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Determination of zeranol and β-zearalanol in calf urine by immunoaffinity extraction and gas chromatography–mass spectrometry after repeated administration of zeranol

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ABSTRACT

A method for the determination of zeranol and its metabolite β-zearalanol in bovine urine is described. It has been applied to samples from calves given multiple subcutaneous doses of zeranol. Samples were extracted with immunoaffinity columns containing antibodies raised against zeranol and were analysed by gas chromatography–mass spectrometry. The immunoaffinity columns were prepared by coupling immunoglobulin G fractions obtained from rabbit antisera with a Sepharose matrix. The immunizing agent was carboxybutylzeranol coupled to bovine serum albumin. Gas chromatography–mass spectrometry was performed in the negative-ion chemical ionization mode, after derivatization of the compounds to their pentafluorobenzyl ethers, and allowed detection of analytes with a sensitivity of 0.01 ppb in spiked urine. The derivatization method and the gas chromatographic determination were also applied to the similar compounds zearalanone, zearalenone and β-zearalenol. A synthesis of dideuterated zeranol and β-zearalanol by isotopic exchange is described. These deuterated analogues had an isotopic purity of more than 99% and were used for quantitation of zeranol and β-zearalanol by isotope dilution mass spectrometry. The recoveries of zeranol and β-zearalanol, using the immunoaffinity columns, were determined after extraction from spiked urine and were 84 and 64%, respectively. The urines of treated calves were collected for several days after treatments and were analysed after hydrolysis with β-glucuronidase and arylsulphatase. The samples showed variable but generally decreasing concentrations of zeranol and β-zearalanol. The levels of β-zearalanol ranged from <0.01 to 98 ppb and were 1.2–3.2 times higher than those of zeranol.

INTRODUCTION

Zeranol (α-zearalanol) is a weak synthetic estrogen obtainable, by chemical reduction, from the mycotoxin zearalenone (a compound produced by *Fusarium*

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moulds and present in contaminated stocks of cereals [1]). Zeranol may be administered to meat-producing animals as a growth promoter [1,2]. However, its use, as well as that of other anabolic agents, is prohibited in the member countries of the EEC and is the subject of a controversial discussion about the risk to public health, owing to the residues of the substances (and their metabolites) in foods coming from treated animals. In particular, estrogens and steroidal sex hormones are under special attention, because of their possible carcinogenicity after chronic low dose exposure [3,4].

Data already acquired about zeranol, zearalenone and their metabolites β-zearalanol (taleranol), zearalanone and β-zearalenol (all of them generally indicated as resorcyclic acid lactones, RALs), seem to indicate that these substances are non-genotoxic and weakly teratogenic and carcinogenic [1,2,5], but the studies so far published do not permit definitive conclusions.

Few data are also available about the levels of zeranol and its metabolites in biological fluids and in tissues of treated animals [1,2,6–10].

To contribute to the knowledge of the disposition of zeranol and other RALs in animals, and to give an accurate method for their detection after possible illegal use or contamination, we describe in this paper a procedure for the analysis of zeranol and β-zearalanol in urine and report the levels of these substances in calf urine after repeated subcutaneous injections of zeranol.

EXPERIMENTAL

Materials

Pure zeranol (α-zearalanol) and zearalanone and a mixture of zeranol and β-zearalanol (*ca.* 1:1, w/w) were from CRC (S. Giovanni al Natisone, Italy). Zearalenone, β-zearalenol, complete and incomplete Freund's adjuvant, and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, U.S.A.). All solvents, of analytical grade, were from Merck (Darmstadt, Germany) or Farmitalia Carlo Erba (Milan, Italy). Ethyl 5-bromovalerate and pentafluorobenzyl bromide (PFBBr) were from Aldrich (Milwaukee, WI, U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), deuterium chloride (20% solution in deuterated water) and monodeuteromethanol (CH_3OD) were from Fluka (Buchs, Switzerland). Protein-A-Sepharose and CNBr-Sepharose 4B were from Pharmacia (Uppsala, Sweden). Polypropylene columns ("Poly-prep") were from Bio-Rad (Richmond, CA, U.S.A.). Enzyme-linked immunosorbent assay (ELISA) microtitration plates were from Nunc (Roskilde, Denmark). Anti-rabbit IgG, peroxidase-conjugated, was from ICN (Cleveland, OH, U.S.A.). β-Glucuronidase–arylsulphatase from *Helix pomatia* was from Boehringer (Mannheim, Germany).

