Quantitative Screening of Stilbenes and Zeranol and Its Related Residues and Natural Precursors in Veal Liver by Gas Chromatography−Mass Spectrometry

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An existing gas chromatography−mass spectrometry-based quantitative screening method for the regulatory analysis of the resorcylic acid lactones zeranol, taleranol, and zearalanone and the stilbene anabolic steroids diethylstilbestrol and dienestrol was extended to include natural precursors of zeranol (zearalenone, α-zearalenol, and β-zearalenol) in veal liver. No changes in sample preparation were required; the instrumental conditions were selected to effect a suitable chromatographic separation and detection of the analytes. Validation experiments were performed to verify the performance and applicability of the extended method for the quantitative screening of the original and additional analytes in veal liver in the concentration range from 0.5 to 2.0 μg/kg. The limits of detection were 0.08−0.19 μg/kg. The limits of quantitation were 0.27−0.64 μg/kg. Recoveries were 29−67%. Combined relative measurement uncertainty estimates were 6−21%.

KEYWORDS: Stilbenes; zeranol; natural precursors; gas chromatography; mass spectrometry

INTRODUCTION

Zeranol is a semisynthetic estrogenic anabolic and growth promoter of the resorcylic acid lactone group of compounds. It is the active ingredient in Ralgro implants, which have been used in cattle production (*1*). Zeranol has been approved for use in Canada, which has established administrative maximum residue limits (AMRLs) of 2 and 10 μg/kg in cattle muscle and liver, respectively (*2*), and in the United States, which has determined that tolerances are not required for cattle tissues (*3*). Maximum residue limits (MRLs) have also been established by the Codex Alimentarius Commission (*4*). However, the use of zeranol has been banned in the European Union (EU) since 1998 (*5*).

Zeranol is metabolized by cattle into taleranol (*β*-zearalanol) and zearalanone and excreted in urine (*6*). The presence of zeranol and its metabolites in cattle urine is often used as a marker for the deliberate use of zeranol (*1*, *6*). However, this determination is complicated by recent detections of zeranol and taleranol in the urine of pasture-fed cattle in New Zealand (*1*) and Ireland (*7*). These studies showed that zeranol can be formed in vivo from zearalenone and α-zearalenol, estrogenic plant toxins produced by *Fusarium* spp. One report indicated that zeranol was present in pasture-fed animals at levels comparable to those in animals deliberately treated with zeranol (*1*). *Fusarium graminearum* causes Fusarium head blight in wheat and barley and also in

wild pasture grasses in parts of the Canadian prairies, where a large proportion of Canada's cattle are raised for market (*8*); therefore, Canadian cattle could also contain zeranol-related residues that may be due to ingestion of prepared feeds or grasses contaminated with *Fusarium* molds rather than from deliberate use of zeranol. Because the conversion of zearalenone and α-zearalenol to zeranol and taleranol is considered to be irreversible, the presence of zearalenone and other known precursors would be evidence of natural contamination rather than the deliberate use of zeranol. The EU has attempted to develop tentative criteria whereby deliberate administration of zeranol and natural contamination of livestock feeds might be distinguished (*9*, *10*). It was concluded that a finding of deliberate administration of zeranol is only possible for those samples in which the presence of zeranol and/or taleranol is confirmed and evidence of natural contamination is absent (*10*). The capability to simultaneously detect residues of zearalenone, α-zearalenol, and the related compound *β*-zearalenol in addition to zeranol and taleranol in meat, organ tissue, and/or urine would enable food regulatory agencies to correctly discriminate between samples indicating deliberate administration of zeranol and related compounds and those samples in which natural contamination of feed renders such a categorization equivocal.

A number of different analytical techniques have been applied to the analysis of zeranol and related residues in animal products (*11*); however, few of these are applicable to the determination of the related natural precursors. Most of the recently

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published methods applicable to these analytes are based on mass spectrometry (MS) coupled either to liquid chromatography (LC) (*6*, *10*, *12*) or to gas chromatography (GC) (*9*). These methods are generally used to quantify and confirm results generated by screening methods that commonly use some form of immunochemical detection (*11*, *13*).

Rather than adopt a method taken from the literature or develop an entirely new method, our strategy was to first explore the possibility of extending a currently used method to include the additional analytes. This approach minimizes the amount of time and resources needed to develop, evaluate, and validate an analytical method, reduces familiarization time for the technical staff, and makes better use of existing analytical equipment. Our regulatory laboratory has a validated gas chromatography–mass spectrometry (GC-MS)-based quantitative screening method, which is based on methods developed by Covey et al. (*14*) and Chichila et al. (*15*) for zeranol, taleranol, and zearalanone and which also includes the stilbene anabolic steroids diethylstilbestrol and dienestrol. This method is used to screen for these five analytes in bovine, porcine, and equine livers and for zeranol and diethylstilbestrol in bovine kidney and muscle; the analytical range is 0.5–2.0 μg/kg. Our approach was to determine if this method, with minimal modifications, could be extended to include zearalenone, α-zearalenol, and β-zearalenol.

