HYDROXYL OXIDATION OF CAFFEINE IN WATER MONITORED BY ELECTROSPRAY IONIZATION MASS SPECTROSCOPY

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ABSTRACT

Caffeine in water undergoes oxidation and is quickly degraded but the organic carbon content of the aqueous solution decreases much more slowly. This presents the problem that there are organic intermediates that remain in solution and are not as efficiently oxidized as caffeine. Electrospray Ionization Mass Spectrometry was used to monitor the advanced oxidation process of caffeine. The analysis showed that caffeine is first oxidized to N-dimethylparabanic acid. N-dimethylparabanic acid is further oxidized to N-hydroxymethylparabanic acid. Caffeine falls under a group of emerging pollutants set for future regulation owing to their potentially harmful effects on human health and the environment that are yet to be extensively studied.¹

INTRODUCTION

Caffeine is ranked as the most widely used legal drug in the world. It is found naturally in more than 60 plant species.² It is consumed in a variety of beverages and numerous food products and is also a constituent of hundreds of prescription drugs. The average adult’s caffeine consumption in the US is estimated to be 210mg/daily.³ Over the years, concern regarding the effects of accumulation of pharmaceutical compounds in the environment has increased. Caffeine as a popular drug has come under scrutiny. Due to its widespread consumption, it is introduced into the sewage system continuously.¹ Its high solubility in water (21.7g L⁻¹) and
negligible volatility, make it likely to persist in the water\textsuperscript{3}. Currently, a number of cities in the US are reporting that caffeine is showing up in their water supply\textsuperscript{4}. Although caffeine presents no large scale threat now, further research is needed on the occurrence of caffeine in natural waters and its chronic toxicity to aquatic organisms\textsuperscript{5}.

Contaminants in water can be degraded either by biotic or abiotic ways. In the article studied, caffeine was degraded chemically via Advanced Oxidation Processes (AOPs). AOPs degrade stable organic compounds in the environment leading to their complete mineralization which forms carbon dioxide, water and other inorganics\textsuperscript{1}. Most AOPs involve the generation of hydroxyl radicals. Hydroxyl radicals are highly reactive, have low selectivity and are short lived\textsuperscript{1}. These properties make it an ideal agent in water treatment\textsuperscript{6}. There are different ways for the generation of hydroxyl radicals in an aqueous environment. In the article studied, the three oxidation systems used were TiO\textsubscript{2}/UV system, Fenton system and UV/H\textsubscript{2}O\textsubscript{2} system\textsuperscript{2}. These systems are considered efficient for persistent organic pollutant degradation in water due to their ability to achieve complete mineralization of the organic contaminants under mild conditions of temperature and pressure. Several different successful laboratory scale applications have been reported for these methodologies. Another advantage of these systems is that the reaction rates are fast\textsuperscript{7}. Production of OH radicals by Fenton reactions occurs by addition of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and Iron (II) salts under acidic conditions in aqueous solutions\textsuperscript{6}. TiO\textsubscript{2}/UV system is a photocatalysis method that uses TiO\textsubscript{2}, a semiconductor metal oxide as a catalyst and UV radiation as the light energy source. Semiconductors are primary light absorbers. When the surface of TiO\textsubscript{2} is illuminated by light energy, the electrons get excited and positive holes and electrons are formed in the valence band and conduction band
respectively. The positive holes oxidize pollutants directly or water to produce hydroxyl radicals\(^8\). In the \(\text{H}_2\text{O}_2\) photolysis system, the pollutant solution containing \(\text{H}_2\text{O}_2\) is irradiated with UV light which causes the homolytic cleavage of \(\text{H}_2\text{O}_2\) to produce 2 free hydroxyl radicals\(^6\).

