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Dairy Sci. Technol. 89 (2009) 485–499
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DOI: 10.1051/dst/2009024

Available online at:
www.dairy-journal.org

Original article

Antioxidant properties of green tea polyphenols encapsulated in caseinate beads

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Received 4 November 2008 – Revised 1st April 2009 – Accepted 27 April 2009
Published online 25 July 2009

Abstract – An aqueous extract of polyphenols was prepared from China green tea under optimised conditions and then freeze-dried (green tea polyphenols extract, GTPE). This extract that contained 28% of total polyphenols had an antioxidant activity above 90% in solution when measured by the *N,N,*-diethyl-*p*-phenylenediamine (DPD) method. The GTPE was encapsulated either in the sodium-caseinate (Na-caseinate) or the calcium-caseinate (Ca-caseinate) beads in order to protect its antioxidant activity. The beads containing GTPE were stored at 21 °C and 44% relative humidity in darkness for 42 days. The antioxidant activity of the encapsulated GTPE was measured during storage using the DPD method. The antioxidant activity of the unencapsulated GTPE and the beads without GTPE was measured as controls under the same environmental conditions. Antioxidant activity of all beads was measured after dissolution in filtered and deionised water (80 °C) at pH 2.4 ± 0.1, before and after removing the hydrolysate caseinates that formed during beads' dissolution. The results showed that the antioxidant properties of unencapsulated tea polyphenols (GTPE) and of both the caseinate beads with GTPE were maintained almost stable over 42 days. No significant difference ($P > 0.05$) was observed between the antioxidant properties of the Na- or Ca-caseinate beads and unencapsulated GTPE. On the other hand, the presence of GTPE either in the Na- or in the Ca-caseinate beads did not change significantly ($P > 0.05$) the antioxidant properties of the protein beads. However, the antioxidant activities of the Na- or Ca-caseinate beads containing GTPE were significantly ($P \leq 0.05$) higher than those of the same beads without GTPE after removing the caseinate hydrolysates present in the bead solutions (82% and 78% vs. 10% and 20%, respectively). These results showed that antioxidant activities of the beads without GTPE in solutions were principally caused by caseinate hydrolysates. Among the beads, Ca-caseinate beads with GTPE showed the best antioxidant properties. Antioxidant properties of caseinate beads were compared with those of Trolox. The results showed that a concentration of 5 mg·mL^{-1} of Na- or Ca-caseinate beads without GTPE had antioxidant activity equivalents to 192 and 205 μmol·L^{-1} of Trolox equivalent per mL of Trolox.

caseinate bead / green tea / polyphenol / antioxidant activity / hydrolysate caseinate

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摘要 – 绿茶多酚酪蛋白胶囊的抗氧化特性。在最优条件下，从中国绿茶中提取茶多酚并进行冷冻干燥 (GTPE)。 该提取物总多酚的含量为 28%，用 *N,N* 二乙基对苯二胺法 (DPD) 检测，其抗氧化活性达 90% 以上。 为了保护其抗氧化活性，以酪蛋白酸钠或酪蛋白酸钙为囊材制成将 GTPE 胶囊，然后将胶囊在 21 °C、相对湿度为 44% 避光的环境中保藏 42 天。 在同样的条件下，以未包裹的 GTPE 和不含 GTPE 的酪蛋白酸钠或酪蛋白酸钙胶囊作为对照，用 DPD 法测定在贮藏期间 GTPE 胶囊抗氧化活性的变化。 将所有的样品(包括酪蛋白水解物除去前后的样品) 溶解在 pH = 2.4 ± 0.1 的去离子水中 (80 °C) 后进行测量。 结果表明，未包埋的茶多酚 (GTPE) 和两种酪蛋白酸盐 GTPE 胶囊的抗氧化性能在 42 天中几乎保持稳定。 GTPE 酪蛋白酸钠或酪蛋白酸钙胶囊和未包埋的 GTPE 之间抗氧化性无显著差异 ($P > 0.05$)。 同时，GTPE 酪蛋白酸钠和 GTPE 酪蛋白酸钙胶囊之间的抗氧化活性没有显著改变 ($P < 0.05$)。 然而， GTPE 酪蛋白酸钠 (82%) 和 GTPE 酪蛋白酸钙胶囊 (78%) 的抗氧化活性显著 ($P \leq 0.05$) 高于不含 GTPE 且去掉酪蛋白水解物的酪蛋白酸钠胶囊 (10%) 和酪蛋白酸钙胶囊 (20%)。 对于不含 GTPE 的酪蛋白酸盐，其抗氧化活性主要是由酪蛋白水解。 在所有样品中，其中 GTPE 酪蛋白酸钙胶囊的抗氧化活性最高。 用 Trolox 当量表示抗氧化能力，浓度为 5 mg·mL^{-1} 的不含 GTPE 的酪蛋白钠和酪蛋白钙的抗氧化能力分别为每毫升 192 μmol·L^{-1} 和 205 μmol·L^{-1} Trolox 当量。

