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Extraction and Quantification of Phytoestrogens in Foods Using Automated Solid-Phase Extraction and LC/MS/MS

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Phytoestrogens are a group of polyphenolic plant metabolites that can induce biological responses. Their bioactivity is based on their similarity to 17β-estradiol and their ability to bind to the β-estrogen receptor. Although epidemiological data are inconclusive, phytoestrogens are considered to be beneficial for a variety of conditions, for example, hormone-related cancers like breast and prostate cancer. To investigate the biological effects of these compounds and to assess the exposure of larger cohorts or the general public, reliable data on the phytoestrogen content of food is necessary. Previously, food analysis for phytoestrogens was performed using either HPLC-UV or GC/MS. Here, we describe the development of the first generic method for the analysis of phytoestrogens in food, using automated solid-phase extraction and liquid chromatography−tandem mass spectrometry. The presented method shows a good reproducibility and can be easily adapted to other phytoestrogens if required.

Phytoestrogens are a group of nonsteroidal polyphenolic plant metabolites that induce biological responses based on their structural similarity to 17β-oestradiol[1,2] and their ability to bind to estrogen receptors.[3] Compared with estradiol, phytoestrogens have only a weak oestrogenic activity (10^{-2}−10^{-3} compared with 17β-estradiol) but can compete with estradiol at the receptor complex; however, they fail to stimulate a full estrogenic response.[4−9] Because of their bioactivity, these compounds receive increasing attention for potentially beneficial effects for a wide range of human conditions such as cancer,[10] cardiovascular disease,[11] osteoporosis,[12] and menopausal symptoms.[13] However, elevated endogenous sex hormone levels are generally associated with an increased risk of breast cancer in women,[14] and a recent study showed an increased risk for breast cancer associated with a high exposure to phytoestrogens.[15] There is also evidence of gene−nutrient interactions between phytoestrogens and estrogen receptor polymorphisms (ESR1 and NR1I2),[16,17] polymorphisms in the gene for the sex hormone binding globulin[18] and probably polymorphisms in the gene encoding aromatase (CYP19),[19] which influence their bioactivity. Despite the large number of studies conducted, there is still no clear evidence whether phytoestrogen intake has a beneficial or detrimental effect on human health and the UK Committee on Toxicity (COT) has recommended further research.[20]

The main source of phytoestrogens are fruits and vegetables, but they are also present in dairy products.[21] The principal classes are isoflavones (mainly in legumes, chickpeas, and soybean), prenylated flavonoids (e.g., in hops), coumestans (e.g., in young sprouting legumes like clover or alfalfa sprouts), and lignans (e.g., in cereals, linseed, and other fruits and vegetables).[20] In plants, phytoestrogens occur predominantly as glucosides,

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which are hydrolyzed by intestinal glucosidases to release the aglycons.[22,23]

Phytoestrogen exposure can be determined either directly by measuring diet or indirectly by using biomarkers in plasma or urine.[15] Although biomarkers are often more reliable due to the limitations in dietary assessment,[24,25] their use is often not feasible, in particular in larger studies, and intake has to be either calculated from dietary information provided by participants or determined by a combination of biomarkers and dietary information. Accurate information on the phytoestrogen content in foods is therefore crucial for the investigation of effects on health; it is also necessary to determine the phytoestrogen intake of the population.

Only limited studies have been conducted to determine the phytoestrogen content in the food, for example, in the UK[26−30] or Finland,[31−35] but these studies only provide data for ~12% of the UK diet[36] and mainly restricted to isoflavones. These studies used mainly HPLC-UV[37] and GC/MS[26,33] for analysis, with either deuterated standards, isomers, or standard addition.[38] The main disadvantage of deuterated standards is the instability of the deuterium label and the reexchange with hydrogen, leading to an overestimation of the analyte concentration.[39−41] We have previously used ^{13}C-labeled standards [42−45] to overcome this problem in plasma and urine analysis.[46−48] Both, GC/MS and HPLC-UV are time-consuming methods, either because of the extended run time necessary (45 min for the HPLC-UV standard method used for the USDA isoflavone databases)[37,49] or because of time-consuming derivatization steps for GC/MS as phytoestrogens are not volatile. Using LC/MS, the analysis time can be greatly reduced as fewer sample preparation steps are required and a shorter run time can be achieved. Although LC/MS has been used successfully for the determination of phytoestrogens in serum,[46] urine,[15] and some foods,[50] no generic LC/MS method is available for the large-scale analysis of phytoestrogens in food. Here, we describe a LC/MS method with automated solid-phase extraction (SPE) for the analysis of phytoestrogens in foodstuff. This is part of a larger study to develop a phytoestrogen database of UK food to investigate intake and exposure and the link between phytoestrogens and health as part of the European Prospective Investigation into Cancer (EPIC).[51]