Preparation of antisera and immunoaffinity columns

To obtain an immunogenic compound, zeranol was carboxylated and conju-

gated to BSA. Zeranol carboxybutyl ether was prepared according to the method of Patel and Dixon [11] and was conjugated to BSA using the carbodiimide method [12]. The conjugate was characterized by UV spectroscopy, using the molar extinction coefficient of free zeranol to calculate the degree of substitution [13]. The zeranol-BSA conjugate was found to have a zeranol/protein molar ratio of 47:1.

Two New Zealand rabbits were immunized with 1 mg of zeranol-BSA in 1 ml of 0.15 *M* NaCl, emulsified with an equal volume of complete Freund's adjuvant. The emulsion was injected subcutaneously in multiple sites of the shaved back. The animals were boosted monthly with 0.5 mg of conjugate in incomplete Freund's adjuvant. One week after the second and subsequent injections blood samples were taken from the ear vein, and the serum titer was checked by ELISA, using zeranol-BSA and BSA as coating antigen and peroxidase-labelled anti-rabbit IgG, as second antibody. The antiserum used for the preparation of the immunoaffinity gel was collected after the third injection, and the titer in ELISA was 1:32 000.

The immunoaffinity gel was prepared as previously reported [14], by coupling purified IgG to CNBr-activated Sepharose 4B. The immunoaffinity columns were prepared by introducing 0.25 ml of this gel into polypropylene columns.

To begin the extraction procedure, the immunoaffinity columns were washed with 5 ml of acetone–water (95:5, v/v) followed by 5 ml of distilled water, then the samples (0.1–2 ml of centrifuged urine, diluted to 10 ml with 0.05 *M* phosphate buffer, pH 7.4) were loaded on the columns. The columns were capped and gently mixed for 5 min, and then the samples were allowed to elute slowly from the gel. After washing with 10 ml of phosphate buffer and 10 ml of distilled water, the immunoadsorbed substances were eluted with two 1.5-ml volumes of acetone–water (95:5, v/v). The columns were finally washed with 10 ml of elution solvent and with distilled water, and were used for other extractions or stored at 4°C in phosphate buffer (0.05 *M*, pH 7.4) containing 0.02% thimerosal.

The columns were used for *ca.* 30 extraction cycles without appreciable decline in performances. Experience in our laboratories, also with other types of immunoaffinity column, has shown that these columns may last for up to 100 extractions, if properly used.

Synthesis of deuterated zeranol and β-zearalanol

Deuterated zeranol and β-zearalanol (zeranol-D_2 and β-zearalanol-D_2) were synthesized for use as internal standards, to allow an accurate quantitation using isotope dilution mass spectrometry. Isotopic exchange of the two aromatic hydrogens in the positions 13 and 15 of the molecules was achieved by aromatic electrophilic substitution in the following way: 2 mg of the standard mixture of zeranol and β-zearalanol were dissolved in 125 μl of a solution of deuterium chloride in deuterated water (*ca.* 0.5 *M*) and 500 μl of monodeuteromethanol; the solution was allowed to stand for six days at 60°C in a tightly closed glass vessel.

The solvents were evaporated, and the residue was redissolved in 1 ml of ethyl acetate. This solution was directly used for the preparation of diluted standards used for spiking of samples.

The isotopic purity of the compounds was checked by gas chromatography–mass spectrometry (GC–MS) and was more than 99%. Under the reaction conditions described no degradation products of either zearalanols were found.

Derivatization and GC–MS

Standard solutions of RALs and samples extracted with immunoaffinity columns were dried in a stream of air at 60°C in conical tubes and derivatized with PFBBr and BSTFA to give bispentafluorobenzyl (PFB_2) or bispentafluorobenzyltrimethylsilyl (PFB_2-TMS) derivatives, which were suitable for high-sensitivity detection with gas chromatography combined with negative-ion chemical ionization mass spectrometry (GC–NICI-MS). The derivatization method was the same as that described for diethylstilbestrol and other estrogens [14]. Briefly, 50 μl of a solution of PFBBr in acetonitrile (1:20, v/v) and 50 μl of a solution of potassium hydroxide in anhydrous ethanol (8 mg/ml) were added to the dried samples and heated at 60°C for 30 min. After evaporation of the solvents the samples were redissolved in 20–50 μl of BSTFA, heated at 60°C for 30 min, and injected into the gas chromatograph.

