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Determination of phytoestrogens in dietary supplements by LC-MS/MS

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Abstract

Labeling data quantifying the exact content of individual phytoestrogen analytes in dietary supplements is generally poor. As these products are commonly used in the management of menopause symptoms, any clinical benefits would be dependent on the exact dosage of isoflavones received. Well established extraction procedures and updated Isotope Dilution Mass Spectrometry LC-MS/MS have been used to accurately quantify the concentrations of 10 common isoflavones in 35 dietary supplement samples on sale in the UK, Canada and Italy. Concentration specific ionization suppression is described for biochanin A and formononetin. All supplements contained phytoestrogens. The soya isoflavones (genistein, daidzein, glycitein) were present in all products and the majority also contained the red clover isoflavones (biochanin A, formononetin) and some the Kudzu isoflavones (daidzein, puerarin). The content of total isoflavones per dose ranged from <1 to 53 mg. Trace amounts of coumestrol were found in 6 products. Other less common analytes, the prenylnaringenins (6-prenylnaringenin, 8-prenylnarigenin, 6,8-diprenylnaringenin) were not found in any of the products. Only 14 of 35 supplements were found to deliver more than or equal to 40 mg/day of aglycone isoflavones, a consensus dose value recognized as delivering therapeutic benefit. 11 did not match label claims. Six delivered less than 10 mg/day isoflavones. There has been little improvement in the overall quality of industry labeling in the five years since this was last investigated. Consequently, the public, retailers and health care professionals should consider using standardised isoflavone supplements, which are supported by analytical measurements.

Keywords: phytoestrogens, isoflavone intake, dietary supplements, soya red clover, menopause.

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Introduction

Vasomotor symptoms (hot flushes) are the most common menopausal symptom experienced by women in Western countries. This is recognised as the main reason women seek advice from their healthcare professional (Guthrie *et al.* 2003 Following the recent controversies regarding Hormone Replacement Therapy (HRT) (Rossouw *et al.* 2002,Beral *et al.* 2005) use has decreased in recent years.

The search for an alternative to HRT has lead to an ever increasing selection of 'natural' alternatives becoming available in retail stores and on the internet. This raises concern, as levels of the active ingredients are not always sufficient to produce the desired effect.

One group of substances receiving a great deal of interest are the phytoestrogens, in particular the sub-group of compounds called the isoflavones. Phytoestrogens are naturally occurring plant components that interact with mammalian endocrine systems. Evidence is accumulating that isoflavones may have beneficial properties, in particular against chronic and degenerative diseases including coronary heart disease and hormone-dependent cancers (Knight and Eden 1995, Tham *et al*. 1998). There is a more substantial body of evidence supporting beneficial clinical effects in non-disease states such as alleviating and lowering the rates of menopausal vasomotor symptoms and maintaining bone health (preventing osteoporosis) (Chiechi *et al* 2005).

Epidemiological data suggest that the ingestion of high isoflavone containing foods (daily intake between 20 –50 mg) can result in certain health benefits. A number of clinical papers and meta analyses of clinical trials using supplements containing at least 40 mg isoflavones have demonstrated reduction in vasomotor symptoms (Nachtigall *et al.* 2006, Howes *et al.*

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2006, Thompson Coon *et al.* 2007). This has lead to their endorsement as first line treatment options in complementing lifestyle changes.

Soya beans and soya containing products are the best known and most widely consumed phytoestrogen-containing foods. Other food sources are generally legumes, for instance; lentils, chickpeas, beans, alfalfa shoots, red clover. The intake of phytoestrogens through the UK diet is generally well below that required to achieve a biologically response. Many consumers who seek to achieve the circulatory concentrations of phytoestrogens that are perceived to produce beneficial effects will do so by dietary supplementation. A diverse range of tablets and capsules are available from a number of manufacturers. The quality of labeling information on many of these products may be inadequate for consumers to compare active ingredients and to make an informed choice.

Dietary isoflavones exist in two forms – as glycosides, attached to a sugar unit - or as aglycones, free forms (table 1, figure 1). Controversy exists as to the bioavailability of isoflavone glycosides compared to the aglycone forms in humans (Setchell *et al*. 2002). Setchell reported that the glycoside form is not absorbed intact across the enterocyte and their bioavailability requires initial cleaving of the sugar moiety by gut flora for uptake into the circulation. The consequence of this is that the aglycone level in supplements needs to be measured to give an indication of the potential biological effect, rather than the total weight of isoflavones, as the glycosides often contribute a large mass fraction of the total. Isoflavones originating from red clover are generally in the aglycone form, whereas those originating from soya are usually in the glycoside forms. As well as simple glucose glycosides (table 1) complex acetyl- and malony-glucosides are present (figure 1).

Soya products contain genistein, daidzein and glycitein as the predominant aglycones, while red clover contains formononetin and biochanin A and sometimes coumestrol, as the major

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components. There are a number of other less common oestrogenic compounds such as the prenylnaringenins and puerarin (daidzein-8-*C*-glycoside) which are also present in dietary supplements. In general the aglycones exhibit much higher potency than the equivalent glycosides.

There is now an extensive literature of many thousands of papers related to phytoestrogens. Methods for the determination of phytoestrogens have been reviewed three times recently (Wang 2002, Wu 2004, Prasain 2004). The procedures for extracting, hydrolyzing and chromatographically separating phytoestrogens from foods are generally well understood and established, but the accepted best practice with reference to the extraction solvent, percentage of water, pH and temperature, varies enormously between various food types and no single ubiquitous method will be suitable to all situations (Murphy 2002, Allaouri 2005, Barbosa 2005). This paper uses pre-existing techniques in these areas (Grace 2003, Antignac 2003, Clarke 2002, 2003a, 2003b, Wiseman 2002). The majority of phytoestrogen analyses are targeted at trace level analysis, this study discusses some of the difficulties encountered when conducting LC-MS measurements at high levels.

Dietary intake estimates are generally based on dietary recall (DR) or food frequency questionnaires (FFQ) rather than the analysis of actual diets. Both methods can be used to correlate dietary intake of isoflavones directly to the consumption of primary soya products. Diet analysis and DR give similar estimates where soya is deliberately consumed (Verkasalo *et al.* 2001). Intakes of 7 and 4 mg day of genistein and daidzein in American diets have been estimated from a detailed examination of 40 soya-containing products, cautioning that many of the established FFQ severely underestimate isoflavone intake (Kirk *et al.* 1999). The Framingham Heart Study reports values as low 0.3 mg day^{-1} each of genistein and daidzein, attributing the major source to peas and beans (de Kleijn *et al.* 2001). The Dutch arm of the

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European Prospective Investigation into Cancer and nutrition (EPIC study) suggests even lower intake values of 0.16 and 0.15 mg day^{-1} each of genistein and daidzein (Boker *et al.* 2002). Dietary intake of isoflavones in Asian soya diets has been estimated on numerous occasions and is now generally accepted to be in the range of 30-50 mg day^{-1} of the combined isoflavone aglycone equivalents (daidzein, genistein and glycitein) (Wakai *et al.* 1999, Messina 1995). The latest estimate (Kikuchi *et al.* 2001), a market basket approach of broadly similar design to the UK Total Diet Study (Peattie *et al.* 1983), analysed Japanese diets for the hydrolysed aglycones. This provided an intake figure for combined aglycones of 38 mg per day. As the intake of soy and isoflavones in the Asian is often cited as a possible explanation of measurable health benefits (e.g. lower disease rates), clinical studies are conducted at similar levels of exposure. This led to a consensus, largely by inference that dietary supplements should be designed to deliver a similar intake of active ingredients i.e. 40 mg day^{-1} isoflavones.

The two previous works in this area that used branded products highlighted problems in the isoflavone supplement industry (Howes and Howes 2002, Setchell *et al*. 2001). Firstly many manufacturers used the weight of glucosides as the measure of potency rather than adjusting downwards for the mass of aglycone. Subsequently many products claimed to deliver an appropriate quantity of the active principle. As an example, in the Howes letter, a label claim of 68 mg isoflavones/dose was supported by an analytical measurement of 50 mg, but this drops to 31 mg when corrected for aglycone content. This product and others like it then deliver less than the stated dose. In that study, only one of the nine products, on sale in Australia, delivered the stated dose measured (38 mg label 40 mg measured), the other eight were found to deliver less than half the stated dose (Howes and Howes 2002). In the other larger study of 33 American dietary supplement products, Setchell measured the total isoflavone weight, including glucosides and found that half deviated by more than 10% from

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the claimed values. Six products were more than 50% from the claimed value. Eight of the 33 products delivered 40-60 mg/day total isoflavones. The majority therefore did not deliver 40 mg aglycones (Setchell *et al*. 2001)

This study has measured the concentrations of isoflavones in a number of isoflavone supplements available in the UK, Canada and Italy. In addition, we have looked at the presence of any other substances that may have an adverse impact on health or interact with pharmaceutical therapies. Previous studies conducted in USA and Australia, showed that there is a great variability in composition of isoflavone supplements, as determined by High Performance Liquid Chromatography (HPLC-UV) profiles, many containing peaks of unknown origin and chemical structure and that for over half the products tested the actual isoflavone levels deviated by more than 10% from the claimed value (Setchell *et al.* 2001, Howes and Howes 2002).

Materials and methods

Collection and preparation of samples

Commercial samples in intact retail packaging were collected by the authors or donated by Novogen Ltd. Samples of red clover, red clover extracts, test certificates and an analysis protocol were supplied by Novogen Ltd. Novogen Red Clover Sample 35 was a older version of sample 14, this three year old formulation sample was assayed as a quality control check to confirm long term product stability at room temperature in the final packaging. Tablets were weighed intact. Capsules were weighed full, then opened and the contents emptied out and the empty capsules and contents weighed separately. All products were weighed in batches of five and the average weights used. All samples were pulverised to powder prior to analysis and stored at ambient conditions.

