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Determination of Five Folate Monoglutamates in Rodent Diets

Filip Kiekens,[†] Jeroen Van Daele,[†] Dieter Blancquaert,[‡] Dominique Van Der Straeten,[‡] Willy E. Lambert,[†] and Christophe P. Stove*[,†]

[†]Laboratory of Toxicology, Ghent University, Ottergemsesteenweg 460, B-9000 Gent, Belgium

[‡]Laboratory of Functional Plant Biology, Department of Physiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

Supporting Information

ABSTRACT: A method for the quantitative determination of folates in rodent diets is very important for correct interpretation of folate intake during feeding trials, given the possible discrepancy between the actual folate concentration in the diet and that mentioned on the product sheet. Liquid chromatography tandem-mass spectrometry is the method of choice to differentiate and quantify the individual folate species present. This discrepancy may be accounted for by, e.g., inaccurate folic acid supplementation and/or the presence of endogenous reduced and substituted folates. We developed a method, validated based on FDA guidelines, that allows the measurement of added and endogenous folates by quantitative determination of 5 folate monoglutamates with linear ranges from 8 μg to 2 mg/kg feed. This information, combined with feed intake data, allows insight into the actual folate intake in animal feeding studies. The relevance of this method was illustrated by the analysis of several feed samples of varying composition, by the investigation of the effect of casein incorporation, and by evaluating the variability of the folate content between pellets and production batches.

KEYWORDS: *folate, rodent diet, liquid chromatography, mass spectrometry*

INTRODUCTION

Over recent years several studies have investigated the bioavailability of folates from various food items. Deficiencies of these essential water-soluble B-vitamins (B9) have been related to several health disorders such as neural tube defects (NTDs),[1] neural damage,[2] and increased cancer risk.[3] Studies investigating the relationship between folate intake and health disorders, e.g., studies in which mice receive a folate-depleted diet to investigate the prevalence of NTDs,[4] typically use an amino acid based diet, supplemented with various amounts of folic acid. However, because of a variety of factors, such as limited chemical stability and the presence of endogenous folates in feed ingredients, the actual folate concentration may differ from the concentration mentioned on the product sheet. Also, folate concentrations may vary as a result of differences in production and storage conditions or feed formulation. Therefore, it is key to quantify the exact amount of folates ingested by the animals as determined in the diet at time of consumption.

Rodent diets used for animal studies can generally be subdivided into three categories. First, unrefined diets are composed of primary ingredients obtained from natural sources. Second, and most used, are purified diets in which the ingredients are purified or consist of refined forms of natural sugars, proteins, and oils as well as purified micronutrients. A third class of diets are composed of chemically pure ingredients such as peptides and reagent-grade micronutrients.[5] Although for open diets the exact formulation is known, this is not the case for a closed formula diet. However, its basic components remain the same and will include some form of protein, peptides, starches, sugars, fibers, oils, and fats. These ingredients are ground when not in powdered form, homogenized, and pressed into pellets.

Although folic acid is generally added to the rodent diet as a source of folate, the addition of natural food constituents such as plant starches and animal- or plant-based protein can result in the presence of endogenous folates.[6] The presence of these non-folic acid folates may impede a correct interpretation of short-term feeding trials due to a difference in absorption characteristics, as observed by Castorena-Torres et al.[7] We recently demonstrated that, during long-term studies, it is essential to precisely determine the folate content to accurately interpret the clinical data resulting from a specific folate intake.[8] Another factor possibly hindering correct interpretation of data from a feeding trial is the labile nature of folates. Because of oxidative and thermal degradation during production and transport, the folate content as specified by the manufacturer may deviate substantially from that actually consumed by the animals.[9]

Several methods have been developed for the analysis of other compounds in rodent diets, for instance, for quantification of estrogenic isoflavones[10] or the vitamins A and E.[11] To our knowledge, we are the first to report a method that not only allows the total folate content to be measured but also enables folate speciation in rodent feed. However, publications describing analytical methods for the analysis of folates in food items are plentiful. One such method for food analysis, i.e., the analysis of folates in potato,[12] features a trienzyme treatment

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Table 1. Concentration of Calibrators, Quality Controls, and Validation Samples[a]

sample	THF [μg/kg]	FA [μg/kg]	5MeTHF [μg/kg]	5,10CH$^+$THF [μg/kg]	10FoFA [μg/kg]	5FoTHF [μg/kg]
blank	0	0	0	0	0	0
calibrator 1	7.980	7.840	8.041	8.161	7.840	8.197
calibrator 2	23.94	23.52	24.12	24.48	23.52	24.59
calibrator 3	59.85	58.80	60.31	61.21	58.80	61.48
calibrator 4	147.6	145.0	148.8	151.0	145.0	151.6
calibrator 5	498.8	490.0	502.6	510.0	490.0	512.3
calibrator 6	997.5	980.0	1005	1020	980.0	1025
calibrator 7	1995	1960	2010	2040	1960	2049
QC1	39.90	39.20	40.20	40.80	39.20	40.99
QC2	718.2	705.6	723.7	734.5	705.6	737.7
LLOQ	7.980	7.840	8.041	8.161	7.840	8.197
3*LLOQ	23.94	23.52	24.12	24.48	23.52	24.59
MID	199.5	196.0	201.0	204.0	196.0	204.9
0.75*ULOQ	1496	1470	1508	1530	1470	1537

