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Biological and Chemical Approaches for the Detection and Identification of Illegal Estrogens in Water-based Solutions

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ABSTRACT

The continuous introduction of new products used as growth promoters in animal husbandry, for sports doping and as products for body-building requires residue laboratories to initiate research on developing a strategy for the identification of 'unknown' components. In this study, a strategy is presented for elucidating the identity, the structure and the possible effects of illegal estrogenic compounds in an unidentified water-based solution. To obtain complete information on the composition and activity of the unidentified product, a multidisciplinary approach was needed. A case-study is described with a 'solution X' found during a raid. First, *in vivo* techniques (animal trials with mice, anatomical and histological research) were combined with *in vitro* techniques (the yeast estrogenic screen (YES)). In a later stage of the investigation, HPLC-fractionation, liquid chromatography–multiple mass spectrometry (LC-MS^n) and gas chromatography-multiple mass spectrometry (GC-MS^n) were used. Finally, the identity of 'solution X' was confirmed in a very low concentration range (10 ng/L estrone and 400 ng/l ethinyloestradiol).

Keywords: animal trials, anatomy, GC-MS^n, histology, LC-MS^n, YES

Abbreviations: CPRG, chlorophenol red-β-galactopyranoside; DAB,3,3′-diaminobenzidine tetrahydrochloride'; βE_2, 17β-oestradiol; E_1, estrone; EE_2, ethinyloestradiol; EEF, estradiol equivalence factor; ER, estrogen receptor; GC-MS^n, gas chromatography–mass spectrometry; HFBA, heptafluorobutylamine; LC-MS^n, liquid chromatography-mass spectrometry; MSTFA, *N*-methyl-*N*-(silyltrimethyl)trifluoroacetamide; YES, yeast estrogenic screen

INTRODUCTION

Nowadays, few 'non-compliant' (positive) samples are found in residue control programmes for the presence of growth promoters (Council Directive 96/23/EC). However, this does not necessarily mean that the use of illegal products has been completely eradicated. Unidentified products are still being found during raids. Moreover, meat inspectors are still convinced that some carcasses of cattle in slaughterhouses show characteristics of the use of growth promoters. Very often, different routine extraction and detection methods are used to check for 'target' compounds. To date, no papers are available on how to approach a survey for 'unknown', probably illegally used, products except for the identification of 'unknowns' in injection sites (De Wasch *et al*., 2002).

In this paper we describe the elucidation of the composition and possible effects of an unknown water-based solution, following a systematic approach based on expertise from different research disciplines (Figure 1). For this research, an unknown water-based solution ('solution X') obtained from a raid and expected to contain growth promoters was used. First, animal trials were set up because the possible effects of 'solution X' *in vivo* were unknown. Parameters such as weight gain, food and water conversion and morphological alterations (determined by anatomical and histological study) were monitored. In the next stage of the investigation, YES (yeast estrogenic screen), which is a widely used *in vitro* assay for the detection of estrogenic activity (Routledge and Sumpter, 1996), provided insights into the *in vitro* binding capacity of compounds in 'solution X' for estrogen receptors, and LC-MS^n and GC-MS^n analyses were used to obtain molecular mass and structural information. For this, 'solution X' was concentrated using Bakerbond C_{18} Speedisk extraction, a technique commonly used in environmental analysis. The extract, multiple dilutions and fractions obtained after HPLC fractionation were investigated using the techniques described.