The existing method was modified to include the additional analytes. No changes in sample preparation were required; the GC-MS instrumental conditions were selected to effect a suitable separation and detection of the analytes. Validation experiments were performed to verify the extended method's performance for the original and additional analytes in veal liver as a model matrix in the concentration range of 0.5–2.0 μg/kg.

MATERIALS AND METHODS

Reagents. Suppliers are listed for reference only. Other brands of equal performance may be substituted except where noted.

All water was purified by reverse osmosis followed by deionization, adsorption, and filtration. Acetonitrile (MeCN), dichloromethane (CH_2Cl_2), hexane, methanol (MeOH), ethyl acetate (EtOAc), and iso-propanol (*i*-PrOH) were of high-purity grade supplied by Caledon Laboratories (Georgetown, Canada). Sodium chloride, potassium chloride, potassium phosphate monobasic and potassium phosphate dibasic, all of ACS grade, and sodium acetate trihydrate, certified reagent grade, were supplied by Fisher (Ottawa, Canada). Glacial acetic acid was of BDH brand supplied by VWR. Sodium hydroxide (NaOH) reagent grade pellets and dextrose monohydrate were supplied by Sigma-Aldrich Canada Ltd. (Oakville, Canada). The derivatization reagents *N*,*O*-bis(trimethyl-silyl)trifluoroacetamide (BSTFA) and trimethylsilylimidazole (TMSI), supplied as 1 mL sealed ampules, were purchased from Pierce Chemical Co. (Rockford, IL). β-Glucuronidase, type H-5, was purchased from Sigma-Aldrich Canada. Nitrogen, purified to a minimum purity of 99.995%, and helium, of ultrahigh pure grade of a minimum purity of 99.999%, were purchased from Air Liquide (Montreal, Canada).

The 2 N NaOH solution was prepared by dissolving 80 g of NaOH pellets in 1 L of water. The 1:1 *i*-PrOH/MeOH solution was prepared by mixing equal volumes of each solvent. This solution was prepared fresh every month. The 5% aqueous acetic acid solution was prepared by diluting 25 mL of glacial acetic acid to a final volume of 500 mL with water. This solution was prepared fresh every 2 weeks. The 25% aqueous MeOH was prepared by diluting 100 mL of MeOH with 300 mL of water. This solution was prepared fresh every 2 weeks. The 0.04 M sodium acetate solution was prepared by dissolving 10.88 g of sodium acetate in 2 L of water. This solution was prepared fresh every month. Phosphate-buffered saline, pH 6.0, was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium phosphate monobasic, 1.15 g of sodium phosphate dibasic, and 0.2 g of dextrose monohydrate in 1 L of water in an autoclaved 1 L glass bottle. The pH was adjusted to 6.0 by the addition of 0.1 N phosphoric acid. The solution was split into 100 mL aliquots, autoclaved at 121 °C for
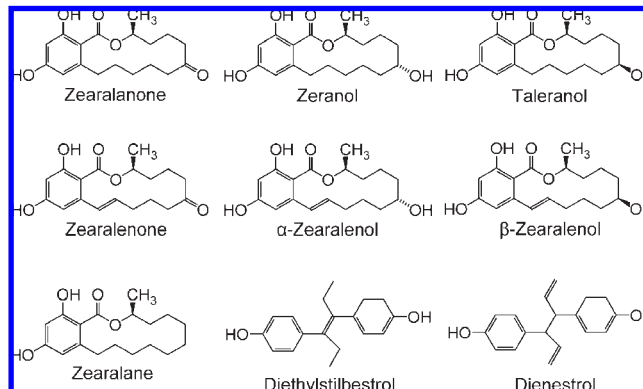

Figure 1. Structures of analytes and internal standards.

15 min at 18 psig, and then stored at 4 °C. Unused aliquots were discarded after 12 months. The β-glucuronidase solution was prepared by weighing the equivalent of 3×10^4 units into a small vial and dissolving in 2 mL of pH 6 phosphate-buffered saline. The solution was prepared fresh just before use. The derivatization reagent was prepared by transferring 2 μL of TMSI and 98 μL of BSTFA using a pipettor to a 100 μL autosampler vial and vortexing to mix. This reagent was prepared fresh before use.

