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Simultaneous determination of residual stilbenes and stilbene metabolites in animal tissue by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatographic (LC)–tandem mass spectrometry (MS/MS) method was developed for simultaneous determination of stilbenes, diethylstilbestrol (DES), hexestrol (HEX), and dienoestrol (DEN) in animal tissue. Sample clean-up and analyte enrichment was performed by automated solid-phase extraction (ASPE) with a silica gel cartridge. Detection capabilities (CCβ) related to the transition products of lowest abundance for the method were 0.04–0.45 ng g^{-1} in tissue and were achieved using atmospheric pressure chemical ionization (APCI) in negative mode. The use of an internal standard in combination with the simplified sample preparation led to a sensitive and reliable analytical method. The recovery level of the method was 84–108% for DES and DEN between 0.5 and 5 ng g^{-1}, and 59–87% for HEX between 0.25 and 2.5 ng g^{-1}.
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Keywords: Liquid chromatographic–tandem mass spectrometry; Stilbenes; Residues; Animal tissue

1. Introdution

Stilbenes were used as growth promotors in animal production in the 1960s [1]. These molecules were proven to induce cancer by toxicology research in the 1980s. Since 1981, the use of stilbenes for growth promotion has been banned in the European Union (EU) [2]. Traditional methods of measuring stilbenes include the screening methods ELISA, RIA and GC [3–6]. A limitation of these traditional approaches is that the techniques do not provide confirmative results, and GC/MS [7], which has detection limits of 1–5 μg/kg, is unable to meet the detection limits specified in EU requirements.

A method for the measurement of residual stilbenes and stilbene metabolites in animal tissue by liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed to identify stilbenes in chicken, shrimp and fish, after an automated solid-phase extraction (ASPE) cleanup. The low detection limits of stilbenes in this method need the preparation of samples with good stability and veracity. The ASPE procedure improved the repeatability and reproducibility than the manual work. The combination of the ASPE sample preparation coupled with the sensitivity and specificity of the optimized LC–MS/MS analysis gave an extremely sensitive and robust method for the detection of stilbenes in a range of food matrices. This technique overcomes the limitations of traditional approaches and enables quantitative and confirmatory detection of stilbenes in a routine and sensitive manner.

2. Experimental

2.1. Reagents and materials

Diethylstilbestrol, *hexestrol*, *dienoestrol* and *diethylstilbestrol* (*DES-d8*): Sigma, USA.
β-Glucuronidase: Type H-2, Helix pomatia, Sigma, USA.
Methanol: LC grade, Merck, Germany.
Acetonitrile: LC grade, Merck, Germany.
Methyl tert-butyl ether (*MTBE*): LC grade, Dikma, Germany.
n-Hexane, *ethyl acetate*, *dichloromethane*, *sodium acetic acid* (*CH3COONa·3H2O*), *acetic acid*, and s*odium hydroxide*: AR grade, China.

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Sodium hydroxide solution: 3 mol/L. Prepared by dissolving 100 g of sodium hydroxide in 1000 mL of water.
Acetate buffer solution: 0.2 mol/L, pH 5.2. Prepared by dissolving 2.52 g of acetic acid and 12.95 g of sodium acetic acid in 800 mL of water. The pH was adjusted to 5.2 ± 0.1 with sodium hydroxide solution and made to 1000 mL with water.
Ultra-pure water: Obtained from a Milli-Q plus system, Millipore, USA.
Filter: 0.45 μm, for organic phase, China.
Solid-phase extraction (*SPE*) *cartridges*: Bakerbond SPE 7086-03 silica gel column, 500 mg, J.T. Baker, USA.
Dissolving solution: *n*-Hexane–dichloromethane (60/40).
Washing solution: Ethyl acetate–*n*-hexane (6/94).
Eluant: Ethyl acetate–*n*-hexane (25/75).
Stock solutions: Single standard stock solutions of DES, DEN, HEX, and DES-d8 at 1 mg/mL were prepared in methanol and stored at 4 °C.
Working standards: 25 ng/mL of DES, 25 ng/mL of DEN, 12.5 ng/mL of HEX were prepared by mixing the DEX, DEN and HEX stock solutions and diluting with methanol. Stored at 4 °C.
Internal working standard: 5 ng/mL of DES-d8. Stored at 4 °C.
LC mobile phase: Acetonitrile (70%) and ultra-pure water (30%).

