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Quantification of Pantothenic Acid and Folates by Stable Isotope Dilution Assays

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ABSTRACT

Stable isotope dilution assays for the quantification of pantothenic acid and folates in foods by using fourfold labeled isotopomers of the vitamins as internal standards (IS) were developed. The use of labeled IS enabled to exactly correct losses during cleanup and derivatization.

Pantothenic acid and its labeled isotopomer were detected as trimethylsilyl derivatives by gas chromatography-mass spectrometry. In starch a detection limit of 44 µg/kg, an intrasample relative standard deviation of 6.7% and recovery values ranging between 97.5 and 99.4 % were determined. Total pantothenic acid contents were analyzed in rice, milk powder and apple juice after enzymatic hydrolysis of the vitamin's conjugates, free pantothenic acid was quantified by omitting enzyme treatment. Almost all results were found to be in good agreement with literature data.

For quantification of folates, 4-fold deuterium labeled folic acid was prepared in a first step and used as starting compound for syntheses of tetrahydrofolate (H_4folate), 5-methyl-H_4folate, and 5-formyl-H_4folate. These compounds were added as IS to food extracts in which pteroylpolyglutamates were subsequently enzymatically deconjugated. After separation by high performance liquid chromatography, folates and their isotopomers were detected by two dimensional mass spectrometry using electrospray ionization. The results revealed good agreement with reported contents in spinach, whereas some differences to the published data for broccoli were found.

INTRODUCTION

Pantothenic Acid

The standard procedure for quantifying pantothenic acid (PA) is a microbiological assay which is tedious and requires sterile working conditions. In a like manner, alternative methods such as enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) or gas chromatography (GC) show major drawbacks. ELISA, up to now, is not commercially available and HPLC is hampered by low UV absorption of PA. Superior sensitivity and accuracy, however, could be achieved by stable isotope dilution assays (SIDAs) using mass spectrometric detection. Particularly for trace analyses, e. g. of flavour compounds (Rychlik and Grosch, 1996) or the mycotoxin patulin (Rychlik and Schieberle, 1999) SIDAs reveal prime benefits, which can be summarized by following statement:

“Addition of an isotopic standard to the sample at the early stages of the analytical method freezes the concentration information as an isotopic ratio that generally is immune to analyte losses during subsequent isolation and derivatization steps.” (Hachey et al., 1985).

Hence, isotopomers are considered the most suitable standards in quantitative analysis. We decided, therefore, to develop a SIDA for the determination of pantothenic acid.

Folates

This group of vitamins is different to PA in that there is a huge variety of vitamers. The most important derivatives in foods are folic acid, tetrahydrofolate (H_4folate), 5-

formyl-H_4folate, and 5-methyl-H_4folate. Besides these monoglutamate forms there are derivatives with typically five to seven glutamyl residues attached.

Analoguously to the analysis of PA, a microbiological assay is the standard method for quantification of folates. Besides being time-consuming, this assay is not able to distinguish the single vitamers. Similarly, the latter constraint holds for assays basing on reaction of folates with folate binding protein (Finglas et al., 1993). Up to now, the only methodology capable of differentiating between the vitamers is HPLC coupled to fluorescence detection (LC/FD) or to mass spectrometry (LC/MS) in order to achieve higher specifity. A first attempt to quantify folates using LC/MS was made by Stokes and Webb (1999), who quantified folic acid and 5-formyltetrahydrofolic acid. As the latter authors did not use an internal standard (IS), correction for losses were not considered. This limitation was circumvented by Pawlosky and coworkers, who used [^{13}C]-labelled folates as IS for quantification of folic acid in fortified foods (2001a) and 5-methyltetrahydrofolate in blood serum (2001b). As we intended to quantify the most important folates occurring naturally in foods, the objective of this study was to synthesize isotopically labelled 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate, and folic acid and use them as IS in SIDAs.

MATERIALS AND METHODS

Synthesis of calcium [^{15}N,$^{13}C_3$]-(R)-pantothenate

Calcium [^{15}N,$^{13}C_3$]-(R)-pantothenate was prepared by adding calcium oxide to [^{15}N,$^{13}C_3$]-β-alanine and reacting the resulting calcium [^{15}N,$^{13}C_3$]-β-alanate with (R)-pantolactone in diethylamine [Rychlik, 2000]

Syntheses of folates

Syntheses are summarized briefly below. Quantities of reagents and spectroscopic data are detailed by Freisleben et al. (2002).

*[$^2H_4$]-4-Aminobenzoic Acid (**2**).* 4-Aminobenzoic acid **1** was reacted with palladium on activated charcoal in deuterium oxide at 200 °C in an autoclav for 2 h to give **2** (yield 58.1 %).

