Journal of Chromatography B, 878 (2010) 509–513

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) for the sensitive determination of folates in rice

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ARTICLE INFO

Article history:
Received 19 August 2009
Accepted 20 December 2009
Available online 4 January 2010

Keywords:
Folates
UPLC–MS/MS
Rice analysis
Biofortification

ABSTRACT

High-performance liquid chromatography, coupled to tandem mass spectrometry (HPLC–MS/MS) has been established as the method of choice for the sensitive and simultaneous determination of different folates in a particular matrix, especially when only minute quantities of material are available. Using a previously developed and validated HPLC–MS/MS method as a starting point, we here report on the development and validation of an ultra-performance liquid chromatography (UPLC–MS/MS) method for analysis of folates in rice, which allows higher throughput and better resolution. UPLC was performed under gradient conditions on an Acquity HSS T3 column, followed by tandem mass spectrometry detection. The method was validated based on linearity, sensitivity, precision, accuracy and matrix effects. The limits of detection and the lower limits of quantification varied between 0.06 and 0.45 μg/100 g and 0.12 and 0.91 μg/100 g, respectively. Two linear calibration curves were established, one for the low and the other for the high concentration range. Analysis of the distribution and levels of folates in wild-type and folate-biofortified rice showed up to 50-fold enrichment in biofortified rice, with total folate levels of up to 900 μg/100 g rice. This is the first successful implementation of a UPLC method for the rapid and sensitive quantitative determination of folates in plant material.

1. Introduction

Folates are a group of water-soluble B vitamins, being essential nutrients for human beings as they play an important role in a wide range of biochemical pathways. They play a major role in one-carbon metabolism, amino acid biosynthesis, replication and growth. It has been suggested that increased folate levels contribute to health benefits such as reduced risk for cardiovascular diseases [1], dementia [2] and certain cancers [3], albeit without a causal relation. Mothers with inadequate folate intake have a higher risk for giving birth to children with neural tube defects [4,5].

Liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) has proven to be a promising technique for the determination of folates in food [6–8] and blood [9–11]. Given the very low concentration of folates in various matrices, such as rice, and given their poor stability, a specific, sensitive and rapid analytical method is required.

Ultra-performance liquid chromatography (UPLC), a novel advance in rapid, sensitive, and high-resolution liquid chromatography, offers the possibility of significantly increased efficiency of the chromatographic separation through the utilization of columns packed with smaller diameter particles (1.8 μm) that can withstand higher pressures compared to the conventional packing materials.

Our prime concern was finding the most suitable analytical method for rapid, simple, sensitive and reproducible analysis of 6 monoglutamate folates in rice. The applicability of this analytical method was demonstrated by analysing wild-type and genetically modified rice samples (biofortified with folates).

2. Experimental

2.1. Chemicals, materials and preparation of standard solutions

Chemicals, materials and preparation of standard solutions are specified in Supplementary File 1. Chemical structures are shown in Supplementary Fig. 1. The 6 folate monoglutamates examined are: tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MTHF), 10-methenyltetrahydrofolate (5,10-CH^+THF), 10-formylfolic acid (10-CHOFA), 5-formyltetrahydrofolate (5-CHOTHF) and folic acid (FA).

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doi:10.1016/j.jchromb.2009.12.032

2.2. Instrumental conditions

The chromatography was performed on a Waters Acquity UPLC system, controlled by Acquity console software (Waters Corp., Milford, USA). The column oven temperature was maintained at 60 °C, the autosampler at 4 °C. The autosampler is equipped with a black door, avoiding samples to be exposed to light.

