

Extraction, Purification and Detection by Liquid Chromatography–Electrospray-Ionization Mass Spectrometry of Tetrahydrofolate Metabolites in *Arabidopsis thaliana*

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Abstract: The interconversions of C₁-substituted folates form the core of plant one carbon metabolism. An analytical method for extraction, purification and liquid chromatography–electrospray-ionization mass spectrometry detection of low level, labile tetrahydrofolate monoglutamates from *Arabidopsis thaliana* was developed in this study. Under gold fluorescent light, folates were extracted by homogenizing the leaves in extraction buffer and heating the homogenate. A portion of leaf homogenate was incubated with crude preparation of rat plasma conjugase to remove the polyglutamate tails in order to simplify the mixture for analysis. The resulting extract containing the monoglutamyl folates was then applied to an agarose affinity column containing folate binding protein which was purified from milk whey. After elution from the affinity column, the folates were separated and identified by liquid chromatography–electrospray-ionization mass spectrometry on a 150×0.32 mm Symmetry 300 C18 column using isocratic elution with mobile phase (0.1% formic acid/95% acetonitrile, containing 0.1% formic acid, 88:12) at a flow rate of 0.3 µl/min. In positive ion mode, ions representing 5-methyl-tetrahydrofolic acid, 5-formyl-tetrahydrofolic acid and 5,10-methenyl-tetrahydrofolic acid were detected and identified. It is concluded that this folate extraction procedure, combined with folate binding protein affinity column purification is applicable to liquid chromatography–electrospray-ionization mass spectrometry and this method is appropriate for analysis of tetrahydrofolate metabolites in plant leaf tissues. [Nature and Science 2003;1(1):32-36].

Key words: liquid chromatography–electrospray-ionization mass spectrometry; rat plasma conjugase; folate binding protein; affinity column; tetrahydrofolate metabolites; *Arabidopsis thaliana*

1. Introduction

One-carbon (C₁) metabolism is essential to plants. Many C₁ transfers are mediated by the coenzyme tetrahydrofolate (THF). C₁ derivatives of THF are interconverted between different oxidation states, ranging from 10-formyl-tetrahydrofolate (10-HCO-THF) through 5,10-methenyl-tetrahydrofolate (5,10-CH⁺-THF) and 5,10-methylene-tetrahydrofolate (5,10-CH₂-THF) to 5-methyl-tetrahydrofolate (5-CH₃-THF). These interconversions of C₁-substituted folates form the core of plant C₁ metabolism (Hanson, 2000). The parent compound, folic acid, is synthesized *de novo* in bacteria and plants, but *id* a dietary requirement in humans and animals. The molecules of THF metabolites consist of pterine ring, para-aminobenzoate that is conjugated to polyglutamyl tails of variable length (typically 5-8) (Konings, 2001).

Analysis of THF metabolites in plant tissues is challenging because of their low level, complexity and general instability (sensitivity to light, oxidation and extremes of pH). We ever employed HPLC method for quantifying the content of THF metabolites in spinach and *Arabidopsis thaliana* by fluorescence detection (Chang, 2003). Our objective in this study is to develop a reliable method for extraction, purification and detection by liquid chromatography–electrospray-

ionization mass spectrometry (LC/ESI-MS) of THF metabolites in plant leaf tissues, in preparation for an investigation on folate flux through the metabolic pathway in the C₁ metabolism engineered plants (Roje, 1999).

Materials and Methods

Rat plasma conjugase and folate binding protein (FBP) affinity columns were prepared by following the procedure described previously (Chang, 2003).

1. Extraction of Folates from *Arabidopsis* Leaves

The *Arabidopsis thaliana* plants (ecotype Columbia) were grown on soil with 16-h-light/8-h-dark fluorescent illumination at 21°C in growth chamber. Leaves from one month-old plants were collected, covered with foil as 1.0 gram per portion and soaked into liquid Nitrogen. One portion of frozen leaves was homogenized in a mortar containing liquid Nitrogen then transferred into a 15 ml centrifuge tube containing 8 ml extraction buffer (50 mM Hepes-50 mM Ches, containing 2% ascorbic acid and 10 mM 2-mercaptoethanol, pH 7.85). The tube was flushed with N₂ gas then kept in boiling water bath for 10 minutes to inhibit the endogenous enzymes and finally in ice-water bath for 3 minutes. The homogenate was adjusted to pH 7.0 with 4 M HCl before one portion of crude rat plasma conjugase preparation (1.0 ml) was

added. The tube was flushed with N₂ gas and incubated for 4 hours in 37°C water bath. The deconjugation reaction was quenched by keeping the tube in boiling water bath for 5 minutes. After kept in ice-water bath for 3 minutes and centrifuged at 4,800×g, 4°C for 30 minutes, the supernatant was transferred into a clean 15 ml centrifuge tube; The residue was suspended in 2 ml extraction buffer and centrifuged, the supernatant was combined with that previously collected and flushed with N₂ gas.