EXPERIMENTAL SECTION

Chemicals. Biochanin A, daidzein, genistein, glycitein, formononetin, shonanin, secoisolariciresinol, matairesinol, and coumestrol were purchased from Plantech (Reading, Berkshire, UK). $^{13}C_3$-Biochanin A $^{13}C_3$-daidzein, $^{13}C_3$-genistein, $^{13}C_3$-glycitein, $^{13}C_3$-formononetin, $^{13}C_3$-matairesinol, $^{13}C_3$-secosiolariciresinol, $^{13}C_3$-enterodiol, and $^{13}C_3$-enterolactone were obtained from Dr. Nigel Botting (University of St. Andrews, Fife, UK).[42−45] β-Glucuronidase (from *Helix pomatia*), β-glucosidase (from almonds), and cellulase (from *Trichoderma reesi*) were purchased from Sigma (Poole, Dorset, UK). Water, methanol, acetic acid, and ammonia were purchased from Sigma and Fisher Scientific (Loughborough, Leicestershire, UK). To inhibit losses of target compounds by adsorption to glassware, only silanized glassware was used.

Purification of β-Glucuronidase. β-Glucuronidase from *H. pomatia* is often contaminated with phytoestrogens, and the enzyme was therefore purified as described by Grace et al.[52] Briefly, after conditioning a Strata-X solid-phase extraction cartridge (1 g, 20 mL, Phenomenex, Macclesfield, Cheshire, UK) with methanol and sodium acetate buffer (140 mM, pH 5), a 10% (*v/v*) solution of *H. pomatia* juice was passed directly through the cartridge and the purified enzyme solution collected in a clean tube.

Sample Collection. Food samples were purchased in local supermarkets, weighed and—if necessary—prepared. Samples were then cut into smaller pieces, frozen (−20 °C), freeze-dried (BOC Edwards, Crawley, Sussex, UK), and stored at −20 °C until analysis.

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Table 1. Molecular Weight (MW) and Precursor and Parent Ion (*m/z*, $[M - H]^-$) for Analyzed Phytoestrogens.

	compound	MW (g/mol)	precursor ion	product ion
1	biochanin A	284.3	283	268
1a	$^{13}C_3$-biochanin A	287.3	286	271
2	daidzein	254.2	253	224
2a	$^{13}C_3$-daidzein	257.2	256	227
3	formononetin	268.3	267	252
3a	$^{13}C_3$-formononetin	271.3	270	255
4	genistein	270.2	269	133
4a	$^{13}C_3$-genistein	273.2	272	135
5	glycitein	284.3	283	268
5a	$^{13}C_3$-glycitein	287.3	286	271
6	matairesinol	358.4	357	83
6a	$^{13}C_3$-matairesinol	361.4	360	84
7	secoisolariciresinol	362.4	361	93
7a	$^{13}C_3$-secosiolariciresinol	365.4	364	122
8	shonanin	344.4	343	328
9	coumestrol	268.2	267	239
10	$^{13}C_3$-enterolactone	301.3	300	255
11	$^{13}C_3$-enteroldiol	305.4	304	255

Sample Preparation. Approximately 100 mg of freeze-dried food was mixed with 1.0 mL of 10% methanol in sodium acetate (0.1%, pH 5), vortex mixed, and extracted for 30 min in a sonicating bath. After centrifugation (30 min, 3000*g*), the supernatant was collected and the pellet resuspended in 1.0 mL of 10% methanol in sodium acetate (0.1%, pH 5), centrifuged as above, and the supernatants combined. To 2 mL of supernatant, 3 mL of hydrolysis reagent (150 μL of purified *H. pomatia* juice, 240 μL of cellulase, and 540 μL of β-glucosidase (10 units/mL) in sodium acetate (0.1%, pH 5) containing 16 ng/L internal standard were added and the mixture was incubated for 16 h at 37 °C.

