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Simultaneous determination of vitamin B_1 and B_2 in complex cereal foods, by reverse phase isocratic HPLC-UV

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ABSTRACT

The evaluation of nutritional or functional components in grain products is an important feature for the industry, especially when recent regulations require a correct nutrition labelling, valid during all the shelf life of the product. For that reason, industry usually makes many efforts to develop simple and reliable analytical methods that can be easily applied in any quality control laboratories for routine analysis. Spectrofluorimetric analysis of thiamine and riboflavin are sensitive, but need specific equipment. A few HPLC-UV methods have been described but they are less sensitive, and present difficulties due to interfering compounds, particularly in complex food matrixes, as grains and derivatives.

A combination of extraction and separation systems, that allows enough sensitivity, precision and accuracy for the analysis of vitamin B_1 and B_2 in complex cereal food products, by isocratic UV-HPLC, in a single wavelength simultaneous separation is presented, with the advantage of using low-cost equipment requirements, simple sample pre-treatment and short time. The achievement of this goal has involved the optimization of compatible extraction and measurement protocols for cereal matrices, comparing seven different separation conditions and six extraction/clarification matrices analysis. The selected method was comparatively validated and compared to reference AOAC spectrofluorimetric methods, providing comparable linearity and accuracy, with better specificity and precision parameters, as well as practical applicability.

1. Introduction

Cereals have been traditionally used as basic foods; however in recent years, their consumption has declined due to new life styles. As the necessity of their recovery in the diet is recognized, some new cereal products have been developed by the industry, adapted to different uses and providing nutritional and functional benefits to the organism, including the intake of energy from complex carbohydrates, as well as the presence of dietary fibre and micro-nutrients as minerals and vitamins.

Thiamine and riboflavin are two vitamins involved in different processes in the human body, such as glucose metabolism, nervous transmission, replication of genes, development of foetal tissues and biosynthesis of corticoids, among others (Illera Martín et al., 2000). These two compounds are unstable to heat, light and other factors, so the technological processes in cereal grains can partially destroy these compounds. For that reason, many industrial cereal products are enriched in B group vitamins to recover their levels. The evaluation of nutritional or functional components in grain products is an important feature for the industry, in relation to final product quality. The recent regulations regarding food nutrition labelling, introduced by the governments and regulatory bodies around the world, require a guaranteed content of nutrients and other bioactive compounds in food products during all their shelf life. Particularly, "European Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006, on the addition of vitamins and minerals and of certain other substances to foods", establishes that not only the added fraction, but the total sum of endogenous (that could need enzymatic extraction) and exogenous vitamins, should be given on the label of the food products. This is the reason why the majority of methods are targeting the determination of total vitamin content, and has led to a growing need for cheap, cost-effective, rapid and reliable analytical monitoring methods for the determination of nutrients and vitamins and other nutrients in food products, that can be easily applied in any laboratories for routine analysis (Heudi et al., 2005; Zafra-Gómez et al., 2006). Many analytical methods have been proposed for thiamine and riboflavine analysis. The choice of

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one method usually depends on the accuracy and sensitivity required and the interferences encountered in the sample matrix. Both vitamin B_1 and B_2 can be easily determined in pharmaceutical products using HPLC-UV methods; but for foods, more specific techniques of analysis should be applied, due to the presence of a high number of interfering compounds, and different food matrixes (Lynch and Young, 2000). For this purpose, chromatographic techniques with ion-exchange or silica columns are of interest to separate vitamins prior to analysis by reverse-phase HPLC or other methods. Most authors use reverse-phase HPLC, with a C18 column and methanol/water as the eluent, although some authors also have reported the use of ion-exchange columns (Hilker and Clifford, 1982) or amide-based columns (Viñas et al., 2003). The presence of buffers is often useful to adjust pH in the solvent and get more reproducible conditions, and the addition of an ion-pair reagent is also applied to improve resolution of the peaks (Ayi et al., 1985; Finglas and Faulks, 1984; Ndaw et al., 2000; Van Niekerk, 1988).

Spectrofluorimetric determination, either directly (AOAC, 2011) or coupled to HPLC separation (Arella et al., 1996; Kyritsi et al., 2011; Ndaw et al., 2000; Reyes and Subryan, 1989; Watada and Tran, 1985) is one of the most recommended methods for the determination of these vitamins in foodstufs. Flavines as riboflavin, FAD and FMN present native fluorescence, either in a neutral isoxazoline or oxidated form. However, the reduced forms do not show fluorescence and their binding to proteins also reduces the fluorescent signal. The oxidized derivative of thiamine (thiocrome) is fluorescent in alkaline medium, so for the HPLC-fluorimetric method, either a pre- or post-column derivatization is needed (Lynch and Young, 2000; Ohta et al., 1993).

