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Investigation of protocols to extraction and quantification of folates in vegetables matrices split into liquor and fiber fraction using factorial design

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ABSTRACT

The main protocols of extraction were investigated for the six folate forms in vegetable matrices, treated in two fractions, liquor and fiber. In a pilot study, it was used ammonium acetate added of 2-mercaptoetanol and ascorbic acid as extraction solution. The condition of use of protease and folate conjugase was evaluated, besides alternative treatments without enzyme use. Based on the results of this stage, it was built the factorial design 2^4, with three replications at the central point, using the following variables: temperature, time for reaction, molar concentration of the extraction solution and ratio sample/solution as independent variables and dependent variable, the amount of each folate form extracted as well as spectral and chromatographic parameters. In the pilot study it was verified that the enzyme use can cause an increase in the variability of the folate content, which enabled to build the factorial design without the enzyme use. The binomial time and temperature showed greatest impact on the extraction profile, besides high concentrations of ammonium acetate resulting in bifurcation of some peaks. 5-Methyltetrahydrofolate was extracted primordially in the liquor fraction, indicating that this treatment on the matrix provoked suitable extraction condition to this folate.

1. Introduction

The importance of folate as an essential micronutrient for prevention of neural tube defect was recognized in the 90s, especially the understanding of its role in the synthesis of nucleic acids and DNA building [1]. The progress of chronic non-communicable diseases in society has given new emphasis for the importance of folate as mediators for DNA repair. In addition, control of folate plasma levels has been used in the prevention of coronary disease [2], neurological disorders and depression [3].

Fruits, peas and others vegetables are good sources of folate, in the polyglutamates form. 5-Methyltetrahydrofolate (5-MTHF), 5-formyltetrahydrofolate (5-FTHF), 10-formylfolate (10-FTHF), 10-formyldihydrofolate (10-FDHF), and tetrahydrofolate (THF) are the main monoglutamates occurring in these foods [4–6].

Vegetables and diet mixtures containing vegetables has been widely chosen as a matrix for the development of methodologies, since they are considered good models for study, especially due to high availability of different folate forms [7–10]. The distribution of these forms in vegetables has been reported in studies using high performance liquid chromatography-HPLC as a method of identification and quantification [5].

Analytical methods for determination of folate in food usually has required three steps: release of folate matrix; deconjugation of polyglutamates to mono- and di-glutamate, identification and quantification of microbial activity by turbidimetric response – official protocol [11], or alternatively, HPLC coupled to detection UV–vis [12], fluorescence [6], mass spectroscopy-MS [13] and radioimmunoassay [14].

HPLC is a tough candidate to substitute the official protocol, since it has an advantageous sensitivity and selectivity for screening the most different folate forms, control of contaminants, precision and repeatability in routine analysis. Among detection techniques employed, fluorescence has been the most used [15], since folate exhibit fluorescence, except for folic acid and pteroic acid, which allows identification and quantification in better sensibility, even on small amounts of analyte [6,15].

However, UV–vis and diode array detector (DAD) have more chances of becoming a routine, especially in developing countries, since they are capable of distinguishing the main folate forms and showing a good correlation with other detection systems [2,6,8,9,15–18].

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Extraction is considered a critical step in the analytical process of folate study, and the use of trienzyme treatment (amylase, protease and folate conjugase) has become a source of variation in results, also the work with enzymes result in more time spent consuming making it difficult the inclusion of the method as a routine [6,8,12,15,18–21].

Recent studies focusing on the intrinsic characteristic of each matrix and also on its chemical composition has guided a better treatment [6,22]. To vegetables, the folate conjugase use remains polemic, due to spontaneous conversion of the polyglutamates to di and monoglutamate during extraction [10,23,24]. However, most studies recommend their use for assure an appropriate conversion to monoglutamate in order to obtain more accurate detection and quantification of these components in the food matrix [25].

Protease and amylase can be obtained commercially. However, folate conjugase is usually extracted from different sources such as chicken pancreas, pig kidney, human and rat plasma, which possibly can generate differences in results. Besides, the costs involved in the extraction steps of enzymes has forced many researchers to reproduce extraction protocols [6,7,9,10,12,15,20,26–28,30], being difficult to standardize the methodology.

Chemometric tools have been applied as strategy for development of analytical methods. It consists of applying mathematical or statistical models to explain the phenomena and chemical processes. Currently the most used models are factorial design and central composite design (CCD), since they reduce the number of experiments and therefore result in economy of time and reagents [31].

The principle of this technique consists in variation all factors, and found the best combination, however, unlike traditional models factorial design allows the investigation of factors at same time [31]. That model is widely used to improve methodologies in pharmaceutical formulations [32], acid mixtures [33] and isomers [34].

