Food Control 138 (2022) 109033

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Antimicrobial residue assessment in 5,357 commercialized meat samples from the Spain-France cross-border area: A new approach for effective monitoring

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ARTICLE INFO

Keywords:
Meat
Antibiotics
Metabolites
Antimicrobial residue control
Biological screening test
QTOF chromatography.

ABSTRACT

Although antimicrobials are valuable allies in animal production, their extended use has led to unexpected threats associated with the emergence and propagation of antimicrobial resistance. Moreover, when withdrawal periods in food-producing animals are not observed, antimicrobial residues can access the food chain, causing direct toxicity, allergies, and/or intestinal microbiota dysbiosis in consumers.

Given that Spain and France are the largest meat producers in the EU and also count among the top consumers of meat, our study's aim was to investigate the presence of antimicrobials in commercialized meat purchased in the Spain-France cross-border area (POCTEFA region). 5,357 meat samples were collected from different animal species and a variety of different retailer types in Spain (Zaragoza, Bilbao, and Logroño) as well as in France (Toulouse and Perpignan). Meat samples were analysed by a screening method (Explorer®+QuinoScan®), yielding 194 positive samples, which were further evaluated by UPLC-QTOF (Ultra Performance Liquid Chromatography-Quadrupole Time of Flight) for confirmation. Chromatographic analyses found antimicrobial residues in 30 samples, although only 5 of them (0.093% of initial samples) were non-compliant according to the current legislation. Further studies suggested that this mismatch between screening and confirmatory analyses might be due to the presence of biologically active metabolites derived from degradation of antimicrobials that were not identified by the targeted UPLC-QTOF method, but which might play a decisive role in the inhibition of the biological Explorer® test. Although chromatographic techniques detect the marker compounds determined by European and national regulations, and although they are the methods selected for official control of antimicrobials in food, certain unknown metabolites might escape their monitoring. This thus suggests that biological tests are the most adequate ones in terms of ideal consumer health protection.

1. Introduction

The current health situation, strongly marked by the pandemic that humanity is going through, has evinced the importance of good global health from a "One Health" perspective. From a holistic point of view, the aim is to ensure a good health status not only of humans but also of

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https://doi.org/10.1016/j.foodcont.2022.109033
Received 29 January 2022; Received in revised form 18 March 2022; Accepted 10 April 2022
Available online 15 April 2022

animals and the environment. In this context, antibiotics have emerged as a major safeguard of human and animal health. Nevertheless, misuse of these compounds or even their utilization for non-therapeutic purposes (Brown, Uwiera, Kalmokoff, Brooks, & Inglis, 2017; Diana, Santinello, Penasa, Sacali, et al., 2020) can lead to serious public health problems.

On the one hand, the consumption of food contaminated with antimicrobials can have worrisome implications for consumers, not only due to direct toxicity, but also in view of possible allergic reactions, changes in the balance of the intestinal microbiota, and the potential emergence of antimicrobial resistance (AMR) in those bacterial communities (Anthony, Burnham, Dantas, & Kwon, 2021; Bacanlı & Başaran, 2019; Pilmis, Le Monnier, & Zahar, 2020). On the other hand, the unexpected delivery of antimicrobial residues from the farm to the environment might likewise contribute to the propagation of AMR (Hamscher, Pawelzick, Höper, & Nau, 2005; Sarmah, Meyer, & Boxall, 2006; Zhou et al., 2020). As the main guarantor of health, the World Health Organisation (WHO) regards AMR as one of the major threats for the next decades (WHO, 2021), since the emergence and dissemination of AMR bacteria renders antimicrobials ineffective against common illnesses.

Since food-producing animals are important consumers of antimicrobials, the eventual appearance of antimicrobials in meat is an important risk to bear in mind. Exhaustive controls are indeed being implemented by health authorities. Most official control plans are performed as a sequential two-step analysis, applying an initial screening of samples with a rapid broad-spectrum method (usually a biological or biochemical test). When a presumptive positive result is obtained, a second analysis is performed with a confirmatory method. Both kinds of methods are foreseen by Regulation (EU) 2021/808, each of them with their own requirements and validation criteria, always observing Regulation 37/2010 regarding Maximum Residue Limits (MRLs) of antimicrobials in meat.

Additionally, the EMA (European Medicines Agency) encourages the rationalization of the use of antibiotics (EMA, 2020). Hence, both the increase of responsible use as well as the implementation of strict legislation have accomplished a reduction in the incidence of antimicrobial residues in food of animal origin for human consumption to percentages as low as a 0.14% in 2019 in Europe (EFSA, 2021), although some studies have revealed a higher incidence. For instance, a study performed in Cyprus on pig meat collected in slaughterhouses showed that 4% of the samples contained antimicrobial residues, although only 1% of them presented amounts of antimicrobials lying above the MRLs (Kyriakides et al., 2020). Even more worrisome are data presented on a review about the presence of antibiotics in muscle tissue of poultry from Bangladesh (Hassan et al., 2021), that shows that up to a 70% of the muscle tissues analysed by biological methods resulted positive to the presence of antimicrobials, and chromatographic analyses reported markedly high amounts of some antibiotics such as amoxicillin.

Even though European data show that current measures are thoroughly effective in ensuring a minimum incidence of antimicrobial residues in meat, agents involved in meat production should not lower their guard. The continuous consumption of compliant meat samples with antimicrobial residues lying below the MRLs, could still lead to side effects of antibiotic consumption, such as liver damage, carcinogenicity, hypersensitivity reactions, gastrointestinal disorders, and reproductive toxicity (Baynes et al., 2016). Despite the considerable amount of data regarding non-compliant samples in the EU, few studies have been recently carried out regarding the levels of antimicrobial residues in commercialized meat including data from compliant and non-compliant contaminated samples (De Wasch et al., 1998; Bartkiene et al., 2020; Kyriakides et al., 2020).

The POCTEFA area is located in the Spain-France cross-border area. These two countries have the highest meat production rate in the EU (Eurostat, 2020). Their meat consumption is also among the highest in Europe (Kanerva, 2013), and even worldwide (more than 80 kg per capita in France, and 90 for Spain in one year according to the Food and Agriculture Organization (FAO, 2021)).

Therefore, in view of the importance of meat production and consumption in Spain and France, the study's aim was to evaluate the incidence of antimicrobial residues in commercialized meat collected in five of the most populous cities of the POCTEFA area, stemming from 12 animal species with different origins and a diverse retailer typology, classifying the trends of residue appearance, and performing a two-step analysis (screening and confirmatory) analogous to the one proposed for official control.

2. Materials and methods

2.1. Meat sample collection

The POCTEFA region comprises territories in Spain (Bizkaia, Gipuzkoa, Araba, Navarra, La Rioja, Huesca, Zaragoza, Lleida, Girona, Barcelona, and Tarragona) and in France (Pyrénées-Atlantiques, Ariège, Haute-Garonne, Hautes Pyrénées, Pyrénées-Orientales). From January 2020 to February 2021, 5,357 commercialized meat samples were collected from five cities in this area: Zaragoza (Aragón, Spain), Logroño (La Rioja, Spain), Bilbao (País Vasco, Spain), Perpignan (Pyrénées-Orientales, France), and Toulouse (Haute-Garonne, France). The retailers selected for sample purchase were representative of the different trade models, and the amount of samples of each species depended on consumption data (Agreste, 2020; MAPA, 2019), availability, and diversity of commercial brands, which was particularly high in bovine meat. All the collected samples were immediately refrigerated until subsequent analysis in the course of the following 24 h.

