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A Microbore High Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry Method for the Determination of the Phytoestrogens Genistein and Daidzein in Comminuted Baby Foods and Soya Flour

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A microbore high performance liquid chromatographic/electrospray/mass spectrometric (HPLC/ESI-MS) method has been developed for the determination of the phytoestrogens daidzein and genistein in soya flours and baby foods. The samples were hydrolysed and extracted with acetonitrile–water prior to analysis. LC was performed on a microbore Primesphere 5C8 column using a water/acetonitrile/acetic acid mobile phase at a flow rate of 60 μl/min. Atmospheric pressure ionization in the form of pneumatically assisted electrospray mass spectrometry (ESI-MS) was used as the method of detection. The limit of detection was 0.2 mg/kg for daidzein and 0.7 mg/kg for genistein in the flour and food samples. The method proved both robust and reliable when operated over a long time period (10 days, 463 injections) generating precision data with a coefficient of variation of 4–15%. © **Crown Copyright**

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Phytoestrogens are plant substances with oestrogenic activity (for example isoflavones) and are found in numerous types of plant material consumed in a typical human diet. Interest in isoflavones and their physiological effects has increased rapidly, particularly over the last fifteen years, with the discovery that these diphenols may be cancer-protective agents. Soybean and it's processed products have been consumed in the Orient for centuries but until relatively recently little was consumed in Western Society. Soybean is high in protein content, contains all essential amino acids, is rich in fatty acids and fat-soluble vitamins and the carbohydrate component is composed mainly of complex polysaccharides and indigestible fibre. As a result, with the increase in health-conscious eating, consumption of soybean products has risen considerably. Soya foods are known to be a rich source of dietary phytoestrogens and a number of papers have been published reflecting the increased interest in phytoestrogens and their presence in soya foods.[1–4]

Several analytical methods have been established for the determination of phytoestrogens. Gas chromatography with electron impact mass spectrometry (GC/MS) has been used to determine phytoestrogens in a number of matrices including plasma[5] and urine.[6] Isotope dilution GC/MS was used in a recent determination of unconjugated lignans and isoflavanoids (including daidzein and genistein) in human faeces.[7] Common dietary phytoestrogens contain at least one hydroxyl group in their structure making them difficult for direct GC/MS analysis. Typically the hydroxyl group is derivatized with N, O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) prior to GC/MS, making the analysis more time consuming. Separation by liquid chromatography obviates the need for derivatization and has been used with ultra-violet and/or fluorescence[8–10] detection. The disadvantage of these detection methods is their non-specificity leading to the possibility of matrix interferents. In the eighties HPLC was successfully coupled to MS detectors via the thermospray interface and an example of the successful application of this technique to the determination of phytoestrogens in soy protein preparations is given in the paper by R. Barbuch *et al.*[11] Thermospray as an HPLC/MS interface has recognized limitations in terms of robustness and the stability of the ion beam. This technique has now been largely superseded by atmospheric pressure ionization (API) as an LC/MS interface. The API techniques of atmospheric pressure chemical ionization (APcI) and electrospray ionization (ESI) are highly sensitive, show greater ionization stability and are more universally applicable than other HPLC/MS techniques. Both APcI and ESI involve the formation and ionization of an aerosol at atmospheric pressure. In APcI the spray is formed by pneumatic nebulization from a capillary in a heated probe and is ionized by a high voltage corona discharge. Coward *et al.* have recently demonstrated the suitability of APcI for the determination of isoflavones in plasma.[12] In pure ESI, which is limited to low solution flow rates the formation of both spray and ions is from a high electric field applied to the stainless steel capillary through which the HPLC column eluent enters the mass spectrometer. Pneumatic assistance can also be used to form a spray and this permits higher solution flow rates (up to 1 ml/min). Aramendia *et al.* have examined synthetic mixtures of isoflavones by capillary electrophoresis/ESI-MS using the negative ionization mode.[13]

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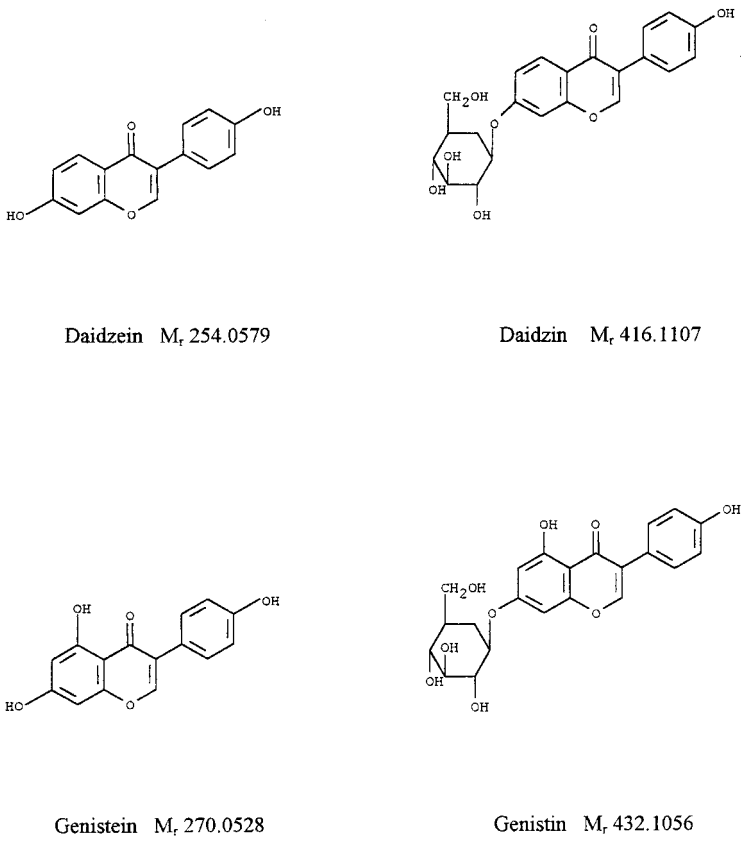

Figure 1. Structures of daidzin, daidzein, genistin and genistein.

