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# Development of a Stable Isotope Dilution LC–MS Assay for the Quantitation of Multiple Polyethylene glycol (PEG) Homologues to be used in Permeability Studies

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## Abstract

A new quantitation method based on a multiple stable isotope dilution assay (SIDA) was developed for polyethylene glycol (PEG) homologues from PEG mixtures with average molecular weights (MW) of 400, 1500, 3000 and 4000 Da in urine. Seven [ $^{13}\text{C}_4^2\text{H}_4$ ] and two [ $^{13}\text{C}_8^2\text{H}_8$ ]-PEG homologues were synthesized and served as labelled internal standards for SIDA. PEG oligomers were resolved by reversed phase high performance liquid chromatography (RP-HPLC) coupled to mass spectrometry (MS) in multiple ion (MI) scan modus. Very low limits of detection (LODs) in a range of 0.4 – 12 ng/mL were achieved for the single homologues. Higher PEG homologues showed increased LODs and LOQs and less effective recovery (77 – 87%) than for PEG with lower molecular masses (95-121%). Precision (relative standard deviation) varied between 3 and 13% and showed no dependence of the chain length. The method was successfully applied to human and mice urine samples. Beside an accurate quantitation of single PEG homologues it was possible to show an alteration in the MW distribution in urine samples compared to the dosed PEG solutions. The highest MW, with which a PEG can pass the intestinal wall (so called “cut off”) for humans appeared to be higher than for mice.

**Keywords:** Polyethylene glycols; PEG reference compounds; stable isotope dilution assay; quantitative NMR; human and mice urine; membrane permeability

## 1. Introduction

Polyethylene glycols (PEG) are synthetic polymers of ethylene oxide with the general molecular formula  $H(OCH_2CH_2)_nOH$ , where  $n$  is the number of ethylene oxide units. They are often used in cosmetic and pharmaceutical products as basis for ointments or as carrier for active agents. The widespread use of PEG is based on their high water solubility, nontoxicity as well as their chemical and microbiological inertness. Furthermore they are available as mixtures of differently sized homologues in broad ranges of molecular weight (MW) distribution and are rapidly excreted into urine. In particular the latter two properties render them suitable markers for assessing intestinal barrier functions, e.g. in studies of inflammatory bowel diseases (IBD). According to the common hypothesis of trans-epithelial penetration in sound biological membranes a general decrease of intestinal permeability can be observed with an increase of PEG molecular weight detectable in urine. To study membrane permeability, Chadwick et al. [1] were the first to report data on PEG 400 as a marker and described its excretion depending on the dose. Later on, the PEG spectrum was extended to mixtures with higher MW ranging between 1000 and 10000 Da [2–5]. With this extension a decrease of permeability with increasing MW as well as differences in permeability of high MW PEG between healthy persons and patients with IBD, e.g. Crohn's disease, were observed [3]. Whereas PEG 400 is readily volatile after derivatization and can be analyzed by gas chromatography (GC) coupled to flame ionization detection (FID) [1], new analytical methods mostly based on reversed phase high performance liquid chromatography (RP-HPLC) for the higher PEG were developed. PEG are very simply structured molecules, which do not possess any chromophores and thus ultra violet or fluorescence detection are not suitable for their analysis. Escott et al. [6] circumvented this shortcoming by using low UV wavelength detection, but in practice evaporative light scattering detection (ELSD) or refractive index (RI) detection are more frequently applied [2,3,5,7]. Lately, detection by mass spectroscopy (MS) found its way into PEG analysis. Using MS technology, short measurement periods with ultra HPLC (UHPLC) or complete waiving of separation in flow injection analysis (FIA) MS are favored, without loss of accuracy. However, with the latter method only short-chained PEG (PEG 300, PEG 400) [8–10] and branched 40 kDa PEG [11] have been examined in biological or pharmaceutical samples. At present, electrospray ionization (ESI) with detection of the protonated molecules or the sodium, ammonium or potassium adducts have been applied most commonly. There are also reports on the successful use of in-source collision-induced dissociation (in-source CID)

of protonated high molecular PEG and methoxyl-PEG (8 and 40 kDa) [11,12] but this detection method lacks information about single PEG homologues. However, ESI is highly susceptible to ionization suppression or enhancement and, therefore, accurate quantitation needs either (a) tedious matrix calibration or (b) standard addition or (c) isotope-labelled internal standards, the latter of which have become the reference methods e.g. in clinical or sections of food analysis [13, 14]. Therefore the purpose of this study was the development of a stable isotope dilution assay (SIDA) as quantitation method for selected PEG homologues out of mixtures with average MW of 400, 1500, 3000 and 4000 Da and an adequate sample preparation for human and murine urine.

## **2. Materials and Method**

### **2.1. Chemicals and reagents**

Polyethylene glycol mixtures with nominal molecular masses ( $M_r$ ) of  $M_r$  400,  $M_r$  1500,  $M_r$  3000 and  $M_r$  4000 Da were obtained from Merck (Darmstadt, Germany). The  $M_r$  number corresponds to the average molecular mass of the mixtures and subscript numbers indicate the repetition of ethylene oxide units, e.g. PEG 400 consists of 10 homologues (PEG<sub>6</sub> – PEG<sub>15</sub>) with a Gaussian distribution and a mean  $M_r$  of 400 Da (mass range: 282 – 678 Da). The other mixtures are characterized as follows: PEG 1500 (PEG<sub>20</sub> – PEG<sub>45</sub>; mass range: 899 – 2000 Da), PEG 3000 (PEG<sub>51</sub> – PEG<sub>90</sub>; mass range: 2264 – 3982 Da) and PEG 4000 (PEG<sub>75</sub> – PEG<sub>115</sub>; mass range: 3322 – 5084 Da). PEG mixtures were the same for permeability studies and analytical procedures. Sodium hydride (60% as dispersion in mineral oil), sodium iodide, lithium aluminum deuteride and D<sub>4</sub>-methanol were from Sigma Aldrich (Steinheim, Germany). [<sup>13</sup>C<sub>2</sub>]-bromoacetic acid was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA) and all solvents were from Merck (Darmstadt, Germany) and were of analytical reagent grade.

### **2.2. Urine samples**

Human and murine urine samples were provided from the Chairs of Nutritional Medicine of the Technische Universität München as part of the human study approved by the Ethics Committee of the Faculty of Medicine of the Technische Universität München (File 5499/12) and the animal experiments performed with permission from the district government of Upper

Bavaria (Regierung von Oberbayern, reference number AZ 55.2.-1-54-2532-116-11), respectively. Human subjects ingested a mixture of PEG 400 (1 mg), PEG 1500 (200 mg), PEG 3000 (4 g) and PEG 4000 (4 g), whereas 12 weeks old BL/6J mice were subjected to dosing of PEG 1500 (3 mg) and PEG 3000 (10 mg). The sampling intervals for urine of humans and mice were 24 h and 14 h, respectively.

### **Purification of unlabelled PEG homologues by preparative HPLC-UV**

Single PEG homologues were isolated from the PEG 400, 1500, 3000 and 4000 mixtures. Therefore, a preparative HPLC-UV system equipped with UV detector (Saphire 600 Variable Wavelength Detector; ECOM spol.s r.o. Americka, Praha, Czech Republic), pumps (HD 2-400; BESTA-Technologie für Chromatographie GmbH, Wilhelmsfeld, Germany), sample injector (FCV-20AH2; Shimadzu, Kyoto, Japan), degasser (FLOM GASTORR VG-25 4; Fischer Analytics GmbH, Bingen am Rhein, Germany) valve unit (KRONLAB high Speed Valve; YMC Europa GmbH, Dinslaken, Germany) and PrepCon 5 software (YMC Europa GmbH, Dinslaken, Germany) was used. The wavelength was set to 190 nm and the mixtures were separated on a semi-preparative HPLC column (YMC, ProC18, 150 x 10 mm I.D., 5 µm) with water (A) and acetonitrile (B) at flow rates of 2 mL/min (PEG 400 and 1500) or 1 mL/min (PEG 3000 and 4000) and different gradients. For PEG 400, the starting conditions of 10% B was held for 1 min followed by raising the content of B to 24% within 19 min, which was held for 3 min. Thereafter, the gradient was brought within 1 min to the starting conditions and the column was equilibrated for 7 min. PEG 1500 were eluted stepwise with 27% B for the first 13 min and 30% (B) until 24 min, followed by 7 min of equilibration. A mixture of PEG 3000 and 4000 was separated into fractions under isocratic conditions at 35% B. After solvent removal and lyophilisation each fraction was rechromatographed with 35% B and separated in purer sub fractions. This procedure was repeated until only one PEG homologue remained in the fraction. Acetonitrile was evaporated and the remaining water content was removed by freeze drying.