酪蛋白胶囊 / 绿茶 / 多酚 / 抗氧化活性 / 酪蛋白水解物

Résumé – Propriété anti-oxydante de polyphénols de thé vert encapsulés dans des billes de caséinate. Un extrait aqueux de polyphénols a été préparé à partir de thé vert chinois dans des conditions optimisées, puis il a été lyophilisé (extrait de polyphénols issu du thé vert, GTPE). Cet extrait, qui contient 28 % de la quantité totale de polyphénols, avait une activité anti-oxydante d'environ 90 % en solution, quand celle-ci a été mesurée au moyen de la méthode *N,N*,-diethyl-*p*-phénylène diamine (DPD). Le GTPE a été encapsulé dans des billes de caséinate de sodium ou de caséinate de calcium, afin de protéger son activité anti-oxydante. Les billes contenant du GTPE ont été stockées dans l'obscurité, à 21 °C et à une humidité relative de 44 % pendant 42 jours. L'activité anti-oxydante du GTPE encapsulé a été mesurée durant le stockage au moyen de la méthode DPD. L'activité anti-oxydante du GTPE non-encapsulé et des billes sans GTPE a été mesurée comme témoin dans les mêmes conditions environnementales. L'activité anti-oxydante de toutes les billes a été mesurée après dissolution dans de l'eau filtrée et déionisée (80 °C) de pH 2,4 ± 0,1 avant et après élimination des caséinates hydrolysés formés pendant la dissolution des billes. Les résultats ont montré que les propriétés anti-oxydantes des polyphénols du thé non-encapsulés et encapsulés sont restées stables plus de 42 jours. Aucune différence significative ($P > 0,05$) n'a été observée, entre les propriétés anti-oxydantes des billes de caséinate de sodium ou de caséinate de calcium et GTPE pendant la période de stockage. D'autre part, la présence de GTPE dans les billes de caséinate n'a pas changé de manière significative ($P > 0,05$) les propriétés anti-oxydantes des billes de protéines. Cependant, les activités des billes de caséinate contenant du GTPE étaient nettement ($P \leq 0,05$) plus élevées que celles des billes qui en étaient dépourvues après que la fraction hydrolysée de caséinate ait été enlevée des billes en solution (respectivement 82 % et 78 % contre 10 % et 20 %). Ces résultats mettent en évidence que les activités anti-oxydantes des billes sans GTPE en solution étaient dues essentiellement à la protéine de caséinate hydrolysée. Les billes de caséinate de calcium montrent de meilleures propriétés anti-oxydantes que celles à base de caséinate de sodium. Les activités anti-oxydantes des billes de caséinate ont été comparées à celle du Trolox. Les résultats montrent qu'une concentration de 5 mg·mL^{-1} de billes de caséinate de sodium ou de calcium sans GTPE avait respectivement une activité anti-oxydante équivalente à 192 μmol·L^{-1} et 205 μmol·L^{-1} équivalent Trolox par mL de Trolox.

billes de caséinate / thé vert / polyphénols / activité anti-oxydante / caséinate hydrolysé

1. INTRODUCTION

Polyphenol compounds have an ideal chemical structure to assure free radical scavenging activities, and in vitro measurements have shown antioxidant properties to be more effective than vitamins E and C on a molar basis [22, 23]. Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolic compounds have attracted great interest in the food industry especially for their protection against lipid oxidation or degradation and thereby improving the quality and nutritional value of food. Among them, green tea (*Camellia sinensis*) contains the most important quantity of polyphenols (about 10–30% w/w) with strong antioxidant properties [15, 25, 33].

Microencapsulation is a process in which unstable compounds like polyphenols are entrapped in a protective polymer. Core materials can thereby be protected from degradation or oxidation in order to stabilise the molecule for a longer period of time and improve the range of applications.

Milk proteins, such as casein, were mainly studied, owing to their excellent nutritional value and their numerous functional properties such as barrier to oxygen transfer [7–9] and their ability to form a complex due to intermolecular hydrogen, electrostatic and hydrophobic bonds as well as for their cross-linking ability via disulphide bonds or bityrosine formation [6, 13, 20].

Edible beads based on casein offer potential solutions to stabilise the molecules, by serving as a barrier to oxygen transfer in food system [8, 9, 30].

Casein is often used as soluble sodium caseinate (Na-caseinate) or as calcium caseinate (Ca-caseinate) [26]. In this study, an aqueous green tea extract rich in polyphenols was encapsulated in the Na- or in the Ca-caseinate beads prepared by the acid coagulation. Then, the protection of the antioxidant activity of green tea polyphenols (GTPs) by the beads was investigated as a function of time at 21 °C and 44% of relative humidity (RH). Since milk proteins can exert certain antioxidative effects [1, 17, 18, 29–32], antioxidant activities of the beads without the green tea extract were studied in order to investigate the possible synergetic effect of the interaction between the two antioxidants. Trolox is a water-soluble analogue of vitamin E lacking the phytyl chain. The Trolox equivalent antioxidant capacity (TEAC) provided an easy way to evaluate the antioxidant properties of other substances. TEAC of the beads and green tea polyphenols extract (GTPE) lost were investigated by the *N*,*N*,-diethyl-*p*-phenylenediamine (DPD) method in which free radical-scavenging capacities of the samples were compared with those of Trolox solutions. Also, in order to verify the effects of encapsulation and bead formulations, the antioxidant capacity of non-encapsulated GTPE was compared to that of encapsulated GTPE using the Na- and Ca-caseinate formulations under standard storage conditions.

2. MATERIALS AND METHODS

2.1. Material and reagents

A commercial dry China green tea was purchased from a local market. Calcium caseinate (Alanate 380) was provided by New Zealand Milk Products (Santa Rosa, CA, USA). Glycerol (99.5%, Reagent Grade) was obtained from American Chemicals Ltd. (Montreal, Quebec, Canada). Sodium caseinate, Xanthan, DPD, catechin (CAT), urea (Acs Reagent), gum Arabic, ammonium persulphate, acrylamide/Bis-acrylamide 30% solution 37.5:1 electrophoresis reagent, Tris base, *N*,*N*,*N*′,*N*′ tetramethyl-ethylenediamine, lauryl sulphate (sodium dodecyl sulphate) and glycine

(G-7126) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Trichloroacetic acid was obtained from Fisher Scientific.

Silver Stain Kit was purchased from Bio-Rad Company. Ferric ammonium sulphate (FAS), *N*,*N*-dimethylformamide and β-mercaptoethanol were obtained from Merck (Darmstadt, W. Germany). Bromophenol blue was obtained from Fluka Company (Sigma-Aldrich Chimie, St. Quentin Fallavier Cedex, France). Tannic acid (TA) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%) were purchased from Sigma-Aldrich (Ontario, Canada). Standard solutions of TA and CAT in DMF-acetate were prepared. Six dilutions were made at concentrations between 10 and 800 μg·mL^{-1}.

