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Analytical Methods

An international quality assurance (proficiency testing) scheme for the quantitated determination of daidzein, genistein and glycitein in infant formula

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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 in FSA funded research projects and 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 were prepared and tested by HPLC with UV detection for homogeneity to establish the concentration of three phytoestrogens, daidzein, genistein, glycitein and their glycoside, acetyl glycoside and malonyl glycoside conjugates in these test materials. Fourteen laboratories participated in the scheme. Test materials for analysis of daidzein, genistein and glycitein were issued to participants in October 2003 and January 2004. The results and methods of analysis were correlated and a report for each round of testing was produced to assess the competency of laboratories. These two rounds of testing were compared with the previous four rounds of testing which had been organised and 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 daidzein, genistein and glycitein in infant formula 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.

Keywords: Quality assurance scheme; Quantification; Daidzein; Genistein; Glycitein, infant formula

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 to human health has recently been comprehensively reviewed by the Committee on Toxicity, an expert advisory committee to the UK Government (COT, 2003).

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

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area (Delmonte & Rader, 2006). Therefore, a quality assurance (proficiency testing) scheme was set up to provide an assessment of the state-of-the-art. The first four rounds of testing were described by Key et al. (2006) and 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 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. This paper reports two further rounds of testing for the analysis of total daidzein, total genistein, total glycitein and their individual conjugates in infant formula matrices. Stable $^{13}C_3$-labelled isoflavone standards of daidzein and genistein were provided to participants to use as internal standards, if they so required. Participants analysed the samples for total daidzein, total genistein and total glycitein and their conjugates using their preferred method of analysis. Samples were issued in October 2003 for Round 5 and January 2004 for Round 6, 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.

The chemical backbone of isoflavones is the 3-phenylchromen-4-one conjugated system where single isoflavones are characterised by the presence of constituents such as methoxy, hydroxyl, and glycoside functional groups on the primary ring. Isoflavones are often present in foods as glycoside conjugates, the 7-O-β-D-glycosides being the most common. Within plants the 6″-O-malonyl-7-O-β-D-glycoside conjugates are the most common form of isoflavone present. These conjugates are chemically unstable and during sample extraction or food processing may undergo decarboxylation to 6″-O-acetyl-7-O-β-D glycosides or are hydrolysed to their 7-O-β-D-glycoside and free isoflavone forms. In part, because of their instability, the 6″-O-malonyl-7-O-β-D-glycoside and 6″-O-acetyl-7-O-β-D-glycoside conjugates have limited availability as reference materials (Delmonte & Rader, 2006).

Soybeans and soy foods contain primarily daidzein, glycitein and genistein. Isoflavones are present as aglycone, glycosides (e.g. daidzin, genistin and glycitin), malonyl-7-O-β-D-glycosides (e.g. malonyl daidzin, etc.) and acetyl-7-O-β-D-glycosides (e.g. acetyl daidzin, etc.) (Delmonte & Rader, 2006). de Pascual-Teresa et al. (2006) and Ren, Kuhn, Wegner, and Chen (2001) all reported that glycosides are hydrolysed by intestinal glucosidases to the aglycones following ingestion. The isoflavones can then be absorbed or metabolised to other biologically active metabolites including, equol, O-demethylangolensin (ODMA) and 6-hydroxy-ODMA. The aglycones and their metabolites are also transported to the liver, where they undergo conjugation via hepatic enzymes to give O-glucuronides and, to a lesser extent, O-sulfates (King & Bursill, 1998 and Setchell et al. 2003). Al-Maharik and Botting (2006) published an efficient method is presented for the synthesis of isoflavone 7-glucuronides of daidzein, genistein and glycitein using a *N*-(4-methoxyphenyl)-trifluoroacetimidate glucuronsyl donor.

Analytical strategies for the analysis of phytoestrogens in foods and dietary supplements have been extensively reviewed by Delmonte and Rader (2006). Wang, Prasain, and Barnes (2002) also reviewed analytical strategies for the determination of phytoestrogens in biological fluids. However, it is often usual practice to simply consider total isoflavone concentrations within a sample which therefore requires a hydrolysis (usually either acid or enzymatic) procedure to convert the glycoside conjugates to the aglycone (i.e. daidzein, genistein and glycitein). To date and to our knowledge, the only method for the quantitation of isoflavones that has been fully validated by a collaborative trial is AOAC Official Method 2001.10.

