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Identification of "unknown analytes" in injection sites: a semi-quantitative interpretation

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Abstract

The analytical approach to the detection of residues of legally used veterinary medicinal products (VMPs) is similar to the approach of forbidden substances. The only difference lies in the quantitative component of the method. Since there is an evolution towards a different strategy in the screening for VMPs in matrices of slaughtered animals, a new approach was developed for determining the residues. The aim of this research was to create an efficient screening approach for determining the identity and/or quantity of legally and illegally used VMPs present at high concentrations in injection sites. The determination of these 'unknown' VMPs is combined with a fast report to the customer. Examples are given of the identification of phenylbutazone, penicillin G benzathine and florfenicol. For quantitative purposes, using a mini-validation procedure, concentrations far above the maximum residue limit (MRL) of the identified VMP can be reported. A quantitative validation normally consists of determining the required validation parameters at three levels: 1/2 MRL, MRL, 2 MRL. For highly concentrated injection sites, an alternative approach is proposed. The alternative validation consists of a comparison of the analyte concentration in the sample with the spike at the MRL and 10 times the MRL concentration.

Keywords: Liquid chromatography with mass spectrometry (LC-MS^n); Veterinary; Maximum residue limit (MRL); Phenylbutazone; Penicillin G benzathine; Florfenicol

1. Introduction

A wide range of VMPs such as antibiotics is administered legitimately to farm animals to treat outbreaks of disease or prevent diseases spreading when modern intensive farming practices are used. In order to reduce the likelihood of harmful levels of these VMPs reaching the human food chain, the European Union (EU) and many other countries have set maximum residue limits (MRLs). Regulatory bodies are required to enforce and verify these requirements. Laboratory testing of food products has to ensure that the regulations are met.

The classical screening test for anti-microbials is the microbial inhibition test. Pieces of frozen kidney are screened on a pH 6 culture medium seeded with *Bacillus subtilis* [1,2]. To report a more specific result immunological methods are used for screening certain

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groups of antibiotics. Finally a quantification by liquid chromatography (LC) with fluorescence, UV absorption, mass spectrometry and other techniques is performed [3–8].

From the beginning of 2001, injection sites are sampled at the slaughterhouse for identification of legally and illegally used VMPs. Based on the results for the detected VMPs and the frequency of detection the approach for screening can be altered. Multiple mass spectrometry is used as an alternative screening procedure for highly concentrated VMPs.

VMPs can legally be used but the detected concentrations can be above (violative or non-compliant) or below the MRL (non-violative or compliant). In addition VMPs can be used illegally with the intention to promote growth. The use of growth promotors, however, is forbidden.

Since it is almost impossible to develop a multi-residue extraction procedure that includes all these pharmacologically active VMPs, an alternative approach is proposed in which a simple extraction and clean-up is combined with a multi-residue (LC)-MS^n identification and/or quantification.

Injection sites very often contain high concentrations of the administered VMP. Injection sites are considered as meat by inspection services and therefor the MRL for meat applies, especially because of the possible consumption of an injection site. To develop and to use very specific confirmation methods takes time and is expensive. In identifying the active compound present in injection sites, regulatory bodies are informed which VMPs are frequently used. Because of the high concentrations there is no demand for the registered VMPs to be quantified in the concentration range of the MRL. A different validation can be used, as proposed in this paper.

2. Experimental

2.1. Reagents and chemicals

Standards were obtained from Sigma (St. Louis, MO) and the injectable solutions from the Clinical Department of the Faculty of Veterinary Medicine (Ghent, Belgium). The injectable solutions were used for identification purposes.

An internal standard, desoximetasone (Dom) (15 μl = 3000 ng per 5 ml of methanol or 1.5 μg kg^{-1}), is added.

2.2. Extraction and clean-up procedure

The extraction and clean-up has been described earlier and is applied as such [9]. The final extract is evaporated to dryness and reconstituted in 50 μl of methanol and 100 μl of mobile phase; 30 μl is injected on column.

2.3. LC-MS^2

Chromatographic separation was achieved using a Symmetry C_{18} column (5 μm, 150 mm × 2.1 mm, Waters, Milford). 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^{-1}. A linear gradient was used. Twenty percent of A was maintained for 7 min and increased to 100% A in 10 min (maintained for 7 min). In between samples there was an equilibration time of 10 min at the initial conditions.

The LC apparatus comprised of a TSP P4000 pump and a model AS3000 autosampler (TSP, San Jose, CA). Separation was carried out on the Symmetry C_{18} column (5 μm, 150 mm × 2.1 mm). The MS detector was a Finnigan LCQdeca ion trap spectrometer (ThermoFinnigan, San José, CA) equipped with an electrospray interface in positive ion mode MS/MS full scan.

For each sample an acquisition is made in positive and negative ion mode to obtain complementary information.

2.4. Some definitions

Unknown: an analyte which is identified in a non-target analysis, for which no specific extraction or confirmation procedure is used or developed, of which there is no information of the group of veterinary drugs or growth promotors to which it belongs.

Suspect ion, during infusion: an ion with a signal-to-noise (s/*n*) ratio >3 that was not present in the previously infused mobile phase or methanol.

Suspect ion, injection on column: a species that gires a chromatographic peak in the total ion current with *s*/*n* >3, or a chromatographic peak of a specific ion trace with *s*/*n* >3.

Layout: option in the software (Xcalibur 1.2) in which mass traces of pseudo-molecular ions of injectable solutions are combined in a window. A layout can be added depending on the knowledge of injectable analytes at that time.

MS^n acquisition: MS^1, MS^2, MS^3, ... MS^n fragmentation of pseudo-molecular ions. Fragmentation in MS^n is performed until the spectrum becomes unstable.

