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Stability of penicillin antibiotic residues in meat during storage Ampicillin

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Abstract

Incurred samples from a pig treated with ampicillin, one of the most important penicillin antibiotic drugs used in food-producing animal treatments, were analyzed at the residue level of the drug in muscle tissue (≈100 μg kg^{-1}) during their freezing storage and using three different techniques: quantitative microbiological assay, HPLC–UV and LC–MS. Two parameters have been specifically monitored: storage temperature (−20 and −75°C) and storage packaging (ground meat or bulk meat). No significant decrease was observed during the first 3 months of storage monitoring at −20 and −75°C. On the contrary, the sample preparation significantly affected the drug concentration in muscle from the very beginning of the storage. Grinding the meat before storage allowed to keep the drug near the higher level of concentration (≈100 μg kg^{-1}) when bulk meat stored frozen systematically led to a decreased value (≈75 μg kg^{-1}). After 8 months of storage at −20°C, a significant decrease arose and was never observed at −75°C. All the results were similarly obtained with the three different techniques used simultaneously, which allows to indicate a good correlation between the techniques.

Keywords: Meat; Food analysis; Stability studies; Penicillins; Antibiotics; Ampicillin; Lactams

1. Introduction

Penicillins, a non-toxic class of antibiotics which are commonly used in food-producing animals, may give rise to residues in meat products that might be harmful for the consumers (allergic reactions). To ensure human food safety, maximum residue limits (MRLs) have been fixed to 50 μg kg^{-1} for ampicillin, benzylpenicillin and amoxicillin in food-producing animals muscle by the EU Council Regulation No. 2701/94 of 7 November 1994 [1].

The control of meat products is performed with both microbiological and chemical methods. The chemical method must be specific and sensitive enough in order to confirm the positive result obtained by microbiological screening. The analysis is not always performed in the vicinity of the slaughter and storage of the carcass in frozen conditions for several weeks is sometimes necessary. The stability of the residues in these conditions must be checked to guarantee the reliability of the results.

Penicillin antibiotics have not been extensively studied regarding their stability during storage. Penicillin-G was investigated under frozen conditions in plasma and lymph by Wiese et al. [2], in plasma and tissues by Boison et al. [3] and in ovine

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liver by Gee et al. [4]. The three studies utilized chemical methods and showed a degradation of the penicillin antibiotic at low freezing temperature (−20°C) along with a relative stability at high freezing temperature (−76°C). Few stability studies are dealing with ampicillin residues. Ampicillin (amphoteric compound) presents a chemical structure slightly different from penicillin-G and thus may behave in a different way compared to penicillin-G. Only one study has been reported in tissues stored at −20°C by O'Brien et al. [5]. This work used a quantitative microbiological method and showed an important decrease for ampicillin.

The work described hereafter is aimed at detecting the effects of different conditions of storage on the stability of residues of ampicillin in muscle tissue. Incurred porcine muscle samples were used. Two different parameters were monitored: the storage temperature (−20 and −75°C) and the sample preparation (ground meat or bulk meat). Three methodologies were used simultaneously to analyze the same material: a quantitative microbiological method (R. Fuselier, N. Cadieu, unpublished data), an HPLC–UV method [6] and an LC–electrospray ionization (ESI)-MS method [8]. They allow us to monitor the ampicillin concentration in the material by three independent parameters: microbiological activity, UV absorption and specific ions in the mass spectrum of the compound, respectively.

2. Experimental

2.1. Reagents and standards

Ampicillin sodium (87.4% as acid form) was obtained from Sigma (St. Louis, MO, USA). For the microbiological analysis, Mueller Hinton medium (Difco, ref. 02552-17-6); European Pharmacopoeia phosphate buffer pH 4.5 and *Bacillus stearothermophilus* spores (Diagnostica Merck, ref. 11499) were used. For HPLC–UV analysis, methanol and acetonitrile were of analytical-reagent grade (Merck, Darmstadt, Germany). Demineralized ultra-pure water was obtained using a Milli-Q ultrafiltration unit from Millipore (Molsheim, France). All the other reagents and solutions used for the HPLC–UV analyses are presented in a previous paper [6]. For LC–MS analysis, cephalexin was obtained from Virbac (Carros, France). Formic acid (98–100% for analysis) and di-sodium hydrogen phosphate were purchased from Merck.