The meat samples purchased stemmed from 12 different species (Table S1) and several production areas (Table S2). The number of samples collected from each city was equivalent (Table S2). The largest percentage corresponded to bovine samples (26.7%), followed by chicken (24.8%), pig (17.5%), turkey (10.3%), lamb (8.9%), and rabbit (5.9%). A small percentage of the samples was reserved for less common species linked to regional dietary habits such as duck, horse, quail, goat, Guinea fowl, and partridge (Table S1).

In terms of origin, 24.2% of all samples were produced in the POCTEFA area. Meanwhile, 61.6% of them were produced in the rest of Spain or France; 7.4% were produced abroad (European and non-European countries, excluding Spain and France), and only 6.8% were of unknown origin (mostly meat purchased in butcher's shops).

The percentage of samples by species varied among cities, depending on cultural trends and habits (Alcalde, Ripoll, & Panea, 2013; Godfray et al., 2018), but overall trends were maintained. Although the percentage of samples by origin varied among cities, the pattern was likewise analogous.

2.2. Sample analysis

Screening was performed on all 5,357 commercial meat samples; only positive samples were re-analysed with chromatographic techniques for confirmation.

2.2.1. Screening analysis

Meat samples were prepared and analysed immediately after collection. Two different methods were used for the screening of antimicrobials in commercialized meat samples: Explorer® (Zeulab, S.L., Zaragoza, Spain) for the detection of a wide range of antimicrobials, and QuinoScan® (Zeulab, S.L.), for the specific detection of quinolones.

2.2.1.1. Sample preparation. A piece of fresh meat of 3 ± 0.5 g was weighed and placed in a tube with a screw top. It was then placed in a thermostatic water bath at 100 °C for 5 min. The meat was squeezed using a pair of tweezers to obtain as much juice as possible and discard the meat from the tube, keeping the juice. The obtained juice was

centrifuged for 3 min at 3,500 r.p.m. using a MiniSpin® (Eppendorf, Hamburg, Germany) centrifuge, and the supernatant was used to perform the analyses. Samples with high collagen content (such as chicken drumsticks) were left sedimenting for 5 min at room temperature instead of being centrifuged. If a sample was gelatinous or difficult to pipette, it was heated in a water bath at 100 °C for 15 s.

In parallel, a 200 g piece of each meat sample was vacuum packaged and kept frozen at −20 °C.

2.2.1.2. Explorer® test. Explorer® (Zeulab, S.L., Zaragoza, Spain), is a biological method for the screening of antimicrobials in meat samples (Mata, Sanz, & Razquin, 2014). It is supplied in small tubes that contain a bacterial growth media and spores of *Geobacillus stearothermophilus*, along with a pH indicator. When the tubes are incubated at 65 °C, spores germinate and cells grow, thereby producing acid compounds that lower the pH of the media, changing its colour from purple to yellowish. Nevertheless, when the sample contains amounts of antimicrobial residues that lie above the method's LoD (limit of detection), microbial growth is slowed down or is even brought to a standstill. During the assay, colour changes due to the microorganism's metabolism are monitored by the e-Reader® (Zeulab, S.L.) device, which is also an incubator. The device automatically determines the assay end time (after approx. 3 h) and then provides a numerical value (ER, Explorer® result) proportional to the blue colour intensity, and a positive/negative binary result indicating the presence or absence of antimicrobial residues. The test was performed according to the instructions provided by the manufacturer, and samples that yielded a positive result were re-tested to confirm the screening result.

2.2.1.3. QuinoScan®. Although the Explorer® test is able to detect a broad spectrum of antimicrobial families, it cannot detect molecules of the quinolone family at levels lower than or close to the MRL. Therefore, to perform a complete screening of antimicrobials in the meat samples, QuinoScan® was added as a complementary test for the detection of quinolones.

QuinoScan® is a competitive lateral flow immunochromatographic test that specifically detects molecules of the quinolone family. It consists of two components: a tube containing the freeze-dried detection reagent (gold nanoparticles functionalized with a specific antibody) and a strip with the capture reagents. If the sample does not contain quinolones, the reagent present in the tube will bind to the test line in the strip when the assay is run, and an intense red line will appear. When quinolones are present in the sample, they will bind the antibodies of the detection reagent instead of binding the test line in the strip, thereby inhibiting the appearance of the red colour of the test line, or decreasing its intensity. The test was performed according to the instructions provided by the manufacturer, and results could be read objectively with the IRIS® (Zeulab, S.L.) device. Samples that yielded a positive result were re-tested to confirm the screening result.

2.2.2. Confirmatory method: UPLC-QTOF

2.2.2.1. Standards and reagents. A full list of the chemicals used for this methodology is provided in the Supplementary Information section (Table S5). All antibiotic standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), and purity was >93%, except for macrolides, for which purities were >86%.

For the measurement of internal standards (see Table S5), ciprofloxacin-d8 (99.9%), roxithromycin (97.6%), demeclocycline (91.0%), and piperacillin (96.4%) were supplied by Sigma-Aldrich, whereas sulfamethoxypyridazine-d3 (99.9%) was acquired from Witega (Berlin, Germany) and cloranfenicol-d5 (100 μg/mL in acetonitrile, ≥ 98%) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Stock standard solutions were dissolved individually to prepare solutions of approximately 1000 μg/mL. Spiking solutions were prepared in methanol and stored at −20 °C.

Acetonitrile (ACN, HPLC grade, ≥ 99.9% and optima LC/MS grade), methanol (MeOH, optima LC/MS grade), formic acid (HCOOH, Optima LC/MS grade) and dimethyl sulphoxide (DMSO, > 99.7%) were supplied by Fisher (Geel, Belgium), ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA, 99-101%) and sodium hydroxide (0.1 N volumetric solution, 99-101%) by Panreac (Barcelona, Spain) and N,N-dimethylformamide anhydrous (N,N-DMF, 99.8%) by Sigma-Aldrich. Ultra-pure water was obtained using a Milli-Q water purification system (Millipore, Merck KGaA, Darmstadt, Germany).

For extraction, a mechanical laboratory shaker (Multi Reax, Heidolph, Schwabach, Germany) and a centrifuge (Sigma 4-16 KS, Osterode am Harz, Germany) were used. Fractions were evaporated in a Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA). After the reconstitution step, the supernatant was filtered through PTFE filters (0.45 μm, 13 mm, Millipore) prior to UHPLC-QTOF-MS/MS analysis.