Instrumental analysis was performed on a VG TS-250 mass spectrometer, equipped with a HP 5890 gas chromatograph and a 11-250J data system. GC conditions were as follows: oven temperature raised from 160°C (1 min) to 325°C at 15°C/min; injector temperature, 240°C; splitless mode. The column was a CP Sil 5 CB (Chrompack Italia, Cernusco sul Naviglio, Italy; 25 m × 0.32 mm I.D., film thickness 0.12 μm) operated with a head-pressure of 30 kPa of helium and with a flow-rate of 2 ml/min. The mass spectrometer conditions were: source temperature, 180°C; electron energy, 60–90 eV (CI negative) and 35 eV (electron impact); emission current, 1000 μA (CI negative); trap current, 500 μA (electron impact). Methane or isobutane, which gave similar NICI mass spectra, was used as the reactant gas for the formation of negative ions by electron capture. Isobutane gave a slightly better detection sensitivity of analytes, but its use required frequent cleaning of the ion source; methane was thus preferred for routine operations.

Treatment of animals

Three calves (two females, one male) were treated six times with subcutaneous injections of zeranol at intervals of ten days, and urine samples were collected every three days. The first three injections were made with 30 mg of zeranol in 1 ml of coconut oil, and the last three injections with 15.6 mg of zeranol. After the sixth injection the urine samples were collected for 28 days following the treatment. Samples were analysed after hydrolysis with β-glucuronidase and arylsulphatase (100 μl/ml of urine, 16 h at 38°C in 0.05 *M* acetate buffer, pH 4.5).

Treatment by multiple subcutaneous injections was used in this study because it is a procedure illegally used in Italy as an alternative to tablet implantations behind the ear (which are more easily detected by legal authority controls).

RESULTS AND DISCUSSION

Immunoaffinity extraction and GC–MS quantitation

The efficiency of the immunoaffinity extraction was checked by measuring the percentage recoveries of the RALs and the capacity of the columns when loaded with large amounts or zeranol. The percentage recoveries obtained are listed in Table I, together with the cross-reactivities (measured by ELISA) of the anti-zeranol antibody with the RALs. The recoveries were measured by extracting 1-ml samples of blank urine spiked with a known amount of each RAL (1 ng) and adding, after extraction, the deuterated zearalanol mixture as an external standard (1 ng of zeranol-D_2 plus 1 ng of β-zearalanol-D_2). Calculations were made by comparing the ratios between the GC peak areas (A) of the non-deuterated and deuterated compounds (A_{D0}/A_{D2}), obtained for these recovery samples and for derivatized standards containing known amounts of all the compounds [14].

The maximum capacity of the columns was measured in the same way by loading 0.5 and 5 μg of zeranol on the columns (containing 0.25 ml of gel) and adding 1 μg of zeranol-D_2 after extraction. In both cases 0.1 μg of zeranol was recovered, corresponding to a capacity of 0.4 μg per ml of gel.

The use of the PFB derivatives of the RALs allowed high sensitivity detection with GC–NICI-MS, as can be deduced from the poor fragmentation of NICI spectra, with respect to EI spectra (Fig. 1). Detection was done by selected-ion recording (SIR) of the most intense NICI fragments, corresponding always to the loss of a PFB group ($M - 181$): m/z 497 for zearalenone, m/z 499 for zearala-

TABLE I

RECOVERIES OF RESORCYLIC ACID LACTONES FROM SPIKED URINE WITH ANTI-ZERANOL IMMUNOAFFINITY COLUMNS, AND CROSS-REACTIVITIES OF THE ANTI-ZERANOL ANTIBODY WITH THE SAME COMPOUNDS

Compound	Recovery (mean ± S.D.; $n = 4$) (%)	Cross-reactivity[a] (%)
Zeranol	84 ± 6.4	100
β-Zearalanol	64 ± 18	30
Zearalanone	25 ± 17	2
Zearalenone	13 ± 10	0.1
β-Zearalenol	40 ± 24	5

[a] Measured by ELISA.