Solid-Phase Extraction. SPE was performed with a Gilson APEX XL4 liquid handling robot (Gilson, Middleton, WI). A Strata C-18E SPE cartridge (50 mg/mL; Phenomenex, Macclesfield, Cheshire, UK) was wetted with 0.95 mL of 100% methanol and activated with 0.95 mL of 5% aqueous methanol. Thereafter, 2 mL of incubated sample was added to the cartridge, washed twice with 0.95 mL of 5% aqueous methanol, and sample eluted with 0.95 mL of 100% methanol. The eluate was then dried using a SpeedVac and reconstituted in 200 μL of 40% aqueous methanol.

Liquid Chromatography–Tandem Mass Spectrometry. Samples were analyzed using an ABI 4000 QTRAP mass spectrometer (Applied Biosystems, Warrington, Cheshire, UK) combined with an Agilent 1100 CapLC system (Agilent, Wokingham, Berkshire, UK) and a CTC HTC PAL autoinjector (CTC, Zwingen, Switzerland). Samples were separated on a diphenyl column (Varian Pursuit, 3 μm, 150 × 2.0 mm; Varian, Oxford, Oxfordshire, UK), kept at 50 °C, using a binary gradient (mobile phase A, 40% ammonium acetate (0.1%, pH 4.8) in methanol; mobile phase B, 100% methanol): 0 min 100% A; 2 min 75% A; 9 min 42% A; 9.01 min 0% A; 10 min 0% A; 10.01 min 100% A; 15 min 100% A (flow rate, 250 μL/min). Samples were analyzed in the negative ion mode with a spray voltage of −4.3 kV and a spray temperature of 600 °C. The transitions used are shown in Table 1.

Quantification. Samples were quantified using the area ratio between analyte and internal standard. For analytes for which no $^{13}C_3$-labeled standard was available, $^{13}C_3$-enterolactone or $^{13}C_3$-enterodiol was used. The area ratio was compared to a calibration line consisting of seven standards (0, 1, 10, 100, 500, 1000, 2000 ng/mL); a $1/x$ weighing factor[52] was applied. As there was no common matrix available, calibration standards were prepared in 40% aqueous methanol containing the same concentration of internal standard as the samples.

Table 2. Method Reproducibility. Phytoestrogen Concentration in Different Preparations of the Quality Control Sample (in ng/mL; Average of Six Samples Each)

	intraday		
	average	% CV	interbatch CV
biochanin A	327	3%	1%
dadizein	13	13%	6%
formononetin	42	1%	1%
genistein	44	4%	3%
glycitein	14	12%	4%
matairesinol	81	3%	3%
secoisolariciresinol	336	4%	4%
coumestrol	41	9%	5%

Quality Control. To monitor the performance of the assay, a quality control sample consisting of equal weights of freeze-dried celery, red cabbage, and orange was prepared and included into each batch.

Recovery. Because of the varied matrix, recovery was determined using extraction buffer. Extraction buffer was spiked with neat standards before and after the SPE step. The recovery was calculated as the ratio of the pre- and postspike area for each compound.

RESULTS AND DISCUSSION

The development of a generic analytical method for the determination of phytoestrogens in foodstuff must meet several criteria if it is to be used on a large number of samples. Apart from the general requirements on robustness, precision, accuracy, and reproducibility, it has to be able to cope with a large variety of different matrixes and should allow for automation to increase throughput. In contrast to the development of bioanalytical methods for compounds in a single matrix like urine or plasma, the development of such a method in foodstuffs is made difficult by the wide variety of matrixes and the lack of proper quality control samples that can be used to ensure accuracy. Furthermore, the determination of recovery and matrix effect is of only limited value since practically it can only be determined for few foods and can vary significantly. The recovery for neat compounds was between 89 and 107%, but this will vary in real samples due to absorption to food material. Liggins et al.[53] described recoveries ranging between 70% and 110%, and we found for some foods recoveries to be as low as 30%. This is partially compensated by the use of $^{13}C_3$-labeled internal standards.