2.2.2.2. Sample extraction. Extraction was performed according to a previously described method (ANSES/LMV/16/02) with minor modifications. Briefly, 2 g of sample were placed together with 8 mL ACN and 800 μL EDTA in a 15 mL vessel, and surrogate standards (sulfamethoxypyridazine-d3, ciprofloxacin-d8, piperacillin, roxithromycin and demeclocycline (40 μL of a 3 μg/mL solution), and chloramphenicol-d5 (60 μL of a 2 μg/mL solution)) were added. For sample extraction, a mechanical laboratory shaker was used for 10 min at 2,000 r.p.m. After the extraction step, sample was centrifuged for 5 min at 3,500 r.p.m and 4 °C. 6 mL of supernatant was evaporated to dryness under a nitrogen stream using a Turbo Vap LV Evaporator and reconstituted in 500 μL of Milli-Q water. The reconstituted extracts were filtered through a 0.45 μm PTFE filter prior to UPLC-QTOF analysis.

2.2.2.3. UPLC-QTOF analysis. Identification of antibiotics was performed using an UPLC Exion LC AD chromatographic system coupled to a SCIEX X500R time of flight (QTOF) mass spectrometer equipped with an electrospray ionization (ESI) turbo V source (SCIEX, Framingham, MA, USA). A Luna Omega Polar C18, 100 Å (1.6 μm, 2.1 × 100 mm) column was used for separation of the target analytes at 40 °C.

Mobile phase A consisted of Milli-Q water (with 0.1% formic acid) and mobile phase B of MeOH (with 0.1% formic acid). The gradient profile started with 95% A (retention time 1 min) and continued with a linear change to 0% An up to 8 min (retention time 1 min). Initial conditions were regained at 9-10 min followed by equilibration until 12 min. The flow rate and the injection volume were set at 0.4 mL/min and 10 μL, respectively.

For data acquisition, Sequential Window Acquisition of All Theoretical Fragment-ion Spectra (SWATH) mode was performed. The method consists of a single TOF-MS experiment over a mass range from m/z 100 to 1000 with an accumulation time of 0.25 s, followed by nine MS/MS experiments distributed across the full mass range as follows: m/z 100-250, 249-275, 274-300, 299-350, 349-400, 399-450, 449-500, 499-650, 649-1000 (accumulation time: 0.03 s). The declustering potential was set to 80 V, and the collision energy to 35 ± 15 eV in each window. Nitrogen was used as nebulizer, drying, and collision gas. ESI in positive mode was carried out using a spray voltage of 5,500 V, a source temperature of 600 °C, a curtain gas pressure of 35 psi, and Ion Source Gas 1 and Ion Source Gas 2 to 55 and 65 psi, respectively. Monitored m/z values of the molecular ions and fragment ions can be found in Table S5.

The LoDs of the 56 identified antibiotics are shown in Table S6. All the analyses are accredited by ENAC (ENAC, 2021). Only aminoglycosides are not included in the list of identified molecules, as due to their chemical characteristics they are not properly identified by the generalist chromatographic technique used herein. The LoD for each antibiotic was calculated as settled in Equation (1), where C_{val} corresponds to the target concentration for validation (μg/kg), *H3xB* is the response (peak height) corresponding to 3 times the mean noise, and *M* is the

mean of responses of the spiked samples for validation (ANSES/LMV/16/02).

$$LoD = Cval \times \frac{H3xB}{M} \quad (1)$$

For data processing, SCIEX OS Software was used (Version 1.6). Antibiotic identification was confirmed with the following criteria: molecular ion minimum peak area ≥3 s/n, molecular ion mass accuracy ≤5 ppm, fragment ion accuracy ≤10 ppm, and molecular ion difference in retention time less than ±2.5% compared to the reference standard. Moreover, library spectrum match and formula finder score were also used for confirmation. Replicates were only performed for quality monitoring of the method.

2.3. Additional analysis

2.3.1. Minimum inhibitory concentrations (MICs) of essential oils

In order to evaluate the MIC of several essential oils and some of their active compounds commonly added to animal feeding, carvacrol (95%; Sigma-Aldrich, Steinheim, Westphalia, Germany), oregano (Bionanoplus SL, Noáin, España), cinnamaldehyde (99%; Acros Organics, Fairlawn, New Jersey, USA), cineole (99%; Sigma-Aldrich), p-cymene (99%; Sigma-Aldrich), thymol (99%; Sigma-Aldrich), and limonene oxide (97%; Sigma-Aldrich) dilutions in antibiotic-free meat juice were prepared and analysed with the Explorer® test. Essential oils are practically immiscible in water; therefore, a vigorous shaking method was applied to obtain homogeneous dispersions and immediately added to the sample (Friedman, Henika, & Mandrell, 2002). Several concentrations ranging from 100 to 1000 μL/L were tested. Three replicates for each essential oil and condition were performed.

2.3.2. Evaluation of the stability of antibiotic residues in meat

Meat samples from the sample bank built by Serrano et al. (2020), originally obtained and characterized in 2018, were re-analysed in 2021 by UPLC-QTOF after having remained vacuum-packaged and frozen for 3 years at −20 °C.

2.3.3. P-aminobenzoic acid assay for the detection of sulfonamides

P-aminobenzoic is a precursor of folate synthesis, the metabolic pathway of which is inhibited by sulfonamides through competitive inhibition. Thus, this property was used to detect sulfonamide molecules or their metabolites with biological activity. Muscle samples were analysed in duplicate by Explorer® test before and after the addition of 50 μg/mL of p-aminobenzoic acid (99%, Sigma-Aldrich) per mL of meat juice (FIL/IDF, 1991; Sanz et al., 2015).

2.3.4. BTScan® test for detection of ß-lactam antibiotics

BTScan® test was used for the specific detection of ß-lactam antibiotics in meat samples. Samples were analysed in duplicate.

BTScan® (Zeulab, S.L.) is a competitive lateral flow immunochromatographic test that specifically detects ß-lactams and tetracyclines in milk. The principle of the assay is similar to that described previously in section 2.2.1.3. for the detection of quinolones. The test was performed following the instructions provided by the manufacturer. Nevertheless, since BTScan® test was designed for the analysis of milk, the protocol of use was accordingly adapted to the analysis of meat juice. After exploring the impact of several dilutions of meat juice in milk, a dilution 1/5 of meat juice in milk was chosen for performing the analyses.

The test's performance in the detection of ß-lactam antibiotics in meat was verified with 4 widely used substances: amoxycillin, ampicillin, benzylpenicillin, and ceftiofur. LoDs (15, 15, 10 and 500 μg/kg, respectively) were lower than the corresponding MRL. BTScan® test was also able to detect tetracyclines; however, the LoD (for oxytetracycline 400 μg/kg) was higher than the MRL and thus it could not be used to confirm the presence of tetracyclines or their active metabolites in the positive screening samples.

2.4. Statistical analysis

PRISM® program was used for data processing and representation, as well as for statistical analysis (GraphPad Software, Inc., San Diego, CA, USA). Two-way ANOVA was used to compare results. Differences were considered statistically significant when $P \leq 0.05$.

3. Results and discussion

3.1. Screening

Explorer® and Quinoscan® tests were simultaneously performed over the 5,357 meat samples, and results of the screening were obtained after considering both outcomes.