An Acquity HSS T3 column (150 mm × 2.1 mm; 1.8-μm particle size, from Waters) with a VanGuard Pre-column (5 mm × 2.1 mm; 1.8-μm particle size, also from Waters) was used. The mobile phase consisted of 0.1% of formic acid in water (solvent A) and 0.1% of formic acid in acetonitrile (solvent B) and was pumped at a flow rate of 0.6 ml/min. The starting condition (100% A) was kept for 1 min. The proportion of B was increased linearly to 10% in 2 min, to 12% in 1 min, followed by an immediate increase to 95%, where it was kept for 1 min. Subsequently, the mobile phase was adjusted to its initial composition and held for 3 min for re-equilibration, resulting in a total time of 8 min. Ten microliter was injected on the column.

The UPLC system was coupled to an Applied Biosystems API 4000 tandem quadrupole mass spectrometer (Foster City, CA, USA), controlled by Analyst 1.4 software. The instrument was operated in the ESI positive mode and the data were acquired in multiple reaction monitoring (MRM) mode. Compound parameters were optimised before [12] and are depicted in Supplementary Table 1. Source parameters were determined using flow injection analysis.

2.3. Sample preparation

Sample preparation was performed as described before [12]. Briefly, ten ml of phosphate buffer (50 mM, with 1% ascorbic acid and 0.5% of dithiothreitol, pH 7.5; containing all internal standards) was added to 1 g of rice, followed by homogenisation by means of an Ultra Turrax and tri-enzyme treatment (α-amylase, protease and deconjugase). After centrifuging the resulting solution for 30 min at 14,000 × *g*, the supernatant was ultrafiltrated at 12,000 × *g* for 30 min on a 5 kDa Millipore filter. All manipulations were carried out under subdued light.

2.4. Matrix-matched calibration and linear regression

A 150-μl volume of standard solution was added to 1.35 ml of rice matrix. As it is not possible to obtain or generate a truly blank rice matrix, we subtracted for each calibrator the signal of the endogenous amount of folates in the matrix (1.1, 5.5, 0.4, 2.5, 1.2 and 0.8 μg/100 g for THF, 5-MTHF, 5,10-CH^+THF, 10-CHOFA, 5-CHOTHF and FA, respectively). Matrix-matched calibration curves were constructed by plotting the ratio of the peak areas of the folates to their internal standard against their concentration. Concentrations ranged from LLOQ concentration (minimum is 0.06 μg/100 g) to ±200 μg/100 g.

Four standard series were set up in 1 day to evaluate different calibration types and weighting factors. Homoscedasticity and linearity were examined as well. Protocols are specified in Supplementary File 2.

2.5. Validation

The analytical method for the determination of these 6 monoglutamate folates in rice was validated according to the requirements of USFDA [13]. For quantitative bio-analytical procedures, the following essential parameters should be evaluated: sensitivity, calibration model, accuracy, precision, and stability [14]. Matrix effects were evaluated as well. Detailed procedures are described in Supplementary File 2.

2.6. Application of the method

This method was applied for determining folate concentrations in Japonica Nippon Bare rice (wild-type and biofortified rice). Two batches of 10 unpolished seeds were analysed. The seeds were harvested when the plant had just matured (4 weeks after flowering). Subsequently, seeds were stored at −80 °C until analysis. Growth and homogenisation conditions are specified in Supplementary File 1.

3. Discussion

3.1. UPLC–MS/MS

Source parameters were optimized to the following conditions: source temperature at 600 °C, ionspray voltage at 3500 V, interface heater was on; gas 1, gas 2, collision activated dissociation (CAD) gas and curtain gas was nitrogen, with pressure settings at 70, 90, 8 and 25 psig, respectively.

Different mobile phases were evaluated; 0.1% of formic acid in water and in acetonitrile provided the most efficient chromatography. Chromatographic conditions were optimized to achieve good resolution and to increase the signal of analytes, as well as to minimize run times. The use of smaller particles of stationary phase allows UPLC to push the limits of peak capacity (due to higher efficiency) and to increase the speed of analysis (due to higher linear velocities). A representative chromatogram is presented in Fig. 1. As their was no complete baseline separation between the peaks for the couples 5-MTHF/5, 10-CH^+THF and 10-CHOFA/5-CHOTHF, we confirmed the absence of cross-interference between these compounds (Supplementary Fig. 2).