Daidzein, genistein and glycitein were chosen for measurement to encourage the maximum participation. For the purpose of the scheme, results were returned for total daidzein, total genistein, total glycitein and their individual conjugates (if quantified). The status of the quality assurance scheme after six rounds of testing is described with particular emphasis on the performance of laboratories and the methods of analysis they used.

2. Materials and methods

2.1. General

Stable $^{13}C_3$-labelled isoflavone standards of genistein and daidzein were previously distributed to participants when they first registered to participate in the scheme to use as internal standards and were synthesised as previously described by Key et al. (2006). Analysis of these standards 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. Laboratories were able to use these standards if they so wished but were not required to report whether they had been used to the scheme co-ordinators.

2.2. Participation

Participation in quality assurance (proficiency testing) schemes provides laboratories with an objective means of assessing and documenting the reliability of the data they are producing. This Quality Assurance Scheme was open to international laboratories. Fifteen laboratories participated at various times in the scheme; they were from the following countries: Australia (2), Brazil (1), Canada (1), Finland (1), France (1), Israel (1), Netherlands (1), UK

(3) and USA. (4). None of the UK participants took part in rounds 5 and 6.

2.3. Test materials

All test materials to be used in these 2 rounds of testing were prepared and tested for homogeneity prior to issuing the materials.

2.3.1. Preparation

For each matrix three different test materials were prepared.

Sufficient amount of commercially available infant formulae (5 kg of each test material) 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 until distribution.

2.3.2. Homogeneity testing

The test materials were assessed for homogeneity using procedures described by Thompson and Wood, 1993. To check homogeneity a random selection of ten units of each of the three test materials was analysed, in duplicate for daidzein, genistein, glycitein and their conjugates. Verification of homogeneity was established using two statistical tests as described by Thompson and Wood, 1993. 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 with UV diode array detection as described by Murphy et al. (1999).

2.3.3. Test materials used in rounds 5–6

For each of these rounds three infant formula test materials were prepared. Some of these test materials had been issued in previous four rounds of testing. A summary of the infant formula test materials issued for each of the six rounds of testing are given in Table 1.

Table 1
Test materials used in rounds 1–6

Round	Matrix Infant milk formula
1	TM 2
2	TM 1 TM 2
3	TM 2 TM 3
4	TM 2 TM 3
5	TM 1 TM 2 TM 4
6	TM 1 TM 2 TM 3

TM1 = Infant milk powder blank (<10 μg/g).

2.4. Data analysis

2.4.1. General

For rounds 5 and 6 participants were issued with three infant formula test materials one blank and two soya. Participants analysed the test materials for daidzein, genistein, glycitein and their conjugates 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 (as received) for the infant formula test materials and the data were returned on a proforma with a brief description of the analytical method.

2.4.2. Statistical analysis and use of z-scores

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

Each laboratory was given *z*-scores calculated from

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

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

Table 2
Comparison of assigned values with homogeneity values (μg/g, as received) for infant formula test materials 2, 3 and 4[*]

	Infant formula test material 2	Infant formula test material 3	Infant formula test material 4
Total daidzein			
Assigned value ± SD	60.3 ± 16.0^{a} ($n = 53$)	87.0 ± 24.4^{b} ($n = 39$)	38.5 ± 9.6^{c} ($n = 7$)
Homogeneity value ± SD	56.5 ± 4.9^{a} ($n = 30$)	83.2 ± 6.8^{b} ($n = 30$)	39.8 ± 6.7^{c} ($n = 20$)
Total genistein			
Assigned value ± SD	$119.8 \pm 28.5^{d**}$ ($n = 53$)	156.0 ± 43.0^{f} ($n = 39$)	83.9 ± 13.5^{g} ($n = 7$)
Homogeneity value ± SD	$106.7 \pm 8.5^{e**}$ ($n = 30$)	143.6 ± 10.9^{f} ($n = 30$)	84.9 ± 7.0^{g} ($n = 20$)
Total glycitein			
Assigned Value ± SD	$13.2 \pm 1.9^{h***}$ ($n = 7$)	$20.0 \pm 8.1^{j**}$ ($n = 16$)	$17.9 \pm 9.4^{l**}$ ($n = 7$)
Homogeneity value ± SD	$18.1 \pm 1.9^{i***}$ ($n = 30$)	$27.3 \pm 2.7^{k**}$ ($n = 30$)	$29.5 \pm 2.8^{m**}$ ($n = 20$)

[*] Values with same letters in each column and for compound are not significantly different ($p < 0.05$).
[**] Significant difference ($p < 0.01$).
[***] Significant difference ($p < 0.005$).