### **2.3. Synthesis of <sup>2</sup>H- and <sup>13</sup>C-labelled standards**

The synthesis of labelled PEG homologous was carried out following the approach of Abello et al. [15] for the synthesis of labelled PEG monomethyl ether with some significant modifications for the synthesis of PEG. Accordingly, [<sup>13</sup>C<sub>2</sub>]-bromoacetic acid (3 equiv),

sodium iodide (0.01 equiv) and sodium hydride (as 60% dispersion in mineral oil; 10 equiv) were added to a solution of the PEG homologous in dichloromethane (1 mL/10  $\mu$ mol). After stirring for 48 h at 50 °C the reaction vessel was cooled to 5 °C and LiAlD<sub>4</sub> (7 equiv) was added. After 3 h the LiAlD<sub>4</sub> excess was quenched with methanol. The obtained suspension was centrifuged (5 min, 14000 rpm) and the residue was extracted twice with 1 mL DCM. The combined organic phases were washed to neutral pH with water, then brought to dryness by means of a mild stream of nitrogen and the residue was dissolved in water/acetonitrile (70/30; v/v) for purification with the preparative HPLC-UV at the same conditions as mentioned above. For labelled standards in the mass ranges of PEG 3000 and 4000 the synthesis and purification was repeated.

## 2.4. Quantitative nuclear magnetic resonance (qNMR) spectroscopy

Isolated PEG homologues were subjected to qualitative and quantitative <sup>1</sup>H-NMR spectroscopy. Lyophilisates were dissolved in 600  $\mu$ L D<sub>4</sub>-methanol (Sigma Aldrich, Steinheim, Germany), transferred into NMR tubes (5 x 178 mm; Bruker BioSpin Corporation, Fällanden, Switzerland) and analyzed with a 500 MHz Avance III NMR spectroscope (Bruker BioSpin Corporation, Fällanden, Switzerland). An external calibration with L-tyrosine and integration of the two protons at 7.1 ppm was carried out for quantitative <sup>1</sup>H-NMR, detailed data are reported elsewhere [16], [17]. PEG homologues were quantitated by the signal of 4 protons at 3.58 ppm. Furthermore the <sup>1</sup>H-NMR of [<sup>13</sup>C<sub>4</sub><sup>2</sup>H<sub>4</sub>]-PEG<sub>30</sub> was recorded.

## 2.5. LC-MS Method

### 2.5.1. Liquid chromatography (LC)

PEG oligomers were partially resolved by a liquid chromatography system consisting of pumps (LC-10Advp), sample injector (SILHTC) column oven (CTO-10Svp) and controller (SCL-10Avp), all components of the LC equipment were from Shimadzu (Kyoto, Japan). The column oven temperature was set to 30 °C. Chromatography was obtained on a LiChrospher 100 RP-18 column (250 x 4.6 mm I.D., 5  $\mu$ m; Alltech Grom GmbH, Rottenburg-Hailfingen, Germany) with a binary water (A) – acetonitrile (B) gradient, both solvents with 5 mmol/L ammonium acetate as additive and a flow rate of 0.5 mL/min. Starting conditions were 17.5% B and a two-step linear gradient to 30% B after 8 min and 38.5% B after 24 min followed.

Thereafter, the gradient was programmed within 1 minute to 100% B, then held for 5 min and returned to starting conditions at 32 min followed by 8 min of equilibration.

### 2.5.2. Mass spectrometry (MS)

LC was coupled to a triple quadrupole mass spectrometer (API 3000; Applied Biosystems Inc.; Foster City CA) operating in ESI positive mode and multiple ion (MI) scan type. The ion source parameters were set as follows: temperature 500 °C, nebulizer gas and curtain gas 12 and 10 psi, ion spray voltage 4800 V.

Intensities of  $m/z$  for the protonated PEG 400 and their ammonium adducts were monitored in one-dimensional MS. For PEG 1500 the  $m/z$  of the one, two and threefold ammoniated homologues were measured, together with the ammonium adducts of multiple charged (3-6 charges) PEG 3000 and PEG 4000. The amount of measured  $m/z$  was limited to the  $m/z$  of PEG<sub>9</sub>, PEG<sub>11</sub> and PEG<sub>13</sub> as key components of the PEG 400 mixture. PEG<sub>25</sub>, PEG<sub>30</sub>, PEG<sub>35</sub> and PEG<sub>40</sub> were chosen as key components of the PEG 1500 mixture and PEG<sub>65</sub>, PEG<sub>70</sub>, PEG<sub>75</sub>, PEG<sub>80</sub>, PEG<sub>85</sub> and PEG<sub>90</sub> as key homologues in the mass range of PEG 3000 and PEG 4000. The  $m/z$  values and ion species of quantifiers and qualifiers used in LC-MS analysis are listed in Table 1 and 2. Data analysis was performed using Analyst 1.5 software (Applied Biosystems Inc.; Foster City CA).

**Place Table 1 here**

**Place Table 2 here**

## 2.6. Sample Preparation

Urine samples were shaken vigorously and centrifuged for 5 min at 3000 g. From human urine 0.5 mL of the supernatant was used for analysis, murine urine was diluted 1:50 with deionized water and 0.5 mL of the dilution was used. 40 µL of the internal standard mixtures (Supplementary Data, Table S-1) were added to the human and 20 µL to the murine urine samples, the mixtures were shaken and purified by means of a C18 SPE cartridge (Discovery, C18, 100 mg/ 1 mL). After conditioning the cartridges successively with 1 mL of acetone, methanol and water, the samples were applied, washed with 1 mL of water/acetonitrile (90/10; v/v) and dried under reduced pressure. PEG and labelled PEG were eluted with 3 mL of water/acetonitrile (30/70; v/v) with a rate of 1 drop per second. The eluate was evaporated

under reduced pressure at 60 °C and the residue was solved in 0.5 mL (human urine samples) and 0.2 mL (murine urine samples) water/acetonitrile (90/10; v/v), respectively. After filtration (PVDF; 0.45 µm) 10 µL were used for LC-MS analysis. All solutions were measured in triplicate. With this procedure 4 human and 4 murine urine samples were prepared.

## **2.7. Method validation**

### *2.7.1. Response curves*

Response solutions with molar ratios of internal standard (S) and analyte (A)  $n(S)/n(A)$  of 1:10, 1:5, 1:2, 1:1, 2:1, 5:1 and 10:1 were prepared, with varying amounts of A. The amounts of S were 15 ng/mL (PEG<sub>9</sub> and PEG<sub>11</sub>), 9 ng/mL (PEG<sub>13</sub>), 60 ng/mL (PEG<sub>25</sub> and PEG<sub>30</sub>), 45 ng/mL (PEG<sub>35</sub> and PEG<sub>40</sub>), 80 ng/mL (PEG<sub>70</sub>) and 150 ng/mL (PEG<sub>80</sub>).

