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Research Paper

Study on Photo-degradation of Sulfamerazin in Honey Samples by Accurate Mass LC-MS/MS

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Abstract: *Antibiotics are used in apiculture as a remedy against bacterial infection of honey bees. Applied antibiotics can eventually come to the final product, honey. Since Apiculture process is performed in open environment, environmental factors such as sunlight, moisture etc. may have an effect in the final concentration of these antibiotics in honey. Hence, modification of antibiotics by photo radiation in honey samples is of great interest. Degradation organic compounds such as antibiotics is slow at room temperature. By absorption of sunlight, which include both UV radiation and IR radiation, antibiotics undergo degradation in presence of moisture. Moisture along with ozone can produce hydroxyl radicals which are believed to be responsible for degradation of these chemical species. In this study, photodegradation of Sulfamerazin is studied by using high resolution Liquid chromatography mass spectrometry. Honey sample is spiked with Sulfamerazin and subjected to sunlight for 20 days. After 20 days sample is subjected to extraction of analyte followed by instrumental analysis. Result showed that Sulfamerazin is getting degraded by the sunlight and degradation products are characterized by generating the fragmentation pattern. Degraded product having molecular formula of $C_{14}H_{19}N$ is identified with 99.1% score of identification. However, the proposed structure of photo modified product, m/z: 201 and its fragment m/z:159 need to be confirmed by other confirmatory tests such as NMR.*

Keywords: photodegradation, Accurate mass LC-MS/MS, Sulfamerazin, honey.

Introduction

'Photo-modification' of chemical structure of organic compounds can be defined as the alteration of those organic compounds by Ultra violet light. Photodegradation usually involves both oxidation and hydrolysis. However, it does not apply to how materials may be degraded via infrared light or heat. Antibiotics are used in apiculture as an antibacterial agent against bacterial infection of honey bees. Their accumulation in the honey samples at higher concentration become harmful to consumer. Hence, there is growing concern regarding the use of antibiotics in apiculture industry. Presence of antibiotics in honey samples take special attention as honey is one of the purest gifts of nature.

Since Apiculture process is performed in open environment, environmental factors such as sunlight, moisture etc. may definitively have an effect in the final concentration of these antibiotics in honey. Hence, modification of antibiotics in honey samples by photo radiation is of interest. Degradation of many organic chemicals such as antibiotics is slow at room temperature. Upon the absorption of light in the atmosphere, they undergone degradation by hydroxyl radicals, which are produced from water and ozone.

Photochemical reactions are initiated by the absorption of a photon, typically in the wavelength range 290-700 nm at the surface of the Earth. The energy of an absorbed photon is transferred to

electrons in the molecule and promotes the molecule from a ground state to an excited state. Excited state molecules are not stable in the presence of O2 or H2O and can spontaneously decompose by either oxidation or hydrolysis. Sometimes molecules decompose to produce high energy, unstable fragments that can react with other molecules around them.

Chen et al. (2011) have recorded that degradation of several pharmaceutical compounds was possible under sunlight, UV, Ozone and in other advanced oxidation conditions. The chief mode of degradation was the photolysis by the absorption of light energy and such rate was directly proportional to the ability of light absorption of the compound.

Degradation of Sulfamerazine, one of the major and long acting sulfa drugs, is studied by liquid chromatography separation coupled to high resolution mass spectrometry analysis. Sulfamerazine inhibits bacterial synthesis of dihydrofolic acid by competing with para-aminobenzoic acid (PABA) for the binding site on dihydropteroate synthase. (https://www.ncbi.nlm.nih.gov/medgen/21389) Inhibition of dihydrofolic acid synthesis decreases the synthesis of bacterial nucleotides and DNA.

