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Comparison of different liquid chromatography methods for the determination of corticosteroids in biological matrices

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Abstract

Various extraction techniques can be combined with column liquid chromatography (LC) and ultraviolet (UV) or mass spectrometric (MS) detection for the determination of synthetic corticosteroids in biological matrices. Target analysis of low concentrations of 25 μg/kg of dexamethasone in feed can be performed by combining immunoaffinity chromatography (IAC) and LC with UV detection. A straightforward multi-analyte procedure is obtained by tandem solid-phase extraction (SPE) and subsequent LC–UV. However, the limits of detection for feed samples are then relatively poor, viz. 100 μg/kg. A multi-analyte method which meets modern demands of about 5 μg/kg detection limit requires one-step SPE combined with LC–MS analysis. As regards urine corticosteroids can be determined down to a level of 0.5 μg/l by either SPE–LC–MS–MS or SPE(IAC)–LC–MS.

Keywords: Solid-phase extraction; Corticosteroids; Steroids

1. Introduction

Within the European Union (EU) the use of synthetic corticosteroids as growth promoters in livestock breeding is prohibited. However, the use of these compounds continues because of commercial reasons, in order to produce meat that is more appealing to consumers because of its juicy and lean look. Corticosteroids are often added illegally during the reconstitution of milk replacers, immediately before feeding of veal calves. They influence both water retention in meat and lipid metabolism by a permissive facilitation of the effects of other illegal growth-promoting agents, such as growth hormones and beta-adrenergic receptor agonists, in inducing lipolysis [1]. To prevent possible consequences on animal welfare and consumer health, analytical strategies are needed to monitor the use of these compounds by the control of animal feeds and biological samples like meat and urine.

For a long time, methods based on gas chromatography (GC), especially in combination with mass spectrometry (GC–MS), have been preferred for the determination of corticosteroids, despite the long sample preparation and the need for derivatisation or oxidation of the analytes. Courteyn et al. [2] described a method for the determination of dexamethasone in urine and faeces of treated cattle with GC and negative chemical ionisation-MS after oxidation of the analyte to the 11,17-diketo derivative

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using pyridinium chlorochromate. The detection limit was 0.2 μg/l for urine. Delahaut et al. [3] reported the quantification of several synthetic corticosteroids by GC–MS after purification of the extracts with solid-phase extraction with immunoaffinity chromatography [SPE(IAC)] and subsequent oxidation to the diketo derivatives.

Recent developments with respect to immunoaffinity chromatography (IAC) [3,4] and the use of new sorbents for solid-phase extraction (SPE) which reduce the time of sample preparation are of great interest if sample throughput has to be improved. In addition, with the advent of mature liquid chromatography–mass spectrometry (LC–MS) techniques, the interest in LC-based separation procedures for the corticosteroids has increased [5,6]. Such an approach makes oxidation or derivatisation superfluous [7–9].

This paper discusses the potential and limitations of different LC-based procedures for the determination of corticosteroids in animal feed and bovine urine. The synthetic corticosteroids selected for the study were dexamethasone (DEX), flumethasone (FLU) and triamcinolone acetonide (TRIA) (see Fig. 1). DEX was selected because it is frequently used as an illegal growth promoter [4], FLU because it is one of the pharmacologically most active drugs among the group of corticosteroids and TRIA because it is resistant to oxidation and can, therefore, not be determined by the frequently used GC–MS methods based on oxidation with pyridinium chlorochromate [2,3].

As regards the sample types selected for the control of the misuse of corticosteroids, animal feed was selected because the corticosteroids are used as feed additives, and bovine urine because residues of the synthetic corticosteroids are excreted in urine. Moreover, the analysis of urine fits well into most residue control programmes, both at the slaughterhouse and in live animals. The methods to be developed for the analysis of animal feed should allow detection at levels as low as 100–500 μg/kg because the addition of such low dosages to feeds has been shown to cause an increased live weight gain and a reduced feed conversion [1]. The expected concentrations in urine are at the low-μg/l level (0.5–5 μg/l).

2. Experimental

2.1. Materials

2.1.1. Chemicals and reagents

The synthetic corticosteroids dexamethasone, flumethasone and triamcinolone acetonide were obtained from Sigma–Aldrich Chemie (Zwijndrecht, Netherlands); acetic acid and sodium acetate were from Merck (Amsterdam, Netherlands); β-glucuronidase/sulfatase (suc d'Helix Pomatia containing 100 000 units β-glucuronidase and 100 000 units sulfatase per ml) was obtained from Brunschwig Chemie (Amsterdam, Netherlands), and methanol, ethanol, *tert.*-butyl methyl ether (TBME), sodium hydroxide and acetonitrile from J.T. Baker (Deventer, Netherlands).

Waters-Oasis SPE columns [Waters-Oasis is a patented, water-wettable hydrophilic–lipophilic-bal-

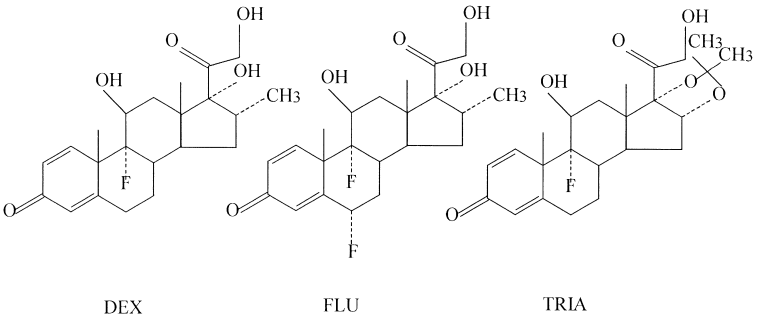

Fig. 1. Structures of the three corticosteroids: Dexamethasone (DEX; M_r 392), flumethasone (FLU; M_r 410) and triamcinolone acetonide (TRIA; M_r 434).

anced (HLB) copolymer] were purchased from Waters (Etten-Leur, Netherlands). Disposable amino-SPE columns (NH2; 1 g) were obtained from Alltech/Applied Science (Breda, Netherlands) and styrene–divinylbenzene PS–DVB columns from J.T. Baker.

Immunoaffinity chromatography extraction columns containing an agarose gel carrying immobilised antibodies which are specific for DEX, FLU and betamethasone were purchased from Randox Labs. (Ardmore, UK).

