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Identification of main corticosteroids as illegal feed additives in milk replacers by liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry

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Abstract

Corticosteroids were proposed as growth promoting agents to improve commercial quality of meat. We developed a liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry (LC–APCI-MS) method able to identify the presence in milk replacers, when given by mouth, of dexamethasone, betamethasone, flumethasone, triamcinolone, prednisone, prednisolone, methylprednisolone, fludrocortisone and beclomethasone, at levels in the range of 20–100 ppb. C_{18} solid-phase extraction, LC-RP C_8 column separation, data acquisition (positive ions) in the scan range m/z 200–550 allowed us to differentiate and identify compounds by protonated molecules, their methanolic adducts and fragmentation patterns.

Keywords: Feed additives; Corticosteroids

1. Introduction

Corticosteroid administration to feedlots as growth promoting agents has been recently introduced in animal production despite the fact that they are unlicensed as feed additives in animal feed [1]. This use has been strongly enhanced for commercial reasons, in order to produce meat more appealing to consumers, due to the juicy and lean look. Corticosteroids are often added illegally during the reconstitution of milk replacers, immediately before feeding of veal calves, effective when given by mouth [2]. They act on 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 [2,3]. To prevent possible consequences on animal welfare and consumer health, we studied the analysis of feeds, particularly milk replacers, as a fundamental step in the control strategy against the illegal use of such growth promoters. To this end, we developed a multi-drug method able to identify in milk replacers corticosteroids used in meat production. We chose a liquid chromatography–mass spectrometry (LC–MS) approach already applied by other authors for residue analysis in urine and plasma [4,5], to avoid the

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time-consuming derivatisation steps required by gas chromatography (GC)–MS methods [1,6].

2. Experimental

2.1. Chemicals and reagents

Betamethasone (Beta), dexamethasone (Dexa), beclomethasone (Beclo), triamcinolone (Triam), flumethasone (Flu), prednisone (Prd), prednisolone (Prn), methylprednisolone (Mpr), fludrocortisone (Fludro) were purchased from Sigma (Milan, Italy).

Milk replacer for finishing veal calves (approximately 21% fat, dry weight) was available on the Italian market.

Water, methanol (HPLC grade), diethyl ether, acetone, acetic acid, *n*-hexane (analytical grade) were supplied by Merck (Darmstadt, Germany).

Clean-up was performed on SPE C_{18} MF columns 500 mg, 3 ml (IST, Mid Glamorgan, UK).

2.2. Apparatus

The following apparatus was used: vacuum dryer centrifuge Univapo (Uniequip, Martinsried, Germany), SPE vacuum manifold (Supelco, Rome, Italy), HPLC column Supelcosil LC-8DB 150×4.6 mm (5 μm) (Supelco), HPLC Pump 616 and 600S controller (Water–Millipore Corporation, Milford, MA, USA), SSQ 710 mass spectrometer with APCI probe (Finnigan-Mat, San Josè, CA, USA)

2.3. Standards solutions

Stock standard solutions (1 mg/ml) in methanol were prepared monthly and stored at −20°C, in the dark. Working solutions (10 μg/ml, 1 μg/ml, respectively) were daily prepared in mobile phase.

2.4. Sample preparation

Milk replacer powder was reconstituted in warm tap water (37°C) under magnetic stirring, according to supplier instruction and fed to a finishing veal calf of about 200 kg body weight (200 g/l water). Five-ml aliquots were drawn in 10-ml glass vials and spiked in triplicate at level of 20, 50 and 100 ng/ml with standard working solutions of each corticosteroid with a 50-μl HPLC syringe. The spiking levels took into account the zootechnical rule of thumb that in 10 l of milk replacer usually delivered to a single calf, we could find 1/7 of the therapeutical dose per head/week (Table 1). Because corticosteroids have different therapeutical dosages, we have focused our attention on flumethasone as the most pharmacologically active drug among the compounds considered. Blank samples ($n=20$) were inserted in the procedure to calculate detection limit. Recovery rates were calculated by external standard method. Calibration graphs were built up by injecting known amounts of corticosteroids standards (5.0, 10.0, 20.0, 50.0 and 100.0 ng) in triplicate into the LC–MS system. Graphs were calculated by least-squares linear fitting of the peak area ratio of the most abundant ion versus concentration. Precision and accuracy were assessed on the same extracted

