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Development and validation of a fast procedure to analyze amoxicillin in river waters by direct injection - LC-MS/MS

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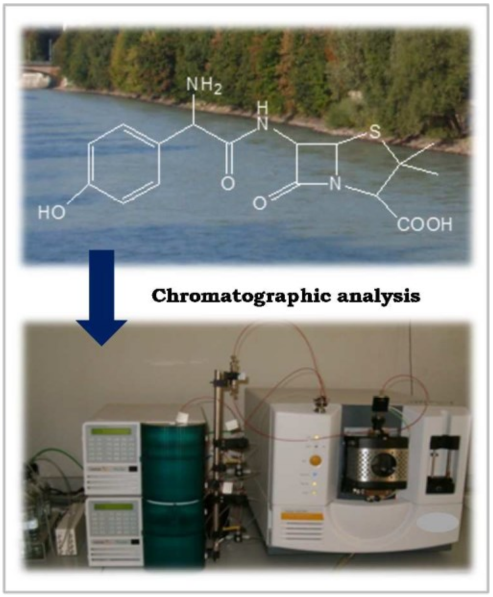
ABSTRACT

A laboratory application with a strong component in analytical chemistry was designed for undergraduate students, in order to introduce a current problem in the environmental science field, the water contamination by antibiotics. Therefore, a simple and rapid method based on direct-injection and high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and optimized for the determination of amoxicillin in river water.

Students learned the main optimization steps for the improvement of the LC-MS/MS methodology and the correct procedure to validate an analytical method (draw a calibration curve, determine detection and quantification limits, precision and accuracy).

This laboratory experiment was successfully applied by students and enables the analysis of a large number of samples in a short period of time, due to the short run time (3 minutes).

ABSTRACT GRAPHIC



KEYWORDS

Second-Year Undergraduate, Upper-Division Undergraduate, Analytical Chemistry, Environmental Chemistry, Laboratory Instruction, Hands-On Learning/Manipulatives, Chromatography, Drugs / Pharmaceuticals, Mass Spectrometry

Amoxicillin is a broad-spectrum β-lactam antibiotic that belongs to penicillin class and is used in veterinary and human medicine, representing one of the most prescribed antibiotics in Europe and in the United States.[1-3] When ingested, 80 to 90% of this antibiotic is excreted unmodified[4] via urine and faeces into the domestic sewage and subsequently discharged to wastewater treatment plants (WWTPs).[4-6] Because most WWTPs are not designed to remove antibiotics, amoxicillin is released into the environment.[7-9] This situation became an emerging issue because the antibiotics can act as persistent and bioaccumulative contaminants, inducing toxic effects in aquatic or terrestrial ecosystems, even in low concentrations levels (in the range of μg-ng.L^{-1}). Besides that, they can produce resistance in microbiological lineages, causing serious problems of public health, namely difficulties in treating diseases and imbalance of microbial ecosystems.[3,6,10,11] Recently, the toxic effects of amoxicillin toward algae and aquatic microorganisms were reported.[12,13]

Amoxicillin has been detected in several environmental matrices since the mid-1990s, when new analytical methods such as liquid chromatography tandem mass spectrometry were developed.[8,9] The LC-MS is an emergent technique with very high sensitivity and selectivity, but that is absent from most undergraduate curricula.[14,15] Therefore, it is essential that undergraduate students learn about this powerful method that allows the analysis of trace environmental contaminants in complex matrices.

The main objective of this laboratory experiment is to provide undergraduate students the opportunity to learn the fundamentals of LC–MS and some analytical principles, employing this technique for the analysis of amoxicillin in water samples. This experiment may be performed in a single 3-h laboratory period (only performing the method validation) or two consecutive 3-h laboratory periods (also learning the LC-MS optimization process) depending upon the complexity level chosen by the instructor. In the end of the experiments the students should be able to:

- understand the fundamentals of LC-MS
- understand the differences between different type of mass analyzers
- operate with a specific equipment, based on ion trap detection - method optimization
- perform a simple validation scheme for a trace analysis
- calculate the validation parameters from obtained data (linearity, limits of detection and quantification, precision and accuracy).

PEDAGOGICAL CONSIDERATIONS

This work was planned for undergraduate students and is ideal for an analytical chemistry lab experiment. In fact, this procedure was tested twice (2012 and 2013) with second-year chemistry engineering and bioengineering students, but may also be conducted by undergraduate students from related areas (chemistry, environmental engineering, pharmaceutical sciences, etc.) or can be adapted for practical classes of LC-MS/MS designed to analysts and laboratory technicians (is this case, the instrumental analysis should be coupled with a sample extraction technique). A total of thirty one students participated in this experiment (15 students in 2012 and 16 students in 2013).