2.1.2. Solutions

Stock solutions containing 1 mg/ml of the corticosteroids were prepared in methanol and stored at −20°C. Working solutions were prepared by sequential 10-fold dilutions of the stock solutions to a single series of appropriate standard solutions. These solutions were stored in the dark at about 4°C (range, 1–10°C) for a period of maximum 6 months.

The 2 mol/l acetate buffer of pH 5.2 used to adjust the pH of the urine samples was prepared by dissolving 25.2 g acetic acid and 129.5 g sodium acetate in 800 ml of water. After adjusting the pH to 5.2±0.1, water was added to a final volume of 1 l. For a 5 m*M* ammonium acetate solution, 0.35 g ammonium acetate was dissolved in 1 l water.

2.1.3. Samples

Samples of animal feed and bovine urine were collected by the Dutch Inspectorate for Health Protection, Commodities and Veterinary Public Health (Inspectie W&V, The Hague, Netherlands) at feed factories and slaughterhouses. The samples of bovine urine were received in frozen condition and were kept frozen (−20°C) until analysis. The feed samples were stored in the refrigerator (4°C) until analysis.

2.1.4. Equipment

Three LC systems were used. One LC system consisted of a WISP (Waters) autosampler, with Pharmacia (Amersham–Pharmacia Biotech, Roosendaal, Netherlands) pumps and a ThermoQuest (Breda, Netherlands) multi-channel UV detector. Separations were obtained under isocratic conditions using a 125×4 mm I.D., 4 μm Superspher 100 RP 18e column (Waters) and methanol–water (50:50, v/v) at 0.8 ml/min as eluent; UV detection was at 242 nm.

The second LC system consisted of a Hewlett-Packard (Amstelveen, Netherlands) 1100 system, a 1100 MSD mass-selective detector and HP LC–MSD Chemstation software for control and data processing. Separations were obtained on a 150×2.1 mm I.D., 5 μm Alltima column (Alltech). The step gradient used (solvent A, water; solvent B, acetonitrile; flow, 0.3 ml/min) was: 0–10 min: 35% B, 10–12 min linear increase to 85% B; 13.0–13.1 min linear increase to 100% B, with final hold of 100% B for 1 min. For MS detection, atmospheric pressure chemical ionisation (APCI) was used, which turned out to be a more sensitive ionisation technique for the corticosteroids than electrospray ionisation. The acquisition parameters were optimised in the APCI(+) mode, by flow injection analysis of 0.01 mg/ml standard solutions. The final conditions were: flow-rate, 0.3 ml/min; vaporiser temperature, 325°C; nebuliser pressure, 60 p.s.i.; drying gas temperature, 300°C; drying gas, 4 l/min; capillary voltage, 2000 V; corona current, 10 μA; fragmentor, 70 (1 p.s.i.= 6894.76 Pa). For selected ion-monitoring (SIM), m/z values of 393 (DEX), 411 (FLU) and 435 (TRIA) were used.

The third LC system consisted of an LC (ion trap) MS^n LCQ (ThermoQuest) system and an Alliance (Waters) pump and autosampler. Separations were obtained at 35°C under isocratic conditions using a 125×4 mm I.D., 4 μm Superspher 100 RP 18e column and acetonitrile–water (35:65, v/v) at 0.8 ml/min as eluent. Washing with methanol was performed after every sample injection. The LCQ was used in the MS–MS mode for screening and in the MS–MS/MS^3 mode for confirmation. Because of the limited sensitivity, no higher MS mode than MS^3 could be used.

Acquisition parameters were optimised by 0.8 ml/min infusion of 0.01 mg/ml standard working solutions. The final conditions were: vaporiser temperature, 500°C; capillary temperature, 150°C; high purity nitrogen, 70 p.s.i. The selection of the diagnostic ions in the ion-trap LCQ is discussed in the relevant section of results and discussion below.

2.2. Methods

2.2.1. Corticosteroids in feed

2.2.1.1. Sample pretreatment and TBME extraction

After homogenisation of the animal feed sample, 2 g were weighed into Corex extraction tubes. The samples were fortified with 100–500 ng of each corticosteroid and equilibrated for 2 h at room temperature. After equilibration the samples were extracted with 10 ml of TBME. After 20 min of rotation (using a Heidolph RAEX2 rotating apparatus) the samples were centrifuged for 10 min at 1000 *g*. The clear TBME phases were collected and the extraction with TBME was repeated.

2.2.1.2. SPE(NH_2) procedure

The combined TBME extracts (approx. 20 ml) were applied to a SPE(NH_2) column which had been conditioned with 5 ml of TBME. After washing the column with 2×5 ml of TBME, the analytes of interest were eluted with 4 ml of methanol–water (80:20, v/v). The eluate was evaporated to dryness at 45°C under a stream of nitrogen and the residue resuspended in 200 μl of methanol and 1.5 ml of water.

2.2.1.3. SPE(PS–DVB) procedure

The SPE(NH_2) eluate was applied to a SPE(PS–DVB) column (conditioned with 1 ml of methanol and 1 ml of water). After washing the column with 1 ml of methanol–water (5:95, v/v), the column was dried by applying vacuum and washed with 1 ml of hexane–dichloromethane (80:20, v/v) and, next, 1 ml of hexane–ethyl acetate (90:10, v/v). The analytes of interest were eluted from the column with 2×1 ml of hexane–ethyl acetate (10:90, v/v).

2.2.1.4. SPE(IAC) procedure

3 ml of water was added to the resuspended SPE(NH_2) extract. The SPE(IAC) procedure was performed as described by the manufacturer. Briefly, the IAC (Randox, article No. DM 2185) column was conditioned with 15 ml of wash buffer and 5 ml of water (wash and storage buffers were delivered with the IAC columns) and next the sample extract was loaded onto the column. After washing with 10 ml of wash buffer and 5 ml of water, the corticosteroids were eluted with 4 ml of water–ethanol (30:70, v/v) of pH 5 (HCl). The IAC column can be regenerated with 10 ml of water–ethanol (70:30, v/v) of pH 5 and 15 ml of diluted wash buffer.