Table 1
Therapeutical doses head/week, zootechnical doses head/day intended for growth promoting use and relative final concentrations in 10 l of reconstituted milk replacers of corticosteroids registered as veterinary drugs

Corticosteroid	Therapeutical dose (mg/head/week)	Zootechnical dose (mg/head/day) per os	Concentration as feed additive (ppb) in 10 l of milk replacer
Beta	20–30	2.9–4.3	290–430
Beclo	5.5–11.1	0.8–1.6	80–160
Triam	12–30	1.7–4.3	170–430
Prn	700–2100	100–300	10 000–30 000
Prd	700–2100	100–300	10 000–30 000
Hydro	7000–10 500	1000–1500	100 000–150 000
Flu	1.2–5	0.2–0.7	20–70
Dexa	35–140	5–20	500–2000

samples used for recovery study, by repeating the same procedure on a second analytical session.

2.5. Solid-phase extraction

Five ml of each sample were processed on SPE C_{18} MF 500 mg, 3 ml cartridges, according to the procedure described by Santos-Montes et al. [7]. After conditioning with 6 ml methanol and 3 ml water, samples were applied to C_{18} SPE columns by the vacuum manifold system. The cartridge was washed with 5 ml of water–acetone (4:1, v/v) and 1 ml of *n*-hexane. Corticosteroids were eluted by 4 ml diethyl ether in 10-ml glass vials (conical bottom). The extracts were vacuum dried in Univapo, re-dissolved in 100 μl mobile phase. A 20-μl aliquot was injected into the LC–(APCI-)MS system.

2.6. Chromatography

Chromatographic analysis was carried out under the following conditions: mobile phase, methanol–water (1% acetic acid) (7:3, v/v); flow, 0.8 ml/min; HPLC column, Supelcosil LC-8DB 150×4.6 (5 μm); loop, 20 μl.

2.7. APCI mass spectrometry

Acquisition parameters were optimised in APCI mode, by direct pump infusion of 10 μg/ml standards working solutions at a flow-rate of 0.8 ml/min in the mass spectrometer. Conditions were: vaporiser 500°C; capillary 220°C; nitrogen (high purity), 70 p.s.i. (1 p.s.i.=6894.76 Pa); lens 130 V, to avoid an extensive fragmentation of the compounds, leading to the presence of the protonated molecule, in the positive ion mode. Data were acquired in scan mode (mass interval *m/z* 200–550), with a dwell time of 0.5 cycle/s. Chromatograms were smoothed (smooth 3) and mass spectra enhanced, by automatic background subtraction.

3. Results and discussion

Standards calibration graph parameters in the range of tested concentrations are shown in Table 2. Different relative response of various corticosteroids to APCI can be evidenced. In Table 3 retention time, nominal molecular mass, most abundant ions of the spectra, in increasing *m/z* order, accuracy and recovery from spiked milk replacers ($n=6$ observation at each concentration) were shown. Precision, expressed as coefficient of variation (C.V.) was within 10% over the two different analytical sessions (data not shown). In Fig. 1 chromatograms (RIC trace, smooth 3) and enhanced APCI mass spectra in the range *m/z* 200–550 are reported for each standard injected at a concentration of 50 ng. In Figs. 2 and 3 respectively, typical chromatograms of: (a) blank sample and (b) positive sample for both hydrocortisone and betamethasone (estimated concentration 32.8 and 13.3 ng/ml, respectively, without recovery correction) with correspondent spectra are shown. Detection limits (LODs), calculated on the basis of a signal-to-noise ratio of 3:1 of the RIC trace in 20 blank samples were 5 ng for Flu, 4 ng for Triam and Fludro, 3 ng for Hydro, Mpr, Beta and Dexa, 2 ng for Prd and Prd, respectively. For flumethasone, the most pharmacologically active drug, the estimated LOD corresponds to an analyte concentration of 12 ng/ml in milk replacer, considering a mean recovery of 80%.

Table 2
Parameters of standards calibration graphs (4 points considered, 6 replicates on two different analytical sessions) calculated by least-squares linear fitting of the peak area ratio of the most abundant ion versus concentration

Compound	*y*	*x*	r^2
Beclo	155±21	45 212±89	0.9989
Dexa	116±26	78 070±92	0.9995
Beta	66±28	58 994±97	0.9998
Flume	320±28	38 916±97	0.9997
Triam	132±16	51 373±56	0.9998
Prn	160±20	182 923±71	0.9989
Prd	62±21	87 531±89	0.9998
Mpr	153±39	76 727±161	0.9998
Hydro	55±21	60 782±88	0.9998
Fludro	127±15	42 604±63	0.9998

Only one milk replacer was tested, because, according to our previous experience, no matrix mismatch were observed between different commercial products, all sharing almost the same chemical composition.

