Collaboration

Analysis of Phenylbutazone residues in horse muscle

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Abstract

Phenylbutazone is a veterinary medicine belonging to the family of non-steroidal anti-inflammatory drugs (NSAID), and is not authorised to be used in the treatment of animals destined for the human food chain. Following the recent identification in the European Union of horsemeat in processed products which, according to the label, only contained beef meat, the European Commission published Recommendation 2013/99/EU on a coordinated control plan with a view to establish the prevalence of fraudulent practices in the marketing of certain foods. In addition to the control of the presence of horsemeat in food products marketed or labelled as containing beef (Action I), the Recommendation includes the control of phenylbutazone in horsemeat destined for human consumption (Action II). To respond to this Recommendation, the National Centre for Food (CNA) of the Spanish Agency for Food Safety and Nutrition (AESAN) has set up and validated in accordance with Commission Decision 2002/657/EC an analytical method of confirmation based on LC-MS/MS, with a Decision Limit (CCα) of 2 µg/kg. The method developed is based on an acidic acetonitrile extraction, followed by a liquid-liquid extraction and subsequent solid phase extraction (SPE) with C_{18} cartridges. Lastly the determination is made using LC-MS/MS with an electrospray probe in negative mode (ESI^-). Quantification uses an external calibration curve without matrix.

In accordance with Recommendation 2013/99/EU, a total of 108 samples were taken from horse muscle in ten different Autonomous Regions in Spain during March. The samples were all analysed at the CNA and no traces of phenylbutazone were detected in any case.

Key words

NSAID, phenylbutazone, horsemeat, horse muscle, SPE, LC-MS/MS.

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Introduction

Phenylbutazone (PBZ) is a non-steroidal anti-inflammatory drug, better known as the acronym NSAID. Phenylbutazone was originally made for use in humans in 1949 for the treatment of gout and rheumatoid arthritis. Today, given its side effects, it is only used for patients that do not respond to other treatments.

For veterinary purposes phenylbutazone is used for the treatment of musculoskeletal disorders in competition horses and pets, never for animals to be used on the food chain.

The use of phenylbutazone can particularly affect bone marrow and has been related to cases of aplastic anaemia and there are even doubts regarding its genotoxic and carcinogenic effects. In 1997 the European Medicines Agency assessed this compound in order to establish a Maximum Residue Limit (MRL) in foods of animal origin. Given the non-existence of information to ensure its food safety, a MRL could not be established and therefore, animals treated with phenylbutazone are not allowed to enter the food chain (EFSA, 2013).

Directive 96/23/EC (EU, 1996) on measures to monitor certain substances and residues thereof in live animals and animal products, transposed in Royal Decree 1749/1998 (BOE, 1998), includes in its annex I the substances that must be monitored by Member States within their national residue monitoring plans (PNIR in Spain). Among these substances are the NSAIDs, specifically classified in group B2e. Up until now there had been very few cases of phenylbutazone detected in horse meat.

In addition, the European Union Reference Laboratories for residues of veterinary drugs in 2007 published a technical guide to improve and harmonise the performance of analytical methods used for substances without an established MRL (BVL, 2007). This document establishes a minimum recommended concentration of 5 μg/kg for the analytical methods of phenylbutazone residues and its metabolite oxyphenbutazone (OPB), in matrices such as muscle, milk, kidney, liver and plasma. In other words, the detection capability (CCβ) in the screening methods, or the decision limit (CCα) in the confirmation methods, must be less than or equal to 5 μg/kg.

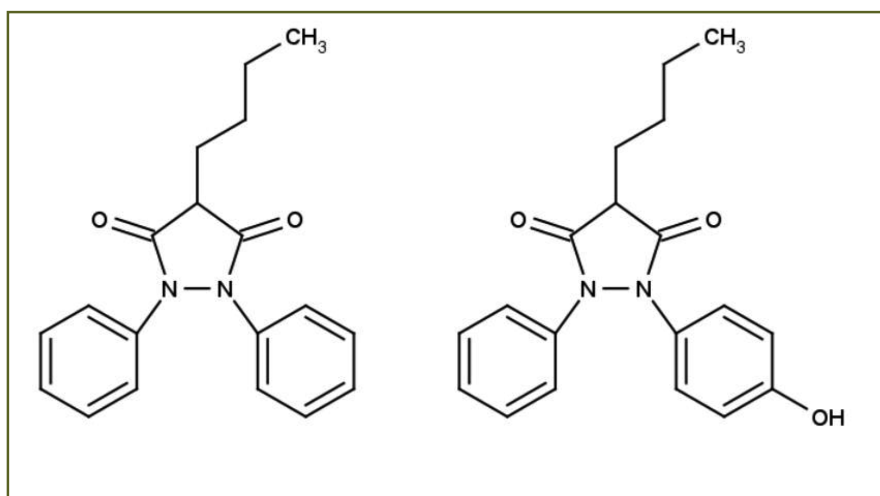

Figure 1. Chemical structures of phenylbutazone and its metabolite oxyphenbutazone.