2.2. Analytical procedures

2.2.1. Microbiological assay (R. Fuselier, N. Cadieu, unpublished data)

Working standard solutions of ampicillin were prepared in buffer, pH 4.5, at concentrations of 25, 50, 100 and 200 μg l^{-1}. Amounts of 15 g of homogenized incurred-muscle tissue sample were blended in stomacher laboratory blender with 15 ml Pharmacopoeia buffer (pH 4.5) and 15 g of blank-muscle tissue were blended in stomacher laboratory blender with 15 ml of each working standard solution of ampicillin. Extracts were obtained after centrifugation and pasteurization. Square plates (23×23 cm) were prepared with 100 ml of Mueller Hinton agar seeded with *Bacillus stearothermophilus* at 10^7 spores ml^{-1}. Stainless steel cylinders were placed on the inoculated agar surface and filled with 200 μl of extracts. Plates were incubated for 15–18 h in an incubator at 55±1°C. Inhibition zones of the incurred samples and of the spiked samples were measured with a vision system AMS 40-10 (Systèmes Analytiques). The precision of the measure was 0.1 mm.

2.2.2. Liquid chromatography with UV detection

This method involves extraction of the residues from the muscle sample by phosphate buffer, clean-up on C_{18} solid-phase extraction cartridge and chemical reaction of the eluate with 1,2,4-triazole and mercuric chloride to obtain derivatized penicillin residues detectable by UV absorption at 325 nm. The entire HPLC–UV method along with its validation has been described elsewhere [6]. The accuracy in terms of recovery and the precision of the method had been evaluated. The mean recovery was estimated to be 75% with a RSD of 7% and a between-day repeatability at 100 μg kg^{-1} level of concentration was assessed to be 5.2%.

The liquid chromatograph was composed of a P1000XR pump (Thermo Separation Products, San Jose, CA, USA), an AS100XR autosampler fitted with a 500-μl loop used in partial volume of

injection (200 μl) and 0.25-ml syringe (Thermo Separation Products), a C_8 analytical column (150× 3.9 mm I.D.; 5 μm) (Symmetry, Waters, Milford, MA, USA); a C_{18} guard column (4×4 mm; 5 μm) (Lichrospher100-RP18e, Merck), a UV3000 multi-wavelength detector (Thermo Separation Products). Data acquisition and the HPLC system were controlled on a Pentium 100 station equipped with a PC1000 software (Thermo Separation Products).

2.2.3. Liquid chromatography with mass spectrometric detection

The LC–ESI-MS method for analyzing ampicillin antibiotic was developed to confirm and identify univocally the ampicillin residue in muscle tissue. Cephalexin was added to samples as internal standard. Tissue samples were homogenized with ammonium acetate buffer (pH 8.5, 0.1 *M*) and further clean-up was performed using Bond-Elut C_{18} cartridges [8]. Following this purification, extracts are injected into the LC–MS system on a C_{18} column (RP 18e, Lichrospher, 125×4 mm I.D.; 5 μm) protected by a guard column (4×4 mm). The LC system consisted of a Hewlett-Packard 1050 pump equipped with a Rheodyne injector and a 100-μl sample loop. The mobile phase was made of methanol–0.2% formic acid in water (45:55, v/v) and a gradient was used (from $t=0$ to 2 min, MeOH was set at 25% then from $t=3$ to 5 min MeOH was increased to 80%). The flow-rate was 0.6 ml min^{-1}. No split was used. A Finnigan SSQ 7000 mass spectrometer was used in positive ion mode with electrospray interface. The capillary temperature was set at 200°C and the spray voltage at 5 kV. The sheath gas pressure was fixed at 80 p.s.i. and the auxiliary gas at 2.5 units (1 p.s.i.=6894.76 Pa). The instrument was operated in selected ion monitoring (SIM) mode, monitoring ions at m/z 350, 351, 372, 373, 382 for ampicillin and ion at m/z 348 for cephalexin (internal standard). As for the HPLC–UV method, the analytical method based on LC–ESI-MS was validated according to the criteria proposed in the European decision No. 93/256/EC [7]. A summary of the main validation parameters are proposed in Table 1.