Material and Methods

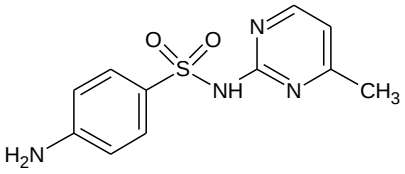

Figure 1: Structure of Sulfamerazin ($C_{11}H_{12}N_4O_2S$)

Sample preparation

10 mg/L of Sulfamerazin solution is prepared in 100% methanol. 5 ml of this solution mixed with 1 ml of the honey sample procured locally. Out of the 6 ml of resultant sample, 3ml is transferred to a test tube made of borosilicate glass. Test tube was covered by parafilm to avoid the evaporation of the solution. Test tube was placed vertically exposed to sunlight. The test tube contents could irradiate with sunlight for 20 days. Rest of the prepared sample of 3 ml volume was kept in dark as a control for this experiment. After 20 days of irradiation, both the samples and the blank were subjected to Liquid-Liquid extraction (LLE) with acidified Acetonitrile as extraction solvent. QuEChERS extraction salts were employed to enhance the layer separation between aqueous and organic phase. Separated organic phase is evaporated and reconstituted in 50/50 (V/V) of aqueous and organic mobile phases as reconstitution solution. Reconstituted solution is then filtered through 0.45 um syringe filter. The main photo degraded products of the sunlight irradiated sample were identified by a Diode Array Detector operated in UV range. The mass of the identified UV peak is measured by using accurate mass LC Quadrupole coupled Time of flight (LC-QTOF) LC-MS/MS system. Flow diagram of the sample preparation is given below in Figure 2.

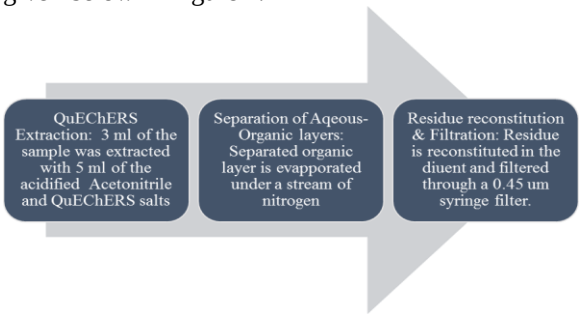

Figure 2: Flow diagram of sample preparation (Colour)

Instrumental parameters

Agilent accurate mass LC-QTOF high resolution LC-MS/MS system consists of an analytical pump, auto sampler with sample cooler and Diode array detector operated at a wavelength of 210 nm instrument. Diode Array Detector is installed with a low dispersion flow cell having a path length of 10mm. Since UV detection is a nondestructive technique, the outlet of this detector is connected in series with a 6545 QTOF LCMS/MS system. QTOF system used for sample analysis is having a mass accuracy of 1 ppm in MS mode and less than 2 ppm mass accuracy in MS/MS mode. Block diagram of the setup is given below. The analytical column used for separation of components in the sample is Agilent Zorbax Eclipse Plus C18 column with a dimension of 100 mm X 2.1 mm having a particle size of 1.8um. A generic reverse phase gradient elution of 18 minutes was performed with 0.1 % Formic acid in Water as aqueous phase and 0.1 % Formic acid in Acetonitrile as organic phase. 0.2 ml/min flow was employed and 2ul injection volume of prepared sample was used for analysis. Agilent QTOF instrument is operated using Electro Spray Ionization (ESI) in positive mode. The dual probe Agilent jet stream ionization source enhances the efficiency of ionization and hence result in higher sensitivity. Moreover, the calibrant solution could be

introduced through the second probe and online mass reference and calibration can be performed. Acquisition modes used were TOF MS full scan and TOF MS full scan followed by a dependent scan TOF MS/MS. Mass range for the TOF MS scan was between 100 Da and 1000 Da. Mass range for the dependent scan were between 50 Da and 1000 Da. Dependent scan, TOF MS/MS is triggered by the user defined intensity threshold. Fragmentor voltage for pulling parent ions kept as 400 V whereas the Collision energies of 10, 20 and 30 eV were used to generate the MS/MS spectra of the predominant masses. Sheath gas and heated gas temperatures were kept at 150 and 250 deg. Celsius respectively. Capillary voltage was kept at 3500V with a nozzle voltage of 500V.

