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Analytical Methods

Development and validation of a near infrared spectrophotometric method to determine total antioxidant activity of milk

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ABSTRACT

In the present study a spectrophotometric method for the determination of total antioxidant activity (TAA) based on ABTS assay was developed and validated on raw milk (RM), whole UHT milk (WUM), partially skimmed UHT milk (SUM), whole pasteurised milk (WM) and partially skimmed pasteurised milk (SM). The most suitable solvent for antioxidant extraction was 80% acetone. Regardless of the type of milk, the coefficient of determination from the linearity test was greater than 0.95. The limit of detection ranged from 0.74 to 6.07 μmol l^{-1} Trolox equivalents. Repeatability, calculated as relative standard deviation of twenty measurements within a day, and reproducibility, calculated as relative standard deviation of sixty measurements across three days, ranged from 1.24 to 4.04% and from 2.18 to 3.52%, respectively. Preservative added to RM had negligible effects on the TAA of milk. The greatest TAA was measured for SM followed by SUM, RM, WM and WUM.

1. Introduction

Free radicals (FR) are defined as highly reactive and unstable molecules, with an unpaired electron in their outer orbit (Gilbert, 2000). These molecules originate in animal cells due to space radiations (Kovalev, 1983) or as by-products of mitochondrial phosphorylative oxidation (Dröge, 2002), in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Although FR have some important roles in animal and human physiology (Valko et al., 2007), in the long term and at high concentrations they can damage or cause complete degradation of essential molecules in cells, including fat molecules, proteins, and DNA, leading to several clinical diseases (Wu & Cederbaum, 2003). Therefore, FR scavenging is essential for human health and involves a wide array of antioxidant substances, defined as molecules able to compete with other oxidizable substrates, preventing their oxidation (Halliwell & Gutteridge, 2015).

Consumers are more inclined to intake natural antioxidants through the daily diet rather than through chemicals, pharmaceuticals or dietary supplements (Gülçin, 2012). Regarding food and diet antioxidants consumption, previous scientific studies have almost always investigated the antioxidant properties of vegetable foodstuffs, whereas very few have dealt with livestock products (e.g. dairy and meat) (Horita et al., 2016; Pereira, Cavalcanti et al., 2016; Pereira, Faria et al., 2016). However, among the latter, milk is one of the most interesting and promising products with regards to its potential antioxidant activity, due to the wide variety of antioxidant molecules. First, the antioxidant activity of milk caseins and milk whey proteins have been investigated (Pihlanto, 2006; Suetsuna, Ukeda, & Ochi, 2000). Second, milk contains a variety of antioxidant molecule traces, such as low molecular weight thiols (Niero, De Marchi, Masi, Penasa, & Cassandro, 2015; Niero et al., 2014), ascorbate (Nielsen, Hald, Kjeldsen, Andersen, & Østdal, 2001), tocopherol, retinol and carotenoids (Jensen & Nielsen, 1996; Nozière et al., 2006). The study of these molecules is difficult, because it implies the fine-tuning of time consuming and expensive HPLC and mass spectrometric methodologies. Moreover, it would be difficult to attribute to each antioxidant molecule its own contribution to the antioxidant power of milk. As reported by Chen, Lindmark-Månsson, Gorton, and Åkesson (2003), it would be more convenient to consider the total antioxidant activity (TAA) of milk, defined as the sum of each antioxidant contribution, related to the aforementioned molecules. Various analytical methods for TAA determination of different food matrices have been developed, and all are based on hydrogen atom transfer (HAT) or on electron transfer (ET) assays. Among HAT assays, the most common are the oxygen radical absorbance capacity and the radical trapping antioxidant parameter, while among ET assays the main versions are the folin ciocalteu reagent, the ferric ion

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reducing antioxidant power, the 2,2-Diphenil-1-picrylhydrazil radical scavenging capacity and the 2,2′-Azino-Bis(3-Ethilbenzotiazolina-6 Sulfonic Acid)Diammonium Salt test (ABTS) (Huang, Ou, & Prior, 2005). The last method, involving a colorimetric reaction, is based on the deactivation of ABTS radical solution and is described in literature as the most effective assay for milk TAA measurement (Chen et al., 2003).

Total antioxidant activity in milk is a new and unexplored trait, and it could have relevant economic and practical applications in dairy sector. The development of an accurate gold standard method for the quantification of TAA in milk is also essential to evaluate the effectiveness of alternative methods, such as infrared techniques (De Marchi, Toffanin, Cassandro, & Penasa, 2014; Penasa, Tiezzi, Sturaro, Cassandro, & De Marchi, 2014; Revilla et al., 2016; Tiezzi, Pretto, De Marchi, Penasa, & Cassandro, 2013) for the prediction of TAA on large amounts of data, in order to assess phenotypic and genetic variation. Therefore, the aim of this study was to develop and validate a simple, robust, fast and cost-effective spectrophotometric assay for the determination of TAA in milk.