### *2.7.2. Synthetic urine*

As blank matrix for the limit of detection (LOD), limit of quantitation (LOQ) and the recovery a synthetic urine solution was prepared as follows (concentration in distilled water given in parenthesis in mmol/L): sodium dihydrogen phosphate monohydrate (53.8), creatinine hydrochloride (6.7), potassium chloride (45.7), ammonium chloride (41.1), sodium sulfate (31), sodium chloride (153), magnesium chloride hexahydrate (6.5), ammonium oxalate (0.4), calcium chloride (7.2) citric acid monohydrate (5.7) and urea (819).

### *2.7.3. Limit of Detection (LOD) and Limit of Quantitation (LOQ)*

LOD and LOQ were determined according to Vogelgesang and Hädrich [18]. Accordingly, four analyte concentrations were spiked to the synthetic urine and 30 µL of the mixtures of labelled standards were added prior to SPE clean up. The analyzed concentrations are given in the Supplementary Data, Table S-2. Each spiking level was analyzed in triplicate. Then, the data obtained from stable isotope dilution assays and spiked amounts were correlated. A subsequent regression calculation provided the calibration line and the confidence interval, which was used to compute the LOD and LOQ.



#### 2.7.4. Precision

Inter-assay and intra-assay precision (relative standard deviation) were determined by extracting and analyzing one human and murine urine sample three times in triplicate within three weeks. Repeatability was evaluated from multiple analyses of one sample extract.

#### 2.7.5. Recovery

The synthetic urine was spiked with low, medium or high PEG concentrations (Supplementary Data, Table S-3) and 40  $\mu\text{L}$  of standard solution followed by sample preparation. Each spiking level was analyzed in triplicate.

### 2.8. Quantification of PEG homologues in the PEG mixtures

The key PEG homologues in the dosed PEG mixtures were quantified in three different concentrations of aqueous solutions of PEG 400, PEG 1500, PEG 3000 and PEG 4000 mixtures. 5.4 ng [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>9</sub> and [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>11</sub> and 1.5 ng [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>13</sub> were added to 50, 75 and 100 ng of PEG 400. 125, 250 and 375 ng PEG 1500 were combined with 30, 30, 60 and 30 ng of [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>25</sub>, [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>30</sub>, [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>35</sub> and [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>40</sub>. 250, 375 and 500 ng PEG 3000 were mixed with 15 and 30 ng of [ $^{13}\text{C}_8^2\text{H}_8$ ]-PEG<sub>70</sub> and [ $^{13}\text{C}_8^2\text{H}_8$ ]-PEG<sub>80</sub>. For the quantification of PEG<sub>80</sub> in PEG 4000 250, 375 and 500 ng of the mixture was spiked with 15 ng [ $^{13}\text{C}_8^2\text{H}_8$ ]-PEG<sub>80</sub>.

Analyses of PEG 1500, PEG 3000 and PEG 4000 showed that they contained lower PEG homologues. For quantification of PEG<sub>9</sub>, PEG<sub>11</sub> and PEG<sub>13</sub> 9 ng of [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>9</sub>, [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>11</sub> and [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>13</sub>, respectively, were added to PEG 1500 (2.5 and 5  $\mu\text{g}$ ), PEG 3000 (5 and 10  $\mu\text{g}$ ) and PEG 4000 (5 and 8  $\mu\text{g}$ ). 6, 6, 9 and 15 ng of [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>25</sub>, [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>30</sub>, [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>35</sub> and [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>40</sub> were spiked to 5 and 10  $\mu\text{g}$  of PEG 3000 and 3.6 ng of [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>25</sub>, [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>30</sub>, [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>35</sub> and [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>40</sub> to 5 and 8  $\mu\text{g}$  of PEG 4000.

All combinations were brought up to 0.5 mL, vigorously shaken and analyzed per LC-MS without further treatment.

## 3. Results

### 3.1. Purification of unlabelled PEG homologues by preparative HPLC-UV

A major constraint of all previously reported methods for PEG homologues is the lack of sufficiently pure reference compounds for calibration of the quantitation method for the single homologues. For this reason, single PEG homologues were isolated from the PEG 400, PEG 1500, PEG 3000 and PEG 4000 mixtures by means of a preparative HPLC-UV system. In this way, pure (> 99% homologues purity, determined with LC/MS) PEG<sub>9</sub>, PEG<sub>11</sub>, PEG<sub>13</sub>, PEG<sub>25</sub>, PEG<sub>30</sub>, PEG<sub>35</sub>, PEG<sub>40</sub>, PEG<sub>70</sub> and PEG<sub>80</sub> were generated as references for analytes and, furthermore, PEG<sub>7</sub>, PEG<sub>9</sub>, PEG<sub>11</sub>, PEG<sub>23</sub>, PEG<sub>28</sub>, PEG<sub>33</sub>, PEG<sub>38</sub>, PEG<sub>66</sub> and PEG<sub>76</sub> were applied as educts for the synthesis of labelled internal standards.

### 3.2. Synthesis of [<sup>13</sup>C<sup>2</sup>H]-labelled standards

Abello et al. [14] prepared [<sup>13</sup>C] labelled PEG monomethyl ethers and other PEG derivatives via chain extension by one ethylene glycol unit with [1-<sup>13</sup>C<sub>1</sub>] or [1,2-<sup>13</sup>C<sub>2</sub>]-bromoacetic acid. This method was modified in our study to synthesize [<sup>13</sup>C<sup>2</sup>H]-labelled PEG. Firstly, both alcohol groups were substituted by [1,2-<sup>13</sup>C<sub>2</sub>]-bromoacetic acid to obtain a chain extension by two ethylene glycol units. Afterwards LiAlD<sub>4</sub> was added to reduce the formed di-acids and to introduce further deuterium labels in one step. This synthesis provided PEG labelled with 8 stable isotopes. The procedure was repeated for the isotopologues of PEG<sub>70</sub> and PEG<sub>80</sub> to obtain 16 stable isotope labels. The yields ranged between 1.5 and 11.7% for [<sup>13</sup>C<sub>4</sub><sup>2</sup>H<sub>4</sub>]-labelled PEG isotopologues and between 0.7 and 3.4% for [<sup>13</sup>C<sub>8</sub><sup>2</sup>H<sub>8</sub>]-PEG<sub>70</sub> and [<sup>13</sup>C<sub>8</sub><sup>2</sup>H<sub>8</sub>]-PEG<sub>80</sub>.

### 3.3. Quantitative nuclear magnetic resonance (q-NMR) spectroscopy

In D<sub>4</sub>-methanol, PEG show two signals in <sup>1</sup>H-NMR, namely a triplet at 3.58 ppm with an integral of 4 protons and a multiplet at 3.66 ppm from the proton of four times the number of ethylene glycol monomers minus 6 protons. With the signal at 3.58 ppm the amounts of isolated PEG homologues were calculated. This signal originates from the four protons at the two carbons in β position at each end of the polymer chain. In the [<sup>13</sup>C<sub>4</sub><sup>2</sup>H<sub>4</sub>]-labelled standard, these four protons couple with <sup>13</sup>C-carbon atoms, which results in a split of the 3.58 ppm

signal into a doublet of doublets signal at 3.38 ppm and 3.74 ppm with integrals of two protons each. This phenomenon was already reported by Asam et al. [19] for multiply  $^{13}\text{C}$ -labelled mycotoxins. Figure 1 shows  $^1\text{H}$ -NMR spectra of  $\text{PEG}_{30}$  and  $[^{13}\text{C}_4^2\text{H}_4]\text{-PEG}_{30}$ .