The purpose of this experiment is to evaluate current treatments employed during folates extraction in vegetables aiming to identify the main factors that generate conflicting results. Factorial design was used as a methodological tool in order to establish a protocol capable of being run in a single day's work, and yet produces sufficient extraction of folates.

2. Material and methods

2.1. Sample

The vegetables used in this work were purchased in local market between the months of September 2011 and January 2012, and conducted to analysis soon after acquisition. The vegetables chosen were those considered by the Brazilian Food Composition Table [35], namely: Broccoli (*Brassica oleracea* var. Itálica), Spinach (*Tetragonia expansa*), Cabbage (*Brassica oleracea* var. capitata), Sauce (*Petroselinum hortense*), Green beans (*Phaseolus vulgaris*) Beet (*Beta vulgaris* L).

2.2. Folates standard, reagents and enzymes

Tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MTHF), 10-formyltetrahydrofolate (10-FTHF), 5-formyltetrahydrofolate (5-FTHF) HPLC grade were provided by Merck (Switzerland). Folic acid and pteroic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were purchased from Merck in HPLC grade (Darmstadt, Germany). All other reagents were of the highest purity available. Ultrapure water was obtained in Milli-Q integral system 5 (Millipore).

Protease (*Streptomyces griseus*) was purchased from Sigma, and folate conjugase was obtained from ratís plasma (project approval by Ethics Committee Animal, 23076.020176/2010-80). Rat's blood (*Ratus noverdicus*) was collected with heparin tubes and centrifugated at 3000 rpm for 20 min [16]. Each 1 mL of plasma was distributed into eppendorfs and lyophilized in order to extend its shelf life. At the time of use, plasma was resuspended in 1 mL of ammonium acetate 8 mmol L^{-1} and centrifuged at 20,000 rpm, according to Gregory et al. [36] in order to use the supernatant as source of folate conjugase.

Prior to its use as a source of folate conjugase, the rat plasma was analyzed for the presence of endogenous folate. The resuspended plasma was injected into the chromatographic column under the same conditions described for the analytical samples, where there was no signal for the presence of folate forms studied.

2.3. Sample preparation—pilot study

In the pilot study, spinach was used as a matrix model and the following conditions were evaluated: use of protease alone; use of folate conjugase alone; dienzime—in which protease and folate conjugase were simultaneously used; trichloroacetic acid – alternative precipitating agent; antioxidants – in this condition the tests were performed without enzymes and trichloroacetic acid; control treatment – in which enzymes, trichloroacetic acid, and antioxidants were not used (Fig. 1).

The whole spinach was washed in Milli-Q water and 400 g was mashed in food processor (Mondial brand, model 1340-01/02 composed of base motor, stainless steel sieve, juice collector, pulp container, plunger and tube feeding) producing two fractions: liquor and fiber.

Separately, 1 mL of the liquor and one gram of fiber were transferred to a test tube and each fraction were added 20 mL of extraction solution (ammonium acetate 50 mmol L^{-1} containing, ascorbic acid 1% and 2-mercaptoethanol 10 mmol L^{-1} for all treatment except control), then stirred in vortex for 20 s and heated at 40 °C for 10 min in water bath. Afterwards, fiber fraction was filtered through qualitative filter paper (whatman paper 50 × 50) and 5 mL of liquor and fiber fraction were transferred individually to a test tube.

Before the enzymatic treatments the pH of the extracts were measured, for tubes with antioxidant the pH was around 6.78 to 6.85 and without antioxidants pH ranged 4.51–4.59. This procedure was performed in order to monitor the extraction solution, since the enzymatic treatments always contain antioxidants.

For the samples submitted to enzyme treatment (protease, folate conjugase and dienzyme) the following protocol was performed: protease was resuspended in extractor solution (2 mg mL^{-1}) and added one ml to test tube [16]. Rat's plasma lyophilized was re-suspended in extractor solution and 250 μL were used to treatments [16]. The tubes were incubated at 37 °C for 1 h in water bath. The enzymes inactivation was carried out at 100 °C for 5 min, and tubes were placed in ice bath for cooling.

In order to reduce the analysis time and variations generated by enzymes trichloroacetic acid [37] was used to make a comparison on proteases use. The concentrations of 2, 5 and 8% triclorocetic acid without the use of folate conjugase were evaluated in the sample.

All treatments (control, antioxidant, tricloracetic acid, folate conjugase, protease and dienzyme) were centrifuged for 15 min at 4 °C, 5000 rpm and supernatants were filtered through cellulose acetate membranes 0.45 μm. One milliliter of the each filtrate (liquor and fiber) was retained for injection into the chromatographic column.

Each protocol was individually tested and all steps were performed in a single analysis, as shown in Fig. 1. From the results of this pilot study it was isolated the main factors that improved folates extraction, in order to building a factorial design model.