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Original Research Article

Fluorometric method based on molecular recognition solid-phase extraction for determination of riboflavin in milk and infant formula

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ABSTRACT

Riboflavin (vitamin B_2) is involved in several biological processes, particularly in energy production, and it is acquired from food ingestion, principally from supplemented food during the first years of life. Therefore, a simple, fast and cost-effective high-throughput method for determination of riboflavin in milk and infant formula is proposed, based on selective extraction using commercially available molecularly imprinted polymers targeted to riboflavin, followed by direct fluorometric determination. Several aspects were studied, namely microplate assay conditions, the composition of eluting solution and the stability of riboflavin in the eluate. Hence, elution using 1% (v/v) acetic acid in methanol or in acetonitrile is recommended, followed by immediate analysis or solvent evaporation, with reconstitution and analysis within 24 h. The proposed method provided a LOD of 0.03 mg L^{-1}, with working range for undiluted samples between 0.125 and 2 mg L^{-1}, and sample throughput of 24 h^{-1}. It was successfully applied to certified reference material NIST-1846 and also to commercial milk and infant formula samples.

1. Introduction

Riboflavin, or vitamin B_2, is a water-soluble vitamin composed of an isoalloxazine ring bound to a ribityl side chain (Powers, 2003). This vitamin is the precursor of flavin mononucleotide (FMN) and flavin dinucleotide (FAD) (Henriques et al., 2010), which act as electron carriers in several biological processes and are also cofactors of flavoenzymes such as flavin oxidases, flavin reductases (*e.g.*, glutathione reductase) and flavin dehydrogenases (Depeint et al., 2006).

However, riboflavin cannot be synthesized by the human body, reflecting the importance of its uptake through the diet (Vergani et al., 1999), in the form of FAD, FMN or riboflavin bound to proteins (Henriques et al., 2010; Powers, 2003). Indeed, riboflavin can be found in its free form in milk and eggs or in cereals, vegetables and meat, mostly as FAD (Powers, 2003). Its absorption occurs in its free form, which is afterward converted to FMN and FAD in ATP-dependent processes (Depeint et al., 2006; Henriques et al., 2010).

Riboflavin deficiency has implications on cellular energy production through mitochondrial fatty acid β-oxidation (Henriques et al., 2010) and mitochondrial respiratory chain (Marriage et al., 2003), on protein synthesis and stabilization (Henriques et al., 2010), haem synthesis, iron mobilization (Fishman et al., 2000; Powers, 2003), protection against oxidative stress (Henriques et al., 2010; Powers, 2003), homocysteine–methionine pathway (Hustad et al., 2000; Powers, 2003), thyroxine metabolism (Powers, 2003) and in other B vitamins metabolism. This vitamin is also important during foetal and infant growth (Ortega et al., 1999) to ensure normal energy production and gastrointestinal tract maturation (Powers, 2003) as well as to guarantee newborn protection against oxidative stress at the time of delivery (Henriques et al., 2010; Powers, 2003). At human body, only liver, cardiac muscle, spleen and kidney are protected organs against riboflavin deficiency (Henriques et al., 2010). For the above stated, adequate riboflavin levels should be assured.

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Therefore, riboflavin quantification in food products is essential and of particular interest in riboflavin-fortified foods, especially for child nutrition, such as milk infant formulas, because riboflavin intake at this age mostly relies on these products (Zand et al., 2012). Furthermore, the decrease of riboflavin content in some foodstuffs and beverages is a quality marker as the degradation of this vitamin is mediated by light exposure (Powers, 2003; Zand et al., 2012). Riboflavin assessment allows to infer about the quality of milk processing and storage (Andrés-Lacueva et al., 1998), as it does for other commodities, because off-flavour formation can occur in such conditions (Manesiotis et al., 2009).

Several methods for quantification of riboflavin in food and beverages have been described, including high-performance liquid chromatography (HPLC) (Gliszczynska-Swiglo and Koziolowa, 2000; Zafra-Gomez et al., 2006) coupled to ultraviolet spectrophotometric (Zand et al., 2012) or to fluorometric (Ndaw et al., 2000) detection, capillary electrophoresis (Cataldi et al., 2002a, 2002b, 2003) coupled to laser-induced fluorescence, and electrochemical methods (Kadara et al., 2006). Generally, quantification of riboflavin is performed by reverse-phase HPLC with fluorescence detection (Koop et al., 2014; Scotter, 2011) preceded by acid hydrolysis and enzymatic treatment in order to convert all riboflavin content into free riboflavin, and by an extraction technique to remove interferences (Ndaw et al., 2000; Segundo et al., 2012; Zand et al., 2012).

Solid-phase extraction (SPE) is widely used for sample pre-treatment (He et al., 2007; Hennion, 1999) and is a suitable technique for riboflavin extraction and pre-concentration from food samples as well as for the elimination of matrix interferences prior to HPLC analysis (Segundo et al., 2012). Furthermore, flow injection techniques have been applied in the automation of SPE procedures, contributing to more accurate, precise, and less laborious processes (He et al., 2007; Oliveira et al., 2010). However, SPE with classic sorbents, namely C18 or conventional polymeric structures, usually leads to co-extraction of interferences due to the non-selective interactions established between the sorbent and the target analyte (Pichon, 2007). In order to overcome this drawback, molecularly imprinted polymers (MIP) have been developed as a revolutionary type of SPE sorbent, allowing higher selectivity due to specific molecular recognition of the analyte (Haupt and Mosbach, 2000).