2.2.4. Animal experiment

Two healthy pigs aged 5–6 months (mass range, 50–60 kg) were held at our experimental farm for a period of 2 weeks before any antibiotic treatments. They were fed with food free of antibiotic. One pig was then injected intramuscularly in the neck area with a 20-mg/kg body mass of ampicillin sodium (Ampiject, Coophavet, St. Herblon, France). The other was left free of antibiotic for blank matrix purposes. They were both slaughtered at our slaughtering room and 1.6 kg of gluteal muscle (left and right sides) was collected and stored immediately at +4°C for one night before sampling. A portion of both matrices (50 g) was also removed before storage and analyzed immediately with the HPLC method in order to evaluate the level of concentration of ampicillin in the contaminated material and to ensure the absence of residues in the untreated material. The samples utilized for the stability study were prepared by first dividing the material into two sets. The first set was ground with a rotary hatcher (Moulinette, Moulinex, France) and conditioned in plastic containers (ca. 130 g) and the other one was cut into large dices of ca. 100 g before their conditioning in the same brand of plastic containers. At this step six containers were randomly removed

Table 1
Validation data for the LC–ESI-MS method

	Ampicillin fortification level (μg kg^{-1})			
	25	50	100	150
Interday repeatability RSD (%)[a]	21.3	14.8	12.7	4.5
Accuracy (%) ($n=4$)	102.0	100.7	98.1	100.7
Recovery (%)±SD ($n=4$)	70±20	76±15	59±15	60±14
Detection limit	16 μg kg^{-1}			
Quantification limit	25 μg kg^{-1}			

[a] Calculated on 4 days with one sample analysed per day and per level of concentration.

from the sets and immediately analyzed to check for material homogeneity. Both sets were then divided into two parts. The first were stored at −20°C and the second at −75°C.

2.2.5. Analytical treatment of the incurred samples

The samples were analyzed in five periods, namely: period 1 (1 week of storage), period 2 (1 month), period 3 (2 months), period 4 (3 months), period 5 (8 months).

For each period the analyses were distributed over two different days: the ground samples stored at −20 and at −75°C on the first day and the diced samples stored at −20 and at −75°C on the second day. The ground samples were thawed at +4°C during one night before the analyses and the diced samples were, in addition, ground in the morning just before the analyses. All the samples were monitored with each of the three methods (microbiological, HPLC and LC–MS) simultaneously and analyzed in triplicate by each method.

2.3. Calculations

2.3.1. Microbiological assay

The standard curve was determined using linear regression and the conditions of validity of the straight line were checked by the analysis of variance. Three portions of 15 g per incurred sample were analyzed by the bioassay procedure. Each of the three results was the mean of nine values measured as diameters collected on three different plates. The results were converted in concentration values by using the standard curve when the conditions of validity were fulfilled.

2.3.2. Liquid chromatography with UV detection

Three portions of 5 g each per incurred sample were analyzed by the HPLC–UV standard operating procedure [6].The area of the derivatized ampicillin peak detected at 325 nm was measured and the concentration of ampicillin residues was determined by interpolation on a calibration curve taking into account the linearity of the detector response and the mean recoveries of spiked samples compared with the same spikes in aqueous solution.

2.3.3. Liquid chromatography with mass spectrometric detection

Three portions of 2 g were analyzed per incurred sample. Quantification was based on the peak area ratio of ion m/z 350 (ampicillin) to ion m/z 348 (cephalexin, internal standard). A calibration curve was constructed from fortified samples at 50, 100 and 150 μg kg^{-1} plotting peak area ratio versus the corresponding concentration. The curve was represented by the equation $y = mx + b$, where y is the peak area ratio and x the fortified concentration. The levels in the incurred samples were calculated using this calibration curve.

2.4. Statistical analyses

2.4.1. Homogeneity of the incurred muscle samples

The evaluation of the homogeneity of the four sets of samples was conducted at the very beginning of the stability study. It was evaluated according to an internationally recognized procedure [9]. Six containers were chosen randomly from the four sets of samples, stored at +4°C and analyzed by HPLC–UV in duplicate within the 2 days following the sample preparation. A comparison of the means was investigated using a one-way analysis of variance with a risk assessment $\alpha = 5\%$ and without exclusion of the outliers.