**Place Figure 1 here**

### 3.4. MS behavior of PEG

In preliminary experiments, the following, remarkable properties of PEG in the MS instrument were observed: (a) The low MW PEG formed adducts with any kind of cations (e.g. proton, ammonium, sodium, potassium) present in the MS source, whereas higher MW PEG preferred ammonium and sodium ions to protons and had (b) the ability to form multiply charged adducts. The higher the PEG homologue was the more charges it could acquire, e.g.  $\text{PEG}_{80}$  was loaded with up to six charges, but (c) in parallel several less charged  $\text{PEG}_{80}$  were present. Even more complex in PEG analysis was that (d) the multiple charges could be formed by different combinations of cations, e.g. the PEG 1500 mixture showed simultaneously  $m/z$  of three fold charged adducts of  $(3x\text{NH}_4)^{3+}$ , along with  $(2x\text{NH}_4 + 1x\text{Na})^{3+}$ ,  $(1x\text{NH}_4 + 2x\text{Na})^{3+}$  and  $(3x\text{Na})^{3+}$ . Possible ion compositions increased with increasing numbers of charges, and (e) fragmentation in the selective reaction monitoring (SRM) mode of the MS was only successful with low MW PEG (PEG 400). Due to preferred formation of the more stable ammonium and sodium adducts [20] of homologues from the PEG 1500, PEG 3000 and PEG 4000 mixtures, in-source collision-induced dissociation (CID) of protonated homologues as described by Warrack et al. [11] and Gong et al. [12] had an extremely low intensity and could not serve as parental ions for CID/SRM experiments. We assume that this disagreement to the studies of the latter authors is due to our mobile phase containing ammonium acetate and the different geometry of our ion source, which obviously enhanced formation of ammonium and sodium adducts. These adducts of high MW PEG showed only the loss of one charge and, thus, ended up in higher  $m/z$  values. These properties of PEG in MS analysis had benefits and drawbacks at the same time. Except for PEG 400, formation of adducts with varying cations (c.f. d) could be suppressed by using ammonium acetate as modifier in the solvents, which led to ammonium adducts with constant charge distribution for one homologue (c.f. c). Measurement with fragmentation provides more selectivity, but, due to the poor fragmentation behavior of PEG (c.f. e), multiple ion (MI) modus was the best

alternative. The lower selectivity was counterbalanced by measuring different adducts for PEG 400 (c.f. a) or different charge stages for PEG with higher MW (c.f. b). On the one hand, the presence of different charges of one homologue reduced the sensitivity and led to overlaps of  $m/z$  from different PEG, on the other hand with multiple charges the  $m/z$  values of high MW PEG lay in the mass range of the MS instrument and, by considering the retention time, clear assignment of  $m/z$  to one PEG was possible.

### 3.5. Method validation

#### 3.5.1. Response curve

Response curves for PEG were linear in a very small molar standard (S) to analyte (A) ratio  $n(S)/n(A)$  from 1:1 to 10:1 except for PEG<sub>11</sub>, which was linear from 1:10 to 10:1. The small linear range is possibly due to the high variability of the formation of adducts and charges. To extend the working range the response points with an excess of S were included and the response curves then fitted an  $x^2$  equation. The respective equations were:

$$\text{PEG}_9: y = -0.0022x^2 + 0.3637x - 0.1197 \quad R^2 = 0.999$$

$$\text{PEG}_{11}: y = 1.1221x - 0.1007 \quad R^2 = 0.999$$

$$\text{PEG}_{13}: y = 0.0040x^2 + 0.2727x - 0.0147 \quad R^2 = 1.000$$

$$\text{PEG}_{25}: y = 0.1904x^2 + 1.5994x + 0.0299 \quad R^2 = 0.999$$

$$\text{PEG}_{30}: y = 0.0779x^2 + 0.7314x + 0.0514 \quad R^2 = 1.000$$

$$\text{PEG}_{35}: y = 0.2083x^2 + 2.4818x - 0.0447 \quad R^2 = 1.000$$

$$\text{PEG}_{40}: y = 0.0103x^2 + 0.7541x - 0.0406 \quad R^2 = 1.000$$

$$\text{PEG}_{70}: y = 0.0318x^2 + 1.5504x - 0.0897 \quad R^2 = 1.000$$

$$\text{PEG}_{80}: y = 0.0968x^2 + 0.7869x + 0.101 \quad R^2 = 0.999$$

with  $y$  = molar ratio  $n(S)/n(A)$  and

$x$  = ratio of areas  $A(S)/A(A)$  of the respective ions of quantifiers listed in Table 1.

#### 3.5.2. Limits of detection and limits of quantitation

The limits of detection (LODs) and limits of quantitation (LOQs) were determined following a standard procedure described in literature [18]. As an authentic urine free from PEG residues could not be obtained, LODs and LOQs were determined in synthetic urine at a reported mean constitution according to Oreopoulos et al. and Tiselius et al. [21, 22]. Homologues from PEG 400 and PEG 1500 revealed LODs between 0.4 and 1.8 ng/mL, LOQs ranged between 1.1 and 5.4 ng/mL. The respective values for PEG<sub>70</sub> and PEG<sub>80</sub> were 5-10 times higher. Detailed data are shown in Place Table .

### 3.5.3. Recovery

Recoveries of PEG in synthetic urine ranged between 77±4% and 121±9% (Table 3). Homologues with high molecular weights (PEG<sub>70</sub>, PEG<sub>80</sub>) had the lowest recoveries.

**Place Table 3 here**

### 3.5.4. Precision

One human and one murine urine sample showing low and high PEG concentrations, respectively, were analyzed three times in triplicate during a period of three weeks. From this data precision (relative standard deviation) was evaluated. Inter-day precision gives the deviation of the results from different days, whereas the variances of analysis within one day are mirrored in intra-day precision. Repeatability describes the differences of repeated injections of the same solution. For the PEG homologues the inter-day and intra-day precision ranged from 3.2 – 15.6% and 3.0 – 9.5% (Table 4). The values for repeatability varied between 2.7 and 9%, there were no differences between the precision of human and murine urine samples.

**Place Table 4 here**

## 3.6. Quantification of PEG homologous in PEG mixtures

The determined concentrations of the PEG homologues represent the Gaussian distribution in the PEG mixtures. Remarkably the mixture of higher PEG contained PEG of lower MW,

which has to be considered in permeability studies using several mixtures. Detailed results are given in the Supplementary Data, Table S-4.

### **3.7. Determination of PEG in human and murine urine samples**

Four human and four murine urine samples were analyzed for PEG using the developed LC-ESI-MS method and concentrations in the sample portions were calculated from isotope ratios and the response curves. To obtain the absolute excreted content of PEG, the concentrations were multiplied with the urine volume. In Table 5 and Table 6 the PEG excretions from humans [in  $\mu\text{g}$  absolute] and mice [in  $\text{ng}$  absolute], respectively, and dose recoveries [%] are listed. Dose recoveries were calculated by dividing the absolute amount found in urine by the administered PEG dose and are given in percentages. In two human urine samples all PEG homologues could be quantitated, whereas in one sample only homologues from the PEG 400 mixture could be determined. Another sample contained homologues from PEG 400 and PEG 1500 mixtures. In all samples signals for PEG<sub>65</sub> and PEG<sub>75</sub> and in two samples signals for PEG<sub>85</sub> and PEG<sub>90</sub> could be detected. In contrast to this, no mice urine sample revealed detectable amounts of PEG<sub>90</sub>, PEG<sub>85</sub> and PEG<sub>80</sub> (except one sample), but in all samples PEG<sub>25</sub>, PEG<sub>30</sub>, PEG<sub>35</sub> and PEG<sub>40</sub> could be quantitated. For both human and murine urine samples, homologues from the PEG 400 mixture had the highest dose recovery. The permeability of homologues from PEG 1500 and PEG 3000 was 10 to 100 times lower and ranged between 0.07 and 1.3% in human urine and 0.03 and 1.1% in murine urine. Figure 2 shows the chromatograms of PEG 400, PEG 1500, PEG 3000 and PEG 4000 standard solutions (A) in comparison to a human urine sample (B). The PEG homologues and their corresponding labelled standards used for quantification are illustrated in (C). PEG 3000 standard solution (A, green) nearly had a Gaussian distribution, which showed an alteration in the human urine sample (B, green) due to worse in vivo permeability of higher MW PEG. Homologues from the PEG 4000 mixture (B, purple) were clearly discriminated or did not permeate the intestinal wall at all.