Following the identification in some EU Member States of undeclared horsemeat in processed products, the European Commission published Recommendation 2013/99/EU on a coordinated control plan with a view to establish the prevalence of fraudulent practices in the marketing of certain foods (EU, 2013). In addition to the control of the presence of horsemeat in food products marketed or labelled as containing beef (Action I), the Recommendation included the control of phenylbutazone in horsemeat destined for human consumption (Action II).

The aim of this document was to comply with the Recommendation, which is why an analytical method was developed and validated to enable the analysis of phenylbutazone residues in horse muscle with a sufficiently low decision limit (equal to or less than 5 µg/kg). The method allowed us to confirm and quantify phenylbutazone residues from 2 µg/kg, a lower concentration than the amount recommended by the European Union Reference Laboratory for NSAID residues (BVL, Berlin) (EU-RL). The validation was performed pursuant to Decision 2002/657/EC (EU, 2002) in an interval of 2 to 30 µg/kg.

Method

1. Analytical standards and reagents

HPLC grade acetonitrile and methanol (Lab Scan), glacial acetic acid, anhydride sodium acetate, n-hexane and ethyl acetate for analysis (Merck), acetic acid 99 % and L-ascorbic acid (Sigma) and demineralised water obtained from a Direct-Q 5 system (Millipore) were used. In order to prepare the sodium acetate and L-ascorbic acid buffer at pH 4.5, 27 g of anhydrous sodium acetate and 1.7 g of L-ascorbic acid were dissolved in 800 mL of demineralised water, the pH was adjusted to 4.5 with glacial acetic acid for analysis and was filled to 1 L. This solution was prepared on a weekly basis and kept in a fridge protected from light. To prepare the 0.02 M solution of L-ascorbic acid, 3.52 g of L-ascorbic acid was dissolved in 1 L of demineralised water on a daily basis and in an amber container. The SPE cartridges used were Bond Elut C_{18} 500 mg 6cc (Varian). The evaporation system used to evaporate the extracts was evaporation in a stream of nitrogen with a controlled temperature TurboVap (Caliper). In order to filter the final extracts before injecting them into the LC-MS/MS system, PVDF 4 mm and 2 µm (Symta) syringe filters were used.

The standards used in this method were as follows: phenylbutazone (Sigma), oxyphenbutazone (supplied by the EU-RL) and phenylbutazone-(diphenyl-$^{13}C_{12}$) (Fluka).

The standard stock solutions (400 ng/µL) were prepared individually in acetonitrile/methanol 90:10. Based on the stock solutions, two intermediate solutions were prepared, a mixture of phenylbutazone and oxyphenbutazone at concentrations of 1 and 10 ng/µL. The intermediate solution, in the case of the internal standard, phenylbutazone-(diphenyl-$^{13}C_{12}$) (FBZ-$^{13}C_{12}$), was prepared at a concentration of 2 ng/µL. Three working mixture solutions were used at different concentrations of 0.04, 0.1 y 0.5 ng/µL, while the concentration of the internal standard working solution was 0.1 ng/µL. An acetonitrile/methanol mixture of 90:10 was used for all the standard solutions as a solvent.

The stock and intermediate solutions were kept at -20 ºC; and the working mixture solutions at 4 ºC.