2. Materials and methods

2.1. Chemicals and equipment

Acetonitrile (purity 99.9%), HCl (purity ⩾ 37%), ABTS (2,2′-Azino-Bis[3-Ethilbenzotiazolina-6 Sulfonic Acid]Diammonium Salt, purity >98%), $K_2S_2O_8$ (purity ⩾ 99%), Trolox ([±]-6-Hidroxy-2,5,7,8-Tetra-Methylchoromane-2-Carboxilic Acid, purity 97%) were purchased from Sigma Aldrich (St Louis, MO, USA). Bronopol (2-bromo-2-nitropropan-1,3-diol; Knoll Pharmaceuticals, Nottingham, UK) was used as milk preservative. Acetone (purity 99.8%) and ethanol (purity 99.9%) were purchased from Carlo Erba (Cornaredo, MI, Italy). Ultrapure water produced by Arium 611UV Sartorius (Sartorius, MB, Italy) was used for the preparation of all solutions. Biospectrometer Kinetic 1.3.6.0 (Eppendorf, Hamburg, Germany) and 1 cm length path plastic cuvettes (Ratiolab, Dreieich, Germany) were used for spectrophotometric assays.

All analyses were carried out in the laboratories of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy).

2.2. Solutions and blank

Stock of ABTS 14 mM (solution A) and stock of 4.9 mM $K_2S_2O_8$ (solution B) were prepared in water. These solutions can be stored safely at 4 °C for 3 months. Before TAA assays, solutions A and B were mixed (1:1) and stored in the dark for 12 h at room temperature to activate the ABTS radical, obtaining a dark blue solution (Huang et al., 2005).

In order to compare different analytical conditions and to find the most effective protocol for milk TAA quantification, four different extraction solvents (ES) were tested by diluting the activated ABTS radical with acetone in water (80:20), ethanol, acetonitrile or HCl 1 M, until reaching an absorbance of 1.10 ± 0.05 at 730 nm (Chen et al., 2003).

For each analytical trial, blank samples were obtained by adding 0.1 ml of water to 1 ml of each ES added with ABTS, and measuring their absorbance at 730 nm.

2.3. Sample collection and preparation

One sample of raw milk (RM), whole UHT milk (WUM), partially skimmed UHT milk (SUM), whole pasteurised milk (WM) and partially skimmed pasteurised milk (SM), for a total of 5 milk samples, were purchased in local commercial stores. Four individual RM samples of Simmental cows were collected in the experimental farm "L. Toniolo" of the University of Padova (Legnaro, Italy), and each sample was divided into two subsamples of 40 ml. One aliquot was added with 200 μl of preservative (Bronopol). All samples were kept at 4 °C until the beginning of analyses.

Before the TAA analyses, the milk was thawed at room temperature for 1 h and diluted in water (1:20). In order to assess the best antioxidant extraction procedure, 0.1 ml of diluted milk was added with 1 ml of each ES added with ABTS. Immediately after milk addition, samples were vortexed to promote antioxidant extraction and incubated at room temperature for 10 min. Samples were then centrifuged at 18,000*g* for 5 min, to promote milk protein precipitation.

2.4. Determination of TAA by ABTS method

The ABTS radical deactivation, depending on milk antioxidant content and on the efficiency of the ES, is appreciable through the clarification of the ABTS solution. Thus, after centrifugation, 1 ml of surnatant was transferred into a cuvette and the sample absorbance was read at 730 nm, in the near infrared spectrum. The difference between the absorbance of blank samples prepared in four different ES and the absorbance of milk samples, treated respectively with the same ES, is directly proportional both to the ABTS radical deactivation and the TAA. Finally this difference was expressed in μmol l^{-1} of Trolox equivalents (TE), which is the most common way to express TAA.

2.5. Linearity, limit of blank and limit of detection

Linearity was evaluated for Trolox diluted at six different concentrations, ranging between 7.30 and 43.60 μmol l^{-1}. In order to perform linearity tests on milk samples, RM, WUM, SUM, WM and SM were diluted at 2, 3, 4, 5, 6, 7 and 8% in water, and an aliquot of 0.1 ml of each dilution was added to 1 ml of ABTS in 80% acetone. Thus, the final concentration of milk samples in the cuvette ranged from 0.17% to 0.67%. Total antioxidant activity of each diluted sample was measured as described above.

Limit of blank (LOB) was defined as the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte were tested, and it was calculated as proposed by Armbruster and Pry (2008) and expressed in raw absorbance units:

$$\mathrm{LOB} = \mathrm{mean}_{\mathrm{blank}} + 1.645 * (\mathrm{SD}_{\mathrm{blank}}),$$

where SD is the standard deviation.

Limit of detection (LOD) was defined as the lowest analyte concentration reliably distinguished from the LOB, and it was calculated following Armbruster and Pry (2008) and Bonfatti, Grigoletto, Cecchinato, Gallo, and Carnier (2008), and expressed in μmol l^{-1} TE:

$$\mathrm{LOD} = 10 * (3 * \mathrm{SD}_{\mathrm{blank}})$$

2.6. Statistical analysis

The normal distribution of milk TAA expressed as absorbance or as TE was checked using Shapiro-Wilk's test. Repeatability of TAA was calculated as the relative standard deviation (RSD_r) of twenty consecutive measurements of RM, WUM, SUM, WM and SM samples within the same day. Similarly, reproducibility of TAA was calculated as the relative standard deviation (RSD_R) of sixty measurements obtained across three days of analyses, as proposed by Biswas, Sahoo, and Chatli (2011) and Sturaro, De Marchi, Masi, and Cassandro (2016).