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Improved Folate Extraction and Tracing Deconjugation Efficiency by Dual Label Isotope Dilution Assays in Foods

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Supporting Information

ABSTRACT: A dual label stable isotope dilution assay was developed to trace the deconjugation efficiency of polyglutamic folate vitamers converted to their monoglutamic analogues. For this purpose, [$^{13}C_5$]-pteroylheptaglutamate was synthesized and added during extraction of foods as a tracer isotopologue along with [$^2H_4$]-5-methyltetrahydrofolate, [$^2H_4$]-5-formyltetrahydrofolate, [$^2H_4$]-tetrahydrofolate, [$^2H_4$]-10-formylfolate, and [$^2H_4$]-folic acid. The [$^2H_4$]-labeled folates were used as internal standards for the monoglutamates. Deconjugation converted the addition tracer [$^{13}C_5$]-pteroylheptaglutamate to the detection tracer [$^{13}C_5$]-folic acid, which was quantified along with unlabeled folic acid using [$^2H_4$]-folic acid as the internal standard. LC-MS/MS enabled the unequivocal differentiation of the three isotopologues. This tracing was used to optimize deconjugation efficiency, which was achieved by using 4-morpholineethanesulfonic acid buffer for extraction at pH 5.0 . The optimized assay revealed limits of detection for the folate vitamers ranging between 2.0 and 5.6 pmol per assay (equivalent to 2.2−6.6 μg/100 g dry mass), recoveries ranging between 98 and 105% and relative standard deviations in inter-assay precision ranging between 2 and 6%. The assay was applied to quantitate folates in spinach, beans, cheeses, bread, wheat germs, and yeast .

KEYWORDS: *deconjugation, folates, stable isotope dilution assay, dual label isotope design, pteroylheptaglutamate*

1. INTRODUCTION

The vitamins of the folate group play a crucial role as coenzymes in the metabolism of one-carbon groups[1] and are decisively involved in DNA synthesis, amino acid metabolism, and methylations, in general. However, intake of this group from natural sources is considered to be below the human dietary recommendations. Consequently, folate deficiency is believed to increase the risk of neural tube defects[2] and is suspected of being associated with the development of certain forms of cancers,[3] Alzheimer's disease,[4] and cardiovascular disease.[5] Therefore, over 50 countries all over the world have introduced mandatory folate fortification, mainly on the American continent but also in Africa, Asia, and the Pacific Region. Folic acid administration was implemented in 1998 in the USA and Canada and most recently in Australia in September 2009. The benefits of this measure with regard to neural tube defects were obvious, as their incidence in Canadian regions was decreased by up to 3.8 cases per 1000 births.[6] However, in the last few years, criticism arose since the decreasing trend of colon cancer reversed in some countries with mandatory folate fortification since its implementation.[7] On a molecular basis, it is suggested that plasma occurrence of folic acid may lead to neoplastic transformations and formation of adenomas.[8] Moreover, upon folic acid supplementation rat studies revealed the progression of aberrant crypt foci (ACF), the earliest precursor of colorectal cancer.[9] In a human study, folic acid supplementation decreased the cytotoxicity of circulating natural killer cells.[10] The latter cells are assumed to play a role in the destruction of neoplastic cells. Therefore, many countries in the EU refuse mandatory fortification and favor the consumption of foods endogenously high in folates or increasing folate content in foods generally. Thus, for dietary recommendations, the content of endogenous folates in foods has to be known. In the last 60 years, the standard assay to quantitate food folates has been the microbiological assay (MA) that generates a total folate figure from a turbidity measurement after the growth of *Lactobacillus casei* ssp. rhamnosus in a folate-deficient medium. Although significant improvement regarding the handling of the MA in microtiter formats has been achieved,[11] the results of the MA lack information on accuracy and vitamer distribution. Therefore, there is increasing application of chromatographic methods, in particular coupled to mass spectrometry. To compensate for losses during cleanup and for ionization interferences in the ion source, internal standards labeled with stable isotopes have been applied.[12,13] The latter were isotopologues of the five most abundant folate monoglutamates, namely, [$^2H_4$]-5-methyltetrahydrofolic acid, [$^2H_4$]-5-formyltetrahydrofolic acid, [$^2H_4$]-tetrahydrofolic acid, [$^2H_4$]-10-formylfolic acid, and [$^2H_4$]-folic acid.[14] Application of the latter standards in stable isotope dilution assays (SIDAs) underwent several improvements, but still the quantitation of

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polyglutamate forms is a critical issue as only the monoglutamates are available as labeled internal standards. Therefore, deconjugation of polyglutamates to the respective monoglutamic forms has to be ensured, which up to date is only possible in additional assays with spikes of polyglutamates.

Therefore, the principle aim of the present study was to assess the best conditions for complete deconjugation and to find a way to monitor the yield of major monoglutamates without performing additional analyses. A further objective was to apply the improved deconjugation conditions to a set of foods high in folates.

2. MATERIALS AND METHODS

2.1. Chemicals. The following chemicals were obtained commercially from the sources given in parentheses: rat serum (Biozol, Eching, Germany), chicken pancreas (Difco, Sparks, USA) acetonitrile, 1,4-dioxane, dichloromethane (DCM), diethyl ether (dried over sodium hydride), dimethylformamide (DMF), formic acid, hexane, 2-propanol, *N*-methylpyrrolidone (NMP), piperidine, sodium hydroxide, methanol, Na_2SO_4, sodium chloride, tetrahydrofuran (Merck, Darmstadt, Germany), ascorbic acid, *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*,*N*′-diisopropylethylamine (DIPEA), folic acid, [$^{13}C_5$]-glutamic acid, 1-hydroxybenzotriazole hydrate (HOBt), 4-morpholineethanesulfonic acid (MES), 2-mercapto ethanol, protease type XIV, sodium acetate, thionyl chloride and trifluoroacetic anhydride (Sigma, Deisenhofen, Germany), tetrahydrofolate, 5-methyltetrahydrofolate, 10-formylfolate, and 5-formyltetrahydrofolate (Schircks, Jona, Switzerland).

[$^2H_4$]-5-Methyltetrahydrofolic acid, [$^2H_4$]-5-formyltetrahydrofolic acid, [$^2H_4$]-tetrahydrofolic acid, [$^2H_4$]-10-formylfolic acid, and [$^2H_4$]-folic acid were synthesized as reported recently.[14]

2.2.1.1. Synthesis of [$^{13}C_5$]-Pteroylheptaglutamate. *Preparation of Fmoc-(γ-Glu)$_6$. Coupling to Resin.* Fmoc protected glutamic acid α-*tert*-butyl ester (1 mmol) was added to tritylchloride resin (0.5 g) in dichloromethane (0.5 g). After the addition of DIPEA (0.54 mL), the mixture was stirred at room temperature for 60 min before further DIPEA (0.5 mL) and methanol (3 mL) were added followed by stirring for further 15 min at room temperature. Thereafter, the resin was filtered and washed successively with dimethyl formamide, dichloromethane, 2-propanol, and diethyl ether (5 mL each) and then dried in a desiccator overnight.