**Place Figure 2 here.**

**Place Table 5 here**

**Place Table 6 here**

## 4. Discussion

To the best of our knowledge this is the first report on PEG quantification achieved by stable isotope dilution assay. The response curves did not fit a linear regression and therefore, had to be adjusted to quadratic regression. This phenomenon already occurred in other studies with external calibration of PEG 300 and PEG 6000, as in one case the distribution and homogeneity in drug formulation was evaluated [10] and in the other case PEG in nano-sized pharmaceutical formulations was determined and the yield of PEGylation was estimated [4]. Zhang et al. [10] described a dependence of the ionization type with a quadratic equation in ESI mode and a linear regression in APCI mode. However, with our quadrupole instrument in APCI mode no multiple charges were formed and the  $m/z$  of single charged PEG 3000 and PEG 4000 fell outside the mass range of the instrument. Hence APCI was no alternative for the envisaged PEG analysis. By contrast, ESI required at least a partial separation of the homologues because of the multiple charges, which caused overlaps of  $m/z$  from smaller PEG homologues in a less charged state and longer PEG homologues with more charges. Examples for overlaps are (1) the ammonium adduct of PEG<sub>13</sub> ( $m/z$  608.7 Da) and PEG<sub>80</sub> with six ammonium charges ( $m/z$  608.4 Da) or (2) PEG<sub>65</sub> with five ammonium charges ( $m/z$  594.3 Da), six fold ammoniated PEG<sub>78</sub> ( $m/z$  593.7 Da) and the adduct of PEG<sub>91</sub> with seven ammonium ( $m/z$  593.3 Da). This restriction could be circumvented by LC separation, but this clearly extended the measurement time in comparison to other methods [8-11]. In the chromatograms a shift of retention time between the analyte and the corresponding labelled standard of about 0.1 min was observed. This is generally the case [23,24] when deuterium is used as stable isotope label. In our method half of the labels (4 and 8 labels respectively) were deuterium, but with the high MW of PEG (414 – 3542 Da) it was surprising, that it resulted in a shift of 6 sec in the retention time. Due to the natural abundance of [<sup>13</sup>C] 16 isotope labels for PEG<sub>70</sub> and PEG<sub>80</sub> and 8 isotope labels for PEG<sub>25</sub>, PEG<sub>30</sub>, PEG<sub>35</sub> and PEG<sub>40</sub> were



necessary. For PEG<sub>9</sub>, PEG<sub>11</sub> and PEG<sub>13</sub> 4 labels would have been suitable, but their ability to form sodium adducts, which have a mass difference from 5 Da to the ammoniated PEG, would have led to *m/z* overlaps with the sodium adducts of the analyte and the ammonium adducts of the 4 fold labelled standards. Because of this, 8 fold isotope labelled standards for homologues from the PEG 400 mixture were synthesized. Labeling with <sup>13</sup>C alone was not feasible because of the low yields (1.5 – 11.7%) obtained in the synthesis. The differences to the higher yields reported in literature ([15]; 75-95%) could be explained, on the one hand, with the use of short chained PEG monomethyl ether (one reactive hydroxyl group) as educts by Abello et al. [15] and, on the other hand, due to the use of medium to long-chained PEG diols in our study. The longer ethylene oxide chains resulted in worse reaction kinetics, which could be compensated for to only a limited extend by prolonged reaction times. Additionally, the PEG diols had to be substituted on both chain ends with [<sup>13</sup>C<sub>2</sub>]-bromoacetic acid and completely reduced with LiAlD<sub>4</sub> to achieve the desired [<sup>13</sup>C<sub>4</sub><sup>2</sup>H<sub>4</sub>]- or [<sup>13</sup>C<sub>8</sub><sup>2</sup>H<sub>8</sub>]-homologues. Nevertheless, the received amounts of labelled PEG were adequate for the purpose of our work, especially due to the high sensitivity of PEG detection in mass spectrometry, which led to very low LODs for the PEG homologues. The determined LODs for homologues in the PEG 400 and PEG 1500 mixtures ranged between 0.4 and 1.8 ng/mL and were 11.8 and 10.0 ng/mL for PEG<sub>70</sub> and PEG<sub>80</sub>, respectively. Direct comparison with reports of LODs of PEG mixtures is difficult, because naturally the LODs of the most abundant single PEG homologues are smaller. But even if the Gaussian distribution of the mixtures was considered, the calculated values were far below the data found in literature, which ranged from 0.1 to 50 µg/mL for PEG 400 with ELSD and RI detection [3,5,7] and 45 to 800 ng/mL for PEG 300 and PEG 400 with MS detection, respectively [8–10]. Data for the larger PEG varied between 0.1 and 5 µg/mL with ELSD and RI [3–5,7], but no values for MS detection of PEG with similar MW could be found. Recovery ranges for differently sized PEG of 87 - 104% were given in literature [1,3,5]. The values were comparable with the calculated

recoveries for PEG homologues in the present study, which varied between 80 – 120%. With RSDs of 3 – 15% the precisions were similar to those reported by Schwertner and Patterson [25] (4.6%) and [1] (7.1%), but higher than the RSDs determined from [2] (1.2 – 3.6%). For both, recovery and precision of PEG, no MS data were available in literature.

The method was successfully applied to urine samples. The dose recoveries found in human urine samples for homologues of the PEG 400 mixture ranged between 0.7 and 38.4%, for PEG 1500 between 0.07 and 1.3% and for PEG 3000 and PEG 4000 between 0.23 and 0.3%. Except for PEG<sub>70</sub> and PEG<sub>80</sub>, the recoveries tended to be lower than reported in literature [3], where 34% of PEG 400, 1.1% of PEG 1500 and 0.05% of PEG 4000 were recovered. A possible explanation for this finding is the lower dose of PEG 400 (1 mg) and PEG 1500 (200 mg) ingested by the subjects in the current study, whereas Parlesak et al. [3] administered 2 g and 1.5 g of PEG 400 and PEG 1500. Nevertheless, a general decrease of permeability with increasing MW of the PEG homologues could be confirmed in human and murine urine samples. The method enables the estimation of the highest MW (“cut off”) with which a PEG homologue can permeate through the intestinal barrier. Therefore, additional m/z values for every fifth homologue of the PEG 3000 and PEG 4000 mixtures (PEG<sub>65</sub>, PEG<sub>70</sub>, PEG<sub>75</sub>, PEG<sub>80</sub>, PEG<sub>85</sub> and PEG<sub>90</sub>) were included into the method and the cut off was determined as the MW between the last visible homologue and the next higher homologue. With this strategy we found that the cut off expectedly was different for the single individuals and ranged from between PEG<sub>65</sub> and PEG<sub>70</sub> (2882 – 3102 Da) to homologues with MW higher than PEG<sub>90</sub> (3983 Da) for humans and ranged between PEG<sub>65</sub> and PEG<sub>85</sub> (2882 – 3763 Da) in mice. However, this is only indicative as our data are not representative.

With the successful validation and application the SIDA presented here offers the perspective as an accurate tool in future permeability studies. Further applications in human studies on alterations of intestinal permeability are under way.

## **Acknowledgment**

We gratefully acknowledge Dr. Oliver Frank, Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, for the q-NMR measurements. We thank Alexander Ebner, Bioanalytik Weihenstephan, Technische Universität München, for assistance in analyzing several samples. Furthermore, we appreciate the cooperation with Beate Ott (Chair of Clinical Nutritional Medicine) and Caroline Kless (Chair of Molecular Nutritional Medicine), who provided human and murine urine samples.

## **Appendix A. Supplementary Data**

Supplementary Data associated with this article can be found in the online version.