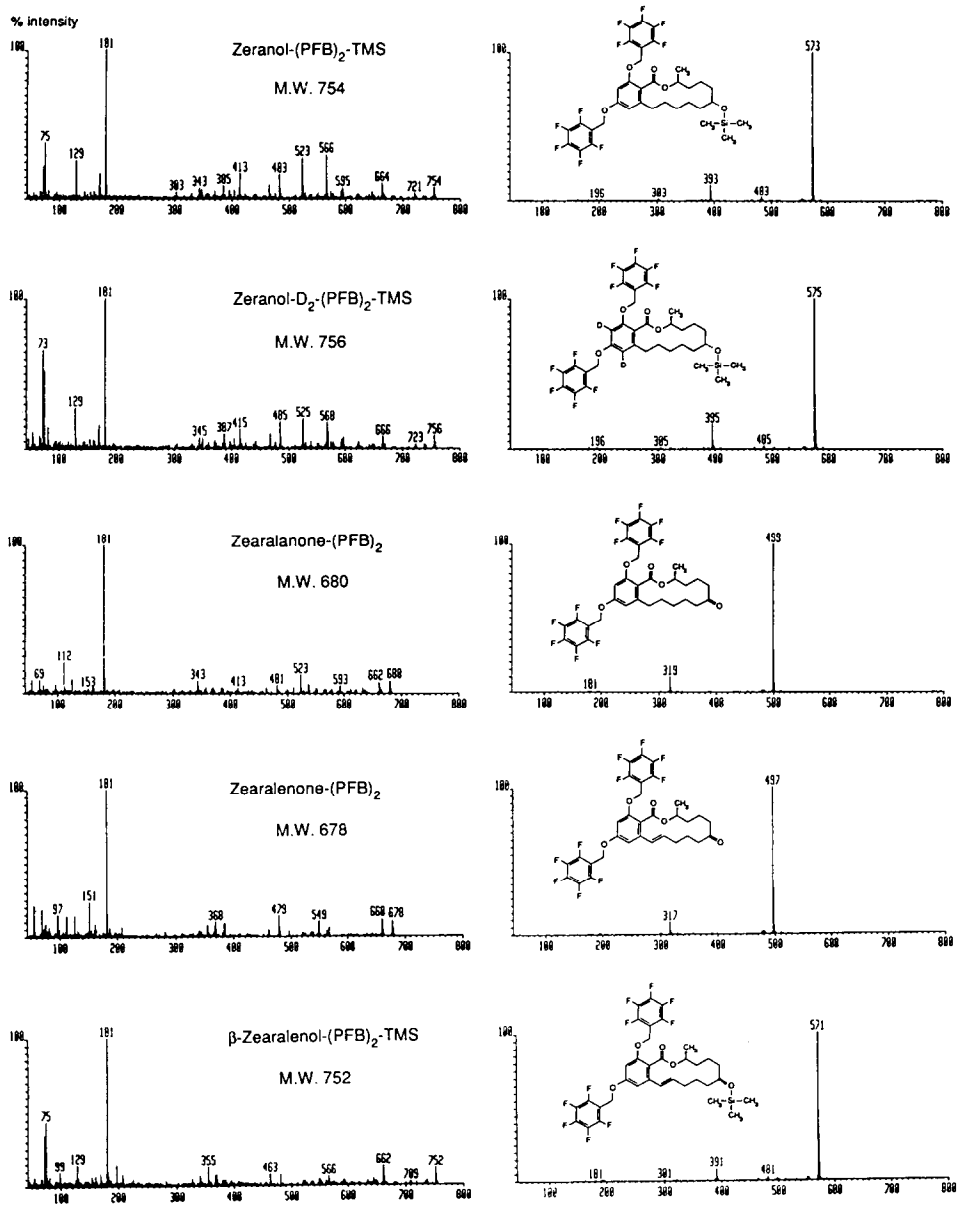

Fig. 1. Negative-ion chemical ionization (right) and electron-impact (left) mass spectra of bispentafluorobenzyl (PFB_2) and bispentafluorobenzyltrimethylsilyl (PFB_2-TMS) derivatives of the resorcylic acid lactones indicated. *x*-Axes, *m/z* values.

none, *m/z* 571 for β-zearalenol, *m/z* 573 for zeranol and β-zearalanol and *m/z* 575 for zeranol-D_2 and β-zearalanol-D_2.

