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SCIENCE DIRECT®
Food Chemistry 96 (2006) 261–272
Food Chemistry
www.elsevier.com/locate/foodchem

An international quality assurance scheme for the quantitation of daidzein and genistein in food, urine and plasma

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Received 22 March 2004; received in revised form 15 February 2005; accepted 15 February 2005

Abstract

This quality assurance (proficiency testing) scheme was commissioned to enable the Food Standards Agency (FSA) to determine the quality of analytical results submitted by researchers measuring the concentrations of phytoestrogens in foods and biological fluids in FSA-funded research projects, and also, to demonstrate that FSA-funded laboratories are producing consistent and precise results. Non-FSA-funded laboratories from around the world were also invited to join in the scheme to increase the number participants. A secondary objective was to highlight the most successful methodologies used to analyse phytoestrogens.

Test materials of infant formula, plasma and urine were prepared and tested by LC-MS/MS for homogeneity to establish the concentrations of two phytoestrogens, daidzein and genistein in these test materials. Twenty one laboratories were recruited to participate in the scheme. Test materials for analysis of daidzein and genistein were issued to participants on a four-monthly basis. The results and methods of analysis were correlated and a report for each round of testing, was produced to assess the competency of laboratories. After four rounds of testing the performances of laboratories were correlated and the validity of the methods of analysis used were assessed.

The performances of most laboratories for analysis of both daidzein and genistein in infant formula and biological test materials varied widely. Suitable methods that performed well for the analysis of daidzein and genistein in infant formula test materials involved a solvent extraction step, followed by reverse phase HPLC with UV detection. For biological matrices, namely plasma and urine, acceptable performance for the analysis of daidzein and genistein was achieved by methods that have an enzyme hydrolysis step before either solid phase extraction or solvent extraction and separation by either GC or HPLC and detection by MS.

Keywords: Quality assurance scheme; Quantitation; Daidzein; Genistein; Infant formula; Urine; Plasma

1. Introduction

Phytoestrogens are compounds naturally present in many foods of plant origin and include isoflavones, coumestans and lignans. Setchell (1995) showed that these compounds and/or their metabolites have estrogenic properties similar to but generally less potent than the human sex hormone, 17β-estradiol. They have been shown to have biological effects when tested in the laboratory and in animals, and this property has prompted research on how phytoestrogens in food may affect humans. Research on the possible risks and benefits of phytoestrogens for human health has recently been comprehensively reviewed by the Committee on Toxicity, an expert advisory committee to the UK Government (COT, 2003).

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doi:10.1016/j.foodchem.2005.02.038

Much of the research on the health implications of phytoestrogens is underpinned by analysis of the phytoestrogen content of foods and biological fluids. However, phytoestrogen analysis is acknowledged to be a difficult area. Therefore, a quality assurance (proficiency testing) scheme was set up to provide an assessment of the state-of-the art.

This scheme has enabled the competency of laboratories to be assessed and allowed some element of validation of the different methods in use to be undertaken. The results have provided an understanding of the effectiveness of the different methods and, in particular, the efficiency of the extraction methods and the detection limits for these compounds.

Initially the scheme was designed for the analysis of total daidzein and total genistein in urine, plasma and soya infant formula matrices and four rounds of testing have been carried out. Stable triply ^{13}C-labelled isoflavones were provided to participants to use as internal standards. Participants analysed the samples for daidzein and genistein using their method of analysis. Samples were issued at regular intervals, contractors returned their results for statistical evaluation and a report was issued for each round of testing. The aim of the scheme was to demonstrate that laboratories were producing consistent and precise results.

Genistein and daidzein are two of the prevalent isoflavones found in leguminous plants and both have been most extensively researched. Daidzein and genistein were chosen for measurement to encourage the maximum participation. For the purpose of the scheme, results were returned for total daidzein and total genistein. Daidzein and genistein or aglucones are usually linked (conjugated) to a sugar molecule to form a glycoside and, following absorption into the body from the gut, they are hydrolysed and subsequently conjugated with glucuronic and sulfuric acids. Analytical strategies usually consider total isoflavone concentrations and thus require a hydrolysis procedure to convert glycoside conjugates to the aglucone.

The status of the quality assurance scheme, after four rounds of testing, is described, with particular emphasis on the performance of laboratories and the methods of analysis that they used.

2. Materials and methods

2.1. Materials

Stable triply ^{13}C-labelled isoflavone standards of genistein and daidzein were synthesised and supplied by Nigel Botting of St Andrews University. The daidzein was labelled at the 3, 4 and 8-positions, using potassium [^{13}C]cyanide and [^{13}C]methyl iodide as the sources of the labels, as described by Oldfield, Chen, and Botting (2004). Similar procedures were used for the synthesis of the ^{13}C-labelled genistein and the ^{13}C atoms were incorporated at the 3, 4 and 1′-positions of the structure, as described by Clarke et al. (2002). Analysis, by LC-MS, showed that the labelled daidzein and genistein contained 7% and 4%, respectively, of the species with only two ^{13}C atoms but neither showed any discernible trace of the species with only one as described by Clarke et al. (2002). This makes them suitable as internal standards as there is very little overlap with the masses from the natural unlabelled analytes.

2.2. Participation

Participation in quality assurance schemes provides laboratories with an objective means of assessing and documenting the reliability of the data they are producing. The Quality Assurance Scheme was open to international laboratories. Twenty one laboratories participated in the Scheme from the following countries: Australia (2), Brazil (1), Canada (1), Denmark (1), Finland (1), Israel (1), the Netherlands (1), UK (6) and USA (7).

After registering for the scheme participants were asked to complete and return a methodological questionnaire describing their method of analysis for each type of matrix. A summary of the methods used by participants describing their method of analysis for each type of matrix is given in Table 1.

2.3. Test materials

2.3.1. Homogeneity

All test materials to be used in the four rounds of testing were prepared and tested for homogeneity prior to issuing the materials in the first round.

2.3.2. Preparation

2.3.2.1. General. For each matrix three different test materials were prepared.

2.3.2.2. Infant formula. Sufficient amounts of commercially available infant formulae were purchased from retail outlets. The infant formulas were thoroughly mixed before being dispensed. Directly after mixing, the infant formula was weighed (10 g) into laminated foil sachets and sealed with a heat sealer. The sachets were individually labelled with random four figure numbers and stored at −20 °C prior to distribution.