The precision and reproducibility of the assay is within the range recommended by the FDA;[15] the intrabatch CV for the quality control sample is between 3 and 14%, the intrabatch CV between 1 and 6% (Table 2). The determination of accuracy of this assay is more difficult. For matrixes like plasma or urine,

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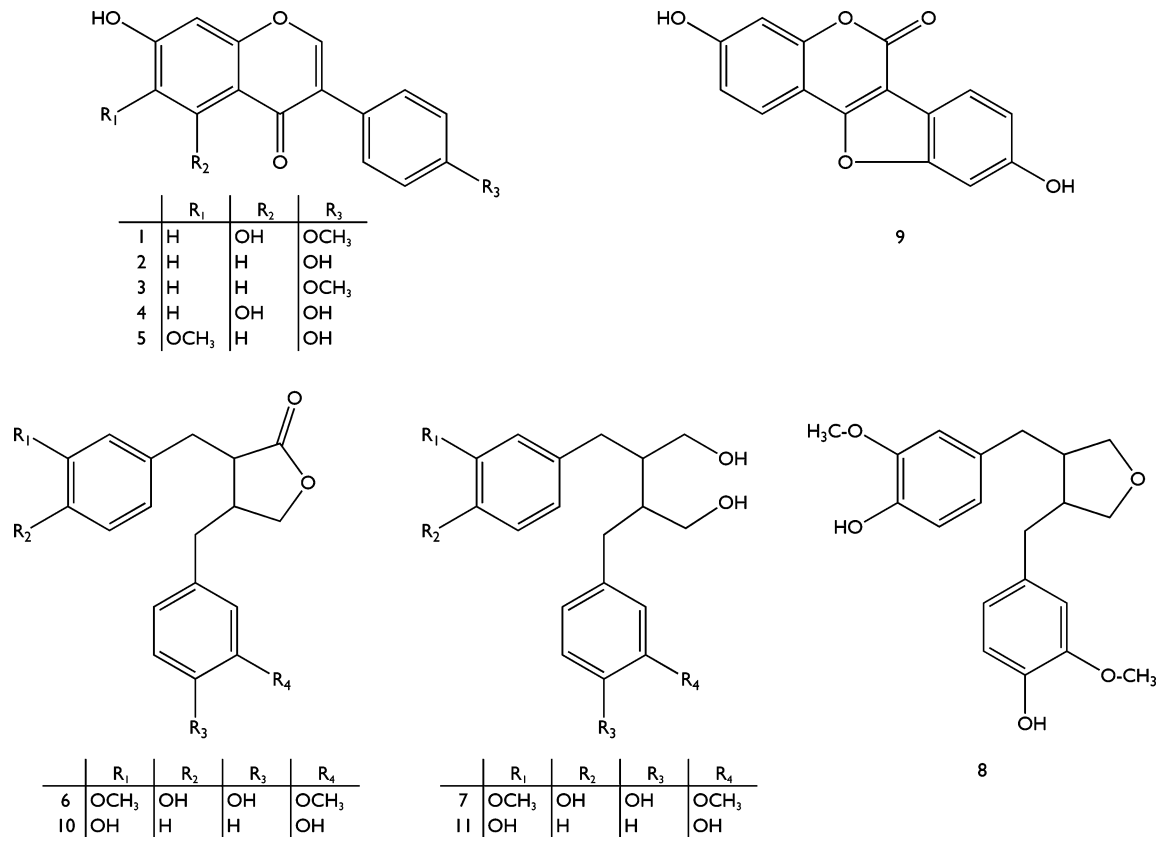

	R_1	R_2	R_3
1	H	OH	OCH_3
2	H	H	OH
3	H	H	OCH_3
4	H	OH	OH
5	OCH_3	H	OH

	R_1	R_2	R_3	R_4
6	OCH_3	OH	OH	OCH_3
10	OH	H	H	OH

	R_1	R_2	R_3	R_4
7	OCH_3	OH	OH	OCH_3
11	OH	H	H	OH

Figure 1. Structures of isoflavones biochanin A (1), daidzein (2), formononetin (3), genistein (4), and glycitein (5); lignans matairesinol (6), secoisolariciresinol (7), and glycitein (8); coumestrol (9) and the enterolignans enterolactone (10) and enterodiol (11).