2.4.2. Stability of the incurred muscle samples

The stability of ampicillin in the incurred muscle samples was estimated for ground and diced samples at −20 and at −75°C over the five periods by testing the slope of the regression line with regard to zero ($\beta = 0$) by means of a Student's test, with a risk assessed to 1%. The kinetics of degradation was then proposed by assuming a law with a first-order rate and plotting the logarithm of the initial concentration (C_0) to the concentration at a given time (C_t) against the time of storage (t) [10].

3. Results

3.1. Homogeneity of the incurred muscle samples

The statistical analysis based on the duplicated results of the six randomly chosen samples showed

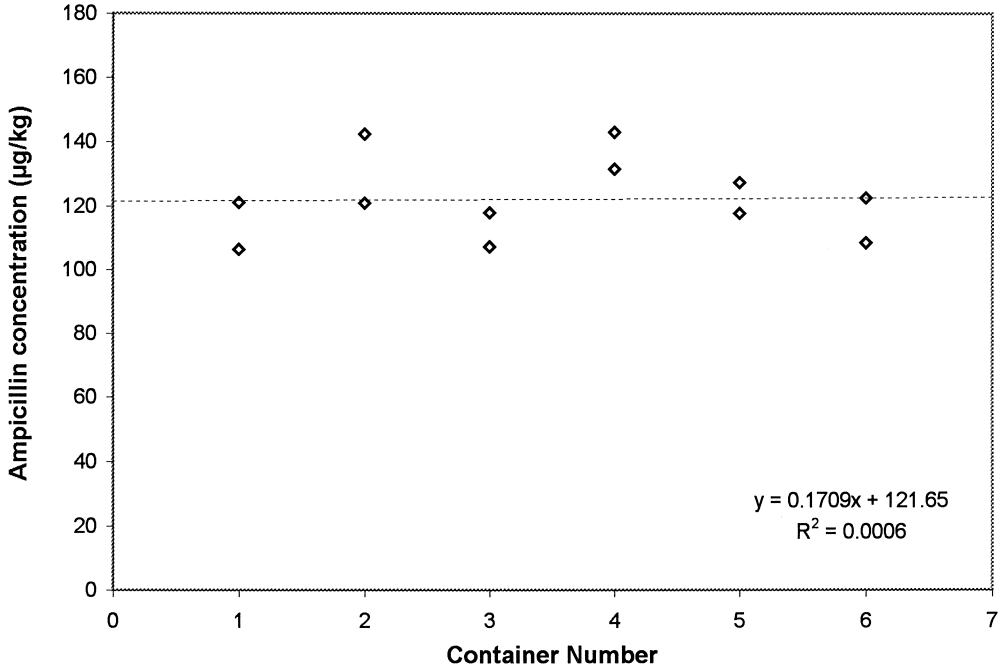


Fig. 1. Homogeneity data obtained by HPLC analysis of six containers (samples) chosen randomly and analyzed in duplicate each.

no significant differences (Fig. 1). The four sets of samples were considered homogeneous with a mean concentration of 122±13 μg kg^{-1}. The sampling standard deviation (S_s) and the analytical standard deviation (S_a) were evaluated to 7.5 and 10 μg kg^{-1}, respectively [9].

3.2. Stability of the incurred muscle samples

Tables 2–4 show the concentrations of ampicillin detected in porcine muscle at different periods of storage. Three major facts can be observed easily.

First, it is clearly demonstrated that ampicillin residues are stable when stored at −75°C over 8 months (237 days) independently from the conditions of sample preparation (ground or diced samples). Second, for the samples stored at −20°C, a large decrease of about 40% can be observed between the results obtained on the eighth month of storage compared to those obtained in the first 3 months. This fact is also independent of the conditions of

Table 2
Effect of storage temperatures (−20 and −75°C) and of sample preparation (ground and diced samples) on the stability of ampicillin residues in muscle tissue from one pig

		Mean concentration±SD (μg kg^{-1})[a]									
		Days of storage									
		5	7	33	35	61	62	96	98	235	237
Ground samples	−20°C	102±7		103±9		98±16		89±9		59±5	
	−75°C	109±12		110±9		111±6		112±7[b]		101±4	
Diced samples	−20°C		75±5		81±11		69±10		78±10		43±3
	−75°C		87±7		88±8		81±15		87±8		92±8

[a] Average of nine analyses carried out with the three methods taking account of three analyses per method
[b] Average of six analyses performed by the microbiological and HPLC–UV methods only (the LC–MS data were outliers).

