MULTI RESIDUE DETERMINATION OF THE PENICILLINS REGULATED BY THE EUROPEAN UNION, IN BOVINE, PORCINE AND CHICKEN MUSCLE, BY LC-MS/MS.

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ABSTRACT

A multiresidue analysis method was developed to determine the content of penicillins in bovine, porcine and chicken muscle tissues. The procedure involves solid phase extraction (SPE) and subsequent analysis by liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) set by the European Union (EU) for all compounds. The method was validated according to EU guideline 2002/657/EC. The LOQ in tissues are below the maximum residue limits (MRL) and appropriate quality parameters in terms of linearity, accuracy (recoveries higher than 70% for all antibiotics and animal tissues except for AMOX with 50% of recovery) and precision (in terms of intra and inter day with values lower than 12% in all cases) are obtained for the developed method..

A study concerning to the matrix effect was made and it was concluded that similar matrix effect could be found in beef, pig and chicken.

The method was applied to the analysis of samples of chicken from animals treated with amoxicillin.

Key Words: Penicillins, beef, pig and chicken muscle, solid phase extraction, tandem mass spectrometry, matrix effect.

1. INTRODUCTION

Penicillins and cephalosporins are β-lactamic antibiotics that are widely used in veterinary medicine (for livestock farming and bovine milk production) to prevent and treat bacterial infections (respiratory, urinary or skin infections). Incorrect use of these veterinary antibiotics represents a potential risk for consumers due to the increasing incidence of microbial resistance and the risk of allergic reactions to residues from antibiotics or their metabolites. Cases of allergic reactions after consumption of foods containing antibiotics residues have been reported in the literature [Dayan, 1993; Marazuela, & Bogialli, 2009]. To protect human health, the EU established safe maximum residue limits (MRLs) for residues of veterinary drugs in animal tissues entering the human food chain [Commission Regulation (EU) No 37/2010, 2010]. Commission Regulation (EU) 37/2010, which repeals Council Regulation (EEC) 2377/90 and its amendments, regulates the drugs authorized for therapeutic veterinary use in animals intended for food production. For reasons of ease of use, all pharmacologically active substances are listed in a single Annex in alphabetical order. Regulation (EEC) 2377/90 established two separate tables: one for authorized substances including penicillins, listed in Annexes I, II and III: and one for prohibited substances, listed in Annex IV. The MRLs established for beef, pig and chicken meat range, from 25 μg/kg for penicillin V (PENV) in pig and chicken muscle to 300 μg/kg for oxacillin (OXAC), cloxacillin (CLOX) and dicloxacillin (DICL) in beef, pig and chicken muscle [Commission Regulation (EU) No 37/2010]. Nafcillin (NAFC) is a penicillin that is only regulated in beef. The low concentrations permitted makes necessary the development of sensitive analytical methods that can be used to confirm and quantify penicillins in different matrices.

A crucial step in the sample treatment process is the extraction of antibiotics from complex matrices. One of the most widely used techniques for the preconcentration and clean-up of samples is solid phase extraction (SPE) [Blasco, Torres, & Pico, 2007; Gentili, Perret, & Marchese, 2005; Kantiani, Farré, & Barceló, 2009; Marazuela, & Bogialli, 2009; Moreno-Bondi, Marazuela, Herranz, & Rodríguez, 2009; Stolker, & Th. Brinkman, 2005]. SPE has the advantages that it is, suitable for small samples, it is not

very time consuming, it only requires small volumes of solvent, and reproducible clean extracts are obtained. For monitoring antibiotic residues in food of animal origin, there are screening methods based on microbiological, receptor or immunological techniques [Alfredsson, Branzell, Granelli, & Lundström, 2005; Benito-Peña, Partal-Rodera, León-González, & Moreno-Bondi, 2006; Cantwell, & O'Keeffe, 2006; McGrath, Baxter, Ferguson, Haughey, & Bjurling, 2005; Myllyniemi, Nuotio, Lindfors, Rannikko, Niemi, & Backman, 2001; Samanidou, Nisyriou, Papadoyannis, 2007]. They are easy to perform and inexpensive but lack specificity. LC-UV can be used to determine antibiotics [Benito-Peña, Partal-Rodera, León-González, & Moreno-Bondi, 2006; Samanidou, Nisyriou, Papadoyannis, 2007], but such methods sometimes present a lack of sensitivity. These techniques have therefore been replaced by methods that use mass spectrometry to provide more specific determination leading to unequivocal confirmation of the compounds studied [Becker, Zittlau, & Petz, 2004; Gentili, Perret, & Marchese, 2005; Granelli, & Branzell, 2007; Hermo, Barrón, & Barbosa, 2008; Kantiani, Farré, & Barceló, 2009; Kantiani, Farré, Sibum, Postigo, López de Alda, & Barceló, 2009; Marazuela, & Bogialli, 2009; Martínez-Huélamo, Jiménez-Gámez, Hermo, Barrón, & Barbosa, 2009; Yamada, Kozono, Ohmori, Morimatsu, & Kitayama, 2006]. Some of these authors report the use of tandem mass spectrometry for the simultaneous identification and quantification of target residues in complex matrices.

This paper describes the optimization of an effective extraction method for the analysis of penicillins in muscle samples of different meats (beef, pig and chicken), which involves solid-liquid extraction, followed by SPE. Determination is carried out by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The matrix effect in beef, pig and chicken meat is studied in order to obtain a single unified method for the three matrices. The method concluded yielded satisfactory in terms of linearity, precision, recovery and limits of quantification, which are lower than the MRLs established by the European Union for beef, pig and chicken muscle.

2. EXPERIMENTAL

2.1. *Reagents and materials*

Penicillin standards: Ampicillin (AMPI), Dicloxacillin (DICL), Penicillin G (PENG) and Penicillin V (PENV) were supplied by the European Pharmacopeia (Strasbourg Cedex, France). Amoxicillin (AMOX), Nafcillin (NAFC) and Oxacillin (OXAC) were from Sigma-Aldrich (St. Louis, MO, USA). Cloxacillin (CLOX) and Piperacillin (PIPE – internal standard (IS)) were provided by Fluka (Buchs, Switzerland).

All reagents were LC grade: Acetonitrile (MeCN), methanol (MeOH), formic acid (HFor), sodium dihydrogenphosphate and sodium hydroxide were supplied by Merck (Darmstadt, Germany) and sodium chloride by Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water generated by the Milli Q system (Millipore, MA, USA) was used.

The SPE cartridges used in this study were Bond Elut C18 (500 mg, 3 ml) obtained from Varian (Harbor City, CA, USA), ENV+ Isolute (200 mg, 3 ml) from Isolute International Sorbent Technologies (Hengoed, UK), and Oasis HLB (60 mg, 3 ml) and Oasis MAX (60 mg, 3 ml) were supplied by Waters (Milford, MA, USA).