Table 3
z-Scores for total daidzein, total genistein and total glycitein in infant formula test materials[a]

No.	Method[b]	Analyte	*z*-Scores										Satisfactory				
			R1	R2	R3 1	R3 2	R4 1	R4 2	R5 1	R5 2	R6 1	R6 2	Scores		TSS[c]	%	Rank
1	SE HPLC-UV	Daidzein	−0.1	0.1	0.1	0.0	0.1	0.1	0.6	−0.8	−0.4	−1.4	10/10	100%	21/24	88	1
		Genistein	0.3	–0.1	–0.1	–0.5	0.0	−0.5	0.0	−1.2	−0.1	−1.2	10/10	100%			
		Glycitein							4.9	6.6	2.7	0.0	1/4	25%			
4	AH-SE HPLC-UV	Daidzein	−1.8	–1.0	–2.6	–1.3	−2.6	−1.5	–2.0	−2.8	−0.5	0.9	7/10	70%	17/24	71	1
		Genistein	−0.9	–0.7	–1.5	–0.7	−1.0	0.3	–1.6	−2.2	1.2	0.8	9/10	90%			
		Glycitein							–4.1	−3.5	−2.3	−1.1	1/4	25%			
5	SE HPLC-UV	Daidzein	0.6		0.4	−0.3	−1.3	−1.6	0.5	−1.5	−0.8	−1.5	9/9	100%	14/22	64	2
		Genistein	−1.8		−1.7	−2.1	4.9	5.3	−1.5	−2.3	−11.4	−2.3	3/9	33%			
		Glycitein							−1.0	−0.8	63.0	−2.5	2/4	50%			
12	EH-SE HPLC-CA	Daidzein	−3.1				−3.1	−2.9	−0.6	−1.1	−1.8	−2.0	4/7	57%	11/18	61	2
		Genistein	−4.7				−1.0	−1.0	1.1	0.9	1.1	−0.1	6/7	86%			
		Glycitein							−3.4	−3.9	0.2	−3.4	1/4	25%			
3	SE HPLC-UV	Daidzein	−0.6	−0.1	−0.8	−0.1	0.1	3.2	8.9	15.0	10.2	8.3	5/10	50%	13/24	54	2
		Genistein	0.5	−0.6	−0.6	−0.9	−0.7	−1.5	6.4	5.5	8.7	6.2	6/10	60%			
		Glycitein							0.6	3.4	4.0	−0.5	2/4	50%			
8	SE HPLC-UV	Daidzein	−0.6	0.3	1.6	−3.0	0.5	2.2			−1.8	−5.1	5/8	63%	8/16	50	3
		Genistein	−2.4	1.4	0.2	−4.7	2.8	3.4			−1.2	−6.2	3/8	38%			
		Glycitein															
10	AH-SE HPLC-UV	Daidzein	1.5	−3.9	−1.6	−1.5	−0.4	−0.1			−0.1	−1.4	7/8	88%	9/18	50	3
		Genistein	4.1	1.8	2.8	3.2	2.8	3.8			1.7	2.8	2/8	25%			
		Glycitein									11.0	3.9	0/2	0%			
6	SE HPLC-UV	Daidzein	−2.0	−5.1	−2.5	−0.3	−1.0	−2.8			3.1	0.9	4/8	50%	8/18	44	3
		Genistein	−2.0	−0.1	−4.3	−5.2	−2.0	−2.1			−2.8	−3.5	3/8	38%			
		Glycitein									4.4	0.9	1/2	50%			
9	SE HPLC-UV	Daidzein	2.3	−1.0	−3.3	−3.1	−3.9	−2.9	−3.6	−3.1			1/8	13%	6/18	33	3
		Genistein	1.4	−5.6	−1.8	−1.8	−4.2	−3.5	−1.9	−0.9			5/8	63%			
		Glycitein							−4.1	−3.1			0/2	0/2%			
11	SE HPLC-MS	Daidzein		2.2	5.8	4.3					4.3	4.3	0/5	0%	4/12	33	3
		Genistein		0.8	2.2	−0.3					−1.5	−1.1	4/5	80%			
		Glycitein									−3.7	−4.6	0/2	0%			
13	AH-SE HPLC-UV	Daidzein	9.7				−10.9	−12.1	1.4	1.1	9.4	9.1	2/7	29%	4/18	22	3
		Genistein	−12.2				−11.9	−13.0	1.1	−0.2	8.7	11.3	2/7	29%			
		Glycitein							7.5	6.0	−5.4	3.6	0/4	0%			
15	ELISA	Daidzein		26.7	15.3	16.3	32.6	23.3			11.5	9.5	0/7	0%	0/14	0%	3
		Genistein		5.0	2.8	2.8	10.3	7.3			6.3	7.1	0/7	0%			
		Glycitein															

