SIMULTANEOUS DETECTION OF TETRACYCLINE AND ITS DEGRADATION BYPRODUCTS USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY WITH DUAL IONIZATION SOURCE

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ABSTRACT: *An accurate and reliable method of measurement is essential to the monitoring of pollutants such as antibiotics in the environment. Antibiotics are considered one of the serious problems since they pose high risk on human health by developing possible antimicrobial resistant pathogenic microorganisms. Tetracycline (TC) is one of the most commonly detected antibiotics in the surface water. This study developed a method of detection and measurement of TC using liquid chromatography-mass spectrometry (LCMS) technique. The general factorial design of experiment was used to compare the signal-to-noise (S/N) ratio of different conditions using the following factors: column characteristics, ionization mode, organic phase, and aqueous phase. The liquid chromatography operation was similar for all conditions. Results show that the 1.9-µm C18 column performed better and faster (1.08 min), which provides higher productivity. This can be due to particle size in the column that provides larger surface but requires higher pump pressure. The best chromatography was attained using a mobile phase composition of 50-50 split of methanol as organic phase and 5 mM oxalic acid solution as aqueous phase flowing at 0.2 mL/min injected with 2 µL of sample. ESI and DUIS modes were almost similar in providing good signals but the use of DUIS has the advantage of detecting a wide rage of compounds and derivatives, in terms of polarity. The method was applied to detect and measure the relative abundance of TC and its byproducts during its photocatalytic degradation.*

Keywords: tetracycline, factorial design, liquid chromatography, mass spectrometry, dual ionization source.

1. INTRODUCTION

Monitoring of antibiotics in the environment is essential to prevent development of antimicrobial resistance (AMR) of microorganism. There are studies being conducted to determine the fate of the antibiotics from the point of use until its accumulation in the receiving environment. Tetracycline (TC) and its derivatives are among the most commonly used antimicrobials worldwide. They are administered to both human and veterinary medication primarily for fighting infections. It is estimated that the uptake of TC in the human body is between 60% to 80% and only about 20% to 50% in the digestive tracts of animals [1,2]. Unabsorbed TC is excreted by the body and eventually ends up in sewage and surface waters. Hence, these drugs are one of the most commonly detected antibiotics in the aquatic environment.

The molecular structure and the different pKa values of the functional groups of TC (MW=444.435) are shown in Fig. 1. These functional groups can exist as cation, zwitterion or anion species, depending on the pH of the solution [3]. Since the pH is a measure of the concentration of H^+ in solution, TC protonation is expected to be favored at lower pH while deprotonation happens at higher pH, forming an anionic TC. At neutral and slightly basic pH, TC deprotonation can be attributed to the β-diketone group (C11-C12) and the dimethylamino group (C4) and these groups are available sites for metal ion interactions [3].

Liquid chromatography-mass spectrometry (LCMS) is an effective tool to separate and detect components in solutions. High-performance liquid chromatography (HPLC) has been used in many applications, which require particular methods to detect and measure specific compounds. Method development involves finding appropriate mobile phase (organic and aqueous phase composition), stationary phase (specifications of the column), and flow characteristics such as sample injection volume and mobile phase flowrate.

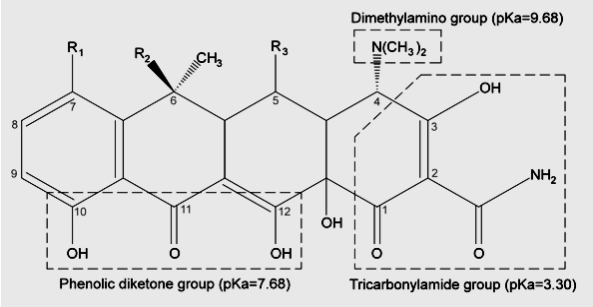

Fig (1) Tetracycline structure

Development of chromatography led to the use of mass spectrometer (MS) in tandem HPLC. Mass spectrometer first ionizes the chemical compound to produce charged molecules or molecule fragments and then measures their mass-to-charge ratios [4]. Conventionally, the ionization can be done by either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Some studies developed MS method using ESI for tetracycline, minocycline, methacycline and among other personal care products in soils and sediments [5-7]. APCI was also used to determine residual TC in foods [8], and TC in water after ozone treatment [9].