Scan event: a mass spectrometer scan that is defined by selecting the required and optional scan event settings. Required settings are scan power, ion polarity and scan mode. Optional settings are source CID (collision induced dissociation) and dependent scan. Multiple scan events can be defined for each segment of time.

Unstable spectrum: when a large fluctuation of ion intensities and ion ratios is observed due to a loss of signal.

Injectable solution: a registered veterinary medicinal product used in veterinary practice of which the concentration of the active component is known.

Specific method: a method containing specific MS^n parameters of the identified analyte, and contains three scan events: MS-full scan 100–1000, MS^2 full scan of the identified analyte, MS^2 full scan DOM.

Violative or non-compliant: the presence of an analyte is proven, according to the analytical procedure, when the general criteria, and the criteria specified for the individual detection method, are fulfilled [10].

3. Results

For the identification of "unknown analytes" two approaches can be used depending on the availability of the instrument. A first approach is infusion-MS^n. A second approach is LC-MS^n. Different injectable solutions of registered VMPs were collected. They were chromatographed after they had been infused and MS^n data were acquired. The injectable solutions were not the active compounds but the registered VMPs as they are used in veterinary practice. This implies that additional impurities will obscure the chromatogram and spectrum, as can also be expected in injection sites. It is important to mention that an ointment base such as poly(ethylene glycol) can mask the pure product when using direct infusion. The collected data will function as a database or library for identification of "unknown analytes".

3.1. Infusion-MS^n

A first approach is infusion-MS^n. Mobile phase is pumped at $0.3\,ml\,min^{-1}$ and mixed with the extract that is connected via a T-piece and pumped at $5\,\mu l\,min^{-1}$. Since the idea is to look for 'unknowns' the parameters for mobile phase and mass spectrometry are default. A default tune file for positive and negative ion mode was used. No mass spectrometric parameters were optimised. ESI and APCI are both soft ionisation techniques but ESI is prefered since fragmentation of the pseudo-molecular ion in full scan MS is not as intense as when using APCI. Fragmentation in MS full scan can mask the presence of the pseudo-molecular ion that is the direct link with the molecular weight of the analyte of interest.

The first acquisition is always the infusion of a blank (methanol). Since a zero signal is only an indication of a serious problem, a blank is considered to contain background ions that can be more or less intense depending on the environmental conditions. Background ions are not taken into consideration for further fragmentation when analysing the sample unless the intensity of the background ions in the sample would be considerably higher than during acquisition of the methanol. Full scan MS^n data in positive and negative ion mode are acquired of the 'suspect' ions.

3.2. LC-MS^n

In addition to the infusion approach a default gradient, as defined in the Section 2, is used in MS full scan in positive and negative ion mode.

The advantage here is that LC-MS^n is automated and data can be acquired overnight while infusion MS^n is an online interpretation.

3.3. Proposed strategy: infusion-MS^n

The acquired data of the infusion are further investigated. Possible molecular masses (M) are calculated from MH^+ or $(M–H)^-$ or, Na^+ or NH_4^+ adducts. A

tentative list of molecular weights is made for each sample. With this list the database of the Merck Index and the steraloids catalogue database is searched [9]. A margin of 4 amu around the molecular weights ($M-2$, $M+2$) is allowed in the search with the objective not to accidentally miss candidate identities. All possible compounds from these databases are taken into consideration but are filtered based on their therapeutic category or intended use. Compounds are considered if used in veterinary practice or if they are prone to illegal application due to a growth promoting or repartitioning effect as compared with already known compounds.

3.3.1. Case I

If a possible identity is found and MS^n data correspond with the MS^n data of the standard or injectable solution, the identity is confirmed based on comparison of spectra. Criteria of identification points are used for a positive identification [10]. The identity of the analyte can be reported if the substance is a forbidden substance. In this case quantification is not mandatory.

3.3.2. Case II

If the identified analyte has a MRL and the standard or injectable solution is available, the concentration must be estimated. A quantitative validation normally consists of determining the required validation parameters at three levels: 1/2MRL, MRL, 2MRL. For highly concentrated injection sites, an alternative approach is proposed. The alternative validation consists of a comparison of the analyte concentration in the sample with the spike at the MRL and 10 times the MRL concentration. The alternative approach is performed as a mini-validation.

Five blank matrices fortified with the MRL concentration of the analyte, five blank matrices fortified with ten times the MRL concentration (10× MRL) of the analyte and one blank matrix are extracted. To all of the spikes, 1500 $\mu g\,kg^{-1}$ DOM is added. A semi-quantitative interpretation allows a concentration >MRL to be reported within 1 or 2 days.

3.3.2.1. Case IIa. If the area ratio of the spike at 10× MRL is ≥4 times the area ratio of the spike at MRL concentration AND the area ratio of the sample is ≥10 times the area ratio of the spike at MRL concentration, the sample is reported as violative with a concentration higher than the MRL.

3.3.2.2. Case IIb. If the area ratio of the spike at 10× MRL is <4 times the area ratio of the spike at MRL concentration, the sample is transferred to the National Reference Laboratory (NRL) for confirmation with a specific method.

3.3.2.3. Case IIc. If the area ratio of the spike at 10× MRL is >4 times the area ratio of the spike at MRL concentration AND the area ratio of the sample is <10 times the area ratio of the spike at MRL concentration, the sample is transferred to the NRL for confirmation with a specific method.

3.3.2.4. Case IId. If the spike at MRL concentration has no response in MS^2-full scan (positive or negative ion mode) and the spike at 10× MRL has a positive response, the concentration can be reported as >MRL if its area ratio is >3 times the area ratio of 10× MRL. If the area ratio of the sample is 10× MRL, the sample is transferred to the NRL for confirmation with a specific method.