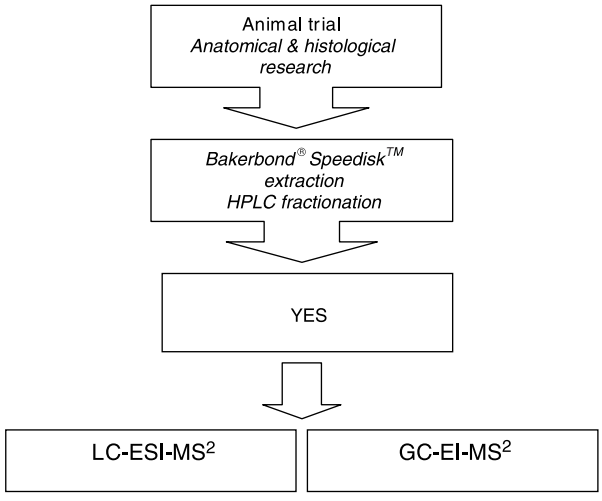

Figure 1. Flow chart summarizing the stepwise approach to elucidating the identity of 'solution X'. YES, yeast estrogenic screen

MATERIAL AND METHODS

Animal trial combined with anatomical and histological investigation

After approval of the local ethical committee, 18 adult mice (9 male, 9 female), 6–8 weeks old, were allocated for each gender into one negative control group and two treatment groups in which the unidentified product was administered orally at 150 μl/100 ml drinking water in one group and at 3 ml/100 ml drinking water, in the other, during the first 6 days. On the following days until the end of the trial, the lower dose, i.e. 150 μl/100 ml drinking water, was increased to 6 ml/100 ml drinking water whereas the higher dose of 3 ml/100 ml drinking water was maintained throughout the trial. Oral administration of the unidentified product was selected in this trial as this was also the intended route of administration in cattle. After 3 days of acclimatization, administration of the unidentified product was started and the daily weight change, food and water intake were measured for each group. Food and water conversion were calculated as the ratios of the weight gain to the food and water intake, respectively. After a treatment period of 17 days, the mice were sacrificed and the effect of the unidentified product on organs was investigated anatomically and histologically in muscles, lymphoid organs, adrenal glands, thyroid gland, jejunum, liver, kidneys and female and male reproductive organs.

To verify the possible estrogenic effect of the unidentified product, a second trial was set up in which 15 prepubertal (21 days old) female mice were divided into three treatment groups, one negative control group and one positive control group in which 17β-oestradiol was administered. In this second trial, a shorter acclimatization period, of 1 day was adopted to prevent the mice from having an oestrous cycle that could influence the results. After the acclimatization, the unidentified product was administered for 10 days to the different treatment groups. At day 11, all mice were sacrificed and female reproductive organs were further investigated anatomically and histologically. Uterine sections were also analyzed immunohistochemically for the presence and distribution of estrogen receptor α (ERα) and the following protocol was used. After rehydration, the uterine sections were pre-treated in an Antigen Retrieval Citra Solution (BioGenex, San Ramon, CA, USA). This pre-treatment consisted of microwaving the slides for 2 min at 700 W and then again for 3, 5 and 5 min at 200 W with 5 min in between. After cooling for 30 min at 4°C and rinsing in distilled water, the slides were incubated for 5 min with 50 μl of a 3% (v/v) hydrogen peroxide–methanol solution to quench endogenous peroxidase activity. All incubations were carried out in a humidified environment. Then the slides were rinsed in TBS (Tris-buffered saline) and incubated consecutively with normal goat serum (1:3) for 30 min at 37°C to reduce non-specific staining. All sections were incubated with 50 μl of a 1:50 concentrated polyclonal rabbit anti-mouse ERα antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in TBS. After rinsing in TBS, the sections were incubated for 30 min at room temperature with 50 μl of a secondary biotinylated goat anti-rabbit antibody (1:500) (Dako A/S, Glostrup, Denmark). The specimens were rinsed in TBS and incubated for 30 min at room temperature with streptavidin–HRP (1:1000)(Dako). Finally, after rinsing in TBS, 50 μl of DAB chromogen substrate (Dako, Carpinteria, CA, USA) was administered for 5 min. Mayer's haematoxylin was applied for 30 s as a nuclear counterstain. Positive and negative controls were included in each staining

procedure. The positive control was a canine uterine section known to contain high numbers of ER-α. The negative controls were a uterine tissue section incubated without the primary antibody and a uterine tissue section incubated without the primary and secondary antibodies.

