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Using calibration approaches to compensate for remaining matrix effects in quantitative liquid chromatography electrospray ionization multistage mass spectrometric analysis of phytoestrogens in aqueous environmental samples

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Abstract

Signal suppression is a common problem in quantitative LC-ESI-MSn analysis in environment samples, especially in highly loaded wastewater samples with highly complex matrix. Optimization of sample preparation and improvement of chromatographic separation are prerequisite to improve reproducibility and selectivity. Matrix components may be reduced if not eliminated by a series of sample preparation steps. However, extensive sample preparation can be time-consuming and risk the significant loss of some trace analytes. The best way to further compensate matrix effects is the use of internal standard for each analyte. However, in a multi-component analysis, finding appropriate internal standards for every analyte is often difficult. In this present study, a more practical alternative option was sought. Matrix effects were assessed using a post-extraction addition method. By comparison of three different calibration approaches, it was found that matrix-matched calibration combined with one internal standard provides a satisfactory method for compensating for any residual matrix effects on all the analytes. Validating experiments on different STP influent samples analysing for a range of phytoestrogens showed that this calibration method provided satisfactory results with concentration ratio 96.1% – 105.7% compared to those by standard addition. It is an easy practical calibration approach to compensate matrix effects in the multi-component LC-ESI-MSn quantitative analysis.

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Using calibration approaches to compensate for matrix effects in quantitative LC-ESI-MS^n analysis of phytoestrogens in aqueous environmental samples

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ABSTRACT

Signal suppression is a common problem in quantitative LC-ESI-MS^n analysis in environment samples, especially in highly loaded wastewater samples with highly complex matrix. Optimization of sample preparation and improvement of chromatographic separation are prerequisite to improve reproducibility and selectivity. Matrix components may be reduced if not eliminated by a series of sample preparation steps. However, extensive sample preparation can be time-consuming and risk the significant loss of some trace analytes. The best way to further compensate matrix effects is the use of internal standard for each analyte. However, in a multi-component analysis, finding appropriate internal standards for every analyte is often difficult. In this present study, a more practical alternative option was sought. Matrix effects were assessed using a post-extraction addition method. By comparison of three different calibration approaches, it was found that matrix-matched calibration combined with one internal standard provides a satisfactory method for compensating for any residual matrix effects on all the analytes. Validating experiments on different STP influent samples analysing for a range of phytoestrogens showed that this calibration method provided satisfactory results with concentration ratio 96.1% – 105.7% compared to those by standard addition. It is an easy practical calibration approach to compensate matrix effects in the multi-component LC-ESI-MS^n quantitative analysis.

INTRODUCTION

The occurrence and fate of estrogenic compounds in the environment has drawn much attention over the past few decades for their potential effects on wildlife and human beings.[1-3] Many analytical methods for identification and quantitation of such compounds in environmental samples have been developed. Among them, high-performance liquid chromatography coupled to an electrospray ion source and a tandem or ion trap mass spectrometer has become a powerful technique for the currently analytical method.[4] Electrospray ionization is a "soft" ionization technique that generally forms protonated or deprontonated molecular ions. ESI-MS has become the technique of choice for the determination of phytoestrogenic compounds due to its high selectivity, sensitivity, and compatibility with HPLC solvent systems[5-7]. However, in LC-ESI-MS^n quantitative analysis matrix effects can present a significant drawback.[8] Different authors have studied this phenomenon in environmental applications.[9-11] Matrix effects, typically result in analyte signal suppression or, less frequently its enhancement. LC-ESI-MS/MS analysis of pharmaceuticals in wastewater samples has reported significant loss of signal on some individual analyte (up to 54% in STP effluent, and up to 60% in STP influent) as compared to a standard solution.[12] A number of options have been used for countering matrix effects in the quantitative analysis, such as to reduce the co-eluting compounds by selective extraction,[13-15]

effective sample clean-up after the extraction,[16] improvement of the chromatographic separation,[17, 18] reduction of flow rate[19] and dilution of the sample extract. [20, 21] In addition, compensating for matrix effects by use of internal standards,[22-24] matrix-matched calibration standards (often referred to as external matrix-matched calibration),[23, 25, 26] standard addition,[27] or other methods such as the ECHO technique[28, 29] have also been used. However, tackling matrix effects is a complex task, and some methods are effective but not very practical. Removing all matrix components is the ideal approach to remove matrix effects, but extensive sample preparation steps may be time-consuming and risk of loss of some trace analytes, especially in the multi-component analysis of environmental samples, such as highly loaded wastewater samples. In many cases it is not possible or practical, particularly if one has a well developed method that needs to be used on a large number of samples which may vary considerably in their background matrix components. To these samples, after optimized sample preparation, the best way to further compensate matrix effects is the use of appropriate internal standard for each analyte.[11, 23, 27] However, appropriate internal standards for every analyte in a multi-component analysis are often not available.