2.2.1.2. Deprotection of the Amine Group. The dried resin was reacted with piperidine in dimethyl formamide (15 mL, 5%) and pure piperidine (3 mL) for 30 min at room temperature. Subsequently, the resin was filtered and washed successively with dimethyl formamide, dichloromethane, 2-propanol, and diethyl ether (5 mL each) and then dried in an desiccator in vacuo.

2.2.1.3. Attaching of Further Amino Acids via γ-Peptide Bonds. HOBt (2 equiv) were added to Fmoc protected glutamic acid α-*tert*-butyl ester (2 equivalents), and the mixture was dissolved in dichloromethane/dimethyl formamide (1 + 1 v/v, 6 mL) and cooled to 0 °C. Subsequently, DCC (2 equiv) was added at stirring for 15 min followed by allowing the solution to warm to room temperature and stirring for further 10 min at room temperature until the insoluble urea derivative precipitated. The suspension was filtered and the filtrate reacted with the resin bound amino acid for 4 h at room temperature. Subsequently, the resin was filtered and washed successively 5 times with dichloromethane, *N*-methyl-2-pyrrolidone, and dichloromethane (5 mL each) and then dried in an desiccator in vacuo.

Deprotecting and attaching was repeated 5 times to obtain a peptide composed of six glutamic acids coupled via γ-peptide bonds.

2.2.1.4. Cleavage from the Resin for Structure Conformation of the Hexapeptide. The resin (100 mg) was deprotected by reacting with piperidine in dimethyl formamide (15 mL, 5%) and pure piperidine (3 mL) for 30 min at room temperature. Filtration of the resin was followed by reaction with a mixture of dichloromethane and glacial acetic acid (9 + 1, v/v) and stirring for 30 min at room temperature. Subsequently, the resin was filtered and washed with dichloromethane. The collected dichloromethane phases were rotary evaporated to give hexaglutamate as the residue.

LC-MS (ESI$^+$): m/z 1130.3. ^{1}H NMR δ_H (400 MHz, $CDCl_3$) 3.361−3.396 (1 α-H, dd), 1.976−2.052 (2 β-H each t), 2.354−2.394 (2 γ-H, dd), 1.382−1.458 (9 *tert*-butyl-H, m).

2.2.2.1. Preparation of Fmoc-[$^{13}C_5$]-L-Glu-OtBu. Butylation of [$^{13}C_5$]-L-Glutamic Acid and Protection with Fmoc According to Lajoie et al..[15] Isobutene gas was liquified in a flask cooled with liquid nitrogen, and 1.4 mL of the liquid was added along with dioxane (1.4 mL) and *p*-toluolsulfonic acid (0.3495 g) to [$^{13}C_5$]-L-glutamic acid (0.1508 g). Subsequently, the mixture was shaken for 23 h at room temperature followed by the addition of aqueous sodium carbonate (2 mL 10%) and dropwise addition of Fmoc-succinimide (0.3191 g in 15 mL dioxane). Thereafter, the solution was stirred for 23 h at 3 °C−6 °C and poured into ice water, followed by washing with portions of diethyl ether (3 × 20 mL), which were discarded. Thereafter, the solution was washed with ethyl acetate (3 × 20 mL). The aqueous phase was then adjusted at 0 °C to pH 5.5 by the addition of aqueous hydrochloric acid (1 mol/L) and extracted with ethyl acetate (3 × 20 mL). The collected ethyl acetate phases were washed with brine (3 × 20 mL), and after drying over sodium sulfate, the solvent was evaporated (yield 72%).

LC-MS (ESI$^+$): m/z = 431.

2.2.2.2. Cleanup of the Mixture of Butyl Esters. Fmoc-L-Glu-OtBu was separated from the byproduct Fmoc-L-Glu(OtBu)-OH by preparative isocratic RP-HPLC using a mixture of 65% methanol and 35% 0,1% trifluoroacetic acid as the mobile phase. From this system, the target compound Fmoc-[$^{13}C_5$]-Glu-OtBu was eluted before Fmoc-[$^{13}C_5$]-L-Glu(OtBu)-OH and collected from several runs before being rotary evaporated and lyophilized (yield 23%).

2.2.3.1. Synthesis of Pteroyl-[$^{13}C_5$]-(Glu-OtBu)$_7$. Deprotection of Resin-Bound Fmoc-(γ-Glu)$_6$. The dried resin coupled to protected hexaglutamate was deprotected with piperidine in dimethyl formamide (15 mL, 5%) and pure piperidine (3 mL) for 30 min at room temperature. Subsequently, the resin was filtered and washed five times successively with dimethyl formamide, dichloromethane, 2-propanol, and diethyl ether (5 mL each) and then dried in an desiccator in vacuo.

2.2.3.2. Coupling of Fmoc-[$^{13}C_5$]-Glu-OtBu to Resin-Bound (Glu-OtBu)$_6$. HOBt (2 equiv) was added to Fmoc protected [$^{13}C_5$]-Glu-OtBu (2 equivalents), and the mixture was dissolved in dichloromethane/dimethyl formamide (1 + 1 v/v, 6 mL) and cooled to 0 °C. Subsequently, DIC (2 equivalents) was added with stirring for 15 min followed by allowing the solution to warm to room temperature and stirring for further 10 min at room temperature until the insoluble urea derivative precipitated. The suspension was filtered and the filtrate reacted with the resin bound hexaglutamate α-*tert*-butyl ester (1 equivalent) for 6 h at room temperature. Subsequently, the resin was filtered and washed successively 5 times with dichloromethane, *N*-methyl-2-pyrrolidone, and dichloromethane (5 mL each) and then dried in an desiccator in vacuo.

2.2.3.3. Synthesis of Pteroyl-[$^{13}C_5$]-(Glu-OtBu)$_7$. After deprotection of the resin-bound Fmoc-heptapeptide as detailed before, HOBt (2 equiv) was added to trifluoroacetyl pteroic acid (2 equiv), and the mixture was dissolved in dichloromethane/dimethyl formamid (1 + 1 v/v, 6 mL) and cooled to 0 °C. Subsequently, DCC (2 equiv) was added with stirring for 15 min followed by allowing the solution to warm to room temperature and stirring for further 10 min at room temperature until the insoluble urea derivative precipitated. The suspension was filtered and the filtrate reacted with the resin bound heptaglutamate α-*tert*-butyl ester (1 equivalent) for 6 h at room temperature.

2.2.3.4. Decoupling from the Resin. The resin-bound product was stirred with a mixture of dichloromethane and glacial acetic acid (9 + 1, v/v) for 30 min at room temperature. After filtering, the resin was washed with dichloromethane, and the dichloromethane phases were collected and rotary evaporated.

2.2.4. Final Deprotection. The residue was reacted with aqueous sodium hydroxide (0.01 mol/L, pH 12) for 18 h, while maintaining a pH of 10 by further addition of aqueous sodium hydroxide (0.01 mol/L, pH 12). The solution was tested by LC-MS for completeness of deprotection and finally lyophilized.