Chemicals

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All reagents, solvents and chemicals were of analytical grade or equivalent. The phytoestrogens; daidzein, genistein, glycitein, biochanin A, formononetin and coumestrol were purchased from Apin Chemicals (Abingdon, UK). [2,3,4-$^{13}C_3$]formononetin, [2,3,4-$^{13}C_3$]biochanin A, [2,3,4-$^{13}C_3$]daidzein, [2,3,4-$^{13}C_3$]daidzein glucoside, [2,3,4-$^{13}C_3$]daidzein glucuronide, [2,3,4-$^{13}C_3$]genistein, [2,3,4-$^{13}C_3$]genistein glucuronide, [2,3,4-$^{13}C_3$]glycitein and [2,3,4-$^{13}C_3$]glycitein glucuronide were supplied by Dr Nigel Botting (St. Andrews University, UK). Puerarin, 6-prenylnarinegnin, 8-prenylnaringenin, 6,8-diprenylnaringenin were purchased form Plantech (Reading UK). The reference compounds were stored as powders at –18°C. Stock solutions were prepared in ethanol, or ethanol/water (polar conjugates) and stored at +4°C. The purity of standards was assessed by HPLC-UV, any containing observable quantities of contaminants or by products were discarded. The purity of the bulk stock of daidzein and genistein analytical standards was further assessed at the beginning of this work against values from by an inter-laboratory trial where five European laboratories provided extinction coefficient data on separately sourced standards (Wiseman *et al.* 2002).

Analysis overview

Due to the high concentrations of isoflavones, and these being presented as pre-extracted and semi-purified active principles, often as the aglycone, these products achieve optimum solubility in a different solvent mix to that used to extract intact soya products. An industry protocol was used to solubilise products. Once solubilised the analysis scheme of acid hydrolysis and Isotope Dilution Mass Spectrometry was similar to that used in previous investigations into phytoestrogen content of food (Clarke *et al*. 2003a and 2003b).

Sample extractions

Powdered samples (0.080 ± 0.001g) were weighed into silyanised glass vials and were quantitatively transferred with solvent into volumetric flasks by the following procedure. Add ethanol (3.5 ml) and sonicate in vial for 5 min, transfer to 50 ml volumetric flasks. Add a ethanol water mixture (40 ml, 1:1), in portions to quantitatively transfer all extract and solid

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residue into the volumetric flask, which was sonicated a further 30 mins. After the solution had cooled, the extracts were made up to the 50 ml mark with further ethanol water. A portion (0.200 ml) of each extract was dispensed into a silyanised glass vial and a solution of internal standard was added (100 µl containing [2,3,4-$^{13}C_3$]daidzein, [2,3,4-$^{13}C_3$]glycitein, [2,3,4-$^{13}C_3$]formononetin [2,3,4-$^{13}C_3$]biochanin A and [2,3,4-$^{13}C_3$]genistein). Internal standardization was not conducted at the first step, as the mg quantities required in each volumetric flask were impractical. Extracts were dried at 30°C under a stream of nitrogen, dissolved in 1M hydrochloric acid (5 ml, aqueous) loosely stoppered and hydrolysed on a hot block at 95 ^{0}C for 2 hrs. The hydrolysed aglycones were extracted with diethyl ether (2 x 5 ml). After careful separation of all aqueous acidic phase, the combined ether extracts were evaporated at 30°C under a stream of nitrogen. Residues were dissolved in 1 ml HPLC mobile phase by adding methanol (0.5 ml) sonicating 5 min then adding water (0.5 ml).

Chromatography and quantitation

Analyses were conducted on a Micromass Ultima triple quadrupole mass spectrometer equipped with an electrospray source operating in negative ion mode (Waters, Manchester, UK). Data were acquired by tandem mass spectrometry using a multiple reaction monitoring (MRM) method that monitored parent to daughter ion transitions for each analyte. Individual transitions, retention times and ionization parameters are listed in Table 2. The desolvation temperature was 300 ^{o}C and the source block temperature was 100 ^{o}C. The desolvation gas flow was 700 L/h, the cone gas flow was 100 L/h and the capillary voltage was 2.8 Kv. Reversed phase separations were carried out at 30° C using a Waters Bridged Ethylene Hybrid (BEH) C18 column (2.1 x 150 mm) (Waters, Manchester, UK). The injection volume was generally 20 µl. A gradient program was used, starting from 10% acetonitrile with 90% aqueous, holding for 3 mins and changing to 30% acetonitrile at 7 min, increasing to 100% acetonitrile at 23 min, with a 2 min hold at 100% acetonitrile and a return to 10% acetonitrile

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at 26 min and a 4 min equilibration before the next cycle. Both mobile phases contained formic acid (0.1%) and the flow ws 0.2 ml/min. The injector was programmed to provide an exterior wash of acetonitrile around the needle on an extended solvent wash program (x-4). The analytes quantified in samples were, genistein, daidzein, glycitein, formononetin, biochanin A, coumestrol, puerarin, 6-prenylnaringenin, 8-prenylnaringenin, 6,8-diprenylnaringenin. Structures are summarized in Figure 1. Analytes were quantified in the range 0.03 to 48 µg/ml.

Analytical quality assurance.

For a specific phytoestrogen to be considered present in a sample extract the following criteria must be met. The relative retention times of the analyte must be comparable to those of a retention time marker,(the first eluting internal standard), the internal standard (used for analyte quantitation i.e. native and $^{13}C_3$-analogue are within ± 0.05 min), and to authentic analytical standards of each analyte. The peak must have the correct mass transition, maximizing at the correct retention time. The signal to noise ratio of any peak must be greater than 3:1. All samples were analysed from duplicate weighings with single analytical runs for each extract, mean values of these duplicate analyses were used. Each analytical batch contained reagent blanks, spiked reagent blanks, replicate analyses and reference samples. As an example, in the definitive analytical batch, the Novogen RedClover and Promensil reference material were each analysed from three separate crushed tablets, with duplicate weighings from each of the three powdered tablets (3 x 2 = 6 replicates).

Samples of known composition were used to validate the analytical approach. A number of raw materials, extracts and formulated products were supplied with the manufacturers analytical test certificates. This was further assessed by an initial comparison to an HPLC-UV method supplied by Novogen Ltd. The two methods were judged as comparable when

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delivering data to within 10% of nominal values. The Novogen product Promensil was used to assess method variability by replicate extractions and analyses in the final experiment.

Glycoside hydrolysis

Hydrolysis of glycosides to aglycones was confirmed by concurrent hydrolysis of labeled glycoside surrogates (figure 4). Triple 13-C labeled daidzein glucoside and a set of similarly labeled glucuronides of daidzein, genistein and glycitein were each hydrolysed within the sample matrix. This is considered proof of quantitative hydrolysis of all glucosylated forms, to their respective native aglycones using the hydrochloric acid hydrolysis protocol. This was a qualitative process conducted with and without matrix, in separate vials, from the samples to be quantified.

Results

Significant concentrations of the isoflavone genistein, daidzein, glycitein, formononetin, biochanin A, were observed in many of the products. Puerarin was observed as a minor component in three products. No products contained significant levels of coumestrol. Many products which are labelled as containing phytoestrogen ingredients contained lower quantities of isoflavones than expected (table 3).

Discussion

Analytical methods

Recent works still continue to successfully use non-labelled and chemically dissimilar, molecules as the internal standard, such as flavone for measuring isoflavones in vegetables (Antonelli 2005), The simpler design of using formononetin as internal standard for

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normalisation of the common soya isoflavones daidzein, genistein and glycitein severely limits applicability of this approach to foods that are already well characterised and known to be free of formononetin (Wu 2004). The use of rarer structural isomers of more common analytes, such as using prunetin an isomer of biochanin A for determination of isoflavones in bovine milk makes this approach more generally applicable (Antignac 2003).

While isotope dilution is a powerful technique, it has many of the same limitations as other internal standardisation techniques, especially when only a limited number of labelled analogues are available and in some cases the labelled standard is being used both as a true isotope dilution internal standard and as a non-labelled internal standard for a wide class of analytes. This has traditionally been the only pragmatic approach for phytoestrogen analysis and this has always been demonstrated to work well at low concentrations. It is often overlooked that papers claiming isotope dilution generally still contain one or more analytes quantified using a labelled analogue as a non-isotope dilution normalisation. A trial and error approach has lead to $^{13}C_3$daidzein being generally accepted as the better choice than $^{13}C_3$genistein for normalisation in such cases, as it displays less intra-run variability, and invariably will give data sets of lower cv%, but these will often be twice as large as those of analytes quantified by true isotope dilution in the same batch. This pragmatic approach has been adopted by the majority of researchers conducting LC-MS/MS measurements and there have been few problems reported for low level quantitation in matrices such as serum and urine (Grace 2003, Clarke 2002).

It was noticed that the two principle red clover analytes, formononetin and biochanin A were failing to give adequate values for a reference material in initial range finding experiments. Further investigation by comparison of UV responses from running the industry HPLC-UV analysis method lead us to believe that the supplied values were correct and that the ratio of

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biochanin A to formononetin in the reference sample was *ca*. 1.6. By LC-MS the calculated values were similar (a 1:1 ratio) and the combined total of formononetin and biochanin A was about half the nominal value. At this point the quantitation method used $^{13}C_3$-daidzein to normalised the native formononetin and biochanin A peak areas. The normalised daidzein response was linear (r^2 = 0.999), while the calibration for biochanin A was non-linear, but achieved an acceptable quadratic calibration (r^2 = 0.994). When only the lower concentration points are used it can be seen that this deviation from linearity at higher concentrations is due to the ratio of biochanin A/$^{13}C_3$-daidzein changing by a factor of 2-3 fold from the predicted value (figure 2). While formononetin exhibited similar properties, the deviations between calculated and true values were larger for biochanin A quantitation at this stage. Using high order quadratic models for fitting calibration lines is normal practice, but this approach is not applicable in this example as it masks ionisation issues.