*[$^2H_4$]-p-Aminobenzoylglutamic Acid (**3**).* [$^2H_4$]-4-Aminobenzoic acid *(**2**)* was trifluoroacetylated and coupled to glutamic acid dimethylester in the presence of dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT). Addition of sodium hydroxide gave deprotected **3** (yield 60.8 %).

*N^2-Acetyl-[$^2H_4$]-folic Acid (**4**).* N^2-acetyl-6-formylpterin was prepared according to Taylor et al. (1978). Addition of [$^2H_4$]-4-aminobenzoylglutamic acid *(**3**)* to glacial acetic acid and subsequent reduction by dimethylaminoborane provided the title compound (yield 35.0 %).

*[$^2H_4$]-folic Acid (**5**).* Hydrolysis of **4** in aqueous sodium hydroxide gave [$^2H_4$]-folic acid *(**5**)* (yield 80.0 %).

Mass spectrum in the ESI mode revealed the degree of labelling as follows: [$^2H_4$]-folic acid : 93%; [$^2H_3$]-folic acid: 7%; [$^2H_{0\text{-}2}$]-folic acid: 0%.

Labelled vitamers of Folic Acid

Hydrogenation of [$^2H_4$]-folic acid *(**5**)* upon platin oxide gave [$^2H_4$]-tetrahydrofolate (yield 62.0 %), formylation of which by treatment with formic acid and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) produced [$^2H_4$]-5-formyltetrahydrofolate (yield 18.0 %).

[$^2H_4$]-5-methyltetrahydrofolate (yield 34.0 %) was prepared by adding formaldehyde and sodium borohydride to [$^2H_4$]-tetrahydrofolate.

Stable isotope dilution assays (SIDAs)

SIDA of pantothenic acid

Rice flour (3g) or milk powder (0.5 g) were stirred in acetate buffer (0.02 mol/L, pH 5.6) for one hour at 20 °C and then filtered. After addition of an aqueous solution of calcium [^{15}N,$^{13}C_3$]-(R)-pantothenate (3 µg) the extracts were incubated with solutions of pigeon liver pantetheinase and alkaline phosphatase for 8 h at 37 °C. The incubated solutions were washed with dichloromethane, then acidified by adding hydrochloric acid (1mL, 18 mol /L) and PA was extracted with ethyl acetate (2 x 15 mL). The solvent was then dried over anhydreous sodium sulphate, evaporated to

dryness and the residue reacted with pyridine (100 µL) and BSTFA (100 µL) for 60 min at 80 °C.

After evaporating the derivatisation reagent, the residue was reconstituted in hexane (100 µL) and subjected to GC/MS (Rychlik, 2000).

SIDAs of folates

Broccoli and spinach were purchased at local markets in the city of Munich, Germany. The samples were frozen in liquid nitrogen and aliquots (2 g) were overlaid with 10 mL of extraction buffer according to Wilson and Horne (1984) containing the [$^2H_4$]-labelled internal standards.

Sample suspensions were then purged with argon and placed in a boiling water bath for 10 min. Subsequently the extracts were rapidly cooled in an ice-bath and incubated with bacterial protease (Sigma P-5147, 6 mg) for 6 h at 37 °C. After enzyme digestion, the samples were heated at 100 °C for 10 min, cooled on ice and spiked with 100 µL of rat serum, respectively. The deconjugation was performed at 37 °C overnight.

At the end of the conjugase treatment, the samples were again heated at 100 °C for 10 min, then cooled on ice and centrifuged at 6000 g for 20 min. After passing the extracts through a syringe filter (0.4 µm, Millipore, Bedford, MA, USA) the solutions were subjected to clean-up by solid phase exctraction according to Gounelle et al. (1989), using Bakerbond SAX cartridges (quaternary amine, 500 mg, No. 7091-3, Baker, Gross-Gerau, Germany). The cartridges were activated with 2 volumes of hexane, methanol and water, successively and then conditioned with 7 to 8 volumes of phosphate buffer (pH 7.5, 0.01 mol/L, containing 0.2 % mercaptoethanol).

After applying the sample extracts, the columns were washed with 6 volumes of conditioning buffer, and the folates were eluted with 3 mL of aqueous sodium chloride (5 %, containing 1 % sodium ascorbate and 0.1 mol/L sodium acetate). 100 µL mercaptoethanol was added to each eluate and the purified extracts were subjected to HPLC/MS/MS.

HPLC/MS/MS

The samples (100 µL) were chromatographed on a spectra series HPLC system (Thermo Separation Products, San Jose, CA, USA) equipped with an Aqua C-18 reversed phase column (250 x 4.6 mm; 5 µm, Phenomenex, Aschaffenburg,

Germany) that was coupled to an UV-Detector and an LCQ ion-trap mass spectrometer (Finnigan MAT, Bremen, Germany).