Using pneumatically assisted ESI we have developed a microbore HPLC/ESI-MS positive ionization method for the determination of total daidzein and genistein in soya flour and baby food. Isoflavones can form a number of different conjugates (glucosides — daidzin and genistin, malonated glucosides and acetylated glucosides) so in order to determine total aglycone levels samples were hydrolysed prior to analyses. Spectra have been acquired for daidzin and genistin but these substances were not quantified in the extracts, however they were monitored as a check for incomplete hydrolysis. Structures of all 4 substances are given in Fig. 1.

EXPERIMENTAL

Materials

Distilled water was prepared in-house. Glacial acetic acid (AnalaR grade) and hydrochloric acid (5 M) were from BDH (Poole, Dorset, UK). Acetonitrile and methanol (both HPLC grade) were obtained from Rathburn Chemicals (Walkerburn, UK). *tert*-Butylhydroquinone (97%) was from Aldrich (Gillingham, UK). Daidzin, daidzein, genistin and genistein were from Apin Chemicals Ltd. (Abingdon, UK). Polyethylene glycols PEG 300, 600, 1000 and 1500 were obtained from Koch Light (Haverhill, UK) and BDH. Soya flour and puréed 'solid' baby food products were purchased from various retail outlets.

Extraction and clean-up of samples

The extraction and clean-up were based on the method

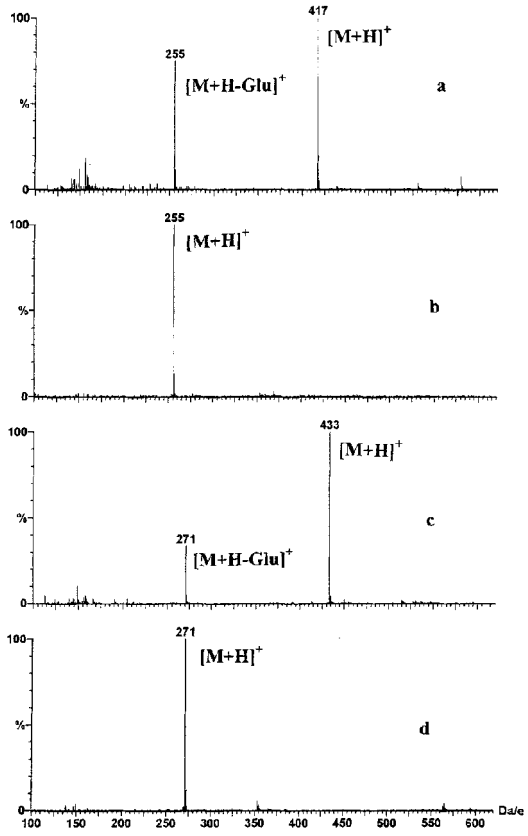

Figure 2. Scanned spectra of the 4 phytoestrogens obtained at low cone voltages. (a) daidzin, (b) daidzein, (c) genistin, (d) genistein.

described by Wang *et al.*[14] To summarize, flour or baby food (2 ± 0.20 g) was combined with *tert*-butylhydroquinone in methanol (1.25% w/v, 1 ml), spiking solution (where necessary), and hydrochloric acid (1M, 25 ml). After gentle stirring the sample was heated over a steam bath for 2 hours. After cooling acetonitrile (100 ml) was added, the sample shaken vigorously for 1 minute, and then left to stand for a further 10–15 minutes. An aliquot of the supernatant (1 ml) was diluted 1:1 with mobile phase (50:50 A/B, see below) and filtered (Whatman SF/A filters, BDH) into a screwcap vial. Extracts were stored at $-20\,^{\circ}C$ prior to LC/MS analysis.

Preparation of standards and spiked samples

All standard concentrations quoted are based on the equivalent sample concentration (mg/kg) prior extraction (assuming 100% recovery). Matrix-matched calibration solutions were prepared for each commodity using pooled extracts of samples previously found to be blank (i.e. concentrations of daidzin, daidzein, genistin and genistein < limit of detection, LOD) and extracted as described above. The solutions contained (post-hydrolysis) daidzein and genistein at 1.3–98.7 mg/kg and 1.4–100.6 mg/kg respectively. An accompanying set of solvent-based (50:50, v/v,

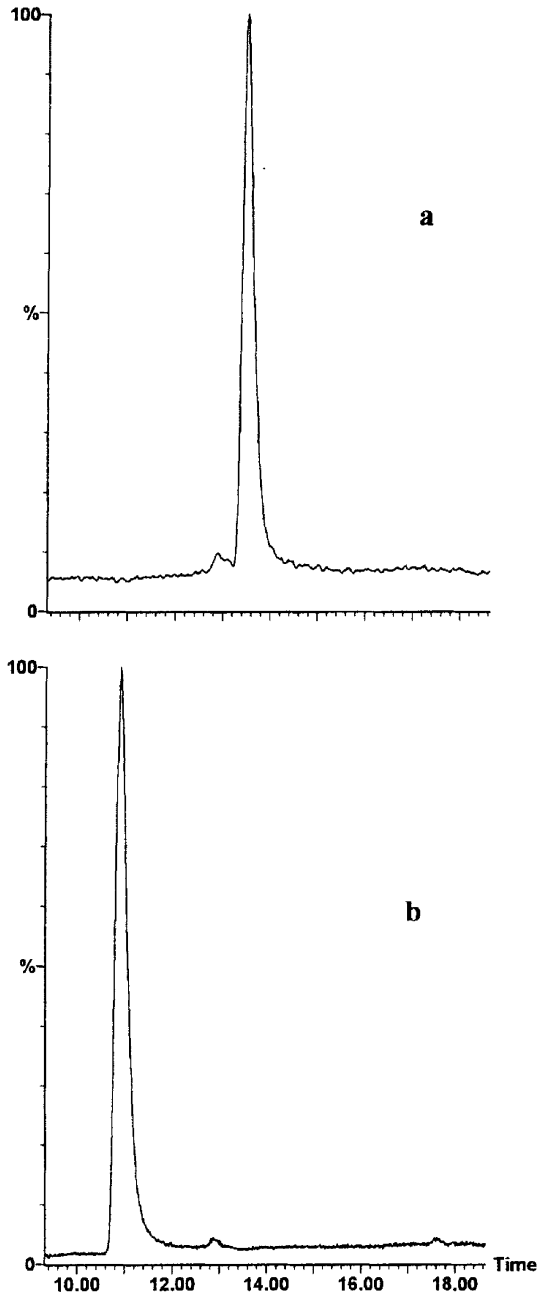

Figure 3. Single ion monitoring (SIM) chromatograms for (a) genistein (*m/z* 217) and (b) daidzein (*m/z* 255) obtained from a 5 µl injection of a solvent-based standard containing 47.5 mg/kg daidzin, 40.1 mg/kg genistin, 98.7 mg/kg daidzein and 100.6 mg/kg genistein.