2.3. Solutions. For UV spectrometry, solid folate vitamers were dissolved in phosphate buffer (0.1 mol/L, pH 7.0), phosphate buffer (0.1 mol/L, pH 7.0) containing 0.2 mol/L 2-mercaptoethanol, or hydrochloric acid (0.1 mol/L, pH 1.0)

The extraction buffer consisted of aqueous MES (200 mmol/L) containing ascorbic acid (20 g/L) and 2-mercapto ethanol (200 mmol/L) adjusted to pH 5.0. The buffer was prepared on the day of use. The phosphate buffer (100 mmol/L) was prepared by dissolving sodium dihydrogen phosphate (100 mmol) in water (1 L) and adjusting the solution with dipotassium hydrogen phosphate (100 mmol) in water (1 L) to pH 7.0. The eluting solution was a mixture of aqueous sodium chloride (5%) and aqueous sodium acetate (100 mmol/L) containing ascorbic acid (1%).

The chicken pancreas suspension was prepared by stirring chicken pancreas powder (5 mg) in diluted aqueous phosphate buffer solution (30 mL, 10 mmol/L) containing 1% ascorbic acid and adjusted to pH 7.

To determine the limits of detection (LOD), limits of quantification (LOQ), and recoveries, a recombinant of bread was developed. This synthetic bread consisted of lyophilized egg white (1.78 g) as the protein component, sunflower oil (0.27 g), wheat starch (11.63 g), cellulose (1.28 g), and NaCl (0.4 g).

2.4. Extraction of Food Samples. Foods were lyophilized. Aliquots (40 mg) were taken from the resulting powder, spiked with [$^2H_4$]-5-methyltetrahydrofolic acid (50 ng), [$^2H_4$]-5-formyltetrahydrofolic acid (25 ng), [$^2H_4$]-tetrahydrofolic acid (75 ng), [$^2H_4$]-10-formylfolic acid (50 ng), and [$^2H_4$]-folic acid (50 ng). The spiked powder was then overlaid with 2 mL of extraction buffer and digested with protease (50 μg/mg sample, no endogenous folates detectable) for 4 h at 37 °C while being constantly agitated. After enzyme digestion, the samples were heated at 100 °C for 10 min, cooled on ice, then spiked with rat serum (150 μL) and chicken pancreas suspension (2 mL, endogenous folate content of rat serum and chicken pancreas: 11 pmol 5-methyltetrahydrofolate in total per assay). After deconjugase treatment of the samples at 37 °C and constant stirring overnight, the samples were heated at 100 °C for 10 min and then centrifuged at 16100*g* for 15 min at 4 °C. After passing the supernatant through a syringe filter (0.45 μm, Millipore, Bedford, MA, USA), the filtrates were subjected to solid phase extraction cleanup as described below.

2.5. Sample Cleanup by Solid-Phase Extraction (SPE). Extracts were purified by SPE using a 12-port vacuum manifold (Merck, Darmstadt, Germany) equipped with Discovery SAX cartridges (quaternary amine, 500 mg, 3 mL, Sigma, Deisenhofen, Germany). The cartridges were successively activated with 2 volumes of hexane, methanol, and diluted aqueous phosphate buffer (10 mmol/L adjusted to pH 7.0 containing 0.2% 2-mercaptoethanol).

After application of sample extracts, the columns were washed with three volumes of diluted aqueous phosphate buffer. Subsequently, the cartridges were dried by vacuum suction and the folates eluted with 2 mL of eluting solution.

In accordance with the recently reported SIDA for plasma folates,[16] SPE cleanup on SAX cartridges showed the best recovery compared to other SPE alternatives such as phenyl phases.

2.6. LC-MS/MS. The samples (10 μL) were chromatographed on a Finnigan Surveyor Plus HPLC System (Thermo electron corporation, Waltham, USA) equipped with a nucleosil C-18 reversed phase column (250 × 3 mm; 4 μm; Phenomenex, Aschaffenburg, Germany) connected to a diode array detector and a triple quadrupole TSQ quantum discovery mass spectrometer (Thermo electron corporation, Waltham, USA).

The mobile phase consisted of a variable mixture of 0.1% aqueous formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B) at a flow of 0.3 mL/min. Gradient elution started at 0% B, followed by raising the concentration of B linearly to 10% within 2 min and to 25% within a further 23 min. Subsequently, the mobile phase was programmed to 100% B within a further 2 min and held at 100% B for 3 min before equilibrating the column for 14 min with the initial mixture.

During the first 11 min of the gradient program, the column effluent was diverted to waste. The spectrometer was operated in the positive electrospray mode using selected-reaction monitoring (SRM) with the mass transitions recently reported.[16] For monitoring [$^{13}C_5$]-PteGlu and [$^{13}C_5$]-pteroylheptaglutamate, the precursor ions were set at m/z 447.00 and 611.00, the both product ions at m/z 295, and the collision energies at 19.0 and 22.0%, respectively. For monitoring 5,10-methenylH_4folate and 10-formylH_2folate, the precursor ions were set at m/z 456.00 and 472.00, the product ions at m/z 412.00 and 297.00, and the collision energies at 27.0 and 19.0%, respectively. The spray voltage was set to 3900 V, capillary temperature to 320 °C. and the capillary voltage to 35 V.

2.7. UV Spectroscopy. The purity of folate solutions were checked by an UV spectrometer UV-2401 PC (Shimadzu, Kyoto, Japan) and also by RP-HPLC-DAD. The samples (10 μL) were chromatographed on an Elite La Chrome L-2130 HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a nucleosil C-18 reversed phase column (250 × 3 mm; 4 μm; Phenomenex, Aschaffenburg, Germany) connected to a diode array detector L-2450 (Merck Hitachi, Darmstadt, Germany) using the gradient elution as detailed in the LC-MS/MS section.

First, the λ_{max} values were determined by measuring the folate solutions with the UV spectrometer and second, the absorption coefficients (Table 1) were determined by measuring five different concentrations at the determined λ_{max}. The absorption coefficients were calculated as the mean of the different concentrations.

Table 1. UV−Vis Absorption Coefficients of Folates in Different Buffers[a]

	ε [m^2/mol]	± SD [m^2/mol]	λ [nm]	buffer
folic acid	2762	±179	282	ME
H_4folate	2771	±65	299	ME
5-CH_3-H_4folate	2614	±99	290	PP
	2371	±121	290	ME
5- CH_3-H_2folate	2714	±54	290	ME
5-HCO-H_4folate	2314	±49	288	ME
5,10-methenyl- H_4folate	1717	±56	259	PP
	1554	±120	259	ME
	985	±79	282	HCl
	2045	±113	354	HCl
5,10-methylen- H_4folate	2386	±36	296	PP
	2373	±68	297	ME
10-HCO-PteGlu	2266	±178	267	PP
	614	±56	350	PP
	2487	±201	252	HCl
	817	±22	322	HCl
10-CH_3-folic acid	2573	±54	305	ME
	2352	±45	309	HCl
$PteGlu_7$	2310	±70	282	PP
	661	±29	347	PP

[a]ME: 0.1 mol/L phosphate buffer containing 0.2 mol/L 2-mercaptoethanol (pH 7.0). PP: 0.1 mol/L phosphate buffer (pH 7.0). HCl: 0.1 mol/L hydrochloric acid (pH 1.0). SD: standard deviation of ε measured at five different concentrations.