[a]THF, tetrahydrofolate; FA, folic acid; 5MeTHF, 5-methyltetrahydrofolate; 5,10CH$^+$THF, 5,10-methenyltetrahydrofolate; 10FoFA, 10-formylfolic acid; 5FoTHF, 5-formyltetrahydrofolate.

followed by ultrafiltration and ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis. Although a trienzyme treatment is indispensable,[13] ultrafiltration is not adequate for feed analysis due to the presence of loosely bound minerals, triglycerides, and proteins within the feed matrix. This can be solved by incorporation of a solid-phase extraction step to purify the raw samples, as was also used for specific food analyses.[14,15] Moreover, an optimized method should have a wide linear range to allow quantification of both high concentrations of folic acid, when added to the diet, and the endogenous folates, which are present at far lower concentrations.

MATERIALS AND METHODS

Reagents and Materials. Tetrahydrofolate (THF), 5-methyltetrahydrofolate (5MeTHF), 5,10-methenyltetrahydrofolate (5,10CH$^+$THF), 10-formylfolic acid (10FoFA), 5-formyltetrahydrofolate (5FoTHF), and folic acid (FA) were purchased from Schircks Laboratories (Jona, Switzerland). ^{13}C-labeled internal standards (ISs), with a labeling yield higher than 98%, were obtained from Merck Eprova (Glattbrugg, Switzerland). $^{13}C_5$−FA was used as internal standard for FA, 10FoFA, and 5FoTHF, while $^{13}C_5$-THF, $^{13}C_5$-5MeTHF, and $^{13}C_5$-5,10CH$^+$THF were used as ISs for their respective isotopologues. All calibrators and IS solutions were prepared in a final concentration of 100 μg/mL in a 50 mM sodium phosphate buffer, pH 7.4, containing 1% L-ascorbic acid (AA) and 0.5% DL-dithiothreitol (DTT)/methanol (MeOH) (50/50 v/v%). Deionized water (H_2O-MQ) was produced in house by means of a Synergy UV water-purification system from Millipore (Billerica, Massachusetts). Acetonitrile (ACN) and MeOH of LC-MS quality were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, phosphoric acid, and ammonium hydroxide were obtained from Merck & Cie. (Darmstadt, Germany). Formic acid, trisodium phosphate, AA, DTT, α-amylase (type Ia, 700−1400 U/mg protein; E.C. 3.2.1.1), and protease type XIV (>3.5 U/mg; E.C. 3.4.24.31) were purchased from Sigma-Aldrich (Diegem, Belgium). The protease powder was dissolved at a concentration of 5 mg/mL in deionized water. As a source of γ-glutamylhydrolase enzyme (GGH; E.C. 3.4.19.9), nonsterile, nonhemolyzed rat serum was obtained from Harlan Laboratories (Horst, The Netherlands), while lyophilized chicken pancreas powder was obtained from Pel-Freez Biologicals (Rogers, Arkensas). Both the protease solution and the rat serum were stirred on ice for 1 h with 100 mg/mL of activated charcoal (Sigma-Aldrich) to remove endogenous folates.[16] Following removal of the activated charcoal by centrifugation at 4500*g* for 15 min at 4 °C, the solutions were filtered over a 0.45 μm syringe filter (CA-S 30/0.45, Whatman, GE Healthcare, Little Chalfont, U.K.), divided into aliquots, and frozen at −20 °C. The chicken pancreas solution was prepared by dissolving 400 mg of powder in 25 mL of a 0.2 M phosphate buffer (pH 4.6), to which a suspension of 0.5 g of activated charcoal and 0.05 g of dextran in 5 mL of H_2O-MQ was added. After a 30 min incubation, centrifugation and filtration was performed as described for protease and rat serum, after which the pH was adjusted to 6.1 and the solution was aliquoted and frozen at −20 °C.[17] Solid-phase extraction (SPE) was performed using Bond Elut SAX tubes containing 500 mg of sorbent in 3 mL tubes, purchased from Agilent (Palo Alto, California).

Five different feed samples were used in this study. TD.06691 is a closed formula chemically pure diet in which casein is replaced by single peptides to remove all endogenous folates. The TD.95247 folic-acid-deficient diet is a purified diet in which the casein has been extracted with ethanol, whereas TD.94045 is an AIN-93G diet in which standard casein is used as a protein source, with folic acid added in a concentration of 2 mg/kg. The 7012 diet is an unrefined diet using primary ingredients without casein and 7 mg/kg added folic acid. To evaluate the influence of casein on the folate speciation in a rodent diet, both standard casein and ethanol-extracted casein were tested. All these samples were obtained from Teklad Diets (Harlan Laboratories, Madison, Wisconsin). As a standard unrefined diet using primary ingredients and casein, a rat and mice maintenance diet (Carfil Quality, Oud-Turnhout, Belgium) was used.

