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Development of stable isotope dilution assays for the quantitation of intra- and extracellular folate patterns of *Bifidobacterium adolescentis*

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ABSTRACT

Folate producing bifidobacteria have been studied extensively but appropriate methods for in detail quantitation of intra- and extracellular pteroylmono- and pteroylpolyglutamate patterns are lacking. Therefore, *B. adolescentis* DSM 20083^T was cultivated in folate-free medium (FFM) for 24 h to develop and validate stable isotope dilution assays (SIDAs) coupled with LC-MS/MS for the determination of 5-formyltetrahydrofolic acid (5-HCO-H_4folate), 10-formylfolic acid (10-HCO-PteGlu), tetrahydrofolic acid (H_4folate), folic acid (PteGlu) and 5-methyltetrahydrofolic acid (5-CH_3-H_4folate) including its di-, tri-, and tetraglutamic vitamers (5-CH_3-H_4PteGlu$_{2-4}$). The respective monoglutamylated isotopologues labelled with deuterium were used as internal standards for quantitation. Limits of detection and quantitation (LOD/LOQ) were sufficiently low to quantify 48.2 nmol·L^{-1} 5-CH_3-H_4folate (5.7/17 nmol·L^{-1}) and 71.0 nmol·L^{-1} 5-HCO-H_4folate (10/30 nmol·L^{-1}) as major folate vitamers extracellularly and 124 nmol·L^{-1} 5-CH_3-H_4folate (3.4/10 nmol·L^{-1}), 213 nmol·L^{-1} 5-HCO-H_4folate (4.8/14 nmol·L^{-1}), and 61.4 nmol·L^{-1} H_4folate (2.3/7.0 nmol·L^{-1}) intracellularly after deconjugation. The major portion of native 5-CH_3-H_4folate vitamer was ascribed to its tetraglutamate (>95%). Concentrations of mono-, di-, tri-, and pentaglutamylated folates were below LOD or LOQ. Intra-assay precision coefficients of variation (CVs) ranged from 7% (at a concentration of 53.9 nmol·L^{-1} for 5-CH_3-H_4PteGlu$_4$), 15% (25.5 nmol·L^{-1} 5-CH_3-H_4folate) to 18% (78.5 nmol·L^{-1} 5-HCO-H_4folate), extracellularly, and from 6% (60.7 nmol·L^{-1} 5-CH_3-H_4PteGlu$_4$), 7% (202 nmol·L^{-1} 5-HCO-H_4folate), 10% (67.1 nmol·L^{-1} H_4folate) to 11% (127 nmol·L^{-1} 5-CH_3-H_4folate), intracellularly. Inter-assay precision CVs ranged from 2% (54.7 nmol·L^{-1} 5-CH_3-H_4PteGlu$_4$), 3% (71 nmol·L^{-1} 5-HCO-H_4folate) to 11% (48.2 nmol·L^{-1} 5-CH_3-H_4folate), extracellularly, and from 1% (61.4 nmol·L^{-1} H_4folate), 5% (213 nmol·L^{-1} 5-HCO-H_4folate), 6% (63.5 nmol·L^{-1} 5-CH_3-H_4PteGlu$_4$) to 10% (124 nmol·L^{-1} 5-CH_3-H_4folate), intracellularly, thus showing excellent reproducibility. Recoveries for all analytes under study ranged

between 81 and 113%. These newly developed methods enable reproducible, precise and sensitive quantitation of eight bacterially synthesized folate vitamers in two totally different matrices, including both monoglutamates and polyglutamates. Furthermore, we here present the first assay using solely monoglutamylated [$^2H_4$]-5-CH_3-H_4folate to quantify native polyglutamate patterns of this vitamer in bacteria which might replace time-consuming determination of monoglutamates in the future.

1. Introduction

Bifidobacteria like *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* have been characterized for their production of vitamins such as thiamine, folate, pyridoxine, nicotinic acid and cobalamine in semi-synthetic medium [1] and, therefore, are considered as important probiotics [2]. In particular, autotrophic bifidobacteria strains of the species *B. adolescentis* are capable of synthesizing folates *de novo* [2]. Folate deficiency is a potential risk factor for inborn errors like spina bifida and anencephaly [3,4] or diseases like Alzheimer´s [5,6] and cancer [7,8]. The relative availability of bacterial folate compared to food folate remains unclear but its contribution to host nutrition is suggested to be substantial because of the longer transit time in the colon [9,10].

Nevertheless, suitable methods for the simultaneous quantitation of bacterially synthesized folates, either mono- or polyglutamic forms, in extra- and intracellular fractions have not been applied recently. In the following the term ´monoglutamate´ (Fig. A.1 of the electronic supplemental material (ESM)) is used to describe the total amount of the respective vitamer in its monoglutamic form after enzymatic deconjugation. The term ´polyglutamate´ (Fig. A.1 of the ESM) refers to the native vitamer distribution including all monoglutamic and polyglutamic forms without deconjugation. Pompei *et al.* [11] analysed the intra- and extracellular folate content after 48 h cultivation in FFM by a microbiological

assay. Unfortunately, the assay did not allow determination of folate patterns. In contrast to this, D´Aimmo *et al.* [12] investigated the intracellular folate production and the folate patterns of bifidobacteria *in vitro* by high performance liquid chromatography coupled with UV and fluorescence (FD) detection (HPLC-UV/FD). They identified 5-CH_3-H_4folate and H_4folate as main vitamers after deconjugation in cells cultivated in FFM. After detecting significant amounts of intra- and extracellular total folate, Deguchi *et al.* [1] claimed high portions of intracellular folate in bifidobacteria to be excreted into the cultivation medium without indicating any underlying mechanism. In this regard, pteroylpolyglutamates form the main storage forms of folate in cells, whereas pteroylmonoglutamates represent transport forms. However, there are neither investigations of the degree of glutamalytion of intra- nor extracellular folate vitamers in bifidobacteria [11,12]. Sybesma *et al.* [13] used HPLC coupled with diode array detection (HPLC-DAD) to identify polyglutamated folate vitamers produced by various lactic acid bacteria. Rong *et al.* [14] investigated polyglutamate patterns of an unspecified microbiota in rat feces after injection of [^{3}H]-p-aminobenzoic acid into the cecum and found mainly monoglutamates, tri- and tetraglutamates. Prior to colonic absorption, pteroylpolyglutamates have to be deconjugated by exogenic conjugase activity. Therefore, precise methods for the combined determination of intra- and extracellular pteroylmono- and pteroylpolyglutamate patterns are urgently needed to shed light on extracellular folate enrichment and folate bioavailability, which depends on the degree of polyglutamylation and conjugase activity.

SIDAs have proven their advantages over conventional microbiological assays, on the one hand, because in the latter assays recoveries of and responses to different folate vitamers strongly depend on the model organism used for detection [15]. On the other hand, methods using HPLC-DAD without using internal standards lack sensitivity in comparison to LC-MS(/MS) methods and calibration has to be carried out by extraction of a spiked sample matrix. In contrast, the use of isotopologic internal standards in SIDAs

compensates for chemical analyte degradation, matrix effects, and losses during extraction and clean-up, which is crucial for accurate folate analysis.

Most recently, SIDAs of folates have been developed in the field of clinical chemistry or food analysis for the quantitation of monoglutamate distribution in erythrocytes [16], dried blood spots [17], and whole blood [18] or polyglutamate distributions in vegetables [19]. However, the SIDAs for folates often use a time-consuming overnight deconjugation. Other methods [18,19] apply UPLC-MS/MS for folate detection and an extraction protocol customized for blood lysates or food. Nevertheless, only 5-CH_3-H_4folate analysis has been validated up to present, which is not sufficient for detailed bacterial folate analysis, because combined, simplified and efficient analysis of all monoglutamates and polyglutamates in both bacteria cells and surrounding medium is needed to understand the physiology of bacterial folate production, accumulation and transport. Moreover, preprocessing steps for sufficient cell lysis have to be established prior to folate extraction and the impact of nutritional medium composition, containing high concentrations of inorganic salts and organic acids, on solid phase extraction (SPE) performance has to be carefully evaluated for polyglutamate extraction. This is particularly critical because the increasing anionic character of the polygutamates is likely to interfere on ion exchange SPE with ionic components of the growth medium. Finally, accuracy has to be verified, e.g. by comparing the total concentration of native polyglutamate distribution to the level of the respective monoglutamate after deconjugation.

Therefore, our first aim was to develop and validate the first stable isotope dilution assay (SIDA) for precise and simultanous determination of extracellular and intracellular folate patterns in two totally different and complex media using *B. adolescentis* DSM 20083^T as model organism. Furthermore, we wanted to established a method for the determination of polyglutamylated 5-CH_3-H_4folate using [$^2H_4$]-5-CH_3-H_4folate as internal standard to compare intracellular and extracellular polyglutamate ratios and to clarify the extent of

glutamylation in the intracellular and extracellular fraction. To the best of our knowledge, the method described herein for the combined analysis of 5 monoglutamates and 3 polyglutamates is unique in its application for screening the physiology of bacterial folate production, accumulation and release.