The development of dual ion source (DUIS) provides the solution of analyzing both highly polar and weakly polar (or non-polar) compounds. In effect, this ionization mode broadens the range of compounds that may be ionized simultaneously [10]. The performance of dual ESI/APCI in

analyzing both highly and weakly polar compounds was evaluated [11,12]. The equipment was modified such that an ESI probe was utilized with an APCI corona discharge needle.

This study was conducted to develop a method of detection and analysis of TC using LCMS comparing the performance of ESI, APCI and DUIS. The method was also used to determine the major byproducts of TC degradation using photocatalysis.

2. EXPERIMENTAL DETAILS

The main factors taken into consideration in the Design of Experiments were column dimensions, ionization mode, organic phase, and aqueous phase. Two columns were used: *1.9-µm column* (50 mm length, 2.1 mm inside diameter (ID), 1.9 µm particle size) and *5.0-µm column* (150 mm length, 4.6 mm inside diameter (ID), 5.0 µm particle size). The performance of the two columns were compared first and used in the succeeding experiments.

General factorial design of experiment was used to compare all possible conditions for the following factors: Ionization Mode (APCI, ESI, DUIS), organic phase (methanol, acetonitrile), aqueous phase (5 mM ammonium acetate, 5 mM oxalic acid, 0.1% acetic acid, 0.1% formic acid). The main response considered was the signal-to-noise (S/N) ratio.

In the actual experimental procedure, the tetracycline (≥98%, Sigma-Aldrich) solutions were dissolved in ultrapure water (Milli-Q™) to obtain three concentrations: 1 ppm, 10 ppm and 100 ppm. The trends were determined and compared in these concentrations.

The prepared solutions were run into Shimadzu LCMS-8030 equipment. The LC operational conditions were the same for all levels in the factorial experiments: 2-µL injection volume, 0.2 mL/min mobile phase flowrate, and 50-50 split aqueous-organic mobile phase composition.

The best condition was used to establish a calibration curve to measure the TC concentration of a prepared stock solution for the TC degradation experiment. The best condition was also used to detect the major byproduct of TC degradation using adsorption and photocatalysis using TiO_2-coated aluminum drum.

For the statistical analysis, analysis of variance (ANOVA) was performed using Design-Expert® 7 software to analyze the significance of the factors and to formulate a factorial model to describe the effects of the factors on the S/N ratio.

3. RESULTS AND DISCUSSION

The first objective was to compare the performance between the two available columns. The LCMS method development utilized a multi-level factorial design of experiment. The response was the S/N ratio. The aim was to attain the best signal received over the noise perceived by the detector. As for the effect of the column length and particle size, the retention time is an additional concern on top of the S/N ratio, provided that there is no issue on the separation of the compounds in the mixture. Thus, the effect of column was determined. There was a pattern on the behavior of the peaks across the other factors. Figure (2) presents the best S/N ratios at 1 ppm TC concentration using DUIS mode and 50-50 split 5 mM oxalic acid solution and methanol.