The chromatographic characteristics of the PFB derivatives were also excellent. All the compounds gave well defined peaks with GC–SIR and had sufficient-

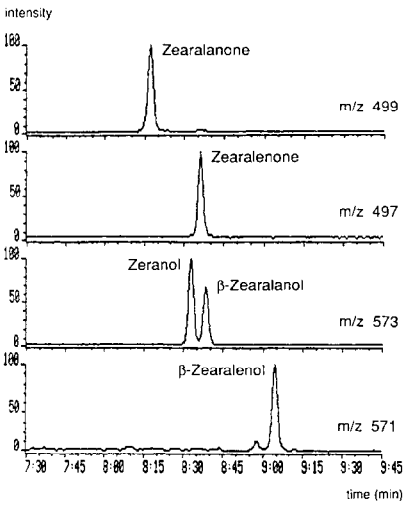

Fig. 2. Selected ion recording tracings of 20 pg of each of the resorcylic acid lactones indicated. Conditions were as described in Experimental.

ly long retention times, to elute after the most chromatographic interferences (Fig. 2). The sensitivity obtained by analysing blank urine samples (2 ml) spiked with zeranol and β-zearalanol was *ca.* 0.01 ppb.

The quantitation of unknown calf urines was made after isotopic dilution of samples with deuterated zeranol and β-zearalanol. Owing to the use of these internal standards, containing only two deuterium atoms, a contribution from the non-deuterated zeranol and β-zearalanol (with the nominal ion m/z 573), arising from natural isotopic abundances, was present in the chromatographic SIR traces of the deuterated analogues at m/z 575. The calculated relative natural abundance (N_a) of the m/z 575 ion of zeranol and β-zearalanol is 11.06% of the most abundant ion (m/z 573). This abundance was also found experimentally, and must be accounted for in a correct quantitation of samples. This was done in the following way: the ratio R of the peak areas of zeranol (or β-zearalanol) and zeranol-D_2 (or β-zearalanol-D_2) calculated from the chromatograms, and which must be used for quantitation, was corrected with the formula $R_c = 100\ R/(100 - N_a R)$, where R_c is the corrected ratio. Calibration curves were obtained by plotting these corrected ratios, obtained for standards containing variable amounts of zeranol and β-zearalanol (0.010–20 ng) and a constant amount of zeranol-D_2 and β-zearalanol-D_2 (2 + 2 ng), against absolute amounts of zeranol and β-zearalanol. These curves gave standard errors of 1.3 and 2.2% for zeranol and β-zearalanol, respectively. For zearalanone, zearalenone and β-zearalenol, a correct quantitation in urine samples was not possible, because the recovery was too low and variable. The determination of these compounds would require the production of their antibodies and immunoaffinity columns and of their deuterated analogues.

Urine samples

Because zeranol and other RALs have been reported to be excreted in urine mainly as conjugates [1,2,6,7,9], the samples collected from treated animals were analysed after hydrolysis with β-glucuronidase and arylsulphatase, as reported in Experimental. Chromatograms obtained from one of these samples are given in Fig. 3.

The levels of zeranol and β-zearalanol found for the three treated calves are reported in Table II. The concentrations of the compounds tend to decrease with time after every treatment, although there are some exceptions, which may be due to variable diuresis in different animals on different days, and to individual char-

TABLE II

ZERANOL AND β-ZEARALANOL LEVELS IN CALF URINE AFTER TREATMENT WITH SUBCUTANEOUS INJECTIONS OF ZERANOL IN OIL

Day	Treatment	Concentration (ppb)					
		Calf 1 (female)		Calf 2 (female)		Calf 3 (male)	
		Zeranol	β-Zearalanol	Zeranol	β-Zearalanol	Zeranol	β-Zearalanol
1[a]	30 mg Zeranol	–[b]	–[b]	<0.01	<0.01	<0.01	<0.01
4		26	31	48	54	12	23
8		27	33	40	48	35	78
11[a]	30 mg Zeranol	0.26	0.64	1.8	2.7	–[b]	–[b]
15		2.1	5.6	7.0	11	7.2	17
18		–[b]	–[b]	51	69	6.0	13
22[a]	30 mg Zeranol	0.17	0.24	2.8	4.2	0.13	0.28
26		30	60	29	43	24	81
29		7.4	8.9	34	57	33	98
32[a]	15.6 mg Zeranol	3.9	5.4	24	32	26	75
36		18	32	40	55	10	30
39		29	46	32	48	3.0	6.2
43[a]	15.6 mg Zeranol	0.63	2.7	0.89	1.7	0.12	0.33
46		5.7	7.7	12	15	–[b]	–[b]
50		0.81	2.7	1.6	2.4	0.27	0.86
53[a]	15.6 mg Zeranol	0.47	0.80	0.12	0.27	0.074	0.23
57		2.1	4.3	22	39	15	30
60		2.3	4.8	14	22	9.3	13
64		0.046	0.11	5.3	8.3	0.22	0.63
67		0.18	0.33	1.8	2.9	–[b]	–[b]
74		0.063	0.14	0.079	0.11	0.066	0.21
81		<0.01	<0.01	0.069	0.13	0.055	0.075

[a] Samples in these days were collected before the treatments with zeranol.