Phosphate buffer was used for those vitamers that are sufficiently stable without the addition of an antioxidant. For, H_4folate and its derivatives, 2-mercapto ethanol has to be added to confer stability during the measurement and further handling of the solution. In contrast to this, folates substituted at N10 were dissolved and measured in hydrochloric acid (0.1 mol/L) as this solvent produces for

Table 2. Response Curves of Folates and the Calibration Curve of [$^{13}C_5$]-PteGlu$_7$[a]

analyte (A)	standard (S)	linear equation using simple linear regression	R^2 for simple linear regression	linear equation using weighted regression, weighting factor $1/y^2$	MS/MS transition analyte	MS/MS transition standard	linear range n(A)/n(S) min	linear range n(A)/n(S) max
folic acid	[$^2H_4$]-folic acid	y = 2.4944 x(S)- 0.0499	0.9996	y = 2.4488 x(S)+ 0.0111	442→295	446→299	0.11	10.919
H_4folate	[$^2H_4$]-H_4folate	y = 1.9793 x(S)+ 0.0309	0.9996	y = 2.0454 x(S)- 0.0161	446→299	450→303	0.10	10.25
5-CH_3-H_4folate	[$^2H_4$]-5-CH_3-H_4folate	y = 0.7984 x(S)- 0.0388	0.9999	y = 0.7805 x(S)- 0.0059	460→313	464→317	0.25	25.34
5-HCO-H_4folate	[$^2H_4$]-5-HCO-H_4folate	y = 0.8527 x(S)- 0.0359	0.9998	y = 0.8478 x(S)- 0.0215	474→327	478→331	0.14	14.18
10-HCO-PteGlu	[$^2H_4$]-10-HCO-PteGlu	y = 2.8383 x(S)- 0.2622	0.9995	y = 2.5564 x(S)- 0.037	470→295	474→299	0.10	9.94
[$^{13}C_5$]-PteGlu	[$^2H_4$]-folic acid	y = 2.5458 x(S)- 0.0345	0.9996	y = 2.5458 x(S)- 0.0345	447→295	446→299	0.04	341
[$^{13}C_5$]-PteGlu$_7$	[$^2H_4$]-folic acid	y = 0.133 x(S)+ 0.0544	0.9999	y = 0.133 x(S)+ 0.0544	611→295	446→299	1.5	24.06

[a] $y = \frac{A(A)}{A(S)}$, $x = \frac{n(A)}{n(S)}$.

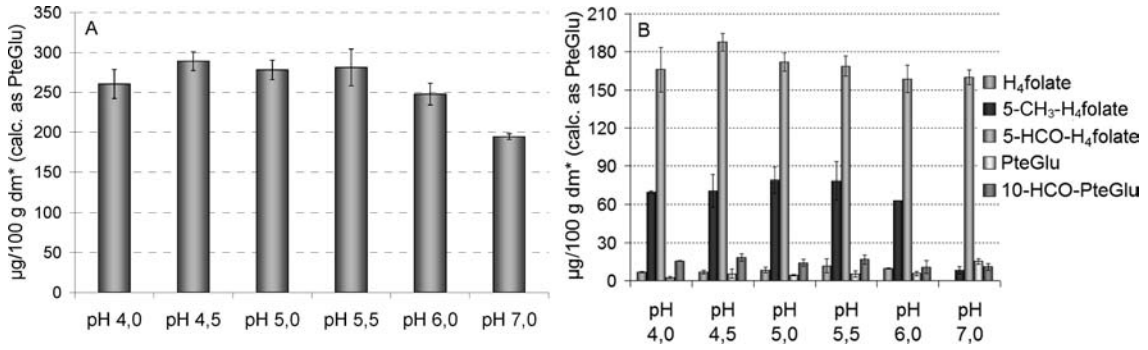

Figure 1. Sum (A) and distribution (B) of folates in mung beans after extraction at different pH (*dm: dry mass).

these vitamers more pronounced maxima. The absorption coefficients were used to determine the concentration of pure stock solutions of labeled and unlabeled folates.

2.8. Calibration and Quantitation. Solutions of deuterated folates as standards (S) were mixed with the respective analytes (A) in 10 molar ratios [n(S)/n(A)] between 1:20 and 5:1 (absolute amounts in mmol: 0.05:1; 0.1:1; 0.2:1; 0.5:1; 1:1; 2:1; 5:1) for H_4folate, 5-formylH_4folate, PteGlu, 10-formyl-PteGlu, 5-methylH_4folate, [$^{13}C_5$]-folic acid, and [$^{13}C_5$]-pteroylheptaglutamate and diluted with elution buffer to obtain a total concentration of 0.1 µg/mL (sum of analyte and internal standard) before LC-MS/MS analysis. For [$^{13}C_5$]-folic acid and [$^{13}C_5$]-pteroylheptaglutamate, [$^2H_4$]-folic acid was used as the internal standard. All concentrations of solutions containing standards or analytes were verified by UV spectroscopy. After mixing, the solutions were measured by LC-MS/MS, and peak area ratios [A(S)/A(A)] were determined. Calibration functions (Table 2) by using all n(S)/n(A) values for each standard/analyte combination were calculated from the obtained A(S)/A(A) ratio using either simple linear regression or weighted linear regression with a weighting factor of $1/y^2$ according to Almeida et al.[17] Stability of response was regularly checked by measuring a randomly chosen n(S)/n(A) value in the linear range.

2.9. Limits of Detection (LODs) and Quantification (LOQs). LODs and LOQs for folates were determined according to Vogelgesang and Hädrich.[18] As blank matrices for foods, a synthetic bread model consisting of lyophilized egg white, sunflower oil, wheat starch, cellulose, and salt was used. LC-MS/MS analysis confirmed that the blank matrix only contained residual 5-methylH_4folate. As for the latter, no folate-free matrix could be found, and extraction buffer was used as the matrix. For the determination of LODs and LOQs, the matrices were spiked (each in triplicate) with four different concentration levels of H_4folate (1.9−19 pmol), 5-HCO-H_4folate (5.5−55 pmol), 10-HCO-PteGlu (2.3−23 pmol), PteGlu (2.9−29 pmol), and 5-CH_3-H_4folate (1.4−14 pmol). After the addition of the respective labeled internal standards, all samples underwent sample preparation and cleanup as described above and were finally analyzed by LC-MS/MS. LODs and LOQs were derived statistically from the data according to a published method.[18]

2.10. Precision. Inter-assay precision was determined by analyzing samples three times in triplicate during 4 weeks.

2.11. Recoveries of Stable Isotope Dilution Assays. Blank synthetic bread and blank extraction buffer (2 mL) were spiked (each in triplicate) with three different amounts of H_4folate (4.0−20 pmol), 5-formylH_4folate (10−50 pmol), PteGlu (6.0−30 pmol), 10-formyl-PteGlu (4.0−20 pmol), and 5-methylH_4folate (3.0−20 pmol), respectively, and were analyzed by stable isotope dilution assay. The recovery was calculated as the mean of the addition experiments.

