Quantification of Total Folate, Folate Species and Polyglutamyl Folate Distribution in Winged Beans (*Psophocarpus tetragonolobus* (L) DC) from Different Cultivars and Growth Stages by Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry

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Summary Winged beans are an important natural source of some micronutrients. This paper presents the first complete characterization of folate derivatives including polyglutamyl 5-methyltetrahydrofolate (5-CH1-H4PteGlu5), folate species and total folate accumulating in pods and immature seeds of winged beans from 9 cultivars and 7 growth stages. 5-CH1-H4PteGlu5 and folate species were determined with a UHPLC-MS/MS method. Accurate determination of 5-CH1-H4PteGlu5 and folate species was optimized and validated according to EMA guidelines including method selectivity, sensitivity, linearity, accuracy, precision, matrix effect and carry-over. The level of total folate is in the range of 73–200 μg/100 g in the pods and 33–61 μg/100 g in the immature seeds. The predominant folate species in winged beans is 5-CH1-H4PteGlu5, 5-CH1-H4PteGlu3 is the major polyglutamyl folate derivative. The level of total folate is increased about 4 fold with advancing maturity. For pods, the chain length is increased with growth which shifts from 5-CH1-H4PteGlu1 in the early stage to 5-CH1-H4PteGlu2 and 5-CH1-H4PteGlu3 in the 7th stage. Our findings demonstrate that winged beans are good source of folate. The validated UHPLC-MS/MS method allows for the determination of 5-CH1-H4PteGlu5 and folate species from other vegetable matrices.

Key Words UHPLC-MS/MS, winged beans, polyglutamyl 5-methyltetrahydrofolate, folate species

Folate is a B group vitamin and is widely distributed in nature. Folate is vital for several biochemical pathways such as acting as essential donors and acceptors of one-carbon transfer reactions (1, 2). Folate is also involved in the methylation and DNA biosynthesis cycle in almost all organisms (2, 3). Epidemiological studies indicate that insufficient dietary folate is one of the most common nutritional deficiencies in the world and is associated with a variety of disorders including neural tube defects such as spina bifida, anencephaly, megaloblastic anemia, occlusive vascular disease and other diseases (4, 5). Humans and animals are unable to synthesize folate de novo, and have to obtain this vitamin from their diet (6, 7).

Chemically, folate species are composed of a pteridine ring, p-aminobenzoate and linked to polyglutamyl chains (Fig. 1). Folate species are differentiated by the reduction state of the pteridine ring (tetra- or dihydrofolate), one-carbon substituent at the N 5 and/or N 10 positions, and the length of the γ-glutamyl chains (8). At the subcellular level, folate is distributed in different plant cell compartments in polyglutamyl forms.

Previous studies have shown that 5-CH1-H4PteGlu5 is a vital cofactor for biosynthesis of secondary metabolites including lignin, alkaloids, betaines and chlorophyll (9). In addition, polyglutamyl folate shows bioavailability equal to or higher than that of monoglutamyl folate (10–12). Pteroylmonoglutamyl folate has shown some adverse effects on human health (13, 14). Therefore, many countries in EU ban the fortification of food with pteroylmonoglutamyl folate and recommend that people consume food with high native folate.

In northern China, people are limited in their consumption of fresh green leafy vegetables and the birth defect rate is high, varying in the different areas from 4.2‰ to 10.6‰ (15). Folates are widely present in fruits, vegetables, legumes, cereals and liver (16). Winged beans (*Psophocarpus tetragonolobus* (L) DC) are one of the important tropical legumes. This little-known tropical plant is grown exclusively in Papua New Guinea, Southeast Asia and Southern China. Winged beans are nutrient rich, and all parts of the plant are edible. The leaves can be eaten like spinach, the flowers are used in salads, the tubers are eaten like other root vegetables, the mature seeds are eaten like soybeans and the pods are eaten like other green beans. Knowledge of the total folate, diversity in polyglutamyl folate profiles and folate...
species in this legume has been lacking. Therefore, the aims of the present study were to determine the distribution of 5-CH₃-H₄PteGlu and folate species in the pods and immature seeds of winged beans from 9 different cultivars and 7 growth stages using a validated UHPLC-MS/MS method. The information obtained can be useful for estimating folate intake for the Chinese population.