Chemical analysis

Reagents and chemicals. All chemicals used for extraction were of analytical grade and those for LC-MS^n of HPLC grade, obtained from VWR (Darmstadt, Germany). All chemicals used for preparation of the media for the YES were research grade biochemicals suitable for cell culture, obtained from Sigma-Aldrich Corp. (St Louis, MO, USA). 17β-Estradiol (98%, βE_2), also purchased from Sigma-Aldrich Corp., was dissolved in absolute ethanol from VWR.

Sample preparation. The sample preparation was the same for YES, HPLC fractionation, LC-MS^n and GC-MS^n. One litre of 'solution X' was extracted using Bakerbond C_{18} Speedisk (J.T. Baker, USA). The disk was conditioned with 20 ml acetone, 20 ml methanol and twice with 10 ml distilled water. When the sample was drawn through the disk, it was dried under vacuum for at least 30 min. The analytes were eluted using 5 ml acetone and 15 ml methanol. The extract was evaporated to dryness and reconstituted in 2 ml ethanol. One millilitre (extract A) was used for the YES. The remaining 1 ml of the extract (extract B) was evaporated to dryness, redissolved in 120 µl ethanol and used for HPLC fractionation. For this 100 µl was injected on column (Beckman ODS Ultrasphere High Performance Column, 10 mm $\times$25 cm, USA) and collected as four fractions using a methanol–water gradient program and a Lachrom Merck Hitachi L-6200 HPLC apparatus and a Hitachi L-4000 UV detector (VWR, Darmstadt, Germany) (Smets *et al.,* 1997).

Yeast estrogenic screen (YES). A major mechanism of endocrine disruption is binding to receptors (estrogen receptor, androgen receptor, arylhydrocarbon receptor, progesterone receptor, thyroid receptor) and subsequent alteration of DNA and protein expression. The YES is a yeast-based assay to detect binding of chemicals in a dose-responsive way to the human estrogen receptor. It is universally accepted as a screening tool for the detection of xeno-estrogens. This bioassay offers an integrated measure of the estrogenic potencies of mixtures without the need to know all relevant compounds beforehand.

The YES was originally developed at Glaxo (Glaxo Group Research Ltd., Middlesex, UK) and was kindly provided by Professor J. Sumpter (Brunel University, UK). Details of the YES have been previously described by Routledge and Sumpter (1996). Briefly, yeast cells (*Saccharomyces cerevisiae*), transfected with the human estrogen receptor (ERα) gene together with expression plasmids carrying the *Lac-Z* expression gene encoding the enzyme β-galactosidase, were incubated in medium containing the test compound and the chromogenic substrate chlorophenol red-β-galactopyranoside (CPRG). Active ligands induce expression of the reporter gene and subsequent secretion of β-galactosidase into the medium, which is quantified through the conversion of the yellow CPRG into chlorophenol red. This conversion can be measured spectrophotometrically at 540 nm. Yeast growth is measured as turbidity at 620 nm. Extract A (not fractionated) and the four fractions of

extract B were tested twice in the YES. A positive control (βE_2, serially diluted from 1×10^{-8} to 1.19×10^{-15} mol/L) and ethanol controls were included in each assay. The multiwell plates were incubated at 32°C and absorbance was read after 7 days. The mean absorbances (corrected for turbidity) of duplicates of all experiments were plotted against the concentration. A response was considered positive when (1) there was a concentration-dependent increase in β-galactosidase production and (2) at least two absorbance values of the concentration–response curve were higher than the limit of detection (solvent control absorbance + 3SD) (McNaught and Wilkinson, 1997). For each extract/fraction eliciting a positive response, the estradiol equivalence factor (EEF) was calculated as the ratio of the EC_{50} of βE_2 to the EC_{50} of the extract/fraction. EC_{50} values, defined as the concentration at which transcriptional response reaches 50% of its maximum value, were calculated using the Probit method (Stephan, 1977).