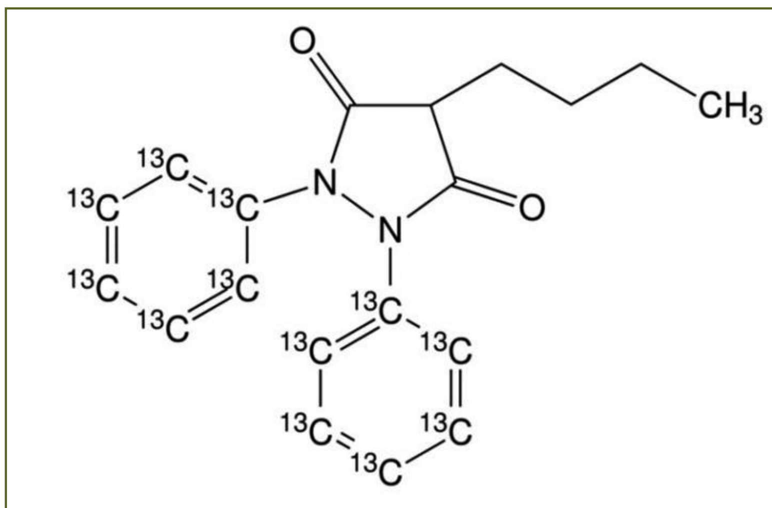

Figure 2. Chemical structure of phenylbutazone-(diphenyl-$^{13}C_{12}$), internal standard used in the method.

2.Technical equipment

LC-MS/MS equipment was used made up of: Agilent 1100 series chromatography system and triple quadrupole detector (AB Sciex 3200 Qtrap). A reversed phase ACE Excel C_{18}-PFP column was used (150 x 3 mm, 3 µm) and an in-line filter (Phenomenex, AF0-8497), with an oven temperature of 40 ºC. The autosampler temperature was kept at 15 ºC. The chromatographic separation was carried out with a mobile phase mixture of solvent A (water with 0.1 % acetic acid) and solvent B (acetonitrile/water 90:10 with 0.1 % acetic acid). The ratio used during the 8 minutes that each analysis lasted was solvent A/solvent B 70:30 (isocratic), with the flow being 400 µL/min. The ionization method used was negative electrospray ionization (ESI^-), with a source temperature of 400 ºC, an ion spray voltage of -4,5 kV, nebulizer gas pressure 25 Psi, turbo gas 70 Psi and curtain gas 30 Psi. A MRM method was used, monitoring two transitions for each analyte and one transition for the internal standard. Table 1 includes the Dwell, DP and CE values for each transition.

Table 1. Mass method used in the phenylbutazone and oxyphenbutazone analysis

Compound	Transitions	Dwell (msec)	DP (V)	CE (V)
Phenylbutazone	307.2 > 131.1*	150.0	-45	-30
	307.2 > 92.0	150.0	-45	-50
Oxyphenbutazone	323.0 > 134.0*	150.0	-50	-34
	323.0 > 295.0	150.0	-50	-26
Phenylbutazone-$^{13}C_{12}$	319.2 > 98.1	150.0	-50	-52

*Transitions used for the quantification.

3. Extraction and purification

50 µL of internal standard working solution is added to 1 g of previously homogenised muscle sample. After 10 minutes, 2 mL of sodium acetate and L-ascorbic acid buffer at pH 4.5 is added and this is shaken vigorously for 20 seconds. Then 5 mL of acetonitrile are added, the mixture is shaken for 10 minutes and centrifuged at 4,750 rpm for approximately 10 minutes. The supernatant is collected in a 50 mL polypropylene tube and set aside. 2.5 mL of acetonitrile is added to the residue and, after shaking and centrifuging, the supernatant is once again collected and mixed with that obtained in the previous step. Then 2.5 mL of n-hexane are added to the extract, it is shaken for 10 minutes and centrifuged at 4,000 rpm for approximately 10 minutes. The top layer is removed by using a Pasteur pipette. The extraction is repeated with another 2.5 mL of n-hexane. The lower layer is transferred to a 12 mL polypropylene tube and it is evaporated in a TurboVap at 50 ºC to an approximate volume of 2 mL. 8 mL of L-ascorbic acid 0.02 M are added and mixed well.

The solid phase extraction is carried out with Bond Elut C_{18} cartridges, which are conditioned by gradually passing through 10 mL of methanol and 10 mL of 0.02 M L-ascorbic acid. Then the sample extract is passed through and washed with 2 mL of 0.02 M L-ascorbic acid and 2 mL of demineralised water. The cartridges are dried under vacuum with a nitrogen stream for approximately 45 minutes. Then 2 mL of n-hexane are passed through and the cartridges are dried again for 2 minutes. Lastly, they are eluted with 4 mL of the n-hexane/ethyl acetate solution 50:50. The eluate is dry evaporated at 50 ºC in a TurboVap. It is re-suspended with 200 µL of the acetonitrile/methanol solution (90:10). Before the injection the final extract is filtered through a PVDF syringe filter. The injection volume in the LC-MS/MS system is 5 µL.

4. Validation

For the validation, 22 horse muscle samples from different sources were used. No PBZ or OPB residues or other interfering peaks were found in any of them.

