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Simple and Rapid Liquid Chromatography−Tandem Mass Spectrometry Confirmatory Assay for Determining Amoxicillin and Ampicillin in Bovine Tissues and Milk

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A simple specific and rapid confirmatory method for determining the two amphoteric penicillins, that is, amoxicillin and ampicillin, in bovine muscle, liver, kidney, and milk is presented. This method is based on the matrix solid-phase dispersion technique with hot water as extractant followed by liquid chromatography (LC)−tandem mass spectrometry. With this instrumentation, the selected reaction monitoring acquisition mode with two fragmentation reactions for each analyte was adopted. After acidification and filtration of the aqueous extracts, 25 μL of the tissue final extracts and 50 μL of the milk final extract were injected into the LC apparatus. Absolute recovery of the two analytes in any biological matrix at the 50 ppb level in tissues and the 4 ppb level in milk was 74−95% with relative standard deviations (RSDs) of no larger than 9%. When penicillin V was used as surrogate internal standard, relative recovery of the targeted compounds present in bovine tissues and milk at, respectively, 25 and 2 ppb levels ranged between 100 and 106% with RSDs of no larger than 11%. When fractionation of analytes by using a short chromatographic run was attempted, remarkable signal weakening for the two analytes was experienced. This effect was traced to polar endogenous coextractives eluted in the first part of the chromatographic run that interfered with the gas-phase ion formation of the two penicillins. Slowing the chromatographic run eliminated this unwelcome effect. Limits of quantification of the two analytes in bovine milk were estimated to be <1 ppb, whereas amoxicillin and ampicillin could be quantified in bovine tissues down to 3.1 and 0.8 ppb levels, respectively.

KEYWORDS: Amoxicillin; ampicillin; bovine; milk; tissues; LC-MS/MS

INTRODUCTION

Amoxicillin and ampicillin are two broad-spectrum β-lactam antibiotics that are widely used in veterinary medicine for the treatment and prevention of primary respiratory, gastrointestinal, urogenital, and skin bacterial infections in food-producing animals. This use can result in the presence of residues in food that can lead to health problems for individuals who are hypersensitive to penicillins. Even more important, low-level doses of antibiotics in foodstuff for long periods has led to the problem with the spread of drug-resistant microorganisms. To ensure the safety of food for consumers, regulation 508/99 of the European Union (EU) Commission (*1*) has laid down maximum residue limits of 50 ppb of ampicillin and amoxicillin in animal tissues and 4 ppb in milk. Bioassays are the most commonly employed methods for determining penicillin residues in food. Although sensitive, these assays are not specific or quantitative and can give origin to false positives. Some years ago (*2*), the National Milk Data Base developed by the U.S. Food and Drug Administration reported that 4,480,530 tests for β-lactam antibiotics were run. Of these, 6148 were positive. None of the positive tests were confirmed by more specific analytical methods. Despite their intrinsic limitations, bioassays continue to be used because of their simplicity and cheapness. However, it is well recognized that these methods need to be supported by highly selective and sufficiently sensitive chemical methods.

The amphoteric and high-polarity characteristics of amoxicillin and ampicillin make their analysis difficult. Thus, only a limited number of liquid chromatography (LC)-based confirmatory methods for their simultaneous determination in different biological matrices are quoted in the literature. Generally, these methods (*3*−*11*) are laborious and time-consuming. In addition, with few exceptions (*8*−*11*), these methods do not use a high specific detector, such as mass spectrometry (MS). Public health agencies in many countries rely on detection by MS for unambiguous confirmation of xenobiotics in foodstuffs. The Commission Decision 2002/657/EEC (12) states that "Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods". LC-MS is thus the ideal technique to determine very polar, thermally unstable

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compounds, such as amoxicillin and ampicillin. None of the LC-MS methods developed by several authors is able to determine amoxicillin and ampicillin residues in both animal tissues and milk.

After the pioneering work of Barker and his colleagues (*13*), many researchers have successfully adopted the so-called matrix solid-phase dispersion (MSPD) technique for extracting contaminants, particularly drugs, from biological matrices (*14*). A fine dispersion of the biological matrix onto a suitable solid support is easily obtained by blending the sample and the support with a mortar and pestle. After blending, this material is packed into a minicolumn and the analytes are eluted by a suitable extractant. Over classical sample treatment procedures, MSPD offers distinct advantages in that (i) the analytical protocol is drastically simplified and shortened, (ii) the possibility of emulsion formation is eliminated, and (iii) solvent consumption is substantially reduced.