LC-MS^n. For LC-MS analysis the extract was evaporated to dryness and reconstituted in mobile phase. Samples of 1, 10 and 50 µl were injected onto the column. Chromatographic separation was achieved using a Symmetry C_{18} column (5 µm, 150 × 2.1 mm, Waters, Milford, CT, USA). A default gradient was used because no optimization of a separation could be developed for an unknown compound or mixture. The mobile phase consisted of a mixture of methanol (A) and 1% acetic acid in water (B). The flow rate was 0.3 ml/min. A linear gradient was used: 20% A was maintained for 7 min and increased to 100% A in 10 min (maintained for 7 min). Between samples there was an equilibration time of 10 min at initial conditions.

The HPLC apparatus consisted of a TSP P4000 pump and a model AS3000 autosampler (TSP, San Jose, CA, USA). The MS-detector was a Finnigan LCQdeca ion trap MS from ThermoFinnigan (San José, CA, USA) equipped with an electrospray interface in positive and negative ion mode MS and MS^n full scan.

GC-MS^n. Extract A and fractions (B1–B4) were evaporated to dryness and derivatized as described by Impens and colleagues (2001). To obtain gas chromatographic and coupled mass spectrometric information, an ion trap mass spectrometer Polaris (ThermoFinnigan, Austin, TX, USA) coupled to a Trace GC 2000 (ThermoFinnigan) was used. A Carlo Erba AS2000 (ThermoFinnigan) autosampler was used to inject the samples. Analyses were performed using a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 GC-column (25 m ×0.22 mm, 0.22 mm, 0.25 µm i.d.) (SGE Inc., Austin, TX, USA). MS measurements were performed in electron impact mode. A temperature gradient was used starting at 100°C, increasing to 250° C in steps of 17° C/min. In a second step, the temperature was increased from 250° C to 300° C in steps of 2° C/min.

RESULTS AND DISCUSSION

In the first animal trial, weight gain, water and food intake, and water and food conversion of the treatment groups (with oral administration of dilutions of 'solution X') were analogous to those in the negative control group. Within the treatment groups, a sex-dependent effect of 'solution X' seemed to be present as female mice showed higher weight gain and food

TABLE I
Mean values for total weight gain, daily water and food intake, and daily water and food conversion per mouse (from the first animal trial for the negative control groups and the different treatment groups[a]

	Negative control (m)	Negative control (f)	P_xC_1 (m)	P_xC_1 (f)	P_xC_2 (m)	P_xC_2 (f)
Weight gain per mouse (g)	+1.5	+1.3	+0.3	+1.8	+0.4	+0.9
Water intake per mouse per day (ml)	5	4.4	5	4	5	4
Food intake per mouse per day (g)	5.3	2.6	3.4	2.5	3.7	2.7
Water conversion per mouse per day (g/ml)	0.30	0.29	0.06	0.45	0.08	0.22
Food conversion per mouse per day (g/g)	0.28	0.50	0.09	0.72	0.11	0.33

m, male; f, female
[a]P_xC_1: 150 µl 'solution X'/100 ml drinking water (after 6 days, 6 ml/100 ml)
P_xC_2: 3 ml 'solution X'/100 ml drinking water (throughout)

and water conversion in comparison with the male mice (Table I). However, no prominent macroscopic or microscopic changes were observed in the investigated organs. For the second trial, i.e. with the prepubertal female mice, animals of the treatment groups showed a weight gain analogous to that of the negative control group, but food and water conversion were higher. These indications were concomitant with anatomical changes of the female reproductive tract in one of the treated mice but were not confirmed histologically. ERα expression was also slightly higher in the treatment groups than in the negative control group, but the difference was minimal. Because the animal tests suggested an estrogenic effect, a strategy directed towards estrogenic properties for the elucidation of the identity of 'solution X' was adopted.