2.3.2.3. Urine. A suitable 24-h human urine sample was obtained and analysed to ensure a low level of isoflavones. This "blank" urine (Urine Test Material 1) was fortified with solutions containing daidzein and genistein in dimethyl sulfoxide to form Urine Test Material 2, fortified to obtain 50 ng/ml of daidzein and 156 ng/ml of genistein and Urine Test Material 3, fortified to obtain 110

Table 1
Summary of methods used by participants

Method			
Matrix	Number of questionnaires returned	Extraction (number of participants)	Detection and quantification (number of participants)
Infant milk formula	15	*Solvent extraction:* Acid hydrolysis (5) Enzymatic hydrolysis (2)	HPLC-UV and/or MS (12) GC-MS (2) ELISA (1)
Plasma	11	Enzymatic hydrolysis with solvent extraction (7) Enzymatic hydrolysis with SPE (2) Enzymatic hydrolysis (1)	HPLC-MS (3) HPLC-ECD (2) Immunoassay (3) HPLC-UV (1) GC-MS (1)
Urine	8	Enzymatic hydrolysis (1) Enzymatic hydrolysis with solvent extraction (3) Enzymatic hydrolysis with SPE (4)	HPLC-UV/MS (4) GC-MS (3) Immunoassay (1)

ng/ml of dadzein and 273 ng/ml of genistein, respectively. The amounts of fortification for daidzein and genistein were based on the concentrations found in urine, as determined in a human metabolic project, namely 10–240 ng genistein/ml and 8–100 ng daidzein/ml in urine.

Directly after mixing in an ultra sonic bath for 10 min, the urine samples were dispensed (3.75 ml) via a Gilson pipette into silanised amber vials and capped with a PTFE septum. The vials were individually labelled with random four figure numbers and stored at −40 °C prior to distribution. Participants received at least 2 vials, both labelled with the same number, for each urine sample i.e. 2 × 3.75 ml = 7.5 ml for each urine sample.

2.3.2.4. Plasma. Sufficient amount of spent plasma (~2 l) was obtained and analysed to ensure a blank level of isoflavones. This ''blank'' plasma (Plasma Test Material 1) was fortified with solutions containing daidzein and genistein in dimethyl sulfoxide to form Plasma Test Material 2 by the addition of 55 ng/ml of daidzein and 138 ng/ml of genistein, and Plasma Test Material 3 by the addition of 110 ng/ml of daidzein and 276 ng/ml of genistein, respectively. The amounts of fortification for daidzein and genistein were based on the concentrations found in plasma as determined in a human metabolic project, namely 10–240 ng of genistein/ml and 8–100 ng daidzein/ml in plasma.

Directly after mixing in an ultra sonic bath for 10 min, the plasma samples were dispensed (3.75 ml) via a Gilson pipette into silanised amber vials and capped with a PTFE septum. The vials were individually labelled with random four figure numbers and stored at −40 °C until distribution.

2.3.3. Homogeneity testing

The test materials were assessed for homogeneity using procedures described by Thompson and Wood (1993a, 1993b). To check homogeneity, a random selection of five units of each of the three test materials for each matrix was analysed, in duplicate for daidzein and genistein. Verification of homogeneity was established using two statistical tests, as described by Thompson and Wood (1993a, 1993b). These tests are the *F*-test and S_s/σ test, where S_s is the square root of the sampling variance and σ is the target value for standard deviation. The infant formula test materials were analysed using solvent extraction and HPLC-tandem mass spectrometry (LC-MS/MS), as described by Coldham and Sauer (2001) and the biological test materials, namely plasma and urine, were analysed using enzymatic hydrolysis, followed by solid phase extraction and detected by LC-MS/MS, as described by Coldham, Zhang, Key, and Sauer (2002).

Table 2
Test materials used in rounds 1–4

Matrix			
Round	Infant milk formula	Plasma	Urine
1	TM 2	TM 1 TM 2	TM 1 TM 2
2	TM 1 TM 2	TM 1 TM 2 TM 3	TM 1 TM 3
3	TM 2 TM 3	TM 2 TM 3	TM 1 TM 2
4	TM 2 TM 3	TM 1 TM 2	TM 2 TM 3

2.3.4. Test materials used in rounds 1–4

For each of the three matrices, there were three different test materials prepared. All of these test materials were issued at least twice throughout the four rounds of testing. A summary of the test materials issued for each of the four rounds of testing is given in Table 2.

3. Results and discussion

3.1. General

Each round of testing consisted of infant formula and biological samples of plasma and urine. Participants

were only issued with the matrices they requested. The majority of participants (11–15) requested infant formula test materials, with fewer participants requesting the biological test materials. Overall 9–12 participants requested plasma test materials and 7–8 participants requested urine test materials over the four rounds of testing. Participants analysed the test materials for daidzein and genistein and returned results for statistical analysis. Participants submitted results using the method they considered appropriate. The concentration of isoflavones in the samples was expressed as μg/g for the infant formula test materials and as ng/ml for the biological test materials. The data were returned on a proforma with a brief description of the analytical method.

3.2. Statistical analysis

3.2.1. General

Results were analysed by the procedure described by Thompson and Wood (1993a, 1993b) in the IUPAC/ISO/AOAC International Harmonised Protocol.

3.2.2. Analysis of z-scores

Each laboratory was given z-scores calculated from

$$z = (x - \hat{X})/\sigma,$$

where x is the measurement of analyte concentration in the test material, $\hat{X}$ is the assigned value, the best estimate of the "true" concentration of the analyte and σ is the target value for standard deviation.

For isoflavones, the assigned value $\hat{X}$ was taken as the consensus value from all results calculated from the Robust Mean, as described by Anon (1989a, 1989b) for infant formula test materials, and the median for biological test materials.

The σ value was obtained by

$$\sigma = b\hat{X},$$

where $b = \%\mathrm{RSD_R}/100$. The $\%\mathrm{RSD_R}$ values were obtained from the Horwitz equation, defined by Horwitz (1982)

$$\%\mathrm{RSD_R} = 2^{(1-0.5\log C)},$$

where C = the assigned value expressed as a decimal, e.g., 115 ng/ml = 115×10^{-9}.

During the four rounds of testing, the same test material was analysed for daidzein and genistein, more than once, in at least two rounds of testing. As the number of participants returning results was limited, <15 in all cases, for each test material in a round of testing, the assigned value was calculated from all results returned for an analyte in a test material over the four rounds of testing to obtain an overall assigned value which was the best estimate of the concentration of the analyte in that test material. This overall assigned value was used to calculate z-scores.