2.2.1.5. LC procedure

The SPE(PS–DVB) or SPE(IAC) eluate was evaporated to dryness at 45°C under a stream of nitrogen and the residue resuspended in 400 μl of the LC eluent. After 30 s of vortexing, 50 μl were injected on the LC column and separated, with detection at 242 nm. When MS detection was used, detection was by means of selected ion monitoring (DEX, *m/z* 393, FLU, *m/z* 411 and TRIA, *m/z* 434) in the APCI(+) mode.

2.2.2. Corticosteroids in urine

2.2.2.1. Hydrolysis

5 ml of bovine urine was fortified with 5–50 μl of the corticosteroid standard mixture (1 ng/μl). After 30 min of incubation at room temperature, the pH of the urine sample was adjusted to 5.2 with dilute acetic acid or 1.0 mol/l NaOH, and 1 ml of 2 mol/l acetate buffer was added followed by 0.1 ml of β-glucuronidase–sulfatase mixture. Next the sample was incubated for 2 h at 37°C.

2.2.2.2. SPE(Oasis)

The hydrolysed samples were applied to a SPE(Oasis) column. The column was conditioned with 3 ml of methanol and 3 ml of water and washed twice with 3 ml of methanol–0.02 mol/l NaOH (40:60, v/v) and, finally, with 3 ml of water. The analytes were eluted with 1.5 ml of methanol.

2.2.2.3. SPE(IAC)

The SPE(Oasis) eluate was evaporated at 45°C under a stream of nitrogen and resuspended in 200 μl of methanol and 4.8 ml of water. The extract was applied to an IAC column and treated as described above for feed analysis.

2.2.2.4. LC–MS

After evaporation of the SPE(Oasis) or SPE(IAC) eluate under a stream of nitrogen at 45°C, the dried extract was redissolved in 50 μl of methanol and vortexed for 30 s. Next, 150 μl of water was added

and a 100 μl aliquot was analysed by LC–MS in the SIM mode. When using MS–MS/MS^3 for confirmation, 100 μl of the final extract was analysed by LC–MS using the LCQ with MS–MS/MS^3 detection for DEX, *m/z* (373/355), FLU, *m/z* (391/371) and TRIA, *m/z* (415/357). Selection of ions is discussed in the relevant section of results and discussion.

2.2.3. Quantification and validation

For quantification, a detector response vs. concentration plot was constructed. To this end, five blank samples of feed or urine were fortified with different concentrations of the specific corticosteroid. The feed or urine samples were analysed together with the calibration samples; concentrations were calculated using the linear regression method.

For the determination of the method validation parameters, repeatability, within-laboratory reproducibility and the recovery percentages, a sample fortified with each of the analytes at 200 μg/kg for feed or 2 μg/l for urine was analysed on three consecutive days by each of the procedures used. Every day five replicates were analysed.

3. Results and discussion

3.1. Analytical strategy

The analytical methods that will be discussed and compared are based on the strategy shown in Fig. 2. Two different types of method can be defined in conformity with the definitions of the revised version of EU document 93/256 [10]. Screening methods are methods that are used to detect the presence of an analyte or class of analytes at the level of interest. Confirmatory methods are methods that provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest.

In the present study liquid–liquid extraction (LLE) and, subsequently, clean-up by SPE and separation and detection by LC–UV or LC–MS were used for screening purposes. For confirmation purposes, combinations of LLE/SPE and IAC-based SPE were used for extraction and clean-up, prior to separation and detection by LC–MS or LC–MS^n.

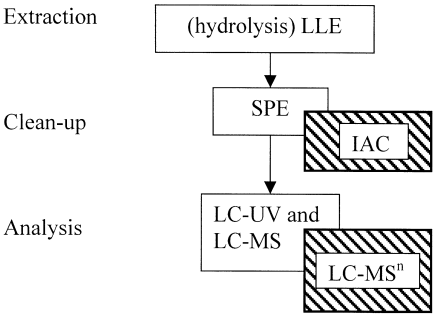

Fig. 2. Strategic approach of trace-level determination of corticosteroids. Shaded blocks: IAC and LC–MS^n, techniques introduced for additional selectivity.

The SPE(IAC) and MS^n procedures were introduced to obtain better selectivity during confirmation. The specific combination of analytical steps selected in each case depended on the matrix to be analysed, the analytes to be detected and the expected level of interest.

The LC separations were performed on a C_{18}-bonded silica column, with mixtures of acetonitrile–water or methanol–water as eluent. With acetonitrile–water, baseline separation of DEX and FLU was difficult to obtain. Although this would not create problems in case of MS detection (DEX $[MH]^+$, *m/z* 393, FLU $[MH]^+$, *m/z* 411), for LC–UV baseline separation was necessary because the coeluting corticosteroids have closely similar UV spectra. Fortunately, with methanol–water (50:50, v/v) baseline resolution of FLU and DEX was obtained. It is interesting to add that the elution order changed from DEX–FLU–TRIA to FLU–DEX–TRIA upon replacing acetonitrile by methanol. With the latter modifier, the retention times in the LC–UV system were 11.0, 12.8 and 13.9 min, respectively. (Retention times for the LC–MS and LC–MS^n systems can be read from Figs. 3c and 5.)

For screening purposes, the MS–MS ion was preferred to the MS ion: although the latter has a higher intensity, its selectivity is insufficient at the low (μg/l) level of interest. The MS–MS ion provides sufficient sensitivity to reach the required detection limits, and has much better selectivity. The strategy for confirmation purposes will be explained in the section on applications below.