In fact, a MIP for selective recognition of riboflavin was developed employing 2,6-bis(acrylamide)pyridine as functional monomer due to its hydrogen-bond interactions with the riboflavin imide group (Manesiotis et al., 2005, 2009). Riboflavin tetra-acetate, an analogue of the target molecule, was chosen as template (Manesiotis et al., 2005), ensuring that the target analyte is strongly retained and that the obtained results are not affected by template bleeding (Pichon, 2007). Pentaerythritol triacrylate was used as cross-linking agent (Manesiotis et al., 2005; Oliveira et al., 2010), permitting specific extraction in aqueous media. A porogen was also employed in the polymerization process to suppress non-specific binding to sorbent in an aqueous medium (Manesiotis et al., 2009).

Considering all the time-consuming steps involved in current methods, a new high-throughput protocol for riboflavin assessment in milk by molecularly imprinted solid phase-extraction (MISPE) followed by direct fluorometric detection under microplate format is proposed. Microplate assay conditions and MISPE parameters were assessed, targeting application towards real samples.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) if not specified. Water was obtained from arium water purification systems (resistivity > 18 MΩ cm, Sartorius, Goettingen, Germany).

Glacial acetic acid was diluted in order to prepare 1% and 2.4% (v/v) CH_3COOH aqueous solution, 1% (v/v) CH_3COOH in CH_3CN and 1% (v/v) CH_3COOH in CH_3OH. CH_3CN and CH_3OH (both HPLC grade) were purchased from Merck (Darmstadt, Germany).

Riboflavin stock solutions were prepared by dissolving 5.00 mg of riboflavin in 100 mL of water, 3.0 mM HCl or 2.4% (v/v) CH_3COOH aqueous solution. This solution was heated at 37 °C for 10 min to ensure complete dissolution of riboflavin. After cooling, riboflavin standard solutions were prepared by dilution of the stock solution in the respective solvent. All riboflavin solutions were kept in the dark at 4 °C.

The MIP selective towards riboflavin was supplied by Supelco (Bellefonte, PA, USA) in cartridges comprising 25 mg of sorbent with a volume capacity of 10 mL. MISPE was performed with 1% (v/v) CH_3COOH in CH_3CN, 1% (v/v) CH_3COOH in CH_3OH or 70% (v/v) CH_3CN in water as elution solvents.

2.2. MISPE protocol

MISPE was performed in a commercial system supplied by Supelco (Bellefonte, PA, USA). The system was coupled to a vacuum pump (Büchi, Flawil, Switzerland) and allowed the simultaneous extraction of 12 samples. Sorbent conditioning was performed by percolation of 1.0 mL of methanol followed by 1.0 mL of ultra-pure water. Then, 1.00 mL of sample or standard solution was loaded into the sorbent column at 0.5 mL min^{-1}, after which the column was dried under vacuum for 10 min. The elimination of matrix interferences was performed by percolation of 2.0 mL of ultra-pure water, followed by column drying under vacuum for 1 min. The elution of riboflavin was performed with 3 portions of 1.0 mL each of elution solvent at 0.2 mL min^{-1}. The eluate was then analyzed by fluorometry or dried and reconstituted in an appropriate solvent before analysis.

2.3. Microplate protocol for fluorometric determination

Fluorometric determinations were performed in a high-throughput 96-well microplate reader (Synergy HT; Bio-Tek Instruments, Winooski, VT, USA; λexc = 380 nm, λem = 528 nm). A 300-μL aliquot of sample or riboflavin standard prepared with the same eluate composition was placed in a 96-well microplate, as depicted schematically in Fig. S1. Using the proposed layout, 13 samples were analyzed in triplicate, with concomitant analysis of 5 standards ($n = 3$). Hence, sample blank was performed with 300 μL of 1% (v/v) CH_3COOH solution in A1–C1 wells, while standards (concentration range of 0.125–2.00 mg L^{-1}) were placed in A2–C6 wells. The remaining wells were filled with samples extracted in duplicate and analyzed in triplicate each.

2.4. Evaluation of the elution conditions and eluate stability

To assess the appropriate solvent for reconstitution of the dried eluate after evaporation, 3 mL of a riboflavin solution (0.33 mg L^{-1}) prepared in 1% (v/v) CH_3COOH in CH_3CN were placed at 90 °C until solvent evaporation and the residue was dissolved in 1 mL of H_2O or 1% (v/v) CH_3COOH in H_2O, providing similar recoveries.

In order to evaluate the possibility of analyte determination on a different day of MISPE performance, a riboflavin solution (1 mg L^{-1}) prepared in 2.4% (v/v) CH_3COOH in H_2O was submitted to MISPE. Elution solvents tested were 1% (v/v) CH_3COOH in CH_3CN, 1% (v/v) CH_3COOH in CH_3OH and 70% (v/v) CH_3CN in H_2O. Thereafter, the obtained eluate was analyzed immediately and on the day after MISPE. Moreover, the eluate was also submitted to the solvent evaporation step described above and analyzed