3.2.3. Interpretation of z-scores

If it is assumed that the parameters $\hat{X}$ and σ correctly describe the variation of the normally distributed variable x, the data are known as "well behaved". In such a case the z-scores and can be interpreted as follows:

$|z| \leqslant 2$ "Satisfactory": will occur in about 95% of cases produced by "well behaved results".
$2 < |z| \leqslant 3$ "Questionable": will occur in about 5% of cases produced by "well behaved results".
$|z| > 3$ "Unsatisfactory": will only occur in about 0.3% of cases produced by "well behaved results".

In other words, the statistics of a normal distribution mean that about 95% of data points will lie between a z-score of −2 and +2. Performance in a quality assurance test is considered 'satisfactory' if a participants z-score lies within this range. If a participants z-scores lie outside $|z| > 2$ there is about a 1 in 20 chance that their results are acceptable result from the extreme of the distribution and if participants z-scores lie outside $|z| > 3$ the chance that their results are actually acceptable is only about 1 in 300.

3.3. Test materials

Each test material was analysed in at least two different rounds of testing. All matrices appeared to be stable although there was greater variation in results received for urine test materials. Over the four rounds of testing, no significant changes were found in results received from individual participants for a given test material.

3.4. Infant formula

Over the four rounds of testing, Infant Formula Test Material 2 was issued in all four rounds and Infant Formula Test Material 3 was issued twice in Rounds 3 and 4. A comparison of the overall assigned value, calculated as the Robust Mean, for daidzein and genistein, in these two test materials established over the four rounds of testing with the homogeneity values, is given in Table 3. The assigned values are comparable with the homogeneity values although, for daidzein, the assigned value is lower than the homogeneity value for Test Material 3 (p-value = 0.036) and, for genistein, the assigned value was higher for Test Material 2 (p-value = 0.004). The methods of analysis used to obtain the concentrations of daidzein and genistein in these test materials are summarised in Table 1. Over the four rounds of testing, satisfactory laboratory performance ($|z| \leqslant 2$) ranged between 36% and 60% for daidzein and between 45% and 70% for genistein. The individual z-scores for daidzein and genistein and method of analysis used by each of the laboratories are given in Table 4.

Table 3
Comparison of assigned values with homogeneity values for Infant Formula Test Materials 1, 2 and 3

	Infant Formula Test Material 1	Infant Formula Test Material 2	Infant Formula Test Material 3
Daidzein			
Assigned value ± SD (μg/g)	ND (<20)	57.7 ± 13.8 (n = 42) p-value = 0.214(NSD)	83.8 ± 20.4 (n = 21) p-value = 0.036(MD)
Homogeneity value ± SD (μg/g)	<400 (n = 10)	62.2 ± 5.2 (n = 10)	92.9 ± 7.0 (n = 10)
Genistein			
Assigned value ± SD (μg/g)	ND (<20)	117.8 ± 25.8 (n = 42) p-value = 0.004(SD)	152.2 ± 41.9 (n = 21) p-value = 0.735(NSD)
Homogeneity value ± SD (μg/g)	<400 (n = 10)	97.9 ± 6.0 (n = 10)	150.1 ± 11.5 (n = 10)

Probability (p-value) calculated using Wilcoxon rank sum test with continuity correction.
SD, significant difference; NSD, no significant difference; MD, moderate difference (not quite significant).

Table 4
z-Scores for daidzein and genistein in infant formula test materials

No.	Method		Analyte	z-Scores[a]						Satisfactory	
	Extraction[b]	Detection		R1	R2	R3 1	R3 2	R4 1	R4 2	Scores	%
1	SE	HPLC-UV	Daidzein	0.5	0.7	0.7	0.5	0.7	0.5	12/12	100
			Genistein	0.6	0.1	0.1	−0.2	0.2	−0.2		
2	SE	HPLC-MS	Daidzein		−0.8					2/2	100
			Genistein		0.9						
3	SE	HPLC-UV	Daidzein	−0.1	0.5	−0.3	0.3	0.5	3.8	11/12	92
			Genistein	0.7	−0.4	−0.4	−0.5	−0.5	−1.2		
4	AH-SE	HPLC-UV	Daidzein	−1.3	−0.5	−2.1	−0.8	−2.1	−1.1	10/12	83
			Genistein	−0.7	−0.5	−1.3	−0.4	−0.8	0.7		
5	AH-SE	HPLC-UV	Daidzein	1.1		1.0	0.1	−0.8	−1.2	8/10	80
			Genistein	−1.6		−1.5	−1.8	5.2	5.8		
6	SE	HPLC-UV	Daidzein	−1.5	−4.7	−2.1	0.2	−0.5	−2.5	8/12	67
			Genistein	−1.9	0.1	−4.2	0.0	−1.9	−1.9		
7	AH-SE	HPLC-MS	Daidzein	3.1						1/2	50
			Genistein	−1.1							
8	SE	HPLC-UV	Daidzein	−0.1	0.8	2.2	−2.6	1.0	2.8	5/12	42
			Genistein	−2.2	1.6	0.4	−4.5	3.1	3.8		
9	SE	HPLC-UV	Daidzein	2.9	−0.5	−2.8	−2.8	−3.5	−2.5	4/12	33
			Genistein	1.7	−5.5	−1.6	−1.5	−4.0	−3.2		
10	AH-SE	HPLC-UV	Daidzein	2.1	−3.5	−1.1	−1.1	0.1	0.3	4/12	33
			Genistein	4.4	2.1	3.1	3.7	3.1	4.3		
11	SE	HPLC-MS	Daidzein		2.8	6.5	5.0			2/6	33
			Genistein		1.1	2.4	0.7				
12	EH-SE	GC-MS	Daidzein	−2.7				−2.7	−2.6	2/6	33
			Genistein	−4.6				−0.8	−0.7		
13	SE	HPLC-UV	Daidzein	−9.5				−10.7	−12.1	0/6	0
			Genistein	−12.2				−11.9	−13.0		
14	SE	GC-MS	Daidzein	7.0						0/2	0
			Genistein	2.3							

[a] R1, Round 1 Infant Formula Test Material 1; R2, Round 2 Infant Formula Test Material 2; R3 1, Round 3 Infant Formula Test Material 1; R3 2, Round 3 Infant Formula Test Material 2; R4 1, Round 4 Infant Formula Test Material 1; R4 2, Round 4 Infant Formula Test Material 2.
[b] SE, solvent extraction; AH, acid hydrolysis; EH, enzyme hydrolysis.