Reference Materials. Diethylstilbestrol [56-53-1], dienestrol [84-17-3], taleranol [42422-68-4], zearalanone [5973-78-0], α-zearalenol [36455-72-8], β-zearalenol [71030-11-0], zearalenone [17924-92-4], and zeranol [26538-44-3] were purchased from Sigma-Aldrich Co. Zearalane [7344-47-0] is not commercially available. It was synthesized by Schering-Plough (Union, NJ). Diethyl-1,1,1′,1′-d_4-stilbestrol-3,3′,5,5′-d_4 (D_8-diethylstilbestrol) [91318-10-4] was purchased from CDN Isotopes (Point-Claire, Canada). The structures of these compounds are presented in **Figure 1**.

Stock solutions of diethylstilbestrol, D_8-diethylstilbestrol, dienestrol, and zearalane (100 μg/mL) were prepared by dissolving 10.0 mg of the solid powder in 100 mL of MeOH. Stock solutions of zeranol, taleranol, zearalanone, zearalenone, α-zearalenol, and β-zearalenol (100 μg/mL) were prepared by dissolving the contents (5 mg) of each ampule in 50 mL of MeOH. Fresh stock solutions were made every 12 months. The mixed working solution of all of the target analytes (100 ng/mL) was prepared by combining 100 μL of each stock solution in a 100 mL volumetric flask and diluting to volume with MeOH. A fresh solution was made every 2 months. The internal standard solution of D_8-diethylstilbestrol (100 ng/mL) and zearalane (20 ng/mL) was prepared by combining 100 μL of D_8-diethylstilbestrol and 20 μL of zearalane stock solution in a 100 mL volumetric flask and diluting to volume with MeOH. A fresh solution was made every 2 months. All stock and working solutions were stored at -20 °C and allowed to warm to room temperature before use.

Apparatus. Suppliers are listed for reference only. Other brands of equal performance may be substituted.

All volumetric glassware used throughout this method was of class A.

The GC-MS used was an Agilent (Santa Clara, CA) 6890N GC-5975 MSD equipped with a LEAP Technologies GC-Pal injector and running Chemstation software, version G170DA revision D.03.00 SP2. The column used was a 30 m $\times$ 0.25 mm i.d., film thickness = 0.25 μm, DB5-MS (J&W Scientific/Agilent, Folsom, CA). The Prep I Automated Sample Processor and the XTRX extraction columns for the Prep I, type AX/S120 mg hydrated polymeric strong anion exchange resin, were supplied by Analtech Inc. (Newark, DE). A Beckman Coulter Allegra 6KR centrifuge, capable of 4000*g* and equipped with bucket adapters for 50 and 15 mL centrifuge tubes, was supplied by VWR Canlab (Mississauga, Canada). A Polytron model PT 3100 homogenizer was supplied by Brinkmann Instruments Canada Ltd. (Mississauga, Canada). An Eberbach two-speed flat-bed mechanical shaker was supplied by VWR. An N-Evap evaporator was supplied by Organomation Associates (Berlin, MA). Falcon brand polypropylene disposable centrifuge tubes, 50 mL capacity, were supplied by VWR. Glass round-bottom centrifuge tubes, 50 mL capacity, and Teflon-lined screw caps were supplied by VWR. Glass centrifuge tubes, 15 mL capacity, were supplied by VWR. Autosampler vials, 100 μL polypropylene, and vial caps with PTFE/silicone septa were supplied by Supelco Canada (Oakville, Canada).

Sample Preparation. The sample preparation procedure closely followed the scheme described by Covey et al. (*14*). A 5.0 g test portion of tissue was transferred into a 50 mL polypropylene centrifuge tube and fortified with 50 μL of the internal standard solution. After the addition of 11 mL of 0.04 M sodium acetate solution, the sample was homogenized for 1 min with the Polytron. The pH of the homogenate was adjusted into the range of 4.3−4.8 by the addition of glacial acetic acid (typically 75 μL). The tube was mixed on a vortex mixer and then left standing for 30 min. A 100 μL aliquot of the β-glucuronidase solution was added to the tube, after which the tube was mixed thoroughly on a vortex mixer then left standing for 30 min. The tube was incubated overnight for 16−18 h at 37 °C in a constant-temperature water bath. After incubation, 16 mL of MeCN was added and the tube shaken on high speed on a mechanical shaker for 5 min. The tube was centrifuged at 4000*g* for 10 min. After the supernatant had been trasnferred into a 50 mL screw-cap glass centrifuge tube, 8 mL of hexane and 2 mL of CH_2Cl_2 were added to the supernatant. The tube was capped and shaken at high speed for 1 min and then centrifuged at 1200*g* for 3 min. Three layers were formed; the middle MeCN layer was transferred using a Eppendorf Repeater Plus pipettor (VWR) to a clean 50 mL screw-cap glass centrifuge tube. The extraction was repeated with another 4 mL of MeCN. The combined MeCN layers were evaporated just to dryness under nitrogen flow at 60 °C on an N-Evap. The residue was redissolved in 2 mL of 1:1 (v/v) *i*-PrOH/MeOH.

