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Evaluation of impact of different antioxidants on stability of dietary folates during food sample preparation and storage of extracts prior to analysis

Johan D.M. Patring *, Madelene S. Johansson, Elena Yazynina, Jelena A. Jastrebova

Department of Food Science, Division of Food Chemistry, Swedish University of Agricultural Sciences, P.O. Box 7051, SE-750 07 Uppsala, Sweden

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Abstract

A troublesome factor during folate analysis is the instability of folates, especially tetrahydrofolate. This investigation was therefore performed to evaluate the relative effectiveness of four antioxidants (2-mercaptoethanol, dithiothreitol, 2,3-dimercapto-1-propanol, and 2-thiobarbituric acid) as stabilizing agents. The antioxidants were tested in the combination with 2% sodium ascorbate in acetate, phosphate, and HEPES/CHES buffers commonly used to extract folates from food samples. Baker's yeast was chosen as a model food matrix. A validated method based on reversed-phase high performance liquid chromatography with fluorescence and diode array detection was used for folate analysis. We showed that sample handling was of critical importance in folate analysis. Heat treatment, long-term storage, and repeated freeze/thaw cycles could impair the stability of tetrahydrofolate in varying degrees depending on buffers and antioxidants used. 2,3-Dimercapto-1-propanol was better than 2-mercaptoethanol in protecting tetrahydrofolate under heat extraction, long-term storage of food extracts and freezing/thawing. The use of 2,3-dimercapto-1-propanol as stabilizing agent in folate analysis may, therefore, be preferable owing to its lower toxicity and higher protective effectiveness. Preparation of food samples should include as few freeze/thaw steps as possible before analysis to prevent degradation of tetrahydrofolate.

Keywords: Folate analysis; Stability; Sample pre-treatment; Storage; Freezing/thawing; Antioxidant

1. Introduction

Folates, a group of water soluble B-vitamins, have received much attention due to their health promoting effects, especially because they reduce the risk of neural tube defects of new-born children [1–3] and prevent cardiovascular disease and certain forms of cancers [4,5]. Folates are required for central cellular functions such as amino acid biosynthesis, replication, and growth [4,6]. Determination of folates is therefore important in order to accurately establish actual folate intake in a population, where many people do not reach the recommended daily intake (400 μg folates for adults, 600 μg for pregnant women, and 500 μg for lactating women) [7,8].

Several analytical techniques have been used over the years to study the folate content in foods [9–12]. However, the analysis of food samples is complicated due to the diversity and instability of dietary folates and complexity of food matrixs [13]. Folates are sensitive to degradation induced by heat, UV light, and oxygen [10,14]. The stabilization of folates during the sample preparation, storage, and analysis is therefore important. Many studies have investigated the relative stability of different folate forms in buffered solutions as a function of pH, oxygen concentration, and temperature [15–24], but the stability of folates in food extracts prior to analysis is less well understood [13].

Today, to minimize degradation of folates induced by oxygen and UV light, the use of subdued light and flushing with nitrogen is recommended [9,16]. However, these precautions are not sufficient to prevent all losses of folates due to breakdown. The use of antioxidants, such as ascorbic acid and 2-mercaptoethanol (MCE), is thus essential for stabilization of folates and greater accuracy in folate analysis [15,16,25,26]. As shown by Wilson and Horne [26] the combination of 2-mercaptoethanol (MCE) and sodium ascorbate provided better stabilization of folates than sodium ascorbate alone. The authors reported that during heat extraction formaldehyde was formed from ascorbate anion and caused interconversion of the various reduced folate forms

* Corresponding author. Tel.: +46 18 672070; fax: +46 18 672995.
E-mail address: johan.patring@lmv.slu.se (J.D.M. Patring).

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[25]. This adverse effect could be totally eliminated by use of both sodium ascorbate (2%) and MCE (0.2 M) in HEPES/CHES buffer, pH 7.85 [26], commonly referred to as the Wilson and Horne extraction buffer. Similar results about the benefits of combining two antioxidants (ascorbic acid and MCE) have also been reported by Vahteristo et al. [27] and Pfeiffer et al. [28] when using phosphate buffer and HEPES/CHES buffer, respectively.

The combination of two antioxidants, ascorbic acid and MCE, is today, by far, the most commonly used approach to stabilize folates during sample preparation and analysis [13]. However, in the literature, we have not been able to find any comparative study of antioxidants, which has investigated alternatives to MCE with respect to their effectiveness as protecting agents for folates. The use of MCE is in fact undesirable for toxicological and ecological reasons (see Section 2.7). Finding a more effective, less toxic agent is therefore of interest and was one of the main purposes of this study. Furthermore, we have not been able to find any extensive study that in detail has investigated how storage and freezing/thawing affect folate stability in food extracts. There are today no official guidelines on how to store and treat an extracted food sample containing folates, which can lead to errors in inter-comparative studies. We wanted therefore to study how sample pre-treatment influenced the folate stability in extracted samples and to suggest a suitable way of acting to prevent significant folate losses due to degradation.