Results of the next step, YES, as shown in Figure 2, indicate a clear estrogenic effect for the extract A (1 ml of the extract obtained after Speedisk extraction) and for the second fraction of extract B (the remaining 1 ml of extract; fractionated) since the response was similar to that of βE_2. The EEF was calculated for extract A and for fraction B2. For extract A an EEF of 85.6 ± 15.1 ng E_2/L was found, while the second fraction B2 resulted in an EEF of 8.5 ± 3.2 ng E_2/L. For the other fractions B1 (see above), B3 and B4, no estrogenic effect was observed.

In an initial survey, extract A was analysed with LC-MS^n and GC-MS^n. Using GC or LC-MS without fractionation, the chromatogram was overloaded with peaks and background noise. Because of the complexity of the chromatogram, it was impossible to identify any analyte. After fractionation, the fraction showing estrogenic activity with the YES was analysed by GC-MS. The fraction was evaporated to dryness and injected after derivatization

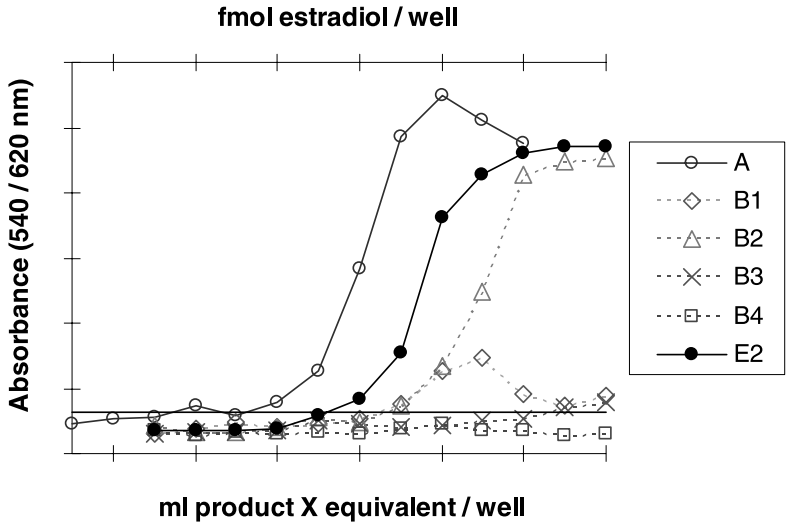

Figure 2. Response of the YES (yeast estrogenic screen) to extract A and to four HPLC fractions (B1–B4) of extract B after 7 days of incubation. E2, estradiol

with a mixture of MSTFA, ethanethiol and NH_4I (1 h at 60°C). After fractionation, the chromatograms were less complex and some substances could be identified. In fraction B2, 10 ng/L estrone (E_1) and 400 ng/L ethinylestradiol (EE_2) were identified (Figure 3). The extraction and fractionation procedure was repeated and the analytes were confirmed by a second laboratory using HFBA-derivatization. By using multiple mass spectrometry the identity was confirmed and structural information was also derived through the MS^n spectra (8,5 Identification Points (IPs), Council Directive 2002/657/EC).

A double check was performed by analysing a solution of 400 ng/L EE_2 and 10 ng/L E_1 with the YES. This double check confirmed that the estrogenic effect in the mixture or fraction was mainly caused by the presence of EE_2.

CONCLUSIONS

The present study showed a clear estrogenic effect of 'solution X' using the YES test, which was also suggested in the first animal trial with adult mice. In this trial, differences in weight gain, food intake and water intake were observed between male and female mice. However, no clear morphological differences were noted between the treatment groups and the control groups. Furthermore, anatomical and histological changes of the reproductive organs of the treated female mice could not be differentiated from normal cyclic reproductive changes of the control mice. This latter observation was further studied in a second trial with prepubertal mice, but no prominent anatomical or histological differences were noted between the treatment groups and the negative control group. Nevertheless, a mild increase