3.2. Linear regression

As high concentrations influence the fitted regression line more than lower concentrations, the accuracy at the lower end of the range is impaired. Therefore, homoscedasticity (the condition of equal variances) is frequently not met for analytical data, and hence should be investigated when optimising an analytical method for quantitative determination [15]. When a broad range of concentrations has to be covered, using weighted least squares linear regression is an option [15], or two or three calibration curves can be constructed, as Chi et al. and Alvarez-Cedron et al. have established previously [16,17]. The %RE at all calibrator levels, as well as the sum of the absolute %RE values, were calculated for each compound and used for evaluating the calibration curves. These data are shown in Supplementary Table 2.

When using only 1 calibration curve and no weighting, the residual plots showed that the error was not randomly distributed around the *x*-axis and the *F*-test demonstrated significantly different variances, revealing heteroscedasticity (data not shown). As there was no significant difference between the use of 2 calibration curves without weighting, or a single calibration curve with weighting; the first option was chosen as it is more comfortable to work with (only Analyst and Excel had to be used for data analysis). When weighting is necessary, also SPSS software had to be applied.

An *F*-test demonstrated linearity of both calibration curves within the evaluated ranges (data not shown).

The sample concentration was calculated from the equation $y = ax + b$. The calibration curve characteristics for each folate are presented in Table 1.

3.3. Validation of the method

LOD and LLOQ varied between 0.06 and 0.45 and 0.12 and 0.91 μg/100 g, respectively. Intra- and inter-day precisions were