2. Materials and Methods

2.1. Chemicals

Agar, MRS bouillon (de Man, Rogosa and Sharpe), urea, magnesium sulphate heptahydrate, manganese chloride dihydrate, sodium molybdate dihydrate, thiamine, calcium pantothenate and L-cysteine were obtained from Carl Roth GmbH (Karlsruhe, Germany). Rat serum (preservative free) was obtained from Biozol (Eching, Germany). Chicken pancreas was purchased from Becton Dickinson and Co. (Sparks, MD, USA). Acetonitrile, potassium dihydrogen phosphate, disodium hydrogen phosphate (anhydrous), methanol, sodium chloride, sodium acetate trihydrate, acetic acid, hydrochloric acid (37%), 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) hydrochloride, Tris base, dipotassium hydrogenphosphate, ferrous sulphate heptahydrate, L-glutamic acid, L-alanine, L-aspartic acid, and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). Dithiothreitol (DTT), Ethylenediaminetetraacetic acid (EDTA), pyridoxine hydrochloride, nicotinic acid, riboflavin, biotin, and glycerine (99.5%) were purchased from Applichem Lifescience (Darmstadt, Germany). Formic acid, formaldehyde solution (37%), sodium borohydride, sodium acetate, ammonium sulphate, Tween 80, glucose, manganese sulphate tetrahydrate, calcium chloride dihydrate, zinc sulphate heptahydrate, copper sulphate pentahydrate, p-aminobenzoic acid, L-arginine,

glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-serine, L-tryptophan, L-valine, Dulbecco`s Phosphate Buffered Saline (PBS) and 4-morpholineethanesulfonic acid (MES) were obtained from Sigma-Aldrich (Steinheim, Germany). Ascorbic acid was obtained from VWR Chemicals Prolabo (Leuven, Belgium). Boric acid was obtained from neoLab Migge Laborbedarf-Vertrieb GmbH (Heidelberg, Germany). L-threonine, L-lysine and L-asparagine were purchased from Serva Feinbiochemica GmbH & Co KG (Heidelberg, Germany). 2-octanol was purchased from Alfa Aesar (Karlsruhe, Germany). Folic acid polyglutamates ($PteGlu_{2-5}$), 5-CH_3-H_4folate, 10-formylfolic acid (10-HCO-PteGlu), 5-formyltetrahydrofolic acid (5-HCO-H_4folate) calcium salt, and H_4folate trihydrochloride were purchased from Schircks Laboratories (Jona, Switzerland). PteGlu was obtained from Fluka (Buchs, Schweiz). The isotopologic standards [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-H_4folate, and [$^2H_4$]-PteGlu were synthesized as reported recently [20].

2.2. Materials and bacteria cultures

Strata strong anion exchange (SAX) (100 mg, 1 mL), Strata strong anion mixed-mode (X-A) (30 mg, 1 mL) and Strata Phenyl cartridges (100 mg, 1 mL) were obtained from Phenomenex (Aschaffenburg, Germany). *Bifidobacterium adolescentis* (DSM 20083^T) was obtained as lyophilisate from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). AnaeroGen 2,5 L (GasPak) was obtained from Oxoid (Hampshire, UK). Chemicals for FFM were mixed according to D`Aimmo *et al.* [12]. LIVE/DEAD BacLight Bacterial Viability Kit L7012 for fluorescence microscopy was obtained from Molecular Probes (Eugene, Oregon, USA). For fluorescence microscopy we used an Axiostar plus (HBO 50) microscope with a red BP 546/12; FT 580; LP 590 and a green BP 475/40; FT 500; BP 530/50 fluorescence filter from Carl Zeiss

Microimaging GmbH (Göttingen, Germany). Zirconium beads (0.1 mm) were obtained from Carl Roth GmbH (Karlsruhe, Germany). The bead beater Fast Prep 24 was manufactured by MP Biomedicals (Solon, OH, USA) The HLC Thermomixer was manufactured by DITABIS AG (Pforzheim, Germany).

2.3. Solutions and nutritional media for the bacteria

Abacterial glycerin solution (40%) was produced by dilution of glycerin with MRS (40/60, v/v). After vortexing the solution was sterile filtered in a sterile centrifuge tube. Sterile L-cysteine solution (2%) consisted of 0.4 g L-cysteine diluted in 20 mL water and sterile filtered in an autoclaved reaction vessel. MRS nutritional medium (MRS, De Man, Rogosa und Sharpe) consisted of 26 g MRS bouillon dissolved in 487.5 mL water and autoclaved at 121°C for 20 min. Finally, 12.5 mL of the L-cysteine solution were added to give a final volume of 500 mL.

2.4. Solutions for folate extraction

Extraction buffer consisted of a 200 $mmol \cdot L^{-1}$ MES hydrate and 20 $g \cdot L^{-1}$ ascorbic acid aqueous solution with 1 $g \cdot L^{-1}$ DTT, adjusted to pH 5 with 7.5 M NaOH. Phosphate buffer (100 $mmol \cdot L^{-1}$) was prepared by adjusting an aqueous solution of disodium hydrogen phosphate (100 $mmol \cdot L^{-1}$) with an aqueous solution of potassium dihydrogen phosphate (100 $mmol \cdot L^{-1}$) to pH 7.0. The equilibration buffer for the SAX cartridges was prepared by adding 0.2 $g \cdot L^{-1}$ DTT to diluted phosphate buffer (10 $mmol \cdot L^{-1}$). Further, the eluting solution was a mixture of aqueous sodium chloride (5%) and aqueous sodium acetate (100 $mmol \cdot L^{-1}$) containing 1 $g \cdot L^{-1}$ DTT and ascorbic acid (1%). The chicken pancreas suspension for pteroylpolyglutamate deconjugation was prepared by stirring chicken

pancreas (30 mg) in aqueous phosphate buffer solution (90 ml, 100 mmol·L^{-1}) containing 1% ascorbic acid adjusted to pH 7 with 7.5 M NaOH.

2.5. Cultivation of bifidobacteria

All glass devices and materials were autoclaved prior to use. Experiments were carried out under a Laminar-Flow. Incubation of the anaerobic bacteria in centrifuge tubes was carried out in an anaerobic jar with activated GasPak.

2.5.1. Cryo-conserved bacterial suspension

The lyophilisate was resuspended in 1 mL MRS medium. Afterwards the suspension was transferred into 24 mL MRS medium for incubation at 37°C for 53 h. 1 mL of the culture was transferred into further 24 mL MRS medium and incubated at 37 °C for further 18 h to reach the stationary phase. The stationary phase was verified by measurement of the optical density (OD) at 600 nm with MRS medium as sample blank. Glycerine solution (40%) was added to the bacteria suspension (1/1 v/v). The mixture was aliquoted in 200 µL portions and stored at -80°C.

2.5.2. Cultivation of bifidobacteria

200 µl aliquots of the cryoconserved culture were suspended in 25 mL medium and incubated for 24 h at 37°C. Afterwards 1.25 mL were diluted to a final concentration of 5% in fresh medium and incubated for further 24 h at 37°C (preculture). The working culture consisted of a further dilution of 0.5 mL preculture in 9.5 mL nutritional medium. The working culture was incubated for further 24 h at 37°C prior to extraction.

2.6. Optimization of intra- and extracellular folate extraction

For the optimization of folate analysis the bacteria cultures of *B. adolescentis* were cultivated as described in point 2.5.2..

2.6.1. Optimization of extracellular pteroylmonoglutamate extraction

Six working cultures were incubated for 24 h and centrifuged at 5000 g at 4°C for 10 min. The supernatants were pooled and aliquoted for extracellular folate extraction as follows. Three aliquots of 1 mL, 2 mL and 3 mL were prepared in triplicate for three different variants and diluted 1+1 with extraction buffer. Afterwards 0.92 ng [$^2H_4$]-5-CH_3-H_4folate was added as internal standard. After vortexing for 10s and equilibrating for 15 min samples were subjected to the following extraction procedures.

For extraction procedure (E1) samples were heated for 4 min at 100°C, centrifuged at 2700 g and 4°C and subsequently subjected to solid phase extraction (SPE) as described in 2.7.7.. Further nine aliquots were mixed with 1 mL chicken pancreas suspension and 150 µL rat serum for extraction procedure (E2). After incubation for 4 h at 37°C under constant agitation samples were heated at 100°C and chilled in an ice bath. Samples were purified via SPE after centrifugation at 2700 g and 4°C for 20 min. Extraction procedure (E3) included an additional cooking step in analogy to procedure (E1) before the addition of the enzyme mixture. The following steps were conducted in analogy to procedure (E2).

2.6.2. Optimization of intracellular pteroylmonoglutamate extraction

4 working cultures were incubated for 24 h and centrifuged (5000 g, 10 min, 4 °C). The cellular residue was washed in 5 mL PBS-buffer. After an additional centrifugation step 2.4 mL extraction buffer were added to the residue. The suspension was spiked with 2.76 ng [$^2H_4$]-5-CH_3-H_4folate and equilibrated for 15 min. Three aliquots of 1 mL for each working culture were transferred into screw tubes with 500 mg beads.