3.1.1. Explorer® results

Table 1 shows the results of the Explorer® analyses of 5,357 commercialized meat samples, classified by species and city (Table 1a), as well as by production origin and city (Table 1b). Globally, 3.5% of the collected meat samples yielded positive results to Explorer®. Percentages in individual cities ranged from 1.3% (Perpignan) to 5.7% (Toulouse). The highest percentages of positive samples were reported for duck (9.2%), turkey (6.3%), and chicken (3.9%), while the lowest was found in bovine meat (1.3%). Moreover, positivity was markedly high in duck samples collected in Logroño as well as in turkey and duck samples collected in Toulouse where over 35.5%, 15.6% and 10.4% of gathered samples were positive to Explorer®, respectively. In general terms, the lowest values were associated with less intensive production systems: bovine, lamb, and horse.

Regarding origin, the lowest percentage of positive results was reported for samples produced in the POCTEFA area (2.7%), closely followed by the ones produced in foreign countries (2.8%). Meat samples produced in the rest of Spain and France showed 3.3% of positivity, while samples from an unknown origin raised the degree of positivity up to 8.2%, a fact that could be linked to a lack of control and/or traceability.

3.1.2. QuinoScan® results

The results of the analysis of the 5,357 samples by QuinoScan® are shown in Table 2a (classified by species and city) and 2b (presented by origin and city). Data presented in Table 2 show that only 0.2% of the samples yielded positive results to QuinoScan®. The highest percentage of positives was reported in Perpignan (0.5% of the samples collected), and, in terms of species, for rabbit (1.3%) and duck samples (1.2%). Regarding sample origin, data obtained evidenced a good control of quinolone utilization in livestock farming in the POCTEFA area, with just one positive out of 1,298 analysed samples. Most quinolone-positive meat samples came from the area designated as "rest of Spain-France" (10 out of 11).

3.1.3. Screening results

Final results from the screening phase are presented in Table 3, organized by species and city (Table 3a), on the one hand, and production origin and city (Table 3b), on the other. In summary, 194 samples were positive to the screening (Explorer® and QuinoScan®), which, in relative terms, corresponds to 3.6% of the samples collected.

The two tests, carried out simultaneously, revealed that antibiotic utilization trends in veterinary medicine can vary considerably among different areas. A good example can be observed in the trends described for duck. We found a high positivity rate in duck samples collected in Logroño and Toulouse according to Explorer® results, while duck samples from Perpignan seemed to be free of antibiotics. In contrast, all the positive results in duck samples found in Perpignan were only detected by QuinoScan®, and were thus exclusively linked to the presence of quinolones. Hence, this complementarity demonstrates the suitability of the in-tandem application of both tests to analyse the

Table 1
Number and percentage of samples of each species (Table 1a) and each territorial origin (Table 1b) positive to Explorer® collected in each city (compared with the total number of samples of each species collected in each city).

1a	Zaragoza		Bilbao		Logroño		Toulouse		Perpignan		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Bovine	7	2.3	3	1.3	4	1.2	5	1.4	0	0	**19**	**1.3**
Chicken	14	4.7	7	2.3	5	1.9	25	7.8	1	0.7	**52**	**3.9**
Pig	10	4.6	12	5.9	2	0.9	11	6.4	0	0	**35**	**3.7**
Turkey	7	6.4	5	4.5	6	5.8	12	15.6	5	3.3	**35**	**6.3**
Lamb	5	3.4	3	6	2	1.6	0	0	1	0.9	**11**	**2.3**
Rabbit	4	4.8	4	6.8	1	0.9	0	0	3	5.8	**12**	**3.8**
Duck					11	35.5	5	10.4	0	0	**16**	**9.2**
Horse					0	0	0	0	2	3.3	**2**	**2.5**
Quail					0	0					**0**	**0**
Goat					2	18.2					**2**	**18.2**
Guinea Fowl							1	50	0	0	**1**	**25**
Partridge					0	0					**0**	**0**
TOTAL	47	4.1	34	3.5	33	2.6	59	5.7	12	1.3	**185**	**3.5**

1b	Zaragoza		Bilbao		Logroño		Toulouse		Perpignan		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
POCTEFA	14	3.8	13	3.3	1	1.3	6	1.9	1	0.7	**35**	**2.7**
Rest of Spain-France	25	4.2	19	3.9	25	2.8	30	5.0	9	1.3	**109**	**3.3**
Abroad	3	3.4	2	2.4	3	2.6	1	5.6	2	2.2	**11**	**2.8**
Unknown	5	4.0			4	2.3	22	23.9			**30**	**8.2**
TOTAL	**47**	**4.1**	**34**	**3.5**	**33**	**2.6**	**59**	**5.7**	**12**	**1.3**	**185**	**3.5**

Table 2
Number and percentage of samples of each species (Table 2a) and from each territorial origin (Table 2b) positive to QuinoScan® collected in each city (compared with the total number of samples of each species collected in each city).

2a	Zaragoza		Bilbao		Logroño		Toulouse		Perpignan		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Bovine	0	0	0	0	0	0.6	1	0.3	1	0.5	**2**	**0.3**
Chicken	0	0	0	0	0	0	0	0	0	0	**0**	**0.0**
Pig	0	0	0	0	0	0.9	1	0.6	0	0	**1**	**0.3**
Turkey	0	0	0	0	0	0	0	0	0	0	**0**	**0.0**
Lamb	0	0	0	0	2	1.6	0	0	0	0	**2**	**0.4**
Rabbit	1	1.2	1	1.7	0	0	0	0	2	3.8	**4**	**1.3**
Duck					0	0	0	0	2	2.1	**2**	**1.2**
Horse					0	0	0	0	0	0	**0**	**0.0**
Quail					0	0					**0**	**0.0**
Goat					0	0					**0**	**0.0**
Guinea Fowl							0	0	0	0	**0**	**0.0**
Partridge					0	0					**0**	**0.0**
TOTAL	**1**	**0.1**	**1**	**0.1**	**2**	**0.5**	**2**	**0.2**	**5**	**0.5**	**11**	**0.2**

2b	Zaragoza		Bilbao		Logroño		Toulouse		Perpignan		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
POCTEFA	1	0.0027	0	0	0	0	0	0	0	0	**1**	**0.0770**
Rest of Spain-France	0	0	1	0.0020	2	0.2265	2	0.3306	5	0.6944	**10**	**0.3032**
Abroad	0	0	0	0	0	0	0	0	0	0	**0**	**0**
Unknown	0	0			0	0	0	0			**0**	**0**
TOTAL	**1**	**0.0009**	**1**	**0.0010**	**2**	**0.4800**	**2**	**0.1944**	**5**	**0.5208**	**11**	**0.2053**

widest possible spectrum.

3.2. Confirmatory analysis

After screening, the 194 positive samples were analysed by UPLC-QTOF (hereafter QTOF) by the Public Health Laboratory of the Basque Government (Derio, Spain), authorized for antibiotic analyses as part of official control. 30 of those samples (Table 4) were confirmed to contain antibiotic residues (15.5%) with the following distribution: tetracyclines (40.6%), sulfonamides (37.5%), quinolones (18.8%), and lincomycin (3.1%). However, only 0.093% of all the samples featured in this study (5 of the 5,357 samples, 2.5% of the QTOF-analysed samples) were considered non-compliant (Table 4), which, compared to the 0.14% reported by the European Food Safety Authority (EFSA, 2021), suggests that the condition of meat commercialized in the POCTEFA area can be regarded as slightly better than the one reported for Europe overall.