3. RESULTS AND DISCUSSION

3.1. Effects of pH on Folate Deconjugation. The previously reported SIDA for food folates[19,20] was based on extraction with HEPES/CHES buffer at pH 7.85 due to the stability of folates. As only monoglutamates are used as labeled standards for folate quantitation by LC-MS/MS, all endogenous folates have to be transformed to the respective monoglutamates, which is achieved by γ-glutamylhydrolases, commonly called deconjugases. According to the literature, the applied conjugases show pH optima ranging between 4.1[21] to 8.5.[22] Therefore, it appeared reasonable to test different pH conditions to obtain a maximum of monoglutamates with the used enzyme combination of chicken pancreas and rat plasma.

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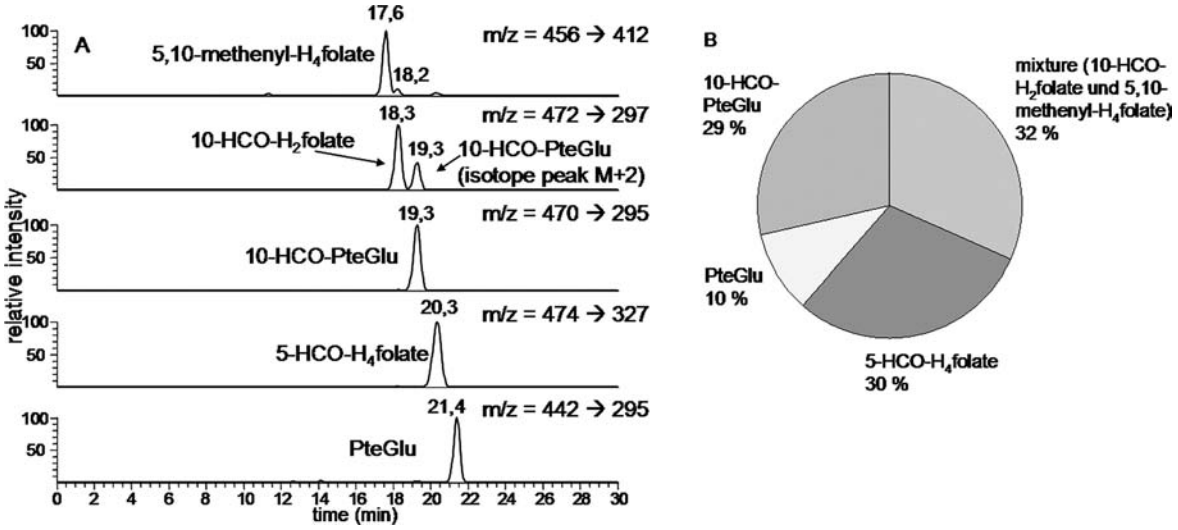

Figure 2. Interconversion products of a mixture of 5,10-methenylH_4folate and 10-HCO-H_2folate after extraction at pH 5.0. (A) LC-MS/MS chromatogram. (B) Molar distribution.

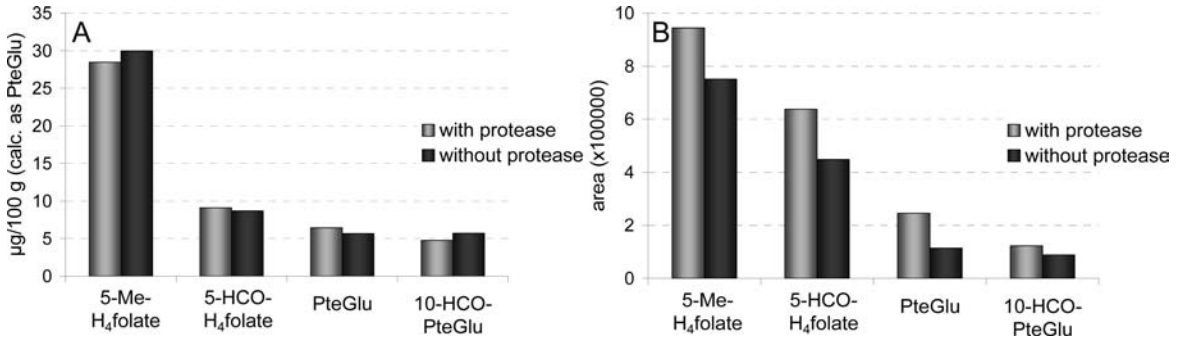

Figure 3. Peak areas (A) and amounts of folates (calculated as PteGlu in 100 μg/g) of camembert cheese extracted with or without additional protease.

As test foods particularly rich in folates, mung beans, wheat germ, and camembert cheese were used.

The studies revealed the highest total folate contents in the range between pH 4.5 and 5.5, which was mainly due to the highest yields for 5-methylH_4folate and 5-formylH_4folate (Figure 1). These improvements obviously were attributable to optimized deconjugation efficiency and improved stability of folates. Therefore, further extractions were performed at pH 5.0 using the best suitable buffer consisting of 4-morpholineethanesulfonic acid (MES).

3.2. Behavior of Labile Folate Vitamers. Folate analysis is mainly restricted to the five major monoglutamic forms H_4folate, 5-formylH_4folate, PteGlu, 10-formyl-PteGlu, 5-methylH_4folate. However, these more labile vitamers have been described to occur in foods. Of the latter, in particular 10-formylH_4folate and 5,10-methenylH_4folate are known to be easily oxidized or to interconvert to other folate forms. To test this behavior during the developed SIDA, the reference compound 5,10-methenylH_4folate was reacted to 10-formylH_4folate in sodium hydroxide according to Stover and Schirch.[23] The HPLC-UV separation on a Hyperclone RP-18 column revealed three peaks (Supporting Information), which were tentatively assigned to 10-formylH_2folate, 10-formylH_4folate, and 5,10-methenylH_4folate by LC-MS. Interestingly, 10-formylH_4folate revealed upon positive ESI only a minor signal of the protonated molecule and a base peak at m/z 137, which can be attributed to 4-aminobenzoylamide. Obviously, this compound already fragments in the ion source of the applied MS equipment. The two other peaks could be clearly assigned by their protonated molecules. The generation of the oxidation product 10-formylH_2folate in the synthetic reaction mixture was suppressable by the addition of mercapto ethanol, but a 90% yield as reported by Stover and Schirck[23] was not achievable at all.