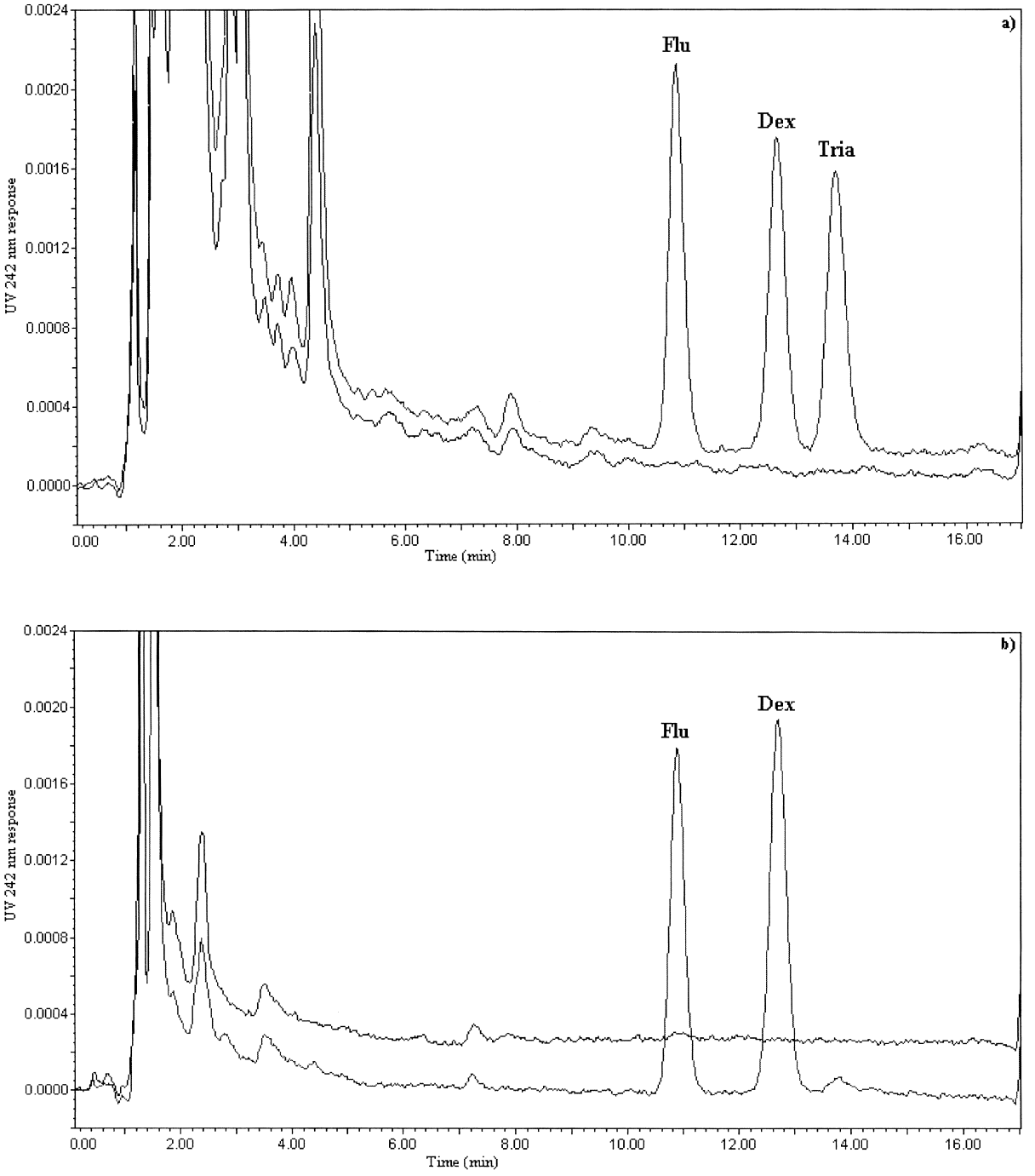

Fig. 3. LC–UV_{242} of (a and b) blank feed and feed fortified with 200 μg/kg of each corticosteroid. Clean-up involved SPE(NH_2) and, next (a) SPE(PS–DVB) or (b) SPE(IAC). LC conditions: 125×4 mm, 4 μm Superspher 100 RP 18e column and methanol–water (50:50 v/v) at 0.8 ml/min. LC–MS using SIM of (c) feed fortified with 10 μg/kg of each corticosteroid. Clean-up SPE(NH2) ions monitored: MS ion of DEX *m/z* 393, MS ion of FLU *m/z* 411 and MS ion of TRIA *m/z* 435. For experimental details, see text.

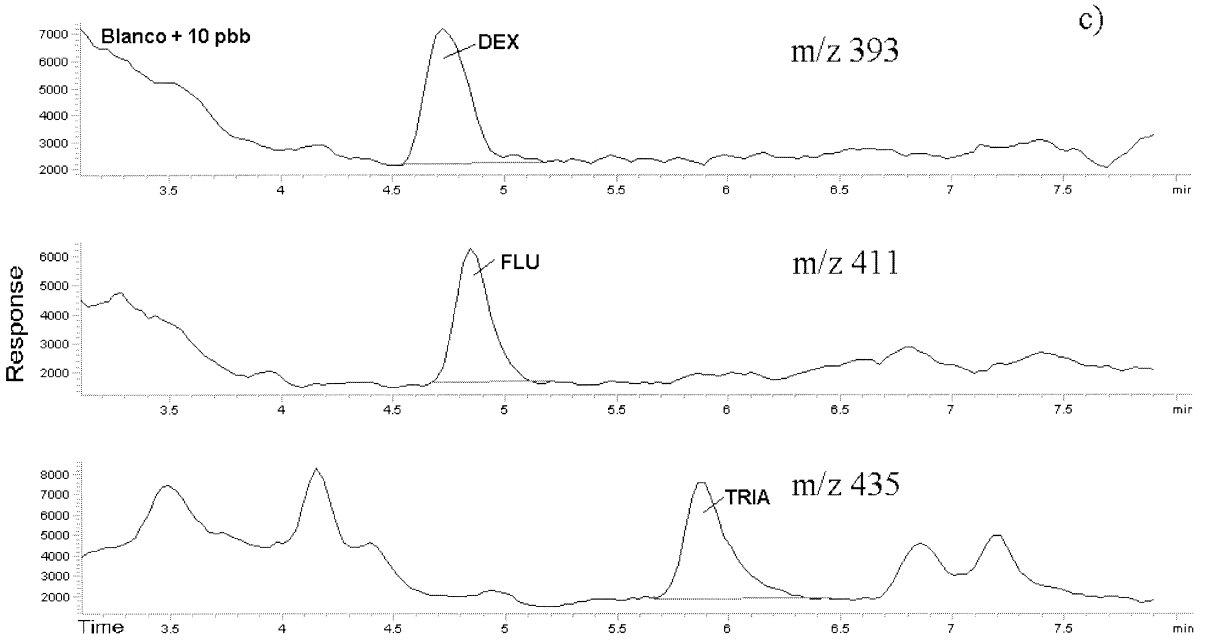

Fig. 3. (*continued*).