[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, R5 1 = round 5 infant formula test material 1, R5 2 = round 5 infant formula test material 2, R6 1 = round 6 infant formula test material 1, R6 2 = round 6 infant formula test material 2.

[b] SE = solvent extraction, AH = acid hydrolysis, EH = enzyme hydrolysis, CA = coulometric array.

[c] TSS = total satisfactory scores: Ranking 1 = high >70% satisfactory; 2 = medium 51–69% satisfactory; 3 = low <51%.

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

No.	Method[a]		Isomer	*z*-Scores				Satisfactory		
	Extraction[b]	Detection		R5 1	R5 2	R6 1	R6 2	Scores	%	Rank[c]
5	SE	HPLC-UV	Daidzin	1.8	0.5	0.8	0.8	43/60	72	1
			Malonyl daidzin	0.8	−0.7	−1.4	−2.2			
			Acetyl daidzin	−0.6	−0.3	−2.0	−0.8			
			Daidzein	1.2	0.3	3.7	2.8			
			Total daidzein	0.5	−2.6	−3.3	−2.6			
			Genistein	0.7	0.8	0.7	0.5			
			Malonyl genistin	−1.3	−1.4	−1.6	−1.5			
			Acetyl genistin	−2.0	−2.4	−2.8	−2.9			
			Genistein	1.1	8.4	2.9	1.9			
			Total genistein	−1.5	−2.5	−11.7	−2.6			
			Glycitin	−0.4	−0.4	−0.1	−1.9			
			Malonyl glycitin	1.1	3.6	0.1	−1.4			
			Acetyl glycitin	−2.2	−0.8	−1.1	5.1			
			Glycitein	0.8	4.9	1.6	4.1			
			Total glycitein	−1.0	−0.4	63.0	−2.2			
9	SE	HPLC-UV	Daidzin	−0.4	0.8			19/30	63	2
			Malonyl daidzin	−3.9	−2.0					
			Acetyl daidzin	−8.2	−8.2					
			Daidzein	−2.0	−1.8					
			Total daidzein	−3.6	−4.2					
			Genistin	−0.2	1.6					
			Malonyl genistin	0.1	1.9					
			Acetyl genistin	−2.1	−1.6					
			Genistein	−0.8	−0.6					
			Total genistein	−1.9	−1.2					
			Glycitin	−3.2	−2.2					
			Malonyl glycitin	−4.1	−0.2					
			Acetyl glycitin	1.5	3.0					
			Glycitein	−3.3	−1.0					
			Total glycitein	−4.1	−2.8					
3	SE	HPLC-UV	Daidzin	2.2	12.4	1.8	0.8	37/60	62	2
			Malonyl daidzin	1.6	2.0	4.5	2.7			
			Acetyl daidzin	0.2	−0.3	−0.8	−0.9			
			Daidzein	2.1	0.3	1.3	1.1			
			Total daidzein	8.9	12.5	5.5	6.3			
			Genistin	0.8	−0.1	1.6	0.4			
			Malonyl genistin	0.3	0.1	2.0	0.8			
			Acetyl genistin	−1.1	−2.2	−1.1	−2.2			
			Genistein	2.6	1.3	1.1	0.6			
			Total genistein	6.4	5.1	6.7	5.7			
			Glycitin	−0.6	0.4	2.6	−2.6			
			Malonyl glycitin	−3.2	1.5	2.7	−1.3			
			Acetyl glycitin	−6.9	−6.7	−7.8	−6.7			
			Glycitein	−1.0	4.9	−1.7	1.0			
			Total glycitein	0.6	3.9	4.0	−0.2			
4	AH-SE	HPLC-UV	Total daidzein	−2.0	−3.9	−3.1	−0.5	7/12	58	2
			Total genistein	−1.6	−2.5	−0.2	0.5			
			Total glycitein	−4.1	−3.7	−2.3	−0.8			
1	SE	HPLC-UV	Daidzin	−0.3	−0.7	−1.1	−1.2	33/60	55	2
			Malonyl daidzin	0.1	1.3	0.1	0.0			
			Acetyl daidzin	3.1	0.9	1.7	1.5			