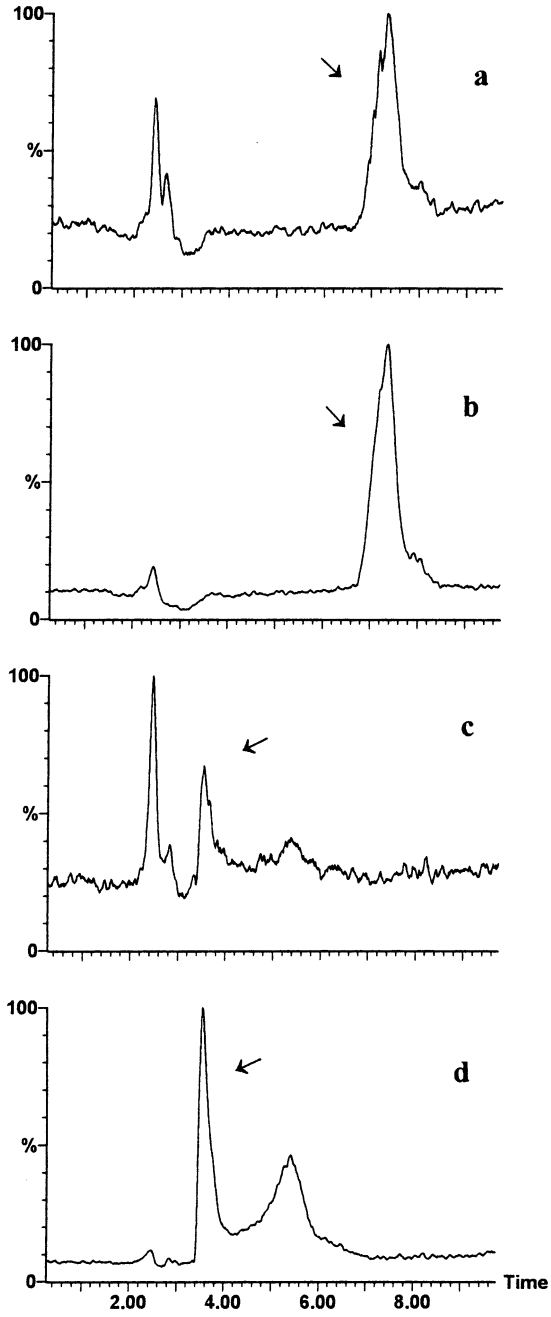

Figure 4. Single ion monitoring chromatograms of the 'check' ions monitored for (a) genistin (*m/z* 433), (b) genistin (*m/z* 271), (c) daidzin (*m/z* 417) and (d) daidzin (*m/z* 255) obtained from a 5 µl injection of a solvent-based standard containing 47.5 mg/kg daidzin, 40.1 mg/kg genistin, 98.7 mg/kg daidzein and 100.6 mg/kg genistein.