3.2. Feed

Using our experiences for the extraction of (anabolic) steroids from feed samples [12], three extraction solvents were selected, TBME, methanol and acetonitrile. Each of these solvents gave extraction efficiencies of over 50% for all corticosteroids. Such recoveries are adequate (registration of use of illegal corticosteroids) as long as the within-lab reproducibility is adequate and the desired limits of detection (LODs) can be obtained. However, TBME proved to be superior in terms of minimal background absorbance when using UV detection. With TBME as extraction solvent, direct clean-up on a SPE(NH_2) column was possible, without the need for evaporation of the solvent. After elution with methanol–water (80:20, v/v), a 50 μl aliquot could be injected directly on the LC column. Analyte concentrations down to about 500 μg/kg could be detected, but such LODs cannot be considered adequate for the present purpose. Moreover, at around that level, quantification was not really possible because of the background interferences. In order to obtain lower LODs with UV detection a more selective (SPE-based) clean-up technique was necessary. A wide range of sorbents from C_2 to C_{18}-bonded silica, a phenyl-bonded phase, Oasis HLB and PS–DVB, were tested. Analyte recoveries (for 1 μg test mixtures) were 80–100% for all SPE columns except C_8-bonded silica (70–85%) and C_2-bonded silica, which gave less than 10% recovery for TRIA and 60–70% for the other two corticosteroids. In the final procedure, desorption was done with hexane–ethyl acetate (10:90, v/v) after a rapid wash step with hexane to elute less polar compounds from the columns, and 1 ml SPE(PS–DVB) columns were preferred for further work because of easy analyte desorption, i.e. a small final extract volume. The desorption solvent mixture can be easily evaporated. The residue was redissolved in methanol–water (50:50, v/v). When using the dual SPE(NH_2)–SPE(PS–DVB) procedure and LC–UV analysis, the LODs of the three corticosteroids improved significantly, viz. to 100 μg/kg.

It is, of course, also possible to introduce even more selectivity by reverting to an immunoaffinity-type procedure. The disadvantage is that SPE(IAC) sorbents often are not available for all analytes of interest. This was also true in the present case: SPE(IAC) could be used for DEX and FLU, but the

material showed little affinity for TRIA (also see below). By substituting SPE(IAC) for SPE(PS–DVB), there was a marked reduction of the background, especially in the early part of the chromatogram (Fig. 3b). The LODs of DEX and FLU now were 50 μg/kg, which implies that the intended goal has indeed been reached. However as regards TRIA, its recovery generally was negligible when relatively high concentrations of DEX and/or FLU were present. At lower concentrations, i.e. when there were a sufficient number of active sites left on the IAC sorbent, TRIA peaks did show up, but peak height precision was very poor. In other words, the procedure cannot be used for a reliable calculation of TRIA concentrations. To prevent clogging and rapid contamination of the (expensive) IAC column, the LLE extract of the feed sample was not directly applied to the SPE(IAC) column. Two procedures can be followed for the pre-clean-up of the extract. One option is to centrifuge the sample, another procedure is to perform a clean-up plus concentration step by means of SPE. The second alternative has the advantage of analyte enrichment so that less solvent has to be applied to the column. With pre cleaning and careful regeneration, the SPE(IAC) columns can be used for at least ten sample analyses.

From the above it will be clear that both extraction approaches, SPE(NH_2)–SPE(PS–DVB) and SPE(NH_2)–SPE(IAC) can be used for screening purposes (the latter procedure not for TRIA) if the analyte concentrations are at or above 50–100 μg/kg. Recoveries determined at the 100–200 μg/kg level were somewhat higher for the SPE(NH_2)–SPE(PS–DVB) than the SPE(NH_2)–SPE(IAC) procedure, viz. 70–85% vs. 70%, but this may partly be due to the co-elution of interfering compounds via the earlier procedure. Repeatability, measured without an internal standard being present, was satisfactory, with RSDs for the two procedures of 10–15% and 7–8% ($n=15$) respectively. For confirmation, SPE(NH_2)–LC–MS was used in the APCI(+) mode. Although the analyte recoveries were somewhat lower, 55–60% ($n=15$) for all three test compounds, repeatability was good with RSDs of 7–8% ($n=15$) and LODs improved to 5 μg/kg for all corticosteroids (Fig. 3c). Again, the quantitative data was obtained without an internal standard being present: deuterated analogues cannot easily be obtained. The most probable explanation for the low absolute recovery of the corticosteroids in LC–MS compared with LC–UV, is ion suppression. The, often high, ion concentration in the ion source (which becomes visible with UV, but not in selected-ion MS monitoring) may well be responsible for the effect [13].

3.3. Bovine urine

Prior to their extraction from urine, the corticosteroids were deconjugated from the sulphates and glucuronidates to which they are bound, by means of conventional treatment with Helix Pomatia juice. For the extraction of the free analytes, SPE(Oasis) was preferred over SPE(C_{18}) and SPE(PS–DVB) because of our good experiences with SPE(Oasis) for the extraction and clean-up of residues of antibiotics. After extraction and subsequent desorption with 1.5 ml of methanol and evaporation of the eluate, the dried extract was redissolved in 50 μl of methanol and 150 μl of water (as described above); next, a 100-μl aliquot was directly injected into the LC–MS system. Because of the very low LODs of 0.5–5 μg/l which are required, LC–UV was not considered to be a reliable alternative. With SPE(Oasis)–LC–MS in the APCI(+) mode, the LODs were ca. 5 μg/l for all three compounds. This was not considered to be fully satisfactory and two approaches were used to improve selectivity and, thereby, enhance detectability. One possibility was to introduce more rigorous clean-up by means of SPE(IAC) according to the procedure described above for feed. This did, indeed, effect the desired increased performance, with LODs of 0.5 μg/l for both DEX and FLU. The approach can, therefore, be recommended for targeted screening but not for more general purposes. Consequently, another option was tested, viz. to improve detection by using the MS^n option of the LCQ system. For the selection of the diagnostic ions in the ion-trap LCQ, first the full mass spectra of the analytes were recorded. The protonated molecules which clearly were the most intense ions, were selected in the ion trap, and application of a collision energy of 16, yielded the MS–MS spectra, as can be seen for DEX in Fig. 4. Again, only one ion, $[MH-20]^+$ was available for identification because of the low intensity of the other fragment ions. Since,