The aims of the present experiments were to evaluate different calibration methods by assessing the extent of matrix effects in the quantitative analysis of phytoestrogens in aqueous environmental samples and find a practical and effective calibration approach to compensate for remaining matrix effects on the analysis, after extensive optimisation of the method and sample preparation.

EXPERIMENTAL

Materials

Daidzein (DAID, 98% purity), genistein (GEN, 98% purity), formononetin (FORM, 99% purity), biochanin A (BIA, 97% purity), glycitein (GLY, 97% purity) enterodiol (END, 95% purity), enterolactone (ENL, 95% purity), and coumestrol (COUM, 97.5% purity) were purchased from Sigma–Aldrich (Sydney, Australia). Deuterated genistein (3',5',6,8-d_4) (98% purity, 95% isotopic enrichment) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Acetonitrile and methanol, both HPLC grade, were supplied by Crown Scientific (Sydney, Australia). Milli-Q water (Milli-Q plus 185, Australia) was used for making up all aqueous solutions. Standard stock solutions of each compound were prepared at a concentration of 100 µg/mL in either acetonitrile or acetonitrile : methanol (80:20 v/v). Concentrated working solutions were prepared in acetonitrile:water (1:3, v/v) and obtained by tenfold dilution of the stock to a concentration of 10 µg/mL. Working solutions were then prepared in acetonitrile/water (1:3, v/v) by successive tenfold dilutions to concentrations of 100 to 1 ng/mL. All these solutions were stored in the dark at -20°C. The cartridges employed for solid phase extraction (SPE) were 6 mL, 200 mg Oasis HLB cartridges, purchased from Waters (Sydney, Australia).

Apparatus

An optimised method for the sample extraction and LC- MS analysis was developed as outlined below. All analyses were carried out on a ThermoElectron Finnigan LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization source. A ThermoElectron Surveyor HPLC system was interfaced to the mass spectrometer for automated LC-MS analysis.

Sample preparation

Samples were collected from different sources: surface water from Mullet Creek (Kanahooka, NSW, Australia), effluent and influent from Wollongong sewage treatment plant (STP) (Wollongong, NSW, Australia) and Gerringong

- Gerroa STP (Gerringong, NSW, Australia). Amber glass bottles rinsed with Milli-Q water were used for this purpose. Before solid phase extraction (SPE), samples were adjusted to pH~5 with dilute H_2SO_4 and vacuum filtered through GF/D and GF/F filters (Whatman, UK). Each aliquot for SPE was 100 mL for STP influent, and 600 mL for STP effluent and creek water. The SPE cartridges, which were placed on a vacuum manifold, were preconditioned with 5 mL methanol and 10 mL Milli-Q water. Subsequently, the samples were drawn through SPE cartridges at a flow rate < 10 mL/min. After that, the cartridges were washed with 15 mL of Milli-Q water and 1mL of a methanol:Milli-Q water (10:90, v/v) solution. The cartridges were then vacuumed dry for a few mins. The retained components were then slowly eluted with 8 mL methanol. The extracts were evaporated to dryness under a gentle nitrogen stream at 40°C and reconstituted with 250 µL mixed solvent of water:acetonitrile (3:1, v/v).

HPLC/MS^n system

The ThermoElectron Surveyor HPLC system, including an auto-sampler and micro-syringe pump, was controlled using Xcalibur 1.3 software (ThermoElectron). An XTerra MS C18 column (2.1×150 mm, 3.5 µm; Waters) and guard column (2.1×20 mm, 3.5 µm; Waters) were maintained at 26°C inside a column oven. A triple solvent mobile phase system was used: 2% formic acid in water as solvent A, Milli-Q water as solvent B, and acetonitrile as solvent C. The gradient program started at 20% of solvent C, 75% of solvent B, and 5% of solvent A (this last component was kept in this proportion during the entire run). The sample was injected at time t=0 min. After 5 min, solvent C was gradually increased to 45% by 30 min. Then solvent C was sharply increased to 90% and kept at constant for 8 min to wash all retained organics from the column. Solvent C was then decreased back to 20% and kept running at this composition for 8 min to re-equilibrate the column. The total running time was 48 min. The flow rate of the mobile phase was controlled at 200 µL/min and sample injection volume was 5 µL. The flow from the HPLC system was fed directly into an LTQ linear ion trap mass spectrometer, via an ESI source. All mass spectra were acquired in negative ion mode with a spray voltage of 3.61 kV, capillary voltage at -11.83V, and a capillary temperature of 274.8C°. Nitrogen was used as both the sheath and auxiliary gas at 29 and 3 arbitrary units respectively. Helium was used as damping and collision gas at a partial pressure of 0.1 Pa. The analytes were monitored by selected MS^2 or MS^3 transitions: DAID, *m/z* 252.9→224.8; GLY, *m/z* 282.9→267.8→239.8; END, *m/z* 301.0→253.0; GEN-d_4, *m/z* 272.9→184.9; GEN, *m/z* 268.9→180.9; COUM, *m/z* 266.8→238.8→210.8; ENL, *m/z* 296.9→252.9→194.9; FORM, *m/z* 266.9→251.9→222.9; BIA, *m/z* 282.9→267.8→238.9. The normalized collision energies were from 36% to 49% for each analyte respectively.