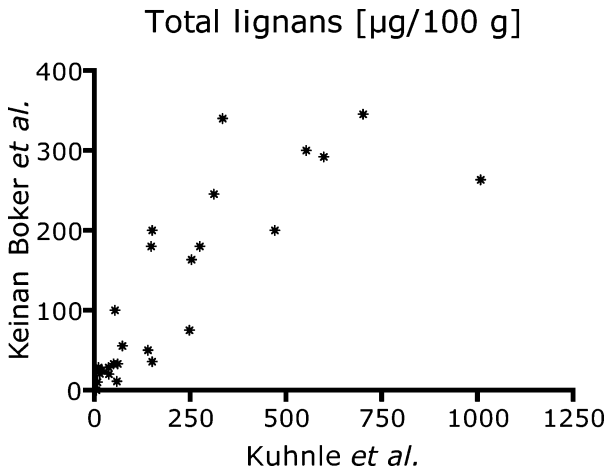

Figure 2. Correlation of total lignan concentration in a selction food between the data from this study and data published by Kleinan Boker et al.[56]

Table 3. Yield (Relative to Treatment with Cellulase (*T. viride*) Only) of Phytoestrogen Extraction from Asparagus, Linseed, and Red Rice Following Hydrolysis Treatments with Different Hydrolytic Enzymes

cellulase (*T.* viride)	cellulase (*T.* reesei)	β-glucosidase (almonds)	β-glucuronidase/ sulfatase (*H. pomatia*)	lignans	isoflavones
X		X		1	1
X		X		1	1
X			X	39	7
X		X	X	29	8
	X			2	2
	X	X		1	2
	X		X	52	12
	X	X	X	42	14

accuracy can be determined by spiking compound-free plasma with known concentrations; for food samples, this is not possible since most foods contain some amounts of phytoestrogens and therefore compound-free samples are virtually not available. A food sample with a known amount of phytoestrogens would be ideal but impossible to obtain because of the large variation of phytoestrogen content.[54,55] The alternative, spiking of food samples with neat compounds, is only of limited use as the spiked compounds will stay mainly outside of the cell and can be absorbed, for example, by the cell wall. Their recovery will be different from the recovery of compounds within the cellular structure of the cell and thereby bias the result. We have therefore compared our results with data published recently and found a highly significant ($p < 0.0001$) correlation (Pearson's r: 0.823) with lignan concentrations published by Kleinan Boker et al.[56] (Figure 1).

In plants, phytoestrogens are often conjugated to one or more carbohydrate moieties,[26,29] impeding the analysis and requiring additional hydrolysis steps to obtain aglycons. Since isoflavones are unstable under strong acidic conditions, enzymatic hydrolysis is generally preferred.[27,29,57] To develop a suitable system, we

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Table 4. Phytoestrogen Content of Fruits and Vegetables[a]