2. FBP Affinity Chromatography Purification

All FBP affinity purification steps were performed at 4 °C, the flow rate was controlled as about 0.3 ml/min. FBP Affi-Gel 10 column (covered by foil) was Rinsed with 5 ml 0.1 M potassium phosphate buffer (pH 7.0). Folate extract was applied onto the FBP column for 2~3 rounds. After washing with 5 ml 0.025 M potassium phosphate (pH 7.0) containing 1.0 M NaCl and with 5 ml 0.025 M potassium phosphate (pH 7.0), folates were eluted into 5 mL volumetric flask containing 40 µl 25% ascorbic acid solution, 5 µl 2-mercaptoethanol and 18.4 µl 600g/l KOH with elution buffer (0.02 M trifluoroacetic acid-0.2% sodium ascorbate, pH 2.0). The elute was saved in 1.5 ml dark centrifuge tubes, flushed with N₂ gas.

3. LC/ESI-MS Analysis

LC/ESI-MS analysis of THF metabolites in *Arabidopsis thaliana* was accomplished using the Waters CapLC system (Waters Corp., Milford, MA) coupled to an LCQ DECA quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) through the picoview nanospray source (New Objective, Cambridge, MA, USA). The chromatographic conditions were modified as to be compatible with analysis by the electrospray ion trap instrument. A 10 µl aliquot of purified sample solution was injected on a 150×0.32 mm Symmetry 300 C18 column. The folates were then eluted from the column using isocratic program of 88% A and 12% B in 30 min at a flow rate of 3 µl/min. Mobile phase A consisted of 0.1% formic acid while mobile phase B consisted of 0.1% formic acid in a 95:5 acetonitrile/water solution. Diode array detector (DAD) was set at 290 nm. The Picofrit column made electrical contact through a precolumn ZDV titanium union, and terminated in a 15 µm tip i.d. outlet spray needle. The mass spectrometer was operated in positive mode and spray voltage was 2.75 KV.

Results and Discussions

1. Extraction and Purification

Extraction and purification are the key steps for successful analysis of folic acid and THF metabolites from plant tissues. Folates are generally not stable. The chemical reactivity of some folates results in losses of

folates during extraction and purification. Oxidative degradation enhanced by O₂, light and heat is responsible for a major part of those losses. This extraction and purification procedure was performed under gold fluorescent light or subdued light. Solutions containing folates were deaerated by flushing with N₂ gas and stored at -80°C in dark tubes. Pfeiffer et al and Vahteristo et al mentioned the superiority of combination of ascorbic acid and 2-mercaptoethanol as antioxidants during the extraction (Pfeiffer, 1997; Vahteristo, 1997). In our study, ascorbic acid and 2-mercaptoethanol were employed as antioxidants in the Hepes/Ches extraction buffer. Dithioerythritol (DTE) or dithiothreitol (DTT) which is usually used as antioxidants in elution buffer (Konings, 2001; Pfeiffer, 1997), was replaced by sodium ascorbate and 2-mercaptoethanol in our analysis.

Different extraction procedures were employed to extract folates from different type of samples (Engelhardt, 1990). Tri-enzyme extraction method (using conjugase, α-Amylase and protease) is the most commonly used method for extraction of folates from food stuff which containing much protein, starch and carbohydrate matrixes (Konings, 2001; Pfeiffer, 1997; Martin, 1990). Konings et al found that for the vegetable extracts, potatoes and meals, no statistically significant increase in folate amounts was observed after addition of protease and amylase (Konings, 2001). Our extraction method was established for analysis of folates from fresh leafy tissues which do not contain much protein and starch matrixes. We applied conjugase only for enzymatic deconjugation of polyglutamates.