Among the 189 remaining samples positive to the screening that were nevertheless compliant according to the confirmatory analyses, 5 of them (Table 4) presented concentrations (56-96 μg/kg) close to the MRLs (mainly lying around 100 μg/kg for muscle). This result is compatible with the LoDs claimed for the screening tests used in the present work (Mata, 2021; Mata et al., 2014). Such results are not unexpected for screening methods such as the ones used in the current work, conceived to achieve a detection capability below the MRL level. The appearance of false positive results should not be of concern, as the main objective of screening methods is to avoid false negatives, and this somewhat elevated sensitivity would provide an extra protection for consumers.

Only 1 out of 11 samples screened as containing quinolones was declared non-compliant (9.1%), and levels below the MRL were

Table 3
Number and percentage of samples of each species (Table 3a) and from each territorial origin (Table 3b) positive to the screening collected in each city (compared with the total number of samples of each species collected in each city).

3a	Zaragoza		Bilbao		Logroño		Toulouse		Perpignan		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Bovine	7	2.3	3	1.3	4	1.8	6	1.7	1	0.5	**21**	**1.5**
Chicken	14	4.7	7	2.3	5	1.9	25	7.8	1	0.7	**52**	**3.9**
Pig	10	4.6	12	5.9	2	1.8	12	7.0	0	0.0	**36**	**3.8**
Turkey	7	6.4	5	4.5	6	5.8	12	15.6	5	3.3	**35**	**6.3**
Lamb	5	3.4	3	6.0	4	3.1	0	0.0	1	0.9	**13**	**2.7**
Rabbit	5	6.0	4[a]	8.5	1	0.9	0	0.0	4[a]	9.6	**16**	**5.0**
Duck	0		0		11	35.5	5	10.4	2	2.1	**18**	**10.4**
Horse	0		0		0	0.0	0	0.0	2	3.3	**2**	**2.5**
Quail	0		0		0	0.0	0		0		**0**	**0.0**
Goat	0		0		2	18.2	0		0		**2**	**18.2**
Guinea Fowl	0		0		0		1	50.0	0	0.0	**1**	**25.0**
Partridge	0		0		0	0.0	0		0		**0**	**0.0**
TOTAL	**48**	**4.2**	**34**	**3.6**	**35**	**2.8**	**61**	**5.9**	**16**	**1.8**	**194**	**3.6**

3b	Zaragoza		Bilbao		Logroño		Toulouse		Perpignan		TOTAL	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
POCTEFA	15	4.1	13	3.3	1	1.3	6	1.9	1	0.7	36	2.8
Rest of Spain-France	25	4.2	19[a]	4.1	27	3.0	32	5.3	13[a]	1.9	119	3.6
Abroad	3	3.4	2	2.4	3	2.6	1	5.6	2	2.2	11	2.8
Unknown	5	5.1			4	2.3	22	23.9			30	8.2
TOTAL	**48**	**4.2**	**34**	**3.6**	**35**	**2.8**	**61**	**5.9**	**16**	**1.8**	**194**	**3.6**

[a] One rabbit sample collected in Bilbao and another collected in Perpignan have been removed from the table as they gave positive results to both tests.

Table 4
Samples positive to the screening with antimicrobial residues (identified by QTOF).

		City	Species	Antimicrobial	Concentration (μg/kg)
Non-compliant		Logroño	Lamb	Enrofloxacin	218,8
				Ciprofloxacin	34,34
			Goat	Sulfadiazine	164,3
			Goat	Doxicyclyne	813
		Toulouse	Pig	Sulfadimethoxine	110
		Perpignan	Rabbit	Sulfadimethoxine	187
Compliant	**Close to MRL**	Zaragoza	Lamb	Sulfadiazine	81.89
		Bilbao	Lamb	Sulfadoxine	96.4
				Oxytetracycline	21.7
		Logroño	Lamb	Sulfadiazine	56.2
		Toulouse	Pig	Enrofloxacin	88
		Perpignan	Rabbit	Sulfadimethoxine	80
	Negative	Zaragoza	Chicken	Doxycycline	< 3
			Chicken	Doxycycline	25.46
			Chicken	Doxycycline	< 3
			Pig	Doxycycline	< 3
			Pig	Doxycycline	9.24
			Lamb	Sulfadiazine	12.4
			Lamb	Sulfadiazine	14.68
			Turkey	Doxycycline	< 3
				Enrofloxacin	10.42
			Turkey	Doxycycline	< 3
			Rabbit	Sulfadimethoxine	22.7
			Rabbit	Enrofloxacin	6.84
		Bilbao	Chicken	Lincomicina	3.4
			Turkey	Doxycycline	7.05
			Rabbit	Enrofloxacin	39.5
		Logroño	Chicken	Doxycycline	27.71
			Lamb	Enrofloxacin	9.7
			Turkey	Doxycycline	6.6
		Toulouse	Chicken	Sulfadimethoxine	12.5
			Chicken	Doxycycline	31.5
			Turkey	Sulfadimethoxine	< 0.4

determined in 5 further samples. Thus, only 55% of the samples pre-identified by QuinoScan® as containing quinolones were confirmed by QTOF. QuinoScan® is an immunochemical test based on an antibody that specifically recognizes the structure of quinolones; thus, the scant number of positive results found by the confirmatory method directly point to certain quinolones or secondary metabolites thereof that could be recognized by the antibody, but would pass unnoticed by the confirmatory targeted QTOF method employed in the present study. Furthermore, as this is a chemical test, it does not provide any information about the antibiotic residues' biological activity, and thus about their eventual consequences for consumer health.

In summary, 20 further samples were shown to contain traces (concentrations lower than the MRLs) of antibiotic residues (Table 4), but no antibiotics were identified in the 164 remaining ones. As both

tests are validated, their LoDs do not legitimate the big gap between the 3.6% of samples positive to the screening and the 0.093% of non-compliant samples confirmed by chromatographic analyses. Although false positives linked to LoDs lower than the MRLs can explain part of this deviation (2.6%, i.e., 5 out 189 screening-positive samples), additional assays would be necessary to explore this matter further in view of these unexpected results.

3.3. Additional studies

Positive results to the Explorer® test are a reflection of the presence of biologically active metabolites in meat samples, with potential adverse effects on human health (toxicity, allergic reactions, or the emergence of AMR), as Explorer® test results stem from the inhibition performed over the growth of a microorganism. Hence, some hypotheses were proposed to further explore the kind of events that can occur due to the presence of certain kinds of molecules that inhibit Explorer® but are not identified by the targeted QTOF:

a) The presence of antibiotics not included in the QTOF list of targeted compounds, such as aminoglycosides. Nevertheless, this should not be the main cause of the gap between Explorer® and QTOF results, as aminoglycoside administration in food-producing animals in Europe is 4 times lower than that of penicillins, and even 5 times lower than that of tetracylcines (EMA, 2021).
b) The presence in meat of alternative and natural antimicrobials, such as essential oils, used by the primary sector.
c) The presence in meat of biologically active metabolites stemming from the degradation of original antibiotics.

Thus, additional experiments based on Explorer® test were performed to elucidate the role played by essential oils and biologically active antibiotic metabolites in the origin of the gap observed between screening and confirmatory analyses.