As this effect appeared to be concentration based, it would be technically feasible to extract differing amounts of each sample and prepare separate extracts for each analyte to measure each individual analyte within a narrow dynamic range, from the LOD (0.03 µg/l) to 10 x LOD (0.3 µg/ml). This would however result in a large number of chromatographic runs.

Further $^{13}C_3$-labelled isoflavones were obtained and this anomaly was investigated in detail. While precision and between batch reproducibility were seen to be influenced by sensitivity and instrument cleanliness, self suppression was observed to correlate with increasing native analyte concentration in the calibration solutions. This was a factor of total analyte load at each point in the charomatogram, andnot of the individual concentrations. The point where self-suppression was induced therefore varied batch by batch and occurred at a lower concentration of each individual analyte as labelled internal standard were introduced, increasing the analyte loading. In solutions containing 4 µg/ml of the internal standards

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($^{13}C_3$daidzein, $^{13}C_3$genistein, $^{13}C_3$glycitein, $^{13}C_3$formononetin, and $^{13}C_3$biochanin A) the internal standard raw area response was static with the addition of up to 0.3 µg/ml of unlabelled analytes (daidzein, genistein, glycitein, formononetin, biochanin A and coumestrol). With increasing analyte concentration of up to 32 µg/ml, the labelled internal standards display decreased ionisation. $^{13}C_3$biochanin A was the most reduced, with 20% signal strength remaining i.e. a five-fold reduction, followed by formononetin 25%, genistein 38% and daidzein 40% (figure 3). If it is assumed that the unlabelled native analyte is similarly suppressed, this affects the ability to accurately calibrate and calculate concentrations of analytes in solution at greater than 0.3 µg/ml. This provides a very narrow range in which quantitation can be achieved. In reality this effect would also be expected to vary markedly between adjacent vials in an analytical run making quantitation impossible.

As quantitation can be conducted by using any similar molecule as an internal standard, we calculated the concentration of analyte in the reference sample (Promensil Red Clover Tablets) using a number of different $^{13}C_3$-isoflavones. As a further comparison a higher extract loading using 10-fold more extract with the same 4 µg of internal standard was conducted to see how ionisations extrapolated at extreme concentrations. Without concentration effects, the measured value of the concentrated solution will be 10-times that of the dilute solution. For biochanin A the quantitation method using $^{13}C_3$-daidzein normalisation was found to be the worst choice for internal standardisation and this gave 60% of the nominal value at the dilute solution and 40% of nominal at the higher concentration (table 4). The methoxylated derivatives of genistein and daidzein, biochanin A and formononetin were each better individual internal standard choices for the normalisation of both biochanin A and formononetin than either of the parent hydroxyl analytes genistein and daidzein as would be expected.

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While much of what we report is academically very obvious, such fundamental ionisation and self-suppression issues resulting in an inability to use chemically dissimilar internals standards has not been previously been reported and we believe these problems are due primarily to operating at the elevated concentrations required for dietary supplements analysis.

In summary, when the method was extended to include further internal standards, one for each of the major analytes being measured the errors introduced through self-suppression were overcome allowing accurate quantitation over a further two orders of magnitude of concentration.

Isoflavone content of supplements

The complete set of analyses data are listed in table 2 by individual analyte (mg/product) and then as a total dose of isoflavones per dose of product and as a percentage of the labelled dose. The results show that there is often variability between the actual content of isoflavones compared to that stated on the label. In addition, the presence of the glycoside form in many of the soya based supplements further complicates the interpretation of the label information. Based on the intake data we have described above, and an allowance of a 20% error, only 14 out of 35 supplements had sufficient levels, i.e. a minimum of 40 mg of aglycone isoflavones to achieve the intended therapeutic benefit. When compared to label claims, only 11 of 35 were within ± 20% of the stated value. There was significant overlap, whereby 8 of the 14 products that contained 40 mg/day of aglycone isoflavones were also within ± 20% of the stated value. 6 products were found to contain less than 50% of the stated dose. Our study shows that there has still been no overall improvement in labelling quality from previous studies (Howes *et al*. 2002, Setchell *et al.* 2001).

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The red clover based products where the aglycones are extracted and solvent partitioned before being added into the formulation are all deliver close to the label claim (84-133%). For soya based products the accuracy of the label claim is considerably poorer (5-69%). This is due to soya extracts being added by weight without specifying if conjugated to glucose or being present in the free form. In the soya source material it is generally assumed that the majority of isoflavones are conjugated, as an illustration, the ratio 1:52:9:38 of aglycone: glucose: acetylglucose:malonyglucose will be used (Wiseman 2002). As the conjugates vary in mass a matrix is used to calculate the contribution of each form to the total of aglycone equivalents (table 5). Daidzein conjugates from soya as weighed out will contribute 57% aglycone equivalents by mass, with genistein contributing 58% and glycitein 60%. As these contributions are essentially the same irrespective of the actual ratio of daidzein : genistein : glycitein in the supplement, a correction factor of 0.58 should be applied to calculate aglycone equivalents. The data suggest that none of the manufacturers have applied this correction. The average % of label claim for products containing soya as the sole isoflavone source average is 55% (excluding the outlier at 5%). After correction for aglycone equivalents against the 0.58 factor (58% aglycone & 42% glucose) this label claim average would have been 95%. In summary, manufacturers of soya supplements are putting in the correct weight, but of the wrong form of isoflavone and these products do not deliver the stated dose. For mixed soya ; red clover or soya kudzu it appears some are correctly formulated (*ca.* 100% of label claim) and some are not (*ca.* 40-60% label claim)

Either soya or soya germ, the hypocotyl portion of the soy bean is used as high concentration starting material Our previous work has shown that soya germ can provide a three fold increase in isoflavone content relative to the whole bean. The ratios of the three isoflavone daidzein, genistein and glycitein changes from 7:10:1 in soya flour to 4:1:3 in soya germ. Changing from a high genistein to high daidzein/glycitein product affects the potency as the

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isoflavone differ in oestrogenic potency. The two different profiles, of high genistein, or high daidzein/glycitein were readily observed in these supplements.

The three year old Novogen Red Clover tablets were found to contain identical quantities of all the measured isoflavones and therefore such products are stable for three years.

Coumestrol content

There has been concern and confusion between coumestans such as coumestrol, a benzofurocoumarin and coumarins such as coumadin (warfarin), which exhibit antithrombotic-anticoagulant effects. Most naturally occurring coumarins have not been evaluated, but some can be harmful. Dicoumarol was found to be the compound responsible for hemorrhagic death of livestock consuming mouldy hay. Standardized red clover has been clinically evaluated and the extract presents little risk of coagulation abnormalities (Booth *et al.* 2004). A dose of 25 to 200 mg/day of dicoumarol is needed to produce clinically significant anticoagulation, this level of intake is virtually impossible to achieve by ingestion of 500 mg dietary supplement tablets. Supplement manufacturers generally consider it cautionary to ensure that no coumestrol is present. None of the products studied contained high levels of coumestrol.

Analytical quality assurance.

Quantitative removal of the glycosides with concomitant appearance of labelled aglycone is taken as proof of successful hydrolysis of native analytes (figure 4). Daidzein glucoside was the only labelled glucoside currently available, therefore a larger set of isoflavone glucuronides surrogates (daidzein, genistein, glycitein) were used alongside daidzein glucoside to probe genistein and glycitein hydrolysis. In the first pair of chromatograms the

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sample is shown to contain the labelled glucoside conjugate (MRM 432>256) and to be clear of the labelled aglycone (MRM 256>226), while after hydrolysis, the conjugate is removed and the aglycone is observed. In the same hydrolysed sample with and without addition of the labelled daidzein glucuronide, the native un-labelled daidzein (MRM 253>223) is observed to be unchanged in concentration while the labelled glucuronide is hydrolysed. Similar profiles are obtained for the daidzein glucoside, the genistein and glycitein glucuronides

For this experiment it was first necessary to update our previous instrumental method to use the same labelled aglycones for the core isotope dilution mass spectrometry quantitation (IDMS) as are released from the hydrolysed conjugates. Due to ongoing improvements in synthesis, the set of available aglycone, glucoside and glucuronide standards available are all now labelled in identical positions [2,3,4- $^{13}C_3$], with the three labelled atoms adjacent to each other in the central ring. It was essential to examine the fragmentation patterns of the aglycones and to establish if the ring cleavage would produce different mass fragments for standards with different labelling patterns. In the two examples synthesised to date our previous internal standard [3,4,1'-$^{13}C_3$]- genistein and the new [2,3,4-$^{13}C_3$] fortuitously yielded the same mass fragment as the predominate ion (figure 5). It is therefore not necessary to make any instrumental alterations in order to utilise the benefit of these new standards. Care must be taken as fragmentation of other labelling such as [3,4,8-$^{13}C_{3]}$]- genistein will result in a need to monitor a different mass fragment.

Precision of the method and experimental uncertainty was due to contributions from four separates processes. Analytical precision was calculated by conducting replicate analyses of sample extracts. With either three or six replicates the instrumental precision was within ± 2%. Where separate triplicate extracts were prepared and these also analysed in triplicate the precision dropped to ± 7 %. Inter-day batch variation was in the order of ± 10 %. Tablet

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weights and contents of capsules varied markedly in weight. Industry figures suggest a 10% variation in weight/contents is considered acceptable. Five of each tablet or capsule (contents) were weighed to give an average value, which was used in the calculations. The structure of the dose vehicle is also important. Capsules generally contained homogenised material that can readily be weighed out without added error. Tablets contained outer coatings of e.g. iron oxide that are difficult to homogenise and even when ground these coatings have a much higher density, affecting homogeneity and making replicate weighings irreproducible. The experimental uncertainty is therefore the sum of thes individual contributions to error and is considered to be 20%, and this is due largely to sampling error not analytical performance.

