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ABSTRACT

Biomarkers of food intake (BFIs) have emerged as a promising objective tool to complement traditional self-reported dietary assessment in nutritional research, with the potential to reduce systematic errors and improve accuracy. The development of comprehensive and robust quantification methods for BFIs is essential for widespread application. However, existing methods typically cover only a moderate number of BFIs per method, hindering their wide application in the field. In this study, we present the development and validation of a method for simultaneous quantification of 80 BFIs in urine reflecting 27 foods. The method utilizes a simple sample preparation procedure, followed by separation using both high-performance liquid chromatography (HPLC) on a C18 column and a hydrophilic interaction chromatography (HILIC) column, combined with tandem mass spectrometry in positive and negative mode (HPLC-MS/MS) (individual runs: 6 min). The working range for each analyte was determined in urine samples from a non-randomized, non-blinded nutritional intervention study. The method was validated with respect to selectivity, linearity, robustness, matrix effects, recovery, accuracy, and precision. In total, 44 BFIs could be absolutely quantified without or with only limitations at low concentrations, while 36 BFIs could only be measured semi-quantitatively, including 16 BFIs with limited validation data due to uncertainties. The 80 BFIs represent 27 foods (6 semi-quantitative) frequently consumed in European diets, including 24 plant-derived and 3 animal-derived items. The future implementation of this large-scale BFI quantification method in nutritional studies is expected to demonstrate the benefits of routinely measuring BFIs to improve the accuracy of dietary assessment.

1. Introduction

In recent years, significant progress has been made in identifying new biomarkers of food intake (BFIs) candidates using metabolomics approaches [1–8]. However, before these BFI candidates can be applied in dietary studies, thorough validation is required [9–11]. Nonetheless, only a limited number of candidates have undergone comprehensive validation, while the majority of BFI candidates have achieved only partial validation, making it challenging to choose the most appropriate BFIs for a specific use [10,12]. The application of valid BFIs in nutritional research presents a promising approach to improve the accuracy of dietary assessment and reduce systematic and random errors. When used as objective measures of dietary intake, BFIs can serve as a valuable complement to traditional methods [13–15]. BFIs can also be used to address compliance in intervention studies.

BFIs combined with self-reported methods could improve accuracy in intake ranking and calibrate self-reported intakes [16,17]. For example, Regal et al. [18] demonstrated an improved estimation of wine

Abbreviations: BFI, biomarkers of food intake; EFSA, European Food and Safety Authority; EMA, European Medicines Agency; FDA, Food and Drug Administration.

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intake with a combination of self-reported intake and quantification of resveratrol in plasma. Similarly, Gibbons et al. [19] and Hu et al. [20] demonstrated an improved estimation of citrus fruit intake through the quantification of proline betaine in urine. To utilize BFIs in nutritional studies, robust methods that quantify a broad range of BFIs in biological samples are crucial.

Currently, the majority of available validated analytical methods either focus on single BFIs, include additional metabolites of the respective BFIs [21–24], or include BFIs associated with one particular food [25–27]. To enhance the quality of food intake assessment for a larger number of foods, multi-target methods are essential. While multi-target approaches are well established in other research fields [28–35], only few were specifically developed for quantification of BFIs [36]. A multi-analyte method is applicable for both randomized controlled trials (RCTs) and large-scale nutritional studies, where a large variety of foods or BFIs needs to be analyzed with high accuracy in small sample volumes and at low cost.

To address the need for a robust multi-target method capable of quantifying BFIs in biological samples for a broad range of foods, a new analytical method was developed and validated. Currently, no validation guidelines or recommendations are available for validating multi-target methods in biological samples, especially for BFI. The most common guidelines for method validation in pharmaceutical drug research, *i.e.* the European Medicines Agency (EMA) guideline [37] and the guideline from the Food and Drug Administration (FDA) of the U.S. Department of Health and Human Services [38], are designed for methods analyzing a limited number of analytes in biological samples. They can be used as guiding principles along with a validation guideline (SANTE 11312/2021) for evaluating multi-residue analysis for foods and feed, which was published by the European Commission [39]. The primary aim of this work was to establish a method suitable for analysis of a wide variety of BFIs with diverse chemical properties. To achieve this, a straightforward dilute-and-shoot approach for sample preparation, liquid chromatography, incorporating both a C18 and a hydrophilic interaction chromatography (HILIC) column, in conjunction with scheduled multiple reaction monitoring (sMRM) on a triple quadrupole mass spectrometer, was employed. Finally, the method was validated for selectivity, linearity, robustness, matrix effects, recovery, intra- and inter-day accuracy, and intra- and inter-day precision.

2. Material and methods

2.1. Selection of BFIs

The method was developed as part of the FoodPhyt project and aimed to investigate BFI candidates for frequently consumed fruits and vegetables in Europe. Consumption data from different national dietary surveys, the European Food and Safety Authority (EFSA) Comprehensive European Food Consumption Database, Statista, as well as expert discussion within the FoodPhyt consortium were used to select those frequently consumed plant foods. Subsequently, information about BFI candidates and their validity were collected from literature. For some of the selected plant foods, biomarker candidates were published together with information on their validity in review articles within the FoodBAll project [40–50]. For those markers, the literature search was updated until December 2021. For the remaining plant foods, a systematic literature search was performed according to the BFIRev guidelines to identify BFI candidates and collect information on their validity [51]. The obtained literature provided the foundation for a BFI candidate inventory, containing more than 500 BFI candidates (data published elsewhere). For the method development, the most promising BFI candidates were included. Therefore, only BFI candidates were included that were plausible, exhibited a high specificity to a small number of foods, and reference substances were commercially available. In addition, a set of BFIs associated with the intake of animal-based foods was added. In subsequent stages of method development, with the aim to enhance the methods sensitivity while keeping the HPLC run-time short, analytes were excluded based on i) the analyte's chromatographic performance, ii) presence in controlled biological samples, and iii) redundancy of BFIs for one food. The workflow of the BFI selection and the method development, illustrated in Fig. 1, resulted in the final list (see Table 1) with 86 analytes for 27 foods.

2.2. Procedures for BFI measurements

2.2.1. Preparation of standard solutions

For each reference standard and internal standard (IS), individual 1 mg/mL stock solutions (see Supplementary table S1 for quality and vendor information) were prepared. Stock mixes and dilutions were prepared from the individual solutions. According to their expected concentrations in urine, the analytes were grouped, and a standard stock mixture was prepared with six concentration levels: 1.0, 2.5, 5.0, 7.5, 10, and 20 µg/mL. The IS stock mix was prepared matching each IS to the corresponding concentration level, resulting in concentrations of 12.5, 50, and 125 µg/mL for the high, medium, and low level, respectively. Final concentrations of stock solutions and dilutions were made in methanol, unless stated differently in Supplementary table S1.

2.2.2. Scheduled MRM

The MRM transition parameters for each analyte were optimized by injecting diluted stock solutions. Subsequently, the declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were optimized for four MRM transitions using the automatic tuning tool provided by the instrument's software (Analyst 1.6.2, AB Sciex, Darmstadt, DE). The means of three measurements were used as MRM parameters for further development. After verification of the MRM transitions under chromatographic conditions, the two most suitable MRM transitions were chosen as quantifier and qualifier ions. The scheduled MRM method was set up using the measured retention times (RT). Some promising BFI candidates are glucuronides, for which no reference compounds are commercially available. In such cases, urine samples containing the glucuronides were employed for the manual optimization of MRM transitions. The identity of the glucuronides was confirmed through a comparison of urine samples measured before and after enzymatic cleavage [65] of the glucuronide group with a reference standard of the aglycon.

2.2.3. Sample preparation

For the HPLC measurement, urine samples (spot and 4 h-collections) were diluted with water to an osmolality of 100 mOsm/kg, according to Supplementary table S2. The diluted urine samples were added to a 2 mL tube on ice, vortexed for 10 s and centrifuged for 10 min at 15,000 × *g* and 4 °C. Two aliquots of 200 µL were transferred into a new tube that was placed on ice for further processing. Subsequently, 190 µL of cold methanol and 10 µL of the IS Mix were added to the sample and vortexed for 10 s. Finally, the samples were filtered through a centrifugal filter (Nanosep with 0.2 µm wwPTFE; Pall cooperation, Crailsheim, DE) for 10 min at 15,000 × *g* and 4 °C, and 150 µL of the supernatant were transferred into vials for HPLC-MS/MS analysis. Samples were freshly prepared each day, those measured on the same day are hereafter referred to as a batch. The analyte stability during analysis (auto sampler stability) was confirmed for a minimum run-time of 48 h for each batch and for all analytes (data not shown).