This result clearly indicated that 10-formylH_4folate is not directly detectable by LC-MS. However, according to Gregory et al.[24] this compound upon heating at pH 4.9 completely converts to 5-formylH_4folate and, therefore, is quantifiable as the sum with the endogenously occurring 5-formylH_4folate by the presented SIDA. Regarding the other folates not yet included in this SIDA, namely, 10-formylH_2folate, and 5,10-methenylH_4folate, we reacted a mixture of the latter at the conditions during extraction and obtained a vitamer distribution depicted in Figure 2. From these results, it could be deducted that 68% of the products would be detected within the existing SIDA as 5-formylH_4folate, 10-formylfolate, and PteGlu. The remaining 32% will remain as the initial mixture of 10-formylH_2folate, and 5,10-methenylH_4folate and will have to be monitored in the existing method in the SRM traces m/z 472/297 and m/z 456/412, respectively. In the case of significant signals, these two compounds would have to be

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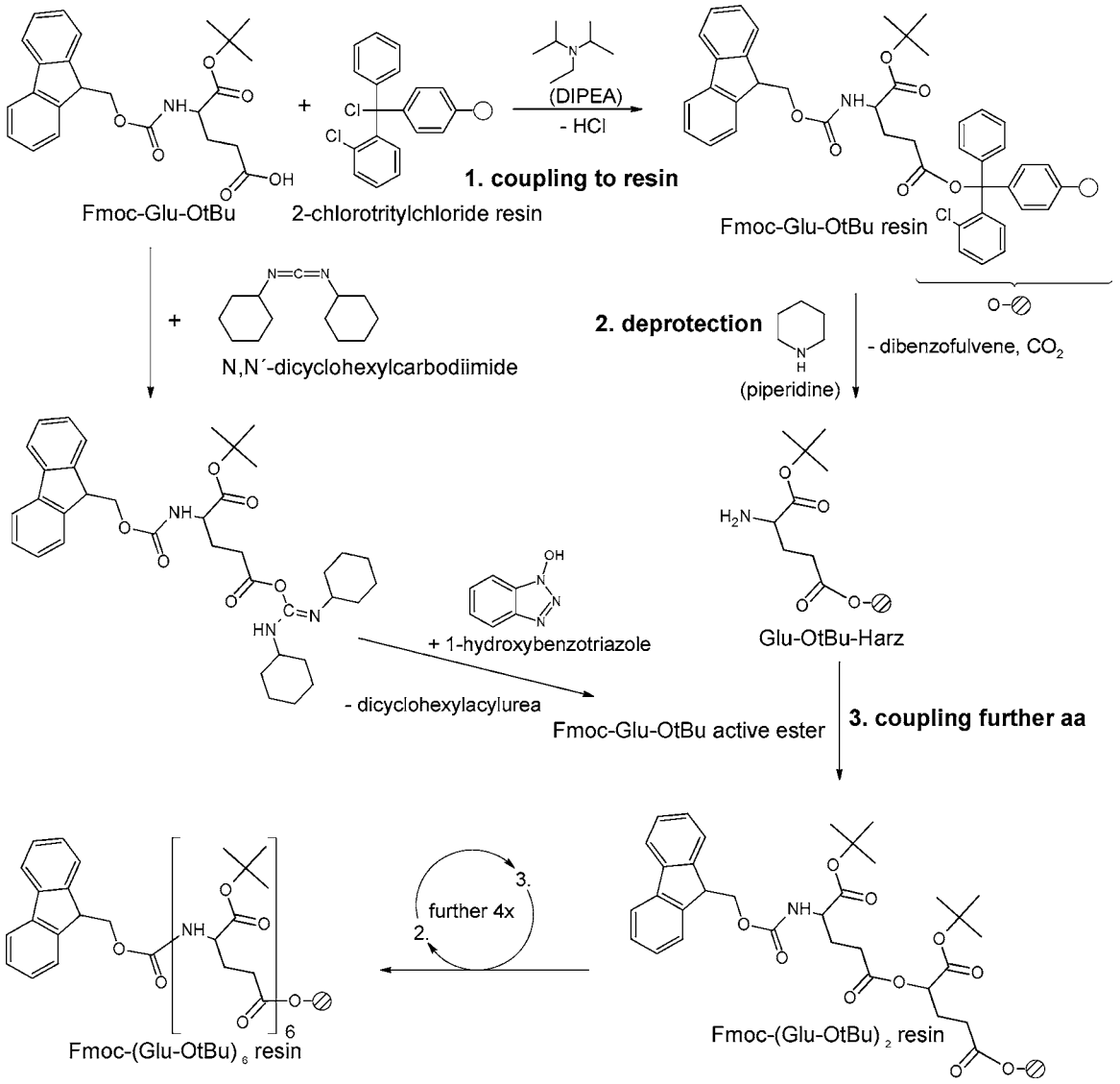

Figure 4. Solid phase synthesis of Fmoc-$(Glu\text{-}OtBu)_6$: 1, Coupling to the resin; 2, deprotection of the amino group; 3, coupling of an active ester of Fmoc-Glu-OtBu (aa: amino acid).

quantified by using suitable deuterated vitamers as IS. A respective study currently is under way.

3.3. Protease Treatment. Besides deconjugation, protease treatment is recommended to degrade the protein matrix and to liberate folates that are entrapped or bound to proteins. For camembert cheese, the effect of protein treatment was tested. A comparison of SIDAs with and without the application of protease is shown in Figure 3 and revealed no significant differences of the calculated amounts for all vitamers. However, when comparing signal intensities of both assays, protease treatment gave higher peak areas. Therefore, it can be concluded that with protease treatment liberation or equilibration between IS and the analytes is not improved, but degradation of proteins might lower matrix effects during subsequent cleanup or detection, and, therefore, leads to increased sensitivity. From this point of view, the use of protease can be recommended.

3.4. Synthesis of Labeled Pteroylheptaglutamate. As food folates are present mainly as polyglutamatic forms, and the SIDA is only able to detect monoglutamates, deconjugation has to be assumed complete for an accurate analytical result. However, until now this only has been tested in separate assays after the addition of a polyglutamate, e.g., pteroyltriglutamate, and determining the yield of the additionally formed folic acid. A more convenient and accurate approach would be to monitor the deconjugation of polyglutamates in the same assay along with quantitation of monoglutamates. For tracing complete deconjugation, on the one hand, the applied polyglutamate, i.e., the addition tracer isotopologue, has to contain the possible maximum of glutamates, i.e., a hepta or an octaglutamate has to be used. However, the resulting monoglutamate, i.e., the detection tracer isotopologue, has to be distinguishable from the endogenously occurring monoglutamates and the isotopologues used as IS for quantitation. As $[^2H_4]$-folates were applied as IS, we chose $[^{13}C_5]$-folic acid labeled in the glutamate residue as the detection tracer isotopologue. As the addition tracer isotopologue, we considered attaching six glutamate residues to the detection tracer isotopologue, which would give $[^{13}C_5]$-pteroylheptaglutamate as the target for synthesis. The synthetic route (Figure 4) started with the generation of an unlabeled hexaglutamate peptide bound to a resin, which was then