Of the participants who analysed infant formula test materials, between 36% and 70% of laboratories performed satisfactorily. Three laboratories consistently performed satisfactorily and all of these laboratories used an HPLC method with C_{18} column and UV detection. The experimental details for the methods of analysis are as described by the participant.

Laboratory 1 consistently performed satisfactorily with 12 out of 12 satisfactory *z*-scores. The method of analysis used by this laboratory was based on that described by Murphy et al. (1999) and involved extraction of the dry sample into 80% acetonitrile with shaking at room temperature for 2 h, followed by separation of isoflavones on a YMCPak C_{18} column with water/acetonitrile gradient elution and detection with a UV diode array detector.

Laboratory 2 performed well with 11 out of 12 satisfactory *z*-scores. The method of analysis used by this laboratory was based on that described by Franke, Custer, Cerna, and Narala (1995) and involved the hydrolysis of samples in acidic ethanol for 2 h at 125 °C, cooling and centrifuging. An aliquot of clear supernatant was filtered through a 0.45 μm filter into an HPLC vial. Samples were analysed by HPLC-PDA using a Waters Nova-Pak C_{18} column (3.9 × 150 mm, 5 μm particle size) with gradient elution of 4% acetic acid and methanol from 60:40 to a 40:60 mix and back to 60:40 over a 30-min run time at constant flow rate of 1.0 ml/min. The analytes were detected by diode array detector set at 258 nm, scanning 200–300 nm.

Laboratory 3 performed well with 10 out of 12 satisfactory *z*-scores. The method of analysis used by this laboratory was an HPLC-UV method and samples (5 g) were extracted in 80% methanol at 65 °C using a platform orbital shaker, followed by saponification at room temperature with dilute sodium hydroxide, filtration through filter paper and centrifugation at 10,000 rpm for 10 min. An aliquot was analysed by HPLC on C_{18} column with methanol/water/2% acetic acid mobile phase and UV detection at 260 nm.

3.5. Plasma

Over the four rounds of testing, Plasma Test Material 2 was issued in all four rounds and Plasma Test Material 3 was issued twice, in Rounds 2 and 3. A comparison of the overall assigned value, calculated as the median, for daidzein and genistein in these two test materials, established over the four rounds of testing with the homogeneity values, is given in Table 5. The assigned values for both daidzein and genistein were generally comparable with the fortification levels for the plasma test materials.

The methods of analysis used to obtain the concentrations of daidzein and genistein in these test materials are summarised in Table 1. Over the four rounds of testing, satisfactory laboratory performance ($|z| \leqslant 2$) ranged between 75% and 100% for daidzein and between 86% and 100% for genistein. Individual *z*-scores for daidzein and genistein and methods of analysis used by each of the laboratories to analyse plasma test materials are given in Table 6. Between 75% and 100% of participants analysing plasma test materials performed satisfactorily and five laboratories (2, 16, 17, 18 and 3) consistently performed satisfactorily. The experimental details for the methods of analysis are as described by the participant.

Laboratory 2 consistently performed satisfactorily with 12 out of 12 satisfactory *z*-scores. The method of analysis used by this laboratory was an HPLC method. Plasma was hydrolysed with β-glucuronidase and aryl sulfatase and extracted with chloroform/propanol (1:1, w/w) and analysed by reverse phase HPLC-MS.

Laboratory 16 consistently performed satisfactorily with 12 out of 12 satisfactory *z*-scores. The method of analysis used by this laboratory was an HPLC-MS method. Plasma (10 μl) was hydrolysed with helix pomatia glucuronidase/sulfatase and extracted by SPE. An aliquot was analysed by HPLC on an Ultracarb ODS (2 × 30 mm) with an isocratic mobile phase of 60% of 0.1% formic acid/40% acetonitrile with electrospray MS positive ion detection.

Table 5
Comparison of assigned values with homogeneity values for Plasma Test Materials 1, 2 and 3

	Plasma Test Material 1	Plasma Test Material 2	Plasma Test Material 3
Daidzein			
Assigned value ± MAD (ng/ml)		58.8 ± 10.7 (n = 31) p-value = 0.015(SD)	179 ± 27.1 (n = 16) p-value = 0.009(SD)
Homogeneity value ± SD (ng/ml)	<20 (n = 10)	53.0 ± 2.8 (n = 10)	217 ± 5.8 (n = 10)
Fortified value (ng/ml)		55.0	110.0
Genistein			
Assigned value ± MAD (ng/ml)		139 ± 39.0 (n = 27) p-value=0.412(NSD)	251 ± 50.0 (n = 14) p-value=0.122(NSD)
Homogeneity value ± SD (ng/ml)	<20 (n = 10)	154 ± 6.1 (n = 10)	318 ± 10.7 (n = 10)
Fortified value (ng/ml)		138	276

Probability (p-value) calculated using Wilcoxon rank sum test with continuity correction.
SD, significant difference; NSD, no significant difference; MAD, median absolute deviation.

Table 6
z-Scores for daidzein and genistein in plasma test materials

No.	Method		Analyte	*z*-Scores[a]						Satisfactory	
	Extraction[b]	Detection		R1	R2 1	R2 2	R3 1	R3 2	R4 2	Scores	%
2	EH-SE	HPLC-MS	Daidzein	1.4	−0.1	−1.0	−0.5	−0.3	0.2	12/12	100
			Genistein	0.4	1.1	1.6	1.8	1.8	1.5		
16	EH-SPE	LC-ES/MS/MS	Daidzein	0.3	0.5	0.3	0.3	0.6	0.8	12/12	100
			Genistein	1.1	1.2	1.3	−0.3	−0.1	0.0		
17	EH-SPE	GC-MS	Daidzein	−0.1	0.6	0.7	−0.5	−0.5	−1.0	12/12	100
			Genistein	−0.4	−1.0	−0.9	−0.3	−0.3	−0.9		
18	EH-SE	HPLC-UV	Daidzein		−0.5	−0.2	−0.2	−0.5	0.0	10/10	100
			Genistein		−0.3	0.0	−0.2	0.0	0.6		
3	EH-SE	HPLC-ECD	Daidzein	−0.1	0.0	0.2	0.0	−0.1		10/10	100
			Genistein	−0.6	−0.4	−0.1	−0.8	−0.6			
12	EH-SE	TR-FIA	Daidzein	0.7					1.6	4/4	100
			Genistein	−0.1					1.1		
14			Daidzein						−0.4	2/2	100
			Genistein						0.3		
19	EH-SE	HPLC-ECD	Daidzein	−1.0						2/2	100
			Genistein	−1.3							
20	EH-SE	ELISA	Daidzein	1.7	−1.0	−2.1	−0.5	−0.4	−0.2	5/6	83
			Genistein								
11	EH-SE	HPLC-MS	Daidzein		−0.3	0.1	3.1	3.3		6/8	75
			Genistein		−1.5	−0.5	1.0	0.8			

[a] R1, Round 1 Plasma Test Material 2; R2 1, Round 2 Plasma Test Material 2; R2 2, Round 2 Plasma Test Material 3; R3 1, Round 3 Plasma Test Material 1; R3 2, Round 3 Plasma Test Material 2; R4, Round 4 Plasma Test Material 2.
[b] EH, enzyme hydrolysis; ES, electrospray; SPE, solid phase extraction; ECD, electrochemical detection; SE, solvent extraction; TR-FIA, time resolved fluoroimmunoassay.