Calibrator and QC Matrix. The blank amino acid defined diet (TD.06691) was used to prepare both the calibrators and QC samples. This diet also contains 1% succinylsulfathiazole, which is used in many rodent feeding trials to limit the influence of intestinal bacteria. The concentration of calibrators, quality controls, and validation samples can be found in Table 1.

Method Optimization. To evaluate the optimal pH during deconjugation with rat serum (5.3−7.4), equal amounts of 5MeTHF di-, tri-, tetra-, and hexaglutamate (Schircks Laboratories) were spiked to blank rodent feed at a total concentration of 500 μg of 5MeTHF/kg while the pH was altered using an 8% phosphoric acid solution. The addition of stripped chicken pancreas extract, as a secondary source of GGH, or oxalic acid, to capture zinc ions that may inhibit deconjugation by rat serum deconjugase,[18] was also evaluated. Finally, the amount and/or incubation time of α-amylase (10, 20, or 50 μL; 15 or 30 min), protease (100 or 200 μL; 30, 60, or 120 min), and rat serum (100, 150, or 200 μL; 120 min) were evaluated by duplicate analysis.

Sample Preparation. All materials used were light-protected, and all manipulations were performed under subdued light from a yellow 15 W bulb. A homogeneous aliquot of 200 mg of rodent diet was weighed in a 15 mL polypropylene centrifuge tube. To this, 5 mL of a phosphate solution (50 mM sodium phosphate, 1% L-AA, 0.5% DTT,

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pH 7.4) including internal standards (ISs) at 250 μg/kg was added. After thorough mixing, the tubes were placed in a boiling water bath for 10 min followed by chilling on ice to room temperature.

Trienzyme Treatment. When at room temperature, 25 μL of the α-amylase solution was added to the suspension of rodent diet and phosphate solution, which was then incubated for 15 min at room temperature. A further incubation at 37 °C for 30 min was performed after addition of 100 μL of protease solution. After incubation, the sample was placed in a boiling water bath for 10 min, chilled on ice to room temperature, and centrifuged for 15 min at 4500*g* and 4 °C. While minimally disturbing the remainder of the rodent feed on the bottom and the fatty layer on top, 3.5 mL of supernatant was transferred to a new light-protected centrifuge tube. The pH was adjusted to 6 ± 0.15 by adding phosphoric acid. The sample was incubated for 2 h at 37 °C after the addition of 200 μL of stripped rat serum. After deconjugation, the pH was adjusted to 7.4, after which the samples were again placed in a boiling water bath for 10 min and subsequently chilled on ice. Prior to centrifugation for 15 min at 4500*g* and 4 °C, 3.25 mL of H_2O-MQ was added to reduce the ionic strength.

Sample Cleanup. SPE was performed using Bond Elut SAX tubes containing 500 mg of sorbent per 3 mL tube. The sorbent was activated with 3 mL of hexane followed by 3 mL of MeOH and conditioned with 4 × 2.5 mL of 1.22 mM sodium phosphate (pH 7.4). Next, 5.5 mL of the supernatant obtained after trienzyme treatment (cfr. Trienzyme Treatment) was loaded onto the sorbent. To remove interferences, the sorbent was washed twice with 1.5 mL of 25 mM sodium phosphate, 0.5% L-AA, 0.25% DTT, and 10% MeOH. The folates were eluted in two steps of 1 mL of 0.52 M ammonium acetate, 1% AA, 0.5% DTT, and 5% MeOH (pH 7.4).

LC-MS/MS Analysis. Samples were analyzed using a Waters Acquity UPLC system coupled to an ABSciex API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray probe. The autosampler was equipped with a 10 μL sample loop, and samples were kept at 4 °C prior to injection. After injection the needle was cleaned with 600 μL of 10/90 v/v% H_2O-MQ/ACN followed by 1200 μL of 90/10 v/v% H_2O-MQ/ACN. Chromatographic separation was achieved using a mobile phase composed of H_2O-MQ (A) and ACN (B), each containing 0.1% of formic acid, with a flow rate of 0.6 mL/min on a Waters HSS T3-column (150 mm × 2.1 mm; 1.8 μm, equipped with Vanguard precolumn) held at 60 °C. The composition of the mobile phase starts with 100% solvent A and was kept constant for 1 min. Thereafter the amount of solvent B was increased linearly to 10% in 2 min and to 12% in 1 min, after which the column was cleaned for 1 min with 95% solvent B and equilibrated to initial solvent composition for 3 min. This amounts to a total time of ∼8.5 min between injections. Mass spectrometric detection was performed in scheduled multiple-reaction monitoring mode. Electrospray ionization was used to ionize the analytes with the ion spray voltage set at 2500 V and a temperature of 600 °C. Nitrogen was used as curtain gas, gas 1, and gas 2, with respective pressure settings at 25, 75, and 90 psig, and as collision gas at setting 11. The interface heater was switched on. The transitions monitored per compound were the same as described before;[12] specific transitions and settings can be found in Tables S1A and S1B. In Figure 1 chromatograms of a blank diet, a blank diet spiked at LLOQ level, and a standard rodent diet are shown. The signal obtained for 5MeTHF in the blank diet originates from incomplete removal from stripped rat serum and is <20% of the LLOQ.