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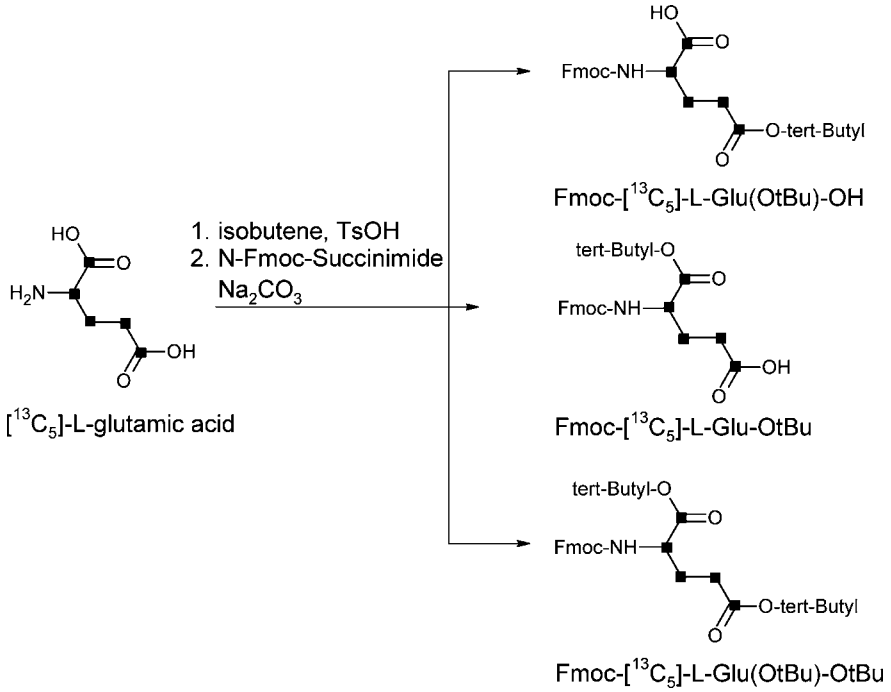

Figure 5. Synthetic route to Fmoc-[$^{13}C_5$]-Glu-OtBu (Fmoc, 9-fluorenylmethyloxycarbonyl; TsOH, *p*-toluenesulfonic acid); ■ = ^{13}C.

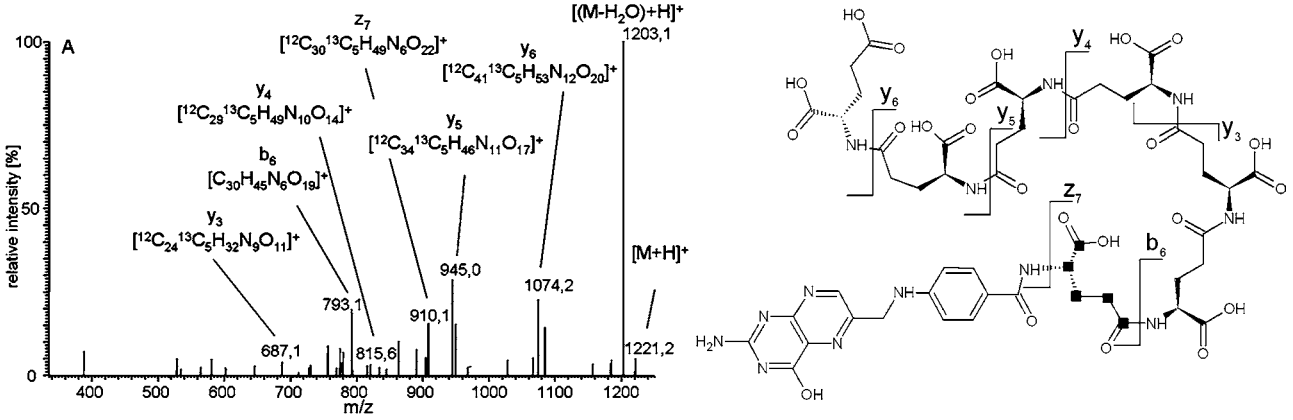

Figure 6. LC-MS/MS spectrum and fragmentation pattern of [$^{13}C_5$]-PteGlu$_7$. (A) LC-MS/MS m/z 1221 → m/z 335−1250 (ESI$^+$, CE 27 V). (B) Structure with fragmentation sites denominated according to the abc or xyz system; ■ = ^{13}C.

coupled to [$^{13}C_5$]-glutamate, and subsequently, the resulting labeled heptaglutamate was bound to pteroic acid.

As in tandem MS the glutamate residue is lost, the detection tracer isotopologue [$^{13}C_5$]-folic acid should be distinguishable from the IS by its differing product ion and from unlabeled folic acid by its different precursor ion.

The synthesis of resin-bound hexaglutamate was achieved by first reacting fluorenylmethoxycarbonyl (Fmoc) protected glutamate *α-tert*-butyl ester with chlorotrityl activated resin followed by Fmoc deprotection and coupling with DCC-activated Fmoc protected glutamate *α-tert*-butyl ester. After deprotection, the latter procedure was repeated until Fmoc-protected and resin-bound hexaglutamate was obtained. For subsequent coupling with *α-tert*-butyl [$^{13}C_5$]-glutamate, the latter had to be protected with Fmoc and *tert*-butylated at the *α* carboxyl moiety, the latter of which could not be prepared selectively (Figure 5). Therefore, the mixture of *α tert*-butyl and *γ tert*-butyl [$^{13}C_5$]-glutamate had to be separated by preparative HPLC. Thereafter, the protected [$^{13}C_5$]-glutamate and trifluoroacetylpteroate were successively coupled to hexaglutamate, and final deprotection gave the target compound.

3.5. Mass Spectrometric Studies of [$^{13}C_5$]-Pteroylheptaglutamate. For characterization of the synthesized heptaglutamate, an ion trap LC-MS/MS spectrum of the protonated molecule was recorded (Figure 6) (ESI$^+$ $[M + H]^+$ m/z = 1221.2, $[(M\text{-}H_2O) + H]^+$ m/z = 1203.1, [y_6-fragment]$^+$ m/z = 1074.2, [y_5-fragment]$^+$ m/z = 945.0, [z_7-fragment]$^+$ m/z = 910.1, [y_4-fragment]$^+$ m/z = 815.6, [b_6-fragment]$^+$ m/z = 793.1, [y_3-fragment]$^+$ m/z = 687.1) . The y fragments y_3 to y_6 are attributable to cleavage of the peptide bonds with a resulting charge at the carbonyl carbon. In contrast to this, upon loss of a hexaglutamate, only the latter was detectable as the b_6 fragment ion. Besides the y and the b fragments, further cleavage between the *α* carbon and the amine moiety yielded the z_7 fragment with the charge at the C- terminus upon loss of pteroic acid.

3.6. Validation Data of Dual Label Isotope Dilution Assay. The applied stable isotope dilution assay allowed

unequivocal identification and quantitation of all folate vitamers along with inherent verification of deconjugation efficiency. Detection of the single substances was unambiguous, as complete chromatographic separation was achieved (Figure 2), and coelution of isotopologic deuterated standards confirmed their identity.

Calibration of the stable isotope dilution assays was performed by measuring mixtures of unlabeled analytes and labeled standards in different ratios. For each analyte, two regression curves were calculated, one using simple linear regression and the other using weighted regression with a weighting factor of $1/y^2$ according to Almeida et al.[17] The latter approach was considered as data from LC-MS are reported to be heteroscedastic. However, as can be seen from Table 2, the two functions were very similar.

For determining LOD and LOQ in foods, an almost folate free matrix could only be obtained by preparing a synthetic bread model for all folates except 5-methylH_4folate, which was detectable in all natural matrices analyzed. Therefore, we used extraction buffer as the matrix for the latter.