For extraction procedure (I1) samples were heated in a Thermoblock (3 min, 95°C, 550 U/min) and subjected to a Bead Beater (3x40 s, 6.5 m/s, on ice) afterwards. Extraction procedure (I2) and (I4) included these steps in reverse order. Procedure (I3) included an additional, analogous cooking step after cell lysis in the Bead Beater. All samples except (I4) were centrifuged (15000 g, 5 min, 4°C). Supernatants were transferred to centrifuge tubes and mixed with 1 mL of chicken pancreas suspension and 150 µL rat serum. Incubation and purification via SPE was conducted in analogy to 2.7.7..

2.6.3. Optimization of extracellular pteroylpolyglutamate extraction

High concentrations of anionic compounds might interfere with analyte and internal standard molecules. Therefore we examined different SPE materials for their suitability compared to an extraction procedure without SPE clean-up. FFM was spiked with the respective polyglutamates to a final concentration of 39, 30 and 61 nmol·L^{-1} for 5-CH_3-$H_4$$PteGlu_{2-4}$ and separated into 9 aliquots. Three aliquots of 1 mL each were spiked with 0.92 ng [$^2H_4$]-5-CH_3-H_4folate and extracted in analogy to the procedure for intracellular monoglutamates with SPE and exclusion of the enzymatic treatment. Further three aliquots were extracted analogously and subjected to SPE with phenyl cartridges (2.6.4) or X-A cartridges (2.6.5). Three additional samples with 0.5 mL each (78, 60 and 122 nmol·L^{-1} for 5-CH_3-$H_4$$PteGlu_{2-4}$ in FFM), were extracted without SPE.

2.6.4. SPE clean-up of pteroylpolyglutamates with phenyl cartridges

Cartridges were activated successively with 2 volumes of acetonitrile, methanol and ammonium formate buffer (8 mmol/L ammonium formate, 0.05 g/L ascorbic acid, pH 3.4). After sample application the cartridges were washed with 3 volumes of ammonium formate buffer and evaporated to dryness by vacuum. Folates were eluted with 0.5 mL of eluting solution (methanol/acetonitrile/water/acetic acid, 40/10/49/1, v/v/v/v with 1 g/L ascorbic acid).

2.6.5. SPE clean-up of pteroylpolyglutamates with Strata X-A cartridges

Cartridges were activated with one volume of methanol and water, successively. After application of the sample cartridges were washed with one volume of an aqueous solution of ammonium acetate (25 mmol/L) and one volume of water, successively. Folates were eluted with 0.5 mL of eluting solution containing 5% formic acid in methanol.

To evaluate the discrimination of each folate vitamer and the internal standard, the results of all samples were compared to a mixture of analyte and internal standard.

2.7. Validation of the folate assay for pteroylmonoglutamates and –polyglutamates

2.7.1. Extraction procedure

Methods for extra- and intracellular folate extraction were developed according to [21] and [11], respectively. Sample purification was based on our previous studies [16,17] and further optimized. Bacteria suspension was centrifuged at 5000 g for 10 min at 4°C. The

supernatant was subjected to the extraction protocol below, whereas the cellular fraction was suspended in 2 mL of sterile water and divided into two aliquots. Aliquot 1 was subjected to fluorescence microscopy, aliquot 2 was resuspended in 4 mL PBS, centrifuged for 15 min at 5000 g and 4°C. The supernatant was discarded and the cells were washed in 5 mL PBS and centrifuged at 5000 g and 4°C for 15 min. Afterwards the cellular fraction was resuspended in 10 mL extraction buffer and subjected to monoglutamate or polyglutamate extraction.

The optimized extraction procedure is summarized in Table A.1 of the ESM.

2.7.2. Fluorescence microscopy

Viability of bifidobacteria was verified via fluorescence microscopy after 24 h. 1 mL of the pellet (aliquot 1) was diluted in 20 mL of sterile water and incubated for one hour at room temperature. The suspension was subsequently centrifuged for 10 min at 5000 g and 21 °C. After repetition of the washing step the pellet was diluted in 10 mL of sterile water. 1 mL was mixed with 3 µL of the colour mixture consisting of SYTO 9 and propidium iodide (50/50 v/v). The cells were incubated at room temperature in the dark for 15 min and counted four times. The percentual amount of living and dead cells was calculated from the mean.

2.7.3. Extracellular pteroylmonoglutamates

250 µl supernatant were transferred into a 5 mL volumetric flask and made up to volume with extraction buffer. 1 mL were transferred into a centrifuge tube with 0.92 ng [$^2H_4$]-5-CH_3-H_4folate, 1.02 ng [$^2H_4$]-5-HCO-H_4folate, and 3.04 ng [$^2H_4$]-H_4folate. After equilibrating for 15 min samples were heated at 100°C for 4 min in a water bath. The chilled samples

were mixed with 1 mL of chicken pancreas suspension and 150 µL of rat serum. After incubation in a water bath at 37°C for 4 h under constant agitation the samples were heated at 100°C for 4 min in a water bath and subsequently centrifuged at 2700 g at 4°C for 20 min. The supernatant was subjected to SPE (2.7.7.).

2.7.4. Intracellular pteroylmonoglutamates

1 mL of the suspension was transferred into Eppendorf tubes with the deuterated internal standards. After equilibrating for 15 min the suspension was transferred into screw tubes filled with 500 mg zirconium beads for cellular destruction in a Bead Beater (3x40 s, 6.5 m/s, on ice). Lysed cells were cooked for 3 min at 95°C (550 U/min) and centrifuged at 15000 g and 4°C for 5 min. The supernatant was transferred into a centrifuge tube and subsequently mixed with 1 mL of chicken pancreas suspension and 150 µL of rat serum. After incubation and heating as described in 2.7.3., the samples were subjected to SPE clean-up (2.7.7.).

2.7.5. Extracellular 5-CH_3-H_4PteGlu$_{2-4}$

0.5 mL supernatant was spiked with 0.92 ng [$^2H_4$]-5-CH_3-H_4folate and diluted with 0.5 mL extraction buffer. After vortexing for 10 s and equilibrating for 15 min the sample was heated at 100°C for 4 min and subsequently centrifuged at 15400 g for 20 min to remove the precipitate prior to LC-MS/MS analysis.

2.7.6. Intracellular 5-CH_3-H_4PteGlu$_{2-4}$

1 mL of the suspension was spiked with 0.92 ng [$^2H_4$]-5-CH_3-H_4folate and equilibrated for 15 min and heated for 3 min at 95°C (550 U/min, Thermoblock). Sample clean-up was performed in analogy to the procedure for intracellular monoglutamates (2.7.4.) except the enzymatic deconjugation step.

2.7.7. SPE clean-up of pteroylmonoglutamates and –polyglutamates with Strata-SAX cartridges

Prior to sample extraction the cartridges were preconditioned with two volumes of methanol and two volumes of equilibration buffer. After adding the sample the cartridges were washed with two volumes of equilibration buffer and dried via vacuum suction. Folates were eluted with 0.5 mL eluting solution.

2.7.8. Synthesis of 5-CH_3-H_4PteGlu$_{2-5}$ for the determination of pteroylpolyglutamates

$PteGlu_2$ (1.79 mg), $PteGlu_3$ (1.67 mg) and $PteGlu_4$ (4.07 mg) were derivatized to the respective methylated forms in a single approach according to Ndaw *et al.* [22]. The completeness of conversion was verified by LC-MS/MS measurement.

2.7.9. LC-MS/MS

Extra- and intracellular pteroylmono- and peroylpolyglutamates were determined separately by means of LC-MS/MS (Finnigan Surveyor Plus HPLC System, Thermo electron corporation, Waltham, USA; triple quadrupole TSQ quantum discovery mass spectrometer, Thermo electron corporation, Waltham, USA). Analyte separation was carried out on a Nucleosil C_{18} reversed phase column (250 x 3 mm; 4 µm; Macherey-

Nagel, Düren, Germany). The mobile phase for gradient elution consisted 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 and ion source parameters have been described earlier by Mönch and coworkers [16]. In brief, gradient elution started at 0% B followed by a linear increase to 10% after 2 min, to 25% within 23 min and to 100% B after further 2 min. After 3 min B was decreased to 0% within two min. Equilibration of the column was carried out for 16 min. Source CID collision energy was set to 10 arbitrary units, spray voltage was set to 3900 V, capillary temperature was 320°C and capillary voltage was set to 35 V. SRM scanning was performed in the positive electrospray ionization mode (ESI^+). 10 µL of the extracts were injected for folate analysis. Eluate was diverted to waste for the first 10 min and scanning of the SRM transitions (Table A.2 and A.3 of the ESM) was performed from 10-25 min.

2.7.10. HPLC-DAD

Dilutions of 10 mg PteGlu in 100 mL extraction buffer and 2 mg of 5-CH_3-H_4folat, H_4folate, 5-HCO-H_4folate, and 10-HCO-PteGlu in 10 mL extraction buffer were prepared. PteGlu and H_4folate had to be dissolved in 100 mmol·L^{-1} phosphate buffer prior to dilution. Analyte concentrations were determined using the HPLC-DAD method published before [17].