Recovery and Calibration experiments

Recovery experiments were carried out by adding 100 µL of a standard working solution (which contained 1 µg/mL of DAID, GEN, GLY, END, ENL, and 0.30 µg/mL of FORM, BIA, COUM) into the sample before SPE. The same amount of analyte standard was also spiked into the replicate extracts after SPE. For a better comparison, a fixed amount of internal standard (100ng/mL of GEN-d_4) was spiked into all sample extracts before injection. The recoveries were calculated by comparing the peak area ratios for each analyte relative to the internal standard in samples that had been spiked before SPE (pre-spiked), with analogous ratios for samples in which the same levels of the analytes were added post-extraction (post-spiked). The peak area ratios obtained from the un-spiked samples were subtracted from the pre-spiked and the post-spiked samples.[30, 31]

The post-extraction addition experiments for assessment of any remaining matrix effects were arranged as follow: the replicate sample extracts from each sample were spiked with different concentration levels of analyte standards (5,

10, 25, 50, 100 ng/mL for surface water extracts and STP effluent extracts; and 10, 25, 50, 100, 500 ng/mL for STP influent extracts) and a fixed amount of the internal standard (100 ng/mL GEN-d_4). Same levels of the analytes and internal standard were also spiked into solvent solutions. The original existence of the analytes in the analysed samples was simultaneously quantified by the standard addition. In order to minimize the possible instrument variations, the spiked real sample extracts, the blanks, and the standard solutions were run alternately.

Matrix-matched standard calibrations curves, consisted of six concentration levels (0, 5, 10, 25, 50, 100 ng/mL for surface water and STP effluent extracts; 0, 5, 25, 50, 100, 500 ng/mL for STP influent extracts), were set up by spiking these different amounts of analyte with the internal standard (100 ng/mL GEN-d_4) into sample extracts. Blanks (samples with zero addition of the analytes) were simultaneously quantified using the standard addition, and the levels of analyte present in the sample were subtracted. To evaluate the performance of the calibration curves, 8 STP influent samples (collected independently from Gerringong Gerroa STP and Wollongong STP) were analysed using a standard addition method with four levels of analyte standards (0, 5, 50, 100, 500ng/mL) and a fixed amount of the internal standard spiked into the replicate sample extracts. Spiked sample extracts and blanks were run consecutively in the instrument.

RESULTS AND DISCUSSION

Method optimisation: sample preparation and chromatography

Optimizing sample preparation is essential to reduce matrix component and thus to improve reproducibility and accuracy of the analysis. In this study, an extraction of phytoestrogens from the water samples was developed and optimised using Oasis HBL SPE cartridges. [30, 32-34] Alternatives such as liquid-liquid extraction (LLE) may sometimes provide clean final extracts the recovery, particularly for polar analytes, may be very low.[35] Sample preparation is an important aspect of any analytical method development and optimising the SPE was a critical step in the sample preparation. To avoid exceeding the retention capacity of SPE cartridge and thus reducing the recoveries of the analytes, the sample loading volume for each single cartridge was trialled by experiment. For those highly concentrated samples such as WWTP influent 100mL as aliquot volume proved optimal for SPE, whilst the lower concentration samples, 600mL of creek water and 1000mL of final STP effluent were found to be suitable. Although Oasis HBL cartridge can be used at wide range of pH, some studies has shown that for some compounds the extraction efficiency was affected by the pH.[21, 36] There was research showed that co-extraction of humic and fulvic acids from water was influenced by the pH of sample extract applied for SPE, they declined rapidly at neutral pH versus extraction at acidic pH.[37] The pH of the samples is important as this would affect the state of the analyzed compounds and the interaction between the analyte and SPE cartridge packing material. Therefore prior to extraction the acidity of samples was adjusted to an appropriate pH range. For our analytes, phytoestrogens, which possess phenolic or diphenolic groups, the acidic level at pH~5 was selected. Keeping this acidity could be helpful in reducing inferences from some alkaline organic compounds and also for the repeatability of the SPE. To reduce the inorganic and some organic interference on the SPE cartridge, the cartridge sorbent was washed with an appropriate solvent before elution. The selection of washing solvent in SPE balanced removal of unwanted compounds and retaining as much of the trace analytes as possible on the SPE cartridge. In this experiment, 15mL of Milli-Q water was used to wash off salt and other very polar chemicals, followed by 1mL of a methanol:Milli-Q water (10:90, v/v) solution to remove comparatively more polar organics. The recovery control experiments showed that this small amount of methanol did not wash off significant amounts of the analytes from the cartridge. The recovery experiments showed good accuracy and repeatability (see Table 1.)