The mobile phase consisted of variable mixtures of aqueous formic acid (0.1 %) and acetonitrile at a flow of 0.8 mL / min. Gradient elution started at 7 % acetonitrile, followed by raising the acetonitrile concentration linearly to 20 % within 9 min and to 80 % within further 4 min. Subsequently, the mobile phase was programmed to 100 % acetonitrile over 4 min before equilibrating the column for 5 min at the initial mixture.

To avoid a contamination of the ion source with buffer salts, the column effluent was diverted to waste during the first 4.5 min of the gradient programme. The spectrometer was operated in the positive electrospray mode using selected-reaction monitoring (SRM). The spray voltage was set to 5.5 kV, the capillary temperature to 200 °C and the capillary voltage to 24.3 V.

RESULTS AND DISCUSSION

Pantothenic Acid

Due to low volatility of pantothenic acid (PA), gas chromatography requires derivatization and so far, lacks a suitable internal standard (IS). Therefore we synthesized an isotopomeric PA by coupling labelled β-alanine to pantolactone (Rychlik, 2000). [^{15}N,$^{13}C_3$]-β-Alanine is commercially available and introduces three carbon-13 isotopes and one nitrogen-15 isotope into PA.

PA occurs in foods in its free form as well as in conjugates, for example as coenzyme A or acyl carrier protein. For quantification of total PA, therefore, it has to be liberated from its bound forms. A flowchart of the SIDA is shown in Figure 1: food samples are extracted at a pH of 5.65 and to the extracts labelled PA is added in a known amount. Subsequent pantetheinase and phosphatase treatment enables cleavage of bound forms and, hence, quantification of total PA. The extract is then acidified and PA extracted into ethyl acetate, trimethylsilylated and finally analysed by GC/MS (Rychlik, 2000). The resulting mass chromatogrammes in case of unpolished rice are displayed in Figure 2. As we reported recently, the trimethylsilyl (TMS) derivatives of the isotopomeric PAs undergo McLafferty rearrangements (Rychlik, 2001) and decompose to give fragments of m/z 291 and 295, respectively. Of these, the internal

standard TMS-[^{15}N, $^{13}C_3$]-pantothenic acid ([^{15}N, $^{13}C_3$]-I) is detected in trace m/z 295, unlabeled TMS pantothenic acid (I) in trace m/z 291. Considering the ratio of areas and the known amount of added standard the content of PA in the sample can be calculated.

A comparison of the new method's characteristics shown in Table 1 with those of the microbiological assay and of the ELISA revealed SIDA to be the most sensitive method, whereas repeatability and recovery were at similar levels.

As detailed in Table 2, quantifications by the new method confirmed the literature data of apple juice and milk powder, whereas lower contents were found in unpolished rice.

Folates

The syntheses of isotopomeric standards started with the production of deuterated folic acid, which was synthesized by deuteration of 4-aminobenzoic acid and subsequent coupling to glutamate and formyl pterine as outlined in Figure 3.

Starting from labelled folic acid, we synthesized the vitamers [$^2H_4$]-folic acid, [$^2H_4$]-H_4folate, [$^2H_4$]-5-formyl-H_4folate, and [$^2H_4$]-5-methyl-H_4folate and used them as IS in the SIDA (Freisleben et al., 2002) schematically shown in Figure 4.

Samples were extracted and treated with protease and rat serum conjugase to liberate the monoglutamates. Thereafter, cleanup was achieved by anion exchange chromatography. Subsequently the folates were detected by LC/MS in the positive electrospray ionization and selected reaction monitoring mode in order to enhance specifity. The new method's detection limit was found to be 0.5, 1.2, 1.5, and 2.6 µg / 100 g fresh weight for 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate, and folic acid, respectively. Evaluating intrasample precision, four repetetive analyses of one sample of frozen spinach revealed a mean value of 45 µg/100 g and a coefficient of variation of 5.3 %.

Figure 5 illustrates the UV and mass traces of a broccoli extract in which only 5-methyl-H_4folate and 5-formyl-H_4folate could be detected. The upper trace represents the signal of the UV detector showing many interferences and no discernable peaks at the retention times of the two folates. In contrast to this, the next lower two traces

at m/z 312.7-313.7 and 316.7-317.7, respectively, show the signals of the isotopomeric 5-methyl-H_4folates and the lowest two traces at m/z 326.5-327.5 and 330.5-331.5 display the peaks of the 5-formyl-H_4folate isotopomers.

The new method was also applied to spinach and revealed the data presented in Table 3. In four different broccoli and six spinach samples, the SIDA showed a high dispersion in the contents of all folate vitamers. As compared to the reported HPLC/FD data of broccoli (Müller, 1993; Vahteristo et al., 1997) the H_4folate and 5-methyl-H_4folate contents as well as the sum of folates were found to be lower. Similarly, our values were below the microbiologically analyzed data (USDA, 2001; Holland et al., 1993; Aiso and Tamura, 1998). Regarding spinach, the literature data basing on HPLC/FD as well as on microbiological assays were in the range of the concentrations analyzed by SIDA in this study.