2.2. *Preparation of standard and stock solutions*

Individual standard solutions of 100 μg ml^{-1} for AMOX, AMPI, PENG, PENV, OXAC, CLOX, NAFC, DICL and 30 μg ml^{-1} for IS were prepared in Milli Q water. All stock standard solutions were stored at –20^{o}C. Working solutions were prepared by mixing the individual standard solutions and diluting them with Milli Q water, to achieve the concentrations used for spiking.

Phosphate buffers (50 mM) at different pH, from pH=4 to pH=11, were prepared for the sample preparation and SPE.

2.3. *Instruments*

A Selecta ultrasound system was used to dissolve the individual penicillin solutions.

An Orion 81025 C Ross combination pH electrode and a Mettler Toledo Inlab 413 pH electrode were used to measure the experimental pH.

SPE was carried out on a Supelco 24-cartridge vacuum manifold (Bellefonte, PA, USA) connected to a Supelco vacuum tank. Finally, evaporation to dryness at room temperature and under a stream of nitrogen was used at the end of the sample treatment.

A Rotanta 460RS centrifuge (Hettich Zentrifugen, Germany) was used to perform the extractions. A Mikro 20 mini-centrifuge (Hettich Zentrifugen, Germany) was used to centrifuge the final extracts.

Chromatographic separation was achieved on a 150 × 4.6 mm Zorbax Eclipse XDB-C8 column from Agilent Technologies (Waldbronn, Germany) with a 20 x 4.5 mm Kromasil C8 guard column supplied by Aplicaciones Analíticas (Barcelona, Spain) and on a 4.0 x 125 mm Lichrospher 100 RP-18 column from Agilent Technologies with a 4 x 4 mm Lichrocart guard column supplied by Akady (Barcelona, Spain).

The LC-UV system was formed of an HP Agilent Technologies 1100 LC system equipped with an autosampler and coupled to a diode array detector (DAD). The system was controlled by Chemstation for LC 3D Rev. A 08.03 (847) software (Agilent Technologies).

LC-MS/MS analyses were performed on an HP Agilent Technologies 1100 LC system equipped with an autosampler and coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex) with a turbo ionspray source. Both the system and the data treatment were controlled by Analyst 1.4.2 software, supplied by Applied Biosystems (Foster City, CA, USA).

2.4. Procedures

2.4.1. Sample preparation method

Different kinds of bovine, porcine and chicken muscle samples were used for the optimization and validation of the method. Upon arrival at the laboratory the samples were ground, homogenized, and stored at –20^{o}C until analysis.

4 g (±0.0001 g) of homogenized raw tissues (bovine, porcine or chicken muscle) was introduced into a 50 ml centrifuge tube, and spiked with appropriate volumes of working solutions of penicillins. The IS was added in order to achieve a 300 μg kg^{-1} final concentration. The samples were allowed to stand for 15 min at room temperature to permit the total interaction between the antibiotics and the muscle matrix. The penicillins

were extracted from the tissues using 2 ml water and shaking for 1 min., and then adding 20 ml MeCN and shaking for 1 min., in order to precipitate the proteins. After extraction, the mixtures were centrifuged at 2685 x g (3500 rpm) at 25^{o}C for 5 min. The suspended solutions were evaporated by nitrogen and 2 ml saturated sodium chloride solution was added to prevent foaming during the MeCN evaporation. 25 ml 50 mM phosphate buffer, at the adequate pH (5-8.5 range) was added to the final solutions, and the extracts were cleaned-up according to the SPE procedure described below.

Reference samples, for recovery studies, were prepared in the same way, except that the spiking solutions were added after SPE, thus ensuring 100% recovery.

2.4.2. Solid phase extraction (SPE)

An exhaustive study of the literature was performed in order to select the cartridge that could yield the best recoveries. The cartridges most commonly used in the literature reviewed were the Oasis HLB (3 ml) [Becker, Zittlau, & Petz, 2004; Blasco, Torres, & Pico, 2007; De Baere, Cherlet, Baert, & De Backer, 2002; Feitosa, Temime, & Chiron, 2007; Gentili, Perret, & Marchese, 2005; Holstege, Puschner, Whitehead, & Galey, 2002; Kantiani, Farré, & Barceló, 2009; Kantiani, Farré, Sibum, Postigo, López de Alda, & Barceló, 2009; Moreno-Bondi, Marazuela, Herranz, & Rodríguez, 2009; Stolker, & Th. Brinkman, 2005], Bond Elut C18 (3ml) [Benito-Peña, Partal-Rodera, León-González, & Moreno-Bondi, 2006; Blasco, Torres, & Pico, 2007; De Baere, Cherlet, Baert, & De Backer, 2002; Gentili, Perret, & Marchese, 2005; Ito, Goto, Oka, Matsumoto, & Takeba, 2004; Kantiani, Farré, & Barceló, 2009; Marchetti, Schwaiger, & Schmid, 2001; Riediker, & Stadler, 2001; Stolker, & Th. Brinkman, 2005] and Oasis MAX (3ml) [Benito-Peña, Partal-Rodera, León-González, & Moreno-Bondi, 2006; Gentili, Perret, & Marchese, 2005; Stolker, & Th. Brinkman, 2005]. The ENV+ Isolute (3 ml) was chosen because good results were obtained in previous work [Clemente, Hermo, Barrón, & Barbosa, 2006; Hermo, Barrón, & Barbosa, 2008; Hermo, Barrón, & Barbosa, 2006]. So the method was applied to all the cartridges (using different activation and elution conditions) to see which of them yielded the best results.

The ENV+ Isolute cartridges were preconditioned with 2 ml MeOH, 2 ml Milli Q water and 2 ml 50 mM phosphate buffer solution (pH 5). After passing the samples, the

cartridges were washed with 3 ml phosphate buffer solution (pH 5) and 1 ml Milli Q water. The analytes were eluted with 2 ml MeOH and 2 ml MeCN.

For Bond Elut C18 and Oasis HLB the following procedure was followed: preconditioning was made with 2 ml MeOH, 2 ml Milli Q water and 2 ml 50 mM phosphate buffer solution (pH 8.5); after passing the analytes, washing was performed with 3 ml phosphate buffer solution (pH 8.5) and 1 ml Milli-Q water [Becker, Zittau, & Petz, 2004]. Analytes were eluted with 3 ml MeCN-H_2O (1:1, v:v).

Oasis MAX cartridges were activated with 2 ml MeOH, 2 ml Milli-Q water and 2 ml 50 mM phosphate buffer solution (pH 8.5). For washing, 2 ml 50 mM, pH7 NaAc:MeOH, (95:5; v:v) was used and the analytes were eluted with 3 ml 2% HFor in MeOH (pH 5).

The extracts were evaporated to dryness using nitrogen, reconstructed with 200 µl Milli Q water and centrifuged at 14170 x g (13000 rpm) for 5 min in order to facilitate injection into the LC system.