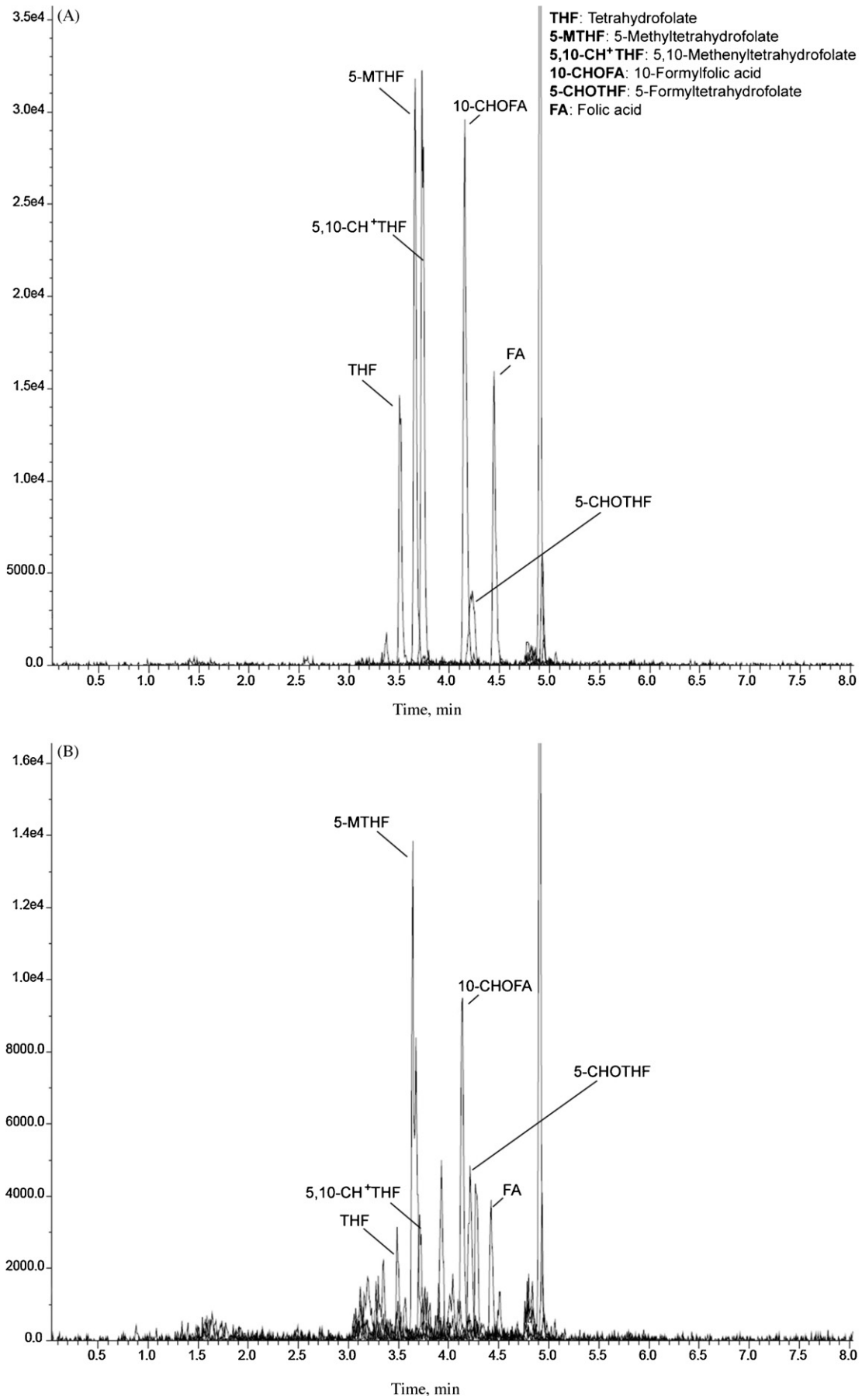

Fig. 1. UPLC–MS/MS chromatogram (A) of a standard mixture of the 6 monoglutamate folates spiked to a ten times diluted rice matrix (concentration of the individual folates varying between 4 and 5 μg/100 g) and (B) of a WT rice sample (total folate content is 19 μg/100 g). For the chromatographic conditions on the HSS T3 column, see text.

Table 1
Calibration characteristics and sensitivity data for individual folates in rice.

	Conc. range (μg/100 g)	*a*	*b*	R^2	LOD	LLOQ	Intra-day precision LLOQ (*n* = 4)	Inter-day precision LLOQ (*n* = 5)
THF	0.79–19.00	10.99	−0.0250	0.999	0.22	0.79	8.99	10.4
	0.79–190.0	10.6	0.0018	0.992				
5-MTHF	0.12–17.53	4.69	−0.0141	0.998	0.06	0.12	7.47	13.0
	0.12–175.3	4.25	0.0147	0.994				
5,10-CH^+THF	0.26–18.54	6.69	−0.0148	0.999	0.07	0.26	4.90	15.5
	0.26–185.4	6.14	0.0107	0.991				
10-CHOFA	0.83–20.00	4.04	−0.0207	0.999	0.26	0.83	4.89	6.56
	0.83–200.0	3.79	0.0016	0.996				
5-CHOTHF	0.91–14.24	9.63	−0.0004	0.998	0.45	0.91	7.20	24.3
	0.91–142.4	9.43	0.0212	0.989				
FA	0.83–20.00	3.87	−0.0222	0.999	0.26	0.83	4.49	16.3
	0.83–200.0	3.55	0.0005	0.997				

determined at LLOQ levels; variation coefficients were all lower than 20%, except for 5-CHOTHF (Table 1).

The method was found to have good accuracy and reproducibility for all analytes at all three levels of concentrations. Accuracy varied between 90.3 and 104.3%, while the intra- and inter-day precision had variation coefficients lower than 15%, except for FA at low concentrations (17.6%). These results are shown in Table 2A. As this method will be used to determine folates in biofortified rice, it may occur that some folate levels exceed the linear range (so-called "over curve samples"), rendering dilution of these samples necessary. Precision and accuracy for these ultra highly concentrated samples, which were diluted 10-fold before or after extraction, were examined on 3 different days. Whereas precision was very good (<2% RSD) for both dilutions, good accuracy (bias < 15%) was only reached when diluting the samples before the extraction (Table 2B). As a consequence, rice samples with potentially high folate concentrations should be diluted before starting the sample preparation.