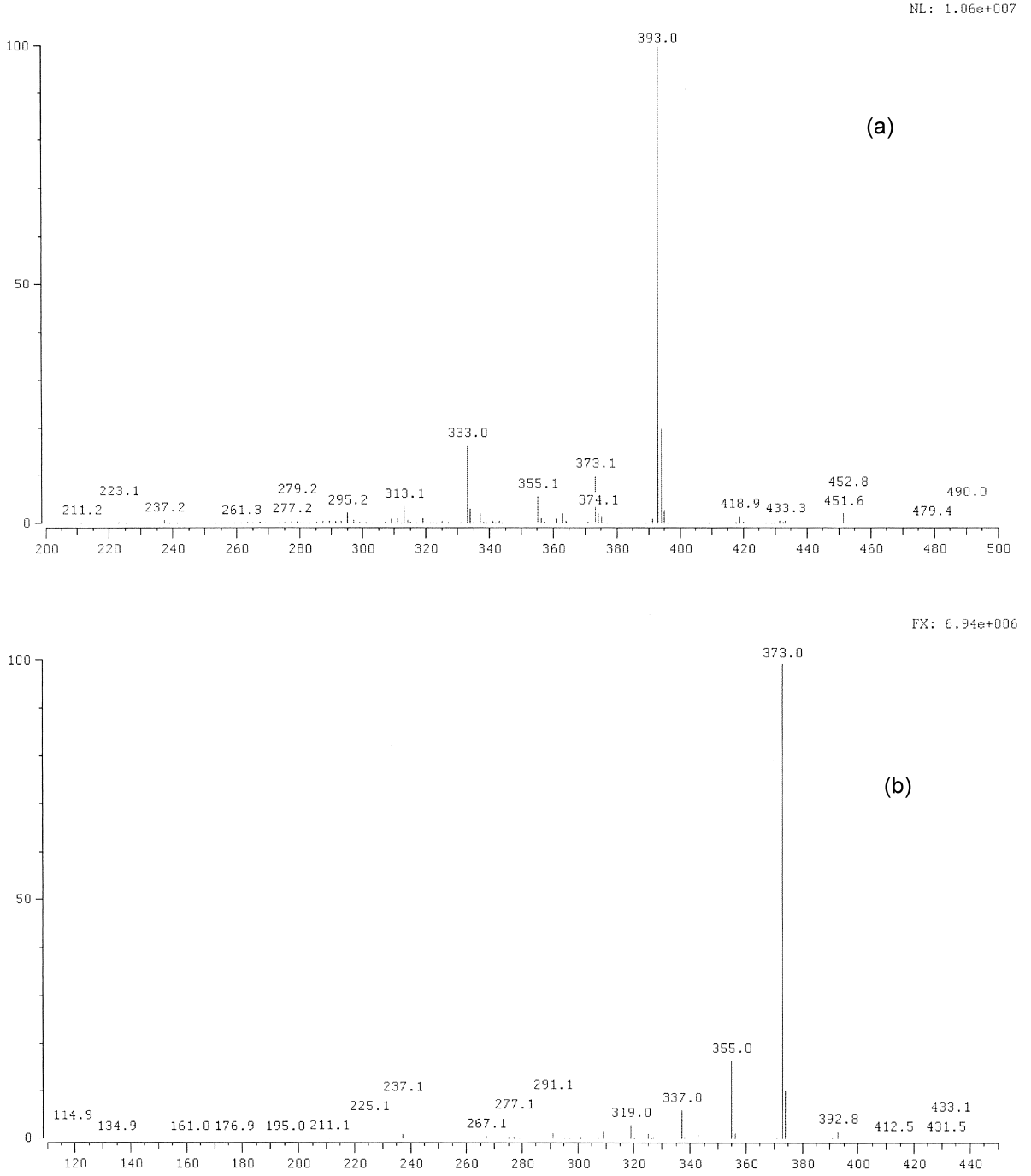

Fig. 4. Full MS, MS–MS and MS^3 spectra (a,b and c respectively) recorded for DEX by means of LC–MS^n on an LCQ mass spectrometer. For experimental details, see text.

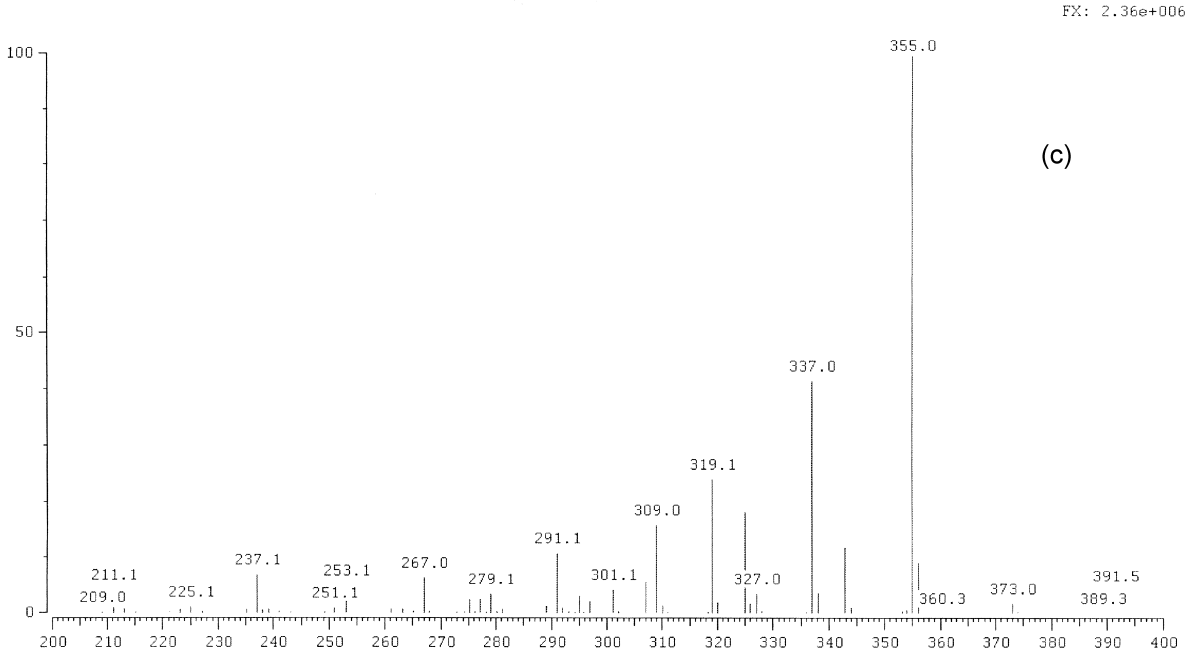

Fig. 4. (*continued*).