The composition and degradation of organic compounds in the environment has been monitored by mass spectroscopy (MS) because of its sensitivity, selectivity and speed\(^2\). Caffeine determination in different matrices has been analyzed by a variety of other techniques such as optical techniques UV, FT-infrared, FT-Raman and NMR spectroscopy\(^9\). Mass spectroscopy works by separating the components of the sample based on the ions that they break down into. The components are first vaporized, and then ionized into gas phase ions. The ionized particles are then accelerated and deflected at speeds dependent on their mass. The ions are then separated in space or time based on each ion’s charge and mass (m/z) by a mass analyzer. Different elements will have different mass-to-charge ratios (m/z). The ion signals are passed onto detector systems and are processed into mass spectra. This process of ion formation and manipulation is carried out in a high vacuum since ion in the gas phase ions are very reactive and short lived\(^9\) and the vacuum phase prevents collision of the ions with air molecules.

Coupling mass spectrometry and Electrospray ionization has made mass spectroscopy an extremely powerful analytical technique. The ESI operating principle is as follows: a dilute analyte solution is injected into the ion source by a mechanical syringe pump at low flow rate. A high voltage is applied to the tip of the metal capillary which causes a strong electric field and in turn disperses the sample solution into a fine spray of highly charged droplets. The applied voltage places a charge on the droplets. The electric field around the solution keeps the
droplets from freezing. Warm dry nitrogen gas around the capillary results in efficient evaporation of the solvent and also helps to direct the spray towards the MS. The charged droplets become smaller as the solvent evaporates which leads to an increase of surface charge density and a decrease of the droplet radius. Ultimately, the electric field strength within the charged droplet reaches a critical point at which the ions at the surface of the droplets can be ejected into the gaseous phase. In positive ion mode, the spraying tip of the ESI is kept at positive potential and the charging occurs via protonation of the analyte and vice versa.

ESI has become a major technique of use in to study reaction mechanisms in solutions of polar solvents. As a result, several key intermediates of the reaction in solution can be intercepted and characterized, in addition to the reaction substrates and products. Subsequently, ESI has helped in consolidating proposed mechanisms of organic reactions. In combination with a tandem mass analyzer mass spectrometry/mass spectrometry (MS/MS) additional structural information regarding the molecule may be gathered. A conventional mass spectrum of the analyte is obtained and then ions of a particular m/z value from the spectrum are chosen. In tandem mass spectrometry, two analyzers are used to detect precursor ions of interest and their product daughter ions. A precursor ion is mass selected by the first mass analyzer then focused into a reaction cell where it undergoes a gas phase chemical reaction in the collision cell producing different ions with different masses. In the collision cell, the mass selected ion is activated by increasing its internal energy which causes the heterolytic or homolytic cleavage of chemical bonds. This is known as collision induced dissociation (CID). The gas used is inert and
neutral such as nitrogen, argon, helium to prevent any side reactions. These product ions are passed to the second mass analyzer that scans the masses of the product ions and generates the product ion spectrum\(^{11}\) (fig1)

Mass spectrometers commonly used with ESI are quadrupole and time of flight spectrometers as they can produce mass spectra at a rapid rate. A quadrupole mass spectrometer uses four charged rods, two negatively and two positively charged, that have alternating direct current (DC) and radiofrequency (RF). The DC and RF fields serve as a mass filter. For a given set of DC and RF voltages, only ions of a specific m/z value avoid collision with the rods and move along the quadrupole filter to reach the detector. All other ions collide with the quadrupole surfaces at the established DC and RF voltage. TOF mass spectrometers involve measuring the time required for an ion to travel from an ion source to a detector located 1-2m from the source. All the ions receive the same kinetic energy during instantaneous acceleration but because of different m/z values, they separate into groups according to velocity as they move along the field free region between the ion source and detector. The ions strike the detector in order of increasing m/z values\(^{10}\). ESI-MS in conjunction with its tandem version ESI-MS/MS is now established as a major tool to study reaction mechanisms in solution (ESI-MS).
Very little information regarding the intermediates and products of caffeine oxidation in water is available despite a plethora of studies of transformation products of oxidation of contaminants in the environment\(^2\). This paper aims to qualify the intermediates arising as a result of advanced oxidation of caffeine and the mechanism involved in their formation.