**MATERIALS AND METHODS**

**Chemicals.** All folate standards employed in this study are shown in Table 1 except (6S)-5-methyl-5,6,7,8-tetrahydrofolate-[¹³C₅]Glu, calcium salt, which was from Merck Eprova AG (Schaffhausen, Switzerland).
Quantification of Folate in Winged Beans by UHPLC-MS/MS

5 g Winged beans pods or seeds

+ 20 mL extraction buffer

Boiling for 5 and 20 min for pods and seeds, respectively

Cooling on ice for 10 min

Homogenization for 5 min

Heating in boiling water for 10 min

Cooling on ice for 10 min

Reconstitute to 50 mL

Take 3 mL aliquot and adjust pH to 2

Centrifuge and the supernatant passing through the 0.45 µm filter

Purification and concentration with HLB cartridge

Elution was dried with N₂ and reconstituted to 300 µL

5-CH₂-H₁₄PteGlu₄ and folate species determination by LC-MS/MS

Fig. 2. Sample preparation flow chart.
The different volumes of Amylase was dissolved in distilled water (10 kU/mL). Isozyme A was treated with 150 U/L folate-stripped rat serum at 37˚C for 6 h to deconjugate the polyglutamyl folate to monoglutamyl form after protease treatment. The complete-ness under a stream of nitrogen, reconstituted in 0.3 mL of the extraction buffer and further filtered through a 0.22 µm nylon filter before injection into the UHPLC-MS/MS. A flow chart describing the sample preparation procedure is shown in Fig.2.

UHPLC-MS/MS. Chromatographic separation of folate species and 5-CH3-H4PteGlu, were carried out by reversed phase on an Agilent 1290 Infinity UHPLC, equipped with a binary pump, autosampler, column oven and degasser (Agilent Corp., Santa Clara, CA). The mobile phase was constituted as follows: 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The 5-CH3-H4PteGlu and folate species were separated with different methods.

For determination of 5-CH3-H4PteGlu, a Sunfire column was kept at 25˚C. The injection volume was 20 µL and the autosampler was kept at 25˚C.

For determination of folate species, an Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 µm; Waters) was used. The flow rate was 0.3 mL/min and the gradient was the same as for the separation of 5-CH3-H4PteGlu. The injection volume was 3 µL and the autosampler was kept at 25˚C.

UHPLC eluate was interfaced with an Agilent 6460 triple quadrupole mass spectrometer (Agilent Corp.) operating in positive ion electrospray mode. An Agilent Mass Hunter workstation was used for the control of equipment, data acquisition and analysis. For the optimization of MS/MS parameters, standard solutions of the external standard (5-CH3-H4PteGlu), internal standard (13C5–5-CH3-H4PteGlu1) and folate species including 5-formyltetrahydrofolate (5-HCO-H4PteGlu1), tetrahydrofolate (H4PteGlu1), 5,10-methenyltetrahydrofolate (5,10-CH=H4PteGlu1) and 10-formylfolic acid (10-CHO-PteGlu1) solutions prepared in methanol were infused into the mobile phase (0.7 mL/min) at a flow rate of 20 µL/min using a syringe pump (Harvard Appa-

| Compounds                          | Precursor ion (m/z) | Qualifier ion (m/z) | Fragmenter voltage (V) | Collision energy (eV) |
|------------------------------------|---------------------|---------------------|------------------------|-----------------------|
| 13C5–5-CH3-H4PteGlu1               | 465                 | 313                 | 110                    | 16                    |
| 5-CH3-H4PteGlu1                    | 460                 | 313                 | 110                    | 16                    |
| 5-CH3-H4PteGlu2                    | 589                 | 313                 | 140                    | 26                    |
| 5-CH3-H4PteGlu3                    | 718                 | 313                 | 180                    | 35                    |
| 5-CH3-H4PteGlu4                    | 847                 | 313                 | 210                    | 39                    |
| 5-CH3-H4PteGlu5                    | 976                 | 313                 | 270                    | 47                    |
| 5-CH3-H4PteGlu6                    | 1,105               | 313                 | 290                    | 53                    |
| 5-HCO-H4PteGlu1                    | 474                 | 327                 | 101                    | 26                    |
| 5,10-CHO-PteGlu1                   | 456                 | 412                 | 115                    | 31                    |
| 10-CHO-PteGlu1                     | 470                 | 295                 | 105                    | 21                    |
| H4PteGlu1                          | 446                 | 299                 | 110                    | 16                    |