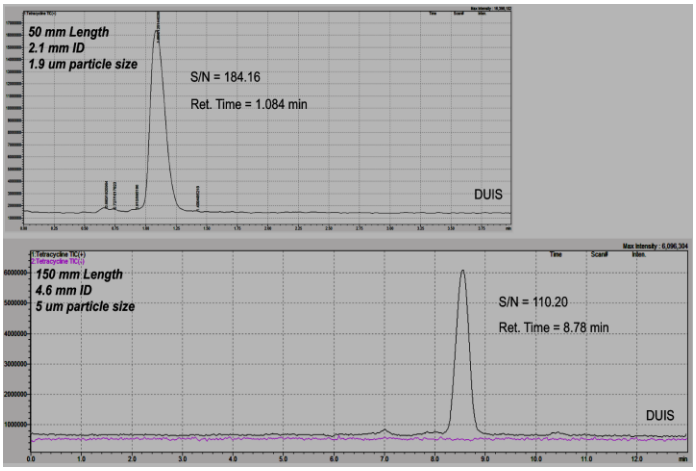

Fig (2) Chromatograms of TC scans using *1.9-µm* (top) and *5.0-µm* (below) columns

The S/N ratios of all the column comparative experiments suggest that smaller particle size is better than the larger particle size. Smaller C18 particles provide more surface area for better analyte-to-particle interaction. However, the operation requires higher pressure in pumping the liquid through the packed column. The column pressure attained was from 6,000 to 7,000 psi, which is below the maximum allowable column pressure (10,000 psi).

The 1.9-µm column provided a much shorter retention time than the 5.0-µm column. This favors the 1.9-µm column for higher productivity, i.e. more analyses can be achieved for the same period of time, than the 5.0-µm column. Therefore, the effect of the column was eliminated from the factorial design and this decreased the number of runs down to (3x2x4), which is equivalent to 24 runs for every TC concentration.

During factorial experiments, the response values for all the TC concentrations were observed and analyzed. The ANOVA describes the model as significant with a *p*-value of 0.0002. The model suggests that the significant factors are aqueous solution (mobile phase A) and the ionization mode. The model also concludes that the best S/N ratio is attained with the following factor levels:

- Mobile phase A = 5 mM oxalic acid
- Mobile phase B = Methanol
- Ionization mode = DUIS
- Column = 50 mm L x 2.1 mm ID x 1.9 µm particle size.

The effect of the ionization modes was also determined in the study. Looking at the individual effects, chromatograms were compared across the other factors. Figure (3) shows the chromatograms comparing the ionization modes used: APCI, ESI and DUIS. The mobile phase is composed of 50-50 split of 5 mM oxalic acid-methanol carrying 2 µL of 1 ppm TC.

It can be generalized that the APCI is not suitable for TC detection and monitoring. There is always a noise in the detection. In the case of ESI and DUIS modes, the effect of noise is always negligible to signal. DUIS gave the highest S/N ratios in all the TC concentrations injected in the LCMS. Its advantage is its applicability to detect a wider range of byproducts of degradation, polar or nonpolar.

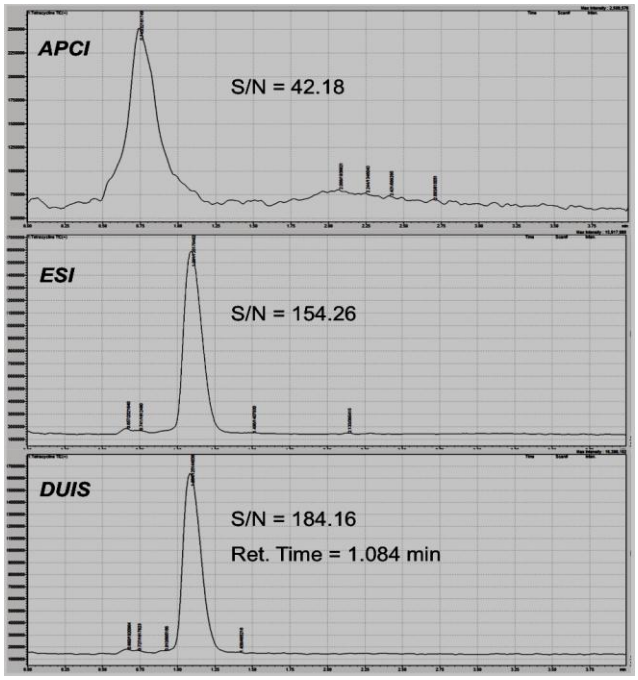

Fig (3) Chromatograms of TC scans using different ion modes

In determining the effect of different aqueous solutions to chromatography, the TC chromatograms are presented in Figure (4). The organic phase for all the conditions is methanol and the ionization mode is DUIS.

