elution of investigated compounds from C-18 columns and the high solubility in water of tannic acid it is possible that the elution from columns is influenced not only by the hydrophobicity but also from other factors such as the molecular mass and the shape of the molecules. We cannot assume that these factors are operative in the curd, decreasing the release of tannic acid. This is an interesting point to be investigate in the future.

The different hydrophilicity affected the distribution of polyphenols between curd and whey. Our results clearly showed that the gallic acid, which had the highest hydrophilicity, exhibited the lowest retention coefficient. Inversely, tannic acid, which had the highest elution time from C-18 column, exhibited the highest retention coefficient in curd.

3.2. The specific binding between polyphenols and casein

Since caseins bind polyphenols to specific binding sites (Tagliazucchi, Helal, et al., 2012), we investigated the binding affinity of polyphenols and the number of molecules of polyphenols that bind to the casein, by fluorescence spectroscopy.

Table 2 shows the *Ksv* for the binding between different polyphenols and casein. The Stern–Volmer constant can be used to gain information about the type of fluorescence quenching. In fact, the fluorophore can be quenched by collision (dynamic quenching) or by complex formation (static quenching) with the quencher. To understand the type of binding, the bimolecular quenching (K_q) constant is calculated and compared to the maximum value possible for diffusion-limited quenching in water (~10^{10} $mol^{-1}s^{-1}L$). K_q was calculated by dividing the experimentally measured K_{SV} for 6×10^{-9} s that is the maximum lifetime (τ_0) of the fluorophore (tryptophan) in the absence of quencher as reported by Lacowitz (2006). It has been shown that in the case of static quenching, the bimolecular binding constant is several magnitude orders higher than the maximum value of diffusion-limited quenching in water.

For all the analyzed compounds the type of quenching was static (K_q 3–4 order of magnitude more than the diffusion-limited quenching in water ~10^{10} mol^{-1} s^{-1} L) suggesting that the quenching involved the formation of a complex between the quencher (phenols) and fluorophore (tryptophan).

The plotting of corrected fluorescence was analyzed by means of non-linear least-square regression fit for the casein–polyphenols models as reported in Rawel et al. (2006).

Fig. 1(A–H) shows the emission spectra of casein before and after the addition of different concentrations of polyphenol. As can be seen, the polyphenols caused a decrease in the tryptophan emission with increasing concentration.

The K_D value is indicative of the affinity between the protein and the polyphenol. The smaller K_D is, higher the affinity is (Table 2). The rank order of polyphenol affinity to caseins is about the same as the order of their retention in the curds.

Linear regression analysis showed an inverse correlation between K_D value and the retention coefficient (Pearson $r = 0.759$; $P = 0.0006$).

The binding affinity of the different polyphenols to casein can largely explain the differences in the retention coefficient in curd. High binding with casein also led to a decrease in the release of the polyphenol during the whey separation, which implies high retention and high recovery in curd.

According to our results, we can conclude that, the retention coefficient of polyphenols in cheese curd is positively affected by their binding affinity versus casein network of curd and negatively affected by their hydrophilicity.

3.3. The binding of polyphenols to curd during milk coagulation

It should be pointed out that the retention coefficients are calculated as percentage of milligrams of phenol added while K_D for casein is reported as μmol/L. There is a large difference about 10 times, between the molecular weight of the *p*-coumaric acid, the compound with the lowest molecular weight we have investigated and the tannic acid. The difference in molecular weight results in relevant differences in the concentration of various phenols used. To compare the data of affinity of polyphenols to casein with the concentration of polyphenols during the various experimental stages we report in Table 3, millimolar concentration of phenols in milk, in whey, as well as their apparent concentration in curd. We measured the volume of curd and used this volume to calculate the apparent polyphenol concentrations. It appears that the apparent concentration of polyphenols in curd was higher than in whey for all polyphenols. The difference was greater for those compounds that had greater affinity for casein (tannic acid and catechin).

From the concentrations of phenols and casein (1.24 mmol/L calculated with a molecular weight of 20 kDa) in the milk, and from the values of K_D it appeared that, in our experimental conditions, no more than 35% of the binding sites of casein for polyphenols could be occupied by the tannic acid, because its concentration (0.30 mmol/L) was lower than that of casein. For the other phenols the 90–98% of the binding site of casein could be occupied. However, the phenol retention coefficient obtained and the concentration of phenols that remained in whey showed that these values were far from being reached. Between 13% (tannic acid) and 37% (gallic acid) of the added compounds remained in the supernatant whey. The presence in milk of compounds bound to phenol binding site of casein, and the partial loss of the capacity of casein to bind phenol during milk coagulation, are two possible reasons for the observed inconsistency. To evaluate the possible role of compounds which in milk compete with the binding of polyphenols to casein we determined the polyphenol retention coefficient of curd prepared from a solution of commercial casein at the same concentration and experimental condition of milk. The catechin retention coefficient significantly increased from 84.0 ± 0.6 to 96.9 ± 0.7 g/100 g with commercial casein, which suggested that some compounds present in milk compete with catechin for polyphenols binding site of casein.