The injection volume was 20 µL and the autosampler was kept at 25˚C.
the sum of 5-CH$_3$-H$_4$PteGlu1–6, was compared with total 5-CH$_3$-H$_4$PteGlu1 was performed in this study so interlaboratory accuracy. In addition, cross validation CRM 485, The Institute for Reference Materials and associated. Certified reference materials (mixed vegetable, earity, precision, accuracy and matrix effect were evaluated. detection (LOD) and limit of quantification (LOQ), linear method validation (18). Selectivity, carry-over, limit of detection (LOD) and limit of quantification (LOQ), linearity, precision, accuracy and matrix effect were evaluated. Certified reference materials (mixed vegetable, CRM 485, The Institute for Reference Materials and Measurements, Geel, Belgium) were analyzed to assure interlaboratory accuracy. In addition, cross validation of total 5-CH$_3$-H$_4$PteGlu1 was performed in this study so that total 5-CH$_3$-H$_4$PteGlu1, which was calculated from the sum of 5-CH$_3$-H$_4$PteGlu1, was compared with total 5-CH$_3$-H$_4$PteGlu1, which was determined after rat serum deconjugation in the pods and winged beans from cultivar Baisha #1.

Calibration curve and linear regression: The ranges of calibration curves were determined from preliminary analysis of folate levels in the pods and immature seeds. Calibration curves were prepared by spiking the internal standard (IS) (20 ng/mL) and external standards into a folate-stripped matrix. For external standards, 7 concentrations were applied as shown in Table 3. The folate-stripped matrix included pods and immature seeds. Calibration curves were prepared by spiking the mixture of standards was spiked into a folate-stripping matrix from pods and immature seed extract. The presence of possible endogenous or exogenous interference was verified by monitoring the MRM chromatograms. MRM chromatograms were specific for each analyte at their expected retention times. The acceptance criterion for the selectivity was that the peak area of interference should be below 20% of the peak area of the LOQ of analytes and 5% for the labeled IS.

carry-over was evaluated by injecting blank samples after the run of highest concentrationibrator. The acceptance criteria for carry-over were the same as for evaluation of selectivity.

Accuracy and precision: To assess accuracy and precision, the mixture of standards was spiked into a folate-stripping matrix from pods and immature seeds to the final concentrations as shown in Table 3. Three Quality Controls (QCs) corresponded to 3 points, LOQ (low QC), medium QC and ULOQ. The 3 QCs were prepared and analyzed on 3 non-consecutive days. Accuracy was determined as% recovery. For evaluating the precision, 5 samples per concentration level at 3 QC samples were analyzed on the same day or 3 different days to evaluate the intra-day precision and inter-day precision.

Matrix effect: The matrix effect was also determined for the method. The sample matrix such as co-eluting compounds can contribute to alter the analyte ionization and overall response. Therefore, complete separation between analytes and the co-eluting matrix could help decrease or increase the ionization of the target analyte. The matrix effect was determined with the following method. Three QCs were spiked into the folate-stripped pods and seed extracts. For each analyte, the peak areas obtained in the matrix were compared with the corresponding peak areas in the extraction buffer reliably so that bias (%) of accuracy and precision was below 20%. In this study, the LOQ is considered as the lowest concentration of calibration curve. In addition, the analyte signal of the LOQ should be at least 5 times that of the signal of a blank sample. The LOD was calculated as one-third of the LOQ.

Selectivity and carry-over: Selectivity refers to the method that can differentiate the target analyte from other matrix components. This was assessed by analyzing 12 folate-stripped pods and immature seed extract samples. The presence of possible endogenous or exogenous interference was verified by monitoring the MRM chromatograms. MRM chromatograms were specific for each analyte at their expected retention times. The acceptance criterion for the selectivity was that the peak area of interference should be below 20% of the peak area of the LOQ of analytes and 5% for the labeled IS.