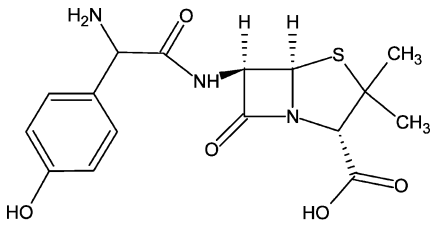
With the exception of a work proposing water at ambient temperature for extracting the highly hydrophilic amino glycoside antibiotics from bovine kidney (*15*), all other methods based on MSPD have used moderate amounts of organic solvents as extractants. This means that problems associated with the use of organic solvents are minimized by MSPD, but not completely removed. Moreover, because no organic solvent is capable of selectively extracting target compounds from complex biological matrices, a sample cleanup step is often included in protocols involving analyte extraction by the MSPD technique. Finally, the use of pure organic solvent restricts direct injection of the extract into a reversed-phase LC column.

Recently, we have developed rapid and simple LC-MS confirmatory methods based on the MSPD technique and heated water as extractant for determining sulfonamide antibacterial residues in bovine muscle (*16*), kidney and liver (*17*), and milk (*18*) at the EU regulatory levels. Over conventional methods employing the MSPD technique, the use of heated water as extractant offers three distinct advantages. Like CO_2 used in supercritical fluid extraction, water is an environmentally acceptable solvent, it is cost-effective, and hot-water conditions are easily achieved with commercial laboratory equipment. Second, because the polarity of water decreases as the temperature is increased, selective extraction of polar and medium-polar compounds can be performed by suitably adjusting the water temperature. Last but not least, the aqueous extract can be directly (*16*), or after little manipulation (*17*, *18*), injected into the reversed-phase LC column. As a consequence, the analytical protocol is simplified and the analysis time is greatly shortened.

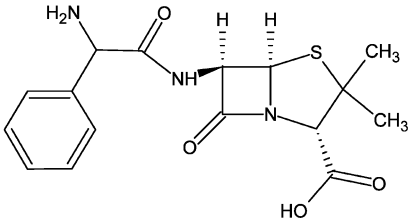
The purpose of this work was to extend the methodology mentioned above to the determination of amoxicillin and ampicillin residues in bovine muscle, kidney, and liver and in whole milk at the EU regulatory levels. Final extracts were analyzed by LC−tandem MS (LC-MS/MS).

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Amoxicillin and ampicillin were purchased from Sigma-Aldrich, Milwaukee, WI. Penicillin V (Sigma-Aldrich) is not used in veterinary medicine and was adopted as a surrogate internal standard (IS). Structures of the three penicillins are visualized in **Figure 1**. Individual standard solutions of the analytes and the IS were prepared by dissolving each compound in water to obtain a 0.2 mg/mL concentration. After preparation, these solutions were stored at 4 °C in the dark to minimize analyte degradation. They were freshly prepared every 2 weeks. A composite working standard solution of the target compounds was prepared by mixing the above solutions and diluting with water to obtain analyte concentrations of 1 μg/mL. The same was done with the IS aqueous solution to obtain a 10 μg/mL final solution. When unused, these two solutions were stored at 4 °C in the dark and renewed after 1 week.



Amoxicillin MW 365.4



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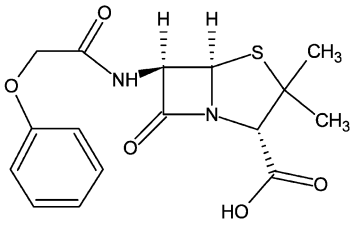

Penicillin V MW 350.4

Figure 1. Chemical structures and molecular weights of selected penicillins.

For LC, distilled water was further purified by passing it through the Milli-Q Plus apparatus (Millipore, Bedford, MA). Methanol "Plus" of gradient grade was obtained from Carlo Erba, Milano, Italy. Sand (Crystobalite, 40−200 mesh size) was from Fluka AG, Buchs, Switzerland. All other solvents and chemicals were of analytical grade (Carlo Erba) and were used as supplied.