The analyte concentrations in the samples do not require a negative-ion measurement to improve

Table 3
Retention time (t_R), nominal molecular mass (M_r), protonated molecules and most abundant ions of the spectra (in bold the base peak), spiking levels, accuracy and recovery rates ($n=6$) of the nine corticosteroids considered

Drug	t_R	M_r	Ions (m/z)	Spiking level (ng/ml)	Recovery (mean±S.D.)
Triam	3.01	394.17	309; **347**; 376; 395	20	82±5
				50	85±4
				100	84±3
Prd	3.38	358.17	**359**; 391;	20	85±3
				50	84±6
				100	86±3
Fludro	4.19	380.20	**381;** 413	20	81±6
				50	81±4
				100	83±5
Hydro	4.24	362.20	353; 359; **363;** 395	20	91±4
				50	93±3
				100	92±2
Prn	4.42	360.19	307; **325**; 343; 361	20	85±3
				50	87±4
				100	82±2
Flu	5.27	410.19	353; 371; **411;** 443	20	81±4
				50	83±5
				100	86±3
Mpr	5.27	374.20	321; **339**; 357; 375	20	85±6
				50	88±3
				100	87±2
Beta	5.29	392.19	337; 355; **373;** 393	20	82±4
				50	85±5
				100	83±4
Dexa	5.39	392.19	337; **355**; 373; 393	20	81±6
				50	83±4
				100	86±3
Beclo	6.44	408.17	337; 355; **373**; 409	20	83±5
				50	85±4
				100	88±5

sensitivity, as for residues level in urine [4]. Determination limit has not been considered in detail, because corticosteroids are not allowed as feed additives, so our main goal is to detect and identify their presence.

Liquid chromatographic conditions allowed us to analyse in the same run a polar compound such as triamcinolone that in a previous paper [4] was not resolved from the solvent front peak. In our case we achieved a good ionization in APCI, at a flow-rate of 0.8 ml/min (Fig. 1). Shibasaki et al. [5], observed a low ionization efficiency for Prd and Prn molecules in thermospray mass spectrometry analysis, using a flow-rate of 0.6 ml/min. Therefore, to enhance protonated molecules they chose to increase the flow to 1.3 ml/min. APCI spectra are characterised by the protonated parent molecules $[MH]^+$, methanolic adducts $[MH+32]^+$ for Prd, Fludro, Hydro and Flu,