Table 3. Calibration, quality control and validation concentrations (ng/mL).

| Compound | 1      | 2      | 3      | 4      | 5      | 6      | 7      | QC1 | QC2 | QC3 |
|----------|--------|--------|--------|--------|--------|--------|--------|-----|-----|-----|
| 5-CH$_3$-H$_4$PteGlu1 | 0.5    | 1      | 2      | 5      | 10     | 20     | 50     | 1.5 | 5   | 20  |
| 5-CH$_3$-H$_4$PteGlu2 | 1      | 2      | 4      | 10     | 20     | 40     | 100    | 3   | 10  | 40  |
| 5-CH$_3$-H$_4$PteGlu3 | 3.5    | 7      | 14     | 35     | 70     | 140    | 350    | 10.5| 35  | 140 |
| 5-CH$_3$-H$_4$PteGlu4 | 12     | 24     | 48     | 120    | 240    | 480    | 1,200  | 36  | 120 | 480 |
| 5-CH$_3$-H$_4$PteGlu5 | 30     | 60     | 120    | 300    | 600    | 1,200  | 3,000  | 90  | 300 | 1,200 |
| 5-CH$_3$-H$_4$PteGlu6 | 45     | 90     | 180    | 450    | 900    | 1,800  | 4,500  | 135 | 450 | 1,800 |
| H$_4$PteGlu1 | 0.5    | 1      | 2      | 5      | 10     | 20     | 50     | 1.5 | 2   | 20  |
| 5-HCO-H$_4$PteGlu1 | 0.5    | 1      | 2      | 5      | 10     | 20     | 50     | 1.5 | 2   | 20  |
| 10-CHO-PteGlu1   | 0.5    | 1      | 2      | 5      | 10     | 20     | 50     | 1.5 | 2   | 20  |
| 5,10-CH$^-$-H$_4$PteGlu1 | 0.5 | 1      | 2      | 5      | 10     | 20     | 50     | 1.5 | 2   | 20  |

QC1, QC2 and QC3 refer to quality control solution 1, quality control solution 2 and quality control solution 3, respectively.
with the same concentration. Each sample was analyzed three times. The matrix effect was calculated as the following:

\[
\text{Matrix effect (\%)} = \frac{A}{B} \times 100
\]

where \(A\) is the mean peak area in the matrix, and \(B\) is the mean peak area in the extraction buffer.

**RESULTS AND DISCUSSION**

**Pre-boiling time optimization**

The main challenge of profiling intact polyglutamyl folates in vegetables is to completely disable GGH during extraction. In this study, a pre-boiling method was applied to inactivate the GGH, allowing for accurate profiling of polyglutamates in the pods and immature seeds of cultivar Baisha #1. The pre-boiling times of 0, 5, 10, and 20 min were chosen for comparison. Figure 3 shows 5-CH₃-H₄PteGlu₅ is enhanced with increasing pre-boiling time in seeds and pods. Comparing no pre-boiling (Fig. 3A) to a 20 min pre-boiling, the relative contribution of 5-CH₃-H₄PteGlu₁ is decreased by about 37%, while the percentage of 5-CH₁-H₄PteGlu₅ is increased by 46% in the seeds. For pods, 5 min pre-boiling is enough to achieve the plateau of maximum polyglutamates. After 5 min pre-boiling, the profile shifts from 5-CH₁-H₄PteGlu₁ (30%) and 5-CH₁-H₄PteGlu₃ (52%) to 5-CH₁-H₄PteGlu₁ (16%) and 5-CH₃-H₄PteGlu₅ (69%) in the pods. Uniquely, around 10% of 5-CH₁-H₄PteGlu₆ is found in the pods. In summary, it is observed that with extending pre-boiling time, the profile of different parts of winged beans shifts from the short-chain form (5-CH₃-H₄PteGlu₁) to the long-chain form (5-CH₃-H₄PteGlu₅) because of the inactivation of GGH. Therefore the optimized pre-boiling time is 5 min for pods and 20 min for seeds. In this study, we monitored the change of 5-CH₁-H₄PteGlu₁₋₆ during different boiling times and found that most of 5-CH₁-H₄PteGlu₁ is cleaved to 5-CH₃-H₄PteGlu₁. An earlier study has examined different cleavage patterns of polyglutamyl folate from various vegetable cultivars in which the long-chain polyglutamyl folate was deconjugated to monoglutamyl folate in turnips and carrots and to triglutamyl folate in some vegetables (such as broccoli and cauliflower) from the Brassica family (19). The reason for different cleavage patterns could be ascribed to the GGH isoforms and their substrate and product specificities. Multiple GGH isoforms were reported in Arabidopsis leaves so that some isoforms could cleave 5-CH₁-H₄PteGlu₅ to 5-CH₁-H₄PteGlu₁ and 5-CH₁-H₄PteGlu₃ and another isoform preferably to form 5-CH₁-H₄PteGlu₁ (19).