2.4.3. Chromatographic and mass spectrometric conditions

The mobile phase used for LC-MS/MS was 0.1% HFor in H_2O (A) and 0.1% HFor in MeCN (B). The injection volume was 20 µl. The flow rate was 1.0 ml min^{-1}. A post column LC split (3:1) was used to reduce the flow rate entering into the electrospray ionization source. The initial mobile phase consists in a mixture of solutions, 80% solution A and 20% solution B. Good chromatographic separation of the penicillins was achieved using the following optimized linear gradient elution: from 0 to 5 min the percentage of organic modifier increased linearly to 50%; from 5 to 10 min it increased to 70%; from 10 to 10.5 min it remained constant at 70%; from 10.5 to 11 min the percentage of organic modifier decreased linearly to the initial conditions and the column remained in initial conditions during 3 min. The program ended at 14 min.

The LC-MS/MS conditions were optimized by direct injection of each penicillin at a concentration of 1 mg l^{-1}.

The turbo ionspray source was used in positive mode with the following settings: capillary voltage, 4500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 15 (arbitrary units). Table 1 shows the optimal values

of the potentials for each of the penicillins studied: DP (declustering potential), FP (focusing potential) and EP (entrance potential). Also shown are the molecular ions of each penicillin, and the ions obtained by collision-activated dissociation (CAD) of the selected precursor ion in the collision cell of the triple quadrupole and analyzed with the instrument's second analyzer. The identification and quantification transitions selected for each penicillin with its optimum collision energy are also shown. Furthermore, base on the literature, this table also shows the mass spectrometry conditions and probable transitions for the AMOX metabolites: amoxicilloic acid (AMA) and amoxicillin diketopiperazine-2',5'-dione (DIKETO) in order to evaluate the presence or absence of these metabolites in the real samples [De Baere, Cherlet, Baert & De Backer, 2002; Reyns, Cherlet, De Baere, De Backer, & Croubels, 2008].

2.4.4. Quality parameters

The methods were validated according to the EU guideline 2002/657/EC and the FDA guideline for bioanalytical assay procedures [Official J. European Communities No. 2002/657/EC, 2002; US Department of Health and Human Services, 2001]. The quality parameters established were the limit of detection (LOD), LOQ, calibration curve, recovery, precision, decision limit (CCα) and detection capability (CCβ).

The LOD was calculated at a signal-to-noise ratio of 3, while the LOQ value was calculated using a signal-to-noise ratio of 10. To determine LOD and LOQ values, spiked bovine, porcine and chicken muscle samples at 8 concentration levels from 0.0005 MRL to 0.05 MRL were injected into the LC-MS/MS system.

The linearity of the analytical methods was verified by analyzing samples at different concentrations in beef, pig and chicken samples (LOQ, 0.05 MRL, 0.075 MRL, 0.1 MRL, 0.3 MRL, 0.5 MRL, 1.0 MRL, 1.5 MRL and 2.0 MRL). Each level was prepared in duplicate and PIPE was used as the IS at a concentration of 300 μg kg^{-1}. By correlating the response of the analyte/IS area ratio to the penicillin/IS concentration ratio the calibration curves were obtained.

Recovery was assessed via a calibration curve and an external curve. For both curves, eight concentration were prepared (between 0.05 and 2 MRL), and injected in duplicate. For the external curve, reference samples were prepared using the exact same procedure

as for the calibration samples, only that they were spiked directly with the extracts, after the SPE, thus ensuring 100% recovery. The slope of the relation between the calibration and external curves, determine the recovery of the substances.

To assess intra-day precision, also referred to as within-day repeatability, three sets (0.5 MRL, 1.0 MRL and 2.0 MRL), each of them with five spiked samples of each one of the three tissues, were prepared and analyzed. The relative standard deviation (RSD) was calculated. The procedure was repeated on 3 different days in order to determine inter-day precision. Each day, different blank muscle samples and separately weighed stock solutions of the analytes were prepared. Finally, RSD values (%) were calculated.

The decision limit (CCα) is the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. Detection capability (CCβ) is the smallest content of a substance that may be detected, identified and/or quantified in a sample with an error probability of β. CCα values were determined by analyzing 20 blank samples fortified with penicillins at MRL level. CCβ was calculated as the decision limit, CCα, plus 1.64 times the corresponding standard deviation.

2.5. Biological sample analysis

Five samples of beef, pig and chicken from different markets in the area of Barcelona (Spain) were analysed. The samples were purchased, and treated according to the optimized method.

The method developed was also applied to four chicken samples provided by "Pondex S.A." in order to quantify amoxicillin and evaluate the presence of its main metabolites AMA and DIKETO. The animals were treated with amoxicillin at 14 mg kg^{-1} in water on 4 consecutive days. Samples (A) correspond to two animals slaughtered the third day during the treatment process; while samples (B) are from two animals slaughtered 48 hours after medication was stopped.

3. RESULTS AND DISCUSSION

To develop an appropriate method for the determination of penicillins in beef, pig and chicken, we first studied several papers in the literature [Becker, Zittlau, & Petz, 2004; Benito-Peña, Partal-Rodera, León-González, & Moreno-Bondi, 2006; Blasco, Torres, & Pico, 2007; Carretero, Blasco, & Pico, 2008; De Baere, Cherlet, Baert, & De Backer, 2002; Gentili, Perret, & Marchese, 2005; Granelli, & Branzell, 2007; Granelli, Elgerud, Lundström, Ohlsson, & Sjöberg, 2009; Holstege, Puschner, Whitehead, & Galey, 2002; Hsieh, Huang, & Lee, 2009; Ito, Goto, Oka, Matsumoto, & Takeba, 2004; Kantiani, Farré, & Barceló, 2009; Kantiani, Farré, Sibum, M., Postigo, López de Alda, & Barceló, 2009; Marchetti, Schwaiger, & Schmid, 2001; Mastovska, & Lightfield, 2008; Moats, & Romanowski, 1998; Moreno-Bondi, Marazuela, Herranz, & Rodríguez, 2009; Msagati, & Nindi, 2007; Riediker, & Stadler, 2001; Samanidou, Nisyriou, & Papadoyannis, 2007; Sorensen, Snor, Elkaer, & Hansen, 1999; Stolker, & Th. Brinkman, 2005; Yamada, Kozono, Ohmori, Morimatsu, & Kitayama, 2006]. Most of the authors study only one matrix. A few papers report more than one matrix from different animals [Carretero, Blasco, & Pico, 2008; Sorensen, Snor, Elkaer, & Hansen, 1999; Yamada, Kozono, Ohmori, Morimatsu, & Kitayama, 2006], but no research into the matrix effect in these tissues was found. The method proposed by Becker et al. [Becker, Zittlau, & Petz, 2004] for the determination of β-lactams in bovine muscle and kidney was chosen as the starting point. The method was modified at various points in order to achieve similar results in all tissues and obtain a method that was fast and robust.