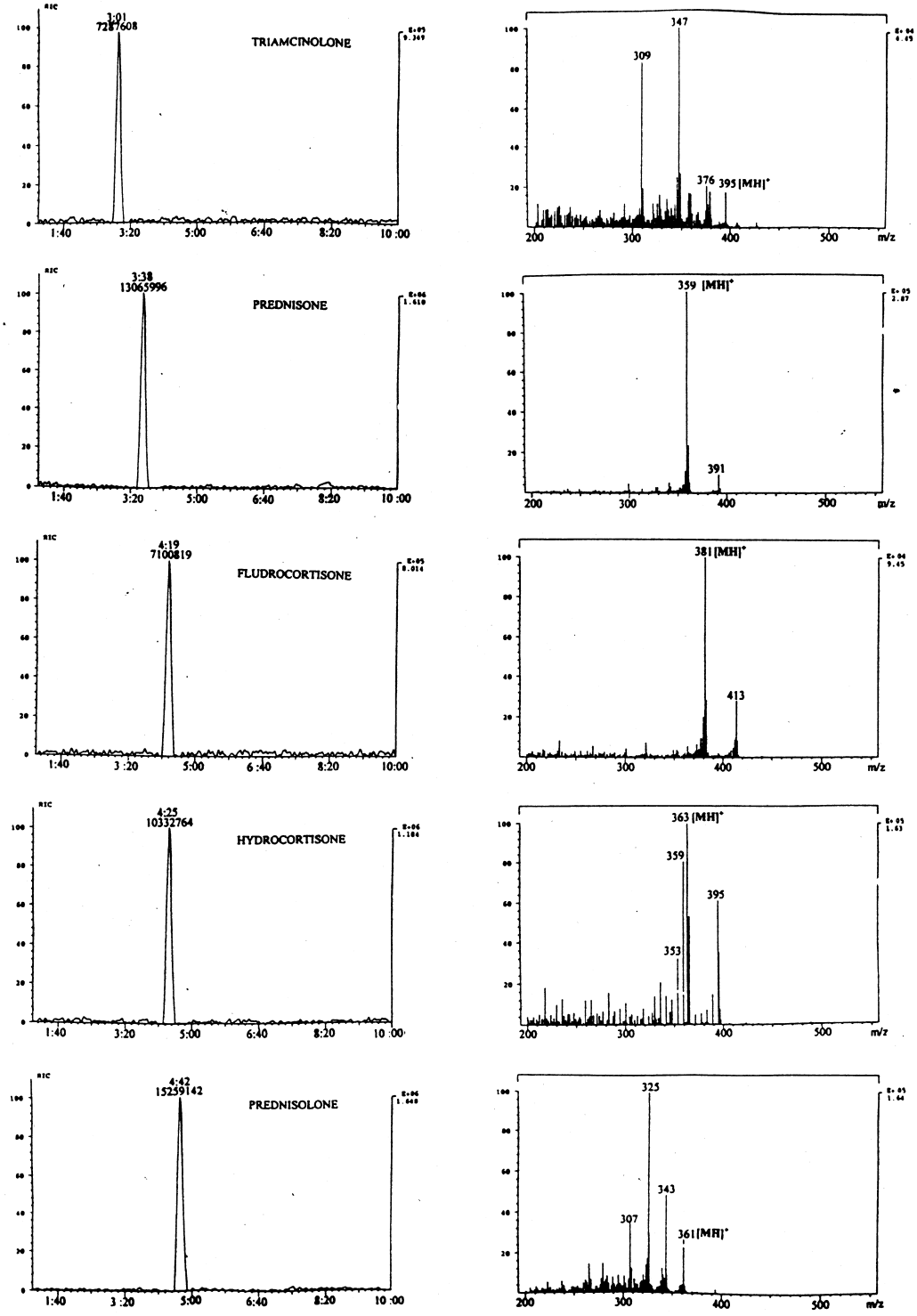

Fig. 1. RIC trace (m/z 200–550, smooth 3) and enhanced APCI spectra of corticosteroids in order of liquid chromatography elution (continued on next page).