In terms of total 5-CH₁-H₄PteGlu₁ (sum of 5-CH₁-H₄PteGlu₁₋₆), folate recovery is increased approximately 98% and 20% after pre-boiling in comparison to the absence of pre-boiling for seeds and pods, respectively. The pre-boiling may liberate folates by denaturing the folate-binding protein to achieve higher extraction efficiency than a no pre-boiling method.

There are several studies about quantitative polyglutamyl folate profiles in vegetables (17, 20–26). An earlier study found pentaglutamyl and heptaglutamyl folate are the major forms in black beans while hexaglutamyl folate is the major form in the tomato fruit; however, the polyglutamyl folate forms in the alfalfa sprouts shift to short-chain forms such as monoglutamyl and tetraglutamyl forms (23). The authors have realized that the polyglutamyl folate of alfalfa shifting towards short chains could be ascribed to the action of GGH activity during extraction. During homogenization, the endogenous polyglutamyl folate is hydrolyzed by plant GGH. So optimization of pre-boiling before homogenization is necessary to quantitate the native polyglutamyl folate profile. Another study profiled folate polyglutamylation of papaya during fruit development and ripening (22). The authors found a uniquely long 5-CH₁-H₄PteGlu₁ profile up to 17. 5-CH₁-H₄PteGlu₁ in vegetables from different families has been profiled (17, 19, 21). The authors found 5-CH₁-H₄PteGlu₁ in Brassicaceae is predominant as 5-CH₁-H₄PteGlu₅ through 5-CH₁-H₄PteGlu₇. Polyglutamyl folate in Asteraceae and Amaranthaceae is mainly in the 5-CH₁-H₄PteGlu₁ and 5-CH₁-H₄PteGlu₅ forms while endive species contain mainly...
the 5-CH\textsubscript{3}-H\textsubscript{4}PteGlu\textsubscript{1} form. Therefore, polyglutamyl folate from different vegetables shows unique profiles. Due to different GGH activity, GGH inhibitors and other food matrices in the vegetables, the extraction method should be optimized to different vegetables in order to profile the polyglutamyl folate correctly. Polyglutamyl folate had not been profiled in the winged beans until now; this new information could be useful for further assessment of folate bioavailability and researching the biological role of polyglutamyl folate in the vegetable.

Di-enzyme optimization

Di-enzyme (amylase and protease) treatment was investigated for possible improvement of folate recovery after pre-boiling. In this study, we found no significant improvement of total 5-CH\textsubscript{3}-H\textsubscript{4}PteGlu\textsubscript{1} after α-amylase and protease treatment although pods, seeds and shoots contain significant protein and starch (Fig. 4A and B). The pre-boiling may already liberate folates from the binding matrix.

Method validation

All analytes were detected in a positive ion mode and
Fig. 5. UHPLC-MS/MS chromatogram of 5-CH₃-H₄PteGlu in the pod. 1, 5-CH₃-H₄PteGlu₁; 1', 1³C₃-5-CH₃-H₄PteGlu; 2, 5-CH₃-H₄PteGlu₂; 3, 5-CH₃-H₄PteGlu₃; 4, 5-CH₃-H₄PteGlu₄; 5, 5-CH₃-H₄PteGlu₅; 6, 5-CH₃-H₄PteGlu₆. Chromatographic conditions were as follows. Flow rate: 1.8 mL/min. Gradient: 0–4 min, 0–20% B; 4–5 min, 20–95% B; 5–6.5 min, 95% B; 6.5–9 min, re-equilibration to initial conditions. The injection volume: 20 μL. Autosampler: 25°C.