Table 3
Content of ampicillin in ground pig muscle samples as a function of the time of storage at −20 or −75°C and detailed with regard to the three analytical methods used

		Mean concentration±SD (μg kg^{-1})[a]				
	Days of storage:	5	33	61	96	235
Bioassay	−20°C	96.3±1.0	99.8±3.7	92.7±5.7	86.0±3.5	55.4±1.7
HPLC	−20°C	102.6±6.2	113.7±2.3	85.7±3.1	97.0±4.4	63.7±1.5
LC–MS	−20°C	107.0±7.9	96.3±5.1	115.3±14.6	83.3±11.0	58.0±6.2
Bioassay	−75°C	93.9±3.4	100.2±3.3	114.7±6.7	104.1±2.0	100.3±2.6
HPLC	−75°C	115.0±1.7	111.7±1.5	106.7±2.1	117.7±3.2	104.7±1.5
LC–MS	−75°C	118.3±7.5	117.0±7.8	n/a[b]	113.5±7.8	97.1±3.8

[a] Average of three analyses carried out on the same sample.
[b] Outlying data.

Table 4
Content of ampicillin in diced pig muscle samples as a function of the time of storage at −20 or at −75°C and detailed with regard to the three analytical methods used

		Mean concentration±SD (μg kg^{-1})[a]				
	Days of storage:	7	35	62	98	237
Bioassay	−20°C	74.9±1.3	73.5±2.6	63.5±1.5	71.1±2.0	43.6±1.3
HPLC	−20°C	79.2±2.8	74.3±6.7	62.7±7.5	72.3±3.1	46.3±0.6
LC–MS	−20°C	82.7±5.5	95.0±4.0	81.7±3.5	92.0±3.6	40.4±1.1
Bioassay	−75°C	79.4±2.4	80.9±2.2	72.3±1.5	85.8±1.3	89.1±1.7
HPLC	−75°C	93.0±3.7	86.4±3.4	69.7±5.1	79.0±2.6	101.0±4.6
LC–MS	−75°C	88.3±6.4	96.7±7.6	100.3±1.5	97.0±5.3	85.3±6.4

[a] Average of three analyses carried out on the same sample.

preparation of the samples (ground or diced samples). Third, and finally, a large difference can be appreciated with regard to the preparation of the samples. Starting with a homogeneous material estimated to 122±13 μg of ampicillin per kg of pig muscle tissue, the analyses performed on the first period of storage (5–7 days) showed a slight decrease for the ground samples (102 and 109 μg kg^{-1}), and a large decrease for the diced samples (79–87 μg kg^{-1}).