3.3.2.5. Case IIe. If the spike at MRL and 10× MRL has no response in MS^2-full scan (positive or negative ion mode), this approach is considered as a *reductio ad absurdum* (reduction to absurdity or contradiction).

If the analyte is identified in an injection site, the concentration can be reported as >MRL without any reasonable doubt.

3.3.3. Case III

If the identity of an analyte can not be illustrated or confirmed using a mini-validation or by comparing MS^n data, extra experiments are performed for the identity elucidation.

Violation of regulatory requirements are those samples in which the concentration of the identified analyte is >MRL, if there is a illegal administration of a registered VMP or if the identified analyte is a prohibited substance. For all cases in which the analyte is identified and if necessary quantified, and the results are in violation with regulatory requirements, the result is reported as violative or non-compliant.

Table 1
Summary of suspect ions in positive ion mode

Layout name	Pseudo-molecular ion	Analyte
VMP-pos1	391	Trimethoprim
	311	Sulfadoxin
	615	Neomycin
	360	Enrofloxacin
	358	Danofloxacin
	255	Ketoprofen
	461	Oxytetracyclin
VMP-pos2	407	Lincomycin
	429	Lincomycin
	365	Spectinomycin
	478	Gentamycin C1
	464	Gentamycin C2
	450	Gentamycin C3
	322	Gentamycin
	988	Chloramphenicol
VMP-pos3	335	Penicillin G
	237	Procain
	241	Benzathin
	297	Flunixin
	279	Flunixin
	869.5	Tilmycosin
	435	Tilmycosin
	899 + 921	Doramectin
VMP-pos4	521	Beclomethasone dipropionate
	407	Flugestone acetate
	321 + 339 + 357	DOM

Table 2
Summary of suspect ions in positive ion mode

Layout name	Pseudo-molecular ion	Analyte
VMP-neg1	309	Sulfadoxin
	333	Penicillin G
	405	Lincomycin
	465	Lincomycin
	350	Meloxicam
	673	Neomycin
	253	Ketoprofen
VMP-neg2	459	Oxytetracyclin
	446	Florfenicol
	356	Florfenicol
	356	Danofloxacin
	392	Florfenicol
	349	Spectinomycin
	331	Spectinomycin
	295	Flunixin
	251	Flunixin
VMP-neg3	357	Chloramphenicol
	957	Doramectin
	897	Doramectin
	216 + 260 + 543	Tolfenamic acid
VMP-negCOST	379 + 469	Flumethasone
	329 + 419	Prednisolone
	343 + 433	Methylprednisolone
	413 + 493	Triamcinolone acetonide
	355 + 435	Fluorometholone
	361 + 451	Dexamethasone
	361 + 451	Betamethasone
	429 + 465 + 525	Clobetasol propionate

3.4. Proposed strategy: LC-MS^n

If during infusion no 'suspect' ions are detected, the sample is injected on column and eluted with a default gradient.

The chromatogram is checked for 'suspect' ions. The total ion current is examined for 'suspect' chromatographic peaks. Different ion traces of 'known' (of which MS^n data are available) compounds are examined by applying a 'layout'. An example of the 'layouts' used in this application is given in Tables 1 and 2. If an identity is suspected the same strategy as in Section 3.3 is followed.

3.5. Examples of identified and/or quantified analytes

3.5.1. Identification of Penicilline G benzathine

Penicillin-G types are beta-lactam antibiotics effective against gram-positive strains of streptococci, staphylococci, enterococci, and meningococci. Benzylpenicillin G is used for treatment of infections when a longtime prophylactic or therapeutic treatment is necessary. It is used in injectable solutions for treatment of cows, horses, pigs, cats and dogs.

An extract of an injection site was directly infused into the mass spectrometer through a T-piece. In positive ion mode major ions, *m*/*z* 241, 575 and 909, with a large signal-to-noise ratio were observed (Fig. 1). In negative ion mode ions with *m*/*z* 333, 573, 907 were acquired.

Since electrospray is a soft ionisation technique the presence of a pseudo-molecular ion (MH^+ or $M–H^-$) or an adduct is to be expected. In this example two molecular weights (908 and 574) can be derived from the positive and negative ions (positive ions: $909-1=908$, $575-1=574$) (negative ions: $907+1=908$,

