Journal of Food Composition and Analysis 57 (2017) 64–72

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original research article

Testing of different extraction procedures for folate HPLC determination in fresh fruits and vegetables

Marta Czarnowska-Kujawska*, Elżbieta Gujska, Joanna Michalak

Department of Commodity and Food Analysis, Faculty of Food Science, University of Warmia and Mazury in Olsztyn, Heweliusza 6, 10-957 Olsztyn, Poland

ARTICLE INFO

Article history:
Received 28 July 2016
Received in revised form 13 December 2016
Accepted 16 December 2016
Available online 19 December 2016

Keywords:
Folate
5-Methyltetrahydrofolate
Folate conjugase
Method evaluation
Di-enzyme treatment
HPLC
Spinach
Food composition
Food analysis

ABSTRACT

There are many critical points in food folate analysis. The differences in analytical procedures used by the authors make comparison of folate data in food composition tables and databases almost impossible. For this reason, the effect of different extraction parameters, including extraction buffers, times and temperatures, on the folate content in fruit and vegetables determined with the HPLC method was investigated. Additionally, the efficiency of two commercially-available folate conjugases (from rat plasma and kidney porcine) was tested. The highest folate content in the tested material was determined when the extraction was carried out in a pH 6.1 phosphate buffer at 100 °C for 15 min using rat plasma conjugase. The method validation included linearity tests with the addition of standard folates for the determination of recovery and repeatability tests on certified reference material. The developed method was successfully applied to the analysis of selected fresh fruit and vegetables and 5-methyltetrahydrofolate was found to be the main folate derivative in the test material with the highest content in spinach and broccoli (180 and 159 μg/100 g, respectively). The results of our study correspond well with the need for the development and optimization of accurate methods for food composition tables and nutritional studies.

1. Introduction

Folate is a group of water-soluble B vitamins. It is a generic term used for naturally occurring food folate and folic acid, which is a fully-oxidized monoglutamate form of vitamin added to foods as a fortifier and used in supplements. Food folate are usually polyglutamates, containing one to eight additional glutamate molecules (Wang et al., 2010) linked through a peptide bond to the γ-carboxyl group of glutamine. Folate vitamers found in food are 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu), 5-formyltetrahydrofolate (5-HCO-H_4PteGlu), 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4PteGlu), 10-formyltetrahydrofolate (10-HCO-H_4PteGlu), 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu), 10-formyldihydrofolate (10-HCO-H_2PteGlu) and tetrahydrofolate (H_4PteGlu) (Blancquaert et al., 2010; Delchier et al., 2016).

Folate are essential cofactors involved in the vital functions of cell metabolism (DNA replication, repair, methylation and synthesis of nucleotides and metabolism of other vitamins such as B_2, B_6, B_{12} and amino acids, e.g. methionine, homocysteine) (Arnesen et al., 1995). Folate deficiency has been linked to a number of health problems, such as megaloblastic anemia, high homocysteine levels, cardiovascular risk, colorectal cancers as well as neurocognitive decline in the elderly. Neural tube defects in the developing foetus are also strongly related to folate deficiency (Cui et al., 2010; Czeizel and Dudas, 1992; Fajardo et al., 2012; Kim, 2007; Lee and Chan, 2011; Morris et al., 2007; Rader, 2002). According to the European Food Safety Authority (EFSA), a Population Reference Intake for folate was established at the level of 330 μg/day, with an increase to 600 μg/day during pregnancy or lactation (EFSA, 2014). However, a folate deficiency in European countries has been observed by De Bree et al. (1997), who estimated that the folate total daily intake among Europeans was 247 μg for women and 291 μg for men.

Vegetables, especially green, leafy varieties and some fruits and berries, are good dietary folate sources with 5-CH_3-H_4PteGlu as the predominant folate derivative (Delchier et al., 2016; Ringling and Rychlik, 2013; Wang et al., 2010). However, it is well-known that food folate data in the literature and databases are inaccurate compared to other nutrients, because they result from different methods for folate extraction and determination, which had a relatively high analytical uncertainty (Bouckaert et al., 2011; Fajardo et al., 2012; Puwastien et al., 2005; Soongsongkiat et al., 2010). The folate analysis in food samples are complicated because

* Corresponding author.
E-mail address: marta.czarnowska@uwm.edu.pl (M. Czarnowska-Kujawska).

http://dx.doi.org/10.1016/j.jfca.2016.12.019
0889-1575/

of their low concentration in the biological material, multiplicity of folate vitamers and their high sensitivity to chemical and physical factors. Reduced folate derivatives found in foods are pH-dependent and considerable losses have been reported due to oxidative degradation enhanced by oxygen, light and heat (Delchier et al., 2016; Gregory, 1996). The loss of folate, at the time of sample preparation, might be affected by the nature of the food matrix in respect to pH and the presence of antioxidants, folate derivatives present in the sample, type and pH of the extraction buffer, extraction time and temperature and the source and amount of γ-glutamyl hydrolase (EC 3.4.19.9, so-called "folate conjugase", "conjugase") for folate deconjugation (Arcot and Shrestha, 2005; Blake, 2007; Delchier et al., 2016; Puwastien et al., 2005; Soongsongkiat et al., 2010; Tamura et al., 1997; Tyagi et al., 2015). Recently, sample preparation stages of folate determination have gained greater attention, after showing their influence on folate content in the final extract and the quantitative assessment. The analytical procedure involves folate extraction from a food matrix using heat treatment, deconjugation of folate polyglutamates to mono- or diglutamyl forms with conjugase and quantitative measurements of total folate or their vitamers (Arcot and Shrestha, 2005; Blake, 2007; Delchier et al., 2016; Puwastien et al., 2005; Soongsongkiat et al., 2010).