3.1. Chromatographic separation

Most of the previous LC methods of penicillin analysis studied used C18 silica particles as the stationary phase. A C8 stationary phase was reported in very few papers [Hsieh, Huang, & Lee, 2009; Samanidou, Nisyriou, & Papadoyannis, 2007]. Thus, the influence of stationary phase type (C18 and C8 silica particles) on penicillin separation was evaluated. In this study, two chromatographic columns of Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm) and Lichrospher RP-18 (125 × 4 mm) were evaluated for the separation of penicillins from a mixture of standard solutions. Several gradient elution conditions were evaluated with both columns. The parameters of width of the peak, resolution and

retention factors were used to select the best separation procedure. Both columns provided good separation of the penicillins, but higher intensity and narrower peaks for penicillins were obtained using the Zorbax XDB-C8 column. Thus, the Zorbax XDB-C8 column was selected for LC-MS/MS studies. Under these conditions, the run time for the separation of the penicillins obtained in LC-MS/MS was approximately 9 min.

3.2 Mass spectrometry detection

MS offers the possibility of selecting the compounds of interest and excluding the presence of interferences, particularly when complex matrices such as bovine, porcine and chicken muscle are analysed. Determination of penicillin residues in muscle was based on monitoring the ions that present the highest relative abundances (highest S/N) in the experimental conditions.

To select the ionization mode and study the product ions from the parent compounds, standard solutions of each analyte were injected at a concentration of 1 mg l^{-1}. Some authors point out that more intense signals are obtained in positive mode [Gentili, Perret, & Marchese, 2005; Kantiani, Farré, Sibum, Postigo, López de Alda, & Barceló, 2009]. Since the purpose of this study was to obtain a method for the simultaneous determination of all penicillins in several matrices, ionization in positive mode was selected.

Table 1 shows the $[M+H]^+$ ion for penicillins and the most abundant product ions. The basic structure of penicillins consists of a thiazolidinic ring condensed on a β-lactam ring, to which a lateral chain is linked [Fagerquist, Lightfield, & Lehotay, 2005]. A common fragment at m/z 160 was obtained for all the analytes, except for AMOX and NAFC. This product ion corresponds to the thiazolidinic ring [$C_6H_{10}O_2NS$]. Also characteristic is the presence of the ion formed due to the loss of this fragment [$M+H^+$-159].

Another characteristic fragment is due to the loss of the carboxylic group from the 160 fragment to obtain a fragment at m/z 114, as happened with several penicillins. For example, in the working conditions, in the case of AMOX, the fragment at m/z 160 is not observed, while the fragment at m/z 114 is found. In order to determine penicillins by LC-MS/MS, the most intense transition was used to quantify penicillins and the second most intense for confirmation. These transitions are also shown in Table 1.

As illustrative examples, the MS spectra of OXAC and NAFC are shown in Figure 1 and possible interpretation of the main fragment ions are also shown.

3.3. pH stability assay

There are some studies in the literature of stability or biodegradability of penicillins during storage [Langin, Alexy, König, & Kümmerer, 2009; Okerman, Van Hende, & De Zutter, 2007; Riediker, Rytz, & Stadler, 2004; Verdon, Fuselier, Hurtaud-Pessel, Couëdor, Cadieu, & Laurentie, 2000]. In a previous study, penicillin freeze-thaw stability, at different stocking temperatures (4 and -20^{0}C) in the presence or absence of 1% MeCN was evaluated [Martínez-Huélamo, Jiménez-Gámez, Hermo, Barrón, & Barbosa, 2009] while pH influence was not studied.

In this work, penicillin stability over time at different pH values (3 to 11) was studied. Mixed penicillin solutions at ten mg kg^{-1} were prepared at different pH values, stored at -20^{o}C and injected into an LC-UV system at λ = 220nm, each day for 5 days. This study showed that the penicillins degrade at the extreme pH: 3, 4, 10 and 11. At pH 3 and 11 no signal was obtained on the first day, and at pH 4 and 10 the signal decreased over the days. So we concluded that a good working pH interval is between pH 5 and pH 9, and thus, these values were set as conditions for our further studies. Moreover, in order to ensure the stability of the penicillins, the storage of solutions of penicillins should be at low temperature, and fresh solutions were prepared each time. The solutions were not used for more than three consecutive days.

3.4. Optimization of the SPE procedure

The optimal sorbent for any given extraction problem is dependent on the properties of the target analyte and the sample/matrix composition [Stolker, & Th. Brinkman, 2005]. In order to establish the optimum conditions for the SPE procedure we have considered the results obtained previously in the pH stability assay and the evaluation of four different SPE cartridges to clean up and preconcentrate the targets in samples: Oasis HLB, ENV+ Isolute, Bond Elut C18 and Oasis MAX.

In the literature, the majority of solid-liquid extractions of β-lactamics antibiotics in tissues are only studied at one pH (between pH 6 to 9.5) and the behaviour across this pH

range is not evaluated [Becker, Zittlau, & Petz, 2004; Benito-Peña, Partal-Rodera, León-González, & Moreno-Bondi, 2006; Feitosa, Temime, & Chiron, 2007; Riediker, Rytz, & Stadler, 2004]. In our work, we studied the solid-liquid extraction of penicillins over the range between pH 5 and pH 8.5 using the cartridges described in this section. Best activation and elution were chosen for each cartridge. Figure 2 shows the peak area obtained by LC-UV for each compound when applying the extraction methods described in sections 2.4.1 and 2.4.2 to the chicken muscle matrix using the four cartridges. The figure shows that ENV+ Isolute cartridge, working at pH=5,0 yield the best results for most part of the penicillins studied. Only AMOX, AMPI and PENG present better results using Bond Elut C18. The same study in beef, also led to the conclusion that the best recoveries are obtained with ENV+ Isolute for all substances except for DICL, which yields similar results using ENV+ Isolute, Oasis HLB and Bond Elut C18. In pig muscle, similar results were obtained using Bond Elut C18 and ENV+ Isolute, except for AMPI and PENG, which present recoveries around 20% higher with Bond Elut C18. From these results for the target penicillins, and in order to select a unified method for the three matrices, the ENV+ Isolute cartridge was selected for subsequent studies.

In order to reduce the sample evaporation time, two kinds of penicillin elution solutions were studied. The first solution consisted of a mixture of MeCN:MeOH:H_2O (3:4:3, v:v:v). Different volumes (2-4 ml) were added for the first method. The second one consisted of a mixture of 2 ml MeOH followed by 2 ml MeCN. In this study, the addition of 4 ml MeCN:MeOH:H_2O (3:4:3, v:v:v) gives a better recovery for AMPI and OXAC. For the rest of the penicillins, better recoveries (more than 10%) were obtained when the second elution solution was used. In consequence, 2 ml MeOH followed by 2 ml MeCN was used for further studies, since it has a shorter evaporation time.

We checked the influence of the air stream in the evaporation step on the stability of the penicillins. Standards of penicillins were evaporated using both air and nitrogen stream. Only the AMPI and AMOX peaks were slightly lower using air instead of nitrogen. Similar results were obtained for both air and nitrogen evaporation in all three tissues matrices. We think that the presence of the matrix stops the slight degradation/oxidation of penicillins, and on this basis, we chose nitrogen evaporation in order to avoid possible degradation/oxidation in some conditions.