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## CAPTIONS TO THE FIGURES

**Figure 1:**  $^1\text{H}$ -NMR spectra of PEG<sub>30</sub> (A) and [ $^{13}\text{C}_4^2\text{H}_4$ ]-PEG<sub>30</sub> (B) with the signals for D<sub>4</sub>-methanol at 3.31 ppm. Enlarged is the relevant segment for PEG<sub>30</sub> with a signal for the 4 protons at the  $\beta$  positioned carbon atoms at 3.58 ppm, which were used for quantification. The signal at 3.66 ppm contains the inner protons and the 4 protons at the  $\alpha$  positioned carbon atoms. In B the signal of the 4 protons at the  $\beta$  positioned carbon atoms is split to 3.38 and 3.74 ppm due to the coupling of the protons with the  $^{13}\text{C}$ -carbon atoms of the labelled standard.

**Figure 2:** LC-ESI-MS chromatograms of PEG 400 (retention time  $r_t$  6-12 min, red;  $m/z = (\text{M}+\text{NH}_4)^+$ ; PEG<sub>7</sub> - PEG<sub>15</sub>), PEG 1500 ( $r_t$  13-18 min blue;  $m/z = (\text{M}+2\text{NH}_4)^{2+/2}$ ; PEG<sub>25</sub> – PEG<sub>40</sub>), PEG 3000 ( $r_t$  22-25 min green;  $m/z = (\text{M}+3\text{NH}_4)^{3+/3}$ ; PEG<sub>60</sub> – PEG<sub>80</sub>) and PEG 4000 ( $r_t$  25-29 min purple;  $m/z = (\text{M}+3\text{NH}_4)^{3+/3}$ ; PEG<sub>81</sub> – PEG<sub>110</sub>); A: Chromatogram of PEG 400 (0.1  $\mu\text{g}/\text{mL}$ ), PEG 1500 (0.25  $\mu\text{g}/\text{mL}$ ), PEG 3000 (3  $\mu\text{g}/\text{mL}$ ) and PEG 4000 (3  $\mu\text{g}/\text{mL}$ ) mixtures. B: Chromatogram of one human urine sample after clean up with SPE. C: Chromatogram of analytes selected from B and the corresponding labelled standards (shown dashed): PEG<sub>9</sub>: 8.26 min; PEG<sub>11</sub> 9.39 min; PEG<sub>13</sub> 10.38 min; PEG<sub>25</sub> 14.01 min; PEG<sub>30</sub> 15.19 min; PEG<sub>35</sub> 16.41 min; PEG<sub>40</sub> 17.66 min; PEG<sub>70</sub> 23.65 min and PEG<sub>80</sub> 25.03 min.