2.2.4. HPLC-MS/MS analysis

Samples were measured on a HPLC system (Nexera; Shimadzu, Duisburg, Germany) coupled to an electro spray ionization (ESI)-triple quad MS (5500 QTrap; AB Sciex, Darmstadt, Germany). The chromatographic separation of non-polar compounds was performed on a Waters Aquity™ PRIMER BEH C18 column (100 mm × 2.1 mm, 1.7 µm; Waters, Eschborn, DE) equipped with a pre-column. Water containing 0.2 vol% acetic acid was used as eluent A and acetonitrile containing

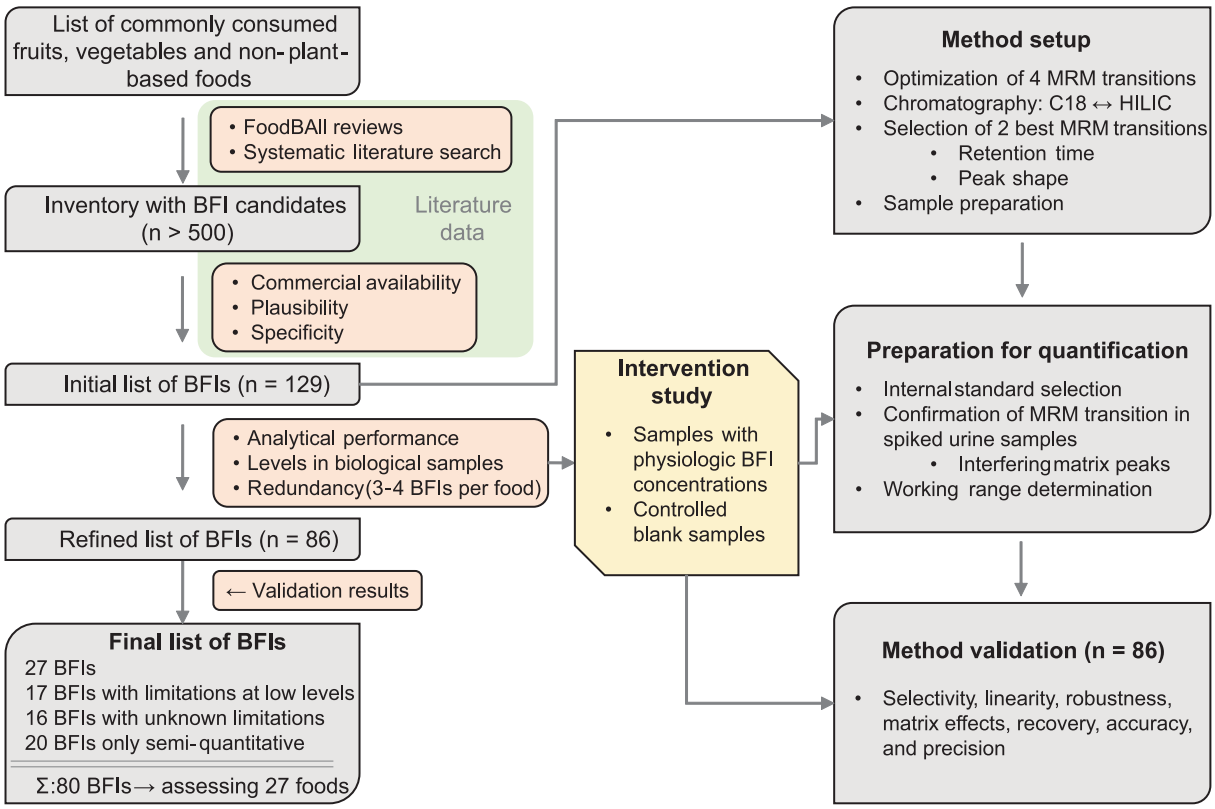

Fig. 1. Workflow for the selection, method development, and analytical validation process. On the left side, the main steps (grey boxes) of selecting and refining the BFI candidates for commonly consumed foods to the final panel of 80 BFIs assessing 27 foods are illustrated. Refinement steps, indicated by light red boxes, involved iterative evaluation using literature data, analytical data from method development, and validation results. On the right side, the main steps (grey boxes) for establishing the HPLC-MS/MS method are detailed, including method setup, preparation for quantification, and validation. The yellow box highlights the integration of samples from the intervention study, which were utilized for method development and refinement of BFI selection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.2 vol% acetic acid and 4 vol% 2-propanol as eluent B at a total flow rate of 0.5 mL/min. A gradient with the following elution profile was used: 0.0–0.1 min isocratic with 5 % B, 0.1–2.5 min from 5 % to 20 % B, 2.5–3.0 min from 20 % to 50 % B, 3.0–3.5 min from 50 % to 95 % B, 3.5–5.0 min isocratic with 95 % B, 5.0–5.1 min from 95 % to 5 % B, 5.1–6.0 min isocratic with 5 % B. The column oven was set to 50 °C and the injection volume was 3 µL.

The chromatographic separation of polar compounds was performed on a M&N Nucleoshell HILIC column (100 mm × 3 mm, 2.7 µm; Macherey & Nagel, Düren, DE) equipped with a pre-column. Aqueous ammonium formate buffer (25 mM with 0.05 vol% formic acid) was used as eluent A and 25 mM ammonium formate buffer in acetonitrile/water/methanol (90/10/3; *v*/v/v) containing 0.05 vol% acetic acid as eluent B at a total flow rate of 0.5 mL/min. A gradient with the following elution profile was used: 0.0–1.0 min isocratic with 98 % B, 1.0–4.5 min from 98 % to 50 % B, 4.5–5.0 min isocratic with 50 % B, 5.0–5.1 min from 50 % to 98 % B, 5.1–6.0 min isocratic with 98 % B. The column oven was set to 40 °C and the injection volume was 3 µL.

MS measurements were performed in positive and negative ESI mode, selecting the following ionization source conditions: curtain gas 40 psi, ion spray voltage 5500 V/−4500 V, ion source gas-1 50 psi, ion source gas-2 60 psi, and ion source gas temperature 400 °C. The optimized sMRM parameters retention time, Q1 mass, Q3 mass, DP, EP, CE, and CXP are listed in Supplementary table S3.

The final HPLC-MS method comprises of consecutive C18 and HILIC runs for each batch. To obtain >12 data points per peak positive and negative polarization had to be measured separately for C18 and HILIC runs on the used instrument. The chromatographic run-time for each of the separate 4 runs was 6 min per sample (including re-equilibration). This resulted in an effective total analysis time of approximately 30 min per sample, including re-equilibration and column/system switching.

2.2.5. *IS selection procedure*

Assigning an isotopically labeled IS to each analyte was not feasible, as only a limited number of labeled counterparts were commercially available, and inclusion of additional analytes would have reduced method sensitivity. Therefore, the analytes were grouped based on their structural properties and the functional group that is ionized. Within the groups, sub-groups based on retention time and double bound equivalents were created. For each group and sub-group, isotopically labeled internal standards were selected (Table 2). Based on this theoretical selection, each analyte was matched with the best suitable IS to have similar retention times, and concentration levels. This results in three concentration levels (Table 2). Final adjustments were made based on the validation results (accuracy and precision). The assignment of each IS to the respective analytes is shown in Supplementary table S4.

2.2.6. *Peak integration settings / data processing*

Processing and integration of chromatograms was done using MultiQuant™ 3.0.2 (AB Sciex, Darmstadt, Germany). The MQ4 integration algorithm was used with the following parameters: gaussian smooth width: 1 point, RT half window: 10 s, min. Peak width: 3 points, min. Peak height: individually set closely below the lowest spiked analyte concentration level, noise percentage: 95 % (with few exceptions), baseline sub window: 1 min, peak splitting: 2 points (with few exceptions). The retention time deviation between standard and sample was below ±0.1 min, and the quantifier/qualifier-ratio tolerance was set

Table 1
Final list of BFI candidates included for method validation, showing the association to the corresponding foods (n = 27). BFIs (initial list n < 500) were collected from previously published FoodBAll review articles and an additional systematic literature search. After selection of most promising BFIs and refinement (see section 2.1 and Fig. 1), 86 BFIs were finally included.