Plant content of supplements

The listed plant ingredients on labels correlate well with the observed individual analytes. The majority of the supplements were labeled as containing soya (*Glycine max*) and this was confirmed by analysis and the co-occurrence of daidzein, genistein and glycitein. A number of supplements list red clover extract (*Trifolium pratense*) and all these products all contained elevated levels of either formononetin or biochanin A. The two supplements which list Kudzu (Japanese arrowroot, *Pueraria lobata)* were observed to contain measurable quantities of puerarin (daidzein-8-*C*-glucoside). As this analyte is contains a carbon-carbon bond between the aglycone and sugar moieties it is resistant to the hydrolysis protocol and was measured as the intact glucoside. One further product was labeled as containing "plants", as this contained puerarin, biochanin A, daidzein, genistein and glycitein it probably contains a mixture of e.g. soya, red clover and kudzu. A further product was labeled as containing hops. Hop flowers contain the potent phytoestrogen 8-prenylnaringenin (8-PNG) and other related chemicals. As no prenyl naringenin analytes were observed in any products, it would appear that this and all other products do not contain appreciable proportions of hop flowers or hop extract amongst the ingredients. The public, retailers and health care professionals should therefore consider using standardised isoflavone supplements, which are supported by analytical measurements and the content of active components adjusted accordingly.

Conclusions

The isoflavone content of 35 dietary supplements intended for treatment of menopausal symptoms have been measured. 14 out of 35 contained concentrations of aglycone isoflavones that provide a comparable intake with a high soya diet and are within the accepted therapeutic range for natural relief of menopausal symptoms by the intake of isoflavones. 11 out of the 35

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products examined did not match label claims and six delivered less than 10 mg/day of isoflavones. This study highlights the need for careful selection of isoflavone containing supplements.

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Table 1. Isoflavone parent aglycones and glucosides

Aglycone	Glycoside
Genistein	Genistin
Daidzein	Daidzin
Glycitein	Glycitin
Biochanin A	Sissotrin
Formononetin	Ononin

Table 2. Acquisition parameters for the quantitation of phytoestrogens

Compounds	Retention time (mins)	MRM transition (m/z)	Dwell time (ms)	Collision Energy (eV)	Cone Voltage (V)
Puerarin	4.7	415→295	0.05	50	30
Daidzein	14.0	253→223	0.05	60	25
$^{13}C_3$-Daidzein	14.0	256→226	0.05	60	25
Glycitein	14.4	283→268	0.05	50	30
$^{13}C_3$-Glycitein	14.4	286→271	0.05	50	20
Genistein	15.6	269→133	0.05	55	30
$^{13}C_3$-Genistein	15.6	272→135	0.05	55	30
Coumestrol	15.7	267→266	0.05	60	27
Formononetin	17.1	267→252	0.05	60	23
$^{13}C_3$-Formononetin	17.1	270→255	0.05	40	22
Biochanin A	18.9	283→268	0.05	50	20
$^{13}C_3$-Biochanin A	18.9	286→271	0.05	50	20
6-Prenylnaringenin	19.0	339→219	0.05	50	20
8-Prenylnaringenin	20.8	339→219	0.05	50	20
6,8-Diprenylnaringenin	23.7	407→119	0.05	50	20

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Table 3. Measured content of phytoestrogens in dietary supplements: daily doses and % of nominal content.

Product type		Weight g/unit	Individual analytes mg/unit* Gen	Daid	Gly	Form	Bio	Cou	Pur	Total isoflavones mg/unit	Units/ Day#	Actual dose mg/day	Label claim mg/day†	% of Label claim
Soya	Tab	1.06	0.2	0.7	0.2	0.0	0.1	0.00	0.00	1	1	1	2.2	45
	Caps	0.34	1.3	6.7	2.1	0.0	0.0	0.00	0.00	10	2	20	35	57
	Caps	0.45	0.3	0.3	0.0	0.0	0.0	0.00	0.00	1	2	2	n/a	-
	Caps	0.54	13.6	10.8	2.4	0.0	0.0	0.01	0.00	27	1	27	40	68
	Tab	1.17	10.3	11.0	1.6	0.0	0.0	0.00	0.00	23	1	23	40	58
	Tab	0.65	1.9	11.7	4.3	0.0	0.0	0.00	0.00	18	1	18	38	47
	Caps	0.31	8.1	4.1	0.2	0.0	0.0	0.00	0.00	12	1	12	60	20
	Tab	1.13	15.6	10.7	1.5	0.0	0.0	0.00	0.00	28	1	28	50	56
	Caps	0.55	0.8	2.3	2.3	0.0	0.0	0.00	0.00	5	3	16	37.5	43
	Tab	1.09	12.6	12.9	2.0	0.0	0.0	0.00	0.00	28	1	28	50	56
	Caps	1.01	0.3	0.9	0.3	0.0	0.0	0.00	0.00	1	1	1	20	5
	Tab	1.10	14.0	12.0	2.5	0.0	0.0	0.00	0.00	29	1	29	55	53
	Caps	0.77	1.7	7.9	3.0	0.0	0.0	0.00	0.00	13	2	25	46	54
	Tab	0.88	14.6	21.1	4.7	0.0	0.0	0.00	0.00	40	1	40	60	67
	Tab	0.69	15.5	8.5	0.5	0.0	0.0	0.00	0.00	25	1	25	37.5	67
	Tab	0.68	16.7	8.8	0.5	0.0	0.0	0.00	0.00	26	1	26	37.5	69
	Tab	0.70	19.3	17.4	2.1	0.0	0.0	0.01	0.00	39	1	39	60	65
	Caps	0.46	5.3	5.1	0.8	0.0	0.0	0.00	0.00	11	2	23	40	58
	Caps	0.58	11.2	10.6	1.5	0.0	0.0	0.00	0.00	23	1	23	40	58
Redclover	Caps	0.47	0.6	0.2	0.4	18.9	16.6	0.00	0.00	37	1	37	40	93
	Tab	0.80	1.6	4.7	5.2	30.2	7.9	0.02	0.00	50	1	50	40	125
	Caps	0.56	1.9	2.9	2.6	24.6	5.5	0.01	0.00	37	1	37	44	84
Novogen - Redclover exp 2008	Tab	0.48	1.0	0.5	0.6	15.8	23.6	0.00	0.00	41	1	41	40	103
Novogen- RedClover exp 2005	Tab	0.54	0.8	0.5	0.5	15.2	23.7	0.00	0.00	41	1	41	40	103
Novogen - Promensil	Tab	0.53	1.0	0.5	0.6	17.1	24.9	0.00	0.00	44	1	44	40	110
	Caps	0.71	1.4	2.6	2.4	36.5	10.5	0.01	0.00	53	1	53	40	133
	Tab	0.54	1.1	0.4	0.4	16.9	25.6	0.00	0.00	44	1	44	40	110
	Tab	0.77	0.3	1.4	1.9	17.7	4.8	0.00	0.00	26	2	52	40	130
	Caps	0.47	0.6	0.1	0.2	24.0	18.6	0.00	0.00	43	1	43	40	108
Soya and Redclover	Caps	0.35	3.3	23.1	7.7	1.2	0.3	0.00	0.00	36	1	36	62.5	58
	Caps	0.72	7.5	11.7	0.6	0.7	0.5	0.00	0.00	21	1	21	20	105
	Caps	0.72	8.1	12.7	0.6	0.7	0.5	0.00	0.00	22	1	22	20	110
	Caps	0.40	0.9	2.4	0.6	1.2	0.3	0.00	0.00	6	2-3	11	20	55
Kudzu	Tab	0.55	2.7	6.4	5.8	0.9	0.5	0.04	0.24	16	1	16	35	46
	Tab	1.05	3.9	8.8	8.9	0.2	0.1	0.05	0.14	22	1	22	55	40
	Tab	1.62	1.7	2.8	2.5	31.1	8.3	0.01	0.02	46	1	46	40	115
Hops	Caps	0.31	1.3	6.1	2.0	0.0	0.0	0.00	0.00	9	1-2	19	17.2	110

*Gen = genistein, Daid = daidzein, Gly = glycitein, Form = formononetin, Bio = biochanin A, Cou = coumestrol, Pur = puerarin.
#Where a dose range is given e.g. 2-3 per day the lower value has been used. † The dose is assumed to be measured as the aglycones.

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Table 4. Differences in quantitation of biochanin A and formononetin in Promensil

Analyte	Internal std	µg/ml Diluted	µg/ml Concentrrated	
Biochanin A	$^{13}C_3$-Genistein	10.6	54	
	$^{13}C_3$-Daidzein	7.2	32	
	$^{13}C_3$-Biochanin A	12.8	131	Correct value
	$^{13}C_3$-Formononetin	10.3	90	
Formononetin	$^{13}C_3$-Genistein	8.9	48	
	$^{13}C_3$-Daidzein	6.1	31	
	$^{13}C_3$-Biochanin A	8.7	78	
	$^{13}C_3$-Formononetin	10.8	103	Correct value

Table 5. Calculation of aglycone equivalents in soya based products

Compound		Molecular weight	Aglycone equivalents	Composition ratio	Aglycone equivalents
Daidzein	Aglycone	254	1.00	0.01	0.01
	Daidzin	416	0.61	0.52	0.32
	6''-O-Acetyldaidzin	458	0.55	0.09	0.05
	6''-O-Malonyldaidzin	502	0.51	0.38	0.19
				1.00	0.57
Genistein	Aglycone	270	1.00	0.01	0.01
	Genistin	432	0.63	0.52	0.33
	6''-O-Acetylgenistin	479	0.56	0.09	0.05
	6''-O-Malonylgenistin	518	0.52	0.38	0.20
				1.00	0.58
Glycitein	Aglycone	284	1.00	0.01	0.01
	Glycitin	446	0.64	0.52	0.33
	6''-O-Acetylglycitin	488	0.58	0.09	0.05
	6''-O-Malonylglycitin	532	0.53	0.38	0.20
				1.00	0.60
		Daidzein/Genistein/Glycitein ratio of 4:1:3			0.58
		Daidzein/Genistein/Glycitein ratio of 7:10:1			0.58