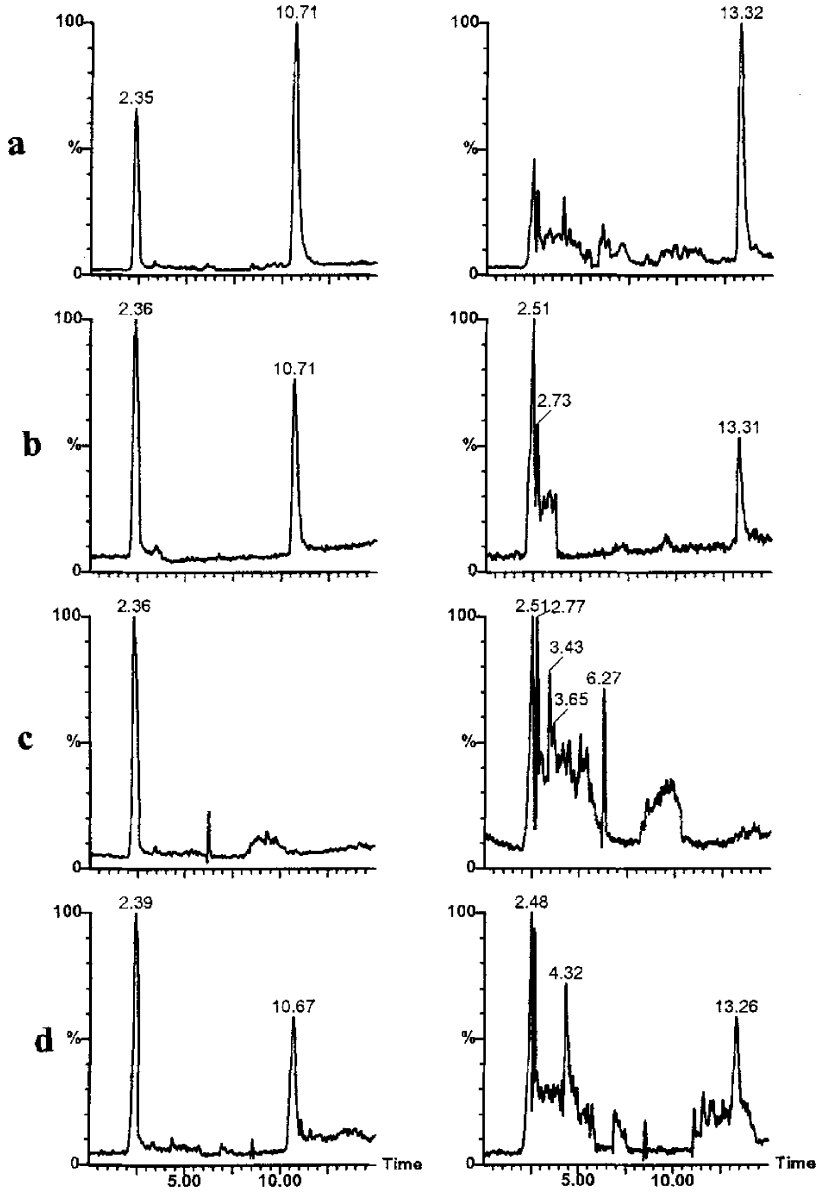

Figure 5. Single ion monitoring chromatograms for daidzein (RT 10.7 mins) and genistein (RT 13.4 mins) obtained from (a) a high spike baby food, (b) a low spike baby food, (c) a matrix blank and (d) a matrix-matched standard.

water/acetonitrile containing 0.5% acetic acid) standards was prepared at the same concentrations.

For recovery determinations blank commodity was spiked with daidzin and genistin before extraction, at two concentrations. The baby food low spike contained 10.0 mg/kg daidzin and 8.5 mg/kg genistin; the high spike contained 50 mg/kg daidzin and 42.5 mg/kg genistin. The flour low spike contained 45 mg/kg daidzin and 52.5 mg/kg genistin; and the high spike contained 90 mg/kg daidzin and 105 mg/kg genistin.

HPLC/ESI-MS

HPLC/ESI-MS analysis of sample extracts was performed on a Platform bench-top mass spectrometer (Micromass, Manchester, UK) coupled to a Gilson 231 XL autosampler (Villiers-le-bel, France) and HP1100 HPLC pump (Hewlett Packard GmbH, Waldbronn, Germany) equipped with an on-line degasser. Gradient elution at 0.5 ml/min used 0.5% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). The gradient profile was such that at times 0.0, 15.0, 15.1 and 20.0 minutes the % B was 20, 65, 20 and 20% respectively. An in-line splitter between the pump and the autosampler reduced the column flow rate to 60 μl/min. The column was a Primesphere 5C18 (250 × 1.0 mm) microbore column (Phenomenex, Macclesfield, UK) with a 2 μm pre-column filter. Eluting peaks were determined by MS using pneumatically assisted ESI operated in the positive ionization mode. The instrument was initially calibrated in the positive APcI mode using a mixture of polyethylene glycols (PEG) 300, 600, 1000 and 1500 over the mass range *m/z* 80–1100. Tuning was then optimized in ESI on the *m/z* 42 background ion followed by optimization on the protonated molecule $[M+H]^+$ ions of daidzein and genistein at *m/z* 255

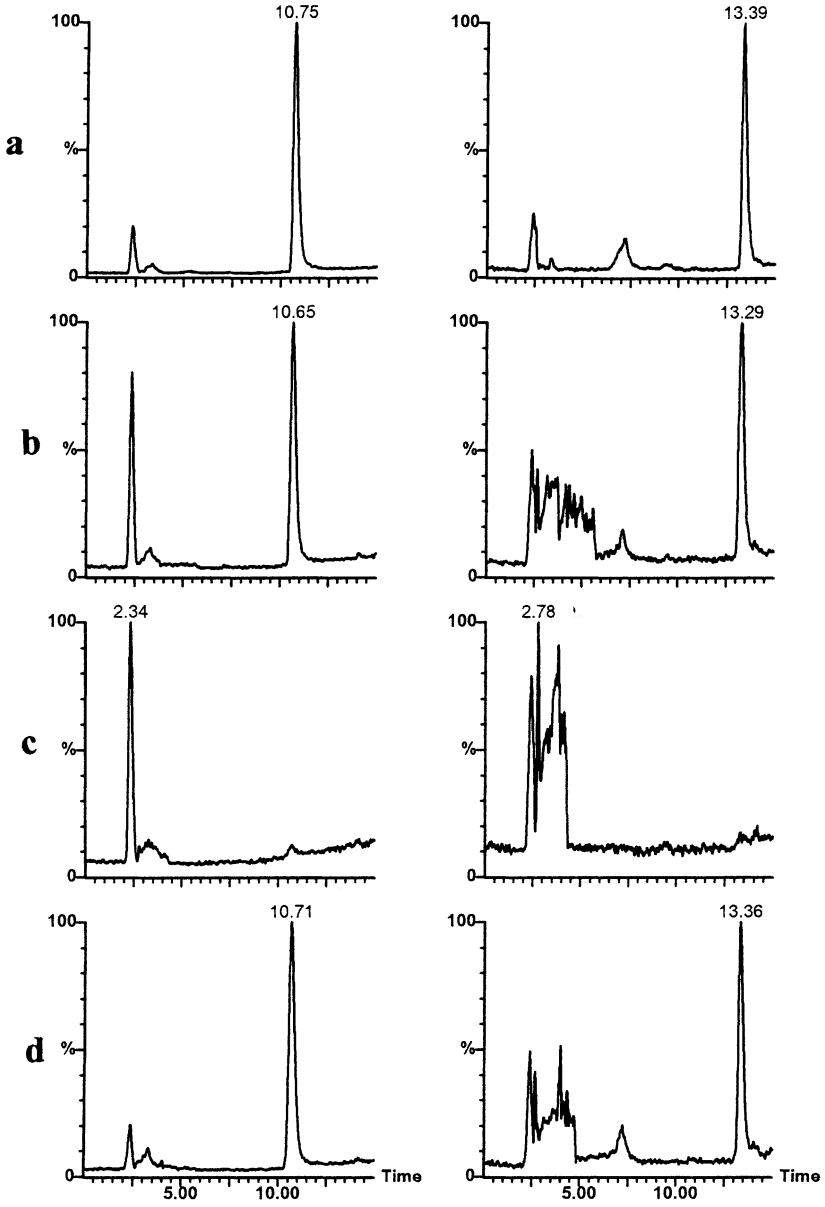

Figure 6. Single ion monitoring chromatograms for daidzein (RT 10.7 mins) and genistein (RT 13.4 mins) obtained from (a) a high spike baby food, (b) a low spike baby food, (c) a matrix blank and (d) a matrix-matched standard.