Despite the enormous success of ESI-LC–MS, this technique is highly susceptible to matrix effects. Matrix effects originate from competition between co-eluting matrix components and analytes for the surface of the solvent droplets. These undetected compounds can enhance or reduce the ion intensity and hence affect the accuracy and reproducibility of the assay [18]. A number of approaches have been developed to compensate for matrix effects [19]. The application of an internal standard in the form of a stable isotopically labelled analogue is one possibility, due to its nearly identical chemical and physical properties as the unlabeled analyte [20]. Nevertheless, Wang et al. showed that even a slight difference in retention time between the analyte and its stable isotopically labelled analogue can result in a different degree of ion suppression between the two analogues, especially in UPLC–MS/MS [21]. Therefore, matrix effects should always be evaluated, in addition to the conventional validation data.

As can be seen from Table 2C, ion suppression or enhancement occurs, with suppression being most pronounced for 5-MTHF and 5,10-CH^+THF (yet the best sensitivity was obtained for these compounds). Isotopically labelled internal standards compensated the ion suppression or enhancement, rendering the matrix effect for all compounds between 85.4 and 103.9%.

3.4. Application of the method

The validated method has been successfully applied to determine folates in real rice samples. In Fig. 2A, total folate concentrations are depicted for wild-type rice (WT) and biofortified lines (B1-7). Some biofortified lines yielded folate levels of up to 900 μg/100 g, which confirms our previously reported results [22], whereas levels in wild-type rice were around 20 μg/100 g. The latter value is lower than previously reported by our lab [12]. From our own experience, however, we know that folate concentrations vary a lot depending on the moment of harvesting and on the way of storage. Folates are very labile and degrade rapidly when stored

Table 2
(A) Intra-day (*n* = 3) and inter-day (over a period of 5 consecutive days) precision and accuracy data for the determination of folates in rice matrix; (B) Inter-day precision and accuracy (*n* = 3) data of ultra highly concentrated samples, diluted 10-fold before or after extraction; (C) Matrix effects of individual folates.

			THF	5-MTHF	5,10-CH+THF	10-CHOFA	5-CHOTHF	FA
(A)								
Intra-day precision (% RSD)	*n* = 3	Low	6.47	9.08	6.16	5.66	13.60	3.33
		Medium	2.81	1.13	3.23	4.31	4.01	2.08
		High	4.51	5.76	5.13	9.84	11.03	5.98
Inter-day precision (% RSD)	*n* = 5	Low	6.47	7.68	7.65	14.19	11.67	17.59
		Medium	3.49	7.32	3.83	10.41	6.96	6.66
		High	2.25	4.36	5.37	3.94	4.09	2.61
Accuracy (%)	*n* = 5	Low	90.31	104.27	101.58	92.93	101.79	103.24
		Medium	96.56	102.67	97.45	101.21	95.92	97.38
		High	100.43	103.56	98.79	95.56	97.00	99.72
(B)								
Inter-day precision (%RSD)	*n* = 3	Before extr	1.28	1.31	1.44	1.63	1.11	1.53
		After extr	0.98	1.05	0.99	1.34	0.86	1.18
Accuracy (%)	*n* = 3	Before extr	94.8	89.6	92.9	97.6	93.9	91.8
		After extr	72.1	71.7	63.8	80.1	72.9	70.9
(c)								
Matrix effect[a]		Without IS	112.0	55.6	72.7	91.6	113.2	113.2
		With IS	103.7	89.6	97.6	85.4	102.2	103.9

[a] Matrix effect % = (B − C)/A × 100 with (A) being the peak area of neat standards in phosphate buffer, (B) the peak area of a rice matrix spiked with the same amount of standards and (C) the peak area of the endogenous amount of folates present in the rice matrix.