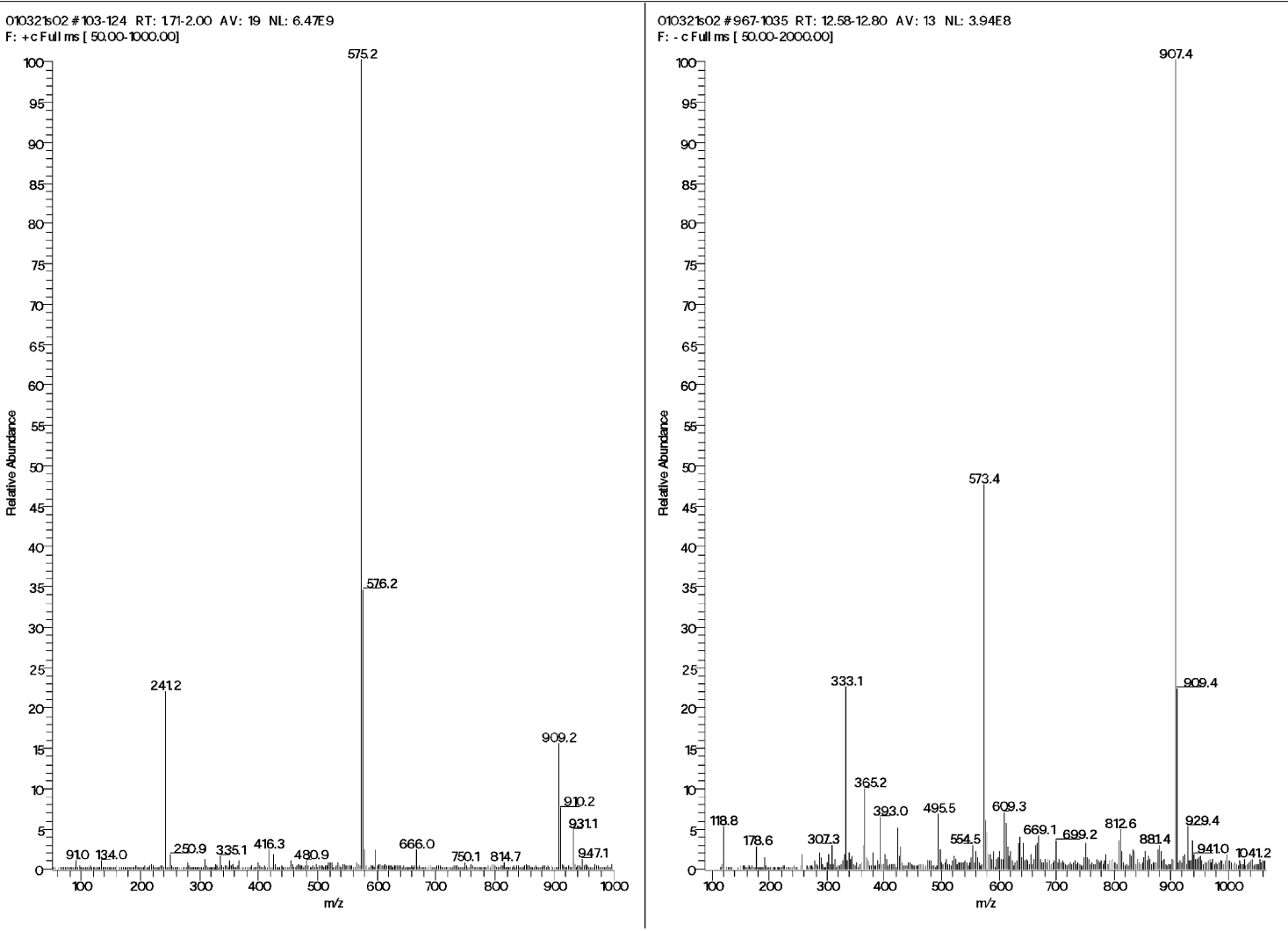

Fig. 1. MS-full scan in positive and negative ion mode.

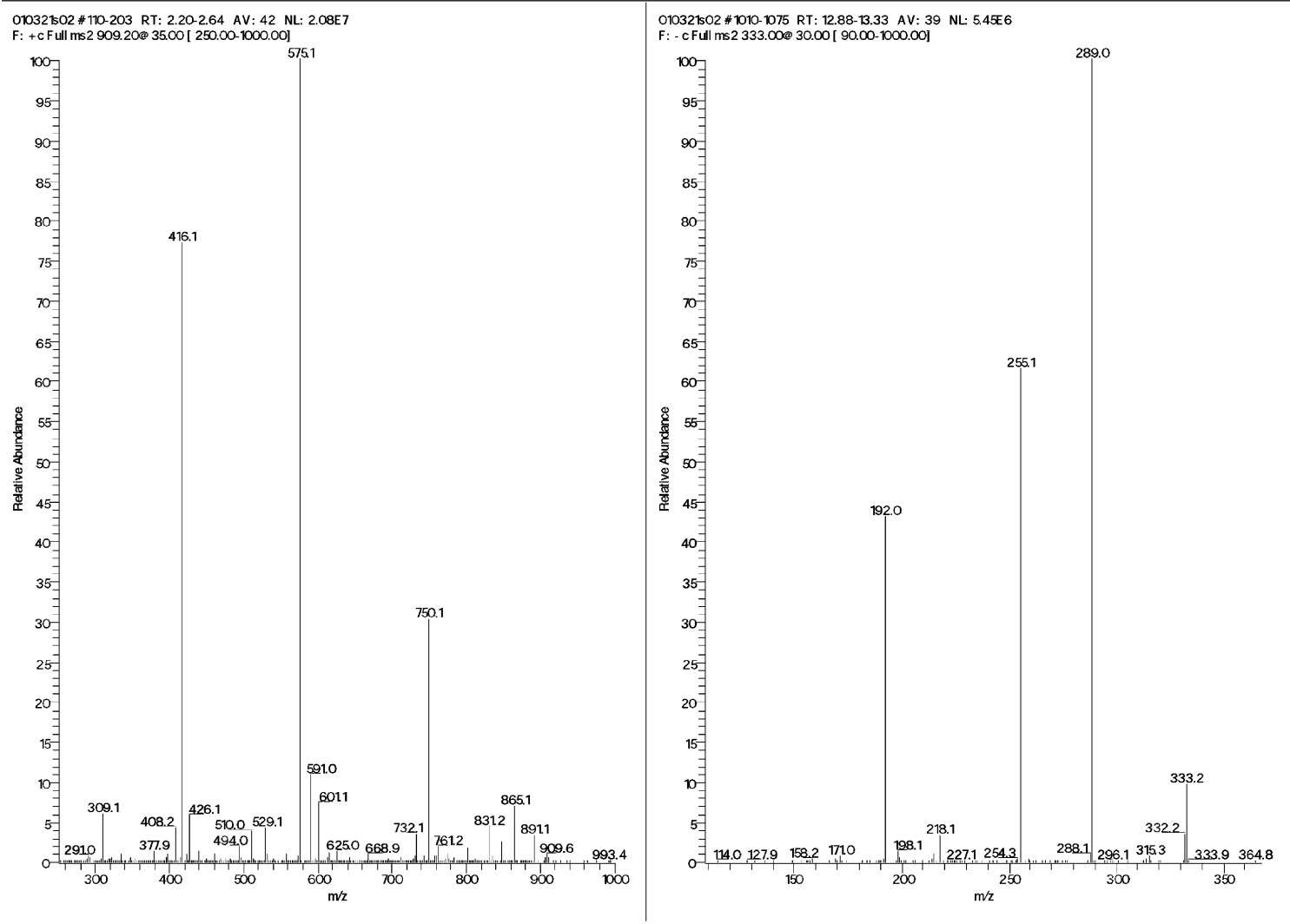

Fig. 2. MS^2 full scan positive ion mode of precursor ion 909 (left) and 333 (right).