and 271 (10 µl injections of a 100 ng/µl solvent-based standard injected directly in to the front of the mass spectrometer). Typical operating conditions were: capillary voltage 2.4 kV, cone voltage offset 5 V, high voltage lens 0 kV, source temperature 150 °C. Scanned acquisitions were made over the range *m/z* 80–620 with a scan time of 2 seconds. Selected ion monitoring (SIM) was performed using the ions listed below, cone voltages are given in brackets: daidzein 255.05 (20 V); daidzin 255.10 (60 V) and 417.10 (20 V); genistein 271.00 (40 V); genistin 271.10 (60 V) and 433.10 (20 V). The dwell time for each channel was 0.08 seconds, the interchannel delay was 0.02 seconds and the mass span was 0.4 u. The retention time (RT) of daidzein was 10.8 minutes and of genistein 13.4 minutes. Daidzein and genistein concentrations were determined by interpolation of peak areas from calibration graphs acquired for each sample batch.

RESULTS

Scanned spectra for the 4 phytoestrogens obtained at low cone voltages (20 V) are given in Fig. 2. Loss of the glucose molecule from daidzin and genistin occurred readily (Fig. 2(a) and (c)), to yield the corresponding aglycone masses at *m/z* 255 and 271 respectively. This loss could be promoted by increasing the cone voltage but the highly conjugated nature of the molecules precluded further induced fragmentation even at high cone voltages. Thus detection is based on a single ion and the retention time. This is obviously a limitation in terms of satisfying confirmation criteria, however detection is mass dependant and therefore this method offers advantages in selectivity over alternative LC-UV methods.

Figures 3 and 4 show the SIM chromatograms for a 5 µl injection of a solvent-based standard containing 47.5 mg/kg

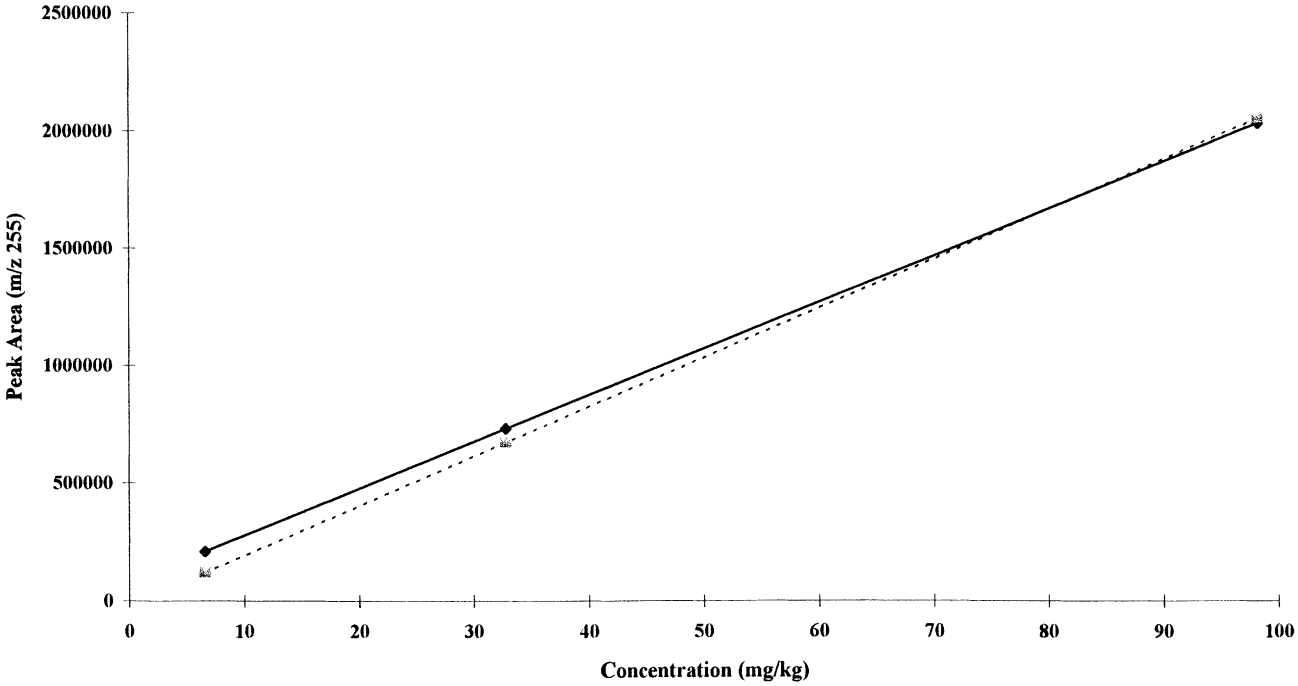

Figure 7. Baby food matrix effects on daidzein signal ion intensity — solvent-based standards; – – – baby food matrix-matched standards.