Samples. Bovine muscle, kidney, and liver tissue samples as well as whole pasteurized bovine milk were purchased from various retail markets. Preliminary analyses showed they were analyte-free.

Extraction Apparatus. The design of the homemade extraction apparatus used in this work was very similar to that shown in a previous paper (*19*), with the exception that nitrogen was bubbled in water to eliminate any trace of dissolved oxygen and the analyte-containing water leaving the extraction cell was collected in a calibrated glass tube instead of a sorbent cartridge. An 8.1 cm × 8.3 mm i.d. stainless steel column was used as extraction cell.

Sample Preparation and Extraction. *Milk Samples*. To evaluate the extraction yield, 2 mL of milk sample was put in a porcelain mortar and spiked with variable volumes of the working standard solution. Vice versa, when the accuracy and precision of the method were assessed, the surrogate internal standard (penicillin V) was added together with the analytes. Under continuous agitation, 10 min was allowed for equilibration at room temperature. Thereafter, 2 mL of milk was poured in a porcelain mortar containing 6 g of sand, and the mixture was blended with the pestle for ~10 min, until an apparently dry material was obtained. This material was then packed into the extraction cell, taking care to tap the tube to avoid loose packing of the particles. Any void space remaining after the solid material had been packed was filled with sand. Stainless steel (2 μm pore size) and polyethylene (20 μm pore size) frits were located, respectively, above and below

the packing. The tube was then put into the oven and heated at 65 °C for 5 min. Three milliliters of water at a 1 mL/min flow rate was then passed through the cell to extract the analytes and, if present, the surrogate internal standard. When experiments were performed to assess the extraction yield by heated water, 300 ng of the IS was added to the extract. To make the aqueous extract injectable into the LC column, the pH of the extract was adjusted to 4.6 with 3 mol/L formic acid and then filtered through a glass fiber (pore size = 0.7 μm, 25 mm diameter; Whatman, Sigma-Aldrich, Milan, Italy). After filtration, a completely uncolored and transparent solution was obtained.

Tissue Samples. Muscle, kidney, and liver samples were finely diced with scissors. For recovery studies, 1 g of tissue was put in a porcelain mortar and spiked with variable volumes of the working standard solution, taking care to uniformly spread it on the sample. An intimate contact between the analytes and the sample was obtained by pounding with the pestle for 2 min. Then, 1 h was allowed for equilibration, storing the mortar at 4 °C. Thereafter, 5 g of sand was added to the mortar, and the mixture was blended with the pestle for <10 min, until an apparently dry material was obtained. From this point, the same procedure as that for milk was followed, with the exception that 150 ng of the IS was added to the extract and the pH of the aqueous extract was adjusted to 3. The muscle extract was filtered as milk, whereas kidney and liver extracts were first filtered through a 1.2 μm pore size glass fiber (25 mm diameter; Whatman, Sigma-Aldrich) and then through a regenerated cellulose filter (pore size = 0.2 μm, 25 mm diameter; Alltech, Sedriano, Milan, Italy). By following the procedure described above, the guard column was replaced with a new one after >90 injections of extracts.

Twenty-five microliters of the tissue final extracts and 50 μL of the milk final extract were injected into the LC apparatus.

LC-ESI-MS/MS Analysis. LC was performed by a PE series 200 binary pump (Perkin-Elmer, Norwalk, CT) equipped with a PE series 200 autosampler. The analytical (250 mm × 4.6 mm i.d.) and guard (7.5 × 4.6 mm i.d.) columns employed were Alltima (Alltech), filled with 5 μm C-18 reversed phase packing. For fractionating the analytes, phase A was methanol and phase B was water. Both phases contained 10 mmol/L formic acid. For chromatographing milk and muscle extracts, the mobile phase gradient profile (where t refers to time in minutes) was as follows: t_0, A = 25%; t_9, A = 60%; t_{10}, A = 80%; t_{20}, A = 100%; t_{25}, A = 100%; t_{27}, A = 0%; t_{35}, A = 0%. For kidney and liver extracts, the mobile phase gradient profile was t_0, A = 0%; t_5, A = 0%; t_6, A = 40%; t_{15}, A = 60%; t_{23}, A = 100%; t_{28}, A = 100%; t_{30}, A = 0%; t_{38}, A = 0%. By following the conditions reported above, retention times of the analytes did not differ by more than 0.5% over two working weeks. A diverter valve led the effluent into the ion source with a flow of 150 μL/min only between 4 and 20 min (milk and muscle) and 10−23 min (kidney and liver) of the chromatographic run.