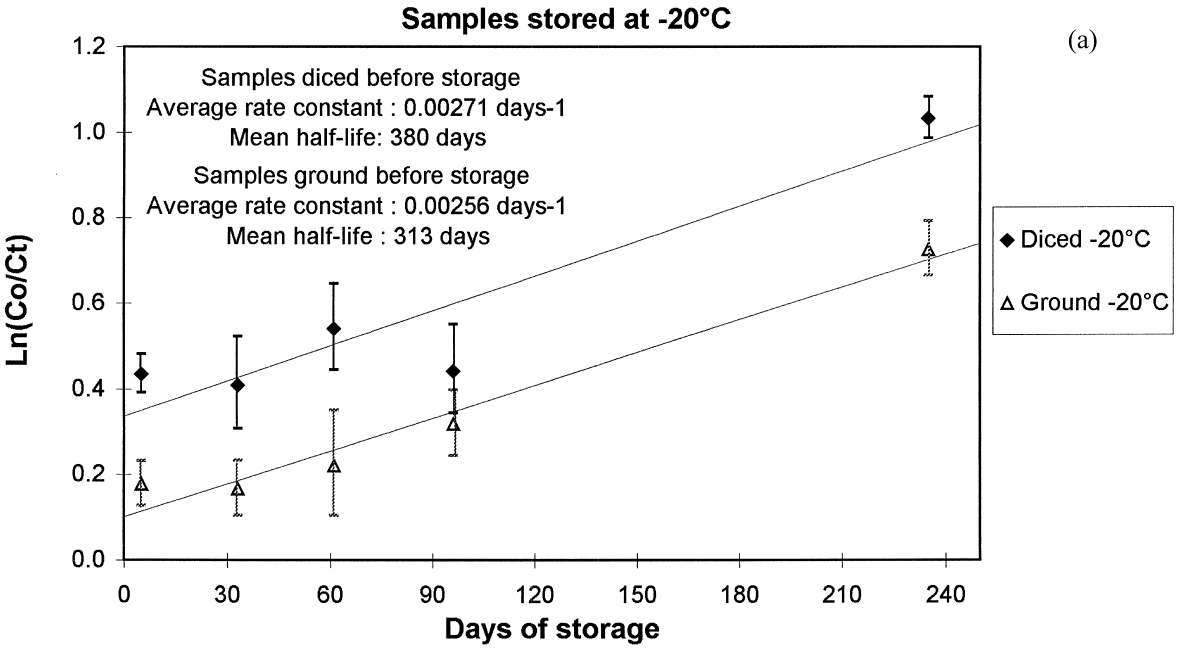
4. Discussion

The results obtained for this study on ampicillin residues in gluteal muscle (Table 5 and Fig. 2a,b)

Table 5
Test of stability and average rate constant values for the degradation of ampicillin residues in ampicillin-incurred muscle tissue samples

	n	Storage temperature (°C)	Variance Sb^2 of the slope	Test of the slope, α=1%	Mean±SD		
					Rate constant (days^{-1})	Half life (days)	90% life (days)
Ground samples	45	−20	3.76473×10^{-8}	Sign[a]	0.00256±0.00019	313±24	83±6
	40	−75	2.26526×10^{-8}	NS[b]	0.00034±0.00015	2346±1317	594±333
Diced samples	44	−20	6.29718×10^{-8}	Sign	0.00271±0.00025	380±36	163±15
	40	−75	4.66463×10^{-8}	NS	−0.00011±0.00022	9568±3403	1848±1179

[a] Sign: Student's t-test is significant and the slope is different from 0.
[b] NS: Student's t-test is non-significant and the slope is not different from 0.