Validation. The method was validated based on the FDA guidelines for bioanalytical method validation. Selectivity, carry-over, precision, accuracy, linearity, matrix effect, recovery, and stability were evaluated.[19]

Selectivity and Carry-over. Method selectivity was tested using blank rodent diet (TD.06691) obtained from Harlan. This is a purified diet without casein, which should therefore contain no endogenous folates. Cross-interferences were evaluated by adding 6 folates and 4 labeled ISs individually to this diet in a concentration of 2 mg/kg. For the analytes, the acceptance criterion for the selectivity assessment was set at 20% of the peak area corresponding to the LLOQ level, whereas for the labeled ISs only 5% was considered acceptable. Carry-over was evaluated by injecting blank samples after calibrators with identical concentrations and acceptance criteria as for the evaluation of selectivity.

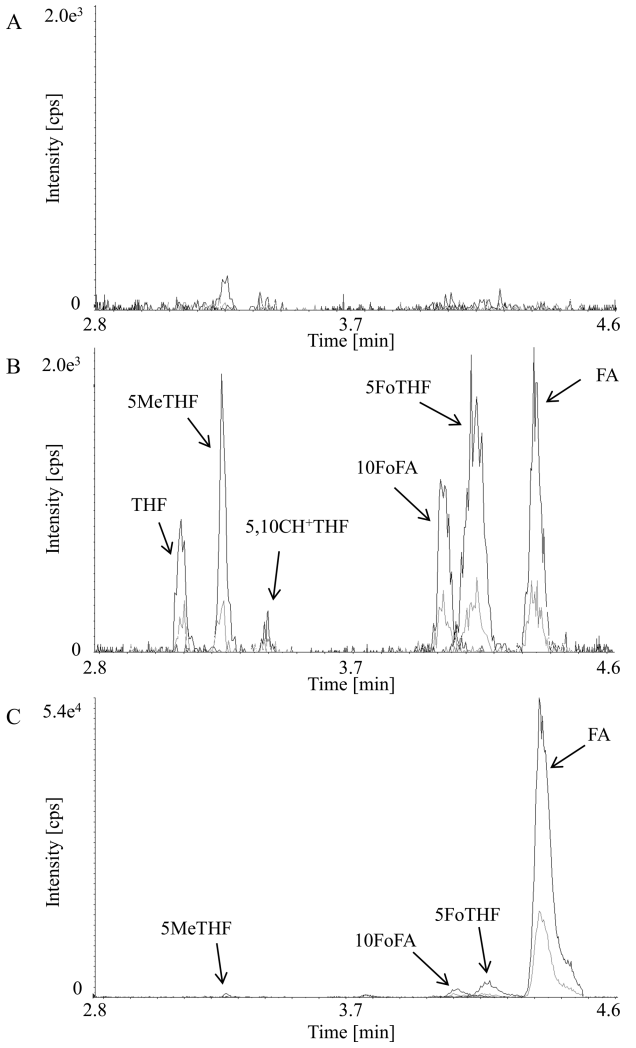

Figure 1. UPLC-MS/MS chromatogram (A) of a "blank" feed sample, (B) a blank feed sample spiked at LLOQ level, and (C) a sample of a standard rodent diet (Carfil). Please note the difference in scale of the *y*-axis in panel C compared to panels A and B.

Linearity, Accuracy, and Precision. The actual LLOQ was verified as the concentration that could be measured with a %bias and relative standard deviation (%RSD) below 20%. Duplicate analysis of 1 zero sample and 7 calibrators was performed on 4 nonconsecutive days to evaluate linearity. Homoscedasticity was evaluated by plotting the residuals versus the nominal values. The sum of the residual error was used to evaluate the need for a weighted or transformed model ($1/x$, $1/x^2$, $1/y$, $1/y^2$, $\sqrt{x}$, $\ln(x)$, $\log(x)$). For the selected model to be acceptable, back-calculated mean concentrations should be within 15% of the nominal value (20% at LLOQ-level). Accuracy and precision were evaluated using 4 spiked samples (LLOQ − 0.75*ULOQ, Table 1), prepared and analyzed in duplicate on 4 nonconsecutive days. A single-factor ANOVA was used to calculate intra- and interbatch variability (%RSD). Accuracy (%bias) was calculated as the measured value divided by the nominal value. Acceptance criteria for %bias and %RSD are 15%, except at LLOQ, where they are 20%. Also, the dilution integrity of samples exceeding the ULOQ was verified by 5-fold dilution of blank matrix spiked at a concentration of 8 mg/kg with sample buffer prior to solid-phase extraction (n = 2).

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Recovery and Matrix Effects. Matrix effect and recovery were evaluated at 3 concentration levels (3*LLOQ − 0.75*ULOQ, Table 1) in blank matrix material TD.06691 according to Matuszewski et al.[20] The analytes were spiked either before (A) or after (B) the extraction and SPE procedure. Also, analytes were spiked in SPE elution buffer to obtain a sample free of matrix (C). Absolute recovery was calculated as the percent ratio of peak areas A over B, while absolute matrix effect was calculated as the percent ratio of the peak areas B over C. Relative recovery and matrix effect are expressed as % RSD of the absolute recovery and matrix effect values and should not exceed 15%.