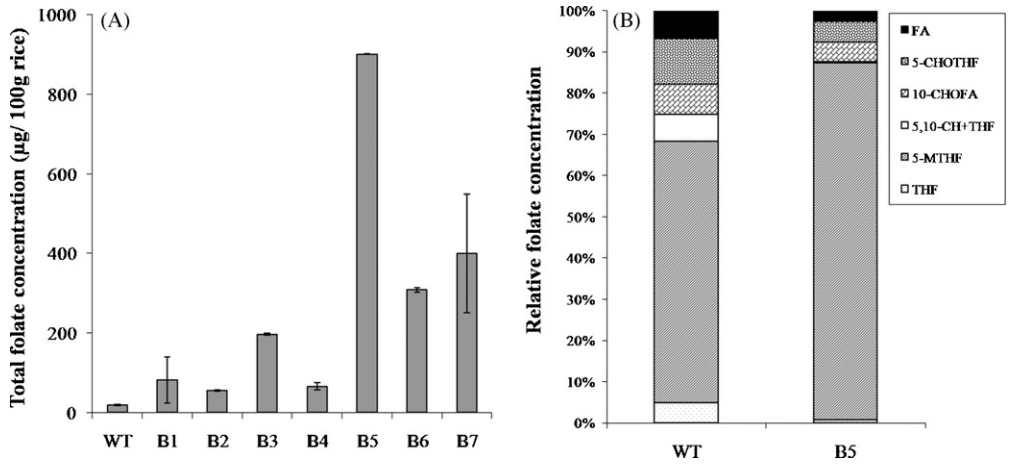

Fig. 2. (A) Total folate concentrations (μg/100 g rice) and (B) folate distribution in wild-type rice (WT) and biofortified rice strains (B1-7). Two times 10 seeds from each line were harvested and analysed separately.

at room temperature, with degradation possibly readily occurring in the plant, before harvesting. Unfortunately, it is very difficult to harvest the rice seeds at exactly the same time point of maturation.

The folate distribution can be seen in Fig. 2B. 5-MTHF is the predominant natural folate form in both wild-type (60%) and transgenic (90%) rice. Only little 5,10-CH^{+}THF and THF can be found in the transgenic rice. These are the most labile folate forms [23]. Rice also contains FA and 10-CHOFA, which are not produced during biosynthesis in the plant but represent oxidation products that arise by degradation.

4. Conclusion

Our newly developed UPLC–MS/MS method has been fully validated and was found to be suitable for determining the concentrations of 6 different folate monoglutamates in wild-type and folate-biofortified rice. The validation experiments showed linearity of the method to determine folate concentrations between 0.12 and 200 μg/100 g rice (dry weight), with acceptable precision and accuracy. Matrix effects were compensated for by use of isotopically labelled internal standards.

This method (LLOQ ranging from 0.12 to 0.91 μg/100 g with total run time of 8 min) is more sensitive for every single folate (up to 10 times) and offers faster separation than our previously developed HPLC method (LLOQ ranging from 0.6 to 4.0 μg/100 g, with total run time of 20 min) [12]. This makes UPLC the method of choice when (very) low levels have to be measured (as in wild-type rice). In addition, the short run time makes UPLC advantageous when analysing very labile compounds, such as folates. Shorter analysis times mean shorter residence times in the autosampler, rendering more reliable results, especially when high throughput (tens of samples) is required.

In conclusion, this work demonstrates that our newly developed UPLC–MS/MS method is a reliable analytical method for the simultaneous determination of 6 folates in rice with high sensitivity and with low matrix interferences.

Acknowledgements

This work was supported by grants GOA 1251204 and GOA 01G00409 (Bijzonder Onderzoeksfonds UGent) and by a bursary to V.D.B. by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.12.032.

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