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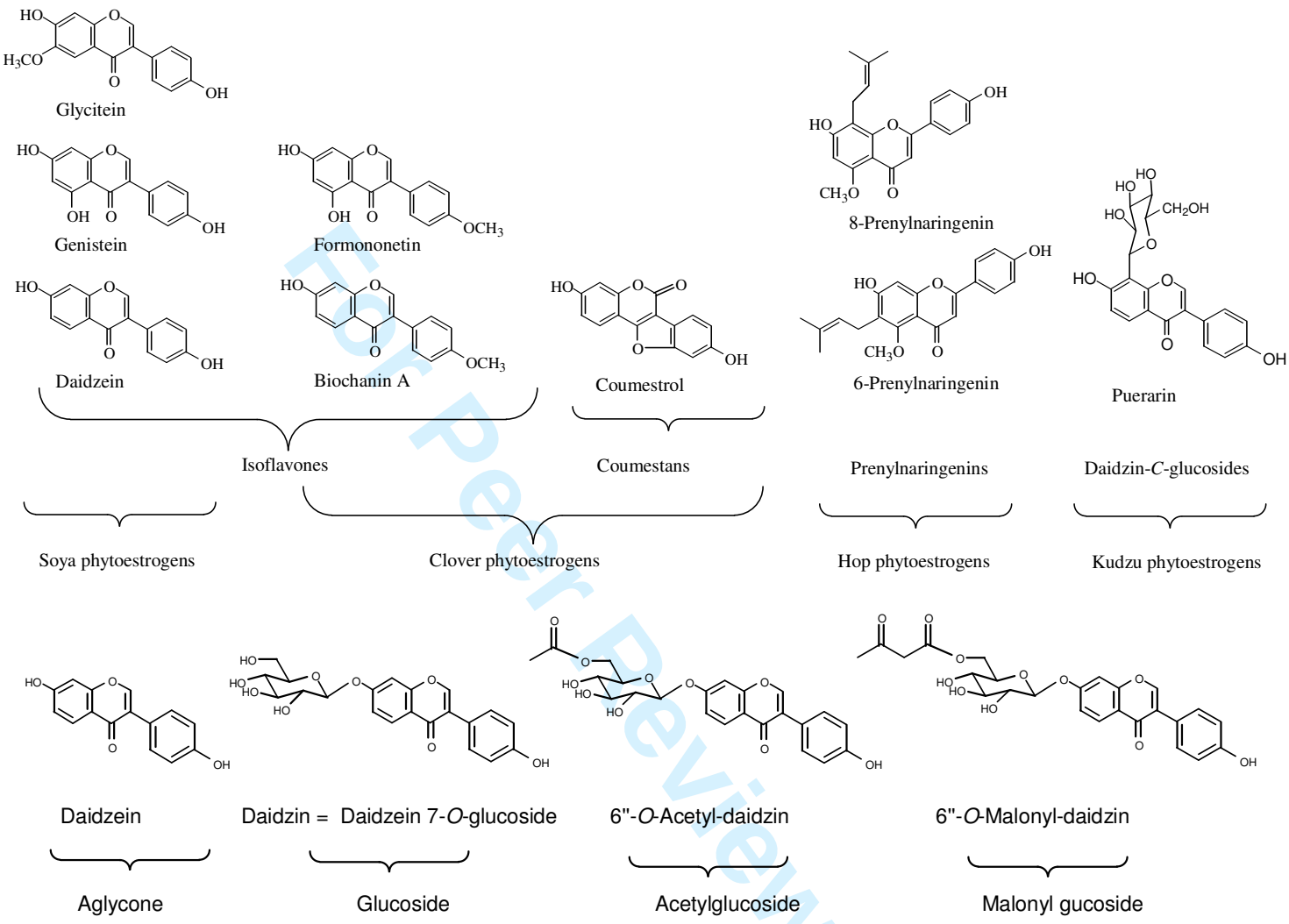

Figure 1. Common dietary sources and chemical structures of phytoestrogens contained in dietary supplements

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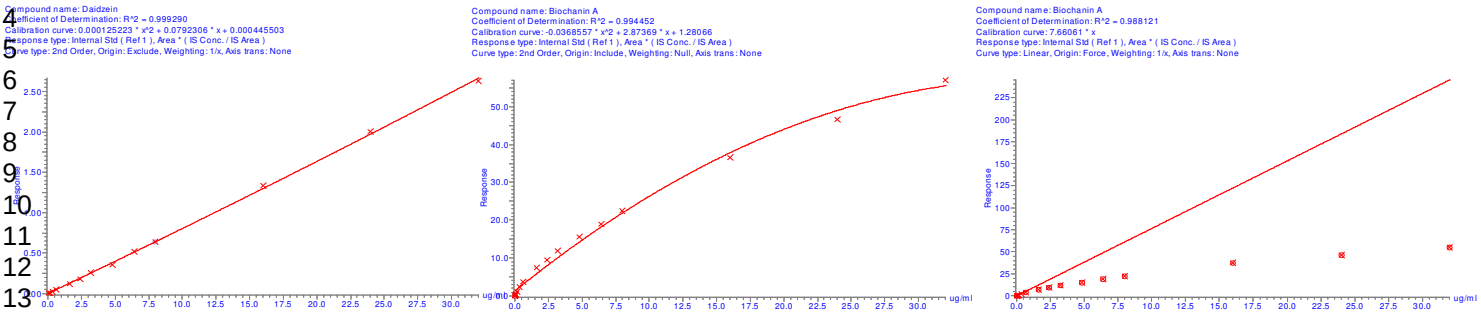

(A) (B) (C)

Figure 2. Calibration lines for A) daidzein and B) biochanin A each normalised to $^{13}C_3$-daidzein and C) the expected linear response for biochanin A

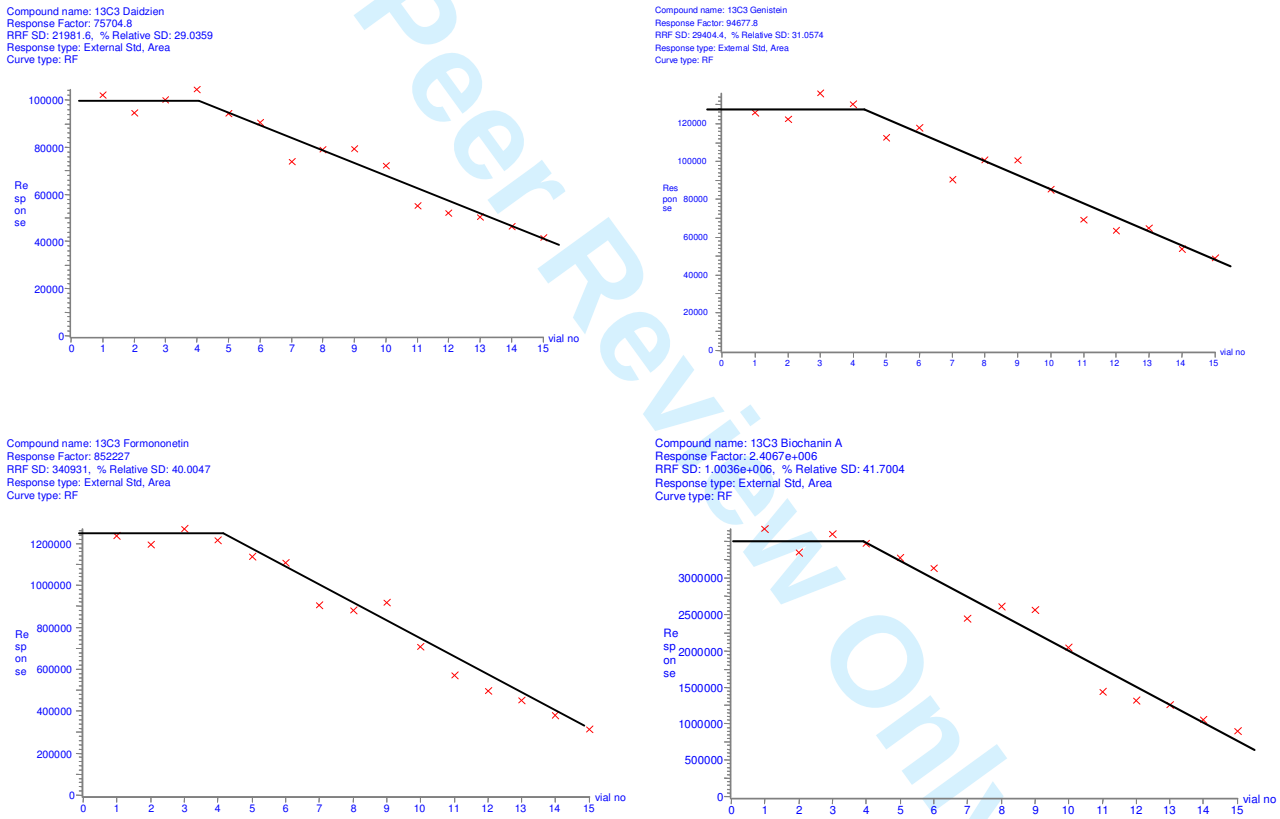

Figure 3. Raw area counts for internal standards A) $^{13}C_3$-daidzein, B) $^{13}C_3$-genistein, C) $^{13}C_3$-formononetin and D) $^{13}C_3$-biochanin A. Each analyte has a different rate of self suppression. An identical amount of internal standard gives a decreased response as the native analyte increases in concentration from vial 1 (0 ug/ml, to 0.03, 0.06, 0.16, 0.3, 0.6, 1.6, 3.2,6.4, 12.8, 16, 19 ug/ml, to vial 15 (32 ug/ml). Displayed on a concentration independent x-axis for clarity.