	Associated food	BFI candidates[#]
Fruits	Apple	phloretin [43]; phloretin glucuronide [43]; xylose [52]
	Banana	methoxyeugenol; methoxyeugenol glucuronide; dopamine 4-*O*-sulfate; salsolinol; salsolinol glucuronide; xanthurenic acid; 5-hydroxyindole acetic acid* [49]
	Orange	hesperetin [6,26]; hesperetin 3'-*O*-glucuronide [6]; synephrine [53]; hydroxyproline betaine [53]; naringenin [6,26]; proline betaine [26,54]; 4-hydroxyphenylacetic acid* [55]
	Pear	hydrochinone glucuronide; hydrochinone sulfate; arbutin [43]
	Blueberry	4-hydroxyphenylacetic acid* [50]
	Strawberry	urolithin A*; urolithin A glucuronide* [50]
Vegetables	Bell Pepper	capsaicin [56]
	Brassicaceae	2-thiothiazolidine-4-carboxylic acid [57]; sulforaphane [23]; sulforaphane-*N*-acetylcysteine [23]
	Celery	apigenin-7-*O*-glucuronide [48]
	Green Beans	3-methylhistidine; kaempferol; pipecolic acid; *S*-methylcysteine; trigonelline* [42]
	Lettuce	11β,13-dihydrolactucin; 11β,13-dihydrolactucopicrin [58]
	Mushrooms	ergothioneine [59]; L-hercynine [59]; *S*-methyl-ergothioneine [60]
	Onion	alliin; *N*-acetyl-*S*-(2-carboxypropyl) cysteine; *S*-allylcysteine [44]
	Peas	asparaginylvaline [2,61]; 2-isopropylmalic acid [61]; trigonelline* [2,42]
	Potatoes	solanidine; α-solanine [46]
	Tomato	*N*-caprylhistidinol; tomatidine; *N*-caproylhistamine; *N*-caproylhistamine glucuronide; *N*-caprylhistamine; *N*-caprylhistamine glucuronide [25]
Other plant foods	Tea	(−)-epigallocatechin; theanine; hippuric acid* [40]
	Soy	daidzein; daidzein 7-β-D-glucuronide; genistein; genistein 7-β-D-glucuronide [42]
	Whole grain	3,5-dihydroxybenzoic acid; 2-(3,5-dihydroxyphenyl)acetic acid; 2-hydroxy-1,4-benzoxazin-3-one; 3-(3,5-dihydroxyphenyl)-1-propanoic acid; avenacoside A; avenanthramide A; *N*-(2-hydroxyphenyl)acetamide [47,62]
	Wine	ethyl glucuronide; trans-resveratrol; trans-resveratrol 3-*O*-sulfate; trans-resveratrol 4'-*O*-glucuronide; tyrosol glucuronide, hydroxytyrosol*; 4-hydroxyphenylacetic acid* [50]
	Walnut	5-hydroxyindole acetic acid*; urolithin A*; urolithin A glucuronide* [45]
	Olive oil	hydroxytyrosol* [45]
	Cocoa	cyclo(pro-val); theobromine*; trimethylamine-*N*-oxide*; 4-hydroxyphenylacetic acid* [41]
	Coffee	1-methylxanthine; atractyligenin; atractyligenin glucuronide 1; caffeine; *N*-(2-furoyl)glycine; hippuric acid*; theobromine*; trigonelline*; choline* [40]
Animal foods	Meat products	acetylcarnitine; anserine; carnosine; guanidinoacetate; indole-3-lactic acid; choline* [63]
	Fish	3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; trimethylamine-*N*-oxide* [63]
	Cheese	4-hydroxyphenylpyruvate; 4-hydroxyphenylacetic acid* [64]

[*] Semi-specific biomarker retained in the method due to absence of more specific alternatives.
[#] The provided literature links the BFI to the respective food, if only one reference is provided, it applies to all listed BFIs.

Table 2
List of internal standards (IS) used in the method, including the respective concentration levels.

Internal standard	Level	Concentration in sample [ng/mL]
DL-sulforaphane-d8	low	25
3-carboxy-4-methyl-5-propyl-2-furanpropionic acid-d5	low	25
capsaicin-d3	low	25
daidzein-d6	low	25
S-methyl-ergothioneine-d3	low	25
indole-3-acetic acid-d2	middle	100
caffeine-13C	middle	100
DL-indole-3-lactic acid-d5	middle	100
4-hydroxyphenylpyruvic acid-13C9	middle	100
acetaminophen-d4	middle	100
resveratrol-13C6	middle	100
benzylisothiocyanate-d7	high	250
arbutin-d4	high	250
genistein-d4	high	250
D-mannose-13C6	high	250

below 20 %. In case the automated peak integration algorithm failed, the RT deviation, or the ion ration was too high, the peak integration was evaluated, and if necessary, the peak was integrated manually. Peaks that failed the RT deviation and quantifier/qualifier-ratio tolerance were excluded. The resulting tables with peak areas were imported to Excel (Microsoft® Excel® 2019) *via* txt-files for further analysis. Calibration equations, calculation of concentrations, and calculation of validation criteria were performed in excel.

2.3. Selection of urine samples for validation & working range determination

A randomized, non-blinded nutritional intervention study with all foods associated with the selected BFIs was performed to ascertain the working range for each BFI and provide controlled blank samples for the method validation (Fig. 2). A detailed description of the study design is provided in the Supplementary Material. In brief, the study investigated 12 individual plant-based foods and three complex meals combining plant-based foods and beverages. Fifteen (11 female, 4 male) healthy, non-smoking participants aged 22 to 40 with a BMI ranging from 19.0 to 30.1 kg/m^2 were recruited, with specific inclusion/exclusion criteria such as no regular medication (except hormonal contraceptives) and no intake of antibiotics or dietary supplements within the previous months. The study was approved by the Ethics Committee at the State Medical Chamber Baden-Württemberg (#F-2021-175) and registered at the *German Clinical Trials Register* (DRKS00029266). Each participant consumed two foods or meals, with a washout period of at least one week between study days. To account for dropouts, three participants completed a third intervention. Before the intervention, participants refrained from consuming the test foods and related items for 24 h, and fasted for 12 h beforehand. Urine samples were collected before and after the intervention, with participants consuming a defined volume of liquid during the 4 h-urine collection period.

The urine samples of the intervention study were prepared using the protocol described in section 2.2.3 above. Since the physiological concentration ranges of analytes in urine after food intake are unknown for many BFIs, a broad calibration range (n = 15; 1–1500 ng/mL) was employed to determine the analyte concentrations. The working range for each analyte was centered around the mean of the concentrations observed in urine samples. Analytes with similar concentrations were grouped, resulting in six concentration groups (A – F) within each calibrator solution (see Supplementary table S4 and Table 3).

2.4. Validation

The validation was performed using urine samples obtained from the

Fig. 2. Study design illustrating the run-in day and the study day. Shown are the timing of spot urine collection (marked in yellow with an arrow), the dietary intervention (green stripe), 4 h-urine collection period (marked in yellow), and the wash-out phase. *Water intake (marked in blue) was adjusted for participants whose intervention foods included coffee, tea, or red wine. The total liquid intake was standardized to 1 L over 4 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Final concentrations (ng/mL) for the eight calibration levels, quality control samples (QC), and levels for the standard addition method (STD add) separated by the six concentration groups for all analytes (A-F; corresponding analytes, see Supplementary tables S4). In addition, the final concentrations (ng/mL) for internal standard levels (IS) is provided. The urine samples (QC, STD add) were spiked with the analytes to obtain the final concentrations.

	A	B	C	D	E	F	IS low	IS medium	IS high
Calibrator 1	1	2.5	5	7.5	10	20			
Calibrator 2	5	12.5	25	37.5	50	100			
Calibrator 3	10	25	50	75	100	200			
Calibrator 4	20	50	100	150	200	400	25	100	250
Calibrator 5	30	75	150	225	300	600			
Calibrator 6	45	112.5	225	337.5	450	900			
Calibrator 7	60	150	300	450	600	1200			
Calibrator 8	75	187.5	375	562.5	750	1500			
QC LLOQ	1	2.5	5	7.5	10	20			
QC low	7.5	18.75	37.5	56.25	75	150	25	100	250
QC medium	25	62.5	125	187.5	250	500			
QC high	65	162.5	325	487.5	650	1300			
STD add 1	80	200	400	600	800	1600			
STD add 2	95	237.5	475	712.5	950	1900	25	100	250
STD add 3	110	275	550	825	1100	2200			

intervention study. Ten urine samples in which the majority of analytes was absent or present in low concentrations were selected (Supplementary table S2) to assess selectivity, recovery, and matrix effects. Analyte concentrations that were non-zero were corrected by the calculated concentrations obtained using the standard addition method (see respective section below). Consistent results for selectivity, recovery, and matrix effects (see respective sections in the results part) over 10 different urine samples enabled the utilization of fewer samples (n = 2) to determine accuracy and precision. Although the urine samples were not free of all analytes, they were designated as blank samples, that were spiked with the analytes to achieve the final concentration as stated in the manuscript (see Table 3 and section 2.4.3–2.4.5).