Although complete separation by HPLC seems not necessary when using selective MS^n detection, it still plays an important role in improving both detectability and reliability. This is because high resolution in chromatographic separation reduces the level of compounds coeluting with the analyte of interest and therefore results in reduced ion suppression. HPLC separation of phytoestrogenic compounds has usually been carried out on a reversed-phase column with a mobile phase of methanol or acetonitrile and water.[5, 6] In this study, both methanol and acetonitrile were tested for their separation performance; it was found that the retention time of the analytes changed slightly, but better peak shape was obtained using acetonitrile with the XTerra MS C18 column. The phytoestrogenic compounds analysed in our work all contain phenolic hydroxyl groups, which exhibit a weak acidic nature. Therefore it would be expected that the use of an acidic modifier would allow the analytes to become dissociated in the solvent system, thus enhancing the chromatographic separation and improvement of peak shapes.[5, 6] To confirm this, three type of mobile phases of acetonitrile-water systems (added 0.1% formic acid, added 0.1% ammonia, and no modifier added) were tested for their chromatographic performance. It was found that better peak shapes were obtained by the addition of 0.1% formic acid. The chromatographic separation was carried out maintaining the column temperature at 26ºC so that the stable retention times were obtained. The gradient program for the mobile phase was optimized by experiment. In the gradient program enough time was given to re-equilibrate the column prior to the next injection. Figure 3 shows the SRM chromatogram obtained from surface water extracts spiked with 100 ng/mL of each analyte standard.

Assessment of remaining matrix effects

Any remaining matrix effects were assessed using a post-extraction addition method. The concept of quantitative assessment of matrix effect used in this paper was adapted from Matuszewski et al.[38]; where matrix effect (ME %) was calculated by comparing the peak area of known amount of a standard solution (A) with that from a sample extract spiked with the same amount of analyte after extraction (B). The ratio (B/A × 100) is defined as absolute matrix effect (ME %). In the current experiments, because the analytes were already present in the samples, it was not possible to obtain a real control matrix sample without any of the analytes. Therefore, the presence and identity of the analytes in the samples was verified and the amount quantified by standard addition methods. The response factor (RF= Peak area / concentration) was used to represent the response of an analyte in a certain matrix,[27] thus the matrix effect (ME %) was calculated by comparing the RFs in the real samples with the RFs in the standard solution: ME % = $[RF(analyte)_{sample} / RF(analyte)_{solvent}] \times 100$.

Three different types of sample (surface water, STP effluent, and STP influent) were used to evaluate the matrix effects on phytoestrogenic compounds in quantitative LC-ESI-MS^n analysis. These were the same matrices used in the method development. These real sample extracts were spiked with a series of concentrations of the analyte standards (5, 10, 25, 50, 100 ng/mL for surface water extracts and STP effluent extracts; and 10, 25, 50, 100, 500 ng/mL for STP influent extracts). The presence and true concentration of the analytes in the samples was simultaneously quantified by standard addition. Samples at each concentration level were run 5 times and in order to minimize the possible instrument variations, the spiked real sample extracts and the standard solutions were run alternately. The ratio of RF obtained in the spiked real sample extract to the RF obtained in the standard solution reflected the degree of matrix effects occurring with the analyte in this type of matrix sample and is represented by the value ME %. Figure 1 shows the signal suppression range of the internal standard (GEN-d_4) in the three types of spiked real sample extract compared with that in the standard solution made with water:acetonitrile (3:1, v/v) as solvent. The data shows the the extent of signal suppression by the three samples matrices. For the control solvent sample, the mean ME% is 100% by

definition. The grey box in this instance represents the reproducibility of the measurement for 25 replicate injections (5 runs of 5 concentration levels).

As all the real sample extracts contained none of the internal standard (GEN-d_4) before it was spiked, the deduction in RFs thus may be attributed to the matrix effect only. The results in Figure 1 confirmed that the sample matrix for this analyte caused signal suppression in the three environmental samples, and that the extent of signal suppression was dependent on the sample background. The more concentrated and complex samples (such as STP influent) suffered greatest signal suppression. The matrix effects for the eight analytes are clearly depicted by the plots in Figure 2 and the same trend in signal suppression is observed and the signal suppression was found to be not equal for all the analytes, with the more polar analytes (which eluted earlier) suffering the most signal suppression. This was especially so at higher analyte concentrations and more complex matrix background samples, such as STP influent. These results are in agreement with the previous literatures.[12, 13 18, 39].