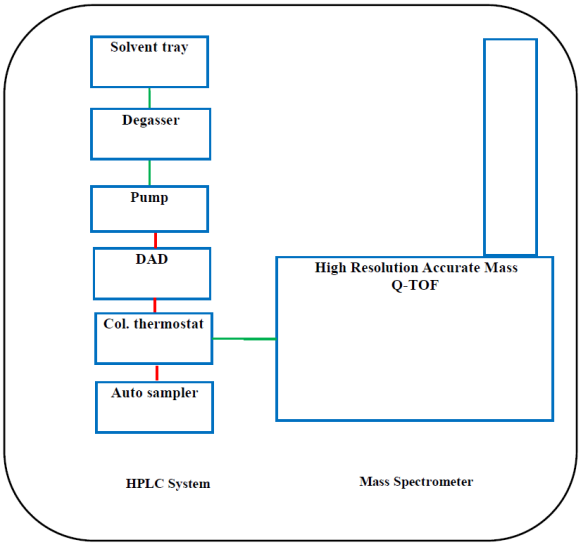

Figure 3: Schematics of LC- MS/MS system used for analysis (Colour)

Nebulization gas pressure was kept 35 PSi for generating a fine spray and thereby ease of evaporation. Agilent MassHunter acquisition software B.06.01 was used for method development and data acquisition. Agilent MassHunter Qualitative software version B.07.00 was used for data analysis. Calibration of the QTOF system is carried out using ESI low tuning mix supplied by the instrument manufacturer. By daily calibration of the instrument, resolution of TOF is set at maximum and mass accuracy of the instrument is maintained below 1 ppm. Moreover, the Quadrupole is also calibrated using the same calibrant solution to increase the mass accuracy especially for the on the fly acquisition of TOF MS/MS data. A solvent blank is injected prior to start of sample analysis in order to make sure that there is no carry over from column and no memory effect from the QTOF source and interface area. Followed by the reagent blank, extracted blank matrix sample is injected and stabilized the column before the sample injection.

Results and Discussion

In comparison to the Total Ion Chromatogram (TIC) of the control sample without photodegradation, the sample kept at Sunlight shown one additional peak in the chromatogram. Control sample clearly show one single peak corresponds to Sulfamerazin at a retention time of 3.7 minutes. The mass spectra in positive ionization mode corresponds to peak at 3.7-minute measure m/z: 265.0753, m/z of Sulfamerazin with a mass accuracy of 0.3 ppm in TOF MS. Actual m/z corresponding to Sulfamerazin (C11H12N4O2S) is 265.0754. Theoretical accurate mass and the m/z in positive ionization mode were calculated from the empirical formula using the mass calculator tool in the Mass Hunter Qualitative software.

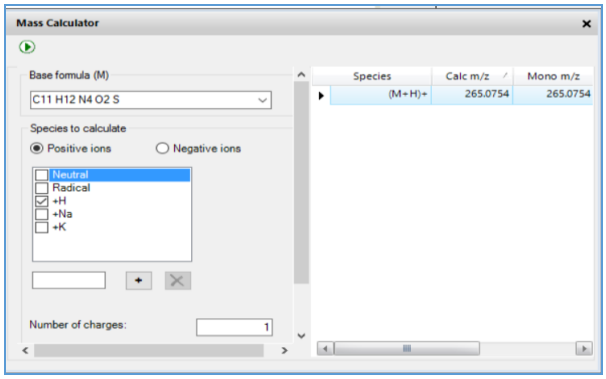

Figure 4: Calculated m/z for Sulfamerazin in positive mode calculated by Agilent MassHunter Mass calculator