according to the EU criteria for confirmatory purposes [12] at least two transition ions in combination with one ion ratio are necessary the MS–MS ion — which had an intensity high enough for a second fragmentation — was subjected to further fragmentation to yield a third diagnostic ion in the MS^3 spectrum. For DEX and FLU, this were the $[MH\text{-}20\text{-}18]^+$ ions, and for TRIA the $[MH\text{-}20\text{-}58]^+$ ion. The MS–MS ions most probably are $[MH–HF]^+$ ions; the MS^3 ions for DEX and FLU probably are $[MH–HF–H_2O]^+$ and for TRIA, $[MH–HF–C_3H_6O]^+$ [7]. As an example, the MS, MS–MS and MS^3 spectra of DEX are shown in Fig. 4.

With SPE(Oasis)–LC–MS^n, the LODs of all three corticosteroids improved to about 0.5 μg/l. Since the analyte recoveries (at the 2 μg/l level) were over 63% (RSD, 8–19%; $n=15$) for the test compounds, it can be concluded that the present procedures are suitable for screening as well as confirmation purposes. It should be added that, for screening purposes, the MS–MS ions are preferred to the MS ions: although the latter have a higher intensity, their selectivity is insufficient at the low μg/l levels of interest. For the test, it is true that the precision is somewhat low but, again, this deficiency can, in the future, be remedied once deuterated internal standards become available. Generally speaking, the recovery data are in the same range as those of published GC–MS [3] and LC–MS–MS [11] procedures.

As an example, Fig. 5 shows results obtained for a suspect urine sample. Comparison of the MS–MS and MS^3 data for standards and samples clearly shows the presence of DEX, and the absence of the other two corticosteroids. The signals observed in the traces of FLU and TRIA do not have the proper retention times and are expanded noise signals. No signals for the specific ions of Fig. 5 were monitored when a blank urine sample was analysed.

Because of the importance of the SPE–LC–MS^n procedure, linear calibration curves were constructed for the 0.25–5 μg/l level of interest. With the data points in all instances, R^2 values ranged from 0.995 to 0.999 for the MS–MS and MS^3 data, which can

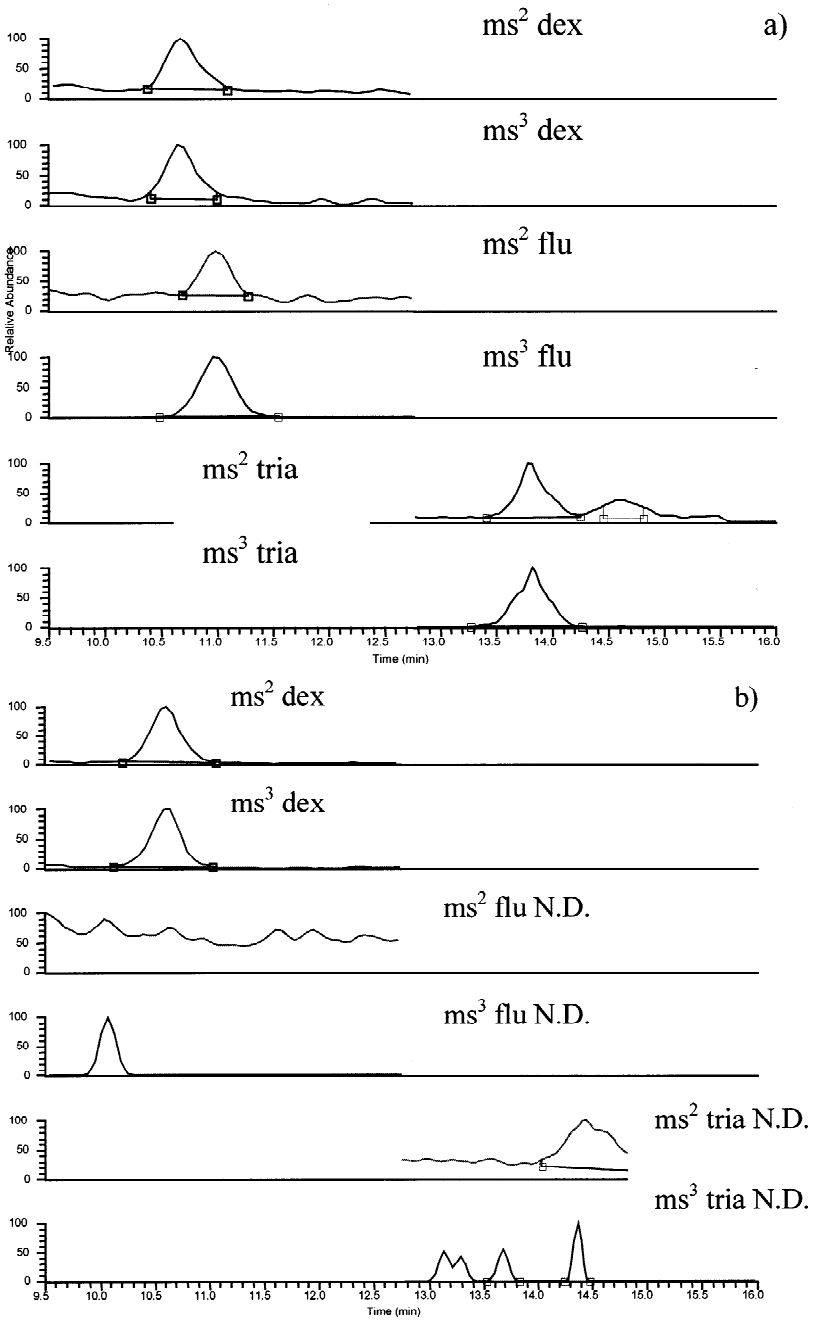

Fig. 5. LC–MS–MS/MS^3 chromatograms of (a) blank urine fortified with 1 μg/l of each corticosteroid, track 1 MS–MS ion of DEX *m/z* 373, track 2 MS^3 ion of DEX *m/z* 353, track 3 MS–MS ion of FLU *m/z* 391, track 4 MS^3 ion of FLU *m/z* 371, track 5 MS–MS ion of TRIA *m/z* 415, track 6 MS^3 ion of TRIA *m/z* 357 (b) sample of urine positive for DEX (1.3 μg/l) analysed after hydrolysis. N.D.=Not detected. LC conditions: 125×4 mm I.D., 4 μm Superspher 100 RP 18e column and acetonitrile–water (35:65;v/v) at 0.8 ml/min as eluent. MS conditions: vaporiser temperature, 500°C; capillary temperature 150°C; nitrogen, 70 p.s.i.; ionisation mode: APCI(+); [MS–MS/MS^3] *m/z* combinations used: DEX [373/355], FLU [391/371] and TRIA [415/357].