The linearity was studied in external calibration curves, without a matrix, to the following concentration levels 0, 2, 5, 7.5, 10, 30 and 40 µg/kg, obtaining a coefficient of determination (R^2) >0.998 and a coefficient for the standard error of the slope (Cm) ≥95 %. Figure 3 shows all the calibration curves obtained in the validation, as well as the global curve and the confidence interval at 95 %.

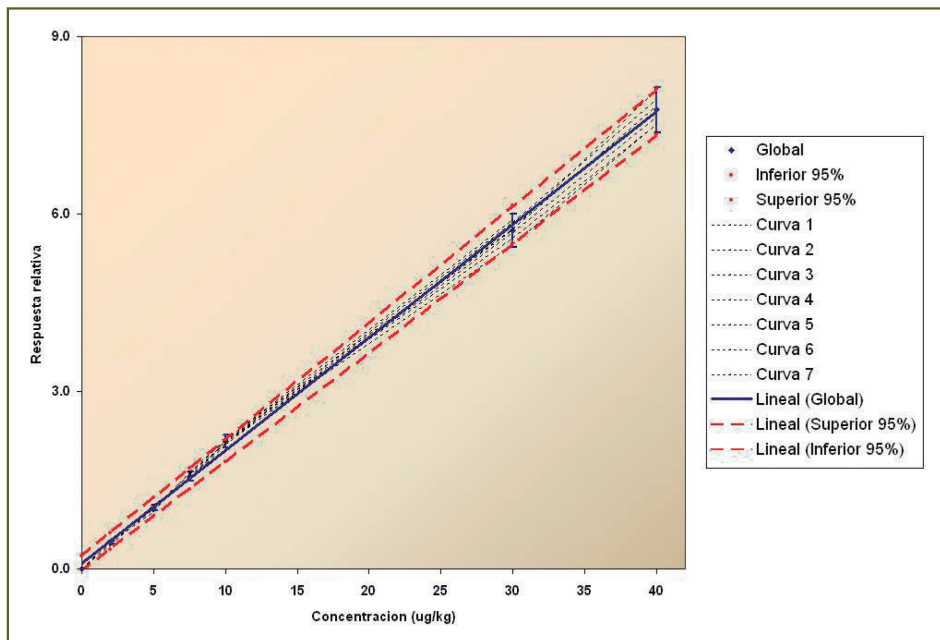

Figure 3. Calibration curves obtained in the validation.

The precision (repeatability and reproducibility) and the recovery on spiked samples at three levels of concentration were studied: 2, 10 and 30 µg/kg (n=18). The results are summarised in Table 2. The decision limit (CCα) was set at 2 µg/kg, with 100 % confirmed additions at this level and the detection capability (CCβ) calculated was 2.19 µg/kg.

Table 2. Summary of the precision results (repeatability and reproducibility) and recovery obtained in the method validation

Parameters	**Phenylbutazone**		
	Concentration studied (µg/kg)		
	2.0	10.0	30.0
Repeatability (CV %)	6.06	3.79	5.29
Reproducibility (CV %)	12.65	8.44	5.71
Recovery (% bias)	-9	9	-2

In order to check how the method worked, bovine muscle supplied by the EU-RL was used as reference material, with a concentration of 23.2±1.7 µg/kg. The result obtained was 23.3±1.4 µg/kg (n=5), in reproducibility conditions, which proved the suitable trueness of the method.

Results and discussion

The method carried out is based on the analytical procedure for determining 14 non-steroidal anti-inflammatory drugs in muscle and liver, implemented by the European Union Reference Laboratory for NSAID (Stoyke and Gowik, 2005) (Stoyke et al., 2006).

This procedure was simplified given that the aim of this research was solely the analysis of phenylbutazone and therefore, the enzymatic hydrolysis process could be avoided as phenylbutazone residues are not conjugated (glucuronates or sulphates), which is the case with carprofen and other NSAIDs (Walton et al., 2001). Also, the fact that an internal standard with chemical behaviour similar to that of the analyte was used, phenylbutazone-(diphenyl-$^{13}C_{12}$) enabled work to be carried out with external calibration curves, without the matrix, which greatly simplified the work sequence with regard to the validation and during the analysis of the samples. Likewise, the acidification step to pH 1.5-2 after the enzymatic hydrolysis included in the EU-RL method was eliminated given that it was shown to cause degradation to the PBZ and OPB. This effect on phenylbutazone was already described by Grippa et al. (2000). Furthermore, it was also discovered that the optimum organic/aqueous ratio (acetonitrile/L-ascorbic acid 0.02 M) to improve the retention in the SPE cartridge was 1:4. In the cartridge elution step a 50:50 mixture of n-hexane/ethyl acetate was chosen used by Jedziniak et al. (2010), instead of the two step elution described in the EU-RL method, since the second elution step used a mixture of acetonitrile/methanol produced extracts with more interference, as indicated by Jedziniak et al. (2010).