# FIGURES

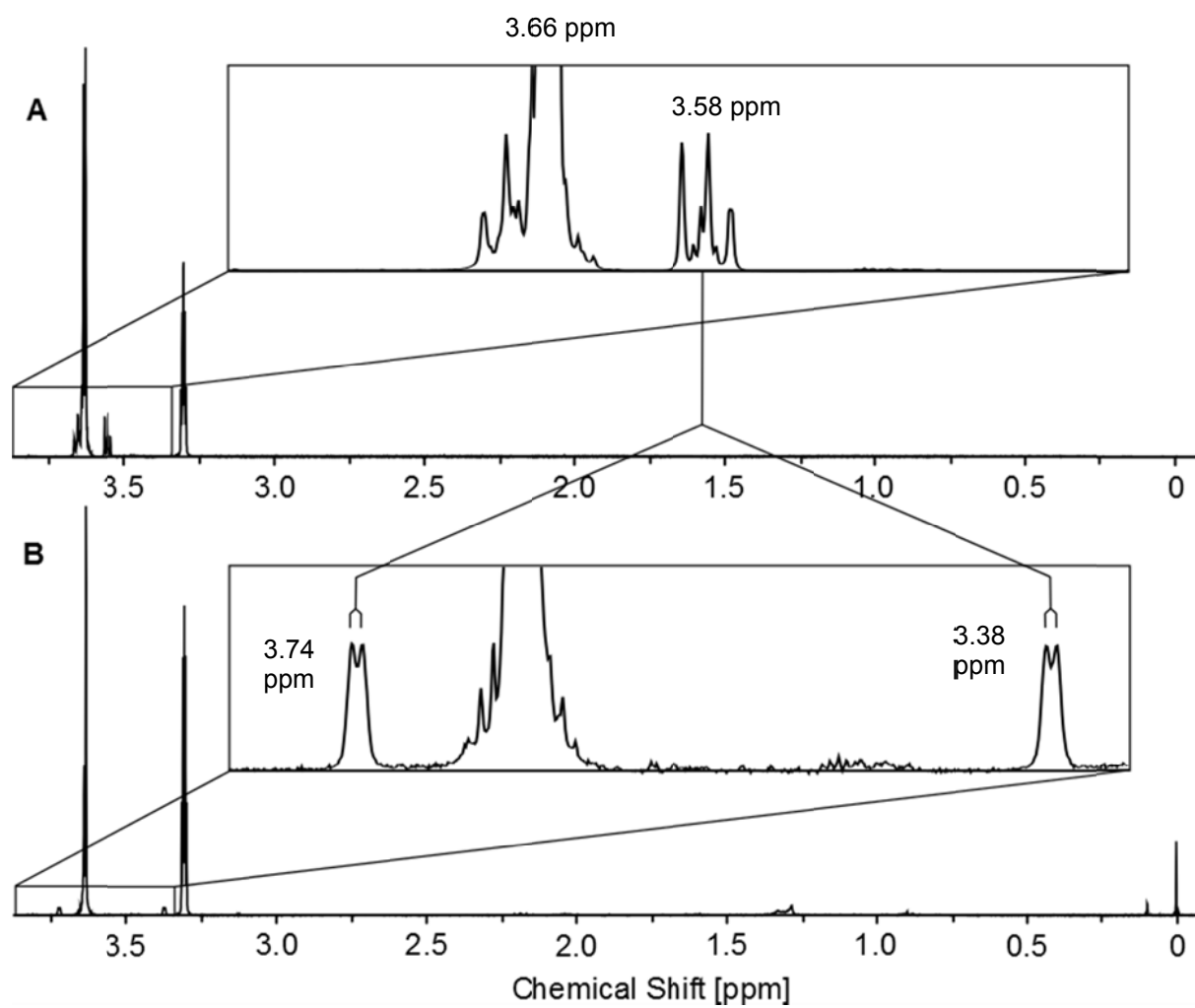


Figure 1

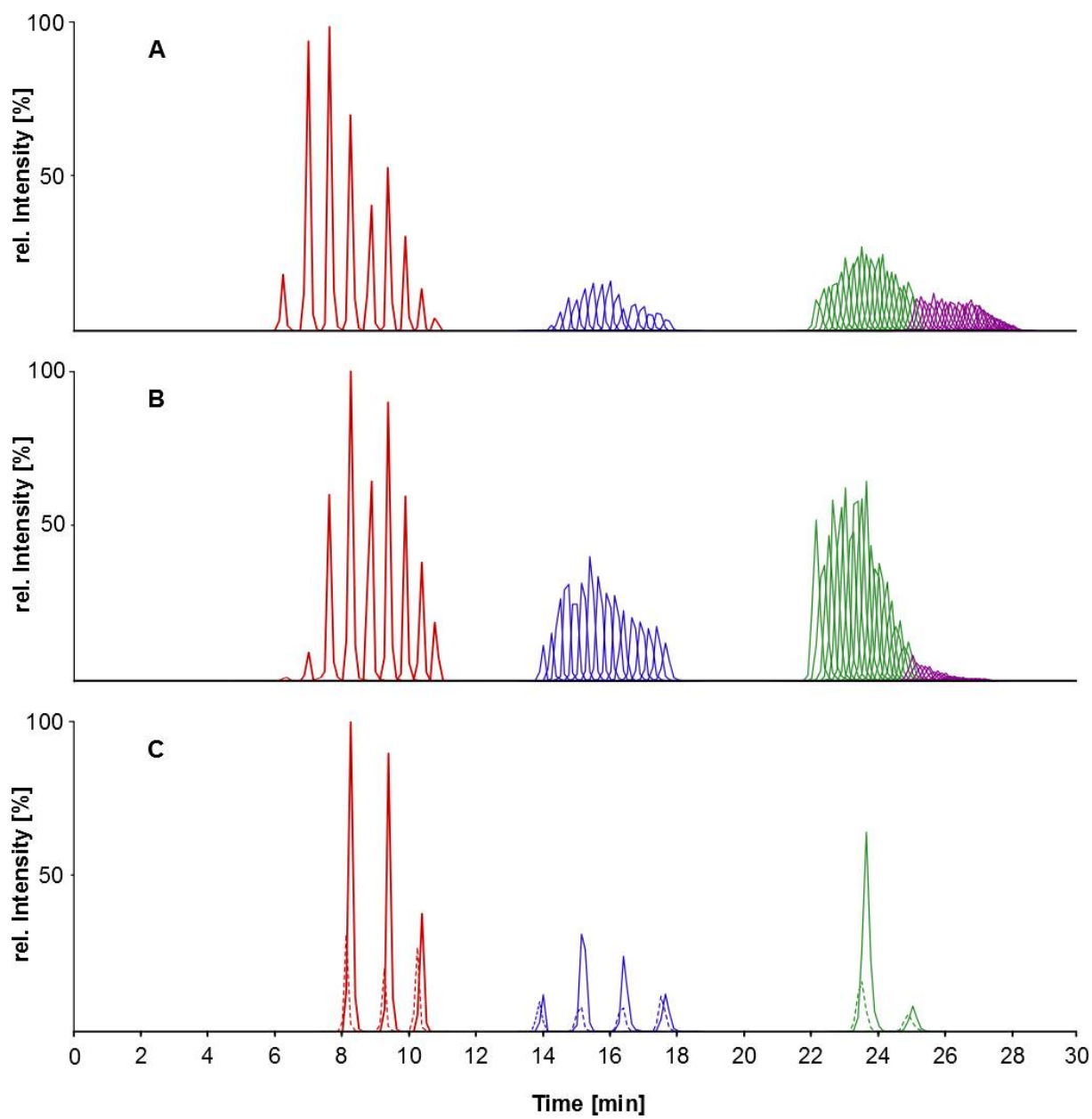


Figure 2

## SUPPLEMENTARY DATA 1

### Development of a Stable Isotope Dilution LC–MS Assay for the Quantitation of Multiple Polyethylene glycol (PEG) Homologues to be used in Permeability Studies

Martina Lichtenegger and Michael Rychlik

The Supplementary Data contain further experimental details in the Tables S-1 to S-4. Additionally, a reagent blank chromatogram (Figure S-1) and a chromatogram of blank human urine (Figure S-2) is shown. The latter reveals several signals of ubiquitously occurring PEGs.

### Post-column infusion experiments to assess matrix effects on LC-MS signal intensity

In order to assess matrix effects on ion-suppression or ion-enhancement we analyzed authentic “blank” urine (extracted by SPE) by post-column infusion of PEG solutions (concentrations) via syringe pump. PEG concentrations of the homologues in the blank urine were low enough not to interfere with the infused PEG. There was no ion-suppression or ion-enhancement in the retention time range from 6 to 34 min after injection (Figure S-3). Therefore, sample clean up obviously was very effective and urine matrix had no influence on the ionization of PEG.



## TABLES

**Table S-1: Concentrations of the labelled internal standards used for human and murine urine samples**

<b>Internal standard</b>	<b>standard concentration for sample preparation [<math>\mu\text{g/mL}</math>]</b>
$^{13}\text{C}_4^2\text{H}_4$ -PEG <sub>9</sub>	0.18
$^{13}\text{C}_4^2\text{H}_4$ -PEG <sub>11</sub>	0.15
$^{13}\text{C}_4^2\text{H}_4$ -PEG <sub>13</sub>	0.09
$^{13}\text{C}_4^2\text{H}_4$ -PEG <sub>25</sub>	0.12
$^{13}\text{C}_4^2\text{H}_4$ -PEG <sub>30</sub>	0.12
$^{13}\text{C}_4^2\text{H}_4$ -PEG <sub>35</sub>	0.18
$^{13}\text{C}_4^2\text{H}_4$ -PEG <sub>40</sub>	0.30
$^{13}\text{C}_4^2\text{H}_8$ -PEG <sub>70</sub>	0.50
$^{13}\text{C}_4^2\text{H}_8$ -PEG <sub>80</sub>	1.00

**Table S-2: Analyte and labelled standard concentrations for the determination of LOD and LOQ**

<b>Analyte</b>	<b>Spiking level 1 [ng/mL]</b>	<b>Spiking level 2 [ng/mL]</b>	<b>Spiking level 3 [ng/mL]</b>	<b>Spiking level 4 [ng/mL]</b>	<b>[<math>^{13}\text{C}^2\text{H}</math>]- Standard [ng/mL]</b>
PEG <sub>9</sub>	0.3	1.2	2.1	3.0	30
PEG <sub>11</sub>	0.3	1.2	2.1	3.0	30
PEG <sub>13</sub>	0.3	1.2	2.1	3.0	30
PEG <sub>25</sub>	1.5	6.0	10.5	15	150
PEG <sub>30</sub>	1.5	6.0	10.5	15	150
PEG <sub>35</sub>	1.2	4.8	8.4	12	120
PEG <sub>40</sub>	1.2	4.8	8.4	12	120
PEG <sub>70</sub>	4.0	16	28	40	400
PEG <sub>80</sub>	4.0	16	28	40	400

**Table S-3: Spiking levels and internal standard concentrations for the determination of PEG recovery in synthetic urine**

Analyte	Spiking level 1 [ng/mL]	Spiking level 2 [ng/mL]	Spiking level 3 [ng/mL]	[ <sup>13</sup> C <sup>2</sup> H]-Standard [µg/mL]
PEG <sub>9</sub>	18	54	90	0.18
PEG <sub>11</sub>	15	45	75	0.15
PEG <sub>13</sub>	18	54	90	0.09
PEG <sub>25</sub>	6	18	30	0.12
PEG <sub>30</sub>	12	36	60	0.12
PEG <sub>35</sub>	18	54	90	0.18
PEG <sub>40</sub>	30	90	150	0.30
PEG <sub>70</sub>	60	180	300	0.50
PEG <sub>80</sub>	40	120	200	1.00

**Table S-4: Quantification of selected PEG homologues in PEG mixtures.**

PEG homologue [µg/mg mixture]	PEG 400	PEG 1500	PEG 3000	PEG 4000
PEG <sub>9</sub>	217	0.15	0.16	0.05
PEG <sub>11</sub>	176	0.23	0.19	0.05
PEG <sub>13</sub>	54	0.35	0.20	0.07
PEG <sub>25</sub>		14	0.63	0.18
PEG <sub>30</sub>		59	0.86	0.22
PEG <sub>35</sub>		76	0.90	0.45
PEG <sub>40</sub>		32	1.35	0.7
PEG <sub>70</sub>			62	
PEG <sub>80</sub>			34	11