573 + 1 = 574). An analyte with molecular weight 240 can become protonated and give a positive ion 241. In a similar way the negative ion with *m*/*z* 333 can indicate an analyte with molecular weight 334.

The above mentioned data contain enough information to perform a targeted search. The Merck Index is used as a starting point. A search is performed in the molecular weight range 906–910. Three possibilities were examined: metocurine iodide, penicillin G benzathine, platonin. Because of the predominant presence of three ions, Penicillin G benzathine of most interest.

The molecular weight of penicillin G is 333.4. Penicillin G, because of the presence of carboxyl-groups, shows a tendency to form negative ions. The negative ion with *m*/*z* 333 is an indication of the presence of penicillin G. Benzathine (MW = 240.35) is a diamine that will preferentially become protonated.

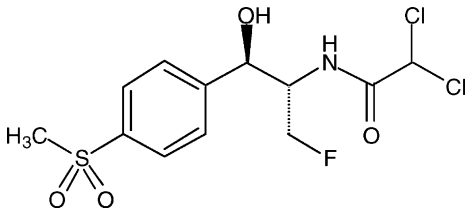

Fig. 3. Structural formula of Florfenicol.

This explains the presence of *m*/*z* 241 in positive ion mode. Penicillin G benzathine contains two penicillin G groups and one benzathine group. Fragmentation and loss of one penicillin gives the positive ion 575 (909–334). An extra confirmation is the presence of a sodium adduct (+23) in the ion with *m*/*z* 931. MS^n fragmentation of the penicillin G fragment

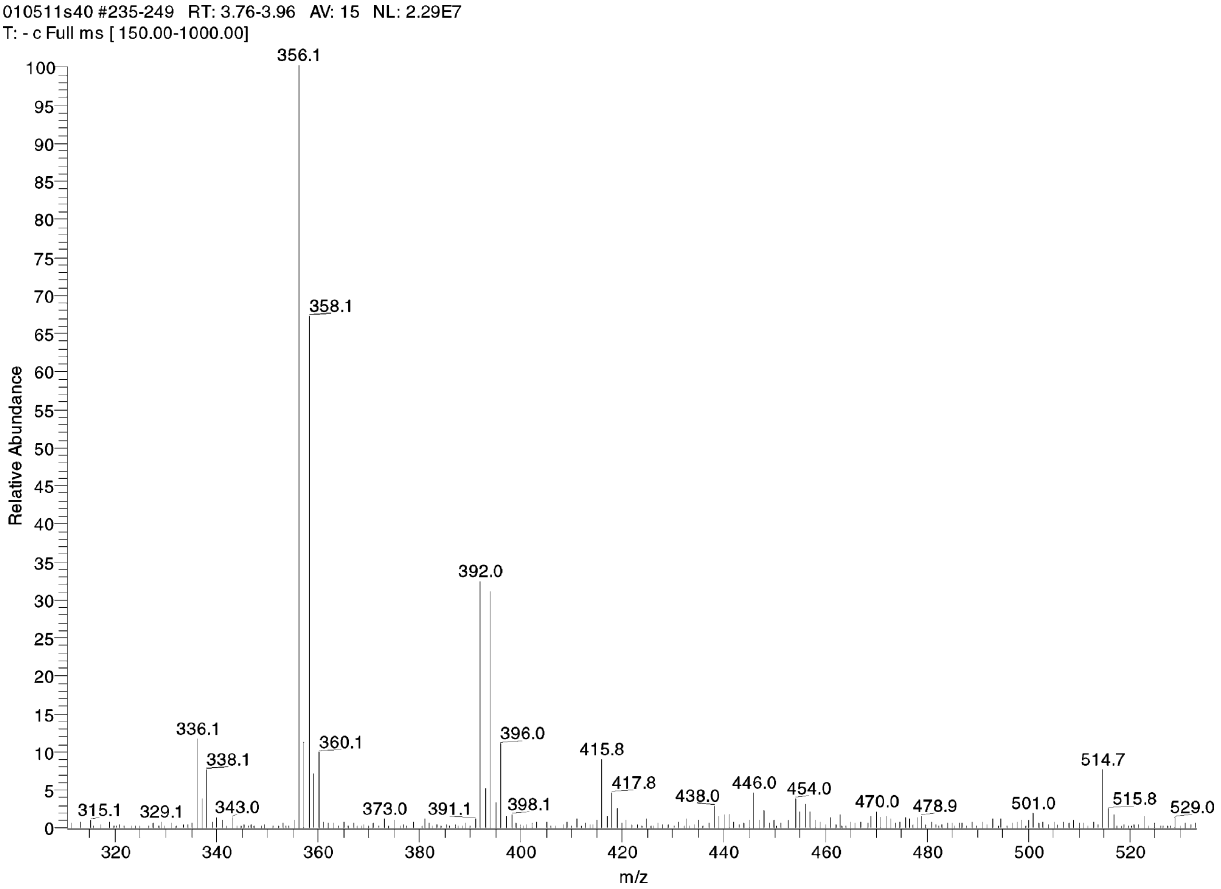

Fig. 4. MS-full scan spectrum of Nuflor 100 ng μl^{-1} Florfenicol in negative ion mode.

corresponds to the standard that was already acquired in a different application (Fig. 2).