CONCLUSIONS

The presented SIDAs turned out to be promising tools for quantification of PA and folates. On comparing the results of SIDAs with those of other methods, conflicting values were found for some foods. In order to resolve these contradictions, direct comparisons of SIDA with the microbiological methods and the HPLC/FD analysis of folates using identical samples are under way.

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Table 1. Comparison of performance data of the stable isotope dilution assay (SIDA) to an enzyme-linked immunosorbent assay (ELISA) reported by Song et al. (1990) and an microbiological assay (MA) reported by Bell (1974) for quantifying pantothenic acid in starch containing foods.

Performance criterion	SIDA	ELISA	MA
Detection limit	44 µg/kg	n.d.	n.d.
Quantification limit	131 µg/kg	400 µg/kg	2000 µg/kg
intrasample RSD of			
free pantothenic acid	6,7 % (n=5)	6 % (n=10)	10 % (n=10)
total pantothenic acid	10,5 % (n=5)	n.d.	20 % (n=10)
in polished rice			
Recovery of pantothenic acid,		95 % [a]	n.d.
Addition level: 6 mg/kg	99.4 % (n=3)	n.d.	n.d.
Addition level: 200 µg/kg	97.5 % (n=3)	n.d.	n.d.

n.d. not determined

[a] mean value

Table 2. Free and total content of pantothenic acid (PA) in foods and blood plasma

Sample	free PA	total PA	total PA, range of literature data
		in mg/kg	
Apple juice	0.23	0.23	0.2 - 1.0 [a, b]
skimmed milk powder	30.3	31.2	32.8 - 36.0 [b, c, d, e]
polished rice	3.93	4.45	6.3 – 13.4 [b, e]
unpolished rice	5.56	20.7	11 - 17 [b, e]
human blood plasma	152 [f]	160 [f]	104 - 197 [f, g]
porcine blood plasma	337 [f]	404 [f]	74.8 [f, h]

[a] Haenel (1956)
[b] USDA (2001)
[c] Holland et al (1999)
[d] Møller (1996)
[e] Souci et al. (1994)
[f] in ng/mL
[g] Song et al., (1985), Srinivasan and Belavady (1976)
[h] Banno et al. (1990)

Table 3. Folate contents in broccoli and spinach determined by stable isotope dilution assays (SIDA) compared to those reported in the literature.

Food	Vitamer	SIDA	Literature data		
µg/100g fresh weight			HPLC-FD[a]	HPLC-FD[b]	Microbiological assay
Spinach (N=6)	5-CH_3-H_4folate	72.8-140.0	46	106.5	
	H_4folate	n.d.-18.7	n.d.	4.6	
	5-CHO-H_4folate	4.8 – 54.7	n.d.	40.7	
	Total folate[c]	127.9 ± 24.5[d]	100 [b]	151.8	150 [e] – 338 [f]
Broccoli (N=4)	5-CH_3-H_4folate	24.6-35.7	98	83.7	
	H_4folate	n.d.	18	14.8	
	5-CHO-H_4folate	n.d.-8.1	n.d.	17.9	
	Total folate[c]	33.8 ± 6.1[d]	114	116.4	71 [g]; 90 [e]– 102.2 [f]

[a] Vahteristo et al. (1997)
[b] Müller (1993)
[c] calculated as folic acid
[d] mean value ± standard deviation
[e] Holland et al. (1993)
[f] Aiso and Tamura (1998)
[g] USDA (2001)