Laboratory 17 consistently performed satisfactorily with 12 out of 12 satisfactory *z*-scores. The method of analysis used by this laboratory was a GC-MS method. Plasma (0.5 ml) was hydrolysed with helix pomatia and derivatised with tertiary butyl dimethyl silyl ether and separated by solid phase using GC-MS with detection at *m*/*z* 425, 470 and 555.

Laboratory 18 consistently performed satisfactorily with 10 out of 10 satisfactory *z*-scores. The method of analysis used by this laboratory was an HPLC method. Plasma (1 ml) was hydrolysed with β-glucuronidase/sulfatase and extracted with ethyl acetate. An aliquot was analysed by HPLC with UV detection at 270 nm.

Laboratory 3 consistently performed satisfactorily with 10 out of 10 satisfactory *z*-scores. The method of analysis used by this laboratory was an HPLC method. Plasma (0.5 ml) was thawed, hydrolysed with β-glucuronidase and extracted with buffered water, then methyl t-butyl ether, dried and reconstituted in aqueous methanol. An aliquot was analysed by HPLC on a C_{18} Hypersil column with gradient elution and detected by an electrochemical detection array system at 390–720 mV.

3.6. Urine

Over the four rounds of testing, Urine Test Materials 1 and 2 were issued in three rounds, namely Rounds 1, 2 and 3 and Rounds 1, 3 and 4, respectively, and Urine Test Material 3 was issued twice in Rounds 2 and 4. A comparison of the overall assigned values, calculated as the median, for daidzein and genistein, in these three test materials established over the four rounds of testing with the homogeneity values, is given in Table 7. The assigned values in Urine Test Materials 1 and 2 are comparable to the homogeneity values for daidzein (p-value = 0.454) and in Urine Test Material 1 for genistein (p-value = 0.332), but considerably lower for genistein in Urine Test Materials 2 and 3 (p-values, 0.056 and 0.026, respectively).

The methods used for the analysis of daidzein and genistein in these test materials are summarised in Table 1. Over the four rounds of testing, satisfactory laboratory performance ($|z| \leqslant 2$) ranged from 40% to 80% for daidzein and from 20% to 80% for genistein. The individual *z*-scores for daidzein and genistein and method

Table 7
Comparison of assigned values with homogeneity values in Urine Test Materials 1, 2 and 3

	Urine Test Material 1	Urine Test Material 2	Urine Test Material 3
Daidzein			
Assigned value ± MAD (ng/ml)	90.0 ± 43.0 (n = 15)	110 ± 62.3 (n = 15)	79.9 ± 40.2 (n = 10)
	p-value=0.454(NSD)	p-value=0.454(NSD)	p-value=0.143(NSD)
Homogeneity value ± SD (ng/ml)	75.6 ± 4.9 (n = 10)	88.3 ± 2.9 (n = 10)	117 ± 4.3 (n = 10)
Fortified value (ng/ml)		50.0	110.0
Genistein			
Assigned value ± MAD (ng/ml)	64.0 ± 29.7 (n = 15)	157 ± 47.4 (n = 15)	224 ± 132.3 (n = 10)
	p-value=0.332(NSD)	p-value=0.056(WD)	p-value=0.026(SD)
Homogeneity value ± SD (ng/ml)	54.3 ± 6.7 (n = 10)	217 ± 8.4 (n = 10)	366 ± 9.9 (n = 10)
Fortified value (ng/ml)		156	273

Probability (p-value) calculated using Wilcoxon rank sum test with continuity correction.
SD, significant difference; NSD, no significant difference; WD, week difference (not quite significant); MAD, median absolute deviation.

of analysis used by each of the laboratories to analyse urine test materials are given in Figs. 1–6.

Of the participants who analysed urine test materials, 20–80% performed satisfactorily. Five laboratories (7, 21, 2, 14 and 11) consistently performed satisfactorily. The experimental details for the methods of analysis are as described by the participant.

Laboratory 7 performed satisfactorily with 4 out of 4 satisfactory z-scores. The method of analysis used by this laboratory was an HPLC-MS method. Urine (1.0 ml) was hydrolysed with helix pomatia, glucuronidase and sulfatase and separated by HPLC on a YMC ODS AM column with aqueous acetonitrile mobile phase and MS–MS detection at MRM 256-226, 272-135, 253-223 and 269-133.

Laboratory 21 consistently performed satisfactorily with 14 out of 16 satisfactory z-scores. The method of analysis used by this laboratory was a GC-MS method modified, as described by Setchell, Zimmer-Nechemias, Cai, and Heubi (1997). Urine (0.5 ml) was hydrolysed with helix pomatia extracted with ether and derivatised with MTBSTFA and analysed using GC-MS on a ZB1 column (15 m × 0.25 mm, 0.1 μm) with He at 25 kPa, source temperature 250 °C, EI +70 eV.