2.2. Preparation of GTPE

An aqueous extract of polyphenols from a commercial dry China green tea was obtained at the optimised conditions, which were reported by Dehkharghanian [9]. Briefly, 12 g of dry commercial green tea (water content: 5.6%) were ground (size range of leaf: 0.080–0.315 mm in length where 53% of them were between 0.200 and 0.315 mm). Phenolic compounds were extracted after dissolution in 900 mL of filtered and deionised hot water (90 °C) and stirring at 150 rpm in a chemical extractor (2 L) for 1 min. The tea extract (880 mL) was chilled immediately, filtered (0.20 μm) and frozen at −80 °C. It was then freeze-dried and stored at −80 °C in the darkness. The yield after freeze-drying from the tea extract was 0.82% (880 mL tea extract gives 7.2 g GTPE).

The quantification of the GTPs was calculated using CAT and TA equivalents on the freeze-dried powder (GTPE) [5]. CAT contains two consecutive hydroxyls adjacent in their benzene rings and TA has three consecutive hydroxyls adjacent to their benzene rings [5].

Determination of polyphenol yield from green tea leaves

Polyphenol yield (mg·TA·g^{-1} dry tea) or (mg·CAT·g^{-1} dry tea) from green tea leaves was calculated as follows:

$$\text{Yield } \left(\text{mg}\cdot\text{g}^{-1}\right) = \frac{V_{\text{TE}} \times \text{TA (or CAT)}}{M_{\text{L}}}, \quad (1)$$

where V_{TE} is the total volume (mL) of extract after 1 min of extraction, TA is the phenolic content of the extract (mg·mL^{-1}) in terms of tannic acid equivalent, CAT is the phenolic content of the extract (mg·mL^{-1}) in terms of catechin equivalent, and M_{L} is the mass of the tea leaves (g) (dry basis).

2.3. Caseinate bead preparation

Four formulations of protein beads named Na-caseinate beads, Ca-caseinate beads, Na-caseinate beads with GTPE and Ca-caseinate beads with GTPE were prepared on the basis of a protocol developed in our laboratory. In order to obtain the same viscosity and thickness, 14 g Na-caseinate or 18.5 g Ca-caseinate were dissolved in 78 and 73.5 mL distilled water at 40 °C, respectively. These solutions were stirred slowly at room temperature overnight. Then, 100 mL of xanthan solution (2%) were prepared with distilled water.

Three grams of glycerol and 5 g of homogenised xanthan solution were added subsequently into the Na- and Ca-caseinate solutions and stirred gently at room temperature for 1 h in order to obtain a homogenised solution.

For the preparation of the Na- and Ca-caseinate beads containing GTPE, 1 g of distilled water was replaced by 1 g of freeze-dried GTPE powder in previous caseinate solutions and stirred for 1 h more to obtain homogenised GTPE-caseinate

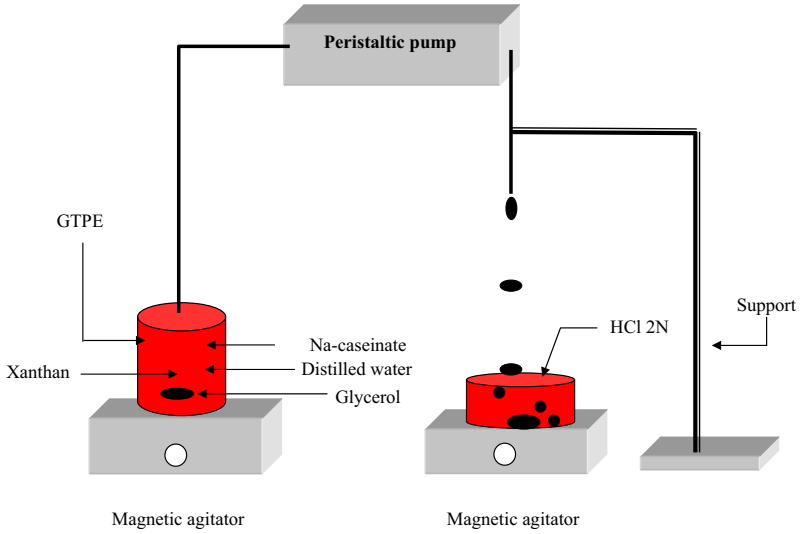

Figure 1. Schematic diagram of the experimental system for preparing Na-caseinate beads with GTPE.

solutions. The composition of the different bead aqueous solutions is as follows:

(a) Na-caseinate bead solution: 14% Na-caseinate, 3% glycerol and 0.1% xanthan;
(b) Ca-caseinate bead solution: 18.5% Ca-caseinate, 3% glycerol and 0.1% xanthan;
(c) Na-caseinate bead solution with GTPE: 14% Na-caseinate, 3% glycerol and 0.1% xanthan, 1% GTPE;
(d) Ca-caseinate bead solution with GTPE: 18.5% Ca-caseinate, 3% glycerol, 0.1% xanthan and 1% GTPE.

All caseinate solutions were centrifuged (1098× *g*) for 10 min at 20 °C in order to remove the entrapped air bubbles.

In order to assure the coagulation of casein and the formation of spherical beads, the whole centrifuged solutions were dropped in 1 L hydrochloric acid (2 N), using a peristaltic pump (F13 pharmacia) connected to a soft plastic tube (Ø = 1.2 mm) by gentle stirring of the mixture (Fig. 1). The solidified beads were recovered by filtration, washed with distilled water and dried at room temperature under darkness.

The bead diameters after drying were 1.5 ± 0.1 and 1.7 ± 0.1 mm for Na- and Ca-caseinate beads, respectively.