LEGENDS TO THE FIGURES

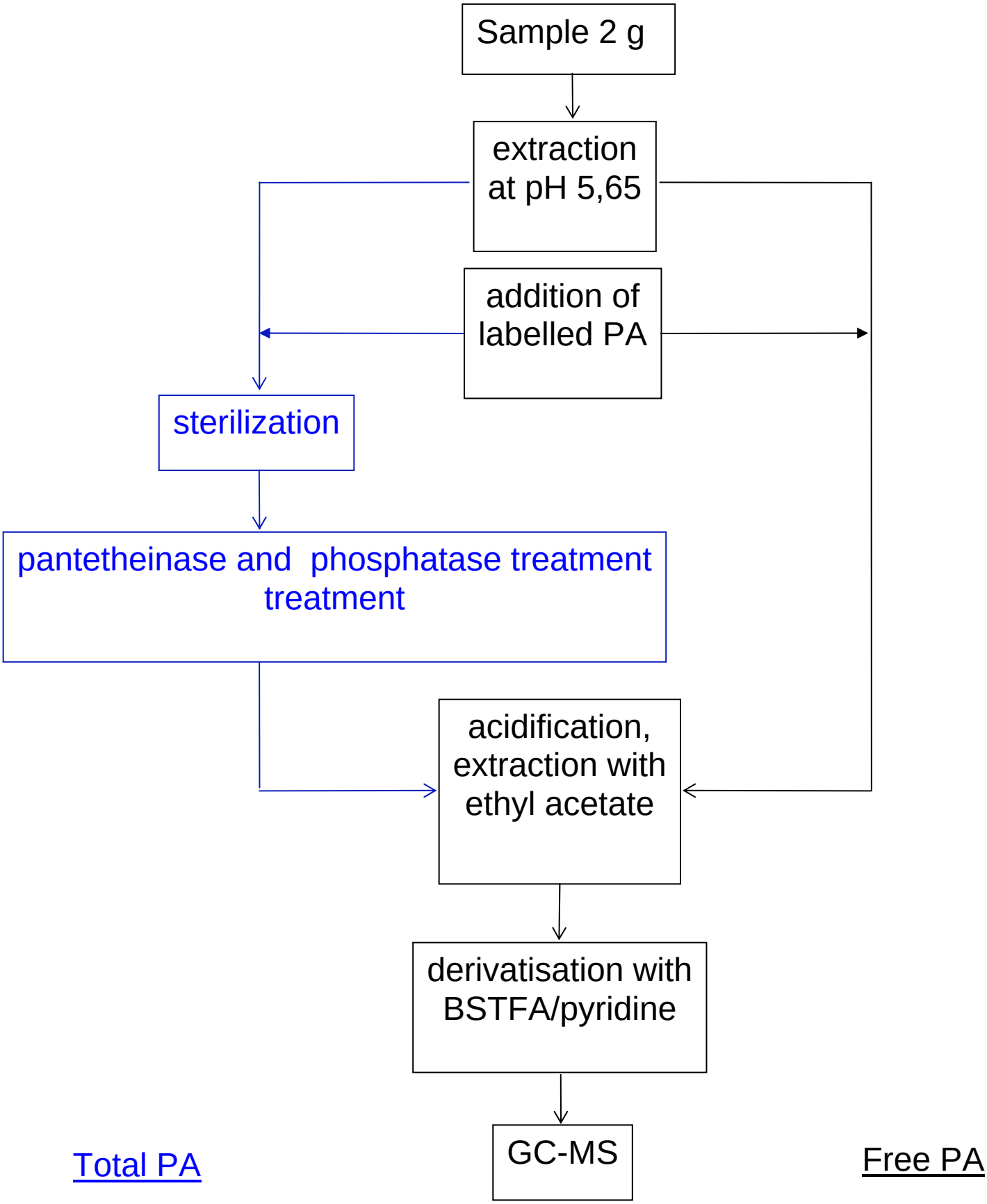
Figure 1. Flowchart of the stable isotope dilution analyses of free and total pantothenic acid (PA).

Figure 2. GC/mass chromatogram of an unpolished rice containing 5.56 mg/kg of free pantothenic acid. The internal standard tris(trimethylsilyl)-[^{15}N, $^{13}C_3$]-pantothenic acid ([^{15}N, $^{13}C_3$]-I) is detected in the trace m/z 295, unlabeled tris(trimethylsilyl)pantothenic acid (I) in trace m/z 291. TIC: total ion current.

Figure 3. Route of synthesis to [$^{2}H_4$]-folic acid

Figure 4. Flowchart of the stable isotope dilution analysis of folates.