A PE Sciex API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Toronto, ON, Canada) equipped with a turbo ion spray source operated in the PI mode was used for the work described herein. High-purity nitrogen was used as drying, curtain, and collision gases; high-purity air was used as nebulizer gas. Drying gas was set at 8 L/min, and the turbo ion spray probe temperature was maintained at 300 °C. The settings for nebulizer and curtain gases were, respectively, 30 and 20 psi, whereas the gas pressure in the collision cell was set at 3 mTorr. Ion spray voltage was 5000 V. Declustering potential, collision energy, and other transmission parameters were optimized for each analyte (data are reported in **Table 1**). Mass axis calibration of each mass-resolving quadrupole Q_1 and Q_3 was performed by infusion of a polypropylene glycol solution at 10 μL/min. Unit mass resolution was established and maintained in each mass-resolving quadrupole by keeping a full width at half-maximum of ~0.7 amu. All of the source and instrument parameters for monitoring penicillins were optimized by standard solutions of 50 pg/mL infused at 5 μL/min by a syringe pump. The selected reaction-monitoring (SRM) mode was used for quantitation by selecting two fragmentation reactions for each analyte. Operative conditions are reported in detail in **Table 1**. Providing injected volumes of the final extracts of tissues and milk were no larger than 25 and 50 μL, respectively, no decrease of the ion signal due to the matrix effect was observed by us.

Table 1. Time-Scheduled Selected Reaction Monitoring Conditions for Detecting Penicillins in Bovine Milk, Muscle, Liver, and Kidney

compound	SRM transition, m/z	declustering potential, V	collision potential, V	retention window, min		dwell time, ms
				A[a]	B[b]	
amoxicillin	366 → 114	20	–40	0–9	0–12	400
	366 → 208		–28			400
ampicillin	350 → 106	25	–35	9–14	12–18	400
	350 → 192		–25			400
penicillin V	351 → 160	25	–30	14–20	18–23	600

[a] Milk and muscle. [b] Kidney and liver.

Quantitation. Absolute recovery of each analyte added to milk and tissue samples at any given concentration was assessed by measuring peak areas resulting from the total ion current relative to the two molecular ion-to-daughter ion transitions, normalizing them to the peak area of the IS, which was added *after* extraction, and comparing these ratios to those obtained by injecting a standard solution. Accuracy and precision data were obtained in a similar way, with the exception that the IS was added *before* analyte extraction**.** The responses of the ESI/MS/MS system were linearly related to injected amounts of the analytes from 1 to 500 ppb (milk) and from 4 to 2000 ppb (tissues) with R^2 ranging between 0.9925 and 0.9987. The mass spectrometry data handling system used was the Analyst 1.2 software from Applied Biosystems.

RESULTS AND DISCUSSION

Critical Analytical Steps. When liver and kidney extracts were injected, chromatographic conditions differed from those adopted for analyzing milk and muscle extracts in that a 5-min initial step with 100% water was inserted in the elution gradient program (see Experimental Procedures). When this step was omitted and the liver and kidney aqueous extracts were injected into the LC column having a methanolic solution as mobile phase, partial clogging of the guard column occurred. Presumably, this effect was caused by the presence of large amounts of salts in the liver and kidney extracts that were coextracted with the analytes and are insoluble in a water/methanol solution.

To achieve high-throughput determination of analytes in biological matrices, analytical protocols based on LC-ESI-MS with short (3−5 cm) LC columns, where analytes are eluted in few minutes, are often adopted. However, it is well recognized that the yield of protonation of targeted compounds in the electrosprayed solution can be remarkably decreased due to competition effects by coextracted and coeluted matrix components. The extent of this unwelcome effect is related to both concentrations and affinities for the proton of the endogenous components. It was shown that ion suppression of the analytes could be minimized or eliminated by adopting an efficient chromatographic separation (*16*, *20*). In the past (*16*−*18*), we observed that endogenous compounds able to interfere with the process of protonation of the analytes were mainly eluted in the first part of the chromatographic run. In this work, a similar unwelcome effect was observed when elution of amoxicillin and ampicillin was attempted with short retention times and/or extract volumes larger than those reported under Experimental Procedures were injected.