2.4.1. Selectivity

For determination of selectivity, ten different urine samples from the intervention study were analyzed without adding standard or internal standards. The extracted ion chromatograms were checked for confounding signals that might interfere with analyte signals. Furthermore, non-blank urine samples were identified. Non-blank urine samples contain the analyte, *e.g.* due to endogenous formation *in vivo*. In these cases, the selectivity of the method was demonstrated with the standard addition method. The consistent linear increase in signal upon spiking, confirmed the absence of interferences.

2.4.2. Linearity

Each day, the calibration solutions were freshly prepared by diluting the stock mix with methanol/water (50/50, v/v). The calibration levels for each of the six concentration groups (see section 2.3) are shown in Table 3. Each of the eight calibration standard solutions contained 25, 100, and 250 ng/mL of IS, depending on the IS concentration level, as shown in Table 3. Calibration standards were measured daily at the beginning and at the end of each sample sequence (bracketing calibration), and linearity was evaluated by accuracy of back-calculated concentrations of the analytes and coefficient of determination (R^2) using a linear fit with a $1/x$ or $1/x^2$ weighting (see Supplementary tables S4 and S5).

2.4.3. Evaluation of extraction recovery and matrix effect

To evaluate potential losses during sample preparation, detector signals (area) from samples spiked with analytes prior to extraction were compared with those from samples spiked after extraction and filtration (n = 10 for each sample set). Recoveries were calculated using the latter as a 100 % reference. Possible matrix effects were evaluated by comparison of detector signals (area) measured in extracted urine samples spiked with the analytes prior to HPLC-MS/MS analysis (recovery samples; n = 10) with detector signals of the respective solvent samples (water/methanol, 50/50, v/v) (n = 6). For analytes present in urine samples, detector signals for spiked samples were corrected for the respective area of blank matrix (selectivity samples; n = 10) as described in section 2.4.4. Recovery and matrix effects were evaluated for low, medium, and high analyte concentrations with individual concentrations for the concentration groups as shown in Table 3 (QC low, QC medium, QC high).

2.4.4. Standard addition to determine blank concentration for correction

For the determination of the blank concentration in the urine samples (n = 10), samples were spiked with four analyte concentration levels (QC high, and STD add 1–3) in equidistant steps as stated in Table 3. A linear regression was used to calculate the concentration of the non-spiked urine samples [66,67]. Blank concentrations of samples

were corrected, when blank concentrations were >1 % of the lower limit of the working range. Within the two urine samples used (see Supplementary table S6), this applied to 28 and 27 analytes (respectively). A consistent water/organic solvent ratio (50/50; v/v) across different concentrations was ensured for the preparation of spiked samples.

2.4.5. Accuracy and precision

Accuracy and precision were evaluated by spiking two urine samples with analytes, each at four different concentration levels, each in five replicates and calculating recovery (theoretical spiking level set as reference) and repeatability (relative standard deviation; RSD). The following spiking levels were selected: LLOQ (lowest calibrator), low, medium, and high. Analyte spiked samples ($n = 5$) were measured on the same day to evaluate the intra-day accuracy and precision. One set (two urine samples with four analyte levels) of spiked samples from the first measurement day, along with four additional sets measured on four subsequent days, was used to assess inter-day accuracy and precision (within-laboratory reproducibility).

3. Results

The aim of this work was to develop a robust and efficient quantitative method for quantification of multiple BFIs assessing a broad range of foods. The method was designed for use in nutritional studies and hence includes a straightforward and simple sample preparation procedure. A total of 86 BFIs were included in the method validation. The majority of the BFIs were commercially available. Some promising glucuronides that were not available were quantified *via* the respective aglycon. The analyte list includes unmetabolized, phase I, and phase II metabolites from various compound classes, such as amino acid derivates (*e.g.* guanidinoacetate, ergothioneine, proline betaine), polyphenols (*e.g.* hesperetin, kaempferol, *trans*-resveratrol), and steroidal alkaloid (*e.g.* solanidine, tomatidine).

3.1. Method development

The initial MRM development resulted in two suitable transitions for each analyte. However, for 4-hydroxyphenylpyruvate, only the MRM transition *m/z* 178.8/106.9 could be established as the quantifier (extracted ion chromatograms are shown in Supplementary Figs. 1–5). The verification of the MRM transitions under chromatographic conditions were additionally employed to assign each analyte to the C18 or HILIC method in order to achieve a sufficient retention on the respective chromatographic columns. Fifty-six of the 86 analytes were measured with the C18 column and 30 with the HILIC column. In addition to column assignment, the peak shapes of each analyte were assessed in solvent and urine samples, revealing no chromatographic issues for analyte signals on the C18 column. Except for methoxyeugenol, which was excluded due to inconsistent peak shape. On the HILIC column, peaks are generally broader than those on the C18 column, as expected. With a few exceptions, analyte peaks showed no chromatographic issues on the HILIC column. The xylose peak was inconsistent and displayed minor tailing, resulting in its exclusion from the method. The hydrochinone derivates arbutin and hydrochinone glucuronide exhibited a double peak, that remained stable over repetitive measurements. In contrast, the observed double peak for theobromine did not remain stable over repeated measurement, hindering the reliable integration of this analyte. As a result, theobromine was excluded from the method. Additionally, theanine had to be excluded due to interfering matrix, and hydrochinone sulfate and ethyl glucuronide due to insufficient peak shapes. Overall, due to the wide variety of analytes, not all analytes could be reliably detected using this straightforward sample preparation approach and the short chromatographic method (see Table 4).

An advantage of using both reversed phase (RP) and HILIC chromatography is their orthogonality in separation. Since the principles of separation in HILIC are based on the hydrophilicity of the analytes, this approach enables the resolution of the polar compounds, which usually

Table 4
Overview of results from method validation. BFI candidates are categorized as having no limitations, concentration-dependent limitations, or being excluded for chromatographic reasons. BFIs with limitations are listed under the corresponding validation parameter not fulfilled according to the acceptance criteria. A candidate may appear multiple times across validation parameters. Unless otherwise stated, a limitation at a given concentration level also applies to all lower levels. Detailed reasons for failure of validation criteria are given as superscript abbreviations.