Table 4 (*continued*)

No.	Method[a]		Isomer	*z*-Scores				Satisfactory		
	Extraction[b]	Detection		R5 1	R5 2	R6 1	R6 2	Scores	%	Rank[c]
			Daidzein	5.0	1.6	4.0	2.0			
			Total daidzein	0.6	−2.1	−3.0	−2.6			
			Genistin	0.3	0.3	0.5	−0.1			
			Malonyl genistin	0.5	0.5	0.4	0.4			
			Acetyl genistin	3.3	0.9	0.9	1.4			
			Genistein	3.7	2.0	6.5	2.7			
			Total genistein	0.0	−1.4	−1.4	−1.5			
			Glycitin	2.3	3.3	8.8	3.7			
			Malonyl glycitin	5.4	7.6	13.4	6.7			
			Acetyl glycitin	30.6	37.5	−7.8	−6.2			
			Glycitein	3.5	20.5	−7.2	−6.8			
			Total glycitein	4.9	7.2	2.7	0.4			
12	EH-SE	HPLC-CA	Daidzin	−0.5	−0.8	−2.0	−1.7	32/58	55	2
			Malonyl daidzin	0.5	1.9	1.4	0.8			
			Acetyl daidzin	3.2	3.1	0.7	1.9			
			Daidzein	−2.0	−2.7	−4.2	2.0			
			Total daidzein	−0.6	−2.3	−4.2	−3.2			
			Genistin	−0.4	−0.1	−0.4	−0.4			
			Malonyl genistin	2.7	4.6	2.5	3.3			
			Acetyl genistin	11.9	10.4	9.7	8.8			
			Genistein	−1.8	−2.5	−3.1	−3.4			
			Total genistein	1.1	0.5	−0.2	−0.4			
			Glycitin	2.9	−3.3	0.3	−3.0			
			Malonyl glycitin	1.8	1.9	4.6	1.3			
			Acetyl glycitin	−1.1	−0.3	−1.5	3.8			
			Glycitein			−1.6	−4.6			
			Total glycitein	−3.4	−3.3	0.2	−3.2			
6	SE	HPLC-UV	Daidzin			2.3	3.0	11/24	46	3
			Acetyl daidzin			−0.1	0.3			
			Daidzein			5.3	3.5			
			Total daidzein			−0.1	−0.4			
			Genistin			−0.4	0.1			
			Acetyl genistin			−8.0	−8.0			
			Genistein			0.9	2.7			
			Total genistein			−3.8	−3.7			
			Glycitin			1.6	−0.7			
			Malonyl glycitin			−5.3	−7.3			
			Acetyl glycitin			8.1	30.1			
			Total glycitein			4.4	1.3			
13	AH-SE	HPLC-UV	Daidzin	−3.9	−4.6	−1.9	−1.0	21/56	38	3
			Malonyl daidzin	−0.9	−1.1	−1.1	−0.9			
			Acetyl daidzin	−1.3	−1.9	17.4	20.1			
			Daidzein	−4.3	−5.1	−8.2	−2.1			
			Total daidzein	1.4	−0.2	4.9	7.1			
			Genistin	−2.2	−3.1	−0.4	−0.3			
			Malonyl genistin	−2.0	−3.3	0.3	−0.7			
			Acetyl genistin	−4.1	−5.4	7.6	24.0			
			Genistein	−5.3	−6.2	−8.2	−3.0			
			Total genistein	1.1	−0.5	6.7	10.7			
			Glycitin	8.6	8.1	−8.5	10.9			
			Malonyl glycitin	−1.1	−0.5	−8.0	−8.8			