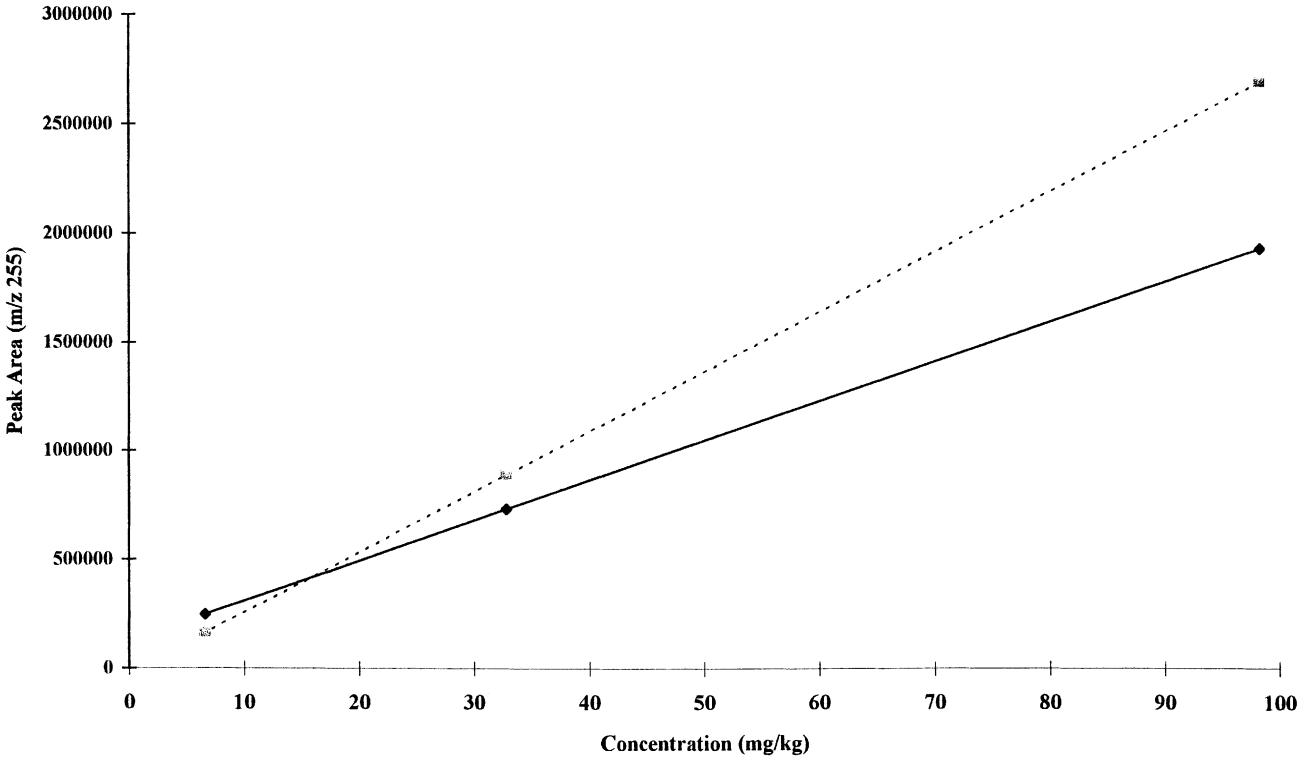

Figure 8. Soya flour matrix effects on daidzein signal ion intensity — solvent-based standards; – – – soya flour matrix-matched standards.

daidzin, 40.1 mg/kg genistin, 98.7 mg/kg daidzein and 100.6 mg/kg genistein. Peak symmetry and sensitivity were poor for daidzin and genistin (Fig. 4) but these ions were included only as 'check-ions' for incomplete hydrolysis and were not quantified. There was no evidence, throughout, of incomplete hydrolysis (i.e. no daidzin or genistin detected) in any of the sample or spike extracts even at high levels of daidzein and genistein.

Figure 5 shows the SIM chromatograms for daidzein (RT 10.7 mins) and genistein (RT 13.4 mins) obtained from (a) a high spike baby food, (b) a low spike baby food, (c) a baby food matrix blank and (d) a baby food matrix-matched

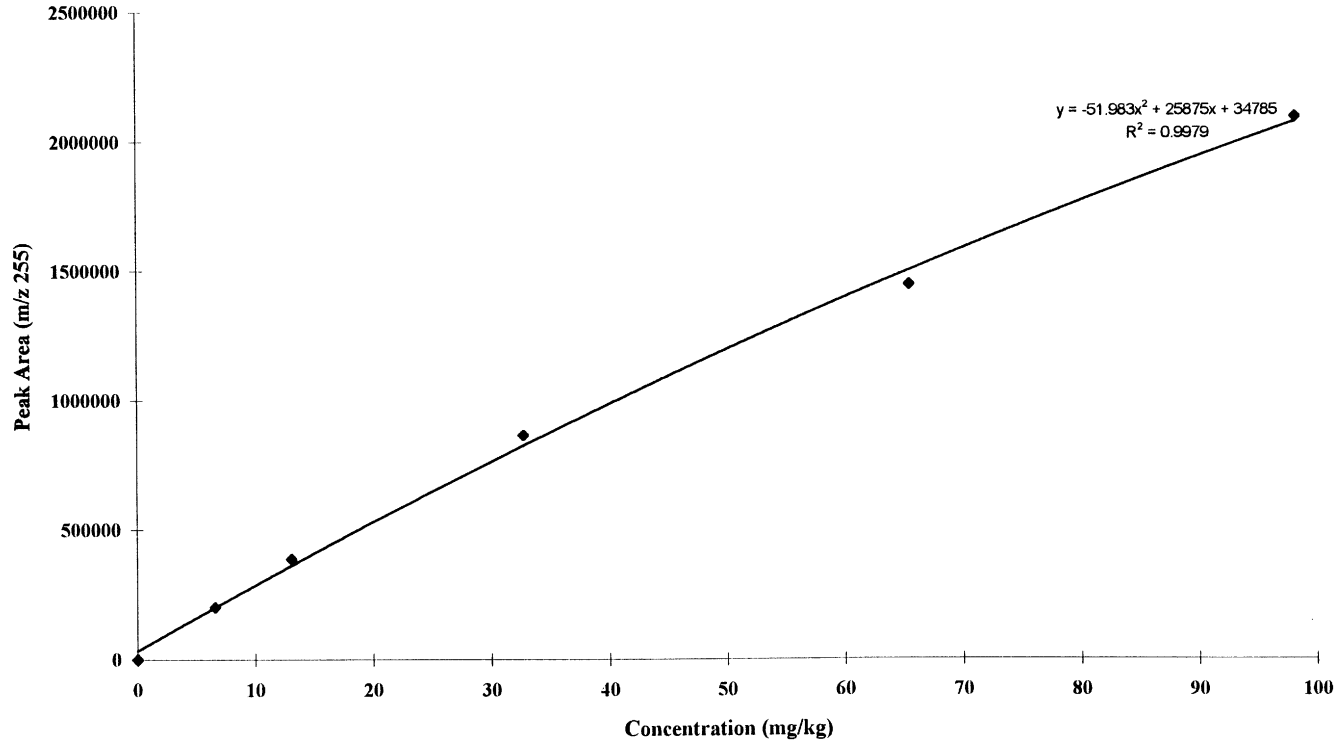

Figure 9. Calibration curve for daidzein over the concentration range 0–98.3 mg/kg.