	Linearity	Matrix effects	Recovery	Accuracy*		Precision (only inter-day)
				intra-day	& inter-day	
No limitations	11β,13-dihydrolactucin; 11β,13 -dihydrolactucopicrin; 2 -(3,5-dihydroxyphenyl)acetic acid; 2 -hydroxy-1,4-benzoxazin-3-one; 4 -hydroxyphenylpyruvate; apigenin -7-*O*-glucuronide; asparaginylvaline; atractyligenin; capsaicin; genistein 7-β-*D*-glucuronide; genistein; hesperetin; hydroxytyrosol; kaempferol; naringenin; *N*-caproylhistamine; *N*-caprylhistamine; *N*-caprylhistidinol; phloretin; solanidine; sulforaphane; tomatidine; trans -resveratrol 3-*O*-sulfate; trans-resveratrol 4' -*O*-glucuronide; trans-resveratrol; tyrosol glucuronide; urolithin A					
Limitations at LLOQ level	3-methylhistidine^{RSD}; arbutin^{RSD}; avenacoside A^{RSD}; guanidinoacetate^{RSD}; *N*-acetyl-*S*-(2-carboxypropyl)cysteine^{RSD}; proline betaine^{RSD}; α-solanine^{RSD}	not determined at LLOQ	not determined at LLOQ	1-methylxanthine^{M}; 2-isopropylmalic acid^{HM}; 3-(3,5-dihydroxyphenyl)-1-propanoic acid^{HM}; 3,5-dihydroxybenzoic acid^{HM}; avenanthramide A; caffeine^{HM}; daidzein 7-β-*D*-glucuronide; N-(2-furoyl)glycine^{HM}; hydrochinone glucuronide^{HM}; indole-3-lactic acid^{H}; sulforaphane-*N*-acetylcysteine	(-)-epigallocatechin; 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; daidzein; salsolinol; *S*-methyl-ergothioneine^{H}; urolithin A glucuronide; avenacoside A; ergothioneine; *S*-allylcysteine	1-methylxanthine^{M}; 2-isopropylmalic acid^{HM}; arbutin; avenacoside A; avenanthramide A; daidzein
Limitations at low level		N-(2-furoyl)glycine^{HM}; sulforaphane-*N*-acetylcysteine; urolithin A glucuronide; xanthurenic acid^{HM}	choline; pipecolic acid; proline betaine^{H}	5-hydroxyindole acetic acid^{HM}; *N*-acetyl-*S*-(2-carboxypropyl)cysteine^{H}; proline betaine^{H}; daidzein 7-β-*D*-glucuronide; hesperetin 3'-*O*-glucuronide; α-solanine	alliin; *N*-(2-hydroxyphenyl)acetamide^{H}; pipecolic acid; salsolinol; xanthurenic acid^{H}	5-hydroxyindole acetic acid^{HM}; hydrochinone glucuronide^{HM}; *N*-(2-hydroxyphenyl)acetamide^{H}; *N*-acetyl-*S*-(2-carboxypropyl)cysteine^{H}; salsolinol; synephrine (only low)
Limitations at medium level		5-hydroxyindole acetic acid^{HM}; caffeine^{H}; choline^{M}; cyclo(pro-val)^{M}; hesperetin 3'-*O*-glucuronide		choline^{HM}; anserine^{Q}; xanthurenic acid^{H}	cyclo(pro-val)^{HM}; 4-hydroxyphenylacetic acid^{HM}	4-hydroxyphenylacetic acid^{HM}; cyclo(pro-val)^{HM}
Limitations at high level	2-thiothiazolidine-4-carboxylic acid^{R}; acetylcarnitine^{RSD}; hydroxyproline betaine^{RSD}; salsolinol^{R,RSD}; *S*-methyl-ergothioneine^{RSD}; trigonelline^{RSD}; trimethylamine-*N*-oxide^{R}	1-methylxanthine^{M}; 2-thiothiazolidine-4-carboxylic acid; 3-methylhistidineTH; acetylcarnitineTH; carnosine^{H}; dopamine 4-*O*-sulfate; (-)-epigallocatechin (only at high); guanidinoacetate^{HM}; hippuric acidTH; hydroxyproline betaine^{HM}; *L*-hercynine; *N*-acetyl-*S*-(2-carboxypropyl)-cysteine^{HM}; proline betaine^{HM}; trigonellineTH; trimethylamine-*N*-oxideTH	(-)-epigallocatechin (only at high)	2-thiothiazolidine-4-carboxylic acid; 3-methylhistidineTH; acetylcarnitineTH; carnosine^{H}; dopamine 4-*O*-sulfate; guanidinoacetate^{HM}; hippuric acidTH; *L*-hercynine; trimethylamine-*N*-oxideTH; arbutin	hydroxyproline betaine^{HM}; trigonellineTH	3-methylhistidineTH; acetylcarnitineTH; alliin; anserine; carnosine; choline^{HM}; ergothioneine; (-)-epigallocatechin; guanidinoacetate^{HM}; hydroxyproline betaine^{HM}; *L*-hercynine; pipecolic acid; *S*-allylcysteine; *S*-methylcysteine; *S*-methyl-ergothioneine; trigonellineTH; trimethylamine-*N*-oxideTH
Chromatographic limitations	ethyl glucuronide; hydrochinone sulfate; methoxyeugenol; theanine; theobromine; xylose					

*BFIs with failed accuracy for both intra- and inter-day accuracy are shaded in grey, or otherwise listed in the respective column for inter- and intra-day accuracy. RSD: the relative standard deviation of the back-calculated concentrations is not in the boundary of ±25 % for C18 and ±30 % for HILIC; R: the correlation coefficient is <0.99; H: high blank level; TH: detector saturated; M: insufficiently matched STD addition levels; HM: both high blank level and insufficiently matched STD addition levels; Q: one of two QCs is matching the criteria.

remain unresolved with RP. The combination of a C18 and HILIC column ensures adequate retention of all analytes on each column, enabling separation from the injection peak and minimizing potential matrix effects. Furthermore, the gradient can start at higher organic percentage in the C18 runs and at higher water content in the HILIC runs, leading to overall faster gradients. This method is easy to apply, even on large samples set, due to the unlaborious sample preparation procedure and its acceptable combined effective total analysis time of 30 min.

3.2. Method validation

The results were evaluated in comparison to the EMA, FDA and SANTE guidelines, as well as to less strict acceptance criteria for some validation parameters. Due to the method targeting a broad panel of analytes with minimal sample clean-up, some degree of analytical variability is unavoidable and applying the strict guideline criteria would have excluded promising BFIs. Instead, acceptance criteria were adapted to fit the purpose of the developed multi-target method, ensuring known deviations remain transparent while maintaining sufficient reliability and robustness to answer research questions related to BFI. The proposed BFI quantification method was validated with respect to selectivity, linearity, matrix effects, recovery of the sample preparation procedure, intra- and inter-day accuracy, and intra- and inter-day precision. BFI candidates fulfilling all validation criteria can be quantified in a reliable manner, *i.e.* with absolute quantitative values. Limitations are given due to failed acceptance criteria for at least one validation parameter or concentration level. BFI candidates exhibiting few and explainable limitations such as failure of accuracy at the lowest quantification level, can still be considered reliable in the acceptable calibration range. However, BFI candidates having failed validation criteria across all concentration levels, are subject to a high degree of uncertainty and should therefore be considered in a semi-quantitative manner, *i.e.* based on relative signal intensities. Summarized validation results for matrix effects, recovery, accuracy, and precision are shown in Figs. 3 and 4, and in Table 4, detailed information is compiled in the Supplementary table S6.

3.2.1. Linearity

The calibration curves for 78 analytes demonstrated linear relationship with $R^2 < 0.99$. Only 2-thiothiazolidine-4-carboxylic acid, salsolinol, acetylcarnitine, and trimethylamine-*N*-oxide showed insufficient linearity. The relative standard deviation (RSD) of the back-calculated concentration should be ≤20 % [39] for 75 % of the measured calibrators and a minimum of 6 calibrators [37]. For HILIC measurement, ≤25 % were tolerated as HILIC chromatography generally has slightly greater variation [68]. Most analytes fulfilled these prerequires. Exceptions were found for avenacoside A, α-solanine, and *N*-acetyl-*S*-(2-carboxypropyl)cysteine analyzed with the C18 column for the lowest calibrator with a RSD of 23 %, 23 %, and 27 %, respectively. Increased variability for the lowest calibrator was observed for the following analytes measured with the HILIC column: arbutin, 3-methylhistidine, guanidinoacetate, proline betaine, and ergothioneine with a RSD of 50 %, 36 %, 32 %, 31 %, and 26 %, respectively. These analytes are limited in their precision at the lowest calibrator level (LLOQ). The analytes salsolinol, hydroxyproline betaine, *S*-methyl-ergothioneine, acetylcarnitine, and trigonelline showed greater variation in back-calculated concentrations at more than one calibration level, and therefore, do not meet the EMA linearity criteria [37].

3.2.2. Recovery & matrix effects

The distribution of results for recovery and matrix effects are illustrated in Fig. 3. According to FDA and EMA guidelines the recovery should be “efficient and reproducible”. According to the SANTE guideline no acceptance criteria for the extraction recovery are suggested. For all analytes and levels, recoveries between 86 % and 116 % with an RSD generally <20 % were considered as satisfactory. The exceptions were: (−)-epigallocatechin (36 % RSD at high levels), choline (31 % RSD at low levels), pipecolic acid (32 % RSD at low levels), proline betaine (26 % RSD at low levels). The SANTE acceptance criteria for matrix effects (±20 %) were met by 48 %, 46 %, and 50 % of the analytes at low,