2.4. Precipitation of caseinate in bead solution

At first, 50 mg of each bead formulation (with or without GTPE) were dissolved in 1 mL of distilled and deionised water at 80 °C in an Eppendorf tube for 6 h. The pH values of beads solution after cooling were 2.4 and 2.5 for Na- and Ca-caseinates, respectively. Then to 500 µL of this solution, 500 µL trichloroacetic solutions were added (20%). The Eppendorf mixture was incubated in an ice bath for 15 min. Then, it was centrifuged at 4 °C and 15 000 rpm for 15 min. Two hundred microlitres

of the supernatant were taken for measuring the antioxidant capacity.

2.5. Storage of the beads and unencapsulated GTPE

Since edible films based on casein salts that are soluble in water generally have a considerable water vapour permeability [2], the Na- and Ca-caseinate beads could hydrate or dehydrate easily depending on the conditions of storage (RH and temperature). In addition, the freeze-dried GTPE could quickly absorb water vapour. In order to maintain all samples under the same conditions, and minimise their weight evolution, the Na- and Ca-caseinate beads with or without GTPE as well as unencapsulated GTPE were reconditioned in a desiccator containing a saturated K_2CO_3 solution, ensuring 44% RH at room temperature (21 °C) under darkness [4]. Mass evolutions ($m_0 - m$) of 2 g (m_0) of the Na- and Ca-caseinate beads with GTPE and unencapsulated GTPE were measured using a balance with 0.01 mg accuracy during storage time. After 14 days, all the samples were brought out of the desiccators and weighted. Then, the samples were returned quickly to the desiccators. Measurements were also performed after 28 and 42 days of storage under the same conditions. The mean values of the measurements at 14, 28 and 42 days of storage named (m). The value of ($m_0 - m$) was the difference between the mass of the beads or unencapsulated GTPE before recondition at 44% RH at 21 °C and the mass of the same samples after recondition, respectively, after 14, 28 and 42 days.

2.6. Total polyphenol analysis

In order to measure the content of total phenols in samples, five dilutions were made at concentrations between 24 and 1.5 $mg{\cdot}mL^{-1}$ for the Na- and Ca-caseinate beads with GTPE and 100–12.5 $mg{\cdot}mL^{-1}$ for unencapsulated GTPE in filtered and deionised water. Then, the total polyphenols content in samples was estimated using the FAS assay [5] at an equilibrium concentration in water (1.5 $mg{\cdot}mL^{-1}$ for the beads and 12.5 $mg{\cdot}mL^{-1}$ for unencapsulated GTPE, respectively).

The ferric ions present in the FAS reagent reacted with phenolic compounds in the GTPE to form a blue-coloured complex with trihydroxybenzene derivatives (galloyl groups, 580 nm) and a green-coloured complex with dihydroxybenzene derivatives (catechol groups, 680 nm). Phenolic content ($mg{\cdot}mL^{-1}$) was expressed in terms of TA or CAT.

2.7. Free radical scavenging capacity determination

Free radical scavenging capacity is a direct method for the measurement of the antioxidant property. Free radical scavenging capacities of the caseinate beads with or without GTPE and unencapsulated GTPE were evaluated following their inhibitory effect on the oxidation of DPD by oxygen free radicals produced by electrolysis [14].

Five milligrams of the beads were dissolved in 1 mL distilled and deionised water at 80 °C for 6 h. The pH of the bead solutions was 2.4 and 2.5 for Na- and Ca-caseinates, respectively. The low pH of the bead solution was due to the remaining chlorohydric acid traces that were utilised for beads' solidification, despite extensive washing. Then, 100 μL of each bead solution were diluted with 900 μL distilled water and homogenised. Two hundred microlitres of the diluted solutions were introduced into a cell containing 3 mL of NaCl solution (0.15 $mol{\cdot}L^{-1}$) and submitted to electrolysis for 1 min (400 V, 10 mA, DC) using a power supply (Bio-Rad, model 1000/500). After electrolysis, a volume of 600 μL of solution was sampled and added to 300 μL of DPD reagent (25 $mg{\cdot}mL^{-1}$ in 0.15 $mol{\cdot}L^{-1}$ NaCl solution). The oxidative species react instantaneously with the DPD

reagent producing a red colouration, which can be measured at 515 nm. The antioxidant capacity describes the bead solution's capacity to prevent the accumulation of oxidative species (able to oxidise DPD) and the red colouration at 515 nm. The reaction mixture was calibrated using the non-electrolysed 0.15 mol·L^{-1} NaCl solution (no oxidative species, ascribed to 100% scavenging) and the electrolysed NaCl solution (0% scavenging in the absence of any antioxidant). Standard solutions of Trolox were prepared in NaCl buffer (0.9%). Eight dilutions were prepared at concentrations between 10 and 2000 μmol·L^{-1} in NaCl solution (0.15 mol·L^{-1}). Two hundred microlitres of each dilution were sampled and their antioxidant capacities were measured by the DPD method, which was explained before.

The scavenging percentage was calculated as follows:

$$\text{Scavenging } (\%) = \left[1 - \left(\text{OD}_{\text{sample}}/\text{OD}_{\text{control}}\right)\right] \times 100, \quad (2)$$

where OD is the optical density at 515 nm and $\text{OD}_{\text{control}}$ represents the OD of electrolysed solution without the sample solution. OD is directly related to the degree of oxidation of the reagent by the oxidative species. Thus, a bead solution that is able to reduce completely the level of reactive oxidative species will have a 100% scavenging capacity.

The antiradical activity of the beads and GTPE was also estimated from the calibration curve ($r^2 = 0.9886$) constructed by plotting known solutions of Trolox (μm) against the scavenging capacity (%).

Determination of the free radical scavenging capacity of unencapsulated GTPE

In order to determine the concentration in which unencapsulated GTPE had maximum antioxidant activity, several dilutions were prepared at concentrations between 0.25 and 20 mg·mL^{-1} in filtered and deionised water. Then, 200 μL of each dilution were sampled for the DPD assay.