2.2. Instruments

LC–MS/MS system: Agilent 1100 HPLC system including an autosampler and a quaternary pump coupled to an Applied Biosystems API4000 triple quadrupole mass spectrometer with an atmospheric pressure chemical ion APCI source, Agilent-Applied Biosystems, USA.
Automatic solid-phase extraction: Aspec Xli, Gilson, USA.
Homogenizer: Ultra-turraxT25, Janke & Kunkel IKA-Labortechnik, Germany.
Nitrogen evaporator: Model N-EVAP 112, Organomation Associates, USA.
Vortex mixer: Model 231, Fisher Scientific, USA.
Centrifuge: MR25, Sifam Instruments Ltd, UK.
LC column: ZORBAX Eclipse XDB-C8, 5 μm, 15 cm × 4.6 mm id, reversed-phase, Agilent, USA.

2.3. Analytical procedure

2.3.1. Sample collection

The edible portions of product were blended until homogeneous, and stored at −20 °C.

2.3.2. Sample preparation, extraction and cleanup

5.0(±0.1) g of the test sample was placed into a 50 mL centrifuge tube. Then 20 mL of MTBE and 1 mL of DES-d8 internal working solution were added. This mixture was homogenized at high speed for 1 min. After centrifugation at 2000 × *g* for 5 min, the supernatant was decanted into a 50 mL screw-cap tube. The pellet was resuspended in 15 mL of acetate buffer solution and homogenized for 1 min. This homogenate was centrifuged at 2000 × *g* for 5 min, and the supernatant decanted into a 25 mL screw-cap tube. The acetate buffer supernatants were evaporated at 40 °C under a nitrogen flow. Then 80 μL of β-glucuronidase was added, mixed, and kept overnight at 52 °C. Sodium hydroxide solution was added to adjust the pH of the solution to 7, then 10 mL of MTBE was added to the solution and mixed well. The tube contents were vortex mixed and then centrifuged at 2000 × *g* for 2 min. The MTBE layer was combined with the initial MTBE extraction and evaporated to dryness at 40 °C under a nitrogen flow. The residue was reconstituted in 1 mL of dissolving solution and vortex mixed for 30 s in preparation for cleanup.

The SPE procedure consisted of the addition of 1 mL of the extract onto a Sep-Pak column that had been conditioned with 2 × 3 mL of *n*-hexane at a flow-rate of 2 mL/min. Then 3 mL of washing solution was added to the tube, mixed and loaded onto the column. This process was repeated and washing solution was passed through the column. After drying the column by passage of air, the cartridge was washed with 6 mL of the eluant, which was collected and evaporated to dryness at 40 °C under a nitrogen flow. The dry residue was dissolved in 1 mL of the HPLC mobile phase, vortex mixed for 30 s and passed through a filter (pore size 0.45 μm). The filtered solution was retained for further LC–MS/MS analysis.

2.3.3. Preparation of matrix calibration standards

Samples of animal tissue without the DES-d8 internal solution were prepared according to analytical procedure A. The standard working solution and the internal solution were mixed in a series of concentrations and evaporated to dryness under a nitrogen flow. The residues were dissolved with the matrix extract and vortex mixed for 30 s.

2.3.4. LC–MS/MS analysis

A 50 μL sample of the filtrate was loaded onto the LC column at a flow-rate of 1.0 mL/min. The MS system was operated in APCI mode with negative ion polarity (−4500 V), the temperature of the ion source was set at 325 °C and the nebulizer current was set at −5 μA. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode throughout the analysis. Nitrogen was used as the collision gas. The collision-activated dissociation gas pressure was 15 psi, the curtain gas pressure was 10 psi and GAS 1 was set to 35 psi. The retention time (RT), precursor and product ions were monitored, the declustering potential, entrance potential, collision energy and collision cell exit potential values are given in Table 1.