Effect of the Temperature on Analyte Recoveries. Progressively increasing the extraction temperature makes water more and more efficient in extracting compounds from matrices. On the other hand, a risk inherent to the use of hot water as extractant is that it could decompose those compounds that are thermolabile and/or prone to hydrolytic attack. Therefore, we evaluated the effect of the temperature on the recovery of the

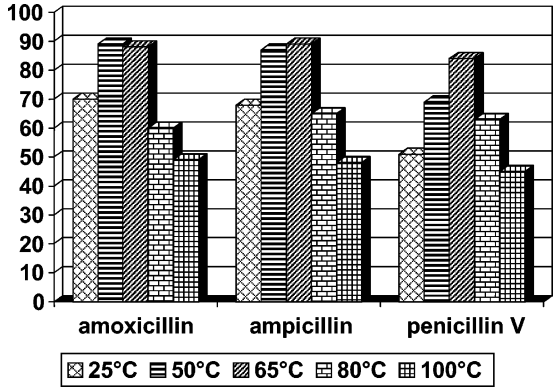

Figure 2. Effect of the extractant temperature on analyte recovery in a bovine kidney sample.

two analytes and penicillin V, the latter being a candidate for use as a surrogate internal standard, by performing extractions at various temperatures. The aim of this study was also that of finding the lowest extraction temperature able to give good recovery of the analytes and penicillin V while minimizing the amount of coextracted medium-polar endogenous components that could interfere with the rest of the analysis. For this study, samples of the matrices considered were spiked with the analytes at the 100 ppb level, whereas penicillin V was added to the samples at the 150 ppb level. In all cases, a water volume equal to 4 mL was allowed to pass through the extraction cell at a 1 mL/min flow rate. Measurements were made in triplicate. For the sake of clarity, results of only the experiments with a kidney sample are presented in **Figure 2**. Increasing the water temperature from 25 to 50 °C had the result of markedly improving the extraction yield of amoxicillin and ampicillin. At this temperature, however, the recovery of penicillin V was still unsatisfactory. The goal of achieving good recovery of all three penicillins was reached by extraction at 65 °C. Higher extraction temperatures would be expected to reduce the water volume needed to quantitatively extract target compounds. Because this method does not include any extract concentration step, reducing the extract volume reflects ultimately an improvement of the method sensitivity. However, when extraction of target compounds at temperatures >65 °C was attempted, a steady decrease of the extraction yield was experienced. This loss was traced to hydrolytic attack of the unstable four-term ring of penicillins. Thus, an extraction temperature of 65 °C was used for subsequent experiments.

Effect of Extractant Volume on Analyte Recoveries. For the reason mentioned above, recovery studies were conducted to find the minimum water volume capable of efficiently extracting the analytes and the surrogate internal standard from all of the biological matrices considered. Experiments were performed by spiking milk and muscle tissue samples with the analytes at 100 ppb level and the surrogate internal standard at the 150 ppb level, and extracting. A 2-mL aliquot followed by two 1-mL aliquots of the aqueous eluent coming out from the extraction cell were separately collected and analyzed. For each matrix, experiments were performed in triplicate. For the sake of conciseness, only results relative to milk and muscle are shown in **Figure 3**. As can be seen, the ability of water to remove penicillins from biological matrices was matrix-dependent, as different volumes of the extractant needed to have satisfactory recovery of the analytes and the surrogate internal standard. One could speculate that this effect is due to different interactions between the analytes and some nonextracted matrix components. Anyway, 3 mL of heated water sufficed to extract