A 1.5 mL portion of 2 M NaOH was added to the sample and the tube immediately mixed on a vortex mixer. The mixture was decanted into the XTRX column. An additional 1 mL portion of *i*-PrOH/MeOH was added to the sample tube, mixed on a vortex mixer, and then added to the XTRX column. The column was loaded into the inner ring of the Prep 1. The processor was run for 5 min to elute the loading solvent mixture to waste. After disposal of the eluted solvent, 4 mL of MeOH wash solvent was added to the cartridge. The processor was run for 5 min to elute the wash solvent to waste. The Prep 1 was then used to automatically wash and elute the samples. The four solvent reservoirs were filled with the following solvents: 18 mL of distilled water, 33 mL of 5% aqueous acetic acid, 22 mL of 25% aqueous MeOH, and 35 mL of MeOH. These volumes correspond to 1.5, 2.75, 1.8, and 2.9 mL/column, respectively. Each column was sequentially washed with the first three solvents; the analytes were eluted with MeOH.

The MeOH eluate in the recovery cup was transferred to a 15 mL glass centrifuge tube. The cup was rinsed with 4 mL of MeOH and combined with the eluate. After the addition of 1 mL of EtOAc, the sample was evaporated to dryness under nitrogen flow at 60 °C on an N-Evap. An additional 0.5 mL of EtOAc was added to the sample and evaporated to dryness. Just prior to analysis, 15 μL of EtOAc was added to the tube and mixed on a vortex mixer to dissolve the residue, and the solution was transferred to a 100 μL autosampler vial.

Each batch of samples included calibration standards, a quality control check sample, and a negative tissue control sample. The calibration standards were prepared by fortifying three blank tissue homogenates with 25, 50, or 100 μL of the mixed working solution to produce concentrations of 0.5, 1.0, and 2.0 μg/kg, respectively. The check sample was prepared by fortifying a blank tissue homogenate with 40 μL of the mixed working standard solution to produce a concentration of 0.8 μg/kg. The negative control was a nonfortified blank tissue homogenate. The standards and quality control check sample homogenates were fortified with the mixed working solution just after pH adjustment and before the addition of β-glucuronidase solution.

GC conditions. were as follows: helium carrier gas head pressure, 82.7 kPa (12 psig); injection port temperature, 275 °C; splitless injector purge time, 1 min; oven temperature program, initial time, 1.5 min; temperature ramp, 20 °C/min to 230 °C; hold, 10 min; temperature ramp, 15 °C/min to 260 °C; temperature ramp, 4 °C/min to 300 °C; hold, 10 min. Analytes were derivatized in the inlet by co-injection of 1.0 μL of BSTFA/TMSI derivatizing solution followed by 1.5 μL of sample solution into the hot GC injection port operating in splitless mode. Injections were made automatically using the autosampler.

MS Conditions. MS with selected ion monitoring; ionization mode, electron impact at 70 eV; solvent delay time, 10 min; electron multiplier, autotune setting plus 400 V. The following ions (dwell times, ms) were selected for monitoring: *cis*-D_8-diethylstilbestrol, *m/z* 420 (50); *cis*-diethylstilbestrol, *m/z* 412 (50), 383 (50), 397 (50); *trans*-D_8-diethylstilbestrol,

m/z 420 (40); *trans*-diethylstilbestrol, *m/z* 412 (40), 383 (20), 397 (20); dienestrol, *m/z* 410 (40), 381 (20), 395 (20); zearalane, *m/z* 435 (100); zearalanone, *m/z* 307 (40), 335 (40), 464 (40); zeranol and taleranol, *m/z* 433 (40), 523 (40), 538 (40); zearalenone, *m/z* 333 (50), 317 (50), 447 (50); α-zearalenol and β-zearalenol, *m/z* 305 (50), 317 (50), 333 (50). The first listed ion for each compound was used for quantitation.

Calculations. Integrated peak areas for analytes and internal standard were transcribed from the Chemstation software output to a Microsoft Excel spreadsheet, which calculated the final results. The response for each analyte was calculated by dividing the integrated peak area of the quantitation ion by the integrated peak area for the appropriate internal standard. For each analyte, a calibration curve was generated by fitting a nonweighted linear regression to the calibration data (response versus concentration). Ion ratios were calculated by dividing the peak area of each qualifier ion by the peak area of the corresponding quantitation ion.