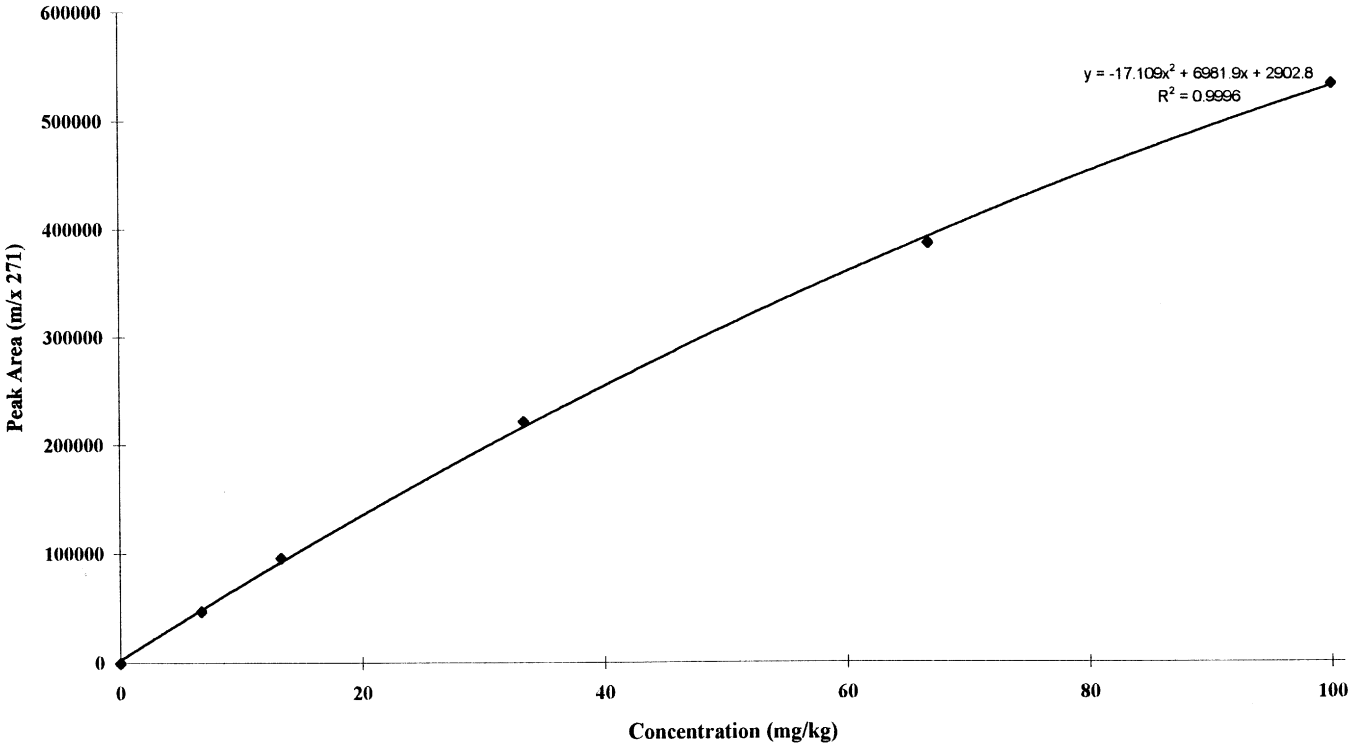

Figure 10. Calibration curve for genistein over the concentration range 0–100.2 mg/kg.

standard. Figure 6 shows the SIM chromatograms for daidzein (RT 10.7 mins) and genistein (RT 13.4 mins) obtained from (a) a high spike soya flour, (b) a low spikesoya flour, (c) a soya flour matrix blank and (d) a soya flour matrix-matched standard.

Figures 7 and 8 compare the calibration curves obtained from solvent-based and matrix-matched standards paired in terms of concentration. There was good agreement for the baby food based standards but a significant difference between the flour matrix-matched standards and the solvent-based standards. To counter such matrix effects matrix-matched standards were used throughout for quantification. Figures 9 and 10 show typical quadratic fit calibration curves for daidzein and genistein respectively.

Table 1. Summary of validation spike recovery (%)

	Baby food[a]		Flour[b]	
Spike	Daidzein	Genistein	Daidzein	Genistein
1	86	115	108	122
2	70	89	83	80
3	82	85	79	94
4	71	76	72	75
5	71	79	78	91
Mean	76	89	84	92
RSD (%)	10	17	17	20

[a] Baby food spiked 10 and 8.5 mg/kg of daidzin and genistin, respectively.
[b] Flour spiked at 50 and 42.5 mg/kg of genistin and daidzin, respectively.

In order to validate the extraction procedure 5 spiked extracts (using a matrix known to be blank) were prepared for each food type. The spiking level for baby food was 10 mg/kg daidzin and 8.5 mg/kg genistin. The spiking level for the flour was 50 mg/kg for daidzin and 42.5 mg/kg for genistin. Table 1 summarizes the validation recovery data. The mean recovery was 76 and 89% for daidzein and genistein respectively in baby food, and 84 and 92% for daidzein and genistein respectively in flour. The relative standard deviation ($n = 5$) was 10 and 17% for daidzein and genistein respectively in baby foods and 17 and 20% for daidzein and genistein respectively in flour.