Most of the available data on folate content in foods has resulted from microbiological assay using *Lactobacillus casei*, as recommended by Association of Official Analytical Communities International (AOAC 992.05). Although it is tedious and time-consuming, it is still the most preferred method of folate analysis (Fajardo et al., 2015; Maharaj et al., 2015; Soongsongkiat et al., 2010). Its main weakness is that it provides one figure for the sum of all folates present without distinguishing diverse derivatives. Therefore, improved chromatographic methods, which are capable of detecting and quantifying each folate derivative separately, are being increasingly used (Azevedo et al., 2016; Czarnowska and Gujska 2012; Delchier et al., 2016; Hefni et al., 2010; Konings et al., 2001). This is of particular importance since data on various chemical folate derivatives are essential to predict their stability in different food products and bioavailability.

For an average folate daily intake which is lower than recommended, especially for fertile women, there is an obvious and urgent need for a critical evaluation of all kinds of dietary folate sources. Firstly, optimization of the folate determination method for different food matrices using rapid, accurate analytical techniques is needed. This study was designed to improve data on the folate content determination method. Various parameters of the analytical process using high performance liquid chromatography (HPLC) were tested. Different extraction buffers with various extraction times and temperatures were investigated on two fruits and six vegetables. Additionally, the efficiency of two commonly used folate conjugases, which differ in price and availability, was also tested. An important aspect of the study was to obtain, with the optimized method, reliable folate concentrations in eight products, which are considered to be good folate sources in a daily diet. The results of the research contribute well to the basic knowledge on folate distribution in commonly consumed fruits and vegetables.

2. Material and methods

2.1. Materials

Fresh fruits were strawberries (*Fragaria* variety *Senga Sengana*) and blackcurrant (*Ribes nigrum* variety *Ojebyn*), fresh vegetables were: green bean (*Phaseolus vulgaris* variety *Unidor*), yellow bean (*Phaseolus vulgaris* variety *Paulista*), green peas (*Pisum sativum* variety *Durango*), cauliflower (*Brassica oleracea Botrytis* variety *Clapton*), broccoli (*Brassica oleracea* variety *Monaco F1*) and spinach (*Spinacia oleracea* variety *Paulista*). The test material was obtained from the company producing frozen fruit and vegetables on the day of its delivery from the field and after pretreatment including washing and non-edible parts discarding. The obtained material, edible parts of vegetables and fruits of the representative portion of 3 kg, were immediately frozen in plastic bags overnight at −20 °C. Subsamples of vegetables and fruits (ca. 100 g) were cut into cubs 1 cm^3 and freeze dried (main drying at −20 °C = 1.0 mbar/24 h; final drying at −76 °C = 0,0010 mbar/1 h) using CHRIST ALPHA 1-2 LD plus equipment (Osterode, Germany).

Certified reference material CRM 485 (a lyophilized mixed vegetable sample) was obtained from the Institute for Reference Materials and Measurements (Geel, Belgium) with the certified total folate content of 3.15 ± 0.28 mg/kg, determined by microbiological assay, and 5-CH_3-H_4PteGlu content of 2.14 ± 0.42 mg/kg, determined with the use of HPLC method with fluorescence detection. CRM was stored at −70 °C until analysis.

2.2. Dry matter

Dry matter was determined in triplicate according to AOAC method 950.46 (2000), using 2–4 g of ground sample.

2.3. Reagents and standards

All reagents were of analytical grade, except of acetonitrile and methanol, which were of HPLC grade. Water was purified using Mili-Q system (Millipore, Austria). Folate standards, folic acid (PteGlu), 5-CH_3-H_4PteGlu, 5-HCO-H_4PteGlu and H_4PteGlu were obtained from Sigma Aldrich (St. Louis, MO, USA) and were of ≥97%, ≥88%, ≥98%, ≥65% purity, respectively; 10-HCO-H_4PteGlu was purchased from Dr Schircks Laboratories (Jona, Switzerland; >98%). These standards were all prepared according to the method described by Konings (1999). 10-HCO-H_2PteGlu was obtained from 5,10-CH^+-H_4PteGlu which was purchased from Dr Schircks Laboratories (>97%) as described by Pffeifer et al. (1997). Standard concentrations were calculated using molar absorption coefficient given by Blakley (1969). Standard solutions were stored in aliquots (1 mL) at −70 °C for no longer than 3 months. The calibration solutions were prepared before use by dilution of the stock solution with the elution buffer (0.1 M sodium acetate containing 10% (w/v) sodium chloride and 0.1% (v/v) 2-mercaptoethanol).

2.4. Enzymes

2.4.1. α-amylase

α-amylase (Sigma Aldrich) was dissolved at the concentration of 20 mg/mL in pH 6.1 or 7.0 phosphate buffer depending on the chosen extraction method (Table 1). 1 mL of α-amylase solution was added to each sample.

2.4.2. Rat plasma folate conjugase

Rat plasma was used as folate conjugase source. 50 mL of fresh rat plasma (Europa Bioproducts Ltd, Cambridge, UK) was dialyzed under stirring in 0.05 M phosphate buffer, pH 6.1 with 0.1% (v/v) 2-mercaptoethanol for 12 h at 4 °C to remove endogenous folate (Patring et al., 2005). During the dialysis buffer was changed three times. Negative result of endogenous folate presence was confirmed by HPLC analysis. Rat plasma was stored in aliquots (1 mL) at −70 °C for no longer than 3 months. The enzyme activity was examined using pteroyltri-L-glutamic acid ($PteGlu_3$) according to Vahteristo et al. (1996).