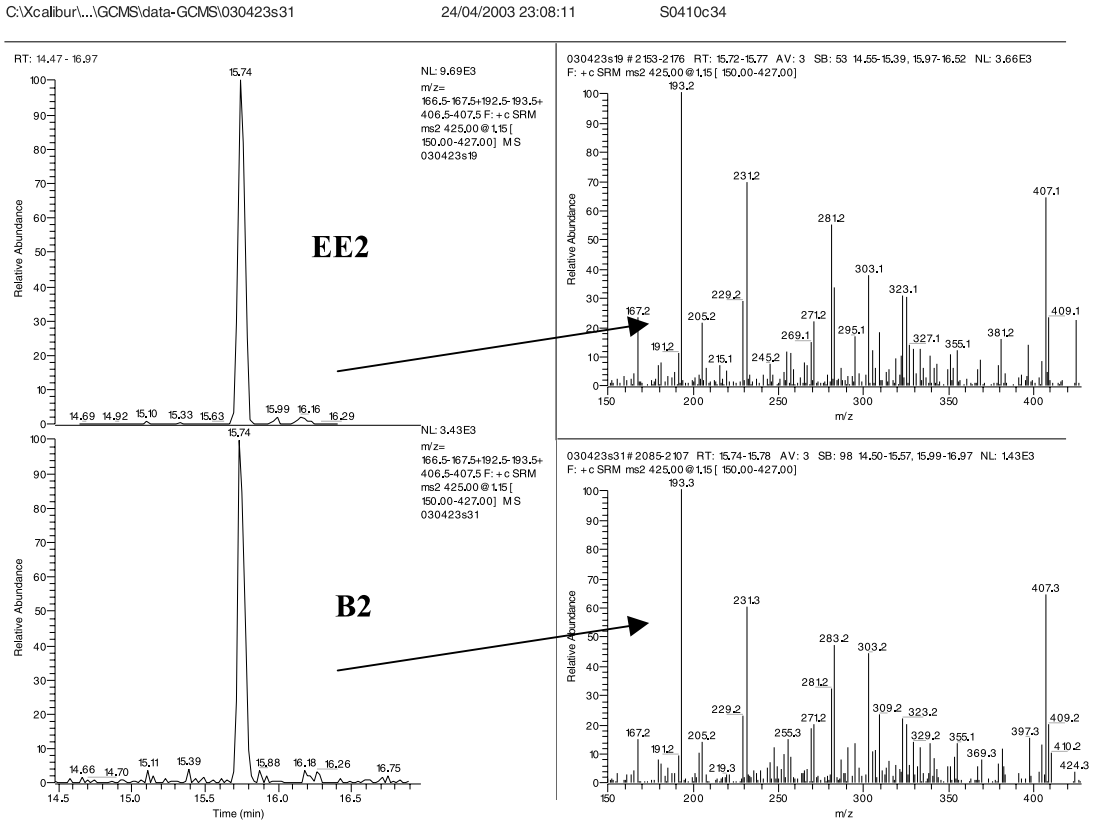

Figure 3. Chromatogram and MS^2 spectrum of the standard ethinylestradiol (EE_2) and the fraction B2 confirming the presence of EE_2 (parent ion, 425; diagnostic ions, 193, 231, 283, 303 and 407; 8,5 IPs, (96/23/EC))

in food and water conversion was present in the treated mice compared to the non-treated mice and ERα expression was also slightly higher in the treated group. For this, the animal trial was obligatory since the purpose of the study was to identify the nature and the possible effects of 'solution X'. HPLC-fractionation of the 'solution X' was necessary for a proper interpretation of the chromatograms obtained. In this way, matrix effects were avoided. A combination of LC-MS^n and GC-MS^n gave complementary information. Finally, the identities and structures of two analytes were confirmed as estrone and ethinyloestradiol. Thanks to the combination of different research disciplines, this survey has led to the successful identification of the analytes present in an 'unknown' product.

These findings confirm the need for a multidisciplinary approach in unravelling the function and structure of new products used in animal husbandry. The strategy can be used in the struggle against the misuse of a variety of products in animal husbandry.

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