In order to avoid the saturation point, the first concentration in which the antioxidant activity of unencapsulated GTPE reached a plateau (2 mg·mL^{-1}) was selected to study the antioxidant activity evolution as a function of time.

2.8. SDS-PAGE

In order to verify the production of hydrolysate of Na- and Ca-caseinates during the bead formation and dissolution in hot water (80 °C) at low pH (2.4–2.5), the bead solutions were analysed after denaturation using β-mercaptoethanol and separation in SDS-PAGE using 15% acrylamide gel according to Laemmli [19]. Gels were silver stained and parent molecular masses of the caseinate bead fractions were compared by Na- and Ca-caseinate solutions as controls in the presence of standard markers.

At first, Na- and Ca-caseinate bead solutions (250 μg·mL^{-1}) were prepared in distilled and deionised hot water (80 °C). The pure Na- and Ca-caseinate beads were solubilised (250 and 125 μg·mL^{-1}) under the same condition as control. Then 20 μL of each sample were deposited on a 15% acrylamide gel electrophoresis with 5 μL of β-mercaptoethanol. The apparent molecular weights of proteins were determined by co-migration of standard markers: phosphorylase b (97 kg·mol^{-1}), albumin (66 kg·mol^{-1}), ovalbumin (45 kg·mol^{-1}), carbonic anhydrase (30 kg·mol^{-1}) trypsin inhibitor (20.1 kg·mol^{-1}) and α-lactalbumin (14.4 kg·mol^{-1}).

2.9. Statistical analysis

Non-parametric Kruskal-Wallis one-way analysis of variance and non-parametric Mann-Whitney test were used to determine whether antioxidant activities of the beads

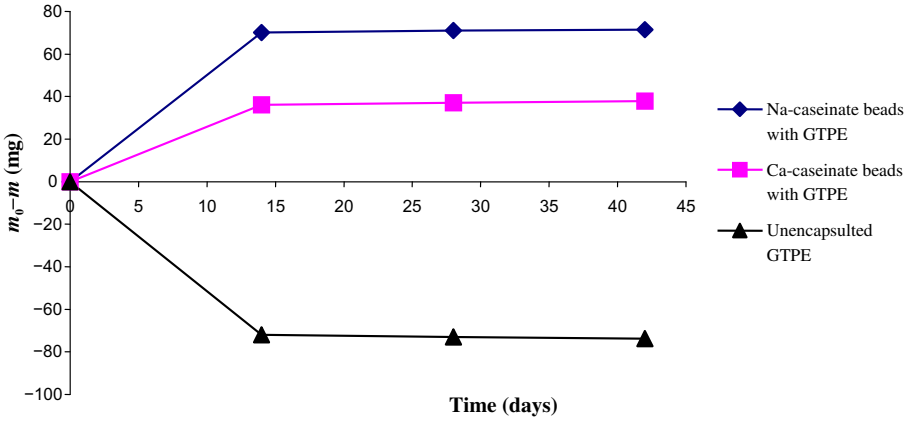

Figure 2. Mass evolution of the Na- and Ca-caseinate beads with GTPE and unencapsulated GTPE at 44% RH and 21 °C during storage time.

and unencapsulated GTPE were affected by time. The Student unpaired *t* test was utilised to test to determine the difference between the antioxidant activities of the Na- or Ca-caseinate beads with and without GTPE as well as the difference between the beads and unencapsulated GTPE during storage time. Non-parametric Mann-Whitney test was utilised to test the difference between antioxidant activities of the Na- or Ca-caseinate beads with and without GTPE before and after removal of casein during storage time. Statistical analyses were carried out by using the software Instat (GraphPad Software, Inc. Version 3). All comparisons were made at a 5% level of significance. The experiments were done in two replicates and for each replicate six samples were tested.

3. RESULTS

3.1. Mass evolution of the caseinate beads and unencapsulated GTPE

The mass evolutions of the caseinate beads and unencapsulated GTPE at 44% RH and 21 °C are shown during storage time (Fig. 2). The results showed that the mass of all samples arrived to equilibrium state after 14 days of storage in the above conditions due probably to the interactions of the bead components with water. Since the beads were reconditioned in controlled environmental conditions (44% RH and 21 °C), the Na- and Ca-caseinate beads were dehydrated only by 1.8% and 2.9%, respectively. However, freeze-dried unencapsulated GTPE hydrated 3.6% in this condition.

The different initial water activities of the samples, which were obtained by the different production conditions (freeze-drying for the GTPE of and natural drying for the beads in the laboratory with 52% RH), explain the hydration or dehydration of the samples during storage at 44% RH. The higher dehydration of Na- and Ca-caseinates was probably due to the higher content of water in those formulations (see Sect. 2). However, the mass evolutions of the samples did not change significantly the results of the free radical scavenging capacity of the GTPE and the beads.

Table I. Total polyphenols content.

Samples	Total polyphenols ($mg \cdot g^{-1}$)*	
	TA	CAT
Green tea leaves	134 ± 8	228 ± 10
Unencapsulated GTPE	226 ± 11	291 ± 13
Na-caseinate beads with GTPE	11 ± 1	13 ± 1
Ca-caseinate beads with GTPE	10 ± 1	12 ± 1

* Dry basis.

3.2. Polyphenols content measurements

Total polyphenols content in commercial China dry green tea leaves, unencapsulated GTPE and caseinate beads including GTPE was analysed and the results are shown in Table I. The results showed that the extraction yields of GTPs in the optimised experimental condition were 228 and 134 mg of CAT and TA equivalents per gram of dry weight of green tea leaves, respectively. These results are in accordance with the polyphenols content indicated by the manufacturer (10–30% w/w). The total polyphenols yields in terms of free GTPE equivalent (non-encapsulated GTPE) were 291 mg CAT per gram and 226 mg TA per gram. The polyphenols content in beads was between 10 and 13 $mg \cdot g^{-1}$ for both the Na- and the Ca-caseinate beads in CAT and TA equivalents (Tab. I). Hence, these contents correspond to polyphenols content in 5.50% and 4.44% of GTPE, which was utilised in the Na- and Ca-caseinate bead composition on theoretical dry weight basis (Tab. II).