Comparison of three calibration approaches

The best method to further compensate for any remaining matrix effects after method development due to sample background variation is the use of internal standard for each analyte.[8, 13, 40] However, in a multi-component analysis, finding appropriate internal standards for every analyte may be rather difficult or impossible. Potential alternatives, such as standard addition methods do not require any internal standards, but is very time consuming in nature and the laborious manipulation involved makes this method often impractical for routine analysis particularly with large batches of samples. By contrast, external matrix-matched calibration may only effectively compensate for matrix effects from moderately loaded samples with uniform matrix.[27, 29] In this present study, a better, more practical alternative option was sought. In order to achieve this, a combination of internal standard with matrix-matched calibration was applied.

In order to evaluate this calibration method, the matrix-matched calibration curves were setup by spiking a series of standards using the three different types of real sample extracts (5, 10, 25, 50, 100 ng/mL). Because the large concentrations of some analytes in influent sample extracts, an additional 500 ng/mL standard was used in the calibration. Figure 4 shows calibration curves of **a**) GEN, **b**) DAID, they were obtained by using a single internal standard (GEN-d_4) in different matrices. The calibration slopes and the correlation coefficient for all analytes are listed in Table 2.

In Figure 4-**a**) calibration curves for GEN obtained from the real sample extracts nearly coincide with the calibration curve from the standard solution, indicating that the internal standard GEN-d_4 can successfully compensate for remaining matrix effects from the three different matrix backgrounds. But in Figure 4-**b**) the calibration slopes from the real sample extracts were lower than that from the standard solution; that means only a single internal standard can not compensate any remaining matrix effects for all analytes and that matrix-matched calibrations are required for quantitation in different matrices. As Figure 4-b and Table 2 show, the internal standard matrix-matched calibration curves were determined on the specific sample matrix, therefore, any variation in the signal suppressions across the different analytes in the sample matrix, such as the severe signal suppression of the three early eluting analytes, has already been taken into account in their calibration curves. With one internal standard to correct the variations of different batch of samples, this calibration method could be an easily practical approach to further compensate the signal suppression from the real samples.

Performance of the selected calibration approach

To evaluate the efficiency of matrix-matched standard calibration with one internal standard in compensating matrix effects in real samples, 8 independent STP influent samples were analysed and the results were compared to those obtained by standard addition. STP influent samples were selected for this assessment because they suffered the most signal suppression. In this assessment, it was assumed that standard addition provided the most accurate value of the analyte concentration and the most effective way to eliminate any matrix effect.[27] The concentrations of the analytes in blank sample extracts were quantitated by three different calibration methods: (i) solvent standard calibration with one internal standard, (ii) external matrix-matched standard calibration and (iii) matrix-matched standard calibration with one internal standard. The concentrations obtained by each calibration curve were compared to the concentrations obtained by standard addition. The ratios [Concentration(analyte)$_{\text{by calibration}}$ / Concentration(analyte)$_{\text{by standard addition}}$] were calculated; they represented the performance of the three standard calibration methods. Figure 5 shows the comparison of the three standard calibration methods in the analysis of 8 different STP influent samples.

It is seen from Figure 5 that the concentrations quantitated by matrix-matched standard calibration with one internal standard were consistently similar to those obtained by standard addition, with the concentration ratios close to 100%. The means of the concentration ratios of all analytes in the 8 different samples were 96.1 – 105.7 % and the coefficient of variation (CV %) for the analytes of the 8 independent samples were 3.0 – 7.6%. These results demonstrate that matrix-matched standard calibration with one internal standard has effectively compensated for any remaining matrix effects in the selected samples.

Use of external matrix-matched standard calibration without the internal standard also achieved better compensation for matrix effects for all analytes compared to solvent standard calibration with a single internal standard, especially for the early eluted analytes DAID, GLY, and END, which had shown severe signal suppression in our earlier experiment (see Figure 3). But unfortunately, this method does not give satisfactory precision with coefficients of variation of 7.6 – 21.3 %. This result is not surprising given the lack of the internal standard which plays a very important role in compensating the variation from samples, instrument and manipulations.

As for the results by use of solvent standard calibration with one internal standard, the attempts to compensate matrix effects were not satisfactory. The amounts of early eluting analytes DAID, GEN, END, were severely underestimated with the means of the concentration ratios in the 8 samples being 70.2%, 68.7% and 76.0%. Although this calibration method provided fair precision (see error bars in Figure 5), it is clearly deficient for the analysis of multiple analytes, especially in complex matrix samples such as STP influent.

In summary, by comparing the three standard calibration methods for compensating matrix effects, matrix-matched standard calibration with one internal standard has been shown to provide the best results. Although the validating samples in this study are limited, it strongly suggests that this calibration method is an easily practical and effective approach to compensate matrix effects.