3.5. Quality parameters

The optimized method of extraction was validated for the penicillins regulated in bovine, porcine and chicken muscles, according to the European Union Regulation 2002/657/EC and including some important parameters from the FDA guideline [Official J. European Communities No. 2002/657/EC, 2002; US Department of Health and Human Services, 2001], using LC-MS/MS.

3.5.1. Calibration curves

In this study, the tandem mass spectrometry calibration curves for all the penicillins were determined from the LOQ to 2MRL in spiked tissue samples of beef, pig and chicken, subject to the treatment samples at the concentration given in section 2.4.4. Table 2 shows showed the calibration curve equations and the corresponding regression coefficients for the three tissues.

In order to determine whether there is a matrix effect in these tissues, the slopes for each penicillin in the different animal species were compared.

Three different types of behaviours can be observed, depending on the analyte: in the first case, the slopes for all three tissues show no significant differences, as for CLOX, OXAC and PENV. Meanwhile, for AMOX, AMPI, DICL and NAFC the slope of the calibration curves for pig and beef are similar but different from that for chicken. Only one substance, PENG, shows different slopes for all three tissues. In order to evaluate the significance of this behaviour, the data were evaluated by one-way ANOVA (with replicates) at a 5% significance level. The slopes of the three calibration curves for each compound in the three different matrices were analysed. A factor is statistically significant when its F-values are greater than F-critical. The results of the statistical analysis show no statistically significant difference between the calibration curves of the penicillins studied in beef, pig and chicken, because the F-values (lower than 2,76) <F-critical (5,14). For PENG, the statistical study indicates that the differences are not statistically significant and because of this, we can declare that all the penicillins present the same behaviour independently of the matrix analysed.

3.5.2. LOD and LOQ

Considering an S/N ratio of 10, by LC–MS/MS methods, the ranges of the LOQ obtained were: 0,2 to 1,25 μg kg^{-1} for beef; 1 to 8 μg kg^{-1} for pig; and 0,3 to 3 μg kg^{-1} for chicken. In order to demonstrate the sensitivity and specificity of the method at low concentrations, a chromatogram of the beef samples spiked at the 0.05 MRL level is shown in Figure 3. Values of LOQ are shown in Table 2 for each substance in each tissue. The values obtained in pig tissues are higher than those obtained in beef and chicken, which may be because more dirty samples were obtained in the pig tissues. The table also shows the LOD values obtained.

All LOQs obtained in the different tissues were lower than the MRLs established in Commission Regulation 37/2010 of the European Union [Commission Regulation (EU) No 37/2010, 2010].

3.5.3. Recovery

Recoveries were calculated by comparing the analytical results of the extracted samples with the matrix spiked after the extraction procedure, which represents 100% recovery.

All the penicillins have recoveries higher than 70%, except for AMOX, which has recoveries of around 50%. The recovery rates are similar in all the tissues analyzed, with slightly higher values in bovine, muscle in some cases (PENG and PENV), as can be observed in Table 2. In general, the recoveries obtained for pig are lower than those for the other matrices.

3.5.4. Inter-day and intra-day studies

Three concentration levels (0.5 MRL, 1.0 MRL and 2.0 MRL) were evaluated by repeatability and reproducibility to assess the precision of the method. Five spiked tissue samples at each level were prepared and analyzed (intra-day precision) and this procedure was repeated on 3 days in order to determine the inter-day precision. The precision results are shown in Table 2, and values lower than 12% were obtained in all cases.

3.5.5. Decision limit (CCα) and detection capability (CCβ)

In order to establish the CCα parameter, 20 samples of each matrix (beef, pig and chicken) were spiked at the corresponding MRL: 50 µg kg^{-1} for AMOX, AMPI and PENG, 300 µg kg^{-1} for CLOX, DICL, NAFC and OXAC, and 25 µg kg^{-1} for PENV. Although PENV is not regulated in beef, samples were spiked at the MRL regulated in the other matrices. The case of NAFC is similar as it is only regulated in beef. In this case, samples of pig and chicken were spiked at 300 µg kg^{-1} (the MRL corresponding to beef). The data obtained were evaluated in order to obtain the RSD of the concentrations found. CCα values were determined, for each penicillin, as the concentration at the MRL level plus 1.64 times the standard deviation at the MRL level. CCβ values were calculated as the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility. Values of CCα and CCβ are shown in Table 2.

3.6. Analysis of tissues samples

After analysing five samples of beef, pig and chicken from different markets by LC-MS/MS, the absence of background peaks coinciding with the corresponding transitions of the target of penicillins showed that the samples did not contain any penicillins. We only observed the IS peak, which we added in order to quantify the penicillins in case of they were present.

3.7. Application to treated chicken samples

The two kinds of samples from animals medicated orally with AMOX were treated and analyzed by LC-MS/MS. In the analysis corresponding to samples (A), from animals slaughtered during the treatment, peaks appear that correspond to AMOX and possibly, its metabolite AMA. Figure 4A shows the chromatogram obtained in the analysis of these samples. The concentrations of AMOX calculated in the two samples were 14 µg kg^{-1} (0.1 R.S.D.) for specimen 1 and 10 µg kg^{-1} (5 R.S.D.) for specimen 2.

In the analysis of samples (B), from animals slaughtered when the treatment had finished, as well as the peak corresponding to AMOX and AMA, the transition corresponding to DIKETO at the retention time of 4.9 min also appears, as can be observed in Figure 4B.

The AMOX concentration in these samples is lower: 6 µg kg^{-1} for both specimens, possibly because of the transformation into its two metabolites, AMA and DIKETO. However, AMA and DIKETO cannot be unequivocally confirmed and quantified because of the lack of more points of identification caused by the absence of commercial reference standards of these metabolites. The concentration of AMOX in the real samples is lower than CCα, established in the validation method and consequently consumer health is ensured.

CONCLUSIONS

A unified method has been developed to determine the penicillins regulated by Commission Regulation 37/2010 below the MRL values in bovine, porcine and chicken muscle.

Four different sorbents, Oasis HLB, Oasis MAX, ENV+ Isolute and Bond Elut C18, were compared for the preconcentration and clean-up of these antibiotics in tissues samples. The best results were obtained with the ENV+ Isolute sorbent. This method allows obtaining a high extraction index and suitability quality parameters for all compounds in all matrices.

From the statistical study of the slopes of the calibration curves for each penicillin in the different matrices, we conclude that similar behaviour is observed for the penicillins and similar matrix effects are observed in all the matrices studied. When applied to biological samples from animals treated with AMOX, the method presents good results for the identification and quantification of the molecular parent. However, because of the low stability of AMOX more studies are needed to be made with techniques that allow to establish unequivocally degradation compounds or metabolites of this substance, in order to obtain better results in terms of recovery.

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FIGURE CAPTIONS

Figure 1. Mass spectra in product ion scan mode of m/z 402 of OXAC (A) and m/z 415 of NAFC (B), obtained by direct infusion of each penicillin at a 1 mg l^{-1} in ESI+. The proposed fragmentation pathways are also included.