3.3. Free radical scavenging capacity of unencapsulated GTPE

Free radical scavenging activities of unencapsulated GTPE as a function of concentration are shown in Figure 3. The results showed that the antioxidant activity of unencapsulated GTPE is concentration dependent. This activity reached its maximum (90%) at a concentration of 2 $mg \cdot mL^{-1}$. This result showed that a concentration of 2 $mg \cdot mL^{-1}$ was enough to scavenge 90% of free radicals (oxidative species) produced in the DPD method during electrolysis.

Table II. Theoretical dry basis composition (%) of Ca- and Na-caseinate beads containing GTPE.

Ca-caseinate beads with GTPE		Na-caseinate beads with GTPE	
Ca-caseinate	81.80	Na-caseinate	77.30
Xanthan	0.44	Xanthan	0.60
Glycerol	13.33	Glycerol	16.60
GTPE	5.50	GTPE	4.44

3.4. Free radical scavenging capacity of beads and unencapsulated GTPE

The free radical scavenging properties of unencapsulated GTPE and beads based on Na- and Ca-caseinates containing GTPE as well as the caseinate beads without GTPE are shown in Figures 4 and 5.

The results showed that generally, antioxidant properties of all beads and unencapsulated GTPE do not vary significantly ($P > 0.05$) during time (42 days of storage at 21 °C and RH: 44%). In the case of unencapsulated GTPE, variation of antioxidant properties between the first and the 42nd day was not significantly different ($P > 0.05$) and varied from 92 to 90 ± 2%. The auto-oxidation of antioxidants like tea polyphenols is a phenomenon, which was favoured normally by some factors such as time and light. In the case of unencapsulated GTPE, the results showed that the short period

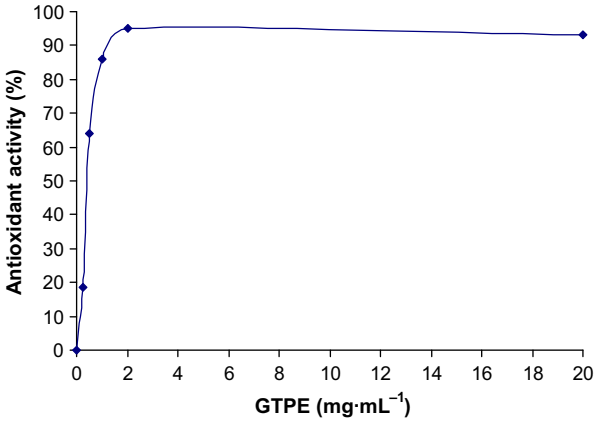

Figure 3. Variation of antioxidant activity of GTPs extracted as a function of concentration.

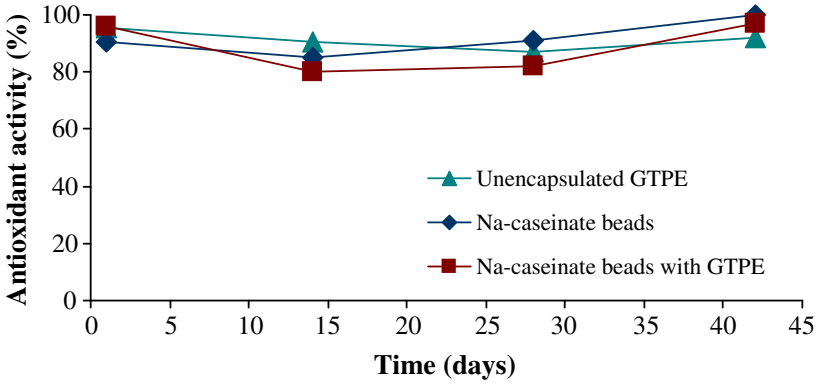

Figure 4. Comparison of the antioxidant activity evolutions among Na-caseinate beads with or without GTPE and unencapsulated GTPE as a function of time.

of storage time under darkness was not enough for which a significant auto-oxidation of polyphenols in GTPE occurred, however, encapsulation of GTPE would probably be indispensable for a longer storage time.

The antioxidant activity of Ca-caseinate beads without and with GTPE was, respectively, 92 ± 2% and 96 ± 3% at day 0 and was relatively stable during storage. For Na-caseinate, the antioxidant was respectively, 88 ± 2% and 90 ± 3% for beads without and with GTPE at day 0 and was also stable during storage. In summary, these values showed an antioxidant property ≥ 80% during storage time in both bead formulations and in unencapsulated GTPE. There were no significant difference ($P > 0.05$) between the antioxidant activity of the Na- or Ca-caseinate beads with or without GTPE during storage time. On the other hand, antioxidant activity of the Na- or Ca-caseinate beads with GTPE were significantly higher ($P \leq 0.05$) than those of the same beads without GTPE

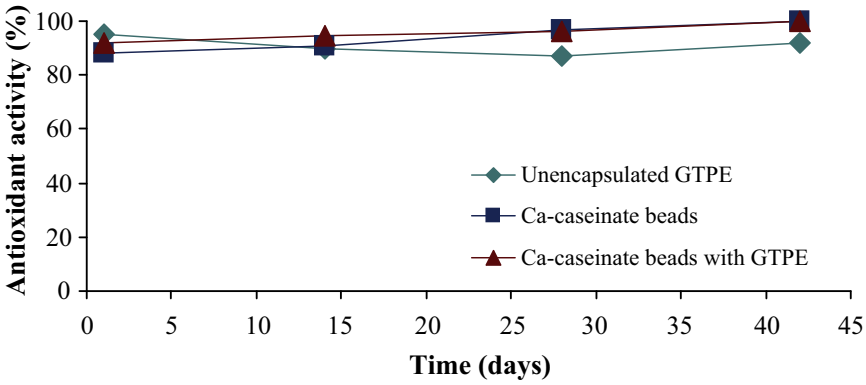

Figure 5. Comparison of the antioxidant activity evolutions among Ca-caseinate beads with or without GTPs and unencapsulated GTPE as a function of time.