In the discipline of analytical chemistry, in which this work is carried out, the laboratory projects (divided in two lab sessions of three hours) are performed on a rotation system by groups of two students.

Students were familiar with the fundamentals of LC-MS/MS and validation of analytical methodologies, since these concepts were obtained in the corresponding lecture course. For that reason, no pre-lab lecture was given and students were expected to understand the lab work. However, some additional information (Background topic) was provided and before the first session, the instructor gave an explanation about LC-MS operation issues. Before starting the experimental session, students were asked to do a brief description of the work they would perform and doubts were clarified.

As mentioned before, the experiment was performed over the course of two three-hour lab periods. On session 1, students received a brief instructor's explanation of the LC-MS operation (20 min) and performed the LC-MS optimization (2h40). Students had adequate time to perform this optimization procedure (capillary voltage, needle voltage, RF loading %, excitation amplitude/CID, drying gas pressure and temperature, nebulizing gas pressure).

The method validation was performed on session 2. One group member prepared the six-calibration standards (according pre-lab calculations), while the other prepared the spiked samples. Students usually need about 30 min to complete this task. Since in the beginning of this session, mobile phase should be running in LC-MS equipment, the injection of standards and samples will be performed in about 1h30. During the waiting time between injections, students should start preparing their worksheet using Microsoft Excel. In the rest of the of the lab session, students should construct the calibration curve, calculate the LOD and LOQ based on signal-to-noise ratios, evaluate the standards and sample precision (repeatability), evaluate the accuracy and determine the concentration of amoxicillin in the real sample.

At the end of the protocol, students will find some questions that will help in the discussion of the obtained results. They should look for responses in complementary bibliography, leaving after work the issues exclusively related to the experimental results. At the end of the two-period sessions, students will present and discuss the results with the instructor, presenting their report in the following lab session.

In the end of this experiment students should be able to:

- understand the fundamentals of LC-MS
- understand the differences between ion trap, quadrupole and time-of-flight mass analyzers
- operate with a specific equipment, based on ion trap detection, and learn the main optimization steps of this equipment (as an example)
- perform a simple validation scheme for trace analysis
- calculate the validation parameters from obtained data (linearity, limits of detection and quantification, precision and accuracy).

EXPERIMENTAL OVERVIEW

Reagents and materials

For these experiments, amoxicillin (≥ 900 µg per mg) was used as well as methanol and water (LC-MS grade) and formic acid (89-91%, p.a.). Standards were filtered through 0.20 µm PTFE syringe filters and mobile phases through 0.45 µm nylon filter membranes.

Standards preparation

An aqueous stock solution of 50 mg.L^{-1} of amoxicillin (AMOX) was prepared and from this, standards of 5 mg.L^{-1} (intermediate standard solution) and 1 mg.L^{-1} (optimization standard). Calibration standards were prepared in water for chromatography at concentrations between 1 and 250 µg.L^{-1} from the intermediate standard. All the solutions were filtered through 0.20 µm PTFE syringes filters and stored at 4 °C.

Instrumentation

Chromatographic analyses were performed using a LC-MS system constituted by a Binary Solvent Delivery Module and an Ion Trap Mass Spectrometer equipped with an electrospray ionization source (ESI). Data was acquired and processed by MS Workstation software.

A C18 column (50 mm x 2 mm i.d., particle size: 5 µm), in combination with a guard column C18 (10 mm x 2.0 mm i.d., particle size: 5 µm) were used. The mobile phase was composed of methanol (70%) and 0.01% v/v formic acid (pH = 2.0) in water (30%), running in isocratic conditions at a flow rate of 0.2 mL.min^{-1}. At this pH value, it is expected that the amoxicillin molecules are in a protonated form (Figure S1 and Figure S2). The injection volume and the run time were 10 µL and 3 minutes, respectively. The analyses were done in the positive ion mode, using the following conditions: multiplier offset – 300 V, shield voltage – 600 V and damping gas – 0.8 mL.min^{-1}. Other relevant MS conditions (such as drying gas pressure and temperature, nebulizing gas pressure, needle and capillary voltage, RF loading and the excitation amplitude) were optimized by students during the laboratory experiment.

Sampling

River waters were collected from the river Cávado (sampling site: Albufeira do Alto do Cávado or Braga) in April 2009. The samples were stored in amber glass bottles. Once in the laboratory, the samples were filtered through 0.45 µm nylon filter membranes before each analysis. All samples were kept at –20 °C and protected from light until they were processed by the students.

PRELAB PREPARATIONS

The stock and intermediate solutions and the optimization standards were prepared by the instructor prior to the start of the lab session. The mobile phase should already be degassed, the LC-MS purged and the mobile phase running in isocratic conditions.