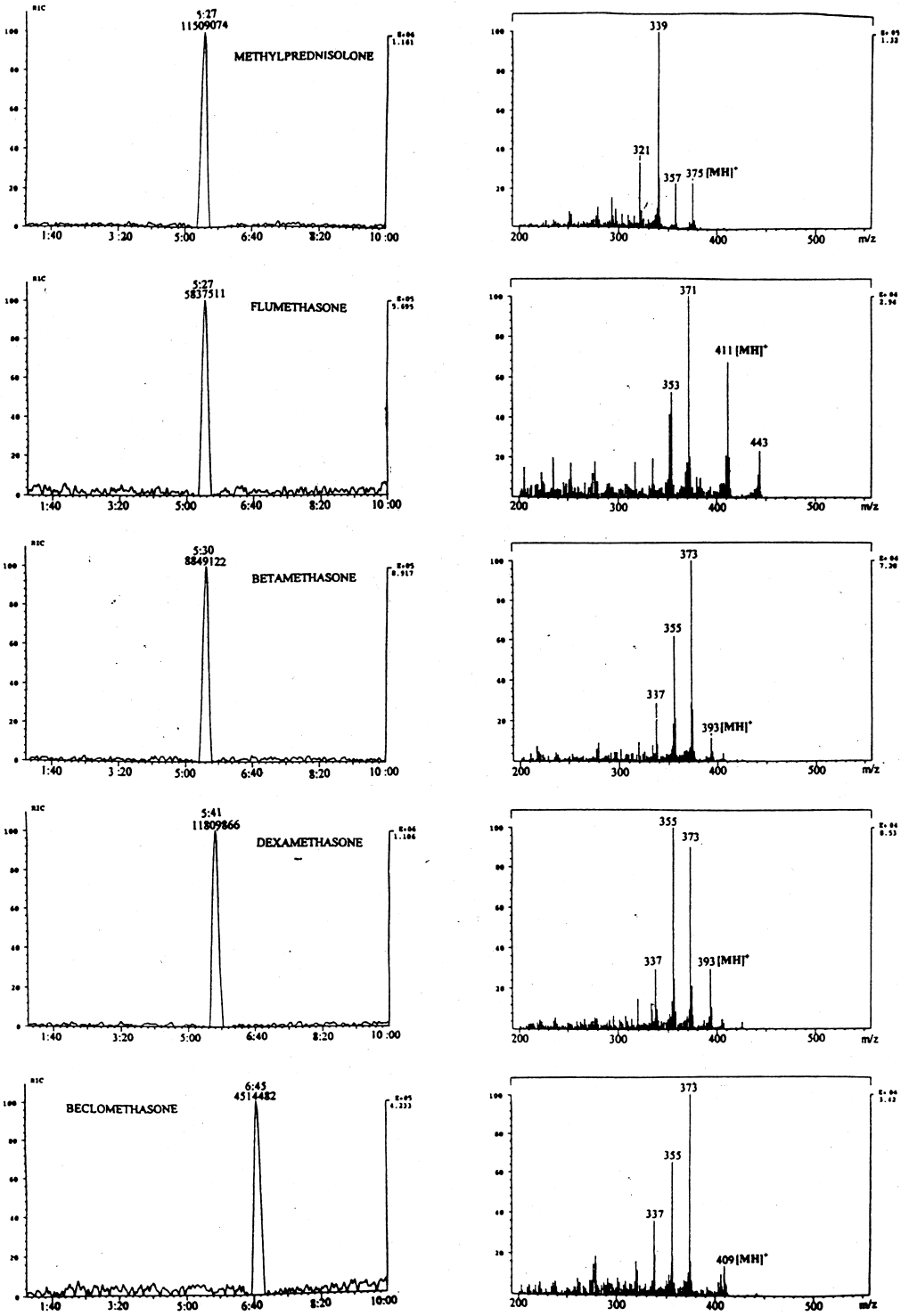

Fig. 1. (*continued*)

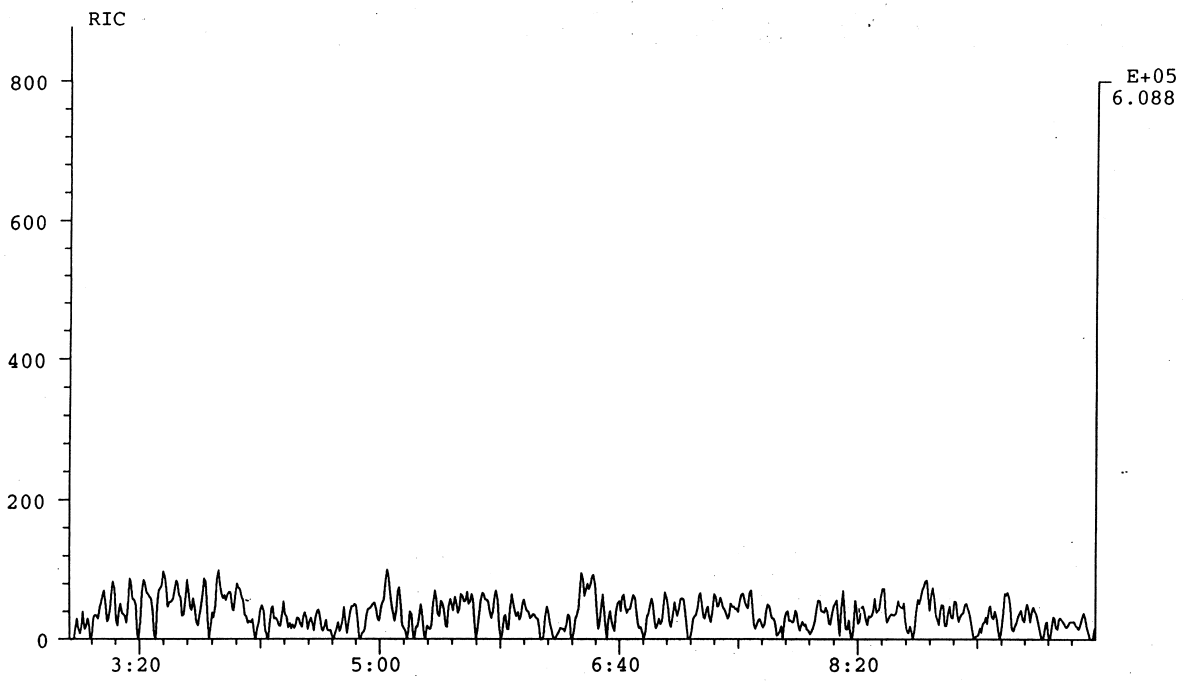

Fig. 2. RIC trace (m/z 200–550, smooth 3) of a blank sample.