in supernatant portions, after precipitation of casein fraction (82 ± 3% and 78 ± 7% vs. 10 ± 7% and 20 ± 5%, respectively) after 42 days of storage (data not shown). In order to explain these results, the protein mass fractions of Na- and Ca-caseinate beads were analysed by SDS-PAGE. A comparison between the protein mass fraction of Na- and Ca-caseinate beads and casein salt (Na and Ca) solutions by SDS-PAGE is given in Figure 6. The results showed major casein salt subunits as α_{s1}- (22–23.7 kg·mol^{-1}), α_{s2}- (25 kg·mol^{-1}), β- (24 kg·mol^{-1}) and κ-caseins (19 kg·mol^{-1}). So, the temperature of the process (80 °C) did not hydrolyse the standard casein salts. On the other hand, these subunits were not observed in caseinate bead portion in the same deposited quantities (5 μg). Therefore, dissolving of caseinate beads in water (80 °C) hydrolysed the casein-based beads composition. These results showed that casein hydrolysed fractions were only due to the presence of remaining hydrochloric acid traces on the surface of beads, which consequently reduced the pH of the dissolution water.

It seems that strong antioxidant activities of the beads without GTPE in solutions were principally caused by the presence of casein hydrolysate. As the beads were formed by the coagulation in HCl 2N, the remaining trace of HCl in the beads will be sufficient to hydrolyse the protein, during dissolution in distilled water even at ambient temperature (21 °C). Also, the low pH (2.5) obtained by dissolving the beads in distilled water could also hydrolyse the protein.

However, the tea polyphenols demonstrate their antioxidant activities in the supernatant portions after 42 days of storage in the environmental conditions. The total quantity of polyphenols, which was measured in the supernatants, confirmed this observation (Tab. III).

On the other hand, a significant difference ($P \leq 0.05$) was observed between the antioxidant activity of the Ca- and Na-caseinate beads with GTPE during storage time (95% vs. 87%, respectively). With regard to theoretical dry basis compositions of these beads, Table II shows that caseinate proportion of the Ca-caseinate beads is more than that of the Na-caseinate beads (81.80% vs. 77.30%).

The antioxidant properties of casein and casein hydrolysate observed are in agreement with those of other reports. Casein hydrolysate is able to inhibit the oxidative

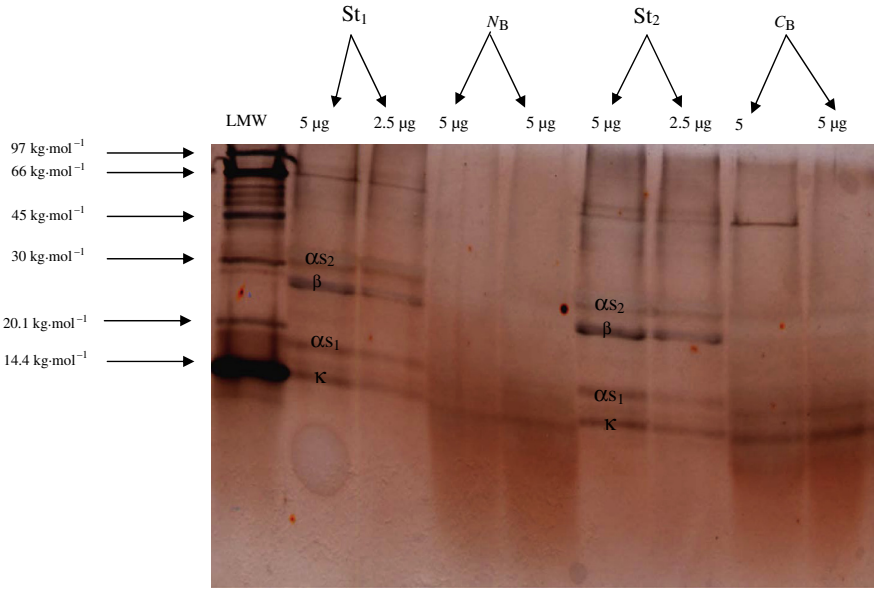

Figure 6. Comparison of SDS-PAGE analysis of Na-caseinate bead (N_B) and Ca-caseinate bead (C_B) fractions with standards of casein sodium salt (St_1) and casein calcium salt (St_2) as control.

Table III. Total polyphenols content (mg·mL^{-1}) in Na- and Ca-caseinate beads with GTPE in solutions (50 mg·mL^{-1}), after removing casein hydrolysates at the 42nd day of storage.

Samples	Polyphenols (mg·mL^{-1})	
	TA	CAT
Supernatant of Na-caseinate bead solution containing GTPE after the removal of casein	0.12 ± 0.04	0.20 ± 0.05
Supernatant of Ca-caseinate bead solution containing GTPE after the removal of casein	0.10 ± 0.04	0.17 ± 0.04

reaction by chelating iron and scavenging free radicals [10]. Casein and casein-derived peptides inhibited enzymatic and nonenzymatic lipid peroxidation, most likely by being a preferred target over fatty acid free radical intermediate(s) [24]. Antioxidant properties of casein and peptides derived from casein might be attributed to chelating metals by phosphoryl residues and scavenging free radicals [11, 16].

Casein was markedly antioxidative on lipid oxidation in emulsion, catalysed by Cu^{2+} ions [1]. A scavenging of oxygen free radicals and reactive oxygen species was found for the film based on the Ca-caseinate which inhibited by 39% of the formation of coloured compounds produced by the reaction of the oxidative species with DPD [30].