(*continued on next page*)

Table 4 (*continued*)

No.	Method[a]		Isomer	*z*-Scores				Satisfactory		
	Extraction[b]	Detection		R5 1	R5 2	R6 1	R6 2	Scores	%	Rank[c]
			Acetyl glycitin			−7.8	−6.7			
			Glycitein			7.8	−6.8			
			Total glycitein	7.5	6.6	−5.4	4.0			
11	SE	HPLC-MS	Daidzin			0.1	0.0	11/30	37	3
			Malonyl daidzin			−1.8	−1.9			
			Acetyl daidzin			−2.7	−3.4			
			Daidzein			−2.4	2.0			
			Total daidzein			0.8	2.7			
			Genistin			−4.1	−3.8			
			Malonyl genistin			−5.3	−4.7			
			Acetyl genistin			−6.3	−5.7			
			Genistein			−1.5	−1.8			
			Total genistein			−2.6	−1.3			
			Glycitin			−4.5	−5.2			
			Malonyl glycitin			−3.9	−4.4			
			Acetyl glycitin			−5.5	−6.3			
			Glycitein			−5.9	−4.9			
			Total glycitein			−3.7	−4.4			
8	SE	HPLC-UV	Total daidzein			−4.1	−6.0	1/4	25	3
			Total genistein			−2.4	−6.4			
			Total glycitein							
15	EH	ELISA	Total daidzein			6.6	7.5	0/4	0	3
			Total genistein			4.6	6.6			
			Total glycitein							

[a] R5 1 = round 5 infant formula test material 1, R5 2 = round 5 infant formula test material 2, R6 1 = round 6 infant formula test material 1, R6 2 = round 6 infant formula test material 2.
[b] SE = solvent extraction, AH = acid hydrolysis, EH = enzyme hydrolysis.
[c] Ranking 1 = high >70% satisfactory; 2 = medium 51–69% satisfactory; 3 = low <51%.

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

The σ value was obtained by

$$\sigma = b\widehat{X} \quad \text{where } b = \%\mathrm{RSD_R}/100$$

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}.

If it is assumed that the parameters $\widehat{X}$ 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" i.e., 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 participant's z-score lies within this range. If a participant's z-score lies outside $|z| > 2$ there is about a 1 in 20 chance that their result is an acceptable result from the extreme of the distribution and if a participant's z-score lies outside $|z| > 3$ the chance that their result is actually acceptable is only about 1 in 300.

3. Results and discussion

During rounds 5 and 6 of testing, infant formula test material 3 and infant formula test material 1 was issued in both rounds and infant formula test material 4 was issued in round 5 and infant formula test material 2 in round 6. A comparison of the overall assigned value, calculated as the Robust Mean, for daidzein, genistein and glycitein in these three test materials established over the six

Table 5
Comparison of suitable methods for determination of diadzein, genistein and glycitein in infant formula with AOAC 2001.10