2.7.11. Calibration of pteroylmono- and pteroylpolyglutamates

Folate calibrator solutions were prepared by mixing the deuterated internal standard solution with the corresponding analyte solutions. Molar ratio ranges [n(S)/n(A)]-[n(S)/n(A)] for the calibration of intra- and extracellular pteroylmonoglutamates were 2.6:1-

1:100 (5-CH_3-H_4folate), 0.5:1-1:10 (10-HCO-PteGlu, 0.82 ng [$^2H_4$]-10-HCO-PteGlu), 2.6:1-1:20 (5-HCO-H_4folate), 10:1-1:34 (H_4folate), and 2.5:1-1:6.1 (PteGlu, 0.74 ng [$^2H_4$]-PteGlu).

Calibrator solutions for 5-CH_3-H_4PteGlu$_{2-4}$ were prepared by adding [$^2H_4$]-5-CH_3-H_4folate as internal standard to the analyte mixture. Molar ratio ranges [n(S)/n(A)]-[n(S)/n(A)] for the calibration of intra- and extracellular pteroylpolyglutamates were 1:157-5:1 for 5-CH_3-H_4PteGlu$_2$, 1:119-7:1 for 5-CH_3-H_4PteGlu$_3$ and 1:244 – 3:1 for 5-CH_3-H_4PteGlu$_4$.

For the calibration functions linear regression was used by combining the molar ratios with the peak area ratios [A(S)/A(A)] measured by LC-MS/MS. Consistency of response was verified by injecting a randomly chosen n(S)/n(A) value in the linear range of the response functions.

2.7.12. Limits of detection (LODs) and quantification (LOQs)

As folates occur ubiquitously in cells and culture medium, folate free surrogates had to be developed. For extracellular LODs and LOQs a folate free medium (FFM) was produced in analogy to D'Aimmo *et al.* [12]. Intracellular matrix surrogate consisted of 11 mg lyophilized egg white suspended in 10 mL extraction buffer. The surrogate was calculated from the wet weight of the cellular residue assuming that bacterial cells contain 40% dry matter [23]. Determination of LODs and LOQs was carried out according to the calibration line procedure proposed by Hädrich and Vogelgesang [24]. For the determination of LODs and LOQs the surrogates were spiked with the respective folates at four different concentration levels starting slightly above the estimated LOD and covering one to tenfold amount of analyte. Extraction and LC-MS/MS analysis was carried out as described in section 2.7.3-2.7.6.

2.7.13. Precision

Inter-assay precision was calculated by analyzing FFM supernatant and cellular residue three times in triplicate during three weeks. Intra-assay precision for intra- and extracellular folate was determined by multiple injection (n=3) of one sample in a row.

2.7.14. Recoveries of analytes in stable isotope dilution assays

FMM and lyophilized egg-white were spiked with three different analyte levels to verify linearity of the response functions. Level 1 and 3 were calculated as the lowest and highest amount expected in the samples. Level 2 was calculated as means of all extra- and intracellular samples. All samples were analysed by stable isotope dilution assay as described in section 2.7.3.-2.7.6.. The recovery was calculated as the mean of the addition experiments. The spiking levels are listed in Table A.4 and A.5 of the ESM.

2.8. Data analysis

Data analysis was carried out using Xcalibur Software vers. 2.0 (Thermo Scientific, Waltham, USA). Significance was calculated by student´s t-Test (two-sided, $P<0.05$).

3. Results

Characterization of bacterial folates is challenging due to the different matrices to be analysed. The extracellular fraction contains small anionic molecules and inorganic salts, which can interfere with the analytes during SAX purification, whereas the intracellular fraction contains macromolecular compounds, which can trap folate vitamers. To meet the requirement of a quantitative folate extraction and to compensate for analyte loss from both matrices, we developed and optimized a SIDA with deuterated internal pteroylmonoglutamate standards. Polyglutamates of 5-CH_3-H_4folate were quantified using [$^2H_4$]-5-CH_3-H_4folate as internal standard. Viability during our experiments was verified by fluorescence microscopy. Green (viable) and red (dead) cells were easily distinguishable from each other. In our experiments we observed a relative mean viability of 48% after 24 h incubation.

3.1. Optimization of intra- and extracellular folate extraction

3.1.1. Optimization of extracellular and intracellular pteroylmonoglutamate extraction

Extracellular folate extraction was optimized according to Lin & Young [21]. Different volumes of supernatant were analysed for pteroylmonoglutamates to evaluate the folate patterns (Fig. 1).

After deconjugation 5-CH_3-H_4folate, 5-HCO-H_4folate and H_4folate were identified. In the chromatogram (Fig. 1) with the mass transition *m/z* 474→327 we observed an additional signal at 16.5 min. According to Pfeiffer *et al.* [25] this molecule was characterized as 4α-hydroxy-5-CH_3-H_4folate (MeFox), the product of 5-CH_3-H_4folate after mild and prolonged oxidation. The identity of MeFox was verified by measuring the *m/z* transition of 474→284.

An enzymatic treatment and a heating step were introduced for quantitative polyglutamate deconjugation and quantitative extraction from the matrix. Fig. 2 shows the concentrations of 5-CH_3-H_4folate depending on cooking (E1), conjugase treatment (E2) and deconjugation after cooking (E3) in 1, 2 or 3 mL of the extracellular fraction.

We observed an enormous increase of the methylated monoglutamate after conjugase treatment of 1 mL and a further significant ($p=0.05$) increase after deconjugation of a pre-heated sample (1 mL). As almost all 5-CH_3-H_4folate is polyglutamylated a deconjugation step is essential to determine extracellular folate. Heating improves release of folates from the matrix. Therefore, we used a combined extraction with heating followed by conjugase treatment. Using SAX-SPE with 100 mg/1 mL cartridges a sample volume of 1 mL seems to be the most suitable as the FFM contains high amounts of inorganic salts and anionic compounds leading to an overloading of the binding sites of the stationary phase. Furthermore, we observed an increasing imprecision of triplicate injection with 8, 23, and 34% after extracting 1, 2, and 3 mL FFM, respectively. Areas for 5-CH_3-H_4folate were 3000000, 660000 and 540000 area units, respectively, thus showing the necessity for further dilution of the sample. Therefore, a twentyfold dilution of FFM was adapted to the extraction procedure to prevent the SPE cartridges from overload.

Intracellular folate extraction was optimized according to Pompei *et al.* [11]. First the cellular fraction was analysed for pteroylmonoglutamates to evaluate the folate patterns (Fig. 3).

After deconjugation 5-CH_3-H_4folate, 5-HCO-H_4folate and H_4folate were identified. The intensity of MeFox is very low compared to the extracellular fraction. Therefore, we conclude that MeFox might be a remnant of oxidative degradation of 5-CH_3-H_4folate in the extracellular medium because of the long incubation period of 24 h.

Four extraction procedures were evaluated for their 5-CH_3-H_4folate content (I1) after cooking and cell destruction in a Bead Beater, (I2) cell destruction and cooking, (I3)

cooking before and after cell destruction and (I4) cell destruction and cooking without centrifugation. The results are shown in Fig. 4.

Our results indicate a significant ($p=0.05$) loss of methylated folate when cooking is performed before mechanic cell lysis (I1). This might be due to folate trapping inside the denaturatedcellular membrane, which is not the case when heating is applied after cell lysis. When cell lysates are centrifuged before the enzymatic deconjugation of pteroylpolyglutamates (I2) slightly lower mean concentrations are obtained as compared to the enzymatic treatment of the lysate as a whole (I4). Nevertheless, method I4 was not suitable for our extraction procedure as the higher amount of matrix contamination led to congestion of the SPE cartridges. Therefore, we chose method I2 for our extraction procedure.

3.1.2. Optimization of extracellular and intracellular pteroylpolyglutamate extraction

Fig. A.2 of the ESM shows that almost all 5-CH_3-H_4folate vitamers are polyglutamylated in the extracellular fraction. Therefore, we qualitatively analysed both fractions for pteroylpolyglutamates to determine the pteroylpolyglutamate patterns. Pteroylpolyglutamates in the extracellular fraction consisted of 5-CH_3-H_4PteGlu$_{2-4}$ (Fig. 5), 5-HCO-H_4PteGlu$_{2-4}$ and to a smaller extent of H_4PteGlu$_{2-4}$ (Fig. A.2 of the ESM).

Due to lack of labelled polyglutamate standards we synthesized the respective 5-CH_3-H_4folate polyglutamates (Glu$_{2-4}$) to evaluate the suitability of [$^2H_4$]-5-CH_3-H_4folate as internal standard and to quantify the intracellular and extracellular polyglutamate pattern of the latter. More than 95% of the intra- and extracellular 5-CH_3-H_4PteGlu$_n$ was represented by the tetraglutamylated vitamer. From our qualitative results we assume that 5-HCO-H_4folate shows a similar polyglutamate distribution as 5-CH_3-H_4folate. However, as the former polyglutamates cannot be obtained as straightforward as those of 5-CH_3-

H_4folate, we focussed our study on the 5-CH_3-H_4folate polyglutamates as model compounds for further improvement of folate quantitation. Nevertheless, the 5-HCO-H_4folate data are valid as sum value over all respective polyglutamates after deconjugation.