We also determined the coefficient of retention of catechin when it was added to milk after coagulation as well as to curd after centrifugation and separation from the whey. In both the experiments, the samples were maintained for 2 h at 35 °C after catechin addition before final centrifugation. When catechin was added to milk after coagulation and to the curd after centrifugation the

Fig. 1. Fluorescence emission spectra of polyphenol–casein interactions. In all samples the casein concentration was 5 μmol/L. (A) Catechin concentrations: 0 (a), 1 (b), 2.5 (c), 5 (d), 7.5 (e), 10 (f), 15 (g), 20 (h), 25 (i), and 30 (l) μmol/L. (B) Tannic acid concentrations: 0 (a), 1 (b), 2 (c), 3 (d), 5 (e), 6 (f), 7 (g), 8 (h), 9 (i), 10 (l), 11 (m) and 12 (n) μmol/L. (C) Chlorogenic acid concentrations: 0 (a), 1 (b), 2 (c), 5 (d), 10 (e), 15 (f), 20 (g), 25 (h), 30 (i), 40 (l), 50 (m) and 100 (n) μmol/L. (D) Coumaric acid concentrations: 0 (a), 2.5 (b), 5 (c), 7.5 (d), 10 (e), 15 (f), 20 (g), 25 (h), 30 (i), 35 (l), and 40 (m) μmol/L. (E) Ferulic acid concentrations: 0 (a), 2.5 (b), 5 (c), 7.5 (d), 10 (e), 15 (f), 20 (g), 25 (h), 30 (i), 35 (l), 40 (m) and 50 (n) μmol/L. (F) Dihydroxyphenylacetic acid concentrations: 0 (a), 5 (b), 7.5 (c), 10 (d), 15 (e), 20 (f), 30 (g), 40 (h), 50 (i), and 60 (l) μmol/L. (G) Vanillic acid concentrations: 0 (a), 5 (b), 7.5 (c), 10 (d), 15 (e), 20 (f), 30 (g), 40 (h), and 60 (i) μmol/L. (H) Gallic acid concentrations: 0 (a), 5 (b), 7.5 (c), 10 (d), 15 (e), 20 (f), 25 (g), 30 (h), 35 (i), 40 (l), 50 (m) and 60 (n) μmol/L.

Table 3
Polyphenol concentration in milk and whey. Concentration in supernatant of diluted curd. Apparent concentration in curd and in pellet of diluted curd.

Polyphenol	Milk mmol/L	Curd mmol/L	Whey mmol/L	Pellet of diluted curd mmol/L	Supernatant of diluted curd mmol/L
Catechin	1.72 ± 0.02[a]	2.61 ± 0.12[a]	0.59 ± 0.05[a]	0.56 ± 0.06[a]	0.17 ± 0.01[a,d]
Tannic acid	0.30 ± 0.01[b]	0.44 ± 0.06[b]	0.09 ± 0.02[b]	0.14 ± 0.03[b]	0.03 ± 0.01[b]
Chlorogenic acid	1.41 ± 0.01[c]	1.77 ± 0.09[c]	0.92 ± 0.06[c]	0.31 ± 0.03[b,c]	0.13 ± 0.02[a]
p-Coumaric acid	3.05 ± 0.02[d]	3.79 ± 0.21[d]	2.09 ± 0.09[d]	1.14 ± 0.10[c]	0.24 ± 0.01[c,e,f]
Ferulic acid	2.57 ± 0.01[e]	3.17 ± 0.19[e]	1.82 ± 0.10[e]	0.92 ± 0.07[d]	0.21 ± 0.02[d,c]
3,4-Dihydroxyphenylacetic acid	2.97 ± 0.01[f]	3.40 ± 0.20[d,e]	2.61 ± 0.11[f]	0.76 ± 0.05[d]	0.23 ± 0.01[c,f]
Vanillic acid	2.97 ± 0.01[f]	3.54 ± 0.14[d,e]	2.24 ± 0.07[d]	0.42 ± 0.05[a,e]	0.28 ± 0.03[e,f]
Gallic acid	2.94 ± 0.02[f]	3.22 ± 0.10[e]	2.55 ± 0.11[f]	0.31 ± 0.05[b,e]	0.27 ± 0.02[f]