Laboratory 14 performed satisfactorily with 12 out of 16 satisfactory z-scores. The method of analysis used by this laboratory was a GC-MS method. Urine (200 μl) was hydrolysed with helix pomatia pH 5, 37 °C for 16 h prior to SPE and derivatisation with TMS, analysed using GC-MS on a GC-1 column programmed from

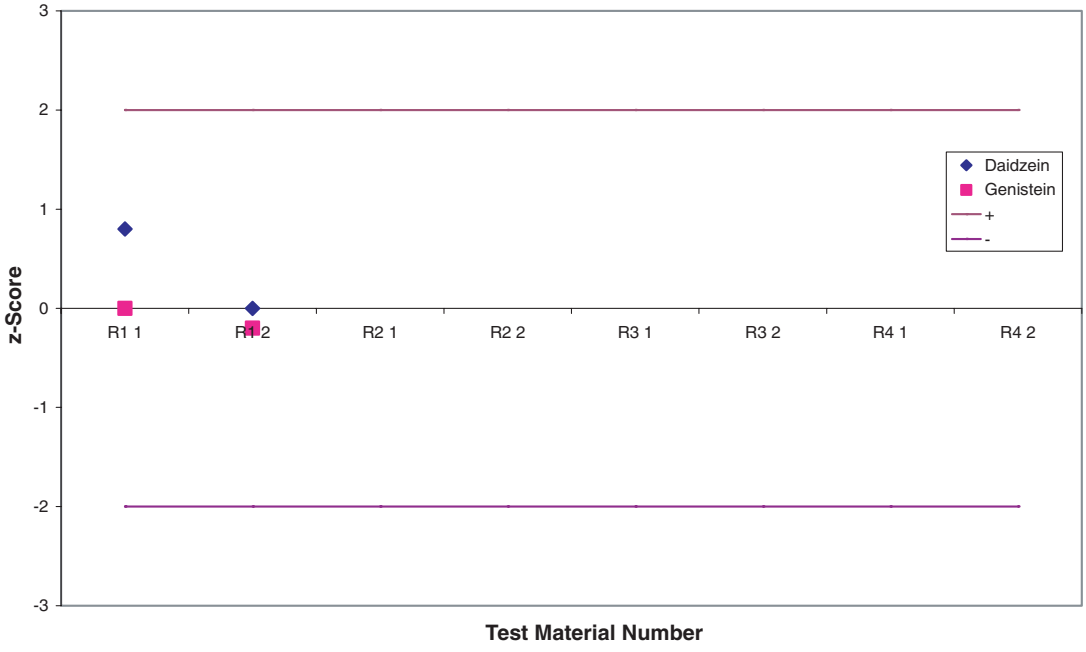

Fig. 1. Laboratory 7: z-scores (4/4, 100% satisfactory) for daidzein and genistein in urine test materials using enzyme hydrolysis and solid phase extraction with HPLC-MS method.

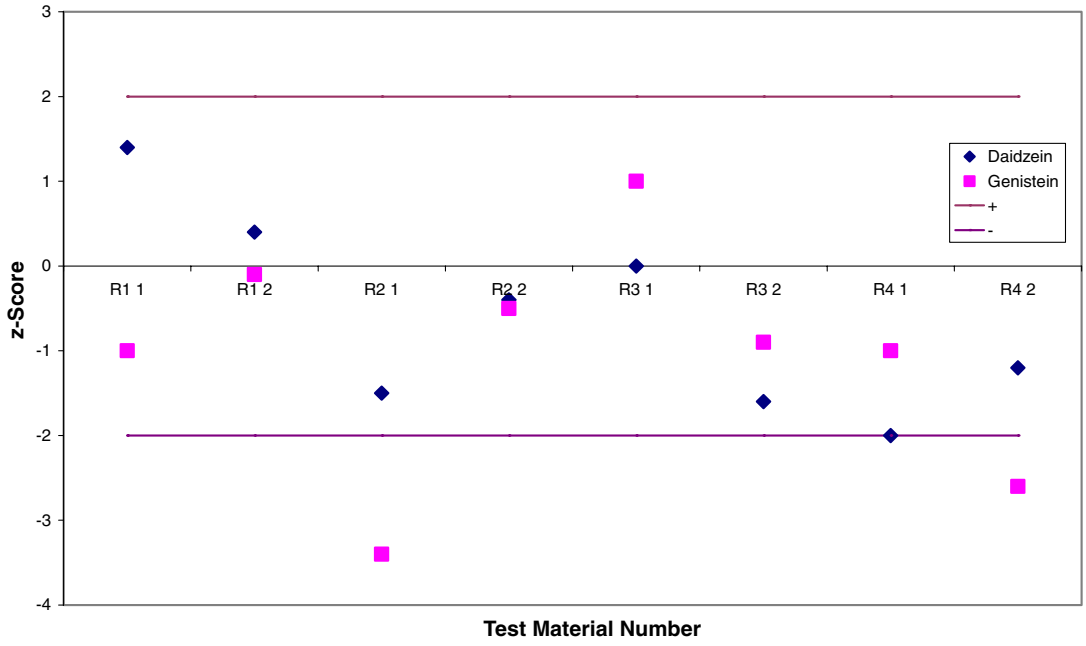

Fig. 2. Laboratory 21: *z*-scores (14/16, 88% satisfactory) for daidzein and genistein in urine test materials using enzyme hydrolysis and solvent extraction with GC-MS method.

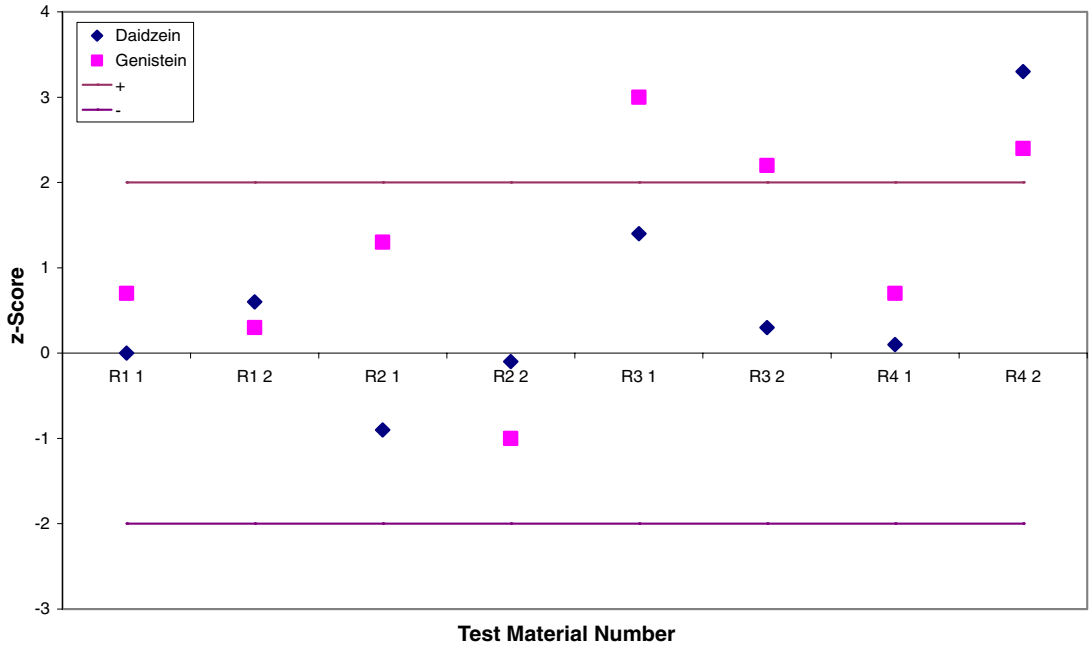

Fig. 3. Laboratory 2: *z*-scores (12/16, 75% satisfactory) for daidzein and genistein in urine test materials using enzyme hydrolysis and solvent extraction with HPLC-MS method.

