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High performance liquid chromatography coupled to mass spectrometry for profiling and quantitative analysis of folate monoglutamates in tomato

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ABSTRACT

Folates are essential micronutrients for animals as they play a major role in one carbon metabolism. Animals are unable to synthesize folates and obtain them from plant derived food. In the present study, a high performance liquid chromatography coupled to mass spectrometric (HPLC–MS/MS) method was developed for the high throughput screening and quantitative analysis of folate monoglutamates in tomato fruits. For folate extraction, several parameters were optimized including extraction conditions, pH range, amount of tri-enzyme and boiling time. After processing the extract was purified using ultra-filtration with 10 kDa membrane filter. The ultra-filtered extract was chromatographed on a RP Luna C18 column using gradient elution program. The method was validated by determining linearity, sensitivity and recovery. This method was successfully applied to folate estimation in spinach, capsicum, and garden pea and demonstrated that this method offers a versatile approach for accurate and fast determination of different folate monoglutamates in vegetables.

1. Introduction

Folate (folic acid) is one of the B groups of vitamins that are essential for the human health. The dietary insufficiency of folate can lead to several health related disorders such as megaloblastic anemia (Gough, Read, McCarthy, & Waters, 1963), exacerbation of cardiovascular disease (Kolb & Petrie, 2013) and some types of cancer (Blount et al., 1997). It is also required for the development of a healthy fetus and its deficiency affects formation of fetus' spinal cord and brain (Blom, Shaw, den Heijer, & Finnell, 2006). Folate cannot be synthesized by animals and therefore it is solely obtained from diet.

Plants are the major source of dietary folates. Green leafy vegetables, legumes and some fruits are among the richest sources of folate. In several countries the cereal based food products are mandatorily fortified with folic acid to prevent folate-related disorders. In addition, transgenic approaches have been used to increase the biosynthesis of folates in tomato fruits, potato tubers and rice grains to remove dietary constraints of low folate levels in diet (Blancquaert, De Steur, Gellynck, & Van Der Straeten, 2014).

Folate is comprised of a pterin moiety attached by a methylene bridge to para-amino benzoic acid, which is coupled to one or more glutamyl residues. *In vivo* folates exist as tetrahydrofolate (THF) and its derivatives (5-methyl, methylene, methenyl, or 10-formyl) which vary in oxidation states, single carbon substituents, and with a variable number of glutamyl residues (Rébeillé et al., 2006), which are collectively called – folate or vitamin B9. THF plays a key role in one-carbon transfer reactions in all living organisms participating in diverse metabolic reactions such as amino acid metabolism, pantothenate synthesis, purines and thymidylate synthesis etc.

For quantitative determination of total folates, microbiological assay is the most commonly used method which is also recommended by Association of Official Analytical Communities International (AOAC, 2000). The main weakness of this method is that it cannot distinguish diverse forms of folate present in food samples. Above limitation has been overcome by using chromatography-based methods allowing separation of different vitamers. The folate determination has been carried out using high-performance liquid chromatography (HPLC) coupled with UV (Pfeiffer, Rogers, & Gregory, 1997), electrochemical (Bagley & Selhub, 2000), and fluorescence detection (Ndaw, Bergaentzlé, Aoudé-Werner,

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Lahély, & Hasselmann, 2001). The folate level in tomatoes was estimated using HPLC coupled with fluorescence detector (Zhang et al., 2003). However, HPLC is unable to quantify folate forms occurring in very low amounts.

In recent years, HPLC/UPLC hyphenated with mass spectrometry has allowed both qualitative profiling and quantification of folate derivatives in several foods, because of its high sensitivity, selectivity, specificity, and accuracy (De Brouwer, Zhang, Storozhenko, Van Der Straeten, & Lambert, 2007; Garratt et al., 2005; Patring & Jastrebova, 2007; Rychlik & Freisleben, 2002; Zhang, Storozhenko, Van Der Straeten, & Lambert, 2005). The gas chromatography mass spectrometry (GC–MS) has also been used for quantitative analysis of total folates using acidic extraction (Dueker et al., 2000). However, GC–MS requires derivatization of all folate to para-amino benzoyl glutamate prior to analysis hindering distinction of different folate forms, thereby limiting its usage.

The precise analysis of folate derivatives present in plants/food samples is a complicated task stemming from multiplicity of folate derivatives, bound/unbound forms, low levels of folate and differences in plant matrix such as seeds, leaf, fruits or tuber. The determination of folate in food is also strongly influenced by the sample preparation and extraction methods. Given that each plant matrix is distinct, there is no standard method for extraction of folate from plant material owing to difficulties in sample preparation, extraction, deconjugation and purification. This entails that for each plant/tissue the extraction protocol has to be independently optimized to enable precise qualitative and quantitative determination of folate levels (Arcot & Shrestha, 2005). Currently limited methods are available to extract and specifically quantify different folate derivatives present in plants, such as in spinach (Zhang et al., 2005), rice (De Brouwer et al., 2008), and potato (Van Daele et al., 2014).