To do this, we first did a comparative study to evaluate the protecting effectiveness of different antioxidants to be used in combination with sodium ascorbate. Antioxidants used were MCE, dithiothreitol (DTT), 2,3-dimercapto-1-propanol (BAL), and 2-thiobarbituric acid (TBA). From this study, two antioxidants were chosen for an extended stability study of folates under different treatment conditions in extracts of baker's yeast. A validated method based on reversed-phase high performance liquid chromatography (HPLC) with fluorescence and diode array (DAD) detection was used for folate analysis.

2. Experimental

2.1. Reagents

Acetonitrile was of isocratic grade for HPLC; the other chemicals were of analytical quality. If not otherwise stated, the chemicals were purchased from Merck (Darmstadt, Germany). DL-Dithiothreitol, 2,3-dimercapto-1-propanol, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) were obtained from Sigma–Aldrich (St. Louis, USA). Water was purified using a Milli-Q system (Millipore, USA). Rat serum was obtained from Scanbur (Sollentuna, Sweden). Dry baker's yeast, *Saccharomyces cerevisae* (trademark: original kronjäst) was a gift from Jästbolaget, a Swedish yeast company in Rotebro, Sweden. It was vacuum packed in plastic bags immediately after delivery and stored frozen at −80 °C until analysis.

(6S)-5,6,7,8-tetrahydrofolate, sodium salt (H_4folate) and (6S)-5-methyl-5,6,7,8-tetrahydrofolate, sodium salt (5-CH_3-H_4folate) were a kind gift from Merck Eprova AG, Schaffhausen, Switzerland. The folate standards were stored at −80 °C until use. The purity of all standards was checked according to the procedure of van den Berg et al. [29] using molar extinction coefficients reported by Eitenmiller and Landen [9]. The standard stock solutions of folates of 200 μg/mL (purity corrected) were prepared under subdued light in 0.1 M phosphate buffer pH 6.1 containing 1% sodium ascorbate (w/v) and 0.1% MCE (v/v). Aliquots of the standard stock solutions were placed in separate tubes, flushed with nitrogen and stored below −80 °C at most 3 months. The calibration solutions were prepared immediately before use by dilution of the stock solution with extraction buffer (0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% MCE (v/v)).

2.2. Stabilization of H_4folate in different buffer solutions

Four antioxidants (DTT, BAL, TBA, and MCE) were tested in different buffer solutions, all containing 2% sodium ascorbate, to evaluate their effectiveness to stabilize H_4folate (the most labile folate form) during boiling at different pH values. The buffers involved were 0.1 M sodium acetate buffer pH 5.0; 0.1 M phosphate buffer pH 6.1 and 0.1 M HEPES/CHES buffer pH 7.8. Each antioxidant was used in the concentration of 0.1% (v/v or w/v) for each buffer, which were all spiked with 100 ng/mL of H_4folate. The prepared volume of each buffer solution being tested was 25 mL. From this solution 10 mL (duplicates) were transferred to glass tubes, which were flushed with nitrogen for 15 s, capped, and placed in a boiling water bath for 1 h. Thereafter, they were cooled on ice and then transferred to vials and placed directly in the thermostated autosampler for analysis on HPLC. The obtained concentrations of H_4folate were compared to the concentrations of H_4folate in the remaining unboiled samples (5 mL) in the respective buffer solutions for each antioxidant. These unboiled samples were placed in the thermostated autosampler directly after the preparations, and the analysis began immediately.

2.3. Stabilization of H_4folate and 5-CH_3-H_4folate in baker's yeast extracts

The effects of freeze/thawing and storage of samples in freezer (−22 °C) were evaluated for the stability of folates in extracts of baker's yeast. Comparison was also made of the relative effectiveness between MCE and BAL. For this purpose, two extraction buffers were compared: 0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% MCE (v/v) (MCE-buffer) and 0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% BAL (v/v) (BAL-buffer).

Extraction of folates from baker's yeast was performed by dissolving 50 mg of yeast in 20 mL extraction buffer (MCE- or BAL-buffer) and boiling for 12 min as described in a validated method for baker's yeast [30]. Deconjugation of folate polyglutamates to monoglutamates was done by adding 50 μL of rat serum containing folate conjugase to 1 mL of the yeast extract in a glass tube and incubation in a shaking water bath at 37 °C for 3 h. The obtained yeast extracts containing folate monoglutamates were analyzed by HPLC.