Figure 2. Comparison of the results obtained with different sorbents for the SPE of penicillins in chicken muscle. Samples analysed by LC-UV, λ=220nm.

Figure 3. Chromatogram of beef muscle spiked at a concentration of 0.05 MRL and obtained by LC-MS/MS.

Figure 4. Ion reconstituted chromatogram obtained for the analysis of medicated chicken muscle samples. A) Animals slaughtered at the third day during the medical treatment. B) Animals slaughtered 48 hours later medication took away.

Table 1. Mass spectrometry parameters for each compound.

Penicillin	Optimized parameters[a]							
	DP[b] (V)	FP[c] (V)	EP[d] (V)	Molecular ion(m/z)	CE[e] (V)	Fragmented ions (m/z)	Quantification transition	Identification transition
AMOX	40	150	6	366	13	349	366→114	366→208
					19	208		
					28	114		
AMPI	65	150	6	350	21	192	350→106	350→192
					22	174		
					17	160		
					40	114		
					26	106		
PENG	65	220	7	335	16	176	335→160	335→176
					16	160		
					45	114		
PENV	40	150	7	351	16	192	351→160	351→192
					17	160		
OXAC	40	160	9	402	13	361	402→160	402→243
					18	243		
					18	160		
CLOX	40	140	7	436	18	395	436→160	436→277
					20	277		
					20	160		
NAFC	50	120	9	415	21	256	415→199	415→256
					19	199		
					44	171		
DICL	50	150	8	470	22	342	470→160	470→311
					22	311		
					32	203		
					21	160		
AMA	25	150	8	384	15	323	384→323	-
					20	189	384→189	-
DIKETO	25	150	8	366	15	160	366→160	-
PIPE(IS)	40	150	9	518	10	500	518→143	518→160
					26	346		
					16	160		
					27	143		

[a] Experiments performed using a collision-activated dissociation (CAD) at 4; [b]Declustering potential; [c]Focusing potential; [d]Entrance potential; [e]Collision energy.

Table 2. Quality parameters obtained for penicillins in beef, pig and chicken tissues.

	AMOX	AMPI	PENG	PENV	OXAC	CLOX	NAFC	DICL
BEEF								
Calibration Curve[a]	y=0.182x-0.0023 (r=0.974)	y=2.2x-0.00473 (r=0.938)	y=3.26x-0.165 (r=0.975)	y=2.34x-0.0159 (r=0.981)	y=1.4x-0.123 (r=977)	y=1.05x-0.0873 (r=0.984)	y=5.96x+1 (r=0986)	y=1.1x-0.115 (r=0.970)
LOD (µg/kg)	<0.5	<0.1	<0.1	<0.1	<0.05	<0.05	<0.05	<0.05
LOQ (µg/kg)	1.25	0.3	0.3	0.3	0.2	0.2	0.2	0.2
Recovery (%)	56	76	93	100	94	91	101	92
Intra day[b] (%, n=15)	1-10	4-8	2 -5	3 - 6	3- 5	3 -6	5 - 7	1 - 4
Inter day[b] (%, n=45)	8-11	7-11	7-11	9-12	9-10	7-8	7-12	6-9
CCα(µg/kg)	62	58	57	26	311	321	309	313
CCβ(µg/kg)	73	66	64	28	323	342	317	326
PIG								
Calibration Curve[a]	y=0.189x-0.000896 (r=0.952)	y=2.26x-0.0656 (r=0.965)	y=5.1x-0.194 (r=0.952)	y=2.62x-0.0116 (r=0.961)	y=1.44x+0.0517 (r=0.946)	y=1x+0.0187 (r=0.944)	y=5.96x+1.85 (r=0.918)	y=0.974x-0.00623 (r=0.938)
LOD (µg/kg)	<0.25	<2.5	<2.5	<1.5	<1.5	<1	<0.5	<1.5
LOQ (µg/kg)	1	8	8	5	5	4	2	5
Recovery (%)	54	70	78	77	91	89	94	84
Intra day[b] (%, n=15)	3 – 7	3 – 4	2 – 6	5 – 12	1 – 1	1 – 1	1 – 2	1 – 2
Inter day[b] (%, n=45)	4 – 9	3 – 5	3 – 10	5 – 12	1 – 2	1 – 2	1 – 3	1 – 3
CCα(µg/kg)	54	56	57	33	308	307	307	310
CCβ(µg/kg)	61	64	69	41	319	317	318	322
CHICKEN								
Calibration Curve[a]	y=0.0781x-0.000223 (r=0.938)	y=1.43x-0.0453 (r=0.991)	y=2.05-0.00595 (r=0.996)	y=2.66-0.0171 (r=0.997)	y=1.35x+.0886 (r=0.996)	y=0.892x+0.0146 (r=0.995)	y=8.85x+0.33 (r=0.994)	y=0.69x+0.0473 (r=0.997)
LOD (µg/kg)	<1	<0.1	<0.1	<0.1	<0.2	<0.2	<0.2	<0.2
LOQ (µg/kg)	3	0.3	0.3	0.3	0.5	0.5	0.5	0.5
Recovery (%)	50	79	83	90	94	92	101	87
Intra day[b] (%, n=15)	2-4	3-4	2-4	2-6	1-3	1-4	1-2	3-4
Inter day[b] (%, n=45)	8-12	5-6	4-12	8-9	4-6	8-10	7-11	7-10
CCα(µg/kg)	61	64	65	40	310	315	311	313
CCβ(µg/kg)	73	78	79	54	321	330	323	326

a) y=penicillin area/IS area, x=penicillin concentration/IS concentration.

b) The intra-day and inter-day precision data corresponding to the minimum and maximum RSD (%) values obtained in the analysis of the samples (prepared at 0.5, 1 and 2 MRL levels).