3.5.2. Interpretation of a florfenicol formulation (Nuflor®, schering-plough animal health)

Nuflor is an injectable solution formulation with the active analyte florfenicol. Florfenicol, proposed for treatment of bovine respiratory disease also called shipping fever or transit fever, is a wide spectrum, synthetic antibacterial substance [11]. The average molecular weight is 358.21, the exact mass is 357. The empirical formula is $C_{12}H_{14}Cl_2FNO_4S$ (Fig. 3). Each chlorine atom occurs as two stable isotopes ^{35}Cl (75.77%) and ^{37}Cl (24.23). Working with the exact mass, the expected positive MH^+ ion has an m/z of 358 and the expected $M–H^-$ has an m/z of 356.

After infusion of a 100 ng μl^{-1} solution florfenicol could only be detected in the negative ion mode (Fig. 4). A distinct 356 (^{35}Cl) ion was observed combined with isotopic peaks 358 ($^{35}Cl^{37}Cl$) and 360 (^{37}Cl). Also a chlorinated adduct with m/z 392 (394, 396) was observed. Fragmentation of the adduct ions produce the original ion of florfenicol. Fluorinated compounds, as do many corticosteroids, lose fluorine in MS^2.

When the infusion concentration is lowered to 10 ng μl^{-1} chlorinated adducts dominate the spectrum. The pseudo-molecular ion is reduced to a background ion (Fig. 5). In positive ion mode the spectrum is dominated by ion clusters with a MW difference of 44. These clusters can be attributed to fragmentions of poly(ethylene glycol). The positive ion spectrum could not be used for further information (Fig. 6).

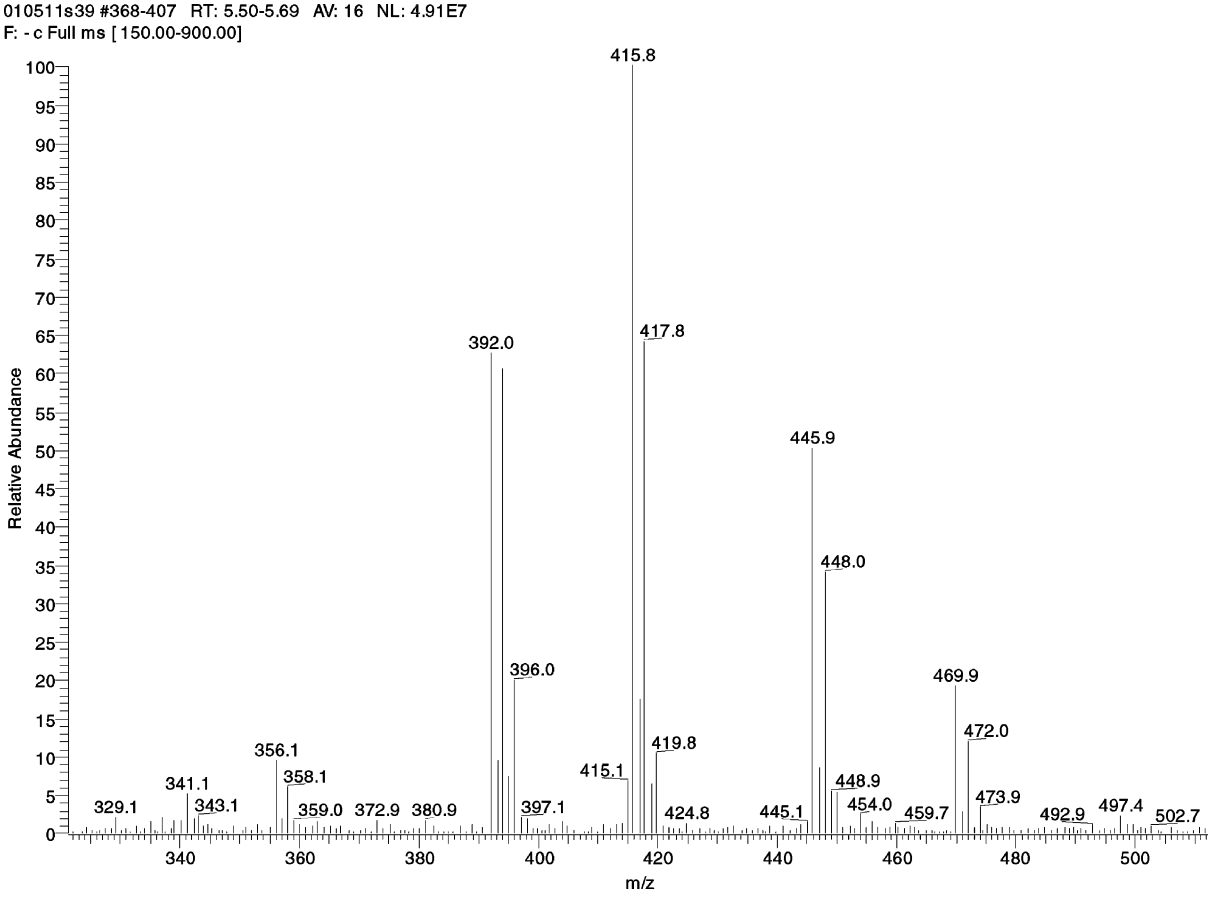

Fig. 5. MS-full scan spectrum of Nuflor 10 ng μl^{-1} Florfenicol in negative ion mode.