accompanied by fragments due to the cleavage on C-21 site (Fig. 1) [8]. For Prd and Prn, our fragmentation patterns are in good agreement with those reported by Shibasaki et al. [5], with thermospray; we recorded ions $[MH-18]^+$, $[MH-30]^+$ and $[MH-60]^+$, derived by loss of water, formaldehyde and glycoaldheyde, respectively, from the protonated molecules.

It is worth noting that we discriminate between corticosteroids eluted with close retention times (t_R), according to the different features of their mass spectra. Flumethasone and methylprednisolone (both eluted with a t_R of 5.27) showed a completely different mass spectra pattern. The two isomers Beta and Dexa (t_R 5.30 and 5.41, respectively), sharing the same protonated molecule at m/z 393, were discriminated according to the different relative abundances of the ions m/z 355 and 373 (Fig. 1 Table 2). Recoveries show no great different among molecules and spiking levels (Table 2); thus procedure can be considered as a multi-drug method. The clean-up proves to be effective in removing possible interferences from the matrix, as results from the RIC trace of blank samples (Fig. 2).

The acquisition of data in scan mode (range m/z 200–550), positive ions, is, in our opinion, an improvement of quality criteria for confirmation of drugs in feedstuffs. In fact, at present official methods of analysis of veterinary drugs in such matrices base the identification on limited structural information, such as the acquisition of UV spectra or, for beta adrenergic agonist drugs, selected ion monitoring on four ions by GC–MS (EI) [9]. The presence of protonated molecules with a relative abundance above 10% in the mass spectra from LC–MS (APCI) technique facilitates drugs identification and suggests this method is suitable to detect other unlicensed corticosteroids in animal productions.

National Residue Plans for Veterinary Drug Residues improved strategies push unscrupulous farmers to choose corticosteroids able to elude conventional immunochemical screening methods. At present, commercially available immunoassays cannot cover all the range of molecules potentially administered to feedlots [10]. The above consideration has been freshly confirmed by our experience: recently we found beclomethasone in feeds, a molecule neither