Pre-study recovery experiments revealed a discrimination of the internal standard compared to the polyglutamates during extraction of spiked FFM when compared to a response mixture. We identified the stationary phases of the SAX cartridges as discriminating factor and compared three different purification procedures with different stationary phases to an extraction procedure without SPE.

SPE of extracellular pteroylpolyglutamates with SAX cartridges led to a vast decrease in the signal of the labelled internal standard. Compared to an extraction without cartridges, the molar ratio of polyglutamates to the monoglutamic standard was about twofold higher in samples after SAX clean-up. Recoveries in the spiked surrogates are shown in table 1.

Table 1. Recoveries of 5-CH_3-H_4PteGlu$_{2-4}$ after extraction and SPE clean-up of 1 mL FFM.

Analyte	conc. [nmol·L^{-1}]	Recovery±SD [%]			
		Strata SAX	no SPE*	Strata X-A	Strata Phenyl
5-CH_3-H_4PteGlu$_2$	39	164±12	85±3*	81±5	78±4
5-CH_3-H_4PteGlu$_3$	30	200±18	87±6*	81±4	53±2
5-CH_3-H_4PteGlu$_4$	61	179±17	99±3*	69±3	25±1

* 78, 60 and 122 nmol·L^{-1} for 5-CH_3-H_4PteGlu$_{2-4}$ in FFM. Extraction of 0.5 mL FFM.

In contrast to this, Strata X-A phases or phenyl cartridges led to a discrimination of polyglutamates. We determined higher amounts of internal standard leading to lower recoveries, especially for 5-CH_3-H_4PteGlu$_4$ after purification with X-A phases and higher losses of 47 and 75% after purification with phenyl cartridges. As the tetraglutamylated

vitamer is the most abundant one in the extracellular fraction and we could not find an appropriate phase, we decided to apply no SPE step because most matrix constituents were separated from the analytes during HPLC and diverted to waste during the first 10 min of elution. Thus we could prevent the MS from major contamination with matrix compounds and ensure reproducible measurement.

3.1.3. Optimized conditions for sample extraction

After separating the extracellular fraction from the cellular residue we included a dilution step for our monoglutamate assay by mixing 250 µL of FFM with extraction buffer (1+19, v/v) and applying 1 mL of this dilution to lower the amount of analyte injected and to minimize matrix effects during extraction. For pteroylpolyglutamate analysis, 0.5 mL FFM were mixed with 0.5 mL extraction buffer and 0.92 ng [$^2H_4$]-5-CH_3-H_4folate was added. Because of severe discrimination of the internal standard (SAX SPE) or the respective polyglutamates 5-CH_3-H_4PteGlu$_{2-4}$ (phenyl SPE) and 99% recovery of 5-CH_3-H_4PteGlu$_4$ without SPE clean-up, extraction was performed using a simple heating step (4 min, 100°C) followed by centrifugation (15400 g, 20 min). Inorganic salts and small organic compounds were separated chromatographically during the first 10 min and diverted to waste. Intracellular monoglutamate and polyglutamate concentration was assayed after resuspending the cellular fraction in 2 mL distilled water. 1 mL of the suspension was resuspended in 4 mL PBS buffer and centrifuged for 15 min at 5000 g and 4°C to remove FFM and extracellular folates. The washing step was repeated in 5 mL PBS buffer and the cellular residue was resuspended in 10 mL extraction buffer. 1 mL of the cell suspension was used for polyglutamate or monoglutamate extraction after adding 0.92 ng [$^2H_4$]-5-CH_3-H_4folate or 0.92 ng [$^2H_4$]-5-CH_3-H_4folate, 1.02 ng [$^2H_4$]-5-HCO-H_4folate, and 3.04 ng

[$^2H_4$]- H_4folate, respectively. The whole extraction protocol is summarized in Table A.1 of the ESM.

3.2. Validation of the folate assay for pteroylmonoglutamates and -polyglutamates

3.2.1. Calibration for stable isotope dilution assays

Calibration curves for 5-CH_3-H_4folate, 5-CH_3-H_4PteGlu$_2$, 5-CH_3-H_4PteGlu$_3$, 5-HCO-H_4folate, PteGlu, 10-HCO-PteGlu and H_4folate were linear in molar ratio ranges [n(S)/n(A)]-[n(S)/n(A)] given in Table 2 along with their response functions calculated according to the following equation.

$$A(\text{labelled standard})/A(\text{analyte}) = R_F * n(\text{labelled standard})/n(\text{analyte}) + b$$

Table 2: Response functions for pteroylmonoglutamates and 5-CH_3-H_4PteGlu$_{2-4}$.

Analyte	Response function	R^2	linear molar ratio range [n(S)/n(A)]-[n(S)/n(A)]
5-CH_3-H_4folate	y=0.6664x+0.0026	0.9955	2.56:1-1:100
5-CH_3-H_4PteGlu$_2$	y=1.602x+0.003	0.9998	5:1-1:60
5-CH_3-H_4PteGlu$_3$	y=2.5084x-0.0162	0.9983	1.7:1-1:30
5-CH_3-H_4PteGlu$_4$	$y=0.0009x^3-0.0157x^2+0.1548x+0.0014$	0.9998	1:244-1:1.2*
5-CHO-H_4folate	y=0.7507x-0.0505	0.9904	2.55:1-1:20
PteGlu	y=0.3721x-0.0383	0.9954	2.5:1-1:6.3
10-HCO-PteGlu	y=0.4112x+0.0275	0.9699	0.5:1-1:10

H_4folate	y=0.9026x+0.0423	0.9994	10:1-1:33

* cubic function

The response equation for 5-CH_3-H_4PteGlu$_4$ followed a polynomial function according to:

$$n(\text{labelled standard})/n(\text{analyte}) = R_{F1} * [A(\text{labelled standard})/A(\text{analyte})]^3 + R_{F2} * [A(\text{labelled standard})/A(\text{analyte})]^2 + R_{F3} * A(\text{labelled standard})/A(\text{analyte}) + b$$

In all equations R_{FN} and b are equivalent to the polynomial factors and the intercept, respectively.

3.2.2. Determination of pteroylmonoglutamates and 5-CH_3-H_4PteGlu$_{2-4}$

Folates are ubiquitous in animals, plants and bacteria. Therefore, a surrogate had to be developed for the determination of LODs/LOQs and recoveries. The extracellular matrix consisted of FFM as used for bacteria cultivation (2.5.2.). Lyophilized egg white was used for the cellular fraction. 11 mg egg white were used as cell surrogate and resuspended in 10 mL extraction buffer. LODs and LOQs were determined using the procedure by Hädrich and Vogelgesang [24]. Results are listed in table 3.

Table 3: LODs and LOQs for pteroylmonoglutamates and 5-CH_3-H_4PteGlu$_{2-4}$.

Analyte	Extracellular fraction		Intracellular fraction	
	LOD [nmol·L^{-1}]	LOQ [nmol·L^{-1}]	LOD [nmol·L^{-1}]	LOQ [nmol·L^{-1}]
PteGlu	6.9	20	5.7	17
10-HCO-PteGlu	8.9	26	3.7	11
5-CH_3-H_4folate	5.7	17	3.4	10

5-HCO-H_4folate	10	30	4.8	14
H_4folate	3.1	9.1	2.3	7.0
5-CH_3-H_4PteGlu$_2$	3.0	9.0	0.4	1.3
5-CH_3-H_4PteGlu$_3$	2.1	6.2	1.0	2.8
5-CH_3-H_4PteGlu$_4$	2.9	8.6	1.6	4.8

LODs and LOQs for extracellular pteroylmono- and polyglutamates were almost twice or three times as high as the values obtained for intracellular folates. This effect can be ascribed to the higher amounts of inorganic salts and anionic compounds in the FFM, which interfere with the folate vitamers during SPE purification.

Recoveries of all analytes using SIDA-LC-MS/MS ranged from 81 to 113% and from 81 to 111% for extra- and intracellular folate, respectively (Table 4).

Table 4: Recoveries for pteroylmonoglutamates and 5-CH_3-H_4PteGlu$_{2-4}$.