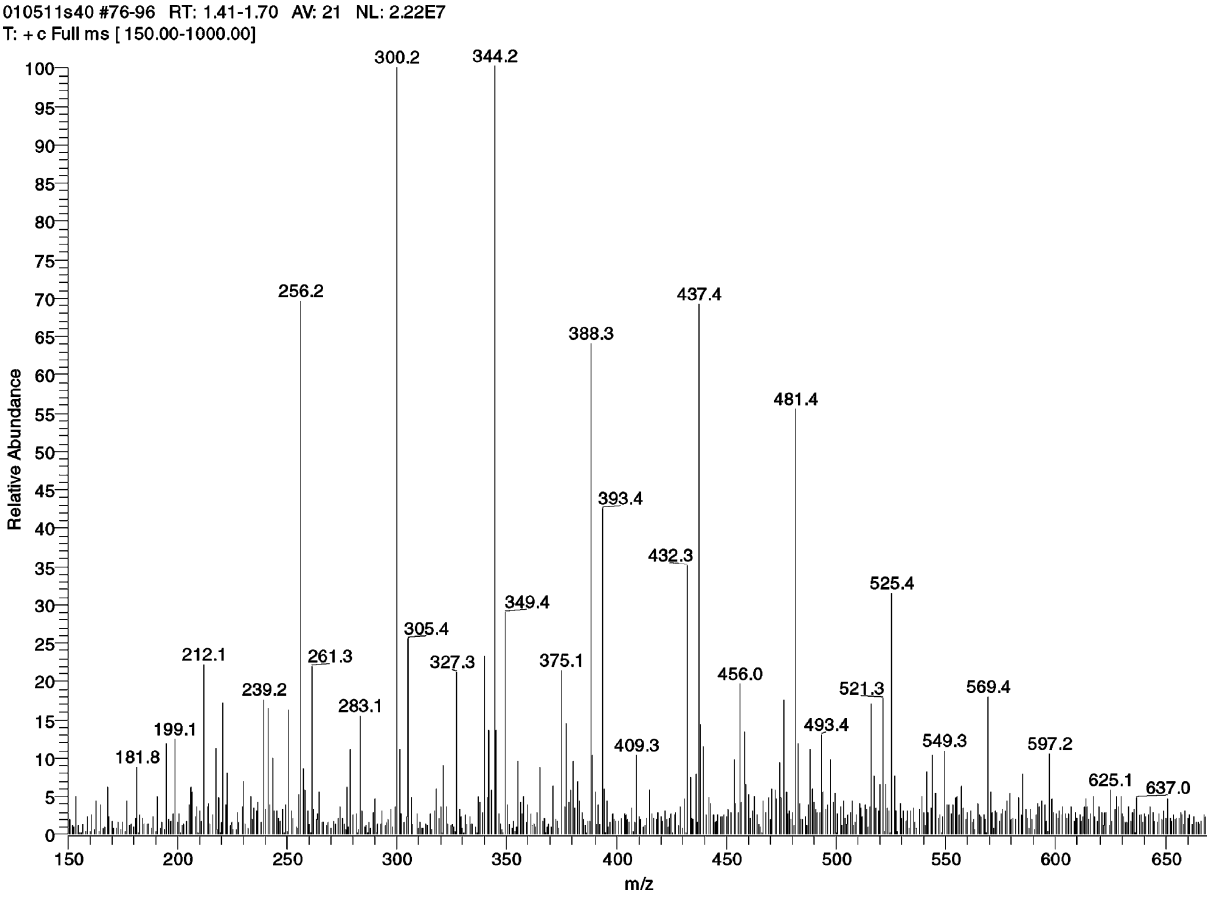

Fig. 6. MS-full scan spectrum of Nuflor 100 ng μl^{-1} Florfenicol in positive ion mode.

If Nuflor® would be present in an injection site it would be very hard to determine the presence of florfenicol because of the interference of poly(ethylene glycol) and formation of adduct ions. Therefore it is better to know the mass spectral data of the commercially available veterinary medicinal products and not the pure standard. It is also important to infuse a low and high (10 and 100 ng μl^{-1}) concentration of the VMPs because of the difference in adduct formation.

Once a database of spectra is established very fast confirmation can be obtained.

3.5.3. Identification of phenylbutazone

Phenylbutazone is a non-steroidal anti-inflammatory drug (NSAID) used in veterinary practice as a treatment used to relieve pain, fever and inflammation. The structural formula is given in Fig. 7. The negative ion spectrum in MS of an extract showed an intense ion with $m/z = 307.3$ (Fig. 8). No complementary positive ion was detected. After searching the

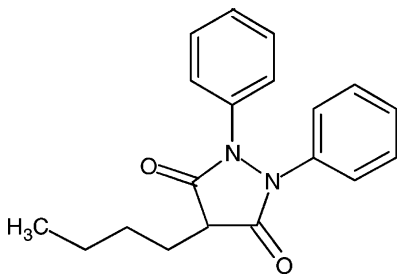

Fig. 7. Structural formula of Phenylbutazone.