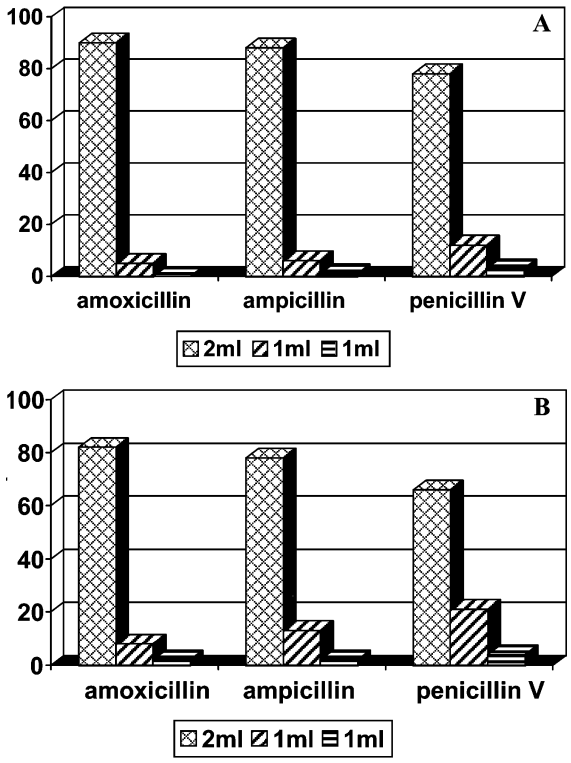

Figure 3. Elution histogram obtained on extraction of three penicillins from (**A**) milk and (**B**) bovine muscle by passing through the extraction cell water heated at 65 °C and collecting separately the first 2 mL and then two 1-mL aliquots of the extracts.

Table 2. Absolute Recoveries of Amoxicillin and Ampicillin by Extraction ($n = 5$) from Various Biological Matrices (Spike Levels = 50 ppb in Tissues and 4 ppb in Milk)

	recovery, % (RSD, %)			
compound	muscle	kidney	liver	milk
amoxicillin	93 (6)	88 (9)	74 (7)	94 (8)
ampicillin	92 (7)	89 (8)	74 (7)	95 (6)

the analytes from any matrix considered, and they were used in the subsequent experiments.

Effect of Extractant Flow Rate on Analyte Recoveries. We evaluated the influence of the flow rate at which water passed through the extraction cell on the extraction efficiency. For this experiment, a bovine muscle sample spiked with the analytes at 100 ppb level and with penicillin V at 150 ppb was submitted to the extraction procedure by passing water through the cell at flow rates ranging between 0.5 and 2 mL/min. Results (not shown here) from duplicate experiments at each flow rate selected evidenced that the analyte extraction yield was substantially not dependent on the extractant flow rate. We chose to extract penicillins at a flow rate of 1 mL/min because at a 2 mL/min flow rate the extraction cell sometimes clogged, especially when analytes were extracted from milk samples.

Extraction Efficiency. Under conditions reported within Experimental Procedures, we evaluated the ability of water to extract amoxicillin and ampicillin from the various matrices considered. This study was conducted by spiking tissue and milk samples with the analytes at the tolerance levels set by the EU, that is 50 and 4 ppb, respectively. Absolute recoveries were estimated by adding penicillin V *after* extraction and *before* extract filtration. For each matrix, five experiments were performed, and results are shown in **Table 2**. The extraction

Table 3. Accuracy and Precision Data from Analysis of Penicillins in Two Selected Matrices at Concentrations Equal or Close to Maximum Residue Limits (MRLs) Set by the European Union (EU)

	accuracy, % (RSD, %)											
	muscle			kidney			liver			milk		
compound	MRL/2[a]	MRL	2 MRL	MRL/2	MRL	2 MRL	MRL/2	MRL	2 MRL	MRL/2	MRL	2 MRL
amoxicillin	103 (5)	107 (8)	110 (11)	101 (6)	104 (5)	103 (7)	100 (9)	105 (8)	103 (9)	106 (7)	105 (3)	108 (9)
ampicillin	102 (6)	103 (10)	106 (7)	100 (4)	102 (5)	105 (8)	105 (6)	108 (9)	105 (4)	104 (11)	106 (6)	109 (7)

[a] Tolerance levels of amoxicillin and ampicillin set by the EU are 50 ppb in bovine tissues and 4 ppb in milk.