Validation Experiments. This validation study included three experiments to verify recovery and precision claims for the method; collectively these are referred to as the "precision experiments". Recognition experiments were also performed in which samples fortified by a third party were submitted "blind" to the analyst. Veal liver was chosen as the target matrix for method development and validation as it is the sample type most commonly received in our laboratory as part of the Canadian domestic survey program for stilbenes, zeranol, and related resorcylic acid lactones.

The design used for the precision experiments is illustrated in **Figure 2**. Three runs were conducted, each on separate days. Each run consisted of the following: A chemical standard containing the analytes and internal standard was used as a system suitability sample. Four matrix-fortified calibration standards were prepared at 0.0, 0.5, 1.0, and 2.0 μg/kg. Two quality control samples, blank tissues fortified at 0.8 μg/kg prior to extraction, were used to estimate bias. Four blank tissue samples were fortified at 0.5 (prepared in duplicate), 1.0, and 2.0 μg/kg, respectively, prior to extraction to estimate precision. Three blank tissue samples were fortified at 0.5, 1.0, and 2.0 μg/kg, respectively, just prior to instrumental analysis to estimate recoveries. The precision and recovery sets were duplicated within each run using two different tissue pools. Three tissue blanks were included, one for each of the tissue pools used for calibration, precision, and recovery. In total, each run consisted of 23 tissue samples and a system suitability sample. Duplicate injections of the first 0.5 μg/kg precision sample for each tissue pool were used to estimate instrumental repeatability precision. The duplicate preparations at 0.5 μg/kg for each tissue pool were used to estimate method preparation precision. A total of 12 determinations at 0.5 μg/kg over 6 tissue pools in 3 runs are used to estimate single-laboratory precision. Recoveries were estimated by calculating the apparent concentration of the recovery set samples using the calibration curve and then dividing by the nominal concentrations. This experimental design, although similar to some hierarchical (nested) experimental designs reported in the literature (*16*, *17*), was not intended to generate data to allow for estimates of within-run, between-run, and intermediate precision; rather, it was designed so that the precision estimates would be based on data acquired over a range of tissue pools and days.

Recognition experiments consisted of two runs of samples. Each run consisted of calibration standards at 0.5, 1.0, and 2.0 μg/kg for each analyte; tissue blank; quality control check samples; and three spiked samples fortified by a third party prior to extraction with one or more analytes plus internal standard, each in duplicate. The recognition experiments were limited to the additional analytes: zearalenone, α-zearalenol, and β-zearalenol.

RESULTS AND DISCUSSION

Method Development. The goal of this work was to make minimal changes to the existing method while extending the scope to include the additional analytes. The first step was to evaluate the current GC-MS method for its applicability to the new analytes and optimize the method, if applicable. Full-scan electron impaction ionization mass spectra were obtained by co-injecting 1.5 μL of each analyte stock solution with the derivatizing reagent. From these data, candidate ions were selected for initial investigation.
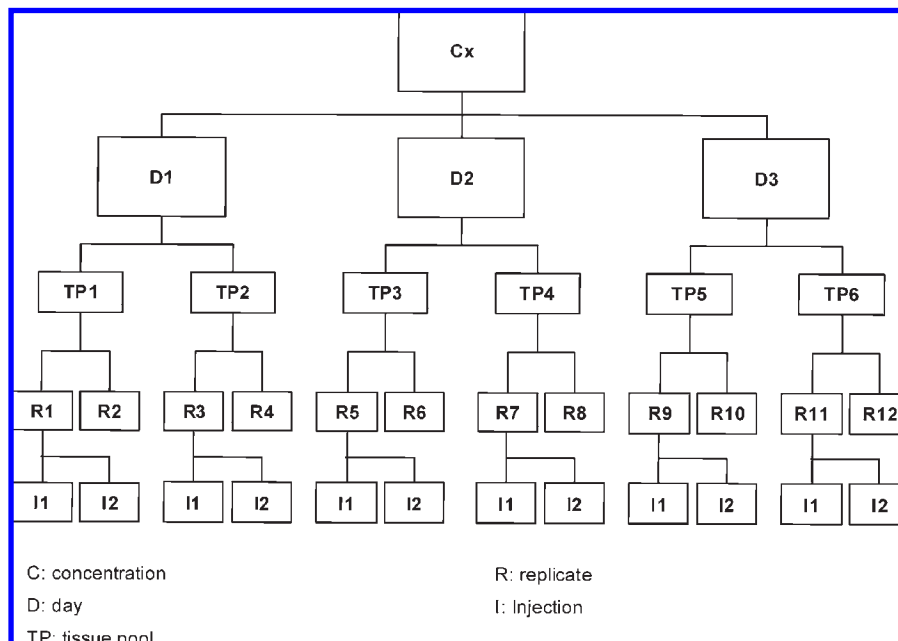
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Figure 2. Experimental design for the precision and recovery experiments.