Figure S-1: reagent blank chromatogram

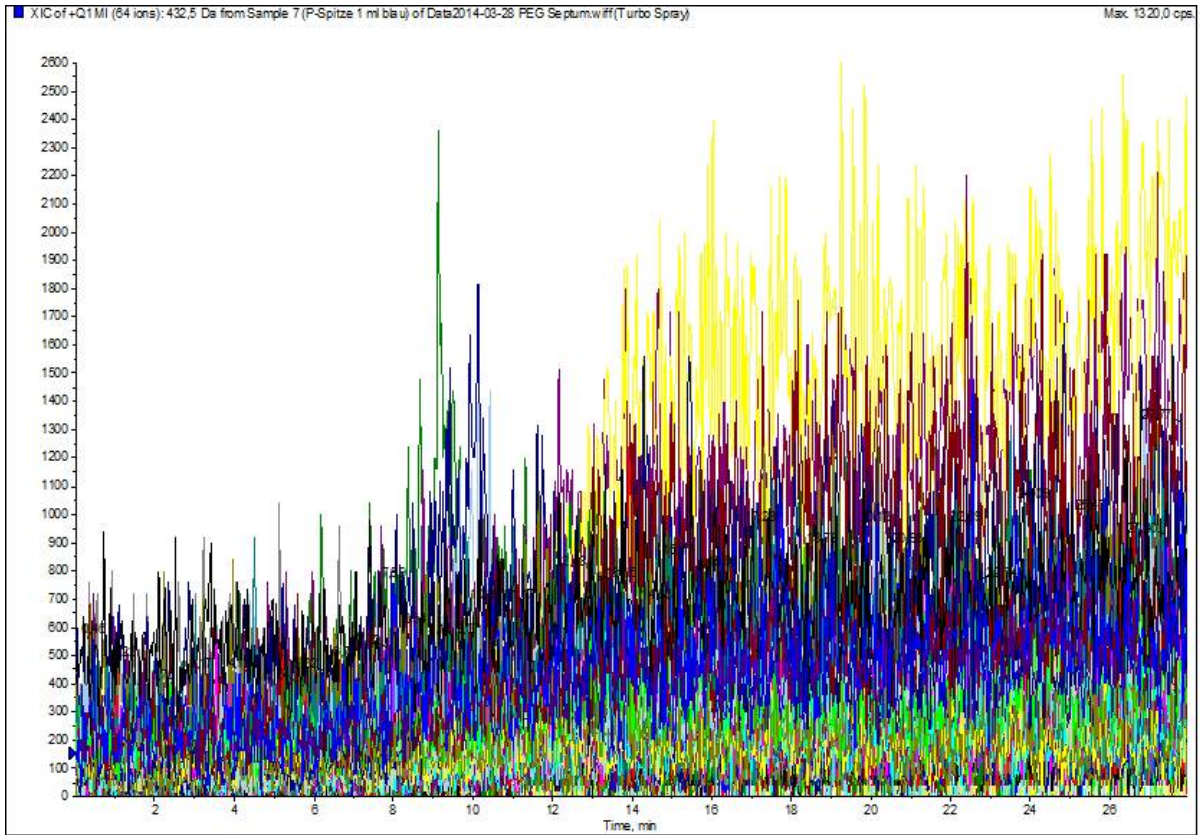


Figure S-2: chromatogram of blank human urine revealing several signals of ubiquitously occurring PEGs

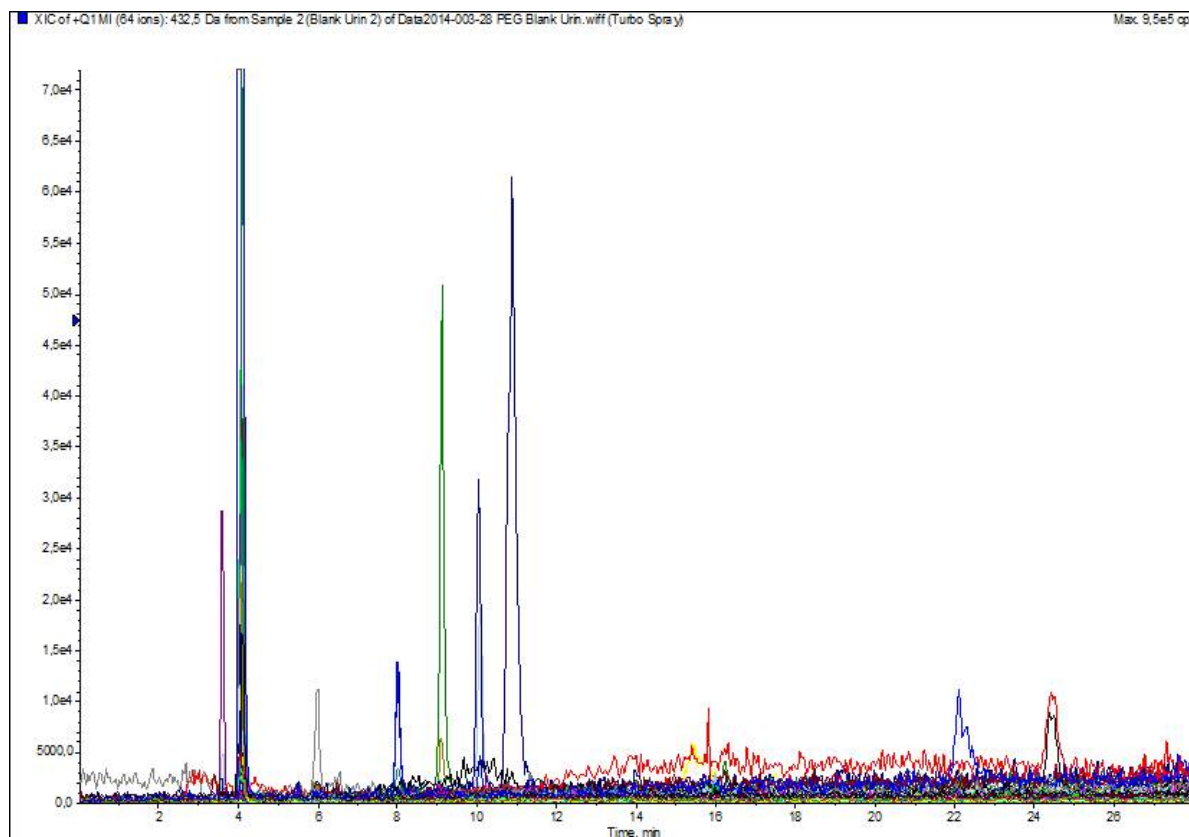


Figure S-3: Post-column infusion of PEG solutions during a LC-MS separation of a human blank urine after clean-up.

## **SUPPLEMENTARY DATA 2 –Details to the Murine and Human Urine Samples**

Development of a Stable Isotope Dilution LC–MS Assay for the Quantitation of Multiple Polyethylene glycol (PEG) Homologues to be used in Permeability Studies

Martina Lichtenegger and Michael Rychlik

In collaboration with Prof. Dirk Haller (Chair of Nutrition and Immunology, ZIEL), Prof. Martin Klingenspor (Chair of Molecular Nutritional Medicine) and Prof. Hans Hauner (Chair of Nutritional Medicine) the human and murine urine samples were generated in the frame of a BMBF-funded collaborative research project (Bundesministerium für Bildung und Forschung, [www.bmbf.de](http://www.bmbf.de); grant number 0315674) at the Institute Food Nutrition and Health (ZIEL) at the Technical University of Munich (TUM) as described in the following publications:

Kless C, Müller V M, Schueppel V L, Lichtenegger M, Rychlik M, Daniel H, Klingenspor M, Haller D (2015) Diet-induced obesity causes metabolic impairment independent of alterations in gut barrier integrity, *Molecular Nutrition and Food Research*, 59 (5): 968-978

Gruber L, Fiamoncini J, Müller VM, Lichtenegger M, Rychlik M, May S, Daniel H, Clavel T, Haller D. (2013) High fat diet aggravates Crohn's Disease-like ileitis independently of obesity via alterations of epithelial barrier homeostasis. *PLOS ONE* 8: e71661, 1-13

Gruber L, Kisling S, Lichti P, Martin FP, May S, Klingenspor M, Lichtenegger M, Rychlik M, Haller D. (2013) High fat diet accelerates pathogenesis of murine Crohn's Disease-like ileitis independently of obesity. *Journal of Crohn's and Colitis Supplements* 02/2013; 7(S1):S22