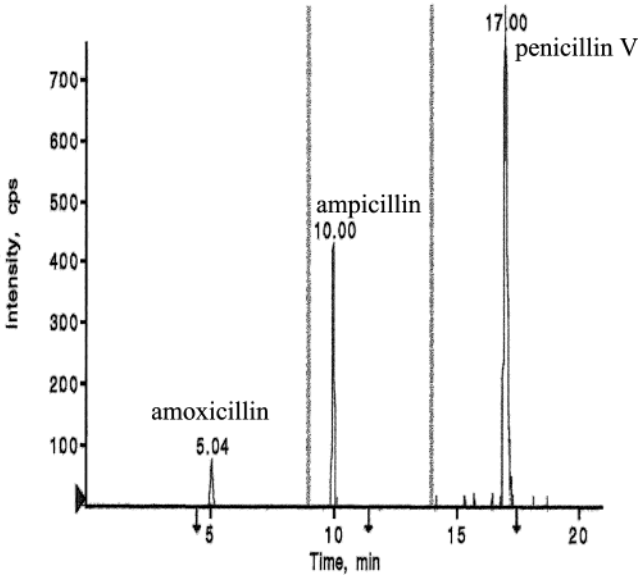

Figure 4. SRM LC-MS/MS trace resulting from analysis of amoxicillin and ampicillin at 4 ppb level in bovine milk.

yields varied from 74 to 95% with relative standard deviations (RSDs) not higher than 9%.

Accuracy and Precision. For amoxicillin and ampicillin, the EU has set maximum residue limits (MRLs) of 50 ppb in edible animal tissues and 4 ppb in bovine whole milk. Following criteria reported in the EU guidelines (*12*), this method was validated at three different concentrations corresponding to half of the MRL, the MRL, and 2 times the MRL. At any analyte concentration and for any matrix considered, five measurements were performed with the criterion of adding the surrogate internal standard (penicillin V) *before* analyte extraction. Results are presented in **Table 3**. To check that the accuracy of the method was not dependent on the analyte concentration and/or the type of matrix, mean accuracy data for each matrix were compared among them by using the two-way analysis of variance (ANOVA) test at the $P = 0.05$ significance level. In both cases, the calculated $F_{2,6}$ and $F_{3,6}$ values (4.18 and 4.54, respectively) were lower than the critical values (5.14 and 4.76), indicating that the extraction method was not influenced by either the concentration of the analyte or the nature of the matrix. The accuracy data varied between 100 and 110% with RSDs not higher than 11%. Thus, this method meets requirements reported in the EU guidelines (*12*) indicating that a method can be considered to be accurate and precise when accuracy data are between 80 and 110% with RSDs not higher than 20%.

Limits of Detection (LOD) and Quantification (LOQ) of the Method. LOQs of the method were estimated from the SRM LC-MS/MS chromatograms resulting from analyses of 4 and 25 ppb of the two penicillins in, respectively, milk (please, note that the resulting chromatographic trace is shown in **Figure 4**) and tissue samples. After extraction of the sum of the ion currents of the two molecular ion-to-daughter ion transitions relative to each analyte, the resulting trace was two-times smoothed by applying the mean smoothing method. Thereafter, the peak height-to-averaged background noise ratio was measured. The background noise estimate was based on the peak-to-peak baseline near the analyte peak. LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise ratio (S/N) of 10. These data are listed in **Table 4**. In the same table, LODs of the method are also presented. When using an MS/MS detector in the SRM mode, the most important condition to be satisfied for ascertaining the presence of a targeted compound is that at least two signals produced by the decomposition reactions are distinguishable from the background ion current. Accordingly, a definition of LOD (S/N 3) of each analyte was adopted considering the transition giving the worst S/N. As can be read, amoxicillin and ampicillin could be detected in milk at levels of <1 ppb.

Table 4. Limits of Detection (LOD) and Quantification (LOQ) of the Method for Determining Penicillins in Milk and Tissues

	milk		tissues	
compound	LOD,[a] ppb	LOQ, ppb	LOD, ppb	LOQ, ppb
amoxicillin	0.5 (366 → 114)	0.8	2.1 (366 → 114)	3.1
ampicillin	0.1 (350 → 192)	0.2	0.5 (350 → 192)	0.8

[a] *m/z* values of the transitions giving the worst S/N ratios are reported in parentheses.

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