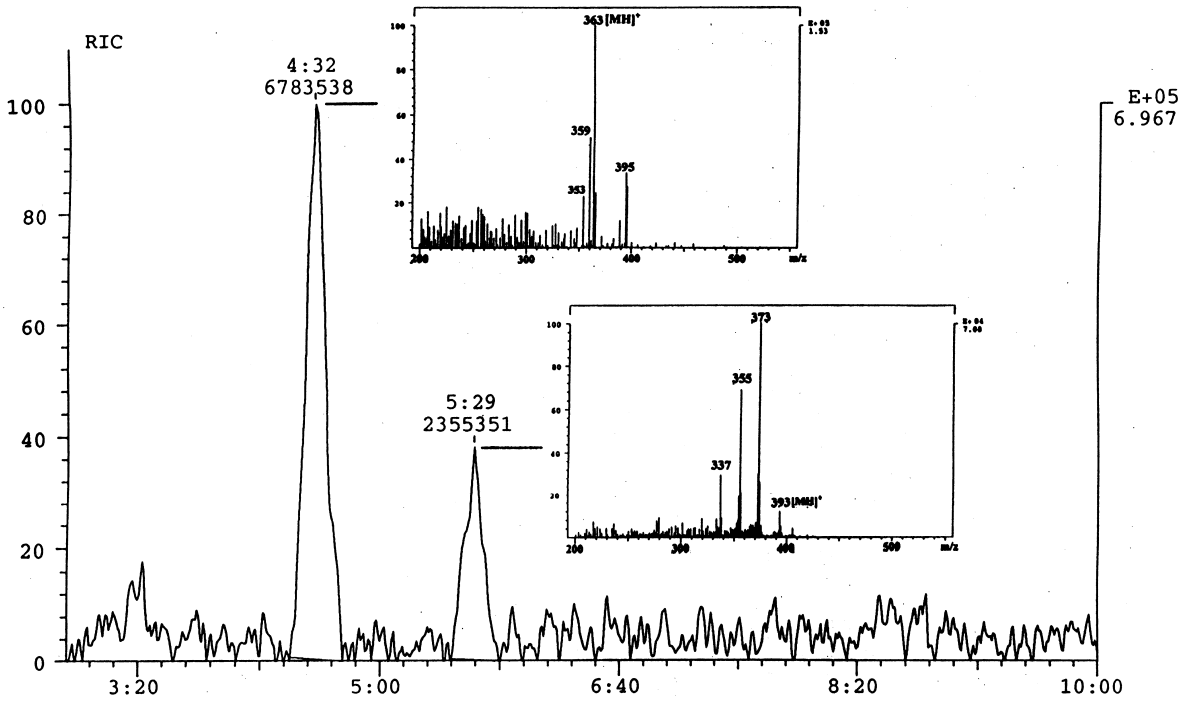

Fig. 3. RIC trace (m/z 200–550, smooth 3) and enhanced spectra of a incurred sample found positive both for hydrocortisone and betamethasone.

licensed as veterinary drug in Italy nor efficiently screened. This finding was strictly related with pathological evidences in veal calves: recorded lymphocytopenia in blood drawn from farmed calves and thymus/surrenal glands atrophy during inspection at slaughter could be clearly addressed to mass corticosteroid treatments [2,11].

4. Conclusions

A multi-drug method is mandatory for the analysis of corticosteroids as illegal growth promoters in feeds. This allows to achieve valuable and reliable information about molecules really used in animal productions. By this way, it could be possible to verify in short time the epidemiological validity of the screening procedures and, as consequence, of the whole analytical strategy to control veterinary drugs residues.

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References

[1] D. Courtheyn, N. Verheye, V. Backeroot, V. Dal, R. Schilt, H. Hooijerink, E.O. Van Beenekom, W. Haasnot, P. Stouten and F.A. Huf, in N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg (Editors), Proceedings of the Euroresidue II Conference, 3–5 May 1993, Veldhoven, Netherlands, p. 251.

[2] P.B. Schimmer and K.L. Parker, in J.G. Hardman and L.E. Limbird (Editors-in-chief), Goodman and Gilman's The Pharmacological Basis of Therapeutics, New York, 1995, Ch. 59, p. 1465.

[3] J.H. Eisemann, in European Commission (Editor), Proceedings of Scientific Conference on Growth Promotion in Meat Production, 29 Nov–1 Dec 1995, Brussels, p. 121.

[4] S. Rizea Savu, L. Silvestro, A. Haag, F. Sorgel, J. Mass Spectrom. 31 (1996) 1351.

[5] H. Shibasaki, T. Furuta, Y. Kasuya, J. Chromatogr. B 692 (1997) 7.
[6] D. Courtheyn, J. Vercammen, R. De Brabander, I. Vandenreyt, P. Batjoens, K. Vanoosthuyze, C. Van Peteghem, Analyst 119 (1994) 2557.
[7] A. Santos-Montes, R. Gonzalo-Lumbreras, A.I. Gasco-Lopez, R. Izquierdo-Hornillos, J. Chromatogr. B 652 (1993) 83.
[8] N.V. Esteban, A.L. Yergey, D.J. Liberato, T. Loughlin, D.L. Loriaux, Biomed. Environ. Mass. Spectrom. 15 (1988) 603.
[9] L. Leyssens, A. Boenke, D. Courtheyn, European Commission BCR Information Chemical Analysis Report EUR 16674 EN, Office for the Official Publications of the European Communities, Brussels, 1995.
[10] P. Stouten, W. Haasnoot, G. Cazemier, P.L.M. Berende and H. Keukens, in N. Haagsma and A. Ruiter (Editors), Proceedings of the Euroresidue III Conference, 6–8 May 1996, Veldhoven, Netherlands, p. 902.
[11] M. Groot, R. Schilt, W. Haasnot, P.L.M. Berende, V. Ramazza, D. Courtheyn, J. Vercammen and M. Logghe, in N. Haagsma and A. Ruiter (Editors), Proceedings of the Euroresidue III Conference, 6–8 May 1996, Veldhoven, Netherlands, p. 440.