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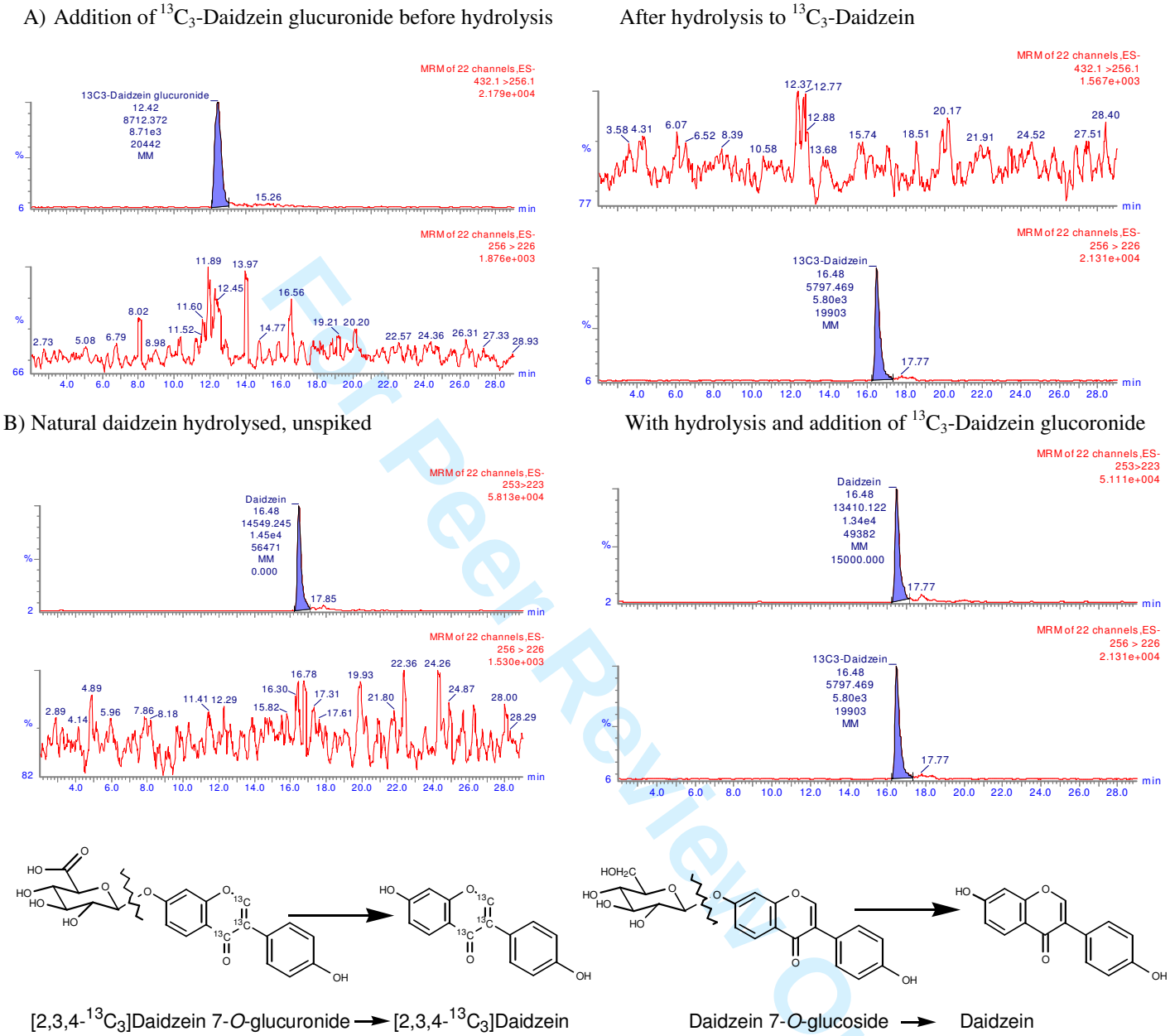

Figure 4. LC-MS/MS chromatograms of extracts of red clover tablets as a proof of quantitative hydrolysis of glycoside conjugates within the sample matrix. Analysis of samples containing native analyte by concurrent acid hydrolysis of $^{13}C_3$ labelled glucuronide to the $^{13}C_3$-aglycone.

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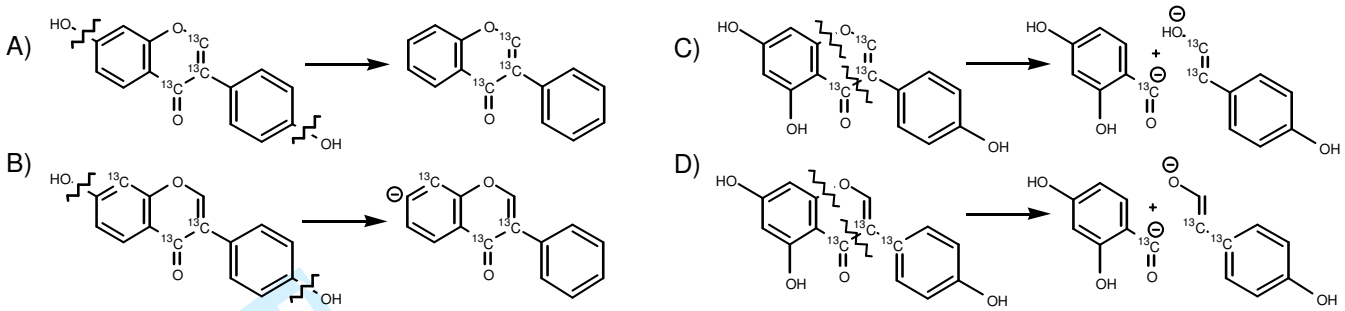

Figure 5. LC-MS/MS fragmentation of labelled standards. Identification; A) [2,3,4-$^{13}C_3$]Daidzein, B) [3,4,8-$^{13}C_3$]Daidzein. For labelled daidzein, as the MRM transitions are a simple double-dehydroxylation the position of labelling is unimportant. For labelled genistein the MRM fragment is derived from a ring cleavage and the positioning of label is important. In the two examples synthesised to date C) [2,3,4-$^{13}C_3$] and D) [3,4,1'-$^{13}C_3$]genistein, fortuitously chemically dissimilar fragments of the identical mass fragment are obtained. Fragmentation of other patterns such as [3,4,8-$^{13}C_3$] genistein will result in a need to monitor a different mass fragment.

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Table 1. Isoflavone parent aglycones and glucosides

Aglycone	Glycoside
Genistein	Genistin
Daidzein	Daidzin
Glycitein	Glycitin
Biochanin A	Sissotrin
Formononetin	Ononin

Table 2. Acquisition parameters for the quantitation of phytoestrogens

Compounds	Retention time (mins)	MRM transition (m/z)	Dwell time (ms)	Collision Energy (eV)	Cone Voltage (V)
Puerarin	4.7	415→295	0.05	50	30
Daidzein	14.0	253→223	0.05	60	25
$^{13}C_3$-Daidzein	14.0	256→226	0.05	60	25
Glycitein	14.4	283→268	0.05	50	30
$^{13}C_3$-Glycitein	14.4	286→271	0.05	50	20
Genistein	15.6	269→133	0.05	55	30
$^{13}C_3$-Genistein	15.6	272→135	0.05	55	30
Coumestrol	15.7	267→266	0.05	60	27
Formononetin	17.1	267→252	0.05	60	23
$^{13}C_3$-Formononetin	17.1	270→255	0.05	40	22
Biochanin A	18.9	283→268	0.05	50	20
$^{13}C_3$-Biochanin A	18.9	286→271	0.05	50	20
6-Prenylnaringenin	19.0	339→219	0.05	50	20
8-Prenylnaringenin	20.8	339→219	0.05	50	20
6,8-Diprenylnaringenin	23.7	407→119	0.05	50	20

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Table 3. Measured content of phytoestrogens in dietary supplements: daily doses and % of nominal content.