To confirm the elemental composition of the measured mass and the corresponding isotopic pattern, Molecular formula generator (MFG) tool was utilized to generate the theoretical formula for the measured m/z: 265.0753. Molecular formula generator tool utilizes the mass accuracy, measured isotopic abundance and measured isotopic spacing and compared to the theoretical mass, theoretical isotopic abundance and theoretical isotopic spacing. Below figure shows the formula generated by the MFG tool which matches with the actual formula for Sulfamerazin. In the below figure 6, the red blocks represent the theoretical isotopic pattern for Sulfamerazin and the middle blue lines represent the measured isotopic pattern. It is very evident that the theoretical isotopic pattern of Sulfamerazin is matching with the measured isotopic pattern. Output of the stationary phase is connected to the DAD detector and the outlet of the DAD is connected in series with the MS. The Total Ion chromatogram (TIC) of the photodegraded sample is given below. From this chromatogram, the main compound Sulfamerazin eluting at 3.7 minute shown a decreased intensity in comparison to the control sample. In figure 7, the degraded compound can be seen and is eluting at 1.998 minute with the same

chromatographic conditions pointing that the degraded product may be more polar in nature compared to Sulfamerazin for it is eluting with more polar composition of the mobile phase applied in the initial gradient condition. As similar chromatogram was found in the diode array detector.

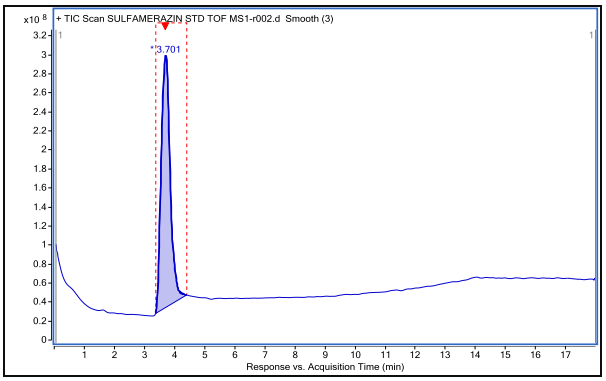

Figure 5: Total ion Chromatogram of Sulfamerazin control sample

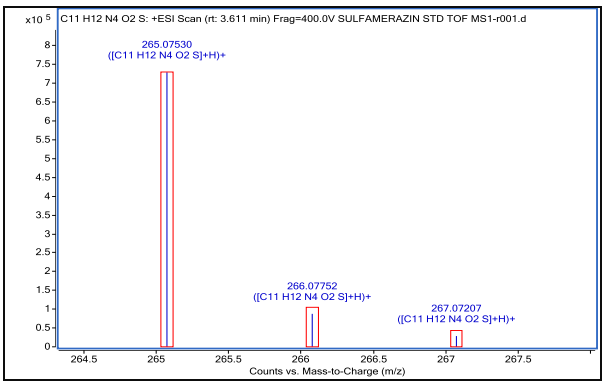

Figure 6: Molecular formula generated from the measured m/z: 265.0753 and isotopic pattern by Molecular formula generator tool in MassHunter Qualitative software

Total ion chromatogram is the total intensity of the ions generated throughout the run. Total ion chromatogram is the plot between the retention time of the components in the X axis against the total ion counts in the Y axis. Certified reference material of Sulfamerazin shown a retention time of 3.7 minutes and at that time the ion counts become maximum.

The MS spectrum of the photo degraded compound is measured by the TOF MS scan. The m/z of this degradant is measured in positive ionization mode is 201. 1562. Like the calculation of elemental composition for main compound Sulfamerazin, molecular formula for degradant is calculated by Molecular formula generator (MFG). The elemental composition found to be $C_{14}H_{19}N$ for the additional peak eluting at 1.998 minutes. The measured isotopic pattern for degraded product is having good matching with the theoretical isotopic pattern of the measured formula, $C_{14}H_{19}N$ with software identification score of 99.05.