Figure 5. LC/MS/MS chromatogram of a broccoli sample after collision-induced dissociation (CID). Upper trace: UV-Signal; trace m/z=312.7-313.7 (product ion after CID of m/z 460.2): unlabelled 5-methyltetrahydrofolate; m/z=316.7-317.7 (product ion after CID of m/z 464.2): labelled 5-methyltetrahydrofolate; m/z=326.5-327.5 (product ion after CID of m/z 474.2): unlabelled 5-formyltetrahydrofolate; m/z=330.5-331.5 (product ion after CID of m/z 478.2): labelled 5-formyltetrahydrofolate.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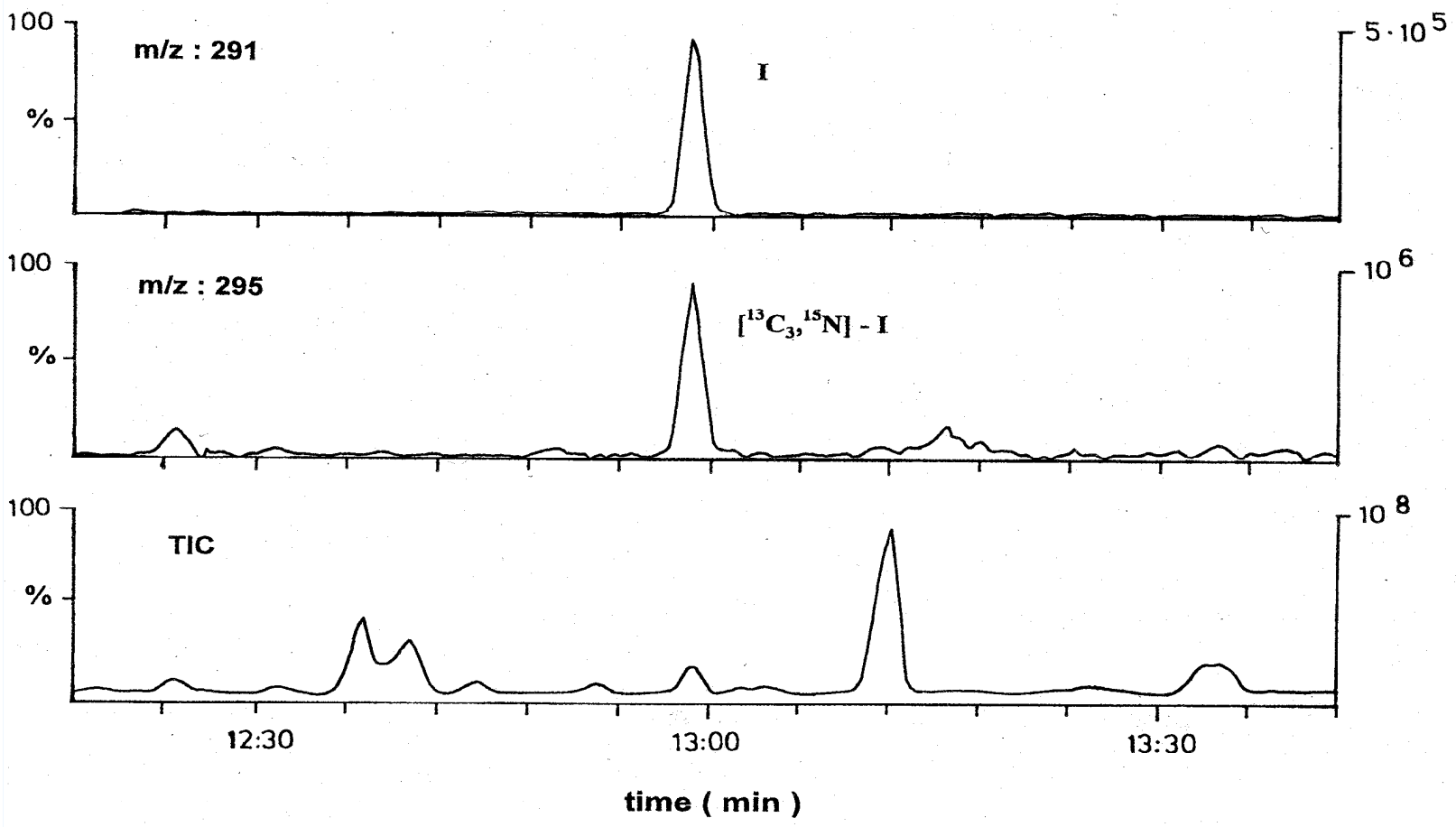
acidification, extraction with ethyl acetate
derivatisation with BSTFA/pyridine
GC-MS
Total PA
Free PA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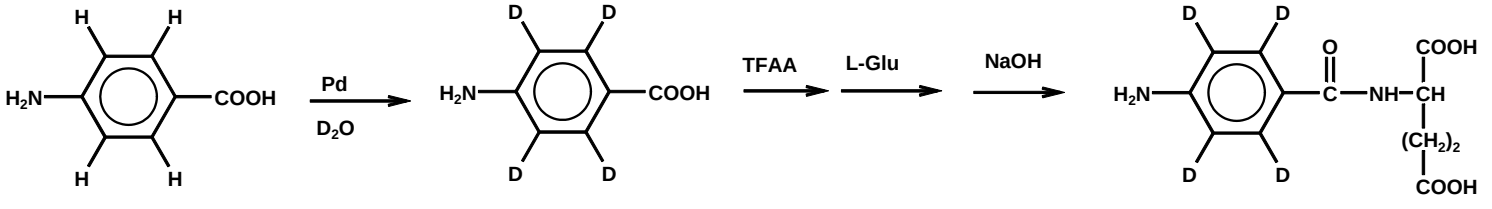