Food	Preparation	Daidzein	Genistein	Glycitein	Biochanin A	Formononetin	Isoflavones	Secoisolariciresinol	Matairesinol	Shonanin	Lignans	Coumestrol
Apple (Bramley)	raw	8 ± 1	4 ± 1	<1	9 ± 4	4 ± 3	25	14 ± 12	3 ± 1	nd	16	nd
Apple (Bramley)	cooked	11 ± 0	2 ± 1	<1	32 ± 13	6 ± 4	51	16 ± 13	3 ± 1	nd	19	nd
Apple (Cox) w skin	raw	2 ± 0	<1	<1	7 ± 3	4 ± 4	13	14 ± 10	1 ± 0	nd	15	nd
Apple (Cox) w/o skin	raw	2 ± 0	2 ± 1	<1	3 ± 1	7 ± 7	13	16 ± 11	<1	nd	16	nd
Apple (Golden Delicious) w skin	raw	1 ± 1	2 ± 1	<1	5 ± 3	6 ± 6	16	21 ± 13	<1	nd	21	nd
Apple (Golden Delicious) w/o skin	raw	2 ± 1	3 ± 1	<1	12 ± 5	4 ± 3	21	15 ± 9	<1	nd	15	nd
Apple (Granny Smith) w skin	raw	2 ± 0	1 ± 1	nd	2 ± 2	7 ± 7	13	13 ± 11	2 ± 2	nd	15	nd
Apple (Granny Smith) w/o skin	raw	5 ± 2	1 ± 1	<1	<1	4 ± 4	11	12 ± 10	2 ± 1	nd	14	nd
Apple (Red) w skin	raw	2 ± 0	1 ± 0	3 ± 0	2 ± 1	<1	7	14 ± 0	nd	nd	14	nd
Apple (Red) w/o skin	raw	3 ± 0	<1	1 ± 0	<1	<1	4	6 ± 0	<1	nd	6	<1
Apricot	raw	3 ± 0	4 ± 1	nd	<1	<1	7	476 ± 4	2 ± 1	<1	479	<1
Aubergine	raw	2 ± 0	1 ± 1	1 ± 0	3 ± 0	<1	8	116 ± 1	<1	nd	116	nd
Aubergine	cooked	<1	<1	<1	<1	<1	0	161 ± 6	<1	nd	161	nd
Banana	raw	<1	3 ± 2	3 ± 0	5 ± 2	<1	11	1 ± 0	2 ± 1	2 ± 0	4	nd
Bean (baked)	raw	6 ± 0	9 ± 2	<1	nd	<1	16	76 ± 2	2 ± 1	nd	79	nd
Bean (baked)	cooked	7 ± 0	10 ± 1	1 ± 0	nd	nd	19	66 ± 2	2 ± 2	nd	67	nd
Bean (Broad)	raw	<1	<1	<1	<1	<1	0	102 ± 3	1 ± 0	nd	103	<1
Bean (Broad)	cooked	<1	<1	<1	3 ± 1	<1	3	80 ± 7	1 ± 0	nd	82	nd
Bean (French)	raw	124 ± 38	381 ± 19	20 ± 1	16 ± 1	<1	541	1009 ± 21	<1	—	1009	31 ± 9
Bean (French)	cooked	82 ± 18	360 ± 24	15 ± 1	23 ± 3	<1	480	1084 ± 7	<1	—	1084	19 ± 4
Beetroot	cooked	<1	<1	<1	nd	<1	0	57 ± 4	<1	nd	57	nd
Beetroot (precooked)	raw	nd	1 ± 0	<1	9 ± 0	<1	11	59 ± 1	<1	nd	59	nd
Broccoli	raw	<1	2 ± 1	<1	<1	nd	2	599 ± 34	2 ± 0	nd	601	nd
Broccoli	cooked	<1	<1	nd	14 ± 4	nd	14	392 ± 8	2 ± 1	nd	394	nd
Cabbage (green)	raw	nd	<1	<1	2 ± 1	2 ± 0	4	98 ± 4	4 ± 0	<1	102	1 ± 0
Cabbage (green)	cooked	nd	1 ± 1	<1	4 ± 2	2 ± 1	7	100 ± 27	5 ± 1	<1	105	2 ± 0
Cabbage (red)	raw	nd	<1	<1	1 ± 1	2 ± 1	3	54 ± 12	4 ± 1	3 ± 0	61	nd
Cabbage (red)	cooked	nd	<1	<1	1 ± 0	2 ± 0	3	73 ± 5	4 ± 0	<1	78	nd
Cabbage (Savoy)	raw	7 ± 6	15 ± 12	<1	15 ± 3	1 ± 0	38	265 ± 115	1 ± 0	nd	266	nd
Cabbage (Savoy)	cooked	1 ± 1	2 ± 1	<1	nd	<1	4	174 ± 96	2 ± 0	nd	175	nd
Cauliflower	raw	<1	2 ± 1	<1	1 ± 1	<1	3	140 ± 41	3 ± 0	nd	143	nd
Cauliflower	cooked	<1	<1	<1	<1	nd	0	118 ± 41	2 ± 1	nd	120	nd
Celeriac	raw	<1	15 ± 14	<1	<1	nd	15	174 ± 165	3 ± 3	26 ± 25	204	nd
Celery	raw	<1	2 ± 1	nd	nd	nd	2	119 ± 36	<1	20 ± 15	140	nd
Celery	cooked	<1	3 ± 1	nd	nd	nd	3	149 ± 59	<1	22 ± 13	171	nd
Cherry	raw	6 ± 1	3 ± 0	17 ± 3	5 ± 3	<1	30	11 ± 3	3 ± 1	nd	13	nd
Chicory	raw	<1	2 ± 1	<1	4 ± 3	<1	5	335 ± 6	14 ± 1	nd	349	nd
Courgette	raw	2 ± 2	<1	nd	<1	nd	2	553 ± 3	<1	nd	553	nd
Courgette	cooked	<1	<1	nd	33 ± 2	3 ± 0	36	619 ± 14	<1	nd	619	nd
Gooseberry	raw	2 ± 1	2 ± 1	3 ± 0	2 ± 1	<1	10	720 ± 2	<1	—	720	<1
Grapefruit	raw	29 ± 1	20 ± 4	nd	99 ± 61	6 ± 5	154	248 ± 25	3 ± 0	nd	251	3 ± 0
Grapes (black)	raw	6 ± 4	21 ± 6	<1	nd	<1	27	39 ± 8	36 ± 4	—	76	nd