STUDENT PROCEDURE

The method development in liquid chromatographic systems coupled to mass spectrometry is not always a simple task, since there are a significant number of parameters that may influence the final results. The development of a methodology for LC-MS/MS consists essentially in three steps (Figure 1): mass, ion source and chromatographic optimization. Initially, students learned how to perform this procedure.

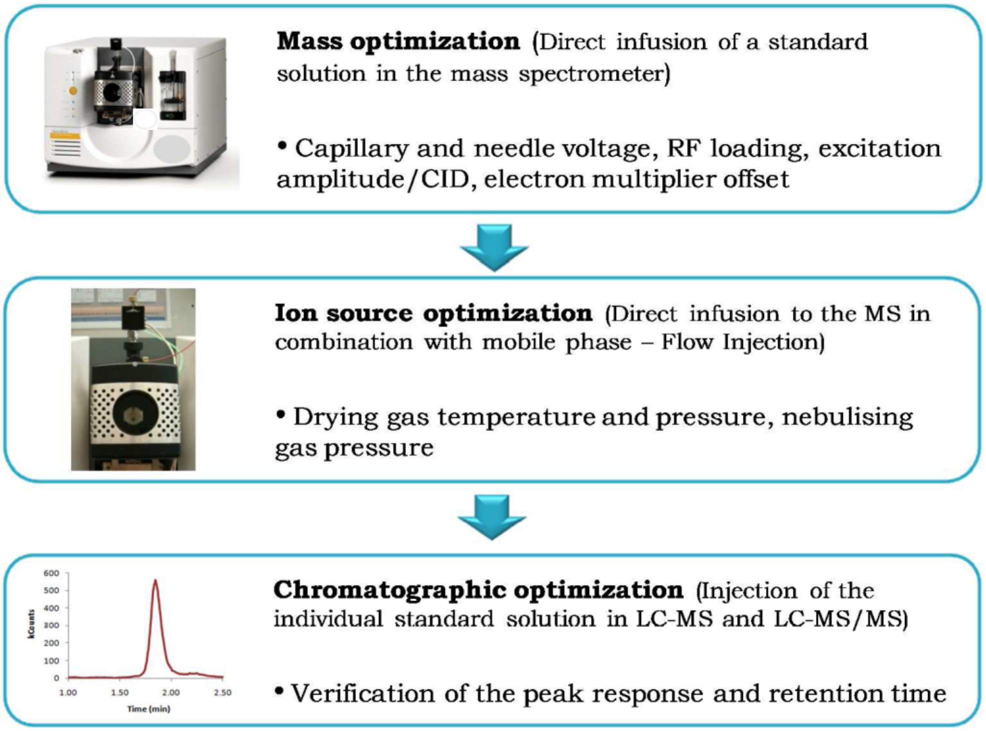


Figure 1. Main steps in the LC-MS/MS method optimization.

After that, students prepared their calibration standards (by diluting the previously prepared intermediate solution of 5.0 mg. L^{-1}) and filtered them with 0.20 µm PTFE syringes filters. Similarly, real samples were spiked (1 and 250 µg.L^{-1}) with the same solution in order to assess the accuracy of the analytical method. All the solutions were injected in LC-MS/MS and the resulting chromatograms were processed to obtain peak areas and retention times. The peak areas were used to perform the instrument calibration.

HAZARDS

There are no significant hazards involved in this experiment. Methanol is a flammable solvent and may be hazardous by ingestion, inhalation or absorption through the skin. It is also an irritant substance. Formic acid has low toxicity, but it is flammable and corrosive. Amoxicillin is a non-toxic and non-flammable compound, but can be harmful if swallowed. Good laboratory practices must be followed, i.e. students should wear gloves, labcoat and protective eyewear. All solvents and standards should be handled in fumehood. The generated waste should be selectively collected in labeled flasks and then, sent to a certified treatment company.

RESULTS AND DISCUSSION

During the first laboratory period, students optimized the MS/MS parameters. The main results are presented in Table 1.

Table 1. Student-Optimized MS/MS Parameters for Amoxicillin Antibiotic

Capillary Voltage/V	*Needle Voltage*^{a}/V	*RF Loading*/%	*Excitation Amplitude*/V	*Drying Gas Temperature*/ºC	*Drying Gas Pressure*/psi	*Nebulizing Gas Pressure*/psi	*Precursor Ion*/(*m*/*z*)	*Product Ion*/(*m*/*z*)
44	3920	90	1.26	250	40	60	366	349, 208

After the MS optimization, students performed the quantification of amoxicillin in LC-MS/MS through the external standard method. Under acidic conditions, the amoxicillin mass spectrum in the positive MS mode was dominated by an abundant $[M+H]^+$ ion. This result indicated that amoxicillin can be protonated with high efficiency, when the mobile phase contains protic solvent and small amounts of formic acid. Thus, the precursor ion was $[M+H]^+$ at m/z 366. Its fragmentation (MS/MS) generated mainly two product ions at m/z 349 (major product) and m/z 208 (Figure 2). The first one resulted from ammonia loss ($[M+H-NH_3]^+$), and the other could be assigned to the opening and cleavage of β-lactam ring with the loss of $C_6H_9NO_2S$ ($[M+H-C_6H_9NO_2S]^+$). These fragmentation ions were also referred by Nägele and Moritz.[16] The most abundant fragment, m/z 349, was used as quantification ion, while m/z 208 as qualification ion.