Analyte	Level	Extracellular fraction		Intracellular fraction	
		Spiked conc. [nmol·L^{-1}]	recovery [%]	Spiked conc. [nmol·L^{-1}]	recovery [%]
PteGlu	I	23.9	104 ± 4	19.9	110 ± 4
	II	35.8	91 ± 8	29.9	89 ± 7
	III	48.0	112 ± 7	36.0	85 ± 6
10-HCO-PteGlu	I	nd	nd	nd	nd
	II	30.2	82 ± 4	18.1	96 ± 5
	III	40.3	99 ± 4	36.2	90 ± 6
5-CH_3-H_4folate	I	24.3	102 ± 2	30.4	95 ± 3
	II	40.1	83 ± 7	120	98 ± 5

	III	80.2	113 ± 2	240	90 ± 4
5-HCO-H_4folate	I	40.3	105 ± 6	40.5	98 ± 4
	II	60.0	112 ± 3	300	91 ± 10
	III	120	92 ± 9	400	84 ± 1
H_4folate	I	11.9	95 ± 6	29.9	91 ± 5
	II	23.9	84 ± 6	60.2	87 ± 7
	III	35.9	81 ± 5	120	81 ± 9
5-CH_3-H_4PteGlu$_2$	I	9.41	86 ± 2	nd	nd
	II	18.8	102 ± 4	3.29	94 ± 11
	III	31.4	96 ± 11	9.41	95 ± 3
5-CH_3-H_4PteGlu$_3$	I	7.16	87 ± 6	nd	nd
	II	14.3	103 ± 7	3.58	94 ± 7
	III	23.9	100 ± 7	7.16	111 ± 7
5-CH_3-H_4PteGlu$_4$	I	14.6	91 ± 5	5.12	95 ± 7
	II	48.7	100 ± 5	73.1	99 ± 1
	III	122	94 ± 5	146	102 ± 6

nd: not determined.

3.2.3. Precision of the assays for pteroylmonoglutamates

Intra-assay precision was determined by multiple injection (n=3) of an extra- and intracellular extract of one bacteria culture. Coefficients of variation (CVs) revealed satisfactory reproducibility of the multiple injection experiment and ranged between 6 and 11% for intracellular and 7 and 18% for extracellular folate (Table 5). High CVs of extracellular 5-CH_3-H_4folate and 5-HCO-H_4folate can be ascribed to their concentrations close to their LOQs. Moreover, higher variations of 5-HCO-H_4folate in these low

concentrations due to possible interconversions to and from 5,10-CH^+-H_4folate and 10-HCO-H_4folate can be assumed.

Table 5. Intra-assay precision for pteroylmonoglutamates and 5-CH_3-H_4PteGlu$_{2-4}$.

Analyte	Extracellular fraction		Intracellular fraction	
	concentration [nmol·L^{-1}]	CV [%]	concentration [nmol·L^{-1}]	CV [%]
PteGlu	< LOD	-	< LOD	-
10-HCO-PteGlu	< LOD	-	< LOD	-
5-CH_3-H_4folate	25.5	15	127	11
5-HCO-H_4folate	78.5	18	202	7
H_4folate	< LOQ	-	67.1	10
5-CH_3-H_4PteGlu$_2$	< LOQ	-	< LOD	-
5-CH_3-H_4PteGlu$_3$	< LOQ	-	< LOQ	-
5-CH_3-H_4PteGlu$_4$	53.9	7	60.7	6

Inter-assay precision was determined by extraction of one sample in triplicate on three days within two weeks. CVs for inter-assay precision were 1–10% for intracellular folates and 2-11% for extracellular folate (Table 6). As different batches of bacteria cultures were used for the validation procedure of pteroylmono- and pteroylpolyglutamates, their concentrations cannot be compared in this table.

Table 6. Inter-assay precision for pteroylmonoglutamates and 5-CH_3-H_4PteGlu$_{2-4}$.

Analyte	Extracellular fraction		Intracellular fraction	
	concentration [nmol·L^{-1}]	CV [%]	concentration [nmol·L^{-1}]	CV [%]
PteGlu	< LOD	-	< LOD	-
10-HCO-PteGlu	< LOD	-	< LOD	-

5-CH_3-H_4folate	48.2	11	124	10
5-HCO-H_4folate	71.0	3	213	5
H_4folate	< LOQ	-	61.4	1
5-CH_3-H_4PteGlu$_2$	< LOQ	-	< LOD	-
5-CH_3-H_4PteGlu$_3$	< LOQ	-	< LOQ	-
5-CH_3-H_4PteGlu$_4$	54.7	2	63.5	6

PteGlu and 10-HCO-PteGlu were not detectable. 5-CH_3-H_4folate and 5-HCO-H_4folate represented the major vitamers intra- and extracellularly. H_4folate was quantifiable solely intracellularly. In an independent approach we quantified 5-CH_3-H_4PteGlu$_{2-4}$. The tetraglutamate was quantified extra- and intracellularly with 54.7 and 63.5 nmol·L^{-1}, respectively. 5-CH_3-H_4PteGlu$_3$ was detectable but not quantifiable in the respective fraction. The diglutamate was detectable only in the supernatant.

3.2.4. Comparison of 5-CH_3-H_4folate and 5-CH_3-H_4PteGlu$_{2-4}$ content

In a single experiment with one culture incubated for 24 h, we compared the accordance of both methods quantitating monoglutamate after deconjugation and polyglutamates of extra- (Fig. 6) and intracellular (Fig. 7) 5-CH_3-H_4folate.

Quantitation showed a good correlation between the total concentrations obtained by either determination of pteroylmono- or pteroylpolyglutamate. 5-CH_3-H_4PteGlu$_2$ and 5-CH_3-H_4PteGlu$_3$ were below LOQ. According to the concentration of monoglutamate, almost 100% of polyglutamylated 5-CH_3-H_4folate can be traced back to the tetraglutamate.

4. Discussion

Because of their probiotic properties and their ability to produce considerable amounts of folate, bifidobacteria became model organisms of particular interest. Pompei *et al.* [11] examined folate production by bifidobacteria in both intracellular and extracellular fractions in folate-free semisynthetic medium containing the folate precursor p-aminobenzoic acid. Most of the 62 human strains tested were auxotrophic for folate. Six of 17 folate-producing strains including strains of *B. adolescentis* and *B. pseudocatenulatum* produced up to 82 $ng \cdot mL^{-1}$ extracellular total folate in FFM. About 9-38% of the produced folate was ascribed to intracellular folate after incubating the cells for 48 h. In contrast to this we quantified 28% 5-CH_3-H_4folate and 25% 5-HCO-H_4folate in the extracellular fraction of *B. adolescentis* DSM 20083^T cultivated in FFM for 24 h corresponding to approximately 72 and 75%, respectively, in the intracellular fraction. Extracellular total folate concentration in our study was 56 $ng \cdot mL^{-1}$ and 185 $ng \cdot mL^{-1}$ were found intracellularly. Comparability between the conflicting results is difficult because of different growth media used, strain-to-strain differences and the time point of sampling. We found H_4folate exclusively in the intracellular fraction, which can be attributed to the lower stability of this vitamer in FFM. Moreover, the determined vitamer distribution is also dependent on many environmental and analytical factors as i.e. 5-HCO-H_4folate might be a conversion product of 10-formyltetrahydrofolate (10-HCO-H_4folate) and 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate) [26,27]. 10-HCO-H_4folate is essential for the synthesis of purines and, on the one hand, is unstable in the presence of oxygen [28] or heat [26]. On the other hand, 5,10-CH^+-H_4folate is converted to 5-HCO-H_4folate when exposed to pH values below 5 [29].

D´Aimmo *et al.* [12] used an HPLC-UV/FD method and identified 5-CH_3-H_4folate and H_4folate to be the most abundant intracellular folate vitamers in bifidobacteria cultivated in FFM. 5-HCO-H_4folate was below their LOD and could not be detected. Methods based on

UV absorption are not suitable for extracellular folates, as aromatic amino acids and further chromophoric substances of the matrix interfere with analyte signals. SPE purification prior to HPLC-UV/FD analysis leads to substantial losses of analytes, which are not compensated for by internal standards. In our assay we observed considerable discrimination of the deuterated internal standard [$^2H_4$]-5-CH_3-H_4folate during extraction and clean-up of extracellular 5-CH_3-H_4PteGlu$_{2-4}$ and identified the purification step via SAX cartridges as the cause. The high content of anions and small, anionic and organic molecules might contribute to a non-proportional release of the monoglutamylated vitamer and its labelled isotopologue from the stationary phase. In contrast to this, the polyglutamylated vitamers possess more than one anionic binding site thus showing higher affinity to the quaternary amine phase. In contrast to this, mixed-mode phases like Strata X-A or Strata Phenyl showed a strong discrimination of tetra- and triglutamylated 5-CH_3-H_4folate. These findings are consistent with the fact that an increasing number of glutamyl residues increases the hydrophilic character of the molecule compared to the monoglutamylated internal standard. Interestingly, almost 100% of 5-CH_3-H_4PteGlu$_4$ were recovered in spiked FFM by only centrifuging the extract without any SPE step. As the recovery results for all polyglutamyl forms of 5-CH_3-H_4folate were satisfactory, no further optimization steps on the different cartridges were performed and SPE was omitted in the final procedure for extracellular folates.