By using the synthetic bread matrix, we determined the LOD and LOQ data given in Table 3 resulting from the calibration with weighted regression. Interestingly, when using the data from simple linear regression, almost identical LOD and LOQ data were obtained at a difference much lower than 1%. These results allow the conclusion that heteroscedascity can be neglected in this case.

Table 3. Validation Data for the New Stable Isotope Dilution Assay

compd	LOD [pmol/assay] (n = 3)	LOQ [pmol/assay] (n = 3)	recovery (±SD) [%] (n = 3)	precision [% RSD] (n = 3) intra assay	precision [% RSD] (n = 3) inter assay
H_4folate	2.0	4.0	105 ± 14	8	3
5-CH_3–H_4folate	1.4	2.8	101 ± 16	9	6
10-HCO-PteGlu	2.1	4.2	103 ± 8	8	4
5-HCO-H_4folate	5.6	11.3	101 ± 21	5	2
PteGlu	3.0	6.0	98 ± 18	4	4

The recoveries (Table 3) for the complete stable isotope dilution assays for all folates were not significantly different from 100% (t test, p = 0.05). These recoveries included inherent correction for losses during the whole procedure. In contrast to this, lower absolute recoveries of 48%, 37%, 32%, 67%, and 88% for folic acid, H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, and 10-HCO-PteGlu, respectively, were measured due to incomplete extraction when the labeled internal standards were added after the extraction step. For SIDA, these losses are compensated for as the internal standards equilibrate with the analytes, and quantitative extraction is not essential.

3.7. Precision of the New Assays for Folates. Precision of real sample analyses was evaluated in an intra-assay study of samples of different foods analyzed several times within one day and in an inter-assay study of the foods analyzed on several days within four weeks. All analyses were performed in triplicate. The results of the precision studies are given in Table 3. Relative standard deviation did not exceed 9% and 6% for the intra-assay and the inter-assay study, respectively.

3.8. Overall Testing of the Improved Method and Application to Different Foods. Along with the [$^2H_4$]-labeled monoglutamates, the synthesized polyglutamate was added during the extraction of foods and underwent enzyme treatment and SPE in parallel to the endogenously occurring folates.

A final LC-MS/MS chromatogram of a dual SIDA of mung beans is shown in Figure 7. In the upper trace, the MRM transition 611/295 for [$^{13}C_5$]-pteroylheptaglutamate is shown and in the next lower trace the transition 447/295 for [$^{13}C_5$]-PteGlu, and the two lower traces reveal the usual ones for unlabeled PteGlu and [$^2H_4$]-PteGlu, respectively. In Figure 7A, referring to the signals of an unreacted standard mixture, [$^{13}C_5$]-pteroylheptaglutamate gives an intense signal, and [$^{13}C_5$]-PteGlu was neglectable. Incomplete deconjugation would be traceable by residual [$^{13}C_5$]-pteroylheptaglutamate and incomplete formation of [$^{13}C_5$]-PteGlu. The signal of [$^{13}C_5$]-PteGlu was detectable independently from the signals of unlabeled Pte Glu and [$^2H_4$]-PteGlu as was proven by the detection of response mixtures of all isotopologues.

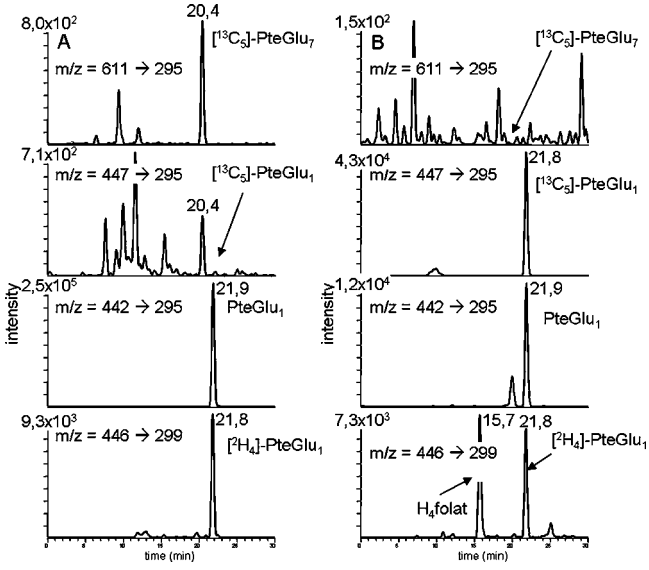

Figure 7. LC-MS/MS chromatogram of a standard mixture before deconjugation (A) and a deconjugated extract of mung beans (B).

The deconjugation tracer was applied to monitoring the analysis of a model solution consisting of [$^2H_4$]-PteGlu, of yeast, and of mung beans.

As can be seen from Table 4, [$^{13}C_5$]-PteGlu was quantified in the purified and deconjugated extracts, which revealed yields exceeding 90% of the added [$^{13}C_5$]-pteroylheptaglutamate. This study confirmed an almost complete deconjugation during sample preparation and the decisive improvement compared to the previously used SIDA,[19] which applied a HEPES/CHES buffer for extraction. Using the improved method presented here, we quantitated folates in several foods (Table 5). These data are intended to be used as the basis for bioavailability studies as the follow-up of a pilot study.[25]

Table 4. Comparison of Deconjugation Efficiency of the Extraction Procedure According to Freisleben et al.[12] with the Optimized Procedure

	$[^{13}C_5]$-PteGlu$_1$ nmol absolute	deconjugated $[^{13}C_5]$-PteGlu$_7$
Extraction According to Freisleben et al.[12]		
model	0.0515	86%
yeast	0.0517	86%
mung beans	2.1	84%
Optimized Extraction		
model	0.0541	90%
yeast	0.0579	97%
mung beans	2.5	100%

Table 5. Total Folate Contents of Different Foods Analyzed by the Optimized Procedure Calculated as μg Pteroyl Glutamate/100g

foods (no. of different samples)	total folate μg/100 g
spinach (n = 2)	105−128
soy beans, dry seeds (n = 1)	290
mung beans, dry seeds (n = 1)	27
camembert cheese (n = 3)	49−286
Edamer cheese (n = 2)	41−43
barley, dry kernels (n = 1)	110
toast bread (n = 1)	23
wheat germs (n = 1)	471
yeast, dry (n = 1)	2210

■ ASSOCIATED CONTENT

Supporting Information

LC-UV chromatogram of 10-HCO-H_4folate obtained from commercial 5,10-methenylH_4folate by treatment with sodium hydroxide[12] and full scan MS (ESI+ m/z = 150−600) of the respective peaks. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

5-CH_3-H_4folate, 5-methyltetrahydrofolate; DCC, *N,N′*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIPEA, *N,N′*-diisopropylethylamine; DMF, dimethylformamide; 5-HCO-H_4folate, 5-formyltetrahydrofolate; 10-HCO-PteGlu, 10-formylfolate; H_4folate, tetrahydrofolate; HOBt, 1-hydroxybenzotriazole hydrate; LOD, limit of detection; LOQ, limit of quantification; MES, 4-morpholineethanesulfonic acid; NMP, *N*-methylpyrrolidone; PteGlu, folic acid; RSD, relative standard deviation; SD, standard deviation; SIDA, stable isotope dilution assay

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