Oxyphenbutazone, a metabolite of phenylbutazone, was included in all the analyses, noting that the internal standard used was not suitable for the quantification thereof. The OPB analysis with this method required the inclusion of another more suitable internal standard for this compound. Nevertheless, no OPB residues were detected in any of the analyses carried out.

The extract is injected into the LC-MS/MS equipment in a 90:10 acetonitrile/methanol mixture, in other words, 100 % organic. Bearing in mind that the composition of the mobile phase had an aqueous percentage of nearly 40 %, the injection volume was reduced to 5 µL to avoid problems in the chromatography. Likewise, the isocratic method of working was suitable for separating the phenylbutazone, the oxyphenbutazone and, also, other chromatographic peaks that were detected in the same transitions as the analytes and the internal standard, although at lower retention times. These peaks could correspond with PBZ and OPB degradation products and also those of the internal standard, PBZ-$^{13}C_{12}$, given that a reduction in its signal and an increase in the peak signal with lower TR were simultaneously detected. These peaks normally showed very small areas (Figures 4 and 5), but occasionally the signal increased to the detriment of the analytes studied, PBZ and OPB (Figure 6). This could be γ-hydroxy derivatives (NTP 1990), which would explain why the retention times were lower (due to the greater polarity) and also that the MRM transitions were equal to the precursor compounds, PBZ, OBP and PBZ-$^{13}C_{12}$, since the hydroxyl group in position γ of the butyl chain would be lost in the ionisation source. These degradation products seem to form in oxidations that take place during the analytical process, since they were not detected in the direct injections of the standard solutions. Gowik et al. (1998) describe the need to use L-ascorbic acid, as an antioxidant agent, during the whole analysis process, to prevent the degradation of some NSAIDs and in particular of PBZ and OPB. This effect was

confirmed during the development of this method. Therefore to prevent the degradation of PBZ and OPB, it is important to pay attention to the expiration date and conservation conditions of the L-ascorbic acid solutions (refrigeration and protection from light). The detection of these higher polarity derivatives was possible thanks to the monitoring of the complete chromatogram. For analysis procedures that divide the mass method into various segments, a reduction in the signal of the analytes could be seen (PBZ, OPB and PBZ-$^{13}C_{12}$) without detecting the degradation products.

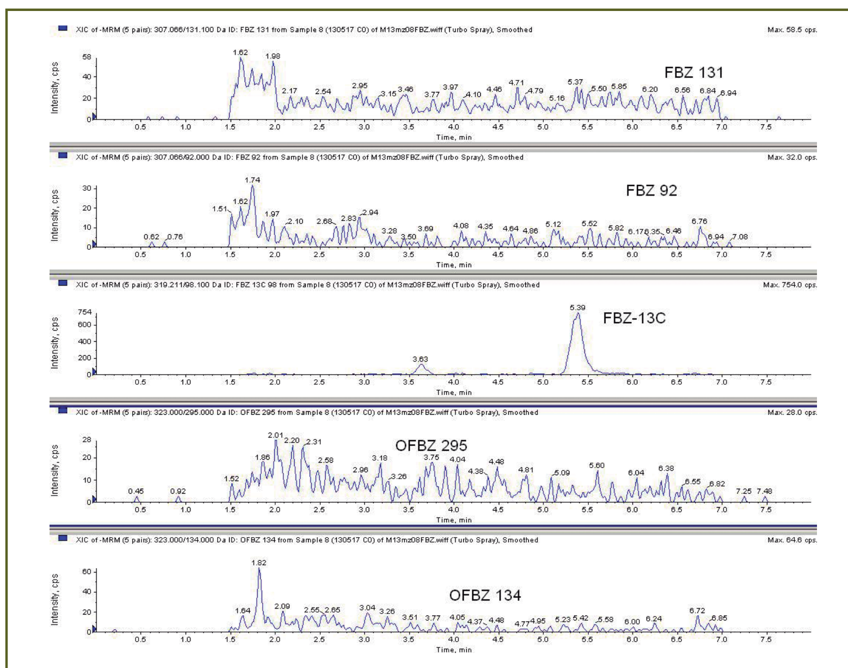

Figure 4. Chromatograms from horse muscle sample without PBZ or OPB residues.