Incorporation of bacterially synthesized folate into host tissue was verified by Rong *et al.* [14] after injection of tritium-labelled p-aminobenzoic acid into the caecum of rats. In the latter study, labelled folate forms synthesized by an undefined fecal microbiota consisted mainly of mono-, tri- and tetraglutamylated folates with small amounts of di- and pentaglutamylated folates. This is particularly important as for assessing folate bioavailability it is necessary to study the degree of polyglutamylation of each folate vitamer. Considering native 5-CH_3-H_4folate vitamers, in our study the intra- and

extracellular fractions of *B. adolescentis* DSM 20083^T consisted mainly of the tetraglutamylated 5-CH_3-H_4folate (>95%) and negligible amounts (<LOD or LOQ) of 5-CH_3-H_4PteGlu$_2$ and 5-CH_3-H_4PteGlu$_3$. 5-CH_3-H_4folate was not found in the FFM during the screening for mono- and polyglutamylated 5-CH_3-H_4folate. Extracellular 5-HCO-H_4folates showed equal polyglutamate patterns with 5-HCO-H_4PteGlu$_4$ being the predominant folate vitamer. Both monoglutamate and polyglutamate assays showed a high correlation. The latter might replace the former in the future, because of facilitated and fast sample clean-up (Table A.1 of the ESM) and quantitating the native folate vitamer distribution. From the extracellular enrichment of tetraglutamylated folates we conclude that intracellular folates might be released in the medium after cell death and progressing lysis. Tetraglutamylated folates have to undergo further deconjugation to be accessible for colonocytes. Therefore, bioavailability of these folates produced by this strain might depend largely on exogenous deconjugase activity in the colon.

5. Conclusions

Herein we present the first SIDA including simultaneous determination of monoglutamylated and polyglutamylated folates for the characterization of bacterial folate synthesis *in vitro*. FFM and cells differ considerably in the complexity of their matrix composition. Cellular components can be separated easily whereas FFM contains small acidic and anionic molecules interfering with the conventional SPE procedures. Monoglutamate losses are compensated for by the respective isotopologic monoglutamate standard. Thus, monoglutamates can be quantified correctly and independent of culture medium composition. The application of these standards may restrict the propagation of the method but their use prevents from tedious matrix calibrations or standard additions, which is even more expensive in terms of work load

and measuring time. With regard to sample clean up, an increasing degree of polyglutamylation of folates leads to an increasing polar character of the molecule thus increasing polyglutamate retention on SAX SPE. Therefore, the isotopic monoglutamate standard is largely displaced by matrix components leading to an overestimation of extracellular polyglutamate content. As most of the interfering salts and molecules are eluted within the first few minutes during LC-MS/MS measurement and small volumes and high dilutions of FFM were used, we were able to exclude the SPE step from our purification. Determination of total 5-CH_3-H_4folate and the respective polyglutamates showed a good correlation of both approaches for both intra- and extracellular fraction independent of matrix composition. These findings might allow further studies on polyglutamylated H_4folate or 5-HCO-H_4folate and their production by bifidobacteria. The quantitation with respective monoglutamylated isotopic standards saves cost and time compared to the purchase or synthesis of the respective isotope labelled polyglutamates and enables sensitive determination of the native bacterial folate vitamer distribution.

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Figure legends

Fig. 1: LC-MS/MS of pteroylmonoglutamates in the extracellular fraction of *B. adolescentis* after deconjugation. *m/z*: SRM transitions of the folate vitamer; monoisotopic mass precursor→monoisotopic mass product ion.

Fig. 2: Concentrations of 5-CH_3-H_4folate depending on cooking (E1), conjugase treatment (E2) and deconjugation after cooking (E3) in 1, 2 or 3 mL of the extracellular fraction analysed in triplicate. * significant difference (Student´s t-test, two-sided, P<0.05).

Fig. 3: LC-MS/MS chromatogram of pteroylmonoglutamates in the intracellular fraction of *B. adolescentis* after deconjugation. *m/z*: SRM transitions of the folate vitamer; monoisotopic mass precursor→monoisotopic mass product ion.

Fig. 4: Intracellular 5-CH_3-H_4folate content after (I1) cooking and cell destruction, (I2) cell destruction and cooking, (I3) cooking before and after cell destruction and (I4) cell destruction and cooking without centrifugation. Samples were analysed in triplicate. * significant difference (Student´s t-test, two-sided, P<0.05).

Fig. 5: LC-MS/MS chromatogram of pteroylpolyglutamates in the extracellular fraction of *B. adolescentis. m/z*: SRM transitions of the folate vitamer; monoisotopic mass precursor→monoisotopic mass product ion.

Fig. 6: Quantitation of extracellular monoglutamate (i.e. after deconjugation) and polyglutamates (i.e. sum of native polyglutamates and monoglutamate without deconjugation) of 5-CH_3-H_4folate.

Fig. 7: Quantitation of intracellular monoglutamate (i.e. after deconjugation) and polyglutamates (i.e. sum of native polyglutamates and monoglutamate without deconjugation) of 5-CH_3-H_4folate.

Appendix A. Supplementary Data

Table A.1: Scheme of intra- and extracellular extraction of pteroylmono- and pteroylpolyglutamates.

extraction step	extraction step	cellular fraction - polyglutamates	cellular fraction - monoglutamates	FFM - monoglutamates	FFM - polyglutamates
purification/ separation	cultivation	24 h, 37°C, anaerobic conditions			
	separation	5000 g, 4°C, 10 min, separate cellular fraction and supernatant			
	dilution	resuspend cellular fraction in 2 mL distilled water		dilute 250 µL supernatant in 5 mL volumetric flask with extraction buffer	
	aliquoting	Aliquot 1 – fluorescence microscopy Aliquot 2 – folate extraction			
	centifugation	resuspend aliquot 2 in 4 mL PBS buffer, centrifugation for 15 min at 5000 g and 4°C, repeat step with 5 mL PBS buffer			
	dilution	resuspend cell fraction in 10 mL extraction buffer			
	internal standards	add 0.92 ng [$^2H_4$]-5-CH_3-H_4folate to 1 mL cell supsension for polyglutamate and 0.92 ng [$^2H_4$]-5-CH_3-H_4folate, 1.02 ng [$^2H_4$]-5-HCO-H_4folate and 3.04 ng [$^2H_4$]- H_4folate to 1 mL cell suspension for monoglutamate extraction		add 0.92 ng [$^2H_4$]-5-CH_3-H_4folate, 1.02 ng [$^2H_4$]-5-HCO-H_4folate and 3.04 ng [$^2H_4$]- H_4folate to 1 mL suspension for monoglutamate extraction	add 0.92 ng [$^2H_4$]-5-CH_3-H_4folat to 0,5 mL supernatant and further 0,5 mL extraction buffer
	equilibration	15 min, transfer suspension into screw tubes with 500 mg zirconia beads			
	heating	3 min, 95°C (550 U/min) Thermoblock			
	cell lysis	cell lysis, Bead Beater (3x40 s, 6,5 m/s, on ice)			
	heating	3 min, 95°C (550 U/min) Thermoblock		4 min, 100°C, ice bath	
	centrifugation	15000 g, 4°C, 5 min			15400 g, 20 min
extraction	deconjugation		150 µL rat serum, 1 mL chicken pancreas suspension (4 h, 37°C)		
	heating		4 min, 100°C, ice bath		
	centrifugation	2700 g, 20 min, 4°C			
	SPE clean-up	SAX-SPE 100 mg, 1 mL			
	washing step	2 volumes methanol, 2 volumes equilibration buffer			
	elution	0,5 mL eluting solution			
detection		LC-MS/MS			

Table A.2: LC-MS/MS transitions for pteroylmonoglutamates.

Analyte	Q1 (*m/z*)	Q3 (*m/z*)
5-CH_3-H_4folate	460	313
H_4folate	446	299
PteGlu	442	295
10-HCO-PteGlu	470	295
5-HCO-H_4folate	474	327
[$^2H_4$]-5-CH_3-H_4folate	464	317
[$^2H_4$]-H_4folate	450	303
[$^2H_4$]-PteGlu	446	299
[$^2H_4$]-10-HCO-PteGlu	474	299
[$^2H_4$]-5-HCO-H_4folate	478	331

Table A.3: LC-MS/MS transitions for pteroylpolyglutamates.