Stability. To investigate the stability of folates in rodent diets, six folate monoglutamates were spiked to blank matrix at a 100 and 1 000 μg/kg level. Triplicate analysis of samples stored at 4 or −20 °C was performed weekly over a total period of 4 weeks. Autosampler stability was assessed by reinjection of samples stored for 24 h in the autosampler while concentrations were calculated based on fresh calibrators. Also, the effect of up to three freeze−thaw cycles was evaluated by freezing and thawing the samples in cycles of 1 h. Stability is acceptable if the concentration measured does not deviate more than 15% from that measured initially.

Application. The accuracy of the method was evaluated via the measurement of a certified reference material, namely, BCR 121 Wholemeal Flour issued by the Institute for Reference Materials and Measurements of the European Commission.[21] Also, the folate content was evaluated in five different rodent diets with varying composition and expected folate content. To evaluate the reproducibility of the folate concentration between different feed batches and between different pellets within one batch, pellets of two different production batches were obtained of which 4 individual pellets were analyzed in duplicate per batch. Also, casein and ethanol extracted casein (Fonterra, Auckland, New Zealand), used as a protein source in many diets, were analyzed to evaluate the efficacy of the ethanol extraction. To correlate the results between feed analysis and casein treatment, casein samples consisted of only 40 mg instead of 200 mg for rodent diets because the rodent diets used in this study contain on average 20% protein.

RESULTS AND DISCUSSION

Method Optimization. Because of the application of LC-MS/MS analysis, the results are not influenced by preservatives and antimicrobial agents that may be present in rodent feed. This is a major improvement over microbial assays commonly used for food and feed analysis. Sample preparation encompasses classical trienzyme treatment with an amylase, protease, and folate conjugase treatment to degrade starches and proteins and to hydrolyze folate polyglutamates, respectively. However, because the feed matrix may have an effect on the activity of the enzyme, the amount of enzyme and length of incubation were optimized.

Optimization of Polyglutamate Deconjugation. Although γ-glutamylhydrolase (GGH) is reported to have a pH optimum between 6.2 and 7.2,[18] deconjugation was found to be optimal at a pH of ∼6.00 with an optimal range between 5.85 and 6.15 (see Figure S1). No compounds exerting an inhibitory effect could be inferred based on literature.[22] The addition of neither oxalate nor chicken pancreas extract improved polyglutamate deconjugation and was therefore not included for further method optimization. The optimized enzyme amounts and incubation times were as described in the Trienzyme Treatment section.

Separation of Oils and Fats. The addition of water-immiscible organic solvents, such as hexane, ethyl acetate, and 1-octanol, followed by vortexing and centrifugation, was evaluated to separate fats and oils from the feed extract prior to trienzyme treatment. Although this indeed led to a better separation after centrifugation, the layer formed at the interface stuck to the pipet tip and fragments were sucked into the aqueous extract upon transfer. Hence, this step did not improve the analytical results and was therefore omitted.

Solid-Phase Extraction. Early during method development, reversed-phase (C_{18} and phenyl) and ion-exchange solid-phase extraction (SAX) were compared. While equivalent folate recovery was obtained, the strong anion-exchange packing gave less background noise and was therefore preferred.

Method Validation. *Selectivity and Carry-over.* No interferences or cross-talk were observed for any of the folates analyzed. However, 5,10CH$^+$THF was partly oxidized to 5FoTHF during initial experiments with a boiling step at pH 6.0. Therefore, it is necessary to adjust the pH to 7.4 prior to performing the boiling step after conjugase treatment. This prevents the oxidation of 5,10CH$^+$THF at elevated temperature, which was also observed by De Brouwer et al. and Jagerstad and Jastrebova.[23,24] Carry-over measured in a blank sample injected after the highest calibrator did not exceed 2.8% of the LLOQ, which is acceptable according to the FDA guideline.

Linearity, Accuracy, and Precision. On the basis of the evaluation of the sum residual error and homoscedasticity, $1/x$ was chosen as the weighting factor for all folates. This transformation also led to the best intra- and interbatch precision and accuracy. The values for both precision (%RSD) and accuracy (%bias) can be found in Table 2 and did not exceed the 15 or 20% acceptance criteria (raw concentration

Table 2. Accuracy and Precision Data ($n = 4 \times 2$)[a]

folate	conc.	intrabatch precision [% RSD]	interbatch precision [% RSD]	accuracy [%bias]
THF	LLOQ	14.1	14.1	−0.8
	3*LLOQ	9.8	9.8	−1.9
	MID	3.9	3.9	−1.4
	3/4ULOQ	4.1	4.1	−0.8
FA	LLOQ	7.4	8.0	−4.5
	3*LLOQ	2.4	4.6	0.8
	MID	2.5	2.5	1.0
	3/4ULOQ	1.4	4.2	−2.9
5MeTHF	LLOQ	10.1	10.1	3.0
	3*LLOQ	6.5	10.7	−2.0
	MID	3.5	6.2	3.4
	3/4ULOQ	4.3	4.3	2.3
5,10CH$^+$THF	LLOQ	16.1	18.7	2.2
	3*LLOQ	5.1	13.8	−3.5
	MID	10.5	10.5	8.1
	3/4ULOQ	3.7	3.7	3.2
10FoFA	LLOQ	7.2	12.3	−5.0
	3*LLOQ	9.4	9.4	−3.0
	MID	5.2	5.2	−4.8
	3/4ULOQ	3.5	6.6	−5.1
5FoTHF	LLOQ	9.0	9.0	5.5
	3*LLOQ	9.9	9.9	3.3
	MID	3.4	3.4	3.0
	3/4ULOQ	1.7	3.0	0.2