CONCLUSIONS

Previous experience has demonstrated that matrix effects are awkward problems that must be tackled to ensure reliable quantitation in LC-ESI-MS. Optimization of sample preparation and improvement of chromatographic separation are essential to improved reproducibility and selectivity of quantitation. Whilst it is desirable to eliminate completely matrix effects this is often not feasible particularly in dealing with environmental samples with trace levels of analytes and wide variation in background matrix. In these circumstances, however, considerable degree of signal

suppression in the real sample extracts, such as wastewater extracts, may be experienced and needs to be compensated by effective calibration approaches. Use of l internal standards is known to be the best way to compensate matrix effects, but in the multi-component analysis one internal standard has been shown to be deficient in compensating the signal suppression on all analytes . That means that each analyte would require an internal standard of its own, but this is not always possible. We have clearly demonstrated in a systematic study that matrix-matched calibration with one internal standard is thus a practical alternative option to compensate matrix effects in multi-component analysis of environmental samples.

Matrix-matched calibration with a single internal standard can retain the main advantages of both standard addition and internal standard methods; it saves laborious work in standard addition and renders unnecessary the requirement to find appropriate internal standards for every analyte in a multi-residue analysis.

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Table 1. Average recoveries of the SPE

Analytes	Surface water		STP effluent		STP influent	
	Average Recovery (%)	R.S.D (%)	Average Recovery (%)	R.S.D (%)	Average Recovery (%)	R.S.D (%)
Daidzein (DAID)	92	5.9	97	7.5	87	4.1
Genistein (GEN)	89	6.1	97	3.0	93	5.2
Formononetin (FORM)	93	6.5	89	6.0	91	6.5
Biochanin-A (BIA)	95	6.0	98	4.8	87	5.2
Glycitein (GLY)	98	6.6	97	5.1	96	7.3
Enterodiol (END)	94	5.1	96	4.5	85	4.8
Enterolactone (ENL)	91	3.9	96	4.3	87	4.1
Coumestrol (COUM)	96	6.6	91	6.5	89	8.1

a. Average recoveries were determined by measuring five replicate aliquots, each aliquot was tested four times.
b. R.S.D. (%) of average recoveries calculated by measure results of the five independent replicate aliquots

Table 2. Slope and the square of the correlation coefficient of internal standard calibrations for the analyzed phytoestrogens in the solvent and in matrix-matched sample extracts.

	Analyte standards							
	DAID	GLY	END	GEN	COUM	ENL	FORM	BIA
In the solvent								
Slope	1.4812	43.803	62.272	5.4695	3.5129	3.1828	31.668	33.893
R^2	0.9996	0.9905	0.9991	0.9979	0.9826	0.9998	0.9987	0.9963
In surface water extract								
Slope	1.2440	38.510	52.867	5.3523	3.6807	3.5745	30.973	35.460
R^2	0.9997	0.9992	0.9947	0.9991	0.9979	0.9986	0.9993	0.9984
In STP effluent extract								
Slope	1.1586	35.671	53.358	5.1512	3.5297	3.2825	29.638	33.177
R^2	0.9990	0.9991	0.9979	0.9993	0.9959	0.9991	0.9904	0.9967
In STP influent extract								
Slope	1.0271	31.336	47.074	5.3487	3.3837	3.4157	32.424	35.774
R^2	0.9999	0.9998	0.9998	0.9988	0.9952	0.9997	0.9999	0.9997