[b] Samples not collected.

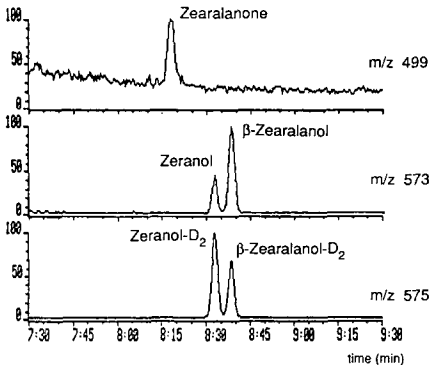

Fig. 3. Selected-ion recording tracings of a urine sample from a male calf treated with zeranol, showing, after 21 days from the last treatment and 74 days from the first treatment, the presence of the metabolites β-zearalanol and zearalanone (see Table II). The sample was hydrolysed with β-glucuronidase and arylsulphatase, and extracted with an immunoaffinity column with deuterated zeranol and β-zearalanol as internal standards. The calculated concentrations of zeranol and β-zearalanol were 0.066 and 0.21 ppb, respectively; zearalanone was not quantitated because its recovery was too low. Internal standards were added at a concentration of 1 ppb. Other conditions were as described in Experimental.

acteristics. The results also show that the relative concentration of β-zearalanol with respect to that of zeranol is variable and ranges from 1.2 to 3.2.

Zearalanone was found in urine samples but not quantitated for the reasons reported above.

The results obtained seem to be in accordance with the data reported in the literature, where β-zearalanol is reported to be a major metabolite of zeranol in cows, rats, rabbits, monkeys, dogs and humans [7]. In all these species zeranol is absorbed and excreted readily after oral doses, but the relative amounts of zeranol and metabolites are reported to vary greatly.

CONCLUSIONS

The method described has been shown to be able to detect zeranol and β-zearalanol in urine with high sensitivity and to be useful for disposition and metabolism studies. The method may be further developed for its applicability to the analysis of other biological samples, such as serum and homogenized tissues, and to the determination of other RALs, provided that the corresponding antibodies can be obtained.

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REFERENCES

1 D. G. Lindsay, *Food Chem. Toxicol.*, 23 (1985) 767.
2 B. Hoffman and P. Evers, in A. G. Rico (Editor), *Drug Residues in Animals*, Academic Press, Orlando, FL, 1986, p. 111.
3 IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 21, *Sex Hormones (II)*, International Agency for Research on Cancer, Lyon, 1979, p. 173.
4 C. A. Sàenz de Rodriguez and M. A. Toro-Solà, *Lancet*, i (1982) 1300.
5 J. J. Li and S. A. Li, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 46 (1987) 1858.
6 M. Metzler, *J. Chromatogr.*, 489 (1989) 11.
7 B. H. Midgalof, H. A. Dugger, J. G. Heider, R. A. Coombs and M. K. Terry, *Xenobiotica*, 13 (1983) 209.
8 R. Maffei Facino, M. Carini, A. Da Forno, P. Traldi and G. Pompa, *Biomed. Environ. Mass Spectrom.*, 13 (1986) 121.
9 T. R. Covey, D. Silvestre, M. K. Hoffman and J. D. Henion, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 45.
10 S. N. Dixon, K. L. Russel, R. J. Heitzmann and C. B. Mallinson, *J. Vet. Pharmacol. Ther.*, 9 (1986) 353.
11 P. J. Patel and S. N. Dixon, *Food Addit. Contam.*, 6 (1989) 91.
12 G. E. Abraham and P. K. Grover, in W. D. Odell and W. Daughaday (Editors), *Principles of Competitive Protein-Binding Assays*, Lippincott, Philadelphia, PA, 1971, p. 140.
13 B. F. Erlanger, F. Borek, S. M. Beiser and S. Lieberman, *J. Biol. Chem.*, 228 (1957) 713.
14 R. Bagnati, M. G. Castelli, L. Airoldi, M. Paleologo Oriundi, A. Ubaldi and R. Fanelli, *J. Chromatogr.*, 527 (1990) 267.