160 to 300 °C and detected by MS using single ion monitoring at *m/z* 838, 398, 399, 401, 402, 386, 471, 474.

Laboratory 2 performed satisfactorily with 12 out of 16 satisfactory *z*-scores. The method of analysis used by this laboratory was an HPLC-MS method. Urine was hydrolysed with β-glucuronidase and arylsulfatase and extracted with chloroform/propanol (1:1. w/w) and analysed by reverse phase HPLC-MS.

Laboratory 11 performed satisfactorily with 6 out of 8 satisfactory *z*-scores. The method of analysis used by

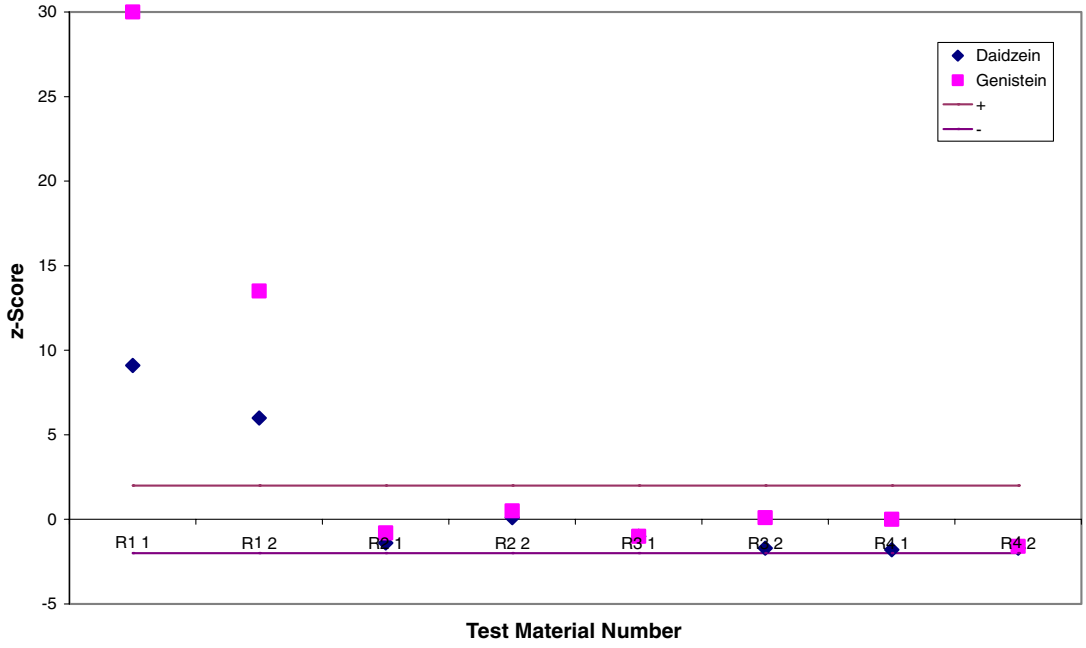

Fig. 4. Laboratory 14: *z*-Scores (12/16, 75% satisfactory) for daidzein and genistein in urine test materials using enzyme hydrolysis and solid phase extraction with GC-MS method.

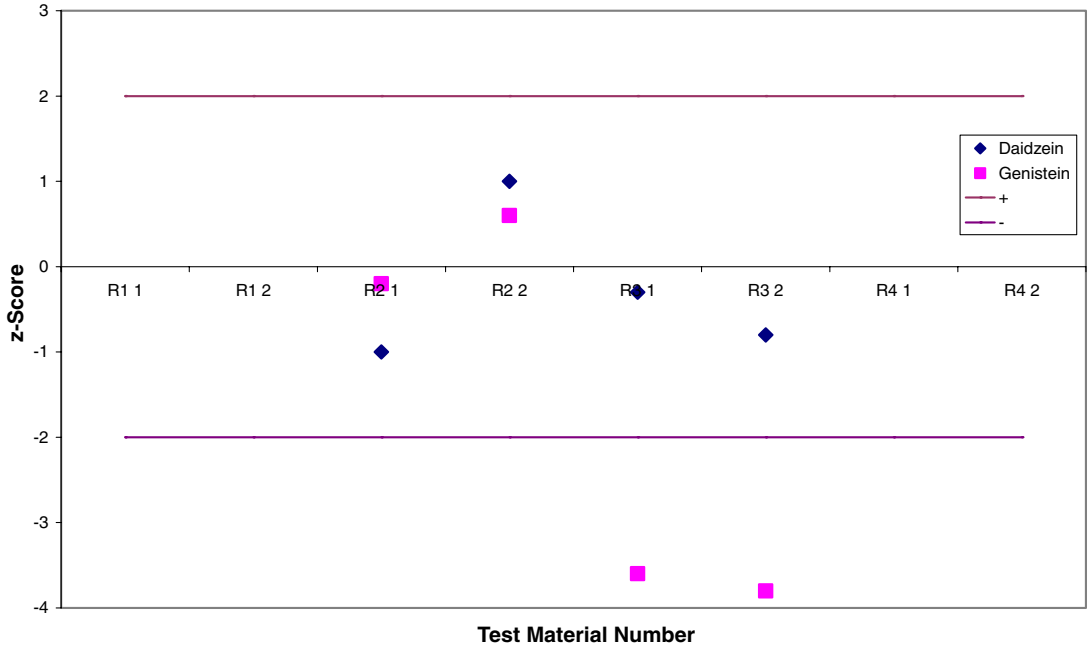

Fig. 5. Laboratory 11: *z*-scores (6/8, 75% satisfactory) for daidzein and genistein in urine test materials using enzyme hydrolysis and solvent extraction with HPLC-MS method.

this laboratory was an HPLC-MS method. Urine was hydrolysed with β-glucuronidase and arylsulfatase and extracted with diethyl ether, evaporated to dryness, redissolved by vortexing with methanol and diluted with 0.2 M acetate buffer (pH 5) and analysed by LC/PDA/ESI/MS using a HydroBond PS C_{18} column with methanol/acetonitrile/0.5% acetic acid as mobile phase with gradient elution and UV detection between 220 and 400 nm, followed by mass spectrometric measurements on line in the negative ion mode after electrospray ionisation with mass screening, covering the range 180–350 amu.