Grapes (red)	raw	3 ± 1	15 ± 2	<1	8 ± 3	nd	26	33 ± 1	34 ± 2	nd	67	nd
Grapes (white)	raw	<1	<1	<1	3 ± 2	<1	3	43 ± 30	50 ± 3	nd	92	nd
Greengage	raw	2 ± 1	3 ± 0	5 ± 0	<1	<1	9	589 ± 21	6 ± 3	—	595	nd
Leek	raw	nd	<1	<1	21 ± 3	<1	21	702 ± 27	3 ± 2	nd	705	nd
Leek	cooked	<1	2 ± 0	<1	103 ± 4	8 ± 0	112	662 ± 19	3 ± 0	nd	665	nd
Lemon juice	raw	1 ± 1	4 ± 1	2 ± 2	4 ± 1	<1	12	23 ± 5	<1	1 ± 0	24	13 ± 1
Lettuce (iceberg)	raw	<1	<1	<1	2 ± 1	nd	2	148 ± 19	6 ± 0	nd	154	<1
Lettuce (round)	raw	<1	<1	<1	2 ± 1	nd	2	151 ± 19	4 ± 1	nd	155	<1
Mango	raw	<1	1 ± 1	<1	5 ± 5	<1	6	85 ± 3	8 ± 1	<1	93	nd
Marrow	raw	<1	2 ± 0	<1	4 ± 0	<1	5	199 ± 7	<1	nd	199	nd
Melon (Cantalupe)	raw	<1	<1	<1	3 ± 1	<1	3	199 ± 5	2 ± 1	nd	200	nd
Melon (water)	raw	1 ± 0	2 ± 1	<1	4 ± 4	<1	7	437 ± 17	<1	nd	437	nd
Blackcurrant	raw	1 ± 0	nd	<1	8 ± 2	<1	9	563 ± 22	9 ± 0	—	573	nd
Nectarine	raw	2 ± 0	1 ± 0	1 ± 0	5 ± 3	<1	10	254 ± 9	<1	—	254	nd
Onion	raw	nd	<1	<1	4 ± 1	<1	4	275 ± 12	2 ± 2	—	277	nd
Onion	cooked	nd	3 ± 0	nd	5 ± 0	<1	8	284 ± 13	3 ± 2	—	287	nd
Orange	raw	25 ± 15	9 ± 1	nd	37 ± 37	2 ± 1	73	151 ± 36	1 ± 0	nd	152	15 ± 10
Parsnip	raw	<1	11 ± 10	<1	23 ± 23	6 ± 6	40	181 ± 13	99 ± 14	16 ± 6	296	nd
Parsnip	cooked	<1	1 ± 0	<1	<1	<1	1	228 ± 6	81 ± 5	14 ± 10	323	nd
Pea	raw	1 ± 0	2 ± 1	3 ± 0	nd	<1	6	5 ± 5	<1	nd	5	nd
Pea	cooked	<1	1 ± 1	1 ± 0	2 ± 0	<1	5	nd	<1	nd	0	<1
Pea (frozen)	raw	<1	<1	2 ± 0	2 ± 1	<1	4	5 ± 2	1 ± 0	nd	6	<1
Pea (frozen)	cooked	<1	<1	2 ± 0	2 ± 1	<1	4	4 ± 2	1 ± 1	nd	5	nd
Peach	raw	<1	<1	nd	1 ± 0	<1	1	312 ± 3	1 ± 1	nd	313	nd
Pear (conference) w skin	raw	nd	<1	5 ± 1	nd	nd	5	28 ± 1	1 ± 1	3 ± 0	32	nd
Pear (conference) w/o skin	raw	nd	1 ± 1	2 ± 1	nd	nd	3	12 ± 5	3 ± 1	3 ± 0	18	nd
Pepper (green)	raw	2 ± 1	<1	nd	nd	nd	2	78 ± 4	<1	nd	78	nd
Plums (red)	raw	2 ± 1	1 ± 1	9 ± 0	3 ± 1	<1	15	37 ± 2	<1	nd	37	nd
Potato (new)	cooked	nd	nd	<1	13 ± 2	<1	13	8 ± 0	2 ± 0	—	10	nd
Potato (sweet)	raw	<1	2 ± 1	<1	1 ± 1	nd	3	517 ± 9	463 ± 13	nd	980	nd
Potato (sweet)	cooked	<1	2 ± 2	<1	<1	nd	2	465 ± 29	521 ± 9	nd	985	nd
Radish	raw	1 ± 0	2 ± 1	1 ± 0	<1	<1	4	62 ± 1	<1	nd	62	nd
Raspberry	raw	<1	<1	<1	10 ± 5	<1	10	182 ± 4	3 ± 1	—	185	nd
Rhubarb	raw	12 ± 9	2 ± 1	2 ± 1	4 ± 3	<1	20	1 ± 1	2 ± 0	nd	3	<1
Rhubarb	cooked	5 ± 3	3 ± 3	<1	4 ± 3	<1	13	2 ± 1	2 ± 0	nd	4	<1
Spinach	raw	<1	nd	<1	15 ± 10	<1	15	50 ± 5	1 ± 0	nd	51	5 ± 2
Spinach	cooked	2 ± 1	2 ± 2	<1	7 ± 7	<1	11	32 ± 3	<1	nd	32	2 ± 0
Spring onion	raw	nd	2 ± 2	nd	163 ± 8	11 ± 0	176	671 ± 12	6 ± 1	<1	677	nd
Sprout	raw	2 ± 0	nd	1 ± 0	<1	nd	3	471 ± 5	<1	nd	471	<1
Sprout	cooked	<1	<1	<1	2 ± 2	<1	2	444 ± 16	<1	nd	444	nd
Strawberry	raw	3 ± 0	2 ± 0	<1	nd	<1	5	73 ± 3	1 ± 1	nd	74	nd
Swede	raw	2 ± 2	1 ± 0	1 ± 0	9 ± 4	<1	13	53 ± 3	2 ± 0	nd	54	nd
Swede	cooked	<1	2 ± 0	<1	3 ± 2	<1	5	48 ± 4	2 ± 1	nd	50	nd
Sweet corn	raw	nd	<1	1 ± 0	7 ± 0	<1	9	14 ± 0	3 ± 0	—	18	nd
Sweet corn	cooked	nd	<1	1 ± 0	6 ± 1	<1	7	17 ± 0	7 ± 1	—	24	nd
Tomato	raw	<1	14 ± 5	<1	2 ± 1	nd	16	61 ± 5	2 ± 1	nd	63	nd
Turnip	raw	nd	2 ± 0	<1	nd	nd	2	131 ± 3	2 ± 1	nd	134	nd
Turnip	cooked	<1	2 ± 0	<1	nd	<1	2	113 ± 2	6 ± 0	nd	119	nd
Watercress	raw	nd	3 ± 0	<1	2 ± 2	<1	5	625 ± 118	3 ± 3	<1	627	nd