Values in the same column with different lowercase letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$).

retention coefficients decreased to 78.7 ± 0.5 and to 50.5 ± 0.7 g/100 g, respectively. We added the catechin to curd of casein solution and also in this case we observed that the catechin retention coefficient decreased to 61.5 ± 0.7 from 96.9 ± 0.7 g/100 g. These data demonstrated that during coagulation the interactions between the molecules of casein decreased the number of binding sites available for the polyphenols. These data also suggested that the coagulation process did not remove polyphenols from casein binding site when they were bound before coagulation.

3.4. Release of polyphenols from curd matrix during simulated gastro-pancreatic digestion

Table 4 reports the phenol released during the digestion steps. For comparison the phenol content in curd before digestion is also reported in the table. The dilution and homogenization of curd in simulated gastric fluid determined a relevant polyphenol release. In the supernatant obtained after centrifugation the amount of polyphenol ranged from 69% (tannic acid) to 90% (gallic acid) of the compound present in the curd. It should be pointed out that the phenols present in curd are in part bound to the solid fraction and in part are present in the liquid fraction of the curd, which has a composition similar to whey.

Table 3 reports the millimolar concentration of polyphenols in the supernatant obtained after curd dilution and centrifugation. The apparent concentration in pellet obtained after curd dilution and centrifugation is also reported in the same table.

With dilution in gastric fluid the concentration of polyphenols and casein decreased 11 times and for some polyphenols such as gallic acid the concentration was near to K_D value.

The rank order of phenol content, in the curd before dilution and homogenization was tannic acid > catechin > chlorogenic acid > *p*-coumaric acid > ferulic acid > vanillic acid > 3,4-dihydroxyphenylacetic acid > gallic acid. The rank order of phenols that remained in pellet after dilution of curd was tannic acid > *p*-coumaric acid > ferulic acid > catechin > 3,4-dihydroxyphenylacetic acid > chlorogenic acid > vanillic acid > gallic acid.

The different rank order may be due to the different pH before (pH 6.70–6.79) and after (pH 2.0) dilution. The decrease of pH results in the protonation of the carboxylic acids of some polyphenols with modification of their solubility in the liquid phase.

The affinity of polyphenols to coagulated casein and to protein-lipid micelles may also change with pH variation.

At the end of gastric digestion some variation in phenols concentration measured in liquid phase of digest respect to the beginning of the digestion were observed. The significant increase in catechin and chlorogenic acid concentrations that was observed, suggested a further release from curd of these phenols. A small, non-significant increase was also observed for tannic acid, *p*-coumaric and vanillic acids.

At the end of the pancreatic digestion the significant decrease in catechin, *p*-coumaric acid, and gallic acid suggested that these compounds were partially degraded during this phase of digestion. Tannic, ferulic, 3,4-dihyddroxyphenylacetic and vanillic acids were quite stable during digestion.

It should be pointed out that the concentration of phenols in the supernatant was the result of the negative effect of their degradation and positive effect of their release from casein. From our digestion data it appears that chlorogenic acid, vanillic acid, catechin and tannic acid are the best candidate as phenol additives to curd since they are recovered in higher amounts in the supernatant after pancreatic digestion.

3.5. Curd antioxidant activity

The antioxidant activities of cheese curds are shown in Table 5. The control curd showed antioxidant activity (102.4 ± 4.7 μmol of TEAC/L). This antioxidant activity occurred as a result of the content of several compounds in milk, especially high molecular weight caseins (Clausen, Skibsted, & Stagsted, 2009).

All the curd samples with polyphenols showed more antioxidant activity than the control sample after homogenization. These results evidenced the higher nutritive value of those polyphenols-enriched cheese.

Table 4
Amount of the phenols retained in curd and in curd pellet after dilution and amount of phenols released from curd after dilution and during *in vitro* digestion. Data referred to curd samples prepared with 20 mL of milk with a specific weight of 1.034 added of 10 mg of phenolic compounds.