Two extraction batches were prepared for each matrix, each batch incorporated a blank, a low spike and a high spike, and each extract was analysed twice non-consecutively. Blank and spike results are given in Tables 2 and 3. Close agreement was obtained between the 2 sets of determinations at each spiking level in both food matrices. With the exception of one flour blank found to contain 2.4 ± 0.4 mg/kg genistein (equivalent to 3.8 ± 0.6 mg/kg genistin) all blanks were at or near the LOD of the analyte.

In order to obtain quality control data a matrix-matched standard was analysed at regular intervals throughout the sample runs. For baby food this was the standard containing 6.5 mg/kg daidzein and 6.8 mg/kg genistein. For the soya flour it was the standard containing 32.8 mg/kg daidzein and 33.4 mg/kg genistein. The relative standard deviation associated with these instrument checks are given in Table 4. This quality control data was acquired over a 10 day period during the course of 463 injections. For daidzein the relative standard deviation ranged from 4–11%, and for genistein it ranged from 10–17%.

Based on $3 \times$ the standard deviation of repeat injections ($n = 5$) of the solvent-based standard containing 1.3 mg/kg daidzein and 1.4 mg/kg genistein, the limit of detection for daidzein was 0.2 mg/kg and 0.7 mg/kg for genistein. The limit of quantification (based on $10 \times$ the standard deviation of the repeat injections) was 0.5 mg/kg for daidzein and 2.2 mg/kg for genistein.

In both analytes the carbonyl and the ether oxygen atoms are potential sites for protonation. The lower response for genistein is possibly due to the close proximity of the hydroxyl group to the carbonyl group on positions 5 and 4 (Fig. 1). Hydrogen bonding between these 2 functional groups could interfere with the protonation of the molecule in the source of the mass spectrometer thus reducing ionization efficiency and decreasing sensitivity. Preliminary work undertaken in this laboratory using capillary electrophoresis MS on these compounds has shown a similar sensitivity ratio and this confirms that the cause of the disparity is mass spectrometric and not chromatographic.

CONCLUSIONS

The coupling of a microbore HPLC system with positive ion electrospray mass spectrometry has given a sensitive and robust technique for the separation and unambiguous identification of phytoestrogens in soya flour and baby foods. This method offers a clear advantage in terms of the low flow rate and small injection volume required, which reduces the amount of extraneous material entering the MS source, negating the need for frequent cleaning of the sampling cone and high voltage lens, and reducing the necessity of frequent calibrations. Limits of detection, based

Table 2. Results for the spikes and blanks associated with each set of baby food sample extractions (low spike 6.0 mg/kg daidzein 5.3 mg/kg genistein; high spike 30.2 mg/kg daidzein, 26.5 mg/kg genistein)

Daidzein	1st Batch				2nd Batch			
	1st Det		2nd Det		1st Det		2nd Det	
Sample	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec
Batch 1								
Blank	<0.2		0.4		<0.2		<0.2	
Low sp	6.2	102	5.8	96	4.2	69	5.0	83
High Sp	22.7	75	24.0	80	27.0	89	27.6	91
Batch 2								
Blank	0.4		<0.2		<0.2		<0.2	
Low sp	5.0	83	5.0	82	5.7	95	5.0	83
High Sp	20.5	68	26.0	86	27.8	92	24.8	82

Genistein	1st Batch				2nd Batch			
	1st Det		2nd Det		1st Det		2nd Det	
Sample	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec
Batch 1								
Blank	<0.7		<0.7		<0.7		<0.7	
Low sp	6.8	128	7.4	140	4.2	79	4.9	92
High Sp	24.6	93	24.9	94	24.1	91	24.6	93
Batch 2								
Blank	2.0		2.8		<0.7		<0.7	
Low sp	4.0	75	4.2	79	4.2	79	5.2	98
High Sp	20.2	76	24.1	91	28.7	109	22.1	83

Table 3. Results for the spikes and blanks associated with each set of soya flour sample extractions (low spike 27.2 mg/kg daidzein, 32.6 mg/kg genistein; high spike 54.4 mg/kg daidzein, 65.3 mg/kg genistein)

Daidzein	1st Batch				2nd Batch			
	1st Det		2nd Det		1st Det		2nd Det	
Sample	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec
Batch 1								
Blank	<0.2		<0.2		0.4		<0.2	
Low sp	23.9	88	25.6	94	23.8	88	24.1	88
High Sp	53.6	98	49.8	91	38.7	71	46.2	85
Batch 2								
Blank	0.4		<0.2		<0.2		<0.2	
Low sp	20.3	75	20.2	74	25.6	94	19.4	71
High Sp	48.0	88	44.7	82	41.4	76	42.7	78

Genistein	1st Batch				2nd Batch			
	1st Det		2nd Det		1st Det		2nd Det	
Sample	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec	Conc (mg/kg)	% Rec
Batch 1								
Blank	<0.7		<0.7		5		15	
Low sp	34.5	106	35.7	110	34.8	107	30.5	94
High Sp	73.5	113	68.0	104	45.7	70	43.0	66
Batch 2								
Blank	<0.7		<0.7		1.6		12	
Low sp	28.2	87	25.8	79	30.9	95	25.6	79
High Sp	100.8[a]	155	79.3[a]	122	46.6	71	46.4	71

[a] The results for this spike were abnormally high, however, this sample had formed an aggregate on extraction which possibly resulted in non-uniform distribution of the spiking material.

Table 4. Quality control data — method performance over 10 days[a]

	Daidzein		Genistein	
	% SD	*n*	% SD	*n*
Baby food validation	7	7	15	7
Baby food 1st batch	11	15	13	15
Baby food 2nd batch	7	15	17	15
Flour validation	4	4	10	4
Flour 1st batch	7	15	10	15
Flour 2nd batch	10	15	14	15

[a] Results from instrument check standards interspersed throughout 463 injections analysed over a 10 day period.

on three times the standard deviation associated with repeat injections of a low concentration standard, were 0.2 mg/kg for daidzein and 0.7 mg/kg for genistein in foods. The limit of quantification (10 × SD) was 0.5 mg/kg for daidzein and 2.2 mg/kg for genistein in foods. The precision of determination was in the range of 4–15% relative standard deviation obtained on data involving 463 injections obtained over a 10 day period.

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