Table 5. Matrix effect, recovery and repeatability.

| Analyte                  | Concentration (ng/mL) | Matrix effect (%) | Recovery (%) | Repeatability (CV%) |
|--------------------------|-----------------------|-------------------|--------------|---------------------|
|                          |                       |                   | Intraday     | Interday            | Intraday | Interday |
| 5-CH₃-H₄PteGlu₁         | 1.5                   | 3.87              | 93.6         | 87.0                | 4.9      | 12.1     |
|                          | 5                     | 7.44              | 96.1         | 79.2                | 9.6      | 8.3      |
|                          | 20                    | 6.9               | 82.9         | 73                  | 11.8     | 8.2      |
| 5-CH₃-H₄PteGlu₂         | 3                     | 8.79              | 104.4        | 89.4                | 8.3      | 6.6      |
|                          | 10                    | 5.5               | 91.9         | 88.9                | 10.5     | 12.5     |
|                          | 40                    | 11.55             | 85.2         | 73.5                | 8.0      | 14.6     |
| 5-CH₃-H₄PteGlu₃         | 10.5                  | 6.30              | 96.9         | 82.1                | 9.4      | 14.0     |
|                          | 35                    | 9.25              | 95.5         | 72.3                | 7.1      | 13.7     |
|                          | 140                   | 6.99              | 88           | 85.4                | 8.3      | 9.4      |
| 5-CH₃-H₄PteGlu₄         | 36                    | 4.77              | 102.7        | 79                  | 3.0      | 14.1     |
|                          | 120                   | 9.97              | 90.5         | 86.9                | 9.2      | 9.8      |
|                          | 480                   | 6.06              | 96           | 89.8                | 6.0      | 10.9     |
|                          | 300                   | 10.65             | 92.9         | 84.6                | 8.0      | 13.2     |
|                          | 1,200                 | 4.78              | 96.1         | 77.5                | 11.5     | 12.4     |
| 5-CH₃-H₄PteGlu₅         | 135                   | 3.53              | 91.9         | 70.6                | 3.3      | 11.1     |
|                          | 450                   | 2.05              | 95           | 88.9                | 10.2     | 13.3     |
|                          | 1,800                 | 3.96              | 94.4         | 79.3                | 12.1     | 11.0     |
| 5-HCO-H₄PteGlu₁         | 1.5                   | 0.76              | 107.1        | 118.9               | 8.6      | 13.5     |
|                          | 5                     | 6.12              | 92.8         | 78.8                | 11.0     | 13.9     |
|                          | 20                    | 5.97              | 91.2         | 75.1                | 7.1      | 12.8     |
| 5,10-CH⁺-H₄PteGlu₁      | 1.5                   | 7.53              | 102.2        | 79.7                | 7.9      | 10.3     |
|                          | 5                     | 8.02              | 90.3         | 77.63               | 8.0      | 14.7     |
|                          | 20                    | 8.87              | 85.0         | 89.5                | 9.0      | 12.6     |
| 10-CHO-PteGlu₁          | 1.5                   | 2.82              | 90.4         | 91.3                | 3.4      | 2.9      |
|                          | 5                     | 5.33              | 85.4         | 85.4                | 11.6     | 11.4     |
|                          | 20                    | 6.45              | 94.3         | 81.3                | 9.8      | 9.7      |
| H₄PteGlu₁               | 1.5                   | 10.24             | 103.8        | 74.3                | 4.6      | 11.2     |
|                          | 5                     | 6.2               | 85.7         | 75.0                | 8.1      | 12.6     |
|                          | 20                    | 7.50              | 88.6         | 82.7                | 10.1     | 12.1     |
were well separated on the reversed phase column used. Each molecule exhibited an adequately separate chromatographic peak that was easily distinguished from the baseline. Quantitation was performed based on retention time, which was compared with the authentic standard and MRM transition for each compound as shown in Table 2 and Table 4. Selection of the transition was based on the precursor ion to the most predominant fragment ions.

Method selectivity was evaluated based on the response of folate-stripped pods and seed extracts. Selectivity met the criterion because there were no interfering peaks observed at the same retention times as the target analytes and the IS.

Calibration curves prepared in the matrix showed an acceptable linearity range and $R^2$ (Table 4). LODs and LOQs ranged from 0.15 to 13.6 ng/mL and from 0.5 to 45 ng/mL, respectively (Table 4). No unacceptable carry-over was observed.