[a]Accuracy and precision data at 4 concentration levels analyzed in 2-fold on 4 nonconsecutive days. THF, tetrahydrofolate; FA, folic acid; 5MeTHF, 5-methyltetrahydrofolate; 5,10CH$^+$THF, 5,10-methenyltetrahydrofolate; 10FoFA, 10-formylfolic acid; 5FoTHF, 5-formyltetrahydrofolate.

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Table 3. Matrix Effect and Recovery (n = 2)[a]

	conc.	THF	FA	5MeTHF	5,10CH$^+$THF	10FoFA	5FoTHF
absolute ME without IS	3*LLOQ	57.3	89.8	72.9	61.4	96.8	88.3
	MID	55.0	84.3	68.2	54.7	88.5	89.3
	3/4ULOQ	54.3	86.5	60.8	50.2	85.6	82.5
absolute ME with IS	3*LLOQ	91.9	101.7	100.2	93.6	109.7	99.9
	MID	96.8	98.3	96.5	99.0	103.2	104.1
	3/4ULOQ	101.5	106.9	88.8	93.3	105.8	101.9
relative ME without IS	3*LLOQ	1.7	1.7	3.4	1.3	7.4	8.2
	MID	6.7	4.3	3.9	3.0	0.7	1.1
	3/4ULOQ	2.1	1.5	4.3	0.2	0.3	0.9
relative ME with IS	3*LLOQ	9.3	2.5	2.1	11.3	7.7	8.8
	MID	7.7	4.4	9.5	7.1	0.2	2.0
	3/4ULOQ	2.4	4.5	4.5	4.1	6.7	5.0
absolute RE without IS	3*LLOQ	46.8	37.7	45.2	4.9	33.1	40.0
	MID	36.6	36.7	38.2	4.5	31.3	39.5
	3/4ULOQ	44.2	37.7	48.2	4.7	35.6	49.6
absolute RE with IS	3*LLOQ	112.6	107.1	111.8	114.5	94.5	112.3
	MID	98.5	102.8	104.6	105.8	88.0	110.3
	3/4ULOQ	98.0	97.5	102.8	118.6	92.2	128.5
relative RE without IS	3*LLOQ	11.9	1.2	6.0	0.0	8.2	5.4
	MID	8.6	2.0	9.8	0.2	3.2	6.9
	3/4ULOQ	3.1	2.5	3.7	0.2	1.1	0.1
relative RE with IS	3*LLOQ	6.6	9.2	12.2	4.1	14.3	1.5
	MID	9.0	0.8	15.9	13.2	5.0	14.1
	3/4ULOQ	0.1	0.6	7.4	18.8	9.5	9.5

[a]Results following the analysis of matrix effects and recovery. The maximal achievable recovery of 53.5% was not taken into account in the values of the absolute recovery without IS. 5,10CH$^+$THF, 5,10-methenyltetrahydrofolate; FA, folic acid; 10FoFA, 10-formylfolic acid; 5FoTHF, 5-formyltetrahydrofolate; IS, internal standard; ME, matrix effect; 5MeTHF, 5-methyltetrahydrofolate; RE, recovery; THF, tetrahydrofolate

Table 4. Long-Term Stability[a]

		weeks at 4 °C					weeks at −20 °C				
folate	conc.	0	1	2	3	4	0	1	2	3	4
THF	low	100	84.9	54.8	32.2	25.3	100	108	98.8	83.6	50.4
	high	100	81.5	78.1	52.1	33.6	100	92.8	96.6	55.3	43.9
FA	low	100	104	89.2	60.8	47.5	100	115	99.0	88.7	49.6
	high	100	100	100	77.2	46.8	100	108	120	75.2	53.9
5MeTHF	low	100	98.4	84.6	65.4	48.5	100	98.5	106	72.7	45.9
	high	100	97.8	108	82.6	50.4	100	90.9	114	91.5	47.5
5,10CH$^+$THF	low	100	112	82.6	79.8	57.0	100	85.1	98.4	77.1	55.5
	high	100	84.2	94.8	82.7	50.1	100	82.7	94.1	78.6	48.3
10FoFA	low	100	112	79.8	55.3	66.3	100	96.9	89.8	67.2	70.6
	high	100	95.8	79.4	73.4	69.9	100	90.3	87.0	70.3	59.6
5FoTHF	low	100	123	82.7	49.9	66.2	100	97.2	90.5	60.9	68.2
	high	100	96.2	80.7	66.8	71.8	100	97.1	94.1	67.1	63.6

[a]Results obtained during the evaluation of long-term stability. Results are depicted as % recovery relative to the concentration measured at the 0 time point. Low concentration = 0.1 mg/kg; high concentration = 1.0 mg/kg. 5,10CH$^+$THF, 5,10-methenyltetrahydrofolate; FA, folic acid; 10FoFA, 10-formylfolic acid; 5FoTHF, 5-formyltetrahydrofolate; 5MeTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate

data in Table S2). Also, the back-calculated concentrations of the calibrators fell within the acceptance criteria. The LLOQ and ULOQ for the different folates are 8 μg/kg and 2 mg/kg, respectively (Table 1).