Folate extracts which may contain many interfering compounds with identical chemical and/or chromatographic properties have to be purified and/or concentrated. FBP affinity chromatograph was employed in recent years for cleanup and concentration of folate extracts (Konings, 1999, 2001; Pfeiffer, 1997). In our experiment, FBP affinity columns were prepared by following the procedure of Konings (Konings, 1999). The folate binding capacity was determined as 5.4 µg folate /column by adding more and more folic acid spiked with ³H folic acid to one FBP column until the breakthrough point. When FBP affinity columns were used for actual samples, it is critical that the folate content in the sample be not more than 30% of the capacity of the column. Pfeiffer et al and Konings mentioned sample folate loading should not exceed 25% of the capacity because of the low 5-formyl-tetrahydrofolate (5-HCO-THF) recoveries (Pfeiffer, 1997; Konings, 1999). This is important because there is competition among folate species for the folate binding sites. If folate content is more than 30% of the capacity, the less avidly bound species would not be adequately retained, thus the content of those species and the total folate content would be underestimated.

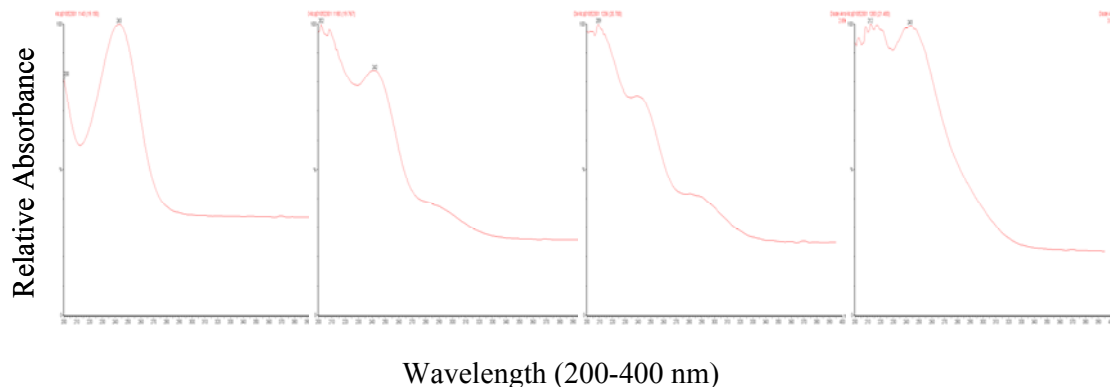


Figure 1. LC/ESI-MS analysis of THF metabolites in *Arabidopsis thaliana* extract. The diode array spectra showing elution of 5-CH₃-THF at 290 nm. The retention time from left to right: 19.15, 19.77, 20.70, 21.48 minute.