The instrumental detection limits for the analytes were estimated by injecting in triplicate a series of 11 mixed solutions of the analytes dissolved in EtOAc ranging in concentration from 0.033 to 0.33 μg/mL, equivalent to 0.1−1.0 μg/kg concentrations in tissue. For these experiments, the original GC temperature program was retained and the mass spectrometer was operated in SIM mode using a 50 ms dwell time for each of four ions. On the basis of signal-to-noise and precision considerations, a working instrumental detection limit of 0.066 μg/mL (0.2 μg/kg of tissue equivalent, 100 pg injected) was assigned for each analyte.

The oven temperature program and the selection of ions to monitor were chosen to reduce interferences from coextracted matrix components while maintaining adequate sensitivity and linearity of response. Test portions of tissue samples from 22 different animals, either left unfortified as blanks or fortified at 2.0 μg/kg TE, were analyzed with different combinations of oven temperature programs and monitored ions to find an optimum method. The original temperature program contained a temperature ramp of 15 °C/min from 230 to 290 °C. This ramp was modified to 4 °C/min from 260 to 300 °C to improve the separation between analytes and coextracted matrix components. A set of three ions for each analyte was chosen for screening analyses to be consistent with the other analytes in the original method. The choice of three ions was considered to be adequate for a screening analysis. Our standard practice is to extract a second test portion of a suspect sample and conduct the instrumental analysis with additional monitored ions (*18*, *19*) or to use a complementary confirmation method to confirm the original detection. In the screening analysis, the ion ratios are used to aid in the interpretation of the chromatograms to ensure that the correct peak eluting within the desired retention time window is chosen for integration. This practice reduces the rate of false-positive determinations. Extracted ion chromatograms from the analysis of a extract of blank veal liver are presented in **Figure 3**, and those from an extract of veal liver fortified with analytes at a concentration of 1.0 μg/kg are presented in **Figure 4**.

A total of 22 different veal liver tissue samples (individual animals) were screened using the new GC-MS method. Four of the samples had detectable levels of the natural precursors of zeranol: zearalenone, α-zearalenol and β-zearalenol. No
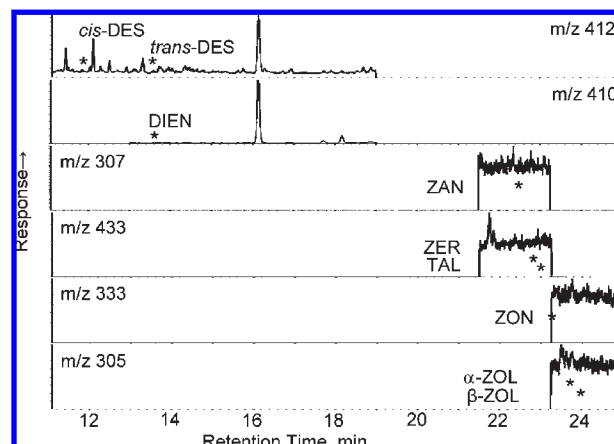

Figure 3. Extracted ion chromatograms from the analysis of an extract of blank porcine liver tissue. The asterisks indicate the retention times of the analytes. Abbreviations: *cis*-DES, *cis*-diethylstilbestrol; *trans*-DES, *trans*-diethylstilbestrol; DIEN, dienestrol; ZAN, zearalanone; ZER, zeranol; TAL, taleranol; ZON, zearalenone; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol.

significant matrix interferences were detected in any of the blank tissue samples. Six composite pools of blank tissue were created from the other 18 tissue samples, 3 samples per pool, to be used for the validation studies.

Calibration and Ion Ratios. Correlation coefficients were 0.998 or greater. The slopes of the three-point calibration curves were for some analytes appreciably different from run to run; however, this did not appear to affect the precision of the determinations. The ion ratios derived from the precision experiments were very reproducible: the relative standard deviations (RSD) ranged from 3 to 12%.

Limits of Detection and Quantitation. The estimates of limits of detection (LOD) and limits of quantitation (LOQ) are presented in **Table 1**. The LOD and LOQ estimates were calculated using the method of Miller and Miller (*20*). Using this method, the LOD is the concentration corresponding to the *y*-intercept plus 3 times the standard deviation of the *y*-intercept, which is estimated from the standard error of the linear regression model fitted to the data. The LOQ is the concentration corresponding to the *y*-intercept

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plus 10 times the standard deviation of the *y*-intercept. The presented values are the highest of three determinations of LOD and LOQ for each analyte, representing the worst-case situation. The LOD estimates were all lower than our minimum performance requirements for the LOD, which are 0.3 μg/kg for diethylstilbestrol and dienestrol and 0.5 μg/kg for the other analytes. The estimates were also lower than the working IDL of 0.2 μg/kg assigned to each analyte. Conservative working LOD values of 0.3 μg/kg for diethylstilbestrol and dienestrol and 0.5 μg/ kg for zeranol and related compounds were assigned. The LOQ estimates were comparable to the lowest calibration concentration of 0.5 μg/kg.