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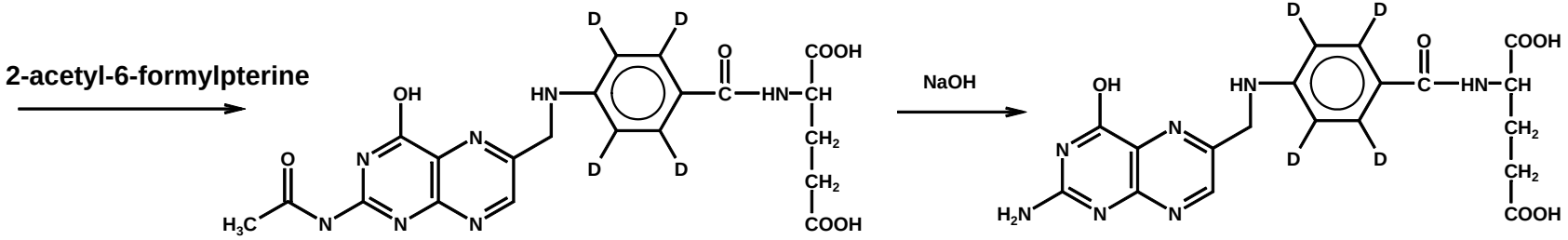
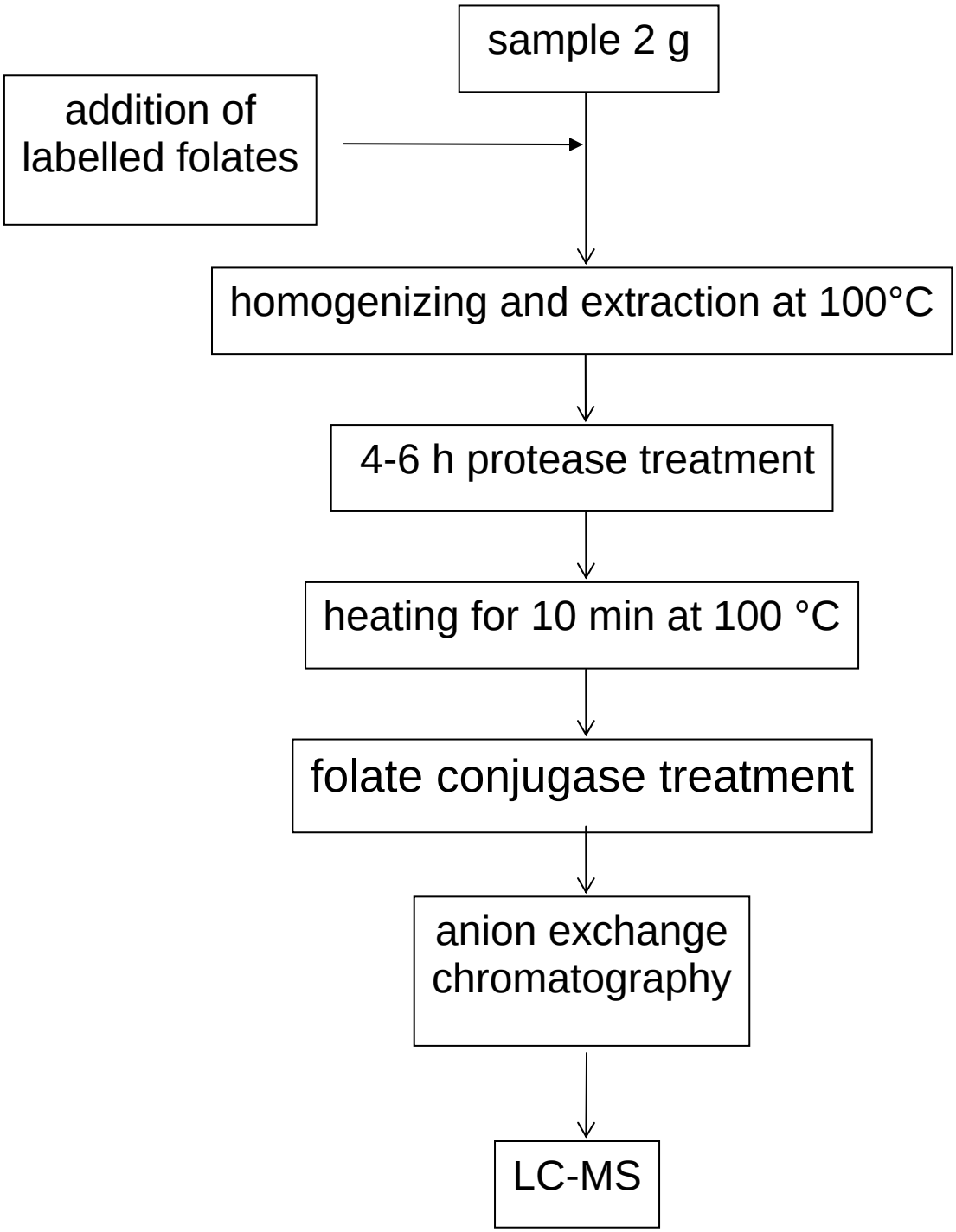