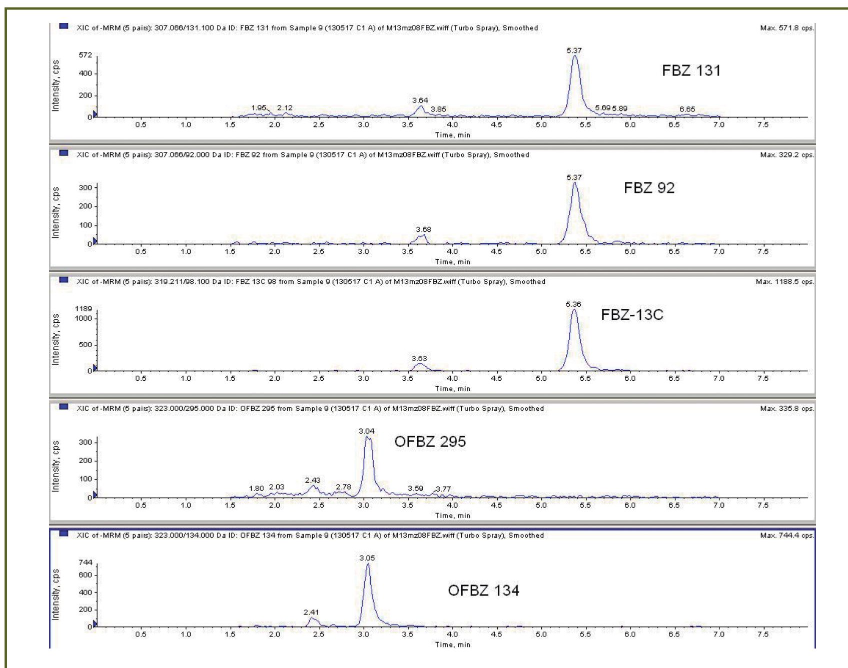

Figure 5. Chromatograms from horse muscle sample spiked at 2 μg/kg.

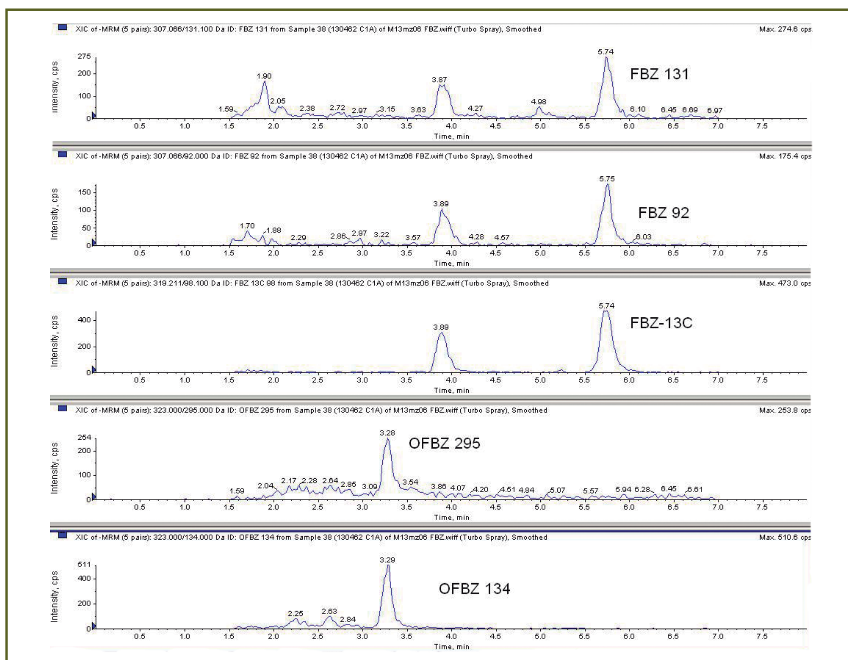

Figure 6. Chromatograms from horse muscle sample spiked at 2 μg/kg, in which an increase in the signal of the degradation products can be seen.

In compliance with Commission Recommendation 2013/99/EU (EU, 2013), the method developed was applied to a total of 108 horse muscle samples from 10 Autonomous Regions. In no cases were found residues of phenylbutazone or its metabolite oxyphenbutazone.

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