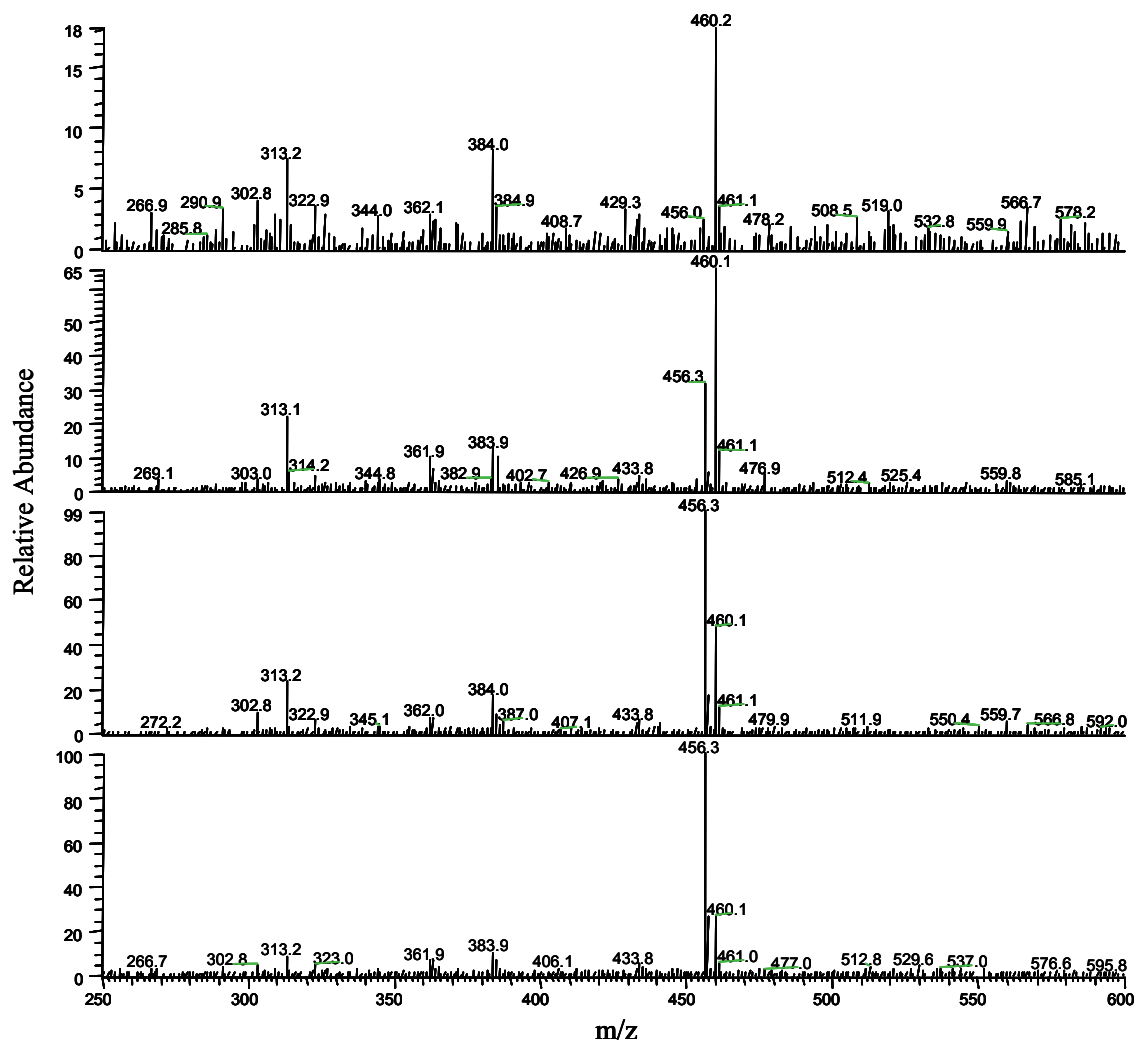


Figure 2. LC/ESI-MS analysis of THF metabolites in *Arabidopsis thaliana* extract. The MS spectra illustrating the sequential detection of 5-CH₃-THF (m/z 460) and 5,10-CH⁺-THF (m/z 456). The retention time from top to bottom: 22.10, 22.17, 22.24, 22.32 minute.

Table 1. Maximum Wavelengths (λ_{\max}), Retention Time (t_R) on Selected ion Chromatograms, Molar Mass (M), ms (MH^+) for THF Metabolites in *Arabidopsis thaliana* Extract

Folates	λ_{\max} (nm)	t_R (min)	M	MH^+ (m/z)
5-CH ₃ -THF	290	22.17	459.4	460.1
5-HCO-THF	285	25.88	473.5	474.4
5,10-CH ⁺ -THF	352	22.32	456.4	456.3