be called as fully satisfactory results at the trace levels of interest. Further information concerning analysis is provided in the next section.

4. Practical applications

4.1. Confirmation

When a sample of feed is found positive (S/N wavelength 242 nm of UV response >3) for one or more corticosteroids, and the concentration is sufficiently high (>200 μg/kg), a UV spectrum is recorded and compared with the spectrum obtained upon adding the standard to a blank sample, with subsequent evaluation according to EU criteria [10]. This situation was never encountered on the course of our study with any feed sample processed in the analysis.

For corticosteroid concentrations below 200 μg/kg in feed, and for urine samples confirmation of suspected positive samples was performed by SPE(Oasis)–LC–MS^n as outlined above. The relative intensities of the two transition ions selected from the MS–MS/MS^3 spectra, expressed as percentage of the most intense ion, must be the same as those of the standard analyte, as recorded during calibration or preferably from fortified samples, at similar concentrations, within the tolerances mentioned in Table 1.

The practicality of the above procedures for the analysis of feed and urine was tested during a EU workshop on corticosteroids held at the RIVM (20 September 1998–1 October 1998). During the workshop, eleven sets of bovine urine samples and the same number of feed samples were analysed by the participants which were trained analysts but without hands-on-experience in the present area of interest. Each set of samples contained two ''positives'': the urine samples were fortified at 1 and 5 μg/l, and the feed samples at 200 and 500 μg/kg. The detailed results of the workshop, which will be published elsewhere [14] showed that the qualitative results obtained were very good. All fortified samples were found positive with all methods under investigation. Quantification was acceptable for the methods involved with only one SPE clean-up step. Introducing a second SPE step caused a decrease of the repeatability which is not unexpected if additional sample manipulation is required from less experienced analysts. As an illustration, the results for a bovine urine sample fortified at the 5 μg/l level and analysed by SPE(Oasis)–LC–MS–MS, are shown in Fig. 6. The range of experimental values for the three analytes is seen to be rather short, viz. between 3.8 and 5.0 μg/l for all but two data points (TRIA sample position 9 and 11). This indicates that the ruggedness of the procedure can be called very satisfactory, especially because eleven operators took part in the exercise. The final results (mean μg/l±SD) were: DEX, 4.5±0.4; FLU, 4.5±0.3; TRI 4.2±0.3. These are good results, especially because no internal standards were available for quantification.

The present procedures were also used routinely within the framework of the Dutch National Monitoring Programme. In 1998 and 1999, 82 urine

Table 1
Maximum permitted tolerances for relative ion intensities using LC–MS–MS/MS^3

Relative intensity of two transition ions (%)	Tolerance (%)
>50	±20
20–50	±25
10–20	±30
≤10	±50

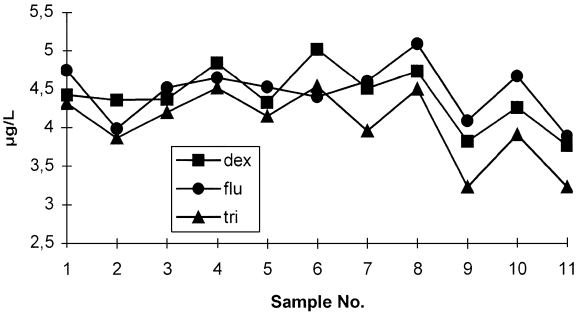

Fig. 6. Results of the SPE(Oasis)–LC–MS^n analysis of eleven samples of bovine urine fortified with 5 μg/l of each DEX, FLU and TRIA, analysed by eleven operators.

samples taken by the Inspectors of the National Veterinary Inspection at several slaughterhouses, were analysed for DEX, FLU and TRIA. Three samples were found positive, viz. for DEX, by using SPE(Oasis)–LC–MS–MS (at 6 μg/l, 1.3 μg/l and 1.7 μg/l). The analyte identity was confirmed in all cases with LC–MS–MS/MS^3. The screening and confirmation data obtained for the 1.3 μg/l sample were introduced in Fig. 5 discussed above. The mean intensity ratio obtained for the MS–MS/MS^3 ions m/z 373/355 in fortified bovine urine samples was 0.24 (n=10; concentration, 1 μg/l). In confirmation with the EU criteria [12] the tolerance interval is ±25%. The intensity ratio of the MS–MS/MS^3 ions in the suspected sample was 0.25, that clearly is within the permitted range of 0.24±0.06. In other words, the presence of DEX was confirmed.

5. Conclusions

A variety of LC based procedures has been studied for the trace-level determination of corticosteroids in feed and urine. In the case of feed samples, modern demands of analyte detectability at or below the 100 μg/kg level cannot easily be met by traditional procedures with on-line UV detection. However novel SPE–LC–UV involving immunoaffinity-SPE provides an efficient solution with LODs of about 50 μg/kg. For confirmation of suspected samples, SPE(NH_2)–LC–MS is recommended which allows detection down to 10 μg/kg. With urine, where demands are very stringent (0.5–5 μg/l), SPE(IAC)–LC–MS is a viable approach for screening. However the use of SPE–LC–ion trap MS with its MS–MS and MS^3 options, is a more powerful alternative for screening plus confirmation.

Several real-life studies, which also included quantification, illustrate that relatively rapid and sensitive procedures are available for corticosteroids which meet the criteria set by the EU for the confirmation of illegal growth promoting agents.

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