**RESULTS AND DISCUSSION**

To identify the presence of transformation products resulting from the reaction of caffeine with hydroxyl radicals in aqueous solution, ESI-MS and ESI-MS/MS was employed. The organic intermediates from caffeine oxidation persist in solution and are not as efficiently oxidized as caffeine. ESI gently transfers key reaction intermediates to the gas phase without inducing undesirable side reactions, even the short lived ones occurring at low concentrations allowing their characterization. The ions generated from ESI have been found to closely reflect that of the solution\(^2\). Strong evidence collected confirms the currently proposed mechanism of oxidation of caffeine in aqueous solution\(^{12}\) by Dalmazio et al\(^2\).

Samples were directly infused into the ESI source via a microsyringe pump\(^2\). The ESI source was operated under positive ion mode producing positively charged ions. Basic samples are usually analyzed in positive ion mode. The nitrogen atoms in caffeine are basic and can pluck protons easily. The ionic fragments observed were singly charged protonated molecules (Figure 2 ). Since caffeine is of low molecular weight, the
masses of the charged fragments were able to provide valuable structural information about
the molecule quite easily as small molecules show only one predominant charge state. High
resolution for the mass spectrometers used, which implies a unit resolution of 10000 or
greater, allowed two adjacent peaks in the mass spectrum to be resolved sufficiently. The
intermediates arising from caffeine oxidation by hydroxyl radicals were dimethylparabanic acid
and di(N-hydroxymethyl)parabanic acid² (scheme 1).

The spectra read out demonstrate the relationship between the mass to charge ratio and the
relative abundance of ions. In mass spectra, the peaks represent the relative abundance of
different charged fragments. The mass in relative atomic units (Daltons) is shown on the
horizontal axis. The charge on the analyte comes from the addition of protons and the ions are
denoted as H⁺. It is observed that very little fragmentation occurs because ESI is a soft
 technique (figure 2). The ionization is soft in that very little residual energy is retained by the
analyte during ionization. This results in the formation of ions without breaking any covalent
bonds. The ESI spectrum provides information about the molecular weights of the fragments
from the peaks. The ions observed represent the ions of the intact molecule and other
components in the solution. The ESI tandem mass spectra of the protonated intermediates
provide a high degree of confidence in characterizing the structure of the oxidation by-products
of caffeine (figure 3). The intermediates undergo further fragmentation breaking their covalent
bonds. By knowing what bonds were likely to break during fragmentation, the molecule could
be reconstructed from the masses of its fragments. Fragmentation was carried out by colliding
the protonated molecules with argon gas, which gave the molecules extra energy to fragment
further.
At zero irradiation time, the molecular ion peak, the ion resulting from the ionization of the analyte molecule, appeared at an m/z value of 195. This is numerically equal to the nominal molecular weight of caffeine in addition with the proton (C₈H₁₀N₄O₂). It is also the base peak having the highest intensity (fig. 2a). The base peak is assigned a relative intensity of 100% and is used as a measure against which all the other peaks are normalized. Being the base peak, caffeine is determined as a stable ion. The more stable an ion is, the more likely it is to form and persist. Its stability arises from its aromaticity. Caffeine is a two ring system with amines,
amides and alkenes present as the functional groups. The five membered ring in caffeine is aromatic. The criteria for aromaticity in a molecule is that: it has to be cyclic, every atom in the ring must be sp² hybridized, the p orbital and the ring must be planar, and delocalization of pi electrons must satisfy Hückel’s rule of 4n+2 where n is an integer. The nitrogens are normally be sp³ hybridized, but since each of the N-CH₃ nitrogens has a lone pair located in a p orbital, the lone pairs can participate in the delocalization and resonance of the molecule by moving the lone pair from an sp³ orbital to a p-orbital. The lone pair of the doubly bonded nitrogen is located in an sp² hybrid orbital and does not participate in resonance. Aromaticity makes the molecule stable despite the energy cost of moving the lone pair to the p-orbital.