2.4. Validation study

For confirmation purposes, the retention time and relative ion intensities, with respect to the maximum permitted tolerances, measured in the samples and the standard were acquired. For quantification of the analytes, the peak areas of quantitative transitions were considered (the content of DES was calculated as the sum of the areas of the two isomers). A series of matrix-matched calibration standards were injected separately and used to generate a calibration curve. The curves were constructed by plotting peak area ratios of the analytes with that of the internal

Table 1
Diagnostic ions, declustering potential, entrance potential, collision energy and collision cell exit potential

Analyte	Diagnostic ions Q1 → Q3	RT (min)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell exit potential (V)
DES	267.0 → 222.1*	2.23–3.25	−90	−10	−40	−10
DES	267.0 → 237.1	2.23–3.25	−90	−10	−40	−10
DEN	265.0 → 221.1	2.32	−90	−10	−35	−7
DEN	265.0 → 235.1*	2.32	−90	−10	−32	−7
HEX	269.0 → 134.0*	2.29	−75	−10	−22	−7
HEX	269.0 → 119.0	2.29	−75	−10	−46	−7
DES-d8	275.1 → 245.0*	2.20–3.19	−73	−5	−40	−15
DES-d8	275.1 → 227.9	2.20–3.19	−74	−5	−40	−15

Quantitive diagnostic ions have the mark of "*".

standard versus the concentration of the analyte. The standard curve was used to calculate the concentrations of analytes in the unknown and validation samples.

The reproducibility and recovery were determined with spiked samples of chicken, shrimp and fish. Three independent series of samples spiked with 0.1, 0.2 or 1.0 mL of the working standard (corresponding to 0.5, 1.0, or 5.0 ng/g of DES and DEN, 0.25, 0.5 or 2.5 ng/g of HEX) and 1 mL of internal working standard were analyzed. For the repeatability study, 10 replicates were considered for each level. The interoperator reproducibility was determined from the results obtained by different operators in the same laboratory. The recovery was expressed as the mean recovery of the three independent series.

CCα was calculated as a signal-to-noise (S/N) ratio of 3:1 on 23 blank samples of chicken, shrimp and fish (10 of chicken, 6 of shrimp and 7 of fish). The signal was determined at the retention time at which the analyte was expected for the less sensitive ion transitions. Table 1 highlights the MRM transitions monitored as both the quantifier and qualifying ions for each stilbene compound. The samples were spiked with standard at the limit of detection and analyzed. The detection capability (CCβ) of the technique was calculated as the CCα plus 1.64 times the standard deviation (SD) of the 23 measurements.

3. Results and discussion

The repeatability, reproducibility and recovery at three levels for each stilbene are given in Table 2. The CCα and CCβ values are given in Table 3.

The LC–MS/MS chromatograms of the spiked samples are shown in Fig. 1(a)–(f).

Table 3
CCαand CCβ

Analytes	CCα as 3S/N (ng/g)	CCβ (ng/g)
DES	0.05	0.06
DEN	0.3	0.45
HEX	0.03	0.04

Table 4
Effect of extraction solvent on recovery

Extraction solvent	Analyte	Recovery (%)
Methanol, ether	DES	32
	HEX	34
	DEN	24
Ethyl acetate	DES	43
	HEX	55
	DEN	46
Methyl tertiary butyl ether	DES	81
	HEX	75
	DEN	72

A variety of extraction methods for stilbenes exist in the literature [7,8]. After extraction of the sample with a mixture of MeOH and acetate buffer as described by Marchand et al. [7], the MeOH must be removed before the addition of β-glucuronidase, which is time-consuming. Marchand et al. also used MeOH and ether to extract stilbenes from samples, with a recovery level of about 30%. In our work, ethyl acetate and MTBE have much better levels of recovery. A comparison of different extraction solvents is given in Table 4. We chose to use ethyl acetate and

Table 2
Repeatability (SDa), coefficients of variation (CVa), within-laboratory reproducibility (SDb), within-laboratory coefficients of variation (CVb), and recovery of substances at the concentrations tested

	DES			DEN			HEX		
	0.5 μg/kg	1.0 μg/kg	5.0 μg/kg	0.5 μg/kg	1.0 μg/kg	5.0 μg/kg	0.25 μg/kg	0.5 μg/kg	2.5 μg/kg
n	10	10	10	10	10	10	10	10	10
Recovery (%)	90.4	92.3	93.4	74.8	87.8	94.0	90.9	80.6	82.5
SDa	4.64	4.07	1.27	12.77	8.95	7.28	7.90	8.32	5.01
CVa	5.1	4.4	1.4	17.1	10.2	7.7	8.7	10.3	6.1
SDb	9.54	11.17	11.92	13.34	14.46	8.53	7.31	15.64	6.25
CVb	10.1	11.4	13.6	15.2	17.0	9.3	7.9	20.2	6.4