Method precision has been evaluated by 5 replicates of three concentrations of standards which were spiked in folate-stripped pods and seed extracts for three consecutive days. The recoveries were under 15% (Table 5).

Accuracy is expressed as recoveries, determined by analyzing 5 replicates of three concentrations of standards which were spiked in folate-stripped pods and seed extracts for three consecutive days. The recoveries were between 70.6% and 118.9%. The chromatogram of representative 5-CH$_3$-H$_4$ PteGlu in the pods is shown in Fig. 5 and the UHPLC-MS/MS chromatogram of folate species spiking in a stripped-pod extraction at LOQ level is shown in Fig. 6. The chromatographic separation of all compounds was achieved in 9 min.

Matrix effects for all of the samples are shown in Table 5 which was compensated with the IS. IS-compensated recoveries were between 0.76 and 11.55% for two different matrices.

In the CRM 485 (mixed vegetable reference), only two folate species, 5-CH$_3$-H$_4$ PteGlu$_1$ and H$_4$ PteGlu$_1$, were detected and quantified in a concentration of 238 mg/100 g and 6.32 mg/100 g, respectively. This concentration was in a concentration range similar to that which has been reported by other researchers (27–29).

In the cross validation study, the sum of 5-CH$_3$-H$_4$ PteGlu$_1$ through 5-CH$_3$-H$_4$ PteGlu$_6$ was not significantly different from total 5-CH$_3$-H$_4$ PteGlu$_1$ after rat serum deconjugation. Total 5-CH$_3$-H$_4$ PteGlu$_1$ ($\mu$g/100 g) without/with deconjugation in the pods and seeds were 107±12/118±21 and 50±4.5/54±6, respectively.
Comparison of the distribution of 5-CH₃-H₄PteGlu₁₀ and folate species and total folate concentrations from pods and immature seeds of winged beans in 9 cultivars

Intact 5-CH₃-H₄PteGlu₁₀ in pods and immature seeds from 9 different cultivars are presented in Fig. 7A and B. For immature seeds, only the forms of 5-CH₃-H₄PteGlu₁ and 5-CH₃-H₄PteGlu₅ were detected for quantification although their distribution was quite different among various cultivars. Significant differences due to cultivars are observed for the seeds: about 71–78% was present as 5-CH₃-H₄PteGlu₅ in the cultivars of Guifeng #1, Guifeng #2, Guifeng #4, Guiai #1, Baisha #1, 6, Baisha #7, 7, Baisha #13; 8, Baisha #9; 9, Wuzhishan #2.

Fig. 7. Comparison the amount of 5-CH₃-H₄PteGlu₁₀ and 5-HCO-H₄PteGlu₁ in the seeds and pods of winged beans (Psophocarpus tetragonolobus (L) DC) from 9 different cultivars. A, immature seeds; B, pods. 1, Guifeng #1; 2, Guifeng #2; 3, Guifeng #4; 4, Guiai #1; 5, Baisha #1; 6, Baisha #7; 7, Baisha #13; 8, Baisha #9; 9, Wuzhishan #2.

Comparison of the distribution of 5-CH₃-H₄PteGlu₁₀, folate species and total folate concentrations from pods and immature seeds of winged beans in 9 cultivars

Intact 5-CH₃-H₄PteGlu₁₀ in pods and immature seeds from 9 different cultivars are presented in Fig. 7A and B. For immature seeds, only the forms of 5-CH₃-H₄PteGlu₁ and 5-CH₃-H₄PteGlu₅ were detected for quantification although their distribution was quite different among various cultivars. Significant differences due to cultivars are observed for the seeds: about 71–78% was present as 5-CH₃-H₄PteGlu₁₀ in the cultivars of Guifeng #1, Guifeng #2, Guifeng #4, Guiai #1, Baisha #1 and Baisha #7. In the cultivars of Baisha #13 and Baisha #9, the distribution of 5-CH₃-H₄PteGlu₁₀ was similar to that of 5-CH₃-H₄PteGlu₁. In the cultivar of Wuzhishan #2, the content of 5-CH₃-H₄PteGlu₁₀ amounted to 60%.