Spectrofluorimetric analysis of vitamin B_1 and B_2 has the advantage of being extremely sensitive, but requires specific equipment (spectrofluorimeter or spectrofluorimetric detector for HPLC) that is not always available for many laboratories (Mondragón-Portocarrero et al., 2011). Some HPLC-UV methods have been described for food products (Albalá-Hurtado et al., 1997; Kamman et al., 1980; Nicholas and Pfender, 1990; Vidal-Valverde and Reche, 1990), but often have the disadvantages of being less sensitive, and with a limited application to various food samples, due to the presence of many interfering compounds absorbing light in the UV range.

To solve these problems, careful hydrolysis and purification procedures have to be applied and also the chromatographic conditions have to be improved. In some cases, this induces very long retention times in the chromatograms (40 min or more), which can be accompanied by poor sensitivity and characteristics of the peaks. HPLC-UV analysis of vitamins B_1 and B_2 requires high amount of sample to make possible the detection of both compounds. In other cases, UV detection is accompanied by a more sensitive and specific technique, as the use of gradients, different wavelengths programs, Diode Array, Coulometric or Mass Spectrometry Detection (Albalá-Hurtado et al., 1997; Aranda and Morlock, 2006; Engel et al., 2010; Heudi et al., 2005; Mandal et al., 2009; Marszałł et al., 2005; Viñas et al., 2003; Zafra-Gómez et al., 2006), or other techniques such as capillary electrophoresis, biosensors or chemiluminescence (Bai et al., 2008; Gao et al., 2008), which often makes necessary the availability of highly complex equipment.

Grains and their derivatives are food matrixes that show special difficulties for this kind of analysis, mainly due to two reasons: first, they are starchy products and often gel when using high amount of sample and hot extraction; and second, they have a lot of interfering compounds, especially when analysing industrial complex cereal mixtures including many different ingredients. For that reason, only a few methods use HPLC-UV, and they often require additional strategies to avoid the problems mentioned above.

Due to the significant challenges that must be faced when a specific equipment is not available for the analysis of vitamins B_1 and B_2 in cereal food products, the contribution of this work has been the establishment of a protocol that combines compatible extraction and separation systems, to get enough sensitivity, precision and accuracy for the analysis of vitamin B_1 and B_2 in a very complex food matrix, with clean, well-resolved analyte peaks, in a single wavelength simultaneous separation, using very simple and easily available equipment.

2. Experimental

2.1. Reagents and samples

Standards of thiamine and riboflavin, as well as enzymes (diastase, *alpha*-amylase, *beta*-amylase, acid phosphatase, papain and pepsin) and other common enzymes were purchased from Sigma (St. Louis, USA). HPLC grade solvents were purchased from Symta (Madrid, Spain). Commercial complex cereal products, consisting of breakfast cereal food (containing rice, oat, whole wheat, wheat flour and wheat bran) with enrichment levels of 2–2.7 mg/100 g of vitamins B_1 and B_2, were purchased from local markets.

2.2. Instrumentation

A Perkin–Elmer LS-3 Fluorescence Spectrophotometer (Massachusetts, USA) was used for this study. The HPLC apparatus consisted of a PU II isocratic pumping system; a Jasco (Tokyo, Japan) AS-1555 autosampler; an ERC-Gecko-2000 (Riemerling, Germany) column heater and a Spectra Series UV100 UV–Vis detector (Micron Analitica, S.A., Madrid, Spain). For data processing and analysis, Biocrom 2000 3.0 version software (Micron Analitica, S.A., Madrid, Spain) was used. The analytical column was a Purospher®-STAR RP-18e (250 × 4 mm), 5 μm pore size, purchased from Merck (Darmstad, Germany).

2.3. Sample preparation

Vitamins B_1 and B_2 are usually bound to macromolecules as proteins and carbohydrates, as well as in phosphorylated forms, less frequent in vegetal origin tissues (Ball, 1994). For this reason, acidic hydrolysis (with HCl or H_2SO_4 together with heating at 60–150 °C for 10–30 min), enzymatic hydrolysis (with at least diastase and protease, and in some cases phosphatase, at about 37 °C overnight), or both, has to be applied to the samples in order to extract these vitamins (Arella et al., 1996; Augustin et al., 1985; Matallana González et al., 1998; Ollilainen et al., 1993).

According to these previous studies, the most suitable extraction procedure for vitamin B_1 and B_2 in this kind of samples were chosen through different acid and enzymatic hydrolysis (with overnight incubation at 37 °C) assays in order to separate both vitamins from other compounds in the seeds (mainly proteins and carbohydrates) (Table 1). Some extracts were concentrated after incubation, using a Savant Speed Vac PD121P concentrator.

2.4. Chromatographic conditions

According to the literature, different solvent systems with methanol, water and acetate buffer have been used for the separation of both vitamins in an endcapped-C18 column, with UV detection (268 nm) (Ndaw et al., 2000; Ottaway, 1993; Vidal-Valverde and Reche, 1990). The use of ion-pair reagents (sodium hexanesulfonate or heptanesulfonate) were tried for the separation of both compounds from other interfering substances in the