Analyte	Q1 (*m/z*)	Q3 (*m/z*)
5-CH_3-H_4PteGlu$_2$	589.3	313.3
5-CH_3-H_4PteGlu$_3$	718.3	313.3
5-CH_3-H_4PteGlu$_4$	847.3	313.3
5-CH_3-H_4PteGlu$_5$	976.3	313.3
[$^2H_4$]-5-CH_3-H_4folate	464	317

Table A.4: Spiking levels for extracellular folate

Analyte	Level 1 [nmol·L^{-1}]	Level 2 [nmol·L^{-1}]	Level 3 [nmol·L^{-1}]
PteGlu	23.9	35.8	48.0
10-HCO-PteGlu	nd	30.2	40.3
5-CH_3-H_4folate	24.3	40.1	80.2
5-HCO-H_4folate	40.3	60.0	120
H_4folate	11.9	23.9	35.9
5-CH_3-H_4PteGlu$_2$	9.41	18.8	31.4
5-CH_3-H_4PteGlu$_3$	7.16	14.3	23.9
5-CH_3-H_4PteGlu$_4$	14.6	48.7	122

nd: not determined

Table A.5: Spiking levels for intracellular folate

Analyte	Level 1 [nmol·L^{-1}]	Level 2 [nmol·L^{-1}]	Level 3 [nmol·L^{-1}]
PteGlu	19.9	29.9	36.0
10-HCO-PteGlu	nd	18.1	36.2
5-CH_3-H_4folate	30.4	120	240
5-HCO-H_4folate	40.5	300	400
H_4folate	29.9	60.2	120
5-CH_3-H_4PteGlu$_2$	nd	3.29	9.41
5-CH_3-H_4PteGlu$_3$	nd	3.58	7.16
5-CH_3-H_4PteGlu$_4$	5.12	73.1	146

nd: not determined

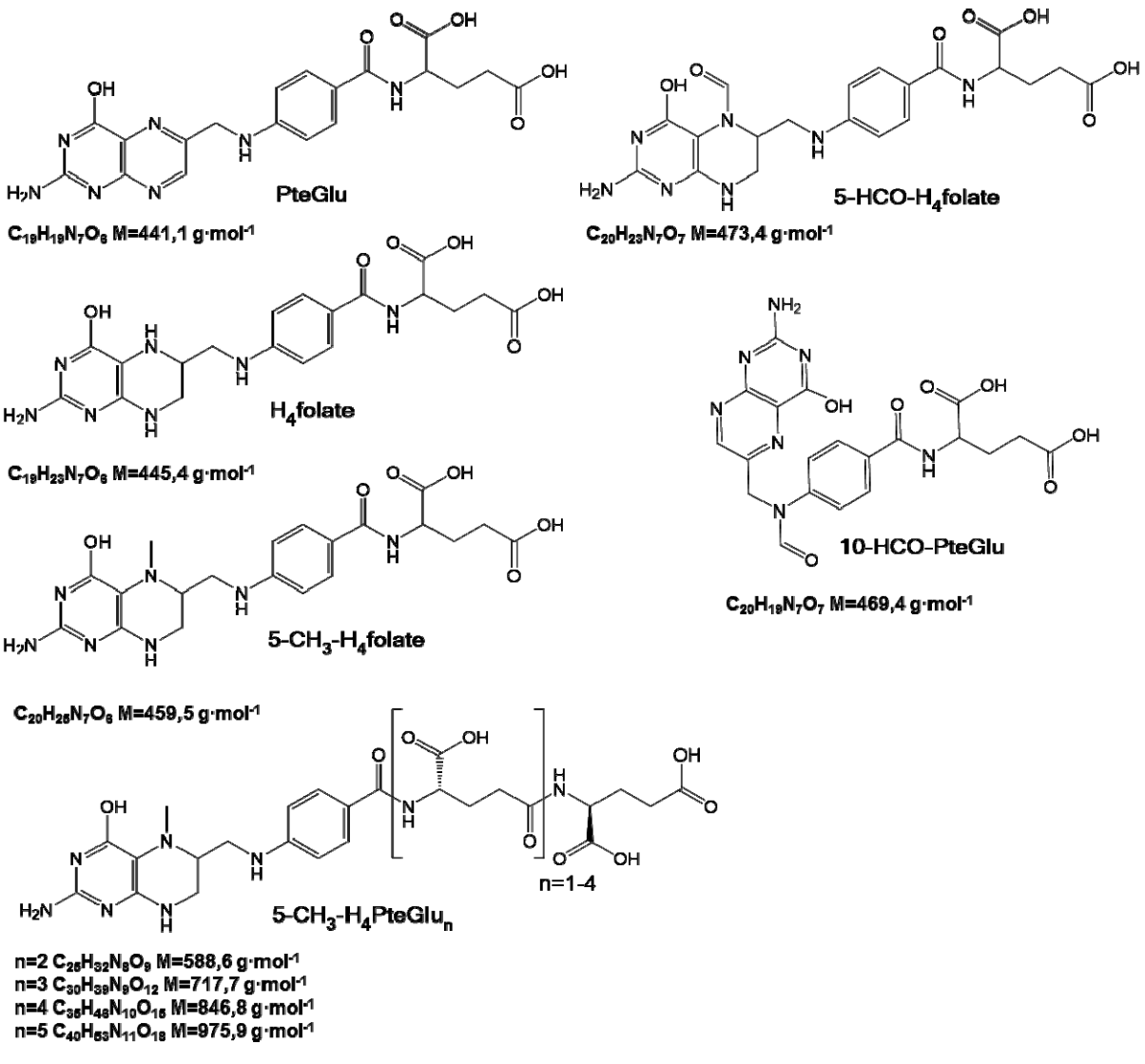

Figure A.1: Structures of all monoglutamates and polyglutamates under study. Monoglutamates: Folic acid (PteGlu), 5-formyltetrahydrofolate (5-HCO-H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), 10-formylfolic acid (10-HCO-PteGlu). Polyglutamates: 5-methyltetrahydropteroylpolyglutamate (5-CH_3-$H_4PteGlu_{2-5}$).

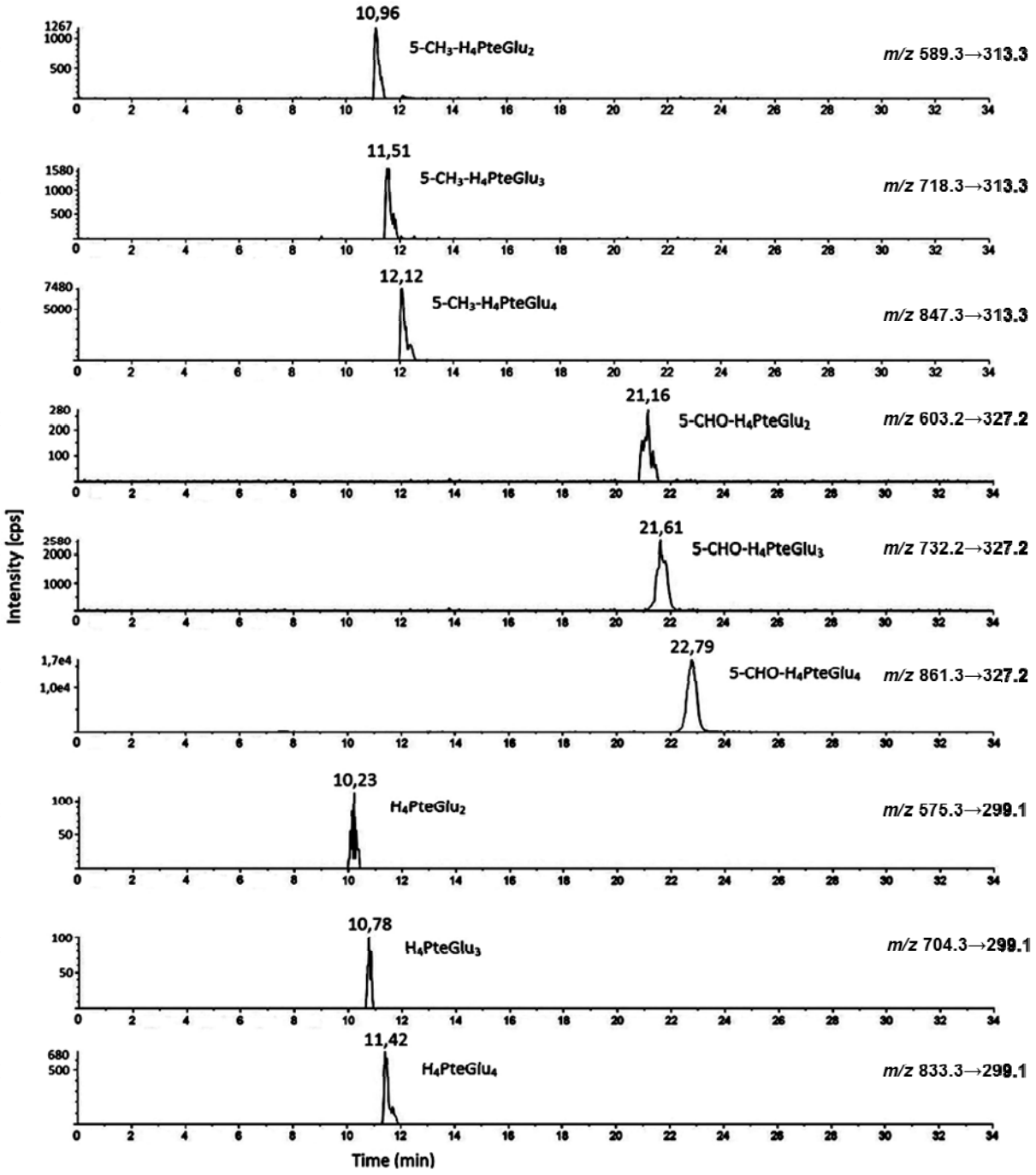

Figure A.2: LC-MS/MS chromatograms of the extracellular pteroylpolyglutamate patterns of *B. adolescentis* DSM 20083^{T}. *m/z*: SRM transitions of the folate vitamer; precursor→product ion.

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