Product type		Weight g/unit	Individual analytes mg/unit* Gen	Daid	Gly	Form	Bio	Cou	Pur	Total isoflavones mg/unit	Units/ Day#	Actual dose mg/day	Label claim mg/day†	% of Label claim
Soya	Tab	1.06	0.2	0.7	0.2	0.0	0.1	0.00	0.00	1	1	1	2.2	45
	Caps	0.34	1.3	6.7	2.1	0.0	0.0	0.00	0.00	10	2	20	35	57
	Caps	0.45	0.3	0.3	0.0	0.0	0.0	0.00	0.00	1	2	2	n/a	-
	Caps	0.54	13.6	10.8	2.4	0.0	0.0	0.01	0.00	27	1	27	40	68
	Tab	1.17	10.3	11.0	1.6	0.0	0.0	0.00	0.00	23	1	23	40	58
	Tab	0.65	1.9	11.7	4.3	0.0	0.0	0.00	0.00	18	1	18	38	47
	Caps	0.31	8.1	4.1	0.2	0.0	0.0	0.00	0.00	12	1	12	60	20
	Tab	1.13	15.6	10.7	1.5	0.0	0.0	0.00	0.00	28	1	28	50	56
	Caps	0.55	0.8	2.3	2.3	0.0	0.0	0.00	0.00	5	3	16	37.5	43
	Tab	1.09	12.6	12.9	2.0	0.0	0.0	0.00	0.00	28	1	28	50	56
	Caps	1.01	0.3	0.9	0.3	0.0	0.0	0.00	0.00	1	1	1	20	5
	Tab	1.10	14.0	12.0	2.5	0.0	0.0	0.00	0.00	29	1	29	55	53
	Caps	0.77	1.7	7.9	3.0	0.0	0.0	0.00	0.00	13	2	25	46	54
	Tab	0.88	14.6	21.1	4.7	0.0	0.0	0.00	0.00	40	1	40	60	67
	Tab	0.69	15.5	8.5	0.5	0.0	0.0	0.00	0.00	25	1	25	37.5	67
	Tab	0.68	16.7	8.8	0.5	0.0	0.0	0.00	0.00	26	1	26	37.5	69
	Tab	0.70	19.3	17.4	2.1	0.0	0.0	0.01	0.00	39	1	39	60	65
	Caps	0.46	5.3	5.1	0.8	0.0	0.0	0.00	0.00	11	2	23	40	58
	Caps	0.58	11.2	10.6	1.5	0.0	0.0	0.00	0.00	23	1	23	40	58
Redclover	Caps	0.47	0.6	0.2	0.4	18.9	16.6	0.00	0.00	37	1	37	40	93
	Tab	0.80	1.6	4.7	5.2	30.2	7.9	0.02	0.00	50	1	50	40	125
	Caps	0.56	1.9	2.9	2.6	24.6	5.5	0.01	0.00	37	1	37	44	84
Novogen - Redclover exp 2008	Tab	0.48	1.0	0.5	0.6	15.8	23.6	0.00	0.00	41	1	41	40	103
Novogen- RedClover exp 2005	Tab	0.54	0.8	0.5	0.5	15.2	23.7	0.00	0.00	41	1	41	40	103
Novogen - Promensil	Tab	0.53	1.0	0.5	0.6	17.1	24.9	0.00	0.00	44	1	44	40	110
	Caps	0.71	1.4	2.6	2.4	36.5	10.5	0.01	0.00	53	1	53	40	133
	Tab	0.54	1.1	0.4	0.4	16.9	25.6	0.00	0.00	44	1	44	40	110
	Tab	0.77	0.3	1.4	1.9	17.7	4.8	0.00	0.00	26	2	52	40	130
	Caps	0.47	0.6	0.1	0.2	24.0	18.6	0.00	0.00	43	1	43	40	108
Soya and Redclover	Caps	0.35	3.3	23.1	7.7	1.2	0.3	0.00	0.00	36	1	36	62.5	58
	Caps	0.72	7.5	11.7	0.6	0.7	0.5	0.00	0.00	21	1	21	20	105
	Caps	0.72	8.1	12.7	0.6	0.7	0.5	0.00	0.00	22	1	22	20	110
	Caps	0.40	0.9	2.4	0.6	1.2	0.3	0.00	0.00	6	2-3	11	20	55
Kudzu	Tab	0.55	2.7	6.4	5.8	0.9	0.5	0.04	0.24	16	1	16	35	46
	Tab	1.05	3.9	8.8	8.9	0.2	0.1	0.05	0.14	22	1	22	55	40
	Tab	1.62	1.7	2.8	2.5	31.1	8.3	0.01	0.02	46	1	46	40	115
Hops	Caps	0.31	1.3	6.1	2.0	0.0	0.0	0.00	0.00	9	1-2	19	17.2	110

*Gen = genistein, Daid = daidzein, Gly = glycitein, Form = formononetin, Bio = biochanin A, Cou = coumestrol, Pur = puerarin.
#Where a dose range is given e.g. 2-3 per day the lower value has been used. † The dose is assumed to be measured as the aglycones.

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Table 4. Differences in quantitation of biochanin A and formononetin in Promensil

Analyte	Internal std	µg/ml Diluted	µg/ml Concentrrated	
Biochanin A	$^{13}C_3$-Genistein	10.6	54	
	$^{13}C_3$-Daidzein	7.2	32	
	$^{13}C_3$-Biochanin A	12.8	131	Correct value
	$^{13}C_3$-Formononetin	10.3	90	
Formononetin	$^{13}C_3$-Genistein	8.9	48	
	$^{13}C_3$-Daidzein	6.1	31	
	$^{13}C_3$-Biochanin A	8.7	78	
	$^{13}C_3$-Formononetin	10.8	103	Correct value

Table 5. Calculation of aglycone equivalents in soya based products

Compound		Molecular weight	Aglycone equivalents	Composition ratio	Aglycone equivalents
Daidzein	Aglycone	254	1.00	0.01	0.01
	Daidzin	416	0.61	0.52	0.32
	6''-O-Acetyldaidzin	458	0.55	0.09	0.05
	6''-O-Malonyldaidzin	502	0.51	0.38	0.19
				1.00	0.57
Genistein	Aglycone	270	1.00	0.01	0.01
	Genistin	432	0.63	0.52	0.33
	6''-O-Acetylgenistin	479	0.56	0.09	0.05
	6''-O-Malonylgenistin	518	0.52	0.38	0.20
				1.00	0.58
Glycitein	Aglycone	284	1.00	0.01	0.01
	Glycitin	446	0.64	0.52	0.33
	6''-O-Acetylglycitin	488	0.58	0.09	0.05
	6''-O-Malonylglycitin	532	0.53	0.38	0.20
				1.00	0.60
		Daidzein/Genistein/Glycitein ratio of 4:1:3			0.58
		Daidzein/Genistein/Glycitein ratio of 7:10:1			0.58

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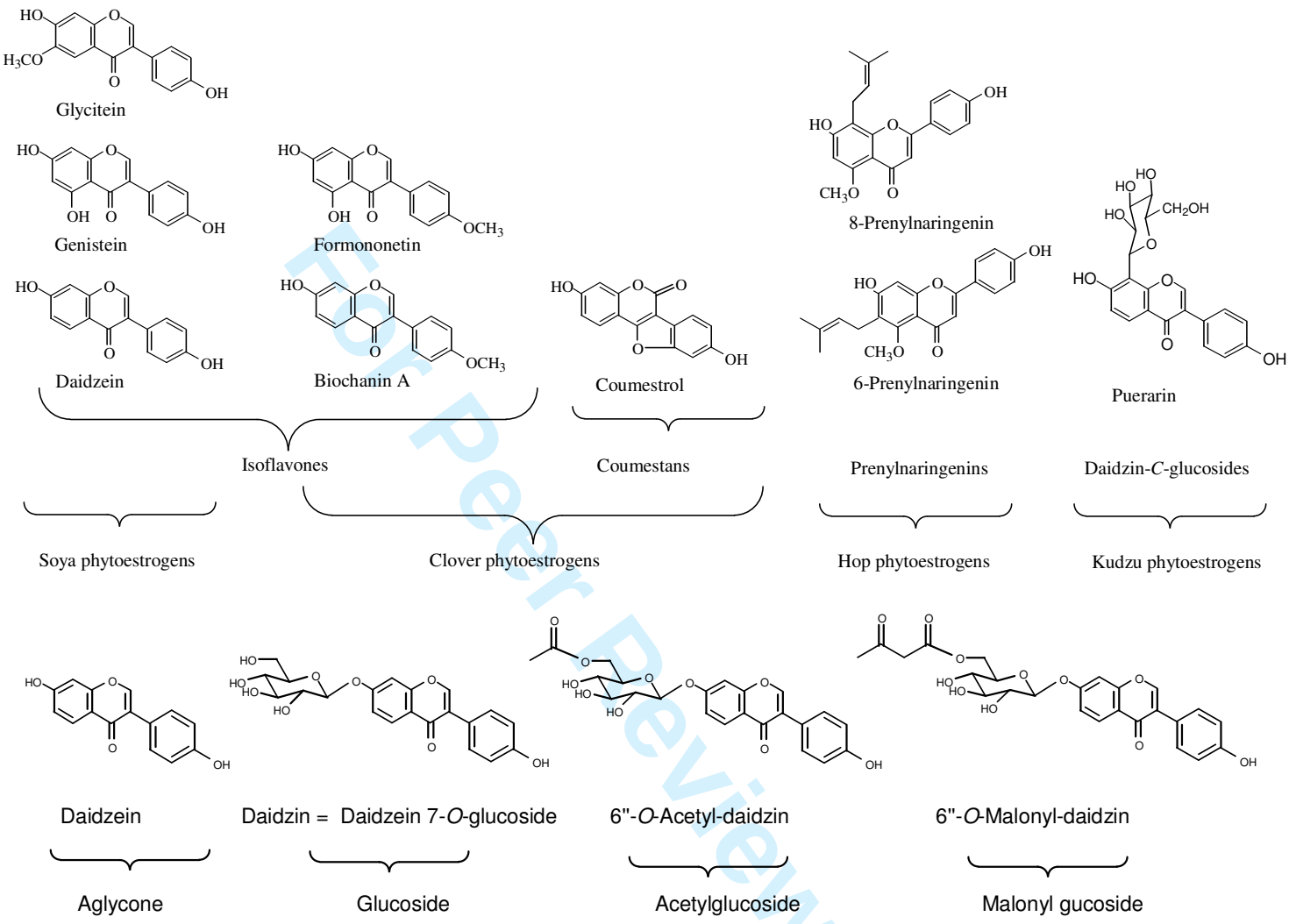

Figure 1. Common dietary sources and chemical structures of phytoestrogens contained in dietary supplements

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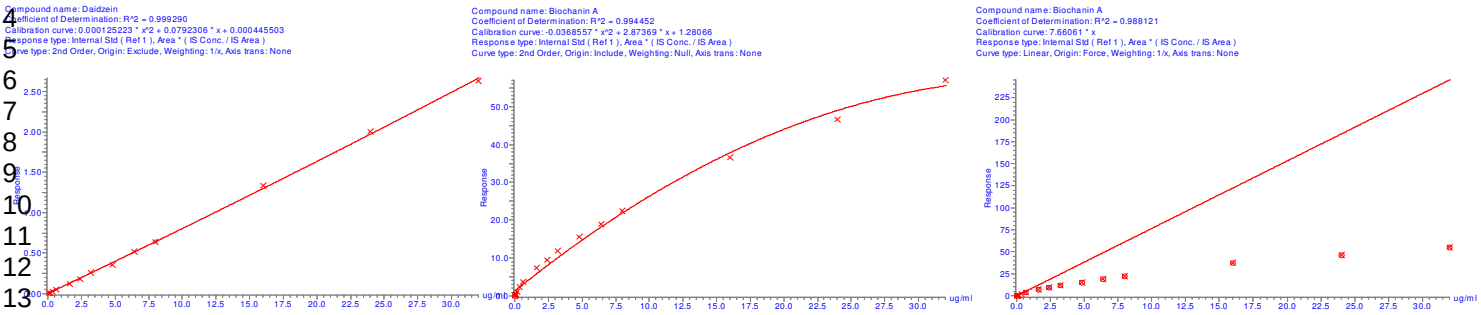