The general trend of the aqueous phase in using methanol as the organic phase is as follows: 5 mM oxalic acid > 5 mM ammonium acetate > 0.1% formic acid > 0.1% acetic acid. Zhu et al. (2001) states that oxalic acid can be used as a complexing agent in the mobile phase to improve peak shape and consistency of tetracycline. However, due to its non-volatility, oxalic acid may accumulate in the capillary interface or skimmer of the ESI or APCI source that may lead to clogging in the capillaries and can cause signal loss.

The effect of organic phase solutions, methanol (MeOH) and acetonitrile (ACN), to the chromatography was also determined. Figure (5) shows the chromatograms of TC scans under DUIS eluted by 5 mM oxalic acid solution as aqueous phase. The figure indicates that the noise has more effect in using ACN than MeOH. The peak using ACN also tends to broaden while the peak of MeOH is narrow and consistent.

The LCMS method developed was applied to detect TC and its degradation byproducts after 1h photocatalytic operation using TiO_2-coated corrugated aluminum drum reactor. Single ion monitoring was initially done to detect and monitor the consistency and concentration of TC in the solution samples during degradation. Precursor scans using DUIS on the positive mode were done to detect the presence of degradation byproducts. Multiple reaction monitoring (MRM) was then carried out to monitor the relative abundance of TC and the major byproducts in the solution. Figure (6) shows the progression of the relative abundance of the compounds in 1h operation.