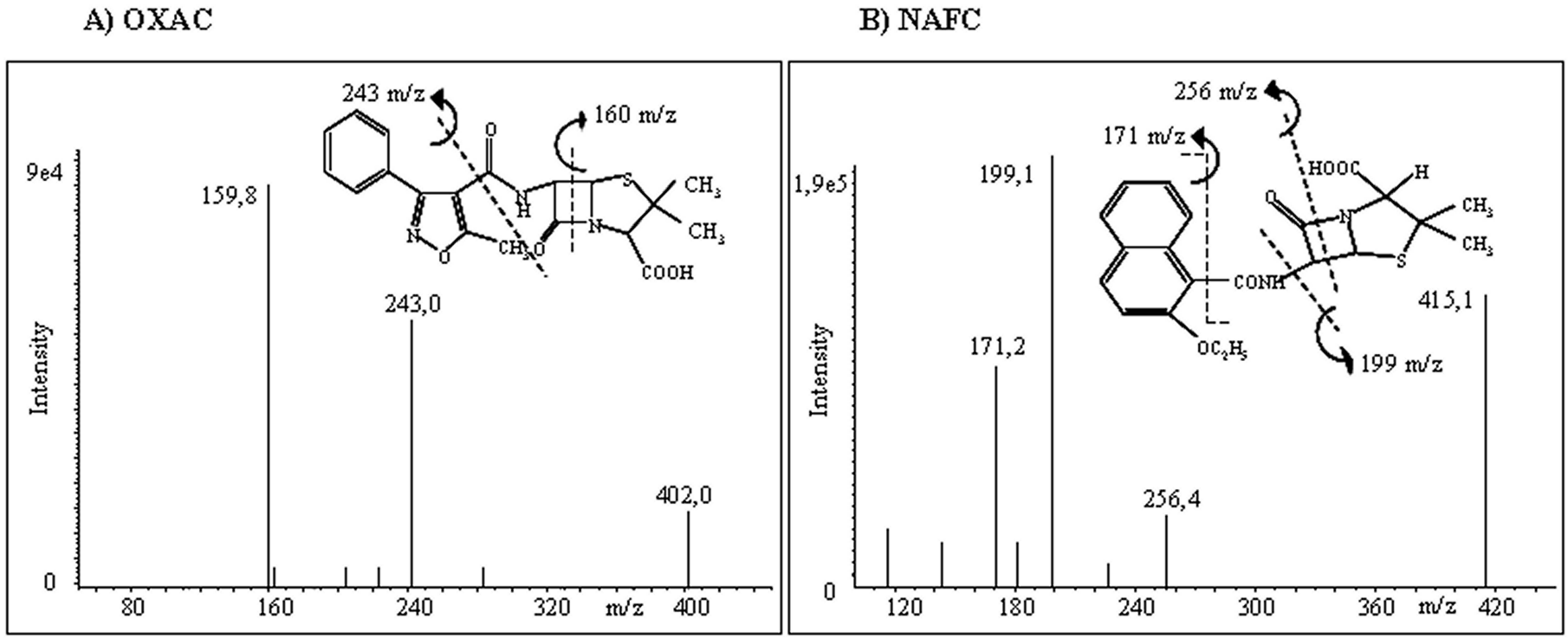
A) OXAC
B) NAFC
243 m/z
160 m/z
9e4
159,8
243,0
402,0
Intensity
0
80
160
240
320
m/z
400
256 m/z
171 m/z
199,1
1,9e5
171,2
256,4
415,1
199 m/z
120
180
240
300
360
420

Figure 1

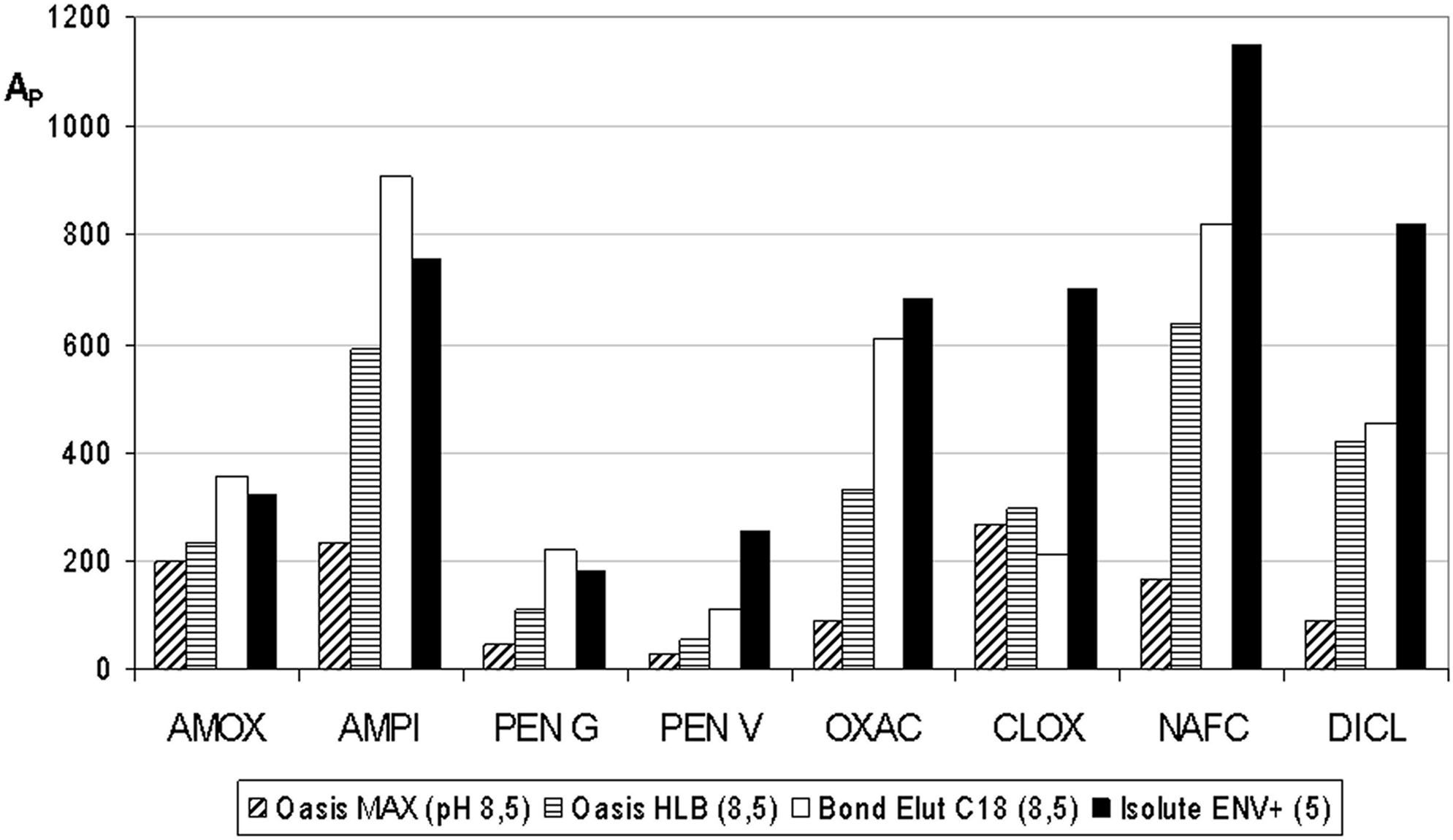
1200
1000
800
600
400
200
0
Ap
AMOX
AMPI
PEN G
PEN V
OXAC
CLOX
NAFC
DICL
Oasis MAX (pH 8,5)
Oasis HLB (8,5)
Bond Elut C18 (8,5)
Isolute ENV+ (5)