Decision Limits and Detection Capabilities. The estimates of decision limits (CCα) and detection capabilities (CCβ) are given in **Table 2**. These values were estimated according to the principles given in EU Commission Decision Document 2002/657/EC (*16*). CCα is "...the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant" (*19*). For banned substances, α is set at 0.01 (1% false-positive rate), so that a truly blank sample would be expected to give a response greater than the critical value (*y*$_c$) corresponding to concentration CCα only 1% of the time. CCβ is the lowest analyte concentration "...at which a method is able to detect truly contaminated samples with a statistical certainty of $1 - \beta$" (*19*). For banned substances, β is set at 0.05 (5% false-negative rate). In this study, *y*-intercepts (*y*$_0$) and the standard errors of the *y*-intercept (SE$_{int}$)
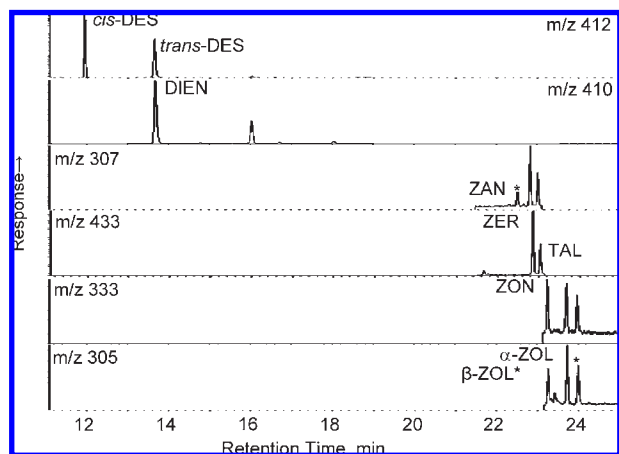

Figure 4. Extracted ion chromatograms from the analysis of an extract of blank porcine liver tissue fortified with analytes at a concentration of 1.0 μg/ kg. See **Figure 3** for abbreviations.

Table 1. Estimates of Limit of Detection (LOD) and Limit of Quantitation (LOQ)

analyte	LOD (μg/kg)	LOQ (μg/kg)	analyte	LOD (μg/kg)	LOQ (μg/kg)
diethylstilbestrol	0.12	0.42	zearalanone	0.12	0.39
dienestrol	0.12	0.56	zearalenone	0.11	0.38
zeranol	0.18	0.62	α-zearalenol	0.15	0.49
taleranol	0.19	0.64	β-zearalenol	0.08	0.27

were calculated by fitting a linear regression model to the precision data sets. For each analyte, the critical value (*y*$_c$) was calculated as $y_0 + 2.33 \times$ SE$_{int}$; CCα was calculated from *y*$_c$ using the linear calibration function. CCβ was calculated as the concentration corresponding to $y_c + 1.64 \times$ SE$_{int}$. Although strict interpretation of the EU document requires that, for banned substances, CCα be determined using concentrations at or above the target level in equidistant steps and that CCβ be determined using concentrations at and below the target level in equidistant steps, the use of the three fortification concentrations (0.5, 1.0, and 2.0 μg/kg) used was considered to be a practical compromise to reduce the number of analyses required to validate the method. The CCα estimates were all below 0.4 μg/kg; the CCβ estimates were all below 0.6 μg/kg.

Precision. The relative precision estimates were based on calculated analyte concentrations derived from the analyses of the 0.5 μg/kg fortified samples. The injection precision was based on six pairs of replicate injections of the first preparation of two for each tissue pool. The preparation precision was based on six pairs of replicate preparations, one for each tissue pool. The laboratory precision was based on the pooled results from the analyses of the 12 replicates (preparations $\times$ tissues) of the 0.5 μg/ kg fortified samples. Before the data were pooled, a single-factor analysis-of-variance procedure was applied to these data to determine if there were any significant tissue or run effects on the precision of the method; no significant effects ($P \leq 0.05$) were observed. The overall method relative precision for each analyte ranged from 4 to 18%.

Recovery. Recoveries were estimated at each of the three fortification levels (0.5, 1.0, and 2.0 μg/kg) from the three precision experiments. Recoveries, averaged over the three concentrations for each analyte, ranged from 29 to 67%.

Bias. Bias was estimated using the duplicate 0.8 μg/kg QC samples included in each of the three runs. The criterion for relative bias was a maximum of 20%. All analytes except taleranol met the criterion for relative bias. The relative bias for taleranol was 21%.