Figure 7B shows the distribution of 5-CH₃-H₄PteGlu₁₀ in the pods from different cultivars. For different cultivars, 5-CH₃-H₄PteGlu₁₀ is the predominant form in the range of 63–79%. Uniquely, 5–13% of 5-CH₃-H₄PteGlu₁₀ was observed in the pods.

Comparison of the distribution of folate species and total folate concentration from pods and immature seeds of winged beans in 9 cultivars

Figure 7 shows the concentration for each folate species as well as the total folate concentration.

Total folate concentration was around 73–200 μg/100 g in the pods, and around 33–61 μg/100 g in the immature seeds. Thus, a normal serving of 35 g pods and seeds would provide 12% and 4.1% of the adult recommended daily allowance (RDA) (400 μg dietary folate equivalents), respectively. Most legumes are identified as good sources of folate (16). For total folate in the pods and seeds, considerable differences among different varieties were observed.

Figure 7A and B also shows a graphic representation of the composition of the folate species in the immature seeds and pods of winged beans from different cultivars. It is observed that only two of the species including 5-CH₃-H₄PteGlu₁ and 5-HCO-H₄PteGlu₁ were submitted to quantification and 5-CH₃-H₄PteGlu₁ was the predominant form, representing 80–99% in the seeds and 85–91% in the pods. 5-CH₃-H₄PteGlu₁ has been identified as the predominant folate species in many vegetables, legumes, fruit, bread, milk and meat (19, 27, 30).

The total amount of folates in the immature seeds measured in this study was similar to that in a report from the USDA database (16).

Comparison of the distribution of 5-CH₃-H₄PteGlu₁₀, folate species and total folate concentration from pods in the different growth stages

Figure 8 shows the concentration for 5-CH₃-H₄PteGlu₁₀, folate species as well as the total folate in the different growth stages of pods from cultivar Baisha #9 in the different growth stages. Stage 1, 27th April; stage 2, 3rd May; stage 3, 10th May; stage 4, 23rd May; stage 5, 30th May; stage 6, 7th June; stage 7, 14th June.

Fig. 8. Comparison the amount of 5-CH₃-H₄PteGlu₁₀ and 5-HCO-H₄PteGlu₁ in the pods of winged beans (Psophocarpus tetragonolobus (L) DC) from cultivar Baisha #9 in the different growth stages. Stage 1, 27th April; stage 2, 3rd May; stage 3, 10th May; stage 4, 23rd May; stage 5, 30th May; stage 6, 7th June; stage 7, 14th June.
of total folate level during fruit developing or the first two ripening stages, while total folate shows a slight increase at late ripening stage (22). Instead of investigating the change in total folate during ripening, our work focused on the growth stage. We also observed a slight decrease trend during ripening at stage 6 and stage 7 from 156 μg/100 g to 108 μg/100 g. In the early stage, 5-CH3-H4PteGlu1 and 5-CH3-H4PteGlu2 account for 90% and 10%, respectively. With the advancing growth, the profile gradually shifts to 5-CH3-H4PteGlu3 with a proportion of 70%. Winged beans accumulate folates in all stages mainly as 5-CH3-H4PteGlu1 and 5-HCO-H4PteGlu1, while 5-CH3-H4PteGlu1 is the predominant form, accounting for 77–98%. 5-CH3-H4PteGlu1, which plays an important role in methionine biosynthesis, is the most abundant form during germination and plant growth, and the methyl group from 5-CH3-H4PteGlu1 is the direct precursor of methylation via S-adenosyl-methionine (SAM) (34). The methyl group from SAM could be transferred by methyltransferase to different substrates such as proteins, lipids, DNA, and hormones (34). SAM also supplies methyl groups to synthesize molecules such as choline, chlorophyll, or lignin (34). In addition, 5-CH3-H4PteGlu1 plays an important role in human health. An increase in homocysteine due to lower 5-CH3-H4PteGlu1 concentration is associated with various diseases (35).

CONCLUSIONS

Winged beans are popular vegetables in many regions, and can contribute substantial amounts of the RDA for folates. The information supplied by this research might be of interest to fresh-vegetable processors, food technologists, dieticians and nutritionists since it illustrates which cultivars, growth stages, and organs of winged beans preserve the highest content of folate and thus their beneficial effects for human health are greatest.

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