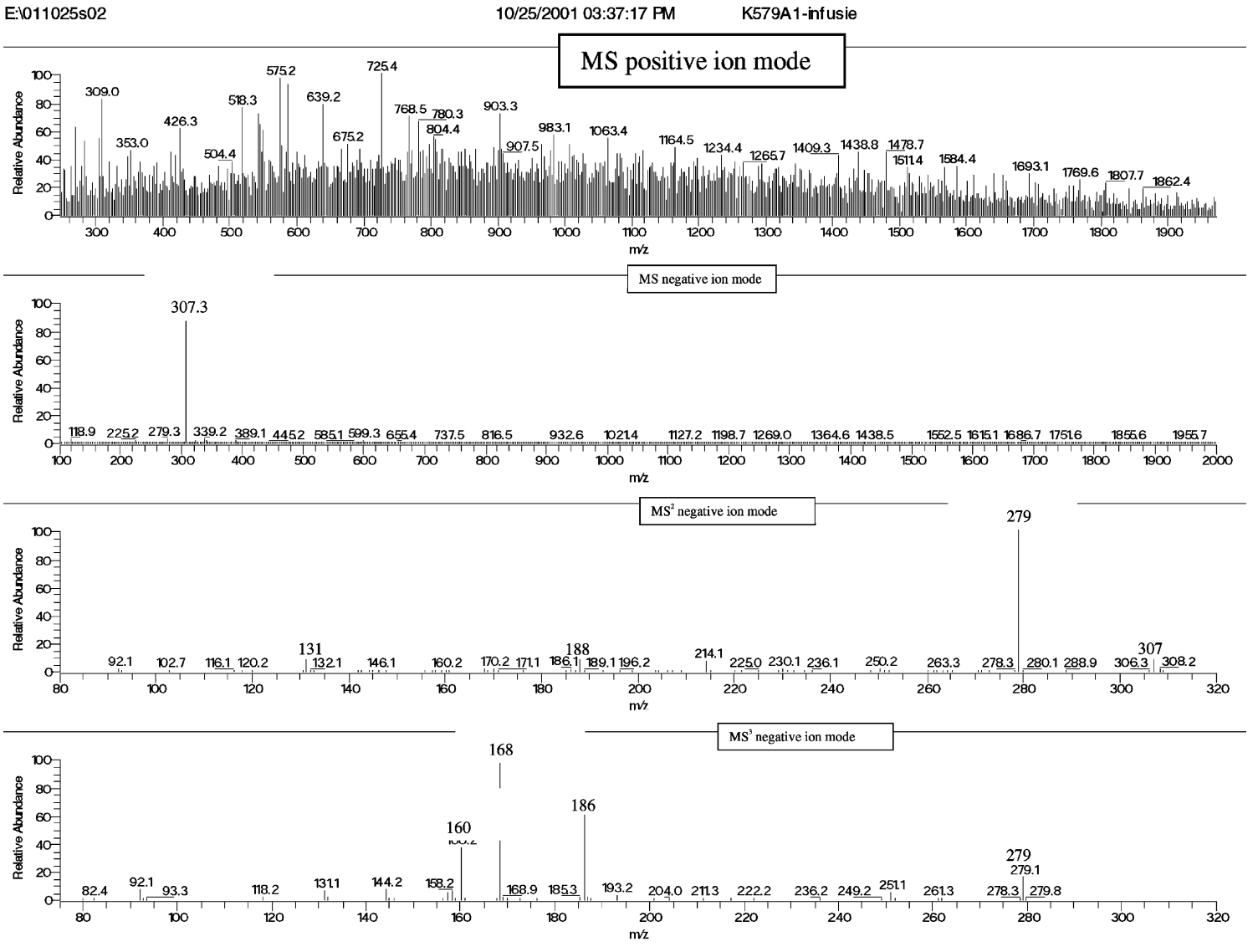

Fig. 8. MS^n data of Phenylbutazone in an injection site.

Table 3
Identity and percentages of the analytes for the total number of analysed injection sites

Analyte	Group of VMPs	Number of violations	Percentage ($n = 169$)
Flunixin	NSAID	13	7.7
Penicillin G	β-Lactam	7	4.1
Florfenicol	Florfenicol and analogues	4	2.4
Fenylbutazone	NSAID	1	0.6
Flugestoneacetate	Progestagen	1	0.6
Oxytetracycline	Tetracycline	6	3.6
Doramectin	Avermectine	1	0.6
Tolfenamic acid	NSAID	1	0.6
Sulfadimethoxin	Sulphonamide	1	0.6
Amoxycillin	β-Lactam	1	0.6
Erythromycin	Macrolide	1	0.6
Prednisolone	Corticosteroid	1	0.6

Merck Index, three compounds that are used in veterinary practice remained; nimesulide, nitrophenide and phenylbutazone. Because of the structure of phenylbutazone (two nitrogen atoms which are likely to become protonated in the positive ion mode), this was the most probable candidate. The identity was confirmed after infusion of the injectable solution.

3.6. Identified analytes in routine samples

From the beginning of 2001 until February 2002, 169 injection sites were analysed for "unknown analytes". In 37% of the injection sites an analyte could be identified. The identity of the analytes is given in Table 3; 14% of the identified analytes were NSAIDs, 8% were beta-lactam antibiotics and 6% were tetracyclines. Other analytes were identified at a smaller percentage and were classified among the following groups of VMPs: florfenicol and analogues, anabolic steroids, avermectines, sulphonamides, macrolides and corticosteroids.

4. Discussion and conclusion

In co-operation with the inspection services it was possible to screen a large number of injection sites for the presence of a variety of VMPs. Since we were working with official samples, a fast and correct way of identifying the analyte and reporting concentrations >MRL was mandatory. Because of the complexity of switching instruments to different specific applications for only one sample, a easier approach was necessary. No specific method development was needed for extraction or clean-up and confirmation. Concentrations can be reported of highly concentrated compounds after a mini-validation. Using this mini-validation it was possible to report a large number of identified analytes as violative. The samples that had to be transferred to the NRL could be analysed using a 'target' specific method. Identification was based on comparison with injectable solutions or previously collected spectra of standard solutions with application of identification criteria [9].

Extraction and identification can be performed within 24 h. If the identified compound needs also to be quantified an extra 12–24 h is necessary before the result can be reported.

This paper illustrates the advantage of using infusion MS^n or LC-MS^n with a default gradient as a fast screening and confirmation technique for highly concentrated compounds in injection sites. It is a 'real' multi-residue approach which is able to detect analytes within structural groups (e.g. penicillin G within the beta-lactam group) but also different structural groups (e.g. tetracyclines and sulphonamides) and even more groups with a different legal application in veterinary practice.

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