[$^2H_4$]-acetyl folic acid **[$^2H_4$]-folic acid**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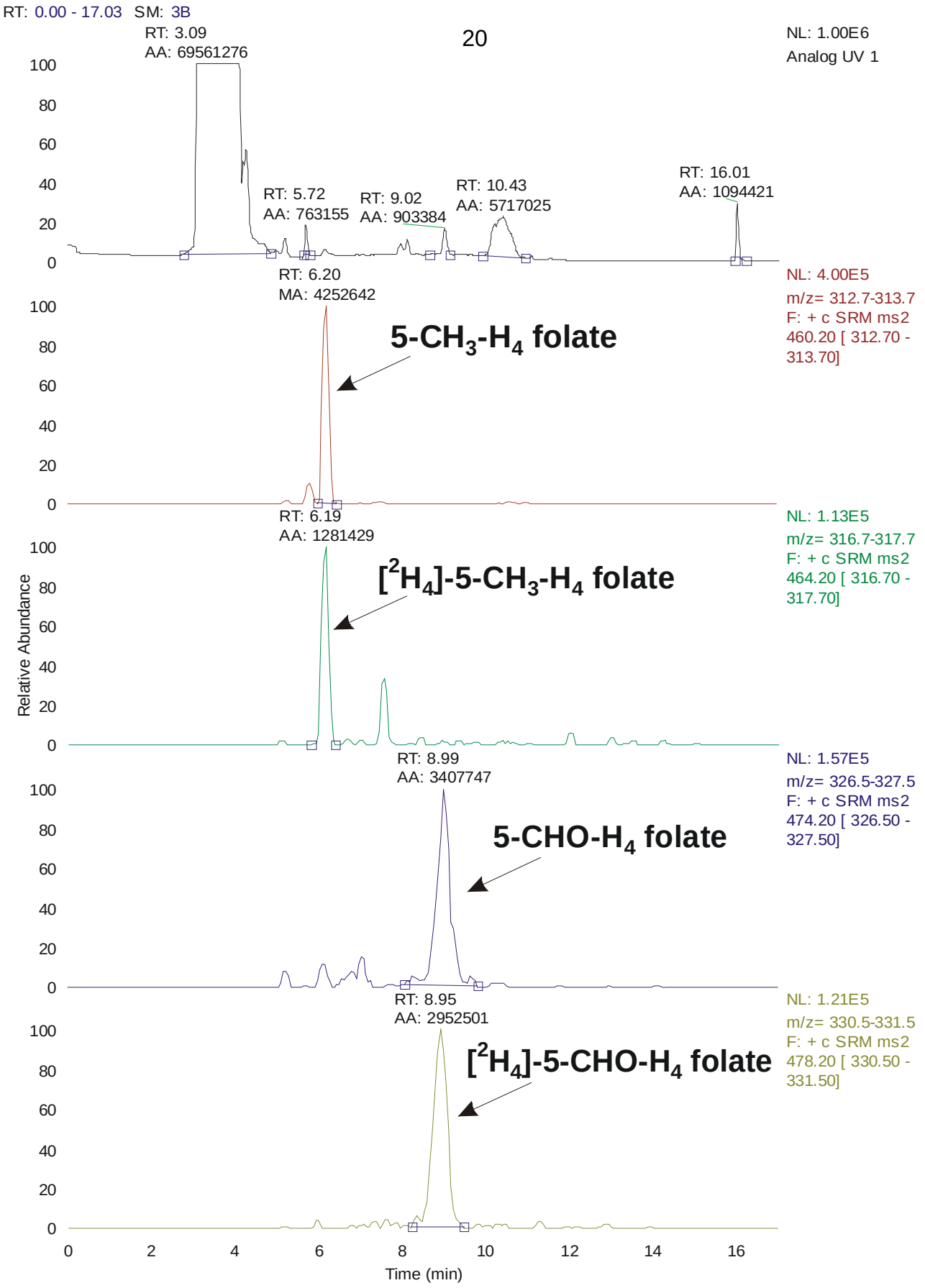
heating for 10 min at 100 °C
folate conjugase treatment
anion exchange chromatography
LC-MS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AA: 69561276
NL: 1.00E6
Analog UV 1
RT: 5.72
AA: 763155
RT: 9.02
AA: 903384
RT: 10.43
AA: 5717025
RT: 16.01
AA: 1094421
RT: 6.20
MA: 4252642
NL: 4.00E5
m/z= 312.7-313.7
F: + c SRM ms2
460.20 [312.70 - 313.70]
5-CH3-H4 folate
RT: 6.19
AA: 1281429
NL: 1.13E5
m/z= 316.7-317.7
F: + c SRM ms2
464.20 [316.70 - 317.70]
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Relative Abundance
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AA: 3407747
NL: 1.57E5
m/z= 326.5-327.5
F: + c SRM ms2
474.20 [326.50 - 327.50]
5-CHO-H4 folate
RT: 8.95
AA: 2952501
NL: 1.21E5
m/z= 330.5-331.5
F: + c SRM ms2
478.20 [330.50 - 331.50]
[2H4]-5-CHO-H4 folate
Time (min)