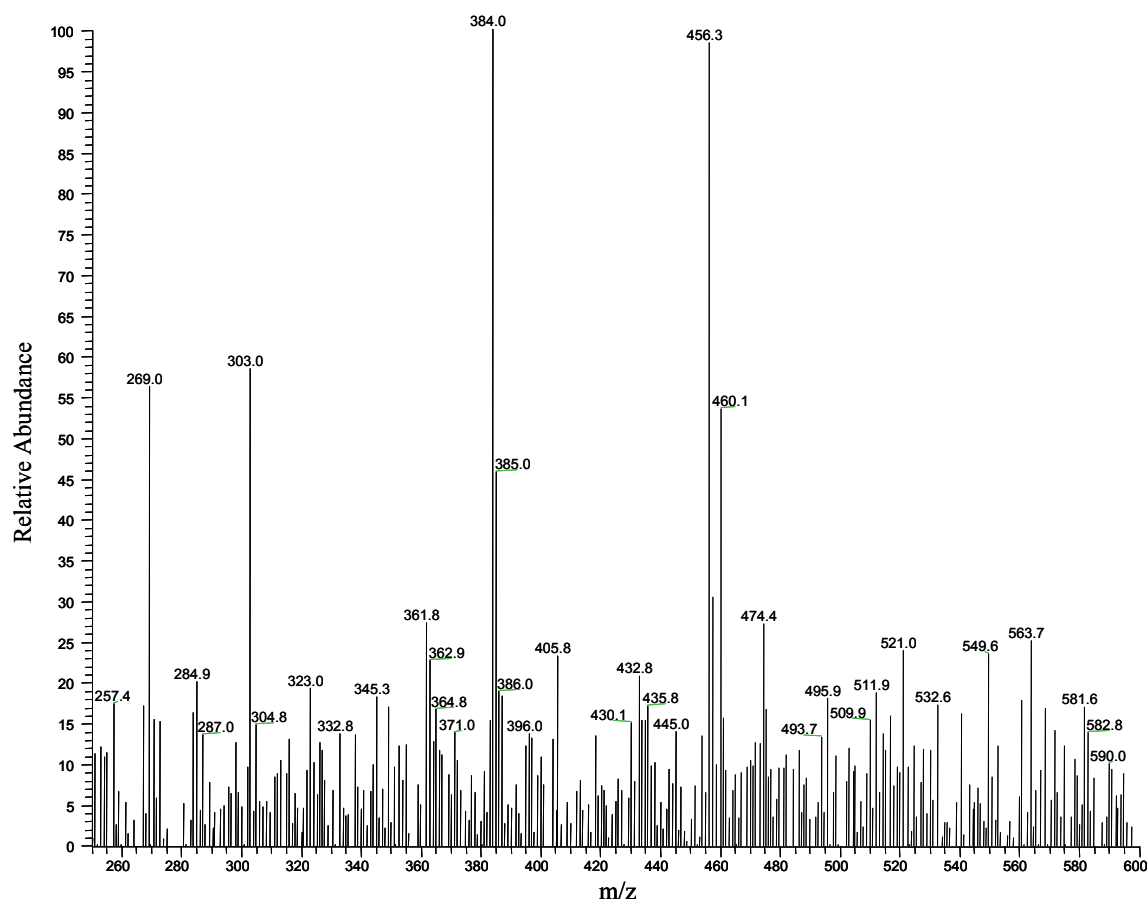


Figure 3. Positive electrospray mass spectrum of THF metabolites from *Arabidopsis thaliana* extract at retention time of 22.58 minute.

2. LC/ESI-MS Analysis

LC/ESI-MS analysis of THF metabolites in *Arabidopsis thaliana* extract was accomplished using 150×0.32 mm Symmetry 300 C18 column, eluted (isocratic program) at a flow rate of 3 μ l /min. Figure 1 showed the diode array spectra of elution of 5-CH₃-THF at 290 nm with retention time 19.15, 19.77, 20.70, 21.48 minute, respectively (from left to right). In positive ion mode, ions representing 5-CH₃-THF, 5,10-CH⁺-THF and a small amount of 5-HCO-THF were detected (Table 1, Figure 2, Figure 3). Figure 2 illustrated the MS spectra of the sequential detection of 5-CH₃-THF (m/z 460) and 5,10-CH⁺-THF (m/z 456) with retention time 22.10, 22.17, 22.24, 22.32 minute, respectively (from top to

bottom). The peak at m/z 313 represented the degraded product of folic acid ($[M-HOOC-CH_2-CH-CO-COOH]^+$). Figure 3 showed the positive ion electrospray mass spectrum (mass range: m/z 250~600) of THF metabolites in *Arabidopsis thaliana* extract at retention time of 22.58 minute. m/z 456, 460, 474 represented protonated molecules of 5,10-CH⁺-THF, 5-CH₃-THF, 5-CHO-THF, respectively. A variable mixture of cationized species including their sodium adduct (m/z 496, 5-CHO-THF +H+ Na), potassium adducts (m/z 512, 5-CHO-THF + H +K; m/z 494, 5,10-CH⁺-THF +K) and sodium-potassium adduct (m/z 521, 5-CH₃-THF+H+Na+K) were also detected.

Conclusion

This paper describes an analytical procedure for the extraction, purification and detection by LC/ESI-MS of THF metabolites in plant leaves. The amount of fresh plant tissues used for folate extraction is as low as 1.0 gram. Liquid chromatography provided separation of folate species using a mobile phase that is compatible with positive ion electrospray mass spectrometric detection. This procedure is suitable for analysis of the naturally occurring THF metabolites in plant tissues. However, it is poorly suited to identification and quantification of 10-HCO-THF and 5,10-CH₂-THF because 10-HCO-THF converts to 5-HCO-THF during heat treatment (Pfeiffer, 1994), while 5,10-CH₂-THF readily dissociates to THF and formaldehyde at physiological pH even with presence of antioxidants (Horne, 2001). Our experiment results indicate that liquid chromatography directly combined with electrospray-ionization mass spectrometry can be used to selectively separate and detect C₁ metabolism relevant folates (5-CH₃-THF, 5-HCO-THF and 5,10-CH⁺-THF) in plant extracts. The combination of folate extraction procedure with FBP affinity chromatography purification and with LC/ESI-MS detection could eventually lead to the development of a quantitative method for selective identification and sensitive quantification of C₁ metabolism relevant folates in plant leaf tissues.

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