(A) (B) (C)

Figure 2. Calibration lines for A) daidzein and B) biochanin A each normalised to $^{13}C_3$-daidzein and C) the expected linear response for biochanin A

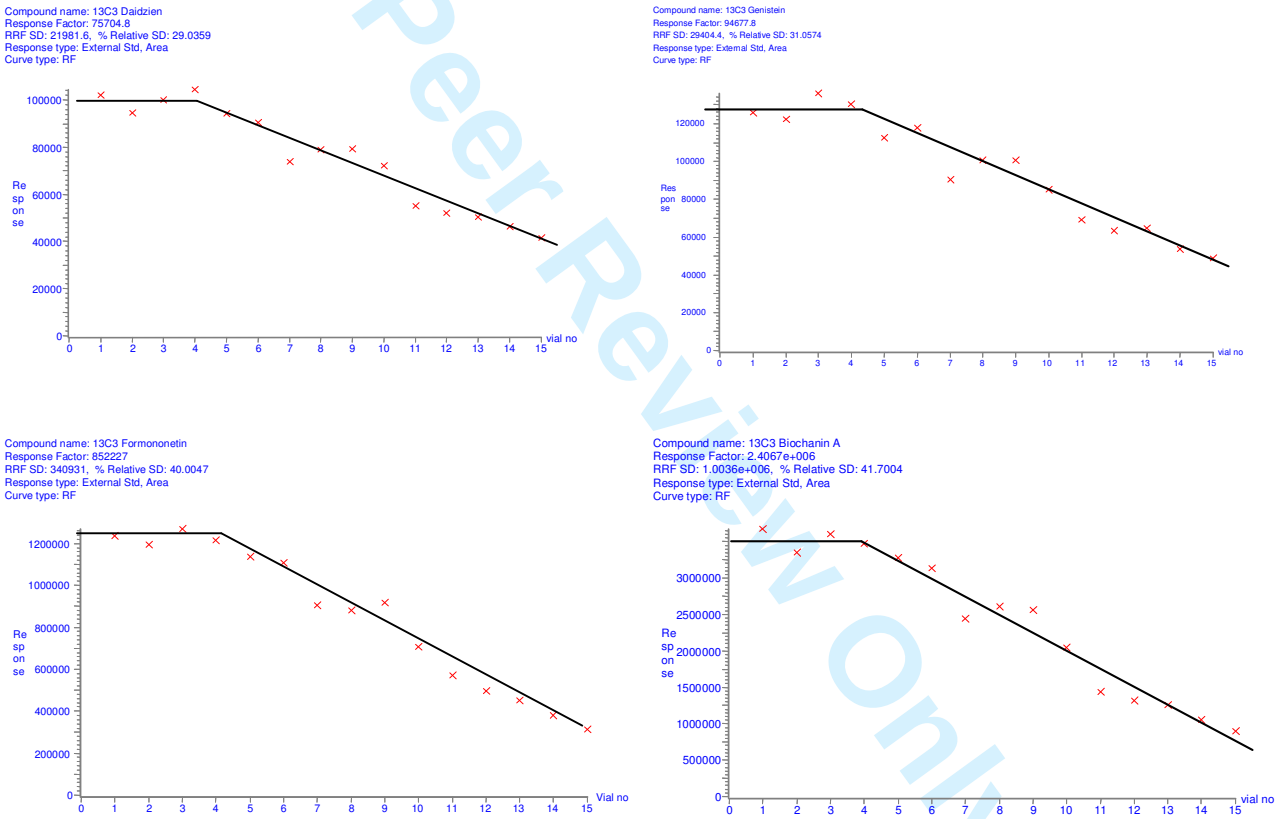

Figure 3. Raw area counts for internal standards A) $^{13}C_3$-daidzein, B) $^{13}C_3$-genistein, C) $^{13}C_3$-formononetin and D) $^{13}C_3$-biochanin A. Each analyte has a different rate of self suppression. An identical amount of internal standard gives a decreased response as the native analyte increases in concentration from vial 1 (0 ug/ml, to 0.03, 0.06, 0.16, 0.3, 0.6, 1.6, 3.2,6.4, 12.8, 16, 19 ug/ml, to vial 15 (32 ug/ml). Displayed on a concentration independent x-axis for clarity.

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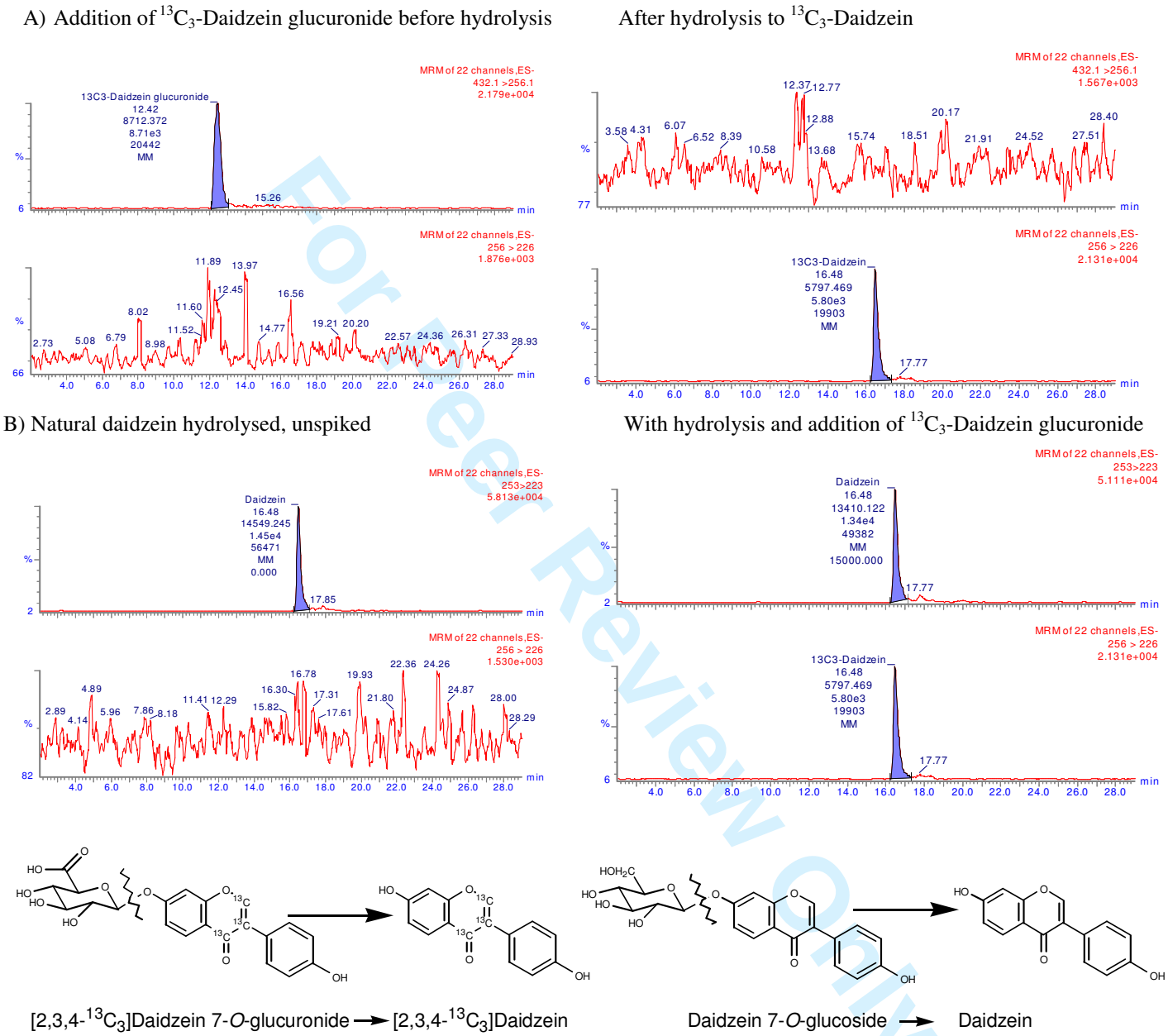

Figure 4. LC-MS/MS chromatograms of extracts of red clover tablets as a proof of quantitative hydrolysis of glycoside conjugates within the sample matrix. Analysis of samples containing native analyte by concurrent acid hydrolysis of $^{13}C_3$ labelled glucuronide to the $^{13}C_3$-aglycone.

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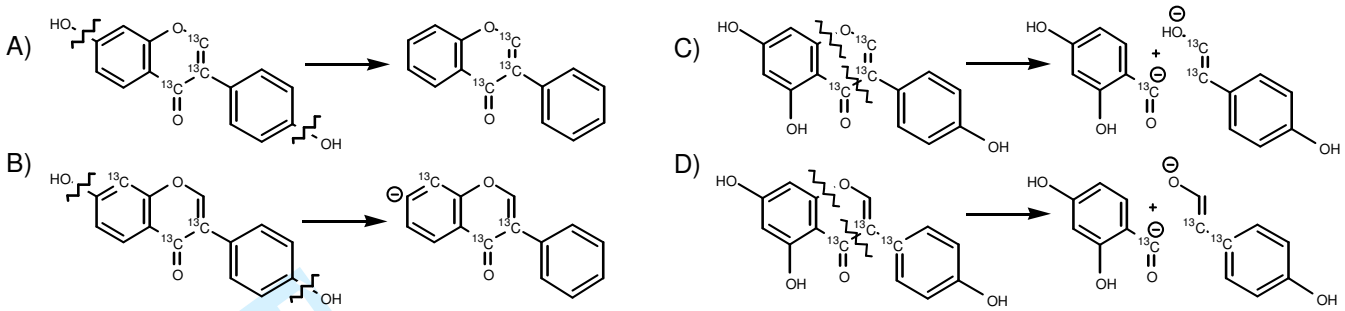

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