3.3.1. Alternative antimicrobials: essential oils

Over the last decades, sanitary authorities, veterinarians, and food producers have focused their efforts on rationalizing the use of antibiotics on the farm (Bourély, Fortané, Calavas, Leblond, & Gay, 2018; Speksnijder & Wagenaar, 2018). In this landscape, essential oils appeared as an alternative: they are added to animal feed and reach the gut, improving animal health, performance, and even meat quality (Franz, Baser, & Windisch, 2010; Omonijo et al., 2018).

Essential oils have a widely acknowledged antibacterial activity (Ait-Ouazzou et al., 2012; Seow, Yeo, Chung, & Yuk, 2014; Evangelista, Corrêa, Pinto, & Luciano, 2021) and might have an impact on the meat quality (Cheng et al., 2017; Smeti, Hajji, Mekki, Mahouachi, & Atti, 2018). In fact, certain studies have revealed that essential oils can accumulate in muscle (De Haro, 2015), and some authors have stated that they can affect the germination and growth of spore-forming bacteria, specifically *G. stearothermophilus* (Voundi et al., 2015). Consequently, the muscle bioaccumulation of essential oils added to animal feed could be regarded as one of the reasons for the positivity to Explorer® of meat samples that were not confirmed by QTOF.

In order to evaluate this hypothesis, it was first needed to determine the MIC of essential oils or essential oil components which have eventually accumulated in meat and which might be responsible for the test inhibition. For this purpose, antibiotic-free meat juice was added with different concentrations of essential oils and analysed with Explorer®. As shown in Table 5, carvacrol, oregano, and cinnamaldehyde inhibited the growth of *G. stearothermophilus*; some of the meat samples containing those essential oils were thus positive to Explorer®, while the other essential oils tested did not show any effect on microbial growth at the chosen levels (data not shown). However, it should be noted that the concentration required for these substances to inhibit the test microorganism was ≥0.02% w/w; their presence would produce a smell and taste so intense that the meat would be unpalatable (Espina, García-Gonzalo, & Pagán, 2014). As meat samples purchased for the current study did not exhibit a special aroma, and essential oil concentrations detected in muscle are usually at levels 1,000 times lower than the inhibitory concentrations described in the present study (De Haro, 2015), inhibition of Explorer® by essential oils can be discarded as an explanation for the positive Explorer® results.

Table 5
Explorer® results obtained from the analyses of antimicrobial-free meat juice added with several different amount levels of the essential oils with inhibitory effects at the concentrations tested. Each experiment was performed in triplicate, and the far right column includes a blank sample.

	0.1% w/w	0.06% w/w	0.04% w/w	0.02% w/w	0.01% w/w	Blank
Carvacrol 1	+	+	+	-	-	-
Carvacrol 2	+	+	+	+	-	-
Carvacrol 3	+	+	+	-	-	-
Oregano 1	+	+	-	-	-	-
Oregano 2	+	+	+	+	-	-
Oregano 3	+	+	+	-	-	-
Cinnamaldehyde 1	+	-	-	-	-	-
Cinnamaldehyde 2	+	+	-	-	-	-
Cinnamaldehyde 3	+	+	-	-	-	-

+: Symbol + stands for a positive result to Explorer® test.
-: Symbol – stands for a negative result to Explorer® test.

3.3.2. Occurrence of biologically active metabolites from degradation of antibiotics

Although the difference in sensitivity between screening and confirmatory methods could explain the deviation of results for the 25 samples containing low levels of antibiotics, further reasons must be responsible for the remainder of the gap between the screening and the confirmatory results (159 samples). Further knowledge about antibiotic residues in compliant samples, or even about molecules with antibiotic properties not included in the target list of the QTOF analysis, such as metabolites of the original antibiotic.

3.3.2.1. Confirmatory analysis of antibiotic residues in random meat samples negative to the screening. To begin with, the feasibility of a relationship between antibiotic traces and positivity to Explorer® was tested by considering the possible presence of Explorer® negatives among meat samples with traces of antibiotics. Thus, 138 samples chosen at random among samples that had been negative to Explorer® as well as to QuinoScan® were analysed with the confirmatory method. As can be observed in Table 6, 17 out of the 138 tested samples (12.3%) contained amounts of antibiotic residues that invariably lay below the MRLs (no false negative results were found). Therefore, although chromatographic analyses proved that all the negative samples were compliant, 12.3% of them contained traces of antibiotics. Even though legislation does not foresee any specific procedure for meat tainted with such small amounts, producers should bear this situation in mind and continue to improve their production systems to reduce even these values and thus achieve excellence. Again, the most frequently identified antibiotics were tetracyclines (63.2%), followed by quinolones (15.8%), lincomycin (10.5%), and sulfonamides and ß-lactam (5.3% for each one of the latter).

The difference between the percentage of screening-positive samples that contained antibiotic residues identified by QTOF (15.5%) and the percentage of screening-negative samples containing antibiotic residues identified by QTOF (12.3%) was 3.2%. This value is close to the percentage of samples positive to the screening, excluding those which were non-compliant (5 samples) and those that lay below but very close to the MRL (5 samples) confirmed by QTOF, which results in a proportion of

Table 6
Samples negative to the screening with antimicrobial residues (identified by QTOF).

City	Species	Antimicrobial	Concentration (μg/kg)
Bilbao	Chicken	Doxycycline	21
	Turkey	Doxycycline	15
	Rabbit	Enrofloxacin	<2.5
		Oxytetracycline	<1.1
	Pig	Lincomycin	14
	Turkey	Doxycycline	<3
	Lamb	Sulfadiazine	16.2
	Turkey	Doxycycline	13
Zaragoza	Rabbit	Oxytetracycline	20.9
	Chicken	Norfloxacin	7.3
	Pig	Doxycycline	11.5
	Pig	Dicloxacillin	10.9
	Rabbit	Oxytetracycline	11
	Pig	Doxycycline	33.5
		Lincomycin	7.8
	Chicken	Flumequine	31.6
	Turkey	Doxycycline	8.27
	Lamb	Doxycycline	<3
	Rabbit	Oxytetracycline	9.8

3.4% (184 out of 5,357). These extra samples, positive to Explorer® and with traces of antibiotic, might contain biologically active metabolites derived from the original antibiotic molecules. Thus, not only original antibiotics, but also undetected metabolites could be responsible for the inhibition of Explorer® test, resulting in the non-confirmed screening-positives observed. Nevertheless, although these findings agree with the hypothesis posed, further experiments were required to demonstrate the presence of these metabolites.

3.3.2.2. The presence in meat of biologically active metabolites derived from original antibiotics. Biologically active metabolites in meat samples might have their source in the degradation of original antibiotics. Although these compounds could maintain biological activity, thus inhibiting microbial growth and yielding positive results to the Explorer® test, their chemical structure would change and thus become undetectable through the routine conventional chromatographic techniques employed for official control, which specifically target marker compounds defined by legislation. To verify this hypothesis, frozen meat samples contained in the samples bank built by Serrano et al. (2020) were studied. These meat samples came from animals bred according to antibiotic-free requirements till they were treated with four antibiotics under controlled conditions. In 2018, meat samples from those animals were obtained and characterized by Explorer® and the confirmatory method, and they had been kept frozen at −20 C since then until they were re-analysed in 2021 by Explorer® and QTOF.