When the folate concentration measured exceeds the ULOQ, 5-fold dilution with extraction buffer without internal standards prior to solid-phase extraction allows one to extend the measurable range up to 10 mg/kg feed. Duplicate analysis of a diluted sample originally spiked at a concentration of 8 mg/kg gave acceptable results, with a difference less than 10.5% compared with the nominal concentration.

As such, it is possible to measure individual folate species in a concentration range of 8 μg/kg to 2 mg/kg. This encompasses the normal range present in rodent feed and allows quantification of natural folates present in feed ingredients and of synthetic folate, generally folic acid, added to the diet. This range can be extended by 5-fold dilution to measure high folic acid diets.

Recovery and Matrix Effects. Given the use of carbon-13 labeled ISs, the IS-corrected matrix effect remained limited (within 15%) for all folates at all three concentration levels. The relative matrix effect ranged from 0.2 to 11.3%. Given the limited recovery of 5,10CH$^+$THF without compensation by the

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IS (±4.7%), it is not possible to reliably obtain a quantitative result for this compound. For all other folates, non-IS corrected absolute recovery exceeded 30% while IS-corrected absolute recovery exceeded 88.0% and was reproducible as relative recovery only exceeded the acceptance criterion of 15% for 5MeTHF at the MID concentration level (15.9%). Detailed data can be found in Table 3, while raw peak area data can be found in Table S3.

Stability. Both storage conditions proposed by the manufacturer were tested, i.e., 4 and −20 °C. As can be seen in Table 4, samples stored at 4 °C were not adequately stable because especially THF was susceptible to degradation with on average 17% loss already after 1 week. Up to 1 week of storage at 4 °C was acceptable for the other folates. Stability at −20 °C was acceptable up to 2 weeks of storage according to the 15% acceptance criterion except for FA at the high concentration level, which was 20% higher than at the start (cfr. Tables S4A and S4B).

The evaluation of the stability of samples in the autosampler of the UPLC instrument revealed no problems during storage up to 24 h (cfr. Table S4C). Only for THF did freeze−thaw cycles affect the measured folate concentration. After 3 cycles, the degradation of THF exceeded the 15% acceptance criterion (cfr. Table S4D).

Application. The analysis of five feed varieties (in duplicate) clearly illustrated the relevance of a method for the analysis of folates in rodent diets. Both the TD.06691 and the TD.95247 diets are reported to be FA-deficient. However, although in the former no folates could be quantified, the incorporation of purified casein in the latter leads to a total folate content of 45.6 μg/kg, with FA and 10FoFA present in quantifiable amounts (28.6 and 17.0 μg/kg, respectively). According to the specification sheet, 7 mg of folate/kg is present in the 7012 diet (as added folic acid). This was confirmed by a measured total folate content of 6.91 mg of total folate/kg. However, only 82.9% of this consists of FA (5.73 mg/kg), while also 5FoTHF (6.7%; 0.465 mg/kg), 5MeTHF (5.8%; 0.402 mg/kg), 10FoFA (4.0%; 0.279 mg/kg), and THF (0.6%; 41.5 μg/kg) are present. Although the product sheet of the TD.94045 diet reports the addition of 2 mg/kg FA, only 1.33 mg/kg FA (95.9% of the total amount of folates present) was measured, which is 31% lower than reported by the manufacturer. However, also trace amounts of 5FoTHF (2.2%; 31.0 μg/kg) and 10FoFA (1.4%; 20.0 μg/kg) were present in this diet, leading to a total folate content of 1.38 mg of total folate/kg. Like the TD.94045 diet, 2.00 mg of FA/kg is added to the rodent maintenance diet obtained from Carfil, according to the product sheet. However, a total folate concentration of 5.63 mg/kg was measured of which 4.98 mg/kg was FA (86.9%), while also 5FoTHF (7.1%; 0.404 mg/kg), 10FoFA (5.3%; 0.303 mg/kg), and 5MeTHF (0.8%; 43.9 μg/kg) were present. These results, including total folate concentration and speciation, are represented graphically in Figure 2A. The variability of folate concentrations between different production batches was evaluated using the diet obtained from Carfil. For two batches, we performed duplicate analysis of four pellets. Though no appreciative difference was observed between pellets of each production batch, there was a significant difference between these batches for FA (p = 0.002), 5MeTHF (p = 0.000), and total folate (p = 0.002) analyzed using an independent t test at the 95% confidence level. An 18.9% difference in folic acid concentration was measured between both batches (4.98 and 4.04 mg/kg), while the 5MeTHF