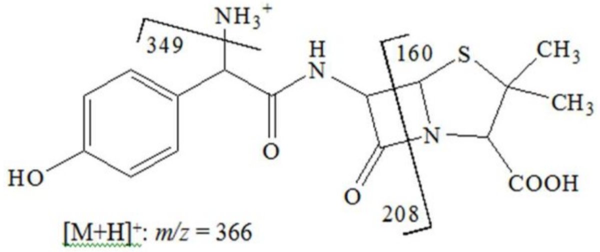


Figure 2. LC-MS/MS fragmentation of amoxicillin molecule.

In the validation procedure, linearity, precision and accuracy were considered. The amoxicillin standards prepared in chromatographic water were injected in LC-MS/MS and it was observed a good linearity range between 1 and 250 $\mu g.L^{-1}$. In Figure 3, the relationship between the detector response and the concentration level of the calibration standards was represented.

The limits of detection and quantification were calculated by the signal-to-noise ratio of 3 and 10 and were 0.6 and 2 $\mu g.L^{-1}$, respectively.

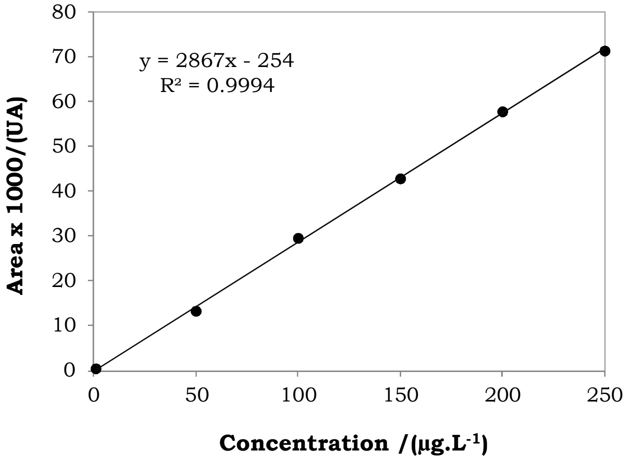


Figure 3. Students-generated calibration curve of amoxicillin by LC-MS/MS.

In this lab experiment, the students evaluated the precision by repeatability (four repeated analyses of the same sample, in the same operational conditions and over a short period of time), at two different levels of concentration (1 and 250 μg.L^{-1}). The coefficients of variation for each concentration level are shown in Table 2 (results are from just one group of students; the variability when different groups of students repeated the experiment was lower than 15%). The precision ranged from 2.9 to 4.7%. As expected, the results indicate that for higher concentration levels the method is more precise.

Accuracy was expressed through analytical recovery experiments (the observed value divided by the expected value). This parameter was evaluated using spiked samples (river water).Two recovery assays were performed for two different concentration levels, 1 μg.L^{-1} and 250 μg.L^{-1}. The results, displayed as average recoveries, are shown in Table 2.For river waters, the recoveries were higher than 66% and for that reason, direct injection could be used for quantification. Replicate measurements showed an average variance of 3%.

Table 2. Precision and accuracy results for amoxicillin analysis by LC-MS/MS

Concentration Levels/(μg.L^{-1})	*Precision* /(%CV)	*Accuracy* /(%Recovery ± RSD)
1	4.7	68 ± 2
250	2.9	87 ± 4

Amoxicillin was detected in one of the river water sample analysed at 24 μg.L^{-1}. This concentration is similar to those recently reported for surface waters.[17-19]

CONCLUSION

This experiment provides students with the opportunity for hands-on experience with an important analytical instrument (LC-MS) for the analysis of a novel class of organic contaminants. Therefore, a rapid and sensitive liquid chromatography-tandem mass spectrometry method was developed and optimized for amoxicillin analysis in river water matrices. This methodology allowed the analysis of a large number of samples in a short period of time and could be also applied in wastewater effluents monitoring. Students have the opportunity to learn the procedure to optimize the LC-MS/MS methodology based on an ion-trap detection and also to validate an analytical method.

ASSOCIATED CONTENT

Supporting Information

Instructor notes and student instructions. This material is available via the Internet at *http://pubs.acs.org.*

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Notes

The authors declare no competing final interest.

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