Figure 2

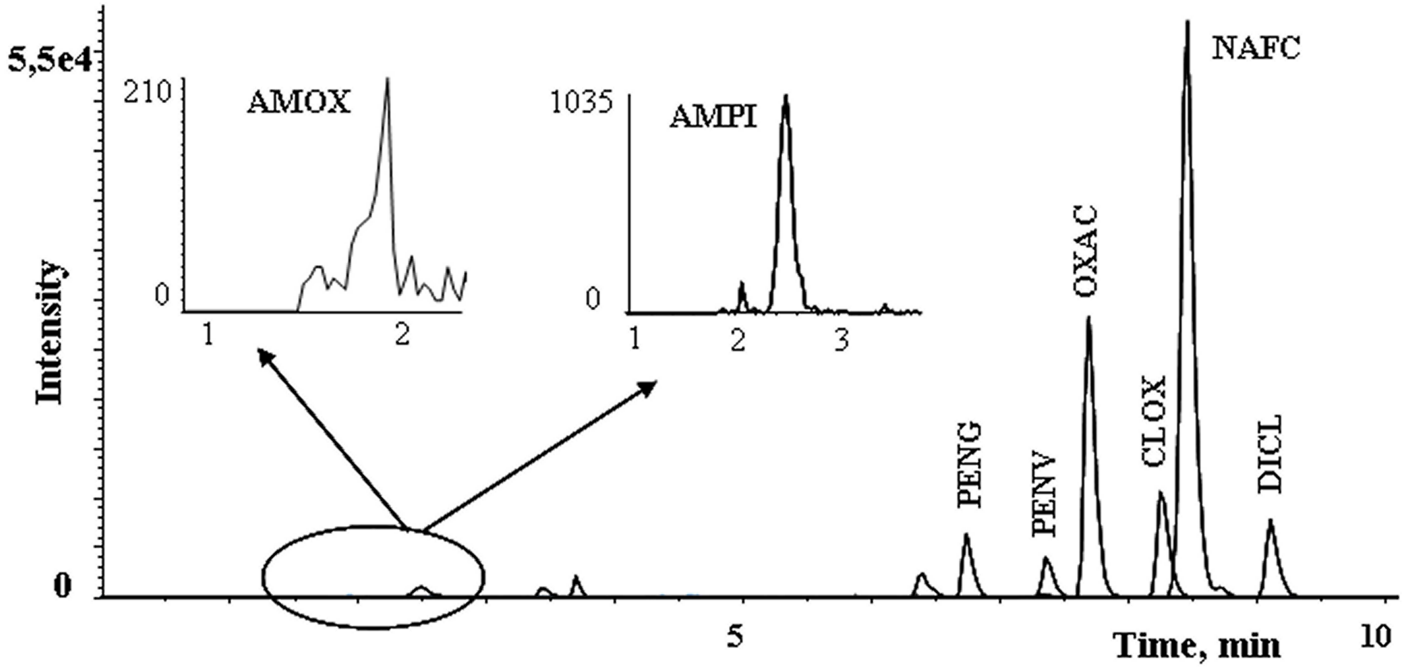
5,5e4
Intensity
0
210
AMOX
0
1
2
1035
AMPI
0
1
2
3
PENG
PENV
OXAC
CLOX
NAFC
DICL
5
Time, min
10

Figure 3

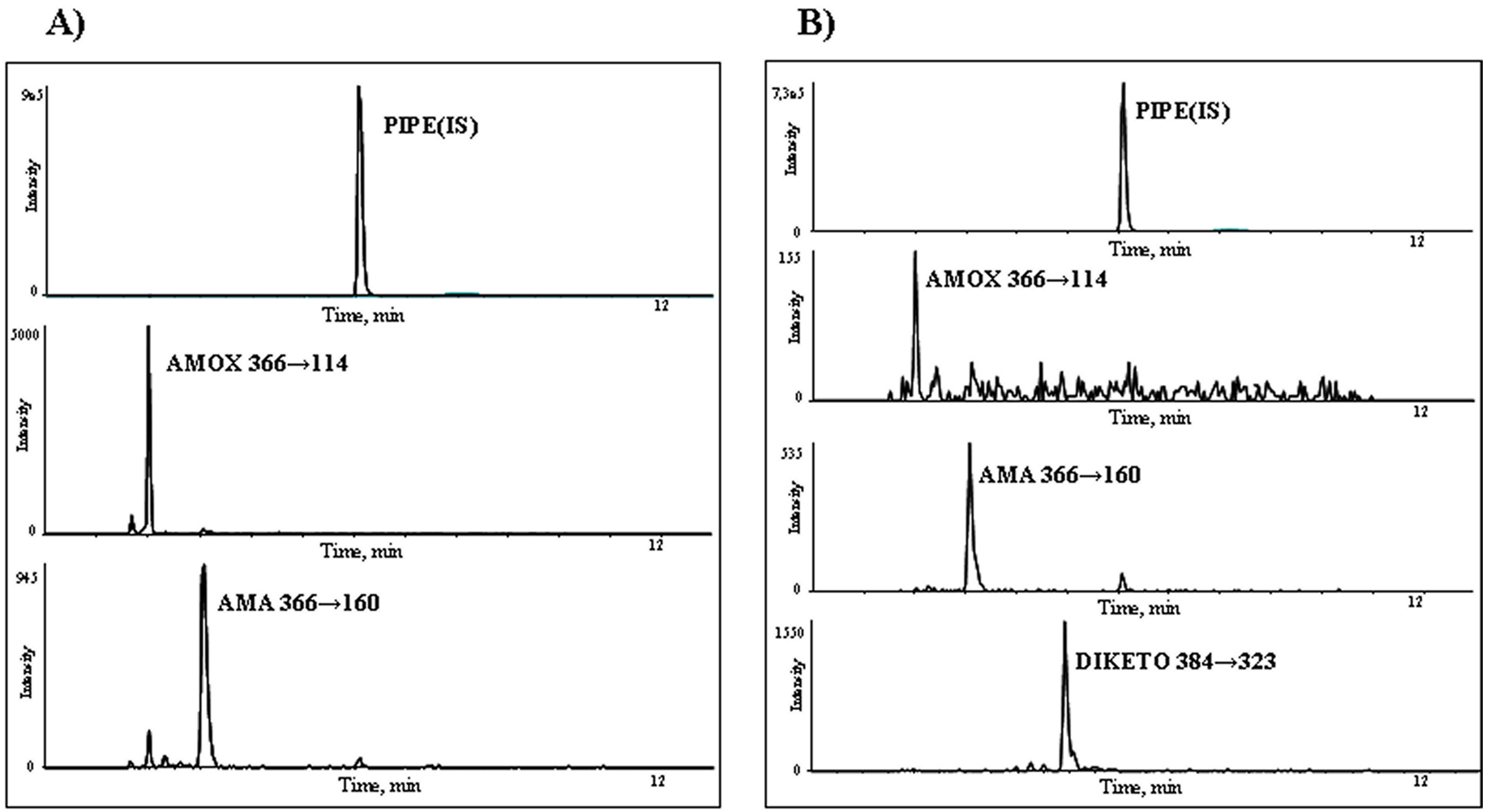
A)
B)
PIPE(IS)
AMOX 366→114
AMA 366→160
DIKETO 384→323
Intensity
Time, min
12
0

Figure 4