Many factors could account for the antioxidant property of milk proteins.

Histidine residues and its derivatives (having an imidazole compound) were reported to have an antioxidant activity that was due to hydrogen donation [17]. Indeed, the hydrogen on the ring nitrogen and on the methylene carbon next to imidazole ring nitrogen is likely donors. Cysteine also inhibits polyphenol oxidase via its SH groups, acting as an agent, coupling quinines and forming stable, colourless compounds [12]. In addition to cysteine, aromatic amino acids like tyrosine and tryptophane are potent free radical targets [3].

The recent studies showed that casein hydrolysates and low molecular weight casein hydrolysates had better peroxyl radical-scavenging activities than the enriched caseinophosphopeptides, which might be explained by a comparison of their amino acid content [10]. Casein calcium peptides, molecular mass of about 3 $kg{\cdot}mol^{-1}$, have been shown to have strong antioxidant activity with the β-carotene bleaching method, and they also showed scavenging activity against radicals such as superoxide, 2,2-diphenyl-1-picrylhydrazyl and hydroxyl radicals. This activity was dose dependent [26].

The industrial process used to separate casein implies protein destabilisation by acidification or by enzymatic treatment of κ-casein, a protein stabilising agent. Heat also induced changes in the casein protein, which are dependent on time, on temperature and on pH. Those changes are related to dephosphorylation, proteolysis, degradation of amino acid side chains, possible cleavages of glycosidic groups from κ-casein, micellar association and cross-linking [27, 28, 34]. Therefore, dissolving caseinate beads in hot filtered and deionised water (80 °C) and low pH (2.4 ± 0.1) may play a part in improving the antioxidant potential of casein on heating due to the hydrolysis of a portion of casein to casein hydrolysate. Peptides generated from the digestion of milk proteins are reported to have antioxidative activities. Milk-derived "antioxidative peptides" are composed of 5–11 amino acids including hydrophobic amino acids, proline, histidine, tyrosine or tryptophan in the sequence [21]. Antioxidant activity of the "hydrolysates" seems to be inherent to the characteristic amino acid sequences of "peptides" derived, depending on the manner of hydrolysis.

On the other hand, some polysaccharides, such as alginate and carboxymethyl chitosan, were shown to delay the hydroproxide accumulation of methyl linoleate by effectively trapping the peroxide radicals [35]. An increase in the antioxidant activity was observed when a casein solution was supplemented with 5% of lactose prior to heat treatment [30]. An improvement in the antioxidant activity of the Ca-caseinate film was observed in the presence of carboxyl methyl cellulose as carbohydrate [30]. Similarly, xanthan present in the bead composition may have the same effect. The strong antioxidant activity of the caseinate hydrolysate beads in solutions may be caused by a synergistic effect of xanthan with casein hydrolysate, used as a stabilising gum in the bead composition (Tab. II).

In order to compare the antioxidant capacity of the GTPE and beads, results of free radical scavenging capacities were presented as $\mu mol{\cdot}L^{-1}$ Trolox equivalent per mL of products and are shown in Table IV. The results showed that a concentration of 5 $mg{\cdot}mL^{-1}$ of Na- and Ca-caseinate beads had antioxidant activity equivalents to 192 and 204 $\mu mol{\cdot}L^{-1}$ per mL of Trolox equivalent, respectively. However, in the case of unencapsulated GTPE, a concentration of 2 $mg{\cdot}mL^{-1}$ had an equivalent activity of 200 $\mu mol{\cdot}mL^{-1}$ of Trolox. This result confirms that GTPs are mostly responsible for the high antioxidant activities.

On the contrary, the proportion of GTPE in the Ca-beads was less than that in the Na-beads (4.44% vs. 5.50%). The caseinate portion difference was more than the GTPE difference, which may explain this significant difference. The Trolox equivalent antioxidant

Table IV. Trolox equivalent antioxidant activity of Na- and Ca-caseinate beads* as well as unencapsulated GTPE** in solutions.

Samples	Concentration ($mg \cdot mL^{-1}$)	TEAC ($\mu mol \cdot L^{-1} \cdot mL^{-1}$)
Na-caseinate beads	5	192 ± 9
Ca-caseinate beads	5	204 ± 10
Unencapsulated GTPE	2	200 ± 9

* The mean values of antioxidant activity of the beads during the 42-day storage were considered.
** The mean values of antioxidant activity of unencapsulated GTPE during the 42-day storage was considered.

activity of Ca-caseinate beads was greater than that of Na-caseinate beads (204 vs. 192 $\mu mol \cdot L^{-1}$ per mL of Trolox), which may also confirm this difference (Tab. IV).

4. CONCLUSIONS

The results suggest that caseinate hydrolysates are free radical scavengers and these substrates could be considered as potential antioxidants. Antioxidant activity of the beads without GTPE was principally caused by the presence of the casein hydrolysate and may be favoured by xanthan, which was used in composition of the beads. Antioxidant activity of Ca-caseinate beads with GTPE was higher than that of Na-caseinate beads with or without GTPE. It may be related to a difference of casein content in the bead compositions. The use of casein hydrolysate as a natural antioxidant is a promising development in the related fields of food science. However, the results showed that the "GTPE" has a high and a stable antioxidant potential during more than 42 days of storage. Encapsulation of aqueous GTPE in the caseinate beads had an additive effect on their antioxidant activities, which could be utilised in bioindustries.

Acknowledgments: We thank Prof. S. Désobry from the Physico-Chemical & Food Engineering Laboratory (ENSAIA, INPL, Nancy, France) for his precious help and advices in some of the experiments and Prof. G. Govaert for his advices in the statistical analysis from the Computer Engineering Department of UTC (Université de Technologie Compiègne). S. Salmieri is acknowledged for reviewing the paper.

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