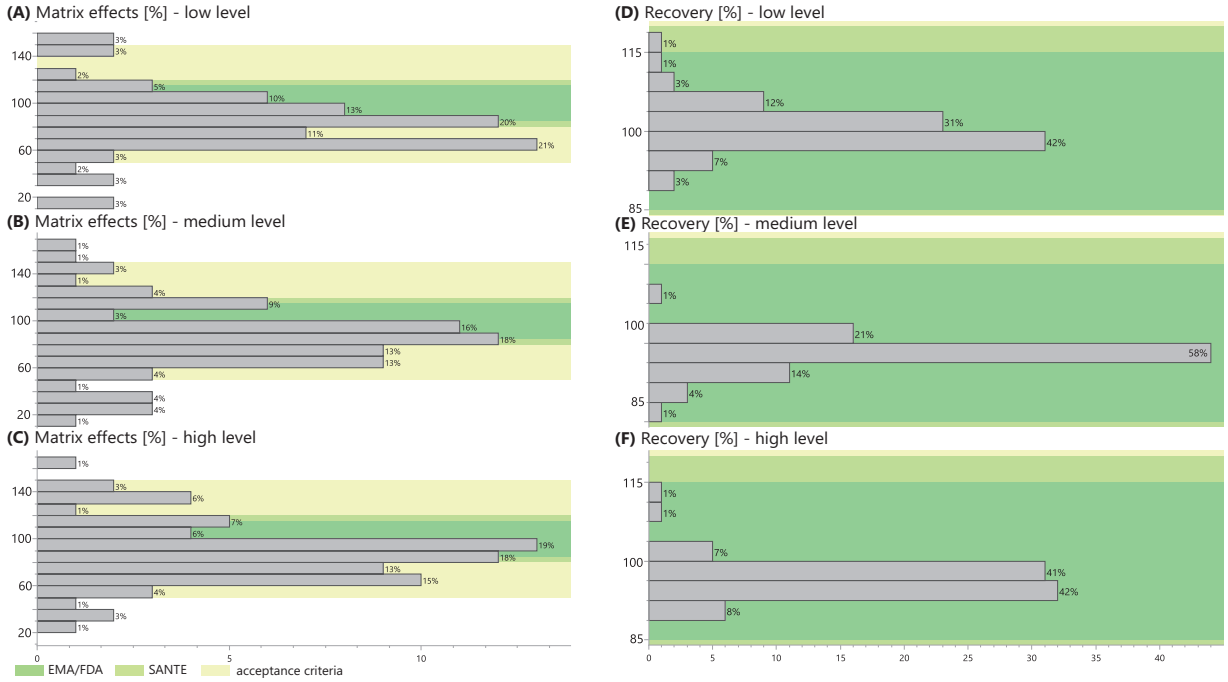

Fig. 3. Overview of the validation criteria matrix effects and recovery depicted as histograms. Panel A: matrix effects at low level; panel B: matrix effect at medium level; panel C: matrix effects at high level; panel D: recovery at low level; panel E: recovery at medium level, and panel F: recovery at high level. The acceptance ranges are indicated as colored background (green for EMA/FDA guidelines, light green for SANTE guideline, yellow for ±50 %). Matrix effects are expressed as percentage of the signal compared to samples without matrix (solvent sample) and recovery as the extraction efficiency. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

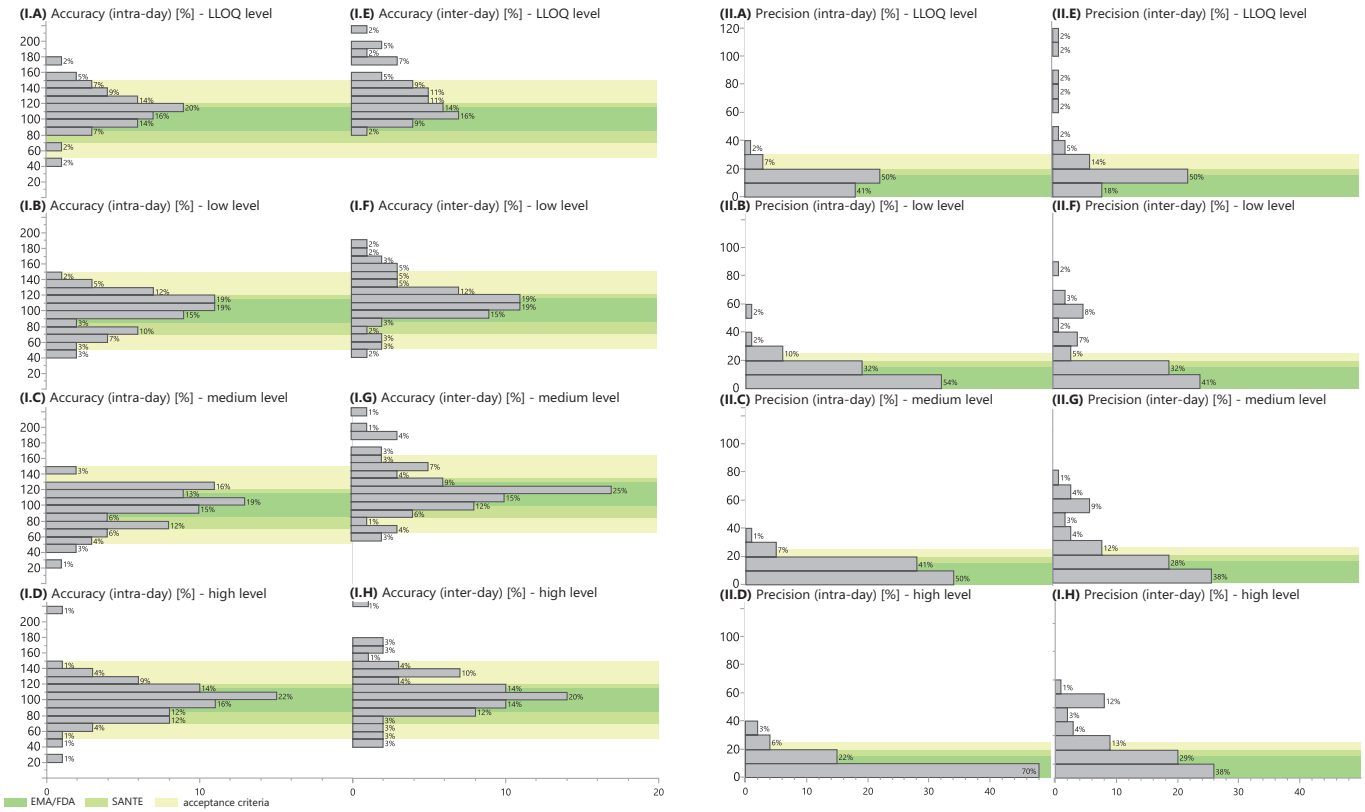

Fig. 4. Overview of the validation criteria intra- and inter-day accuracy, and intra- and inter-day precision depicted as histograms. Panels I.A to I.D: intra-day accuracy at LLOQ, low, medium, and high levels; panels I.E to I.H: inter-day accuracy at LLOQ, low, medium, and high levels; panels II.A to II.D: intra-day precision at LLOQ, low, medium, and high levels; panels II.E to II.H: inter-day precision at LLOQ, low, medium, and high levels. The acceptance ranges are indicated as colored background (green for EMA/FDA guidelines, light green for SANTE guideline, yellow for ±50 % in case of accuracy, ±30 % in case of precision at LLOQ, and ±25 % in case of precision at all other concentration levels). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

medium, and high levels, respectively. Matrix effects that exceed ±20 % must be uniform across different matrix samples and must be considered, *e.g.* by calculating a matrix factor [37]. As this was the case, higher matrix effects up to ±50 % were tolerated and the analytes were kept for further validation. For 13 of the 86 analytes at low and medium levels, and 7 analytes at high levels (details in Supplementary table S6), no matrix effects were determinable due to missing blank urine or chromatographic reasons. 6 analytes (sulforaphane-*N*-acetylcysteine (low), proline betaine (med), choline (med), and (−)-epigallocatechin (high)) showed non-uniform matrix effects across the 10 urine samples as the RSD exceeded 50 %. For these analytes, quantitative values should only be reported when they exceed the next higher concentration level, *e.g.* sulforaphane-*N*-acetylcysteine concentrations should only be reported if they are above the low level. Values below this threshold are considered semi-quantitative.

3.2.3. *Accuracy and precision*

Estimation of accuracy and precision of analyzed BFIs and their comparison with the EMA/FDA and SANTE guideline criteria are shown in Fig. 4. An overview of the analytes having fulfilled or failed the criteria at a certain level is provided in Table 4, whereas the detailed information is provided in the Supplementary table S6. The acceptance criteria of the SANTE guideline for the recovery, which is also termed as accuracy, should range from 70 % to 120 %. The intra-day accuracy criteria were met by 57 %, 66 %, 65 %, and 76 %, and the inter-day criteria were met by 41 %, 58 %, 67 %, and 63 % of the analytes at LLOQ, low, medium, and high levels, respectively. In exceptional cases, we propose that average accuracy outside the specified range may be tolerated, if they fall in a broader range of 50 % to 150 %, provided that i) the precision remains ≤20 %, and ii) at least one additional BFI without limitations is available for the corresponding food. Otherwise, only semi-quantitative values should be reported. Summarized results on a food level are provided in Table 5. The ±50 % criteria for intra-day accuracy were fulfilled by 89 %, 95 %, 94 %, and 95 %, and for the inter-day accuracy by 72 %, 86 %, 86 %, and 87 % of the analytes at LLOQ, low, medium, and high levels, respectively. Compared to the stricter SANTE criteria 27 % and 26 % more analytes met the higher threshold for the intra-day and the inter-day accuracy, respectively.