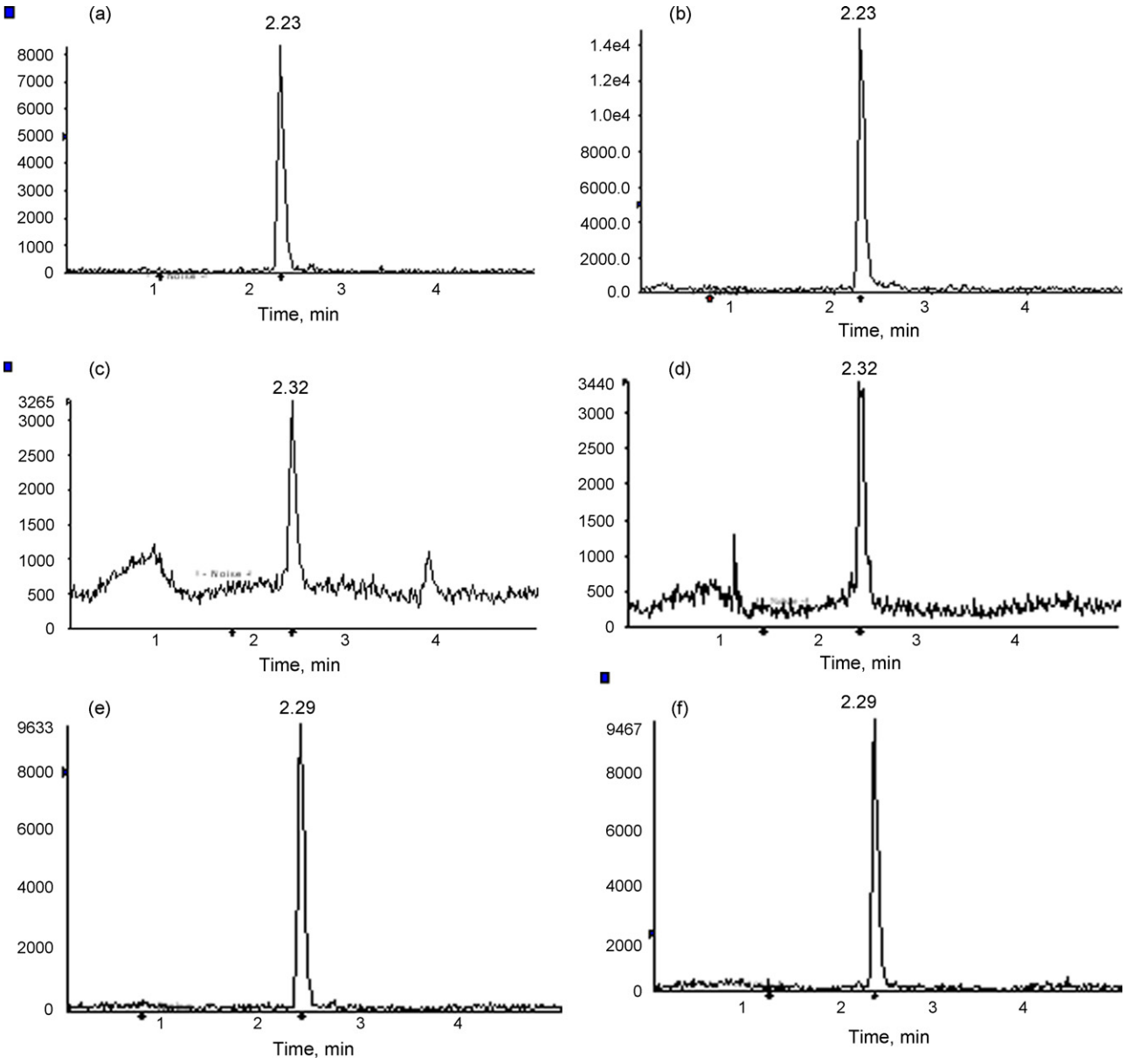

Fig. 1. LC–MS/MS chromatograms of samples spiked with 0.5 ng/g stilbens. (a) DES, diagnostic ion 267.0 → 222.1. (b) DES, diagnostic ion 267.0 → 237.1. (c) DEN, diagnostic ion 265.0 → 235.1. (d) DEN, diagnostic ion 265.0 → 221.1. (e) HEX, diagnostic ion 269.0 → 134.0. (f) HEX, diagnostic ion 269.0 → 119.0.