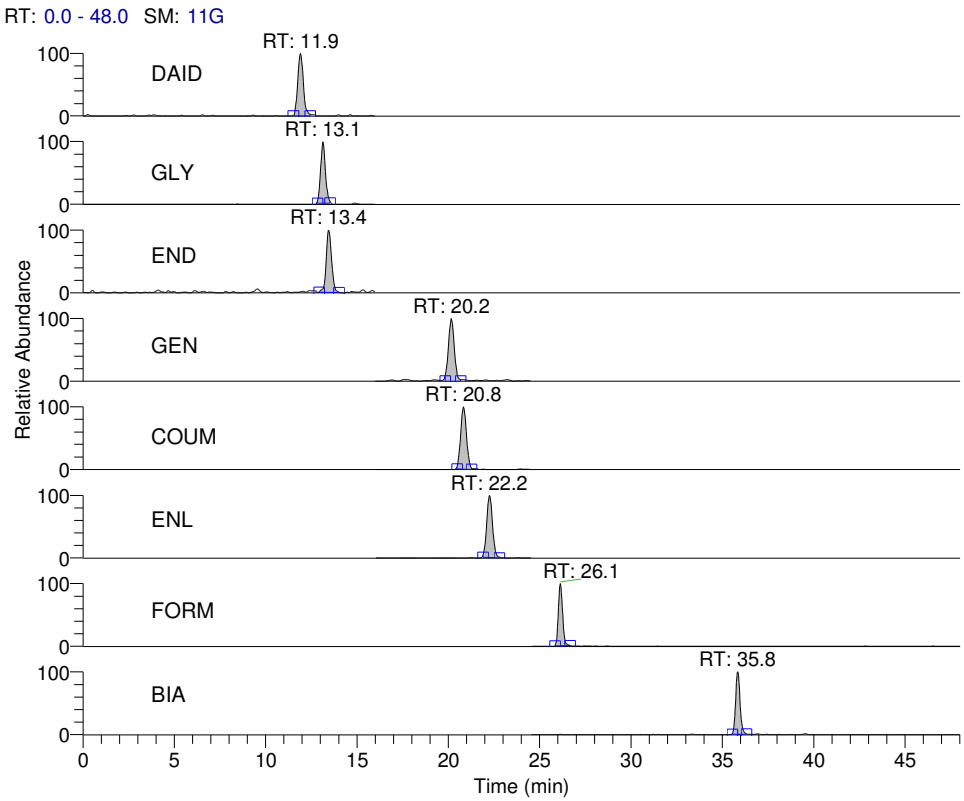

Figure 1. SRM chromatogram obtained from surface water spiked with 100ng/mL of each analyte standard.

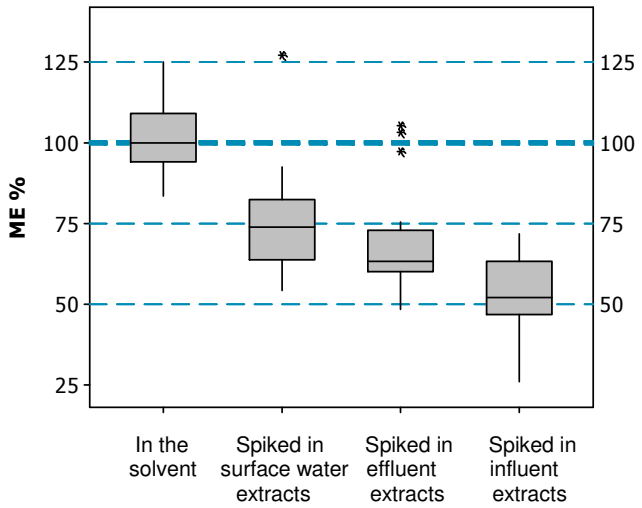

Figure 2. Signal suppression ranges of GEN-d_4 (internal standard) in three sample extracts. The RFs of GEN-d_4 spiked in the sample extracts (n=25) compared with the mean value obtained in the solvent (n=25). The *line in boxes* represents the median, the *boxes* the 25-75% percentile, the *whisker* extends to the extreme, and the *asterisks* are statistical outliers (distance to box exceeds 1.5 times the box).

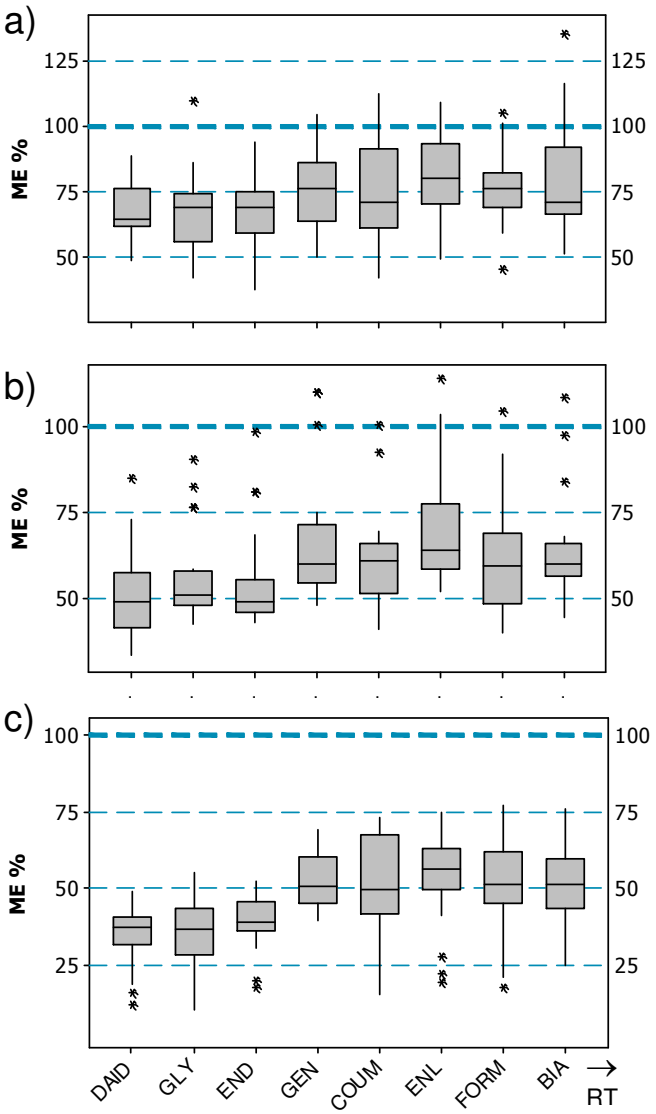

Figure 3. Signal suppression ranges of each analyte in **a)** surface water extracts **b)** STP effluent extracts **c)** STP influent extracts. The RFs of analytes spiked in each of the sample extracts (n=25) compared with the mean values obtained in the solvent (n=25). Refer to Fig.1 for explanation of the boxes in the plot.