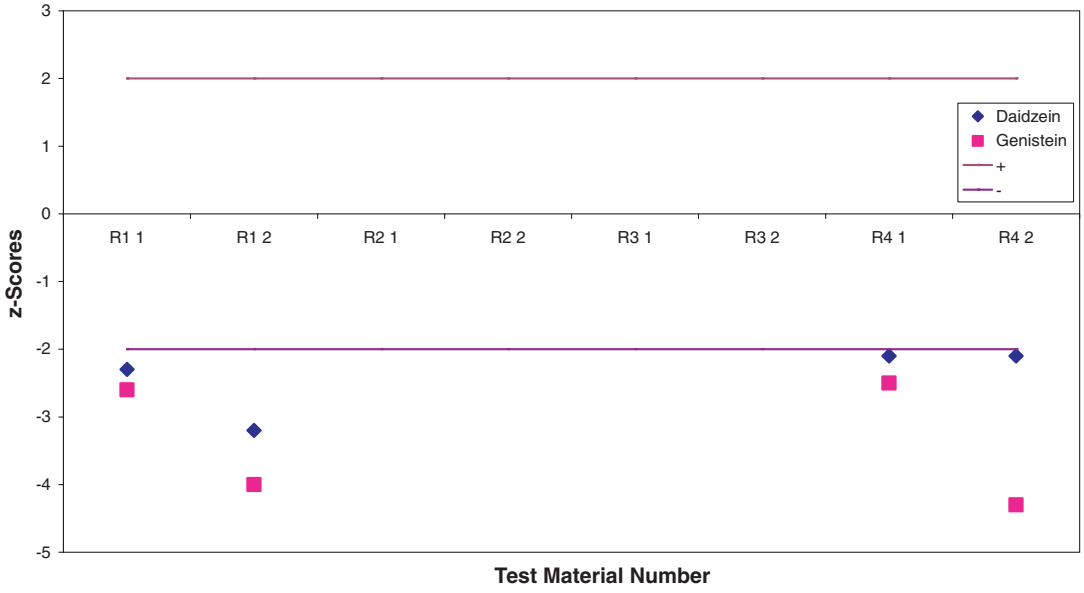

Fig. 6. Laboratory 12: *z*-scores (0/8, 0% satisfactory) for daidzein and genistein in urine test materials using enzyme hydrolysis and solid phase extraction with GC-MS method.

4. Discussion

The infant formula test materials contained naturally occurring daidzein and genistein in the product whereas the biological test materials were fortified to obtain realistic concentrations of these analytes at levels usually found in human plasma and urine. Both urine and plasma test materials were fortified at levels of 10–240 ng genistein/ml and 8–100 ng daidzein/ml. These concentrations are comparable with those found in a Japanese study by Yamamoto et al. (2001), where mean levels of 120 nmol/l daidzien and 475 nmol/l genistein were found in human plasma. The levels of both free genistein and free daidzein found in human plasma are generally a factor of 10 times lower than their respective conjugates.

The homogeneity values and the assigned values for the majority of the test materials analysed in this scheme were comparable, with no significant differences between the values. There was a significant difference in the values for daidzein in Plasma Test Materials 2 and 3 (p-value 0.015 and 0.009, respectively) with the assigned value being higher than the homogeneity value in Plasma Test Material 2 and lower for Plasma Test Material 3, as shown in Table 5. There was a significant difference in the values for genistein in Infant Formula Test Material 2 and Urine Test Material 3, (p-values 0.004 and 0.026, respectively) with the assigned value being higher than the homogeneity value for Infant Formula Test Material 2, as shown in Table 3 and lower in Urine Test Material 3, as shown in Table 7.

The results obtained from participants analysing infant formula materials for daidzein and genistein in this quality assurance scheme can be compared to results for a soya flour test material in an earlier quality assurance scheme carried out for FSA by Central Science Laboratory, as described by Clarke (2001). The soya flour test material had daidzein and genistein values 10 times greater than the concentrations of these analytes in the two infant formula test materials used in this scheme, as shown in Table 8. The coefficient of variation for

Table 8
Comparison of assigned values for infant formula test materials with soya flour test materials

	Infant Formula Test Material 2	Infant Formula Test Material 3	Soya Flour Test Material[a]
Daidzein			
Assigned value (μg/g)	57.7	83.8	1094
SD	±13.8	±20.4	±107
CV	29.9%	24.3%	9.5%
Genistein			
Assigned value (μg/g)	117.8	152.2	1376
SD	±25.8	±41.9	±24.6
CV	21.9%	27.5%	17.6%

SD, standard deviation; CV, coefficient of variation.
[a] Clarke (2001).

the infant formula test materials ranged from 22% to 30%, which was higher than those for the soya flour which ranged from 9.5% to 18% The higher coefficient of variation was probably due to the lower concentration of isoflavones in the infant formula test materials, as would be expected.

5. Conclusions

There is a wide variation in analytical performance between laboratories and, for some laboratories, between rounds of testing. Some laboratories performed poorly and some performed well. Performance differed between matrices and analytes. The scheme allowed laboratories to measure their performance and make improvements. The scheme allowed identification of laboratories with satisfactory and poor performance and laboratories improving or maintaining performance. The data highlighted the need for a quality assurance scheme and highlighted some successful methods. Suitable methods that perform well for the analysis of daidzein and genistein in infant formula test materials involve a solvent extraction step, followed by reverse phase HPLC with UV detection, as previously described by Murphy et al. (1999) and Franke et al. (1995). For biological materials, namely plasma and urine, methods that give good performance for the analysis of daidzein and genistein require an enzyme hydrolysis step before either solid phase extraction or solvent extraction and separation by either GC or HPLC and detection by MS.

Acknowledgements

Participation of laboratories in this quality assurance scheme is gratefully acknowledged. This work was funded by the Food Standards Agency within the Phytoestrogen Research Programme. The authors thank Tom Barlow (FSA) for helpful comments in the preparation of this manuscript.

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