Phenol added	Phenols retained in curd (mg)		Phenols released in supernatant (mg)		
	Before dilution	After dilution (pellet)	After dilution	After gastric digestion	After pancreatic digestion
Catechin	8.5 ± 0.1[a]	1.8 ± 0.02[b]	6.6 ± 0.3[c]	7.3 ± 0.1[d]	6.0 ± 0.3[e]
Tannic acid	8.7 ± 0.1[a]	2.7 ± 0.05[b]	6.0 ± 0.1[c]	6.3 ± 0.5[c]	5.8 ± 0.2[c]
Chlorogenic acid	7.3 ± 0.1[a]	1.4 ± 0.02[b]	6.0 ± 0.3[c]	7.0 ± 0.1[a,d]	6.6 ± 0.5[a,c,d]
p-Coumaric acid	7.0 ± 0.2[a]	2.2 ± 0.04[b]	4.9 ± 0.1[c]	5.3 ± 0.1[c]	3.9 ± 0.1[d]
Ferulic acid	6.9 ± 0.1[a]	2.0 ± 0.03[b]	4.9 ± 0.2[c]	4.6 ± 0.1[c]	4.4 ± 0.1[d]
3,4-Dihydroxyphenylacetic acid	6.4 ± 0.2[a]	1.4 ± 0.04[b]	4.9 ± 0.1[c]	4.6 ± 0.2[c,d]	4.5 ± 0.1[d]
Vanillic acid	6.7 ± 0.1[a]	0.7 ± 0.01[b]	5.9 ± 0.2[c]	6.1 ± 0.1[c,d]	6.3 ± 0.1[d]
Gallic acid	6.3 ± 0.1[a]	0.5 ± 0.01[b]	5.7 ± 0.1[c]	5.5 ± 0.1[c]	4.9 ± 0.2[d]

Values in one row not sharing the same superscript letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$).

Table 5
Antioxidant activity of supernatant of *in vitro* digested curds determined by ABTS assay at pH 2.0. Results are expressed as μmol of TEAC/L.

Treatment	Before digestion	Post-gastric	Post-pancreatic
Control	102.4 ± 4.7[a]	260.4 ± 51.8[b]	808.2 ± 126.0[c]
Catechin	560.3 ± 3.6[a]	885.1 ± 68.7[b]	1317.3 ± 129.3[c]
Tannic acid	323.7 ± 25.5[a]	506.9 ± 56.2[b]	1116.8 ± 75.8[c]
Chlorogenic acid	268.9 ± 24.5[a]	560.9 ± 52.8[b]	1101.3 ± 111.1[c]
p-Coumaric acid	576.1 ± 51.5[a]	739.8 ± 30.3[b]	1137.8 ± 119.7[c]
Ferulic acid	592.4 ± 32.5[a]	729.4 ± 136.2[a]	1202.3 ± 51.5[c]
3,4-Dihydroxyphenylacetic acid	579.9 ± 56.4[a]	808.0 ± 48.9[a]	1332.3 ± 80.3[c]
Vanillic acid	396.4 ± 25.9[a]	496.3 ± 43.8[a]	1047.2 ± 105.5[c]
Gallic acid	637.4 ± 27.9[a]	964.3 ± 78.8[b]	1474.9 ± 97.0[c]

Values in one row not sharing the same superscript letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$).

The curd with gallic acid exhibited the highest value followed by ferulic acid, 3,4-dihydroxyphenylacetic acids, *p*-coumaric acid and catechin. Vanillic, tannic and chlorogenic acids containing curd were those with the lowest antioxidant activity. However, it should be taken into account that there are differences in the millimolar concentration of phenols as it results from Table 3, column "supernatant of diluted curd". For example, the concentration of gallic acid was 1.56 and 8.3 times higher than that of catechin and tannic acid, respectively.

Following simulated gastric digestion phase, we found a significant increase in the control curd antioxidant activity, which was more evident after pancreatic digestion. This increase was due to the release of antioxidant peptides from milk protein during digestion.

During digestion (gastric and pancreatic) all the supernatants of cheese enriched with polyphenols maintain a higher antioxidant activity than control.

3.6. Conclusion

The polyphenol retention coefficients of enriched cheese curds were positively related to polyphenol affinity to a single high affinity binding site on casein molecules, while was negatively affected by their hydrophilicity. The polyphenols should be added before milk coagulation since the binding decreases as casein molecules aggregate. The polyphenols released from enriched curds at the end of digestion depend from their stability in gastro and pancreatic fluids and from their affinity to casein.

All the tested polyphenols increased the antioxidant activity of enriched curds. This antioxidant activity is released during gastric and pancreatic digestion.

This study represents a model for further investigations at molecular level, for the preparation of cheese enriched with bioactive compounds.

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