According the SANTE guidelines, the RSD of the intra-day and the inter-day precision should not exceed 20 %. The SANTE intra-day criteria were met by 91 %, 86 %, 91 %, and 92 %, and the inter-day criteria were met by 68 %, 73 %, 66 %, and 67 % of the analytes at LLOQ, low, medium, and high levels, respectively. As the SANTE guidelines are not intended for multi-target analysis in biological samples, criteria similar to those typically applied in metabolomics methods of 25 % and at LLOQ level of 30 % are reasonable [69]. Applying these criteria for intra-day precision, 98 %, 91 %, 94 %, and 95 %, and for the inter-day precision, 82 %, 75 %, 72 %, and 73 % of the analytes met the criteria at LLOQ, low, medium, and high levels, respectively. Compared to the stricter SANTE criteria 4.5 % and 7 % more analytes met the higher threshold for the intra-day and the inter-day precision, respectively. Details can be found in Table 4 and in the Supplementary table S6.

3.2.3.1. Pseudo-MRMs and detection in urine sample. For some phase II metabolite BFIs no reference standards were commercially available. Therefore, the cleaved form of the BFIs was included into the method to indirectly quantify phloretin glucuronide, atractyligenin glucuronide,

Table 5
Summarized validation results showing in columns the BFIs that can be quantitatively determined without any limitations (i), with limitations at low concentration levels (ii), with potential limitations (iii; validation data entail uncertainty *e.g.* no blank matrix or reference standard available), and BFIs that only allow semi-quantitative determination (iv). The colour code* next to the food summarizes, which foods have sufficient well validated BFIs.

	Food incl. colour code*	BFIs for quantitative determination (i)	BFIs for quantitative determination with limitation at LLOQ/low levels (ii)	BFIs with potential limitations (iii)	BFIs for semi-quantitative determination (iv)
Fruits	Apple	phloretin		phloretin glucuronide	
	Banana#			methoxyeugenol glucuronide; salsolinol glucuronide; xanthurenic acid; 5-hydroxyindole acetic acid	dopamine 4-*O*-sulfate; salsolinol
	Orange	hesperetin; naringenin	synephrine	4-hydroxyphenylacetic acid	hydroxyproline betaine; proline betaine; hesperetin 3'-*O*-glucuronide;
	Pear		hydrochinone glucuronide		arbutin
	Blueberry#			4-hydroxyphenylacetic acid	
	Strawberry	urolithin A	urolithin A glucuronide		
Vegetables	Bell pepper	capsaicin			
	Brassicaceae	sulforaphane	sulforaphane-*N*-acetylcysteine		2-thiothiazolidine-4-carboxylic acid
	Celery	apigenin-7-*O*-glucuronide			
	Green beans	kaempferol	pipecolic acid		S-methylcysteine; 3-methylhistidine; trigonelline
	Lettuce	11β,13-dihydrolactucin; 11β,13-dihydrolactucopicrin			
	Mushrooms#				ergothioneine; *S*-methyl-ergothioneine; *L*-hercynine
	Onion#			*N*-acetyl-*S*-(2-carboxypropyl)cysteine	alliin; *S*-allylcysteine
	Peas	asparaginylvaline	2-isopropylmalic acid		trigonelline
	Potatoes	solanidine	α-solanine		
	Tomato	*N*-caprylhistidinol; tomatidine; *N*-caproylhistamine; *N*-caprylhistamine		*N*-caproylhistamine glucuronide; *N*-caprylhistamine glucuronide	
Other plant foods	Tea		(-)-epigallocatechin		hippuric acid
	Soy	genistein; genistein 7-β-*D*-glucuronide	daidzein 7-β-*D*-glucuronide; daidzein		
	Whole grain	2-(3,5-dihydroxyphenyl)acetic acid; 2-hydroxy-1,4-benzoxazin-3-one	3,5-dihydroxybenzoic acid; 3-(3,5-dihydroxyphenyl)-1-propanoic acid; avenacoside A; avenanthramide A; *N*-(2-hydroxyphenyl)acetamide		
	Wine	trans-resveratrol; trans-resveratrol 3-*O*-sulfate; trans-resveratrol 4'-*O*-glucuronide; tyrosol glucuronide; hydroxytyrosol		4-hydroxyphenylacetic acid	
	Walnut	urolithin A	urolithin A glucuronide	5-hydroxyindole acetic acid	
	Olive oil	hydroxytyrosol			
	Cocoa#			4-hydroxyphenylacetic acid; cyclo(pro-val)	trimethylamine-*N*-oxide
	Coffee	atractyligenin		1-methylxanthine; atractyligenin glucuronide 1; caffeine; choline; *N*-(2-furoyl)glycine; cyclo(pro-val)	hippuric acid; trigonelline
Animal foods	Meat products	4-hydroxyphenylpyruvate	indole-3-lactic acid	choline; guanidinoacetate	anserine; carnosine; acetylcarnitine; trimethylamine-*N*-oxide
	Fish		3-carboxy-4-methyl-5-propyl-2-furanpropionic acid		
	Cheese#			4-hydroxyphenylacetic acid	

* Dark green – fully validated: ≥ 1 BFI in column i; light green – validated but limitations at low levels (higher levels reliable): 1 BFI in column ii; yellow – validation limited, semi-quantitative: ≥ 1 BFI in column iii; red – validation failed, semi-quantitative: BFIs in column iv; # limitations at medium or high levels (only semi-quantitative determination).

N-caproylhistamine glucuronide, *N*-caprylhistamine glucuronide, methoxyeugenol glucuronide, and salsolinol glucuronide. Two MRM transitions for the glucuronides were successfully integrated into the method. Then, the precision in a urine sample from one individual (5 aliquots) was determined, as spiking of blank samples was not possible. With the exception of methoxyeugenol glucuronide, which could not be detected in the sample, all other glucuronides showed acceptable precision ranging from 7 % to 21 % across the measurements. Consequently, the validation parameters matrix effects, recovery, linearity, and accuracy could not be determined, as no reference standard was available for spiking. Therefore, these metabolites are quantified in a semi-quantitative manner.

4. Discussion

This method was specifically developed to meet the requirements for analyzing large sample sets, making it suitable for application in large cohort studies. The majority of analytes can be quantified without limitations, except at low concentrations, where some validation criteria failed. For several other BFIs, validation parameters were determined, however, certain uncertainties remain due to the lack of a blank matrix and/or elevated blank levels, which limited the reliability of spiking. Due to these uncertainties and to ensure conservative data handling, these BFIs can only be quantified on a semi-quantitative basis and should be interpreted with caution, because they might have potential unknown limitations (see Table 5 column iii). To elaborate whether these BFIs could be suitable for absolute quantification in the future, identification of more appropriate blank matrices is necessary to fully determine validation parameters. Only six out of the 86 analytes had to be excluded from the method due to chromatographic challenges. For two of the six BFIs, alternative BFIs were available to measure the corresponding food intake. While the remaining four BFIs account for a food (tea, pear, banana, cocoa), for which no alternative BFIs without any other limitation is available. *E.g.* for tea, (−)-epigallocatechin determination is less precise independent of concentration level (inter- and intra-day precision ~30 %), however, with the exception of LLOQ level, inter-day accuracy was <120 %. The purpose of our validation is to provide information that enables proper interpretation of the resulting data. Depending on the specific research question, certain deviations may be acceptable under defined conditions, *e.g.* the less precise determination of (−)-epigallocatechin. Some additional analytes have limitations at medium ($n = 9$) or high ($n = 25$) concentration caused by various factors, such as matrix effects that could not be corrected by an appropriate IS, high concentration reaching upper detector limit, or higher variability in repeated measurements (see Table 4 for summarized validation results).