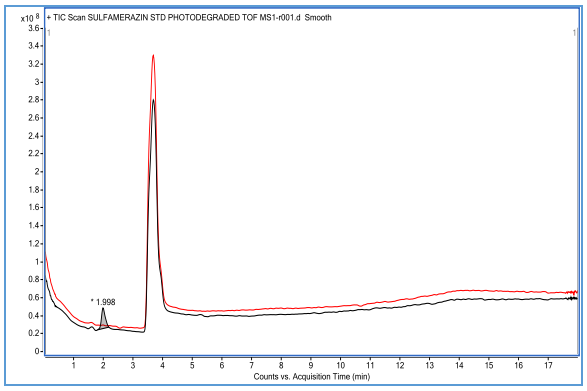

Figure 7: Comparison of TIC of Control versus degraded sample. Degraded product at 1.998 min showing that it is more polar in nature (Color)

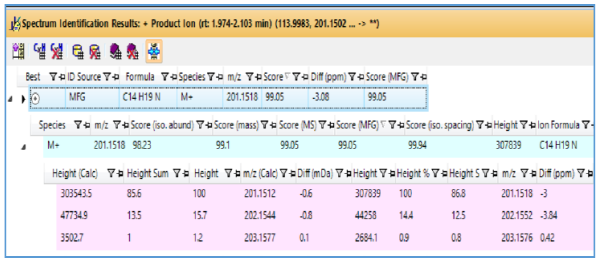

Figure 8: Molecular formula generation result by MFG with an overall score of 99.05

Discovery of sulfonamides occurred rapidly in the 1940s and 1950s, and widespread use in the decades since then has resulted in high levels of resistance. Most of the sulfa drugs are co dosed with trimethoprim. Some of the available literatures discuss about the aerobic biodegradability of Sulfamerazin. 60% of the Sulfamerazin solution undergoes degradation in closed bottle test within 2 days of time.

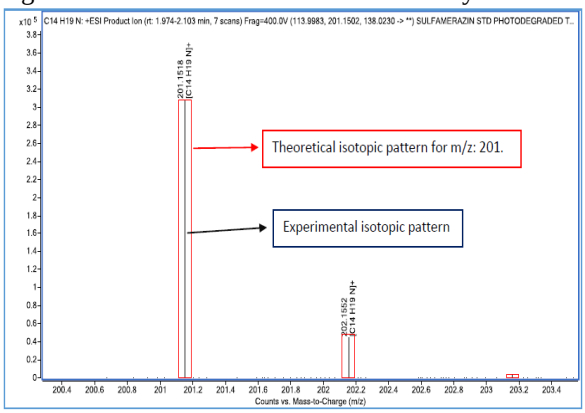

Figure 9: Mass spectra of the degraded sample in TOF MS acquisition mode. The measured formula $C_{14}H_{19}N$ can be seen in the figure (Color)

(http://opus.uniluebeburg.de/opus/volltexte/2013/14278/pdf/Thesis_Waleed_Ahmed_05122013_final2.pdf) A. Van Epps et.al (DOI: 10.1007/s40726 016-0037-1) May 2016 explained about degradation of antibiotic residues in animal waste. In this paper author discussed about the abiotic process of removal of antibiotics residues such as adsorption or absorption and biotic processes such as aerobic and anaerobic treatment for the degradation of antibiotic residues. Selvam et al reported that the rate of decomposition of antibiotics depends on the initial concentration of the antibiotics. However, very limited data is available on the photodegradation of antibiotics in general. The fragmentation pattern for the m/z: 201.1518 (with formula of $C_{14}H_{19}N$) is generated along with the fragmentation pattern of Sulfamerazin by auto MS/MS acquisition mode. Major Fragments of m/z: 201.1518 are m/z: 151.1291, m/z: 184. 1248, 119.0964 etc. as given below.

Major fragment found for sulfamerazin is m/z: 156, characteristic fragment for the Sulfa drugs. However, the fragmentation pattern of the photodegraded compound, m/z: 201.1491 also shown m/z: 156, however the intensity of this fragment in the photodegraded compound is found to be very less compared to that of sulfamerazin. There is a probability that this fragment experience matrix suppression from the honey matrix and hence appear in low intensity. Proposed hypothetical structure for m/z: 201 is given below which shows the destruction of the second ring completely. As per this structure and the MS/MS formula details calculated by the LCMS instrument, m/z:159, the major fragment of m/z: 201 has elemental composition as $C_{11}H_{13}N$ with a loss of C_3H_6 group.