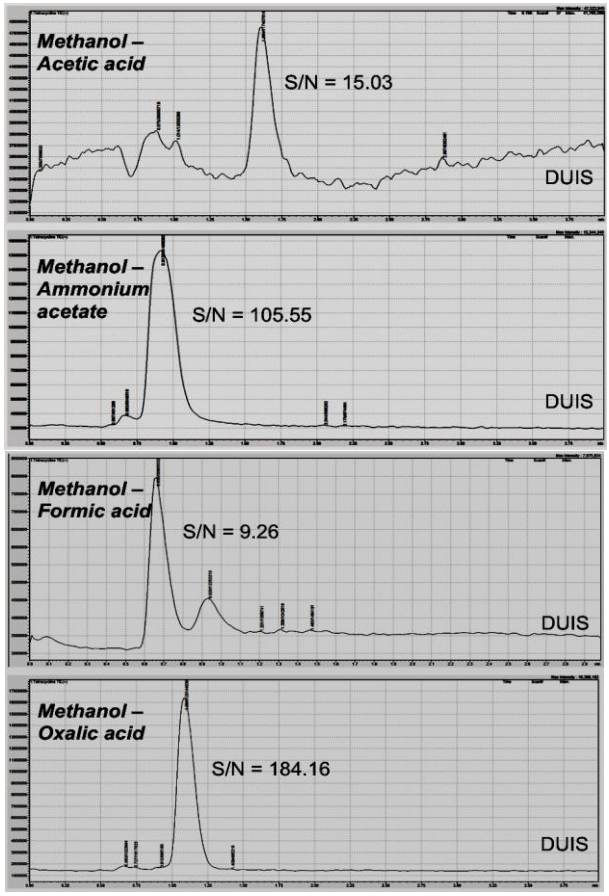

Fig (4) Chromatograms of TC scans using different aqueous phase

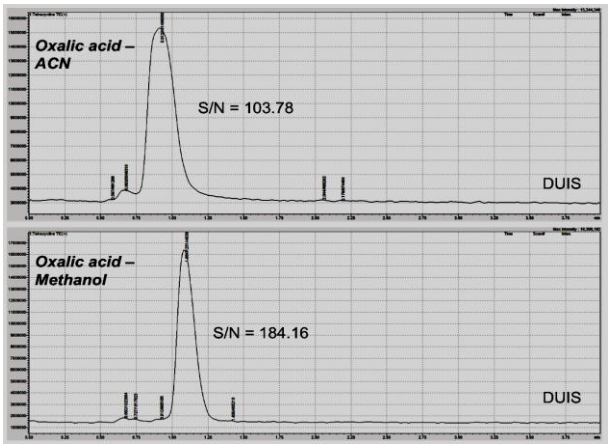

Fig (5) Chromatograms of TC scans using different organic phase

The Figure (6) shows the initial MRM scan of the solution, where the actual TC concentration based on the calibration curve was 66.63 ppm. After 10 min operation, the TC concentration dramatically decreased to 10.15 ppm with the detection of new compounds with m/z values of 195.00 and 234.95. After 60 min operation, the TC concentration continued to go down to 2.27 ppm with the increasing abundance of the other compounds that are assumed to be the degradation byproducts.

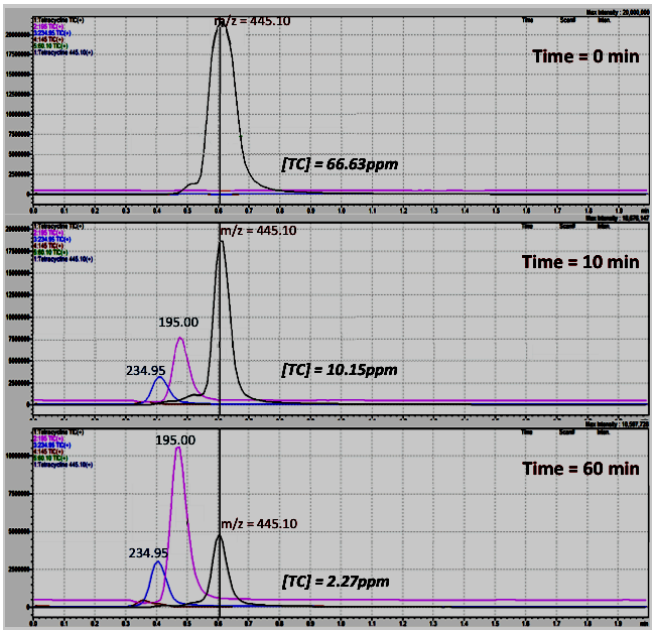

Fig (4) Relative abundance of TC and its byproducts during degradation

The experiment shows that the LCMS method used to best detect the TC in the solution can also be used to detect and monitor its degradation byproducts. The identities of the byproducts, however, were not determined and cannot be determined by the LCMS.

4. CONCLUSIONS

An LCMS method was developed to detect and monitor TC in the solution. The best S/N ratio was attained with the following conditions: C18 50mm L x 2.1mm ID x 1.9µm particle size column, DUIS ionization mode, 50-50 split of methanol as organic phase and 5mM oxalic acid solution as aqueous phase flowing at 0.2 mL/min carrying 2 µL of injected sample.

The short C18 column was able to perform a good chromatography at a very quick retention time at 1.08 min, which can accommodate more samples in a given time. The good performances can be attributed to the small particle size in the column that provides a large surface are for a given column volume. The drawback, however, is the required high pressure to attain required flowrate though the column.

The developed LCMS method was applied to the detection and monitoring of the degradation byproducts of TC during photocatalytic process. It was found that there were two major byproducts detected and found to be increasing in concentration as the TC concentration diminishes. However, the limitation of LCMS was that it has no capability of identifying the structure or the empirical formula of the compounds.

The use of DUIS has the advantage of detecting a wide rage of precursor compounds and derivatives. This will be useful in the detection and monitoring of degradation byproducts during wastewater treatment of a target pollutant, whether polar or nonpolar.

5. ACKNOWLEDGMENT

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