Upon irradiation of the aqueous solution, of caffeine in the presence of TiO₂, by monochromatic UV lamp, 2 additional abundant ions of m/z 175 and 143 were observed in the ESI mass spectrum at 90 and 150 minutes respectively (figure 2b). The molecular ion is always further to the right because its mass is the heaviest therefore it is deflected most. After 150 minutes of reaction time, the second protonated molecule was more abundant than the third one (figure
2c). This confirms di(N-hydroxymethyl)parabanic acid as an unstable compound. Unstable compounds appear as low intensity peaks in MS. Oxidation of caffeine monitored by GC-MS has not been able to detect this compound citing its relative instability and polarity\(^2\). GC-MS is an offline technique that requires extraction and sometimes purification pre-steps of the sample. Offline studies of reactions in solution also require the intermediates to be screened at pre-defined intervals when determining the solution composition over time as the reactants are progressively transformed into products over time. Thus GC-MS will only detect intermediates that have reasonable concentration in solution and if the species are not degraded within a few seconds/minutes\(^{12}\). GC-MS is limited in this way because short-lived, relatively unstable compounds in solution are likely to be missed. With ESI-MS, reactors are coupled to the ion source. The microsyringe is the simplest online reactor\(^{12}\), which was used in the experiment in the article studied. ESI-MS can provide mass-specific characterization of stable products and reactive intermediates that are transient and short-lived as the reaction is screened in real time. Consequently, di(N-hydroxymethyl)parabanic acid was detected.

The ion of \(m/z\) 143 is the protonated form of dimethylparabanic acid which is a known oxidation product of caffeine degradation\(^2\). It indicates a reduction peak of 52Da with respect to protonated caffeine. Tandem mass spectrometry selects and fragment ions from the normal mass spectrum to form radicals and cations. The major peaks in the mass spectrum explained by reasonable cleavage processes for the proposed structure verify the presence of the structure. Fragmentation occurs across the weakest bonds or bonds near the site of ionization. The ion of \(m/z\)143 dissociates mainly by the loss of \(\text{CH}_3\), a neutral group which is typical of all fragmentations and a peak is observed of \(m/z\)128 (Fig 3a). The second cleavage occurs at the \(\alpha\)
bond of the amide. The bond to the carbonyl carbon is cleaved and another neutral molecule of \( \text{NC}_2\text{O}_2 \) composition is lost and a peak with the \( m/z \) 58 observed (figure 3a). These dissociations are consistent with both the presence of N-methyl group and the OCNCO connectivity of dimethylparabanic acid (\( \text{C}_5\text{H}_7\text{N}_2\text{O}_3 \)). The \( m/z \) ion peak of 175 indicates a mass increase of 32Da with respect to 143. The reduced mass of 32Da between the two peaks suggests incorporation of two oxygens and that the previous molecule, dimethylparabanic acid, has further been oxidized. This is the di(N-hydroxymethyl)parabanic acid. The ion of \( m/z \) 175 dissociates mainly by the losses of \( \text{H}_2\text{O} \) (\( m/z \) 157) and \( \text{CH}_3\text{OH} \) (\( m/z \) 143), which are consistent with the presence of N-CH\(_2\)OH groups for di (N-hydroxymethyl) parabanic acid (\( \text{C}_5\text{H}_7\text{N}_2\text{O}_3 \)) (figure 3b).

Dalmazio, et al\(^2\) proposed a mechanism for the conversion of caffeine to its intermediates following its oxidation in water. Advanced Oxidation Processes involve three steps: firstly, the OH radicals are formed. Secondly, the target molecules are attacked by the OH radicals and broken down into fragments and finally, subsequent attacks by OH radicals follow\(^13\).