MTBE as extraction solvents to maximize recoveries with our method.

Stilbenes exist in animal tissues alone or in combination with proteins [1,7,8]. The stilbene–protein combination can be disrupted by β-glucuronidase and the stilbene is released. We confirmed this, as the peak area of stilbene MRM transitions was enhanced by about 30% upon addition of β-glucuronidase, and the use of this enzyme is necessary for the determination of stilbenes in samples. The two-step extraction can effectively extract the stilbenes combined with proteins, and improve the efficiency and sensitivity of the analysis.

A good clean-up procedure leads to stable ionization, a cleaner response and better repeatability in the LC–MS/MS analysis. We tried different types of silica gel columns and Oasis cartridges (Waters) in the development of the SPE procedure and finally chose to use the Bakerbond cartridge. In this study, we used ASPE, which improved the repeatability of the SPE procedure markedly compared with that of the traditional manual SPE procedure.

The SPE conditions (proportion, volume of wash solution or eluant) were optimized by using the ASPE procedure (Tables 5–7). We found that in our SPE procedure, 6 mL of ethyl acetate/*n*-hexane (6/94) as wash solution could remove the impurity effectively, and the analytes could not be washed off. The recoveries of analytes were highest when using ethyl acetate/*n*-hexane (25 + 75). A volume of 6 mL was effective and minimized waste solvent and time.

Electrospray ionization (ESI) is used in many research fields, but mostly in the analysis of drugs of low molecular weight. The MS/MS conditions were first optimized by infusion of stilbene

Table 5
Effect of the wash solution on recovery

Ethyl acetate/*n*-hexane (volume ratio)	Recovery (%)
0:100	0
2:98	0
4:96	0
6:94	0
10:90	24.6

Table 6
Effect of elution on recovery (after washing with 6 mL ethyl acetate/*n*-hexane (6/94))

Ethyl acetate/*n*-hexane (volume ratio)	Recovery (%)
20:80	27.2
25:75	85.3
30:70	63.9

Table 7
Effect of elution volume on recovery (elute three times, 3 mL every time)

Elution volume (mL)	Recovery (%)
0–3	21.2
4–6	72.0
7–9	0

standards using the ESI mode and then by injection of standards into the mobile phase using the APCI mode. The optimization was first performed in the MS mode and then in the MRM mode. We obtained several-fold better sensitivity when using the APCI mode for the analysis of stilbenes.

In each case, the dominant precursor stilbene ion was obtained from APCI, then fragmented into several product ions. We chose two of the most sensitive transitions for each analyte in each case. The use of two transitions gave the identification points of 4.0, in accordance with the EU requirement for confirmation of banned substances [9]. The more sensitive transition of each analyte was used for quantitative measurement.

The optimization of LC conditions was performed using both C18 and C8 columns, which have been used traditionally for analysis of stilbenes [10,11]. In our LC–MS/MS analysis, the C8 column achieved better separation of the stilbenes. Acetonitrile is the preferred mobile phase in APCI analysis, with a flow-rate of 1 mL/min [12]. We tried different solvent compositions and gradient programs, and finally chose to use acetonitrile/water (70/30) as the mobile phase.

The CCα values related to the less sensitive ion transitions were determined as 3:1 S/N [7]. The ability to detect stilbenes at the minimum required performance limit (MRPL) of 0.5 ng/g was validated. The standard deviation (SD) of analysis showed good repeatability, considering the complexity of the matrix. Moreover, the coefficients of variation (CV) are within the precision requirements of the EU [9].

The quantification of stilbenes is susceptible to interference by the complex matrix in animal tissues, which can lead to unsatisfactory reproducibility. In recent years, the use of isotopically labeled analytes as internal standards has been advocated in the EU and in the USA for trace analysis of residues. Their use allows a more sensitive and reliable analysis. In our procedure, we used DES-d8 as the internal standard and spiked it into the samples before the extraction procedures. The use of an internal standard in this way gives us far more reliable results by determining the recoveries accurately and improving the reproducibility of the method.

4. Conclusions

We describe a method for the simultaneous qualitative and quantitative determination of stilbene residues in animal tissues. We carried out a number of experiments to determine the most appropriate ASPE conditions for the analysis. The combination of the ASPE sample preparation coupled with the sensitivity and specificity of the optimized LC–MS/MS analysis gave an extremely sensitive and robust method for the detection of stilbenes in a range of food matrices.

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