Antibiotic degradation ($P \leq 0.05$) was observed by chromatographic analysis as a function of the antibiotic tested (Fig. 1). While oxytetracycline did not show any degradation, amoxicillin was completely degraded. Enrofloxacin (26-55% degradation) and sulfamethoxipyridazine (5-39% degradation) displayed an intermediate behavior. The degradation of certain antibiotics during frozen storage has been previously described (Shaltout, Shatter, & Sayed, 2019).

According to chromatographic analyses, certain amoxicillin and sulfamethoxypyridazine samples (A1, A3, A4 and S3; Fig. 1), suffered from degradation up to concentrations even lower than the LoD of Explorer®; in contrast, they were still positive to the biological test. These results thus pointed to antibacterial activity derived from degradation compounds stemming from antibiotics and not identified by conventional targeted confirmatory methods. Further trials were performed to investigate this likelihood.

3.3.2.2.1. ß-lactam metabolites in amoxicillin incurred samples: detection based on biochemical structure recognition. BTScan® is a specific lateral flow immunochromatographic test that detects the presence of ß-lactams and tetracyclines in milk. Detection of ß-lactam antibiotics is based on the structural biorecognition of a specific protein receptor (a penicillin-binding protein) related to the mechanisms of antibiotic resistance: it is therefore able to bind exclusively to the molecules of this family.

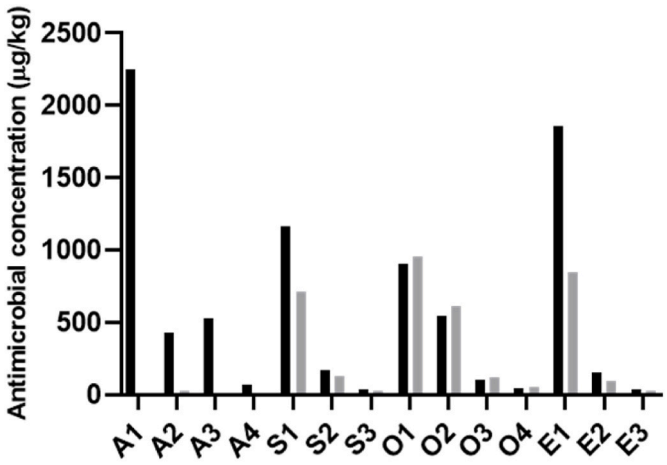

Fig. 1. Antimicrobial concentrations described in 2018 (■) and 2021 (■) for 4 meat samples tainted with amoxicillin (A1, A2, A3, A4), 3 with sulfamethoxypyridazine (S1, S2, S3), 3 with oxytetracycline (O1, O2, O3, O4), and 3 with enrofloxacin (E1, E2, E3).

With the aim of evaluating the potential presence of active ß-lactam metabolites in meat samples, 4 samples tainted with high levels of amoxicillin, obtained from the sample bank built by Serrano et al. (2020) and characterized by chromatography LC-MS/MS in 2018, were re-analysed by QTOF, Explorer® and BTScan® in 2021 (Table 7). QTOF analyses showed that the antibiotic was nearly completely degraded after 3 years of frozen storage. Nevertheless, Explorer® was able to detect microbial inhibition in all the analyses performed, not only in 2018, when chromatographic analysis identified high concentrations of amoxicillin, but also in 2021, when the same samples were quantified by QTOF and only one sample presented quantifiable concentrations of amoxicillin. BTScan® test also showed the presence of ß-lactam molecules in all the samples tested. It is worth mentioning that the LoD of this biochemical test for amoxicillin is around 15 μg/kg. This LoD is higher than that of QTOF (Table S6), thus pointing to a recognition not only of the parent molecule, but also of derived metabolites that maintain their biological and structural properties. These findings reveal the presence of biologically active metabolites of amoxicillin in meat samples, stemming from amoxicillin degradation during frozen storage, and which might be responsible for positive results obtained in biological tests such as Explorer®, even if they go unnoticed by conventional routine chromatographic techniques employed for official control,

Table 7
Chromatographic (LC-MS/MS) and Explorer® results of 4 meat samples tainted with amoxicillin, obtained after the analysis in 2018 and in 2021, when BTScan® test was likewise performed. Numerical values are presented in μg/kg; positive results are presented with the symbol +, and results close to positive with the symbol ±.

	LC-MS/MS		Explorer®		BTScan® 1/5[a]
	2018	2021	2018	2021	2021
A1	2254	<10	+	+	+
A2	429	29	+	+	+
A3	532	<10	+	+	±
A4	72	<10	+	+	±

[a] As the BTScan® technique is optimized for the analysis of ß-lactam in milk, a dilution 1/5 of meat juice in milk was performed in order to avoid the matrix effect. Samples marked as ± have results that indicate the presence of ß-lactam, taking the 1/5 dilution into account.

which specifically target certain marker compounds as required by legislation.

3.3.2.2.2. Detection of sulfonamide metabolites based on competitive inhibition of their biological activity. Sulfonamides are antibiotics with bacteriostatic activity related to the inhibition of DNA synthesis. Concretely, they are analogous to p-aminobenzoic acid (pABA), required for folic acid synthesis in bacteria, thus acting as competitors and supressing the synthesis of DNA (Campbell, 1999). Hence, the *in vitro* addition of pABA to a meat sample tainted with sulfonamides might also revert its inhibitory activity over bacteria. This is the reason why pABA is commonly used for the identification of sulfonamide residues in food (Ferrini, Mannoni, & Aureli, 2006; Stead et al., 2008).

In order to test this approach, 8 meat samples coming from the sample bank built by Serrano et al. (2020), tainted with sulfonamides at concentrations lower than the MRL, were analysed by Explorer® before and after the addition of pABA. It should be noted that the Explorer® test is validated for sulfonamides (Mata et al., 2014), and its LoDs are close to the MRLs determined by legislation (ranging from 50 to 100 μg/kg). Fig. 2a illustrates these samples' positivity prior to the addition of pABA, and it shows how their inhibition disappeared after the addition of pABA ($P \leq 0.05$), which reflects the competition with sulfonamide metabolites whose biological activity is present in meat, presumably degradation products of the original sulfonamide that cannot be identified by the chromatographic technique.