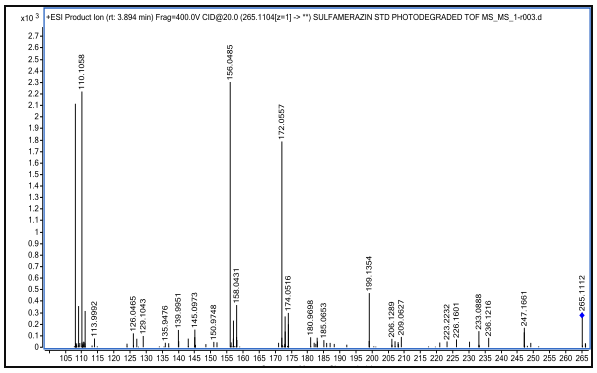

Figure 10: Fragmentation pattern of Sulfamerazin eluting at 3.7 min

The MassHunter MSC (Molecular Structure Correlator) program correlates accurate mass MS/MS fragment ions for a compound of interest with one or more proposed molecular structures for that compound. MSC achieves this by matching each observed fragment ion into the proposed structure using a "systematic bond breaking" approach as described by Hill and Mortishire-Smith. Input can be either a molecular formula entered manually, or have MSC select the n most probable molecular formulas it calculates for a compound using the accurate mass MS and MS/MS information.

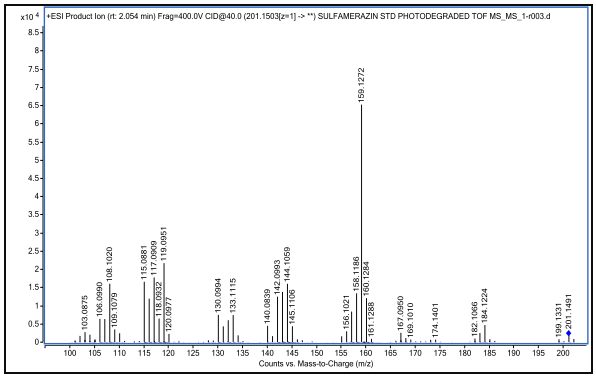

Figure 11: Fragmentation pattern of Photo degraded compound formed and eluted at 1.99 min

For each fragment ion one or multiple substructure candidates may be suggested and a "penalty" assigned based on how many and what bonds need to be broken to generate that substructure. Breaking two bonds, a double bond or even an aromatic ring carries a higher penalty (requires more energy and therefore is less likely) than just breaking one single bond. Two other factors impacting the overall correlation score are the mass accuracy of the observed fragment ions and the overall percentage of fragment ion intensity that can be plausibly explained with substructures.

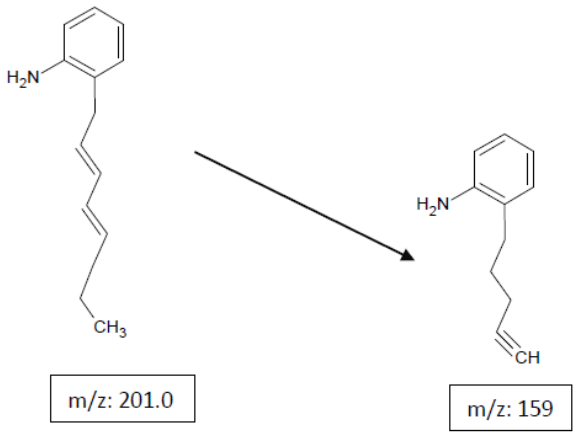

Figure 12: Hypothetical structure of the photo-modified product and its major fragment

With the help of MSC, the following structure is proposed for the degraded product of Sulfamerazin and its major fragment of m/z: 159. The structure of photo modified product, m/z: 201 and its fragment m/z:159 need to be confirmed by other confirmatory tests. It

will be interesting to have the data on the toxicity of this photodegraded product. It can be either less harmful or more harmful than the parent compound, Sulfamerazin. More toxic nature of any modified chemical species is a concern and its health effects need to be evaluated.

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