Radicals are very reactive because they have unpaired electrons and will attack any molecule in order to balance their unpaired electron configuration. Caffeine is classified as an alkaloid as it contains basic properties from the electron rich nitrogen atoms. Radicals attack the closest reactive site the most readily. In the caffeine molecule, radicals will attack double
bonds. The proposed mechanism of caffeine oxidation to N-dimethylparabanic acid is likely via initial 2 OH radical insertions to the C4=C5 bond. The homolytic cleavage of breaking the π bond provides the second pair of electrons to pair up with the two hydroxyl radicals (scheme 3). Other studies have postulated a C8-OH radical adduct as the first intermediate product of caffeine oxidation using a different oxidation system, Fe³⁺-EDTA ascorbate and Fe³⁺-EDTA/polyphenolics suggesting a direct reaction with ozone instead of hydroxyl mediated reactions. Instead of the radicals attacking the C4=C5 bond as was in the article studied, the initial attack takes place on the N9C8 double bond which is a less hindered position. This difference is in the severity of oxidation systems.

In the second oxidation step, the double ring is opened up following a free radical oxidation mechanism. AOPs are much less understood as environmental restoration methods compared to other techniques due to the complex chemical and physical processes involved in oxidation reactions. The opened up ring sets up the molecule for an intramolecular reaction. The nucleophilic atom and the electrophilic atom are present in the same molecule. The advantage of this reaction is that it leads to better entropy. In intermolecular reactions, two molecules come together to form one thus increasing the order in the system and decreasing the entropy. In an intramolecular reaction there is one molecule to start and one at the end which does not change the entropy of the system in an unfavorable way as is seen in intermolecular reactions.

The intramolecular reaction proceeds in two mechanistic steps via an addition elimination mechanism to form a cation at the nucleophilic nitrogen atom. Addition elimination is the mechanism involved with carboxylic acid derivatives. It entails the addition of a nucleophile and elimination of a leaving group. In caffeine, amides are present, one of the four carboxylic acid
derivatives. The lone pair of electrons on N3 attacks the carbonyl carbon C5 (scheme 4). C5 is much preferred to C6 as the electrophilic site since a 5-membered ring and not a four membered ring would form. Larger rings have less ring strain and better enthalpy. Ring strain causes heat to be released due to an increase in energy. a lot of that energy is stored in the bonds of the molecule causing the ring to be unstable and reactive. Water in this step protonated the terminal amine in the molecule to give it its full octet configuration. The cation of the amide formed is still very reactive as it has more than 8 electrons in its outer shell. The amide undergoes hydrolysis. Hydrolysis involves another addition elimination reaction forming a carboxylic acid as is typical of hydrolysis reactions with carboxylic acid derivatives. The known oxidation product of caffeine degradation, N-dimethylparabanic acid is the other product of this reaction (scheme 4). N-dimethylparabanic acid undergoes further oxidation via a free radical mechanism at both of its N-methyl groups to form di(N-hydroxymethyl)parabanic acid, an unprecedented intermediate in the degradation of caffeine\(^2\) (scheme 4).

**CONCLUSION**

This study found that caffeine is oxidized to dimethylparabanic acid and di(N-
hydroxymethyl)parabanic acid in water. Caffeine readily degrades and is consumed in water but its organic intermediates persist in water. If organic products and intermediate products overstay in water, this can arise as a concern as it accumulates over time in the environment. Chemical compounds in the environment degrade by direct sunlight absorption or by reaction with strong oxidizing species. The oxidation systems employed in this experiment were not as severe. They employed hydroxyl mediated reactions to oxidize caffeine. Different oxidation systems have been used to study caffeine degradation in aqueous environments. The results of the products and reaction intermediates arising from the caffeine oxidation differed citing the severity of oxidation processes\textsuperscript{1}. Ozonation when used in mineralizing organic compounds causes a significant decrease in the Chemical Oxygen Demand and Total Organic Carbon of many effluents.

For future work, a method to propose is the conversion of these organic intermediates into biodegradable compounds that would not require chemical oxidation processes to degrade them.
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CITATION