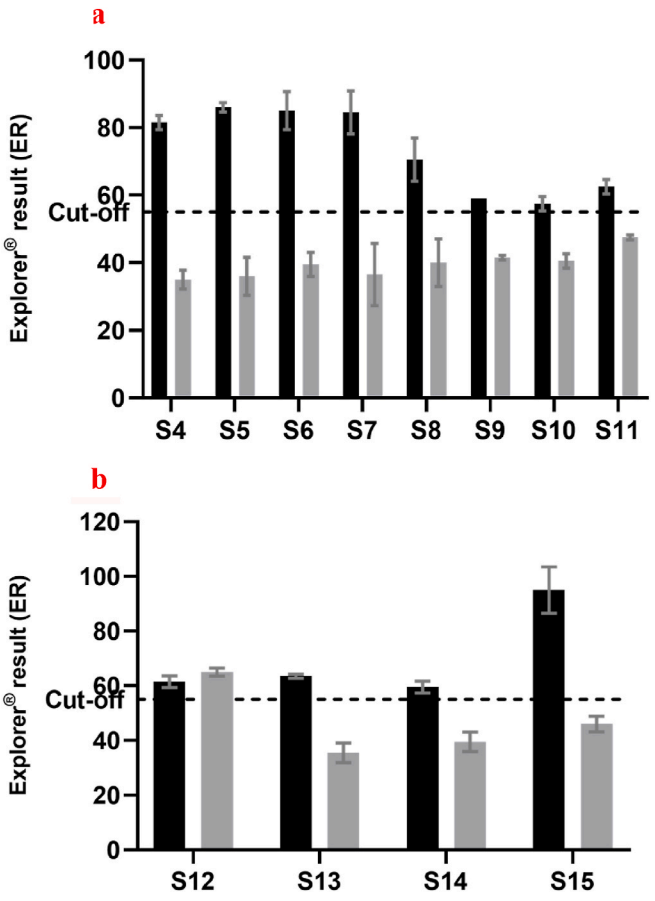

Fig. 2. Explorer® results (ER) (values presented are a reflection of the colour changes of the growth media) obtained: Fig. 2a, after the analysis of 8 meat samples coming from animals injected in vivo with sulfamethoxypyridazine before (■) and after (■) the addition of pABA; Fig. 2b, after the analysis of 4 meat samples collected for the study of the presence of antimicrobial residues in commercialized meat from the POCTEFA area, before (■) and after (■,) the addition of pABA. The dotted line represents the cut-off value settled for Explorer® test (see Table S3) above which results are considered positive.

The existence of biologically active metabolites of sulfonamides was proved in 3-year-frozen meat samples stemming from sulfonamide-treated animals. Furthermore, 18 commercialized meat samples collected for the current study, positive to Explorer® but negative according to QTOF analyses, were re-analysed by Explorer® 4 and 8 months post collection and frozen storage. After 4 months, only 7 samples remained positive to the biological test, while only 3 remained positive after 8 months (S12–S14, Fig. 2b). In order to reveal the presence of metabolites present in these 3 samples, they were added with pABA. Fig. 2b shows that, in 2 out of the 3 tested samples (S13 and S14), the addition of pABA reverted the inhibition over Explorer® ($P \leq 0.05$), which pointed to the presence of sulfonamides in those 2 samples. The accuracy of pABA trials to evince sulfonamide activity was proved with sample S15, a commercialized meat sample characterized by QTOF to have 82 μg/kg of sulfadiazine at the moment of its collection, and positive to Explorer® even after 8 months of frozen storage, as Fig. 2b shows loss of sulfonamide activity after addition of pABA. This finding reinforces the hypothesis of biologically active metabolites stemming from the degradation of sulfonamides as responsible for Explorer® inhibition.

Henceforth, the presence in meat of biologically active metabolites stemming from the degradation of original antibiotics has been proved. Consequently, although chromatographic analyses offer the advantage of recognizing and quantifying the compound present in a sample, their monitoring does not evince active metabolites of the original compound, due to their targeted approach. Biological screening tests thus offer certain benefits, as they are a direct measure of biological activity of antibiotics as well of their unknown metabolites.

Moreover, these results, together with the degradation of antibiotics as observed in samples coming from the sample bank, evince the importance of carrying out confirmatory analysis as soon as possible after sample collection, as degradation during frozen storage could also be involved in loss of positivity between screening and confirmatory analysis.

4. Conclusions

The two-step analysis of the presence of antibiotic residues in 5,357 commercialized meat samples of the POCTEFA area showed that 3.6% of the samples (194 out of 5,357) were positive to screening (biological Explorer® + immunochemical Quinoscan® tests). Targeted QTOF analyses of those samples confirmed the presence of antibiotics in 15.5% of the screening positives (30 out of 194). 20 of them contained antimicrobials below the MRL, 5 below but close the to the MRL; remarkably, only 5 lay over the MRL (0.093% of the collected samples). The data are thus even better than those reported by the EFSA for Europe (EFSA, 2021). The most frequently identified antibiotic families, by order of incidence, were: tetracyclines, sulfonamides, quinolones, lincomycin, and ß-lactams. Nevertheless, a large gap could be observed between the 3.4% of samples that were unexplainably positive to the initial screening, and the 0.093% of confirmed non-compliant samples. Further analyses proved that meat samples, although compliant, contained unknown biologically active metabolites derived from original antibiotics, detectable by the Explorer® biological test, but undetectable by the targeted QTOF method. Henceforth, the election of a test for antibiotic residue control will depend on the purpose of the assessment. When legal requirements are prioritized, targeted chromatographic confirmatory analyses that only include marker compounds defined in Regulation 37/2010 should be carried out. Their monitoring, however, does not reveal most metabolites; biological screening tests should thus be of choice in the interest of preserving consumer health.

CRediT authorship contribution statement

M.J. Serrano: Conceptualization, Methodology, Data curation, Visualization, Investigation, Supervision, Writing – original draft, preparation, Writing – review & editing. **J. Elorduy:** Funding

acquisition, Methodology, Data curation, Investigation, Supervision, Writing – review & editing. **I. Zabaleta:** Methodology, Data curation, Investigation, Writing – original draft, preparation. **G. Istamboulie:** Funding acquisition, Supervision, Writing – review & editing. **E. González Fandos:** Funding acquisition, Conceptualization, Data curation, Supervision, Writing – review & editing. **A. Bousquet-Melou:** Funding acquisition, Supervision, Writing – review & editing. **L. Mata:** Funding acquisition, Methodology, Data curation, Visualization, Investigation, Supervision, Writing – review & editing. **C. Aymard:** Data curation, Investigation, Writing – review & editing. **A. Martínez Laorden:** Data curation, Investigation. **J. Da Silva Guedes:** Data curation, Investigation. **M. Lacroix:** Data curation, Investigation. **D. García-Gonzalo:** Conceptualization, Methodology, Writing – review & editing. **S. Condón:** Conceptualization, Methodology, Writing – review & editing. **E. Abilleira:** Methodology, Data curation, Investigation, Supervision, Writing – review & editing. **R. Pagán:** Funding acquisition, Conceptualization, Methodology, Data curation, Visualization, Investigation, Supervision, Writing – original draft, preparation, Writing – review & editing.

Declaration of competing interest

None.

Acknowledgements

This project was 65% cofinanced by the European Regional Development Fund (ERDF) through the Interreg V-A Spain-France-Andorra programme (POCTEFA (Programa INTERREG V-A España-Francia-Andorra) 2014-2020) (EFA (España-Francia-Andorra) 152/16). POCTEFA aims to reinforce the economic and social integration of the French–Spanish–Andorran border. Its support is focused on developing economic, social, and environmental cross-border activities through joint strategies favouring sustainable territorial development.

Alba Martínez-Laorden acknowledges and extends her thanks to the University of La Rioja for her predoctoral fellowship (UR-CAR-2019).

Jessica Da Silva Guedes has received funding from the European Union's H2020 research and innovation programme under Marie Sklodowska-Curie grant agreement No 801586.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109033.

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