On the food level, the method can reliably quantify at least one BFI for each of the 18 foods out of the 27 selected foods: five vegetables (lettuce, tomato, Brassicaceae vegetables, bell pepper, celery), three legumes (soy, green bean, peas), three fruits (apple, orange, strawberry), and seven further foods (whole grain, potatoes, coffee, wine, meat products, olive oil, walnuts). BFIs for pear, tea, and fish are subject to limitations at low concentrations. Due to uncertainties associated with the determined validation parameters and resulting limitations at medium or high concentrations, BFIs for two fruits (banana, blueberry), onion, cocoa, and cheese should be considered semi-quantitative. Only in case of mushroom intake, all BFIs failed the validation criteria and must therefore be considered on a semi-quantitative basis. Foods with

only semi-quantitative BFIs were kept in the method, even though they might not be suitable for food intake calibration as proposed by Gormley et al. [16,17]. However, semi-quantitative BFIs may be applicable for classification into consumers and non-consumers as well as ranking of food intake within a study population *e.g.* to establish mean differences. To ensure appropriate application of BFIs in epidemiological studies, validation status of the respective BFI has to be evaluated (validation criteria such as specificity, variability, or dose-response relationship), all of which might influence the applicability of BFIs for ranking dietary intake [9–11]. Table 5 gives a detailed overview, which BFIs can be used for quantitative BFI determination, which BFIs encounter limitations at low concentrations, and which BFIs can only be assessed semi-quantitatively.

Compared with previously published methods [32,36], this method includes twice as many foods (14 and 12 food types) while having 71 % (57 BFIs) and 24 % (19 BFIs) of the analytes in common. While the method by Beckmann et al. [36] includes a larger number of analytes (62 BFIs), which often feature multiple, potentially redundant metabolites for around 15 single food sources and non-specific biomarkers for *e.g.* polyphenol-rich foods or protein intake, our method focuses on a streamlined set of BFIs per food. This targeted selection was made to optimize sensitivity and facilitate robust quantification across a broad range of foods. Compared to the method described in this manuscript, 19 BFIs and 12 foods are in common. The sample preparation technique is similar except for the normalization of urine samples. Beckmann et al. [36] normalized by refractive index, while we normalized by osmolality of the urine samples. Both methods use C18 and HILIC columns to account for the wide polarity range of the analytes, which is resulting in overall similar validation results for both methods. With our method, we provide a comparable method that includes an enlarged set of BFIs assessing more than 10 additional foods.

González-Domínguez et al. [32] presented a large-scale multi-target method investigating a wide polarity range of 667 analytes of which 57 can also be detected with the presented method. However, a significant number of analytes are not sufficiently retained on the applied reversed phase column, making them susceptible to matrix interferences as they elute in the solvent peak, and polar analytes are insufficiently resolved. To address these issues, our method utilized both a C18 column and a HILIC column. Comparing precision of analytes measured with HILIC and C18 columns, analytes measured on the HILIC column generally have slightly greater variation, which is acceptable due to better separation of polar compound and lower matrix effects [36].

Another key feature of our method is the matching of the expected BFI concentration ranges to measured concentrations in urine samples from an intervention study that used realistic portion sizes in complex meals (accounting for 17 of 27 foods), and moderately greater portion sizes for five individual foods (Supplementary table S7). In contrast, many previous intervention studies have relied on large, non-representative portions, often several hundred grams [2,3,23,24,55] to induce measurable BFI responses. While such designs are useful for biomarker discovery, they may overestimate BFI concentrations under free-living conditions.

To reflect real-world conditions, the working range for each BFI was individually defined based on measured concentrations in urine samples from the aforementioned intervention study, in which participants consumed customary portion sizes of each food. This required the establishment of analyte-specific concentration ranges, a step that goes beyond standard practice and involved considerable additional effort. However, this tailored approach enhances the method's applicability to free-living conditions and increases its relevance for large-scale nutritional studies. A similar strategy was previously applied by Zheng et al. [70], yielding positive results, as the established working ranges align well with expected urinary concentrations following food consumption. Compared to other multi-target methods [30,32,33], our method incorporates a higher degree of analytical refinement selecting individual concentration ranges per analyte. While this increases complexity in standard preparation, it allows for more accurate quantification within the physiologically relevant concentration spectrum. Given the broad dynamic range of analyte concentrations, some compounds were close to the lower and upper detection limits of the instrument. To balance analytical performance with efficiency, we opted against multiple dilutions per sample. Instead, we assigned an individual concentration level to each analyte, constraining the precision and accuracy for certain analytes. This strategy ensured the practical feasibility of the method.

Isotopically labeled standards are a common way to compensate matrix effects in LC-MS methods (EMA/SANTE). As with other multi-target methods [32,33,35], it was not feasible to include isotopically labeled counterparts for all analytes, as in many cases labeled standards are commercially not available. Moreover, adding a large number of additional analytes would increase the overall method costs and potentially decrease the sensitivity of MRM methods, which is not favorable, as many analytes are close to lower detection limits of the instrument. Nonetheless, some analytes experience matrix effects that alter quantitative measurements, which could not be explained by non-zero blank concentrations or insufficient matching of standard addition concentrations. Due to the lack of isotopically labeled counterparts for these analytes, the matrix effects cannot be compensated and BFIs are considered semi-quantitative only, leaving room for improvement by adding suitable internal standards or adjustment of the chromatographic method to overcome matrix effects for those analytes. For example, proline betaine and hydroxyproline betaine, both BFIs for citrus fruit intake, exhibit higher matrix effects that could not be compensated by any included IS. To improve the quantification for these BFIs, an isotopically labeled counterpart of the analytes could be added to the method. Modifying the chromatographic method may help reduce matrix effects for proline betaine, but may at the same time cause negative effects for another BFI. Additionally, this would require a revalidation of the method.

One of the main challenges in validating quantitative methods for endogenous compounds or substances to which individuals are frequently exposed is the lack of true blank samples, which is a common issue when analyzing endogenous metabolites or analytes originating from commonly consumed foods [32–34]. This complicates the assessment of selectivity, potential matrix effects, and accuracy, particularly when the spiked analyte concentration is lower than the endogenous level already present. Consequently, the quantification of the spiked amount might lack the required precision. In such cases, apparent limitations may be misleading, quantitative measurements for these analytes carry inherent uncertainty and should be interpreted with caution. Furthermore, the simple sample preparation protocol, while advantageous for throughput, may not sufficiently eliminate interfering matrix components. This necessitated a compromise between analysis time and measurement accuracy for certain analytes.

The impact of these limitations depends on the intended application. For monitoring dietary compliance, semi-quantitative data may be acceptable. For ranking intake and comparison of mean differences, depending on the research question, semi-quantitative data may be similarly sufficient. However, for quantitative dietary intake assessment, precise quantification is required, particularly for BFIs that exhibit variability. Overall, despite some constraints, the method provides a valuable foundation for large-scale BFI quantification for a large number of foods.

5. Conclusion

Quantitative measurement of BFIs in nutritional studies represents a crucial next step towards establishing more reliable and robust relations between diet and health in both observational studies and dietary interventions. The developed HPLC-MS/MS method provides a robust and efficient tool to quantify BFIs for 27 widely consumed plant- and animal-based foods with an acceptable combined effective total analysis time of 30 min per sample together with a simple sample preparation

procedure. Twenty-one of the 27 foods can be absolutely quantified, while the remaining 6 foods can be quantified semi-quantitatively. The method encompasses analyte-specific working ranges tailored to physiologically relevant concentrations obtained under realistic habitual intakes. Its reliability was confirmed through validation according to common validation guidelines. While the presented setup already demonstrates high analytical performance, further development is possible by adding new or recently identified BFIs that were unavailable at the time of method development. For BFIs currently determined semi-quantitatively, the inclusion of suitable isotopically labeled standards, the use of dedicated blank matrices, or chromatographic method adjustments to better accommodate these BFIs might improve validation results and ultimately enable absolute quantification. In addition, the use of more sensitive LC-MS/MS instruments could improve peak resolution, and the implementation of polarity switching may reduce total run-time by half.

The next steps will involve applying this quantitative multi-BFI method to larger cohort studies to demonstrate its suitability for routine use in nutritional research. This approach offers a complementary tool to enhance diet assessment, potentially advancing the integration of BFI quantification into nutritional research. Moreover, the simultaneous quantification of a wide range of BFIs will generate valuable new validation data, further strengthening the evidence base for the included biomarkers.

CRediT authorship contribution statement

Christoph Hassenberg: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Sebastian T. Soukup:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Marina Armeni:** Writing – review & editing, Methodology, Investigation. **Achim Bub:** Writing – review & editing, Resources. **Jil V. Cannas:** Writing – review & editing, Resources. **David Fuentes:** Writing – review & editing, Formal analysis. **Otto Savolainen:** Writing – review & editing, Methodology. **Stephanie Seifert:** Writing – review & editing, Resources. **Rikard Landberg:** Writing – review & editing. **Sabine E. Kulling:** Writing – review & editing, Funding acquisition, Conceptualization. **Carina I. Mack:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2025.124793.

Data availability

Data will be made available on request.

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