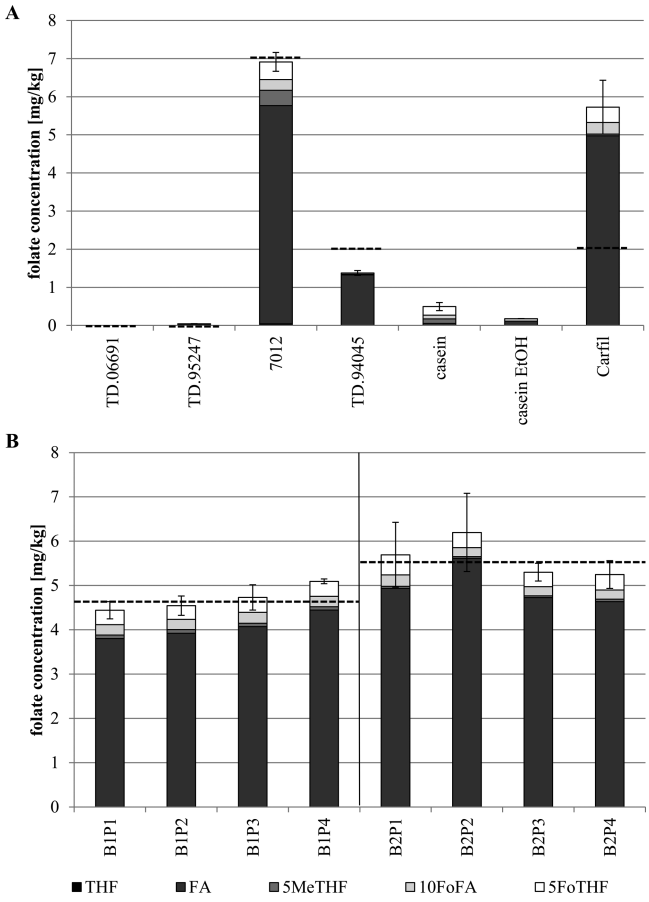

Figure 2. Measured folate species in various rodent diets, analyzed in duplo, including standard deviation on total folate content and a dotted line indicating the folic acid concentration mentioned by the manufacturer (A) and evaluation of homogeneity of the folate concentration of 2 production batches and 4 different pellets per batch indicated as B(#batch)P(#pellet). The average total folate concentration was indicated for each batch by a dotted line. Significant differences were observed for FA, 5MeTHF, and total folate (B). THF, tetrahydrofolate; FA, folic acid; 5MeTHF, 5-methyltetrahydrofolate; 10FoFA, 10-formylfolic acid; 5FoTHF, 5-formyltetrahydrofolate.

concentration differed by 41.2% (45.7 and 79.5 μg/kg, respectively), as can be seen in Figure 2B. As such, the use of pellets from different production batches during a long-term feeding trial may exert an influence because of variable folate concentrations, especially because the concentrations of the distinct folates do not differ equally. These results again illustrate the importance of an analytical method capable of quantifying individual folate species.

When analyzing casein without the rest of the ingredients present, a total folate concentration of 0.494 mg/kg casein was found. Here, mainly 5FoTHF (45.5%; 0.225 mg/kg), 5MeTHF (24.3%; 0.120 mg/kg), 10FoFA (19.5%; 0.101 mg/kg), and FA (10.8%; 53.2 μg/kg) were present. While commonly performed to remove endogenous compounds, among which are folates, ethanol extraction only succeeds partly in removing folates from casein, with a measured remaining total folate concentration of 0.179 mg/kg. Although 5FoTHF was the major folate form present in untreated casein, only FA (63.4%; 0.114 mg/kg) and 10FoFA (36.6%; 65.5 mg/kg) could be quantified in ethanol-extracted casein. As such, ethanol extraction is demonstrated to be insufficient to completely remove all endogenous folates.

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The BCR 121 certified reference material consists of a ground wholemeal flour with a certified total folate concentration of 0.50 mg/kg with an uncertainty of 0.07 mg/kg. Although wholemeal flour differs from rodent feed in terms of fat, protein, and starch content and consistency, it was used to evaluate the accuracy of the method. Following duplicate analysis, we measured a total folate content of 0.443 mg/kg with a standard deviation of 0.004 mg/kg, which corresponds to the certified range. Also, individual folate concentrations (0.059, 0.025, 0.048, 0.111, and 0.199 mg/kg for THF, FA, 5MeTHF, 10FoFa, and 5FoTHF, respectively) were comparable to those found in literature.[12,14,25]

It is clear that, for the correct interpretation of results obtained during animal feeding trials, it is very important to determine the actual feed intake and the folate concentration at the time of feeding. This conclusion is supported by our analysis of various feed samples, in which we observed substantial differences between the specified folate content and the actual folate content and the presence, up to 17%, of reduced and substituted folates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b04075.

Mass spectrometric settings, raw data for the assessment of accuracy and precision, raw data for the assessment of matrix effect and recovery, stability data, and influence of pH on the deconjugation efficiency using rat serum γ-glutamylhydrolase (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: christophe.stove@UGent.be. Tel.: +32 9 264 81 35. Fax: +32 9 264 81 83.

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Notes

The authors declare no competing financial interest.

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