	Method			
	Method used by Lab 1	Method used by Lab 5	Method used by Lab 4	AOAC 2001.10
Standards				
Supplier of standards	In house (purified) and LC Laboratory	LC Laboratory	Indofine Chemical Co	Indofine Chemical Co
Nature of standards	Natural and synthetic	Natural	Synthetic	
Extraction				
Processing procedures	Freeze dried if not free flowing	None	Solid samples ground to pass through 0.5 mm mesh	
Quantity (g)	2	1–2	0.5–0.6	<5
Extraction solvents	53% acetonitrile	70% acetonitrile	95% ethanol containing internal isoflavone standard	80% methanol
Mechanical extraction	2 h shaker	Shaking	Refluxed in 125 °C sand bath	Shake for 2 h at 65 °C
Method for hydrolysis of conjugates	None	None	Concentrated hydrochloric acid	Saponified with NaOH solution. Acidified. filtered, diluted with water to 50% methanol
Separation				
Separation technique	HPLC	HPLC	HPLC gradient mobile phase and constant flow rate	HPLC gradient mobile phase and constant flow rate
HPLC conditions				
Column	YMC Pak C_{18}	250 mm × 3.0 mm YMC ODS Am	Waters Nova-Pak C18, 3.9 mm × 150 mm, 5 μm	C18 reverse phase
Mobile phase	Water–acetonitrile gradient with acetic acid acidification	10% acetonitrile to 30% acetonitrile at 0.65 mL/min	Mobile phase A = 4% acetic acid	Mobile phase A = Water–methanol–acetic acid (88 + 10 + 2)
			Mobile phase B = HPLC methanol	Mobile phase B = Methanol–acetic acid (98 + 2)
Detection				
Type of detector	Photo diode array	UV	UV/VIS absorbance detector Photo diode array	UV
Detector settings (nm)	200–350	260	Monitor at 258 Scanning between 200 and 300	260
Detection limit (μg/g IF)				
Total daidzein	0.925	0.01	8.0	20
Total genistein	0.500	0.01	8.0	20
Total glycitein	2.708	0.01	4.0	20

IF = Infant formula.

rounds of testing with the homogeneity values is given in Table 2. The assigned values are comparable with the homogeneity values although for glycitein the assigned value is lower than the homogeneity value for test materials 2, 3 and 4 (p-value = 0.012, 0.095, 0.038), respectively and for genistein the assigned value was higher for test material 2 (p-value = 0.006).

Over the six rounds of testing satisfactory laboratory performance ($|z| \leqslant 2$) ranged between 0–100% for daidzein, 0–100% for genistein and 0–50% for glycitein. The individual z-scores for total daidzein, total genistein and total glycitein with the method of analysis used by each of the laboratories are given in Table 3. The individual z-scores for the various conjugates are given in Table 4. The z-scores for glycitein conjugates were variable possibly due to low levels (<10–32 μg/g) of the conjugates in the test material.

One of the participants who analysed infant formula test materials, between 0% and 88% of laboratories performed satisfactorily. Three laboratories 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. Although the AOAC Official Method 2001.10 was available to participants, the laboratories who performed best in the scheme all used their own in-house validated methods. Details about the methods used are given in Table 5 as are the characteristics of the AOAC method.

Laboratory 1 performed satisfactorily with 21 out of 24 satisfactory z-scores. The method of analysis used by this laboratory was as described by Murphy et al. (1999) and involved extraction of the dry sample into 53% acetonitrile with shaking at room temperature for 2 h, followed by separation of isoflavones on YMCPak C_{18} column with water/acetonitrile gradient elution and detection on UV diode array detector.

Laboratory 4 performed well with 17 out of 24 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, cooled and centrifuged. An aliquot of clear supernatant was filtered through 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 5 performed well with 14 out of 22 satisfactory z-scores. The method of analysis used by this laboratory was an HPLC-UV method and samples (1–2 g) were extracted in 70% acetonitrile with shaking, followed by separation of isoflavones on YMCPak C_{18} column (250 mm × 3.0 mm) with water/acetonitrile gradient elution (10% acetonitrile to 30% acetonitrile at 0.65 ml/min) and UV detection at 260 nm.

The homogeneity values and the assigned values for the majority of the test materials analysed in this scheme were comparable. In general, no significant difference between the values was observed. However, there was a significant difference in the values for genistein in infant formula test material 2 (p-value 0.006) with the assigned value being higher than the homogeneity value for infant formula and for glycitein the assigned value was lower than the homogeneity value for Test Materials 2, 3 and 4 (p-value = 0.012, 0.095, 0.038) respectively.

Laboratories were able to choose the methods of analysis used in this exercise. For the laboratories 1, 4 and 5 the methods used had detection limits between 0.01–8 μg/g for total daidzein; 0.01–8 μg/g for total genistein; and 0.01–4 μg/g for total glycitein. Although variable they were significantly lower than the assigned values used in this proficiency testing scheme.

4. Conclusions

There is a wide variation in analytical performance between laboratories, and for some laboratories, between each round of testing. Some laboratories performed consistently poorly and some performed consistently well. Performance differed between analytes. The scheme allowed laboratories to measure their performance and to make improvements. The scheme allowed identification of laboratories with either satisfactory or poor performance and of 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).

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