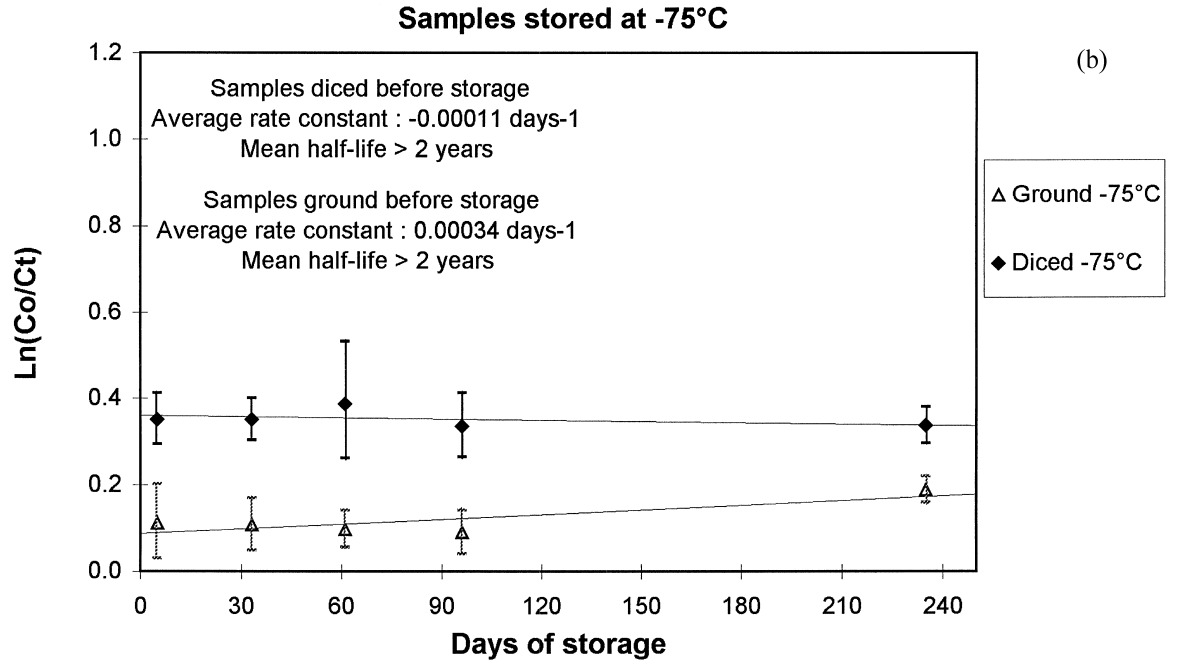


Fig. 2. (a) Plots of the logarithm of the initial concentration, C_0, to the concentration at a given time (t), C_t, of ampicillin in muscle tissue against time. (△) samples ground before storage at −20°C and (◇) samples diced before storage at −20°C. (b) Plots of the logarithm of the initial concentration, C_o, to the concentration at a given time (t), C_t, of ampicillin in muscle tissue against time. (△) samples ground before storage at −75°C and (◇) samples diced before storage at −75°C.

Table 6
Analytical variations recorded during the entire study

	n	RSD Min (%)	RSD Max (%)	Mean RSD[a] ± SD (%)
Microbiological assay	60	1	6	3.0 ± 1.3
HPLC–UV	55	2	12	3.8 ± 2.7
LC–ESI-MS	54	2	13	7.1 ± 4.5

[a] The mean RSD of a method was calculated considering 60 analyses distributed on three analyses per factor (four factors studied) and per period (five periods).

corroborate adequately the experiments presented earlier for penicillin-G residues in muscle, liver and kidney by Boison et al. [3]. They particularly underline the assertion pretending that a large amount (50%) of the initial concentration of the penicillin-G residue in gluteal muscle stored at −20°C is systematically lost after 10 days. A significant lost occuring within the first week of storage was effectively observed for ampicillin residues. This seems to be essentially due to a degradation taking place in bulk tissue (30–35% loss), and can be reduced considerably by grinding the muscle matrix (only 10–15% loss). In fact, grinding the meat before its storage in freezing conditions seems to protect ampicillin from a previous depletion appearing when freezing the bulk material. Some further experiments could probably establish the protecting reactions that may take place in the ground matrix, or reversely underline the degrading actions of endogeneous compounds in the bulk matrix that are desactivated by grinding the meat.

Comparing the stability of ampicillin with regard to the one of penicillin-G in tissues recorded by Boison et al. [3], and according to the rate constants of degradation measured at −20°C, it is predicted that it takes more than 300 days for loss of 50% of the ampicillin residues in muscle tissue, while less than 150 days are sufficient for depleting the same amount of penicillin-G in liver and kidney. The rate constants measured for the kinetics of degradation at −20°C (Table 5) prove that ampicillin residues in gluteal muscle behave the same as those for penicillin-G in plasma and muscle. Consequently, it can be assumed that kidney and liver should be less stable matrices compared to muscle and plasma for ampicillin as for penicillin-G. Comparing diced samples and ground samples, similar rate constants are obtained, but diced samples present a slightly higher stability for long-range storage at −20°C.

This study also indicates that the three different technologies lead to relatively well-correlated results when the analyses are performed simultaneously. Although the analytical variabilities show slightly different levels of precision (Table 6), it is thus demonstrated that the microbiological activity in the agar diffusion test, the UV absorption of a derivatized ampicillin molecule in HPLC and the specific ions in LC–MS, allow us to monitor in a similar manner the ampicillin degradation in muscle tissue.

5. Conclusion

This study contributes to the determination of the best way of analyzing ampicillin residues and, further, penicillin antibiotic residues in muscle tissue. To avoid any degradation for a period of storage of more than 1 year, it is clearly demonstrated that ampicillin-incurred samples must be stored at high freezing temperature (ca. −75°C). But considering slaughtering house procedures and the controlling systems used, it is possible to store the samples at a low freezing temperature, providing analyses are carried out within 3 months, in order to keep the loss under the 10% level.

Finally, when accurate and realistic measurement is required for the control of the content of ampicillin residues in the carcass, the use of ground muscle samples is particularly recommended. Bulk material or diced-cut samples stored frozen may result in higher losses, ranging from 30 to 50% within a week of storage.

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