Measurement Uncertainty. Relative measurement uncertainty (RMU) was estimated for each analyte using the approach given in example A4 of the appendix of the *Eurachem/CITAC Guide* (*21*).

Table 3. Magnitude of Relative Uncertainty Factors Contributing to Combined Relative Measurement Uncertainty (RMU)

analyte	factors			
	precision (%)	recovery (%)	bias (%)	combined RMU[a] (%)
diethylstilbestrol	4	1	1	6
dienestrol	10	3	3	12
zeranol	18	8	5	21
taleranol	13	8	7	17
zearalanone	12	9	4	16
zearalenone	11	10	5	16
α-zearalenol	17	9	6	21
β-zearalenol	10	8	8	16

[a] Includes factors of homogeneity (3%), calibration preparation (3%), and sample weighing (0.5%) that are common for each analyte.

Table 2. Estimates of Decision Limit (CCα) and Detection Capability (CCβ)

analyte	CCα (μg/kg)	CCβ (μg/kg)	analyte	CCα (μg/kg)	CCβ (μg/kg)
diethylstilbestrol	0.10	0.17	zearalanone	0.26	0.45
dienestrol	0.15	0.25	zearalenone	0.33	0.57
zeranol	0.27	0.45	α-zearalenol	0.33	0.56
taleranol	0.32	0.55	β-zearalenol	0.31	0.53

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Table 4. Results of Analyses of Duplicate Fortified Samples Prepared by a Third Party

run	sample	analyte	fortified concn (μg/kg)	avg (μg/kg)	bias (%)	precision (RSD, %)
1	1	zearalenone	0.6	0.71	18	1
		α-zearalenol	1.0	0.94	−6	6
		β-zearalenol	1.7	1.7	−2	2
	2	zearalenone	1.7	1.7	0	6
		α-zearalenol	0.0	nd[a]	na[b]	na
		β-zearalenol	0.0	nd	na	na
	3	zearalenone	0.0	nd	na	na
		α-zearalenol	1.8	1.9	4	6
		β-zearalenol	0.6	0.11	17	6
2	1	zearalenone	1.5	1.3	−16	11
		α-zearalenol	0.0	0.26	na	3
		β-zearalenol	1.5	1.1	−29	7
	2	zearalenone	0.0	0.33	na	na
		α-zearalenol	0.7	0.64	−9	0
		β-zearalenol	0.0	nd	na	na
	3	zearalenone	0.0	0.35	na	8
		α-zearalenol	0.9	0.84	−7	12
		β-zearalenol	0.9	0.78	−15	10

[a] nd, not detected. [b] na, not applicable.

In this model, the overall method relative precision is combined with other factors to provide an estimate of overall RMU. The other major factors included in the estimate are the relative standard error of recovery and the relative standard error of bias. Additional factors, which were identified by Holland et al. (22) when applying the Eurachem example to shellfish toxins, were incorporated into the calculations of RMU. These factors were homogeneity, calibrant preparation, and sample weighing. The relative uncertainty of these factors could not be derived from the validation experiments, so the conservative estimates of Holland et al. were used: 3, 3,and 0.5%, respectively. The magnitude of the factors used to estimate RMU, and the overall RMU values of the method for each analyte are presented in **Table 3**. RMU values ranged from 6% for diethylstilbestrol to 21% for zeranol and α-zearalenol.

Recognition Experiments. Although it is preferred that incurred samples be used for recognition experiments, these were not available for this study. Samples were prepared by fortification of blank tissue samples by a third party and submitted as unknowns to the analyst. The results of the recognition experiments are presented in **Table 4**. The acceptability criteria for the method are as follows: no individual or systematic bias of > 20%; repeatability precision of ≤25% RSD. Except for one sample, the blind spiked samples meet the acceptability requirement for bias. The average absolute bias was 11%, and the range was 0−29%. The blind spiked samples meet the acceptability requirement for precision. The average RSD for the fortified blank samples was 6.0%, and the range was 0−12%.

The extended GC-MS method was shown to be fit for the purpose and acceptable as a quantitative screening method for the original analytes and the additional natural precursors of zeranol, zearalenone, α-zearalenol, and β-zearalenol in veal liver in the analytical range of 0.5−2.0 μg/kg. The ability to detect and quantify these analytes will enable food regulatory agencies to correctly discriminate between natural contamination of feed and the abuse of zeranol as the source of residues of zeranol and related compounds in food animals.

SAFETY

The anabolic and stilbene hormones used in this method are very physiologically active. Avoid direct contact and/or inhalation of hormone drug standards. Follow appropriate safety precautions as described in the appropriate Material Safety Data Sheet.

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