[a] In μg/100 g of dry weight ± standard error. nd denotes not detected.

hydrolyzed three different foods—linseed, red rice, and asparagus—with a combination of several enzymes (Table 3). The results show that cellulase from *T. reesei* results in a higher yield of phytoestrogen than that from *Trichoderma viride* and that best results were achieved when both β-glucosidase and β-glucuronidase were added.

The extraction and LC/MS/MS method developed here is a modification of a method used routinely for the analysis of phytoestrogen in plasma and urine.[46,47] This method allows the detection of less than 1 ng/mL phytoestrogen, which corresponds to a concentration 1.5 μg/100 g in food. Table 4 shows the phytoestrogen content of a selection of foods determined with the method described.

CONCLUSION

We described here a generic LC/MS method with automated solid-phase extraction for the analysis of phytoestrogens in food. This method shows a good precision and reproducibility and allows the analysis of a wide variety of foodstuff. Previous studies relied on HPLC-UV or GC/MS for analysis, with either deuterated standards, isomers, or standard addition. These methods are time-consuming, and the instability of the deuterium label can lead to an overestimation of the analyte concentration. In this method, ^{13}C-labeled standards are used that overcome this limitation.

The method described here is this first generic LC/MS method for the determination of phytoestrogens in food and will be used to determine phytoestrogens in the UK food table.

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