The aim of this study was to develop an accurate, reproducible, and quantitative method for high throughput screening of large populations of different cultivars, mutants and natural accessions of tomato. We report here optimization of extraction, validation, and application of a high performance liquid chromatographic method with mass spectrometric (HPLC–MS/MS) detection for quantification of folate monoglutamates in tomato. The all ion fragmentation approach of the Orbitrap mass spectrometer allowed confirmation of all targeted folate monoglutamates at the same time without pre-selection. The above method was also used for quantification of folates from plants such as capsicum fruits and spinach leaf.

2. Materials and methods

2.1. Chemicals and reagents

5-Methyltetrahydrofolate (5-CH_3-THF), tetrahydrofolate (THF), 5,10-methenyltetrahydrofolate (5,10-CH^+THF), 5-formyltetrahydrofolate (5-CHO-THF) and 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) were purchased from Schirck's Laboratory (Jona, Switzerland). Folic acid (FA) and methotrexate (MTX) were purchased from Sigma–Aldrich Co. (St. Louis, USA). Supplementary Fig. 1 shows the structure of various folate forms and MTX.

LC–MS grade acetonitrile was purchased from Sigma–Aldrich Co. (St. Louis, USA). Ultrapure water (18.2 mΩ at 25 °C) was obtained from Milli-Q water purification system (Millipore, Bradford, USA). Ascorbic acid and 2-mercaptoethanol (ME) were purchased from Sigma–Aldrich Co. (St. Louis, USA). Formic acid (HCOOH) and acetic acid (CH_3COOH) of LC–MS grade were purchased from Fisher Scientific (Loughborough, UK). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate and activated charcoal were purchased from HiMedia (Mumbai, India).

2.2. Preparation of standard and enzyme solutions

Stock solutions of folate standards (1 mg/mL) were prepared in 50 mM potassium phosphate solution, pH 4.5 containing 1% (w/v) of ascorbic acid and 0.5% (w/v) of 2-mercaptoethanol except FA and MTX, which were dissolved in neutral or basic pH buffer. The standard stock solutions were diluted appropriately in the extraction buffer to prepare working solutions. The remaining stock solutions were flushed with nitrogen gas, and small aliquots were stored at −80 °C.

Protease (from *Streptomyces griseus*, RM6186) and α-amylase (from *Bacillus* sp., A6814) were purchased from HiMedia (Mumbai, India) and Sigma–Aldrich Co. (St. Louis, USA) respectively. Protease (2 mg/mL) and α-amylase (20 mg/mL) were dissolved in ultrapure water, and aliquots were stored at −20 °C. Rat plasma was purchased from National Institute of Nutrition (NIN), Hyderabad, India. To remove endogenous folate from rat plasma, 100 mL of rat plasma and α-amylase were mixed with 5 g of activated charcoal separately, stirred on ice for 1 h followed by centrifugation at 5000*g* for 10 min at room temperature. The supernatant was filtered through a 0.22 μm filter, divided into 1 mL aliquots, and stored at −20 °C. Protease was used without pre-treatment and was stored at −20 °C. For ultra-filtration, 10 kDa membrane filters (Pall Corporation, USA) were used.

2.3. Plant material: growth conditions and tissue harvesting

Tomato plants (*Solanum lycopersicum* L. cv. Arka Vikas) were grown in a greenhouse of University of Hyderabad at 25 ± 2 °C day and ambient temperature in night. For extraction procedure, method development, and validation experiments, different tissues were selected including tomato fruit and leaf tissue, garden pea seeds, capsicum fruit and spinach leaf. Fresh garden pea, spinach and capsicum were purchased from the local market. The juvenile leaves from six-weeks-old plants and fruit tissue at mature green and red ripe stage were harvested from tomato plants. Tomato fruits, pea seeds, and capsicum fruits were homogenized to powder after freezing in liquid nitrogen using a homogenizer (IKA A11, Germany). The tomato and spinach leaves were manually homogenized in liquid nitrogen using pre-chilled mortar and pestle. Homogenized powder was used for extraction of folates.

2.4. Sample extraction: homogenization, enzyme treatments and purification

The basic extraction procedure for folates was adopted from De Brouwer et al. (2008) with modifications. Several parameters were evaluated to obtain the best recovery of folates from tomato fruit and leaf. These modifications included selection of appropriate pH and concentration of extraction buffer, amount of enzyme and different boiling treatment to deactivate enzyme activity. The extraction buffer (pH 4.5) consisting of 50 mM potassium phosphate, 1% (w/v) ascorbic acid, 0.5% (v/v) β-mercaptoethanol and 1 mM calcium chloride was freshly prepared for the sample extraction and flushed with nitrogen for 20 s. Initially, 650 μL of extraction buffer was mixed with 100 mg of plant homogenate in 2 mL Eppendorf tube. To the extract 2.7 ng/mL of individual folates were added at the beginning of extraction process for recovery calculation.

The tubes were capped tightly and after mixing by vortexing were placed in a boiling water bath for 10 min. After cooling on ice, the extract was incubated with 10 μL of α-amylase at room temperature for 10 min to digest complex carbohydrates. Thereafter, 2.5 μL of protease was added and tubes were incubated at 37 °C for 1 h, then transferred to boiling water bath for 5 min. After cooling on ice, the deconjugation of folate polyglutamates