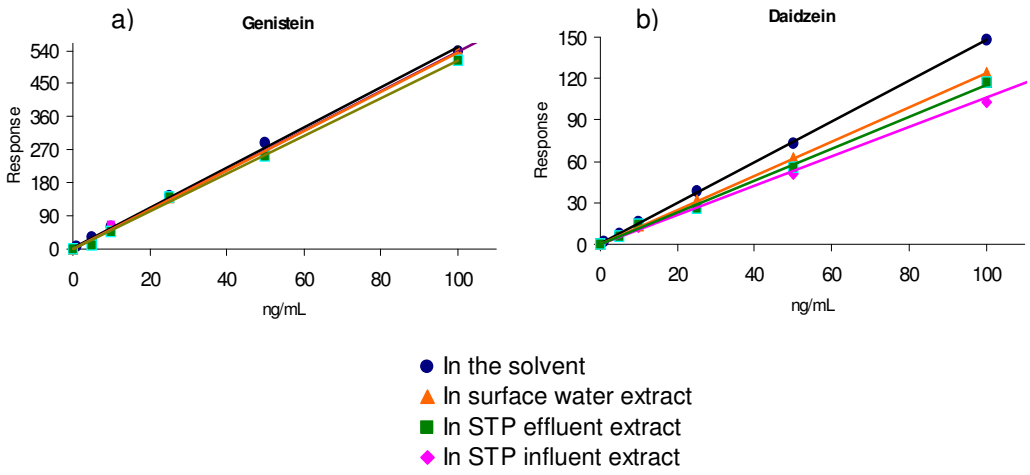

Figure 4. Internal standard calibration curves in the solvent and in matrix-matched sample extracts for: a) GEN (GEN-d_4 as internal standard), b) DAID (GEN-d_4 as internal standard).

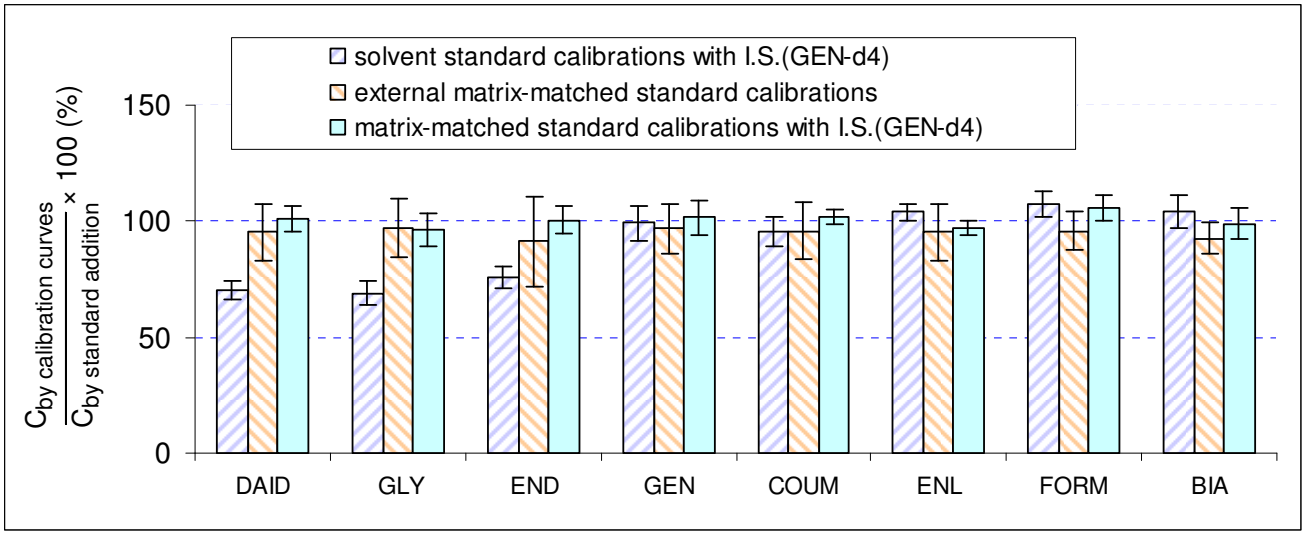

Figure 5. Ratios of the concentrations by the three standard calibration methods to those by standard addition in the analysis of STP influent samples (mean±SD, n=8).